Analysis of Myeloid Derived Suppressor Cells and the Role of Inducible Nitric Oxide Synthase in Renal Transplantation Across MHC Mismatched Mice

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Analysis of Myeloid Derived Suppressor Cells and the Role of Inducible Nitric Oxide Synthase in Renal Transplantation across MHC Mismatched Mice

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A Dissertation Submitted to the Faculty of Harvard Medical School in Partial Fulfillment of the Requirements for the Degree of Master of Medical Sciences in the Department of Immunology

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Abstract

Despite recent advances in immunology and post-operative care, transplant recipients continue to face major complications. This is due to immune-mediated destruction of graft tissue and drug toxicity caused by chronic immunosuppression. A solution to both of these issues is the induction of long-term tolerance to donor antigens. Recently, renal allograft tolerance has been achieved between HLA-disparate individuals in the clinic without the need for immunosuppression using a mixed chimerism protocol. However, the mechanisms and cellular components involved in the initiation of regulatory and deletional tolerance in this model remains to be understood. The aim of this study is to identify suppressive mechanisms that myeloid-derived suppressor cells (MDSCs) utilize to induce regulatory tolerance. MDSCs were enriched from the bone marrow using a negative selection protocol, and cultured with stimulating cytokines. Flow cytometry was then used to assess the expression of surface and intracellular molecules. Moreover, DBA donor kidneys were transplanted into B6 recipients and were euthanized after 1, 3, and 6 weeks. Nitrosylation was assessed by immunohistochemistry (IHC) using anti-nitrotyrosine and anti-iNOS unconjugated antibodies. CD11b+ cells were isolated from 6 week kidney allografts, and analyzed with IHC and flow cytometry. Cell culture showed that when MDSCs are stimulated they upregulate PD-L1, podoplanin, and F4/80. Interestingly, Ly6C expression is dramatically reduced. Additionally, when these cells are cultured with naïve T cells they are able to promote the induction of T regulatory cells (Tregs). Moreover, accepted renal allografts are distinct from rejected allografts with respect to nitrosylation. The presence of iNOS+ cells in the graft shows that nitric oxide (NO) production is
occurring within the kidney microenvironment. CD11b+ cells within the Treg-rich organized lymphoid structures (TOLS) of the graft are one source of NO production. Our findings provide insight into the phenotype and potential role of these tissue effector cells in solid organ transplantation.
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**Figure 4.1 Model of MO-MDSCs Function in Renal Allografts.** CD11b\(^+\) Ly6C\(^{hi}\) MO-MDSCs circulate in the blood and localize to renal allografts. Mediators in the allograft, such as M-CSF, IL-4, and HMGB1 promote the differentiation of naïve MO-MDSCs into M2-like macrophages. Here, these cells promote tolerance through the inhibition of T cells by secreting RNS and depleting L-arginine. Additionally, these terminally differentiated cells promote regulatory tolerance through the induction of Tregs via the presentation of allopeptide. *Adopted from: Ochando et al. doi:10.1016/j.immuni.2015.05.009*
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Chapter 1: Biology and Regulatory Functions of Myeloid-Derived Suppressor Cells

1.1 Introduction

1.1a) Historical Perspectives

In the past half century we have made significant strides in the field of transplantation. In order to appreciate these developments, it is important to discuss the work of individuals who have brought transplantation from a primitive therapeutic measure to an accepted means of clinical practice. In 1954, Dr. Joseph Murray and his team conducted the first kidney transplant between identical twins\(^1\). The physicians had to overcome two major roadblocks before attempting the procedure, which were the development of an appropriate surgical protocol and a means to determine genetic compatibility. Surgical aptitude was achieved by renal autograft experiments in domestic dogs. The selected site for graft implantation was the retroperitoneal space in the pelvis, which allowed for successful ureteroureterostomy and optimal thermal environment for the organ\(^1\). To limit cellular or humoral mediated immune rejection, it was crucial to make certain that the twins were genetically identical. This was assessed through the acceptance of skin allografts. The effective surgical model and knowledge that the twins were monozygotic gave the surgeons confidence to move forward with the procedure. The transplantation was ultimately realized, and the subjects survived. Although the efficacy of this procedure was confirmed, it has become practice to prescribe immunosuppressive drugs to the
recipient. This is because even in HLA-matched individuals minor histocompatibility antigens (miHA) may still contribute to immune mediated rejection$^2$.

The induction of long-term graft tolerance remains to be a significant challenge puzzling transplant immunologists. Dr. Peter Brian Medawar and his team have made tremendous contributions to understanding the graft rejection process and immune tolerance. Before Murray and his team conducted the first transplant, little was known about why grafts were rejected. In a landmark paper, Medawar and colleagues showed that the immune system contributed to graft rejection between MHC-disparate humans$^3$. The subject of this study was a burn victim who received 52 autografts from her own thigh, and 50 allografts from her brother. At day 8 post-transplantation, the skin allografts were completely inflamed and started to deteriorate, while the autografts showed no sign of rejection. Histological analysis showed that the allografts contained abundant amounts of lymphocytic infiltrate when compared to the autografts. These experiments indicated that there are intrinsic factors in allografts that stimulate the infiltration of immune cells and promote graft destruction. A few years later, Medawar also demonstrated a method to induce allograft tolerance$^4$. He showed that when donor embryonic cells were transplanted into the embryos of the recipient, the offspring were tolerant of donor antigens. This was validated by means of skin transplantation. Moreover, tolerance was ablated after the adoptive transfer of lymphocytes, which were developed from the same recipient strain vaccinated against donor skin antigens. This was the first achieved account of actively acquired tolerance, and indicated that immunological plasticity can be exploited to induce long-term transplant tolerance.
We have made significant advances in post-operative care and transplant immunology since the first successful transplant by Joseph Murray’s team at Brigham and Women’s Hospital. However, major challenges remain which result in less than optimal outcomes after transplantation. Currently, organ procurement networks across the United States indicate that there are thousands of patients in need for a lifesaving organ transplant. Unfortunately, the demand for vital organs exceeds the supply of suitable donors. The organ shortage is compounded by the loss of organs due to rejection or chronic graft dysfunction. In particular, the 10 year survival after heart transplantation is in the order of 50%. After successful graft implantation, cell-mediated and humoral immunity are major contributors to eventual organ attrition in HLA-mismatched individuals. While these are controlled with a good degree of efficacy in the short term, under chronic immunosuppression patients are prone to developing diabetes mellitus, kidney disease, hypertension, and obesity\(^5\). Additionally, immunocompromised patients have a higher likelihood of infection and malignancy\(^6\). In the long term, immunosuppression has largely failed to control chronic allograft dysfunction or rejection. Prolongation of allograft survival through the induction of mixed chimerism is a possible solution to these challenges. There is evidence that mixed chimerism results in allograft tolerance. However, the molecular events by which this takes place remains elusive. Understanding this will help to tailor more effective therapies for transplant patients. It is for this reason that it is important to further dissect what hematopoietic populations determine the mechanisms of tolerance induction.
1.1b) Allograft Rejection

A major barrier to a successful transplantation is immune-mediated rejection. The initiation of the innate immune system and subsequent amplification of the adaptive axis results in the activation of molecular pathways that facilitate graft destruction. The manipulation of tissue during the surgical procedure of organ engraftment produces an inflammatory response that identifies the allograft as a site for leukocyte infiltration. Through invariant pattern recognition receptors (PRRs), innate immune cells can sense damage associated molecular patterns (DAMPs) that are released from injured cells. The recognition of nucleic acids and chromatin associated proteins active intracellular NOD-like receptors (NLRs), which promote the formation of inflammasomes that upregulate pro-inflammatory mediators. The secretion of IL-1, IL-6, and TNF-α ultimately cause an increase in hydrostatic pressure and permeability of post-capillary venules, facilitating the infiltration of professional antigen presenting cells (APCs) and other leukocytes into the allograft. After the uptake of antigen, conventional dendritic cells (cDCs) travel to secondary lymphoid organs and active alloreactive T cells through three distinct pathways. Initially, donor derived APCs interact with recipient naïve T cells in the lymph nodes and spleen through recognition of alloantigen presented on major histocompatibility molecules (MHC). This is the direct-allorecognition pathway, and is the main mechanism promoting T cell expansion early post-transplantation. As donor derived APCs reduce in number, the indirect pathway takes on more of an effect. Here, recipient APCs engulf donor cellular debris and present donor derived MHC peptide or miHA to recipient T lymphocytes. Moreover, the most uncommon mechanism of alloreactive T cell activation is conducted through the semi-direct pathway, were
recipient APCs capture donor MHC-peptide complexes via the exchange of fragments of the cell membrane. Upon APC stimulation, activated T and B lymphocytes are then able to promote graft destruction through a variety of innate and adaptive mediated pathways. Cytotoxic CD8 T cells recognize their targets through allogeneic MHC:peptide complexes and induces apoptosis through the upregulation of granzyme B, perforin, and FAS-L13. Additionally, the secretion of alloantibodies by B cells aid in innate mediate graft damage through activation of the classical complement pathway and NK cell cytotoxicity14,15. Many molecular pathways trigger tissue injury. Understanding these pathways have helped in the identification of novel therapeutic measures that promote graft survival.

1.1c) Mixed Chimerism

Advances in clinical immunology and organ preservation techniques have established transplantation as a standard medical practice. However, chronic immunosuppression and poor long-term survival of the graft have contributed to drug induced toxicity and eventual rejection. A solution to both of these issues is the induction of long-term tolerance of donor antigens. Theoretically, extended tolerance can be achieved by three strategies: T-cell depletion, co-stimulatory blockade, and chimerism16. Interestingly, animals that lack T cells are unable to reject allografts17. So, the depletion of this lymphocyte through total body irradiation or α-CD4 and α-CD8 antibodies is one method to reduce an immune response to alloantigens. Although these studies were confirmed in mice, using this protocol in non-human primate (NHP) models proved to be very difficult due to the lack of T cell depleting strategies 18. Furthermore, the
administration of antibodies that block CD28, CD80, and CD86 or CTLA-4 agonists have aided in the prolongation of graft survival by means of immunosuppression, but not tolerance\textsuperscript{19,20}. Currently, mixed chimerism is a successful method to induce the tolerance and survival of allografts.

Mixed chimerism is the transplantation of donor hematopoietic system in irradiated recipients, which leads to a coexistence of both donor and recipient leukocytes\textsuperscript{21}. In this model, deletional tolerance is achieved by the localization of both donor and recipient hematopoietic cells in the thymus to delete alloreactive T cells. Mixed chimerism is preferable to full chimerism because immunocompetence is maintained by not ablating the hosts T cells\textsuperscript{22}. The establishment of mixed-chimerism in murine models has been done; however, translating these procedures to larger mammals has been difficult. Mixed-chimerism protocols in NHPs and humans have shown to be transient in nature. Fortunately, the survival of even a small percentage of donor bone marrow is sufficient enough to allow tolerance of donor antigens\textsuperscript{23}. Currently, the acceptance of renal allografts across HLA-disparate individuals has been achieved with a short mixed chimerism protocol\textsuperscript{24}. As the level of mixed chimerism is reduced in recipients, regulatory mechanism become important in promoting graft acceptance. It is for this reason that is may be important to elucidate what hematopoietic populations are responsible for the induction of regulatory tolerance in allografts.
1.1d) Spontaneous Renal Allograft Acceptance

Pioneering studies conducted by Dr. Paul Russell and his team lead to the discovery that fully mismatched kidney transplantation between certain mouse strains led to spontaneous acceptance without the need for preconditioning or immunosuppression\textsuperscript{25}. Moreover, long-term acceptance resulted despite the development of alloreactive lymphocytes\textsuperscript{26}. This striking observation resulted in significant study of this phenomenon, particularly between DBA/2J and C57BL/6 mice, to further define what mechanisms drive immunological tolerance of allografts.

In 2008, Dr. Charles Orosz \textit{et al.} showed that spontaneous renal allograft acceptance was associated with TGF-\(\beta\) production and possibly Tregs\textsuperscript{27}. Here the researchers showed that relative FoxP3\(^+\) mRNA peaked in renal allografts 30 days post-transplantation and was reduced, but persisted, to 150 days after acceptance. As the transcription of FoxP3 was slightly downregulated in the long-term, relative expression of the tryptophan depleting enzyme called indoleamine 2,3-dioxygenase (IDO) increased. This enzyme has been shown to modulate the immune response by suppressing T cell function. The mammalian target of rapamycin (mTOR pathway), which couples amino acid sensing to ribosomal translation, detects the depletion of tryptophan and results in reduced protein production and accumulation of uncharged tRNA molecules. Also, the downstream metabolites of tryptophan may also lead to apoptosis and arrest of proliferation\textsuperscript{28}. These results were further validated by Colvin \textit{et al.} who showed that the depletion of Tregs in B6 recipients of DBA allografts promoted CD8 interstitial mononuclear inflammation, endarteritis and eventual graft rejection\textsuperscript{29}. Together these results show that spontaneous renal allograft acceptance is promoted by the induction of regulatory mechanisms.
In the clinic, tolerance of kidney allografts has been achieved in both nonhuman primates and in humans. However, these same protocols are unable to induce the acceptance of cardiac allografts in the same models. Remarkably, the co-transplantation of both heart and kidney allografts results in cardiac allograft acceptance. In these studies the kidney shows the ability to confer systemic tolerance to donor derived antigens. Although, the formation of Treg structures in kidneys seem to be important in the induction of organ tolerance, data from other groups propose that Tregs alone are unable to explain this regulation. A greater understanding of the immunological mechanisms, which result in spontaneous renal allograft acceptance, are important to reveal new ways to induce immune tolerance.

1.1e) Immature Myeloid Cells

Immature myeloid cells (IMCs) can differentiate into granulocytes, dendritic cells, and macrophages. Under pathological conditions, such as transplantation, the maturation process is partially blocked and results in the expansion of immunoregulatory cells (chapter 1.2) characterized by the upregulation of the inducible isoform of nitric oxide synthase (iNOS), arginase-1, transforming growth factor- β (TGF-β), and reactive oxygen species (ROS). These leukocytes can modulate the immune response through the manipulation of amino acid metabolism. Recent findings indicate that the depletion of L-arginine and release of nitric oxide (NO) into the microenvironment suppresses the immune system by impairing T-cell function. Myeloid-derived suppressor cells (MDSCs) have been studied predominantly in the context of tumor progression; however, their potential to induce transplant tolerance remains to be
defined. Ochando et al. recently showed that mononuclear MDSCs accumulate into cardiac allografts 5 days after transplantation, where they differentiate into DC-SIGN+ macrophages and induce Treg expansion. The immunomodulatory role of MDSCs is evident in transplantation. Myeloid-derived suppressor cells function through direct cell-to-cell contact and are responsible for the release of biological mediators that alter the metabolism of other leukocytes. These cells have possible therapeutic potential to promote extended graft survival in patients, and should be further studied in the context of solid organ transplantation.

1.2 Ontogeny of Immature Myeloid Cells

1.2a) Myelopoiesis

Phagocytic cells play a paramount role in host defense mechanisms. These mediators of the innate immune system are grouped into two categories: polymorphonuclear (granulocytic) and mononuclear phagocytes. Collectively, these progenitors are referred to as myeloid cells, and make up most of the cellular population in the bone marrow. Mononuclear phagocytes constitute mature macrophages, monocytes, and subsets of immature progenitor cells. In the late 19th century, Metchnikoff was the first to classify these phagocytic cells as macrophages, and demonstrated that they were not only involved in engulfing, but facilitated the destruction of microorganisms. Today, researchers have identified a variety of activating stimuli that promote macrophage function. Macrophage activation is divided into two general categories: classical and alternative. The former is mediated by interferon-γ (IFN-γ), while interleukin-4 (IL-
4) and IL-13 signaling are responsible for the alternative pathway. Classically activated macrophages (M1) promote a pro-inflammatory immune response, and alternatively activated macrophages (M2) account for anti-parasitic immunity and tissue repair\textsuperscript{37}. Moreover, other exogenous and endogenous ligands also promote the maturation of macrophages. During an innate immune response, microbial peptide binding to PRRs on macrophages and stimulate the production of pro-inflammatory cytokines, ROS, and NO\textsuperscript{37,38,39,40}. Macrophages effector functions may also be activated through humoral mechanisms, such as the binding of antibodies to Fc or complement receptors. The activation of these receptors promote increased actin polymerization and particle uptake via intricate phosphorylation events\textsuperscript{37,39,40}. In order to elucidate how macrophages are activated and distributed throughout the various physiological compartments it is important to discuss their origin.

The mononuclear phagocyte system that Metchnikoff postulated has helped guide research for many years. This paradigm established the concept of a “layered” immune system, which suggests that there are several types of pluripotent hematopoietic stem cells (HSCs) that give rise to immune cells during different developmental stages. The layered myeloid system consists of tissue-resident macrophages that arise from progenitor cells in the yolk sac, while passenger myeloid cells are produced and maintained from bone marrow HSCs\textsuperscript{41}. Tissue-resident macrophages have the capability to self-renew within their localized area, and may differentiate into osteoclasts, microglia, Langerhan cells, and Kupffer cells\textsuperscript{37}. Moreover, recruited macrophages have also been known to reside in the skin, heart, liver, and other areas of the body. The development of macrophages depends on cytokine signals and cell interactions with stroma in the bone marrow. Key regulatory cytokines and molecules include: macrophage
colony-stimulating factor (M-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF), IL-2 and KIT\textsuperscript{37}. Studies of these mononuclear cells indicated that pluripotent HSCs give rise to multipotent intermediates called common myeloid progenitors (CMPs). Additionally, within the bone marrow CMPs differentiate into monoblasts, which required G-CSF signaling. Following G-CSFR activity, monoblasts develop into promonocytes and ultimately to monocytes\textsuperscript{36}. The identification of these cells was accomplished by van Furth \textit{et al.} using the metabolic incorporation of \textsuperscript{3}H-thymidine to monitor cell proliferation\textsuperscript{42}.

Interestingly, there is no single “master” transcription factor that is expressed exclusively during myeloid differentiation, but a combination of several transcription factors\textsuperscript{34}. PU.1, a member of the \textit{ets} transcription factor family, binds to the promoters of M-CSFR and GM-CSFR. Moreover, C/EBP\(\alpha\), \(\beta\), and \(\delta\) isoforms are upregulated in early myeloid cells, and also regulate M-CSFR and GM-CSFR levels\textsuperscript{43}. As described above, signaling through these receptors is crucial for CMPs to differentiate into monocytes that are released from the bone marrow\textsuperscript{44}. In the bloodstream the monocytes use adhesion receptors to localize to many tissue compartments where various cytokines and growth factors contribute to further cell differentiation\textsuperscript{37}. Stimulatory cytokines such as M-CSF and the upregulation of PU.1 and C/EBP are required for normal myeloid development.

Under pathological conditions, such as chronic microbial infection, cancer, or transplantation the myelopoiesis process is affected. Here, the maturation of antigen presenting macrophages is inhibited and a subset of immature myeloid cells begins to develop\textsuperscript{45}. MDSCs are a heterogeneous group of myeloid progenitor cells that have the ability to suppress T cell function and, in mice, are characterized by the expression of both Gr-1 and CD11b. Antibodies that target
Gr-1 bind to two different epitopes, Ly6G and L6yC. About 30% of cells in the bone marrow are Gr-1+ CD11b+. Furthermore, there are two subsets of MDSCs: CD11b+ Ly6G+ Ly6C- granulocytes (G-MDSCs) and CD11b+ Ly6G- Ly6C- monocytes (MO-MDSCs)\(^46\). They differ not only in phenotype and morphology, but effector mechanisms. MDSCs were first identified in tumor models, and where show to make up almost 40% of leukocytes within the spleens of transgenic mice overexpressing oncogenes\(^47\). Additionally, they have been detected in the blood of human cancer patients. In these models, G-MDSCs were shown to preferentially expand when compared to MO-MDSCs\(^48\). The expansion of MDSCs is directly caused by the secretion of growth factors and cytokines by cells in these pathological conditions. For example, in bacterial infections, T cells producing IFN-γ stimulated the proliferation of MDSCs\(^49\). Moreover, the number of these cells have also been shown to increase in autoimmune diseases such as experimental autoimmune encephalomyelitis and autoimmune uveoretinitis\(^50,51\). Investigation of MDSCs in different disease models has pointed toward an immunomodulatory and suppressive role for MDSCs.
1.2b) MDSC Expansion

During an inflammatory response, the stromal milieu secretes soluble mediators that induce the expansion of MDSCs. These cytokines and growth-factors promote myelopoiesis and prevent the maturation of myeloid cells. Some factors that induce the expansion of suppressive immature myeloid cells include: stem-cell factor (SCF), prostaglandin E2 (PGE2), M-CSF, and many other molecules. MDSCs that infiltrate into cancer tissue may stimulate the development of intratumoral T regulatory cells and promote T cell anergy. These mechanisms are used by various cancers to avoid immunosurveillance. Chen et al. have identified various factors that are upregulated during tumor growth, and one was SCF. The receptor for this cytokine is CD117 (c-KIT). Using tumor-bearing mice, SCF siRNA knockdown, and CD117 antagonizing antibodies the group was able to demonstrate a substantial decrease in MDSC expansion and restored proliferative capability of tumor infiltrating T cells. Therefore, outlining the importance of the SCF cKIT interaction in promoting MDSC expansion during inflammation. Moreover, the Ostrand-Rosenberg group generated data that implicates pro-inflammatory lipid-mediators in MDSC expansion. In vitro work using PGE2 agonists showed the expansion of Gr-1+ CD11b+ MDSCs in vitro. Additionally, the importance of PGE2 in MDSC expansion was validated using in vivo models where MDSC proliferation was reduced in BALB/c PGE2 receptor knockout transgenic mice and mice treated with COX-2 inhibitor that were subject to 4T1 mammary carcinoma cell transplantation. Finally, using a heterotopic heart transplantation model Ochando et al. realized that the interference with M-CSF receptor signaling prevented the induction of MDSCs.
mediated regulatory tolerance\textsuperscript{32}. Together this data demonstrates that soluble factors that are secreted during the inflammatory are implicated in promoting MDSC expansion.

The cytokines described above mediate their activity through Janus kinase (JAK) protein family members and signal transducer and activator of transcription 3 (STAT3). Upon engagement, JAK2/STAT3 promote cell survival and differentiation signals through signal transduction events\textsuperscript{54}. Gabrilovich \textit{et al.} showed that using a STAT3 inhibitor, JSI-124, the MDSC infiltrate levels in a mouse CT26 colon carcinoma model decreased, and promoted the accumulation of mature macrophages\textsuperscript{55}. These results were confirmed by the Yu group, which showed that inhibiting STAT3 signaling in Cre-STAT3\textsuperscript{lox/loxp} transgenic mice, which were inoculated with MB49 or B16 tumor cells, enhanced immunosurveillance and the function of effector leukocytes\textsuperscript{56}. Together, these results associate STAT3 as a main transcription factor needed for MDSC expansion and function.

One mechanism that JAK2/STAT3 system utilizes to expand MDSCs is through the upregulation of S100 calcium-binding proteins A8 and A9 (S100A8 and S100A9). The presence of these proteins prevent myeloid progenitor cell differentiation and aid in the homing of MDSCs into tumor sites\textsuperscript{31}. The Gabrilovich group showed that the overexpression of S100A9 in stem cells or transgenic mice promoted the differentiation of MDSCs, and inhibited development of mature macrophages and dendritic cells. Additionally, the STAT3 dependent upregulation of S100A9 was correlated with enhanced MDSC accumulation in tumor-bearing mice\textsuperscript{57}. Elevated levels of S100A8 and S100A9 are common in pro-inflammatory conditions such as cell damage or infection. These proteins bind to receptors for advanced glycation end-products (RAGE), such as carboxylated N-glycans that are expressed on the surface of MDSCs. Srikrishna \textit{et al.} showed that
blocking S100A8 and S100A9 binding to RAGE using anti-carboxylated glycan antibodies reduced the levels of circulating MDSCs in the blood of mice with metastatic disease. S100A8/A9 mediated autocrine signaling promotes the induction of MDSCs from immature myeloid cells. In conclusion, molecules such as IL-6, M-CSF, and PGE2 that are secreted during an inflammatory response bind to JAK2/STAT3 receptors on IMCs. This event promotes MDSC development through the upregulation of survival genes, such as cyclin D1, B-cell lymphoma XL (Bcl-XL), and MYC. Additionally, STAT3 dependent upregulation of S100A8/A9 further enhance MDSC development and accumulation in sites of inflammation.

1.2c) MDSC Activation

Signaling through JAK2/STAT3 promotes the expansion of MDSCs, but does not upregulate genes that mediate suppressive activity. Molecules that are secreted by inflamed stromal cells, stimulated T cells, and pathogens alter the gene expression of MDSCs, and induce iNOS, arginase-1, and TGF-β activity. The signal transduction pathway is mediated through STAT1, STAT6, and MyD88. Both MO-MDSCs and G-MDSCs have the capability to suppress T cell function and contribute to immune evasion mechanisms utilized by a variety of cancer. However, monocytic and granulocytic MDSCs use different effector mechanisms to do this. Binding of IFN-γ to its cognate receptor promotes the docking of STAT1 to the intracellular domain of the receptor. A cascade of phosphorylation events mediated by STAT1 promotes the production of iNOS mRNA, which is expressed in MO-MDSCs. Thus, blocking IFN-γ signaling disrupts only MO-MDSC function and promotes expansion of CD8+ intratumoral T cells. Moreover, this was
confirmed by showing iNOS transcript was unable to be detected by quantitative reverse transcription PCR (q-rt-PCR) from STAT1−/− MDSCs61. Collectively, this data shows that iNOS function in MO-MDSCs is STAT1 dependent, and nitrosylation events facilitated by this enzyme abrogate T cell function (this will be discussed in the following section).

Monocytic and granulocytic MDSCs share a common mechanism to suppress lymphocyte function and that is through the expression of arginase-1. This enzyme is regulated by STAT6, which binds to the intracellular domain of a type I heterodimeric cytokine receptor. T helper 2 (Th2) cytokines, IL-4 and IL-13, bind to this receptor and are required for STAT6-dependent arginase-1 activity62. In line with these observations, the blockade of STAT6 via STAT6+/− transgenic mice, inhibits arginine metabolism by activated MDSCs63. Interestingly, MDSCs use the STAT6 pathway to also secret TGF-β, which promotes regulatory tolerance and reduced immunosurveillance64. The depletion of essential amino acids has a negative effect on T cell function, and is one mechanisms used by suppressive myeloid cells collectively.

PRRs play an essential role in detecting microbial derived pathogen associated molecular patterns (PAMPs) and DAMPs. The engagement of either surface or intracellular PRRs initiates an innate immune response characterized by inflammation and an anti-viral response. Under septic conditions, antigen presenting cells and T lymphocytes are dysfunctional65. This observation has put tremendous focus on identifying what leads to the loss of immunocompetence. In chronic inflammation models the population of immature myeloid cells tends to increase in secondary lymphoid organs66. The Moldawer group not only showed that MDSCs are the primary cell type contributing to T cell suppression in septic mice, but also adaptor proteins of PRRs are necessary for the activation of these MDSCs31,67. MyD88 and TIR domain-containing adaptor-inducing IFN-
β (TIRF) adaptor proteins bind to the domains of PRRs and recruit other proteins that activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). This transcription factor is responsible for upregulating both arginase and iNOS enzyme in MDSCs\(^3\). Altogether, this work indicates that the activation of MDSCs is a direct outcome of the innate immune response and cytokines secreted by both stromal and immune cells.

1.3 MDSC Effector Functions

1.3a) L-Arginine Metabolism

Amino acids are essential subunits that make up proteins, which are important in a myriad of biological functions. Amino acids, such as L-arginine, are the precursors for other organic molecules that play a crucial role in the normal physiology of mammals, such as nitric oxide, urea, and proline\(^6\). The importance of this amino acid in the diet of humans has made it a subject of extensive investigation. Recently, the metabolism of this amino acid has been shown to play a role in abrogating the immune response\(^6\). However, before exploring the mechanism of amino acid metabolism, it is crucial to define how L-arginine is synthesized and metabolized under normal physiological conditions. L-glutamine and L-proline are the main precursor amino acids for the synthesis of L-arginine, which is catalyzed by phosphate-dependent glutaminase, ornithine aminotransferase (OAT), argininosuccinate synthase (ASS), argininosuccinate lyase (ASL), aspartate aminotransferase, carbomoyl-phosphate synthase I (CPS I), ornithine carbamoyltransferase (OCT), and N-acetylglutamate\(^7\). The distribution of these enzymes varies
in different cell types throughout mammals, and the complete pathway for net arginine in adult mammals occurs through the intestinal-renal axis. The intestinal mucosa synthesizes L-citrulline, which is then secreted into the blood where the conversion to L-arginine occurs in the kidney\(^7\). L-arginine can be metabolized through multiple pathways, and the two enzymes that compete for L-arginine as a substrate are arginase and nitric oxide synthase\(^6\).

Mammals encode two isoforms of the arginase enzyme, which are under the control of the \(\text{ARG1}\) and \(\text{ARG2}\) genes\(^6\). Although these enzymes share homology in their genetic sequence, they are localized to different organs in mammals. Arginase-1 is found in the cytosol of hepatocytes, while arginase-2 is constitutively expressed and located in the mitochondria of various cells\(^6\). Moreover, under STAT6 signaling, arginase-1 is upregulated in myeloid cells when exposed to cytokines, such as IL-4, IL-13, GM-CSF, and TGF-\(\beta\)\(^7\). Another group of enzymes that bind to L-arginine are the 3 isoforms of NOS, which include: neuronal NOS (nNOS or \(\text{NOS1}\)), inducible NOS (iNOS or \(\text{NOS2}\)), and endothelial NOS (eNOS). These dimeric enzymes require calmodulin and numerous co-factors for their full activation. \(\text{NOS2}\) has been shown to be upregulated in many leukocytes, and just like arginase-1, requires signaling through STAT6 or STAT1\(^6\).

Signal transduction through STAT6 upregulates the mouse cationic amino-acid transporters CAT2A, CAT2B, CAT3 and CAT4, which transport various amino acids including L-arginine\(^7\). Under normal conditions, when L-arginine is transported inside cells, arginase-1 binds the amino acid and catalyzes the production of L-ornithine and a by-product, urea. Downstream reactions mediated by ornithine decarboxylase (ODC) and OAT, induce cell division and collagen synthesis\(^6\). However, when L-arginine is scarce an alternative pathway for catabolism is
activated. Here, NOS enzymes bind L-arginine to produce the L-arginine precursor L-citrulline and NO. This reaction generates superoxides as a by-product, which reacts with NO to produce reactive nitrogen species (RNS) that cause oxidative damage to macromolecules. The depletion of L-arginine and generation of oxidative molecules have been implicated in the immunoregulatory functions of MDSCs.

NOS2 and arginase-1 expression in MO-MDSCs are regulated by T helper 1 (TH1) and TH2 cytokines. The TH1 cytokine IFN-γ promotes the production of NO by NOS2, while TH2 cytokines like IL-4 and IL-13 inhibit NOS2 and induces arginase-1 activity. This is a simplified scheme, as studies have also shown both STAT1 and STAT6 pathways activate NOS2 and arginase-1 expression. Ochoa et al. showed that tumor infiltrating myeloid cells, CD11b+ ARG1+ CAT2B+, impaired T cell function by reducing the expression of the T cell receptor component CD3 ζ. The injection of arginase inhibitors reversed this effect in 3LL lung carcinoma model. Additionally, the depletion of L-arginine and the production of urea have been shown to negatively impact the translation of proteins. Amino acid starvation results in the activation of general control non-depressible 2 (GCN2) and catalyzes the removal of a phosphate group on eukaryotic translation initiation factor 2 (EIF2α), which effectively stops translation. Moreover, the activity of arginase-1 effects the mTOR pathway, which regulates metabolic function through the sensing of nutrients. Aberrations in the MTOR pathway and the suppression of protein synthesis caused by arginase-1 activity is another mechanism that reduced T cell proliferation and function. NO effects T cell function through the regulation of downstream T cell receptor (TCR) intracellular singling. This is accomplished through the nitrosylation of tyrosine or cysteine residues or by the activation of other kinases, which mediate the blockade of JAK/STAT signaling of TCRs. MDSCs
expression of arginase-1 and NOS2 mediate T cell dysfunction and promote a state of immunosuppression.

1.3b) Generation of Reactive Oxygen Species

Reactive oxygen species (ROS), such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl (OH•) are by-products of aerobic respiration$^{83}$. These molecules are involved in initiating oxidative stress, which leads to pathology, and serve as signaling molecule that regulate normal biological processes$^{84,85}$. Superoxides are produced by redox reactions in the electron transport chain of mitochondria during normal respiration, and by the membrane-bound enzyme called NADPH oxidase. Superoxide dismutases 1 and 2 (SOD1/2), located in the cytosol and the mitochondrial membrane, convert superoxides into hydrogen peroxide$^{83,86}$. Finally, hydrogen peroxide is produced from the reaction between hydrogen peroxide and ferrous ions. At normal physiological levels ROS function as signaling molecules, while the excessive accumulation of these molecules in the environment result in the oxidation of lipids, proteins, and DNA, leading to cellular and DNA damage$^{87}$.

Growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), promote cell proliferation. This event is facilitated by autophosphorylation events downstream of receptor tyrosine kinase (RTK) activation$^{88}$. During this process, the RTK pathway is regulated by phosphatases including: protein tyrosine phosphatases (PTPs) and PTEN$^{89,90}$. Interestingly, growth factors mediate the production of ROS through the activation of NADPH oxidases. The secretion of hydrogen peroxide promotes cell proliferation by oxidizing and
effectively disabling phosphatases that reduce mitogenic signaling. Under normal conditions, cell proliferation by growth factors require ROS secondary messengers\(^91\). In addition to cell expansion, ROS are essential redox messengers for the activation of immune cells. Innate immune cells respond to pathological conditions through sensing PAMPs and DAMPs. When danger signals bind to their respective PRR, ROS production is increased and activate inflammasomes, which promote the production of pro-inflammatory cytokines\(^83\). Moreover, ROS production from the cell surface and mitochondria have been shown to promote T and B lymphocyte proliferation\(^92,93\). Prolonged production of ROS, however, can promote tumorigenesis and autoimmunity through sustained cellular growth and chronic inflammation\(^83\). Additionally, in the absence of antioxidants, the dysregulation of NADPH oxidase enzymes promotes apoptosis by inducing mitochondrial stress and the release of pro-apoptotic mediators\(^94\).

During a normal immune response, mature phagocytes utilize ROS to reduce infection, enhance PRR signaling, and promote adaptive immune function\(^95\). Granulocytic MDSCs produce ROS through STAT3 signaling, and the upregulation of S100A8 and S100A9 proteins. Additionally, growth factors and pro-inflammatory cytokines have been shown to induce ROS production by G-MDSCs\(^96\). Superoxide anions produced by G-MDSCs react with NO to produce peroxynitrite, which is one of the most powerful oxidants produced in mammals\(^31\). This reaction augments the immunosuppressive effect of T cell nitration caused by NO discussed earlier.

**1.3c) Induction of T Regulatory Cells**
Thymic generation of T cells from common lymphoid progenitor cells is accomplished through a wide-array of elaborate cellular reactions. The random rearrangement of variable (V), joining (J), and diversity (D) segments in the formation of immunoglobulins (Ig) and TCRs is possibly the most important event in the generation of B and T cells respectively\textsuperscript{97}. This method of producing a highly variable TCR repertoire often times may result in the production of autoreactive T lymphocytes. Fortunately, most are deleted during negative selection event in the thymus; however, some do escape into the periphery\textsuperscript{80}. The expansion of these self-reactive cells may result in the development of diseases, such as type I diabetes, asthma, and inflammatory bowel disease\textsuperscript{98}. In order to maintain homeostasis many regulatory mechanism are in place to prevent these ailments. Principal among these mechanisms is the generation of Tregs through deletional and regulatory tolerance.

Tregs utilize four main mechanisms to suppress aberrant immune cells function through the production of inhibitory cytokines, cytolysis, metabolic disruption, and targeting stimulatory molecules on APCs. The three main inhibitory molecules produced by Tregs are IL-10 and TGF-β\textsuperscript{98}. Upon secretion, IL-10 binds to the IL-10 receptor and signals through a JAK/STAT signaling pathway, where STAT3 is the required downstream transcription factor for IL-10 mediated suppression\textsuperscript{99}. The dimerization of this transcription factor induces the expression of genes in apoptotic, cell migration, cell cycle, and cytokine pathways. In particular STAT3 activates the upregulation of Bcl-XL, IL-10, TGF-β, PD-1, and the downregulation of genes required for antigen presentation\textsuperscript{100}. TGF-beta binding to its respective receptor induces apoptosis through the SMAD pathway, reduced cell proliferation by downregulating the gene MYC, and blocks the activation of leukocytes\textsuperscript{101}. Moreover, Tregs secret granzyme-b, serine protease, and perforin to induce
programmed cell death of target cell. This is done through destabilizing genomic material and organelles, which in turn promote the upregulation of pro-apoptotic genes\textsuperscript{102}. Tregs are characterized by high expressions of CD25, which is the receptor of IL-2, and rapidly consume IL-2 in the microenvironment effectively starving activated T cells from this essential cytokine necessary for T cell proliferation\textsuperscript{103}. Finally, Tregs induce suppression of target cells by direct cellular contact. Tregs express cytotoxic T-lymphocyte antigen 4 (CTLA-4), which competes for CD80 and CD86 co-stimulatory molecules with CD28. CTLA-4 prevents T cell activation by preventing co-stimulatory signals and singling through the TCR\textsuperscript{104}. Treg function and development are dependent on the expression of the key transcription factor, forkhead box P3 (FoxP3)\textsuperscript{98}.

Tregs are generated by mechanisms of deletional and peripheral tolerance, which occur in the thymus (tTregs) and periphery (iTregs) respectively. After naive thymocytes pass through the selection process in the cortex of the thymus and upregulated the CD4 co-receptor they interact with medullary thymic epithelial cells (mTECs), DCs, macrophages, and other antigen presenting cells in the medulla. In this instance, naive thymocytes undergo another round of selection dependent on the TCR signal strength via interaction with cognate peptide-MHC. A strong signal through the TCR promotes deletion; a low signal supports the formation of conventional T cells, while a moderate TCR signal strength stimulates FoxP3 induction and the development of Tregs\textsuperscript{105}. Additionally, cytokine signaling through the common gamma chain (\(\gamma_c\)) with cytokines such as IL-2, IL-7, and IL-15 are also important for FoxP3 induction\textsuperscript{106}. Signal transduction through this receptor activates STAT5, which binds to a nuclear region of the FoxP3 gene and encourages expression of this transcription factor\textsuperscript{107}. Moreover, the induction of iTregs
from peripheral CD4+ T cells is dependent on the binding of high-affinity antigen to the T cell receptor, along with reduced co-stimulation brought about by increased CTLA-4 expression and reduced CD28 activation\textsuperscript{108,109}. As in tTreg induction IL-2 is crucial; however, in iTreg development, TGF-β is also important. This cytokine promotes SMAD3 and nuclear factor of activated T-cells (NFAT) binding to FoxP3, survival factors, and antagonizing the recruitment of maintenance DNA methyltransferase I (Dnmt1)\textsuperscript{105}. Deletional and regulatory tolerance mechanisms are important in maintaining immune homeostasis.

Recent studies have demonstrated the ability of MDSCs to promote suppression through the induction of Tregs in tumors\textsuperscript{110}. MDSC mediation of iTreg development was found to require CTLA-4 expression, and was independent of TGF-β secretion by MDSCs\textsuperscript{111,112}. Conversely, another group found that MDSCs were not involved in the expansion of Tregs\textsuperscript{60}. In this study we showed that MDSCs promoted the induction of Tregs (Figure 2.3). The role that MDSCs play in the induction of peripheral tolerance is not clear. However, it seems possible that these cells can induce Tregs through the processing of alloantigen and direct cell-to-cell contact.
1.4 MDSCs and the Current State of Transplant Research

Currently, many groups are studying MDSCs and exploring their role in preventing allograft rejection. Dr. Zhiqiang Pan and colleagues are investigating the potential of sepsis induced MDSCs in prolonging allograft survival, particularly in corneal and skin transplant models. In this model, female B6 mice were donors and female B6 mice were recipients. Sepsis induced MDSCs were isolated from cohorts of B6 mice, which underwent a cecal ligation and puncture procedure. Here, the cecum is punctured, promoting the release of fecal material into the peritoneal cavity. This event stimulates an exacerbated immune response. MDSCs were isolated from the bone marrow and stained with anti-CD11b and anti-Gr-1 monoclonal labeled antibodies. MDSCs were then enriched using fluorescence activated cell sorting. These sepsis induced MDSCs were adoptively transferred into either corneal or skin transplant recipients. Graft survival was scored, and results show that group which received MDSCs significantly prolonged graft survival. This data suggests that MDSCs may be used as a novel cellular based therapeutic method to promote graft acceptance\textsuperscript{113}.

Moreover, clinical studies have shown that patients who show an increase in the expression of S100A8/A9 proteins have a better clinical outcome after transplantation\textsuperscript{114}. As we have discussed earlier in chapter 1, these proteins are essential components for MDSCs activation. In fact, Dr. Eikmans’ group who published this recent study showed that these proteins also correlated with MDSCs. Here, peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors, and MDSCs were enriched and cultured with M-CSF, IL-4, and LPS.
Functional assays showed that increased production of ROS by these cells reduced the activity of DCs, and prevented DC mediated alloactivation of T cells. The effect of MDSCs on antigen presentation as well as T cell activation proves that MDSCs are of significant importance to transplantation outcome.

Finally, MDSCs have also been implicated in the efficacy of new drugs used for transplantation. Hematopoietic cell transplantation has become a common curative practice for patients with hematological malignancies. Graft-versus-host disease (GvHD) is a common concern after bone marrow transplantation and can be avoided through the administration of drugs, such as cyclophosphamide. However, it has recently been shown that this drug is associated with increased relapse. For this reason the feasibility of other drugs were investigated. Dr. Katsanis and colleagues have recently shown that bendamustine significantly reduces GvHD and improves survival rates when compared to cyclophosphamide. Here they show that bendamustine is less myelosuppressive than other medication, and promotes the development of CD11b+ Gr-1+ MDSCs. In culture these suppressive MDSCs impair T and B cell proliferation, which may contribute to GvHD. Together this data shows that the efficacy of bendamustine is associated with the expansion of MDSCs. MDSCs have been studied mostly in the context of cancer immunology; however, recently data suggest that these cells play a paramount role in allograft acceptance. Further studies of these cells are necessary to develop new novel therapeutics to improve clinical outcomes of transplant recipients.
1.5 Conclusion

We have made significant advances in transplant immunology over the past few decades. Dr. Medawar group was the first to identify cell-mediated rejection of allografts, and demonstrated adaptive tolerance through the first chimerism experiments. Using this information and developing new surgical techniques Dr. Murray's team was able to conduct the first successful transplant. However, despite our increased knowledge of the immune system and the pathology of organ rejection transplant recipients continue to face major complications, which can be attributed to chronic immunosuppression and the lack of tolerance toward donor antigens. A promising solution that can solve both of these problems is the induction of mixed chimerism. In this model, the donor and recipient immune systems live in harmony and donor APCs aid in the deletion of alloreactive T cells in the thymus. The efficacy of this model has been demonstrated in the clinic, but chimerism is transient. This implies that the long-term acceptance of allografts in mixed chimerism patients is attributed to regulatory rather than deletional tolerance. For this reason it is important to dissect what components of the hematopoietic system influence regulatory tolerance. Possible candidates are a group of heterogeneous cells of myeloid origin called MDSCs.

MDSCs arise from normal CMPs produced during myelopoiesis, however under pathological conditions such as chronic microbial infection, cancer, or transplantation stromal cells release pro-inflammatory cytokines which mediate the suppression of myeloid maturation and the development of MDSCs. These cells promote suppression through a variety of mechanisms, such as the depletion of essential amino acids, production of reactive oxygen
species, and the induction of regulatory T cells. MDSCs have been shown to be important in the
development of tumors, and their depletion results in increased activity of tumor infiltrating
leukocytes. The exploitation of the suppressive activity of MDSCs may prove to be therapeutic to
individuals who are recipient of allografts.
Chapter 2: Myeloid-Derived Suppressor Cells Alter Phenotypic Expression Pattern and Induce the Development of T Regulatory Cells In Vitro

2.1 Abstract

Myeloid-Derived Suppressor Cells are a heterogeneous population of immature myeloid cells. The two major subsets are granulocytic and monocytic MDSCs, which differ in morphology and effector functions. CD11b⁺ Ly6G⁺ Ly6C⁻ G-MDSCs are expanded in tumor-bearing mice, and suppress immune function through the secretion of ROS and depletion of L-arginine from the microenvironment. CD11b⁺ Ly6G⁻ Ly6C⁺ MO-MDSCs also mediate the suppression of lymphocytes, but do so by the production of RNS such as peroxynitrite. Although these cells have been implicated in the production of IL-10 and the induction of T regulatory cells, it is unclear if MO-MDSCs can do the same through direct cell-to-cell interaction. The aim of this study is to develop an in vitro culture technique to produce suppressive MO-MDSCs, and elucidate their role in promoting regulatory tolerance. MO-MDSCs were enriched using a negative selection protocol, and cultured with stimulating cytokines such as IL-4 and M-CSF. To engage the MyD88 pathway, the myeloid cells were also cultured with the TLR4 ligand, LPS. Flow cytometry and naïve T cell cultures were used to determine suppressive MO-MDSC phenotype and the capability to induce Tregs. After stimulation with IL-4, M-CSF, and LPS, MO-MDSCs dramatically altered their gene expression profile. Ly6C, podoplanin, CD80, and CD86 markers were reduced, while PD-L1 and F4/80 gene expression increased. These MDSCs were also able to induce T regulatory cells in an allogeneic fashion. Moreover, histological analysis showed that CD11b⁺ Ly6C⁺ and
F4/80+ myeloid cells accumulate in accepted renal allografts. Together, these results indicate that stimulation with IL-4, M-CSF, and LPS cause the differentiation of these immature myeloid cells to macrophage like cells. The induction of T regulatory cells was dependent on MyD88 activity, and we hypothesize that it is achieved through direct alloantigen presentation to naïve T cells. The localization of CD11b+ Ly6Clo cells and the functional characteristics of cultured MO-MDSCs provide initial evidence that these cells may play a role in the induction of regulatory tolerance to promote graft survival.

2.2 Introduction

A significant challenge in transplant immunology is the establishment of a protocol, which promotes tolerance to donor antigens without the continued use of immunosuppressive drugs. A successful technique, such as this, would reduce immune mediated graft destruction and the prescription of immunosuppressant medication. To date, one method has been quite successful in the clinic, mixed chimerism115. However, there are a few issues with this approach, which prevent it from becoming standard practice. One such problem is the harsh conditioning required before bone marrow engraftment21. Accordingly, it is important to identify what hematopoietic cells are necessary to prevent rejection, so updated methods of intervention can be developed. Gabrilovich and colleagues have made substantial contributions to our understanding of myeloid-derived suppressor cells. These immature progenitors have been mostly studied within the context of cancer immunology, and have been shown to contribute to tumor metastasis48.
Recently, their role in transplantation has been described by Ochando et al., where they provide evidence showing mechanisms MO-MDSCs utilize to promote regulatory tolerance\textsuperscript{32}. MDSCs suppress the immune system through the secretion of cytokines and reactive species; moreover, their potential to promote tolerance through cell-to-cell contact is unclear. The goal of our study was two-fold, which was to design an MO-MDSC induction protocol, and to elucidate their capacity to induce T regulatory cells through direct TCR interaction.

2.3 Results

2.3a) Enrichment of Monocytic Myeloid-Derived Suppressor Cells

*Hypothesis:* Labeling unwanted cells with biotinylated antibodies directed against non-monocytes will provide a higher yield of MO-MDSCs compared to enrichment protocols that target the non-specific Gr-1 marker.

Myeloid-derived suppressor cells are a heterogeneous group of immature cells. As described earlier, they are characterized by the expression of CD11b and lymphocyte antigen 6 (Ly6)\textsuperscript{116}. The former makes up a subunit of the heterodimeric integrin $\alpha_M\beta_2$, also known as macrophage-1 antigen (Mac-1)\textsuperscript{117}. Ly6 can be subdivided into groups, Ly6G and Ly6C. MDSCs with granulocytic morphology express high levels of Ly6G, while Ly6C is a common surface marker for the monocytic subtypes\textsuperscript{31}. These progenitor cells are created from the normal myelopoiesis
process in the bone marrow, and differentiate into suppressive cells within pro-inflammatory environments\textsuperscript{34}. MO-MDSCs have been shown to play an important role in transplant tolerance and the induction of T regulatory cells\textsuperscript{118}. In order to develop an \textit{in vitro} culture technique, we first needed to appropriately enrich CD11b\textsuperscript{+} Ly6C\textsuperscript{+} cells from the bone marrow. Immunohistochemical analysis showed that Gr-1\textsuperscript{+} cells (anti-Gr-1 monoclonal antibody is reactive to both Ly6G and Ly6C epitopes) accumulate in renal allografts that were harvested six weeks after transplantation (\textit{Figure 2.1 A}). The Gr-1 cells organized themselves within the lymphocytic infiltrate of TOLS. Interestingly, cells expressing Ly6C were not found in the TOLS, but in the vasculature that TOLS surround (\textit{Figure 2.1 B}). The validation that our cells of interest can be found in renal allografts, prompted us to isolate MDSCs based on the Gr-1 marker. MDSCs were collected in two fractions. Using flow cytometry, the mean fluorescence intensity (MFI), with respect to the Gr-1 surface marker, indicated that two separate populations were isolated (\textit{Figure 2.1 C}). Due to the cross-reactivity of the Gr-1 antibody to both Ly6 antigens, it was necessary to employ a different technique that would allow for the enrichment of a more pure population of Ly6C monocytes. This was achieved using a negative selection protocol, where the unlabeled fraction collected was almost all positive for Ly6C\textsuperscript{hi} monocytes, and the labeled fraction was completely void (\textit{Figure 2.1 D}). Here we were able to successfully enrich a population of CD11b\textsuperscript{+} Ly6C\textsuperscript{hi} monocytes from the bone marrow of non-pathogenic mice using a negative selection protocol.
Figure 2.1 Enrichment of CD11b<sup>+</sup> Ly6C<sup>hi</sup> Monocytic Myeloid-Derived Suppressor Cells. Transplants were conducted between MHC-disparate mice (DBA→B6), and allografts were harvested after 42 days. Histological slides were fixed in formalin and embedded in paraffin. Immunohistochemical analysis of renal allograft was conducted using A) anti-Gr-1 and B) anti-Ly6C antibodies. C) Bone marrow cells were obtained from B6 mice, and a positive selection protocol was enacted to isolate Ly6G and Ly6C myeloid cells. This was done using antigen specific biotin antibodies and streptavidin beads to Gr-1. The first positive fraction contained G-MDSCs, while the second positive fraction contained MO-MDSCs. D) Ly6C<sup>hi</sup> monocytes were enriched using a negative selection protocol via the addition of biotinylated and tetrameric magnetic antibodies directed against non-monocytes. Gr-1 and Ly6C fluorescently labeled antibodies were used for flow cytometric analysis. Quadrant boundaries were set by isotype controls.
2.3b) Phenotypic Analysis of Cultured Monocytic Myeloid-Derived Suppressor Cells

Hypothesis: Culturing MO-MDSCs with the MyD88 stimulant, LPS, will result in the increased expression of co-inhibitory markers.

The successful enrichment of CD11b$^+$ Ly6C$^{hi}$ monocytes allowed us to develop proper in vitro culture, which would allow the differentiation of suppressive MO-MDSCs. As described in chapter 1, adequate myelopoiesis events required growth factors, such as M-CSF and GM-CSF. These cytokines stimulate pathways that lead to the expression of PU.1 as well as C/EBPα, β, and δ isoforms, which are important myeloid lineage transcription factors$^{34}$. Moreover, MDSC development requires additional signals to promote expansion and activation. M-CSF and IL-4 mediated signal transduction events results in the expression of survival genes (MYC, BCL-XL, and cyclin D1) and the expression of arginine metabolizing enzymes (iNOS and arginase-1) respectively$^{31}$. Interestingly, in addition to the cytokines mentioned, pro-inflammatory pathways governed by the adaptor protein MyD88 also need to be activated so that the full suppressive capacity of MDSCs can be realized$^{67}$. Taking this information into account an in vitro culture technique was developed. Here, CD11b$^+$ Ly6C$^{hi}$ monocytes were enriched from the bone marrow of mice, and cultured with IL-4, M-CSF, and LPS. Co-stimulatory, co-inhibitory, morphological, and intracellular markers were analyzed using flow cytometry.

Phenotypic analysis of respective markers were conducted in three separate groups: naïve MO-MDSCs, MO-MDSCs cultured with growth factors, and MO-MDSCs cultured with growth factors and LPS. Two co-stimulatory molecules that were of great interest were CD86
(B7.2) and CD80 (B7.1). The interaction between these co-stimulatory molecules and CD28 on T cells lowers the threshold of activation required for T lymphocyte stimulation\textsuperscript{119}. CD80 and CD86 are expressed on professional antigen presenting cells, and we wanted to analyze the relative expression patterns of these molecules across our indicated three groups. Naïve CD11b\textsuperscript{+} Ly6C\textsuperscript{hi} cells isolated from naïve mice did not express either of these molecules. However, upon culture with M-CSF and IL-4 both CD80 and CD86 expression increased, with CD86 upregulation nearly twice as abundant when compared to CD80. Furthermore, addition of LPS to the culture promoted cells to dramatically reduce expression of these co-stimulatory molecules to levels comparable with the naïve compartment (Figure 2.2 A and B). Interestingly, PD-L1 expression increased with the addition of M-CSF and IL-4. The same result was seen with the LPS group, but the induction was not as intense (Figure 2.2 C). Ochando and colleagues, using microarray, showed that monocytes infiltrating the myocardium increased the expression of a PRR, DC-SIGN, which was explained to be necessary in the secretion of IL-10 and induction of regulatory tolerance\textsuperscript{32}. Our culture failed to show any increase in DC-SIGN expression across all groups, suggesting that other mediators that are present within the graft are needed for expression (Figure 2.2 D).

In addition to innate stimulatory and inhibitory molecules, we examined the expression of macrophage markers. Interestingly, we found that Ly6C expression is reduced after culture with cytokines, with and without LPS (Figure 2.2 E). Additionally, enriched MDSCs prior to culture did not express F4/80, a mature macrophage marker; however, they seemed to upregulate this surface protein in the presence of cytokines regardless LPS addition (Figure 2.2 F). Some studies have shown that CD11b\textsuperscript{+} inflammation induced macrophages promote lymphangiogenesis, and
we wanted to examine if MO-MDSCs express the lymphatic endothelium marker podoplanin\textsuperscript{120}. We saw that prior to culture our cells of interest did not express this marker, but after stimulation with the respective growth factors, about 25% of CD11b\textsuperscript{+} cells upregulate podoplanin. Levels were reduced after LPS addition, but were still greater than naïve MO-MDSCs (Figure 2.2 G). Finally, one mechanism in which monocytic MDSCs suppress T cell activity is through regulation of L-arginine metabolism. So, we examined intracellular iNOS levels, which were not substantially increased after culture (Figure 2.2 H). Our results indicate that after stimulation with M-CSF, IL-4, and LPS there is alteration in the expression of both surface and intracellular genes. Groups stimulated with LPS, which are theoretically suppressive, increased their expression of CD86 and podoplanin when compared to the group not cultured with the MyD88 stimulant.
Figure 2.2 Phenotypic Analysis of Cultured CD11b⁺ Ly6C⁺ Monocytic Myeloid-Derived Suppressor Cells. MO-MDSCs were enriched using the protocol described in Figure 2.1, and were plated at 5x10⁴ cells per well. These monocytes were supplemented with 10ng/mL of IL-4 and M-CSF. After 48 hours 100ng/mL of LPS was added to respective groups. Surface and intracellular markers were then analyzed using flow cytometry. The fluorescently labeled antibodies there were used, include: A) anti-CD86 B) anti-CD80 C) anti-PD-L1 D) anti-DC-SIGN E) anti-Ly6C F) anti-F4/80 G) anti-podoplanin H) anti-iNOS. Quadrant boundaries were set by isotype controls.
2.3c) Effect of Monocytic Myeloid-Derived Suppressor Cells on the Induction of Tregs

Hypothesis: MO-MDSCs cultured with IL-4, M-CSF, and LPS have the potential to promote regulatory tolerance through the induction of Tregs.

T Regulatory cells play an important role in modulating the immune response. These cells function through the expression of inhibitory molecules, secretion of cytotoxic mediators, release of anti-inflammatory cytokines, and the depletion of local IL-2\textsuperscript{105}. MDSCs have recently been shown to induce Tregs in tumor models, but other studies suggest that these immature myeloid cells are not involved in Treg induction\textsuperscript{31,112}. Previously we have shown that allograft tolerance requires the development and maintenance of FoxP3\textsuperscript{+} Tregs\textsuperscript{6}. We hypothesize that MO-MDSCs contribute to allograft tolerance through the promotion of regulatory tolerance mechanisms. To study this, using our culturing technique, we examined the effect of MO-MDSCs on the induction of Tregs.

CD11b\textsuperscript{+} Ly6C\textsuperscript{hi} monocytes were enriched from the bone marrow of both DBA and B6 background mice. The cells were cultured according to the protocol described in Figure 2.2, and were then cultured with B6 CD25\textsuperscript{-} CD4\textsuperscript{+} Naïve T cells. The purpose of isolating MO-MDSCs from two genetically distinct mice was to model syngeneic and allogenic Treg induction. T regulatory cells require IL-2 and CD28 engagement for their proper development. Non-suppressive (-LPS) and suppressive (+LPS) MO-MDSCs were cultured with or without IL-2 and TGF-β in conjunction with naïve B6 T cells. Across all combinations of cytokines and LPS, B6 MO-MDSCs were unable to adequately induce B6 Tregs (Figure 2.3 A). Interestingly, un-suppressive DBA MO-MDSCs that
were supplemented with IL-2 and TGF-β were able to induce a minute amount of Tregs. However, even more surprising, suppressive DBA MO-MDSCs were able to induce Tregs with and without the addition of IL-2 and TGF-β. FoxP3 induction was lower in the cohorts that were not supplemented with Treg stimulating cytokines (*Figure 2.3 B*). These results indicate that the MO-MDSCs produced in culture are able to induce Tregs in an allogenic manner. This is most likely due to the presentation of allopeptide and activation of the TCR.
Figure 2.3 Cultured CD11b⁺ Ly6C⁺ Monocytic Myeloid-Derived Suppressor Cells Induce T Regulatory Cells in an Allogeneic Fashion. MO-MDSCs were enriched and cultured using the protocol described in Figure 2.1 and Figure 2.2 respectively. MO-MDSCs were transferred to new 96 well plates and 1.5x10⁵ CD25⁻ CD4⁺ naïve B6 T cells were added per well (1 MO-MDSC: 3 Naïve T cell). The wells were supplemented with or without 10ng/mL of IL-2 and TGF-β (cytokines). After 96 hours, the cells were fixed and analyzed using flow cytometry. A) B6 MO-MDSCs effect on B6 Treg induction. B) DBA MO-MDSC effect on B6 Treg induction. Quadrant boundaries were set by isotype controls.
2.3d) Accumulation of F4/80\(^+\) and Ly6C\(\text{lo}\) CD11b\(^+\) Cells in Renal Allografts

**Hypothesis:** MO-MDSCs accumulate in the TOLS of accepted renal allografts.

Establishing a new protocol for the induction of MO-MDSCs helped us reveal that these cells alter their expression pattern of certain genes and have the capability to induce Tregs in an allogeneic manner. We next wanted to determine if we can isolate these cells from renal allografts that were harvested after 42 days. Histochemical analysis showed that F4/80 expressing cells localize to periphery of TOLS (*Figure 2.4 A*). Moreover, CD11b\(^+\) Ly6C\(\text{lo}\) cells were found to infiltrate to renal allografts, but not the native kidney of the graft recipient. Moreover, CD11b\(^+\) Ly6C\(\text{lo}\) myeloid cells were not identified in in naïve controls (*Figure 2.4 B*). Next, to we wanted to know if any F4/80\(^+\) cells could be found in the graft. Here, the renal allograft was digested and all lymphocytes were extracted out of the fraction. Gating on only CD11b\(^+\) cells we found that about a fifth of all CD11b\(^+\) cells were F4/80\(^+\) (*Figure 2.4 C*). These results indicate that myeloid cells consistent with our phenotypic analysis of suppressive MO-MDSCs are located within the allograft environment. However, more techniques need to be employed to definitively state if our cells of interest in fact accumulate in renal allografts.
Figure 2.4 Accumulation of F4/80⁺ and Ly6C⁺ CD11b⁺ Myeloid Cells within Renal Allografts. A) Harvested DBA→B6 renal allograft was harvested after 6 weeks and a frozen histological section was produced, which displays the result of anti-F4/80 staining. B) 6 week post-transplant renal allograft was digested using collagenase. The cells were fixed in paraformaldehyde and stained with anti-Ly6C and anti-CD11b fluorescein conjugated antibodies. C) Following the same graft isolation protocol, the lymphocytes were removed from the cell suspension and the obtained fraction was stained with anti-F4/80 and anti-CD11b fluorescein labeled antibodies. Quadrant boundaries were set by isotype controls.
2.4 Discussion

Here we demonstrated that MO-MDSCs that are cultured with IL-4, M-CSF, and LPS alter their gene expression patterns and cells that are supplemented with the MyD88 stimulant are capable of inducing T regulatory cells. Very few Gr-1⁺ cells were identified to accumulate in renal allografts, however, Ly6C⁺ cells were detected in the vasculature that TOLS surround. This is quite interesting, because this tells us that Ly6C⁺ cells are possible localizing to the graft. Moreover, because little Ly6C⁺ cell can be found in the graft, we hypothesize that our cells of interest may infiltrate the graft, but dramatically reduce expression of the Ly6C surface antigen. To further elucidate the function of MO-MDSCs, an in vitro culture technique was developed.

As described above, IL-4 and M-CSF stimulating cytokines were given as growth factors, which, according to literature, promote the upregulation of survival genes as well as iNOS and argianase-1. Additionally, a MyD88 stimulant was also needed to fully activate MO-MDSCs. We chose to use LPS because of cost and availability, however, the nuclear protein high mobility group box 1 (HMGB1) is most likely the TLR4 agonist in in vivo models. Cells that were supplemented with or without LPS during the 72 hour culture dramatically changed their phenotypic expression. Most notably, consistent with our hypothesis, MO-MDSCs reduced Ly6C expression. Interestingly, F4/80, a marker for macrophages, was significantly upregulated. This data points toward the possibility that, in culture, MO-MDSCs are maturing and differentiating into macrophages. The promotion of F4/80 expression on the surface of cells seemed to be independent of LPS and dependent on IL-4 and M-CSF. We hypothesize that the terminally differentiated cells are M2 like macrophages, principally on the observation that IL-4 has been known to promote the M2 axis. The next steps would be to identify the cytokine secretion profile.
of our cells and definitively identify what an M1 or M2 pattern. This would be done by culturing the cells in vitro and for the last hours add a protein transport inhibitor, such as brefeldin A. We would then stain using antibodies to pro or anti-inflammatory cytokines.

Recently, we have demonstrated that TOLS contain FoxP3+, CD4+, CD8+, B220+, CD11c+, and CD11b+ cells. These structures have been identified in the kidneys across species, and lack high endothelial venules as well as distinct T and B cell zones. So, these structures do not share the same characteristics of tertiary lymphoid structures. Interestingly, these TOLS are also podoplanin+. CD11b+ macrophages have been shown to express podoplanin and promote lymphangiogenesis. We hypothesize that MO-MDSCs may be promoting TOLS formation. Cells that were cultured with stimulating cytokines upregulated podoplanin on their surface, but quickly reduced them when LPS was added. Furthermore, a prominent feature of MDSC activity is iNOS activity. MO-MDSCs cultured with all combinations of factors failed to upregulated iNOS. This is most likely do to the observation that iNOS regulation is linked to STAT1 signaling, which is governed by IFN-γ. In the future we would like to deplete MO-MDSCs using anti-CD169 monoclonal antibodies in transplant recipients and examine whether or not TOLS are broken. Additionally, we would like to supplement our cultured with interferon and examine iNOS expression, as this cytokine is most likely present within the microenvironment of transplant recipients.

Co-stimulatory and co-inhibitory cytokines regulate T cell responses through the stabilization of TCR mediated signaling. We choose to look at CD80, CD86, and PD-L1 expression of our MO-MDSCs after culture. Cells cultured without LPS were different than cells cultured with LPS with respect to CD86 expression. B7.2 was upregulated with the addition of only growth factors, but reduced when LPS was added. This is most likely because suppressive MDSCs do not want to co-stimulate and activate T cells, rather their physiological role is to reduce T cell function. CD80 expression was similar between the two groups. Interestingly, PD-L1 expression was also upregulated in LPS stimulated cells. This is consistent with the downregulation of B7 molecules to inhibit T cell activation. Moreover, Ochando and colleagues...
showed that MO-MDSCs upregulate the PRR DC-SIGN, which is explained to be of importance during the induction of regulatory tolerance. After culture, we did not see the increase expression of this PRR. This may be due to the fact that protein mediators other than M-CSF and IL-4 are necessary for the regulation of this gene. After phenotypic analysis, we conducted functional assays to determine the ability of these cells to promote Treg induction.

A syngeneic and allogenic model were used to examine Treg induction. Cells across the same strain combination were not able to induce the differentiation of FoxP3^+ Tregs from naïve T cells. However, DBA MO-MDSCs cultured with B6 naïve T cells were able to induce Tregs. Presumable this was mediated by direct presentation of allopeptide and engagement of the TCR. During the Treg induction, cells supplemented with or without TGF-β and IL-2 were able to induce regulatory cells. However, when these cytokines were added to culture, a greater abundance of FoxP3 was recorded. To identify if this is occurring through an MHC TCR interaction new experiments are being developed where B6 MO-MDSCs are cultured through our normal protocol, and for 24 hours after this, cells are pulsed with DBA lysate. Also we are going to use flow cytometry to examine the expression of MHC I and II genes after culture. Finally, we digested a kidney that was grafted into a MHC dissimilar mouse, and after 42 days it was harvested and digested. We were able to clearly identify the presence of Ly6C^lo and F4/80^+ CD11b^+ cells. To clearly identify if our cells of interest are localizing to the graft we will label suppressive MO-MDSCs in vitro with CFSE and adoptively transfer them to a recipient. Where after an appropriate amount of time the graft will be harvested and digested to look for the accumulation of these cells within the graft.

In conclusion, we developed a culturing technique, which stimulated the differentiation of MO-MDSCs with the ability to induce T regulatory cells. To date, the induction of Tregs through direct cell-to-cell contact is unclear. Here, we provide a potential new mechanism for the induction of regulatory tolerance. The data provides a framework for developing new therapeutic measures for transplant recipients involving the administration of matured MO-MDSCs.
2.5 Materials and Methods

Mice

C57BL/6 (H2Db) and DBA/2J (H2Dd) strains were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were maintained under pathogen-free conditions in filter-top cages throughout the experiments with an automatic water system and were cared for in accordance with the methods approved by the American Association for Accreditation of Laboratory Animal Care. All animal experiments were approved by the Center for Comparative Medicine’s at Massachusetts General Hospital.

Surgical Protocol

Bilateral nephrectomy accompanied with left kidney transplantation were performed on recipient B6 mice with a donor DBA/2 (H-2d) kidney. This procedure was performed as previously described6. Blood urea nitrogen (BUN) and urea output was monitored daily (score).

Buffer Preparation

Three different buffers were used throughout the experiments. MACS buffer was used for isolation of MDSCs using the Miltenyi protocols, and to make this 2.5 grams of bovine serum
albumin (BSA) and .29 grams ethylenediaminetetraacetic acid (EDTA) were added to 500mL of 1x PBS and mixed thoroughly. EasySep Buffer was used for the STEMCELL protocols, and was prepared by adding 0.2922g EDTA and 20mL of 2% fetal bovine serum (FBS) to 980mL of 1x PBS. Moreover, FACS staining buffer was prepared by the addition of 50ml 10x PBS, 5g BSA, and 0.5 g sodium azide (NaN₃) to 450mL of de-ionized water. This buffer was used during flow cytometry.

**Histological and Immunopathological Assessment**

Transplanted kidney grafts were removed upon mouse euthanasia and cross-sectioned. One section was fixed in 10% formalin and embedded in paraffin. The other section was embedded and frozen in OCT compound (Sakura Finetek USA Inc., Torrance, CA) and stored at -20°C. Paraffin sections were further processed for staining of CD11b, Gr-1, and Ly6C at 10% formalin fixation; 3% H₂O₂ to block endogenous peroxidases; citrate buffer pH 6.0 for 1 min at 100°C as antigen retrieval treatment.

**Hematopoietic Blood Cell and Splenocyte Isolation**

Mouse femurs and tibias were extracted and stored in 1mL of RPMI (Sigma-Aldrich, St. Louis, MO). Using 19-gauge needles, the bone marrow was flushed out into a sterile petri dish containing 8mL of RPMI. Isolated bone marrow cells in the petri dish were then transferred to a
15mL Falcon tube (Fisher Scientific, Waltham, MA). Suspension was then centrifuged at 1300 RPM for 8 minutes. Supernatant was discarded and 1mL of Red Blood Cell Lysis Buffer (Sigma-Aldrich, St. Louis, MO) was added. After 2 minutes, 9mL of RPMI was used to re-suspend the cell mixture. Spleens were isolated from sacrificed animals and placed in 1mL of RPMI in a 15mL conical tube on ice. Under the hood, a sterile plunger was used to smash the spleen. The suspension of splenocytes was collected and passed through a sterile cotton filter into a 15mL Falcon tube. The cells were centrifuged at 1300 RPM and lysed similarly to the bone marrow cells.

Myeloid-Derived Suppressor Cell Enrichment

MDSCs were isolated using two methods. First, Gr-1$^{hi}\text{Ly6G}^+$ and Gr-1$^{lo}\text{Ly6G}^-$ MDSCs were obtained following Miltenyi Biotec’s Myeloid-Derived Suppressor Cell Isolation Kit, mouse (Cambridge, MA). Briefly, bone marrow cells were isolated using the protocol above, and the resulting cell suspension was incubated with anti-Ly6G Biotin antibody and anti-Biotin microbeads. The cells are then passed through a MACS column (Miltenyi Biotec, Cambridge, MA), which attracts the magnetically labeled Ly6G$^+$ cells, while the Ly6G$^-$ cells are collected in the flow through. The Ly6G$^+$ cells are eluted from the column, and the Ly6G$^-$ cells are purified through the addition of an anti-Gr-1 Biotin antibody and Streptavidin Microbeads. The labeled population are then passed through another MACS column, and are magnetically retained and subsequently eluted. CD11b$^+$ Ly6C$^{hi}$ cells were isolated from the bone marrow using StemCell Technologies’ EasySep Mouse Monocyte Enrichment Kit (Vancouver, CA). This negative selection protocol, labels unwanted cells using biotinylated antibodies against tetrameric antibody complexes.
recognizing biotin and dextran-coated magnetic particles. The cells are fractionated using an EasySep magnet.

*Myeloid-Derived Suppressor Cell Culture*

MDSCs were isolated from the bone marrow using the previously described enrichment protocol of Ly6C cells. The cells were plated at 5x10^4 cells per well in a round bottom 96-well polystyrene plate. The cells were suspended in 200µL of complete medium and supplemented with 10ng/mL of IL-4 and M-CSF for 72 hours. To obtain suppressive MDSCs, 100ng/mL of LPS was added to respective wells for the last 24 hours.

*Myeloid-Derived Suppressor Cell Induction of T Regulatory Cells*

To isolate naïve CD4^+ CD25^- T cells were isolated from the spleen of a B6 mice. T cells were then purified from this cell suspension using StemCell’s Mouse Naïve CD4^+ T cell Isolation Kit. Here, biotinylated antibodies and streptavidin-coated magnetic particles were directed against a multitude of markers to remove unwanted cells. Isolated MDSCs were added to round bottom 96 well plates at 5x10^4 cells per well. Also, 1.5x10^5 naïve T cells were added to each respective well. The cells were supplemented with IL-2 and TGF-β at a concentration of 10ng/mL. As a control, naïve T cells were plated alone with cytokines and CD3 and CD28 beads. The cells were cultured for a total of 96 hours before analysis.
Flow Cytometric Analysis of Myeloid-Derived Suppressor and T Regulatory Cells

Cells were stained with FITC-conjugated anti-mouse CD11b, PerCP-Cyanine5.5-conjugated anti-mouse Ly-6C, FITC-conjugated Ly-6G (Gr-1), PE-eFluor 610 conjugated anti-mouse Ly-6G (Gr-1), FITC-conjugated anti-mouse F4/80, PE-conjugated anti-mouse podoplanin, PerCP-conjugated anti-mouse DC-SIGN, FITC-conjugated anti-mouse NOS2, PE-conjugated anti-mouse CD80, PE-conjugated anti-mouse CD86, and PerCP-conjugated anti-mouse PD-L1, PE-conjugated anti-mouse CD3ε, and PerCP-conjugated anti-mouse FoxP3 mAbs to identify myeloid-derived suppressor cell population. All cells were analyzed on the BD Accuri C6 Cytometer (BD Biosciences) with FlowJo software (Tree Star Data Analysis Software, Ashland, OR).
Chapter 3: Accepted Allografts Across Mismatched Mammals are Characterized by Increased Nitrosylation

3.1 Abstract

Leukocytes can modulate the immune response through manipulation of amino acid metabolism. Recent findings indicate that the depletion of L-arginine and release of nitric oxide (NO) into the microenvironment suppresses the immune system by impairing T-cell function. iNOS expressing cells, such as CD11b+ subsets, have been studied in the context of tumor progression and infectious disease; however, their potential to induce transplant tolerance remains unclear. The aim of this study is to examine nitrosylation levels and iNOS expressing cells across murine renal allografts. DBA donor kidneys were transplanted into B6 recipients and were euthanized after 1, 3, and 6 weeks. Additionally, cardiac tissue from NHP MHC-mismatched grafts were harvested and analyzed. Nitrosylation was assessed by immunohistochemistry (IHC) using anti-nitrotyrosine and anti iNOS unconjugated antibodies. CD11b+ cells were isolated from 6 week kidney allografts, and analyzed with IHC and flow cytometry. IHC confirmed that the tyrosine residues of accepted allografts were heavily nitrosylated, while rejected cohorts from the same strain combination following Treg depletion were not. Histological analysis showed that iNOS positive cells were present in lymphocytic infiltrate as early as 1 week and persisted at 6 weeks. Moreover, IHC and flow cytometry indicated that CD11b+ cell localize to Treg-rich organized lymphoid structures (TOLS), which are signature formations of kidney acceptance, and express iNOS. Accepted renal allografts are
distinct from rejected allografts with respect to nitrosylation. The presence of iNOS+ cells shows that NO production occurs within the kidney microenvironment. CD11b+ cells within the TOLS of the graft are one source of NO production. Our results show the possibility of NO playing a role in graft acceptance and implicate myeloid progenitors in this process.

3.2 Introduction

Nitric oxide synthase is commonly associated with the innate axis of the immune system, and is responsible for antimicrobial and tumoricidal activity. However, the development of new technologies, such as NOS directed antibodies and knock-out mice, reveal a broader function of this enzyme in regulating lymphocyte function\textsuperscript{121}. This is due to NO strong reaction with thiol groups of amino acid residues, superoxide anions, soluble cations, and nucleic acids\textsuperscript{122,123,124}. The different isoforms of nitric oxide synthase all produce NO using L-arginine as a substrate, but differ in their regulation at the transcriptional level. Both eNOS and nNOS are expressed constitutively in endothelial cells and neurons respectively; however, iNOS is upregulated in leukocytes during pro-inflammatory settings\textsuperscript{68}. Although the role of eNOS has been established, the specific function of iNOS in immune cells is unclear and is currently the subject of extensive research. Today, emerging studies are implicating iNOS in transplant tolerance.

The majority of renal allograft infiltrating leukocytes that express iNOS are of myeloid origin, but recent studies have shown that iNOS is constitutively expressed in renal tubular cells, glomeruli, and segments of both the interlobular and arcuate arteries\textsuperscript{125}. The expression of this
enzyme in macrophages requires the intervention of pro-inflammatory cytokines and microbial stimulants, such as IL-1β, TNF-α, and LPS. Moreover, the production of NO by iNOS in myeloid cells have been shown to prevent intratumoral infiltration of antigen-specific T cells through the nitration of amino acids on chemokines and the inhibition of adhesion receptors. Additionally, iNOS deficiency has been shown to promote graft rejection in a syngeneic model of transplantation. Collectively, this data points towards iNOS as a regulator of T cell infiltration and possibly cytotoxic function. The aim of our study is to examine the role of nitrosylation in allograft acceptance, and the potential contribution of MDSCs in this process.

3.3 Results

3.3a) Nitrosylation Patterns of Accepted and Rejected Allografts

Hypothesis: Accepted renal allografts will show more tissue nitrosylation than rejecting allografts.

Using our spontaneous acceptance model, we wanted to assess the levels of nitrosylation in renal allografts. We stained naïve, rejecting, and accepted renal allografts. In the past, we have shown that graft acceptance requires Tregs. We simulated graft rejection by transplanting a kidney from a DBA/2J into a B6.Foxp3-DTR mouse. After the operation was completed, a sequential dose of DTR was administered to deplete Tregs. In naïve and rejecting models we saw no nitrosylation of graft tissue. Interestingly, allografts that were accepted were characterized by
increased nitrosylation (*Figure 3.1 A*). Next, we wanted to examine if this phenomenon was conserved in samples obtained from NHP cardiac rejecting models. Here, we observed that as the time course of rejection proceeds, nitrosylation decreases (*Figure 3.1 B*). Our results show that NO is deposited on the tyrosine residues of renal allograft tissue, and that this phenomenon is reduced in NHP hearts as rejection proceeds.
Figure 3.1 Anti-Nitrotyrosine Staining of Accepted Murine Renal Allografts and Rejecting NHP Cardiac Allografts. A) DBA donor kidneys were transplanted into B6 recipient mice. The same strain combination was used in the rejecting model, except the recipient was a B6.FoxP3-DTR mouse, and diphtheria toxin was administered before and after transplantation at a constant dosage. Naïve, rejected, and accepted murine renal allografts were fixed in paraffin embedded sections and stained with anti-nitrotyrosine antibodies. B) Cardiac tissue was obtained from NHP heart allograft models and also stained using anti-nitrotyrosine. The clinical score of transplant rejection was assessed by physician tending to the animal.
3.3b) iNOS+ Cells Accumulate in Accepted Renal Allografts

Hypothesis: Increased graft nitrosylation is associated with the accumulation of iNOS+ cells within the TOLS.

We next wanted to look at what cells in the allograft are producing nitric oxide. To do this, we stained histological samples with anti-iNOS antibodies. Naïve renal samples only showed moderate staining within tubular cells; however, after the transplant procedure iNOS+ cells accumulated within the TOLS. These cells peaked in number at 3 weeks post-operation, and persisted to 6 weeks (Figure 3.2 A). Since myeloid cells are the major type of white blood cells that express this enzyme, we digest the allograft with type II collagenase and stained total cells for CD11b and iNOS. Using out isotype control, we gated on cells that were CD11b+, and found that more than 10% of CD11b+ cells expressed iNOS. Accepted renal allografts are distinct from rejected allografts with respect to nitrosylation. The presence of iNOS+ cells shows that NO production is occurring within the kidney microenvironment. CD11b+ cells within the TOLS of the graft are one source of NO production. Our results show the possibility of NO playing a role in graft acceptance and implicate myeloid progenitors in this process.
Figure 3.2 Accumulation of CD11b⁺ iNOS⁺ Cells in Accepted Renal Allografts. Transplants were conducted between MHC-mismatched strain combinations (DBA→B6). A) Histological sections of the kidney were obtained from naïve and 1, 3, and 6 week post-operation recipients. Samples were fixed, and stained with anti-iNOS antibodies. B) The same allograft was digested, after histological samples were obtained, and digested with type II collagenase. Cells were stained with either isotype controls or anti-CD11b and anti-iNOS. Quadrant boundaries were set by isotype controls.
3.4 Discussion

The role of metabolism in modulating the immune system is currently of great interest. Specifically, the metabolism of L-arginine has been shown to be essential in lymphocyte function. Two enzyme of interest that use L-arginine as a substrate are arginase-1 and iNOS. Nitric oxide is a by-product of the reactions carried out by these enzymes. Interestingly, NO and other reactive species have been shown to regulate cellular function, and under great concentration cause tissue damage. Here, we show that nitric oxide is deposited on tyrosine residues of tissue in accepted allografts, and that myeloid cells that accumulate in grafts contribute to NO production.

Histological analysis of an accepted renal allograft tissue showed excessive nitrosylation, while rejected cohorts showed none. Moreover, we show that as the rejection process proceeds in NHP hearts nitrosylation levels decrease. Taken together, these observations shows a correlation between graft acceptance and nitrosylation. Studies in cancer models have shown that nitrosylation of tumors contributes to intratumoral T cell dysfunction through inhibition of chemokine, integrin, and TCR function. Our hypothesis is that the diffuse production of NO contribute to graft tolerance through similar mechanisms.

Nitric oxide is produced in a gaseous form and may diffuse through different compartments of the body. We wanted to look at where NO is being secreted. Histological analysis using anti-iNOS antibodies show that both infiltrating mononuclear and renal tubular cells express this enzyme. This shows that NO production is occurring within the kidney microenvironment. Furthermore, characterization of these cells showed that a portion of
accumulating immune cells are of myeloid origins. We hypothesize that our cells of interests are matured MDSCs, which have also been implicated in nitrosylation of tumor tissue\textsuperscript{128}. To further support his hypothesis new experiments will be conducted. In particular we plan on experimental models where NOS2 deficient kidneys from DBA mice will be transplanted into wild-type B6 mice. Studies from this experiment will show if donor, recipient, or both combinations are contributing to graft nitrosylation. These results will be analyzed in conjunction with control transplants among the same strain combinations. Our hypothesis is that the graft will be rejected, and tolerance is dependent on iNOS\textsuperscript{+} infiltrating leukocytes. The recapitulation of tolerance will be assessed through the adoptive transfer of suppressive MO-MDSCs. Moreover, the transfer of MO-MDSCs deficient in L-arginine transport into recipients will further evaluate the importance of MO-MDSCs in tolerance induction.

Today, recipients of allografts are often prescribed immunosuppressive drugs to prevent immune mediated graft rejection. Nitrosylation of graft tissue could serve as a maker for transplant tolerance, and could be used to asses when patients should be taken off immunosuppression. Also, nitrosylation of graft tissue through localized administration could possibly be used as a new immunosuppressant. In theory, this intervention would prevent T cell infiltration, which are the major mediators of rejection. Chronic immunosuppression using common mediation is associated with the development of malignancies, diabetes, and other disease. The production of new therapeutics is necessary to improve the lives of allograft recipients. Studying MDSCs is the first step in this process.
3.5 Materials and Methods

Mice

C57BL/6 (H2D\textsuperscript{b}) and DBA/2J (H2D\textsuperscript{d}) strains were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were maintained under pathogen-free conditions in filter-top cages throughout the experiments with an automatic water system and were cared for in accordance with the methods approved by the American Association for Accreditation of Laboratory Animal Care. All animal experiments were approved by the Center for Comparative Medicine’s at Massachusetts General Hospital.

Surgical Protocol

Bilateral nephrectomy accompanied with left kidney transplantation were performed on recipient B6 mice with a donor DBA/2 (H-2\textsuperscript{d}) kidney. This procedure was performed as previously described\textsuperscript{6}. Blood urea nitrogen (BUN) and urea output was monitored daily (score).

Buffer Preparation

Three different buffers were used throughout the experiments. MACS buffer was used for isolation of MDSCs using the Miltenyi protocols, and to make this 2.5 grams of bovine serum albumin (BSA) and .29 grams ethylenediaminetetraacetic acid (EDTA) were added to 500mL of
1x PBS and mixed thoroughly. EasySep Buffer was used for the STEMCELL protocols, and was prepared by adding 0.2922g EDTA and 20mL of 2% fetal bovine serum (FBS) to 980mL of 1x PBS. Moreover, FACS staining buffer was prepared by the addition of 50ml 10x PBS, 5g BSA, and 0.5 g sodium azide (NaN₃) to 450mL of de-ionized water. This buffer was used during flow cytometry.

**Histological and Immunopathological Assessment**

Transplanted kidney grafts were removed upon mouse euthanasia and cross-sectioned. One section was fixed in 10% formalin and embedded in paraffin. The other section was embedded and frozen in OCT compound (Sakura Finetek USA Inc., Torrance, CA) and stored at -20°C. Paraffin sections were further processed for staining of iNOS and nitrosylated tyrosine residues at 10% formalin fixation; 3% H₂O₂ to block endogenous peroxidases; citrate buffer pH 6.0 for 1 min at 100°C as antigen retrieval treatment.

**Flow Cytometric Analysis of iNOS⁺ Cells**

Cells were stained with FITC-conjugated anti-iNOS and APC-conjugated anti-CD11b monoclonal antibodies. All cells were analyzed on the BD Accuri C6 Cytometer (BD Biosciences) with FlowJo software (Tree Star Data Analysis Software, Ashland, OR).
Chapter 4: Myeloid-Derived Suppressor Cells and the Current State of Transplant Research

4.1 Concluding Remarks

In the past years we have made significant strides in advancing the fields of transplantation and immunology. Together, a greater understanding of these subjects has led to improved patient care through the development of better organ transportation systems, immunosuppressive medication, and noninvasive clinical diagnosis. However, despite our progresses, we continue to face many challenges, such as poor long-term graft survival and increased morbidity due to chronic immunosuppression\textsuperscript{16}. Both of these problems can be attributed to immune-mediated graft destruction. This event is facilitated by allore cognition of donor antigen by the adaptive axis and heightened recruitment of leukocytes to the graft. More specifically, cytotoxic lymphocytes, alloantibody production, and the deposition of complement proteins all enhance an immune response against a newly implanted graft\textsuperscript{12}. A solution to preventing graft destruction is to develop techniques that limit alloantibody recognition. Today, this can be achieved through T cell depletion, co-stimulatory blockade, and chimerism.

Successful tolerance protocols enacted in the clinic, such as mixed chimerism, require harsh preconditioning regimens to partially ablate the hematopoietic compartment and deplete lymphocytes, which may react against donor bone marrow engraftment. Moreover, initial studies in mice have shown that the elimination of T cells alone contribute to prolonged graft acceptance; however, the translation of this protocol in the clinic has proven to be disappointing\textsuperscript{18}. 
Furthermore, the treatment of patients with other lymphocyte antagonizing drugs suggest that the reduction of T cell activity may not be sufficient in inducing transplant tolerance alone\textsuperscript{24}. For this reason, it is important to examine the potential of other cells, such as those in the innate arm of the immune system, to induce regulatory or deletional tolerance\textsuperscript{130}.

Myeloid-derived suppressor cells are a very heterogeneous population, and the lack of consensus concerning their identifiable markers and naming make studying these cells quite difficult. Taking this into consideration, MDSCs have been examined in the context bacterial infection, malignancy, and transplantation. These cells can be divided into two populations, granulocytic and monocytic MDSCs. They differ not only in morphology, but suppressive function. The latter through the production of ROS, and the former in manipulating the metabolism of L-arginine. G-MDSCs have been shown to expand in the context of uncontrolled cell division (cancer), and protect tumors by mediating the prevention of immunosurveillance\textsuperscript{127}. While, M-MDSCs have been implicated in promoting regulatory tolerance in transplantation through the production of anti-inflammatory cytokines\textsuperscript{32}. The recent studies on M-MDSCs suggest that they may be relevant in facilitating allograft acceptance. Here, we show that M-MDSCs are able to induce T regulatory cells in an allogenic background, and that they may be contributing dysfunction of T cell function and/or recruitment.

During the normal myelopoiesis process, M-CSF and GM-CSF are important in promoting normal myeloid development. However, under pathogenic conditions monocytes stop their differentiation into mature macrophages or DCs and gain suppressive function. These cells are called MDSCs. The monocytic cohort are defined by their expression of CD11b and Ly6C. The expansion of these cells in pathogenic environments, mostly due to the engagement of the
MyD88 pathway, prompting us to examine the expression of genes of interest. We wanted to phenotypically characterize M-MDSCs that have been cultured with and without the MyD88 agonist, LPS. We observed that cells cultured without LPS had a marked increase in CD86 and PD-L1 expression when compared to LPS treated groups. These cells, which we believe to be naïve M-MDSCs because of a lack of exposure to activating stimulants, most likely upregulate CD86 due to the culture with LPS. PD-L1 could be providing signals to T cells promoting reduced function via immunoreceptor tyrosine-based inhibitory motif (ITIM) signaling, which could potentially override signals from CD86 engaging the immunoreceptor tyrosine-based activation motif (ITAM). Groups supplemented with or without LPS, however, are similar with the reduced expression of Ly6C and increase the F4/80 surface protein. The significant difference between these two groups is that cells cultured with LPS are the only ones that are able to induce T regulatory cells. Moreover, this phenomenon is only observed when M-MDSCs are cultured with naïve T cells of different genetic origin. It is for this reason, we suspect that induction may be prompted through direct TCR interaction with MHC and allopeptide. *In vitro* studies aside, we also showed that CD11b+Ly6C+ and F4/80+ cells accumulate in accepted renal allografts. This data shows that M-MDSCs that are exposed to inflammatory stimuli can promote Tregs in culture across varying MHC backgrounds.

Past studies have shown that the production and deposition of NO on proteins have a modulatory effect on components of the immune system. Groups have shown that NO produced by MDSCs bind to chemokines and integrins, inhibiting their normal function in recruiting T cells\textsuperscript{127,128}. In a separate series of experiments we have noticed a trend that accepted allograft tissue is nitrosylated, while rejecting grafts lose NO deposition as the score of rejection increases.
The major subtype of leukocytes that produce NO are of myeloid origin, and MDSCs which have been studied in cancer have been shown to produce NO and prevent cytotoxic T cell function\textsuperscript{127}. We wanted to further examine this observation in our spontaneous renal allograft acceptance model. Since tubular cells also express iNOS, we prepared histological slides to analyze if cell infiltrate also expressed iNOS. Our data shows that after 1 week iNOS\textsuperscript{+} cells other than tubular cells are present, and persist to 6 weeks after transplant. This data shows that along with tubular cells, other components, possible leukocytes, are locally producing NO and leading to graft nitrosylation. Interestingly, we found that CD11b\textsuperscript{+} cells enriched from 6 week accepted allografts expressed iNOS. Together, this data shows that iNOS producing myeloid cells may be important in the nitrosylation process and subsequent graft acceptance. This data, along with the evidence of Treg induction point toward the potential of M-MDSCs as playing a role in transplantation. We hypothesize that blood CD11b\textsuperscript{+} Ly6C\textsuperscript{hi} monocytes localize to the allograft site, and once inside mature into CD11b\textsuperscript{+} F4/80\textsuperscript{+} Ly6C\textsuperscript{lo} MDSCs via signals mediated through M-CSFR, IL-4R, and PRRs. After their differentiation, M-MDSCs promote tolerance through two mechanisms: Treg development and the inhibition of T cell function and accumulation. Here, we believe that direct cell-to-cell contact and cytokine secretion promote Treg development. Moreover, nitrosylation of chemokines, adhesion receptors, and components of the TCR reduce T cell mediated graft destruction. In rejecting models, increased infiltration of T cells and other leukocytes may overwhelm M-MDSC mediated regulation and promote loss of the allograft. More studies will need to be conducted to validate these hypotheses (comprehensive model Figure 4.1).

In the future we would like to conduct microarray analysis and specifically analyze adhesion receptors, cytokines, and enzyme mRNA levels to form a clear distinction between M-
MDSCs stimulated with HMGB-1 and other PRR ligands. Researchers have yet to fully characterize these cells and identification of new markers will be novel. Additionally, it will be important to cytokines and other molecules that are present in the graft microenvironment that may contribute to M-MDSC expansion and activation. Identifying if these factors are secreted within the graft will help us elucidate if the M-MDSCs are differentiating in the graft or prior to infiltration. Next, we need to validate that our cells of interest are actually accumulating within grafts. This can be assessed with bioluminescence imaging, adoptive transfer of CFSE labeled M-MDSCs, and histological analysis. Developing a model where we are able to deplete M-MDSCs will also be important to assess the contribution of these cells in graft acceptance. In the M-MDSC knockout model we can assess the amount of FoxP3+ cells in the graft as well nitrosylation patterns. Moreover, contribution of NO secretion to graft acceptance can be further looked at using NOS2 KO mice, where the recipient is void of iNOS+ cells. Finally, we need to look at what proteins are being nitrosylated and that can be achieved through enrichment of our cells of interest from the graft and co-immunoprecipitation experiments to examine NO deposition. Here, we have laid out preliminary work showing that M-MDSCs may be major contributors to transplant tolerance, and in the future more experiments will be conducted to validate this claim.

Myeloid-derived suppressor cells have been studied mostly in the context of cancer immunology; however, recently these immunomodulatory cells have been under intense investigation in transplant immunology. In particular, MDSCs have been shown to suppress T cell function through a variety of mechanisms, such as the depletion of essential amino acids, secretion of anti-inflammatory cytokines, and the inhibition of TCR mediated signal transduction. The discovery of these functions has made MDSCs a prime candidate for cellular
therapy in the induction of allograft tolerance and autoimmunity. Our new insights into the function of MO-MDSCs further add to the potential validity of these cells as therapeutic agents to improve the lives of patients.
Figure 4.1 Model of MO-MDSCs Function in Renal Allografts. CD11b\(^+\) Ly6C\(^{hi}\) MO-MDSCs circulate in the blood and localize to renal allografts. Mediators in the allograft, such as M-CSF, IL-4, and HMGB1 promote the differentiation of naïve MO-MDSCs into M2-like macrophages. Here, these cells promote tolerance through the inhibition of T cells by secreting RNS and depleting L-arginine. Additionally, these terminally differentiated cells promote regulatory tolerance through the induction of Tregs via the presentation of allopeptide.

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