CD4-positive T-helper cell responses to the PASD1 protein in patients with diffuse large B-cell lymphoma

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ABSTRACT

Background
Vaccine development targeting the novel immunogenic Per ARNT Sim Domain containing 1 (PASD1) cancer testis antigen represents an attractive therapeutic approach for the significant number of patients with diffuse large B-cell lymphoma who are refractory to conventional treatment. Since CD4-positive T helper cells have crucial roles in promoting and maintaining immune responses to tumor antigens, the presence of a CD4-positive T-helper immune response to the PASD1 antigen in patients with diffuse large B-cell lymphoma was investigated in the current study.

Design and Methods
Thirty-one patients with diffuse large B-cell lymphoma (25 with *de novo*, five with transformed and one with T-cell-rich B-cell lymphoma) were studied. Five immunogenic PASD1 peptides predicted to bind to several major histocompatibiliy complex, class II *DR beta 1* alleles were identified using web-based algorithms. Peripheral blood mononuclear cells from patients were used to investigate the immunogenicity of these DR beta 1-restricted peptides *in vitro* using both gamma-interferon release enzyme-linked immunospot and cytolytic assays.

Results
Two of the five PASD1 peptides, PASD1(6) and PASD1(7), were shown to be immunogenic in 14 out of 32 patients studied in a gamma-interferon release assay. CD4-positive T-helper cell lines from two patients raised against PASD1 peptides were able to lyse cell lines derived from hematologic malignancies expressing endogenous PASD1 protein.

Conclusions
This is the first report of a CD4-positive T-helper response to the PASD1 protein in patients with lymphoma. The immunogenic peptides described here represent valuable additional candidates for inclusion in a vaccine to treat patients with PASD1-positive diffuse large B-cell lymphoma whose disease is refractory to conventional therapies.

Key words: diffuse large B-cell lymphoma, CD4 T-helper cells, immune response, PASD1, cancer-testis antigen.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common form of adult non-Hodgkin's lymphoma and is heterogeneous with respect to morphology, clinical features and immunophenotype.[1] Despite advances in the definition of clinically relevant subtypes and treatment, a significant proportion of patients with DLBCL fail to achieve long-term remission.[2] The development and use of cancer vaccines boosting the immune response of patients represents an attractive approach to improve therapeutic options for high-risk patients.

A prerequisite for immunotherapeutic approaches is the identification of tumor-associated antigens, of which the cancer-testis antigens are of particular interest.[3-6] Cancer-testis antigens, especially the CT-X antigens, have a restricted distribution in normal tissues (being predominantly expressed in testis, an immunoprivileged site with low or even absent HLA expression) but are present in a range of tumors.[6,7] Such an expression pattern raises the possibility of targeted treatment while minimizing any attendant potential autoimmune problems for patients.

Although cytotoxic T cells (CTL) are considered to be the major effector cells in tumor immunity, there is increasing evidence for the importance of the CD4+ T helper (CD4 Th) cell population in maintaining tumor immunity.[8] Possible mechanisms of these cells include roles in maintenance and support of the CTL response, the cross-presentation of tumor antigens, their interaction with other effector cells such as natural killer cells and the release of immunologically relevant cytokines involved in anti-tumor effects, such as interleukin-2 and interferon-γ.[9-14] Evidence from human and animal studies has indicated that optimal cancer vaccines require the participation of both CD4+ and CD8+ T cells.[15-17] A recent report highlighted the successful use of tumor-associated antigen-specific CD4 T-cell clones as therapeutic agents in the treatment of melanoma.[18]

We initially identified PASD1 as a tumor-associated antigen in DLBCL[19] and acute myeloid leukemia[20] while later studies showed PASD1 to be a novel cancer-testis antigen expressed in a wide range of hematologic malignancies.[21,22] Our subsequent identification of a CTL response to PASD1 in DLBCL[23] supports this molecule as a candidate for vaccine development, not only for DLBCL, but also for use in a generic vaccine for the treatment of other PASD1-positive malignancies. The current study was performed to investigate the CD4 Th response in DLBCL patients to obtain further support for the inclusion of PASD1 in a lymphoma vaccine.

Design and Methods

Subjects

Peripheral blood was obtained from 31 patients with DLBCL attending the Haematology Departments of the John Radcliffe Hospital (n=28) and the Milton Keynes General Hospital (n=3). Of these 31 patients, 25 had *de novo* DLBCL, five had transformed DLBCL and one had T-cell rich B-cell lymphoma. The clinical details of these patients are given in Table 1. Peripheral blood was also obtained from five healthy subjects. Tissue typing was done by polymerase chain reaction as previously described.[24] 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 immunolabeling studies.

Peptides

The TEPITOPE prediction algorithm[25] and SYPETHI (www.syfpeithi.de) programs were used to select PASD1 peptides predicted to be immunogenic in the context of HLA-DRB1 *0101, *0301, *0401, *0701,*1101 and *1501 (the most prevalent alleles among the Caucasian population).[26] The following peptides were identified: PASD1(6)$_{31-50}$(DYFNQVTLQLLDGFMITLST); PASD1(7)$_{42-61}$ (DGFMITLSTDGVIICVAENI); PASD1(8)$_{58-77}$ (AENISSLL-GHLPAEIVGKKL); PASD1(9)$_{170-189}$ (VGNVCILRTQLLQQLYTSKA) and PASD1(10)$_{599-618}$ (NHPVRFLQAQPIVPVQRAAE). The PASD1(6)$_{31-50}$ peptide encompasses the immunogenic PASD1(1)$_{38-47}$ CTL epitope while PASD1(8)$_{58-77}$ encompasses another immunogenic PASD1(3)$_{63-71}$ CTL epitope.[23] The location of the PASD1 peptides in the PASD1 protein is illustrated in Figure 1. The irrelevant control peptide was HIV-1$_{121-140}$ (DESFRKYTAFTIPSMNNETP) (Invitrogen, UK). The PASD1 peptides were synthesized by standard chemistry and were more than 90% pure (Invitrogen, Paisley, UK).

Preparation and culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) in RPMI 1640 containing 10% fetal calf serum (RPMI 1640/FCS) were prepared as previously described.[23] PBMC (2×10^5) in 200 μL were then added to each well of a 96-well flat-bottomed plate and incubated for 10-14 days with 10 μM of one of following: one of the PASD1 peptides, the irrelevant HIV$_{121-140}$ peptide, 10 μg/mL phytohemagglutinin (Sigma-Aldrich Co. Ltd., Dorset, UK) or RPMI 1640/FCS only. Recombinant interleukin-2 (20 IU/mL; Roche Diagnostics, Indianapolis, IN, USA) and recombinant human interleukin-7 (25 ng/mL; R&D Systems, Minneapolis, MN, USA) were added on days 2, 5, and 7. In some experiments, PBMC were co-cultured with the PASD1(1) CTL epitope for 8 days before being used in an enzyme-linked immunospot (ELISPOT) assay.

Enzyme-linked immunospot assay

After 10-14 days of culture, the 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 (10 μM), HIV control peptide (10 μM), phytohemagglutinin (10 μg/mL) or medium only. Interferon-γ release assays were performed according to manufacturer's instructions (Mabtech, Stockholm, Sweden). Results were considered positive if the number of spots in the test wells was at least twice the number present in the control cultures (media only or in the presence of the HIV-1 irrelevant peptide) and assays were excluded if there were more than 25 spots per well in the absence of peptides.

Generation of CD4 T-helper cell lines, depletion and blocking experiments

PBMC cultured at a density of 2×10^6 cells/mL were stimulated with 10 μM of either the PASD1(6) or PASD1(7) peptides. After 72 h, an equal volume of RPMI 1640/ FCS containing 50 IU/mL of recombinant interleukin-2 was added to each culture well. Half of the medium was removed and replaced with fresh medium every 3 days thereafter for up to 6 weeks before being used in ELISPOT and cytolytic assays. Cells were also used to produce cytocentrifuge preparations for immunophenotyping at 4 weeks. In some experiments, a CD4+ T-cell purification step was performed using magnetic beads coated with anti-human CD4 antibody according to the manufacturer's instructions (Dynabeads, Dynal, Oslo, Norway) prior to being used in an interferon-γ release ELISPOT assay as described above. In other experiments, 2 μg/mL of the HLA-DR-specific WR18 antibody (Abcam, Cambridge, UK) were added to the CD4 Th cell population for 1 h prior to the interferon-γ release ELISPOT assay.

Table 1. Clinical details of the DLBCL cases studied.

"Patients	Diagnosis	Subtype#	Stage	IPI	Sex	Age	Treatment	Outcome
1	DLBCL(dn)	NGC	1	1	F	23	CHOP-R + MTX (CNS prophylaxis)	CR (40 months)
2	DLBCL(dn)	GCB	3	3	M	67	CHOP-R + MTX+RX	CR (44 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 (73 months)
8	DLBCL(dn)	GCB	1	0	M	63	CHOP-R	CRU (48 months)
9	DLBCL(dn)	NGC	3	2	F	71	CHOP-R	PR (47 months)
10*	DLBCL(dn)	GCB	1	0	F	60	CHOP-R + RICE + ESHAP + BEAM + TX	CR (37 months)
11	DLBCL(dn)	GCB	1	1	M	38	CODOX-M + RX	PR (41 months)
12	DLBCL(dn)	GCB	1	0	F	59	CHOP-R	CR (46 months)
13	DLBCL(dn)	GCB	3	3	M	67	CHOP-R + MTX	PR (41 months)
14	DLBCL(dn)	NGC	3	2	M	63	CHOP-R + MTX	Died of disease (14 months)
15	DLBCL(dn)	NGC	3	3	M	85	VIN/PRED	Died of disease (6 months)
17	DLBCL(dn)	GCB	3	4	M	60	CHOP-R	Died –cause unknown (21 months)
18	DLBCL(dn)	NGC	4	4	M	74	CNOP-R	Died of buccal cancer (31 months)
19	DLBCL(dn)	GCB	4	2	M	56	CHOP-R + RX	Died of disease (19 months)
20	DLBCL(dn)	NGC	2	2	F	70	NONE	Died of disease (2 months)
21	DLBCL(dn)	GCB	1	3	M	73	CHOP-R	PR (47 months)
24	DLBCL(dn)	NGC	1	1	F	62	CHOP-R	CR (35 months)
25	DLBCL(dn)	GCB	2	2	F	74	CHOP-R	Died (6 months)
27	DLBCL(dn)	GCB	1/2	2	M	>60	CHOP-R	CR (52 months)
28	DLBCL(dn)	GCB	1	2	M	>60	CHOP-R	CR (44 months)
29	DLBCL(dn)	GCB	3	4	F	71	CHOP-R	Died of disease (7 months)
30	DLBCL(dn)	NGC	3	3	M	62	CHOP-R	CR (48 months)
31	DLBCL(dn)	NGC	1	1	M	63	CHOP-R	CR (47 months)
32	DLBCL(dn)	GCB	1	3	M	75	CNOP-R	CR (47 months)
37	DLBCL(t)	ND	1	2	M	59	CHOP-R + RX	CR (46 months)
39	DLBCL(t)	ND	4	2	F	39	CHOP-R	Died of disease (6 months)
40	DLBCL(t)	ND			M	64	CHOP-R	Died of disease (4 months)
41	DLBCL(t)	ND	4	4	F	60	CHOP-R + CNOP-R	CR (53 months)
43	DLBCL(t)	ND	4	2	F	60	CHOP-R + RX	CR (48 months)
47	TCR	ND	3	2	F	80	PMITCEBO-R	CR (36 months)

*Patients previously reported in the description of a CTL response to PASD1.[22] DLBCL(dn): De novo diffuse large B-cell lymphoma; DLBCL(t): diffuse large B-cell lymphoma trans-formed; TCR: T cell rich B-cell lymphoma; # subtyped according to expression of CD10, BCL-6 and MUM1; GCB: germinal center-derived; NGC: non-germinal center-derived; CHOP -R: cyclophosphamide, doxorubicin, vincristine, prednisolone, rituximab; MTX: intrathecal methotrexate; RX: radiotherapy; PRED: prednisolone; VIN: vinblastine; RICE: rituximab, ifos-famide, carboplatin, 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; PMITCE-BO-R: prednisolone, mitoxantrone, cyclophosphamide, etoposide, bleomycin, vincristine, rituximab; IVAC: ifosfamide, etoposide, cytarabine. *Sample at relapse; CR: complete response; PR: partial response: CRU: complete remission unconfirmed.*

Antibodies
Monoclonal
Major histocompatibility complex (MHC) class II expression was studied using the monoclonal anti-HLA-DP, DQ, DR anti-body (CR3/43) (DAKOCytomation, Glostrup, Denmark). Antibodies to 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 (4KB5, IgG1 isotype) were gener-ated in the authors' laboratory.

Polyclonal
The rabbit/mouse Envision-HRP labeling system was obtained from DAKOCytomation. Isotype-specific goat anti-rabbit immunoglobulin (Ig) and anti-mouse Ig-isotype specific antibod-
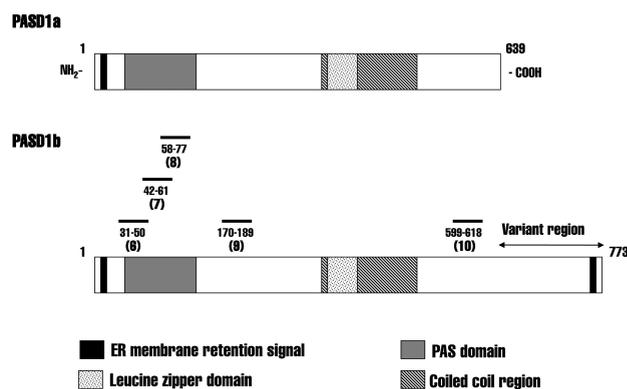
Figure 1. Diagram to show the positions of the CD4 PASD1 peptides investigated here across the PASD1a and PASD1b protein isoforms.

ies conjugated to either fluorescein isothiocyanate or Texas Red™ (diluted 1:100) were obtained from Invitrogen (Paisley, UK).

Immunolabeling studies

Immunoperoxidase

Paraffin-embedded tissue sections were de-waxed and antigen retrieval was performed using 50 mM Tris:EDTA at pH 9.0. Immunolabeling for anti-MHC-class II was carried out using the Envision-HRP labeling kit (DAKO, Ely, UK)[26] The staining for PASD1 protein expression (using the monoclonal antibodies PASD1-1 and PASD1-2) and subtyping of these DLBCL cases as non-germinal or germinal center subtypes had been performed previously.[23]

Immunofluorescence

Cytocentrifuge preparations of CD4 Th cell lines cultured for 4 weeks were fixed in acetone for 10 min and air-dried. The slides were then incubated for 30 min with one of the following combinations: (i) a mixture of rabbit anti-CD3 with one of the following mouse monoclonal antibodies to CD4, CD8, CD20, CD45RA, CD45RO, CD56 or CD68 or (ii) a combination of two mouse monoclonal antibodies: anti-CD4 or CD8 (both isotype IgG1) and anti-CD45RO (isotype IgG2a) or CD20 (isotype IgG2a) and CD45RA (isotype IgG1). After washing in phosphate-buffered saline, the slides were incubated for a further 30 min with a mixture of: (i) fluorescein isothiocyanate-conjugated goat anti-rabbit Ig and an isotype-specific anti-mouse Ig conjugated to TexasRed™ or two anti-mouse Ig isotype-specific antibodies conjugated to either fluorescein isothiocyanate or TexasRed™ where relevant. A minimum of 300 cells were counted per sample. The slides were washed and mounted in an anti-fade mountant Vectorshield containing 4',6-diamine-2'phenylindole dihydrochloride (Vector Laboratories, Peterborough, UK). Results were then visualized as previously described.[22]

Cell lines

The PASD1-positive (HLA-DRB1*0301) OCI-Ly3 (DLBCL-derived), PASD1-positive (HLA-DRB1*0401,*1101) Thiel (myeloma-derived), and PASD1-negative (HLA-DRB1*0101, *0401) SUDHL-6 (DLBCL-derived) cell lines were obtained and cultured as described previously.[27]

Cytolytic assay

A ^{51}Cr-labeling release assay was used to investigate the ability of CD4 Th cell lines generated from DLBCL patients to lyse PASD1-positive tumor target cells. The OCI-Ly3, SUDHL-6 and Thiel target cell lines were radio-labeled for 4 h with 100 μCi ^{51}Cr and then added to the CD4$^+$ Th lines at effector:target ratios of 1:10, 1:20 and 1:40. The cells were incubated for 18 h (rather than the 4 h used for CTL assays) at 37°C in 5% CO_2. ^{51}Cr release was determined following the addition of 10% Triton-X to the effector:target cell cultures as previously described.[23]

Statistical analysis

Student's t-test was used to analyze the results obtained in the ELISPOT and cytolytic assays.

Results

Detection of interferon-γ release

As shown in Table 2, 15 patients displayed a significant response to at least one of the PASD1 peptides, with respect to the control peptide ($P<0.05$), after 10-14 days culture. Three patients (1, 41 and 47) responded to four peptides while patients 8, 14 and 24 recognized only one peptide. These patients included eight of the 16 patients previously reported to have a CTL response to PASD1.[23] Peptide PASD1(7) also elicited a CD4 Th response in patient 24, despite a previous observation that no CTL response was detected in this HLA-A*0201-positive patient.[22] Furthermore, an interferon-γ response was also detected in six other DLBCL patients who were HLA-A*0201-negative. While the number of patients studied here was relatively small, ten of the 15 patients with a CD4 Th response remain in complete remission, one is in partial remission and four have died (one of carcinoma). These results contrast with the outcome of those patients unable to immunologically recognize the PASD1 peptides of whom only five patients are in remission, four in partial remission and seven have died (one of a carcinoma). These results are summarized in Table 3.

It is noteworthy that the PASD1(6) peptide which encompasses the immunogenic 10-mer CTL epitope PASD1(1)[23] was also immunogenic eliciting interferon-γ release from stimulated PBMC in the majority of patients studied here. Data from the ELISPOT assays enabled PASD1(6) and PASD1(7) to be identified as the most immunogenic of the five PASD1 peptides studied.

The interferon-γ responses were investigated in two high responder patients (patients 4 and 47) at the time of diagnosis and 1 year after diagnosis (Figure 2A). In both patients, a significant interferon-γ response to the two PASD1 peptides PASD1(6) and PASD1(7) was sustained 1 year after diagnosis. Both of these patients were considered to be in complete remission at this time with the absence of obvious minimal residual disease (suggesting the absence of tumor cells expressing PASD1).

CD4-enriched cell immune response

CD4$^+$ enriched T-cell lines were generated following the weekly stimulation with PASD1(6) and PASD1(7) peptides of PBMC from patient 4 (with de novo DLBCL) and patient 47 (with T-cell rich B-cell lymphoma) in order to enable further study of their functional activity. The cell lines demonstrated a significant response to both PASD1 peptides (Figure 2B). These responses were abrogated after the removal of the CD4 Th cell population or the addition of anti-HLA-DR antibody (Figure 2B) thus demonstrating that the response is dependent on both CD4 and MHC class II.

Cytolytic activity

CD4 Th cell lines specific for PASD1(6) and PASD1(7) raised from patients 4 and 47 lysed PASD1-positive Thiel and OCI-Ly3 cell lines in a dose-dependent fashion (Figure 4). The lysis of these cells expressing different HLA-DRB1 alleles demonstrates the promiscuity of the PASD1 peptide epitopes. No lysis was detected in the PASD1-negative cell line SUDHL-6 despite its expression of HLA-DRB1*0101. Since the HLA-A*0201-negative OCI-Ly3 cell line was shown to be resistant to lysis by CTL raised against the PASD1(1) and PASD1(2) peptides in our previous study,[23] lysis of this cell line in the current study was not due to CTL raised against these peptides in the PASD1(6) and PASD1(7) peptides. This factor, combined with the immunophenotyping results of the cell lines, and the cell purification and blocking steps shown in Figure 2, provides additional evidence to support CD4 Th cell killing in the cell lines used here.

● Table 2. Summary of the CD4 Th immune response to the PASD1 peptides by DLBCL patients and normal donors.

*Patients	Sub-type	CTL response in HLA-A*0201 + patients[22]	MHC class II	Reactivity with antibody PASD1-1	Reactivity with antibody PASD1-2	MHC class II expression in tumor	Interferon-γ response to PASD1 peptides/50,000 cells							
							PASD1(6) (31-50)	PASD1(7) (42-61)	PASD1(8) (58-77)	PASD1(9) (170-189)	PASD1(10) (599-618)	No peptide	HIV-1 (121-140)	PHA
Significant response														
Patient 1 DLBCL (dn)	NGC	+	DRB1*0102,*1104	50% +/-	-	+	52±6	28±4	32±3	30±2	40±2	10±4	2±1	98±15
Patient 2 DLBCL (dn)	GCB	+	DRB1*0101, *0701	-	-	+	32±4	46±8	36±6	24±2	40±4	14±2	16±2	106±18
Patient 3 DLBCL (dn)	GCB	+	DRB1*0301, *0401	++	+/-†	+	34±4	26±4	18±2	24±2	32±2	10±2	8±2	62±8
Patient 4 DLBCL (dn)	NGC	+	DRB1*0102, *0301	+<50%,†,#	-	+	58±8	44±4	28±4	18±2	32±4	16±2	14±2	98±6
Patient 8 DLBCL (dn)	GCB	+	DRB1*1501, *0803	+/-,†	†	+	36±6	18±2	20±4	18±2	18±4	10±2	12±4	48±4
Patient 12 DLBCL (dn)	GCB	+	DRB1*0401, *1401	+	†	+	44±6	36±4	24±2	20±4	32±4	14±2	12±2	72±10
Patient 14 DLBCL (dn)	NGC	+	DRB1*0403, *0401	-	+/-	+/-	34±4	16±2	28±4	22±2	36±4	10±2	14±2	108±12
Patient 24 DLBCL (dn)	NGC	-	DRB1*1301, *1301	+	-	+	22±4	38±4	28±4	26±4	16±4	14±4	8±2	86±14
Patient 25 DLBCL (dn)	GCB	nd	DRB1*0401, *2401	+/-	-	+	42±4	34±2	26±4	16±4	26±4	12±4	10±2	66±8
Patient 27 DLBCL (dn)	GCB	nd	DRB1-0102, 0301	++	-	+/-	40±4	24±2	36±4	26±2	16±2	14±4	12±2	82±10
Patient 29 DLBCL (dn)	GCB	nd	DRB1*0101, *0405	+50%	+/-	+	24±2	22±4	10±2	32±2	10±2	6±2	8±2	58±6
Patient 32 DLBCL (dn)	GCB	nd	DRB1*1501, *1501	++,#	†	+	44±2	32±4	12±4	22±2	20±2	12±2	6±4	88±16
Patient 41 DLBCL (t)	ND	nd	DRB1*0901, *1113	-	-	+	34±4	26±2	30±6	18±2	36±2	8±2	10±2	76±12
Patient 43 DLBCL (t)	ND	nd	DRB1*0101, *1101	-	-	+	26±4	28±2	16±6	20±2	14±2	6±2	8±2	80 ±12
Patient 47 TCR	ND	+	DRB1*0301, *0801	++,#	+/-†	+	48±4	56±6	40±6	26±2	38±2	14±2	12±2	84±10
No significant response														
Patient 9 DLBCL (dn)	NGC	+	DRB1*0301, *1601	+/-,†	+/-,†	+	22±6	18±2	12±3	20±2	14±2	8±2	4±2	118±18
Patient 10 DLBCL (dn)	GCB	+	DRB1*0101	†	+/-†	+	30±8	26±2	36±4	10±2	32±2	14±2	16±4	78±8
Patient 11 DLBCL (dn)	GCB	+	DRB1*1404, 1501	+	+/-	+	24±4	16±2	20±4	16±2	12±2	8±2	10±2	58±6
Patient 13 DLBCL (dn)	GCB	+	DRB1*0103, *0401	-	+/-	+/-	16±2	14±2	18±6	16±2	10±2	14±2	10±2	92±12
Patient 15 DLBCL (dn)	NGC	+	DRB1*1301, *1101	+,†	†	na	28±4	32±2	10±1	8±2	18±4	12±4	14±4	58±6
Patient 17 DLBCL (dn)	GCB	+	DRB1*0301, *0401	-	-	-	20±2	18±4	14±2	8±2	10±4	12±2	10±2	54±12
Patient 18 DLBCL (dn)	NGC	+	DRB1*0401, 0401	+	-	+/-	14±2	26±2	20±2	24±2	22±2	16±2	14±2	62±12
Patient 19 DLBCL (dn)	GCB	-	DRB1*0401, *0901	-	-	+	22±2	26±2	24±2	18±2	12±2	16±2	12±2	58±14
Patient 20 DLBCL (dn)	NGC	-	DRB1*1104, *1501	-	-	-	10±2	14±2	18±2	8±2	16±2	12±2	10±2	52±10
Patient 21 DLBCL (dn)	GCB	-	DRB1*0301, *1303	-	-	na	18±2	10±2	14±2	16±2	18±2	8±2	10±2	102±10
Patient 28 DLBCL (dn)	GCB	nd	DRB1*0701	+50%	-	na	28±4	22±6	16±2	22±4	10±2	14±1	6±2	45±8

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Patient 30 DLBCL (dn)	NGC	nd	DRB1*0401, *1104	-	-	+	8±2	12±2	14±2	18±4	28±2	16±1	12±2	76±10
Patient 31 DLBCL (dn)	NGC	nd	DRB1-0701, 1301	-	-	na	22±2	20±1	18±2	12±4	24±2	12±1	4±2	48±4
Patient 37 DLBCL (t)	ND	+	DRB1*0103, *1101	+/-,†	†	+	38±4	32±6	16±2	28±4	34±2	10±2	16±2	126±22
Patient 39 DLBCL (t)	ND	+	DRB1*0701, *1301	-	-	+/-	24±4	22±4	26±2	26±4	16±2	12±1	10±2	92±12
Patient 40 DLBCL (t)	ND	nd	DRB1*0401, *0401	+	-	+	12±2	20±4	20±2	14±2	16±2	10±3	8±2	64±8
Healthy Donors														
HD 1	-		DRB1*0102, *0301				14±2	20±2	18±2	14±2	10±2	10±2	8±2	86±14
HD 2	-		DRB1*0401, *0701				10±2	8±2	10±2	1±1	4±2	4±2	6±2	66±10
HD 3	-		DRB1*0401, 0701				5±2	10±2	6±2	8±2	2±1	4±2	1±1	46±6
HD 4	-		DRB1*10301, *0101				14±2	20±2	10±2	19±2	18±2	8±2	10±2	74±16
HD 5			DRB1*0301, *0701				24±2	22±2	10±2	18±2	16±2	12±2	8±2	63±10

GCB: germinal center-derived; NGC: non-germinal center-derived; +/-, + and ++ denotes intensity of cytoplasmic labeling; † denotes from 5-30%nuclear labeling # denotes labeling of some smaller lymphocytes and vessels in tumor; nd: not done; na: tissue not available. ∞ denotes the same patients previously reported in the description of a CTL response to PASD1.[22] The results +/- are from triplicate ELISPOT cultures. The SD was calculated using standard techniques. Shading of figures denotes significant interferon γ responses.

Immunolabeling studies

Table 2 summarizes the presence of MHC class II antigens in the DLBCL cases studied. Tumor cells of 25 of the 27 patients tested expressed MHC class II. No interferon-γ response to PASD1 was detected in those two patients whose tumors were MHC class II-negative. With the exception of three patients (patients 2, 41 and 43), PASD1 protein was detected in the tumor cells of those patients with an interferon-γ response (Table 2). PASD1 protein was undetectable in seven of those 12 patients who did not mount an interferon-γ response to any of the PASD1

Table 3. Comparison of the presence of a CD4 Th response to PASD1 in DLBCL patients with clinical outcome.

	Clinical outcome of patients		
	Complete remission	**Partial remission**	**Died**
Patients with a significant CD4 response	1, 2, 8, 12, 24, 27, 32, 41, 43, 47	4	3, 14, 25, 29
Patients with no significant response	10, 28, 30, 31, 37	9, 11, 13, 21	15, 17, 18, 19, 20, 39, 40
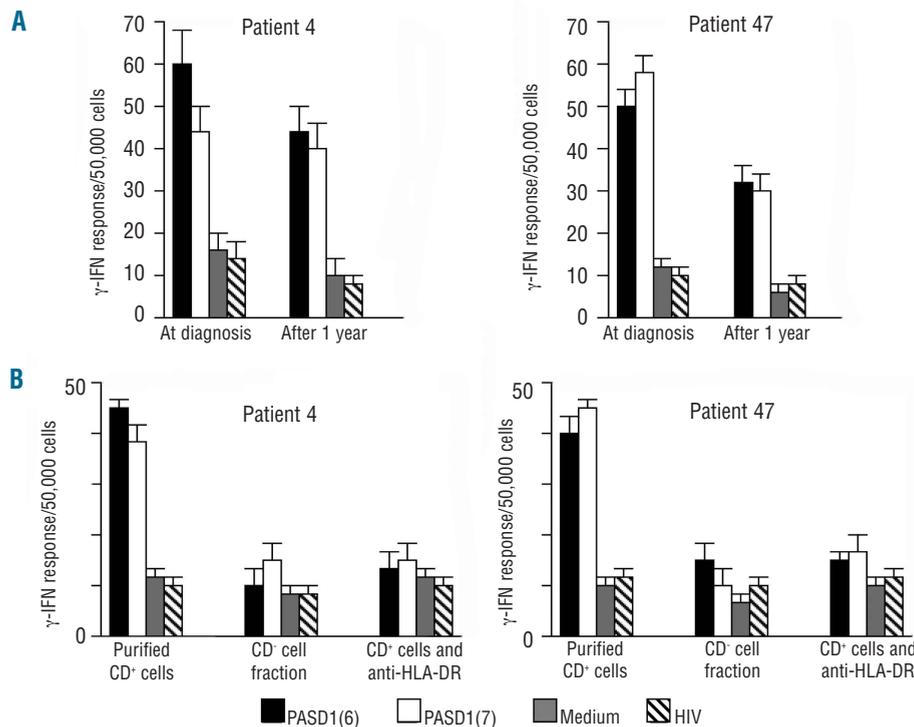
Figure 2. Interferon-γ ELISPOT studies showing CD4 Th responses to PASD1 peptides of patients 4 and 47. (A) A study monitoring the CD4 Th responses of these two patients at the time of diagnosis and 1 year after-diagnosis showed the presence of significant immune interferon-γ responses to PASD1(6) and PASD1(7) peptides even after 1 year in remission (P<0.05). In contrast, no significant responses to the control HIV peptide or medium only were observed. (B) CD4 Th cell lines were cultured from patient 4 and patient 47 and CD4+ cell populations were obtained using a magnetic purification step. After overnight incubation with either PASD1(6) or PASD1(7) peptides, the purified CD4+ cells from both patient 4 and 47 responded to these PASD1 peptides. No significant response was detected to the irrelevant control peptide or to medium only. The removal of the CD4+ cells or the addition of anti–HLA-DR antibody abrogated the interferon-γ response to both peptides. The results are the mean ± SD and were from triplicate cultures.
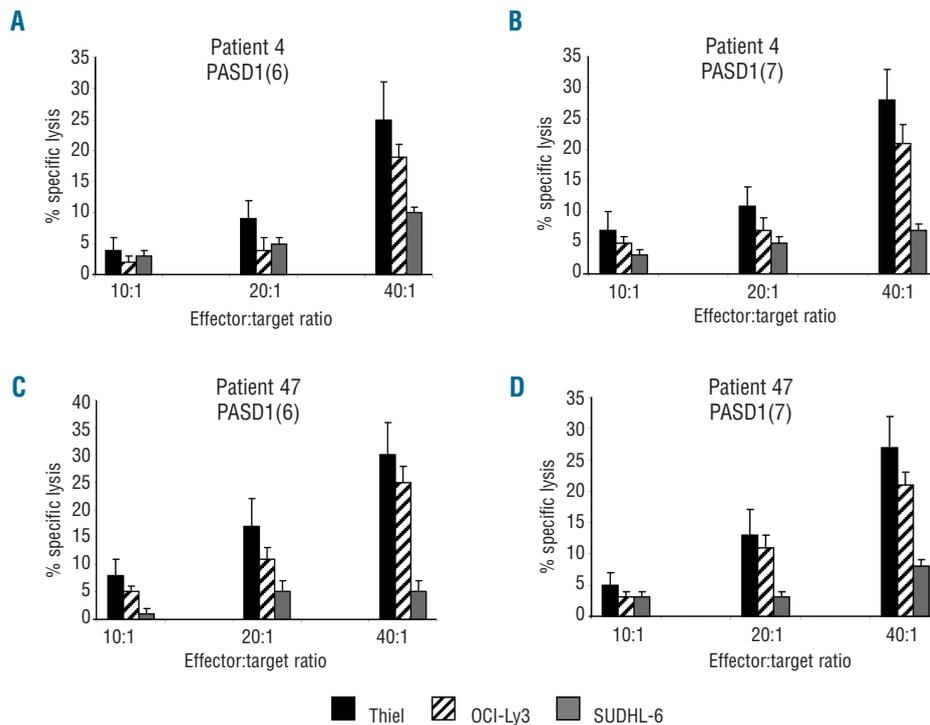
Figure 3. Cytolytic activity of PASD1-specific CD4 Th cell lines derived from two DLBCL patients 4 and 47. The cytolytic activity of CD4 Th cell lines raised from patient 4 (DRB1*0102,*0301) and patient 47 (DRB1*0301,*0801) against PASD1(6) (A) and PASD1(7) (B) peptides was tested in an 18 h ^{51}Cr release assay on the PASD1-positive Thiel (DRB1*0401,*1101) and OCI-ly3 (DRB1-0301) cell lines. Significant lysis was observed only in those cultures containing the PASD1-positive Thiel or OCI-Ly3 cells. This was in contrast to the lack of lysis observed of the PASD1-negative SUDHL-6 (DRB1*0101,*0401) cell line (P<0.001). Similarly, significant lysis was also observed for CD4 Th cell lines raised from patient 47 against (C) the PASD1(6) and (D) PASD1(7) peptides. The results are the mean ± SD and were from triplicate cultures.

peptides. Of these, two of the five cases tested were negative for MHC class II.

Immunophenotyping of the CD4 T-cell lines showed that 93.3±2.6 % of the cells were CD3^{+} T cells of which 86.6±2.4% expressed CD4 and 15.4±4.3% were CD8^{+}: 88.0±2.1% of the CD4^{+} T cells were CD45RO^{+} with only 12.0±4.1% expressing CD45RA. Representative examples of the labeling results are shown in Figure 4.

Discussion

Previous reports have described limitations in using short peptide sequences from tumor-associated antigens to elicit long-lasting CTL responses to the target molecule. Repeated boosting with peptides may be necessary to achieve a sufficient immune response, a strategy potentially leading to the induction of immunotolerance.[28,29] Indeed, Lehe *et al.* reported the undesirable recognition of a 15 amino-acid sequence of WT1 by immunosuppressive T regulatory cells.[30] There is, however, accumulating evidence that longer peptides containing CD4 Th epitopes are essential for obtaining and maintaining a long-lasting CTL response.[31,32] The inclusion of such peptides in a vaccine should increase the efficacy of the resulting immune response. The current study was performed to identify longer immunogenic PASD1 peptides capable of eliciting CD4 Th cell responses. Such peptides could then be included in a lymphoma vaccine for the treatment of DLBCL as well as other hematologic malignancies expressing PASD1.

Five potential CD4 Th PASD1 peptides of 20 amino acids in length that would be recognized by a number of HLA-DRB1 subtypes were selected by a stringent method using two highly reliable search tools: the TEPITOPE and the SYPEITHI programs.[17] The identification and use of promiscuous MHC class II epitopes has been previously reported for the NY-ESO-1 cancer-testis antigen.[33,34] The presence of such epitopes, recognizable in the context of a variety of different MHC class II molecules, expands the population of patients for whom the peptides could be immunogenic beyond that determined by their MHC class I allele. All five peptides in the current study were able to elicit interferon-γ responses in DLBCL patients, with the PASD1(6) and PASD1(7) peptides being identified as the most immunogenic peptides. It is notable that these peptides were able to expand the numbers of patients recognizing PASD1 since they elicited interferon-γ responses in a patient who, despite being HLA-A*0201-positive, was previously unable to respond to the PASD1 CTL peptides and also in seven HLA-A*0201-negative patients.[23] The lack of significant homology of the PASD1 peptides with other molecules combined with the absence of any significant response with the irrelevant peptide demonstrates the specificity of the response to PASD1.

Although interferon-γ responses to the PASD1 peptides were present in 12 of the 15 patients whose tumors expressed PASD1 protein, three patients recognizing the PASD1 peptides lacked detectable PASD1 protein. A disparity between PASD1 expression and CTL activity has been described previously[23] and it is possible that the immunolabeling techniques used here may not constitute a sufficiently sensitive tool to identify low levels of PASD1 protein expression.[35] Importantly, high levels of target protein expression may not be necessary for immune recognition.[36] There is also evidence that additional isoforms of PASD1 may exist[21] and some of these proteins may lack the epitopes recognized by the PASD1-1 and PASD1-2 antibodies that are currently being used to study PASD1 protein expression. The development of more sensitive assays for PASD1 expression, such as western blotting and/or the production of additional antibodies, should assist in the identification of the maximum number of PASD1-positive patients in future clinical trials targeting

this antigen. Another possible explanation for the lack of detection in the present study is that PASD1 expression may have been lost through immunoediting by the tumor cells.[37]

The tumor cells of only two patients in the current study lacked MHC class II expression and PBMC from neither patient were able to respond to the PASD1 peptides. Although cross-presentation of antigen by antigen-presenting cells can occur at the tumor site in the absence of MHC class II,[12] loss of MHC class II has been linked to poorer prognosis.[38,39] and it will be of interest in future studies to determine whether PASD-positive MHC class II-negative patients have a significantly worse prognosis than PASD1-positive MHC class II-positive patients.

CD4 Th cell lines raised from two patients were able to lyse PASD1-positive tumor cell lines (but not the PASD1-negative cell line) confirming that these T cells were able to recognize endogenously expressed PASD1. Of note is the ability of the CD4 Th cell lines to lyse both the PASD1-positive Thiel and OCI-Ly3 cells, despite the fact that these two cell lines express different HLA-DRB1 alleles. This could be explained by the high degree of homology present between the DRB1 molecules[25] and further demonstrates the promiscuity of the PASD1 peptides, which enables them to be recognized by closely related DRB1 molecules.

The persistence of PASD1-specific CD4 Th cell populations in patients after 1 year in remission is an indication of the immunogenicity of the PASD1 protein and is suggestive of pre-existing memory T cells able to recognize PASD1. Such cells could play an important role not only in protective tumor immunity[40] but also in the maintenance of the CTL previously reported to be present in PASD1-positive DLBCL patients.[23] In contrast to the previous study on the CTL immune response to PASD1 (in which 79% and 37% of the cultured cells were CD8^{+} and CD45RO^{+}, respectively), the majority of the cell lines cultured from these patients were CD4^{+} and expressed CD45RO, an antigen present on memory cells. It will be of interest to look at the *ex vivo* phenotype (memory or naïve) of circulating T cells from patients in future experiments.

Previous studies have shown the potential of using single peptides containing epitopes predicted to bind to both MHC class I and class II to obtain more effective immune responses.[41,42] The PASD1(6) peptide, encompassing a CTL epitope previously shown to be immunogenic in PASD1-positive patients,[22] was highly immunogenic in the majority of patients and could, therefore, represent an attractive
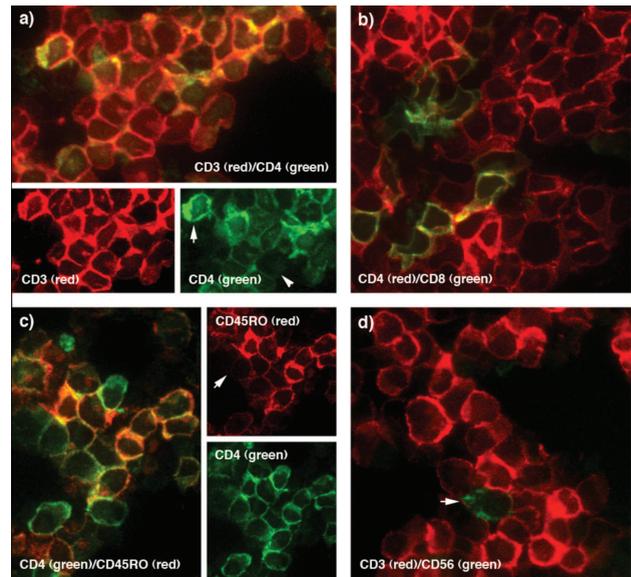
Figure 4. Double immunofluorescent labeling of the cultured CD4^{+} T-cell lines. In a) the majority of cultured cells express both CD3 and CD4 antigens (arrow). Heterogeneity was observed in CD4 expression. An example of a CD3^{+} cell CD4^{-} T cell is shown (arrowhead). b) Shows the presence of a small population of CD8^{+} cells (green) c) Co-expression of CD45R0 on the majority of CD4^{+} cells can be observed. An example of a CD4^{+} CD45RO^{-} cell is arrowed. d) Only the occasional cell was CD56^{+} (arrowhead) confirming the presence of only a small number (<1%) of natural killer cells in the cultured cells.

candidate for inclusion in a vaccine formulation.

In conclusion, this study is the first report of a CD4 Th response to a cancer-testis antigen by patients with lymphoma. These CD4 Th peptides hold future potential for use in conjunction with PASD1 peptides eliciting CTL in a vaccine for relapsing DLBCL patients or those who are refractory to conventional treatments.

Authorship and Disclosures

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