

Identification of Glycopeptides as Posttranslationally Modified Neoantigens in Leukemia



Stacy A. Malaker¹, Sarah A. Penny², Lora G. Steadman², Paisley T. Myers¹, Justin C. Loke², Manoj Raghavan², Dina L. Bai¹, Jeffrey Shabanowitz¹, Donald F. Hunt^{1,3}, and Mark Cobbold^{2,4}

Abstract

Leukemias are highly immunogenic, but they have a low mutational load, providing few mutated peptide targets. Thus, the identification of alternative neoantigens is a pressing need. Here, we identify 36 MHC class I-associated peptide antigens with O-linked β -N-acetylglucosamine (O-GlcNAc) modifications as candidate neoantigens, using three experimental approaches. Thirteen of these peptides were also detected with disaccharide units on the same residues and two contain either mono- and/or di-methylated arginine residues. A subset were linked with key

cancer pathways, and these peptides were shared across all of the leukemia patient samples tested (5/5). Seven of the O-GlcNAc peptides were synthesized and five (71%) were shown to be associated with multifunctional memory T-cell responses in healthy donors. An O-GlcNAc-specific T-cell line specifically killed autologous cells pulsed with the modified peptide, but not the equivalent unmodified peptide. Therefore, these posttranslationally modified neoantigens provide logical targets for cancer immunotherapy. *Cancer Immunol Res*; 5(5); 376–84. ©2017 AACR.

Introduction

The role of antitumor immunity has been an intense focus of research for many decades (1–4). Although it is clear from strong correlative clinical data, combined with definitive experimental evidence from mouse cancer models, that T cells mediate this protection, the nature of the antigens targeted remains poorly characterized. Over the past decade, the role of altered-self antigens, termed neoantigens, has become clear (5–9). Tumor-specific neoantigens act as targets for spontaneously arising adaptive immunity to cancer and thereby determine the ultimate fate of developing tumors (7). Nonsynonymous mutations in coding regions of expressed proteins are termed mutational neoantigens and, perhaps critically, are not subject to central tolerance. In patients with cancers that have high muta-

tional loads, such as non-small cell lung cancer and melanoma, CD8⁺ T cells can be identified within the tumor that are specific for MHC class I-restricted neoantigens in response to immunotherapy (10, 11). However, tumor-resident immunity against mutational neoantigens occurs at very low frequencies and it would be surprising if this magnitude of immunity could be responsible for the dramatic reductions in tumor volume seen (7, 12). Additionally, some of the tumors with the best clinical responses to immunotherapy have some of the lowest mutational loads, for example, renal cell carcinomas and leukemias (12–14). Hematological malignancies, in particular, are known to be among the most immunogenic cancers (15). Therefore, it is likely that the antigens in these malignancies derive from other classes of antigens.

An alternative source of neoantigens may be the posttranslational modifications (PTM) that occur in malignant and not healthy cells, particularly as dysregulated signaling is a hallmark of cancer (16). Indeed, a number of phosphorylated peptides have been identified as potent cancer antigens (17). Immunity to these antigens was seen in healthy donors, but lost in a subset of leukemia patients with poor clinical outcome and restored after stem cell transplant, suggesting a role for these antigens in the graft-versus-leukemia response. Dysregulation of cell signaling pathways in cancer is also caused by another PTM, β O-linked N-acetylglucosamine (O-GlcNAc), which is involved in cross-talk with phosphorylation (18–20). As such, aberrant O-GlcNAcylation can correlate with augmented cancer cell proliferation, survival, invasion, and metastasis (21). Synthetic O-GlcNAc-modified peptides can bind MHC class I complexes and elicit glycopeptide-specific T-cell responses in mice, with X-ray structures confirming that the O-GlcNAc group was solvent exposed and accessible to the T-cell receptor (22–25). However, up until recently, limitations in proteomic

¹Department of Chemistry, University of Virginia, Charlottesville, Virginia. ²Department of Clinical Immunology, University of Birmingham, Birmingham, United Kingdom. ³Department of Pathology, University of Virginia, Charlottesville, Virginia. ⁴Massachusetts General Hospital Cancer Center and Department of Medicine, Harvard Medical School, Charlestown, Massachusetts.

Note: Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

S.A. Malaker, S.A. Penny, D.F. Hunt, and M. Cobbold contributed equally to this article.

Current address for Stacy A. Malaker: Department of Chemistry, Stanford University, Stanford, CA.

Corresponding Author: Mark Cobbold, Massachusetts Cancer Center, 149 13th Street, Boston MA 02129. Phone: 617-320-4243; Fax: 617-726-5611; E-mail: mcobbold@mgh.harvard.edu

doi: 10.1158/2326-6066.CIR-16-0280

©2017 American Association for Cancer Research.

technology made it impossible to characterize O-GlcNAcylated peptides from biological samples.

Here, we report three experimental approaches that allowed the detection and sequencing of O-GlcNAcylated peptides from a complex mixture of peptides presented by HLA-B*07:02 molecules on various primary leukemia samples. These methods allowed for the identification of 36 unique glycopeptides in several different states of glycosylation and, surprisingly, methylation. We go on to show that healthy donors have heterologous immunity to a number of these complex neoantigens and that T cells from these donors can specifically target and kill cells displaying only the modified peptide. Ultimately, we believe that these glycopeptide antigens will prove pivotal in the design of novel cancer immunotherapeutics.

Materials and Methods

Leukemia samples and cell lines

Leukemia samples were the same as those used previously (Supplementary Table S1; ref. 17). All cell lines were grown at 37°C with 5% CO₂ in medium consisting of RPMI 1640 supplemented with 10% fetal bovine serum (all from Sigma-Aldrich). The JY cell line (ECACC, 94022533) was grown continually between 2006 and 2014 with its authenticity verified by HLA-typing and also in-house peptide profile.

Isolation of HLA-associated peptides

Class I MHC molecules were immunoaffinity-purified from cell lines or tumors and their associated peptides were extracted as described previously (17). Cells (10⁸–10⁹) were lysed in 10 mL of CHAPS buffer (Sigma-Aldrich) and the lysate was centrifuged at 100,000 × g for 1 hour at 4°C. Supernatants were passed over protein A-sepharose preloaded with HLA-B7-specific antibody (ME1). Peptides were eluted from the purified MHC class I molecules with 10% acetic acid and separated by ultrafiltration (Ultrafree-MC, Millipore).

Enrichment of HLA-associated peptides

POROS20 AL beads (Applied Biosystems) were derivatized with amino-phenyl boronic acid (APBA; Thermo Fisher Scientific). Briefly, POROS20 beads (7 mg) were dispersed into 200 μL of PBS (pH 6-7) containing 40 μmol of APBA. Following the addition of NaCNBH₃ (1.3 μmol in 1 μL of PBS), the reaction was allowed to proceed with agitation for 2 hours at room temperature and then quenched by washing the beads with water on a spin column (pore size < 20 μm). Water was removed under vacuum, and the dried beads were stored at 4°C.

Class I MHC peptides from 2 × 10⁸ to 5 × 10⁸ cells in 0.1% acetic acid were desalted by loading the solution onto a fused-silica column (360 μm o.d. × 150 μm i.d.) packed in-house with 5 cm of irregular C18 (5–20 μm diameter) particles at a flow rate of 0.5 μL/minutes. After washing the column with 25 μL of 0.1% acetic acid, peptides were eluted into Eppendorf tubes with a 40-minute gradient (0–80%) solvent B (A: 0.1 mol/L acetic acid, B: 70% acetonitrile, 0.1 mol/L acetic acid). Fractions were screened by MS and those that contained peptides, but not CHAPS detergent, were combined, taken to dryness and stored at –35°C.

APBA beads were washed 3 × with 100 μL of anhydrous DMF and then allowed to react with desalted peptides in 20 μL of anhydrous DMF for 1 hour with agitation. Solvent was removed

by centrifugation, and the beads were washed 2 × with 100 μL of anhydrous acetonitrile. Bound peptides were released by agitating the beads in 20 μL of 0.1 mol/L acetic acid for 30 minutes. Supernatant was collected, taken to dryness, and reconstituted in 10 μL of 0.1 mol/L acetic acid for loading onto an in-house packed C18 column for MS analysis.

RP-HPLC-MS

In-house, packed C18 columns were prepared as previously described (26). Peptides were eluted by a 2-hour 0–60% B gradient (A: 0.1 mol/L acetic acid, B: 70% acetonitrile (ACN), 0.1 mol/L acetic acid). Without enrichment, samples were loaded directly onto the C18 column. The RP-HPLC elution was electrospray ionized into an Orbitrap Velos, or Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific), the former equipped with an in-house front-end ETD ion source. On the Orbitrap Velos, the instrument method was a top-10 collision activated dissociation (CAD) with electron transfer dissociation (ETD) only when loss of dehydro-GlcNAc neutral loss of [203]+2 or [203]+3 was detected. On the Fusion, the instrument method was a top-speed higher-energy collisional dissociation (HCD) triggered ETD when three of six O-GlcNAc fingerprint ions (m/z 204, 186, 168, 144, 138, and 126) were detected at >5% relative abundance. Peptide sequences were determined by manual interpretation of HCD, CAD, and ETD mass spectra.

Synthetic peptides

O-GlcNAc- and O-GalNAc-peptides were synthesized using Fmoc chemistry and purified by HPLC to >90% purity by Pierce Biotechnology. Sequences and purity for all synthetic peptides were confirmed by online HPLC MS/MS and manual interpretation of the resulting spectra. Immunodominant HLA-B*0702-restricted antigens from human cytomegalovirus (pp65_{417–426}, TPRVTGGGAM); Epstein-Barr virus (EBNA-3A_{247–255}, RPPI-FIRRL) and Influenza A virus (PB1_{329–337}, QPEWFRNVL) were synthesized at >90% purity by GenScript.

Selective transfer of N-azidoacetyl galactosamine (GalNAz) to O-GlcNAcylated peptides

A solution of the modified β1-4-galactosyltransferase, GalT1 (Invitrogen), was dried to 5 μL in a vacuum concentrator at 40°C. Excess synthetic glycopeptides or tumor peptides (3e8 cell equivalents) were taken to dryness, redissolved in a mixture containing 1 μL MnCl₂, 5 μL uridinediphosphate N-azidoacetyl galactosamine, UDP-GalNAz (Invitrogen), and 5 μL enzyme and allowed to react for 5 hours at room temperature. After the reaction was quenched by addition of 0.2 μL glacial acetic acid, the solution volume was increased to 15 μL with 0.1% acetic acid, and then loaded directly onto an HPLC column for analysis by LC-MS/MS.

Intracellular cytokine staining

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors and resuspended (10⁶ cells/mL) in AIM-V medium (Invitrogen). Synthetic peptide antigens were added to the wells (10 μg/mL), and cells were expanded for 6 days. The positive control was stimulated with phytohemagglutinin (1 μg/mL). On day 6, cells were washed and restimulated with peptide antigen overnight or, for the positive control, with phorbol 12-myristate 13-acetate/ionomycin (4 ng/mL and 500 ng/mL, respectively), in the presence of anti-CD107a-FITC. Cells were harvested, washed with PBS, and stained with

fixable viability dye (APC-Cy7) and surface antibodies: anti-CD3 and anti-CD8. Cells were fixed using 2% formaldehyde, permeabilized using 0.5% saponin, and stained with anti-IFN γ -PE, anti-IL2–Pacific blue, and anti-TNF α -PE-Cy5.5 for 30 minutes at room temperature. Cells were washed, lightly fixed, and analyzed on the LSRFortessa X20 flow cytometer (BD Bioscience). A list of antibodies used in the study is shown in Supplementary Table S2.

Establishment of a peptide-specific T-cell line

PBMCs (10^7) were stimulated with synthetic peptide and cultured for a week. They were subsequently restimulated overnight, in the presence of anti-CD107a-FITC and washed and labeled with anti-CD137-PE (Miltenyi Biotec) and counterstained with anti-CD8-APC (Biolegend). Cells were sorted using a FACS Aria cell sorter (BD Bioscience), collected, and expanded using the rapid expansion protocol previously described (27). The T-cell line was subsequently reassessed, using a similar protocol.

Europium release killing assay

The Delfia EuTDA cytotoxicity assay (Perkin Elmer) was used according to the manufacturer's instructions. Briefly, autologous transformed B-cell lines were used as target cells. These were washed and resuspended at 10^6 cells/mL in RPMI 10% FCS, and the relevant peptide antigen was added at 10 μ g/mL and the mixture was incubated at 37°C, 5% CO $_2$ in a humidified environment for 40 minutes. Subsequently, 2.5 μ L/mL of the BATDA fluorescence enhancing ligand was added and the cells were incubated for a further 20 minutes. Cells were then washed 5 \times in excess medium. Target cells (10^4) were added to each well of a V-bottomed 96-well plate. T cells at varying effector to target (E:T) ratios were added to the test wells. All well volumes were made up to 200 μ L. The plate was incubated for 2 hours at 37°C, 20 μ L of each supernatant was transferred to a flat bottomed, white, 96-well plate and 200 μ L of Europium solution was added, incubated for 15 minutes, with shaking, at room temperature. Fluorescence was measured with a time-resolved fluorometer (Tecan Infinite 200 PRO).

Results

Experimental approaches that allow identification of O-GlcNAcylated peptide antigens

Three different experimental approaches were used to identify peptides with O-GlcNAc modifications from leukemia patient samples. The first approach used higher energy collision-induced dissociation (HCD) mass spectrometry (MS) to visualize the loss of a dehydro-*N*-acetyl-glucosamine moiety (203Th) from fragment ions. The HCD mass spectrum of the first O-GlcNAcylated class I MHC peptide, XPVsSHNSX (where X = I or L), detected during analysis of HLA B*07:02 peptides presented on ALL, is shown in Fig. 1A. The amino acid sequence, XPVsSHNSX, is uniquely present as IPVSSHNSL in a single human protein, myocyte-specific enhancer factor 2C. This approach is limited by the ability of nonglycosylated peptides to suppress electrospray ionization of co-eluting O-GlcNAcylated peptides (28).

The second approach overcomes this limitation and allows detection and characterization of O-GlcNAcylated peptides from attomole quantities, using an enrichment procedure to

selectively pull down the glycosylated peptides from the pool of unmodified peptides. The enrichment allowed selective esterification of glycosylated peptides, linking them to aminophenylboronic acid-derivatized POROS AL 20 beads. This O-GlcNAc enrichment procedure allowed us to achieve quantitative yields from femtomoles of starting material and may be critical for efficient identification of these antigens from patient samples. This methodology was used in combination with an instrument method that selectively recorded electron transfer dissociation (ETD) when the collision-activated dissociation (CAD) spectrum contained a doubly charged ion corresponding to the loss of dehydro-*N*-acetyl-glucosamine (203Th; ref. 29).

The third experimental approach extended the sensitivity of the method and allowed the user to obtain spectral information from extremely low-level peptide species. This was achieved using an instrument method that triggered an ETD spectrum whenever three of six O-GlcNAc fingerprint ions (m/z 204, 186, 168, 144, 138, and 126) were detected at >5% relative abundance in a particular HCD spectrum when the Orbitrap Fusion Tribrid was set to record HCD spectra at top speed. All of the fingerprint ions in Fig. 1B were the result of the O-GlcNAc oxonium ion at m/z 204 undergoing further fragmentation as a result of multiple collisions with the background nitrogen gas in the collision chamber. The use of multiple fingerprint fragments created a reliable trigger that minimized false positives.

36 O-GlcNAcylated peptides identified on HLA B*07:02 leukemia samples

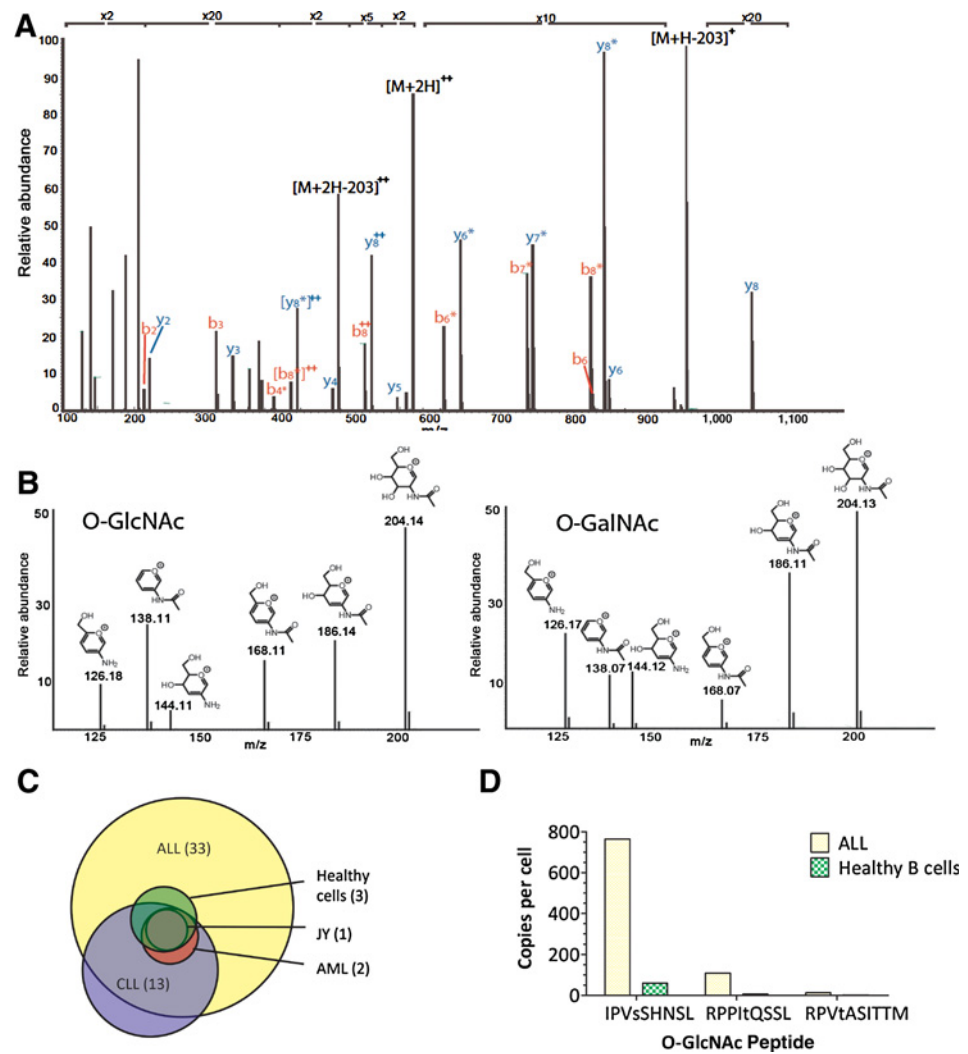
In total, using the three experimental approaches outlined, 36 O-GlcNAcylated peptides were identified from leukemia samples (Table 1). Ninety-two percent (33/36) of the O-GlcNAcs identified were found only on the leukemia samples tested and not the healthy tissue samples, making them potential leukemia neoantigens (Table 1, Fig. 1C, Supplementary Fig. S1). Just under a quarter (7/32) of the proteins that the O-GlcNAcylated peptides derived from were associated with key cancer pathways (as defined by the NCI pathway interaction database, which is now accessible via the NDEx database at <http://www.ndexbio.org/#/>). These pathways included many classical cancer signaling pathways, involving proteins such as p38, p53, c-Myc, Notch, Wnt, Rb, ErbB1, and MAPK. Of note, IPVSSHNSL, which derives from Mef2c, a transcription factor implicated in leukemogenesis (30), was identified on nearly all of the leukemia samples tested (1/1 ALL, 3/3 CLL, and 1/1 AML) and although it could be detected on healthy B cells, it was present in far lower amounts (Fig. 1D). An incidental, but significant, finding was that one of the O-GlcNAcylated peptides detected, RPPItQSSL, contained another PTM—a methylated Arg residue at P1 and was also found with an asymmetrically dimethylated Arg residue (Supplementary Fig. S2).

Distinguishing O-GlcNAc from O-GalNAc

Because O-GlcNAc and O-GalNAc are isobaric, but have different biological properties, it was important to confirm that the peptides we identified indeed contained O-GlcNAc modifications, and not O-GalNAc. Furthermore, some MHC class I peptides we observed contained disaccharide units (see footnotes for Table 1), so they might have been derived from degradation of O- and N-linked glycans synthesized in the Golgi and ER, rather than true cancer neoantigens. To validate that the peptide antigens we had tested were O-GlcNAcylated, an *in vitro* enzyme

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, IPV_sSHNSL. 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. β 1-4-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 β 1-4 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, APRG_nVTSL 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 β 1-4 galactosyltransferase). 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

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	Astroctactin-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(ssst)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	TPAsRAQTL	2320–2329	Q01082	CLL1	Spectrin beta chain, non-erythrocytic 1
29	TPAsSSSAL	875–883	Q9NPG3	ALL, CLL1	Ubinuclein 1
30	TPIsQAQKL	3024–3032	Q96L91	ALL	E1A-binding protein p400
31	VPAStSTSL	576–584	Q9NYV4	ALL, CLL1	Cyclin dependent kinase 12
32	VPTSSSL	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.

^aPeptide 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.

^bN5 is modified by N-linked hexose-GlcNAc.

^cPeptide was detected in two forms, GlcNAc on S4 and two GlcNAcs on S4 and S5.

^dPeptide was detected in two forms: GlcNAc (S4) and hexose-GlcNAc (S4).

^ePeptide was detected in four forms: GlcNAc (S4), double GlcNAc (S4, S5), single hexose-GlcNAc (S4), and an acetyl-GlcNAc (S4).

^fPeptide was detected in two forms: GlcNAc and hexose-GlcNAc (T4).

^gS5 is modified by O-linked hexose-GlcNAc.

^hN7 is modified by N-linked hexose-GlcNAc.

ⁱPeptide was detected in two forms: hexose-GlcNAc and asymmetric di-methyl (R4) + hexose-GlcNAc (T7).

^jT4 or S5 is modified by O-linked hexose-GlcNAc.

^kPeptide 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).

^lS5 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 IFN γ 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).

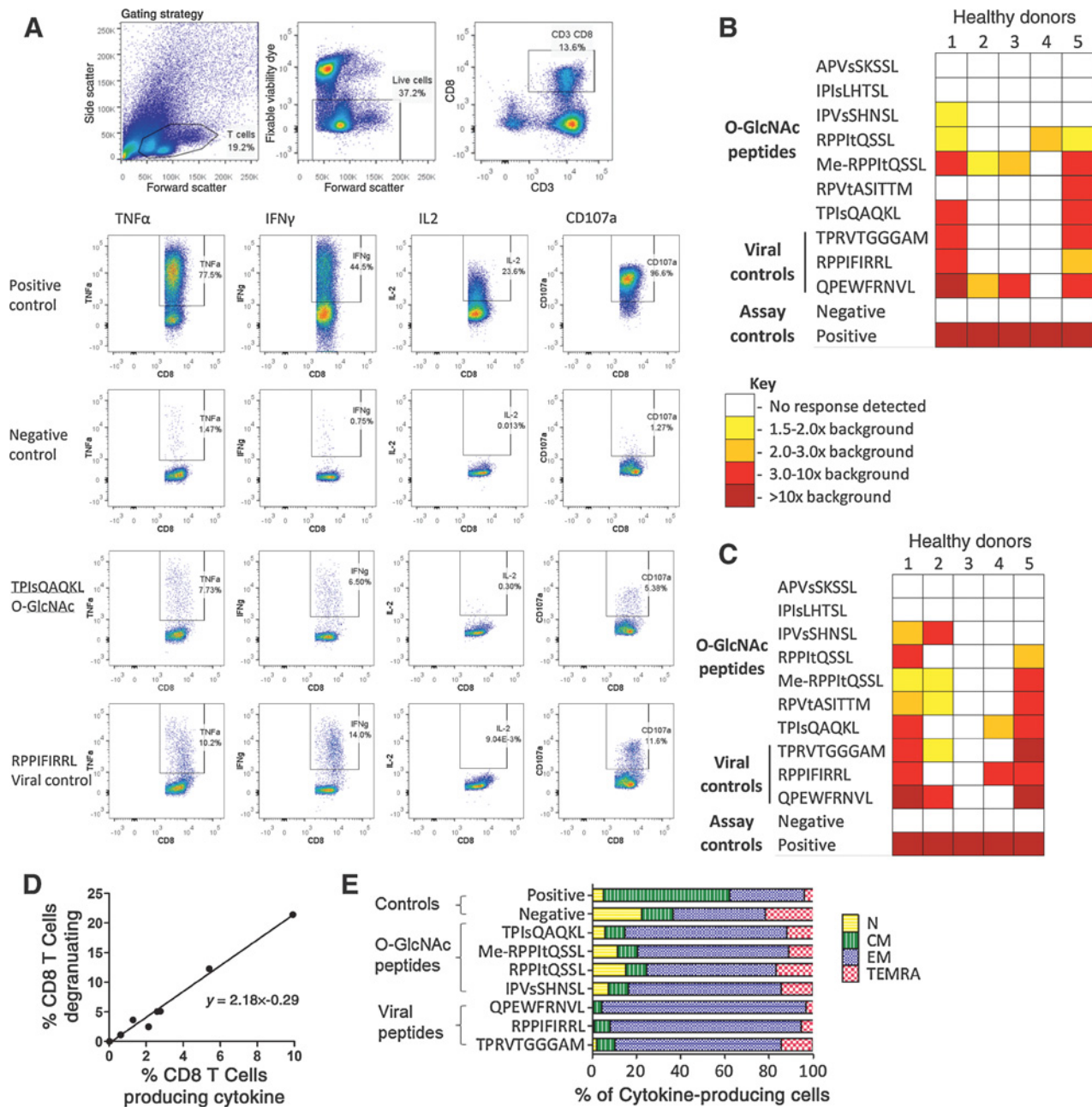


Figure 2.

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

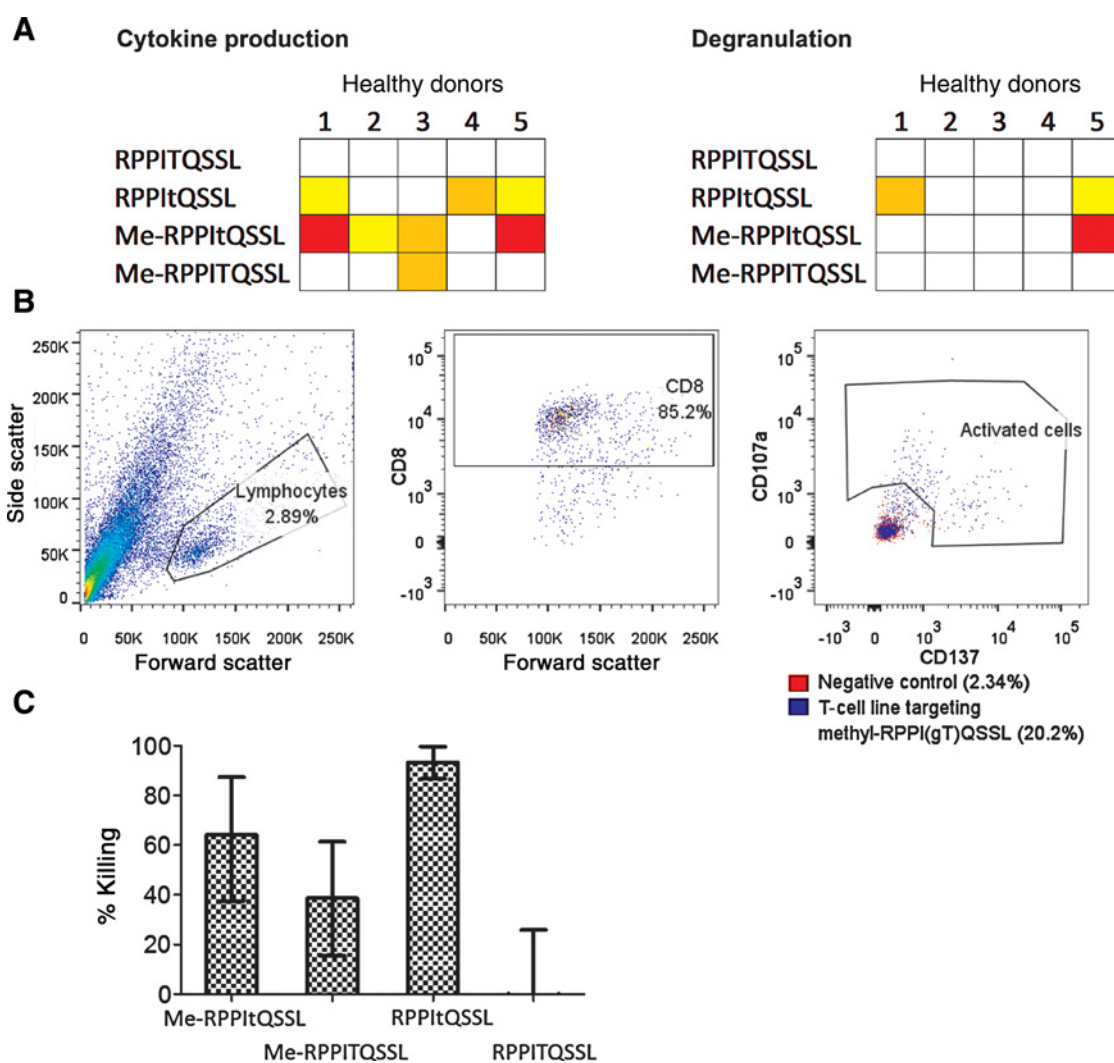


Figure 3.

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 *GlcNAc*ylated 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

Conception and design: S.A. Malaker, D.F. Hunt, M. Cobbold

Development of methodology: S.A. Malaker, S.A. Penny, J. Shabanowitz, D.F. Hunt, M. Cobbold

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.A. Malaker, S.A. Penny, P.T. Myers, J.C. Loke, M. Raghavan, D.F. Hunt, M. Cobbold

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

Grant Support

This work was supported by NIH grants GM037537 and AI033993 (to D.F. Hunt). Kay Kendall Leukaemia Research grant KKL3227 and Bloodwise grant 08038 (to M. Cobbold).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 19, 2016; revised November 10, 2016; accepted March 9, 2017; published OnlineFirst March 17, 2017.

References

- Gillis S, Smith KA. Long term culture of tumour-specific cytotoxic T cells. *Nature* 1977;268:154-6.
- Coulie PG, Lehmann F, Lethé B, Herman J, Lurquin C, Andrawiss M, et al. A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. *Proc Natl Acad Sci U S A* 1995;92:7976-80.
- Cox AL, Skipper J, Chen Y, Henderson RA, Darrow TL, Shabanowitz J, et al. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. *Science* 1994;264:716-9.
- Chen YT, Scanlan MJ, Sahin U, Türeci O, Gure AO, Tsang S, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci U S A* 1997;94:1914-8.
- Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell* 2015;160:48-61.
- Linnemann C, van Buuren MM, Bies L, Verdegaal EM, Schotte R, Calis JJ, et al. High-throughput epitope discovery reveals frequent recognition of neoantigens by CD4+ T cells in human melanoma. *Nat Med* 2015;21:81-5.
- McGranahan N, Furness AJ, Rosenthal R, Ramskov S, Lyngaa R, Saini SK, et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Science* 2016;351:1463-9.
- Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 2015;348:124-8.

9. Segal NH, Parsons DW, Peggs KS, Velculescu V, Kinzler KW, Vogelstein B, et al. Epitope landscape in breast and colorectal cancer. *Cancer Res* 2008;68:889–92.
10. Van Allen EM, Miao D, Schilling B, Shukla SA, Blank C, Zimmer L, et al. Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science* 2015;350:207–11.
11. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med* 2014;371:2189–99.
12. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature* 2013;499:214–8.
13. Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott EC, Gutierrez M, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N Engl J Med* 2015;372:311–9.
14. Motzer RJ, Escudier B, McDermott DF, George S, Hammers HJ, Srinivas S, et al. Nivolumab versus Everolimus in Advanced Renal-Cell Carcinoma. *N Engl J Med* 2015;373:1803–13.
15. Nagorsen D, Scheibenbogen C, Marincola FM, Letsch A, Keilholz U. Natural T cell immunity against cancer. *Clin Cancer Res* 2003;9:4296–303.
16. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–74.
17. Cobbold M, De La Peña H, Norris A, Polefrone JM, Qian J, English AM, et al. MHC class I-associated phosphopeptides are the targets of memory-like immunity in leukemia. *Sci Transl Med* 2013;5:203ra125.
18. Hart GW. Minireview series on the thirtieth anniversary of research on O-GlcNAcylation of nuclear and cytoplasmic proteins: Nutrient regulation of cellular metabolism and physiology by O-GlcNAcylation. *J Biol Chem* 2014;289:34422–3.
19. Slawson C, Hart GW. O-GlcNAc signalling: implications for cancer cell biology. *Nat Rev Cancer* 2011;11:678–84.
20. Wells L, Kreppel LK, Comer FI, Wadzinski BE, Hart GW. O-GlcNAc transferase is in a functional complex with protein phosphatase 1 catalytic subunits. *J Biol Chem* 2004;279:38466–70.
21. de Queiroz RM, Carvalho E, Dias WB. O-GlcNAcylation: The Sweet Side of the Cancer. *Front Oncol* 2014;4:132.
22. Glithero A, Tormo J, Haurum JS, Arsequell G, Valencia G, Edwards J, et al. Crystal structures of two H-2Db/glycopeptide complexes suggest a molecular basis for CTL cross-reactivity. *Immunity* 1999;10:63–74.
23. Haurum JS, Tan L, Arsequell G, Frodsham P, Lellouch AC, Moss PA, et al. Peptide anchor residue glycosylation: effect on class I major histocompatibility complex binding and cytotoxic T lymphocyte recognition. *Eur J Immunol* 1995;25:3270–6.
24. Haurum JS, Arsequell G, Lellouch AC, Wong SY, Dwek RA, McMichael AJ, et al. Recognition of carbohydrate by major histocompatibility complex class I-restricted, glycopeptide-specific cytotoxic T lymphocytes. *J Exp Med* 1994;180:739–44.
25. Kastrop IB, Stevanovic S, Arsequell G, Valencia G, Zeuthen J, Rammensee HG, et al. Lectin purified human class I MHC-derived peptides: evidence for presentation of glycopeptides in vivo. *Tissue Antigens* 2000;56:129–35.
26. Udeshi ND, Compton PD, Shabanowitz J, Hunt DF, Rose KL. Methods for analyzing peptides and proteins on a chromatographic timescale by electron-transfer dissociation mass spectrometry. *Nat Protoc* 2008;3:1709–17.
27. Dudley ME, Wunderlich JR, Shelton TE, Even J, Rosenberg SA. Generation of tumor-infiltrating lymphocyte cultures for use in adoptive transfer therapy for melanoma patients. *J Immunother* 2003;26:332–42.
28. Wang Z, Udeshi ND, O'Malley M, Shabanowitz J, Hunt DF, Hart GW. Enrichment and site mapping of O-linked N-acetylglucosamine by a combination of chemical/enzymatic tagging, photochemical cleavage, and electron transfer dissociation mass spectrometry. *Mol Cell Proteomics* 2010;9:153–60.
29. Zhao P, Viner R, Teo CF, Boons GJ, Horn D, Wells L. Combining high-energy C-trap dissociation and electron transfer dissociation for protein O-GlcNAc modification site assignment. *J Proteome Res* 2011;10:4088–104.
30. Cante-Barrett K, Pieters R, Meijerink JP. Myocyte enhancer factor 2C in hematopoiesis and leukemia. *Oncogene* 2014;33:403–10.
31. Suzuki T, Huang C, Fujihira H. The cytoplasmic peptide:N-glycanase (NGLY1) – structure, expression and cellular functions. *Gene* 2016;577:1–7.
32. Mohammed F, Cobbold M, Zarling AL, Salim M, Barrett-Wilt GA, Shabanowitz J, et al. Phosphorylation-dependent interaction between antigenic peptides and MHC class I: a molecular basis for the presentation of transformed self. *Nat Immunol* 2008;9:1236–43.