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Order number: _____

UNIVERSITY OF TLEMCE – ABOU-BEKR BELKAÏD

FACULTY SNV-STU – DEPARTMENT OF BIOLOGY

LABORATORY OF APPLIED MOLECULAR BIOLOGY AND IMMUNOLOGY

- BIOMOLIM-

THESIS

Submitted in fulfilment of the requirements for a

Master's degree in Biological Sciences

Specialty: Immunology

By:

HASSANI Meriem

Defended on:

14/06/2026

Topic:

Arginase/NO axis signature in human monocytes exposed to autologous erythrocytes with reduced oxygen transport capacity.

_____ Under the supervision of **Professor Mourad ARIBI** _____

Jury

Pr. Mohammed CE Ismet SMAHI	Professor	University of Tlemcen, Algeria	President
Pr. Mourad ARIBI	Professor	University of Tlemcen, Algeria	Thesis director
Dr. Zineb HADJIL	MCB	University of Tlemcen, Algeria	Examiner



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Abstract

Background: Red blood cells have recently been recognized as more than mere oxygen transporters, with growing evidence pointing to their active role in influencing the functional behaviour of neighbouring immune cells. Through their capacity to shape local oxygen availability, they constitute a key microenvironmental factor capable of influencing monocyte behaviour and functional responses. However, the specific impact of haemoglobin-mediated oxygen transport on monocyte functional orientation through the NO/Arginase axis remains poorly understood, representing the central question addressed in the present study.

Materials And Methods: This study was conducted on four experimental groups, in which monocytes isolated from anaemic donors characterized by reduced haemoglobin levels were treated with or without their intact autologous red blood cells (RBCs), in the presence or absence of LPS stimulation. The NO–arginase axis in monocytes was subsequently assessed by measuring iNOS activity through nitric oxide (NO) production using the Griess assay, and Arginase-1 activity through urea formation using a colorimetric urea-based assay, performed on both cell lysates and culture supernatants.

Results: Exposure of human monocytes to red blood cells with reduced haemoglobin levels resulted in a significant increase in NO production and iNOS activity, while decreasing ARG1 activity. Consequently, the iNOS/ARG1 ratio was significantly elevated, indicating a shift toward the iNOS pathway.

Conclusion: In conclusion, this study establishes that alterations in haemoglobin-mediated gas transport can influence the balance of NO–arginase in human monocytes.

Key words: RBC, Monocyte, Haemoglobin, gas transport, NO/Arginase axis.

Résumé

Introduction : Les globules rouges ont récemment été reconnus comme bien plus que de simples transporteurs d'oxygène, avec des preuves croissantes soulignant leur rôle actif dans l'influence du comportement fonctionnel des cellules immunitaires voisines. Par leur capacité à façonner la disponibilité locale en oxygène, ils constituent un facteur microenvironnemental clé capable d'influencer le comportement et les réponses fonctionnelles des monocytes. Cependant, l'impact spécifique du transport d'oxygène médié par l'hémoglobine sur l'orientation fonctionnelle des monocytes à travers l'axe NO/Arginase reste mal compris, représentant la question centrale abordée dans la présente étude.

Matériel et méthodes : Cette étude a été menée sur quatre groupes expérimentaux, dans lesquels des monocytes isolés de donneurs anémiques présentant une diminution du taux d'hémoglobine ont été traités avec ou sans leurs propres globules rouges autologues intacts (GR), en présence ou en l'absence d'une stimulation par le LPS. L'axe **NO–arginase** des monocytes a ensuite été évalué en mesurant l'activité de l'iNOS par la production de monoxyde d'azote (NO) à l'aide du test de Griess, ainsi que l'activité de l'arginase-1 par la quantification de l'urée à l'aide d'un dosage colorimétrique, réalisé à la fois sur les lysats cellulaires et les surnageants de culture.

Résultats : L'exposition des monocytes humains à des globules rouges à faible teneur en hémoglobine a induit une augmentation significative de la production de NO et de l'activité de l'iNOS, associée à une diminution significative de l'activité de l'ARG1. Par conséquent, le rapport iNOS/ARG1 a été significativement augmenté, indiquant une orientation vers la voie de l'iNOS.

Conclusion : En conclusion, cette étude établit que des altérations du transport des gaz assuré par l'hémoglobine peuvent influencer la balance NO–arginase dans les monocytes humains.

Mots clés : Globules rouges, monocytes, hémoglobine, transport gazeux, axe NO/arginase.

ملخص

الخلفية: لطالما اعتُبرت كريات الدم الحمراء مجرد ناقلات للأكسجين، غير أنها باتت تُعرف حديثاً بدور أكثر نشاطاً، إذ تشير الأدلة المتزايدة إلى مساهمتها الفعلية في التأثير على السلوك الوظيفي للخلايا المناعية المجاورة. من خلال قدرتها على التأثير في توافر الأكسجين المحلي، تُشكّل عاملاً بيئياً دقيقاً رئيسياً قادراً على التأثير في سلوك الخلايا وحيدة النواة واستجاباتها الوظيفية. غير أن الأثر المحدد لنقل الأكسجين بواسطة الهيموغلوبين على التوجه الوظيفي للخلايا وحيدة النواة عبر محور أكسيد النيتريك/الأرجيناز لا يزال غير مفهوم بشكل كافٍ، وهو التساؤل المحوري الذي تسعى الدراسة الحالية إلى معالجته.

المواد والطرق: أُجريت هذه الدراسة على أربع مجموعات تجريبية، حيث غُولجت الوحيدات المعزولة من متبرعين مصابين بفقر الدم يتميزون بانخفاض مستويات الهيموغلوبين، مع أو بدون كريات الدم الحمراء الذاتية السليمة الخاصة بهم، وذلك في وجود أو غياب التحفيز بالليبوبوليسكاريد (LPS) بعد ذلك، جرى تقييم محور **NO-الأرجيناز** في الوحيدات من خلال قياس نشاط إنزيم **iNOS** عبر إنتاج أكسيد النيتريك (NO) باستخدام اختبار **غريس (Griess)**، وقياس نشاط إنزيم **الأرجيناز-1** من خلال تقدير تكوّن اليوريا باستخدام اختبار لوني، وذلك على كلّ من مستخلصات الخلايا والسوائل الفوقية للزراعة الخلوية.

النتائج: أدى تعريض الوحيدات البشرية لكريات دم حمراء منخفضة المحتوى من الهيموغلوبين إلى زيادة معنوية في إنتاج أكسيد النيتريك (NO) ونشاط إنزيم **iNOS**، مصحوبة بانخفاض معنوي في نشاط إنزيم **ARG1**. ونتيجة لذلك، ارتفعت نسبة **iNOS/ARG1** بشكل ملحوظ، مما يعكس توجهاً نحو هيمنة مسار **iNOS**.

الاستنتاجات: في الختام، تُثبت هذه الدراسة أن التغيرات في نقل الغازات الذي يؤمنه الهيموغلوبين يمكن أن تؤثر في توازن **NO-الأرجيناز** في الوحيدات البشرية.

الكلمات المفتاحية: كريات الدم الحمراء، الخلايا الوحيدة، الهيموغلوبين، نقل الغازات، محور أكسيد النيتريك/الأرجيناز.

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Dedication

To my beloved **parents**,
whose boundless love, sacrifices, and unwavering support have been the foundation of everything I have achieved. Your patience, strength, and steadfast belief in me have guided me through every challenge. I am forever grateful, and I owe you more than words can ever express.

To my dear **grandmother**,
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To those who believed in me when I doubted myself,
this work is dedicated to you.

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List of Abbreviations

Abbreviation	Full Term
aDMA	Asymmetric Dimethylarginine
ARG	Arginase
ATP	Adenosine Triphosphate
BFU-E	Burst-Forming Unit–Erythroid
CCL2	C-C Motif Chemokine Ligand 2
CCR2	C-C Chemokine Receptor 2
CD47	Cluster of Differentiation 47
CFU-E	Colony-Forming Unit–Erythroid
CMPs	Common Myeloid Progenitors
cMoP	Common Monocyte Progenitor
CpG-rich microbial DNA	Unmethylated CpG microbial DNA
DAMPs	Damage-Associated Molecular Patterns
DARC	Duffy Antigen Receptor for Chemokines
DNA	Deoxyribonucleic Acid
DRYLAIV	Conserved motif in chemokine receptors (GPCR signaling)
EPO	Erythropoietin
GMPs	Granulocyte-Macrophage Progenitors
Hb	Haemoglobin
HSCs	Hematopoietic Stem Cells
IFN	Interferon
iNOS	Inducible Nitric Oxide Synthase

List of abbreviations

Abbreviation	Full Term
IRF8	Interferon Regulatory Factor 8
LPS	Lipopolysaccharide
MDPs	Monocyte-Dendritic Cell Progenitors
MEP	Megakaryocyte–Erythroid Progenitor
MHC class II	Major Histocompatibility Complex class II
moDC	Monocyte-derived Dendritic Cells
moMφ	Monocyte-derived Macrophages
MPPs	Multipotent Progenitors
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NO	Nitric Oxide
NOHA	Nω-hydroxy-L-arginine
OAT	Ornithine Aminotransferase
ODC	Ornithine Decarboxylase
PU.1	Transcription Factor PU.1
RBCs	Red Blood Cells
RONS	Reactive Oxygen and Nitrogen Species
ROS	Reactive Oxygen Species
STAT6	Signal Transducer and Activator of Transcription 6
TGF	Transforming Growth Factor
Th	T helper cells
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor

Introduction

As the most prevalent cells in human circulation, red blood cells (RBCs) have long been recognized for their fundamental role in oxygen delivery and carbon dioxide clearance. Their distinctive biconcave shape and high haemoglobin content maximize surface area for efficient gas exchange, thereby supporting physiological equilibrium (Pretini et al., 2019).

At the core of erythrocyte function, haemoglobin (Hb) serves as a sophisticated molecular mediator of physiological homeostasis. It is characterized by a tetrameric quaternary structure composed of two α -globin and two β -globin chains. Each subunit contains a heme group with a central ferrous iron (Fe^{2+}), enabling reversible oxygen binding through cooperative allosteric interactions (Lukin and Ho, 2004a).

Beyond gas transport, RBCs are increasingly recognized as active participants in innate immunity. Despite lacking nucleus and organelles, they express a diverse array of surface receptors that enable them to bind and clear immune complexes, modulate cytokine availability, and interact directly with pathogens (Xie et al., 2022).

These multifunctional properties redefine RBCs as dynamic regulators of inflammation and immune homeostasis, challenging their traditional classification as inert carriers and highlighting their functional interplay with key immune effectors such as monocytes (Risso and Antonutto, 2025).

Monocytes are central effectors of the innate immune system, serving as vigilant patrollers that rapidly respond to infection and tissue damage. Derived from bone marrow progenitors and can differentiate into macrophages or dendritic cells upon migration into peripheral tissues (Cellular and Molecular Immunology, 2021). This population is inherently heterogeneous, comprising functionally distinct subsets specialized in pathogen sensing, cytokine production, and vascular surveillance.

Furthermore, monocytes exhibit remarkable functional plasticity, allowing them to adapt to microenvironmental cues and adopt distinct phenotypes through dynamic metabolic and phenotypic reprogramming (Guilliams et al., 2018). A key determinant of this polarization is cellular metabolism, particularly the utilization of L-arginine through two competing enzymatic pathways: inducible nitric oxide synthase (iNOS), which generates nitric oxide and supports pro-inflammatory responses, and arginase-1 (ARG1), which promotes tissue repair and the resolution of inflammation (Thomas and Mattila, 2014a). Through this metabolic checkpoint,

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monocytes serve as a decisive regulator of the balance between acute inflammation and tissue homeostasis.

In this study, we aimed to investigate the effect of autologous red blood cells on the NO–arginase balance in human monocytes, specifically under conditions of diminished gas transport capacity resulting from low haemoglobin levels. By quantifying nitric oxide production and arginase activity, we evaluated how this impaired erythrocyte functionality modulates monocyte functional responses under in vitro culture conditions.

I.Literature review

I.1 Red blood cells

RBCs, or erythrocytes, represent the most abundant cellular component of human blood and have traditionally been viewed as highly specialized cells dedicated to the transport of respiratory gases. Their cytoplasm is dominated by haemoglobin, the oxygen- and carbon dioxide-carrying protein that enables respiratory gas transport throughout the body (Armstrong and Mason, 2025; Giri and Tamgadge, 2024).

I.1.1 Structure

Healthy erythrocytes exhibit a characteristic biconcave discoid shape, with a diameter of approximately $7.2\text{--}8\text{ }\mu\text{m}$ and a peripheral thickness of about $2\text{ }\mu\text{m}$. This specific geometry increases the surface-to-volume ratio by 20–30% compared to a sphere of the same volume, facilitating rapid diffusion of oxygen and carbon dioxide across the membrane (Himbert and Rheinstädter, 2022).

The absence of a nucleus and organelles in mature erythrocytes provides more intracellular space for haemoglobin, effectively transforming the cell into a specialized and highly efficient gas carrier (Mei et al., 2020).

Beyond gas exchange, the biconcave shape provides reversible deformability, allowing erythrocytes to traverse narrow capillaries as small as $3\text{--}4\text{ }\mu\text{m}$, stretching and folding as needed to deliver oxygen to deep tissues without compromising membrane integrity (Diez-Silva et al., 2010).

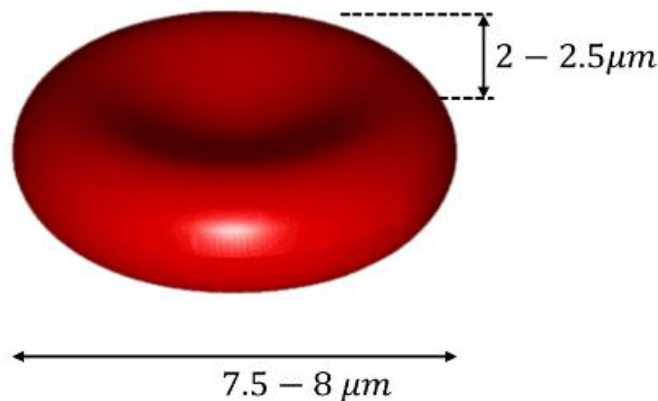


Figure I-1: Illustration of the native biconcave erythrocyte shape (Puthumana Melepattu, 2025).

I.1.2 Membrane architecture

The erythrocyte membrane is a highly sophisticated, asymmetric lipid–protein bilayer that functions as a viscoelastic shell. Its unique structural organization provides the cell with exceptional flexibility, mechanical resilience, and the ability to traverse narrow capillaries without rupturing (Huang, 2026). The membrane is composed of two main interdependent domains:

I.1.2.1 The lipid bilayer

Composed of nearly equal proportions of lipids and proteins, displays a strictly maintained phospholipid asymmetry. Neutral phospholipids are primarily located in the outer leaflet, whereas amino phospholipids, such as phosphatidylserine, are confined to the inner leaflet. This organization is vital; the disruption of this asymmetry can trigger blood coagulation or signal for macrophage-mediated destruction (Mohandas and Gallagher, 2008).

I.1.2.2 The membrane cytoskeleton

Beneath the lipid bilayer lies a dense, proteinaceous meshwork known as the cytoskeleton responsible for maintaining the cell's remarkable deformability and mechanical stability. Unlike most cells, erythrocytes rely on a spectrin-based network rather than microtubules, in which α - and β -spectrin tetramers form a flexible hexagonal lattice that preserves the characteristic biconcave shape during microcirculatory transit (Hammer, 2018). This network is anchored to the membrane through proteins such as ankyrin and Protein 4.1R, which connect spectrin to Band 3 and glycophorins while stabilizing actin junctional complexes (Lux, 2016; Salomao et al., 2008). Additional structural proteins, including adducin and tropomyosin, reinforce actin filament organization and membrane resilience. Beyond structural support the plasma membrane is densely populated with functional transmembrane proteins that regulate the cell's internal environment, notably Band 3 represents the predominant membrane protein, and mediates bicarbonate/chloride exchange essential for carbon dioxide transport. GLUT1 facilitates glucose uptake, while Aquaporin-1 regulates transmembrane water movement and osmotic equilibrium. Although present in lower quantities, ion pumps such as Na^+/K^+ ATPase and Ca^{2+} -ATPase maintain ionic homeostasis and electrochemical gradients necessary for erythrocyte survival and deformability (Bernhardt and Ellory, 2003).

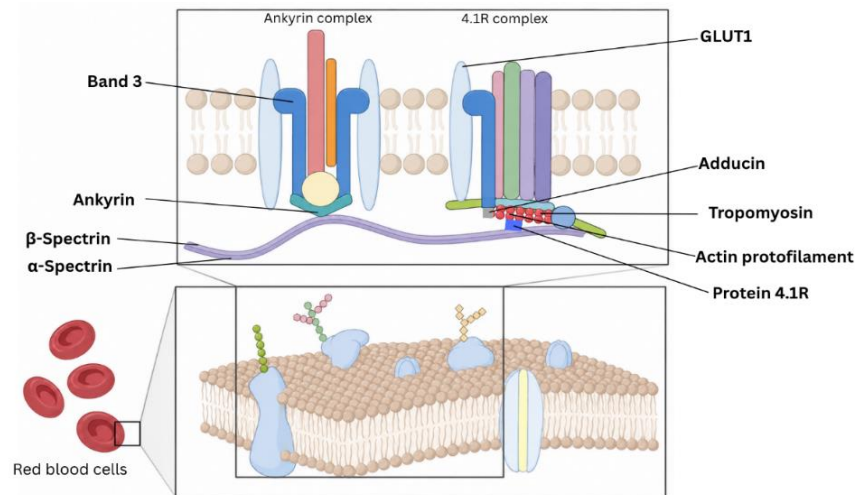


Figure I-2: Structural model of the erythrocyte plasma membrane and cytoskeletal scaffold.

The synergy between these two domains allows the erythrocyte to withstand significant hydrodynamic shear and undergo the extreme, reversible deformations required for microcirculatory transit (Barbalato and Pillarisetty, 2022).

I.1.3 Erythropoiesis

Within the adult systemic circulation, red blood cells represent the specialized final stage of a complex hematopoietic hierarchy, arising from a finely tuned seven-day maturation program known as erythropoiesis. Triggered by erythropoietin (EPO) within the bone marrow (Koury and Ponka, 2004). This process involves a series of distinct morphological and biochemical transitions, through the following stages:

I.1.3.1 Hematopoietic hierarchy

The process begins as multipotent hematopoietic stem cells (HSCs) differentiate into common myeloid progenitors (CMPs), which further commit to the megakaryocyte-erythroid progenitor (MEP) lineage. These MEPs give rise to burst-forming unit–erythroid (BFU-E) cells, the earliest erythroid-committed progenitors, which undergo extensive proliferation to meet the body’s daily demand for red blood cells. Subsequently, they mature into colony-forming unit–erythroid (CFU-E) cells (Koury, 2014).

I.1.3.2 Enucleation and reticulocyte formation

During terminal erythroid differentiation, precursors progress sequentially through proerythroblast, basophilic, polychromatophilic, and orthochromatic erythroblast stages, characterized by haemoglobin accumulation and progressive nuclear condensation (pyknosis), culminating in enucleation and reticulocyte formation (Moras et al., 2017).

I.1.3.3 Reticulocyte maturation and circulation

Reticulocyte maturation occurs within erythroblastic islands, where central macrophages support differentiation, supply iron, and remove extruded nuclei. Mature erythrocytes then enter circulation for approximately 120 days before being cleared by splenic and hepatic macrophages (Klei et al., 2017).

This efficient system ensures the continuous renewal of the body's gas-transport vessels, producing approximately 2×10^{11} new erythrocytes every day to replace aging cells (Dzierzak and Philipsen, 2013).

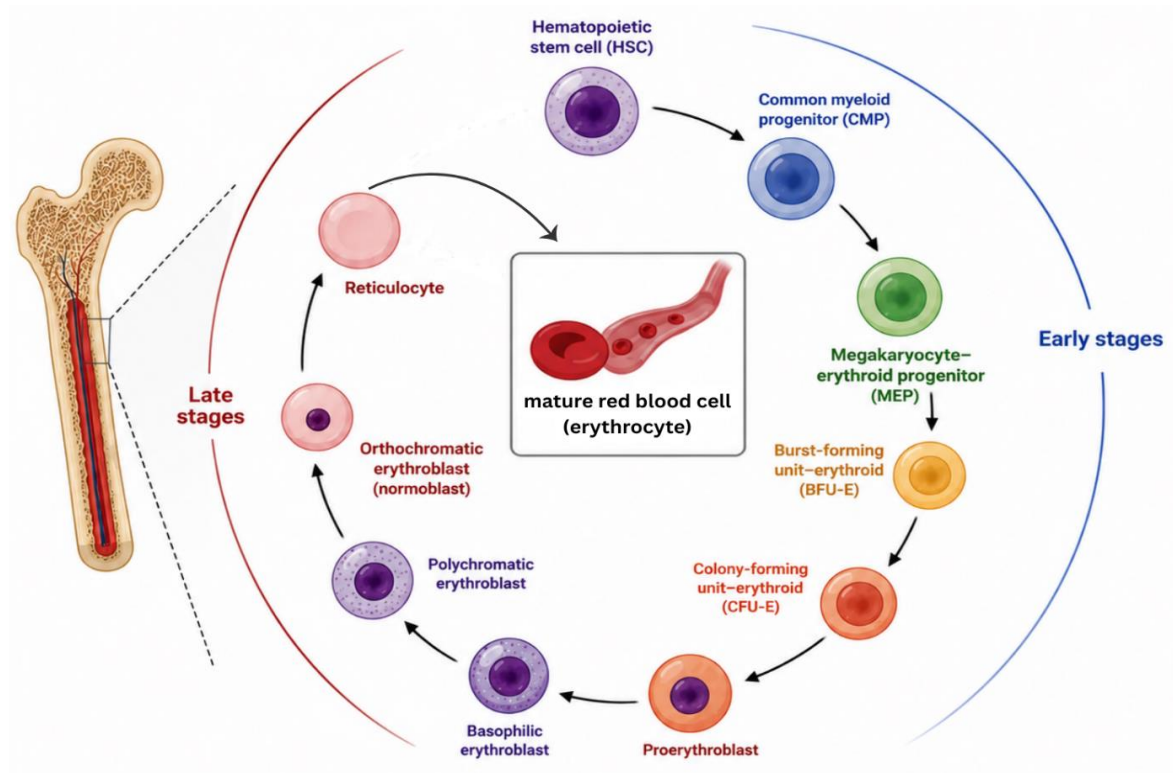


Figure I-3: Schematic Representation of Human Erythropoiesis.

I.1.4 Haemoglobin: The engine of gas transport and erythrocyte homeostasis

As the functional engine of the erythrocyte, haemoglobin (Hb) occupies approximately one-third of the cell's volume ($\sim 90 \mu\text{m}^3$) and serves as the primary driver of both gas transport and cytosolic viscosity. In its adult isoform (HbA), haemoglobin adopts a quaternary configuration composed of two α -globin and two β -globin chains arranged into a tetramer, each globin subunit contains a heme group composed of a protoporphyrin IX ring with a central ferrous iron (Fe^{2+}) atom, enabling reversible oxygen binding (Ahmed et al., 2020).

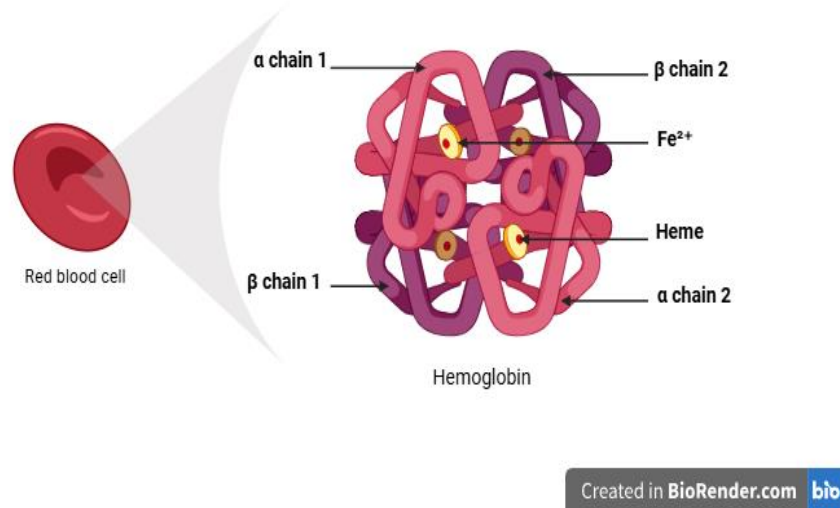


Figure I-4: Molecular structure of haemoglobin.

This specialized molecular architecture allows each tetramer to bind up to four oxygen molecules (O₂) (Lukin and Ho, 2004b), a process governed by cooperative allosteric dynamics, whereby ligand (O₂) binding induces a conformational transition from the low-affinity T (tense) state, characterized by low oxygen affinity, to the high-affinity R (relaxed) state, which exhibits high affinity. This critical allosteric mechanism ensures maximal oxygen saturation within the pulmonary capillaries and efficient, targeted O₂ unloading in hypoxic, acidic microenvironment of metabolically active peripheral tissues (Bringas et al., 2017).

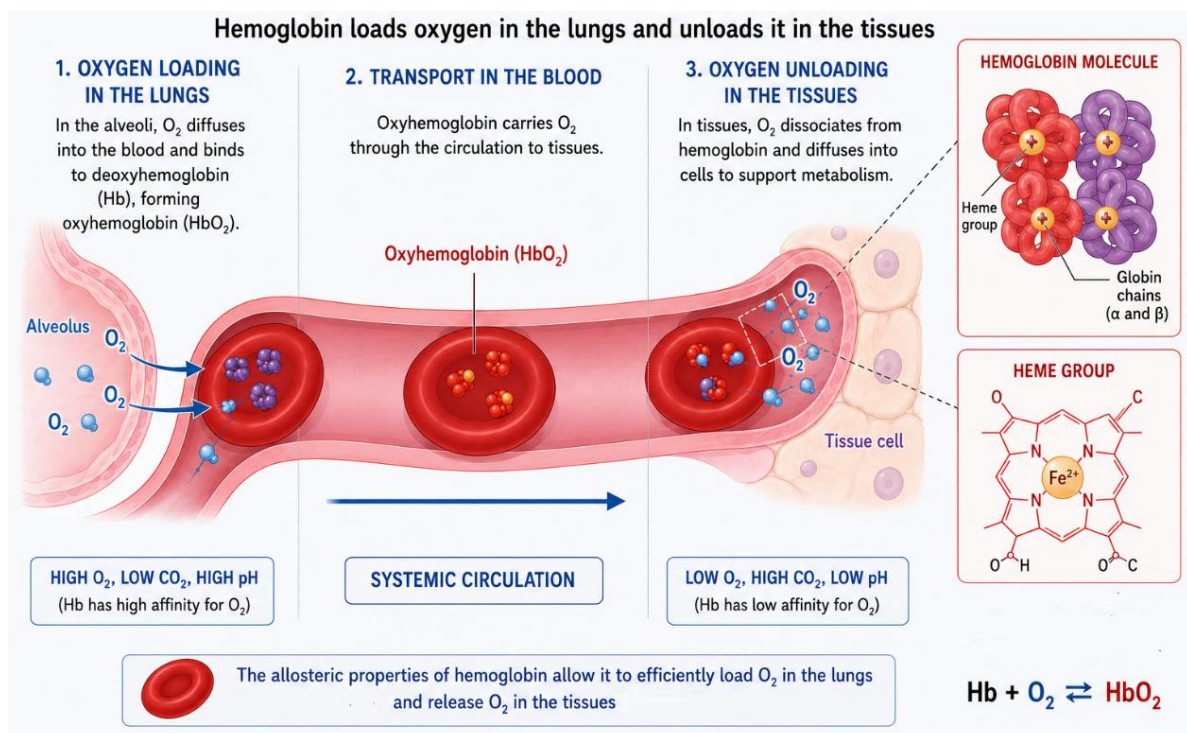


Figure I-5: O₂ delivery by haemoglobin.

Carbon dioxide transport is equally sophisticated: approximately 70% of tissue-derived CO₂ enters the erythrocyte and is rapidly hydrated by carbonic anhydrase II into bicarbonate (HCO₃⁻) and protons (H⁺), with bicarbonate exported into plasma via the Band 3 anion exchanger (AE1, Cl⁻/HCO₃⁻ exchanger) (Jensen, 2004). The remaining CO₂ is transported either in physical solution in plasma (~10%) or bound covalently to the N-terminal amino groups of globin chains as carbaminohaemoglobin (~20%), a form that additionally stabilizes the T state which helps the red blood cell release its oxygen more efficiently to the tissues that need it (Geers and Gros, 2000).

At an intracellular haemoglobin concentration of ~33 g/dL, the erythrocyte increases blood oxygen-carrying capacity 50-fold over plasma dissolution alone, enabling delivery of approximately 1,000 mL of O₂ per minute under resting conditions (Barbalato and Pillarisetty, 2022; Peter Klinken, 2002). Each gram of haemoglobin binds 1.34 mL of O₂ when fully saturated, making haemoglobin concentration the primary determinant of total oxygen delivery (Pittman, 2011) — a relationship directly reflected in the clinical consequences of anaemia, where even modest reductions in Hb concentration substantially impair tissue oxygenation.

Beyond its canonical respiratory role, haemoglobin acts as a redox-active protein and molecular sensor, capable of cycling between multiple oxidation states (Fe²⁺, Fe³⁺, and ferryl)(Alayash, 1999). Through interactions with nitric oxide (NO), nitrite, and reactive species, it functions as both a nitrite reductase and an NO dioxygenase depending on local O₂ tension, thereby contributing to the regulation of nitric oxide homeostasis and vascular tone (Gardner, 2012).

1.1.5 Immunomodulatory functions of red blood cells

As the most abundant cellular constituents of the circulatory system, erythrocytes maintain a continuous and intimate proximity with circulating immune cells. Rather than acting as passive gas transporters, their high-density presence within the vascular space positions them as active participants in systemic immunity (Anderson et al., 2018).

Their primary immunological functions encompass immune sensing, scavenging and immunoregulatory activities mediated by a diverse repertoire of surface molecules, including CD47, Toll-like Receptor 9 (TLR9), and the Duffy Antigen Receptor for Chemokines (DARC). Within this framework, TLR9 confers the ability to sense and bind extracellular DNA, including microbial CpG-rich DNA and host-derived mitochondrial DNA released during tissue injury allowing erythrocytes to function as a circulating DNA sink that limits excessive immune activation (Lam et al., 2021). In contrast, DARC serves as a non-signalling chemokine receptor that binds a broad range of inflammatory chemokines (such as CXCL8), thereby controlling their bioavailability and influencing leukocyte trafficking (Darbonne et al., 1991).

Beyond these surface-mediated interactions, erythrocytes exert direct effector functions through redox-mediated antimicrobial activity. Their high intracellular haemoglobin concentration continuously generates reactive oxygen species (ROS) as a consequence of haemoglobin autooxidation, which produces superoxide radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) as byproducts of oxygen binding and release. Under physiological conditions, these oxidants are efficiently neutralized by the erythrocyte antioxidant machinery (Gwozdziński et al., 2021). However, during oxidative stress or pathogen exposure, this balance is disrupted, and erythrocyte-derived ROS spill into the extracellular compartment. These reactive species are highly microbicidal, allowing erythrocytes to capture and kill circulating bacteria and fungi, thereby influencing the immune microenvironment and providing a front-line defence in the bloodstream (Zhong and Yazdanbakhsh, 2018).

In addition, stressed or damaged erythrocytes release Damage-Associated Molecular Patterns (DAMPs), including free heme, haemoglobin and ATP. These molecules can trigger pro-inflammatory pathways, such as TLR4 activation, effectively transitioning the erythrocytes from a protective homeostatic buffers into a potent danger signal that coordinates and amplifies innate immune responses (Bozza and Jeney, 2020; Janciauskiene et al., 2020). Through these activities, erythrocytes participate in innate immunity and influence the behaviour of neighbouring immune cells, including monocytes.

I.2 MONOCYTE

Monocytes are a distinct population of circulating mononuclear leukocytes of myeloid origin, making up nearly 10% of the total nucleated cells in peripheral blood (Cormican and Griffin, 2020). They originate from bone marrow-derived hematopoietic stem and progenitor cells (Dash et al., 2024), and are characterized morphologically by a wide diameter (15–20 μm) approximately twice the size of red blood cells, a distinctive reniform or horseshoe-shaped nucleus, and a granule-rich basophilic cytoplasm (Goasguen et al., 2009; Prinyakupt and Pluempitiwiriyawej, 2015). They are further distinguished by a high degree of functional heterogeneity and phenotypic plasticity, enabling them to dynamically recalibrate their effector functions according to the local requirements of the tissue microenvironment (Dash et al., 2024).

I.2.1 Monocytes origin

Monocyte development is a dynamic process of monocytopoiesis that originates from HSC in the bone marrow (Salnikova et al., 2024). These pluripotent cells progressively differentiate through Multipotent Progenitors (MPPs) and Common Myeloid Progenitors (CMPs) into Granulocyte-Macrophage Progenitors (GMPs), which give rise to monocytes through intermediate precursor stages. However, recent evidence emphasizes a specialized developmental pathway in which Monocyte-Dendritic Cell Progenitors (MDPs) generate monocytes via a Common Monocyte Progenitor (cMoP), as schematically depicted in Figure I-8. This stepwise commitment is orchestrated by a tightly regulated network of transcription factors, among which PU.1 serves as a key determinant of early myeloid lineage commitment. Together with IRF8, it drives the differentiation of myeloid progenitors toward a monocyte lineage (Williams et al., 2023).

Before their mobilization via the CCR2-CCL2 axis, monocytes undergo an intramedullary maturation phase governed by a CXCR4 gradient, during which they transition from immobilized CXCR4^(high) precursors to mature CXCR4^(low) cells ready for systemic circulation (Chong et al., 2016). Once in the periphery, monocytes exhibit remarkable plasticity, with classical subsets capable of converting into patrolling cells to maintain vascular homeostasis and respond to inflammatory stimuli (Trzebanski and Jung, 2020).

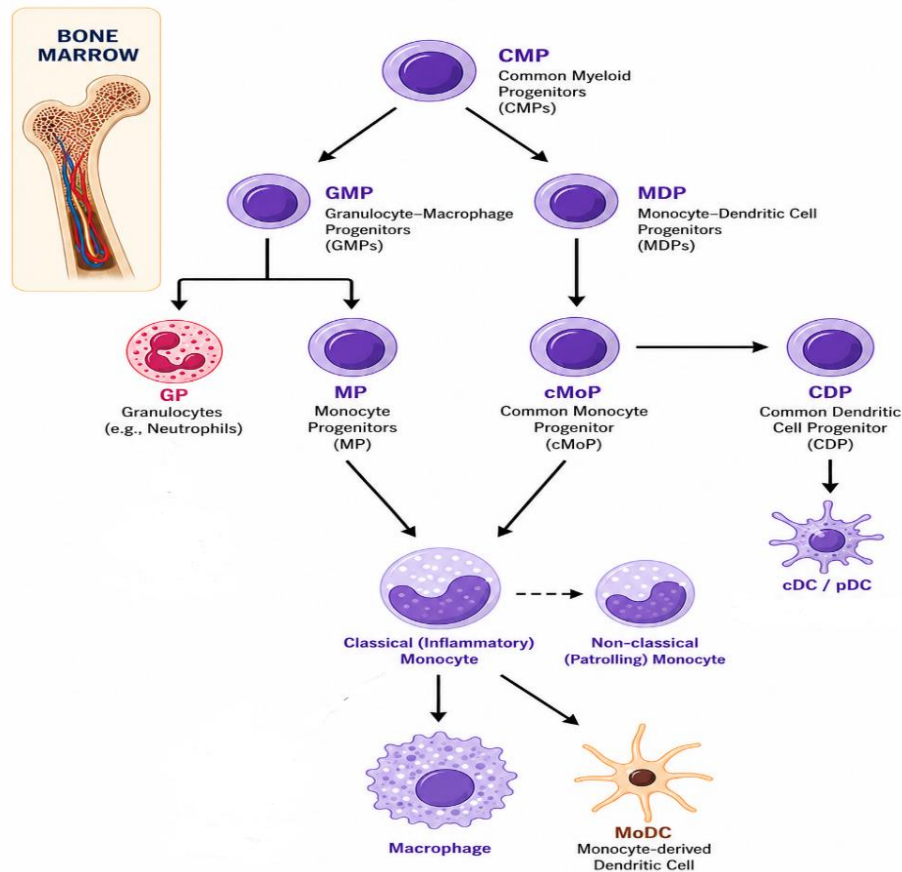


Figure I-6: Visual summary: Monocyte Lineage and Differentiation Pathways. *Monocytes arise from distinct progenitor populations, including granulocyte-monocyte progenitors (GMPs) and monocyte-dendritic cell progenitors (MDPs). Once in circulation, classical inflammatory monocytes can differentiate into non-classical patrolling subsets or further give rise to macrophages and monocyte-derived dendritic cells (moDCs).*

I.2.2 Monocyte heterogeneity and circulating phenotypes

The understanding of monocyte heterogeneity has evolved from historical classifications based on nuclear morphology or density toward a nuanced appreciation of distinct circulating phenotypes. Today, these cells are recognized as a diverse population categorized into three primary functional subsets defined by their unique surface protein signatures and specialized roles in host defence and tissue homeostasis (Williams et al., 2023).

I.2.2.1 Classical monocytes (CD14⁺⁺CD16⁻)

Comprising the vast majority of the circulating pool (85–90%), this population serves as the primary inflammatory effector subset and exhibits pronounced pro-inflammatory properties (Sampath et al., 2018). They display high surface expression of CD14 and absence of CD16, they are specialized for rapid mobilization and the robust production of pro-inflammatory

cytokines upon activation (Yang et al., 2014). Furthermore, they are sustained by a predominantly glycolytic metabolism that enables rapid activation and effector responses (Kozlov et al., 2025).

I.2.2.2 Non-Classical monocytes (CD14⁺CD16⁺⁺)

Non-classical monocytes represent a mature, terminally differentiated subset defined by low CD14 and high CD16 expression. Functionally specialized in vascular surveillance and homeostatic maintenance, they act as anti-inflammatory regulators that prioritize tissue repair and the resolution of inflammation over acute activation (Thomas et al., 2015). Unlike classical monocytes, they rely predominantly on oxidative phosphorylation, and prioritize tissue repair, regulation of neutrophil–endothelial interactions, and antiviral responses over acute inflammation (Cormican and Griffin, 2020; Khalaf et al., 2025; Xuan et al., 2023).

I.2.2.3 Intermediate monocytes (CD14⁺⁺CD16⁺)

Intermediate monocytes co-express high levels of CD14 and low-to-moderate levels of CD16, placing them phenotypically and functionally between the two major subsets. This transitional population is primarily distinguished by its potent antigen-presenting capabilities, driven by high surface expression of MHC class II molecules (HLA-DR) and costimulatory markers CD80 and CD86, which enhance T cell activation. they display a dualistic cytokine signature positioning them as key modulators at the interface of inflammatory initiation and resolution (Lee et al., 2017).

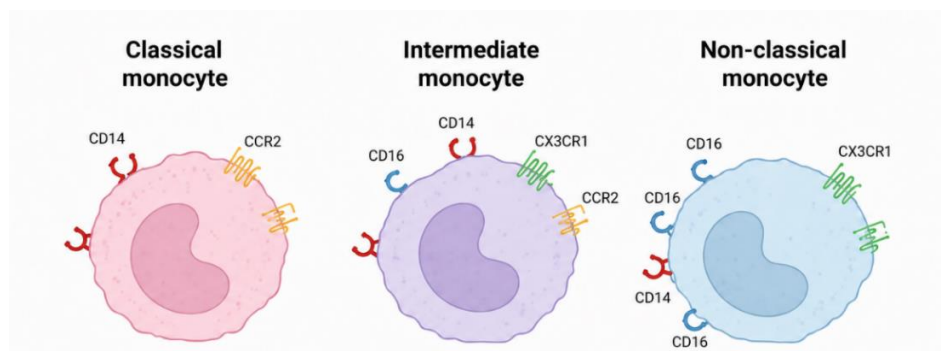


Figure I-7 Major Human Monocyte Subsets: Classical, Intermediate, and Non-Classical.

I.2.3 Monocyte functional duality in homeostasis and inflammation

Monocytes are circulating innate immune cells that exert a dual major role within the immune system, depending on their physiological state, they continuously patrol the bloodstream but also can serve as precursors to macrophages and dendritic cells (Espinoza and Emmady, 2025).

I.2.3.1 Homeostatic maintenance and sentinel surveillance

Under steady-state conditions, the monocyte pool maintains a constant equilibrium between the bloodstream and peripheral tissues. While some subsets specialize in long-term vascular patrolling to maintain endothelial integrity, others contribute to the replenishment of specific tissue-resident macrophage and dendritic cell populations (Ginhoux and Jung, 2014). This baseline "sentinel" activity ensures that tissues are equipped for debris clearance and trophic support without the induction of an overt inflammatory response (Hernandez and Iruela-Arispe, 2020).

I.2.3.2 Contextual differentiation and inflammatory plasticity

The most striking feature of monocyte biology is their "contextual plasticity". In response to inflammatory or infectious signals, circulating precursors extravasate into affected tissues where they undergo terminal differentiation into monocyte-derived macrophages (moMφ) or dendritic cells (moDC) within hours (Dash et al., 2024). This process is not a fixed path; rather represents a dynamic spectrum of functional polarization. During the early phase of infection, these cells adopt a pro-inflammatory effector phenotype characterized by potent pathogen-directed cytotoxicity, antigen presentation, and the production of reactive oxygen species and cytokines such as TNF- α and IL-1 β (Randolph et al., 2008). Subsequently, to limit tissue damage and promote resolution, they transition toward anti-inflammatory and pro-resolving phenotypes that facilitate tissue remodelling and wound healing through the secretion of immunoregulatory mediators (Arnold et al., 2007).

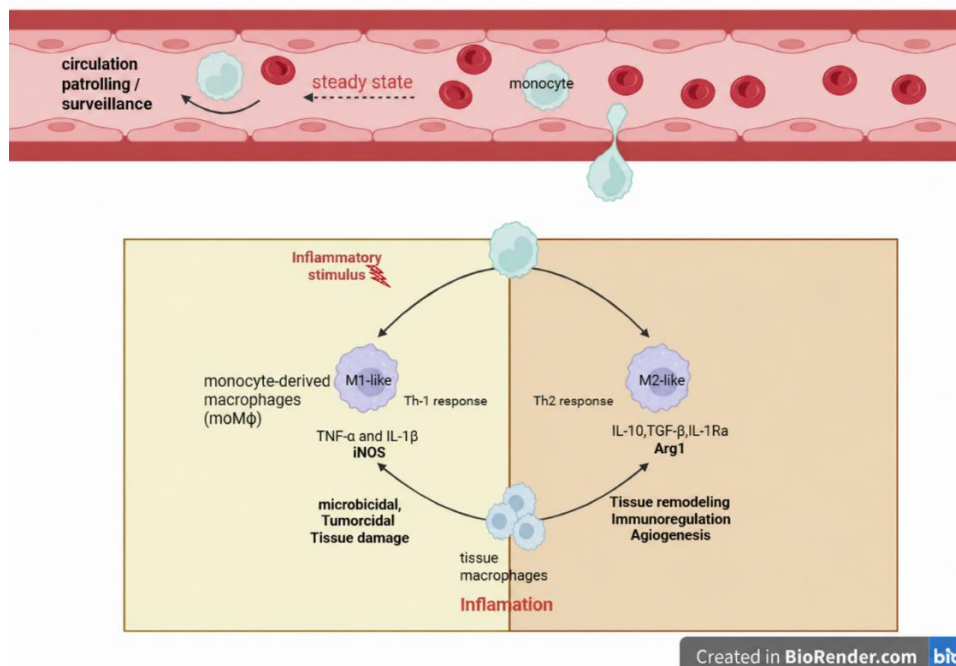


Figure I-8 : Monocyte Dynamics in Tissue Homeostasis and Inflammatory Responses.

I.3 The Role of L-Arginine in Monocyte-Lineage Plasticity

L-arginine is a conditionally essential amino acid that serves as a pivotal metabolic checkpoint controlling myeloid cell fate and effector function (McRae, 2016).

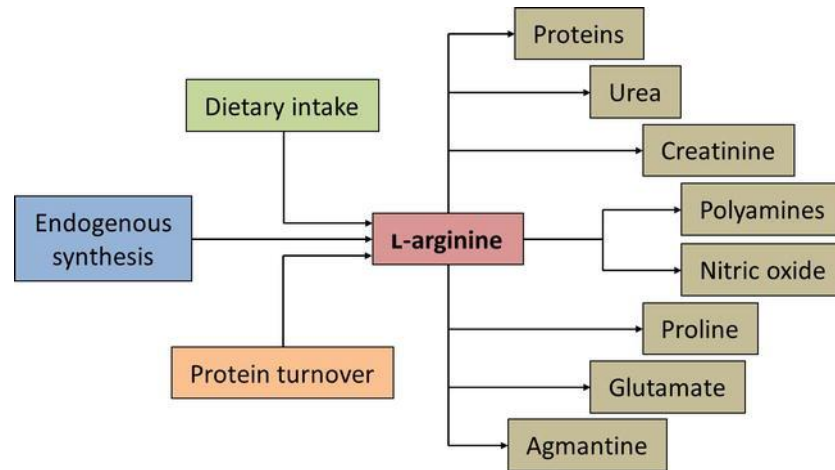


Figure I-9 L-Arginine metabolism: Key Metabolites and Sources (Gutiérrez-Kobeh and Wilkins-Rodríguez, 2019).

Within the monocyte-macrophage lineage, L-arginine serves as a shared substrate for two competing enzymatic pathways: inducible nitric oxide synthase (NOS2/iNOS), which catalyses nitric oxide (NO) production to drive pro-inflammatory and microbicidal responses, and arginases (ARG1/2), which generate ornithine to support immunoregulatory programs and tissue-repair mechanisms. This metabolic competition constitutes a central axis that governs monocyte plasticity in response to microenvironmental cues (Bronte and Zanoello, 2005).

I.3.1 The Pro-Inflammatory Pathway: iNOS

In the context of the pro-inflammatory immune response, monocytes undergo "classical" activation, adopting a pro-inflammatory (M1-like) phenotype characterized by the transcriptional induction of Nitric Oxide Synthase 2 (NOS2 or iNOS) (Thomas and Mattila, 2014b). This activation state is primarily driven by Th1 associated signals such as IFN- γ , TNF- α and IL-1 β , as well as microbial products including LPS. Once activated, monocyte-derived NOS2 catabolizes L-arginine into Nitric Oxide (NO) and L-citrulline in the presence of NADPH and molecular oxygen (Alderton et al., 2001; MacMicking et al., 1997). This reaction occurs through a two-step mechanism involving the intermediate N ω -hydroxy-L-arginine (NOHA). The resulting NO can react with superoxide anions — generated by other cellular sources such as NADPH oxidase — to form peroxynitrite, a highly reactive nitrogen species (Radi, 2013). Together, these reactive oxygen and nitrogen species (RONS) constitute a potent

cytotoxic arsenal that enables monocytes and macrophages to eliminate pathogens and contribute to tumoricidal activity (Gutiérrez-Kobeh and Wilkins-Rodríguez, 2019). This enzymatic activity is tightly regulated and can be endogenously antagonized by the arginine analog asymmetric dimethylarginine (ADMA), which acts as a metabolic brake on NO production (Förstermann and Sessa, 2012).

I.3.2 The anti-inflammatory Pathway: Arginase 1

In the context of immune resolution and tissue homeostasis, monocytes and their derivatives can transition toward an alternative, anti-inflammatory (M2-like) phenotype characterized by the induction of Arginase 1 (ARG1). This pathway is primarily driven by Th2 cytokines such as IL-4 and IL-13, which signal through the STAT6 and C/EBP β transcriptional pathways (Weinberg et al., 2016); and is further upregulated by anti-inflammatory mediators including IL-10 and TGF- β (Gordon and Martinez, 2010). Functionally, ARG1 hydrolyses L-arginine into urea and ornithine, effectively diverting the amino acid away from the pro-inflammatory NOS2 pathway. Ornithine then serves as a precursor for polyamines via ornithine decarboxylase (ODC), facilitating cell proliferation, and for L-proline via ornithine aminotransferase (OAT), supporting collagen synthesis and tissue repair (Wynn, 2004).

This metabolic shift from NOS2-dominant to ARG1-dominant pathways reflects the transition from a pro-inflammatory, microbicidal state to an immunoregulatory, tissue-repairing phenotype (Rodriguez et al., 2017).

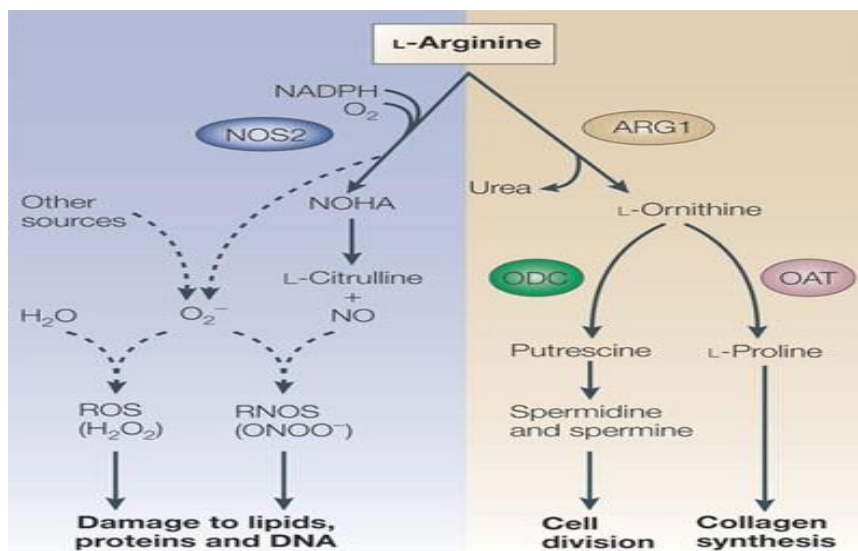


Figure I-10: Metabolic Pathways of L-Arginine: iNOS- and Arginase-1-Mediated Processes (Bronte and Zanovello, 2005). *L-arginine is metabolized by two main pathways: ARG1, which produces ornithine and supports cell growth and tissue repair; and NOS2, which generates nitric oxide (NO)*

involved in antimicrobial and inflammatory responses. When L-arginine is limited, NOS2 shifts to producing superoxide, which reacts with NO to form highly reactive molecules such as peroxynitrite. These reactive oxygen and nitrogen species constitute an important defence mechanism used by monocytes and macrophages (Bronte and Zanovello, 2005).

I.5 Problematic

Red blood cells (erythrocytes) are classically recognized as the primary carriers of respiratory gases. However, beyond this canonical function, accumulating evidence suggests that they may also participate in the modulation of immune responses notably through their influence on oxygen availability and other bioactive mediators within the cellular microenvironment, thereby exerting a substantial influence on the response of adjacent immune cells. At the same time, circulating monocytes behave as key modulators of innate immunity. Their inflammatory functions are tightly regulated by the balance between two competing enzymatic pathways within L-arginine metabolism: the iNOS/Arginase axis. Predominant activation of inducible Nitric Oxide Synthase (iNOS) promotes nitric oxide (NO) production, contributing to host defence and pro-inflammatory responses, whereas increased arginase activity favors regulatory processes and tissue repair.

The major issue lies precisely in the disruption of gas transport. As oxygen carriers, a decrease in haemoglobin levels compromises the efficiency of gas delivery to tissues. This limitation in oxygen supply constitutes a critical microenvironmental signal for monocytes.

Although interactions between respiratory gases and immune cells are well established, the direct link between reduced erythrocyte haemoglobin levels and the shift of the NO/Arginase axis in humans remains to be elucidated.

Aim

Our goal is to assess the impact of haemoglobin-mediated gas transport on the NO/Arginase balance in human monocytes.

Objective

To evaluate the activity of the NO and Arginase pathways in human monocytes in relation to variations in gas transport efficiency (as measured by haemoglobin levels).

II. Materials and methods

Confidential

III. Results and interpretation

Confidential

IV. Discussion

Confidential

V. Conclusion

Confidential

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