Characterization of T-cell responses against IκBα in cancer patients

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The nuclear factor κ light chain enhancer of activated B cells (NFκB) is constitutively active in most cancers, controlling multiple cellular processes including proliferation, invasion and resistance to therapy. NFκB is primarily regulated through the association with inhibitory proteins that are known as inhibitors of NFκB (IκBs). Increased NFκB activity in tumor cells has been correlated with decrease stability of IκB proteins, in particular IκBα. In responso to a large number of stimuli, IκB proteins are degraded by the proteasome. Cytotoxic T lymphocytes (CTLs) recognize HLA-restricted antigenic peptides that are generated by proteasomal degradation in target cells. In the present study, we demonstrate the presence of naturally occurring IκBα-specific T cells in the peripheral blood of patients suffering from several unrelated tumor types, i.e., breast cancer, malignant melanoma and renal cell carcinoma, but not of healthy controls. Furthermore, we show that such IκBα-specific T cells are granzyme B-releasing, cytotoxic cells. Hence, the increased proteasomal degradation of IκBα in cancer induces IκBα-specific CTLs.

Introduction

Nuclear factor κ light chain enhancer of activated B cells (NFκB) contributes to cell proliferation, immunity, inflammation and-apoptosis. Thus, NFκB activity is indispensable for lymphocyte survival and activation, as well as for the development of immune responses. Transformation of normal cells into malignant cells leads to a number of distinctive phenotypic alterations, including the capacity to proliferate autonomously and to invade neighboring as well as distant tissues. Additional hallmarks of cancer include the ability to evade cell death as well as to stimulate angiogenesis. Finally, cancer cells must be able to escape immunosurveillance.[1] These features are initiated in part through alterations of cell-intrinsic signaling pathways. In this respect, NFκB is a crucial transcription factor for the development of cancer. Indeed, NFκB is involved in transformation, proliferation, invasion, metastasis, inflammation, angiogenesis, as well as in resistance to therapy, which, all are of vital importance for oncogenesis. Constitutive activation of NFκB has been reported in a wide range of hematological and solid malignancies including melanoma, head and neck, breast and ovarian cancer.[2-4] The critical importance of NFκB in cancer has led to the theory of "NFκB addiction."[5]

NFκB proteins are normally kept under control in the cytoplasm by the association with inhibitory proteins (known as inhibitors of NFκB, IκBs).[6] Activation of NFκB mainly occurs when IκBs are degraded by the proteasome, in response to a plethora of distinct stimuli. The degradation of IκBs permits NFκB proteins to translocate to the nucleus. There are numerous human IκB proteins including IκBα, IκBβ, IκBε and IκBζ. Increased NFκB activity in tumor cells has been correlated with decreased stability of IκB proteins, in particular IκBα.[7,8] Thus, elevated activation of NFκB is correlated with a decrease in the half-life of IκBα.

Cytotoxic T lymphocytes (CTLs) have a central role in the examination of all the cells in the body. CTLs recognize antigenic peptide epitopes on the surface of target cells when they are presented by MHC Class I molecules. The processing and presentation of peptide-MHC complexes requires MHC Class I to sample peptides derived from intracellular proteins, upon their degraqdation by the proteasome. Thus, the major source of Class I-restricted peptide epitopes is the proteasomal degradation of proteins in the cytosol. Such peptides bind to MHC Class I molecules in the endoplasmic reticulum before being transported to the cell surface.[9] When they recognize a MHC/peptide complex, CTLs become activated and can mediate the death of target cells. CTLs are thought to provide a major contribution to immunosurveillance against tumors by virtue of their ability to detect quantitative and qualitative antigenic differences in transformed cells.[10]

In the present study, we analyzed if the decreased half-life of IκBα in cancer cells due to increased proteasomal degradation triggers a measurable CTL-response against IκBα-derived peptides in cancer patients.

Results

Spontaneous immune responses against IκBα. The IκBα protein sequence was examined for potential HLA-A2-restricted

nona- and decamer peptide epitopes by means of the previously described HLA-A2 peptide binding motifs.[11] Eighteen IκBα-derived peptides (entitled IκB1–18) were selected for further investigation. We scrutinized peripheral blood mononuclear cells (PBMCs) from healthy individuals and cancer patients for the presence of specific T-cell responses against such IκBα-derived peptides by means of an interferon γ (IFNγ) secretion ELISPOT assay. The ELISPOT has previously been employed for the identification of novel tumor antigens based on spontaneous immunity in cancer patients.[12–14] Thus, HLA-A2+ PBMCs from patients with breast cancer, renal cell carcinoma or melanoma were stimulated once with the different peptides in vitro before examination by ELISPOT. IFNγ secretion was detected against IκB18 and IκB10 (**Fig. S1**).

Apparently, the most immunogenic IκBα-derived peptide among those that we tested was IκB10. Thus, IκB10 was examined for its ability to bind to HLA-A2 by the comparison with two HLA-A2-restricted, high affinity epitopes, i.e., HIV-1 pol$_{476–484}$ (ILKEPVHGV) and CMV pp65 pos$_{495–503}$ (NLVPMVATV) using peptide exchange technology followed by ELISA. IκB10 bound HLA-A2 with an affinity that was comparable to that of control epitopes (**Fig. 1A**). Next, the reactivity against IκB10 was examined in a larger cohort of patients by means of ELISPOT assays. **Figure 1B** exemplifies IκB10-specific T-cell responses in two melanoma patients (MM1 and MM3). Overall, the presence of IκB10-reactive T cells in the blood of HLA-A2+ cancer patients were revealed by ELISPOT (**Fig. 1CD**). In addition, reactivity against IκB10 was examined in PBMCs from healthy individuals (**Fig. 1C**). A Mann-Whitney test illustrated that there was a significant difference in T-cell reactivity against IκB10 between healthy donors and cancer patients (p = 0.001). Thus, the spontaneous reactivity toward IκB10 was restricted to patients affected by malignancies.

Functional capacity of IκB-specific T-cells. Next, we used PBMCs from patients hosting responses against the IκB10 peptide to examine if IκB10-specific T cells exert cytotoxic functions. Thus, PBMCs from three melanoma patients (MM1, MM3 and MM5) bearing IκB10-specific IFNγ-secreting T cells were analyzed for further reactivity against IκB10 using a granzyme B (GrB) ELISPOT assay. Responses against IκB10 could be detected in all three patients with a frequency at about 80–250 IκB10-specific, GrB-releasing cells per 10^5 PBMCs (**Fig. 2A**).

IκB10 bound to HLA-A2 with a high binding affinity, allowing for the assembly of stable HLA-A2/ IκB10 tetramers. We utilized such tetramers to detect and isolate IκB10-reactive CTLs. Thus, for further T-cell cytotoxicity tests, we established a short IκB10-specific bulk culture by co-stimulating PBMCs from a melanoma patient (MM2) with IκB10 and the Staphylococcal enterotoxin B (SEB). The presence of HLA-A2-restricted, IκB10-specific CD8+ T-cells was verified by HLA-A2/IkB10 double tetramer-staining (**Fig. 2B**). Next, T cells recognizing IκB10 were isolated using HLA-A2/IκB10 tetramers-coupled microbeads. Thus, CTL clones were generated upon the expansion of isolated, tetramer+ single cells. The reactivity of the growing T-cell clones was analyzed after a short expansion step using ^{51}Cr release assays. A total of 138 T-cell clones were expanded and, subsequently,

analyzed for cytotoxicity against T2 cells loaded with either IκB10 or an irrelevant peptide, namely, HIV-1 pol$_{476–484}$. Thirty-seven of the T-cell clones were IκB10-specific. **Figure 2C** exemplifies the lytic capacity of two of the IκB10-specific clones. Unfortunately, we were not successful in further expanding any of the IκB10-specific T-cell clones for further analysis. Instead, we generated specific T-cell cultures against IκB10 in vitro by re-stimulating PBMCs with autologous IκB10-pulsed dendritic cells (DCs) four times. A standard cytotoxicity assay against T2 cells pulsed with IκB10 or HIV-1 pol$_{476–484}$ illustrated that only T2 cells loaded with IκB10 were recognized and killed by the T-cell bulk culture (**Fig. 3A**). To increase the frequency of specific T cells, clones were established from the bulk culture by the limiting dilution technique. The specificity of the growing T-cell clones was analyzed in standard ^{51}Cr release assays after a short expansion step. To this end, T2 cells and autologous DCs either loaded with IκB10 peptide or HIV-1 pol$_{476–484}$ served as targets. This assay revealed that T2 cells and DCs were only killed by a T-cell clone when pulsed with IκB10 (**Fig. 3B**). Next, the IκB10-specific T-cell clone was further examined for its capacity to kill the HLA-A2+, highly immunogenic melanoma cell line FM3. **Figure 3C** illustrates that the IκB10-specific T-cell clone was indeed able to kill such melanoma cells. Killing of FM3 cells was further increased by pre-treatment of melanoma cells with IFNγ (**Fig. 3C**). However, we were not successful in the generation of long-term stable cultures of IκB10-specific T-cell clones, which may reflect the concurrent auto-recognition and self-toxicity of such T cells in culture.

Discussion

The proteasome is an essential player in the adaptive immune system. Proteasomal degradation of cytosolic proteins generates peptide epitopes that are presented on the cell surface in the context of MHC Class I molecules. The proteasome has been involved in inflammatory and autoimmune diseases, due to the activation of NFκB upon IκB degradation.[15] In cancer, the proteasome activity have been correlated with increased NFκB signaling, as well as progressive disease.[16] In this regard, proteasome inhibitors like bortezomib display significant preclinical and clinical efficacy and have therefore been approved for the treatment of multiple myeloma.[17] Along similar lines, IKKβ antagonists have revealed potent anticancer effects in pre-clinical studies.[18] IKKβ is a subunit of the kinase (i.e., IKK, for IκB kinase) that phosphorylates IκBα leading to its proteasomal degradation. In the present study, we analyzed if the increased degradation of IκBα by the proteasome in cancer cells gives rise to spontaneous CD8+ T-cell responses against IκBα-derived peptides. The intracellular degradation of proteins by the proteasome is the major source of peptide epitopes that are presented on the cell surface by MHC Class I molecules. We therefore synthesized 18 IκBα-derived peptides and examined PBMCs from HLA-A2+ cancer patients for reactivity against these peptides by means of ELISPOT assays. We found that T cells from melanoma, breast cancer and renal cell carcinoma patients react in a HLA-restricted manner against a peptide derived from IκBα. Importantly, such a spontaneous
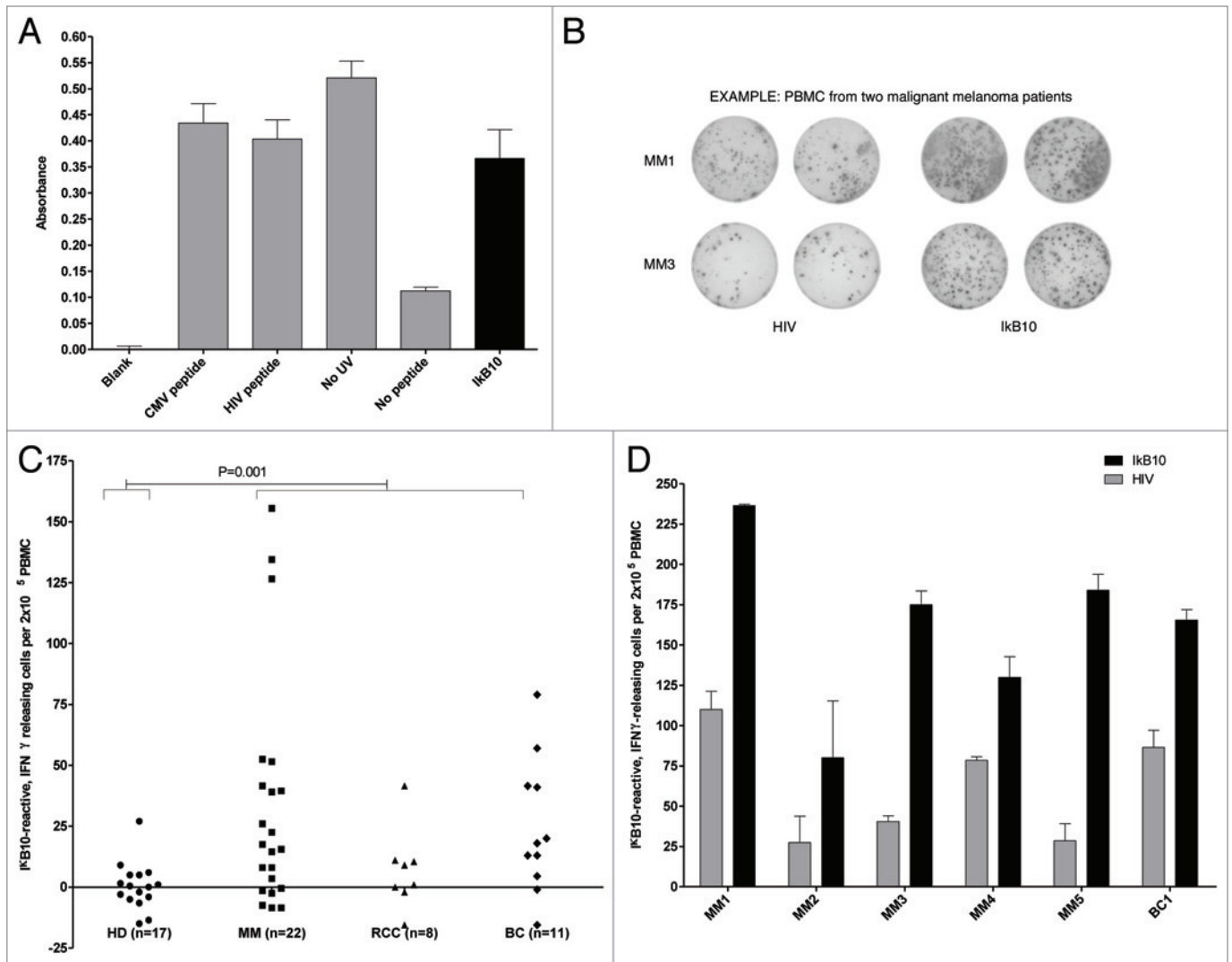
Figure 1. T-cell responses against IκB10 (IκB$_{188-196}$, HLASIHGYL) as measured by interferon γ (IFNγ) ELISPOT. (**A**) ELISA analysis of UV-sensitive ligand (KILGFVFJV) exchanged with various peptides: CMV/HLA-A2 (pp65 pos$_{495-503}$, NLVPMVATV), HIV-1/HLA-A2 (pol$_{476-484}$, ILKEPVHGV) and IκB10 (IκB188–196, HLASIHGYL), no-UV (not exposed to UV light) and no peptide (without rescue peptide). (**B**) Examples of ELISPOT responses against IκB10 and HIV pol$_{476-484}$ in peripheral blood mononuclear cells (PBMCs) from two melanoma patients (MM1 and MM3). (**C**) The average number of IκB10-specific spots (after subtraction of spots obtained with an irrelevant HIV peptide) was calculated per 2×10^5 PBMCs for each patient. PBMCs from 17 healthy donors (HD, black circles), 22 malignant melanoma patients (MM, black squares), 8 renal cell carcinoma patients (RCC, black triangles) and 11 breast cancer patients (BC, black diamond) were analyzed. All individuals were HLA-A2[+]. T cells were stimulated once with peptide before being plated at 2×10^5 cells per well in duplicates with the IκB10 or HIV peptide. A Mann-Whitney test illustrated a significant difference in T-cell reactivity toward IκB10 between healthy donors and cancer patients (p = 0.001). (**D**) Examples of IFNγ-releasing cells in response to IκB10 as well as a HIV peptide among PBMCs from five melanoma patients and one breast cancer patient.

T-cell reactivity was significantly restricted to cancer patients, as we could not detect it in healthy HLA-A2[+] healthy donors.

The IFNγ ELISPOT assay is a very sensitive and solid method for monitoring T-cell responses. Reactivity in the IFNγ ELISPOT most often correlates with the ability of CTLs to exert cytotoxicity against target cells. However, since IFNγ secretion is not restricted to cytotoxic cells, the proof hereof needs to be established directly. One means whereby CTLs mediate target cell death involves the release of GrB. Hence, a GrB ELISPOT assay can be employed to estimate the frequency of cytolytic effector cells.[19] By this approach, we confirmed that

IκBα-specific CTLs detected in cancer patients were indeed cytolytic effector cells. Furthermore, we generated an IκBα-specific T-cell bulk culture by re-stimulating patient-derived PBMCs with the IκB10 peptide in vitro, and found the resulting T-cell line to be IκBα-specific. These IκBα-specific CTLs were able to lyse HLA-A2^{+} melanoma cells. Hence, these findings indicate that, upon proteasomal degradation, IκB peptide epitopes are processed and presented on the cell surface in a HLA-A2-restricted fashion.

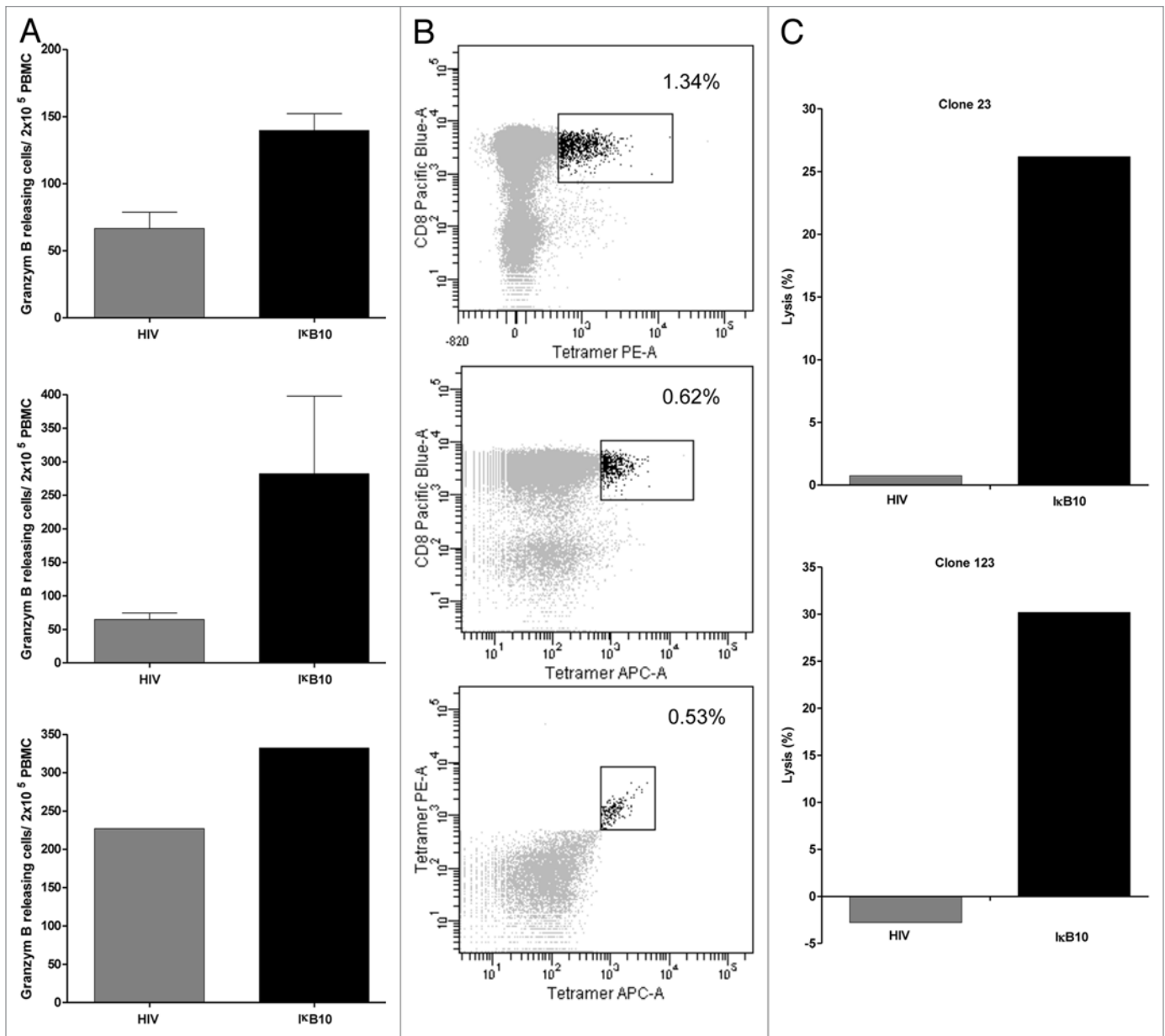Upon exposure to inflammatory stimuli, such as tumor necrosis factor α (TNFα), IκB is phosphorylated and targeted

Figure 2. Cytotoxic functionality of IκB10-specific T cells. (**A**) Examples of granzyme B (GrB) releasing cells in response to IκB10 as well as a HIV peptide among peripheral blood mononuclear cells (PBMCs) from three melanoma patients (MM3, MM1 and MM5) as measured by GrB ELISPOT. (**B**) Tetramer staining visualizing IκB10:tetramer⁺ cells among PBMCs from a melanoma patient (MM2) cultured with IκB10 and the Staphylococcal enterotoxin B (SEB). CD8-PB and HLA-A2/IκB10-PE (top), CD8-PB and HLA-A2/IκB10-APC (middle) andHLA-A2/IκB10-PE and HLA-A2/IκB10-APC double tetramer staining (bottom). (**C**) Lysis of T2-cells pulsed with IκB10 peptide or an irrelevant peptide (HIV-1 pol$_{476–484}$) by two T-cell clones expanded as single cells after isolation from the IκB10-specific bulk culture using HLA-A2/IκB10-tetramer-coupled microbeads.

for degradation via the ubiquitin-proteasome pathway. Due to the important functions of NFκB in normal cells, including immune cells, the existence of a IκBα-specific T-cell response in cancer patients may seem counterintuitive. Thus, the immune system apparently lacks tolerance toward IκBα. Of note, NFκB signaling in activated T cells may result in presentation of IκBα-specific peptide/HLA ligands on the cell surface, perhaps allowing for self-recognition and fratricidal activity. This perhaps explains why we failed to generate long-term, stable cultures of IκB10-specific CTL clones. In all experiments, IκB10-specific

cells very suddenly died after a short expansion time. In this regard, it has recently been described that lymphocytes expressing high-avidity transgenic T-cell receptors recognizing survivin underwent extensive apoptosis over time due to the autologous expression of survivin.[20]

NFκB plays a major role in coordinating the expression of a wide variety of genes that control immune responses, and affects multiple cells involved in both adaptive and innate immune responses. Accordingly, IκBα-specific CTLs might participate in the inflammatory process as regulatory cells. In this respect,

we have recently shown spontaneous T-cell reactivity against the immune regulatory protein indoleamine 2,3-dioxygenase (IDO).[21,22] IDO-specific CTLs enhanced T-cell immunity by eradicating IDO+ regulatory cells. Furthermore, we have previously demonstrated that heme oxygenase-1-specific CD8+ suppressor T cells[23] were present among PBMCs from cancer patients. These data suggest that antigen-specific T cells may contribute to immune regulation in this setting. Thus, it could be further speculated that IκBα-specific CD8+T cells may not arise just as a result of increased IκB degradation but that such cells play a specific function in the regulation of immune responses.

The data presented here suggest that an unusual high expression (or rather proteasomal degradation) of a given self-antigen may give rise to the induction of specific T cells. NFκB is not just constitutively activated in cancer but in multiple pathological settings including inflammatory diseases such as asthma, multiple sclerosis and rheumatoid arthritis. The data presented here warrant further examination of a potential role for IκBα-reactive T cells in these settings.

Materials and Methods

Patients. Peripheral blood mononuclear cells (PBMCs) were collected from cancer (renal cell carcinoma, melanoma and breast cancer) patients and healthy controls. Blood samples were drawn a minimum of four weeks after termination of any kind of anti-cancer therapy. The majority of renal cell carcinoma patients had previously been treated with interleukin -2 (IL-2) and IFNα, most melanoma patients had received high dose IL-2 and IFNα, while all breast cancer patients were pre-treated with several kinds of chemotherapy, (e.g., epirubicin, docetaxel, cabecitabine), trastuzumab and/or endocrine therapy. PBMCs were isolated using the lymphoprep separation, HLA-typed (Department of Clinical Immunology, University Hospital) and frozen in fetal calf serum supplemented with 10% dimethylsulfoxide (DMSO). The protocol was approved by the Scientific Ethics Committee for The Capital Region of Denmark and conducted in accordance with the provisions of the Declaration of Helsinki. Written informed consent from all patients was obtained before enrollment.

Peptides. Epitopes from IκBα were predicted using the "Database SYFPEITHIP"[24] in combination with manual examination of the protein sequence for MHC Class I anchor residues. Twelve 9mer and six 10mer synthetic peptides were produced: IκB1 (IκB$_{197-205}$; GIVELLVSL), IκB2 (IκB$_{191-199}$; SIHGYLGIV), IκB3 (IκB$_{235-243}$; LLLKCGADV), IκB4 (IκB$_{201-209}$; LLVSLGADV), IκB5 (IκB$_{12-20}$; AMEGPRDGL), IκB6 (IκB$_{195-203}$; YLGIVELLV), IκB7 (IκB$_{44-52}$; QMVKELQEI), IκB8 (IKB$_{81-89}$; AIIHEEKAL), IκB9 (IκB$_{149-157}$ HLACEQGCL), IκB10 (IκB$_{188-196}$; HLASIHGYL) andIκB11 (IκB$_{264-272}$; RIQQQLGQL), IκB12 (IκB$_{248-256}$; YQGYSPYQL), IκB13 (IκB$_{226-235}$; DLQNPDLVSL), IκB14 (IκB$_{88-97}$; ALTMEVIRQV), IκB15 (IκB$_{130-139}$; LLGAGCDPEL), IκB16 (IκB$_{25-34}$; LLDDRHDSGL), IκB17 (IκB$_{268-277}$; QLGQLTLENL) and IκB18 (IκB$_{271-280}$; QLTLENLQML). The peptides were dissolved in DMSO (final concentration 10 mM) or distilled water (final concentration 2 mM). The HLA-A2 high affinity binding
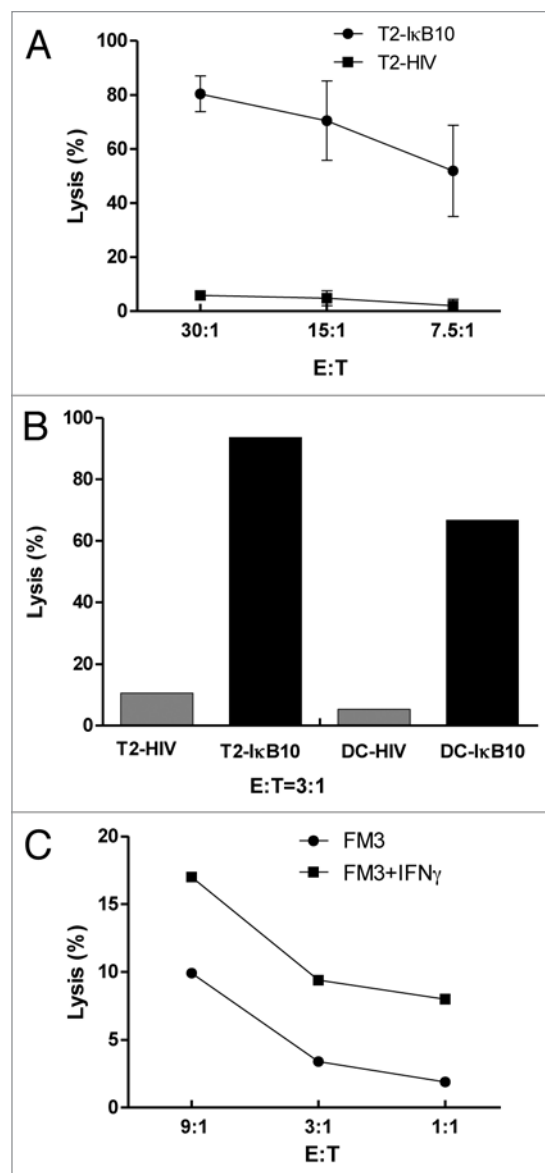

Figure 3. Functional capacity of IκB10-specific T cells. (**A**) Lysis by a T-cell bulk culture of T2-cells pulsed with IκB10 peptide (black circles) or an irrelevant peptide (HIV-1 pol$_{476-484}$) (black squares) at different effector to target ratios as measured by ^{51}Cr-release assay. (**B**) Lysis by a IκB10-clone of T2 cells as well as autologous dendritic cells (DCs) either pulsed with IκB10 peptide or an irrelevant HIV peptide (HIV-1 pol$_{476-484}$). (**C**) Lysis of T2-cells pulsed with IκB10 peptide or an irrelevant peptide (HIV-1 pol$_{476-484}$) and of HLA-A2+ melanoma FM3 cells with or without interferon γ (IFNγ) pre-treatment by the IκB10-clone, as measured by ^{51}Cr-release assay.

epitopes HIV-1 pol$_{476-484}$ (ILKEPVHGV) was used as irrelevant control.

ELISPOT assays. The ELISPOT assay was used to quantify epitope-specific IFNγ-releasing effector cells as described previously.[12] PBMCs were stimulated once in vitro with peptide prior to analysis as described[25] to extend the sensitivity of the assay. Briefly, nitrocellulose bottomed 96-well plates (MultiScreen MSIPN 4W50; Millipore) were coated overnight with IFNγ capture monoclonal antibodies (Mabtech). The wells were washed,

blocked with X-vivo medium and the effector cells were added in duplicates at different cell concentrations, with or without 10 µM peptide. The plates were incubated overnight. The following day, medium was discarded and the wells were washed prior to addition of the relevant biotinylated secondary antibody (Mabtech). The plates were incubated at room temperature (RT) for 2 h, washed andavidin-enzyme conjugate (AP-Avidin; Mabtech) was added to each well. Plates were incubated at RT for 1 h and the enzyme substrate NBT/BCIP (Invitrogen Life Technologies) was added to each well and incubated at RT for 5–10 min. Upon the emergence of dark purple spots, the reaction was terminated by washing with tap water. The spots were counted using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers).

Establishment of antigen specific T-cell cultures and clones. Peripheral blood lymphocytes (PBLs) isolated from a melanoma patient were stimulated with irradiated (25 Gy) autologous IκB10-loaded DCs (PBL:DC ratio = 3×10^6:3×10^5). The following day IL-7 (5 ng/mL) and IL-12 (10 ng/mL) (PeproTech) were added. Stimulation of the cultures were performed every 8 d with IκB10-loaded irradiated autologous DCs (2×) followed by IκB10-loaded irradiated autologous PBLs (3×). After each stimulation, 120 U/mL IL-2 (PeproTech) was added. The established cultures were tested for specificity for IκB10 after 4th stimulation.

PBLs from a specific culture were cloned by limiting dilution in the presence of cloning mix containing 10^6 irradiated (25 Gy) lymphocytes/mL from three different healthy donors in X-vivo medium supplemented with 5% heat-inactivated human serum, 25 mM HEPES buffer (GibcoBRL) and 120 U/mL IL-2 (PeproTech). The plates were incubated at 37°C and 5% CO_2. Every 2–3 d 25 µL fresh media were added containing IL-2 to a final concentration of 120 U/mL. Growing clones were expanded using 120 U/mL IL-2. After expansion the clones were tested for specificity and cytotoxic potential in a standard ^{51}Cr-release assay.

A short antigen specific bulk culture was established where peptide-stimulated PBMCs were co-cultured with super antigen Staphylococcal enterotoxin B (SEB, Sigma Aldrich). Thus, PBMCs from a melanoma patient were stimulated with IκB10 peptide and 40 U/mL IL-2. Five days after peptide stimulation, 1 µg/mL SEB was added. On day 14 tetramer staining was performed where cells were stained with HLA-A2/IkB10 tetramer conjugated with APC or PE fluorochromes. Subsequently, tetramer⁺ cells were isolated using anti-PE microbeads according to manufacture protocol (Miltenyi Biotec). The purified cells were cloned and growing clones tested for specificity in a standard ^{51}Cr-release assay.

Cytotoxicity assay. Conventional ^{51}Cr-release assays for CTL-mediated cytotoxicity were performed as described elsewhere.[26] Target cells were T2 and the HLA-A2⁺ melanoma cell line FM3.

In some assays, target cells were treated with 100 U/mL IFNγ for 2 d.

Tetramer staining. For tetramer staining, IκB10 cells co-cultured with SEB for 14 d were analyzed. Tetramers coupled with PE and APC were prepared using MHC-peptide exchange technology as described in.[27,28] Cells were stained with tetramers in PBS plus 2% FCS for 15 min at 37°C and 5% CO2. Subsequently, surface staining was performed with CD3-FITC, CD8-PB, CD4-PECy7 (BD Bioscience) in PBS plsu 2% FCS for 20 min at 4°C in the dark. The cells were analyzed on FACSCanto, using FACSDIVA software (BD Bioscience).

HLA peptide exchange technology and ELISA. To evaluate the affinity of the HLA-peptide complex a UV exchange method was used in combination with a sandwich ELISA as previously described.[27] In short, HLA-A2 light and heavy chains were produced in *Escherichia coli* and refolded with a UV-sensitive ligand. This conditional ligand was cleaved upon 1 h of UV light exposure and substituted with the peptide of interest. After adding the HLA-A2-peptide complex to an ELISA, the affinity of the complex was measured as the absorbance. Two strong binder-peptides, namely, HLA-A2/CMV pp65 pos$_{495–503}$ (NLVPMVATV) and HLA-A2/HIV-1 pol$_{476–484}$ (ILKEPVHGV), and a sample not exposed to UV light were used as positive controls, while a sample without rescue peptide was used as a negative control. Positive controls were made in quadruplicates and IκB10 in duplicates.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/oncoimmunology/article/21625

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