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The role of the CD28-C86 signaling module in maintaining myeloma cell viability

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The role of the CD28-CD86 signaling module in maintaining myeloma cell viability

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B.S., University of the Philippines, 2003

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Abstract

Multiple myeloma is an incurable hematologic malignancy of long-lived antibody secreting cells. Despite advances in therapeutics in the past decade that have led to vastly improved patient outcomes, there is still no known cure. Myeloma cells resemble their normal counterpart, long-lived plasma cells that reside in the bone marrow, to such an extent that survival factors that regulate plasma cell survival have also been shown to be required to maintain myeloma cell viability. A reliance on the bone marrow microenvironment for growth and survival signals is a characteristic shared by normal and malignant plasma cells. These signals are mediated by interactions between the myeloma cells and other components of the bone marrow stroma. We hypothesize that disrupting these interactions can induce apoptosis in myeloma cells, providing another means to target this disease.

We propose that one interaction that could be targeted for clinical benefit in myeloma is that between CD28 and CD86. These molecules are better characterized for their role in T-cell costimulation, however the signaling pathway/s they mediate have been shown to be important in maintaining plasma cell longevity in murine models. Previous studies from our lab have shown that activation of the CD28 pathway can protect myeloma cells against different death signals. These molecules are frequently expressed in myeloma cells, thus research into the signals relayed by this module is important to determine the role/s it plays in myeloma pathogenesis.

Our data shows that blockade of this pathway by inhibiting expression of either CD28 or CD86 with shRNAs leads to myeloma cell death. When we investigated gene expression changes when either molecule was silenced, we found that one of the genes that was down-regulated with CD28 or CD86 silencing was IRF4, a well-known myeloma survival factor. We also found that modulation of this signaling pathway led to downregulation in the expression of integrins, and solute carrier (SLC) family members, showing that this pathway regulates multiple aspects of myeloma physiology that are important in maintaining viability and function.

Interestingly, our data also shows that CD86, the canonical ligand in this signaling module, has signaling capacity. Overexpression of full-length CD86 protects myeloma cells against different death signals. In contrast, a “tail-less” version of CD86 could not protect against the same insults, indicating that CD86 is relaying a pro-survival signal in myeloma cells that is dependent on its cytoplasmic tail. Altogether, our data indicates that further investigation of the signals mediated by the CD28-CD86 signaling module is warranted in the context of myeloma, given that these two molecules are regulating important myeloma survival factors.

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Chapter 2: CD86 regulates myeloma cell survival

I. Introduction

Multiple myeloma (MM) is a malignancy of long-lived plasma cells, and is the second most common hematologic malignancy. Like most cancers, the primary risk-factor for the disease is age, as myeloma is most commonly diagnosed in people over the age of 60. The criteria upon which diagnoses of myeloma are based include elevated calcium levels, renal dysfunction, anemia, bone lesions (aka CRAB) in patients, as well as high M protein levels in urine and/or sera[1]. While the median survival of myeloma patients has vastly improved over the past decade, the disease is still considered incurable, as patients eventually succumb to drug-resistant disease.

Unlike other cancer types, MM cells retain most of the physiologic characteristics of their normal counterpart, the long-lived bone marrow plasma cell[2,3]. MM cells have well-developed protein secretory machinery, as they still secrete a form of immunoglobulin. They home and reside in the bone marrow, where their ability to survive and proliferate is heavily dependent on signals coming from the surrounding stroma. Because myeloma cells so closely resemble normal long-lived plasma cells, understanding the biology, stress response, and survival pathways upon which these cells rely could lead to new therapeutic avenues that we can exploit for even better prognoses for patients. Importantly, as plasma cell dysfunction impact other diseases, such as cancer (multiple myeloma, Waldenstrom's macroglobulinemia), autoimmunity (lupus, rheumatoid arthritis), as well as transplantation-associated disorders, learning how to better target myeloma cells could also lead to therapeutic avenues for other diseases.

A. Myeloma cells are long-lived bone marrow plasma cells gone awry

Myeloma cells retain plasma cell function and longevity

B cells are lymphocytes that develop in the bone marrow and express surface membrane-bound antibodies. Long-lived plasma cells are terminally differentiated B-cells that are the cornerstones of humoral immunity (Figure 1), and the primary function of these cells is to secrete immunoglobulin, which recognize pathogenic factors and act as molecular tags to facilitate neutralization. Plasma cell differentiation involves suppression of the B-cell program, which results in metamorphosis of the B-cell from a sentinel into a specialized protein secreting machine that can survive for the lifetime of the host[4-6].

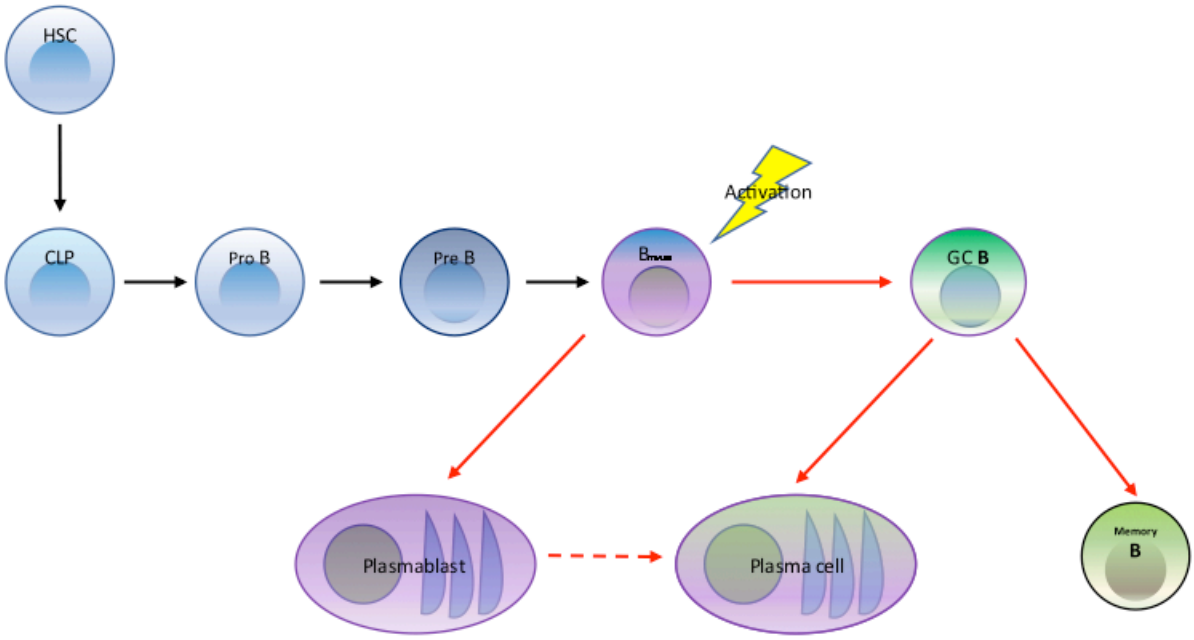

Figure 1. The B cell lineage. B cells develop from common lymphoid progenitors (CLP), a precursor that also gives rise to T cells. B cells then undergo further differentiation, which is dependenet on the ability of the cell to make a function immunoglobulin molecule. Upon activation, B cells can undergo further specialization depending on the stimulus received, as well as CD4 T cell help

Plasma cells had been initially characterized to be short-lived, with a lifespan of only a few hours to a few days[7-9]. In these studies, rodents were vaccinated with albumin from a different species, injected with tritiated thymidine after secondary challenge, then cellular components of various lymph nodes were characterized at different time points post-injection. Plasma cell numbers were measured via meticulous visualization of cellular morphology. Both groups were unable to find labeled plasma cells past 12 hours post-injection of tritiated thymidine. From these, they surmised that plasma cells were short-lived "end cells" with little proliferative capacity[7,8]. Their data indicated that antibody titers were likely maintained by the constant activation and differentiation of precursors into antibody secreting cells. These data are in stark contrast to what is known about plasma cells and their longevity, likely because their search was limited to those cells found in the lymph nodes. Both groups, however, did point out that migration out of the nodes, and cell death could well explain the disappearance of cells. However, their results did characterize that plasma cells do not proliferate much, if at all.

One of the first studies indicating that plasma cells may have longer lifespans than originally thought also involved systemic labelling with tritiated thymidine post-challenge of rats (this time with a *Salmonella* strain)[10]. This study was focused on determining if plasma cell lifespans lasted longer than a few days. J. Miller directly compared the number of plasma cells between unimmunized, primary-challenge, and post-secondary challenge up to 24 weeks after the last dose of label, and found that there were still plasma cells even at longest time point tested (1 year, according to his addendum)[10]. Importantly, J. Miller found that the plasma cells still secreted functional

antibody as long as 3 months after challenge (longest time point post challenge tested), indicating their important role in maintaining (protective) antibody titers.

The question regarding plasma cell longevity was further investigated by Manz *et al.* in the 1990s, and their data indicated that long-lived plasma cells (LLPCs) likely had the same lifespan as memory B cells[4]. They immunized mice with ovalbumin, and then administered a boost. After secondary challenge, they injected bromodeoxyuridine (BrdU) systemically into mice either for the next 19 days (from time of boost), or starting at 19 days post-boost, and until around 110 days after[4]. They found that the number of labelled plasma cells between the 2 groups were fairly similar, and that plasma cell numbers reached the peak 2 months post-boost and were maintained from then on, with little to no cell proliferation, indicating that these cells could not have come from the rapid proliferation of primed precursors. Subsequently, it was shown by Slifka *et al.* that antibody titers were maintained for greater than a year despite the in vivo depletion (via total body irradiation) of memory B-cells[5], showing that bone marrow plasma cells are a distinct lineage from memory B cells, and that they are fairly long-lived.

The longevity of plasma cells and their important role in mediating long-term humoral immunity is well characterized, particularly in the field of vaccinology[6,11]. The half-lives of plasma cells have been measured to surpass even the life-expectancy of the host[6,12]. The factors that maintain the longevity of these cells is still widely researched today, given the proven positive impact of vaccines in human health. However, the intrinsic longevity of myeloma cell precursors is an "advantage" in the pathogenesis of myeloma, given that in the normal process of carcinogenesis the ability to evade apoptosis is "earned" by mutations[13]. Plasma cells despite being long-lived, are essentially non-

proliferative. However, processes involved in normal plasma cell development leads to acquisition of the next requirement for oncogenesis – the ability to grow.

Myeloma cells, like most long-lived plasma cells, arise from the germinal center reaction

Bone marrow plasma cells arise mainly from activated B-cells that undergo the germinal center reaction. Upon antigen stimulation, B cells can go through different routes of specialization, depending on the nature of the antigen it recognizes (Figure 2). Some will develop into short-lived plasmablasts, and become the primary source of antibody early in the immune response. Those B cells that receive sufficient T cell help can home to germinal centers (GCs), sites in secondary lymphoid organs where T and B cell interactions result in the proliferation and development of different effector cell subsets[14,15].

B cells that home to GCs can undergo class switch recombination (CSR) and affinity maturation. These processes are mediated by controlled DNA damage and repair mechanisms with the end-goal of producing cells with higher affinity antibodies. CSR is a process that leads to isotype switching of the antibody heavy chains, resulting in distinct antibody effector functions[16,17]. The process of affinity maturation involves somatic hypermutation (SMH) specifically at the regions involved in antigen binding, which results in higher affinity antibodies[18]. B cells that express antibodies with higher affinity for antigen have an advantage in terms of their ability to more strongly interact with the follicular dendritic cells and T follicular helper cells in the GC. Because the

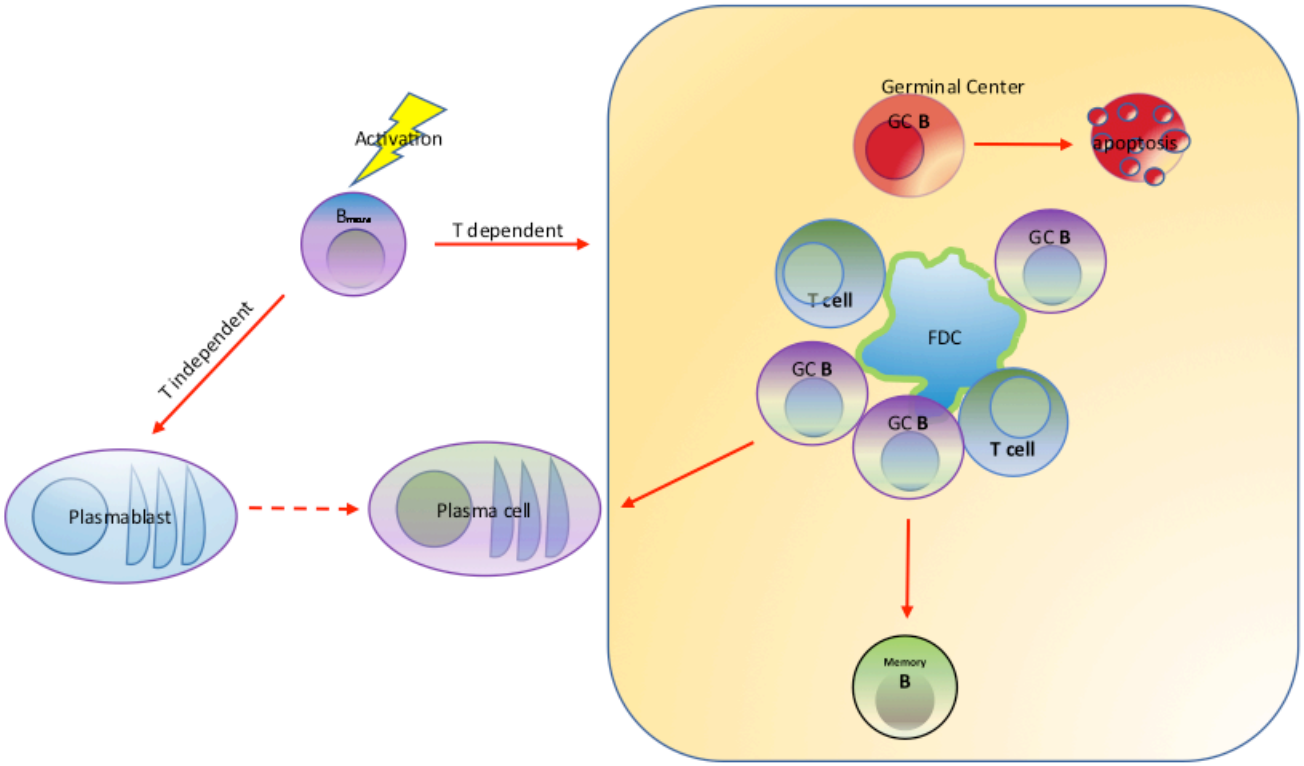

Figure 2. The determination of B cell fates post-antigen recognition. T independent antigens lead to a shorter path of B cell differentiation, as B cells differentiate into plasmablasts, short-lived secretory cells that can differentiate further into long-lived plasma cells under the right conditions. In the case of T-dependent stimuli, B cells can home to germinal centers, where interactions with CD4 T cells and follicular dendrtitic cells can drive them to differentiate further, depending on strength of signal received. Germinal centers are the sites where B cells can undergo affinity maturation via somatic hypermutation and class switch recombination, producing higher affinity antigen receptors. These cells can go down a further path of specialization and differentiate either into memory B cells, or long-lived plasma cells, depending on affinity of the antibody they now produce, as well as on signals from the milieu.

survival signals in this niche is limited, these B cells are thought to have a higher chance of differentiating into long lived plasma cells[14,15].

Importantly, the germinal center reaction is where a high rate of DNA mutagenesis occurs, as the processes mentioned above involve inducing DNA breaks and point mutations in immunoglobulin coding regions to produce higher affinity clones[17]. These processes are intrinsic to lymphocyte (both B and T cell) biology, as these are the same pathways involved in the generation of the diverse antigen receptor repertoire. However, despite how tightly regulated these processes are, errors can occur. These mistakes can result in an array of hematologic malignancies that are classified according to the stage of lymphocyte development in which the mistake occurred (Figure 3).

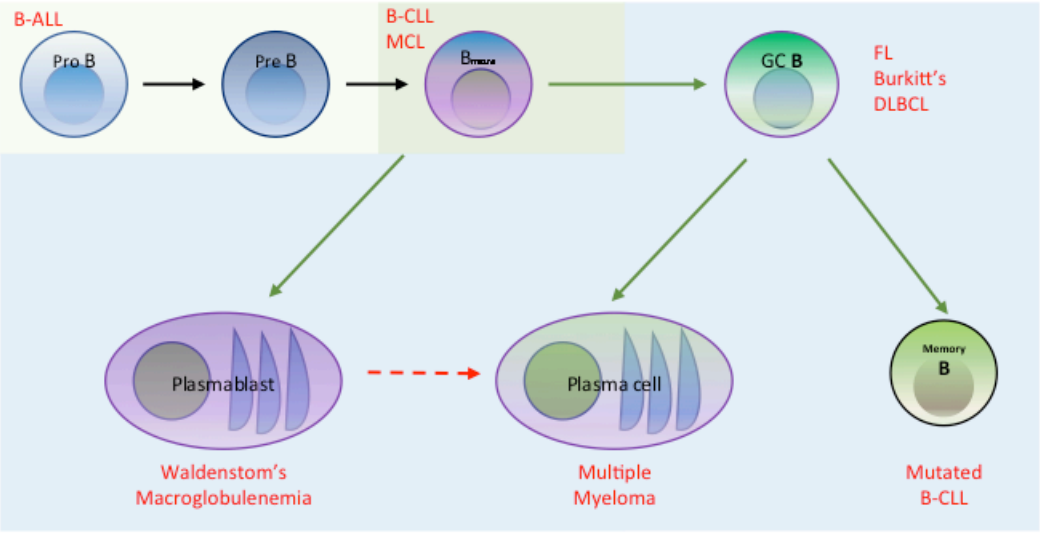

Figure 3. B cell malignancies derived from their lineage counterparts. Diagram showing different B cell lineages and the different hematologic malignancies that can arise from each B cell stage. Definitions of acronyms are as follows: ALL (acute lymphocytic leukemia); CLL (chronic lymphocytic leukemia); MCL (mantle cell lymphoma); FL (follicular lymphoma); Burkitt's (Burkitt's lymphoma); DLBCL (diffused large B-cell lymphoma)

Myeloma is a disease characterized by chromosomal abnormalities, most of which involve juxtaposition of an oncogene near enhancers for antibody-coding loci, resulting in overexpression of factors that can drive proliferation (reviewed[3,19-21]). The initiation of myeloma pathogenesis can thus be linked to the GC reaction and processes involved in antibody affinity maturation.

Plasma cell programming is maintained in myeloma cells

As mentioned previously, plasma cell differentiation occurs mainly via suppression of B-cell programming. The complexity of this process is highlighted by the simplified schematic of the transcriptional networks involved (Figure 4). The transition from B cell to plasma cell is regulated primarily by two transcriptional repressors that antagonize the functions of each other. Plasma cell differentiation is held back by PAX5, a transcriptional repressor that targets the BLIMP-1 gene *PRDM1*[22-24].

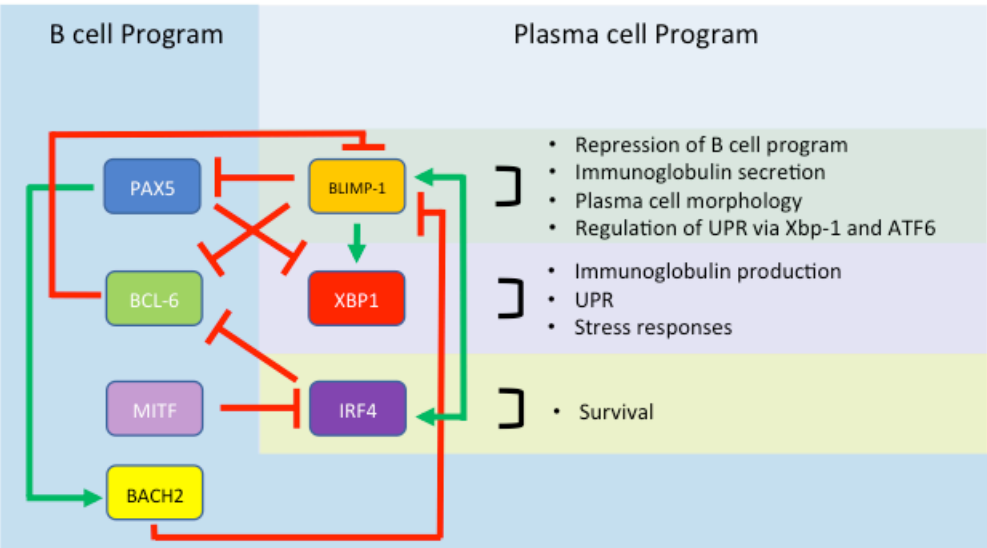

Figure 4. B cell vs. Plasma cell network. Interplay of the regulatory networks involved in maintenance of B cell vs plasma cell fates. Red lines indicate repressive activity, green arrows indicate activation. Highlighted at right are the physiological outcomes upon activation of the plasma cell regulators transcriptional networks.

BLIMP-1 is the master regulator of plasma cell differentiation, and one of its main functions to repress PAX5, which controls the B cell fate[25]. Most recently, BLIMP-1 has been shown to initiate transcriptional programs that lead to immunoglobulin secretion, by regulating expression of immunoglobulin gene and Elongation Factor for RNA Polymerase II 2 (*ELL2)*, a transcription elongation factor that promotes the switch between membrane-bound to secreted antibody forms[26]. BLIMP-1 had been characterized to be required for the maintenance of plasma cells[27]. While it has been shown to be unnecessary for the survival of plasma cells, BLIMP-1 has been shown play a role in the regulation of the unfolded protein response (UPR) (via regulation of *XBP1* and *ATF6*) and antibody secretion (via regulation of *MTOR* (mechanistic target of rapamycin) and important amino acid transporters)[28], indicating that maintenance of antibody generation and secretion in plasma cells is closely tied to BLIMP-1 function.

XBP1 is another transcription factor that has been shown to be required for plasma cell differentiation, and its primary function relates to coping with the sudden high protein-load of the differentiating B-cell[29-31]. XBP1 plays an important role in cellular protein homeostasis, and is activated by the endonuclease activity of IRE1α, a UPR sensor that gets activated when excessive misfolded protein leads to its release from BIP. IRE1α mediates splicing of XBP1 mRNA to produce the active form XBP1s. XBP1s binds to UPRE (unfolded protein response elements) and ERSE (endoplasmic reticulum stress elements) in target genes, leading to upregulated expression of factors that help the cell adapt to proteotoxic stress. During plasma cell differentiation, XBP1s functions also by initiating transcriptional programs that results in cellular remodeling that enables the cell to perform its function as an antibody factory[29,30].

The third node in the plasma cell differentiation program is IRF4, a transcription factor that was characterized as potential oncogenic factor downstream of aberrant chromosomal translocations in multiple myeloma[32], and was later found to be expressed in GC B cells, plasma cells, and activated T cells[33]. IRF4 has been shown to play an important role in plasma cell differentiation, as deletion of IRF4 from GC B cells led to ablation of plasma cells[34]. As a transcription factor, IRF4 has multiple binding partners and temporal regulation of its expression mediates different downstream effectors that determine the fate of the activated B cell[35].

As previously mentioned, myeloma cells retain the physiologic characteristics of plasma cells, and because these transcription factors play key roles in the development and maintenance of normal long-lived plasma cells, their roles in myeloma pathogenesis have been investigated. Functional ablation of these 3 key transcription factors via inhibition with a pharmacologic agent (IRE1 inhibitor to prevent *XBP-1* splicing)[36,37] or via silencing (either of BLIMP-1 or IRF4) have all been shown to lead to myeloma cell death[38-41]. IRF4 has been shown to be a viable target for myeloma as silencing this transcription factor has been shown to be an Achilles heel of myeloma cell lines[39,42]. This indicates that viability of myeloma cells is tied closely with maintenance of plasma cell identity, likely because these factors broadly regulate physiologically important networks.

Targeting transcription factors has proven to be a challenging pharmacologic problem. There are agents, however, that have shown efficacy in myeloma that work via modulating key transcription factors. Lenalidomide, a less teratogenic analog of thalidomide, belongs to a new class of agents used to treat myeloma that are referred to as

"IMiDs", or immunomodulatory agents. The mechanism of thalidomide teratogenicity was characterized to be due to its effect on Cereblon (CRBN), an E3 ligase[43], and lenalidomide has been shown to function similarly. Lenalidomide binds to CRBN, regulating its activity, which results in downregulation of genes *IKZF1* and *IKZF3* which encode Ikaros and Aiolos[44] respectively, key transcription factors for the lymphocyte lineage that play roles in regulating IRF4 expression in myeloma[45]. While the mode of IRF4 regulation by these agents is indirect, this is further evidence that modulating the activity of transcription factors that play important roles in normal plasma cell physiology are viable therapeutic avenues for the treatment of myeloma.

Plasma cells rely on protein processing pathways, as do myeloma cells

The ability of long lived plasma cells to maintain antibody titers throughout the lifetime of the host is facilitated by physiological changes that enable them to perform their function, from their extensive ER and Golgi networks, to their primed state for response to accumulation of misfolded proteins. Because of their function as secretory vessels, plasma cells require the ability to cope with cellular misfolded proteins, given the rate at which they synthesize, process, and secrete antibodies. The unfolded protein response (UPR) is a pathway that serves to maintain cellular protein homeostasis[46]. Upon detection of excessive misfolded proteins in the ER lumen, the UPR is activated, and cellular programs are initiated that help the cell adapt to the high protein load, through modulation of protein translation, initiation of autophagy, and activation of stress pathways to cope with the excessive protein burden(reviewed[31,46,47]). If the cell is unable to relieve the stress, cell death by apoptosis is a potential outcome. One of the key mediators of the UPR is the transcription factor XBP1[29,30].

As myeloma cells retain and require maintenance of plasma cell programming, their sensitivity to dysregulation of cellular protein content is well known, highlighted by the effects of proteasome inhibitors (PIs) on their viability[48]. PIs are small molecule inhibitors of the 26s proteasome, a multimeric protein complex responsible for the degradation of ubiquitinated substrates in the cell, and the sensitivity of myeloma cells to these agents has contributed to vastly improved patient outcomes (reviewed[49,50]). The proteasome functions not only in degrading excessive protein, but also in the regulation of signaling pathways that maintain cell homeostasis.

The mechanism of myeloma sensitivity to PIs was initially attributed to the NFκB signaling pathway, which mediates viability by facilitating adhesion and IL-6 pro-survival responses[51], however it was later found that this could not be the sole mechanism of PI-treatment efficacy in myeloma[52]. Myeloma cell sensitivity to the PI Bortezomib is linked to their function as antibody secreting machines[53]. Being secretory cells, myeloma cells constitutively express factors associated with the UPR (GRP74, GRP78), similar to their normal counterpart[54]. Thus, the high protein load and the accumulation of misfolded proteins leads to overactivation of the UPR, leading to cell death from stress[54,55].

Autophagy is a process that also functions as a means of coping with proteotoxic stress, but also as an adaptation to nutrient deprivation, via the degradation of cellular components through lysosomal acidification. Perturbations in the rate of autophagic flux can lead to cell death that can be inhibited by Bcl-2 family member[56-58] In the context of the humoral immune response (reviewed[59,60]), autophagy has been found to impact B cell differentiation[61], and has also been found to be required for the maintenance of normal long-lived plasma cells[62].

Myeloma cells are also very heavily reliant on autophagy for their survival, and require a baseline level of autophagic activity for homeostasis[42]. Disruption of this pathway has been shown to significantly impact myeloma cell viability, in normal and chemotherapeutic contexts[40,63,64]. Interestingly, IRF4, an important myeloma survival factor, has been shown to play a role in the regulation of autophagy in myeloma[42], again indicating that targeting IRF4 or its regulators would be a viable therapeutic option for myeloma as it regulates multiple pathways required for myeloma survival.

B. Survival signaling requirements for bone marrow plasma cells are also necessary for myeloma cells

Aside from physiological processes that maintain cell viability and function, the effectors of long-term survival of plasma cells also involve both cellular and soluble components. There are intrinsic survival factors whose primary function is to regulate cell death. Interactions with cells in the bone marrow stroma have been shown to facilitate longevity and residency of these cells in the bone marrow. Stromal cells provide adhesion substrates, as well as soluble factors that provide plasma cells with a pro-survival niche that can influence sensitivity to therapeutic agents. Soluble factors such as IL-6 and APRIL have been shown to be required for plasma cell survival. That these factors have been shown to also affect myeloma cell survival indicates that further understanding of normal plasma cell niches and requirements, and how these factors maintain plasma cell viability, may give us novel therapeutic avenues to provide even better prognoses for patients with plasma cell dyscrasias.

MCL-1 is required for both normal and malignant plasma cell survival

Cell survival can be facilitated by Bcl-2 family members, proteins which play important roles in either promoting or inhibiting cell death. For example, different immune cell subsets have been shown to be dependent on different members of the pro-survival Bcl-2 family, such that inhibition of specific members leads to loss of specific cell subsets[65-67]. This dependence can be exploited to treat different hematologic malignancies, as malignant counterparts of the different cellular subsets seem to retain their dependence on specific Bcl-2 family members[68,69], rendering them sensitive to small-molecule inhibitors that target specific interactions[70,71] (Figure 5).

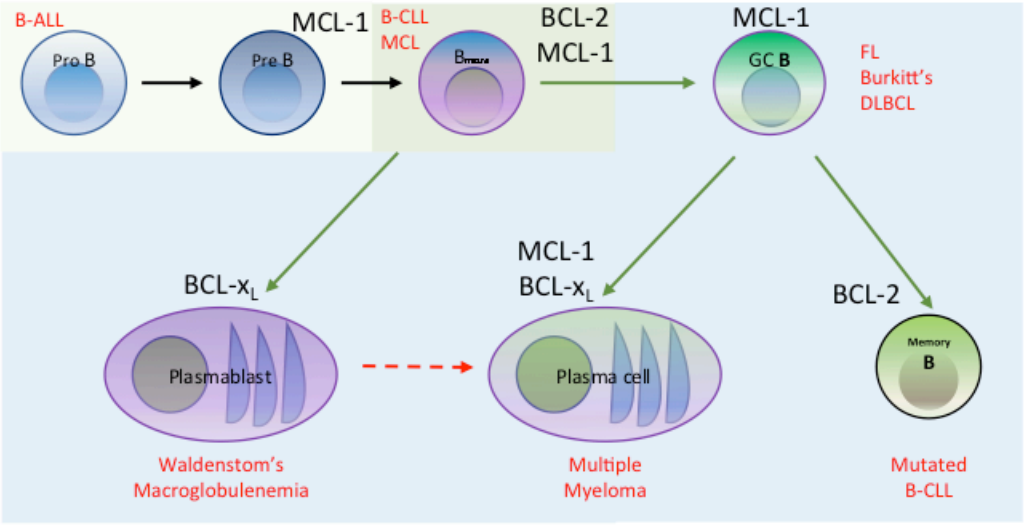

Figure 5. B cell malignancies are derived from their lineage counterparts and retain their unique dependence on specific Bcl-2 family members. Diagram showing how different B-cell malignancies, which are derived from distinct B-cell developmental stages, share dependencies on pro-survival Bcl-2 family members with their normal counterpart.

MCL-1 (myeloid cell leukemia 1) is a pro-survival Bcl-2 family member that has been shown to be important in plasma cell survival[67]. MCL-1 binds and sequesters the pro-apoptotic activator proteins BIM, and PUMA, preventing these from activating BAX and

BAK, which mediate mitochondrial outer membrane permeabilization, a key step in the apoptotic cascade (Figure 6). Malignant plasma cells have also been shown to be dependent on MCL-1, and inhibition of MCL-1 is a promising therapeutic avenue for myeloma patients. Our lab has also shown that while MCL-1 is a requirement for the survival of myeloma cell lines, these cells are also co-dependent on the other pro-survival family members BCL-2 and BCL-X_L. Treatment with a small molecule inhibitor ABT-737, a BCL-2, BCL-xL and BCL-w inhibitor, has shown that sensitivity of different myeloma cell lines is dependent on where BIM is preferentially bound. Overall, learning about specific dependencies on Bcl-2 family members is an avenue to exploit to initiate cell death in the context of aberrant plasma cells. In particular, newly developed MCL-1 inhibitors are promising therapeutics for myeloma, as well as other cancers, since MCL-1 dependency is common in different tumor types[71,72].

Receptor-Ligand interactions play important roles in plasma cell survival

The reliance of both normal and malignant plasma cell on the bone marrow microenvironment is well characterized, and this reliance is facilitated by interactions with stromal components (Figure 7). These interactions can take many forms, such as via receptor-ligand interactions that signal directly into the cell, or via the soluble factors released into the stroma[23,73,74]. The following are examples of these factors that facilitate viability of plasma cells in the bone marrow.

An example of an important soluble factor is IL-6, a cytokine known to be important for maintenance of normal plasma cells[75,76]. High IL-6 levels in sera is a poor prognostic indicator for myeloma patients[77], as the dependence of myeloma cells on stromal IL-6 facilitates both survival and drug resistance via a variety of mechanisms[68,77-80].

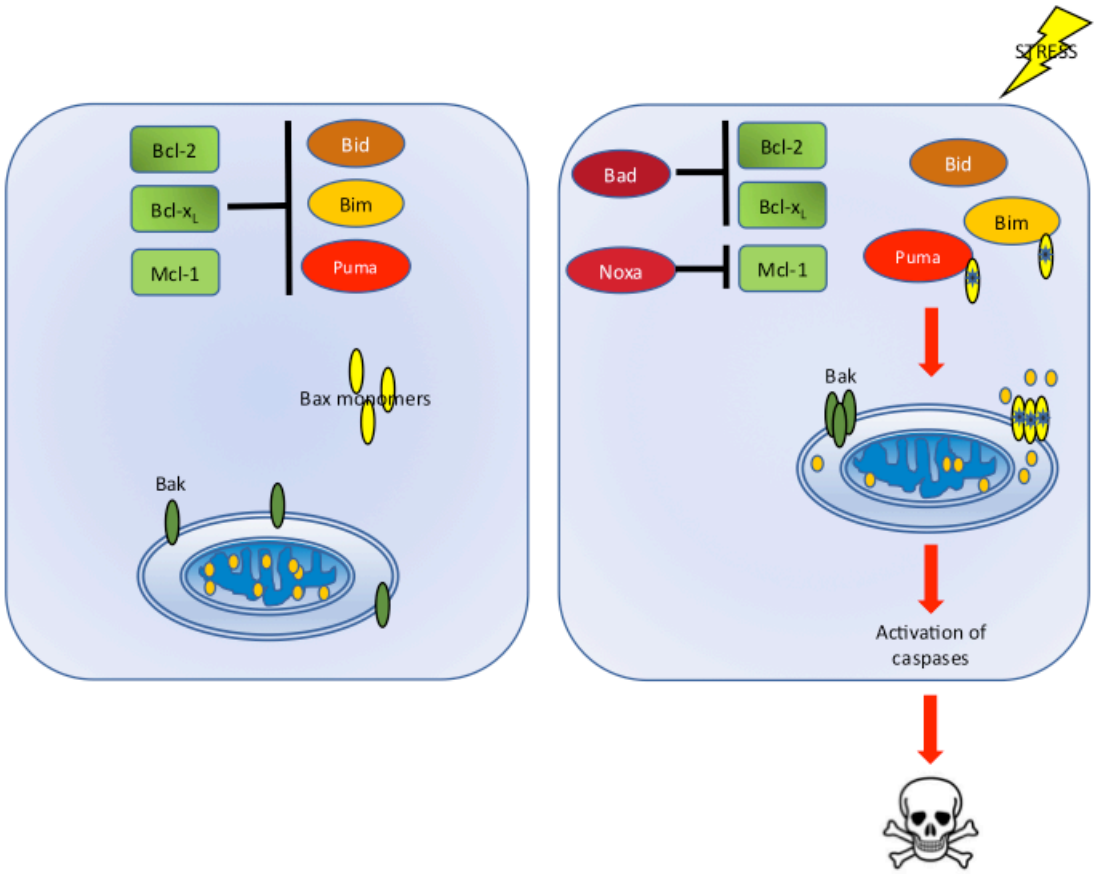

Figure 6. The intrinsic apoptotic cascade. The left shows homeostatic conditions, while the right panel shows the activation of cell death under stress conditions. Under normal conditions, the BH3-containing activators Bid, Bim, and Puma are sequestered by the pro-survival family members Bcl-2, Bcl-x_L and Mcl-1. Stressors such as DNA damage, etc. leads to induction of the sensitizer proteins (e.g. Bad and Noxa) which disrupt the interactions between the activators and pro-survival family members. The activators are thus free to activate Bax and Bak, which then oligomerize on the mitochondrial membrane, leading to permeabilization. This leads to cytochrome C release, subsequent caspase activation, and cell death.

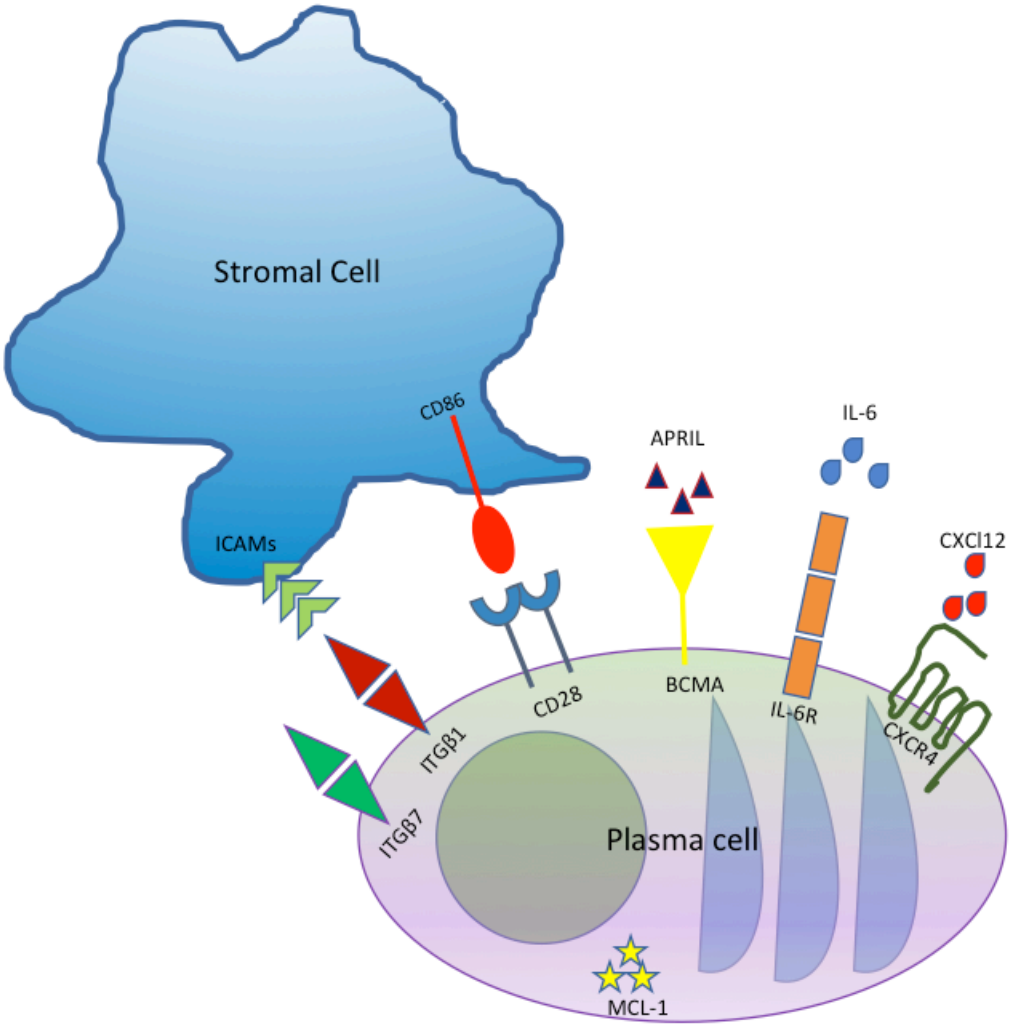

Figure 7. Survival signals that mediate plasma cell survival. Illustrated are the various factors known to be required in maintaining longevity of long-lived plasma cells. Integrins (ITGβ1 is a subunit of VLA-4, while ITGβ7 forms dimers with α4 or αE integrins) mediate adhesion interactions with stromal cells via ICAMs. The other surface receptors interact with ligands that are either expressed on stromal cells, or secreted into the bone marrow milieu.

Integrins are cell-surface molecules that mediate cell-cell or cell-matrix interactions, and are well characterized in their ability to mediate survival signals. In normal plasma cells, integrins have been shown to facilitate setting up residency in the bone marrow. Integrins have been shown to facilitate drug resistance in myeloma cells[81-83], as ablation of integrin

expression has been shown to exacerbate the cell-death induced via treatment with different drugs. Interestingly, integrins have also been shown to be a potential marker for clones present in patients with minimal residual disease (MRD)[84], indicating that these molecules can provide a survival advantage for myeloma cell.

Aside from adhesion molecules, another class of cell-cell interaction that plasma cells utilize for survival signaling is via costimulatory molecules, which refer to their function as co-activators (alongside antigen recognition) during an immune response. Costimulation can work both ways, either to send further activation signals during an immune response, but can also be a means of curtailing an existent response, mainly as a mode of preventing excessive immune pathology.

BCMA (B cell maturation antigen) is a member the TNF (tumor necrosis factor) family of costimulatory molecules. It is a surface receptor for BAFF and APRIL (also TNF family members), and has been shown to be important for the survival of normal LLPCs[85]. Most myeloma cell lines express this surface protein[86], and there have been several studies showing this is a viable therapeutic target for myeloma, as its expression is restricted to plasma cells and because it mediates expression of Mcl-1 upon activation, making it an attractive target[86,87]. Recently, reports of targeting BCMA via a bispecific antibody[88] or via T cells expressing chimeric antigen receptors specific to BCMA[89,90] have shown promise in pre-clinical testing, validating that targeting of this protein is a viable myeloma therapeutic option.

A key signal that functions to maintain viability of plasma cells involve the CD28 signaling pathway. CD28 is the canonical receptor for the ligands, CD80 and CD86.

Recently, it has been shown that long-lived plasma cells require CD28 signaling for their survival[12]. CD28 high expression has been shown to be a negative indicator for myeloma patients, as it has been found to correlate with disease progression[91]. Interestingly, CD28 is expressed in the majority of human myeloma cell lines, which are all derived from extramedullary disease[91]. And while myeloma cells rarely express CD80, CD86 is co-expressed by most myeloma cell lines. High expression of CD86 has also been shown to be a poor prognostic indicator for myeloma patients[92]. Altogether, there seems to be indication that this costimulatory pathway may play an important role in myeloma cell survival and disease progression.

C. CD28-CD86 signaling regulates a prosurvival signal in normal and malignant plasma cells

CD28 mediates immune activation in T cells

CD28 signaling is well studied in T cell activation, where it has been associated with mediating survival and proliferation upon ligation[93,94]. Initially referred to as Tp44 (based on the molecular weight of the monomer at 44KDa), it was characterized to primarily be a T-cell marker, but was later shown to also be highly expressed in plasmacytomas, and on maturing plasma cells[95]. CD28 has subsequently been found to be expressed on other cell types, however the role this molecule plays in these cells is unclear. CD28 is the most well-characterized costimulatory molecule, in terms of the multiple signaling cascades associated with its activation.

The CD28 molecule forms a homodimer on the surface, and binds to 2 ligands, CD80 and CD86. Another member of the costimulatory family, CTLA-4, is established to have a higher affinity for both ligands, and the dichotomy between CD28 and CTLA-4 are well

established modes of controlling T-cell responses. The interactions between these 4 molecules (CD28, CD80, CD86, and CTLA-4) have been extensively studied, however consensus as to whether CD28 prefers either CD80 or CD86, and whether it is bivalent or not, remains unclear. It has been proposed that CD28 preferentially binds monomers, and thus CD86 may be the initial immune activator and preferred binding partner, as it fits the structural requirements. In terms of expression kinetics, CD86 is constitutively expressed and upregulated quickly by APCs upon activation, while CD80 is only upregulated at much later time points. CD86 also has a lower affinity for CTLA-4 compared to CD80, and altogether, these data suggest that there may be ligand preferences in this costimulatory module[96-98](reviewed[99]).

Upon activation, CD28 has been shown to induce NFAT, NFκB, and PI3-Akt pathways. The cytoplasmic tail of CD28, while lacking in enzymatic activity, possesses multiple tyrosine-phosphorylation sites that adapter-proteins can bind to, leading to the activation of a myriad of signaling pathways. Specifically, the membrane-proximal YMNM and carboxy-terminal PYAP domains in the cytoplasmic tail have been extensively studied. Analysis of CD28 mutants in both the YMNM and PYAP domains have shown that while both contribute to IL-2 and Bcl-xL induction[93,94], loss of both domains does not result in complete abrogration of CD28 activity, suggesting that other domains may still play a role in signaling downstream of CD28 activation[100]. Of note, the cytoplasmic domain of CD28 is very highly conserved across species, indicating that activation of this molecule likely results in similar signaling cascades in murine and human systems.

The 2 well-known downstream effectors of CD28 activation are IL-2 and Bcl-x_L[94], leading to proliferation and survival of T-cells. The role of CD28 activation and its

effects on T cell metabolism has also been investigated[101]. The CD28 signaling pathway has thus been shown to play an important role in T cell physiology by coordinating different aspects of biology that facilitate proliferation, survival, and differentiation, enabling T cells to respond to pathogenic factors.[102]

CD28 in normal plasma cells

Loss of CD28 signaling has been shown to impair germinal center (GC) formation[103], which subsequently results in lower plasma cell numbers and antibody titers. It has recently been shown that this pathway is also intrinsically important in maintaining the longevity of normal plasma cells, such that loss of CD28 in the B cell lineage results in the decline of long lived bone marrow plasma cells and antigen specific antibody titers. This was attributed to a shortening of plasma cell half-lives from over 400 days (the lifetime of a mouse) to around 63 days[12], which indicates that this pathway plays an important role in plasma cell survival. Interestingly, while knocking out CD28 in mice adversely affected bone marrow plasma cell survival, the splenic subset was unchanged, suggesting different CD28-signaling requirements for the 2 subsets of plasma cells.

The specific downstream mediators in CD28 signaling are currently being characterized in bone marrow plasma cells, although it is known that the PI3K-AKT pathways are involved. Recently, it has been shown that CD28 activation in plasma cells plays a role in the induction of Blimp-1, one of 3 key transcription factors that plays a role in plasma cell differentiation and survival[104]. Further characterization of the CD28 pathway as it affects metabolism of normal long-lived plasma cells is ongoing, and indicates it may differ from the T-cell program in that it induces mitochondrial respiration dependent on production of reactive oxygen species.

CD28 and its role in malignant plasma cells

As previously mentioned, CD28 was found to be expressed not only in T cells but also in plasmacytomas[95], aggregates of neoplasmic plasma cells that can be found in the bone marrow or in soft tissue. High expression of CD28 appears to correlate with myeloma progression[91], and has been proposed to be used as a marker for monitoring disease progression in patients[84,105,106]. Our lab has shown that, blockade of this signaling pathway leads to sensitization of malignant plasma cells to different therapeutic agents[107]. Conversely, activation of this pathway can protect myeloma cells against cell death induced by different insults[108]. These data indicate that CD28 activation in myeloma cells can provide a survival advantage.

Characterization of the signaling mediated by CD28 activation in malignant plasma cells via treatment with an agonistic antibody showed activation of the PI3K-AKT and NFκB pathways, leading to protection against cell death, but also having a negative effect of the proliferative capacity of myeloma cells. Interestingly, unlike what is seen in T-cells, CD28 activation did not result in the induction of the pro-survival factor Bcl-x_L [108].

In contrast, blockade of this pathway exacerbated cell death from different chemotherapeutic agents[107]. Co-culture of myeloma cells with dendritic cells (DCs) can protect against cell death induced by different pharmacologic agents. Conversely, preventing DC-myeloma interaction via CTLA-4-Ig abrogated the protection, indicating that CD28 interaction with CD80/CD86 on DCs can provide a survival advantage. Blockade of this interaction induces higher expression of Bim due to inactivation of the PI3K-Akt pathway, leading to cell death.

Sine there are therapeutic agents that are used to target this pathway to modulate immune activation in the context of transplant and autoimmunity, we propose that blockade of this signaling pathway is a therapeutic avenue for myeloma patients whose myeloma cells express CD28 (Figure 8).

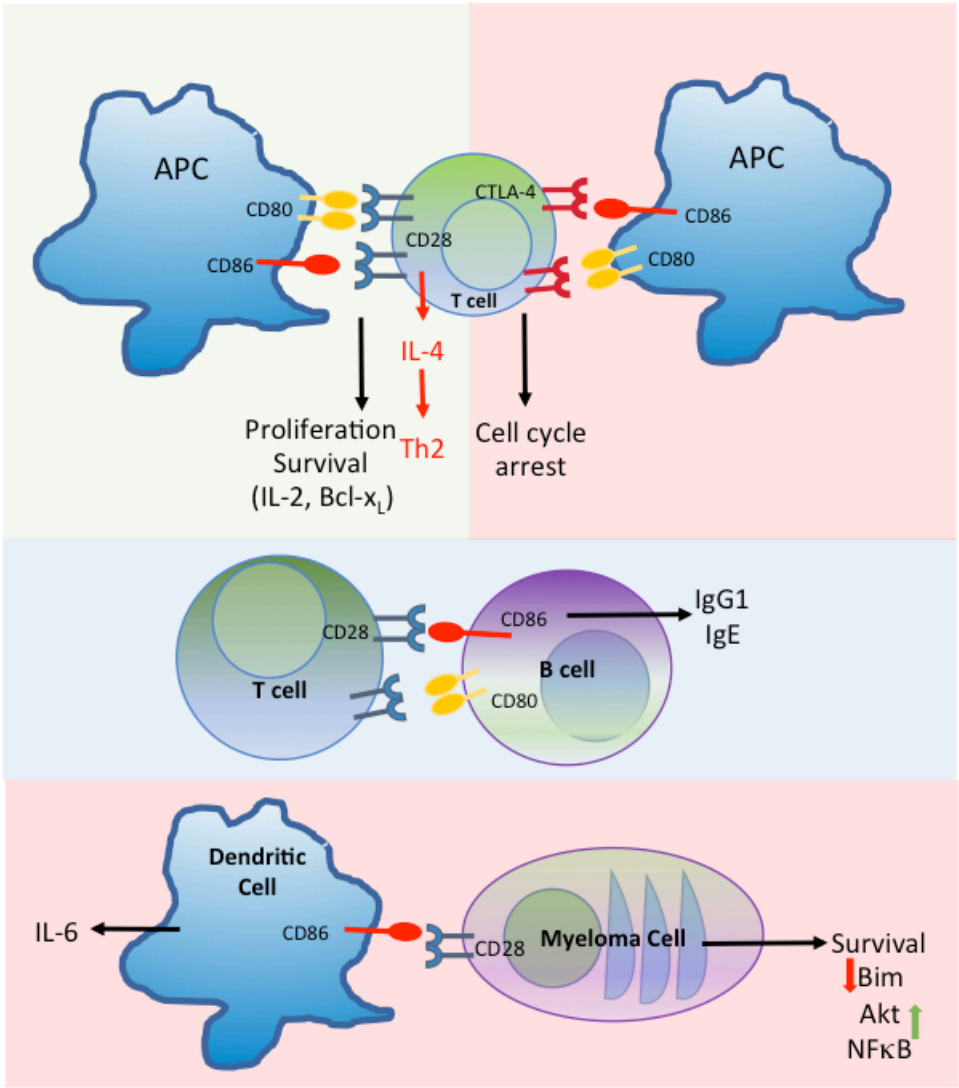

Figure 8. The CD28-CD86 pathway. (*Top)* In T cells, CD28 ligation to CD80 or CD86 mediates survival and proliferation via the NFκB, NFAT and AKT pathways, while CTLA-4 leads to cell cycle arrest. *(Middle)* In B cells, ligation of CD86 with CD28 leads to IgG1 and IgE secretion. (*Bottom)* In the bone marrow stroma interactions between CD86 on stromal DCs and CD28 on myeloma cells leads to secretion of IL-6, a myeloma growth factor, by the DC. Myeloma cells also induce lower Bim expression via activation of the Akt, NFκB pathways, facilitating a cytoprotective effect.

CD86 is a CD28 ligand and has a distinct function from CD80

CD86 (originally known as B70/B7-2) is one of 2 ligands known to bind both CD28 and CTLA-4, its existence being extrapolated from observations showing that blockade of CD80 was insufficient for costimulatory blockade. CD86 was first described as being found on activated B-cells[109-112], and was found to be constitutively expressed at low levels on resting monocytes, DCs, and B-cells. Upon activation, CD86 is upregulated much earlier than CD80 in all APCs. In murine cells, CD86 has been shown to be important for antibody responses, such that B-cell intrinsic ablation of CD86 results in lower antibody titers to vaccinia virus[113]. Because of the expression pattern of CD86, it has been hypothesized that it may have a preferred role versus CD80 in activation of an immune response.

CD86 belongs to the immunoglobulin superfamily of molecules, alongside its costimulatory partners. While the signaling cascades that CD28 mediates have been thoroughly investigated, the role of CD86 beyond its ability to activate CD28 is not fully characterized, and the signaling cascades downstream of CD86 ligation are unclear. In the case of T cell costimulation, ligation of CD28 with CD86 was shown to induce a greater amount of IL-4 secretion than with CD80, skewing T cells toward the T_{H2} lineage[114], indicating there is a difference in the way CD80 and CD86 bind to their shared receptor CD28.

CD80 and CD86 share only 25% sequence homology, thus one would predict that ligation with their receptors would mediate different effectors downstream into the cells in which they are expressed. In mice, the role of CD86 in costimulation has been shown to differ from CD80, particularly in terms of B cell function. Early reports showed that

$CD80^{-/-}$ mice can generate antibody responses even without the presence of adjuvant compared to $CD86^{-/-}$ mice[115], suggesting that CD86 mediates a signal required for either plasma cell differentiation or maintenance. Anti-CD86 antibodies have also been shown to have differential effects on murine B cells and their ability to proliferate and induce pro-survival factors[116]. In human B cells, CD86 ligation has been shown to impact synthesis of IgE and IgG4 antibody isotypes[117], indicating that CD86 signaling upon ligation to CD28 results in antibody effector outcomes different from CD80 ligation.

CD86 and what is known about its downstream signaling effectors

The previous study showing that CD86 can induce B cells to secrete different antibody isotypes compared to CD80 was the first indication of their differential signaling capacities[117]. Importantly, the role of CD86 signaling in the development of antibody responses has been heavily investigated. Studies have shown that CD86 activation induces IgG1 and IgE production in an IL-4 dependent manner[118], and that this increase is mediated by increasing IgG1 transcription[119], indicating that CD86 ligation activates transcriptional changes in the activated B cell. This was subsequently found to be mediated by increase of Oct-2 transcription factor binding to the IgH enhancer mediated by the PKC and NFκB pathways[120]. Follow up studies showed that this was mediated by dual action of the CD86 cytoplasmic domain and prohibitins, scaffolding proteins that mediate a plethora of signaling pathways[121,122]. However, the role of the CD86 cytoplasmic tail remained unclear as their data indicated that while the cytoplasmic domain was required, prohibitins bound to the transmembrane domain, leaving the direct downstream effectors upon CD86 activation still undefined.

In human DCs, it has been shown that CD86 ligation leads to activation of the PI3K-Akt pathway via novel cross-talk with Notch signaling, leading to secretion of IL-6 by DCs upon ligation[123]. Again, this indicates that CD86 is mediating signaling pathways downstream of its activation. Importantly, single nucleotide polymorphisms in the CD86 cytoplasmic tail are associated with differential effects on immune function. A polymorphism (rs1129055) has been associated with increased cancer susceptibility[124-126], as well as increased tolerance for transplants[127,128], indicating that this polymorphism may alter signaling pathways mediating activation of the immune response.

These data implicate numerous signaling pathways are activated upon CD86 ligation, but indicate there may be differences in murine vs. human signaling pathways involved. This could be a function of the different cellular contexts investigated. However, while murine systems have proven thoroughly useful in immunology, as manipulation of different mouse models have allowed for better characterization of what occurs during an immune response, there are acknowledged caveats to the use of murine models, foremost being that mice are fundamentally still different from humans, such that even minute differences in sequence homology in protein orthologs can have vastly different biological outcomes[129]. Inspection of the cytoplasmic domain of CD86 shows that it is not as well-conserved across species compared to its extracellular domain. The murine sequence is quite divergent from that of other species, indicating that studies in murine models may not be as informative in terms of comparing it to the human immune system. This indicates that while its role in binding to either CD28 or CTLA-4-Ig are well conserved, what happens downstream of CD86 ligation may vary across species.

CD86 in B cell differentiation

CD86 was also shown to be intrinsically required by B cells to acquire the germinal center phenotype[113]. CD86 may have a role in B cell differentiation in germinal centers, as one of the markers used to differentiate between light zone (LZ) and dark zone (DZ) cells in the germinal center in CD86[14,130]. High expression of CD86 (as well as CD83 and CXCR4) are characteristic of murine LZ B cells, which indicates that CD86 may play a role in relaying positive signals that allow the B cell to undergo somatic hypermutation (SMH) or affinity maturation, which can lead to differentiation into antibody secreting cells. While LZ vs DZ GC B cells are not as clearly defined in humans via CD86, this is mainly because CD86 is not down-regulated in DZ cells in human GC B cells to the same extent as in murine GC B cells[131], and its high expression in human GC B cells may indicate it plays a role in the further differentiation of this B cell subset.

CD86 in hematologic malignancies

CD86 is characterized to be a negative prognostic indicator for myeloma patients, as it was shown to be indicative of poor patient outcome when myeloma cells express it at high levels[132]. Curiously, a soluble form of CD86 has been found to circulate in humans, and is a poor prognostic indicator myeloma B-CLL, and AML patients[133,134]. However, the mechanisms as to how it provides an advantage is unclear, given the disparate effects of CD86 ligation to its cognate receptors, CD28 or CTLA-4.

Blockade of CD28-CD86 as a therapeutic avenue for myeloma

Our lab had previously shown that CD28 activation can provide a survival advantage to myeloma cells, and that blocking CD28 can sensitize myeloma cells to death signals[107,108]. Of particular interest is that both the canonical receptor (CD28) and ligand

(CD86) are expressed on the same cell in myeloma, indicating the potential for autocrine or paracrine signaling.

Because of the highly characterized role of this pathway in T cell activation, modulation of this pathway to achieve beneficial host outcomes is a therapeutic strategy for a variety of conditions. For example, the blockade of this pathway is used in the context of transplantation to modulate T cell responses to the allograft. In contrast, re-invigoration of T cell mediated immunity is the goal for treatment of chronic infections and cancers. While myriad pathways are associated with either activation or blockade of the CD28 signaling pathway, it is still incompletely understood. The role of the CD28-CD86 signaling axis in myeloma cells is the focus of this study, and the therapeutic potential of blocking this pathway is investigated.

II. CD86 regulates myeloma cell survival

Abstract

While multiple myeloma patient prognosis has improved over the past decade, research towards discovery of new therapeutic avenues is important, and could lead to a cure for this plasma cell malignancy. Here we show that blocking the CD28-CD86 pathway via silencing either CD28 or CD86 leads to myeloma cell death. Inhibiting this pathway leads to downregulation of integrins and IRF4, a known myeloma survival factor. Our data also indicate that CD86, the canonical "ligand" in this pathway, has pro-survival activity that is dependent on its cytosolic domain. These findings indicate that targeting this pathway is a promising therapeutic avenue for myeloma, as it leads to modulation of different processes important in cell viability.

Introduction

Multiple myeloma is a malignancy of long lived plasma cells, and is the second most common hematologic malignancy. While recent therapeutic advances have led to an increase in overall survival rates, most patients will eventually succumb to drug-resistant disease[135,136]. Because myeloma cells retain much of the physiological characteristics of their normal counterpart – the bone marrow plasma cell (BMPC), further understanding of the survival mechanisms of plasma cells are important, and could lead to knowledge that will help in developing agents to potentially cure myeloma[2].

Recently, it has been shown that long lived BMPCs require CD28 signaling for their generation and survival[12]. While the signals downstream of CD28 activation have been thoroughly investigated in T cells, the downstream mediators in plasma cells are only

beginning to be characterized[93,94,104]. CD28 is expressed in the subset of bone marrow cells to which the most long-lived fraction of human plasma cells belong[137], and has been reported to be a poor prognostic indicator for myeloma patients. It is highly expressed at diagnosis in patients with the MAF translocations, and expression correlates with disease progression[91,138,139]. We have previously shown that CD28 activation can protect myeloma cells against cell death induced by different death stimuli, including different chemotherapeutic agents[107,108] Thus, this pathway may be a feasible therapeutic avenue in myeloma, especially since FDA-approved agents that target and block CD28-CD86 interactions (CTLA-4-Ig) exist and are being used to treat autoimmune disorders[140,141] and to facilitate transplant acceptance[142,143].

Most CD28-positive myeloma cells also express its ligand CD86, and like CD28, CD86 has also been reported to be a poor prognostic indicator for myeloma patients[132]. Little is known about what happens downstream of CD86 ligation, although recent work has shed some light on the matter, particularly in terms of B cell function and activation in mice[113,118-120,144-146]. Therefore, we investigated the role of CD86 on human myeloma cells.

Materials and Methods

Cell lines

Cell lines used in these studies are described in Supplementary Table 1 and were cultured as previously described[70].

Patient sample processing

All samples were collected following an Emory University Institutional Review Board-approved protocol. Mononuclear cells from bone marrow aspirates from myeloma patients were collected as previously described[70].

Lentiviral shRNA preparation and transduction

shRNA clones were obtained from Open Biosystems and Sigma Aldrich. Clones used are listed in Supplementary Table 2. Viral particles were prepared and myeloma cells were infected as previously described[107].

Flow cytometry and analysis

Cell surface expression of CD28, CD86, ITGB7 and ITGB1 (Cat.Nos. listed in Supplementary Table 3) were measured via flow cytometry. Live cells (100,000) were collected, washed with 1x PBS, and stained with appropriate antibodies in 100 µL 1X Annexin Staining Buffer. After incubation (15 minutes) at 4°C in the dark, cells were washed in 1x PBS, resuspended in 400µL Annexin Staining Buffer containing 1µL of Annexin V. Samples were collected in a BD FACS Canto II. Analysis of flow cytometry data was done using FlowJo.

RNA extraction, cDNA synthesis, and qRT-PCR

RNA was extracted and qRT-PCR was performed as previously described[70]. All data are functions of relative quantity compared to pLKO.1 empty vector control. GAPDH and beta-actin were used as endogenous control genes. qRT-PCR probes are from Applied Biosystems, and are listed in Supplementary Table 4.

Protein extraction, Western blotting

Cell pellets were lysed in RIPA buffer with protease and phosphatase inhibitors as previously described[70]. Lysates were quantified using the BCA Assay, and 15-30μg of lysate were run in SDS-PAGE gels, then blotted as previously described[70]. Antibodies used for detection are listed in Supplementary Table 3.

RNA preparation for RNA-seq analysis

RNA was isolated using the Qiagen RNEasy Kit as described, quality control tested at the Emory Integrated Genomics Core, and 1 μg was sent to Hudson Alpha for RNA-seq library construction using the Illumina TruSeq mRNA protocol. RNA-seq libraries were sequenced using 50 bp paired-end reads on an Illumina Hi-seq 2500 to a depth of approximately 25 x 10^6 paired-end reads.

Alignment and quantification of RNA-seq data, and Differential and bioinformatic analysis of RNA-seq data

Please refer to Supplementary Methods for detailed description of RNA-seq analysis.

Cell adhesion assays

Please refer to the Supplementary Methods for detailed description of cell adhesion assay methodology.

Statistical Analysis

Statistical significance was assessed using two-tailed student's t-test using GraphPad Prism.

Results

CD28 and CD86 expression influence patient outcomes

Previous studies demonstrated that individually, CD28 and CD86 expression correlate with poor prognosis[91,132]. Using data from UAMS, we had previously shown that the high-risk MF (MAF) subtype of myeloma expressed high levels of CD28[139], and found similar results with CD86 (Supp.Fig. 1A), indicating that high expression of these molecules is associated with a form of high-risk disease. We confirmed these findings using data from CoMMpass (Clinical outcomes in Multiple Myeloma to personal assessment) (Supp.Fig. 1B). CoMMpass is a prospective study which follows 1,000 newly diagnosed myeloma patients, linking clinical, genomic, and transcriptomic data.

We next determined if CD28 or CD86 expression alone influenced patient outcome. In contrast with previous reports, we found that high expression of CD28 or CD86 alone did not impact progression-free or overall survival (data not shown). We next evaluated if combined expression of CD28 and CD86 was prognostic of outcome. Patients who were in the top quartile of both CD28 and CD86 expression (n=53/645) were compared to those in the bottom quartile(n=47/645) (Figure 1A). Significant differences between these 2 groups were observed in both the progression-free and overall- survival (Figure 1B), suggesting that the combination of CD28 and CD86 expression on myeloma cells significantly impacts patient prognosis. However, these differences were due to patients in the bottom quartile (Figure 1B) having a better prognosis compared to the rest of the patient cohort, indicating that low expression of both CD28 and CD86 positively impacts patient outcome. We compared data generated from CoMMpass to similar analyses done on the Arkansas patient database (Supp.Fig. 1C,D), and found a similar trend, showing

that patients whose myeloma cells express low levels of both CD28 and CD86 had better overall survival than the rest of the patient cohort. While these data demonstrate that expressing high levels of both CD28 and CD86 is associated with a worse outcome than expressing low levels, they are not consistent with CD28 and CD86 being markers of high risk disease.

Previous analyses demonstrating a prognostic role for CD28 and CD86 were generated from cell surface expression as measure by flow cytometry while our current study used RNA-seq data. Therefore we compared values obtained via sequencing (RNA-seq) to flow cytometric data available. CD86 cell surface expression was not performed for CoMMpass, however data was available for CD28. We analyzed 141 patient samples where CD28 staining had been performed, and found 101/141 (71%) were samples wherein 100% of the myeloma cells were characterized to be CD28-positive (Figure 1C). We compared CD28 mRNA expression between this group (100% CD28-positive) to the rest of the patients for whom we had myeloma cell surface CD28 expression data available (<100% CD28-positive), and found that surface expression correlated with higher mRNA levels (Figure 1C). We then compared the progression-free- and overall-survival between these 2 groups, and found that no statistically significant differences between the 2 groups, however the CD28 low expressers again look to have slightly better prognoses (Figure 1D). Taken together, data from CoMMpass shows that low expression of CD28 and CD86 provides a clinical advantage to myeloma patients, and therefore CD28 and CD86 may contribute to myeloma patient outcomes.

Blockade of CD86 expression leads to myeloma cell death

We previously demonstrated that silencing of CD28 resulted in significant cell death in the RPMI8226 (8226) myeloma cell line[107]. We extended these findings using additional myeloma cell lines and determined the effects of CD86 silencing. We found that silencing of CD28 resulted in significant levels of cell death compared to empty-vector controls in 2 of the 3 additional CD28+/CD86+ lines tested (Figure 2A, and Supp.Fig. 2A,B). Silencing of CD86 also resulted in significant cell death in 4/4 CD28+/CD86+ cell lines tested. Cell death in 3/4 (MM.1s, 8226, H929) lines was greater with CD86 silencing than with CD28 (Figure 2A, Supp.Fig. 2A,B). Additionally, in both 8226 and MM.1s cell lines, CD86 expression was increased when CD28 was silenced both at the protein (Figure 2B, Supp.Fig.3) and mRNA level (Figure 2B). Similar results were observed with independent shRNAs against both CD28 and CD86 (Supp.Fig. 2C), however no death was observed in these short-term assays when GAPDH was silenced, indicating that the effects are not non-specific. (Figure 2A, Supp.Fig. 2A,B). Finally, silencing of CD28 or CD86 had no effect in the CD28-negative cell line, KMS12BM (Supp.Fig. 2B). Taken together, these data suggest that myeloma cells that express both CD28 and CD86 are dependent on CD28-CD86 signaling for survival. Moreover, signaling through this pathway may be self-regulated, where CD28 signaling is a negative regulator for CD86 expression.

We next evaluated whether CD86 silencing would have the same effect on myeloma cells from a freshly isolated patient sample. We infected cells from myeloma patient bone marrow aspirates with lentivirus containing shRNA against CD86. shCD86 could induce cell death in the CD38-positive cells from the sample, despite incomplete silencing at day

3 post-infection, similar to results obtained using myeloma cell lines (Figure 2C-D). No difference was observed in the CD38-negative subset (Figure 2D). In contrast, in patient samples where myeloma cells were found to lack either CD28 or CD86, no effect was observed with shCD86 on CD38-positive cells (Supp.Fig. 4).

Gene expression changes in CD28 versus CD86-silenced cells are consistent with regulation of both distinct and common pathways, including expression of IRF4

To determine potential mechanisms of CD28-CD86 cell survival signaling, both CD28 and CD86 were silenced by shRNA in MM.1s, 8226, and KMS18 myeloma cell lines and compared to mock-silenced cells (pLKO.1 vector) using RNA-seq. Hierarchical clustering showed that RNA expression separated samples by cell type as expected, but that CD86-silenced cells clustered more closely to controls than CD28-silenced cells (Supp.Fig. 5A). Additionally, RNA-Seq confirmed our qRT-PCR and flow cytometry analyses, and showed that CD28 silencing resulted in upregulation of CD86 in MM.1s and 8226 cells (Supp.Fig. 5B). To determine genes affected by silencing of CD28 or CD86, differential analysis was performed while controlling for cell line differences. This analysis yielded 390 genes regulated by CD28 silencing and 207 genes regulated by CD86 silencing, and shows that they regulate few genes in common (Supp.Fig. 5C). However, gene set enrichment analysis indicated that overlaps did occur with respect to pathways affected by the expression changes. Interestingly, the primary overlap between CD28 and CD86 silencing appear to involve the downregulation of IRF4 and c-Myc targets (Fig. 3A, Supplementary Table 5), transcription factors known to play important roles in myeloma cell survival.

We confirmed RNA-Seq results via qRT-PCR and protein analyses. We focused on genes where a consistent pattern was observed in the 3 cell lines tested. First, we looked at IRF4 expression, and confirmed down-regulation upon CD28 and CD86 silencing in MM.1s, 8226 (Figure 3B-C) and KMS18 (data not shown).

We also confirmed changes in expression of different integrin subunits. *ITGB7* mRNA was down-regulated when either CD28 or CD86 was silenced, while *ITGB1* was downregulated with CD86 silencing and upregulated with CD28 silencing, in a pattern similar to CD86 itself (Figure 3D). However, we did not observe significant changes at the cell surface except for the increase in ITGß1 following CD28 silencing in MM.1s. To determine if these changes in expression of adhesion molecules could be of functional significance, we next determined if silencing CD28 or CD86 would affect the ability of myeloma cells to adhere to stromal HS-5 cells. We performed cell adhesion assays with cells that were infected with either shCD28 or shCD86 or the control vector. We found that in both MM.1s and RPMI8226 cells that were infected with either shCD28 or shCD86 adhered less compared to pLKO.1 controls (Figure 3E, post-wash) despite similar numbers of live cells loaded into wells (Figure 3E, pre-wash).

Given the differential pattern in changes in integrin expression, where ITGß1 seems to be following the same pattern as CD86 (Figure 3D), we hypothesized that CD86 may be mediating downstream signals that involve regulation of ITGß1 expression. In addition, because silencing of CD86 alone had a profound effect on myeloma cell viability, and our RNA-Seq data shows that this leads to expression changes in genes distinct from when CD28 is silenced, we next investigated the role of the cytoplasmic domain of CD86 in myeloma cells.

The cytoplasmic tail of CD86 plays a role in CD28-CD86 function

Alignment of the cytoplasmic tail of CD86 from 8 mammalian species shows only 3.3% identity and 9.8% similarity (Supp.Fig. 6) for the 61-residue cytoplasmic region (human). Upon closer inspection, the lack of conservation is primarily due to rodent species, and removal of rat and mouse sequences results in an increase to 21.3% identity and 50.8% similarity (Figure 4A). This suggests that the CD86 cytoplasmic tail may have a role in human cells that is distinct from that in rodents. Therefore, to test the role of the CD86 cytoplasmic tail in myeloma cell survival we expressed both a full length (CD86FLm) and a truncated CD86 (CD86TLm) that lacked all but the first 7 residues of the cytoplasmic tail (Figure 4B). These residues were previously reported to be required to stabilize CD86 surface expression[147]. Additionally, silent mutations were introduced to render the constructs resistant to the shCD86 used for CD86 silencing. After transfection into RPMI8226 cells, we sorted CD86-high expressing cells, and used these cells in subsequent experiments (Figure 4B). We reasoned that with these cell lines, we could differentiate between signals coming from CD28 versus CD86, as the CD86TLm would have little to no signaling capacity. After silencing of endogenous CD86, we would then have a means by which to determine the role of the CD86 cytoplasmic tail.

We found that overexpression of CD86FLm and CD86TLm resulted in a downregulation of surface CD28 (Figure 4C). This occurs irrespective of the presence of the CD86 cytoplasmic tail, suggesting modulation of CD28 levels is due primarily to a negative feedback loop, where over-stimulation of CD28 via ligation with surface CD86 results in subsequent downregulation of CD28 expression.

To determine if overexpression of CD86FLm or CD86TLm would affect expression of genes regulated by CD86 silencing, we looked at surface levels of ITGß7 and ITGß1 (Figure 4C). We found that overexpression of CD86FLm resulted in an induction of integrin expression. The induction of integrin expression only occurred with CD86FLm but not CD86TLm, which indicates that the cytoplasmic domain of CD86 is necessary for regulation of integrin surface expression.

Since silencing of CD86 results in myeloma cell death, we next determined if there would be a survival advantage with CD86 overexpression. We treated the cell lines with a Bcl-2/Bcl-x_L inhibitor (ABT-737), proteasome inhibitors (Bortezomib, Carfilzomib), a corticosteroid (Dexamethasone) and an alkylating agent (Melphalan) (Figure 4D). We found that CD86FLm but not CD86TLm, provided a distinct survival advantage for cells treated with ABT-737 or dexamethasone. In the case of the proteasome inhibitors, a small but reproducible shift in the dose response curves is observed while with melphalan, there was no effect. Overall, our data indicate that the cytoplasmic domain of CD86 is relaying a signal in myeloma cells that can provide a survival advantage.

Overexpression of shRNA-resistant full length CD86 protects against CD28- and CD86- silencing, but not against loss of IRF4

To determine if overexpression of CD86FLm or CD86TLm could protect against CD28-CD86 blockade, we silenced endogenous CD28 or CD86 in cells that overexpress these constructs. As seen in Figure 5A, silencing of CD28 and CD86 in the vector control cells yields similar results as in parental 8226. We saw a 50% decrease in CD86 expression when CD86 is silenced, while CD28 silencing resulted in a nearly two-fold increase in CD86. In contrast, CD86 expression in the cells overexpressing the CD86TLm was

completely resistant to the effects of shCD86. While shCD86 did lower CD86 in the CD86FLm-expressing cells, these cells still expressed higher levels than endogenous CD86 expression. Overexpression of full length CD86 completely blocked shCD28-induced death, and significantly protected against shCD86-induced death. In contrast, CD86TLm protected against both hairpins reproducibly, however the level of protection was modest (Figure 5B).

Since IRF4 is a well-characterized myeloma survival factor[39,40], we investigated whether IRF4 levels changed in cells that were overexpressing either the CD86FLm or CD86TLm constructs. We found that overexpression of CD86FLm led to a modest increase in IRF4 at both the mRNA and protein levels (Figure 5C-D). Downregulation of IRF4 in these lines was less pronounced after silencing of either CD28 or CD86 compared to vector-control cells (Figure 5D). These data are consistent with CD28-CD86 signaling promoting myeloma cell survival via the regulation of IRF4 expression. To determine if overexpression of CD86FLm could protect against cell death from IRF4 loss, we silenced IRF4 in these cells. While CD86FLm could protect against shCD86, it could not significantly protect against shIRF4 (Figure 5E). Silencing with shIRF4 resulted in comparable loss of IRF4 at day 4 post-infection in both empty-vector and CD86FLm-overexpressers (Figure 5F).

Overexpression of pro-survival Bcl-2 family members leads to partial inhibition of CD86-knockdown induced death

To characterize the mechanism(s) of cell death induced by blockade of the CD28-CD86 pathway, we next determined whether overexpression of the pro-survival Bcl-2 family members could protect against silencing of either molecule. Compared to pCDNA3.1

(empty-vector controls), we found that overexpression of Bcl-2, Bcl-x_L or Mcl-1 significantly protected against cell death induced by silencing of either CD28 or CD86 (Figure 6A, Supp.Fig. 7). In the case of CD28 silencing, overexpression of any of the pro-survival proteins abrogated cell death (Figure 6A, top). Silencing of CD86 in the cells that overexpress pro-survival proteins resulted in significantly lower levels of cell death as compared to empty-vector controls (Figure 6A, bottom), however cell death was only partially blocked.

Since overexpression of pro-survival Bcl-2 family members was unable to completely protect against cell death induced by CD86 silencing, we treated cells with pan-caspase inhibitors to determine whether shCD86-induced cell death was caspase dependent. After infection with lentivirus containing either shCD28 or shCD86, cells were treated with the caspase inhibitor Q-VD-Oph at day 2 post-infection, and viability was determined after 48 hours. We found that inhibition of caspase activity could not protect against cell death induced by CD86 or CD28 silencing (Figure 6B). To ensure that the lack of protection following caspase inhibition was not simply due to incomplete blockade, we determined the effect of silencing and Q-VD-Oph on caspase-3 and PARP cleavage (Figure 6C). In both MM.1s and 8226 cells, silencing of CD28 or CD86 resulted in caspase-3 cleavage to the mature p17 form and cleavage of the caspase-3 substrate PARP. Q-VD-Oph treatment resulted in a complete block of PARP cleavage suggesting that effector caspase (caspase-3,7) activity is completely blocked under these conditions. However, for CD86 silencing, there appears to be some residual initiator caspase activity as demonstrated by the appearance of the pro-p20 intermediate band that is the expected product of partially

processed caspase-3. Since PARP cleavage is ablated, this product does not have activity under these conditions.

As our data indicates that IRF4 expression is being regulated by CD86 cytoplasmic tail activity, we next determined if shIRF4 induced death would phenocopy that of shCD86. Silencing of IRF4 in MM.1s and 8226 cell lines led to high levels of cell death that could only be partially inhibited by treatment with Q-VD-Oph (Figure 6E). Consistent with CD86 silencing, we find that treatment with the pan-caspase inhibitor can only partially block caspase-3 cleavage, but effectively blocked effector caspase function as shown by lack of cleavage of PARP in the presence of Q-VD-Oph (Figure 6F). Overall, our data indicates that CD86 is mediating a signal that involves regulation of myeloma survival via the regulation of IRF4 expression.

Discussion

We previously characterized how CD28 on myeloma cells can interact with dendritic cells that express CD80/CD86 which leads to secretion of IL-6 by DCs[139], indicating how myeloma cells can influence the tumor microenvironment to their advantage. However, progression in myeloma involves the development of independence from stroma, and co-expression of both CD28 and CD86 is a means to gain that independence, as it allows for survival signaling to be mediated either between myeloma cells, or via autocrine activation of the pathway. This could explain the sensitivity of CD28-CD86 myeloma cell lines and patient samples to blockade of this pathway, as once either component is partially silenced, a key survival signal is lost. This is the first demonstration that a receptor-ligand pair on myeloma cells mediates stroma-independent survival.

Our data also indicates that the cytosolic domain of CD86 is relaying a signal that is separate but not independent from its role as a ligand for CD28 activation. While previous reports have indicated that CD86 has signaling capacity, these studies were primarily done using murine models. The extracellular domain of the different CD86 orthologs are highly conserved, however the cytosolic domains are highly variable, with the rodent orthologs showing the greatest divergence. This indicates that while the function of CD86 as a CD28 activator is conserved, what happens downstream of its ligation to CD28 may differ across species. Thus, studies in murine systems may not completely inform what signals are mediated in human cells by CD86. Given the differences in the cytoplasmic tail, and our finding that the cytoplasmic tail is required for maximal CD86 activity, further studies of the downstream signaling events from human CD86 are warranted.

We were surprised that overexpressing CD86FLm, could only partially protect against cell death induced by shCD86. We hypothesize that this could be due to the variant of CD86 we are overexpressing in 8226. This cell line expresses the CD86-A304 allele, however the cDNA in the overexpression construct (CD86FLm) contains the CD86-T304 allele. This change is a result of a polymorphism (rs1129055) that has been linked to increased cancer risk[124-126], and graft acceptance[127,128], suggesting this version of CD86 may be less effective in an immunological setting. Interestingly, A304 is in the cytoplasmic tail and is conserved in all species analyzed except rat and mouse (Supp.Fig. 4).

The survival signal being mediated by CD28-CD86 is multifaceted, as shown by how this pathway seems to be regulating expression of different gene families that play important

roles in normal myeloma cell physiology. Interestingly, the top gene ontologies downregulated for CD28 (RNA processing) and CD86 silencing (UPR, protein processing) are processes wherein mutations were found to be prevalent in the initial genome sequencing analysis done in myeloma[148]. This could explain why we saw more significant gene expression changes with silencing of CD28, but saw a higher impact on viability with silencing of CD86, given that myeloma cells are so reliant on protein metabolic pathways for function and survival. While our RNA-seq data shows limited overlap in the list of genes whose expression changed with silencing of either CD28 or CD86, gene set enrichment analyses indicate that these 2 molecules regulate common pathways important in maintaining cell viability. Additionally, differences in expression could be due to the availability of other ligands. CD28 can also bind to CD80, and ICOSLG[149]. While CD80 is not observed on myeloma cells, ICOSLG has been detected in myeloma[150], and appears to be more highly expressed than CD86 at the mRNA level in the KMS18 cell line (data not shown).

CD86 also plays a role in regulating surface expression of integrins, which are important molecules for facilitating cell-cell and cell-stroma interactions. Myeloma cells are dependent on integrin-mediated interactions with the bone marrow stroma components[81-83]. Consistent with altering integrin levels, silencing of either CD28 or CD86 resulted in decreased adhesion to stromal cells, demonstrating a role for this pathway in regulating myeloma cell adhesion. Additionally, our studies demonstrate that ITGβ1 expression is regulated in a similar fashion as CD86 itself, and importantly the cytoplasmic tail of CD86 is required for increased ITGβ1. These data suggest that ITGβ1 is regulated downstream of CD86 and is not a consequence of CD28 signaling. Interestingly,

upregulation of ITGβ1 when CD28 is silenced does not appear to compensate for the decrease in expression of ITGβ7, as we still observe a decrease in the ability of myeloma cells to adhere to the HS-5 stromal cells. This indicates that ITGβ7 may play a more significant role in adhesion to HS-5 cells than does ITGβ1.

Myeloma cells require IRF4 for survival[39]. Our data show that CD28-86 signaling plays a role in the regulation of this transcription factor, as silencing of CD28 or CD86 results in down-regulation of IRF4. IRF4 regulates homeostatic autophagy in myeloma cells[42]. Autophagy is a catabolic pathway that cells use to compensate for nutrient deficiency, or extreme protein burden. If overactivated, however, it can lead to cell death that is caspase independent but inhibitable by Bcl-2/Bcl-x_L[56,57] as we observed with CD86 silencing or blockade.

Our data do not support previous findings that CD28 and/or CD86 are associated with poor prognosis. Several potential reasons could account for these differences, including the number of patients evaluated, the length of follow up observations, differences in treatment regimens, as well as the proportion of t(14:16) patients in each cohort. This translocation is both associated with poor prognosis and significantly higher CD28 expression. While we see a similar pattern in CoMMpass, of the 645 patients analyzed, seq-FISH on 552 samples indicate that only 17 (3.02%) have c-MAF translocations. Since flow cytometry data for CD28 expression was available for 141 of 645 patients, we were able to compare RNA and protein expression. While unable to perform a direct comparison of expression levels, we were able to demonstrate that nearly all have myeloma plasma cells that express CD28 at diagnosis. Of these, we observed 100% CD28-positive staining in 71% of the samples (101 of 141). This is significantly higher

than previously reported (47.8% positive)[151], and suggests CD28 may play a more important role in myeloma pathogenesis than previously appreciated. While cell surface expression of CD86 was not available for comparison, RNA expression also suggests that CD86 expression is more prevalent at diagnosis than previously appreciated[132].

Taken together, our data strongly indicates the combination of CD28-CD86 signaling plays an important role in mediating myeloma cell survival. Data from CoMMpass shows that low expression of CD28 and CD86 is prognostic of a better outcome for patients as compared to the rest of the patient cohort (i.e. intermediate or CD28- CD86- high expressers). However, this represents a minority of newly diagnosed patients. Therefore, blocking this pathway may prove beneficial for most myeloma patients. Since a pharmacologic agent that inhibits this pathway is already FDA-approved, we believe this is a promising therapeutic addition for the treatment of myeloma.

Authors' Contributions

C.M.G. and L.H.B. conceptualized and designed all experiments. C.M.G. performed all experiments. B.G.B. performed in depth computational analyses of the sequencing data. C.M.G., B.G.B., S.N., P.N., A.N., S.L., K.P.L. and L.H.B. analyzed and interpreted data. C.M.G. and L.H.B. wrote the paper. C.M.G., B.G.B., S.N., P.N., A.N., S.L., K.P.L. and L.H.B. reviewed and edited the final manuscript.

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Conflicts of Interest

- S. Lonial is a Consultant for Millennium, Celgene, Novartis, BMS, Onyx, Janssen; and gets research support from BMS, Janssen, Takeda, Celgene.
- A. Nooka is a consultant/advisory board member for Spectrum Pharmaceuticals, Novartis, and Amgen.
- L. Boise is a consultant for Abbvie.

Figure Legends

Figure 1. CD28 and CD86 expression influence patient outcomes. (A) Plot of CD28 vs CD86 expression in patients enrolled in CoMMpass. Vertical and horizontal dashed lines represent the 25 and 75 percentiles (B) Kaplan Meier survival curves for patients in the top CD28 and CD86 expression quartile (red), bottom quartile of CD28 and CD86 expression (blue), and all other patients (gray) in the CoMMpass study. Both progression-free survival (PFS; left) and overall survival (OS; right) are shown. (C) Histogram of percent myeloma cells positive for CD28 by flow cytometry data from 141 patients (left) and boxplot of CD28 mRNA expression for patients with myelomas that are less than 100% CD28+ and those that are 100% CD28+ (right). (D) PFS (left) and OS (right) for 141 patients with both CD28 flow cytometry and outcome data in the CoMMpass study.

Figure 2. Silencing or blockade of CD86 results in myeloma cell death. *(A,* **left***)* Myeloma cell lines were infected with lentiviral particles carrying empty vector (pLKO.1) or the individual shRNAs, and cell death was monitored by Annexin V-FITC staining for 4 days. Data for different time points were all compared to pLKO.1 controls. (*A*, **middle**) mRNA quantification as measured via qRT-PCR comparing levels of CD28 or CD86 to vector-controls. (*A*, **right**) Representative histograms show CD28 or CD86 surface levels at Day 4 post-infection. Thin grey histograms at left are isotype controls. (*B*, **left**) mRNA quantification as measured via qRT-PCR comparing levels of CD28 or CD86 to vector-controls. (*B*, **right**) Representative histograms show CD28 or CD86 surface expressionat Day 4 post-infection. Thin grey histograms at left are isotype controls. All data are presented as the mean $\pm$ SEM of at least 3 independent experiments.

(*p<0.05, **p<0.01, ***p<0.005). All qRT-PCR data are normalized to ß-Actin as an endogenous control, and then compared relative to mRNA levels in pLKO.1 empty-vector control. The RNA was extracted on Day 3 post-infection. For flow cytometry data, histograms are representative of the Annexin-V-negative set in the population. *(C)* Gating strategy for cells from the buffy coat from a bone marrow aspirate of a myeloma patient. Total cells were separated into CD138-positive (purple) versus CD138-negative (black). Histograms show that CD138-positive cells are also CD38-, CD28-, CD86-positive. *(D)* Cells from the buffy coat from the same myeloma patient were infected with lentivirus containing shCD86, or pLKO.1 empty-vector control. Cell death of CD38+ vs. CD38- cells were assessed via staining with Annexin-V at indicated time points post infection. Representative histograms for CD86 surface expression are from day 3 post-infection.

Figure 3. Gene expression changes in CD28 versus CD86-silenced cells are consistent with regulation of both distinct and common pathways, including expression of IRF4. (*A*) Gene set enrichment analysis showing upregulated (top) and downregulated (bottom) gene sets in shCD28 (blue) and shCD86 (red) treated myeloma cells. For each gene set, the enrichment score is shown above the ranked change in gene expression where genes that overlap the gene set are denoted by blue and red ticks for shCD28 and shCD86, respectively. *(B)* qRT-PCR showing IRF4 mRNA levels when CD28 or CD86 was silenced in MM.1s or 8226 cells. (*C)* Representative Western blots showing IRF4 levels with silencing of CD28, or CD86 in cell lines indicated at different time points. *(D*, **left panels***)* Integrin levels were measured via qRT-PCR (*ITGB7* and *ITGB1*). (*D*, **right panels**) Representative histograms showing ITGß1 and ITGß7 on day 4 following

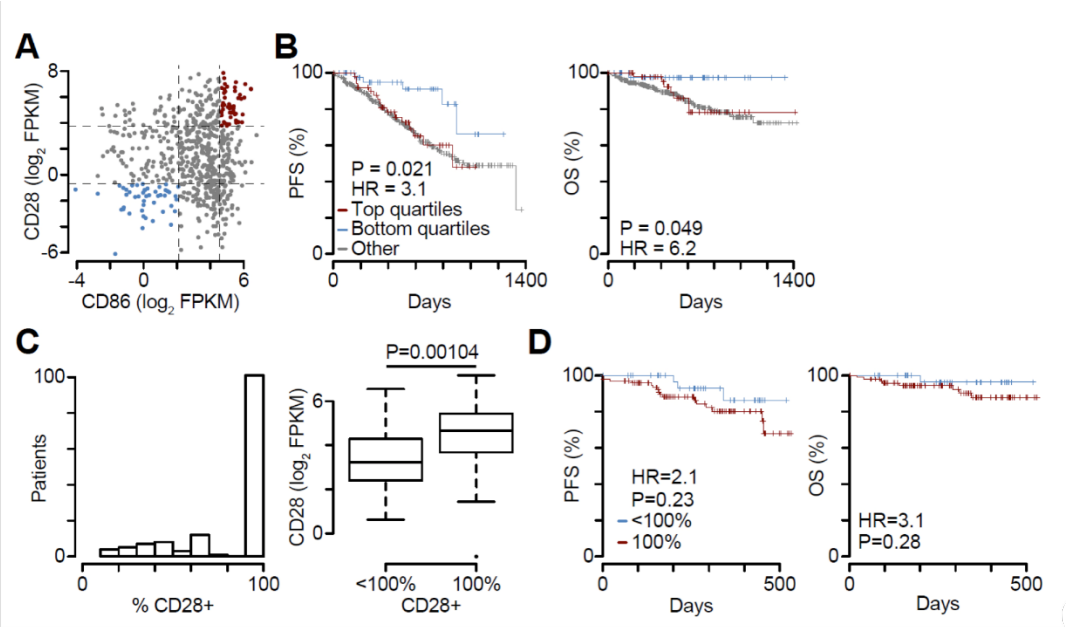
shRNA treatment. For qRT-PCR, all data are normalized to ß-Actin as an endogenous control, and then compared relative to mRNA levels in pLKO.1 empty-vector control. RNA was extracted at Day 3 post-infection. Data shown are mean ± SEM of at least 3 independent experiments. Histograms showing surface levels of indicated molecules. Grey histograms at left represent unstained or isotype controls. Flow cytometry data shown are from Day 4 post-infection with lentiviral vectors. Flow cytometry and Western blot data shown are representative of at least 3 independent experiments. (*E*) Cell adhesion 3 days post-infection with lentivirus containing the indicated shRNA. Myeloma cells stained with calcein-AM were co-cultured with HS-5 cells for 2 hours. Fluorescence was measured (485/528 emission/excitation) with BioTek Synergy H1 multi-well plate reader, and data are presented as fluorescence relative to pLKO.1 controls (mean ± SEM). Pre-wash readings were taken to ensure similar number of live cells were added to each well. All samples were plated in triplicate. Data are mean of 3 independent experiments.

Figure 4. Overexpression of CD86 provides a survival advantage against different cell death signals. *(A)* Alignment of CD86 cytoplasmic domains using Clustal. (* - denotes identity; :,. – similarity) *(B)* Diagrams showing structure of the two different CD86 constructs. CD86FLm represents full-length CD86, while CD86TLm represents the tail-less version, wherein the cytosolic domain was shortened to 7 amino acids to abrogate signaling capacity. The histograms at the right show surface levels of stable transfection of CD86 constructs compared to pCDNA3.1-vector control and parental cells. *(C)* Representative histograms showing surface expression of indicated molecules in RPMI8226 CD86FLm- and CD86TLm- expressing cells. *(D)* Concentration curves for

CD86 transfectants and vector controls with indicated pharmacologic agents. Cells were treated for 24 hours (except in the case of Dexamethasone – 48 hours), and cell death was measured via Annexin V-PI staining. Data shown as percent of untreated control for each cell line. Data shown are mean ± SEM of at least 3 independent experiments.

Figure 5. Overexpression of CD86FLm provides a survival advantage against silencing of CD86 and CD28, but not against silencing of IRF4. *(A)* Representative histograms showing the levels of surface CD86 in 3 different cell lines when either CD28, CD86, or GAPDH are silenced. Thin black histograms at left are unstained pLKO.1-infected controls. *(B)* Cell death measured at Day 4 post-infection via Annexin V staining, shown as percent of pLKO.1-infected controls. Data shown (A-B) are from day 4 post-infection, representative of at least 3 independent experiments. *(C)* qRT-PCR was performed to determine levels of *CD86*, *CD28*, and *IRF4* to compare the different cell lines. Data are normalized to ß-Actin as endogenous control, and then compared relative to mRNA levels in pLKO.1 empty-vector control. Data shown are mean ± SEM of at least 3 independent experiments *(D)* Representative Western blots showing levels of IRF4 in cells overexpressing CD86FLm or CD86TLm when either CD86 or CD28 are silenced. Lysates are from Day 4 post-infection. *(E)* Cell death as measured by Annexin-V staining at day 4 post-infection in 8226-pCDNA3.1 or 8226-CD86FLm cells where CD86 or IRF4 was silenced, shown as percent of pLKO.1-infected controls. *(F)* Representative Western blots showing levels of IRF4 and β-Actin in lysates from experiments in (*E*). Data shown are mean ± SEM of at least 3 independent experiments. (*$p<0.05$, **$p<0.01$, ***$p<0.005$).

Figure 6. Cell death induced by CD86 blockade is only partially caspase-dependent, and has similarities with death induced by loss of IRF4. (*A*, **left***)* Panels showing cell death represented as percent of pLKO.1 infected controls over time in RPMI 8226 cells overexpressing the indicated Bcl-2 family members. (A, right) Representative histograms showing surface expression of CD28 or CD86 in 8226-pCDNA3.1 controls and 8226-Mcl-1 transfectants. *(B)* Cell death levels measured via percent of Annexin V-positive cells at day 4 post-infection. For Q-VD-Oph treated cells, the caspase inhibitor was added at day 2 post infection. All data are from day 4 post-infection *(C)* Representative Western blots showing Caspase-3 and PARP cleavage with silencing of CD28 or CD86, with or without Q-VD-Oph. *(D)* Cell death with IRF4 or CD86 silencing was measured via Annexin V staining, in the presence or absence of Q-VD-Oph. *(E)* Representative Western blots showing expression of IRF4, Caspase 3, PARP when CD86 or IRF4 are silenced in MM.1s, 8226 cells. Data shown are mean ± SEM, or representative, of at least 3 independent experiments. (*$p<0.05$, **$p<0.01$).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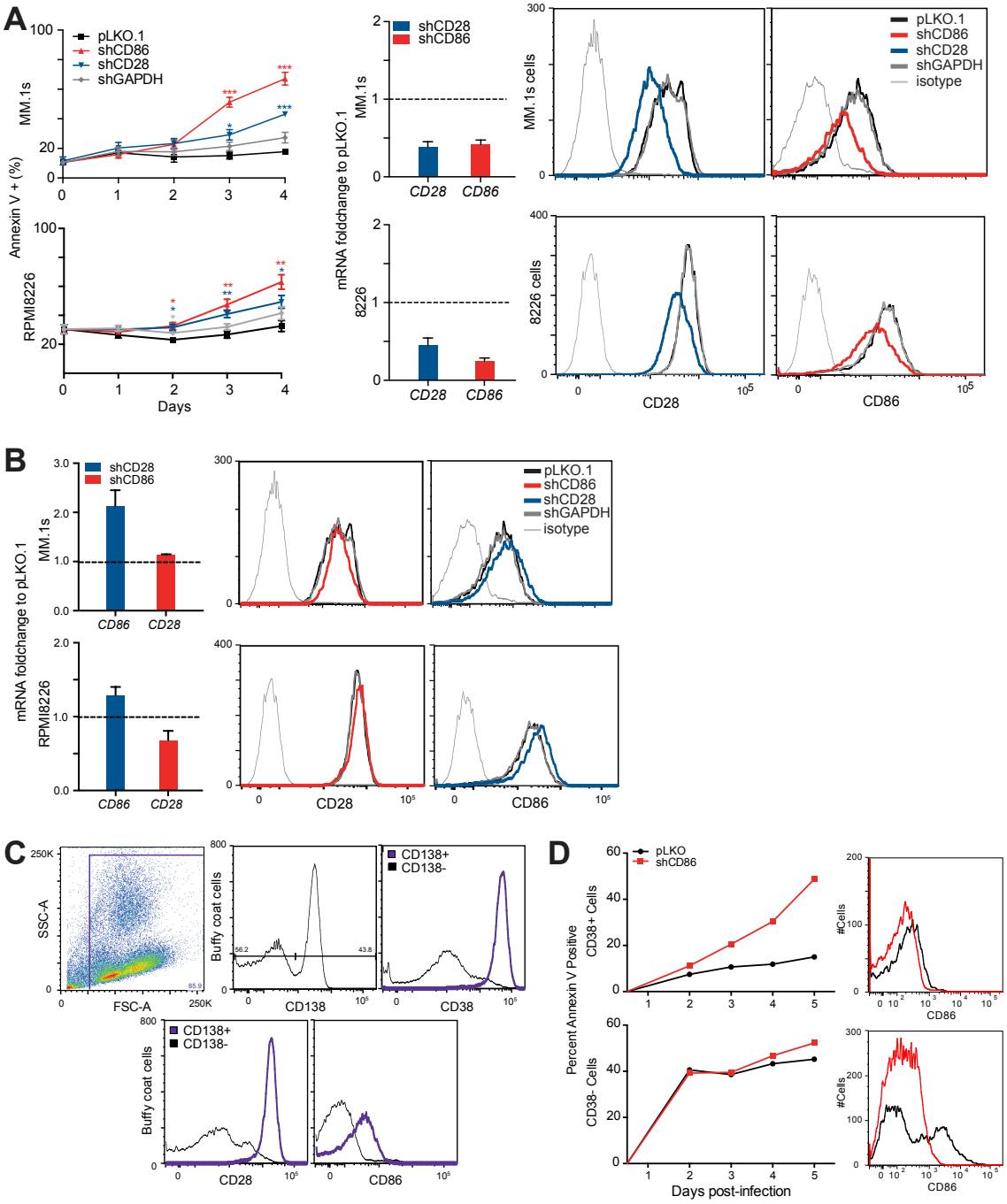
CD28 (log2 FPKM)
CD86 (log2 FPKM)
B
PFS (%)
P = 0.021
HR = 3.1
Top quartiles
Bottom quartiles
Other
Days
OS (%)
P = 0.049
HR = 6.2
Days
C
Patients
% CD28+
P=0.00104
CD28 (log2 FPKM)
<100%
100%
CD28+
D
PFS (%)
HR=2.1
P=0.23
<100%
100%
Days
OS (%)
HR=3.1
P=0.28
Days

Figure 1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shCD86
shCD28
shGAPDH
MM.1s
RPMI8226
Annexin V + (%)
Days
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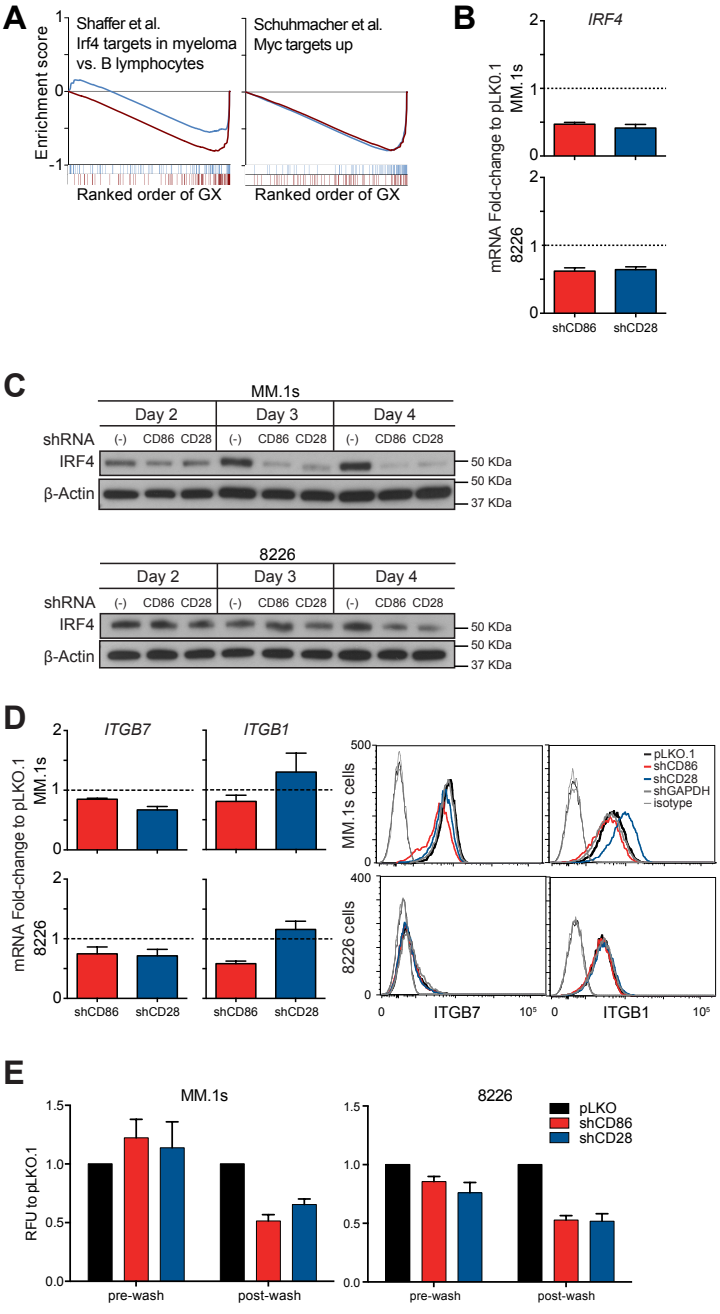
shCD86
mRNA foldchange to pLKO.1
CD28
CD86
MM.1s cells
8226 cells
isotype
B
C
SSC-A
FSC-A
Buffy coat cells
CD138+
CD138-
CD38
D
pLKO
shCD86
Percent Annexin V Positive
CD38+ Cells
CD38- Cells
Days post-infection
#Cells

Figure 2


A
Enrichment score
Shaffer et al.
Irf4 targets in myeloma
vs. B lymphocytes
Schuhmacher et al.
Myc targets up
Ranked order of GX
Ranked order of GX
B
IRF4
mRNA Fold-change to pLKO.1
MM.1s
8226
shCD86
shCD28
C
MM.1s
Day 2
Day 3
Day 4
shRNA
(-) CD86 CD28 (-) CD86 CD28 (-) CD86 CD28
IRF4
β-Actin
50 KDa
50 KDa
37 KDa
8226
Day 2
Day 3
Day 4
shRNA
(-) CD86 CD28 (-) CD86 CD28 (-) CD86 CD28
IRF4
β-Actin
50 KDa
50 KDa
37 KDa
D
ITGB7
ITGB1
mRNA Fold-change to pLKO.1
MM.1s
8226
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shCD28
MM.1s cells
8226 cells
pLKO.1
shCD86
shCD28
shGAPDH
isotype
ITGB7
ITGB1
E
MM.1s
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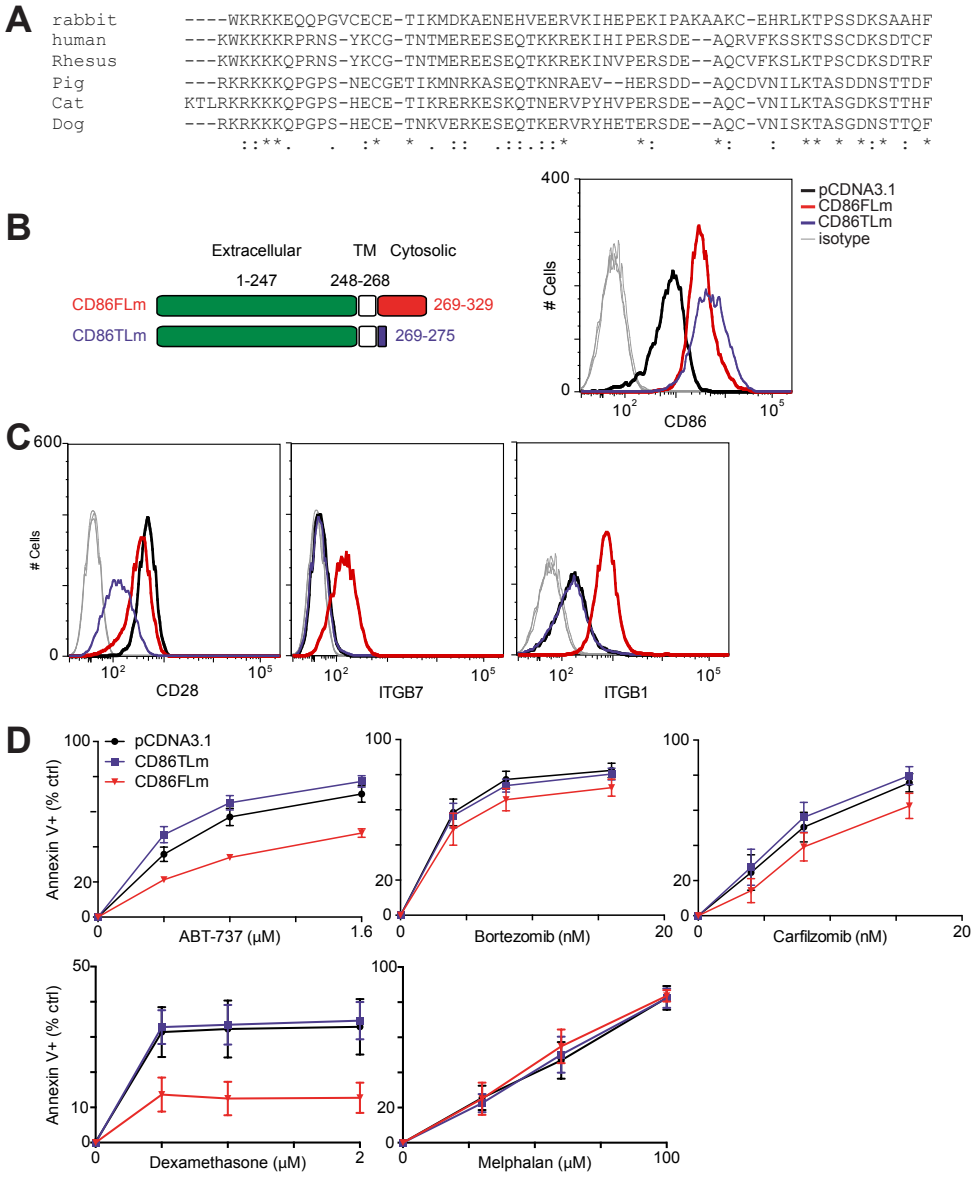
pLKO
shCD86
shCD28
RFU to pLKO.1
pre-wash
post-wash
pre-wash
post-wash

Figure 3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Dog ---RKRKKKQPGPS-HECE-TNKVERKESEQTKERVRYHETERSDE--AQC-VNISKTASGDNSTTQF
B
Extracellular
TM
Cytosolic
1-247
248-268
CD86FLm
269-329
CD86TLm
269-275
400
0
Cells
pCDNA3.1
CD86FLm
CD86TLm
isotype
CD86
C
600
0
Cells
CD28
ITGB7
ITGB1
D
Annexin V+ (% ctrl)
pCDNA3.1
CD86TLm
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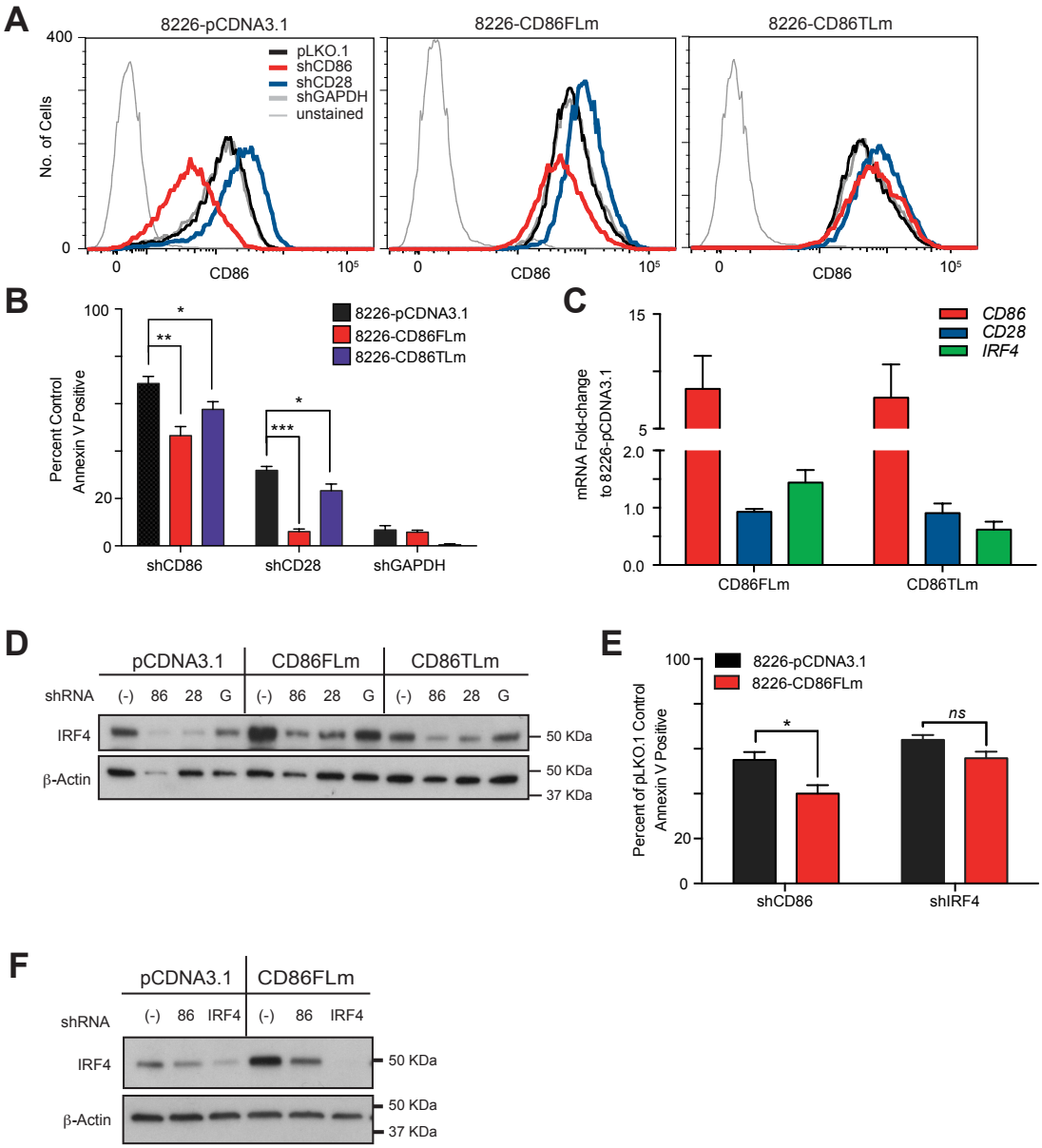
ABT-737 (μM)
Bortezomib (nM)
Carfilzomib (nM)
Dexamethasone (μM)
Melphalan (μM)

Figure 4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8226-CD86FLm
8226-CD86TLm
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shCD86
shCD28
shGAPDH
unstained
No. of Cells
400
0
CD86
B
Percent Control Annexin V Positive
8226-pCDNA3.1
8226-CD86FLm
8226-CD86TLm
shCD86
shCD28
shGAPDH
C
mRNA Fold-change to 8226-pCDNA3.1
CD86
CD28
IRF4
CD86FLm
CD86TLm
D
pCDNA3.1
CD86FLm
CD86TLm
shRNA
IRF4
β-Actin
50 KDa
37 KDa
E
Percent of pLKO.1 Control Annexin V Positive
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shCD86
shIRF4
F
pCDNA3.1
CD86FLm
shRNA
IRF4
β-Actin

Figure 5

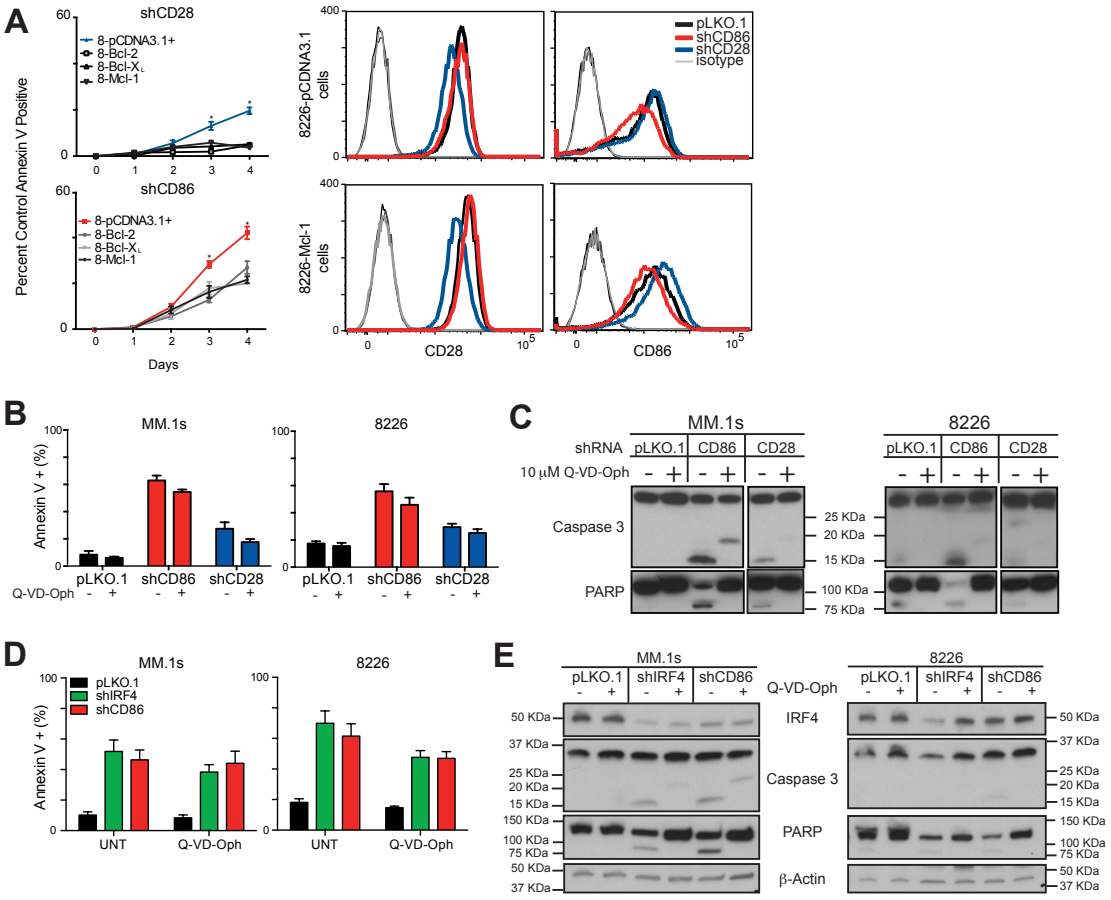
A
shCD28
8-pCDNA3.1+
8-Bcl-2
8-Bcl-XL
8-Mcl-1
shCD86
Percent Control Annexin V Positive
Days
8226-pCDNA3.1 cells
8226-Mcl-1 cells
pLKO.1
shCD86
shCD28
isotype
CD28
CD86
B
MM.1s
8226
Annexin V + (%)
pLKO.1
shCD86
shCD28
Q-VD-Oph
C
MM.1s
8226
shRNA
pLKO.1
CD86
CD28
10 μM Q-VD-Oph
Caspase 3
PARP
25 KDa
20 KDa
15 KDa
100 KDa
75 KDa
D
MM.1s
8226
pLKO.1
shIRF4
shCD86
Annexin V + (%)
UNT
Q-VD-Oph
E
MM.1s
8226
pLKO.1
shIRF4
shCD86
Q-VD-Oph
IRF4
Caspase 3
PARP
β-Actin
50 KDa
37 KDa
25 KDa
20 KDa
15 KDa
150 KDa
100 KDa
75 KDa

Figure 6

Supplementary Methods and Data

Alignment and quantification of RNA-seq data

RNA-seq data was mapped back to the UCSC human genome (hg19) using Tophat2[1] (v.2.1.1) using the following parameters "-p 14 -N 2 --bowtie1 --max-multihits 1 --read-gap-length 1 -- transcriptome-index" where the Gencode GRCh37 (v19) transcription database[2] was used. PCR duplicates were identified using Picard[3] (http://broadinstitute.github.io/picard/) and removed from subsequent analyses. Reads that uniquely overlapped Gencode GRCh37 exons were determined in R (v.3.2.3) using the 'summarizeOverlaps' function in mode 'IntersectionNotEmpty' of the 'GenomicAlignments' package54 (v.1.6.3). Reads per million (RPM) were calculated for each gene based on the number reads in all potential exons for a given gene and the total number of uniquely mappable reads per sample. Fragments per kilobase per million (FPKM) were calculated based on RPM and the total size of non- overlapping exons for a gene. Both raw and summarized data were deposited in GEO under accession GSE89511.

Differential and bioinformatic analysis of RNA-seq data

Differentially expressed genes (DEGs) were determined using the generalized linear model function in edgeR[4] (v.3.12.1) where a co-variate was added for cell line. P-values calculated by EdgeR were corrected for multiple hypothesis testing using Benjamini-Hochberg FDR correction and those with an FDR ≤ 0.01 were considered significant. Heatmaps and hierarchical clustering of gene expression data used an 'average' or unweighted pair group method with arithmetic mean agglomeration method applied to the Z-score normalized gene expression (FPKM) using

the R/Bioconductor functions 'hclust' and 'image' as previously described[5] (R code available upon request). Gene ontology analysis was conducted on differentially expressed genes (DEGs) using the R/Bioconductor package GOstats[6] (v2.36.0). Gene Set Enrichment Analysis[7] (GSEA v2.1.0) was performed using the pre-ranked list option where the rank was determined by the - log10(FDR) x sign(fold-change). Data were plotted using custom R scripts, and all code is available upon request.

Cell adhesion assay

In a 96-well plate, $1.25x10^4$ cells were plated in each well in 200 μL of media for 48 hours prior to adhesion assay.

For the adhesion assay, $6x10^5$ live cells (Trypan blue negative) were collected for each sample. Cells were washed thrice in plain RPMI1640 (without L-glutamine and phenol red, Cellgro 17-105-CV), and then resuspended in 1.2 mL of 5 μM calcein-AM (Molecular Probes C3100MP) for 30 minutes at 37°C. After incubation, cells were washed in plain RPMI three times, resuspended in 1.2 mL complete media, and applied to the 96-well plates containing HS-5 cells. Co-cultures were incubated for 2 hours, after which fluorescence readings were taken using a BioTek Synergy H1 plate reader (pre-wash readings). After initial readings, wells were gently washed three times with plain RPMI to remove non-adherent cells. After washing, 100 μL of plain RPMI was applied to each well, and fluorescence readings were again taken (post-wash readings).

Table S1. List of Cell lines and sources

Cell line Used	Source from which cell line was procured
MM.1s	Dr. Steven Rosen (City of Hope, CA)
RPMI8226	ATCC
KMS18	Dr. P. Leif Bergsagel (Mayo Clinic, AZ)
NCI-H929	ATCC
KMS12-BM	Japanese Collection of Research and Bioresources Cell Bank

Table S2. List of shRNA clones used in this study

Target gene	TRC Clone #, and Source
CD28	TRCN0000057679 and TRCN0000057678 (Open
CD86	TRCN0000007646 and TRCN0000007644 (Open
GAPDH	TRCN0000221342 and TRC0000221343 (Open Biosystems)
IRF4	TRCN0000014767 (Sigma Aldrich)

Table S3. List of antibodies used for flow cytometry and Western Blotting

Flow Cytometry Antibodies and Reagents	• CD28-PE (BD 555729) • CD28-PercCP Cy5.5 (BD 337181) • CD28-BV510 (BD 563075) • CD28-PECy7 (BD560864) • CD38-V450 (BD 646851) • CD45-APC-Cy7 (BD 348795) • CD86-APC (BD 555660) • CD86-PE (BD 555658) • CD86-Percp Cy5.5 (BD 561129) • CD98-FITC (BD 556076) • CD98-PE (BD 556077) • CD138-FITC (BD552723) • ITGB7-PE (BD 555945) • ITGB1-APC (559883) • Annexin V (FITC) – Biovision 1001 • Annexin V (Pacific Blue) – Life Technologies (A35122)
List of Antibodies used for Western blotting	• rabbit anti-Bim pAb (AB17003, EMD Millipore) • rabbit anti-Mcl-1 pAb (ADI AAP-240D, Enzo Life Sciences) • rabbit anti-Bcl-xL pAb (27648), rabbit anti-IRF4 pAb (4964S), and rabbit anti-caspase 3 pAb (9662S) (Cell Signaling Technology) • mouse anti-Bcl-2 mAb (sc509, Santa Cruz Biotechnology) • rabbit anti-LC3B pAb (L7543), mouse anti-PARP mAb (C-2-10 clone P248) and mouse anti-β-actin mAb (030M4788) (Sigma-Aldrich)

Table S4. List of qRT-PCR probes used for measuring mRNA expression levels

List of qRT-PCR probes used (all purchased from Applied Biosystems	• CD86 (Hs01567025_m1, Hs00199349_m1) • CD28 (Hs00174796_m1, Hs01007422_m1) • SLC7A5 (Hs00185826_m1) • ITGß1 (Hs00559595_m1) • ITGß7 (Hs00168469_m1) • Bim (Hs00708019_s1) • Mcl-1 (Hs01050896_m1) • Bcl-x_L (Hs00236329_m1) • Bcl-2 (Hs0060823_m1) • GAPDH (4332649) • ß-Actin (4333762-1108032) • IRF4 (Hs01056533_m1)

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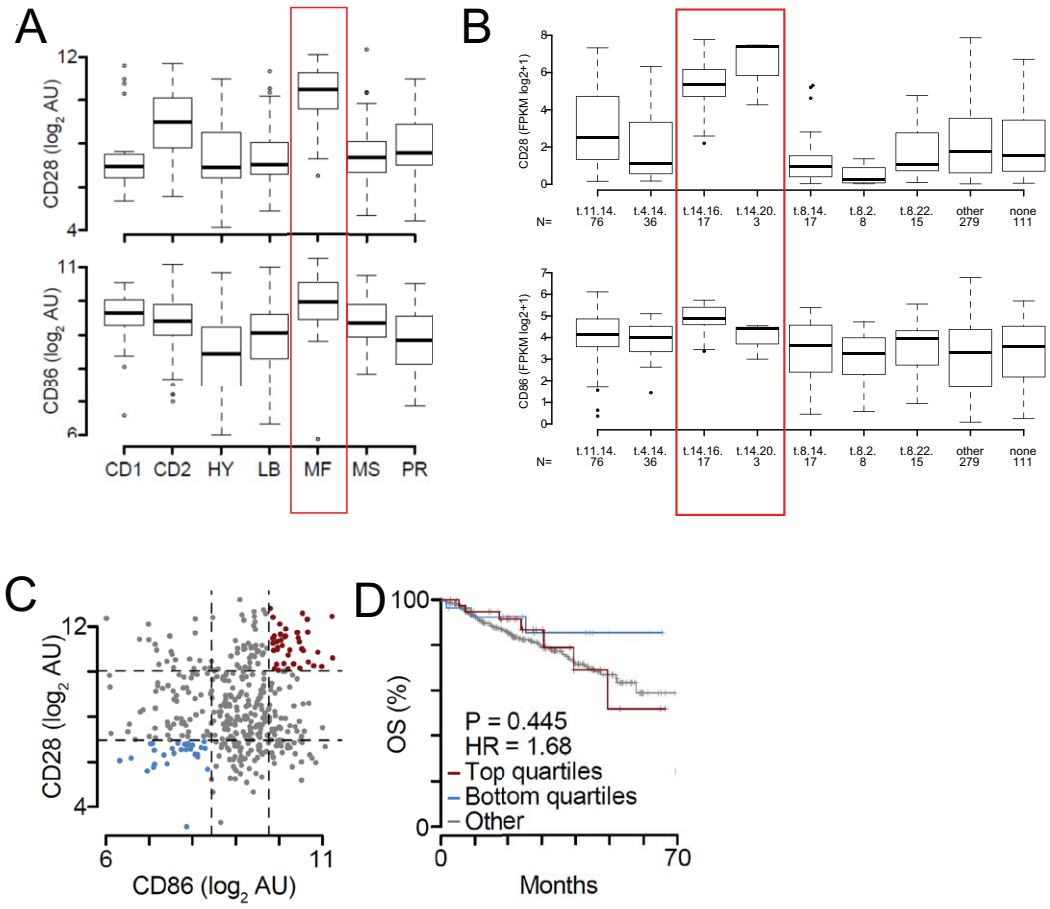
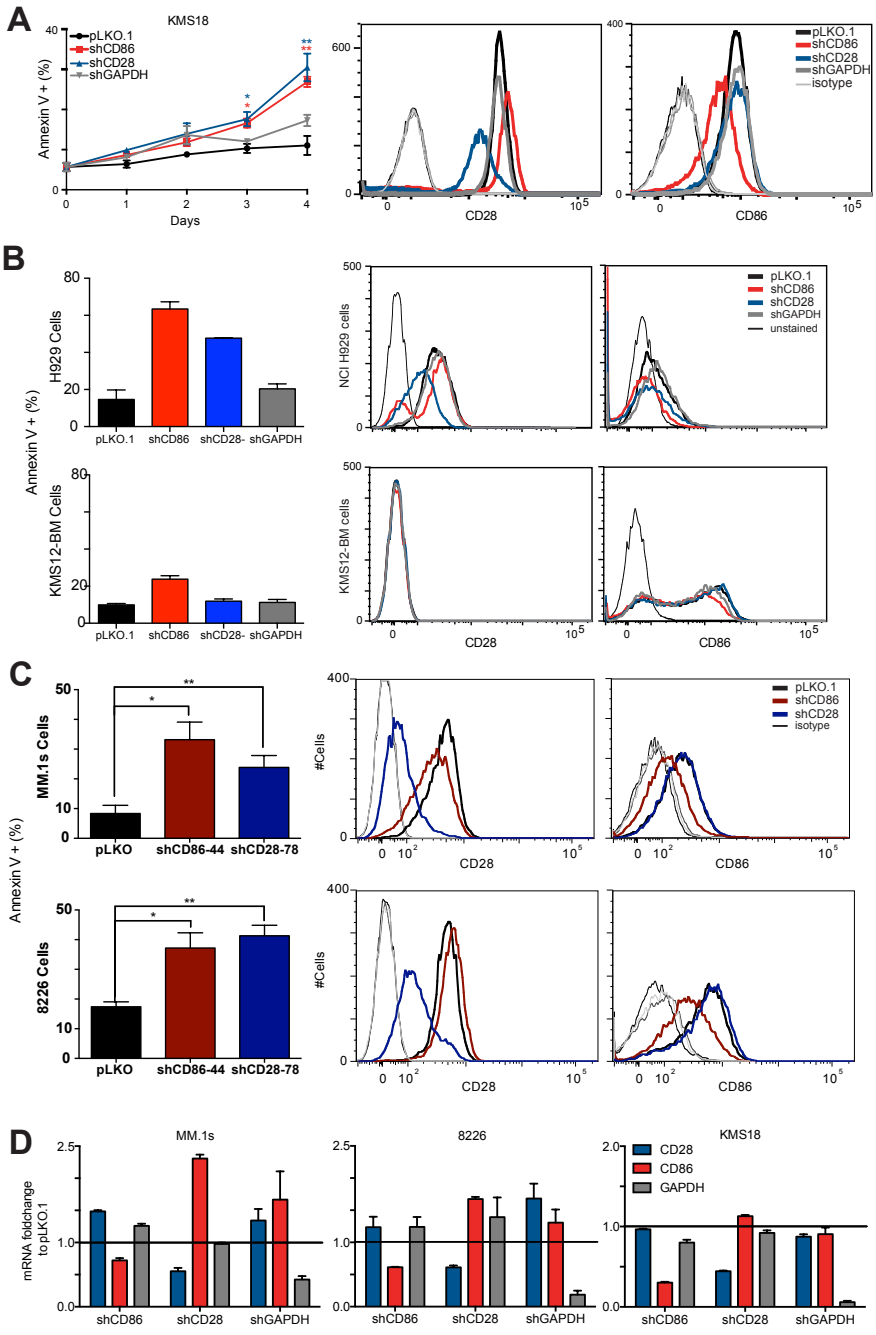


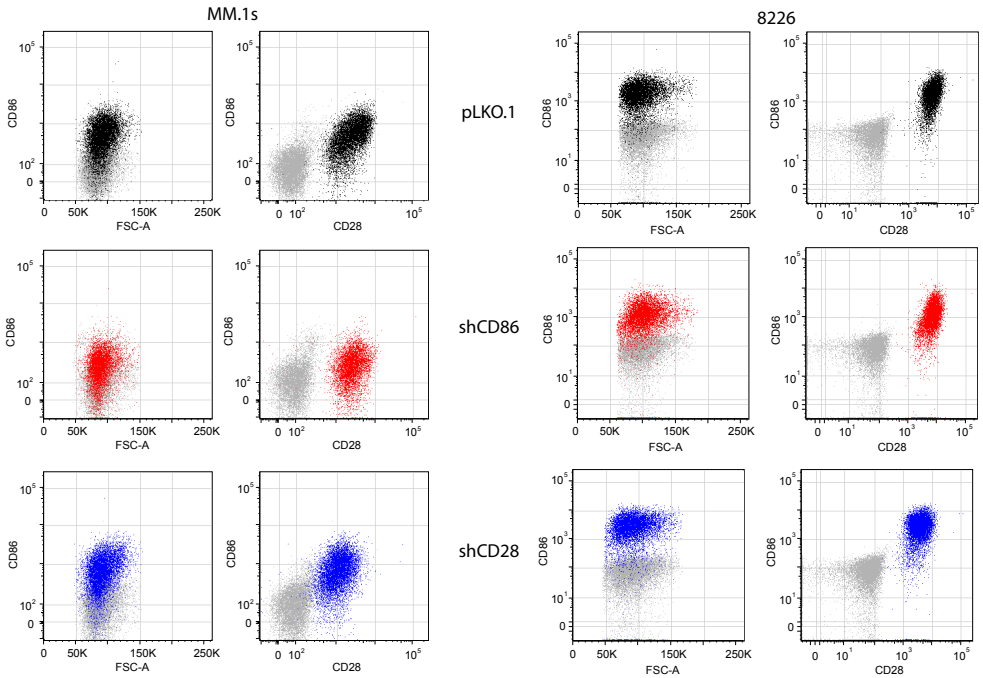
Supplementary Figure 1. CD28 and CD86 expression analysis of the different myeloma subtypes and clinical impact. Data analyzed are from a previously published cohort ffrom UAMS (Zhan *et al*. PMID:11861292) in (A), and CoMMpass in (B). (C) Plot of CD28 vs CD86 expression in a previously published Arkansas cohort of myeloma patients (Zhan et al. PMID: 11861292). Vertical and horizontal dashed lines represent the 25 and 75 percentiles
(D) Kaplan Meier survival curve for patients in the top CD28 and CD86 expression quartile (red), bottom quartile of CD28 and CD86 expression (blue), and all other patients (gray) in the Arkansas study.

Supplementary Figure 1



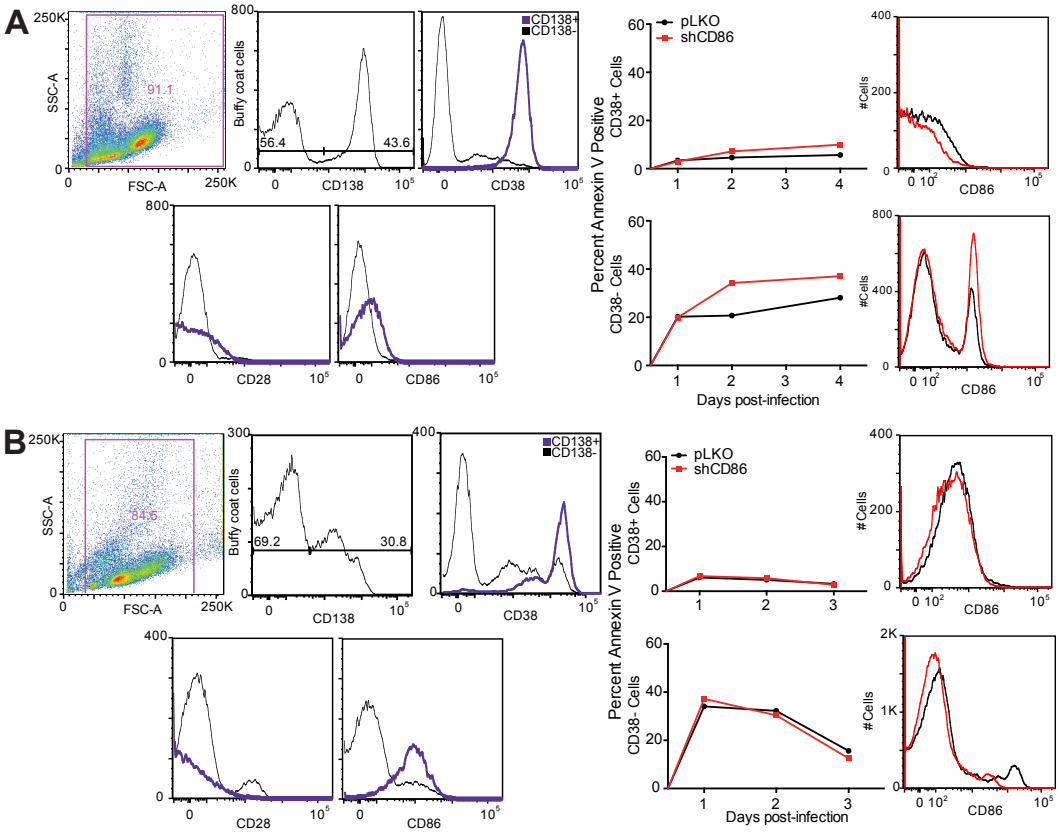
Supplementary Figure 2

Supplementary Figure 2. (A-B) Additional cell lines tested show that CD28-CD86 double positive lines are sensitive to silencing of CD28 or CD86 (KMS18, H929), while those that are negative for one of the markers are less sensitive to the shRNAs (KMS12BM). The panels on the left show levels of cell death as measure by Annexin V staining. Histograms at right depict surface levels of CD28 or CD86. (C) Testing other shCD28 or shCD86 resulted in partial knockdown of either molecule, and myeloma cell death. (D) shGAPDH results in modulation of GAPDH expression at the mRNA levels. All data are shown as mean + SEM of 3 independent experiments, except for D, where 8226 and KMS18 data are n=2.



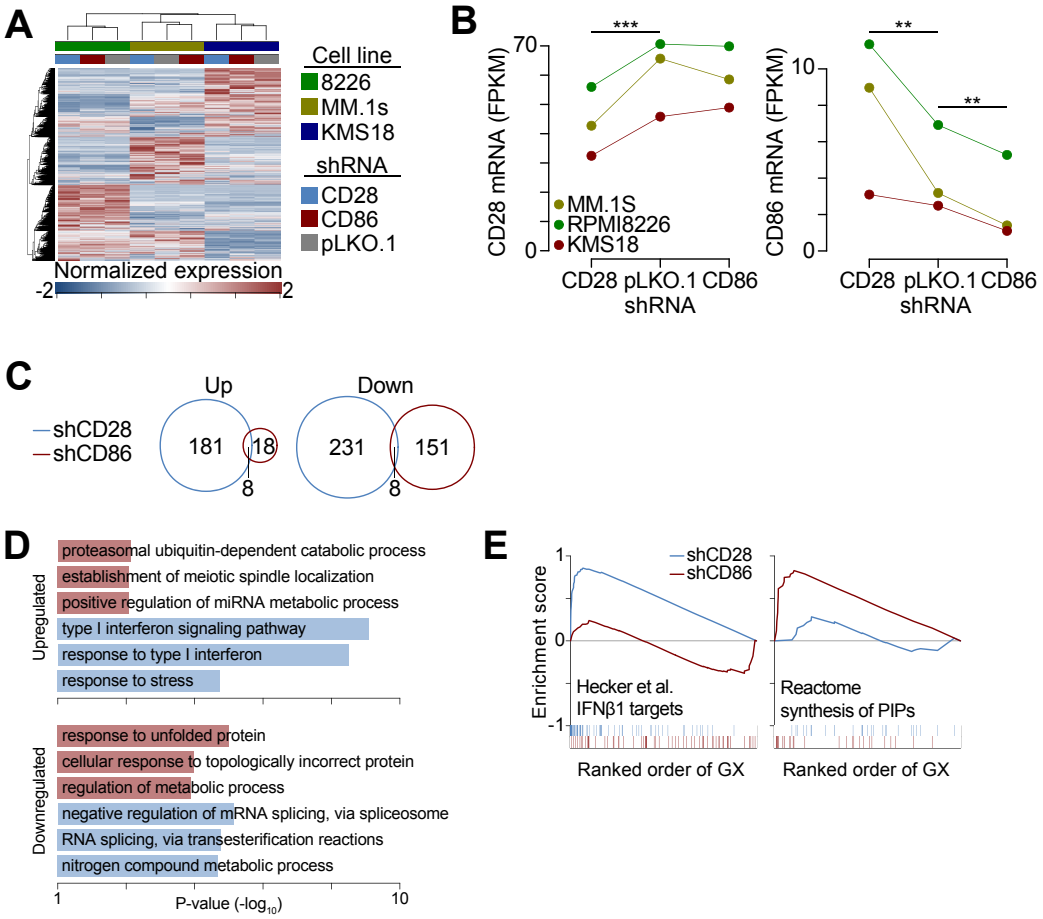
Supplementary Figure 3. Bivariate contour plots showing Forward Scatter, CD86 and CD28 levels in MM.1s and 8226 cells where either CD86 (red) or CD28 (blue) are silenced. Grey plots represent unstained samples. Data are from cells that were negative for Annexin V. All plots are representative of at least 3 independent experiments. Flow cytometry analysis was done in FlowJo.

Supplementary Figure 3



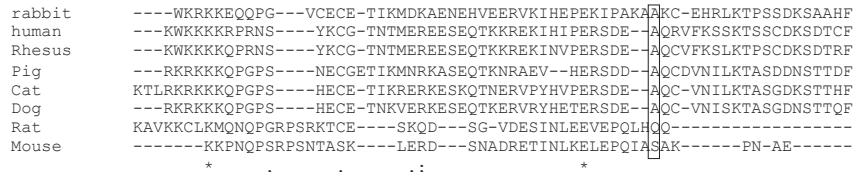
Supplementary Figure 4. Silencing of CD86 in patient samples that are either (A) CD28low-CD86low, or (B) single-positive (in this case, CD28negative-CD86positive) show no effect of viability of CD38-positive cells from bone marrow aspirates. Histograms at right show CD86 levels on cells indicated at day 4 (A) or day 3 (B) post infection.

Supplementary Figure 4



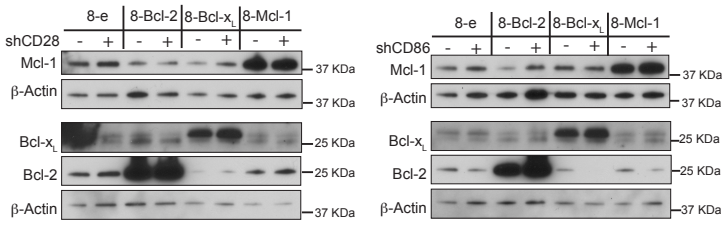
Supplemental Figure 5. CD28 and CD86 modulate gene expression. (A) Hierarchically clustered heatmap of gene expression in MM.1s, KMS18, and RPMI8226 cell lines transfected with vector (pLKO.1) or shRNA against CD86 or CD28. (B) Gene expression of CD28 (left) and CD86 (right) in myeloma cells transfected with shCD28, pLK0.1 vector, or shCD86. ***P-value ≤0.001, **P-value ≤0.01, negative binomial likelihood test in edgeR. (C) Venn diagram of upregulated (top) and downregulated (bottom) differentially expressed genes upon knock-down of CD28 (shCD28, blue) and CD86 (shCD86, red). (D) Top 3 gene ontology terms of upregulated (top) and downregulated (bottom) differentially expressed genes. (E) Gene set enrichment analysis showing differential results for CD28 and CD86 treated myeloma cells. For each gene set, the enrichment score is shown above the ranked changed in gene expression where genes that overlap the gene set are denoted by blue and red ticks for shCD28 and shCD86 respectively.

Supplementary Figure 5



Supplementary Figure 6. Alignment of the cytoplasmic domains of CD86 from 8 mammalian species using Clustal. Residues in box are at position 304, in the cytoplasmic domain of the human CD86 protein. (* - denotes identity; :,. – similarity)

Supplementary Figure 6



Supplementary Figure 7. Analyses of Bcl-2 family member expression in different cell lines with CD28 or CD86 silencing. (A) Representative Western blots showing levels of Bcl-2 family member when CD28 or CD86 are silenced in cell lines that overexpress the indicated pro-survival Bcl-2 family members.

Supplementary Figure 7

III. The CD28-CD86 signaling pathway plays a role in the regulation of SLC7A5

Introduction

We have shown that the CD28-CD86 signaling pathway can provide myeloma cells with a survival advantage that is detrimental to patient outcome. Given that our data point to this pathway playing a role in the regulation of IRF4, an intrinsic survival factor for myeloma cells, this indicates that this pathway is a viable therapeutic target for myeloma (Chapter 2).

Our RNAseq results indicate that the CD28-CD86 pathway regulates different physiological aspects that are crucial to maintaining myeloma viability. UPR and protein catabolism are necessary for maintaining myeloma homeostasis especially as myeloma cells still work to fulfill their function as antibody secreting machines. The solute carrier family members (SLC) were found to exhibit expression changes with silencing of either CD28 or CD86. We focused on *SLC7A5*, a gene which encodes one of the subunits of CD98, a large neutral amino acid transporter, and found that its expression is regulated by the CD28-CD86 pathway.

Results

Silencing of CD28 or CD86 results in downregulation of SLC7A5

To confirm our RNA-seq results, we silenced CD28 or CD86 with shRNAs, and then determined whether *SLC7A5* was downregulated. We found that silencing of either CD28 or CD86 results in downregulation of *SLC7A5* transcripts in both MM.1s and RPMI8226 cell lines. The surface expression of CD98 (the heterodimer formed by SLC7A5 and

SLC3A2) was also downregulated with silencing of CD28 (in both cell lines) and CD86 (in MM.1s). In the case of 8226, shCD86 only modestly downregulated surface CD98.

Overexpression of CD86 results in modest protection against cell death induced by silencing of SLC7A5

We have previously shown that overexpression of CD86-full-length (CD86FLm) can protect cells against different pharmacologic agents, as well as silencing of either CD86, or CD28. A tail-less version of the CD86 (CD86TLm) construct could not protect against the different drugs, and only modestly protected against silencing of CD86 or CD28.

We next determine if silencing of *SLC7A5* would differentially affect viability of cell overexpressing either CD86FLm or CD86TLm. We found that both constructs modestly protected against cell death induced by silencing of *SLC7A5*.

Discussion

We performed RNA-seq on cells where we silenced either CD28 or CD86 in multiple myeloma cell lines to get a global view of gene expression changes when we block this signaling module. While overlap between the two treatments was not extensive, we focused on genes whose expression changed when either CD28 or CD86 was silenced, and was in common across all the cell lines tested. IRF4 was one, which indicates that this pathway mediates myeloma cell viability via the regulation of this important myeloma survival factor. Interestingly, there were multiple SLC (solute carrier) family members whose expressions changed with blockade of the CD28-CD86 signaling pathway.

We focused on *SLC7A5*, a gene that codes for one of the subunits of LAT-1/CD98. CD98 transport activity is necessary for protein expression of c-Myc[152], a transcription factor that has a myriad of targets, most of which are proteins that play a role cell proliferation. Interestingly, c-Myc is a well characterized oncogenic factor, and myeloma cells have been found to be addicted to c-Myc signaling[153], as it forms a pro-survival feedback loop with IRF4[39].

CD98 was recently found to be an important amino acid transporter that provides a vital metabolic switch that facilitates T-cell activation, and interestingly this was also found to require CD28-costimulation[152]. Because of its role in the transport of amino acids across membranes, CD98 thus has dual roles in myeloma. First, secretion of antibodies requires protein synthesis and a constant source of amino acids. Second, the proliferative capacity of myeloma cells also requires continuous biomolecule transport to facilitate protein synthesis. Because CD98 activity is required for c-Myc expression, blockade of this transporter could induce myeloma cell death via loss of Myc and subsequent loss of IRF4[39]. Notably, IRF4 also plays a role in regulation of CD98, via regulation of *SLC3A2*, the gene that encodes the other half of the heterodimer. Since CD28-CD86 signaling regulates IRF4 expression (Chapter 2), there are multiple mechanisms modulation of this pathway can affect CD98 expression (Figure 2).

SLC7A5 thus plays a role in maintaining not only cell viability, but also the ability of myeloma cells to keep performing their function of immunoglobulin secretion. Interestingly, this gene is also a target of Blimp-1[28]. Blimp-1 is the master transcription factor in plasma cell differentiation that has recently been shown to be regulated by CD28 signaling in normal long-lived plasma cells[104]. This indicates that a mechanism by which

the CD28-CD86 signaling pathway is regulating viability and function of myeloma cells is via *SLC7A5*.

Figures

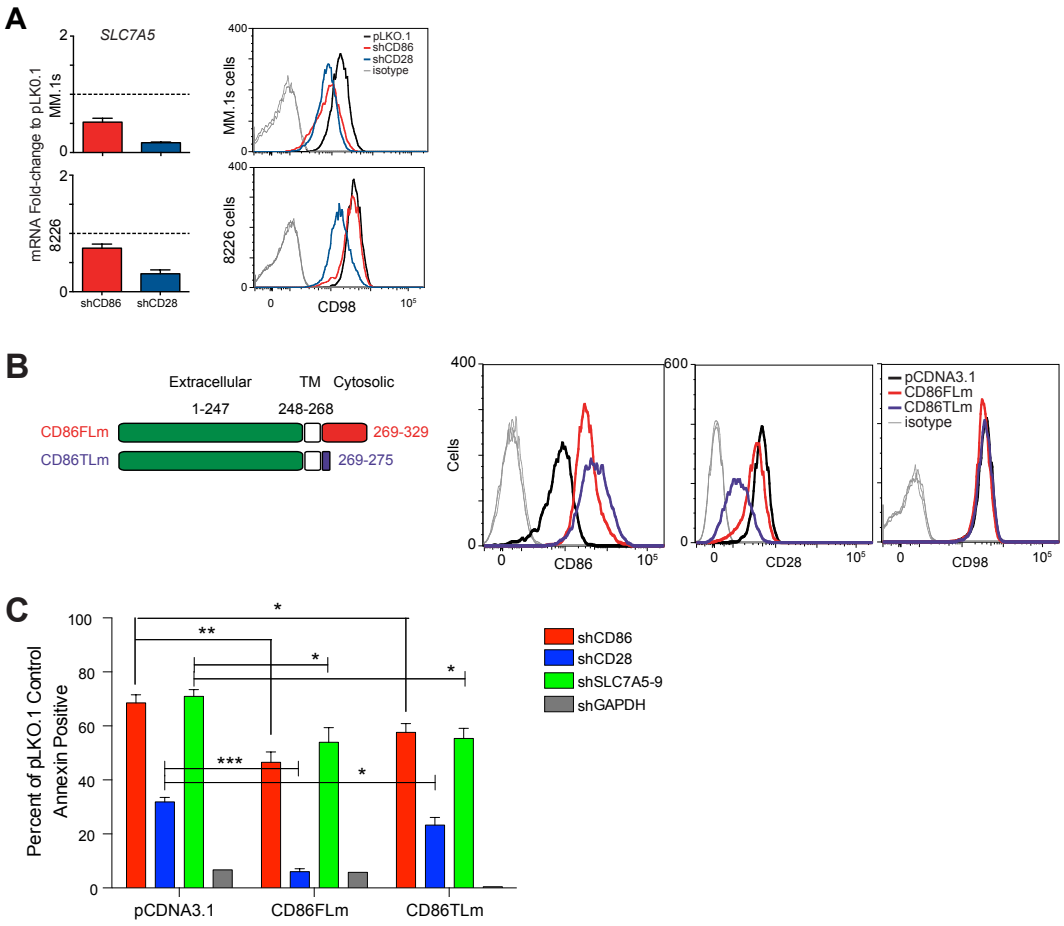


Figure 1. *SLC7A5* is regulated by the CD28-CD86 signaling pathway. *(A)* qRT-PCR and flow cytometry data showing *SLC7A5* mRNA and CD98 surface expression when CD28 or CD86 are silenced in MM.1s or 8226 cells. *(B)* Diagram of CD86 constructs. Histograms at right show surface expression levels of CD86, CD28 and CD98 when the different constructs are overexpressed in the RPMI8226 cell line. *(C)* Cell death as measured by Annexin V staining when *SLC7A5* is silenced in the different cell lines described in (B). Data shown are mean ± SEM of at least 3 independent experiments. Flow cytometry data showing surface levels of indicated molecules are representative of at least independent experiments. Grey histograms at left are unstained or isotype controls.

Figure 1

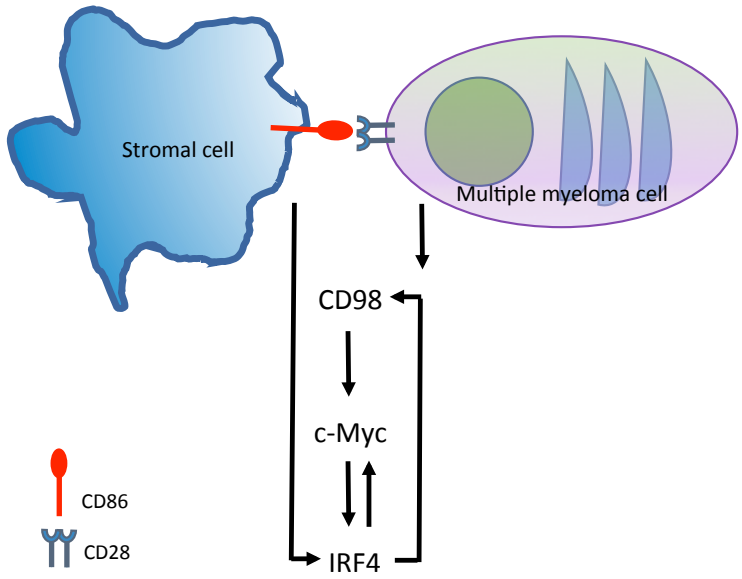


Figure 2. Signaling cascade from CD28-CD86 interaction regulates CD98. CD98, a heterodimer of SLC7A5 and SLC3A2, is regulated (1) by CD28-CD86 signaling via direct regulation of SLC7A5 expression, and (2) by regulation of IRF4, a transcription factor regulated by CD28-CD86 signaling that targets SLC3A2. Note that myeloma cells could also potentially interact via CD28 and CD86 in an autocrine manner, or between myeloma cells.

IV. CTLA-4-Ig and it's potential for therapeutic use in myeloma

Introduction

The discovery of the CD28 costimulatory pathway showed that aside from antigen recognition, a host of other signals were required to fully engage a T-cell to mediate the appropriate immune response. Antigen recognition alone led to anemic T-cell responses, whereas co-activation of CD28 via ligation to CD80 or CD86 led to induction of pro-survival and proliferative factors, allowing the T-cell to go further down the path of differentiating into the appropriate effector subset[93]. The discovery of another receptor for the B7 ligands (as CD80 and CD86 were referred to) in the form of CTLA-4 led to the hypothesis that the system may require 3 signals for full activation. Because CTLA-4 was shown to bind CD80 and CD86, a soluble receptor form was developed to test its role in T cell activation[154]. It was later found that this soluble form of CTLA-4 had a much higher affinity than CD28 for the B7 ligands. It was subsequently shown by work from James Allison's group that CTLA-4 blocked T-cell proliferation[155]. The development of CTLA-4 knockout mice further illustrated the inhibitory role of CTLA-4, as these mice developed uncontrolled lymphoproliferative disease and autoimmunity[156].

The important role of this signaling axis in T-cell co-stimulation has been heavily investigated, and manipulation of the outcome (whether activation or inhibition) for use in the treatment of patients has had varying degrees of success. The most well known clinical trial involved testing the therapeutic potential of the CD28 superagonist, the end goal of which was to induce T-regulatory cells to treat various autoimmune diseases[157,158]. The adverse events from this trial led to further investigation of the mechanism of action of the super-agonist on CD28, highlighting that there is still much to

be learned about the signaling pathways mediated by these molecules. In contrast, blockade of this signaling axis has been more successful in clinical contexts, and treatment with the blocking reagent CTLA-4-Ig has led to improved outcomes for patients who undergo transplants or have autoimmune dysfunctions. CTLA-4-Ig is the soluble form of CTLA-4 developed by BMS. To date, there are several iterations used in the clinic administered to patients in facilitate graft acceptance, and to block inflammatory signals that mediate autoimmune diseases such as lupus and rheumatoid arthritis.

Manipulation of the CD28-pathway (as well as other costimulatory pathways) is also being explored as a means of treating cancer, mainly by inducing the immune system to mount a response against the tumors. Immunotherapy, as this class of treatment is referred to, has 2 arms. Immune checkpoint blockade agents are so-called as they are administered with the goal of blocking inhibitory signals, and this class of agents are comprised mainly of antibodies (anti-PD-1, anti-PD-L1, anti-CTLA-4) that block inhibitory receptors on T-cells from interacting with their ligands, leading to release of the T-cells from their unresponsive states. The other side of immunotherapy involves activation of an anti-tumor response by facilitating recognition of the tumor as aberrant self-antigen, via antibodies specific against tumor antigens, or by genetically modifying patient cells to express a chimeric antigen receptor that are specific to the tumor.

In terms of myeloma treatment, immunotherapy has primarily consisted of the development of antibodies that can detect myeloma-associated surface markers to activate an immune response against the tumor cells (MAGE, anti-CD38)[159]. Recently, the development of T cells expressing chimeric antigen receptors against BCMA[8], or of a

bispecific antibody against BCMA[88,90] and Fchr5[160] have shown efficacy in inducing a durable cytotoxic anti-myeloma response, leading to disease regression.

Myeloma cells are heavily dependent on the tumor microenvironment for growth and survival signals. Our data showing that the CD28-CD86 signaling pathway mediates myeloma survival suggests that it would be of clinical benefit for myeloma patients if we can apply some of the agents used to manipulate this pathway for the patients benefit. Importantly, because CD28 and CD86 are expressed on myeloma cells, targeting this pathway directly is possible. Given that CD28 and CD86 are mediating pro-survival signals in myeloma, we next determined if inhibiting the interaction between these molecules on myeloma cells would induce cell death. We found that blockade using CTLA-4-Ig can have varying effects on the viability of myeloma cells, potentially due to the different ways this reagent can bind to CD86.Based on how our CTLA-4-Ig data initially corroborated our results from silencing either CD28 or CD86, the effects of this biologic on myeloma cells requires further investigation.

Materials and Methods

Cell lines

Cell lines used in these studies are described in Supplementary Table 1 (Chapter 2) and were cultured as previously described[70]

CTLA-4-Ig treatment

Cells were collected and washed in 1x PBS. Cells were then resuspended at 500,000 live cells/mL. CTLA-4-Ig (R&D Systems Cat. No 325-CT, in 1X PBS at 1μg/μL) was applied to 0.5 mL of the cell suspension in 24 well plates. Samples were collected at 16 hours, and viability was measured via Annexin V staining.

Flow cytometry and analysis

Cell surface expression of CD28, CD86, CD98, ITGB7 and ITGB1 (Cat.Nos. listed in Chapter 2, Supplementary Table 3) were measured via flow cytometry. Live cells (100,000) were collected, washed with 1x PBS, and stained with appropriate antibodies in 100 μL 1X Annexin Staining Buffer. After incubation (15 minutes) at 4°C in the dark, cells were washed in 1x PBS, resuspended in 400μL Annexin Staining Buffer containing 1μL of Annexin V. Samples were collected in a BD FACS Canto II. Analysis of flow cytometry data was done using FlowJo.

RNA extraction, cDNA synthesis, and qRT-PCR

RNA was extracted and qRT-PCR was performed as previously described[70]. All data are functions of relative quantity compared to pLKO.1 empty vector control. GAPDH and beta-actin were used as endogenous control genes. qRT-PCR probes are from Applied Biosystems, and are listed in Supplementary Table 4 (Chapter 2).

Protein extraction, Western blotting

Cell pellets were lysed in RIPA buffer with protease and phosphatase inhibitors as previously described[70]. Lysates were quantified using the BCA Assay, and 15-30μg of

lysate were run in SDS-PAGE gels, then blotted as previously described[70]. Antibodies used for detection are listed in Supplementary Table 3 (Chapter 2).

Results

CTLA-4-Ig induces cell death in myeloma cells in vitro

To determine if blocking endogenous CD28-CD86 signaling would recapitulate the cell death seen with silencing, we used CTLA-4-Ig, a soluble receptor for CD86, to block the ability of these 2 molecules to interact. CTLA-4 binds to CD86 with a 20-fold greater affinity than CD28, thus one would expect complete blockade at the concentration added. Indeed, CTLA-4-Ig addition also blocked **α**CD86 binding (Figure 1A, right panels). We found that blockade of CD28-CD86 interaction alone led to myeloma cell death (Figure 1A), such that greater than 60% of cells were Annexin V positive after overnight incubation with CTLA-4-Ig. We also saw that treatment with CTLA-4-Ig resulted in an increase of surface CD28 (Figure 1A, left histograms). This was recapitulated at the mRNA level (Figure 1B) in both cell lines.

Treatment with CTLA-4-Ig leads to downregulation of many of the same genes as silencing of CD28 or CD86

To characterize cell death induced by treatment of CTLA-4-Ig in terms of downstream mediators, we next determined if the same genes that looked to be regulated by CD28-CD86 signaling were perturbed by treatment wwith CTLA-4-Ig. We found that IRF4 and SLC7A5 were downregulated at the protein level upon treatment with CTLA-4-Ig (Figure 1C,D). In the case of the integrins, we saw only a modest down-regulation in

MM.1s for both ITGβ7 and ITGβ1, however this may be due to the short-term nature of the experiment.

Cell death induced by physical blockade of CD28-CD86 interaction is both caspase -dependent and -independent

We next determined if caspase inhibition could protect myeloma cells against death induced by CTLA-4-Ig. We co-treated myeloma cell lines with CTLA-4-Ig and the pan-caspase inhibitor. As a control, we used ABT-737, which induces caspase-dependent apoptotic death (Figure 1F). While the caspase inhibitors could completely protect against death from ABT-737, we found that treatment with the caspase inhibitors could only partially protect against cell death induced by CD86 blockade. Together these data indicate that CD86 blockade induces cell death that can be inhibited by Bcl-2 pro-survival members (Chapter 2, Figure 6), but is only partially caspase dependent.

Overexpression of CD86FLm could not protect against cell death induced by CTLA-4-Ig

We next investigated whether CD28 ligation to CD86FLm was necessary for mediating survival. We treated cells that overexpress different versions of CD86 with CTLA-4-Ig, and monitored apoptosis. We found that neither CD86FLm nor CD86TLm overexpression could protect against CTLA-4-Ig treatment, as cell death in these cell lines were at the same level as parental or empty vector controls (Figure 1G). This suggests that CD86FLm requires ligation to CD28 for survival signaling to occur.

Variability in myeloma cells response to CTLA-4-Ig

We next determined if treatment of myeloma patient samples with CTLA-4-Ig would recapitulate our silencing data. Our initial results showed that treatment of mononuclear cells isolated from myeloma patient bone marrow aspirates led to cell death in the subset identified to be plasma cells (i.e. CD38 high). In contrast, CTLA-4-Ig treatment had no effect on the rest of the cells in the Buffy coat. As treatment controls, we used the 8226 cell line to confirm the effects of CTLA-4-Ig on myeloma cell viability. We got the expected results in the first set of samples we tested (Figure 2A), however, subsequent lots of CTLA-4-Ig did not recapitulate our initial findings (Figure 2B). Once we had exhausted our supply of the reagent, the new master lot seemed to lose efficacy. Notably, the reagent was no longer inducing cell death in our control cell line (8226 panels, Figure 2A,B).

Discussion

Our initial data with CTLA-4-Ig largely confirmed our silencing data, in that use of this reagent effectively led to myeloma cell death, which we reasoned was due to down-regulation of IRF4. Notably, because of the more complete nature of the blockade with the use of this reagent, cell death in both MM.1s and 8226 lines occurred at a much shorter time course. Interestingly, complete blockade of CD28 access to CD86 led to the upregulation of CD28, at both the protein and mRNA levels. This again confirmed that there is regulatory cross-talk between the 2 molecules, and indicated that the expression of CD28 is somehow regulated by CD28 signaling (just as we propose based on our overexpression of CD86FLm or CD86TLm), as in this case blockade of CD28 ligation by CD86 led to a potential compensatory mechanism via upregulation of CD28.

The variability in our results with respect to CTLA-4-Ig and its' ability to induce myeloma cell death is due to a change in manufacturing protocols. Specifically, we were informed that to more efficiently purify out the biologic, a change in detergents had been made. While this did not affect the results in their quality control assays (as determined by the efficacy of the reagent to block IL-2 secretion in Jurkat cells *in vitro*), this greatly diminished the efficacy of CTLA-4-Ig in killing myeloma cells. Notably, the reagent was still able to block CD86 antibody from detecting CD86 on the surface of the myeloma cells, indicating that it still bound CD86. However, we no longer observed myeloma cell death, or upregulation of CD28, suggesting that while it bound CD86, it was binding differently.

Because the change in the phenotype was observed only upon the change in purification methods, one would logically conclude that the previous effects are artifacts of leftover detergent in the milieu. However, we don't believe this is the case, as the manufacturer assured us that the levels of detergent in all the preparations of the reagent they sell are so low as to be below the limit of detection of their quality control testing. Also, because we had gotten multiple lots over 5 years (30+ experiments worth) to show consistent results (with only 3 experiments showing no effect), we feel that our initial results, while currently not reproducible due to technical issues, merit further investigation. Of note, if the cell death effect was only due to leftover detergent in the system, then the non-plasma cells (CD38-) in our patient sample data should have also died compared to our untreated controls (Figure 2A), as well as our cell line control (8226). Since this was not the case, this indicates that cell death was due to CD86 blockade via CTLA-4-Ig, and not to the lethality of the detergent.

Figures

Figure 1. CTLA-4-Ig treatment recapitulated effects seen with CD86 silencing. (***A****, left*) Cells were treated with CTLA-4-Ig (R&D 325-CT) for 16 hours and cell death determined by Annexin V staining. (***A****, right*) Representative histograms show CD28 and CD86 surface levels following indicated treatment. (***B***) qRT-PCR data from mRNA collected after cells were treated with CTLA-4-Ig. (***C***) Representative Western blots showing effect of CTLA-4-Ig treatment on IRF4 levels in myeloma cell lines. (***D***) CD98 surface expression was determined by flow cytometry 16 hours following the addition of CTLA-4-Ig. (***E***) Representative histograms showing ITGβ7 and ITGβ1 surface expression levels after treatment with CTLA-4-Ig. (***F***) Cells were treated for 16-24 hours with CTLA-4-Ig or ABT-737 in the presence or absence of 10 μM Q-VD-Oph. Cell death was measured via Annexin V staining. (***G***) Cell death (measured as Annexin V staining) in cell lines that overexpress either pCDNA3.1 control or the CD86FLm or CD86TLm overexpressers when treated with CTLA-4-Ig. All data are presented as the mean ± SEM of at least 3 independent experiments. (*$p<0.05$, **$p<0.01$, ***$p<0.005$). All qRT-PCR data are normalized to β-Actin as endogenous control.

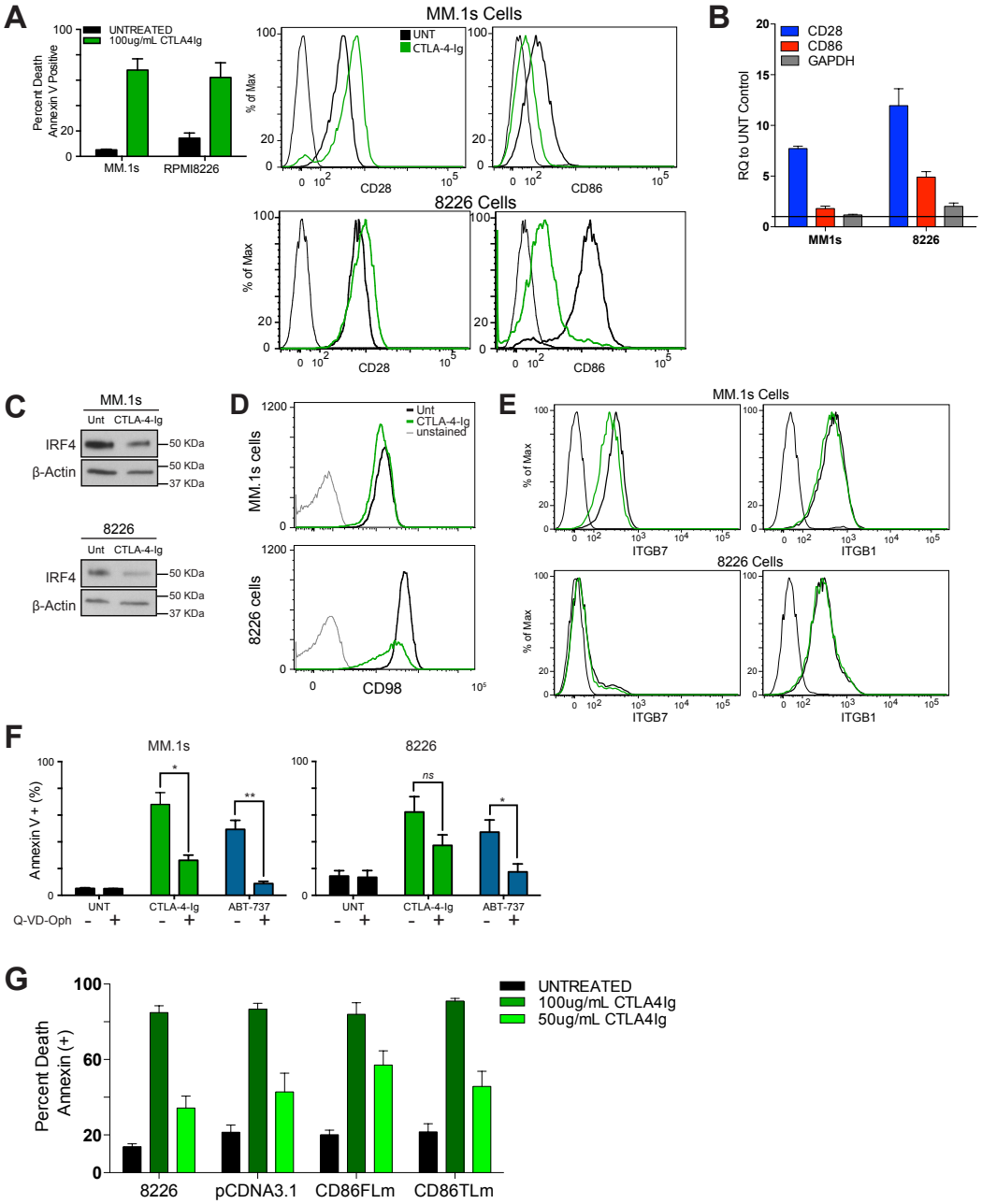

A
UNTREATED
100ug/mL CTLA4Ig
Percent Death Annexin V Positive
MM.1s
RPMI8226
MM.1s Cells
UNT
CTLA-4-Ig
% of Max
CD28
CD86
8226 Cells
B
CD28
CD86
GAPDH
RQ to UNT Control
MM1s
8226
C
MM.1s
Unt
CTLA-4-Ig
IRF4
β-Actin
50 KDa
37 KDa
8226
D
Unt
CTLA-4-Ig
unstained
MM.1s cells
8226 cells
CD98
E
MM.1s Cells
ITGB7
ITGB1
8226 Cells
F
MM.1s
8226
Annexin V + (%)
UNT
CTLA-4-Ig
ABT-737
Q-VD-Oph
ns
G
UNTREATED
100ug/mL CTLA4Ig
50ug/mL CTLA4Ig
Percent Death Annexin (+)
8226
pCDNA3.1
CD86FLm
CD86TLm

Figure 1

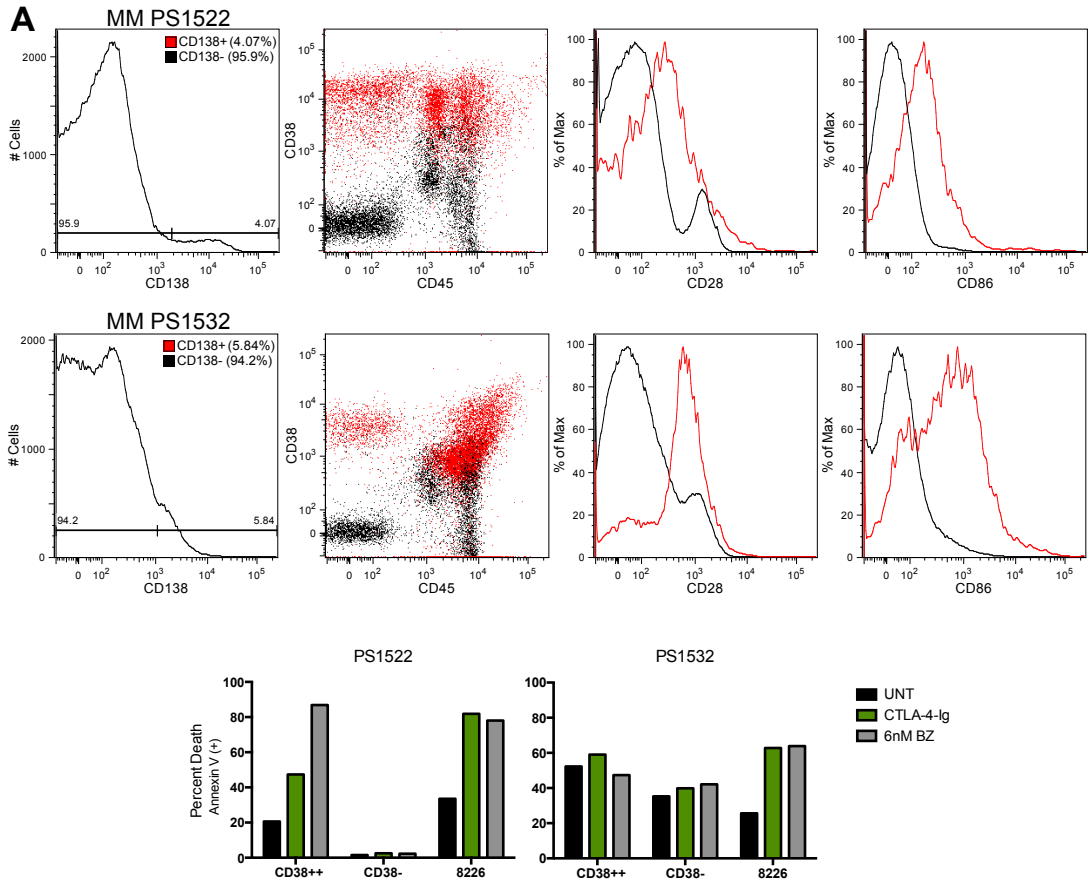


Figure 2A. Patient samples treated with CTLA-4-Ig from Master Lot No. XC34 (R&D Cat No. 325-CT). Top panels show histograms depicting staining profiles comparing the CD138-positive (red) versus the CD138-negative (black) cells in the Buffy coat post-Ficoll separation of bone marrow aspirates at Day 0. As treatment controls, the RPMI8226 cell line was treated alongside the patient samples for comparison of response to different agents. Cells were plated at 0.5 x 10^6 per mL, and treated for 24 hours with 100 υg per mL CTLA-4-Ig, or 6nM Bortezomib (Bz). Cell death was measured via Annexin V staining. For cells from patient samples, the populations that were CD38++ (CD38-high) versus CD38- were compared.

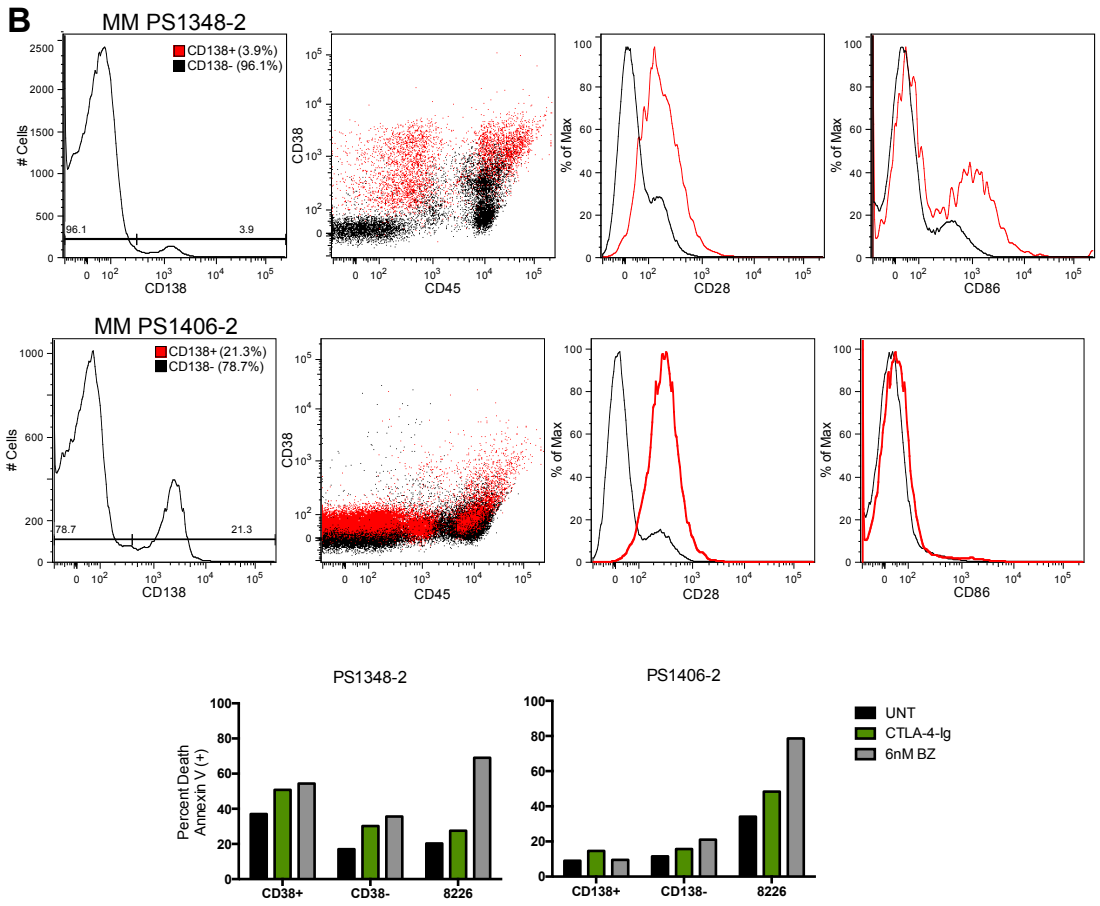


Figure 2B. Patient samples treated with CTLA-4-Ig from Master Lot No. XC35 (R&D Cat No. 325-CT). Top panels show histograms depicting staining profiles comparing the CD138-positive (red) versus the CD138-negative (black) cells in the Buffy coat post-Ficoll separation of bone marrow aspirates at Day 0. As treatment controls, the RPMI8226 cell line was treated alongside the patient samples for comparison of response to different agents. Cells were plated at 0.5 x 10^6 per mL, and treated for 24 hours with 100 υg per mL CTLA-4-Ig, or 6nM Bortezomib (Bz). Cell death was measured via Annexin V staining. For PS1406-2, because Buffy coat cells could not be separated according to CD38 staining, CD138 was used as the marker to identify plasma cells in culture.

V. Discussion

CD28 and CD86 are commonly expressed on myeloma cells from primary cells

Our results show that the CD28-CD86 signaling module plays an important role in maintaining myeloma cell viability, and is thus a viable therapeutic target, especially since reagents exist that function to block the interaction between these two molecules. We postulate that because CD28 and CD86 form a signaling module that mediates a pro-survival signal, this may facilitate stromal independence since if both are present on myeloma cells, ligation of these two surface molecules (whether autocrine or between myeloma cells) can replace the need for signals from the microenvironment. Previous reports showed that individually, high expression of CD28 and CD86 are indicators of poor prognosis for patients, and strongly correlated with disease progression[91,132], such that studies have indicated that CD28 may be a useful marker for monitoring myeloma progression. However, analysis of data from CoMMpass (a myeloma patient database containing sequencing data from ~1,000 myeloma patients) shows that only CD28-low expression was statistically significant (compared to the rest of the patient cohort) in prognosticating a positive patient outcome. There are several potential reasons for the differences in results. First, the sample sizes for the studies cited are much smaller than patient data sets currently available, lending current studies more statistical power. Next, myeloma patient prognoses are vastly different between now and 25 years ago, when the initial reports on CD28 and CD86 in myeloma came out, due to the advent of new therapies. Our current data could also be a measure of the length of the study thus far, as CoMMpass is only in its fourth year. Another potential reason could be that while CoMMpass is based on mRNA levels, previous studies that defined these two molecules

as poor prognostic indicators used flow cytometry as a means of measuring CD28 or CD86 levels.

When comparing patient outcome based on the presence of both molecules, we found that CD28-CD86 double high expressers cluster with the rest of the patient cohort, whereas the low overexpressers fare much better than all the patients combined. While the difference between the two extreme ends (double high vs low) is statistically significant, our data primarily indicates that low activity from this signaling module confers clinical benefit for myeloma patients (Chapter 2, Figure 1). We analyzed data from the UAMS patient database (Zhan *et al*. PMID:11861292) and showed the same pattern of outcome (Chapter 2, Figure S1), that low expression of both CD28 and CD86 indicates better clinical outcome for myeloma patients, indicating that blockade of this signaling cascade may be a viable therapeutic option for myeloma.

The question becomes, how many patients will blockade of CD28-CD86 potentially benefit? Our RNA-seq data shows that most myeloma cells express CD28 and/or CD86 mRNA to some degree (Chapter 2, Figure 1). Flow cytometry data is only recently becoming available for these two markers, and is limited to what we have collected throughout the course of this study, as most staining protocols frequently do not include CD28 or CD86. For CD28, available data from CoMMpass (Chapter 2, Figure 1) corroborates the RNA-seq data, as it shows that at least 71% of myeloma patients have 100% CD28-positive myeloma cells at diagnosis, with the rest having between 10-80% CD28-positive myeloma (0/141 patients were CD28-negative). While this is a small sampling (141 of 645 patients in CoMMpass to date), this does indicate that most myeloma patients have some percentage CD28-positive myeloma cells. We were able to

perform CD28 and CD86 staining in 27 myeloma samples, and found that 81% (22/27) of these had CD138+ cells that were CD86-positive (16/27 (59%) were CD28-positive, 13/27 (48%) were CD28-CD86 double positive) compared to the CD138- subset. Thus, while high expression may not indicate high risk disease, our data does suggest that blockade of this signaling pathway may benefit most myeloma patients, given that expression of these markers is common in myeloma.

Blockade of CD28-CD86 signaling in primary myeloma cells results in cell death

One way to block this pathway is via inhibiting expression of CD28 and/or CD86. Our data shows that using lentiviral vectors to silence CD86 results in myeloma cell death over time in patient samples when these have been characterized to be CD28-CD86 double high (Chapter 2, Figure 2), while having no effect on the rest of the cells in the population. In contrast, silencing of CD86 does not seem to adversely affect CD28-negative myeloma, or those myeloma cells that express low levels of both (Chapter 2, Figure S3). Because of the short nature of our studies (4 days), it would be interesting to determine whether blocking expression of these molecules over a longer period would start to have an effect even on myeloma cells that express these at a lower level. Because of their role in maintaining cell viability, it may be that while myelomas that express high levels of CD28 and/or CD86 are more sensitive to blockade, low-expressers are still dependent on the signal, only it may require longer to induce cell death. A caveat to blockade via silencing is that we were only able to partially silence expression of CD28 or CD86. In addition, blockade of signaling using shRNAs mediated by lentivirus may also not be the best way to target this in patients, due to challenges of optimization.

Because CD28 and CD86 are expressed on the cell surface, blockade can be achieved using reagents that bind to either molecule, which would block activation on either side. The well-characterized role of these two molecules in immune costimulation has made targeting this pathway of great interest. There are currently FDA-approved agents that specifically block this interaction, and these have been used to facilitate graft acceptance[143], and to treat autoimmune diseases[141]. These agents are those we are proposing could be viable additions to myeloma treatment regimens. Importantly, because our data shows that this signaling pathway mediates an intrinsic survival signal, such that even partial loss of CD28 or CD86 leads to myeloma cell death, this suggests that drug resistance may not affect sensitivity of myeloma cells to blockade.

CD28-CD86 signaling regulates different factors involved in myeloma cell survival

Despite forming a receptor ligand pair, (partial) silencing of CD28 and CD86 had little overlap in terms of the genes with significant expression changes, which could be a function of the incomplete inhibition of signaling. Another factor could be that silencing of CD28 led to upregulation of CD86, which adds another layer of complexity. Also, there are other potential ligands for CD28 that may be expressed on myeloma cells. ICOSLG, another costimulatory molecule, can bind to CD28[149], and has been found to be expressed in myeloma cell lines[150]. Analyses of the expression of ICOSLG on the myeloma cell lines using the TGen database showed that it is expressed at higher levels in KMS18, a cell line we used for our RNA-seq analyses.

Initial RNA-seq analysis of the global gene expression changes when either CD28 or CD86 resulted in around ~1500 transcripts that were statistically significantly changed for each treatment. While we initially focused on genes that were significantly changed

with both treatments, we did find hits that were unique to either, and these differences in expression changes indicated to us that signals may be emanating from both molecules. We first narrowed down the list of significant hits to those that were common across silencing of either CD28 or CD86 across the 3/4 cell lines tested (MM.1s, RPMI8226, KMS18), and we found several genes that were of great interest, based on their roles in maintaining myeloma cell viability.

We consistently saw downregulation of IRF4 expression at the protein and mRNA levels upon silencing of CD28 or CD86 (Chapter 2, Figure 3), indicating that this pathway plays a role in the regulation of this myeloma survival factor[39]. IRF4 is a transcription factor that is important in the survival and differentiation[28,34,35] of normal plasma cells, and data indicates that maintenance of plasma cell physiology is a requirement for myeloma cell survival, as demonstrated by the induction of cell death in myeloma cell lines *in vitro* when co-regulators (XBP-1, BLIMP-1) of plasma cell programming are ablated[36,38-40].

Aside from intrinsic factors that maintain plasma cell programming, survival signals within the bone marrow are facilitated by interaction with other stroma residents. Integrins are surface proteins that facilitate cell-cell and cell-stroma interactions, which can provide protection against cell-death, such that integrin function has been linked to cell-adhesion mediated-drug resistance (CAM-DR)[81]. We found 2 integrin subunit genes, *ITGB1* and *ITGB7*, which are potential downstream targets of CD28-CD86 signaling (Chapter 2, Figure 3). Interestingly, *ITGB1* is a target that we think is regulated by downstream signals mediated by the cytoplasmic tail of CD86, as expression of this factor parallels that of CD86 in the different conditions we have tested (down when CD86 is silenced, up via shCD28 since CD86 is also induced; up when CD86FLm is

overexpressed but not with CD86-tailless). Expression of ITGβ1 has been shown to be upregulated in myeloma clones found in patients with minimal residual disease[84], indicating that ITGβ1 can provide a survival advantage to myeloma cells. Expression of ITGβ7 has also been shown to be protective against myeloma cell death induced by treatment with bortezomib[82]. This shows that aside from regulating IRF4, the CD28-CD86 pathway regulates integrin expression, and by doing so could affect response to therapeutics and provide myeloma cells with a survival advantage.

Because the changes in integrin expression were quite modest, we determined if this would be of any biological significance by looking at how it would affect the ability of myeloma cells to interact with stromal cells. Using an *in vitro* adhesion assay, our data shows that silencing of CD28 or CD86 resulted in a decreased ability of myeloma cells to adhere to HS-5 stromal cells (Chapter 2, Figure 3). Interestingly, this occurred despite the upregulation of ITGβ1 when CD28 is downregulated. This may be a function of integrin substrates, as HS-5 cells are characterized to express e-cadherin, a substrate of ITGβ7, which suggests that interactions with stromal cells may be mediated primarily by this integrin subunit. Because myeloma cells are known to de dependent on stromal interactions for positive signals, this is another aspect of myeloma survival signaling the CD28-CD86 pathway is regulating.

Aside from genes that facilitate pro-survival interactions, we found that CD28-CD86 signaling module also plays an important role in the regulation of many of the SLC family members, heterodimeric nutrient transporters that maintain metabolic homeostasis. Because myeloma cells retain the immunoglobulin secretion phenotype, the SLC family members help maintain plasma cell function by facilitating the transport of amino acids

used as building blocks for antibodies. LAT-1 or CD98 is a heterodimeric large neutral amino acid transporter comprised of SLC3A2 and SLC7A5 subunits. The *SLC7A5* gene is one we found to be consistently downregulated when either CD28 or CD86 was silenced (Chapter 3, Figure 1). The relevance of *SLC7A5* in myeloma has primarily been associated with its function in melphalan transport into the cell[161,162]. However, in activated T cells, activity of this transporter has been shown to play an important role in regulating c-MYC[152]. Because c-MYCis also an important myeloma factor, indirect modulation of c-MYC expression may be another way this signaling module functions in the regulation of myeloma viability.

Interestingly, despite cell death being the most prominent phenotype we observed with silencing of either molecule, analysis of global gene expression changes show that there was no consistent pattern of changes in expression of the Bcl-2- family members, proteins that regulate apoptosis. Notably, only the gene *BCL2L1*, which encodes BCL-X_L, was significantly down-regulated, but only when CD28 was silenced. This had previously been reported to be one of the pro-survival factors induced upon CD28 activation in T cells[94]. Activation of CD28 in myeloma cells had previously been shown to have no effect on BCL-X_L levels despite having protective effects against death signals (serum withdrawal and treatment with dexamethasone), making the relevance of change in mRNA upon silencing of CD28 difficult to interpret.

We also found that when CD28 is down-regulated, expression of *MYC* and *IDH1* were also down. MYC is a known myeloma survival factor[153,163] and a well-characterized oncogene, while IDH1 (isocitrate dehydrogenase) is a protein involved in metabolism and

is frequently mutated in multiple cancer types[164]. These data indicate that modulation of CD28 signaling leads to downregulation of factors that play a role in proliferation.

Our data also shows that there are transcripts that are downregulated specifically when CD86 is silenced. *TNFRSF17* encodes for BCMA, a costimulatory receptor that is known to be important for normal and malignant plasma cell survival[85-87,165]. *MAP1LC3B* encodes LC3, a protein that mediates autophagy, a catabolic process that allows the cell to adapt to nutrient deprivation and/or accumulation of misfolded protein. Autophagy is known to be tightly regulated in myeloma[40,42,64], is required for myeloma cell survival, and is also down with shCD86. Based on analyses of gene expression changes, it appears that CD86 modulation leads to down-regulation of factors that play a role in maintaining cell viability.

GSEA (gene set enrichment analysis) was performed to elucidate what cellular pathways are most affected by silencing of CD28 or CD86. Because of the limited overlap in genes affected in silencing of either CD28 or CD86, we found unique biological pathway hits between the 2 treatments. However, gene sets that were affected for both validated our RNA-seq expression analyses data, in that the top pathway hits involved downregulation of the IRF4 and c-MYC transcription networks. The differential pathway hits involved upregulation of the Type I interferon response and stress responses, and downregulation of RNA processes involved in splicing for CD28 silencing. In contrast, silencing of CD86 led to upregulation of proteasome-dependent protein catabolism and downregulation of responses to misfolded proteins (Chapter 2, S4). These results could explain the higher levels of cell death when CD86 is silenced compared to CD28, given their high protein burden and dependence on pathways involved in regulation of protein catabolic pathways

(autophagy, UPR). Because of the effects of protein catabolic pathways, blockade of CD86 may also further sensitize myeloma cells to proteasome inhibitors, which are already effective agents in myeloma treatment.

Our data also indicate that blockade of the CD28-CD86 pathway results in pleiotropic modes of myeloma cell death. When we tried to determine the mechanism of cell death induced upon silencing CD28 or CD86, we found that overexpression of pro-survival Bcl-2 family members, pan-caspase inhibitors (Chapter 2, Figure 6) and exogenous addition of IL-6 (data not shown) were ineffective at blocking cell death. Our data Indicate that cell death induced via blockade of this pathway is both Bcl-2 inhibitable and partially caspase dependent. We hypothesize that we are inducing dysregulated autophagic flux when we silence either CD28 or CD86. Autophagy is a physiological process that can provide the cell a means of adapting to nutrient-deficient conditions, but is also a means by which cells can die in a Bcl-2-inhibitable and caspase-independent manner[56,57]. Myeloma cells are known to require some level of autophagy[64], just like normal plasma cells[62]. IRF4 has been characterized to play a role in the regulation of homeostatic autophagy in myeloma[42], and our data show that it is downregulated when CD28 or CD86 is silenced. This suggests that blockade of CD28-CD86 signaling induces aberrant autophagic flux, prompted by deregulation of autophagy-regulators, and exacerbated by loss of the nutrient transporter function of the SLC family members, leading to myeloma cell death.

CD86 has signaling capacity

The novel finding of our study involves the characterization of CD86 as regulating downstream effectors important in myeloma cell survival via its cytoplasmic tail. While

the costimulatory role of CD86 has been extensively studied, little is known what happens downstream of CD86 upon ligation to CD28. The low conservation of the cytosolic domain of CD86 across species (Chapter 2, Figure 4, S6), and in particular the divergence from the rodent orthologs, suggest that the knowledge we have gained from murine models when studying the signaling outcomes from this molecule may not fully inform what happens in the context of human cells.

Analysis of the CD86 cytoplasmic tail indicates that there are several motifs that can bind adaptor proteins, but experimental data on these are sparse. Even though CD86 is primarily characterized to be express on APCs (antigen presenting cells), it is also known to be induced on activated human T cells upon exposure to IL-2[166]. If the downstream effects upon ligation of CD86 to CD28 are similar to that in myeloma cells (IRF4, survival), then this pathway may also play a role in mediating a positive signal in activated T cells. Because T cells also express the canonical receptors for CD86 (CD28 and CTLA-4), this has implications in T cell physiology, albeit data show CD86 expression on T cells is much lower than that in DCs[166]. While this could indicate that CD86 signaling may play a minor role in human T cell physiology, the potential downstream signals warrant further investigation, as we can readily manipulate the pathway using FDA-approved biologics.

The differences between ligation of CD86 vs. CD80 to either CD28 or CTLA-4 have been thoroughly investigated, but still incompletely characterized. These two molecules have long been acknowledged to mediate different effects upon ligation to both CD28 and CTLA-4[114-116,167]. CD86 is expressed at much higher levels than CD80, and is more readily induced upon activation in DCs[97]. With respect to T cell costimulation, the

expression pattern of CD86 and effects of CD86-blockade studies indicates that CD86 is a stronger immune activator than CD80[168]. Ligation of CD86 on murine B cells and murine lymphoma cells were demonstrated to induce proliferation and pro-survival signals[116]. Human myeloma cell lines express CD28 and CD86, but not CD80 or CTLA-4. If CD86 is indeed the preferred activating ligand for CD28, then selective expression of this molecule on myeloma cells may be due to its binding affinity to CD28, as well as the positive signals we have shown it can relay.

Our data showing that overexpression of full length CD86 (CD86FLm) induces higher levels of integrins (ITGβ1 and ITGβ7) (Chapter 2, Figure 4) and IRF4 (Chapter 2, Figure 5), as well as being able to protect against different cell death signals (Chapter 2, Figure 4,5), supports our hypothesis that CD86 has signaling capacity. We can attribute this to the cytosolic domain of CD86 since CD86TLm did not have a similar effect. IRF4 as a downstream target of CD86 signaling is a novel finding, and has implications in myeloma since it provides another means of targeting this myeloma survival factor.

The pathways mediated by CD86 signaling are currently being investigated. Previous work from our collaborators have shown that in DCs, CD86 ligation with CD28 leads to activation of PI3K-Akt pathways that also involve cross-talk with Notch signaling induction of IL-6 secretion[123]. While we have no direct evidence that CD86 ligation in myeloma cells leads to similar pathways activated as that in DCs, one of the gene sets that were downregulated upon CD86 signaling are the components of the pathway involved in the synthesis of phosphoinositides (PIPs), a family of membrane lipids that can serve as signaling scaffolds in biological pathways such as PI3-Akt. We are also

currently investigating what proteins can bind the CD86 cytosolic domain so as to define what pathways are induced in myeloma upon CD86 ligation.

One of the effects of overexpression of CD86 on the surface is downregulation of CD28 expression levels. These data indicate that regulation of CD28 expression can be attributed to CD28 signaling activity, as over-stimulation of CD28 (from the overexpression of either CD86FLm or CD86TLm) leads to concomitant downregulation.

Our data also show that allelic variants in CD86 may have different signaling capacities. Specifically, our CD86-full-length cDNA construct contains the CD86-T304 allele which results from a polymorphism (rs1129055), whereas the parental 8226 cell line endogenously expresses the A304 allele. The polymorphism (T304 in the cysolic domain) has been linked to increased cancer risk[124-126] and better rates of graft acceptance[127,128], suggesting that this variant may be hypomorphic and thus mediates a weaker signal. Curiously, A304 is in the cytoplasmic tail and is conserved in all species analyzed except rat and mouse.

Comparing silencing of CD28 or CD86 with blockade using CTLA-4-Ig

Because myeloma cells express both CD28 and CD86, identifying the effectors downstream of ligation can be difficult to interpret. Silencing of CD86 led to higher levels of cell death, and given that our data shows that CD86 can signal, the cell death levels could be attributed to the combined loss of CD28 and CD86 downstream survival mediators. Silencing of CD28 led to cell death as well, the levels of which were presumably modulated by CD86 upregulation.

This indicates that CD28 signaling may be a negative regulator of CD86 expression. A mechanism by which this could occur is via regulation of the transcriptional repressor, BLIMP-1. There is data that shows BLIMP-1 represses CD86 in plasmablasts[26] and myeloma cells[41]. It was recently shown that in normal long-lived plasma cells, CD28 activity via the Vav1 motif was responsible for the regulation of BLIMP-1 expression[104]. Thus, loss of CD28 signals could lead to upregulation of CD86 via loss of BLIMP-1. Looking at our RNA-seq data, there was a slight downregulation of *PRDM1* (the gene that encodes BLIMP-1) when CD28 is silenced (30% in MM.1s; 11% in RPMI8226; 13% in KMS18; 38% in 8226-Mcl-1 all relative to vector-control), but this did not reach significance. Again, this may be due to incomplete silencing, and that CD28 downregulation led to upregulation of CD86, potentially blunting the loss of CD28 signal by providing more ligand. Based on current knowledge about CD28 signaling in the context of (normal) plasma cells, this warrants investigation, as understanding the cross-talk between these two molecules is important in order to fully understand the potential effects of manipulation of this pathway.

There are FDA-approved agents available that function to block the interaction between CD28 and CD86, and these biologics are used most often to dampen immune responses by disallowing the interaction between these 2 molecules. We initially had success recapitulating our silencing data using CTLA-4-Ig (Chapter 4). Treatment with CTLA-4-Ig of myeloma cell lines *in vitro* completely blocked CD28 access, leading to cell death in under 24 hours. Using primary cells from myeloma patient samples, we also were able to show cell death of the myeloma cell upon CTLA-4-Ig treatment, with no effect on the rest of the cells in the bone marrow aspirate. Treatment with CTLA-4-Ig also

recapitulated our findings with blockade of the CD28-CD86 pathway via silencing, as downregulation of the main hits we got from RNA-seq analysis (*IRF4*, *ITGB1*, *ITGB7* and *SLC7A5*) also occurred.

However, the reagent we were using to block CD28-CD86 interaction subsequently became unavailable (Chapter 4, Figure 2) and these technical issues are still in the process of being resolved. Based on our data using 2 shRNAs (Chapter 2, Figure 2, S2) for each target (CD28 and CD86), our data is still biologically relevant, and thus our hypothesis that CD28-CD86 blockade could be a potential therapeutic avenue for the treatment of myeloma requires investigation.

Once the technical issues with the reagents are resolved, determining if there are similarities between silencing of CD28 or CD86 versus CTLA-4-Ig treatment of myeloma cell lines should be performed, since CTLA-4-Ig treatment looks to be a more complete blockade compared to silencing of CD86. This would potentially allow us to more clearly identify which genes are regulated by CD86 signaling. Interestingly, our data (when the reagent was working) showed that complete blockade of CD86 led to upregulation of CD28 (at the protein and mRNA levels), which suggests there is indeed cross-talk between these two molecules. This upregulation of CD28 could be due to a more significant loss of CD86 signaling (total vs. ~50%) compared to silencing. This was not enough to abrogate cell death, however, since CD86 was not free to bind CD28, suggesting that survival signals coming from the CD86-end requires activation by CD28.

Manipulation of the CD28-CD86 pathway as a therapeutic avenue in myeloma

The CD28-86 signaling axis is an attractive clinical target because of its highly-described role in T-cell costimulation. A vast amount of research has been done elucidating the mechanisms by which this pathway can be blocked or activated, depending on the physiological context required. Our data shows that manipulation of this pathway, specifically blockade of the signaling between CD28 and CD86, has therapeutic potential in myeloma. While this approach has led to improved patient outcomes in transplant and some autoimmune diseases, there are potential advantages and pitfalls that need to be considered in the context of myeloma.

Because myeloma cells express both CD28 and CD86, there is potential for autocrine ligation of these two molecules, and our *in vitro* data with the cell lines would indicate that this occurs, given that even partial loss via silencing of either molecule led to myeloma cell death. However, these molecules can also influence the microenvironment in which myeloma cells reside, and the complexity of this milieu needs to be considered so that any manipulation can lead to the most optimal outcome for the patient (Figure 1).

CD28 on myeloma cells can interact with DC in the bone marrow microenvironment, leading to induction of IL-6[139], and blockade of this would deprive myeloma cells of an important growth factor. It has also been reported that CD28 on myeloma cells binding to CD86 on DCs leads to modulation of tumor-antigen-presentation, leading to decreased susceptibility of myeloma cells from CD8+ T cell killing[169]. Thus, blockade of CD28 on myeloma cells from interacting with CD86 on DCs in the microenvironment would be to the patient's advantage.

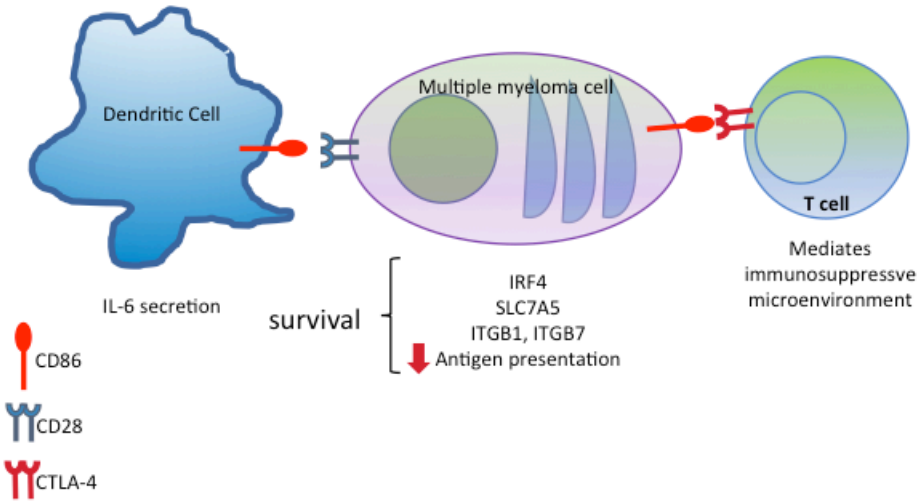


Figure 1. Signaling cascade from CD28-CD86 interaction regulates pro-myeloma mediators. Interaction between CD28 and CD86 with their ligands mediates a pro-myeloma signal on multiple fronts. CD28 on myeloma cells interacting with CD86 on DCs induces IL-6 secretion by the DCs, an important myeloma growth factor. CD86 on MM cells can interact with CTLA-4 on CD4 T cells, inducing a more immunosuppressive microenvironment that can mediate immune evasion. CD28 and CD86 ligation on myeloma cells, whether autocrine or via paracrine interactions with other MM cells, or stromal components, can mediate survival via factors regulated by the signaling pathway. Note that myeloma cells could also potentially interact via CD28 and CD86 in an autocrine manner, or between myeloma cells.

In contrast, CD86 on myeloma cells can interact with CD28 or CTLA-4 in T cells present in the stroma. T cell subsets can express CD28 or CTLA-4 differentially, depending on their effector function. In the case of the tumor microenvironment, there are conflicting reports as to whether there is a link between myeloma disease progression and the presence of T regulatory cells (Tregs), a T cell subset that can modulate effector T cell function. The presence of this immunosuppressive subset is reportedly increased in myeloma patients vs. healthy controls[170-172], and the lower numbers in myeloma long term survivors (compared to patients who progress faster) is thought to relate to a less

immunosuppressed microenvironment and a better outcome in this patient subset[171]. Regulatory T cells express higher levels of CTLA-4, the other ligand to which CD86 can bind, and since a microenvironment that ablates effector T cell function would be advantageous to myeloma, blockade of CD86 may promote not only cell death in myeloma, but restore immune effector function by blocking T regulatory cells. This may be a viable combination with immune checkpoint blockade agents, which have so far not shown efficacy in the treatment of myeloma.

Because CD28 and CD86 form a signaling module that is mediating an intrinsic pro-survival signal in myeloma cells that is also potentially responsible for altering the tumor microenvironment to the advantage of myeloma cells, targeting this pathway is a viable therapeutic avenue for this hematologic malignancy since it regulates a plethora of physiological pathways that regulate cell function, residence in the bone marrow, and viability.

Future directions

While our data indicate that CD86 is mediating a pro-survival signal in myeloma, the molecular effector/s that mediate induction of the downstream factors that play a role in myeloma cell survival are still unknown. Like CD28, the cytoplasmic domain of CD86 does not have any intrinsic enzymatic activity, therefore adaptor protein(s) must exist that mediate this, leading to activation of signaling pathways. A previous study showed that there is a KKKK (Lysine) motif that is required to maintain the ability human CD86 to interact with the cytoskeleton, the mutation of which affected the ability of CD86 to co-stimulate CD28[147]. In addition, murine and human CD86 have been shown to be regulated by the activity of an E3 ligase (MARCH1) to bind to the transmembrane and

cytosolic domains, leading to downregulation of CD86[173]. Our collaborators showed that CD86 ligation results in PI3K-Akt activation via cross-talk with Notch signaling. While these previous studies have hinted at the capacity of CD86 to signal, there is no direct interaction between the cytosolic domain of CD86 and a known adaptor reported to date. Preliminary analysis of the human CD86 cytoplasmic tail via different bioinformatics tools suggest putative phosphorylation sites predicted to be substrates for PKC, and MAPKAPK2/3/5 (data not shown from iGPS39 analysis of the CD86 cytoplasmic domain)[174]. There is also a putative PDZ domain at the carboxy terminus of CD86, short amino acid motifs that mediate a myriad of biological processes via signal transduction pathways (reviewed[175]).

CD138, a surface marker used to identify plasma cells (both normal and malignant), has been shown to bind the PDZ-containing adaptor syntenin, an adaptor that contains a PDZ domain that has been shown to be involved in multiple signaling pathways[176-179]. Because CD138 expression is linked to myeloma cell viability, in that CD138 is shed by myeloma cells once they start dying, this signaling complex may be involved in signaling cascades from CD86. Interestingly, syntenin expression correlates with patient prognosis in the same pattern as CD86, in that there are higher levels of syntenin in patients whose myeloma cells express high CD86 (data from Dr. Boise CoMMpass analyses). Our RNA-seq analysis shows that *SDC1 transcripts*, (encodes for CD138) are down when CD86 is silenced, indicating that CD86 may play a role in regulating the syntenin-CD138 signaling axis in myeloma cells. Whether CD138 or syntenin bind to CD86 could be tested, to determine if these factors form a signaling complex in myeloma.

Because of the cell lines we have generated, we have a good system in which to test different binding partners for CD86. Co-immunoprecipitation studies to pull down CD86FLm versus CD86TLm (full-length vs tail-less) will allow us to test interactions between different candidate adaptors and the cytoplasmic tail. Because we have different truncation mutants, we can also preliminarily test which domains of CD86 are important for different interactions. Importantly, we can also determine if there is a difference in signaling mediators when the polymorphism (rs1129055) is present, as we have the construct where we have mutated back (G1057A) the T at position 304 back to an A. These studies will allow us to identify putative binding partners for the CD86 cytoplasmic tail, and then trace which pathways these potentially play roles in. This should allow us to delineate the specific pathways mediated by CD86 signaling, which is also facilitated by the fact that we have several candidates (*IRF4*, *ITGB1*, *TNFRSF17*, *MAP1LC3B*) already identified.

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