CHARACTERIZATION OF PORCINE MYELOID DERIVED SUPPRESSOR CELLS

A THESIS

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Abstract

Myeloid derived suppressor cells (MDSCs) are heterogeneous population of immature myeloid cells having immunosuppressive properties in tumor and sepsis conditions. MDSCs are characterized in mice and human disease conditions and also reported in dogs. Recently, MDSCs are gaining increased attention in non-tumor settings including viral, bacterial and parasitic diseases. In pig, MDSCs are not characterized but they might be playing a role in immunosuppressive conditions. In humans, treatment of peripheral blood mononuclear cells (PBMCs) with GM-CSF and IL-6 induces MDSCs phenotype and function. Thus, we made an attempt to characterize porcine MDSCs by treatment of myeloid cells with GM-CSF and IL-6. For this study, CD172+ myeloid cells were magnetically sorted from porcine PBMCs and cultured in presence of the cytokines GM-CSF + IL-6; GM-CSF + IL-4 and medium control. After 7 days, cells were harvested, stained for cell surface markers CD172, SLAI, CD80/86, CD11R3, CD11c and DC-SIGN and analyzed by flow cytometry. Cytokine treated myeloid cells were also co-cultured for additional 4 days with autologous lymphoid cells to define their functional characteristics related to T cell proliferation, induction of CD25+Foxp3+ regulatory T cells (Tregs) and IL-10 production. The expression of the cell surface marker CD11b in mice and human MDSCs is predominant, and CD11R3 is the porcine
homologue for CD11b. In our study, GM-CSF + IL-6 treatment did not induce significantly higher CD11R3 level on porcine myeloid cells, suggesting that CD11R3 expression alone is insufficient to define porcine MDSCs. Moreover, there was no difference in the frequencies of cells expressing the combination of cell surface markers, CD172^+SLAII^+, CD172^+CD80/86^+, CD172^+CD11c^+, CD172^+DC-SIGN^+ in GM-CSF + IL-6 treated sorted CD172^+ cells compared to untreated control cells. When cytokine treated myeloid cells were co-cultured with autologous lymphoid cells for additional 4 days, we did not find significant suppression of lymphoid cells proliferation by GM-CSF + IL-6 treated cells. There was a numerical increase in the frequency of Tregs in co-cultured lymphoid cells, probably induced by GM-CSF + IL-6 treated myeloid cells, compared to untreated control cells. However, the production of IL-10 in the co-cultured supernatant was comparable among the cytokines treated cells.

Porcine reproductive and respiratory syndrome virus (PRRSV) is known for its immunosuppressive role in infected pigs. Therefore, we analyzed whether GM-CSF + IL-6 treatment of myeloid cells of PRRSV infected pigs will induce the generation of MDSCs ex vivo. For this analysis, magnetically sorted CD172^+ myeloid cells of PRRSV infected pigs were treated with GM-CSF + IL-6 for 7 days and analyzed by flow cytometry for the expression of cell surface markers, and also co-cultured with autologous lymphoid cells for additional 4 days. Our results suggested that the frequencies of the cells expressing the myeloid cell markers, CD172^+SLAII^+, CD172^+CD80/86^+, CD172^+CD11c^+, CD172^+DC-SIGN^+ were comparable to similarly cultured PRRSV uninfected pig cells. Functionally also we did not find significant
differences in terms of proliferation of lymphoid cells expansion of Tregs and IL-10 production, mediated by myeloid cells of PRRSV infected pigs cultured with GM-CSF + IL-6 compared to PRRSV uninfected pig cells. In conclusion, unlike human PBMCs, the pig myeloid cells treated with GM-CSF + IL-6 did not induce generation of typical MDSCs, either phenotypically or functionally. However, before we declare the presence or absence of typical MDSCs in pigs, we still need to measure the expression of effector molecules involved in immunosuppression in mice and human MDSCs, such as nitric oxide, reactive oxygen species, arginase 1 and nitric oxide synthase. Our pilot study results have created increased interests to explore porcine MDSCs.
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List of abbreviations

MDSCs.................................. Myeloid derived suppressor cells
M-MDSCs.......................... Monocytic myeloid derived suppressor cells
G-MDSCs............................Granulocytic myeloid derived suppressor cells
PMN-MDSCs...................... Polymorphonuclear myeloid derived suppressor cells
PBMCs............................... Peripheral blood mononuclear cells
GM-CSF............................ Granulocyte-macrophage colony stimulating factor
IL-6......................................Interleukin-6
IL-4......................................Interleukin-4
IL-10.....................................Interleukin-10
TGF-β..................................Tumor growth factor β
IFN-γ..................................Interferon γ
tregs..................................T regulatory cells
PRRS.................................Porcine reproductive and respiratory syndrome
PRRSV..............................Porcine reproductive and respiratory syndrome virus
Arg..................................Arginase
iNOS.................................Inducible nitric oxide synthase
ROS..................................Reactive nitric oxide synthase
APC.................................Antigen presenting cells
BSL 2.......................... Bio safety level 2
MACS....................... Magnetic-activated cell sorting
IMC.............................. Immature myeloid cells
CMP.............................. Common myeloid progenitor
CLP.............................. Common lymphoid progenitor
G-CSF.......................... Granulocyte colony stimulating factor
M-CSF.......................... Macrophage colony stimulating factor
Chapter 1: Literature Review

Myeloid derived suppressor cells (MDSCs)

From early 1900s several researchers reported the existence of an unknown cell population with immune-suppressive feature in mice. Such cells accumulated mainly in bone marrow and spleen of tumor bearing mice; suppressed T cell response and also induced tolerance (1-4). These suppressor cells were described as neither T cells nor macrophages. Various names were assigned to these cells including, ‘veto cells’, ‘natural suppressor cells’, ‘immature myeloid cells’, and ‘myeloid suppressor cells’. Since they lack membrane markers of T cells, B cells, NK cells as well as macrophages, they were also called as null cells. However, in 2007, they were classified into ‘myeloid derived suppressor cells’ (MDSCs) (4-9). Now, MDSCs refer to a heterogeneous population of myeloid cells, comprised of myeloid progenitors and immature macrophages, immature dendritic cells and immature granulocytes (10).

MDSCs are derived from immature myeloid cells (IMC). In bone marrow, hematopoietic stem cells (HSCs) generate multipotent progenitor cells (MPP). HSCs are the cells which are capable of self-renewal (produce other HSCs) as well as multi-potent (produce
multiple lineage of blood cells). MPPs however, are multi-potent but do not have self-renewal property. MPPs produce oligopotent progenitors namely common lymphoid progenitor (CLP) and common myeloid progenitor (CMP) cells. Under normal conditions, CMPs produce immature myeloid cells (IMC) in bone marrow, which are then released to blood circulation and reach peripheral lymph nodes where they mature into dendritic cells, macrophages and granulocytes. However, in certain conditions like trauma, infections and tumors IMCs may fail to mature and remain in immature state with the ability of suppressing host immune response known as MDSCs (10-12).

MDSCs are broadly categorized into two subtypes: monocytic and granulocytic. Monocytic MDSCs have single, large, round nucleus as in monocytes while granulocytic MDSCs bear multi-lobed nucleus as in polymorphonuclear cells (13).

![Fig 1: Schematic diagram showing origin of MDSCs (Adapted from: Gabrilovich and Nagaraj, 2009)](image-url)
MDSCs in mouse

In mice, MDSCs are broadly characterized by co-expression of Gr1 and CD11b surface markers (5, 14). Gr-1 antigen is a myeloid differentiation antigen and expressed on macrophages and granulocytes. It is expressed on mouse bone marrow from early myeloid development to lineage commitment (15). CD11b, also referred as Mac-1, is a member of β2 integrin family of adhesion molecules. It is expressed mainly on monocytes, neutrophils, dendritic cells as well as few lymphocytes (16). In healthy mice, cells positive for Gr-1 and CD11b are present in bone marrow (20-30%); to a lesser extent in spleen (2-4%), but not in lymph nodes. However, in tumor bearing mice, the frequency of these cells can increase dramatically and also bear immunosuppressive potential, which is absent in healthy state (10, 14). Mouse MDSCs are further divided into two categories based on two markers associated with Gr1 viz. Ly6C and Ly6G. Ly6C is a macrophage marker while Ly6G is a neutrophil marker. Therefore, cells with the phenotype $\text{CD11b}^+\text{Ly6C}^{\text{high}}\text{Ly6G}^-$ are monocytic MDSCs (M-MDSCs) and cells with phenotype $\text{CD11b}^+\text{Ly6C}^{\text{low}}\text{Ly6G}^+$ are granulocytic MDSCs (G-MDSCs). M-MDSCs preferentially express iNOS and have increased T cell suppressive activity, whereas G-MDSCs express high levels of Arginase-1 (ARG-1). Since G-MDSCs have polymorphonuclear (PMN) morphology, they are also known as PMN-MDSCs. G-MDSCs represent larger fraction over M-MDSCs in tumor bearing mice (17-19).
MDSCs in human

Human MDSCs are less well characterized compared to murine MDSCs due to lack of uniform markers and homologue of Gr-1 marker in human (20). Human MDSCs are much more diverse. For example, in head and neck cancer patients Lin^-CD33^+CD34^+CD15^+ immune suppressive immature myeloid cells are reported, while in renal cancer patients CD11b^+CD14^+CD15^+HLA-DR^+ cells are found in peripheral blood (21, 22). In case of melanoma, CD14^+CD11b^+HLADR^{low} cells mediate immune suppression via TGF-β production (23). In hepatocellular carcinoma, CD14^+ MDSCs suppress autologous T cells through arginase synthesis and via expansion of IL-10 producing Treg cells (24). In general, human MDSCs express both or either of the common myeloid markers CD11b and CD33 and lack expression of myeloid maturation markers including CD80, CD83, HLA-DR and CD40. Attempts have been made to classify human MDSCs as well into monocytic and granulocytic MDSCs. CD14^+ cells are suggested as monocytic MDSCs, while CD15^+ cells are taken as granulocytic MDSCs (20, 25). Lechner et. al., characterized MDSCs from normal human PBMCs by treating with various cytokines in vitro. GM-CSF + IL-6 treatment of PBMCs generated high quality population of MDSCs followed by GM-CSF + IL-1β, PGE2, TNFα, or VEGF. In vitro generated MDSCs were CD33^+CD11b^+CD66b^+HLADR^{low}IL-13Rα2^{int} large mononuclear cells. These cells suppressed autologous T cells in contact dependent manner and expressed iNOS, TGFβ, NOX2, VEGF and/or ARG-1 (26).
MDSCs in dog

In recent years, attempts have been made to characterize MDSCs in dogs as well. Goulert et. al., investigated the presence of MDSCs in dogs with naturally occurring cancer. They analyzed PBMCs from tumor bearing dogs and compared with age matched control dogs by flow cytometry for different cellular markers. They found significant increase in CD11b+CD14-MHCII- cell population in tumor bearing dogs compared to control dogs. Morphologically these cells sub-population were polymorphonuclear granulocytes. Such cells possess ability to suppress T cell proliferation and IFN-γ production, and they also expressed ARG1, iNOS2, TGF-β and IL-10, which are known immune suppressors, reported in both mice and human MDSCs (27). Sherger et. al., reported increased CD11b^{low}CAD048^{low} cell populations in tumor bearing dogs, which had potential of suppressing proliferation of responder canine lymphocytes, likely to represent canine MDSCs (28). Using microarray, Mucha et. al., examined changes in gene expression in canine mammary cancer cells after co-culture with Gr1/CD11b/CD33 positive cells. They found that co-culture of cancer cells with MDSCs enhances IL-28 signaling pathway that promotes angiogenesis, epithelial-mesenchymal transition, invasion and migration of tumor cells (29). Further studies are needed to pinpoint the specific MDSCs phenotype and function in dogs, so that dogs can be used as a large animal model for clinical trials of drugs targeting MDSCs.
MDSCs in cancer/ tumor microenvironment

Chronic inflammatory mediators accumulated during tumor progression favor the expansion, recruitment and activation of MDSCs. These mediators include cytokines like IL-1β, IL-4, IL-6; growth factors like granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and macrophage colony-stimulating factor (M-CSF); chemokines and other factors (30). Besides tumor environment leading to MDSCs accumulation, it is also shown that accumulated MDSCs may also directly support tumorigenesis (31, 32). MDSCs facilitate tumor immune evasion by limiting infiltration and immune response of T cells at tumor microenvironment (33). Youn et. al., attempted to identify specific subset of MDSCs responsible for immunosuppression in mice using 10 different tumor models. They found increased population of MDSCs in all tumor models which consist G-MDSC (Ly6G⁺Ly6Clow) as well as M-MDSC (Ly6G⁻Ly6Chigh) (34). In various mouse tumor models, 20-40% of the splenocytes have shown MDSCs markers compared to 2-4% in spleen of normal mice. MDSCs are also found in tumorous tissue and lymph nodes of tumor bearing mice (10).

In humans, enhanced MDSCs population is negatively correlated with prognosis and overall survival of cancer patients. However, due to heterogeneous nature of MDSCs and lack of consensus on definitive MDSCs phenotype, using MDSCs as possible immunotherapeutic target is difficult (35). Khaled et. al., have reviewed and summarized patient demographics based on clinical studies of MDSCs in human cancer, which shows
that MDSCs involvement is present in different types of human cancers including but not limited to breast cancer, oesophageal cancer, colon cancer, pancreatic cancer, gastric cancer, and hepatocellular cancer (36). In glioblastoma multiforma, which is one of the most frequent brain tumors, Lin-CD33⁺HLADR⁻ cells were identified in PBMCs of the patients, which had phenotype similar to immunosuppressive monocytes generated in vitro (37). Iclozan et. al., reported MDSCs with phenotype Lin⁻HLADR⁻ in PBMCs of patients with lung cancer (38). Montero et. al., have pointed out the possibility of using MDSCs for prognostic and predictive purpose in breast cancer patients. They have identified MDSCs as Lin⁻HLA-DR⁻CD11b⁺CD33⁺ cells (39, 40). Hoechst et. al., have reported CD14⁺HLADRlow/- M-MDSCs in hepatocellular carcinoma that leads immunosuppression through regulatory T cell mediated mechanism (24).

**MDSCs in bacterial infection**

Fewer studies are available on involvement of MDSCs in bacterial diseases. *Staphylococcus aureus* is the common cause of biofilm infections on orthopedic implants (41). It was observed that, *S. aureus* biofilms alternatively activate macrophages, produce Arg-1 and subvert immune-mediated clearance (42). Heim et. al., found involvement of CD11b⁺Gr1⁺ MDSCs in *S. aureus* biofilm associated immune suppression. MDSCs were the main cellular infiltrate in *S. aureus* mediated orthopedic biofilm formation, which lead to enhanced expression of Arg-1, iNOS, IL-10 and also inhibited T cell proliferation (41). Involvement of MDSCs has also been studied in *Pseudomonas aeruginosa* mediated cystic fibrosis patients. *P. aeruginosa* induces granulocytic MDSCs to escape T cell
immunity in cystic fibrosis patients. Flagellin of \( P. \text{ aeruginosa} \) is responsible for induction of MDSCs population in cystic fibrosis as well as \( P. \text{ aeruginosa} \) associated chronic lung diseases in human (43).

**MDSCs in parasitic infection**

Involvement of MDSCs have been studied in various parasitic diseases including \textit{Trypanosoma cruzi, Leishmania sp., Toxoplasma gondii, Plasmodium sp., Schistosoma sp.}, and \textit{Taenia sp.}, (44). Parasite induced inflammatory cytokines like IL-1 and IFN-\( \gamma \) have been linked with MDSCs expansion (44). CD11b\(^+\)Gr1\(^+\) MDSCs were accumulated in spleen of BALB/c mice infected with \textit{Leishmania major}. MDSCs accumulation was dependent on concentration of IL-1 and suppressed T cell mediated immunity in mice (45). Acute \textit{Trypanosoma cruzi} infection is associated with severe unresponsiveness to mitogens and antigens which facilitates dispersal and persistence of parasite in the host. CD11b\(^+\)Gr1\(^+\) MDSCs are involved in spleen cells unresponsiveness to mitogens in \textit{T. cruzi} infected mice. MDSCs mediated immune suppression in \textit{T. cruzi} infection through production of NO (46). In \textit{Schistosoma mansoni} infected mice, cells with non-B, non-T cell phenotype were involved in suppressing cytotoxic T cell response suggesting involvement of MDSCs (47). \textit{Toxoplasma gondii} infection induces transient hypo responsiveness of leukocytes in blood and spleen of mice and human and also in lungs of mice. Gr1\(^+\)CD11b\(^+\) myeloid cells are responsible for immune hypo responsiveness in lungs of \textit{T. gondii} infected mice (48).
MDSCs in viral infection

The discovery on the list of viruses that use MDSCs as mechanism of immunosuppression is growing. Since many viruses induce tumor formation and chronic infection, role of MDSCs in viral infection is generating more interest (49). The core protein of hepatitis C virus (HCV) is a potent inducer of MDSCs. Tacke et. al., showed that core protein of HCV can induce CD33^+CD11b^+HLADR^{lo/cd}CD14^+ MDSCs in in vitro treatment of healthy human PBMCs. Such MDSCs were able to suppress CD4^+CD8^+ T cell proliferation and IFN-γ production in ROS dependent manner (50). T cell dysfunction is the hallmark of chronic progressive HIV-1 infection. Vollbrecht et. al., observed significantly higher CD11b^+CD14^-CD33^+CD15^+ MDSCs in PBMCs of chronically HIV infected patients as compared to controls. MDSCs population was correlated positively with HIV load and negatively with CD4 count. When magnetically separated and co-cultured, such MDSCs reduced proliferative potential of CD8 T cells (51). In another experiment it was observed that Tat protein of HIV-1 is involved in induction of MDSCs (52). There are reports indicating involvement of CD33^+CD11b^+CD14^-CD15^+ granulocytic MDSCs as well as HLA-DR^{-/low}CD11b^+CD33^{+/high}CD14^+CD15^- monocytic MDSCs in HIV-1 infection (51, 52). Vesicular stomatitis virus was also shown to induce MDSCs in spleen of C57BL/6 mice (53). Likewise, Vaccinia virus induced MDSCs at the site of infection in C57BL/6 mice (54). Similarly involvement of MDSCs is also known in adenovirus infection. Zhu et. al.,
have shown that granulocytic MDSCs population is responsible for suppression of NK cell function in adenovirus infection (55).

**Mechanisms of MDSC mediated immunosuppression**

MDSCs suppress innate as well as adaptive immune responses of host (13). Various mechanisms of MDSCs mediated immunosuppression are investigated, some of which are described below:

1) **Metabolism of L-arginine**

One of the main mechanisms for MDSCs mediated immunosuppression is via depletion of L-arginine. L-arginine is an amino acid generally non-essential but conditionally required in pathological and physiological conditions. Two enzymes: arginase (Arg) and nitric oxide synthase (NOS) compete in metabolism of L-arginine and produce urea/L-ornithine and nitric oxide/L-citrulline, respectively. Two isoforms of arginase (Arg 1 and Arg 2), with 58% sequence homology, are known in mammals while three isoforms of NOS with nearly 50% homology are known namely, NOS1, NOS2 and NOS3. NOS 2 is also referred as inducible isoform (iNOS) (56). MDSCs enhance Arg-1 production and in turn suppress T cell proliferation and function. Arginase 1 metabolizes and depletes extracellular L-arginine. Once level of extracellular L-arginine is depleted, T cell proliferation will be arrested. It also alters post-transcriptional and translational mechanisms for CD3ζ chain, which is the main signal transduction component of T cell
receptor complex and necessary for proper assembly of T cell receptors, and also blocks re-expression of internalized CD3ζ chain on activated T cells (57). MDSCs may also induce production of iNOS which suppresses T cells through inhibiting intracellular IL-2 signaling pathway. iNOS blocks phosphorylation of various signaling proteins involved in IL-2 cascades and also alters stability of IL-2 mRNA (56).

2) Production of reactive oxygen and nitrogen species

MDSCs also mediate immunosuppression through production of reactive oxygen and nitrogen species. Kusmartsev and Gabrilovich, showed the role of reactive oxygen species (ROS) in inhibition of myeloid cell differentiation in cancer. Gr1⁺CD11b⁺ immature myeloid cells had a significantly higher production of ROS than Gr1⁻CD11b⁺ macrophages (58). Follow up study by the same group showed that immature myeloid cells in tumor bearing mice were capable of inhibition of CD8⁺ T cells through ROS production. Inhibition of ROS activity abrogated the immature myeloid cells mediated suppression of CD8⁺ T cell response (59). Up-regulation of NADPH oxidase (NOX2) enzyme was found to be responsible for MDSCs mediated enhanced ROS production in mice tumor models (60).

In human squamous cell carcinomas, MDSCs produce excessive nitric oxide that inhibits vascular E-selectin expression followed by T cell recruitment (61). The role of peroxynitrite is also crucial for MDSCs mediated immunosuppression (10). Peroxynitrite (ONOO⁻) is produced by reaction of nitric oxide (NO) and superoxide (O₂⁻) radicals.
Peroxynitrite anions are strong oxidant in biological system that can readily oxidize amino acids like cysteine and tyrosine to produce corresponding cysteinyl and tyrosyl radicals. Peroxinitrites mediated posttranslational protein modifications in T cell cytoplasm influences various enzyme activity, cell proliferation and survival (62). MDSCs mediated T cell tolerance in cancer was linked to nitration of T cell receptor and CD8 T cells due to hyperproduction of ROS and peroxynitrite (63).

3) **Induction of regulatory T cells**

Regulatory T cells (Treg), also known as suppressor T cells, are the counter mechanism in immune system to avert unwanted immune responses either by direct contact (expression of inhibitory molecules on the surface) or through production of soluble factors (64, 65). MDSCs also mediate immune suppression through induction of Treg population. Serafini et. al., have shown MDSCs mediated induction of Treg cells in B cell lymphoma. MDSCs function as APCs in induction of Treg which in turn responsible for immune tolerance. When MDSCs function was inhibited, Treg proliferation and lymphoma associated T cell tolerance was abrogated (66). In murine colon carcinoma study, Huang et. al., have shown that Gr1+CD115+ MDSCs were able to induce Treg cells. MDSCs mediated Treg induction was dependent on IL-10 and IFN-γ (67).
4) Production of anti-inflammatory cytokines

MDSCs also enhance production of anti-inflammatory cytokines like IL-10 and TGF-β, which lead to immunosuppression. MDSCs interact with macrophages in tumor microenvironment and change tumor immunity towards tumor promoting type 2 phenotype. Such mechanism is induced by increasing the production of IL-10 and decreasing the macrophage production of IL-12 (68). Zhang et. al., also looked upon correlation of MDSCs, IL-10 and IL-12 level in children with asthma. The percentage of MDSCs in PBMCs had positive correlation with serum IL-10 level and negative correlation with IL-12 (69). TGF-β is another potent immunosuppressive cytokine, and it is associated with initiation, progression and metastasis of several tumors (70). Li et. al., have shown involvement of membrane bound TGF-β 1 on MDSCs in inducing NK cell anergy in mouse tumors (71).

PRRS disease overview

Porcine reproductive and respiratory syndrome (PRRS) is caused by PRRS virus (PRRSV), an enveloped positive stranded linear RNA virus, belonging to family Arteriviridae. Two broad categories of PRRSV: the European (type I) and the North American (type II) exist with antigenic diversity (72). PRRSV causes significant economic loss annually to the US pork industry. Economic losses are mainly due to reduced growth, infertility, abortions and high mortality in pre-weaned piglets (73). PRRSV is transmitted through infected pigs and semen. Virus is excreted intermittently
in all body secretions like saliva, nasal secretions, urine, milk, colostrum, feces, semen, etc. Transportation by physical means like farm personnel and airborne transportation is also known. At present, mainly killed vaccines are being used to prevent PRRSV infection in pregnant sows and breeding boars (74-76).

**PRRSV mediated immunosuppression**

PRRSV has strong tropism for macrophages in lungs as well as in other tissues which play important role in host to provide protective immunity (77). PRRSV has developed various strategies to overcome host innate and adaptive immune responses (78, 79). Innate immune response provides protection against viral infections, and type I interferon (mainly IFN-α and IFN-β) are the major players in innate immunity (80). It is already shown in *in vitro* and *in vivo* experiments that PRRSV is sensitive to type I IFNs. Buddaert et. al., pre-treated PAM cells with porcine IFN-α and infected with PRRSV and found significant reduction in PRRSV yield (81). Similarly, when MARC 145 cells and pulmonary alveolar macrophages (PAM) were treated with IFN-β by Overend et. al., inhibition of PRRSV replication was observed (82). In an *in vivo* experiment, Brockmeier et. al., inoculated pigs with recombinant adenovirus for IFN-α expression and after one day infected with PRRSV. They observed reduction in fever and lung lesions, and the viremia was delayed in IFN-α induced pigs compared to control infected pigs (83). Since PRRSV is sensitive to type I IFN, it has developed mechanisms to inhibit production of type I IFN in host. In PRRSV infected pigs, IFN-α is not detectable or very low (81). Different PRRSV proteins are involved in inhibition of IFN induction including nsp1,
nsp2, nsp11 and N. Some of the known mechanisms these proteins involve to inhibit IFN production are: (i) downregulating interferon regulatory factors (IRF); (ii) interfering with phosphorylation of IRF and (iii) interfering nuclear translocation of IRF (84). PRRSV can inhibit NK cell cytotoxicity as well (85). An in vitro experiment has shown reduced susceptibility of PRRSV infected PAMs towards NK cell cytotoxicity (86).

PRRSV is known to modulate host immune response particularly at early stages of infection. Cell mediated immunity in PRRSV infected pigs is detected in between 2 to 8 weeks and develops gradually. PRRSV specific IFN-γ producing cells also become detectable around 2 weeks. However, the PRRSV specific cytotoxic T cells are low in PRRSV infected pigs (79, 87-91). Induction of Treg is one of the various mechanisms used by viruses to escape host immunity. Silva-Campa et. al., have shown that PRRSV infection increases the frequency of CD4+CD8+CD25+Foxp3[^high] Treg in pigs. Such cells also produce TGF-β which might be involved in dampening host immune response (92). Cytokines are critical in induction and maintenance of host immune response. Production of anti-inflammatory cytokine IL-10 is another probable mechanism used by PRRSV for host immune evasion. In vitro treatment of porcine PBMCs with PRRSV significantly increased IL-10 gene expression (93). Similar increment on IL-10 gene expression on PBMCs was observed on in vivo infection of pigs with PRRSV as well (94). IL-10 has potent immunosuppressive effect through inhibition of antigen presenting cell maturation and T cell activation (95). Probable role of PRRSV nucleocapsid protein in induction of IL-10 and Treg cells has been elucidated (96).
Chapter 2

Characterization of porcine myeloid derived suppressor cells

1. Introduction

Myeloid derived suppressor cells (MDSCs) are a heterogeneous population of myeloid origin comprising macrophages, dendritic cells and granulocytes at various stages of differentiation (10). MDSCs have been recognized for their immunosuppressive ability in murine and human tumor and sepsis models, based on their ability to suppress T cell response as well as modulating cytokine production (10, 68).

The phenotype and function of MDSCs in pigs have not been identified yet, though they might have been playing similar important role in various immunosuppressive conditions. In this study, we have attempted to delineate the phenotypic and functional characteristics of pig MDSCs using magnetically sorted myeloid (CD172+) cells isolated from peripheral blood mononuclear cells (PBMCs) and cultured in the presence of cytokines GM-CSF and IL-6. The porcine reproductive and respiratory syndrome virus (PRRSV) induces suppression of innate and adaptive immune responses in pigs (106, 107), but the
mechanisms adapted by the virus is not completely understood. Since the PRRSV mediated immune-evasion strategy is comparable to MDSCs mediated activity in mice and human diseases, we were also interested to look at the effect of cytokines treatment on myeloid cells of PRRSV infected pigs, and determine whether PRRSV infection has any additive or synergistic effect on myeloid cells mediated immune suppression or not. In this study, we used magnetically sorted CD172\(^+\) myeloid cells isolated from PBMCs of PRRSV infected pigs and performed cytokine treatments and coculture experiments.

2. Materials and methods

Animals used in the study

PRRSV antibody-free uninfected pigs and pigs infected with PRRSV strain MN184 (108), or SD95-21 (110) were used in this study. 4 pigs were infected with MN184 (10\(^6\) TCID\(_{50}\) in 1 ml, intranasal) and 3 pigs were infected with SD95-21 (10\(^6\) TCID\(_{50}\)/ml virus 1 ml intramuscular and 1ml intranasal routes). The PBMCs isolated from the blood of approximately 2 month old pigs used in another experiment was used in this experiment. In total, we used PBMCs of 7 PRRSV infected pigs at 14 days post-infection and 6 animals from uninfected mock group. Pigs were raised in BSL2 facility at Food Animal Health Research Program (FAHRP), Ohio Agricultural Research and Development Center (OARDC), The Ohio State University. Animals were maintained, infected with PRRSV, blood samples collected and euthanized in accordance with the standards of the Institutional Laboratory Animal Care and Use Committee, The Ohio State University.
Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Blood was collected in EDTA directly from heart puncture of euthanized healthy as well as PRRSV infected pigs, and processed using density gradient medium in Sepmate-50 tubes (Stemcell, BC, Canada) following the manufacturer's instructions. In general, blood was diluted with PBS-FBS [PBS containing 2% FBS at room temperature (RT)] in 1:1 ratio. 15 mL of density gradient medium was added to Sepmate-50 tube and diluted blood was poured gently from the side of the tubes. Tubes were centrifuged at 1200xg for 20 min at RT (20°C). The top layer was poured-off and washed with PBS-FBS two times. Pellet was harvested and RBCs were lysed using 0.84% ammonium chloride solution. After washing with PBS-FBS, cells were resuspended in enriched RPMI (ERPMI) medium [10%FBS, gentamicin (100 µg/ml), ampicilin (20 µg/ml), 20 mM HEPES, 2 mM l-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 50 nM 2-ME], counted and the viability was tested by trypan blue dye-exclusion method.

Magnetic sorting of CD172⁺ myeloid cells

PBMCs were suspended in MACS buffer (PBS pH 7.2 containing 0.5% BSA and 2mM EDTA) at the ratio of 10 million cells per 80µl buffer, and immunolabeled with biotin conjugated CD172 antibody (Southern Biotech, AL) at 1µg/10 million cells, mixed gently and incubated for 30 min at 4°C. Cells were washed twice with MACS buffer at 300xg for 5 min at 4°C and resuspended in MACS buffer (10 million cells per 80µl
buffer). Cells were treated with Streptavidin microbeads (Miltenyi Biotec, CA) at 20 µL/10 million cells, incubated for 15 min at 4°C and gently flicked the pellet every 5 min. Cells were washed twice with MACS buffer (300xg for 5 min at 4°C) and finally suspended in MACS buffer maintaining the concentration of 100 million cells per 1000µl buffer.

Magnetic separation was done by using MACS Large cell column (Miltenyi Biotec, CA) as per the manufacturer's instructions. Magnetic columns were adjusted on MACS separator. Each column was rinsed with 500µl of MACS buffer and the cell suspension was passed through the column. Flow through was collected by washing the column thrice with 500 µl of MACS buffer. Then column was removed from the magnetic field and washed with 500µl MACS buffer 3 times to collect the sorted CD172+ cells. Both sorted cells (rich in myeloid cells) and flow-through (rich in lymphocytes) were centrifuged (at 300xg for 5 min at 4°C), resuspended in ERPMI and counted. Cells from both fractions (sorted as well as flow-through) were subjected to staining with CD172 and analyzed by flow cytometry to check the efficiency of cell sorting. The aliquots of lymphoid cells were frozen for later use.

**In vitro culture of CD172+ cells with cytokines**

Five million magnetically sorted CD172+ cells were cultured per well in 6 well cell culture plates (Costar, NY) in ERPMI medium at 37°C with the following treatments: (i) medium only; (ii) GM-CSF (10ng/mL, Kingfisher Biotech, MN) + IL-4 (25ng/mL,
Kingfisher Biotech, MN); and (iii) GM-CSF (10ng/mL) + IL-6 (10ng/mL, Kingfisher Biotech, MN). Cells in culture were harvested on day 7 and subjected to flow cytometry as well as for co-culture with autologous lymphoid cells. The cytokines GM-CSF and IL-4 combination was selected based on their ability to induce differentiation of monocytes into dendritic cells, whereas GM-CSF and IL-6 combination was selected as this combination is previously shown to induce MDSCs in human PBMCs (26, 111, 112).

**Co-culture of cytokine treated CD172⁺ myeloid cells with autologous lymphoid cells**

Magnetically sorted CD172⁺ cells from PBMCs were cultured with cytokines as explained above for 7 days, and cells were harvested and counted. The frozen lymphoid cells thawed, washed, resuspended in ERPMI and counted by trypan blue dye exclusion method. Myeloid cells (50,000) were co-cultured with autologous lymphoid cells at ratios 1:2, 1:5, 1:10 and 1:20 in round bottom 96 well cell culture plates (Costar, NY) in ERPMI media. Separate plates were maintained for flow cytometry analysis as well as for cell proliferation assay. After 4 days of co-culture of myeloid and lymphoid cells, supernatants were collected and stored at -20°C for cytokine assay, and cells were subjected to cell proliferation assay and flow cytometry analysis.

**Flow cytometry analysis**

The magnetically sorted CD172⁺ cells cultured with cytokine mixtures as described above for 7 days were evaluated for the cell surface expression of CD172, CD11R3,
CD11c, SLA II, CD80/86 and DC-SIGN markers. Antibodies were purchased commercially: mouse anti-porcine monocyte/granulocyte PE (IgG1, Southern Biotech); mouse anti-pig CD11R3 (IgG1, AbD Serotec); human CD152 (CTLA-4)-muIg (IgG2a, Ancell); mouse anti-human CD11c APC (IgG2b, Becton Dickinson); mouse anti-pig SLA II DR FITC (IgG2b, AbD Serotec); goat anti-mouse IgG2a FITC (AbD Serotec); Streptavidin PerCP (BD Pharmingen); and goat anti-mouse IgG1 APC/Cy7 (Southern biotech). Porcine DC-SIGN antibody was generously gifted by Dr. X-J. Meng (Virginia Polytechnic Institute and State University, Blacksburg, VA).

For staining, cells (0.5 to 1 million) were suspended in fluorescence activated cell sorting (FACS) buffer (0.1% BSA, 0.035% sodium bicarbonate and 0.02% sodium azide in HBSS) and plated in U-bottom 96 well plates. Cells were treated with 2% pig serum in FACS buffer to block the Fc receptors for 10 min at 4°C. After blocking, cells were washed twice with FACS buffer and treated with fluorochrome tagged purified or biotin labeled pig specific or cross-reactive antibodies for 30 min at 4°C. Cells were washed twice with FACS buffer and treated with appropriate secondary antibodies diluted in FACS buffer and incubated for 30 min at 4°C. Cells were washed twice with FACS buffer and fixed in 100µl of 1% paraformaldehyde, washed again twice with FACS buffer and resuspended in 200µl of FACS buffer and kept at 4°C to protect from light until acquired.
Similarly, the day 4 co-cultured cytokine treated CD172\(^+\) myeloid cells with lymphoid cells were also evaluated for the expression of markers CD25 and FoxP3. Antibodies were purchased commercially: mouse anti-pig CD25 (IgG1, AbD Serotec); goat anti-mouse IgG1 PerCP (Santa Cruz) and anti-mouse/rat Foxp3 APC (eBiosciences). Staining procedures were similar as mentioned above for the cell surface CD25 labeling. For staining intracellular marker Foxp3, after fixing, cells were permeabilized using permeabilization buffer (85.9% deionized water, 11% PBS with no Ca or Mg, 3% formaldehyde solution, and 0.1% saponin) overnight at 4\(^\circ\)C. Cells were washed with saponin wash twice and treated with Foxp3 antibody and incubated for 30 min at 4\(^\circ\)C. Cells were washed twice with saponin wash and finally suspended in 200\(\mu\)l of FACS buffer and kept at 4\(^\circ\)C to protect from light until acquired. The immunostained cells were acquired using FACS AriaII (BD Biosciences) and 10,000 to 100,000 events were acquired depending on different ratios of cells cultured. Data were analyzed using the FlowJo software (TreeStar Inc., OR).

**Cell proliferation assay**

To determine the effect of cytokine treated CD172\(^+\) myeloid cells on proliferation of autologous lymphoid cells, myeloid cells (50,000) were co-cultured with lymphoid cells for 4 days at ratios 1:2, 1:5, 1:10 and 1:20 in ERPMI media. Cell proliferation assay was carried out by using cell titer 96 aqueous non-radioactive proliferation assay kit (Promega, WI) as per the manufacturer's instructions. Briefly, day 4 co-culture supernatants were collected by centrifugation of plates at 2000 RPM for 5 min at 4\(^\circ\)C and
stored at -20°C until cytokine ELISA was done. Cells were maintained in 100µL EPRMI medium per well and 20µL of MTS/PMS solution was added and incubated at 37°C in 5% CO2 incubator. After 4 hrs of incubation optical density (OD) at 490 nm was recorded using the ELISA plate reader (Spectramax plus384, Molecular Devices, CA), the recorded OD value is directly proportional to metabolic activity of cells.

**IL-10 ELISA on culture supernatants**

Ninety-six well microplates (F-bottom, from Greiner Bio-one®) were coated with 50µl of anti-pig IL-10 antibody (R&D systems, MN) diluted at concentration of 4µg/ml in coating buffer (1.59 gm sodium carbonate anhydrous and 2.92 gm sodium bicarbonate in 1L distilled water) and incubated overnight at 4°C. The plates were washed twice with 200µl PBST (PBS containing 0.05% Tween-20) and blocked with 200 µl/well of blocking buffer (2% bovine serum albumin in PBS-T) by incubating at RT for 2 hrs. Plates were washed twice using PBS-T and 50µl of samples were added to respective wells. Samples were added in duplicates and incubated at RT for 2 hrs. Plates were washed 4 times with PBS-T and 50µl of detection antibody (mouse anti-swine IL-10 biotin; Invitrogen; 1µg/ml) was added and incubated at RT for 2 hrs. The plates were washed 4 times with PBS-T, 50µl of Streptavidin-HRP conjugate (Invitrogen) was added to each well and incubated for 60 min at RT. Again plates were washed 4 times with PBS-T and 50µl/well TMB-substrate (TMB Peroxidase Substrate and TMB Peroxidase Substrate B mixed 1:1; KPL) was added and incubated for 5-15 min till blue color was developed. Finally, 50µl/well of stop solution (1M Phosphoric acid) was added to stop
the reaction and the plates were read using ELISA reader (Spectra max plus; Molecular Devices®) at 450nm wavelength.

Statistical analysis

Data were analyzed using the GraphPad Prism 5.02 software, and showed as the mean +/− SEM of the number of pigs included in each study. Nonparametric Kruskal-Wallis test was used followed by Dunn's post-hoc test for statistical analysis and considered the level of significance at p<0.05.

3. Results

Efficiency of magnetic sorting for CD172+ myeloid cells

CD172a/SWC3 is an established porcine myelomonocytic marker, which is expressed on all the porcine myeloid cells from early stage of myelomonocytic lineage and maintained until their complete differentiation (113). In our study, CD172+ cells were magnetically sorted from porcine PBMCs and the sorted cells were analyzed by flow cytometry for the purity of CD172 stained cells, and observed the average purity of 90% (Fig. 2).
Expression of cell surface markers on CD172\(^+\) cells cultured with cytokines

Magnetically sorted CD172\(^+\) cells were cultured with the mixture of cytokines GM-CSF + IL-6, GM-CSF + IL-4 or without any cytokines for 7 days. After 7 days, cells were harvested and stained for different myeloid cell markers. Using the FlowJo software the acquired events by Flow cytometry were gated for myeloid population, and for cells double positive for CD172/SLAII, CD172/CD80/86, CD172/CD11R3, CD172/DC-SIGN and CD172/CD11c. Cell sub-populations were expressed as percentage of myeloid cells or parent population.

CD172\(^+\)SLAII\(^+\) expression was significantly higher in GM-CSF + IL-4 treatment group compared to both GM-CSF + IL-6 and medium control. However, there was no difference between medium control and GM-CSF + IL-6 treatment. Also comparable frequency of CD172\(^+\)SLAII\(^+\) cells was observed in PRRSV uninfected and infected pig groups (Fig. 3).

CD172\(^+\)CD80/86\(^+\) cells were significantly increased in GM-CSF and IL-4 treated myeloid cells compared to both medium only and GM-CSF and IL-6 treated cells. However, there was no difference in the expression of CD172\(^+\)CD80/86\(^+\) markers in between medium control and GM-CSF and IL-6 treatment of myeloid cells. Similar results were observed in PRRSV infected pigs derived myeloid cells when GM-CSF and IL-4 treatment was compared with GM-CSF and IL-6 treatment; but the data was not significant when GM-CSF and IL-4 treatment was compared to medium control (Fig. 4).
Expression of CD172⁺CD11c⁺ on myeloid cells was numerically higher in GM-CSF and IL-6 treated cells compared to both medium only and GM-CSF and IL-4 treatments; and a similar trend was also observed in PRRSV infected pig cells (Fig. 5). In PRRSV infected (but not uninfected) pig myeloid cells, cells expressing the markers CD172⁺CD11R3⁺ and CD172⁺DC-SIGN⁺ were numerically increased in both GM-CSF and IL-6 and GM-CSF and IL-4 treated cells compared to untreated cells (Fig. 6 and 7).

**Proliferation of autologous lymphoid cells in co-culture with cytokine treated myeloid cells**

Magnetically sorted CD172⁺ cells were treated with the mixtures of cytokines for first 7 days, and co-cultured with autologous lymphoid cells at different cell ratios for an additional 4 days. Optical density (OD) values were compared for cell proliferation, and observed statistically higher proliferation of lymphoid cells in GM-CSF and IL-4 treated myeloid cells containing culture at 1:2 and 1:5 myeloid to lymphoid cell ratios (but not at higher ratios) compared to lymphoid cell alone. There was no statistical difference in cell proliferation in medium control, GM-CSF/IL-4 and GM-CSF/IL-6 treatments. While there was overall decrease in the proliferation of autologous lymphoid cells when co-cultured with PRRSV infected myeloid cells treated with either GM-CSF and IL-6 or GM-CSF and IL-4 but no statistical difference was observed (Fig. 8).
CD25^+Foxp3^+ Regulatory T cell phenotype in co-culture of myeloid and autologous lymphoid cells

Cytokines treated CD172^+ cells were harvested after 7 days and co-cultured with autologous lymphoid cells in different ratios for 4 more days. 50,000 myeloid cells were cultured with autologous lymphoid cells at 1:2, 1:5, 1:10 and 1:20 ratios. Cells were immunostained for CD25 and Foxp3 markers and analyzed by flow cytometry. For gating, total lymphocytes were selected first and then looked for cells double positive for CD25 and Foxp3 markers. Isotype control was used to make basis for deciding negative and positive cell population. In PRRSV uninfected pigs, GM-CSF + IL-6 treated CD172^+ cells induced numerical increase in CD25^+FoxP3^+ regulatory T cells present in the autologous lymphoid cells compared to other treatment groups at cell ratios 1:2, 1:5 and 1:10, indicating that there might be presence of immunomodulatory effect induced by GM-CSF and IL-6 treated myeloid cells. While in PRRSV infected pigs, any such trend in Tregs was not detected in any of the treatments (Fig. 9).

Production of IL-10 in co-culture of myeloid and autologous lymphoid cells

Supernatants harvested from day 4 co-culture of cytokines treated CD172^+ myeloid cells with autologous lymphoid cells were assayed for porcine IL-10 levels. In PRRSV uninfected pigs, production of IL-10 was higher both in medium control and GM-CSF and IL-6 treatment groups compared to lymphoid cells not cultured with any cytokines. Though, GM-CSF and IL-6 treatment group had higher level of IL-10 secretion
compared to GM-CSF and IL-4 group, the data was not statistically different. In PRRSV infected pigs, IL-10 production in medium control was relatively higher compared to other groups (Fig. 10).

**Discussion**

MDSCs are a heterogeneous population of myeloid cells comprised of cells at various stages of their differentiation. Though heterogenous in nature, MDSCs share some common properties including myeloid origin, immature state and remarkable ability to suppress T cell activity (10). MDSCs induced immunosuppression has been studied in cancer and sepsis conditions. In addition, their significant suppressive role in bacterial, parasitic and viral diseases is also documented (49). The mechanisms of MDSCs mediated immunosuppression are varied and range from suppressing innate response of NK cells to the adaptive responses of CD4+ and CD8+ lymphocytes (10, 55). There are studies characterizing the phenotypic and functional characteristics of MDSCs in mice and humans but not in pigs. Lechner *et al.*, have characterized MDSCs from healthy human PBMCs by treating the cells with different combinations of cytokines and found that cytokine mixture GM-CSF and IL-6 has remarkable potential to induce CD33+ MDSCs (26).

An attempt to characterize porcine MDSCs by stimulating porcine PBMCs with GM-CSF and IL-6 combination was also made previously in our lab, which resulted in generation of a subpopulation of cells expressing the phenotype CD172+CD11R3+ and producing
higher level of IL-10 (114). In order to refine it and explore more towards functional attributes of cell population induced by GM-CSF and IL-6 treatment on porcine myeloid cells, we used magnetically sorted CD172$^+$ myeloid cells. To explore if in vivo PRRSV infection status has any effect on inducing MDSCs during GM-CSF + IL-6 treatment, we also included CD172 sorted cells from PRRSV infected pigs.

CD172 is a porcine monocyte/granulocyte marker, and it is expressed on the myelomonocytic lineage from early stage of differentiation to its completion (113). Sorted CD172$^+$ myeloid cells were cultured with GM-CSF + IL-6 for 7 days and phenotypically characterized for the maturation markers SLA II and CD152 (CD80/86), as well as for cellular markers CD11c and CD11b (CD11R3). Further, cultured myeloid cells for 7 days were co-cultured with autologous lymphoid cells at different ratios for 4 days and subjected to cell proliferation assay, phenotypic analysis of regulatory T cells, and IL-10 production. Since GM-CSF + IL-4 treatment to porcine PBMCs induces generation of monocyte derived dendritic cells (MoDC) (111, 112, 115), we included that stimulation of myeloid cells for comparison with GM-CSF + IL-6 and medium only treatments.

In mice there are two major lineages of MDSCs, granulocytic (CD11b$^+$GR-1$^{hi}$Ly-6G$^+$Ly-6C$^{lo}$CD49d$^+$) and monocytic (CD11b$^+$GR-1$^{hi}$Ly-6G$^+$Ly-6C$^{hi}$CD49d$^+$), while in humans CD11b$^+$CD33$^+$ lineage is regarded as the MDSCs. CD11b is commonly expressed in both human and mice MDSCs. CD11b is expressed generally on granulocytes, monocytes and
alveolar macrophages (10, 101). CD11R3 is a pig cell surface glycoprotein of α-integrin family (116), expressed on granulocytes, monocytes and on alveolar macrophages (117), and it is the human homolog of CD11b (117). An earlier study in our lab attempted to characterize porcine MDSCs and showed expansion of sub-population of CD172\(^+\)CD11R3\(^+\) cells in GM-CSF and IL-6 treatment of porcine whole PBMCs, suggesting the possible presence of MDSCs (114). In this study, using magnetically sorted CD172\(^+\) myeloid cells, we observed only a numerical increase in the expression of CD172\(^+\)CD11R3\(^+\) phenotype bearing cells in GM-CSF and IL-6 treated group compared to the medium control in both PRRSV uninfected and infected pigs. In previous study, plate adhered myeloid cells were used while in recent study sorted CD172\(^+\) cells were used. In previous study, PRRSV strain VR2332 was used to treat myeloid cells \textit{in vitro}; while in this study cells were used from pigs infected \textit{in vivo} with PRRSV strains MN184 and SD95-21 which might define the differences between these two studies. Altogether, this study suggests that CD11R3 expression alone may not be sufficient enough to define porcine MDSCs in PBMCs. Further, GM-CSF + IL-6 environment itself, as was in human PBMCs, might not be enough to induce porcine MDSCs.

It is known that GM-CSF and IL-4 treatment of PBMCs differentiates monocytes into MoDC (111, 112), which enhances expression of maturation markers SLAII and CD80/86 on dendritic cells (111, 112, 115). In our experiment, we also found differentiation of CD172\(^+\) myeloid cells into MoDC, with increased population of double positive CD172/SLAII and CD172/CD80/86 cells when sorted myeloid cells were treated
with GM-CSF and IL-4 for 7 days. While, CD172<sup>+</sup>SLAI<sup>+</sup> and CD172<sup>+</sup>CD80/86<sup>+</sup> cell frequencies were significantly lower in GM-CSF + IL-6 treatment group compared to GM-CSF + IL-4. However, both untreated and GM-CSF and IL-6 treated cells had similar low level of SLA II and CD80/86 expression. Overall, our results suggested that GM-CSF and IL-6 treatment of sorted porcine CD172<sup>+</sup> cells did not induce a typical MDSCs phenotype, like observed earlier in similarly cultured human myeloid cells.

MDSCs are known for suppression of T cell sensitization and function in tumor microenvironment leading to reduced host immune response (17, 118). The mechanism of host immune suppression can be diverse and interlinked (10, 101). In order to characterize the functional aspect of GM-CSF and IL-6 induced cell sub-population, we co-cultured them with the autologous lymphoid cells. Our results indicated that proliferation of autologous lymphoid cells was not reduced by GM-CSF + IL-6 treated myeloid cells.

Expansion of regulatory T cells is one of the various mechanisms involved in MDSC mediated immunosuppression (97-99). In this study, the GM-CSF and IL-6 treated group had higher expression of CD25<sup>+</sup>FoxP3<sup>+</sup> phenotype referring to expansion of regulatory T cells at higher frequency compared to GM-CSF and IL-4 treated group. However, owing to high pig to pig variation, data were not statistically significant. Production of immunosuppressive cytokines IL-10 and TGF-β is another well studied mechanism of immunosuppression by MDSC (97-99). We observed slightly higher IL-10 production in GM-CSF and IL-6 treatment group compared to GM-CSF and IL-4 treatment, with no
statistical significance. Since the lymphoid cells produced low level of IL-10 and media treated cells also produced higher IL-10 level, it appears that besides Treg cells, myeloid cells also contributed in IL-10 synthesis.

Immunosuppression is one of the features of PRRSV infection, but the viral pathogenesis and the key immune players involved are not clearly understood. For example, PRRSV infection induces suppression of innate immunity by increased IL-10 and TGF-β production (119-123) and inhibition of NK cell cytotoxicity (107, 124). Likewise, suppression of adaptive immune system results from dampened functional ability of CD8$^+$ T cells (91, 124), decreased T cell proliferation and expansion of Treg cells (125). Since these features are quite comparable to the mechanism by which MDSCs are expressing their immunosuppressive function, we wanted to know whether in vivo PRRSV infected pig derived myeloid cells will have any effect on GM-CSF + IL-6 treatment in vitro. Our results suggested that, there was no difference in phenotypic characteristics of myeloid cells (CD172/SLAII; CD172/CD80.86; CD172/CD11R3; CD172/CD11c and CD172/DC-SIGN) after 7 days of GM-CSF and IL-6 treatment, and the frequencies were comparable to PRRSV uninfected pig cells. Functionally, GM-CSF and IL-6 treated myeloid cells did not significantly inhibit autologous T cell proliferation, enhance expansion of Tregs and increase IL-10 production, as we expect in MDSCs mediated immune-modulation.

CD11R3 and other available porcine myeloid markers used in this study were found insufficient to phenotypically define porcine MDSCs in PBMCs. Functionally, T cell
proliferation, regulatory T cell frequencies and IL-10 induction in autologous T cell cocultures were comparable in untreated and GM-CSF + IL-6 treated cells. Thus, none of the evaluated physical and functional characteristics of GM-CSF and IL-6 treated pig myeloid cells showed satisfactory immunosuppressive nature of porcine MDSCs. Therefore, future studies should be aimed to examine the levels of important effector molecules of MDSCs, such as nitric oxide, RNS (ONOO’) and ROS (H2O2) and intracellular enzymes like nitric oxide synthase, arginase 1 and NADPH oxidase, which might help at least to characterize functional porcine MDSCs. Further, our data showed that, PRRSV infected pig myeloid cells were not synergistically induced to acquire immunosuppressive functions upon GM-CSF + IL-6 treatment. However, similar studies using bronchoalveolar lavage (BAL) cells or lung mononuclear cells (LMNC) might help to delineate PRRSV mediated changes in myeloid cell phenotype and functions in vivo. Though, our study has not confirmed the presence of porcine MDSCs in PBMCs, it made a beginning to understand this immunosuppressive subset using new immune reagents and advanced technologies.
Fig 2: Efficiency of CD172\(^+\) cell sorting. Porcine PBMCs were first stained with CD172 biotin antibody followed by Streptavidin microbeads and passed through MACS large column separator. Labeled PBMCs before and after sorting (myeloid fraction) were stained for the cell surface marker CD172 and analyzed by Flow cytometry. Representative figure from one pig shows: percentage of CD172\(^+\) cells in (A) PBMCs before sorting and (B) myeloid cells after sorting.
Fig 3: Frequency of CD172^SLAII^ cells. CD172^+ myeloid cells were magnetically sorted using MACS column separator (Miltenyi biotech) and cultured with medium only, GM-CSF + IL-4 or GM-CSF + IL-6 for 7 days. Cells were harvested, immunostained and analyzed by flow cytometry: (I) Gating strategy for CD172^+SLAII^ cells in (a) medium (b) GM-CSF + IL-4 and (c) GM-CSF-IL-6 treatments. Frequency of CD172^+SLAII^ cells in (II) PRRSV uninfected and (III) PRRSV infected pigs. Each bar is an average percentage of cells from 6 to 7 pigs +/- SEM. Statistical significance was determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test.
Fig 4: Frequency of CD172⁺CD80/86⁺⁺ cells. CD172⁺ myeloid cells were magnetically sorted using MACS column separator (Miltenyi biotech) and cultured with medium only, GM-CSF + IL-4 or GM-CSF + IL-6 for 7 days. Cells were harvested, immunostained and analyzed by flow cytometry: (I) Gating strategy for CD172⁺CD80/86⁺ cells in (a) medium (b) GM-CSF + IL-4 and (c) GM-CSF-IL-6 treatments. Frequency of CD172⁺CD80/86⁺ cells in (II) PRRSV uninfected and (III) PRRSV infected pigs. Each bar is an average percentage of cells from 6 to 7 pigs +/- SEM. Statistical significance was determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test.
Fig 5: Frequency of CD172+CD11c+ cells. CD172+ myeloid cells were magnetically sorted using MACS column separator (Miltenyi biotech) and cultured with medium only, GM-CSF + IL-4 or GM-CSF + IL-6 for 7 days. Cells were harvested, immunostained and analyzed by flow cytometry: (I) Gating strategy for CD172+CD11c+ cells in (a) medium (b) GM-CSF + IL-4 and (c) GM-CSF-IL-6 treatments. Frequency of CD172+CD11c+ cells in (II) PRRSV uninfected and (III) PRRSV infected pigs. Each bar is an average percentage of cells from 3 to 4 pigs +/- SEM. Statistical significance was determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test.
Fig 6: Frequency of CD172⁺CD11R3⁺ cells. CD172⁺ myeloid cells were magnetically sorted using MACS column separator (Miltenyi biotech) and cultured with medium only, GM-CSF + IL-4 or GM-CSF + IL-6 for 7 days. Cells were harvested, immunostained and analyzed by flow cytometry: (I) Gating strategy for CD172⁺CD11R3⁺ cells in (a) medium (b) GM-CSF + IL-4 and (c) GM-CSF-IL-6 treatments. Frequency of CD172⁺CD11R3⁺ cells in (II) PRRSV uninfected and (III) PRRSV infected pigs. Each bar is an average percentage of cells from 3 to 4 pigs +/- SEM. Statistical significance was determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test.
Fig 7: Frequency of CD172\(^+\)DC-SIGN\(^+\) cells. CD172\(^+\) myeloid cells were magnetically sorted using MACS column separator (Miltenyi biotech) and cultured with medium only, GM-CSF + IL-4 or GM-CSF + IL-6 for 7 days. Cells were harvested, immunostained and analyzed by flow cytometry: (I) Gating strategy for CD172\(^+\)DC-SIGN\(^+\) cells in (a) medium (b) GM-CSF + IL-4 and (c) GM-CSF-IL-6 treatments. Frequency of CD172\(^+\)DC-SIGN\(^+\) cells in (II) PRRSV uninfected and (III) PRRSV infected pigs. Each bar is an average percentage of cells from 3 to 4 pigs +/- SEM. Statistical significance was determined by non-parametric Kruskal-Wallis test followed by Dunn's post hoc test.
Fig 8: Proliferation of autologous lymphoid cells. Magnetically sorted CD172$^+$ myeloid cells were cultured with indicated cytokines for first 7 days, and 50,000 myeloid cells were cocultured with autologous lymphoid cells at ratios of 1:2, 1:5, 1:10 and 1:20 for an additional 4 days. The cocultured cells were subjected to cell proliferation assay using cell titer 96 non-radioactive cell proliferation assay kit (Promega) and OD values are compared. Lymphocyte proliferation analysis in (A) PRRSV uninfected pig cells and (B) PRRSV infected pig cells is shown. Each bar is an average percentage of cells from 6 to 7 pigs +/- SEM. Statistical significance between the groups was determined by non-parametric Kruskal-Wallis test followed by Dunn's post-hoc test.
Fig 9: Frequency of CD25⁺Foxp3⁺ regulatory T cells. Magnetically sorted CD172⁺ myeloid cells were cultured with indicated cytokines for first 7 days, and 50,000 myeloid cells were cocultured with autologous lymphoid cells at ratios of 1:2, 1:5, 1:10 and 1:20 for an additional 4 days. The cocultured cells were immunostained for CD25 and Foxp3 markers and subjected to flow cytometry. (A) Representative gating of CD25⁺Foxp3⁺ cells in the coculture of myeloid cells and lymphoid cells of (i) mock-treated; (ii) GM-CSF + IL-4 treated; and (iii) GM-CSF + IL-6 treated groups. Frequency of CD25⁺Foxp3⁺ Treg cells in (B) PRRSV uninfected pig cells and (C) PRRSV infected pig cells is shown. Each bar is an average percentage of cells from 6 to 7 pigs +/- SEM. Statistical significance between the groups was determined by non-parametric Kruskal-Wallis test followed by Dunn's post-hoc test.
Fig 10: The cytokine IL-10 production in the supernatants of myeloid/lymphoid coculture. Magnetically sorted CD172⁺ myeloid cells were cultured with indicated cytokines for first 7 days, and 50,000 myeloid cells were cocultured with autologous lymphoid cells at ratios of 1:2, 1:5, 1:10 and 1:20 for an additional 4 days. Supernatants from the cocultured cells were collected and the levels of secreted cytokine IL-10 was determined by ELISA in: (A) PRRSV uninfected pig cells and (B) PRRSV infected pig cells. Each bar is an average percentage of cells from 6 to 7 pigs +/- SEM. Statistical significance between the groups was determined by non-parametric Kruskal-Wallis test followed by Dunn's post-hoc test.
References


