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Hypersialylation and multiple myeloma

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There is growing recognition of the importance of sialylation as a critical post translational modification in cancer. In this article we review the role of increased cell surface sialylation (hypersialylation) in Multiple Myeloma as it relates to cellular trafficking and immune evasion. Knowledge of the specific effects of sialic acid on cell trafficking machinery and modulation of immune cell interactions will identify opportunities for therapeutic interventions. The available evidence indicates that hypersialylation facilitates disease progression and negatively impacts on response to treatment and overall survival. Further research is required to fully elucidate the mechanisms through which hypersialylation influences disease biology and therapy resistance with the ultimate goal of developing new treatment approaches to improve the outcomes of patients with Multiple Myeloma.

KEYWORDS

multiple myeloma, glycosylation, sialic acid, selectin, Siglec, immune evasion, immunotherapy, cellular trafficking


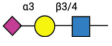

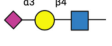
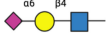


1 Introduction to multiple myeloma and sialylation

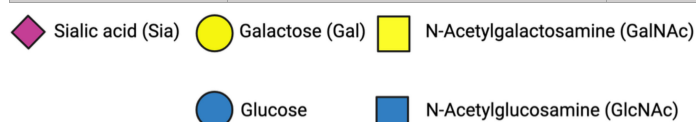
Multiple Myeloma (MM) is a clonal malignancy of antibody producing plasma cells, involving the bone marrow (BM) and associated with clinical features of anemia, bone destruction, renal impairment and hypercalcemia. Despite advances in the treatment of MM the majority of patients will eventually develop resistance and die from their disease, with significantly shorter survival in patients with high-risk genetic features (1). The BM microenvironment has a major influence on the pathogenesis of MM, in which malignant plasma cells have bidirectional interactions with the BM cellular and non-cellular elements. The multiple and complex interactions between MM cells and various BM cell types, contribute to neoplastic plasma cell growth, proliferation, and drug resistance (2). A frequently overlooked mediator of cell-to-cell interactions and communication in the tumor microenvironment (TME) is glycosylation. All cells are covered by a dense coating of

glycans, which post-translationally modify multiple proteins or lipids, significantly impacting their function (3). Glycosylation of extracellular structures plays a critical role in cell-to-cell interactions and is known to influence cellular trafficking, signaling and the immune response. Sialic acid residues, which decorate the end of glycan (sugar) structures on the cell surface play a key role in many of these processes (4). Glycosylation is not a template driven process and glycans on proteins or lipids are produced by combined action of a number of different enzymes.

In the case of sialic acid-coated glycans (sialoglycans), the final transfer of sialic acids to the underlying glycan chain is catalyzed by an enzymatic family of sialyltransferases (Table 1), and takes place in the Golgi apparatus (5). Glycan binding proteins are known as lectins and the best-known family of sialoglycan binding lectins are the Selectins. E, P and L-Selectin, expressed predominantly on endothelial cells, platelets and lymphocytes, play an important role in cellular trafficking and platelet adhesion. Well known Selectin ligands, include P-selectin glycoprotein ligand-1 (PSGL-

TABLE 1 Human sialyltransferases, focusing on those more highly expressed in Multiple Myeloma, based on an expression cut off > 10 transcripts per million (nTPM) in cell lines in Human Protein Atlas and also detected in > 1% cells by single cell RNA seq in bmbrowser.org.

Sialyltransferases	Sialylated oligosaccharide sequence(s) synthesized	Expressed in Myeloma	Glycan specificity	Functional significance
ST3GAL1	ST Antigen 	Y	O-glycan	Can generate Siglec ligands
ST3GAL2	GD1a GT1b, ST antigen	N	Glycolipid > O-glycan	
ST3GAL3		N	O-glycan, N-glycan, glycolipid	
ST3GAL4		Y	O-glycan, N-glycan	Contributes to formation of Siglec-9 and Selectin ligands
ST3GAL5	GM3 	Y	Glycolipid	
ST3GAL6		Y	O-glycan, N-glycan	Contributes to formation of Siglec and Selectin ligands
ST6GAL1		Y	N-glycan	Can influence cell signaling, apoptosis via Fas sialylation
ST6GAL2		N	N-glycan	
ST6GalNAc1		N	O-glycan	
ST6GalNAc2		N	O-glycan	
ST6GalNAc3	GD1a	N	Glycolipid > O-glycan	
ST6GalNAc4	Di-sialyl T 	Y	O-glycan > glycolipid	Contributes to formation of Siglec ligands
ST6GalNAc5	GD1a	N	Glycolipid	
ST6GalNAc6	GD1a, GT1aα, GQ1bα	Y	Glycolipid	Can contribute to Siglec ligand synthesis on glycolipids
ST8SIA1	GD3	N	Sialic acid on glycolipid	
ST8SIA2	Polysialic acid	N	Sialic acid on N-glycan on CD56 (NCAM)	
ST8SIA3		N	Sialic acid on glycolipid and N-glycans	
ST8SIA4	Polysialic acid 	Y	Sialic acid on N-glycan on CD56 (NCAM)	Generates polysialic acid on CD56
ST8SIA5	GD1c, GQ1b, GT1a, GT3	N	Sialic acid on glycolipid	
ST8SIA6	GD3	N	Sialic acid on N-glycan > glycolipid	



Selected oligosaccharides generated and glycan specificity of individual sialyltransferases are shown along with functional significance. For preferred substrates refer to the original. [adapted from Hugonnet M, et al. (5)].

Grey shading indicates those sialyltransferases not significantly expressed in Myeloma cells.

1), CD43 and CD44, which when appropriately post translationally modified by sialic acid and fucose (to generate sialyl Lewis X/sLe^X) can bind to E and P-Selectin (6). The other main family of sialoglycan binding lectins are the Siglecs (4). The Siglecs are sialic acid binding immunoglobulin like lectins. This is a large family of receptors, which are expressed by immune cells. While some are activating receptors, containing an immunoreceptor tyrosine-based activation (ITAM) motif in their intracytoplasmic tail, most Siglecs are inhibitory receptors, with an intracellular immunoreceptor tyrosine-based inhibitory (ITIM) motif, analogous to PD-1 and CD47. It is now well recognized that increased sialylation (hypersialylation) correlates with advanced stage and inferior prognosis in many forms of cancer. Indeed, by exploiting sialic binding receptors, such as the Selectins and Siglecs, hypersialylation promotes cancer cell survival by enabling dissemination, enhancing survival and by modulating immune cell responses (7).

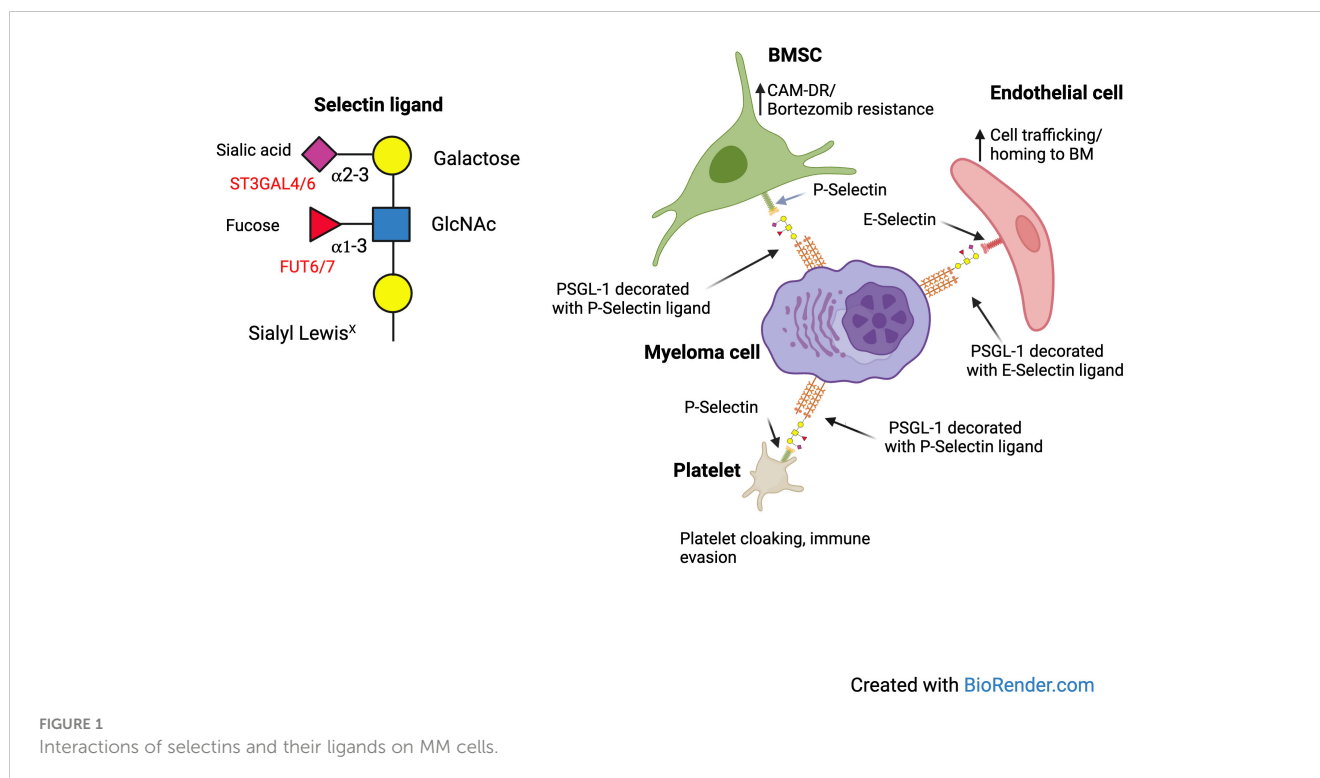
2 Role of sialoglycans in cellular trafficking and adhesion

Many glycosyltransferases have important roles in regulation of leukocyte trafficking, through regulation of the expression of selectin ligands. These include α 1,3 fucosyltransferases and α 2,3 sialyltransferases (8). These glycosylation dependent mechanisms of immune cell trafficking are also functional in hematological malignancies. We first identified the potential importance of hypersialylation in disease progression of MM, when we discovered an important role for the sialyltransferase ST3GAL6 in the biology of MM. In the UK MRC Myeloma IX trial overexpression of ST3GAL6 was an independent prognostic marker of poor overall survival (OS) (9). We identified an important role for ST3GAL6 in cellular trafficking and drug resistance and found that it played a major role in generation of E-selectin ligands, influencing trafficking in and out of the bone marrow niche (10). This suggests that there may be benefit in blocking the interaction of E-selectin and its ligands to overcome drug resistance. Indeed, the success of this strategy has been demonstrated in Acute Myeloid Leukemia (AML) with the E-selectin inhibitor uproleselan (GMI-1271) having entered clinical trials with encouraging results (11). A study was also conducted to assess the efficacy of uproleselan in preventing gastrointestinal toxicity in patients receiving high dose melphalan as conditioning for stem cell transplantation. The rationale for this study was that the expression of E-selectin is increased at sites of inflammation, resulting in increased extravasation of inflammatory leukocytes into inflamed tissue associated with chemotherapy toxicity. Blockade of E-selectin could therefore reduce this inflammation (11). While this study has been completed, results have not yet been reported (NCT04682405). We also found that sialylation influenced cell adhesion not only by mediating Selectin binding but also α 4 integrin mediated binding to VCAM1 and MadCAM. In an *in-vivo* model we found that pretreatment with a sialyltransferase inhibitor improved survival of SCID Beige mice injected with

MM.1S cells expressing sLe^X (12). In effect, using cell sorting with the HECA452 antibody we were able to generate a subclone of MM.1S cells which were enriched for the expression of E-selectin ligands. While low dose bortezomib was largely ineffective in this model, the combination with sialyltransferase inhibitor treatment was synergistic. Thus, we concluded that de-sialylation of MM cells likely overcame cell adhesion mediated drug resistance (CAM-DR) *in vivo*. Conceivably, de-sialylation could be relevant for cell adhesion mediated immune resistance as well. In more recent work we found that MM cells can bind platelets in a selectin dependent manner, which can be blocked using a P-selectin antibody and is more pronounced with disease progression (13). Platelet cloaking is a potential mechanism of immune evasion as it can lead to inhibition of NK mediated killing. In summary, there are several different ways in which sialoglycans can influence cellular adhesion and drug resistance. Figure 1 demonstrates potential interactions of sialoglycans on MM cells with E and P Selectin.

3 Siglec ligands and immune evasion in MM

Following the discovery of the Siglec family of receptors, it became clear that sialoglycans behave as self-associated molecular patterns (SAMPs), which are recognized by inhibitory Siglec receptors to suppress autoimmune responses. It is now well accepted that this axis could also be exploited by tumor cells to mediate cancer immune evasion (14). Tumor cells and stromal cells in the TME can induce the expression of Siglec receptors on NK cells, T cells and macrophages. There is now an extensive literature linking hypersialylation of tumor cells with immune evasion in many different cancers. These interactions can induce macrophage polarization, inhibit dendritic activation, leading to reduced antigen dependent T cell responsiveness, as well as inhibit direct activation of T cells and induce exhaustion. Presentation of sialylated antigen by dendritic cells has also been reported to induce T cell tolerance with expansion of antigen specific regulatory T cells (Tregs) (15). Reprogramming of tumor stroma by tumor cells can also lead to enhanced sialylation and induction of Siglec ligands, which induce T cell exhaustion (16). Among the first insights in this regard, were reports that interaction of sialoglycan SAMPs on tumor cells with Siglecs on NK cells could lead to inhibition of NK cell function (17, 18). This prompted us to explore whether MM cells expressed sialoglycans capable of acting as Siglec ligands and whether the expression of these ligands influenced the cytotoxicity of NK cells (19, 20). We found that MM cell lines, as well as primary samples from patients with MM, strongly expressed ligands for Siglec-7 and Siglec-9. Siglec ligands were also present on plasma cells from individuals with MGUS. While the Siglec-7 receptor was equally expressed on NK cells from healthy donors and MM patients, we found that expression, based on the mean fluorescent intensity (MFI), was significantly higher on NK cells from MM patients ($p = 0.0043$). When the surface of MM cells was desialylated using a sialidase enzyme (neuraminidase), co-culture with NK cells led to significantly enhanced killing under different conditions (naïve, IL-

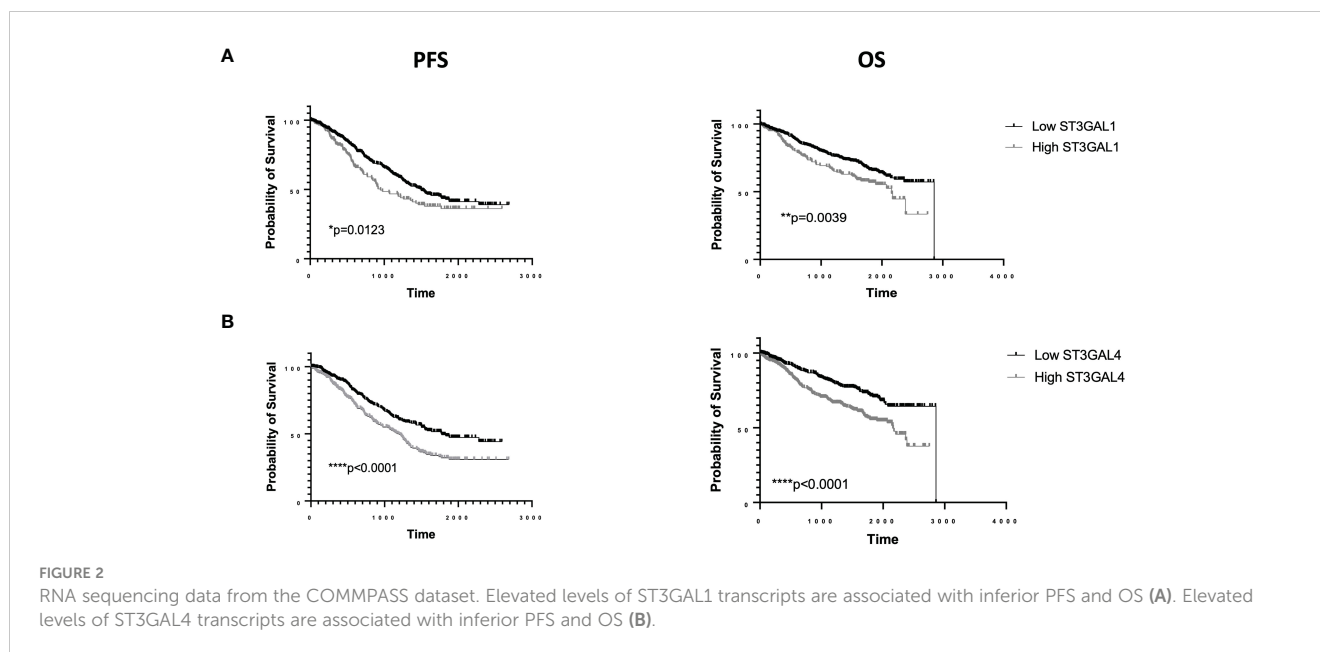


2 activated and expanded NK cells). Using an alternate approach, blockage of sialic acid incorporation on glycoproteins in the Golgi, using STI, we again observed enhanced killing by NK cells following co-culture and also ADCC in the presence of Daratumumab. Interestingly, pre-treatment of MM cells led to enhanced detection of CD38 by flow cytometry, suggesting that sialylation of CD38 could potentially mask this important therapeutic epitope in some cells. We did not observe this to be the case for BCMA, though previous work suggested BCMA could be internalized following sialylation (21). From this work, we concluded that sialylation of MM cells could protect from NK cell mediated cytotoxicity and reasoned that this was at least in part due to interactions between Siglec-7 and its ligands. Indeed, knock out (KO) of Siglec-7 in NK cells led to significant enhancement of killing of MM cells in co-culture, supporting this conclusion.

Given an apparent important role for Siglec-7 ligands in immune evasion, we then sought to determine the identity of potential glycoproteins that could function as Siglec-7 ligands. To elucidate the identity of glycoproteins that could function as Siglec-7 ligands in MM, mass spectrometry was carried out on proteins bound to Siglec-7 Fc chimera-magnetic bead complexes after incubation with cell lysates from MM cell lines untreated or treated with neuraminidase. PSGL-1 emerged as the primary Siglec-7 ligand expressed in 3/3 cell lines. Furthermore, CD43 was also identified as a Siglec-7L expressed in one cell line, consistent with previous work showing that CD43 is a high affinity ligand for Siglec-7 (22). These results identified PSGL-1 as a new, broadly expressed Siglec-7 ligand in MM. We have also confirmed PSGL-1 to be the main E-selectin ligand on MM cells, expressing sLe^x (23). PSGL-1 along with CD43 recently emerged in a genome wide CRISPR screen as being associated with resistance to NK cells

(24). PSGL-1 is of particular interest in MM. In the Human Protein Atlas, proteomic analysis shows the highest level of PSGL-1 in MM patients and MM cell lines expressed the highest levels of mRNA for PSGL-1 (25). The expression of PSGL-1 has previously been shown to increase with disease progression in MM (26, 27).

Siglec-7 predominantly binds sialylated O-glycans. In addition to PSGL-1 and CD43, O-linked glycoproteins such as CD34, CD44, CD45, and MUC-1 have the potential to function as Siglec-7 ligands provided they possess the appropriate post translational glycan modifications (28). On the other hand, Siglec-9 ligands appear to be predominantly expressed on N-linked glycans, though sialylated MUC-1 has been reported to be a Siglec-9 ligand. It is important to recognize that sialylated glycolipids (gangliosides), such as GD2 and GD3 expressing disialyl T antigens can also function as ligands for both Siglec-7 and Siglec-9 (29). GD2 for example, was recently demonstrated to be a Siglec-7 ligand (30). In addition to functioning as a Siglec-7 ligand, it has also been reported that PSGL-1 can bind Siglec-5 (31, 32). Siglec-5 was identified as a new inhibitory T cell immune checkpoint molecule and studies suggest that blockade of Siglec-5 could serve as a new strategy to enhance anti-tumor T cell functions. Cancer cell lines bind Siglec-5 indicating the presence of Siglec-5 ligands and it is conceivable that MM cells may express ligands for Siglec-5, given the strong expression of PSGL-1, though this remains to be explored. Other potential Siglec receptors that could be important in MM include Siglec-10, which plays an important role in regulating the phagocytic function of macrophages (33). The best recognized ligand of Siglec-10 is CD24. CD24 has been reported to be associated with a favorable prognosis in MM but this preceded the use of CD38 monoclonal antibodies, for which macrophages are important effector cells (34). A re-evaluation of the role of CD24 in the era of CD38 antibodies is



warranted. Siglec-15 has recently emerged as a new target for cancer immunotherapy (35). Interestingly, Siglec-15 is expressed on osteoclasts, and engagement of Siglec-15, which contains an ITAM motif, leads to differentiation and function of osteoclasts, which are central feature to myeloma bone disease. The function of Siglec-15 seems to be sialic acid dependent since treatment with sialidase inhibited osteoclast development (36). Finally, treatment of 5T2MM mice with a Siglec-15 inhibitor led to prevention of bone loss (37).

4 Sialyltransferases involved in Siglec-7 ligand synthesis and potential role in myeloma

The mere expression of PSGL-1 or CD43 does not necessarily imply the expression of Siglec ligands. The expression of Siglec ligands depends on the presence of appropriate post translational modifications, which are mediated by glycosyltransferases. The generation of Siglec ligands is dependent on the combined activity of a number of glycosyltransferases, including sialyltransferases which deposit a disialic structure on a carbohydrate backbone, typically on O-linked glycans. Gene knockout (KO) and knock-in (KI) studies suggest an important role for specific sialyltransferases in the generation of Siglec-7 ligands (38). The α 2,3 sialyltransferase ST3GAL1 or ST3GAL2 can generate sialyl T (ST) antigen on an O-glycan, which via further sialylation via ST6GALNAC1/2/3/4 can add an α 2,6 linked sialic to a core N-Galactosamine to generate a disialyl T (dST) antigen, capable of binding to Siglec-7. ST3GAL1, ST6GALNAC4 and ST6GALNAC1 have all been reported to be involved in generation of Siglec-7 ligands in cancer cells (22, 39, 40). As noted above, glycolipids can also function as Siglec-7 ligands. ST3GAL1 and ST6GALNAC4 are both highly expressed in a subset

of MM patients. The Human Protein Atlas proteomic analysis shows that of all cancers ST3GAL1 is most highly expressed in the serum of MM patients and MM cell lines have the highest mRNA expression of all cancer cell lines screened. This is similarly the case for ST6GALNAC4 (24). While a causal link remains to be proven, ST6GALNAC4 has recently been shown to be induced by MYC, an oncogene strongly associated with disease progression in MM patients (41). Publicly available single cell RNA seq data (www.bmbrowser.org) shows the presence of transcripts for a number of sialyltransferases, including ST3GAL1 and ST6GALNAC4 transcripts in MM cells from patients (42). We have also seen strong cytoplasmic staining for ST3GAL1 in plasma cells of MM patients on immunohistochemistry, especially in those with t(4,14) (unpublished data). High levels of ST3GAL1 have been associated with inferior PFS +/- OS in a number of studies. In the UKMRC Myeloma IX study, expression was independent of ISS but elevated levels of ST3GAL1 were associated with inferior PFS ($p = 0.0147$) with a trend for poor OS ($p = 0.0695$). Interestingly, in this study, increased ST3GAL1 expression was associated with hypodiploidy ($p = 1.2 \times 10^{-5}$, 1q gain ($p = 0.01$) and t(4,14) (0.009) (43, 44). Meanwhile, within the COMPASS dataset elevated ST3GAL1 expression was associated with both inferior progression free survival (PFS) ($p = 0.0123$) and OS ($p = 0.0039$) (45) (Figure 2A). Once again in this study we saw significantly higher expression of ST3GAL1 in patients with t(4,14) ($p = 0.006$). We also observed that elevated levels of expression of ST3GAL4, which may be involved in either Selectin or Siglec-9 ligand synthesis, were associated with inferior PFS and OS (Figure 2B).

Supporting this hypothesis, ST3GAL1 was recently identified as the main contributor to Siglec-7 ligand synthesis in pancreatic cancer and was associated with driving tumor associated macrophage differentiation (38). There is indeed abundant literature linking expression of Siglec ligands with macrophage polarization in cancer (39, 46, 47). This has yet to be addressed in MM. Conceivably, Siglec-7 and Siglec-9 ligands expressed by MM

cells facilitate the accumulation of M2 polarized macrophages within the BM, leading to immune suppression and reduced response to immunotherapies, such as CD38 monoclonal antibodies. Preliminary analysis of BM samples from patients with MM and compared to normal controls suggests a significantly higher percentage of Siglec-7 positive monocytes/macrophages as well as much higher intensity of expression as determined by MFI (unpublished data). Data from the bone marrow browser confirm that all relevant immune subsets in MM patients, including NK cells and monocytes express Siglec-7 receptors (41).

Using single cell RNA sequencing (scRNA seq) analysis, Francesco Maura and colleagues recently demonstrated that factors associated with sustained measurable residual disease (MRD) negativity in patients receiving intensive quadruplet induction with Carfilzomib, lenalidomide and dexamethasone in combination with Daratumumab (KRD-Dara) included a sufficient number of functional CD16 positive NK cells at baseline and evidence of increased monocyte activation and expansion following Daratumumab exposure (48). We have already shown that hypersialylation and expression of Siglec ligands can inhibit Daratumumab induced ADCC. Our preliminary observations suggest the possibility that interaction between Siglec ligands on MM cells and Siglecs on monocyte/macrophages could result in failure to achieve the necessary activation and expansion of monocytes required for optimal efficacy of Daratumumab. Clonal T cell expansion is also important for sustained responses in Dara treated patients. Hypersialylation and expression of Siglec ligands also has the potential to negatively affect T cell responses as well as negatively affect the function of antigen presenting dendritic cells. Apart from potential negative effects on both innate and adaptive cellular immune responses, hypersialylation has the potential to inhibit complement dependent cytotoxicity. A recent study reports that sialylation of the complement inhibitory protein CD55 by ST3GAL1 facilitated immune evasion in breast cancer (49). O-linked desialylation of CD55 by ST3GAL1 silencing resulted in increased C3 deposition and complement mediated lysis of breast cancer cells and enhanced sensitivity to ADCC. This could be relevant to MM as Daratumumab resistance is accompanied by increased expression of complement inhibitory proteins, including CD55 (50).

5 Sialoglycans and T cell redirected therapies

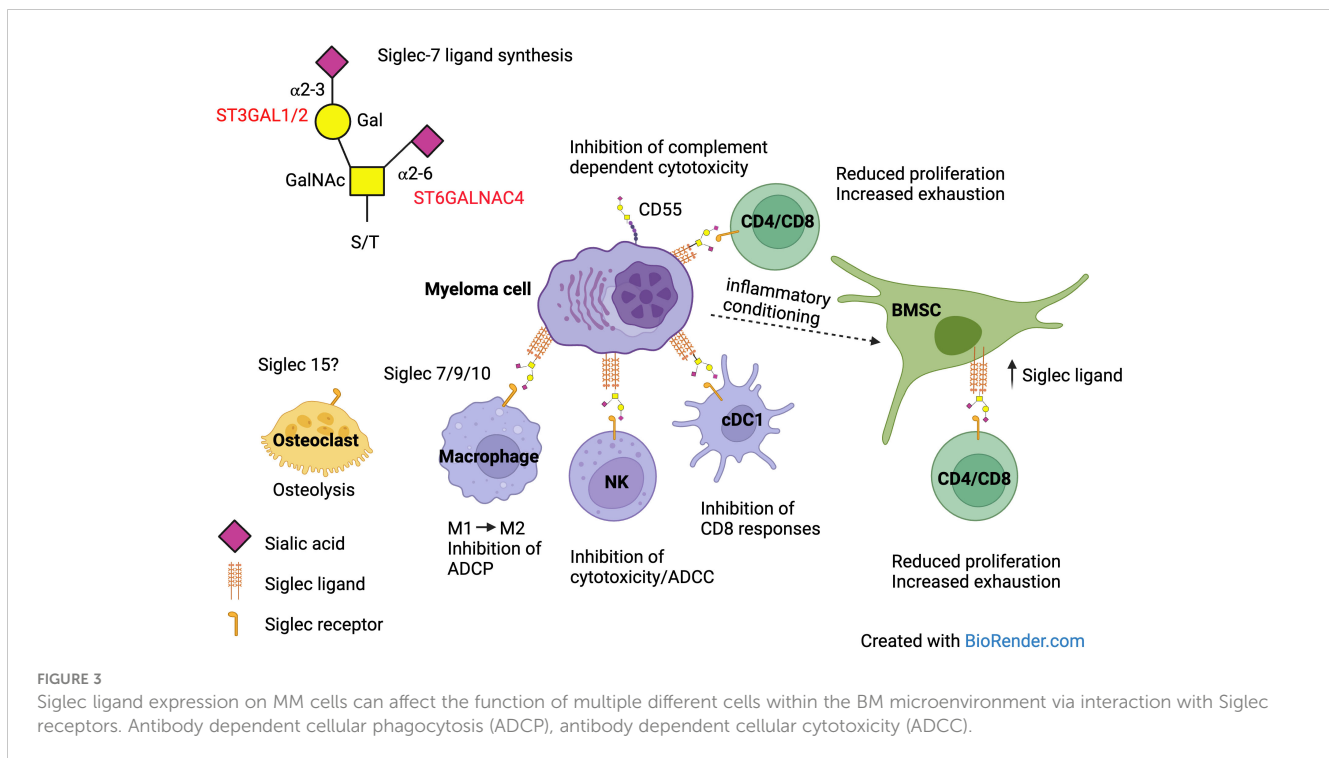
Given the emerging importance of T cell redirected therapies such as CAR-T and bispecific antibodies (BsAbs) in the treatment of MM, consideration of the potential impact of sialylation is worth considering. In other cancers there is growing evidence implicating Siglec ligands in immune evasion of T cells (51–53). Increased expression of Siglec receptors has been documented on intratumoral T cells, co-expressed with other exhaustion markers e.g. PD-1. Importantly, desialylation leads to enhanced T cell activation and cytotoxicity. Sialylation of the Fas receptor could

also inhibit apoptosis induction by Fas-L expressing T cells (54). Sialic acid-containing glycans on the surface of both T cells and antigen presenting cells (APCs) are alternative ligands of CD28 that compete with binding to CD80 on the APC, resulting in attenuated co-stimulation (55). Tumor cell glycosylation can negatively impact the function of CAR-T cells and strategies to reduce tumor cell glycosylation can enhance the efficacy of CAR-T cells. It has been proposed that this could be due to interference of glycans with formation of a proper immunological synapse. Apart from the potential benefit of reducing expression of Siglec ligands it is also known that N-glycosylation of PD-L1 is critical to its function. Therefore, it is possible that disruption of tumor glycosylation could lead to reduced expression of functional inhibitory ligands. In one study treatment of tumor cells with the glucose/mannose analogue 2-deoxy-D-glucose (2DG) to disrupt the N-glycan coating on the cell surface resulted in enhanced CAR-T cell activity in different xenograft mouse models of pancreatic adenocarcinoma (56). It is quite likely that interactions of sialylated glycans with Siglecs exposed on activated CAR-T cells could contribute to CAR-T cell inhibition. Indeed, a high throughput CRISPR screen implicated key enzymes involved in Siglec ligand biosynthesis (GNE and GALE) in resistance to BCMA CAR-T cells (57). The top hit GNE is a rate limiting enzyme involved in sialic acid biosynthesis (58). GALE is required for glycoprotein and glycolipid biosynthesis and GALE KO cells have greatly reduced sialylation (59). Increased activity of sialidase secreting CAR-T cells in the setting of solid tumors has been reported (60). Another study utilizing a genome wide CRISPR activation screen identified increased expression of CD43, recently described as a Siglec-7 ligand, as being associated with resistance to a CD20/CD3 bispecific antibody. Importantly, resistance due to CD43 was dependent on its sialylation (61). A recent report showed that sialic-acid removal was synergistic with a HER2 targeting bispecific T cell engager treatment *in vitro* (62).

In the context of MM, factors currently associated with resistance to T cell redirected therapies include loss of antigen/target epitope, loss of MHC I, low diversity of pre-therapy TCR repertoire, hyper-expanded CD8 clones with an exhausted phenotype, bone marrow stromal cell (BMSC) mediated immune resistance and increased numbers of immune suppressive cells within the BM microenvironment (63, 64) BMSCs may impair the activity of T cell-redirecting bispecific antibodies against tumor cells by suppressing T-cell activation, forming a physical barrier that hampers T-cell penetration and by inducing cell adhesion mediated resistance in tumor cells (65–67). Understanding precisely how BMSCs hinder immune function is currently an area of intense research.

6 A potential role of sialoglycans and inflammatory stromal cells in myeloma

It is becoming increasingly clear that an inflammatory BM microenvironment is associated with resistance to T cell redirected therapy in patients with MM. The BM microenvironment is composed of a number of discrete cell types, many of which are



known to play an immune suppressive role and promote disease progression in MM. These include myeloid derived suppressor cells (MDSCs), Tregs and of course mesenchymal stromal cells (MSCs)/BMSCs. However, while the tumor promoting role of the immune suppressive BM microenvironment was well recognized in MM, until recently a detailed characterization of the individual cell types was lacking. Following a detailed analysis of matched MM and control bone marrows, both of CD138 positive and CD138 negative fractions by scRNA seq, Tom Cupedo and colleagues have recently shown the presence of a distinctive inflammatory signature in BM stromal cells in patients that is absent in BM stromal cells of normal controls (68). This inflammatory signature is driven by TNF- α and IL-1 β signaling, as a result of chronic inflammation in the bone marrow, which may be amplified by neutrophils. Intriguingly, this inflammatory stromal signature persists following treatment, even in patients who achieve MRD negativity with induction therapy. The authors speculated whether this MM specific inflamed stroma could play a role in MM pathobiology and contribute to disease persistence and relapse. It has previously been reported that MSCs can induce resistance to CAR-T cells (67). BM-cancer associated fibroblasts (CAFs) were found to suppress CAR-T cells through both contact-dependent and cytokine-mediated effects. They also noted that when BCMA-CAR-T cells were stimulated and cocultured with BM-CAFs, the surface expression of inhibitory receptors such as PD-1 was significantly upregulated on CAR-T cells, whereas BM-CAFs simultaneously overexpressed inhibitory ligands such as PD-L1. Contact dependent intrinsic resistance was previously reported (66). Adhesion of MM cells to stroma led to induction of anti-apoptotic molecules, which protected MM cells from CAR mediated cytotoxicity. Since integrin mediated

attachment to stroma in MM is dependent on sialylation of $\alpha 4$ integrins, there is the potential this could be blocked using desialylation approaches (12).

We recently reported that stromal cells with an inflammatory signature have an immune suppressive phenotype, which is, at least in part, sialic acid dependent (16). In murine models of both MM and colorectal cancer we found that MSCs grown in tumor conditioned media induce T cell exhaustion in a sialic acid dependent manner. Tumor conditioning led to upregulation of sialyltransferases, increases in cell surface sialic acids and expression of Siglec-E ligands. Siglec-E is the murine homolog of Siglec-7 and 9. When co-cultured with activated T cells these tumor conditioned MSCs blocked T cell proliferation and induced T cell exhaustion. Treatment of MSCs with a sialyltransferase inhibitor reduced sialylation of MSCs and restored T cell proliferation along with increases in CD25 expression and Granzyme B content. Similarly, evaluating biopsies from patients with colorectal cancer, we found that the secretome of tumor cells induced a similar phenotype in human MSCs/CAFs with induction of ligands for Siglec-7 and Siglec-9. Intratumoral T cells had an exhausted phenotype with increased expression of Siglec-7 and Siglec-9 along with increased expression of PD-1. When cocultures were performed with intratumoral T cells and conditioned MSCs/CAFs, T cell activation and proliferation could be restored following STI treatment of MSCs/CAFs with a decline in expression of Siglec receptors and other exhaustion markers such as PD-1. Analysis of Tom Cupedo's scRNA seq data generated from inflamed stromal cells of MM patients indicates close similarity to the pattern of sialyltransferases induced in our model. We believe that ongoing inflammation in the bone marrow, could be a major driver of immune suppression in a sialic dependent manner.

From the above, it is clear that the interaction of Siglec ligands on MM cells has the potential for multiple different consequences, facilitating disease progression in MM (Figure 3).

7 Therapeutic targeting of the sialoglycan-Siglec axis

Could this sialoglycan-Siglec axis be therapeutically targeted? Based on an original concept developed by Carolyn Bertozzi, sialidase conjugated antibodies are being developed as a means of desialylating tumor cells at the cell surface (69, 70). In preclinical models of HER2 positive breast cancer, the delivery of sialidase HER2 conjugated antibodies proved more active than HER2 antibody treatment alone, with the combination with PD-1 blockade adding synergism. This approach is now being tested in the clinic with non-targeted antibodies conjugated with sialidase in solid tumors (NCT05259696) and promising initial clinical data were recently presented (71). In the context of MM sialidase conjugated antibodies could be tested in combination with CD38 monoclonal antibodies or even bispecific antibodies, should further preclinical data support this.

An alternative approach is sialyltransferase inhibition. Shortly after the sialyltransferase inhibitor 3FaxNeu5Ac was first described we started to evaluate the activity of this drug in MM. This drug, originally developed by Paulson and colleagues, was the first cell permeable sialyltransferase inhibitor (72). In its peracetylated form it readily diffuses across the cell membrane, is deacetylated by cellular esterases and subsequently form CMP-3Fax-Neu5Ac, which acts as a poor substrate for sialyltransferases by virtue of the electron withdrawing effects of the fluorine substituent. As a result, CMP-3Fax-Neu5Ac builds up to high levels inside the cell, impairing the action of all sialyltransferases, both *in vitro* and *in vivo* (6). Our initial interest was to assess the ability of this drug to inhibit cellular trafficking and overcome cell adhesion mediated drug resistance (CAM-DR) in MM models, both *in-vitro* and *in-vivo*, as noted above, before moving on to study its effect on immune modulation. In initial *in-vivo* studies, with systemic administration of free drug, we confirmed previous observations that at higher doses the drug had potential for nephrotoxicity (12, 73). Bull and colleagues reported that intratumoral injections with the same sialyltransferase inhibitor blocked tumor sialic acid expression *in vivo* and suppressed tumor growth in multiple tumor models (74). Sialic acid blockade had a major impact on the immune cell composition of the tumor, enhancing tumor-infiltrating NK cell and CD8⁺ T-cell numbers while reducing regulatory T-cell and myeloid regulatory cell numbers. Sialic acid blockade enhanced cytotoxic CD8⁺ T-cell-mediated killing of tumor cells in part by facilitating antigen-specific T-cell-tumor cell clustering. Sialic acid blockade also synergized with adoptive transfer of tumor-specific CD8⁺ T cells *in vivo* and enhanced CpG immune adjuvant therapy by increasing dendritic cell activation and subsequent CD8⁺ T-cell responses. Their data emphasize the crucial role of sialic acids in tumor immune evasion and provided proof of concept that sialic acid blockade creates an immune-permissive TME for CD8⁺ T-cell-

mediated tumor immunity, either as single treatment or in combination with other immune-based intervention strategies. Local intratumoral delivery is however not practical for treatment of disseminated cancers. Perhaps this issue can be overcome by targeted delivery to the BM using nanoparticles encapsulating the inhibitor (75, 76). Tumor targeting could also be enhanced by the functionalization of nanoparticles with monoclonal antibodies, though it may also be necessary to also target the stromal compartment for optimal efficacy (16).

Finally, an alternative novel approach would be to specifically target MM specific mucins, such as PSGL-1, by conjugating the secreted protease of C1 esterase inhibitor (StcE) to a nanobody targeting a MM specific antigen, such as BCMA. StcE is a bacterial protease from *Escherichia coli* and cleaves mucin domains by recognizing a discrete peptide- and glycan-based motif. Carolyn Bertozzi and colleagues have shown StcE to be highly effective in eliminating mucins, such as PSGL-1 and CD43 from the cell surface and abolishing Siglec-7 binding (28). Her group recently developed an anti HER2 conjugate, termed α HER2-eStcE, which exhibited high on-target activity as quantified by mixed cell assays in a variety of cell types expressing different mucin proteins (77). In cell lines, functional mixed cell assays replicated the biophysical and immunological effects of treatment with the wild-type StcE enzyme and showed high on-target selectivity. In an orthotopic mouse model of breast cancer, previously shown to be driven by sialic acid-mediated immunological silencing, α HER2-eStcE treatment decreased tumor burden and increased survival.

8 Conclusions

Emerging evidence implicates hypersialylation in the biology of MM, suggesting hypersialylation could play an important role in cellular trafficking, drug resistance and resistance to immune therapies, through modulation of the tumor microenvironment. Future research is required to extend these findings and establish the true importance of sialylation in cell-cell communication, immune cell infiltration and effective anti-tumor immunity in the tumor microenvironment. disease progression. Understanding the impact of sialylation in the TME and how it affects MM disease progression is likely to uncover new therapeutic targets. Hopefully, this will lead to new innovative approaches to improve the outcome of MM patients, particularly in those with high-risk MM.

Author contributions

MO'D: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. SG: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. RM: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. AN: Writing – original draft, Writing – review & editing. AR: Writing – original draft, Writing – review & editing.

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Conflict of interest

MO'D is a founder of ONK Therapeutics, a company developing engineered NK cell therapies and a member of its Board of Directors.

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