Spontaneous presence of FOXO3-specific T cells in cancer patients

Stine Kiaer Larsen1, Shamaila Munir Ahmad1, Manja Idorn, Özcan Met, Evelina Martinenaite, Inge Marie Svane, Per thor Straten, and Mads Hald Andersen*

Center for Cancer Immune Therapy (CCIT); Department of Hematology; Copenhagen University Hospital; Herlev; Herlev, Denmark

1These authors contributed equally to this work.

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**Abbreviations:** APC, antigen presenting cell; CTL, cytotoxic T lymphocyte; CTLA4, cytotoxic T-lymphocyte associated protein 4; DC, dendritic cell; FOXO3, forkhead box O3; IDO, indoleamine-2,3-dioxygenase; PBMC, peripheral blood mononuclear cell; TADC, tumor-associated DCs; TGFβ, tumor growth factor β; TNFα, tumor necrosis factor α; Tregs, regulatory T cell.

In the present study, we describe forkhead box O3 (FOXO3)-specific, cytotoxic CD8+ T cells existent among peripheral-blood mononuclear cells (PBMCs) of cancer patients. FOXO3 immunogenicity appears specific, as we did not detect reactivity toward FOXO3 among T cells in healthy individuals. FOXO3 may naturally serve as a target antigen for tumor-reactive T cells as it is frequently over-expressed in cancer cells. In addition, expression of FOXO3 plays a critical role in immunosuppression mediated by tumor-associated dendritic cells (TADCs). Indeed, FOXO3-specific cytotoxic T lymphocytes (CTLs) were able to specifically recognize and kill both FOXO3-expressing cancer cells as well as dendritic cells. Thus, FOXO3 was processed and presented by HLA-A2 on the cell surface of both immune cells and cancer cells. As FOXO3 programs TADCs to become tolerogenic, FOXO3 signaling thereby comprises a significant immunosuppressive mechanism, such that FOXO3 targeting by means of specific T cells is an attractive clinical therapy to boost anticancer immunity. In addition, the natural occurrence of FOXO3-specific CTLs in the periphery suggests that these T cells hold a function in the complex network of immune regulation in cancer patients.

**Introduction**

The immune system responds effectively to infecting pathogens (non-self), while remaining unresponsive, and thus tolerant, to the body’s own cells (self).1 Several different cell types are involved in a complex network of central and peripheral tolerance mechanisms that ensure immune homeostasis, including regulatory T cells (Tregs), dendritic cells (DCs) and myeloid-derived suppressor cells. DCs are essential to the initiation of a productive immune response by presenting antigen to T cells and by the secretion of pro-inflammatory cytokines. However, DCs are also responsible for controlling overactive immunity by regulating immune tolerance. While tolerance is an essential component of protecting the host from autoimmune disease, it is a major complication and obstacle in the pursuit of better immune-based therapies for cancer. Tumor-associated DCs (TADCs) are central to immunosuppressive mechanisms constraining tumor-specific immunity.2,3 Many different molecules are responsible for the tolerogenic role of TADCs and it was recently shown that the enzyme indoleamine-2,3-dioxygenase (IDO) plays an important role in the occurrence of DC-mediated T-cell tolerance.4 Tolerogenic DCs may also express inhibitory ligands on their cell surface (e.g., PD-L1 and PD-L2), produce immunosuppressive factors such as interleukin-10 (IL-10), tumor growth factor β (TGFβ), and Arginase (ARG1), all factors that suppress T-cell immunity.5

Recently, pioneering work by Hurwitz and colleagues showed that increased expression of forkhead box O3 (FoxO3) was associated with or even responsible for TADC-induced T-cell tolerance in both mice and humans.6,7 Additionally, they described elevated levels of FOXO3 expression in TADCs compared with non-tumor-associated DCs in prostate cancer patients. Elevated expression of FOXO3 in DC may be mediated by cytotoxic T-lymphocyte-associated protein 4 (CTLA4) interaction with B7 molecules.8 FoxO3 induction may inhibit the production of immunostimulatory and inflammation-associated molecules, such as IL-6 and tumor necrosis factor (TNF). In contrast, attenuation of FoxO3 expression leads to reduced levels of the suppressive factors TGFβ, IDO and Arginase, as well as increased expression of the co-stimulatory molecule CD80 and the pro-inflammatory cytokine IL-6, molecular changes culminating in reduced DC tolerogenicity.6 In support, downregulation of FoxO3 expression resulted in more stimulatory DCs.6,9
FoxO3 is a member of the Forkhead box transcription factor class-O family and was originally described as a tumor suppressor gene. In general, FoxO transcription factors play a crucial role in the regulation of a number of cellular functions, including cell death, protection from stress, and cell cycle arrest. In this regard, FoxO3 inactivation leads to deregulated cell proliferation and has been associated with tumorigenesis. FoxO3 overexpression has been shown to inhibit tumor growth. However, it was recently suggested that FoxO3a may in addition promote invasive tumor migration, allowing tumors to invade neighboring tissues and ultimately metastasize to distant organs. Furthermore, importantly, nuclear exclusion of FoxO3a contributes to cell survival, and cytoplasmic location of FoxO3 seems to correlate with many forms of cancer and has been associated with poor survival in breast cancer. Cytotoxic T lymphocytes (CTLs) recognize HLA-restricted, antigenic peptides, which are generated by proteosomal degradation in the cytoplasm. We recently described specific CTL-responses in cancer patients toward another transcription factor, forkhead box P3 (FOXP3) that is generally expressed in Tregs. Due to the important function of FoxO3 in cancer and immunity, in the current study we examined whether FOXO3 is a natural target for CTLs in human cancer patients and characterized the cytolytic capacity of these effector T cells.

**Results**

**Spontaneous T-cell reactivity against FOXO3 in cancer patients**

The amino acid sequence of the FOXO3 protein was screened using the database “SYFPEITHI” to predict the best HLA-A2 nona-peptide epitopes. Two peptides were predicted to be top candidates with a predictive score of 28; FoxO3 92–100 (LLLED-SARV) and FoxO3 118–126 (GLSSGTQAL). These 2 peptides were synthesized for further studies. We examined peripheral blood mononuclear cells (PBMCs) from different HLA-A2+ cancer patients as well as healthy donors for the presence of specific T-cell responses against the peptides by means of the IFNγ ELISPOT assay. Previously, we have made use of this assay for the identification of antigen-specific T-cell responses in cancer patients. In total, we scrutinized PBMCs from 27 HLA-A2+ cancer patients (i.e., 5 patients with renal cell carcinoma, 16 patients with melanoma and 6 patients suffering from breast cancer) for reactivity against the 2 FOXO3-derived peptides (Fig. 1A and B). Frequent and strong responses were detected against both peptides in several cancer patients and in all 3 forms of cancer. Notably we did not detect any responses against FoxO3 92–100 or FoxO3 118–126 among PBMCs from healthy individuals. The difference between immune responses in healthy donors and cancer patients was illustrated by a Mann-Whitney test showing that the difference was significant for both peptides ($P = 0.0001$ and $P = 0.0125$, respectively). Examples of significant responses against each peptide are depicted in bar plots in Figure 1C and D, in which responses are compared to background for each patient.

Next, we examined 3 responding patients for the presence of FoxO3 118–126-specific cells directly ex vivo, without prior peptide stimulation in vitro (Fig. 2A). Using the IFNγ ELISPOT assay, 2 renal cell carcinoma patients (RCC41 and RCC44) showed statistically significant T-cell responses to FoxO3 118–126 ex vivo. The ELISPOT response of RCC44 is depicted in Figure 2B. Next we utilized an HLA-A2/FoxO3 118–126 tetramer to detect and examine reactive T cells by flow cytometry. However, upon staining PBMCs from RCC44 with the HLA-A2/FoxO3 118–126 tetramer, we failed to detect tetramer-positive cells directly ex vivo (Fig. 2C). This discrepancy might be explained by the presence of non-HLA-A2 restricted CD4+ and CD8+ T cells contributing to the apparent ELISPOT response. However, it could also be caused by a low T-cell avidity. Next, we used PBMCs from RCC44 to generate T-cell bulk cultures against FoxO3 118–126 in vitro. We stimulated PBMCs from the patient with FoxO3 118–126–pulsed autologous DCs in vitro and stained these cultures for HLA-A2/FoxO3 118–126-positive cells. After several rounds of peptide-stimulation in vitro, a clear population of tetramer-positive T cells became detectable (Fig. 2D). The culture was enriched for HLA-A2/FoxO3 118–126-positive T cells by fluorescence-activated cell sorting (FACS) isolation of tetramer-specific cells. Expansion of the isolated cells resulted in T-cell cultures of very high specificity (90–100% specific T cells) (Fig. 2D).

**Cytotoxicity of FoxO3-specific CD8+ T cells**

To analyze the functional capacity of ex vivo expanded FoxO3-specific T-cells we first analyzed the effect of FoxO3 118–126 peptide stimulation of a tetramer-positive T-cell culture (Fig. 3A). In this regard, upon FoxO3 118–126 recognition the HLA-A2/FoxO3 118–126-positive T cells produced both IFNγ and TNFα as measured by intracellular staining and cytofluorimetric analysis (Fig. 3B). In addition, the cells displayed the cytolytic marker CD107a on their surface (Fig. 3C and D). Next, the cytotoxic functional properties of different FoxO3-specific bulk cultures was tested in standard 51Cr release assays using transporter associated with antigen processing (TAP)-deficient T2 cells as target cells either loaded with FoxO3 118–126 or an irrelevant control peptide from human immunodeficiency virus (HIV). T2 cells pulsed with FoxO3 118–126 were lysed by the FoxO3 118–126 specific T cells, whereas T2 cells pulsed with the irrelevant peptide from HIV were not lysed (Fig. 3E). As expected, the specific lysis of FoxO3 118–126–pulsed T2-cells was more efficient after sorting of the HLA-A2/FoxO3 118–126-specific cells (Fig. 3F and G).

K562 cells are leukemic cells that express FoxO3 but do not express any HLA molecules. To establish the HLA-restriction as well as potential NK-cell mediated killing by the FoxO3 118–126 specific T cells, we utilized either K562 cells transduced to express HLA-A1 or HLA-A2 or non-transduced K562 cells as target cells in 51chromium-release assays (Fig. 4A). The HLA-A2 transduced FoxO3 expressing cells were effectively lysed by the FoxO3 118–126-specific CTL, whereas the HLA-A1 transduced, as well as the non-HLA expressing cells, were not recognized. Hence, the killing by FoxO3 118–126-specific CTL was, indeed, HLA-A2-restricted. Additionally, FoxO3 siRNA-mediated downregulation of FOXO3 protein expression in the HLA-A2-transduced K562 cells...
reduced the killing of the transduced K562 cells, implicating a link between the level of intracellular FOXO3 in target cells and FoxO3-specific T-cell cytotoxicity (Fig. 4B). The expression levels of FOXO3 in the various K562 cells was confirmed by Western Blot and was not affected by the transduction with either HLA-A1 or HLA-A2 (Fig. 4C). Furthermore, Western Blot confirmed that the use of FoxO3 siRNA reduced the level of FOXO3 protein expression in FoxO3 siRNA-treated K562 cells, although the levels were only partially knocked down (Fig. 4C).

**FoxO3-specific CD8 T cells recognize dendritic cells**

We have previously described that self-reactive T cells, i.e., IDO- and PD-L1- specific CTLs, are able to specifically recognize standard prostaglandin E2 (PGE-2) matured autologous DCs.
generated in vitro. First, the high expression of FoxO3 in our PGE2-matured DCs was confirmed by Western Blot analysis of DCs derived from 5 different patients. Thus, we next examined if FoxO3 118–126-specific CTLs recognize autologous DCs acquired by this standard in vitro technique. We found that FoxO3 118–126-specific CTLs released both IFNγ as well as TNFα in response to autologous, standard matured DCs in contrast to autologous monocytes, as determined by intracellular staining and fluorescence cytometry (Fig. 5A and B). Similarly, FoxO3 118–126-specific CTLs effectively lysed autologous DCs generated in vitro as determined by 51chromium-release assay (Fig. 5C). Additionally, FoxO3 siRNA-mediated FoxO3 protein knockdown in autologous DCs attenuated the T-cell mediated killing of dendritic target cells (Fig. 5C). This was confirmed in 3 independent assays. Western Blot analysis confirmed that the use of FoxO3 siRNA reduced the level of FOXO3 protein in the DCs, although the level was only partially downregulated (Fig. 5D).

**FoxO3 118–126-specific CTL skill cancer cells of different origin and B cells that cross present the epitope**

FoxO3 is reportedly overexpressed in many cancers. We therefore examined if the FoxO3 118–126-specific CTLs were able to recognize cancer cells of different origins. First, we examined the melanoma cell line FM55M1. We found that FoxO3 118–126-specific CTLs released both IFNγ as well as TNFα in response to FM55M1, as determined by intracellular staining and fluorescence cytometry (Fig. 6A). In order to examine the ability of the FoxO3 118–126-specific CTL culture to kill cancer cells, FM55M1...
cells were used as targets in chromium-release assays. We found that the CTL culture successfully recognized and effectively killed the HLA-A2+ FM55M1 cells (Fig. 6B). Next, we examined 2 additional HLA-A2 positive melanoma cell lines, namely FM6 and FM28. The FoxO3\textsubscript{118–126}-specific CTL efficiently lysed cells from both cell lines (Fig. 6C and D).

We next sought to examine the universal character of the FoxO3\textsubscript{118–126}-specific CTL-mediated lysis by evaluating the ability of the FoxO3-specific T cells to recognize cancer cells of differing origins. Hence, the HLA-A2 positive breast cancer cell line MDA-MB-231 and the HLA-A2 positive colon cancer cell line SW480 were subsequently examined. The FoxO3\textsubscript{118–126}-specific CTLs recognized and killed both of these HLA-A2 positive cancer cell lines (Fig. 6E and F). B cells are known to cross-present peptides. Hence, we utilized the FoxO3-negative Epstein Barr virus...
(EBV)-transformed B cell line KIG-Bcl to show that B cells are able to take up and cross-present a 23 amino acid long peptide (FoxO3116–138) that included the sequence of FoxO3118–126 (Fig. 6G). To this end, the FoxO3118–126-specific CTLs were examined against KIG-Bcl cells pulsed with either the long FoxO3116–138 peptide, an irrelevant HIV peptide (as negative control) or the actual epitope FoxO3118–126 (as positive control). The long peptide FoxO3116–138 was efficiently presented by HLA-A2 on the surface of KIG-Bcl cells, since these cells were killed by FoxO3118–126-specific CTLs almost as effectively as the positive control cells. Of note, the KIG-Bcl cells did not appear to express FOXO3 protein as determined by Western Blot (Fig. 6H). The expression of FOXO3 in the cancer cell lines used here have been previously described28–30 and was confirmed by Western Blot (Fig. 6H).

Discussion

In the present study we examined whether the transcription factor FOXO3 is a natural target for CTLs in cancer patients. In this respect, we found that peripheral blood from cancer patients harbors CD8+ T cell reactivity toward FOXO3 expressing cells by means of the enzyme-linked immunospot (ELISPOT) assay. We detected reactivity against 2 HLA-A2-restricted FoxO3-derived peptides in patients suffering from unrelated tumor types—i.e., melanoma, renal-cell carcinoma and breast cancer. Even more remarkably, we showed high numbers of specific
T cells in a few cancer patients in whom we were able to measure specific T-cell responses directly ex vivo, which is highly unusual. In contrast we were unable to detect immune responses against FoxO3 peptides in healthy controls. Hence, the immune responses toward FOXO3 were restricted to patients suffering from a malignant disease.

It has recently been described that FoxO3+ DCs play a critical role in the regulation of tumor immunity, i.e., modulating host responses to tumors. Hence, FoxO3 has been described as a crucial player in the programming of the inflammatory versus the tolerogenic potential of dendritic cells. Further support for the regulatory role of FoxO3 in immune cells was recently published, showing that increased expression of FOXO3 in human monocytes leads to production of fewer pro-inflammatory cytokines and more IL-10 by these cells. Interestingly, the single nucleotide polymorphism that regulates this increased expression of FOXO3 was associated with a less severe course of the autoimmune diseases Crohn’s disease and rheumatoid arthritis.

Here, we used FoxO3+, PGE2-matured DCs generated from blood monocytes to demonstrate the cytotoxicity exerted by FoxO3-reactive CTLs. Despite the fact that this strategy was primarily chosen in order to surmount the impracticality of isolating and labeling TADCs with 51Cr for cytotoxicity assays, it nevertheless illustrates that FoxO3-specific CTLs are capable of recognizing and killing FOXO3-expressing immune cells. In addition, we established that FoxO3-specific CTLs recognized and killed FOXO3-expressing cancer cells. Thus, FoxO3-specific T cells effectively lysed FoxO3+ solid cancer cell lines of different origin. Hence, in both DCs and cancer cells the intracellular FOXO3 protein was degraded and the peptide epitope subsequently processed and presented on the cell surface in the context of HLA-A2 molecules. We further showed that a long peptide derived from FOXO3 was cross-presented on the surface of non-professional antigen-presenting cells (APCs), namely EBV-transformed B cells. Non-professional APCs have previously been shown to cross-present HLA class I-restricted epitopes from exogenous NY-ESO or PD-L1 polypeptides.

The noticeable lack of tolerance against FOXO3 peptides in cancer patients could suggest a more general role for FoxO3-specific CTLs in the comprehensive regulation of the immune system. Hence, FoxO3-specific CTLs may represent a feedback

Figure 5. FoxO3118–126-specific T cells recognize and kill autologous dendritic cells. (A-D) Dendritic cells were derived from cancer patient peripheral blood monocytes enriched by adherence to plastic and cytokine stimulation ex vivo. FoxO3118–126-specific T cells response to autologous matured DCs and monocytes were determined by intracellular staining and fluorescence cytometry (A and B) or cytotoxicity assay (C). (A) Intracellular interferon-γ (IFNγ) staining of FoxO3118–126-specific T cells stimulated with either directly isolated monocytes (left), or in vitro generated DC (right) for 5 h. (B) Intracellular tumor necrosis factor α (TNFα) staining of FoxO3118–126-specific T cells stimulated with either directly isolated monocytes (left), or in vitro generated DC (right) for 5 h. (C) Chromium51-release assay to determine the FoxO3118–126-specific T cell-mediated lysis of autologous, in vitro generated DCs transfected with control siRNA (circles) versus a FoxO3-specific siRNA (squares). Statistical analysis was performed by unpaired Student’s t-test; *P < 0.05. (D) Expression level of FOXO3-protein examined by Western Blot analysis in the autologous, in vitro generated DC transfected with a FoxO3-specific siRNA or autologous, in vitro generated DC (mock). Densitometric quantification and blotted membranes are shown.
mechanism to suppress the function of immunosuppressive APCs. In this respect, FOXO3 is not the only protein expressed in regulatory immune cells that have been described as potential targets for CTLs in recent years. Thus, spontaneous CD8+ T-cell responses toward epitopes derived from proteins such as FOXP3, IDO as well as PD-L1 have been described.16,27,34–37 These are all normal self proteins inducible in immune cells under many different physiological and/or pathological conditions, such as inflammation, infection and cancer. In normal ontogeny and tissue homeostasis in healthy organisms, the elimination of certain cells by cytolytic events is a fundamental regulatory mechanism.38,39 Thus, self-reactive CTL responses may be important to maintain the homeostasis of the immune system. We have previously suggested the term “supporter T cells” for similar IDO-specific, CD8+ T cells.35 Hence, FoxO3-specific CTLs could play an important role by eliminating regulatory APCs, thereby boosting effector immune responses. To understand this it will be important to consider the expression of FoxO3 in normal cells, especially since FoxO3 is expressed in the hippocampus, cortex and cerebellum40 as well as in the placenta41 and the intestinal epithelia.42 FoxO3 is a critical transcription factor for normal cell cycle regulation. It is involved in numerous processes such as angiogenesis, cardiovascular development, vascular tone, oxidative stress, stem-cell proliferation, fertility, immunity and cancer. Thus many of the overall biological functions of self-reactive FoxO3-specific CTLs may vary depending on the microenvironment and the state of the immune response.

In terms of using FOXO3 as a target in clinical cancer therapy, attenuating FOXO3 protein expression by the silencing of the FOXO3 gene is complicated by the fact that FoxO3 is not only important for modulating DC function but may also impact cancer cell survival via targeting by FoxO3-specific CTLs. Thus, FOXO3 knockdown should somehow specifically target FOXO3
gene expression in dendritic cells, thus preventing downregulation of FOXO3 in tumor cells. Alternatively, FOXO3-specific CTLs may be immensely useful for immune therapy. The induction of FOXO3-specific immune responses could function highly synergistically with additional anticancer immunotherapy, not only by eliminating cancer cells but especially TADCs, which suppress anticancer immunity. Obviously, one should be especially careful of autoimmune reactions when aiming at a self protein such as FOXO3. However, the circulation of a detectable number of FOXO3-specific T cells measurable directly ex vivo did not seem to have caused autoimmunity in the patients. The questions remain under what conditions FOXO3-specific T cells become activated and what the potential role of such autoreactive T cells is in regards to immune regulation and cancer. Thus, the data presented here justify further examination of the role for FOXO3-specific CTLs in immunity and the potential anticancer therapeutic use of FOXO3-specific CTLs in the clinic.

Materials and Methods

Patients
Peripheral blood mononuclear cells (PBMCs) were collected from healthy individuals (average age of 41 years) and cancer patients (melanoma, renal cell carcinoma and breast cancer patients with an average age of 64 years). Blood samples were drawn a minimum of 4 weeks after termination of any kind of anticancer therapy. PBMCs were isolated using Lymphoprep (Fresenius Kabi, Oslo, Norway) separation, HLA-typed and frozen in fetal calf serum (FCS) with 10% dimethyl sulfoxide (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 the patients was obtained before study entry.

Peptides
To identify HLA-A2 restricted CTL epitopes for FOXO3, the amino acid sequence of FOXO3 was analyzed using the database SYFPEITHI (http://www.syfpeithi.de) and the epitope prediction search option.17 Two nonamer peptides, FoxO392–100 (LLLEDASARV) and FoxO3118–126 (GLSGGTQALLQPQPLPPPQP) that included the amino acid sequence of FoxO3 (AGGLSGGTQALLQPQPLPPPQP) and FoxO3 118–126 (GLSGGTQAL) were used as control antigens and scored highest according to the SYFPEITHI algorithm, and thus came out as the top candidate epitopes. The HLA-A2 high affinity binding epitope HIV-1 polK76-K84 (ILKEPVHGV) was used as a control. In addition a 23 amino acid long peptide FoxO3116–138 (AGGLSGGTQALLQPQPLPPPQP) that included the sequence of FoxO3118–126 was synthesized for cross presentation assays.

ELISPOT assay
The ELISPOT assay was used to quantify cytokine (IFNγ) release by peptide specific T cells, as previously described.18 PBMCs from healthy donors or patients were stimulated once in vitro with peptide prior to analysis to increase the sensitivity of the assay, as previously described.43 Briefly, nitrocellulose bottomed 96-well plates (MultiScreen MSIPN4W; Millipore) were coated overnight with the capture anti-human IFNγ antibody (Clone 1D1K, Mabtech). The wells were washed and subsequently blocked by X-VIVO medium (Lonza). The PBMCs were added in triplicates or duplicates at different cell concentrations either with relevant or irrelevant peptide and incubated overnight. The wells were washed prior to addition of the biotinylated secondary monoclonal antibody (mAb 7-B6–1-Biotin; Mabtech), followed by the avidin-alkaline phosphatase conjugate (AP-avidin; Mabtech). Finally, the enzyme substrate nitro-blue tetrazolium/ 5-bromo-4-chloro-3’indolyphosphate (NBT/BCIP; Invitrogen Life Technologies) was added to develop spots. The spots were counted using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers). The definition of an ELISPOT response was based on the guidelines provided by the CIMT Immunoguiding Program (CIP) panel (http://www.cimt.eu/workgroups/cip) as well as Moodie et al.44 using either an empirical or statistical approach. The empirical approach is based on the “signal-to-noise” ratio and suggests that the threshold for response definition should be defined as >6 specific spots per 10⁶ PBMCs. The non-parametric distribution free resampling (DFR) method can be used when experiments are performed in triplicates and allows statistical comparison of antigen-stimulated wells with negative control wells. This method was utilized in Figure 1C and D. The non-parametric unpaired Mann-Whitney test was used to compare FOXO3-specific responses between cancer patients and healthy donors. P-values <0.05 were considered significant.

Generation of FOXO3-specific T-cell cultures
Peripheral blood lymphocytes (PBLs) from a renal cell carcinoma patient were stimulated with irradiated (30 Gy) autologous DCs pulsed with FoxO3118–126 peptide (PBL:DC ratio 10:1) and 40 U/mL IL-7 (PeproTech, London, UK). 20 U/mL IL-12 was added the next day (PeproTech, London, UK). Three subsequent stimulations with FoxO3118–126-loaded irradiated autologous DC followed, at weekly intervals with addition of 20 U/mL IL-12 (PeproTech, London, UK) the next day. The culture was then stimulated 3 times with FoxO3118–126-loaded irradiated autologous PBLs (culture:PBL ratio 1:1) at weekly intervals with addition of 120 U/mL IL-2 (Proleukin, Novartis) the next day. The culture was tested for specificity in an IFNγ ELISPOT assay and stained with tetramers for cytfluorimetric analysis.

The culture was enriched for specific cells by staining with the fluorescein-conjugated tetramers HLA-A2/FoxO3118–126-PE and HLA-A2/FoxO3118–126-APC followed by fluorescence activated cell sorting (FACS) on a FACS Aria flow cytometer (BD Biosciences). The sorted cells were expanded by incubation with 0.6 µg anti-CD3 antibody (eBioscience, clone OKT3), 3000 U/mL IL-2 (Proleukin, Novartis) and allogeneic PBMCs as feeder cells. Sorting was performed twice, starting with a larger gate during the initial sort and, after culturing for 28 d to expand the enriched cells, with a more restrictive gate in a subsequent sort.

Flow cytometry
Staining with tetramers was done in PBS + 2% FCS for 15 min at 37°C, followed by staining with CD4-FITC and
CD8-PerCP (BD Biosciences) fluorophore-conjugated antibodies and the dead cell marker LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen) for 30 min on ice. The tetramers were prepared using the MHC-peptide exchange technology as described. The fluorophore-conjugated MHC tetramer complexes were used: HLA-A2/FoxO3118-126-PE, HLA-A2/FoxO3118-126-APC and as a control HLA-A2/HIVpol476-484 -PE and HLA-A2/HIVpol476-484 -APC. Samples were analyzed on a BD FACS Canto using FACS DIVA software (BD Biosciences) and FlowJo software.

**Generation of dendritic cells**

Dendritic cells (DCs) were generated from monocytes isolated from cancer patient-derived PBMCs by plastic adherence. The monocytes were cultured for 6 d in RPMI-1640 with 10% FCS with 250 U/mL IL-4 and 1000 U/mL GM-CSF (PeproTech). DCs were matured for 2 d by addition of 1000 U/mL IL-1β, 1000 U/mL IL-6, 1000 U/mL TNFα (PeproTech) and 1 µg/mL PGE2 (Cayman Chemical).

**Cytotoxicity assay**

CTL-mediated cytotoxicity was measured by ⁵¹Chromium-release assays, as previously described. Target cells included: T2 cells with HIV-1 pol476-484 (ILKEPVHGV) or T2 cells with FoxO3118-126 the melanoma cell lines FM55M1, FM6, FM28,⁴⁷ the breast cancer cell line MDA-MB-231, or the colon cancer cell line SW480, all available at the American Type Culture Collection (ATCC), the EBV-transformed B-cell line KIG-Bcl,⁴⁸ the natural killer target cell line K652 or transduced K562 cells expressing HLA-A1 or HLA-A2 (a kind gift from Mariam Heemskerk, University Hospital Leiden, The Netherlands), and autologous DCs generated in vitro. FoxO3 expression has previously been described for the cell lines: MDA-MB-231,⁵⁹ SW480,³⁰ K562.²⁶ For the cross-presentation assay KIG-Bcl cells were pulsed for 1 h, with or without the long FoxO3116-138 peptide before being examined in a 4 h cytotoxicity assay.

All cell lines included in the study were tested and authenticated by HLA genotyping. The cell lines were routinely confirmed with their HLA typing and antigen expression by flow cytometry and coculture assays, respectively.

**Intracellular cytokine staining**

For detection of cell subpopulations producing cytokines, FoxO3-specific T-cell cultures were stimulated with 5 µg/mL of relevant, or irrelevant, peptide for 5 h at 37°C with 5% CO₂. Cells were stained with CD107a-FITC (eBioscience) and Golgi-Plug (BD Bioscience) at a dilution of 1:200 was added after the first hour of incubation. After 4 additional hours cells were washed twice with PBS, stained with fluorochrome conjugated antibodies for surface markers (CD3-Amcyan, CD4-PerCP and CD8-Pacific Blue, all from BD Bioscience). Cells were washed an additional time and thereafter fixed and permeabilized with Fixation/Permeabilization and Permeabilization Buffer (eBioscience), according to manufacturer’s instructions. Cells were subsequently stained with the following fluorochrome-conjugated antibodies for detection of intracellular cytokines: IFNγ-PE (eBioscience), TNFα—APC (eBioscience). Relevant isotype controls were used to enable correct compensation and confirm antibody specificity. At least 10⁵ events were acquired. Stained cells were analyzed using a BD FACS Canto II flow cytometer. Analysis was performed with BD FACSDiva Software. Finally, cytokine production by FoxO3-specific T-cell cultures stimulated with monocytes, matured DC or melanoma cells FM55-M1 (effector: target =10:1) was analyzed.

**Downregulation of FoxO3 using siRNA**

SignalSilence FoxO3a siRNA I (#6302S) was obtained from Cell Signaling. Medium GC Duplex control stealth RNAi was obtained from Invitrogen. For downregulation of FoxO3, mDC or K562 cells transfected with HLA-A2 (3 x 10⁶) were transfected with 100 pmol FoxO3 siRNA using electroporation parameters, as previously described.⁵⁹ Mock-transfected DCs used as controls underwent electroporation using the same conditions.

**BCA protein ELISA**

Cell lines were collected and analyzed for expression of siRNA knock down and mRNA transfection with FoxO3 by protein gel blot. Cells were lysed by addition of 100 µL RIPA buffer + protease and phosphatase inhibitors per 10⁶ cells, and mixed by inverting 30 min at 4°C. RIPA buffer, protease and phosphatase inhibitors were prepared according to Abbacm “Western blotting— a beginner’s guide.” To remove DNA and cell debris, cell lysate was sonicated (Branson, Digital sonifier) for 20 sec on ice followed by centrifugation at 18000 x g for 10 min at 4°C. Protein concentration in cell lysate was measured by Pierce BCA Protein Assay kit (Thermo Scientific) according to manufacturer’s recommendations. In short, 200 µL of reagent mix (50:1, reagent A: reagent B) and either 25 µL standard or sample were added per well to a 96F microwell plate (Nunc, Thermo Fisher Scientific). The plate was incubated 30 min at 37°C and read by ELISA plate reader (Synergy HT, Biotek) at a wavelength of 450 nm.

**Western Blot**

Western Blot was set up with either 3 or 5 µg total protein of each sample, a Magic Mark XP Western Standard Ladder (Invitrogen) and a Benchmark Prestained Protein Ladder (Invitrogen). Laemmli sample buffer (BioRad) + 5% 2-mercaptoethanol (BioRad) was diluted 1:1 in cell lysate and samples denatured at 100°C for 5 min. Denatured samples and ladders were loaded onto a 10% Mini protein TGX Stain-free gel (BioRad) and run at 200 V in 10x Tris/Glycine/SDS buffer (BioRad) (diluted 1:10 in d₂H₂O). Protein was transferred to 0.2 µm nitrocellulose membrane (Transblot turbo transfer pack, BioRad) by semidried transfer at 150 A. The membranes were blocked for 1 h in blocking buffer containing PBS + 0.05% Tween20 (Sigma-Aldrich) + 5% skimmed milk powder (BioRad). The membranes were stained with the following primary antibodies: rabbit anti-human FoxO3 (clone 75D8, Cell Signaling Technologies) and mouse anti-human α-tubulin (clone DM1A, Abcam) overnight at 4°C. The next day, membranes were washed and incubated with appropriate horseradish peroxidase conjugated secondary
antibodies, including anti-rabbit IgG HRP-linked antibody (Cell Signaling Technologies) and peroxidase labeled goat anti-mouse IgG (KPL) for 1h at room temperature. Between each staining the membrane was washed 6 x for 10 min each in blocking buffer. Following the last wash, the membrane was washed twice in PBS + 0.05% Tween-20. For signal development Super Signal West Femto Max Sensitivity Substrate (Thermo Scientific) was used according to manufacturer’s recommendations, and the membranes were photographed by a C-DigIt® Chemiluminescent Blot Scanner (Li-Cor).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5. Comparison of Elispot responses between healthy donors and cancer patients was performed using a two-sided Mann-Whitney test. For individual patients, the difference between elispot responses against control HIV peptide and FOXO3 peptides was tested using a one-sided unpaired Student’s t-test. In all analyses, a P < 0.05 was considered to be significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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