Induction of arginase-1 in MDSC requires exposure to CD3/CD28 activated T cells

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by

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ABSTRACT

Tumor-induced myeloid derived suppressive cells (MDSC) have been reported to inhibit T cell responses. In our study MDSC isolated from tumor bearing mice showed potent inhibition of T-cell proliferation. However, surprisingly we observed that freshly isolated MDSC from the bone marrow of tumor bearing mice do not constitutively express arginase-1 until after exposure to T-cell proliferation. The aim of this study is to determine the mechanism by which arginase-1 is induced in MDSC following exposure to a proliferative T cell environment. We showed that treatment of MDSC with culture supernatant isolated from T cells activated with CD3/CD28 antibodies successfully induced arginase-1 expression and this process is independent of IL-10 and IFNγ. This suggested that arginase-1 induction in MDSC can occur independently of cell-cell contact. Interestingly IL-2, ConA or PMA activated T-cell supernatant as well as supernatant from multiple cancer cell lines failed to induce arginase-1 in MDSC. We also showed that M-MDSC
expressed higher levels of arginase-1 than G-MDSC after co-culture with CD3/CD28 activated T cells as well as its supernatant. In addition, other bone marrow cells have shown the potential to express arginase-1 following exposure to the same conditions. For example, we observed that healthy Ly6C+ monocytes but not mature granulocytes successfully expressed arginase-1. These data demonstrated that T cells activated through stimulation of TCR but not other means of activation induced arginase-1 enzyme expression.

INDEX WORDS: Myeloid derived suppressor cells, CD3, T cells, Arginase-1.
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by

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DEDICATION

This work is dedicated to my family. Mom, Dad, Eng. Mohammed, Drs. Islam and Eman who have always been my strengths. I appreciate all of your patience and encouragement.
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LIST OF ABBREVIATIONS

BM-Bone Marrow
MDSC-Myeloid-derived suppressor cells
IMC-Immature myeloid cells
IL-Interleukin
VEGF-Vascular endothelial growth factor
МΦ-Macrophage
IFN-γ-Interferon gamma
ROS-Reactive oxygen species
RNS-Reactive nitrogen species
TCR-T cell receptor
MHC-Major histocompatibility complex
NF-κB-Nuclear factor kappa B
HSC-Hematopoietic stem cell
JAK-Janus kinase
STAT-Signal transducer and activator of transcription
Treg-Regulatory T cell
GM-CSF-Granulocyte/Macrophage colony-stimulating factor
G-CSF-Granulocyte colony-stimulating factor
CMP-Common myeloid progenitor
iNOS-Nitric oxide synthase
Arg-Arginase
CAT-2B Cationic amino acid transporter 2B
G-MDSC-Granulocytic MDSC
M-MDSC-Monocytic MDSC
NO-Nitric oxide
ELISA-Enzyme-linked immunosorbent assay
HRP-Horseradish peroxidase
RBC-Red blood cell
FACS-Fluorescence activate cell sorting
CFSE-Carboxyfluorescein succinimidyl ester
CD-Cluster of differentiation
ConA- Concanavalin A
iNOS- Inducible nitric oxide synthase
COX2- Cyclooxygenase-2
SCF- Stem cell factor
CXCR- chemokine receptor
PD/PDL- Programmed death receptor/ligand
CTLA- Cytotoxic T-lymphocyte antigen
HVEM- herpes virus entry mediator
PKC –Protein kinase C
1 INTRODUCTION

1.1.1 Myeloid Derived Suppressor cells
Myeloid derived suppressor cells are a heterogeneous population of immature myeloid cells that have been associated with a potent suppression of the immune system including both innate and adaptive immunity[1, 2].

1.1.2 History of MDSC
Myeloid derived suppressor cells (MDSC) were first observed associated with tumor evasion in the early 1900s[3]. This observation was explained as an extreme deprivation of neutrophils (neutropenia) and an extramedullary hematopoiesis, which was also associated with abnormal myeloid cells differentiation [3]. Null cells, Veto or natural suppressor cells were used to describe this population of cells as they do not have any distinct cell surface markers that distinguish them from other cells as T cells, B cells, NK cells or macrophages [3]. A few years later, these cells were reported to inhibit T-cell proliferation and activity [4]. In 2007, the name “Myeloid derived suppressor cells” was formally used to describe this population of suppressor cells[5]. Recently, many reports describe the biology and the significance of this population of cells in different disease conditions[6].

1.1.3 MDSC origin
Different factors including granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), stem-cell factor (SCF) control myelopoiesis. During normal myelopoiesis, Hematopoietic stem cells (HSCs) form common myeloid progenitor
(CMP) cells which differentiate into immature myeloid cells (IMCs)\[2\]. Lastly IMCs differentiate into macrophages, granulocytes and dendritic cells. Under different disease conditions such as cancer, trauma and inflammation, immature myeloid cells (IMCs) lose their ability to form mature cells and instead give rise to myeloid derived suppressor cells (MDSC)\[2\] (Fig. 1-1).

**1.1.4 MDSC phenotypic markers**

One of the most challenging issues in the field of MDSC, is the lack of specific surface marker to distinguish suppressive granulocytes from non-suppressive granulocytes [7]. In mice, CD11b and Gr-1 protein, which include two isoforms (LY6C and LY6G) still the most useful markers to describe MDSC. However, these markers are not MDSC specific [7]. MDSC involve two populations of myeloid leukocytes: Granulocytic MDSC (G-MDSC), which can be described as CD11b\(^+\), Ly6\(^{\text{low}}\), Ly6G\(^+\) cells, and monocytic MDSC (M-MDSC), which can be described as CD11b\(^+\), Ly6\(^{\text{high}}\), Ly6G\(^{-}\) [8]. Both M-MDSC and G-MDSC have been reported to inhibit T cell responses including T-cell proliferation and functions [2]. In addition to the difference in phenotype, M-MDSC and G-MDSC are also different in the mechanisms of their immunosuppressive function[9].

**1.1.5 Characteristics of M-MDSC**

There are 2 subpopulations of MDSCs, the monocytic MDSC, and the granulocytic MDSC. As previously mentioned M-MDSC and G-MDSC have different surface markers as well as different immunosuppressive mechanisms. Monocytic MDSC immunosuppressive function is believed to be generated primarily by the upregulation of both arginase-1 (Arg-1) enzyme and inducible nitric oxide synthase (iNOS) as well as by suppressive cytokines[10]. Arginase-1 enzyme acts by depleting L-arginine from the environment by degrading it into L-ornithine and urea. Inducible
nitric oxide synthase (iNOS) breaks down L-arginine into nitric oxide (NO) which induces T cell apoptosis[10].

1.1.6 Characteristics of G-MDSC
Granulocytic-MDSC, which is characterized by high expression of the neutrophilic marker Ly6G+, shares some common characters with the mature neutrophils including the formation of intracellular granules and the expression of NADPH oxidase, the enzyme responsible for the production of reactive oxygen species (ROS)[2]. ROS works in combination with NO to form peroxynitrite radicals, that inhibit cytotoxic T cell responses through nitration of the T-cell receptor making T-cells unresponsive to antigen stimulation[10, 11]. In addition, it has been reported that G-MDSC produces TGF-β, which enhances tumor invasion, metastasis and suppresses the immune response[10, 12].

1.1.7 MDSC in different disease conditions
As previously discussed, MDSCs were first discovered in patients with cancer or in tumor-bearing mice[2]. It also has been reported that there is a marked expansion of myeloid derived suppressor cells in patients with different cancers [2, 13]. However, MDSCs are also found in other diseases such as trauma, acute and chronic inflammation, transplantation and sepsis[2]. Expansion of MDSCs is also associated with different autoimmune diseases such as inflammatory bowel disease (IBD), which suggests that MDSCs are observed under different pathological conditions[2, 14].
Under healthy conditions, hematopoietic stem cells (HS) give rise to common myeloid progenitor cells (CMP), then into immature myeloid cells (IMC) that finally differentiate into mature granulocytes, macrophages or dendritic cells. In the case of tumor, trauma or infections, IMC are forced by the diseases environment to stay in the immature state and become myeloid derived suppressor cells (MDSC).

**Figure 1-1MDSC origin.**
1.1.8 MDSC expansion and activation mechanisms

Many studies have shown that many factors are able to regulate MDSCs expansion or activation. An interesting review by Dmitry I. Gabrilovich et al. showed that these factors can be classified into two main groups: 1) those that control MDSCs expansion, and 2) those that control MDSCs activation. The first group are produced mainly by tumor cells, and work by preventing the formation of mature myeloid cells. The second are produced by tumor stromal and activated T cells [2].

1.1.8.1 Factors that control MDSC expansion

Factors reported to control MDSC expansion include vascular endothelial growth factor (VEGF)[2, 15], cyclooxygenase-2 (COX2)[2, 16], granulocyte/macrophage colony-stimulating factor GM-CSF[2, 17] and IL-6 [2, 18], stem-cell factor (SCF)[19] and prostaglandins[2]. Interestingly, most of these factors activate the JAK-STAT3 signaling pathway. Stat-3 phosphorylation is also upregulated in MDSCs from tumor bearing mice [2, 20]. It has been reported that selective STAT3 inhibitors successfully enhance T-cell responses by reducing MDSCs expansion[2, 20]. A recent study from our laboratory showed that, CXCR2-expressing granulocytic MDSC (G-MDSC) but not monocytic MDSC (M-MDSC) isolated from tumor bearing mice expand during tumor progression. This suggests that G-MDSC are the most important immunosuppressive cells regulated during tumor growth.
1.1.8.2 Factors that control MDSC activation

In addition to the factors that promote MDSCs expansion, MDSCs require factors to induce their activation. These factors include IL-4, IL-13, Transforming growth factor-β (TGFβ) and IFNγ[2]. Treatment of freshly isolated MDSCs from the spleen of tumor bearing mice or cloned MDSCs cell lines with IL-4 upregulate arginase-1 expression[21].

It has been demonstrated that IL-13 and IL-4 treatment upregulate arginase-1 expression in bone marrow derived macrophages (BMDM)[22]. Importantly, IL-13 and IL-4 binds to (IL-4Rα) and this pathway involves STAT-6 activation[2]. However, in a different study using a breast tumor model, IL-4Rα deficient mice contain MDSCs after surgery[23].

Another study has attributed the upregulation of Arg-1 and iNOS in MDSCs isolated from spleen of tumor bearing mice to STAT-1 signaling pathway which is mediated by IFNγ[24].

1.1.9 Mechanisms of MDSC suppressive activity.

MDSCs use different mechanisms to suppress both adaptive and innate immunity components including depletion of L-arginine, ROS and RNS production or induction of Treg. Many studies have shown that MDSCs suppressive function require direct contact with T cells[2].

1.1.9.1 Depletion of L-arginine

L-arginine plays an important role in T-cell proliferation, activation, metabolism and functions [25, 26]. L-arginine can act as a substrate for many enzymes including arginase- I, arginase- II, and nitric oxide synthase [25].

It is now well established that arginase-1 activity promotes tumor growth and escape from immune-surveillance through depletion of L-arginine [27]. Arginase-1 activity has been detected
in the tumor microenvironment of skin, breast and prostate cancer and is considered as a marker of tumor progression in patients with different tumor models[27].

In a study using 3LL murine lung carcinoma model, it has been reported that mature myeloid cells isolated from the tumor microenvironment; successfully inhibit T-cell proliferation and expression of signaling molecules in an arginase-1 dependent manner[28]. However, arginase-1 expression and suppressive activity is not restricted to mature myeloid cells.

Using arginase-1 and iNOS, MDSCs break down L-arginine into L-ornithine and urea [2, 25].Arginase-1 expression in MDSC is associated with inhibition of T-cell responses including down regulation of CD3ζ expression [2, 29]while NO inhibits T cell responses through the induction of T-cell apoptosis or inhibition of MHC class II expression [2, 30, 31]. Interestingly, many reports showed that inhibition of arginase-1 was able to rescue T-cell proliferation [32-34][33]and is also to inhibit tumor growth which suggests that maintaining L-arginine levels in T-cell environment is very important in preventing the immunosuppression by MDSCs[32].

Importantly, Murine MDSCs deplete the extracellular L-arginine levels by increasing the uptake of L-arginine through the cationic amino acid transporter 2B (CAT-2B) while human MDSCs do not uptake L-arginine but release arginase-1 enzyme within granules into the microenvironment. This induces L-arginine depletion which inhibits T-cell proliferation [28, 35, 36].

1.1.9.2 ROS

In the tumor microenvironment, reactive oxygen species (ROS) are produced by MDSCs, neutrophils, eosinophils, regulatory T-cells, macrophages and tumor cells. ROS are associated with the inhibition of T-cell responses and the enhancement of tumor growth [37, 38]. One of the main characteristics of MDSCs in cancer patients and tumor-bearing mice is elevated ROS[2,
Inhibition of ROS production by MDSCs in these cancer patients and tumor-bearing mice completely rescues T-cell functions[2, 39, 40]. It has been shown that, integrins on the MDSCs surface play a role in ROS production since blocking integrins (CD11b, CD18, and CD29) by specific antibodies completely abolishes the suppressive function of MDSCs [2, 40].

ROS inhibit T-cell responses through different mechanisms such as blocking the interaction between T-cell receptor (TCR) and the major histocompatibility complex (MHC)[2, 11, 41], inhibiting the differentiation of MDSCs into macrophages so MDSCs will stay in the immature state [2, 42] or depleting cystine and cysteine which causes inhibition of T-cell activation and function [37, 43]. In addition, it has been reported that MDSCs can induce T-cell apoptosis via ROS dependent mechanisms [37, 44, 45]. However, low levels of ROS can induce T-cell activation and proliferation [37, 46, 47].

1.1.9.3 Reactive nitrogen species (RNS)

RNS are considered as an important immune-suppressive instrument used by MDSCs to suppress T cell responses. For example peroxynitrite (ONOO⁻) induces cysteine, tryptophan, methionine and tyrosine nitration and nitrosylation [2, 48]. It has been shown that peroxynitrite makes T-cell unresponsive to different stimuli through the nitration of TCR and CD8 molecules [11]. Another study showed that peroxynitrite induces T-cell apoptosis through impairment of tyrosine phosphorylation [49].
1.1.9.4 Other mechanisms

In addition to previous mechanisms by which MDSCs inhibit T-cell responses, several studies demonstrate that MDSCs can also induce regulatory T-cell (Treg) development which is shown to be independent of NO but requires the presence of TGF-β and IL-10 [2, 50]. Furthermore, arginase-1 is reported to enhance Treg expansion in lymphoma bearing mice [51].
Figure 1-2: MDSC mechanisms of suppression.

- Nitration of TCR and CD8 molecules.
- Induces T-cell apoptosis.

- Block the binding between TCR and MHC complex.
- Prevent the differentiation of MDSC into macrophages.
- Depletion of cystine and cysteine.
- Induces T-cell apoptosis.

- Down regulation of CD3ζ expression.
- Cell cycle arrest.
1.2 Overview on T cells

1.2.1 T cell development and maturation

T cell development takes place in the thymus where the bone marrow hematopoietic stem cells (HSCs) generate lymphoid progenitor cells which give rise to thymocytes[52]. Two characteristics are required for the bone marrow cells to generate T cell lineage progenitors. First, the ability to move to the thymus[52, 53]. Second, they must have a potential to generate T cells in the thymus[52, 54]. Based on the expression of either CD4 or CD8 surface marker, T cells undergo different selection steps in the thymus[55]. The earliest thymocytes are double negative which mean that no expression of either CD4 or CD8 surface markers (CD4− CD8−)[55]. These double negative cells can be further classified based on the expression of CD117, CD44 or CD25[55]. Then they become double positive (CD4+CD8+) through their development and end as single positive thymocytes (CD4+CD8− or CD4−CD8+) which leave to the peripheral organs [55].

T cell fate is determined by the strength of the interaction between the major histocompatibility complex (MHC) and the T cell receptor[56]. Double positive thymocytes that express T cell receptor and have intermediate interaction with the major histocompatibility complex of the cortical epithelial cells, undergo positive selection process to form a mature single positive naïve T cell. If the T cell receptor of the double positive thymocytes failed to interact with the major histocompatibility complex, the thymocytes will die by neglect. The thymocytes will be deleted by negative selection if the interaction is too strong. Each day, about 50 million double positive thymocytes (CD4+CD8+) are generated in the mouse thymus[57]. More than 90 % of these cells have T cell receptor that lack positive selection ability thus these cells undergo death by neglect as they failed to interact with the major histocompatibility complex[56].
1.2.2 T cell activation

T cell activation requires two signals. The first signal is driven after the interaction between the T cell receptor (TCR) and the major histocompatibility complex (MHC) on the surface of antigen presenting cell. The second signal is a costimulatory signal between co-stimulatory molecules on the surface of T cells and costimulatory receptor on the surface of the antigen presenting cell [58]. For example, the costimulatory molecule CD28 binds to CD80 or CD86 receptors on the surface of the antigen presenting cell [58]. In addition to the costimulatory molecules, there are co-inhibitory molecules as CTLA-4 and PD-1 which lead to T cell exhaustion after the binding with CD80, CD86 or PDL-1 receptors [58].

The TCR consists of α and β-chain that is linked with CD3 subunits [59]. The binding between TCR and (MHC) complex lead to initiation of downstream signaling pathways that cause T cell proliferation, differentiation and cytokines secretion [58, 59]. Although the first signal through TCR is enough for T cell activation, the costimulatory signals are very important to sustain the proliferation of naïve T cells through the activation of NF-κB signaling pathway [59, 60]. The CD28 role in enhancing T cell proliferation was confirmed using CD28 deficient T cells [61]. In addition to the antigen (Ag)-dependent activation of T cells through the binding of TCR to the MHC complex, T cells activation can occur independently of the binding between TCR and MHC complex in an Ag-independent way. Different substances as anti-CD3 monoclonal antibody, PMA plus ionomycin or concanavalin A (ConA) are known to activate T cells [62]. Anti-CD3 monoclonal antibody activates T cells by stimulating the signaling downstream of TCR, while PMA plus Ionomycin induce protein kinase C (PKC) activation [62]. Concanavalin A (ConA) binds to the TCR and other cell surface glycoproteins [62, 63] and its role in T cell activation is shown to be
regulated by herpes virus entry mediator (HVEM)[62], which promotes T cell activation, proliferation and cytokine production by co-operating with CD3/TCR signaling[64].
2 MATERIALS and METHODS

2.1 Mouse tumor models

C57BL/6J mice (6-8 week, 20-22 g, Jackson Laboratory) were subcutaneously (s.c.) engrafted (allograft) with B16 melanoma with \(2 \times 10^5\) B16 (B16F10) cells injected in 300 µl sterile PBS. B16 cells (ATCC) were cultured in DMEM supplemented with 10% FBS. After engraftment, tumor developed in 2-3 weeks.

2.2 Isolation of bone marrow

The femur and tibia of euthanized mice were removed from each mouse and the surrounding tissue was removed. The bone ends were removed from the diaphysis with scissors surgically, so the bone marrow rich medullary cavity was exposed. A syringe was filled with 4mls ice-cold PBS. The PBS washed out all the bone marrow into a 15ml BD Falcon conical collection tube as it pass through the medullary cavity. The cell suspension was centrifuged at 1000RPM, 4°C for 10 minutes. The pellets were resuspended in sodium bicarbonate, ammonium chloride, EDTA lysis buffer to lyse any red blood cells from the bone marrow. After lysis of the RBCs, the cells were washed in PBS and centrifuged again.

2.3 Separation of leukocytes from bone marrow

Bone marrow leukocytes were collected by flushing the femur and tibia of previously euthanized mice using iced-cold PBS followed by lysis of RBCs using RBCs lysis buffer. Enrichment and separation of the myeloid cells from the collected leukocytes were performed using percoll dentistry gradient as previously described. In brief, Percoll gradient was prepared using Percoll (Sigma) by mixing of 9 mls of Percoll with 1 ml of 10 × HBSS followed by filtration and dilution by 1 × HBSS to prepare different Percoll solutions with different densities: 70%
(1.097 g/mL), 60% (1.083 g/mL), 50% (1.071 g/mL), 40% (1.058 g/mL). Five-layer discontinuous density gradients were prepared by adding one layer (2 ml/layer) upon one another starting with the layer with the highest density at the bottom in a 15 ml tube using a syringe fitted with a 22G needle. About $7 \times 10^7$ bone marrow leukocytes were suspended in 1 mL HBSS and then placed on top of the gradients using a syringe with 22 G needle, followed by centrifugation at 600 g x g for 40 min in a swinging bucket rotor. After the centrifugation, the cells were separated and formed bands at the interface between each 2 layers. Cells in band III were collected, washed and analyzed by FACS. PE-conjugated anti-Ly6G antibody and PE-conjugated magnetic microbeads were used to isolate granulocytes (LY6G$^+$) by positive selection, while the rest without binding to the beads contained about 90 % of Ly6C$^{\text{high}}$ monocytic leukocytes.

2.4 Isolation of splenocytes and T cells

To isolate cells from tumor tissues or the spleen, tumor tissues or spleen were grounded and were digested with collagenase D (400 U/ml) at 37°C (45 min) in HBSS with agitation following by filtration through nylon mesh with the pore size of 70 µm diameter and collection in ice-cold RPMI 1640. Spleen tissues were grinded in HBSS. The pellets were resuspended in red blood cell lysis buffer to lyse any red blood cells from the spleen. After lysis of the RBCs, the cells were washed in HBSS and centrifuged again followed by collection in ice-cold RPMI 1640 with 10% FBS. To purify CD4+ and CD8+ T cells, PE-conjugated anti-CD4+ antibody and PE-conjugated anti-CD8+ antibody, PE-conjugated magnetic microbeads (Miltenyi Biotec.) were used for positive selection and the finally purified CD4+ and CD8+ T-cells were confirmed to have a purity of > 95% by using of fluorescence microscopy.
2.5 T-cell proliferation assay

Carboxyfluorescein diacetate succinimidyl ester (CFSE) was used to label splenocytes \((6 \times 10^5)\) following their isolation from mice or purified T cells \((6 \times 10^5)\) after further purification as mentioned above, followed by washing. Labeled splenocytes \((6 \times 10^5)\) or purified T cells were seeded in 96-well pre-coated with anti-CD3 \((1 \mu\text{g/ml, clone 145-2C11, Biolegend})\). RPMI1640 with 10% FBS and soluble anti-CD28 \((0.5 \mu\text{g/ml, clone 37.51, Biolegend})\) was added followed by the incubation of cells at 37°C, 5% CO2 for 4 days. For the co-culture experiment, MDSC isolated from the bone marrow were added to splenocytes at the ratio of 1:3 or to purified T cells at the ratio of 1:1. After 4 days, Cells were collected and labelled with fluorescent anti-CD8 and CD4 antibodies (Biolegend) and analyzed by FACS to evaluate T-cell proliferation.

![Diagram](image.png)

**Figure 2 1T cell proliferation assay.**
Splenocytes labelled with CFSE, activated by CD3 and CD28 antibodies, after each cell division, the intracellular CFSE will become more diluted.
2.6 Western blot (WB) analysis of arginase

Freshly isolated bone marrow derived M-MDSC or G-MDSC that were separated by Percoll, BM Ly6G$^+$ granulocytes and Ly6C$^+$ monocytes after co-culture with splenocytes or treated with CD3/CD28 activated T cell supernatant were lysed in a buffer containing 100 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, protease inhibitors (Sigma, Product Code: P8340) and 3 mM PMSF following by centrifugation at (10,000 rpm, 10 min), cell lysates were separated by 10% SDS-PAGE after adding of 5x loading buffer , followed by transferring onto nitrocellulose for 2 hrs , constant current at 0.3 A then blocking with 5% non-fat milk. The membrane was incubated with anti-arginase 1 (mAb H-52, Santa Cruz Biotechnology), secondary antibody and ECL. B-actin in cell lysates was detected by using anti- β-actin (Santa Cruz Biotechnology).

2.7 Flow cytometry analysis

FACS analysis was performed to the leukocytes following their separation by percoll. After lysis of red blood cells, leukocytes were resuspended in PBS following by labelling with fluorescence-conjugated antibodies (Ly6G (1A8), Ly6C (HK1.4), Gr-1 (RB6-8C5) and CD11b (M1/70) (all from Biolegend or R&D systems) and analyzed using BD LSR Fortessa (BD Biosciences). Data analysis was performed using FlowJo software.

2.8 Measurement of the cytokines

Splenocytes isolated from the spleen of healthy mice were seeded to 96 well plate at the density of (6 × 10^5) per well that had been coated or not with anti-CD3e antibodies (BioLegend).Cells were resuspended in complete RPMI (10% FBS, 0.1% β-mercaptoethanol and CD28, 0.5 µg/ml,
clone 37.51, Biolegend). For non-activated T cells, cells were cultured in complete RPMI (10 % FBS) without CD3 or CD28 antibodies. In case of Concanavalin A (ConA), PMA+Ionomycin and IL2 activated T cells, T cells were cultured in RPMI (10 % FBS) + (ConA) at the final concentration of (0.5 μg/ml), PMA (30ng/ml)/Ionomycin (80ng/ml) or IL-2 (1000 IU/ml) . Cell supernatant was collected after 1 or 4 days. Cytokines concentration was measured using standard sandwich ELISA technique. In brief, antibodies against IL-6, IL-10, IFNγ and GM-CSF were used to coat a 96-well flat-bottom ELISA plate. Following washing, both activated and non-activated T cells media were incubated in the wells, each sample in duplicate. Following by the incubation of Biotin labelled antibodies and HRP-conjugated streptavidin antibodies. Wells were washed with 0.005% Tween-20 PBS following the incubation of each antibody. Color change was detected at wavelength 450nm following the addition of HRP substrate, o-phenylenediamine dihydrochloride (OPD, Sigma) to each well. All of the antibodies were purchased from BD Biosciences and BioLegend. Data analysis was performed using SoftMax Pro microplate data software.
3 RESULTS

Although aginase-1 is believed to be an important enzyme used by myeloid derived suppressor cells to inhibit T cell responses, the mechanisms that control aginase-1 expression has been subjected to major debate and research. One study using 3LL tumor model cells showed that aginase-1 expression in tumor infiltrating MDSC is independent of T cell cytokines but requires tumor released factors[65]. A second study using C26 colon carcinoma tumor model attributed aginase-1 expression in blood MDSC to IFNγ signaling [66] while a third study proved that IFNγ does not control MDSC suppressive function and targeting of IFNγ as a potential therapy will not limit MDSC number[67]. The variability of these results suggests that, MDSC formed under different tumor conditions or isolated from different tissues, might be controlled differently. Understanding this mechanism will help to rescue of T cell responses as well as to understand the interplay between MDSC and T cells. In this study we hypothesized that aginase-1 expression in bone marrow derived MDSC requires the exposure to T cells that activated through ligation of CD3 and is independent of tumor released factors.

3.1 Bone marrow-derived MDSC from tumor bearing mice suppressed T cell proliferation

We had previously shown that bone marrow-derived Ly6C+ monocytes, both in bone marrow and the peripheral blood of C57BL6 mice, are natural M-MDSC, and display potent inhibition in CD3 and CD28 ligation-induced T cell proliferation. In addition, tumor conditions induced an extensive expansion in bone marrow of low-density granulocytes (Ly6G+), which displayed characteristics of G-MDSC and as well potently inhibited T-cell proliferation. Different from M-MDSC, which are present constitutively, G-MDSC appear only under pathogenic conditions such as tumor. Using Percoll density gradients, M-MDSC and G-MDSC (both in band III) can be separated from mature
granulocytes, especially mature neutrophils (PMN), which displayed high cell density (band IV) and non-myeloid cells in bone marrow (band I-II) (Fig. 3.1A).

Because mice MDSCs express both CD11b and Gr-1 protein, flow cytometry analysis using both CD11b and Gr-1 antibodies was used to identify cell types from bone marrow of both B16 bearing mice and control mice (healthy) following the separation by percoll. Flow cytometry analysis results showed that Both band III and IV from B16 bearing mice contain CD11b$^+$Gr-1$^+$ leukocytes and then further gating using Ly6G and Ly6C antibodies in fraction III show that band III contains immature myeloid cells that have the same phenotype of M-MDSC or G-MDSC while fraction IV contains about 98% mature granulocytes (Fig. 3.1A).

Tumor-induced myeloid derived suppressive cells have been strongly correlated with the inhibition of T cell proliferation in different tumor models[2, 68]. To confirm that fraction III formed in mice engrafted with B16 melanoma (s. c.) are immunosuppressive, fraction III separated cells were co-cultured with total mice splenocytes from healthy C57BL6 mice, labelled with CFSE and activated by incubating with CD3 and CD28 antibodies. As expected, CD4 and CD8 T-cell proliferation was potently inhibited while fraction IV displayed no inhibition (Fig.3.1B). In addition, the co-culture of either purified Ly6G$^+$ granulocytic cells or Ly6C$^{high}$ monocytic cells from fraction III with CD3/CD28 activated T cells showed inhibition of T-cell proliferation. Same fraction III from tumor bearing mice, inhibited Con.A, PMA or IL-2 induced T-cell proliferation (Fig.3.1D, E). These results were also repeated using different tumor models as B16 melanoma, MC38 colon carcinoma and EL4 lymphoma (Fig.3.1C). These results suggest that these cells resemble MDSC phenotypically and functionally.
Figure 3-1 Bone marrow-derived MDSC from tumor bearing mice suppress T cell proliferation.

A) BM collected from tumor-bearing mice were added to percoll density gradients in 15 ml tube. After centrifugation the cells separated into four fractions (I, II, III and IV) and MDSC present in fraction III followed by FACS analyses for cells in fraction III and IV using LY6C<sup>high</sup> and LY6G<sup>+</sup> antibodies. C) Testing of MDSC suppressive activity. Cells from fraction III or IV were co-cultured with splenocytes that activated by anti CD3/CD28 antibodies. After 4 days cells were stained for CD4 and CD8 T cells and cell proliferation was determined by FACS by the evaluation of CFSE signal. MDSC were added to splenocytes at the ratio of 1:3. Ctr is only T cells without any leukocytes. D, E) ConA, IL-2 and PMA activated T cells inhibited by Cells from Fraction III. Cells from Fraction III were co-cultured with T cells in RPMI (10% FBS+ 0.2ug/mL ConA, PMA 20ng/mL / Ionomycin 60ng/mL or IL-2 500 IU/mL followed by FACS analysis of T cell proliferation. Percentage of Ly6C<sup>high</sup> monocytes and Ly6G<sup>+</sup> granulocytes in fractions III and IV were calculated as a percent of total BM cells. Data are presented as mean ± SEM (n=3). ***, p ≤ 0.001.
3.2 Tumor bone marrow MDSC express arginase-1 upon exposure to TCR-activated T cells

Since arginase-1 is believed to be a major feature of MDSC suppressive activity, we tested arginase-1 expression in MDSC isolated by percoll using western blotting analysis. Although MDSC isolated from tumor bearing mice, profoundly inhibited T-cell proliferation, surprisingly, no arginase-1 expression was detected in MDSC following their isolation from the bone marrow. Interestingly, the incubation of MDSC from tumor bearing mice with CD3/CD28 activated T cells induced arginase-1 expression. As shown in figure 3.2A, the incubation of MDSC with CD3/CD28 activated splenocytes but not non-activated splenocytes, induced arginase-1 expression.

Since splenocytes consists of different cell types such as T cells, B cells, macrophages and dendritic cells, we purified T-cells using CD4 and CD8 positive selection that affinity isolated T cells (> 97% purity). Incubation of MDSC with only CD3/CD28 activated T cells induced arginase-1 expression while the incubation with CD3/CD28 activated splenocytes (depleted T cells) failed to induce arginase-1 expression). As shown in Fig.3. 2B, MDSC co-cultured with purified T cells in the presence of CD3/CD28 ligation were induced to express arginase-1. Depletion of T cells from splenocytes in the co-culture, or removal of antibodies for TCR ligation, failed to induce arginase-1 expression in MDSC.

We next tested weather Ly6C\textsuperscript{high} monocytes (M-MDSC) and Ly6G\textsuperscript{+} granulocytes (G-MDSC) have the same potential to express arginase-1. Our results show that, both M-MDSC and G-MDSC isolated from the bone marrow of tumor bearing mice tend to express arginase-1 only after the exposure to CD3/CD28 activated T cells. As shown in Figure 2C, the co-culture of either M-MDSC or G-MDSC with CD3/CD28 activated T cells successfully induced arginase-
1 expression that was not detected following the isolation from tumor-bearing mice. Consistent with that, no arginase-1 expression was detected in the mature granulocytes from tumor bearing mice before or after the exposure to CD3/CD28 activated T cells (Fig. 3. 2C). Arginase-1 expression was detected after (18 – 24 hrs.) but not shorter time (3 or 6 hrs.) (Fig. 3. 2D).
Figure 3-2 Bone marrow-derived MDSC express no arginase but until after exposure to activated T cells.

A) Western blotting to detect arginase-1 expression in MDSC before (-) or after (+) co-culture with activated or non-activated splenocytes after incubating for overnight (18 hrs.). B) Arginase-1 expression in MDSC requires activated T cells. Western blotting of arginase-1 expression before or after co-culture with activated splenocytes (SP), splenocytes without T cells or purified T cells. C) Fraction III Ly6G^+ granulocytes and Ly6C^{high} monocytes, and fraction IV mature PMN isolated from B16 melanoma-bearing mice were tested for arginase expression before (-) and after (+) addition into the CD3/CD28 activated T cells for 18 h. D) Time course for arginase-1 expression by MDSC after co-culture with CD3/CD28 activated T cells.
3.3 Arginase-1 expression by bone marrow derived MDSC is independent of direct contact with T cells.

We next investigated whether the induction of arginase-1 expression in bone marrow derived MDSC that primarily triggered by CD3/CD28 activated T cells requires direct contact with T cells. We found that arginase-1 expression is induced in bone marrow derived MDSC by a mechanism that does not necessarily require contact between MDSC and CD3/CD28 activated T cells. As shown in figure (3.3.A), the culture of MDSC isolated from bone marrow of tumor-bearing mice in culture supernatant collected from CD3/CD28 activated total splenocytes or purified CD4 and CD8 T cells successfully induced arginase-1 expression while the incubation in culture supernatant collected from non-activated T cells, B16 tumor cells failed to induce arginase-1 expression. Furthermore, incubation of MDSC in culture supernatant isolated from either CD3/CD28 activated CD4 or CD8 T cells induced arginase-1 expression (Fig. 3.3.B). In addition, both M-MDSC and G-MDSC expressed arginase-1 only after being treated with CD3/CD28 activated T cells supernatant which is similar to the result that we got from the co-culture system (Fig.3.3C). This result suggested that, bone marrow derived MDSC from tumor bearing mice do not constitutively express arginase-1 but instead arginase-1 expression induced once MDSC was exposed to activated T cell environment.
3.4 Arginase-1 induction in BM derived MDSC by activated T cells requires ligation of TCR co-receptor (CD3).

Since different activators such as Concanavalin A (ConA), PMA plus Ionomycin and IL-2 have the ability to induce T-cell proliferation, we sought to determine whether T-cells activated by ConA, PMA plus Ionomycin or IL-2 induce arginase-1 expression in MDSC. Although ConA, PMA plus Ionomycin or IL-2 successfully activated T cell proliferation, interestingly, only T cells activated via ligation of CD3 induced arginase-1 expression. As shown in figure (3.4A,B), incubation of MDSC with culture supernatant isolated from T cells that had been activated by IL-2, ConA or PMA plus Ionomycin failed to induce arginase-1 expression but only MDSC that incubated with cell culture supernatant isolated from CD3/CD28 activated cells successfully induced arginase-1. This result suggested that T cells activation through ligation of CD3 but not other ways of activation can induce arginase-1 enzyme expression in myeloid derived suppressor cells.
**Figure 3-3** Arginase-1 expression by BM derived MDSC is independent of direct contact with T cells.

A) Arginase-1 expression in MDSC (FIII) isolated from tumor bearing mice after the treatment using 50% CD3/CD28 activated splenocytes, T cells or B16 cells supernatant. B) Induction of arginase-1 expression by either CD3/CD28 CD4 or CD8 activated T cells supernatant. C) Arginase-1 expression detection in fraction III Ly6G<sup>+</sup> granulocytes and Ly6C<sup>high</sup> monocytes. Fraction III Ly6G<sup>+</sup> granulocytes or Ly6C<sup>high</sup> were tested for arginase-1 expression after overnight treatment with supernatant collected from T cells after overnight activation with CD3/CD28 antibodies. B-actin was used as a loading control.
Figure 3-4 Arginase-1 induction in BM derived MDSC by activated T cells requires ligation of TCR co-receptor (CD3).

A) T cell proliferation after incubating of T cells with PMA (30 ng/ml)/Ionomycin (80 ng/ml), ConA (0.5 µg/ml) or IL-2 (1000 IU/ml). B) Arginase-1 expression in F III (MDSC) after treatment with supernatant collected from activated T cells showed in (A).
3.5 Healthy Ly6C<sup>high</sup> monocytes but not Ly6G<sup>+</sup> granulocytes express arginase-1 upon the exposure to CD3/CD28 activated T cells.

We had shown that Ly6C<sup>high</sup> monocytes but not Ly6G<sup>+</sup> granulocytes isolated from the bone marrow of healthy C57BL6 mice, inhibited T cell proliferation in a similar manner to their counterpart isolated from tumor bearing mice (fig. 3-5.A). We next sought to determine whether Ly6C<sup>high</sup> monocytes isolated from bone marrow of healthy mice express arginase-1 after being exposed to CD3/CD28 activated T cell or its supernatant. As shown in figure 3-5B, Ly6C<sup>high</sup> monocytes but not Ly6G<sup>+</sup> granulocytes from healthy mice expressed arginase-1 after co-culture with CD3/CD28 activated T cells. Furthermore, treatment with supernatant isolated from T cells activated by CD3/CD28 antibodies, induced arginase-1 expression while no arginase-1 expression was detected in mature granulocytes before or after treatment with similar supernatant. This suggested that not only MDSC that formed under tumor or disease conditions have the potential to express arginase-1 but also Ly6C<sup>high</sup> monocytes can express arginase-1 after being exposed to CD3/CD28 activated T cells and can be considered as natural MDSCs.
**Figure 3-5 Healthy Ly6C<sup>high</sup> monocytes but not Ly6G<sup>+</sup> granulocytes express arginase-1 only after the exposure to CD3/CD28 activated T cells.**

A) Evaluation of LY6C<sup>high</sup> suppressive activity by inhibiting T cell proliferation. Splenocytes were labelled with CFSE and incubated in plates immobilized with anti-CD3 and in the presence of soluble anti-CD28 antibodies, in presence of myeloid cells isolated from healthy mice or without any myeloid cells (ctl.) for 4 days before FACS analyses. The ratio of splenocytes: myeloid cells was 3:1. B) Arginase-1 detection in LY6C<sup>high</sup> or Ly6G<sup>+</sup> cells from band III isolated by percoll from BM of healthy mice before and after co-culture with CD3/CD28 activated T cells. Data are presented as mean ± SEM (n=3). ***, p ≤ 0.001.
4 DISCUSSION

MDSC are one of the suppressive cell types found in the tumor microenvironment associated with suppression of T cell responses and enhancing tumor progression[69]. Blocking MDSC activity had been shown to have a negative effect on tumor progression[70]. Suppression of T cell responses by MDSC had been shown to be associated with arginase-1 enzyme[71]. However, We found that arginase-1 enzyme is not constitutively expressed in bone marrow derived MDSC isolated from tumor-bearing mice, instead required to be induced .In our study we showed that T cells activated via the ligation of CD3 induced arginase-1 expression in BM derived MDSC. Although many reports had shown that MDSC inhibition of T-cell proliferation require direct contact between MDSC and T cells [21, 72], our results showed that direct contact between MDSC and activated T cells is not required for arginase-1 enzyme induction in bone marrow derived MDSC. Supernatant collected from CD3/CD28 activated T cells, induced arginase-1 expression suggesting the presence of diffusible factors that are released after T cell activation leading to the induction of arginase-1 expression in bone marrow derived MDSC. The culture of bone marrow derived MDSC with resting T cells failed to induce arginase-1 enzyme expression. This result is consistent with previous results that showed that arginase-1 suppressive activity required the presence of activated T cells[72]. We also showed that both monocytic MDSC (M-MDSC) and granulocytic MDSC (G-MDSC) expressed arginase-1 after being treated with CD3/CD28 activated T cell supernatant.

Even though tumor released factors had been shown to induce arginase -1 expression in tumor infiltrating myeloid suppressor cells [65], we did not see any arginase-1 expression in bone marrow derived MDSC after treatment with supernatant collected from B16 cells. But instead, our data demonstrated that soluble factors released from CD3/CD28 activated T cells induced
arginase-1 in bone marrow derived MDSC in vitro. Thus, we hypothesized that bone marrow derived MDSC express no arginase-1, but once they enter the tumor microenvironment and encounter activated T cells, MDSC will express arginase-1 as a result of CD3 activated T cell released factors.

Interestingly, our data showed that arginase-1 expression required CD3/CD28 activated T cells but not other ways of activation. In our study, the co-culture of MDSC with ConA, IL-2 or PMA activated T cells potently inhibited T-cell proliferation. However, we failed to detect arginase-1 expression in MDSC after the co-culture with IL-2, ConA or PMA activated T cells. This result suggested the presence of a different mechanism of suppression rather than arginase-1 enzyme activity which is believed to be an NO-dependent mechanism[72]. This was confirmed by the use of an iNOS inhibitor (l-NMMA) which profoundly rescued T-cell proliferation[72]. Furthermore, neither IL-2, ConA nor PMA activated T cells culture supernatant induced arginase-1 expression in bone marrow derived MDSC suggesting a difference in soluble factors released from activated T cells using different activators.

We and others had shown that LY6C<sup>high</sup> monocytes isolated from healthy mice suppressed T-cell proliferation[73]. Our results demonstrated that in a similar manner to their counterparts from tumor-bearing mice, LY6C<sup>high</sup> monocytes from healthy mice expressed arginase-1 only after the exposure to CD3/CD28 activated T cells. Indeed, this observation might provide a mechanism to treat autoimmune diseases.
REFERENCES


