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The Construction of a Plasmid for Detecting the Pathway of Arginine Metabolism in Human Macrophages: a Real-Time Assessment of Macrophage Polarity

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THE CONSTRUCTION OF A PLASMID FOR DETECTING THE PATHWAY OF ARGININE METABOLISM IN HUMAN MACROPHAGES: A REAL-TIME ASSESSMENT OF MACROPHAGE POLARITY.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science.

By

BENJAMIN ANDREW HOLMES
B.S., Wheaton College, 2009

2012
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY
SUPERVISION BY Benjamin Holmes ENTITLED The Construction of a Plasmid for
Detecting the Pathway of Arginine Metabolism in Human Macrophages: a Real-Time
Assessment of Macrophage Polarity BE ACCEPTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT


Macrophages are “first responders”, innate immune system cells which quickly arrive to a site of infection and injury, consuming cell debris and foreign matter and recruiting other immune system cells to the area. While historically they have been thought to react uniformly to all challenges, the discovery of toll-like receptors has shown that macrophages actually work closely with the adaptive immune system in fine-tuning the immune response. Furthermore, it has recently been discovered that macrophages can become polarized to one of two subtypes – M1 or M2.

M1 macrophages are efficient producers of reactive oxygen species, nitrogen intermediates, and inflammatory cytokines. They are especially effective at mediating resistance against intracellular parasites and tumors. Arginine metabolism in M1 macrophages is characterized by high levels of inducible nitric oxide synthetase (iNos), and this is used as a marker for polarization of macrophages to the M1 phenotype. M2 macrophages, by contrast, produce anti-inflammatory molecules, have high levels of scavenger, mannose, and galactose-type receptors, and arginine metabolism is shifted to production of ornithine and polyamines via arginase. Arginase, encoded by the ARG1 gene, is considered to be one of the hallmarks of the M2 phenotype, and is one of the most specific markers used to determine polarization to that phenotype.
Polarization to one phenotype or another is not permanent, and macrophages can be polarized directly from one state to the other directly by addition of appropriate cytokines (IFNγ, LPS, TNFα for M1, IL-4, IL-13, IL-10, TGFβ for M2). The state of macrophage polarization can be determined by examining a population of macrophages for tell-tale products of one state or another (ROS, RNS, TNFα, IL-1, IL-6, IL-12, or IL-23 for M1, IL-10, TGFβ, PDGF, VEGF, EGF, and arginase for M2). Determining macrophage polarization has implications in health outcomes- M1 macrophages excel at fighting parasites and fighting tumorous growth, while M2 macrophages assist in wound healing and nerve re-growth. The problem is that the methods of detecting macrophage polarization – flow cytometry, western blots, or ELISA tests, are not real time and kill the cells involved. This paper describes the theory and methods behind creating a plasmid which combines the promoter for genes whose transcription indicates the M1 or M2 phenotype with a GFP or RFP, when transfected into a colony of RAW macrophages, will enable real time, quantitative visualization of the production of inducible nitric oxide synthetase or arginase, markers for the M1 and M2 phenotype. A clearer understanding of macrophage polarity in the course of illness, wounding, and cancer might lead to diagnostic or therapeutic discoveries in those areas.
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LIST OF ABBREVIATIONS

ARG1 – Arginase gene

CCL – Chemokine (C-C motif) ligand

CD – Cluster of differentiation

CXCL – C-X-C motif chemokine

ECM – Extracellular matrix

FGF – Fibroblast growth factor

IFN – Interferon

IGF – Insulin-like growth factor

IL – Interleukin

iNOS – inducible nitric oxide synthase

IP – Interferon-gamma producing protein

LPS – Lipopolysaccharide

LPB- Lipopolysaccharide-binding protein

MCR1 – Macrophage mannose receptor
MD-2 - Lymphocyte antigen 96

MDC/CCL22 – Macrophage-derived chemokine

MIP – Macrophage inflammatory protein

MMP – Matrix Metalloproteinase

NK cells- Natural Killer cells

NO – nitric oxide

PARC/CCL18 – Pulmonary and activation-regulated chemokine

PDGF – platelet-derived growth factor

TARC/CCL17 – Thymus and activation-regulated chemokine

TGF-β – Transforming growth factor beta

TLR – Toll-like receptor

VEGF – Vascular endothelial growth factor

WT – Wild Type
INTRODUCTION AND PURPOSE

Traditionally, macrophages have been thought of as “first responders”, members of the innate immune system that serve the body both by engulfing and processing foreign threats when possible, and by helping to direct the rest of the immune system in combatting threats (Gordon, 1999). One way the macrophage helps direct the immune system is by secreting pro-inflammatory and antimicrobial mediators. It is known that, through toll-like receptors, macrophages can be made to secrete cytokines that induce or repress inflammation, recruit other immune system cells, and prime elements of the adaptive immune response for a fine-tuned response (Stein et al., 1992).

Fig. 1: Macrophages have an important role in the immune system – they modify and direct both the innate and adaptive immune system, through antigen presentation and TLRs. Adapted from Scott-Algara et al., 2008
Nevertheless, it was previously thought that macrophages existed as essentially single-phenotype antigen-presenting cells which played a role in the development of the immune response through the secretion of various cytokines (Gordon, 1999).

Today, these macrophages are known to be “classically activated”, or M1 macrophages. In order to become classically activated, macrophages require priming with interferon gamma (IFN-γ) (Dalton, 1993), by way of the IFN-γ receptor (Huang, 1993), or through lipopolysaccharide (LPS). When a macrophage thus primed by IFN-γ or LPS encounters a recognized stimulus, for example a bacterial product, it initiates classical activation. If the macrophage encounters LPS, the LPS is bound by soluble lipopolysaccharide-binding protein (LBP) and then by CD14, which can be either soluble or membrane-bound. CD14 delivers LPS to the LPS recognition complex (Janeway and Medzhitov, 2002), TLR4 and MD-2. (Nagai et al., 2002) The foreign pathogens and their components are subsequently taken up by phagocytosis (Greenberg and Grinstein, 2002). Once inside the macrophage, they are delivered to lysosomes where they are degraded by a variety of enzymes, such as cathepsin cysteine proteases. (Honey and Rudensky, 2003). Once degraded by enzymes, the antigens are processed and loaded onto MHC class II molecules. On the surface of the macrophage, late endocytic compartments and antigen/MHC class II complexes are presented to T cells, along with co-stimulatory B7 family members (Harding, 2003).
Antigen presenting cells, like macrophages, serve a vital role in stimulating the proliferation of T cells, here shown presenting their antigens to T cells along with costimulatory B7 molecules. Adapted from Zou and Chen, 2008

Once classically activated, the macrophage undergoes significant changes in its morphology and in the content of molecules secreted from the cell. To attract neutrophils and limit angiogenesis, C-X-C- Motif protein 8 (CXCL8) is released. CXCL10, Chemokine (C-C-motif) ligand 3 (CCL3), and CCL4, chemoattractants for polymorphonuclear leukocytes and natural killer cells, and CCL5, a chemokine for T-cells, eosinophils, and basophils, are also produced, (Luster, 2002) along with cytokines designed to promote inflammation, such as IL-1β/IL-1F2, IL-6, and TNF-α/TNFSF1A.
(Duffield, 2003, Gordan, 2003, Ma et al., 2003, Mosser, 2003). In addition to being proinflammatory, TNF-α is a phagocytic agent for the macrophage (Boyle et al., 2003, Duffield et al., 2001, Song et al., 2000). Other hallmarks of classical activation are nitric oxide release as a result of inducible nitric oxide synthase (iNOS) upregulation (Hesse et al., 2001, Thomassen and Karvau, 2001, Duffield et al., 2000, Munder et al., 1998), and Fas Ligand/TNFSF6 secretion (Boyle et al., 2003). Proteolytic enzymes are also released by the classically activated macrophage; these include MMP (matrix metalloproteinase)-1, -2, -7, -9, and -12, which degrade extracellular matrix (ECM) components such as collagen, elastin, and fibronectin (Chizzolini et al., 2000, Gibbs et al., 1999 (1), Gibbs et al., 1999 (2)).

![Diagram](image)

Fig. 3: Elastic, collagen, and Gelatin can all be degraded with the use of MMPs, shown here released by a classically activated macrophage. Adapted from Elkington and Friedland, 2006
The release of these molecules is both a boon and a potential hazard. They are important for the protection of the host, and also help to direct the course of the innate and adaptive immune response. However, they also have the potential to cause significant damage to the local host environment. These cytokines are designed to cause infiltration by other leukocytes, and can do so on a massive scale. These invading leukocytes and the classically activated macrophages themselves can flood the area with pro-apoptotic and pro-inflammatory factors, and matrix degrading proteases. In this way, M1 macrophages can disrupt tissue structure, to the point of causing serious damage. Not only are there the obvious short-term negative consequences of this issue destruction, but tissue destruction caused by chronic inflammation has been associated with more long-term negative effects, including the development of tumors, type 1 autoimmune diseases, and glomerulonephritis (Gordon, 2003, Ma et al., 2003, Mosser, 2003).

Fig. 4: Macrophage accumulation is a prominent feature in most types of human glomerulonephritis: here macrophages, stained brown, are shown in the kidney. Adapted from Busey et al., 2012
The understanding about macrophage states was broadened in 1993 in the seminal paper by Stein, Keshav, Harris, and Gordon. This paper was the first to describe macrophages having a phenotype different from the “classically” activated macrophage. They noted that when stimulated with IL4, macrophages would display relatively few characteristics commonly found in classically activated cells (up-regulation of proinflammatory cytokines, recruitment of other immune system cells to the area), but did display the upregulation of the macrophage mannose receptor (MRC1), otherwise known as CD206, as measured by the binding and degradation of I-mannose-bovine serum albumin. They performed PCR analysis of cDNA from purified primary macrophage populations to confirm that the macrophage mannose receptor, but not lysozyme or tumor necrosis factor alpha levels were increased.

Fig. 5: The two types of macrophage polarization can lead to differing health outcomes, as shown here relating to inflammation. Adapted from Tidball, 2011
Macrophages taken from wild-type mice in vitro or in vivo, would develop this alternate activation when a TH2 response was induced by challenge with Schistosoma mansoni eggs. However, in a knockdown strain of mice, that was -/- for IL-4Rα, the common α receptor chain for IL-4 and IL-13 (Th2 cytokines), a similar challenge would elicit a classically activated or Th1-type response, not the Th2 response. The mannose receptor was expressed by granuloma macrophages in WT mice but not in IL-4Ralpha -/- mice, as determined by antibody staining (Linehan et al, 2003). This indicates that the alternate response was dependent on Th2-type cytokines, as knocking down the receptor for Th2 cytokines also stopped expression of the mannose receptor, the then-prototypical marker for alternate activation. Later experiments have shown that the patterns of activation shown with the Th2-type cytokines are also dependent on the transcription factor STAT6, indicating that there were transcriptional changes associated with an alternative activation. This was shown when CD4+ T cells transferred from H. polygyrus-primed mice induced alternatively activated macrophages in wild-type (d,e) but not Stat6-/- recipients (Anthony et al., 2006).

These observations have led to the understanding that “alternate activation” is not simply a deactivation of macrophages caused by Th2-type cytokines, but is an alternate pattern of activation, a different phenotype designed to accomplish separate goals than a classically activated macrophage. “Classically activated”, or M1 macrophages display characteristics consistent with a Th1-type phenotype, including chronic inflammation, recruitment of other immune system cells to the affected area, and tissue damage,
whereas “alternatively activated” or M2 macrophages resolve inflammation and facilitate wound healing.

Fig. 6: The products of the M1 and M2 phenotypes of macrophages dictates their roles: outputting NO and citrulline makes M1 macrophages killers, while M2 macrophages are anti-inflammatory “healers”. Adapted from Sly et al., 2007

Alternatively activated, or M2 macrophages, are activated differently than M1 macrophages. For instance, differentiation of alternatively activated macrophages does not require any priming. IL-4 (Stein et al., 1992) or IL-13 (Doherty et al., 1993) are sufficient to cause the differentiation. Once these factors are bound to their receptors, fluid-phase pinocytosis of soluble antigen may take place. (Brombacher, 2000, Conner and Schmid, 2003, Montaner et al., 1999) The next step is somewhat like the classical activation, wherein soluble antigen is coupled with MHC class II molecules and the
antigen/MHCII complexes are subsequently displayed to T cells, along with co-stimulatory B7 family members. (Harding et al., 2003)

Fig. 7: The process of antigen presentation in macrophages, from uptake and endocytosis to degradation of the invading molecule and presentation of the peptide to T cells. The macrophage also releases proinflammatory cytokines. Adapted from Nicchitta, 2003

Also, similar to the classically activated macrophage, changes are seen in the alternatively activated macrophage’s cellular morphology and secreted molecules as a result of appropriate stimulation. These macrophages release chemokines which attract leukocytes, including pulmonary and activation-regulated chemokine (PARC/CCL18) (Kodelja et al., 1998, Goerdt et al., 1999) Macrophage-derived chemokine (MDC/CCL22) (Andrew et al., 1998, Imai et al., 1999) and TARC/CCL17. (Imai et al., 1999) Where the real differences between classical and alternative activation become
evident, though is when anti-inflammatory cytokines such as IL-1ra/IL-1F3, (Mantovani et al., 2001) Ym1, Ym2, resistin-like molecule A (RELMα), (Raes et al., 2002, Loke et al., 2002) IL-10, (Mosser, 2003) and TGF-β are released. In addition to being an anti-inflammatory mediator, TGF-β also indirectly promotes ECM accumulation by inducing nearby fibroblasts to produce ECM components. (Song et al., 2000) ECM accumulation is aided by the M2 macrophage itself, which secretes the ECM components Fibronectin and bIG-H3, (Gratchev et al., 2001) Trans-glutaminase (a ECM cross-linking enzyme), (Haroon et al., 1999) and Osteopontin, which functions by facilitating cell adhesion to the ECM. (Murry et al., 1994)

In addition, alternatively activated macrophages upregulate expression of the enzyme Arginase I, which is involved in proline as well as polyamine biosynthesis. Proline promotes ECM construction while polyamines are involved in cell proliferation. (Hesse et al., 2001) Other factors secreted by the alternatively activated macrophage that promote cell proliferation include platelet-derived growth factor (PDGF), insulin-like grown factor (IGF), and transforming-growth factor beta (TGF-β). (Song et al., 2000, Cao et al., 2000) As well as promoting cell proliferation, these factors participate in angiogenesis, along with fibroblast growth factor (FGF basic), TGF-α, and vascular-endothelial growth factor (VEGF). (Cao et al., 2000, Sunderkotter et al., 1991)

Taken as a whole, the profile of the molecules secreted by the alternatively activated macrophage contribute to an immune response characterized by a lessening of
inflammation and promotion of wound repair. The response can be characterized as anti-inflammatory, proliferative, fibrotic, and angiogenic. In keeping with its profile as similar to that of a Th2 response, M2 macrophages are also efficient at combating parasitic infections, like schistosomiasis and filariasis. While the alternatively activated macrophage is beneficial for the body, and for wound repairing in this way, it is also true that M2 macrophages are not as effective at eliciting a strong immune Th1 immune response as M1 macrophages (by design), and by themselves they have been implicated in several pathologies, the most prominent of which are allergy and asthma (Duffield, 2003, Gordon, 2003).

![Diagram](image)

Fig. 8: Parasites, by activating receptors like the chitin receptor on macrophages, induce a Th2 differentiation. Adapted from Akira, 2010

One of the key discoveries in the current distinction between M1 and M2 macrophages was the discovery that they metabolize the amino acid arginine differently.
Macrophages take up arginine from the surrounding environment by cationic amino acid transporters (cat) embedded in their plasma membrane (Schapria et al., 1998). Once taken into the cytosol, the cell has two basic options for metabolism of arginine – it can use the nitric oxide (NO) pathway, which is what happens if the cell is in the M1 phase, or the arginase pathway, used by M2 macrophages. These pathways produce different results – the NO pathway produces NO and L-cituline, while the arginase pathway produces urea and L-orthinine (Mills, 2001). This branching pathway, in which one phenotype metabolizes arginine through one pathway, and the other phenotype through another, generates a number of biological molecules which are unique to each pathway – thus making good markers for the phenotypic state of the macrophage.
The NO pathway uses inducible nitric oxide synthase (iNOS) in the generation of nitric oxide. This pathway is upregulated by Th1 cytokines, especially IFN-γ and TNFα. Because of this, the NO pathway is also present in M1 or classically activated macrophages (Bogdan, 2001). iNOS is expressed constantly during the time a macrophage spends in the M1 phenotype, so massive amounts of NO can accumulate, up to 1000 times the levels of NO present in unstimulated, or M0 macrophages (Bogdan, 2001). NO inhibits cell replication (Nathan et al, 1991) and inhibits mitochondrial respiration. NO also triggers apoptosis (Albina et al, 1993; Saio et. Al, 2001).

Additionally, NO can interact with superoxide to create peroxynitrite. Superoxide is an oxygen radical also generated by macrophages. While in some cases superoxide can increase the cytotoxic effects of M1 macrophages, it may inhibit NO toxicity (Bautista and Spitzer, 1994), – an effect which can be reversed by the production of superoxide dismutase (Tohyama et al., 1996).
Fig. 10: The signaling pathway of iNOS releases toxic NO, and is mediated by Jak/Stat signaling. Adapted from Howard et al., 2010

Instead of the NO pathway, M2 macrophages use the arginase pathway. While macrophages contain both arginase I and arginase II, arginase I is the enzyme used in the NO pathway. It is present in the cytosol (arginase II is present in the mitochondria). Arginase I (ARG1) expression is upregulated in response to Th2 cytokines: IL-4, IL-13, TFGβ, and IL-10 (Morris et. Al, 1998). Arginase I converts arginine to orthinine, which is then converted to polyamines. Polyamines are required for DNA replication, so their production favors cell proliferation (Pegg, 1988). This is part of the reason that the arginase pathway seems to favor tumor proliferation. The arginase pathway also favors the proliferation of lymphocytes (Bowlin et al., 1987)
These same properties also make the M2 macrophages ideal for tissue and nerve regrowth, and may help in wound healing after nerve damage (Kigerl, 2009).

While the M1/M2 nomenclature will be used in the scope of this paper, it is important to note that there are certain shortcomings to the nomenclature. Some transcriptional studies (Biswas et al., 2006; Ghassabeh et al., 2002) have shown that some populations of macrophages are intermediates between the two phenotypes, and have transcriptional profiles that don’t match a simple definition as M1 or M2 macrophages – they have characteristics of each. It is not currently known if these macrophages represent other discrete phenotypes, or if there exists a gradient of M1-like and M2-like characteristics, which a macrophage might express many combinations of. One solution
to this problem taken by Mantovani et al. (2004), is to further divide M2 macrophages. In
this system, M1 refers to macrophages which are activated by LPS, IFN\(\gamma\), and other
Th1 type cytokines, and have the following profile: IL-10low, IL-12high, IL-23high, and
are good antigen-presenting cells. They produce high levels of NO and other reactive
oxygen intermediates. In contrast to this, the M2 category is a catchall for “not M1”,
including macrophages that have a profile of IL-12low, IL-10high, with enhanced tissue
remodeling properties. M2 macrophages are further divided into M2a and M2b. M2a
macrophages are activated by IL-4 or IL-13, and M2b macrophages are activated by IL-
10 or glucocorticoid hormones (Mantovani et al., 2004). Future genomic and proteomic
analysis may clarify the various phenotypic states that macrophages can exist in. For this
paper, M1 indicates a more aggressive, proinflammatory, proliferative, pro-apoptosis
phenotype, differentiated by the presence of iNOS, and M2 indicates an anti-
inflammatory, tissue regeneration-promoting phenotype differentiated by the expression
of the arginase gene (\(\text{Arg1}\)). It is known that iNOS levels are very low in macrophages in
the M2 phenotype, while Arg1 levels are very low in M1 macrophages. This is intuitively
sensible, as these represent divergent paths in the metabolism of arginine, and are
unlikely to coexist in large quantity. Macrophages that shift from an M1 phenotype to an
M2a phenotype experience an increase in arginase expression that is concomitant with
their reduction in inducible nitric oxide synthase (iNOS) expression (Munder et al., 1998,
Munder et al., 1999).
Both the M1 and M2 populations of macrophages can be shown to have environments in which they are greatly helpful, and microenvironments in which their presence is either less helpful or actively harmful. M1 macrophages are important both in fighting infection and in the reduction and destruction of tumors. Because of their pro-inflammatory, immune cell-recruiting cytokines, and because of their proliferation and activation of the rest of the immune system, M1 macrophages are associated with increased odds of survival during cancer, and in general anti-tumor activities (Ma et al., 2010). Additionally, due to the anti-microbial properties of M1 macrophages, the common immunological response during acute infection (by bacteria or viruses, not parasites) is to polarize macrophages towards an M1 response.
Fig 13: IFN-gamma, TNF, LPS polarize towards the M1 phenotype, while parasites, specifically Chitin, or IL-4 polarizes macrophages towards the M2 phenotype. Adapted from Satoh et al., 2010

As an example, during infection with *Listeria monocytogenes*, which causes disease in pregnant women and the immunocompromised, the body induces the M1 phenotype in macrophages. The pro-phagocytic cytokines act to prevent bacterial phagosome escape. This allows for increased intracellular killing of bacteria both in vitro and in vivo (Shaughnessy and Swanson, 2007). Studies done using mice that do not have IFN-γ and TNF and their receptors (two markers of M1 polarization) die from *L. monocytogenes* infection (Pfeffer et al., 1993). Similarly, *Salmonella typhi*, the agent of typhoid fever, and *Salmonella typhimurium*, a gastroenteritis agent, induce the M1 polarization of human and murine macrophages, and this induction is associated with the control of the infection (Benoit and Mege, 2008).
M2 macrophages show the most positive effects when mediating wound healing and nerve regrowth. M2 macrophages aid in tissue healing, nerve regrowth, and angiogenesis by releasing cytokines which promote angiogenesis and matrix remodeling while simultaneously suppressing destructive immunity (Sica et al., 2006). In one exploratory study, it was found that in mice who received an electromechanical contusion in their central nervous system (CNS), M2 macrophages promoted nerve regrowth with axons regrowing to more than two times the length of axons in tissues where M1 macrophages predominated. It was hypothesized that, since the population of M1 macrophages persists for far longer than the M2 population, which lasts about 3-7 days, M2 macrophages were responsible for wound healing and nerve re-growth in the damaged region, and M1 macrophages might protect the area from secondary infection. The authors suggested that understanding the diverse roles that M1 and M2 macrophages played in the course of CNS wound healing would be informative. They also suggested that the ability to modulate the phenotypic profiles of the macrophages involved in the wound after nerve damage might be highly valuable in producing more desirable outcomes (Kirgerl et al., 2009)
Fig 14: Through upregulation of TNF-alpha and infiltration of macrophages into wound tissue, Keratinocytes and Fibroblasts are stimulated through the TNFR1 and facilitate wound healing. Adapted from Lia et al., 2009

The subject of macrophage polarization is a complex one, and not everything is known about the ways in which macrophages become polarized to one phenotype or another. Nonetheless, some generalizations can be drawn. It is known that macrophages become polarized to the M1, or classically activated phenotype, after stimulation with LPS or IFN-γ or both, which induces TNF-α, and M2 or alternatively activated macrophages were produced after stimulation with IL-4 and IL-13 (Gordon, 2003). It is also known that LPS + IL-1β, IC + IL-1ra, TGFβ, and IL-10 can stimulate M2 macrophages (Martinez et al., 2008), and other
proinflammatory cytokines such as IFNs, IL-1, IL-2, and hypoxia can induce M1 macrophages polarization. Obviously, then, there are a diverse set of pathways that can lead to their activation (Melillo, 1995, Kleinart et al., 2003).

Fig. 15: In general, Th1 cytokines, LPS, and IFN-gamma stimulate the M1 phenotype, responsible for inflammation and further stimulation of the immune system, while Th2 cytokines stimulate the repair-centered M2. Adapted from Sica et al., 2006

Furthermore, macrophage phenotype is plastic – macrophages can be polarized to the M2 phenotype from the M1 phenotype, vice versa, and will revert to an unpolarized M0 phenotype in the absence of stimulus to one or the other phenotype (Devaraj and Jialal, 2011, Mantovani et al, 2002). To convert one macrophage phenotype to the other simply requires that the macrophage be stimulated with the appropriate polarizing signals, and taken out of the environment which initially polarized it to the opposite phenotype (Kirgerl et al., 2009).
Because understanding macrophage polarization might give insight into disease progression and wound healing, because there are selectable markers that indicate the state of the macrophage phenotype (iNOS for M1, ARG1 for M2), and because all the current tests for macrophage phenotype rely on lengthy detection of proteins through immunological assay (i.e. ELISA tests, flow cytometry, western blots), it is desirable to develop a quantitative, real-time test for the state of the macrophage using the markers mentioned above, which can be performed on live cells.

MATERIALS AND METHODS

The goal of this project is to create a macrophage cell line which allows visualization of macrophage polarity in real-time on live cells. This is to be done by the creation of a plasmid which detects transcription of proteins indicating one or the other phenotypic state, coupled to a reporter

![Plasmid 2](image)

Fig 16: A standard plasmid has an ori with multiple restriction sites and selection marker for detection. Adapted from Hoesl, 2011.
The reporter for this system was to be either green or red fluorescent protein (GFP and RFP, respectively). GFP and RFP are relatively well-characterized molecules that fluoresce either a green or a red color when excited with a different UV light wavelength (Inouye and Tsuji, 1994). Their expression is non-toxic to cells, easily detected through non-lethal doses of UV radiation, and able to be quantified using a spectrophotometer (Chalfie et al., 1994).

In addition to the reporter system, a means of detecting the phenotypic state of macrophages is needed for this plasmid. As mentioned above, the levels of iNOS and ARG1 are inversely related, with iNOS being high and ARG low in M1 macrophages, and vice versa. This is because they play opposite roles in the metabolism of arginine, with the MO pathway being used in M1 macrophages, and the arginase pathway in M2. The promoter regions for the iNOS and ARG1 genes, therefore, will be used in the construction of the plasmid. Fortunately, both of these promoters have previously been isolated, the ARG1 plasmid by Pauleau et al (2004),
Fig 17, the Arg1 promoter plasmid – note the firefly luciferase for identification, and the MhuI and XhoI sites for restriction digests. Adapted from Pauleau, 2004 who found that both the promoter region and an enhancer ~kb upstream from the promoter start site were required in a plasmid for stable and significant activation when the host cell promotes ARG1 transcription. The iNOS plasmid was found to be sufficient without enhancer regions to be activated on a plasmid when the host cell activated iNOS transcription (Lowenstein et al., 1993)
The iNOS promoter plasmid also includes an upstream enhancer region. Adapted from Andresen et al., 2004.

Both plasmids were graciously provided by the respective labs that developed them.

Once the plasmids were obtained, a vector and *E. coli* line were chosen. This project will use the pBluescript SK+ plasmid vector (PBSSK+) for bacterial expression, both for its stable expression in a variety of bacteria, and also for its MCS, which contains the appropriate restriction sites. The PBSSK+ plasmid is capable of holding both the ARG1 and iNOS promoter. Appropriate cloning sites must be chosen – ideal sites should be found on either end of the promoter section of the promoter plasmid and on the MCS of the PBSSK+ plasmid for proper insertion of the promoter into the bacterial...
expression vector. Additionally, an appropriate host strain of *E. coli* must be selected. Stbl3 *E. coli* were chosen for this project, because they proved able to uptake the plasmid and were able to be grown on a miniprep afterwards.

Following selection of restriction sites, and *E. coli* population, a concentration of the appropriate promoter plasmid is transfected into the stbl3 *E. coli*. This is grown in culture overnight. The culture is isolated, and the plasmid DNA is isolated through a miniprep. This DNA is subjected to a restriction digest with the predetermined restriction enzymes, which are then run in an electrophoresis gel. Assuming the correct size bands are found, which indicates a successful restriction digest, the *E. coli* is grown up and the plasmid DNA isolated in a maxiprep (which gives much greater plasmid yield as compared to the miniprep).

**FUTURE WORK**

After growing the *E. coli* and harvesting the plasmid DNA in a maxiprep, the DNA is then run again on a gel. Assuming the correct size bands are found, the DNA will be isolated from the gel and added to a solution of PBSSK+ plasmid which will be cut with the appropriate restriction enzymes to encourage uptake on plasmid, along with ligase. This ligation mix will be incubated, and added to a culture of *E. coli* which will be subjected to heat shock. If successful, this procedure will introduce the PBSSK+ promoter into the culture of *E. coli*. This will yield a plasmid which confers bacterial
ampicillin resistance to the *E. coli*, and will become activated along with the appropriate protein (iNOS or ARG1) because of the promoter element. The procedure to insert the promoter section into the plasmid will then be repeated to add GFP or RFP to the 5’ end of the promoter plasmid on the plasmid, meaning that activation of the promoter element on the plasmid will yield GFP or RFP.

Fig. 19: The final shape of the PBSSK+ plasmid

Meanwhile, a culture of the macrophages (in this case, RAW 277.97 macrophages) are transfected with a PBSSK+ plasmid with the GFP or RFP sequences to be used in the experiment attached to a constitutive promoter, and one with GFP and RFP with no attached promoter. These macrophages are examined under a fluorescent microscope, and used as baselines for total expression and non-expression of the marker. These endpoints will be used to evaluate the strength of the expression of the GFP and RFP in the plasmid with the promoter element. The *E. coli* transformed with the promoter plasmid + GFP or RFP are selected by growing on amp+ media. These *E. coli* are grown
overnight in amp+ broth followed by a miniprep experiment to determine that the plasmid has been inserted correctly, in the proper orientation in the *E. coli*. These plasmids containing the promoter of interest and either GFP or RFP are then transfected into the macrophage cell line with lipofectamine, which increases the efficiency of transfection. This yields the cell line of interest – a culture of macrophages that will fluoresce green or red when the iNOS or ARG1 is promoted in culture. Next, the macrophages which have successfully taken up the plasmid must be isolated and cloned. This involves isolating macrophages that fluoresce under appropriate UV radiation, and clonally growing them. The copy number of the plasmids in these macrophages must now be determined. This can be performed by Southern blot, by comparing the signal intensity of the unknown copy number to a known copy number. This process should then be repeated using the other promoter/reporter, transforming the promoter + reporter into the macrophages that already contain one such construct. The final macrophage will express GFP if one promoter is activated, and RFP if another is activated. This plasmid system is then tested by incubating these macrophages with cytokines known to polarize the macrophage to one phenotype or another (LPS and IFN-γ for M1, IL-4 and IL-10 for M2), and the fluorescent patterns are observed under a fluorescent microscope.
POSSIBLE PROBLEMS/SOLUTIONS

There are a few foreseeable problems that could arise during the construction of these transgenic macrophages. First, and most potentially troublesome, is the difficulty of cloning the macrophages after isolation based on GFP expression. Macrophages can be difficult to cultivate following isolation, as individual macrophage cells frequently have trouble expanding, and die, even in media with sufficient nutrients. This could be solved, if necessary, by using a macrophage line which has been shown to be able to clonally expand after the uptake of a transgene, such as mouse alveolar macrophages (Joshi et al., 2008). Additionally, the reporters might have to be altered. There is some spectral overlap between the range of UV wavelengths that GFP and RFP respond to, and there could potentially be confusion in interpreting fluorescence. If this is a problem, markers with excitation spectra further from GFP, such as brilliant violet or aqua.

FUTURE IMPLICATIONS

Once the system is in place, the macrophages can be given any number of challenges – infection with foreign agents, bacterial or viral, addition of cytokines whose polarizing effects on macrophages are unknown, polarization profiles after being affected by a number of different cytokines. Of initial interest would be visualizing the polarization of macrophages in the presence of herpes simplex virus – it is possible that
due to their persistence in cells long-term, they might act to induce an M2 polarization of macrophages. Likewise, both acute and long-term bacterial infectious agents could be investigated, to see if different ones induce different polarization profiles in macrophages. It would also be interesting to see if different classes of infectious agents – bacteria, viruses, and parasites, tend to induce different profiles, especially because parasites are mostly greatly affected by th2 mechanisms, it would be interesting to see if M2 macrophages predominate, and over what time periods they predominate, in parasitic infection.

The real-time nature of the expression of GFP and RFP means that the time a cell remains polarized, and the speed with which it becomes depolarized, could be explored in much greater detail than has yet been observed. Additionally, using this system as a model, promoters for other proteins of interest during macrophage polarization could be detected using expression of GFP and RFP, as an example, the SOCS pathway could be investigated in some detail. It is also possible that the polarization of macrophages in vivo could be investigated. In this situation, promonocytes could be isolated from murine bone marrow, and transformed with the promoter/reporter system described above. If reintroduced into the mouse they were taken from, these cells could produce macrophages which expressed the promoter/reporter construct. While GFP/RFP would be inappropriate for this task, noninvasive bioluminescent imaging with luciferase has been shown to be able to image selected cells in whole animals. This could show real-time images of the
progression of macrophage polarization in whole animals during the course of injury or infection (Mezzanotte et al., 2011).

The use plasmids to solve other biological problem is expanding on multiple fronts. One other possibility for recombinant DNA is the construction of computational plasmids. In computational plasmids, assume that $P$ is a plasmid, $k$ is a positive integer and $s_1$ to $s_k$ are $k$ pairwise non-overlapping subsegments of $P$. Also assume that for each $i$, the nucleotide sequence of $s_i$ is unique within the plasmid $P$. These unique segments chosen in this way are ‘stations’ of the plasmid. Once this setup is developed, a computation is begun. It begins with a test tube of or buffer that contains many identical $k$-station plasmids. The plasmids are modified in such a way as to be readable later, and only modified at the stations. Because of this binary modification, each station on the plasmid is either modified or unmodified, which can be thought of as one of the bits – a 1 or a 0.

<table>
<thead>
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<th>Variation</th>
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Fig. 20: By dividing the strand of DNA into “stations” with specific restriction enzymes, operations can be performed independently on separate stations and the final results can be computed by examining stations later. Adapted from Head et al., 2000.
When conceived of in this way, each station is similar to a k-bit data register in a computer. The “memory” in this kind of computing is simply the totality of the system – the water plus the plasmid content. This is done because water helps with rapid partitioning of memory into subsets. This can be accomplished through stirring or diffusion. It makes the assumption that each of the members of such a partition contains the same variety of molecules. Different, separate members of the partition can be modified in different ways and brought back later into a single test tube. Once brought back together, the final “solution”, or computational answer, is read from the solution – the water plus plasmid. Many separate methods for modifying the plasmid might exist, but cutting and pasting sequences is a valid way to modify them. In this way, the speed and accuracy of biological processes can be brought to bear to solve complex mathematical problems (Head et al., 2000).

Another problem that can be solved with plasmids, in this case larger-scale ones, is to create a DNA library. In a DNA library, a collection of DNA fragments that come from one organism, and are stored in another, often a bacterium, are collected. This is first achieved by extracting and purifying DNA from human cells. This collected DNA is likely made of extremely long strands of DNA, so it is digested with restriction enzymes. Because DNA is so large, each restriction enzyme is likely to cut the DNA in multiple places. Thousands of fragments are thus generated, each of which might contain one or more genes. Each fragment is then inserted into a plasmid vector – this can be done because researchers know the profile of restriction enzymes they used to create the
fragments, thus they know the profiles of the sticky ends of the pieces of DNA, and can cut plasmid vectors with the matching restriction enzymes. The fragments are then put into *E. coli*, which is grown to large numbers, creating many copies of each fragment of DNA.

**Genomic Library**

![Diagram of genomic library](image)

**cDNA Library**

![Diagram of cDNA library](image)

Fig 21. Construction of a plasmid-based DNA library involves cutting the DNA from a cell into pieces, ligating them into the vector, and storing the vector in bacteria – the process is similar for a cDNA library, but mRNA is converted through reverse transcriptase into cDNA which is then inserted into the vectors. Adapted from Winning, 2012.

The fragments of DNA can then be isolated and studied, or the researcher can further isolate the particular genes they are interested in for further study. (Cain et al., 2006)

There has been a great amount of interest lately in recombinant DNA and molecular biology – one source for models of molecular and sensing biology is the international genetically engineered machine (iGem) competition. This competition, for
undergraduate biology students, involves being a standard set of biological building blocks (standardized plasmids, promoters, reporting systems, etc.) taken from the registry of standard biological parts. These parts are combined to create biological models that accomplish creative and useful tasks (igem.org). Part of the inspiration for this project was taken from a project in which *E. coli* was modified to detect environmental mercury through production of GFP. This was accomplished through a plasmid that detected changes in the host cell commonly associated with the cell responding to environmental mercury, and designed a plasmid that would express green fluorescent protein when this happened in the cell (http://2012hs.igem.org/Team:WarrenCentral_WCC_IN).
Sources


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Career Focus

Microbiologist and computer scientist. Lab experience, both with self-directed and team-based research. Wet lab experience includes ELISA, Western and Southern blots, gel electrophoresis, PCR, culturing techniques, plasmid transformation, vacuum filtration, flow cytometry, titration. Computing experience includes proficiency in java, C++, ruby, and perl. Designed programs to interface with online databases, match genetic profiles by similarity, and record results.

Core Qualifications

- Knowledge of in vitro culture and immunohistochemistry techniques, such as sterile technique, culturing and colony isolation, ELISA, western and southern blots, plasmid transfection, antibody staining, confocal microscopy, and gel electrophoresis.

- Ability to construct plasmids by selecting appropriate genes, promoters, and vector systems. Experience with maxi and mini-preps, transfection, transformation, and plasmid insertion by viral vector or lipofectamine.

- Excellent communication, documentation, and presentation skills. Experience managing ordering for a lab, documentation of procedures and techniques, and management of lab stores. Ability to communicate and work effectively with a team.

Employment Summary

05/2011 – Present Forensic Bioinformatics Dayton, OH
Genetic analyst/programmer

06/2010 – 08/2010  Wright State University Dayton, OH
Clinical Laboratory Science graduate teaching assistant and
inventory

management

03/2010 – 05/2010  Wright State University Dayton, OH
Microbiology Graduate Teaching Assistant

10/2008 – 06/2009  Wheaton College Wheaton, IL
Undergraduate Researcher, biology/microbiology

08/2004 – 05/2009  Wheaton College Wheaton IL
Wheaton ResNet, Senior Call Center Tech Support

06/2008 – 08/2008  IDCAST, Dayton OH
Researcher

06/2007 – 08/2007  Yellow Springs Instruments, Yellow Springs, OH
Research Assistant

Education

2012  Wright State University Dayton, OH
(Fall) expected graduation date
Microbiology MS

2009  Wheaton College Wheaton, IL
Microbiology BS
Computer Science BS