Indoleamine-2,3-dioxygenase in an Immunotherapy Model for Ewing Sarcoma

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Abstract. *Background/Aim: Interleukin-2 (IL2) transgenic Ewing sarcoma cells reduce tumor growth in vivo and in vitro. In the present study we analyzed the expression of immune suppressive indoleamine-2,3-dioxygenase (IDO) in this model. Materials and Methods: Expression of IDO was analyzed by polymerase chain reaction. The impact of the cluster of differentiation 137 (CD137)/CD137 ligand (CD137L) co-stimulatory system on expression of IDO and different cytokines was analyzed both in vivo and in vitro. Results: Tumors that developed in vivo in the presence of IL2 transgenic tumor cells expressed IDO. The presence of CD137L transgenic tumor cells led to down-regulation of IDO. Further in-vitro analysis of this phenomenon indicated that IDO was expressed in tumor cells as a consequence of interferon-gamma produced by lymphocytes in response to IL2. Depending on the concentration of IL2, stimulation of CD137 increased or reduced cytokine production in lymphocytes. Conclusion: Our data indicate that the CD137/CD137L pathway can modulate the immune response against Ewing sarcoma cells.*

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Key Words: Interleukin-2, immune escape, indoleamine-2,3-dioxygenase, CD137, Ewing sarcoma.

Ewing sarcomas (also named Ewing family tumors, EFT) comprise a biologically distinct group of bone and soft tissue neoplasias in children and young adults. More than half of patients with localized EFTs can be cured with multimodal regimens with chemotherapy and radiotherapy. In contrast, the prognosis for patients with advanced disease remains poor with conventional therapy (1). For these patients, it is necessary to establish new treatment strategies. EFT can be characterized at the molecular level by expression of tumor-specific onco-fusion proteins (2). Such fusion proteins are highly tumor-specific and might be interesting for the development of immunological treatment strategies. Using high-density DNA microarrays, we identified additional potential tumor antigens expressed in EFT, *e.g.* lipase I (3-6). Despite the presence of such tumor-specific antigens, the immunostimulatory activity of EFT is usually low. Employing an established murine xenotransplantation model for EFT (7), we observed that the presence of interleukin-2 transgenic (IL2Tg) EFT cells enhanced the immune response against wild-type tumor cells. Transgenic expression of IL2 induced an EFT-specific T- and natural killer (NK) cell response and reduced tumor growth *in vivo* and *in vitro* (8), however, tumor growth was not completely inhibited. Therefore, we asked whether additional targeting of co-stimulatory pathways can increase antitumor efficacy. In the present study, we analyzed the effect of stimulation of the immune system *via* the cluster of differentiation 137 (CD137)/CD137 ligand (CD137L) co-stimulatory pathway on the immune response against EFT. CD137L and CD137 are members of the tumor necrosis factor (TNF) and TNF receptor superfamilies, respectively. CD137 is expressed on activated T-cells, activated NK cells, macrophages, granulocytes and dendritic cells. CD137L is expressed on activated B-cells, activated macrophages and activated

dendritic cells. Cross-linking of CD137 by *CD137L* transgenic (CD137LTg) cells or agonistic antibodies against CD137 promotes proliferation, activation, cytokine production [IL2 and interferon gamma (IFNγ)] and survival of T-cells (9-12). Interestingly, agonistic antibodies against CD137 can induce suppression of T-dependent humoral immunity (13). Similarly, these antibodies can inhibit the development of autoimmune disease, and can reverse established autoimmune disease (14-16). One of the discussed mechanisms for these observations is that the antibodies against CD137 generate anergy in antigen-specific CD4-positive T-cells as a result of IFNγ production and the expression of immune-suppressive indoleamine-2,3-dioxygenase (IDO) in macrophages and dendritic cells. On the other hand, the presence of CD137LTg cells or agonistic antibodies against CD137 can induce rejection of different tumor types *in vivo* (17, 18). Failure of immunotherapy can be a consequence of immune-suppressive activity of tumor cells. IDO catalyzes an important step in tryptophan metabolism. Tumor cells can express IDO and low tryptophan concentrations in the proximity of the tumor can result in cell-cycle arrest of T-cells (19). A small population of dendritic cells expresses IDO and can also inhibit the proliferation of T-cells *in vitro* (20). Given the importance of IDO for the regulation of antitumor immune responses, we investigated the expression of IDO in our immunotherapy model for EFT.

Materials and Methods

Cell lines and cell culture. EFT cell lines SK-N-MC, SK-ES1 and TC-71 (21-23), neuroblastoma cell lines SH-SY5Y and IMR-32 (21, 24), Hodgkin's lymphoma cell lines L-1236, L-540, HDLM-2 and KM-H2 (25-28), leukemia cell lines THP1, K562 and Jurkat (29-31), fibrosarcoma cell line HT-1080 (32), and the cervical carcinoma cell line HeLa (33) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). The EFT cell lines A673 and RD-ES (34-36) were purchased from the American Type Culture Collection (Manassas, VA, USA). Leukemia cell line HL-60 (37) was a kind gift from J.-U. Hartmann (Halle, Germany). Thyroid carcinoma cell lines B-CPAP, FTC-133, 8505C and HTh 74 (38-41) were a kind gift from T. Mueller (Halle, Germany). EFT cell lines STA-ET8, TC-32 and TTC-446 (22, 42, 43) were a kind gift from B. Schmiedel (Halle, Germany). Melanoma cell lines 518A2 (44) and 31IGR1 were a kind gift from J. Wohlrab (Halle, Germany). Epstein-Barr virus (strain B95-8)-immortalized lymphoblastoid cell lines (LCL) were established from peripheral blood mononuclear cells (PBMCs) using standard methods (45). CD137LTg A673 cells were established by transfection of A673 Ewing sarcoma cells with CD137L in vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). IL2Tg A673 cells and mock-transfected A673 cells were established as described elsewhere (8, 36). Mesenchymal stem cells (MSCs) were a kindly gift from L. Mueller (Halle, Germany). PBMCs were isolated from buffy coat from healthy donors with informed consent and approval of the Ethics Committee of the Martin Luther University Halle-Wittenberg (14.09.2005/24.11.2005) using Pancoll human (PAN-Biotech, Aidenbach, Germany) gradient centrifugation.

Cells were cultured in RPMI-1640 or Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and penicillin/streptomycin. For *in vitro* experiments, different cell types were separated by tissue culture inserts with 0.02 μm pore size (TPP, Trasadingen, Switzerland). The cells were cultured in 6-well plates containing 4 ml RPMI-1640 per well. In the tissue culture inserts, 5×10^5 A673 cells were cultured. In the bottom chamber, 5×10^5 IL2Tg A673 cells or vector control cells were cultured together with 2×10^6 human leukocyte antigen (HLA)-matched PBMCs. The cells were cultured in the presence or absence of 26G.6 antibody to human CD137 (a kind gift from R. S. Mittler, Atlanta, GA, USA). Six-well plates were coated with antibody (10 μg/ml) either overnight at 4˚C or for 4 hours at 37˚C. Thereafter, the wells were washed with phosphate buffered saline (PBS) (Lonza, Cologne, Germany) and cells were added to the wells. In experiments with defined concentrations of IL2, proleukine (Chiron, Amsterdam, the Netherlands) was used in concentrations between 3,000 and 48,000 U/ml. In this setting, only PBMCs were cultured in the bottom chamber. The cells were cultured for three days. Thereafter, A673 cells were harvested and RNA was isolated. The culture supernatants were harvested, centrifuged at 200 × *g* for 10 minutes and stored at −20˚C.

Different cell lines were incubated with 500 U/ml IFNγ (R&D, Wiesbaden, Germany) for three days. Thereafter, stimulated and unstimulated control cells were harvested and RNA was isolated.
For allostimulation assays, 1×10^5 HLA-mismatched PBMCs and 5×10^4 IFNγ-stimulated A673 cells were incubated in 200 μl medium in the presence of 3,000 U/ml IL2. A673 cells without IFNγ stimulation served as controls. The proliferation was measured with a Cell Proliferation ELISA (Roche, Grenzach-Wyhlen, Germany) on day one, three, five, seven and nine. To this end, the cells were incubated with 5-bromo-2-deoxyuridine (BrdU) for 2 hours at 37˚C. After centrifugation for 10 min at 300 ×*g* the medium was carefully removed. After fixation and denaturation of the cells, they were incubated with antibody to BrdU for 90 minutes. After three washing steps, the wells were incubated with a substrate solution for 10 minutes. The reaction was stopped with 1 M H_2SO_4 and the absorbance measured photometrically at 450 nm (reference wavelength: 690 nm).

Cytometric bead array (CBA). The presence of cytokines [IL2, IL4, IL5, IL10, IFNγ, TNF alpha (TNFα)] in culture supernatants was analyzed with the CBA Human Th1/Th2 Cytokine Kit II (BD Bioscience, Heidelberg, Germany) following the manufacturer's protocol.

Animal model. NOD/scid mice were bred and maintained under defined pathogen-free conditions in sterilized cages with filter tops and fed with sterilized food and water in our animal facility. Mice were used at the age of 6-20 weeks. Animal experiments were approved by the Animal Care Committee of the local government (2-728/I13G6). For transplantation of tumor cells into NOD/scid mice, tumor cells were recovered from tissue culture flasks using PBS containing 5 mM EDTA. IL2Tg cells (cell line SBVGA1), vector-control transfected A673 cells (cell line SBVGA3), or CD137Tg A673 cells were irradiated with a single dose of 30 Gy directly before cell transfer. HLA matched PBMC/tumor cell mixtures were injected subcutaneously into the right back of the

animals. Tumor growth was measured until the animals were sacrificed for ethical reasons. The tumors were carefully dissected and RNA prepared as described below.

Conventional and quantitative reverse transcriptase-polymerase chain reaction. RNA from cell lines and tumors was isolated by using Trifast reagent (PeQLab, Erlangen, Germany) following the manufacturer's protocol. After reverse transcription of 2 μg of RNA, polymerase chain reaction (RT-PCR) was performed as described elsewhere (5). The following primer combinations were used: β-actin (ACTB): 5'-GGC ATC GTG ATG GAC TCC G-3', 5'-GCT GGA AGG TGG ACA GCG A-3'; IDO: 5'-TCA AAG CAC TGA AAG ACG CTG C-3', 5'-CCC CTG ACT TAT GAG AAC ATG G-3'. Two microliters of cDNA was mixed with 2.5 μl 10× Taq Buffer, 1.5 μl $MgCl_2$ (25 mM), 0.5 μl dNTP (10 mM), 0.25 μl of both primers (25 μM), 0.2 μl Taq polymerase (5 U/μl), and 17.8 μl water. The PCR conditions were: 94˚C for 30 s; 60˚C for 30 s; 72˚C for 45 s (ACTB, 25 cycles; IDO, 27 cycles). PCR products were subjected to agarose gel (1.5%) electrophoresis in the presence of ethidium bromide. Quantitative RT-PCR was carried out using the Maxima SYBR Green qPCR Master Mix (Fermentas, St Leon-Rot, Germany) as described previously (5). Each reaction was subjected to melting temperature analysis to confirm the presence of the expected amplified products. Specific gene amplification was normalized to that of ACTB. IDO and ACTB were amplified with 40 cycles using a Rotor Gene RG-3000 (Corbett Research, Cambridgeshire, UK) and Rotor-Gene 6 software. Relative gene expression values were calculated by using the standard $2^{-\Delta\Delta ct}$ method (46).

Results

In an earlier investigation, we observed that transgenic expression of IL2 induced EFT-specific T- and NK cell response and inhibited tumor growth *in vivo* and *in vitro* (8). To improve the efficacy and specificity of this immunotherapy model, we tested the combination of IL2 and the co-stimulatory CD137/CD137L system. In combination with IL2, the presence of irradiated CD137L transgenic tumor cells or antibodies against CD137 induced an enhanced tumor-specific T-cell response *in vitro* (Figure 1). For validation of these results *in vivo*, wild-type EFT cells (cell line A673) were transplanted together with HLA-matched PBMCs, irradiated IL2 transgenic (IL2Tg) A673 cells, and irradiated CD137L-transgenic (CD137LTg) A673 cells into immune-compromised mice. As shown in Figure 2A, the presence of CD137LTg cells only slightly reduced tumor growth.

We analyzed the expression of IDO in tumors from this xenotransplantation model for EFT. Expression of IDO in the growing tumors was analyzed with conventional RT-PCR and quantitative RT-PCR. The expression of IDO was detected only in tumors that had been grown in the presence of PBMCs and IL2Tg tumor cells (Figure 2B and C). Interestingly, IDO expression was undetectable in the presence of CD137LTg tumor cells.

The *in vivo* observations were further analyzed *in vitro* using semi-permeable tissue culture inserts, antibodies

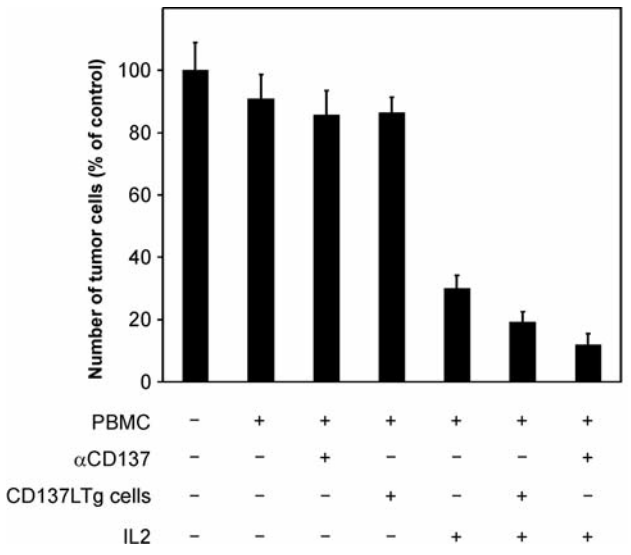

Figure 1. *Cluster of differentiation 137 (CD137) stimulation enhances anti-tumor reactivity in a model for Ewing sarcoma immunotherapy in vitro. Wild-type A673 cells were cultured in the presence or absence of human leukocyte antigen (HLA)-matched peripheral blood mononuclear cells (PBMCs). During co-culture, interleukin-2 (IL2), irradiated CD137 ligand transgenic (CD137LTg) A673 cells or antibodies to CD137 (αCD137) were present or absent. After 5 days, plastic-adherent living tumor cells were counted. The number of cells in cultures without PBMCs was set as 100%. Presented are means and standard deviations from three experiments.*

against CD137, and PBMCs from different donors. For some donors, we found the same behavior as in the *in vivo* experiments (Figure 3; donor A). On the other hand, the response of some donors differed from the *in vivo* results (Figure 3; donors B, C and D). There were donors where IDO was expressed in the presence of IL2Tg tumor cells and the addition of antibodies to CD137 did not reduce the expression of IDO (donor B, Figure 3). There were other donors where none of the culture conditions resulted in IDO expression (donor C, Figure 3) or where IDO was expressed under all culture conditions (donor D, Figure 3). We observed IDO expression only in cultures with IFNγ production by PBMCs. In contrast, no link between IDO expression and IL2 production from the transgenic tumor cells was observed (Figure 3).

It seems possible that the IL2Tg tumor cells produce varying amounts of IL2. Therefore, we used defined concentrations of IL2 for the next experiments. We used 3,000 U/ml IL2 in the presence or absence of antibodies against CD137. Using quantitative RT-PCR, for this concentration of IL2, we found the same result as in the *in vivo* experiment (Figure 4A): induction of IDO in the wild-type tumor cells in the presence of IL2 and reduced expression of IDO in the presence of CD137 antibodies. By varying the IL2

concentration, a variation in the expression of IDO occurred (Figure 4B). At low IL2 concentrations (0 to 12,000 U/ml), IDO was reduced in the presence of the antibody against CD137. However, at high IL2 concentrations (24,000 to 48,000 U/ml), IDO was more highly expressed in the presence of the CD137 antibodies. The cytokine analysis showed a correlation between IDO expression by tumor cells and IFNγ secretion by PBMCs (Figure 4C).

In addition to IFNγ, we measured the concentration of TNFα, IL10 and IL6 (Figure 5). IL2-alone had no influence on TNFα secretion, but the additional presence of antibodies to CD137 increased the concentration of TNFα at higher IL2 concentrations. Different IL2 concentrations had no influence on IL10 secretion in our system, but IL10 concentrations were always lower when antibodies to CD137 were present (Figure 5). Similarly, the concentration of IL6 was always reduced in the presence of antibodies to CD137. Here, increasing IL2 concentrations led to increased IL6 secretion. For IFNγ, we observed increased concentrations at high IL2 concentrations. At low IL2 concentrations, antibodies to CD137 blocked INFγ secretion, whereas at high IL2 concentration, the CD137 antibodies led to increased INFγ secretion.

We also analyzed the influence of INFγ on IDO expression in different tumor cell lines. It is known that MSCs express IDO after incubation with IFNγ (47). We observed that different cell lines from EFT and other solid tumor types (melanoma, neuroblastoma, thyroid carcinoma, fibrosarcoma, cervix carcinoma) expressed IDO after incubation with IFNγ (Figure 6). In contrast, the investigated hematopoietic cell lines (Epstein Barr virus-immortalized lymphoblastoid cell lines, lymphoma and leukemia cell lines) did not express IDO.

Finally, we investigated whether IDO-expressing EFT cells can inhibit the proliferation of PBMCs in co-culture experiments. As shown in Figure 7, IDO-expressing A673 cells inhibited the growth of allogeneic PBMC in a similar way as is known for MSCs (47).

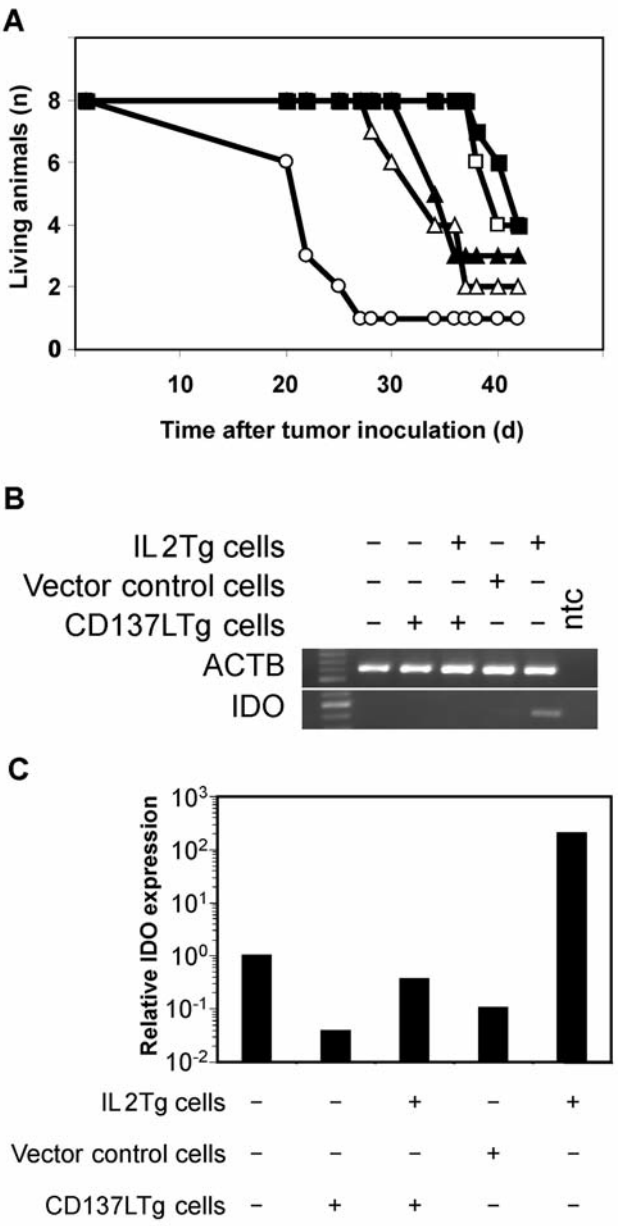

Figure 2. *Indoleamine-2,3-dioxygenase (IDO) expression in tumors grown in a model for Ewing sarcoma immunotherapy in vivo. A: NOD/scid mice (8 animals per group) were transplanted with wild-type A673 cells (open circles); wild-type A673 cells and human leukocyte antigen (HLA)-matched peripheral blood mononuclear cells (PBMCs) (open triangles); wild-type A673 cells, HLA-matched PBMCs, and irradiated interleukin-2 transgenic (IL2Tg) SBVGA1 cells (open squares); wild-type A673 cells, HLA-matched PBMCs, and irradiated cluster of differentiation 137 ligand transgenic (CD137LTg) A673 cells (closed triangles); or wild-type A673 cells, HLA-matched PBMCs, irradiated IL2Tg SBVGA1 cells, and irradiated CD137LTg A673 cells (closed squares). Mice were sacrificed after tumor volumes had exceeded 2.5 ml. IDO expression in xenografted tumors from animals with different treatments was analyzed by conventional (B) and quantitative (C) reverse transcription-polymerase chain reaction. For comparative analyses, expression values from the tumor in the presence of PBMCs without additional cells were set as 1. ntc: No template control.*

Discussion

Transgenic expression of IL2 enhanced the immunostimulatory activity of EFT cells *in vivo*, but in contrast to our *in vitro* results, this enhancement could not completely inhibit the growth of the tumor cells (8). It is known that the growth of tumor cell lines can be inhibited by antibodies against CD137 (17, 18). In patients with CD137L-expressing EFT, CD3/CD137-positive cytotoxic T-cells have been observed which were found to control primary tumor growth and the development of metastases in a xenograft model (48). Therefore, we combined both stimuli in one model, but here the tumor growth was also not completely inhibited. It is possible that the tumor cells used tumor-escape mechanisms to outwit the immune system. As mentioned above, IDO expression by

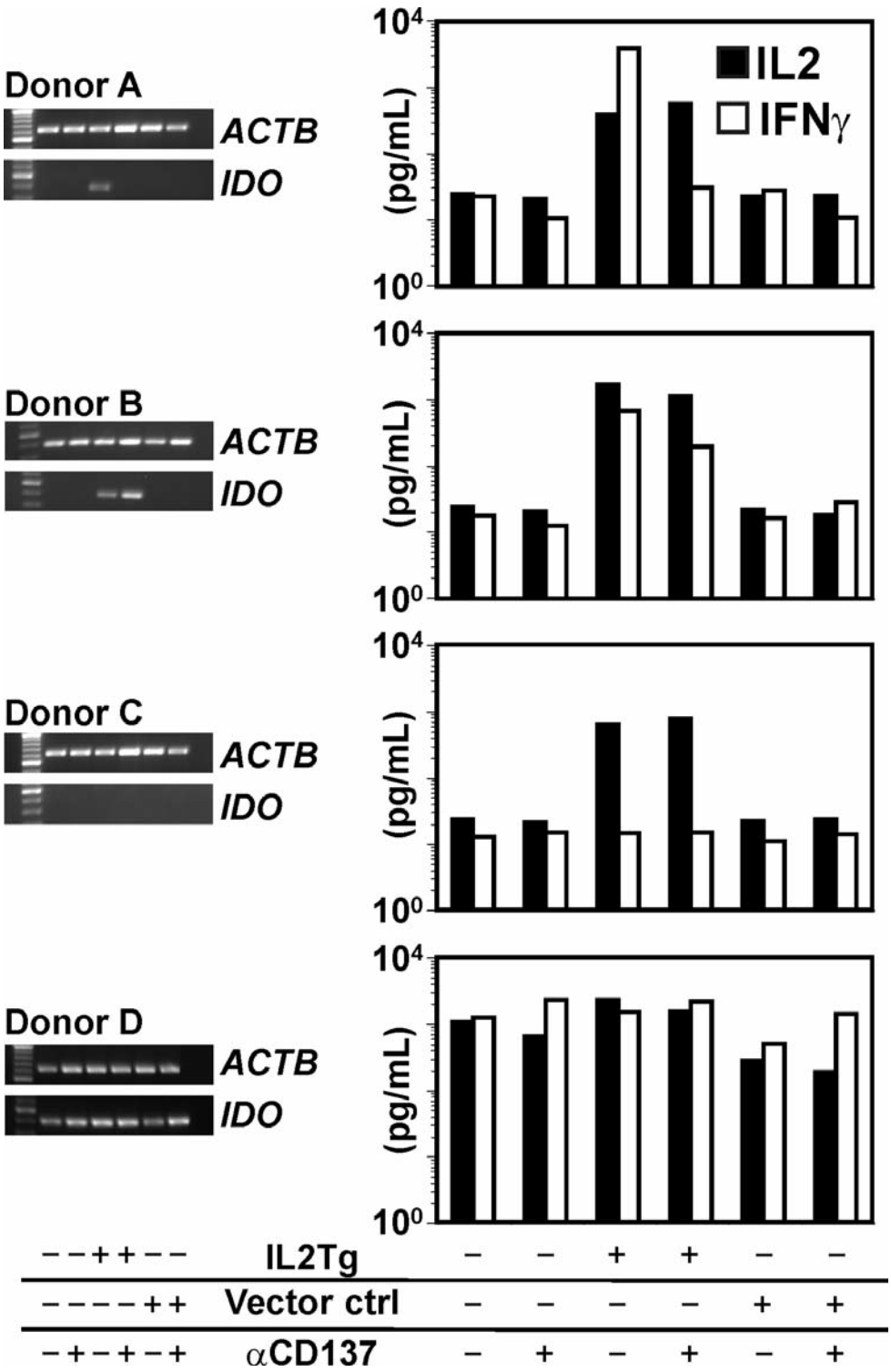

Figure 3. *Expression of indoleamine-2,3-dioxygenase (IDO) and secretion of interferon-gamma (IFNγ) in co-cultures of Ewing sarcoma (EFT) cells and peripheral blood mononuclear cells (PBMCs) in the presence or absence of interleukin-2 (IL2) and antibodies against cluster of differentiation 137 (CD137). Left panels: Expression of IDO was analyzed by reverse transcription-polymerase chain reaction in A673 cells after co-culture with PBMCs from four different donors (A-D). During co-culture, interleukin-2 transgenic (IL2Tg) A673 cells, vector-transfected control cells (Vector ctrl) and antibodies to CD137 were present or absent. β-Actin (ACTB) was used as housekeeping control gene. Right panel: Supernatants from the same cultures were tested by cytometric bead array for the presence of IL2 (closed bars) and IFNγ (open bars).*

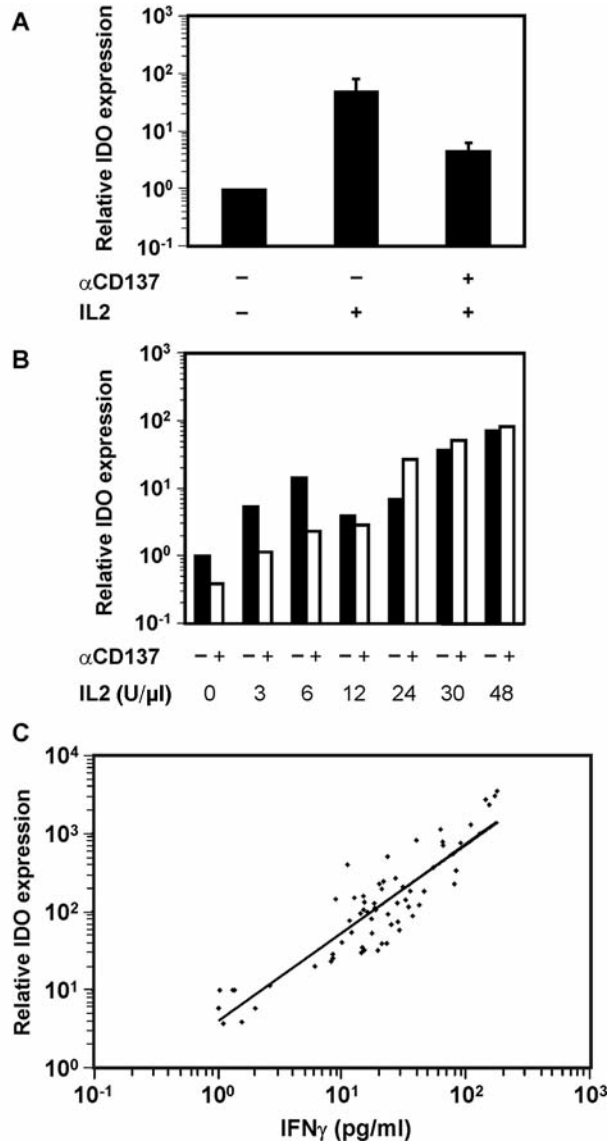

Figure 4. *Expression of indoleamine-2,3-dioxygenase (IDO) in A673 cells after co-culture with peripheral blood mononuclear cells (PBMCs) in the presence or absence of interleukin-2 (IL2) and antibodies to cluster of differentiation 137 (CD137). Expression of IDO was analyzed by quantitative reverse transcription-polymerase chain reaction (RT-PCR) in A673 cells after co-culture with PBMCs in the presence or absence of IL2 in the presence or absence of antibodies to CD137. For comparative analyses, the expression of IDO in the absence of IL2 and antibodies to CD137 was set as 1. β-Actin (ACTB) was used as housekeeping control. A: A673 cells were cultured with PBMCs in the presence or absence of 3000 U/μl IL2 and presence or absence of antibodies to CD137. Data are the means and standard errors from five different donors B: A673 cells were cultured with PBMCs from a healthy donor in the presence or absence of varying concentrations of IL2 and in the presence or absence of antibodies to CD137. C: Correlation between IDO expression and IFNγ secretion after co-culture of PBMCs and A673 cells. A673 cells were cultured with PBMCs from five different donors in the presence or absence of IL2 and in the presence or absence of antibodies to CD137. The concentration of IFNγ was determined in culture supernatants by cytometric bead array. During co-culture, IL2 and anti-CD137 antibodies were present or absent.*

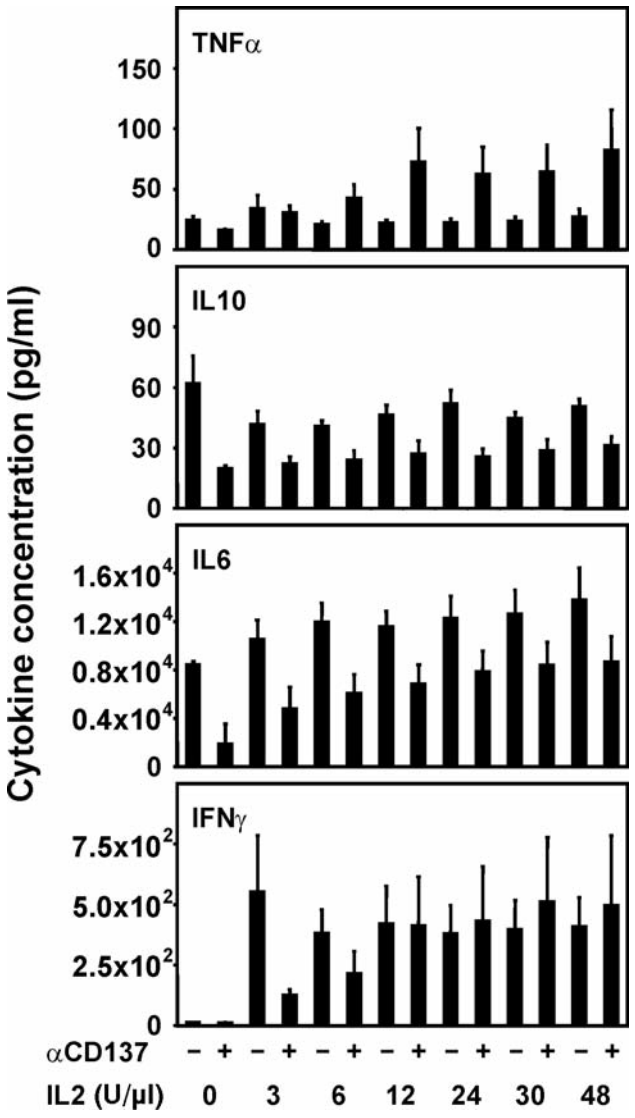

Figure 5. *Analysis of tumor necrosis factor-alpha (TNFα), interleukin-6 (IL6), IL10 and interferon-gamma (IFNγ) in cell culture supernatants. The presence of the indicated cytokines in culture supernatants was quantified after co-culture of peripheral blood mononuclear cells and A673 cells in the presence of different IL2 concentrations and the presence or absence of antibodies to CD137. Data are the means and standard errors from five independent experiments.*

tumor cells is one known tumor immune escape mechanism. In the *in vivo* model examined here, we found expression of IDO in the tumor which grew in the presence of PBMCs and IL2Tg tumor cells. Tumors that grew in mice under control treatment (treatment only with PBMCs, or with PBMCs and control vector-transfected cells) showed no expression of IDO. In the presence of IL2Tg and CD137LTg tumor cells, we also did not observe IDO expression. A possible explanation for this

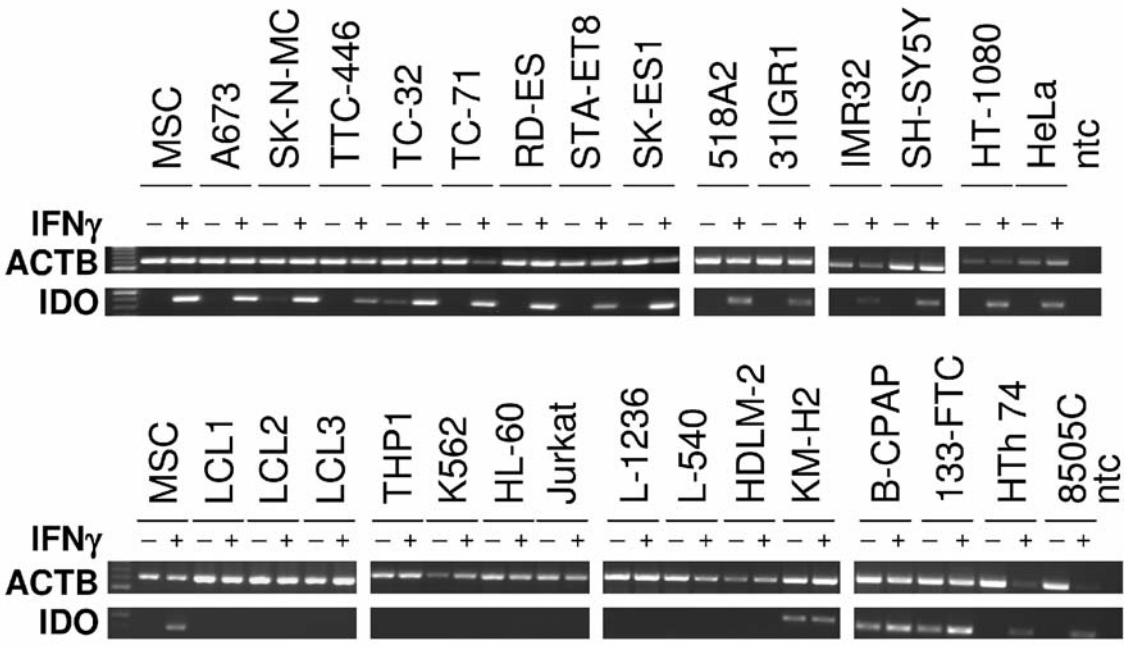

Figure 6. *Expression of indoleamine-2,3-dioxygenase (IDO) in different tumor cell lines after stimulation with interferon-gamma (IFNγ). Expression of IDO and β-actin (ACTB) was analyzed by conventional RT-PCR in the indicated cell lines. Cells were incubated with or without IFNγ for three days. Results are from EFT (A673, SK-N-MC, TTC-446, TC-32, TC-71, RD-ES, STA-ET8, SK-ES1), melanoma (518A2 and 31IGR1), neuroblastoma (IMR32 and SH-SY5Y), Epstein-Barr virus-immortalized lymphoblastoid (LCL1, LCL2, LCL3), leukemia (THP1, K562, HL-60, Jurkat), Hodgkin's lymphoma (L-1236, L-540, HDLM-2, KM-H2), thyroid carcinoma (B-CPAP, 133-FTC, HTh 74, 8505C), fibrosarcoma (HT-1080) and cervical carcinoma (HeLa) cell lines. IFNγ-stimulated mesenchymal stem cells (MSCs) were used as positive control. ntc: No template control.*

phenomenon might be the interaction between IL2, IFNγ and IDO: The secretion of IL2 by transgenic tumor cells induces the secretion of IFNγ by the PBMCs. IFNγ induces the expression of immunosuppressive IDO in the wild-type tumor cells and inhibits T-cell activity against the tumor cells. Furthermore, it seems that the antibodies against CD137 modulate IFNγ secretion by the PBMCs.

To test this model, an *in vitro* system was used. In these experiments, cells from different donors showed different behaviors. In the cytokine measurement, we observed that the donor cells produced different amounts of IFNγ after IL2 contact. It seems possible that differences in histocompatibility between the tumor cells and PBMCs are partially responsible for this behavior. We also observed that small differences in IL2 produced by the transgenic tumor cells resulted in different amounts of IFNγ. When we used high concentrations of IL2 with a fixed concentration of antibodies against CD137, the expression of IDO was higher than without the antibody. It was shown that cross-linking of CD137 on CD8-positive T-cells and NK cells induces secretion of IFNγ and TNFα (49, 50). Furthermore, macrophages and monocytes express TNFα after incubation with antibodies against CD137 (51, 52). TNFα is known to be an inducer of IDO expression (53, 54).

In the present study, we observed that the secretion of IL10 and IL6 was down-regulated in the presence of antibodies against CD137. Altered secretion of IL10 after inhibition of bi-directional CD137/CD137L signaling has also been described in other models (55-58). Seo and colleagues demonstrated in a mouse autoimmune disease model that an antibody against CD137 induces immune suppression through IFNγ-dependent enhanced expression of immune-suppressive IDO (15). In animal models, an antibody-mediated stimulation of CD137 shifted the immune response to a T-helper 1 (TH1) type and away from TH2 (57, 59). In our experiments, only cross-linking of CD137 at low IL2 concentrations led to a reduction of IFNγ and IDO expression. No such inhibition was found at high concentrations of IL2. One explanation of our findings is that immune modulation through the CD137/CD137L pathway is dependent on the activation status of the involved cells. Our results are in agreement with observations from Zhang and co-workers showing that antibodies against CD137 can induce T-cell activation or T-cell death depending on the time point after virus infection (57).

It is known that different leukocyte sub-populations, different tumor cell lines and MSCs express IDO after

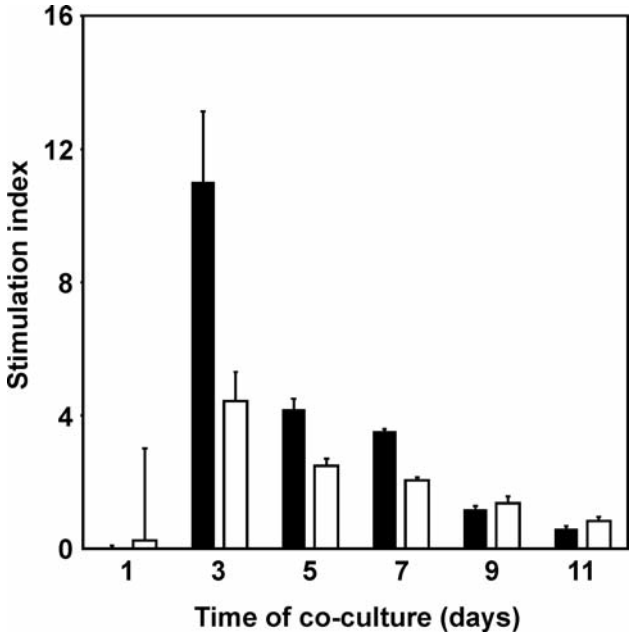

Figure 7. *Inhibition of peripheral blood mononuclear cell (PBMC) proliferation in co-cultures with interferon gamma (IFNγ)-treated A673 cells. Human leukocyte antigen-mismatched PBMCs were cultured in the presence of interleukin-2 (IL2) and wild-type A673 cells (closed bars) or in the presence of IL2 and IFNγ-treated indoleamine-2,3-dioxygenase (IDO)-expressing A673 cells (open bars). The proliferation was measured by 5-bromo-2-deoxyuridine assay on days 1, 3, 5, 7, 9, and 11. Data are the means and standard errors from triplicate determinations. For calculation of stimulation indices, proliferation of unstimulated PBMCs was set as 1.*

incubation with IFNγ (47, 60, 61). The induction of IDO by IFNγ seems to be dependent on the investigated cell type. For few of the examined cell lines from the present study, induction of IDO by IFNγ has been described [HeLa cells (62); neuroblastoma cell line SH-SY5Y (61)]. For different melanoma cell lines, expression of IDO after IFNγ incubation was described (63). The cell lines analyzed in our study showed the same induction. For some leukemia and lymphoma cell lines it was observed that they do not express IDO after IFNγ stimulation (62, 64). We found no expression of IDO after IFNγ stimulation of cells, but others studies report loss of tryptophan in the culture supernatant of THP1 cells after using high concentrations of IFNγ (64, 65). Nevertheless, tryptophan degradation was clearly lower than the degradation by other examined cell lines (64). It was postulated by Carlin *et al*. that the neuroblastoma cell line IMR32 does not express IDO after IFNγ stimulation (60), but IDO expression was not directly analyzed, the product of its metabolism (kynurenine) was measured. By the sensitive RT-PCR method used in our experiments, we observed that this cell line expressed IDO at the RNA level.

To our knowledge, expression of IDO after IFNγ stimulation has not been analyzed in Hodgkin's lymphoma cells, EFT, thyroid carcinoma cell lines and Epstein Barr virus-immortalized B-cell lines (LCL). We observed that cell lines with a neuro-ectodermal, endodermal, or mesenchymal origin express IDO after IFNγ stimulation (EFT, neuroblastoma, melanoma, fibrosarcoma, cervix carcinoma, and thyroid carcinoma cell lines). In contrast, most cell types of hematopoietic origin do not express IDO after IFNγ stimulation (Hodgkin's lymphoma, leukemia, LCL). This hypothesis is supported by detection of IDO expression after IFNγ stimulation in cell lines with a neuro-ectodermal, endodermal or mesenchymal origin (66-70). Several studies have analyzed the expression of IDO in peripheral blood and bone marrow of patients with leukemia. Expression of IDO was found in material from some patients (71-73). In such material from patients, malignant cells and normal cells are also present. It is possible that such normal cell types express IDO in blood and bone marrow. It is known for many different cell types, that IDO-expressing cells inhibit proliferation of immunocompetent cells. IDO-expressing EFT cells inhibit the proliferation of allogeneic PBMCs. Whether inhibition of IDO can increase the efficacy of immunotherapeutic approaches for EFT requires further investigation.

We have shown that IDO is induced in EFT in response to leukocyte-derived IFNγ and that this IDO expression might be immunosuppressive and be responsible for the growth of tumor cells in the presence of IL2-stimulated PBMCs. The modulation of this pathway by engagement of the CD137/CD137L bi-directional signaling system seems possible but our results suggest that this strategy is dependent on the activation status and might be difficult to control.

Acknowledgements

We are grateful to Ines Volkmer and Corinna Giersberg for technical assistance. We thank Robert S. Mittler for kind support.

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Received July 8, 2014
Revised August 27, 2014
Accepted September 1, 2014