PASD1: A Promising Target for the Immunotherapy of Haematological Malignancies

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Abstract

In general, there is a lack of good immunotherapy targets within the spectrum of haematological malignancies. However haematopoietic stem cell transplants and continuing antigen discovery have allowed further insight into how further improvements in outcomes for patients might be achieved. Most patients with haematological malignancies can be treated with conventional therapies such as radio- and chemotherapy and will attain first remission. However the removal of residual diseased cells is essential to prevent relapse and its associated high mortality. PASD1 is one of the most tissue restricted cancer-testis (CT) antigens with expression limited to primary spermatagonia in healthy tissue. However, characterisation of PASD1 expression in cancers has been predominantly focussed on haematological malignances where the inappropriate expression of PASD1 was first identified. *PASD1* has one of the highest frequencies of expression of all CT antigens in acute myeloid leukaemia, with some suggestion of its role as a biomarker in diffuse large B-cell lymphoma. Here we describe the characterisation of the function and expression patterns of *PASD1* in cell lines and primary tissues. Development of DNA vaccines targeting PASD1 epitopes demonstrate effective *ex vivo* T-cell responses in terms of IFNγ secretion and tumour cell killing. Of particular note these vaccines have led to the destruction of cells which process and present endogenous PASD1 indicating that effectively primed CTLs could kill PASD1-positive tumour cells.

Keywords: PASD1; Immunotherapy; Cancer; Tumour antigen; Cancer-testis antigen

Abbreviations: AA: Amino-Acid; AML: Acute Myeloid Leukaemia; CT: Cancer-testis; CTL: Cytotoxic T-lymphocyte; DLBCL: Diffuse Large B-cell Lymphoma; IFNγ: Interferon-gamma; MLR: Mixed Lymphocyte Reaction; MM: Multiple Myeloma; PASD1: Per Arnt Sim Domain Containing 1; pMHC: Peptide Major Histocompatibility Complex; RT-PCR: Reverse Transcription Polymerase Chain Reaction; RQ-PCR: Real-time Polymerase Chain Reaction; SEREX: Serological Identification of Antigens by Recombinant Expression Cloning.

Introduction

The quest to identify novel antigens has been the focus of a number of studies over the last four decades [1,2]. Autologous typing had been used to identify some antigens including alpha fetoprotein (in hepatoma and germ cell tumours), carcinoembryonic antigen (CEA) (in gastrointestinal cancers), prostate-specific antigen (in prostate cancer), cancer antigen 125 (CA-125) (in ovarian cancer) and AU (in melanomas)(reviewed in [3,4]). However the technique has limitations, including a requirement that the cells under examination can be cultured. Furthermore, the recognition of antigens by low titre antibodies in patients often prevents their further characterisation. The Boon group developed representational difference analysis and were successful in identifying a number of cancer-testis (CT) antigens from melanoma patients, the MAGE family of CT antigens, and one antigen from renal cancer, RAGE [5,6].

In 1995 Sahin et al. described the use of the serological identification of antigens by recombinant expression cloning (SEREX) to discover antigens in a range of cancer tissues [7,8]. In this technique patient immunoglobulins (IgG) from peripheral blood sera were used to immunoscreen cDNA from tumours, cell lines or normal testis tissues in the form of polypeptides on the surface of the phage. There are over 2,000 tumour antigens detailed in the Cancer Immunome database (http://ludwig-sun5.unil.ch/CancerImmunomeDB/) each identified using the SEREX technique [9]. SEREX was validated by the identification of known antigens such as SSX2 (synovial Sarcoma X 2) [10], mutated p53 [11] and the AKT oncogene [12]. To maximise the chance of finding new CT antigens cDNA libraries made from healthy testis cDNA have been immunoscreened with patient sera. This has led to the identification of a number of CT antigens including cTAGE-1, NY-ESO-1, SSX2 [13,14] and PASD1 [15,16]. One-third of all antigens identified by SEREX were found to be novel and have led to the development of a number of clinical trials, most notably those targeting NY-ESO-1 positive tumour cells [17,18].

Tumour antigens are classified into the following categories: CT, mutational, differentiation, amplified/overexpressed, splice variant and viral antigens [7]. CT antigens show restrictive expression, their presence only in tumours and in testis rendering them very attractive therapeutic targets. The testis is an immunologically protected site i.e. lacking in MHC class I expression, therefore targeting CTAs should not lead to catastrophic auto-immune responses against healthy tissue [19].

The debate now remains as to whether we have found enough tumour antigens and whether there is a need to continue the search for

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more. Supported by the National Cancer Institute, Cheever et al. [20] reported a short-list of antigens which demonstrated properties that would support their further investigation as targets for immunotherapy clinical trials. From a list of 75 antigens identified as the most promising for translational therapies, the authors ranked antigens by criteria such as, but not limited to, therapeutic function, immunogenicity, their role in cancer development, specificity to cancer, expression levels and percent of antigen-positive cells within a cancer type, stem cell expression, number of patients with antigen-positive cancers, number of antigenic epitopes and the cellular location of antigen expression. The aim was to focus funding and research effort on antigens that showed the most potential to make it through to clinical trials and benefit patients. However in some cancers, and most notably haematological malignancies, few of the antigens on this short-list were expressed with a frequency that would justify targeting them in clinical trials.

Today techniques including the Serological identification of antigens by Recombinant Expression cloning (SEREX) [8], Serological Proteome Analysis (SERPA) [21] and peptide elution from MHC for mass spectrometry analysis [22] are commonly used to identify which antigens are important in a given cancer. There are of course pros and cons to each including cost and labour intensity. Most of the focus concerns tumour types which lack suitable targets for immunotherapy and for which conventional or current therapies still do not overcome poor survival rates. An additional major benefit of new antigen identification is that such antigens can provide invaluable insight into disease mechanisms [23], act as biomarkers of disease stage [24] and predict survival rates at disease presentation [25-28].

Here we review the CT antigen, PASD1 and whether it has the hallmarks of an "ideal" cancer antigen which meets the criteria as outlined by Cheever et al. [20]. If so then this would justify the progression of PASD1 into clinical trials.

The *PASD1* Gene

The CT antigen database (http://www.cta.lncc.br) indicates there are more than 250 CT antigens categorised as either CT-X and located on the X-chromosome or located on an autosome [29,30]. Approximately 10% of genes on the X-chromosome appear to be CT antigens [29,31,32]. Antigens on the X chromosome, such as the MAGE antigens, tend to group in families and are usually expressed in the testes in a co-ordinated way [2,31]. However spermatagonia and cancer share a number of features including rapid proliferation and global promoter hypomethylation and as such share expression of a number of CT antigens. Like many CT antigens, the gene encoding *PASD1* also maps to the X chromosome at Xq28 [33], near NY-ESO-1 and LAGE-1.

Two SEREX antigens were found to be encoded by the per ARNT SIM (PAS) domain containing 1 (*PASD1*) gene: OX-TES-1, identified through the immunoscreening of a healthy donor testis cDNA library with diffuse large B-cell lymphoma (DLBCL) [34] and the SEREX antigen GKT-AML20 found through the immunoscreening of a healthy donor testis cDNA library with acute myeloid leukaemia (AML) sera [15].

Although *PASD1* is transcribed from 16 exons [35], the *PASD1_v1* transcript is longer than the *PASD1_v2* transcript due to a retained intron between exons 14 and 15. The presence of a stop signal in the retained intron leads to the translation of a shorter PASD1-1 protein (PASD1a) from *PASD1_v1*. Such retained introns are relatively common in cancer [36,37] and have been described previously in

tumour antigens [38]. The *PASD1_v1* transcript encodes a 639 aa protein product named PASD1a while *PASD1_v2* encodes the PASD1b protein which is 773 aa long, with the first 638 aa being common to both proteins [35].

The PASD1 protein contains two PAS domains in the N-terminal region between aa 32-94 and aa 41-137 [35]. PAS domains are found on proteins which are critical regulators of developmental and metabolic processes, including transcriptional responses to stimuli such as hypoxia and environmental pollutants, mediated by factors including hypoxia inducible factors (HIF-α) and the dioxin (aryl hydrocarbon) receptor (DR) [39]. A family of 23 known members, the PAS domain genes contain a basic helix-loop-helix (bHLH) sequence, and are involved in signal transduction in a wide number of organisms [40]. Incorrect signalling by PAS domains has been associated with diseases including heart arrhythmias [41] as well as myocardial and cerebral ischemia [42].

PASD1 homologues have been identified in 16 additional species including gorilla, cow, chicken and mouse and referred to as GM1141 (Entrez Gene). In mice, *PASD1* shows most similarity to the *CLOCK* gene essential for circadian behaviour. Circadian rhythms are biological processes that show endogenous oscillations which are adjusted to the environment-often natural light levels. Such processes may include daily fluctuating hormone levels or sleeping patterns.

We have previously examined whether PASD1 levels of expression were affected by the cell cycle. Briefly we synchronised K562 cells using either serum starvation (10^5 cells/ml in 0.1% foetal calf serum for 5 days to cause a block at G_0) or using 0.3 mM hydroxyurea for 3 days to block at the G_1/S interface of the cell cycle, as demonstrated by flow cytometry [43]. We detected no significant changes in the number of PASD1 expressing cells within the synchronised population over a 24-hour period (with hourly sampling). However PASD1 expression was only found within a subset(s) of the K562 cell population throughout the period of analysis.

Sub-Cellular Localisation of PASD1 Expression in Healthy and Cancerous Tissue

PASD1 expression in normal tissues was restricted to the nuclei of a subpopulation of spermatogonia [44] and labelling intensity decreased with maturity of the spermatogoa. Both PASD1-1 and PASD1-2 monoclonal antibodies gave comparable labelling of testis tissue.

In a cohort of haematological malignancy derived cell lines, the sub-cellular localisation of PASD1, as determined by immunostaining with monoclonal antibodies, was variable [44] (Table 1). Cooper et al. showed that in the non-germinal centre DLBCL-derived cells OCI-Ly3 [45] PASD1-1 strongly labelled the cell membrane and cytoplasm while there was no staining of these cells with PASD1-2. KM-H2, established from the pleural effusion of a patient with Hodgkin's disease of mixed cellular type [46] and the mantle cell lymphoma (MCL)-derived cell line Granta519 showed nuclear staining with PASD1-1 and PASD1-2 antibodies while strong cytoplasmic labelling with PASD1-2 was also observed in a subpopulation of Granta519 cells. K562 cells derived from a patient with myeloid leukaemia [47] and the Thiel multiple myeloma (MM) cell line all exhibited nuclear staining. The detection of nuclear staining was not unexpected and likely reflects the presence of a nuclear localisation signal in the common region of the PASD1-1 and PASD1-2 proteins. Expression of PASD1 was also demonstrated in the FEDP (ALK-negative anaplastic large-cell lymphoma) cell line.

The antibodies PASD1-1 and PASD1-2 both label the 100 kDa

A.

Cell line name and tissue derived from	Method of analysis	Frequency and subcellular localisation of PASD1 expression	Reference
FEDP (ALK-negative anaplastic large-cell lymphoma)	Immunostaining	PASD1-1, but not PASD1-2, positive	[44]
G361 (melanoma)	Tissue arrays	PASD1 positive	[35]
Granta519 (mantle cell lymphoma cell line)	Immunostaining	Nuclear staining with PASD1-1 and PASD1-2 antibodies, while strong cytoplasmic labelling with PASD1-2 was also observed in a subpopulation of Granta519 cells.	[44]
H1299 (lung cancer)	RQ-PCR Immunostaining	PASD1 positive >99% expression of PASD1-2	[15] [63]
Hn5 (head and neck cancer)	RQ-PCR	PASD1 positive	[15]
Jurkats (T cell leukaemia)	RQ-PCR	PASD1 positive	[15]
K562 (CML)	Immunostaining	17.6 ± 3.6% nuclear staining	[63]
KH-M2 established from the pleural effusion of a patient with Hodgkin's disease of mixed cellular type	Immunostaining	Nuclear staining with PASD1-1 and PASD1-2 antibodies	[44]
KMS-12-BM MM cell line	Immunostaining	Expression of PASD1-1 and PASD 1-2. Expression in >95% nuclei of cells	[48]
MOLP-8 MM cell line			
OCI-LY3 (non-germinal centre diffuse large B-cell lymphoma)	Immunostaining	PASD 1-1 strongly labelled the cell membrane and cytoplasm while there was no staining of these cells with PASD1-2	[44]
Thiel (MM)	Immunostaining	Nuclear staining PASD 1-1 and PASD1-2 staining 5 of 11 multiple myeloma cell lines, of which THIEL and RPMI8226 expressed both PASD1 mRNA and protein.	[49]
RPMI8226 (MM)			[44]
SW480 (colon cancer)	Tissue arrays RQ-PCR Immunostaining and flow cytometry	PASD1 positive Expression of PASD1-2	[15,35,63]

B.

Tissue	Technique	Reference
Expression in 25 of 68 solid tumours	Probing Northern blot arrays	[35]
A range of normal tissues including brain, liver, kidney, placenta, breast, uterus or ovary	RT-PCR and ICC	[15,44]
4 of 12 AML patients, and 1 of 6 CML patients	RT-PCR and RQ-PCR	[15]
Normal testicular tissues expression was only found in the nuclei of a subpopulation of spermatogonia. Labelling intensity decreased with maturity of the spermatogoa.	Immunostaining	[44]
Examples of PASD1-1: 21 of 51 DLBCL, 4 of 9 mantle cell lymphoma, 4 of 15 follicular lymphomas, 4 of 12 Burkitt's lymphoma. PASD1-2: 11 of 52 DLBCL, 2 of 4 MM, 4/10 peripheral T cell lymphoma and a range of other tumour cells from patients with haematological malignancies. 41% overall. PASD1-1 was mostly cytoplasmic and weak nuclear staining in DLBCL and MM. Stronger labelling towards the periphery of the tumour. PASD1-2 was nuclear staining.	Immunostaining	[44]
14 of 16 primary MM samples including 9 presentation and 7 previously treated cases	RQ-PCR	[49]
Two of four primary MM tumour samples	ICC	[49]
PASD1 (22/25) cell lines derived from 21 B- and 4 T-cell malignancies	RT-PCR	[50]
3.4% of 320 newly diagnosed and 264 relapse cases of MM	Microarray using Affymetrix GeneChips	[51]
Not found in 78 basal cell carcinoma	RQ-PCR	[53]

AML: Acute Myeloid Leukaemia; CML: Chronic Myeloid Leukaemia; DLBCL: Diffuse Large B-cell Lymphoma; MM: Multiple Myeloma

Table 1: PASD1 expression in human cells **(A)** cell lines and **(B)** tissues.

PASD1b protein in lysates from Thiel and KM-H2 cells by Western blotting, while the PASD1-1 antibody also identified a 40kDa protein in OCI-Ly3 and FEPD cells. The PASD1-2 antibody did not recognise any proteins in these two cell lines. This 40 kDa protein is smaller than was predicted from the translation of the *PASD1_v1* or *PASD1_v2* transcripts and suggests the presence of an additional novel PASD1 isoform in these cells, not least due to its cytoplasmic expression [44]. In 2010, Joseph-Pietras et al. showed that in addition to a full length PASD1 protein found in Thiel cells there was a higher molecular weight protein labelled by the antibody to PASD1-2 in KMS-12-BM again suggesting the existence of additional variants of PASD1 [48].

Comparison of the Expression of PASD1 with Other CTs

PASD1 mRNA expression was seen to be highest in G361 (melanoma) and SW480 (colorectal adenocarcinoma) of the nine cell lines tested on matched tumour/normal tissue Northern blot arrays

[35]. Using real-time polymerase chain reaction (RQ-PCR), *PASD1* transcripts expression were detected in the K562 (chronic myeloid leukaemia (CML), Jurkats (T-cell leukaemia) and H1299 (lung cancer) cell lines. *PASD1* levels were significantly higher in the lung cancer cell line H1299 (88 times higher than K562 and more than twice the levels seen in the testis) [15]. *PASD1* transcripts have also been found in 33% (4 of 12) AML patients, 1 of 6 CML patients and 4 of 16 cell lines, (Hn5, Jurkats, K562 and H1299) by RQ-PCR [15] as well as in primary MM samples at presentation and following treatment [49]. Reverse transcription (RT)-PCR analysis of the expression of 32 antigens in cell lines derived from 21 B- and 4 T-cell malignancies indicated that eight CT genes had the broadest expression profiles. Of the 25 cell lines examined expression of CT antigens was found as follows: *Sp17* (25/25, 100%), *PRAME* (25/25, 100%), *CSAGE* (24/25, 96%), *PASD1* (22/25, 88%), *CAGE/DDX53* (19/25, 76%), *CTAGE*1 (19/25, 76%), *HAGE/DDX43* (16/25, 64%) and *PLU-1/JARID1B* (15/25, 60%) [50].

Previous data also showed *PASD1* expression in 25 of 68 solid

tumours on matched tumour/normal tissue array [35]. Of note in this study, the highest levels of hybridisation of *PASD1* cDNA was observed with stomach and uterine cancer samples when compared with normal adjacent tissues samples.

Cooper et al. (2006) [44] also showed PASD1 expression in 21 of 51 diffuse DLBCL patients, 4 of 9 MCL, 4 of 15 follicular lymphomas and a range of other tumour cells taken from patients with haematological malignancies. Notably PASD1a expression, was found more often than PASD1b, and was often cytoplasmic. PASD1 protein expression has been shown by immunostaining 2 of 4 primary MM tumour samples [49] and 5 of 11 MM cell lines, of which THIEL and RPMI8226 expressed both *PASD1* mRNA and protein.

Despite these results, *PASD1* did not achieve the elevated expression in the required cut-off of ≥ 5% of 320 newly diagnosed and 264 relapse MM patients when analysed by Affymetrix GeneChips. In contrast 87 of the 123 CT antigens analysed did merit further evaluation in this study by van Duin et al. [51]. We have also found variation between the relative expression of antigens on microarray [52] compared with detection of antigens by SEREX [15] in AML samples. The results of Van Duin may, in part, be explained by the propensity for probe sets on microarrays to favour 3' transcripts, the most variable end of most transcripts, especially for genes like *PASD1* which are anticipated to have multiple transcriptional variants. In addition *PASD1* is not present on some microarray chips including U133A perhaps leading to a small under estimate in the frequency of expression. In addition *PASD1* transcript expression was not found in 78 basal cell carcinoma by RQ- PCR [53].

PASD1 as a Biomarker

Insufficient evidence is available to determine whether PASD1 is a suitable prognostic biomarker. To date, two of the four DLBCL patients in whom antibodies to PASD1 were found also had poor survival markers [35]. Germinal centre markers CD10 and BCL6 are associated with better survival while expression of the non-germinal centre marker MUM1 suggests worse overall survival [54]. Patients with non-germinal centre type showed serum reactivity with PASD1, and *PASD1_v2* expression was restricted to non-germinal cell lines.

Immune Responses to PASD1

Humoral immune responses

Humoral responses to PASD1 have been demonstrated through the use of SEREX [15,16] indicating that at disease presentation 4 out of 10 patients with DLBCL, 6 out of 17 AML and 1 of 6 CML patients had already mounted cellular immune responses releasing antibodies into the peripheral blood volume of these patients. Similar antibody responses were not found in the periphery of 20 and 10 healthy donors in these studies, respectively. The question remains why these antibody responses were insufficient to kill PASD1 positive tumour cells. One

theory is that tumour cells may induce some immune response in the mid-stages of the cancers' development, when there are enough tumour cells to be seen by the immune response, but as these tumour cells proliferate they downregulate the immune system of the patient and escape effective killing [55]. Many of the antigens recognised by SEREX are nuclear transcription factors with low immunogenicity. It is proposed that those antigens with high enough immunogenicity would have already induced tumour cell killing. Only antigens with poor immunogenicity would have escaped immune surveillance. Immunotherapy, therefore, offers a way to enhance immune recognition of CT and tumour-associated antigens by the immune system and induce effective residual tumour cell killing.

Indeed we have shown that the elevated expression of some tumour antigens at disease presentation is associated with enhanced patient survival [25,27,28]. We have proposed that, during conventional therapy, the resulting tumour lysis would lead to inflammation and provide the requisite "danger signals" [56] which in turn leads to the induction of effective anti-tumour immune responses. Patients with elevated antigen expression would be better able to induce immune responses which would lead to the killing of antigen positive tumour cells and subsequent epitope spreading [57]. There is increasing evidence that chemotherapy may have a synergistic effect on the immune response to support this possibility [58].
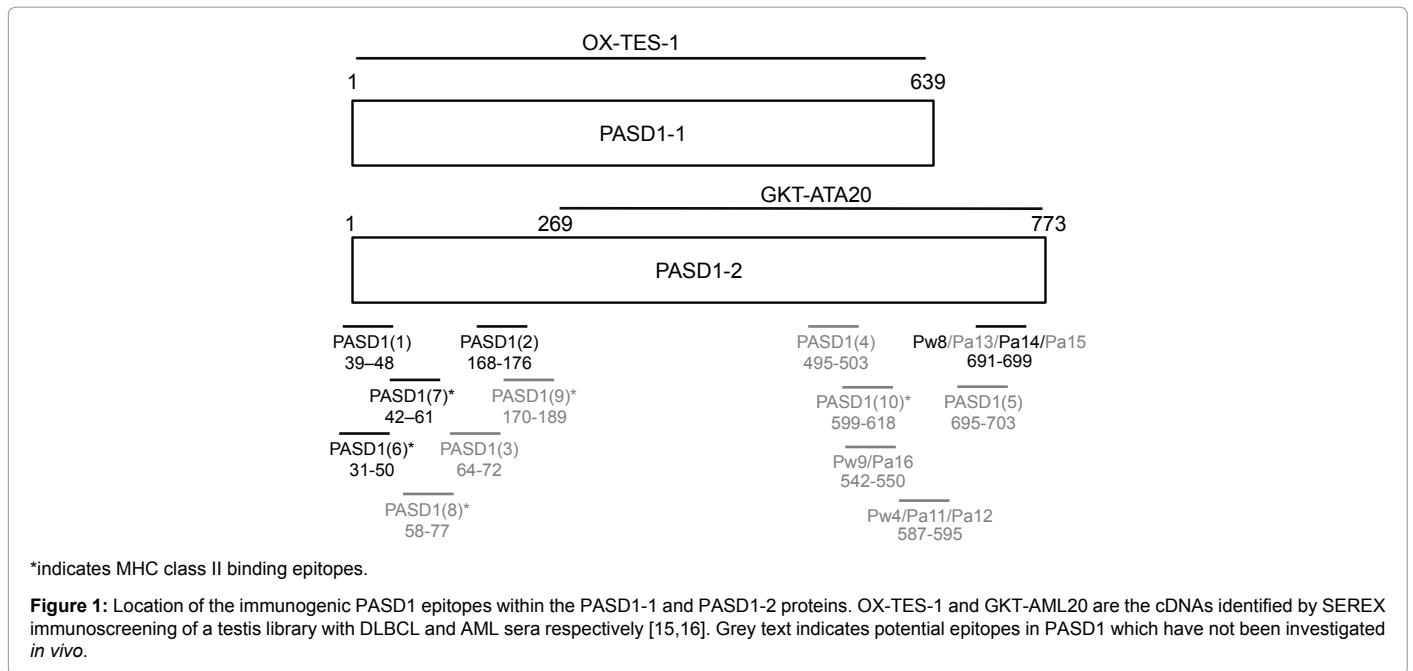
T cell responses

T-cell immunogenic epitopes within PASD1a and PASD1b were identified using the TEPITOPE [59], SYFPEITHI [60] and BIMAS [61] prediction programmes (Table 2 and Figure 1). The capacity of the MHC class I epitopes to bind HLA-A2 were confirmed using T2 assays (assay detailed in [62]) which can predict A2-peptide off-rates. T2 cells are TAP-deficient and only stabilize the HLA-A*0201 molecule on the surface of the cell when binding peptide. The avidity and half-life of peptide binding to MHC were detected by fluorescent activated cells (FACs) analysis using anti-HLA-A2. In each case the criteria used required that epitopes under investigation should not be similar to epitopes from other known human proteins. A cut-off of similarity across the 9-10 a.a. peptides was ≥ 40% to any other known human protein and utilisation of SYFPEITHI predicted epitopes in favour of BIMAS ones [63].

Cytotoxic T cells are considered to be the major effector cells in tumour immunity. A cytotoxic T-cell (CTL) response to PASD1 could be detected in DLBCL patients [64]. Furthermore, interferon-gamma (IFNγ) release was detected in 21 out of 29 HLA-A*0201-positive DLBCL patients following short-term culture of their peripheral blood mononuclear cells stimulated with five HLA-A*0201-restricted PASD1 peptides. However, there was no response in the 21 patients who were HLA-A*0201-negative.

CD4 T helper cells are known to promote immunity in a number

Name of epitope	MHC binding	Peptide sequence	Immunogenicity
PASD1(1)$_{38-47}$	HLA-A*0201	QLLDGFMIT	IFNγ secretion following MLR with patient T cells [64]. Demonstrated by IFNγ ELISpot and CTL assays *ex vivo* [48]
PASD1(2)$_{167-175}$	HLA-A*0201	YLVGNVCIL	IFNγ secretion following MLR with patient T cells [64]. Demonstrated by IFNγ ELISpot and CTL assays *ex vivo* [48]
PASD1(6)$_{31-50}$	HLA-DRB1	DYFNQVTLQLLDGFMITLST	IFNγ secretion following MLR with patient T cells [65]
PASD1(7)$_{42-61}$	HLA-DRB1	DGFMITLSTDGVIICVAENI	IFNγ secretion following MLR with patient T cells [65]
Pw8/Pa14	HLA-A*0201	RLWQELSDS/RLWQELSDI	IFNγ secretion following MLR with patient T cells. Demonstrated by IFNγ ELISpot and CTL assays *ex vivo* [63]

Table 2: Details of the most immunogenic PASD1 epitopes.

Figure 1: Location of the immunogenic PASD1 epitopes within the PASD1-1 and PASD1-2 proteins. OX-TES-1 and GKT-AML20 are the cDNAs identified by SEREX immunoscreening of a testis library with DLBCL and AML sera respectively [15,16]. Grey text indicates potential epitopes in PASD1 which have not been investigated *in vivo*.

of ways such as stimulating production of antibodies. CD4 responses against PASD1 epitopes were investigated by Ait-Tahar et al. [65] in patients with DLBCL. They showed that immunogenic PASD1 epitopes predicted to bind several class II DR beta 1 alleles were able to induce CD4+ T helper responses to PASD1-positive cells from patients with DLBCL. Two of the five peptides (PASD1(6) and PASD1(7)) were shown to be immunogenic in 14 of the 32 patients tested and T-helper cell lines generated from two patients were able to lyse PASD1 positive cell lines derived from haematological malignancies. CD4+ T helper cell lines raised from two patients were able to lyse PASD1-positive tumour cell lines corroborating that these T cells recognized intracellular expressed PASD1. The PASD1-negative cell line was not lysed.

None of the seven MHC class I binding nonamers (named sequentially Pw4 through to Pw10) identified using predictive programmes were able to stabilise HLA-A*0201 in T2 assays and all had poor SYFPEITHI scores [63]. However, modification of one of the anchor residues at a.a. 2 or 9 (to a lysine, valine or isoleucine) did lead to epitopes with improved SYFPEITHI scores and enhanced and/or extended periods of binding to HLA-A*0201 in T2 assays. One of these modified peptides, named Pa14, was shown to be able to stimulate patient T cells. This caused a very limited expansion in CD8+ T cell numbers from two of three HLA-A*0201 positive, PASD1-positive AML patient samples. This corresponds with the findings of others [66] who also found limitations in the expansion that can be achieved with AML T cells *ex vivo*. A 2-3 week limited expansion is the maximum that has been achieved prior to cell death. Reasons for the limited responses may be due to the presence of myeloid suppressor cells in mixed lymphocyte assays [67], interleukin-6 (IL-6) secretion by myeloid leukaemia cells [68] and/or defects in T cell populations in myeloid leukaemia patients [69]. However stimulation of T cells from a single colon cancer patient, in this study, led to a substantial increase in the number of Pa14-specific T cells to 13.6% of the CD8+ cell population after 4 rounds of weekly Pa14 stimulation, with Pa14-specific IFNγ responses being evidenced [63].

The combined treatment of conventional (surgery and chemotherapy) and immunotherapy in a patient with metastatic melanoma has been recently described [70]. Palliative treatment with radiation had eliminated the patient's primary tumour and in-transit non-irradiated metastases. This abscopal effect was shown to be due, at least in part, to a systemic anti-MAGEA3 immune response. When the patient relapsed, they were treated with radiosurgery and anti-CTLA4 (ipilimumab) immunotherapy (reviewed in [71]). After this treatment the patient entered a complete response and, notably, in addition to an increase in MAGEA3 antibodies the patient also showed a new anti-PASD1 response.

pMHC arrays

To compare the T-cell responses in patients to the PASD1 epitopes identified by Hardwick et al. [63] and Ait-Tahar et al. [64] we have used peptide major histocompatibility complex (pMHC) or tetramer arrays. pMHC arrays were validated originally with mouse T cells by Soen et al. [72] and then shown to be able to detect tumour antigen specific T-cell responses in melanoma by Chen et al. [73]. The technique is able to simultaneously detect multiple T- cell populations, without haplotype restriction, using small numbers of CD8+ T cells (~1.2×10^6 cells/array) incubated for 20mins with pMHC (1ng per spot) spotted onto polyacrylamide gels. Briefly CD8+ T cells were negatively isolated from the peripheral blood of patients with cancer. When we performed the pMHC arrays, T cells were not expanded or stimulated with peptide. These "untouched" T cells were dyed with the lipophilic tracer DiD and incubated with arrays printed with pMHCs from more than 50 tumour-associated antigen and viral epitopes (including HLA-A*0201/ CMV and Flu controls). Positive scoring of T-cell populations was only made when T cells were consistently bound to three of six of the same pMHC spots in two distinct regions on the array. We have now analysed PASD1 epitope recognition by T cells from patients with leukaemia, colon cancer, head and neck cancer and pancreatic cancer and compared the frequency of T cell recognition of other well-known tumour antigen epitopes including Wilm's tumour-1 (WT1), Proteinase 3, receptor for hyaluronan-mediated motility (RHAMM) and Surviving.

Development of Immunotherapy Strategies Targeting PASD1

Joseph-Pietras et al. [48] used the pDOM-epitope DNA vaccine design [74] to compare the efficacy of the whole *PASD1_v1* cDNA (FL) in vaccination studies compared with the CTL $PASD1(1)_{38-47}$ and $PASD1(2)_{167-175}$ epitopes [64] (Table 2). The group found a greater T-cell response in HHD mice [75] to PASD1(1) than PASD1(2) in IFNγ ELISpot assays and significant CTL killing of loaded and endogenously PASD1 positive myeloma cell lines. Vaccines containing the FL PASD1 induced greater anti-PASD1(1) responses compared with anti-PASD1(2) suggesting immunodominance. In addition the FL PASD1 vaccine could induce CTL killing of MM cells.

The DNA fusion gene vaccine has also been used to assess PASD1 analogue peptides in HHD humanized mice [63]. One of the epitopes Pa14 was shown to be capable of inducing immune responses against the modified (Pa14) and wild type (Pw8) peptides in studies using mixed lymphocyte reaction (MLR) and CTL assays. Splenocytes from vaccinated mice demonstrated *in vitro* cytotoxicity against myeloid leukaemia tumour cells, which were either exogenously loaded with the corresponding wild type peptide (Pw8) or presented endogenously processed PASD1 peptides. Of note mice immunised with a pDOM-Pw8 DNA vaccine were unable to mount a significant immune response but mice immunised with the modified peptide pDOM-Pa14 killed Pw8 loaded and endogenous PASD1 presenting targets.

Further epitopes could be identified through the immunoscreening short overlapping peptide libraries [76,77] although some of the already predicted PASD1 epitopes [63-65] remain to be more thoroughly studied *in vivo*.

Summary

Clinical trials targeting PASD1 have yet to be initiated however there is accumulating evidence of the utility of DNA vaccines in such settings [78,79]. The expression of *PASD1* in a range of tumour types, especially haematological malignancies, suggests that PASD1 specifically has a role as a target for the immunotherapy of these difficult to treat cancers that do not respond well to conventional therapy and frequently lack a more suitable treatment for the removal of minimal residual disease.

Although further studies of the frequency of PASD1 protein expression in cancers will help determine the suitability of PASD1 as a target for immunotherapy; major issues are why PASD1 has such variable expression in some cell lines and primary tissues; and whether this indicates more progressed cancer cell populations within affected tissues. The role of PASD1 as a biomarker will help us to better understand its role in cancer progression. Furthermore, PASD1 expression has been shown to exist after treatment in some patients [49] highlighting it as a potential robust post-conventional therapy target. In addition increasing our understanding of the functional role of PASD1 in cancer development will help determine whether killing tumour cells which express PASD1 will impact on cancer progression in a more meaningful way than just acting as a target for immunotherapy strategies.

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