Mesenchymal stromal cells protect mantle cell lymphoma cells from spontaneous and drug-induced apoptosis through secretion of B-cell activating factor and activation of the canonical and non-canonical nuclear factor κB pathways

Daniel J. Medina,[1,2] Lauri Goodell,[1,3] John Glod,[1,4] Céline Gélinas,[1,5,7] Arnold B. Rabson,[1,3,4,6,7,8] and Roger K. Strair[1,2]

[1]The Cancer Institute of New Jersey and the [2]Departments of Medicine, Pathology and [3]Laboratory Medicine, [4]Pediatrics, [5]Biochemistry, Molecular Genetics, [6]Microbiology and Immunology, and, [7]Center for Advanced Biotechnology and Medicine, and [8]The Child Health Institute of New Jersey, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, New Brunswick, New Jersey, USA

ABSTRACT

Background

There is increasing evidence that stromal cell interactions are required for the survival and drug resistance of several types of B-cell malignancies. There is relatively little information regarding the role of the bone marrow/lymphoid microenvironment in the pathogenesis of mantle cell lymphoma. In this study we investigated the interaction of primary mantle cell lymphoma cells with stromal cells in an *ex vivo* co-culture system.

Design and Methods

The murine stromal cell line MS-5 and human bone marrow mesenchymal stromal cells were each co-cultured with primary mantle cell lymphoma cells for up to 7 months. Mantle cell lymphoma cultures alone or combined with human stromal cells were analyzed for cell number, cell migration, nuclear factor-κB activation and drug resistance.

Results

Co-culture of mantle cell lymphoma cells and human stromal cells results in the survival and proliferation of primary mantle cell lymphoma cells for at least 7 months compared to mantle cell lymphoma cells cultured alone. Mantle cell lymphoma-human stromal cell interactions resulted in activation of the B-cell activating factor/nuclear factor-κB signaling axis resulting in reduced apoptosis, increased mantle cell lymphoma migration and increased drug resistance.

Conclusions

Direct mantle cell lymphoma-human stromal cell interactions support long-term expansion and increase the drug-resistance of primary mantle cell lymphoma cells. This is due in part to activation of the canonical and non-canonical nuclear factor κB pathways. We also demonstrated the ability of B-cell activating factor to augment CXCL12- and CXCL13-induced cell migration. Collectively, these findings demonstrate that human stromal cell-mantle cell lymphoma interactions play a pivotal role in the pathogenesis of mantle cell lymphoma and that analysis of mantle cell lymphoma-human stromal cell interactions may help in the identification of novel targets for therapeutic use.

Key words: mantle cell lymphoma, mesenchymal cell, drug resistance, BAFF, NF-κB.

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Correspondence:
Daniel J. Medina, Ph.D.,
The Cancer Institute of New Jersey, 195 Little Albany Street, New Brunswick, New Jersey, 08901, USA.
Phone: international + 1.732.2358066.
Fax: international + 1.732.2358098.
E-mail: medinadj@umdnj.edu

The online version of this article has a Supplementary Appendix.

Introduction

Mantle cell lymphoma (MCL) is a distinct subtype of non-Hodgkin's lymphoma derived from naïve CD5$^+$ B cells originating from the mantle zone surrounding reactive germinal centers. MCL accounts for 5% to 8% of adult non-Hodgkin's lymphomas in the United States and Europe. The disease is aggressive, but with newer therapeutic approaches, the median survival of affected patients is 5 to 7 years.[1]

Despite their apparent longevity in patients, MCL cells typically undergo spontaneous apoptosis *in vitro*. This implies that some factors essential for proliferation and survival are not intrinsic to the MCL cell. There is now clear evidence that several types of leukemias and lymphomas, such as acute myelogenous leukemia, chronic lymphocytic leukemia, follicular lymphoma, and multiple myeloma, are dependent on the bone marrow/lymphoid microenvironment for proliferation, survival and drug resistance.[2-5]

Compared to the situation for the hematopoietic malignancies described above, there is relatively little information regarding the role of the bone marrow/lymphoid microenvironment in the pathogenesis of MCL. Several studies using a variety of non-Hodgkin's lymphoma cell lines and some primary MCL cells have demonstrated that lymphoma cell-stromal cell interactions increase cell survival and drug resistance.[6-8] It was recently reported that co-culture of MCL cells (cell lines and primary cells) with human or murine bone marrow stromal cell lines resulted in MCL cell adhesion. Adhesion of the MCL cells to the stromal cells was due in part to the surface expression of CXCR4, CXCR5 and VLA-4.[6,7,9] The increased cell survival was due to elevated expression of the cyclin-dependent kinase inhibitors p21 and p27, which resulted in reversible G1 cell cycle arrest.[7,10] These same groups demonstrated that co-culturing MCL cells with stromal cells resulted in a significant increase in MCL drug resistance, associated with increased expression of B-cell activation factor (BAFF) with concomitant activation of the nuclear factor-κB (NF-κB) survival pathway.[9,11,12]

The above studies provide intriguing evidence that MCL-microenvironment interactions play a critical role in the survival and drug resistance of MCL. One caveat to the above studies is that they were conducted mostly with MCL and stromal cell lines in short-term cultures (12-24 h).

Based on the above studies and the knowledge that tumor-microenvironment interactions contribute significantly to the pathogenesis of other hematologic malignancies, we tested the hypothesis that normal purified bone marrow-derived human mesenchymal stem/stromal cells (hMSC) play a pivotal role in the pathogenesis of MCL and may provide a robust *ex vivo* model for understanding MCL-niche interactions.

Design and Methods

Patients' specimens

Samples from six patients with MCL (samples UPN-1 to UPN-6) were included in this study. The diagnosis of MCL was based on the immunophenotype (CD5$^+$, CD19$^+$, and CD23$^-$) of the malignant cells in conjunction with the expression of cyclin D1 and/or detection of the translocation t(11;14) by cytogenetics or fluorescence *in situ* hybridization analysis. Samples of blood or spleen were obtained under an exemption protocol approved by the University of Medicine and Dentistry of New Jersey Institutional Review Board. MCL patients had active disease at the time of sampling. Because the protocol prohibited collection of any identifying information, the samples collected were only accompanied by the diagnosis, but no information regarding disease stage, treatment history or treatment response.

Mononuclear cells were isolated from 10 to 25 mL of fresh, heparinized blood or spleen biopsy by Histopaque centrifugation. Isolated cells were counted, re-suspended in freezing medium [90% fetal bovine serum and 10% dimethylsulfoxide (Sigma-Aldrich, St Louis, MO, USA)] and stored in liquid nitrogen until use. Cells were further purified by negative selection using a B-cell isolation (B-CLL) kit (#130-093-660, Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions, which resulted in the isolation of untouched (not labeled) MCL cells. The purity of the MCL cells was determined by staining the isolated cells with CD19-PE, CD5-ECD, CD14-FITC and CD3-FITC (Beckman Coulter, Miami, FL, USA), and analyzing them on a Coulter Cytomics FC500 flow cytometer (Beckman Coulter). For comparison, normal CD19$^+$ cord blood B cells were purchased from Lonza (Walkersville, MD, USA). The CD19$^+$CD5$^+$ B-cell subset (CD5$^+$ cord blood B cells, CBBC) was isolated by positive selection by incubating the cells with an anti-human CD5 biotinylated (Miltenyi Biotec) antibody followed by magnetic separation using streptavidin-coated magnetic beads (Miltenyi Biotec). CBBC purity was evaluated as described above for MCL cells. The purity of MCL and CBBC was determined to be at least 93%.

The mouse stromal cell line MS-5 was obtained from Dr. Joseph Bertino (CINJ) and human primary bone-marrow stromal cells (hMSC) were purchased from Lonza (Walkersville, MD, USA). In all experiments MCL and stromal cells were maintained in α-MEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Sigma-Aldrich) and supplemented with 2 mM glutamine, penicillin (100 U/mL) and streptomycin 10 U/mL) (Invitrogen). Fetal bovine serum was screened and selected for optimal *in vitro* culture of human colony-forming units fibroblasts and myeloid colony-forming units. hMSC were used from passages 3 to 6. As we have previously reported,[13] the expanded hMSC population had the capacity to be induced to differentiate into chondrocytes, adipocytes and osteoblasts and were positive for the expression of surface antigens Stro-1-FITC (Invitrogen), PE-conjugated CD90, CD105, CD106, HLA-ABC, CD44 (Beckman Coulter) and negative for CD45, CD11b and HLA-DR (Beckman Coulter) (*data not shown*).

Co-culture of mantle cell lymphoma cells with MS-5 or human mesenchymal stem/stromal cells

MCL cells and CBBC were plated at a density of $4x10^4$ cells/cm^2 on a pre-established confluent layer of irradiated (25 Gy) MS-5 cells or hMSC (MCL-to-stromal cell ratio of 10:1) and maintained at 37°C in 5% CO_2. Once a week, culture populations were halved by carefully removing half of the medium from each culture vessel and adding an equal volume of fresh α-MEM. As controls, MCL cells and CBBC were also cultured in the absence of stromal cells. In the experiments in which adherent MCL cells or CBBC were removed from the stromal cell layer, the cells were first treated with enzyme-free cell disassociation solution (Invitrogen) and then washed twice in α-MEM. MCL cells and CBBC were then isolated using anti-human CD45-conjugated magnetic beads (Miltenyi Biotec). The CD45-labeled beads were used in place of CD19-beads to minimize potential inadvertent activation of NF-κB due to CD19 cross-linking.[14]

Information on BAFF receptor expression, BAFF quantitation, apoptosis analysis, chemotaxis and cell adhesion assays, NF-κB

activation, quantitative RT-PCR, drug toxicity and long-term culture is available in the *Online Supplementary Appendix*.

Statistical analysis

Statistical analyses were carried out using Student's t-test or one-way ANOVA as appropriate. A P value less than 0.05 was considered to be statistically significant. All statistical analyses were conducted using the GraphPad Prism software program (version 5, Graphpad Software).

Results

Mouse and human stromal cells sustain the viability of mantle cell lymphoma cells

Several groups have shown that adherence of MCL cells to stromal cells results in increased survival and drug resistance of the former compared to non-adherent MCL cells.[15] To increase our understanding of the role that stromal cells play in MCL pathogenesis, we compared the survival of primary MCL cells cultured in medium alone to that of such cells co-cultured with either human primary bone-marrow MSC or the well-characterized mouse stromal cell line MS-5.[16] The MS-5 cell line has been shown to support the growth and survival of human hematopoietic stem cells and some types of leukemia/lymphoma cells.[16,17]

As shown in Figure 1, primary MCL cells from six patients showed a significant time-dependent loss in cell number when maintained in medium alone (Figure 1). A similar decrease in cell number was observed when normal CBBC from three independent donors were cultured in medium alone (*Online Supplementary Figure 1S*). In contrast, when MCL cells were co-cultured with either MS-5 cells or hMSC, the numbers of MCL cells increased significantly (Figure 1). Conversely, CBBC treated in a similar manner, showed only a minimal increase in cell number followed, over time, by a slow but consistent decrease (*Online Supplementary Figure 1S*).

Using this co-culture system, we have maintained primary MCL cells in culture for up to 7 months (*Online Supplementary Figure S2*). The MCL cells maintained a $CD5^+$, $CD19^+$, $CD23^-$ immunophenotype and were positive for the t(11;14) translocation throughout the time in culture and were negative for Epstein-Barr virus as determined by PCR (*data not shown*). We next evaluated the ability of MCL cells maintained in long-term culture to grow in the absence of hMSC support. As shown in *Online Supplementary Figure S2*, MCL cells harvested on days 84 and 197 and cultured in α-MEM alone demonstrated a significant decrease in cell number, while cells co-cultured with hMSC exhibited a significant increase in cell number, demonstrating that MCL cells grown long-term in our co-culture system maintained their dependence on hMSC support.

Stromal cells inhibit spontaneous apoptosis of primary mantle cell lymphoma cells

In order to begin to understand how stromal cells increase the survival of MCL cells, we evaluated the onset of spontaneous apoptosis in MCL cells and CBBC cultured in medium alone or co-cultured with MS-5 or primary hMSC. As shown in Figure 2A, 53% of MCL cells grown in α-MEM alone were apoptotic, while only 9% and 12% of primary MCL co-cultured with either hMSC or MS-5 cells, respectively, were apoptotic. In contrast, CBBC grown in α-MEM alone had 86% apoptotic cells, while 33% and 24% of the MSC co-cultured with either MS-5 cells or hMSC, respectively, were apoptotic (Figure 2B).

We next evaluated the cells for PARP cleavage and

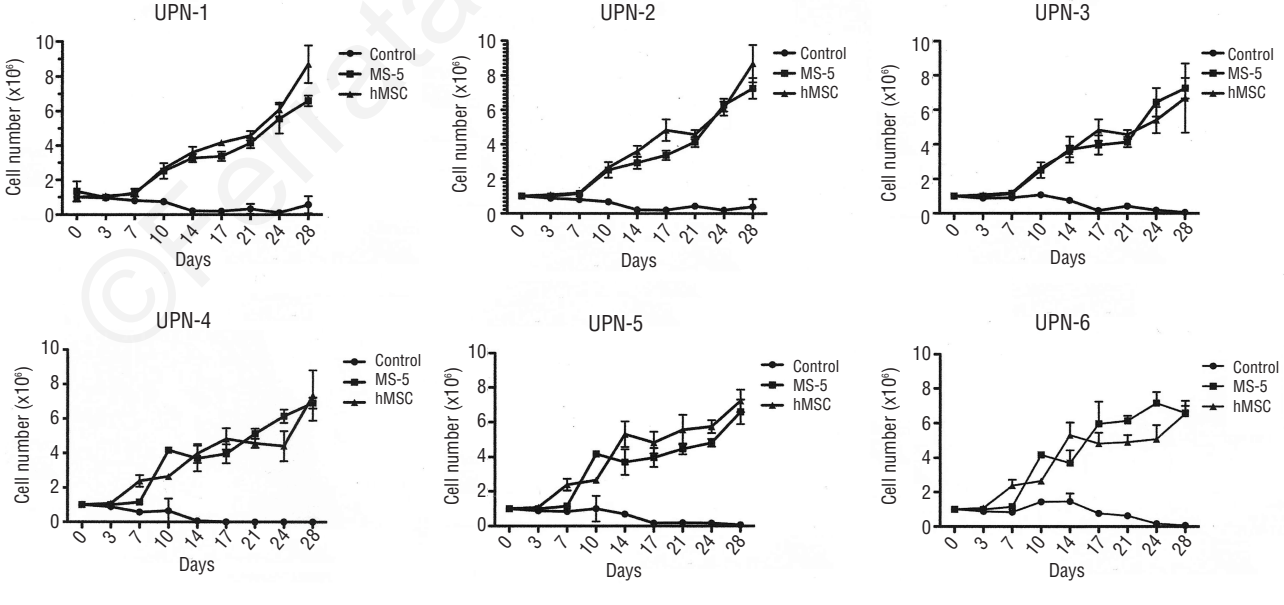

Figure 1. Stromal cells increase the number of primary MCL cells. MCL cells from six patients (UPN1-UPN6) were cultured in medium alone (α-MEM) or with an adherent layer of murine MS-5 cells or normal hMSC. At the indicated times, MCL cells were removed and cell viability and cell number were evaluated by trypan blue exclusion using a ViCell Cell Viability Analyzer. Data shown are the mean ± SD from three independent experiments.

expression of the anti-apoptotic protein bcl-2 known to be over-expressed in MCL cells.[18-20] Western blot analysis of protein lysates from MCL cells grown in medium alone had significantly less bcl-2 expression compared to cells co-cultured with either hMSC or MS-5 cells (Figure 2C). In contrast, there was significantly less PARP cleavage in MCL cells that were co-cultured, consistent with reduced apoptosis in these cells (Figure 2B). These results demonstrate that stromal cells significantly affect MCL cells, in part, by maintaining expression of the anti-apoptotic protein bcl-2 and suppression of apoptosis.

Human mesenchymal stem/stromal cells produce both bound and secreted B-cell activating factor

Several recent reports have described the role of BAFF in the survival and leukemia and lymphoma cells.[12,21-23] Three BAFF receptors have been identified on the surface of normal and malignant B-cell lymphoma cells, namely BCMA, TACI and BAFF-R/BR3. Only BAFF-R is expressed on MCL cells.[24] Based on this information we also evaluated primary stromal cells from three independent healthy donors for both expression of BAFF localized to cell membranes and secreted BAFF. As shown in *Online Supplementary Figure S3A*, these three samples of normal primary human MSC had variable but significant surface expression of BAFF. To further examine BAFF production, we measured BAFF secretion from these stromal cells. *Online Supplementary Figure S3B* shows that the three primary hMSC lines produced high levels of secreted BAFF (concentrations between ~1000-2000 pg/mL).

Human mesenchymal stem/stromal cells - mantle cell lymphoma interactions result in increased B-cell activating factor secretion and survival of mantle cell lymphoma cells

We next evaluated the impact of hMSC-MCL cell interactions on BAFF secretion and MCL survival. In this experiment hMSC and MCL cells were cultured either alone or together in serum-free α-MEM. Cell-free supernatants were evaluated for soluble BAFF. As shown in Figure 3A, MCL cells secreted low amounts of BAFF (200-650 pg/mL) while stromal cells alone secreted 1.5- to 10-fold more BAFF (1000-2000 pg/mL). In contrast, stromal cell–MCL cell interactions resulted in a marked increase in secreted BAFF (3000-4300 pg/mL). To confirm the role of BAFF in the survival of MCL cells in our system, we maintained MCL cells in serum-free α-MEM alone, or with recombinant BAFF, or with BAFF plus BAFFR-Fc. As shown in Figure 3B, primary MCL cells cultured in the presence of BAFF produced significantly greater numbers of cells compared to MCL cells grown in α-MEM alone or in BAFF-supplemented cultures containing BAFFR-Fc. In addition, experiments in which MCL cells maintained in serum-free α-MEM were co-cultured with hMSC with or without addition of BAFFR-Fc demonstrated that inhibition of MSC-derived BAFF resulted in a significant reduction of MCL viability. These results underscore a critical role for hMSC-MCL interactions in promoting BAFF secretion and MCL cell survival.

We next wanted to determine the relative contribution of MCL and hMSC to BAFF secretion. MCL and hMSC were fixed with 1.0% paraformaldehyde. The corresponding cell populations, MCLFX and hMSCFX, were used in co-culture experiments. As shown in *Online Supplementary Figure S4A,* MCL cells (545 pg/mL), and MCL co-cultured with hMSCFX (600 pg/mL) produced equivalent amounts of secreted BAFF. When unfixed MCL cells were co-cultured with hMSC or hMSCFX cells, the MCL-hMSC cultured cells secreted the greatest amount of BAFF into the medium (3200 pg/mL). MCLFX cells co-cultured with hMSC produced 2700 pg/mL of BAFF. These results suggest that the primary source of BAFF secretion in our co-culture system was hMSC.

To determine whether the increase in BAFF secretion in

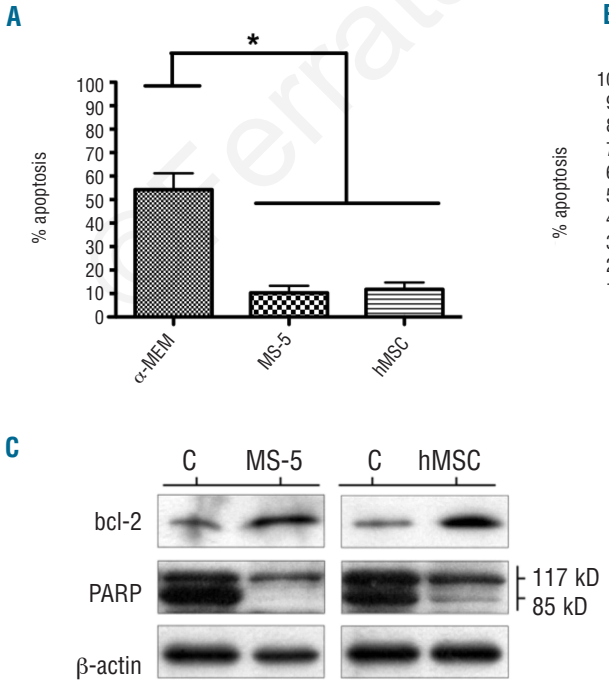

Figure 2. Stromal cells protect MCL cells from apoptosis by maintaining bcl-2 expression and preventing PARP cleavage. Primary MCL cells (A) and CBBC (B) were cultured in α-MEM alone or co-cultured with either hMSC or MS-5 cells. On day 10, aliquots were removed and stained for apoptosis with annexin V/propidium iodide. Apoptotic cells were quantitated by flow cytometry. (C) On day 10, aliquots were removed and lysed for western blot analysis with monoclonal antibodies to bcl-2 or PARP. A representative blot is shown. Data shown are representative of three independent experiments conducted on MCL samples from UPN-1 to UPN-6. Data from UPN-3 are shown.

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hMSC after co-culture with MCL was due to transcriptional activation, we used quantitative PCR to measure mRNA expression from MCL cells alone, hMSC alone or MCL cells and hMSC from the co-cultures isolated by magnetic beads. hMSC showed 3-fold more mRNA expression compared to MCL cells. In contrast, the MCL cells isolated from co-culture exhibited a small increase (1.5-fold) compared to MCL cells cultured alone, while the hMSC isolated from co-culture showed a 5.3-fold increase in mRNA expression compared to MCL cells alone, and a 2-fold increase compared to hMSC cultured alone (*Online Supplementary Figure S4B*).

To further investigate hMSC-MCL cell interactions and the production of BAFF, we evaluated MCL cell survival using a transwell system. As shown in Figure 3C, MCL cell survival was dependent upon the cell-cell interaction. We next evaluated the possibility that the increased BAFF production observed in the experiments presented in Figure 3A in response to hMSC-MCL interactions has the capacity to increase survival of MCL cells not directly in contact with stromal cells. As shown in Figure 3C, the percentage of apoptotic cells grown in the lower chamber, exposed to media conditioned by MCL-MSC interactions in the upper chamber (18%), was significantly decreased compared to that of MCL cells maintained either alone (68%), separated from hMSC by the insert (60%), or containing the decoy receptor (53%). Apoptosis of the MCL cells in the lower chamber exposed to the hMSC-MCL cell media from the upper chamber was, however, higher than that of MCL cells in direct contact with hMSC (32%). These results indicate that soluble BAFF, induced by MSC-MCL interactions, partially protects MCL cells not in direct contact with hMSC from apoptosis.

Human mesenchymal stem/stromal cells - mantle cell lymphoma interactions activate the canonical and non-canonical nuclear factor-κB signaling pathways

Several studies have shown that the canonical and non-canonical NF-κB signaling pathway is constitutively activated in MCL cells.[11,20,25-27] We, therefore, tested the hypothesis that increased BAFF production as a consequence of hMSC-MCL cell interactions results in activation of the NF-κB pathway. Nuclear extracts from MCL cells obtained from four patients (UPN-1, UPN-2, UPN-5 and UPN-6) were evaluated for NF-κB activation as previously described.[28] Due to the limited number of cells, we were unable to evaluate NF-κB activation from samples UPN-3 and UPN-4. For these experiments nuclear extracts were produced from MCL immediately after thawing and 3 days after culturing in either serum-free α-MEM alone, co-cultured on hMSC or co-cultured on hMSC with the addition of BAFFR-Fc. As shown in Figure 4, MCL cells co-cultured with hMSC had a significant increase in nuclear NF-κB activity compared to pre-culture samples or cells cultured in serum-free α-MEM. In contrast, co-cultures containing BAFFR-Fc demonstrated a significant reduction in NF-κB activation, indicating that NF-κB activation was due primarily to BAFF. Examination of the relative activation of canonical NF-κB proteins p65 and p50 showed 2.8- to 6.5-fold and 2.3- to 6.2-fold increases in activity, respectively, when co-cultured with hMSC. The non-canonical NF-κB proteins p52 and RelB were activated 3.3- to 6.0-fold and 1.9- to 4.0-fold, respectively. REL was increased 2.1- to 4.8-fold. Samples from patients UPN-1 and UPN-6 had relatively greater increases in the non-canonical pathway, while the canonical pathway was dominant in the UPN-5 sample. In UPN-2 both pathways were activated equally. These data demonstrate that co-cultivation of MCL cells with hMSC leads to activation of both the canonical and non-canonical NF-κB pathways. To further demonstrate a key role of the NF-κB pathways in MCL cell-hMSC driven survival we inhibited NF-κB activation by infecting MCL cells from patients UPN-2 and UPN-5 with either an adenovirus containing the dominant-negative form of IKK1 (AdIKK1DN), adenovirus containing the dominant-negative form of IKK2 (AdIKK2DN), or a control virus containing green fluorescent protein GFP (AdGFP). IKK2DN is known to inhibit the activation of the canonical NF-κB pathway (p50) while IKK1DN is pre-

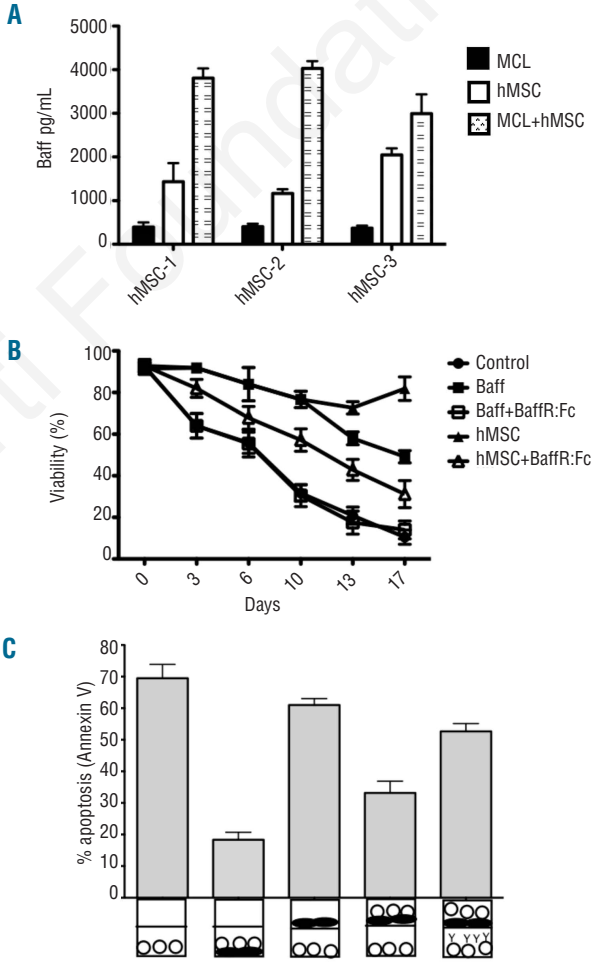

Figure 3. hMSC-MCL interactions result in increased BAFF secretion and MCL survival. (A) Primary MCL cells were grown in serum-free α-MEM for 72 h in the presence or absence of hMSC, after which the culture medium was removed and analyzed for secreted BAFF by ELISA. (B) Effect of BAFF neutralization on MCL survival. MCL cells were cultured in α-MEM alone, with 100 ng/mL recombinant BAFF or co-cultured with hMSC. BAFF was neutralized by the addition of 5 μg/mL of the decoy receptor BAFFR-Fc. (C) BAFF secretion requires direct contact with hMSC. hMSC (solid oval cells) and MCL (empty circles) were cultured alone, together (direct contact) or separated via a 0.4 μm transwell-membrane. After 72 h in culture the cells were carefully removed from the stromal cell layer and the percentage of apoptotic MCL cells was determined by annexin V staining followed by flow cytometry. The data are mean±SD from three independent experiments on samples from six MCL patients.

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dicted to inhibit the activation of the canonical and non-canonical NF-κB pathways (p52). The average infection rate of primary MCL cells with AdGFP was 92±3%, which is consistent with our previously published results.[29] As shown in *Online Supplementary Figure S5A*, MCL cells infected with either AdIKK1DN or AdIKK2DN showed a significant increase in apoptosis (60-70%) in the presence or absence of BAFF compared to control or AdGFP-infected MCL cells. In addition, infection with AdIKK1DN or AdIKK2DN also significantly reduced the activation of the NF-κB (canonical and non-canonical) pathways compared to AdGFP-infected cells as measured by enzyme-linked immunosorbent assays for p50 and p52 (*Online Supplementary Figure S5B and S5C*).

B-cell activating factor increases CXCL12- and CXCL13-dependent chemotaxis of mantle cell lymphoma cells via nuclear factor-κB activation

A recent study demonstrated that BAFF enhances chemotaxis of primary memory B cells to the chemokines CXCL12 and CXCL13.[30] Based on this study and other reports that the stromal cell-derived chemokines CXCL12 (SDF-1) and CXCL13 induce chemotaxis in MCL,[31,32] we hypothesized that BAFF may also enhance CXCL12 and CXCL13 chemotaxis of primary MCL cells. Using the chemotaxis assay, we compared MCL cell migration responses to CXCL12 and CXCL13 following incubation with medium only, BAFF or BAFF plus BAFFR-Fc. The percentages of MCL cells migrating in BAFF and BAFF/BAFFR-Fc were comparable to that of the untreated cells (*Online Supplementary Figure S6A, S6B*). In contrast, the percentage of MCL cells specifically migrating in response to CXCL12 or CXCL was more than tripled (*Online Supplementary Figure S6A, S6B*).

We next tested whether the BAFF-mediated increase in MCL cell chemotaxis to CXCL12 and CXCL13 was due, at least in part, to activation of the NF-κB pathway. As a control, migration toward CXCL12 (*Online Supplementary Figure S7C*) and CXCL13 (*Online Supplementary Figure S7D*) was similarly inhibited by the addition of pertussis toxin. We next used adenovirus containing either GFP (control virus) or the dominant-negative forms of IKK1 and IKK2 to specifically inhibit activation of the NF-κB pathway. As shown in Figures 5C and 5D infection with AdGFP did not impair the CXCL12- or CXCL13-mediated migration of mock and BAFF-treated cells. In contrast, infection of MCL cells with AdIKK1dn or AdIKK2dn virus significantly inhibited CXCL12- (Figure 5C) and CXCL13-mediated (Figure 5D) migration of BAFF-treated cells compared to mock-treated cells. Similar results were obtained for normal CBBC (*Online Supplementary Figure S6*). These data suggest that BAFF-treated MCL cells and CBBC are more dependent than mock-treated MCL cells and CBBC on NF-κB-activated chemotaxis.

Co-culture of mantle cell lymphoma cells with human mesenchymal stem/stromal cells leads to increased drug resistance

We next evaluated the effects of primary hMSC on the sensitivity of MCL cells to drugs. In this series of experiments, MCL cells from six patients were treated with the indicated concentration of doxorubicin, bortezomib or fludarabine. MCL cells were cultured in either α-MEM (control) or together with hMSC ± BAFFR-Fc. As shown in

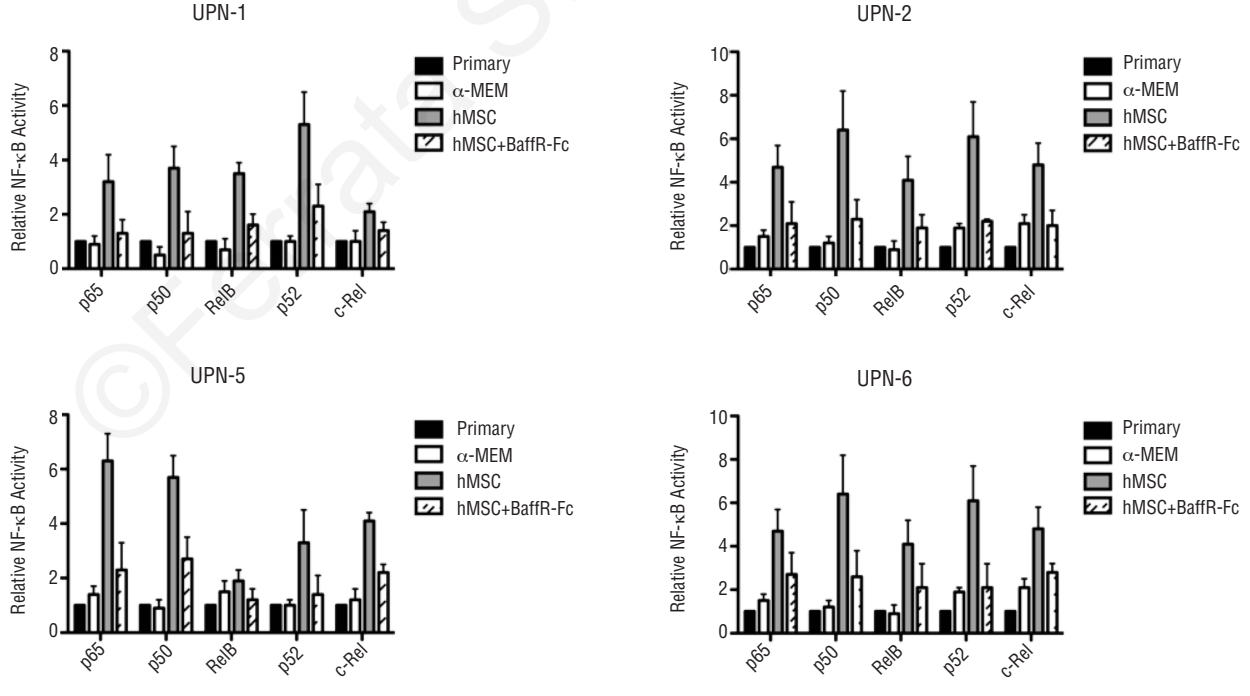

Figure 4. The MCL-stromal cell interaction activates both the canonical and non-canonical NF-κB pathways. NF-κB activation in four patients was determined by co-culturing MCL cells with hMSC for 24 h. Nuclear extracts were made and evaluated for nuclear NF-κB activation by ELISA. Data are presented as the mean±SD from three independent experiments.

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Figure 5, primary MCL cells maintained in α-MEM and treated with either doxorubicin, bortezomib or fludarabine showed a dose-dependent decrease in cell viability. We next compared the effects of these drugs on MCL cells co-cultured with hMSC. Adherent MCL cells demonstrated no to low drug cytotoxicity compared to treated control cells. In contrast, the non-adherent cells demonstrated intermediate sensitivity (i.e. they showed significantly more cytotoxicity when compared to the adherent cells, but significantly less toxicity compared to the treated control cells).

To determine whether the observed drug-resistant phenotype was dependent on BAFF, drug-treated cells were cultured with medium containing BAFFR-Fc. As shown in Figure 6, the addition of the decoy receptor had minimal effect on the drug cytotoxicity of the adherent cell population. In contrast, addition of the decoy receptor abrogated the intermediate drug-resistant phenotype, demonstrating that BAFF plays an important role in maintaining drug resistance in non-adherent MCL cells.

To help to ensure that the increase in survival of the adherent population was not the result of adherent apoptotic cells migrating into the suspension cells and skewing the data, we determined the ratio of adherent MCL cells to non-adherent cells, finding that only 1:333 to 1:143 of the MCL cells adhered to the stromal layer. This suggests that any adherent apoptotic MCL cells that migrated into the non-adherent population would have had a negligible impact on the toxicity data.

Discussion

In many types of leukemia and lymphoma, the tumor microenvironment has been shown to play a vital role in the regulation of cell survival, proliferation, dissemination and drug resistance. In this study we characterized the interactions of primary MCL cells with primary hMSC. Our experiments demonstrated enhanced survival of adherent and non-adherent MCL cells in the presence of the murine stromal cell line MS-5 and primary hMSC. Using primary hMSC and MCL cells in our co-culture system we demonstrated for the first time that MCL cells can be maintained *ex vivo* for at least 7 months and that the MCL cells retain their original phenotype and continue to require MCL-hMSC interactions for proliferation and survival. We also demonstrated that MCL-hMSC interactions stimulate increased secretion of the B-cell survival factor BAFF and that increased BAFF is associated with activation of the BAFF-NF-κB axis, reduced apoptosis, enhanced chemotactic response to CXCL-12 and CXCL-13 and increased drug resistance.

Studies evaluating the interactions of MCL cells with stromal cells have clearly demonstrated the importance of this interaction for MCL survival and drug resistance.[6,7,9,10] A major constraint of these studies is the fact that MCL cell survival and drug resistance were evaluated only in short-term (12-24 h) cultures.[6,7,9,10] In the present study, we investigated the survival and drug resistance of MCL cells maintained in a long-term MCL-hMSC co-culture system. In this system, MCL cell-hMSC cultures maintained for approximately 4 weeks demonstrated a significant increase (6- to 8-fold) in MCL cell number while MCL cells maintained in the absence of stromal cell support resulted in a precipitous decrease in cell number due to increased apoptosis. Importantly, by isolating MCL (adherent and non-adherent) cells every 4 weeks from the stromal cell layer and plating on a fresh layer of hMSC we successfully maintained MCL cells for longer than 7 months. It is important to note that beyond passage 6 hMSC maintain their original immunophenotype and ability to differentiate but lose their ability to maintain MCL proliferation and survival. These results are similar to those reported by Briquet *et al.*, demonstrating that extended culture of mesenchymal stem cells, with normal immunophenotype and differentiation capacity, significantly reduced the ability of these cells to support normal hematopoietic progenitor cells.[33]

Accessory cells (i.e. nurse cells, macrophages, dendritic cells and mesenchymal stromal cells) in the tumor microenvironment have been reported to secrete BAFF, which acts as a survival factor.[34-37] We demonstrate that human stromal cells produce bound and secreted forms of BAFF and that the MCL cell-stromal cell interaction results in increased BAFF secretion and MCL survival. We also believe that the increased survival observed in co-cultures of MCL cells and MS-5 (a murine stromal cell line) was due to Baff as murine BAFF binds to and activates the BAFFR.[38] The role of BAFF in MCL survival was further demonstrated by the ability of the decoy receptor BAFFR-Fc to inhibit survival of MCL cells cultured with exogenous BAFF or co-cultured with stromal cells. These observations are consistent with those of other studies showing that BAFF increases the survival of malignant B cells by inhibiting spontaneous apoptosis and that inhibiting BAFF using decoy receptors, blocking antibodies or siRNA restores the apoptotic process in malignant B cells.[11,22,39]

Our studies have shown that direct contact of MCL cells with stromal cells was associated with increased BAFF secretion and enhanced MCL survival. Even MCL cells not in direct contact with the stromal cells had better survival when cultured in a transwell system that contained hMSC cells and primary MCL cells co-cultured on the opposite sides of the membrane. This phenomenon may play an important role in MCL pathogenesis by allowing MCL cells in other potentially non-supportive microenvironments (e.g. blood) to be sustained by systemic factors such as BAFF and/or other cytokines or chemokines that result from primary MCL-stromal cell interactions at distant sites. This may also be the source of elevated serum BAFF levels observed in patients with non-Hodgkin's lymphoma.[24,40,41] Furthermore, these results demonstrate that in addition to myelo-monocytic cells, which are thought to be the primary source of BAFF, stromal cell-derived BAFF also contributes to the constitutive activation of NF-κB in MCL. These data are consistent with previously published reports that BAFF/NF-κB forms an important regulatory loop that is vital for the pathogenesis of MCL.[11,12]

Numerous studies have shown that BAFF acts as a B-cell survival factor by activating the NF-κB pathway with subsequent up-regulation of several anti-apoptotic proteins including bcl-2.[12,42,43] Recent studies have shown that the NF-κB pathway is constitutively activated in MCL.[26] However, the mechanisms involved in regulating this activation are still unclear. Our results demonstrate that co-culture of MCL cells with hMSC results in the activation of both the canonical and non-canonical NF-κB pathways. Although there was heterogeneity of the patients' samples with regard to expression of individual NF-κB family

members, it is clear that both pathways are activated in samples from MCL patients. The activation of both pathways raises several important questions about the roles and interactions of these pathways in the pathogenesis and treatment (drug sensitivity) of MCL. Additional research will be required to address this important area.

Several reports have described that stromal cell adherence of leukemia and lymphoma cells, including MCL cells, contributes to drug resistance in MCL.[6,7,9,10,23] In a recent study evaluating the expression of several chemokine receptors and adhesion molecules it was found that MCL cells display high levels of functional CXCR4 and CXCR5 chemokine receptors and VLA-4 adhesion molecules and that these molecules were important for MCL-stromal cell adhesion and resulting drug resistance.[6] In this study we demonstrated that BAFF increases the chemotactic activity of CXCL-12 and CXCL-13, the ligands for CXCR4 and CXCR5, respectively. We hypothesized that the increased chemotactic activity results in MCL cells migrating to the appropriate niche which, in turn, may act as a protective sanctuary. Additional research will be required to test this hypothesis. In addition we demonstrated that adherent MCL are highly resistant to the cytotoxic effects of doxorubicin, fludarabine and bortezomib and that the addition of a BAFF decoy receptor had only marginal effects on MCL sensitivity in this subpopulation of cells. In contrast, MCL cells cultured in the absence of stromal cells were highly sensitive to the cytotoxic affects of the drugs. Interestingly, in contrast to other studies,[6,7,9] we showed that non-adherent MCL cells in the co-culture system had an intermediate drug resistance phenotype that can be overcome by neutralization of BAFF, suggesting that BAFF contributes to the drug-resistant phenotype of these cells. Importantly, this study further highlights the potential therapeutic value of targeting this regulatory loop. By blocking NF-κB activation and MCL cell adhesion to stromal cells it may be possible to break this regulatory loop. For example, combining the NF-κB inhibitor bortezomib or the BAFF-specific inhibitor belimumab with either the CXCR4 antagonist plerixafor or the VLA-4 antibody natalizumab (both prevent binding to stromal cells) may result in a more robust cytotoxic response.

In summary, our studies demonstrate the importance of stromal cells for the *ex vivo* proliferation, migration, survival and chemotherapy-resistance of primary MCL cells in association with expression of bcl-2 and nuclear canonical and non-canonical NF-κB activation. Stromal cell production of BAFF was shown to play a key role in the support of MCL, and cell-cell contact between stromal cells and primary MCL cells is required for maximum apoptosis suppression and proliferation. MCL cell resistance to the chemotherapeutic agents doxorubicin, fludarabine and bortezomib was also dependent on stromal cell interactions with co-cultured MCL cells compared to MCL cells cultured in the absence of hMSC. Interestingly, MCL cells co-cultured with stromal cells may either adhere to the stromal cells or remain non-adherent. We, like others, observed that the greatest degree of drug resistance occurred in the adherent population. Further characterization of this subset of MCL cells is underway.

Finally, this study provides a convenient and reproducible method to achieve prolonged *ex vivo* maintenance and expansion of primary MCL cells from patients. This system should be particularly amenable to studying the roles of various accessory cell components found in the MCL-associated niche in the pathogenesis of MCL. In addition, the system may be a better model for evaluating MCL-targeted therapeutic agents especially those that target cell adhesion and/or the BAFF/NF-κB signaling pathway.

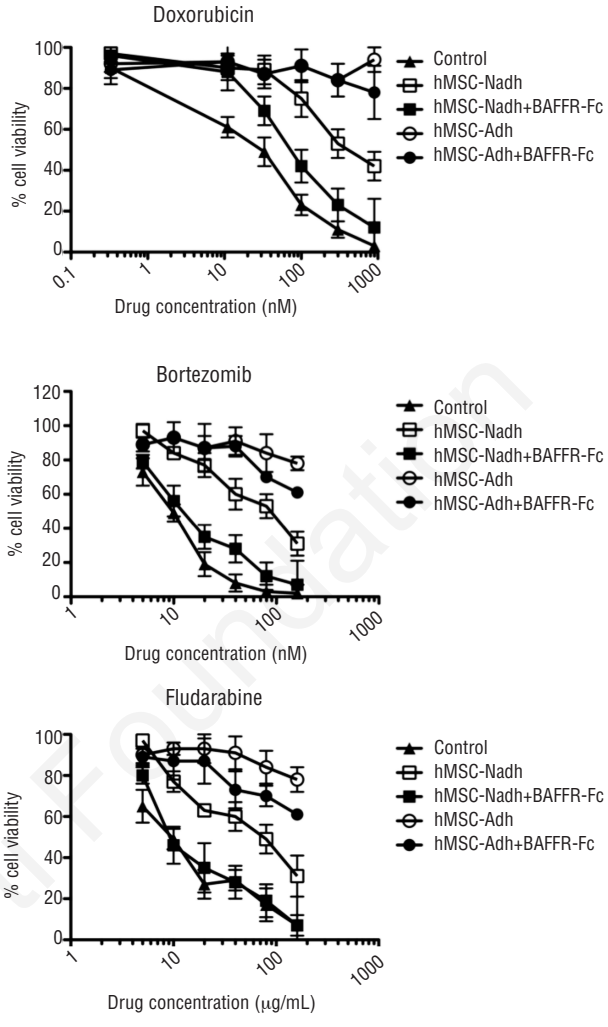

Figure 5. MCL adherence to stromal cells increases MCL drug resistance. MCL cells were co-cultured with stromal cells in the presence or absence of various concentrations of doxorubicin, (A), bortezomib, (B) or fludarabine (C) for 72 h. Cell viability of the adherent MCL (hMSC-Adh) and non-adherent MCL (hMSC-Nadh) populations was determined by staining the cells with hCD45-Cy7 and 7-AAD followed by flow cytometry. Data are expressed as mean ± SD from three independent experiments.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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