Mast cells in Waldenstrom’s macroglobulinemia support lymphoplasmacytic cell growth through CD154/CD40 signaling

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Bone marrow (BM) mast cells (MC) are commonly found in association with lymphoplasmacytic cells (LPC) in patients with Waldenstrom’s macroglobulinemia (WM). We therefore sought to clarify the role of MC in WM. Co-culture of sublethally irradiated HMC-1 MC, KU812 basophilic cells, or autologous BM MC along with BM LPC from WM patients resulted in MC dose-dependent tumor colony formation and/or proliferation as assessed by 3H-thymidine uptake studies. Furthermore, by immunohistochemistry, multicolor flow cytometry and/or RT-PCR analysis, CD40 ligand (CD154), a potent inducer of B-cell expansion, was expressed on BM MC from 32 of 34 (94%), 11 of 13 (85%), and 7 of 9 (78%) patients, respectively. In contrast, MC from five healthy donors did not express CD154. By multicolor flow cytometry, CD154 was expressed on BM LPC from 35 of 38 (92%) patients and functionality was confirmed by CD154 and CD40 agonistic antibody stimulation, which induced proliferation, support survival and/or pERK phosphorylation of LPC. Moreover, MC induced expansion of LPC from 3 of 5 patients was blocked in a dose dependent manner by use of a CD154 blocking protein. These studies demonstrate that in WM, MC may support tumor cell expansion through constitutive CD154-CD40 signaling and therefore provide the framework for therapeutic targeting of MC and MC-WM cell interactions in WM.

Key words: mast cells, Waldenstrom’s, macroglobulinemia, cd154, cd40, lymphoplasmacytic cells

Introduction

Waldenstrom’s macroglobulinemia (WM) is a B-cell disorder characterized by bone marrow (BM) infiltration by lymphoplasmacytic cells (LPC) with a predominantly intertrabecular pattern, fulfilling the REAL and WHO criteria of a lymphoplasmacytic lymphoma, along with IgM monoclonal gammapathy [1, 2]. Subsequent to the first description of the disease by Jan Waldenström in 1944, BM mast cells (MC) have been reported in association with LPC in WM patients with increasing frequency [3, 4]. This striking association has become characteristic of WM and is now used widely as a supportive basis for diagnosing WM by pathologists [5, 6].

In addition to WM, increased numbers of MC have also been reported in other B-cell disorders including chronic lymphocytic leukemia (CLL) and Hodgkin’s disease [7–11]. A role for MC in supporting tumor growth was suggested more than a century ago by Westphal, and more recent evidence has implicated a role for MC supported angiogenesis in promoting solid tumor growth [12, 13]. Among the B-cell disorders, studies by Molin et al. showed that MC from patients with Hodgkin’s disease expressed the tumor necrosis factor (TNF) family member CD153 (CD30 ligand), and in vitro promoted proliferation of Reed-Sternberg cells via CD30 signaling [14]. In addition to CD30L, the expression of CD40 ligand (CD154), another member of the TNF family has been reported on activated MC in anaphylaxis [15, 16]. The role of CD154 as a potent inducer of both normal and malignant B- and plasma cell growth has previously been described by us and others. We therefore investigated the role of MC in the pathogenesis of WM, and sought to delineate the role of putative TNF family members in supporting WM cell expansion.

Materials and methods

Patients and clinical characteristics

BM aspirates, clots, and trephine biopsies were obtained from 44 patients with the consensus panel diagnosis of WM [2] who were followed at the Dana-Farber Cancer Institute following informed consent. Thirty-two patients were male, and 15 were female, with a median age of 62 (range...
confirmed by tryptase staining. Cytomics RXP software (Beckman Coulter). MC were identified first by cell sorting using a Cytomics FC500 cytometer (Beckman Coulter) and the fraction of the BM MNC was used for MC analysis by flow cytometry and analysis which demonstrated >90% clonal cells by staining for CD20 and manufacturer’s instructions. Clonality was confirmed by flow cytometric CD20-PE, LPC and MC were analyzed by direct immunofluorescence using multicolor infiltrates and tryptase-positive MC patterns were assessed at 40× magnification with 3% goat serum for 30 min. After further washing, immunoperoxidase staining was developed according to the manufacturer’s instructions, followed by washing in 50 mM Tris–HCl (pH 7.4), secondary (rabbit) anti-mouse conjugated antibody (Envision detection kit; DAKO) was applied for 30 min. After further washing, immunoperoxidase staining was developed by using a DAB chromogen kit (Dako) according to the instructions of the manufacturer and counterstained with Harris hematoxylin. The LPC fragments were detected by 2% (w/v) agarose gel electrophoresis and staining with 0.3 mg/ml of ethidium bromide (Sigma, St. Louis, MO).

historical examination of BM trephine biopsies and clots

BM trephine biopsies were fixed in neutral-buffered formalin, decalcified using Zenker’s acetic acid solution and processed into paraﬁn wax-embedded blocks for histology by standard methods. Sections were cut at 4 μm and stained with hematoxylin and eosin (H&E) staining. Immunohistochemistry was performed using the mouse monoclonal antibody AA1 (Dako, USA), reactive with MC tryptase, at the pre-determined optimal titration of 1/1000. Briefly, slides were deparafﬁnized and pretreated with 10 mM sodium citrate buffer (pH 6.0) (Zymed, South San Francisco, CA) in a steam pressure cooker (Decloaking Chamber; BioCare Medical, Walnut Creek, CA) according to the manufacturer’s instructions, followed by washing in distilled water. All further steps were performed at room temperature in a hydrated chamber. Slides were pretreated with Peroxidase Block (Dako) for 5 min to quench endogenous peroxidase activity followed by a 1:5 dilution of goat serum in 50 mM Tris–HCl (pH 7.4) for 20 min to block nonspeciﬁc binding sites. Primary mouse anti-human tryptase (AA1) (Dako, USA), the primary antibody (Dako) was applied at a 1:1000 dilution in 50 mM Tris–HCl (pH 7.4) with 3% goat serum for 1 h. After washing in 50 mM Tris–HCl (pH 7.4), secondary (rabbit) anti-mouse antibody (Dako) was applied at a 1:7500 dilution in 50 mM Tris–HCl (pH 7.4) with 3% goat serum for 30 min. Slides were washed again in 50 mM Tris–HCl (pH 7.4), and (goat anti-rabbit) horseradish peroxidase-conjugated antibody (Envision detection kit; DAKO) was applied for 30 min. After further washing, immunoperoxidase staining was developed by using a DAB chromogen kit (Dako) according to the instructions of the manufacturer and counterstained with Harris hematoxylin. The LPC infiltrates and tryptase-positive MC patterns were assessed at 40× or 100× magniﬁcations. In order to evaluate the MC density, on each BM biopsy at least 10 non-overlapping ﬁeld of cellular bone marrow tissue were analyzed at 40× magnification with a reticulated eye piece. BM aspirate clots were obtained after allowing the ﬁrst 3 ml of BM aspirate to ﬁrmly coagulate. BM aspirate clots were then ﬁxed in 10% neutral-buffered formalin and processed into paraﬁn wax-embedded blocks. Sections were cut at 4 μm and stained for human tryptase and CD154.

flow cytometric analysis

LPC and MC were analyzed by direct immunofluorescence using multicolor flow cytometry with the following monoclonal antibodies: CD20-FITC, CD20-PE, k-PE, λ-FITC, CD40-PE-Cy5 for LPC; CD154-PE, CD117-PE-Cy5 (all from Becton Dickinson, San Jose, CA) and FcεRI (Upstate) for MC. A FITC conjugation kit (Pharmacia) was used to label FcεRI. Mouse IgG1-PE, IgG1-FITC and IgG1-PE-Cy5 (Becton Dickinson, San Jose, CA) were used as isotype controls. Cell viability was assessed using Apo 2.7-PE (Immunotech, Marseille, France). Patients were deemed to be positive if at least 20% of gated cells were antigen expressive.

reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from 0.1–2 × 109 cells using RNeasy Mini Kit following the manufacturer’s instructions (Qiagen, Valencia, CA). 0.3 μg of RNA was reverse transcribed in a 20 μl reaction by oligo-dT(12),( priming using Superscript III reverse transcriptase according to the protocol provided by Invitrogen (Invitrogen, Carlsbad, CA). Two microliters of ﬁrst-strand cDNA was used as template for PCR ampliﬁcation. PCR was performed using the PTC-200 DNA EngineThermal Cycler (MJ Research Inc., Waltham, MA). For CD154 ampliﬁcation, a 278 bp fragment was generated using the sense primers 5′-GCC GCA CAT GTC ATA AGT-3′ (406–423) and antisense primer 5′-GCC GCC AAG GTT TGG CCG AA-3′ (678–697). The β-actin ampliﬁcation producing a 620 bp fragment using the primers 5′-TCA CCC ACA CTG TCG TCA T-3′ (sense) and 5′-GCA TTT GGG GTG GAC GAT G-3′ (antisense) was also used in the PCR as control of each cDNA and to compare the amount of transcript. After an initial denaturation at 95°C for 5 min, 35 cycles of PCR ampliﬁcation were performed, each consisting of a denaturing step of 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, followed by a ﬁnal step at 72°C for 10 min. Thirty cycles were used for β-actin ampliﬁcation in order to keep the reaction in a linear range. The ampliﬁed fragments were detected by 2% (w/v) agarose gel electrophoresis and staining with 0.3 mg/ml of ethidium bromide (Sigma, St. Louis, MO).

cell lines and cell cultures

The human MC-derived cell line HMC-1 [17] was kindly provided by J. Butterﬁeld (Mayo Clinic Rochester, MN, USA) and cultured in Iscove’s modiﬁed Dulbecco medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Harlan, Indianapolis, IN, USA). The human basophilic-derived cell line KU812 was obtained from ATCC (American Type Culture Collection, Manassas, VA) and cultured in complete Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated FBS. In every above culture media were added 2 mmol/l L-glutamine, 50 μg/ml streptomycin and 100 IU/ml penicillin.

in vitro proliferation assays

LPC were placed in 96-well plates at a concentration of 50 000 cells/well. Samples were run in duplicate or triplicate, depending on the number of sorted LPC available. Proliferation was evaluated using a 3H-thymidine incorporation assay, and cells exposed to 3H-thymidine during the last 12 h of experiments. Cells were then harvested onto glass-ﬁber ﬁlters using an automatic cell harvester (Tomec Harvester 96 Mach III, Hampsden, CT, USA) and radioactivity counted using the Wallac Trilux Bepatlab scintillation counter (Turku, Finland). Cell proliferation was also assessed in some experiments by comparative photomicrography using a Pentax Optio 330 (3.34 megapixel) camera through the eyepiece of an inclined microscope at 10× or 20× magniﬁcation.

stimulation of LPC by mast cells

To investigate if LPC proliferation could be triggered by MC or basophils, we performed co-culture assays using either HMC-1, KU812 cell lines or autologous BM MC which were sublethally irradiated (8.5 Gy) in order to inhibit autonomous MC growth. LPC were co-cultured with MC in 96 well plates at a concentration of 50 000 cells/well for 48 h at various MC:LPC
ratios in Stem StemPro-34 serum free media supplemented with 100 ng/ml recombinant human stem cell factor (rhSCF). In certain experiments, CD40L expression on MC cell lines was further triggered by pre-incubation with 20 ng/ml of phorbol-myristate ester (PMA) (Sigma) and 1 µg/ml of calcium ionophore A23187 (Sigma) at the concentration of 20 ng/ml and 1 µg/ml respectively for 24 h. MC were then harvested by mild scraping, washed three times and re-suspended in RPMI before use in co-culture studies with LPC [15, 16].

Stimulation of LPC with CD40 agonists and prevention of serum starvation induced apoptosis
To evaluate the CD154/CD40 signaling pathway, sorted LPC were cultured in RPMI 1640 plus 2.5% FBS in the presence or absence of the murine anti-CD40 agonistic monoclonal antibody G28.5 or control IgG1 monoclonal antibody at 0.1, 1.0, 10 µg/ml (R&D Systems, Minneapolis, MN); recombinant soluble trimeric CD40L at 0.1, 1.0 10 µg/ml (R&D Systems, Minneapolis, MN); or transfected CD40L expressing Chinese hamster ovary (CHO) cells fixed in PFA (0.5%) at a CHO: LPC ratio of 1:1, and 2:1. Sorted LPC were cultured overnight in RPMI 1640 plus 1% FBS. Soluble trimeric CD40L or fixed CD40L-expressing CHO were added to the culture media for 48 and/or 72 h. Cells were harvested and apoptotic LPC were assessed by Apo 2.7 staining.

Cell lysis and western-blot assays
Sorted LPC were washed twice with PBS and suspended in lysis buffer (10 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% triton X-100, 1 mM sodium vanadate, 1 mM phenylmethyl sulfonyl fluoride and 2 mg/ml aprotinin). After 40 min on ice, lysates were cleared by centrifugation at 13 000 g for 30 min at 4°C and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis prior to electrophoretic transfer onto an Hybond C super membrane (Amersham, Arlington Heights, IL). Blots were probed overnight with either with antibodies to β-actin or

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Results are given for median fold difference in tritiated thymidine uptake versus BM LPC alone for each experimental condition. p-values depicted are for comparison of differences in median tritiated thymidine uptake versus LPC alone.

Figure 1. Proliferation of BM LPC from a WM patient in response to MC quantity dependent co-culture with sublethally irradiated HMC-1 MC as assessed by by 3Hthymidine uptake studies (A) and tumor colony formation (B).
CD154-CD40 targeted inhibition of co-culture induced proliferation

We next investigated if LPC proliferation induced by contact with MC could be inhibited by disrupting CD154/CD40 signaling. MC were co-cultured with LPC at an MC:LPC ratio of 1:5 using sublethally irradiated HMC-1 or KU812 cell lines which were incubated 2 h prior to co-culture with the CD154 blocking CD40:Fc chimeric protein (Axxora, LLC San Diego, CA) at concentrations of 0.1, 1 or 10 μg/ml.

Results

MC induce proliferation of WM LPC

Following 48 h co-culture of BM LPC with either sublethally irradiated HMC-1 and KU812 cells, or paraformaldehyde fixed LAD2 cells, mast and basophilic cell quantity-dependent increases in LPC proliferation were observed as assessed by tritiated thymidine uptake (Table 1) and/or tumor colony formation (Figure 1). Similar to our observations with MC and basophilic cell lines, co-culture of BM LPC with autologous MC from a WM patient for whom sufficient MC could be collected to perform this assay resulted in >150-fold increase in BM LPC proliferation (Figure 2).

Expression of CD154 (CD40L) on MC in WM

After determining that MC induced proliferation of BM LPC from WM patients, we next sought to identify ligands which could promote growth of LPC. The expression of the TNF family member CD154 (CD40L), which previously was described by us and others as a potent inducer of malignant B-cell growth, was investigated for expression on WM patient MC, particularly since its expression had earlier been described on ‘activated’ MC [15,16]. By immunohistochemical staining of BM aspirate clots, CD154 was found to be expressed on tryptase positive BM MC from 32/34 (94%) patients (Figure 3).
Expression of CD154 on BM MC ranged from moderate to bright as compared to the weak CD154 expression detected on background megakaryocytes.

In order to further delineate expression of CD154 on BM MC, we performed flow cytometric analysis and/or RT-PCR analysis of BM MC (CD117^+ FceRI^+ ) taken from WM patients and healthy donors (Figure 4). RT-PCR analysis confirmed presence of CD154 transcripts in BM MC taken from 7/9 (78%) WM patients; in contrast, CD154 transcripts were present in BM MC in only 1/5 healthy donors, wherein weak transcript expression was detected (P = 0.09). By multicolor flow cytometry, CD154 was detected on BM MC from 11/13 (85%) patients (Figure 5), but on none of 5 normal donors (P = 0.002).

Among WM patients expressing CD154, the median percentage of CD154 antigen expressing BM MC was 27% (range 2–62%).

expression of CD154 on MC on HMC-1, LAD2 and KU812 cell lines

We also observed CD154 expression by flow cytometry on HMC-1 MC and KU812 basophils, which became more pronounced following PMA/ionomycin exposure (Figure 6), as previously described by others [15, 16].

functional expression of CD40 on WM LPC

We next determined if the receptor for CD154 was expressed on WM LPC. Flow cytometric analysis demonstrated CD40 expression on BM LPC from 35/38 (92%) WM patients. The median percentage of BM LPC expressing CD40 among antigen
positive patients was 63.7 (range 22.5 to 98.1%). To determine if CD40 receptors on WM LPC were functional, we next evaluated the effect of the CD40 directed agonistic monoclonal IgG1 antibody G28.5 to stimulate proliferation of BM LPC. At concentrations of 0.1, 1.0, and 10 μg/ml, the G28.5 antibody induced a median 1.8 (range 1–15.5), 2.2 (range 1.2–35.3) and 3.0 (range 1.5–50.6) fold increase in tritiated thymidine uptake, respectively among CD40+ BM LPC taken from seven patients versus untreated or IgG1 control treated control cells (Figure 7A). Similar observations were also made following stimulation of one WM patient CD40 expressing BM LPC, wherein 0.5% PFA fixed CD154 expressing CHO cells were used as a source for CD40 stimulation (Figure 7B). Moreover, soluble CD154 ligand (sCD154) promoted survival of WM BM LPC from 3 of 4 patients in a dose dependent manner as assessed by staining with Apo2.7 (Figure 7C). Lastly, stimulation of BM LPC taken from one WM patient resulted in pERK 1/2 phosphorylation following simulation with either the G28.5 antibody or trimeric human recombinant CD154 (Figure 7D).

Figure 6. Expression of CD154 by multicolor flow cytometry at baseline (I) and following PMA/ionomycin stimulation (II) on HMC-1 MC and KU812 basophils cell lines.

Figure 7. Dose dependent proliferation and survival of BM LPC from a WM patient in response to CD154 stimulation using the CD40 agonistic antibody G28.5 (A), CD154 transfected CHO cells (B), and recombinant human soluble CD154 (C), as well as pERK activation following culture of WM BM LPC in response to soluble CD154, and G28.5 stimulation (D).

To determine if CD154 expression on MC was responsible for MC induced proliferation of WM patient LPC, we performed

blockade of CD154/CD40 signaling partially inhibits MC induced proliferation of WM LPC

To determine if CD154 expression on MC was responsible for MC induced proliferation of WM patient LPC, we performed
co-culture assays of MC and WM BM LPC in the presence or absence of a CD40:Fc chimeric protein which blocks CD154/CD40 signaling by binding to the CD154 signaling domain. PMA/ionomycin pretreated and sublethally irradiated HMC-1 and KU816 cells were co-cultured with CD40 expressing BM LPC taken from five WM patients at a ratio of 1:5 (HMC-1 or KU816 to BM LPC). Following addition of the CD40:Fc chimeric protein, a dose-dependent decrease in BM LPC proliferation, as assessed by tritiated thymidine analysis, was observed in LPC co-cultured with HMC-1 and KU812 cells taken from 3/5 (60%) and 2/4 (50%) patients, respectively (Figure 8). The median decrease in proliferation for responding BM LPC co-cultured with HMC-1 and KU812 cells following addition of the CD40:Fc chimeric protein was 50% and 28% at 10 μg/ml.

**discussion**

In these studies, we sought to clarify the role of MC in WM. The presence of excess MC in WM has served as a classic hallmark of the disease, having even been remarked upon by Jan Waldenström, though the functional significance for excess MC presence in WM remained to be clarified. Using MC and basophilic cell lines, and paired autologous BM MC from a WM patient, we demonstrated for the first time the ability of the MC to induce proliferation of WM patient BM LPC. Importantly, these studies demonstrated that MC expressed CD154, a potent inducer of malignant B-cell proliferation, while BM LPC functionally expressed the CD154 receptor, CD40. Moreover, the use of a CD154/CD40 signal inhibitor partially inhibited MC mediated BM LPC proliferation and/or tumor colony formation.

The results of these studies may have important therapeutic implications, and may also raise further questions about the teloelogical relevance of mast cells in B-cell homeostasis. From a therapeutic perspective, these studies suggest a potentially novel approach to the treatment of WM, such as direct targeting of BM MC and/or BM MC-WM LPC interactions. As part of an effort to target both BM MC and LPC in WM patients, we recently demonstrated the expression of CD52 and CD70, target antigens for the Campath-1H and SGN-70 monoclonal antibodies. Both antibodies led to robust antibody dependent cell mediated cytotoxicity (ADCC) killing of WM patient MC and LPC in these studies, which may explain the unusually high level of clinical activity observed with Campath-1H in WM, even among patients previously treated with rituximab which targets CD20, an antigen expressed on BM LPC but not BM MC. In addition to serotherapy, the use of agents which target growth signals vital to MC survival, such as stem cell factor receptor (c-kit) may be therapeutically relevant in WM and include those targeting CD154, a proliferation inducing ligand (APRIL), B-lymphocyte stimulator (BLYS) protein, platelet derived growth factor alpha (PDGFα) and vascular endothelial growth factor (VEGF). Conversely, agents which may block vital signaling of WM LPC to BM MC may be important therapeutically and include, by example, the SGN-70 monoclonal antibody which blocks CD27-CD70 signaling. As we recently demonstrated, high levels of soluble CD27 are found in circulation of WM patients, which induces both CD154 and APRIL expression on WM patient BM MC through CD70 signaling, which is blocked by SGN-70 [18]. A clinical trial examining SGN-70 in WM patients is contemplated. In addition to the therapy of WM, the above studies may be relevant to other malignancies wherein MC dependence has previously been suggested.

These studies coupled with others also suggest a teloological role for MC in B-cell homeostasis. The demonstration of CD154, as well as BLYS and APRIL expression on WM MC raises the possibility that MC, in the normal context, serve to regulate B-cell differentiation, immunoglobulin production and even heavy chain class switching: events possibly regulated by CD27/CD70 signaling, WM LPC express transcripts and secrete CD27 while high levels of soluble CD27 are found in WM patients, which might therefore provide the trigger for CD154 and APRIL expression by BM MC through CD70 signaling. Why WM LPC disobey such differentiation and immunoglobulin regulatory signals remains to be clarified, but in recently reported studies, we observed frequent mutations in the APRIL and BLYS receptor TACI in patients with WM. Both B-cell differentiation and immunoglobulin heavy chain class switching are mediated through TACI signaling in response to APRIL and BLYS.

In conclusion, these studies demonstrate that MC may support tumor cell expansion in WM through constitutive CD154-CD40 signaling, and therefore may provide a framework for therapeutic targeting of MC and MC-WM cell interactions in Waldenström's.
acknowledgements

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