Spontaneous Cytotoxic T-Cell Reactivity against Indoleamine 2,3-Dioxygenase-2

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Abstract
Several lines of data have suggested a possible link between the indoleamine 2,3-dioxygenase (IDO)-like protein IDO2 and cancer. First, IDO2 expression has been described in human tumors, including renal, gastric, colon, and pancreatic tumors. Second, the apparent selective inhibition of IDO2 by the D stereoisomer of the IDO blocker 1-methyl-tryptophan (1MT), which tends to be more active than the L-isomer in a variety of biological assays for IDO function, suggests that IDO2 may be important to sustain immune escape and growth of tumors. Especially, D-1MT heightens chemotherapeutic efficacy in mouse models of cancer in a nontoxic fashion. Here, we describe the immunogenicity of IDO2 by showing the presence of spontaneous cytotoxic T-cell reactivity against IDO2 in peripheral blood of both healthy donors and cancer patients. Furthermore, we show that these IDO2-specific T cells are cytotoxic effector cells that recognize and kill tumor cells. Our data suggest that IDO2 might be a useful target for anticancer immunotherapeutic strategies.

Introduction
Indoleamine 2,3-dioxygenase (IDO) is an immunoregulatory enzyme that is implicated in suppressing T-cell immunity in normal and pathological settings, including cancer (1). IDO1 suppresses effector T-cell function and favors the differentiation of regulatory T cells (Tregs) through the capacity to degrade the essential amino acid tryptophan to kynurenine and other downstream metabolites (2). IDO is widely deregulated in tumors and in tumor-draining lymph nodes. Consistent with a role for IDO in mediating tolerance to tumor, preclinical studies have shown the promise of IDO- inhibitors in targeting several cancers (3–7). In particular, the racemic compound 1-methyl-tryptophan (1MT) composed of L- and D-isomers has been widely studied as an inhibitor of IDO activity. Interestingly, recent studies have shown that the racemere D-1MT has superior antitumor activity compared with L-1MT (8). The D-1MT preferentially target a recently discovered paralog of IDO named indoleamine 2,3-dioxygenase-2 (IDO2; ref. 9). This suggests that IDO2 may be important to sustain immune escape and growth of tumors. In this respect, IDO2 expression has been found in human tumors, including gastric, colon, renal, and pancreatic tumors. Immunohistochemical analysis done in pancreatic tumors reveals IDO2 expression both at the level of the tumor cell as well as immune cells in tumor-draining lymph nodes (10).

We have recently described the presence of IDO1-reactive CTL in peripheral blood of cancer patients and that these cells can recognize and kill tumor cells (11). In this study, we examined the immunogenicity of IDO2. Our data show that spontaneous CTL reactivity against IDO2 exists in the peripheral blood of both cancer patients and healthy donors, and that these cells can recognize and kill tumor cells.

Materials and Methods

Donors
Peripheral blood mononuclear cells (PBMC) were collected from healthy individuals and cancer patients (renal cell carcinoma and melanoma). Blood samples from cancer patients were drawn a minimum of 4 weeks after termination of anticancer therapy. Informed consent was obtained from the patients prior to any of these measures.

MHC-ELISA assay for peptide binding to HLA class I molecules
Peptide affinity for HLA-A2 was measured using an ELISA assay as previously described (12). Controls included in the assay were a low affinity peptide [MART-1 wt (EAAGIGILTV)], two high affinity peptides [MART-1 A2L (ELAGIGILTV) and CMV pp65 (NLVPMVATV)], an HLA-A3-restricted peptide [HIV-1 gag20–28 (RLRPGKKK)], HLA-A2 with no peptide bound, and HLA-A2 bound to the conditional ligand.

IFN-γ ELISPOT assay
The ELISPOT assay was used to quantify peptide-specific IFN-γ or Granzyme B-releasing effector cells as described previously (13).
MHC-tetramer staining

MHC-tetramers were prepared using the MHC-peptide exchange technology as described (14). The MHC-tetramer complexes used were: HLA-A2/IDO2273–281 (VLHAFDEFL), HLA-A2/IDO2386–394 (AVMSFLKSV), and HLA-A2/HIV-1 pol476–484 (ILKEPVHGV).

Establishment of antigen-specific CTL cultures and clones

Specific CTL cultures and clones were generated as described (15). Tetramer positive cells were sorted as single cells by FACS, expanded and tested for specificity and cytotoxic potential in standard 51Cr-release assays. IDO2 expression in cancer cell lines was examined by RT-PCR.

Results

The amino acid sequence of the IDO2 protein was screened for the most probable HLA-A2 nona- and deca-mer peptide epitopes. The following 10 peptides were selected: IDO228–36 (LLPDSLKEL), IDO2110–118 (NLALPFVEV), IDO2199–207 (ALLQALQRL), IDO2224–232 (YVDPDFYFA), IDO2273–281 (VLHAFDEFL), IDO2321–329 (ILSSGQDHL), IDO2329–337 (LLTAYNQCV), IDO2354–362 (YLITAAAKA), and IDO2386–394 (AVMSFLKSV). We examined the IDO2-derived peptides for their binding affinity to HLA-A2 by MHC-ELISA. Seven of the selected IDO2-derived peptides (IDO228–36, IDO2199–207, IDO2224–232, IDO2273–281, IDO2329–337, IDO2354–362, and IDO2386–394) displayed high affinity for HLA-A2, whereas the remaining peptides displayed intermediate affinity for HLA-A2 (Fig. 1A). PBMC from HLA-A2+ healthy individuals and late-stage cancer patients (melanoma and renal cell carcinoma) were examined for the presence of T-cell responses against HLA-A2 binding, IDO2-derived peptides by IFN-γ ELISPOT assay. To extend the sensitivity of the assay, PBMC were stimulated once with the peptides in vitro before examination by ELISPOT. T-cell reactivity toward several IDO2-derived peptides was observed, in PBMC from

Figure 1. A, HLA-A2 binding affinity of IDO2-derived peptides and spontaneous T-cell reactivity toward these peptides in cancer patients. HLA-A2 molecules, with a photocleavable, conditional ligand in the binding groove, were exposed to UV light for 0 or 60 minutes in the presence of either no peptide, the HLA-A3-restricted control peptide HIV-1 gag20–28 (RLRPGGKKK), the low affinity control peptide MART-126–35 wt (EAAGIGILTV), the high affinity control peptides MART-126–35 A2L (ELAGIGILTV), CMVpp65495–503 (NLVPVMATV), or an IDO2-derived peptide, and analyzed by MHC-ELISA. B, HLA-A2+ PBMC were plated at 2 × 10⁵ cells in duplicates either alone or with IDO2 peptide. The average number of IDO2-specific spots (after subtraction of spots in wells without added peptide) was calculated.
several melanoma and renal cell carcinoma patients. T-cell reactivity was detected against the high affinity peptides: IDO2_{219-207}, IDO2_{224-232}, IDO2_{273-281}, IDO2_{329-337}, IDO2_{354-362}, and IDO2_{386-394} (Fig. 1B). The most frequent and most dominant responses were observed against IDO2_{273-281} and IDO2_{386-394}. PBMC from a larger panel of patients and healthy donors were examined for reactivity against these peptides (Fig. 2A and B). A Mann–Whitney test illustrated that there apparently was no difference in T-cell reactivity toward a specific peptide between healthy donors and tumor patients (IDO2_{273-281}: \( P = 0.88 \) and IDO2_{386-394}: \( P = 0.98 \)). However, when looking at the frequency of immune reactivity against one or the other peptide within the same group of donors, it seemed that while the reactivity toward both peptides was correlated in healthy donors (Spearman rho = 0.61, \( P = 0.004 \)), it seemed to be independent in cancer patients (Spearman rho = 0.1, \( P = 0.67 \)). T-cell reactivity toward IDO2_{273-281} was additionally detectable by Granzyme B ELISPOT (Fig. 2B and D). The high binding affinity of IDO2_{273-281} and IDO2_{386-394} to HLA-A2 enabled us to make stable HLA-A2/IDO2 tetramers, which were used to detect IDO2-reactive T cells by flow cytometry. These analyses confirmed the

![Graph A](image1.png)

**Figure 2.** Spontaneous cytotoxic T-cell reactivity toward IDO2. Spontaneous T-cell reactivity against IDO2-derived peptides in PBMC from HLA-A2^+^ healthy donors (HD), renal cell carcinoma patients (RCC), and melanoma patients (MM) visualized by IFN-\( \gamma \) ELISPOT assay (A, B), flow cytometry (C), and Granzyme B ELISPOT assay (B, D) after one in vitro peptide stimulation. For IFN-\( \gamma \) ELISPOT assay, PBMC were plated at 2 \( \times 10^5 \) PBMC in duplicates either alone or with IDO2_{273-281} (top) or IDO2_{386-394} peptide (bottom). The average number of IDO2-specific spots (after subtraction of spots in wells without added peptide) was calculated (A, B). IDO2-specific T cells were identified using the tetramer complex HLA-A2/IDO2_{273-281} or HLA-A2/IDO2_{386-394} with CD8 mAb. For comparison, cells were stained with the tetramer complex HLA-A2/HIV-1 pol476-484 and CD8 mAb (C). For Granzyme B ELISPOT assay, PBMC were plated at 2 \( \times 10^5 \) PBMC in duplicates either alone or with IDO2_{273-281} peptide (B, D).
presence of IDO2-specific T cells in the blood of HLA-A2+ cancer patients after in vitro peptide stimulation. Figure 2C illustrates examples of IDO2-specific T cell stainings of PBMC from a renal cell carcinoma and a melanoma patient, respectively.

To examine the functional capacity of IDO2-specific T cells, we generated CTL bulk cultures in vitro against IDO2273–281 and IDO2386–394 using PBMC from the 2 patients described in Figure 2C. The IDO2-specificity of the bulk cultures was confirmed by tetramer staining and 51Cr-release assay as depicted in Figure 3A and B. The IDO2-specific CTL bulk cultures effectively killed IDO2-pulsed TAP-deficient T2 cells compared with T2 cells pulsed with an irrelevant peptide [HIV-1 pol476–484 (ILKEPVHGV); Fig. 3A and B]. Finally, CTL clones were established from the IDO2273–281-specific CTL bulk culture by FACS single-cell sorting of IDO2273–281 tetramer positive cells. IDO2273–281-specific and HLA-A2 restriction of the established CTL clones were confirmed by tetramer stainings (Fig. 3C). Figures 3 and 4 show the lytic capacity of representative IDO2273–281-specific CTL clones analyzed by 51Cr-release assay. The IDO2273–281-specific CTL clones effectively killed IDO2273–281-pulsed T2 cells, whereas T2 cells pulsed with an irrelevant peptide (HIV-1 pol476–484) were not lysed (Fig. 3D).

The CTL clones were of intermediate-to-high avidity, because they lysed T2 cells pulsed with peptide concentrations down to 10⁻⁷ mmol/L (Fig. 3D). Most importantly, IDO2273–281-specific CTL also killed the HLA-A2+/IDO2+ colon cancer cell lines SW480 and HCT116, as well as the HLA-A2+/IDO2+ breast cancer cell line CAMA-1 (Fig. 4A). In contrast, no cytotoxicity was observed against the HLA-A2+/IDO2-...
IDO2⁺ melanoma cell line FM6 (Fig. 4A) or against the K562 cell line lacking MHC complexes (Fig. 4B). HLA restriction was further confirmed by blocking HLA-A2 using a HLA-A2-specific mAb, which abolished lysis of the SW480, HCT116, and CAMA-1 cells (Fig. 4B). Cold target inhibition assays using unlabeled T2 cells pulsed with IDO2₂₋₃₋₂₈₁ peptide confirmed HLA-A2/peptide specificity of the killing: The addition of cold (unlabeled) IDO2₂₋₃₋₂₈₁-pulsed T2 cells completely abrogated the killing of CAMA-1 breast cancer cells, whereas the addition of cold T2 cells pulsed with the irrelevant HIV-1 pol₄₇₆₋₄₈₄ peptide only showed a limited dilution effect (Fig. 4C). Importantly, no cytotoxicity was observed against the HLA-A₂⁻/IDO2⁻ lymphoblastic cell line T1, unless IDO2₂₋₃₋₂₈₁ peptide was added (Fig. 4C). Furthermore, we show that the IDO2₂₋₃₋₂₈₁-specific CTL clones were not cross-reacting with or influenced by IDO1 expression, because they were able to kill both the IDO1⁺ SW480 cells as well as the IDO1⁻ HCT116 cells (Fig. 4A). Furthermore, SW480 cells transfected with IDO1 shRNA (IDO1⁺ SW480 cells) were killed in a similar manner as SW480 by the IDO2₂₋₃₋₂₈₁-specific CTL clones (data not shown). Finally, we tested the capacity of the IDO2₂₋₃₋₂₈₁-specific CTL clones to lyse HLA-A₂⁺ immune cells, that is, monocytes, lymphocytes, and DC. For this purpose, we isolated CD14⁺ monocytes and CD3⁺CD19⁺ lymphocytes directly ex vivo from PBMC, and generated DC in vitro. The isolated cells were subsequently used as target cells in a ⁵¹Cr-release assay. Neither autologous nor allogeneic immune cells were lysed (Fig. 4D). Similarly, no killing was observed against an HLA-A₂⁻/IDO2⁻ B-cell line (Fig. 4D).

Target cells were examined for IDO2 expression by RT-PCR. As mentioned above, SW480, HCT116, CAMA-1, FM6, and K562 were all IDO2⁺, whereas the T1 cells (and TAP-deficient T2 cells) were IDO2⁻ (Fig. 4A). Additionally, autologous and allogeneic monocytes, lymphocytes as the B-cell line were IDO2⁺/IDO2⁻ in vitro, whereas matured DC were IDO2⁻ (data not shown).

Discussion

In this study, we set out to examine if IDO2 may serve as target for immune responses, which may be exploited for anticancer immune therapy. We identified HLA-A2 peptides within the IDO2 protein to which spontaneous T-cell reactivity were detected in patients suffering from unrelated tumor types. However, surprisingly healthy individuals also host spontaneous immunity against IDO2. These naturally occurring T-cell responses could be readily visualized by flow cytometry using HLA/peptide tetramers as well as
ELISPOT assays. Furthermore, we confirmed that IDO2-reactive T cells are indeed peptide-specific, cytotoxic effector T cells. Hence, isolated and expanded IDO2-specific T cells effectively lysed IDO2\textsuperscript{+} cancer cell lines of different origin, that is, colon carcinoma cells as well as breast cancer cells. The killing of cancer cells of different origin by IDO2-specific T cells underlines the immunotherapeutic potential of IDO2.

Not all cancer cells are IDO2\textsuperscript{+}. Here, we show that an IDO2\textsuperscript{--} lymphoblastic cell line was only killed by the IDO2-specific T cells when loaded with the IDO2 epitope. Hence, these data strongly indicate that the expression of IDO2 is a necessity for T-cell recognition although several additional factors certainly influence the level of killing by T cells; the avidity of the T cells, the HLA-expression of the target cell, the antigen-processing machinery, the expression of activating and inhibitory NK-receptors as well as adhesion molecules have all been shown to be involved in efficient target cell recognition by effector T cells. The ligands of these receptors could be differentially expressed on the tumor lines used in this study and it would, consequently, be very difficult to directly correlate IDO2-expression level with the level of killing.

Recent data from the clinic suggest a synergistic effect of anticancer immunotherapy and chemotherapy. This is supported by the report that the IDO-blocker 1MT works synergistically with different chemotherapy drugs in established murine cancers (16). Many anticancer vaccination strategies are already focusing on the combination with other immunotherapeutic strategies. However, so far most peptide-based vaccination trials have targeted only a single antigen. Recently, we showed that IDO1-specific T cells are able to recognize and kill tumor cells. To maximize the impact of immunotherapy, an exciting strategy would be to cotarget both IDO1 and IDO2. It is still not known to what extent each isoform contributes to tumor-related immunosuppression and how much clinical benefit (or autoimmune toxicity) targeting one isoform over another confers. Another unknown is how much IDO and/or IDO2-specific T cells influence other pathways not directly linked to IDO1/IDO2. Although IDO1 and IDO2 are upregulated in almost all cancers, there may be significant quantitative divergences concerning the amount of each protein in individual patients.

By definition most anticancer immunotherapeutic strategies irrespective of their molecular targets aim at the induction of an immunological activation. Virtually, within the limits of acceptable toxicity as much immune activation as possible is the goal; hence, counterregulation is not desired. In this regard, in treatment with anti-CTLA-4 antibodies an association between autoimmune reactions have been correlated with clinical efficacy (17). Naturally, one should be extremely cautious of the possible introduction of autoimmunity when targeting a self-protein–like IDO2. In this regard, IDO2 is expressed in a variety of antigen-presenting cell types and human IDO2 mRNA have been detected in several organs including lever, intestine, spleen, and lung (18). In vivo data with 1-MT may suggest that the inhibition of IDO2 in vivo does not lead to evident toxicity. However, one thing is to inhibit one molecule of a cell and the other to kill an entire cell. The circulation of a measurable number of IDO2-specific T cells does not seem to cause autoimmunity. Furthermore, the data presented here suggest that normal immune cells are not lysed by IDO2-specific T cells. Similar findings have previously been described for other tumor antigens (19). It might be related to the affinity of the induced T cells and to higher presentation of IDO2-derived peptides by tumor cells. Another explanation might be that nonmalignant cells present a different repertoire of T-cell epitopes as compared with tumor cells. Hence, it is still a question how IDO2-specific T cells are activated in healthy individuals in vivo and what, if any, potential role such IDO2-specific T cells play in immune regulation. It may be possible that the sizable reactivity to this antigen in normal individuals contributes to immune surveillance against cancer as we have recently shown that IDO1-specific T cells isolated from healthy individuals were cytotoxic toward IDO1-expressing cells in a similar manner as IDO1-specific T cells isolated from cancer patients (20). However, it still needs to be verified that the IDO2-specific class I–restricted lymphocytes in peripheral blood of healthy subjects are indeed cytolytic for IDO2-expressing cells in vivo.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We would like to thank M. Jonassen and T. Seremet for excellent technical assistance, as well as S.R. Hadrup for providing tetramers to the study. We thank T.W. Klausen for statistical analysis.

Grant support

The Danish Cancer Society, Novo Nordisk Foundation, Danish Medical Research Council, Lundbeck Foundation, and Herlev Hospital.

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Received September 20, 2010; revised December 13, 2010; accepted December 21, 2010, published online March 15, 2011.

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