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Exploiting the glioblastoma peptidome to discover novel tumour-associated antigens for immunotherapy

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Peptides presented at the cell surface reflect the protein content of the cell; those on HLA class I molecules comprise the critical peptidome elements interacting with CD8 T lymphocytes. We hypothesize that peptidomes from *ex vivo* tumour samples encompass immunogenic tumour antigens. Here, we uncover >6000 HLA-bound peptides from HLA-A*02$^+$ glioblastoma, of which over 3000 were restricted by HLA-A*02. We prioritized in-depth investigation of 10 glioblastoma-associated antigens based on high expression in tumours, very low or absent expression in healthy tissues, implication in gliomagenesis and immunogenicity. Patients with glioblastoma showed no T cell tolerance to these peptides. Moreover, we demonstrated specific lysis of tumour cells by patients' CD8$^+$ T cells *in vitro*. *In vivo*, glioblastoma-specific CD8$^+$ T cells were present at the tumour site. Overall, our data show the physiological relevance of the peptidome approach and provide a critical advance for designing a rational glioblastoma immunotherapy. The peptides identified in our study are currently being tested as a multipeptide vaccine (IMA950) in patients with glioblastoma.

Keywords: glioblastoma; immunotherapy; peptidome; tumour antigen; tumour-infiltrating lymphocytes

Abbreviations: GBM = glioblastoma multiforme; HLA = human leukocyte antigen; LC-MS = liquid chromatography-mass spectrometry

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Introduction

T lymphocytes monitor peptides presented by major histocompatibility complex molecules [human leukocyte antigen (HLA) in humans] at the cell surface; those bound by HLA class I molecules, the HLA class I peptidome, are recognized by CD8 T lymphocytes. The identity of such HLA-associated peptidomes remains largely unknown, although their precise knowledge could lead to valuable information about the biology of cells, and particularly tumour cells. This would enhance understanding of tumorigenesis and tumour–immune system interactions. In the last 10 years, studies using proteomics have looked to tumours to identify biomarkers of disease or tumour-associated proteins for targeted therapies including immunotherapy, but few studies (Weinschenk *et al*., 2002) have thus far analysed tumours *ex vivo* at the peptide level. Indeed, isolating and identifying such peptides requires sophisticated techniques that are today available with the development of highly sensitive liquid chromatography followed by mass spectrometry analysis (LC–MS) (Weinschenk *et al*., 2002; Lemmel and Stevanovic, 2003).

In the identification of tumour-associated targets for immunotherapy, the advantage of peptidomics over proteomics is the guarantee of selecting peptides that are naturally present at the cell surface. To date, the vast majority of antigens routinely used for peptide vaccination have been identified using prediction algorithms, reverse immunology techniques, *in vitro* cultured tumour cell lines or complementary DNA libraries (Sampson *et al*., 2010; Okada *et al*., 2011). However, these *in vitro* or *in silico* techniques do not investigate direct peptide presentation *in vivo* and do not ensure that the selected peptides are naturally present at the tumour cell surface at levels sufficient to allow cytotoxic T lymphocyte recognition.

Glioblastoma multiforme (GBM, grade IV astrocytoma) is a deadly tumour of the brain for which current treatments only marginally improve patients' prognosis. Thus, it is imperative to develop new treatment modalities, among which immunotherapy is very promising. Therefore, to assess the potential of using HLA-associated tumour peptidomes as a source of tumour-associated antigens to be used in immunotherapy, we submitted HLA/peptide complexes isolated from HLA-A*02$^+$ GBM samples to peptide elution and identification using LC–MS (Weinschenk *et al*., 2002; Singh-Jasuja *et al*., 2004). We then selected tumour-associated peptides with high GBM-associated expression and strong immunogenicity and characterized them.

Materials and methods

Additional information is available in the online Supplementary material.

Patients and samples

HLA-A*02-positive patients with GBM (stage IV glioma) were included in this study, which conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board of all centres concerned. All patients gave written informed consent. Material for peptide elution was collected at the time of surgery before any treatment. Brain tumour samples were processed immediately and frozen in liquid nitrogen for peptide elution. Normal brain samples and non-CNS tissues were processed similarly. Peripheral blood was collected in heparin tubes and peripheral blood mononuclear cells were isolated using a Ficoll gradient (PAA). The T2, U118 and K562 cell lines (ATCC) were maintained in RPMI medium containing 10% foetal calf serum (Invitrogen). T2 cells were verified for HLA-A2 expression by antibody staining. The K562 cell line was verified for natural killer-dependent killing using natural killer cells cultured with IL-2 and IL-15 in a 4 h cytotoxicity assay. The GBM tumour cell lines (Ge 258, 479 and 518) used were early passage (7–10) lines derived in our laboratory from brain tumour resections.

Isolation of HLA class I-bound peptides

Shock-frozen tumour samples were essentially processed as described previously (Schirle *et al*., 2000) according to standard protocols (Falk *et al*., 1991). Briefly, HLA-A*02 peptide pools from shock-frozen tissue samples were obtained by immune precipitation from solid tissue using HLA-specific antibodies, acid treatment and ultrafiltration. To obtain samples containing HLA-A*02-restricted peptides the antibody BB7.2 was used (Parham and Brodsky, 1981).

Liquid chromatography–mass spectrometry

The HLA peptide pools as obtained were separated according to their hydrophobicity by reversed-phase chromatography (nanoAcquity UPLC® system, Waters) and the eluting peptides were analysed in an LTQ Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization (ESI) source.

Data analysis and peptide sequence identification

The LC–MS data were collected and automatically processed by analysing the LC–MS survey (mass signals of unfragmented peptides) as well as the tandem-MS (MS/MS) data (fragment spectra containing peptide sequence information). Automated data analysis had been optimized and adapted for identification of HLA-restricted peptides. Sequences of peptide vaccine candidates were additionally confirmed by manual inspection of the fragment spectra. The identity of these peptides was further assured by comparison of the recorded natural peptide fragmentation pattern with the fragmentation pattern of a synthetic sequence-identical reference peptide.

Relative peptide quantification

Label-free relative LC–MS quantitation was performed by ion counting i.e. by extraction and analysis of LC–MS features (Mueller *et al*., 2007). The method assumes that the peptide's LC–MS signal area correlates with its abundance in the sample. Extracted features were further processed by charge state deconvolution and retention time alignment (Mueller *et al*., 2007; Sturm *et al*., 2008). Finally, all LC–MS features were cross-referenced with the sequence identification results to combine quantitative data of different samples and tissues to peptide presentation profiles. The quantitative data were normalized in a two-tier fashion according to central tendency to account for variation within technical and biological replicates. Thus each identified

peptide can be associated with quantitative data allowing relative quantification between samples and tissues. In addition, all quantitative data acquired for peptide candidates were inspected manually to assure data consistency and to verify the accuracy of the automated analysis.

Messenger RNA isolation

Total RNA was prepared from snap frozen GBM samples ($n = 20$) using TRIzol® (Invitrogen) followed by a cleanup with RNeasy® (QIAGEN); both methods were performed according to the manufacturer's protocol. Total RNA from healthy human tissues ('bulk' RNA isolations = mixture of all cell types contained in the respective tissue) was obtained commercially (Ambion, Clontech, Stratagene and BioChain). Peripheral blood mononuclear cells were isolated from blood samples of four healthy volunteers. RNA quality for all RNA samples was determined using the RNA 6000 Pico LabChip kit on a 2100 Bioanalyzer (Agilent Technologies).

Gene expression analysis

Gene expression analysis of all tumour and normal tissue RNA samples was performed by Affymetrix Human Genome (HG) U133A or HG-U133 Plus 2.0 oligonucleotide microarrays (Affymetrix). The same normal kidney sample was hybridized to both array types to achieve direct comparability of all samples. All steps were carried out according to the Affymetrix manual. An empirical messenger RNA over-expression score was calculated for each gene considering expression levels in the analysed GBM samples versus expression in normal tissues. An empirical cut-off was set in order to prefilter for genes over-expressed, qualifying the HLA-A*02-derived peptides from these genes for more detailed analysis as potential targets for GBM immunotherapy.

Tissue microarray, immunohistochemistry and immunofluorescence staining

The tissue microarray consisted of 250 formalin-fixed paraffin-embedded glioblastoma WHO grade IV and four normal brain tissue samples as described elsewhere (Campos *et al*., 2011). Informed consent was obtained from each patient according to the research proposals approved by the Institutional Review Board at Heidelberg Medical Faculty. Antigen retrieval, incubation with primary and secondary antibodies as well as detection with VECTASTAIN® Laboratories Elite ABC Kit (Vector Laboratories) was carried out as described (Campos *et al*., 2011). Each tumour biopsy was evaluated at × 20 magnification by two independent investigators.

In vitro immunogenicity experiments

$CD8^+$ T cells were stimulated three times with artificial antigen-presenting cells according to Walter *et al*. (2003) and stained at Day 21 with HLA/peptide tetramers incorporating either the cognate peptide or a control peptide. Each peptide was tested in four to six healthy individuals (9–12 wells per peptide) and in 7 to 11 patients with GBM (2–5 wells per peptide). For analysis of naïve and memory T cell populations, fluorescence-activated cell sorted $CD8^+$ $CD45RA^+$ $CCR7^+$ and $CD45RA^-$ $CCR7^{+/-}$ fractions were similarly stimulated with artificial antigen-presenting cells.

Flow cytometry and cell sorting

Cultures were stained with HLA/peptide tetramers (5 μg/ml) and CD8 antibodies (Beckman Coulter) and analysed using a Gallios flow cytometer and the Kaluza software (Beckman Coulter) or sorted using a Vantage SE cytometer (BD Biosciences). Cultures were considered positive when >1% of $tetramer^+$ cells among $CD8^+$ cells were detected. For intracellular staining experiments, cells were incubated for 4 h with PMA (phorbol myristate acetate) and ionomycin (both 1 μg/ml, Invitrogen) in the presence of Brefeldin A (1 μg/ml, GolgiPlug, BD Biosciences), fixed with 1% formaldehyde, permeabilized with 0.5% saponin and stained with antibodies to IFN-γ, IL-2, CD8 (Beckman Coulter) and IL-4, IL-10, IL-17 (ebioscience).

Generation of T cell clones

Flow cytometry-sorted $CD8^+$ $tetramer^+$ cells were plated in limiting dilution condition (0.3 cells/well) in Terasaki plates (Greiner BioOne) in Iscove's Modified Dulbecco's Medium containing 8% human serum (Laboratoires Jacques Boy), penicillin, streptomycin, non-essential amino acids, sodium pyruvate and HEPES (all from Invitrogen) (cytotoxic T lymphocyte medium) containing 150 IU/ml IL-2, 1 μg/ml PHA (phytohaemagglutinin) and allogeneic irradiated (3000 rad) peripheral blood mononuclear cells (10 000 cells/well). Allogeneic peripheral blood mononuclear cells were obtained from healthy individuals from the local blood bank. Cells were further maintained in 96-well plates in cytotoxic T lymphocyte medium containing 150 IU/ml IL-2 and restimulated periodically (every 3–4 weeks) with 1 μg/ml PHA and allogeneic irradiated peripheral blood mononuclear cells (100 000 cells/well).

Functional T cell assays

For peptide titration experiments, T2 cells were stained with Vybrant® DiD (Invitrogen) and incubated with cytotoxic T lymphocyte clones and increasing concentration of peptide for 4 h at 37°C at an effector to target ratio of 10:1. Cell death was measured by addition of 7AAD (Beckman Coulter) and detection of $7AAD^+$ Vybrant® DiD^+ T2 cells by flow cytometry. For tumour and K562 cell killing, target cells were similarly incubated with cytotoxic T lymphocyte clones at an effector:target ratio of 10:1 unless stated otherwise in presence or absence of peptide.

Detection of tumour-infiltrating lymphocytes

Tumour-infiltrating lymphocytes were stained with HLA/peptide tetramers (5 μg/ml) and CD8 after 1 week of culture with PHA (1 μg/ml), IL-2 (150 IU/ml) and allogeneic irradiated peripheral blood mononuclear cells.

Results

Identification of the HLA-associated glioblastoma multiforme peptidome

In order to identify peptides involved in the composition of the GBM peptidome, we submitted 32 $HLA\text{-}A*02^+$ GBM samples to HLA class I peptide elution and sequenced the isolated peptides by mass spectrometry. We identified 6820 HLA-restricted peptides, comprising 3686 different HLA-A*02-restricted sequences eluted

with the HLA-A*02-specific BB7.2 antibody. At this point, analysis was discontinued as the rate of newly identified sequences from the last samples dropped below 15% (i.e. 85% of identified peptides had been identified on other GBM samples before) suggesting that a relevant portion of the accessible HLA-A*02-restricted GBM peptidome had been discovered. The number of identified peptides varied between samples (median sequences identified per sample: 170; range: below 100 to >2000 sequences), which was due to different amounts of tissue available and possibly different levels of HLA expression. The peptide composition of peptidomes also varied among GBM samples, but was more conserved among GBMs (Pearson correlation $R = 0.40$) than when comparing GBM ($n = 32$) and non-GBM ($n = 101$) tumours (Pearson correlation $R = -0.08$). HLA-A*02 restriction was verified for all peptides using the SYFPEITHI prediction algorithm (www.syfpeithi.de). This comprehensive description of the glioblastoma peptidome is the first study of its kind on GBM *ex vivo* and offers a basis for identification of relevant targets for immunotherapy.

In order to identify the tumour-associated fraction of the peptidome, we compared expression of the source genes in GBM samples, normal brain and non-CNS normal tissues using messenger RNA microarrays. We calculated an empirical messenger RNA over-expression score for each gene considering expression of each gene in GBM compared to all normal tissues as well as to the normal tissue with highest expression. This led to the selection of 309 peptides (8.4% of the total peptidome). Most of these 309 peptides could unambiguously be assigned to a single human source gene and were in total derived from 148 different genes, providing evidence that a broad range of proteins is involved in the composition of the tumour-associated fraction of the HLA-associated GBM peptidome.

We performed a protein database and literature search on the 148 proteins identified (Supplementary Table 1) and observed that 39 proteins (26%) were associated with cell proliferation (with 24, 16%, directly involved in cell cycle progression), 27 (18%) with cell migration and 16 (11%) with cell adhesion, showing that a large proportion of proteins (>50%) is associated with the malignant phenotype (Fig. 1A). An important fraction of proteins (22%) was related to nervous system development at the embryonic stage, reflecting re-expression in GBM of genes normally expressed during foetal life. Interestingly, 67 proteins (45%) have previously been described in the literature to be involved in non-CNS malignancies, suggesting that an important fraction of the HLA-associated GBM peptidome is not specific to CNS tumours but implicated in common tumorigenic mechanisms. In addition, the large majority ($n = 115$, 78%) of identified proteins had not been previously described for GBM to our knowledge, opening new avenues to explore the biology of this tumour.

Selection and characterization of glioblastoma multiforme-associated peptides for immunotherapy

One immediate application provided by a comprehensive knowledge of the HLA-restricted GBM peptidome is to focus on the tumour components that are seen by the immune system and which could be ideal targets for immunotherapy. We selected the most attractive candidates based on high messenger RNA over-expression in GBM (Fig. 1B and C) and potential implication in gliomagenesis, leading to the identification of 35 peptides. We further tested these for their ability to elicit strong T cell responses *in vitro* using peripheral blood mononuclear cells from HLA-A*02$^+$ healthy individuals stimulated by artificial antigen-presenting cells loaded with HLA/peptide complexes, and selected the 10 most immunogenic candidates (Tables 1 and 2) (Walter *et al*., 2003). The HLA-A*02 restriction of these peptides was confirmed using an HLA refolding assay (not shown). As foreseen with the design of the selection strategy, the 10 GBM-associated antigens are involved in tumourigenic mechanisms (Supplementary Table 2) such as tumour cell motility, proliferation, invasion or angiogenesis (Sehgal *et al*., 1998; Kim *et al*., 2000; Chekenya *et al*., 2002; Yang *et al*., 2004; Phillips *et al*., 2006; Mita *et al*., 2007; Hu *et al*., 2008). Interestingly, the expression of most of these antigens is known to correlate with higher tumour grade or decreased patient survival (Chekenya *et al*., 1999; Herold-Mende *et al*., 2002; Ulbricht *et al*., 2003; Liang *et al*., 2005). In addition, tenascin C can be induced by hypoxia and TGF-β, which play major roles in GBM progression (Lal *et al*., 2001; Hau *et al*., 2006). Finally, protein tyrosine phosphatase, receptor-type, Z polypeptide 1 (PTPRZ1) is expressed by embryonic stem cells (Soh *et al*., 2007), and brevican has been described to be over-expressed in GBM stem cells (Gunther *et al*., 2008).

To further validate the choice of these peptides, we performed an in depth expression analysis at the messenger RNA, peptide and protein levels. Result of the messenger RNA analysis (representative example in Fig. 1B) showed a highly enhanced expression in GBM compared to non-CNS healthy tissues (Fig. 1C) and normal brain (Fig. 1C). A crucial step was then to confirm that the selected antigens are also preferentially presented as peptides at the tumour cell surface. Using a novel mass spectrometry-based peptide quantification method (Weinschenk *et al*., 2010; poster can be downloaded at http://www.immatics.com/index.php?page=78&modaction=detail&modid=228&modid2=), we compared natural peptide presentation on GBM, normal brain and non-CNS normal tissue samples. As depicted in Fig. 1E for a representative example, we showed that peptides are only weakly presented at the surface of normal tissues. In contrast, peptides were presented at high levels at the surface of GBM, with some heterogeneity among samples. Remarkably, 6 out of 10 peptides were detected only on tumour cells and the remaining four were over-presented in the range of 7- to 160-fold on tumour cells as compared to normal tissues (Fig. 1D). Interestingly among the four antigens displaying the lowest degree of messenger RNA over-expression in GBM versus normal brain (Fig. 1C), three were detected at the peptide level only on tumour cells, emphasizing the critical value of direct peptide presentation analysis for accurate antigen selection and the poor correlation between messenger RNA and HLA ligand levels (Weinzierl *et al*., 2007).

Finally, to get a wide appraisal of antigen expression, we analysed tissue microarrays containing 221 GBM and 29 recurrent GBM samples (Campos *et al*., 2011) by immunohistochemistry and compared them with normal brain samples. We observed

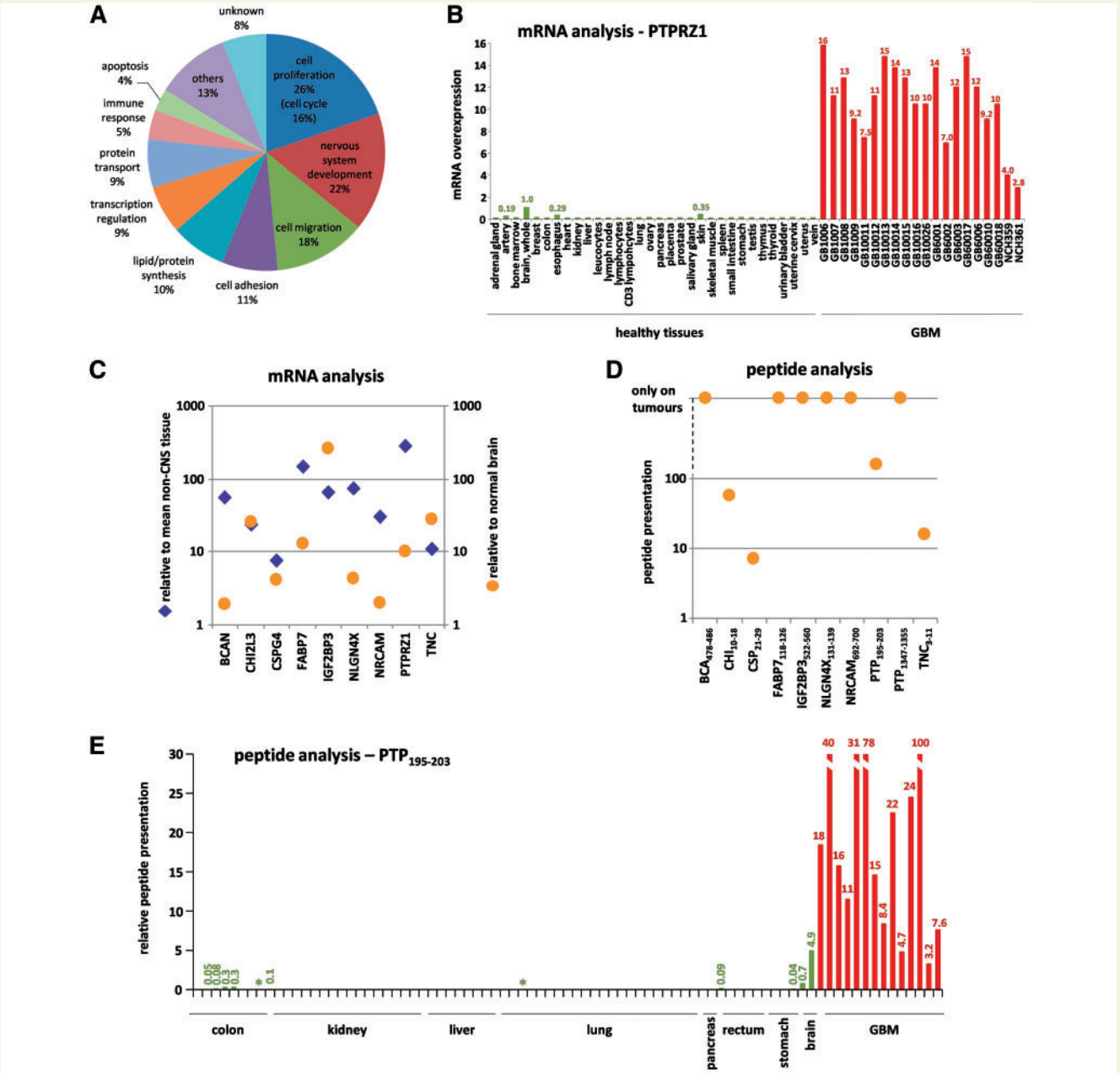

Figure 1 Identification of the HLA-associated GBM peptidome and characterization of selected tumour-associated antigens. (**A**) Pie chart displaying the processes in which the tumour-associated source proteins of the HLA-associated GBM peptidome are involved. The total percentage exceeds 100, as one protein can be involved in several processes. (**B**) Messenger RNA expression profile of PTPRZ1 plotted relative to normal brain. Values for normal tissues (green bars) and GBM (red bars) are shown. Each bar represents a single microarray measurement. (**C**) Average messenger RNA expression of the peptides in GBM ($n = 20$) compared to the mean messenger RNA expression in normal tissues (*left* y-axis, $n = 32$) or in normal brain (*right* y-axis, $n = 3$). (**D**) Average peptide presentation on GBM samples relative to mean of normal tissues. 'Only on tumours' indicates that the peptide could not be detected on any normal tissue sample ($n = 69$) including additional samples for which peptide quantification was not possible. (**E**) Peptide presentation profile of the PTPRZ1-derived $PTP_{195–203}$ peptide. Relative peptide presentation levels for $PTP_{195–203}$ in all GBM (red bars, $n = 12$) and all normal tissue samples (green bars, $n = 69$) that qualified for relative quantification by objective quality control check. Asterisk indicates not quantifiable. Each bar represents a single peptide presentation measurement.

intense staining in most GBM samples (representative examples in Fig. 2A), with absent or faint signals in normal brain. Importantly, eight out of nine proteins were expressed by 75–100% of both primary and recurrent GBM samples (Fig. 2A and C; Herold-Mende *et al*., 2002), demonstrating an almost ubiquitous expression in GBM samples. Furthermore, we performed co-localization studies for the PTPRZ1, CHI3L2 and CSPG4 proteins and showed that expression was restricted to $GFAP^+$ tumour cells

Table 1 Description of the 10 A*02-restricted GBM-associated peptides

Peptide code	Source protein	Accession number	Position	Sequence	Functional relevance
$BCA_{478-486}$	Brevican (BCAN)	NP_068767	478–486	ALWAWPSEL	Brain-specific extracellular matrix molecule involved in invasion; specifically deglycosylated in glioma; stem-cell associated
CHI_{10-18}	Chitinase 3-like 2 (CHI3L2)	NP_003991	10–18	SLWAGVVVL	Extracellular protein with unclear function
CSP_{21-29}	Chondroitin sulphate proteoglycan 4 (CSPG4)	NP_001888	21–29	TMLARLASA	Transmembrane proteoglycan; role in neovascularization; over-expressed by tumour cells and pericytes
$FABP7_{118-126}$	Fatty acid-binding protein 7, brain (FABP7)	NP_001437	118–126	LTFGDVVAV	Cytoplasmic protein involved in fatty acid metabolism; associated with increased motility of GBM cells and short survival
$IGF2BP3_{552-560}$	Insulin-like growth factor 2 messenger RNA-binding protein 3 (IGF2BP3)	NP_006538	552–560	KIQEILTQV	Messenger RNA turnover and translational control; oncofoetal protein; over-expressed in several cancers; associated with poor survival
$NLGN4X_{131-139}$	Neuroligin 4, X-linked (NLGN4X)	NP_065793	131–139	NLDTLMTYV	Cell-adhesion molecule; role in invasion and tumorigenesis
$NRCAM_{692-700}$	Neuronal cell adhesion molecule (NRCAM)	NP_001032209	692–700	GLWHHQTEV	Involved in β-catenin signalling; major role in invasion, tumour growth and tumorigenesis; high expression correlates to poor survival
$PTP_{195-203}$	Protein tyrosine phosphatase, receptor-type, Z polypeptide 1 (PTPRZ1)	NP_002842	195–203	AIIDGVESV	Type I transmembrane protein; role in tumorigenesis; gene amplification occurs frequently in GBM and other tumours
$PTP_{1347-1355}$			1347–1355	KVFAGIPTV	
TNC_{3-11}	Tenascin C (TNC)	NP_002151	3–11	AMTQLLAGV	Role in angiogenesis, tumour transformation and proliferation; cancer stem-cell associated

and absent from adjacent endothelial ($CD31^+$) cells (Fig. 2B). We detected some $PTPRZ1^+$ microglial ($CD68^+$) cells (Fig. 2B), however, only in areas with a high PTPZR1 expression in tumour cells while it was absent in PTPRZ1-negative tumour areas, as well as in cases of PTPRZ1-negative tumours ($n = 35$). Therefore, we interpret this positivity in some microglial cells as phagocytosis of $PTPRZ1^+$ tumour cells. Finally, and as expected, we noted some heterogeneity in antigen expression by individual tumour cells (Fig. 2D), arguing for the use of a panel of tumour-associated antigens in contrast to single antigens in future vaccines to minimize the risk of tumour escape by immunoediting.

Absence of tolerance to the selected peptides in patients with glioblastoma multiforme

In order to confirm the choice of the selected peptides for immunotherapy, we investigated and compared the presence and function of specific T cells in the blood of HLA-$A*02^+$ patients with GBM, and HLA-$A*02^+$ healthy individuals. We stimulated peripheral blood mononuclear cell-derived $CD8^+$ T cells with HLA/peptide complex-loaded artificial antigen presenting cells (Walter *et al*., 2003) and detected antigen-specific T cells by tetramer staining. We tested each peptide in peripheral blood mononuclear cells from 7 to 11 patients, with two to five replicates, depending on peripheral blood mononuclear cell availability. We detected antigen-specific T cell responses for all peptides in patients with GBM (representative example in Fig. 3A), with 6 out of 10 peptides inducing a response in >50% of the patients (Fig. 3B and Table 2). Importantly, peptide immunogenicity, as defined by combining percentage of responding individuals and positive wells of the culture plate, was comparable in patients and healthy donors (Fig. 3C). Overall, these results suggest that a comparable frequency of antigen-specific T cells is present in patients with GBM and healthy donors.

In order to further explore the functional status of GBM-specific T cells, we generated T cell clones with six different antigen specificities. We then compared those derived from patients with those from healthy individuals. T cell clones from both (37 analysed in total) shared similar properties regarding cytokine secretion (Table 3) and avidity of peptide recognition (Fig. 3D and Table 3). We performed a direct comparison of clones specific for $NLGN4X_{131-139}$ or $PTP_{1347-1355}$ (five clones from patients with GBM and five from healthy individuals for both specificities, Table 3) and confirmed the similar avidity of antigen recognition for patients and healthy donors (mean $EC_{50} \pm$ SD: $NLGN4X_{131-139}$: patients: 34 ± 29 nM, healthy donors: 37 ± 30 nM; $PTP_{1347-1355}$: patients: 31 ± 39 nM, healthy donors: 31 ± 23 nM). In addition, this avidity was comparable to that of antigen–specific T cells spontaneously primed *in vivo* by melanoma (Valmori *et al*., 2000; Dutoit *et al*., 2001) or even viruses (Couedel *et al*., 1999; Yang *et al*., 2003; Trautmann *et al*., 2005). We next assessed the critical issue of cytotoxicity against GBM cells using patient-derived T cell clones specific for $NLGN4X_{131-139}$ and $PTP_{1347-1355}$. As shown in Fig. 3E, $PTP_{1347-1355}$-specific T cells were able to specifically lyse antigen-expressing $HLA-A*02^+$ tumour cells but

Table 2 ***In vitro*** **immunogenicity of the 10 glioma-associated peptides in A*02$^+$ healthy individuals and patients with GBM**

Antigen	Healthy individuals				Patients with GBM			
	Donors		Wells		Donors		Wells	
	Total	Positive (%)	Total	Positive (%)	Total	Positive (%)	Total	Positive (%)
$BCA_{478–486}$	6	6 (100)	66	66 (100)	8	8 (100)	22	21 (95)
$CHI_{10–18}$	6	6 (100)	60	60 (100)	7	7 (100)	22	18 (82)
$CSP_{21–29}$	4	4 (100)	42	25 (60)	9	4 (44)	26	6 (23)
$FABP7_{118–126}$	6	6 (100)	55	33 (60)	7	5 (71)	22	10 (45)
$IGF2BP3_{552–560}$	6	4 (66)	60	15 (25)	10	2 (20)	29	2 (7)
$NLGN4X_{131–139}$	5	5 (100)	48	31 (65)	8	5 (63)	23	12 (52)
$NRCAM_{692–700}$	6	6 (100)	58	10 (17)	9	6 (67)	25	8 (32)
$PTP_{195–203}$	6	2 (33)	71	9 (13)	11	4 (36)	30	5 (17)
$PTP_{1347–1355}$	4	4 (100)	37	25 (68)	7	6 (86)	20	13 (65)
$TNC_{3–11}$	6	6 (100)	65	10 (15)	11	5 (45)	30	9 (30)

Peptide immunogenicity was assessed by repetitive stimulation of CD8$^+$T cells with polystyrene beads coated with HLA/peptide complex monomers and anti-CD28 antibodies as described in the 'Materials and methods' section. T cell responses were detected by staining with phycoerythrin-labelled HLA/peptide tetramer complexes and a response was considered positive when >1% of tetramer$^+$ cells among CD8$^+$ cells were observed. Each peptide was tested in 4 to 6 healthy individuals, with 9–12 wells per peptide and in 7 to 11 patients with 2–5 wells per peptide.

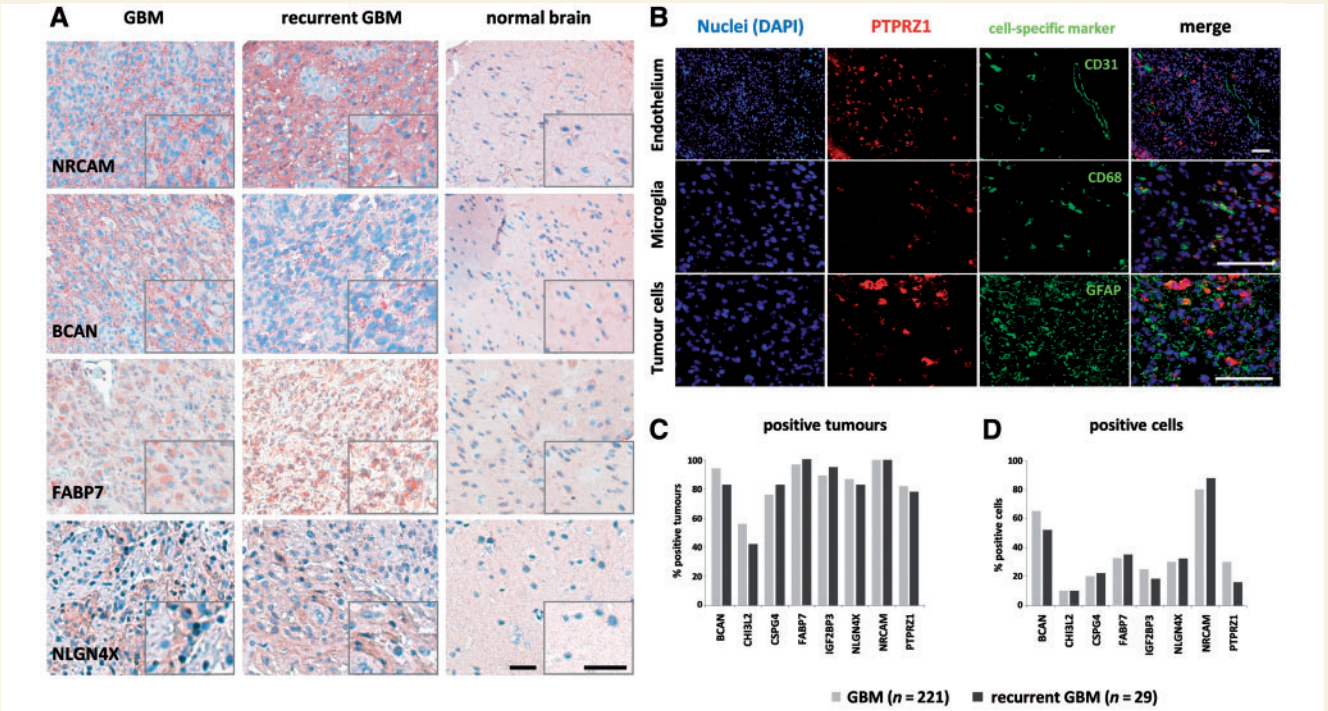

Figure 2 Protein expression analysis. (**A**) Immunohistochemical analysis of representative proteins on GBM (n = 221), recurrent GBM (n = 29) and normal brain (n = 4) tissues. Inserts show higher magnification. Scale bars: 50 μm. (**B**) Immunofluorescent staining of the PTPRZ1 protein in GBM. Nuclei are shown in blue, PTPRZ1 staining in red and markers specific for endothelial cells (CD31, *upper row*), microglial cells (CD68, *middle row*) and astrocytic tumour cells (GFAP, *lower row*) in green. Merge is shown on the *right*. Scale bars: 100 μm. (**C** and **D**) Analysis of the percentages of positive tumours (**C**) or positive individual tumour cells (**D**).

not HLA-A*02$^-$ antigen$^+$, HLA-A*02$^+$ antigen$^-$ or HLA-A*02$^-$ antigen$^-$ tumour cells, nor the natural killer-sensitive K562 cell line. Furthermore, tumour killing was proportional to the effector to target ratio (not shown). In addition, similar results were obtained for $NLGN4X_{131–139}$-specific T cell clones (not shown).

Finally, to assess whether antigen-specific T cells had been spontaneously elicited by the tumour in patients, we analysed tumour-infiltrating lymphocytes obtained from GBM resections. The small size of samples available for research after diagnostic procedure precluded a direct *ex vivo* T cell analysis. We therefore

Table 3 Functional analysis of antigen-specific T cell clones derived from patients with GBM and healthy individuals

Antigen	Donor[a]	Clone name	Cytokine secretion[b]					Avidity of antigen recognition
			IFN-γ	IL-2	IL-17	IL-4	IL-10	
$BCA_{478–486}$	Ge 540	3E4	+ + +	+ +	–	+	–	100
	Ge 549	1F10	+ + +	+ + +	–	–	–	13
	Ge 549	1E8	+ + +	+ +	–	–	–	20
						mean ± SD[d]		**44 ± 48**
	HD 057	2A7	+ + +	+ +	–	–	–	25
	HD 058	5D1	+ + +	+ + +	–	–	–	80
$CHI_{10–18}$	Ge 549	1B3	+ + +	+ +	–	–	–	10
	HD 057	1C3	+ + +	+ + +	–	–	–	35
	HD 057	3D3	+ +	+ + +	–	–	–	50
$CSP_{21–29}$	Ge 549	10B9	+ + +	+ +	–	–	–	1
	Ge 570	1A1	+ +	+ +	–	–	–	20
$FABP7_{118–126}$	Ge 540	1D1	+ + +	+ + +	–	–	–	38
	Ge 549	5F3	+ + +	+ + +	–	–	–	20
	Ge 570	1D1	+ + +	+ +	–	–	–	38
						mean ± SD		**32 ± 10**
	HD 055	3A7	+ + +	+ + +	–	–	–	105
	HD 055	1E4	+ + +	+ + +	–	–	–	12
	HD 057	2A4	+ + +	+ +	–	–	–	100
	HD 058	3A8	+ + +	+ + +	–	–	–	110
						mean ± SD		**82 ± 47**
$NLGN4X_{131–139}$	Ge 540	2E5	+ + +	+ + +	–	–	–	12
	Ge 540	3E4	+ +	+ + +	–	–	–	45
	Ge 540	1F8	+ +	+ +	–	–	–	25
	Ge 549	1E8	+ + +	+ + +	–	–	–	10
	Ge 549	2E5	+ + +	+ + +	–	–	–	80
						mean ± SD		**34 ± 29**
	HD 057	1A7	+ + +	+ +	–	–	–	40
	HD 057	5F5	+ + +	+ + +	–	–	–	10
	HD 058	5A2	+ + +	+ + +	–	–	–	50
	HD 058	3B4	+ + +	+ +	–	–	–	80
	HD 058	3B6	+ + +	+ +	–	–	–	5
						mean ± SD		**37 ± 30**
$PTP_{1347–1355}$	Ge 242	4B4	+ + +	+ +	–	–	–	15
	Ge 242	2B7	+ +	+ +	–	–	–	10
	Ge 522	3B9	+ + +	+ + +	–	–	–	10
	Ge 522	5C6	+ + +	+ + +	–	–	–	20
	Ge 522	2C5	+ + +	+ +	–	–	–	100
						mean ± SD		**31 ± 39**
	HD 055	4A6	+ + +	+ +	–	–	–	38
	HD 057	4E4	+ + +	+ +	–	–	–	10
	HD 057	1E6	+ + +	+ + +	–	–	–	60
	HD 057	1B1	+ + +	+ +	–	–	–	40
	HD 058	3D2	+ + +	+ + +	–	–	–	5
						mean ± SD		**31 ± 23**

a Ge = patients with GBM; HD = healthy individuals.
b Cytokine production by: +, 5–10%; + +, >10–50%; and + + +, >50% of the cells.
c Avidity of antigen recognition as defined by the dose of peptide giving 50% maximal lysis [nM] on T2 cells at an effector to target ratio of 10:1.
d SD = standard deviation; mean and SD are given when at least three clones were tested.

amplified tumour-infiltrating lymphocytes ($n = 12$) using PHA and interleukin (IL)-2, a procedure that does not bias representation of antigen-specific T cells (Arenz *et al*., 1997; Jason and Inge, 1996), and analysed them after 1 week of culture with tetramers incorporating the 10 GBM-associated peptides. Among the tumour-infiltrating lymphocytes tested, we observed one culture displaying 3% of T cells specific for the $BCA_{478–486}$ peptide, which corresponds to 12.6% of $CD8^+$ T cells (Fig. 3F). Considering that brevican protein expression is known to be lost in culture (Jaworski *et al*., 1994), we tested antigen recognition and killing ability with T2 cells. As shown in Fig. 3G, $BCA_{478–486}$-specific tumour-infiltrating lymphocytes were able to specifically kill T2 cells loaded with the $BCA_{478–486}$ peptide but not the control peptide. To our knowledge, these results are the first demonstration that GBM is able to spontaneously induce an antigen-specific immune response in the brain microenvironment. To strengthen

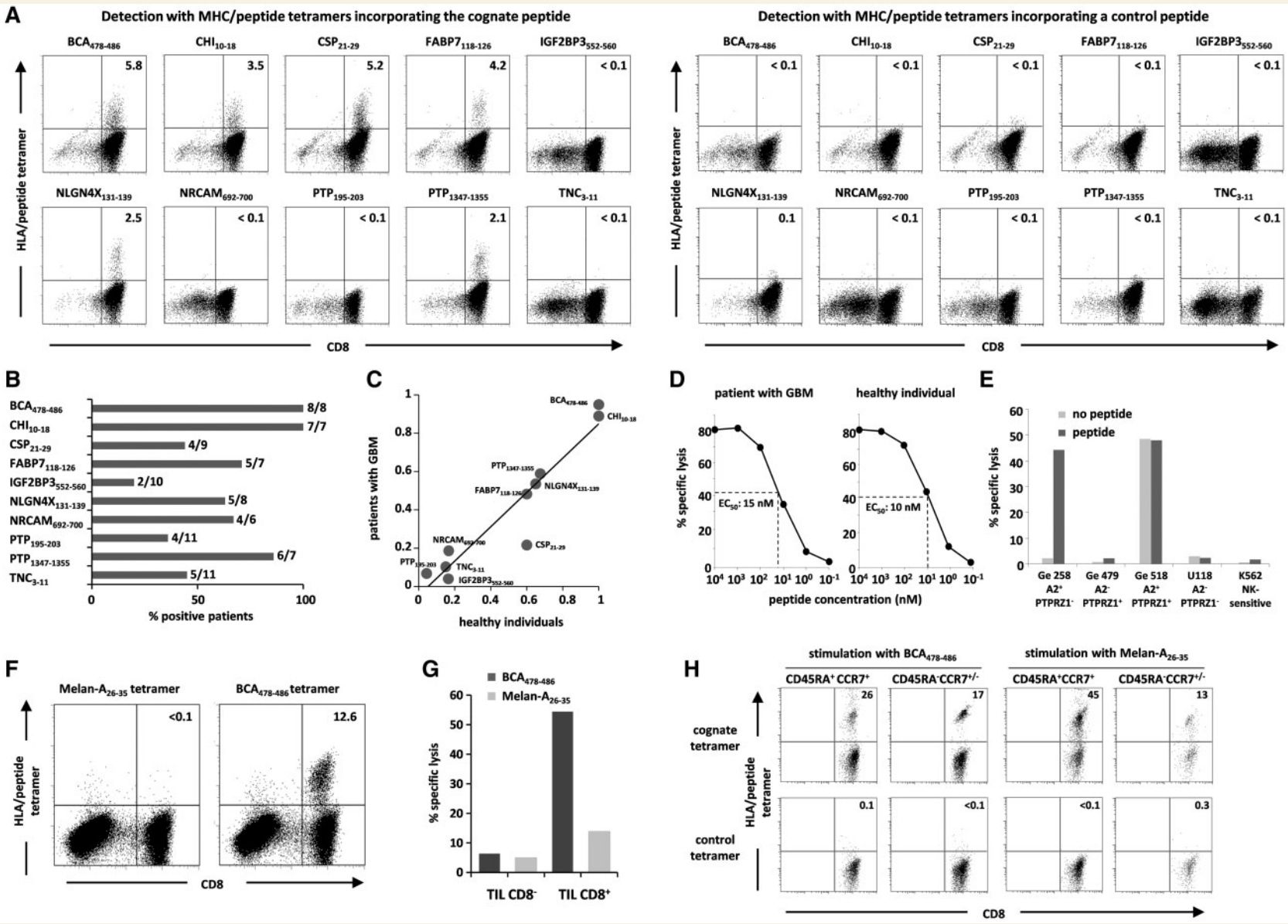

Figure 3 Functional characterization of the novel GBM-associated peptides. (**A**) Multipeptide *in vitro* T cell response in GBM Patient Ge 549. Cultures were tested with HLA/peptide tetramers incorporating the cognate (*left*) or a control (*right*) peptide. Numbers in the upper right quadrant represent the percentage of tetramer$^+$ cells among CD8$^+$ T cells. Representative example of two independent experiments. Cultures were considered positive when >1% of tetramer$^+$ cells among CD8$^+$ cells were detected. (**B**) Percentage of

(continued)

Figure 3 Continued
responding patients for all peptides tested. (**C**) Peptide immunogenicity in patients with GBM and healthy individuals. Peptide immunogenicity was defined by multiplying the percentage of positive donors by that of positive wells, each peptide being tested in 7 to 11 patients (2–5 wells per experiment) and four to six healthy individuals (9–12 wells per experiment, Table 2). The Pearson's correlation coefficient for the two sets of values is 0.95. (**D**) Peptide titration on T2 cells with $PTP_{1347–1355}$-specific T cell clones derived from one patient with GBM (*left*) and one healthy individual (*right*). EC_{50}: amount of peptide required to achieve 50% maximal lysis. Representative example of two independent experiments for each clone tested. (**E**) Tumour killing of GBM cell lines using a $PTP_{1347–1355}$-specific T cell clone derived from a patient with GBM at an effector to target ratio of 10:1. Representative example of three independent experiments. (**F**) Staining of tumour-infiltrating lymphocytes from Patient Ge 533 with tetramers incorporating the Melan-$A_{26–35}$ control or $BCA_{478–486}$ peptides after 1 week of *in vitro* culture. The number in the *upper right* quadrant is the percentage of $tetramer^+$ T cells among $CD8^+$ tumour-infiltrating lymphocytes. Representative example of two independent experiments. (**G**) T2 killing with sorted $CD8^+$ tumour-infiltrating lymphocytes at an effector to target ratio of 100:1 (final calculated ratio of $BCA_{478–486}$-specific to target cells = 8:1) in the presence of $BCA_{478–486}$ or Melan-$A_{26–35}$ control peptide. The $CD8^-$ T cell fraction was used as control. This experiment could be performed only once due to the limited amount of material available. (**H**) The precursor origin in the naive ($CD45RA^+$ $CCR7^+$) or memory ($CD45RA^-$ $CCR7^{+/-}$) population for $BCA_{478–486}$ and Melan-$A_{26–35}$-specific T cells is shown for Patient Ge 549. This experiment was performed twice on each patient with similar results, with a total of three patients tested.

this observation, the origin of the precursors of $BCA_{478–486}$-specific T cells was analysed in peripheral blood. $CD45RA^+$ $CCR7^+$ naïve and $CD45RA^-$ $CCR7^{+/-}$ memory $CD8^+$ T cell subsets were sorted by fluorescence-activated cell sorting and stimulated with artificial antigen-presenting cells incorporating the $BCA_{478–486}$ peptide or Melan-$A_{26–35}$ control (Dutoit *et al*., 2002) peptide. As shown in Fig. 3H, a response was detected both in the naïve and memory compartments for $BCA_{478–486}$-specific T cells, corroborating a spontaneous amplification of these cells by the tumour *in vivo*.

Discussion

The originality and power of the approach used in this report to reveal the *ex vivo* HLA-associated GBM peptidome is a major advance in tumour immunology. It provides a window on how T cells can recognize and interact with a brain tumour *in vivo*. This was possible thanks to the sensitive technologies now available for elution and identification of individual peptides in highly complex mixtures and sophisticated bioinformatics analysis.

A first level of interpretation of the present results is that they allow a comprehensive view of the spectrum of peptides and genes expressed by GBM *in vivo*. While several proteins are already known to be involved in invasion and many others are shared by non-CNS tumours, we have now uncovered 115 proteins not previously described to be associated with GBM, opening new opportunities to explore GBM biology and identify therapeutic targets. In addition, we show for the first time that a significant fraction (8.4%) of the peptides presented at the surface of GBM cells actually derive from tumour-associated proteins. This number remains an estimate since: (i) tumour biopsies are not pure populations of tumour cells; (ii) there is a certain degree of heterogeneity between individual tumour cells; and (iii) there are variations between patients.

The LC–MS approach undertaken here does not allow us to ensure that the identified peptides originate from tumour cells only, as biopsies also contain microglial, endothelial and T cells, which all express major histocompatibility complex class I. Since GBM biopsies are usually small in size, using microdissection to isolate GBM cells would result in a too low cell yield that would not allow peptidome analysis by LC–MS for the majority of samples (at least 0.3 g required). *Ex vivo* sorting of given cell types (such as GBM or microglial cells) from GBM biopsies would have the same limitation in the cell number obtained. Using cell lines or primary cell cultures may be a possibility but resulting peptides are unlikely to reflect the *in vivo* situation, which is the strength of our study. However, despite these technical limitations, we showed that the cellular origin of these peptides is predominantly the GBM cells (immunostainings shown in Fig. 2B). Thus, even taking into account this complexity of tumours *in vivo*, GBM cells are likely to present at their surface a large number of tumour-associated peptides from which peptides used as tumour rejection antigens can be selected for immunotherapy. Here, we report 10 peptides derived from proteins involved in tumorigenesis (Supplementary Table 2) (Sehgal *et al*., 1998; Kim *et al*., 2000; Chekenya *et al*., 2002; Mita *et al*., 2007; Hu *et al*., 2008), which

are highly expressed in the large majority of the GBM samples analysed *ex vivo*, but poorly or not expressed in normal brain and non-CNS tissues. Interestingly, non-tumoural tissues displayed some antigen expression at the messenger RNA level but were devoid of peptide presentation at the cell surface, confirming the strength of tumour peptidome for antigen selection. We further validated antigen expression at the protein level in 250 primary or recurrent GBM samples, a critical step considering the limited number of initial samples compatible with thorough peptidome analyses.

Data from this study, together with the knowledge gained in the last 10 years on the rules of antitumour responses in the brain (Grauer *et al*., 2009), provide a scientific background to construct rational therapies. One obstacle to immunotherapy of GBM is that research on defining tumour-associated targets for immunotherapy has until now relied on *in vitro* or *in silico* techniques, which do not necessarily reflect the *in vivo* situation. Also, research has also often focused on a limited list of particular oncogenes in glioma. In contrast, the unbiased generation of knowledge of a HLA-associated tumour peptidome provides direct access to the fraction of cellular peptides that are seen by the immune system. In this regard, it is interesting to note that the set of 3686 peptides identified here did not include previously reported HLA-A*02-restricted GBM peptides from the EGFRvIII, Eph-2A, gp100, HER2, IL-13Rα, MAGE-1, TRP-2, SOX-2, -3, -11 and WT1-derived peptides (Dunn *et al*., 2007). Messenger RNA analysis demonstrated expression of EGFR, HER2, IL-13Rα, TRP-2, SOX-2, and -11 by the majority of GBM samples analysed here, whereas expression of Eph-A2, gp100, MAGE-1, SOX-3 and WT1 was rare or absent. Interestingly, for EGFR and Eph-A2, peptides other than the previously published ones were identified, confirming that these proteins are produced and processed *in vivo*. Thus, it could be speculated that the previously reported HLA-A*02-restricted peptides are poorly presented by GBM cells *in vivo*. The lack of identification of these peptides could alternatively be due to the limits of our detection technique; however, the high sensitivity of the LC–MS used here increases the chance that peptides presented even at very low levels are identified.

We additionally show that the 10 peptides investigated in this study are highly immunogenic not only in healthy individuals but also in patients with GBM. Moreover, the presence of the tumour in the patient group did not change functional properties of specific T cells tested *in vitro*. This suggests that no tolerance was induced by the tumour in patients. This is further supported by the demonstration of specific tumour killing by patient-derived GBM-specific T cell clones. In addition, we consistently observed inducible T cell responses *in vitro* against several antigens in patients for whom $\geqslant$3 peptides could be tested. If similar multipeptide immunogenicity is achieved *in vivo* in the first clinical trials, multipeptide vaccination could be a first step to avoid tumour escape by immunoediting. This is particularly relevant considering the recent observation that immunization of patients with GBM with a single epitope (EGFRvIII) led to antigen loss at the time of tumour progression (Choi *et al*., 2009; Sampson *et al*., 2010). In addition, the future identification of major histocompatibility complex class II-derived peptides would be valuable to provide CD4 T cell help. However, identification of class II ligands by LC–MS is more challenging due to the structural characteristics of HLA class II molecules and a usually low HLA class II expression by GBM.

Furthermore, our results seem to indicate that the immune system of patients with GBM is ignorant of the antigens identified here, except for the $BCA_{478–486}$ peptide for which we could detect antigen-specific tumour-infiltrating lymphocytes in one patient and precursors in the memory compartment in the periphery for some individuals. Whilst it is clear that cellular techniques have a threshold of sensitivity, it still appears that the immune system is able to detect some antigens (even if they are a minority) and not others at a significant level. One explanation would be that the brevican antigen is recognized by the immune system through molecular mimicry, a phenomenon that has been already described for other antigens such as Melan-A (Dutoit *et al*., 2002). A wider analysis of tumour-infiltrating lymphocytes and of the origin of the antigen-specific precursor in the naïve/memory compartment (which we are currently undertaking in healthy individuals and patients with GBM) would help answer these questions. Whether this ignorance by the immune system can be overridden by an efficient peptide vaccination is currently being investigated in the undergoing multi-peptide vaccine trial incorporating 9 out of the 10 peptides described here.

Finally, the detection of $BCA_{478–486}$-specific T cells at the tumour site in one patient (Fig. 3F and G), and in the periphery among memory T cells in three patients tested (Fig. 3H) suggests that one of the selected peptides was able to spontaneously elicit an immune response *in vivo*, providing support for the tumour peptidome approach for antigen identification. Apart from being the first time that spontaneously occurring GBM-specific tumour-infiltrating lymphocytes are reported, these results also suggest that GBM-specific T cells have the potential to target the brain and reach the tumour.

Altogether, this comprehensive inventory of the peptides presented by GBM cells *in vivo* is a valuable resource for exploring the biology of GBM and gives evidence that peptidomes are important sources of multiple tumour antigens with high potential for immunotherapy. Regarding the dismal prognosis of patients with GBM, our results will help to improve the targeting of vaccination and cell therapy approaches. In a first step towards the development of efficient immunotherapies, 9 of the 10 peptides (one was omitted due to poor solubility) are now being developed in a multipeptide therapeutic vaccine designated IMA950. Recently, two phase I studies in first-line patients with GBM have started in the UK and USA (clinicaltrials.gov identifier codes NCT01403285 and NCT01222221). This will allow us to assess the immunogenicity of the vaccine and set the base to improve immunization strategies by combining multipeptide vaccines with the most efficient immunomodulator and other therapeutic interventions aimed at targeting the immunosuppressive mechanisms taking place in the brain microenvironment.

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Conflict of interest

All authors with affiliation immatics biotechnologies GmbH are employees of this company, which also has a commercial interest in a glioma vaccine comprising the antigens described here (IMA950). H.G.R. holds shares in immatics biotechnologies GmbH.

Supplementary material

Supplementary material is available at *Brain* online.

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