Cancer Immunol Immunother (2012) 61:169–179
DOI 10.1007/s00262-011-1095-2

ORIGINAL ARTICLE

Human kallikrein 4 signal peptide induces cytotoxic T cell responses in healthy donors and prostate cancer patients

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Received: 29 October 2010 / Accepted: 30 July 2011 / Published online: 27 August 2011

Abstract Immunotherapy is a promising new treatment for patients with advanced prostate and ovarian cancer, but its application is limited by the lack of suitable target antigens that are recognized by $CD8^+$ cytotoxic T lymphocytes (CTL). Human kallikrein 4 (*KLK4*) is a member of the kallikrein family of serine proteases that is significantly overexpressed in malignant versus healthy prostate and ovarian tissue, making it an attractive target for immunotherapy. We identified a naturally processed, HLA-A*0201-restricted peptide epitope within the signal sequence region of *KLK4* that induced CTL responses in vitro in most healthy donors and prostate cancer patients tested. These CTL lysed HLA-A*0201^+ *KLK4*$^+$ cell lines and KLK4 mRNA-transfected monocyte-derived dendritic cells. CTL specific for the HLA-A*0201-restricted *KLK4* peptide were more readily expanded to a higher frequency in vitro compared to the known HLA-A*0201-restricted epitopes from prostate cancer antigens; prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA) and prostatic acid phosphatase (PAP). These data demonstrate that *KLK4* is an immunogenic molecule capable of inducing CTL responses and identify it as an attractive target for prostate and ovarian cancer immunotherapy.

Keywords Kallikrein · Prostate cancer · Immunotherapy · Tumor antigen · Cytotoxic T cells

Electronic supplementary material The online version of this article (doi:10.1007/s00262-011-1095-2) contains supplementary material, which is available to authorized users.

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Introduction

Metastatic prostate cancer is a disease for which there are currently no effective treatments. Harnessing the therapeutic potential of the immune system's cellular response using strategies such as dendritic cell (DC) immunotherapy or adoptive T cell therapy is an attractive treatment for patients with metastatic prostate cancer because of its potential efficacy, low toxicity and ability to be combined with other therapies [1, 2]. Testimony to this approach is a recent phase III clinical trial of a vaccine comprised of antigen-presenting cells (APC) loaded ex vivo with a prostatic acid phosphatase (PAP)-GM-CSF fusion protein

(Sipuleucel-T, Dendreon) [3]. This has been one of the few new treatments for metastatic prostate cancer to significantly improve patient survival and the first cell-based immune therapy to receive US FDA approval.

Tumor-specific cytotoxic T lymphocyte (CTL) responses are central to the induction of anti-tumor immune responses. The identification of tumor-associated antigens (TAA) that can serve as CTL targets is essential to the development of clinical immune therapies and monitoring their outcomes. The ideal TAA should be strongly expressed in tumors and be capable of inducing both $CD8^+$ and $CD4^+$ T lymphocyte responses to multiple MHC alleles (i.e., have broad applicability) but have limited expression in normal tissues (reducing peripheral TAA tolerance and minimizing the risk of induction of autoimmunity). Ideally, a TAA should also play a significant role in the oncogenic process or cancer cell survival so as to minimize selection of TAA loss tumor variants during immunotherapy. A number of TAA have been identified for prostate cancer, including PAP, prostate-specific antigen [PSA, also known as kallikrein 3 (KLK3)] and prostate-specific membrane antigen (PSMA) [4–9], but none of them meet all the above criteria. Furthermore, the use of multiple TAA and several epitopes to maximize responses and minimize immune escape by antigen loss tumor variants is now favored by clinicians [10]. Thus, the identification of additional, more tumor-specific TAA remains an important priority for clinical immunotherapy.

Human kallikrein 4 (*KLK4*) is a serine protease belonging to the PSA-related kallikrein family [11]. *KLK4* is expressed in virtually 100% of prostate cancers and is significantly overexpressed in malignant versus normal prostate, with weak expression in a limited range of healthy tissues [12–16]. The *KLK4* gene consists of five coding exons, and alternative splicing in prostate cancer cells results in two major transcripts: the full-length *KLK4-254* transcript and the exon 1-deleted *KLK4-205* transcript [17]. The *KLK4-254* transcript encodes a 254 amino acid preproenzyme (KLK4-254) that is predominantly localized to the cytoplasm and can be secreted, whereas the *KLK4-205* transcript encodes the more abundant *N*-terminally truncated 205 amino acid isoform (KLK4-205) that is devoid of a signal peptide and proregion, and is localized to the nucleus. Both protein isoforms are overexpressed in prostate cancer.

Expression of *KLK4* is associated with an epithelial to mesenchymal transition and proliferation of cell growth in prostate cancer cell lines, suggesting involvement in cancer progression [18, 19]. This and the overexpression of *KLK4* in most prostate cancers make it an attractive candidate to target for immune intervention. The detection of KLK4-specific antibodies in the serum of prostate cancer patients [20] and the identification of naturally processed $CD4^+$ T helper cell epitopes [21] provide evidence that KLK4-specific immune responses can be induced. Given the importance of CTL responses in the induction of tumor immune responses, we investigated whether *KLK4*-specific $CD8^+$ CTL responses could be generated in healthy donors and prostate cancer patients. We identified a HLA-A*0201-restricted naturally processed epitope residing within the signal peptide region of the molecule (within the *KLK4-254* isoform) that stimulated the expansion of specific $CD8^+$ CTL. These data demonstrate that *KLK4* is an immunogenic molecule and justifies its use as an immunotherapeutic vaccine target.

Materials and methods

Blood samples

Peripheral blood mononuclear cells (PBMCs) were obtained by venepuncture or apheresis from HLA-A*0201-positive healthy donors and prostate cancer patients following informed consent obtained under ethical approval from the Mater Health Services Human Research Ethics Committee. Human AB serum was obtained from the Australian Red Cross Blood Service.

Antibodies, reagents and cell lines

IL-2 was provided by Roche (Basel, Switzerland), and IL-7 and β-2-microglobulin were purchased from Sigma. Prostate cancer cell lines LNCaP (HLA-A*0201^+) and PC3 (HLA-A*0201^-), ovarian carcinoma cell lines OVCAR-3 and CaOV3 (both HLA-A*0201^+), the myelogenous leukemia cell line K562 and the TAP-deficient T2 cell line were obtained from the ATCC (Manassas, VA) and maintained in the recommended media.

Peptides

Two separate computer algorithms were used to analyze the KLK4 amino acid sequence for potential HLA-A*0201-binding nonamer peptide sequences. The algorithm from the BioInformatics and Molecule Analysis Section of the NIH (BIMAS, http://www-bimas.cit.nih.gov) [22] scores potential HLA-A*0201-binding peptides based on the estimated half-time of dissociation of complexes containing peptide at 37°C and pH 6.5, while the SYFPEITHI algorithm [23] (access via http://www.syfpeithi.com) scores peptides according to amino acids in anchor and auxiliary anchor positions based on published motifs. Peptides with the highest scores obtained from both programs were synthesized using standard solid-phase synthesis (Auspep, Parkville, Australia) and stocks were reconstituted at 10 mg/ml.

HLA-A*0201-restricted epitopes from other known prostate cancer TAA [PAP-$5_{299–307}$ ALDVYNGLL; PSA-$3_{154–163}$ VISNDVCAQV; PSMA-$2_{711–719}$ ALFDIESKV (Bachem AG)] were also synthesized.

T2-binding assay

Peptide binding to HLA-A*0201 was assessed by stabilization of HLA-A*0201 molecules on the surface of T2 cells by flow cytometry. T2 cells were washed twice in RPMI 1640 and resuspended in X-vivo 15 serum-free medium (Cambrex) in the presence of β-2-microglobulin. Serial dilutions of KLK4 peptides (50–0.75 μg/ml in dH_2O) were added and cells incubated at room temperature for 2 h with occasional gentle resuspension. Cells were then washed and stained with an anti-HLA-A2 (HB82 supernatant, ATCC) followed by sheep anti-mouse FITC (Chemicon) and analyzed by flow cytometry. The stabilization ratio was calculated as the mean fluorescence intensity (MFI) of HLA-A2 staining of T2 cells pulsed with test peptide (for the peptide concentration with the highest MFI) divided by the MFI of control T2 cells without peptide at the same peptide concentration.

CMRF-56^+ DC preparation

CMRF-56^+ DC preparations were isolated as previously described [24]. Briefly, PBMCs were isolated by Ficoll density gradient separation and cultured for 16 h in RPMI 1640 containing 2 mM glutamine, 10 mM HEPES, 1 mM pyruvate, 0.1 mM nonessential amino acids, 50 μM 2-mercaptoethanol and 10% human pooled AB serum (complete medium, CM). Cells were harvested and resuspended in cold separation buffer (PBS, 2 mM EDTA, 1% human pooled AB serum). Biotinylated CMRF-56 mAb (MMRI) was added for 15 min at 4°C. Cells were incubated with anti-biotin microbeads (Miltenyi) for 15 min at 4°C. After washing once, cells were passed over a MACS LS magnetic column (Miltenyi) according to the manufacturer's instructions. For some experiments, CMRF-56^+ cells were isolated on the CliniMACS machine using the enrichment 1.1 program and TS Tubing set (Miltenyi). The positively selected cell fraction was referred to as the CMRF-56^+ preparation.

Generation of *KLK4* mRNA

cDNA coding for the complete *KLK4* sequence was ligated into the RNA expression vector pGEM4Z/A64 (kindly provided by Dr Eli Gilboa, Duke University Medical Center, Durham, North Carolina, USA), using the restriction enzymes *Hind* III and *Xba* I. The mRNA expression vector was linearized by overnight restriction enzyme digestion with *Spe* I followed by purification using the Qiaquick PCR purification kit (Qiagen). Capped antigen-encoding mRNA was generated by in vitro transcription using the mMESSAGE mMACHINE T7 RNA in vitro translation kit (Ambion, Austin, TX, USA) and purified by column centrifugation using the MEGAclear kit (Ambion), according to the manufacturers' instructions. The quality and quantity of antigen-encoding mRNA were established by denaturing agarose gel electrophoresis and spectrophotometry.

Transfection of DC

DC isolated from donor PBMC by CMRF-56^+-positive selection as described above or monocyte-derived DC (MoDC) differentiated in the presence of GM-CSF and IL-4 were transfected with mRNA using a BioRad GenePulsar II. Briefly, DC were mixed with mRNA in a 0.2-cm-gap cuvette (BioRad). Cells were electroporated at 250 V, 150 μF, (BioRad GenePulsar II) and then incubated on ice for 1 min, prior to resuspension in CM.

Induction of CTL cultures

The CMRF-56^+ preparation was resuspended in X-vivo 15 medium and pulsed with peptide (5 μg/ml) for 2 h at room temperature. Either these peptide-pulsed cells or cells transfected as described above were washed once in CM and incubated with autologous PBMC responders at a 1:5 ratio in CM, containing IL-7. IL-2 was added on day 3 and every 3 days thereafter. Cultures were restimulated with autologous irradiated peptide-pulsed PBMC, or transfected CMRF56^+ DC on day 12 and then at weekly intervals.

IFN-γ ELISPOT

The induction of peptide-specific T lymphocyte responses in the cultures was measured prior to each restimulation using an IFN-γ ELISPOT assay kit (Mabtech) according to standard operating procedures and criteria for establishing positive responses. In brief, nitrocellulose filtration plates (Millipore) were coated overnight at 4°C with IFN-γ capture antibody. After washing and blocking with CM, 1:4 serial titrations of harvested CTL cultures, commencing at 1×10^5 per well for polyclonal cultures and 10,000 cells or less for CTL clones, were incubated with individual test, control or no peptide-pulsed T2 cells in the presence of β-2-microglobulin. After 18-h culture (37°C, 5% CO_2), the plates were washed with PBS/0.01% Tween followed by PBS and incubated with biotinylated IFN-γ detection antibody for 2 h at 37°C before washing again. Spots were visualized by the addition of streptavidin–alkaline

phosphatase for 1 h at 37°C, followed by Sigma FAST™ substrate. Color development was terminated with water, plates were dried and spots counted on an automated ELISPOT reader (Autoimmun Diagnostika). All dilutions were performed in triplicate against individual peptides and controls. The frequency of antigen-specific CTL was calculated from dilutions which fell within the linear sensitivity range of the assay (greater than 10 spots per well and less than 300 spots per well). Responses were classified as antigen-specific only where the number of spots in the test wells was significantly greater ($P \leq 0.05$ by Student's unpaired t test) than those in the control wells at the same CTL dilution. The percentage of $CD8^+$ T lymphocytes within the cultures was monitored by flow cytometry. Positive responses were then expressed as number of IFN-γ-producing cells per 10^4 or 10^6 $CD8^+$ T lymphocytes from the means of triplicate test minus control wells. A representative example is shown in Online Resources 1.

Clonal expansion of in vitro-generated CTL

When the CTL cultures generated greater than 1% specific CTLs detected by ELISPOT, attempts were made to isolate and expand clonal antigen-specific CTL using an IFN-γ secretion assay (Miltenyi). Briefly, cultures were incubated with specific peptide- or control-pulsed T2 cells overnight and then stained with IFN-γ catch reagent followed by incubation in CM for 45 min at 37°C to trap IFN-γ on the surface of secreting cells. Cells were stained with a PE-conjugated IFN-γ detection antibody along with anti-CD3-FITC, CD4-APC, CD8-PerCPC5.5 and CD56-PECy7. Single IFN-γ^+ $CD3^+CD8^+CD56^-$ events were sorted by flow cytometry into round-bottom plates and expanded with irradiated allogeneic PBMC in the presence of phytohemagglutinin (PHA) and IL-2. Specificity of the expanded clones was tested by ^{51}Cr release assay using peptide-pulsed T2 cells as targets. Clones were maintained in CM containing 100 U/ml IL-2 with intermittent restimulation with irradiated allogeneic PBMC and PHA as required.

Cytotoxicity assay

A standard 4-h chromium (^{51}Cr) release assay was used to examine the CTL cytotoxic capacity against peptide-pulsed T2 cells and tumor cell lines. Washed targets were labeled with 100 μCi ^{51}Cr and incubated with titrated doses of CTL for 4 h in the presence of excess K562 cells to saturate any nonspecific NK-mediated lysis. Culture supernatants were harvested and counted on a Microbeta Trilux β-scintillation counter (Wallac). Percent specific lysis was calculated as 100 × (test count − spontaneous release control count)/(total release control count − spontaneous release control count).

Pentamer analysis

Induction of peptide-specific MHC class I-restricted CTL was determined using pentamer technology prior to each stimulation. These assays were validated and performed according to standard operating procedures that were established in the laboratory for immune monitoring of a phase I immunotherapy clinical trial. Briefly, CTL cultures were stained with the relevant peptide/MHC APC-conjugated pentamers (Proimmune) in PBS/0.2% human serum albumin for 10 min at room temperature followed by staining with anti-human CD4-APC-H7, CD8-PE, CD3-FITC, CD14-PerPC, CD19-PerCP and 7-aminoactinomycin D (all BD). A no pentamer-stained control was used to set the gating strategy. Cells were washed and analyzed on a BD LSRII flow cytometer following standard QC checks. Settings were based on BD anti-mouse Ig, k/negative control (FBS) compensation particles set compensation controls that were set up for each experiment. A minimum of 2×10^5 events were acquired per sample. Data were analyzed using FlowJo software. Pentamer-positive cells were determined as those that were $CD3^+CD14/19/7AAD^-$, $CD8^+pentamer^+$ and data expressed as the percentage of $pentamer^+$ cells within the $CD8^+$ population. A representative example is shown in Online Resource 2.

Reverse transcription–PCR (RT–PCR)

Total RNA was isolated from cancer cells using TRIzol reagent (Invitrogen, Mount Waverley, VIC, Australia), treated with RNasefree DNase I (Invitrogen) and reverse-transcribed with random hexamers pd(N)6 (Roche Diagnostics, Brisbane, QLD, Australia) and Superscript II (Invitrogen). PCR with KLK4-specific primers K4Ex 1ATGS (5′-ATGGCCACAGCAGGAAATCCC-3′) and K4Ex4AS (5′-CACGCACTGCAGCACGGTAG-3′) (detects full-length *KLK4-254* transcript that includes the signal peptide codon), or K4Ex2S (5′-GCGGCACTGGTC ATGGAAAACG-3′) and K4Ex5AS (5′-CAAGGCCCTG CAAGTACCCG-3′ was performed with 1 μl cDNA, 0.5 units platinum Taq DNA polymerase (Invitrogen) and 35 cycles with annealing temperatures of 62°C. PCR for β2-microglobulin, which gave a 249-bp amplicon used as an internal control for RNA integrity, was performed using forward (5′-TGAATTGCTATGTGTCTGGGT-3′) and reverse (5′-CCTCCATGATGCTGCTTACAT-3′) primers for 32 cycles with similar PCR conditions. The PCR products were electrophoresed on a 1% (w/v) agarose gel and visualized by ethidium bromide staining.

Results

Induction of KLK4 peptide-specific $CD8^+$ T lymphocyte responses in healthy donors and prostate cancer patients

We used two computer algorithms to identify several nonamer peptide sequences from KLK4 that were predicted to bind efficiently to HLA-A*0201. Five peptides with the highest binding scores from both algorithms (designated KLK4A-E) were synthesized. The KLK4A peptide sequence is located in the signal sequence region of exon 1 that is only present in the *KLK4-254* isoform, while the peptide sequences B–E are located downstream in regions common to both *KLK4-254* and *KLK4-205* isoforms. The five peptides all bound HLA-A*0201 with similar or higher affinity compared to the control strong HLA-A*0201-binding epitope from influenza A matrix protein (FluMP), and known HLA-A*0201 epitopes from prostate cancer TAA PAP, PSA and PSMA, as indicated by stabilization ratios of greater than 1.4 in a T2-binding assay (Table 1). The KLK4A exon 1 signal sequence peptide had the highest affinity for HLA-A*0201 as predicted by both algorithms.

To induce KLK4-specific $CD8^+$ CTL responses, we pulsed $CMRF\text{-}56^+$ blood DC from $HLA\text{-}A*0201^+$ donors with the pooled KLK4A-E peptides and used the pulsed DC to stimulate autologous PBMC. Cultures were restimulated with peptide-pulsed PBMC at weekly intervals, and the induction of individual peptide-specific responses was monitored by IFN-γ ELISPOT (Fig. 1a). A response to a given peptide was considered positive only if the number of IFN-γ spots was significantly higher ($P < 0.05$ by student's t test) than the number of spots following restimulation with no peptide (see Online resource 1). We observed strong IFN-γ production specific for the KLK4A peptide in 2 healthy donors and significant but lower responses in a further 2 healthy donors (Fig. 1a). These responses were observed in both women (donors 1, ∇ and 3, $\bigcirc$, Fig. 1a) and men (donors 2, $\square$ and 4, $\diamond$ Fig. 1a). The maximum percentage of specific IFN-γ-producing cells was observed between 2 and 5 stimulations. Weaker responses specific for the KLK4B, KLK4C, KLK4D and KLK4E peptides were induced in some, but not all, healthy donors (Fig. 1a, open symbols).

Cancer patients' immune systems are often compromised as a result of the malignancy itself or treatment regimens [25]. We therefore examined whether prostate cancer patients at various stages of their disease could generate KLK4-specific immune responses. Strong KLK4A-specific responses, similar in magnitude to the responses induced in healthy donors, were induced in 4/6 prostate cancer patients and weak responses induced in a further two patients (Fig. 1a, closed symbols). We also observed responses to the KLK4B, KLK4C, KLK4D and KLK4E peptides in several donors; however, these were considerably lower than the responses specific for KLK4A. These data demonstrate that HLA-A*0201-restricted responses specific for KLK4 can be generated in both male and female healthy donors as well as prostate cancer patients, with the strongest response being to the KLK4A epitope.

To confirm the immunogenicity of KLK4A, KLK4A/HLA-A*0201 pentamers were synthesized and used to monitor the induction of KLK4A-specific responses in vitro (Fig. 1b). Consistent with the IFN-γ ELISPOT data, KLK4A/HLA-A*0201-specific CTL were expanded in a healthy male donor and 7/9 prostate cancer patients (Table 2; Fig. 1b). Therefore, KLK4A appears to be an immunogenic peptide that induces CTL responses in most healthy donors and prostate cancer patients.

Table 1 Peptide sequences, predicted binding and stabilization of HLA-A2 on T2 cells

Name	Peptide position	Peptide sequence	Peptide purity (%)	SYFPEITHI score	BIMAS score	HLA-A2 stabilization ratio
KLK4 A	$KLK4_{11–19}$	FLGYLILGV	87	27	736	2.2
KLK4 B	$KLK4_{95–103}$	QMVEASLSV	89	23	306	1.6
KLK4 C	$KLK4_{164–172}$	VLQCVNVSV	70	25	118	1.5
KLK4 D	$KLK4_{160–168}$	RMPTVLQCV	91	22	116	1.5
KLK4 E	$KLK4_{59–67}$	VLVHPQWVL	91	22	112	1.8
PSA-3	$PSA_{154–163}$	VISNDVCAQV	95	21	16.26	1.7
PAP-5	$PAP_{299–307}$	ALDVYNGLL	99	23	1.11	2.2
PSMA-2	$PSMA_{711–719}$	ALFDIESKV	95	26	1,055	1.5
FluMP	$FMP_{58–66}$	GILGFVFTL	95	30	551	1.4

The sequences and amino acid positions of 5 KLK4 peptides are shown. Predicted HLA-A2-binding based on two online algorithms is shown (arbitrary scores), as well as HLA-A2 stabilization ratios in a standard T2-binding assay. The KLK4 peptide HLA-A2-binding scores and stabilization ratios were compared with those of previously used proven HLA-A2-specific peptides

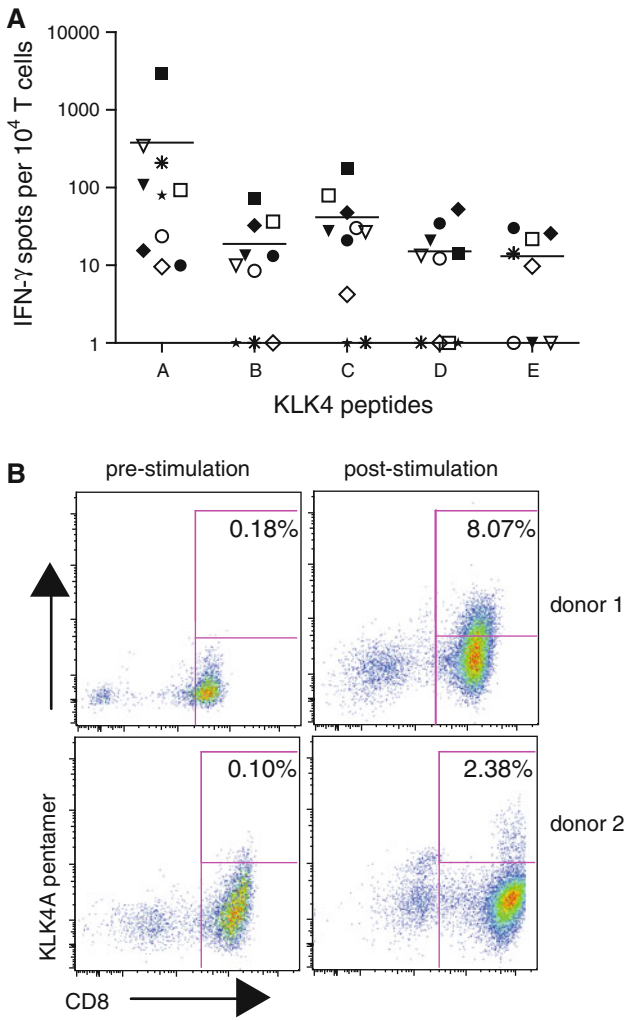

Fig. 1 Induction of KLK4-specific CTL responses in healthy donors and prostate cancer patients. DC were loaded with pooled KLK4 peptides A-E and used to stimulate autologous PBMC. Induction of peptide-specific responses was assessed by ELISPOT (**a**) or pentamer (**b**). **a** Maximum responses specific for each KLK4 peptide induced after 2–5 stimulations in healthy donors (*open inverted triangle*—40-year-old woman; *open square box*—46-year-old man; *open circle*—25-year-old woman; *open diamond*—45-year-old man) and prostate cancer patients (*asterisk*—70-year-old man with hormone refractory disease and bony metastases; *filled circle*—76-years-old man who had undergone radical prostatectomy followed by biochemical recurrence and hormone sensitive disease; *filled diamond*—68-year-old man with hormone sensitive disease; *filled square*—53-year-old man with hormone naïve disease; *star*—74-year-old man with stage T2 a disease; *filled inverted triangle*—73-year-old man with hormone refractory disease and nodal metastases). Data represent the number of specific IFN-γ-producing CD8$^+$ T cells (KLK4 peptide minus T2 control). **b** Induction of KLK4A-specific CTL was analyzed using a KLK4A/HLA-A*0201-specific pentamer from whole PBMC (prestimulation) or after 2–3 rounds of stimulation. Two individual prostate cancer donors are shown. Values in the *upper right* quadrants are the number of pentamer-positive events as a percentage of the total CD8$^+$ T cells

Isolation and expansion of CTL clones specific for KLK4A and KLK4C

CTL specific for the KLK4A and KLK4C peptides were isolated from the polyclonal cultures derived from a male healthy donor using an IFN-γ secretion assay and cloned by limiting dilution. Two CTL clones specific for KLK4A and four clones specific for KLK4C were expanded and analyzed to establish their specificity and functionality. Specificity of the KLK4A clones was confirmed by pentamer staining (Online resource 3). All CTL clones secreted IFN-γ in response to T2 cells pulsed with their respective peptides, but not in response to control peptide-pulsed T2 cells in ELISPOT assays (Fig. 2a, b). Each clone also lysed T2 cells pulsed with the respective KLK4A or KLK4C peptides but not control T2 cells in a ^{51}Cr release assay (Fig. 2c, d). Clones specific for the KLK4A peptide did not cross-react with other HLA-A*0201-restricted peptides KLK4B-E in IFN-γ ELISPOT assays (Fig. 2e). Likewise, anti-KLK4C CTL clones recognized the KLK4C peptide exclusively and did not cross-react with peptides KLK4A, KLK4B, KLK4D or KLK4E (Fig. 2e). Next, we investigated the affinity of the CTL clones by examining their ability to recognize T2 cells pulsed with titrating doses of specific peptide (Fig. 2f). Clones specific for KLK4C showed reactivity down to 5–50 pg/ml of specific peptide, whereas KLK4A clones had an even higher affinity, reacting to as little as 0.5 pg/ml of specific peptide. These data demonstrated that functional CTL clones with specificity for the HLA-A*0201-restricted peptides KLK4A and C could be expanded after in vitro stimulation.

CTL specific for KLK4A but not KLK4C peptides recognize and lyse *KLK4*-expressing tumor cells

To determine whether the KLK4A and KLK4C peptides are endogenously processed and presented by tumor cells, we tested the ability of the KLK4A- and C-specific CTL clones to lyse *KLK4*-expressing tumor cell lines. First, we confirmed mRNA expression of both *KLK4-254* and *KLK4-205* transcripts in the target HLA-A*0201$^+$ prostate cancer cell line LNCaP and ovarian carcinoma cell lines CaOV3 and OVCAR-3 (Fig. 3a). KLK4A-specific CTL lysed the CaOV3 cell line with a similar percentage of specific lysis as induced by other HLA-A2-restricted ovarian cancer CTL [26] (Fig. 3b). In contrast, KLK4C-specific CTL did not lyse CaOV3 cells, despite their capacity to lyse KLK4C peptide-pulsed T2 cells very efficiently at low effector/target ratios in the same assay (Fig. 2d). Neither CTL clones lysed control PC3

Table 2 KLK4A-specific CTL responses in healthy donors and prostate cancer patients

Donor type	$CD8^+$ $pentamer^+$ cells	
	Precursor	Max response
Healthy male	0.58	11.00
PC	0.10	0.15
PC	0.18	8.07
PC	0.13	1.30
PC	0.23	4.46
PC	3.13	5.13
PC	0.28	4.31
PC	0.10	7.25
PC	0.40	3.37
PC	0.30	0.6

Induction of KLK4A-specific CTL was analyzed using a KLK4A/HLA-A*0201-specific pentamer from whole PBMC (precursor) or after a number of restimulations which gave the maximum response. Data shown represent the percentage of $CD8^+$ T cells specific for KLK4A. A pentamer-specific response was demonstrated in a healthy male donor and 7/9 PC donors

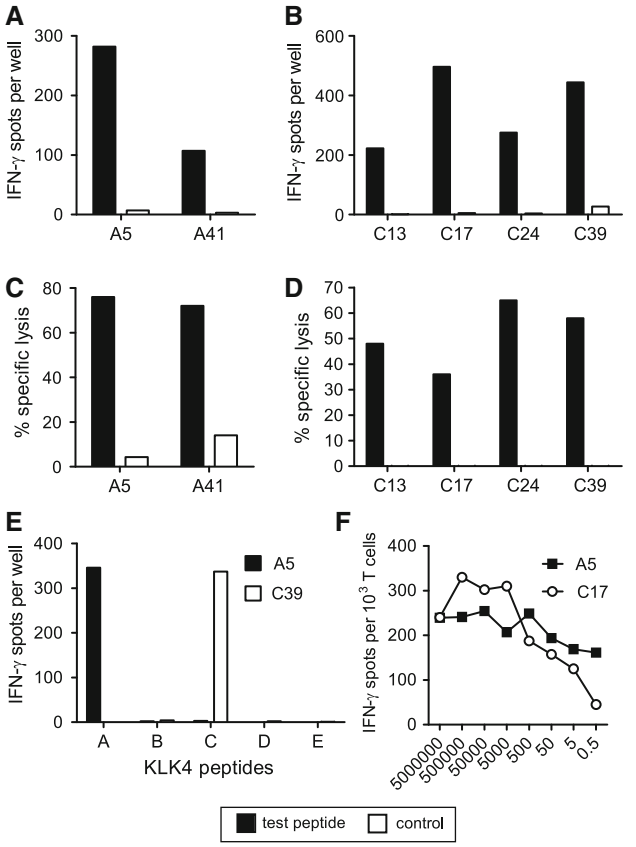

Fig. 2 Isolation of $CD8^+$ CTL clones specific for KLK4 peptides A and C. CTL clones specific for KLK4A (**a**, **c**) or KLK4C (**b**, **d**) were isolated from polyclonal CTL by an IFN-γ secretion assay followed by flow cytometry sorting and cloning by limit dilution. The specificity of each clone was assessed by examining the number of IFN-γ-producing cells by ELISPOT following stimulation with T2 cells pulsed with specific peptide or control (**a**, **b**) and by their capacity to lyse T2 cells pulsed with specific but not control-pulsed peptide by ^{51}Cr release assay (**c**, **d**) E/T ratios were A5 = 29:1, A41 = 46:1, all C clones = 25:1. **e** Clones specific for KLK4A (A5) or KLK4C (C39) were screened for reactivity against KLK4 peptides A-E by ELISPOT and shown not to cross-react with other KLK4 peptides. **f** KLK4A (A5) and KLK4C (C17) clones recognize target MHC/peptide complexes with high affinity down to peptide concentrations of 5 pg/ml

(HLA^*0201^-) cells or OVCAR-3 cells that have previously been reported to be resistant to CTL lysis [26] (Fig. 3b). We also used LNCaP cells as targets for the KLK4-specific CTL (Online Resource 4). Despite marginal expression of HLA-A2 on their surface, LNCaP cells were weakly lysed by CTL specific for a HLA-A2-restricted epitope from influenza matrix protein, following pulsing of LNCaP with the cognate peptide, and by KLK4A- but not KLK4C-specific CTL (Online Resource 4). These data suggest that the KLK4A epitope, but not the KLK4C-epitope, is naturally processed and presented by $HLA\text{-}A^*0201^+$*KLK4-254*$^+$ tumor cell lines. KLK4A-specific CTL also lysed MoDC targets that had been transfected with mRNA encoding the *KLK4-254* transcript (Fig. 3c). KLK4 mRNA expression following transfection of MoDC was confirmed up to 24 h post-transfection (Online Resource 5). Furthermore, KLK4A pentamer-specific $CD8^+$ T cells could be expanded in a prostate cancer patient using *KLK4-254* mRNA-transfected autologous MoDC as stimulators (Fig. 3c). This demonstrates that the KLK4A epitope is also endogenously processed by MoDC and may therefore be a relevant vaccine candidate.

KLK4A-specific $CD8^+$ T cells are more readily expanded in vitro compared to $PSMA_{711–719}$, $PSA_{154–163}$ and $PAP_{299–307}$ peptides

HLA-A*0201-restricted epitopes that induce CTL responses in prostate cancer patients and healthy donors have been identified in prostate cancer TAA PSMA, PSA and PAP [4–9]. We compared the ability to expand $CD8^+$ T cells specific for these epitopes with the induction of KLK4A-specific $CD8^+$ T cells in prostate cancer patients. Similar percentages of KLK4A and $PSMA_{711–719}$-specific T cells could be detected directly ex vivo in the PBMC of most donors (Fig. 4a). In contrast, the frequency of $PSA_{154–163}$- and $PAP_{299–307}$-specific T cells was much lower or below the limit of detection in the same donors (Fig. 4a). After several rounds of peptide specific in vitro stimulation, significantly higher frequencies of KLK4A-specific $CD8^+$ T cells were induced compared to $PSA_{154–163}$- and $PAP_{299–307}$-specific T cells (Fig. 4b). The percentage of KLK4A-specific $CD8^+$ T cells was also higher than that of $PSMA_{711–719}$-specific T cells in 5/6

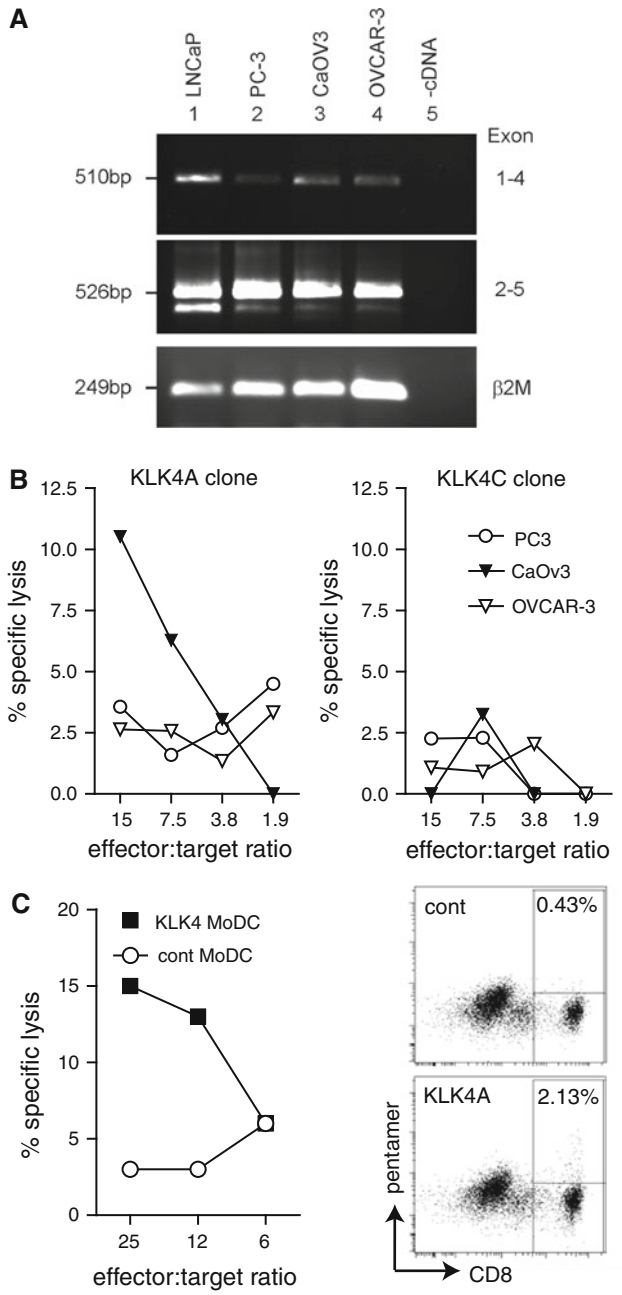

Fig. 3 KLK4A but not KLK4C is endogenously processed and presented by tumor cells and DC. **a** Expression of *KLK4-254* (Exon 1–4) and *KLK4-205* (Exon 2–5) transcripts in the prostate cancer cell lines LNCaP (HLA-A*0201^{+}), PC-3 (HLA-A*0201^{-}) and ovarian cancer cell lines CaOV3 and OVCAR-3 (both HLA-A*0201^{+}) by PCR. **b** CaOV3 cells are lysed by a CTL clone specific for KLK4A but not by a CTL clone specific for KLK4C in a ^{51}Cr release assay. **c** KLK4A is endogenously processed by MoDC. *Left*: A KLK4A CTL clone lysed *KLK4* mRNA-transfected MoDC but not mock-transfected (control) MoDC in a ^{51}Cr release assay. *Right*: MoDC were transfected with *KLK4* mRNA used to stimulate autologous CD8^{+} T cells. After three rounds of stimulation, the induction of KLK4A-specific CTL was monitored using a KLK4A-specific pentamer (*lower panel*) or no pentamer as a control (*upper panel*). The percentage of CD8^{+} cells that are pentamer^{+} are shown

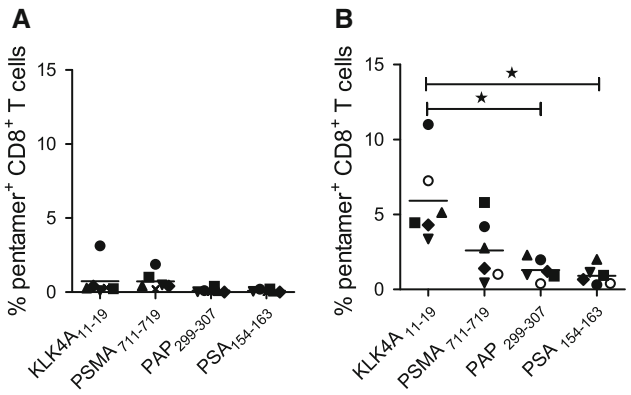

Fig. 4 KLK4A induces similar responses compared with PSMA$_{711–719}$ and superior responses compared to PSA$_{154–163}$ and PAP$_{299–307}$ peptides. **a** Precursor frequency of pentamer^{+}CD8^{+} T cells in prostate cancer patients' PBMC measured using specific MHC/peptide pentamers. **b** Maximum induction of peptide-specific T cells after up to five stimulations in prostate cancer patients (*filled symbols*) and a male healthy donor (*open symbol*). Each *symbol* represents the same donor for each peptide. *Asterisk* $P < 0.05$ by ANOVA

donors (Fig. 4b). In a further nine healthy donors and seven prostate cancer patients, we were unable to expand PSA$_{154–163}$- and PAP$_{299–307}$-specific T cells after up to 6 rounds of stimulation (data not shown). These data demonstrate that CTL specific for the KLK4A epitope are more readily expanded in vitro than other HLA-A2-restricted prostate cancer epitopes in current clinical use.

Signal peptide sequences are rich in putative HLA-A*0201-restricted epitopes

One of the distinguishing features of the KLK4A peptide, compared to the KLK4B-E, PSMA$_{711–719}$, PSA$_{154–163}$ and PAP$_{299–307}$ epitopes, is its locality within the signal sequence region of the molecule. Hydrophobic signal peptides are known to associate with HLA-A*0201 [27, 28], and a few have been shown capable of inducing tumor-specific CTL responses [29–32]. We reasoned that the signal peptide regions of *KLK4* and related kallikrein family members would be a source of putative immunogenic TAA. To investigate this, we identified the top 20 predicted HLA-A*0201-binding peptides from the full-length sequences of *KLK2,3(PSA),4,5,6,7,8,11,14* and *15* using computer algorithms BIMAS and SYFPEITHI. The TAA tyrosinase, MUC1 and calcitonin that have known immunogenic HLA-A*0201-restricted epitopes within their signal peptide regions were also included. Despite the signal peptides comprising only a small proportion (mean 9%) of the full-length molecules, they contained a high percentage (mean 27%) of the top 20 putative

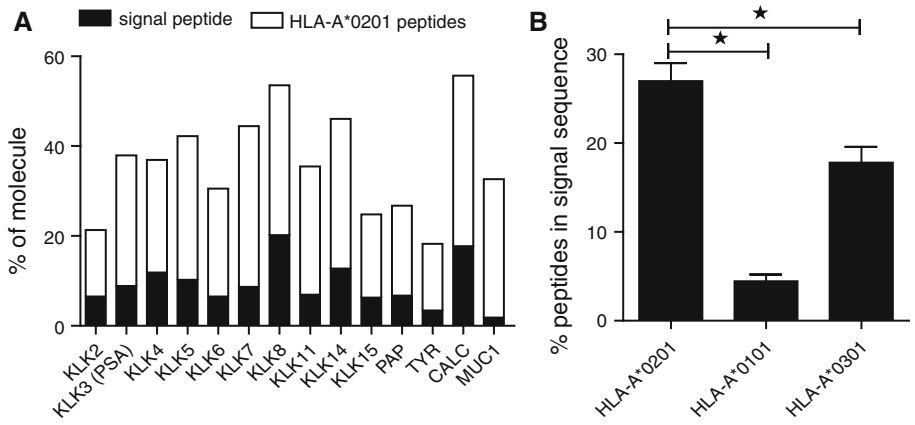

Fig. 5 Signal sequence peptides from human kallikrein molecules are rich in putative HLA-A*0201-restricted epitopes. **a** Sequences from *KLK2,3,4,5,6,7,8,11,14* and *15* were analyzed for their top 20 putative HLA-A*0201-binding peptides using computer algorithms. Included for comparison were PAP, tyrosinase (TYR), preprocalcitonin (*CALC*) and MUC1. The *filled portions* of the histograms represent the percentage of the signal sequence expressed as a proportion of the full-length molecule. The *open portions* represent the percentage of the top 20 putative HLA-A*0201-binding peptides that are located within the signal sequence. **b** Signal sequences contain significantly lower percentages of HLA-A*0101- and HLA-A*0301 putative–binding epitopes compared to HLA-A*0201. Data represent the mean ± SEM of the percentage of putative top 20 binding epitopes for each allele located within the signal sequence for the molecules analyzed in **a**

HLA-A*0201-binding epitopes (Fig. 5a). HLA-A*0101- and HLA-A*0301-binding peptides were also identified within the signal sequence molecules; however, there were significantly lower percentages of these compared to HLA-A*0201-binding peptides (Fig. 5b). These data suggest that signal sequence peptides of *KLK4* and other kallikrein family molecules could be a rich source of HLA-A*0201-restricted epitopes to harness for immunotherapy.

Discussion

The identification of new immunogenic prostate cancer TAA is essential to develop effective immunotherapy for this disease. Using the "reverse immunology" approach, we identified an immunogenic HLA-A*0201-restricted CTL epitope within the signal peptide region of *KLK4* which we termed KLK4A. CTL specific for KLK4A could be expanded from most healthy donors and prostate cancer patients in vitro. KLK4A-specific CTL were more readily expanded to higher frequencies than HLA-A*0201-restricted epitopes from known prostate cancer TAA, PSA and PAP and were at least as effective as PSMA.

The KLK4A epitope was endogenously processed and presented by tumor cells and MoDC, allowing the KLK4A-specific CTL to lyse target cancer cell lines expressing *KLK4* and HLA-A*0201. Although the levels of lysis of the tumor targets were low, these were comparable to lysis of these cell lines by other prostate and ovarian TAA [26, 33, 34] and consistent with a low-affinity T cell receptor (TCR) that is typical of CTL for most TAA. The identification of the naturally processed KLK4A epitope can now allow the development of new adoptive T cell therapies targeting this epitope where the functional avidity might be enhanced by TCR cloning and affinity maturation [35], in addition to vaccine strategies incorporating appropriate adjuvants that maximize sensitivity of low-affinity TCRs [36]. *KLK4* thus represents a new immunogenic prostate cancer target that could be utilized as an alternative or in addition to those prostate cancer TAA used in current clinical trials.

In addition to its ability to induce CTL responses, *KLK4* is a particularly attractive therapeutic vaccine candidate for the following reasons. Its overexpression in the majority of prostate cancers but much reduced levels in healthy tissue limits the potential for deleterious autoimmune side effects. The documented role of *KLK4* in contributing to prostate cancer development and progression minimizes the risk of immunoselecting *KLK4*-negative variants. Furthermore, $CD4^+$ T helper responses are now considered crucial in maintaining effective CTL responses and a number of MHC class II-restricted epitopes within *KLK4* have been identified [21]. *KLK4* is highly expressed in ovarian cancers [37, 38], and our data showed that KLK4A-specific CTL could lyse an ovarian cancer cell line. *KLK4* is elevated in breast cancer stromal cells [39], which are also relevant vaccine targets [40]. Thus, the utility of *KLK4* as an effective TAA could be applicable to immunotherapy not only for prostate cancer but for the wider application to hormone-dependent cancers.

Interestingly, the immunogenic KLK4A epitope was located within the signal sequence region that is expressed only by the less abundant but more prostate cancer specific *KLK4-254* isoform. The location of the epitope within the

signal sequence may explain why KLK4A-specific CTL responses were readily induced in both healthy donors and prostate cancer patients. Signal peptide molecules are known to associate with HLA-A*0201 molecules [27, 28], and their capacity to induce CTL has been documented in a few cases [29–32, 41, 42]. Epitopes derived from signal peptides can be directly processed and presented on MHC class I when expressed endogenously by tumor cells or DC (direct presentation) [42]. However, they are not efficiently cross-presented by DC, which is proposed as the mechanism by which peripheral tolerance to self-antigens is generated [42]. Naïve T cells specific for epitopes that are not cross-presented by DC would be unlikely to be deleted during thymic selection or induced to be tolerogenic. This would imply that a repertoire of naïve CTL precursors specific for signal peptides may be available for expansion in both healthy donors and cancer patients. Our data provide one example since we were able to more readily detect and expand CTL specific for the KLK4A epitope compared to other HLA-A*0201-restricted peptides such as $PSA_{154–163}$, $PAP_{299–307}$ and KLK4B,D,E that are located in their respective core proteins. In support of this theory, HLA-A*0201-restricted immunogenic epitopes have been identified within the signal peptides of other human TAA, including the melanoma antigen tyrosinase [30, 31] and the medullary thyroid and lung carcinoma–associated *CALCA* gene [29]. Notably, a HLA-A*0201-restricted epitope is located within the signal sequence of MUC1 and is immunodominant in breast cancer patients compared to a HLA-A*0201-restricted epitope located in the MUC1 core protein tandem repeat [32, 43].

Our data suggest that in addition to *KLK4*, signal sequences of other kallikrein family members may also be a rich source of HLA-A*0201-restricted peptides, which may potentially be immunogenic for the reasons outlined above. An array of kallikrein family members are overexpressed in hormone-dependent cancers and many are associated with disease progression and poor prognosis [11, 44]. The majority (*KLK4-11,14,15*) are overexpressed in ovarian cancer, and many of these (*KLK4-7, 10,15*) are associated with later stage disease and decreased survival. *KLK14* and *15* are associated with disease progression in prostate cancer, while *KLK5* and *14* are associated with breast cancer. *KLK5,6,7,10,11,14* have also been associated with poor prognosis in colorectal cancers [44]. For these malignancies, there are few suitable TAA currently available. Based on our data, exploration of the signal peptide regions of the kallikrein family may reveal a new source of TAA that could extend the potential of immunotherapy as a treatment for these diseases.

Acknowledgments We thank Sonia Hancock, Georgina Crosbie, Stephanie Diaz-Guilas and Peter Swindle for provision of patient samples. This work was supported by funding from the Cancer Council Queensland, the Prostate Cancer Foundation Australia and the Mater Foundation, Mater Hospitals, Brisbane Australia.

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