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Mesenchymal Stem Cells Employ IDO to Regulate Immunity in Tumor Microenvironment

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Abstract

Mesenchymal stem cells (MSCs) are present in most, if not all, tissues and are believed to contribute to tissue regeneration and the tissue immune microenvironment. Murine MSCs exert immunosuppressive effects through production of the nitric oxide synthase iNOS, while human MSCs utilize indoleamine 2,3-dioxygenase (IDO). Thus, studies of MSC-mediated immunomodulation in mice may not be informative in the setting of human disease, although this critical difference has been mainly ignored. To address this issue, we established a novel humanized system to model human MSCs, employing murine iNOS^{-/-} MSCs that constitutively or inducibly express an ectopic human IDO gene. In this system, inducible IDO expression is driven by a mouse iNOS promoter that can be activated by inflammatory cytokine stimulation in a similar fashion as the human IDO promoter. These IDO-expressing humanized MSCs (MSC-IDO) were capable of suppressing T lymphocyte proliferation in vitro. In melanoma and lymphoma tumor models, MSC-IDO promoted tumor growth in vivo, an effect that was reversed by the IDO inhibitor 1-methyl-tryptophan. We found that MSC-IDO dramatically reduced both tumor-infiltrating CD8⁺ T cells and B cells. Our findings offer an important new line of evidence that interventional targeting of IDO activity could be used to restore tumor immunity in humans, by relieving IDO-mediated immune suppression of MSCs in the tumor microenvironment as well as in tumor cells themselves.

Keywords

Mesenchymal stem cell; indoleamine 2,3-dioxygenase; inducible nitric oxide synthase; immunosuppression; melanoma tumor model

Introduction

Mesenchymal stem cells (MSCs) are a population of non-hematopoietic stem cells that exist in almost all tissues. These cells have the potential to differentiate into osteoblasts, chondrocytes, adipocytes and other cell types depending on ambient culture conditions or

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No potential conflicts of interest were disclosed.

the in situ niche¹⁻³. Although MSCs are not known for their physiological functions⁴, recent studies have demonstrated that these cells have great immune modulating capacity. The immunoregulatory effects do not occur spontaneously, however; only when they are stimulated by inflammatory cytokines such as interferon- γ (IFN γ) in combination with tumor necrosis factor- α (TNF α) or interleukin-1 (IL-1) do MSCs acquire their immune regulatory functions⁵⁻⁹. This characteristic of MSCs can generate an immune-tolerant environment where lymphocyte proliferation, cytokine production, and other functions are affected.

The exact mechanisms of MSC-mediated immunosuppression are still debated; many different factors are believed to be involved, such as inducible nitric oxide synthase (iNOS), indoleamine 2,3-dioxygenase (IDO), tumor necrosis factor-inducible gene 6 (TSG6), CC-chemokine ligand 2 (CCL2), interleukin-10 (IL-10), and prostaglandin E2 (PGE2) among others¹⁰. In fact, it has been revealed that MSC-mediated immunosuppression varies among different mammalian species^{11,12}. We previously demonstrated that iNOS mediates immunosuppression by mouse MSCs, while IDO plays a similar role in human MSCs¹¹. This species variation makes it difficult to extend to humans any information derived from studies of the murine system. Although human MSCs have been studied to some extent and even successfully applied clinically such as in the treatment of acute steroid-refractory GvHD¹³, most findings on MSCs are largely derived from the murine system. The species differences in MSC-mediated immunomodulation should be considered when utilizing mouse models, and one approach is to adopt a humanized murine system using murine MSCs that express transfected human IDO, and then apply it to mouse models of disease.

IDO, which is produced by many cell types including dendritic cells, macrophages, human MSCs, and most tumor cells, is the first and rate-limiting enzyme in the degradation of tryptophan^{14,15}. IDO potently suppresses immune responses by depleting local tryptophan and allowing the accumulation of tryptophan metabolites, including kynurenine, 3-hydroxyanthranilic acid, and quinolinic acid^{16,17}. However, the actual molecular mechanisms underlying the immunoregulatory function of IDO and its overall role in the immune response remain largely unknown.

Although human IDO (403 amino acids) shares 62% sequence homology with mouse IDO (407 amino acids)¹⁸⁻²⁰, the induction of IDO expression in response to inflammatory cytokines seems to be highly variable between species. Interestingly, genetic knockout of IDO in mouse does not result in any detectable phenotype; homozygous mice are viable and fertile and have normal immune system development and function. They exhibit no spontaneous autoimmune disorders²¹. The only observed changes are diminished responses to CTLA4-Ig and IFN α , and reversal of CpG-ODN induced-suppression of T cell expansion^{21,22}. In the human system, however, IDO expression is highly responsive to IFN γ and plays an important role in immune responses. Taken together with the difference in IDO induction in mouse and human MSCs¹¹, it is apparent that the mouse system may not accurately mimic the human system.

To resolve this predicament, we developed a novel strategy to humanize murine MSCs by incorporating the human IDO gene into MSCs derived from mice lacking the iNOS gene (iNOS^{-/-}). These IDO-expressing mouse MSC transfectants (MSC-IDO) provide a unique opportunity to study the role of human IDO without interference from iNOS. Since MSCs have been shown to create an immunosuppressive microenvironment and thus promote tumor growth, we examined the effects of MSC-IDO transfectants in the B16-F0 melanoma and EL4 lymphoma tumor models. We found indeed that MSC-IDO transfectants dramatically enhance tumor growth, and the effect is exerted through the modulation of immune responses. Thus, we have established a novel humanization strategy to circumvent

some issues by using mouse models to study human MSC function. This unique system has the potential to greatly improve our understanding of the role of IDO in MSC-mediated immunomodulation and support the clinical application of MSC therapy.

Materials and Methods

Mice

C57BL/6 mice, iNOS^{-/-} mice, Rag1^{-/-} mice, CIITA^{-/-} mice, and β 2-microglobulin^{-/-} mice at 6–8 weeks old were all from Jackson Laboratory (Bar Harbor, ME). They were maintained in the Robert Wood Johnson Medical School Vivarium. Animals were matched for age and gender in each experiment. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Robert Wood Johnson Medical School.

Reagents

RPMI 1640 medium, α -MEM, trichloroacetic acid (TCA), Ehrlich's reagent and 1-methyl-DL-tryptophan (1-MT) were from Sigma-Aldrich (St. Louis, MO). Anti-IDO monoclonal antibody (clone 10.1) was from Upstate Cell Signaling Solutions (Lake Placid, NY). FBS was from Invitrogen (Carlsbad, CA). Recombinant mouse and human TNF α , IL-1 β , IFN γ , and IL-2 were from R&D Systems (Minneapolis, MN). Anti-CD3 and anti-CD28 monoclonal antibodies were from R&D Systems. G418 was from Invitrogen. FITC-conjugated anti-CD3, PE-conjugated anti-CD19, FITC-conjugated anti-CD4, PE-Cy5-conjugated anti-CD8, FITC-conjugated anti-F4/80, PE-conjugated anti-NKG2D, and their respective isotypes were all from eBioscience (La Jolla, CA).

Cells

Mouse MSCs were generated in house from bone marrow of tibia and femur of 6- to 8-week old mice following established protocols⁵. Human bone marrow-derived MSCs were from ScienCell Research Laboratories (Carlsbad, CA). B16-F0 melanoma cells and EL4 lymphoma cells were tested by IDEXX Laboratories, Inc. (Columbia, MO) and certified to be negative for potential viruses. Details are provided in the Supplementary Materials and Methods.

Expression vector construction

Human MSCs (ScienCell Research Laboratories) were stimulated with recombinant human IFN γ , TNF α , and IL-1 β (20 ng/ml each) for 48 hr. Cells were harvested and RNA was extracted and reverse transcribed into cDNA. The following human IDO primers were used to amplify the IDO cDNA fragment. Forward, 5'-AATTTCTCACTGCCCCTGTG-3'; Reverse, 5'-AATGGGTAATGACAGGAATGC-3'. The human IDO cDNA fragment was inserted into pcDNA3.0 vector or pCMS-EGFP vector, as described below.

Constitutive IDO expression

IDO under the control of the constitutively active CMV promoter in the pcDNA3.0 vector were transfected into mouse iNOS^{-/-} MSCs by AmaxaTM NucleofectorTM Kit V (Lonza, Basel, Switzerland). Briefly, After culture to 70–85% confluence, mouse iNOS^{-/-} MSCs (2×10^6 /sample) were pelleted and resuspended at Nucleofector Solution (100 μ l) and 5 μ g of highly purified IDO-expressing plasmid DNA was added. For a negative control, empty pcDNA3.0 vector was similarly transfected. Transfection efficiency was traced using 2 μ g maxGFPTM. After transfection, cells were immediately placed in culture. The medium was changed after 24 hr and then replaced with complete medium plus G418 (1 mg/ml). After two weeks, single clones were selected and sub-cultured in 24-well plates. After

confirmation of IDO expression at both RNA and protein levels, The MSC-IDO clones were maintained in complete medium with G418 (400 μ g/ml).

Inducible IDO expression

Human IDO cDNA fragment was inserted into the pCMS-EGFP vector (Clontech Laboratories Inc. Mountain View, CA). The original CMV promoter in pCMS-EGFP vector was removed, and three different lengths of mouse iNOS promoter were amplified from genomic DNA of C57BL/6 by PCR, and then inserted 5' to the IDO coding sequence to generate different constructs. These were transfected into mouse iNOS^{-/-} MSCs using the AmaxaTM NucleofectorTM Kit V (Lonza), as described above. After transfection, GFP-positive cells were sorted using the BD FACS Aria III cell sorter and single IDO-expressing cells were expanded as clones. Expression of IDO upon treatment with IFN γ , TNF α and IL-1 β was verified by real-time PCR and western blotting analysis.

Cell proliferation assay

Cell proliferation was assayed by a standard ³H-thymidine (Tdr) incorporation assay. See details in the Supplementary Materials and Methods.

Detection of kynurenine

Since IDO catalyzes the metabolism of tryptophan in the kynurenine pathway, IDO activity was determined by spectrophotometric assay for kynurenine in supernatant from cultures of inducible IDO-expressing mouse iNOS^{-/-} MSCs with or without cytokine stimulation ²³. See details in the Supplementary Materials and Methods.

Real-time PCR

mRNA was quantitated by real-time PCR (MX-4000 from Stratagene) using SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Primer sequences for mouse β -actin gene were: Forward, 5'-GCTGTATTCCCCTCCATCGT-3', Reverse, 5'-TCCCAGTTGGTAACAATGCC-3'. Primer sequences for human IDO gene were: Forward, 5'-GCCCTTCAAGTGTTTCACCAA-3', Reverse, 5'-CCAGCCAGACAAATATATGCGA-3'. See details in the Supplementary Materials and Methods.

Western blotting analysis

The expression of specific protein was detected by western blotting analysis. See details in the Supplementary Materials and Methods.

Flow cytometry analysis

Cells were stained with fluorescence-conjugated antibodies and examined by flow cytometry on a FACScan flow cytometer (BD Biosystems), using CellQuest software for data acquisition and analysis. Non-specific binding was determined using isotype control antibodies. See details in Supplementary Materials and Methods.

B16-F0 melanoma and EL4 tumor models

Age-matched wild type C57BL/6 mice or genetically-modified mice (iNOS^{-/-} mice, Rag1^{-/-} mice, CIITA^{-/-} mice, or β 2M^{-/-} mice) were randomly assigned to groups of at least 5 mice each and injected intramuscularly in the leg with B16-F0 melanoma or EL4 lymphoma cells (0.5×10^6 cells). MSCs (1×10^6) were then injected intraperitoneally every 2 days. Mice were examined thrice weekly and tumor growth was evaluated by measuring

the tumor volume. Animals were euthanized and blood, spleen and tumor tissues were harvested for further analysis.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 or Microsoft Excel software. Significance was assessed by unpaired two-tailed Student's t test or analysis of variance (ANOVA). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

Generation of constitutive IDO expression in iNOS-deficient mouse MSCs

We have demonstrated that immunosuppression by mouse MSCs is mediated by iNOS, while human MSCs instead utilize IDO¹¹. This difference in effector molecules makes studies using mouse MSCs less relevant to humans. In addition, the physiological function of IDO in humans remains largely uncertain due to limitations on experimentation in human subjects. Herein, we describe a humanized murine system in which mouse MSCs were rendered to express human IDO instead of iNOS. We cloned IDO cDNA from human MSCs, inserted it into the pcDNA 3.0 vector under the control of the constitutively-active CMV promoter, and transfected this construct into mouse MSCs (Fig. 1A). Using this novel approach we established a constitutive IDO expression system (MSC-IDOc) to study the function of human IDO in the mouse system. Importantly, since iNOS is a key mediator of MSC-mediated immunosuppression in mouse, we excluded interference from iNOS by employing MSCs derived from iNOS-deficient mice as targets of the IDO transfection. Successful transfectants were selected using neomycin, and two different clones (MSC-IDOc1 and MSC-IDOc2) were checked for IDO expression and functionality. Both clones expressed high levels of human IDO mRNA and protein (Fig. 1B, C), while control cells transfected with vector- alone did not. It is noteworthy that mouse IDO was not detectable in iNOS^{-/-} MSCs (Fig. 1C), which is consistent with our previous report¹¹.

Constitutive IDO-expressing MSCs potentially inhibit lymphocyte proliferation

We next determined whether our murine iNOS^{-/-} MSC constitutive IDO-expressing transfectants (MSC-IDOc) were functional *in vitro*. Since IDO is reported to strongly suppress T cell proliferation²⁴⁻²⁶, this function was tested by co-culturing MSC-IDOc transfectants with human T cell blasts supplemented with IL-2. The resultant proliferation of human T cell blasts was strongly inhibited by MSC-IDOc (Fig. 2A). The effects on mouse T cell blasts were similarly tested in co-cultures with freshly-isolated mouse splenocytes supplemented with soluble antibodies against mouse CD3 and CD28. The proliferation of mouse lymphocytes was also inhibited by MSC-IDOc (Fig. 2B). Negative control vector-only transfectant suppressed neither human nor mouse T cell blasts in the presence of IL-2, as expected (Fig. 2A, B). Therefore, these MSC-IDOc were immunosuppressive *in vitro* for both mouse and human T lymphocytes.

To further examine the strength of their immunosuppressive effect in the murine system, MSC-IDOc transfectants were co-cultured with mouse T cells blasts at graded ratios from 1:10 to 1:80 (MSC-to-lymphocyte ratios) in the presence of IL-2. We found, even at ratios as low as 1:80, that MSC-IDOc transfectants were still potentially immunosuppressive (Fig. 2C). To verify the specificity of IDO in this immunosuppression, we added 1-methyl-tryptophan (1-MT), a competitive inhibitor of IDO, into co-cultures, and found that it reversed the inhibitory effect of MSC-IDOc (Fig. 2C). These results were verified in both MSC-IDOc clones. (Data for MSC-IDOc2 shown in Supplementary Fig. S1). Negative control vector-only transfectants did not inhibit lymphocyte proliferation (Fig. 2D).

Therefore, the observed immunosuppression by human IDO-expressing mouse MSCs is dependent on IDO. We next examined the potential function of these cells *in vivo*.

Constitutive IDO-expressing MSCs promote tumor growth

We have shown that mouse *iNOS*^{-/-} MSCs constitutively expressing human IDO (MSC-IDOc) are strongly immunosuppressive in both mouse and human systems *in vitro*. To examine their potential functionality *in vivo*, we employed the B16-F0 melanoma tumor model to determine whether MSC-IDOc transfectants could influence the tumor microenvironment and affect tumor growth. After B16-F0 tumor cell implantation, MSC-IDOc1 transfectants were injected i.p. every two days (this clone was utilized since it demonstrated greater immunosuppression). On day 14, mice were sacrificed and tumors were weighed. Mice treated with MSC-IDOc showed dramatically increased tumor mass in comparison to those receiving negative control MSCs (Fig. 3A). To verify that this tumor-promoting effect was indeed due to IDO, 1-MT (an IDO inhibitor) was administered in drinking water starting on the day of MSC injection and continuing for the duration of the experiment. We found that 1-MT reversed the tumor-promoting effect of MSC-IDOc1 (Fig. 3A), while it had no effect in mice treated with negative control MSCs (Fig. 3B). These results show that tumor promotion by MSC-IDOc transfectants was due to the activity of IDO. Therefore, these constitutive human IDO-expressing mouse *iNOS*^{-/-} MSCs not only inhibited the proliferation of lymphocytes *in vitro*, they also promoted the growth of B16-F0 melanoma tumor *in vivo*.

Inducible expression of human IDO in mouse MSCs

Since IDO expression by human MSCs occurs only after exposure to inflammatory cytokines such as $\text{IFN}\gamma$ ¹¹²⁴, synthetic constitutive expression of human IDO in mouse MSCs may not completely recapitulate natural IDO expression in human MSCs. Therefore, to better mimic the pathophysiological role of human IDO, we established an inducible IDO expression system. Since human IDO and mouse *iNOS* both require similar inflammatory cytokines for normal expression induction¹¹²⁷⁻²⁹, we designed an inducible system, again in *iNOS*^{-/-} MSCs, in which human IDO expression is controlled by the promoter of mouse *iNOS* gene, so that it would more closely resemble the induction of IDO expression in human MSCs. Because the human IDO promoter may not respond to mouse $\text{IFN}\gamma$ in the same way that it does to human $\text{IFN}\gamma$ ³⁰, we cloned an essential 1750 base pair fragment of the *iNOS* promoter, *iNOS_p2*, which has been reported to be necessary for the induction of *iNOS* expression²⁹³¹. We then cloned both *iNOS_p1* and *iNOS_p3* fragments and the human IDO gene into the pCMS-EGFP vector from which the CMV promoter had been removed (Fig. 4A). In order to explore the efficiency of the induction system, we extended the *iNOS* promoter elements further upstream to include possible enhancers or suppressors, thus generating *iNOS_p1* (1750bp+1650bp) and *iNOS_p3* (1750bp+1650bp+1650bp) (Fig. 4A). With these promoter fragments, we successfully constructed three different inducible human IDO expression systems: *iNOS_p1*/IDO, *iNOS_p2*/IDO, and *iNOS_p3*/IDO (Fig. 4A). These three constructs were then separately transfected into *iNOS*^{-/-} mouse MSCs, and several clones generated. Next, these clones were stimulated *in vitro* with inflammatory cytokines (TNF α , IL-1 β and $\text{IFN}\gamma$) and screened for human IDO expression to select clones with inducible IDO expression. Among the positive clones, three of them (MSC-IDOi1, MSC-IDOi2 and MSC-IDOi3, each with a different promoter) showed dramatic upregulation of IDO mRNA and protein after inflammatory cytokine stimulation, achieving levels similar to those observed with the constitutive CMV promoter. IDO expression was undetectable in the absence of inflammatory cytokines (Fig. 4B, C). In negative controls, we found that neither non-transfected mouse *iNOS*^{-/-} MSCs (Fig. 4B, C) nor vector-only *iNOS*^{-/-} MSCs (data not shown) expressed detectable levels of IDO before or after inflammatory cytokine stimulation. To verify the inducible expression of human IDO in

these MSCs (MSC-IDOi1, MSC-IDOi2, MSC-IDOi3) are indeed controlled by the iNOS promoter, we also stimulated these cells with inflammatory cytokines at different doses and incubated for different time (0 hr, 12 hr and 24 hr). The expression of IDO was then examined, and demonstrated to be in a dose- and time-dependent manner, similarly to the iNOS expression in bone marrow-derived MSCs from wild type mice (Supplementary Fig. S2A).

In order to verify the enzymatic activity of the IDO protein produced by these iNOS promoter-regulated human IDO-transfected mouse MSCs, the cells were stimulated with inflammatory cytokines *in vitro*, and the supernatant was assayed for the IDO metabolite, kynurenine, using Ehrlich's reagents³². We found remarkably increased kynurenine concentrations after cytokine stimulation (Fig. 4D). These data verified that all three constructs were functional, since IDO expression was potently inducible and fully functional in the corresponding MSC clones.

MSCs with inducible IDO expression potently inhibit T lymphocyte proliferation

To determine whether these iNOS promoter-regulated human IDO-expressing mouse iNOS^{-/-} MSCs (MSC-IDOi) could also inhibit lymphocyte proliferation, MSC-IDOi1 transfectants were co-cultured with freshly isolated murine splenocytes at various ratios (MSC-to-splenocyte ratios from 1:10 to 1:80) in the presence of soluble anti-CD3 and anti-CD28. Notably, MSC-IDOi1 transfectants effectively suppressed activation-induced T cell proliferation at ratios as low as 1:80 (Fig. 5A). Transfectants with the other two constructs (MSC-IDOi2 and MSC-IDOi3) were similarly immunosuppressive (Supplementary Fig. S2B). Furthermore, 1-MT completely reversed the observed immunosuppression with all three clones. As negative controls, we observed no effect on lymphocyte proliferation by mouse iNOS^{-/-} MSCs (Fig. 5B) or pCMS-EGFP vector-only transfected iNOS^{-/-} MSCs (data not shown). The effect of wild type mouse bone marrow-derived MSCs was also examined as a control. When co-cultured with splenocytes in the presence of anti-CD3 and anti-CD28, lymphocyte proliferation was dramatically inhibited due to iNOS induction in the wild type MSCs; however, this effect was not reversed by 1-MT (Fig. 5C). These results reveal that mouse MSCs with inducible IDO expression are indeed immunosuppressive and acquire this function only upon cytokine stimulation. To assess lymphocyte proliferation, we performed DNA content analysis on splenocytes co-cultured with MSCs as above. We found that lymphocyte proliferation at 24 hr and 48 hr was inhibited by both wild type MSCs and MSC-IDOi. Addition of 1-MT reversed only the effect of MSC-IDOi, but not wild type MSCs (Supplementary Fig. S2C). To extend these results to human lymphocyte proliferation, MSC-IDOi transfectants were co-cultured with human T cell blasts (MSC-to-human PBMC ratios 1:10 to 1:80) in the presence of mouse IFN γ , TNF α , and IL-1 β . As expected, the resulting IL-2-driven human T cell blast proliferation was dramatically inhibited, even at 1:80 (Fig. 5D). Interestingly, mouse iNOS^{-/-} MSCs had no effect on human T cell proliferation (Supplementary Fig. S3).

Mouse MSCs with inducible human IDO promote tumor growth by modulating the immune response

Although we have demonstrated that mouse MSCs with *constitutive* IDO expression (MSC-IDOc) promote B16-F0 melanoma tumor growth (Fig. 3), mouse MSCs with *inducible* IDO (MSC-IDOi) may better reflect the physiology of human MSCs. To examine the effects *in vivo*, C57BL/6 mice were co-injected with B16-F0 melanoma tumor cells and MSC-IDOc or MSC-IDOi transfectants. We found that tumor growth was enhanced to a similar extent whether IDO expression by MSCs was inducible or constitutive. In addition, concurrent 1-MT administration reversed the effects of both MSC types (Fig. 6A). Thus, inducible IDO-transfected MSCs also potently promote tumor growth *in vivo*.

To determine whether IDO-expressing MSCs indeed infiltrate into B16 melanoma tumors, we conducted experiments *in vivo*. B16-F0 melanoma tumor cells were injected into the thigh of C57BL/6 mice. This was followed after one week with intraperitoneal injection of either iNOS^{-/-} MSCs or MSC-IDO transfectants. Tumor samples were collected after MSC injection. Real-time PCR of mRNA isolated from tumor tissue revealed the presence of human IDO mRNA, but not mouse IDO (Supplementary Fig. S4A). To further verify the presence of these human IDO-expressing MSCs *in situ*, tumor samples were collected 24 hr after intraperitoneal injection of either iNOS^{-/-} MSCs or MSC-IDOi transfectants (with GFP) in B16-F0 tumor mice, and cryosections of these tumor samples were prepared and visualized. As MSC-IDOi transfectants contain the GFP reporter gene, we examine the presence of these cells in tumors directly by confocal microscopy (Zeiss LSM 700). Indeed, we confirmed that these cells were present in tumor tissues (Supplementary Fig. S4B). The immunohistochemical staining of human IDO further demonstrated the *in situ* localization of human IDO from MSC-IDOi in tumor tissues (Supplementary Fig. S4C).

It has been reported that wild type mouse MSCs pretreated with the inflammatory cytokines IFN γ and TNF α can also promote B16-F0 melanoma growth *in vivo* owing to iNOS induction³³. Therefore, to exclude the influence of iNOS in the wild type C57BL/6 host mice, we replicated the B16-F0 melanoma tumor model in iNOS^{-/-} hosts. Interestingly, these iNOS^{-/-} mice were somewhat resistant to B16-F0 melanoma tumor growth, since the time required for the tumors to reach a palpable size was significantly longer, verifying the role of iNOS in promoting tumor growth in wild type mice. Nevertheless, co-transplantation of B16-F0 melanoma cells with either inducible or constitutive IDO-transfected MSCs promoted tumor growth in these iNOS-deficient mice. Furthermore, 1-MT reversed these effects (Fig. 6B). Thus, in replicating the B16-F0 melanoma tumor model in iNOS-deficient mice, we further confirmed that tumor promotion was due to upregulated IDO activity.

To determine if our findings IDO-expressing MSCs promote B16-F0 melanoma tumor growth could be extended to other tumor types, we examined the effects in the EL4 lymphoma model. When EL4 lymphoma cells were co-transplanted with MSC-IDOi or MSC-IDOc, the resultant tumors grew larger and more rapidly compared with negative vector- transfectants or wild type MSCs. Again, these effects were reversed by 1-MT (Fig. 6C). Therefore, our MSC-IDO provide a good model with which to study the role of IDO in tumor immunity and cancer immunotherapy in mouse models.

To assess the effect of IDO-expressing MSCs on the cellular immune response to B16-F0 melanoma tumor described above (Fig. 6A), we further examined these mice for the distribution of several immune cell populations in the peripheral blood and in tumor tissues. Although we found no significant changes in the peripheral blood (Supplementary Fig. S5), MSC administration significantly altered the distribution within tumor-infiltrating lymphocytes (Fig. 6D-I). In mice administered with either constitutive or inducible IDO-transfected MSCs, CD3⁺ T cell numbers in tumors were reduced by 70% in comparison to mice treated with negative control-transfected iNOS^{-/-} MSCs. Furthermore, the vast majority of this reduction was in the CD8⁺ T cell fraction, rather than CD4⁺ T cells (Fig. 6E, F). The frequency of regulatory T cells (Tregs) was not significantly affected (Fig. 6G), a surprising result since Tregs have been reported to play an important role in tumor immunity^{34,35}. Similarly, changes in NK cell numbers did not achieve statistical significance (Fig. 6H). There was also significant diminution in the tumor-associated B cell population (Fig. 6I), a finding that warrants further investigation. In the groups treated with IDO inhibitor, 1-MT, in which tumor growth enhancement was prevented, the changes in CD3⁺ T cell, CD 8⁺ T cell, and B cell frequencies were not observed (Fig. 6D, F, and I). MSC-IDO caused similar changes in immune cell populations when the B16-F0 melanoma tumor model was carried out in iNOS^{-/-} mice (Supplementary Fig. S6, 7). Taken together

these studies of human IDO-transfected murine MSCs in mouse tumor models *in vivo* demonstrate that IDO is a key factor in promoting tumor growth, and that it acts mainly through modulation of CD8⁺ T cells in the tumor microenvironment.

Critical role of adaptive immunity in the modulation of anti-tumor immune response by IDO-expressing MSCs

We have demonstrated that administration of IDO-expressing MSCs promotes dramatic tumor growth in wild type mice and iNOS^{-/-} mice. Importantly, the frequency of certain types of immune cells in the tumor was reduced, suggesting that it may have a role in this effect. To determine which immune cells might be involved in tumor promotion by MSCs, we examined the effect of IDO-expressing MSCs on B16-F0 tumors in mice lacking particular immune cells. First, in Rag1^{-/-} mice, which lack both T cells and B cells, tumor-promotion by MSC-IDO transfectants was completely abolished, since there was no difference in tumor size between mice given IDO-expressing MSCs or control MSCs (Fig. 7A). This result demonstrates that the effect of IDO is dependent on cells of the adaptive immune system. To narrow it down to specific T cell populations, we next used β 2M^{-/-} and CIITA^{-/-} mice, which are deficient in CD8⁺ and CD4⁺ T cells, respectively. We found that, like in the Rag1^{-/-} mice, tumor growth in β 2M^{-/-} mice was unaffected by IDO-transfected MSCs (Fig. 7B), clearly implicating CD8⁺ T cells. Since CD4⁺ T cells numbers were unchanged by IDO-expressing MSCs, we expected that CIITA^{-/-} mice would show a response similar to that of wild type mice. Instead, the result with CIITA^{-/-} mice was inconclusive, as CIITA^{-/-} mice given *constitutive* IDO-expressing MSCs showed only a small, statistically insignificant increase in tumor growth, while *inducible* IDO producers had no effect at all (Fig. 7C). Thus, it is possible that these helper T cells are required for IDO-mediated tumor growth enhancement, possibly to provide essential help to the CD8⁺ T cells. Overall, the resultant abolishment of tumor-promotion by IDO-expressing MSCs in Rag1^{-/-}, β 2M^{-/-} and CIITA^{-/-} mice strongly suggests that the adaptive immune system plays a vital role in IDO-mediated tumor progression.

Discussion

The species variation in MSC-mediated immunomodulation between human and mouse hinders the application of findings from studies using mouse MSCs to human pathophysiological conditions¹¹. Our study partially addresses this issue by incorporating the human IDO gene into mouse iNOS^{-/-} MSCs, and placing it under control of the mouse iNOS promoter, thus establishing a more relevant experimental system to study the effects of human IDO *in vivo*. By transfecting IDO-expressing plasmids into mouse iNOS^{-/-} MSCs, these cells were not only able to express human IDO, but also any potential interference by mouse iNOS was eliminated. Using these human IDO-transfected mouse MSCs, we employed B16-F0 melanoma and EL-4 lymphoma tumor models in mouse and demonstrated that IDO, whether expressed in a constitutive or inducible manner, can potently promote tumor growth by modulating the immune response. Therefore, this unique system provides a new platform to mimic the physiological role of MSC-mediated immune modulation in human diseases.

Our previous studies have shown that human and mouse MSCs are not innately immunosuppressive, but rather acquire this capability when induced by inflammatory cytokines. For inducible IDO expression in mouse MSCs, the human IDO gene was placed under control of the mouse iNOS promoter, which can be switched on by stimulation with inflammatory cytokines, much like the human IDO promoter is activated^{27,28}. The upstream region of the human IDO promoter contains several important regulatory elements, such as IFN-stimulated response elements (ISRE-1 and ISRE-2) and gamma activation sequences

(GAS-1 and GAS-2). These regulatory elements are crucial for IFN γ responsiveness since they can interact with IFN regulatory factor-1 (IRF-1) and signal transducer and activator of transcription 1 α (Stat1 α), respectively ²⁷. Like the human IDO promoter, there are two regions of the mouse iNOS promoter known to regulate the induction of iNOS by inflammatory cytokines: a proximal region I (RI) and a more distal region II (RII). It has been reported that IFN γ only targets to RII. Interestingly, the RII region also contains a GAS element and ISREa and ISREb elements ²⁸. The strong similarity between the human IDO promoter and mouse iNOS promoter suggests that both are responsive to IFN γ , a fact borne out experimentally. Therefore, using the mouse iNOS promoter to direct inflammation-induced expression of human IDO mimics to some extent the function of the human IDO promoter, since they have similar regulatory mechanism.

In the murine system, iNOS is found mainly in MSCs, macrophages, and some other cell types. In contrast to iNOS, which is inducible, expression of the other two forms of NOS (nNOS, found in neurons, and eNOS, found in endothelial cells) are relatively constitutive ³⁶³⁷, and do not have a role in MSC-mediated immunosuppression. When stimulated by inflammatory cytokines, mouse MSCs express iNOS, thus generating nitric oxide (NO), a potent immunosuppressive molecule ⁵³⁸³⁹. On the other hand, human MSCs mediate immunosuppression by up-regulation of IDO in response to inflammatory cytokines. Interestingly, in most human tumors, IDO is believed to be expressed continuously ⁴⁰, which may explain why tryptophan catabolism is elevated in cancer patients. Since a tumor can be considered to be a wound that never heals, tissue damage signals result in continuous recruitment of MSCs into the tumor ⁴¹⁴². Using our inducible IDO-transfected mouse MSCs in tumor models, we observed that the ongoing inflammatory microenvironment also induces the expression of IDO, leading to its immunomodulatory effect.

Employing mouse iNOS^{-/-} MSCs to express the human IDO gene avoided potential interference by iNOS-mediated immunomodulation. Since normal MSCs promote tumor growth ³³, while iNOS^{-/-} MSCs actually inhibit tumor growth in the mouse B16 melanoma tumor model ⁴³, it was critical to exclude iNOS from the system. Accordingly, we selected iNOS^{-/-}MSCs for transfection with our constitutive or inducible human IDO expression constructs. These cells became immunosuppressive when human IDO was expressed, significantly inhibiting the proliferation of both mouse and human lymphocytes. Since IDO is known to regulate the activation and proliferation of T cells, B cells and NK cells ²⁴⁴⁴⁴⁵, these results verified that these IDO-expressing humanized murine MSCs are functionally equivalent to human MSCs.

We applied our humanized mouse MSCs in a melanoma model and a lymphoma model *in vivo*, and demonstrated that human IDO from these MSCs do indeed promote tumor growth in both models, an effect reversible by the IDO inhibitor, 1-MT. We found the tumor-favoring effect of IDO-expressing MSCs may be mediated by immune cells, including CD8⁺ T cells and possibly CD4⁺ T cells. We also found that MSC-mediated tumor promotion is absent in Rag1^{-/-} mice, a finding that appears to contradict a recent report that IDO-overexpressing tumor cells display tumor growth promotion in a xenograft model in nude mice, which, like Rag1^{-/-} mice, are also immunodeficient ⁴⁶⁴⁷. It may be possible, however, that IDO production by tumor cells themselves promotes their own growth independent of any adaptive immune cell effects, while tumor promotion by IDO-producing MSCs requires an intact immune system. We would postulate that, since immune surveillance is always present in the physiological milieu, it is more relevant to investigate the biology underlying its role. In our model, MSCs are the source of IDO. These cells also produce several chemokines that attract immune cells, which, when in close proximity with the MSCs, are then subject to inhibition by IDO. Although the immune cell populations involved need

further investigation, our *in vivo* data support the concept that, in humans, IDO is produced by MSCs in response to inflammatory cytokines present within the tumor microenvironment and this IDO is hijacked by tumor to escape immune surveillance.

MSCs have shown great clinical potential in cell-based therapy for wound healing, tissue regeneration, and cancer treatment. The emerging field of cancer-MSC biology has recently gained much attention, providing new insights into tumor biology and novel therapeutic targets. Since accumulating evidence indicates that IDO plays a critical role in tumor immune escape⁴⁸⁻⁵⁰, it is thus important to determine how MSCs exert their IDO-mediated functions in humans. Here, we have described a unique strategy that provides a means to more relevant investigation of human IDO function and regulation in murine models. Application of this new tool should lead to a better understanding of the role of IDO-mediated immunosuppression by MSCs in the tumor microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MSC	mesenchymal stem cell
IDO	indoleamine 2,3-dioxygenase
1-MT	1-methyl-tryptophan
iNOS	inducible nitric oxide synthase
TNFα	tumor necrosis factor- α
IL-1	interleukin-1
IFNγ	interferon- γ

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Figure 1. Constitutive expression of human IDO in mouse MSCs deficient in iNOS

(A) Design of the human IDO constitutive expression construct. Human IDO (hIDO) cDNA fragment was inserted into the pcDNA3.0 plasmid under control of the CMV promoter. Successful transfectants were selected using Neomycin.

(B) Human IDO expression by MSCs transfected with IDO constitutive expression vector. Vectors with or without the human IDO gene construct were transfected into mouse iNOS^{-/-} MSCs. Cells were harvested after two days in culture, total mRNA was extracted, and IDO message was assayed by real-time PCR, normalized to β-actin mRNA (defined as 1000 arbitrary units). MSC-IDOc1 and MSC-IDOc2: two representative human IDO constitutive expression clones. MSC-V: MSCs transfected with pcDNA3.0 vector only (negative control).

(C) Human IDO protein was determined by western blotting analysis. MSCs were cultured for two days, harvested, and total cell lysates assayed for human IDO. MSC-V served as a negative control; human MSCs stimulated with inflammatory cytokines (TNFα, IL-1β, IFNγ; 20 ng/ml each) served as positive control. Results are representative of three independent experiments.

Figure 2. MSCs constitutively expressing human IDO inhibit the proliferation of both human and mouse lymphocytes

(A-B) Effect of MSC-IDOc on lymphocyte proliferation. MSC-IDOc were co-cultured with human PBMCs stimulated with OKT3 antibody (A) or mouse splenocytes stimulated with anti-CD3 and anti-CD28 (B) at a MSC-to-lymphocyte ratio of 1:10. Cell proliferation was assessed by ³H-thymidine incorporation after 48 hr.

(C-D) 1-MT reversed the immunosuppressive effect mediated by MSC-IDOc. MSCs were co-cultured with mouse splenocytes at graded ratios of MSCs-to-splenocytes with or without 1-MT (0.5 mM), and cell proliferation was assessed by ³H-thymidine incorporation after 48 hr (C). MSC transfected with pcDNA3.0 empty vector (MSC-V) were not immunosuppressive (D). Values represent means $\pm$ SD from a representative of three experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. Human IDO constitutively expressed by MSCs Promotes Tumor Growth

(A-B) Wild type C57BL/6 mice were injected i.m. in the thigh with B16-F0 melanoma cells (0.5×10^6), and then administered intraperitoneally with MSC-IDOc or vector-transfectants (1×10^6 per mouse) every 2 days, with or without 1-MT (2 mg/ml) supplementation in drinking water. After 14 days, mice were sacrificed and resultant tumors were excised and weighed (A). 1-MT had no effect on mice treated with vector-transfectants (MSC-V) (B)

Figure 4. Inducible expression of human IDO in mouse MSCs deficient in iNOS

(A) Design of the human IDO inducible expression constructs. The original CMV promoter of the pCMS-EGFP vector was removed. Mouse iNOS promoter and human IDO cDNA fragments were then inserted into the vector. Three different versions of mouse iNOS promoter were incorporated: mouse iNOS promoter *p2* (1750 base pairs upstream from ATG) was the essential part of the entire promoter; mouse iNOS promoter *p1* was extended from *p2* (1750+1650 base pairs); mouse iNOS promoter *p3* was further extended from *p1* (1750+1650+1650 base pairs). GFP was included as a reporter.

(B) Each of the three inducible IDO expression constructs (iNOS-p1/IDO, iNOS-p2/IDO, iNOS-p3/IDO) were transfected into mouse iNOS^{-/-} MSCs to generate three different MSC transfectants with inducible human IDO (hIDO) expression (MSC-IDOi1, MSC-IDOi2 and MSC-IDOi3, respectively). These transfectants were cultured with or without inflammatory cytokines (TNF α , IL-1 β , IFN γ ; 20 ng/ml each) for 24 hr, cells were harvested, and total mRNA was extracted. human IDO message was assayed by real-time PCR, normalized to β -actin mRNA (defined as 1000 arbitrary units). Non-transfected iNOS^{-/-} MSCs served as negative control.

(C) The same inducible hIDO-expressing MSC transfectants were assayed for hIDO expression at the protein level by western blotting analysis after culture, as in (B). Results are representative of three experiments.

(D) To determine IDO enzyme activity, MSCs were cultured with or without inflammatory cytokine (TNF α , IL-1 β , IFN γ ; 20 ng/ml each) stimulation for 24 hr. IDO activity was assayed by spectrophotometric detection of the tryptophan metabolite, kynurenine, a product of IDO catabolism.

Figure 5. MSCs with inducible human IDO expression inhibit proliferation of both mouse and human lymphocytes

(A-D) Effects of MSC-IDOi on lymphocyte proliferation. MSCs were co-cultured with freshly-isolated mouse splenocytes at graded ratios of MSCs-to-splenocytes in the presence of soluble anti-CD3 and anti-CD28, and IFN γ , TNF α , and IL-1 β (10 ng/ml each), with or without 1-MT (0.5 mM). After 48 hr, proliferation was assessed by ^3H thymidine incorporation (A). Negative control mouse iNOS^{-/-} MSCs were not immunosuppressive under identical conditions (B). The effects of wild type MSCs were similarly tested (C). The effects of MSC-IDOi on human lymphocyte proliferation was tested using human T cell blasts (D).

Figure 6. MSCs with inducible expression of human IDO promote tumor growth through modulation of immune cells

(A-C) Effects of MSCs with inducible human IDO (hIDO) expression on tumor growth. Wild type C57BL/6 mice or *iNOS*^{-/-} mice were injected i.m. in the thigh with B16-F0 melanoma cells (0.5×10^6) (A, B) or EL4 lymphoma cells (0.5×10^6) (C), and then administered i.p. with MSCs having inducible hIDO-expression (MSC-IDOi1), or constitutive hIDO-expression (MSC-IDOc1; positive control), wild type (wt) MSCs, or negative control MSCs (MSC-control) (1×10^6 cells) every 2 days, with or without 1-MT (2 mg/ml) supplementation in drinking water. After 14 days, mice were sacrificed and resultant tumors were excised and weighed. (D) Distribution of immune cell subpopulations within tumor tissue. Cells were harvested from the tumors in (A), stained for the indicated markers, and analyzed by flow cytometry. The number of CD3⁺ T cells, CD4⁺ T cells, CD8⁺ T cells, CD4⁺ Foxp3⁺ T cells (regulatory T cells, Tregs), NKG2D⁺ cells (NK cells) and CD19⁺ cells (B cells) as a percentage of total cells within the tumor are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 7. Tumor promotion by human IDO-transfected MSCs is abolished in immunodeficient mice

Rag1^{-/-}, β2M^{-/-} or CIITA^{-/-} mice were treated as described in (Fig. 6A) and tumor growth measured. MSCs expressing either constitutive or inducible human IDO did not promote tumor growth in these mice.