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# Regulation of Polyamine Synthesis and Transport in the Context of Macrophage Polarization and Pathogen Invasion

A Thesis submitted in partial satisfaction
of the requirements for the degree of

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in

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by

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education, working hard, and being dedicated to a cause. I am especially thankful to my mom for her optimism, encouragement, and patience. She is always there for me and helps me to see the best in every situation. And, I am very thankful to my dad for always having so much confidence in me. I'll never forget the efforts he made to motivate me during my first year of graduate school.

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DEDICATION

TO THE ONE WHO MADE THE JOURNEY WORTH TAKING

AND

TO THE ONES THAT I LOVE THE MOST

ABSTRACT OF THE THESIS

Regulation of Polyamine Synthesis and Transport in the Context of Macrophage Polarization and Pathogen Invasion

by

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University of California, Riverside, March 2014
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Macrophages play a vital role in early defense against pathogens and are required for tissue repair following injury. In response to various stimuli, macrophages become activated/polarized towards pro-inflammatory or anti-inflammatory states, known as M1 and M2 states respectively. Arginase1 (Arg1), the most commonly used diagnostic marker of M2 macrophage polarization, is also the enzyme that catalyzes arginine into ornithine, which feeds into the polyamine biosynthetic pathway. Polyamines, positively charged, organic aliphatic compounds; are necessary for cell growth and survival in all living organisms. In M1/M2 macrophages, arginine/polyamine synthesis and transport are tightly regulated. Higher intracellular levels of polyamines can promote tumor formation in cells where mutations have already occurred. Still, the mechanisms underlying the control of these responses in the context of the polyamine metabolism have not been completely elucidated. When Arg 1 expression is upregulated, it is

expected that the synthesis and export of polyamines will subsequently increase. Yet, it is still not clear if polyamines serve a physiological function in M2 macrophages or are merely bystanders in the entire M2 macrophage activation/ polarization process. The usage of novel polyamine synthesis inhibitors provides a useful approach to understanding the role of polyamines in both macrophage states. The inhibition of polyamine synthesis has been used as a treatment for pathogen invasion, because many pathogens rely on arginine and polyamines for growth and survival. In other words, arginine/polyamines are not only indispensible resources for their hosts, but also for the pathogens that prey upon them.

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| Abbreviation | Meaning |
|---|---|
| ABH | 2(S)-amino-6-boronohexanoic |
| AcSPD | Acetylspermidine |
| AcSPM | Acetylspermine |
| ADC | Arginine decarboxylase |
| ADI | Arginine deiminase |
| AdoDATO | S-adenosyl-1,8-diamino-3-thiooctane |
| AdoMac | S-[5'-deoxy-5'-adenosyl]-1-ammonio-4-[methylsulfonio]-2-cyclopentene |
| AMA | S-[5'-deoxy-5'-adenosyl]-1-ammonio-4-[methylsulfonio]-2-cyclopentene |
| AOE-PU | N-[2[aminooxyethyl]-1,4-diaminobutane |
| APA | 1-aminooxy-3-aminopropane |
| AP-APA | 1-aminooxy-3-N-[3- aminopropyl]-aminopropane |
| APCHA | N-[3-aminopropyl]cyclohexylamine] |
| APC | Amino acid/polyamine/organocation transporter |
| Arg 1 | Arginase 1 |
| AZ | Antizyme |
| AZI | Antizyme inhibitor |
| A549 cells | Adenocarcinomic human alveolar basal epithelial cells |
| BALB/c | M2 mice strain |
| BEC | S-(2-boronoethyl) – L- cysteine |
| BDAP | N-(n-butyl)-1,3-diaminopropane |
| BEHSpm | bis(ethyl) homosperimine |
| BENSpm | bis(ethyl)norspermine |
| BESpm | bis(ethyl)spermine |
| BE-444 | N1,N14-bis(ethyl)homospermine |
| bIG-H3 | Transforming growth factor beta-induced |
| BMDMs | Bone Marrow Derived Macrophages |
| BOC | tert-Butyloxycarbonyl (protects amino acid from polymerization) |
| B10D2 | M1 strains |
| CAD | Cadavarine |
| CAT | Cationic amino acid transporter |
| CBENSpm | N1-ethyl-N11-(cyclobu-tyl)methyl-4,8-diazaundecane |
| CCL | Chemokine (C-C motif) ligand |
| CD | Cluster of differentiation |
| CGC-11047 | PG-11047, SL-11047 |
| CGC-11144 | SL11144 |

| | |
|---|---|
| CGP39937 | [2,2-bipyridine]-6’6’-dicarboximidamide |
| CGP40215, CGP40215A | N'',N'''-bis[(1E)-[3-(aminoiminomethyl)phenyl]methylene]) |
| CGP48664 | 4 amidinoindanon-1-[2’amidino]hydrazine, SAM 364A |
| CHENSpm | N1-ethyl-N11 -((cycloheptyl)methyl)-4,8-diazaundecane |
| CHEXENSpm,25 | N-(Cyclohexylmethyl)-N'-(3-{[3-(ethylamino)propyl]amino}propyl)-1,3-propanediamine |
| CHE-3-7-3 | N-{3-[(Cycloheptylmethyl)amino]propyl}-N'-[3-(ethylamino)propyl]-1,7-heptanediamine |
| CHO | Chinese hamster ovary |
| CK | Carbamate kinase |
| Cldn11 | Claudin 11 |
| CNS | Central nervous system |
| CPC-200 | N1,N4-bis(2,3-butadienyl)-1,4-butanediamine, MDL 72527 |
| C57BL/6 | M1 mice strain |
| CPENSpm | N1-ethyl-N11(cyclopropyl)-methyl-4,8-diazaundecane |
| CPENTSpm | Protein subunit encoded by Slc3A2 |
| CXCL | Chemokine (C-X-C motif) ligand |
| DAX | Diamine exporter |
| DBA/2 | M2 mice strain |
| DCHA | dicyclohexylamine sulfate |
| DCL-1 | Dicer Like 1 |
| DC-SIGN | Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin |
| DENSpm (aka. BENSpm) | bis(ethyl)norspermine |
| DFMO | alpha-difluoromethylornithine |
| DL-HAVA | DL-alpha-Hydrazino-delta-aminovaleric acid |
| Genz-644131 | 8-Methyl-5′-{[(Z)-4-Aminobut-2-enyl]-(Methylamino)}Adenosine |
| gpaAT | glycoprotein-associated amino acid transporters |
| GPI | Glycosylphosphati-dylinositol |
| dcSAH | Decarboxylated S- adenosylhomocysteine |
| DEGBG | Diethylglyoxal bis(guanylhydrazone) |
| Dex | Dexamethasone (glucocorticoid) |
| ear11 | Eosinophil-associated ribonuclease A family, member 11 |
| EGBG | Ethylglyoxal-bis(guanylhydrazone) |
| FABH,. | 2-amino-6borono-2-(difluoro-methyl)-hexanoic acid |
| FN1 | Fibronectin 1 |

| | |
|---|---|
| 4F2hc | Arginine transporter encoded by SLC3A2 |
| FVB | M2 mice strain |
| GM-CSF | Granulocyte macrophage colony-stimulating factor |
| HSPG | Heparin sulfate peptidoglycan |
| IEC-6 | Normal rat small intestine epithelial cells |
| IFN | Interferon |
| iGF-1 | Insulin-like-growth factor 1 |
| IL | Interleukin |
| IPENSpm | N1-ethyl-N11-((isopropyl)methyl)-4,8-dia-Zaundecane |
| J774 cells | Macrophage like cells derived from female BALB/c mouse tumor |
| LdAAP3 | Arginine permease |
| LmPOT | Polyamine transporter |
| *L. donovani* | *Leishmania donovani* |
| L-HOArg | NOHA acetate salt, $N^G$-Hydroxy-L-arginine acetate salt |
| *L. major* | *Leishmania major* |
| *L. mexicana* | *Leishmania mexicana* |
| L-Nor-Valine | (S)-2-Aminovaleric acid, (S)-(+)-2-Aminopentanoic acid |
| LPS | Lipopolysaccharide |
| L1210 | Mouse lymphocytic leukemia cells derived from ascitic fluid of 8-month-old female mice |
| M1 | Pro-inflammatory state |
| M2 | Anti-inflammatory |
| MABH | 2-amino-6-borono-2 methyl-hexanoic |
| MAOEA | S-adenosylmethionine inhibitor |
| MCP-1 | Monocyte chemotactic protein -1 |
| 4 MCHA | *trans*-4-methylcyclohexylamine |
| MDL 72521 | $N^1$-Methyl-$N^2$-(2,3-butadienyl)-1,4-butanediamine |
| MDL 72527 | N1,N4-bis(2,3-butadienyl)-1,4-butanediamine |
| MDL 73811 | 5'-{(Z)-4-amino-2-butenyl]methylamino, AbeAdo |
| MGBG | Methylglyoxal bis(guanylhydrazone) |
| MHC II | Major histocompatibility complex class II |
| MHZEA | 5'-deoxy-5'-[(2-hydrazinoethyl)-methylamino] adenosine |
| MHZPA | 5'-deoxy-5'-[N-methyl]-N-[(3-hydrazinoproplyl)amino]adenosine |
| MMTA | S-methyl-5'-methylthioadenosine |
| MR | Mannose receptor |
| mrc1 | Mannose receptor c type1 |
| MSP | Macrophage stimulatory protein |
| MO | alpha-methylornithinchloride |
| NEM | N-Ethylmaleimide |
| NO | Nitric oxide |

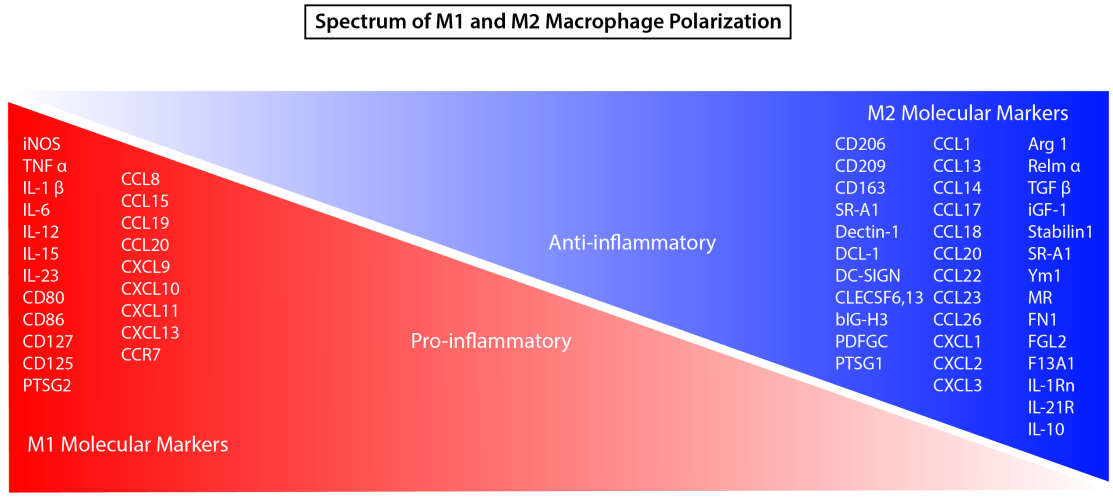
| | |
|---|---|
| iNOS | Nitric Oxide Synthase |
| $N^1$OSSpm | $N^1$-[n-octanesulphonyl]spermine |
| NOHA | NG-hydroxy-L-arginine |
| NorNOHA | Nω-hydroxy-nor-Arginine |
| ODC | Ornithine decarboxylase |
| OCT | Ornithine carbamoyltransferase |
| OvAZ | Antizyme of O. volvus |
| OAZ | ODC antizyme |
| POB | N-(4′-Pyridoxyl)-Ornithine(BOC)-OMe, BOC-protected pyridoxyl-ornithine conjugate |
| PSV | Polyamine sequestering vesicle |
| PTSG1 | PTS system glucose-specific transporter 1 |
| PTSG2 | PTS system glucose-specific transporter 2 |
| Put | Putrescine |
| RAW 264.7 | Mouse leukaemic monocyte macrophage cell line |
| rBAT | Arginine transporter encoded by Slc3A1 |
| RON | Receptor tyrosine kinase recepteur d' origine nantais |
| Retnla | resistin like alpha |
| SAMDC | S-adenosylmethionine decarboxylase |
| SAM 486A | S-adenosylmethionine |
| SL11144 | CGC-11144 |
| Slc | Gene encoding solute carrier family transporter |
| Slc3A2 | Gene encoding solute carrier family |
| Slc7A2 | Gene encoding solute carrier family |
| SMO | Spermine oxidase |
| Spd | Spermidine |
| Spm | Spermine |
| Spd-$C_2$-BODIPY | Flourprobe that reports polyamine transport |
| SR-A1 | Steroid receptor RNA activator |
| SSAT | spermidine/spermine acetyl transferase |
| TGF β | Transforming growth factor beta |
| TcPOT1.1 | Polyamine transporter |
| TLR | Toll-like receptor |
| TNF α | Tumor necrosis factor alpha |
| TREM1 | Triggering receptor expressed myeloid cells 1 |
| *T. brucei* | *Tympanosoma brucei* |
| *T. cruzi* | *Tympanosoma cruzi* |
| *T. gondii* | *Toxoplasma gondii* |
| V-ATPase | Vacuolar-type H+-ATPase |
| WT | Wild type |
| Ym1 | chitinase 3-like 3 |

# CHAPTER ONE

## Introduction

## 1.1 Establishing Macrophage Polarization

M1 polarized macrophages, associated with cytotoxicity and pathogen defense, induce pro-inflammatory responses. M2 polarized macrophages, involved in anti-inflammatory responses, are traditionally associated with cytoprotective responses, wound healing, allergic responses, and anti-parasite responses. Both of these activation/ polarization states are defined by a distinct set of molecular markers. Molecular markers of M1 activation/polarization include (but are not limited to) iNOS, TNF α, IL-1 β, IL-6, IL-12, CD80, and CD86; while molecular markers of M2 activation/polarization include (but are not limited to) Arg1, Relm α, TGF β, stabilin1, MR, Ym1, and CD206 (Figure 1.1-1).

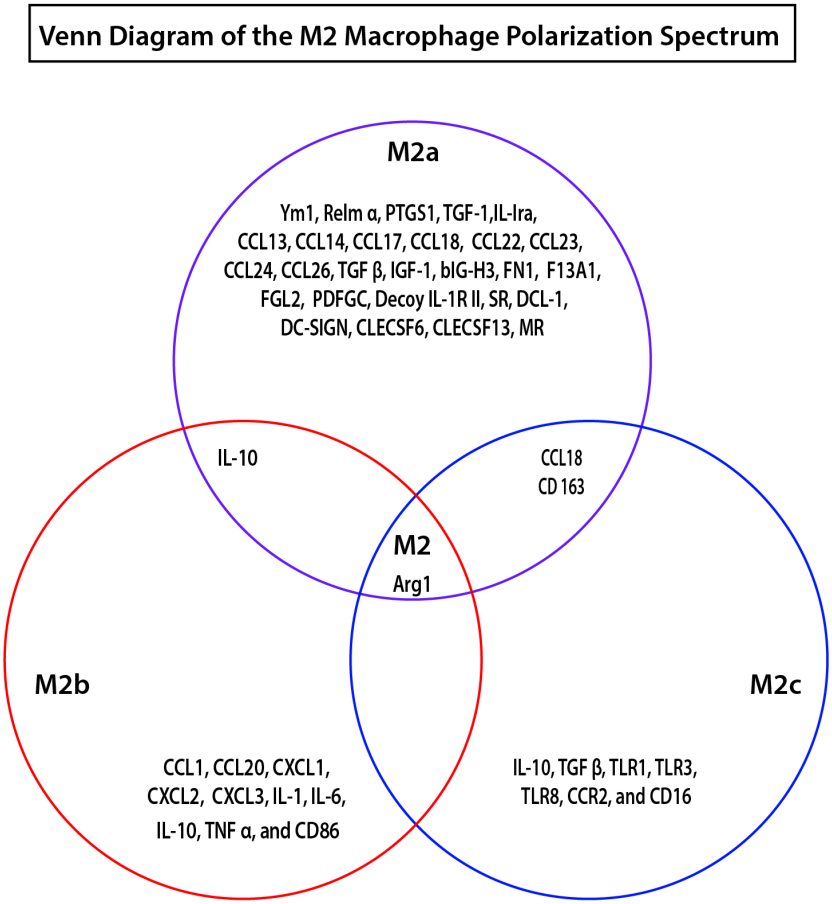



*Customized from Hao et al., 2012, Martinez, 2008

**Figure 1.1-1 M1 and M2 molecular markers.**

M2 activated/polarized macrophages can be further sub-categorized into three subtypes. IL-4/IL-13 induce the M2a subtype, which is characterized by the expression of IL-10, TGF β -1, IL-Ira, CCL17, CCL22, CCL24, Decoy IL-1R II, SR, MR, and CD 163.

Immune complexes are responsible for the induction of the M2b, which is characterized by the expression of CCL1, IL-1, IL-6, IL-10, TNFαlpha, and CD86. LPS, TLRs, or IL-1ra can also induce the M2b subtype. IL-10, glucocorticoids, or TGF β can induce the M2c subtype, which is characterized by the expression of IL-10, TGF β, TLR1, TLR8, CCR2, and CD163 (Hao et al., 2012) (Figure 1.1-2).




*Customized from Hao et al., 2012, Martinez, 2008

**Figure 1.1-2 M2 molecular markers.**

Although sometimes used interchangeably, macrophage activation and macrophage polarization are two separate events that can occur simultaneously or apart from each other. Activated macrophage express specific molecular markers (ie. If activated towards M2 states, they express Arg1, Relm α, TGF β, stabilin1, MR, Ym1, CD206 and more) at higher levels than resting macrophages, while polarized macrophages express this specific set of molecular markers to a higher degree than the markers of the opposing state (ie. iNOS, TNF α, IL-1 β, IL-6, IL-12, CD80, CD86, and more) (Figure 1.1-3).

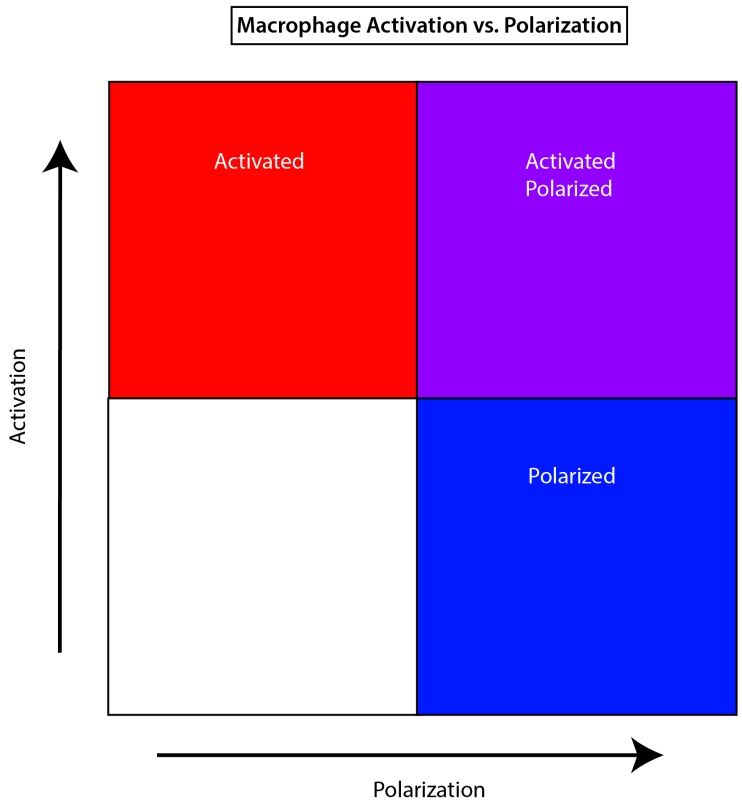



**Figure 1.1-3 Macrophage activation and polarization are two separate events that can occur simultaneously or apart from each other.**

Here I provide an example of data showing bone marrow derived macrophages that are both activated and polarized. They are activated because they express higher levels of the M1 or M2 macrophage molecular markers than the untreated bone marrow derived macrophages express (Figure 1.1-4). The graphs on the left side of figure 1.1-4 depict bone marrow derived macrophages polarized towards M1 states, expressing higher levels of M1 molecular markers than M2 Molecular markers. The graphs on the right side of figure 1.1-4 depict bone marrow derived macrophages polarized towards M2 states, expressing higher levels of M2 molecular markers than M1 Molecular markers (Figure 1.1-4).

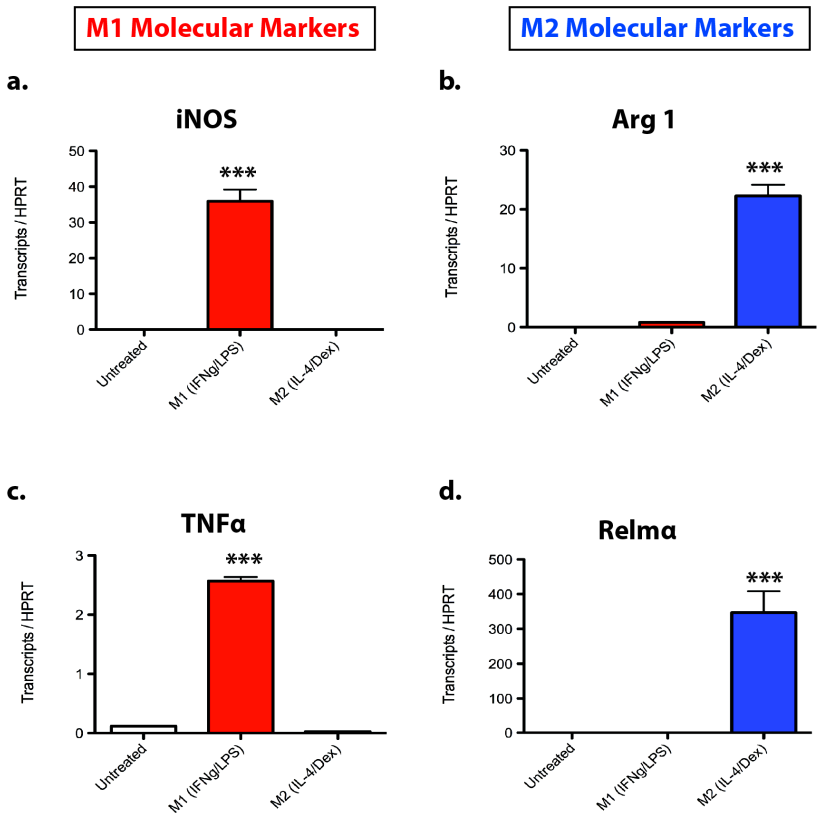



**Figure 1.1-4 BMDMs (bone marrow derived macrophages) treated with IFNγ /LPS or IL-4/Dex to induce an M1 or M2 response, respectively. BMDMs given M1 treatments express higher levels of iNOS (a) and TNFα (c) demonstrating M1 polarization, while BMDMs given M2 treatments express higher levels of Arg1 (b) and Relm α (d) demonstrating M2 polarization.**

Macrophage activation and polarization are variable. Even when macrophages are treated with the same pretreatments, they can be activated to different degrees (Figure 1.1-5). This makes establishing macrophage polarization states challenging. Fortunately, iNOS: Arg1 and Arg1: iNOS ratios can be used to determine the degree of M1 or M2 polarization, respectively. For example, if the M2 pretreated macrophages express higher levels of Arg1 than iNOS, then the ensuing higher Arg1: iNOS ratio is indicative of M2 macrophage polarization (Figure 1.1-6).

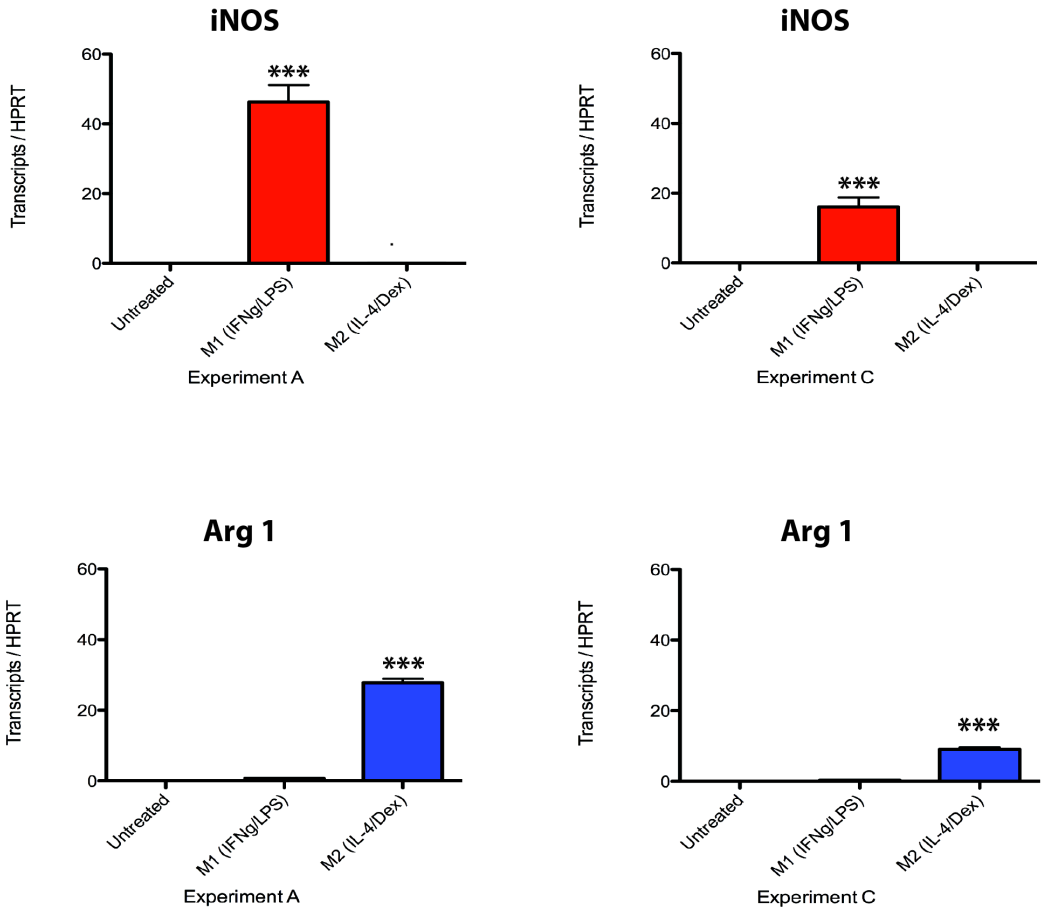



**Figure 1.1-5 Example of two experiments with different degrees of macrophage activation, but the same pretreatments.**

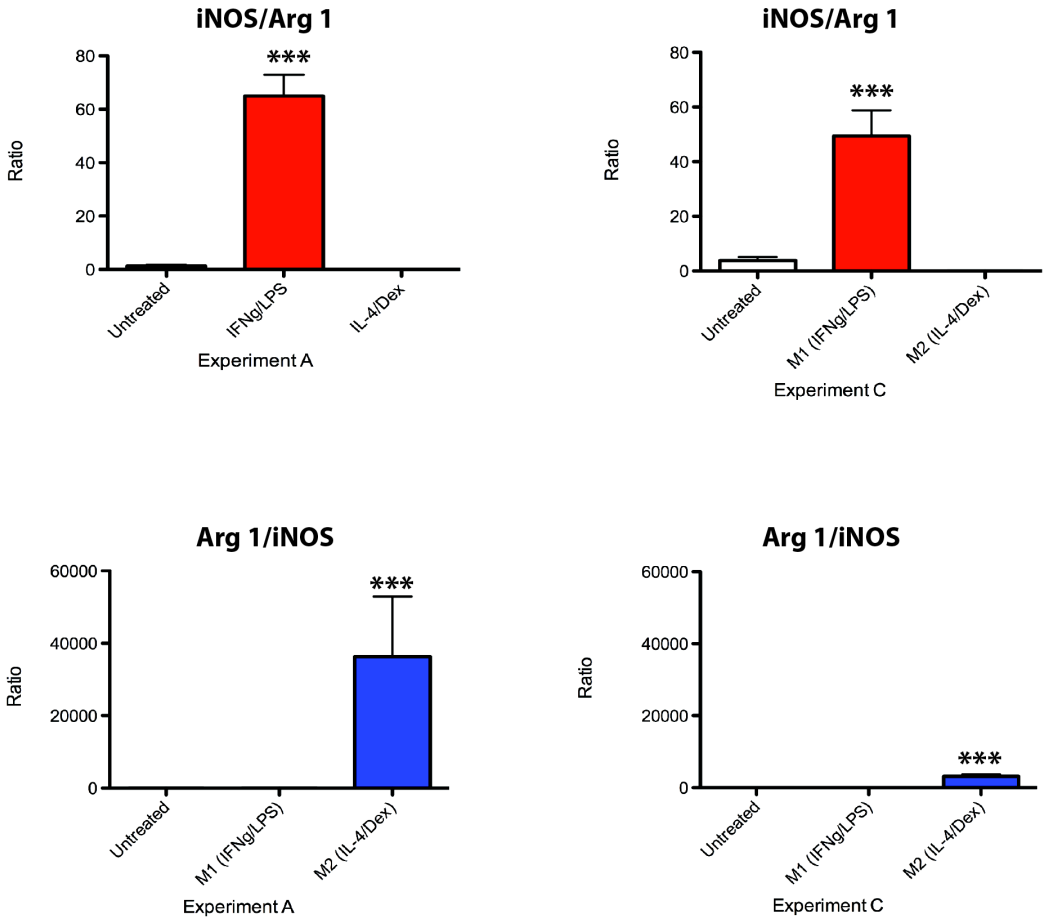



**Figure 1.1-6 Ratios are a good benchmark for macrophage polarization, but varying degrees of M1 and M2 activation can alter the degree of M1 or M2 polarization.**

M1/M2 defines not only the activation/polarization state, but also the metabolic state of the macrophage; M1 macrophages are characterized by high production NO in response to INFγ/LPS and subsequent inhibition of cell division, while M2 macrophages are characterized by increased production of polyamines and subsequent cell division (Mills, et al., 2000). During the fifth step of the urea cycle, L-arginine is metabolized by arginase, the most diagnostic marker of M2 macrophages, into urea and L-ornithine. Nitric oxide synthase2 (iNOS), the most diagnostic of differential activation along the classical states, competes with Arg1 for substrate L-arginine. Arg1 has faster catalytic activity, but iNOS has a higher binding affinity. Arginine is a valuable resource that is imported at higher levels in activated macrophages. Polyamines are also taken up and exported at different levels in polarized macrophages as opposed to resting macrophages. Excessively high polyamines levels have been observed in rapidly proliferating cancer cells. Despite the therapeutic potentials of regulating intracellular polyamine levels, there is much unknown about how polyamines synthesis and transport are modulated in M1 and M2 polarized macrophages.

### 1.2. Generating Bone Marrow Derived Macrophages

Femurs and tibias were aseptically removed from the 6-week to 6-month-old C57/bl6 mice. A 25G needle attached to a syringe containing DMEM, 1X, supplemented with 10% FBS was used to flush the femurs and tibias of bone marrow. Cells were pelleted, seeded in poly-d-lysine coated 100 mm X 15mm polystyrene plates, and cultured in DMEM, 1X, supplemented with 10% FBS and 10mg/ml Recombinant Mouse M-CSF

(R&D Systems) [differentiation media] over a time course of 7 days. On the third day, the media was replaced with new differentiation media. On the $5^{th}$ or $6^{th}$ day, when the cells were no longer in log phase, they were split and seeded onto 100 mm X 20 mm tissue culture plates.

### 1.3 Polarization of Macrophages Towards M1 and M2 States

On the $8^{th}$ day, the bone marrow derived macrophages that were given one of two types of treatments or left untreated. The first set of bone marrow derived macrophage tissue cultures were treated with 11.86ng/ml recombinant mouse IFNg (R&D Systems) and 10ng/ml LPS (Sigma Aldrich) to induce an M1 response. The second set of bone marrow derived macrophage tissue cultures were treated 10ng/ml recombinant mouse IL-4 (R&D Systems) and $10^{-7}$M Dexamethasone (Sigma Aldrich) to induce an M2 response. For the untreated set of bone marrow derived macrophages, the differentiation media was replaced with DMEM, 1X, supplemented with 10% FBS.

### 1.4 RNA isolation and Reverse Transcription

Trizol Reagent was used to isolate the RNA from the macrophage tissue cultures 24 hours following each treatment. RNA was further purified with alcohol precipitations. Genomic DNA was digested by Deoxyribonuclease I, Amplification Grade (Invitrogen). Measuring the RNA's absorbance at A260, A230, and A280 assessed its quantity and quality per each sample. The A260/280 and A230/260 ratios were used to evaluate the quality of the RNA. The A260 was used to determine the concentration of the RNA.

Using Not I-d$(T)_{18}$ bifunctional primer and Bulk first strand cDNA reaction mix as described in the manufacturer's protocol, 2 µg of RNA from each biological replicate was reverse transcribed into first strand cDNA (GE Healthcare First-Strand cDNA Synthesis kit). PCR and gel electrophoresis for hypoxanthine phosphoribosyl transferase (HPRT) was used to assess the quality of first strand cDNA from each sample.

**1.5 Quantitative Real Time PCR (qRT-PCR)**

M1 and M2 molecular marker expression was quantified by amplifying the bone marrow derived macrophage HPRT, iNOS, Arg1, TNF α, and Relm **α** genes via qRT-PCR. Template first strand cDNA was diluted in HPLC water (1:12 dilution). Each of the three biological replicates was assayed in triplicates. cDNA standard serial dilutions were made (5, 0.5, 0.05, 0.005, 0.0005, and 0.00005 pg / µl) and assayed in duplicates. 15 µl reaction mix of iQ SYBR Green Supermix (Bio Rad), 0.150 µl forward primer, 0.150 µl reverse primer (Primers sequences are listed below), and 2.2 µl HPLC water combined with 10 µl template first strand cDNA was used to form a 25 µl volume reaction. Each of the genes were amplified in the Bio-Rad CFX 96 Real Time PCR system using an 8 minute initial denaturation period followed 39 cycles. Each of the cycles consisted of a 15 second 95°C denaturation period, a 1 minute 60 °C annealing period, and a 5 second 65°C extension period. The melt curve and threshold values (Ct) were generated and analyzed using Excel. HPRT, a housekeeping gene expressed endogenously, was used to normalize the values with a standard curve. Analysis was completed using Prism graph pad with a one-way ANOVA followed by a Dunnet to determine statistical significance.

The following primer sequences were used:

HPRT F: CCCTCTGGTAGATTGTCGCTTA

HPRT R: AGATGCTGTTACTACTGATAGGAAATCGA

Arg1 F: CAG AAG AAT GGA AGA CTC AG

Arg1 R: CAG ATA TCG AGG GAG TCA CC

iNOS F: GGC AGC CTG TGA GAC CTT TG

iNOS R: GCA TTG GAA GTG AAG CGT TTC

TNF alpha F: CTGTGAAGGGAATGGGTGTT

TNF alpha R: GGTCACTGTCCCAGCCATCTT

Relm alpha F: CAAGGAACTTCTTGCCAATCCAG

Relm alpha R: CCAAGATCCACAGGAAAGCCA

# CHAPTER TWO

## Arginine Regulation and Transport

## 2.1 Arg1/iNOS Crossroads

NO production and polyamine biosynthesis (from ornithine) are the two primary competing metabolic pathways that utilize arginine in macrophages. Here, I will address the importance of arginine as the substrate for both Arginase and iNOS; the most diagnostic markers of M1 and M2 polarized macrophages and its questionable role in macrophage polarization. Arginine is necessary for T cell function and proliferation, but arginine's possible role in macrophage activation and function is yet to be completely elucidated. Arginine is a "nutritionally essential" amino acid, which is consumed at high levels in meats and nuts (Wu et al., 2009, Popovic et al., 2007). Studies have shown that when arginine was consumed or taken intravenously, it was helpful in enhancing immune functions, promoting the wound healing process, and sustaining tissue integrity (Wu et al., 2009). This is most likely a primary consequence of arginine being metabolized into NO or ornithine, depending on the preexisting polarization states at the time of the arginine treatment. However, Arginine can also be utilized to synthesize proteins, creatine, agmatine, ciruline, and urea (Bronte and Zanovello, 2005). Creatine, predominantly synthesized in the kidney and liver, is utilized to supply energy to the muscles. Human studies have shown that creatine can function as an anti-oxidant, decrease inflammation, and aid glucose tolerance (Wu et al., 2009). Agmatine, another product of arginine, is also associated with anti-inflammatory responses in myeloid cells. It also has been shown to function as a protective agent of chronic neuropathic pain and ischemic injury (Regunathan and Piletz, 2003).

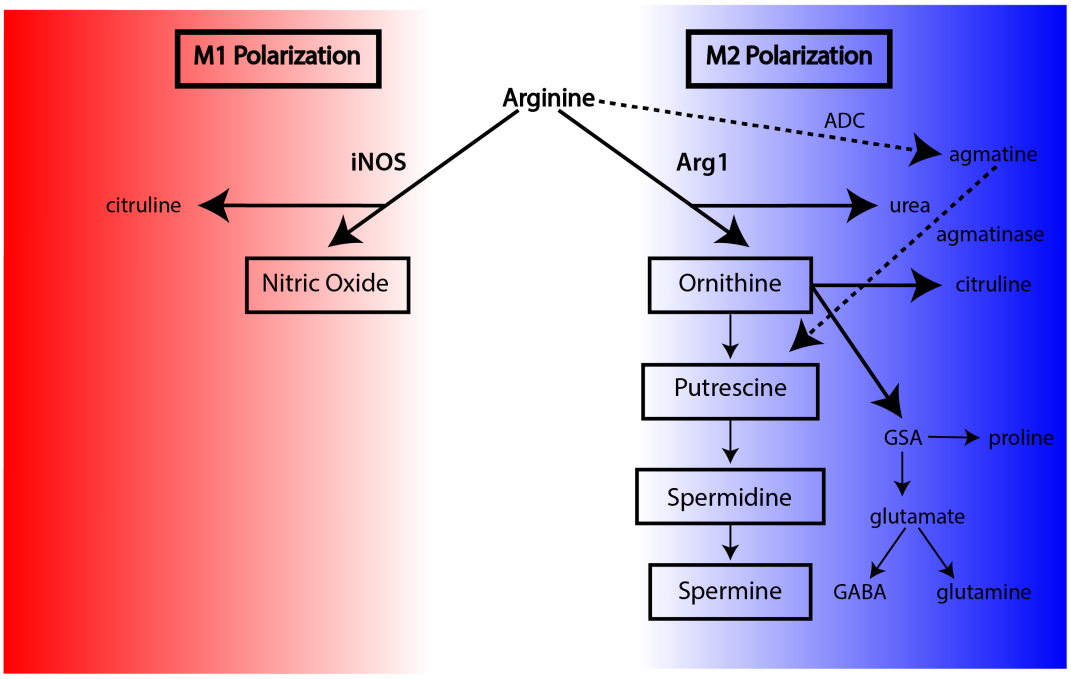



**Figure 2.1-1 Arginine usage in the context of M1 and M2 macrophage polarization.**

### *2.1.1 Arginine/Agmatine Relationship*

Arginine can be alternatively catalyzed by Arginine decarboxylase (ADC) into Agmatine. M1 Macrophage activation initially increases agmatine levels, but by depriving the cell of arginine gradually drives ADC activity down. Studies have shown that LPS treatments in RAW264.7 cells (Mouse leukaemic monocyte macrophage cell line) decrease ADC activity, while the addition of agmatine inhibits NOS catalytic activity. Increasing concentrations of agmatine decrease nitrite accumulation in LPS treated RAW264.7 cells. In cells treated with LPS, there is initial increase ADC activity followed by a gradual decline in ADC activity with greater exposure time. High Performance Liquid Chromatography (HPLC) measurements indicated that LPS/cytokine (IL-1β, IFNγ, and

TNFα) treatments increase agmatine levels within 6 hours following treatments. Yet, after 12 hours there was no significant difference between agmatine levels in control cells and treated cells (Regunathan and Piletz, 2003). This data suggests that ADC is one of the enzymes upregulated in activated macrophages. The gradual decrease in ADC may be a consequence of decreased availability of arginine associated with M1 macrophage activation or a possible negative feedback loop that was triggered by excessive amounts of intracellular agmatine. Since after 12 hours the agmatine levels return to those comparable to resting macrophages, it is conceivable that the macrophages utilize the agmatine to supply a metabolic need or export the agmatine.

The addition of agamatine, an amino acid associated with the M2 macrophage activation pathway, to M1 activated macrophages; may induce a phenotypic switch to M2 activated macrophages. In LPS treated RAW 264.7 cells, iNOS activity and protein levels decrease with exprosure to agmatine, while iNOS mRNA expression increases (Regunathan and Piletz, 2003). Although iNOS is transcriptionally upreguated by the LPS treatment, the increase in agmatine negatively alters its rate of translation and activity. This may be explained by the concept that agmatine can contribute to wound healing/repair processes associated with M2 activation/polarization. Agmatine can be converted by agmatinase into ornithine, which is subsequently converted into polyamines or proline. Perhaps, if the authors observed iNOS transcription levels after 48 hours, they would be much lower signifying a phenotypic switch from M1 to M2. Granted, further studies would need to be

conducted to determine if arginase transcriptional expression, activity, and protein levels (of these macrophages) are higher than those of iNOS.

### *2.1.2 Arginine/LPS Connection*

Studies show that L-arginine may work synergistically with LPS to augment the induction of intracellular signaling pathways associated with pro-inflammatory responses. In mice injected with LPS alone, L-arginine plasma concentrations decreased. In these mice injected with LPS alone, nitrite and nitrate levels in the plasma increased. Nitrite and nitrate levels increased still further in mice injected with LPS and given arginine treated water (Pekarova et al, 2012). Further studies were performed on peritoneal macrophages. When compared to peritoneal macrophages, from mice injected with LPS alone; peritoneal macrophages, from mice injected with LPS and given arginine treated drinking water, doubled their production of ROS (Pekarova et al, 2012). Showing that both in vitro and vivo, arginine and LPS may work synergistically to increase the products from iNOS catalyzed reactions. Arginine treatments alone appear to have no effect on the production of the products associated with the M1 response. Pekarova et al showed that iNOS protein expression and NO production remained undetectable in RAW 264.7 cells treated with L-arginine alone at different doses, but increased in LPS/L-arginine treated RAW 264.7 cells. When peritoneal macrophages were isolated and cultured in vitro with LPS and L-arginine, iNOS expression and NO production increased (Pekarova et al, 2012). Yet, their study did not observe the effects of inducing M2 responses in combination with arginine. Perhaps, the arginine would also work

synergistically with the IL-4 and dexamethasone (a glucocorticoid typically used in combination with IL-4 to M2 activation/polarization) to increase the production of ornithine and polyamines. If this was the case, then arginine may be responsible for enhancing both M1 and M2 macrophage activation/polarization.

### *2.1.3 Arginine Deprivation*

The expression of M1 and M2 molecular markers in macrophages may be independent of arginine. Since arginine is consumed for production of both NO and polyamines, it is expected that arginine deprivation would decrease the ability of macrophages (exposed to pro-inflammatory and anti-inflammatory stimuli) to become polarized in either direction. Choi et al performed studies monitoring the expression M1 and M2 molecular markers in M1 and M2 polarized bone marrow derived macrophages, deprived of arginine. Contrary to previous presumptions, the absence of L-arginine during M1 and M2 polarization did not significantly change the expression of activation markers. L-arginine deprivation (in IL-4 treated bone marrow derived macrophages) did not change the expression of Arg 1,YM1 or Relm α (Choi et al. 2009). Note that they used IL-4 and/or IL-10 alone, but did not use dexamethasone, which is typically used to polarize macrophages towards M2 states. Macrophages treated with IL-4, but not dexamethasone are nonresponsive to TGFβ -1, which is responsible for inducing the expression of 111 genes involved in transcriptional/signaling regulation, immune regulation, and the atherosclerosis process. Dexamethasone is necessary for increased expression of cell surface TGFβ- RII (Gratchev et al., 2008). If the macrophages were treated with IL-4 alone, perhaps other

M2 molecular markers were not expressed. This brings into question whether the same results would be obtained if the macrophages were deprived of arginine and treated with IL-4/Dex. Perhaps, arginine is necessary for the induction of M2 molecular markers when they are treated with dexamethasone in addition to IL-4. It is expected that more M2 molecular markers would be expressed at higher levels with dexamethasone. At this point I can only speculate that the addition of arginine to IL-4/Dex treated macrophages would enhance the expression of molecular M2 markers.

Arginine may be necessary for macrophages to reach a complete state of M1/M2 activation/polarization. Choi et al shows that L-arginine deprivation does not change the protein expression of iNOS (in IFNγ/TNFα treated bone marrow derived macrophages), but decreases the production of NO (Choi et al. 2009). When the griess reaction was performed on classically (M1) and alternatively (M2) activated macrophages left in the presence (400mM) or absence of arginine, NO production was lower than in classically activated macrophages deprived of arginine than those with arginine. NO was not detected in resting bone marrow derived macrophages or alternatively activated macrophages (Choi et al. 2009). This means that although iNOS protein expression may be independent of arginine, the production of NO is dependent on the presence of arginine as a substituent of NO synthesis. So, although arginine deprived macrophages still manifest the molecular markers of M1 or M2 polarized macrophages, they may not be metabolically the same as those cultured in the presence of arginine. This brings into question how macrophages are categorized as M1 or M2 activated/polarized. It brings us

to question whether it is appropriate to rely solely on the expression of Molecular M1 and M2 markers or whether we should also be considering the production of downstream metabolic products such as NO and ornithine/polyamines.

M2a molecular markers may be independent of arginine, while M2a/b molecular markers are dependent on the presence of arginine. Choi et al mentions that IL-10, an M2a/M2b marker, was not detected under arginine-depleted conditions (Choi et al. 2009). This brings into question whether IL-10 was detected in the control cells. If that was the case, then perhaps IL-10 expression is dependent on dexamethasone treatments. Studies have shown that dexamethasone induces monocyte derived dendritic cells to produce IL-10 (Xia et al., 2005). Since macrophages and monocyte derived dendritic cells are of the monocyte lineage, it is expected that macrophages would also express higher levels IL-10, when treated with dexamethasone. Unfortunately, this data from Choi et al's study is not shown. Thus, there is no way of visually comparing IL-10 levels in arginine vs. arginine depleted BMDMs. Nevertheless, if IL-10 expression is enhanced in presence of arginine, then M2a/M2b molecular markers may be regulated by a different mechanism from pure M2a molecular markers (Arg1, Ym1, and Relm α).

Arginine may not be the primary source for polyamine synthesis in M1/M2 activated/polarized macrophages. From this study, it can be inferred that the majority of M1 and M2 macrophage activation/polarization molecular markers are not dependent on the availability of arginine. If the arginine has no effects on the expression of M1 and M2

markers, then perhaps polyamines are being synthesized predominantly via alternative pathways. Perhaps, ornithine is being synthesized from proline that is catalyzed by ornithine dehydrogenase. Or, perhaps ornithine is being synthesized from agmatine, which is catalyzed by ADC. Surveying the transcriptional levels proline dehydrogenase, ornithine aminotransferase, ADC, and ODC in M2 polarized macrophages would determine if the polyamines are being synthesized via other pathways at higher levels than via the ODC associated pathway.

In summary, the addition or subtraction of arginine does not significantly alter the expression levels of M1 or M2 molecular markers in polarized macrophages, but the amounts of product increase or decrease, respectively. Arginine is needed for the synthesis of NO, but it does not induce the cell to increase iNOS expression or activity. During activated/polarized macrophage states, there are higher levels of both Arg1 and iNOS expression. Perhaps, Arg1 and iNOS are expressed at such high levels that they are the reactants in excess, while arginine is the limiting reactant.

## 2.2 Arginine Transport Systems

Activated/polarized macrophages are more metabolically active. Thus, they must rely not only on arginine synthesized intracellularly, but also on arginine imported from the external melleui. This leads us to question how arginine transport occurs and is regulated in M1 and M2 polarized macrophages. Here, I will discuss the mechanisms of arginine transport and how these mechanisms are affected by the presence of pro-inflammatory or anti-inflammatory stimuli.

### *2.2.1 Arginine Transport Mechanisms*

Solute Carrier Family 7 and 3 (SLC7 and SLC3) proteins have thus far been implicated in arginine transport. The SLC7 family can be split into two subfamilies, "the cationic amino acid transporters (the CAT family, SLC7A1-4) and the glycoprotein-associated amino acid transporters (the gpaAT family, SLC7A5-11)." The SLC3 family consists of "associated glycoproteins (heavy chains) 4F2hc (CD98) or rBAT (D2, NBAT). Intracellular signaling via trans-stimulation induces CAT family members to transport cationic amino acid via facilitated diffusion. gpaAT family members must associate with SLC3 family members to be expressed. These transporters can be defined by their selectivity. System L is selective for large neutral amino acids. System asc is selective for small neutral amino acids. Whereas, y+L and $B^{0,+}$-like systems transport cationic amino acids in addition to neutral amino acids (Verrey et al., 2004). Specifically, system y+L transports cationic amino acid, independent of $Na^+$. And, it transports neutral amino acids, dependent on $Na^+$. System $b^{0,+}$, independent of $Na^+$, transports neutral and

cationic amino acids. System $B^0$ takes up neutral and cationic amino acids in $Na^+$ and $Cl^-$ dependent manner. System y+ is involved in the transport of cationic amino acids and weakly neutral amino acids, with or without $Na^+$(Martín et al., 2006). "High affinity/low-rate y+L transporter and the low-affinity/high- rate y+ transporter" are both utilized for cationic amino acid uptake (Nel et al., 2012). "Uptake mediated by the y+ transporter is sodium-independent, pH-insensitive, sterioselective and inhibited by N-Ethylmaleimide (NEM) and certain neutral amino acids." Cationic amino acid uptake mediated by the y+L transporter is also sodium-independent, but transports neutral amino acids at high affinity when sodium is present. Nel et al also showed that both y+ and y+L transporters were not completely inhibited by the presence of varying concentrations of leucine. y+ transporter arginine uptake decreased significantly in response to decreases in sodium, while y+L transporter arginine uptake was not affected by alterations in sodium concentrations. However, in the absence of sodium, y+L transport was uncompetitively inhibited. The affinity of the y+ transporter for arginine was not affected by the changes in sodium concentrations indicating noncompetitive inhibition. Their studies also confirmed that NEM (N-Ethylmaleimide) completely inhibited the y+ transporter, but did not affect the y+L transporter. On the other hand, BCH (2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid) significantly decreased y+L transport activity, but did not affect y+ transport activity (Nel et al., 2012).

**Table 2.2-1 Arginine Transporter Systems**

| Gene | Protein | Transport System | References |
|---|---|---|---|
| SLC7A1 | CAT1 | y+ system | http://www.ncbi.nlm.nih.gov/gene/11987 |
| SLC7A2 | CAT2 | y+ system | http://www.ncbi.nlm.nih.gov/gene/11988 |
| SLC3A1 | rBAT | $b^{0+}$ system | D et al., 1999, http://www.ncbi.nlm.nih.gov/gene/20532 |
| SLC3A2 | 4F2hc | y+L system | D et al., 1999, http://www.ncbi.nlm.nih.gov/gene/17254 |

*2.2.2 Arginine Transport Regulation*

CAT transporters play a key role in modulating the synthesis of products such as NO by regulating the rate or L-arginine uptake (Verrey et al., 2004). More metabolically active, activated/polarized macrophages express higher levels of the enzymes that consume Arginine, and thus demand higher levels of Arginine transport. In this section, I will focus on how changes in macrophage activation/polarization states alter arginine transporter expression and activity levels.

First, it is important to identify which transport systems are responsible for arginine uptake in macrophages. Former studies, using macrophage-like J774 cells (derived from

female BALB/c mouse tumor), suggested that "CAT1, CAT2, and 4F2hc mRNAs; but not rBAT or CAT2a mRNAs" were involved in l-arginine uptake. Northern blots and RT-PCR were unable to detect the presence of rBAT mRNA in untreated and activated J774 macrophages. Studies using radioactivity and liquid scintilation showed that the y+L and $b^{0+}$ transport systems (encoded by 4F2hc and rBAT, respectively) were not involved in arginine transport in activated J774 macrophages. They showed that the competitive inhibitor of y+L and $b^{0+}$, l-leucine, was in ineffective at inhibiting l-arginine uptake in the J774 macrophage cell line (D et al., 1999).

One notable hallmark of macrophage differentiation is that it can induce macrophages to shift from one type of arginine transport system to another type arginine transport system. Martin et al conducted the following arginine transport studies on BMDMs (more physiologically relevant than J774 cells). Their experiments focused on elucidating the role of GM-CSF in arginine transport systems. Initial experiments showed that under basal conditions, arginine entered through y+L system (75%) and the y+system (10%)(Martín et al. 2006). Traditionally, GM-CSF has been associated with pro-inflammatory functions in macrophages. Interestingly, this paper points to another study done by Jost et al, where GM-CSF treated BMDMs show higher expression of Arg1 than untreated BMDMs (Jost et al., 2003). If expression Arg1, the most diagnostic marker of M2 polarization, is upregulated in these BMDMs; then it is expected that these cells are exhibiting the metabolism of macrophages with an M2 like phenotype. With this knowledge, they treated BMDMs with GM-CSF and monitored arginine transport

activity. Transport activity was measured by using radioactive arginine and a beta scintillation counter to quantify the radioactivity. GM-CSF treatments induced 10x increase in y+ system (became responsible for 40% of import), which correlated to an increase in CAT2 mRNA and protein (Martín et al. 2006). This means that the GM-CSF treatments induced the BMDMs to shift to the transport system responsible for a higher rate of arginine uptake. When macrophages become activated towards M1 or M2 states, they shift from using predominantly one type of transport system to having another type of transport system.

Since GM-CSF induces differentiation by activating the transcription of different genes, it follows that a differentiated macrophage will have a different arginine transporter. Here, I propose the mechanism by which the macrophage upregulates the CAT2 expression, when treated with GM-CSF. First, GM-CSF induces the translocation of PKC to the plasma membrane of the macrophage. Next, PKC triggers a signal transduction pathway leading to the upregulation of c-jun, followed by AP-1 enhancer activity and increased SLC7A2/CAT2B mRNA transcription. Early studies show that when monocytic leukemia cells U937 are treated with GM-CSF, protein kinase C rapidly translocates to the plasma membrane and triggers signal transduction leading increased c-jun mRNA expression followed by the activation of AP-1 enhancer activity (Adunyah et al., 1991). Furthermore previous studies report that in A549 lung carcinoma cells (adenocarcinomic human alveolar basal epithelial cells), PKC activation induced an increase in SLC7A2/CAT2B mRNA. With this knowledge, Visigalli et al set up a study

to determine if PKC was involved in controlling arginine transport in endothelial cells. Using PKC inhibitors and thymeleatoxin to activate PKC, they determined that SLC7A2 was being transcriptionally expressed at higher levels when PKC was activated. Western blots revealed that PKC activated this system y+ arginine transporter via the ERK1/2 AP-1 signaling pathway (Visigalli et al., 2010).

Consistent with the work of Martin et al, D et al used northern blots to show that LPS/IFNγ treated J774 macrophages expressing iNOS also expressed CAT2 RNA at higher levels, but expressed CAT1 RNA at lower levels than untreated macrophages. Western blot protein expression analyses revealed that IFNγ treatments slightly increased CAT2 protein levels, while LPS alone or LPS/IFNγ increased CAT2 protein levels more (D et al., 1999). Since LPS/IFNγ are pro-inflammatory stimuli, it could be presumed that GM-CSF in the previously mentioned studies is also functioning as a pro-inflammatory stimulus. However, considering that Arg1 expression is expected to be upregulated, it would be of great utility to understand the effects of M-CSF. M-CSF has traditionally been associated with inducing M2 responses in macrophages. By using M-CSF, they would have been able to determine if the CAT2 expression of M-CSF treated macrophages is upregulated to the same extent, a lesser extent, or a greater extent than that of GM-CSF treated macrophages. If CAT2 expression was upregulated to a lesser extent, then perhaps CAT2 expression is an additional molecular marker of M1 polarization.

When one type of arginine transport system is not expressed, the activated macrophage compensates by upregulating the other arginine transport system. Another question posed by Martin et al was whether BMDMs lacking CAT2 would still import arginine. They showed that the BMDMs from CAT2 KO, treated with GM-CSF, were unable to upregulate CAT2 and instead induced increased CAT1 mRNA (Martín et al. 2006). This suggests that when differentiating, the BMDMs must import more arginine even if it means overexpressing another arginine transporter to compensate for the knocked out transporter.

Macrophage activation regulates arginine transporter expression, but arginine availability has no effect on arginine transport system expression. Myeloid cells, in the absence of immune stimuli, consume low levels of arginine, because they do not express "high affinity cell membrane transporters." Thus, when these stimuli are not present, myeloid cell function cannot be enhanced by the dietary arginine supplementation (Popovic et al., 2007). In other words, the upregulated expression of the arginine transporter is direct consequence of the macrophage becoming activated/polarized. Yeramian et al showed that the uptake of arginine and the induction of Slc7A2 are independent of arginase and iNOS activity. "Induction of Slc7A2 was independent of arginine available and of the enzymes that metabolize it (Yeramian et al. 2006)." In addition D et al. verified that a deficiency of exogenous l-arginine deceased NO synthesis in activated J774 macrophages. Further studies done using CAT 2 antisense oligodeoxynucleotides to block induced l-arginine transport decreased NO synthesis, indicating that CAT2 protein

expression plays a role in NO synthesis (D et al., 1999). Changes in arginine transporter expression are dependent upon immune stimuli rather than changes in the concentration of extracellular arginine. This is not surprising, because inactive macrophages do not need to synthesize more NO as a pathogen defense response or ornithine to contribute to the wound healing process. Furthermore, increasing dietary consumption of arginine will have no effect on the levels Arg1 or iNOS expression, and subsequently no effect on the degree of macrophage polarization. If the arginine transport is inhibited and macrophages are deprived of exogenous arginine, they synthesize less NO. From these results, it can only be inferred that ornithine synthesis also decreases. For confirmation, studies using HPLC to examine the levels of polyamines synthesized by macrophages deprived of exogenous arginine would need to be performed. Perhaps, the macrophage would compensate polyamine synthesis by deriving ornithine from some other amino acid source. Nevertheless, it is now clear that an increase in the expression arginine transporters is a direct response of macrophage activation/polarization, and not changes in levels of available exogenous arginine, endogenous Arg1, or endogenous iNOS.

### *2.2.3 Inconsistencies with Experimental Techniques*

Unfortunately, many studies done on y+L transporters and y+ have met several challenges and inconsistencies. First, the utilization of different cell types and experimental techniques has lead to discrepancies in measuring transport activity and comparing it among studies. In fact, some "in vitro kinetic uptake studies" have only observed activity of y+ transport systems and disregarded that of the y+L transport

systems (Nel et al., 2012). Studies done by D et al and Martin et al observed transport in not only y+ transport systems, but also in y+L transport systems.

Secondly, the majority of previous studies using radiolabeled amino acids have adopted the Michaelis-Menten kinetics. Further disparity is rooted in the fact that they have based their calculation constants on "Lineweaver-Burk reciprocal plots, Eadie-Hofstee plots and non-linear modeling (Nel et al., 2012)." Unfortunately, the paper by D et al does not mention how they obtained the calculation constants or analyzed the kinetics. Nel et al. goes on to describe a number of other assumptions that previous studies have made. Instead, Nel et al. used raw data in nonlinear regression format in their study to analyze the kinetics of the arginine transporters (Nel et al., 2012). When researching this area, the reader must be aware of these inconsistencies and consider for them, before embarking on future arginine transport studies.

# CHAPTER THREE

## Regulation of Polyamine Synthesis and Transport

## 3.1 Polyamines and Mechanisms of Transport

Polyamines are positively charged (at a physiological pH), aliphatic, organic compounds found in all living organisms. They are ubiquitous and essential for life. Higher ODC and polyamine levels are characteristic of rapidly growing cells, whereas cells that are growing slowly or not growing at all have lower levels. Since polyamines are involved in so many biochemical mechanisms, it is difficult to determine which specific processes they control. Studies have shown that polyamines interact with DNA and RNA, and are involved in protein biosynthesis as well as the activation of protein kinases (Tabor and Tabor, 1984).

### *3.1.1 Polyamine Import*

Intracellular polyamines levels can be manipulated not only by changes in the rate of biosynthesis, but also by uptake, export, and degradation (Tjandrawinata et al., 1994). Unlike $Ca^{2+}$ and $Mg^{2+}$, polyamines have a homogenous allocation of charges across the carbon chain (Reguera et al., 2005). This quality prevents them from being passively transported across the cell membrane. As of 2005, transporters of polyamines have been found in bacteria and yeast. Yet, there is no clearly defined mechanism of polyamine import in mammalian cells. At least four different groups have investigated (mammalian) polyamine import using fluorescent polyamine probes (Cullis et al., 1999, Belting et al., 2003, Soulet et al., 2004, Poulin et al., 2006). The flourophore was conjugated to the polyamine backbone at either the "terminal ($N^1$) amino group or central ($N^4$) amino group (Poulin et al., 2006)."

Two models of polyamine uptake have been proposed. In the first model, a plasma membrane carrier imports the polyamines and then polyamine-sequestering vesicles isolate the polyamines. In the second model, "receptors undergoing endocytosis" capture the polyamines. According to studies done with fluorescent probes (Spd-C2-BODIPY, BODIPY FL transferrin, and LysoTrackerRed), polyamines are not taken up by binding to the plasma membrane receptor as described in the second model. "End1 CHO (chinese hamster ovary) mutants that are strongly defective in receptor-mediated endocytosis and exhibit pleiotropic defects at the non-permissive temperature" (40 degrees Celsius) were compared to WT CHO cells. Results revealed that there was no difference in Spd-$C_2$-BODIPY (fluorprobe that reports polyamine transport) uptake indicating that receptor - mediated endocytosis is not required for uptake. Thus a new model was proposed, in which plasma membrane transporters/channels (membrane potential-dependent), possibly assisted by glypican, transport polyamines into the cytosolic compartment. Next, V-ATPase in combination with a polyamine/$H^1$ antiporter enables polyamine vesicular internalization. Then, in the late endocytic compartment, polyamine-sequestering vesicles fuse with acidic vessicles to be degraded (Soulet et al., 2004).

Belting et al proposes a similar mechanism. Uniquely, their model portrays heparin sulfate as a plasma membrane polyamine carrier (Belting et al., 2003). Cheng et al. shows that non-S-nitrosylated glypican carring side chains rich in N- unsubstituted glucosamines colocalize with caveolin-1 implicating caveolin-1 involvement in the polyamine import process. Glycosylphosphatidylinositol-anchored proteins usually have

corresponding plasma membrane domains. When these domains are associated with caveolin-1, they form caveolae, which are responsible for non-clathrin dependent endocytosis (Cheng et al., 2002). Unlike classical caveolar endocytosis, which involves glycosylphosphati- dylinositol (GPI)-anchored proteins, non-classical caveolar endocytosis is thought to involve GPI-linked HSPG glypican (Belting, 2003). Heparin sulfate chains are complexed with the polyamine, and then the glycosylphosphatidylinositol-bound glypican-1 internalizes the complex via endocytic caveolation (Belting et al., 2003). Glypican 1 can be present in different forms depending on the time point in the internalization process. It can be in the S-nitrosylated form or the "slightly charged glypican-1 glycoforms containing heparan sulfate chains rich in N-unsubstituted glucosamines." When NO is released from nitrosothiols at the core of glypican-1, heparin sulfate undergoes deaminative cleavage at the N-unsubstituted glucosamines and $Cu^{+}$ becomes oxidized to $Cu^{2+}$. Cleavage results in not only heparan sulfate fragments with anhydromannose residues (Ding et al., 2002), but also the release of the polyamines due to the weakening of the interactions between the heparin sulfate and the polyamines (Belting et al., 2003).

With this knowledge, I propose a more complete model in which heparin sulfate proteoglycan serves as a plasma membrane polyamine carrier. The extracellular polyamine becomes complexed with the heparin sulfate and the complex is internalized in its S-nitrosylated form. Once internalized, NO is released from nitrosothiols and heparin sulfates get cleaved liberating polyamines within the cytosol. V-ATPase works

synergistically with a polyamine/$H^1$ antiporter on the surface of the polyamine sequestering vesicles (PSV) to internalize the polyamines. Next, the PSVs enter the late endocytic compartment and merge with acidic vesicles where the polyamines are degenerated.

*3.1.2 Polyamine Export*

In order to maintain the necessary and sufficient intracellular polyamine levels, the cell must not only synthesize and import arginine/polyamines, but also export polyamines. Diamines, putrescine and cadavarine, are exported from RAW264 cells by facilitative diffusion system with the aid of integral membrane protein(s). Although it was initially speculated that putrescine was exported via a putrescine-calcium "bidirectional electronegative antiporter system," further studies have demonstrated that putrescine export does not directly involve intracellular calcium and sodium (Tjandrawinata et al., 1994).

More recently, studies were done using HPLC to monitor polyamine transport by inside-out membrane vessicles in SLC3A2 knock down cells to confirm that SLC3A2 (which encodes 4F2hc) was involved in polyamine export (Uemura et al., 2008). But, 4F2hc is only a glycoprotein associated amino acid transporter protein and must be colocalized with another transporter system to function successfully. For example, 4F2hc is often paired with CD98 light subunit protein (which is encoded by SLC7A5) forming an LAT-1 transporter (Verrey et al., 2004). Experiments using co- immunopreciptiations showed

that the polyamine transporter is not that simple. They showed that SAT1 colocalizes with SLC3A2 on the cell's plasma membrane. These studies led to the development of the first molecular model of a polyamine exporter in animal cells. Polyamines are exported via a diamine exporter (DAX) consisting of a SLC3A2 transporter heterodimered with a y+LAT light chain. In this model, DAX exports putrescine, AcSPD, and AcSPM in exchange for arginine. SLC3A2 also complexes with SAT1 to acetylate and export acetylated polyamines (Uemura et al., 2008).

### 3.2 Intracellular Polyamine Level Regulation

Since activated/polarized macrophages are metabolizing and consuming polyamines at different rates than resting macrophages, it follows that polyamine synthesis and transport is regulated differently in these macrophages. Yet, little is known about how polyamine synthesis, import, and export are controlled in these states. Do activation/polarization states play a role modulating the control polyamine import and export?

#### *3.2.1 Polyamines and the M1 response*

Studies have shown that macrophages treated with M1 stimulants or M2 stimulants exhibit a higher polyamine metabolism than their untreated counterparts. According to Putambekar et al, polyamine metabolism is upregulated in macrophages/microglia in M1 and M2 activated states. Primary microglia treated with LPS/IFNγ for 24 hours, expressed higher levels of ODC (ornithine decarboxylase), AZ (antizyme), and SSAT (Spermidine/spermine-$N^1$ –acetyltransferase) than untreated primary microglia. Based on

the expression of TREM1, CD40, MHC class II, microglia and macrophages isolated from mice brains injected with LPS and DFMO are just as activated as those isolated from LPS injected brains (Puntambekar et al., 2011). In the absence or presence polyamines synthesis, the macrophages and microglia still become activated when stimulated, and thus activation may not be dependent on polyamines. This is consistent with Choi et al data, which shows that the expression of M1 and M2 molecular markers is not dependent on arginine, the predecessor of ornithine and polyamines (Choi et al., 2009). Yet, our lab shows that polyamines increase the expression of CCL2 in astrocytes and microglia. LPS induced recruitment of macrophages in mice brains was suppressed by a co-injection of DFMO, showing that a lack of polyamines suppresses the M1 response of fluxing macrophages into the CNS. This was confirmed when the addition of putrescine and spermine to mixed glial cultures increased the concentrations of TNF and CCL2 secreted. In mice brains injected with LPS and DFMO, the CCL2 expression decreased in comparison to LPS alone injected mice brains, showing that polyamines are needed in addition to an LPS response to get effective macrophage influx (Puntambekar et al., 2011). This data suggest that polyamines are needed to achieve M1 responses associated with the recruitment of more macrophages to the site of assault.

### *3.2.2 Polyamines and the M2 Response*

When polyamine levels are low, the macrophage is more inclined to express higher levels of M1 molecular markers than M2 molecular markers. Bossche et al shows that polyamine depletion decreases the expression of some M2 markers, while increasing the

expression of some M1 markers. When balb/c BMDMs were treated for 24 hours with IL-4/DENSPM (potent spermine analogue) treatment, there was a downregulation of 2/3 of these genes; including retnla, ym, cldn11, mrc1, or ear11. However, there was no effect on the expression of 1/3 of the tested genes that encode markers of alternatively activated macrophages, including the gene arg1, which encodes Arg1 (Bossche et al., 2012). Although polyamines are required to obtain a M1 response, more polyamines are needed to achieve a M2 response. This suggests that stringent control of polyamine levels is necessary to get the appropriate level of M1or M2 response for the particular condition.

Bossche et al also shows that putrescine levels are significantly lower in BALB/c thio-PEM macrophages pretreated with DFMO (an ODC inhibitor) and stimulated with IL-4 than in those not pretreated with DFMO. However, in these same conditions there is no significant difference in spermidine or spermine levels (Bossche et al., 2012). This suggests that higher putrescine levels in M2 activated/polarized macrophages induced by IL-4 treatments are a result of increased Arg1 expression and subsequent availability of ornithine. Ornithine concentration were very low in IL-4 treated Arg1-deficient thio-PEM macrophages (same as untreated), while polyamines levels in the same conditions were much higher than untreated Arg1-deficient thio-PEM macrophages (Bossche et al., 2012) It can be speculated that if ornithine is not available, IL-4 induces the macrophage to upregulate agmatine transcription/activity, which is also used for the synthesis of putrescine.

*3.2.3 Polyamine Level Regulation*

To elucidate how polyamine levels are controlled within activated/polarized macrophages, the biosynthetic polyamine pathways must be examined. As mentioned previously, polyamines are synthesized when arginase binds to arginine catalytically converting it into ornithine, which is in turn is converted by ODC (ornithine decarboxylase) into putrescine, and subsequently the polyamines. Thus intracellular polyamine levels are dependent on the amount of active arginase and ODC present. ODC antizyme (OAZ), an endogenous inhibitor of ODC, also modulates polyamine synthesis by degrading ODC. OAZ functions by binding to ODC and transporting it to the 26 S proteasome for degradation (Liao et al., 2009). Thus, if ODC is degraded, it follows that there will be a decrease in the intracellular levels of polyamines. Moreover, OAZ also appears to be associated with a decrease in polyamine import. Although the mechanism is still unclear, it is known that antizymes inhibit polyamine transport across the plasma membrane (Kahana, 2009). A more recent review speculates that OAZ interacts directly with polyamine permeases to regulate polyamine transport, but this remains to experimentally proven or disproven (Poulin et al., 2012).

Another protein, the Anti-enzyme inhibitor (AZI) functions by binding to OAZ. Together OAZ and AZI regulate polyamine transport. Overexpression of AZI has been shown to increase polyamine import (Liao et al., 2009). It appears that when OAZ is bound to AZI, it can no longer inhibit polyamine transport or bind to ODC and prevent putrescine synthesis. An overexpression of AZI would be responsible for an increase in

intracellular polyamine levels. It can be speculated that AZ and AZI expression are transcriptionally regulated by responses to M1 and M2 stimuli. It would be of utility to conduct studies observing the levels of AZ and AZI transcriptional expression and protein expression after inducing macrophages with M1 (IFNγ/LPS) or M2 (IL-4/Dex) treatments. I expect that AZ expression will be higher in unpolarized macrophages than in M1 and M2 activated/polarized macrophages. While, AZI expression will be lower in unpolarized macrophages than in M1 and M2 activated/polarized macrophages.

Polyamine transport is regulated distinctly in M1 activated/polarized macrophages. Thus far, HPLC experiments have been done to monitor the quantities of polyamines exported from macrophages treated with M1 stimulants. In RAW264 cells treated with LPS, intracellular and extracellular putrescine levels increased after 24 hours, while spermidine and spermine levels were equivalent to those of untreated cells (Tjandrawinata et al., 1994). Since ornithine is a predecessor of polyamines, it is expected that the addition of ornithine alone would increase intracellular polyamine levels. The addition of ornithine to LPS treated RAW 264 cells, increased intracellular and extracellular putrescine levels over a time course of 5-25 hours. Intracellular putrescine levels initially only increased slightly in response to treatments of ornithine alone, but later were nearly equivalent to those of untreated cells. Increasing extracellular putrescine levels reveal that ornithine alone is synthesized into putrescine, which is rapidly exported from the cells over a time course of 5-24 hours (Tjandrawinata et al., 1994). Polyamines start getting exported at higher levels as early as 4 hours after the cells are treated with LPS. LPS induced ODC

activity peaks at 4 hours post treatment and plateaus at 6 hours post treatment (Tjandrawinata et al., 1994). Cadavarine, a diamine derived from lysine, is also intracellularly synthesized and selectively exported from RAW 264 cells. Intracellular cadavarine was detected at very low levels, while extracellular cadavarine was detected at high levels indicating that intracellular cadavarine was being rapidly exported or converted into other downstream products. RAW 264 cells treated with LPS for 24 hours exported 7x more cadavarine than untreated RAW 264 cells. Cardavarine export in these LPS treated cells was inhibited by the addition of ornithine at a concentration of 1.0 mM for 4 hours (Hawel et al., 1994).

Regarding polyamine transport in M1 and M2 macrophages, most of the studies discussed have been at 24 hour time points to ensure that the macrophages have been completely polarized towards M1 or M2 states, but what happens to the polyamines as the macrophages progresses towards becoming M1 or M2 polarized is unknown. So far, no studies have been done to show the time points and rates at which the polyamines are exported from IL-4/Dex treated macrophages. However, Bossche et al showed that polyamine depletion was associated with an increase in mRNA expression of nos2, ccl2, ccl5, tnf, il-6, and il-12 p40 (Bossche et al., 2012). Perhaps polyamine export is more upregulated early on in the advance towards M1 polarization. Later in the progression, polyamine synthesis and export become downregulated allowing for the utilization of arginine by iNOS. In contrast to M1 polarized macrophages, macrophages treated with IL-4/Dex would have an upregulated polyamine metabolism throughout much of their

progression towards an M2 state. It can be speculated that in macrophages going through the process of becoming M1 polarized, AZ expression would increase following the rapid increase in ODC expression. AZI expression would remain low throughout the process and afterwards as well. While, in macrophages going through the process of becoming M2 polarized, AZ expression would remain low allowing for continual polyamine synthesis and export. AZI expression would remain low throughout the process and afterwards as well.

**Table 3.2-3 Polyamine Regulation**

| Polyamine Regulatory Molecules | Mechanism | Polyamine Synthesis | Polyamine Import | Intracellular Polyamine Levels | References |
|---|---|---|---|---|---|
| OAZ (ODC antizyme) | Binds to ODC and transports it to the 26 S proteasome for degradation | ↓Putrescine | ↓Import | ↓Putrescine<br>↓Spermidine<br>↓Spermine | Liao et al., 2009 |
| AZI (Anti-enzyme inhibitor) | Binds to OAZ and prevents it from binding to ODC | ↑Putrescine | ↑ Import | ↑Putrescine<br>↑Spermidine<br>↑Spermine | Liao et al., 2009 |

### 3.3 Polyamine Synthesis and Inhibition

Polyamine synthesis inhibition has traditionally been used for the purpose of chemotherapeutic treatments. However polyamine synthesis inhibitors have also been used as treatments for many other pathological states. Here I seek to provide a better understanding of how these pharmaceuticals work and identify which are the best candidates to obliterate parasite infections. The synthesis of polyamines can be inhibited at many different points in the polyamine metabolic pathway, but wherever it is inhibited there will be different repercussions. The farther down in the pathway inhibition occurs, the fewer polyamines will be inhibited unless the catabolic enzymes are inhibited. Perhaps, the most ideal polyamine inhibitor would inhibit the polyamine synthesis early on at the point of Arginase, preventing the synthesis of not only polyamines, but also ornithine. Otherwise, ornithine could be used by the parasite to synthesize its own polyamines. For the sake of chronological order, I will focus on each target enzyme in the pathway in the order that it occurs. But, remember these enzymes are active simultaneously.

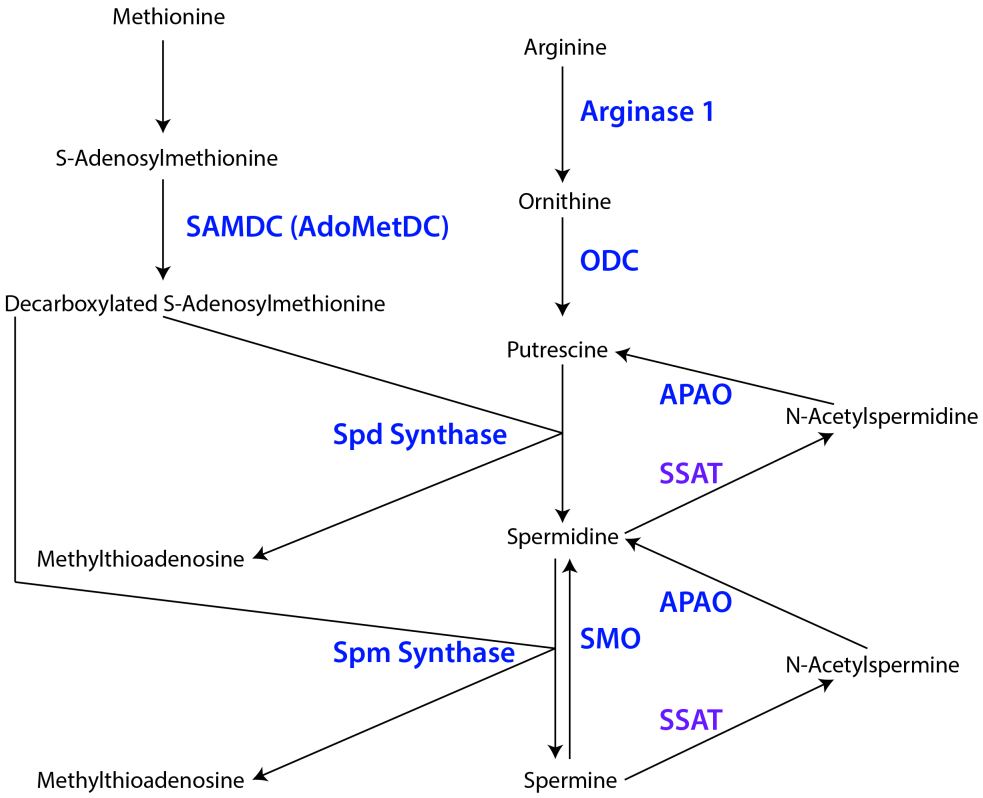



**Figure 3.3-1 Polyamine metabolism and enzymes targeted for polyamine synthesis inhibition.**

*3.3.1 Arginase Inhibitors*

Arginase is the first enzyme involved in polyamine synthesis, and it is responsible for catalyzing arginine into ornithine decarboxylase (ODC). In order to identify which inhibitor is the most effective, it is important to understand how arginase interacts with its inhibitors. Initially, studies on inhibitors were done using mice and rat arginase. The problem with this is that human arginase does not have the same amino acid sequence identity. Human Arginase 1 is a 322- residue, "trimeric binuclear manganese

metalloenzyme," that shares 87% amino acid sequence identity with rat arginase 1 and 55% amino acid sequence identity with human arginase 2 (Di Costanzo, 2005). Thus rat arginase was a relatively good target for arginase inhibition studies projected to humans. But, X ray crystallography studies have now elucidated the human arginase structure complexed with (S)-amino-6-boronohexanoic acid (ABH) (Di Costanzo, 2005), providing us with most appropriate target. ABH is the arginase inhibitor that is "most widely used and reported in the literature (Golebiowski et al., 2013)." ABH inhibits arginase1 activity in human and mice myeloid cells and has a Kd of 55 nM (Di Costanzo, 2005) suggesting that human and mice Arg1 share the same active sites. Human arginase 1 has an active site that is stereoselective for L-stereoisomers of amino acids (Ilies et al., 2011). When human arginase1 is complexed with ABH, it forms an asymmetric unit consisting of "2 monomers from three different trimmers." The ABH chemical reaction with arginase mimics that of arginine and arginase1 (Di Costanzo, 2005). ABH interacts predominantly via hydrogen bonds with the Ser137A, His141A, Asp128A, Asp232A, and Asp183A residues of Arginase (PDB accession code 1D3V) (Cox et al., 1999).

Other related arginase inhibitors include 2-amino-6-borono-2 methyl-hexanoic (MABH) and 2-amino-6borono-2-(difluoro-methyl)-hexanoic acid (FABH). They also inhibit human Arg1, but are not as potent as ABH (Golebiowski et al., 2013). FABH and MABH have 1889 and 49 times lower binding affinity human arginase 1 than ABH. Another inhibitor, BEC, resembles ABH in that when it binds to arginase, it has the same

conserved hydrogen bond arrangement involved in recognizing its alpha amino and alpha carboxylate groups (Ilies et al., 2011).

Nor NOHA (Nω-hydroxy-nor-Arginine), another inhibitor of arginase, stands out because it interacts with arginase in a very distinct way from ABH. Leishmania Arginase-nor NOHA complex intramolecular interactions (PDB accession code 3KV2) are similar to those of human arginase-nor NOHA complex. nor NOHA interacts with Leishmania arginase by displacing the arginase metal-bridging hydroxide ion with its hydroxyl group. Although not a transition state analogue, nor-NOHA binds with high affinity to arginase. This is consistent with both human and Leishmania arginase, because "residues important for substrate and inhibitor binding are strictly conserved between the two enzymes." Unfortunately, when compared to ABH, nor NOHA has 40 times weaker inhibitory potency. The article also mentions that nor-NOHA is inefficiently taken up by *L. mexicana*, and is thus unable to interact with its arginase (D'Antonio et al., 2013).

When aiming to inhibit parasite growth and survival by blocking arginase synthesis, it is important to remember that the parasite is not only synthesizing/using its own arginase, but also that of the host. That is why it is important to use an inhibitor that inhibits both the human and parasite arginase activity simultaneously. If you only inhibit the arginase of the parasite, the parasite will still have the arginase of the host to synthesize ornithine and subsequently polyamines. Unlike human cells, Leishmania only expresses one Arg enzyme. nor-NOHA, NOHA, ABH, BEC, boric acid, trypanothione, L-lysine, L-leucine,

and L-ornithine have been shown to block human Arg1 by greater than 50%, while nor-NOHA, NOHA, ABH, BEC, boric acid, trypanothione, DL-homocysteine, L-cysteine, and 8-amino-guanine have been shown to block leishmania Arg by greater than 50%. nor-NOHA, NOHA, ABH, and BEC were determined to completely inhibit human Arg1 and Leishmania Arg activity. Based on previous studies, the authors impart that both human and leishmania Arg enzymes are needed for parasite to progress successfully within the host. They suggest targeting an inhibitor of both the parasite and its host as a potential treatment. Under assay conditions, both human and Leismania Arg activity were completely blocked by nor-NOHA, NOHA, ABH, or BEC. When tested on cultured Leishmania promastigotes, nor NOHA and NOHA were the only inhibitors that exhibited toxicity (Riley et al., 2011). This means that the most effective Leishmania treatment with Arginase inhibitors would be nor-NOHA, NOHA, ABH, or BEC. The next question to ask is how these inhibitors compare based on binding affinity. Of the four compounds, ABH has the strongest binding affinity (a Ki of 1.3 uM for Leishmania arginase and a Ki of 3.5 uM for human arginase1) for arginase, while NOHA has the weakest binding affinity for arginase (a Ki of 85 uM for Leishmania arginase and a Ki ~70 uM of for human arginase1) (Riley et al., 2011). Based on binding affinity and the ability to be taken by the parasite, ABH is the most effective arginase inhibitor to use for the treatment of leishmania inffections.

More recent studies have placed an emphasis on improving ABH inhibitory potency by adding a new functional group to this alpha-hydrogen projecting outward. This group was

able to improve potency for both Arg1 and Arg2 by substituting the alpha center with "a tertiary amine linked via a 2 carbon chain." X-ray crystallography showed that Asp181 or Asp 200 (amino acid residue at entrance of the active site pocket of arg1 or arg2, respectively) interact with the substituted nitrogen demonstrating improved potency. Potency was improved by up to 5-10 fold, when elylene-linked tertiary amines were used to substitute the alpha center (Golebiowski et al., 2013). With these developments, Golebiowski et al laid down the groundwork for the development of future Arg1 inhibitors, which are sufficiently potent to obliterate all human and parasite arginase activity and prevent the synthesis of polyamines all together.

**Table 3.3-1 Arginase Inhibitors**

| Arginase Inhibitor | Molecular Formula | Mechanism | Applications | References |
|---|---|---|---|---|
| ABH (2-(S)-amino-6-boronohexanoic acid) | $C_6H_{13}BNO_4$ | Slow binding competitive inhibitor of human Arginase 2. | Inhibitory properties with Leishmania. | Di Costanzo, 2005, Golebiowski et al., 2013, Ilies et al., 2011, https://www.caymanchem.com/app/template/Product.vm/catalog/10006862/promo/emolecules |
| 2-AIAP ((2S)-(+)-Amino-5 iodoacetamidopentanoic acid) | $C_7H_{13}N_2O_3I$ | Irreversible Inhibitor of Arginase. | NA | Trujillo-Ferrara et al., 1992, http://www.scbt.com/datasheet-202409-2s-amino-5-iodoacetamidopentanoic-acid-html |
| BEC (S- (2-boronoethyl)- L-cysteine) | $C_7H_{15}$ $BNO_4$ S | Slow binding competitive inhibitor of Arginase. | Inhibitory properties with Leishmania. | Ilies et al., 2011, https://www.caymanchem.com/app/template/Product.vm/catalog/10170, http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=446122 |

| | | | | |
|---|---|---|---|---|
| FABH (2-amino-6-borono-2-(difluoromethyl)-hexanoic acid) | NA | Slow binding competitive inhibitor Arginase. | Inhibitory properties with *Plasmodium falciparum* arginase. | Golebiowski et al., 2013, Ilies et al., 2011 |
| L-Nor-Valine ((S)-2-Aminovaleric acid, (S)-(+)-2-Aminopentanoic acid) | $C_5H_{11}NO_2$ | Enhances NO production. | Antifungal Agent, Involvement in Trypanosome killing. | Gobert et al., 2000, http://www.google.com/patents/US20110318271?dq=L-HOArg+arginase+inhibitor, http://www.chemspider.com/Chemical-Structure.58608.html, http://www.sigmaaldrich.com/catalog/product/sigma/n7627?lang=en&region=US, |
| L-HOArg (NOHA acetate salt, $N^G$-Hydroxy-L-arginine acetate salt) | $C_6H_{14}N_4O_3 \cdot C_2H_4O_2$ | Intermediate in NO biosynthesis. | NA | http://www.sigmaaldrich.com/catalog/product/sigma/h7278?lang=en&region=US |

### *3.3.2 Ornithine Decarboxylase (ODC) inhibitors*

Ornithine Decarboxylase (ODC) is the first committed and rate limiting step of polyamine synthesis in mammalian cells. It is responsible for the rapidly catalyzing the conversion of ornithine to putrescine. There are many inhibitors of polyamine synthesis, but DFMO (alpha-dimethylornithine) is probably the most common. It functions by acting on the ODC active site at the lys 69 and lys 360 residue. ODC cleaves DFMO into a product that cannot be released, so that ODC becomes irreversibly inactive. The L-enantiomer of DFMO has 20 times higher chances of forming an enzyme-inhibitor complex than the D-enantiomer form.

In vitro studies have demonstrated that DFMO effectively depletes cells of putrescine and spermidine, but does not affect spermine levels. DFMO triggers cell growth arrest in cells chiefly during the G1 phase of the cell cycle (Wallace and Fraser, 2004). It is not surprising that ODC activity has its first peaks at G1 phase (Wallace et al., 2003). IEC-6 cells (normal rat small intestine epithelial cells) treated with DFMO undergo cell cycle arrest with increased expression of p21, p27, p53, and MAPK (Ray et al., 1999). When ODC activity is inhibited, the cell becomes depleted of putrescine and spermidine. It is suggested that in response to this depletion, the MAPK signaling pathway induces cyclin dependent kinase inhibitors preventing cell cycle progression. Unfortunately, DFMO has a relatively low binding affinity (Ki f 40 Amol/L) for ODC and is easily displaced by orntihine. An upregulation of ODC expression has been reported with DFMO treatments (Wu et al., 2007). This has led to development of new ODC inhibitors that will be discussed here in more detail.

Although more commonly used as a cancer chemotherapeutic, DFMO has also been used as an anti-parasitic infection treatment. It has been successful in the treatment of *Trympanosoma brucei brucei*. Since spermidine is utilized in the production of trypanothione (used by parasite to withstand oxidative stress), DFMO makes the parasite more vulnerable to oxidative stress. Without the supply of putrescine and spermidine, trypanosomes enter a non-dividing stage, which does not allow for surface glycoprotein changes making them less resistant to immune forces of the host (Wallace and Fraser, 2004). According to Landfear et al, *T. brucei* naturally live in environments with low

polyamines and rely on their own ODC to synthesize polyamines (Landfear, 2011). Studies have demonstrated that DFMO treatments cure *T. brucei gambiense*. Since the host and the parasite enzymes have the same ODC active site (Jackson et al., 2003), DFMO also inhibits the ODC of the parasite. However, there are exceptions. DFMO would be ineffective against *Tympanosoma. cruzi*, because "its genome does not encode the gene for ornithine decarboxylase (Landfear, 2011)." This means ODC inhibitors in general would be ineffective as treatments against *T. cruzi*.

Other ODC inhibitors of interest in treating parasite infections include APA (1-aminooxy-3-aminopropane) and pentamidine (p-p'-[pentamethylenedioxy] dibenzamidine). APA decreases ODC activity (Khomutov et al., 1985). When *L. donovani* was treated with APA, in vitro promastigote growth was inhibited and in the macrophage (J774A.1) model amastigote growth was also inhibited. This study demonstrated that "APA is a potent inhibitor of *L. donovani* growth and that its leishmaniacidal effect is due to inhibition of ODC (Singh et al., 2007)." It is responsible for decreasing putrescine, spermidine, and trypanothione levels in the cell (Heby et al., 2007).

Pentamidine decreases ODC activity and putrescine levels, but does not change spermidine levels (Libby and Porter, 1992). Pentamidine has been most commonly used to treat *T. brucei* infections (sleeping sickness) (Basselin et al., 2002). It is the second most prescribed treatment for visceral leishmania (Roberts et al., 2002). It is also used to

treat *Pueumocystis carinii pneumonia*. Pentamidine and other diamidines enter African trypanosomes via the P2 nucleoside transporter. Loss of this transporter can render them resistant to diamidines. Since *T. brucei* appears to have additional diamidine transporters, it remains sensitive pentamidine. Unlike in African trypanosomes, diamidines enter Leishmania cells via a carrier-mediated process (a transporter with an unidentified physiological role in the Leishmania cell). Contrary to previous hypothesis, a decrease in the diamidine transporter functionality is not responsible for resistance development. Leishmania cells develop resistance when their mitochondria no longer accumulate diamidines and instead export it (Basselin et al., 2002). It is clear that diamidines, particularly pentamidine, have other functional mechanisms of cytotoxicity against Leishmania than purely ODC inhibition. As trypanosomas develop resistance to pentamidine and other diamidine pharmaceuticals, it is of essence that alternative drugs be designed.

POB (N-(4′-Pyridoxyl)-Ornithine(BOC)-OMe), a more recently developed inhibitor may be the most effective ODC inhibitor yet (5-10 times more potent than DFMO). POB was designed based on the discovery of an additional hydrophobic pocket on the human ODC. Between the two ODC subunits, aromatic residues TyrA389, TyrA331, PheB397, and TyrB323 form this hydrophobic pocket where ornithine fits. With this knowledge, it was determined that "1-methylaminoethanol, 2-aminopyrrole, triethylamine, and trimethylamine" fragments would fit into this pocket creating a more complimentary interaction. So, POB was designed with an "additional hyrdrophobic BOC group

(protects amino acid from polymerization) of the q-amino group of ornithine." This also gives it no negative charge and the feasibility of crossing the cell membrane. Further studies revealed that POB completely inhibits newly induced ODC activity and strongly inhibits DNA synthesis (Wu et al., 2007). This leads us to the question whether POB would be an appropriate treatment for Leishmania and other Trypanosomas. The answer to this question would be based on the molecular structure of Trypanosomas ODC and how it is different from human ODC. Using the appropriate software, one could compare the dynamics of the human aromatic residues TyrA389, TyrA331, PheB397, and TyrB323 in ODC (PDB accession code 1D7K) to those of the trypanosome (PDB accession code 1F3T). This would allow us to determine if this newly discovered hydrophobic pocket is also present in Trypanosoma ODC and if it is located in the same position as that of the human ODC. If this were the case, then the additional hydrophophic BOC group of POB would fit into the Trypanosomas hydrophobic pocket rendering similar inhibitory activity. Further studies will need to be done with POB and Trypanosomas, but it certainly does represent a promising direction for treatment of Trypanosoma infections.

**Table 3.3-2 ODC Inhibitors**

| ODC Inhibitor | Molecular Formula | Mechanism | Effects on polyamine metabolism | Applications | References |
|---|---|---|---|---|---|
| 2-AIPA (2-aminoindan-2-phosphonic acid) | $C_9H_{12}NO_3P$ | Irreversible inhibitor of ODC, inhibitor of phenylalanine ammonia lyase.<br><br>*Also inhibits Arginase | NA | NA | Trujillo-Ferrara et al., 1992, http://www.chemspider.com/Chemical-Structure.13077856.html |
| APA (1-aminooxy-3-aminopropane) | $C_3H_{10}N_2O$ | Competitive inhibitor of ODC.<br><br>*Also inhibits SAMDC and Spd Syn. | ↓ODC activity | Growth inhibitory properties with *Leishmania donovani.* | Khomutov et al., 1985, Singh et al., 2007, http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=65020 |
| AP-APA (1-aminooxy-3-N-[3-aminopropyl]-aminopropane) | $C_6H_{17}N_3O$ | Competitive inhibitor of ODC.<br><br>*Also inhibits SAMDC and Spd Syn. | NA | NA | Eloranta et al., 1990, http://www.brenda-enzymes.org/php/ligand_flatfile.php4?brenda_ligand_id=240999 |
| AOE-PU (N-[2[aminooxyethyl]-1,4-diaminobutane) | $C_6H_{17}N_3O$ | Inhibits ODC<br><br>*Competitive Inhibitor of Spermine Synthase.<br><br>*Also inhibits SAMDC and Spd Syn | NA | NA | Eloranta et al., 1990, http://www.brenda-enzymes.org/php/ligand_flatfile.php4?brenda_ligand_id=241000 |
| DFMO (alpha-diflouromethylornithine) | $C_6H_{12}F_2N_2O_2$ | Suicide, irreversible inhibitor of ODC. | ↓Putrescine, ↓Spermidine, ↔ Spermine | Treatment of *Pueumocystis carinii, Trypanosoma brucei, Plasmodia, Eimeria tenella.* | Landfear, 2011, Wallace and Fraser, 2004, Jackson et al., 2003, Coons et al., 1990 |
| DL-HAVA | $C_5H_{13}N_3O_2$ | Competitive | ↓Putrescine | NA | Peter McCann – |

| | | | | | |
|---|---|---|---|---|---|
| (DL-alpha-Hydrazino-delta-aminovaleric acid) | | Inhibitor of ODC. | | | 2012, Kato,1978, Kato et al., 1976, Harik et al., 1974, http://www.chemspider.com/Chemical-Structure.149435.html?rid=78f1eae2-c6b4-44e7-aaa8-5d11abd2770e |
| MO (alpha-methylornithine) | $C_6H_{14}N_2O_2$ | Competitive Inhibitor of ODC. | ↓↓Putrescine, ↓Spermidine ↔Spermine | Inhibitory properties with cell proliferation. | Mamont et al., 1976, http://www.scbt.com/datasheet-291873.html |
| POB (N-(4′-Pyridoxyl)-Ornithine(BOC)-OMe, BOC-protected pyridoxyl-ornithine conjugate) | $C_{19}H_{31}N_3O_6$ | Coenzyme substrate analogue precursor inhibitor of ODC. | NA | NA | Wu et al., 2007, http://www.millipore.com/catalogue/item/497985-10MG&cid=BIOS-A-LINS-1219-1204-RC |

*Adapted from Wallace and Fraser, 2004

### *3.3.3 S-adenosylmethionine decarboxylase (SAMDC) Inhibitors*

S-adenosylmethionine decarboxylase (SAMDC) is another tightly regulated enzyme with a quick turn over rate. It is responsible for the catalysis of S-Adenosylmethionine into Decarboxylated S-Adenosylmethionine. In 1898, Thiele and Dralle synthesized Methylglyoxal bis(gua nylhydrazone) (MGBG) (Hoff, 1994). MGBG has similar structure to that of spermidine, and is thus a competitive inhibitor of SAMDC. It depletes the cells of spermidine and spermine, but induces the cells to accumulate high levels of putrescine (Wallace and Fraser, 2004). Studies have shown that MGBG enters the cells via the polyamine transport system. MGBG functions by interfering with uracil

incorporation. And, after a 3-hour lag period, DNA synthesis is inhibited. MGBG inhibits not only nucleic acid synthesis, but also polyamine synthesis (Bachrach et al., 1979). The problem with SAMDC inhibitors is that they do not inhibit putrescine synthesis and result in incomplete polyamine depletion. When administered in combination DFMO, MGBG induces spermidine and spermine depletion, and is taken up more by the cell. DFMO and MGBG have been shown to work synergistically to counteract "childhood leukaemia and P388 leukaemia in mice (Agostinelli et al., 2010). Since DFMO depletes putrescine and spermidine, while MGBG depletes spermidine and spermine; MGBG seemed promising. Furhermore, MGBG inhibits leishmania Adomet DC activity. The addition of spermidine and spermine reverses the growth inhibition triggered by MGBG (Mukhopadhyay and Madhubala, 1995). After clinical trials, MGBG was discontinued due to the side effects of mucositis and other toxicities (Hoff, 1994). More recent studies show that MGBG causes ultrastructural damage to mitochondria associated with metabolic impairment (Agostinelli et al., 2010).

EGBG, a cogener of MGBG, decreases spermine levels. In response to that, ODC activity and putrescine levels increase (Sjöholm et al., 1994). EGBG has a stronger effect than MGBG on most SAMDCs except for trypanosomal SAMDCs. MGBG has a stronger effect on trypanosomal SAMDC than EGBG does (Tekwani et al., 1992). Another SAMDC inhibitor, SAM 486A is 200 times more active than its parent analogue, MGBG. Like MGBG, SAM486A functions by inhibiting SAMDC and subsequently depleting spermidine and spermine intracellular levels, while increasing putrescine intracellular

levels (Eskens et al., 2000). CGP48664 (SAM 364A) is responsible for depleting L1210 cells (mouse lymphocytic leukemia cells derived from ascitic fluid of 8-month-old female mice) of spermidine and spermine, while increasing intracellular putrescine levels (Svensson et al., 1997). CPG 40215A, a more potent inhibitor than Berenil and MGBG, was shown to inhibit leishmania promastigote growth in dose dependent manner. CPG 40215A is responsible for an accumulation of intracellular putrescine and a decrease in intracellular spermidine (Mukhopadhyay et al., 1996). AbeADO (MDL 73811), another irreversible SAMDC inhibitor, is effective at eliminating mice *T. brucei* infections and inhibits the growth of *L. donovani* promastigotes. Roberts et al showed that AbeADO specifically inhibits *T. brucei* intracellular SAMDC. In contrast, other inhibitors (pentamidine, berenil, and MGBG) may have alternative or additional drug targets, making them more toxic to the host (Roberts et al., 2002). Althourgh MGBG was initially used extensively for the treatment of trypanosomes, the development of more potent and specific SAMDC inhibitors with less host toxicity suggests that AbeADO and some of the CPGs are superior for the treatment of trypanosome infections.

**Table 3.3-3 SAMDC Inhibitors**

| SAMDC Inhibitors | Molecular Formula | Mechanism | Effects on Polyamine Metabolism | Applications | References |
|---|---|---|---|---|---|
| AdoDATO (S-adenosyl-1,8-diamino-3-thiooctane) | $C_{18}H_{29}N_7O_3S$ | Competitively inhibits SAMDC.<br><br>*Also inhibits Spermidine Synthase. | ↑Putrescine<br>↑Spermidine<br>↓Spermine | NA | Holm et al., 1989, Birkholtz et al., 2011 |
| AdoMac (S-[5'-deoxy-5'-adenosyl]-1-ammonio-4-[methylsulfonio]-2-cyclopentene) | $C_{16}H_{26}N_6O_{12}S_3$ | Enzyme-activated, irreversible inhibitor of SAMDC. | ↔Putrescine<br>↔Spermidine<br>↔Spermine | Inhibitory properties with *E. coli* SAMDC. | Wu and Woster, 1992, Wu and Woster, 1993, http://www.guidechem.com/cas-142/142697-76-5.html |
| AMA (S-[5'-deoxy-5'-adenosyl]methylthioethylhydroxylamine) | $C_{13}H_{20}N_6O_3S$ | Active Site Directed-Irreversible inhibitor of SAMDC. | ↑Putrescine<br>↓Spermidine<br>↓Spermine | NA | L et al., 1989, Marton and Pegg, 1995, http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=134872 |
| APA (1-aminooxy-3-aminopropane) | $C_3H_{10}N_2O$ | Inhibits SAMDC.<br><br>*Competitive Reversible Inhibitor of ODC.<br><br>*Also inhibits ODC and SpdSyn. | ↓ ODC activity<br>↓Putrescine<br>↓Spermidine | Growth inhibition of Leishmania promastigotes in vitro and amastigotes in macrophage model. | Khomutov et al., 1985, Singh et al., 2007, Heby et al., 2007, http://www.chemspider.com/Chemical-Structure.58535.html |
| AP-APA (1-aminooxy-3-N-[3-aminopropyl]-aminopropane) | $C_6H_{17}N_3O$ | Inhibits SAMDC.<br><br>*Competitive inhibitor of ODC.<br><br>*Also inhibits Spd Syn. | NA | NA | Eloranta et al., 1990, http://www.brenda-enzymes.org/php/ligand_flatfile.php4?brenda_ligand_id=240999 |
| AOE-PU (N-[2[aminooxyethyl]-1,4-diaminobutane) | $C_6H_{17}N_3O$ | Inhibits SAMDC.<br><br>*Competitive | NA | NA | Eloranta et al., 1990, http://www.brenda- |

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| | | Inhibitor of Spermine Synthase.<br><br>*Also inhibits ODC. | | | enzymes.org/php/ligand_flatfile.php4?brenda_ligand_id=241000 |
| CGP39937 ([2,2-bipyridine]-6'6'-dicarboximidamide) | $C_{12}H_{12}N_6$ | Competitive Specific Inhibitor of SAMDC. | ↑ODC activity | NA | Stanek et al., 1993, Regenass et al., 1994 http://www.chemspider.com/Chemical-Structure.8258541.html |
| CGP40215, CGP40215A, (N",N'"-bis[(1E)-[3-(aminoiminomethyl)phenyl]methylene]) | $C_{17}H_{19}N_9$ | Inhibitor of SAMDC. | ↑Putrescine<br>↓Spermidine | Inhibitory properties with the growth of leishmania promastigotes, effectively cures acute laboratory infections of *T. b. brucei*, *T. b. rhodesiense, T. b. gambiense,* and *T. congolense.* | Mukhopadhyay et al., 1996, Bacchi and Yarlett, 2002, http://www.chemspider.com/Chemical-Structure.7851239 |
| CGP48664 (SAM 364A) (4 amidinoindanon-1-[2'amidino]hydrazone) | $C_{11}H_{14}N_6$ | Competitive, Specific inhibitor of SAMDC.<br><br>*Does not seem to utilize the polyamine transport carrier system since it competes poorly with spermidine for uptake into L1210 cells. | ↑Putrescine<br>↓Spermidine<br>↓Spermine<br>↑ODC activity | NA | Regenass et al., 1994, Svensson et al., 1997, http://www.medkoo.com/Anticancer-trials/Sardomozide.htm |
| Genz-644131 (8-Methyl-5′-{[(Z)-4-Aminobut-2-enyl]-(Methylamino)} Adenosine) | $C_{16}H_{25}N_7O_3$ | Inhibitor of SAMDC.<br><br>*Analogue of MDL 73811. | NA | Inhibitory properties with *T. brucei.* | Barker et al., 2009, Bacchi et al., 2009 http://brenda-enzymes.org/php/ligand_flatfile.php4?brenda_ligand_id=292151 |

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|---|---|---|---|---|---|
| EGBG (ethylglyoxal bis[guanylhydrazone]) | $C_6H_{14}N_8$ | Competitive, Specific inhibitor of SAMDC. | ↑Putrescine ↓Spermine ↑ODC activity | Inhibitory properties with *T. brucei* SAMDC. | Sjöholm et al., 1994, Tekwani et al., 1992, http://www.chemicalbook.com/ChemicalProductProperty_EN_CB11390561.htm |
| DEGBG (diethylglyoxal bis(guanylhydrazone)) | $C_8H_{18}N_8$ | Competitive inhibitor of SAMDC. | ↑Putrescine | NA | Elo et al., 1988, Marton and Pegg, 1995, http://www.chemblink.com/moreProducts/more116173-27-4.htm |
| MAOEA (5'-deoxy-5'-{N-methyl-N-[2-(aminooxy)ethyl]amino}adenosine) | $C_{13}H_{21}N_7O_4$ | Active Site-directed irreversible inhibitor of SAMDC. | ↑Putrescine, ↓Spermidine ↓Spermine | NA | Pegg, 1988, Wallace and Fraser, 2004, http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=3081018 |
| MGBG (methylglyoxal bis[guanylhydrazone]) | $C_5H_{12}N_8$ | Competitive, Reversible inhibitor of SAMDC. | ↑Putrescine, ↓Spermidine ↑ ODC activity and SAMDC activity | Inhibitory properties with Leishmania. | Porter et al., 1980, Tekwani et al., 1992, Mukhopadhyay and Madhubala, 1995 |

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| MDL 73811 (AbeAdo) (5'-{(Z)-4-amino-2-butenyl]methylamino | $C_{15}H_{23}N_7O_3$ | Irreversible inhibitor of SAMDC. | ↑Putrescine, ↓Spermidine ↓Spermine | Inhibitory properties with *L. donovani* promastigote growth, decrease *T. cruzi* ability to infect and proliferate, treatment for mice with *T. brucei* infection, arrest *P. falciparum* growth in vitro. | L et al., 1993, Barker et al., 2009, Birkholtz et al., 2011, http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=6474401 |
| MHZEA (5'-deoxy-5'-[(2-hydrazinoethyl)-methylamino] adenosine) | $C_{13}H_{22}N_8O_3$ | Active Site-directed irreversible inhibitor of SAMDC. | ↑Putrescine, ↓Spermidine ↓Spermine | Inhibitory properties with Trypanosome SAMDC. | Pegg, 1988, Tekwani et al., 1992 Wallace and Fraser, 2004, http://www.lookchem.com/cas-144/144224-28-2.html |
| MHZPA (5'-deoxy-5'-[N-methyl]-N-[(3-hydrazinopropl yl)amino]adenosine) | $C_{14}H_{24}N_8O_3$ | Active Site-directed irreversible inhibitor SAMDC. | ↑Putrescine, ↓Spermidine ↓Spermine | NA | Pegg, 1988, Marton and Pegg, 1995, https://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=596146 |
| Pentamidine (p-p'-[pentamethylenedioxy] dibenzamidine) | $C_{19}H_{24}N_4O_2$ | Competitive reversible inhibitor of SAMDC<br><br>*Also inhibits SSAT and PAO | ↓Putrescine, ↔Spermidine, ↓ODC activity | To treat Leishmania, *Pueumocystis carinii* pneumonia and *T. brucei*<br><br>*Competitively inhibits arginine transport and non-competitively inhibits putrescine and spermidine | Libby and Porter, 1992, Basselin et al., 2002, http://www.chemspider.com/Chemical-Structure.4573.html?rid=60ae2071-6927-4c10-9a2f-4e04a889eb51 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | transport in *Leishmania infantum L. donovani,* and *L. mexicana.* | |

*Adapted from Wallace and Fraser, 2004

### *3.3.4 Spermidine/spermine-$N^1$ –acetyltransferase (SSAT) Inhibitors*

Spermidine/spermine-$N^1$ –acetyltransferase (SSAT) is responsible for the catalysis of spermidine into $N^1$-acetylspermidine and spermine into $N^1$-acetylspermine. Spermidine/Spermine N1 Acetyltranferase (SSAT) functions by adding acetyl groups to the aminopropyl ends of spermidine and spermine. SSAT has substrate specificity for "N1-acetylspermine, sym-norspermine, and sym-norspermidine", but not putrescine, N1-acetylspermidine, and sym-homospermidine. Although the Km value for spermine is lower than the Km value for N1-acetylspermine, in vivo SSAT more readily acetylates N1-acetylspermidine than spermine. SSAT levels change in response to polyamine levels in order to maintain polyamine homeostasis (Pegg, 2008).

SSAT has no specific inhibitors, but it has been demonstrated that berenil and pentamidine (aromatic diamines) inhibit it (Wallace and Fraser, 2004). Both of these pharmaceuticals inhibit SAMDC, noncompetitively inhibit arginine/polyamine uptake, and replace spermidine from nucleic acids (Reguera et al., 2005). Berenil treatments increase polyamine levels, especially spermine (Libby and Porter, 1992).

Unfortunately, *L. donovani* are not readily treated with SSAT inhibitors. Studies have proved that *L. donovani* does not have the complete polyamine back conversion

pathways. The addition of exogenous spermine did not rescue null mutant parasites demonstrating that spermine cannot be converted back into spermidine (Müller et al., 2001). This means that *L. donovani* most likely lacks SSAT or PAO. The fact that *L. donovani* lacks one of these enzymes may be an anomaly, since N-acetylated spermidine and spermine N-acetyltransferase activity have been reported in Leishmania (Rojas-Chaves et al., 1996, Rojas-Chaves et al., 1996). Since SSAT is present in other trypanosomes, SSAT inhibition may be an effective mode of treatment for other trypanosome infections. Still, there remain no specific inhibitors of SSAT and the development of novel SSAT inhibitors may prove useful.

**Table 3.3-4 SSAT Inhibitors**

| SSAT Inhibitors | Molecular Formula | Mechanism | Effects on Polyamine Metabolism | Applications | References |
|---|---|---|---|---|---|
| Berenil (N-Acetylglycine - 4,4'-[(1E)-1-triazene-1,3-diyl]dibenzenecarboximidamide) | $C_{18}H_{22}N_8O_3$ | Competitive Inhibitor of Spermidine Acetylation. | ↑Polyamine accumulation, esp. ↑↑↑spermine | Inhibitory properties with Leishmania. | Libby and Porter, 1992, Mukhopadhyay and Madhubala, 1995, http://www.chemspider.com/Chemical-Structure.58571.html |
| Pentamidine (p-p'-[pentamethylene dioxy] dibenzamidine) | $C_{19}H_{24}N_4O_2$ | Inhibits SSAT.<br>*Competitive reversible inhibitor of SAMDC.<br>*Also inhibits PAO. | ↓Putrescine, ↔Spermidine ↓ODC activity | To treat Leishmania, *Pueumocystis carinii* pneumonia and *T. brucei.*<br>*Competitively inhibits arginine transport and a non- competitively inhibits putrescine and spermidine transport in *Leishmania infantum L. donovani,* and *L. mexicana.* | Libby and Porter, 1992, Basselin et al., 2002, http://www.chemspider.com/Chemical-Structure.4573.html?rid=60ae2071-6927-4c10-9a2f-4e04a889eb51 |

* Adapted from Wallace and Fraser, 2004

*3.3.5 Spermidine/Spermine Synthase (Spd/Spm Syn) Inhibitors*

Spermidine synthase is responsisble for the catalysis of putrescine into spermidine and spermine synthase is responsible for the catalysis of spermidine into spermine.

Since *T. brucei* lacks the capability to take up spermidine, and must utilize its own spermidine synthase to synthesize spermidine, spermidine synthase make an ideal pharmaceutical target. In studies where *T. brucei* spermidine synthase was downregulated

using tetracycline-inducible RNAi, intracellular spermidine levels decreased and parasite growth stopped (Taylor et al., 2008).

In 1989, Holm and Pegg conducted the first wave of studies on spermidine synthase inhibitors: S-adenosyl-1, 8-diamino 3-thiooctane (AdoDATO), S-methyl-5'-methylthioadenosine (MMTA) and S-adenosyl-1,12-diamino-3 thio-9-azadodecane (AdoDATAD) (I et al., 1989, Pegg et al., 1989). AdoDATO treatments cause spermidine levels to decrease, while putrescine and spermine levels increase. AdoDATO has an inhibitory effect on trypanosome spermidine synthase (Bitonti et al., 1984). MMTA treatments lead to an increase in spermidine and a decrease in spermine (I et al., 1989). AdoDATAD treatments result in a decrease in the synthesis of spermine followed by a compensatory increase in spermidine synthesis (Pegg et al., 1989). Since most of these in inhibitors have compensatory increases in the other polyamines, they would not completely obliterate polyamines and are thus not effective at treating trypanosome infection.

Beppu et al was involved in conducting the second wave of studies on spermidine synthase inhibitors: trans-4-methylcyclohexylamine (4 MCHA) and N-[3 aminopropyl] cyclohexylamine (APCHA) (Beppu et al., 1995). 4MCHA or APCHA treated HTC tumor cells had a decrease in spermidine or spermine (respectively) followed by a compensatory increase in putrescine (APCHA) (Beppu et al., 1995). APA, a potent ODC inhibitor, also inhibits SpdSyn and is a potent leishmaniacidal therapeutic. It is

responsible for a decrease in putrescine and spermidine levels (Singh et al., 2007). DCHA, another spermidine synthase inhibitor, had similar effects on 9L cells (rat brain tumor cells), except spermine levels remained the same (Feuerstein et al., 1985). This compensatory increase in putrescine makes these drugs less than ideal treatments for trypanosomes. However, perhaps if paired with an ODC inhibitor, such as DFMO, they would be effective at treating trypanosome infections.

Furthermore, in studies observing the effects of spermidine synthase inhibitors on trypanosome spermidine synthase, DCHA (3 uM yields 50% inhibition) is a much more potent inhibitor of trypansosomal spermidine synthase than AdoDATO (20 uM yields 50% inhibition). Unfortunately, the authors have reason to believe that DCHA is not effectively absorbed by the parasite. They conclude that there was no difference in trypanosome putrescine and spermidine levels in DCHA-treated mice in comparison to those of parasites from untreated mice. They also note that there was also no difference in the progression of the infection or how long the mice lived (Bitonti et al., 1984). If DCHA is not being taken up by the parasites, its potency does not matter. This reminds us of the many factors (i.e. potency, toxicity, parasite uptake) that must be considered, when selecting a polyamine synthesis inhibitor to treat a trypanosomal infection. SpdSynthase inhibitors (ie. APA), that also inhibit ODC or other enzymes earlier in the pathway, prove to be more effective treatments against trypanosomal infections, because they also inhibit putrescine synthesis.

**Table 3.3-5 Spd/Spm Syn Inhibitors**

| Spermidine Synthase Inhibitors | Molecular Formula | Mechanism | Effects on Polyamine Metabolism | Applications | References |
|---|---|---|---|---|---|
| AdoDATO (S-adenosyl-1,8-diamino-3-thiooctane) | $C_{18}H_{29}N_7O_3S$ | Inhibitor of Spd Syn.<br>*Also competitively inhibits SAMDC. | ↑Putrescine<br>↑Spermidine<br>↓Spermine | NA | Holm et al., 1989, Birkholtz et al., 2011 |
| AdoDATAD (S-adenosyl-1,12-diamin0-3-thio-9-aza-dodecane) | $C_{21}H_{38}N_8O_3S$ | Inhibitor of Spm Syn. | ↓Putrescine<br>↑Spermidine<br>↓Spermine | NA | Pegg et al., 1989, http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=50999529 |
| AOE-PU (N-[2[aminooxyethyl]-1,4-diaminobutane) | $C_6H_{17}N_3O$ | Competitive Inhibitor of Spm Syn.<br>*Also inhibits ODC and Spd Syn. | NA | NA | Eloranta et al., 1990, http://www.brenda-enzymes.org/php/ligand_flatfile.php4?brenda_ligand_id=241000 |
| APA (1-aminooxy-3-aminopropane) | $C_3H_{10}N_2O$ | Inhibits Spd Syn.<br>*Competitive inhibitor of ODC.<br>*Also inhibits SAMDC | ↓ ODC activity,<br>↓Putrescine<br>↓Spermidine | Growth inhibitory properties with *Leishmania donovani.* | Khomutov et al., 1985, Singh et al., 2007, http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=65020 |
| AP-APA (1-aminooxy-3-N-[3-aminopropyl]-aminopropane) | $C_6H_{17}N_3O$ | Inhibits Spd Syn.<br>*Competitive inhibitor of ODC.<br>*Also inhibits SAMDC. | NA | NA | Eloranta et al., 1990, http://www.brenda-enzymes.org/php/ligand_flatfile.php4?brenda_ligand_id=240999 |

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|---|---|---|---|---|---|
| APCHA (N-[3-aminopropyl]cyclohexylamine]) | $C_9H_{20}N_2$ | Competitive inhibitor of Spd and Spm Syn. | ↑Putrescine ↓Spermidine ↑Spermine ↑SAMDC activity | NA | Beppu et al., 1995, http://www.scbt.com/datasheet-202715-n-3-aminopropylcyclohexylamine.html |
| BDAP (N-(n-butyl)-1,3-diaminopropane) | $C_7H_{18}N_2$ | Inhibitor of Spm Syn. | ↓Putrescine ↑Spermidine ↓Spermine | NA | Marton and Pegg, 1995, http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=95733 |
| Cyclohexylamine | $C_6H_{13}N$ | Inhibitor of Spd Syn. | NA | NA | Marton and Pegg, 1995, http://www.chemspider.com/Chemical-Structure.7677.html |
| DCHA (dicyclohexylamine sulfate) | $C_{12}H_{25}NO_4S$ | Inhibitor of Spd Syn. | ↑Putrescine ↓Spermidine ↔Spermine | Inhibitory properties with *P. falciparum* cell growth in vitro (dicyclohexylamine was used). | Ito et al., 1982, Feuerstein et al., 1985, Bitonti et al., 1984, Birkholtz et al., 2011, http://www.chemicalbook.com/ChemicalProductProperty_EN_CB6332688.htm |
| 4 MCHA (*trans*-4-methylcyclohexylamine) | $CH_3C_6H_{10}NH_2$ | Inhibitor of Spd and Spm Syn. | ↑Putrescine ↓Spermidine ↑Spermine ↑SAMDC activity | Inhibitory properties with *P. falciparum* cell growth in vitro. | Beppu et al., 1995, Birkholtz et al., 2011, http://www.sigmaaldrich.com/catalog/product/aldrich/177466?lang=en&region=US |

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|---|---|---|---|---|---|
| MMTA (S-methyl-5'-methylthioadenosine) | $C_{12}H_{18}N_5O_3S^+$ | Inhibitor of Spm Syn. | ↑Putrescine ↑Spermidine ↓Spermine ↑SAMDC activity | NA | Pegg et al., 1989, I et al., 1989 http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=167321 |
| n-butylamine | $C_4H_{11}N$ | Inhibitor of Spd Syn. *May also inhibit ODC | NA | NA | Marton and Pegg, 1995, Roberts et al., 2007, http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=8007 |

*Adapted from Wallace and Fraser, 2004

### *3.3.6 Polyamine Oxidase (PAO) Inhibitors*

Polyamine oxidase is responsible for the catalysis of $N^1$ Acetylspermidine into putrescine and $N^1$ Acetylspermine into spermidine. A few polyamine oxidase inhibitors include MDL 72521, MDL 72527, and MDL73811. These irreversible inhibitors of polyamine oxidase result in the synthesis of more putrescine and/or spermidine. Treating Leishmania and other Trypanosoma infections with polyamine oxidase inhibitors would most likely exacerbate the infections by increasing parasite survivability and proliferation. Thus PAO inhibitors are not effective treatments for trypanosome infections.

**Table 3.3-6 PAO Inhibitors**

| Polyamine Oxidase Inhibitors | Molecular Formula | Mechanism | Effects on Polyamine Metabolism | Applications | References |
|---|---|---|---|---|---|
| MDL 72521 ($N^1$-Methyl-$N^2$-(2,3-butadienyl)-1,4-butanediamine) | $C_9H_{20}Cl_2N_2$ | Specific, potent, enzyme-activated, irreversible inhibitor of polyamine oxidase. | ↓↓Putrescine ↓Spermidine | N A | Bolkenius et al., 1985, http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=135104195&viewopt=PubChem |
| MDL 72527 (N1,N4-bis(2,3-butadienyl)-1,4-butanediamine) (CPC-200) | $C_{12}H_{20}N_2 \cdot 2HCl$ | Specific, potent, enzyme-activated, irreversible inhibitor of polyamine oxidase. | ↓↓Putrescine ↓Spermidine | NA | Bolkenius et al., 1985, http://www.sigmaaldrich.com/catalog/product/sigma/m2949?lang=en&region=US |
| $N^1$OSSpm ($N^1$-[n-octanesulphonyl]spermine) | $C_{18}H_{42}N_4O_2S$ | Potent inhibitor of SMO. *Vujcic et al does not mention mechanism. | NA | NA | Vujcic et al., 2002, http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=136352258&viewopt=PubChem |

*Adapted from Wallace and Fraser, 2004

### *3.3.7 Polyamine Analogues (Mimicry)*

There are also polyamine analogues, which resemble spermidine and spermine structures, but lack their functions. Polyamine analogues were synthesized in succession of three generations: the bis (ethyl) polyamines, the unsymmetrically substituted alkylpolyamines, and the last group (consisting of the conformationally restricted, cyclic and long-chain oligoamine analogues) (Wallace and Niiranen, 2007). They can also be separated into two categories, depending how they alter or fail to alter polyamine levels. The first category, antimetabolites, function by being uptaken by the cell as a polyamine analogue,

inducing a highly active catabolism, and exporting natural polyamines. The second category, the mimetics, functon by displacing "natural polyamines at intracellular sites so causing cytotoxicity," but do not alter polyamine levels (Wallace and Fraser, 2004). Several symmetrically substituted analogues (include bis (ethyl) polyamine analogues)) enter the cell using the natural polyamine uptake system and induce the dowregulation of ODC and SAMDC, resulting in a decreased rate of polyamine synthesis. Yet, they differ from natural polyamines in the fact that they cannot take their place functionally in cell growth and survival (Woster, 2006). Common bis (ethyl) polyamine analogues include bis(ethyl)norspermine (BENSpm), bis(ethyl) homosperimine (BEHSpm), and bis(ethyl)spermine(BESpm) (Wallace and Fraser, 2004)." The second generation of polyamine analogues, the unsymmetrically substituted alkylpolyamine analogues, were derived from the polyamine backbone structures of some of the bis (ethyl) polyamine analogues (Woster, 2006). They were designed by adding alkyl groups to the C-terminus and a substituted group to the N-terminus of a spermine analogue (Wallace and Niiranen, 2007). Common unsymmetrically substituted alkylpolyamine analogues include CBENSpm (N1-ethyl-N11-(cyclobu- tyl)methyl-4,8-diazaundecane), CHENSpm (N1-ethyl-N11-((cycloheptyl)methyl)-4,8-diazaundecane), and PENSpm (N1-ethyl-N11-propargyl). 228Interestingly, Bellevue discovered that 3-7-3 analogues have anti-trypanosomal activity, but little anti-cancer activity; while 3-3-3 have anti-proliferative activity and little anti-trypanosomal activity. 3-3-3 analogues include PENSpm, CPENSpm, CHENSpm, and MDL 2697, while 3-7-3 analogues include CHE-3-7-3 and

bis-CH-3-7-3 (Bellevue III et al., 1996). Yet, no explanation is provided for why 3-7-3 analogues inhibit trypanosomal activity more effectively than 3-3-3 analogues.

Conformationally restricted analogues are designed by restricting the rotation of the central region of the polyamine chain in BESpm, and include conformational restriction of "cis-and trans-cyclopropyl or cyclobutyl ring, a cis-and trans-double bond, a triple bond, and a 1,2-disubstituted aromatic ring (Woster, 2006)." Oligamine analogs are chains with NH2+ residues separated by CH2 residues. Trans-oligamines (trans-octamine SL-11158, trans-decamine SL-11144, trans-docecamine SL-11150) had trans-unsaturation introduced at the center, while Cis oligamines (cis octamine SL-11157, cis-decamine SL-11150) had cis-unsaturation. Their cytotoxicity is correlated to their ability to induce DNA aggregation (Valasinas et al., 2003). So far five budmunchiamines (also known as macrocyclic polyamines) have been designed. They are imported by cells and have the ability to selectively deplete polyamine adenosine triphosphate resulting in polyamine depletion (Woster, 2006).

When rat liver HTC cells (hepatocarcinoma cells) are treated with some polyamine analogs (bisethylnorspermine (BENSpm), bisethylhomospermine (BEHSpm), 1,19-bis-(ethylamino)-5,10,15-triazanonadecane (BE-4444), "longer analogues and many conformationally constrained analogues of these compounds"), they are induced to synthesize antizyme at different levels, often producing more antizyme than if they had been induced by spermine. Results revealed that long oligamines were able to induce

antizyme at higher levels than spermine was able to. Other analogues with conformational restrictions, such as "three-, four and five-membered rings or triple bonds in the carbons between the central nitrogen" were not so successful at inducing antizyme. Their levels of induction were comparable to that spermine or lower. Here the authors propose that polyamine analogues may alter antizyme not at the transcriptional level (it is thought to be expressed constituitively), but at the point where it undergoes +1 translational frameshift (Mitchell et al., 2002). Thus, this is the mechanism by which these inhibitors most likely inhibit putrescine synthesis and subsequently polyamine synthesis. They also discuss oligamine cytoxicity and explain that cytotoxicity may not be induced by polyamine depletion, but rather the oligamine having an unknown secondary site of action. Other studies have shown that antizyme may be involved in inducing the degradation of proteins needed for the cell cycle (Mitchell et al., 2002). There still remain mechanisms to discover, to elucidate how these inhibitors work.

**Table 3.3-7a Bis(ethyl) Polyamine Analogues (Symmetrically Substituted Analogues) (First Generation)**

| Bis(ethyl) polyamine analogues (Symmetrically substituted analogues) | Molecular Formula | Effects on Polyamine Metabolism | Applications | References |
|---|---|---|---|---|
| BEHSpm ($N^1$, $N^{14}$ -bis(ethyl)-homospermine) | $C_{16}H_{38}N_4$ | ↑antizyme activity<br>↓ODC activity<br>↓SAMDC<br>↓Putrescine<br>↓Spermidine<br>↓Spermine | NA | Mitchell et al., 2002, Woster, 2006, https://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=60702 |
| BENSpm ($N^1$, $N^{11}$-bis(ethyl)norspermine) (aka. DENSpm) | $C_{13}H_{32}N_4$ | ↑↑SSAT activity<br>↑antizyme activity<br>↓ODC activity<br>↓SAMDC<br>↓Putrescine<br>↓Spermidine<br>↓Spermine<br>↑Polyamine excretion | Readily imported by Trichomonads and possess inhibitory properties with Trichinomad SSAT. | Jr et al., 1995, Bernacki et al., 1995, Fogel-Petrovic et al., 1997, Mitchell et al., 2002, Bacchi and Yarlett, 2002, Woster, 2006, https://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=4282&loc=ec_rcs |
| BESpm (N1, N12-bis(ethyl) spermine) | $C_{14}H_{34}N_4$ | ↑SSAT activity<br>↓SAMDC activity<br>↓ODC activity<br>↓Putrescine<br>↓Spermidine<br>↓Spermine | NA | Ghoda et al., 1992 Shappell et al., 1992, Woster, 2006, https://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=4283&loc=ec_rcs |
| BE-444 (N1,N14-bis(ethyl)homospermine) | $C_{16}H_{38}N_4$ | ↓SAMDC activity<br>↑antizyme activity<br>↓ODC activity<br>↓Putrescine<br>↓Spermidine<br>↓Spermine | Likely to have little anti-trypanosomal activity.<br><br>* 3-3-3 analogues have antiproliferative properties in relation to cancer | Ghoda et al., 1992, Mitchell et al., 2002,Woster, 2006, https://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=60702 |

*Mechanism of action for Bis-ethyl polyamine analogues: They downregulate ODC and SAMDC (Woster, 2006).

**Table 3.3-7b Unsymmetrically Substituted Alkylpolyamine Analogues (Second Generation)**

| Unsymmetrically substituted alkylpolyamine analogues | Molecular Formula | Effects on Polyamine Metabolism | Applications | References |
|---|---|---|---|---|
| CBENSpm (N1-ethyl-N11-(cyclobu-tyl)methyl-4,8-diazaundecane) | $C_{16}H_{36}N_4$ | ↑SSAT activity ↓ODC activity | Likely to have little anti-trypanosomal activity.<br>* 3-3-3 analogues have antiproliferative properties in relation to cancer | Jr et al., 1995, Woster, 2006, http://www.chemspider.com/Chemical-Structure.9953331.html?rid=8c9cc2d5-28ca-44c6-b53b-e9d2c1889109 |
| CHENSpm (N1-ethyl-N11-((cycloheptyl)methyl)-4,8-diazaundecane) | $C_{15}H_{38}Br_4N_4$ | Weak ↑SSAT activity | Little anti-trypanosomal activity.<br>* 3-3-3 analogues have antiproliferative properties in relation to cancer | Nairn et al., 2000, Woster, 2006, https://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=127816 |
| CHEXENSpm,25 N-(Cyclohexylmethyl)-N'-(3-{[3-(ethylamino)propyl]amino}propyl)-1,3-propanediamine | $C_{18}H_{40}N_4$ | Likely to ↑SSAT | Likely to have little anti-trypanosomal activity.<br>* 3-3-3 analogues have antiproliferative properties in relation to cancer | Woster, 2006, http://www.chemspider.com/Chemical-Structure.8239677.html |
| CHE-3-7-3 (N-{3-[(Cycloheptylmethyl)amino]propyl}-N'-[3-(ethylamino)propyl]-1,7-heptanediamine) | $C_{23}H_{50}N_4$ | ↑SSAT activity ↓ODC activity | Anti-trypanosomal activity.<br>* 3-7-3 analogues have little in relation to cancer | Casero and Woster, 2000, http://www.chemspider.com/Chemical-Structure.23137350.html |
| CPENSpm (N1-ethyl-N11(cyclopropyl)-methyl-4,8-diazaundecane) | $C_{15}H_{38}Br_4N_4$ | ↑SSAT activity ↓ODC activity | Little anti-trypanosomal activity.<br>* 3-3-3 analogues have antiproliferative properties in relation to cancer | Jr et al., 1995, Nairn et al., 2000, Woster, 2006, Casero and Woster, 2009, https://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=127816 |

| | | | | |
|---|---|---|---|---|
| CPENTSpm | $C_{17}H_{38}N_4$ | ↑SSAT activity | Likely to have little anti-trypanosomal activity.<br><br>* 3-3-3 analogues have antiproliferative properties in relation to cancer. | Woster, 2006, https://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=16048115 |
| IPENSpm (*S*)-$N^1$-(2-methyl-1-butyl)-$N^{11}$-ethyl-4,8-diazaundecane | $C_{16}H_{38}N_4$ | ↓Putrescine<br>↓Spermidine<br>↓Spermine<br>↑SSAT activity<br>↑Polyamine export | Likely to have little anti-trypanosomal activity.<br><br>* 3-3-3 analogues have antiproliferative properties in relation to cancer. | Fraser et al., 2002,Woster, 2006, http://www.brenda-enzymes.info/php/ligand_flatfile.php4?brenda_ligand_id=213049 |
| PENSpm ( N1-ethyl-N11-propargyl - 4,8-diazaundecane) | $C_{14}H_{34}Br_4N_4$ | ↑SSAT activity<br>↓ODC activity | Little anti-trypanosomal activity.<br><br>* 3-3-3 analogues have antiproliferative properties in relation to cancer. | Jr et al., 1995, Woster, 2006, Casero and Woster, 2009, https://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=134000 |

*Mechanism of action for Unsymmetrically substituted alkylpolyamine analogues (Second Generation): They decrease ODC activity, while inducing SSAT (Wallace and Fraser, 2004, Woster, 2006).

**Table 3.3-7c Third Generation Polyamine Analogues**

| Third Generation | Molecular Formula | Mechanism | Effects on Polyamine Metabolism | Applications | References |
|---|---|---|---|---|---|
| Conformationally restricted analogs of BESpm ie. PG-11047 (SL-11047, CGC-11047), ($N^1$, $N^{12}$bis(ethyl)-6,7-dehydrospermine tetrahydrochloride) | $C_{14}H_{36}Cl_4N_4$ | Thought to compete with polyamines and thus inhibit pro-liferation/other cellular functions. | ↓Putrescine<br>↓Spermidine<br>↓Spermine | Treatment of *Cryptosporidium parvum* infections in mice model. | Woster, 2006, Kuo et al., 2009, https://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=9822383 |
| Oligamines ie. SL11144 (CGC-11144) | $C_{40}H_{90}N_{10}$ | Aggregate DNA. | Modest<br>↓Putrescine<br>↓Spermidine<br>↓Spermine | Reduce spermine uptake and interconversion *Encephalitozoon cuniculi.* | Valasinas et al., 2003, Bacchi et al., 2004, Woster, 2006, http://www.brenda-enzymes.info/php/ligand_flatfile.php4?brenda_ligand_id=213061 |
| Macrocyclic polyamines (Budmunchiamines) ie. N, N',N''-Tritosyldiethylenetriamine disodium salt | $C_{25}H_{29}N_3$ $Na_2O_6S_3$ | Selectively deplete polyamine adenosine triphosphate. | ↓Putrescine<br>↓Spermidine<br>↓Spermine | NA | Newkome et al., 1983, Woster, 2006, https://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=3338046&loc=ec_rcs |

# CHAPTER FOUR

## Host Pathogen Interactions

## 4.1 How Pathogens Exploit the Host to Synthesize More Resources for Their Growth and Survival

Arginine and polyamines are not only necessary for the growth and survival of the host, but also for the parasites to utilize as resources. Parasites utilize their own biosynthetic enzymes for these pathways or induce their host to synthesize them. If a parasite can use most of the available arginine; then not only is the parasite's growth fueled, but also the host's arginine source is limited decreasing its NO production and T cell proliferation.

### *4.1.1 Leishmania and Other Manipulative Pathogens*

An M2 environment may be more advantageous for the invader than for the host. When arginase activity increases within the host's cells, more ornithine and subsequently polyamines are synthesized supplying more energy to the invader. Since increased arginase activity is characteristic of an M2 response in macrophages, this brings into question whether an M2 response is more beneficial to the pathogen than the host. Traditionally M2 responses have been considered beneficial to the host due to the healing and repair processes that take place simultaneously. However, in many cases the pathogens appear to take advantage of macrophages in the M2 state. Ornithine and polyamines are also important sources of energy for invading pathogens. When the parasites' fuel production machinery is obliterated, its survivability and reproducibility decrease. For example, Leishmania lacking the arginase gene, cultured in vitro in the absence of any source of polyamines, have their survival and proliferation severely compromised (Muleme et al., 2009).

"*Mycobacterium*, *Trypanosoma*, *Helicobacter*, *Schistosoma*, and *Salmonella spp*." have been demonstrated to decrease host arginine availability mainly by upregulating host arginases (Stadelmann et al., 2012). This work will discuss the host/pathogen interactions of a number of these parasites, but will focus predominantly on those of trypanosomes, particularly Leishmania. According to the World Health Organization, there are approximately 1.3 million new leishmania cases per year and it is responsible for the death of 20,000-30,000 humans annually (http://www.who.int/mediacentre/factsheets/fs375/en/). Leishmania is an intracellular protozoan parasite, transmitted by the bite of vector (phlebotomine and lutzomyia) sandflies. Leishmania is capable of exhibiting a number of pathologies including visceral, cutaneous, and mucocutaneous forms (Santos et al., 2008). The pathology exhibited is dependent on the leishmania species and the host immune response. There are a number of different leishmania species, but *Leishmania donovani, Leishmania major, Leishmania mexicana*, and *Leishmania amzonensis* are the most studied species. *Leishmania donovani* and *Leishmania infantum* are the two main species responsible for viceral leishmania. Visceral leishmania affects predominantly the liver and spleen resulting in splenomegaly and eventually mortality (Santos et al., 2008). *Leishmania major* and *Leishmania tropica* are responsible for cutaneous leishmania (Balaña-Fouce et al., 1998). Cutaneous leishmania is manifested in skin ulcerations, especially on the face resulting in disfiguration (Santos et al., 2008). *Leishmania brazilensis, Leishmania panamensis*, and *Leishmania guyanensis* are responsible for mucocutaneous leishmania. Mucocutaneous leishmania starts in the cutaneous form and metastasizes to the mucosal areas of the nasal

and oral cavities. It can eventually damage "neighboring skin, oropharynx, pharynx, and even trachea (Balaña-Fouce et al., 1998)."

**Table 4.1-1 Forms of Leishmania**

| Forms of Leishmania | Pathologies of Leishmania | Species of Leishmania | Number of New Cases | Locations affected | References |
|---|---|---|---|---|---|
| Visceral Leishmania | Affects predominantly the liver and spleen resulting in splenomegaly and eventually mortality. | *Leishmania donovani, Leishmania infantum.* | 200,000 to 400, 000 | Bangladesh, Brazil Ethiopia, India, South Sudan, Sudan. | Santos et al., 2008, http://www.who.int/mediacentre/factsheets/fs375/en/ |
| Cutaneous Leishmania | Manifested in skin ulcerations, especially on the face resulting in disfiguration. | *Leishmania major, Leishmania tropica.* | 0.7 million to 1.3 million | Afghanistan, Algeria, Brazil, Colombia, Iran (Islamic Republic of) and the Syrian Arab Republic. | Balaña-Fouce et al., 1998, Santos et al., 2008, http://www.who.int/mediacentre/factsheets/fs375/en/ |
| Mucocutaneous Leishmania | Starts in the cutaneous form and metastasizes to the mucosal areas of the nasal and oral cavities. | *Leishmania brazilensis, Leishmania panamensis, Leishmania guyanensis.* | NA | Plurinational State of Bolivia, Brazil, Peru. | Balaña-Fouce et al., 1998, http://www.who.int/mediacentre/factsheets/fs375/en/ |

### *4.1.2 Changes in Host Systems in Response to the Pathogen*

Interestingly, macrophages can also utilize the parasite's arginase. Regarding the expenditure of arginase, it appears that in some cases the host and the parasite may manifest a symbiotic relationship. 96 hours post infection and beyond, BMDMs infected with arginase deficient *Leishmania major* show significantly lower arginase activity than those infected with WT *L. major*. Moreover, IL-4 treated BMDMs infected with arginase deficient *L. major* show significantly lower arginase activity than IL-4 treated BMDMs infected with WT *L. major* (Muleme et al., 2009). This means that during *L. major* infections, BMDMs are also utilizing the arginase that the parasite synthesizes. It is expected that when more arginase is available to bind to the arginine present in the macrophage, there is less arginine available for iNOS to bind to, leading to decreased iNOS activity and less NO production. However BMDMs, treated IFNγ/LPS and infected with arginase deficifient *L. major*, did not produce more NO than BMDMs treated with IFNγ/LPS and infected with WT. *L. major*. Griess assays revealed that culture media nitrite levels were not different in these two conditions (Muleme et al., 2009). The increase in arginase activity associated with an *L. major* infection does not appear to influence the BMDMs' ability to synthesize NO suggesting that NO is not the macrophages' primary mechanism for eliminating the parasites. Perhaps the lower parasite burden observed in BMDMs infected with arginase deficient *L. major*, is due instead to decrease polyamine levels.

Macrophages infected with arginase deficient Leishmania may become less M2 polarized and more M1 polarized. When *L. major* lacks the arginase gene, there is less arginase available for not only the parasite, but also the host. Arginase deficient *L. major*, studied in vitro and in vivo exhibited impaired survival and proliferation when phagocytosed by macrophages. This led to "delayed disease onset, reduced pathology, and lower parasite burden (Muleme et al., 2009)." It is tempting to conjecture that since less arginase was available for the macrophages, they were less M2 polarized and expressing higher levels of M1 molecular markers. Since, the authors failed to use any other molecular markers of M2 polarization, we have no caliber by which to quantify the degree of polarization in the IL-4 treated BMDMs infected with arginase deficient or WT *L. major*. This also brings into question whether these macrophages can be classified as M2 polarized, considering they are deficient in the arginase, the most diagnostic marker of M2 polarization. Furthermore, since parasite burden was lower, it is also conceivable that the decreased arginase availability and subsequently decreased sustenance rendered the parasites less productive and less virulent.

### *4.1.3 Mechanisms of Pathogen Exploitation of the Host*

Spd synthase is essential for Leishmania to perpetuate a strong infection, but not necessary to sustain *T. brucei* infection. One study was constructed on D spdsyn knockouts from the WT Ld Bob strain of *L. donovani*. In this study, parasites were starved for 2 days of exogenous polyamines prior to the initiation of the growth assay. Final parasite densities of spdsyn1 *L. donovani* KO parasites were significantly lower

than the densities they were initally inoculated at. When putrescine was added, spddsyn1 *L. donovani* KO parasites still had significantly lower densities than they had when they were initally inoculated. However, when spermidine was added, there was no significant difference between spdsyn1 *L. donovani* KO parasite densities and WT *L. donovani* parasite densities, because the synthesis of spermidine was no longer necessary (Gilroy et al., 2011). This demonstrates that spermidine is more important than putrescine for the reproduction and survival of *L. donovani*. BALB/c mice liver and spleen parasite burdens were lower in mice inoculated with Spdsyn1 KO *L. donovani* parasites (Gilroy et al., 2011), because the parasites had a decreased supply of spermidine. This is not the case for *T. brucei*. Another study used RNA interference to silence ODC and SpdSyn in *T. brucei*, and showed that the addition of putrescine rescued ODC RNAi cells, but the addition of spermidine did not (Xiao et al., 2009). Putrescine is more important for *T. brucei* survival, while spermidine is more important for *L. donovani* survival.

Spermine Oxidase (SMO) is another enzyme that pathogens may utilize to manipulate the resources of the host. Over a time course of 6-18 hours, *H. pylori* induced RAW 264.7 cells transfected SMO containing cDNA vector to maintain lower spermine levels than the *H. pylori* induced RAW 264.7 cells transfected with the empty vector. Under the same conditions, more arginine was taken up by the *H. pylori* induced RAW 264.7 cells, transfected the SMO containing cDNA vector, than by those transfected with the empty vector (Chaturvedi, et. al. 2013).

*H. pylori* infected RAW264.7 cells expressing higher levels of SMO catabolize spermine into spermidine, decreasing the intracellular levels of spermine. But, decreases in spermine levels could be triggering the cells to take up more arginine.

Pathogens may manipulate host cell viability by depriving it of arginine. Parasites can decrease arginine availability to the host, by consuming the arginine. Prokaryotic parasites, such as Giardia, actually utilize the arginine themselves "via the arginine dihydrolase pathway." Unlike eukaryotes, prokaryotes utilize arginine deiminase (ADI) to convert arginine into citrulline, which is then converted by ornithine carbamoyltransferase (OCT) into ornithine and carbamoylphosphate. Next, carbamate kinase (CK) uses the carbamoylphosphate in the process of phosphorylation generating ATP, while synthesizing amonia and CO2. Giardia uses its host arginine as a source of energy. Although glucose can also be used by Giardia to produce energy, arginine usage produces “7-8 times more energy” than glucose usage. Several proteins, including ADI and OCT are released during the first 30 minutes of interacting intestinal epithelial cells. Previous studies have shown that intestinal epithelial cells produce reduced NO levels, when "ADI expressed in E. coli" is added. Furthermore, Giardia consumes arginine during its interactions with host's intestinal epithelium cells triggering growth arrest and decreased cell proliferation (Stadelmann et al., 2012).

*T. gondii* infection increases the expression of arginase1. Recent studies done using qRT-PCR have revealed that CD11b+ brain mononuclear cells isolated from *T. gondii* infected brains express nearly 2 times more arginase1 than those isolated from naive brains (Nance et al., 2012). Furthermore, when *T. gondii* infected macrophages were compared to non-infected macrophages, no significant difference in ODC or ADC activity was detected (Henrique Seabra et al., 2004). It is expected that if arginase was being transcribed at higher levels during a *T. gondii* infection, then it would be actively catalytically converting the host arginine into ornithine. But, if ODC and ADC are remaining constant, infected host cells are not using this newly synthesized ornithine to synthesize more polyamines or agmatine. Instead, perhaps the *T. gondii* is taking up the ornithine and utilizing it to produce its own polyamines and/or some other products that are essential for its growth and survival.

Appropriate putrescine and spermidine are important for promoting *T. gondii* virulence. Macrophages treated with putrescine or spermidine for 24 hours followed by 2 hours of exposure to *T. gondii* had significantly higher adhesion indexes than those not treated with polyamines prior to infection. When macrophages were treated with MO (a competitive, reversible inhibitor of ODC) prior to exposure to *T. gondii*, intracellular *T. gondii* failed to grow indicating that polyamines are also needed for *T. gondii* to reproduce (Henrique Seabra et al., 2004). However, excessive amounts of polyamines are not beneficial to *T. gondii*'s virulence. *T. gondii* treated with putrescine 24-hours and 48-hours prior to macrophages exposure had significantly lower infection indexes than those

not treated with putrescine. Putrescine treated *T. gondii* had impaired ability to inhibit NO synthesis or inhibit "fusion of acidic compartments with parasitophorous vacuoles." However, similar to non-treated *T. gondii*, they were capable of preventing respiratory burst (Henrique Seabra et al., 2004). This suggests that *T. gondii* needs polyamines to proliferate, but when exposed to excessively high levels of polyamines, it is functionally impaired.

## 4.2 Pathogen's Utilization of Arginine and Polyamine Transport Systems to Hijack the Host's Resources

When considering pathogen infection in the context of M1 and M2 macrophage polarization, arginine is the amino acid of interest. If the parasite is taking up the host's arginine, then it is not being used by the macrophage.

### *4.2.1 Manipulation of Arginine Transport Mechanisms*

Arginine is an essential amino acid for the survival of most pathogens. Just as it is vital for the host's macrophages to import arginine, it is also essential for the parasite to import arginine. A number of Kinetoplastid parasites (Tympanosomata and Bodonita) have had their genome sequenced. In these parasites, amino acid permeases account for the majority of transporters. 30 to 40 genes encode for amino acid tranporters in *L. major*, *T. brucei*, and *T. cruzi* (Landfear, 2011) demonstrating the importance of arginine import and usage for the growth and survival of the parasite.

Some parasites are capable of regulating arginine import, based on how much intracellular arginine they already have. In Leishmania donavani, LdAAP3 (an amino acid permease composed of 480 amino acids and 11 predicted trans-membrane domains) was cloned and identified as a "high affinity arginine-specific transporter (Shaked-Mishan et al., 2006, Darlyuk et al., 2009)." Further studies revealed that the LdAAP3 arginine transporter expression and activity are dependent on intracellular amino acid levels. When the *L. donovani* promastigotes were deprived of all amino acids except glucose, LdAAP3 expression and activity became upregulated (after 2 and 4 hours of amino acid starvation, arginine transport was 2 times higher and 5 times higher, respectively). Note that glucose was kept in the medium to provide the energy needed for the arginine transport to occur. The same results were achieved when proline was used as an energy source instead of glucose. When each of the amino acids was evaluated individually, arginine was the only amino acid that inhibited LdAAP3 upregulation (Darlyuk et al., 2009). Leishmania parasites must rely on arginine import from the hosts, because arginine is an essential amino acid for its survival (Landfear, 2011). Other parasites do not rely so heavily on arginine transport.

### *4.2.2 Manipulation of Polyamine Transport Mechanisms*

A number of organisms, such as Leishmania, are capable of both synthesizing their own polyamines and importing their hosts' polyamines via their plasma membranes. *T. cruzi* is completely dependent on polyamines imported from its host cell's cytosol, because its genome lacks the gene that encodes for ODC and ADC (Landfear, 2011, Hasne et al.,

2010). On the other hand, *T. brucei* must rely completely on its own polyamine synthesis mechanisms, because it habitats environments low in polyamines (Landfear, 2011).

To elucidate the molecular mechanism of polyamine transport in eukaryotic parasites, the gene encoding the *L. major* transporter (LmPOT) was cloned and identified as a transporter of putrescine and spermidine (insect form). LmPOT is a high affinity APC (amino acid/polyamine/organocation) transporter localized primarily to the cell's plasma membrane. They reported that there was was no evidence of amino acid transport via LmPOT (Hasne and Ullman, 2005). In *T. cruzi*, two high affinity transporters, TcPOT1.1 and TcPOT1.2, are responsible for the recognition of "putrescine and cadavarine but not spermidine or spermine (Hasne et al., 2010)." Previous studies imply that *T. cruzi* also takes up the spermidine and spermine of the host. Further studies have revealed that there are more homologs of TcPOT1.1 and TcPOT1.2, which have not been researched (Landfear, 2011). Perhaps, they are responsible for sperimidine and spermine transport in *T. cruzi*. This is an area where more research could be done.

ODC antizyme is found widely throughout the Eukaryotic domain (Coffino, 2001). However, there is variation. In *T. brucei*, the binding affinity between ODC and antizyme is much lower than that of humans. Specifically, human ODC has several residues (resides119, 124,125, 129, 136, 137 and 140), which make it different from that of *T. brucei* and give it a stronger binding affinity for OAZ (Liu et al., 2011).

Most parasites only have antizyme, a negative regulator of intracellular polyamine levels, but lack antizyme inhibitor, the positive regulator. Anomalously, *B. malay* (a filarial parasite) possesses an antizyme, which appears to function as antizyme inhibitor. This antizyme is known as AZ of *O. volvus (Ov*AZ). In silico experiments revealed that only one ODC gene exist in the *B. malay* genome. Surprisingly, this gene encodes a non-functional ODC. Pull down assays revealed that OvAZ interacts with several heterlogous ODCs. The authors speculate that these ODC-like proteins serve the same function as AZI (which the parasite does not have), regulating the polyamine transporters of the filaria (Kurosinski et al., 2013).

### 4.3 Host Genetic Variability and the Control of M1 and M2 Responses

Research has shown that genetic variability renders some hosts more susceptible to particular pathogens than other hosts. Humans with different genetic backgrounds may be more susceptible to parasites (or perhaps even different disease states).

#### *4.3.1 Genetic Variability Among Mice Strains*

Genetic inconsistencies among distinct mice strain influence the ability of macrophages from different mice strains to become polarized and transport arginine. Just as there are prototypical Th1 and Th2 strains, there are also M1 and M2 strains that correspond to them. C57BL/6 and B10D2 are M1 strains, while BALB/c and DBA/2 are M2 strains (Mills, et al., 2000). Recent studies have demonstrated that M2 mice strains are more susceptible to parasite infection than M1 mice strains. This leads us to question why one

strain of mice would be more resistant to infection than another strain. The major difference is that M1 strains are primed to respond to pathogenic attack by setting up the prototypic Th1/M1 pro-inflammatory environment, while M2 strains are primed to respond by setting up the prototypic Th2/M2 anti-inflammatory environment. The genetic differences that separate these two mice strains are minor, yet they render the macrophages of the M2 strains more susceptible to infection.

One reason for higher susceptibility to Leishmania in macrophages from M2 mice strains is that their macrophages exhibit higher arginase activity, when exposed to the parasites. BALB/c mice (an M2 mouse strain) CD4 T cells produce high levels of IL-4 inducing arginase activity and subsequently higher levels of polyamines, which can be readily taken up by the parasites (Muleme et al., 2009). The higher levels of arginase activity, followed by the subsequent increase in the synthesis of ornithine and polyamines, (resources that the parasite can utilize to survive and proliferate) render the prototypical M2 strains more susceptible to parasite infections.

Leishmania parasite replication relies heavily on arginase availability and decreases when Nor NOHA inhibits arginase activity (drives the synthesis of ornitine/polyamines, which are beneficial to parasite (Tympanosomatid) growth) (Heby, Persson, and Rentala 2007). According to Duleu et al, *T. Brucei brucei*- infected BALB/c macrophages are less effective at killing *T. brucei brucei* than C57BL/6 macrophages, because arginase is induced at higher levels in BALB/c macrophages. They show that *T. brucei brucei*

induced high Arg activity inhibits NO production, and subsequently decreases the effectiveness of the macrophage to kill the parasites. M1 strains synthesize more NO, which is toxic to the pathogen making the macrophages less susceptible. When Nor NOHA was used to inhibit Arginase activity in both mouse strains, BALB/c macrophages started producing equivalent levels of NO and killing *T. brucei* at the same levels (Duleu et al., 2004). In addition, spermidine/spermine inhibits M1 associated cytokines (Kropf et al. 2005).

Another reason for susceptibility of the BALB/c mice macrophages is that they transport more arginine, because the promoter of the SLC7A2 transporter is "complete," whereas a sequence is deleted in that of the C57/bl6 mice macrophages. Sans Fons et al came to this conclusion by showing that BALB/c mice macrophages are more susceptible to high Leishmania parasite burden than those of C57/bl6 mice, because C57/bl6 mice macrophages exhibit lower levels of IFNγ and IL-4 induced SLC7A2 transporters (transport arginine into the macrophage) than BALB/c mice. This study showed that one of 4 AGGG repeats in the SLC7A2 promoter was deleted in the C57/bl6 mice. Therefore, C57/bl6 macrophages (treated with M1 or M2 stimuli) have decreased arginine import in comparison to BALB/c mice macrophages (treated with M1 or M2 stimuli) (Sans-Fons et al. 2013). This leaves not only the macrophages with less arginine to metabolize into polyamines, but also the Leishmania amastigotes with less arginine to metabolize into polyamines/prolines for growth and trypanothione, which protects against oxidative stress.

When macrophages from both mice strains were treated with M1 (IFNγ or IFNγ/LPS) or M2 (IL-4 or IL-4/IL-10) stimululi, BALB/c macrophages expressed higher levels of SLC7A2 and took up more arginine than C57/bl6. In studies done with radioactive labeling and thin layer chromatography, IL-4 treated BALB/c mice macrophages synthesized more ornithine, citruline, spermine, and proline than IL-4 treated C57/bl6 macrophages. This was accounted for by the higher levels arginine transport. Note that intracellular levels of putrescine, spermidine, and glutamate were the same in IL-4 treated macrophages from both strains of mice (Sans-Fons et al. 2013). This may be attributed to the fact that putrescine does not accumulate in macrophages, and is instead rapidly catalyzed by SpdSyn into Spermidine or exported from the cell. SpmSyn also rapidly catalyzes spermidine into spermine. Perhaps, the glutamate is being catalyzed by glutaminase into glutamine at a rapid pace so that it does not accumulate. When si RNA was used to silence the SLC7A2 expression, arginine uptake and usage decreased. In addition, Leishmania amastigote burden decreases in response to the silencing of SLC7A2 (Sans-Fons et al. 2013) and subsequent decrease in arginine uptake. When macrophages from both mice strains were treated with M1 and M2 inducing stimuli, both isoforms of SLC7A2 expressed at higher levels in BALB/c macrophages (Sans-Fons et al. 2013).

In contrast to the studies done by Duleu et al, their data showed that there is no difference in the levels of Arg1 transcription and expression in BALB/c mice macrophages and C57/Bl6 mice macrophages, regardless if they were treated or left

untreated. This may be due to the fact that macrophages were infected with *T. brucei brucei* in the studies done by Duleu et al, while macrophages were infected with Leishmania in the studies done by Sans-Fons et al. Perhaps, macrophages respond differently to different types of parasites.

TLR responsiveness is another difference between M1 and M2 mice strains, which effects how they respond pro-inflammatory stimuli such as LPS. This may be another reason why M2 mice strains are more susceptible to parasite infection than M1 mice strains. Macrophage stimulatory protein (MSP) activates the receptor tyrosine kinase recepteur d' origine nantais (RON). RON is involved in a number of macrophage functions, including phagocytic activity and motility. Specifically, RON controls TLR4 signaling. When LPS treated C57/BL6 macrophages were treated with MSP; TNFα and IL-12p40 transcription were not suppressed, while CSF-2 transcription increased. However, when LPS treated FVB (M2 mice strain) macrophages were treated with MSP, TNF α and other "TLR4 dependent Type 1 Interferon" associated genes were suppressed. In other words, RON was less effective at modulating TLR4 signaling in C57/BL6 macrophages than in FVB macrophages (Chaudhuri et al., 2013). This means that RON has a tighter control of TLR4 signaling in M2 mice strains than M1 mice strains. The distinction between the way the macrophages of M1 and M2 mice strains respond to pathogen attack is dependent on the variabilities discussed above as well as variability in other mechanisms that are yet to be discovered.

*4.3.2 Genetic Variability Among Different Mammalian Species*

Recent studies have focused on how genetic variability among different species affects their immune responses to infection. For example, Arginase1 expression is also regulated differently in mice and hamsters. Significantly higher levels of parasite burden accompanied by lethality were found in hamsters compared to mice. Faulty macrophage activation and parasite killing abilities may be accounted for by the low iNOS expression and NO production. In this study, they found that arg1 activity and expression in the spleen of infected hamsters was significantly higher than that of infected mice spleens. Higher Arg1: iNOS ratios were found in hamster splenic macrophages, whereas higher iNOS: Arg1 ratios were found in mice splenic macrophages. As expected, infected hamster spleens and livers had synthesized higher levels of polyamines (putrescine, spermidine, and spermine) than uninfected hamster spleens and livers (Osorio et al., 2012). The macrophages of hamsters appear to be more prone to become M2 activated/polarized than those of mice. Perhaps, their entire immune system is skewed towards more anti-inflammatory states. This brings into question whether hamster models are really valid when using them to project on human disease models.

The next animal model to consider is the mouse model, which is probably the most common animal model. As mentioned previously while discussing the role of genetics in susceptibility to viceral leishmania (in mouse leishmania models), Th1 responses are associated with resistance, while Th2 responses are associated with susceptibility in various mouse strains. Genes encoding IL-4 and other M2 associated cytokines are

located on chromosome 11 of mice and chromosome 5q23-q33 of humans. These genes located on chromosome 11 are associated with increased visceralization following *L. major* infection in BALB/c (Blackwell et al., 2009). The article does not mention if increased viceralization is also associated with genes located on chromosome 5q23-q33 of humans.

Whole genome microarrays were done to compare IL-4 activated human and mice macrophages. When human datasets were compared, 489 transcripts were found to be expressed at high levels in distinct donors and models. When mice datasets were compared, 459 transcripts were also found to be expressed at high levels. When the human list of transcripts and the mice list of transcripts were compared, 231 (50% of the human list and 50% of the mice list) genes overlapped (Martinez et al., 2013). Only 50% of the molecular markers of M2 activation/polarization are the same in humans and mice. Interestingly, some of the most recognized molecular markers of M2 macrophage activation are not induced in human macrophages. As stated in one article, IL-4 and IL-13 do not "induce arginase-1 in human monocytes and monocyte-derived macrophages." It also mentions that eosinophil chemotactic factor (the most equivalent homologue of mouse Ym1) is not induced by IL-4 (Raes et al., 2005). Another study showed that Arg 1 is constitutively expressed only in human granulocytes, but not macrophages. In fact, they used immunoblotting to show that human macrophages treated with IL-4 or IL-4/IL-10 were unable to induce Arg1, while mice macrophages treated with IL-4 or IL-4/IL-10 did induce Arg1 (Munder et al., 2005). Considering that Arg1 and Ym1 are among the

most commonly observed M2 molecular markers in mice macrophages, it is questionable whether mice models are most physiogically appropriate models for macrophage immune response studies.

Furthermore, when proteomic analyses were done on human macrophages, 977 proteins were detected in both the M-CSF macrophage differentiation model and autologuous serum macrophage differentiation model. When proteomic analyses were done on mice macrophages, 1038 proteins were detected in both the M-CSF macrophage differentiation model and the autologous serum macrophage differentiation model. When human list of proteins and mice list of proteins were compared, 513 proteins overlapped. "Overlap between the conserved mRNA and protein signatures; 231 highly expressed genes and 513 detected proteins, respectively, amounted to 87 genes (Martinez et al., 2013)." Generally, mouse macrophage models for determining activation states are based on functional assays, while human macrophage studies rely on "surface molecules or cytokine expression" to identify activation states. A recent study showed that in vitro differentiated human macrophages levels of nitrite production and arginase activity were not consistent with the activation states established by "surface staining of CD14 and CD163 (Geelhaar-Karsch et al., 2013)."

This leads us to question whether human genetic backgrounds play a role in resistance to infection and if some humans are more prone to anti-inflammatory states or vice versa. The answer to this question would have implications that extend beyond parasite

infections to other diseases associated with excessive inflammation or lack of inflammation. However, since this review focuses on in invading pathogens, I will return to the topic of Leishmania in humans. Studies showed that in Brazilialian and Indian Viceral Leishmania human patients, IL-4 was detected at much higher levels (13X higher in Indian patients) than controls. Blackwell et al goes into further detail about these variations in genetics; unfortunately lack of power prevents further conclusions about the correlations between mice and human genetic variations and Th2 responses to viceral leishmania (Blackwell et al., 2009). Further studies will need to be done to determine how extensive of a role genetics plays in controlling resistance to infection and disease.

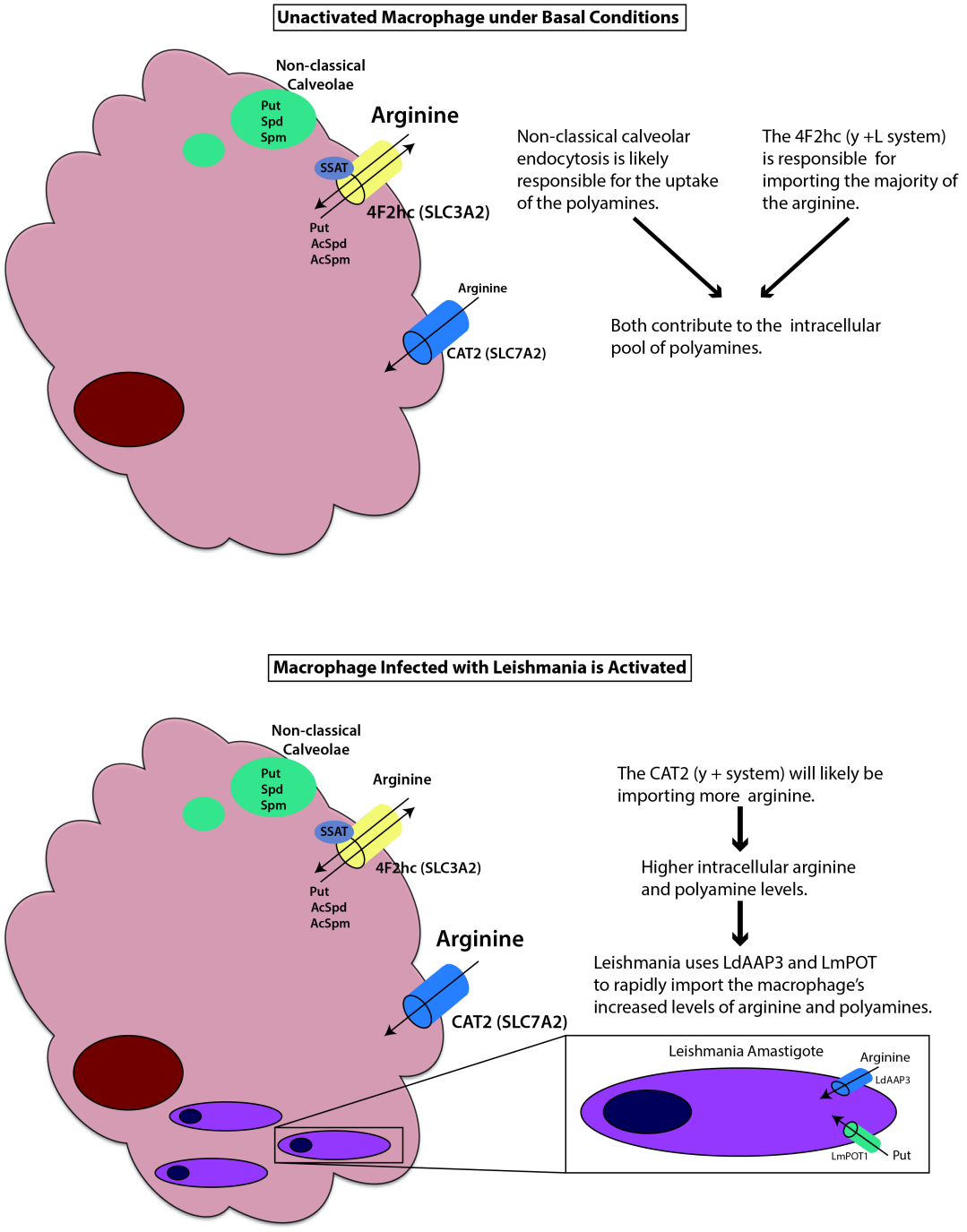
# SUMMARY

M1 and M2 macrophage activation/polarization states are not the clear-cut phenotypes that they so easily get categorized into. Macrophage activation and polarization are two distinct events that can occur in concert or separately from each other. Since macrophage activation and polarization are so variable, it is of essence that the researcher carefully examines the macrophage states before making conclusions. Remember, M1 and M2 are defined not only by their activation state, but also by their metabolic state.

Since iNOS and Arg1, the most diagnostic markers of M1 and M2 activated/polarized macrophages (respectively), compete for L-arginine; it is evident that arginine is important for both macrophage states. For an investigator in the field of macrophage biology (specifically M1 and M2 macrophage activation/polarization research), it is important to understand how arginine is transported into the cell and how its levels are regulated in distinct activation/polarization states. Furthermore, since polyamines are involved in so many cellular processes and also happen to be a metabolic product of arginine in M2 activated/polarized macrophages, it is also vital to understand how polyamine transport and levels are regulated during these two activation/polarization states.

Arginine and polyamines are not only valuable resources to the macrophages of the hosts, but also to the invading pathogens that exploit the hosts. Understanding how the transport of these compounds is regulated in the parasite provides a lead for those seeking to develop treatments against infection. The usage of arginase/polyamine synthesis

inhibitors to manipulate polyamine levels in the host as well as the invader has led to the formulation of novel pharmaceuticals that have proven promising for the treatment of trypanosomes and other parasites. Granted, genetic variabilities among hosts will need to be considered to design the most effective treatments against parasite infection.




**Summary Figure: Proposed model of the changes in arginine/polyamine transport regulation that are expected to occur when an unactivated macrophage becomes infected with Leishmania.**

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