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Cytolytic T-cell response to the PASD1 cancer testis antigen in patients with diffuse large B-cell lymphoma

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Summary

The identification of immunogenic cancer testis antigens (CTAs) as immunotherapeutic targets represents one approach to improve treatment options for diffuse large B-cell lymphoma (DLBCL). We previously identified PASD1 [PAS (Per ARNT Sim) domain containing 1 (PASD1)], a DLBCL-associated CTA that was expressed in a range of hematopoietic malignancies. The aim of the present study was to investigate the presence of a cytotoxic T-cell (CTL) response to PASD1 in DLBCL patients. A significant γ-interferon (IFN) release was detected in 21/29 HLA-A*0201-positive DLBCL patients (18 *de novo* DLBCL, two transformed DLBCL and one T-cell rich B-cell lymphoma) following short-term culture of their peripheral blood mononuclear cells stimulated with five HLA-A*0201-restricted PASD1 peptides. No significant responses were detected in 21 HLA-A*0201-negative DLBCL patients (12 *de novo* DLBCL, seven transformed DLBCL, two T-cell rich B-cell lymphoma) or six normal subjects. CTL cell lines were able to lyse PASD1-positive tumour cells in a major histocompatibility complex-Class I dependent manner. The presence of a γ-IFN response correlated with PASD1 protein expression in the tumour cells in 12/15 cases studied. This is the first report of a CTL response to a CTA in DLBCL. Our results provide additional valuable evidence supporting PASD1 as a potential immunotherapeutic target for the treatment of DLBCL and other malignancies.

Keywords: cancer testis antigen, cytotoxic T-cell, diffuse large B-cell lymphoma, immunotherapy, lymphoma.

Diffuse large B-cell lymphoma (DLBCL), representing 40% of non-Hodgkin lymphoma (NHL), is heterogeneous with respect to morphology, clinical features and immunophenotype. Whilst most patients respond initially to combination chemotherapy, a large proportion fail to achieve long term remission (Coiffier, 2005; Stein *et al*, 2008). Although there have been significant advances in defining clinically relevant subtypes of DLBCL and improving treatment (Shipp *et al*, 2002; Hans *et al*, 2004; Banham *et al*, 2005; Haarer *et al*, 2006), the identification of high-risk patients and improved therapeutic options still remains a priority. The detection of tumour-associated antigens (TAAs), recognized by the immune system of the patient, represents one important approach to achieve these objectives. Evidence in support of this has been provided by autologous bone marrow transplantation and donor lymphocyte infusion studies, demonstrating that donor cells can recognize and respond to TAAs in a variety of haematological malignancies, such as multiple myeloma and myeloid leukaemia (Porter & Antin, 2006; Atanackovic *et al*, 2007). Furthermore, vaccination studies have reported an increased immune response to TAAs (Schmitt *et al*, 2008). It is also of note that the immune response signature has been identified as being of importance in predicting survival in DLBCL and follicular lymphoma (FL) (Rosenwald *et al*, 2002; Dave *et al*, 2004).

Tumour-associated antigens that are of current particular interest for improving treatment regimens are the cancer testis antigens (CTAs). Their restricted normal tissue distribution but widespread expression in tumours makes them attractive immunotherapeutic targets, while minimizing potential problems with autoimmunity (Scanlan *et al*, 2004; Simpson *et al*, 2005; Suri, 2006). Initial studies of CTA expression focused on

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solid tumours (Simpson *et al*, 2005), but there are increasing reports of CTAs being expressed in haematological malignancies, such as multiple myeloma (Pellat-Deceunynck *et al*, 2000; Lim *et al*, 2001; Goodyear *et al*, 2005; Jungbluth *et al*, 2005; van Rhee *et al*, 2005), lymphomas (Eichmuller *et al*, 2003; Xie *et al*, 2003) and myeloid malignancies (Adams *et al*, 2002; Zhang *et al*, 2003). A particularly relevant gene expression profiling study recently reported transcripts of multiple CTAs in myeloma tumour cells (Condomines *et al*, 2007). Other studies have also reported the presence of cytotoxic T cells (CTLs) to CTAs, such as NY-ESO-1 and Sp17, in multiple myeloma patients, suggesting the presence of spontaneous immunity to these CTAs (Goodyear *et al*, 2005; van Rhee *et al*, 2005). A subsequent investigation has shown that this immunity can be boosted through vaccination with CTAs such as NY-ESO-1 and clinical trials are ongoing using CTAs as vaccine targets (Szmania *et al*, 2006).

We previously used the serological analysis of recombinant cDNA expression libraries (SEREX) technique to identify the PAS (Per ARNT Sim) domain containing 1 (PASD1) protein (or CT63) as a lymphoma-associated antigen and candidate CTA (Liggins *et al*, 2004a,b). The production of monoclonal antibodies allowed us to confirm PASD1 as a novel CT-X antigen (a CTA encoded on the X chromosome) with a highly restricted distribution in normal tissues but expression in a wide range of haematological malignancies (Cooper *et al*, 2006; Sahota *et al*, 2006). The aim of the current study was to investigate the presence of a CTL response to PASD1 in DLBCL patients in order to ascertain the potential of PASD1 as a vaccine candidate.

Materials and methods

Subjects

Peripheral blood was obtained from 50 patients with B-cell lymphoma attending the Haematology Departments of the John Radcliffe Hospital, Oxford ($n = 44$) and Milton Keynes General Hospital ($n = 6$). The patient cohort consisted of 36 patients with *de novo* DLBCL (two with relapsed DLBCL), 10 patients with transformed DLBCL and four patients with T-cell rich B cell lymphoma. The patients presented with differing stages of disease and their clinical details and treatment protocols are summarized in Table I. Peripheral blood samples were also obtained from six healthy subjects. Tissue typing was done by polymerase chain reaction (PCR) as previously described (Bunce *et al*, 1995). Ethical approval and written consent was obtained from the Oxfordshire Research Ethics Committee B for all blood samples collected and tissue sections used in the immunolabelling studies.

Peptides

Five 9–10 amino acid sequences, predicted to be immunogenic in the context of the major histocompatibility complex (MHC) Class I HLA-A*0201 allele, were identified using the web-based BIMAS (http://bimas.drct.nih.gov/molbiol/hla_bind/index.html) and SYPETHI (http://www.syfpeithi.de) programmes. The peptides identified were as follows: PASD1(1)$_{38–47}$ (QLLDGFMITL); PASD1(2)$_{167–175}$ (YLVGNVCIL); PASD1(3)$_{63–71}$ (LLGHLPAEI); PASD1(4)$_{494–502}$; QLREQLQQL; PASD1(5)$_{694–702}$ (ELSDSLGPV). A control irrelevant peptide from HIV-1 reverse transcriptase (ILKEPVHGV) predicted to bind to HLA-A*0201 was also used. All peptides were synthesized by standard chemistry on a multiple peptide synthesizer (Invitrogen, Paisley, UK) and were >90% pure. Lyophilized peptides were diluted in dimethyl sulfoxide and stored at −20°C. The peptide sequences of PASD1(1), PASD1(2), PASD1(3) and PASD1(4) were common to both PASD1a and PASD1b protein isoforms while PASD1(5) was specific for the PASD1b isoform, which represents a longer protein with a unique C-terminus that is absent in PASD1a. The positions of the peptide sequences in the PASD1 isoforms are shown in Fig 1.

Antibodies

Monoclonal antibodies. Both of the anti-PASD1 monoclonal antibodies, PASD1-1 (recognizing a region common to both PASD1a and PASD1b) and PASD1-2 (recognizing an epitope in the C-terminus of PASD1b) were produced in the authors' laboratory (Cooper *et al*, 2006). Antibodies to BCL6 (PG-B6p), CD10 (56C6), CD4 (T4-10, IgG1 isotype), CD20 (DAKO-L26, IgG2a isotype), CD45RO (UCHL1, IgG2a isotype), CD56 (T199, IgG1 isotype) and CD68 (PGM1, IgG3 isotype) were purchased from Dako (Ely, Cambridgeshire, UK) while anti-CD8 (X-107, IgG1 isotype) and anti-CD45RA (4KB4, IgG1 isotype) were generated in the authors' laboratory. Anti-MUM1 was a kind gift from Prof B. Falini (Perugia, Italy). The anti-HLA-A*0201 (BB7.2) was purchased from BD BioSciences (Oxford, UK).

Polyclonal antibodies. Rabbit anti-CD3 (DAKO-CD3 – diluted 1:100) and the Envision-horseradish peroxidase (HRP) labelling system were obtained from DAKO. The Mach Three-HRP kit was purchased from BD Biosciences. Isotype specific goat anti-rabbit immunoglobulin (Ig) and anti-mouse Ig-isotype specific antibodies conjugated to either fluorescein isothiocyanate (FITC) or Texas Red^{TM} (diluted 1:100) were obtained from Invitrogen Ltd, Paisley, UK.

Cell lines

The following cell lines were obtained and cultured as described previously (Cooper *et al*, 2006), namely: the PASD1-positive and HLA-A*0201-positive Thiel (myeloma-derived), PASD1-positive and HLA-A*0201-negative OCI-Ly3 (DLBCL-derived) KM-H2 [Hodgkin lymphoma (HL)-derived] and the PASD1-negative and HLA-A*0201-positive SUDHL-6 (DLBCL-derived).

Table I. Clinical details of DLBCL cases.

Patient ID	Diagnosis	Subtype*	Stage	IPI	Sex	Age (years)	Treatment	Current status from time of diagnosis
1	DLBCL(dn)	NGC	I	1	F	23	CHOP-R	CR (21 months)
2	DLBCL(dn)	GCB	3	3	M	67	CHOP-R + MTX + RX	CR (20 months)
3	DLBCL(dn)	GCB	3	3	M	81	VIN/PRED	Died (22 months)
4	DLBCL(dn)	NGC	1	2	F	76	CHOP-R	PR (29 months)
5	DLBCL(dn)	GCB	1	0	M	52	CHOP-R + RX	CR (12 months)
6†	DLBCL(dn)	NGC	2	1	M	21	CHOP-R	Died (19 months)
7	DLBCL(dn)	GCB	2	0	M	49	CHOP-R + MTX	PR (19 months)
8	DLBCL(dn)	GCB	1	0	M	63	CHOP-R	CRU (24 months)
9	DLBCL(dn)	NGC	3	2	F	71	CHOP-R	PR (23 months)
10†	DLBCL	GCB	1	0	F	60	CHOP-R + RICE + ESHAP + BEAM + TX	CR (13 months)
11	DLBCL(dn)	GCB	1	1	M	38	CODOX-M + RX	PR (17 months)
12	DLBCL(dn)	GCB	1	0	F	59	CHOP-R	CR (22 months)
13	DLBCL(dn)	GCB	3	3	M	67	CHOP-R + MTX	PR (17 months)
14	DLBCL(dn)	NGC	3	2	M	63	CHOP-R + MTX	CR (12 months relapse 2 months)
15	DLBCL(dn)	NGC	3	3	M	85	VIN/PRED	Died (6 months)
16	DLBCL(dn)	GCB	2	2	M	59	CHOP-R	CR (22 months)
17	DLBCL(dn)	GCB	3	4	M	60	CHOP-R	CR (17 months)
18	DLBCL(dn)	NGC	4	4	M	74	CNOP-R	CR (14 months)
19	DLBCL(dn)	GCB	4	2	M	56	CHOP-R + RX	Died (19 months)
20	DLBCL(dn)	NGC	2	2	F	70	NONE	Died (2 months)
21	DLBCL(dn)	GCB	1	3	M	73	CHOP-R	PR (23 months)
22	DLBCL(dn)	GCB	3	1	M	53	CHOP-R	PR (24 months)
23	DLBCL(dn)	NGC	4	4	F	68	CHOP-R	Died (2 weeks)
24	DLBCL(dn)	NGC	1	1	F	62	CHOP-R	CR (11 months)
25	DLBCL(dn)	GCB	2	2	F	74	CHOP-R	Died (6 months)
26	DLBCL(dn)	GCB	2	2	F	62	CHOP-R	PR (29 months)
27	DLBCL(dn)	GCB	1/2	2	M	>60	CHOP-R	CR (28 months)
28	DLBCL(dn)	GCB	1	2	M	>60	CHOP-R	CR (26 months)
29	DLBCL(dn)	GCB	3	4	F	71	CHOP-R	Died (7 months)
30	DLBCL(dn)	NGC	3	3	M	62	CHOP-R	CR (24 months)
31	DLBCL(dn)	NGC	1	1	M	63	CHOP-R	CR (23 months)
32	DLBCL(dn)	GCB	1	3	M	75	CNOP-R	CR (23 months)
33	DLBCL(dn)	GCB	3	3	M	46	CHOP-R	CR (22 months)
34	DLBCL(dn)	NGC	2	1	M	61	CHOP-R	CRU (21 months)
35	DLBCL(dn)	GCB	3	2	M	45	CODOX-M + CHOP + MTX + RX + IVAC + R + RICE + ESHAP	PR (15 months)
36	DLBCL(dn)	NGC	4	2	M	58	CHOP-R + RICE + BEAM + TX	PR (15 months)
37	DLBCL (t)	ND	1	2	M	59	CHOP-R + RX	CR (22 months)
38	DLBCL (t)	ND	3	2/3	M	71	PMitCEBO + PRED + RX + VIN	CR (12 months)
39	DLBCL (t)	ND	4	2	F	39	CHOP-R	Died (6 months)
40	DLBCL (t)	ND			M	64	CHOP-R	Died (4 months)
41	DLBCL (t)	ND	4	4	F	60	CHOP-R + CNOP-R	CR (29 months)
42	DLBCL (t)	ND	2	1	F	54	CNOP-R	PR (5 months)
43	DLBCL (t)	ND	4	2	F	60	CHOP-R + RX	CR (24 months)
44	DLBCL (t)	ND	1	0	M	56	CHOP-R + MTX ESHAP + BEAM + TX	CR (24 months)
45	DLBCL (t)	ND	2	3	M	65	CHOP-R	CR (21 months)
46	DLBCL (t)	ND	4	4	F	47	CHOP-R	CR (19 months)
47	T-cell rich	ND	3	2	F	80	PMITCEBO-R TO MARCH 06	CR (12 months)
48	T-cell rich	ND	2	0	M	51	CHOP-R	CR (18 months)
49	T-cell rich	ND	3	4	M	74	CHOP-R	PR (18 months)
50	T-cell rich	ND	4	4	F	39	CODOX + IVAC + MTX + R	CR (27 months)

*Subtyped according to expression of CD10, BCL-6 and MUM1 according to Hans *et al* (2004).
†Sample at relapse.
IPI, International Prognostic Index; DLBCL(dn), diffuse large B cell lymphoma *de novo*; DLBCL (t), diffuse large B-cell lymphoma transformed; T-cell rich, T-cell rich B cell lymphoma; GCB, germinal centre derived; NGC, non-germinal centre-derived; CHOP-R, cyclophosphamide, doxorubicin, vincristine, prednisolone, Rituximab; MTX, intrathecal methotrexate; RX, radiotherapy; PRED, prednisolone; VIN, vinblastine; RICE, rituximab, ifosfamide, carbplatin, etoposide; ESHAP, etoposide, methyprednisolone, cytarabine, cisplatin; TX, autologous transplant; CODOX-M, cyclophosphamide, vincristine, doxorubicin, methotrexate; BEAM–BCNU, (bis-chloro-ethyl nitrosourea), Etoposide, cytarabine, melphalan; CNOP-R, cyclophosphamide, mitoxantrone, vincristine, prednisolone, rituximab; PMitCEBO, prednisolone, mitoxantrone, cyclophosphamide, etoposide, bleomycin, vincristine; CODOX, cyclophosphamide, doxorubicin, vincristine; IVAC, Ifosfamide, etoposide, cytarabine; CR, complete response; PR, partial response; CRU, complete remission unconfirmed.

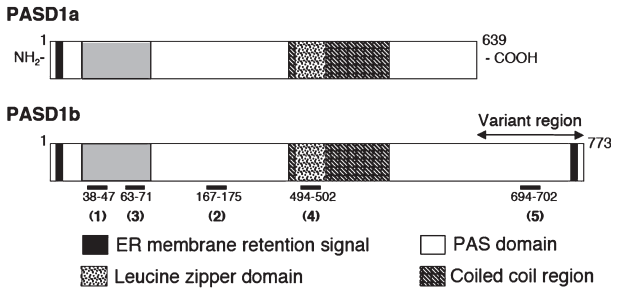

Fig 1. Schematic diagram of the PASD1 protein isoforms. The positions of the PASD1 peptides are shown: 1 = PASD1(1); 2 = PASD1(3); 3 = PASD1(2); 4 = PASD1(4); 5 = PASD1(5).

Preparation and culture of PBMCs

Peripheral blood mononuclear cells (PBMCs) were prepared in RPMI 1640 medium containing 10% fetal calf serum (FCS) (RPMI 1640/FCS; Invitrogen Ltd) as described previously (Ait-Tahar *et al*, 2006). PBMCs ($0{\cdot}5 \times 10^5$) in 200 μl of RPMI 1640/FCS were added to each well of a 96-well round-bottomed plate and incubated for 8–10 d with 1–10 μmol of one of the following: the PASD1(1), PASD1(2), PASD1(3), PASD1(4), PASD1(5) or the control HIV peptides (10 μl), 10 μg/ml phytohaemagglutinin (PHA; Sigma-Aldrich Co. Ltd, Dorset, UK) or tissue culture media only. Recombinant interleukin-2 (rIL-2: 20 iu/ml; Roche Diagnostics, Indianapolis, IN, USA) and rIL-7 (25 ng/ml; R&D Systems, Minneapolis, MN, USA) were added on days 2, 5 and 7.

Enzyme-linked immunosorbent spot (ELISPOT) assay

After 8–10 d of culture, cells were washed and incubated for 18 h with RPMI 1640/FCS at 37°C in 5% CO_2 with one of the PASD1 peptides, HIV control peptide, PHA or medium only. Peptides were used at 10 μmol and all cultures were carried out in triplicate. γ-interferon (IFN) release assays were performed according to manufacturer's instructions (Mabtech, Stockholm, Sweden). Spots were counted using an automated ELISPOT reader (Autimmun-Diagnostika, Strasberg, Germany). Results were considered positive if the number of spots in the test wells were at least twice those present in the control cultures (media only or containing the irrelevant HIV-1 peptide) and assays were excluded if there were more than 25 spots per well in the absence of peptides.

Generation of CTL lines

PBMCs (2×10^6) were cultured in RPMI 1640/FCS containing 10 μmol of the appropriate PASD1 peptides. After 72 h, an equal volume of RPMI 1640/FCS containing 50 i/u of rIL-2 per ml was added. Half of the medium was removed and replaced with fresh medium every 3 d. The cells were restimulated weekly for 6 weeks with PASD1 peptides before being used in an ELISPOT assay. Cells were used to prepare cytocentrifuge preparations for immunophenotyping. In some experiments, CD8-positive T-cells were enriched from the CTL lines using magnetic beads coated with anti-human CD8 antibody according to the manufacturer's instructions (Dynabeads, Dynal, Oslo, Norway) before assay. In other experiments, the anti-HLA-A*0201 antibody (BB7.2) was added at a concentration of 10 μg/ml to block γ-IFN release. The remaining cells were tested in a cytolytic assay.

Cytolytic assays

A conventional ^{51}Cr-labelling release assay was used to investigate the ability of CTL lines generated from DLBCL patients to lyse PASD1-positive tumour target cells. The target cell lines, consisting of the OCI-Ly3, SUDHL-6, KM-H2 and Thiel, were radiolabelled with 3·7 MBq ^{51}Cr for 90 min. After washing, the target cells were added to the CTL lines (at effector:target ratios of 3:1, 5:1 and 10:1) in 96-well microplates and incubated for 4 h at 37°C in a humidified atmosphere in 5% CO_2. Maximum ^{51}Cr release was determined following the addition of 10% Triton-X to the radiolabelled target cells and spontaneous release was assessed by adding RPMI 1640/FCS to the target cells. The supernatant was harvested and counted in a gamma-counter (Beckmann, Heidelberg, Germany). The percentage of specific lysis was calculated as follows:

$$\frac{(\text{experimental cpm} - \text{spontaneous cpm})}{(\text{maximum cpm} - \text{spontaneous cpm})} \times 100.$$

Immunoperoxidase labelling studies

Paraffin-embedded tissue sections were de-waxed and antigen retrieval was performed using 50 mmol Tris: 2 mmol EDTA at pH 9·0 as previously described (Cooper *et al*, 2006). PASD1 protein expression was detected following overnight incubation of the tissue sections with the antibodies PASD1-1 (diluted 1:50) and PASD1-2 (diluted 1:25) and labelling with the Mach-Three detection kit following the manufacturer's instructions. Subtyping of the DLBCL cases was performed using antibodies to MUM1, BCL6 and CD10 and the Envision detection system. Cases were identified as being of germinal centre (GCB) or non-germinal centre (NGC) origin according to Hans *et al* (2004).

Immunofluorescent labelling study

Cytocentrifuge preparations were fixed in acetone for 10 min, air-dried and incubated for 30 min with a mixture of either (i) rabbit anti-CD3 and one of the mouse monoclonal antibodies (anti-CD45RO, anti-CD8 or anti-CD56) or (ii) a combination of two mouse monoclonal antibodies: anti-CD8 (isotype IgG1) and anti-CD45RO (isotype IgG2a) or anti-CD56 (isotype IgG1) and anti-CD45RO (isotype IgG2a). After washing in phosphate-buffered saline, the slides were incubated for a further 30 min with a mixture of either FITC-goat anti-rabbit Ig and a goat isotype specific anti-mouse IgG conjugated to TexasRed™ or,

where two primary monoclonal antibodies had been used, a mixture of anti-mouse IgG isotype specific antibodies conjugated to either FITC or TexasRed™. The slides were then washed and mounted in an anti-fade mountant Vectorshield containing 4′,6-diamine-2′phenylindole dihydrochloride (Vector Laboratories, Peterborough, UK). Results were then visualized on a Zeiss Axioskop microscope (Hamaatsu, Hamatsu City, Japan) using Improvision Openlab software (Improvision, Warwick, Warwickshire, UK).

Statistical analysis

The student's *t*-test was used to analyse the results obtained in the ELISPOT and cytolytic assays. P values $< 0{\cdot}05$ were considered significant.

Results

γ-IFN release assay

The results of the γ-IFN response ELISPOT assay are summarized in Table II. A significant γ-IFN response was detected in 21/29 (72%) HLA-A*0201-positive DLBCL patients after short-term culture with PASD1 peptides compared to those results obtained from the control cultures (cells stimulated with the irrelevant HIV peptide or medium only, $P < 0{\cdot}05$). Of these, 18 patients had *de novo* DLBCL of which two patients had relapsed DLBCL, two patients developed DLBCL via transformation of their FL and one patient had T-cell rich B-cell lymphoma. Thirteen patients responded to two or more peptides and, of these, two patients responded to all five PASD1 peptides, one patient to four peptides and six patients to three peptides. The frequencies of PASD1-responding T cells varied between patients, ranging from 1:600 (0·17%) PBMCs in Patient 1 to 1:2000 (0·05%) in Patient 2. No significant γ-IFN responses were obtained from the HLA-A*0201-negative patients with either *de novo* ($n = 12$), transformed ($n = 8$) DLBCL or T-cell rich B-cell lymphoma ($n = 1$). Results are shown in Table SI. Furthermore, none of the PBMCs obtained from the four HLA-A*0201-positive and 2 HLA-A*0201-negative healthy subjects recognized the PASD1 peptides. Although only relatively small numbers of patients were studied, it is notable that of those patients who were able to recognize the PASD1 peptides, 12 achieved complete remission, six are currently in partial remission while three patients have died. This is in contrast to the situation with the eight HLA-A*0201-positive patients who were unable to recognize PASD1 peptides where only one achieved complete remission, three are in partial remission and four have died during the course of this study.

The results from the γ-IFN release assay permitted the PASD1 peptides to be listed in the following order of immunogenicity, namely, PASD1(1), PASD1(2), PASD1(5), PASD1(3) and PASD1(4) with PASD1(1) being the most immunogenic. Subsequent studies on the CTL response therefore focused on the more immunogenic PASD1(1) and PASD1(2) peptides present in both PASD1a and PASD1b proteins and the PASD1(5) peptide limited to the C-terminus of PASD1b.

Persistence of the γ-IFN response to PASD1

Blood was collected from three HLA-A*0201-positive patients, two with *de novo* DLBCL (Patients 1 and 12) and one patient with T-cell rich B cell lymphoma (Patient 47) on their return to clinic 1 year after initial diagnosis. A significant γ-IFN response to PASD1 peptides following short-term culture was still detected in all three patients after 1 year. Results from two patients are shown in Fig 2A. This response suggested the persistence of a pool of memory CD8-positive T cells to the PASD1 protein.

Patient 3, who initially exhibited a γ-IFN response to four PASD1 peptides, died 22 months after diagnosis. It is of note that no significant γ-IFN response to the PASD1 peptides was detected in peripheral blood collected from this patient at the time of relapse.

Generation of CTL lines specific for PASD1 peptides.

PASD1-stimulated PBMCs from four HLA-A*0201-positive patients (three with *de novo* DLBCL and one with T-cell rich DLBCL) were maintained in long-term culture to permit further analysis of their functional activity. PBMCs were re-stimulated weekly with rIL-2 and with one of the following: PASD1(1), PASD1(2), or PASD1(5) or the irrelevant HIV peptide. After 6 weeks, the cell lines were tested for their γ-IFN secreting activity to the PASD1 and control peptides in an overnight ELISPOT assay. The CTL cell lines demonstrated a γ-IFN response to the PASD1 peptides that was abrogated by the removal of CD8-positive T cells or the addition of the anti-HLA-A*0201 monoclonal antibody BB7.2 (Fig 2B). These results confirm the CD8-positive MHC Class I restricted nature of the response. Immunophenotyping studies showed 90 ± 3% of the cell lines to be CD3-positive, of which 79 ± 7% were CD8-positive, 37 ± 7% of the CD8-positive cells were also CD45RO-positive. Less than 0·5% of the cells were CD56-positive.

Cytolytic activity of the CTL lines

The abilities of the CD8-positive CTL lines specific for PASD1(1), PASD1(2) and PASD1(5) to recognize and lyse tumour cells expressing endogenous PASD1 protein were tested in a standard ^{51}Cr release assay. CTL lines from four patients raised against the PASD1(1), PASD1(2) and PASD1(5) peptides demonstrated a dose-dependent lysis of the HLA-A*0201-positive PASD1-positive Thiel cell line but not the HLA-A*0201-positive PASD1-negative (SUDHL-6) or the HLA-A*0201-negative PASD1-positive (OCI-Ly3 and KM-H2) cell lines. The cytolytic effect was significant even at an

Table II. Summary of the γ-IFN release by DLBCL patients to PASD1 peptides predicted to be immunogenic in the context of HLA-A*0201.

Patient	Diagnosis	Sub-type	HLA-A*0201 status	Reactivity with antibody PASD1-1	Reactivity with antibody PASD1-2	γ-IFN response to peptides per 50 000 cells							
						PASD1(1)	PASD1(2)	PASD1(3)	PASD1(4)	PASD1(5)	No peptide	HIV-1	PHA
Significant response													
1	DLBCL(dn)	NGC	+	50% ±	−	68 ± 8	76 ± 12	84 ± 14	56 ± 12	64 ± 10	12 ± 2	14 ± 4	96 ± 18
2	DLBCL(dn)	GCB	+	−	−	34 ± 2	38 ± 4	24 ± 4	30 ± 4	44 ± 8	8 ± 2	8 ± 2	72 ± 12
3	DLBCL(dn)	GCB	+	++	±*	44 ± 2	38 ± 6	24 ± 4	30 ± 4	44 ± 8	8 ± 2	8 ± 2	72 ± 12
4	DLBCL(dn)	NGC	+	+ <50%*†	−	54 ± 6	48 ± 4	42 ± 6	38 ± 8	12 ± 4	10 ± 2	14 ± 6	52 ± 12
5	DLBCL(dn)	GCB	+	NA	NA	44 ± 2	38 ± 4	24 ± 4	30 ± 4	42 ± 6	10 ± 2	12 ± 2	82 ± 12
6	DLBCL(dn)	NGC	+	NA	NA	40 ± 4	34 ± 8	38 ± 4	52 ± 6	28 ± 4	14 ± 4	12 ± 2	78 ± 18
7	DLBCL(dn)	GCB	+	NA	NA	34 ± 4	28 ± 4	42 ± 6	36 ± 4	28 ± 4	10 ± 2	12 ± 2	54 ± 8
8	DLBCL(dn)	GCB	+	±*	*	46 ± 12	24 ± 4	36 ± 6	16 ± 4	18 ± 4	10 ± 2	12 ± 2	58 ± 10
9	DLBCL(dn)	NGC	+	±*	±*	34 ± 4	38 ± 4	18 ± 2	30 ± 6	28 ± 4	12 ± 2	10 ± 2	42 ± 10
10	DLBCL(dn)	GCB	+	‡*	±*	30 ± 4	28 ± 2	32 ± 2	18 ± 4	22 ± 4	8 ± 2	10 ± 4	48 ± 10
11	DLBCL(dn)	GCB	+	+	±	54 ± 8	32 ± 6	38 ± 2	30 ± 4	28 ± 2	12 ± 2	14 ± 4	86 ± 12
12	DLBCL(dn)	GCB	+	+	*	25 ± 16	22 ± 2	26 ± 4	28 ± 2	32 ± 4	10 ± 4	12 ± 2	86 ± 10
13	DLBCL(dn)	GCB	+	−	−	30 ± 4	26 ± 4	22 ± 6	26 ± 4	38 ± 4	8 ± 2	14 ± 2	74 ± 8
14	DLBCL(dn)	NGC	+	−	±	52 ± 8	28 ± 2	32 ± 8	34 ± 8	36 ± 2	14 ± 4	16 ± 2	108 ± 14
15	DLBCL(dn)	NGC	+	+*	*	18 ± 4	24 ± 6	22 ± 2	32 ± 4	44 ± 6	12 ± 2	16 ± 4	128 ± 24
16	DLBCL(dn)	GCB	+	NA	NA	12 ± 2	14 ± 4	22 ± 4	26 ± 2	36 ± 4	10 ± 2	12 ± 4	58 ± 6
17	DLBCL(dn)	GCB	+	−	−	36 ± 6	30 ± 4	34 ± 6	44 ± 2	28 ± 2	10 ± 2	14 ± 2	86 ± 8
18	DLBCL(dn)	NGC	+	‡+	−	36 ± 6	42 ± 4	28 ± 4	20 ± 4	32 ± 8	16 ± 4	12 ± 4	54 ± 8
37	DLBCL (t)	−	+	±*	*	56 ± 12	48 ± 8	36 ± 6	12 ± 2	26 ± 4	10 ± 2	12 ± 4	108 ± 16
38	DLBCL (t)	−	+	NA	NA	38 ± 2	24 ± 4	18 ± 2	28 ± 6	26 ± 4	12 ± 2	14 ± 2	100 ± 12
47	T-cell rich	−	+	++†	±*	48 ± 8	56 ± 12	36 ± 8	62 ± 12	22 ± 4	12 ± 4	10 ± 4	92 ± 18
No significant response													
19	DLBCL(dn)	GCB	+	−	−	32 ± 4	20 ± 6	22 ± 6	28 ± 2	ND	12 ± 4	8 ± 6	44 ± 8
20	DLBCL(dn)	NGC	+	−	−	28 ± 4	34 ± 10	18 ± 2	38 ± 8	22 ± 6	14 ± 2	10 ± 4	54 ± 12
21	DLBCL(dn)	GCB	+	−	−	20 ± 4	16 ± 2	18 ± 2	22 ± 4	12 ± 2	8 ± 2	10 ± 2	38 ± 6
22	DLBCL(dn)	GCB	+	+*	*	32 ± 8	34 ± 6	18 ± 4	28 ± 4	16 ± 2	12 ± 2	14 ± 4	76 ± 16
23	DLBCL(dn)	NGC	+	+	−	18 ± 2	12 ± 4	16 ± 2	10 ± 4	12 ± 2	14 ± 2	10 ± 2	36 ± 4
24	DLBCL(dn)	NGC	+	+	−	26 ± 4	24 ± 2	18 ± 2	16 ± 4	22 ± 4	8 ± 2	10 ± 4	52 ± 8
39	DLBCL (t)	−	+	−	−	28 ± 4	22 ± 2	12 ± 2	8 ± 4	18 ± 4	12 ± 2	14 ± 4	42 ± 8
49	T-cell rich	ND	+	±§	−	28 ± 4	28 ± 4	30 ± 4	10 ± 2	16 ± 1	12 ± 2	10 ± 4	94 ± 12
HD 1		−	+			22 ± 4	18 ± 2	20 ± 2	14 ± 4	16 ± 2	12 ± 2	14 ± 6	48 ± 8
HD 2		−	+			18 ± 2	10 ± 2	16 ± 2	12 ± 2	22 ± 4	8 ± 2	10 ± 2	62 ± 14
HD 3		−	+			8 ± 1	6 ± 2	10 ± 2	12 ± 2	12 ± 2	8 ± 2	10 ± 2	78 ± 10
HD 4		−	+			22 ± 0	12 ± 0	14 ± 1	6 ± 2	12 ± 2	8 ± 2	10 ± 2	112 ± 14
HD 5		−	−			18 ± 2	14 ± 1	10 ± 1	16 ± 2	18 ± 2	10 ± 1	8 ± 2	56 ± 10

Shading of figures denotes significant γ-IFN response. The results ± are from triplicate ELISPOT cultures. The SD was calculated using standard techniques.
±, + and ++ denotes intensity of cytoplasmic labelling.
*Denotes nuclear labelling from 5–30% of tumour cells.
†Denotes labelling of some smaller lymphocytes and vessels in tumour.
‡Biopsy taken at time of relapse.
§Dependent on biopsy.
DLBCL(dn), diffuse large B cell lymphoma *de novo*; DLBCL (t), diffuse large B cell lymphoma transformed; T-cell rich, T-cell rich B cell lymphoma; GCB, germinal centre derived; NGC, non-germinal centre-derived; HD, healthy donor; NA, tissue not available; PHA, phytohaemaglutinin.

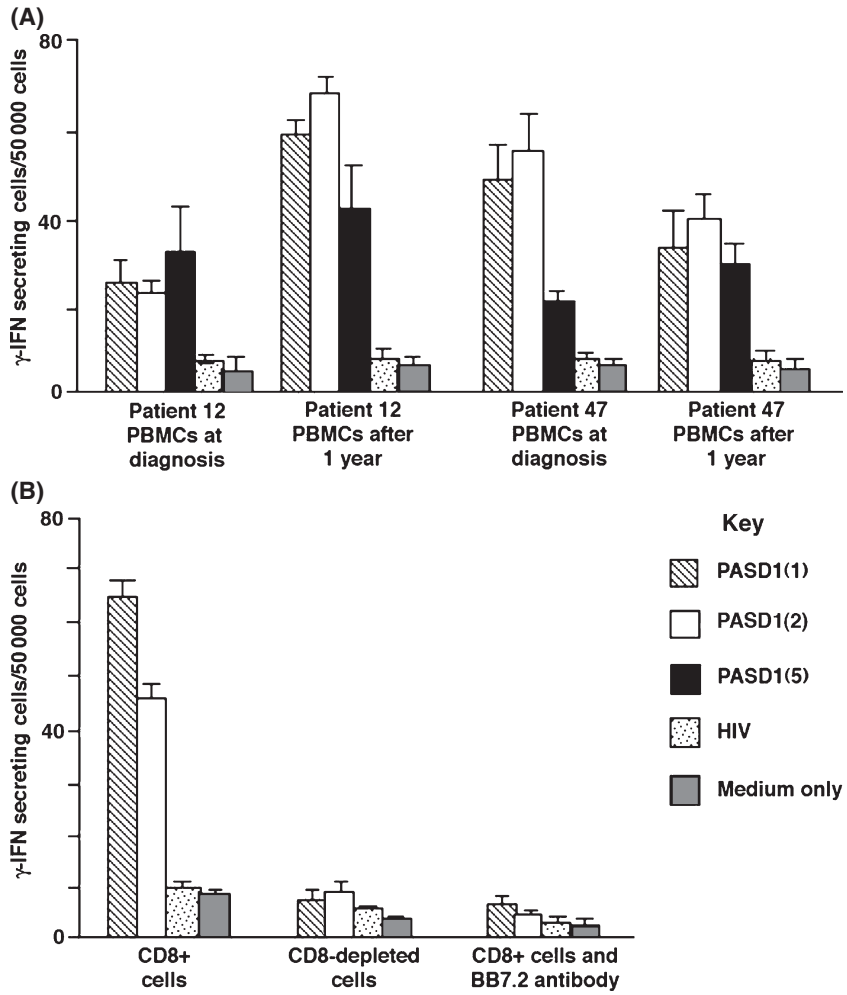

Fig 2. γ-IFN responses of patients with *de novo* DLBCL (Patient 12) and T-cell rich lymphoma (Patient 47) to PASD1 peptides. In (A) peripheral blood mononuclear cells (PBMCs) obtained from Patients 12 and 47 at time of diagnosis and after 1 year from start of treatment were maintained in short term culture. A significant γ-IFN response to peptides PASD1(1), PASD1(2) and PASD1(5) was observed in cells from both patients obtained at time of diagnosis and after 1 year ($P < 0{\cdot}05$). No significant response was detected in cultures stimulated by the HIV peptide or containing medium only. (B) CTL cell lines (generated from Patient 12) after 6 weeks of culture were either enriched for CD8-positive cells using anti-CD8 antibody-coated magnetic beads or incubated with an anti-HLA-A*0201 monoclonal antibody (BB7.2). A significant γ-IFN response was observed only in the culture containing the CD8-positive cells in the absence of anti-MHC Class I ($P < 0{\cdot}05$). No significant responses were detected in the control cultures or the irrelevant peptides. The results are the mean ± SD and were obtained from triplicate ELISPOT cultures.

effector:target ratio of 5:1 using cells stimulated with PASD1(1) peptide in all four patients. The results obtained from three patients are shown in Fig 3.

Immunoperoxidase labelling of DLBCL

Results obtained from the DLBCL subtyping and PASD1 immunolabelling studies of tumour biopsies from patients are summarized in Table II. Tissue sections from diagnostic biopsies were available for 16 of the patients who mounted a significant γ-IFN response to PASD1 peptides. Labelling with one or both of the PASD1 antibodies was detected in the tumour cells of 13 of these patients. Examples of results are shown in Fig 4. Moderate to strong labelling of the cytoplasm of the tumour cells was observed in eight patients using antibody PASD1-1 (recognizing an epitope common to both PASD1 isoforms), while weaker labelling was present in four other cases. Nuclear labelling of a small number of tumour cells was also seen in biopsies from six patients using this reagent. Antibody PASD1-2 (recognizing the unique region of PASD1b) stained either a subpopulation of nuclei or weakly labelled the cytoplasm of the tumour cells in 10 cases of DLBCL. Labelling of the tumour cells using the PASD1-1 and PASD1-2 antibodies was also observed in 11/24 of the HLA-A*0201-positive or HLA-A*0201-negative patients studied whose cells did not mount a γ-IFN response. In addition to the tumour cells, occasional smaller lymphoid cells and vessels were also labelled by antibody PASD1-1 in a case of *de novo* DLBCL and a case of T-cell rich DLBCL.

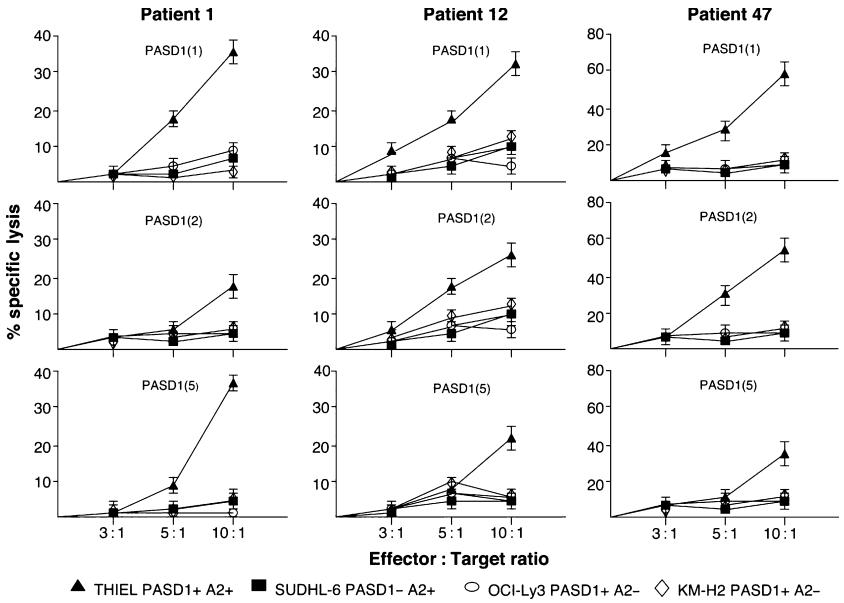

Fig 3. Cytolytic activity of the PASD1-specific CTL cell lines derived from patients with DLBCL. The functional activity of CTL lines obtained from Patients 1, 12 (*de novo* DLBCL) and Patient 47 (T-cell rich B-cell lymphoma) were studied in a conventional ^{51}Cr release assay on a range of haematological cell lines. Significant dose-dependent lysis of the HLA-A*0201-positive PASD1-positive Thiel (myeloma) cell line was observed by cells from all three patients. In contrast no significant lysis was observed of the SUDHL-6 (DLBCL; HLA-A*0201-positive but PASD1-negative) or the OCI-Ly3 (DLBCL) and KM-H2 (HL; HLA-A*0201-negative but PASD1-positive) cell lines. Results are the mean ± SD from triplicate cultures.

Discussion

We previously identified PASD1 as a novel DLBCL-associated CTA using the SEREX technique (Liggins *et al*, 2004a,b). This approach, which relies upon the presence of a co-ordinated cellular and humoral response, has been used to identify immunogenic CTAs and other molecules that represent potential immunotherapeutic targets (Preuss *et al*, 2002; Scanlan *et al*, 2004). PASD1, encoded by the gene *PASD1* on Xq28, is a member of the CT-X group of CTAs (Scanlan *et al*, 2004). Its restricted distribution in normal tissue but expression in a variety of haematological malignancies highlighted PASD1 as a potential immunotherapeutic target in both DLBCL and other haematological malignancies (Cooper *et al*, 2006; Sahota *et al*, 2006). This was of particular importance given previous reports of the paucity of CTA expression in B-cell lymphomas (Huang *et al*, 2002; Xie *et al*, 2003). The potential of PASD1 as an immunotherapeutic target was further supported by a study reporting that PASD1 not only represented a SEREX antigen in acute myeloid leukaemia but also that PASD1 mRNA elicited a proliferative CD4-positive T-cell response in normal subjects (Guinn *et al*, 2005). The current paper describes the presence of circulating functional CTLs to PASD1 in DLBCL patients thus providing the first experimental validation of PASD1 as a potential vaccine candidate that is recognized by a T-cell response in patients with B-cell tumours.

Cytotoxic T cells recognizing PASD1 peptides were detected after short-term culture using the functional ELISPOT technique in 72% of HLA-A*0201-positive DLBCL patients; a result suggestive of the presence of circulating PASD1-specific cells in these patients. Such spontaneous immunity to CTAs, including NY-ESO-1 and MAGE-A3, has been previously reported in multiple myeloma (Goodyear *et al*, 2005; van Rhee *et al*, 2005). The percentage of T cells recognizing PASD1 after short-term culture varied from 0·17% to 0·05%, thus comparing favourably with the results obtained for NY-ESO-1, MAGE-A(1–4), MAGE-A3, LAGE-1 and NY-ESO-1 in haematological and non-haematological malignancies (Goodyear *et al*, 2005; van Rhee *et al*, 2005; Inokuma *et al*, 2007). The presence of CTLs recognizing CTAs has also been reported in patients following allogeneic transplantation (Atanackovic *et al*, 2007) and provides additional support for the use of CTAs as immunotherapeutic targets.

Correlations have been reported between antibody responses and prognosis in myeloma (Goodyear *et al*, 2005; van Rhee *et al*, 2005). We previously detected autoantibody responses to PASD1 only in patients with poor prognosis DLBCL (identified through immunolabelling techniques) (Liggins *et al*, 2004a,b). In the present study, however, a γ-IFN response to PASD1 peptides was detected in 10 patients with GCB-derived DLBCL in addition to the nine patients with poor-prognosis DLBCL (six with NGC-derived DLBCL, two patients with transformed DLBCL and one with T-cell rich B-cell lymphoma). These results suggest that PASD1 may be applicable as a therapeutic target regardless of DLBCL subtype. It is of interest that T-cell rich B-cell lymphoma, representing a variant of DLBCL with an aggressive outcome (El Weshi *et al*, 2007; Stein *et al*, 2008) is characterized by the presence of

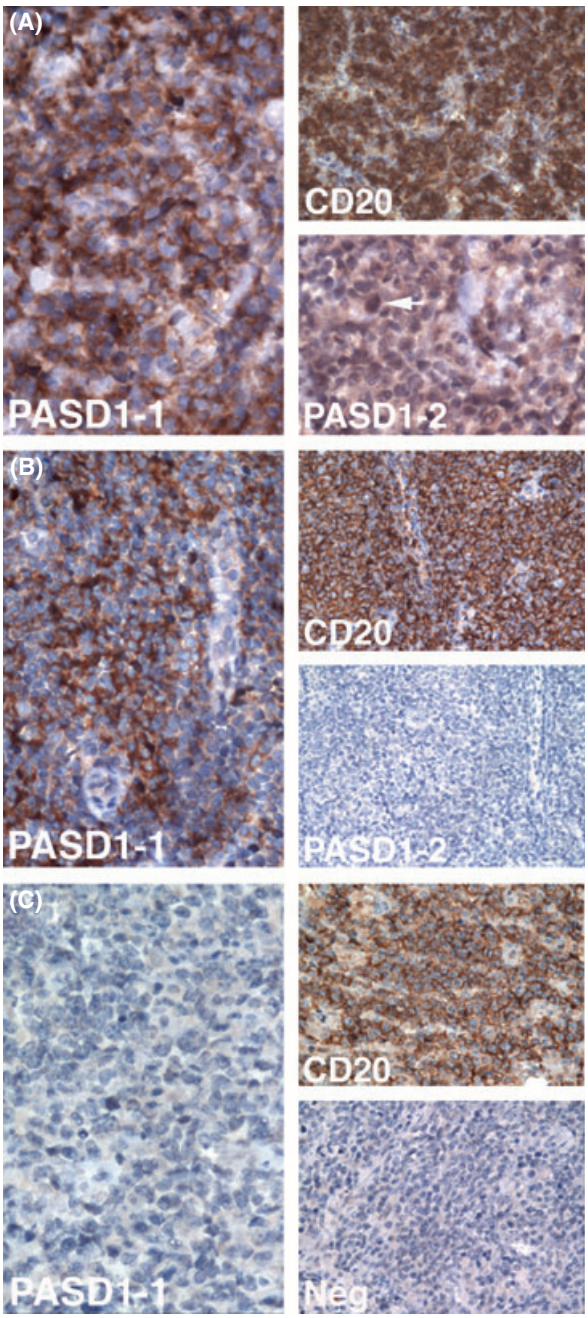

Fig 4. Immunoperoxidase labelling studies of biopsy sections from patients with *de novo* DLBCL. (A) Antibody PASD1-1 strongly stains the cytoplasm of tumour cells from HLA-A*0201-positive Patient 3, whose PBMCs exhibited a significant γ-IFN response to PASD1 peptides. Antibody PASD1-2 stained a subpopulation of nuclei (arrowed) as well as cytoplasm of the tumour cells. (B) and (C) show the immunolabelling results obtained from two HLA-A*0201-negative patients in whom no PASD1 T-cell response was detected. Whereas the tumour cells from Patient 27 were labelled strongly with antibody PASD1-1 (B), no labelling was detected with antibody PASD1-2. Neither of the antibodies PASD1-1 (C) or PASD1-2 (not shown) stained the tumour cells of Patient 17. The sections were viewed using a bright field microscope (Axioskop; Zeiss, Welwyn Garden City, UK) fitted with a 20× or 40× lens. The images were captured using a Micropublisher 5MP TRV camera (QImaging, Surrey, UK).

infiltrating inflammatory cells suggestive of a 'host response' to the tumour (Abramson, 2007).

A study of sequential blood samples from three DLBCL patients in the present paper demonstrated a persistent CTL response to PASD1 peptides a year after diagnosis. All three DLBCL patients remained in remission by the end of this study. Sustained CTL responses to TAAs have been reported in myeloma (Goodyear *et al*, 2005) and anaplastic large cell lymphoma (Ait-Tahar *et al*, 2006; Passoni *et al*, 2006). The persistence of these T cell responses suggests the presence of memory T cells, which might be involved in protective immunity and which also represent potential populations of T cells that could be further stimulated following vaccination (Baumgaertner *et al*, 2006).

As PASD1 constitutes a potential immunotherapeutic target, it is important to correlate the presence of a γ-IFN response to the expression of PASD1 in tumours. van Rhee *et al* (2005) and Goodyear *et al* (2005) were able to confirm NY-ESO-1 and MAGE protein expression in those myeloma patients who mounted a CTL response to these CTAs. Immunohistochemical labelling with anti-PASD1 monoclonal antibodies confirmed PASD1 expression in the majority (12/15) of the patients who possessed circulating T cells recognizing PASD1 peptides. As previously described (Cooper *et al*, 2006), variations in the labelling patterns of the tumour cells given by the two antibodies PASD1-1 and PASD1-2 were observed, providing support for the differential expression of PASD1 isoforms in the tumour cells. Furthermore, heterogeneity of labelling was observed within a tumour. Intratumoural variation of CTA expression has been previously described in solid tumours (Scanlan *et al*, 2004; Barrow *et al*, 2006; Theurillat *et al*, 2007) and in myeloma (Dhodapkar *et al*, 2003). Possible explanations for such heterogeneity include the presence of CTA isoforms (Nakagawa *et al*, 2005), epigenetic phenomena such as hypermethylation (Simpson *et al*, 2005; Inokuma *et al*, 2007) and post-translational modifications. Increased expression of CTAs has also been linked to the aggressiveness of the tumours (Dhodapkar *et al*, 2003; van Rhee *et al*, 2005; Barrow *et al*, 2006).

Heterogeneity in PASD1 expression may also explain the absence of labelling by both of the antibodies to PASD1 in the three cases of DLBCL in which a CTL response was detected. Discrepancies in NY-ESO-1 expression have been linked to the size of the tissue sections studied indicating that CTA expression can vary in different regions of the tumour (Theurillat *et al*, 2007). Needle biopsy sections were available for two of these DLBCL cases and it is possible that PASD1-positive regions of tumour were absent in the tissue sections studied. It is also possible that immunolabelling may not constitute a sufficiently sensitive technique to identify low levels of PASD1 protein expression. This was found to be the case in a study on CTA expression in haematological malignancies (A.P. Liggins, S. Lim, K. Pulford & A.H. Banham, unpublished observations) and in breast tumours where Western blotting, rather than immunolabelling techniques,

was necessary to confirm CTA expression in the tumours (Sugita *et al*, 2004). It has also been reported that it is the turnover rate, rather than the protein levels of a TAA in tumour cells, that may be important for CTL recognition (Vierboom *et al*, 2000).

Although *PASD1* transcripts and PASD1 proteins were undetectable in normal non-reproductive tissues (apart from a few salivary gland nuclei stained by antibody PASD1-2) in previous studies (Liggins *et al*, 2004a; Guinn *et al*, 2005; Cooper *et al*, 2006), *PASD1* mRNA was detected in the histologically normal tissues present in a matched tumour/normal expression array (Liggins *et al*, 2004a). It is possible that PASD1 expression in these normal tissues could be due to early genetic changes occurring in the cells before morphological abnormalities become obvious. Such a situation may explain the labelling of scattered small lymphoid cells detected in two of the PASD1-positive patients who responded to the PASD1 peptides in the current paper. It is also noteworthy that CTA protein expression has been reported in benign hyperplastic prostate tissue (Hudolin *et al*, 2006).

A γ-IFN response to the PASD1 peptides was not detected in those patients who were HLA-A*0201-negative even when PASD1 protein was detected in their tumour cells. These results, combined with the abrogation of the γ-IFN response through depletion of CD8-positive cells or the addition of an anti-MHC Class I reagent to CTL lines provides further evidence for an MHC Class I-dependent PASD1 CTL response.

It was possible that the γ-IFN response of the expanded CTL lines investigated here is limited to the recognition of the exogenous PASD1 peptides and that endogenous PASD1 peptides may not be processed appropriately by the tumour cells for CTL recognition (Luckey *et al*, 1998). Using cell lines derived from a range of haematological malignancies, we were able to confirm that the CTL cell lines raised against PASD1(1), PASD(2) and PASD1(5) peptides were able to recognize endogenously expressed PASD1 peptides and lyse PASD1-positive tumour cells in an MHC Class I-dependent manner.

Other studies have described the presence of more than one CTA antigen in solid tumours and in haematological malignancies such as myeloma and plasmacytoma (Atanackovic *et al*, 2006; Condomines *et al*, 2007). The presence of more than one CTA within a tumour, combined with their loss and/or heterogeneity in their protein distribution, provides support for the inclusion of multiple CTAs in vaccine development. This approach should maximize the eradication of the tumour cells while minimizing the escape variants of the tumour.

Given that other studies suggest the importance of the immune microenvironment of the tumour on outcome (Rosenwald *et al*, 2002; Rimsza *et al*, 2004), additional studies will include the investigation of the identities and distribution of immune cells within DLBCL biopsies and their correlation with the immune response of the patient. Further work is also ongoing on the CD4 T-helper response to PASD1, as a number of studies have demonstrated the potential of using peptide epitopes binding to both MHC Class I and Class II to achieve optimal immune responses on vaccination (Zeng, 2001).

This study is the first to define immunogenic PASD1 peptides and describe a CTL response to PASD1 in DLBCL. It is also the first description of a CTL response to a CT-X antigen in DLBCL. The current results support PASD1 as a potential immunotherapeutic target for patients with PASD1-positive DLBCL and other malignancies that express this CTA. Since tumours may express more than one CTA, the inclusion of PASD1 in a polyepitope vaccine should increase the chances of successful treatment of malignancies.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table SI. Summary of the γ-IFN release by HLA-A*0201-negative DLBCL patients to PASD1 peptides predicted to be immunogenic in the context of HLA-A*0201.

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