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ORIGINAL RESEARCH

Gemcitabine alters the proteasome composition and immunopeptidome of tumour cells

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ABSTRACT

The antigenic makeup of tumour cells can have a profound effect on the progression of cancer and success of immunotherapies. Therefore, one strategy to improve the efficacy of cancer treatments is to augment the antigens displayed by tumours. The present study explores how the recognition of tumour cells may be altered by non-cytotoxic concentrations of gemcitabine (GEM). Testing a panel of chemotherapeutics in human cancer cell lines *in vitro*, it was found that GEM increased surface expression of HLA-A,B,C and that underlying this were specific increases in β-2-microglobulin and immunoproteasome subunit proteins. Furthermore, the peptide antigen repertoire displayed on HLA class I was altered, revealing a number of novel antigens, many of which that were derived from proteins involved in the DNA-damage response. Changes in the nature of the peptide antigens eluted from HLA-A, B,C after GEM treatment consisted of amino acid anchor-residue modifications and changes in peptide length which rendered peptides likely to favour alternative HLA-alleles and increased their predicted immunogenicity. Signalling through the MAPK/ERK and NFκB/RelB pathways was associated with these changes. These data may explain observations made in previous *in vivo* studies, advise as to which antigens should be used in future vaccination protocols and reinforce the idea that chemotherapy and immunotherapy could be used in combination.

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Introduction

The recognition of antigenic molecules on the surface of tumour cells plays an important role in $CD8^+$ T-cell-mediated clearance of cancer. Part of this recognition is dependent upon the MHC class I-antigen complex which indicates the health of the cell by displaying signs of aberrant protein expression to the immune system. Loss of MHC class I can hide tumour cells from the adaptive immune response, preventing detection by removing antigenic evidence of cancer-related proteins from the plasma membrane. HLA class I downregulation has been observed in numerous human tumour types,[1-4] and can be mediated through defects in α-heavy chain or β-2-microglobulin (β2 m).[5,6] Downregulation of MHC class I is strongly associated with poor prognosis in cancer,[7-9] and so reversing this is a promising strategy to enhance or reengage an anti-cancer immune response, especially in cancers characterised by low MHC class I, such as colorectal cancer.[10] MHC class I expression has been shown to be increased on tumour cells in response to stress stimuli, including chemotherapeutic treatments such as 5-fluoracil (5-FU) and gemcitabine (GEM).[11,12]

In addition to the absolute level of MHC class I, the peptide antigens expressed in conjunction with MHC class I are vital in the detection of cancer by immune cells and as such, antigen-specific tumour immunotherapy will be enhanced by the identification of putative tumour-associated immunogenic HLA-ligands. Many factors influence the make-up of these peptide ligands but an important part of this process is the cleavage of peptide bonds which can be catalysed by constitutively expressed proteasomal subunits or the interferon (IFN)-γ-inducible immunoproteasomal subunits LMP2 (β1i), MECL-1 (β2i) and LMP7 (β5i).[13,14] Compared with their constitutively expressed counterparts, immunoproteasomal subunits confer increased trypsin and chymotrypsin-like activity and generate peptides with distinctive C-termini.[15-17]

GEM is a nucleoside analogue that has a broad spectrum of anti-tumour activity against solid tumours, it exerts its antiproliferative effects via "masked-termination" of DNA replication and targeting of ribonucleotide reductase, an enzyme required for DNA replication and repair.[18] GEM has been successfully combined with a number of different immunotherapies in cancer. It is reported that GEM improves dendritic cell (DC) vaccination in clinical and pre-clinical settings, possibly by encouraging a cytotoxic T-cell response against subdominant immune epitopes.[19-23] GEM selectively removes myeloid-derived suppressor cells (MDSC) in mice;[24,25]and this may link to the potentiation of immunotherapy that is observed in

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combination with GEM. However, this has not been extensively studied in humans where there are conflicting reports on the ability of treatments involving GEM to reduce the percentage of Lin−DR−CD11b$^+$ MDSC in patients with advanced adenocarcinoma.[26] GEM is not associated with suppression of lymphatic activity in cancer patients,[27-29] and is shown to expand the T-lymphocyte subset and increase tumour infiltration in mice by enhancing cross-priming of tumour-specific CD8$^+$ T-cells.[30] Additionally, GEM increases the absolute numbers and percentage of peripheral CD14$^+$ monocytes and DCs in pancreatic cancer patients,[31] and in mice broadens the range of tumour antigens seen by CD8$^+$ T-cells by shifting the CD8$^+$ T-cell response towards subdominant epitopes.[32]

Considering the capacity of GEM to upregulate MHC class I on the surface of tumour cells in *in vitro* and *in vivo* settings;[12] and the coordinated regulation of MHC class I and the antigen processing machinery (APM), we suggested that in addition to influencing MHC class I expression, other changes in antigen presentation may be caused GEM. In the present study we confirm GEM-mediated upregulation of cell surface HLA-A,B,C and demonstrate that this is influenced by altered expression of β2 m. Moreover, consistent with our hypothesis, GEM also induced upregulation of immunoproteasomal subunits and altered the peptide antigens displayed by tumour cells in *in vitro* cell cultures.

Results

GEM altered expression of HLA-A,B,C at the surface of tumour cells

Surface expression of HLA-A,B,C was measured on a panel of tumour cell lines after culturing with equi-active concentrations of chemotherapeutic drugs for 24 hours. Representative plots are shown in Fig. 1a. GEM significantly increased expression of HLA-A,B,C in all three cell lines (Fig. 1b). The mean change in HLA-A,B,C expression in response to culture with GEM, as measured by a change in MFI from untreated controls, was 81.3% for HCT116 cells, 67.7% for A549 cells and 41.5% for MCF-7 cells. For comparison, 1000 IU/ml IFNγ increased HLA-A,B,C by 62.2% in HCT116 cells, 142.9% in A549 cells and 367% in MCF-7 cells (data not shown). Cyclophosphamide (CPM) did not influence HLA-A,B,C expression while oxaliplatin (OXP) had no effect on expression of HLA-A,B,C in A549 and HCT116 cells but reduced expression in MCF-7 cells by 24.8%. Increased surface expression of HLA-A,B,C was

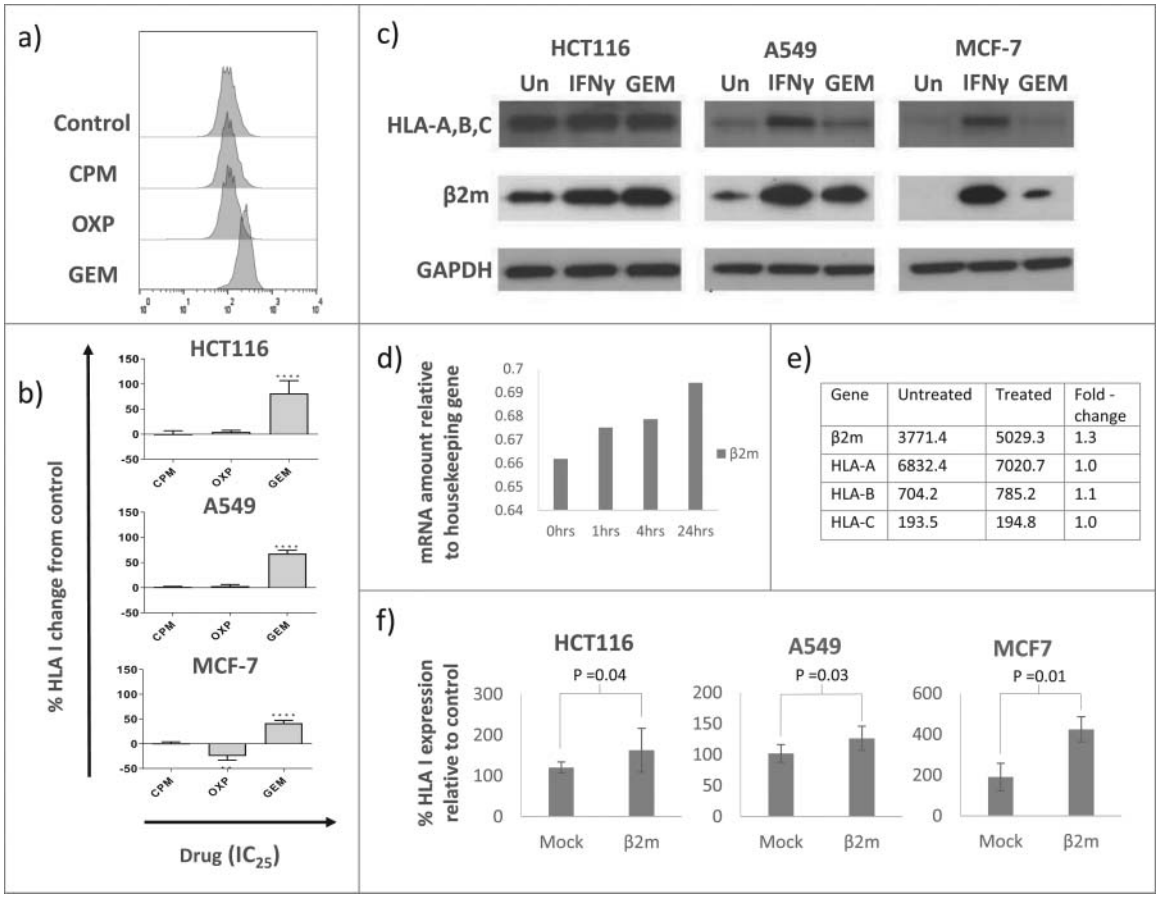

Figure 1. GEM increased HLA class I expression on tumour cells in a β2 m-associated manner. a) Representative histograms showing change in HLA-A,B,C MFI in response to culture with drugs at IC_{25}. b) The effect of chemotherapy drugs on surface expression of HLA-A,B,C on tumour cells as measured by flow cytometry. Data are expressed relative to untreated controls. Mean and standard deviation is plotted and values are significantly different (**** = p<0.0001, ** = p<0.01) to controls by one-way ANOVA with Dunnett's multiple comparisons test. n = 3. c) Blots representative of three experiments showing expression of HLA α-heavy chains and β2 m proteins in untreated (Un), IFNγ-treated (1000 IU/ml) or GEM-treated (100 nM) tumour cells. d) Transcription of the *β2 m* gene was increased in HCT116 cells in response to GEM, as assessed by qPCR. n = 1. e) Mean fold-change in mRNA for HLA class I genes in response to GEM. n = 3. f) Tumour cells transfected with human β2 m expressing plasmid had increased surface expression of HLA class I 48 hours after transfection, as measured by flow cytometry. Means and standard deviations are plotted and mock-transfected and β2 m-transfected are significantly different by student's paired t-test. For A549 and MCF-7, n = 3, for HCT116 n = 6.

associated with increased levels of intracellular β2 m but not α-heavy chain proteins. Western blots showed that the amount of β2 m protein detected increased in response to GEM in all three cell lines (Fig. 1c). In contrast expression of HLA class I α-heavy chains A, B and C were not altered by GEM treatment. Real-time PCR and microarray data, performed only on HCT116 cells (Fig. 1d and e), suggested that the increase in β2 m may be partially due to increased gene transcription. These gene analyses also corroboratively indicated that α-heavy chain expression remained unchanged by culturing with GEM. Transfection of A549, HCT116 and MCF-7 cells with a β2 m-expressing plasmid was sufficient to increase surface expression of HLA-A,B,C (Fig. 1f). Generally, transfected cells displayed a smaller increase in HLA-class I compared to GEM-treated cells.

Immunoproteasome subunits are expressed upon treatment with GEM

In addition to changes in HLA-A,B,C, expression of the immunoproteasome catalytic subunits LMP2 and MECL-1 was also induced by GEM in all three cell lines (Fig. 2a). Expression of the third immunoproteasome subunit, LMP7, was not detectably increased at this concentration and time-point.

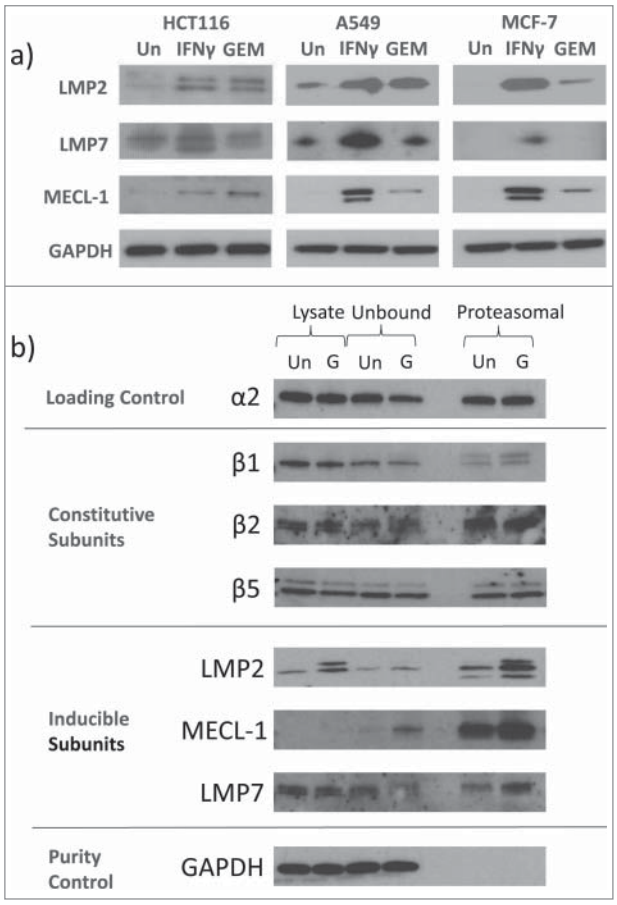

Figure 2. GEM induced immunoproteasome subunits in tumour cells. a) Immunoproteasome expression was assessed by Western blot after 24 hour culture with 100 nM GEM. IFNγ (1000 IU/ml) was used as a positive control. Blots are representative of three separate experiments. b) Proteasomes were isolated from HCT116 cells either untreated (Un) or treated with 100 nM GEM (G) for 24 hours. Expression of proteasome and immunoproteasome subunits in crude cell lysate, unbound and proteasome pull-out fractions was then measured. Representative blots from three separate experiments are shown.

Proteasome pull-outs from HCT116 cells showed that LMP2 and MECL-1 were incorporated into proteasomes in increasing amounts after GEM treatment. Fig. 2b shows expression of the constitutive proteasome subunits β1, β2 and β5, the immunoproteasome subunits LMP2, LMP7 and MECL-1, and α2, which is present in both forms of proteasome, in control and HCT116 cells treated with GEM for 24 hours. Shown are the relative amounts of each protein in crude cell lysate, unbound and proteasomal fractions. LMP2 and MECL-1 coimmunoprecipitated with proteasomes in GEM-treated cells, LMP7 was also present in this fraction. Subunit α2 was used as a loading control, indicating the total amount of proteasome present. There was little change in the expression of the constitutive proteasome subunits, β1, β2 and β5 in the crude lysate or proteasomal fractions.

The types of peptides displayed on HLA-A,B,C are altered by GEM

The induction of LMP2 and MECL-1 in tumour cells in response to culture with GEM suggests that there may also be changes to the HLA-expressed peptidome of these cells. To assess this, HCT116 cells were cultured alone or with GEM and peptides eluted from HLA-A,B,C molecules before being sequenced by mass spectrometry. The sequences derived were analysed by comparing the peptide antigens present only on the surface of the GEM-treated cells versus those found on only control cells.

In the presence of GEM, the peptide repertoire displayed on the surface of tumour cells was altered. There was a noticeable change in the length of peptides, with 8-mers making up 9.8% of peptides exclusively displayed on control cells but 17.1% of peptides exclusively displayed on GEM-treated cells. In addition, there were fewer peptides with a length of 10 amino acids or more after GEM treatment (26.2% versus 16.1%) (Fig. 3a). GEM also increased the proportion of leucine and phenylalanine residues at the C-terminus of the peptide sequences (Fig. 3b). Of the peptides that appeared only on control cells, 7.6% had C-terminal leucine. For peptides that appeared only after GEM treatment, this was increased to 12.2%. The proportion of phenylalanine residues at the C-terminus was increased to an even greater extent after GEM-treatment, from 2.2% to 8.0% and this change reached statistical significance. Conversely, after treatment with GEM the numbers of peptides with alanine or proline at the C-terminus was decreased, from 27.8% to 13.2% and 10.1% to 5.0%, respectively, the latter reaching statistical significance.

HLA-binding preferences for individual peptides were analysed using HLA binding prediction servers IEDB analysis resource and SYFPEITHI. The percentage of peptides predicted to bind most strongly to HLA-B*18:01 was larger in the GEM-treated group than the untreated group (Fig. 3c). On average, 10.6% of peptides were predicted to bind to HLA-B*18:01 in control cells but this was increased to 24.9% in GEM-treated cells. Conversely, the percentage of peptides predicted to bind most strongly to HLA-B*45:01 decreased from 38.7% in control cells to 18.7% in GEM-treated cells. This preference for peptides to bind HLA-B*18:01 after GEM was underpinned by changes in the anchor residues occurring at binding position

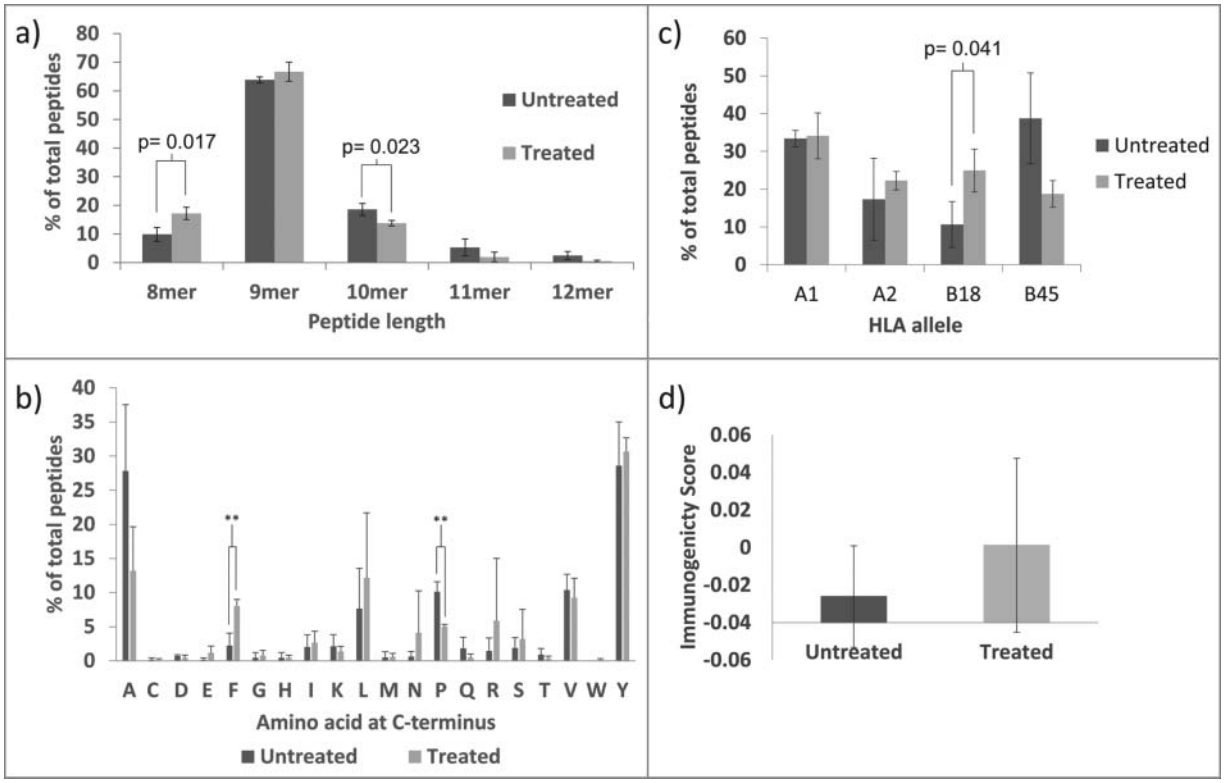

Figure 3. Peptide ligands eluted from HLA-A,B,C on HCT116 cells treated with 100 nM GEM were different from those found on untreated control cells. All panels represent mean and standard deviation values of three separate peptide elution experiments and show differences in the proportion of peptides with various characteristics between those found exclusively on treated or exclusively on control cells in terms of: a) Peptide length, b) C-terminal amino acid, c) Predicted HLA-allele binding, d) Predicted immunogenicity. Values significant different from controls by student's t-test are indicated (** = $p<0.01$). Due to the anomalous appearance of a number of 11 and 12mer peptides in one of the three peptide elution experiments, data from this particular experiment was removed from the analysis of a) and replaced by peptide length data from a small pilot study.

P2 and was especially true for peptides with an F or L residue at the C-terminal position which were now more likely to be associated with an acidic amino acid at the P2 position (Supplemental Figure S1). A prediction model was used to assess the immunogenicity of the eluted peptides.[33] Peptides displayed on HLA class I exclusively after culture with GEM were, on average, predicted to be more immunogenic than those peptides found on control cells (Fig. 3d).

GEM treatment causes new protein representation in the peptidome

Eluted peptides were used to identify proteins expressed in HCT116 cells and then we asked whether new epitopes were generated from within these proteins in the presence of GEM. Table 1 lists a sample of proteins from which the peptidome of HCT116 cells was derived. Only proteins with novel GEM-exclusive peptides found in at least two of three peptide elution experiments are shown.

The peptide SEQETLVRP from the oncoprotein Mdm2, was found before and after GEM treatment. However, after cells were cultured with GEM, additional peptides from Mdm2 were also detected in the immunopeptidome of HCT116 cells: DEVYQVTVY, YTMKEVLFY and DEKQQHIVY. Using peptide spectral matches (PSMs) as a measure of abundance, SEQETLVRP made up 0.26% of peptides eluted from control cells but 0.56% in GEM-treated cells (Fig. 4a). Intracellular expression of MDM2 remained unchanged by GEM treatment (Fig. 4c), though transcription may have increased, 144.1 versus 207.5 units, measured by microarray.

The presentation of new antigens to the immune system was also observed with other proteins, including DNA Topoisomerase 1 (TOPO1). Expression of *TOPO1* decreased slightly in the presence of GEM, as assessed by microarray (390.7 versus 365.5) and Western blot (Fig. 4c), but an additional peptide, YLDPRITVA, was generated in GEM-treated cells (Table 1). Interestingly, in control cells peptides from TOPO1 made up only 0.013% of total peptides but with GEM treatment this was increased nearly tenfold to 0.11% (Fig. 4a). These data imply that expression level alone does not drive the appearance of new epitopes within individual proteins. As well as an increasing diversity of peptides from a particular protein, peptide antigens from proteins not represented on HLA-A,B,C at the surface of control HCT116 cells also appeared after culturing cells with GEM. For example, the lung cancer oncoprotein, kanadaptin, was not represented in the immunopeptidome of HCT116 cells under basal conditions, however, upon culture with GEM the peptide EENPIVLEF was displayed on HLA class I (Table 1). No peptide exclusive to GEM treatment was found in all three peptide elution experiments.

However, there were 31 peptide sequences present on both control *and* GEM-treated HCT116 cells that were conserved between all three experimental repeats. The relative fold-change in the abundance of these peptides in response to GEM (as measured by changes in PSMs) is shown in Fig. 4b. Two peptides showed a fold-difference greater than two and were

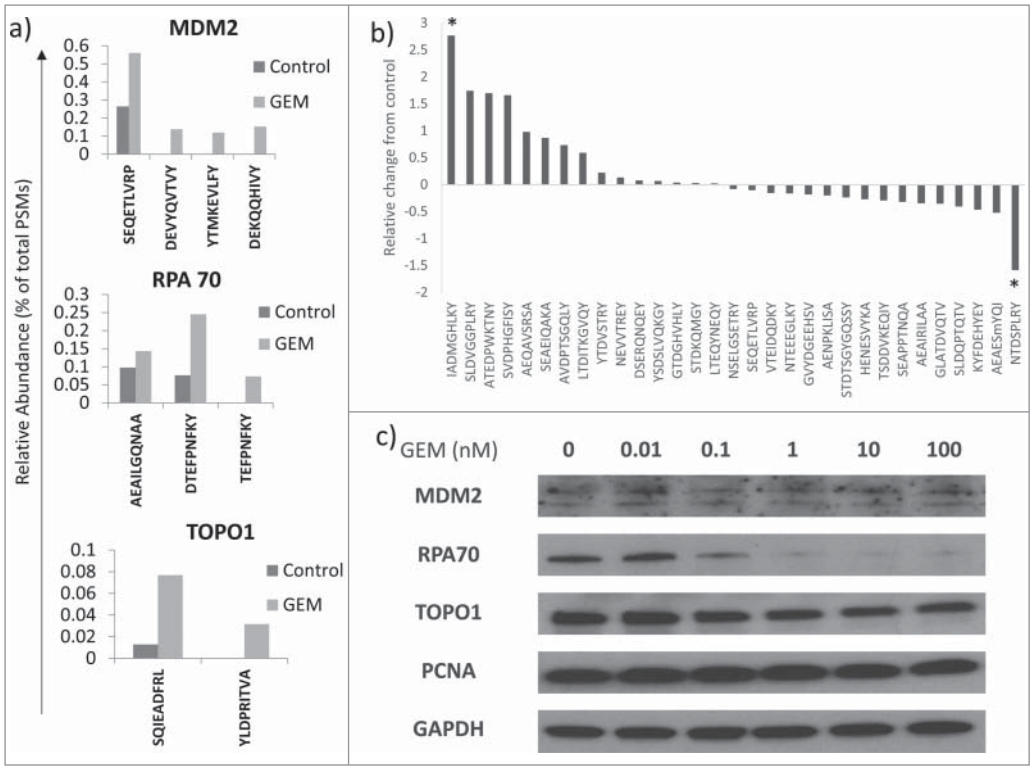

Figure 4. GEM alters the immunopeptidome of tumour cells. a) Relative abundance of peptides from MDM2, RPA70 and TOPO1 proteins was assessed in control and GEM-treated HCT116 cells. n = 2. b) The relative abundance of all peptides present in both control and GEM treated cells in all three experiments is shown. Values significantly different from controls by student's paired t-test are indicated (* = $p<0.05$). c) Western blots showing the expression of proteins that have an altered peptide representation in GEM-treated cells are shown. Blots are representative of three separate experiments.

statistically different from no change, NTDSPLRY, from 40 S ribosomal protein SA, which was downregulated in response to GEM treatment, and IADMGHLKY, from the protein proliferating cell nuclear antigen (PCNA), which was upregulated. The intracellular expression of PCNA was unchanged by GEM treatment (Fig. 4c).

Of the proteins measured by Western blot, only RPA70 showed major changes in expression in response to GEM treatment (Fig. 4c). The loss of RPA70 protein alongside increases in the amount of RPA70 peptides at the surface of the cell upon GEM treatment may suggest increased degradation of this protein as part of the response to GEM.

Table 2 lists the peptides found exclusively after GEM treatment in more than one of the peptide elution experiments. References to these sequences were then investigated in the scientific literature. Of 86 GEM-specific peptides conserved between two experiments, 53 (61.63%) were novel (no reference could be found), whereas 33 had been reported previously.

MEK and NFκB are involved in GEM mediated HLA-A,B,C upregulation

In accordance with previous reports, culturing tumour cells with GEM was found to activate the MEK signalling pathway, increasing phosphorylation of ERK1/2 (Fig. 5a). To test whether this was involved in upregulation of HLA-A,B,C and the changes in the peptide repertoire, phosphorylation of ERK1/2 was inhibited by U0126 in GEM-treated HCT116 cells and the effect on HLA-A,B,C surface expression and intracellular LMP2 measured. Inhibition of ERK1/2 phosphorylation was demonstrable by Western blot (Fig. 5d) and reduced GEM-induced HLA-A,B,C in a dose-dependent manner (Fig. 5b). GEM alone increased expression of HLA-A,B,C by a mean of 78% compared to matched untreated control but this was reduced to 57% when 1 μM U0126 was added to the culture medium and 23% at 10 μM of U0126. It should be noted that treatment with U0126 alone did increase basal expression of HLA-A,B,C.

Western blots showed that GEM-mediated LMP2 upregulation was also reduced when inhibiting phosphorylation of ERK1/2 (Fig. 5c and d). GEM alone increased relative expression of LMP2 1.8-fold above untreated controls, however, this was reduced to 1.3-fold and to basal levels by 1 μM and 10 μM U0126, respectively. As with HLA-A,B,C, use of the MEK inhibitor alone was associated with increased LMP2 (Fig. 5d).

In addition to the MEK pathway, NFκB was also involved in GEM-mediated HLA-A,B,C upregulation. Culture with GEM caused nuclear translocation of the NFκB subunits RelB and p52 in HCT116 cells. No effect on the p65 subunit of NFκB was observed (data not shown). The involvement of NFκB in GEM-mediated HLA-A,B,C upregulation was confirmed by inhibiting NFκB with IKK-16. Expression of HLA-A,B,C was reduced from a mean MFI of 3288 units with GEM alone to 1698 units with 2.5 μM IKK-16 also present. These data suggest that GEM activates non-canonical NFκB signalling and that this is important in the regulation of HLA-A,B,C at the surface of tumour cells.

Discussion

In this study it was found that the chemotherapeutic drug, GEM, increased expression of HLA-A,B,C on the surface of a number

Table 1. How proteins were represented in the immunopeptidome of HCT116 cells was altered by GEM, which induced new peptide antigens from proteins which were displayed on HLA-A,B,C. In addition to proteins having altered peptide representation after GEM treatment, new proteins were also represented in the immunopeptidome of HCT116 cells after GEM treatment. Microarray data is also shown indicating the relative amount of mRNA present for each gene. A sample of proteins with consistently expressed GEM-novel peptides in at least two peptide elution experiments are shown.

Protein	Peptide(s) from Control	Peptide(s) from Treated	Microarray score Control	Microarray score Treated
Oncoprotein Mdm2	SEQETLVRP	SEQETLVRP DEVYQVTVY YTMKEVLFY DEKQQHIVY	144.1	207.5
Replication protein A 70 kDa DNA-binding subunit	AEAILGQNAA DTEFPNFKY	AEAILGQNAA DTEFPNFKY TEFPNFKY	2977.9	3072.2
Pyruvate dehydrogenase [acetyl-transferring]]-phosphatase 1	VGDPNSFLNY	VGDPNSFLNY SLLPHETLL	1672.9	1389.5
DNA Topoisomerase 1	SQIEADFRL	SQIEADFRL YLDPRITVA	390.7	365.5
Cullin-9		LLLDLERVL	189.4	211.8
Kanadaptin		EENPIVLEF	255.8	226.8
Serine/threonine-protein kinase PLK2		NEDRISTTF	235.9	246.3
Exosome component 10		DEYDFYRSF	1608.8	1580.5
Spermatogenesis-associated serine-rich protein 2		SSEKGGMNGY	864.9	715.0
Dynamin-2		TLIDLPGITKV ETERIVTTY	292.8	286.8
Antigen peptide transporter 1 (TAP 1)		LLYESPERY	726.0	1235.8
Sestrin-1		SLAELVHAV QMDGPLPLHY	212.0	639.4

of different tumour cell lines. This upregulation occurred using low concentrations of GEM and was associated with increased levels of β2 m. Moreover, this study demonstrated that GEM induced immunoproteasome subunit expression and that this corresponded to changes in the immunopeptidome of HCT116 cells, which included the display of novel potential T-cell epitopes not present on control cells.

MHC class I is often down-modulated in tumour cells, rendering them less susceptible to $CD8^+$ T-cell-mediated killing, and it is presently unclear whether the changes in HLA-A,B,C observed in response to GEM represent an increase above basal levels or some correction of previous downregulation. However, culturing cell lines with GEM upregulated β2 m but not α-heavy chain expression and reduction of MHC class I expression in cancer frequently occurs through defects in β2 m which are often reversible.[34,35] It is interesting to note that transfection with β2 m protein alone was able to increase HLA-A,B,C at the surface of the tumour cells tested here. Because of the role of β2 m in the MHC class I folding, mutation of the β2 m gene leads to the gradual loss of HLA class I and immune escape during tumour progression.[36] β2 m deficiency, through mutation or other means, is reported in a number of cancer types[37-39] and is proposed as a reason for the failure of check-point blockade therapy.[40] Our data demonstrate that through modulation of β2 m, GEM increases MHC class I expression, suggesting the potential to correct non-mutational MHC class I deficiency in tumour cells by using GEM.

MHC class I expression increases in response to various chemotherapy drugs and radiation.[11,12,41,42] This may form part of a normal stress response and represents an increase in the quantity of the MHC class I-antigen complex on the surface of tumours. Increasing the number of antigens on a tumour cell is, on balance, a good strategy to elicit an immune response against tumour. However, what may be equally or even more important than the quantity of the antigens presented, is the quality of the antigens and a major finding of the present study is that culturing with GEM altered the nature of peptide antigens displayed on HLA class I by tumour cells.

Exposure to 100 nM GEM increased expression of the immunoproteasomal subunits LMP2 and MECL-1 in tumour cells and these proteins were detected in proteasomes using α4 pull-outs. No increases were found in the total amount of LMP7 present in tumour cells in response to GEM, though LMP7 was found in proteasomes. Proteasomes can exist as constitutive, mixed, or immunoproteasomes, dependent on which catalytic subunits are present and so the precise composition of proteasomes in GEM-treated HCT116 cells is not currently known. However, the induction of immunoproteasomal subunits by GEM suggests an alteration in the way in which proteins are degraded and may help explain the new pattern of peptides within the tumour cell immunopeptidome.[43] It is suggested that immunoproteasomally cleaved peptides may be distinguished by their tendency to have particular hydrophobic amino acids, such as phenylalanine or leucine, at the C-terminus.[44-46] Data presented in the present study also indicate an increase in the proportion of peptides with phenylalanine or leucine at the C-terminus eluted from HLA-A,B,C after culture with GEM. An interesting further study would be to compare the peptide antigens eluted from GEM-treated cells to

Table 2. References to the sequences of peptides exclusive to GEM treatment and conserved in two of three experiments were searched for in the scientific literature. "Novel" indicates that the present study is the first time the peptide has been reported. Otherwise, peptides found previously in: A = Patent - Cytotoxic T-lymphocyte-inducing immunogens for prevention treatment and diagnosis of cancer,[56] B = Patent - Comparative ligand mapping from MHC class I positive cells,[57] C = Toward a Definition of Self: Proteomic Evaluation of the Class I Peptide Repertoire,[58] D = Characterization of spontaneous tumor antigen-reactive T cell responses in melanoma patients and treatment of human melanoma with optimized T cell receptor transgenic T cells in a xenotransplantation model,[59] E = Patent - MHC molecule-binding tumor-associated peptides,[60] F = Features of TAP-independent MHC class I ligands revealed by quantitative mass spectrometry.[61] IEDB = Found in the immune epitope database.

	Peptide	Protein	Description
1	LSLENLEKI	Phosphatidylinositide phosphatase SAC2	A (2008)
2	NEDRISTTF	Serine/threonine-protein kinase PLK2	Novel
3	DEYDFYRSF	Exosome component 10.	B and C
4	SLAELVHAV	p53 regulated PA26 nuclear protein	Known immune epitope IEDB
5	DEFEFLEKA	E3 ubiquitin/ISG15 ligase TRIM25	B and C
6	DEVYQVTVY	E3 ubiquitin-protein ligase Mdm2	B and known CLL ligand
7	YTMKEVLFY	E3 ubiquitin-protein ligase Mdm2	Novel
8	DEGLIIHVF	Protein kinase C, zeta	Novel
9	SSEKGGMNGY	Spermatogenesis-associated serine-rich protein 2	Novel
10	VVEQLKDWLY	MAD2 mitotic arrest deficient-like 1	Novel
11	MEVEVDGQKF	Interleukin enhancer-binding factor 3	Known immune epitope IEDB
12	SEIELFRVF	U5 small nuclear ribonucleoprotein 200 kDa helicase	known immune epitope IEDB and A
13	TLWVDPYEV	B-cell Translocation Gene 1	Known immune epitope IEDB
14	NEAIMHQY	Protein FAM111B	Novel
15	MEQVIFKY	ARP3 actin-related protein 3 homolog;	B
16	ETERIVTTY	Dynamin 2	Novel
17	NQVIFPVSY	Mak3 homolog	Known immune epitope IEDB
18	EENPIVLEF	Kanadaptin	Known immune epitope IEDB
19	LTEIKGSVY	Zinc finger with UFM1-specific peptidase domain protein	Novel
20	ADKVHLMY	E3 ubiquitin/ISG15 ligase, Tripartite motif-containing 25	Novel
21	TVDDPYATFV	Cofilin-1	Novel
22	DVDPETLSY	Exonuclease I	Novel
23	TEFPNFKY	Replication protein A 70 kDa DNA-binding subunit	Novel
24	TVDPASLWEY	Fascin homolog 1	Novel
25	VEIITKEF	PMPCA protein	Novel
26	YTELLAQVY	Solute carrier family 25 member 35	Novel
27	ASDGTVRL	Heterogeneous nuclear ribonucleoprotein H3	Novel
28	ASEIAVGHQY	Predicted Putative solute carrier family 25 member 35	Novel
29	DENFILKH	Peptidyl-prolyl cis-trans isomerase A	B
30	LLYESPERY	Antigen peptide transporter 1	Known immune epitope IEDB
31	NEYLNPEL	Histone chaperone ASF1B	Novel
32	DEAGGRFVAF	Ubiquitin fusion degradation 1 like	Novel
33	DEWKAIQN	SERPINE1 mRNA binding protein 1	Novel
34	EEFETIERF	Chromodomain-helicase-DNA-binding protein 1	B and C
35	DEKQQHIVY	E3 ubiquitin-protein ligase Mdm2	D and known CLL ligand
36	AEQKKLEAA	NAD(P)H dehydrogenase [quinone] 1	Novel
37	VTEAIQAVL	WD repeat-containing protein 72	Novel
38	NLAEKLIGV	Ral GTPase-activating protein subunit beta	A (2011)
39	DEKSIITY	Plectin	E
40	KLLEVQILE	GRIP & coiled-coil domain-containing protein 2	A (2011)
41	FGGLGGGSVR	Keratin, type I cytoskeletal 19	Novel
42	GLGGGSVRFG P	Keratin, type I cytoskeletal 19	Novel
43	YTSGPGSRIS	Keratin, type II cytoskeletal 8	Novel
44	YTSGPGSRISS	Keratin, type II cytoskeletal 8	Novel
45	VKLAKAGKN	Nucleolin	Found on HEK293 cells
46	ILIDWLVQV	G2/mitotic-specific cyclin-B1	Known immune epitope
41	KMDASLGNLFA	Protein FAM3 C	F
42	DEKPLVLEm	N-acetyltransferase 14	Novel
43	AEISAMLKA	Pop1	Novel
44	RTLAEIAKV	Non-POU domain-containing octamer-binding protein	Known immune epitope IEDB
45	KIFEMGPVFTL	Cytochrome c oxidase subunit II	Novel
46	SEIYIHGL	Ribonuclease P protein subunit p20	Novel
47	QAEFQILKA	MORC family CW-type zinc finger protein 4	Novel
48	KEMPVKVEA	Importin-8 isoform 2	Novel
49	TELLIRKL	Histone H3.3 C	Known immune epitope IEDB
50	SEYQWITSP	Centrosomal protein of 78 kDa	Novel
51	GSDDGTVKL	38kDa splicing factor	Novel
52	TLTEEGVIKV	GTP binding protein 4	Known immune epitope IEDB
53	DEVVWVRA	Aspartyl-tRNA synthetase	Novel
54	DEMNVKVL	MYL6 protein	Found in thymus
55	SEAEIFYNA	Plakophilin 3	Novel
56	LTDDDLLRY	1,4-alpha-glucan-branching enzyme	Known immune epitope IEDB
57	NETDILSQY	Metastasis-associated protein MTA2	Novel
58	LEAHRDAPGA	Serine/threonine-protein phosphatase 6 regulatory subunit 2	Novel
59	QEYSEFVKA	PTPL1-associated RhoGAP 1 variant	Novel
60	AEILSEMRA	2 '-5 ' oligoadenylate synthetase 3	Novel
61	ATEYKNEEY	YTH domain-containing protein 1 isoform2	Known immune epitope IEDB
62	VEHKVETF	40 S ribosomal protein S7	Known immune epitope IEDB

(*Continued on next page*)

Table 2. (*Continued*)

	Peptide	Protein	Description
63	NEVPVKEL	Leucine-rich repeat-containing protein 59-like	Novel
64	DELEVIHL	Ro ribonucleoprotein	Known immune epitope IEDB
65	FLLGPRLVLA	transmembrane emp24 domain-containing protein 10 precursor	Novel
66	EEEFFYEKA	BRCA2 and CDKN1 A interacting protein	Novel
67	LLLDLERVL	Cullin-9	Novel
68	VMAPRTLVL	HLA class I histocompatibility antigen, A-2 alpha chain precursor	Known immune epitope IEDB – HLA-E binding HLA-2 leader sequence
69	PDPIRGFGS	Putative poly(ADP-ribosyl) transferase	Novel
70	KTDKTLVLL	Profilin-1	A (2011)
71	DEHEGPALY	Proteasome subunit beta type-2	Known immune epitope IEDB and B
72	QMDGPLPLHY	Sestrin-1	Novel
73	SSDRHLTQY	Chromodomain helicase DNA binding protein 1	Novel
74	GEKRFADAA	Nuclear pore complex protein Nup85	Known immune epitope IEDB
75	SLLPHETLL	Pyruvate dehyrogenase phosphatase catalytic subunit 1	Novel
76	HSDPSILGY	GIGYF1 protein	Novel
77	YLDPRITVA	DNA topoisomerase	Novel
78	AVLELKNEL	Protein-tyrosine kinase 2-beta	Novel
79	EPAQVSLLY	Ubiquitin carboxyl-terminal hydrolase 40	Novel
80	ATLVRSPGP	Junction-mediating and -regulatory protein	Novel

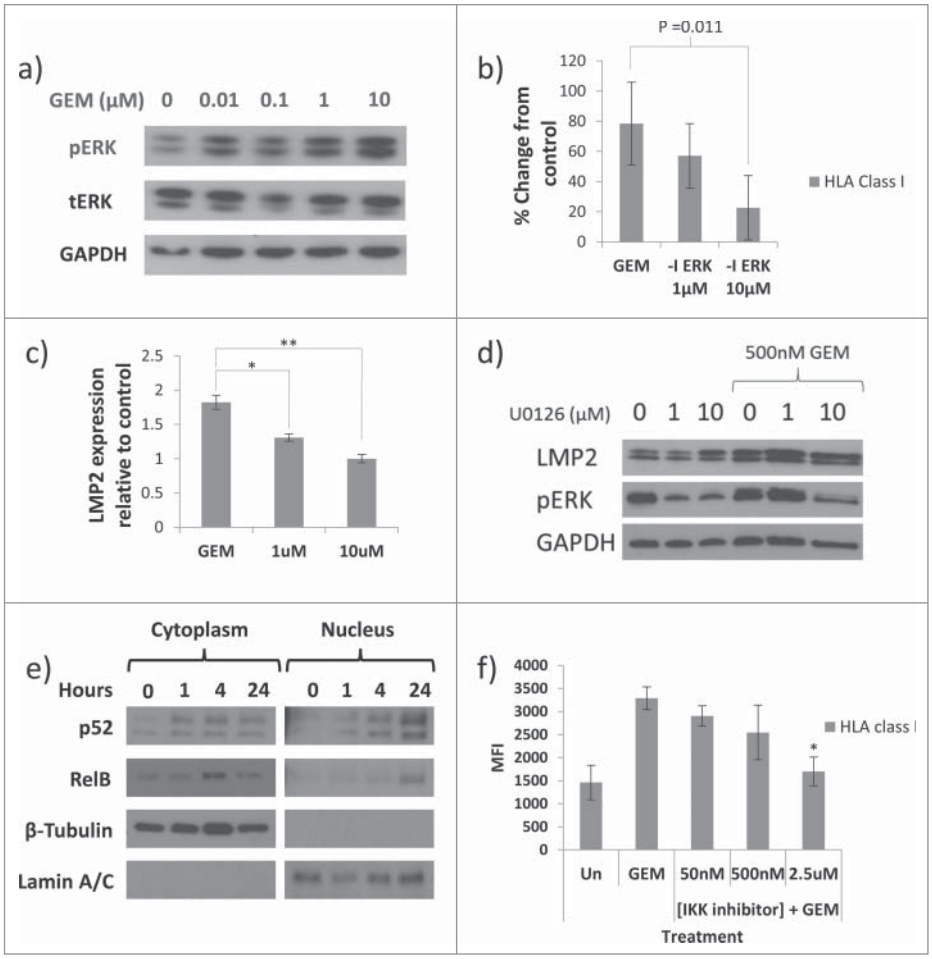

Figure 5. MEK and NFκB are associated with GEM-mediated HLA-A,B,C upregulation. For all panels n = 3 and involve HCT116 cells unless otherwise stated. a) Phosphorylation of ERK1/2 was increased in response to GEM in HCT116, A549 and MCF-7 cells. Representative blots from MCF-7 cells are shown. b) GEM-mediated HLA-A,B,C upregulation was reduced by inhibiting ERK signalling. HCT116 cells were untreated (Un) or cultured with 100 nM GEM, +/− ERK inhibitor U0126 (-I ERK) at 1 μM or 10 μM and HLA-A,B,C measured by flow cytometry. n = 4. c) As b) but measuring intracellular levels of LMP2 expression by Western blot. d) Representative blot showing the effect of U0126 on expression of LMP2 and pERK. e) Expression of p52 and RelB were measured in the cytoplasmic and nuclear fractions of HCT116 cells treated with GEM for various durations. Representative blots are shown. Anti-β-tubulin and lamin A/C antibodies used as loading and purity controls for cytoplasmic and nuclear fractions, respectively. f) HCT116 tumour cells were treated with 100 nM GEM +/−IKK-specific NFκB inhibitor. The effect on upregulation of HLA-A,B,C at the surface of the tumour cells was then assessed. b), c) and f) Values significantly different from GEM-treated by one-way ANOVA with Dunnett's test for multiple comparisons are shown. * = p<0.05, ** = p<0.01.

those eluted from IFNγ-treated cells which should have a full immunoproteasome.

Peptides eluted from GEM-treated HCT116 cells had shifted in character so that a higher proportion were predicted to bind HLA-B*18. This may be a consequence of an increase in shorter peptides found after GEM-treatment, as HLA-B*18 molecules have a preference for binding shorter peptides in their binding clefts.[47] Shorter peptides may allow a broader response, as is the case for an HLA-B*18 bound 8-mer from EBV which has been reported to initiate stronger T-cell responses to a wider range of peptide epitopes compared to the 12-mer peptide displayed on HLA-B*44.[48] Additionally, CTLs have been shown to lyse cells expressing HLA-B*18 bound 8-mer peptides more strongly than other peptides bound to other HLA-allotypes.[49] HLA-B*18 may be of particular importance in immunity. It is associated with autoimmune diseases such as multiple sclerosis, coeliac disease and diabetes mellitus;[50-52] and multiple cancers present with a loss or downregulation of HLA-B*18 expression.[53-55] Increasing the proportion of peptides that bind to "immune-relevant" alleles may enhance immunity. However, GEM-induced changes to antigen binding could also be detrimental to an immune response if the resultant peptides are designated for HLA alleles lost in loss of heterozygosity mutations. Here, peptides would be generated but unable to be displayed efficiently, potentially undermining an antigen-specific immune response. These types of mutations are common in cancer and may limit the use of GEM as an immune potentiator in this disease.[62,63]

Altered proteasomal cleavage specificity may explain the differences observed in peptides eluted from control or GEM-treated cells; however, TAP-independent mechanisms may play a role, as could changes in proteasome entry requirements or changes to peptide trimming, transport and loading.[64-66] Whatever the true mechanism, GEM-induced changes to the conformation of HLA-ligands have the potential to increase the ability of the adaptive immune system to propagate an effective immune response towards them.

It is reported that a discrepancy exists between the epitopes cross-presented by DCs and those displayed on tumour cells.[67] GEM may overcome this by inducing immunoproteasomes in cancer cells and shifting the peptides displayed on HLA class I molecules to be more similar to those generated and cross-presented by APCs. Interestingly, in the above study involving NY-ESO-1, the efficiently cross-presented, immunoproteasomally generated NY-ESO-1_{88-96} peptide was HLA-B*18 restricted, while the non-immunogenic, NY-ESO-$1_{157-165}$, was HLA-A*02 restricted.

Numerous peptides were exclusively found after GEM treatment and not on control cells, raising the possibility of using some of these novel, GEM-inducible antigens in future immunotherapy-GEM combinations. Some GEM-inducible peptides were derived from tumour-associated proteins, suggesting that GEM-treatment may reveal tumour antigens to immune cells. There were also a number of novel peptides from proteins intimately associated with the DNA-damage response (DDR) and these may represent a signal to the immune system further to the well-known innate signals induced by DNA-damage, such as increased expression of CD95 or NKG2D ligands. Many of the peptides found only on GEM-treated cells have previously been observed on other cell types, including: patented immunogens and tumour-associated peptides. The GEM-inducible peptide, ILIDWLVQV, has been identified by reverse immunology and shown to elicit spontaneous T-cell reactivity in blood from cancer patients and healthy donors. Additionally, there were GEM-inducible peptides previously described as "unique" to HIV MN-1-infected human T-cells; an interesting finding in the context of the work presented here, as GEM-treatment and viral-infection both induce immunoproteasomes and provide danger signals to immune cells. Further to those previously reported immunogens, the 53 novel peptides found in response to GEM represent new HLA-ligands that may be chemotherapy-specific and novel epitopes for cytotoxic T-cells.

A primary function of the adaptive immune response is to protect the integrity of the genome and as such the antigenic targets of T-cells are often evidence of some defect in the DNA of a cell, the most pertinent example of this in cancer being the "targetability" of neoantigens. Neoantigens, tumour-associated antigens, and antigens corresponding to other forms of DNA damage have consistently been shown to elicit robust and effective $CD8^+$ T-cell responses. In the present study, many of the GEM-inducible novel peptides appearing on HLA-A,B,C were linked to the DDR, which may suggest that self-antigens from DDR proteins are immunogenic. A theory conceptually advantageous as it would allow rapid responses by a less clonally diverse pool of T-cells against cells harbouring DNA damage, and plausible, given that these antigens will often be perceived by the immune system under inflammatory conditions and the existence of T-cells specific to DDR-related protein antigens such as MDM2 and TOPO1 in the periphery.[68,69] If GEM is providing a mechanism for these danger signals to be displayed on tumour cells then this helps explain the reported synergy between the drug and immunotherapy. Renovating the peptidome in this way may be a prime strategy to reengage immune responses against tumours and could also play a role in autoimmune disease and toxicities associated with GEM.

GEM-mediated cellular damage, genomic or otherwise, may generate peptides normally only seen on diseased or damaged cells making these cells targetable by the immune system. This concept increases understanding about the improved efficacy of chemotherapies in immunocompetent individuals and helps to identify mechanisms by which GEM has been shown to alter the antigen-specificity of primed $CD8^+$ T-cells towards subdominant epitopes. GEM may not be unique in this action but understanding the relationship between drug treatment and altered immunopeptidome may be important in boosting T-cell responses to cancer and poses questions as to which antigens should be chosen to combine with chemotherapy. This work suggests a rationale for combining GEM-treatment with immunotherapies, such as anti-PD-1, as increasing the antigenicity of the tumour will render it more susceptible to antigen-specific $CD8^+$ T-cells that may be unconstrained by checkpoint blockade therapy.

Methods

Tumour cell lines

The human cancer cell lines: A549 (lung), HCT116 (colon) and MCF7 (breast) (all Public Health England, Porton down, UK),

were grown in DMEM (Sigma-Aldrich, Dorset, UK) medium supplemented with 10% foetal bovine serum (Invitrogen, Paisley, UK) and 1% penicillin/streptomycin (Sigma). All cell lines were incubated in a humidified atmosphere with 5% CO_2 in air at 37 °C. For *in vitro* experiments, cells were seeded at 1×10^5 cells/ml and allowed to attach overnight before addition of experimental reagents. All experiments represent 24 hour treatment of cells with 100 nM GEM unless otherwise stated.

Drugs, cytokines and inhibitors

GEM, OXP, CPM (all Sigma) and IFNγ (R & D biosystems, Abingdon, UK) were reconstituted in phosphate buffered saline (PBS) (Sigma). IC_{25} values used for GEM, OXP and CPM were: 100 nM, 500 nM and 1μM, respectively. Cell signalling was inhibited by U0126 (New England Biolabs (NEB), Hitchin, UK) and NFκB by IKK-16 (Selleck Chemicals).

Flow cytometry

Cells were stained with HLA-A,B,C-FITC (Becton Dickinson (BD), Oxford, UK) using manufacturer's instructions prior to analysing with LSRII flow cytometer (BD Biosciences).

Immunoblotting

Cells were solubilised in lysis buffer (NEB) and resolved by tris-glycine electrophoresis. Following transfer of proteins onto nitrocellulose membranes (ThermoFisher Scientific, Dartford, UK), staining was performed with primary and then horseradish peroxidase (HRP)-conjugated anti-species secondary antibodies. Primary antibodies used were specific for: proteasome subunits α2, β1 and β2, β5 (all Enzo Life Sciences, Exeter, UK), β2 m (Abcam, Cambridge, UK), HLA-A, B and C (all Santa-Cruz Biotechnologies, California, USA), LMP2 (β1i), LMP7 (β5i) and MECL-1 (β2i) (all Abcam), pERK, tERK, RelB, p50, p52, p65 (all NEB). Bands were visualised on Hyperfilm™ ECL (GE Healthcare, Buckinghamshire, UK) using SuperSignal™ West Pico chemiluminescent substrate (Thermo). The housekeeping protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (NEB) was used as a loading control.

Separation of Nuclear and Cytoplasmic Fractions

Cell lysates were separated into nuclear and cytoplasmic fractions using NE-PER® Nuclear and Cytoplasmic Extraction reagents (Thermo) and following manufacturer's instructions. Briefly, Cytoplasmic Reagent (CER) I was added to cells before vortexing vigorously and addition of CER II. Cells were then centrifuged at 16000 g for 5 minutes and the supernatant (cytoplasmic extract) isolated and stored. The cell pellets were resuspended in Nuclear Extraction Reagent and incubated on ice for 40 minutes with vigorous vortexing at regular intervals. The resulting lysate was then centrifuged at 16000 g for 10 minutes and the supernatant (nuclear extract) stored at -80°C. After separation, cytoplasmic and nuclear fractions were treated as total cellular lysates in the previous section. Lamin A/C (NEB) and β-tubulin (NEB) were used to determine the quality of the separation and as loading controls for the nuclear and cytoplasmic protein fractions, respectively.

Real-time reverse transcriptase polymerase chain reaction

High purity RNA was isolated from HCT116 cells with the Qiagen RNA extraction mini-prep kit (Qiagen, Manchester, UK) using manufacturer's instructions. This was converted to cDNA by reverse transcription using precision RT all-in-one mix (Primer Design Ltd, Southampton, UK). Gene expression of $\beta 2\,m$ was then measured by qPCR, using QuantiTect® Sybr® Green-based qPCR primers (Qiagen, Manchester, UK). Approximately 40 ng cDNA (4 μl), 1 μl QuantiTect® primer and 10 μl SsoFast™ EvaGreen® Supermix (Bio-rad, Hertfordshire, UK) were added to the qPCR reaction mix. 18 S ribosomal RNA was used as the reference gene.

Illumina microarray

RNA was isolated from HCT116 cells using the Qiagen mini-kit protocol as before. Microarrays were performed by Dr Jayne Dennis at the SGUL Biomics Centre as described previously.[70]

Transfections

Tumour cells were transfected with a human β2 m (NM_004048) expressing, pCMV6-XL5 plasmid (OriGene, Herford, Germany) using Lipofectamine® LTX (HCT116 cells) or Lipofectamine® 3000 (A549 and MCF-7 cells) (Invitogen) and following manufacturer's instructions. Briefly, DNA-lipid complexes were added to cells before incubating for 48 hours at 37°C and 5% CO_2 in a humidified atmosphere. Expression of HLA-A,B,C at the cell surface was then determined by flow cytometry. Cells "mock transfected" with a non-encoding plasmid were used as a negative control

HLA ligand elution and sequencing

HCT116 cells were lysed in buffer containing PBS, 0.6% CHAPS, and complete protease inhibitor (Roche). Following centrifugation to remove debris supernatant was applied on affinity columns overnight. Columns were prepared by coupling W6/32 antibodies to CNBr-activated Sepharose (GE Healthcare) (1 mg antibody/40 mg Sepharose). On the second day the columns were eluted in 8 steps using 0.2% TFA. Filtration of the eluate through a 10 kDa filter (Merck Millipore, Darmstadt, Germany) yielded the HLA ligands in solution. The filtrate was desalted with C18 ZipTips (Merck) and subsequently concentrated using a vacuum centrifuge (Bachofer, München, Germany). Sample volume was adjusted for measurement by adding 1% ACN/0.05% TFA (v/v). With an injection volume of 5 μl HLA ligands were loaded (100 μm x 2 cm, C18, 5 μm, 100 Å) and separated (50 μm x 25 cm, C18, 3 μm, 100 Å) on Acclaim Pepmap100 columns (Dionex, Sunnyvale, CA) using an Ultimate 3000 RLSCnano uHPLC system (Dionex). A gradient ranging from 2.4-32% of ACN/H_2O with 0.1% formic acid was used to elute the peptides from the columns over 90 min at a flow rate of 300 nl/min. Online electrospray ionization (ESI) was followed by tandem MS analysis in a LTQ Orbitrap XL instrument (Thermo Fisher Scientific, Bremen, Germany). Survey scans were acquired in the Orbitrap mass analyser with a resolution of 60.000 and a mass range of 400 to 650 m/z. Peptides with a charge state other

than 2+ or 3+ were rejected from fragmentation. Fragment mass spectra of the 5 most intense ions of each scan cycle were recorded in the linear ion trap (top5 CID). Normalized collision energy of 35%, activation time of 30 ms and isolation width of 2 m/z was utilized for fragment mass analysis. Dynamic exclusion was set to 1s. The RAW files were processed against the human proteome as comprised in the Swiss-Prot database (www.uniprot.org, status: Dec12th, 2012; 20.225 reviewed sequences contained) using MASCOT server version 2.3.04 (Matrix Science, Boston, MA) and Proteome Discoverer 1.4 (Thermo). Oxidation of methionine was allowed as dynamic peptide modification. A mass tolerance of 5 ppm or 0.5 Da was allowed for parent and fragment masses respectively. Filtering parameters were set to a Mascot Score<20, search engine rank = 1, peptide length of 8–12 AA, achieving a false discovery rate (FDR) of 5% as determined by an inverse decoy database search.

Peptide sequence analysis

Peptides was stratified into two groups: those sequences that were unique to control HCT116 cells and those that were unique to GEM-treated HCT116 cells. These peptide groups were then subjected to various tests, including analysis of: length, C-terminal amino acid, predicted HLA-allele binding and predicted immunogenicity. The length and C-terminal amino acid of each peptide was determined manually. MHC class I binding predictions were made with IEDB Analysis Resource (http://tools.immuneepitope.org/mhci/) and SYFPEITHI Epitope Prediction (http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm) using the HLA-alleles HLA-A*01:01, HLA-A*02:01, HLA-B*18:01 and HLA-B*45:01. Predicted Immunogenicity of the peptides was determined using a model, IEDB Analysis Resource (http://tools.iedb.org/immunogenicity/), where the peptides were analysed for the presence of particular immunogenicity-associated amino acids at specific positions in the peptide sequence.[33] N.B. Due to a technical issue with the peptide length data in one of the three peptide elution experiments, this data was removed from the analysis of Fig. 3a and replaced by peptide length data from a small pilot study.

Proteasome isolation

Proteasomes were purified using the Enzo Protein Purification Kit (Enzo Life Sciences, Exeter, UK) and following manufacturer's instructions. Briefly, cells were lysed for 15 minutes on ice before centrifuging at high speed to clear the supernatant. A sample of this crude lysate was put aside for analysis and proteasome purification matrix (agarose immobilised anti-proteasome subunit-α4 antibodies) added to the remaining supernatant (25 μl matrix/100 μg protein). After incubation overnight, the matrix was isolated by centrifugation for 30 seconds at 5000 g and the supernatant (unbound fraction) set aside for analysis. Proteasomes were eluted from the matrix by the addition of LDS and boiling for 10 minutes.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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