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ORIGINAL ARTICLE

A naturally processed HLA-DR-bound peptide from the IL-9 receptor alpha of HTLV-1-transformed T cells serves as a T helper epitope

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Abstract Human T cell leukemia virus type 1 (HTLV-1) induced adult T cell leukemia/lymphoma (ATLL) is usually a fatal lymphoproliferative malignant disease. Thus, the enhancement of T cell immunity to ATLL through the development of therapeutic vaccines using characterized T cell peptide epitopes could be of value. We isolated and characterized HLA-DR-bound peptides from HTLV-1-transformed T cells by fractionating on reverse-phase high performance liquid chromatography and Edman NH_2-terminal sequencing and were able to identify five independent peptide sequences. One of the identified peptide sequences corresponded to a fragment of the human interleukin-9 receptor alpha (IL-9Rα), which is commonly expressed by HTLV-1-infected T cell lymphoma cells. Using a synthetic peptide corresponding to the identified IL-9Rα sequence, we generated antigen-specific CD4 helper T lymphocytes in vitro, which were restricted by HLA-DR15 or HLA-DR53 molecules and could recognize and kill HTLV-1+, IL-9Rα+ T cell lymphoma cells. These results indicate that IL-9Rα functions as T cell leukemia/lymphoma-associated antigen for CD4 T cells and that synthetic peptides such as the one described here could be used for T cell-based immunotherapy against IL-9Rα positive ATLL.

Keywords HTLV-1 · IL-9 receptor · Adult T cell leukemia/lymphoma · Tumor antigens · Major histocompatibility complex class II · CD4 helper T lymphocytes

Introduction

Major histocompatibility complex (MHC) class II molecules (MHC-II) are heterodimeric peptide receptors that bind antigenic peptides processed in the endosomal compartments, which can be presented to CD4 helper T lymphocytes. Although MHC class I-restricted CD8 cytotoxic T lymphocytes (CTLs) can be effective in recognizing and destroying tumor cells, CD4 T cells play an important role in the priming and maintenance of tumor-specific CD8 CTLs [10]. Moreover, in some instances, CD4 T cells are also effective in killing MHC-II expressing tumor cells [17]. Several MHC-II-restricted CD4 T cell epitopes have been identified by cDNA tumor expression cloning using patient-derived CD4 T cells [29]. Additional CD4 T cell epitopes have been identified by reverse immunology using computer-based algorithms that predict MHC-II binding peptides from putative tumor-associated antigens (TAAs), followed by validation through the in vitro induction of anti-tumor CD4 T helper responses against the predicted epitopes [16]. Lastly, in some instances, researchers have identified CD4 T cell epitopes by elution and sequencing of MHC-II-bound self-peptides derived from melanoma or Epstein-Barr virus-transformed B lymphoblastoid cell lines (EBV-LCLs) [13, 25, 27]. In the present report, we have followed the last strategy to identify endogenous

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HLA-DR-associated peptides in adult T cell leukemia/lymphoma (ATLL) cells with the goal of developing CD4 T cell peptide immunotherapy against this hematological malignancy. We describe here that a peptide derived from the IL-9Rα that was isolated from HLA-DR molecules purified from HTLV-1-transformed T cells was able to induce in vitro CD4 T helper responses to T cell lymphoma cells.

Materials and methods

Cell lines

EBV-LCLs were produced from peripheral blood mononuclear cells (PBMCs) of HLA-typed volunteers using culture supernatant from the EBV-producing B95-8 cell lines, obtained from American Type Culture Collection (ATCC, Manassas, VA). Mouse fibroblast cell lines (L cells) transfected and expressing individual human MHC-II molecules were kindly provided by Dr. Robert W. Karr (Idera Pharmaceuticals, Essex, CT). The HTLV-1 infected T cell lymphoma cell lines, TL-Su, TCL-Kan, Hut102 and TL-Hir were supplied by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). The HTLV-1 infected T cell lymphoma cell line OKM-2T was purchased from Dainippon Sumitomo Pharma (Osaka, Japan). MT2 is an HTLV-1-transformed T cell line [23]. The Jurkat T cell lymphoma cell line and prostate tumor cell line PC3 were purchased from ATCC. To generate anti-CD3 monoclonal anitibody (mAb)-induced T cell blasts, PBMCs (2×10^6/ml) were treated with 30 ng/ml OKT3 and recombinant human IL-2 (50 U/ml) in medium consisting of RPMI-1640 medium supplemented with 10 % FCS. The expression of HLA-DR molecules and IL-9Rα on tumor cells was evaluated by flow cytometry using anti-HLA-DR mAb conjugated with phycoerythrin (PE) (BD Pharmingen, San Diego, CA) and anti-human IL-9Rα conjugated with PE (BioLegend, San Diego, CA), respectively.

Purification of HLA-DR molecules

HLA-DR molecules from MT2 cells were purified by means of affinity chromatography using a mAb L243 (anti-HLA-DR, IgG2a, prepared from supernatants of the hybridoma HB-55 and obtained from ATCC)-coupled CNBr-activated Sepharose (GE Healthcare, UK) column [9]. MT2 cells were lysed in phosphate-buffered saline (PBS) containing 1.5 % Nonidet-P40, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM EDTA for 2 h at 4 °C. Detergent-insoluble materials were removed by centrifugation at 10,000*g* for 20 min. The supernatant containing the HLA molecules were precleared first by bovine γ-globulin and then the effluent applied for L243-coupled CNBr-activated Sepharose. The column was washed with 10 column volumes of PBS containing 0.1 % Nonidet-P40, 2 column volumes of PBS and 2 column volumes of PBS containing 1 % octylglucoside. The column was eluted with 0.05 M diethylamine in 0.15 M NaCl containing 1 % octylglucoside (pH 11.5). The eluates were immediately neutralized with 0.1 M Tris–HCl (pH 6.8), concentrated by ultrafiltration on a Minicon (Amicon, Denvers, MA) and stored at −30 °C. Protein content was evaluated by means of a bicinchoninic acid protein assay (Pierce Chemical Co., Rockford IL) and confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Preparation of bound peptides

The elution of HLA-DR-bound peptides was performed in 10 % acetic acid at 37 °C for 30 min. The released peptides were separated from proteins by using ultrafiltration on a 3 kDa cartridge (Centricon SR3, Amicon) and stored at −70 °C until HPLC separation. The acid-eluted crude peptide fraction was separated by reverse-phase HPLC using a C2/C18 RPC column (Pep-S 4.0 × 250 mm, GE Healthcare) under the following conditions: Buffer A was 0.06 % trifluoroacetic acid (TFA) in H_2O and buffer B was 0.052 % TFA in 80 % acetonitrile in H_2O. Elution was done with a gradient system of 0–80 % B for 5–84 min with the flow rate of 0.5 ml/min. Absorbance was measured at 210 nm. Fractions were collected and stored at −70 °C until sequencing. The amino acid sequence of each peptide peak was determined by automated Edman microsequencing (477A; Applied Biosystems, Foster City, CA).

Synthetic peptides

The peptides for the induction of antigen-specific CD4 T cells were synthesized by solid-phase organic chemistry and purified by HPLC. The purity (>80 %) and identity of peptides were assessed by HPLC and mass spectrometry, respectively. Biotinylated peptide for binding assay was purchased from Hokkaido System Science (Sapporo, Japan).

Binding assay

L cells expressing individual HLA-DR molecules were used for direct binding of biotinylated peptides to antigen presenting cells (APCs) [8, 9]. The expression of HLA-DR molecules was evaluated by flow cytometry using biotinylated mAb L243 (BioLegend), followed by PE-streptavidin. L cell transfectants (3×10^5/well) were incubated with

biotinylated peptide (50 μM) in Dulbecco's modified Eagle's medium and incubated at 37 °C for 16 h in 96-flat-bottomed-well culture plate. After collecting L cell transfectants, cells were incubated with 50 ng of PE-streptavidin for 30 min at 4 °C. After washing, specific peptide binding was measured by flow cytometry. For inhibition of binding, mAb L243 (final 20 μg/ml) was added to the first incubation mixture together with the biotinylated peptide.

In vitro induction of antigen-specific CD4 T lymphocytes with synthetic peptides

The procedure utilized for the generation of human IL-9Rα peptide-reactive CD4 T cell lines using peptide-stimulated lymphocytes from PBMCs of normal human volunteers has been described in detail [18]. Briefly, dendritic cells (DCs) were produced from purified CD14 monocytes (using antibody-coated magnetic microbeads from Miltenyi Biotech, Auburn CA) that were cultured for 7 days at 37 °C in a humidified CO_2 (5 %) incubator in the presence of 50 ng/ml GM-CSF and 1,000 IU/ml IL-4. Peptide-pulsed DCs (3 μg/ml for 2 h at room temperature) were irradiated (4,200 rads) and co-cultured with autologous purified CD4 T cells (Miltenyi Biotech Inc, Auburn, CA) in 96-flat-bottomed-well culture plates. One week after peptide stimulation, the CD4 T cells were restimulated in individual microcultures with peptide-pulsed irradiated autologous PBMCs, and 2 days later, recombinant human IL-2 was added at a final concentration of 10 IU/ml. One week later, the T cells were tested for antigen reactivity using a cytokine-release assay as described below. Those microcultures exhibiting a significant response of cytokine-release to peptide (at least 2.5-fold over background) were cloned by limiting dilution and expanded in 24- or 48-well plates by weekly restimulation with peptides and irradiated autologous PBMCs. Complete culture medium for all procedures consisted of AIM-V medium (Invitrogen/GIBCO, Carlsbad CA) supplemented with 3 % human male AB serum. All blood samples were obtained after the appropriate informed consent.

Measurement of antigen-specific responses with CD4 T cell clones

CD4 T cells (3×10^4/well) were mixed with irradiated APCs in the presence of various concentrations of antigen in 96-well culture plates. APCs consisted of autologous PBMCs (1×10^5/well), HLA-DR-expressing L cells (3×10^4/well), HTLV-1 infected T cell lymphoma, MHC-typed EBV-LCL, anti-CD3 mAb-induced T cell blasts and Jurkat T cell lymphoma (3×10^4/well). Culture supernatants of the T cell activation assays were collected after 48 h for measuring antigen-induced lymphokine (GM-CSF or IFN-γ) production by the CD4 T cells using ELISA kits (BD Pharmingen, San Diego, CA). To demonstrate antigen specificity and MHC restriction, blocking of antigen-induced responses were assessed by adding anti-HLA-DR mAb L243 or anti-HLA-A/B/C mAb W6/32 (IgG2a, ATCC) at 10 μg/ml throughout the 48 h antigen stimulation period. All ELISA determinations were carried out in triplicates and results correspond to the mean values with the standard deviation (SD) of the means.

Western blot analyses

One million tumor cells were washed in PBS and lysed in NuPAGE LDS sample buffer (Invitrogen). The cell lysate was subjected to electrophoresis in a 4–12 % NuPAGE bis–Tris SDS-PAGE gel (Invitrogen) under reducing condition and then transferred to Immobilon-P (Millipore, Bedford, MA) membrane. The membrane was then blocked in PBS containing 0.01 % Tween 20 and 5 % nonfat dry milk for 1 h at room temperature and incubated with mouse anti-human IL-9Rα mAb (clone 33423, R&D Systems, Minneapolis, MN) and mouse anti-β-actin (C4) (Santa Cruz Biotechnology, Santa Cruz, CA) as the control overnight at 4 °C. After washing, the membrane was incubated with horseradish peroxidase-labeled sheep anti-mouse IgG and subjected to the enhanced chemiluminescence assay using the ECL detection system (Amersham, Buckinghamshire, UK).

Cell-mediated cytotoxicity assays

Cytotoxic activity of CD4 T cells was measured using colorimetric CytoTox 96 assay (Promega, Madison WI). This system quantifies the release of lactate dehydrogenase (LDH) from target cells. T cells were mixed with 2×10^4 targets at different effectors to target (E:T) ratios in 96-round-bottomed-well plates. After 6–9 h incubation at 37 °C, a 50 μl sample of supernatant was collected from each well to measure LDH content. To correct for spontaneous LDH release from effector cells, LDH levels were measured for each individual effector cell concentration used in the experimental set up (effector spontaneous). All measured values were assayed in triplicate and corrected for the culture medium LDH background. The percentage of specific LDH release was determined as $\%\ \text{cytotoxicity} = [(\text{experimental} - \text{effector spontaneous} - \text{target spontaneous})/(\text{target maximum} - \text{target spontaneous})] \times 100$.

Results

Purification and sequencing of self-peptides derived from HLA-DR molecules from HTLV-1 infected T cells

The HLA-DR-bound peptides, which were eluted from 800 μg of HLA-DR molecules purified from the HTLV-1 infected T cell line MT2 (HLA-DR4/-15/-53), were separated by reverse-phase HPLC (Fig. 1). Several prominent peptide peaks were observed broadly in the HPLC chromatogram. Single-fraction sequencing was performed by Edman degradation, and the resultant five definitive sequences were obtained (Table 1). Of these five sequences, peaks 3, 4 and 5 were identified by homology search with the SwissProt protein sequence database as fragments of human von Willebrand factor, α-1-antitrypsin and human IL-9Rα, respectively (Table 1). The origins of the two remaining peptides were not identified.

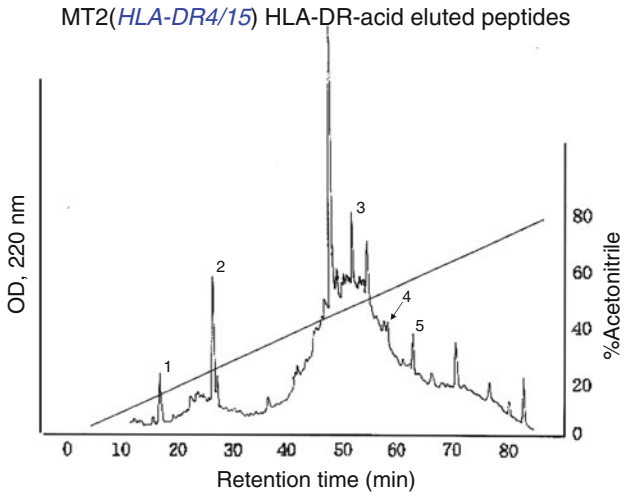

Fig. 1 HLA-DR molecules were purified by immunoaffinity chromatography using the HLA-DR specific mAb L243 from the detergent lysate of an HTLV-1-transformed T cell line MT2. Reversed-phase HPLC separation of HLA-DR-bound peptides isolated from MT2 cells was performed using C2/C18 column. Elution times of 5 and 84 min correspond to 0–80 % acetonitrile in eluates, respectively

Table 1 Amino acid sequences of endogenous peptides eluted from HLA-DR molecules of the HTLV-1 infected human T cell line, MT2

Source protein	Position	Sequence	Peak
Unknown		FRRGK	1
Unknown		NIVMTQSP	2
Human von Willebrand factor	1,336–1,347	RPXELRRIASQV	3
α-1-antitrypsin	383–398	PPDVEFNRRFLXILYD	4
Human IL-9 receptor	168–182	ISPALEPMTTLLSYE	5

Binding capacity and specificity of peptide IL-9R$\alpha_{168–182}$ to HLA-DR molecules

MT2 cells constitutively express IL-9R because of cis/transactivation of the IL-9Rα gene by integration of HTLV-1 into the 5′ coding sequence of the IL-9Rα gene [19]. In addition, it has been shown that IL-9R is expressed on some HTLV-1 negative T cell leukemias [2, 6]. Thus, the above findings suggest that the IL-9R$\alpha_{168–182}$ peptide eluted from purified HLA-DR molecules from HTLV-1 infected T cells could function as a naturally processed antigen recognized by CD4 T cells either on the context of the HLA-DR4, HLA-DR15 and/or HLA-DR53 molecules. To evaluate this possibility, we first determined the capacity of synthetic peptide, IL-9R$\alpha_{168–182}$ to bind to the individual MHC-II molecules. Biotinylated peptide was used to examine the direct binding to the cell surface MHC-II molecules on HLA-DR gene-transfected L cells by measuring the fluorescence intensity on the cells by flow cytometry. As shown in Fig. 2a, L-DR15 and L-DR53 cells were capable of binding peptide IL-9R$\alpha_{168–182}$. On the other hand, no significant peptide binding was observed with L-DR4 and L-DR9 cells. The peptide binding to L-DR15 or L-DR53 cells was blocked to a great extent by the addition of anti-HLA-DR mAb (L243), confirming that the IL-9R$\alpha_{168–182}$ peptide was binding to cell surface HLA-DR molecules (Fig. 2b).

IL-9R$\alpha_{168–182}$ is an immunogenic epitope for stimulating CD4 T cells

We next examined whether IL-9R$\alpha_{168–182}$ could function as a T cell lymphoma/leukemia CD4 T cell epitope. A peptide corresponding to IL-9R$\alpha_{168–182}$ was synthesized and evaluated for its capacity to elicit CD4 T cell responses by in vitro vaccination of PBMCs from four healthy individuals (HLA-DR1/15, HLA-DR4/9, HLA-DR4/15 and HLA-DR9/14) as described in "Materials and methods". Three peptide-reactive CD4 T cell clones were isolated from three separate donors. As shown in Fig. 3, the IL-9R$\alpha_{168–182}$-reactive CD4 T cell clones, K25 (from the HLA-DR1/15 donor), KS19 (from the HLA-DR4/15 donor) and H21 (from the HLA-DR4/9 donor) responded to the synthetic peptide presented by autologous PBMCs in a dose-dependent manner. It should be noted that all three CD4 T cell clones predominantly secreted GM-CSF and IFN-γ in response to the peptide stimulation and that no IL-4 or IL-5 could be detected (data not shown), indicating that these T cells behaved as typical Th-1 CD4 T cells.

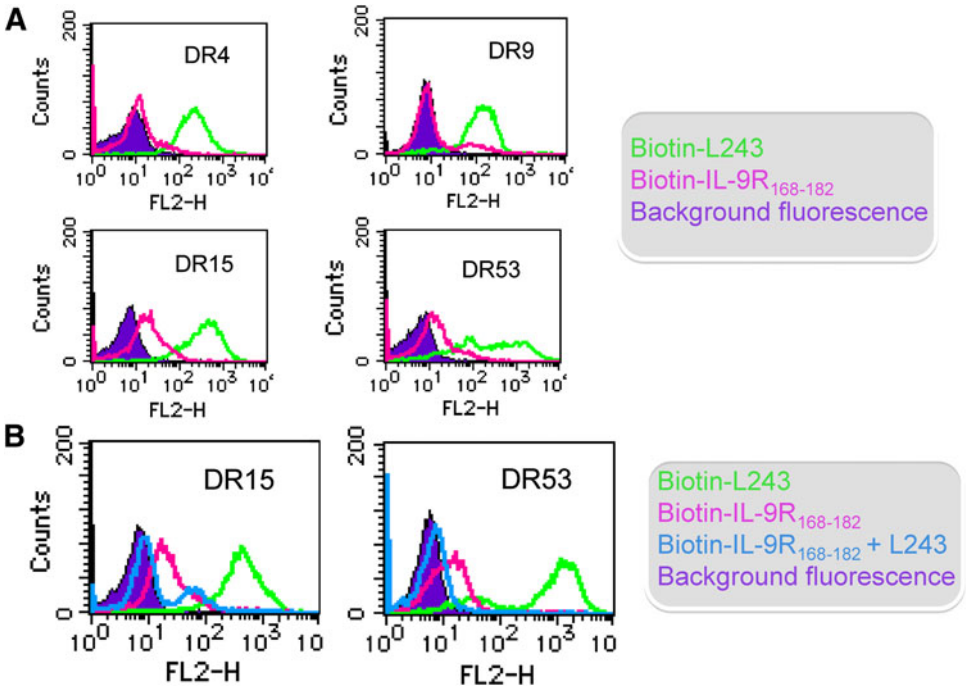

Fig. 2 Binding of biotinylated peptide IL-9R$\alpha_{168–182}$ to HLA-DR gene-transfected mouse L cells. **a** L cells expressing individual HLA-DR alleles (L cell transfectants) were incubated with the biotinylated peptide (50 μM) for 16 h and stained with PE-streptavidin (*red lines*). Background fluorescence in the absence of biotinylated peptide (*filled histograms*). **b** Inhibition of binding of the biotinylated IL-9R$\alpha_{168–182}$ by anti-HLA-DR mAb L243. L cell transfectants were co-incubated with the biotinylated peptide and anti-DR mAb L243 (20 μg/ml) for 16 h and stained thereafter with PE-streptavidin (*blue lines*). Staining of the cells incubated with the biotinylated peptide (*red lines*). Background fluorescence in the absence of biotinylated peptide (*filled histograms*). Expression of HLA-DR molecules on L cell transfectants evaluated by stained with biotinylated mAb L243 (*green lines*)

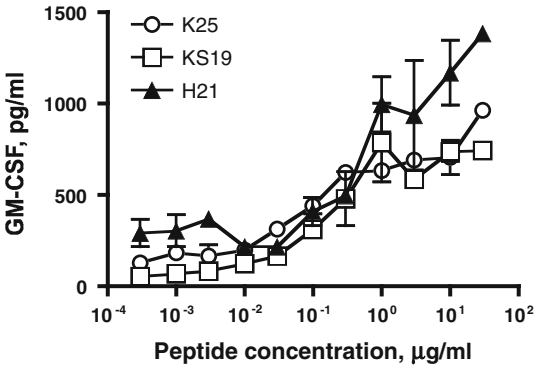

Fig. 3 Induction of CD4 T cell responses using peptide IL-9R$\alpha_{168–182}$. Peptide-elicited CD4 T cell clones were induced from three donors (K25 from HLA-DR1/15 donor, KS19 from HLA-DR4/15 donor and H21 from HLA-DR4/9 donor) and tested for their capacity to recognize autologous PBMCs as APCs in the presence of various concentrations of peptide. *Points* means of triplicate determinations, *bar* SD. *Points without bars* had SD < 10 % the values of the mean. Results are representative of two experiments that were done with the same sample

MHC restriction analysis of IL-9R$\alpha_{168–182}$-reactive CD4 T cells

The IL-9R$\alpha_{168–182}$-reactive T cell clones were analyzed for their MHC-II-restriction elements first by using mAbs specific for HLA-DR molecules (L243) or HLA class I molecules (W6/32, as a negative control) to inhibit the recognition of peptide presented by autologous PBMCs. Lack of inhibition by both of these antibodies would suggest possible restriction by HLA-DQ or HLA-DP molecules, which are not recognized by the L243 mAb. As shown in Fig. 4, recognition of peptide-pulsed autologous PBMCs by all CD4 T cell clones was inhibited by the addition of anti-HLA-DR mAb but not by the anti-HLA class I mAb, indicating that the presentation of IL-9R$\alpha_{168–182}$ to these CD4 T cell clones is via HLA-DR molecules and not HLA-DQ, HLA-DP, or HLA class I. In view of these results, T cell responses to IL-9R$\alpha_{168–182}$ were evaluated using a panel of HLA-DR-transfected mouse L cells as APCs to determine the specific HLA-DR alleles capable of presenting peptide to the T cells. The results revealed that IL-9R$\alpha_{168–182}$-reactive CD4 T cell clones K25 and KS19 recognized antigen in the context of HLA-DR15 while clone H21 was restricted by HLA-DR53 (Fig. 4). Moreover, all three IL-9R$\alpha_{168–182}$-reactive T cell clones did not react with irrelevant peptides (peptide HTLV-1 $Tax_{305–319}$ restricted by HLA-DR15 or DQ9 molecules and peptide EBV $LMP1_{159–175}$ restricted by HLA-DR9, DR15, or DR53 molecules, ref. 16), demonstrating that the recognition of IL-9R$\alpha_{168–182}$ was antigen-specific.

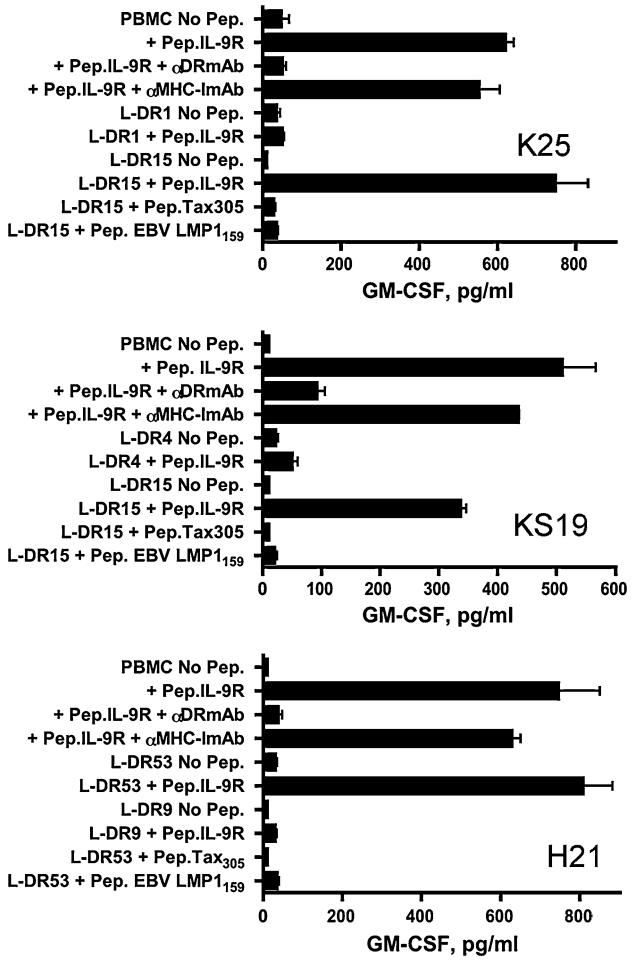

Fig. 4 MHC restriction analysis of peptide IL-9R$\alpha_{168–182}$-reactive CD4 T cell clones. MHC restriction molecules were identified using antibody-blocking assays. Monoclonal Abs specific for HLA-DR (L243) used at 10 μg/ml but not for HLA-A, B and C (W6/32) inhibited the lymphokine production from the all CD4 T cell clones. In addition, CD4 T cell responses were also evaluated using various HLA-DR expressing L cell transfectants to define the restricting MHC class II molecules. *Columns* means of triplicate determinations, *bars* SD. *Columns without bars* had SD < 10 % the values of the mean. Results are representative of two experiments that were done with the same sample

Recognition of HTLV-1+ T cell lymphomas by IL-9R$\alpha_{168–182}$-specific CD4 T cells

Next, we assessed the capacity of the peptide-reactive T cells to directly recognize HTLV-1+ T cell lymphoma cells expressing IL-9R. Only by demonstrating that the peptide-reactive CD4 T cells are able to recognize MHC-II-bound naturally processed antigen would indicate that this epitope would be a potential immunotherapeutic target. However, before performing these experiments, we first evaluated the expression of IL-9Rα and MHC-II molecules on the cell surface of several HTLV-1+ T cell lymphoma cell lines that would be used as APCs. As shown in Fig. 5a, IL-9Rα was expressed on HTLV-1+ MT2 cells and also on the HTLV-1 negative Jurkat T cell lymphoma. On the other hand, IL-9Rα was not observed on other HTLV-1+ T cell lymphoma cell lines (Hut102, Kan, OKM2T, Hir and Su), on EBV-LCL, PC3 (prostate adenocarcinoma), PBMCs and on anti-CD3 mAb-induced T cell blasts. The expression of IL-9Rα protein on various cell types was also evaluated by Western blot analysis using whole cell lysates. As shown in Fig. 5b, the IL-9Rα protein was detected in all the HTLV-1+ T cell lymphoma cell lines tested (MT2, HUT102, Kan, OKM2T, Hir and Su) and in HTLV-1 negative Jurkat T cell lymphoma. On the other hand, IL-9Rα protein was not detected in EBV-LCLs and PC3 cells. Using Western blots, the IL-9Rα protein was also detected, but in much lower amounts (compared to MT2 cells), in PBMCs and anti-CD3 mAb (OKT3)-stimulated T cells from healthy individuals (Fig. 5c). These observations indicate that although low expression levels of surface IL-9Rα were observed on some HTLV-1+ T cell lymphomas, this protein could be present intracellularly in other tumor cells. As expected, expression of surface MHC-II was clearly observed in all the HTLV-1+ T cell lymphoma cell lines (and on the EBV-LCLs) using HLA-DR specific monoclonal antibodies (data not presented).

With this information on hand, we assessed the capacity of IL-9R$\alpha_{168–182}$-reactive CD4 T cells to recognize various T cell lymphoma cell lines that express or not the IL-9Rα and the appropriate MHC-II molecules. Both HLA-DR15-restricted CD4 T cell clones, K25 and KS19 were effective in recognizing DR15+, IL-9R+ HTLV-1 infected T cell lymphoma cells (MT2 and Hut102), but as expected, were unable to react with DR15+, IL-9Rα negative EBV-LCL, DR negative, IL-9R+ Jurkat T cell lymphoma or with anti-CD3 mAb-stimulated T cells (Fig. 6). Similarly, the DR53-restricted T cell clone H21 was able to recognize the DR53+, IL-9R+ HTLV-1+ T cell lymphomas, MT2 and Kan in an antigen-specific manner. Moreover, the anti-tumor reactivity of the T cells was significantly inhibited by anti-HLA-DR mAb (L243), indicating that the IL-9R$\alpha_{168–182}$ epitope is recognized by the T cells in the context of MHC-II.

Cytotoxic activity of peptide IL-9R$\alpha_{168–182}$-specific CD4 T cell clones

It is known that CD4 T lymphocytes may exhibit cytolytic activity against MHC-II+, virally infected or tumor cells [14, 17]. Thus, we investigated whether the IL-9R$\alpha_{168–182}$-specific CD4 T cell clones were capable of killing HTLV-1+, IL-9R+ T cell lymphoma cells. The results presented in Fig. 7 demonstrate that all three CD4 T cell clones were

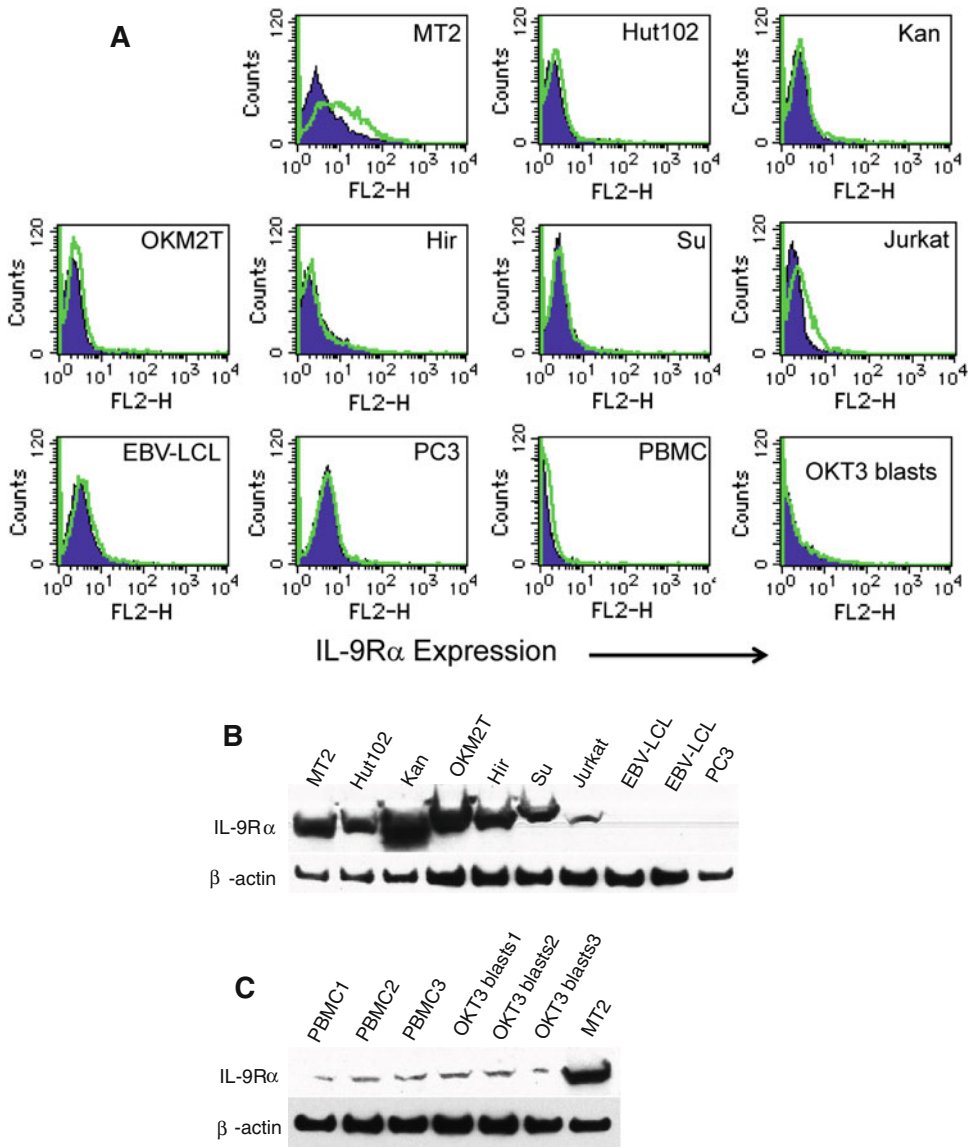

Fig. 5 Evaluation of the expression of IL-9R protein in various cells. **a** Expression of cell surface IL-9R on various cells was evaluated by flow cytometry using anti-human IL-9R mAb conjugated with PE (*green lines open histograms*). Staining with the isotype-negative control (*filled histograms*). **b** and **c** Western blot analysis was done using a human IL-9R-specific mAb and *β*-actin-specific mAb as the control as described in "Materials and methods". The IL-9R protein has a mass of approximately 57 kDa

quite effective in killing the T cell lymphomas in an MHC-II-restricted and antigen-specific manner.

Recognition of naturally processed IL-9Rα protein presented by DCs

Lastly, we evaluated whether professional APCs such as DCs would be able to capture and process antigens derived from dead IL-9Rα-expressing T cell lymphoma cells and present the IL-9R$\alpha_{168–182}$ epitope to antigen-specific CD4 T cell clones. The results in Fig. 8 demonstrate that all three CD4 T cell clones, K25, KS19 and H21 were capable of recognizing antigen prepared from cell lysates of IL-9Rα positive T cell lymphomas (MT2, Kan and Jurkat) and that these responses were inhibited by the addition of anti-HLA-DR mAbs. On the other hand, almost no reactivity was evident when the CD4 T cell clones were stimulated with unpulsed DCs or DCs pulsed with EBV-LCL lysate. These results indicate that professional APCs are effective in processing IL-9Rα protein derived from dead tumor cells and present the IL-9R$\alpha_{162–182}$ epitope to CD4 T cells in the context of HLA-DR15 and DR53.

Discussion

One way of improving current therapeutic cancer vaccines that solely stimulate CTLs is concurrently inducing CD4 T cell responses that would provide immunological help and perhaps assist with the destruction of the tumor cells. Thus, there is a pressing need to identify the respective tumor-specific CD4 T helper epitopes. Most TAA and their HLA class II-restricted CD4 T cell epitopes have been identified by either T cell-monitored cDNA expression cloning [29]

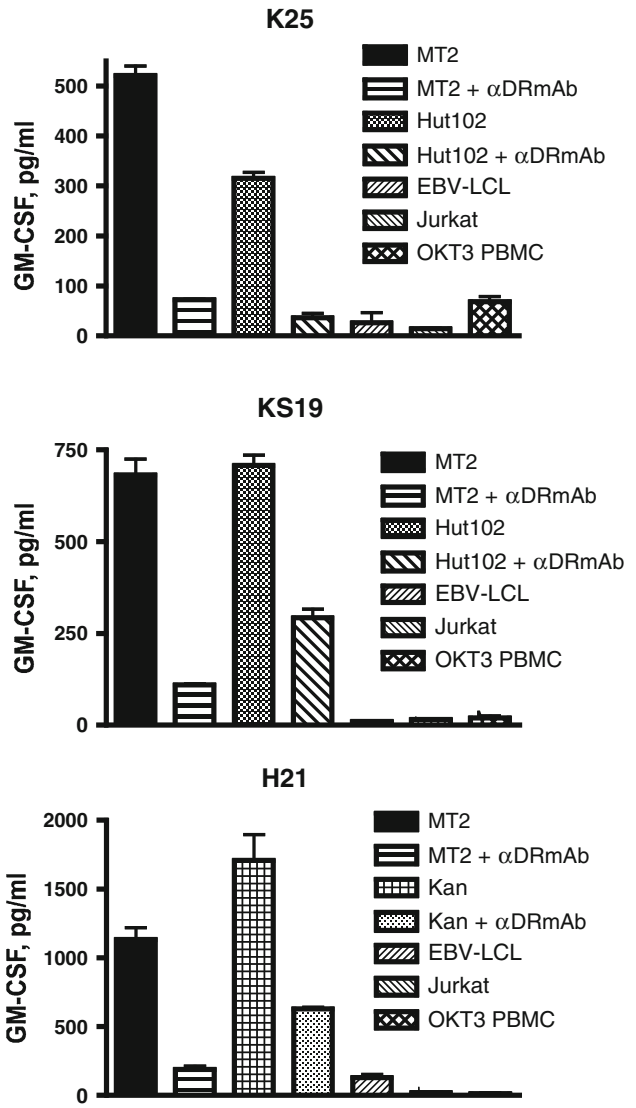

Fig. 6 Direct recognition of IL-9Rα-expressing HTLV-1 infected T cell lymphoma cells by IL-9R$\alpha_{168–182}$-reactive CD4 T cell clones. HLA-DR15-restricted CD4 T cell clones K25 and KS19 (*top* and *middle*, respectively) and HLA-DR53-restricted CD4 T cell clone H21 (*bottom*) were tested for their capacity to recognize antigen directly on HLA-DR15 or DR53 expressing, IL-9R+ T cell lymphoma cells, IL-9 negative EBV-LCLs, DR negative, IL-9R expressing Jurkat T cell lymphoma, or autologous anti-CD3 mAb-induced T cell blasts. The antigen specificity of these responses was demonstrated by blocking tumor recognition with anti-DR mAb L243. *Columns* means of triplicate determinations, *bars* SD. *Columns without bars* had SD < 10 % the values of the mean. Results are representative of two experiments that were done with the same sample

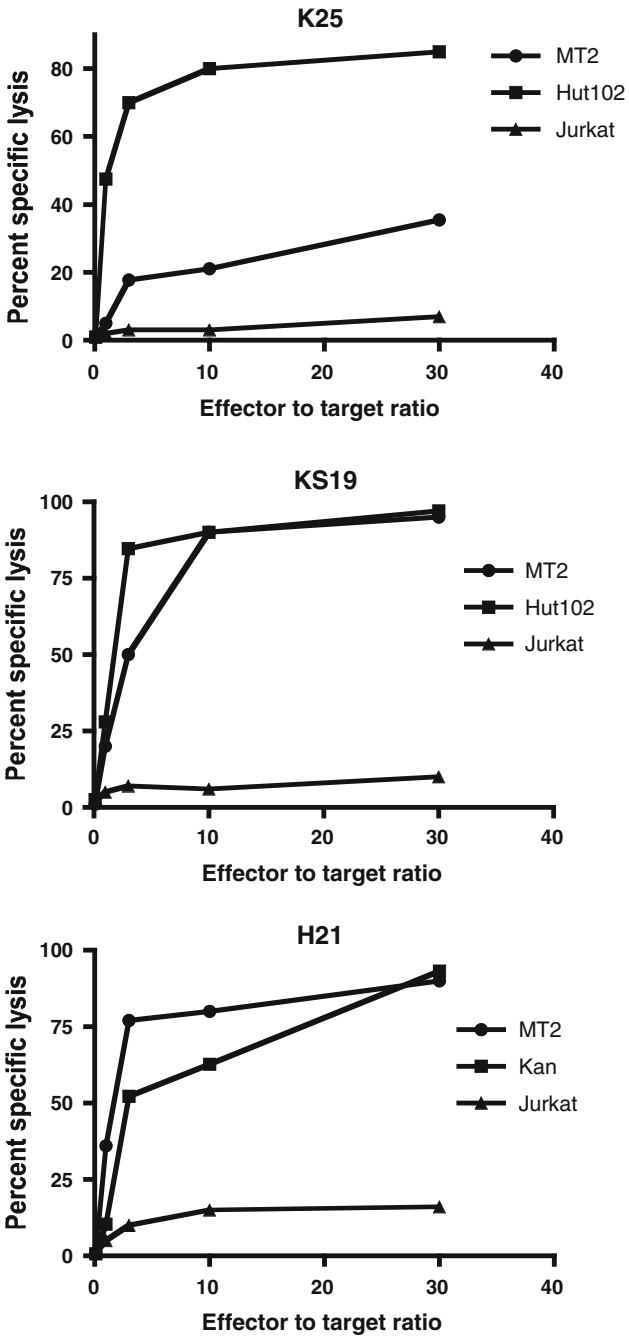

Fig. 7 Assessment of cytolytic activity of IL-9R$\alpha_{168–182}$-specific CD4 T cell clones. The DR15-restricted CD4 T cell clones K25 and KS19 (*top* and *middle*, respectively) and DR53-restricted clone H21 (*bottom*) were evaluated for their capacity to kill IL-9R expressing, DR15+ or DR53+ T cell lymphomas (MT2, Hut102, or Kan) or DR negative, IL-9R expressing Jurkat T cell lymphoma (negative control). *Points* means of triplicate determinations, *bar* SD. *Points without bars* had SD < 10 % the values of the mean. Results are representative of two experiments that were done with the same sample

or reverse immunology using computer-based algorithms that predict peptide binding to HLA class II molecules [16]. A more direct, but difficult, way to identify potential T cell epitopes is to directly isolate and sequence MHC-binding peptides from the surface of tumor cells [13, 15, 27]. However, it is evident that not all MHC-binding peptides on tumor cells will represent tumor-reactive T cell epitopes, since in many instances these peptides will correspond to sequences of proteins expressed by many cell

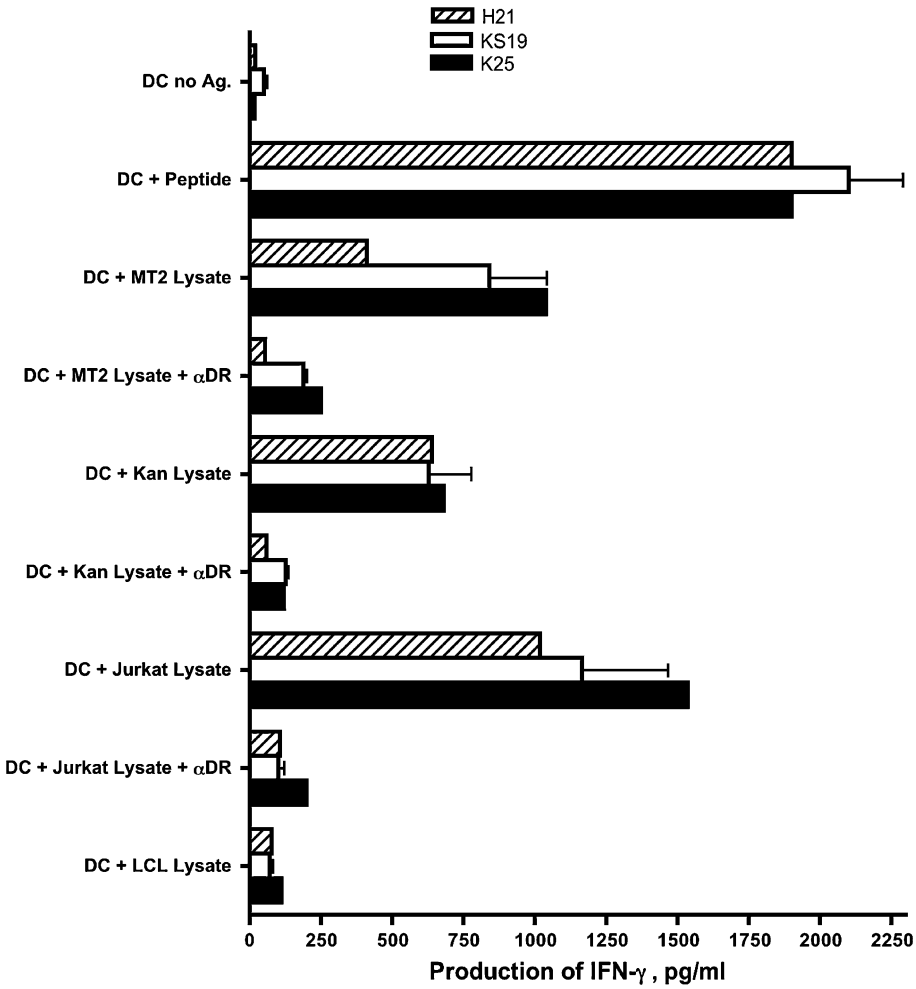

Fig. 8 Presentation of naturally processed IL-9R$\alpha_{168–182}$ epitope to CD4 T cell clones by DCs. Autologous DCs were pulsed with tumor cell lysates or IL-9R$\alpha_{168–182}$ peptide and then tested for their ability to stimulate CD4 T cell clones K25, KS19 and H21. EBV-LCL lysate was used as negative control and peptide-pulsed DCs as positive control. The capacity of anti-DR mAb L243 (αDR) was assessed to determine that T cell reactivity was through TCR/MHC interactions. *Columns* mean of triplicate determinations; *bars* SD. *Columns without bars* had SD < 10 % the value of the mean. Results are representative of at least two experiments

types and throughout various tissues. In these cases, problems such as immune tolerance or potential autoimmune diseases would preclude the use of broadly expressed MHC-binding peptides as components for T cell epitope-based immunotherapies. Nevertheless, our results indicate that an MHC-II binding peptide derived from the IL-9Rα chain, which has been reported to be expressed by numerous cell types such as T and B lymphocytes, mast cells, hematopoietic progenitor cells, some epithelial and smooth muscle cells [12], was able to generate in vitro CD4 T cell responses against T cell leukemia/lymphomas. Thus, it appears that immune tolerance to this T cell epitope is not sufficiently strong to prevent the induction of anti-tumor responses. On the other hand, little if any, T cell reactivity was observed against PBMCs and T cell blasts (Fig. 6), which express IL-9Rα. Furthermore, throughout our studies, we did not observe significant background responses in the absence of peptide or tumor cells, indicating that the CD4 T cells generated with epitope IL-9R$\alpha_{168–182}$ do not express sufficient MHC class II/peptide complexes themselves to become autoreactive. These results may be explained simply due to our observations that the T cell leukemia/lymphoma cells express much higher levels of IL-9Rα as compared to the normal cells (Fig. 5). In addition, it is also possible that HTLV-1 infected/transformed T cells produce and process the IL-9Rα molecule through their MHC-II pathway in a different manner as compared to normal cells and that normal cells do not generate the IL-9R$\alpha_{168–182}$ proteolytic product. The observations that some HTLV-1 infected/transformed T cells contain a cis/trans-activation of the IL-9Rα gene would agree with these possibilities [19]. However, caution will have to be taken to assess the possibility of deleterious autoimmune reactions if peptide IL-9R$\alpha_{168–182}$ (or other IL-9Rα T cell epitopes) was to be used in the clinic. Nonetheless, one should keep in mind that many of the currently approved anti-tumor immune-based therapies are directed against cell surface receptors that are expressed by normal cells such as CD20, CD25 and CD3 [1, 11].

IL-9 is a multifunctional cytokine produced mainly by antigen-activated Th2 lymphocytes [5]. IL-9 was initially described as a T cell growth factor [28], but in humans, IL-9 is also thought to affect the differentiation of hematopoietic progenitors and B cells [5] and to promote the

proliferation of not only T cell lines but also mast cell progenitors [21]. Furthermore, IL-9 is thought to have a functional role in some malignant lymphomas, such as Hodgkin lymphoma, anaplastic large cell lymphoma and NK/T cell lymphoma [7, 22, 24, 26], because it promotes oncogenesis and acts as an autocrine growth factor for Hodgkin and Reed-Sternberg cells, anaplastic lymphoma kinase positive anaplastic large cell lymphoma cells and NK/T cell lymphoma cell lines, which express high amounts of IL-9R. By contrast, cell surface expression of IL-9R and IL-9 responsiveness appeared to be infrequent in HTLV-1-transformed T cells [20]. However, our results revealed that HTLV-1 positive ATLL cell lines contained high amounts of IL-9Rα protein intracellularly, suggesting that IL-9Rα protein could be processed in the T cell lymphoma and presented MHC-II-bound peptide epitopes on the cell surface. In addition, primary ATLL cells or smoldering ATLL cells have a capacity to respond IL-9 [3, 20], indicating that IL-9 and its receptor could be venues of new therapeutic approaches for ATLL.

Lastly, this study is the first report of T helper responses to the peptide-epitope from human IL-9Rα protein. HTLV-1 infection is a potent inducer of IL-9 and most of these tumor cells constitutively express IL-9 [4]. Furthermore, HTLV-1-transformed human T cell line (MT2) showed a cis/trans-activation of the IL-9R gene [19], raising the hypothesis that such leukemia need genetic rearrangement of the IL-9R gene to be able to use IL-9 as an autocrine factor. These findings indicate that IL-9R and its signaling pathway may be a new target for the treatment of ATLL.

Conflict of interest The authors declare no conflict of interest.

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