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Gli3 Is Required for M2 Macrophage Polarization and M2-Mediated Waldenström Macroglobulinemia Growth and Survival

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Abstract/Summary

The macrophage polarization paradigm has become at the forefront of cancer research in recent years; however, the effects of this phenomenon have yet to be investigated in Waldenström Macroglobulinemia (WM). WM is an indolent, B-cell lymphoma, characterized by increased IgM production and infiltration of the bone marrow niche by malignant cells. Macrophages may polarize to one of 2 phenotypes: M1, which is inflammatory or M2, which is inhibitory. While macrophage polarization is typically investigated as a singular point in time, it is important to understand that M2-type macrophages can switch to an M1 phenotype, or vice versa, based on environmental changes. M1 macrophages are typically pro-inflammatory and generally have an anti-tumor role (although some cancers have reported an increased presence of M1 macrophages leading to worse clinical outcomes), while M2 macrophages are typically anti-inflammatory and have a pro-tumorigenic role. Understanding which phenotype is prevalent in WM, as well as the mechanism behind the increased tumorigenesis is important for guiding WM research and future therapies.

To assess the effects of macrophages on WM cell proliferation, we examined the effects of macrophage polarization using THP-1 cells, $CD14^{+}$-derived macrophages from peripheral blood mononuclear cells (PBMCs) and bone marrow-derived macrophages (BMDM) from C57BL/6 mice on WM cell growth and viability. WM cells grown in direct co-culture with macrophages exhibited increased proliferation compared to WM cells grown alone. WM cell viability was also enhanced when cells were directly co-cultured with macrophages. To investigate whether M1 or M2 macrophages were responsible for this increased proliferation and viability, we performed a qPCR analysis of macrophages in indirect and direct co-culture with WM cells. We found that WM cells induce a M2 phenotype upon direct co-culture, but this

effect was not seen in indirect co-cultures. Additionally, upon the polarization of macrophages towards an M2 phenotype, we observed that the expression of transcription factor GLI3 was increased, indicating a role for GLI3 in macrophages polarization. In previous work, we found that the transcription factor GLI3 plays a role in regulating cytokine expression and secretion in response to LPS stimulation. In previous work, we performed RNA-seq on macrophages derived from mice lacking Gli3 in myeloid cells (*M-Gli3*$^{-/-}$) stimulated with or without LPS. Using a generalized linear model in edgeR, we identified 495 genes with significant interaction effects between genotype and LPS treatment. Ingenuity Pathway Analysis of the interaction genes revealed "Inflammatory Response" and "Immune Cell Trafficking" pathways as most significantly enriched. The 25 significant interaction genes on these pathways included 9 with a positive interaction and 16 with a negative interaction. Analysis also suggested Gli3 may play a role in M2 macrophage polarization. Bone marrow-derived macrophages were isolated from *M-Gli3*$^{-/-}$ and *WT* mice and were cocultured with WM cells. We found that *M-Gli3*$^{-/-}$ macrophages could not increase the proliferation and viability of WM cells cocultured with these macrophages.

Our findings identify a novel role for Gli3 in regulating M2 polarization and subsequently a role for M0 and M2 macrophages, but not M1, in promoting WM cell growth and survival. Taken together, these results suggest that therapeutic targeting of Gli3 in the tumor microenvironment may be beneficial in the treatment of WM.

Introduction/Prior Research

Macrophages are myeloid cells that are essential members of the innate immune response [1]. These heterogenous cells originate from monocyte precursors in the blood and differentiate in the presence of cytokines and growth factors in the tissues they infiltrate [2,3]. Macrophages are found in every human tissue in the body and exhibit anatomical and functional diversity [4]. These cells have three key functions: phagocytosis, exogenous antigen presentation, and immunomodulation through cytokine and growth factor secretion. As one of the earliest immune cells to encounter antigens at the site of infection or injury, their response is critical to the remainder of the immune response. Antigen presentation, the ability to phagocytose, digest, and present antigen through the major histocompatibility complex class II (MHC II) system, is crucial for the activation of the adaptive immune system and highlights one of the key roles that macrophages play in the immune response [5]. T-cells (specifically T-helper cells) can recognize these MHC II complexes on classically activated macrophages through their T-cell receptors (TCRs), leading to further activation of the adaptive immune response [5]. Through phagocytosis, macrophages are also able to assist in the resolution of inflammation by effectively eliminating pathogen materials as well as assist in the clearance of apoptotic host cells such as neutrophils [6]. Without macrophages at the site of infection/tissue damage, an elevated apoptotic neutrophil population and elevated and prolonged inflammation has been demonstrated [7]. Macrophages are not only responsible for phagocytosis of foreign antigen; they also coordinate processes that initiate new tissue formation of the extracellular matrix and new blood vessel formation through angiogenesis under normal physiological conditions [4,8]. Macrophages, therefore, play a key role in tissue homeostasis under normal physiological conditions as well as after tissue damage. Macrophages also make key hematopoietic decisions

by engulfing cells exuded from the bone marrow that do not express the CD47 ligand [9]. Macrophages engulf dead cells after infection or injury through recognition of the phosphatidylserine that is externalized on apoptotic cells. Phosphatidylserine is typically confined to the inner plasma membrane, but during apoptosis, it becomes externalized on the cell surface [10]. The engulfment of neutrophils and erythrocytes in the spleen and liver resolves the problems of neutropenia, splenomegaly, and reduced body weight [6].

Macrophages also regulate angiogenesis through different mechanisms. Macrophages can identify vascular endothelial cells and instruct them to undergo apoptosis if they do not receive countersignals from pericytes to survive. Macrophages have been shown to instruct functional angiogenesis in normal vessel maturation [11], wound healing [12], and development [13] and non-functional angiogenesis in many types of cancer [14] and chronic inflammatory conditions [15]. The protein, Wingless-Type MMTV Integration Site Family Member 7B (WNT7B), is secreted by macrophages and triggers apoptosis of vascular endothelial cells and, in the absence of WNT7B-secreting macrophages, there is significant vascular overgrowth. WNT7B secretion by macrophages is triggered by the presence of ANG2 secreted by mature blood vessels [13]. ANG2 secretion by blood vessels causes macrophages to upregulate WNT7B, which induces the proliferation of vascular endothelial cells and allows them to be targeted by ANG-2-induced apoptosis [13]. In response to Wnt7b, vascular endothelial cells enter the cell cycle and die in the G1 phase due to ANG2-mediated withdrawal of survival signals [16]. To induce angiogenesis, macrophages secrete WNT11 and WNT5A, which induce the expression of soluble vascular endothelial growth factor (VEGF) receptor 1 (VEGFR1) in an autocrine fashion. Soluble VEGFR1 regulates the levels of VEGF so vascular complexity is reduced, and the vasculature is more properly organized [17]. Wnt5a and Wnt11 are associated with non-canonical Wnt

signaling. In a study of angiogenesis regulation by the non-canonical Wnt–Flt1 pathway, soluble and membrane-tethered VEGFR1 was measured by quantitative PCR in the RAW264.7 myeloid cell line after Wnt treatment, and both soluble and membrane-tethered VEGFR1 expression were significantly increased after RAW264.7 treatment with Wnt5a [17]. Macrophages are not limited to just blood vessel formation, but also play a key role in lymphangiogenesis during development and inflammation [18]. Macrophages can promote lymphangiogenesis by transdifferentiating into lymphatic endothelial cells and becoming incorporated into growing lymphatic vessels or by secreting growth factors and proteases that lead to lymphatic vessel formation [18]. Lymphangiogenesis can lead to tumor metastasis [19], making the role of macrophage regulation important to understand and further investigate.

Macrophage Markers

Regardless of origin, the major lineage regulator of almost all macrophages is the macrophage colony-stimulating factor 1 receptor in both humans (CSF1R) and mice (Csf1r). This is a class III, transmembrane tyrosine kinase receptor that is expressed on most mononuclear phagocytic cells [4]. While CSF1R/Csf1r is important in macrophage differentiation, $Csf1r^{-/-}$ mice have still exhibited some tissue macrophages, indicating the importance of other macrophage growth factors such as granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-3, which act as macrophage growth factors in tissue culture [4]. Macrophages also constitutively express the surface marker F4/80 in mice [4,13] and the equivalent constitutive markers CD14 and CD68 in humans [20–23].

Human Macrophage Markers

In humans, M1 macrophages are typically identified by the surface markers CD86 and CD64 [23,24]; the macrophage receptor with collagenous structure MARCO [23,25,26]; C-X-C chemokine ligand (CXCL) 9, 10, 11 (CXCL9, 10, 11) [23]; nitric oxide synthase 2 (NOS2) [27], the suppressor of cytokine signaling 1 (SOCS1) [27]; and secretion of IL-6, IL-12, IL-1α [27], and TNF-α [24]. M2 macrophages typically express the surface markers CD206 [23–25,28,29] and CD163 [23,29] and express/secrete transforming growth factor-beta (TGF-β), peroxisome proliferator-activated receptor gamma (PPARγ), C-C motif chemokine ligands 14 and 22 (CCL14 and CCL22) [28], and arginase-1 (ARG-1) (Table 1) [18,22].

Mouse Macrophage Markers

While most macrophage markers are the same in both humans and mice, some exceptions exist. Murine M1 macrophages express macrophage receptors with collagenous structures (Marco) [23,25,26], Cxcl9, Cxcl10, Cxcl11 [23], Nos2, and Socs1, and secrete Il-6, Il-12, Il-1α [27], and Tnf-α [24], all comparable to their human counterparts. Murine M1 macrophages do not express surface markers CD86 or CD64. Murine M2 macrophages typically express the surface markers Cd206 [17–19,21,22] and Cd163 [23,29] and express/secrete Tgf-β, Pparγ, Ccl14 and Ccl22 [28], and Arg-1 [24,29], similar to human M2 macrophages; however, they also have Chitinase-3-like protein 3 (Chil3), unique from human M2 macrophages (Table 1) [29].

Species	M0	M1	M2
Mouse	Csf1r, F4/80, CD11b	Marco, Cxcl9, Cxcl10, Cxcl11, Nos2, Socs1	Cd206, Tgm2, Fizz1, Chil3, Arg1, Ccl22, Cd163, Arg2
Human	CSF1R, CD14, CD68, CD11B	CD86, MARCO, CXCL9, CXCL10, CXCL11, NOS2, SOCS1, CD64	TGM2, CD23, ARG1, CCL22, CD163, CD206, ARG2, PPARγ, CCL14

Table 1. Macrophage polarization markers on M0, M1, and M2 mouse and human macrophages.

Macrophage Polarization

Many phenotypes of macrophages have been characterized based on their in vitro characteristics in cell culture experiments. Primarily, the classically activated M1 phenotype and the alternatively activated M2 phenotype are differentiated based on different surface receptor expression, secretory profiles, and functions [4,30]. Recent studies of gene expression of *in vivo* wound healing have shown that macrophages exhibit a pro-inflammatory M1 secretory profile during the early stages and then transition to an anti-inflammatory M2 gene expression profile during the later healing stages [31]. Macrophage polarization refers to the activation state of a macrophage at a singular point in time, but due to the plasticity of macrophages, their polarization state is not fixed and can be altered based on the integration of multiple signals from other cells, tissues, and pathogens [32]. While macrophage polarization is typically discussed as a singular point in time, it is important to understand that M2-type macrophages can switch to an M1 phenotype, or vice versa, based on environmental changes such as cytokine and growth factor secretion, inflammation, infection, injury, hypoxia, and other conditions. Macrophage polarization is more complex than the M1 and M2 binary classification, with those subtypes representing the extremes on the spectrum of macrophage polarization (Figure 1). Many of these subsets express combinations of M1 and M2 cell markers and have yet to be formally defined.

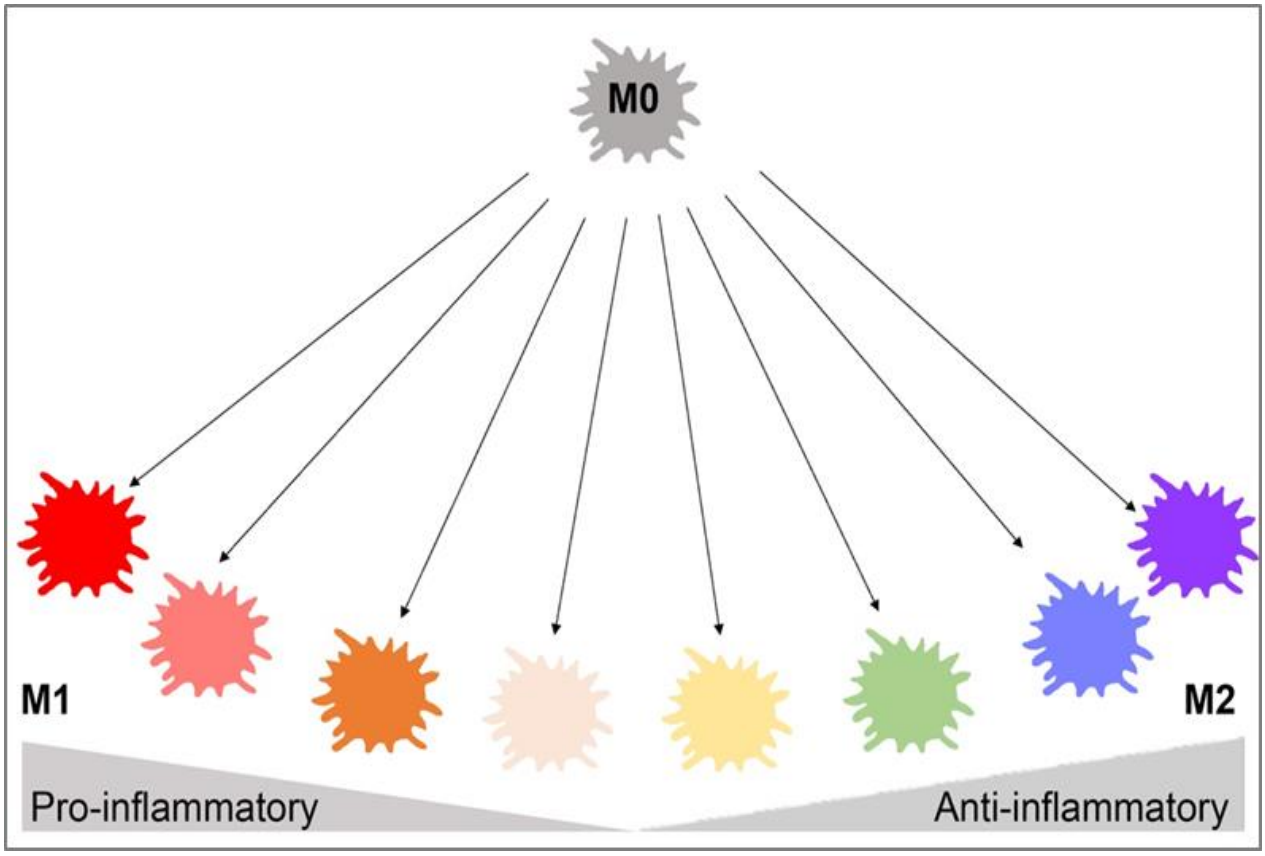

Figure 1. Macrophage polarization gradient. This figure illustrates the heterogeneity of macrophage polarization in place of binary M1/M2 classifications. The pro-inflammatory M1 and anti-inflammatory M2 cells lie on opposite ends of the polarization axis, but many macrophages with mixed pro- and anti-inflammatory characteristics exist in between. Environmental changes may cause macrophages to shift from M1 to M2, vice versa, or to a hybrid of both cells. This highlights the plasticity of macrophages and interdependence on the surrounding environment. This figure was created with Biorender.com.

M1 and M2 Macrophages

M1 macrophages are pro-inflammatory in nature and are characterized by their high capacity to present antigens, produce interleukin 12 and 23 (IL-12 and IL-23) [33], and activate type-I T-cell responses [5]. They inhibit cell proliferation and cause tissue damage through the secretion of pro-inflammatory cytokines and nitric oxide (NO) and are induced by T-helper type-1 cytokines including interferon-γ (IFN-γ), interleukin-1β (IL-1β), and lipopolysaccharide (LPS) [1,3,4,34]. M2 macrophages are typically anti-inflammatory in nature and are characterized by

their poor ability to present antigen; having low IL-12 and high IL-10, IL-4, and IL-13 secretory profiles; and immunosuppressive effects [5]. These cells promote cell proliferation, tissue repair, angiogenesis, and phagocytosis to downregulate inflammation and "clean up" after inflammatory events and are T-helper type-2 activators and TH1 inhibitors [1,3–5,34]. While macrophage polarization is often defined as a specific moment in time, it is important to note that these markers are often present on many subtypes of macrophages in varying expression levels. For example, M2 macrophages can still express M1 markers but with lower levels than M1 macrophages, and vice versa [35].

Extrinsic Polarization

Extrinsic polarization is a primary method of macrophage polarization and is mediated by cytokine secretion by other cells such as CD4+ TH1 or TH2 cells (Table 2). Some non-cytokine, extrinsic pathways of macrophage polarization do exist, however, including hypoxia as well as the production of lactate within tumors, which drive M2 polarization [36].

Table 2. Overview of extrinsic mechanisms of macrophage polarization.

Protein/Gene	Normal Function	Effect on Polarization
Interleukin-4 and Interleukin-13	Cytokines	M2-favored
Interleukin-4 receptor alpha	IL-4 and IL-13 signaling	M2-favored
Signal transducer and activator of transcription 6	Transcription factor	M2-favored
Peroxisome proliferator activated receptor gamma	Transcription factor	M2-favored
Tubular sclerosis 1	Inhibitor of mTOR	M2-favored
AKT Serine/Threonine Kinase 1	Signaling	M2-favored
AKT Serine/Threonine Kinase 2	Signaling	M1-favored
Src homology region 2 domain-containing phosphatase-1/2	Phosphatases	M1-favored
SH2-containing Inositol 5′-Phosphatase	Phosphatase	M1-favored

Phosphatase and tensin homolog	Lipid phosphatase	M1-favored
Myeloid differentiation primary response 88	Signaling adapter	M1-favored
Tumor necrosis factor	Cytokine	M1-favored
Tumor necrosis factor receptor 1	Cytokine receptor	M1-favored
Interferon-gamma, Interleukin-12	Cytokines	M1-favored

To detail Table 2 and Figure 2, TH1 cells secrete IFN-γ, which drives polarization of macrophages towards an M1 phenotype, while TH2 cells secrete IL-4 and IL-13, which drive M2-phenotype polarization [34,37]. IL-4 and IL-13 inhibit the production of nitric oxide, an inflammatory mediator, through the depletion of arginine, which serves as the substrate for iNOS/Nos2. This inhibition of NO production in macrophages was found to be dependent on IL-4 or IL-13 through the depletion of Arg-1 through a Stat6-dependent pathway [38]. This inhibition of NO production leads to a loss of the M1 phenotype and polarization toward the M2 phenotype through cytokines IL-4 and IL-13. IL-4 and LPS signaling can also target the mechanistic target of rapamycin (mTOR) and Akt to trigger polarization. In LPS-mediated M1 polarization, Toll-like receptor 4 (TLR4) activates phosphoinositide 3-kinase (PI3K) followed by Akt and mammalian target of rapamycin complex 1 (mTORC1) activation, leading to M1 polarization [39,40]. Additionally, pharmacological and genetic inhibition of Akt1/2 has shown that Akt1 inhibits M1 activation, and Akt2 leads to the activation of M1 genes, favoring polarization to the M1 phenotype [39,40]. Akt signaling is likely to control macrophage polarization through downstream effectors; for example, Akt signaling inhibits transcription factor Foxo1, which is a key gene in M1 macrophages. Additionally, Akt1 has been implicated as a negative regulator of the nuclear factor, kappa-light-chain-enhanced activity of activated B cells (NF-κB), while Akt2 is a positive regulator. NF-κB is a master regulator of M1 activation [41]. Src homology region 2 domain-containing phosphatase 1/2 (SHP-1/2) inhibits CD11b

activity, therefore inhibiting M2 polarization and leading to an increase in M1-type macrophages [42]. Src homology 2 (SH2) domain-containing inositol polyphosphate 5-phosphatase (SHIP) is another phosphatase that inhibits the activation of M2-like macrophages. $SHIP^{-/-}$ peritoneal and alveolar macrophages have been found to be profoundly M2-skewed, with high arginase I levels and impaired LPS-stimulated NO production [43]. Phosphatase and tensin homolog (PTEN) plays a key role in regulating the inflammatory response through M1-polarization. Mice with a myeloid-specific PTEN knockout have been shown to have levels of M2 macrophages and produce lower TNF-α and higher IL-10 in response to TLR ligands, indicating that PTEN plays a key role in M1 macrophage differentiation [44]. Tumor necrosis factor (TNF) has been found to be a positive regulator of M1 polarization through its activation of the NF-κB pathway. Tumor necrosis factor receptor (TNFR) signaling was found to be a negative regulator of M2 polarization in tumor-associated macrophages (TAMs), and myeloid differentiation primary response 88 (MyD88) was shown to suppress M2 gene expression in TAMs, leading to an M1 phenotype [45].

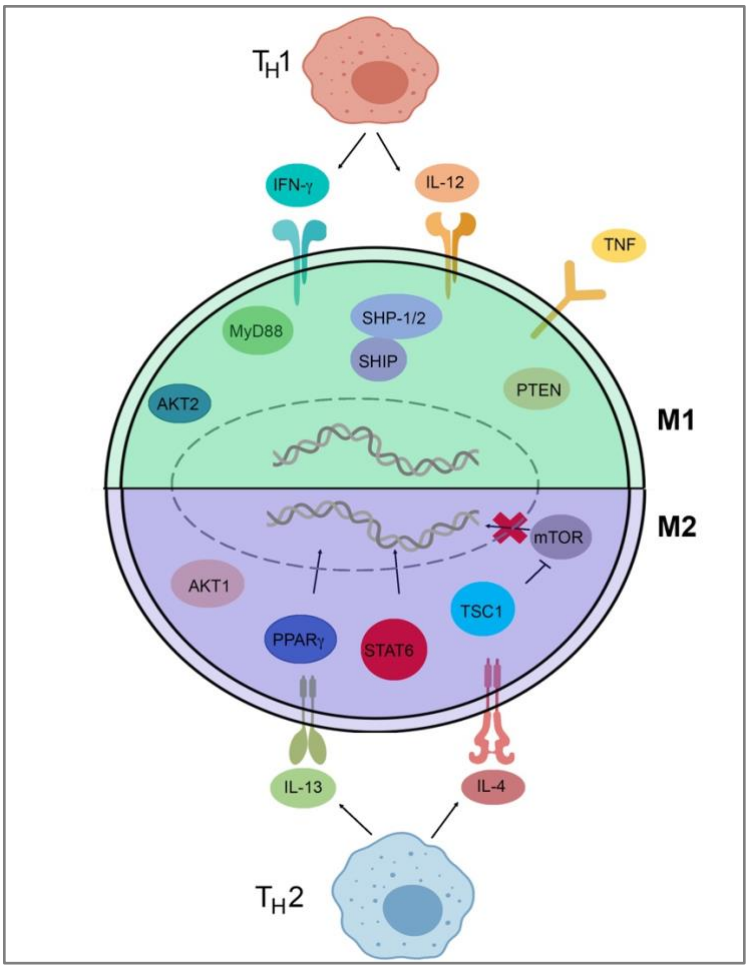

Figure 2. Signaling pathways of macrophage polarization. This figure illustrates several of the various mechanisms that drive extrinsic macrophage polarization. Those pathways include IFN-y and IL-12 secretion by TH1 T-cells, LPS signaling through mTOR/Akt or TLR4, Akt2/NF-κB activation, SHP-1/2 inhibition of Cd11b, SHIP and MyD88 inhibition of M2 genes, PTEN activation, and TNF/TNFR/NF-κB activation to induce M1 gene expression. Induction of M2 genes is directed by the secretion of IL-4 and IL-13 from TH2 T-cells and IL-4Rα receptor activation as well as downstream Stat6-dependent arginase-1 inhibition, PPARγ activation, and TSC1 inhibition of mTOR. This figure was created with Biorender.com.

Hypoxia-Induced Polarization

Hypoxia can be a key driver of macrophage recruitment and polarization in the TME. Hypoxia is common in most solid tumors, and TAMs are found in higher concentrations in hypoxic areas. Due to the high concentrations of chemokines, HIF-1/2, and endothelin-2 secreted from hypoxic tissues, macrophages are drawn to the hypoxic areas [46]. Damage-associated molecular pattern (DAMP), high-mobility group box 1 protein (HMGB1) is most commonly associated with hypoxia-induced macrophage polarization. HMGB1 has been shown to be overexpressed in many solid tumors and correlated with the development of hepatocellular

carcinoma [47] as well as colon [48] and skin cancers [49]. In metastatic melanoma, serum HMGB1 levels in human patient samples have been shown elevated compared to healthy controls. [50]. Additionally, a murine model of metastatic melanoma analysis of dissociated tumors by flow cytometry showed a significant increase in the total number of TAMs exhibiting a M2 phenotype in HMGB1-positive tumors [50]. Using short-hairpin RNA (shRNA) to target HMGB1, a higher number of M1-polarized macrophages were found at the tumor site, indicating that HMGB1 led to the M2 polarization of recruited macrophages. In the same study, HMGB1 was found to induce IL-10 production in M2-like macrophages through receptor for advanced glycation end product (RAGE)-dependent signaling [50]. HMGB1 had no effect on IL-6, TNF, or IL-1β expression but significantly increased IL-10 expression in bone-marrow derived M2-like macro-phages [50]. $RAGE^{-/-}$ mice did not show an upregulation of IL-10 signaling, indicating that this induction was through RAGE-dependent signaling [50]. These hypoxia-associated macrophages secreted higher levels of pro-angiogenic factors VEGF and TNFα [50].

Intrinsic Polarization

Intrinsic macrophage polarization refers to the origin of the macrophage. Macrophages have classically been described as being derived from bone marrow-derived circulating monocytes. However, additional sources of macrophage progenitors have been discovered. Many organs harbor embryonic-derived populations of resident macrophages that can self-renew and maintain throughout adulthood [4,36]. Most TAMs have been shown to be from either an embryonic precursor (either the fetal liver or yolk sac) or a monocyte precursor from an adult origin. Historically, TAMs have been observed as being exclusively from a circulating monocyte origin that undergoes differentiation upon tissue infiltration, although a higher fraction of

resident macrophages have been discovered in solid tumors [51]. TAM recruitment is highly linked to the CCL2/CCR2 axis [52], and in many cancer models, blocking this axis has led to a significant decrease in TAM populations [53]. The theory of monocyte-derived TAMs was tested in a mouse model of pancreatic ductal adenocarcinoma. $Ccr2^{-/-}$ mice showed no difference in tumor weight, but a depletion of resident TAMs using an anti-CSF1R antibody and clodronate showed a significant reduction in weight [54], indicating that resident macrophages made up a larger part of TAM populations than previously hypothesized. In mice, embryonic macrophages begin to develop at embryonic day 8 and give rise to macrophages that do not have a monocyte progenitor [4]. The fetal liver serves as the site of hematopoiesis of circulating monocytes originally, but then primary hematopoiesis is shifted to the bone marrow later in development, significantly increasing the bone marrow-derived monocyte population and minimizing the importance of embryonic macrophages [4]. The developmental origin of macrophages has been linked to some changes in polarization state. At any tissue site, there is always a mixture of both bone marrow-derived and embryonic macrophages [55]. The importance of origin in polarization is heavily debated, as hematopoietic depletion in lethal irradiation, chemotherapy, and systemic infection has shown that macrophage populations can fully return from a bone marrow-derived origin [56]. However, data has suggested that bone marrow-derived macrophages are more susceptible to local signals and subsequent polarization than embryonic macrophages, which appear to exhibit less plasticity than BM-derived macrophages [57]. Additionally, tissue signals appear to trump the embryonic developmental signals, and polarization states reflect the signals received from the environment rather than signals received from embryonic macrophages [58].

The mechanism of macrophage polarization is important to understand because of the potential ability to therapeutically manipulate the interchangeable polarization states of macrophages and subsequently promote or inhibit inflammation in cancer and other inflammation-related diseases.

Influence of the Tumor Microenvironment on Macrophage Polarization

The polarization process of TAMs is directly controlled by cancer cells within the TME [59], and the phenotypic ratio changes drastically as cancer progresses. At early stages, the ratio is more favorable for M1 macrophages, but as cancer cells hijack this process, the M2-like population drastically increases. M1-like macrophages are essential tumor-suppressing cells that initially act in the tumor microenvironment to suppress tumor cell growth [59,60]. M1-like macrophages achieve this suppressing effect by recruiting $CD8^+$ T and NK cells to the TME through antigen presentation to the T-cell receptor (TCR) [61] and the tumor-derived chemokine secretion of CXCL9, CXCL10, and CXCL11 to recruit and activate NK cells [62]. These $CD8^+$ T and NK cells express high levels of cytokines such as IFN-γ, GM-CSF, and TNFα as well as chemokines such as CCL4, CCL5, and CCL23 that assist in the further recruitment of immune cells and the signaling of anti-tumorigenic pathways [59,60]. The M1 phenotype is also associated with the expression of IL-12, IL-1, and inducible nitric oxide synthase (iNOS) [63,64] The M1 phenotype is well-characterized for its anti-tumorigenic properties, and an increased M1/M2 TAM ratio has been linked to an improved 5-year prognosis in ovarian cancers [63].

Most commonly, M1 macrophages are positively associated with longer survival times and most positive clinical outcomes in many cancers such as small cell lung cancer [65], non-small cell lung cancer [66], colorectal cancer [67], ovarian cancer [63], breast cancer [68], oral squamous cell carcinoma [69], and more. However, in some cancers such as renal cell carcinoma

(RCC), several markers of M1 macrophages have been found alongside M2 markers in TAMs isolated from patients, indicating that some TAMs can exhibit a hybrid phenotype in some cancers [70]. In the skin, during early stages of tumor development, M1 TAMs shifted to the M2 phenotype in melanoma, but the presence of either M1 or M2 TAMs was associated with poor prognosis [71].

Malignant cells can secrete M2-like cytokines such as IL-10, CCL2/3/4/5/7/8, CXCL12, VEGF, and platelet derived growth factor (PDGF) in order to recruit more monocytes and M0 macrophages to the area and differentiate them into the M2 phenotype [63]. The majority of intratumoral macrophages exhibit an M2 phenotype and are correlated with poor prognosis in several malignancies [72,73].

Tumor-associated macrophages are implicated in cancer cell latency, growth, and metastasis through the secretion of cytokines, chemokines, and growth factors. In the tumor microenvironment (TME), TAMs are most frequently found in the M2-like, pro-tumor phenotype [74,75]. M2-like macrophages characteristically assist the cancer cell in metastasis, angiogenesis, and proliferation through various anti-inflammatory mechanisms.

Pro-Tumorigenic Outcomes

Immune Suppression

Current literature supports the theory that most TAMs originate from either tissue-resident embryonic macrophages or macrophages derived from circulating monocytes that originate in the bone marrow [73]. Monocytes are recruited to the tumor by various growth factors and cytokines such as CCL2, CCL5, and CSF1 [76,77]. While the tumor microenvironment can polarize both tissue-resident and bone marrow-derived macrophages

depending on the tissue type, it is hypothesized that tissue-resident macrophages are the first to be affected [76]. These tissue-resident macrophages primarily cause DNA damage, survival of transformed cells, and cancer-related inflammation. Monocyte-derived macrophages that are recruited to the tumor site usually promote the proliferation and survival of tumor cells and angiogenesis [78].

M2-type macrophages play a significant immunosuppressive role and have been found to secrete immunosuppressive molecules into the TME including IL-10, TGF-β, and human leukocyte antigen G (HLA-G) [79]. Additionally, M2-type cells interact directly with myeloid-derived suppressor cells (MDSC) and actively suppress T-cell-mediated anti-tumor responses [80]. Myeloid-derived suppressor cells are a heterogeneous population of non-defined myeloid cells that typically expand during inflammation, infection, and cancer. In mice, these cells are characterized by GR1 and CD11b expression and in humans, characterized by the phenotype $CD14^{-}CD11b^{+}$ [81]. MDSCs are associated with the metabolism of L-arginine, providing the substrate for iNOS and arginase-1. MDSCs express high levels of both iNOS and arginase-1, which both play a direct role in suppression of T-cell function [81]. MDSC are elevated in most individuals with cancer and are key producers of IL-10, reducing the macrophage production of IL-12, skewing macrophages towards the M2-phenotype, and contributing to MDSC suppression [80]. Other cell–cell interactions induce STAT3 activation, which adds many different immunosuppressive cytokines to the TME [82]. M2 macrophages also play a significant role in recruiting regulatory T-cells into the TME through the chemokine receptor CCR4 as well as M2-derived CCL17/CCL22 [82]. M2 TAMs also show increased programmed cell death 1 ligand 1 (PD-L1), also known as B7-H1, and increased cytotoxic T-lymphocyte antigen 4 (CTLA4) ligand expression. Both PD-L1 and CTLA4 are well-characterized immune checkpoints for

cytotoxic T-cells, inhibiting their ability to eliminate cancer cells [83-85]. In studies from patients with hepatocellular carcinoma (HCC), elevated levels of PD-L1 expression by TAMs correlated with poorer clinical outcomes compared to patients with lower PD-L1 expression [86]. Glioblastoma patient samples showed TAMs having in-creased expression of PD-L1 compared to circulating monocytes, which had minimal PD-L1 expression. Circulating monocytes with low levels of PD-L1 expression were co-cultured with the glioblastoma cancer cell line U251, both through direct contact between cells and by using a 0.2μm filter. After 24h, the number of PD-L1 expressing cells increased by more than 2-fold (48.0 ± 5.2% vs. 13.0 ± 3.9%) through the 0.2μm filter and more than 4-fold (83.9 ± 6.2% vs. 13.0 ± 3.9%) in direct cell-to-cell contact [86]. In a mouse model of Lewis lung carcinoma (LLC), tumor-infiltrating T-cells in LLC grown in mice were analyzed to determine how TAMs affected the defense response. It was found that CD4 and CD8α T-cell infiltration was significantly increased after tumors were depleted of TAMs [83].

Additional immunosuppressive surface ligands expressed by M2 TAMs include the PD-L1 [84] and B7-H4 [85] immunosuppressive surface ligands. In a study of human gastric cancer patient tissue, PD-L1 and B7-H4 expression on circulating monocytes was significantly higher than normal tissue controls, and advanced stage tissues experienced higher levels of B7-H4 expression than earlier stage cancers on circulating monocytes [84]. Further studies showed that B7-H1 expression was significantly higher on TAMs in the gastric cancer tissues than circulating monocytes [84]. $B7^{-}H4^{+}$ TAMs were shown to suppress $CD4^{+}$ T-cell proliferation and IFN-γ secretion more than $B7^{-}H4^{-}$ TAMs [84], leading to greater immune evasion and suppression by TAMs. After surgical removal of the whole gastric tumor, B7-H4 expression decreased substantially, from 7.9 ± 6.9% to 2.8 ± 1.3% one month after surgery [84]. Finally, *in vitro*, it was demonstrated that gastric cell lines could induce B7-H4 expression on monocytes. Two

gastric cancer cell lines, MKN-45 and MKN-74, were directly cocultured with PBMCs for 24h, resulting in a significant upregulation of B7-H4 expression. In an indirect coculture using supernatants from both cell lines, this upregulation was not observed, indicating that direct cell-to-cell contact was necessary for this induction [84].

Proliferation

Uncontrollable proliferation is one of the hallmarks of cancer. TAM M2 macrophages express molecules that can directly affect cancer cell proliferation including members of the fibroblast growth factor (FGF) family, namely transforming growth factor beta (TGF-β) and epidermal growth factor (EGF) [75,87]. The epidermal growth factor is a tyrosine kinase that typically plays an important role in normal physiological conditions, causing downstream activation of molecules that allow for the avoidance of apoptosis, the promotion of proliferation and invasion, and metastasis [88]. In many human cancers, EGFR is overexpressed, leading to an increase in cell proliferation, angiogenesis, metastasis, and inhibition of apoptosis [89]. Fibroblast growth factors and their receptors lead developmental signaling pathways responsible for cell survival, migration, and proliferation [89]. TGF-β is a growth regulatory protein that typically inhibits cell proliferation. In cancer, there is a marked upregulation of TGF-β, and this is linked to advanced stages of cancer and decreased survival rates [90]. Cancer cells often lose their response to the inhibitory proliferative effects of TGF-β. The additional functions of TGF-β include angiogenesis and immunosuppressive effects, allowing for immune evasion and metastasis [90].

A study done on the effects of M2 macrophages in an orthotopic nude mouse model of liver cancer showed that M2 macrophages injected into the liver promoted tumor growth,

increasing the tumor volume by 3.26-fold compared to the negative control. Injected M1 macrophages showed a 2.31-fold decrease compared to the control [30].

Lymphangiogenesis, Angiogenesis, and Metastasis

Angiogenesis is an essential process for the survival of malignant tissue, providing nutrients and oxygen for growth. While angiogenesis theoretically provides all necessities for tumor survival, tumor angiogenesis is not a perfect process, leading to many dysfunctional vessels and the perpetuation of hypoxia [91]. TAMs play a key role in tumor angiogenesis and have been described in animal models of ovarian cancer, cervical cancer, prostate cancer, breast cancer, and melanoma [87]. TAMs can sense hypoxia in tumors and react with the production of VEGFA, which can stimulate the chemotaxis of endothelial cells and macrophages and lead to an elevated expression of MMP9 from TAMs [87]. This elevated MMP9 mediates extracellular matrix degradation and the release of bioactive VEGFA [87]. Hypoxia is strongly associated with adverse prognosis in cancer, and hypoxia pathways are frequently activated during cancer development. In non-small cell lung cancer (NSCLC), TAMs were shown to enhance tumor hypoxia in mouse subcutaneous tumors and in patients. In mouse models of NSCLC, TAMs exhibited increased gene expression in hypoxic pathway-signaling molecules Vegfa, Slc2a1, Pdk1, and Cxcr4. Interestingly, M1 marker Nos2 was upregulated, and M2 marker arginase-1 was also upregulated, indicating a mixed phenotype in angiogenesis-promoting TAMs [90]. TAMs can also influence hypoxia in the TME because of their aberrant, pro-angiogenic factor secretion, leading to leaky blood vessels that lose normal structure and function. These blood vessels are often leaky, with loose endothelial junctions, defective basement membranes, and

lacking pericyte coverage. Macrophages and hypoxia exist in a positive feedback loop, as hypoxia drive TAM polarization, and TAMs drive hypoxia through poor vessel formation [86]. The epithelial–mesenchymal transition (EMT) is a process where epithelial tumor cells lose their epithelial characteristics and gain mesenchymal function [92]. This transition contributes to overall metastasis through increased invasiveness and motility of the cancer cells themselves [93]. This EMT process enables cancer cells to leave the tissue site, enter the bloodstream, and infiltrate other body sites.

M2-like macrophages play an important role in EMT during cancer progression [94]. They can induce EMT through various signaling pathways such as the TLR4 and IL-10 pathways [95], the TGF-β/Smad2 pathway [95], and the miR-30a/NF-κB/Snail [96] signaling pathways. Additionally, high expression of M2 marker CD68 has been linked to loss of E-cadherin expression, an essential tumor suppressor protein that prohibits EMT and metastasis [92].

M2 macrophages play a key role in the initiation of metastasis through the secretion of pro-angiogenic cytokines and growth factors [97] (Table 3). Neovascularization of the tumor microenvironment is crucial for not only nutrient supply, but also for initiation of metastasis, as cancer cells can enter the bloodstream and travel throughout the body to establish foothold in other areas. The angiogenic involvement of M2 macrophages can be further subcategorized into M2a, or alternatively activated macrophages, and M2c, or regulatory macrophages. M2a-induced angiogenesis is regulated by fibroblast growth factor (FGF) signaling, and the M2c-induced angiogenesis is regulated by placental growth factor (PlGF) signaling [97]. TGF-β also plays a key role in the angiogenic progression of malignant cells. Early in tumor development, TGF-β is a tumor suppressor factor and it inhibits proliferation and induces apoptosis. Tumor cells eventually overcome the TGF-β-induced suppressive effects, and TGF-β induces the epithelial–

mesenchymal transition, (EMT) which facilitates invasion and metastasis. Overexpression of TGF-β is reported in many human cancers and is constitutively expressed by M2 TAMs. This overexpression is correlated with tumor progression, metastasis, angiogenesis, and poor prognosis [98]. Additionally, M2-like macrophages promote blood vessel formation through their close association with endothelial cells in the TME. M2-macrophages have been found to co-localize with these endothelial cells at the branching points and merge into tubes to become part of the tubular network [97].

Factors Secreted by M2 TAMs	**Pro-Tumorigenic Outcome**
EGF, IL-8, IL-10, CCL2	Tumor growth
IL-10, TGF-b, MMP-7, PD-1, PDE-2, arginase	Immune suppression
CCL18, CCL22, MMPs, TGF-b, EGF, CCL20, IGF-1	Tumor invasion and metastasis
VEGFA, PDGF, COX2, HIF, MMPs, IL-10, adrenomedullin	Tumor angiogenesis and lymphangiogenesis
TGF-b, MMPs, IL-6, IL-10	Anti-cancer therapy resistance

Table 3. Soluble factors secreted by M2-polarized TAMs, influencing various pro-tumor outcomes.

TAMs support tumor lymphangiogenesis by secreting pro-lymphangiogenic factors and by trans-differentiating into lymphatic epithelial cells. TAMs produce matrix metallopeptidase 9 (MMP9) abundantly, which leads to the development of lymphatic vessels [87] in addition to VEGFR-3 and its ligands VEGF-C and VEGF-D, leading to lymphangiogenesis. TAMs can also integrate directly into peritumoral lymphatic vessels, where they lose their macrophage functions and become a part of the lymphatic vessel wall. Indirectly, TAMs produce the enzymes plasmin,

urokinase plasminogen activator, and MMP, which regulate matrix remodeling and growth factor regulation [5, 99]. Similar to angiogenesis, lymphangiogenesis provides an additional avenue for malignant cells to travel through the body and establish footholds in other areas [100].
Both lymphangiogenesis and angiogenesis can play key roles in the initiation of metastasis, allowing malignant cells to travel via new formed vessels to various tissues in the body and causing complications in therapy as well as decreased survival [89,100]. Understanding the role of TAMs in these processes allows for the development of therapeutic targets that inhibit metastasis through these mechanisms.

Proposed therapies to treat macrophage polarization

The complex biology of TAMs and their involved role in tumor proliferation, angiogenesis, EMT, metastasis, immune suppression, and therapy resistance makes developing anti-cancer therapies that consider all mechanisms of pro-tumor activity difficult. Some therapies aiming to either reprogram TAMs toward an M1 phenotype, kill existing TAMs, or inhibit the recruitment of new TAMs have been developed. CSF-1 has been described as the most important tumor-derived factor leading to monocyte recruitment through CCL2/CCR2 interaction, so CCR2 blockade therapies have been effective in suppressing TAM recruitment [101]. Both CCR2 inhibitors and anti-CCL2 monoclonal antibodies have been used in pre-clinical murine models to disrupt this CCL2/CCR2 interaction, showing efficacy both on their own and in combination with other anti-cancer therapies [102,103]. While this has shown promise, in murine breast cancer models, a rebound effect was shown after the withdrawal of anti-CCL2 treatment, increasing the infiltration of bone-marrow monocytes into the tumor and accelerating lung metastasis [104]. Another key pathway in monocyte recruitment and differentiation into TAMs is

the CXCL12/CXCR4 interaction. In breast cancer, expression of CXCL12 by tumor cells increased the number of macrophages and blood vessel density, contributing to metastasis [105]. Inhibition of CXCR4 with AMD3100 reduced the formation of metastasis [106].

Depletion of TAMs is another therapy group being developed to help combat the multifaceted functions of TAMs in tumor progression and resistance. CSF-1 or CSF-1R expression in the TME has been associated with poor prognosis in many types of cancer. Because CSF-1 plays a key role in the proliferation and survival of monocytes and macrophages, the CSF-1/CSF-1R interaction is an attractive target for reducing the number of TAMs. A monoclonal antibody, emactuzumab, targets CSF-1R, decreasing the number of TAMs and increasing the $CD8^+/CD4^+$ T-cell ratio in the TME in a pre-clinical mouse model [107]. Small molecule CSF-1R blockades such as PLX3397 have been developed as well and have shown increased $CD8^+$ T-cell infiltration and improved therapy response in murine models of several different tumor types [108]. While targeting the CSF-1/CSF-1R pathway appears to be an attractive target, some studies have shown that long-term CSF-1R inhibition can lead to activation of the PI3K pathway and therapy resistance over time. A combination PI3K blockade and CSF-1R inhibition has shown positive results in pre-clinical trials [109].

Reprogramming M2 TAMs toward a more pro-inflammatory M1 phenotype is the third category of anti-TAM cancer therapy. Toll-like receptors are key players in M1 programming, as upon binding of a ligand to these receptors, macrophages are activated and exhibit an M1 phenotype [110]. Targeting of TLR3, TLR7, TLR8, and TLR9 have all been evaluated in the past few years but currently, a TLR7 agonist, imiquimod, is the only FDA-approved, topical-only treatment for squamous and basal cell carcinomas [111]. TLR3 stimulation with poly I:C has been shown to be more effective than imiquimod in reprogramming M2 TAMs to an M1

phenotype [112], but this type of treatment has not yet been fully developed. A nanoparticle containing poly I:C targeted for M2 TAMs was developed and *in vitro*, TNF-α and iNOS expression was upregulated and NO secretion was increased [113].

Tumor-associated macrophages play a key role in the development, metastasis, and reoccurrence of human malignancies, contributing to nearly every step of tumorigenesis. TAMs contribute to malignant cell proliferation, inflammation, host cell immunosuppression, angiogenesis and lymphangiogenesis, and therapy resistance. While there is still much to understand about macrophage regulation in the tumor microenvironment, it may prove to be a potentially effective anti-tumor therapeutic target if we gain the ability to control the switching of tumor-associated macrophages from M2 to M1 phenotypes.

Waldenström macroglobulinemia

Waldenström macroglobulinemia (WM) is a rare, indolent B-cell malignancy, characterized by the infiltration of plasma cells, plasmacytoid lymphocytes, and small lymphocytes to the bone marrow [114-116]. The median age of diagnosis is 63-68 years old [117] and this disease accounts for 1-2% of hematological neoplasms, with an age-adjusted incidence rate of 3.4 per million among male and 1.7 per million among female populations in the United States [117]. The World Health Organization defines WM as a lymphoplasmacytic lymphoma (LPL) with an immunoglobulin M (IgM) paraprotein [115, 118]. The most frequently observed cytogenetic abnormality in WM is deletion of the long arm of chromosome 6 (6q). This deletion is correlated with poor prognostic features, such as higher levels of beta2-microglobulin and a greater prevalence of hypoalbuminemia and anemia [117, 119]. There is no standard treatment for WM and treatment programs are variable. To date, no cure has been discovered for

WM [120]. Because most WM/LPL patients eventually relapse, there is a need to balance benefit of treatment and the side effects of that treatment. Some patients with WM will relapse with the aggressive form of non-Hodgkin lymphoma [121, 122]. Approximately 75% of WM patients have symptoms at the time of diagnosis and anemia is common, as WM cells growth in the bone marrow progresses [115]. Hyperviscosity syndrome, caused by abnormal IgM secretion and accumulation in the blood, occurs in 10-30% of patients, and may be life-threatening [115]. Lymphoma cells typically express CD19, CD20, CD22, CD79α and lack CD5, CD10, and CD23 which aids in discriminating WM from follicular lymphoma, chronic lymphocytic leukemia (CLL), and mantle cell lymphoma (MCL). It is almost always possible to document CD138+ plasma cells in WM either by immunohistochemistry or flow cytometry. The expression of CD5, CD10, and CD23 may be found in 10-20% of lymphoma cases and doesn't exclude the diagnosis of WM. WM has to be separated from the CD5+ lymphoplasmocytoid lymphoma as these cases are B-CLL variants. A diagnosis of WM requires the differentiation from IgM myeloma, which is a rare disease presenting as a homogenous plasma cell population in the bone marrow, characterized by symptomatic clonal plasma cell proliferation, 10% or more plasma cells on bone marrow biopsy, plus the presence of lytic bone lesions and/or translocation.

Tumor Microenvironment in WM

The tumor microenvironment has recently become an emerging area of research, with a growing number of studies looking at the tumor microenvironment in not only WM, but in other cancers as well. Homing to the bone marrow is a key characteristic of WM and the mechanisms by which WM cells home to the bone marrow have been investigated. Chemokine stromal

derived factor-1 (SDF-1) is highly expressed in the bone marrow of WM patients and is involved in increased migration by WM cells *in vitro* [123].

The bone marrow is made up of a collection of immune and non-immune cells, including T-cells, B-cells, macrophages, myeloid-derived suppressor cells, mast cells, mesenchymal stem cells, osteoclasts, osteoblasts, natural killer cells, and dendritic cells (Figure 3) [124]. While the full function and mechanism of these cells in the progression of WM has not been described, some efforts have been made to quantify the importance of these cells in WM prognosis. Recently, reports on the role of mast cells, T-cells, monocytes, and endothelial cells in WM have been published. Mast cell hyperplasia is a characteristic of WM. It has been previously demonstrated that mast cells in the bone marrow of WM patients induce proliferation of malignant B cells through CD40L and CD40 interactions [125].

T-cells have also been examined in WM and the expression of PD-1 and the ligands PD-L1 and PD-L2 have been characterized. PD-L1 and PD-L2 gene expression was induced by IL-21, interferon-γ, and IL-6 expression in WM cell lines and patient bone marrow cells. Increased expression of PD-L1 and PD-L2 in the bone marrow of WM patients increased the proliferation of malignant B cells and reduced T-cell proliferation [126].

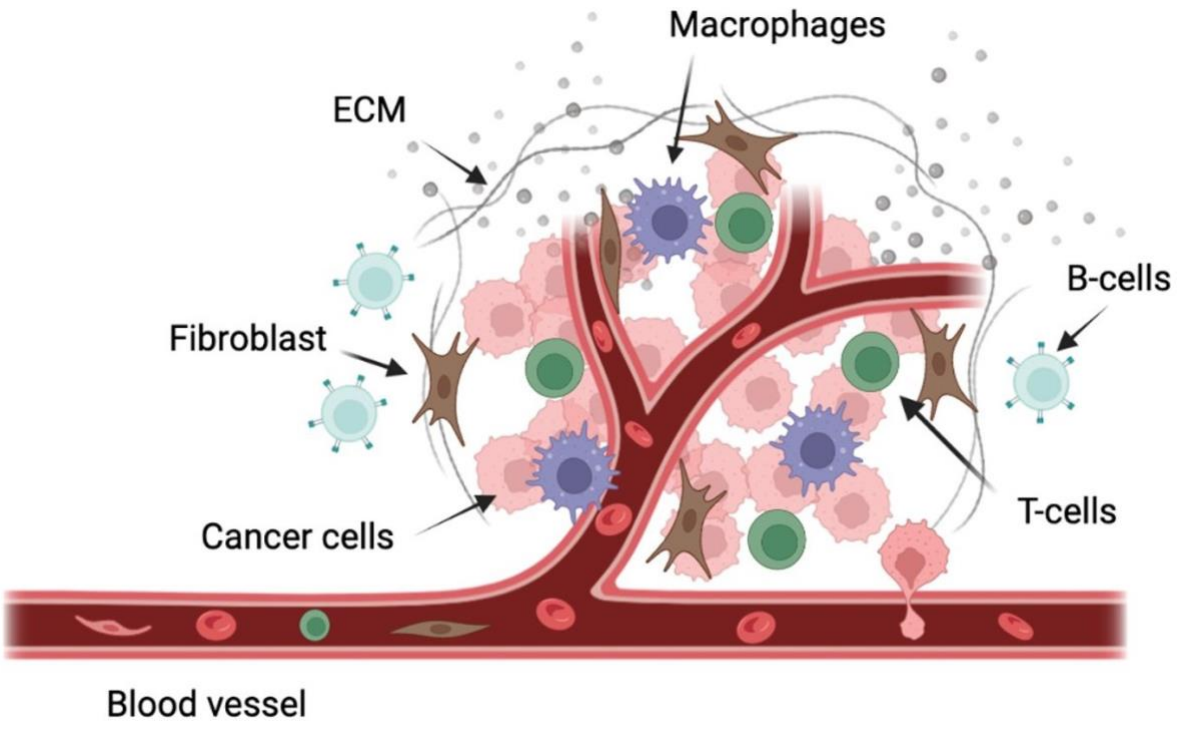

Figure 3: Schematic representation of some of the cells present in the WM tumor microenvironment. This figure was generated using www.biorender.com.

Bone marrow stromal cells (BMSC) are a heterogenous population that have been shown to play an important role in normal and malignant cell biology [127]. Mesenchymal stem cells (MSCs) serve as the progenitor for most bone marrow stromal cell populations, including osteoblasts, chrondrocytes, fibroblasts, endothelial cells, and myocytes [128]. In WM, BMSCs have been shown to regulate the proliferation of tumor cells while contributing to increased drug therapy resistance [129].

Endothelial cells have been shown to increase WM cell adhesion and proliferation through the Ephrin receptor B2 (Eph-B2), which is found upregulated on WM cells [130]. The Eph-B2 receptor was found to be activated in WM patients compared with healthy samples. Endothelial cells in the bone marrow express high levels of Ephrin-B2 ligand. Blocking of either

Ephrin-B2 or Eph-B2 inhibited the increased adhesion and proliferation caused by the endothelial-WM cell interaction [129].

Mechanisms of Disease Progression in WM

Proliferation

IL-21 is a type I cytokine commonly found in the WM tumor microenvironment that rapidly induces the phosphorylation of STAT3 in WM cells [130]. MWCL-1 cells cultured in IL-21 for 72 hours displayed a dose-dependent increase in cellular proliferation and phosphorylated STAT3 levels [130]. In MWCL-1 cells, stimulation with IL-21 for 10 minutes yielded significant increases in the phosphorylation of STAT3 [130]. Treatment with a STAT3 inhibitor eliminated the IL-21-mediated increase in proliferation [130].

Fibroblast growth factor receptor 3 (FGFR3) is a member of the FGFR family that interacts with fibroblast growth factor 3 (FGF3), inducing a cascade of downstream signals that influence cell proliferation. This is well documented in many types of cancer, including tongue, colorectal, breast, bladder, and oral cancers [131-136]. In WM, the expression of FGFR3 on $CD19^{+}$ cells from WM patients was greater than expression on B cells from healthy subjects and FGFR3 was also overexpressed in the cell lines BCWM.1 and MEC-1 [137].

In cancer, overexpression of the Akt and mTOR pathways play an important role in the progression of malignancies through the phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway. This pathway can enhance cell survival by inhibiting cell death and stimulating cell proliferation [138,139]. The activation of this pathway ultimately leads to growth, angiogenesis, resistance to apoptosis and therapy resistance [140,141]. In WM, constitutive activation of the PI3K/Akt pathway exists and leads to increased cell proliferation

and resistance to apoptosis [142]. Phosphatases and tensin homolog (PTEN) is a haploinsufficient tumor suppressor, therefore partial loss-of-function mutations can have a dramatic effect on cancer progression. PTEN acts to deactivate the PI3K/Akt/mTOR pathways, therefore loss-of-function can lead to constitutive activation. Studies in mouse models have shown that even a small reduction in PTEN expression can significantly increase cancer risk [143,144]. Unfortunately, PTEN loss-of-function mutations are frequent in human cancers, leading to the perpetual activation of AKT. Furthermore, the role of PTEN in WM has not been reported.

IL-6 is known to play an important role in normal B cell proliferation and maturation as well as in B-cell malignancies including diffuse large B-cell lymphoma [145], Hodgkin lymphoma [146], and multiple myeloma [147], where it has been shown to regulate the growth of malignant cells. Previous studies have shown that serum IL-6 levels are in-creased in patients with WM compared to healthy patients [148]. IL-6 has shown a significant upregulation of IgM secretion by WM cells through the CCL5-IL-6-IgM axis in the TME [149,150]. CCL5 signaling has been shown to induce expression of the transcription factor GLI2 through the PI3K-AKT-IκB-p65 pathway. GLI2 is required to modulate IL-6 expression in vitro and in vivo through this pathway [151]. Targeting the IL-6 receptor with Tocilizumab to block IL-6 effects on WM tumor cells was shown to reduce IgM levels and deter tumor growth in WM, while not inducing toxicity [152]. This suggests that blocking IL-6 may provide therapeutic efficacy in WM. Despite this, targeting IL-6 in WM patients has not been investigated.

The role of bone marrow stromal cells has been extensively studied in WM and are attributed to the growth of WM cells [142, 152-154]. Ephrin-B2 was demonstrated to be highly expressed on endothelial cells from the bone marrow of patients with WM com-pared with

healthy controls [129] and activation of the Eph-B2 receptor did not directly increase the proliferation of WM cells, but it increased adhesion of WM cells to endothelial cells, promoting WM cell proliferation [155]. This increase in WM cell proliferation is dependent on downstream activation of focal adhesion kinase (FAK) and Src and inhibition of ephrin-B2 on endothelial cells or inhibition of Eph-B2 on WM cells reduced the adhesion of WM cells to endothelial cells, preventing the proliferative induction from occurring [155].

B-lymphocyte stimulator (BLyS) is a TNF family member expressed by dendritic cells, neutrophils, monocytes, and macrophages and has been shown to be necessary for normal B-cell development. BLyS binds to the receptors B-cell-activating factor of the TNF family receptor (BAFF-R), transmembrane activator and CAML interactor (TACI) and B-cell maturation antigen (BCMA) on WM patients. Expression of BLyS in WM patient bone marrow and elevated serum BLyS levels have also been noted, as well as upregulated IgM secretion upon BLyS addition. In vitro, BLyS was shown to enhance proliferation and survival of WM cells [156].

Bone marrow mast cells are commonly associated with malignant cells in patients with WM. CD40 ligand (CD40L/CD154) is an inducer of B-cell proliferation and is expressed on malignant cell-associated mast cells in 94% of WM patients, in contrast with 0% of healthy patient mast cell samples. It was found that co-culture of mast cells and lymphoplasmacytic cells (LPC) induced LPC proliferation and tumor colony formation [125]. Increased Erk phosphorylation and cell growth in malignant B-cells co-cultured with CD40L-expressing stromal cells has also been reported. GLI2 induced increased CD40L expression and GLI2 knockdown decreased CD40L expression. GLI2 has been shown to directly bind to and regulate the activity of the CD40L promoter [157].

Survival

Myeloid differentiation factor 88 (MYD88) L265P somatic mutation is frequent in WM, with whole genome sequencing results showing 91% of WM/LPL patients expressed MYD88L265P [158,159]. The presence of MYD88L265P has also been reported in IgM monoclonal gammopathy of undetermined significance [160], mucosa-associated lymphoid tissue lymphoma (9%) [161] and diffuse large B-cell lymphoma [162]. Inhibition of MYD88/IRAK signaling induced apoptosis of MYD88L265P-expressing WM cells. This was done using a cell-permeating peptide that blocks MYD88 homodimerization which is essential for IRAK1 and IRAK4 signaling. This treatment induced significant apoptosis in BCWM.1, MWCL-1 cell lines as well as primary WM patient cells. Induction of apoptosis did not occur without the MYD88L265P mutation [158]. Due to the activation of NF-kB, increased anti-apoptotic Bcl-xL expression has been observed in both MYD88L265P and MYD88 L265RPP mutations, promoting increased survival of malignant cells [163].

Angiogenesis

Angiogenesis plays an essential role in wound healing and bone repair and regeneration. This process forms new blood vessels from existing ones, which allow the body to re-establish normal blood flow, oxygen/nutrient/growth factor delivery to the injured or proliferating area [164-167]. In cancer, tumor cells can develop an angiogenic phenotype through the up-regulated pro-angiogenic or down-regulated anti-angiogenic pathways [168, 169]. This causes endothelial cells to enter a rapid growth phase, forming new blood vessels, providing nutrients, oxygen, and growth factors to the tumor cells [170]. This process is often rushed in cancer and endothelial cells do not have the time to form perfect blood vessels, leading to leaky, disorganized blood

vessels [171,172]. This an essential step of disease progression and serves to initiate the process of metastasis in many types of cancer [124, 171]. VEGF is a well-established growth factor, known for its role in both physiological and pathological angiogenesis. VEGF-A is the main member of the VEGF family and plays a key role in promoting angiogenesis during embryonic development and tissue repair under physiological conditions [167]. In cancer, VEGF-A production from tumor cells results in an angiogenic switch, leading way to vasculature growth and as a result, tumor growth and metastasis [167]. As the tumor mass increases, the oxygen availability of decreased and hypoxia occurs, leading to the release of proangiogenic factors such as VEGF-A [166]. Angiopoietin-1 (Ang-1) and its antagonist, angiopoietin-2 (Ang-2) are ligands for receptor tyrosine kinase Tie-2 and are also essential for angiogenesis in physiological and malignant conditions [173]. Fibroblast growth factors (FGF) are a family of heparin-binding growth factors. Basic FGF (bFGF) interacts with endothelial cell surface receptors and has pro-angiogenic activity [174]. The crosstalk between bFGF, VEGF and other inflammatory cytokines plays an important role in mediating angiogenesis in the tumor microenvironment.

In WM, the bone marrow microvessel density is only elevated in 30-40% of patients [175]. In a study of 56 patients with WM, it was reported that increased levels of angiogenin, vascular endothelial growth factor (VEGF), vascular endothelial growth factor A (VEGFA), and basic fibroblast growth factor in sera of patients, compared with healthy controls [176]. A lower level of the angiogenesis antagonist, angiopoetin-1 (Ang-1), was also reported in WM sera versus healthy controls [176].

Hypoxia

Hypoxia plays an important role in the progression of many malignancies and activated hypoxia pathways are strongly associated with adverse prognosis in cancer [124]. Tumor hypoxia in multiple myeloma activates HIF1α that promotes cell survival, motility, invasiveness, drug resistance, and neoangiogenesis [177,178] and is associated with a more aggressive tumor [179]. In multiple myeloma, the egress of bone multiple myeloma cells from the bone marrow into the circulation and into new niches was also demonstrated [180].

In a study demonstrating hypoxia in WM cells, the WM cell line, BCWM.1, was genetically engineered to express luciferase and mCherry fluorescent protein. The cells were injected into SCID mice via the tail vein and allowed to grow for 3 weeks to establish tumor burdens in the bone marrow of the mice [179]. This growth in the bone marrow was confirmed by flow cytometry. The mean fluorescent intensity (MFI) of hypoxia marker pimonidazole hydrochloride signal was analyzed and a direct correlation between the tumor burden in the bone marrow and hypoxia in the WM cells was found. Other cells in the bone marrow were tested for hypoxic signs as well and found that the mCherry-negative population was less hypoxic than the WM cells, but still showed hypoxic signs, and hypoxic signs were more greatly shown at higher tumor burdens [179]. In addition, the effect of tumor hypoxia on the egress of WM cells from the bone marrow was tested and a direct linear correlation between the hypoxia in the bone marrow and the number of circulating WM cells was found [179]. This indicated that the mechanism of WM cell entry to the circulation is regulated by hypoxia.

Hypoxia also plays a major role in regulating WM cell proliferation. BCWM.1 and MWCL.1 WM cell lines were exposed to normoxic and hypoxic conditions for 24 hours in vitro and found that after 24 hours of normoxia, the BCWM.1 and MWCL.1 cells had nearly doubled,

and the hypoxic cells only increased by 1.3-fold [179]. This suggests that hypoxic conditions do not promote WM cell growth but play a role in other aspects of WM biology.

Epithelial-Mesenchymal Transition

The epithelial-mesenchymal transition (EMT) is a process where cells lose their epithelial characteristics and gain mesenchymal characteristics [181]. This process can lead to increased invasiveness of the cancer cells, leading to overall metastasis [182]. This process allows cancer cells to leave the primary tissue site, enter the bloodstream, and infiltrate other tissues [125].

In a study of WM cells and hypoxia, the effect of hypoxia on the expression of EMT markers E-cadherin, CXCR4, and VLA-4 was assessed using flow cytometry. BCWM.1 cells were exposed to either normoxic or hypoxic conditions for 24 hours, then analyzed for expression of EMT markers by flow cytometry [179]. Additionally, in a study looking at the effect of Eph-B2 in WM cells, it was found that inhibition of Eph-B2 on WM cells reduced bone marrow infiltration by WM cells [155]. This indicates that the expression of Eph-B2 is essential to the bone marrow infiltration of WM cells, which leads to disease progression.

The adhesion ability of WM cells to bone marrow stromal cells and to each other was assessed *in vitro* and incubation of BCWM.1 or MWCL.1 cells in hypoxic conditions reduced their adhesion to a bone marrow stromal cell monolayer by 50% and 25%. This decrease in adhesion was linked to reduced expression of the epithelial marker E-cadherin in WM cells [179].

Metastasis

Ephrin receptors (Eph) represent the largest family of receptor tyrosine kinases (RTK) and are divided into 2 classes: Eph-A and Eph-B, depending on their affinity to ligands ephrin-A and ephrin-B [183]. These receptors are important in embryogenesis and development but are rarely found in adult tissue [184]. The Eph receptors and ephrin ligand serve as a guide during embryogenesis to position cells and modulate cell morphology [185]. EphA1/A2 and ephrin-A1 have been correlated with tumor malignancy and prognosis but can be over-expressed or down-regulated in various types of cancer. For example, higher ephrin-A1 expression in liver and colorectal cancer is associated with worse prognosis [186,187], but in stage I non-small cell lung cancer patients, higher expression levels of EPHA2 and ephrin-A1 improved their prognosis [188]. In WM patient samples, Eph-B2 receptor was overexpressed in primary WM cells and inhibiting ephrin-B2 on endothelial cells led to decreased adhesion of WM cells to endothelial cells and decreased proliferation, cell-cycle progression, signaling, and tumor progression in WM cells [130].

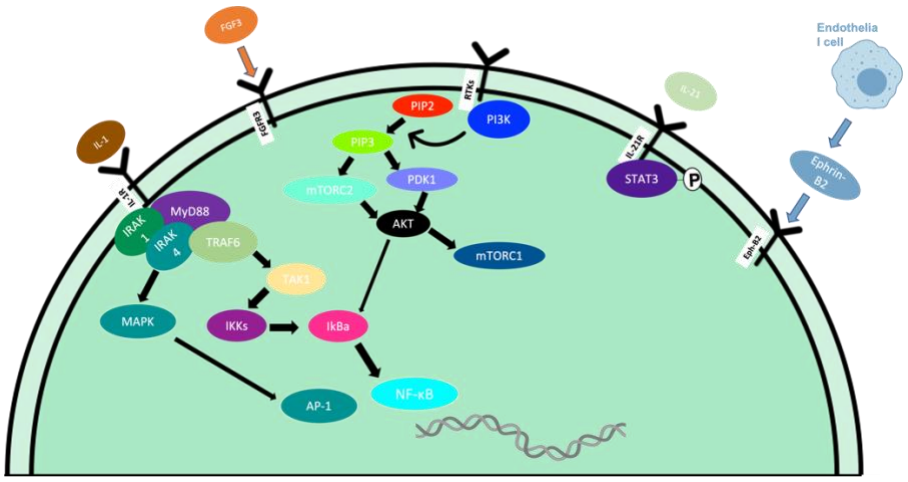

Figure 4: Signaling pathways contributing to tumor progression in WM.

Treating WM

There is no standard therapy for the treatment of WM [137] and only two FDA approved treatments, Zanubrutinib and Ibrutinib, exist [189]. Most treatments were originally derived from other lymphoproliferative diseases such as multiple myeloma and chronic lymphocytic leukemia [190].

Due to the crucial role of B cell receptor (BCR) signaling in B cell development and pathogenesis of B cell malignancies, efforts to drug the BCR signaling pathway has been extensively researched for treatment of B cell malignancies [191]. Bruton's tyrosine kinase (BTK) is a key component of BCR signaling, making BTK an important therapeutic target. Several BTK inhibitors have shown remarkable results in treating other B cell malignancies, such as chronic lymphocytic leukemia (CLL) [192,193], mantle cell lymphoma (MCL) [192], marginal zone lymphoma (MZL) [194], and Waldenström macroglobulinemia (WM) [194].

GLI3

The transcription factor GLI3 is a member of the GLI family and is classically regulated by the hedgehog (Hh) signaling pathway. Gli3 can exist as a full length (Gli3-FL) or repressor (Gli3-R) form. Gli3 targets the Gli1 promoter upon activation of Hh signaling and is phosphorylated and partially degraded when Hh is inactive. Gli3 is important in embryonic development and has shown important roles in tissue, brain, and lung development. Additionally, Gli3 regulates B-, T-, and NK-cells and has been shown to play a role in inflammation through LPS-TLR4 signaling. Gli3 has also been shown to play a role in cancer and has been shown upregulated in multiple cancers. Gli3 influences pro-tumorigenic behaviors such as anchorage-independent growth, angiogenesis, proliferation, and migration [195].

Methods

Cell lines and human monocytes

BCWM.1 cells were provided by Dr. Steven Treon (Dana Farber Cancer Institute, Boston, MA). MWCL-1 cells were provided by Dr. Stephen Ansell (Mayo Clinic, Rochester, MN), and RPCI-WM1 cells were provided by Dr. Asher Chanan-Khan (Mayo Clinic, Jacksonville, FL). WM cell lines were maintained in RPMI 1640 supplemented with 10% FBS and 1% antibiotic-antimycotic (AA). Cells were passaged every 3-4 days. THP-1 cells were purchased from ATCC (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% FBS, 1% AA and 1% L-glutamine.

Primary monocytes were sorted from leukoreduction cones obtained from healthy donors from the Oklahoma Blood Institute (OBI). Cells were diluted with DPBS (1:5) and separated on a Ficoll-hypaque gradient as previously published [196] to isolate peripheral blood mononuclear cells (PBMCs). Monocytes were isolated from total PBMCs by magnetic cell sorting using the EasySep human buffy coat $CD14^+$ negative selection kit (Stemcell Technologies, Seattle, WA) according to the manufacturer's protocol.

Mouse bone marrow derived macrophages (BMDMs)

BMDM were isolated and generated from C57BL6/J mice as previously published [197]. Briefly, bone marrow was flushed from mouse femurs and tibias and was plated in 85% RPMI 1640 supplemented with 10% FBS and 1% antibiotic-antimycotic (AA) plus 15% L929 media to generate BMDMs.

We have previously generated mice with conditional knockout of Gli3 in myeloid cells (*M-Gli3*$^{-/-}$) [198]. Total bone marrow cells were harvested from femurs and tibias post-mortem under sterile conditions.

For murine bone marrow derived macrophage differentiation, C57BL/6J mice were euthanized and the bone marrow was harvested. Bone marrow cells were then plated in 6 well plates at $0.25x10^6$ cells per well and treated with media containing 15% L929 and the media was changed on day 4. On day 7, macrophages were successfully generated and ready to use.

Monocyte differentiation

Human THP-1 monocytic cell line was seeded in 6-well plates in 2mL of media at $1x10^6$ cells/well. To differentiate into macrophages, 50 ng/mL phorbol 12-myristate 13-acetate (PMA) was added to the media and incubated for 24 hours. After 24 hours, the cells were washed with complete media to remove PMA and let rest for 24 hours to generate M0 macrophages.

For $CD14^+$ M0 differentiation from monocytes, recombinant MCSF (50ng/mL) was added to the media for 7 days and the medium was supplemented every 2-3 days. After 7 days, $CD14^+$ M0's were washed with complete media to remove MCSF and then used in further experiments.

Macrophage polarization

Both human THP-1 M0 and CD14+ M0 were differentiated into M1 and M2 phenotypes by cytokine treatment after generation of M0 cells. M1 macrophages were generated using 10ng/ml lipopolysaccharide (LPS) and 100 ng/ml interferon-gamma (IFN-γ). M2 macrophages

were generated using 20 ng/mL recombinant human interleukin-4 (IL-4) and 20ng/mL recombinant human interleukin-13 (IL-13).

Murine macrophages were generated on day 7 post-harvest. M1 macrophages were generated using 1 µg/mL LPS and 300 ng/mL IFN-γ, while M2 macrophages were generated using 40ng/mL murine recombinant IL-10 and 40 ng/mL murine recombinant IL-13. All recombinant proteins were purchased from Peprotech (Cranbury, NJ).

RNA isolation and RT-qPCR

Total RNA was extracted using TRIsure reagent (Bioline, London, UK), according to the manufacturer's instructions. Briefly, 1mL of TRIsure was added to cells upon harvest and incubated for 5 minutes at room temperature. Then, 0.2mL of chloroform was added to each sample, spun, and incubated for 10 minutes at room temperature. These samples were then spun at 12,000xg for 15 minutes at 4°C, and the aqueous phase was transferred to a new tube. Isopropyl alcohol was added to the aqueous phase and re-spun at 12,000xg for 15 minutes at 4°C. The supernatant was discarded upon the identification of a pellet. The pellet was then washed with ethanol, spun at 12,000xg for 5 minutes at 4°C. The ethanol was discarded, and the pellets were set to dry overnight. Once dry, the pellets were resuspended in 20µL nanopure water and RNA concentrations were calculated and the samples were adjusted to the lowest sample concentration using nanopure water. cDNA was synthesized using Promega M-MLV reverse transcriptase following the manufacturer's instructions (Promega, Madison, WI). Briefly, RNA and nuclease free water was added to a final volume of 12 µL and 1 µL of dNTPs (Invitrogen, Waltham, MA) and random decamers (IDT-DNA, Coralville, IA) were added to the tube. The samples were heated to 65°C before adding 4µL 5x M-MLV buffer (Promega, Madison, WI),

1µL nuclease free water, and 1µL M-MLV reverse transcriptase (Promega, Madison, WI) to each reaction. Each reaction was run under a reverse transcription program on a thermal cycler to complete the reaction, and cDNA was then used directly in qPCR.

For M1 and M2 marker expression, quantitative PCR reaction (qPCR) was performed, and the results were analyzed using Applied Biosystems ViiA 7 Real-time PCR Instrument (Life Technologies, Grand Island, NY). The primers used were purchased from IDT-DNA (Coralville, IA). Oligonucleotide primer sequences can be found in Table 4.

Target	**Species**	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
CD206	Human	CTACAAGGGATCGGGTTTATGGA	TTGGCATTGCCTAGTAGCGTA [12]
CCL2	Human	GCCACCTTCATTCCCCAAGGG	GCTTCTTTGGGACACTTGCTGC
CCL22	Human	ATTACGTCCGTTACCGTCTG	TAGGCTCTTCATTGGCTCAG [12]
IL-1β	Human	GGACAGGATATGGAGCAACAA	CCCAAGGCCACAGGTATTT
GAPDH	Human	CTCGACTTCAACAGCGACA	GTAGCCAAATTCGTTGTCATACC
CD163	Mouse	GCAAAAACTGGCAGTGGG	GTCAAAATCACAGACGGAG [13]
ARG2	Mouse	GAAGTGGTTAGTAGAGCTGTGTC	GGTGAGAGGTGTATTAATGTCCG [13]
TNFa	Mouse	CTTCTGTCTACTGAACTTCGGG	CACTTGGTGGTTTGCTACGAC [13]
iNOS	Mouse	CAGCACAGGAAATGTTTCAGC	TAGCCAGCGTACCGGATGA [13]
GAPDH	Mouse	CGTCCCGTAGACAAAATGGT	TTGATGGCAACAATCTCCAC

Table 4. Sequence of the oligonucleotide primers used for qPCR.

Reagents and recombinant proteins

Dimethyl sulfoxide (DMSO) and trypan blue dye were purchased from Sigma-Aldrich and Corning Incorporated, respectively. All recombinant proteins were purchased from Peprotech (Cranbury, NJ), including murine recombinant IL-13, IL-10, and IFN-γ, and human recombinant IL-4, IL-13, IFN-γ, and M-CSF.

Cell viability and proliferation assay

Cell viability was determined using trypan blue exclusion. Cells were counted using a Luna II automated cell counter (Logos Biosystems, Annandale, VA) based on manufacturer's instructions. $CD14^+$ or human THP-1 monocytes (0.5×10^6 cells/well) were differentiated into M0, M1 and M2 phenotypes and then co-cultured with WM cell lines (0.5×10^6 cells/well) in 24-well plates for 120 hours. Every 24 hours, cell viability was analyzed.

Cell proliferation was evaluated using TACS XTT Cell Proliferation Assay (R&D Systems, Minneapolis, MN). $CD14^+$ and human THP-1 monocytes were seeded in 48-well plates at 0.25×10^6 cells/well. Cells were polarized into M0, M1 and M2 phenotypes and WM cells were added (0.25×10^6 cells/well) and co-cultured for 3 days. After 3 days of co-culture, 100µL of WM cells were removed from each well and re-plated in a new, 48-well plate. XTT activator and XTT reagent were then added in a 1:50 ratio and incubated at 37°C for 3 hours. After 3 hours, samples absorbance was determined using a SpectraMax M2e microplate reader (Molecular Devices, San Jose, CA) at 490nm with reference at 630nm.

Statistical analysis

Statistical analysis was performed by using one-way analysis of variance or Student's t test using GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA). For more than 2 variables, a 2-way analysis of variance (ANOVA) was used followed by a Tukey's post-hoc test to determine significantly different groups. Data are presented as mean ± standard error of the mean (SEM), and $p < 0.05$ was considered statistically significant.

Results

Co-culture of THP-1-derived-M0 and M2 macrophages and WM cells increased the proliferation and viability of WM cells

M2 macrophages have demonstrated pro-tumorigenic affects in several other malignancies, although the influence of macrophage polarization in WM has not been investigated. To determine if M1 or M2 macrophages played a role in WM, we generated M0, M1, and M2 macrophages from THP-1 human monocytes as described in materials and methods and co-cultured them for 72 hours in direct contact with WM cell lines, BCWM.1, MWCL-1, and RPCI-WM1, and an XTT assay was performed to determine if there was a change in cell proliferation. Both M0 and M2 populations significantly increased the proliferation of WM cells, and co-culture with M1 macrophages either slightly decreased or did not affect proliferation (Figure 5A). To determine if M0, M1, or M2 macrophages influenced the viability of WM cells, M0, M1, and M2 macrophages were generated from THP-1 human monocytes and co-cultured with WM cell lines, BCWM.1, MWCL-1, and RPCI-WM1, for 72 hours. After 72 hours, the WM cells were harvested and counted via Trypan Blue exclusion. Both the M0 and M2 phenotypes increased the viability of WM cells after 3 days of co-culture, while the M1 phenotype did not affect cell viability (Figure 5B). To ensure that M1 and M2 macrophages were successfully polarized to their respective phenotypes, qPCR of these phenotypes was performed using known M1 and M2 markers. The M1 markers, CCL2 and IL-1ß, were used to confirm that M1 macrophages were successfully polarized (Figure 5C) and the M2 markers, CCL22 and CD206, were used to confirm that M2 macrophages were successfully polarized (Figure 5D).

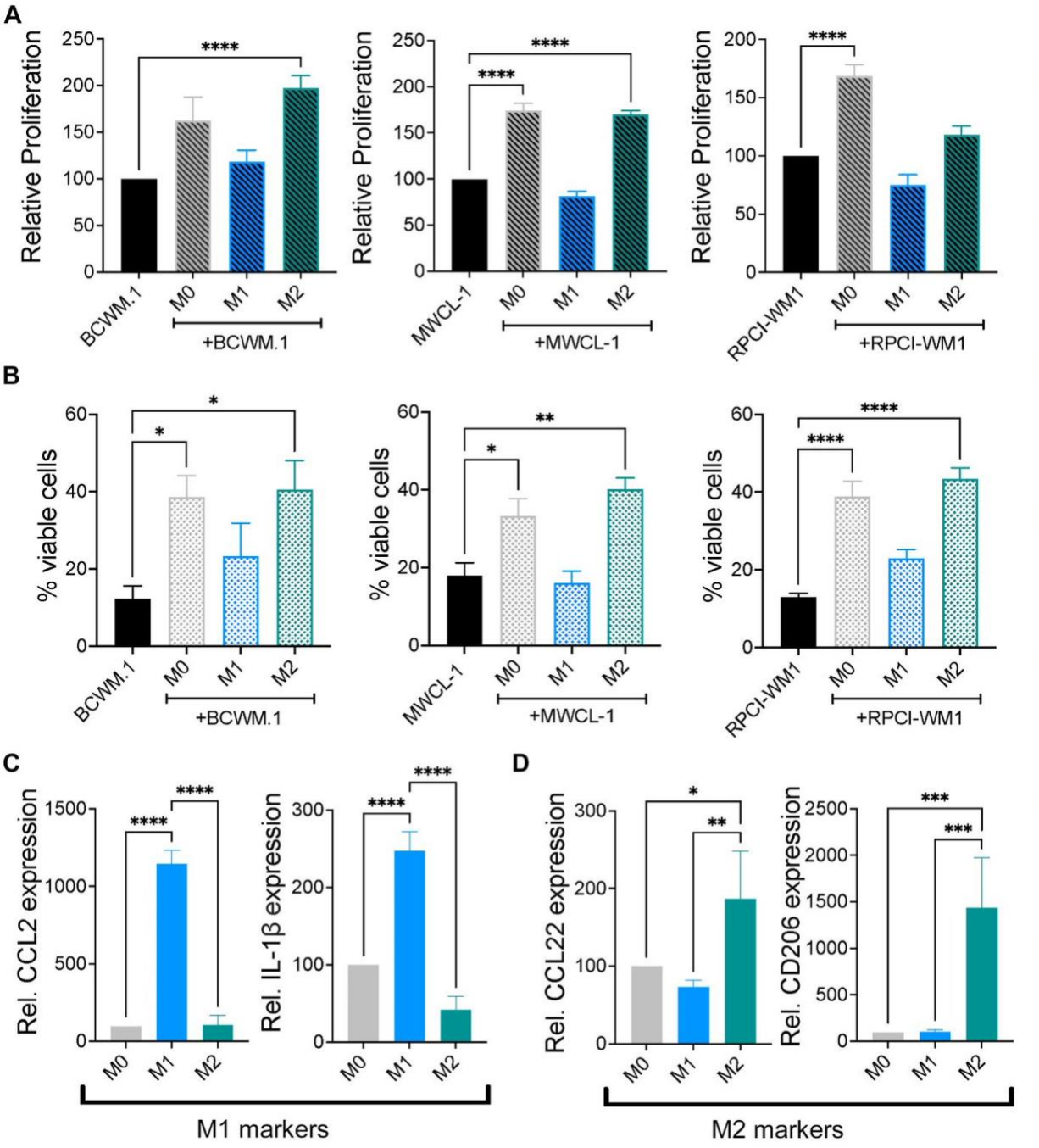

Figure 5. THP-1-derived M0 and M2 macrophages promote WM cell growth and survival. M0, M1 or M2 macrophages were co-cultured with WM cell lines (BCWM.1, MWCL-1, or RPCI-WM1). **(A)** Relative proliferation of WM cells was measured via XTT assay after 72h of co-culture with M0, M1, or M2 macrophages. **(B)** Viability of WM cells was measured via Trypan Blue Exclusion after 72h of co-culture with M0, M1, or M2 phenotypes. qPCR was used to confirm THP-1 cells were successfully polarized into an **(C)** M1 phenotype or **(D)** M2 phenotype. These experiments were repeated at least 3 times and the results are presented as the average of 3 independent experiments, each performed in triplicate wells +/- SEM.

Co-culture of $CD14^+$-human peripheral blood-derived-M0 and M2 macrophages increased the proliferation and viability of WM cells

To confirm the results obtained using the THP-1 cell line, we utilized primary monocytes isolated from peripheral blood of healthy donors. Monocytes were isolated from total peripheral blood mononuclear cells (PBMCs) using $CD14^+$ magnetic beads. These monocytes were then differentiated into macrophages (M0) as described in the methods section. M1 and M2 macrophages were differentiated similar to THP-1 cells. To confirm that M0 and M2 macrophages influenced the viability and proliferation of WM cells, we co-cultured M0, M1 and M2 cells for 72 hours in direct contact with WM cell lines, BCWM.1, MWCL-1, and RPCI-WM1. An XTT assay was performed to determine if there was a change in proliferation. Both M0 and M2 populations increased the proliferation of WM cells, and co-culture with M1 macrophages either slightly decreased or did not affect proliferation (Figure 6A and B). To determine if M0, M1, or M2 macrophages influenced the viability of WM cells, M0, M1, and M2 macrophages were generated from $CD14^+$-human peripheral blood donors and co-cultured with WM cell lines, BCWM.1, MWCL-1, and RPCI-WM1, for 72 hours. After 72 hours, the WM cells were harvested and counted via Trypan Blue exclusion. Both the M0 and M2 phenotypes increased the viability of WM cells after co-culture, while the M1 phenotype did not increase the viability of WM cells (Figure 6C).

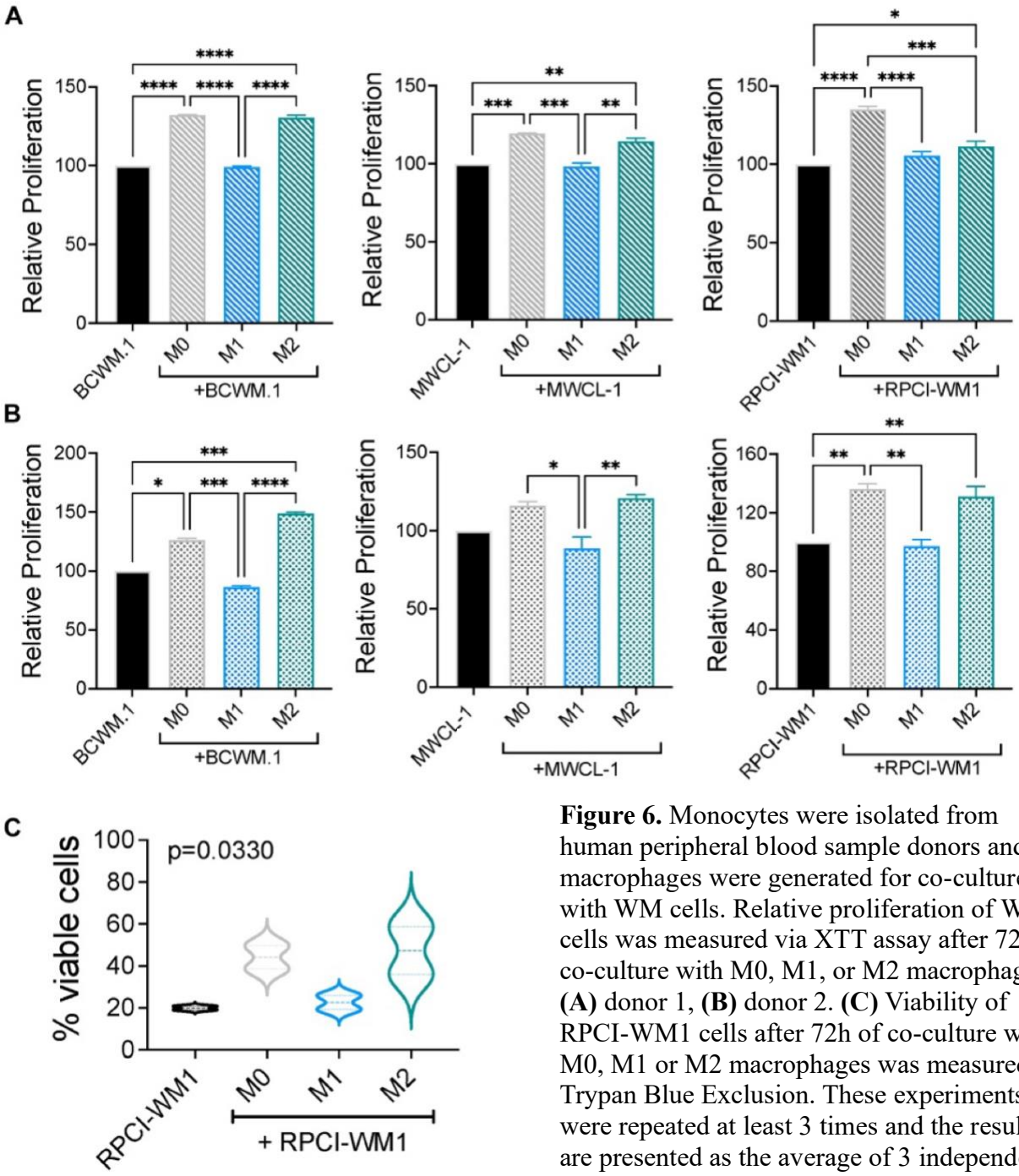

Figure 6. Monocytes were isolated from human peripheral blood sample donors and macrophages were generated for co-culture with WM cells. Relative proliferation of WM cells was measured via XTT assay after 72h of co-culture with M0, M1, or M2 macrophages: **(A)** donor 1, **(B)** donor 2. **(C)** Viability of RPCI-WM1 cells after 72h of co-culture with M0, M1 or M2 macrophages was measured via Trypan Blue Exclusion. These experiments were repeated at least 3 times and the results are presented as the average of 3 independent experiments, each performed in triplicate wells +/- SEM.

Co-culture with mouse bone marrow-derived- macrophages (BMDMs) increased the viability of WM cells

To confirm that M0 and M2 macrophages influence proliferation and viability, we generated M0, M1, and M2 macrophages from C57BL6/J bone marrow cells and co-cultured them for 72 hours in direct contact with WM cell lines, BCWM.1, MWCL-1, and RPCI-WM1. After 72 hours, the WM cells were harvested and counted via Trypan Blue exclusion. Both the M0 and M2 phenotypes increased the viability of WM cells after co-culture, while the M1 phenotype did not increase the viability (Figure 7A). To ensure that M1 and M2 macrophages were successfully polarized to their respective phenotypes, qPCR of these phenotypes was performed using known murine M1 and M2 markers. M1 markers, *iNos* and *Tnfα*, were used to confirm that M1 macrophages were successfully polarized (Figure 7B) and M2 markers, *Arg2* and *Cd163*, were used to confirm that M2 macrophages were successfully polarized (Figure 7C).

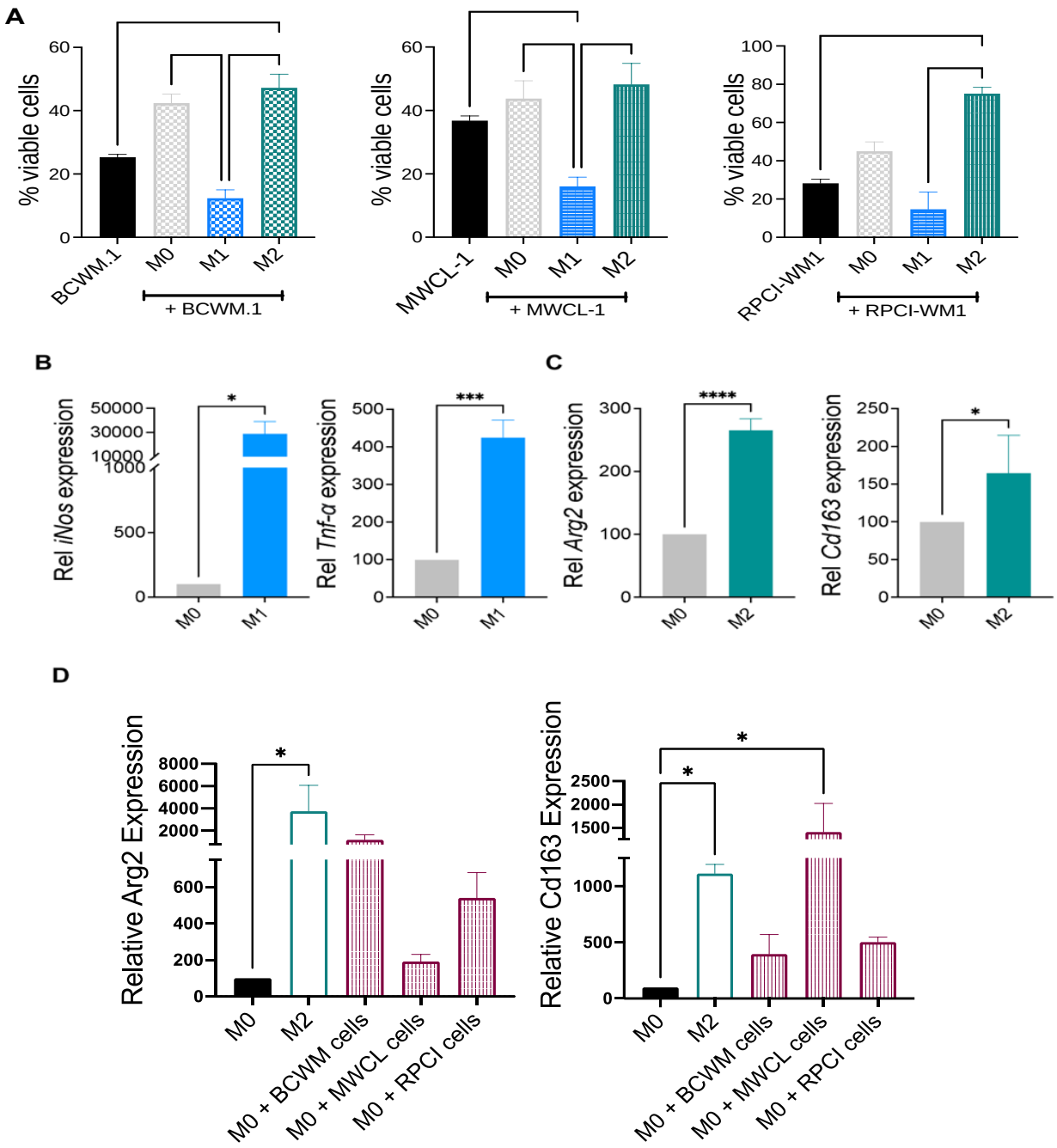

Figure 7. Bone marrow-derived macrophages were generated from C57BL/6J mice and co-cultured with WM cells. **(A)** Viability of WM cell lines were measured after 72h of co-culture with M0, M1, or M2 macrophages. qPCR was used to confirm C57BL/6J bone-marrow derived macrophages could successfully polarize to **(B)** M1 phenotype or **(C)** M2 phenotype. **(D)** qPCR of M2 genes was performed after 24h of co-culture of M0 macrophages and WM cells to confirm that WM cells influence macrophage polarization. These experiments were repeated at least 3 times and the results are presented as the average of 3 independent experiments, each performed in triplicate wells +/- SEM.

WM cells modulate macrophage polarization toward M2 phenotype.

Due to the increase of viability and proliferation of WM cells observed with co-culture with both M0 and M2 phenotypes, M0 macrophages were co-cultured with WM cell lines, BCWM.1, MWCL-1, and RPCI-WM1 for 24 hours to determine if the WM cells were influencing polarization towards an M2 phenotype. WM cells lines were removed and discarded after 24 hours, and macrophages were harvested for qPCR. qPCR for known M2 markers, *Arg2* and *Cd163*, were used to determine if M0 macrophages were transforming into an M2 phenotype because of WM cell influence. We found an increase in Cd163 expression in macrophages after co-culture with WM cells. However, although the pattern was consistent, this only reached statistical significance in M0 macrophages that were cocultured with MWCL-1 cells. This suggests that WM cells are causing a polarization of macrophages towards an M2 phenotype (Figure 7D).

Indirect co-culture does not induce the polarization of macrophages

To assess if indirect co-culture would affect the polarization of macrophages, we generated M0 macrophages from C57BL6/J bone marrow cells and co-cultured them for 72 hours with supernatants from WM cell lines, BCWM.1, MWCL-1, and RPCI-WM1. After 72 hours, the macrophages were harvested, and qPCR was performed to look at M1 and M2 markers. Indirect co-culture did not have a polarizing effect on the macrophages towards an M2 phenotype (Figure 8A) or an M1 phenotype (Figure 8B).

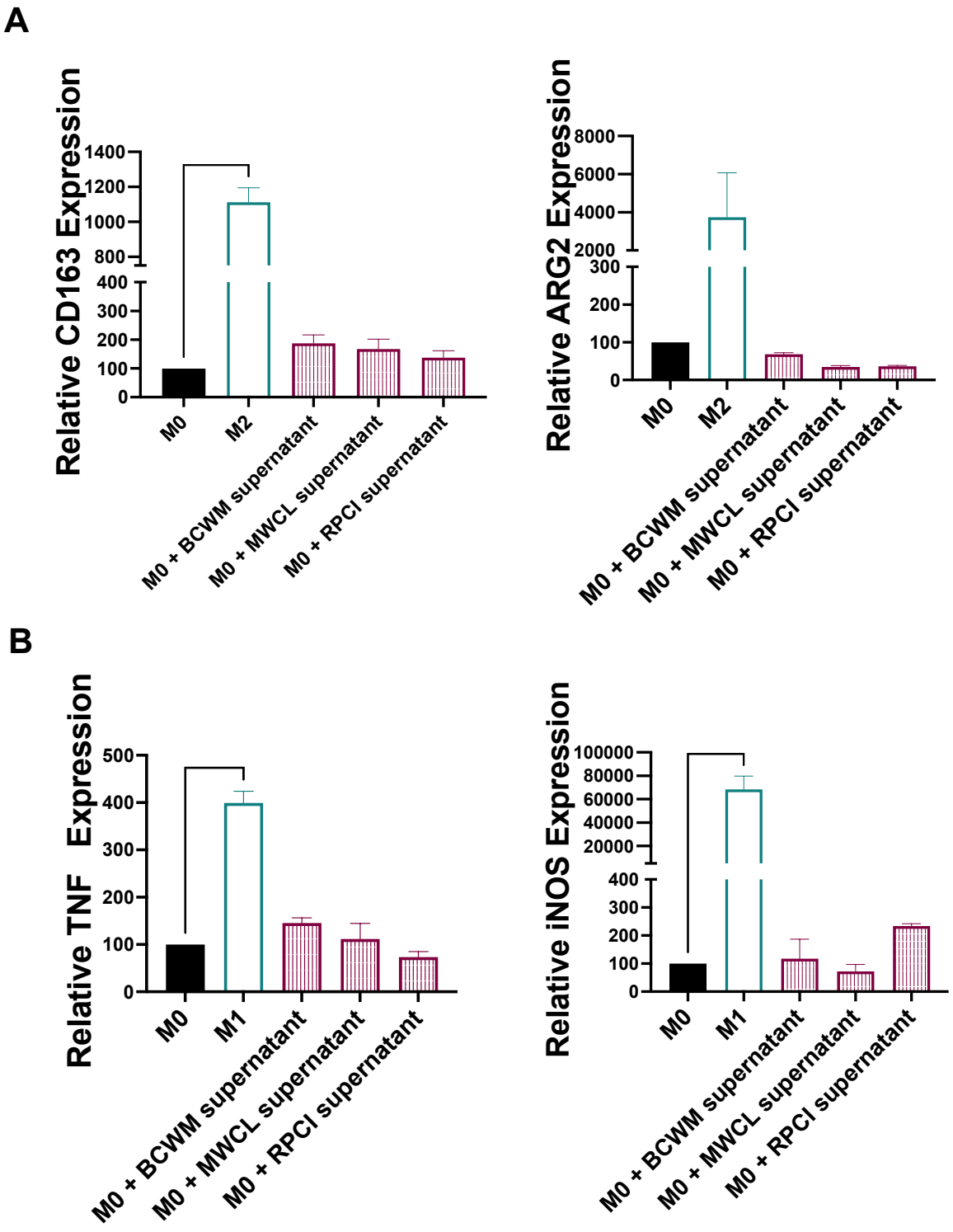

Figure 8. Bone marrow-derived M0 macrophages were generated from C57BL/6J mice and indirectly co-cultured with WM supernatants. Indirect co-culture did not affect the polarization state of macrophages towards an M2 **(A)** or M1 phenotype **(B)**. These experiments were repeated at least 3 times and the results are presented as the average of 3 independent experiments, each performed in triplicate wells +/- SEM.

Gli3 is required for increased proliferation from direct co-culture of M0 macrophages and WM cells

In previous work, we have identified a novel role for the transcription factor Gli3 in regulating cytokine expression and secretion in response to LPS stimulation. We performed RNA-seq on macrophages derived from mice lacking Gli3 in myeloid cells (*M-Gli3*$^{-/-}$) that were stimulated with LPS or DPBS (control). Using a generalized linear model in edgeR, we identified 495 genes with significant interaction effects in the absence of Gli3 upon LPS stimulation (Figure 9A). Interestingly, further analysis showed 25 inflammatory genes (Adipoq, Acacb, Nqo2, Tnfsf13, Lrrc8a, Tlr8, Rnf122, Cgas, Hmgn5, Rapgef3, Cd226, IL12b, CCl25, Mertk, Tnfrsf18, Tnfsf13b, Slc6a4, Cx3cl1, Ccl1, Fry, Sort1, Gstk1, Timd4, Ccne1, Trem2) being affected in the absence of Gli3 (Figure 9B). The 25 significant interaction genes on these pathways included 9 with a positive interaction and 16 with a negative interaction. Ingenuity Pathway Analysis of the interaction genes revealed "Inflammatory Response" and "Immune Cell Trafficking" pathways as most significantly enriched (Figure 9C). Analysis also suggested Gli3 may play a role in M2 macrophage polarization (Figure 9D).

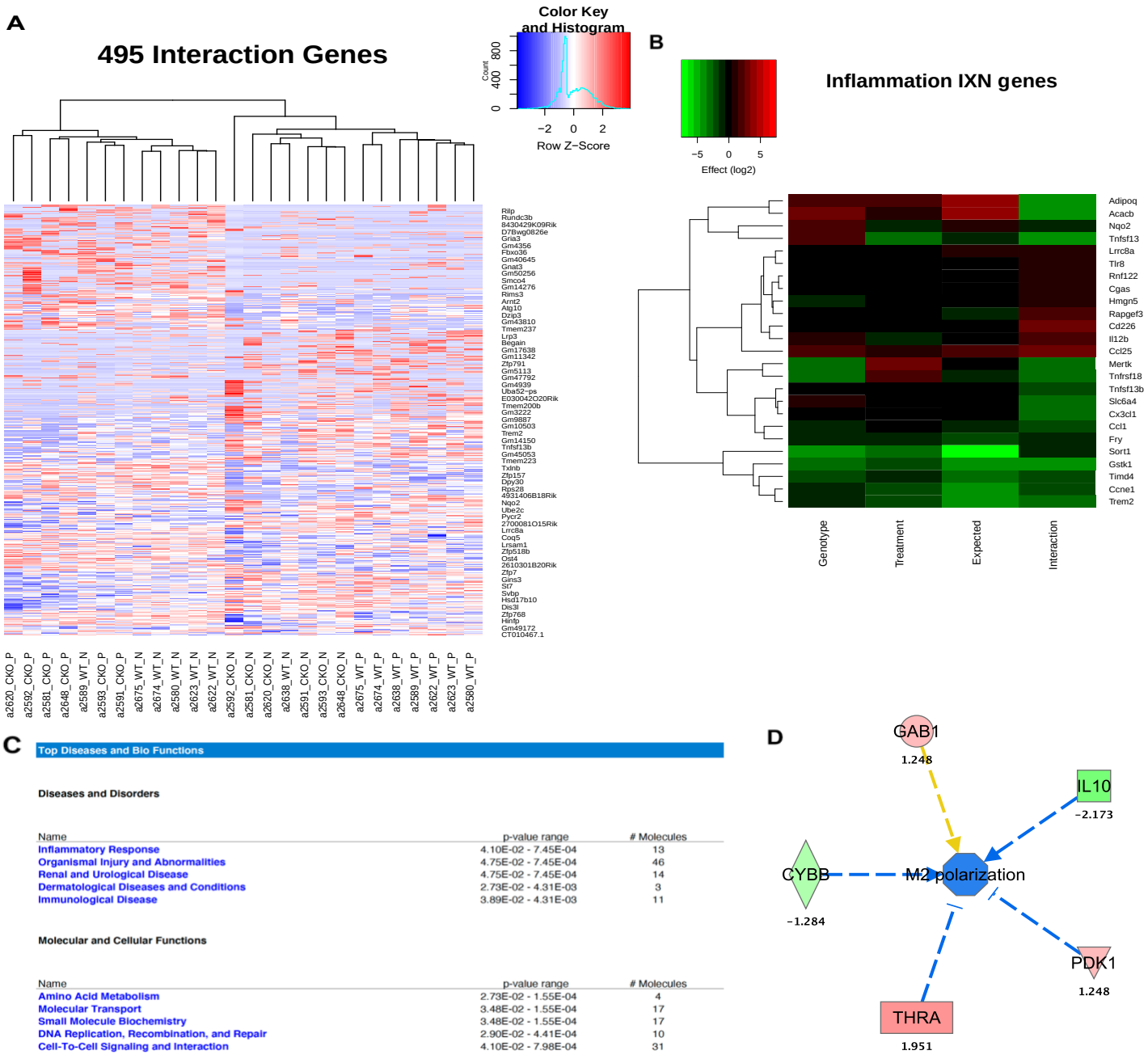

Figure 9. RNA-seq on macrophages derived from *M-Gli3*$^{-/-}$ mice challenged with either LPS or DPBS was performed. 495 genes with significant interactions in the absence of Gli3 with LPS stimulation were identified **(A).** Further analysis showed 25 inflammatory genes that were affected in the absence of Gli3 **(B).** Ingenuity Pathway Analysis of the interaction genes revealed "Inflammatory Response" and "Immune Cell Trafficking" pathways as most significantly enriched. The 25 significant interaction genes on these pathways included 9 with a positive interaction and 16 with a negative interaction **(C)**. Analysis suggested Gli3 may play a role in M2 macrophage polarization **(D)**.

To address the possibility that Gli3 modulates macrophage polarization, bone marrow-derived macrophages were isolated from C57BL/6J mice and M0 and M2 macrophages were generated followed by examination of Gli3 expression by qPCR. We found a significant increase in Gli3 expression in M2 macrophages compared with M0 macrophages. This suggests that Gli3 may play a role in macrophage polarization (Figure 10A). Next, bone marrow-derived macrophages were isolated from *M-Gli3*$^{-/-}$ and *WT* mice and M0 and M2 macrophages were generated *in vitro* and co-cultured with WM cell lines, BCWM.1 and MWCL-1. After 72 hours of co-culture, WM cell proliferation was determined using an XTT cell proliferation assay. We found that *M-Gli3*$^{-/-}$ M2 macrophages were unable increase the proliferation of BCWM.1 cells while *WT* M2 macrophages increased WM cell proliferation (Figure 10B). A similar observation was found when MWCL-1 cells were cocultured with *M-Gli3*$^{-/-}$ and *WT* M0 macrophages, however, this was not statistically significant (Figure 10B). While these results were obtained from cocultured experiments from 1 mouse per group, as these studies are replicated using additional mice, we will examine statistical significance again. Taken together, these results suggest that Gli3 is required for M2 polarization (Figure 10B).

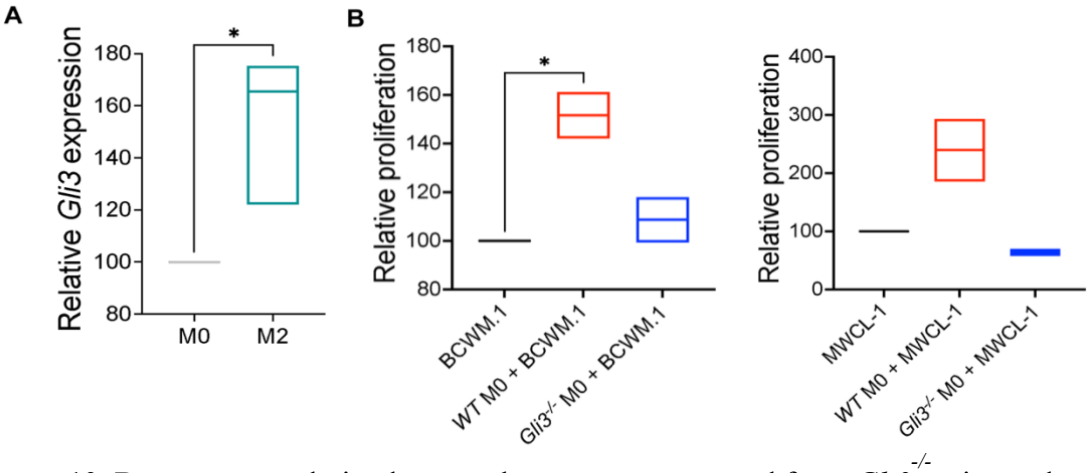

Figure 10. Bone marrow-derived macrophages were generated from *Gli3*$^{-/-}$ mice and co-cultured with WM cells. **(A)** GLI3 gene expression was assessed via qPCR after polarization into M2 phenotype. **(B)** Relative proliferation of BCWM.1 and MWCL-1 cells was assessed via XTT assay after 72h of co-culture with *WT* or *Gli3*$^{-/-}$ M0 macrophages. These experiments were repeated at least 3 times and the results are presented as the average of 3 independent experiments, each performed in triplicate wells +/- SEM.

Discussion

The WM tumor microenvironment consists of a collection of immune and non-immune cells, T-cells, B-cells, macrophages, myeloid-derived suppressor cells, mast cells, mesenchymal stem cells, osteoclasts, osteoblasts, natural killer cells, and dendritic cells [124]. The full pro- or anti-tumorigenic effects of these cells on the progression of WM is not quite complete, but some efforts have been made to quantify the effects of these cells in WM. Recently, reports on the role of mast cells, T-cells, monocytes, and endothelial cells in WM have been published. It has been previously demonstrated that mast cells in the bone marrow of WM patients induce proliferation of malignant B cells through CD40L and CD40 interactions [125]. T-cells have also been examined in WM and the expression of PD-1 and the ligands PD-L1 and PD-L2 have been characterized. PD-L1 and PD-L2 gene expression was induced by IL-21, interferon-γ, and IL-6 expression in WM cell lines and patient bone marrow cells. Increased expression of PD-L1 and PD-L2 in the bone marrow of WM patients increased the proliferation of malignant B cells and reduced T-cell proliferation [126]. Bone marrow stromal cells (BMSC) are a heterogenous population that have been shown to play an important role in normal and malignant cell biology [127]. Mesenchymal stem cells (MSCs) serve as the progenitor for most bone marrow stromal cell populations, including osteoblasts, chondrocytes, fibroblasts, endothelial cells, and myocytes [128]. In WM, BMSCs have been shown to regulate the proliferation of tumor cells while contributing to increased drug therapy resistance [129]. Endothelial cells have been shown to increase WM cell adhesion and proliferation through the Ephrin receptor B2 (Eph-B2), which is found upregulated on WM cells [130]. The Eph-B2 receptor was found to be activated in WM patients compared with healthy samples. Endothelial cells in the bone marrow express high levels of Ephrin-B2 ligand. Blocking of either Ephrin-B2 or Eph-B2 inhibited the increased

adhesion and proliferation caused by the endothelial-WM cell interaction [129]. Our research has shown that M0 and M2 macrophages increase the proliferation and viability of WM cells, playing a potent pro-tumorigenic role, which only adds to our knowledge of the WM tumor microenvironment. Future studies should investigate the presence of macrophages in the WM microenvironment and determine whether they are M1 or M2-like cells. By enumerating and understanding the role of these macrophages, better therapies may be used in combination with conventional therapies to achieve better clinical outcomes in WM patients.

The Gli family of proteins, Gli1, Gli2, and Gli3, have been shown to play a role in embryonic development and homeostasis of stem cells in normal tissues through hedgehog signaling. The role of Gli3 in development has been well-described. Gli3 plays a key regulatory role in preventing diseases such as Greig cephalopolysyndactyly syndrome, Pallister-Hall syndrome and tibial hemimelia [195]. Additionally, Gli3 has been shown to play a role in the development of the Dentate gyrus, hippocampus, and lungs. Several studies have also suggested a role for Gli3 in the immune system, specifically in B- and T-cell development, as well as the expression of CD155 on NK cells [198].

Additionally, studies have shown that Gli3 may play a role in regulating the inflammatory response. *WT* and *M-GLI3*$^{-/-}$ mice were challenged with LPS and peritoneal macrophages were isolated. *M-GLI3-/-* macrophages showed a reduction of LPS-induced CCL2, IL-6, and TNFα secretion in the absence of Gli3 [198]. This effect has been shown to be modulated through the TLR4/TRIF/IRF3 signaling axis.

The effect of Gli3 on macrophage polarization is a novel field of research. Gli3 has been shown to play a role in inflammation and in cancer development, but we now show that Gli3 plays a role in macrophage polarization and the subsequent pro-tumorigenic effects of M2

macrophages. Therefore, in addition to its role in embryonic development and in cancer, we now show a novel role for Gli3 in the development or differentiation of macrophages. This postulates that Gli3 may play a role in the differentiation of other immune cells such as B cells and T cells and future studies investigating the role of Gli3 in these cells may shed some light on this possibility.

While the effects of Gli3 on macrophage function have been primarily unknown previous to this study, mounting evidence has supported that one of the main immunosuppressive roles of the Hedgehog pathway is the polarization of macrophages toward the M2 phenotype. In a breast cancer mouse model, M2 macrophage polarization was reduced upon the inhibition of the Hh signalling pathway [199]. Furthermore, the inhibition of Hh signaling influenced the polarization of M2 macrophages back towards their inflammatory counterpart, M1, indicating a potential therapeutic target for treating tumor-associated macrophage polarization. Another study investigating the role of Gli1, Gli2, and Gli3 in pancreatic ductal adenocarcinoma showed that the genetic depletion of Gli2 and Gli3 in fibroblasts reduced the infiltration of M2 macrophages, while the complete deletion of Gli1, Gli2, and Gli3 promoted M2 macrophage infiltration, indicating that Gli1 may be responsible for increased M2 populations, while Gli2 and Gli3 are responsible for decreasing the M2 population [200]. Additionally, we discovered that Gli3 is required for M2 macrophages to exert its proliferative effects on WM cells, and without Gli3, we do not see an increase in WM cell proliferation.

Due to the abnormal B-cell receptor signalling in disease progression in WM, Bruton's Tyrosine Kinase (BTK) inhibitors have proved successful in treating these malignancies [89]. BTK inhibitors work by blocking BTK activation, therefore inhibiting NF-κB and MAP kinase activation, leading to reduced survivability and proliferation. Ibrutinib and Zanubrutinib are the

only two FDA-approved treatment options for WM and are both BTK inhibitors. In a phase 1/2 study of patients with WM, either treatment naïve or relapsed/refractory, the overall response rate was 95.9% at 36 months post-treatment initiation for Zanubrutinib treatment [201]. In a randomized phase 3 trial of Zanubrutinib versus Ibrutinib in WM, patients with MYD88 L265P disease were randomly assigned to treatment with Ibrutinib or Zanubrutinib. More patients in the Zanubrutinib group (28%) versus the Ibrutinib group (19%) achieved a very good partial response, and side effects of BTKi therapy, including contusion, diarrhea, edema, atrial fibrillation, and other adverse effects leading to treatment discontinuation were lower in Zanubrutinib patients versus Ibrutinib [203]. The full mechanism of action of ibrutinib has yet to be fully investigated and has shown to impact tumor-associated macrophages. In a study, Ibrutinib treatment suppresses the production of CXCL12, CXCL13, CCL19 and VEGF by TAMs. Additionally, ibrutinib did not impact the viability of TAMs, but did significantly decrease the macrophage-dependent adhesion, invasion, and migration of malignant cells. This indicates that the therapeutic effects of ibrutinib may be linked to both direct cytotoxic effects of malignant cells and immunomodulatory effects on TAMs [202]. Zanubrutinib, however, has been shown to promote M2 macrophage activation through the regulation of the JAK/STAT6 and PI3K/AKT pathways. In a study of LPS-induced acute lung injury, it was found that zanubrutinib could inhibit M1 polarization and encourage M2 polarization via inhibition of JAK2/STAT1 and TLR4/MyD88/NF-κB signalling pathways and the activation of STAT6 and PI3K/Akt signaling pathways [203].

This data is critical to further influence and inform tumor-associated macrophage-related research in WM, which could lead to an increased understanding of the WM tumor microenvironment and aid diagnosis and treatment in WM patients.

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