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Loss of SIMPL Compromises TNFα Dependent Survival of Hematopoietic Progenitors

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Abstract

Objective—Emerging work has revealed an integral role of the TNFα-NF-κB pathway in the regulation of hematopoiesis. TNFα inhibition of hematopoietic stem/progenitor cell growth involves the type I TNFα receptor (TNF RI) and the type II TNFα receptor (TNF RII). However the role of TNF RI versus TNF RII in mediating this response is less clear. Full induction of NF-κB dependent gene expression through TNF RI requires the transcriptional co-activator SIMPL. To address the role of SIMPL in TNFα dependent signaling in hematopoiesis, endothelial cells and hematopoietic progenitors expressing SIMPL shRNA were characterized.

Material and Methods—In vitro gene expression and progenitor assays employing SIMPL shRNA were used to examine the requirement for SIMPL in TNFα dependent effects upon cytokine gene expression and hematopoietic progenitor cell growth. Competitive repopulation studies were used to extend these studies in vivo.

Results—SIMPL is required for full TNF RI dependent expression of NF-κB controlled cytokines in endothelial cells. Hematopoietic progenitor cell expansion is not affected if progenitors lacked SIMPL or if progenitors are treated with human TNFα which signals through TNF RI. In the absence of SIMPL, human TNFα leads to a dramatic decrease in progenitor cell expansion that is not due to apoptosis. Loss of SIMPL does not affect the activity of TGF-β1 and IFNγ, other known suppressors of hematopoiesis.

Conclusions—The suppression of myeloid progenitor cell expansion requires signaling through TNF RI and TNF RII. Signals transduced through the TNFα-TNF RI-SIMPL pathway support hematopoietic progenitor cell survival, growth and differentiation.

INTRODUCTION

Tumor necrosis factor-α (TNFα) is a pleiotropic cytokine, produced daily as a homeostatic control mechanism to limit uncontrolled expansion of the hematopoietic system [1,2].

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CONFLICT OF INTEREST DISCLOSURE

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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Physiologically, TNFα production is intricately controlled at the systemic and local level in a time- and concentration dependent manner. TNFα regulates many aspects of hematopoiesis including activation of mature cells as well as the regulation of hematopoietic cell proliferation, differentiation and survival. TNFα affects nearly all blood cell types from the most primitive hematopoietic stem cell (HSC) to the terminally differentiated cell (e.g. the neutrophil). The cellular response to TNFα varies as a function of blood cell maturity. TNFα treated HSCs and hematopoietic progenitor cells (HPCs) arrest in the G_0/G_1 phase of the cell cycle [2–5], while terminally differentiated cells (e.g. neutrophil) are activated and subsequently undergo apoptosis [6–8]. HSCs derived from TNFα$^{-/-}$ mice live 4-times longer than HSCs derived from wild-type animals [9]. When comparable phenotypic populations of bone marrow cells from wild-type and TNFα$^{-/-}$ mice are enriched for HSCs (lin^{-}sca^{+}c-kit^{+}) or HPCs (lin^{-}sca^{-}c-kit^{+}), TNFα knock-out mice contain 4-fold higher numbers of functional myeloid progenitors [10]. Analysis of mice lacking either the type I or the type II TNFα receptor (TNF RI, TNF RII, respectively) revealed that lack of TNF RI dependent responses leads to an expansion in the HSC pool with a concomitant loss in HSC function (reconstitution ability) and expansion in HPCs. These effects are not seen in mice lacking TNF RII [2,11]. Together these data suggest that TNFα through TNF RI regulates the expansion and differentiation of HSCs and HPCs.

The inflammatory response is up-regulated as a function of the normal aging, a process termed inflammaging (for review see [12]). Physiologically this manifests as a low-grade increase in the circulating levels of pro-inflammatory cytokines such as TNFα. As a negative regulator of HSC proliferation the increased TNFα, imparts a selection pressure for the outgrowth of pre-leukemic cells. Thus long term exposure of HSCs to TNFα, as seen in myelodysplastic syndrome can serve as a selective pressure for the pre-leukemic clonal expansion by inducing differentiation, limiting self-renewal of the HSCs and HPC and providing an environment that enhances the selection of TNFα resistant stem cells.

TNFα dependent responses in large part are mediated through changes in NF-κB controlled genes whose products regulate the cell cycle, cell survival/apoptosis, differentiation and differentiated cell function (chemokines, cytokines, cognate receptors). An imbalance in TNFα dependent signals contributes to the pathophysiology associated with several diseases including septic shock, arthritis, atherosclerosis, congestive heart disease and many cancers (leukemias, lymphomas, prostate, and colorectal cancers) [13–19]. In hematopoietic cells full activation of TNFα dependent gene expression requires signaling through the type I and the type II receptor. TNFα signaling is concentration dependent, at low concentrations the type I receptor is preferentially activated; higher concentrations are needed to activate the lower affinity type II receptor [20]. Analysis cells derived from mice lacking TNF RI or TNF RII has revealed that each receptor contributes induction of NF-κB activity, although induction through TNF RII is significantly weaker [21,22].

We have identified a TNFα specific co-activator of NF-κB termed SIMPL (substrate that interacts with mouse pelle-like kinase; also known as interleukin-1 receptor associated kinase binding protein 1) [23,24]. SIMPL synergistically enhances p65 (NF-κB subunit) activity but not the activity of either c-jun or C/EBPβ [24]. Since SIMPL activity is restricted to the TNF RI signaling pathway modulation of SIMPL protein levels can be used to study the requirement for the TNF RI-NF-κB signaling in hematopoiesis. The studies herein examine the requirement for SIMPL in hematopoietic progenitor cell growth. Our results reveal that loss of SIMPL alone does not affect hematopoietic progenitor cell growth. However in the absence of SIMPL there is a dramatic decrease in TNFα dependent progenitor cell proliferation that is not the result of an increase in apoptosis. The loss of SIMPL is seen specifically in TNFα dependent TNF RI dependent responses. SIMPL loss has no effect upon the activity of other negative regulators of hematopoiesis. These studies reveal that TNF RI induction of NF-κB activity is important

for hematopoietic progenitor cell growth. Together with our previous work we can define a signaling pathway, TNFα-TNF RI-SIMPL-NF-κB that modulates hematopoietic cell function.

RESULTS

SIMPL shRNA decreases SIMPL mRNA and protein levels

Expression of the SIMPL shRNA construct (SIMPL 18) in mouse embryo fibroblasts and in primary mouse bone marrow cells decreased SIMPL mRNA (Figure 1A and C) and protein levels (Figure 1B). SIMPL mRNA levels were decreased by 70% in the mouse embryo fibroblasts and were decreased by 65% in the hematopoietic progenitor cells ($lin^{-}sca^{-}c\text{-}kit^{+}$). Similar results were obtained in a human embryonic kidney cell line (HEK 293) expressing either SIMPL 18 or another SIMPL shRNA construct (SIMPL 640; data not presented). Steady-state levels of the IRAK-1 protein were unaffected by the expression of either the SIMPL or scrambled shRNA, thus confirming the specificity of the SIMPL shRNA (Figure 1B). Thus, SIMPL protein is expressed in early hematopoietic progenitors and steady-state levels of SIMPL protein are specifically reduced in cells expressing the SIMPL 18 shRNA.

SIMPL is required for full TNFα dependent expression of NF-κB controlled genes whose products regulate hematopoiesis

Loss of SIMPL significantly reduces TNFα induced IκBα gene expression [24]. To determine whether SIMPL is required for the expression of other TNFα dependent NF-κB controlled genes, a mouse endothelial cell line stably expressing the SIMPL 18 shRNA (or its scrambled counterpart) was generated as the vascular endothelium is known to play a key role in the modulation of HSC and HPC function [25]. SIMPL transcript levels were reduced >95% in the SIMPL 18 expressing cell line (data not presented). Endothelial cells expressing either the scrambled (scr) or SIMPL 18 shRNA (sh) were treated with recombinant human TNFα (rhu TNFα, 10ng/ml; 1h), which signals through the mouse type I but not through the mouse type II TNFα receptor. Cultures were harvested and total cellular RNA was analyzed in a qRT-PCR based NF-κB Signaling Pathway Array (RT^2 Profiler™ PCR Array; Biosciences). The array includes several known NF-κB-responsive genes. 19 genes were induced (≥ 2-fold) in response to a one hour treatment with human TNFα, 18 of which are known NF-κB controlled genes (NF-κB.org and references within). In cells expressing the SIMPL 18 shRNA, the TNFα induced response was significantly reduced for 13 of the 19 genes, including several NF-κB controlled genes necessary for hematopoiesis: interleukin (IL)-6, TNFα, granulocyte colony stimulating factor (G-CSF), CD-40 and IL-1α (Figure 2). These data reveal that SIMPL is required for full TNFα induced expression of several NF-κB controlled genes whose products are known to regulate hematopoiesis.

Loss of SIMPL has no effect on steady-state CFU-GM

To examine the role of SIMPL directly in hematopoietic cell growth, *in vitro* colony forming assays were performed. Bone marrow-derived $c\text{-}kit^{+}$ cells were infected with retrovirus expressing green fluorescent protein (GFP) under the control of the cytomegalovirus immediate early gene promoter and either the SIMPL 18 shRNA or a scrambled version under the control of the rRNA U6 gene promoter. $GFP^{+}c\text{-}kit^{+}$ cells were then analyzed in CFU-GM assays. Decreased levels of SIMPL had no effect upon CFU-GM formation, size or proliferation potential as determined by quantitating the numbers of high proliferative potential and low proliferative potential (HPP and LPP respectively) progenitors (see Materials and Methods). Thus, in parallel to progenitors derived from $TNF\alpha^{-/-}$ or TNF $RI^{-/-}$ mice [11], reduced levels of SIMPL do not compromise CFU-GM progenitor cell proliferation.

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Loss of SIMPL sensitizes CFU-GM to the inhibitory effects of TNFα

TNFα is a widely recognized as an inhibitor of mouse hematopoietic progenitor cell expansion [2,26,27]. To determine if loss of SIMPL would affect the inhibitory activity of TNFα, mouse bone marrow-derived c-kit$^+$ cells were infected with retrovirus expressing the SIMPL shRNA (or control scr shRNA); GFP$^+$c-kit$^+$ cells were analyzed in CFU-GM assays in the absence or presence of increasing amounts of mouse TNFα. As described above, in the absence of TNFα, decreased levels of SIMPL had no effect upon the generation of CFU-GM progenitors or their proliferation (Figure 3A). A modest inhibition of CFU-GM formation is seen in TNFα treated GFP$^+$c-kit$^+$ bone marrow derived cells expressing the scrambled shRNA. At the highest dose of recombinant mouse TNFα tested (10 ng/ml) significant inhibition of CFU-GM growth was seen in GFP$^+$c-kit$^+$ bone marrow derived cells expressing the SIMPL shRNA (Figure 3A). The rather modest inhibitory effect of mouse TNFα was initially unexpected and contrasted results of others [28]. A comparison of assay conditions revealed that our assays were performed in the presence of defined growth factors (GM-CSF, IL-3 and SCF) but in the absence of pokeweed mitogen-stimulated spleen cell conditioned medium (PWMSCM), a complex mixture of cytokines including mouse TNFα [29,30]. To determine if the modest inhibitory effect seen with mouse TNFα was due to the absence of PWMSCM, GFP$^+$c-kit$^+$ cells expressing the SIMPL shRNA (or scr shRNA) were assayed for CFU-GM progenitor formation/proliferation and PWMSCM was added to the assays. In the presence of PWMSCM significant inhibition of CFU-GM formation was seen in the control GFP$^+$c-kit$^+$ (scr shRNA) cells treated with low doses of mouse TNFα (1 or 5 ng/ml) (Figure 3B, solid line). The inhibitory effect was enhanced in cells lacking SIMPL (Figure 3B; dashed line). Analysis of control cultures revealed that the addition of PWMSCM alone did not significantly affect the growth of progenitors expressing either SIMPL shRNA or the scr shRNA (data not presented). Thus loss of SIMPL sensitizes hematopoietic progenitors to the inhibitory effects of mouse TNFα.

The data presented in Fig. 3A and B was generated in assays performed with mouse TNFα, which signals through TNF RI and TNF RII. Our previous studies linked SIMPL to TNF RI dependent activation of NF-κB dependent responses [23, 24]. Thus we examined whether diminished levels of SIMPL would influence the inhibitory effect of human TNFα, which activates only mouse TNF RI on progenitor cell formation and growth. Mouse bone marrow-derived c-kit$^+$ cells expressing the SIMPL shRNA (or control scr shRNA) and GFP$^+$c-kit$^+$ cells were analyzed in CFU-GM assays in the presence of increasing amounts of rhuTNFα. As seen previously in the absence of TNFα, loss of SIMPL does not affect CFU-GM formation/ proliferation (Figures 3A–C). As observed when GFP$^+$c-kit$^+$ cells were analyzed with mouse TNFα, in the absence of PWMSCM, CFU-GM formation is slightly inhibited in cells expressing the scrambled shRNA at the highest dose of human TNFα tested (10 ng/ml). However, in contrast to the results obtained with mouse TNFα, diminished levels of SIMPL did not enhance the inhibitory effect at the higher dose of human TNFα (compare Figs 3A and C). To determine whether supplementing the assays with PWMSCM would enhance the inhibitory effect of human TNFα, as was seen with mouse TNFα (Figure 3B) PWMSCM was added to the assays. In contrast to the results obtained with mouse TNFα, where loss of SIMPL enhanced the inhibitory effect of TNFα, addition of PWMSCM did not enhance the inhibitory effect of human TNFα (Fig 3D). In the presence of PWMSCM and human TNFα, CFU-GM formation was significantly inhibited in progenitors expressing the SIMPL shRNA at all concentrations of human TNFα tested (range 40–60% inhibition, Figure 3D). Thus, the inhibitory effect of TNFα on the growth of CFU-GM progenitors is most evident when assays are performed in the presence of PWMSCM and requires signaling through TNF RII. The inhibitory effect of TNFα is also seen in the presence of PWMSCM if TNF RI dependent activation of NF-κB is compromised (compare Figure 3B and D).

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Diminished SIMPL levels do not sensitize CFU-GM to the inhibitory effects of TGF-β1 or IFNγ TGF-β1 and IFNγ, two other known inhibitors of hematopoiesis are found in PWMSCM [31,32]. Thus, we examined whether $GFP^{+}c\text{-}kit^{+}$ cells expressing either the scr shRNA or the SIMPL shRNA generated CFU-GM in the presence of either TGF-β1or IFNγ. Both TGF-β1 and IFNγ inhibited CFU-GM colony formation by $GFP^{+}c\text{-}kit^{+}$ cells expressing either the scr shRNA or SIMPL shRNA; however similar levels of inhibition were seen for the scr shRNA and the SIMPL shRNA expressing cells (data not presented). Thus the absence of SIMPL does not enhance suppression by all inhibitors of HPC proliferation.

In vivo hematopoietic potential of HSCs and HPCs lacking SIMPL

To determine the requirement for SIMPL *in vivo,* hematopoietic reconstitution assays were performed. Lethally irradiated mice were reconstituted with $GFP^{+}c\text{-}kit^{+}$ cells expressing either the SIMPL shRNA or the scrambled shRNA. All mice survived the transplant. At 4 months post-transplant all animals were sacrificed and hematopoietic parameters were quantified. Bone marrow cellularity was similar in animals reconstituted with either the $c\text{-}kit^{+}$ cells expressing the SIMPL shRNA or the scrambled shRNA (Figure 4A). In neither group were statistically significant changes in the numbers of HSC ($lin^{-}sca^{+}c\text{-}kit^{+}$) or in HPCs ($lin^{-}sca^{-}c\text{-}kit^{+}$) detected (Table 1). Cells with antigenic phenotypes ($lin^{-}sca^{+}c\text{-}kit^{+}$) and ($lin^{-}sca^{-}c\text{-}kit^{+}$) that are enriched for stem and progenitor cells respectively were comparable in both groups. Analysis of the expression of differentiated hematopoietic markers (Gr-1 and Mac-1) revealed no significant differences as a result of diminished SIMPL levels. Consistent with our *in vitro* assays, analysis of mouse bone marrow cells isolated from the reconstituted animals revealed that colony formation by the $c\text{-}kit^{+}$ SIMPL shRNA as compared to the $c\text{-}kit^{+}$ scr shRNA expressing cells was inhibited in a dose-dependent manner by mouse TNFα (Figure 4B).

Loss of SIMPL does not increase apoptosis of differentiated hematopoietic cells

TNFα treatment results in the induction of apoptosis in cells lacking NF-kB activity such as following viral infection or inhibition of protein synthesis [33–39]. In the hematopoietic system exposure to low dose TNFα does not cause apoptosis in HSC or HPCs, but does increase apoptosis of more differentiated cells [6–8]. TNFα can also inhibit HSC or HPC colony formation through induction of a cell cycle arrest at the G1/S border [2,11]. The growth inhibitory effect diminishes proliferation, but allows cell differentiation and maturation without inducing apoptosis. Thus we were interested in determining whether the decrease in CFU-GM observed for the TNFα treated $GFP^{+}c\text{-}kit^{+}$ cells expressing the SIMPL shRNA was due to an increase in apoptosis. $c\text{-}kit^{+}$ cells derived from the lethally irradiated animals transplanted with either the $GFP^{+}c\text{-}kit^{+}$ SIMPL shRNA or scr shRNA cells were cultured in liquid media (no methylcellulose) with rmuTNFα and the same complement of growth factors used to assay CFU-GM. After rmuTNFα exposure (48h; 10ng/ml) cells were harvested and analyzed for markers of apoptosis. Based upon Annexin V staining, rmuTNFα did not induce apoptosis in cells expressing the scr shRNA or the SIMPL shRNA (Figure 5).

DISCUSSION

TNFα is a potent inhibitor of hematopoiesis, an effect thought to be mediated in large part through type II TNF receptor [2,26,27]. Our data reveal that signals mediated through the type I TNF receptor are also involved. Under defined conditions, the presence of GM-CSF, IL-3 and SCF, TNFα has a modest inhibitory effect upon $c\text{-}kit^{+}$ hematopoietic progenitor cell growth. Under defined conditions and in the presence of PWMSCM TNFα responsiveness is altered: in $c\text{-}kit^{+}$ hematopoietic progenitors, signaling through TNF RI alone does not elicit an inhibitory effect whereas signaling through TNF RI and TNF RII does elicit an inhibitory effect (Fig 3D vs. 3B). Consistent with these observations, when progenitors from $TNF\text{-}RI^{-/-}$ mice

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are assayed *in vitro*, CFU-GM colony formation is not inhibited if bone marrow cells are treated with mouse TNFα [2]. Why addition of PWMSCM modulates TNFα responsiveness *in vitro* is not clear. In parallel to adding fetal bovine serum for the propagation of cultured cell lines, PWMSCM is a complex mixture of soluble factors that in general facilitates cell growth/differentiation/survival. In addition to TNFα, other known inhibitors of hematopoietic progenitor cell growth are present in PWMSCM including TGFβ and INFγ. However neither TGFβ nor INFγ is responsible for the TNFα dependent inhibitory effects, as addition of PWMSCM to c-kit$^+$ progenitors treated with human TNFα does not enhance its' inhibitory effects. While PWMSCM does contain mouse TNFα, the concentration present is not sufficient to trigger a sufficiently strong TNF RII signal, as inhibition of colony formation is not seen when progenitors expressing scr shRNA are assayed in media containing PWMSCM in the presence or absence of human TNFα which supplies a TNF RI dependent signal. Thus our data reveal that the inhibitory effect of TNFα on progenitor cell proliferation requires signals generated through the type I and II receptors and involves alterations in the cellular responsiveness to other cytokines. Mechanistically this could be mediated directly through TNFα induced changes in the expression of receptors for cytokines/chemokines present in PWMSCM or indirectly through a change in the survival and/or proliferation/differentiation potential of the progenitors which in turn affects their ability to respond to additional soluble mediators.

Loss of SIMPL enhances TNFα dependent inhibition of CFU-GM progenitor cell proliferation, an effect detected in the presence mouse TNFα or in the presence of a TNF RI signal plus PWMSCM. Others have reported that culturing wild type mouse hematopoietic progenitors in the presence of human TNFα, at concentrations higher than used herein, has no effect upon CFU-GM colony proliferation [40]. We have advanced these observations by demonstrating that the type I TNF receptor is functionally active on hematopoietic progenitors, as human TNFα can inhibit progenitor growth if NF-κB dependent responses are simultaneously compromised.

Discerning the differences between TNF RI and TNF RII dependent responses has been facilitated through characterization of knock-out mice. Loss of TNF-RI$^{-/-}$ as compared to TNF-RII$^{-/-}$ results in a greater loss in TNFα induced NF-κB activity [21,22]. However TNFα induction of NF-κB activity is entirely prevented only in fibroblasts derived from the TNF-RI$^{-/-}$/TNF-RII$^{-/-}$ animals. As shown in Figure 2, SIMPL is required for full expression of several TNFα induced NF-κB dependent genes. These data are consistent with our previous work demonstrating that SIMPL functions as a transcriptional co-activator for p65 activated through TNF RI [24]. As discussed above the relative contribution of TNF RI dependent signaling to NF-κB is much greater than TNF RII [21]. Thus in an intact animal where TNF RI and RII would be activated by TNFα, it would be reasonable to predict that the absence of SIMPL would prevent full induction of TNFα dependent gene expression. Treatment of progenitors expressing the SIMPL shRNA with TNFα did not induce apoptosis. These data suggest that TNF RII dependent activation of NF-κB can prevent the apoptosis that normally occurs if cells lacking NF-κB activity are treated with TNFα [22]. Integration of our data with others suggest a model in which progenitor cell expansion, through changes in proliferation and/or differentiation, is inhibited through a response that involves TNF RI along with TNF RII dependent responses. However progenitor survival is assured through induction of a TNF RI dependent response. Physiologically a role for TNF RI in the control of hematopoiesis has been described. In young animals ($<$ 3 mon) the hematopoietic profile of bone marrow derived cells isolated from TNF-RI$^{-/-}$, TNF-RII$^{-/-}$ and wild-type mice is either similar [11] or there is a slight expansion in progenitors in the TNF-RI$^{-/-}$. However as the animals age ($>$6 mon) there is a diminished capacity of an expanded pool, as compared to control, of HSCs and HPCs derived from TNF RI$^{-/-}$ but not the wild-type or TNF RII$^{-/-}$ mice to form CFU-GM or to reconstitute bone marrow function in lethally irradiated hosts [11].

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Myelodysplastic Syndrome (MDS) may be the most prevalent clonal hematopoietic neoplastic disease in adults in the Western world and is thought to be linked to the aging process [41]. MDS comprises a collection of TNFα linked diseases in which hematopoietic cells are functionally and morphologically aberrant. Bone marrow cellularity in these patients is either normal or elevated with peripheral pancytopenia (global loss of all peripheral blood cell types), a condition known as ineffective hematopoiesis [18,41–44]. MDS is linked to impaired hematopoietic cell differentiation, and can progress from less severe Refractory Anemia (RA) to in the most severe cases acute myeloid leukemia (AML) [18,41]. MDS is a disease of accumulating mutations; elevated TNFα and soluble TNF-RI levels are often present. Levels of TGF-β1, another inhibitor of hematopoietic differentiation are normal in MDS [42]. In RA, an early stage of MDS, TNF RI expression is significantly increased. As the disease transforms into RA with excess blasts (RAEB), the level of TNF RII expression increases, which is associated with an increase in apoptosis of peripheral blood cells [18,45]. TNFα levels and the amount of apoptosis are the highest in patients with RA [42,43], and are most likely the result of the chronic inhibition of HPC proliferation elicited by TNFα [1]. As indicated above the MDS often progresses to acute myeloid leukemia (AML). In AML myeloid blasts cells fail to differentiate, leading to rapid loss of life within 1–3 years for those over 60 years of age [46]. NF-κB (p65/p50 heterodimer) is upregulated and constitutively active in AML, usually as a result of a mutation at the level of the IκB kinases (IKKs) (for review [47,48]. Thus as MDS evolves, and TNFα levels increase leading to inhibition of normal, wild-type HSCs and early progenitor cell proliferation and apoptosis is induced among the more differentiated hematopoietic cell types [13]. Constitutive activation of NF-κB dependent gene expression leads to increased production of inhibitors of apoptosis such as: XIAP, survivin, cIAP1, cIAP2, cFLIP, Bcl-2, and Bcl-xL [49–51] which in turn selects for the outgrowth of pre-leukemic/leukemic cells.

In MDS patients, cA2 anti-TNFα antibody treatment decreases activated $CD4^+$ and $CD8^+$ T-lymphocyte populations which contribute to the MDS and TNFα release [44], but also diminishes the elderly patients cell mediated immune response. Previous studies from our laboratory demonstrated that loss of SIMPL targets NF-κB activity induced specifically by TNFα [23,24] enabling NF-κB activity controlled by other cytokines to proceed unencumbered. Additionally loss of SIMPL still allows for TNFα dependent activation of other signaling pathways such the JNK and p38 pathways. Our results presented herein support the concept that SIMPL is a target for the development of therapeutics aimed at eliminating a hyper-active TNFα dependent response. A disruption of SIMPL function could be used to reduce a hyper-activated immune system characteristic of septic shock, arthritis, atherosclerosis, congestive heart failure and certain cancers without compromising normal daily hematopoietic and immune cell function.

MATERIALS AND METHODS

Antibodies

SIMPL antibody was commercially generated (Covance, Denver, PA). IRAK-1 antibody and Anti-FLAG® M2 monoclonal antibody (F3165) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-mouse IgG F $(ab')_2$ fragment specific-HRP was from Upstate Biotechnology (Lake Placid, NY; 115-36-006). Goat anti-mouse IgG $F(ab')_2$ fragment specific-HRP was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Fluorochrome-labeled antibodies were from BD Bioscience (BD Biosciences, San Diego, CA) except as noted.

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Cytokines, Growth Factors, Chemokines

rhuTNFα and rmuTNFα were from Sigma-Aldrich, Inc. (Sigma-Aldrich, St. Louis, MO). RmuGM-CSF, rmuSCF, rmuG-CSF, rmuIL-3, rhuTGF-β1, rhIL-6, rmuIFNγ were from PeproTech Inc. (PeproTech, Rocky Hill, NJ).

Cell culture and transfection

Mouse embryo fibroblasts and the human embryonic kidney epithelial cell line (HEK 293) were maintained and transfected as described previously [24].

Bone marrow cells and LDMNC separation

Bone marrow was isolated from 8–12 week old C57BL-6 mice. Low density mononuclear cells (LDMNCs) enriched using Histopaque 1119 (Sigma-Aldrich) were plated at a density of 4×10^6 cells/mL in media containing stem cell factor (SCF; 100 ng/mL) and IL-6 (25 ng/mL).

Progenitor assays

c-kit$^+$ (1000 to 2000 cells/mL), whole bone marrow (50,000 nucleated cells/mL), or spleen cells (~200,000 LDMNCs) containing shRNA (scrambled or SIMPL) were cultured in methylcellulose containing FBS (30%), β-mercaptoethanol, glutamine (1%), mSCF (100 ng/mL), mGM-CSF (10 ng/mL), mIL-3 (20 ng/mL) with the indicated amount of recombinant mouse or recombinant human TNFα. Each condition was plated in triplicate (1 ml final volume/35 mm dish). Six 35 mm dishes were placed in a 150 mm petri dish that also contained a 35 mm dish filled with autoclaved water and no lid. Cell cultures were incubated in a 37°C humidified incubator with 5% CO_2; colonies (number, size and type) were counted 7 days later. The data is reported as percent change from control (untreated but expressing the same type of retrovirally encoded shRNA).

High and low proliferative potential (HPP, LPP) assays

Green fluorescent protein$^+$c-kit$^+$ cells transduced with retrovirus expressing either SIMPL shRNA or the scrambled shRNA were plated in methylcellulose containing FBS (30%), β-mercaptoethanol, glutamine (1%), mSCF (100 ng/mL), mGM-CSF (10 ng/mL) and mIL-3 (20 ng/mL). Cell cultures were incubated in a 37°C humidified incubator with 5% CO_2. Seven days later HPP (>0.5 mm with a dense core/nucleus of cells) and LPP (greater than 50 cells but do not meet HPP criteria) colonies were counted.

Generation of SIMPL shRNA constructs

SIMPL short hairpin RNAs (shRNAs) were designed using the Clontech siRNA Sequence Selector program. The identified sequences were screened against the mouse genome/transcriptome using Primer-Blast (NCBI). Of the four siRNAs dispersed throughout the SIMPL sequence shRNA 18 spanning nucleotides 557–575 and shRNA 640 spanning nucleotides 640–658 successfully decreased SIMPL protein levels. siRNAs were converted to shRNAs using the siRNA Hairpin Oligonucleotide Sequence Designer™ program by Clontech. Oligonucleotides were commercially generated and purified by polyacrylamide gel electrophoresis (Sigma Genosys). Sequence 18 (SIMPL 18 shRNA) is identical and sequence 640 is 84% identical to human SIMPL.

Oligonucleotides were subcloned into a RNAi-Ready pSIREN-RetroQ-ZsGreen (Clontech) and into RNAi-Ready pSIREN-RetroQ vectors (Clontech). Vectors were transfected into the packaging cell line which were sorted for by fluorescent activated cell sorting (FACS) using the co-expressed ZsGreen (GFP-like) fluorescent tag. A scrambled shRNA (negative control) plasmid was generated and included in all experiments.

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Retroviral production and transductions

Retrovirus was generated in the HEK 293 based Phoenix GP MMULV-based retroviral packaging cell line following introduction of the pSIREN plasmid, and plasmids containing the envelop gene and the gag/pol genes using the Calcium Phosphate Transfection Kit (Invitrogen). Transfection mixtures also contained chloroquine (Sigma-Aldrich, St. Louis, MO). Retroviral containing supernates were collected every 24 h for a total of 72h. Collected media was centrifuged to remove detached cells and frozen (−80°C). Cell lines at 50–80% confluency were transduced with the retrovirus in growth media supplemented with polybrene (8 μg/mL). Dishes were centrifuged (2000 rpm, 30 min., 32°C) and then incubated at (37°C, 30 min). Twenty-four hours later, the media was replaced with fresh growth media. For non-adherent cell lines, after the 30 min incubation, the cells were collected by centrifugation, resuspended in fresh growth media and returned to the incubator.

Petri dishes coated with Retronectin (Takara) were pre-treated with supernates containing retrovirus and centrifuged (2000 rpm, 30 min, 32°C). Supernates were removed and fresh media containing retrovirus supplemented with SCF (100ng/mL), IL-6 (25 ng/mL) and Polybrene was added. LDMNCs grown for 2–3 days in the pre-stimulation conditions (media containing SCF plus IL-6), were added to the retronectin treated petri dished. Dishes were centrifuged (2000 rpm, 30 min, 32°C) and incubated for 30 min at 37°C, 5% CO_2. Retroviral containing media was removed, fresh media [IMDM with 20% FBS, mSCF (100ng/mL), hIL-6 (25 ng/mL)] was added and cultures were returned to a 37°C, 5% CO_2 incubator.

In the absence of TNFα, c-kit$^+$ cells expressing either SIMPL shRNA or the scrshRNA grew equivalently (120.8 to 120.6 respectively; n=13). Additionally HPP and LPP did not differ between SIMPL shRNA or scrshRNA expressing cells (HPP 42 vs. 41 and LPP 85 vs. 83 respectively).

Staining for c-kit$^+$ cells containing shRNA

Retrovirally transduced LDMNCs were collected by centrifugation (1500 rpm, 5 min, room temp) and resuspended in PBS containing 0.5% BSA. Non-transduced control cells were incubated with isotype control antibody; GFP only control LDMNCs were transferred to a fresh tube containing PBS with 0.5% BSA. All LDMNCs were treated with Mouse FC Block (5 min, 4°C). shRNA LDMNCs were incubated with the APC-c-kit antibody; control LDMNCs were incubated with APC lineage isotype control antibody and APC-c-kit antibody isotype control antibody. Cells were collected by centrifugation (1500 rpm, 5 min, RT) and cell pellets were resuspended in PBS containing 0.5% BSA.

Lineage cell depletion

LDMNCs transduced with retrovirus containing the shRNAs were subject to magnetic cell sorting using a mouse lineage cell depletion kit (Miltenyi Biotec, Auburn, CA, 130-090-858) according to the manufacturers' protocol. After lineage depletion, the cells were incubated in Mouse BD Fc Block™ (5 min, 4°C, 1 μL/million cells) followed by the APC-lineage cocktail, PE-Cy7 Sca-1 and PE-c-kit antibody. Surplus lineage positive cells containing the scrambled shRNA served as the GFP positive cells. Controls stained with individual antibody were generated as described above. Lin$^-$sca$^+$c-kit$^+$ and lin$^-$sca$^-$c-kit$^+$ populations were isolated by FACS at the IUSM Flow Cytometry Facility.

Apoptosis Assay

APC conjugated Annexin V and 7-amino-actinomycin were obtained from BD Biosciences (San Diego, CA). Apoptosis assays were performed as described by the manufacturers (BD Biosciences) and fluorescence was quantitated by FACSCalibur or the FACScan (BD

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Biosciences). For the whole bone marrow apoptosis assay of transplanted mice, CD45.2$^+$ cells were sorted with PE-CD45.2 and PE IgG2A isotype by FACS eBioscience (San Diego, CA).

Transplant experiment

C57BL/6 mice (female; 8 to 10 weeks) were irradiated as previously described [52] and injected intravenously (lateral tail vein) with c-kit$^+$ cells (250,000) containing either the SIMPL or the scrambled shRNA. Four months post-transplant, all the mice were alive and subject to analysis. Whole blood cells from a portion of spleen (cut and weighed) were harvested by homogenizing the spleen in a nylon cell strainer (70μm; BD Bioscience, Bedford, MA).

Whole bone marrow cellularity assay using transplanted animals

Bone marrow cells (tibias, femur, pelvic flat bones) from transplanted and control (non-transplanted) mice were added to PBS containing 0.5% BSA and collected by centrifugation (1500 rpm, 5 min, RT). Cell pellets were resuspended in PBS containing 0.5% BSA. One group of transplanted cells (100,000 cells) was left unlabeled to assess GFP (ZsGreen) percentage by FACs. A modified BD Bioscience protocol (no BD FC Block) provided with antibodies was used to characterize the cells using PE conjugated rat anti-mouse CD117 (c-kit), APC mouse lineage antibody cocktail, and PE-Cy7 rat anti-mouse Sca-1 or PE-Cy7-conjugated rat anti-mouse CD11b (Mac-1 α chain) and PE conjugated rat anti-mouse Gr-1 antibodies.

Real-Time PCR

Total RNA was isolated using the RNeasy Plus Mini Kit (QIAGEN). GeneAmp Gold RNA PCR kit (Applied Biosystems, Foster City, CA) was used to generate cDNAs that were quantitated using the SYBR Green kit (Applied Biosystems) on an Eppendorf Mastercycler EP instrument (Eppendorf, Westbury, NY). The uniformity of the PCR products was determined using the melt curve function.

qRT-PCR Array

RNA isolated from retrovirally infected endothelial cells was analyzed according to manufactures' protocol in an NF-κB Signaling Pathway qRT-PCR Array (PAMM-025; Biosciences). Individual assays were normalized to a pool of five housekeeping genes, and then normalized to expression in the untreated (scr) or (sh) expressing cells. The results are derived from three independent assays for each experimental condition. *$p<0.01$, **$p<0.001$.

Western Blot analysis

Western blots were performed as previously described (Kwon et al., 2004).

Statistical analysis

Statistical significance was calculated using Student 's t test [53].

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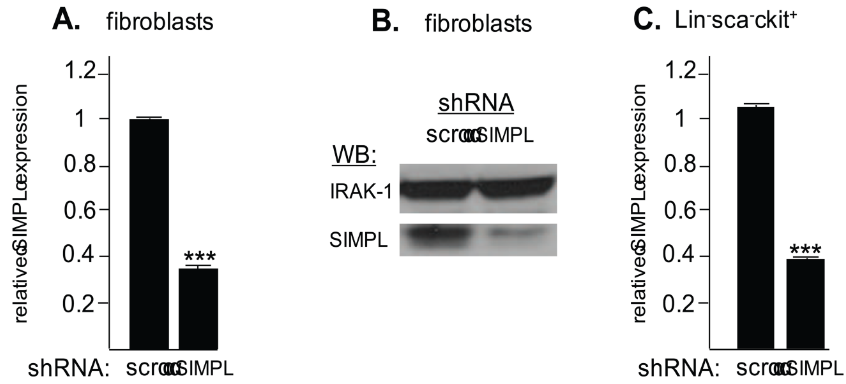

Figure 1.

SIMPL levels are diminished in cells expressing SIMPL shRNA. (A and B) Mouse embryo fibroblasts (fibroblasts) were transfected with a plasmid encoding a SIMPL shRNA or a scrambled shRNA, 48 h later, cells were harvested. **(A)** SIMPL mRNA levels were quantitated by real-time PCR, were normalized to GAPDH transcript levels and are represented as the change in SIMPL gene expression relative to that detected in cells expressing the scr shRNA construct. **(B)** Cell lysates from the transfected fibroblasts (panel A) were subject to Western analysis for expression of IRAK-1 and SIMPL protein. **(C)** $Lin^{-}Sca^{-}ckit^{+}$ progentiors were transduced with retrovirus encoding the scr or SIMPL shRNA. SIMPL mRNA expression quantitated by real-time PCR, was normalized to GAPDH transcript levels. Data are represented as the change in SIMPL gene expression relative to that detected in cells expressing the scr shRNA construct. (n=3) ***p<0.001

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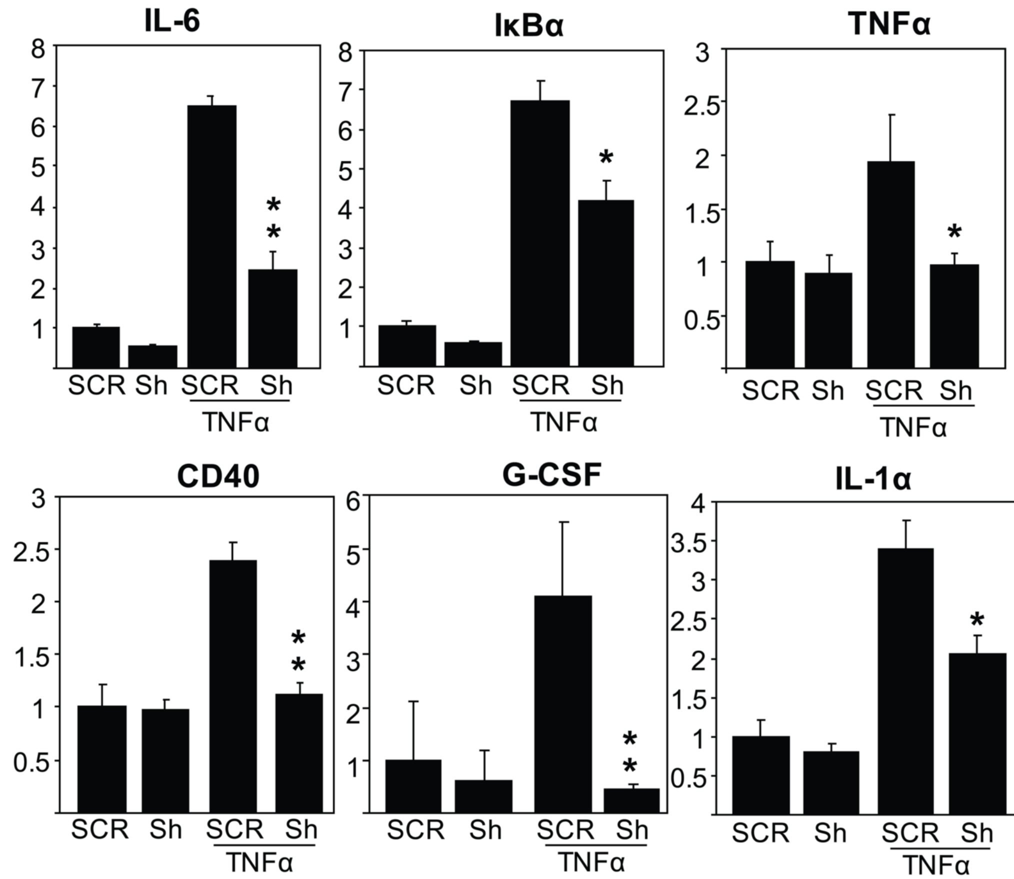

Figure 2.

SIMPL is required for full induction TNFα induction of NF-κB controlled genes. Mouse endothelial cells infected with retrovirus encoding either either SIMPL shRNA or a scr shRNA were left untreated or were treated with recombinant human TNFα (10ng/ml) for 1h. Total cell RNA was isolated, cDNAs were generated and used in qRT-PCR assays (SA Biosciences) to measure the expression of the indicated genes. The results are derived from three independent assays for each experimental condition. Data were normalized to the averaged expression of 5 different housekeeping genes and are presented as the fold difference relative to the untreated, cells expressing the scrambled shRNA. Error bars are the standard deviation.

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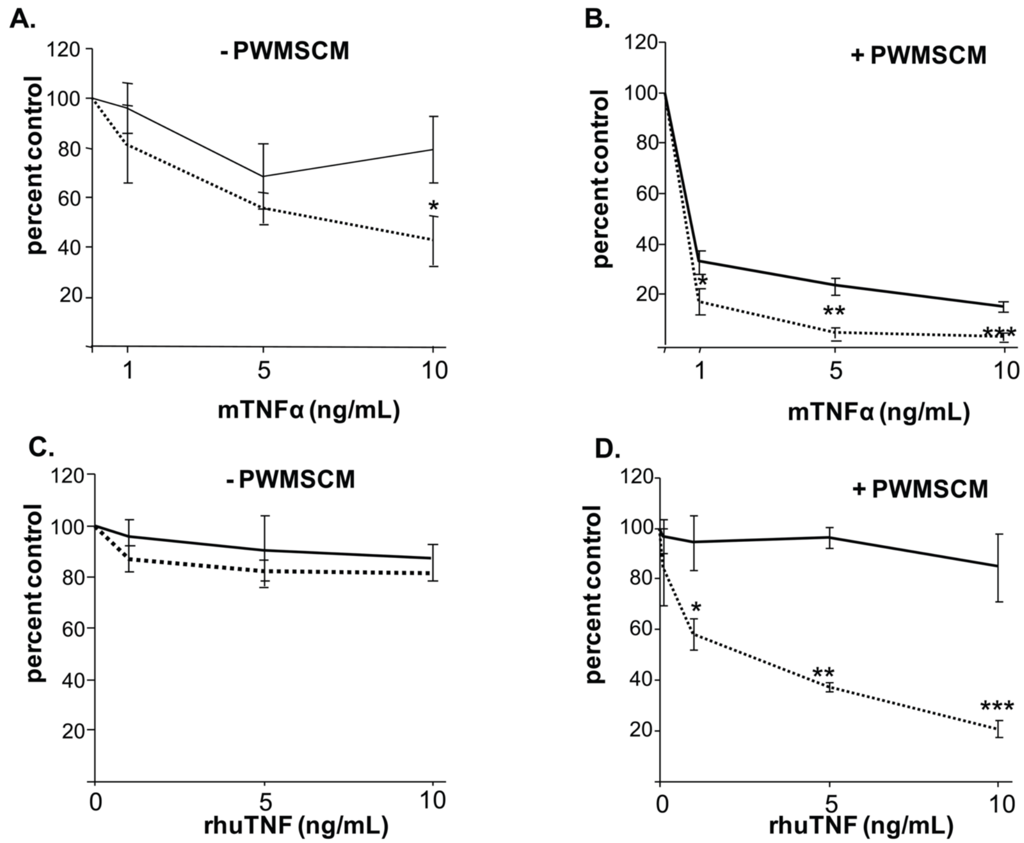

Figure 3.

Loss of SIMPL decreases CFU-GM colony formation in a TNF-RI dose dependent manner requiring TNF-RII activity. Scramble and SIMPL shRNA c-kit^{+} cells were plated into a colony assay on methylcelluose containing mGM-CSF, mIL-3, mSCF with (A) mTNFα only *p<0.04 (mean ± SEM; n=3); (B) PWMSCM and mTNFα *p<0.05; **p<0.006; ***p<0.006 (mean ± SEM; n=3); (C) rhuTNFα only (mean ± SEM; n=3). (D) PWMSCM and rhuTNFα *p<0.03; **p<0.0002; ***p<0.05 (mean ± SEM; n=3). (A–D) Independent experiments performed in triplicate at each dose. Percent colony formation determined by comparing colonies in TNFα treated to the untreated control within a respective experimental group (scr or SIMPL shRNA). p-values were calculated from Student's t test, and are based on a comparison between scr and SIMPL shRNA expressing cells.

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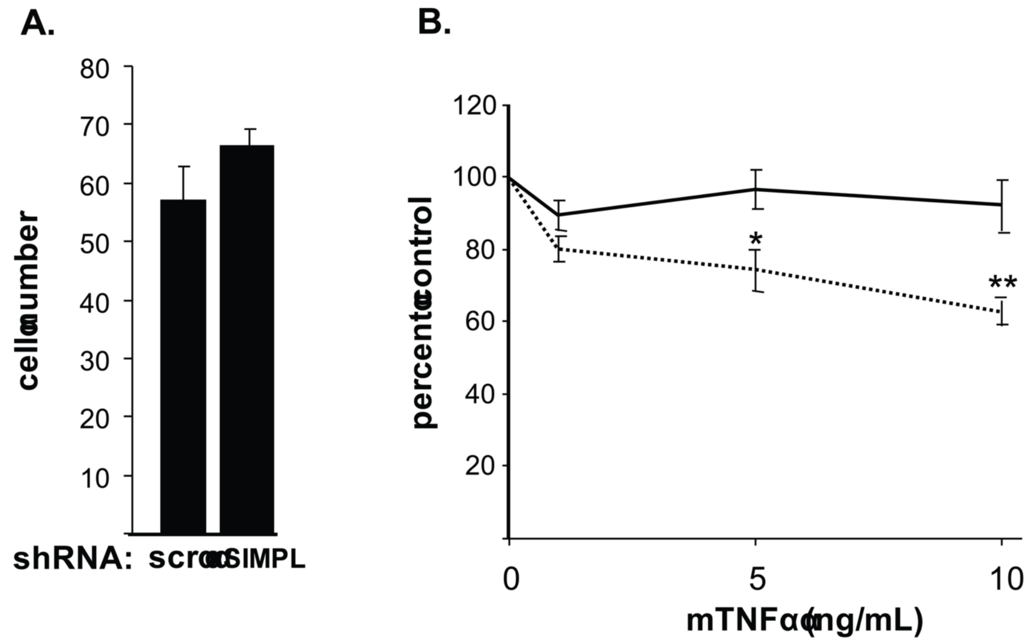

Figure 4.

Diminished CFU-GM formation by the BM of mice reconstituted with c-kit$^+$ SIMPL shRNA cells. LDMN cells were harvested 4 months after C57BL/6 mice (n = 6) were transplanted with c-kit$^+$ scr (indicated by solid lines) or SIMPL shRNA expressing cells (dashed lines). (A) Total numbers of LDMN cells/animal (tibia, femur and pelvis; mean ± SEM). (B) Scrambled or SIMPL shRNA cells (50,000 LDMNCs/mL) were assayed for CFU-GM in methylcellulose containing media supplemented with mGM-CSF, mIL-3, mSCF and increasing concentrations of rmTNFα. The percent colony formation is based on the ratio of the numbers of colonies formed in the presence of rmTNFα to the number of colonies formed in the absence of TNFα colonies within a respective experimental group (scr or SIMPL shRNA) (mean ± SEM; independent experiments were performed in triplicate at each concentration) *p<0.02 **p<0.04; calculated by the Student's t test and are based on a comparison between scr and SIMPL shRNA expressing cells.

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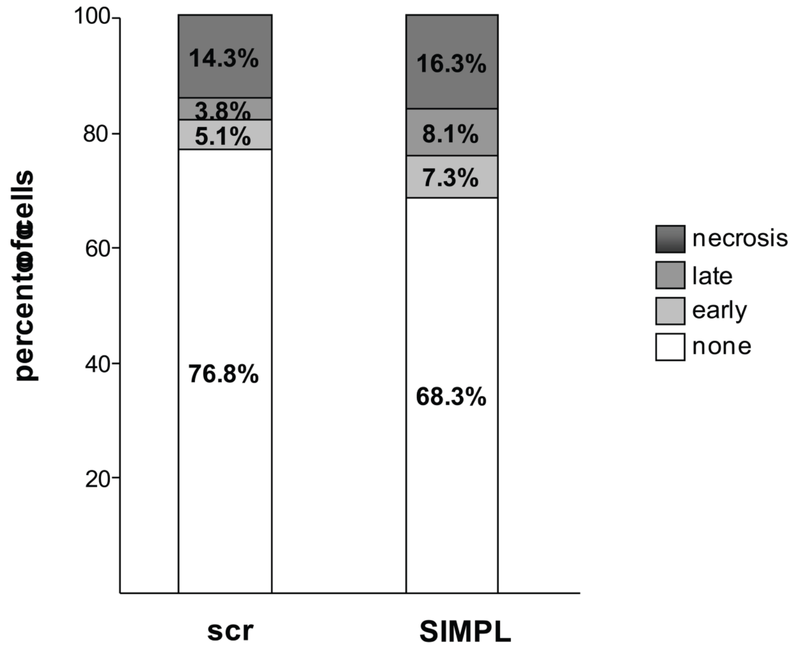

Figure 5.

Comparison of apoptosis from whole bone marrow cells containing either SIMPL or scramble shRNA using Annexin V/PE FACs analysis. c-kit$^+$ cells derived from the lethally irradiated animals transplanted with either the GFP$^+$c-kit$^+$ SIMPL shRNA or scr shRNA cells were cultured for 48 h in liquid media (no methylcellulose) with rmuTNFα (10 ng/ml) and the same complement of growth factors used to assay CFU-GM. Cells were harvested and were analyzed by FACScan for Annexin V staining.

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Table 1

Bone marrow cellularity of mice transplanted with c-kit$^+$ expressing scr or shRNA to SIMPL.

	Scrambled		SIMPL	
Fraction	**% of BM**	**SEM**	**% of BM**	**SEM**
Lin$^-$ Only	3.660	0.651	5.410	1.979
Lin$^-$Sca$^+$	0.468	0.060	0.422	0.109
Lin$^-$c-kit$^+$	0.260	0.054	0.390	0.087
Lin$^-$Sca$^+$c-kit$^+$	0.203	0.134	0.047	0.010
Lin$^-$ Total	4.592	0.751	6.268	1.980

	Scrambled		SIMPL	
Fraction	**% of BM**	**SEM**	**% of BM**	**SEM**
MAC-1$^+$	1.878	0.129	1.757	0.131
GR-1$^+$	14.648	1.542	13.107	1.904
MAC-1$^+$/GR-1$^+$	33.437	1.959	26.022	4.727