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Characterization of HLA-DR-restricted T-cell epitopes derived from human proteinase 3

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ABSTRACT

Human proteinase 3 (PRTN3) is a leukemia-associated antigen specifically recognized by CD8+ cytotoxic T-lymphocytes (CTL). PRTN3 also has been shown to elicit both antibody responses and T-cell proliferation in patients with Wegener's granulomatosis. In order to improve current vaccines that aim to stimulate CTL without inducing harmful autoimmune disease, it is necessary to study the role of PRTN3-specific CD4+ T-helper (TH) and CD4+ T-regulatory (Treg) cells. Since both TH and Treg cells recognize antigens in the context of HLA-class-II-molecules, identification of HLA-class-II-associated peptide-epitopes from self-antigens such as PRTN3 is required. Here, we analyzed T-cell responses against proteinase 3 using synthetic peptides predicted to serve as HLA-DR-restricted epitopes. We first screened a panel of ten epitope peptide candidates selected with the TEPITOPE program and found that nine out of ten peptides induced PRTN3 peptide-specific proliferation of T-cells with precursor frequencies of 0–1.1 $\times 10^{-6}$. For one peptide-epitope, $PRTN3_{235}$, T-cell-clones were demonstrated to be capable of recognizing naturally processed protein antigen in a HLA-DR-restricted fashion. $PRTN3_{235}$-specific T-cells could be stimulated from the blood of healthy individuals with multiple HLA-DR-genotypes. In summary, the identified $PRTN3_{235}$-epitope can be used to study the role of CD4+ TH- and Treg-cells in immune responses against PRTN3 in leukemia patients and patients with Wegener's disease.

1. Introduction

Proteinase 3 (PRTN3) is a myeloid-tissue restricted serine protease that is over-expressed in myeloid leukemia cells and is important for maintenance of the leukemia phenotype [1,2]. Previously, PRTN3-specific CD8+ cytotoxic T lymphocytes (CTL) cultured from blood of healthy donors and patients with leukemia have been demonstrated to selectively kill chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) cells [3–6]. Accordingly, PRTN3 represents a candidate target antigen for vaccination and T-cell adoptive immunotherapy in the treatment of AML and CML. To date, specific T-cell based immunotherapy of human cancer overall has shown limited clinical success [7]. The focus of these immunotherapeutic approaches has been to induce HLA-class-I restricted CD8+ CTL responses. Despite the importance of HLA-class-II-restricted CD4+ T lymphocytes in the modulation of anti-tumor immunity, their specific role in this context remains to be studied for many tumor-associated antigens including PRTN3.

CD4+ T-cells play critical roles in initiating and regulating immune responses in infectious disease, autoimmunity and cancer [8,9]. CD4+ T helper (TH) cells provide help for the priming, maintenance and expansion of CD8+ cytotoxic effector T lymphocytes (CTL), thus enhancing the overall anti-tumor immunity [10]. On the contrary, CD4+ T regulatory (Treg) cells induce self-tolerance and can profoundly inhibit host immune responses [8,9]. Both, CD4+ TH and antigen-induced CD4+ Treg cells recognize peptide epitopes presented by HLA-class-II-molecules. Interestingly, previous studies have demonstrated that phenotypically and functionally distinct TH and Treg cells can recognize the same HLA-class-II-associated epitopes [11,12]. In order to study and to potentially manipulate the opposing roles of CD4+ TH and CD4+ Treg cells in anti-tumor immunity and autoimmune disease, it is necessary to identify HLA-class-II-restricted tumor antigens and to characterize the corresponding epitopes.

In the present study, we applied our previously established "reverse immunology" approach [13–16] and characterized HLA-DR-restricted peptide epitopes for stimulation of PRTN3-specific CD4+ T lymphocytes.

2. Materials and methods

2.1. Blood donors, synthetic peptides

The study was approved by the Ethical Committee of the University of Göttingen. Heparinized blood was collected from healthy volunteers. HLA-typing was performed by PCR-SSP-DNA-based

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procedures (Institute of Immunology, University of Göttingen). TEPITOPE [17] was used to predict potential HLA-DR-binding peptides. The prediction threshold was set at 3% and peptides were selected based on their ability to bind to at least two of the following HLA-DR molecules: DRB1*0101, DRB1*0301, DRB1*0401, DRB1*1101, DRB1*1301, and DRB1*1501. Based upon the predictions, ten peptides were synthesized in the MD Anderson Cancer Center Peptide Core (peptide purity above 95%). Proteinase-3-peptides used in the study were $PRTN3_{58}$ (GTLIHPSFVLTAAHALRDI), $PRTN3_{77}$ (PQRLVNVVLGAHNVRTQE), $PRTN3_{87}$ (AHNVRTQEPTQQHFS), $PRTN3_{117}$ (NDVLLIQLSSPANLSAS), $PRTN3_{166}$ (PAQVLQELNVTVVTF), $PRTN3_{187}$ (IATFVPRRKAGIAFG), $PRTN3_{204}$ (GGPLIADGIIQGIDSFVIWGAA), $PRTN3_{216}$ (IDSFVIWGAATRLFPDFF), $PRTN3_{235}$ (RVALYVDWIRSTLRR), and $PRTN3_{241}$ (DWIRSTLRRVEAKGRP).

2.2. Recombinant protein, tumor cell lysates, monoclonal antibodies, and tissue culture reagents

Recombinant PRTN3 protein with an amino-terminal histidine tag was produced in *SF9* insect cells by use of a baculovirus expression system (Gibco, Grand Island, NY), purified using nickel-chelating affinity chromatography (Qiagen, Germany), and tested by Western blot analysis using a monoclonal mouse antibody against $(Histidine)_6$-tags (clone: 6-His, BabCo, Richmond, CA) as described previously [15,18]. Lysates were prepared by repetitive freeze–thaw cycles of PRTN3-expressing (K562, HL-60) and PRTN3-negative (HEK293, PM-1) tumor cells as described [13,14]. The following hybridomas were used to produce monoclonal antibodies: HB55 (L243, anti-human HLA-DR, ATCC) and HB95 (W6/32, anti-human HLA-class-I, ATCC). Anti-human CD4 (RPA-T4, FITC labeled) and anti-human CD8 (HIT8a, PE labeled) were purchased from BD PharMingen (San Diego, CA). Media used for cell culture were AIM-V serum-free medium (Life Technologies, Inc., Grand Island, NY) and RPMI 1640 supplemented with 10% FBS (Life Technologies, Inc., Grand Island, NY) and L-glutamine/penicillin/streptomycin. Human recombinant IL-2 was purchased from Boehringer Roche (Germany).

2.3. Culture of PBMC-derived human dendritic cells

Human dendritic cells (DC) were prepared as recently described [19]. Briefly, PBMC (peripheral blood mononuclear cells) were resuspended in serum-free DC medium (CellGenix) and incubated at 37 °C in humidified 5% CO_2. The cell fraction adherent to plastic was cultured in serum-free DC medium with 1000 IU/ml recombinant human GM-CSF (rhGM-CSF; R&D Systems) and 1000 IU/ml rhIL-4 (R&D Systems). On day 5, DC were matured by stimulating with a cytokine cocktail consisting of recombinant human tumor necrosis factor alpha (rhTNF-α; 10 ng/ml, R&D Systems), rhIL-1β (1000 ng/ml, R&D Systems), rhIL-6 (10 ng/ml, R&D Systems), and prostaglandin E_2 (PGE_2; 1 μg/ml, Sigma).

2.4. Peptide-specific T-cell clones, analysis of T-cell responses

T-lymphocytes were cultured with synthetic peptides under serum-free condition [14,15]. Proliferative responses with peptides in the presence of autologous irradiated PBMC (60 Gy) were tested one week later in [^{3}H]-thymidine assays. Peptide-reactive T-cell lines with a stimulation index above 3.0 were cloned by limiting dilution [14,15]. T-cell clones were co-cultured with peptide, protein-, and tumor lysate-pulsed irradiated DC [19] (60 Gy). The HLA-restriction-pattern was determined by HLA-antibody inhibition of T-cell responses.

3. Results and discussion

Proteinase 3 (PRTN3) is a promising antigen for immunotherapy of myeloid leukemia. In previous studies, HLA-class-I-restricted CD8+ T-cell responses against the HLA-A2-binding epitope PR1 have been demonstrated in the peripheral blood of healthy donors and patients with acute and chronic myeloid leukemia [5,6,20]. Furthermore, the autoimmune disease Wegener's granulomatosis is associated with an anti-neutrophil cytoplasmic antibody (ANCA) specific for PRTN3 [21], and T lymphocytes taken from patients with active Wegener's disease show proliferative responses on exposure to purified PRTN3 [22]. In order to enhance the clinical activity of PRTN3-based immunotherapy strategies against leukemia and to further address the role of T-cell immunity in Wegener's disease, studying the function of PRTN3-specific CD4+ T lymphocytes in these situations is warranted. For this purpose, HLA-class-II-restricted T-cell epitopes in PRTN3 remained to be identified.

Here, we used the TEPITOPE program [17] to analyze PRTN3 regarding HLA-class-II-restricted epitope candidates. At a prediction threshold of 3%, ten sequence motifs were predicted to contain binding motifs for different HLA-DR molecules (Fig. 1). $PRTN3_{58}$, $PRTN3_{77}$, $PRTN3_{87}$, $PRTN3_{117}$, $PRTN3_{166}$, and $PRTN3_{235}$ were predicted to bind to HLA-alleles DR3 and/or DR4, respectively. Sequence motifs $PRTN3_{187}$, $PRTN3_{204}$, $PRTN3_{216}$, and $PRTN3_{241}$ were expected to bind to HLA-alleles DR1, DR11, DR13 and/or DR15,

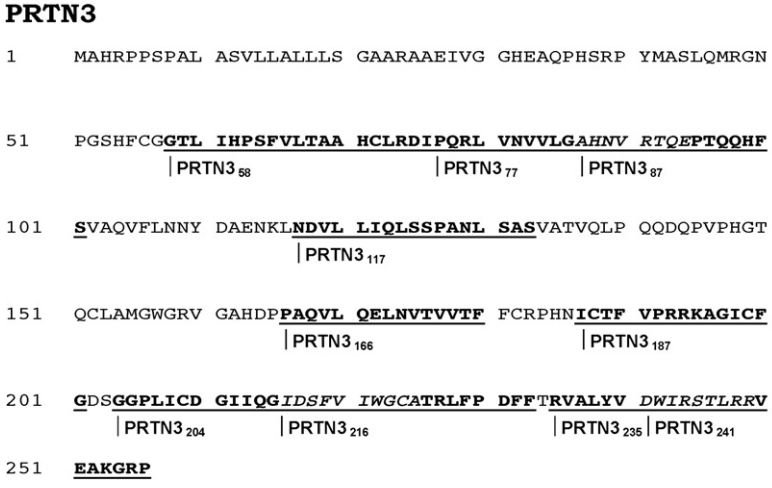

Fig. 1. Schematic representation of predicted HLA-class-II-epitopes within PRTN3. The HLA-DR epitopes in PRTN3 predicted with the TEPITOPE program at the 3% threshold are depicted within the complete amino acid sequence of human PRTN3.