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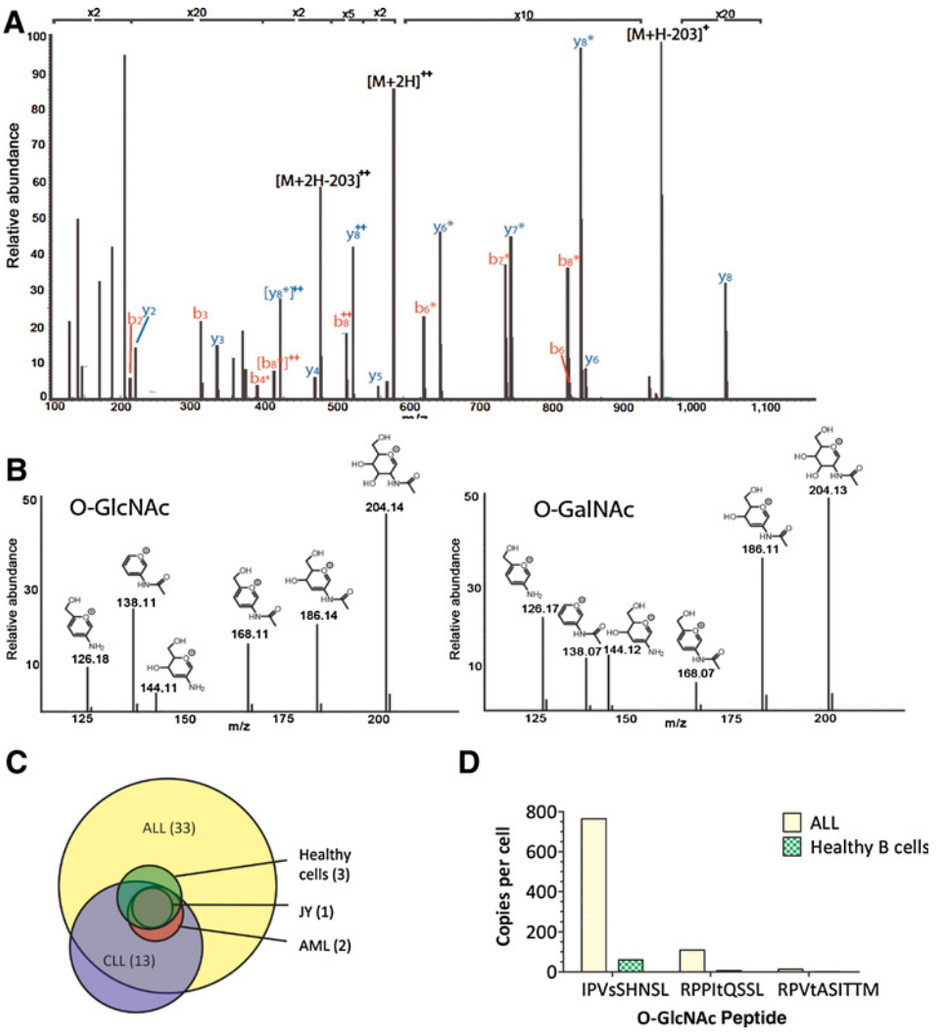

Figure 1.

The discovery of MHC class I–associated glycopeptides on primary leukemia cells. **A,** HCD mass spectrum of the first *O*-GlcNAcylated peptide detected in ALL, IPVsSHNSL. Fragment ions that define the complete amino acid sequence are labeled as b and y. Those that have lost the *O*-GlcNAc moiety are labeled with an asterisk. **B,** Fingerprint ions in the HCD spectra of *O*-GlcNAcylated and *O*-GalNAcylated peptides. Relative abundances of fragment ions derived from secondary fragmentation of the oxonium ion at m/z 204 are substantially different for *O*-GlcNAcylated and *O*-GalNAcylated peptides. **C,** Distribution of 36 HLA-B*07:02–restricted glycopeptides among the different leukemia and healthy cells analyzed. ALL, acute lymphoblastic leukemia; healthy cells, healthy donor tonsil/spleen cells; LCL, lymphoblastoid cell line; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia. **D,** Number of copies per cell of the *O*-GlcNAcylated peptides identified on ALL versus healthy B cells (purified from a healthy spleen).

reaction was used. β1-4-galactosyltransferase (GalT1) was shown to transfer *N*-azidoacetylgalactosamine (GalNAz) to four peptides [IPVsSHNSL and (me-)RPPItQSSL] in the ALL sample. Additionally, we found that synthetic *O*-GlcNAcylated versus *O*-GalNAcylated peptides could be differentiated based on the relative ion abundances observed for fragments derived from the oxonium ion at m/z 204 in the corresponding fingerprint region of the HCD mass spectra (Fig. 1D; ref. 29). All of the peptides observed (Table 1) produced HCD spectra with the necessary fingerprint region to confirm their identity as *O*-GlcNAc peptides.

Ten of the peptides detected were also found with disaccharide units attached to the same residues that were *O*-GlcNAcylated. It was determined that these correspond to a hexose bound to a HexNAc, because the oxonium ion observed for all of these peptides occurred at m/z 366 (204 +162). This was likely the result of the transfer of galactose to the *O*-GlcNAcylated peptide by a β-*N*-acetylglucosamine β1-4 galactosyltransferase; however, the remote possibility that this instead could involve the O-glycan synthetic pathway, in which the first residue to be added is a GalNAc and the second is either galactose or GlcNAc, needed to be excluded. Again, using synthetic peptides (IPVsSHNSL modified with Gal-GalNAc and Gal-GlcNAc), the fingerprint patterns for fragmentation of the oxonium ion at m/z 204 in HCD mass spectra could be distinguished, confirming that none of the disaccharide-modified peptides in Table 1 were derived from the O-glycan synthetic pathway.

Two of the glycosylated peptides in Table 1, APRGnVTSL and KPTLLYnVSL, have disaccharide units, Hexose-HexNAc, attached to Asn residues. Both peptides have consensus sequences, NX(S/T), for attachment of N-linked oligosaccharides. We conclude, therefore, that the observed Hexose-GlcNAc disaccharide units attached to Asn in these peptides probably result from degradation of the N-linked oligosaccharide structures to a single N-linked GlcNAc that then accepts a hexose such as galactose (from a β-*N*-acetylglucosamine β1-4 galactosyl-transferase). This is a novel finding, as the enzyme *N*-glycanase1 is responsible for removing all N-linked glycosylation prior to loading onto MHC class I molecules, potentially suggesting a new source of neoantigens in leukemia (31).

Leukemic glycopeptides elicited potent memory T-cell responses in healthy donors

Previous studies have highlighted how posttranslationally modified antigens can be immunogenic, with immunity against

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Table 1. *O*-GlcNAcylated peptides presented by HLA B*0702 class I MHC molecules on leukemia

#	Sequence	Start–Stop	UniProt	Tumor	Source protein
1[a]	APP(sts)AAAL	405–414	Q86TM6	ALL, CLL1	E3 Ubiquitin-protein ligase synoviolin
2[b]	APRGnVTSL	60–68	Q9NR96	CLL1, CLL2	Toll-like receptor 9
3	APRtNGVAM	187–195	Q92567	ALL, CLL1, CLL2	Protein FAM168A
4	APTsAAAL	1116–1123	Q86Z02	ALL	Homeodomain-interacting protein kinase 1
5	APVsASASV	1807–1815	Q9Y520	ALL	Protein PRRC2C
6	APVsSKSSL	850–858	Q86Z02	ALL, CLL1, CLL2	Homeodomain-interacting protein kinase 1
7	EP(sst)VVSL	1076–1085	O75129	ALL	Astrotactin-2
8	HPMsTASQV	345–353	Q13492	ALL	Clathrin assembly lymphoid myeloid leukemia
9[c]	HP(sss)AAVL	740–748	Q86XN7	ALL, CML	Proline and serine-rich protein 1
10	HP(sst)ASTAL	3041–3050	Q96T58	ALL	Msx2-interacting protein
11	IPIsLHTSL	1959–1967	Q5JSZ5	ALL	Protein PRRC2B
12	IPTsSVLSL	710–718	O15027	ALL	Protein transport protein Sec 16A
13[d]	IPVsKPLSL	104–112	Q16621	AML, ALL, CLL1	Leucine zipper protein 1
14[e]	IPVsSHNSL	147–155	Q06413	AML, ALL, CLL1, JY, S, To	Myocyte-specific enhancer factor 2C
15[f]	KPP(ts)QSSVL	411–420	Q5T6F2	ALL	Ubiquitin-associated protein 2
16[g]	KPPVsFFSL	95–103	Q6PKC3	ALL	Thioredoxin domain containing protein 11
17[h]	KPTLLYnVSL	373–381	P04220	CLL1, CLL2	Ig Mu heavy chain disease protein
18	LPRN(st)MM	335–342	Q9NPI6	ALL	mRNA-decapping enzyme 1A
19	LPTsLPSSL	2464–2472	P46531	ALL	Neurogenic locus notch homolog protein 1
20[i]	MPVRPTtNTF	218–227	Q7Z3K3	ALL	pogo transposable element with ZNF domain
21	NPVsLPSL	831–838	Q6VMQ6	ALL	Activating transcription factor 7-interacting protein
22[j]	PPS(ts)AAAL	405–414	Q86TM6	ALL	E3 Ubiquitin-protein ligase synoviolin
23[k]	RPPItQSSL	382–390	Q9P2N5	ALL, S	RNA binding protein 27
24[l]	RPPQsSSVSL	937–946	O15027	ALL	Protein transport protein Sec 16A
25	RPP(sss)QQL	1758–1766	Q8WYB5	ALL	Histone acetyltransferase KAT6B
26	RPPVtKASSF	341–350	Q9Y2K5	ALL, CLL1	R3H domain containing protein 2
27	RPVtASITTM	927–936	Q9ULH7	ALL, CLL1, CLL2, S	MKL/myocardin-like protein 2
28	TPASsRAQTL	2320–2329	Q01082	CLL1	Spectrin beta chain, non-erythrocytic 1
29	TPAsSSSAL	875–883	Q9NPG3	ALL, CLL1	Ubinucleain 1
30	TPIsQAQKL	3024–3032	Q96L91	ALL	E1A-binding protein p400
31	VPAsSTSTL	576–584	Q9NYV4	ALL, CLL1	Cyclin dependent kinase 12
32	VPTtSSSL	1284–1291	Q14004	ALL	Cyclin dependent kinase 13
33	VPVsGTQGL	93–101	P23511	ALL	Nuclear transcription factor Y subunit alpha
34	VPVsNQSSL	146–154	Q14814	ALL	Myocyte-specific enhancer factor 2D
35	VPVsSASEL	596–603	Q7Z2W4	ALL	Zinc finger CCCH-type, antiviral 1
36	VPVsVGPSL	1157–1164	Q86Z02	ALL	Homeodomain-interacting protein kinase 1

NOTE: Thirty-six peptides, often with multiple forms of glycosylation, were isolated from class I MHC molecules on several leukemias, cell lines, and healthy tissue. These sources are indicated as follows: CML; chronic myeloid leukemia, 1 and 2; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; J, JY cell line; S, spleen; To, tonsil; see Supplementary Table S1. Small letters, s, t, and n specify Ser, Thr, and Asn residues that are modified by O-GlcNAc unless otherwise indicated in a footnote. Parentheses enclose s and t residues that could be a site of GlcNAcylation. Samples were independently analyzed by MS at least 3 times.

[a]Peptide was detected in a total of five forms: single GlcNac, double GlcNAc, single hexose-GlcNAc, single GlcNAc (S6) + hexose- GlcNac (T5), and double hexose-GlcNAc.

[b]N5 is modified by N-linked hexose-GlcNAc.

[c]Peptide was detected in two forms, GlcNAc on S4 and two GlcNAcs on S4 and S5.

[d]Peptide was detected in two forms: GlcNAc (S4) and hexose-GlcNAc (S4).

[e]Peptide was detected in four forms: GlcNAc (S4), double GlcNAc (S4, S5), single hexose-GlcNAc (S4), and an acetyl-GlcNAc (S4).

[f]Peptide was detected in two forms: GlcNAc and hexose-GlcNAc (T4).

[g]S5 is modified by O-linked hexose-GlcNAc.

[h]N7 is modified by N-linked hexose-GlcNAc.

[i]Peptide was detected in two forms: hexose-GlcNAc and asymmetric di-methyl (R4) + hexose-GlcNAc (T7).

[j]T4 or S5 is modified by O-linked hexose-GlcNAc.

[k]Peptide was detected in four forms: GlcNAc (T5), mono-methyl (R1) + GlcNAc (T5), asymmetric di-methyl (R1) + GlcNAc (T5), and asymmetric di-methyl (R1) + acetyl-GlcNAc (T5).

[l]S5 is modified by O-linked hexose-GlcNAc.

leukemia-associated MHC class I phosphopeptides having been shown to be present in healthy individuals. Immunity against naturally processed MHC class-I *O*-GlcNAc or methylated peptides has not been studied, but we hypothesized that it may exist in healthy individuals. Immunogenicity in healthy donors was assessed using seven of the *O*-GlcNAcylated peptides discovered on leukemic cells (Fig. 2A–E; Supplementary Figs. S2–S5). Five of the seven (71%) HLA-B*0702 glycopeptides were immunogenic—heterogeneous responses were seen, with both intra- and inter-donor variation (Fig. 2B and C). The responses were further validated using IFNγ ELISpot (Supplementary Fig. S5). All healthy donors had immunity to at least one of the glycopeptides and two had strong responses, similar to the magnitude of responses against chronic viral antigens. Degranulation was assessed as a proxy for killing (Fig. 2C) and despite some background staining, degranulation significantly correlated with multifunctional cytokine responses (Fig. 2D), suggesting that these T cells targeting *O*-GlcNAcylated peptide antigens have a cytotoxic phenotype. Furthermore, these T cells appeared to be largely the memory phenotypes (Fig. 2E).

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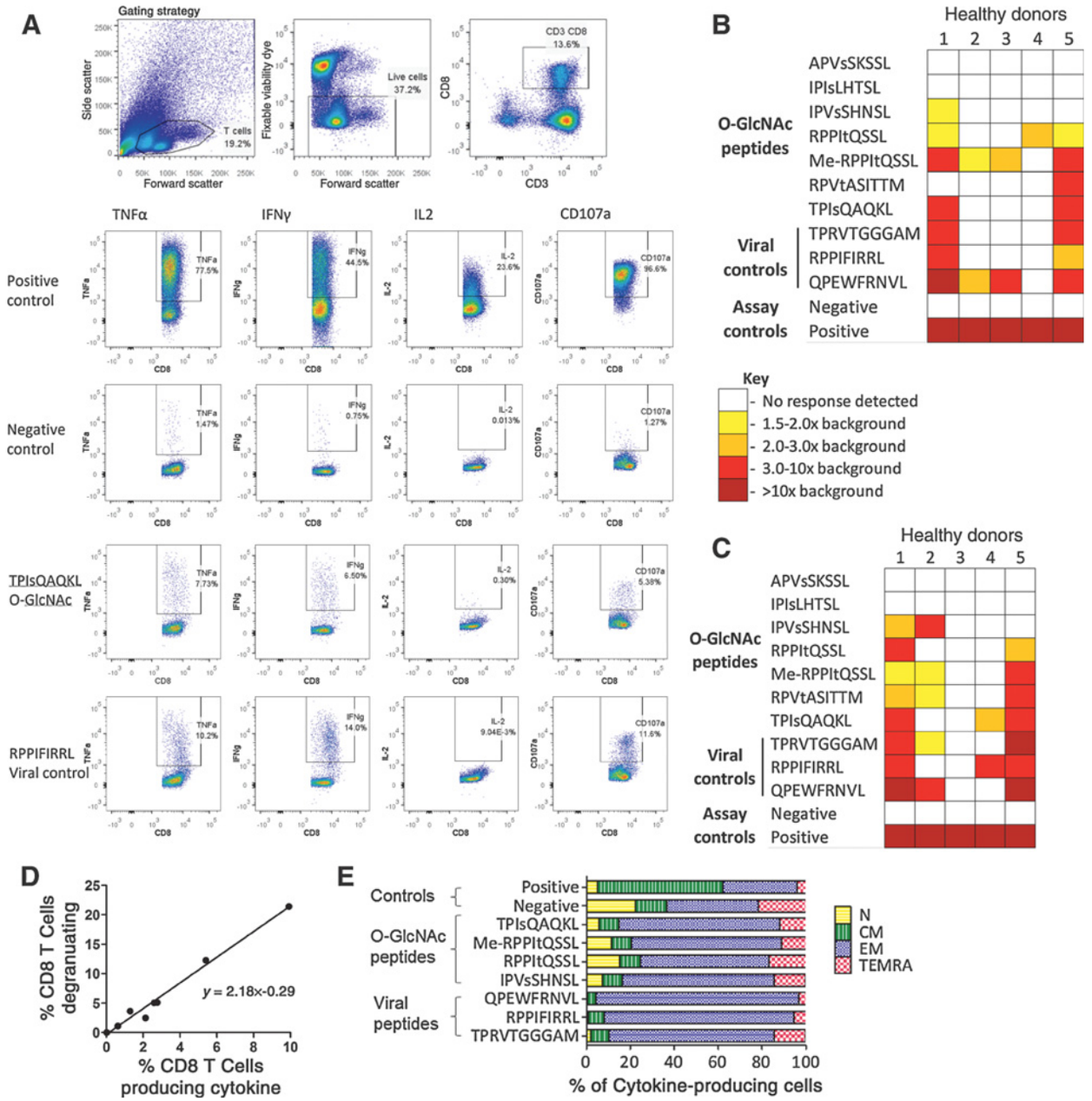

Figure 2.

Healthy donor immunity to leukemia-associated posttranslationally modified neoantigens. **A,** Flow cytometry plots showing the gating strategy used in the ICS protocol to determine healthy donor immunity to the *O*-GlcNAcylated peptides (Supplementary Fig. S4 contains additional plots). Immunity to viral antigens was used as an internal control, for comparison. Collated results of cytokine production (**B**) and degranulation (**C**) by healthy donor T cells in response to stimulation with posttranslationally modified leukemia neoantigens. **D,** The correlation between the percentage of cells producing cytokine and degranulating for HD1. **E,** HD1 T cells that produced cytokine in response to stimulation with peptides were also stained with surface antibodies for phenotyping (CD27 and CD45RA; Supplementary Fig. S5). CM, central memory; N, naïve; EM, effector memory and TEMRA, terminal effector memory. Responses were independently verified at least twice.

Modifications of a methylated glycopeptide specifically targeted by cytotoxic T cells

As responses were seen against the intriguing methylated glycopeptide ((me-R)PPI(GlcNAc-T)QSSL) in 4 of 5 (80%) of healthy donors tested, two being potent, these responses were further analyzed using peptides that were either methylated or glycosylated. Whereas no T-cell responses were seen against the unmodified peptide, responses in different individuals were seen targeting either the glycosylated or the methylated peptide (Fig. 3A; Supplementary Fig. S6). In the two donors

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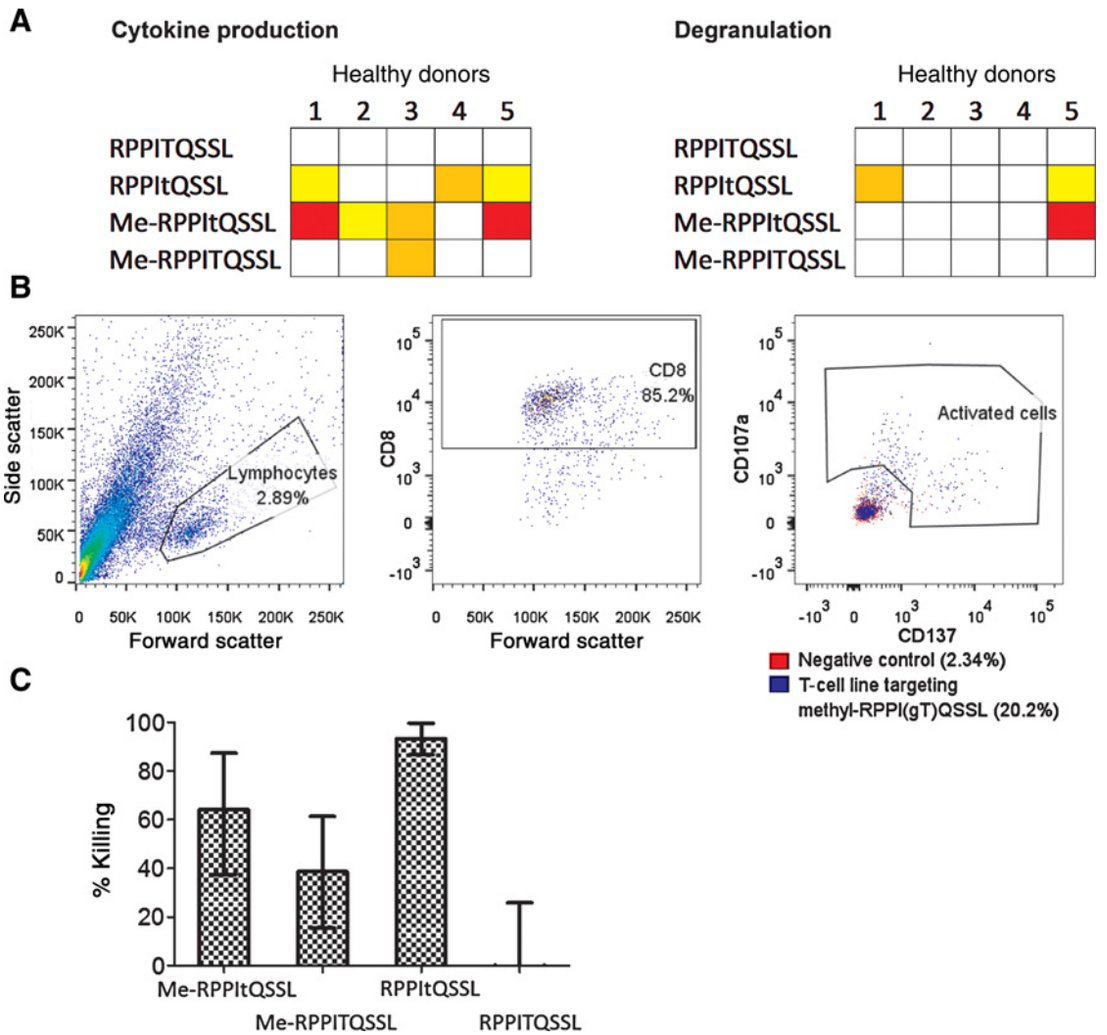

Figure 3.

Investigating T-cell recognition of the methylated *O*-GlcNAc peptide. **A,** Healthy donor immunity to the unmodified, *O*-GlcNAcylated, methylated and both *O*-GlcNAcylated and methylated peptide, measured by cytokine production and degranulation. **B,** A T-cell line was grown from HD5 against the methylated RPPItQSSL peptide. The percentage of cells recognizing the peptide was assessed by overnight stimulation with the peptide and detection of CD137 and CD107a surface markers. **C,** This T-cell line was using a europium release killing assay to assess killing of autologous transformed B cells pulsed with different modifications of the peptide. Responses were independently verified at least twice.

with potent responses to the methylated glycopeptides, fewer T cells recognized the glycopeptide alone, suggesting that the methylation may somehow increase immunogenicity. To assess this further, a T-cell line was initiated using the methylated glycopeptide. After culture, around 18% of T cells were shown to be specific for the methylated glycopeptide (Fig. 3B; Supplementary Fig. S6). Autologous transformed B cells were pulsed with modified and unmodified peptides and killing by the T-cell line assessed. Specific killing was seen of the B cells pulsed with methylated, *O*-GlcNAcylated, and doubly modified peptide, but not with the unmodified peptide (Fig. 3C). These results suggest that we may have identified modified peptides targeted by the endogenous antileukemia T-cell response, which may lead to fruitful targets for novel immunotherapeutics.

Discussion

We outline here three methodologies for the identification of MHC class I peptides containing a little-known PTM, *O*-GlcNAc, a potential class of cancer neoantigens. Utilizing these methods, we identified 36 GlcNAcylated peptides from primary leukemia samples and showed that a memory T-cell response against a subset of these antigens could be found in healthy donors. We have also identified peptides that contained other moieties—not previously seen on MHC class I peptides from cancer samples—namely, methyl, disaccharide, and N-linked GlcNAc groups. Peptides containing these PTMs offer a hitherto untapped source of neoantigens in leukemia.

These neoantigens created by PTMs may be found on leukemic cells because of their aberrant cell signaling. This has

been reported for phosphopeptide leukemia antigens, and *O*-GlcNAcylation sites are usually identical, or in close proximity, to those that get phosphorylated (17, 20). Aberrant *O*-GlcNAcylation has been shown to correlate with augmented cancer cell proliferation, survival, invasion, and metastasis (21). The essential nature of these pathways to the leukemic cells suggests that these PTM neoantigens may not be patient specific, as seen with the mutated neoantigens, but common across patients of the same HLA-type (7, 8). Indeed, we identified many of them on multiple samples from leukemia patients, even those with different clinical types. Around a quarter (7 of 32) of the proteins that the PTM peptides derived from are associated with key cancer pathways (as defined by the NCI pathway interaction database). Antigens from these key signaling pathways are ideal targets for immunotherapies because the leukemic cell is unlikely to be able to survive without these pathways, reducing the risk of immune escape. Although further work is required to ensure that these PTM peptides are truly cancer neoantigens and not found in healthy tissues, they may provide an attractive new avenue for immunotherapeutic targeting.

Not only are these neoantigens present on leukemia samples, but positional analysis indicates that the GlcNAc residues may be optimally positioned for T-cell recognition. The GlcNAc group is in the middle of the peptide (up to 34/36; 62% P4, 18% P5, 21% equivocal P4/P5; Supplementary Fig. S7), identical to the preferred position of phosphate groups in phosphopeptides, and where structural studies have revealed that the CDR3 regions of the T cell receptor (TCR) loops around the center of the peptide (32). Indeed, previous structural studies in mouse of TCR binding have demonstrated that GlcNAc-modified antigens are recognized in this manner (22).

We saw potent multifunctional memory T-cell responses against these *O*-GlcNAcylated leukemia antigens in healthy donors, suggesting that these neoantigens may reflect an endogenous immunosurveillance system against leukemia. Not only did healthy donor T cells recognize the PTM neoantigen, but we also showed that they could specifically kill cells presenting modified peptides. Therefore, we would not expect targeting of these antigens to be compromised by self-tolerance, as may be seen with overexpressed antigens. What is more, if healthy donors have cytotoxic memory T cells targeting these PTM neoantigens without autoimmunity, targeted therapies against these neoantigens may have low toxicity. The most immunogenic peptide identified was me-RPPItQSSL, containing both a methylated arginine and *O*-GlcNAcylated serine. It is tempting to speculate that combined modifications lead to the most dramatic structural change and, therefore, peptides more antigenically distinct from self. We showed that T cells may recognize and kill cells presenting this peptide with either the methylation, or the *O*-GlcNAc modification, but not the unmodified peptide. This potent antigen, targetable by T cells from several healthy donors, is an attractive target for the development of immunotherapeutics. We are expanding this work to identify *O*-GlcNAcylated antigens from patients with other HLA types and cancers and in the process of developing methods that allow for the identification of methylated peptide antigens from MHC class I.

Overall, this work identified both glycosylated and methylated residues as potent classes of tumor antigens, broadening the availability of immunotherapy targets, and potentially yielding safe and effective therapeutics for leukemia.

Disclosure of Potential Conflicts of Interest

J. Shabanowitz has ownership interest (including patents) in Agenus Inc. and is a consultant/advisory board member for the same. D. Hunt is a consultant for, has ownership interest (including patents) in, and is a consultant/advisory board member for Agenus. M. Cobbold reports receiving commercial research grant from Agenus and is a consultant/advisory board member for Agenus. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.A. Malaker, S.A. Penny, D.L. Bai, J. Shabanowitz, D.F. Hunt, M. Cobbold
Writing, review, and/or revision of the manuscript: S.A. Malaker, S.A. Penny, D.L. Bai, J. Shabanowitz, D.F. Hunt, M. Cobbold
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.G. Steadman, D.L. Bai, D.F. Hunt
Study supervision: D.F. Hunt, M. Cobbold

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Identification of Glycopeptides as Posttranslationally Modified Neoantigens in Leukemia

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