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Glycosylation as regulator of human B-cell leukaemias in bone marrow

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Leukemic B-cells are lodged in the bone marrow [BM], a complex organ composed of many cell types and extracellular matrix. Determining how the reciprocal interactions between these components are regulated is critical to our understanding of the factors that allow leukemia cells to survive, multiply and withstand treatment. All cells in the bone marrow are surrounded by a glycocalyx, a glycan-rich layer of high complexity, which regulates such cell-cell and cell-matrix interactions. However, the structure and function of the glycan components of the biomolecules that constitute this layer have not been explored in much detail. Gaps are difficult to fill due to technical limitations as well as the fact that the composition of the BM in health, disease and aging is not static. This also applies to B-lineage malignancies that develop or persist in BM such as B-cell precursor acute lymphoblastic leukemia and Multiple Myeloma, and the effects of their treatment. In contrast, the proteomes and transcriptomes of different human bone marrow cells have been studied more extensively. A combination of technologies now increasingly allows correlations to be made between the expression of glycosyltransferases and glycan structures in cell lines, which could be extrapolated to RNAseq data from primary cells. Glycopeptide analysis will also be invaluable in providing details of specific glycan occupancy on glycoproteins, even if only as a snapshot in time. Functional studies on CD19, CD138/SDC1 and BCMA/TNFRSF17 have already demonstrated the importance of their glycosylation. Additional studies using such approaches are likely to find many more other instances in which malignant B-cell homeostasis is regulated by glycosylation, and lead to the identification of new targets to treat B-cell malignancies.

KEYWORDS

B-cell precursor acute lymphoblastic leukemia, multiple myeloma, bone marrow, scRNAseq, glycans, glycosaminoglycans, proteoglycans

1 Introduction

This review focuses only on human glycans and bone marrow B-cells. Mouse studies, which have provided substantial insight into the functional contribution of glycans to normal murine B-cells, were recently reviewed (1). Since there are no published studies that focus on the glycan component of human bone marrow, main sources of information are

isolated primary cells or cells in tissue culture. Unfortunately, there are no glycomic studies on normal human bone marrow B-cell development, with older studies frequently using human leukemia cell lines such as NALM6 as a stand-in for normal early B-cells (2). Now that it has become more feasible to modify human hematopoietic cells genetically with Cas9/CRISPR technology, questions about the function of capping glycans can also be partly addressed, both *in vitro* as well as after transplant of glycosyltransferase-modified human B-lineage leukemia cells into immunocompromised mice (3). However, such *in vivo* experimentation introduces the variable of cross-species differences in glycosylation (4) and it is unclear if results from rodent cells can always be directly extrapolated to human cells (5, 6). Such limitations also apply to tissue culture. Nguyen et al. (7) showed that human leukemia cells take up N-glycolylneuraminic acid, a non-human glycan, as a component of the commonly used fetal bovine serum. Such incorporation clearly has functional consequences for immune cells (8–10). Additionally, the complexity of cell-cell and extracellular matrix [ECM]-cell interactions is only captured to a limited extent in cultured cells, and the culture of hematopoietic cell types at the non-natural oxygen conditions of tissue culture as compared to bone marrow also can induce changes (11). Thus, an optimal data set for human cells would include information from tissue culture or animal experimentation combined with primary cell analysis.

2 Glycan modifications add tremendous diversity to biomolecules

Glycan modifications add a staggering degree of diversity to biomolecules. Proteins, lipids and [more recently shown for] RNA (12) can all be modified by glycosylation, but the contributions of glycosylation to normal and abnormal B-cell development in the BM remain largely unknown. Glycosylation is regulated by glycosyltransferases, enzymes that are dedicated to one or more biosynthetic pathways of the glycosylation machinery. Its importance is illustrated by the fact that around 2% of all human protein-encoding genes are reserved for this activity: Growth et al. identified 403 human glyco-enzymes involved in biosynthesis of glycans (13).

Details of glycan biosynthesis can be found in reviews on glycosylation (14–17), their analysis (18–21) and in the *Essentials of Glycobiology* (22). The different forms of glycosylation that result in the formation of glycoconjugates are broadly classified based on the molecules to which they are attached, to include proteins [glycoproteins and glycophosphatidylinositol-linked proteins] and lipids [glycolipids]. Protein-linked glycans are also classified based on their attachment to asparagine [N-linked] or serine/threonine [O-linked, including Glycosaminoglycans, GAGs] residues, or on the type of initial sugar attachment such as mannose, fucose or N-acetylglucosamine (4). The contribution of monosaccharide N-acetyl glucosamine modification of serine/threonine residues on mostly intracellular proteins [termed O-GlcNAcylation (23)] in leukemias has been reviewed (24, 25). While biosynthetic steps involved in the different forms of glycosylation are frequently shared, many individual key steps are known to be unique to just one specific pathway (26).

The initial forms of glycan attachment to proteins, such as N-glycosylation, developed early on in the evolution of eukaryotes and remain highly conserved across all eukaryotic species studied to date (22). Individuality and diversification of glycosylation between species was largely introduced by the so-called glycan capping steps such as sialylation and fucosylation (4, 26). These terminal variations have produced highly antigenic epitopes and species-specific functions. In addition, post-glycosylation modifications such as acetylation of sialic acid attached to glycoproteins or glycolipids add further diversity and specificity (27, 28).

3 The human bone marrow is a nursery for normal and abnormal precursor B-cells

The "B" in B-cells designates the bone marrow [BM], the site at which immature B-cells are generated from hematopoietic stem cells. Early B-cell development proceeds from the common lymphoid progenitor, via pro-B1, pro-B2, pre-B1, pre-B2 and immature B cell stages (29). Immature B cells subsequently exit the bone marrow to undergo further differentiation and maturation (30, 31). This developmental process is regulated by changes in the expression of transcription factors (32) and by interactions within the niche of the hematopoietic precursors with bone marrow stromal cells and ECM (33).

The contribution of protein glycosylation to normal human B-cell development remains largely unexplored, although it seems certain that the glycan components of biomolecules are involved. For example, modification of the transcription factor c-Myc by O-GlcNAc in mice stimulates division of pre-B cells (34) suggesting a similar regulation of c-Myc in human B-cell development (35). Table 1 lists some of the important cell surface proteins involved in B-cell development/homeostasis and the contribution of glycosylation to their function, if any. For example, IL7 signaling is essential for early human B-cell development (76). *In vitro*, IL7 binds 300-fold more tightly to N-glycosylated IL7Rα than to the non-glycosylated protein (37). CRLF2, shown to be important for normal human B-cell development (77), needs N-linked glycosylation for cells surface expression and signal transduction after stimulation with TSLP (40).

Not surprisingly, the bone marrow is also the initiating site of precursor B-cell acute lymphoblastic leukemia [BCP-ALL], of which treatment has changed substantially over time (78). BCP-ALL has peaks of incidence in childhood and in older adults. It is the most common childhood malignancy but in recent years has become largely treatable, with survival in children of around 85-92% (79, 80). Older adults still have a much less favorable prognosis (81, 82).

All age groups of BCP-ALL can be divided into different subgroups and risk categories based on transcriptomes and genetic abnormalities (83). These leukemias represent different immature B-lineage precursor cells with a block in normal development and they differ in gene expression profiles to include glycosyltransferases and other glycan-relevant genes such as lectins, which are glycan-binding proteins. For example, the lectin Galectin-1 [Lgals1] is most highly expressed in one specific subgroup of BCP-ALL characterized by KMT2A rearrangements (84, 85), is implicated in other BCP-ALLs (43, 84, 86) and is overexpressed in the ECM of Multiple Myeloma [MM] (87).

4 The human bone marrow is also a retirement home for normal and abnormal plasma B-cells

Bone marrow is also a long-term repository for normal mature hematopoietic cells such as plasma cells, which confer immunological memory (88). Normal BM plasma cells are quiescent solitary cells that depend on, among others, SDF1α/CXCR4 and APRIL/BCMA (Table 1) signaling for retention in BM and survival (89–91). Based on single-cell RNA sequencing, different clusters of normal plasma cells co-exist in BM (92).

TABLE 1 Major glycoproteins important for normal and malignant human BM B-cell homeostasis and development.

[1]expression	receptor/ ligand	[2]glycosylation sites	references	effect of glycosylation	as target
early B-lineage	IL-7Rα receptor	N151	(36) [[3]DLBCL cell lines]	IL-7Rα -IL7 binding (37)	
	IL-7 ligand	predicted	(38, 39)		
early B-lineage	CRLF2 receptor	N55	(36)	needed for cell surface expression and TSLP-stimulated signal transduction (40)	
	TSLP ligand	predicted	(38, 39)		(41)
pre-B	pre-BCR CD179b subunit/Λ5/ IGLL1	T117, S193	(42)	Binding of Lgals1 to the pre-BCR results in pro-survival signalling (43–45)	
	Galectin-1 ligand	N119 S30 [O-GlcNAc]	(36, 39)		
B-lineage	CD20/MS4A1	N9	(36)	not reported	rituximab (46)
B-lineage	CD19	N86, N125, N138	(36, 47)	Hyper and hypoglycosylation inhibits CAR T-cell effector function (48)	CAR T cells (49),
B-lineage	CD22	N61, N65, N67, N99, N101, N112, N135, N231, N445, N546, N574, N575, N621, N633, N634	(50) [Daudi B-cell line] (36)	inhibits B-cell receptor signalling (51) N-glycans needed for epratuzumab engagement (52)	epratuzumab (53)
B-lineage, plasma cells	CXCR4 receptor	S18 [CS]	(54) [cell lines]	no effect on CXCL12 binding (54)	(55, 56)
	SDF1α/CXCL12 ligand	none reported		binds GAGs *in vitro* (57, 58)	
B-lineage	CD44	N57, N100, N110, N282, N350, S180, Y412, N597, N599	(36, 59) (60) [gastric cancer cell lines]	receptor for HA; sialylation of N-glycans inhibits HA binding	binds rVAR2 lectin (61)
plasma cells	CD138/SDC1	S37, S45, S47, S206 [CS/HS] N-linked sialylated	(62, 63) (64)[glioblastoma multiform tissues]	HS essential for binding APRIL (65) 3-O-sulfation mediates APRIL binding [mouse] (66)	binds rVAR2 lectin (67)
plasma cells, B-lineage	BCMA/ TNFRSF17 receptor	N42	(36)	sialylation of N42 promotes internalization (68)	many (69, 70)
	APRIL/TNFSF13 ligand	N174	(71) [human milk]	not reported	
plasma cells pre-B cells	CD38	N100, N209, N219, N229	(72) [[4]liver] (36)	structure stability (73)	daratumumab and others (74)

[1]Markers often expressed in multiple stages of B-cell development (75).
[2]Based on experimental data as indicated in GlyGen (38, 39).
[3]Diffuse large B-cell lymphoma.
[4]It is generally unknown to which extent glycosites are occupied or occupied with similar glycans across human tissues and/or whether this is modulated by age or disease.

MM cells are the malignant counterparts of such plasma cells. However, the initiating mutations in MM may not have occurred in plasma cells located in the BM, but in those in germinal centers, where plasma cells develop (93, 94). MM is a lymphoid malignancy that is more prevalent in older adults and has an overall survival rate in the USA of 58% (79). MM is also subdivided into genetic subgroups with associated risk categories (95) and different treatments (96). Plasma cells and MM cells no longer express a B-cell receptor but can secrete copious amounts of antibodies [between ~10 and 10,000 Ab molecules per cell per second] (97). Since all immunoglobulins are glycosylated (98), this poses unique metabolic (99) and glycome-related challenges to these cells. Glycosylation has also been linked to specific aspects of MM pathology: osteolytic bone disease in patients correlated with reduced levels of galactose and sialic acid on the Fc part of the immunoglobulin and increased formation of immunoglobulin aggregates (100).

5 The ever-changing involvement of glycans to B-cell homeostasis in bone marrow

Whereas glycosylation is a wide-spanning modification, the environment of B-cells in the bone marrow is equally complex: BM is an organ consisting of bone as well as a large number of different hematopoietic cell types, stromal cells and extracellular matrix. These together regulate homeostasis and development of B-cells. Therefore, a comprehensive study of B-cell glycosylation would not only involve analysis of the B-cells themselves, but also incorporate glycan-based interactions and instructions from the cells and ECM that constitute the B-cell microenvironment.

Moreover, the hematopoietic compartment is a picture of constant change: development from hematopoietic stem cells into mature lineages such as the myeloid and lymphoid branches is not a stochastic process, but instead is represented by a continuum (101). For the B-lineage, this includes proliferation and resting phases which distinguish themselves by different metabolic states (102). Seeing that glycosylation is also regulated by metabolic flux (103, 104), one can anticipate that the different states will have different glycosylation as well. In addition, hematopoiesis is dynamically regulated by age (105–107) and inflammation (108, 109), as well as the presence of hematological malignancies (89, 110–112). Thus, glycan analysis of BM B-cells unavoidably represents a captured still image of what is essentially a motion picture.

Not surprisingly, analytical studies of human bone marrow in health and disease are less abundant than those of mice, because bone marrow aspiration and trephine biopsies are the only primary materials available for analysis. Generally, there are two approaches for analysis of BM glycans. One involves isolation and characterization of individual component cell types and ECM, which can provide significant detail. Although not glycan-focused, single-cell proteomic or transcriptomic analysis of bone marrow (Table 1) can generate subclassification of stromal and hematopoietic cell clusters in unprecedented detail. This provides some information on the potential or probable glycans expressed by these cells.

Imaging techniques, in contrast, allow visualization at less granularity but can capture potential glycan-based interactions of B-cells, if appropriate tools such as antibodies or lectins are available. Indeed, advances in microscopy techniques using immunostaining have greatly refined our view of the 3-dimensional structure of human bone marrow (111, 113), although this has not yet focused on the glycan component.

6 Tools for identification of glycans: general considerations

A first step towards analyzing contributions of glycans to normal and abnormal B-cell functioning in bone marrow would be an inventory of what is actually there, and where the glycans are located. Direct analysis of N-linked glycans has perhaps been the most widely used. An analysis of this type was performed on MM peripheral blood and bone marrow plasma using MALDI-TOF-MS for N-glycans. This technology employs enzymatic digestion to separate the N-glycans from the protein backbone, followed by mass spectrometry, which can yield the overall composition of the glycan and, through further analysis, glycosidic linkages. The study reported changes in all main glycosylation features in the MM samples compared to normal plasma (114).

Lectins and antibodies with specificity towards glycan and/or glycoconjugate structures have been widely employed to identify glycosylation traits *in situ* (115, 116). Indirect mass spectrometry imaging [MSI] approaches such as imaging CyTOF allow multiplexing of up to 40 different protein markers within a single experiment (117). Using a panel of scFv antibodies against heparin sulphate, Piszczatowski et al. (118) tracked differentiation from human CD34+ HSPCs into the erythroid and megakaryocyte lineages. Recently, lectins have also been used for cell identification in time-of-flight mass cytometry [CyTOF] to characterize human T-cell subsets (119). However, the vast majority of lectins employed for analytical purposes are of plant origin and unfortunately show a diverse range of affinities for specific glycan structures (120). The complex interaction preferences of the most widely applied lectins have just recently been critically re-evaluated (121), confirming that most lectins exhibit affinities to more than one specific glyco-epitope. While this does not preclude their use to visualize and study glycosylation, an observed change in lectin binding can be caused by more factors than just the simple presence/absence of one specific glyco-epitope. On the other hand, viral lectins and engineered sialyl-O-acetylesterases have been applied with exquisite specificity to allow discrimination of O-acetylation on the 9-, 7- or 4- position of sialic acid as biologically important post-glycosylation modifications (122, 123).

Direct MSI overcomes issues of multiplexing and probe specificity as it does not make use of lectins or antibodies (124). Using this technology, more than 100 biomolecules can be routinely imaged with a resolution down to 1-10 μm within a single

experiment (125). Microdissection glycomics/glycoproteomics combines the benefits of microscopy and histopathology with the power of -omics technologies. In principle, cellular structures of interest can be isolated using microdissection and further processed for -omics analyses (126–128). Microdissection of areas of interest in formalin-fixed, paraffin-embedded tissue sections in combination with nano-scale liquid chromatography coupled to Mass Spectrometry [nanoLC] of N- and O-glycans was shown to be robustly possible down to around 1000 cells (129, 130), opening up novel opportunities to obtain detailed glycan structures and quantitative distribution information in a microenvironment context. To date no study has focused on normal or abnormal B-cells in human BM using any of the above-mentioned methods.

7 The transcriptome -a largely untapped source of information on potential glycotraits

Glycosylation is a non-template driven process that is also regulated by factors beyond direct transcriptional control such as metabolomic flux, glycosyltransferase localization, intracellular transport, and complex enzymatic specificities. This poses specific and unique analytical challenges. However, some inferences can be drawn from transcriptome studies, which in depth and number vastly exceed the direct and indirect glycan analysis mentioned above. In particular, there are numerous transcriptomic studies on both stromal and non-stromal bone marrow cells which could be analyzed for glycosyltransferase transcripts and used to generate hypotheses concerning possible glycotraits expressed on these cells.

As noted, a significant limitation of this approach is that transcriptomics data are only partially indicative for the composition of the glycome: expression levels of glycosyltransferases do not necessarily reflect their enzymatic activity and the presence of final products (18). One example of an early study is that of Bret et al. (131) who analyzed Affymetrix-based signatures of 100 heparan sulphate and chondroitin sulphate-relevant genes in normal and abnormal plasma cells. Jöud et al. (132) also used glycosyltransferase gene expression data to predict carbohydrate blood group loci.

An inherent limitation of analysis of bulk RNA expression data is that signals from cell types which constitute only a minor percentage of the total number may be lost. For example, BM stromal stem/progenitor cells constitute only around 0.001–0.01% of the total amount of BM cells (133). Since glycosyltransferase RNAs are not always highly expressed (134), bulk RNA sequencing is likely to therefore miss their expression in cell types with a low representation. This can be mitigated to some extent by purification of specific cell types using antibody markers. More recently, single-cell RNA sequencing [scRNAseq] techniques, often with prior purification or selection of certain cell types, have been applied to generate unprecedented resolution of human bone marrow in normal and diseased states. Table 2 lists some of these studies [for BCP-ALL, also see (75)]. However, with the increased resolution on a cellular level, there is again potentially a loss of ability to detect glycosyltransferase transcripts due to their low expression combined with technical limitations of scRNAseq to detect all transcripts present in a cell (146). Such data, in combination with other data described below, could be used to generate a prediction of glycotraits expressed on cells that constitute only a minute fraction of the total bone marrow compartment including Minimal Residual Disease (MRD), the [frequently low percentage of] leukemia cells that can persist after chemotherapy.

8 Combinomics provide missing links between transcriptomes and glycotraits

One way to substantiate links between transcriptomes/proteomes and glycotraits is by multi-omics analysis of the same cells. In the only study of this type reported so far, analysis of proteins, RNA and glycans was performed on the same primary BCP-ALL and control samples (18). The study found increased sialylated N-glycans in the leukemia samples. A CRISPR survival screen on a leukemia cell line showed that MGAT1, the enzyme that initiates complex N-linked carbohydrate synthesis, is needed for survival of these cells in a tissue culture setting. Interestingly, compared to normal precursor B-cells, the leukemia cells showed a higher complexity of O-glycans, which could be a reflection of normal B-cell development (147–149). However, expression of mRNA and protein for GALNT7, an enzyme involved in O-glycan synthesis was also higher in the leukemia samples. This enzyme is one of the O–GalNAc glycosylation-initiating enzymes with a substrate preference for follow-up glycosylation of previously glycosylated regions (150–153). Glycopeptide studies with other cell types such as prostate cancer linked higher GALNT7 levels to the presence of the Tn antigen (152), but there is no evidence for Tn expression in BCP-ALL cells.

A landmark paper by Huang et al. (154) provided a critical framework in which RNAseq expression data for glycosyltransferases and the actual glycans expressed in HEK293, an immortalized human embryonic kidney cell line, were linked. The authors identified 38 N-glycan composition structures [complex 13.7%, hybrid 1.7%, oligomannose 84.7%] and 14 O-glycan compositions. Importantly, they validated these correlations by knocking out genes related to N-glycan processing and analysis of the remaining structures. Their GlycoMaple tool is available for analysis of other RNAseq data as well (155). Nielsen et al. (152) used a similar approach to correlate specific O-glycosyltransferases to O-glycan structures. Such studies and those reported by Zhu et al. (156) are likely to ultimately result in a degree of predictability, as reviewed (134) and could be applied to anticipate glycotraits of human BM B-cells based on transcriptome data (Table 2).

Other studies also combined different omics to investigate glycan traits in other cell types and organisms. A systems-based glycobiology approach integrating RNAseq and ChIP-Seq data was used to link transcription factor expression to that of glycogenes in breast cancer (157). Studying Alzheimer's disease, Tang et al. (158) analyzed publicly available data sets for glycosyltransferase expression, with a confirmation through Q-PCR and subsequently

TABLE 2 scRNAseq on primary normal and malignant human B-cells in bone marrow.

	cells	selection	numbers analyzed	goal/analyzed cell types	clusters/cell types	reference
Normal human bone marrow						
1	human BM	[1]BM MNC; Adipocytes excluded. FACS for CD45^{low} CD235a- and CD272+	25,067 cells	stromal cells	42 clusters incl. some hematopoietic cells	(135)
2	human BM	none	100,000 cells; 8 donors	mainly hematopoietic	35 clusters	(136)
3	human BM	analyzed existing data sets	673,750 cells; 145 donors	hematopoietic	54 cell types	(137)
4	human BM	thirteen-colour flow cytometry using 5 customized panels (T, B, NK; monocytes, [2]DC)	20 donors	hematopoietic		(138)
5	human BM	CD34-enriched	6 donors	97 cell surface markers+ scRNAseq [3]Abseq		(139)
6	human BM	BM MNC	4 donors; 18,751 cells	hematopoietic		(140)
7	human BM	BM MNC; negative selection for CD138+, flow sorting with CD19, CD38 and CD138	5 donors; 17,347 antibody-secreting cells	normal BM antibody secreting/plasma cells	15 clusters	(92)n
Leukaemia -Multiple Myeloma and BCP-ALL						
8	MM diagnosis	BM MNC	18 patients;164,521 cells	MM	MM	(141)
9	MM relapsed/ refractory	BM MMC	8 patients; 6955 cells	MM	MM	(55)
10	MM	BM MNC	53 MM samples from 41 patients and 8 normal BM samples	MM	MM and normal PC	(57)
11	MM diagnosis	BM MNC, CD38-enriched, sorted CD38 +CD138+	29 MM and 11 normal BM; 20,568 cells	MM		(142)
11	BCP-ALL	review	different			(143)
12	BCP-ALL	BM MNC	8 different cALL patients, three normal BM samples; 39,375 cells	BCP-ALL cells		(144)

[1]MNC, mononuclear cells; standardly obtained after red blood cell lysis and Ficoll density centrifugation [2]Dendritic cells, [3]Abseq is a high-throughput method to characterize cells using antibodies (145).

N-glycan analysis of changes predicted by the transcriptome changes. Single-cell RNAseq has been combined with lectin-based glycan cell identification (159) for analysis of human induced pluripotent stem cells (160), which could be applied to human BM B-cells. Thus, a combination of many empirical and computational approaches is expected to lead to an increased ability to predict major glycotraits.

9 Glycosaminoglycans

GAGs are a unique type of carbohydrate consisting of very long, pole-like linear carbohydrate chains that are built of repeated disaccharide units of varying repeat length. GAGs can be attached to proteins [proteoglycans] or secreted, like hyaluronic acid (HA). GAG chains are hydrophilic, with a strong negative charge. They also can be modified by the attachment of sulfur groups (sulfation). Heparin sulfate (HS) and chondroitin sulfate (CS) are two main distinct types of GAGs attached to cell surface proteins. GAGs regulate a variety of cellular interactions in the bone marrow because they are expressed by both the bone marrow stroma and by normal hematopoietic cells (161–163). To date, only 77-78 mammalian/human proteoglycan core proteins have been identified (61, 164).

Because core proteins of proteoglycans expressed in human bone marrow can reasonably be expected to have attached GAGs, their mRNA and protein expression can be used to extrapolate the presence of some GAGs. However, this data is limited in that it does not provide information about GAG modification. Main subclasses of proteoglycans include heparin-sulfate and chondroitin sulfate proteoglycans [HSPG and CSPG, respectively] and numerous proteins that interact with them have been identified (165, 166). In a study of the normal human bone marrow proteome, which included hematopoietic cells and mesenchymal stromal/stem cells (MSC), 12,000 proteins were identified. Interestingly, of the age-related changes in older MSC, abundance of proteins tightly linked to glycosaminoglycan metabolism were significantly changed (167).

10 Glycosaminoglycans in MM: CD138 presentation of APRIL to BCMA

Significant differences have been found in GAGs and proteoglycans in MM. For example, on a transcriptional level, increased expression of B4Galt7, involved in the synthesis of CS, was found in comparison with normal BM plasma cells (57, 131). Studying 56 trabecular bone tissue samples, Ho et al. found that ECM proteoglycans were higher in control and monoclonal gammopathy of undetermined significance samples, compared to smoldering and active MM (168). HSPG also have a specific significance to normal BM plasma cells and MM cells as reviewed in (161). In particular, HSPGs can bind and present chemokines and survival factors to hematopoietic cells in the BM. HSPGs were shown to bind SDF1α/CXCL12 (169). This is the key chemokine/receptor axis that attracts and anchors different hematopoietic cell types including normal progenitors, plasma cells as well as malignant hematopoietic cells [BCP-ALL, MM] in the bone marrow and mediates MM drug resistance (169).

APRIL [TNFSF13] is the key cytokine for maintaining viability of bone marrow plasma cells (170) and MM cells (171). APRIL is secreted by myeloid cells in bone marrow (172). It is sequestered by the proteoglycan CD138 [also known as Syndecan-1, SDC1] (173, 174) which is expressed on and is the defining hallmark of plasma cells. In this way, APRIL can be presented to its cell surface receptor BCMA [TNFRSF17], which can be located on the same cell or on other MM cells. Human CD138 exhibits a mixed glycosylation profile (62) (Table 1). Interestingly, using heparitinase and chondroitinase, Matthes et al. showed that the sequestration of APRIL by SDC1 is dependent only on the HS (65). Moreover, Baert et al. recently determined, using murine MM cells, that 3-O-sulfation of the HS on SDC1, as detected by a specific anti-HS antibody, mediates APRIL binding and was associated with increased drug resistance. Human diagnosis MM samples also contained cells reacting with this specific antibody (66). APRIL itself contains one N-linked glycosylation site, Asn174 that, in human milk (71), contains different complex sialylated and core fucosylated structures (38, 39) of unknown function.

BCMA is currently one of most well-studied targets for treatment of MM: clinically approved treatments that target BCMA include CAR-T cells, bispecific T-cell engagers and toxin conjugates (69, 175). Interestingly, glycosylation of BCMA (Table 1) is important for its function, although this does not appear to be studied in the context of therapies that target it, or resistance to or relapse on treatment. BCMA contains one N linked glycosylation site, N42, and sialylation of the glycan structure attached to this site promotes internalization of BCMA (68). The importance of sialic acids to cell surface expression of BCMA was recently confirmed in a Cas9/CRISPR screen, which also surprisingly showed SDC1 as important for BCMA cell surface expression (176).

11 Glycosaminoglycans in BCP-ALL

GAGs and proteoglycans also are important to BCP-ALL, with the process of transformation to malignancy as well as chemotherapy modulating their representation. For example, based on transcriptomic data, it is likely that KMT2A-rearranged BCP-ALL cells undergo a shift from CS towards HS proteoglycans compared to normal BCP cells (18). Some proteoglycans and glycan structures have potential diagnostic and therapeutic applications. For example, CD44, the receptor for HA (177) in bone marrow, is widely expressed on BCP-ALL cells (178). It is a marker for MRD leukemia cells in both BCP-ALL (179, 180) and MM (181). Interestingly, CD44 is also functionally important: low molecular weight HA killed BCP-ALL cells and cell lines by a mechanism involving CD44 (182). CD44 is a complex glycoprotein (Table 1) with many isoforms generated through alternative splicing. Up to 9 potential N-linked glycosylation sites are present, and sialylation interferes with HA binding (183). A specific glycoform of CD44 that forms a ligand for L-selectin and is detected by the HECA-452 antibody (184) is expressed on human hematopoietic cells (185). In MM, relapsed samples had a high degree of HECA-452-positivity (186).

In addition, CD44 is a proteoglycan. A lectin called rVAR2 from P. *falciparum* detects an oncofetal CS structure on many cancer cell types. Candidate proteins expressed on malignant bone marrow B-cells that could bind this lectin include CD44 as well as CSPG4 and SDC1 (164, 187). Indeed, this special structure was detected on NALM-6, a BCP-ALL cell line, and MOLP-2, a MM cell line (164). Other proteoglycans could also present targets for immunotherapy: the proteoglycan CSPG4 is highly and specifically expressed in one of the subtypes of BCP-ALL (188).

12 Glycotraits as targets and modulators of immunotherapy in B-cell malignancies in the BM

Thus, glycosylation is strongly connected to normal and abnormal B-cell survival and development in bone marrow, among others through the regulation of adhesion and of cytokine/chemokine presentations. There are also therapeutic implications: immunotherapy targets are, for the most part, glycoproteins expressed on the cell surface (189). They could also be glycolipids (190) (191–193). Overall, immunotherapies based on antibodies and their derived products such as chimeric antigen receptors (CARs) have made a tremendous impact on treatment of human B-cell malignancies (82, 194–196) but are mainly targeted against CD19, CD22 or CD20 protein epitopes (78). However, there are also therapeutic antibodies specifically directed against glycan epitopes on other targets. It would be outside of the focus of this review to discuss this in-depth, and glycan-focused applications including targets of immunotherapy (164, 197–199) and drug delivery (200), have been recently reviewed, albeit not specifically for BCP-ALL or MM. Antibodies that detect acetylation of sialic acid on the 9-O position in glycoproteins and gangliosides could be the basis for immunological treatment in BCP-ALL (27, 28, 191, 201). Therapeutic applications related to proteoglycans and GAGs were already noted.

A relatively unexplored question is to which extent glycosylation of immunotherapy target proteins on the B-cell

malignancies regulates the way the immune system could react with that target other than direct target recognition. For example, if and how glycosylation regulates the tertiary and quaternary structure of immunotherapy targets is mostly unknown. That glycosylation can regulate different aspects of immune responses is illustrated by the mAb camrelizumab, which interferes with binding of PD-L1 to its receptor PD-1 on T-cells. This approach represents an important breakthrough in treatment of solid tumors (202) although PD-1 blockade did not have a beneficial effect in MM (203). The crystal structure of PD-1 alone or in complex with blocking mAbs was also determined [for example (204–206)]. Since glycosylation was regarded as problematic for efficient crystal formation and structure analysis, crystallography of PD-1 and of almost all other cell surface immunotherapy targets has been traditionally performed on proteins expressed in bacteria, which lack eukaryotic glycosylation, or on proteins in which asparagine (Asn) residues, the sites of N-linked glycosylation, have been mutated. In the case of PD-1, this clearly has limited structure-function insights, seeing that glycosylation was found to regulate PD-1 interaction with PD-L1 as well as with some of the different therapeutic mAbs (207–209).

CD38 is an established treatment target for MM (Table 1). Its glycosylation was reported to be important for the assembly of homodimers and tetramers on the plasma membrane (73, 210) suggesting that glycosylation regulates features of the quaternary structure of this protein. Daly et al. (211) in fact reported that de-sialylation of CD38 increases NK-mediated antibody-dependent cytotoxicity of MM cell lines by daratumumab. A more direct line of evidence for the effect of glycosylation on immunotherapy was presented by Heard et al. (48). The authors performed a Cas9/CRISPR screen to identify factors that contribute to CART19 resistance. They reported that knockout of the Golgi protease SPPL3 led to branched N-glycan changes and increased glycosylation of CD19 in NALM6 BCP-ALL cells. Interestingly, these cells were also partly resistant to CART-19 mediated killing. Overexpression of SPPL3 reduced glycosylation of CD19 and its cell surface expression, also leading to CART19 treatment resistance.

13 Concluding remarks

The glycocalyx of B-cells and bone marrow stromal cells, as well as glycosylation of the ECM is ubiquitous, likely in constant flux, and diverse in composition. Although analysis of only the glycan component of this layer gives a global overview and inventory of what is present and what has changed, the ultimate goal would be to understand specific effects of specific glycans on the biology of these cells. To accomplish this, it will be necessary to analyze the composition of glycans in the context of glycopeptides or other glycoconjugates. Remarkably, this has become possible at the molecular level using electrospray deposition combined with scanning tunneling microscopy (212). However, sorting out which glycosylated sites on a protein are important from a functional point of view will be challenging, because those individual sites may also have variable site occupancy (152). Although it is unlikely that all glycotraits carry equal biological significance (213), constant features/trends do seem to exist: using a combination of analytical techniques, key glycan structures that regulate important biological functions are beginning to emerge. Glycoproteins such as CD44 and CD138 show how specific glycan components can play an essential functional role. Similar studies on other proteins will ultimately lead to a better understanding of how these highly diverse structures contribute to regulation of normal and abnormal B-cell homeostasis in the bone marrow.

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Conflict of interest

The author declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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