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14. ABSTRACT

Therapies aimed at activating breast cancer patient's adaptive immune response to metastatic tumor cells are being developed; however, most patients with advanced disease are immune suppressed, making it unlikely that active immunotherapies will be effective. Myeloid-derived suppressor cells (MDSC) are a major player in tumor-induced immune suppression seen in many patients and experimental animals with breast cancer and inhibit immunity by blocking T cell activation. Indoleamine 2,3 dioxygenase (IDO), a tryptophan degradation enzyme, also contributes to immune escapes by suppressing T cell activation. To determine if MDSC activity is regulated by IDO, wild type BALB/c and IDO-/- mice were challenged with 4T1 mammary carcinoma cells. MDSC from IDO-/- mice were less suppressive than MDSC from IDO+/+ mice, and treatment with the IDO inhibitor 1-D-MT partially restored T cell proliferation. Western blots demonstrated that MDSC do not contain IDO, suggesting that IDO indirectly affects MDSC suppression. IDO-induced tryptophan starvation is known to act through the IL-6 signaling pathway by activating nuclear factor-IL-6. Since IL-6 is an inducer of MDSC, we tested MDSC from IDO+/+ mice carrying IL-6 transfected 4T1 cells (4T1/IL-6). 4T1-IL-6-induced MDSC from IDO-/- mice were equally suppressive to 4T1-induced MDSC from IDO+/+ mice demonstrating that IL-6 overcomes IDO deficiency and restores MDSC suppression. We conclude that IDO enhances MDSC suppressive activity by inducing IL-6 production which then activates MDS

15. SUBJECT TERMS
Immune Suppression, Myeloid-Derived Suppressor Cells, Indoleamine 2,3 Dioxygenase, Interleukin-6 (IL-6), Nuclear factor-IL-6

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Introduction

The concept that tumor development was suppressed by immune system was originally proposed by Paul Ehrlich in 1909 (1). Immunotherapy, aimed at enhancing efficient immune responses to defend against tumors, offers great promise as a new treatment for advanced cancer (2, 3). However, attempts to target tumors by immunotherapy are less successful than expected. Tumor induced immune suppression in patients with metastatic disease limits the effect of this novel therapy.

Tumor escape from immune surveillance is partly due to the accumulation of an immature precursor cell population called myeloid-derived suppressor cells (MDSC) seen in many patients and experimental animals with breast cancer (4, 5). MDSC are present at basal levels in healthy individuals, but can accumulate to very high levels in spleen, blood and lymphoid organs in tumor-bearing individuals. MDSC are characterized by the $Gr\text{-}1^{+}CD11b^{+}$ cell surface markers in mouse and $CD33^{+}CD11b^{+}$ markers in human (6, 7). MDSC are becoming a therapeutic cellular target in cancer immunotherapy because they have potent immunosuppressive activity and block

tumor immunity by preventing the activation of $CD4^+$ and $CD8^+$ T cells (5, 6), inhibiting natural killer (NK) cell activity (8) and by skewing immunity towards a tumor-promoting type 2 response (9).

Indoleamine 2,3 dioxygenase (IDO), a tryptophan degradation enzyme, is another contributor to immune escape by suppressing tumor immunity and T cell activation (10), blocking NK cell activation (11), and by enhancing the suppressive activity of Tregs (12). The immunosuppressive activity of IDO was first demonstrated by Munn and colleagues who showed that IDO in the placenta is crucial to prevent immunological rejection of an allogeneic fetus (13). IDO is generally inactive in immune cells, but functional IDO expression is chronically induced or activated by specific signals in many patients and mice with various cancers (14-16). IDO was found to have another isoform, named IDO2, to be distinguished from the previously identified IDO1. The *IDO2* gene is situated just downstream of *IDO1* on human and mouse chromosome 8. These two protein have similar enzymatic activities (17).

We hypothesized that IDO may also contribute to MDSC suppression. To test this hypothesis, we have studied IDO and MDSC in the spontaneously metastatic BALB/c-derived 4T1 mouse mammary carcinoma system because 4T1 is a poorly immunogenic tumor that closely resembles human breast cancer (18). To determine if IDO is involved in MDSC function, we have used wild type BALB/c ($IDO1^{+/+}$) and IDO1 knockout ($IDO1^{-/-}$) mice challenged with 4T1 mammary carcinoma cells. MDSC from $IDO1^{-/-}$ mice were less suppressive than MDSC from $IDO1^{+/+}$ mice, and treatment with the IDO inhibitor 1-D-MT partially restored $CD4^+$ and $CD8^+$ T cell proliferation, consistent with our hypothesis that MDSC activity is regulated by IDO. To determine if IDO is present in MDSC, western blots have been performed on lysates of MDSC induced by 4T1 using IDO1-specific antibodies as described by Munn et al. (15). Western blots demonstrated that MDSC do not contain IDO, suggesting that IDO indirectly affects MDSC suppression. IDO-induced tryptophan starvation is known to act through the IL-6 signaling pathway by activating nuclear factor-IL-6 (17). Since IL-6 is an inducer of MDSC (19), we tested MDSC from $IDO1^{-/-}$ mice carrying IL-6-trasnfected 4T1 cells (4T1/IL-6). 4T1/IL-6-induced MDSC from $IDO1^{-/-}$ mice were equally suppressive to 4T1-induced MDSC from $IDO1^{+/+}$ mice. The finding that IDO deficiency partially eliminates MDSC suppressive activity and 4T1/IL-6 overcomes IDO deficiency in tumor bearing mice has led us to propose the causal relationship that IDO enhances MDSC suppression by inducing IL-6 production which then induces MDSC, thereby blocking immune surveillance.

Body

Note: Text appearing in the original statement of work (SOW) is underlined.

<u>Task 1: Determine if IDO is generally used by mammary carcinoma-induced MDSC to block T cell activation.</u>

1a: Test the suppressive activity of MDSC from IDO1$^{-/-}$ BALB/c mice carrying TS/A or EMT 6 mammary tumors.

1b: Using IDO$^{-/-}$ NeuT $^{+/-}$ mice test if MDSC induced by spontaneous mammary tumors in NeuT$^{+/-}$ or NeuNT$^{+/-}$ mice use IDO to mediate suppression.

1c: Determine if MDSC from breast cancer patients use IDO to suppress T cell activation. [Blood samples will be obtained from patients at the University of Maryland, Baltimore, Hospital, Breast Cancer Clinical under an approved IRB protocol]

Not as yet attempted.

Task 2: Determine if inflammation increases MDSC activity by increasing MDSC production of IDO.

2a: Determine if de letion of th e IDO gene elim inates inc reased suppression induced by inflammation.

2b: Determine if the IDO inhibitor 1-D-MT alters the suppressive activity of inflammation-induced MDSC.

Results for 2a: Since chronic inflammati on, as induced by the pro-inflamm atory cytokine IL -6, increases MDSC accum ulation (19), we co mpared the suppressiv e activity of MDSC induced by 4T1/IL-6 tumor cells vs. 4T1 tumor cells in IDO1$^{-/-}$ and IDO1$^{+/+}$ mice to determ ine if inflam mation increases MDSC suppressive activity by up-regulating IDO (Figure 1). T o test for the suppressive differences between MDSC induced by 4T1 and 4T1/IL-6 tum ors in IDO1 $^{+/+}$ and IDO1 $^{-/}$- m ice, CD8+ T cell proliferation using HA-specific clone 4 transgenic T cells was m easured by ^{3}H-thymidine uptake. T ransgenic T cells we re incubated with specific peptide and MDSC from IDO1$^{+/+}$ or IDO1$^{-/-}$ mice carrying 4T1/IL-6 or 4T1 tumors.

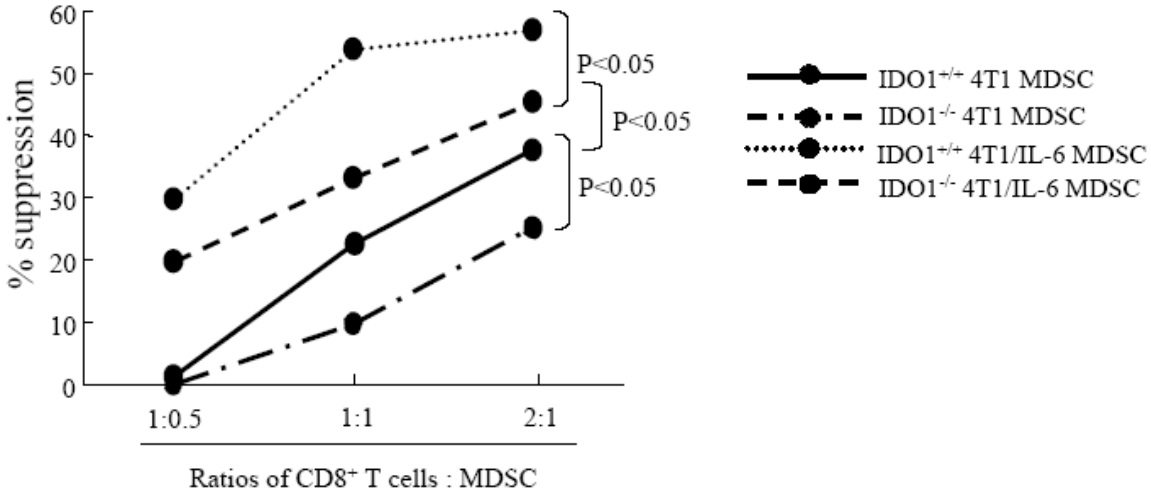

Figure 1: 4 T1/IL-6-induced MDSC from IDO1 $^{+/+}$ m ice (IDO1 $^{+/+}$ 4T1/IL-6 MD SC) were m ore suppressive than 4T 1/IL-6-induced MDSC from IDO $^{-/-}$ mice (IDO1$^{-/-}$ 4T1/IL-6 MDSC), consistent with our hypot hesis that IDO is involved in MDSC suppression of T cell activation. IDO1 $^{-/-}$ 4T1/IL-6 MDSC were e qually suppressive to 4T1-induced MDSC from IDO1 $^{+/+}$ m ice (IDO1 $^{+/+}$ 4T1 MDSC) dem onstrating that IL-6 overco mes IDO deficiency and restores MDSC suppression. Blood-derived Gr1^{+}CD11b^{+} MDSC were prepared as describe d (9). Briefly , blood was collected from m ice with lar ge 4T1 tum ors (~10- 12 m m in diam eter) in 5m l of 0.008% heparin solution dissolved in ddH $_2$O. Red blood cells were rem oved by Gey' s treatment.. More than 85% of the white blood cells in m ice with 4T1 or 4T1/IL6 tumors of 10-12 mm i n diam eter are Gr1 $^{+}$CD11b^{+} MDSC as quantified by flow cytometry. Splenocyte s f rom transgenic m ice were cultu red with th eir r espective peptide in the presen ce of gr aded doses of MDSC from IDO1 $^{+/+}$ or IDO1 $^{-/-}$ 4T1 or 4T1/IL-6 tumor-bearing mice, pulsed with tritiated thymidine on day 4, and the cells harvested and counted as counts per m inute (cpm) 18 hours later . CD8 $^{+}$ clone 4 transgenic T cells [H-2K $_d$-restricted, influenza hem agglutinin-peptide 518-526 (IYSTVASS)] were used for the experim ents. CD4^{+} T cell proliferation assays using HA-specific TS1 transgenic T cells are in progress. % Suppression= 1-[cpm (T cells + peptides + MDSC) /cpm (T cells + peptides)] * 100. Data are representatives of one of three experiments.

Results for 2b: Not as yet attempted.

Final results for Task 2– partially completed, and more in progress

Task 3: Determine if IDO is involved in the cross-talk between MDSC and macrophages.

Results: Since MDSC skew tumor immunity towards a tumor-promoting type 2 response by down-regulating macrophage production of IL-12 (9), we evaluated whether IDO is involved in the cross-talk between MDSC and macrophages. We compared macrophage production of IL-12 in the presence of MDSC generated from IDO1$^{-/-}$ mice vs. IDO1$^{+/+}$ mice (Figure 2a)，as well as the IL-12 production of IDO1$^{-/-}$ macrophage vs. IDO1$^{+/+}$ macrophage in the presence of IDO1$^{+/+}$ MDSC (figure 2b). The results suggested that the absence of IDO facilitates the down-regulation of macrophage production of IL-12 in the cross-talk between MDSC and macrophages.

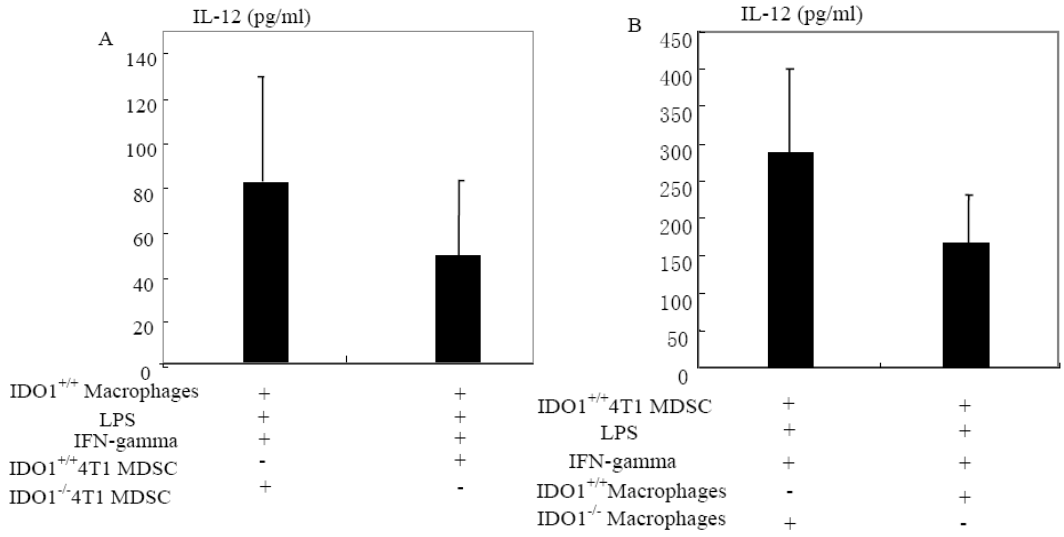

Figure 2: IDO1 deficiency up-regulates macrophage production of IL-12 in MDSC-macrophage cross-talk. Blood-derived $Gr1^{+}CD11b^{+}$ MDSC were prepared and quantified as described in Figure 1.$IDO1^{+/+}$ or $IDO1^{-/-}$ BALB/c mice were inoculated with thioglycolate (1ml of 3% Brewer thioglycolate in distilled water) and peritoneal exudate cells (PEC) were harvested and red blood cells (RBC) removed by lysis. The resulting cells were plated, the non-adherent cells removed, and the remaining attached macrophages were harvested and activated with LPS （100ng/ml） and IFN-gamma (2ng/ml). Peritoneal macrophages and MDSC co-culture assays were performed as previously described (9). Briefly, MDSC were irradiated (2500 rad) and added to wells ($1.5x10^6$ MDSC/well/500 µl macrophage medium) containing peritoneal macrophages ($7.5x10^5$ macrophages/well). Culture supernatants were collected 18h later and assayed for IL-12 by ELISA. (A) $IDO1^{+/+}$ macrophage co-cultured with MDSC generated from $IDO1^{-/-}$ mice vs. $IDO1^{+/+}$ mice. (B) Macrophages from $IDO1^{-/-}$ mice or $IDO1^{+/+}$ mice co-cultured with $IDO1^{+/+}$ MDSC. More repeats are in progress. In Figure 2 (B), it is the first time here showing that $IDO1^{-/-}$ macrophages make more IL-12 than $IDO1^{+/+}$ macrophage, which requires more investigation.

Final results for Task 3– Partially completed, and more in progress

Task 4: Determine which isoform(s) of IDO is present in MDSC.

4a: Perform western blot analyses of lysates of MDSC induced by 4T1, 4T1/IL-1 beta and 4T1/IL-6 tumor cells, using IDO1 and IDO2 specific antibodies (months 2-4)

4b: Alternatively, perform RT-PCR on mRNA from MDSC induced by 4T1, 4T1/IL-1 beta, and 4T1/IL-6 tumor cells using RT-PCR primers specific for IDO1 and IDO2 (month 4-6, if task 4a is not successful).

Results: Similar to IDO1, IDO2 protein also degrades tryptophan and has similar structure to IDO1. IDO2 is a preferred target of IDO competitive inhibitor 1-D-MT (17) suggesting that IDO2 might also be involved in MDSC suppression. To test which isoform(s) of IDO exist in MDSC (15), IDO1- specific antibodies were used in western blots of lysates of MDSC induced by 4T1, 4T1/IL-1 beta or 4T1/IL-6 (Figure 3). Western blots using IDO2- specific antibodies are in progress. RT-PCR analyses of IDO1 and IDO2 mRNA expression in MDSC generated from 4T1, 4T1/IL-1 beta and 4T1/IL-6-induced MDSC are also in progress.

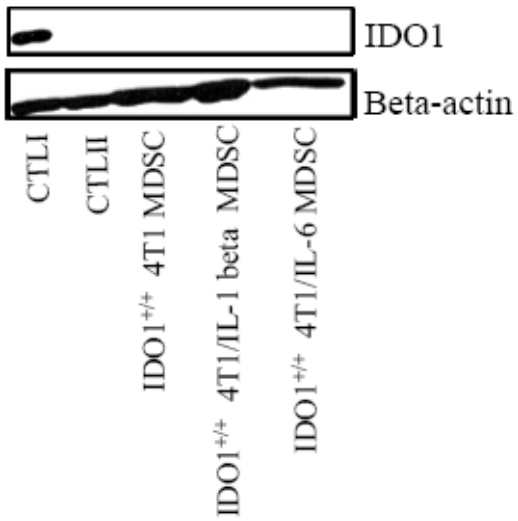

Figure 3: IDO1 $^{+/+}$ MDSC do not contain IDO1 protein, suggesting that IDO1 indirectly affects MDSC suppression. MDSC (1x10 ^{7} cells/ml) were lysed using RIPA lysis buffer and 10ug protein was loaded in wells of SDS-PAGE gels (10% separation gel, 5% stacking gel) and electrophoresesed for 1 hr at 100V, and transferred for 70 min at 100V. Membranes were incubated with either mouse anti-IDO1 antibody (Upstate) or mouse anti-beta actin antibody (Sigma Aldich) for 1 hr at room temperature. CTL I, IDO1 transfected fibroblasts; CTL II, vector only transfected fibroblasts, used as IDO1 negative control. CTL I and CTL II were obtained from Dr. David Munn and the same amount of protein was loaded per well.

Final results for Task 4– Partially completed, and more in progress

Besides the preliminary data described in the project objectives, more repeated experiments were performed to confirm the hypothesis that IDO is involved in MDSC suppression by comparing the ability of MDSC to block $CD4^{+}$ and $CD8^{+}$ T cell activation from IDO1$^{+/+}$ vs. IDO$^{-/-}$ 4T1 tumor bearing mice (Figure 4a and 4b). IDO inhibitor experiments were also repeated using two different isoforms of the inhibitor (1-D-MT and 1-L-MT) because both inhibitors can block the IDO-mediated tryptophan pathway, further supporting the finding that the IDO inhibitor reduces MDSC suppression of T cell activation (Figure 5).

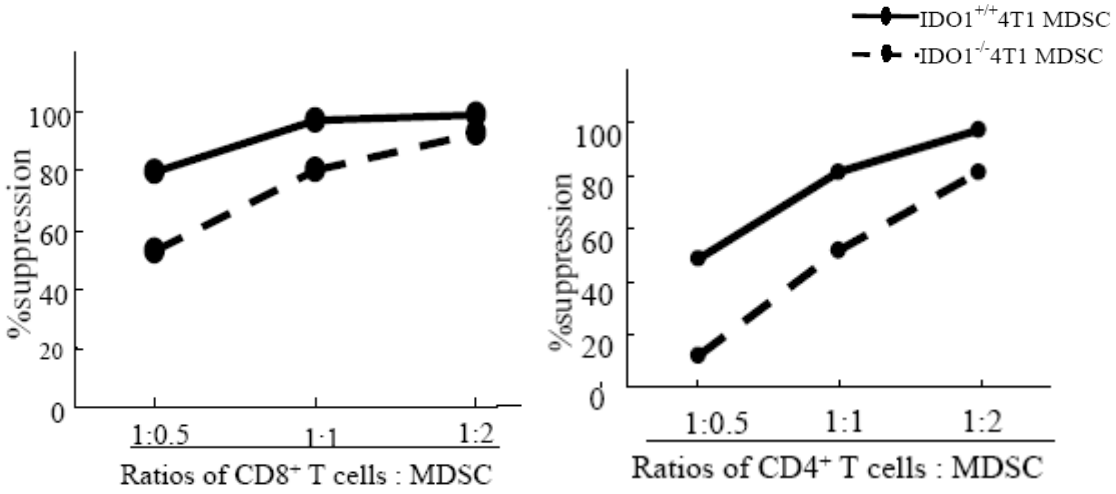

Figure 4: 4T1 induced-MDSC from IDO1 $^{-/-}$ tumor bearing mice are significantly less suppressive than 4T1 MDSC from IDO$^{+/+}$ mice. Suppression assays were performed as in Figure 1. (A) CD8^{+} clone 4 transgenic T cells [H-2Kd-restricted, influenza hemagglutinin-peptide 518-526 (IYSTVASS)]. (B) CD4^{+} TS1 transgenic T cells [I-E^{d}-restricted, HA peptide 110-119(SFERFEIFPK, HA1 110-119). Data are representatives of one of more than 6 experiments.

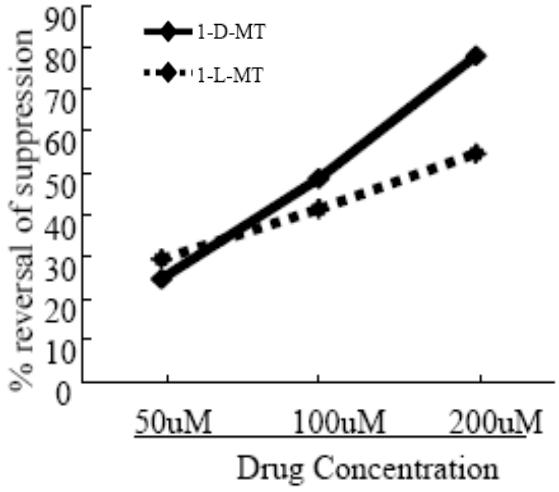

Figure 5: IDO inhibitor 1-D-MT or 1-L-MT reduces MDSC suppression of T cell activation. 4T1 induced-MDSC from IDO1$^{+/+}$ tumor-bearing mice were obtained as described in Figure 1 and were co-cultured with clone 4 transgenic CD8^{+} splenocytes plus HA$_{110-119}$ (T cells : MDSC=1:1) in the presence of graded doses of the IDO inhibitors. CD8^{+} T cell activation was measured as in Figure 1. Data are representatives of one of more than 6 experiments.

Additional Work

While pursing the above experiments, additional experiments were performed to further evaluate the IDO-tryptophan pathway in MDSC suppression. Since the completed task 4a suggested that IDO does not directly affect MDSC suppression, there must be some other mediator(s) involved in the IDO-MDSC suppression. IL-6 is known to induce MDSC accumulation in tumor bearing mice (19), and IDO over-expression-induced tryptophan starvation causes nuclear factor-IL-6 activation which up-regulates IL-6 (17). Since completed task 2 (Figure 1) demonstrated that IL-6 overcomes IDO deficiency and restores MDSC suppression, we hypothesized that IL-6 is the critical factor that links IDO and MDSC suppression. To further

determine whether IL-6 has a direct ef fect on MDSC, activated 4T1 MDSC fr om IDO1$^{-/-}$ and IDO1 $^{+/+}$ tumor bearing m ice were com pared for IL-6 production by ELISA (Figure 6). Since IDO is over -expressed by antigen presenting cells (APCs) such as IFN gamm a-induced macrophages in tumor bearing individua ls (20), we also tested peritoneal macrophages from IDO1$^{-/-}$ and IDO1$^{+/+}$ mice for production of IL-6.

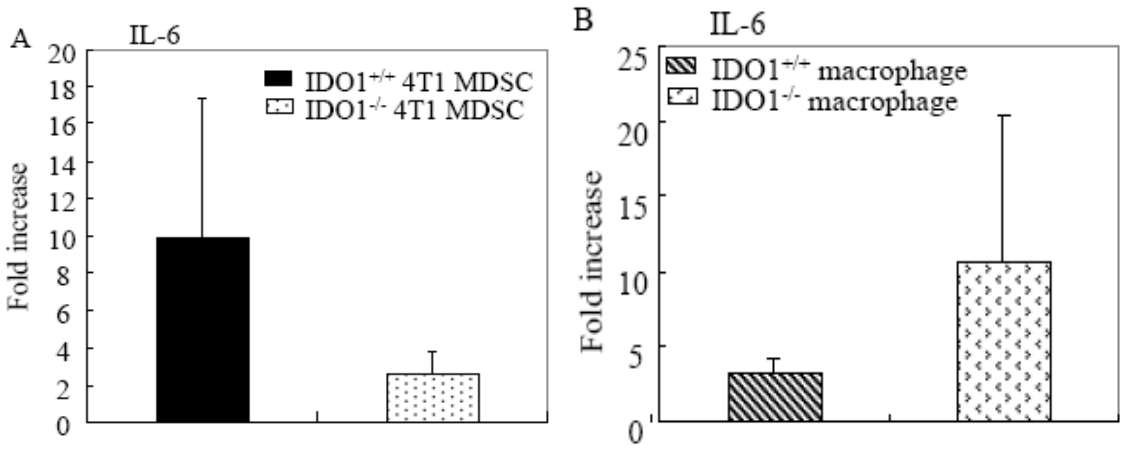

Figure 6: (A). 4T1-induced MDSC from IDO1 $^{+/+}$ m ice produce m ore IL-6 than 4T-induced MDSC from IDO1 $^{-/-}$ m ice. Blood-derived Gr1 $^{+}$CD11b^{+} MDSC and peritoneal m acrophages were prepared a nd activated by IF N-gamma a nd/or LPS as described in Figure 1 and 2. Culture supernat ants were collected and sto red at -80^{o}C. Thawed supernatants were assayed for IL -6 using a m ouse IL-6 ELISA duo set kit according to the m anufacturer's p rotocols (R&D System s, MN). Plates were read at 420 nm on a Bio-T ek 311 m icroplagte reader and quantified using a standard curve. Data are the mean ± SD of quadruplicate wells. Fold Increase = (IFN-gamma and LPS activated MDSC) / (MDSC without IFN-gamma and LPS). (B). IDO1 $^{-/-}$ macrophages produce m ore IL-6 th an IDO1 $^{+/+}$ m acrophages, which agrees with F igure 2b that IDO1$^{-/-}$ m acrophages produce m ore IL-12 than IDO1 $^{+/+}$ macrophages in the presence of IDO1 $^{+/+}$ MDSC. The m echanism of the findings is under investigation. More repeats will be performed.

Future Directions

We wish to extend these experim ents to IL-6 deficient BALB/c mice (IL-6$^{-/-}$) to determine whether IL-6 is essential fo r the IDO-involved-MD SC suppression. IL-6 knockout mice on a BALB/c background were recently o btained fro m Dr. Manfred Kopf (Institut f . Integr ative Bio logie, Switzerland) and are now breeding in our animal facility. IL-6 $^{-/-}$ mice will be inoculated with 4T1 or 4T1/IL-6 tumor cells, and MDSC suppressive activities will be com pared. If I DO af fects MDSC suppression through the IL-6 pathway, we expect 4T1-induced MDSC from IL-6$^{-/-}$ mice to have equivalent suppressive ac tivity as MDSC from IDO1 $^{-/-}$ m ice. We would also exp ect MDSC from 4T1/IL-6 inoculated ID O1$^{-/-}$ m ice to be equivalently suppressive to 4T1 IDO$^{+/+}$ MDSC. Especially , 4T1 IDO $^{+/+}$ MDSC are expected to be m ore suppressive

than MDSC from 4T1- inoculated $IDO^{+/+}$ $IL\text{-}6^{-/-}$ mice.

IDO-induced tryptophan starvation also activates the nuclear factor –IL-6 (NF-IL-6) pathway, up-regulating the NF-IL-6 isoform called liver-enriched transcriptional inhibitory protein (LIP). LIP increases IL-6 production (17, 21). Therefore, western blots will be performed on lysates of MDSC induced by 4T1 and 4T1/IL-6 tumor cells in $IDO1^{+/+}$, $IDO^{-/-}$ and $IDO1^{+/+}$ $IL\text{-}6^{-/-}$ mice using commercial anti-LIP antibodies to determine whether LIP has a direct effect on MDSC suppression. We would expect LIP levels to be the highest in MDSC from $IDO1^{+/+}$ mice with 4T1/IL-6 tumors; and MDSC from $IDO1^{-/-}$ and $IL\text{-}6^{-/-}$ mice with 4T1 tumors to have approximately equal levels of LIP .. If western analyses for LIP protein are not definitive, we will perform RT-PCR as described (22) with LIP primers.

Key Research Accomplishments

- IDO function has been statistically proved to be involved in MDSC suppression of T cell proliferation: $IDO1^{-/-}$ MDSC are less suppressive than $IDO1^{+/+}$ MDSC
- Statistically, addition of IDO inhibitor (1-D-MT or 1-L-MT) restores $CD4^+$ and $CD8^+$ T cell proliferation.
- Inflammation increases MDSC activity by increasing IDO production: IDO1 deficiency reduced the increased suppression induced by inflammation caused by IL-6 induction.
- IDO is involved in the cross-talk between MDSC and macrophages: IDO1 deficiency up-regulates macrophage production of IL-12 in the presence of MDSC.
- IDO1 is not present in MDSC suggesting that IDO1 has an indirect effect on MDSC suppression.
- IL-6 was proposed to be a crucial mediator to link IDO and MDSC: IL-6 overcomes IDO deficiency and restores MDSC suppression.

Reportable Outcome

The reported data will be presented as a poster at the following meeting:

- Keystone Symposia Meeting on Molecular and Cellular Biology of Immune Escape in Cancer in February 2010 in Keystone, CO.

Conclusions

The purpose of this project is to determine the tumor tolerance mechanism by which indoleamine 2,3 dioxygenase or inflammatory factors such IL-6, promote MDSC-mediated immune suppression of T cell activation in breast cancer. Immune suppression caused by MDSC is a significant barrier to efficient immunotherapy for

patients with metastatic breast cancer, and successful immunotherapy will require elimination or reduction of MDSC activity. Thus far, we have identified that IDO is one of the key factors to induce MDSC suppression and that IL-6 is a candidate intermediary for IDO effects on MDSC. The next phase of this research project will focus on the mechanism by which IDO-IL-6 enhances MDSC suppressive activity.

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