Differentiation of regulatory myeloid cells and the potential for therapeutic applications

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

Zachary Curtis VanGundy

Graduate Program in Comparative Veterinary Medicine

The Ohio State University

2014

Master's Examination Committee:

Tracey L. Papenfuss, DVM, PhD, ACVP, “Advisor”, Prosper N. Boyaka, PhD, Eric M. Bachelder, PhD, Abhay R. Satoskar, MD, PhD.
Abstract

Subsets of myeloid cells (MC) have potent regulatory abilities but factors influencing the development of such regulatory myeloid cells (MC\textsubscript{regs}) remain poorly understood.

Retinoic acid (RA) is a metabolite of vitamin A that we have shown produces MC\textsubscript{regs} that expressed increased IL-10, induce T\textsubscript{reg} cells, and were able to suppress the proliferation/cytotoxicity of CD4\textsuperscript{+}/CD8\textsuperscript{+} T cells respectively. We hypothesize that encapsulation of RA within acetalated dextran (Ac-Dex) microparticles (MPs) can be used for targeted generation of MC\textsubscript{regs} \textit{in vivo}. We first utilized a C57BL/6 macrophage cell line \textit{in vitro} to determine that RA MPs will induce a regulatory phenotype. It was found that RA MPs were able to increase the regulatory (PD-L1) phenotype of the macrophage cell line \textit{in vitro} compared to either control or E3 encapsulated MPs. We then used MPs containing encapsulated FITC-labeled BSA to determine the relative uptake of Ac-Dex MPs by myeloid cell populations \textit{in vitro} and \textit{in vivo}. We found that labeled MPs were readily taken up by macrophages, DCs and myeloid progenitors \textit{in vitro}. Following \textit{in vivo} injection, increased percentage of FITC\textsuperscript{+} cells was seen in both CD11c\textsuperscript{+}CD11b\textsuperscript{−} and CD11c\textsuperscript{−}CD11b\textsuperscript{+} cells in draining lymph nodes (DLN) over controls of free FITC or empty MP administration. When RA MPs were injected into mice it was found that myeloid cells within the DLNs had an increase expression of regulatory co-stimulatory marker PD-L1. Also the DLNs of mice receiving RA Ac-Dex MPs had reduced proliferation of responder immune cells when analyzed via proliferation assay compared
to control mice. These results suggest that *in vivo* targeted delivery of regulatory compounds such as RA by Ac-Dex MPs can induce MC\textsubscript{regs} which may have therapeutic application to treat inflammatory and immune-mediated diseases.
Dedication

This document is dedicated to my family and friends that have supported me through this degree and life.
Acknowledgments

I would like to thank my advisor Dr. Papenfuss for giving me the chance to learn, and discover my passion. With her support I have persevered to gain my degree and discovered myself.

I would like to thank my committee members for helping guide me though my degree and advice in circumstances that were beyond my comprehension.

I would like to thank my friends that helped me inside and out of my degree to shape the person I am today.

Last but not least, I would like to thank my family (Mom, Dad, and Wife) for loving me and supporting me through everything that life has thrown at me. I will be forever grateful.
Vita

2005.................................................................Lancaster High School

2009...............................................................B.S. Biology, Wilmington College

Publications

First author:


2. VanGundy, Z. Markowitz, J. Strange, H. Papenfuss, T. An in vitro model system to generate breast cancer MDSCs and study immune cell interactions in immunocompetent C57BL/6 mice.

Co authors:


Fields of Study

Major Field: Comparative Veterinary Medicine

Emphasis in Immunology
# Table of Contents

Abstract ............................................................................................................................... ii

Dedication .......................................................................................................................... iv

Acknowledgments............................................................................................................... v

Vita..................................................................................................................................... vi

List of Figures .................................................................................................................... xi

Chapter 1: Introduction and Literature Review ............................................................... 1

Chapter 1.1.1 Myeloid cells ........................................................................................ 1

Chapter 1.1.2 Myeloid progenitors ............................................................................. 1

Chapter 1.1.3 Granulocytes ........................................................................................ 2

Chapter 1.1.4 Monocytes ............................................................................................ 4

Chapter 1.1.5 Macrophages ........................................................................................ 7

Chapter 1.1.6 Dendritic cells ...................................................................................... 9

Chapter 1.2 Regulatory and inflammatory myeloid cell development ....................... 11

Chapter 1.2.1 Regulatory cells and their differentiation .............................................. 11

Chapter 1.2.2 Factors to induce MC_{regs} ................................................................ 14

Chapter 1.2.3 Estrogens ............................................................................................ 16
Chapter 1.2.4 Retinoic Acid. ................................................................. 17
Chapter 1.2.4a Vitamin A metabolism and RA signaling ................. 17
Chapter 1.2.4b Role of RA in immunoregulation............................... 19
Chapter 1.3 In vivo delivery Methods. .................................................. 20
Chapter 1.3.1 Non-degradable microparticles. ................................. 21
Chapter 1.3.2 PLGA. ................................................................. 21
Chapter 1.3.3 Liposomes ................................................................. 22
Chapter 1.3.4 Acetalated dextran. ..................................................... 23
Chapter 1.5: Study objectives and hypothesis................................. 25
Chapter 2: Materials and Methods .................................................... 26
Chapter 3 Differentiation of regulatory myeloid cells and the potential for therapeutic applications. .......................................................... 33
Chapter 3.1.1: The differentiation of regulatory myeloid cells.......... 33
Chapter 3.1.2: CD11b but not CD11c cells were the suppressive population. ....... 37
Chapter 3.1.3: CD11b+CD11c-Ly6C_{low/intermediate} were the primary population responsible for suppression. .......................................................... 42
Chapter 3.1.4: Generation of MC\textsubscript{regs} \textit{In vitro} by Ac-Dex MPs. .......................................................... 45
Chapter 3.1.5: Ac-Dex Microparticle trafficking ................................ 47
Chapter 3.1.6: Inducing MC\textsubscript{regs} with MPs \textit{in vivo}. .......................................................... 50
Chapter 4: Conclusion and discussion ................................................................. 53

References: ........................................................................................................... 64
List of Figures

Figure 1 Myelopoiesis............................................................................................................... 2
Figure 2 Monocyte differentiation............................................................................................ 6
Figure 3 Acetalated dextran chemical structure........................................................................ 24
Figure 4 RA treatment of bone marrow myeloid cells produces a MC_reg population. ..... 36
Figure 5 RA treatment of BM-MCs produces mature MCs. .........................................................37
Figure 6 RA mediated suppression of T cell proliferation is not mediated by CD11c^+ BM-MCs................................................................................................................................................. 40
Figure 7 GM-CSF induced myeloid cells. ............................................................................... 40
Figure 8 RA generated CD11b^+ BM-MCs suppress T cell proliferation and express a regulatory phenotype. .............................................................................................................. 41
Figure 9 CD11c^- IL-10 and T regulatory induction................................................................. 42
Figure 10 RA CD11b^+CD11c^-Ly6C^low/intermediate are the suppressive population........... 44
Figure 11 CD11b^+CD11c^- Ly-6C^low cells have an activated regulatory phenotype. ........ 44
Figure 12 RA CD11b^+CD11c^-Ly-6C^intermediate cells trend suppressive but are not significant............................................................................................................................................. 45
Figure 13 RA MPs induce increased expression of PD-L1 on C57BL/6 macrophages in vitro. ..................................................................................................................................................... 46
Figure 14 RA MPs induce increased expression of PD-L1 on C57BL/6 macrophages in vitro compared to E3 MPs. ................................................................................................................................. 47
Figure 15 Trafficking of FITC uptake within dendritic cells. ................................................. 49

Figure 16 Trafficking of FITC uptake within monocytes/macrophages. ........................... 50

Figure 17 RA MPs induce increased expression of PD-L1 on myeloid cells *in vitro*
compared to blank MPs. ..................................................................................................... 51

Figure 18 RA MPs induce increased regulatory function of myeloid cells *in vitro*
compared to blank MPs. ..................................................................................................... 52
Chapter 1: Introduction and Literature Review

1.1.1 Myeloid cells.

Myeloid cells (MCs) are a heterogeneous and diverse population of non-lymphoid cells formed during hematopoiesis. Myeloid cells are comprised of granulocytes, mononuclear phagocytes and their precursors. MCs are innate immune cells that have an important role in promoting and resolving inflammation, development, and tissue regeneration(1). Myeloid cells also play a key role in both inducing and regulating the adaptive immune response. MCs are a part of the innate immune system that play a role in the first line of defense and are known to play a critical role in host defense and the induction of the regulatory response.

1.1.2 Myeloid progenitors.

The vast majority of myeloid cells originate from the self-renewing hematopoietic stem cells (HSC) residing within the bone marrow. These cells then differentiate to specific myeloid cell populations (myelopoiesis) from a HSC progress through various precursors. There has been much discussion about the specific precursor origins of various mature myeloid populations but, in general these can be identified as three subsets(2). The three subsets include the macrophage dendritic cell progenitor (MDP), granulocyte macrophage progenitor (GMP), and common dendritic cell progenitor (CDP) depicted in figure 1(2). The GMPs are a subset of cells that only differentiate into
granulocytes while MDPs differentiate into macrophages, monocytes, and monocyte derived dendritic cells but are considered to not give rise to granulocytes(3). The CDP subset is a group of cells that generally differentiate into pre-conventional dendritic cells and plasmaeytoid dendritic cells (pDCs). The CDPs are considered to commonly not to give rise to monocytes or granulocytes(3).

Figure 1 Myelopoiesis.

1.1.3 Granulocytes.

Granulocytes are circulating leukocytes that play crucial roles in the immune defense against pathogens and participate in various other processes. Neutrophils, basophils and eosinophils are the three populations of granulocytes which have relatively distinct roles in immune responses(4-6). Blood neutrophils comprise approximately 60%
of the circulating leukocytes and live approximately less than 72 hours once released from bone marrow. Neutrophils are known to participate in numerous processes including viral and mycobacteria clearance, diabetes, atherosclerosis, allergic skin reactions, and influence cell populations such as B cells, plasmacytoid dendritic cells, T cells, and NK cells(7). Neutrophils are short lived (~1 day) cells that have three main mechanisms of immunomodulation, the use of the granule enzymes(azurophils, peroxidase-negative granules, and secretory vesicles), the release of cytokines (TNF-α, IL-1, and IL-12) and the formation of neutrophil extracellular traps or “NETs”(8-11). Neutrophils can phagocytose infectious pathogens and release intracellular granules and reactive oxygen species to kill the pathogen collected(12). They have also been shown to release a fibrillar network of nuclear components (NETs) to trap pathogens and dispose of them with the assistance of hydrogen peroxide(10, 13).

Eosinophils compose approximately 1-6% of circulating leukocytes and are short lived cells, circulating in the blood for approximately 8-12 hours and residing in the tissues for around 6-12 days. Eosinophils are activated by IL-5 to release toxic and pro-inflammatory mediators, such as IL-2, IL-12, IL-13, and TGF-β, that contribute to a Th2 response(14, 15). Eosinophils are known to play roles in allergic responses, gastrointestinal disorders, anti-tumor responses and parasitic infection.

Basophils compose nearly 1% of the blood leukocytes, and play a role in response to parasitic infections and allergic disorders(16). They are short lived cells, only estimated to live 2-3 days. Basophils are known to be matured and differentiated by IL-3
like many other myeloid cells (17, 18). The hallmark for basophils are their production of IL-4 and IL-13, two cytokines important for the induction of a Th2 response (4, 19).

1.1.4 Monocytes.

Monocytes are circulating myeloid cells that give rise to tissue macrophages and DCs. Murine monocytes comprise approximately 5% of the circulating leukocytes, and have a half-life of 24-60 hours (20, 21). Monocytes have recently been recognized as a contributor to the inflammatory responses, and are now known to contribute to immune regulation (22). Monocytes are produced in the bone marrow and can vary in size, morphology, and granularity making them heterogeneous in nature (Figure 2) (23). The ability of monocytes to traffic from the bone marrow to the blood was demonstrated as early as 1939. Monocytes remain in circulation for multiple days and then establishing themselves in the tissue (24). Monocytes differentiate from the bone marrow into a Ly-6C\(^+\) expressing cell that is immature in nature but passes through an intermediate phenotype denoted by a Ly-6C\(^\text{intermediate}\) phenotype and finally entering into a Ly-6C\(^-\) phenotype with insufficient inflammatory cues, where they become resident monocytes (25). Ly-6C is a common phenotypic marker identifying a cell as monocyte, Ly-6C can also be correlated with relative functional status. Ly-6C is a murine-specific glycoprotein that is used to identify monocytes, though it can also be expressed on other immune cells such as macrophages (26). Ly-6C has been shown to also correlate with inflammatory and maturation status of monocytes and its expression can be related to CX3CR1 on humans (22, 27-29). Sunderkotter et al. showed that Ly-6C\(^\text{high}\) monocytes were the only Ly-6C subset that was recruited to inflammatory sites and that Ly-6C\(^\text{high}\) monocytes were
immature in nature(28). This data suggests that the Ly-6C expression correlates with the functional characterization of monocytes(28). The enrichment of Ly-6C\textsuperscript{high} cells have been associated with earlier onset and increased severity of autoimmune disease, implicating the role of monocytes in inflammatory disease(30). Others have also shown that Ly-6C\textsuperscript{high} monocytes are known to influence multiple models of infection and atherosclerosis(27, 31, 32). Recently published studies from our studies demonstrate similar findings where Ly-6C may correlate with inflammatory status since Ly-6C\textsuperscript{low} monocytes induced by retinoic acid were regulatory in nature(33). In addition to Ly-6C, other monocytic phenotypic markers have been associated with monocyte function have been used. Monocytes expressing the C-C chemokine receptor type 2 (CCR2) are inflammatory in nature while, non-inflammatory/regulatory monocytes (CCR2\textsuperscript{-})(34). However CCR2 expression is not limited to monocytes, it has also been reported to be expressed on up to 2-15% of T cells(34). The expression of specific receptors CD62L or L-selectin and CX3CR1 and multiple other markers can be variably expressed and associated with monocyte function(35).

The inflammatory monocyte or classical monocyte is known for its anti-infectious properties and is recruited to sites of inflammation by CCL2 or CCL7 to produce tumor necrosis factor, IL-1, and reactive oxygen species(36, 37). In chronic inflammation monocytes differentiate into inflammatory monocytes and are destructive to surrounding tissues, but also inhibit T cell proliferation by reducing the T cell receptor zeta chain(38). There is much debate about the differentiation of a classical monocyte. One theory is that after a classical monocyte engulfs a dying cell, it differentiates into or promotes the
induction of, a non-classical monocyte and participates in wound healing(39). Another theory of the plasticity of monocytes is that classical monocytes, without the proper inflammatory stimulation, will return to the bone marrow and become a non-classical monocyte. The non-classical monocyte will then patrol the bloodstream to participate in wound healing (excretion of VEGF), clearance of dead cells (phagocytosis), and the influence of the adaptive immune system (cytokines and chemokines)(40). Alternatively, non-classical monocytes may arise directly to patrol blood vessel walls without becoming inflammatory and eventually accumulate in the peripheral tissue to differentiate into resident macrophages(27).

Figure 2 Monocyte differentiation scheme. The ratio of Ly-6C$^{\text{low}}$ to Ly-6C$^{\text{high}}$ monocytes are 6:4 respectively.
1.1.5 Macrophages.

Macrophages play multiple roles in immune responses and are likely the most widely studied innate immune cells. Macrophages are rarely present within the bloodstream and are more typically found within the tissue, often from differentiated monocytes. The majority of macrophages arise from blood-borne monocytes although recent studies show that select tissue-resident macrophage populations are derived from embryonic progenitors in the yolk sac(41, 42). Macrophages differentiate under environmental influences and cytokines such as M-CSF, GM-CSF and others. M-CSF is one of the primary cytokine that drives the differentiation of macrophages from HSCs and has been shown to be important in the proliferation and maintenance of macrophages. Although GM-CSF is a common method to generate large numbers of DCs, it can also drive the differentiation of a small population of macrophages and is critical for lung macrophage homeostasis(43-45). The cytokine IL-34 has been shown to be necessary for the differentiation of specialized skin macrophages while IL-4 and IL-13 induce proliferation of macrophages and promote alternatively activated macrophages in mice(46, 47).

In addition to general macrophage differentiation, additional factors contribute to the variety of macrophage subsets within the body. Macrophages can be categorized based on location, function and/or phenotype. Tissue resident macrophages, such as Kupffer cells within the liver and microglial cells within the brain, have unique nomenclature and often unique function due to the environmental landscape of the organ.
of residence. Macrophages within these tissues fulfill tissue specific functions, ranging from homeostasis, immune surveillance, tissue repair and iron acquisition\(^{(48\text{, }49)}\).

One of the most commonly used functional delineations of circulating macrophages are the M1 and M2 macrophages which are correlative to classical Th1 and Th2 immune response. The M1 macrophage or classically activated macrophage is characterized by high production of inflammatory cytokines IL-12 and IL-23, production of nitric oxide, reaction oxygen intermediates and its ability to present antigens\(^{(50)}\). M1 macrophages are known to kill indirectly due to the secretion of IL-1\(\beta\), TNF-\(\alpha\), and ROS\(^{(51)}\). The M2 macrophage or alternatively activated macrophage is characterized by high production of IL-10, low IL-12, and the promotion of an overall Th2 response\(^{(52)}\). These macrophages respond to exogenous factors within the environment and typically resolve inflammatory status by promoting a regulatory response\(^{(53)}\). Although the categorization suggest that these are distinct population, there is much debate regarding the relative stability and malleability of different functional subsets of macrophages\(^{(54)}\). M2 macrophages play an important role in regulating immune responses. Data suggests that M2 macrophages are induced by the down regulation of NF-\(\kappa\)B and STAT1, reducing the pro-inflammatory chemokines produced. The M2 macrophage can be further broken down into three subcategories; M2a, M2b and M2c. The three subcategories are determined by the mechanism by which the M2 population is induced; M2a is induced under the influence of cytokines IL-4 and IL-13, M2b is induced by the exposure to immune complexes or toll-like receptors and M2c is differentiated by IL-10 and hormones\(^{(55)}\).
1.1.6 Dendritic cells.

Dendritic cells (DCs) play a critical role in the immune system, influencing and bridging both the innate and adaptive immune response, preservation of self/non-self, and the immunity/tolerance balance(56). First described in 1973 by Dr. Steinman, DCs are a relatively new addition to the myeloid cell family but similar to other myeloid cell populations with a variety of described subsets and functional roles(57).

DC subsets are defined based on tissue location, function and /or phenotype like macrophages. Langerhans and follicular DCs are DC subsets present within unique locations (skin and secondary lymphoid organs) that play very specific roles in these organs including controlling immune responses(58, 59).

Previously considered to be either of myeloid or lineage origin, the current understanding is that two distinct DC populations exist, the conventional and plasmacytoid DCs (cDC and pDC). Numerous studies have shown a wide array of functional sub-categorizations of primarily cDC based on phenotypic marker expression and functional abilities. Both cDC and pDCs are considered to arise from the CDPs within the bone marrow differentiating into subsets based on the microenvironment influences(60). The more commonly studied cDC plays a key role at recognizing and phagocytosing pathogens(57). Immature cDC express high MHC and co-stimulatory molecule levels and often migrate to secondary lymphoid organs to present extra-cellular antigens to T cells and induce adaptive immunity. The cDC can be broken down into two subsets the CD8+ lineage, that do not express CD11b and are efficient at presenting
antigens through the MHC class I to CD8+ T cells(59, 61-63). The second subset is the CD4+ DCs that are able to present antigen on the MHC class II but not MHC class I efficiently (59, 64). The plasmacytoid dendritic cell or pDC is a distinct but well studied subset of DCs that is known for its secretion of IFN-α following viral infection, its morphology and relative lack of antigen presentation(65-67). The murine pDC expresses an array of markers including the traditional CD11c marker, the B cell marker B220, Sca-1, Siglec-H, Bst2 CCR9, CD45RA and T cell markersCD4 and CD8(67, 68).

Inflammatory DCs or inf-DCs are most often differentiated from monocytes that are recruited to the site of inflammation. Inf-DCs are identified phenotypically by numerous extracellular markers including MHCII, CD11b, CD11c, Ly-6C, CD64 and many others(44, 69). While there are many markers that are associated with inflammatory DCs there is debate that FcεRI and CD64 are the most optimal markers for the identification of inf-DCs in tissue and lymphoid organs(70). Key characteristics of inflammatory DCs are the ability to differentiate and activate naïve T cells, being CCR7 dependent (homing of memory T cells), and secreting a variety of cytokines important in inducing inflammatory responses in bacterial, fungal and viral pathogen defense (IL-12, IL-23, type 1 IFN, IL-1α and IL-1β)(71).

Of more recent interest are the DCs that are important in regulating the immune responses and maintaining homeostasis. Such DCs have a variety of names including patrolling, steady-state, regulatory or tolerogenic(72, 73). The steady-state or regulatory DCs are often defined by their ability to produce immunoregulatory soluble mediators IL-10, TGFβ, and indoleamine 2,3-dioxygenase(25). DCs have been shown to mediate
negative selection of T cells(74, 75) and also induce peripheral tolerance(76). Immature DCs, their immediate precursors or exhausted DCs are often regulatory in nature and this regulatory state can be synthetically accomplished with various molecules including IL-10, vitamin D3 and corticosteroids(77-79). DCs exposed to such molecules typically have reduced MHC class II and co-stimulatory molecules and have reduced abilities to process and present antigens. DCs can express numerous pattern recognition receptors including toll like receptors, C-type lectin receptors, retinoic acid-inducible gene-1 and many more allowing the reaction with various microbial/non-microbial agents(80-82).

1.2 Regulatory and inflammatory myeloid cell development.

1.2.1 Regulatory cells and their differentiation.

Myeloid cells which are comprised of non-lymphoid cells such as granulocytes, mononuclear phagocytes and their precursors are important contributors to inflammation in response to pathogens or other insults. Inflammatory MCs are induced and increased in numbers following exposure to exogenous (pathogens) or endogenous “danger” (post-necrotic release of high-mobility group box 1 or HMGB1) signals(27). These and other environmental factors present within peripheral tissue and bone marrow impact granulopoiesis, monopoiesis and dendropoiesis to influence the ultimate fate of inflammatory granulocytes, monocytes/macrophages and DCs, respectively. Similarly environmental factors play an important role at imparting regulatory abilities in many MC populations. Factors such as inflammatory cytokines or TLRs often contribute to the induction of M2 macrophages, regulatory DCs and other regulatory MC populations.
Nearly all MC populations have the potential for regulatory abilities and similar mechanisms (IL-10) can be used to induce regulatory macrophages and DCs.

In order to avoid the model-specific or method-specific means used to induce regulatory, tolerogenic or steady state myeloid populations (monocyte, macrophage, and DCs) we have chosen to utilize the term regulatory myeloid cell or MC_reg(27, 33, 83, 84). MC_regs are a diverse population of cells with the ability to control inflammation and, thus, are a potential target to treat a wide array of inflammatory diseases. To date, however, factors involved in the differentiation of various MC_reg populations remain poorly understood. MC_reg subsets are particularly diverse, both in terminology and in function. The regulatory potential of MC populations was first recognized in macrophages over 30 years ago. Alternatively activated macrophages are able to promote wound healing and resolve inflammation rather than promoting inflammation(54, 85). Over the last 10 years the regulatory abilities of DCs and their therapeutic potential have been the focus of many studies(56, 83).

MC_regs are regulators of the immune responses through various methods including the production of soluble regulatory factors (IL-10, TGF-beta, indoleamine 2,3 deoxygenase, arginase, nitric oxide), expression of inhibitory or regulatory cell surface molecules (PD-L1, PD-L2) the induction of other regulatory cells (regulatory T cells) or the enhancement of regulatory feed-back loops(86, 87). At present, MC_regs are identified based on combination of phenotype and function, with no equivalent to T_reg FoxP3 marker being identified(88-90). In addition to the lack of a definitive regulatory marker,
the inherent heterogeneity and various states of differentiation and activation of various
MC populations make identification and therapeutic application of MC$_{\text{regs}}$ difficult.
Through cell-cell interactions and the production of soluble immunoregulatory
molecules, MC$_{\text{regs}}$ have very potent and diverse means of inducing immune regulation.
However, much remains to be characterized about factors controlling MC$_{\text{reg}}$ induction
and how different MC$_{\text{reg}}$ subsets regulate immune responses. Given that MC$_{\text{reg}}$ therapy
has the potential to diminish disease in the 100+ millions of individuals impacted by
immune-mediated, chronic inflammatory and autoimmune diseases worldwide, it is
critical to determine the factors which govern the induction and function of these
cells(91-93). The therapeutic potential of MC$_{\text{regs}}$, has been described in several
experimental models of inflammatory and autoimmune disease. Specifically, MC$_{\text{regs}}$,
including MDSC, conventional DCs, lung-resident tissue macrophages, monocytes, and
plasmacytoid DCs have all been shown to impact disease course in animal models of
diabetes(94), colitis(95), allergic asthma(96), experimental autoimmune disease(97), and
rheumatoid arthritis(98) respectively.

For many MC$_{\text{regs}}$, an arrest in immature and/or altered functionality contributes to
their regulatory abilities(99, 100). Glucocorticoids, vitamin D and IL-10 are the most
common means to induce these immature MC$_{\text{regs}}$. These altered MC$_{\text{regs}}$ cells have
decreased expression levels of maturation/activation markers CD80, CD86 and MHC
class II(83, 101-105). Additionally, these immature MC$_{\text{regs}}$ can have reduced
inflammatory cytokine expression(106, 107), overall blunted function, induce T$_{\text{regs}}$ and
suppress the action of other immune cells. While these immature cells can regulate
immune responses, a primary concern with using immature MC$_{\text{regs}}$ for therapy is that they may mature into inflammatory MCs under inflammatory disease conditions. Such inflammatory MCs could then actually exacerbate the very inflammatory disease they were used to treat(83, 99, 100, 108). Thus, mature (and stable) MC$_{\text{regs}}$ may avoid such concerns but, to date only a handful of studies have significantly explored the induction of such mature MC$_{\text{regs}}$(95, 99, 109). Typically, mature MC$_{\text{regs}}$ have been induced by combining traditional immature MC$_{\text{reg}}$ induction protocols with the addition of inflammatory stimuli such as LPS or TNF-alpha (110, 111). To avoid the use of inflammatory stimuli and the potential differentiation of inflammatory cells we sought out alternative methods to induce MC$_{\text{regs}}$.

1.2.2 Factors to capable of inducing MC$_{\text{regs}}$.

A wide array of factors can induce a regulatory myeloid cell. Each factor has a particular advantage or disadvantage including toxicity, induction of immature cells, or changes in phenotype. Three commonly used methods to induce MC$_{\text{regs}}$ are vitamin D, glucocorticoids, and IL-10.

Vitamin D (VD) is a secosteroïd hormone that is important in many factors of growth and differentiation of numerous cell types(112, 113). The biological effects of VD are mediated through the vitamin D receptor(VDR), specifically through the heterodimer of retinoid X receptor (RXR). Once activated the VDR/RXR heterodimer induces a histone-modification influencing the rate of RNA polymerase II(113). Due to antigen presenting cells (APCs) expressing the vitamin D receptor, vitamin D influences the cell by reducing or inhibiting differentiation and maturation(114, 115). Treatments with
vitamin D consistently show a decrease in activation markers and a decrease in pro-inflammatory cytokine IL-12 and increase in anti-inflammatory cytokine IL-10(114, 115). This change in cytokine production and reduction in activation markers leads to an immature regulatory cell population. The negative characteristic associated with the use of an immature regulatory cell is the potential for this population to be forced into differentiation, hypothetically switching into an inflammatory myeloid cell.

Glucocorticoids (GC) are endogenously secreted hormones that are potent anti-inflammatory agents, commonly used in autoimmune and allergic disease(116). GCs are also known to affect the immune response, specifically by reducing IL-12, IL-6, TNF-α and inducing secretion of IL-10(103). It has also been shown that the combination of glucocorticoids, such as dexamethasone or vitamin D can enhance IL-10 secretion and attenuate IL-2 and IFN-γ(101). This attenuation has been shown to reduce the proliferation of T cell proliferation, thereby reducing the adaptive immune response’s pro-inflammatory ability. GCs have also been shown to reduce the activation of monocytes and macrophages by reducing there phagocytic capability and expression of MHC class II(117). This once again induces an immature cell with the potential to differentiate into an inflammatory cell.

IL-10 is an anti-inflammatory cytokine that is produced by multiple cells (B cells, DCs, NK cells, Macrophages, etc) and affects multiple cells alike. IL-10 has recently been established as participating largely in the onset and development of numerous autoimmune diseases(118). It was found that when peripheral blood mononuclear cells were differentiated in the presence of IL-10 they were able to produce a tolerogenic DC(102).
IL-10 itself is an immunosuppressive agent that was identified as a suppressor to MC function along with IFN-γ and IL-2 particularly inducing the down-regulation of MHC class II on APCs(118, 119). The down-regulation MHC class II is generally accompanied by the impeding of differentiation producing an immature regulatory cell, which has the potential to differentiate into a mature inflammatory cell.

1.2.3 Estrogens.

Estrogens are known to influence immune cells with estrone (E1), 17-β estradiol (E2) or estriol (E3) being the most well-known immunomodulatory estrogens. Estrogens have been shown to elicit rapid non-genomic changes in various cell populations, including signaling pathway alterations and calcium fluxes(120). E1 and E2 are endogenous and found present in adults, while E3 is only present at high levels during pregnancy(121). Estrogens signal through estrogen receptor genes and the associated hormone response elements(122). There are three subtypes of estrogen receptors, alpha, beta and gamma and are located in the nucleus and cell membrane. Both the alpha and beta are found on hematopoietic progenitors while only alpha is typically found to be expressed by professional APCs(123, 124). Gamma has recently been discovered, but has been detected in various cellular models including spermatozoa(125, 126). Our laboratory has previously focused on identifying non-inflammatory systems to induce mature MCregs and have found that E3 produces a mature activated DCreg(127). When DCs are expanded \textit{in vivo} in the presence of E3, DCregs were produced(127). These E3 DCregs maintained their regulatory abilities within an inflammatory environment and protected mice against
the inflammatory autoimmune disease, experimental autoimmune encephalomyelitis (EAE)(127). Although E3 shows promise, there are potential limitations on the broad use of estrogens in the human patient population necessitated investigating alternative means of inducing mature stable MC_{reg} populations. We have also found that another steroid hormone, retinoic acid is able to induce regulatory cells and may be more potent in its ability to induce regulatory cells than estriol(33).

1.2.4 Retinoic Acid.

1.2.4a Vitamin A metabolism and RA signaling

Retinoic acid is an active metabolite of vitamin A, a fat-soluble vitamin essential for the development and maintenance of many body tissues. Vitamin A has been shown essential in skin, bone, vasculature, vision, cell proliferation, embryonic growth, and immune functions(128). Lack of vitamin A can lead to numerous growth deformities including underdeveloped growth, iron deficiency, blindness, and numerous immunological malfunctions(129). RA regulates myeloid cell survival and promotes the differentiation of immature MCs into mature populations of DCs, macrophages and granulocytes(27, 95, 130, 131). Additionally, RA appears to be required for the production of mature phagocytes in the bone marrow through its effects on MHC class II and co-stimulatory molecule expression(132).

Retinoic acid is an essential component to both plants and animals. The cleaving of carotenoids can form retinoids (analogs of retinol), carotenoids can be found in
vegetables, fruits, or in flowers.(133) There are 6 active isoforms of retinoic acid found in plants and animals, all-trans, 11-cis, 13-cis, 9,13-di-cis, 9-cis and 11,13-di-cis.(134, 135). The two most widely used active forms of RA are all-trans, and 9-cis respectively. The precursors of RA are known to be converted into active forms in the gut, liver, and target tissues.(136) The metabolic process for retinoic acid is long and complicated and only narrowly covered here. Cellular retinoic acid binding protein 2 (CRBP2) is used as a method to regulate RA uptake by determining the levels of intracellular retinol accumulation and esterification.(137) This then leads to either further processing to retinyl esters for storage or the conversion to retinol for intracellular delivery. Approximately 80% of the total retinoids that are within vertebrates are stored in the liver in ester form. Intracellular deliver is initiated using retinol binding protein (RBP). RBP binds to retinol protecting it from being metabolized and mobilizing it for storage or use.(138). Transthyretin (TTR) also is associated with RBP to prevent elimination from the kidney.(133). The RBP, TTR and retinol are specific for the cell surface receptor identified as stimulated by retinoic acid gene 6 or STRA6. This is a multi-trans membrane protein that mediates cellular uptake of retinol.(139). Once in the cell retinol dehydrogenases oxidize retinol to retinaldehyde (retinal), then to retinoic acid.(140). Once retinoic acid is formed it is bound to cellular retinoic acid binding proteins 1 or 2 and is either transported into the nucleus for active transcription or to nearby target cells.(134). There are two subgroups of receptors, the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). These nuclear receptors are two main modules the DNA binding domain or DBD and the ligand-binding domain or LBD(141). In vertebrates there are three forms
of both receptors the α, β, and γ forms. RARs will bind to all-trans and 9-cis while the RXRs will only bind to 9-cis. Although, the retinoid X receptor can heterodimerize with other nuclear receptors such as peroxisome proliferator-activated receptor, and the previously mentioned vitamin D receptor(142). The heterodimer of RAR/RXR is shown to induce transcription of the active gene. The RXR protein interacts with a zinc finger on RAR that is bound to the retinoic acid response element or RARE on the target DNA causing transcription(143). Target genes that are included in transcription are many self-regulating genes, CRBP, CRABP, CYP26 (oxidation of RA to metabolites) and other non-self-regulating genes such as the Hox genes(144, 145).

1.2.4b Role of RA in immunoregulation

RA has received great attention for its important role in maintaining immunoregulation at mucosal sites (gut). It has also long been used to promote myeloid cell differentiation in patients with myeloid cell leukemia(146, 147). Due to this, it is possible that RA would induce a mature but regulatory MC population(148-150). Within the gut, RA influences the balance between T_{reg} and Th17 cells, B cell isotype switching, antibody production and mucosal homing of numerous immune cells(56, 149, 151-155). Mucosal MCs are largely responsible for producing local RA which acts in a paracrine and autocrine manner to regulate mucosal immune responses(56, 149). Although mucosal DCs produce much of the RA required for immune regulation at mucosal sites, much less is known about RA’s direct impact on MC populations at both an mucosal and non-mucosal sites(87, 96, 151, 152).
As with any excess, excessive amounts of vitamin A can be problematic and result in toxicities to multiple organs including skin, liver, the central nervous system and many others (133). Individuals that undergo retinoic acid treatment also have the possibility of developing differentiation syndrome (DS). Differentiation syndrome, previously known as retinoic acid syndrome is the main life threatening complication with retinoic acid therapy. Differentiation syndrome is thought to be accompanied by renal failure due to capillary leak syndrome that is prompted by the large influx in cytokine release. This near cytokine storm has also been linked to cellular migration and endothelial activation (146, 147). The percentage of individuals that develop DS is approximately 2-27%, of that nearly half develop severe DS associated with increased mortality (147). This wide range of individuals reported to develop DS is most likely due to variability in the identification and reporting of DS in the earlier trials.

1.3 In vivo delivery Methods.

Like many drugs, retinoic acid can exert its effects on numerous cells types once within the body. However the potential complication of systemic RA administration (DS and early metabolism), is cause of great interest in how to effectively deliver RA to differentiate myeloid cells into MCregs. RA can currently be delivered via oral, intraperitoneal injection, intramuscular injection, subcutaneous injection, intradermal injection, and many more but these are systemic in nature. There are new the technologies that can potentially target RA delivery to myeloid or other immune cells such as microparticle, nanoparticle or scaffoldings.
1.3.1 Non-degradable microparticles.

Deposition of an inert, non-dissolvable molecule can be effective in creating a depot-like effect that provides continuous delivery of antigen or other associated materials. Examples include metals such as aluminum-based, iron or gold based microparticles. Such non-degradable microparticle (MP) delivery systems are common and take advantage of the phagocytic properties of many MC populations. Iron oxide MPs are a commonly used delivery system while gold MPs are less commonly used (156). Numerous methods can be used to produce MPs but the self-assembled monolayer (SAM) method has become popular within the non-degradable MP field. This is when the use of SAMs (thiol, carboxylate, isocyanide, etc.) are attached to gold particulates and are incorporated into assembly of the microparticle (157). This delivery method has many advantages including molecular imaging, magnetic modifications to cells, induction of heat, and the induction of inflammatory responses (158-160). Due to the imperfections that can occur during the self-assembly of the MPs the release of product can be non-specific. Additionally, these MPs often induce inflammation which, while helpful for inducing vaccine responses, would limit their effectiveness in inducing regulatory MCs.

1.3.2 PLGA.

Polylactic-co-glycolic acid or PLGA is another commonly used polymer delivery system. PLGA is a biocompatible material with the ability to show sustained release of encapsulated materials (161). When PLGA MPs degrade they are reduced to biocompatible products lactic and glycolic acid. The advantages to using PLGA MPs over various other well accepted bioengineering encapsulation method is the ability of
these MPs to be customized, specifically in size, charge, and surface display(162, 163). Importantly, PLGA is FDA approved and is already used in human medicine, specifically in the form of biodegradable sutures, leading to the notion that it would be ideal for other biodegradable encapsulation uses(162). The two common disadvantages to the use of PLGA are its lengthy release of materials (~30 days) and its unwanted side effect of shifting the pH of the surrounding environment upon degradation(164). The pH shift has important immunological implications due to the fact that if antigen (peptide) is part of the payload, pH shifts have the potential to destroy important immunogenic epitopes.

1.3.3 Liposomes.

Liposomes are micro-particulate lipoidal vesicles that range in size commonly between 0.05-5.0μm in diameter. These micro-particulates form spontaneously when lipids are hydrated in aqueous media(165). Due to their ability to encapsulate either hydrophobic or hydrophilic, they are ideal for the delivery of many drugs occurring in either an entrapped aqueous volume or in the bilayer itself. Liposomes can be classified by many methods including composition (natural or synthetic lipids), size, surface charge, and hydration. The most common method for liposome preparation is the use of thin-film hydration, however this method provides relatively poor encapsulation efficiency. Another issue with the use of liposomes is the consistency in manufacturing causing varied particle size, drug entrapment, and half-lives(166). While the liposome is ideal for encapsulating many drugs it has variable manufacturing and poor encapsulation efficiency.
1.3.4 Acetalated dextran.

Acetalated dextran is a biocompatible polymer that is insoluble in water, only soluble in organic solvents, allowing the processing of the MPs to be developed with standard emulsion techniques, maintaining an advantage similar to PLGA. Acetalated dextran is a homopolysaccharide of glucose that is biocompatible, biodegradable and approved by the food and drug administration (FDA)(167). The reaction of dextran with 2-methoxypropene in the presence of an acid catalyst forms both cyclic and acyclic acetals forming the backbone of acetalated dextran (Figure 3), the basis for the MPs(168). Microparticles are developed by using a standard double-emulsion technique, isolated by centrifugation, washed with basic water (pH~8) then lyophilization and assessed by scanning electron microscopy and dynamic light scattering for size and dimensions. Microparticles have previously been shown to encapsulate numerous sensitive materials including DNA, antigen, and peptides(169, 170). The encapsulation provides significant protection of the payload from the local environment. Due to the composition of the Ac-Dex microparticles they are pH sensitive, specifically as a result of the acetal hydrolysis. At a pH of 7 (cellular) the microparticles are relatively stable, once entering a pH of 5 they become less stable and are likely to degrade releasing the payload. This provides us with the ability to passively target specific environments to release the payload, such as a lysosomal condition a pH of 5(171). The MPs are also tunable for degradation time, by varying ratios of acyclic acetal backbones the degradation time can be manipulated between minuets to months(172). Also MPs are tunable for size, directing the uptake of these microparticles by either pinocytosis or phagocytosis(173). The particle size is
influenced by the emulsion techniques used, depending on size desired either an emulsion (larger) or a microemulsion (smaller) can be used(173). Acetalated dextran microparticles have the ability to reduce the local environmental effects on its payload it can reduce the catabolism of retinoic acid.

Due to retinoic acid having a rapid decrease in half-life because of the increased catabolism products, a method for the targeting of RA to myeloid cells is needed. Previously RA encapsulated liposomes have been used to passively deliver RA to myeloid cells(174). With the use of Ac-Dex MPs the possibility of increased metabolism products is reduced. Also the use of Ac-Dex MPs provides reduce toxicity, little to no change in pH, and a passive targeting system that will deliver retinoic acid directly to antigen presenting cells reducing the exposure to bystander cells.

Figure 3 Acetalated dextran chemical structure.
1.5: Study objectives and hypothesis.

The goal of this study was to evaluate the ability of retinoic acid to induce regulatory myeloid cells in vivo. We have established two hypotheses relative to this project.

Hypothesis 1: Retinoic acid will induce regulatory myeloid cells

Hypothesis 2: Delivery of retinoic acid by Ac-Dex microparticles will provide a passively targeted delivery system to myeloid cell, inducing regulatory myeloid cells.
Chapter 2: Materials and Methods.

Mice.

C57BL/6 (H-2^b^) mice (4-8 wk old), C57BL/6-Tg (TcraTcra)425Cbn/J, C57BL/6-Tg(Tcra2D2,Tcra2D2)1Kuch/J and reporter Foxp3EGFP (B6.Cg-Foxp3^tm2Tch^) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) or bred in-house. Mice were housed five per cage and maintained on a 12 hr. light/dark cycle, maintained under specific pathogen-free conditions and were housed and cared for according to the institutional guidelines of the Ohio State University’s Institute for Animal Care and Use Committee.

Cell lines.

EG7 and EL4 cells are T lymphoma cell lines used to study the MHC class I restricted response of CTLs in mice. The EL4 lymphoma cell line, the EG7 cells which are EL4 cells transfected to constitutively secrete OVA 257-264 peptide and the NR-9456 macrophage cell line on the C57/BL6 background were kindly provided by Dr. Prosper Boyaka (the Ohio State University). The DC2.4 cell line is a murine bone marrow derived dendritic cell line kindly provided by Dr. Kenneth Rock (Dana-Farber Cancer Institute).
BM-MC differentiation and development of regulatory MC differentiation model.

Bone marrow (BM) cells were collected from the femurs, tibias and humeri of C57Bl/6 mice. After erythrocyte lysis (AKC or in-house lysis buffer), cells were cultured with RPMI 1640 (Invitrogen) supplemented with 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 5 x 10^{-5} M 2-mercaptoethanol and 200U/ml recombinant murine GM-CSF (R&D Systems) ± 100 nM of either estriol (E3), all-trans retinoic acid (RA) or E3/RA microparticles (Sigma-Aldrich/Bachelder group) for 6-7 days at a density of 2x10^6 cells/ml. Day 6-7 cells were considered differentiated BM-MCs (media control) and BM-MC_{regs}(RA and E3). Cells were challenged with inflammatory stimulus LPS (1 μg/ml, 055:B5, Sigma-Aldrich) during \textit{in vitro} culture as indicated at day 6 or later for BM-DCs.

**Functional immunosuppressive assays: T cell proliferation assay.**

Myeloid cells (BM-MCs or BM-MC_{regs}) were cultured with responder spleen cells from antigen-specific T cell receptor transgenic (TCR Tg; where antigen was either OVA323-339 or MOG35-55) or Foxp3+EGFP mice as indicated. To assess T cell proliferation co-cultures were stimulated with anti-CD3 (BD Bioscience), T cell-receptor specific antigen MOG35-55 (Bio Matic) or T cell-receptor specific antigen OVA 323-339 (Anaspec). To assess the effects of myeloid cell activation, co-cultures were stimulated with LPS from Escherichia coli, 055:B5 (Sigma-Aldrich) for 96 hours, pulsed with (H^3 thymidine) (Perkin Elmer Life Sciences or MP Biomedicals) in the last 18 hours, harvested and counted, data is expressed as counts per million (cpm) ± SEM (127).
Functional immunosuppressive assay: CD8+ cytotoxic assay.

To generate cytotoxic T lymphocytes (CTLs), spleen (SPL) and lymph nodes (LN) were removed from antigen-specific CD8+ T cell receptor transgenic OT-1 mice specific for SINFEKL (OVA 257-264) peptide and pooled for use as responder cells. Responder cells were cultured with OVA (257-264) pulsed DC2.4 cells for 4 days, removed and cultured with mrIL-2 (R&D Systems) for 2 days. OVA-expressing (EG7) and non-transfected control cells (EL4) were seeded at 2x10^4 cells per well and co-cultured with CTLs (1x10^5) and control or RA treated monocytes (2x10^4) for 6-18 hours (175, 176). An MTT assay (Sigma-Aldrich) was used to determine quantity of live cells adapted from methods described in(177). Briefly, after incubation, cells were centrifuged (1500RPM for 5 min) media was decanted and 100ul of fresh media was added. 10ul of 5mg/ml thiazolyl blue tetrazolium bromide (MTT) was added to each well for 2 hours at 37°C. After incubation cells were centrifuged (1500 RPM for 7 min) and media was decanted, cells were allowed to dry for 15-30 min before 100ul of DMSO was added, mixed well and read at 570nm wavelength on a Spectra Max 2. The absorbance levels were calculated by averaging the non-specific and specific absorbance levels of five separate data sets. Media control is compared to RA treated cells.

In vitro Treg induction.

Bone marrow (BM) cells were collected from femurs, tibias and humeri of C57Bl/6 mice as previously described. After erythrocyte lysis, BM cells were cultured with supplemented RPMI (as previously described) for 7 days at a density of 2x10^6
cells/ml +/- RA. Spleens from mice with reporter Foxp3EGFP (B6.Cg-Foxp3^{tm2Tch}) were harvested, passed through cell strainers (70µm, BD Falcon), collected by centrifugation (1500 RPM for 7 Min at 4°C) and subjected to erythrocyte lysis. Responder cells and MCs or CD11c- MCs were cultured for 4-6 days and aliquots from cultures assessed for Foxp3 expression by flow cytometry.

Flow Cytometry.

In vivo and in vitro derived MC populations were labeled and evaluated by three-color flow cytometry using combination of the following conjugated directly antibodies (clone): CD11c (HL3), CD11b (M1/70), Gr-1 (RB6-8C5), Ly6G (1A8), Ly6C (AL-21), MHC class II (AF6-120.1), CD80 (16-10A1), CD86 (IT 2.2), PD-L1 (MIH5), and PD-L2 (YT25) with appropriate isotype controls. (BD Bioscience, eBiosciences or Miltenyi Biotec). Cells were stained with fluorochrome-labeled antibodies or isotype controls for 20 min in the dark at 4°C, washed twice in FACS buffer and re-suspended in 300 µl FACS buffer for flow cytometry analysis.

Intracellular IL-10, IL-4, IL-17 and INF-γ levels were measured after incubating myeloid cells with 1 µg/ml LPS overnight (IL-10) or Ionomycin (1 mg/ml) and PMA (25 ng/ml) for 4 hours (IL-4, IL-17, and INF-γ). 2 µM of Monensin (eBioscience) added 2-4 hours before harvesting cells. Cells were removed from culture, washed with 2 ml of supplemented RPMI and blocked with 0.5 µg/ml Fc block (anti-CD16/CD32) for 15 minutes. Cells that required extracellular markers were re-suspended in FACS buffer and stained with anti-CD4 (0.2 mg/ml) and incubated in the dark at 4°C for 20 min. Cells were washed with FACS buffer (2x with 1ml) fixed and permeabilized using FIX/PERM
solution (BD Bioscience), briefly vortexed and incubated in the dark at 4°C for 20 min. Cells were then washed twice with 1 ml of PERM/WASH buffer (BD Bioscience), re-suspended in PERM/WASH buffer and stained with 0.2 mg/ml anti-IL-10 (BD Bioscience) for 30 min. in the dark at 4°C. All flow samples were processed on an Accuri C6 flow cytometer and results analyzed using the Accuri C6 Flow software (BD Biosciences).

**Myeloid cell Purification.**

Day 6-7 differentiated BM cells were incubated with manufacturer suggested amounts of CD11c/CD11b microbeads (Miltenyi Biotec) for 15 minutes in the dark at 4°C. Cells were washed with running buffer (10% FBS in PBS with 900 mg of NaN₂ per 1L of PBS), and centrifuged (1500 RPM, 7Min). Cell separation was performed using either the Auto Macs (Miltenyi Biotec) magnetic separation instrument or the FACS Aria III 12 color, 4 laser cell sorter. The Auto Macs was used according to the manufacturer’s instructions. Cell sorting with the FACS Aria III was performed at the OSU Flow Cytometry Core and isotype control antibodies were included to determine detection levels. CD11b⁺CD11c⁻ Ly-6C<sup>low</sup> monocyte populations were serially gated on CD11c⁻ cells, followed by CD11b⁺ with gates set around distinct populations of Ly-6C low, intermediate and high. The purity of the cell populations was ≥95%.

**Microparticles.**

All chemicals pertaining to the microparticles were purchased from Sigma (St. Louis, MO) and used as received, unless otherwise indicated. Dextran (C₆H₁₀O₅)ₙ 71
kDa, was used to synthesize acetalated dextran (Ac-Dex, 71 kDa, 59% cyclic acetal coverage) using the methods described in previous work(171, 172, 178).

**Ac-Dex Synthesis.**

Lyophilized dextran (1g, MW = 10 400) and pyridinium p-toluenesulfonate (0.0617mmol) were dissolved in anhydrous dimethyl sulfoxide (DMSO, 10 mL) and reacted with 2-ethoxypropene (37 mmol, Waterstone, Carmel, IN) under nitrogen gas at room temperature. At 50 min aliquots were withdrawn and quenched with triethylamine, precipitated with basic water (0.02% v/v triethylamine in water, pH 9), vacuum filtered, and lyophilized for two days. The products were purified by dissolving them in ethanol (200 proof) and centrifuging (5 min, 9,000 × RPM, Thermo Legend Micro 21). The supernatants were precipitated again in basic water and lyophilized for two days to yield Ace-Dex polymer (2 g), a powdery white solid.

**Retinoic acid emulsion particle fabrication.**

RA (2mg) was dissolved in 2ml of dichloromethane and added to Ace-Dex (100mg). A 3% polyvinyl alcohol (PVA) solution was added the mixture was homogenized for 30 s at 18,000 RPM using Polytron PT 10–35 Homogenizer (Westbury, NY). The particles were added to 0.3% PVA solution and stirred on a magnetic stir plate for 2 h to evaporate the organic solvent. The particles were isolated through centrifugation for 10 min at 4°C and 9160 rpm. The supernatant was decanted, the particles re-suspended in basic water, washed two more times with basic water, freeze-dried without additional compounds added (cryoprotectants) and stored under at −20°C until use.
**Encapsulation efficiency.**

ATRA was measured in particles by suspending microparticles (RA or Blank) (1mg/ml) in DMSO to dissolve particles creating a solution. A standard curve was created with the use of free drug measured on a plate reader at 350nm. The ATRA or Blank MPs dissolved in DMSO were also read on the plate reader and compared to the standard curve.

**Statistical analysis.**

Data are represented as mean +/- SEM or fold change. Statistical significance was determined using a Student’s t-test or 1 way ANOVA with a significance level (p-value) < 0.05 and the Wilcoxon signed-rank test. All analyses were performed using Excel and/or GraphPad Prism software (La Jolla, CA).
Chapter 3 Differentiation of regulatory myeloid cells and the potential for therapeutic applications.

3.1.1: The differentiation of regulatory myeloid cells.

Given that RA is a regulator of mucosal immunity and influences myelopoiesis, we hypothesized that RA would induce a population of mature MCregs that was more potent than the known E3 differentiated regulatory DCs. To determine whether RA could influence the differentiation of myeloid cells we differentiated freshly isolated bone marrow cells with GM-CSF and RA or E3 for 6-7 days and then assessed for a regulatory phenotype and function. On day 7 BM cells that were differentiated with GM-CSF and RA were able to suppress the proliferation of responder immune cells and this suppression was markedly greater than either control or E3 treated cells (Figure 4A). The ability of RA differentiated cells to suppress proliferation was apparent regardless of whether responder immune cells were stimulated with either antigen specific peptides or non-antigen specific stimuli anti-CD3. Interestingly, cells treated with E3 suppressed proliferation after stimulation with peptide but not anti-CD3 (Figure 4A). We next determined whether the RA differentiated cells remained regulatory when exposed to the inflammatory stimulus LPS. Figure 4B shows that RA differentiated cells maintained their ability to suppress proliferation even after exposure to LPS challenge and that this
was present following stimulation of co-cultures with either peptide or anti-CD3. E3-treated cells were no longer able to suppress proliferation upon exposure to inflammatory stimulus *in vitro*. These results suggest that differentiation under the influence of RA myeloid cells were able to maintain their regulatory ability following exposure to an inflammatory stimulus and that RA may generate MCregs, which are more potent and stable than E3 differentiated cells.

MCregs have a variety of mechanisms by which to regulate immune responses with production of IL-10 and induction of Tregs being two important mechanisms. IL-10 is a potent immunoregulatory cytokine whose production has been shown to be increased in E3 DCregs and other MCreg populations (127, 179, 180). To evaluate whether RA induced an increased number of IL-10 secreting cells, we evaluated RA differentiated cells for intracellular IL-10 production by flow cytometry. Figure 4C shows that RA differentiated cells had an increased percentage of IL-10-producing cells compared to either media or E3 control cells. To evaluate whether RA differentiated cells increased Treg numbers, we co-cultured RA differentiated cells with naïve immune cells from EGFP Foxp3 reporter mice. We found that RA differentiated cells were able to induce a significant increased percentage of FoxP3+ cells following a 5 day culture with naïve immune cells (Figure 4D). Cells differentiated *in vitro* in the presence of E3 failed to significantly increase either IL-10+ cells or induce Treg cells (Figures 4C, D). These results show that RA differentiated cells suppressed the proliferative abilities of responder immune cells that RA induced increased percentages of IL-10+ cells and that RA differentiated cells promoted the induction of FoxP3+ (Treg) cells.
Maturation status of MCregs can influence the therapeutic potential with mature MCregs considered to be more stable in regulatory phenotype. To determine whether these RA differentiated cells were mature in nature, we evaluated the cell surface expression of maturation markers CD80, CD86 and MHC class II and inhibitory markers PD-L1 and PD-L2. RA differentiated cells demonstrated an increased percentage of CD80^+, CD86^+ and MHC class II^+ (Figure 5A), indicating that an increased proportion of the cells were mature and/or activated in comparison to E3 or control cells. Additionally, there were increases the mean fluorescence intensity (MFI) of CD80, CD86 and MHC class II in RA differentiated cells as depicted in Figure 5C and Figure 5D, indicating that the relative expression levels on a per cell basis were increased in RA differentiated cells. Although E3 differentiated cells had mildly increased expression levels of CD80, CD86 and MHC class II, RA differentiated cells had consistently higher levels than either E3 differentiated or control cells. To confirm whether RA differentiated cells demonstrated an “activated regulatory” phenotype as previously described for E3, we evaluated the expression of inhibitory co-stimulatory molecules PD-L1 and PD-L2 (127). RA increased the percentage of PD-L1^+ cells (but not PD-L2^+) (Figure 5B) and the MFI of both PD-L1 and PD-L2 (Figure 5C, D) compared to E3 or media controls. These results suggest that the presence of RA during differentiation induces a population of mature activated MCregs that suppress the proliferation of responder immune cells even in the face of inflammatory challenge. Additionally, our data shows that RA MCregs appear to have superior regulatory abilities compared to E3 MCregs when presented with an inflammatory challenge (Figure 4A).
Figure 4 RA treatment of bone marrow myeloid cells produces a MC$_{reg}$ population.
Figure 5 RA treatment of BM-MCs produces mature MCs.

3.1.2: CD11b⁺ but not CD11c⁺ cells were the suppressive population.

DCs are a primary MC population that have been of great interest for tolerogenic cell therapy and many studies have looked to generate immature DCs and their myeloid precursors for therapeutic purposes(95, 98). To generate MC population for cell therapy applications, we utilized GM-CSF to differentiate MCs. *In vitro* differentiation of bone marrow cells with GM-CSF is a commonly used protocol to produce large numbers (>80%) of highly enriched CD11c⁺ immature DCs that, upon inflammatory stimulation in the final days of differentiation can generate mature DCs(150, 154, 181). To investigate
whether the MC<sub>regs</sub> induced by RA were DC<sub>regs</sub>, we purified CD11c<sup>+</sup> cells from day 7 differentiated cells and cultured them with responder immune cells. Although RA induction of mucosal “DC<sub>regs</sub>” have been described (87, 148, 182), surprisingly we found that RA-treated CD11c<sup>+</sup> cells were not the suppressive cell population (Figure 8A). In all experiments, RA treated CD11c<sup>+</sup> cells failed to suppress proliferation and had variable effects of either no proliferation or in some experiments, RA CD11c<sup>+</sup> DCs actually enhanced proliferation. Phenotypic evaluation of these CD11c<sup>+</sup> cells showed no difference in percentage (Figure 6B) or expression levels of CD80<sup>+</sup>, CD86<sup>+</sup>, MHC class II<sup>+</sup>, PD-L1<sup>+</sup> and PD-L2<sup>+</sup> compared to media controls. To determine the source of the suppressive MC<sub>regs</sub>, we evaluated the CD11c<sup>-</sup> population and confirmed that phenotypic changes similar to previously described cells in the E3 model were present within the CD11c<sup>-</sup> fraction (Figure 6C). These CD11c<sup>-</sup> cells had a marked (>30%) increase in the percentage of CD80<sup>+</sup>, CD86<sup>+</sup>, MHC class II<sup>+</sup> and PD-L1<sup>+</sup> cells (with no differences in PD-L2<sup>+</sup> cells) (Figure 6D) when differentiated with RA. This phenotype is consistent with an activated regulatory phenotype in these cells described previously (127). In contrast, levels of CD80, MHC class II and PD-L1 did not change, remaining consistently high (>80%) in RA versus control MCs. (Figure 6B). These data suggest that RA present during GM-CSF differentiation increased an activated regulatory phenotype in the CD11c<sup>-</sup> (non-DC) populations.

Both differentiated and precursor populations within the bone marrow are predominantly (>90%) but not completely CD11b<sup>+</sup>(Figure 7). Our data demonstrated that while approximately 80-90% of the cells were CD11c<sup>+</sup>, an additional >95-99% were
CD11c− but CD11b+ (Figure 7). To definitively isolate the effects of CD11b+CD11c− cells, we serially purified CD11b+ cells from the CD11c− fraction and evaluated their phenotype and function. As expected, the increases in the percentage of CD80+, CD86+, MHC class II+ and PD-L1+ cells seen in figure 3D was also seen in the CD11b+CD11c− purified population (Figure 8A). We then evaluated the ability of these cells to influence CD4+ and CD8+ responses. We found that the CD11b+CD11c− population was able to suppress the proliferation and modify the cytokine profile of responder immune cells and of T cells (Figure 8B). The proliferating CD4+ responder immune cells cultured with RA CD11b+CD11c− cells were also shown to have reduced expression of IL-17 and IFN-γ (Figure 8C) and IL-10 (Figure 9) but no change in IL-4 production as determined by intracellular cytokine staining (Figure 8C). Intracellular IL-10 and FoxP3+ cells were also increased as expected (Figure 9A and 9B, respectively). We also evaluated the ability of RA CD11b+CD11c− cells to influence CD8+ T cell responses. Using an OVA specific activation of CD8+ T cells, we demonstrated a reduced cytotoxicity in CD8+ T cells cultured with RA CD11b+CD11c− cells (Figure 8D). Taken together, these results suggest RA induced an activated regulatory population of CD11b+CD11c− cells that were able to suppress both CD4+ and CD8+ adaptive immune responses.
Figure 6 RA mediated suppression of T cell proliferation is not mediated by CD11c+ BM-MCs.

Figure 7 GM-CSF induced myeloid cells.
Figure 8 RA generated CD11b+ BM-MCs suppress T cell proliferation and express a regulatory phenotype.
3.1.3: CD11b+CD11c-Ly6C<sub>low/intermediate</sub> were the primary population responsible for suppression.

Although used primarily to induce large numbers of DCs, differentiation with GM-CSF can potentially promote the differentiation of a mixture of granulocytes, monocytes, macrophages and DCs (154, 181, 183-186). In our GM-CSF cultures, we found that Ly-6G<sup>+</sup> granulocytes were no longer present in CD11b<sup>+</sup> cells at day 7 of differentiation (Figure 7B), indicating that granulocytes were not responsible for the suppression seen in CD11c<sup>-</sup>CD11b<sup>+</sup> cells (187, 188). Monocytes are typically phenotypically labeled as CD11b<sup>+</sup>CD11c<sup>-</sup>Ly-6C<sup>+</sup>. To determine whether monocytes were present and may be responsible for the suppressive effects, we evaluated day 7 non-adherent cells sorted based on their relative expression of the monocyte marker Ly-6C. Ly-6C expression levels have been shown to correlate with cellular function and maturation level where Ly-6C<sup>high</sup> monocytes are inflammatory and Ly-6C<sup>low</sup> monocytes...
are steady-state or regulatory (22, 28). Figure 10A shows that the presence of RA during differentiation increased the percentage of cells expressing low to intermediate levels of Ly-6C. To determine whether the increase in these cells was responsible for the suppression seen in the CD11b<sup>+</sup>CD11c<sup>-</sup> population, we sorted cells based on relative Ly-6C expression into Ly-6C<sub>low</sub>, Ly-6C<sub>intermediate</sub>, and Ly-6C<sub>high</sub> expression patterns. Figure 10B demonstrates that both Ly-6C<sub>low</sub> and Ly-6C<sub>intermediate</sub> cells were able to suppress up to a 6-fold decrease in proliferation of responder cells following antigenic stimulation while Ly-6C<sub>high</sub> cells failed to influence peptide-specific proliferation. Similarly, Ly-6C<sub>low</sub>, Ly-6C<sub>intermediate</sub> cells maintained their ability to suppress proliferation (Figure 10C) even when co-cultures were stimulated with LPS. In contrast, Ly-6C<sub>high</sub> actually significantly increased the proliferation of responder immune cells (Figure 10C) following stimulation with LPS. These results demonstrate that RA Ly-6C<sub>low</sub> and Ly-6C<sub>intermediate</sub> cells are the suppressive population and are able to maintain suppressive abilities even in the presence of inflammatory (LPS) challenge. RA Ly-6C<sub>low</sub> cells also showed the most marked increase in regulatory marker PD-L1<sup>+</sup>, as well as maturation and activation markers CD86<sup>+</sup> and MHC class II<sup>+</sup> cells, with over 90% of the Ly-6C<sub>low</sub> cells expressing PD-L1 (Figure 11A). A similar but less dramatic phenotype was seen in the Ly-6C<sub>intermediate</sub> cells (Figure 12). Taken together, these data show that RA induces a small but potent population of CD11b<sup>+</sup>CD11c<sup>-</sup>Ly-6C<sub>low/intermediate</sub> MCregs consistent with an activated regulatory monocyte phenotype that are able to suppress immune cell proliferation.
Figure 10 RA CD11b⁺CD11c⁻Ly6C<sub>low/intermediate</sub> are the suppressive population

Figure 11 CD11b⁺CD11c⁻Ly-6C<sub>low</sub> cells have an activated regulatory phenotype.
3.1.4: Generation of MC\textsubscript{regs} \textit{In vitro} by Ac-Dex MPs.

While adoptive cell therapy to treat inflammatory disease has therapeutic potential, the ability to induce MC\textsubscript{reg} populations in vivo would provide a useful therapeutic option obviating the need for in vitro methodologies. Ac-Dex MPs are a means to passively target delivery of regulatory molecules such as RA to MCs \textit{in vivo} to induce MC\textsubscript{regs}. To determine whether RA encapsulated within Ac-Dex MPs have the ability to induce MC\textsubscript{regs}, we first evaluated RA Ac-Dex MP effects on MCs \textit{in vitro}.

To accomplish this we used a macrophage cell line from mice with a background strain of C57Bl/6. Macrophages were plated and exposed to RA or empty MPs for 48 hours to determine whether phenotypic or functional characteristics of MC\textsubscript{regs} developed.
Figure 13 shows the increase in PD-L1 expression on macrophages exposed to RA MPs after 48 hours. Specifically, blank MP treatment had similar PD-L1 levels as control media treated cells of 63% and 65%, respectively. Addition of both free RA and RA MPs significantly increased the percentage of PD-L1 cells to 79% and 82%, respectively with the increase in RA MP being significantly increased over that of free RA. When evaluated MPs containing encapsulated E3, we see no significant increase in the percentage of PD-L1+ cells in either E3 MPs or free E3 treated macrophages although free E3 appeared to have a slight ability to increase PD-L1+ cells but never to the level of either RA MPs or free RA (Figure 14).

Figure 13 RA MPs induce increased expression of PD-L1 on C57BL/6 macrophages in vitro.
Figure 14 RA MPs induce increased expression of PD-L1 on C57BL/6 macrophages *in vitro* compared to E3 MPs.

### 3.1.5: Ac-Dex Microparticle trafficking.

Although our data suggests that Ac-Dex MPs may be useful to modify MCs phenotypes, nothing is known about their relative trafficking following *in vivo* administration. Intravenous administration results in primarily delivery to the liver and lung through first-pass metabolism and other non-parenteral (subcutaneous or intradermal administration) routes are of therapeutic interest we wanted delivery of the payload to the myeloid cell rich draining lymph nodes. We next wanted to determine the relative trafficking patterns of Ac-Dex MPs *in vivo* following intradermal and subcutaneous administration. To accomplish this we encapsulated FITC labeled BSA into MPs and compared *in vivo* distribution of FITC+ cells in mice given free FITC BSA and FITC
BSA MPs. Figure 15 and 16 show the percent FITC expression in the CD11c+ and CD11b+ cells 24 hours post injection within the draining lymph nodes. Figure 15A and B shows a statistically significant increase (p<0.05) in FITC+ CD11c+ cells in the draining lymph nodes of mice injected with the FITC BSA MPs over that of the free FITC injected mice in both the subcutaneous (Figure 15A) and intradermal (Figure 15B) delivery methods. Similar trends were seen in other lymph nodes (axillary and popliteal) but were not statistically significant. When CD11b+CD11c- (macrophage/monocyte) cells were evaluated, a significant increase in FITC+ cells was seen in mice given FITC MPs over free FITC, regardless of injection route (Figure 16A,B). Additionally, significant increased levels of FITC+ CD11b- cells were seen in all potential draining lymph nodes (axillary, inguinal and popliteal; Figure 16B). These findings indicate that the intradermal injection of MPs are the most efficient way to deliver a payload to draining lymph nodes by MPs in vivo.
Figure 15 Trafficking of FITC uptake within dendritic cells.
3.1.6: Inducing MC\textsubscript{regs} with MPs \textit{in vivo}.

To determine whether RA MPs could induce MC\textsubscript{regs} \textit{in vivo}, we injected mice with RA or blank MPs. Figure 17 shows that mice given RA MPs had an increased percentage of PD-L1\textsuperscript{+} cells, however this increase was not significant. Importantly, the proliferation response of cells within the draining lymph nodes of mice receiving RA MPs were significantly decreased when stimulated with a non-antigen specific stimulus, ant-CD3(Figure 18). Also of interest is that the RA MP differentiated MCs were able to
maintain their regulatory capability when face with an inflammatory stimulus (LPS). These results show that RA MP administration decreased proliferation in draining lymph nodes and suggests that the induction and maintenance of MC\textsubscript{regs} may be able to maintain their regulatory abilities within an inflammatory environment.

Figure 17 RA MPs induce increased expression of PD-L1 on myeloid cells \textit{in vitro} compared to blank MPs.
Figure 18 RA MPs induce increased regulatory function of myeloid cells *in vitro* compared to blank MPs.
Chapter 4: Conclusion and discussion.

The principal objective of this study was to evaluate the ability of RA, a steroid hormone known to play important roles in regulating both mucosal immune responses and differentiation of myeloid cells, to generate an activated/mature MC\textsubscript{reg} population \textit{in vitro} and \textit{in vivo}. We demonstrate that RA influences myelopoiesis to differentiate MC\textsubscript{regs} that were surprisingly not DC\textsubscript{regs} but a CD11b\textsuperscript{+}CD11c\textsuperscript{-}Ly-6Clow/intermediate regulatory monocyte. These regulatory monocytes decreased both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell responses and promoted FoxP3\textsuperscript{+} (T\textsubscript{reg}) cell induction. Our data suggest that RA has distinctly different effects on monopoiesis and dendropoiesis to promote the generation of regulatory monocytes \textit{in vitro}. We furthered our study to determine if the use of RA to differentiate MC\textsubscript{regs} \textit{in vivo} was viable. We next showed that with the use of Ac-Dex MPs we can passively deliver RA to mature myeloid cells (macrophages) \textit{in vitro} to promote phenotypic and functional changes consistent with a regulatory macrophage. We also demonstrated that MP delivery \textit{in vivo} is a potentially viable means to deliver regulatory molecules such as RA. Both CD11b\textsuperscript{+} and CD11c\textsuperscript{+} MCs are targets of subcutaneously and intradermally delivered MPs. Finally, we also show that when RA is encapsulated within Ac-Dex MPs and delivered intradermally into mice we increased PD-L1 expression on MCs and that the proliferation of responder immune cells within the draining lymph
nodes is decreased upon stimulation with LPS, a commonly used treatment to stimulate MCs.

MCregs are a diverse population of cells, considerable attention has been focused on the in vitro generation and clinical application of MCregs. While the in vitro generation of such MCreg populations has great therapeutic potential, much remains to be learned regarding the factors which contribute to MCreg induction. Many in vitro generated MCregs are arrested in an immature or hypo-functional state. An emerging concern is that these immature MCreg populations may mature to become inf-DCs or macrophages and, thus, contribute to inflammatory disease pathology (83, 99, 100, 106-108, 189). A more recent approach is to induce mature MCregs which would be stable and maintain regulatory potential in an inflammatory environment (99, 109, 190, 191). Anderson and colleagues have demonstrated that human DCregs (generated with dexamethasone, vitamin D and LPS) maintain tolerogenic activity and actually induce significantly higher levels of IL-10 production by resultant T cells (110). However, the relative stability and ability of MCregs (such as DCregs) to maintain regulatory abilities during inflammation may still be in question. For example, a study by Voigtlander et al. suggests that DCregs induced by TNF-α do not maintain their regulatory abilities upon a secondary stimulation with TNF-α in vivo (111). Obviously, this is of considerable concern given that TNF-α is present in a large array of inflammatory conditions where such DCregs (or other MCreg populations) may be applied therapeutically. Much work remains to determine critical factors important in generating mature MCregs for anti-inflammatory therapies but we have focused on non-inflammatory pathways to induce mature MCregs.
We have shown that mature MCregs can be generated with the use of steroid hormones alone (127). Our previous work has shown that the sex steroid hormone estriol (E3) induces a mature activated MCreg population of CD11c⁺ DCregs that protects against inflammatory challenge in vitro and in an in vivo disease model (127). In the present study, we have extended our research to identify additional means of normal homeostatic induction of mature MCregs by investigating the ability of the steroid hormone RA to induce mature MCregs that are resistant to inflammatory challenge. Our results show that RA is more effective than E3 in vitro in generated MCregs and that these MCregs are resistant to LPS inflammatory challenge.

RA is known for its ability to promote the differentiation, and maturation, of myeloid cell populations. This ability, along with its known immunoregulatory role at mucosal sites, made it a logical candidate for these studies (130, 192). RA is present in relatively large concentrations within mucosal sites and is largely produced by local antigen presenting cells (APCs) residing within these mucosal sites. Specifically, mucosal CD103⁺ DCs are the primary immunoregulatory myeloid cells within the gut. These DCs have up-regulated Raldh2 gene expression, constitutively produce RA, and produce increased TGF-β. They also significantly induce Foxp3⁺ Tregs, mucosal homing receptors CCR9 and α4β7 expression on lymphocytes and enhance antibody production and Ig isotype switching (56, 87, 148, 182). These mucosal DCs are the most common MCs investigated regarding RA biology and induced mucosal DCs have been generated from monocytes or splenic DCs with GM-CSF with IL-4 (86, 155) or bone marrow precursors with RA (95, 153, 182, 193, 194). Increasingly, the non-mucosal and therapeutic
applications of RA (cancer) are being investigated (87, 96, 130, 155, 195) and this study focused on RA’s ability to induce mature activated MCregs that are able to suppress responder immune cell proliferation (86, 127, 153, 155, 182, 196).

Given RA’s critical role in DC-mediated immunoregulation within the gut, it was quite surprising that RA CD11c⁺ cells were not suppressive in our study. One possibility is that DCs differentiated with RA could generate mucosal DCs but wouldn’t generate mature activated DCregs that could suppress proliferation as seen with E3 DCregs. While induction of mucosal DCs can be accomplished with RA (95, 155), the immunomodulatory abilities of these DCs were not the focus of this study. Alternatively, the timing of RA administration may have resulted in the lack of DCreg induction as described by Feng and colleagues (153). Specifically, their studies showed that the presence of 1 µM RA from day 0 throughout differentiation failed to induce mucosal DCs. Although our study utilized different dosages and evaluation criteria than those in the Feng study, it is possible that the continuous presence of RA during differentiation may have resulted in the inability to induce DCregs in our study. Similarly, Wada’s group showed that the use of a synthetic RARα and β agonist (AM-80) could differentiate human peripheral blood monocytes into DCs with tolerogenic phenotype and function (95). The use of AM-80 versus ATRA in our study or the differentiation of human monocytes versus murine myeloid progenitor could explain the differences in DCregs versus regulatory monocytes seen in our study (95).

It could be argued that CD11c⁻ DC precursors existed within the population of CD11b⁺CD11c⁻ suppressive cells rather than the cell population being regulatory
monocytes. Given the described effects of RA in promoting differentiation and maturation, in conjunction with our data demonstrating an activated phenotype, we believe this to be unlikely (40, 182, 197, 198). Rather, our data on Ly-6C expression strongly support that the suppressive cells were regulatory monocytes with an activated regulatory phenotype (increased CD80, CD86, MHC class II and PD-L1) consistent with previous work within our lab with other MC_reg populations. Given that the CD11b^+ CD11c^- population is comprised of less than 20% of the entire population, the ability of these cells to suppress both CD4^+ and CD8^+ responses is noteworthy. The specific contribution of cell contact-dependent (PD-L1) versus cell contact-independent (IL-10, TGF-β, etc.) mechanisms responsible for the regulatory abilities of these cells was beyond the scope of this study. However, we did see increases in regulatory markers including PD-L1, IL-10 and the percentage of FoxP3^+ cells with RA MC_reg.

Monocytes are circulating myeloid cells which give rise to tissue DCs and macrophages, their regulatory abilities have recently been recognized(22). Although numerous markers can be present on mouse monocytes (CD11b, CD115, CCR2, CX3CR1 and Ly-6C), we chose to investigate Ly-6C expression levels given that it has been correlated with monocyte function (22, 28, 40, 175). Specifically, Ly-6C_high represents an inflammatory monocyte while Ly-6C_low/^- monocytes have been shown to play important roles in tissue repair, patrolling vasculature and potentially resolving inflammation(22, 28, 60, 199, 200). Ly-6C is also down-regulated following differentiation which is consistent with our findings where RA, a molecule known to promote differentiation and maturation, increases the percentage of cells that are Ly-
Although levels of Ly-6C haven’t been directly correlated with the functional abilities of BM-derived monocytes, our data suggest that Ly-6C levels correlate with suppressive abilities with the lowest Ly-6C expression associated with the most suppressive ability. Given that Ly-6C$_{\text{high}}$ monocytes are typically inflammatory monocytes, it is not surprising that proliferation is actually enhanced following LPS stimulation in this cell population (Figure 10C). Taken together, these data showed a progression from Ly-6C$_{\text{high}}$ to Ly-6C$_{\text{low}}$ associated with increasing regulatory abilities. These results are consistent with the association seen between Ly-6C expression and blood monocyte function described by others (22, 28, 40, 201). Currently, the mechanisms and pathways by which RA maturation of monocytes imparts them with increased regulatory abilities remain undefined. Whether a specific signal during differentiation drives monocytes to become regulatory in an active process or whether differentiation under homeostatic or regulatory (RA) conditions in the absence of inflammatory stimuli is a default mechanism for regulatory monocyte induction is unknown. Additionally, whether these RA Ly-6C$_{\text{low/intermediate}}$ monocytes have the potential to further differentiate into DC$_{\text{reg}}$ or regulatory macrophage populations remains to be determined and is the subject of ongoing studies within our laboratory (22).

Due to the known side effects of systemic RA administration, an alternative method for delivering RA for therapeutic induction of MC$_{\text{regs}}$ was a focus of the half of this study. The use of MP for the delivery of RA \textit{in vivo}, to induce MC$_{\text{regs}}$ was chosen for many reasons. Firstly, we wanted to use MPs to diminish the effects of systemic RA on non-targeted immune cells such as non-phagocytic cells. Individuals that undergo RA
therapy have the possibility of developing differentiation syndrome (DS), this is often accompanied by renal failure due to capillary leak syndrome, associated with an excess of RA in the epithelial cells(146, 147). With the use of specific MPs we have the ability to target or passively target specific cell types. Secondly we chose to use MPs to protect RA from early metabolism. Retinoic acid has been observed to have rapid decrease of half-life with continuous oral administration or intravenous injection due to the increase cytochrome P450s an enzyme implicated in metabolizing RA(202, 203). Utilization of MPs to target delivery to MCs was considered a means to avoid such complications. There are various materials available to encapsulate RA, we specifically chose Acetalated dextran.

We chose to utilize Ac-Dex MPs due to multiple key characteristics of Ac-Dex. First, Ac-Dex is FDA approved and unlike FDA approved PLGA, the metabolites of Ac-Dex are nontoxic and fail to induce a potentially harmful pH shift(167). Additionally these MPs have been shown to effectively encapsulate numerous sensitive materials including DNA, antigen, and peptides(169, 170). Finally, the size and release characteristics of Ac-des MPs can be modified. Alterations in the size of MPs can promote either pinocytosis or phagocytosis by professional phagocytic MCs to passively target intracellular (lysosomal) compartments(171, 173). Degradation times can be modified by varying ratios of acyclic acetal backbones which can create degradation times ranging from minutes to months(172). Such key characteristics allow for an adjustable system to deliver regulatory molecules, such as RA, directly to MCs in a precise controlled and passively targeted manner.
It is important to understand that MCs are not only influenced within the bone marrow but are also “educated” within by the microenvironment to become functionally specific. RA is known for its ability to promote the differentiation of MC populations and play a immunoregulatory role at mucosal sites, making it a logical candidate for these studies(130, 192). To confirm that RA will be able to induce MC\textsubscript{regs} when encapsulated in Ac-Dex MPs we first utilized an \textit{in vitro} model system. It has been shown that RA induces increased expression of co-stimulatory molecules CD80, CD86, and CD40 along with MHC class II on DC lines, specifically NB4 cells(132). Although it has not been shown that RA has an effect on our particular cell line(NR-9456), it is known that RA has that ability to enter and alter macrophages(139). Given the correlation between PD-L1 expression and regulatory function we focused on phenotype expression of the regulatory marker PD-L1 on the RA MP differentiated macrophages. We added RA MPs or blank MP treatments to the macrophage cell line for 48 hours and then assessed for PD-L1 expression. Our data showed two unique findings. First, due to the lack of induction of the regulatory marker PD-L1 on cells treated with blank MPs we show that that Ac-Dex does not have a immunological effect, particularly on the induction of a regulatory phenotype within the macrophage cell line after 48 hour. Secondly we also show that RA encapsulated within MPs has an equal effect on macrophages as RA in terms of inducing a regulatory effect only altering the percentage of PD-L1 expression by as much as 2%.

Our data shows that RA has an effect on the MC population within 48 hours, indicating that RA is a rapidly acting molecule and that when encapsulated in Ac-Dex and delivered to professional phagocytes it can increase PD-L1 expression, a molecule
associated with regulatory abilities in MCs. There is a possibility that the RA has already escaped the MPs and affected the cell at the site of injection, but because the MP payload release is dependent on pH, it is unlikely that RA is released before becoming phagocytosed by MCs(167). However, it cannot be entirely ruled out that non-phagolysosomal breakdown did not occur.

To further confirm that RA is superior to E3 in its ability to induce MC$_{\text{regs}}$ in-vivo we compared E3 and RA encapsulated into Ac-Dex MPs and their effect on macrophages for 48 hours(33, 127). Figure 14 shows that E3 MPs had little to no effect on the expression of PD-L1. It is interesting that E3 MPs had little effect on the induction of MC$_{\text{regs}}$, especially due to the previous knowledge that E3 and Flt3L differentiated cells were able to reduce EAE(127). The differences between in vivo versus in vitro induction methods and the shorter time course of these studies versus previous studies may explain the limited ability of E3 to induce MC$_{\text{regs}}$(127).

With the utilization of MPs we were able to circumvent many of the issues that free systemic RA inflicts on off-target cells (epithelial cells). Delivery of RA either intravenously, intraperitoneal, or intramuscular has multiple unwanted side effects on epithelial cells and an activation of regulatory metabolites (P450’s) that cause many side effects(146, 147, 202, 203). The goal of the use of the MPs was the passive targeting of myeloid cells to avoid the previously listed complications and induce MC$_{\text{regs}}$.

One unknown with the application of Ac-Dex MPs is that trafficking patterns following in vivo administration has not been studied. Obviously, therapeutic application requires an understanding of where these MPs traffic. To determine the trafficking
patterns of Ac-Dex MPs we chose to explore the trafficking patterns of BSA-FITC encapsulated MPs in vivo. The uptake of free FITC and Fite-labeled BSA in labeled MC populations was a useful means to evaluate the relative uptake of labeled MPs by different cell populations. Previous work had demonstrated the professional phagocytes were the primary target but evaluation of DC versus macrophage uptake in vivo had not been evaluated (170, 204, 205). We found that MPs are consistently taken up by MCs within local draining lymph nodes within 24 hours and that FITC uptake was increased in the MP-treated group over that of free FITC. Although statistical significance was seen only in the local draining lymph node (inguinal), a similar trend in other lymph nodes and spleen suggest that local MP administration may have far-reaching effects within the peripheral immune system. The lack of increased FITC+ MCs within the spleen is most likely due to cellular dilution and sensitivity in detection issues. One interesting finding is that we saw a significant increase in FITC+ expression in CD11b+ cells following ID versus SQ administration of MPs.

Given our experience and abundant literature that PD-L1 expression is associated with regulatory abilities, up-regulated PD-L1 expression was empirically used to suggest MCreg induction by RA MPs. (206, 207). Our data suggests a trend towards increased expression of PD-L1 in mice that were injected with RA MPs. Although not statistically significant, it is possible that the increased PD-L1 expression may be biologically significant. Additional increases in PD-L1 expression may be possible by altering RA MP dosing strategies. Ultimately, to assess the utility of RA MP use for treating inflammatory disease, we need to assess whether in vivo MP administration would elicit
regulatory responses within MCs. Our initial studies begin to assess the functional ability of the immune cells extracted from mice injected with either RA MPs or blank MPs by a T cell proliferation assay. The data shows that mice injected with RA MPs have a reduced proliferation in a non-antigen specific manner. We also shown that when faced with an inflammatory stimulus (LPS), immune cells from mice spleen and lymph nodes injected with RA MPs are able to maintain their regulatory capability. This indicates that when mice are injected with RA MPs, even within an inflammatory environment, would maintain a regulatory ability, potentially reducing autoimmune disease.

In summary, we show that continuous RA has significant effects on myeloid cell populations that may be useful for therapeutic purposes. Specifically, we show that exposure during myelopoiesis promotes the induction of a regulatory monocyte that suppresses the proliferation of immune cells. We also explore a novel technology (Ac-Dex) for delivering RA to MCs which may have significant applications in treating inflammatory disease. Ongoing studies are evaluating the in vivo application within a disease model. RA is an ideal immunomodulatory compound given that is a naturally occurring hormone essential for homeostasis and immune regulatory and that it promotes mature MCregs. While much remains to be learned about dosages, variable effects in different cell populations and the potential of biphasic actions of different cell types, our data suggest the utility of RA for inducing regulatory immune responses. A more thorough understanding of how RA mediates these differential effects has important implications in our understanding of MCreg biology and the potential application of RA MPs to treat a wide variety of inflammatory diseases.
References:


70.
71.
72.
73.
74.
75.
76.
77.
78.
79.
80.
81.
82.
83.
84.
85.
86.
87.
88.
89.
90.
91.
92.
93.


202. Achkar CC, Bentel JM, Boylan JF, Scher HI, Gudas LJ, Miller WH, Jr. Differences in the pharmacokinetic properties of orally administered all-trans-retinoic


