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CD27-CD70 interactions in the pathogenesis of Waldenstrom's Macroglobulinemia

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Abstract

Waldenström's macroglobulinemia (WM) is a B-cell malignancy characterized by an IgM monoclonal gammopathy and bone marrow (BM) infiltration with lymphoplasmacytic cells (LPC). Excess mast cells (MC) are commonly present in WM, and provide growth and survival signals to LPC through several TNF family ligands (CD40L, APRIL, BLYS). As part of these studies, we demonstrated that WM LPC secrete soluble CD27 (sCD27), which is elevated in patients with WM ($p < 0.0009$ versus healthy donors), and serves as a faithful marker of disease. Importantly, sCD27 stimulated expression of CD40L on 10/10 and APRIL on 4/10 BM MC samples obtained from WM patients as well as on LAD2 MC. Moreover, the SGN-70 humanized monoclonal antibody which binds to CD70 (the receptor-ligand partner of CD27), abrogated sCD27 mediated upregulation of CD40L and APRIL on WM MC. Lastly, treatment of SCID-hu mice with established WM using the SGN-70 antibody, blocked disease progression in 12/12 mice, whereas disease progressed in all 5 untreated mice. The results of these studies demonstrate a functional role for sCD27 in WM pathogenesis, along with its utility as a surrogate marker of disease and a target in the treatment of WM.

Introduction

Waldenström's macroglobulinemia (WM) is a distinct B-cell lymphoproliferative disorder characterized primarily by bone marrow (BM) infiltration with lymphoplasmacytic cells (LPC), along with demonstration of an IgM monoclonal gammopathy¹. This condition is considered to be lymphoplasmacytic lymphoma as defined by the REAL and WHO classification systems.^{2,3} An interesting feature of the disease is the finding of increased number of mast cells (MC) in the BM of patients with WM, most typically in association with LPC⁴⁻⁷. This striking association has become characteristic of WM, and is often widely used as a supportive basis for making the diagnosis of WM^{2,3,6}.

Recently, we demonstrated that BM MC provide important growth and survival cues to WM LPC through multiple TNF-family ligands including CD40L (CD154), A Proliferation Inducing Ligand (APRIL), and B-lymphocyte stimulator factor (BLYS)⁸⁻¹⁰. Importantly, MC-induced expansion of WM LPC was inhibited by use of blocking proteins to CD40L, APRIL and BLYS. Moreover, direct therapeutic targeting of BM MC with alemtuzumab and imatinib mesylate have also resulted in remissions among patients with WM^{11,12}.

While the above studies have shown that MC can induce WM cells through multiple ligand-receptor signals, the mechanism(s) by which WM cells may potential facilitate such supportive signaling through MC remains to be clarified. One potential pathway for

WM-MC signaling is via CD70, a TNF-family member which is found on activated lymphocytes, stromal cells of the thymic medulla and mature dendritic cells but which is absent from other normal tissues including all vital organs¹³. CD70 has been shown to play a role in B lymphocyte regulation through binding to CD27, a TNF-family member expressed by thymocytes, natural killer, T, and B cells, including memory B-cells from which WM LPC may have derived¹⁴⁻¹⁸. As such, we sought to establish the expression of CD27 and CD70 in WM, and delineate their interactions between WM LPC and MC.

Materials and Methods

Approval for human studies was obtained from the Dana-Farber Cancer Institute IRB. Informed consent was provided according to the Declaration of Helsinki.

Cell lines and cultures

BCWM.1 and LAD2 cell lines were used in these studies. BCWM.1 is a cell line derived from an untreated patient with WM¹⁹, whereas the LAD2 cell line is a MC line derived from a patient with untreated mast cell sarcoma²⁰. Cells were maintained as previously described^{19,20}. Sorted lymphoplasmacytic cells (CD19⁺) and mast cells (FcεRI⁺, CD117⁺) were obtained from consenting WM patients and isolated as previously described^{9,18,21}.

RT-PCR Analysis

Total RNA was extracted using RNase Mini Kit (Qiagen, Valencia, CA, USA). 0.3 µg of RNA was reverse transcribed in a 20µL reaction by oligo-p-(dT)15 priming using Superscript III reverse transcriptase according to the protocol provided by Invitrogen (Carlsbad, CA, USA). First-strand cDNA was synthesized using Superscript III reverse transcriptase according to the protocol provided by Invitrogen. Two µL of first-strand cDNA was used as template for PCR amplification. PCR was performed using the PTC-200 DNA Engine Thermal Cycler (MJ Research Inc., Waltham, MA, USA).

Flow cytometric analysis

Direct immunofluorescence flow cytometric analysis was performed using a Coulter Epics XL with data acquisition software (Cytomics FC500-CXP; Beckman Coulter, Fullerton CA) as described previously^{9,18,21}. Peripheral blood mononuclear cells (PBMC)

or natural killer (NK) cells treated with antibody alone or in combination were washed, followed by dual immunostaining with specific anti-FITC-, or anti-phycoerythrin (PE)-conjugated mAbs to CD27, CD70 (Beckman Coulter) or APRIL, BLYS or CD40L (R&D Systems, Minneapolis MN).

ELISA analysis of WM patient serum

To evaluate cytokine production, 100 μ l of patient or age matched healthy donor serum was collected and analyzed for sCD27 by an ELISA kit per manufacturer's instructions (Bender Medsystems, Burlingame CA).

Functional Assays for sCD27

BCWM.1 LPC, LAD2 MC, as well as primary BM LPC and MC isolated from 10 WM patients were cultured for 24-48 hours with recombinant human sCD27 (0.1-50 ng/mL). Proliferation and apoptosis were assessed by tritiated thymidine analysis, and APO2.7 staining as before^{9,18,21}. Changes in cell surface expression of APRIL, BLYS and CD40L were determined by flow cytometric analysis following culture for 24 hours with sCD27 (10 μ g/mL) in the presence or absence of the anti-CD70 human monoclonal antibody SGN-70 (1 μ g/mL) (Seattle Genetics, Bothell Washington)¹³.

Antibody-dependent cell-mediated cytotoxicity (ADCC) assays

WM target cells labeled with calcein-AM were co-cultured with normal donor NK cell enriched peripheral blood mononuclear cells as before²¹ at various E/T ratios for 2 hours at 37°C in either medium alone or with SGN-70 (0-20 μ g/mL). Following incubation

with or without SGN-70, cells were centrifuged and absorbance in supernatants measured by spectrophotometry. Percentage of specific lysis was calculated using the equation $S_p - S / M - S$, where S_p to denote experimental lysis caused by antibody. S denotes spontaneous lysis, and M is maximum cell lysis in Triton X-100. $\{(M - \text{medium control release}) / (S - \text{medium control release})\} > 7$ validated each experiment. Experiments were performed at least twice.

Mouse model

WM SCID-hu mice were generated as previously described²². The mice were housed and monitored in a VA Animal Research Facility. The Institutional Animal Care and Use Committee approved all experimental procedures and protocols. Increasing levels of circulating human paraprotein in mice sera were used to monitor tumor engraftment and growth of BCWM.1 WM cells in SCID-hu mice. Mouse blood was collected from tail vein, and sera were serially tested for circulating IgM, IgG, and κ and chain by enzyme-linked immunosorbent assay (ELISA; Bethyl, Montgomery, TX).

Results

Expression of CD27 and CD70 in WM LPC and MC

We first sought to establish CD27 and CD70 expression on WM BM LPC and MC. By reverse transcriptase polymerase chain reaction (RT-PCR) analysis, CD27 transcripts were expressed in BM LPC from 7 of 7 WM patients and on the BCWM.1 WM cell line (Table 1). However, in contrast to the wide expression of CD27 transcripts by RT-PCR analysis, cell surface expression of CD27 on BM LPC was observed on only 5/12 (42%) WM patients, and was absent on BCWM.1 WM cells. CD27 was less commonly expressed on BM MC, with expression observed by RT-PCR and flow cytometric analysis in 4/7 (52%) and 2/8 (25%) of WM patients, respectively but not in LAD2 mast cells (Table 1). In contrast to the heterogeneous expression of CD27 on WM LPC and MC, CD70 was found to be widely expressed on BM LPC and MC, as well as on BCWM.1 WM and LAD2 MC by both RT-PCR and multicolor flow cytometric analysis (Table 1).

Soluble CD27 in WM patients and healthy donors

In view of the above results demonstrating constitutive CD27 transcript expression in WM LPC, but expression at the cell surface level in only a subset of patients, we next sought to delineate if soluble sCD27 was produced by WM LPC. By ELISA, soluble

CD27 was detectable in supernatants taken from BCWM.1 cells, which express CD27 transcripts by RT-PCR but are devoid of cell surface CD27 expression (data not shown). Given these results, we next sought to determine if sCD27 levels were elevated in patients with WM, IgM monoclonal gammopathy of unknown significance (MGUS), multiple myeloma (MM) as well as healthy donors. As shown in Figure 1, significantly higher levels of sCD27 were observed in the serum of patients with active WM (n=67, median 120.1, range 27.2-626.2 U/mL) and IgM MGUS (n=3, median 71.3, range 51.3-156.7 U/mL) versus age matched healthy donors (n=16, median 45.7, range 24.7-87.7 U/mL); $p=0.0009$, and 0.01 for comparison by student's t-test of sCD27 levels in WM and IgM MGUS patients versus healthy donors, respectively. In contrast, sCD27 levels for MM patients with active disease (n=25, median 54.2, range 32.3-87.7 U/mL) did not differ from those observed in healthy donors ($p=0.2$).

Soluble CD27 as a marker of disease burden in WM

We next investigated if sCD27 served as a marker of disease burden in patients with WM. Soluble CD27 levels were serially measured in 31 patients whose clinical outcomes (responder, stable disease, or progressive disease) were determined using consensus panel criteria based on concurrently measured changes in serum IgM levels²³. As shown in Figure 2, changes in sCD27 levels paralleled those of serum IgM levels in all outcome categories, and highly correlated to serial changes in serum IgM levels ($r= 0.9760$; $p<0.0001$ by Pearson's correlation test).

Sequence analysis of CD27 and CD70 in WM

As part of studies delineating CD27-70 interactions in WM, we sequenced CD27 and CD70 from genomic DNA isolated from BM LPC and MC isolated from 15 WM patients. All exons with flanking intronic regions were sequenced on both strands. These studies demonstrated no novel sequence variants in CD27 and CD70 when compared to consensus sequences (NCBI human genome build 36.2) and all known polymorphisms appeared at their expected frequency with normal distribution.

Functional role of soluble CD27 in WM

To define a functional role for sCD27-CD70 interactions in WM, we cultured BCWM.1 (CD27⁻CD70⁺) WM cells, LAD2 (CD27⁻CD70⁺) MC, as well as primary BM LPC and MC (CD70⁺) isolated from WM patients with recombinant human sCD27 (0.1-50 µg/mL). We observed no direct effect of sCD27 on the proliferation of BCWM.1 and LAD2 cells (as assessed by tritiated thymidine analysis) nor on the induction of direct apoptosis (as assessed by APO2.7 staining) on BCWM.1 and LAD2 cells as well as BM LPC and MC from 10 WM patients (Figure 3). However, as shown in Figure 3, culture of LAD2 MC as well as primary MC with sCD27 from 10/10 and 4/10 WM patients resulted in marked up-regulation of CD40L and APRIL, respectively. Importantly, SGN-70 (1 µg/mL), a CD70 directed monoclonal antibody which blocks CD27-CD70 signaling, abrogated sCD27 up-regulation of CD40L and APRIL (Figure 4). In contrast, we observed no changes in levels of BLYS on either WM MC or LPC, nor changes in

CD40L or APRIL levels on CD70 expressing WM LPC following sCD27 stimulation (data not shown).

Direct targeting of WM LPC by the CD70 directed human monoclonal antibody SGN-70.

We next assessed the SGN-70 monoclonal antibody for its ability to kill WM LPC by direct induction of apoptosis, by complement dependent cytotoxicity (CDC), and through antibody-dependent cell-mediated cytotoxicity (ADCC). We did not observe induction of apoptosis nor CDC activity for SGN-70 against BCWM.1 LPC at dose levels up to 20 $\mu\text{g/mL}$ (data no shown). However, as shown in Figure 5, SGN-70 mediated robust dose-dependent ADCC activity against BCWM.1 cells. We further evaluated the therapeutic potential of SGN-70 in an *in vivo* WM model utilizing SCID-hu mice bearing BCWM.1 cells. SCID-hu mice were engrafted with BCWM.1 cells and treated with the SGN-70 antibody after demonstrating progressive WM, as assessed by serial increases in human IgM and sCD27 serum levels. Treatment of WM SCID-hu mice every other day with SGN-70 (1 mg/kg, intraperitoneally) blocked disease progression, as assessed by serial human IgM and sCD27 serum levels in 12 of 12 mice, whereas disease continued to progress in all 5 of 5 untreated mice; $p=0.0002$ by Fisher's exact test (Figure 6).

Discussion

The results of these studies demonstrate an important role for sCD27-CD70 signaling among BM LPC and MC in WM, and demonstrate a novel mechanism of action for sCD27 as a regulator of two principal TNF family members (APRIL and CD40L) whose role as growth and survival factors has previously been established by us and others in WM and other B-cell malignancies. Additional to its functional role, these studies also suggest that sCD27 may serve as a faithful marker of disease in patients with WM. Notably, our data also suggest that targeting CD70 and sCD27-CD70 interactions may produce important clinical benefits for patients with WM, and possibly other B-cell malignancies such as chronic lymphocytic leukemia (CLL), and Hodgkin's disease (HD), as well as autoimmune related disorders wherein either elevated sCD27 levels have been reported²⁴⁻³⁰ or the presence of excess MC has been observed in association with lymphoid aggregates³¹⁻³⁴. Investigation, therefore, of a role for sCD27-CD70 interactions among lymphocytes and MC in these disorders would appear warranted.

It remains unclear at present whether the high levels of sCD27 detected in WM patients is a result of a malignant event, or part of a normal homeostatic mechanism for control of LPC expansion. Normally CD27 is expressed on the cell surface of memory B-cells from which WM is thought to derive. However, in WM, CD27 is heterogeneously expressed, and more often is absent on the cell surface of WM LPC^{7,13,17}. Both APRIL and CD40L play important roles in normal LPC differentiation, and it remains plausible that their

expression on MC (and possibly other regulatory cells) are induced by sCD27 as a normal countermeasure to curtail LPC expansion by inducing their differentiation.

The release of sCD27 by CLL cells through the action of matrix metalloproteases (MMP) has been suggested in one report³⁵. It remains possible that over-expression of MMP may contribute to the cell surface loss of CD27 and subsequent release of sCD27 in WM. Investigation for a role of MMP in modulating sCD27 release is therefore warranted as is also the potential therapeutic role of MMP inhibitors.

The lack of cell surface CD27 observed in this and other studies of LPC in most patients with WM^{7,14,18} may also have important implications for the deregulation of LPC homeostasis in WM. Upon binding of CD70 to CD27, the pro-apoptotic adaptor proteins SIVA-1, and SIVA-2 bind to the intercellular domain of CD27 and trigger apoptosis via a caspase dependent mitochondrial pathway^{36,37}. The loss of cell surface expression of CD27, or inhibition of CD70 signaling by tumor released sCD27, or loss of SIVA-1 and SIVA-2 may represent pathways by which normal homeostasis of LPC is lost in WM³⁸. Our sequencing studies did not detect any variants in CD27 to suggest that a solubilized form of this molecule resulted from mutation inducing truncation of this protein. However, it does remain possible that post-transcriptional or post-translational events may still be shifted as an epigenetic malignant phenomenon resulting in the overproduction of sCD27. Investigation into these potential mechanisms of WM pathogenesis is currently ongoing in our laboratory.

Additional investigation is also warranted to uncover the detailed molecular mechanisms underlying the upregulation of CD40L and APRIL by sCD27 on mast cells. Recent work has shown that CD70 activates the PtdIns-3 kinase, NFκB, protein kinase B and Erk MAP kinase pathways^{16,39}. CD70 signaling affects cell cycle entry and progression in activated B cells and impedes plasma cell differentiation and IgG production in response to hapten–protein conjugates *in vivo*³⁹. Signaling through CD70 on WM mast cells by sCD27 resulted in the upregulation of CD40L and APRIL; yet, this CD70 specific upregulation was not observed in WM LPCs. This implies that there may be different signaling properties for CD70 between WM LPCs and mast cells, or potentially an inhibitory network in WM LPCs. Moreover, our lack of observation of a proliferative or survival effect of sCD27 on CD70 expressing WM LPC may also reflect the lack of other co-stimulatory signals such as CD40L as suggested by the studies of Arens et al¹⁶. Further studies to characterize a direct functional role for sCD27 on WM LPC are underway in our laboratory.

Lastly, the results of this study suggest that disruption of the CD27-CD70 signaling pathway may yield important clinical benefits for patients with WM, and possibly in patients with other malignancies and autoimmune disorders in which sCD27-CD70 interactions may have a pathogenic role. Use of the SGN-70 monoclonal antibody, which is in early clinical development¹³, blocked disease progression in SCID-hu mice with established WM. The mechanism(s) by which SGN-70 blocked disease progression in the SCID-hu WM mouse may be multifactorial (i.e. immunological and biological) and further studies to characterize its *in vivo* activity model are needed. ADCC or CDC

activity or some other immune effector function (i.e. phagocytosis) may be possible as suggested by our previous studies using rituximab in this mouse model²² as is also blockade of sCD27-CD70 interactions. We propose in future experiments to use anti-SGN-70 Fab fragments in SCID-hu mice to further clarify this point.

In summary, the results of these studies suggest a functional role for sCD27 in WM pathogenesis, along with its utility as a surrogate marker of disease and a potential target for the development of therapeutics for WM.

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Authorship

AWH, EH, ARB, ZRH, XL, OT, LX, KO, performed basic science experiments including ELISA assays, flow cytometry, RT-PCR. AWH, DDS, MC performed animal studies. CJP, JS and RJM provided clinical correlative data. JAM, CLL, ISG provided SGN-70, and supported therapeutic antibody studies. NCM, SPT oversaw research. SPT analyzed data and wrote manuscript.

Conflict of Interest Disclosure: ISG, JAM and CLL are paid employees of Seattle Genetics, Inc, manufacturer of SGN-70.

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	Flow Cytometry		RT-PCR	
	LPC	MC	LPC	MC
CD27	5/12 (42%)	2/8 (25%)	7/7 (100%)	4/7 (52%)
CD70	6/6 (100%)	10/11 (91%)	7/7 (100%)	7/7 (100%)

Table 1. CD27 and CD70 expression on bone marrow lymphoplasmacytic cells (LPC) and mast cells (MC) as assessed by flow cytometric and reverse transcriptase polymerase chain reaction (RT-PCR) analyses.

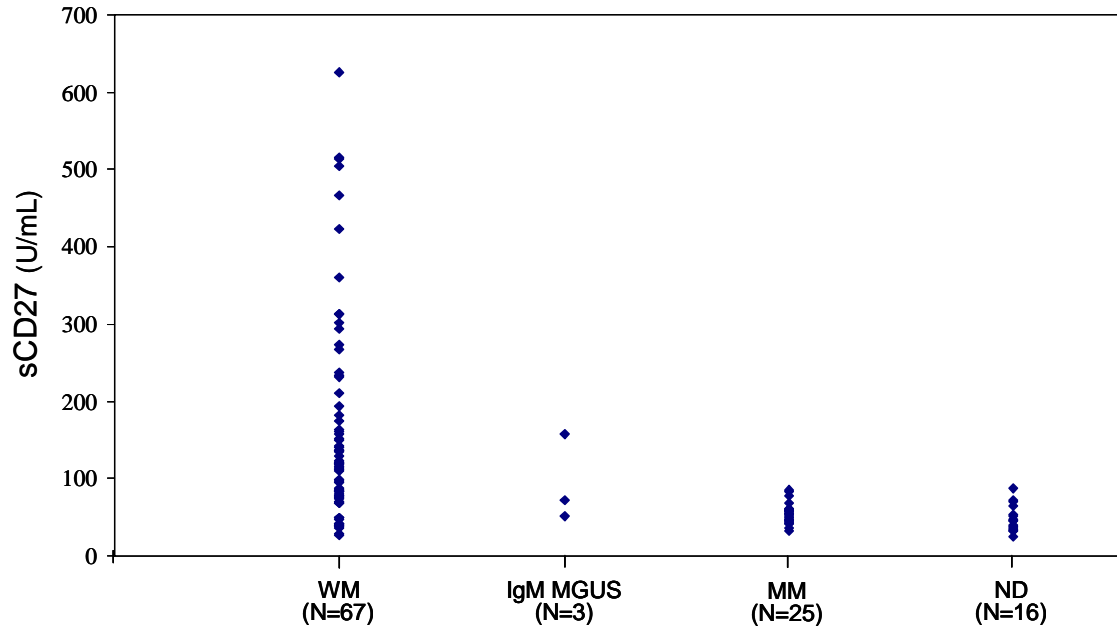
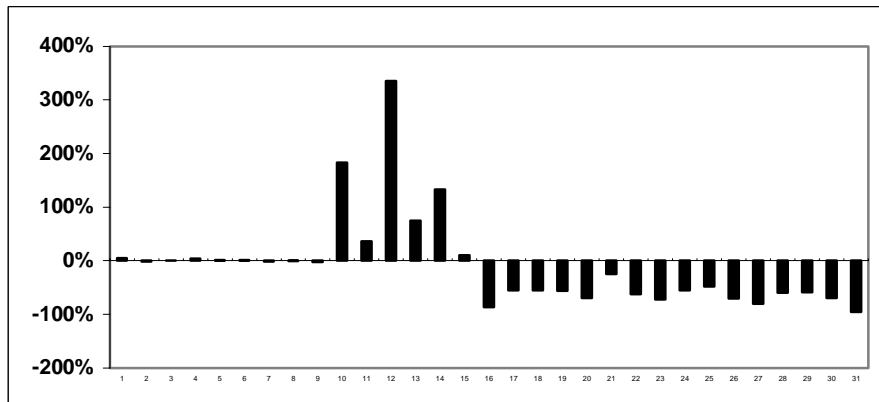
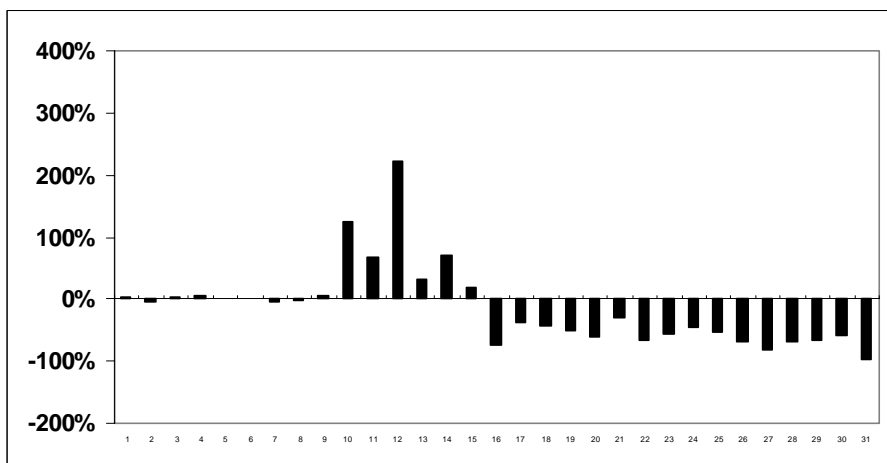


Figure 1. Soluble CD27 (sCD27) levels in serum of patients with Waldenstrom's macroglobulinemia (WM), IgM monoclonal gammopathy of unknown significance (MGUS), multiple myeloma (MM) and healthy donors.

A. Serum IgM levels



B. Serum sCD27 levels



C. Correlation of changes in serum IgM and sCD27 levels

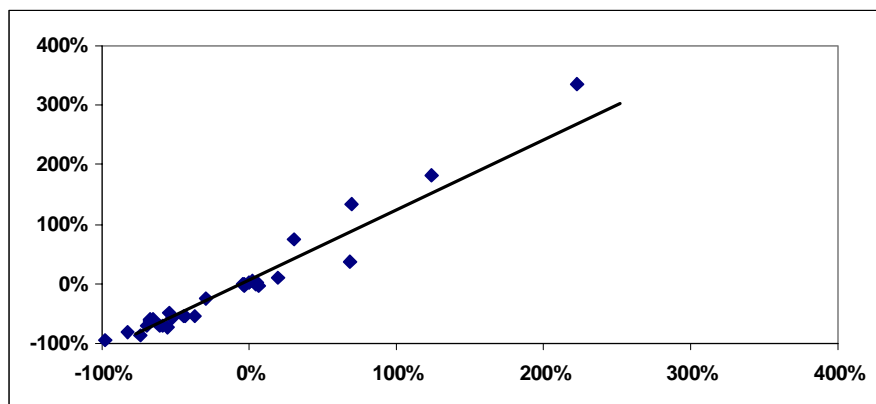
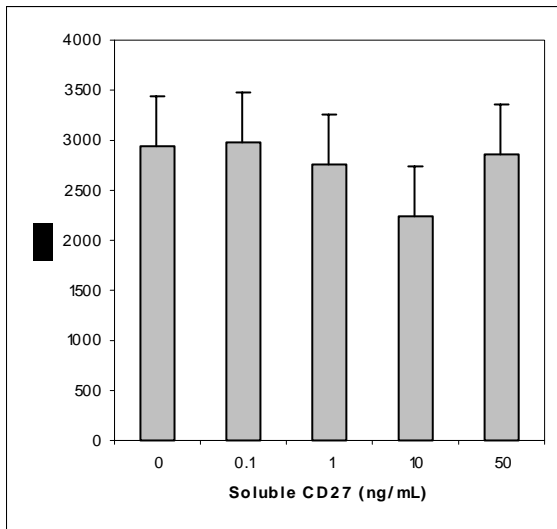
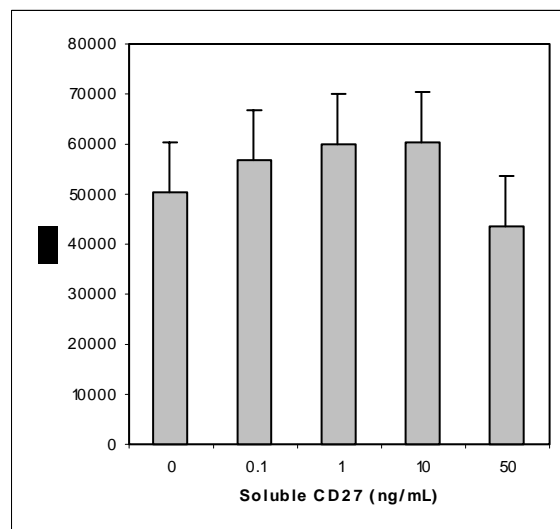


Figure 2. Serial (A,B) and correlative (C) changes in serum IgM and sCD27 levels in 31 WM patients (WM 1-9, stable disease; WM 10-16, progressive disease; and WM 17-31, responders) whose outcomes were determined by consensus panel response criteria²³.

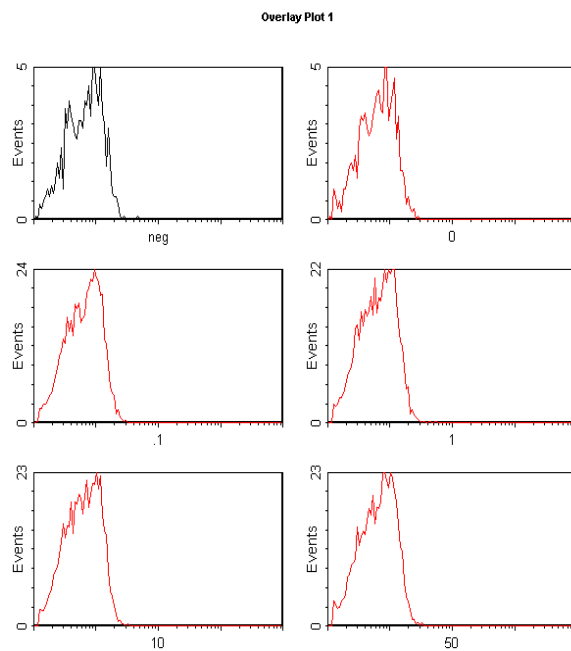
A.



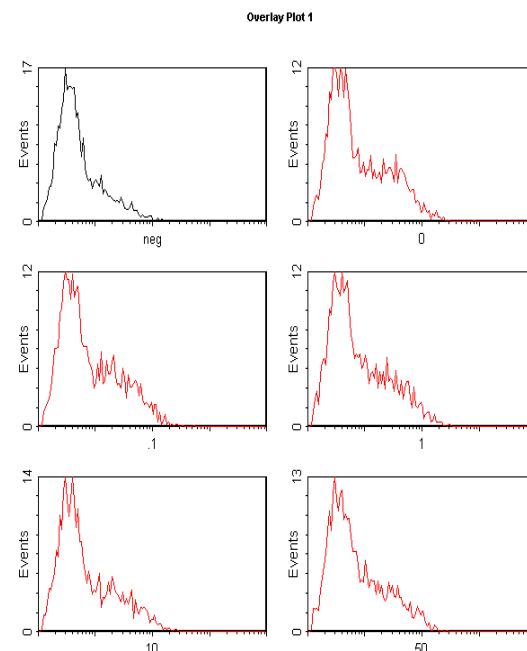
B.



C.



D.



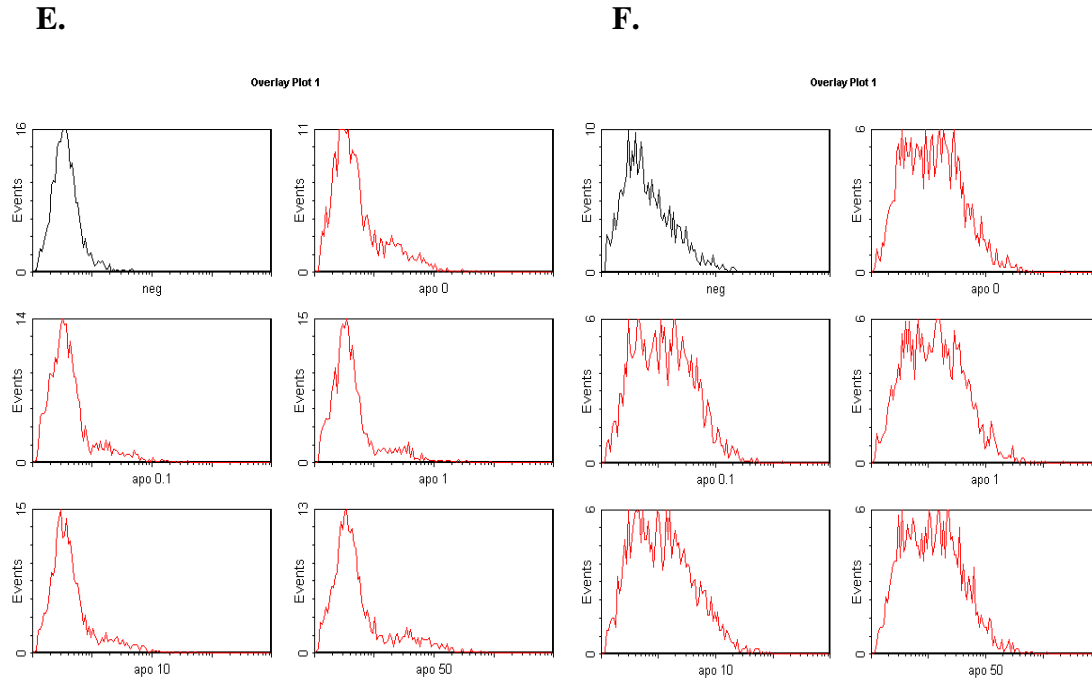


Figure 3. Effect of soluble CD27 (0-50 ng/mL) on proliferation of BCWM. 1 LPC (A) and LAD2 MC (B) as assessed by tritiated thymidine uptake, as well as on induction of apoptosis of BCWM.1 LPC (C) and LAD2 MC (D), and on bone marrow LPC (E) and MC (F) from a representative WM patient as assessed by APO2.7 staining. For Figures C-F, first histogram represents unstained control cells, while histograms 2-5 represent cells stained with APO2.7 following treatment with sCD27 at 0, 0.1, 1, 10 and 50 ng/mL.

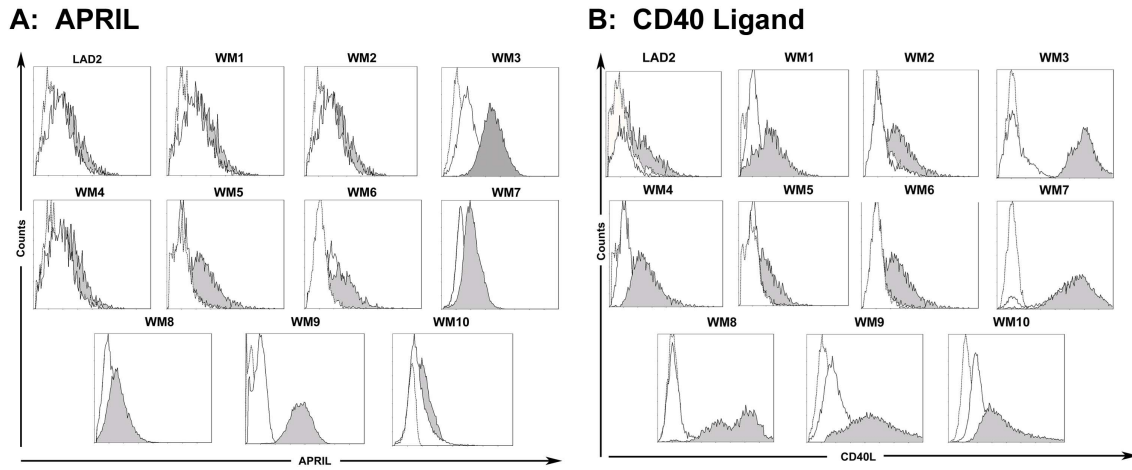


Figure 4. Expression of APRIL (A) and CD40 ligand (B) on CD70 expressing LAD2 mast cells, and primary bone marrow mast cells from 10 patients with Waldenstrom's macroglobulinemia following stimulation with sCD27 (10 $\mu\text{g}/\text{mL}$) alone (shaded histogram) or sCD27 (10 $\mu\text{g}/\text{mL}$) after pre-incubation with the CD70 directed SGN-70 (1 $\mu\text{g}/\text{mL}$) monoclonal antibody (clear histogram, solid line). Dotted, clear histograms denotes isotype control.

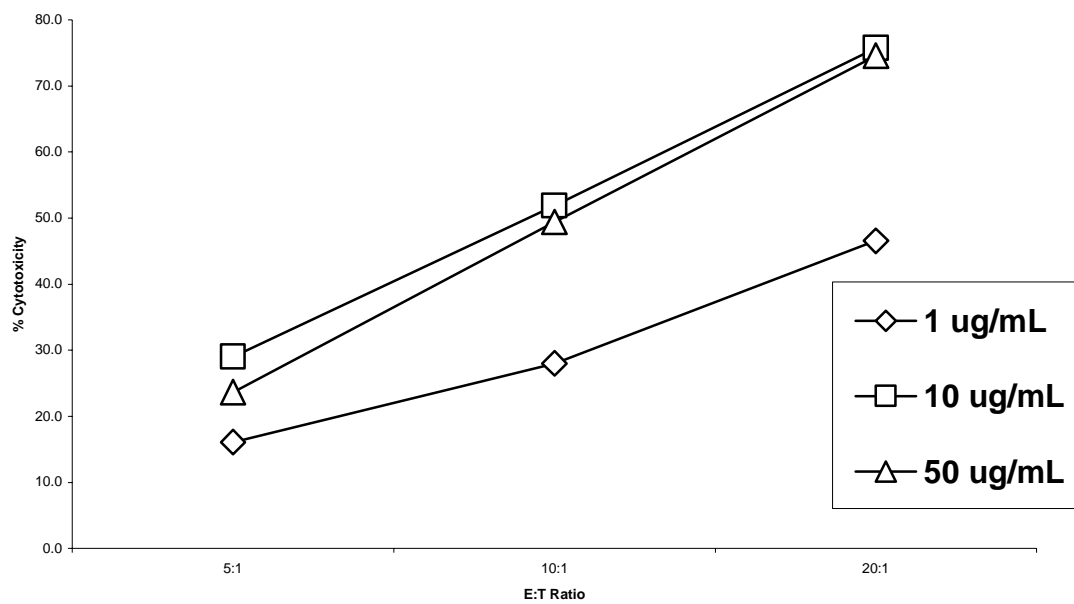
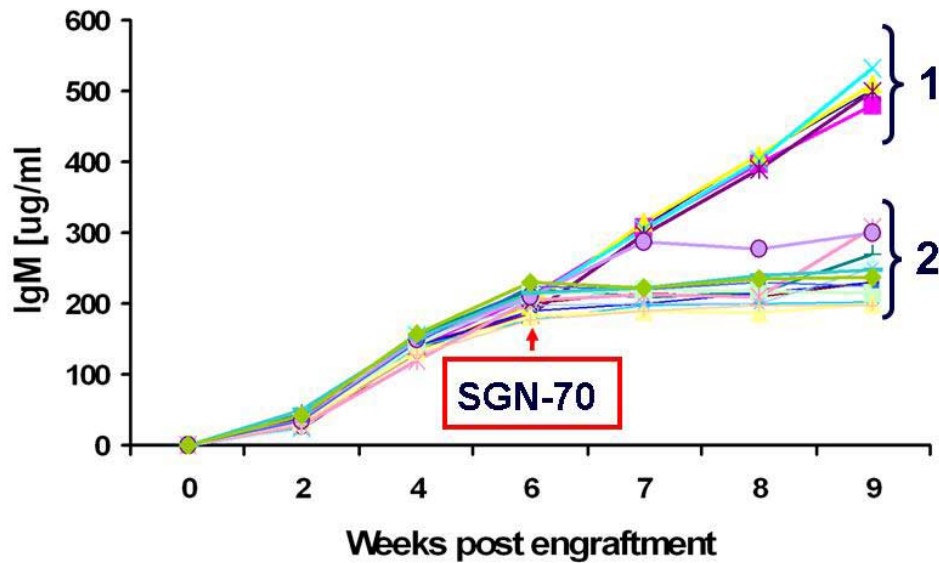


Figure 5. Specific lysis (%) for SGN-70 mediated antibody-dependent cell-mediated cytotoxicity (ADCC) against CD70 expressing BCWM.1 WM lymphoplasmacytic cells at effector: target (ET) ratios of 5:1 to 20:1.

A. Serial changes in serum IgM levels in BCWM.1 engrafted SCID-hu mice



B. Serial changes in serum CD27 levels in BCWM.1 engrafted SCID-hu mice

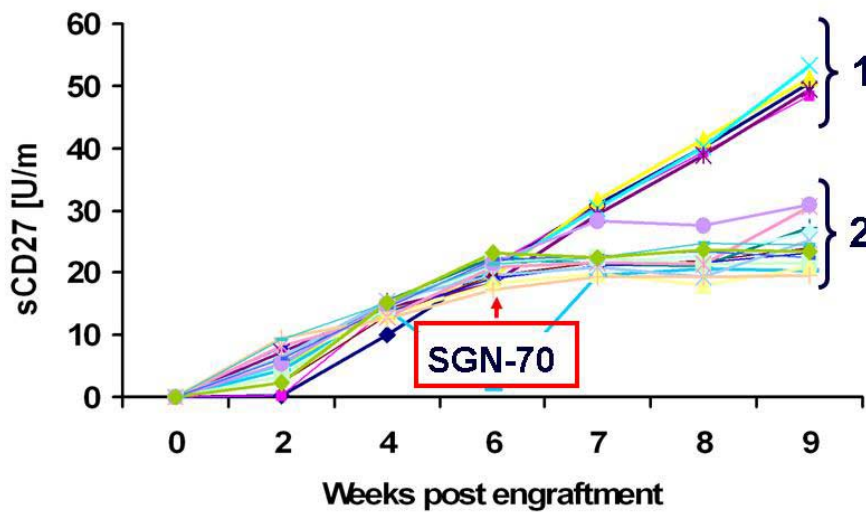


Figure 6. Effect of SGN-70 on disease progression as assessed by serial changes in serum IgM (A) and sCD27 (B) levels in SCID-hu mice engrafted with BCWM.1 WM lymphoplasmacytic cells. Group 1 denotes untreated mice (n=5), and Group 2 denotes mice treated with SGN-70 (n=12).