Indoleamine 2,3-dioxygenase-expressing leukemic dendritic cells impair a leukemia-specific immune response by inducing potent T regulatory cells

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ABSTRACT

Background
The immunoregulatory enzyme indoleamine 2,3-dioxygenase, which catalyzes the conversion of tryptophan into kynurenine, is expressed in a significant subset of patients with acute myeloid leukemia, resulting in the inhibition of T-cell proliferation and the induction of regulatory T cells. Acute myeloid leukemia cells can be differentiated into dendritic cells, which have increased immunogenicity and have been proposed as vaccines against leukemia.

Design and Methods
Leukemic dendritic cells were generated from acute myeloid leukemia cells and used as stimulators in functional assays, including the induction of regulatory T cells. Indoleamine 2,3-dioxygenase expression in leukemic dendritic cells was evaluated at molecular, protein and enzymatic levels.

Results
We demonstrate that, after differentiation into dendritic cells, both indoleamine 2,3-dioxygenase-negative and indoleamine 2,3-dioxygenase-positive acute myeloid leukemia samples show induction and up-regulation of indoleamine 2,3-dioxygenase gene and protein, respectively. Indoleamine 2,3-dioxygenase-positive acute myeloid leukemia dendritic cells catabolize tryptophan into kynurenine metabolite and inhibit T-cell proliferation through an indoleamine 2,3-dioxygenase-dependent mechanism. Moreover, indoleamine 2,3-dioxygenase-positive leukemic dendritic cells increase the number of allogeneic and autologous $CD4^+CD25^+$ $Foxp3^+$ T cells and this effect is completely abrogated by the indoleamine 2,3-dioxygenase-inhibitor, 1-methyl tryptophan. Purified $CD4^+CD25^+$ T cells obtained from co-culture with indoleamine 2,3-dioxygenase-positive leukemic dendritic cells act as regulatory T cells as they inhibit naive T-cell proliferation and impair the complete maturation of normal dendritic cells. Importantly, leukemic dendritic cell-induced regulatory T cells are capable of *in vitro* suppression of a leukemia-specific T cell-mediated immune response, directed against the leukemia-associated antigen , Wilms' tumor protein.

Conclusions
These data identify indoleamine 2,3-dioxygenase-mediated catabolism as a tolerogenic mechanism exerted by leukemic dendritic cells and have clinical implications for the use of these cells for active immunotherapy of leukemia.

Key words: acute myeloid leukemia, dendritic cells, T regulatory cells, immunotherapy, indoleamine 2,3-dioxygenase.

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The online version of this article has a Supplementary Appendix.

Introduction

Indoleamine 2,3-dioxygenase (IDO1) is a key enzyme in tryptophan metabolism, catalyzing the initial rate-limiting step of tryptophan degradation along the kynurenine pathway.[1] Tryptophan starvation by IDO1 consumption inhibits T-cell activation,[1,2] while products of tryptophan catabolism, such as kynurenine derivatives and oxygen free radicals, negatively regulate T-cell proliferation and survival.[1,3] More recently, IDO1-expressing cells, including dendritic cells (DC), have been demonstrated to have a tolerogenic effect on T-cell-based adaptive immune response by expanding/inducing a population of regulatory T cells (T_{reg}).[4-6] Interestingly, human monocyte-derived DC have recently been demonstrated to induce a population of T_{reg} in both allogeneic and autologous culture systems.[7,8]

A wide variety of human solid tumors express IDO1.[9] We and others have also demonstrated that acute myeloid leukemia (AML) cells express an active IDO1 protein, which converts tryptophan into kynurenine and inhibits allogeneic T-cell proliferation.[10-13] Moreover, we demonstrated that modulation of tryptophan catabolism by AML cells results in the *de novo* induction of T_{reg} by conversion from $CD4^+CD25^-$ naïve T cells.[14]

AML samples have been used to generate, *in vitro*, DC-like cells (AML-DC) which stimulate T-cell proliferation and cytotoxic T lymphocyte activity against autologous leukemia cells more efficiently than undifferentiated blasts.[15-18] Thus, AML-DC have been proposed for use as a cellular vaccine in leukemia patients for the treatment of minimal residual disease.[19] However, we previously demonstrated that AML-DC are not fully competent to revert T-cell impairment induced by the leukemic microenvironment,[20] and it remains to be elucidated whether AML-DC retain some intrinsic tolerogenic features, including the capacity of inducing T_{regs}. To this end, in the present study, we tested the expression of IDO1 by AML-DC and the functional role of this enzyme in the development of T_{reg} cells.

Design and Methods

Cells

Buffy coats were obtained during the preparation of transfusion products from healthy adults. Peripheral blood samples including at least 70% leukemic cells were harvested from AML patients at diagnosis and used for AML-DC generation. Normal and leukemic mononuclear cells were obtained by gradient centrifugation (Lymphoprep; 1.077 g/mL; Nycomed Pharma, Oslo, Norway). $CD14^+$, $CD3^+$, $CD8^+$ and $CD4^+$ cells were purified from the mononuclear cell fraction in a MiniMacs high-gradient magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions (the purity of cell populations was always greater than 95%). $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were isolated using a MiniMacs $CD4^+CD25^+$ regulatory T-cell isolation kit (Miltenyi) according to the manufacturer's instructions. To achieve the highest possible purity, positive and negative cell fractions were separated over a second column. $CD4^+CD25^+$ cells routinely accounted for more than 90% of total cells, as evaluated by flow activated cell sorting analysis. $CD4^+CD25^-$ T cells accounted for more than 98% of the cells collected in the negative fraction.

Generation and maturation of leukemic dendritic cells

AML-DC were derived from AML blasts of monocytic lineage, which are known to give rise to AML-DC more efficiently.[17] However, similar results were also obtained with AML cells of lineages other than monocytic (*Online Supplementary Table S1*). AML-DC were generated from eight patients with AML. Briefly, 10^6/mL AML blasts were cultured for 6 days in RPMI 1640 (Whittaker Biproducts, Walkersville, MD, USA), supplemented with 10% fetal calf serum (Sera Lab, Sussex, UK), antibiotics, L-glutamine, HEPES buffer (Whittaker Biproducts), and non-essential amino acids (Whittaker Biproducts), hereafter referred as complete medium, 50 ng/mL granulocyte-macrophage colony-stimulating factor (Endogen, Woburn, MA, USA), 800 U/mL interleukin (IL)-4 (Endogen) and 10 ng/mL tumor necrosis factor-α (Endogen). For final maturation, day 6 AML-DC (immature DC) were resuspended at a density of 10^6 cells/mL in complete medium plus granulocyte-macrophage colony-stimulating factor (50 ng/mL), IL-4 (800 U/mL), tumor necrosis factor-α (10 ng/mL), IL-6 (Endogen;10 ng/mL), IL-1-β (Endogen; 10 ng/mL), and prostaglandin E_2 (Endogen; 1 μg/mL) and incubated for 48 h (mature DC). The same protocol was followed to generate and mature normal DC obtained from highly purified $CD14^+$ cells, as previously reported.[21] The generation of functionally active AML-DC was assessed by phase-contrast microscopy, immunophenotyping and mixed lymphocyte reaction.

Construction of the standard curve for absolute quantification of IDO1 transcript

To create a standard curve for the absolute quantification of *IDO1* gene transcript copy number, the polymerase chain reaction product obtained by amplification with the following forward and reverse primers (forward: 5'-ACA GAC CAC AAG TCA CAG CG-3'; reverse: 5'-AAC TGA GCA GCA TGT CCT CC-3') of cDNA from human placenta (Clontech, BD Biosciences Italia) was cloned into the pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Serial 10-fold dilutions of the plasmid from 10^5 to 10^0 plasmid copies were prepared and used to create the standard curve of *IDO* transcript. The standard curve for *ABL* transcript was obtained using FusionQuant plasmid standards commercially available from Ipsogen (Ipsogen, New Haven, CT, USA), starting from 10^5 and ending at 10^3 plasmid copies.

Real-time quantitative polymerase chain reaction analysis of IDO1

Quantitative polymerase chain reaction amplification was performed with an ABI-PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The reaction was performed in a final volume of 25 μL, consisting of 5 μL cDNA (diluted 1:3) or plasmid, 12.5 μL of TaqMan Universal PCR Master Mix (Applied Biosystems), 0.75 μM primers plus 1 μM probe specific for *IDO1* (forward: 5'-GGT CAT GGA GAT GTC CGT AA-3', reverse: 5'-ACC AAT AGA GAG ACC AGG AAG AA-3', probe: 5'-6-FAM-CTG TTC CTT ACT GCC AAC TCT CCA AGA AAC TG-TAMRA-3')7 and 0.37 μM primers plus 1.2 μM probe specific for ABL (forward: 5'-TGG AGA TAA CAC TCT AAG CAT AAC TAA AGG T-3'; reverse: 5' GAT GTA GTT GCT TGG GAC CCA-3'; probe: 5' FAM-CCA TTT TTG GTT TGG GCT TCA CAC CAT T-TAMRA-3'). The quantitative polymerase chain reaction conditions consisted of an initial step at 50°C for 2 min, a denaturation step at 95°C for 10 min, followed by 40 cycles, each for 15 s at 95°C and 1 min at 60°C.[22] Absolute quantification of the transcript copy number was achieved for the *IDO1* and *ABL* genes from the corresponding standard curves. Results were expressed as a ratio: (*IDO1* copy number/*ABL* copy number)x10^4. All real-time polymerase chain reactions were performed at least in triplicate.

IDO1 expression and activity

AML cells were tested for IDO1 expression both at mRNA and protein levels. Polymerase chain reaction analysis of human *IDO1* was performed as described above. For detection of IDO1 protein, rabbit anti-human IDO1 polyclonal antibody (Alexis Biochemicals, NY, USA) was used. Western blot analysis was performed as previously reported.[23] Serum concentrations of kynurenine and tryptophan were quantified using reversed-phase high performance liquid chromatography. The chromatographic procedure was similar to a method previously described, with minor modifications.[24] In brief, sample aliquots (100 μL) were de-proteinized with $HClO_4$ (0.3 M final concentration). After centrifugation (14000 rpm for 15 min), the supernatants were spiked with 50 μM 3-L-nitrotyrosine and analyzed using a ReproSil-Pur C18-AQ (4x250 mm, 5 μm granulometry) reversed-phase high performance liquid chromatography column (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany), using a double-pump mod. 2080 HPLC apparatus from Jasco (Tokyo, Japan) equipped with a model 2070 UV spectrophotometric detector and a FP-2020 fluorescence detector. Both detectors were connected in series to allow simultaneous measurements. The chromatographic peaks were detected by recording UV absorbance at 360 nm and emission fluorescence at 366 nm, after excitation at 286 nm. The elution solvent was 2.7% CH_3CN in 15 mM acetate buffer, pH 4.0 (both reversed-phase high performance liquid chromatography-grade from Fluka, Milan, Italy). Borwin 1.5 and MS Excel software were used to control the set-up and for peak quantification. The concentrations of components were calculated according to peak heights and were compared with both 3-nitro-l-tyrosine as the internal standard and the reference curves constructed with genuine kynurenine and L-trypthophan, both purchased from Sigma-Aldrich (St. Louis, MO, USA).

Induction of allogeneic and autologous regulatory T cells by leukemic dendritic cells

AML-DC (10^5/mL) were cultured in complete medium with 10^6/mL allogeneic or autologous $CD3^+$ T cells for 7 days in the presence or absence of optimal concentrations (1000 μM) of the IDO1-inhibitor, 1-methyl-L-tryptophan (Sigma-Aldrich).[10,14] At the end of the culture period, cells were collected and used for phenotypic and functional assays (see below).

T-cell proliferation and in vitro suppression assays

Standard mixed lymphocyte reactions were performed as previously described.[23] Briefly, naive $CD4^+CD25^-$ T cells (10^5/well) were incubated for 5 days with 10^4/well irradiated (3000 cGy) mature AML-DC in the presence or absence of 1-methyl-L-tryptophan. The cells were then pulsed with 1 μCi (0.037 MBq) per well of [^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and tested as previously described.[23] The stimulation index (SI) was calculated for each individual experiment as follows: SI= cpm (counts per minute)(T-cell responders+stimulators)/cpm (T-cell responders). To test their suppressive activity, purified $CD4^+CD25^+$ T cells (10^4/well), which had been obtained after culture with AML-DC (see above), were added to cultures consisting of the same donor-derived $CD4^+CD25^-$ T cells (10^5/well) as responders and irradiated allogeneic mononuclear cells as stimulators (10^4/well). After 5 days, cultures were pulsed with 1 μCi per well of [^{3}H]thymidine (Amersham Pharmacia) and tested as previously described.[21] To test the capacity of AML-DC-induced $CD4^+CD25^+$ T_{reg} to inhibit the maturation of DC, normal immature DC were matured in the presence of an optimal concentration of lipopolysaccharide (Sigma-Aldrich; 1 μg/mL), as determined in preliminary experiments, and highly purified $CD4^+CD25^+$ T_{reg}, obtained after culture with AML-DC (DC:T cell=1:3). Purified $CD4^+CD25^-$ T cells, obtained from the same culture, were used as control samples.

Generation of leukemic lysate and dendritic cell pulsing

AML cells were resolved in complete medium at a density of 10^7 cells/mL. Cells were treated with three cycles of heating and freezing. Each cycle consisted of 10 min at 37°C followed by another 10 min at -80°C. After each cycle, the necrotic cell material was filtered through an insulin syringe. At the end of the third cycle the cell suspension was used as a soluble lysate and added to normal immature DC at an AML:DC ratio of 2:1. After overnight incubation, pulsed DC were washed and matured. The endocytic activity of immature DC was assessed with PKH26 red fluorescent cell linker kit (Sigma-Aldrich). Briefly, AML cells were washed twice with RPMI without serum and antibiotics and resolved in diluent C (10^6 cells in 200 μL). Diluted PKH26 was added to the AML cell suspension v/v and incubated for 4 min in the dark. The reaction was blocked with 10 mL RPMI 1640 supplemented with 10% fetal calf serum for 4 min in the dark. After pulsing, DC were analyzed using FACScan equipment (Becton Dickinson, Franklin Lakes, NJ, USA). At least 10,000 events were collected in list mode on FACScan software.

Inhibition of leukemia-reactive $CD4^+$ T cells by regulatory T cells induced by leukemic dendritic cells

Freshly purified $CD3^+$ T cells, obtained from healthy donors, were co-cultured in complete medium with irradiated (3000 cGy) autologous DC, previously loaded with allogeneic necrotic AML blasts, at a T cell:DC ratio of 10:1. During the priming phase, 20 U/ml IL-2 (Proleukin, Novartis, Basel, Switzerland) was added every 2 days for 8 days. Then, every 8-10 days, T cells were re-stimulated with AML-cell-loaded DC and IL-2 was added at day 2 after re-stimulation. After three rounds of re-stimulation, T cells were collected and used for functional suppression assays. Cultured T cells were incubated with autologous normal DC, previously loaded or not with same patient's AML lysate, in the presence or absence of AML-DC-induced T_{regs} (see above).

Suppression of Wilms' tumor-specific $CD8^+$ T cells by autologous regulatory T cells induced by leukemic dendritic cells

AML patients were preliminarily screened for HLA-A0201 positivity. AML-DC were pulsed or not with 10 μg/mL HLA-A0201-restricted Wilms' tumor (WT1)-derived peptide (PRIMM, Milan, Italy; WT1 126-134) for 4 h in complete medium. Then, pulsed or not pulsed AML-DC were cultured with 10^5 autologous $CD3^+$ T cells in 96-well, round-bottomed plates at a T cell:DC ratio of 10:1 in complete medium with 10% autologous serum supplemented with recombinant human IL-15 (10 ng/mL; R&D Systems, Minneapolis, MN, USA), IL-7 (5 ng/mL; Endogen) and IL-2 (20 U/mL; Proleukin) at day 2 and day 4. AML-DC-induced T_{regs} were added or not to cell cultures at a 1:1 ratio with stimulating AML-DC. According to the manufacturers' instructions, T cells were tested for antigen-specific intracellular interferon-γ production (FastImmune, CD8 Intracellular Cytokine Detection Kit; Becton Dickinson) and for the frequency of WT1-specific $CD8^+$ T cells (Pro5® Recombinant Human MHC Pentamers; Proimmune, Oxford, UK), after 24 h and 7 days of culture, respectively.

Immunophenotype studies

Dual-color immunofluorescence was performed using the following panel of monoclonal antibodies: phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 (Pharmingen; clone UCHT1); PE-conjugated anti-human Foxp3 (Biolegend, San Diego, CA, USA; clone 259D); PE- or FITC-conju-

gated anti-human CD4 (Pharmingen; clone RPA-T4); PE- or FITC-conjugated anti-human CD8 (Pharmingen; clone HIT8a); PE-conjugated anti-human CD25 (Pharmingen; clone M-A251); PE- or FITC-conjugated anti-human HLA-DR (Pharmingen; clone L242). PE- or FITC-conjugated antihuman CD1a (Biolegend; clone HI149); PE- or FITC-conjugated antihuman CD86 (Biolegend; clone IT2.2); PE-conjugated antihuman CD80 (Biolegend; clone 2D10); PE- or FITC-conjugated antihuman CD14 (Biolegend; clone HCD14); FITC-conjugated antihuman CD83 (Biolegend; clone HB15e); and FITC-conjugated antihuman CD40 (Biolegend; clone HB14). Negative controls were isotype-matched irrelevant monoclonal antibodies (Pharmingen, Biolegend). Cells were analyzed by using FACScan equipment (Becton Dickinson). At least 10000 events were collected in list mode on FACScan software.

Statistical analysis

Results are expressed as mean ± SD. Where indicated, differences were compared using Student's t-test and χ^2 analysis.

Results

Immature and mature leukemic dendritic cells express an active form of IDO1 protein

As previously reported,[20] AML blasts can be differentiated into AML-DC, maintaining leukemia-specific molecular markers (*Online Supplementary Figure S1*), with a typical DC morphology, high expression of HLA-DR, CD86, CD80, CD40 and intermediate expression of CD1a and CD83. As expected, the addition of maturation stimuli to day-6 AML-DC (see *Design and Methods*) resulted in the up-regulation of CD86, CD80, CD40 and CD83 levels, and in the down-modulation of CD1a (*Online Supplementary Figure S2*).

We previously demonstrated that a significant fraction of AML blasts expresses IDO1 mRNA and protein.[10,13] We, therefore, tested whether DC differentiation of AML cells might influence IDO1 expression. As shown in Figure 1A and B, DC differentiation of AML cells resulted in a significant up-regulation of IDO1 both at molecular and protein levels. IDO1 expression was not influenced by AML lineage (*Online Supplementary Table S2*). Interestingly, immature AML-DC also showed high levels of IDO1 gene and protein although the levels were lower than those of mature AML-DC. Moreover, both baseline IDO1$^+$ and IDO1$^-$ AML cells gave rise to AML-DC expressing IDO1 at high and comparable levels (Figure 1C).

To test the enzymatic activity of IDO1 protein expressed by AML-DC, the supernatants of cultured cells were analyzed for L-kynurenine concentrations. As shown in Figure 1D, IDO1 from AML-DC was active, resulting in the increase of L-kynurenine in the culture medium. This increase was more prominent in mature AML-DC than in immature AML-DC, thus reflecting higher mRNA and protein levels (Figure 1A and B). The addition of the IDO1-inhibitor 1-methyl-L-tryptophan resulted in a significant decrease of kynurenine production by IDO1-expressing AML-DC.

To test whether IDO1 expression by AML-DC results in the inhibition of their ability to stimulate T-cell proliferation, IDO1-expressing mature AML-DC were used as stimulators for allogeneic CD3$^+$ T cells in a standard mixed lymphocyte reaction in the presence or absence of 1-methyl-L-tryptophan. As shown in Figure 1E, the addition of 1-methyl-L-tryptophan resulted in a significant increase of T-cell proliferation. Taken together, these data demonstrate that both immature and mature AML-DC express IDO1 at molecular and protein levels. The presence of kynurenine in AML-DC cultures is strong evidence of functional IDO1 activity, which is also supported by its capacity to inhibit T-cell proliferation.

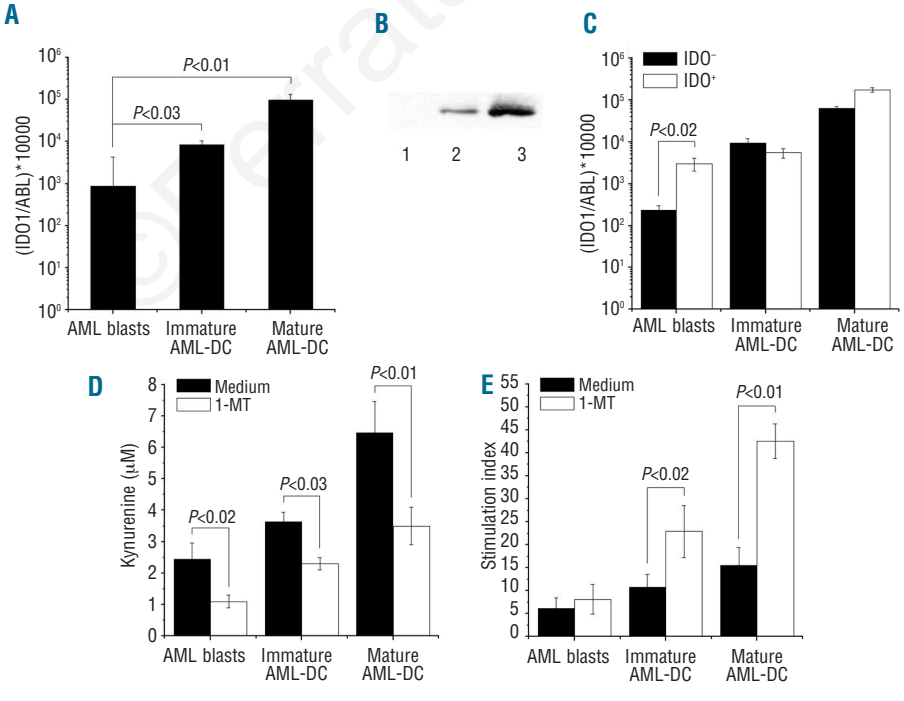

Figure 1. AML-DC express a functionally active IDO1 protein. (A) Real-time quantitative polymerase chain reaction of *IDO1* mRNA in AML blasts and after differentiation into DC (immature AML-DC, mature AML-DC). Results represent the mean ± SD of eight different experiments. (B) Western blot analysis of IDO1 protein. Lanes are as following: 1) IDO1- AML blasts; 2) immature AML-DC; 3) mature AML-DC. The results are representative of six different experiments. (C) Real-time quantitative polymerase chain reaction of *IDO1* mRNA in IDO1$^+$ and IDO1$^-$ AML samples before and after differentiation to DC. The results are the mean ± SD of six different experiments. (D) L-kynurenine production of AML blasts and AML-DC at different stages of maturation. The results are the mean ± SD of six different experiments. AML blasts include IDO1$^-$ and IDO1$^+$ AML samples. (E) Allogeneic T-cell proliferation induced by AML blasts and AML-DC in the presence and absence of 1-methyl tryptophan (1-MT) (1000 μM). The results are the mean ± SD of six different experiments.

IDO1-expressing leukemic dendritic cells increase CD4+CD25+Foxp3+ T cells

We previously demonstrated that IDO1-expressing AML samples induce a population of fully functional CD4+CD25+Foxp3+ Treg by conversion from the CD4+CD25+ T-cell fraction.[14] To investigate the role of IDO1 expression by AML-DC on Treg-development, we co-cultured IDO1-expressing AML-DC with highly purified allogeneic CD3+ T cells, obtained from healthy donors, in the presence or absence of 1-methyl-L-tryptophan. The viability of cells cultured in the presence of 1-methyl-L-tryptophan was not different from that of cells cultured in medium alone (79%±7 and 85%±8, respectively); furthermore, CD4+ and CD8+ T-cell frequencies were not modified by the addition of 1-methyl-L-tryptophan (*data not shown*). Co-culture of T cells with IDO1-expressing AML-DC increased the percentage of CD4+CD25+ T cells (Figure 2A, $P<0.01$) and the surface expression of CD25 (mean fluorescence intensity [MFI] 150±45 and 980±390 before and after co-culture, respectively; $P=0.0003$). Moreover, CD4+CD25+ T cells collected after culture with IDO1-expressing AML-DC had an increased percentage of Foxp3+ cells, as compared to baseline (Figure 2B, $P<0.01$). These data are similar to those obtained with normal IDO1-expressing monocyte-derived DC, which have recently been shown to induce, *in vitro*, Foxp3+ T cells.[7,8] This phenotypic pattern suggests that CD4+ T lymphocytes, after co-culture with IDO1-expressing AML-DC, acquire a CD4+CD25+ Treg phenotype. Importantly, the addition of 1-methyl-L-tryptophan to co-cultures of T cells with IDO1-expressing AML-DC completely restored the expression of CD25 and Foxp3 to that observed before culture (Figure 2A and B). This finding strongly suggests that IDO1 represents the main mechanism by which AML-DC induce CD4+CD25+Foxp3+ T cells.

Leukemic dendritic cell-induced CD4+CD25+ T cells have regulatory activity

An additional set of functional experiments was performed to validate the Treg nature of the cells induced by IDO1-expressing AML-DC. Naive CD4+CD25− T cells were stimulated by allogeneic mononuclear cells in the presence of AML-DC-cultured T cells. As shown in Figure 3, T-cell proliferation was significantly reduced when AML-DC-cultured CD4+CD25+ T cells were added to cell cultures ($P<0.03$). No effect was observed when naïve T cells were added to cell cultures (*data not shown*). These data support the hypothesis that CD4+CD25+Foxp3+ T cells induced by IDO1-expressing AML-DC retain immunosuppressive activity and may be considered *bona fide* Treg.

Regulatory T cells induced by IDO1-expressing leukemic dendritic cells inhibit the proliferation of autologous leukemia-specific CD4+ T cells

To test the ability of AML-DC-induced Treg to inhibit the leukemia-specific immune response, we first induced a population of T cells, obtained from healthy donors, with reactivity against leukemia cells. Immature DC were loaded with necrotic AML blasts and then, after maturation, used as stimulators for autologous total CD3+ T cells. As shown in Figure 4A, immature DC were capable of internalizing necrotic leukemia cells. Moreover, leukemia cell-loaded DC showed increased allostimulatory capacity of T-cell proliferation as compared to unloaded DC (*data not shown*). At the end of the culture, the proliferation of total CD3+ T cells, as well as that of fractionated CD4+ and CD8+ T cells, was tested in a secondary mixed lymphocyte reaction against DC, which had been pulsed with the same leukemia cells used for T-cell stimulation. Unloaded DC were used as negative controls. As shown in Figure 4B, only CD4+, but not CD8+ T cells proliferated specifically in response to leukemia cell-loaded DC ($P<0.01$). Indeed, CD3+, CD4+ and CD8+ cells gave 2603±951, 677±271 and 435±118 cpm in response to not pulsed DC and 3073±1028, 1299±556 and 635±240 cpm in response to pulsed DC, respectively. Thus, further experiments were performed with CD4+ T cells obtained after culture with leukemia cell-loaded DC. In particular, AML-DC-induced Treg were added to a mixed lymphocyte reaction consisting of leukemia-reactive CD4+ T cells as responders and autologous leukemia cell-loaded DC as stimulators. As shown in Figure 4C, AML-DC-induced Treg significantly reduced leukemia-specific CD4+ T cell-proliferation ($P<0.01$; 471±206 *versus* 1299±556 cpm). This effect was dose-dependent (*data not shown*) and blocked by anti-HLA-class II antibody (*Online Supplementary Figure S3*). These data demonstrate that IDO1-expressing AML-DC may induce a population of Treg which is capable of inhibiting the proliferation of leukemia-specific CD4+ T cells.

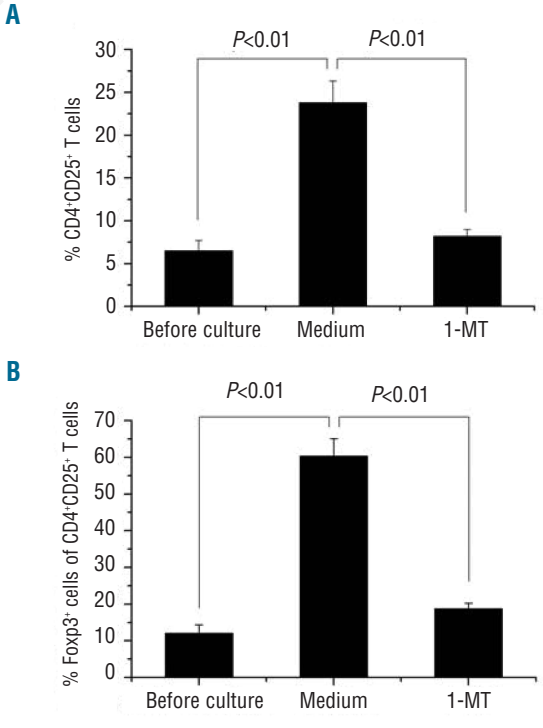

Figure 2. IDO1-expressing AML-DC induce CD4+CD25+Foxp3+ T cells. Phenotypic analysis of CD3+ T cells cultured for 7 days in the presence of mature AML-DC with or without the addition of 1-methyl tryptophan (1-MT) (1000 μM). The results are expressed as percentage of positive cells. The results are the mean ± SD of five different experiments.

Regulatory T cells induced by IDO1-expressing leukemic dendritic cells impairs the generation of antigen-specific CD8⁺ T cells

WT1 has recently been demonstrated to be over-expressed in the vast majority of AML samples and may be used as a leukemia target antigen given its immunogenicity.[25] In the attempt to reproduce a model of anti-leukemia vaccination *in vitro*, AML-DC were used to induce a population of autologous WT1-specific $CD8^+$ T cells in the presence of IL-15, IL-7 and IL-2. To test the capacity of T_{reg} induced by AML-DC to reduce the generation of antigen-specific $CD8^+$ T cells, WT1-specific T cells were stimulated by antigen-loaded AML-DC in the presence or absence of autologous T_{reg} induced by AML-DC. As shown in Figure 5A and B, AML-DC were highly efficient in stimulating IFN-γ-secreting and WT1-specific $CD8^+$ T cells. This effect was more prominent when the stimulating AML-DC were loaded with WT1-derived peptide. Interestingly, the addition of AML-DC-induced T_{reg} to cell cultures resulted in a significant reduction of interferon-γ-secreting and WT1-specific $CD8^+$ T cells to levels which were comparable to those of the negative control samples. Hence, T_{reg} expanded by IDO1-expressing AML-DC significantly suppressed the development of leukemia antigen-specific $CD8^+$ T cells.

Regulatory T cells induced by IDO1-expressing leukemic dendritic cells impair the full maturation of normal dendritic cells

To test the capacity of AML-DC-induced $CD4^+CD25^+$ T_{reg} to inhibit the maturation of DC, normal immature DC were matured in the presence of an optimal concentration of lipopolysaccharide and with the addition of highly purified $CD4^+CD25^+$ T_{reg}, obtained after culture with AML-DC. Purified $CD4^+CD25^-$ T cells, obtained from the same culture, were used as control samples. As expected, addition of lipopolysaccharide resulted in marked increases of CD80, CD86, CD40 and CD83 on the surface of the DC, as evaluated both as percentage of positive cells and as mean fluorescence intensity (*data not shown*). As shown in Figure 6, the addition of T_{reg} significantly inhibited the maturation of DC by reducing the expression of maturation-related markers on DC. These data suggest that T_{reg} induced by AML-DC may have a tolerogenic effect also through the inhibition of the complete maturation of normal DC.

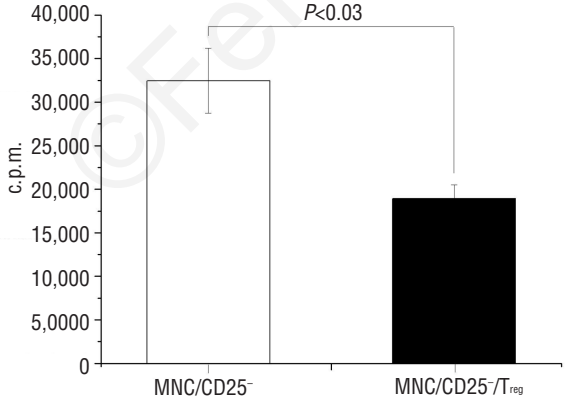

Figure 3. AML-DC-induced $CD4^+CD25^+$ T cells act as T_{reg} by inhibiting allogeneic T-cell proliferation. Naive $CD4^+CD25^-$ T cells were stimulated to proliferate by allogeneic mononuclear cells (MNC) in the presence or absence of AML-DC-cultured $CD4^+CD25^+$ T cells. The results are mean ± SD of three different experiments.

Discussion

The demonstration that AML cells can be differentiated *in vitro* into DC-like cells has provided a new tool for anti-leukemia vaccination.[19] The rationale relies on the concept that *ex-vivo* differentiation of AML blasts to DC has the advantage of obtaining antigen-presenting cells in the absence of *in-vivo* acting immunosuppressive factors. However, we previously demonstrated that a significant portion of AML-DC have impaired IL-12 production and

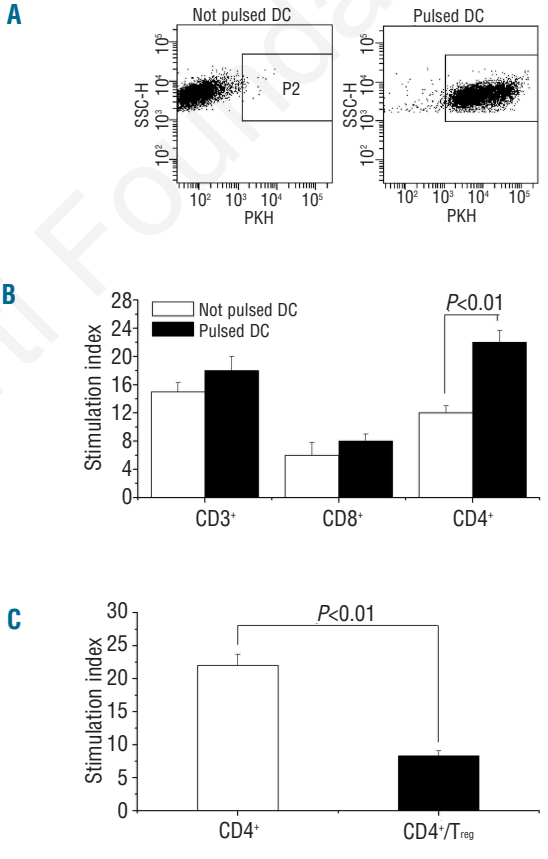

Figure 4. T_{reg} induced by IDO1-expressing AML-DC inhibit leukemia-specific T-cell proliferation. (A) AML cells were lysed and used for the pulsing of immature normal monocyte-derived DC. The endocytic activity of immature DC was assessed with a PKH26 red fluorescent cell linker kit. Pulsed and not pulsed DC were tested by flow cytometry. The results are representative of three different experiments. (B) Freshly purified normal $CD3^+$ T cells were co-cultured with autologous DC, previously loaded with allogeneic necrotic AML blasts, at a 10:1 T cell–to–DC cell ratio. After three rounds of re-stimulation, T cells were collected, purified into different T-cell subsets and used for proliferation assays in response to autologous monocyte-derived DC previously loaded with necrotic AML blasts. As control samples, unloaded monocyte-derived DC were used. AML cells used for DC pulsing were the same cells as those with had been used to generate AML-DC. The results are the mean ± SD of four different experiments. (C) T_{regs} induced by IDO1-expressing AML-DC were added to cultures consisting of leukemia-specific $CD4^+$ T cells and autologous DC previously loaded with necrotic AML cells. The results are the mean ± SD of three different experiments.

are not capable of contrasting the inhibitory effect of the leukemic microenvironment on T cells.[20] Moreover, in AML patients, circulating DC belonging to the leukemic clone had impaired functional capacity[26] and induced apoptosis of tumor-specific T cells by a Fas-FasL interaction.[27] These data suggest that leukemic DC from AML patients retain some immunosuppressive features and may contribute to leukemia escape from immune control.

The role of IDO1 in the induction of immunological tolerance has been extensively studied.[28] Normal DC have the capacity to modulate IDO1 expression in response to external pro-inflammatory stimuli, such as interferon-γ,[29-32] and the interaction of DC with CTLA-4-expressing T_{reg} is known to up-regulate a tolerogenic pathway, including IDO1 expression.[33,34] Moreover, recent studies have also demonstrated that certain subsets of human myeloid DC might constitutively express IDO1 protein.[35,36] These data highlight the physiological existence of a subset of normal regulatory DC, which are functionally defined by the expression, either constitutive or inducible, of IDO1 protein (IDO1$^+$DC). In this study, we show that leukemic DC may represent another example of IDO1-expressing DC. We and others[10-13] have previously demonstrated that a subset of AML samples from newly diagnosed patients constitutively expresses significant amounts of IDO1 gene and protein. Our current results demonstrate that during DC generation IDO1 expression is markedly induced/up-regulated in all AML samples. Interestingly, both immature and mature AML-DC express IDO1, although the expression is greater in the later stage of differentiation. These data are partially in contrast to those obtained from normal myeloid DC, in which it is known that IDO1 levels increase significantly only after maturation.[7,8] It remains to be elucidated whether this difference derives from the leukemic origin of AML-DC as the result of leukemia-derived molecular alterations of the IDO1 pathway.

Tumor cells, including leukemia cells, are known to create an inhibitory microenvironment for the immune system.[37] Among the different mechanisms by which tumor cells escape the immune control, the induction of T_{reg} is emerging as a very important one. A significantly increased number of T_{regs} has been observed in several tumors, such as lung, breast, pancreatic, and ovarian carcinomas, and melanoma, and T_{reg} have been found to suppress tumor-specific immune responses.[38-40] In hematologic malignancies, an increase in circulating CD4$^+$CD25$^+$ T_{reg} has been demonstrated in Hodgkin's and non-Hodgkin's lymphomas, chronic lymphocytic leukemia, AML, multiple myeloma and high-risk myelodysplastic syndromes.[41-46] In particular, we demonstrated that AML cells favor the *de novo* emergence of a population of T_{regs} through the aberrant over-expression of IDO1 protein.[14] In the present study, we show that IDO1-expressing AML-DC expand, *in vitro*, a population of CD4$^+$CD25$^+$ Foxp3$^+$ T cells which act as *bona fide* T_{reg} cells. These data are in agreement with recent reports that, both in mice and humans, tryptophan catabolism by normal DC mediates the emergence of CD25$^+$Foxp3$^+$ T_{reg} cells by conversion from CD25$^-$Foxp3$^-$ cells.[4-6] Our observations in leukemic DC support the notion that IDO1 expression may be considered a general tolerogenic pathway by which normal and malignant DC suppress T-cell-mediated immune responses through the induction of T_{reg}.

Since AML-DC have been proposed as a means of active

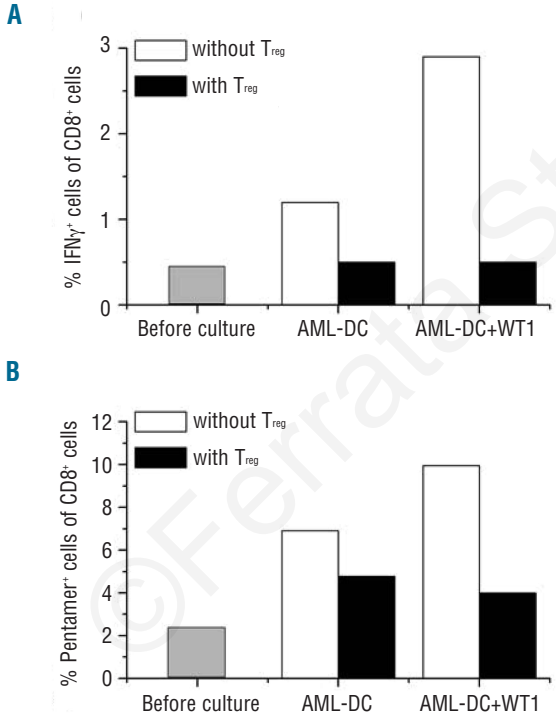

Figure 5. T_{reg} induced by IDO1-expressing AML-DC inhibit the WT1-specific CD8 T-cell response. AML-DC were pulsed or not with 10 mg/mL HLA-A0201-restricted WT1 126-134 for 4 h in complete medium. Then, pulsed or not pulsed AML-DC were cultured with 10^5 autologous CD3$^+$ T cells at a ratio of 10:1 (T:DC) with 10% autologous serum supplemented with IL-15, IL-7 and IL-2. AML-DC-induced T_{reg} were added or not to cell cultures at a 1:1 ratio with stimulating AML-DC. (A) CD8$^+$ T cells were tested for WT1-specific intracellular interferon-γ production after 24 h of culture. (B) CD8$^+$ T cells were tested for the frequency of WT1-specific CD8$^+$ T cells, by using recombinant human MHC pentamers, after 7 days of culture. Results are representative of three independent experiments.

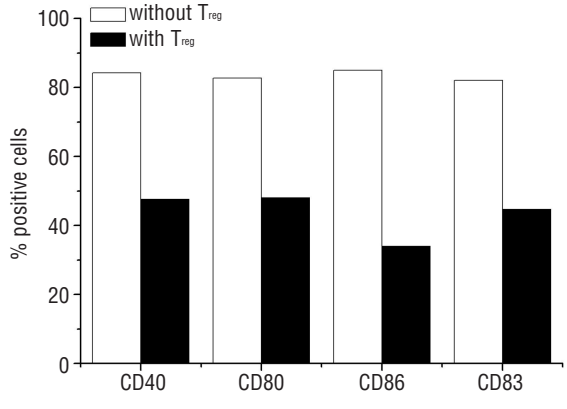

Figure 6. T_{reg} induced by IDO1-expressing AML-DC inhibit the full maturation of normal monocyte-derived DC. Normal immature monocyte-derived DC were matured in the presence of an optimal concentration of lipopolysaccharide (1 mg/mL) and CD4$^+$CD25$^+$ T_{regs}, obtained after culture with AML-DC and highly purified. As control samples, purified CD4$^+$CD25$^-$ T cells, obtained from the same culture, were used. Surface expression of maturation-related markers was tested on DC and expressed as the percentage of positive cells. Results are representative of three independent experiments.

immunotherapy,[19] our results have some clinical implications. The initiation of a T-cell immune response depends strictly on the balance between different activating or inhibiting pathways, which act during DC/T cell encounters. Mature AML-DC, with their high expression of costimulatory molecules, have been shown to have better antigen-presenting capacity than that of undifferentiated AML cells or immature AML-DC.[15-18] In line with these data, the results of our study clearly demonstrate that AML-DC are highly efficient at inducing a $CD8^+$ T-cell response, which is specific for the leukemia-associated antigen, WT1. However, our results demonstrate that, through the expression of IDO1, AML-DC may also induce a population of $CD4^+CD25^+Foxp3^+$ T_{reg}, which are capable of suppressing allogeneic T-cell proliferation and, in the autologous setting, of inhibiting both CD4 and CD8 T-cell functions in response to leukemia-specific antigens, including WT1. Moreover, our data suggest that T_{reg} induced by IDO1-expressing AML-DC inhibit the maturation of normal DC, which in turn may have a decreased antigen-presenting capacity. Altogether, these findings indicate that T_{reg} which are induced by IDO1-expressing AML-DC may play their tolerogenic role at different levels during the induction of a leukemia-specific immune response. In particular, T_{reg} may inhibit the direct priming of T cells by negatively affecting their capacity to proliferate/expand in response to leukemia-associated antigens and they may impair the cross-priming of T cells by suppressing the full maturation of normal DC, thus resulting in impaired antigen presentation. Indeed, although AML-DC have the potential to elicit leukemia-specific immunity by expanding the frequencies of the antigen-specific T-cell repertoire, the use of IDO1-expressing AML-DC as part of a cellular vaccine against leukemia may be hampered by the consensual activation/expansion of a population of suppressive T_{reg}, which are well-known to reduce the efficacy of vaccination strategies.[47]

In summary, our study demonstrates that: (i) AML-DC invariably express IDO1 gene and protein, which is enzymatically and functionally active; (ii) AML-DC are capable of inducing a population of T_{reg} through IDO1; and (iii) T_{reg} which are induced by AML-DC act as suppressors both by inhibiting a leukemia-specific T-cell immune response and by reducing the antigen-presenting capacity of normal DC. These results have clinical implications as they demonstrate a novel immunological tumor escape mechanism for AML, *in vivo*, and question the use of AML-DC as an anti-leukemia vaccine.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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