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Tyler J. Moser-Katz Date

The Role of CD86 Trafficking and Signaling on Myeloma Survival and Proliferation

By

Tyler J. Moser-Katz

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences Biochemistry, Cell and Developmental Biology

_____

Lawrence H. Boise, Ph.D. Advisor

_____

Mandy L. Ford, Ph.D. Committee Member

_____

Michael Koval, Ph.D. Committee Member

_____

Kenneth H. Moberg, Ph.D. Committee Member

_____

Nicholas T. Seyfried, Ph.D. Committee Member

Accepted:

_____

Kimberly Jacob Arriola, Ph.D, MPH. Dean of the James T. Laney School of Graduate Studies

_____

Date

The Role of CD86 Trafficking and Signaling on Myeloma Survival and Proliferation

By

Tyler J. Moser-Katz
B.A., University of North Carolina

Advisor: Lawrence H. Boise, PhD

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Abstract

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By Tyler J. Moser-Katz

Multiple myeloma is an incurable hematologic malignancy of long-lived antibody secreting plasma cells. Myeloma cells retain numerous features of plasma cell biology including a reliance on signals within the bone marrow microenvironment. In advanced stages, myeloma cells can become extramedullary and survive independently of the bone marrow due to autocrine cytokine signals and cell-cell interactions. One such physical interaction involves the CD28-CD86 module which signals bi-directionally to confer myeloma cell survival and drug resistance. My studies investigate how myeloma cells regulate this interaction and the expression of the CD86 at the plasma membrane. I also demonstrated a role for CD86 in proliferation and further elucidated downstream molecular changes induced by CD28 signaling.

I identified that while several regions of the tail are required for proper trafficking of CD86 out of the Golgi, a specific region for proper transport is a three amino acid-long PDZ binding motif at the C-terminus of the tail. BioID analysis uncovered two PDZ-domain containing proteins, SCRIB and DLG1 as proximal to the CD86 cytoplasmic tail. Deletion of SCRIB and DLG1 in myeloma cell lines results in a decrease in CD86 surface expression, myeloma proliferation and viability. These proteins are important for generating CD86 to the surface where it binds to CD28 and is stabilized. My studies also expand upon the role for CD86 in myeloma, showing a role for CD86 in myeloma proliferation and IMiD resistance. Furthermore, they demonstrate a potential CD28 pro-survival signaling mechanism via SYNTENIN upregulation and may reveal a compensatory role for the ICOS-L-CD28 axis in the absence of CD86.

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Chapter 1

Introduction

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A. Game of Bones: How Myeloma Manipulates its Microenvironment

Abstract: Multiple myeloma is a clonal disease of long-lived plasma cells and is the second most common hematological cancer behind Non-Hodgkin's Lymphoma. Malignant transformation of plasma cells imparts the ability to proliferate, causing harmful lesions in patients. In advanced stages myeloma cells become independent of their bone marrow microenvironment and form extramedullary disease. Plasma cells depend on a rich array of signals from neighboring cells within the bone marrow for survival which myeloma cells exploit for growth and proliferation. Recent evidence suggests, however, that both the myeloma cells and the microenvironment have undergone alterations as early as during precursor stages of the disease. There are no current therapies routinely used for treating myeloma in early stages, and while recent therapeutic efforts have improved patients' median survival, most will eventually relapse. This is due to mutations in myeloma cells that not only allow them to utilize its bone marrow niche but also facilitate autocrine pro-survival signaling loops for further progression. This review will discuss the stages of myeloma cell progression and how myeloma cells progress within and outside of the bone marrow microenvironment.

Introduction

Multiple myeloma (MM) is defined as a clonal proliferation of malignant plasma cells, and it accounts for roughly 10% of all hematological cancers[1]. Myeloma cells retain numerous features of plasma cell biology including a reliance on signals within the bone marrow microenvironment[2]. Interestingly, myeloma's precursor states share the same genetic alterations observed in symptomatic MM patients in both the plasma cells and the microenvironment[3,4]. In advanced stages, myeloma cells can extravasate from the bone marrow leading to extramedullary plasmacytomas and/or circulating plasma cells in the blood[5]. Currently, there are ~70 patient-derived myeloma cell lines (HMCL), representing the most advanced stage of myeloma progression whereby myeloma cells survive independently of the bone marrow microenvironment. To this effect, myeloma cells can be compared to an expansive civilization that strategically taps the resources of its niche and when left unchecked will colonize and overtake its host. The malignant cells compete in a "Game of Bones" against the host's innate defenses and utilize the microenvironment in as a means of gaining an advantage. This review will examine progression of disease from asymptomatic precursor states to MM while shining a light on the changes myeloma cells induce in themselves and within the microenvironment to enable such progression. It will also address the signals that allow myeloma to survive independently of the bone marrow microenvironment in their quest for further growth and expansion.

Spectrum of plasma cell dyscrasias

Multiple myeloma: Historically, establishing the diagnosis of multiple myeloma required both documented bone marrow plasmacytosis (BMPC) ≥10% or an extramedullary plasmacytoma with evidence of end organ damage defined by the CRAB criteria (elevated serum calcium levels, renal insufficiency, anemia, and lytic bone disease). However, in 2014, the International Myeloma Working Group (IMWG) revised the diagnostic criteria to include ultra high-risk patients previously classified as having pre-myeloma or SMM given the ~80% risk of progression to symptomatic disease at two years. These risk factors include BMPC ≥60%, involved to uninvolved serum free light chain ratio >100, and >1 focal lesion on whole body MRI or PET-CT[6–9].

Management and therapy selection for myeloma patients can be determined based on risk stratification. Previously, the International Staging System (ISS) divided disease burden of myeloma into three stages. Serum levels of beta-2 microglobulin (ß2M) and albumin were determined to be the most accurate predictors of disease burden and median survival. Stage I is defined as ß2M < 3.5mg/L and serum albumin ≥ 3.5g/dL. In Stage III, ß2M ≥ 5.5mg/L and serum albumin < 3.5g/dL. Stage II refers to the intermediate stage of neither Stage I or III[10,11]. Recognizing the important role cytogenetics play in risk stratification, revised ISS (R-ISS) was recently developed still utilizing the ISS, but incorporating both serum lactate dehydrogenase (LDH) levels as well as high risk cytogenetics defined by IgH-MMSET/FGFR3 [t(4,14)] translocations, IgH-MAF [t(14,16)] translocations and deletion of the p arm of chromosome 17 (del17p) **(Table 1)**.

Outside of the R-ISS risk stratification model, there are additional favorable and adverse risk factors that aid clinicians in appropriate stratification and subsequent treatment of myeloma patients. These factors include additional cytogenetic features, presence of extramedullary disease, gene expression profiling, and plasma cell proliferation. Standard risk myeloma encompasses 80 to 85% of newly diagnosed myeloma (NDMM) patients and portends good prognosis with a median overall survival (OS) not reached at 10 years[12]. Cytogenetic features indicative of standard risk disease includes trisomies of chromosomes 3,5,7,9,11,15,19, and 21 that is referred to as hyperdiploidy as well as IgH-Cyclin D1 [t(11,14)], and IgH-Cyclin D3 [t(6,14)] translocations. High risk patients encompass 15 to 20% of NDMM patients and have median OS of 5 years[13]. In addition to high risk cytogenetic features previously described, additional markers of aggressive disease include complex karyotype, defined as 3 or more changes on standard karyotype analysis. Another marker of high risk disease is amplification of the q arm of chromosome 1 (amp1q). Co-occurrence of 1q gain with other high risk markers portends poor prognosis, and patients with >4 copies of 1q are at very high risk for progression following initial treatment[14]. Another marker of high risk includes the least common IgH translocation with MAFB [t(14,20)] which makes up <1 % of myeloma patients [13]. High risk myeloma patients are also identified by disease burden (ISS stage III), proliferation (BMPC labelling index ≥ 3% via thymidine kinase and C-reactive protein[15]), and presence of extramedullary disease[16].

MM Precursor Stages: The presence of a precursor state is not known for most NDMM patients as most diagnoses occur at symptomatic stages. However, studies in 2009

from Drs. Michael Kuehl and Ola Landgren used molecular and biological markers to show that myeloma is preceded in virtually all cases by a premalignant state[17,18]. The following two subsections will refer to these precursor states.

Monoclonal Gammopathy of Undetermined Significance (MGUS): MGUS was first described in 1961 by Dr. Jan Waldenström who identified a subset of patients with elevated serum and urine immunoglobulin levels without displaying symptoms of malignancy[19]. Waldenström labelled this phenomenon a gammopathy, and the term, MGUS, was later coined in 1978 by Dr. Robert Kyle and colleagues[20]. The IMWG now defines MGUS as the presence of a serum monoclonal (M) protein or M-protein at <3g/dL concentration and <10% BMPC with the absence of CRAB criteria [6].

MGUS is found in 3% of Caucasians over the age of 50 and occurs at a 2 to 3-fold higher rate in African Americans[21,22]. Patients diagnosed with MGUS have a 1% risk per year of progressing to symptomatic myeloma, and therefore the standard of care is surveillance without intervention[23]. Risk of patient progression can be further stratified using three risk factors: presence of a non-IgG M protein (IgA or IgG), M-protein >1.5 g/dL, and abnormal serum free light-chain (FLC) ratio[24] **(Table 1)**. Recently, advancement of technology allowed for detection of precursor cells to MGUS, labelled pre-MGUS[3,25]. As many genomic alterations in MGUS originate in the germinal center, an aberrant clonal population of plasma cells can be formed prior to migration into the bone marrow[26,27]. Furthermore, microenvironment changes present in MGUS have shown to be key regulators in progression to symptomatic stages, and can be targeted in these early stages[3,28].

Smoldering Multiple Myeloma (SMM): SMM is an intermediate clinical stage in progression between MGUS and multiple myeloma initially described in 1980 after observing a series of six patients with BMPC >10% that continued to have stable disease without treatment for >5 years[29]. SMM is defined as the presence of an M-protein at ≥3 g/dL, and/or BMPC percentage of >10% with no evidence of end organ damage defined by the CRAB criteria (hypercalcemia, renal failure, anemia, bone lesions)[30]. After the IMWG revised the diagnostic criteria of myeloma, a subset of patients previously classified as having SMM were now reclassified as having symptomatic myeloma. However, this reclassification ultimately only affected a small proportion of SMM patients, and the challenge still remained how to appropriately risk-stratify the remaining patients. SMM is a very heterogeneous disorder encompassing patients that will progress in the first two years and patients with stable low-level disease more than ten years after diagnosis. How then, do we identify which patients are at the highest risk of progression, and how do we safely manage them?

The Mayo 2018 model, also known as the 20/2/20 model, uses three independent risk factors of progression to myeloma: (1) a serum FLC ratio >20, (2) M-protein >2 g/dL, and (3) BMPC >20%. Depending on whether the patient has either 0, 1, or 2-3 of these factors, they are categorized as having either low, intermediate, or high risk SMM corresponding to a 5%, 17%, or 46% risk of progression at 2 years[31] . The IMWG validated this model using a retrospective cohort, but added the high-risk cytogenetic features t(4,14), gain(1q), del(17p), and del(13q). Interestingly hyperdiploidy has been shown to be an adverse prognosticator in SMM despite its opposite meaning

in MM[32]. In this model, SMM patients were grouped into four risk categories (low risk, low-intermediate risk, intermediate risk, high risk) associated with a 2-year progression rate of 3.7%, 25%, 49%, and 72% respectively[33] **(Table 1)**.

Historically, observation was also the standard of care for SMM as with MGUS. However, recently published data has shown the benefit of early intervention with the immunomodulatory agent (IMiD) lenalidomide in high-risk SMM in terms of delaying progression to myeloma[34]. The efficacy of using IMiDs in SMM illustrates the role that the microenvironment has in facilitating MM progression. Ongoing clinical trials continue to investigate different therapeutic strategies in SMM, as this continues to be an evolving area of research.

Extramedullary Multiple Myeloma: Extramedullary multiple myeloma (EMM) refers to hematogenous spread of clonal plasma cell tumors leading to soft tissue tumors at anatomic sites outside the bone marrow[35]. This is a separate diagnosis from solitary plasmacytomas which originate from the underlying bone marrow and grow through the cortical bone[36,37]. EMM can present in the liver, skin, central nervous system, pleura, kidneys, lymph nodes, and pancreas and is present in 6-8% of NDMM cases and 10-30% of relapsed myeloma patients[38,39]. EMM may also present as plasma cell leukemia (PCL), an aggressive variant of the disease with >20% or ≥ $2*10^9$ circulating plasma cells in the blood[40]. PCL can either present de novo, known as primary PCL, or more commonly as a progression from already diagnosed myeloma, known as secondary PCL.

Extramedullary disease and PCL are considered high risk entities and associated with a poor prognosis with a median OS of less than 6 months[37]. Profiling of extramedullary tumors reveals differences from malignant bone marrow plasma cells. Cytogenetics that indicate standard risk myeloma such as hyperdiploidy and t(11,14) are mainly found in BMPC and rarely found in extramedullary plasmacytomas whereas t(4,14) is more commonly seen in EMM[35,41]. However, PCL, while heterogeneous, does have a higher incidence of t(11,14) translocations[40 42]. In relapsed patients, EMM cells undergo a shift from secretion of intact IgG to light chain, and most HMCL secrete only light chain, demonstrating its correlation with myeloma progression[43]. The changes in molecular and protein expression that allow myeloma cells to survive and spread outside of the microenvironment will be addressed in a subsequent section of this review.

The role of the bone marrow microenvironment

In 1889, Stephen Paget introduced his “seed and soil” hypothesis which postulated that tumor cells (seed) grow preferentially in selective microenvironments (soil)[44,45]. We have seen that plasma cells undergo genomic alterations in the germinal center prior to MGUS[26,27]. Once this clonal population arrives in the bone marrow it gains access to a wide array of microenvironment signals that facilitate plasma cell survival. Recent studies have found little difference between the microenvironments of MGUS and myeloma, demonstrating that the “soil” has a role in shaping the malignant progression[3,46]. The bone marrow microenvironment produces pro-survival signals for

non-malignant long-lived plasma cells, which can live throughout the lifetime of the host, and secrete antibody titers as part of the adaptive immune response[2]. Myeloma cells, the aggressive counterparts, use the supportive surrounding stromal cells, osteocytes and endothelial cells to further their growth. Myeloma precursor states have been shown to mediate progressive growth *in* vivo in humanized mouse models supporting a dominant role for the microenvironment or tumor-extrinsic signals in regulating tumor growth[46]. Initial small changes in the microenvironment or molecular changes to myeloma cells themselves cause an expansion of the plasma cell niche throughout the bone marrow.

Molecular Changes Driving Myeloma Growth: Myeloma cells undergo numerous molecular changes and genetic events which allow proliferation and induce further changes in the bone marrow microenvironment. One family of proteins commonly dysregulated in myeloma are D-type cyclins[47]. D-type cyclins are cell cycle proteins that activate cyclin dependent kinase 4 (CDK4) and CDK6, which phosphorylate and inactivate Rb allowing for E2F activation and cell cycle progression[48]. Primary genetic translocations such as t(11,14) and t(6,14) directly drive constitutive expression of cyclin D1 and D3 respectively[47,49,50]. Another translocation t(4,14) which increases the expression of the histone methyltransferase MMSET (NSD2) also indirectly drives activation of cyclin D2[47,51]. Cyclin D2 can also be dysregulated through t(14,16) and t(14,20) translocations which drive transcription factors that target Cyclin D2[47]. Although infrequent, biallelic inactivation of Rb itself is a subclonal mutation that occurs in 3% of tumors[52]. Rb is found on chromosome 13q, and this deletion of this region is the most

common mutation in myeloma, frequently accompanying t(4,14), t(14,16), and t(14,20) translocations[53,54]. Recently it was shown that monoallelic deletion of two other genes on 13q which code for Mir15A and Mir16-1 resulted in development of MGUS in wild type C57BL/6 mice and progression of myeloma in the Vk*Myc multiple myeloma mouse model[55].

Myeloma upregulates oncogenes that are typically associated with proliferation in cancer. One such gene is MYC, and its deregulation typically leads to a more aggressive disease phase[56]. MYC translocations are found in 15% of human myeloma tumors[57] and include both IgH-MYC translocations [t(8,14)] and IgL-MYC translocations [t(8,22)]. The MYC locus is the most common source of light chain translocations accounting for 40% of these anomalies, and lambda light chain translocations portend a particularly poor outcome compared to kappa light chain translocations[58]. Another pathway that is involved in myeloma proliferation is RAS signaling. A secondary mutation that is uncommon in MGUS, KRAS and NRAS mutations are each found in ~20% of NDMM patients[27,59]. KRAS and NRAS mutations appear to not uniformly activate MAPK signaling pathways and actually lead to distinct downstream transcriptional signatures[60]. Interestingly, FGFR3 mutations, which are mutually exclusive with RAS mutations appear to induce MAPK signaling more effectively[60,61]. Finally, the MAPK pathway can be activated by BRAF mutations. BRAF is mutated in 4% of patients with the V600E mutation being the most common[62]. Additionally, recent studies have shown a role for cytidine deaminases such as AID and APOBEC in mediating genomic instability in MM cells[63]. The expression of these genes, however, is also dependent on interactions with the microenvironment[64].

Extracellular Matrix: The bone marrow microenvironment provides a layered structure called the extracellular matrix (ECM) which acts as the "home base" for myeloma cells. Homing to the bone marrow is mediated by interaction of myeloma receptor CXCR4 with the chemokine SDF1α[65]. This causes a subsequent migration to the stromal compartment of bone marrow. There, it will interact with ECM proteins or other native bone marrow cells.

The ECM consists of proteins such as fibronectin, collagen, osteopontin, hyaluronan, and laminin. Adhesion of myeloma cells has been shown to be important for survival and drug resistance[66,67]. A method of cell-ECM adhesion is activation of integrins, and myeloma cells have shown preference toward very large antigen-4 (VLA-4) aka integrin α4ß1 and integrin ß7 (ITGB7)[68–70]. Binding of VLA-4 to fibronectin of the ECM induces activation of nuclear factor κB (NFκB) leading to cell adhesion-mediated drug resistance (CAM-DR) and pro-survival signaling[71]. ITGB7 can be regulated by the MAF gene, and as a result, patients with t(14,16) have elevated levels of ITGB7[51]. ITGB7 is necessary for myeloma cell survival and CAM-DR, and has been shown to be constitutively active in myeloma cells[70,72]. Additional integrins such as VLA5 and the beta 5 integrin CD56 play a smaller but active role in myeloma progression[68,73] **(Fig 1)**.

Syndecan-1 or CD138 is a heparan sulfate proteoglycan and a surface marker of myeloma cells. It binds to type I collagen and induces expression of matrix metalloproteinase 1 (MMP1) to promote tumor invasion, bone resorption, and angiogenesis[74,75]. Additionally, syndecan-1 levels on cells correlate with cell survival and growth[76]. Heparanase has an intricate interplay with syndecan-1, either causing its

clustering and increased adhesion to the ECM or inducing its shedding[77,78]. Soluble syndecan-1 has been shown to promote myeloma tumor growth *in vivo*[79]. Finally, CD44, RHAMM, and CD38 are hyaluronan receptors[67]. Hyaluronan is a secreted scaffold protein in the bone marrow. While certain splice variants of CD44 and RHAMM are active in the bone marrow as receptors for hyaluronan and osteopontin, they are generally more involved in extramedullary myeloma. Both proteins regulate the SDF1α/CXCR4 axis, and cell-ECM adhesion with RHAMM is more involved in cell motility[67]. CD38 is another hyaluronic acid interacting partner that is expressed in high levels in plasma cells but low levels in other lymphoid and myeloid cells making it an effective target for antibody therapies like daratumumab and isatuximab[80]

Myeloma-Stroma Cell-Cell Contact: Binding of VLA-4 of a myeloma cell to VCAM of an adjacent stromal cell promotes downstream signaling pathways that activate NFκB and cause cellular survival and proliferation[74]. Another myeloma receptor, lymphocyte function-associated antigen 1 (LFA1), will bind to ICAM-1 of an adjacent bone marrow stromal cell (BMSC). LFA1 is an integrin composed of αL and ß2 subunits and is associated with poor prognosis and disease progression in patients as well as increased proliferation in mice[81,82]. Mucin 1 (MUC1) is another transmembrane binding partner of ICAM-1 that has been shown to drive myeloma progression **(Fig 1)**. MUC1 will induce proliferation in multiple myeloma by signaling via a ß-catenin/TCF4 mechanism to drive MYC gene expression[83].

Plasma and myeloma cells also express CD28, a transmembrane protein classically known for its role in T cell co-stimulation. During this process, MHC of an

antigen presenting cell (APC) will first bind to the T cell receptor. The T cell is not fully activated unless CD80/86 of an APC binds to CD28 of a T cell, inducing survival, proliferation, and effector function in T cells[84]. Plasma cells retain this CD28 pro-survival signaling capacity, and binding with CD80/86 of a BMSC e.g. dendritic cell confers survival throughout the lifetime of the host[85,86]. Plasma and myeloma cells are dependent on CD28 signaling through both the PI3K and Vav signaling pathways[87,88]. Knockout of CD28 leads to decreased antibody titers of long-lived plasma cells in mice, and knockdown of CD28 or CD86 with short hairpin RNA leads to myeloma cell death in HMCL[86,88,89] **(Fig 1)**.

Myeloma-BMSC Pro-Survival Cytokines: Multiple myeloma cells also pave the way for their own survival and proliferation by inducing cytokine secretion in BMSC. Direct binding of plasma and myeloma cells to BMSC leads to downstream pathways such as MAPK, NOTCH, and PI3K and cause subsequent transcription and secretion of numerous cytokines. One such cytokine is interleukin-6 (IL6), which has roles in myeloma growth, survival, migration, and drug resistance. IL6 binds to its cognate IL6-receptor (IL6R) and signals through MEK/MAPK, JAK/STAT, and PI3K/Akt pathways[90–92]. It also increases dependence on Mcl-1, an anti-apoptotic Bcl-2 protein that is essential for plasma and myeloma cell survival. IL6 upregulates Mcl-1 in a STAT3 dependent manner and induces phosphorylation of Bim, thus increasing affinity of Bim for Mcl-1 over Bcl-2/Bcl-x. This increased binding of the two proteins ultimately leads to stabilization of Mcl-1[93,94].

In the absence of IL6, two other cytokines, B-cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) have been shown to have a protective effect on myeloma cells particularly from treatment with corticosteroid[95]. BAFF is a member of the tumor necrosis factor (TNF) family and is expressed on the surface of BMSC as well as in a cleaved soluble form. It has been shown to stimulate B cell growth, and, additionally, ligation of its receptors BAFF-R and TACI leads to increased proliferation and survival in myeloma cells[96,97]. APRIL is a secreted protein that will bind to TACI and B-cell maturation antigen (BCMA), a protein which has recently become a target for myeloma CAR-T cell therapy with an 88% response rate[98,99]. BAFF and APRIL-mediated signals also impact survival and growth signals to MM from surrounding dendritic cells[100]. They are overexpressed in myeloma cells compared to normal plasma cells illustrating the importance of these cytokines[95]. APRIL and BCMA promote cell growth (via MAPK and NFκB) and immunosuppression (via PD-L1, TGF- ß, and IL10) in myeloma cells[98].

Another member of the TNF family involved in myeloma growth and survival in the bone marrow microenvironment is TNFα. TNFα is a mediator of inflammation and has been found to be significantly higher in supernatants of patients with bone disease than those without[101]. While TNFα signaling itself causes a modest increase in proliferation, it induces expression of adhesion molecules resulting in a 2-4 fold increase in binding of myeloma cells to BMSC. It also results in a significant increase in IL6 secretion. Interestingly, TNFα levels decrease with thalidomide treatment which may be a result of downstream effects of the drug's immunomodulatory effects on bone marrow myeloma cells[102].

Myeloma cells induce BMSC to secrete numerous growth factors. Among them, insulin-like growth factor (IGF) appears to have a sustained and pronounced effect on myeloma proliferation and antiapoptotic signaling. IGF binds to the tyrosine kinase receptor IGF-1R, and additionally influences proteasome and telomerase activities in myeloma cells. IGF is also implicated in drug resistance to cytotoxic chemotherapy, dexamethasone, and proteasome inhibitors[103]. It primes myeloma cells to respond to other cytokines and to produce pro-angiogenic cytokines. BMSCs also produce other growth factors such as hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) which influence osteoclast activation and angiogenesis[74] **(Fig 1)**.

Osteoclast Interactions: Bone lesions result from osteoclast activation to enable further space for myeloma proliferation in the bone marrow. To directly activate osteoclasts, myeloma cells secrete macrophage inflammatory protein-1α (MIP1α) and MIP1ß. MIP1α binds to C-chemokine receptor 1 (CCR1) and CCR5 while MIP1ß binds to CCR5 and CCR8 to induce osteoclast formation and activity[104–106]. MIP1α has been shown to lead to bone destruction, BMSC adhesion, and tumor burden in SCID mice with multiple myeloma[104]. In turn, osteoclasts secrete IL6 to stimulate proliferation and growth of not only myeloma cells but other osteoclasts as well[107]. Myeloma-osteoclast interaction also upregulates Chondroitin synthase 1 (CHSY1), which induces Notch signaling promoting the survival of myeloma cells[108]. Notch signaling, particularly Notch3 and Notch4 stimulation leads to recruitment of osteoclast precursors and increased bone resorption[109,110].

Interactions between myeloma cells and BMSCs also leads to production of cytokines that stimulate osteoclastogenesis. Binding of VLA4 with VCAM promotes secretion of cytokines such as IL1, IL6, TNFα, and parathyroid hormone related peptide (PTHRP) which promote osteoclast growth[111]. Binding of VLA4 and VCAM also lead BMSC to produce receptor activator of NFκB ligand (RANKL). RANKL will bind to its receptor RANK to stimulate osteoclast activation and differentiation and bone lysis[111,112]. RANKL, MIP1α, and IL11 are upregulated by p38 MAPK in BMSCs, and inhibiting p38 MAPK decreases osteoclastogenesis and bone resorption[113] **(Fig 1)**. The bone matrix glycoprotein, osteopontin, and the pro-inflammatory cytokine IL17 have also been implicated in osteoclastogenesis and bone resorption. They have been shown to be associated with poor prognosis and osteolytic lesions in patients[114–116].

Osteoblast interactions: Myeloma cells also disrupt bone homeostasis by inhibiting osteoblast production and activation. Osteoblasts and BMSC produce osteoprotegerin (OPG) which inhibits the development of bone disease by competing for binding of RANK with RANKL[117]. Binding of OPG with RANK prevents osteoclast maturation and activation[118]. The ratio between RANKL and OPG is important prognostic indicator in patients and can be influenced in numerous ways[119–121]. One way is binding of VLA4 on myeloma cells to VCAM of BMSCs which decreases secretion of OPG and increases secretion of RANKL, thereby tipping the balance in favor of osteoclasts[111,112]. Other factors which augment the RANKL/OPG ratio are activin A and sclerostin[122,123]. Sclerostin is cysteine knot protein which induces apoptosis in osteoblasts and inhibits bone formation[124]. Activin A, a member of the TGF- ß superfamily, signals through

numerous pathways to promote osteoclast differentiation and is a marker of poor prognosis[122,125]. Interestingly, IL3 can increase osteoclastogenesis by regulating activin A levels[126].

Myeloma cells can also prevent the maturation of osteoblast progenitor cells. Binding of VLA4 of myeloma cells to VCAM of osteoblast progenitors downregulates the activity of RUNX2, a transcription factor that is necessary for the differentiation of osteoblastic cells[127]. In addition to increasing the RANKL/OPG ratio, IL7 secretion by BMSC also decreases RUNX2 activity and osteoblast differentiation[119,127,128]. Recent studies from the Croucher lab have shown that MM-osteoblast interactions may also be important for maintaining dormancy of tumor cells[124]

Secretion of the cytokines Dickkopf 1 (DKK1) and Frizzled related protein 2 (sFRP-2) by myeloma cells contributes to bone resorption as well. DKK1 and sFRP-2 inhibit the canonical Wnt pathway which is responsible for the differentiation of osteoblast progenitor cells[127,128]. DKK1 and sFRP-2 are expressed in multiple myeloma cells of patients with bone lesions. Recombinant DKK1 and sFRP-2 or conditioned media containing either of the two cytokines inhibit differentiation of osteoblast precursor cells *in vitro* and suppress *in vitro* bone mineralization[130,131]. Interestingly, immunodepletion of sFRP-2 led to increased bone restoration suggesting it is necessary for bone resorption. Osteoblast differentiation may take place via the bone morphogenic protein 2 (BMP2) pathway. sFRP-2 as well as IL3 inhibit this pathway, thereby stunting osteoblast activation. Additionally, secretion of the cytokines TGF- ß and HGF by BMSC promote osteoclast generation while limiting osteoblast activity[74,132] **(Fig 1)**.

Endothelial cell interactions: Angiogenesis is the creation of new blood vessels through the use of endothelial cells. Patients with progressive myeloma disease show increased level of microvessel density (MVD), a measure of angiogenesis, when compared to those with inactive MGUS[133]. This is because myeloma cells crowd the bone marrow microenvironment and generate hypoxic tumors, so they upregulate angiogenesis to deliver oxygen and nutrients while removing catabolites. In the presence of hypoxic conditions, myeloma cells upregulate hypoxia induced factor 1α (HIF1α), which regulates transcription of pro-angiogenic cytokines including HGF, bFGF, VEGF, and Angiopoietin-2 (Ang-2). Myeloma cells may also constitutively produce these cytokines due to genetic mutations or oncogene activation[134].

Adhesion of myeloma to the ECM increases angiogenesis. Expression of adhesion molecules VLA4, LFA1 and CD44 have been shown to correlate with increased angiogenesis in active myeloma[135]. Syndecan-1 has been shown to have a prominent role in bone marrow angiogenesis as well. Syndecan-1 is correlated with MVD and facilitates binding of growth factors, particularly HGF, to cells. Not only can syndecan-1 potentiate the surface binding of HGF to cells, but it can also be shed in a soluble form that complexes with HGF to increase potency[136,137]. Myeloma cells also facilitate degradation of the ECM using matrix metalloproteinases (MMP) and heparanase to allow migration of endothelial cells into the surrounding tissue[138,139].

Myeloma cells stimulate BMSCs to secrete HGF, VEGF, and IL8 to induce neovascularization[140]. In turn, endothelial cells will produce IGF1 and IL6 to promote myeloma cell growth. This process can induce an autocrine loop in endothelial cells as

they produce VEGF, platelet-derived growth factor (PDGF), Ang-1, HGF, and IL1 to further promote angiogenesis[141] **(Fig 1)**.

Immune cells: While the previous subsections have addressed allies that myeloma uses to advance itself in the Game of Bones, myeloma cells have an antagonist in the form of antitumor cells in the bone marrow. To overcome this, MM and its precursor MGUS are associated with several alterations in both innate and adaptive immunity. Immune cells increased in MM include regulatory T cells, IL-17-producing T cells, and terminally differentiated effector T cells, however, immunosuppression and exhaustion of these cells was present as early as the MGUS stage[3,142]. The bone marrow increases CD4(+) regulatory T cells and decreases CD4(-)CD8(-) regulatory T cells, and this correlates with increased disease burden[143]. Myeloma cells produce proteins such as TGF- ß, PD-L1, LAG3, TIM3, and IL10 that contribute to the immunosuppressive phenotype and T cell anergy **(Fig 1)**. Interestingly, these proteins are upregulated in myeloma cells by binding of APRIL to BCMA[98]. CD28 ligation with CD80/86 has also been shown to cause BMSC secretion of IL6 and IDO. This occurs via “back signaling” of CD80/86 to activate the PI3K pathway. While IL6 normally activates T cells, IDO catabolizes tryptophan in the microenvironment into the toxic metabolite kynurenine. This results in T cell anergy via GCN2 kinase-mediated sensing of depleted intracellular tryptophan pools[144,145]. Interestingly, a subset of endothelial cells express low levels of CD80/86 as well as CD40 and ICOS-L in myeloma patients which can trap a population of T cells and stimulate them to induce immunosuppressive proteins[146]. Autologous dendritic cells stimulated with tumor antigen can be used to activate T cells *ex vivo* to expand and

attack the tumor. Emerging treatments such as targeted antibodies, checkpoint inhibitors and CAR-T cell therapy have aimed to increase the potency of the immune response[28,142]. Currently, advances in mass cytometry and RNA sequencing single cell analyses are being used to identify the immune checkpoint signature of the microenvironment[25]. These methods have identified immunosuppressive phenotypes such as regulatory T-cell suppression, secretion of suppressive cytokines and interferons, and increased expression of PD-1 on CD8(+) T and NK cells as early as MGUS[147].

Myeloid derived suppressor cells (MDSC) have been shown to promote immune suppression and angiogenesis in multiple myeloma. They induce myeloma cell survival and proliferation by causing AMPK phosphorylation in myeloma cells. This increases levels of the anti-apoptotic proteins MCL-1 and BCL-2 and the autophagy marker LC3II[148]. Myeloma cells in turn will cause an increase of MCL-1 expression and survival in MDSC[149]. Another cell type, plasmacytoid dendritic cells (pDC), contribute to immunosuppression of the microenvironment when in direct contact with myeloma cells. While pDC can normally be activated to cause apoptosis of myeloma cells, pDC-myeloma binding via E-cadherin can convert pDC into tumor promoting cells[150]. Myeloma cells use cell-cell contact to court the pDC to their advantage and signal downstream to inhibit pDC secretion of interferon-α (IFN- α).[150]

Natural killer (NK) cells induce cell death in myeloma cells via granzyme and perforin release and other proapoptotic ligands[151]. Myeloma cells express CD1d and are also highly sensitive to lysis by NK cells. PD-L1 of a myeloma cell can bind PD-1 of NK cells to suppress their cytotoxic effect of myeloma cells. NK cells are a target of

numerous therapies aimed at the immunosuppressive microenvironment. Lenalidomide can be added to checkpoint inhibitors to abrogate this effect and stimulate NK to target myeloma cells[152]. The anti-SLAMF7 antibody elotuzumab can also be used to activate NK cells and mediate their activity in myeloma[153]. In addition to targeting myeloma cells, the anti-CD38 antibody, daratumumab, also depletes CD38(+) regulatory cells in the bone marrow thus promoting an immune response[154]. Recently, daratumumab has been shown to specifically stimulate NK cell activity in myeloma by selectively targeting CD38(+) NK cell populations[155]. NK cells and other bone marrow resident immune cells are avenues for immunotherapy and have yielded some initial success in treating myeloma patients at precursor stages[28,156–159].

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Myeloma takeover beyond the microenvironment

While myeloma cells can be seen circulating in peripheral blood in advanced stages, most EMM is characterized by plasmacytomas in adjacent tissues and organs. Myeloma cells must develop the capacity to extravasate through stroma and ECM into the blood and navigate challenges such as building their own microenvironment in sites outside of the bone marrow. As the disease advances, cells undergo molecular and genomic alterations to promote autocrine loops that facilitate survival and proliferation away from its bone marrow sanctuary. It is notable that although extramedullary growth is a feature of advanced MM, circulating tumor cells can be detected even in early stages of MM[160]. This section will explore a new landscape for the Game of Bones and

how myeloma cells can undergo changes to survive and expand their niche independently of bone marrow signals.

Extravasation model: While little is known about myeloma extravasation from the bone marrow, we can follow an adaptation of the leukocyte multistep model of extravasation and homing **(Fig 2)**. In the standard model, cells first home to an environment as a result of chemoattractants. This is followed by adhesion of the cell to vascular endothelial cells and reorganization of the cytoskeleton to migrate through gaps between these endothelial cells. During this process, the cell degrades basement membrane and extracellular matrix to allow passage until its penetration through[161] **(Fig 2A)**. We can reverse this first step for the myeloma cell extravasation model as they must first shed homing signals which tether them to the bone marrow. They must also reduce their affinity to ECM and cells that are specific to the bone marrow and upregulate migratory proteins. Finally, myeloma cells must also degrade the basement membrane to allow passage through gaps created in the bone marrow structure **(Fig 2B)**. Once the myeloma cells are in circulation, they may re-enter the vasculature in other marrow compartments via the standard leukocyte model of extravasation. They may also form tumors in organs or remain circulating in the blood in the case of PCL.

In order to home to the bone marrow, myeloma cells depend on chemokine signaling. The SDF1α/CXCR4 axis is the myeloma homing pathway most extensively characterized, and impairment of signaling between these molecules is associated with extramedullary transformation[39]. Myeloma also depends on CCR1 and CCR2 signaling to regulate migration. Patients with active disease express significantly lower amounts

of CXCR4, CCR1, and CCR2 than those with non-active disease, and expression of at least one of these receptors portends favorable clinical outcome[162]. Additional chemokine receptors such as CXCR5 and CCR7 are downregulated to promote cell motility and decrease sensitivity to B and T cell cytokines[163].

Myeloma cells alter their adhesion properties to extravasate and migrate through the ECM. EMM plasma cells decrease expression of CD56 while increasing expression of certain CD44 isoforms that are important for proliferation and motility[164]. In murine models, decreased expression of P-selectin and VLA4 are associated with increased extramedullary disease[165]. Extramedullary myeloma cells also favor focal adhesion kinase (FAK), a protein that mediates an invasive and migratory phenotype. Patients with EMM have significantly increased expression of FAK mRNA compared with patients without extramedullary disease[166]. The tetraspanin family of proteins is another family that modulates myeloma adhesion and migration. Two such proteins, CD81 and CD82, are downregulated in HMCL, and exogenous overexpression of these proteins reduces cell motility, invasion, and secretion of MMP-9[167].

Finally, myeloma cells must degrade the ECM to allow passage through. MMP-9 will degrade basement membrane, and its secretion leads to increased invasion of tumor cells. HMCL have been shown to constitutively secrete MMP-9, and its expression is enhanced by HGF secretion by endothelial cells. Moreover, some HMCL can produce HGF, thus sustaining a loop of increased MMP-9 degradation. Interestingly, SDF1α stimulates MMP-9 production in mouse myeloma model suggesting that SDF1α may have pleiotropic effects in both myeloma cell homing and invasion[168]. Myeloma cells also produce heparanase, an enzyme that cleaves

heparanase sulfate chains of adhesive proteoglycans such as syndecan-1. Production of heparanase increases motility of myeloma cells and induces a migratory phenotype[169]. In part, heparanase and syndecan appear to regulate one another throughout the progression of myeloma and EMM **(Fig 2B)**.

EMM Molecular changes: Comparison of myeloma cells at extramedullary sites with bone marrow myeloma cells revealed increased subclonal mutations in the extramedullary sites. Morgan et al. proposed a model of myeloma progression that follows the Darwinian mechanism of species evolution. In this model, myeloma cells undergo primary mutations that underlie their growth and expansion. When a bottleneck is applied, subsequent mutations of cells are selected for, thus resulting in surviving subclonal genetic populations[170]. In addition to drug treatment, bottlenecks may refer to hypoxia, cell-competition in the microenvironment, and other novel environmental differences in extramedullary sites. Myeloma cells can migrate to other less populated marrow compartments or soft tissue sites and extravasate into these sites or form adjacent plasmacytomas to bone[168].

EMM cells upregulate the adhesion molecules platelet/endothelial cell adhesion molecule-1 (PECAM-1), secreted protein and rich in cysteine (SPARC), and endoglin (ENG), illustrating the shift in adhesion specificity in EMM[171]. EMM cells also upregulate nestin, an intermediate filament implicated in metastasis and invasion[172].

A mechanism for myeloma autocrine pro-survival loop lies in the co-expression of CD28 and its ligand CD86. Ligation of CD28 promotes cellular survival and drug resistance in HMCL, and silencing of CD28 and CD86 leads to respective increases in

cell death[88,89]. Recent work from our lab has shown that CD86 can signal to confer survival and drug resistance in HMCL. CD86 overexpression induces molecular changes such as increased expression of integrin ß1 and ß7 and interferon regulatory factor 4 (IRF4), a transcription factor necessary for myeloma survival which directly targets MYC[89,173]. Another autocrine loop involved in myeloma survival is secretion of HGF. HMCL and primary myeloma express the tyrosine kinase HGF receptor, c-Met, and produce HGF at variable levels. By this means, EMM cells can signal to stimulate c-Met thus preventing apoptosis and inducing proliferation through autocrine cytokine production[174,175]. Advances in screening technology using CRISPR offer new tools for future elucidation of genes necessary for HMCL survival and proliferation.

EMM Angiogenesis signaling: One of the processes highly relied upon in the bone marrow microenvironment, angiogenesis, also has an important role in EMM. Hypoxia in the bone marrow causes myeloma cell upregulation of HIF1α which regulates secretion of proangiogenic cytokines. HIF1α is also upregulated in circulating plasma cells and is associated with myeloma EMT[176]. Additional angiogenic factors are upregulated in EMM including VEGF, MMP-9, PECAM-1, and Ang-1. Other angiogenesis related genes such as PDGF, SPARC, NOTCH3, thrombospondin 2, TIMP3, and fibronectin 1 are overexpressed[163]. Increased expression of these proteins indicates an important role for angiogenesis in EMM. Although current standard therapies for EMM such as lenalidomide[177] and bortezomib[141] are antiangiogenic, the role of angiogenesis in EMM remains largely unknown.

PCL Molecular Alterations: PCL is an aggressive variant of EMM marked by rapidly proliferating circulating plasma cells and poor prognosis of patients. Primary immunoglobulin translocations are common in PCL with MAF translocations [t(14,16) and t(14,20)] being the most common followed by t(11,14) and t(4,14). Other common mutations in PCL are MYC translocations which can be found on IgH (5%), IgK(10%), and IgL (10%) loci respectively[178].

PCL also overexpresses certain genes compared to plasmacytomas including RPL17, CD14, TRAF2, TRAF3, and CCL2. Other affected cancer driver genes include those involved in cell-matrix adhesion and membrane organization (SPTB, CELA1), cell cycle and apoptosis (CIDEC), genome stability (KIF2B), and protein folding (CMYA5). PCL cells are also enriched in functional pathways including Cadherin/Wnt signaling, ECM-receptor, and G2/M cell cycle checkpoint. As PCL are circulating in the blood, there is a downregulation of integrins (CD11a, CD11c, CD29, CD49, CD49e) and other adhesion molecules (CD33, CD117, CD138, CD81) in comparison to EMM. PCL also expresses decreased markers of plasma cells (CD28, CD38) and increased markers of B cells (CD19, CD20, CD45) due to their high prevalence of t(11,14) translocations[178,179].

Conclusion

In the Game of Bones, myeloma cells are manipulators that identify allies within the bone marrow microenvironment to exploit and thereby enable their neutralization and

evasion of their opposition, the host's immune defenses. Data that show microenvironment changes as early as MGUS propose that the microenvironment is susceptible to myeloma growth in precursor stages. Mutations in precursor stages beckon a "chicken or the egg" conundrum between myeloma cells and the microenvironment in assessing the advancement of this malignancy. By the time symptomatic myeloma develops, the Game of Bones has already tipped in favor of the cancer. This is because the disease is already quite evolved with numerous means of drug resistance and proliferation in its arsenal, and the cancer has a substantial advantage against innate defenses and chemotherapeutic intervention. In advanced stages, myeloma can readily proliferate in the bone marrow and develop the capacity to transcend the bone marrow.

Recent studies have aimed to tip the advantage back to the side of the host's defense system, either by effectively targeting myeloma cells and the microenvironment or by strengthening the immune response. As technology and detection tools improve, myeloma cells can be combatted at their early stages before treatment of myeloma or EMM is necessary. Modern genomic approaches such as single cell genomics, mass cytometry, ATAC-seq, whole genome bisulfite sequencing, and integrated phosphoproteomics can elucidate properly tailored treatments for improved efficacy and decreased toxicity of patients. The rise of IMiDs and targeted antibody treatments represent our growing understanding of the therapeutic role of targeting the microenvironment.

Mobilizing the body's own immune system also improves its odds at winning the Game of Bones. IMiDs, as well as CAR-T cell therapy are of particular interest as they

can be utilized to bolster the body's defenses against its adversary. IMiDs are a potent frontline treatment for MM, and have even been shown to improve patient outcome in the SMM stage[34]. Overall, studies of the cancer biology in myeloma cells and their surrounding microenvironment using *ex vivo* patient studies, murine models, and HMCL provide insight to future treatment options and increased efficacy of therapy.

B. Initial Treatment Strategies

We have identified the strategies that myeloma employs to spread and takeover supporting its role as the antagonist of the Game of Bones. Similarly, therapy to combat multiple myeloma has progressed and expanded over recent years. Early treatment of multiple myeloma involved high dose chemotherapy until the 1960's when the DNA alkylating agent melphalan became an effective therapeutic treatment together with the corticosteroid, prednisone[180]. This was the gold standard of patient treatment until the mid-1990's when the introduction of autologous stem cell transplant (ASCT) drastically improved patient's survival[181]. Further advances emerged in the 2000's with introduction of the immunomodulatory drug (IMiD) thalidomide and the proteasome inhibitor bortezomib[182]. Since the 1990's, patient's 5-year survival rate has more than doubled, and numerous next generation drugs have emerged for myeloma treatment. This section will review the current treatment course for NDMM patients in each stage of myeloma detection. It will identify methods to manage progression of the disease by targeting the cancer biology of myeloma or mobilizing the surrounding immune environment for combat.

MGUS/pre-MGUS: Treatment trials for patients with MGUS are complicated as the patients are relatively healthy and have low risk of progression. Intervention toward eradicating the MGUS clone must be specific with a causal relationship between MGUS and myeloma. In IgM associated MGUS, patients with neuropathy such as POEMS syndrome may be treated with a short dose of the CD20 specific agent Rituximab[183]. Treatment of MGUS patients with nontoxic agents such as curcumin has also been utilized[184]. The introduction of mass spectrometry based technology has allowed detection of MGUS precursor cells. Furthermore, with recent studies revealing immunosuppression of the microenvironment as early as MGUS, clinical trials are ongoing using tumor antigen specific dendritic cells in this stage[28]. Future studies of MGUS and pre-MGUS will allow targeted preventative treatments to combat the disease in its early stages.

SMM: SMM is an asymptomatic disease with a risk of progression to myeloma of 10%, and as with MGUS, the standard of care has been careful observation. This is due to the lack of data supporting improvement of intervention in overall survival (OS) and quality of life. Conditions such as toxicity of therapy, and chance that the disease may remain asymptomatic are taken into account as well as risk of selecting for resistant clones in early treatment. There is, however, a subset of high-risk patients with a risk of progression of ~50% in 2 years that are considered for clinical trials[30,31]. Small, randomized trials using the bisphosphonates pamidronate or zoledronic acid did not appear to significantly improve time to progression (TTP)[185,186]. Thalidomide plus zoledronic acid improved TTP compared to zoledronic acid alone where there was no

difference in progression to symptomatic myeloma and OS[156]. This suggests that the effect on TTP was likely to be immune mediated. Treatment of high-risk SMM by the Spanish Myeloma Group with the IMiD lenalidomide plus low dose corticosteroid dexamethasone (Rd) yielded a significant increase in TTP, OS, and even generated a complete response in 26% of patients[157]. While Rd is the widely prescribed treatment course, some patients may elect for a more aggressive combination treatment with the proteasome inhibitor carfilzomib, lenalidomide, and dexamethasone (KRD). Treatment using this regimen has been relatively effective, and may be considered as another course of action in treating high-risk SMM[158]. Recent phase three clinical trials have also implicated single agent lenalidomide as effective treatment for high-risk SMM. Patients treated with lenalidomide had a significant improvement in progression free survival (PFS) when compared to observed patients[159] **(Fig 3A)**. Importantly, lenalidomide treatment did not change disease burden of patients further implicating it as a safe and viable option for treating high risk SMM patients.

Multiple Myeloma: Advances in myeloma cytogenetics and molecular characterization have allowed stratification into 2 risk subgroups: standard and high risk. Treatment for standard risk patients eligible for ASCT begins with 3-4 cycles of a triplet therapy of lenalidomide, bortezomib, and low dose dexamethasone (RVd). Following this initial frontline therapy, patients can undergo early ASCT or continue RVd treatment until they stop responding and then undergo ASCT upon relapse[13]. ASCT consists of high dose therapy (HDT) of 200mg/m^2 melphalan followed by reintroduction of autologous stem cells. ASCT has been shown to increase patient's OS by 12 months with early ASCT

showing a significant improvement in PFS compared with late ASCT[187]. Following ASCT, patients receive a maintenance therapy of RVd at day +60 and are monitored until relapse **(Fig 3B)**. In standard risk patients who do not qualify for ASCT either due to age or comorbidity, RVd treatment for 8-12 cycles is preferred followed by lenalidomide-based maintenance therapy (**Fig 3C)**. The CD38 targeting antibody, Daratumumab is also used in clinical trials to treat standard risk patients in combination with lenalidomide and dexamethasone (DRd). Daratumumab is combined with lenalidomide as both drugs have synergistic immunomodulatory effects. There is no cycle recommendation for DRd, and treatment is continued until progression[188].

Patients with high risk myeloma also receive an initial treatment of 3-4 cycles RVd and early ASCT following diagnosis[13,16]. Quadruplet regimens combining daratumumab with RVd (Dara-RVd) have shown increased rate and depth of response in both standard and high risk patients in ongoing clinical trials. However, as no data has monitored long-term costs of this treatment, Dara-RVd is suggested for high-risk patients with secondary or tertiary mutations[189]. Following early transplant, a bortezomib-based maintenance therapy is suggested for patients with t(4,14) and a lenalidomide based therapy for patients with other high risk factors. Lenalidomide treatment may be switched to thalidomide should the patient develop renal failure[16] **(Fig 3B)**. Transplant ineligible patients receive 8-12 cycles of RVd treatment followed by lenalidomide or bortezomib based maintenance as determined above **(Fig 3C)**.

EMM: Data from patients with EMM are more limited than those with non-extramedullary disease due to lower incidence in NDMM patients. PET/CT imaging is

an effective tool in diagnosis and monitoring of extramedullary sites. Similar to high-risk myeloma, a triplet therapy regimen of IMiD, proteasome inhibitor and low-dose corticosteroid are recommended. This can include RVd or KRd for 4 cycles before HDT and ASCT. In younger/fit patients with extensive disease burden, a more aggressive initial therapy can be administered of RVd/KRd with cisplatin, doxorubicin, cyclophosphamide, and etoposide (PACE) for 2 cycles[163]. This therapy can provide rapid tumor reduction prior to transplant. Additionally, tandem ASCT has shown to be more effective than single ASCT in certain high risk patients and may be preferred[190]. Following transplant, patients receive a lenalidomide/bortezomib based maintenance therapy until relapse **(Fig 3B)**. Transplant-ineligible EMM patients will resume RVd/KRd treatment for 8 additional cycles or RVd/KRd-PACE for 2 additional cycles before receiving maintenance therapy **(Fig 3C)**. As with high-risk myeloma, ongoing trials with Daratumumab are assessing efficacy in combination with frontline therapies for EMM[163]. Approach for treatment of PCL follows the same model as EMM. However, increased prevalence of t(11,14) translocation has provided rationale for single agent treatment with the Bcl-2 inhibitor, venetoclax, which has been effective in treating t(11,14) myeloma patients[191]. Since dexamethasone causes an upregulation in the proapoptotic protein, Bim, venetoclax and dexamethasone is a promising combination therapy[192]. Other targeted treatments such as HDAC inhibitors, exportin inhibitors, and SLAMF7 antibodies have proven effective in high-risk myeloma and merit further investigation.

C. CD28-CD86 signaling

CD28 in T-cell co-stimulation

CD28 has classically been characterized as a costimulatory molecule in T cell activation. It is expressed as a homodimer on T cells, and following T cell receptor binding (TCR) to MHC peptide complex of an antigen presenting cell (APC), it will bind to either CD80 or CD86 of an APC to maximally activate T cell growth and survival[84]. Another molecule expressed on T cells, CTLA-4, binds to CD80/86 at a higher affinity than that of CD28[193] (**Fig 4)**. CTLA-4 binding generates an immunostatic phenotype, and CTLA-4 inhibitors are involved in numerous immunotherapy treatment regiments[194].

Signaling downstream of CD28 activation in T cells has been well-characterized. The CD28 cytoplasmic tail contains numerous signaling motifs including the PYAP and YMNM domains. Phosphorylation of the tyrosine in the membrane-proximal YMNM motif, leads to binding of the p85 subunit of PI3K and downstream PI3K activation[195]. The C-terminal PYAP motif also contains a tyrosine whose phosphorylation leads to binding of Grb2 and Lck and subsequent Vav signaling[195]. Activation of these domains leads to induction of IL-2 and Bcl-xL leading to proliferation and survival in T cells[196]. However, loss, of both domains does not completely abrogate CD28 signaling, suggesting there are other motifs involved in CD28 cytoplasmic tail[197]

CD86 downstream signaling in APCs

While CD86 was classically defined as a ligand for CD28 in co-stimulation, numerous findings have implicated that CD86 has distinct signaling properties. Chapter 1A addresses the capacity of CD86 activation in human DCs to induce IL6 and IDO secretion[145]. In human B cells, CD86 ligation affects the synthesis of IgG4 and IgE antibody titers while CD80 ligation did not have such an effect[198]. In B cells, CD86

activation also leads to increased IgG1 and IgE production in an IL-4 dependent manner[199]. Additionally, polymorphisms found in the cytoplasmic tail of CD86 are implicated in discrepancies in immune function. Some variants of CD86 contain a SNP of G1057A coding for an amino acid change of A304T. This SNP is associated with increased cancer susceptibility[200,201] and decreased graft rejection[202] in patients. Taken together, these studies indicate that CD86 cytoplasmic tail contains numerous regions that may be important for signaling and cellular functions.

CD28 and CD86 in normal and malignant plasma cells

CD28 has been shown to be important for survival of murine bone marrow plasma cells. In CD28 knockout mice, the half-lives of these long-lived plasma cells decreased from around 400 days to 63 days[86]. CD28 activation has also been shown to induce Blimp-1, a key transcription factor involved in plasma cell differentiation and survival[87]. Recently it was shown that CD28 has a role in a ROS-dependent metabolic programming necessary for plasma cell survival. CD28 promotes survival signaling in plasma cells through differential SLP76 signaling. CD28 signaling also increases glucose uptake and mitochondrial mass/respiration[203].

In malignant plasma cells, high expression of CD28 and CD86 appear to correlate with poor patient outcome[89]. We have previously shown that blockade of CD28-86 signaling using CTLA-4-Ig sensitizes myeloma cells to therapeutic treatment[88] while activation of the pathway protects myeloma cells from death signals[89]. CD28 survival signaling appears to activate the PI3K and NFκB pathways but, unlike T cells, does not induce Bcl-xL[85]. Blockade of this interaction also increases expression of Bim

as PI3K inactivation leads to FOXO3a translocation to the nucleus and increased Bim transcription[88].

We recently showed that CD86 is important for myeloma cell survival, and knockdown of CD86 results in higher levels of cell death than CD28 silencing, illustrating a role for CD86 pro-survival signaling in myeloma. Indeed, we found that the CD86 cytoplasmic tail confers a protective phenotype against chemotherapeutic agents. CD86 signaling induces pro-survival molecular changes in myeloma cells including increased expression of IRF4, ITGB1, and ITGB7[89]. While CD86 signaling appears to play an important role in myeloma viability, it has remained largely unstudied. Further research of CD28 and CD86 signaling pathways may augment the therapeutic potential of blocking this pathway, particularly as available CTLA-4-Ig FDA approved agents are being used to treat autoimmune disorders[204,205].

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Table 1: Defining Stages of Myeloma

MGUS				
	Risk Factors	Risk Group	Risk of progression at 20 years (%)	Reference
	(1) Serum M-protein <1.5 g/dL; (2) non-IgG subtype (IgM or IgA); (3) serum FLC ratio <0.26 or >1.65)	Low-risk (0 factors)	5	
		Low-intermediate risk (any 1 abnormal factor)	21	
		High-intermediate risk (any 2 abnormal factors)	37	
		High-risk (all 3 factors abnormal)	58	Rajkumar et al (Lancet Oncology, 2014)
SMM				
Mayo 2018 model	Risk Factors	Risk Group	Risk of progression at 2 years (%)	
	(1) M-protein >2 g/dL, (2) BMPC >20%, (3) FLC ratio >20	Low-risk (0 factors)	5	
		Intermediate risk (1 factor)	17	
		High risk (2-3 factors)	46	Lakshman et al (BCJ, 2018)
IMWG 2019 model	(1) M-protein >2 g/dL, (2) BMPC >20%, (3) FLC ratio >20 (4) HR-CTG			
		Low (0 factors)	3.7	
		Low-Intermediate (1 factor)	25	
		Intermediate-High (2 factors)	49	
		High (3+ factors)	72	San Miguel et al (JCO, 2019)
MM				
	Criteria	Stage	Median OS (months)	
ISS	B2M<3.5, Alb >3.5	1	62	
	Not meeting criteria for either ISS 1 or 3	2	44	
	B2M >5.5	3	29	Griepp et al (J. ASCO, 2005)
R-ISS	ISS stage 1 without HR-CTG and LDH WNL	1	Not reached	
	Not meeting criteria for either R-ISS 1 or 3	2	83	
	ISS stage 3 with either LDH >ULN and HR-CTG	3	43	Palumbo et al (J. ASCO, 2015)

MGUS, monoclonal gammopathy of undetermined significance; SMM, smoldering multiple myeloma; MM, multiple myeloma; BMPC, bone marrow plasma cells; OS, overall survival; ISS, International Staging System; R-ISS, revised ISS; B2M, beta2-microglobulin; Alb, Albumin; FLC, free light chain; LDH, lactate dehydrogenase; HR-CTG, high risk cytogenetics; WNL, within normal limit; ULN, upper limit of normal

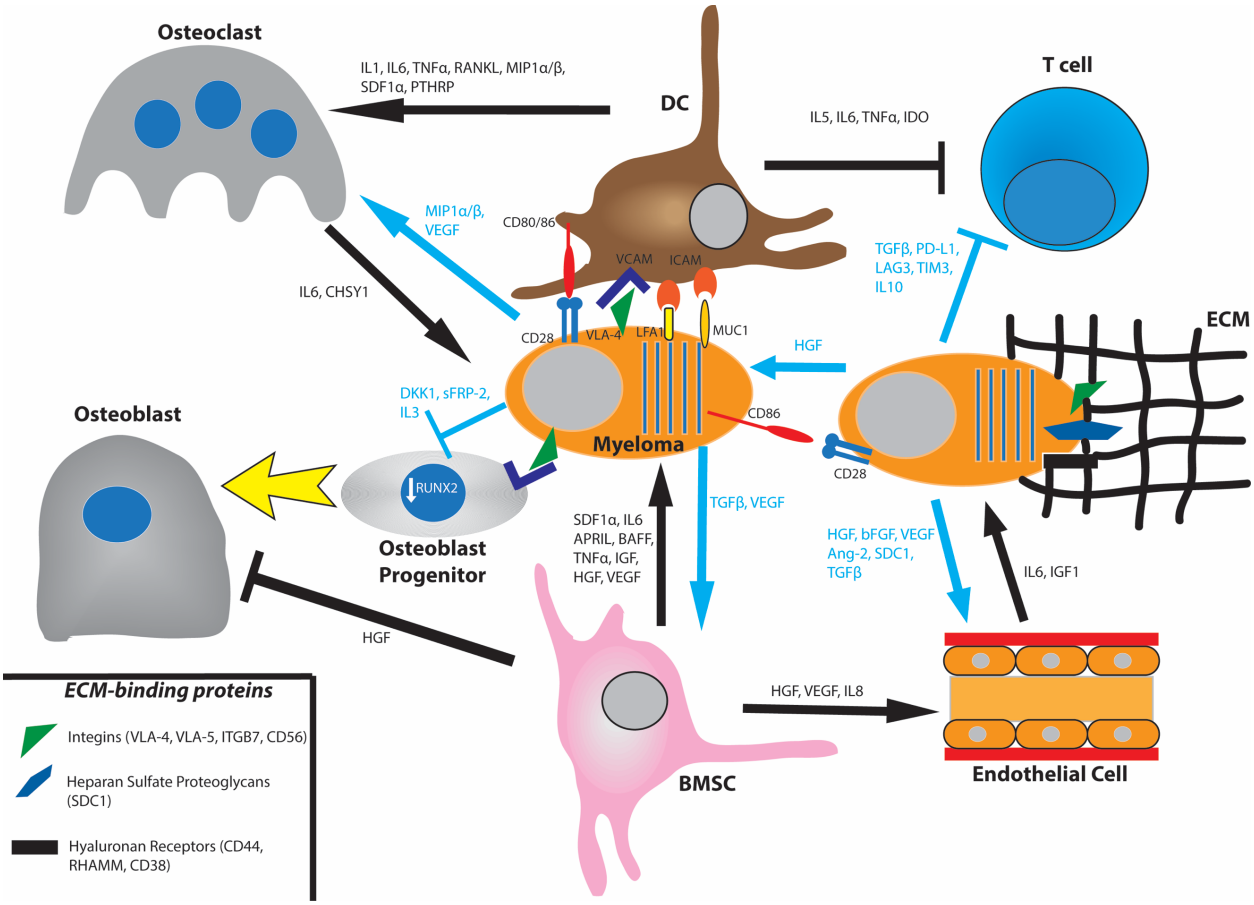

Figure 1: Bone marrow interactions that promote myeloma growth and survival. Myeloma cells bind to the ECM via integrins, proteoglycans, and hyaluronan receptors. They also directly bind to BMSC such as dendritic cells (DC) via the VCAM-VLA4, ICAM-LFA1, ICAM-MUC1, and CD80/86-CD28 axes. BMSC will produce the cytokines SDF1**α**, IL6, APRIL, BAFF, TNF**α**, IGF, HGF, and VEGF. In turn, myeloma cells secrete TGF**β** and VEGF for BMSC. Myeloma cells promote osteoclast activation by secreting MIP1**α**/**β** and VEGF and by promoting BMSC secretion of IL1, IL6, TNF**α**, RANKL, MIP1**α**/**β,** SDF1 **,** and PTHRP. They also prevent osteoblast differentiation by downregulating RUNX2 via direct binding of the VLA-VCAM axis and secretion of DKK1 and IL3. They also secrete sFRP-2 which also suppresses osteoblast differentiation. Osteoclasts produce IL6 and CHSY1 to promote myeloma cell survival. Myeloma cells

induce angiogenesis in the bone marrow by secreting HGF, bFGF, VEGF, Ang-2, cleaved syndecan-1 (SDC1), and TGF**β**. They also promote BMSC secretion of HGF, VEGF, and IL8. Endothelial cells produce IL6 and IGF1 to influence myeloma cell survival. Myeloma cells promote an immunosuppressive environment by inhibiting T cell function through production of TGF**β**, PD-L1, LAG3, TIM3, and IL10. They also signal to BMSC to produce IL5, IL6, TNF**α,** and IDO.

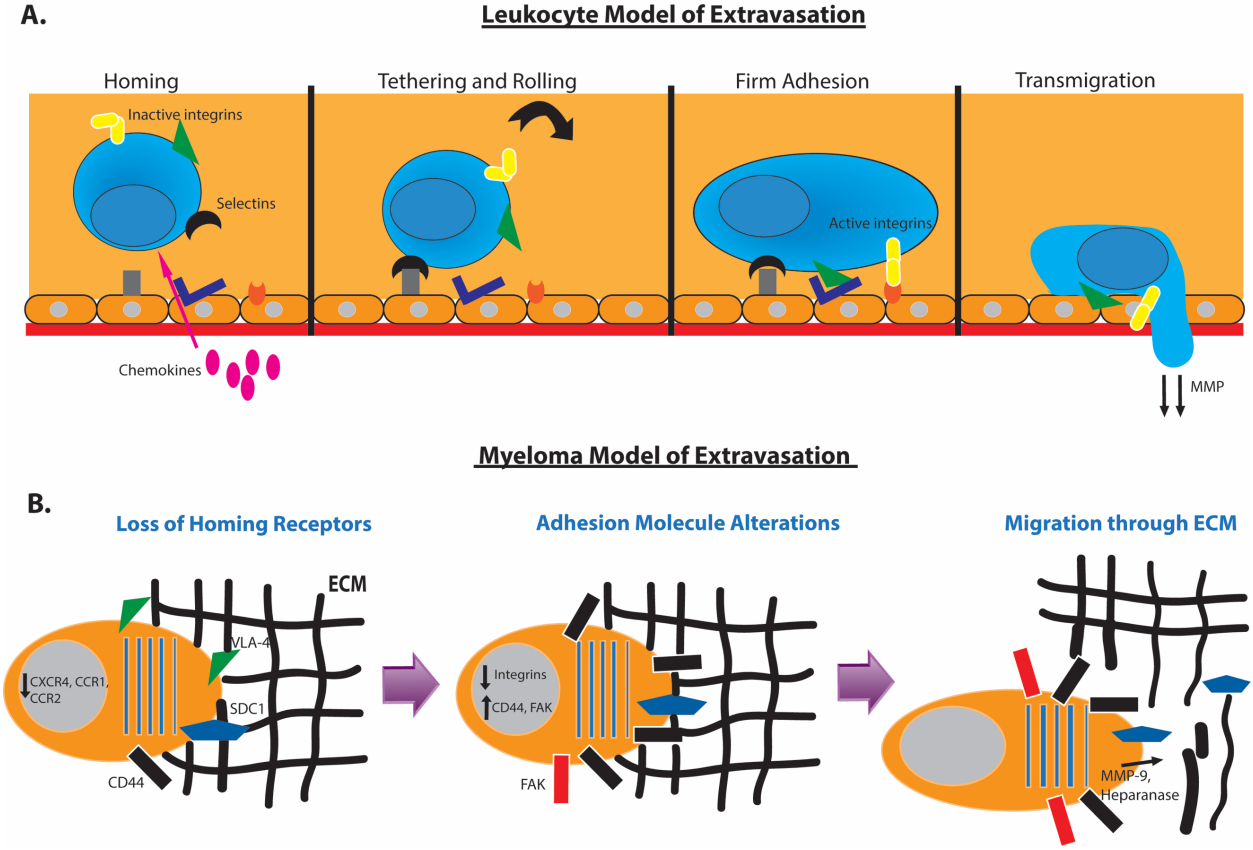

Figure 2: Models for leukocyte and myeloma cell extravasation. (A.) *Standard leukocyte multistep model of extravasation*. The leukocyte in the bloodstream receives homing signals from chemokines. This is followed by weak adhesion to the endothelium and rolling along the surface. Integrins such as VLA4 and LFA1 are activated to form tight adhesion to the endothelium. The leukocyte then reorganizes its cytoskeleton and degrades the basement membrane to transmigrate through. (B.) *Model of myeloma extravasation out of the bone marrow.* Myeloma cells downregulate receptors used for homing to the bone marrow. They alter adhesion molecules by downregulating integrins and increasing hyaluronan receptors such as CD44 and RHAMM and expression of FAK. The myeloma cell will secrete MMP-9 and heparanase as well as induce production of MMP-9 via endothelial cells to degrade the ECM. Heparanase secretion

can cause shedding of SDC1 which also contributes to cell motility. The myeloma cell will then reorganize its cytoskeleton and migrate through the ECM.

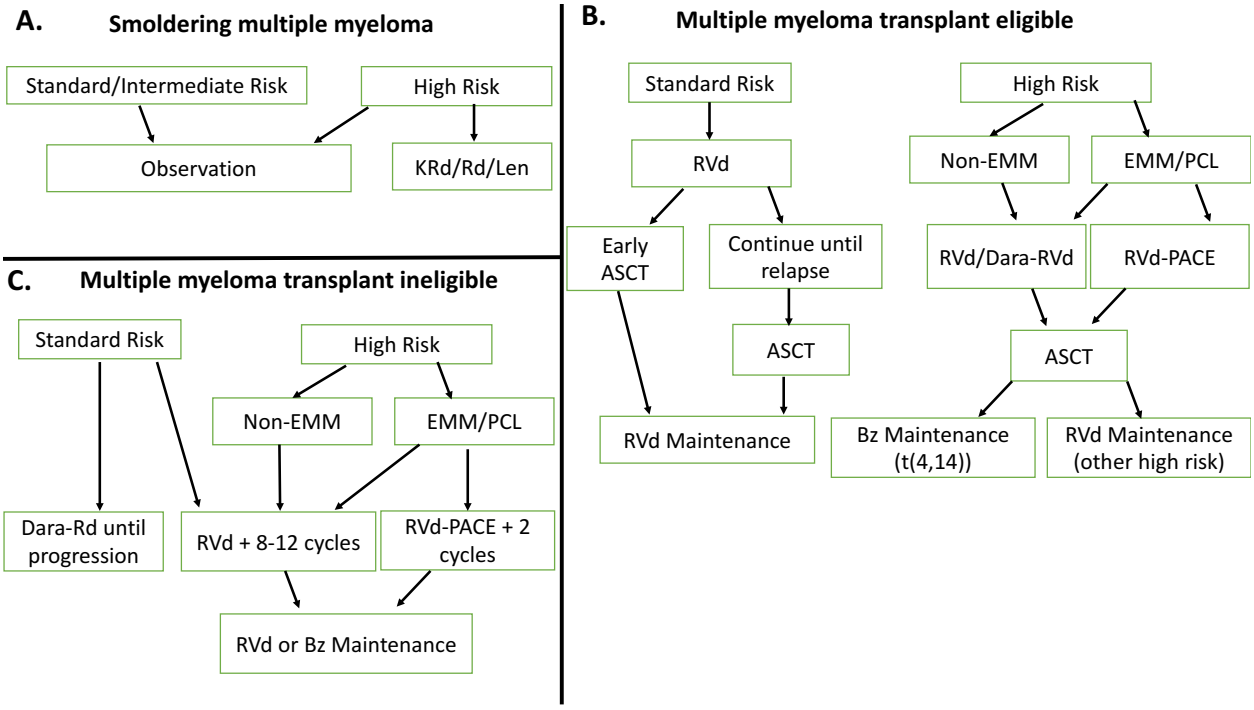

Figure 3: Suggested course of action for newly diagnosed SMM and multiple myeloma patients. (A.) *Smoldering multiple myeloma patient course of action*. Rd, Lenalidomide+Dexamethasone; Len, single agent lenalidomide (B.) *Multiple myeloma transplant eligible patient course of action*. ASCT, autologous stem cell transplant; Dara-RVd, Daratumumab+Lenalidomide+Bortezomib+Dexamethasone ; EMM, extramedullary multiple myeloma; PCL, plasma cell leukemia; RVd, Lenalidomide+Bortezomib+Dexamethasone; PACE, cisplatin, doxorubicin, cyclophosphamide, etoposide; Bz, bortezomib (C.) *Multiple myeloma transplant ineligible patient course of action*. Dara-Rd, Daratumumab+Lenalidomide+Dexamethasone

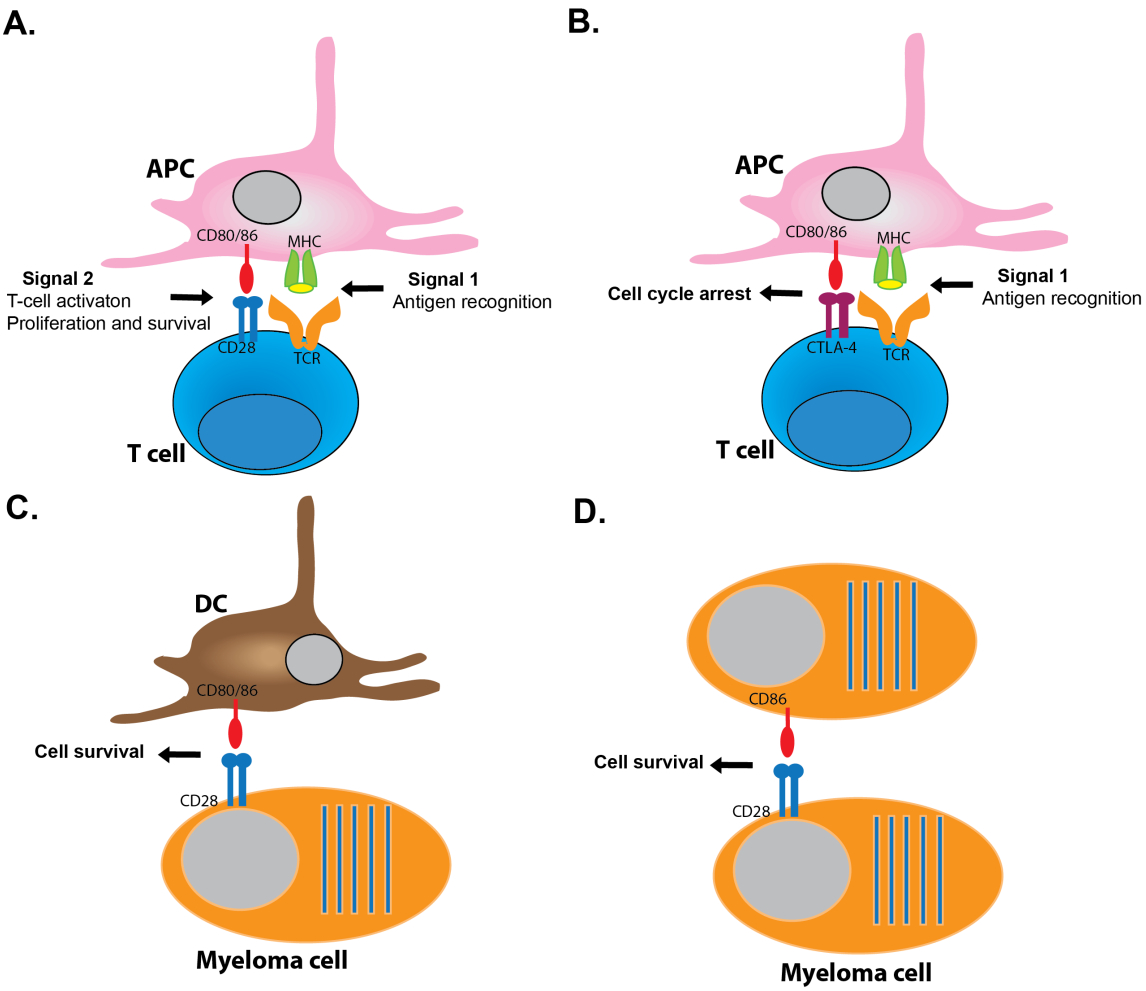

Figure 4: CD28-CD86 binding in different immune cell types. (A.) T Cell co-stimulation interaction: First, Major histocompatibility complex (MHC) presents antigen peptides to T cell receptor (TCR). Then CD80/86 of an antigen presenting cell (APC) binds to CD28 of a T cell to maximally activate and induce proliferation and survival. (B.) Binding of CTLA-4 receptor of a T cell to CD80/86 of an APC confers cell cycle arrest of a T cell. (C.) Binding of CD28 of a myeloma cell to CD80/86 of a dendritic cell (DC) results in

myeloma cell survival. (D.) Myeloma cells express both CD86 and CD28. Binding of CD28 to CD86 induces myeloma cell survival.

Chapter 2

PDZ proteins, SCRIB and DLG1, regulate myeloma surface CD86 expression, growth, and survival

Tyler Moser-Katz, Catherine M. Gavile, Benjamin G. Barwick,

Kelvin P. Lee, and Lawrence H. Boise

A version of this manuscript is in review at Molecular Cancer Research

Abstract

Despite advances in the treatment of multiple myeloma (MM) in the past decades, the disease remains incurable, and understanding signals and molecules that can control myeloma growth and survival are important for the development of novel therapeutic strategies. One such molecule, CD86, regulates MM cell survival via its interaction with CD28 and signaling through its cytoplasmic tail. Although the CD86 cytoplasmic tail has been shown to be involved in drug resistance and can induce molecular changes in MM cells, its function has been largely unexplored. Here, we show that CD86 cytoplasmic tail has a role in trafficking CD86 to the cell surface. This is due in part to a PDZ-binding motif at its C-terminus which is important for proper trafficking from the Golgi apparatus. BioID analysis revealed 10 PDZ-domain containing proteins proximal to CD86 cytoplasmic tail in myeloma cells. Among them, we found the planar cell polarity proteins, SCRIB and DLG1, are important for proper CD86 surface expression and the growth and survival of myeloma cells. These findings indicate a mechanism by which myeloma cells confer cellular survival and drug resistance and indicate a possible motif to target for therapeutic gain.

Introduction

Multiple myeloma, a disease of antibody-producing plasma cells, is the second most common hematologic malignancy in the U.S.[1] In 2020, approximately 32,000 patients were diagnosed with myeloma and 13,000 deaths occurred[2]. Although therapeutic agents such as proteasome inhibitors, immunomodulatory drugs, and targeted antibody treatments have increased median survival rates, efficacy of these agents is still limited, and the majority of patients become drug-resistant and relapse[3–5]. While normal plasma cells are highly reliant on interactions with bone marrow stromal cells for survival, myeloma cells may become extramedullary in advanced stages and can survive and proliferate outside of the bone marrow microenvironment[6–9]. Thus the mechanisms that allow MM cells to become independent of the bone marrow microenvironment are central to understanding how the disease progresses to this terminal treatment refractory stage.

Some clues underlying myeloma cell progression can be found within the bone marrow microenvironment where the pro-survival interactions of myeloma cells with stromal cells and the extracellular matrix have been previously studied[6,10–12]. One such stromal-myeloma cell interaction involves the binding of myeloma cell receptor CD28 to CD80/CD86 of a dendritic cell[13]. This CD80/CD86-CD28 interaction is primarily known in context of T-cell co-stimulatory response. During this process, the T cell receptor (TCR) is first activated by MHC peptide complex of an antigen presenting cell (APC), and CD80/86 expressed on APCs binds to T cell CD28 to provide co-stimulation to maximally activate T cell proliferation, and survival[14–18]. Since most myeloma cells co-express CD28 and CD86, we hypothesized a similar role for the proteins in myeloma

cells. Specifically, because the CD86-CD28 interaction promotes survival in T cells, this suggests a possible mechanism by which myeloma cells survive independent of stromal cell signals. This pathway could represent a therapeutic target in myeloma, especially as the U.S. Food and Drug Administration has already approved inhibitors of the CD86-CD28 interaction (CTLA4-Ig) for treatment of graft-host rejection and autoimmune disorders[19–22].

CD86 has been primarily characterized as a ligand of CD28, and numerous CD28 signaling motifs and pathways have been identified[23–26]. However, recent work on B cell function in mice[27–33] and dendritic cells[34] demonstrates that CD86 also signals upon ligation to initiate specific responses. Consistent with this, our lab has recently shown that CD86 is necessary for myeloma cell survival and drug resistance[13,35]. The cytoplasmic region of CD86 is important for conferring these effects as well as inducing molecular changes in myeloma cells such as upregulation of IRF4, ITGB1, and ITGB7[35]. This suggests that proper surface expression of CD86 and its downstream signaling is important in myeloma, While a specific polylysine motif in the cytoplasmic tail has been shown to associate CD86 with the cytoskeleton to maintain surface expression in APCs[16], other motifs in the cytoplasmic tail are not known. CD86 contains numerous sites of N-linked glycosylation in its extracellular domain that can direct trafficking to the plasma membrane. However, additional means of regulating CD86 surface expression have remained understudied. Therefore, we set out to further elucidate mechanisms of proper CD86 trafficking in myeloma via its cytoplasmic tail.

Materials and Methods:

Cell Lines

Human Embryonic Kidney 293T (HEK293T) cell line was purchased from American Type Culture Collection (ATCC). Cells were cultured and seeded in 6-well plates containing 2 mL Dulbecco's modified Eagle's medium (DMEM; Corning) supplemented with 10% fetal bovine serum (Gemini) at 37 °C in an incubator with 5% CO_2.

Myeloma cell lines were cultured as previously described[36]. MM.1s were provided by Dr. Steven Rosen (City of Hope, Duarte, CA), KMS18 were purchased from the Japanese Cell Resource Bank (JCRB), and RPMI8226 were purchased from ATCC. Cells were cultured in RPMI1640 media supplemented with 10% fetal bovine serum at 37 °C in an incubator with 5% CO_2.

Transient Transfection

HEK293T cells were seeded on coverslips on a six-well plate. The next day, they were transfected using Lipofectamine-2000 (Invitrogen) according to manufacturer's instructions after reaching a confluency of 70–90%. Forty-eight hours after transfection, cells were either lysed for protein extraction or fixed for immunofluorescence. Cells were transfected with CD80 (Genscript) and CD86 constructs cloned into pcDNA3.1+ plasmid as previously described[35].

Protein Extraction and Immunoblotting

Cell pellets were lysed in radioimmunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitors as previously described[9]. Lysates were quantified using the

bicinchoninic acid assay (ThermoFisher), and lysates were run in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels, then blotted as previously described[9]. Primary antibodies used included: Mouse monoclonal α-CD86 (R&D), mouse monoclonal α-β-actin (Sigma), Streptavidin HRP (Millipore) and rabbit polyclonal α-HA (Abcam). The following secondary antibodies were used: anti-mouse immunoglobulin G–horseradish peroxidase (IgG-HRP) and anti-rabbit IgG-HRP (Santa Cruz Biotechnology)

Immunofluorescence

Cells were grown on glass coverslips (Fisher) coated with 5 μg/cm^2 fibronectin unless otherwise specified (Millipore). Twenty-four hours after transfection, cells were fixed with PHEMO buffer (68 mM PIPES, 25 mM HEPES, 15 mM EGTA-Na_2, 3 mM $MgCl_2$.$6H_2O$, 10% DMSO, pH 6.8) supplemented with 3.7% formaldehyde (Fisher), 0.05% glutaraldehyde (Fisher) and 0.2% Triton X-100 (Bio-Rad) for permeabilization when indicated. CD86 was labelled by staining with CD86-APC antibody (Caprico Biosciences). CD80 was labelled by staining with CD80-FITC antibody (BD Biosciences). Mannose-6 phosphate receptor was labelled overnight at 4 degrees Celsius using a rabbit polyclonal antibody α-Mannose-6 phosphate receptor provided as a gift from Dr. Paul Luzio (Cambridge Institute for Medical Research)[37]. Other antibodies used were Rabbit α SYNTENIN (Abcam), Rabbit α SCRIB (Cell signaling) and Rabbit α DLG1 (Thermo Fisher), Mouse α EEA1(BD Biosciences). Goat α rabbit Alexa Fluor 488 (Invitrogen) or Goat α Mouse Alexa Fluor 488 secondary antibody was used to stain cells for 1 hour at room temperature. Coverslips were then mounted on microslides

(Fisher) using Prolong Gold containing 300 nM 4′-6-diamidino-2-phenylindole dilactate (DAPI;Invitrogen). Cells were imaged using a Leica TCS SP8 inverted confocal microscope (63X oil HC PL APO, NA 1.4). Mander's correlation coefficient was analyzed for five cells in three independent assays (15 cells total) and determined using Co-localization tool on FIJI ImageJ.

BioID

For BioID analysis, 50 million cells of MM.1s BirHA or MM.1s CD86-BirHA were cultured in 150 cm^2 flasks. Subsequent BioID was performed as previously described[38]. Gene ontology was performed using String.db. PDZ domain-containing proteins were identified using the HUGO nomenclature online database.

In Situ Proximity Ligation Assay (PLA)

SCRIB/CD86 and DLG1/CD86 interactions were detected in situ using Duolink™ II secondary antibodies and detection kits (Sigma–Aldrich, #DUO92002, #DUO92004, and #DUO92008) according to the manufacturer's instructions. Briefly, PLA probes and primary antibodies: Rabbit α HA (Abcam), Mouse α SYNTENIN (Abnova) Mouse α SCRIB (GeneTex), and Mouse α SAP97/DLG1 (Santa Cruz) were applied to fixed cells. Then, Duolink™ secondary antibodies were added. Polymerase and amplification buffer were added to amplify a positive signal (red dot) and detected by confocal microscopy. DAPI was used for counterstaining of the nucleus.

Real Time Polymerase Chain Reaction (RT-PCR)

RNA was extracted and quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed as previously described using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies)[36]. Resulting cDNA was amplified using the TaqMan Gene Expression Master Mix (Life Technologies) on the CFX96 Real-Time PCR System following the manufacturer's protocol (Bio-Rad). Probes used were Glyceraldehyde-3-phosphate dehydrogenase, SYNTENIN, CD86, SCRIB, DLG1, and IRF4 (Applied Biosystems).

Flow Cytometry and Analysis

Cell-surface expression CD86-APC (Caprico Biosciences) and integrin β7-PE (BD Biosciences) were measured via flow cytometry. 100,000 live cells were collected, washed with 1× PBS, and stained with appropriate antibodies in 50 µL FACS staining buffer. After incubation of 15 min at 4°C in the dark, cells were washed in 1× PBS and resuspended in 400 µL FACS staining buffer. Samples were run on a FACS Symphony A3 Flow Cytometer (BD Biosciences) and analyzed using FlowJo software.

Generation of Cas9-inducible cell lines and determination of sgRNA

Inducible Cas9 expressing cells were generated using the pCW-Cas9 plasmid through lentiviral infection[39]. Virus was produced by transfecting HEK293T cells with pCW-Cas9 (gift from Eric Lander and David Sabatini: Addgene plasmid # 50661) and packaging plasmids DR8 and VSVg using Lipofectamine 2000[40]. Lentiviral infection and selection of cells were performed as previously described[39]

CD86, SYNTENIN, SCRIB, and DLG1 sgRNA clones were generated by designing specific sgRNAs using the online CRISPOR tool[41] and cloning them into pLX-sgRNA (gift from Eric Lander and David Sabatini: Addgene plasmid # 50662) using the following primers: GTCGAGTGTGCTACTCAACTCGTTTTAGAGCTAGAAATAGCAA (forward) and GAGTTGAGTAGCACACTCGACGGTGTTTCGTCCTTTCC (reverse). The sgRNA sequences are provided in Supplemental Methods. Virus generation and infection of inducible Cas9 cells were as described[39]. Twenty-four hours after infection, 10 μg/mL blasticidin was added to select cells for 7 days.

Cell Count Proliferation Assay

KMS18 and RPMI8226 Cas9 inducible cells were infected with sgRNA containing lentiviral particles as described above. The following day, live cells were counted using 100 μL of cells and 100 μL of FITC-conjugated counting beads (Thermo Fisher) and run on a FACS Symphony A3 Flow Cytometer (BD Biosciences). 100,000 live cells were then seeded on 24-well plates in 2 mL of complete media. Doxycycline hyclate (1 μg /mL; Sigma) was then added to cells and counts were repeated using FITC-conjugated counting beads as per the manufacturer's instructions for seven days.

Apoptosis Assays

Cell death was measured by annexin V-fluorescein isothiocyanate (BioVision) and propidium iodide (PI) staining as previously described[36].

Statistical Analysis

Statistical significance was assessed using two-tailed Student *t* test using GraphPad Prism.

Results

CD86 cytoplasmic tail is important for trafficking to cell surface

We previously generated RPMI8226 myeloma cell lines which stably overexpress full length CD86 (CD86FL) or a "tail-less" mutant of CD86 (CD86TL)[35]. CD86TL expresses the extracellular and transmembrane domain and 7 amino acids of the cytosolic domain (**Fig 1A**). We used vectors coding for CD86FL and CD86TL and transiently transfected them into HEK293T, a cell line which does not endogenously express CD86. We then stained fixed cells with a CD86 antibody to determine CD86 localization 2 days after transfection (**Fig 1B**). While CD86FL can be observed at the perimeter of the CD86FL cells, CD86TL formed a more diffuse staining pattern with several puncta inside the cells. To verify that this phenotype was not due to a discrepancy in transfection efficiency, we took lysates of these cells and performed western blot analysis. There was no decrease in CD86 total protein expression and in fact, the CD86TL expression was significantly higher than CD86FL (**Fig 1C**).We noticed that our stable RPMI8226-CD86FL myeloma cells also produced a uniform stain around the perimeter while the RPMI8226-CD86TL myeloma cell lines had more punctate staining (**Fig 1D**). In order to determine what stage CD86 trafficking to the surface is inhibited, we co-stained the HEK293T cells with Mannose-6 phosphate receptor (M6PR), a Golgi marker, as well as CD86 (**Fig 1E**). We then analyzed co-localization of these proteins using a Mander's correlation coefficient and observed a significant increase in co-localization between M6PR and CD86 in the CD86TL compared to CD86FL transfected HEK293T (**Fig 1F**). Since CD86 is glycosylated, we would expect it to be exported from the Golgi under normal conditions following its synthesis and post-

translational modifications. The presence of CD86 in the Golgi apparatus could indicate a defect in anterograde trafficking or increased retrograde trafficking. Therefore, we used a pulse-chase experiment utilizing cell surface biotin labeling to measure rates of CD86 delivery to the plasma membrane following trypsin cleavage. We observed that CD86FL was able to repopulate the cell surface after 1 hour while CD86TL exhibited delayed recovery (**Fig S1A**). To determine if truncation of the CD86 cytoplasmic tail results in increased retrograde trafficking, we also co-stained with CD86 and an endosomal marker, early endosomal antigen-1 (EEA1) and saw no significant difference in co-localization with CD86 in CD86FL and CD86TL transfected lines (**Fig S1B**). Together, these data show that the cytoplasmic tail has a role in conferring proper transport of CD86 from the Golgi apparatus to the membrane.

Multiple regions of the CD86 cytoplasmic tail are important for anterograde trafficking

The cytoplasmic region of CD86 is composed of a 61 amino acid long tail. Since we detected a trafficking defect in the CD86TL-transfected HEK293T, we wanted to define which areas of the tail are important for CD86 trafficking. We used homology modeling of human CD86 compared to other higher mammalian species and identified numerous conserved and potentially important areas of the cytoplasmic tail to focus on[35]. Full length CD86 is 329 amino acids in length, and there may be multiple motifs working in conjunction along the protein to regulate trafficking. Therefore, instead of mutating specific motifs, we developed CD86 truncation mutants that were 282 (CD86Δ282) 298 (CD86Δ298), and 315 (CD86Δ315) amino acids long respectively (**Fig**

2A). We then transfected the truncation mutants into HEK293T cells and observed CD86 trafficking 2 days following transfection (**Fig 2B**). While we noticed that each of the truncation mutants trafficked CD86 more effectively than CD86TL, they were never able to fully phenocopy CD86FL. To determine what point the trafficking is inhibited, we co-stained M6PR and CD86 in pcDNA3.1 vector control, CD86FL, CD86Δ282, CD86Δ298, and CD86Δ315 transfected cells (**Fig 2C**). When we quantified co-localization using a Mander's correlation coefficient, we observed that the truncation mutants each had significantly higher co-localization of M6PR and CD86 compared to CD86FL (**Fig 2D**). Furthermore, there is less co-localization the longer the tail, suggesting that there are several regions of the tail that are important for proper CD86 trafficking.

CD86 contains a PDZ binding motif important for surface expression

Since full-length CD86 contains 329 total amino acids, and the CD86Δ315 mutant improperly traffics, we identified 14 amino acids at the C-terminus of CD86 to further dissect. Among higher order mammalian species, CD86 contains a conserved class I Psd95/DLG1/zo-1 (PDZ) binding motif at its C-terminus (**Fig 3A**). A class I PDZ binding motif is composed of a S/T-X-**ϕ** motif where X represents any amino acid and **ϕ** represents a hydrophobic residue[42], and human CD86 C-terminus meets these criteria with TCF at its C-terminus. This motif is commonly found at the C-terminus of receptor proteins and is recognized by adaptor proteins that contain a PDZ domain. PDZ proteins can then perform numerous signaling or scaffolding functions specific to cell and context[42].

To determine whether the PDZ binding motif has a role in cellular trafficking of CD86, we developed a truncation mutant which lacks the final 3 amino acids of CD86, effectively truncating the PDZ binding motif (CD86Δ326). We then transfected this construct into HEK293T and co-stained with M6PR and CD86 2 days after transfection (**Fig 3B**). After quantification of co-localization using a Mander's correlation coefficient, we observed significantly more co-localization of M6PR and CD86 in the CD86Δ326 HEK293T compared to CD86FL, illustrating that the PDZ binding motif is important for proper CD86 trafficking to the cell surface (**Fig 3C**).

Like CD86, CD80 is another receptor protein classically recognized to be involved in the T-cell co-stimulatory response[14]. Unlike CD86, CD80 is not expressed in myeloma cells, so the two proteins may not have redundant roles. Interestingly human CD80 does not contain a PDZ binding motif, thereby suggesting a further difference in trafficking between the two proteins in immune cells (**Fig S2A**). We developed a "tail-less" mutant of CD80 to assay whether CD80 cytoplasmic tail also confers a proper trafficking phenotype (**Fig S2B**). When we transfected HEK293T with CD80FL and CD80TL we observed no notable difference in trafficking between the two proteins, illustrating a different mechanism of transport between the two proteins (**Fig S2C**).

BioID proximity assay identifies numerous CD86 interacting partners

In order to determine possible interacting partners for the CD86 cytoplasmic tail, we utilized the BioID proximity-based labeling assay. This method utilizes a constitutively active biotin ligase (BirA) that is engineered to biotinylate proteins within 10 nm of it[38]. We developed a construct with BirA containing an HA tag (BirHA) cloned

into the CD86 tail prior to the PDZ domain (CD86-BirHA) (**Fig S3A**). We transfected soluble BirHA or CD86-BirHA into the myeloma cell line, MM.1s, and developed stable cell lines expressing either protein (**Fig 4A, Fig S3B**). We then used streptavidin-conjugated beads to pull down proximal proteins in either cell line (**Fig S3C**). On-bead trypsin digestion was performed and unique peptides were analyzed using mass spectrometry.

Proteins were then stratified based on peptide-spectrum match (PSM) score. This assigns a numerical value that expresses the likelihood that fragmentation of a peptide is contained in the experimental spectrum[43]. Proteins with a PSM score of greater than 5 met the determined threshold for a positive hit. Proteins were then deemed to be enriched in the CD86 cytoplasmic tail if a threefold higher PSM score in MM.1s CD86-BirHA was observed in comparison to MM.1s BirHA. In total, there were 225 proteins that were associated with the CD86 cytoplasmic tail (**Supplementary Table 2**). Using the online database, string.db, we performed gene ontology analyses for KEGG pathway analysis and Reactome Pathways (**Fig 4B**) and found that our top hits for each pathway were Endocytosis in KEGG (FDR=9.34×10^{-6}) and Membrane Trafficking in Reactome Pathways (FDR=1.13×10^{-5}). The Reactome Pathways analysis also revealed additional functions such as Receptor Tyrosine Kinase signaling, Rho GTPases, glycosylation, intra-Golgi and retrograde Golgi-ER traffic, and COPI-mediated anterograde transport which providing additional information about the biological processes controlled by CD86 binding partners.

We next determined how many of the stratified hits were PDZ domain-containing proteins. Using the HUGO nomenclature online database, we identified 152 known PDZ

domain-containing proteins and cross-referenced them with our 225 enriched proteins. Ten proteins with PDZ domains were found among our hits: SLC9A31, SCRIB, SYNTENIN, PDLIM, PARD3, GORASP2, CASK, AHNAK, DLG1, and ERBB2IP (**Fig 4C**). Using the CoMMpass dataset, a longitudinal study which follows patient gene expression throughout treatment, we determined that all 10 PDZ domain proteins are expressed to varying degrees in primary myeloma patients (**Fig S4A**). To stratify proteins to focus, we analyzed patient subtype expression. Both CD86 and CD28 have increased expression in the MAF myeloma subtype[7,35]. We identified SCRIB, AHNAK, and PARD3 as genes with the highest expression in the MAF subtype and DLG1 having the lowest expression in this subtype (**Fig S4B**). Among the three highest expressing proteins, we decided to focus on SCRIB as it is prognostic of poor patient progression-free and overall survival (**Fig S4C**). We also wanted to test a PDZ protein that has median relative expression in the MAF subtype and selected SYNTENIN (encoded by SDCBP) as it has been shown to regulate surface levels CD138. CD138 or SYNDECAN is a marker of myeloma cells with a role in myeloma cell adhesion, and we therefore were interested in the potential role of SYNTENIN in myeloma[44]. Furthermore, using the CoMMpass dataset, we observed a positive correlation between SDCBP and CD86 mRNA expression in patients (**Fig S4D**). Taken together, these proteins may have implications in CD86 expression as well as myeloma cell survival and bone marrow localization, and warranted further exploration.

SCRIB and DLG1 are apical-basal polarity proteins that have been shown to regulate surface levels of numerous proteins[45,46] and have been shown to form complexes with one another to regulate polarity axes of cells[47]. Using a proximity

ligation assay (PLA), we verified that SCRIB and DLG1 interact with CD86 while SYNTENIN does not (**Fig 4D**). We also co-stained CD86-overexpressing RPMI8226 cell lines with CD86 and either SYNTENIN, SCRIB, DLG1, or a secondary antibody-only control (**Fig 4E**). We compared co-localization of CD86 with either a non-specific secondary antibody-only control, SYNTENIN, SCRIB, or DLG1. While there was no significant difference between SYNTENIN co-localization and the secondary-only control, there was a significant increase in SCRIB and DLG1 co-localization compared with SYNTENIN (**Fig 4F**). Identification of SCRIB and DLG1 co-localization with CD86 led us to further explore their function in multiple myeloma.

SCRIB and DLG1 regulate CD86 surface expression

We next wanted to elucidate the role of SCRIB and DLG1 in CD86 trafficking to the cell surface. While they have been involved in numerous cancers, their roles in multiple myeloma have been unexplored[48,49]. We utilized myeloma cell lines, KMS18 and RPMI8226, which we engineered to stably express a doxycycline-inducible Cas9 enzyme. Single guide RNA targeting either CD86 (sgCD86), SCRIB (sgSCRIB), and DLG1 (sgDLG1) were generated based on specificity and efficiency using the online CRISPOR tool[41]. As a control, a single guide vector targeting the viral incorporation site, AAVS1, was used (plx-sgRNA)[40]. We used lentiviral particles to infect myeloma cells with sgRNA and then selected for cells containing the plasmids for seven days using blasticidin. Following selection, cells were plated and doxycycline was added to activate the Cas9 enzyme expression (**Fig 5A**). We have previously determined a role for CD86 in myeloma cell survival and hypothesized that SCRIB and DLG1 may be involved as

well. We therefore did not establish stable cell lines and instead performed a transient assay measuring cell counts, cell death, and CD86 surface expression for seven days.

We first verified that CD86 surface expression was decreased with the addition of CD86 guides. Indeed, we observed reduced CD86 expression in two guides targeting CD86 as soon as three days after doxycycline addition (**Fig 5B**). At day three, we also observed decreased CD86 surface expression with three of our SCRIB guides in KMS18 (**Fig 5C-D, 5G, S5A**). Similarly, we observed decreased CD86 surface expression in the sgDLG1 KMS18 cells after three days (**Fig 5E-G, S5A)**. While surface levels were decreased, we did not observe any difference in total CD86 protein levels (**Fig S5B**). We repeated this experiment using two guides against SYNTENIN (sgSYNTENIN) with the hypothesis that CD86 surface expression would not be affected as SYNTENIN does not co-localize with CD86. Indeed, we observed no change in CD86 surface expression (**Fig S6A-B**). These data suggest that SCRIB and DLG1 affect CD86 surface expression in myeloma, and we wanted to further explore what effect SCRIB KO and DLG1 KO would have on myeloma cells.

SCRIB, DLG1, and SYNTENIN are important for myeloma cell growth and viability

To determine whether knockout of CD86, SYNTENIN, SCRIB, or DLG1 resulted in less proliferation of myeloma cells, we used FITC-conjugated beads and flow cytometry to count live cells 0 to 7 days following doxycycline addition. We conducted these experiments in KMS18 and RPMI8226 myeloma cell lines and found that knockout of CD86, SCRIB, and DLG1 (**Fig 6A-C**) resulted in significantly diminished cell growth compared to plx-sgRNA control cells. We hypothesized that this decrease in cell

count could be largely due to an increase in cell death. We have previously shown using shRNA knockdown that loss of CD86 results in significantly higher cell death in both KMS18 and RPMI8226[35]. Indeed, we saw that knockout of CD86 via CRISPR-Cas9 resulted in increased cell death in both lines (**Fig 6D**). When we tested the viability of SCRIB KO and DLG1 KO, we saw an increase in cell death in both cell lines (**Fig 6E-F**). We also observed decreased cell proliferation and increased cell death in SYNTENIN knockout lines despite the lack of change in CD86 surface expression (**Fig S6C-D**). This suggests a role for SYNTENIN in myeloma viability that is independent of control of CD86 trafficking.

Interestingly, the majority of cell death occurred during days 1-3 in all knockout lines. This is particularly evident in RPMI8226 lines where approximately 80% of all knockout cells die by day 3 (**Fig 6D-F, Fig S7**). RPMI8226 are largely dependent on CD86 for survival[35], and the majority of CD86 KO cells die by day 3 (**Fig 6D)**. In the RPMI8226 SCRIB KO and DLG1 KO, a "CD86 low" population fails to grow out, and the few cells that do survive past day 3 have normal protein expression of CD86 (**Fig S7B**). Furthermore, in the KMS18 SCRIB KO and DLG1 KO, the "CD86 low" populations do not grow out past day 3, and CD86 resurfaces in days 4-7 (**Fig S5C**). This points to a population of cells that did not efficiently edit CD86 as the population that was able to effectively grow out.

SCRIB and DLG1 facilitate CD86 pro-survival signaling

We wanted to further investigate the role that SCRIB and DLG1 had in CD86-mediated cell survival. Using the RPMI8226-CD86FL lines, we co-stained the cells with CD86 and

either SCRIB or DLG1 (**Fig 7A**). Since CD86 signals via cell-cell contact, we hypothesized that there would be a difference in co-localization of CD86 and SCRIB or DLG1 at points of cell contact compared to non-contact sites. Surprisingly, we observed a decrease in SCRIB/DLG1 co-localization with CD86 at sites of contact compared to non-contact sites (**Fig 7B**). We also noticed that these contact sites had higher expression of CD86 in the RPMI8226-CD86FL cell lines (**Fig 4E, 7C**). We hypothesized that binding of CD86 to CD28 on myeloma cells stabilizes CD86 expression and allows SCRIB and DLG1 to leave. Consistent with this possibility, CD86FL-transfected HEK293T, a cell line which does not express CD28, displayed no difference in CD86 expression in contact sites compared to non-contact sites (**Fig 7C-D**). We also wanted to verify whether the cell death in the SCRIB and DLG1 knockout lines was due in part to the lack of a CD86-intrinsic signal. We have previously shown that loss of CD86 in myeloma cell lines leads to decreased expression of IRF4 and Integrin $\beta 7$[35], two proteins which have been implicated in myeloma cell survival[50,51]. We quantified mRNA expression of IRF4 in our CD86, SCRIB, and DLG1 RPMI8226 knockout lines and observed a significant decrease with every sgCD86 and sgSCRIB line and 2 out of 3 DLG1 sgRNAs (**Fig 7E**). When we assayed integrin $\beta 7$, we found a decrease in all CD86, SCRIB, and DLG1 sgRNAs 2 days following doxycycline addition (**Fig 7F**). This suggests that the decrease in CD86 surface expression observed with the loss of SCRIB or DLG1 results in a decrease in CD86-mediated growth and survival signaling. To verify that loss of CD86 signaling is important for SCRIB KO and DLG1 KO-induced cell death, we overexpressed CD86FL in SCRIB KO and DLG1 KO RPMI8226 cells and

observed that dominant CD86 expression rescues cell death induced by SCRIB/DLG1 ablation (**Fig 7G, S7C**).

Discussion

Interaction between CD28 and CD86 on myeloma cells facilitates myeloma cell survival in a bone marrow independent niche. We previously demonstrated a role for the CD86 cytoplasmic tail in drug resistance and induction of molecular changes in myeloma cells. This study illustrates how the CD86 cytoplasmic tail mediates effective trafficking of the protein to the cell surface. We took advantage of a cell line lacking endogenous CD86 to monitor trafficking of full-length CD86 or a tail-less mutant of CD86 and observed a clear difference in efficiency of CD86 to traffic to the plasma membrane. We have shown that lacking the tail results in accumulation of CD86 at the Golgi apparatus, and KEGG pathway analysis has revealed numerous proteins involved in endocytosis in proximity to CD86 cytoplasmic tail. While retrograde trafficking may play a role in CD86 surface expression, the lack of difference in EEA1 co-localization between CD86FL and CD86TL transfected HEK293T suggest that the tail primarily affects anterograde trafficking.

The CD86 cytoplasmic tail is 61 amino acids long and makes up almost 20% of the protein sequence. It follows that there may be numerous regions that can be possible binding regions for intracellular proteins to influence trafficking. Since the length of the tail inversely corresponds with the amount of CD86 that co-localizes with M6PR, we hypothesize that a variety of proteins may be binding to the tail at different locations to influence CD86 localization both inside and outside of the Golgi. Additionally, KMS18 cytoplasmic tail contains a SNP resulting in a A304T change whereas MM.1s and RPMI8226 have an allele that is conserved among higher order mammalian species. This polymorphism is associated with increased cancer risk and

allograft rejection, illustrating the importance for further study of various regions of the CD86 cytoplasmic tail[52–54].

The discovery of a PDZ-binding motif at the C-terminus provides clues as to how the protein is being regulated. PDZ domain proteins have been largely studied and have numerous context-dependent roles in a variety of cell types. The difference in trafficking effectiveness between full-length CD86 and CD86 lacking the PDZ binding motif in HEK293T shows the importance of this motif for proper surface transport. The existence of the PDZ binding motif on CD86 but not CD80 and lack of importance of CD80 tail in trafficking to the surface may also provide clues in antigen presenting cells toward the preferential expression of either CD80 or CD86 during T-cell co-stimulation. While the CD80 cytoplasmic tail does not seem to be necessary for trafficking to the surface, it has previously been shown to influence CD80 localization at the cell surface during T cell co-stimulation[15,55]. Interestingly, ICAM-1, another receptor protein that is involved in both myeloma cell adhesion[56] and immunological signaling[57] contains a PDZ binding motif. SYNDECAN-1 (CD138) is yet another PDZ binding motif-containing protein whose surface levels have previously been shown to be regulated by SYNTENIN[44]. CD138 is a heparan sulfate proteoglycan important for myeloma cell adhesion, and this may be a means by which SYNTENIN affects myeloma cell growth and survival[58]. The presence of a three-amino acid motif on proteins that are implicated in myeloma growth and survival represents a specific target for potential therapeutic treatment.

We mapped the interaction network of CD86 cytoplasmic tail using the BioID method followed by liquid chromatography-mass spectrometry. Among our enriched proteins, ten of them contained PDZ domains. Of these ten PDZ domain proteins, half

of them (CASK, DLG1, PARD3, PDLIM, SCRIB) are involved in cell polarity. All of these five proteins except PDLIM have been shown to interact with one another[59–61]. A recent study found that SCRIB can regulate CD86 surface levels in activated antigen presenting cells[62]. Our findings expand this research to malignant plasma cells, identify a role for DLG1 in CD86 regulation as well, and determine a novel outcome of this process in regulating myeloma growth and survival.

One notable enriched protein in the interactome without a PDZ domain was SLC3A2. This protein makes up the heavy chain of CD98, which is a determinant of IMiD activity in multiple myeloma[63]. IMiDs are cytostatic drugs that halt proliferation in myeloma. We have also previously found that CD98 light chain (SLC7A5) is significantly downregulated in both CD28 and CD86-silenced cells. Since our data suggest that CD86 may have a role in proliferation, the functional interaction between CD86 and CD98 in myeloma warrants further investigation.

The similar phenotype of decreased CD86 surface expression in SCRIB KO and DLG1 KO cells suggests that these proteins may be forming a complex with one another. Alternatively, they could be part of a larger complex which regulates CD86 membrane trafficking and localization. The classical function of these two proteins may underlie that not only the amount of CD86 surface expression but also its location in time and space is a means of cellular survival. We notice changes CD86 surface expression in different locations of individual myeloma cells as points of cell-cell contact appear to have higher expression of CD86 (**Fig 4E, 7C-D**). Furthermore, we found a decrease in co-localization between CD86 and SCRIB/DLG1 in areas of cell contact (**Fig 7A-B**). This suggests that SCRIB and DLG1 may be helping to traffic CD86 to

areas of the cell surface where it can bind to CD28. Binding to CD28 then stabilizes CD86 surface expression and can allow SCRIB and DLG1 to leave the site of contact to perform other roles in the cell.

Our data also show that SCRIB and DLG1 are important for cell growth and viability in multiple myeloma cell lines, KMS18 and RPMI8226. The majority of cell death appears to take place within the first four days of doxycycline addition in both cell lines. Interestingly, in KMS18 we start to see expression of CD86 decrease by day 2 in our CD86 KO, SCRIB KO, and DLG1 KO. However, following day 4, the "CD86-low" population in the SCRIB KO and DLG1 KO do not grow out while a population of cells with normal expression of CD86 survives and proliferates. We see this to a greater effect in RPMI8226, a cell line that is more dependent on CD86. In this line, a CD86-low population is unable to grow out and roughly 80% of SCRIB/DLG1 KO cells die by day 3. The decrease of IRF4 and Integrin β7 expression in the SCRIB/DLG1 KO cells and rescue of cell mortality via dominant CD86-expression point to the lack of CD86 signaling as a mechanism by which these cells die. Since we have also shown a role for CD28 in both normal and malignant plasma cell survival, we cannot rule out a role for diminished CD28 signaling as a result of lower CD86 surface expression contributing to cell death as well[26]. The presence of a population of normally expressing CD86 cells is likely due to incomplete gene editing, however it remains possible that activation of a compensatory mechanism to maintain CD86 surface expression in a small fraction of cells may occur. Additionally, SCRIB and DLG1 have pleiotropic roles and may also be conferring myeloma cell survival via additional mechanisms.

Taken together, our data identify a means of CD86 transport and expression at the surface of myeloma cells. It illustrates that SCRIB and DLG1 are two polarity proteins expressed in myeloma that can transport CD86 to the membrane. Proper transport is reliant on a complete cytoplasmic tail with a PDZ binding motif at its C-terminus. Ablation of SCRIB or DLG1 results in a decrease in CD86 surface expression, myeloma cell survival, and proliferation. These results elucidate a role for PDZ proteins in regulation of myeloma growth and may provide new insights for targeted therapeutic advances in multiple myeloma.

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Figures

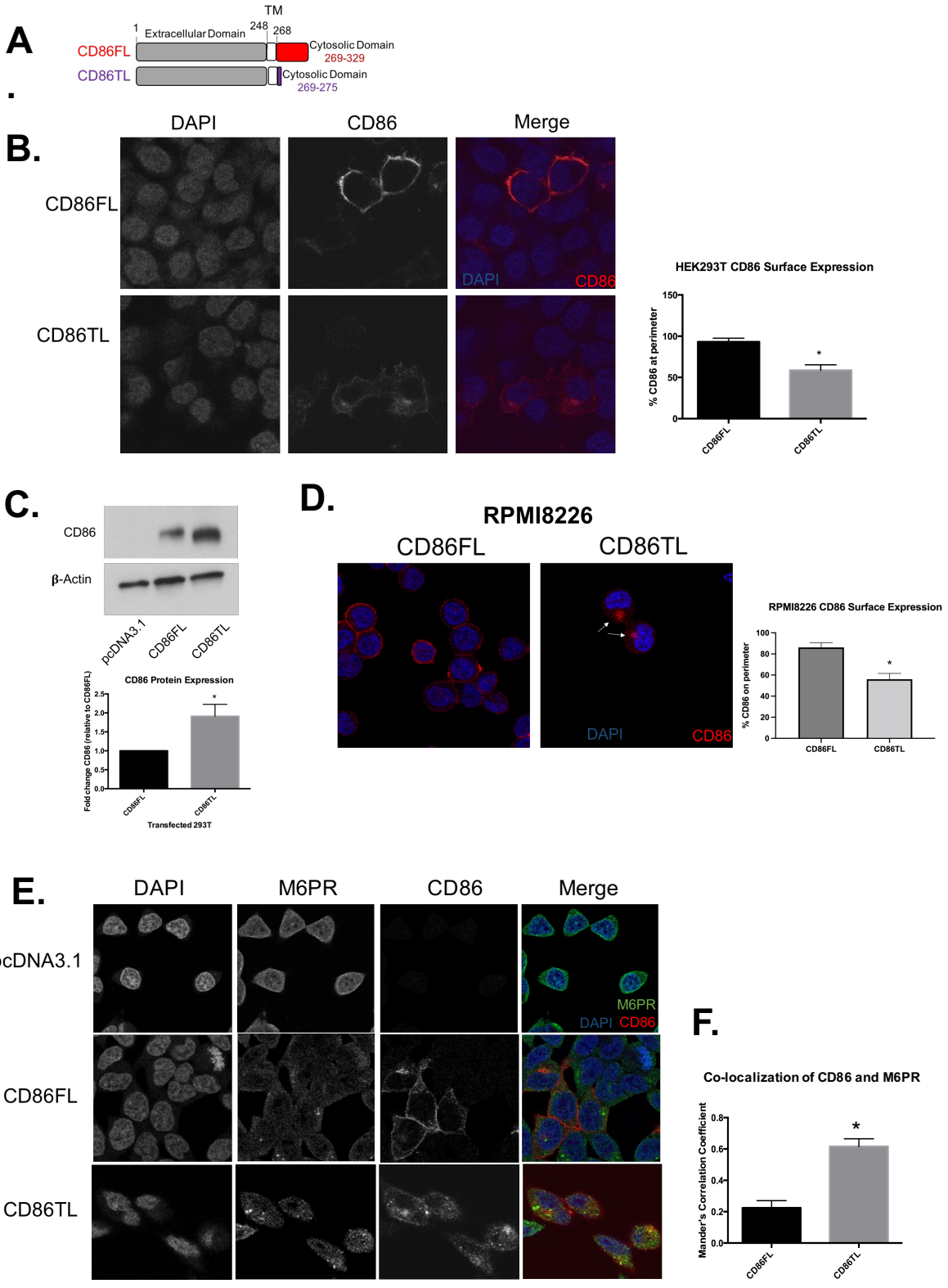

A.
TM
1
Extracellular Domain
248
268
CD86FL
Cytosolic Domain
269-329
CD86TL
Cytosolic Domain
269-275
B.
DAPI
CD86
Merge
CD86FL
CD86TL
DAPI
CD86
HEK293T CD86 Surface Expression
% CD86 at perimeter
150
100
50
0
*
CD86FL
CD86TL
C.
CD86
β-Actin
pcDNA3.1
CD86FL
CD86TL
CD86 Protein Expression
Fold change CD86 (relative to CD86FL)
2.5
2.0
1.5
1.0
0.5
0.0
*
CD86FL
CD86TL
Transfected 293T
D.
RPMI8226
CD86FL
CD86TL
DAPI
CD86
RPMI8226 CD86 Surface Expression
% CD86 on perimeter
100
80
60
40
20
0
*
CD86FL
CD86TL
E.
DAPI
M6PR
CD86
Merge
pcDNA3.1
CD86FL
CD86TL
M6PR
DAPI
CD86
F.
Co-localization of CD86 and M6PR
Mander's Correlation Coefficient
0.8
0.6
0.4
0.2
0.0
*
CD86FL
CD86TL

Figure 1: CD86 cytoplasmic tail is important for trafficking to cell surface (A) Diagram of full length CD86 (CD86FL) and a "tail-less" CD86 (CD86TL) which lacks all but 7 amino acids of the cytosolic domain. TM refers to transmembrane domain. (B) Confocal microscopy analysis of HEK293T cells transfected for 48h with either CD86FL or CD86TL constructs. In merge images, blue staining denotes DAPI nuclear staining, and red staining denotes CD86. Quantification of percentage of CD86 at the perimeter of the cells shown on right. Data quantification are representative of 15 individual cells taken from 3 independent experiments. (C) Protein lysates of HEK293T transfected with either empty vector pcDNA3.1, CD86FL or CD86TL were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting using anti-CD86 and anti **β**-actin antibodies. Quantification of CD86 expression in CD86FL and CD86TL transfected HEK293T. CD86 protein expression was normalized to **β**-actin expression and then normalized to CD86FL protein expression. N=3. (D) Confocal microscopy of RPMI8226 lines stably overexpressing either full-length (CD86FL) or tail-less CD86 (CD86TL). Blue staining denotes DAPI nuclear staining while red staining denotes CD86. Arrows refer to regions of punctate CD86 expression in CD86TL. Quantification of percentage of CD86 at the perimeter of the cells shown on right. Data representative of 3 independent experiments. (E) HEK293T cells transfected for 48h with either pcDNA3.1, CD86FL or CD86TL constructs and co-stained with CD86 and Mannose-6 Phosphate Receptor (M6PR). In merge images, blue staining denotes DAPI nuclear staining, green staining denotes M6PR, and red staining denotes CD86. (F) Mander's Correlation Coefficient was used to determine co-localization of M6PR

with CD86 in CD86FL and CD86TL HEK293T cells. This reports findings from 15 individual cells taken from 3 independent experiments

*$p<0.05$

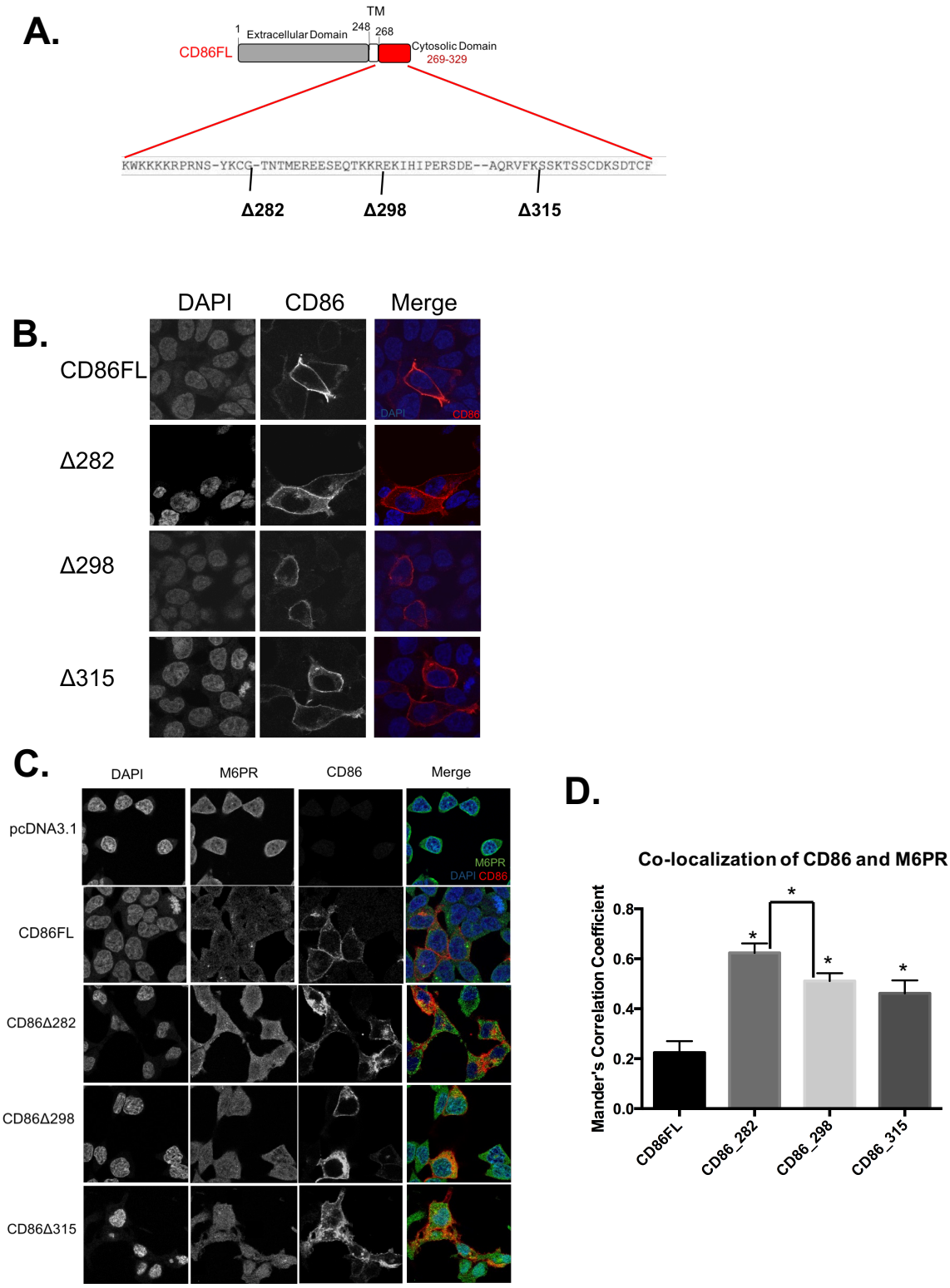


Figure 2: Multiple Regions of CD86 cytoplasmic tail are important for trafficking to cell surface (A) Diagram of CD86 cytoplasmic region and truncation mutants. Δ282

refers to truncated CD86 containing 282 amino acids. Δ298 and Δ315 refer to truncated CD86 containing 298 and 315 amino acids respectively. (B) Confocal microscopy analysis of HEK293T cells transfected for 48h with either CD86FL, Δ282, Δ298, or Δ315 constructs. In merge images, blue staining denotes DAPI nuclear staining, and red staining denotes CD86. Data are representative of 3 independent experiments. (C) HEK293T cells transfected for 48h with either empty vector pcDNA3.1, CD86FL or CD86 truncation mutant constructs were co-stained with CD86 and M6PR. In merge images, blue staining denotes DAPI nuclear staining, green staining denotes M6PR, and red staining denotes CD86. (D) Mander's Correlation Coefficient was used to determine co-localization of M6PR with CD86 in CD86FL, CD86Δ282, CD86Δ298, and CD86Δ315 HEK293T cells. The graph represents results from 15 individual cells analyzed from 3 independent experiments.

*$p<0.05$

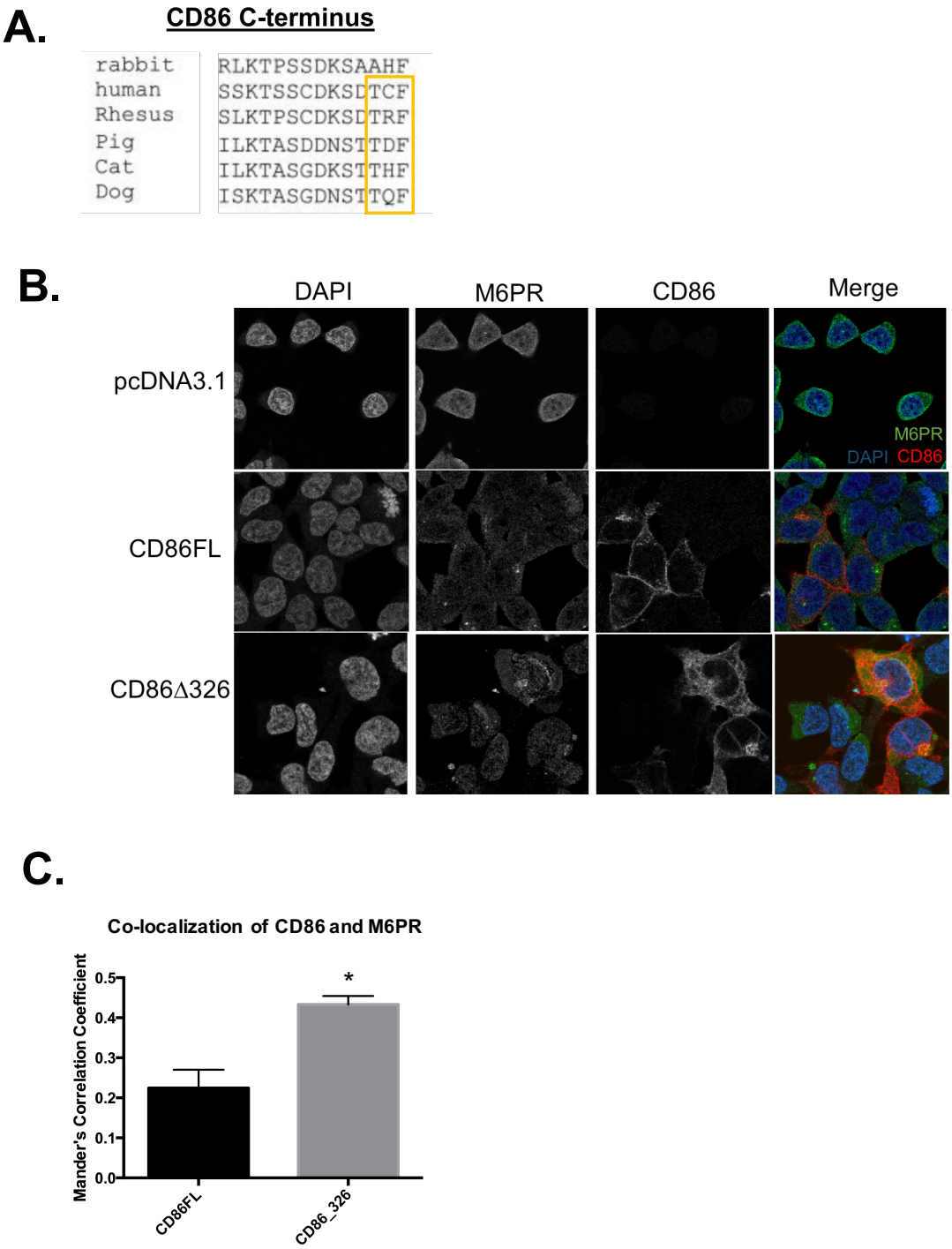
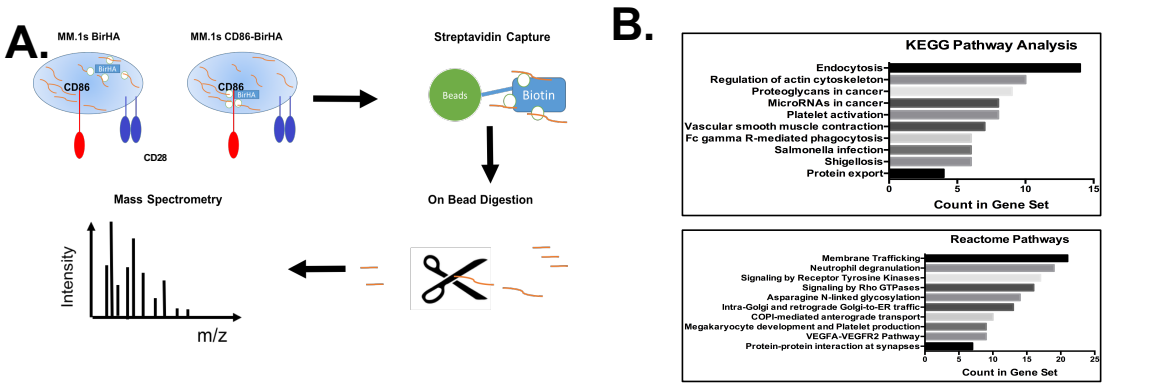
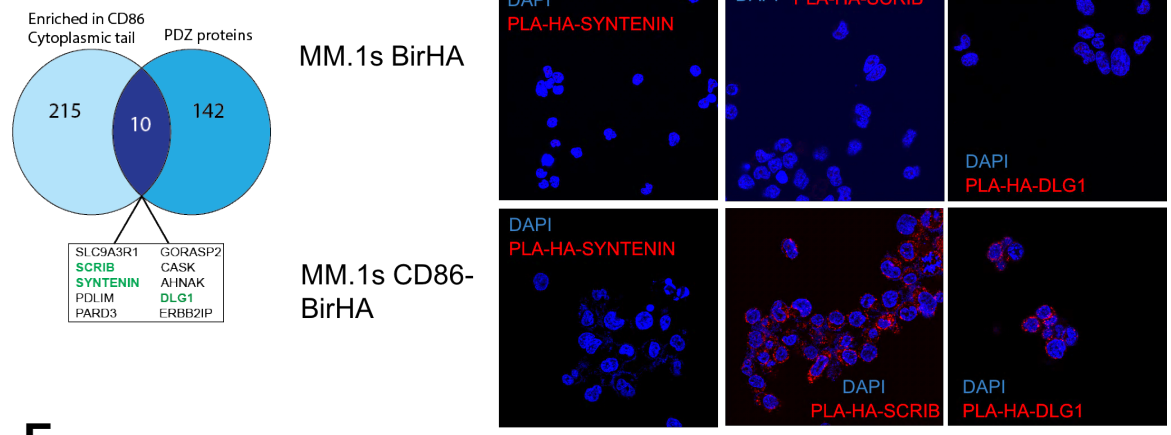


Figure 3: CD86 contains a PDZ binding motif important for surface expression (A) Alignment of the 14 amino acids at the C-terminus of CD86. Highlighted is the conserved Class I PDZ binding domain. (B) HEK293T cells transfected for 48h with

either empty vector pcDNA3.1, CD86FL or CD86 truncation mutant constructs lacking the PDZ binding motif (CD86Δ326) were co-stained with CD86 and Mannose-6 Phosphate Receptor (M6PR). In merge images, blue staining denotes DAPI nuclear staining, green staining denotes M6PR, and red staining denotes CD86. (C) Mander's Correlation Coefficient was used to determine co-localization of M6PR with CD86 in CD86FL and CD86Δ326 HEK293T cells. The graph represents results from 15 individual cells analyzed from 3 independent experiments.

*$p<0.05$


A.
MM.1s BirHA
MM.1s CD86-BirHA
Streptavidin Capture
CD86
CD28
Beads
Biotin
Mass Spectrometry
On Bead Digestion
Intensity
m/z
B.
KEGG Pathway Analysis
Endocytosis
Regulation of actin cytoskeleton
Proteoglycans in cancer
MicroRNAs in cancer
Platelet activation
Vascular smooth muscle contraction
Fc gamma R-mediated phagocytosis
Salmonella infection
Shigellosis
Protein export
Count in Gene Set
Reactome Pathways
Membrane Trafficking
Neutrophil degranulation
Signaling by Receptor Tyrosine Kinases
Signaling by Rho GTPases
Asparagine N-linked glycosylation
Intra-Golgi and retrograde Golgi-to-ER traffic
COPI-mediated anterograde transport
Megakaryocyte development and Platelet production
VEGFA-VEGFR2 Pathway
Protein-protein interaction at synapses
Count in Gene Set


C.
Enriched in CD86 Cytoplasmic tail
PDZ proteins
215
10
142
SLC9A3R1
SCRIB
SYNTENIN
PDLIM
PARD3
GORASP2
CASK
AHNAK
DLG1
ERBB2IP
D.
MM.1s BirHA
MM.1s CD86-BirHA
DAPI
PLA-HA-SYNTENIN
PLA-HA-SCRIB
PLA-HA-DLG1

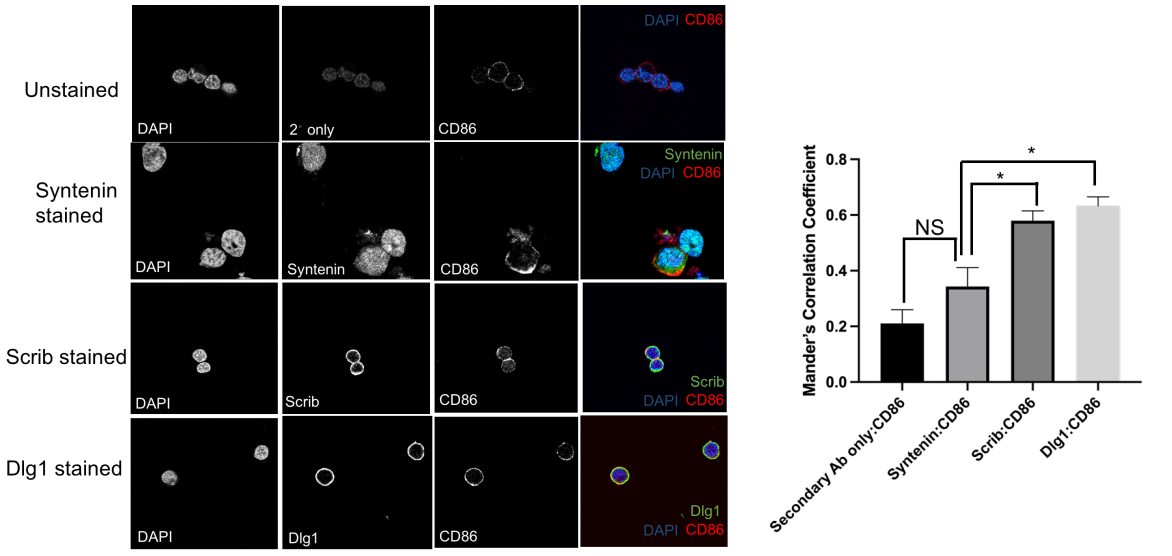

E.
RPMI8226-CD86FL
Unstained
Syntenin stained
Scrib stained
Dlg1 stained
DAPI
2° only
CD86
DAPI CD86
Syntenin
Scrib
Dlg1
F.
Mander's Correlation Coefficient
NS
*
*
Secondary Ab only:CD86
Syntenin:CD86
Scrib:CD86
Dlg1:CD86

Figure 4: BioID proximity assay identifies numerous CD86 cytoplasmic tail interacting partners (A) Schematic of study design: MM.1s cells stably expressing soluble BirHA control or CD86-BirHA were cultured with 50μM biotin, lysed, and quantified. Biotinylated proteins were then captured using equal amounts of streptavidin beads. Peptides were generated via on bead digestion, and these resulting peptides were then purified and analyzed by LC-MS. (B) Gene ontology KEGG pathway analysis and Reactome Pathway analysis of the high confidence CD86 cyotplasmic tail binding partners. (C) Venn Diagram of CD86 cytoplasmic tail enriched proteins and PDZ-domain proteins determined from the HUGO nomenclature database. (D) Interaction between SYNTENIN, SCRIB, or DLG1 with CD86 (MM.1s CD86-BirHA) or soluble BirHA control (MM.1s BirHA) were analyzed by PLA. Blue staining denotes DAPI nuclear staining, red staining denotes PLA staining. (E) RPMI8226 cells stably overexpressing CD86FL (RPMI8226-CD86FL) were co-stained with CD86 and either SYNTENIN, SCRIB, DLG1 or a secondary antibody only control. In merge images, blue staining denotes DAPI nuclear staining, green staining denotes SYNTENIN/SCRIB/DLG1, and red staining denotes CD86. Data are representative of 3 independent experiments. (F) Mander's Correlation Coefficient was used to determine co-localization of SYNTENIN/SCRIB/DLG1 with CD86 in RPMI8226-CD86FL as well as cells stained only with a secondary antibody. The graph represents results from 15 individual cells analyzed from 3 independent experiments.

*$p<0.05$

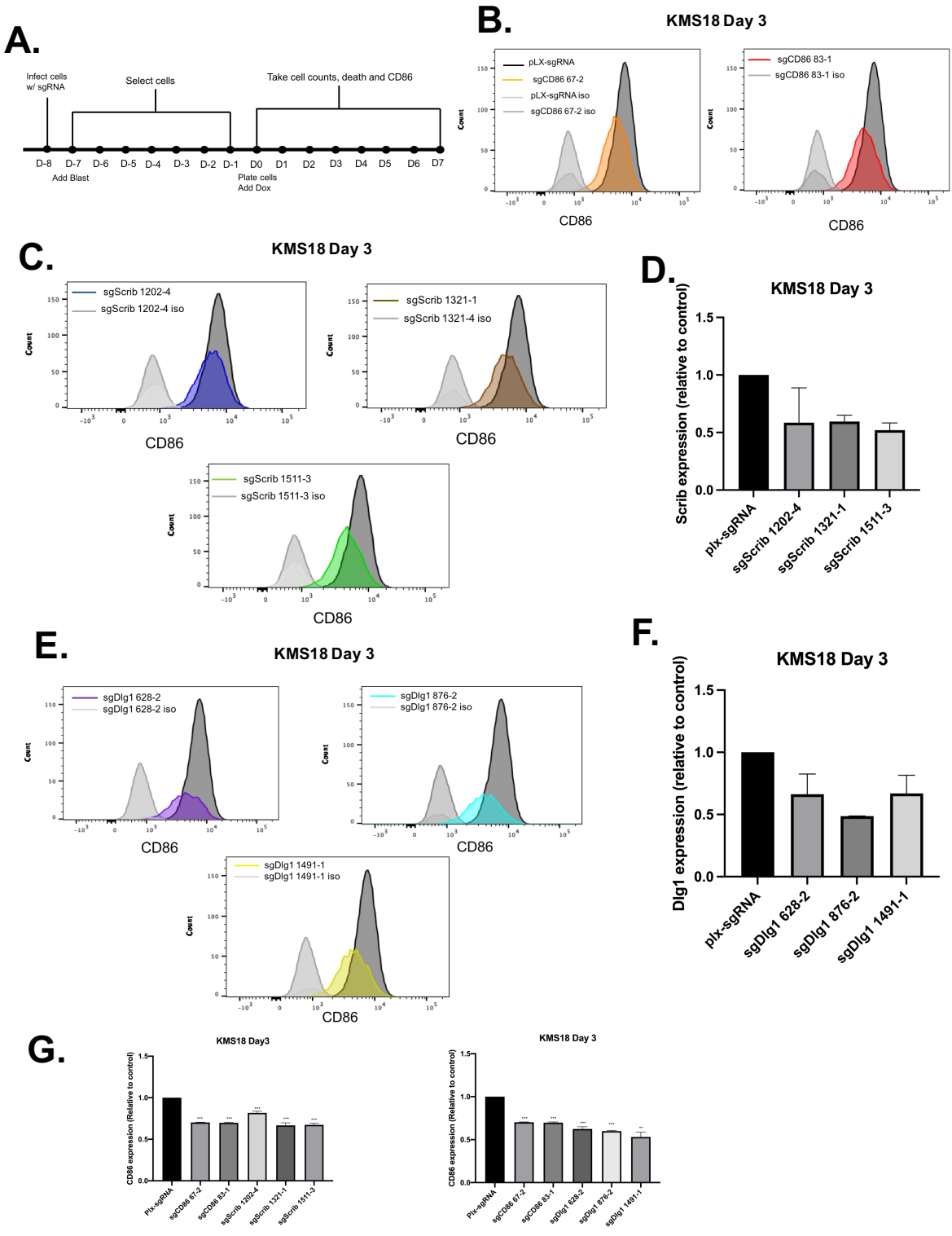


Figure 5: SCRIB and DLG1 regulate CD86 surface expression (A) Study design: Doxycycline-inducible Cas9 containing KMS18 or RPMI8226 myeloma cells were

infected with lentiviral particles containing CRISPR single guide RNA (sgRNA) targetting coding regions in SCRIB (1202-4, 1321-1, 1511-3), DLG1 (628-2, 876-2, 1491-1) or a control sgRNA targetting the viral incorporation site AAVS1 (plx-sgRNA). The next day, blasticidin was added to select cells for seven days. Following this, doxycycline was added to activate the Cas9 enzyme and cell counts, cell death, and CD86 expression were measured from days 0-7. (B,C,E) Representative histograms showing CD86 surface expression was measured via flow cytometry three days after doxycycline addition for (*B)* sgCD86*,* (*C*) *sgSCRIB*, and (*E)* sgDLG1 KMS18 cells. (D,F) mRNA expression for plx-sgRNA and (*D*) SCRIB and (*F)* DLG1 sgRNA guides were measured using quantitative reverse transcription PCR (qRT-PCR) 3 days after doxycycline addition in KMS18 cell lines. (G) Quantification of CD86 surface expression for 3 independent experiments 3 days after doxycycline addition in plx-sgRNA, sgCD86, sgSCRIB (left) and sgDLG1 (right) in KMS18 myeloma cell line. N=3

*p<0.05

**p<0.01

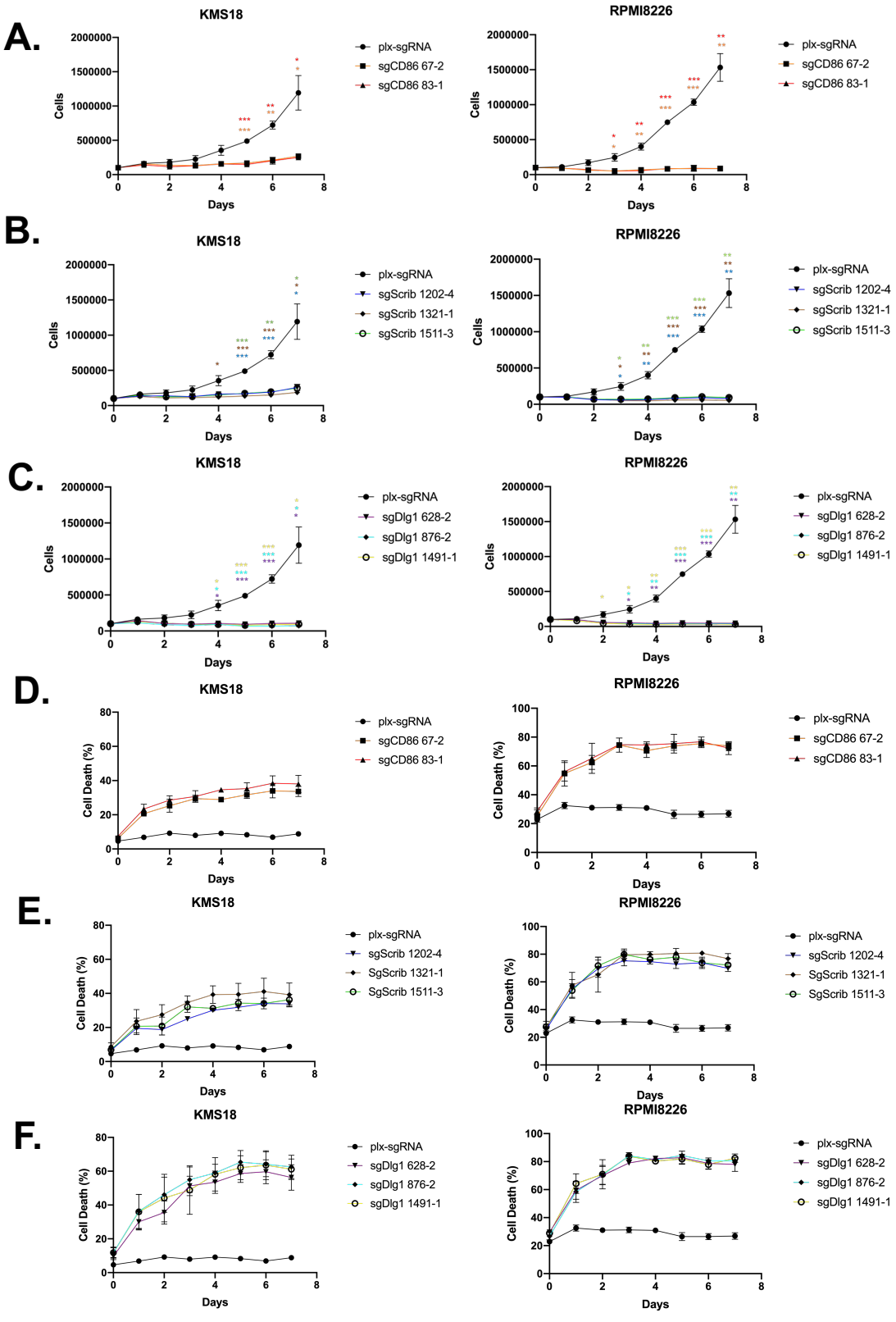

A.
KMS18
RPMI8226
Cells
Days
plx-sgRNA
sgCD86 67-2
sgCD86 83-1
B.
KMS18
RPMI8226
Cells
Days
plx-sgRNA
sgScrib 1202-4
sgScrib 1321-1
sgScrib 1511-3
C.
KMS18
RPMI8226
Cells
Days
plx-sgRNA
sgDlg1 628-2
sgDlg1 876-2
sgDlg1 1491-1
D.
KMS18
RPMI8226
Cell Death (%)
Days
plx-sgRNA
sgCD86 67-2
sgCD86 83-1
E.
KMS18
RPMI8226
Cell Death (%)
Days
plx-sgRNA
sgScrib 1202-4
SgScrib 1321-1
SgScrib 1511-3
F.
KMS18
RPMI8226
Cell Death (%)
Days
plx-sgRNA
sgDlg1 628-2
sgDlg1 876-2
sgDlg1 1491-1

Figure 6: SCRIB and DLG1 are important for cell growth and viability (A-C) Doxycycline inducible Cas9 containing KMS18 or RPMI8226 myeloma cell lines were infected with lentiviral particles containing CRISPR single guide RNA (sgRNA) targetting exons in CD86 (67-2, 83-1) SCRIB (1202-4, 1321-1, 1511-3), DLG1 (628-2, 876-2, 1491-1) or a control sgRNA targetting the viral incorporation site AAVS1 (plx-sgRNA). After blasticidin selection for seven days, doxycycline was added and cell counts were assessed for Day 0-7. (A) Counts of plx-sgRNA control cells with sgCD86. (B) Counts of plx-sgRNA control cells with sgSCRIB. (C) Counts of plx-sgRNA control cells with sgDLG1 cells. (D-F) Annexin V–fluorescein isothiocyanate and propidium iodide staining staining was used to assess cell viability in KMS18 and RPMI8226 cell lines receiving either plx-sgRNA, sgCD86, sgSCRIB, or sgDLG1 guides for day 0-7 following doxycycline treatment. (D) Cell death of plx-sgRNA control cells with sgCD86 cells. (E) Cell death of plx-sgRNA control cells with sgSCRIB cells. (F) Cell death of plx-sgRNA with sgDLG1 cells. N=3

*$p<0.05$

**$p<0.01$

***$p<0.001$

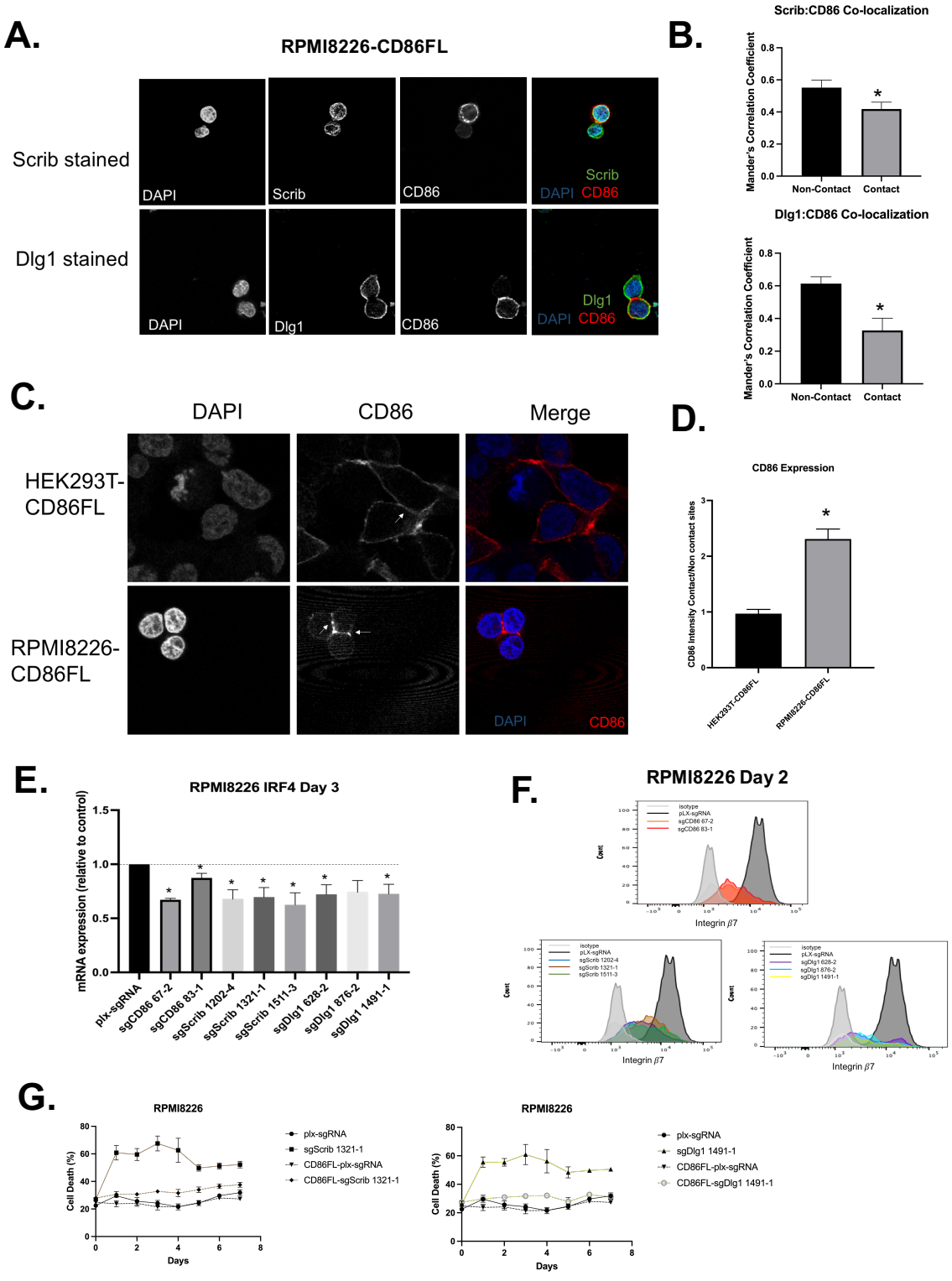
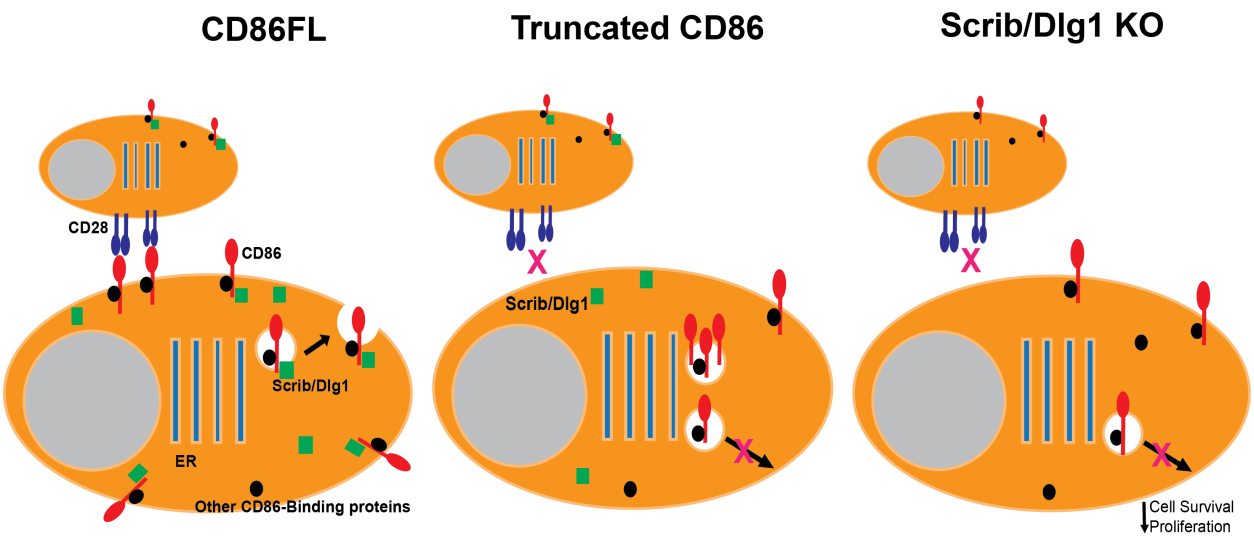


Figure 7: SCRIB and DLG1 regulate CD86 prosurvival signaling (A) RPMI8226-CD86FL cells were co-stained with CD86 and either SCRIB (top) or DLG1 (bottom). In

merge images, blue staining denotes DAPI nuclear staining, green staining denotes SCRIB/DLG1, and red staining denotes CD86. (B) Mander's Correlation Coefficient was used to determine co-localization of SCRIB/DLG1 with CD86 in areas of cell-cell contact compared to areas of no contact between adjacent RPMI8226 cells. The graph represents results from 15 individual cells analyzed from 3 independent experiments. (C) Confocal microscopy analysis of HEK293T cells transfected for 48h with CD86FL and RPMI8226 cells stably overexpressing CD86FL. In merge images, blue staining denotes DAPI nuclear staining and red staining denotes CD86. (D) Ratio of relative intensity of CD86 staining was determined between areas of cell contact and non-contact between adjacent CD86FL-transfected HEK293T and RPMI8226-CD86FL cells. The graph represents results from 15 individual cells analyzed from 3 independent experiments. (E) mRNA expression of IRF4 for plx-sgRNA, CD86, SCRIB, and DLG1 sgRNA guides were measured using quantitative reverse transcription PCR (qRT-PCR) 3 days after doxycycline addition in RPMI8226 cell lines. N=3. (F) Surface Integrin $\beta 7$ levels were measured in RPMI8226 for sgCD86 (left), sgSCRIB (center), and sgDLG1 (right) 2 days after doxycycline addition. Data are representative of 3 independent experiments. (G) Cell viability in RPMI8226 or CD86FL-overexpressing RPMI8226 cell lines receiving either plx-sgRNA, sgSCRIB, or sgDLG1 guides for day 0-7 following doxycycline treatment. N=3

*$p<0.05$


CD86FL
Truncated CD86
Scrib/Dlg1 KO
CD28
CD86
Scrib/Dlg1
ER
Other CD86-Binding proteins
Scrib/Dlg1
Cell Survival
Proliferation

Supplemental Methods

Analysis of RNA-seq from CoMMpass

RNA-seq data from the Clinical Outcomes in Multiple Myeloma to Personal Assessment (CoMMpass) study (NCT01454297) was performed similarly to previously described[11]. Briefly, FPKM normalized read counts were downloaded from the Genomic Data Commons and analyzed in R / Bioconductor (v3.6.3). Gene expression subtypes were determined by consensus clustering and based on those defined by Zhan et al[2]. Analysis of outcome used a cox-proportional hazards analysis from the R 'survival' package (v3.2-11) and outcome data from CoMMpass IA17.

Biotin labeling in pulse-chase experiments

CD86FL and CD86TL cleavage and recovery experiments were conducted as previously described[3]. Briefly, Lenti-X 293T cells (Takara Bio) were grown to confluence in 6-well culture plates (Corning). Cells were trypsinized (Corning) for ~8 min and suspended. After the indicated refractory period, surface proteins were biotinylated using PBS containing 0.5 mg/ml EX-Link Sulfo-NHS-SS-Biotin (Thermo Scientific) for 30 min at 4°C. Unbound biotin was quenched in PBS containing 50 mM Tris pH 8.0 for 1 min. Cells were washed with PBS, lysed in RIPA buffer, and incubated for 15 min on ice. Lysate was cleared via centrifugation at 16,000 × *g* at 4°C for 10 min. Biotinylated protein was captured on streptavidin-coated beads (Invitrogen) during overnight incubation at 4°C. Beads were collected via centrifugation at 2500 × *g* at 4°C for 2 min. Protein was released from beads using Laemmli buffer containing 5% β-mercaptoethanol for 5 min at 95°C. Eluates were run on SDS-PAGE gel and western

blot analysis was conducted using Mouse α CD86 (R&D) and Mouse α E-Cadherin (BD Biosciences).

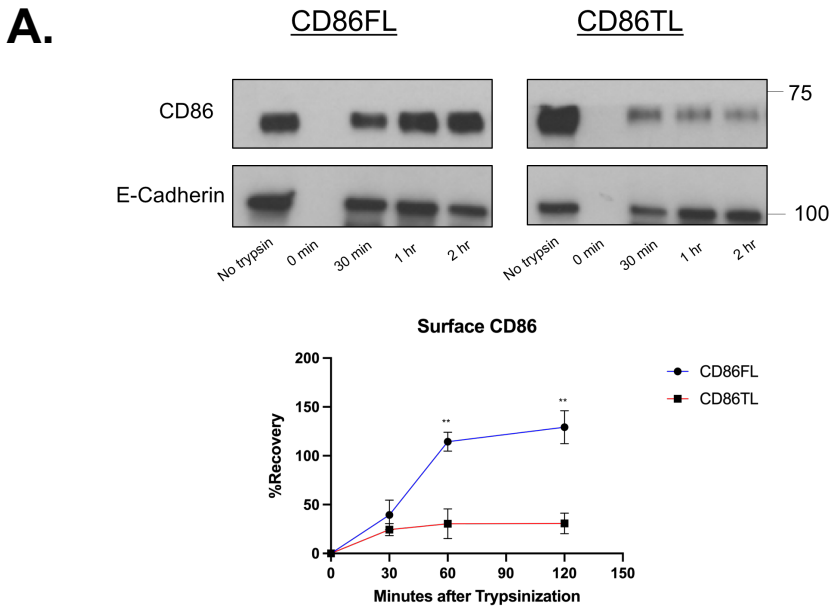
Supplemental Tables/Figures

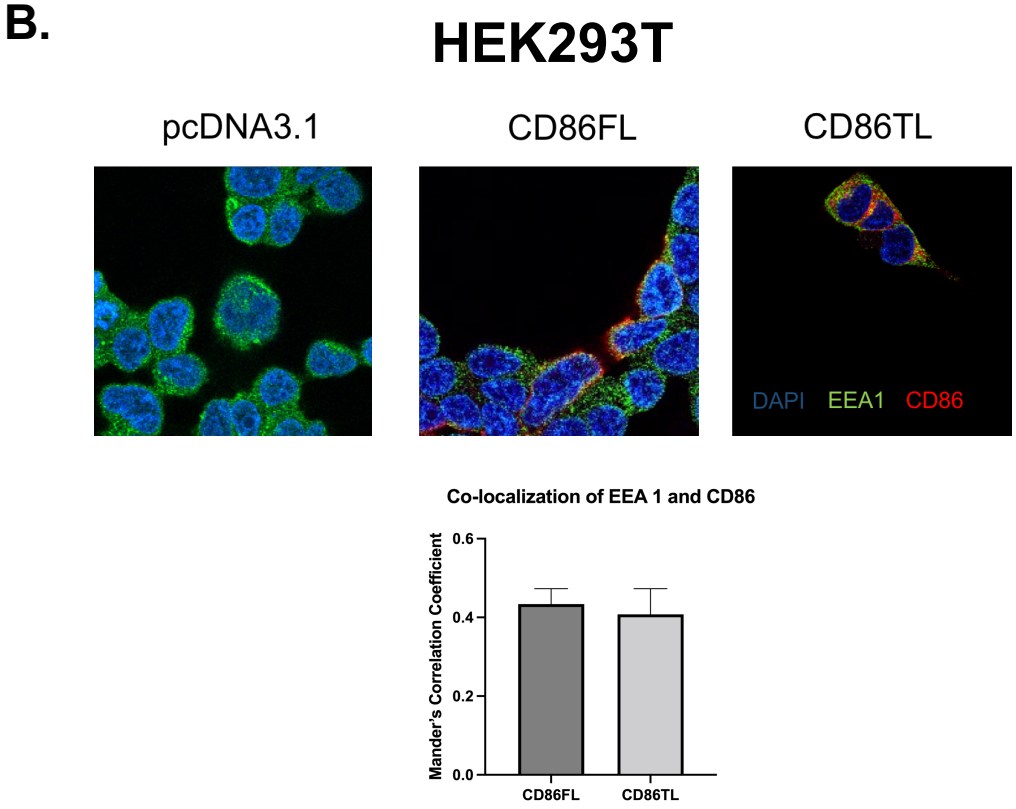
Table S1

CRISPR Guide	sgRNA Sequence
CD86 67-2	GTAACCGTGTATAGATGAGC
CD86 83-1	TTGACCTGCTCATCTATACA
SYNTENIN 42-1	CAGAGCTCTCTCAATACATG
SYNTENIN 108-1	TTTCTGGTGCACCACTTCAG
SCRIB 1202-4	CATGCTCCGGTTCCAGACGG
SCRIB 1321-1	ATGACGCTGACGCGGCTCGG
SCRIB 1511-3	TGGGTATGGCCCTTCCAACG
DLG1 628-2	GTCTGTGCCATTAACGTAAGT
DLG1 876-2	TATTGTACAGGGGGATACGC
DLG1 1491-1	GGGCTAACATGGTTATCAAC

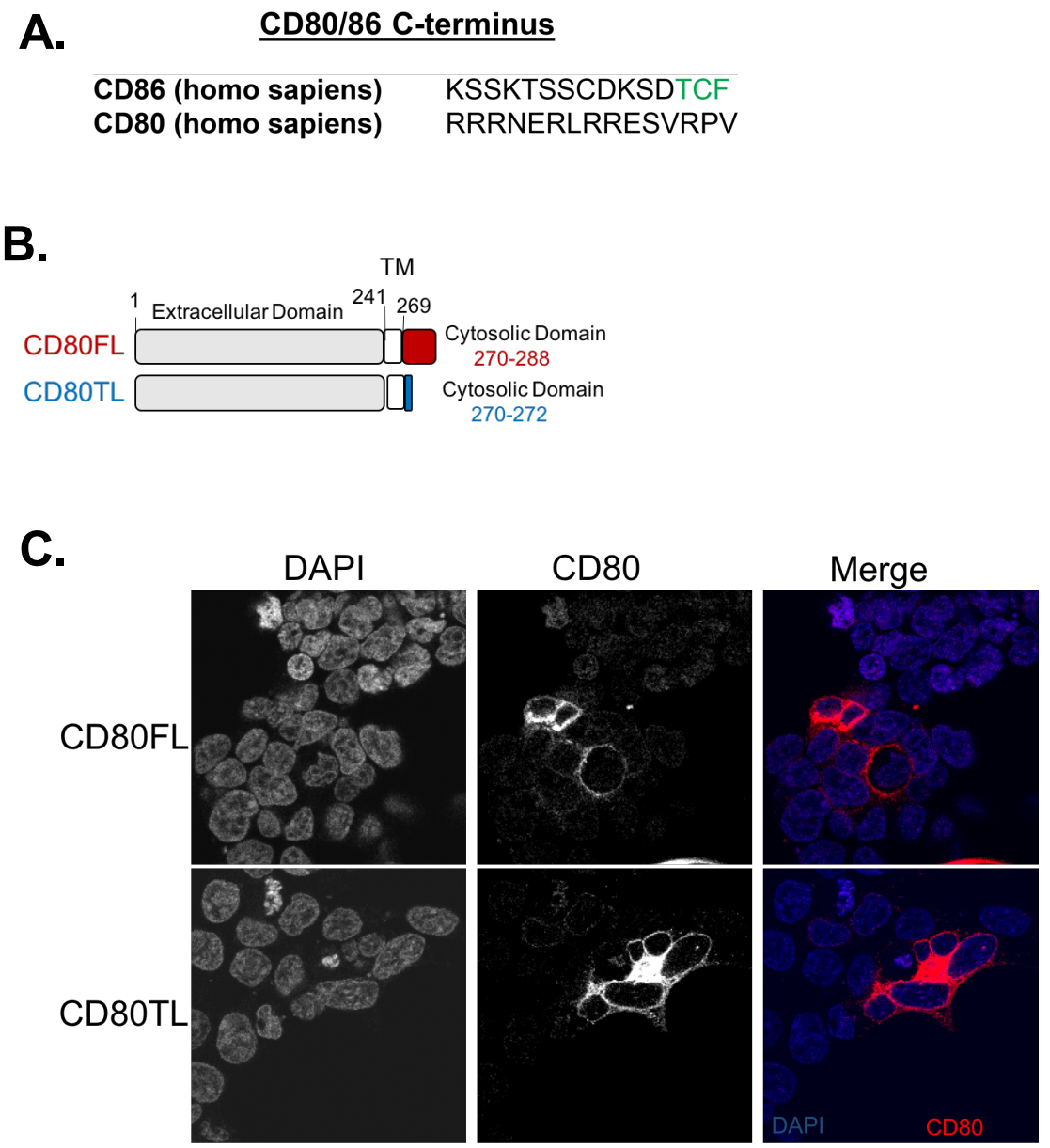
Table S2

In attached excel file


A.
CD86FL
CD86TL
CD86
E-Cadherin
75
100
No trypsin
0 min
30 min
1 hr
2 hr
Surface CD86
%Recovery
Minutes after Trypsinization
CD86FL
CD86TL

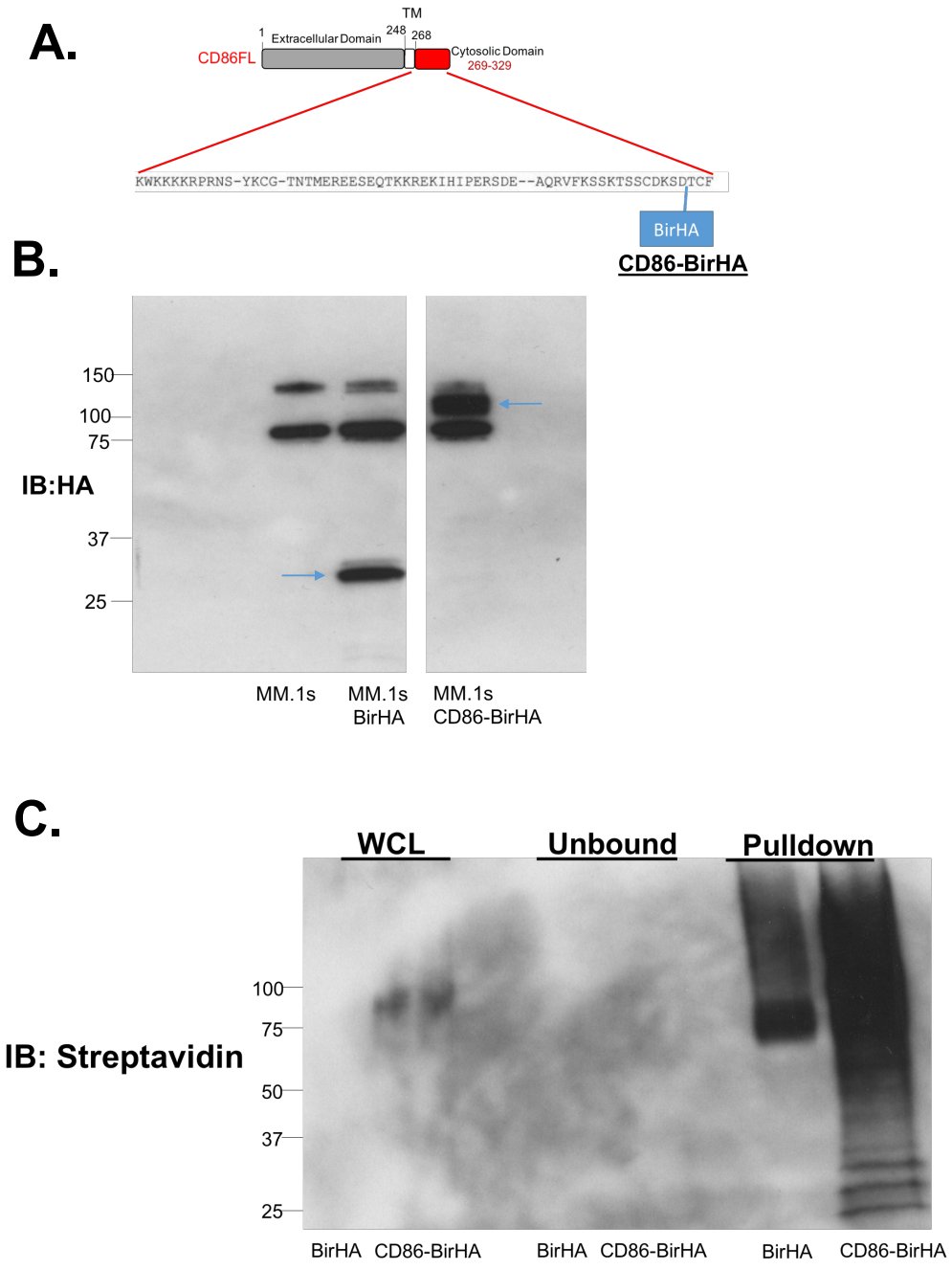

B.
HEK293T
pcDNA3.1
CD86FL
CD86TL
DAPI
EEA1
CD86
Co-localization of EEA 1 and CD86
Mander's Correlation Coefficient
CD86FL
CD86TL

Supplementary Figure 1 (A) Cell surface proteins were cleaved using trypsin at t=0. Trypsin was removed and cells were incubated for either 0 min, 30 min, 1 hour, or 2 hours. The amount of CD86 was assayed using biotin labeling and capture using streptavidin beads and Western Blot analysis. Quantification using densitometry analysis of the ratio of CD86 to E-Cadherin surface protein control shown below representative Western blot. N=3 (B) HEK293T cells transfected for 48h with either empty vector pcDNA3.1, CD86FL or CD86 truncation mutant constructs were co-stained with CD86 and endosomal marker Early Endosome Antigen 1(EEA1). In merge images, blue staining denotes DAPI nuclear staining, green staining denotes EEA1, and red staining denotes CD86. Data are representative of 2 independent experiments.



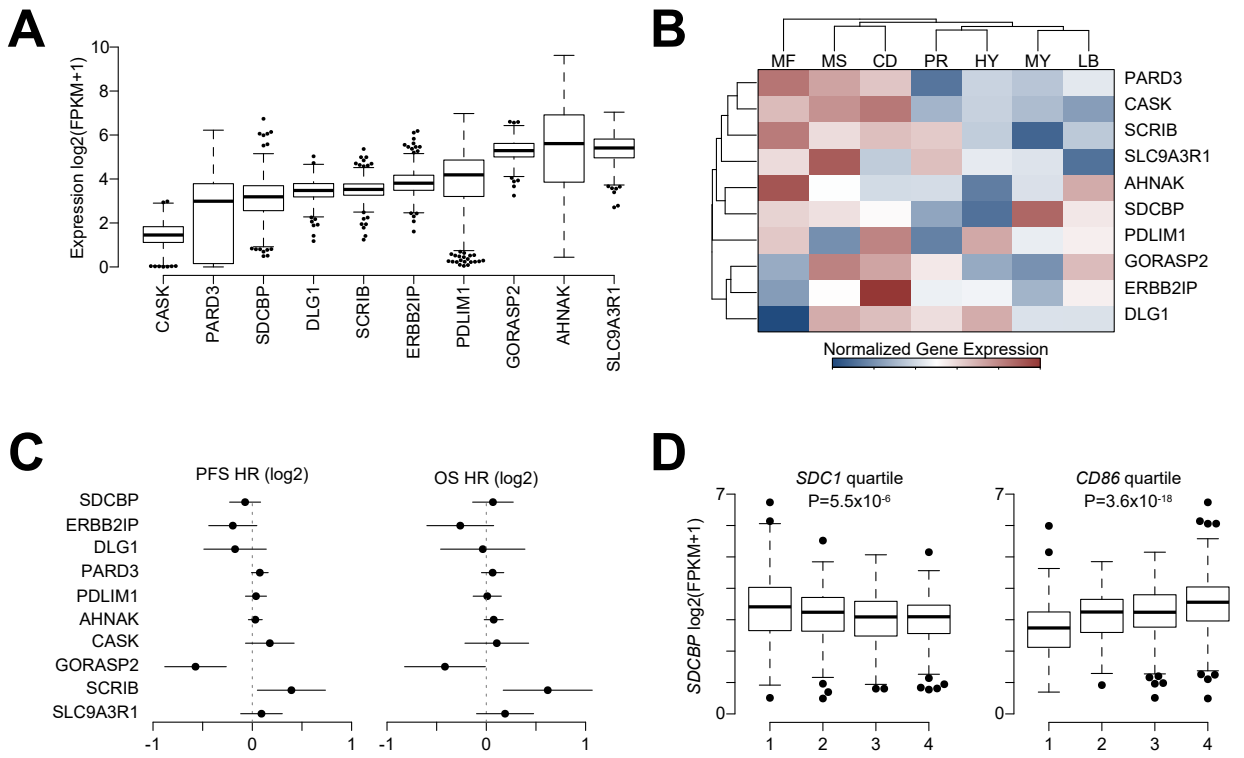
Supplementary Figure 2: (A) Alignment of C termini human CD86 and human. Green text refers to PDZ binding motif in CD86. (B) Diagram of full length CD80 (CD80FL) and a “tail-less” CD80 (CD80TL) which lacks all but 3 amino acids of the cytosolic domain. TM refers to the transmembrane domain. (C) Confocal microscopy analysis of HEK293T cells transfected for 48h with either CD80FL or CD80TL constructs. In merge

images, blue staining denotes DAPI nuclear staining, and red staining denotes CD80. Data are representative of 2 independent experiments.

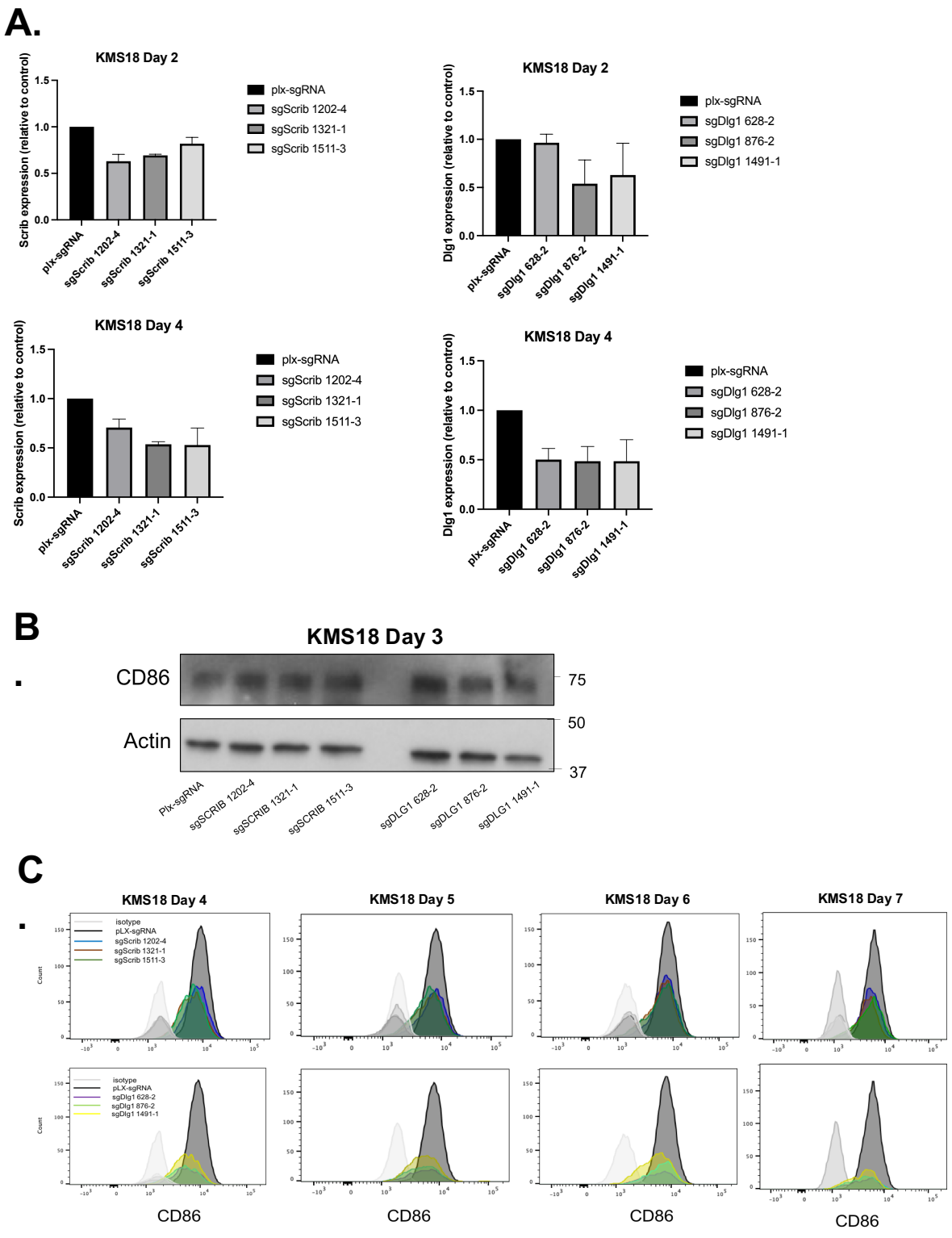


Supplementary Figure 3: (A) Model of CD86-BirHA construct with BirHA cloned just prior to PDZ binding motif. (B) Western blot analysis of MM.1s (left) and MM.1s lines stably expressing either a soluble BirHA construct (MM.1s BirHA) or the CD86-BirHA construct (MM.1s CD86-BirHA). Probe with HA antibody reveals a soluble BirHA

(MW=35 kD) in center lane and overexpressed CD86 containing a BirHA in the far right lane (MW CD86-BirHA≈110 kD). Bands at 75 and 130 kD represent non-specific protein. (C) Verification that biotinylated protein in MM.1s BirHA and MM.1s CD86 BirHA were pulled down using streptavidin beads. Western blot was performed for whole cell lysates (left) unbound fraction (center) and a fraction of bead eluates (right). Measurement of biotinylated protein using streptavidin HRP is shown.

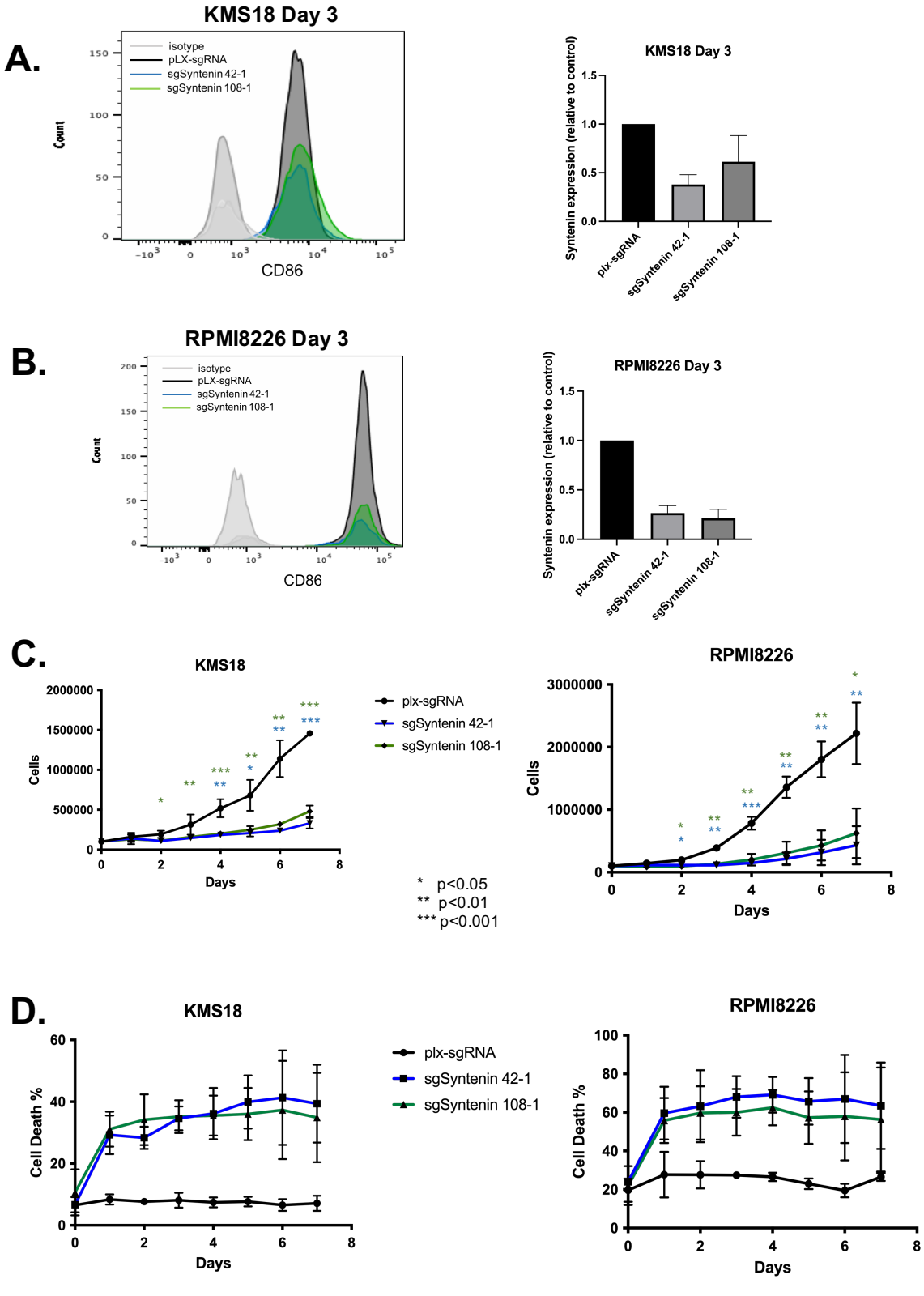


Supplementary Figure 4: (A) Expression of 10 genes identified by BioID that encode PDZ domain containing proteins in 644 primary myeloma samples from newly diagnosed patients as part of the CoMMpass trial. (B) Heatmap of gene expression summarized by gene expression subtype in CoMMpass patients. MF: MAF, MS: MMSET, CD: Cyclin D, PR: Proliferation, HY: Hyperdiploid, MY: Myeloid, LB: Low Bone Disease. Data are Log2(FPKM+1) and z-score normalized for each gene. (C) Hazard ratio (HR) of gene expression associated with progression-free survival (PFS; left) and overall survival (OS, right). Error bars represent 95% confidence interval. (D) Expression of SDCBP (encodes SYNTENIN) stratified by SDC1 (encodes CD138) expression quartile (left) or CD86 quartile (right). P-values indicate association of SDCBP expression with SDC1 and CD86 as determined by analysis of variance.

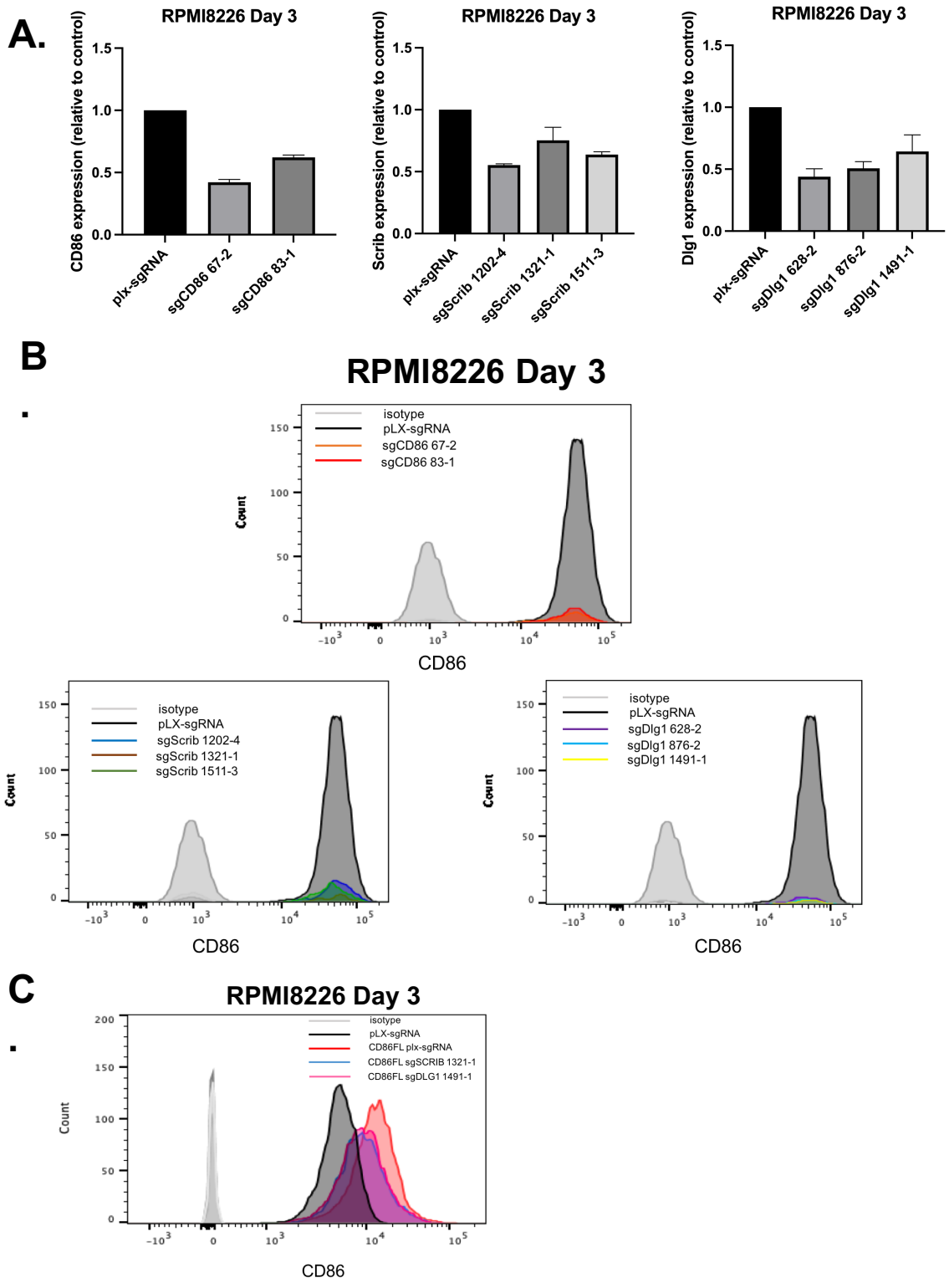


Supplementary Figure 5: (A) mRNA expression for plx-sgRNA and SCRIB (left) or DLG1 (right) sgRNA guides were measured using quantitative reverse transcription

PCR (qRT-PCR) 2 and 4 days after doxycycline addition in KMS18 cell lines. (B) Protein lysates of KMS18 cell lines 3 days following doxycycline addition were subjected to (SDS-PAGE) followed by western blotting using anti-CD86 and anti **β**-actin antibodies. (C) Representative histograms showing CD86 surface expression was measured via flow cytometry four to seven days after doxycycline addition for sgSCRIB (top) and sgDLG1 (bottom) KMS18 cells.


A.
KMS18 Day 3
isotype
pLX-sgRNA
sgSyntenin 42-1
sgSyntenin 108-1
Count
CD86
KMS18 Day 3
Syntenin expression (relative to control)
plx-sgRNA
sgSyntenin 42-1
sgSyntenin 108-1
B.
RPMI8226 Day 3
RPMI8226 Day 3
C.
KMS18
RPMI8226
Cells
Days
plx-sgRNA
sgSyntenin 42-1
sgSyntenin 108-1
* p<0.05
** p<0.01
*** p<0.001
D.
KMS18
RPMI8226
Cell Death %
Days

Supplementary Figure 6: (A) CD86 expression in KMS18 cell lines 3 days after doxycycline addition (left). Black curves denote plx-sgRNA while blue and green represent sgSYNTENIN lines (42-1, 108-1). mRNA expression (right) for plx-sgRNA and SYNTENIN sgRNA guides were measured using quantitative reverse transcription PCR (qRT-PCR) 3 days after doxycycline addition in KMS18 cell lines. (B) CD86 expression in RPMI8226 cell lines 3 days after doxycycline addition (left). mRNA expression for plx-sgRNA and SYNTENIN sgRNA guides were measured using quantitative reverse transcription PCR (qRT-PCR) 3 days after doxycycline addition in RPMI8226 cell lines (right). N=3. (C-D) Doxycycline inducible Cas9 containing KMS18 or RPMI8226 myeloma cell lines were infected with lentiviral particles containing CRISPR single guide RNA (sgRNA) targetting exons in SYNTENIN (42-1, 108-1) or plx-sgRNA. After blasticidin selection for seven days, doxycycline was added and cell counts and cell death were assessed for Day 0-7. (C) Counts of plx-sgRNA control cells and sgSYNTENIN in KMS18 and RPMI8226. (D) Cell death of plx-sgRNA control cells and sgSYNTENIN in KMS18 and RPMI8226. N=3



Supplementary Figure 7: (A) mRNA expression for plx-sgRNA and CD86, (*left*) SCRIB (middle) or DLG1 (*right)* sgRNA guides were measured using quantitative reverse

transcription PCR (qRT-PCR) 3 days after doxycycline addition in RPMI8226 cell lines. N=3 (B) CD86 expression in RPMI8226 cell lines 3 days after doxycycline addition. CD86 guides shown on top. SCRIB guides shown on bottom-left and DLG1 guides shown on the bottom-right. Data are representative of 3 independent experiments. (C) Representative CD86 expression in RPMI8226 parental and RPMI-CD86FL cell lines 3 days after doxycycline addition.

Supplemental Bibliography

1. Barwick, B. G. *et al.* Multiple myeloma immunoglobulin lambda translocations portend poor prognosis. *Nat Commun* **10**, 1911 (2019).
2. Zhan, F. *et al.* The molecular classification of multiple myeloma. *Blood* **108**, 2020–2028 (2006).
3. Lewis, J. D. *et al.* The desmosome is a mesoscale lipid raft-like membrane domain. *Mol Biol Cell* **30**, 1390–1405 (2019).

Chapter 3

Differential expression of CD28 interacting partners CD86 and ICOS-L induces molecular changes in myeloma cells

Tyler Moser-Katz, Catherine M. Gavile, and Lawrence H. Boise

This work is unpublished

Introduction:

While molecular changes induced by the interaction between CD28 on T cells and CD86 on APCs have been well characterized, downstream effects of their interaction in normal and malignant plasma cells have been largely unstudied. A large subset of myeloma cells express both CD28 and CD86[1,2]. This suggests a means in which myeloma cells can exist outside of the bone marrow niche and even a possible autocrine signaling mechanism between the two proteins on the same cell. While we have shown that interaction between these two proteins facilitates myeloma cell survival, cell growth, and adhesion (**Chapter 2**)[3,4], other functional outcomes may result from this interaction.

We have shown a localized increase in CD86 expression at sites of contact in myeloma cells. CD86 expression, however, did not change at contact sites in HEK293T, a cell line which does not express CD28 supporting a possible role for stabilization of CD86 via CD28 binding (**Chapter 2**). CD86 confers pro-survival signaling and contains numerous important sites on its cytoplasmic tail including a PDZ binding motif at its C-terminus. We previously identified SYNTENIN as an important PDZ domain protein expressed in myeloma patients and cell lines. SYNTENIN is an adapter protein that is highly involved in intracellular trafficking and has been

shown to regulate surface levels of the myeloma marker CD138[5,6]. While SYNTENIN mRNA expression correlates with CD86 in patients, SYNTENIN does not regulate CD86 surface expression in myeloma cell lines. However, SYNTENIN is necessary for myeloma cell survival and proliferation, indicating a possible unexplored mechanism for myeloma progression.

T cell co-stimulation may also occur via binding of inducible T-cell costimulator (ICOS) on an activated T cell with ICOS-ligand (ICOS-L) of B-cells or monocytes[7]. While ICOS-L only binds to ICOS in mice, it has been shown in human T cells to also be a ligand for CD28, inducing a co-stimulatory response and upregulation of Bcl-xL[8]. ICOS-L is expressed on myeloma cells and can stimulate proliferation and inhibit anti-tumor immune response[9]. However, how ICOS-L induces these functional changes is not known. These studies reveal a possible regulation of SYNTENIN levels through CD28 signaling and also implicate ICOS-L as a secondary binding partner for CD28 in the absence of CD86 in myeloma cells.

Materials and Methods:

Cell Lines

Human Embryonic Kidney 293T (HEK293T) cell line was purchased from American Type Culture Collection (ATCC). Cells were cultured and seeded

in 6-well plates containing 2 mL Dulbecco's modified Eagle's medium (DMEM; Corning) supplemented with 10% fetal bovine serum (Gemini) at 37 °C in an incubator with 5% CO_2. MM.1s were provided by Dr. Steven Rosen (City of Hope), RPMI8226 were purchased from ATCC. Stable RPMI8226 CD86-overexpressing cell lines were generated as previously described[3]. Cells were cultured in RPMI1640 media supplemented with 10% fetal bovine serum at 37 °C in an incubator with 5% CO_2.

Lentiviral shRNA preparation and infection

Short hairpin RNA (shRNA) clones were obtained from Open Biosystems and Sigma Aldrich. Viral particles were prepared and myeloma cells were infected as previously described[10]. Clones listed in Supplemental Table 1.

Protein Extraction and Immunoblotting

Cell pellets were lysed in radioimmunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitors as previously described[11]. Lysates were quantified using the BCA assay (ThermoFisher), and lysates were run in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels, then blotted as previously described[11]. Primary antibodies used included: rabbit α-β-actin (Sigma) and mouse monoclonal α-SYNTENIN

(Abcam). The following secondary antibodies were used: anti-mouse immunoglobulin G–horseradish peroxidase (IgG-HRP) and anti-rabbit IgG-HRP (Santa Cruz Biotechnology).

Real Time Polymerase Chain Reaction (RT-PCR)

RNA was extracted and quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed as previously described using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies)[3]. Resulting cDNA was amplified using the TaqMan Gene Expression Master Mix (Life Technologies) on the CFX96 Real-Time PCR System following the manufacturer's protocol (Bio-Rad). Probes used were Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), SYNTENIN, and CD86 (Applied Biosystems).

Flow Cytometry and Analysis

Cell-surface expression CD86-APC (Caprico Biosciences) and CD275-PacBlue (BD Biosciences) were measured via flow cytometry. 100,000 live cells were collected, washed with 1× PBS, and stained with appropriate antibodies in 50 µL FACS staining buffer. After incubation of 15 min at 4°C in the dark, cells were washed in 1× PBS and resuspended in 400 µL FACS

staining buffer. Samples were run on a FACS Symphony A3 Flow Cytometer (BD Biosciences) and analyzed using FlowJo software.

Statistical Analysis

Statistical significance was assessed using two-tailed Student *t* test using GraphPad Prism.

Results:

We have previously observed that CD86 mRNA expression in patients correlates with SYNTENIN expression (**Chapter 2**). We observed SYNTENIN mRNA levels in stable RPMI8226 myeloma cell lines which overexpress either full length CD86 (CD86FL) or a tail-less mutant of CD86 (CD86TL)[3] with the hypothesis that increased CD86 expression would result in an increase in SYNTENIN transcription. Indeed, we noticed an increase in SYNTENIN transcription in both the CD86FL and CD86TL lines compared to our empty vector controls (**Fig 1A**). To determine whether SYNTENIN protein levels correlate with increased CD86, we took lysates of the cells and performed Western blot analysis (**Fig 1B**). Similarly, there was increased expression of SYNTENIN in CD86FL and CD86TL lines (**Fig 1C**). This suggests that expression of CD86 extracellular domain correlates with

SYNTENIN levels, and CD28 signaling may be a mechanism for SYNTENIN expression in myeloma.

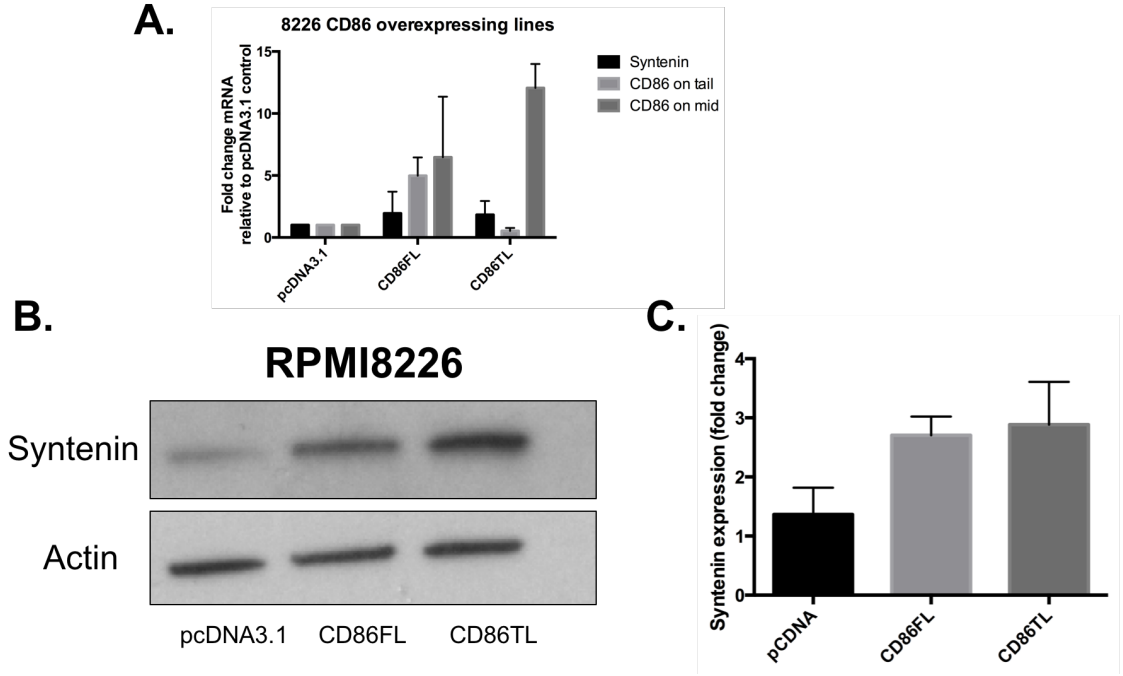


Figure 1: Overexpression of CD86FL and TL results in increased syntenin expression. (A.) SYNTENIN mRNA expression for pcDNA3.1, CD86FL, and CD86TL RPMI8226 lines (shown in black) were measured using quantitative reverse transcription PCR (qRT-PCR). Also shown is measurement of CD86 mRNA using two separate targets for either the transmembrane domain (CD86 on mid) or the cytoplasmic tail of CD86 (CD86 on tail). N=3 (B.) Protein lysates of RPMI8226 stably expressing either empty vector pcDNA3.1, CD86FL or CD86TL were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting using anti-SYNTENIN and anti **β**-actin antibodies. (C.) Quantification of SYNTENIN total protein expression in cell lines.

SYNTENIN protein levels were normalized to **β**-actin expression and then normalized to pcDNA3.1 protein levels. N=3.

To verify that SYNTENIN expression decreases with lower CD86 expression, we used shRNA to silence CD86 (shCD86) in the myeloma cell line, MM.1s Interestingly, knockdown of CD86 results in increased expression of SYNTENIN mRNA expression 4 days following lentiviral infection (**Fig 2A**). We observed this same increase in SYNTENIN protein expression 4 days following silencing of CD86 (**Fig 2B-D**). Increase of SYNTENIN in the absence of a CD86 signal could indicate that CD28 is receiving an alternative signal that results in increased SYNTENIN expression.

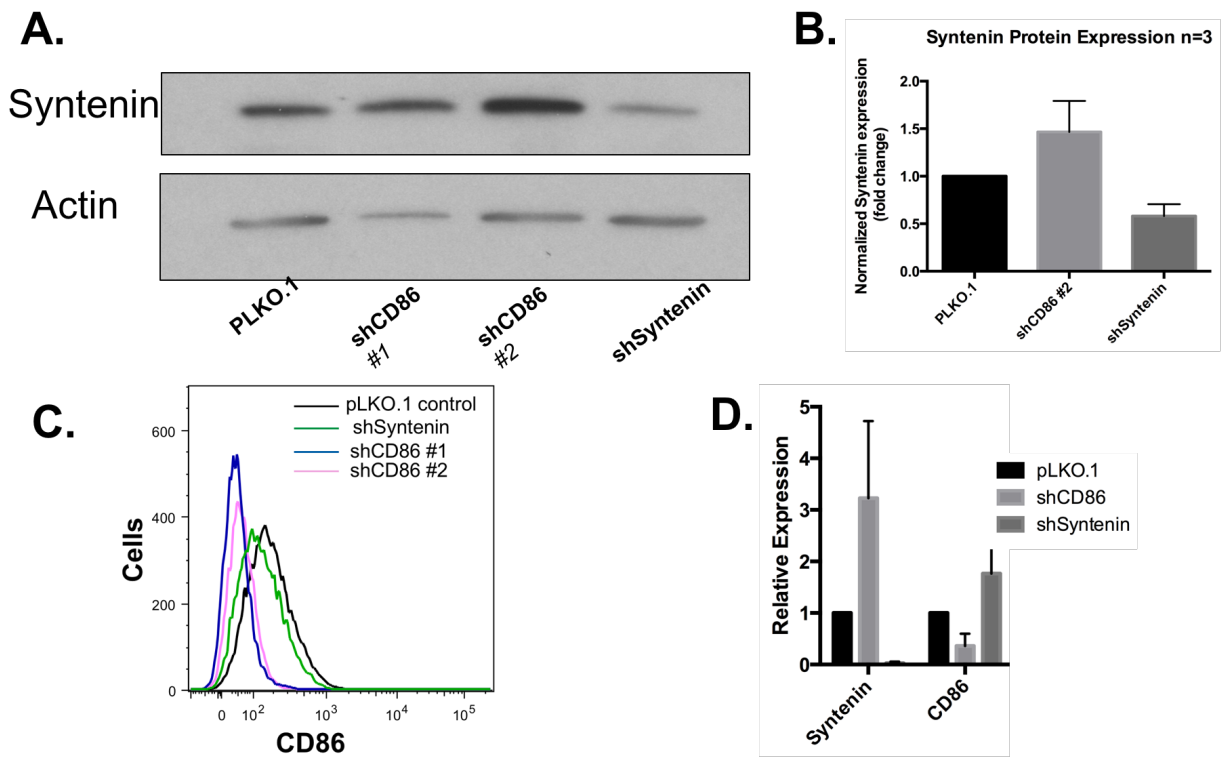


Figure 2: Silencing of CD86 results in increased syntenin expression. (A.) Protein lysates of MM.1s which received short hairpin RNA against CD86 (shCD86), SYNTENIN (shSyntenin) or an empty vector control (PLKO.1) were subjected to SDS-PAGE followed by western blotting using anti-SYNTENIN and anti **β**-actin antibodies. (B.) Quantification of SYNTENIN expression in silenced cells. SYNTENIN total protein expression was normalized to **β**-actin expression and then normalized to plKO.1 protein expression. N=3. (C.) CD86 expression in PLKO.1, shSyntenin (green), and two shCD86 hairpins (blue and pink). (D.) SYNTENIN (left) and CD86 (right) mRNA expression for plKO.1, shCD86, and shSyntenin MM.1s were measured using qRT-PCR. N=3

While myeloma cells do not express the CD28 binding partner CD80, they do express ICOS-L which has also been shown to bind to CD28 in

human T cells[8]. ICOS-L is a B7 family member and shares homology with both CD80 (21%) and CD86 (21%)[12]. Interestingly, unlike CD86, ICOS-L does not contain a PDZ binding motif, suggesting a differential regulation in transport to the plasma membrane in plasma cells and APCs (**Fig 3**).

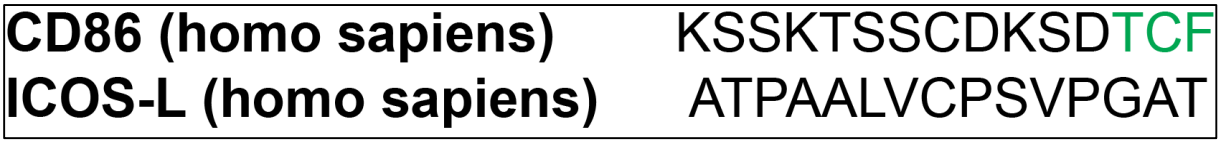


Figure 3: ICOS-L does not contain a PDZ-binding motif. Alignment of the final 15 amino acids at the C-terminus of CD86. PDZ domain of CD86 is highlighted in green.

To determine whether ICOS-L may be involved in CD28 signaling in the absence of CD86, we used flow cytometry to measure surface levels of ICOS-L 3 days after infection with shCD86. We observed an increase in ICOS-L surface level expression following CD86 silencing in both RPMI8226 and MM.1s indicating that myeloma cells may upregulate ICOS-L in the absence of a CD86 signal (**Fig 4A-B,** *blue*). Interestingly, 3 days after silencing of SYNTENIN, we noticed a similar increase in ICOS-L expression (**Fig 4A-B**, *green*).

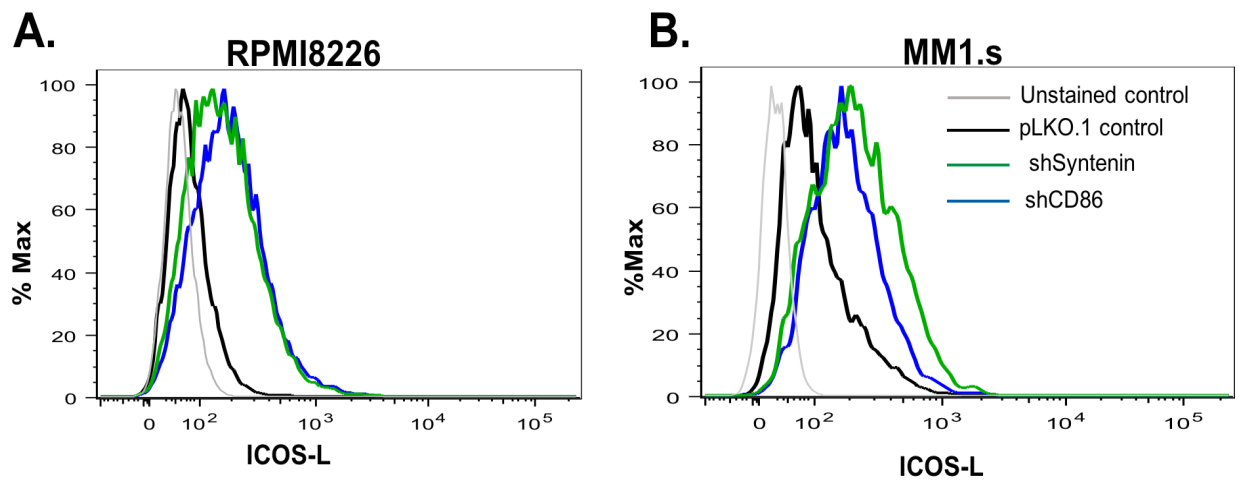


Figure 4: ICOS-L is upregulated with CD86 and SYNTENIN silencing. Representative histograms showing ICOS-L protein surface expression were measured via flow cytometry four days after infection with plKO.1 empty vector control (black) or hairpins against SYNTENIN (green) and CD86 (blue) in (A.) RPMI8226 and (B.) MM.1s. N=3

We next observed the protein surface expression of ICOS-L in the RPMI8226-CD86 overexpressing lines. Strikingly, there is a decrease in ICOS-L surface expression in the RPMI8226-CD86FL line compared to the empty vector pcDNA3.1 control. We also noticed a slight decrease in ICOS-L expression in the RPMI8226-CD86TL line but not to the effect of the CD86FL (**Fig 5**).

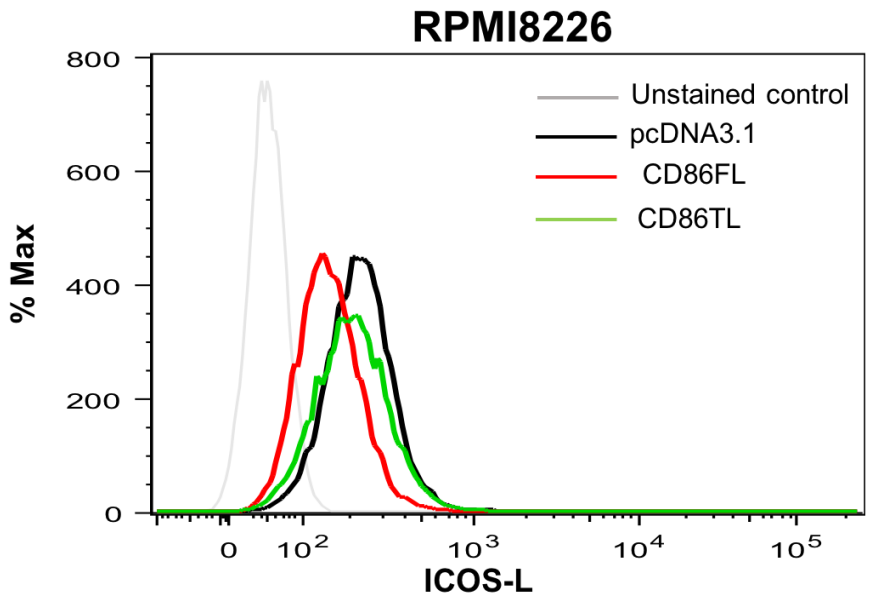


Figure 5: ICOS-L has decreased expression in CD86-overexpressing cell lines. Representative histogram showing ICOS-L surface expression was measured via flow cytometry in RPMI8226 which stably overexpress CD86FL (red), CD86TL (green) or an empty vector pcDNA3.1 control (black). N=3

Discussion:

We have previously shown that SYNTENIN is important for myeloma cell survival, despite not affecting CD86 surface expression. Here, we show that overexpression of the extracellular domain of CD86 results in increased SYNTENIN expression, suggesting a possible role for CD28 signaling in regulating SYNTENIN levels. Consistent with this, increased SYNTENIN levels are shown to be associated with activation of the PI3K/AKT pathway, a known CD28 signaling module[13,14]. While CD28 has also been shown to

activate NFκB, studies diverge as to whether SYNTENIN has a role in in its activation[14,15]. Future studies will be needed to elucidate the mechanism of SYNTENIN on myeloma survival.

We have previously shown that SYNTENIN gene expression in patients positively correlates with that of CD86 (**Chapter 2)**. The higher mRNA and protein expression of SYNTENIN in the CD86FL overexpressing line mirrors this phenotype. However, our data shows that CD86 knockdown in MM.1s also results in an increase of SYNTENIN transcription and protein expression. Since these data were measured at day 4 following knockdown, over half the cells had died, and in the cells that survived, a compensatory mechanism may have generated increased SYNTENIN expression as a means of maintaining pro-survival signaling. We have also previously seen that knockdown of CD86 does not change mRNA or protein expression of CD28[3]. This observation along with the increase in SYNTENIN expression suggest a possible alternative interacting partner to maintain survival signaling in myeloma cells. Since myeloma cells do not express CD80, the other known extracellular binding partner is ICOS-L[8]. The affinity of ICOS-L-CD28 binding compared to CD28-CD86 in myeloma cells remains to be determined. Additionally, ICOS-L binding may induce differential signaling

pathways and molecular changes in myeloma cells such as increased SYNTENIN expression.

The upregulation of ICOS-L in CD86 silenced MM.1s and RPMI8226 verifies a potential competition of binding with CD86 for CD28 interaction. These cell lines are dependent on CD28 signaling for survival and may be preferential toward CD86 as the primary ligand for CD28 activation. Furthermore, ICOS-L is upregulated in SYNTENIN-silenced cells, suggesting that SYNTENIN may have a role in regulating ICOS-L expression in the absence of sufficient CD28 activation. SYNTENIN has been shown to regulate surface expression of proteins by recycling from the cell surface (CD138)[5] or inhibiting their internalization (CD63)[16] and may be regulating ICOS-L surface expression by either of those means. However, this is unlikely as most of SYNTENIN's receptor interacting partners have PDZ-binding motifs while ICOS-L does not. SYNTENIN has also been shown to translocate to the nucleus where it regulates transcription factors and may therefore be affecting ICOS-L expression in this manner[17].

The decrease in ICOS-L surface expression in the CD86-overexpressing lines further verifies the competition these proteins have for surface expression. Interestingly, while there is a slight decrease in ICOS-L expression in the CD86TL line, the CD86FL line had increased reduction of

ICOS-L compared to pcDNA3.1 control and CD86TL. This suggests that both CD28 and CD86 signaling may have roles in regulating ICOS-L expression.

Taken together we have shown that CD28 signaling may affect SYNTENIN expression and, to an extent, ICOS-L expression. Future studies will need to be performed to further elucidate the molecular changes in malignant plasma cells associated with CD28 activation. The potential regulation of ICOS-L by SYNTENIN may also represent an interesting avenue to explore in myeloma cells as well as APCs. Further research will also be needed to assess the effects of silencing or knocking out ICOS-L expression in myeloma cells. The effects of combining knockdown of CD86 with ICOS-L may be have a synergistic effect on cell viability by blocking CD28 activation. Exploration of ICOS-L and its role in CD28-CD86 interaction in myeloma cells could represent an important route for future therapeutic avenues.

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Chapter 4

The Role of CD28 and CD86 in myeloma cell proliferation

Tyler Moser-Katz, Benjamin G. Barwick, and Lawrence H. Boise

This work is unpublished

Introduction:

CD86 is a receptor protein classically known for its role in T cell co-stimulation. During this process, the TCR is first activated by the MHC peptide complex of an APC, and either one of the B7 family proteins, CD80 or CD86, on an APC, binds to T cell CD28 to maximally activate T cell proliferation, and survival[1]. Both CD28 and CD86 are expressed on normal and malignant plasma cells, and we have shown that ablation of either of these proteins results in decreased myeloma cell viability[2–5]. Interestingly, while CD28 appears to have an anti-proliferative on myeloma cells, CD86 has a role in cell growth (**Chapter 2**)[4,6,7].

Myeloma patients who co-express high levels of CD28 and CD86 had significantly worse progression-free and overall survival compared to those who express low amounts of the genes[8]. CD86 expression is more common in the malignant plasma cells compared to those in a precursor stage or non-malignant cells[6]. Furthermore, CD86 on myeloma cells can induce T cells to produce soluble factors such as IL-10 that stimulate myeloma cell proliferation[6].

One means of targeting myeloma cell proliferation is the immunomodulatory Imide Drug (IMiD) Lenalidomide. Lenalidomide was first approved for use in myeloma based on two studies in 2007 demonstrating

its effectiveness in treating relapsed patients[9,10]. It functions by binding the E3 ubiquitin ligase Cereblon to promote the degradation of transcription factors Ikaros and Aiolos[11]. This results in growth arrest of myeloma cells and activation of T cells. Treatment with IMiD also leads to a downregulation of the transcription factor IRF4[12], a phenotype we also observe when CD86 expression is ablated (**Chapter 2**)[8]. Finally, IMiD treatment results in phosphorylation of CD28 and downstream PI3K activation in T cells, suggesting IMiD treatment may affect CD28-mediated signaling in myeloma cells as well[13].

One determinant of IMiD sensitivity in multiple myeloma is the glycoprotein CD98[14]. CD98 is composed of a heavy chain element (SLC3A2) and a light chain element (SLC7A5). Using RNA-seq, we determined that SLC7A5 is significantly downregulated in both CD28 and CD86 silenced myeloma cells. Using BioID analysis, we also found that SLC3A2 was part of the interactome of the CD86 cytoplasmic tail (**Chapter 2**). Taken together, these studies suggest an underexplored role for CD86 in cellular proliferation and a possible molecular target of IMiDs.

Materials and Methods:

Cell Lines

MM.1s were provided by Dr. Steven Rosen (City of Hope), RPMI8226 were purchased from ATCC. Stable RPMI8226 CD86-overexpressing cell lines were generated as previously described[8]. Cells were cultured in RPMI1640 media supplemented with 10% fetal bovine serum at 37 °C in an incubator with 5% CO_2.

Immunofluorescence

Cells were grown on glass coverslips (Fisher) coated with 5 μg/cm^2 fibronectin unless otherwise specified (Millipore). Twenty-four hours after transfection, cells were fixed with PHEMO buffer (68 mM PIPES, 25 mM HEPES, 15 mM EGTA-Na_2, 3 mM $MgCl_2$.$6H_2O$, 10% DMSO, pH 6.8) supplemented with 3.7% formaldehyde (Fisher), 0.05% glutaraldehyde (Fisher) and 0.2% Triton X-100 (Bio-Rad) for permeabilization when indicated. CD86 was labelled by staining with CD86-APC antibody (Caprico Biosciences). Coverslips were then mounted on microslides (Fisher) using Prolong Gold containing 300 nM 4′-6-diamidino-2-phenylindole dilactate (DAPI;Invitrogen). Cells were imaged using a Leica TCS SP8 inverted confocal microscope (63X oil HC PL APO, NA 1.4).

Reagents

Lenalidomide was a gift from Lenny Lechowitz (Cayman Chemicals)

Flow Cytometry and Analysis

Cell-surface expression CD86-APC (Caprico Biosciences) and CD28-PE (BD Biosciences) were measured via flow cytometry. 100,000 live cells were collected, washed with 1× PBS, and stained with appropriate antibodies in 50 µL FACS staining buffer. After incubation of 15 min at 4°C in the dark, cells were washed in 1× PBS and resuspended in 400 µL FACS staining buffer. Samples were run on a FACS Symphony A3 Flow Cytometer (BD Biosciences) and analyzed using FlowJo software.

Results:

We took advantage of RPMI8226-CD86FL a myeloma cell line engineered to stably overexpress CD86 to observe CD86 expression at various stages of the cell cycle. Using immunofluorescence and confocal microscopy, we observed an increase in CD86 expression at the cell surface in dividing cells compared to non-dividing ones (**Fig 1**). This increase appears to take place during the mitotic phase of the cell cycle.

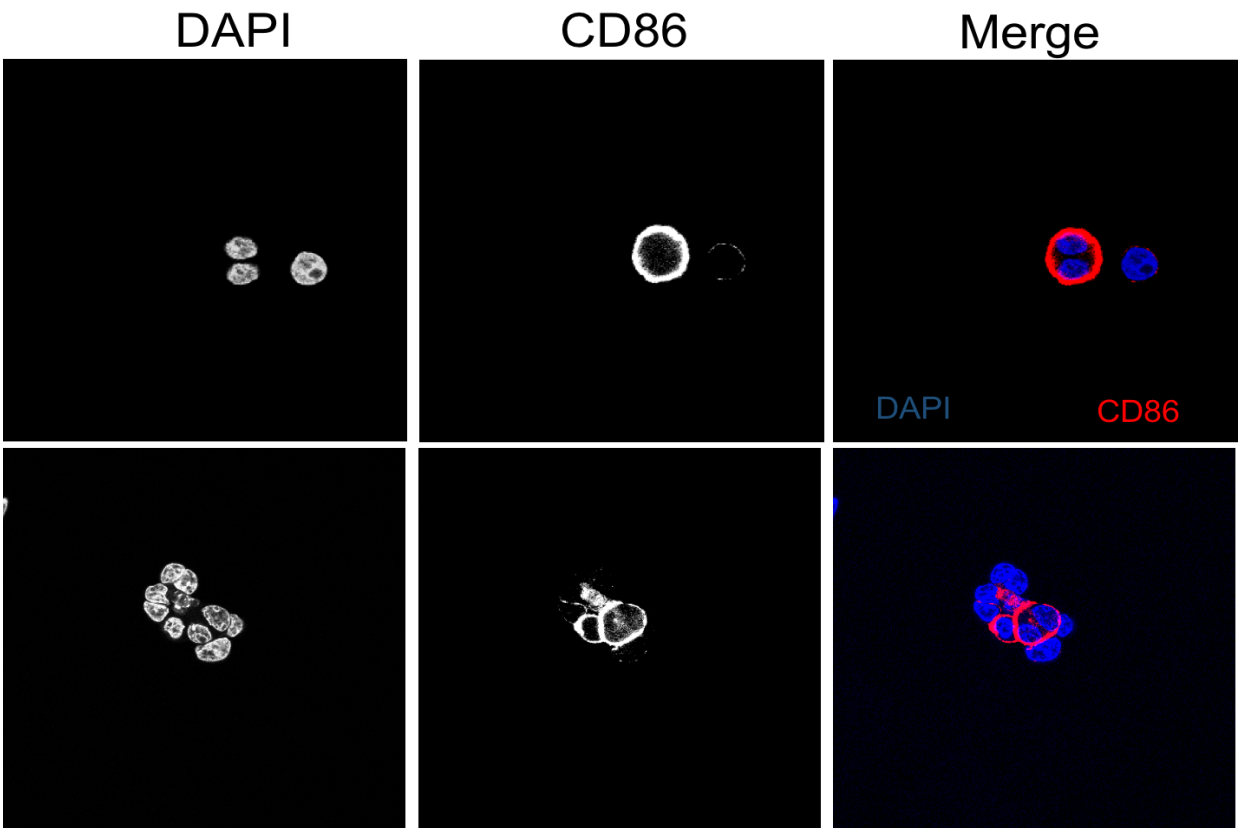

Figure 1: Dividing cells express higher CD86. Confocal microscopy analysis of RPMI8226 cells stably overexpressing CD86FL. In merge images, blue staining denotes DAPI nuclear staining, and red staining denotes CD86. Data are representative of 4 independent cells undergoing division.

This increase in CD86 expression in dividing cells led to hypothesize that inhibiting cell division may result in decreased CD86 expression. We were also interested in the effect of IMiD treatment on CD28 due to research showing that IMiDs can activate CD28-mediated co-stimulation in T cells[13]. We treated the myeloma cell line MM.1s with either 10, 100, 1000, or 10000 nM Lenalidomide and measured CD28 and CD86

expression 3 days following treatment. Interestingly, we noticed a decrease of CD28 and CD86 expression levels in a dose dependent manner compared to our untreated cells (**Fig 2**).

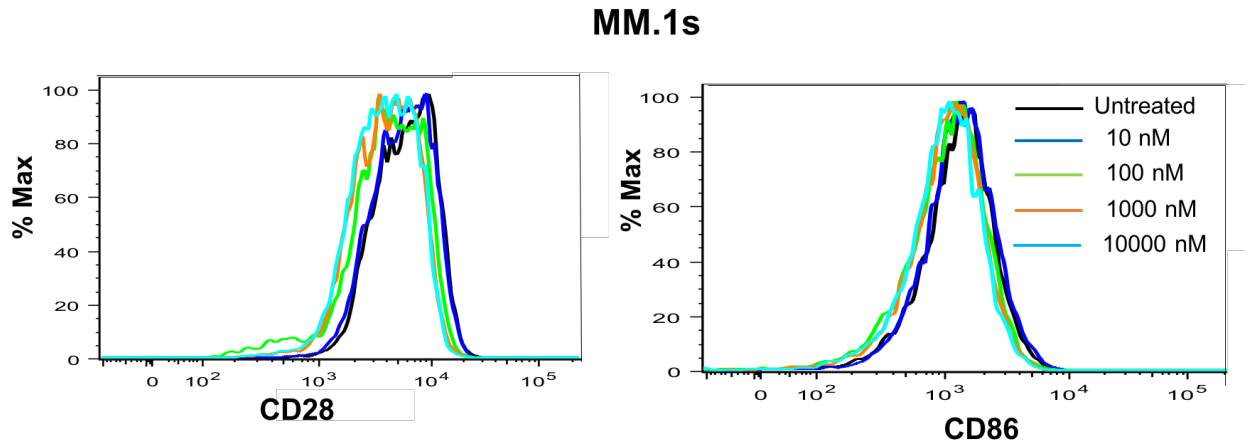

Figure 2: Lenalidomide treatment decreases CD28 and CD86 expression levels. Representative histograms showing CD28 (left) and CD86 (right) surface expression measured via flow cytometry after 3 days of treatment with Lenalidomide in MM.1s. N=2

Discussion:

We have previously demonstrated a role for CD86 in myeloma cell growth (**Chapter 2**). These preliminary data provide a more nuanced role for CD86 in myeloma proliferation. The increased expression of CD86 in actively dividing cells suggests that CD86 expression may also fluctuate during particular phases of the cell cycle. While we have previously shown that overexpression of full-length CD86 protects myeloma cells from chemotherapeutic agents[8], treatment with cyclin-dependent kinase (CDK)

inhibitors may sensitize CD86FL overexpressing cells to therapy[15]. Further research is essential to determine CD86 expression during various stages of the cell cycle.

Treatment of myeloma cells with the IMiD, lenalidomide, has a subtle effect on the expression of both CD28 and CD86 after 3 days. These studies will be continued to 5 and 7 days to test for increased difference in expression. This is consistent with unpublished data from our lab showing that treatment with IMiD eliminated the disparity in prognosis between patients with high or low CD86 expression. Other IMiDs such as pomalidomide will need to be tested as well as the CD38 monoclonal antibody, daratumumab, which has been shown to have immunomodulatory effects[16,17].

These initial studies demonstrate a more specific role for CD86 in myeloma cell proliferation. They point to a potential function for CD86 at specific times during the cell cycle. These data have clinical relevance as they provide a rationale for co-treatment with cell-cycle inhibitors or immunomodulatory drugs with CTLA-4-Ig in multiple myeloma patients. Future studies will need to be conducted to further elucidate the role of CD86 in myeloma cell growth and provide a more effective targeted treatment of the disease.

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Chapter 5

Discussion

A. Summary

Importance of cell-cell contact for myeloma cells

The bone marrow microenvironment provides a multitude of cytokine signaling and receptor-mediated signals which allow normal and malignant plasma cells to survive throughout the lifetime of the host[1]. Mutations in myeloma cells and alterations in the microenvironment during precursor stages promote myeloma proliferation in the bone marrow[2–4]. Myeloma cells can physically interact with BMSC, osteoblast progenitors, and T cells to facilitate growth and survival (CD80/86-CD28, VLA-4-VCAM, LFA1-ICAM, MUC1-ICAM) (**Chapter 1**)[1]. In advanced stages, myeloma cells can forgo signals from cells in the microenvironment and sustain themselves using autocrine cytokine feedback loops and pro-survival cell-cell contact[5–8]. CD28 and CD86 signaling represents the first known example of pro-survival cell-cell contact that occurs in a stroma-independent manner.

Characterization of the CD86 cytoplasmic tail

CD80 and CD86 are members of the B7 family of receptor proteins and were classically thought to have functionally redundant roles as co-stimulators of CD28[9]. Recent studies in immunized mice illustrated that ablation of CD86 but not CD80 resulted in failed class switch of antibody

titers without adjuvant, indicating a different function between these proteins[10]. Studies in human B cells and DCs have also shown downstream antibody and cytokine production following activation of CD86[11,12]. The CD86 cytoplasmic tail is not highly conserved between mice and humans, and since numerous immunological studies are conducted in murine models, the human CD86 cytoplasmic tail represents a largely unstudied region in APCs and plasma cells.

The CD28 cytoplasmic tail has numerous regions for adapter proteins and includes two characterized motifs for tyrosine phosphorylation, the YMNM and PYAP motifs[13]. The downstream signaling pathways of these motifs have been characterized in T cells and plasma cells[13,14]. We previously showed the importance of the CD86 cytoplasmic domain in generating functional downstream results such as drug resistance and molecular changes (IRF4, ITGB1, ITGB7 expression)[8] in multiple myeloma cells. My studies represent the first known classification of a CD86 cytoplasmic motif important for proper myeloma trafficking. I have identified numerous proteins such as SCRIB and DLG1 that may have a role in CD86 signaling and surface expression.

Elucidation of a mechanism of CD86 surface expression

We took advantage of a cell line which does not express CD86 to observe trafficking of exogenous CD86 at early stages of expression. Using truncation mutants of differing length, we were able to discern numerous regions of the cytoplasmic tail that are important for CD86 trafficking. These regions may be sites of interaction for scaffolding proteins and merit further research. Identification of a PDZ-binding motif led us to stratify for candidates that are involved in CD86 surface expression[15,16]. By identifying two such proteins, SCRIB and DLG1, we have gained insight to the nature of CD86 localization. SCRIB and DLG1 have been largely studied in apical-basal polarity, and this points to the importance of proper localization of CD86 to generate viability[17,18].

We observed that CD86 has a higher localized expression at points of contact with other myeloma cells. This is presumably due to its binding with CD28 as we did not observe an increase in binding at contact points in HEK293T. We hypothesize that binding with CD28 stabilizes CD86 and engages it in signaling that facilitates further survival as CD86 accumulates at regions of contact (**Figure 1**). These studies represent the first observation of localized CD86 expression in myeloma cells and suggest a more nuanced explanation to the function of the protein at distinct areas of

the cell. Additional studies are being conducted to observe localization of CD28 in these cells as well as determine the effects of CD28 knockout.

We observed that expression of the extracellular domain of CD86 correlates with SYNTENIN expression, a protein that we show is important for myeloma survival and proliferation (**Chapter 2, Chapter 3**). This suggests that CD28 signaling may be potentiating the increased expression of SYNTENIN. In the absence of CD86, myeloma cells compensate for lack of CD28 signal by increasing expression of another CD28 binding partner, ICOS-L[19]. ICOS-L may be negatively regulated via SYNTENIN as knockdown of SYNTENIN also results in an increase of ICOS-L in MM.1s and RPMI8226. The role of ICOS-L signaling, especially in the absence of CD86 represents another promising area of research to pursue.

While these studies were conducted in myeloma cells, they provide clues toward regulation of co-stimulatory proteins in other immunological cell types. Activated B cells differentially regulate CD80 and CD86 depending on the context and signal provided[11]. We have shown that CD80 does not contain a PDZ-binding motif, suggesting a differential means of generating surface expression. Furthermore, while the CD80 tail is important for localization to areas of co-stimulation while already on the surface, the lack of a tail appears to not be necessary for proper trafficking

to the surface (**Chapter 2**)[20]. Another protein involved in co-stimulation that competes for CD28 is ICOS-L[19]. Like CD80, ICOS-L does not contain a PDZ-binding motif, further illustrating its difference in generating surface expression from CD86 in plasma cells and APCs (**Figure 1, Chapter 3**).

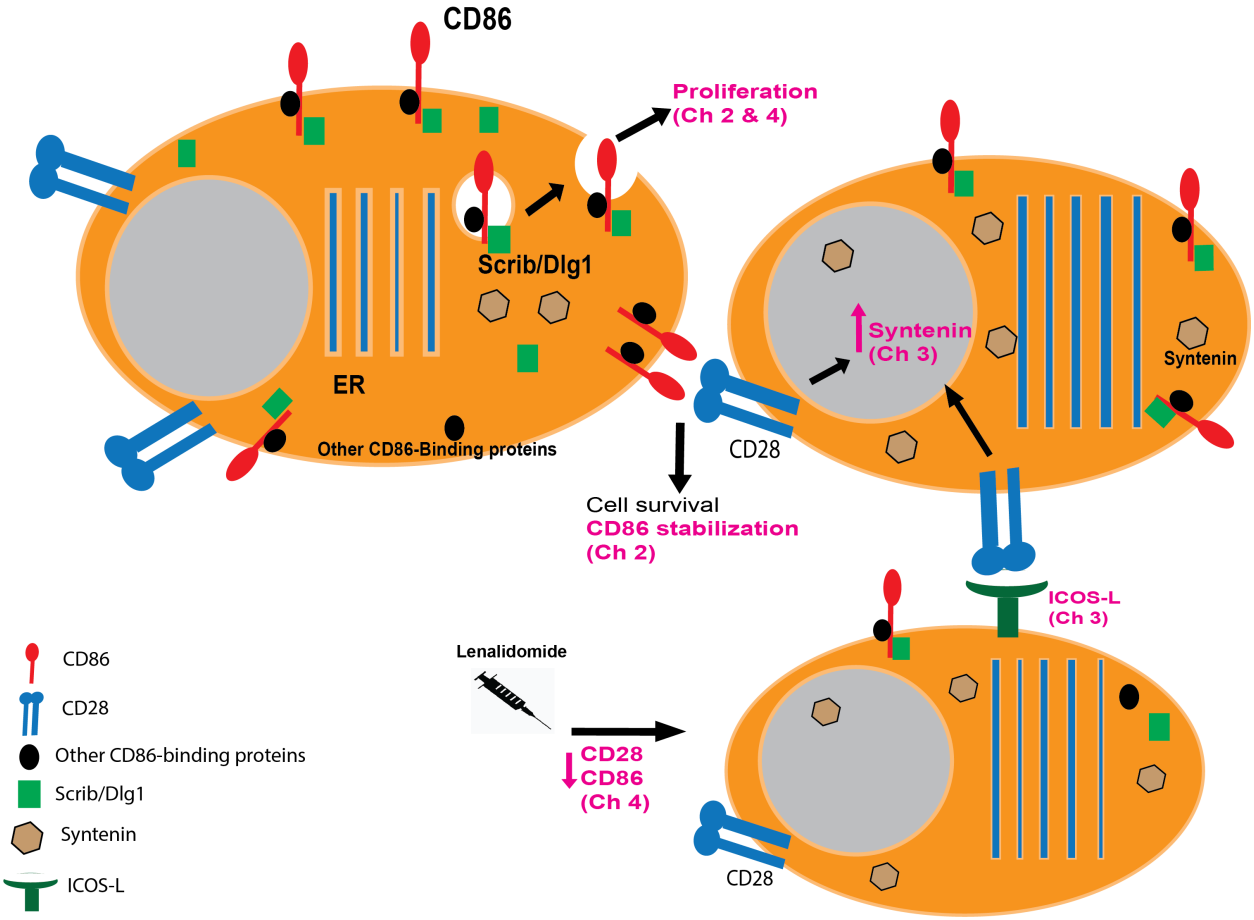


Figure 1: Summary of main research findings. **Chapter 2** addresses how CD86 surface expression is regulated. PDZ proteins, SCRIB and DLG1 facilitate CD86 surface expression and localization. There, it binds to CD28 resulting in stabilization of CD86, increased cell survival, and proliferation. Preliminary data in **Chapter 3** suggests that CD28 signaling results in an increase in SYNTENIN expression. In the absence of CD86, myeloma cells upregulate ICOS-L, another known CD28 binding partner.

Chapter 4 further explores the role for CD86 in the induction of proliferation**.** We found an increase of CD86 in actively dividing cells, and treatment with Lenalidomide decreases CD28 and CD86 expression.

CD86 role in cell viability and proliferation

Using shRNA, we have previously shown that CD86 is necessary for myeloma survival[8]. Here, I have verified the importance of CD86 on myeloma viability using a doxycycline-inducible CRISPR-Cas9 knockout system (**Chapter 2**). Utilization of CRISPR-Cas9 is often associated with off-target effects on the genome, and these could contribute to cell death[21]. However, we accounted for this by using an online CRISPOR tool to minimize predicted off-target effects[22]. Additionally, we used two distinct guides in multiple cell lines and a non-targeting control sgRNA and can more confidently determine that the cell death we observed was likely not due to off-target effects.

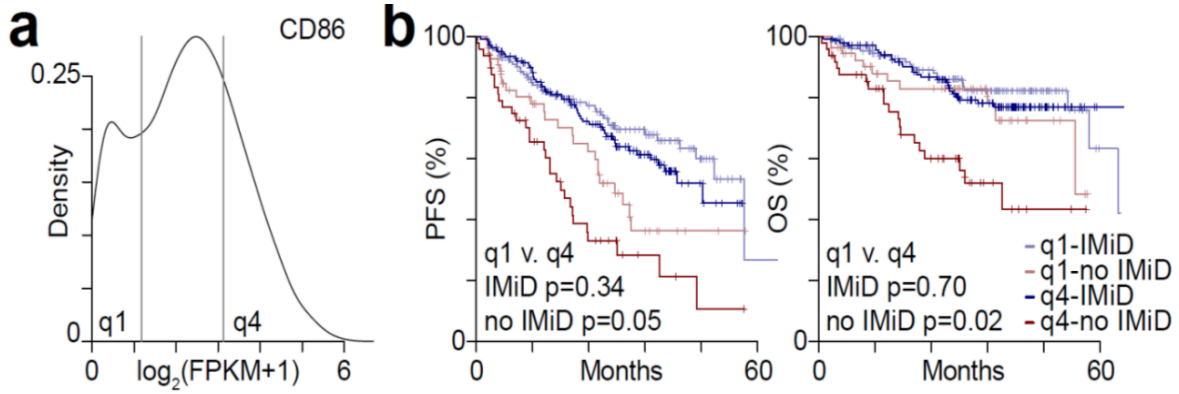
My work also addresses the previously unstudied role of CD86 in myeloma proliferation. Knockout of CD86 results in lower cell counts in days 1-7 following activation of the Cas9 enzyme (**Chapter 2**). While the lower cell numbers are due in part to an increase in cell death, the majority of cell death takes place in the first 3 days of the experiment. In the subsequent 4 days, less cell death occurs and a small population of cells

grow out. This population represents cells which have not effectively incorporated the sgRNA plasmid or ones who have developed a compensatory mechanism to survive in the absence of CD86. These cells grow at slower rate in both KMS18 and RPMI8226 cell lines and future studies will be performed using stable CD86 knockout lines to directly compare rates of cell growth and molecular changes to lines with normal CD86 expression.

While CD86 may have roles in numerous stages of cell growth, we observed an increase in CD86 in actively dividing cells (**Chapter 4**). As mentioned above, regulation of CD86 by SCRIB and DLG1 indicates the importance of CD86 expression in specific areas. This upregulation of CD86 during mitosis points to a cell cycle context for CD86 expression, and future studies using cell cycle analysis will need to be conducted. Therefore, cell cycle inhibitor drugs such as CDK6 inhibitors may represent a beneficial co-treatment with CTLA-4-Ig. Furthermore, recent work has shown that CDK6 inhibitors sensitize myeloma cells to cytotoxic killing through loss of IRF4[23].

Another class of drugs associated with CD86 in myeloma are IMiDs. IMiDs are cytostatic drugs that prevent the growth of myeloma cells while mobilizing neighboring immune cells[24]. Data from our lab has shown that

high expression of CD86 portends poor prognosis in patients who did not receive an IMiD. However, treatment with an IMiD trumps high CD86 expression, and patients' prognosis improves regardless of CD86 expression (**Figure 2**). Treatment of cell lines with IMiD decreases CD28 and CD86 expression, illustrating the importance of IMiD treatment on CD86 function in myeloma (**Chapter 4**). However, the decrease in CD28 and CD86 protein expression occurred in a Lenalidomide-sensitive line, MM.1s, with the highest dosages of the drug. CD28 and CD86 both signal to upregulate IRF4, a molecule important for myeloma survival that has been shown to generate resistance to IMiD treatment[8,25]. IMiDs downregulate IRF4 expression and may result in the cell downregulating CD28 and CD86 to prioritize an alternative pro-survival signaling pathway[24]. Future studies using IMiD treatment on CD86FL and CD86TL myeloma cell lines will be performed to tease out the effects of IMiD treatment on CD86 signaling.



***Data courtesy of Dr. Benjamin G. Barwick**

Figure 2: Myeloma CD86 expression and IMiD outcome. a) Distribution of CD86 RNA expression in myeloma from 746 newly diagnosed patients participating in the CoMMpass (IA13) study. First and third quartiles are denoted by gray lines. b) Progression-free (PFS) and overall survival (OS) of patients from the CoMMpass study who had CD86 expression in the 1st (q1) and 4th (q4) quartiles stratified by whether or not they received an IMiD.

PDZ proteins and multiple myeloma treatment

Using the BioID proximity assay, we identified ten PDZ-domain containing proteins that are proximal to the CD86 cytoplasmic tail. All ten of these proteins are expressed in patients and may have important roles in CD86 function. We further characterized three of these proteins, SCRIB, DLG1, and SYNTENIN as important for myeloma survival and proliferation. SCRIB and DLG1 are involved in regulating CD86 surface expression while SYNTENIN may be associated with CD28 signaling and ICOS-L regulation. Additionally, five other PDZ proteins that are proximal to the tail (PARD3, PDLIM1, AHNAK, CASK, and SLC9A3R1) portend poor progression free and overall survival in patients although they did not reach significance (**Chapter 2**). PARD3, PDLIM, and CASK have roles in apical-basal polarity and may be associating with SCRIB and DLG1 to regulate CD86 as well as

performing other functions in myeloma cells[26–28]. Both CD28 and CD86 also have highest expression of the MAF myeloma subtype[8,29], and PARD3, SCRIB, and AHNAK also have the highest expression of this subtype (**Chapter 2**). We also found that AHNAK has higher expression in peripheral blood myeloma cells compared to bone marrow plasma cells, indicating a possible role in myeloma progression. Taken together, we have identified several PDZ proteins as a promising avenue for further studies. This is particularly relevant as numerous PDZ domain-targeting peptides including those against SCRIB and SYNTENIN are being tested for treatment of oncological diseases[30].

The majority of PDZ-targeting drugs inhibit the protein-protein interaction functions between PDZ domains and the PDZ-binding motif[16]. The PDZ-binding motif is the recognition sequence which PDZ-domains bind to perform subsequent downstream functions. There are three different classes of PDZ domains that correspond to three recognition sequences. A class I PDZ domain recognizes a sequence S/T-X-**ϕ** motif where X represents any amino acid and **ϕ** represents a hydrophobic residue. Class II and class III domains recognize the X-**ϕ**-X and D/E-X-**ϕ** motifs respectively[15]. CD86 contains a class I PDZ domain while other proteins involved in myeloma growth, survival, and adhesion such as

ICAM-1, CD138, and ITGB7 contain class II PDZ domains. We have shown that truncation of these three amino acids was enough to have an effect on the expression of CD86 (**Chapter 2**). Therefore, the relative size of this motif, its importance in myeloma growth and survival, and its location at the terminus of the protein may render it an effective specific target for peptide or small molecule development.

Myeloma cells are able to take advantage of their hospitable bone marrow niche through a wide array of cell-cell interactions and cytokine signaling. Targeting of PDZ domains can enable a multi-pronged approach to combatting myeloma cells before they reach advanced stages of myeloma. In **Chapter 1**, I referred to a "Game of Bones" that occurs between myeloma cells and immune cells. Myeloma cells are able to tip the balance in their favor through several means including binding of CD86, ICAM-1, CD138, or ITGB7 to neighboring cells or the ECM[1]. We have shown that PDZ proteins may have a role in regulating localized expression of these proteins which is important for generating proper cytokine signaling and adherence to the ECM. Without proper coordinated expression of these proteins, myeloma cells' ability to signal for increased angiogenesis, osteoclast activation and immune suppression is impaired, tipping the Game of Bones back in favor for the immune cells. We have

also observed that IMiDs can deliver a multi-pronged attack against myeloma cells by opposing myeloma growth, inhibiting angiogenesis, and stimulating immune activity[31,32]. We have found a mechanism by which IMiDs perform these roles by impairing CD28 and CD86 signaling in myeloma. As such, IMiD treatment may have a synergistic effect with PDZ inhibitors as well as CTLA-4-Ig and warrants further exploration.

B. Future Directions

My findings have brought forth many questions concerning fundamental biology of trafficking, cell signaling, cancer progression, and immunology. This section will address avenues for future researchers to pursue.

Regions of the CD86 cytoplasmic tail

We have uncovered a PDZ binding motif of the cytoplasmic tail that is important for proper trafficking of CD86 to the cell surface. This region, however, is small and makes up approximately 5% of the cytoplasmic region. We have additionally shown that numerous regions of the tail confer CD86 transport from the Golgi as co-localization of CD86 and M6PR negatively correlates with tail length. A particular region of interest for future

studies is between amino acid 282 and 298 where we observe a significant difference in co-localization between CD86 and M6PR.

Use of HEK293T allowed us exogenously express CD86 and observe its localization 1-2 days following initial expression. We would, however, like to observe CD86 expression in a myeloma cell line in the absence of endogenous CD86. To do this, we have developed stable CD86FL and CD86 truncation mutants in NCI-H929, a cell line which has low endogenous levels of CD86 that will enable us to determine CD86 localization in a myeloma context.

We would also like to test the effect of novel chemotherapeutic agents such as IMiDs, venetoclax, and daratumumab on these truncation mutants to further elucidate what regions of the tail play a role in drug sensitivity. Another question remains as to what proteins may be involved in binding throughout the cytoplasmic tail. To address this, BirHA constructs can be engineered into the truncation mutant plasmids and compared to one another for possible interacting partners at each region. We can also perform RNA-sequencing analysis of these truncation mutants to determine if any differences in gene expression occur as a result of incomplete CD86 cytoplasmic tail expression.

CD86, SCRIB, and DLG1 in myeloma proliferation

Using the doxycycline-inducible CRISPR-Cas9 system we have demonstrated that CD86, SCRIB, and DLG1 have roles in myeloma cell growth. However, the mechanisms by which these proteins influence proliferation is unknown. Study of possible downstream markers of proliferation such as Ki67 and PCNA as well as cell cycle dependent proteins (cyclin D1) and transcription factors (FOXM1) merit further research to tease out mechanisms of cell growth.

We have also identified that treatment with lenalidomide negates the effects of CD86 in patients and cell lines. This begs the question as to the effects of IMiDs on SCRIB and DLG1 expression. Does treatment with an IMiD mirror the phenotype we see on CD86 with SCRIB and DLG1? Analysis of SCRIB and DLG1 in patients treated with IMiDs and in cell lines represent an interesting potential avenue for future studies in the lab.

ICOS-L and other compensatory signaling

While we have found that knockdown of CD28 and CD86 results in myeloma cell death, there is a subset of cells which are able to survive. One molecule that is upregulated in the absence of CD86 is ICOS-L. This molecule remains largely unstudied in myeloma and its effect on cell

viability and signaling could be promising. By designing guides against ICOS-L, we can knock out the protein and determine any molecular changes that may occur. The relationship between ICOS-L and SYNTENIN remains unexplored and may be an interesting area of research.

Other compensatory signals may take place in the absence of CD28 and CD86 signals in cell lines. While knockout of CD86 results in an initial increase in cell death, a small population of CD86-depleted cells begins to grow out in KMS18 and RPMI8226. We are currently using CD28 and CD86 guides to determine the molecular changes in CD28-KO or CD86-KO populations compared to CD28 or CD86-normal populations and cells which do not receive guides. We will conduct RNA-sequencing to analyze gene expression changes that occur in the knockout populations.

Further study of PDZ proteins

These studies have elucidated a previously unstudied role for PDZ proteins in myeloma. In addition to further study of SCRIB and DLG1, we would like to determine the mechanism by which SYNTENIN induces cell death in myeloma. SYNTENIN has been shown to regulate levels of CD138 and our preliminary data show that it may be associated with CD28 signaling (**Chapter 3**)[33]. We have successfully generated SYNTENIN guides that we

will use in future studies to tease out signaling pathways affected by SYNTENIN ablation.

In addition, we identified AHNAK as a potentially important protein for myeloma progression. Other PDZ proteins such as PDLIM, CASK, and PARD3 have roles in polarity and may be associating with SCRIB and DLG1 to regulate CD86. Moreover, small peptides against other PDZ proteins such as Disheveled 1/2 have shown promise in treatment of other cancers and may be a target for therapeutic avenues[30].

Drug treatment combinations

Our work has implications for several novel applications of therapy. Currently, the CD80/86 blocking antibody CTLA-4-Ig has been approved for treatment of autoimmune disorders and graft rejection[34–36]. In light of our recent studies of the role of CD86 in proliferation, there may be a synergistic effect of treatment of CTLA-4-Ig in combination with IMiD or CDK6 inhibitors. CTLA-4-Ig may also be effective in co-treatment with Daratumumab, which has an immunomodulatory effect[37]. Our elucidation of the role of PDZ proteins render them a promising avenue for therapeutic treatment, particularly as two of the proteins, SCRIB and SYNTENIN, have available peptides in development for cancer trials[30]. These peptides can

also be used in combination with any of the aforementioned drugs to determine the effect on myeloma proliferation and survival.

C. Concluding Remarks

Taken together, my studies have uncovered a more nuanced function for CD86 in myeloma growth and survival. My work has shown how CD86 surface expression is generated via its intracellular domain, and in doing so, uncovered a role for a class of PDZ proteins that may be a promising avenue of clinical discovery. We showed the importance of CD86 localization and that its contact with neighboring cells stabilizes localized expression. We have also revealed a role for CD86 in myeloma proliferation and cell division. Finally, this work lays the groundwork for compensatory mechanisms the cell uses in the absence of CD86 and CD28 signaling including the role of another CD28 binding partner, ICOS-L[19].

These data have advanced the fields of cell and cancer biology, clinical cancer therapy, and immunology. They will be a springboard for future researchers to tease out new mechanisms of cancer viability and how immune cells regulate receptor trafficking. They have important implications for clinical research and provide basis for novel combinations

of drug treatments in myeloma. Together, these data provide a richer understanding for us to help tip the Game of Bones to the side of the immune system and generate healthier, longer-living patients.

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