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ORIGINAL RESEARCH REPORT

Mesenchymal Stromal Cells Protect Against Caspase 3-Mediated Apoptosis of CD19$^+$ Peripheral B Cells Through Contact-Dependent Upregulation of VEGF

Marc E. Healy, Ronan Bergin, Bernard P. Mahon, and Karen English

The immune suppressive and anti-inflammatory capabilities of bone marrow-derived mesenchymal stromal cells (MSCs) represent an innovative new tool in regenerative medicine and immune regulation. The potent immune suppressive ability of MSC over T cells, dendritic cells, and natural killer cells has been extensively characterized, however, the effect of MSC on B cell function has not yet been clarified. In this study, the direct effect of MSC on peripheral blood B cell function is defined and the mechanism utilized by MSC in enhancing B cell survival in vitro identified. Human MSC supported the activation, proliferation, and survival of purified CD19$^+$ B cells through a cell contact-dependent mechanism. These effects were not mediated through B cell activating factor or notch signaling. However, cell contact between MSC and B cells resulted in increased production of vascular endothelial growth factor (VEGF) by MSC facilitating AKT phosphorylation within the B cell and inhibiting caspase 3-mediated apoptosis. Blocking studies demonstrated that this cell contact-dependent effect was not dependent on signaling through CXCR4-CXCL12 or through the epidermal growth factor receptor (EGFR). These results suggest that direct cell contact between MSC and B cells supports B cell viability and function, suggesting that MSC may not represent a suitable therapy for B cell-mediated disease.

Introduction

Mesenchymal Stem or Stromal Cells (MSCs) are a heterogeneous cell population first described in the bone marrow but subsequently identified in almost every tissue [1,2]. MSC form plastic-adherent colonies in vitro and are capable of osteocyte, adipocyte, or chondrocyte differentiation [3]. However, the conceptual focus of using MSC in regenerative medicine has shifted with an appreciation of the wide range of MSC secreted trophic factors capable of promoting tissue repair and potent immunemodulation [1]. The ability of allogeneic MSC to suppress T cell proliferation [4,5], dendritic cell (DC) [6–8] and natural killer (NK) cell function [9,10] has now been extensively characterized. In contrast to the detailed understanding of how MSC modulate T, DC, and NK cell function, the effect of MSC on B cells is poorly characterized and published studies present conflicting results [11–18].

B cells develop from hematopoietic progenitor cells in the fetal liver and postnatal bone marrow [19] before migrating to the lymph nodes where the recognition of foreign antigen signals their development. B cell maturation is a complex and tightly controlled process [20], however, dysregulation in B cell development can result in the production of self-recognizing antibodies and subsequent autoimmune diseases.

The effect of human MSC on the B cell immune response has been analyzed over the last number of years; however, the effect of MSC on B cell biology in vitro is poorly characterized and studies into MSC modulation of B cell biology have presented conflicting results [13–18]. While the majority of the published data suggest that MSC inhibit B cell function [13,14,18], other publications have demonstrated a supportive role for MSC in B cell expansion and differentiation [15,16,21]. The mechanism by which MSC either support or inhibit B cell function has yet to be identified; however, a requirement for cell contact has been suggested [15,16,21].

Recent studies have demonstrated that MSC are capable of either inhibiting or supporting B cell proliferation and antibody production depending on the presence of T cells in the coculture [21,22]. MSC inhibit the proliferation and differentiation of stimulated B cells in whole peripheral blood lymphocyte populations or in coculture with CD4$^+$ T cells, but support proliferation and antibody production when cultured with pure B cell populations [21,22].

The immunemodulatory ability of MSC offers considerable possibilities for regenerative medicine and a potential treatment for a variety of immune disorders. However, to further the development of MSC toward clinical application it is essential to clarify exactly how MSC interact with all cells of the immune response. In this study, the effect of MSC on B

Institute of Immunology, Maynooth University, National University of Ireland Maynooth, Maynooth, Ireland.

cell function is defined and the mechanism utilized by MSC in enhancing B cell survival in vitro identified.

Materials and Methods

Human MSC isolation and culture

The research was approved by the Institutional Review Boards and Biological Ethics Committee of the National University of Ireland, Maynooth. All human participants were provided written informed consent. Human bone marrow MSC were generated as previously described [23] in collaboration with REMEDI at the National University of Ireland Galway. Human MSC conformed to criteria established by ISCT and were capable of differentiation to adipocyte, chondrocytem, and osteocyte lineages. All experiments were conducted using MSC between passages 4–7 ($\sim$10–21 population doublings). Human MSC were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (BioSera), 200 U/mL penicillin, and 200 μg/mL streptomycin (Sigma-Aldrich).

Isolation of PBMC and separation of CD19$^+$ B cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy packs, generously provided by the Irish Blood Transfusion Service. PBMC were isolated by Ficoll-density centrifugation and peripheral B cells were isolated using CD19$^+$ positive selection MACS beads (Miltenyi Biotec). The purity of peripheral CD19$^+$ B cell isolations was verified using flow cytometry for human CD20.

Cell cultures

For each culture condition 9×10^5 B cells were cultured in tissue culture grade six-well plates preseeded with or without 1.8×10^5 MSC in Iscove's modified Dulbecco's medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (BioSera), 1% penicillin-streptomycin (Sigma-Aldrich), 1% L-Glutamine (Sigma-Aldrich), and 0.01% 2-βmercapthoethanol (Life Sciences). For studies requiring B cell activation and proliferation, B cells were activated using a cytokine cocktail of 25 ng/mL recombinant interleukin-10 (IL-10) (Peprotech), 100 U/mL recombinant IL-2 (Peprotech), 100 ng/mL recombinant IL-21 (Peprotech), insulin-transferrin-selenium (1:1,000) (Gibco), 250 ng/mL recombinant CD40L (Peprotech), and 2.5 μg/mL CpG-ODN (Invivogen).

For experiments investigating B cell activation, purified CD19$^+$ B cells were stained with CD19 and CD69. For experiments examining the effect of MSC on B cell proliferation, purified CD19$^+$ B cells were stained with 10 μM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen). After 5 days, B cell proliferation was determined using an Accuri C6 flow cytometer (BD biosciences). For experiments investigating B cell viability, dual staining with FITC-conjugated Annexin V and Propidium Iodide (PI) was performed using a commercially available kit (eBioscience) to visualize apoptotic B cell populations on an Accuri C6 flow cytometer.

Antibody and cytokine production

Quantitative analysis of IgG and IgM production by CD19$^+$ B cells was performed by ELISA (eBioscience) according to manufacturer's instructions. Vascular endothelial growth factor (VEGF) production by MSC was analyzed by ELISA (Peprotech) according to manufacturer's instructions. VEGF production was also examined by intracellular cytokine staining.

Following B cell and MSC coculture in the presence of the activation cocktail, 1× brefeldin A (eBioscience) was added for the last 4 h of the 48 h culture. Cells were surface stained with CD45, CD19, and CD73. Cells were then incubated in fixation/permeabilization buffer according to manufacturer's instructions (eBioscience), followed by incubation in permeabilization buffer (eBioscience), blocking with 2% rat serum (Sigma-Aldrich) and incubation with mouse anti-human VEGF or matched isotype control (R&D Systems). MSC were gated on CD73$^+$CD45$^-$ populations and B cells were gated on CD45$^+$CD19$^+$ populations and expression of intracellular VEGF analyzed using an Accuri C6 flow cytometer.

Cell contact-dependent assays

Assays investigating cell contact dependence were performed using transmembrane inserts with 0.4 μm pores (Greneir Bio-One). 9×10^5 CD19$^+$ B cells were seeded in the upper chamber of six-transwell tissue culture plates. About1.8×10^5 MSC were seeded in the lower chamber. Cells were cultured as previously described and cell proliferation and viability were analyzed using an Acurri C6 flow cytometer.

In vitro neutralization experiments

In vitro neutralization assays were carried out to determine whether Notch, BAFF, CXCR4-CXCL12, or epidermal growth factor receptor (EGFR) signaling were required for MSC support of peripheral B cell proliferation and survival. Inhibition of Notch signalling was established by adding increasing concentrations (1–10 μM) of GSI XII (Merck Millipore) to B cell:MSC cocultures. BAFF inhibition was achieved using specific neutralizing antibody (R&D Systems). CXCR4 signaling was blocked via the addition of AMD3100 (Sigma-Aldrich) to B cell:MSC coculture experiments and EGFR stimulation was inhibited using specific blocking antibodies (Tocris Bioscience). Inhibition of VEGF signaling on peripheral B cells was achieved by adding vascular endothelial growth factor receptor (VEGFR) (R&D) to B cell:MSC cocultures.

Western blot analysis

Western blot analysis was performed to analyze the expression BAFF by human MSC and the expression of intracellular proteins by B cells following MSC coculture. B cells were carefully removed from coculture by gentle aspiration. Samples were centrifuged at 300*g* for 5 min and pellet resuspended in lysis buffer [50 mM HEPS, 1 mM EDTA, 10% Glycerol, 0.05% CHAPS, 0.5% Triton X, 250 mM NaCl, 1 mM NaVO3, 1 mM PMSF, and complete protease inhibitor ''cocktail'' (Roche)] and incubated on ice for 30 min. Samples were centrifuged at 300 *g* for 5 min at 4°C and cell lysate was removed and stored at −20°C until required for use. Cell lysates were probed with anti-human antibodies for BAFF (R&D Systems), phosphorylated AKT, cleaved Caspase 3 (both Cell Signalling) with β-actin (Cell Signalling) used as a control.

Real-time PCR analysis

Real-time polymerase chain reaction (PCR) analysis was performed to determine the mRNA expression levels of *BAFF* in resting and stimulated MSC. TriReagent (Life Technologies) was used to isolate total RNA and samples were reverse transcribed to cDNA using Tetro cDNA synthesis kit (Bio-Line). Real-time PCR analysis was performed using SYBR Green reagent (Sigma-Aldrich) and specific primers were utilized to determine gene expression. The following PCR predesigned primers (Sigma-Aldrich) were used to determine *BAFF* expression: 5′–3′ AATTTAACAGACAGCCACAG and 3′–5′ TGTCCTTCCTCCAAGATAAG. *GAPDH* expression: 5′–3′ ACAGTTGCCATGTAGACC and 3′–5′ TTTTT GGTTGAGCACAGG was used as housekeeping gene.

Results

MSCs support the activation and significantly enhance proliferation of CD19$^+$ peripheral B cells

B cell activation following antigen recognition or appropriate CD4$^+$ T cell signaling, triggers B cell clonal expansion, isotype switching, and the development of antibody-producing plasma cells and memory B cells [24]; however, previous studies investigating the ability of MSC to modulate B cell activation, proliferation, and antibody production have provided contradictory results [13–18,22]. This study was designed to determine how MSC directly affect purified CD19$^+$ peripheral B cell biology.

To determine the effect of MSC on the activation of B cells, CD19$^+$ peripheral B cells were cocultured with MSC (5:1 B cell:MSC) for 48 h in the presence of human IL-10, IL-2, IL-21, and activating molecules CD40L and CpG. The upregulation of the early lymphocyte activation marker CD69 was analyzed by flow cytometry. Stimulation of CD19$^+$ B cells significantly increased CD69 expression on B cells as expected. However, expression of CD69 was further enhanced when CD19$^+$ B cells were activated in the presence of MSC (Fig. 1A). Notably, CD69 expression was not enhanced on CD19$^+$ B cells cultured in the presence of MSC in the absence of B cell stimulation cocktail suggesting that MSC supported but did not induce the activation of B cells (Fig. 1A).

The proliferation of activated CD19$^+$ B cells was determined using CFSE analysis. Following activation in the presence of MSC, there were significantly less nonproliferating (0 divisions) B cells and significantly more B cells, which had gone through at least five divisions compared to B cells activated without MSC (Fig. 1B). The dependence of cell contact in MSC support of B cell proliferation was examined using a transwell coculture system, separating B cells from the MSC. Inhibition of cell contact completely abrogated MSC support of B cell proliferation demonstrating the requirement for a cell contact-dependant mechanism (Fig. 1C). IgG and IgM production by B cells during MSC cocultures was analyzed after 72 h by ELISA. In contrast to results observed for B cell proliferation and activation, total levels of IgG and IgM were not affected by MSC coculture (Fig. 1D, E).

MSCs protect CD19$^+$ peripheral B cells from apoptosis through a contact-dependent mechanism

A role for MSC in maintaining the hematopoietic stem cell niche has previously been identified [25]. MSC are capable of supporting the survival of immune cells through contact-dependent and soluble mechanisms and the ability for MSC to support B cell viability in vitro may explain the enhanced activation and proliferation of B cells after coculture with MSC (Fig. 1A, B).

To investigate the effect of MSC on the survival of CD19$^+$ peripheral B cells, B cells were isolated from PBMC and cultured in the presence or absence of MSC (5:1 B cell:MSC ratio) for 72 h. In the absence of MSC, the viability of B cells was very low with $\sim$20% of cells negative for apoptosis markers Annexin V/PI, as analyzed by flow cytometry (Fig. 2A). However, B cells cultured in the presence of MSC demonstrated significantly elevated viability illustrated by the significant increase in the percentage and number of Annexin V$^-$ PI$^-$ B cells (Fig. 2). Preventing cell contact abrogated the protection of CD19$^+$ B cell survival by MSC and the percentage and cell number of Annexin V$^-$ PI$^-$ B cells was restored to a similar level of B cells cultured alone (Fig. 2).

MSC support of B cell survival is not dependent upon BAFF or Notch signaling

In addition to the data presented in the previous section other studies examining the effect of MSC on B cell biology have also demonstrated the requirement of a cell contact signal [16], however, this contact signal has not yet been elucidated. B cell activating factor (BAFF) is a member of the tumor necrosis factor (TNF) superfamily of receptors, which functions both as a surface bound protein and soluble factor, and is known to bind TNSFR13 on B cells and support proliferation, activation, and survival [26].

The expression of BAFF by human adipose-derived MSC has previously been shown [27]. Therefore, to determine the importance of BAFF in mediating the support of B cell survival and proliferation by MSC, the ability of bone marrow-derived MSC to produce *BAFF* was probed by real-time PCR and verified by immunoblotting. MSC constitutively express low levels of *BAFF* but stimulation of MSC with proinflammatory cytokines interferon gamma (IFN-γ) or TNF-α clearly indicate that *BAFF* expression is inducible on MSC (Fig. 3A, B). To determine whether BAFF-BAFFR was the signal used by MSC to promote B cell proliferation, BAFF signaling was neutralized during B cell:MSC coculture (Fig. 3C). However, inhibition of BAFF signaling did not prevent MSC from enhancing B cell proliferation (Fig. 3C).

Another important cell contact-dependent signal known to play a key role in B cell survival is notch. Notch signaling is essential for interactions between immune cells and their environment [28] and is critical during peripheral B cell development [29]. Notch signaling is known to upregulate the antiapoptotic Bcl-2 pathway and support B cell survival [30]. Over the last number of years, the importance of the notch signalling pathway in immune modulation by MSC has been clearly identified. The roles for notch signaling in MSC modulation of DC maturation and antigen presentation [31] and in the induction of regulatory T cells [32,33] have now been established. Therefore, it was hypothesized that notch signaling between MSC and CD19$^+$ B cells increased peripheral B cell viability.

Notch signaling was inhibited during B cell:MSC cocultures using the specific notch signaling inhibitor GSI XII.

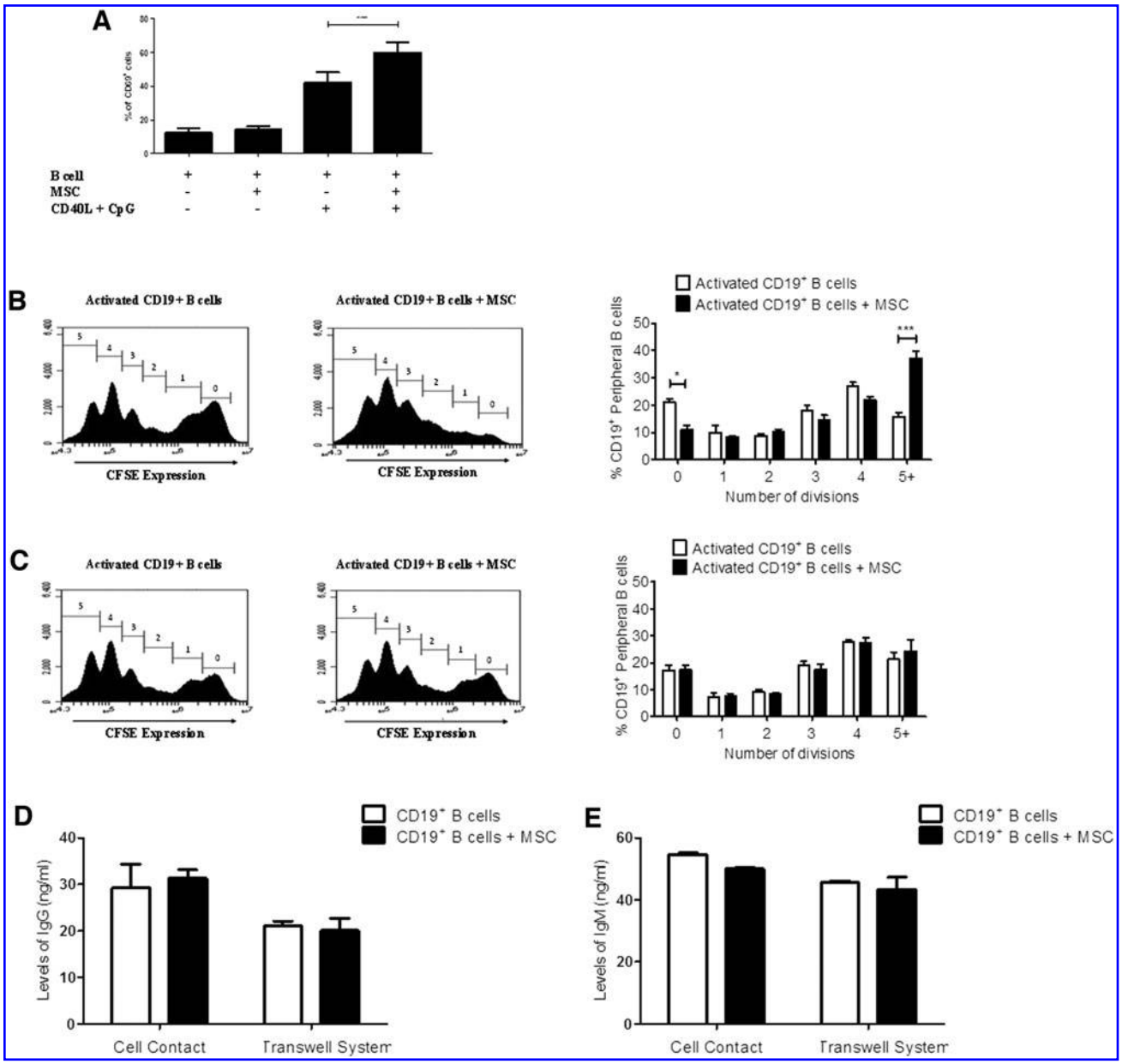

FIG. 1. Mesenchymal stem or stromal cell (MSC) supported the activation and enhanced the expansion of $CD19^+$ peripheral B cells. $CD19^+$ B cells isolated from peripheral blood mononuclear cell (PBMC) using positive selection $CD19^+$ MACS beads were cultured with or without MSC and analyzed for the expression of early lymphocyte activation marker CD69 after 48 h. The expression of CD69 was also analyzed on $CD19^+$ B cells, which were activated with CD40L and CpG in the presence or absence of MSC **(A)**. $CD19^+$ peripheral B cells were stained with carboxyfluorescein succinimidyl ester (CFSE) and activated with CD40L and CpG in the presence of interleukin-10 (IL-10), IL-2, and IL-21, with or without MSC for 120 h. Proliferation of $CD19^+$ B cells was then analyzed by flow cytometry **(B)**. Transwell membrane inserts (0.4 μm pore size) were used to prevent cell contact between MSC and CFSE-labeled $CD19^+$ B cells during coculture before the proliferation of B cells was analyzed by flow cytometry **(C)**. IgG and IgM production was analyzed from supernatant isolated after 72 h coculture by ELISA **(D, E)**. $n = 5$ PBMC donors with 2 MSC donors for A and 8 PBMC donors with 2 MSC donors for **(B, C)**. $n = 4$ PBMC donors with 2 MSC donors for **(D, E)**. Student's *t*-test was used to determine significance where $* < 0.05$, $*** < 0.001$.

The presence of GSI XII during MSC:B cell cocultures had no effect on the viability of peripheral B cells, suggesting that the ability of MSC to support the survival of $CD19^+$ B cells was independent of the notch signaling pathway (Supplementary Fig. S1A, B; Supplementary Data are available online at www.liebertpub.com/scd).

Cell contact-dependent production of VEGF by MSC promotes B cell survival

VEGF was originally identified as a proangiogenic factor [34] and its production by a number of stromal cells including MSC has since been reported [35]; however, one of the most

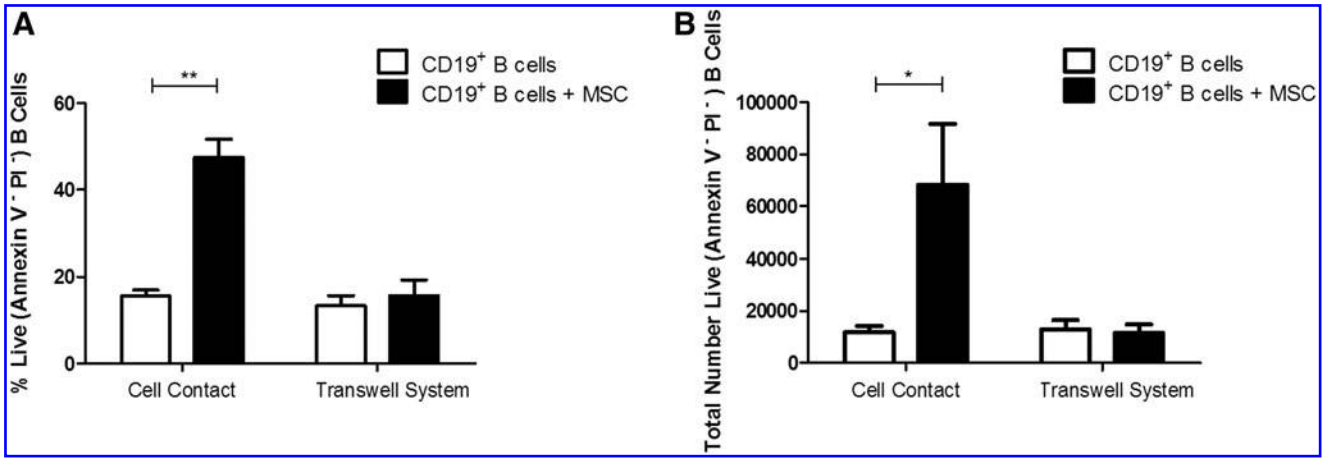

FIG. 2. MSC significantly increased B cell survival in a contact-dependent manner. $CD19^+$ peripheral B cells were cultured with or without transwell inserts in the presence or absence of MSC for 72 h. B cell viability was determined by analyzing the percentage **(A)** and total cell number **(B)** of Annexin V^- PI^- B cells after coculture with MSC. Viable B cells were distinguished from MSC by gating on $CD45^+$ cells before Annexin V and Propidium Iodide (PI) analysis. Total cell numbers were generated using counting beads. $n = 3$ PBMC donors with 3 MSC donors. Statistical significance was determined using paired Student's *t*-test where $* < 0.05$, $** < 0.005$.

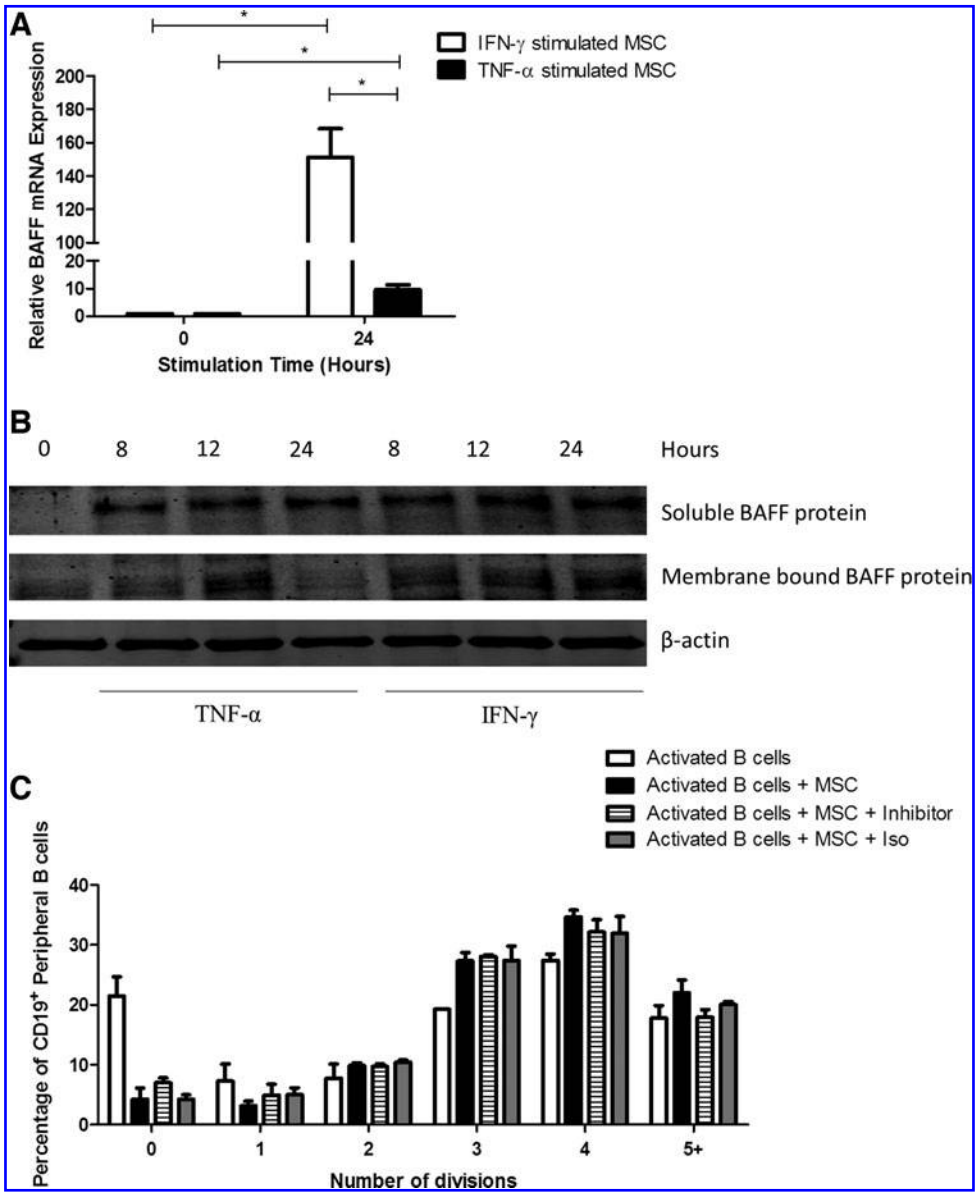

FIG. 3. BAFF signaling was not required for MSC support of B cell proliferation or survival. The expression of total BAFF mRNA **(A)** and both membrane bound and soluble BAFF protein **(B)** expression by MSC stimulated with 50 ng/mL interferon gamma (IFN-γ) or 20 ng/mL tumor necrosis factor alpha (TNF-α) for 8, 12, or 24 h was determined by western blot and real-time polymerase chain reaction, respectively. $CD19^+$ peripheral B cells were isolated, labeled with CFSE, and cultured with or without MSC and in the presence or absence of 50 μg/mL BAFF inhibitor for 120 h. B cell proliferation was measured by flow cytometry **(C)**. $n = 3$ for **(A)** and **(B)** and $n = 4$ for **(C)**. Statistical significance was determined using paired Student's *t*-test. $* < 0.05$.

potent functions of VEGF is in antiapoptotic signaling [36]. The antiapoptotic effect of VEGF has previously been demonstrated by Spyridopoulos et al. They demonstrated that recombinant VEGF was capable of protecting up to 90% of epithelial cells from TNF-α-induced apoptosis in vitro. Therefore, the potential role for MSC-produced VEGF in protecting B cells from apoptosis was examined.

VEGF production by B cells cultured alone was very low at 24, 48, and 72 h (Fig. 4A–C) while MSC cultured alone produced 1 ng/mL VEGF at 24, 48, and 72 h. However, when MSC were cultured in the presence of B cells VEGF production was significantly increased after 48 and 72 h (Fig. 4B, C). Interestingly, the increase in VEGF production was not present in samples where B cells were separated from MSC by transwell membranes (Fig. 4B, C). To further clarify the source of VEGF production following MSC and B cell cocultures in the presence of the activation cocktail, intracellular cytokine staining for VEGF was carried out on the coculture after 48 h. While only a small proportion of B cells produced low levels of VEGF, MSC produced significantly higher levels of VEGF (Fig. 4D) demonstrating that MSC were the major source of VEGF produced in the coculture.

To determine the significance of the increased VEGF production on the survival of B cells, a VEGF inhibitor (SU5416) was added to B cell:MSC cocultures for 72 h before B cell viability was determined by Annexin V/PI staining. Inhibition of VEGF signaling during direct B cell:MSC coculture completely abrogated the promotion of B cell survival by MSC (Fig. 5A, B). Inhibiting VEGF signaling resulted in a significant reduction in the percentage and number of Annexin V and PI-negative B cells after MSC coculture, restoring them to similar levels as observed when B cells were cultured alone (Fig. 5A, B). These results demonstrate that cell contact-dependent upregulation of VEGF by MSC induced B cell survival.

VEGF production by MSC increases AKT phosphorylation and prevents caspase 3 activation

Following the demonstration of the importance of MSC-derived VEGF, this study sought to investigate the signaling cascade involved in promoting B cell survival. VEGF signaling has previously been shown to induce cell survival through the phosphorylation of AKT [37]. Phoshphorylation of AKT

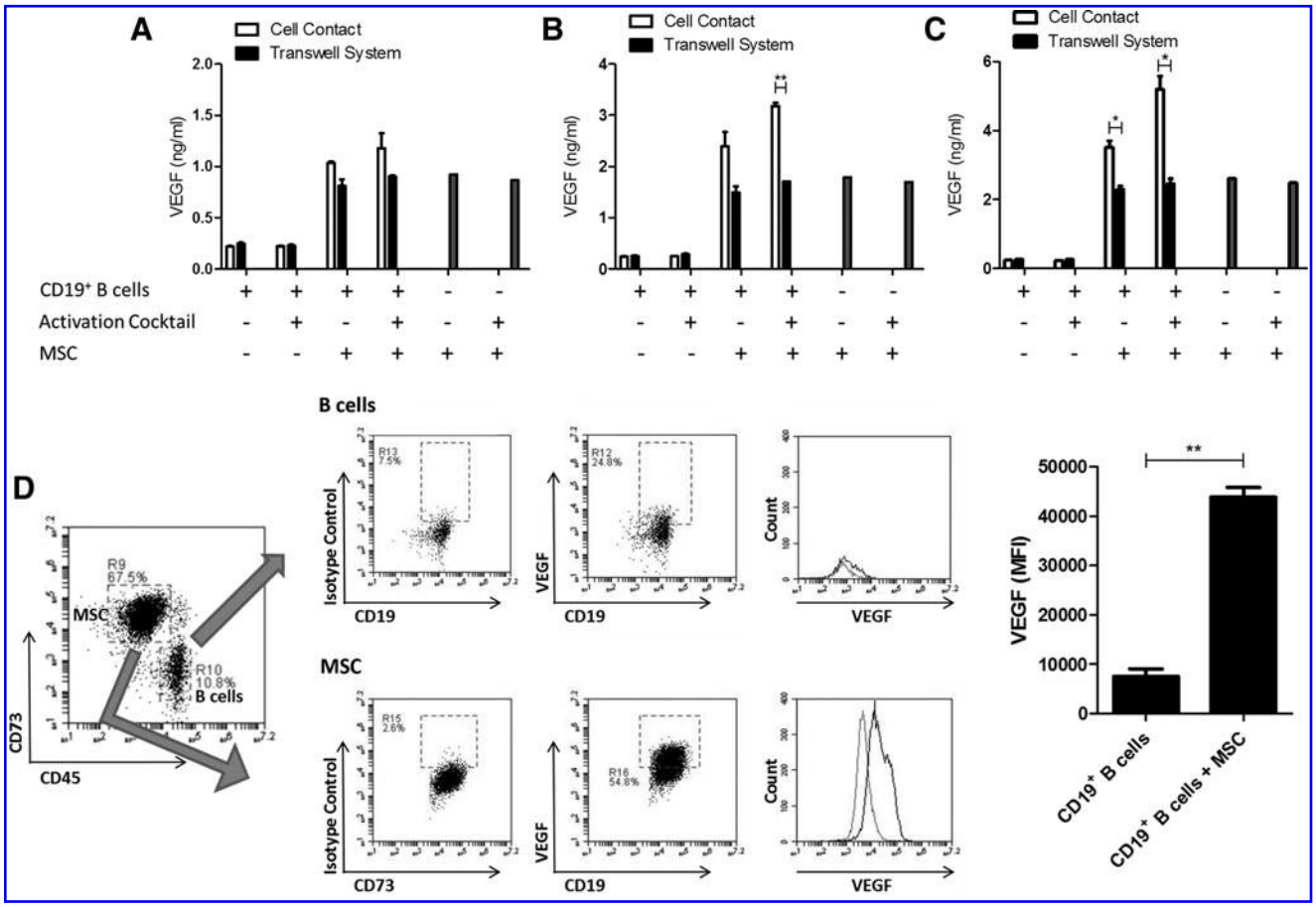

FIG. 4. MSC secrete significantly higher levels of vascular endothelial growth factor (VEGF) following coculture with B cells. $CD19^+$ peripheral B cells were cultured with or without transwell inserts in the presence or absence of MSC for 72 h. Supernatant was removed after 24 **(A)**, 48 **(B)**, and 72 **(C)** h and analyzed for VEGF production by ELISA in triplicate. The source of the VEGF production following MSC and $CD19^+$ B cell cocultures in the presence of the activation cocktail was examined by intracellular cytokine staining for VEGF or the appropriate isotype control by flow cytometry. The MSC population was gated on $CD45^-CD73^+$ cells and the B cell population was gated on $CD45^+CD19^+$ cells. The bar chart represents VEGF mean fluorescence intensity (MFI). Statistical significance was determined using either two-way ANOVA **(A–C)** or a paired Student's *t*-test **(D)**. $*<0.05$, $**<0.005$. **(A–C)** $n=4$, **(D)** $n=3$.

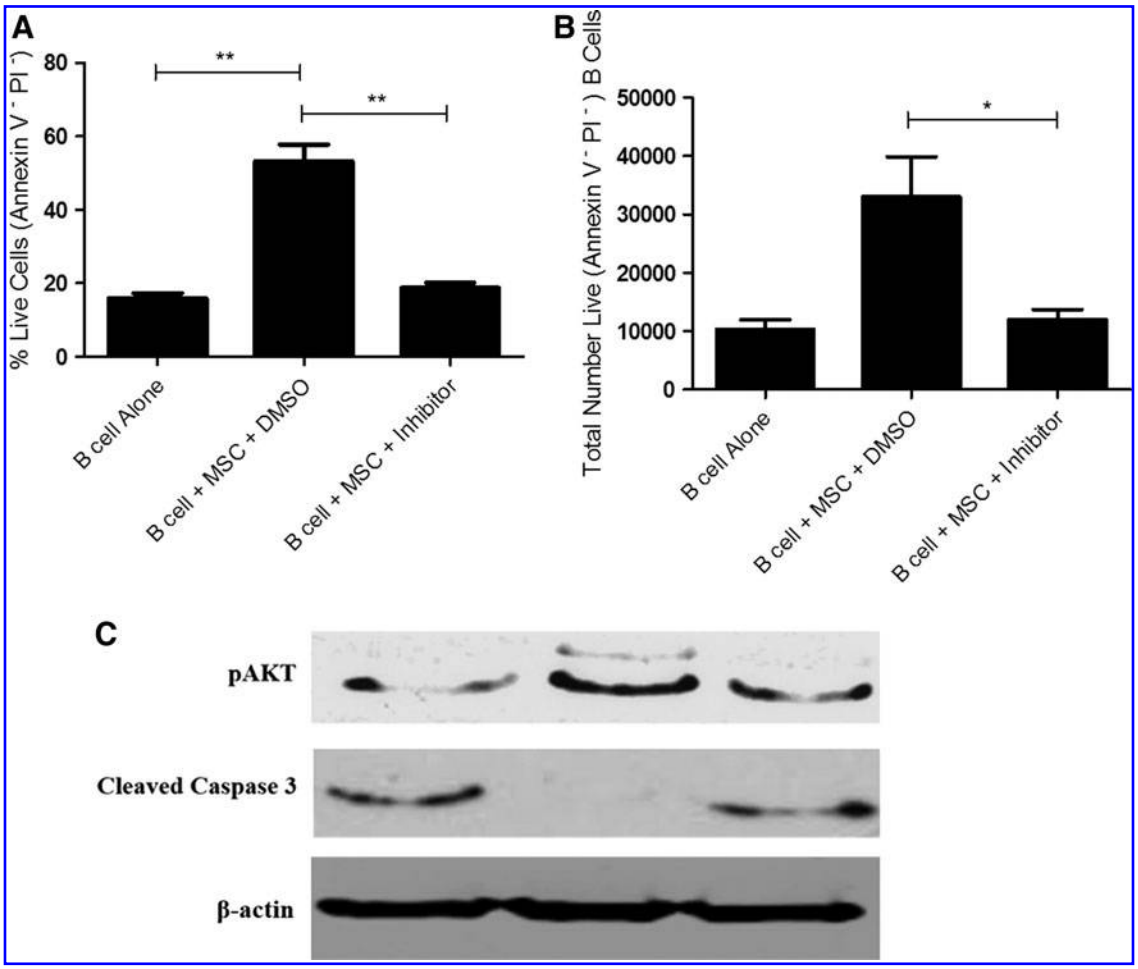

FIG. 5. VEGF production by MSC is essential for promoting B cell survival. VEGF inhibitor (SU5416) was added to $CD19^+$ B cell and MSC cocultures for 72 h. B cell survival was determined by analyzing the percentage **(A)** and total cell number **(B)** of Annexin V^- PI^- B cells after coculture with MSC. Total cell numbers were determined using counting beads. Viable B cells were distinguished from MSC by gating on $CD45^+$ cells. $CD19^+$ B cells were recovered from coculture and analyzed for pAKT, AKT, and active Caspase 3 expression by western blot **(C)**. **(A–C)** Two PBMC donors and two MSC donors. VEGF inhibitor was reconstituted in DMSO and so DMSO was added to B cell:MSC coculture as a control. **(A, B)** Four PBMC donors and two MSC donors. **(C)** Representative image of two separate experiments using four PBMC donors and four MSC donors. Statistical significance was determined using a paired Student's *t*-test where $* < 0.05$, $** < 0.005$.

(pAKT) is known to inhibit the activation of the caspase cascade preventing the expression of cleaved (active) caspase 3 [38].

Therefore, the expression of pAKT and cleaved caspase 3 were analyzed in B cells after 72 h coculture with or without MSC by western blot. The role of VEGF signaling in pAKT and cleaved caspase 3 expression was also determined using VEGF inhibitor. B cells cultured in the presence of MSC had a marked increase in pAKT expression and displayed little cleaved caspase 3 (Fig. 5C). B cells that were cultured alone expressed low levels of pAKT but strong levels of cleaved caspase 3 (Fig. 5C). The inhibition of VEGF signaling during cocultures with MSC clearly reduced the level of pAKT expression and restored levels of cleaved caspase 3 to that of B cells cultured alone (Fig. 5C). These data suggest that the increased VEGF production by MSC during coculture with B cells induces the phosphorylation of AKT and inhibits the caspase cascade and subsequent cleaved caspase 3 expression resulting in reduced apoptosis.

MSC enhancement of B cell survival is not dependent on CXCR4-CXCL12 signaling or EGFR stimulation

As described above, cell contact between MSC and B cells results in significantly increased VEGF production and inhibition of VEGF signaling prevents MSC support of B cell survival and restores caspase 3 expression (Fig. 5). To elucidate the cell contact signal responsible for the increased VEGF production and subsequent support of B cell survival by MSC, CXCR4-CXCL12 signaling was identified as a possible candidate. CXCR4 is constitutively expressed on peripheral B cells [39] and binding of CXCR4 to its ligand CXCL12 has previously been shown to induce VEGF production [40]. The expression of CXCL12 on MSC has previously been established and therefore, a competitive inhibitor to CXCR4 (AMD3100) was used to inhibit CXCR4-CXCL12 signaling during B cell:MSC cocultures. $CD19^+$ B

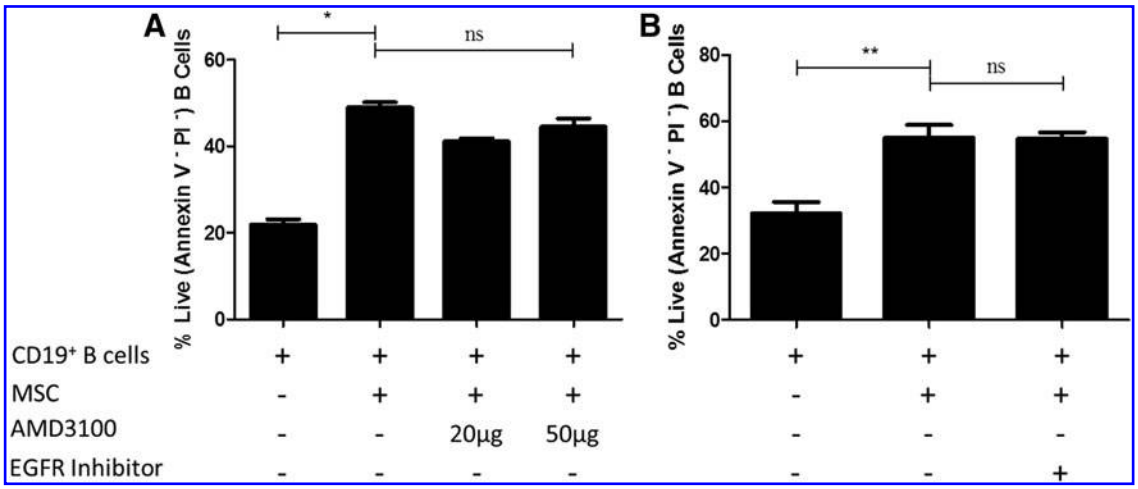

FIG. 6. CXCR4-CXCL12 contact or epidermal growth factor receptor (EGFR) stimulation is not essential for VEGF-mediated support of B cell survival by MSC. $CD19^+$ peripheral B cells were isolated from PBMC using positive selection MACS beads and cultured in the presence or absence of MSC with or without 20 or 50 µg/mL CXCR4 inhibitor (AMD3100) (**A**). $CD19^+$ B cells were cultured in the presence or absence of MSC with or without 10 nM EGFR inhibitor (**B**). B cell viability was determined by analyzing the percentage Annexin V^- PI^- B cells after coculture with MSC for 72 h. Viable B cells were distinguished from MSC by gating on $CD45^+$ cells before Annexin V and PI analysis. $n = 2$ PBMC donors with 2 MSC donors. Statistical significance was determined using paired Student's *t*-test where * < 0.05, ** < 0.005.

cells were isolated from PBMC and cultured in the presence or absence of MSC (5:1) with or without AMD3100 for 72 h. B cell viability was determined using Annexin V and PI viability staining and analyzed by flow cytometry.

Inhibition of CXCR4 did not prevent MSC support of B cell survival (Fig. 6A) suggesting that the B cell–MSC interaction is not dependent on CXCR4-CXCL12 signaling. The production of proangiogenic factors by MSC, including VEGF, is mediated by the EGFR [41]. Stimulation of EGFR is capable of significantly increasing the production VEGF by bone marrow-derived human MSC [41]. To investigate whether B cell–MSC interactions were mediated through EGFR stimulation, a neutralizing antibody to EGFR was added to the B cell:MSC (5:1) cocultures. Neutralization of the EGFR resulted in no detectable differences in B cell survival after coculture with MSC (Fig. 6B), suggesting that B cell stimulation of the EGFR is not essential for the increase in VEGF production and the subsequent enhancement of B cell survival by MSC.

Discussion

Bone marrow-derived MSC are currently being investigated as potential new therapies against a wide range of immune disorders and their ability to suppress T cell [4,5], DC [6–8], and NK cell function [9,10] has been well characterized in vitro and in vivo. However, the direct effect of MSC on B cell biology remains controversial [18]. Variability among experimental protocols is proposed as one of the main factors leading to these conflicting findings [22]. In addition, the mechanism by which MSC support or suppress B cell biology has not yet been established. In this study, we sought to determine how MSC affect B cell biology in terms of activation, proliferation, and survival and to elucidate the mechanisms involved in a well-defined system. MSC support the activation, proliferation, and survival of $CD19^+$ B cells through the cell contact-dependent upregulation of VEGF and subsequent inhibition of caspase 3-mediated apoptosis.

Clinical trials have demonstrated the beneficial potential of MSC as a novel cell therapy, particularly against GvHD, Crohn's disease, and myocardial infarction [42–44]. Despite these recent advances, there are outstanding questions that need to be addressed before the full potential of MSC therapy can be realized. The main unresolved issues include the optimal conditions for large scale manufacturing of MSC, the persistence and biodistribution of MSC in vivo and the widespread clinical use of MSC therapy without a complete understanding of their mechanisms of action. B cells are major regulators of the adaptive immune system and their activation and rapid proliferation in response to antigen recognition is essential to the development of a functional immune response [24]. Accordingly, to develop an effective cell therapy, a complete understanding of how MSC interact with B cells is essential.

Studies on the effect of MSC on peripheral B cell biology in vitro have reported conflicting results [13–16,18]. The majority of the key in vitro published data suggest that MSC inhibit the proliferation and antibody production of adult B cells [13,14,17]; contrary to this, however, a number of published studies suggest that MSC support proliferation and antibody production [15,16].

More recently, two studies have elegantly demonstrated that MSC promote the survival of purified B cells, but MSC inhibition of B cell proliferation and differentiation requires the presence of purified T cells or whole PBMC [21,22]. Here, we demonstrate that MSC enhanced the activation and proliferation of purified $CD19^+$ peripheral B cell populations through a contact-dependent mechanism. This parallels previous observations [16,21], but importantly identifies that enhanced B cell activation was not influenced by allogeneic recognition of MSC.

The clonal expansion of activated B cells is essential for the development of antibody producing effector B cells and generally occurs within secondary lymphoid tissues. It is

now clear that transplanted MSC have the capacity to migrate to lymph nodes [45]. Thus, the administration of MSC in concurrence with B cell activation may lead to enhanced expansion of B cells, which in the case of B cell driven or idiopathic disease may be detrimental to the patient. This premise is consistent with experimental data; the administration of allogeneic MSC in a murine model of lupus resulted in exacerbated disease [46] and is a good example of the potential adverse effects associated with MSC therapy if applied without adequate experimental understanding of the functional mechanisms.

Antibody production by activated B cells is one of the hallmarks of B cell-mediated disease; however, the ability of MSC to support or inhibit antibody production is also a contentious issue. The studies conducted by Traggiai et al. observed increased IgG and IgM when B cells from lupus patients were cultured in the presence of MSC, while others reported increased IgG production by healthy B cells following culture with MSC [15,16,21]. In contrast to these studies, our data showed that MSC had no effect on the IgG or IgM production by $CD19^+$ B cells. These differential findings are probably caused by variances in experimental setup as the ratio of B cell to MSC, activating molecules and the purity of B cell populations all differ between studies.

The results presented here were obtained using a B cell:MSC coculture ratio of 5:1 in an attempt to create a more physiologically relevant situation than ratios of 1:1 or 1:2 and to determine how MSC directly modulate B cell biology using B cell instead of whole PBMC. Another possibility is that the cytokine cocktail used to activate B cells in this experiment may have been sufficient to maximize B cell production of IgG and IgM, making it impossible to detect any increases in the presence of MSC. IL-21 is known to induce plasma cell differentiation and IgG production [47] and is notably absent from the studies that reported increased IgG [15,16].

In vivo, MSC function as stromal cells and are essential for maintaining the hematopoietic stem cell niche within the bone marrow [25,48]. In adults, B cell development occurs within this MSC supported environment [49] and we hypothesized that in vitro B cell:MSC cocultures mimic the HSC niche where MSC support the survival of B cells. Indeed, B cell viability was significantly increased in the presence of MSC and this was mediated through a cell contact-dependent mechanism. This result supports previous work [14,21], but further demonstrates MSC influence on purified peripheral B cell populations and highlights the requirement for cell contact.

In contrast to this study, however, Tabera et al. demonstrated inhibition of B cell proliferation during MSC coculture [14]; however, the difference in results between these two studies likely lies in the activation status of the B cells. Increased B cell survival when exposed to MSC may well offer an explanation to the increased activation and proliferation, as a more viable population of B cells would be more responsive to the activation cytokines. Thus, the B cell:MSC interaction is likely to be further modulated by T cells in vivo; but the major effect of MSC on activated B cells is supportive of expansion when mature T cells are absent. This finding has consequences for cell therapy in terms of disease targets and especially the route and timing of administration.

While a requirement for cell contact has previously been identified for MSC support of B cell proliferation [15,16] no further advances in defining how MSC modulate B cell biology have been reported. Identifying the mechanisms behind MSC modulation of T cell function has provided a platform from which to design effective MSC therapies. Similarly, identifying the mechanism involved in MSC support of B cell survival would further our understanding and provide valuable information for future in vivo studies.

With cell contact dependence already established, potential cell surface interacting proteins between MSC and B cells were investigated. Under normal physiological conditions, BAFF signalling is a potent inducer of B cell expansion and survival [26]. In addition to this, the expression of BAFF on the surface of adipocyte-derived MSC has been described [27]. Although low levels on constitutive BAFF expression were detected on our bone marrow-derived MSC, this expression was significantly upregulated following stimulation with proinflammatory cytokines IFN-γ or TNF-α suggesting that in the presence of the correct signals our MSC could express high levels of BAFF. In line with Franquesa et al. [21], neutralizing BAFF signaling in our system did not prevent MSC from enhancing B cell proliferation, indicating that MSC support of B cell in vitro expansion was not dependent on BAFF.

An alternative cell contact-dependent signaling pathway known to have a role in B cell survival is notch. Notch signaling is a highly conserved immune regulatory pathway with established roles in immune cell development, proliferation, differentiation, and survival [29,50]. MSC inhibition of DC maturation [31] and induction of regulatory T cells [32] required notch signaling. The role of notch signaling during MSC–immune cell interactions and in B cell development highlighted notch as a potential contact signal mediating MSC support of B cell survival. In addition, MSC have previously been shown to support the survival of B acute lymphocyte leukemia (B-all) cells via signaling through notch 3/4 [30]. In this study, however, blocking studies demonstrated that notch signaling was not essential for MSC support of B cell survival. The recent study by Franquesa et al. also ruled out MSC-B cell contact signaling through IL-6, PGE-2, and a proliferation-inducing ligand but failed to identify the mechanism of action involved [18].

To elucidate the cell-contact dependent mechanism behind MSC modulation of B cell biology it was necessary to explore more indirect pathways. MSC are known to modulate immune cells through the secretion of a wide range of immunemodulatory soluble factors [1]. Hence, it was hypothesized that cell contact between MSC and B cells was triggering the production of a soluble antiapoptotic signal by the MSC, which in turn was promoting B cell survival.

VEGF has been described as one of the most important factors controlling angiogenesis [51,52] but a role for VEGF in antiapoptotic signaling has also been established [37]. In fact, the addition of recombinant VEGF to TNF-α-induced apoptotic epithelial cells in vitro was sufficient to induce survival in 80%–90% of cells [53]. VEGF production by MSC has also been identified as a major contributor to MSC support of angiogenesis [35]. Therefore, it was hypothesized that B cell contact stimulated VEGF production by the MSC and that increased levels of VEGF induced antiapoptotic signaling in the B cell in our system.

Interestingly, a significant upregulation in VEGF was detected following MSC contact with B cells. MSC support of B cell survival was completely abrogated when VEGF signaling was blocked. The antiapoptotic signaling pathway associated with VEGF signaling is mediated through the phosphorylation of AKT and results in a reduction in caspase 3 cleavage [37,38]. MSC support of B cell survival mirrored this increase in phosphorylated AKT (pAKT) and dramatically reduced cleaved caspase 3 expression; this expression was completely restored by the addition of the VEGF inhibitor.

Expression of CXCR4 by B cells and CXCL12 by the MSC [54] in addition to studies demonstrating that CXCR4-CXCL12 binding facilitates upregulation of VEGF production [40] identified CXCR4-CXCL12 as a potential contact signal required for MSC production of VEGF. However, inhibiting CXCR4 binding in our system had no effect on the ability of MSC to promote B cell survival.

An interesting alternative contact signal involved stimulation of the EGFR, a member of the tyrosine kinase family of receptors, which play an important role in cell survival and proliferation [55]. Human MSC expression of the EGFR has previously been reported and its activation results in significant upregulation of proangiogenic factors including VEGF [41]. The addition of the EGFR inhibitor to MSC and B cell cocultures did not prevent MSC support of B cell survival, suggesting that the elusive cell contact signal between MSC and B cells was neither CXCR4 nor EGFR.

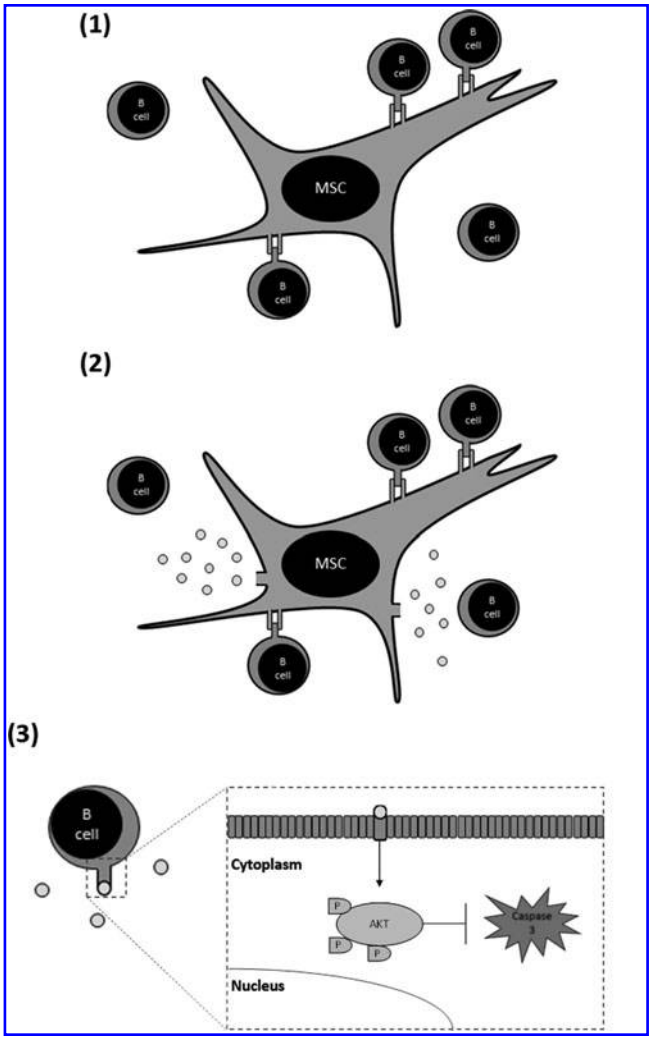

FIG. 7. Model of mechanism of action in MSC promotion of B cell survival. Graphical depiction of proposed mechanism of action behind MSC support of B cell survival. **(1)** In our system, MSC bind to $CD19^+$ peripheral B cells (contact signal yet to be identified). **(2)** Cell contact signal induces a significant upregulation of VEGF production by MSC. **(3)** Soluble VEGF is bound by vascular endothelial growth factor receptor (VEGFR) on $CD19^+$ B cells and induces phosphorylation of AKT. pAKT inhibits the proapoptotic caspase cascade significantly inhibiting caspase 3 cleavage and B cell apoptosis.

Conclusion

In conclusion, our data demonstrate the capacity for MSC to alter B cell biology and presents a step forward in understanding the mechanism behind how MSC support the activation, proliferation, and survival of $CD19^+$ B cells. Although the critical cell contact signal between MSC and B cells remains to be elucidated, the data presented here represent a significant progression in understanding the mechanism by which MSC support the activation, proliferation, and survival of $CD19^+$ peripheral B cells.

This study reveals that cell contact between B cells and MSC induces a significant upregulation of VEGF production by MSC. VEGF induces the upregulation of pAKT and inhibits caspase 3-mediated $CD19^+$ B cell apoptosis (Fig. 7). To further the development of MSC toward clinical application it is essential to clarify exactly how MSC interact with all cells of the immune response and design more tailored approaches to MSC treatment. This study has furthered our understanding of how MSC interact directly with peripheral B cell populations in vitro and has provided key information for future studies into the relationship between MSC and B cells.

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Author Disclosure Statement

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Address correspondence to:
Dr. Karen English
Institute of Immunology
Maynooth University
National University of Ireland Maynooth
Maynooth
County Kildare
Ireland

E-mail: karen.english@nuim.ie

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