INVESTIGATING THE EFFECT OF HEMOGLOBIN-BASED COMPLEXES ON MACROPHAGES FOR USE IN CHRONIC WOUND HEALING

by

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ABSTRACT OF THE DISSERTATION

Investigating the Effect of Hemoglobin-based Complexes on Macrophages for Use in Chronic Wound Healing

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Chronic skin wounds are hypoxic and are stalled in a pro-inflammatory state. Hemoglobin(Hb)-based oxygen carriers have shown promise in increasing local oxygen delivery to aid in healing. Less considered in these previous studies is the ability of macrophages to take up extracellular Hb. There is evidence that macrophages that internalize Hb have an anti-inflammatory phenotype due to activation of the heme oxygenase 1 (HO-1) pathway, which may be beneficial to wound healing. Because free extracellular Hb is not stable, here we investigated two novel Hb-based oxygen carriers with respect to their ability to modulate macrophage function, and in turn promote wound healing. These include: 1) crosslinked polymerized hemoglobins (PolyHbs), and 2) Hb and haptoglobin (Hp) complexes.

PolyHbs were manufactured by polymerizing Hb in its relaxed (R) or tense (T) quaternary state, and by varying the ratio of glutaraldehyde crosslinking agent to Hb. Alternatively, Hp, Hb, or Hb-Hp complexes were tested. In our studies, primary human macrophages were stimulated with lipopolysaccharide (LPS) to create a highly-inflammatory environment, and incubated with experimental treatments. The resulting secretion profiles

consisting of 27 cytokines and growth factors related to inflammation were analyzed within a wound healing context.

For the PolyHb studies, Hb decreased the secretion of most measured factors. PolyHb treatment resulted in generally similar secretion profiles to one-another, however Hb had more similar trends with R-state rather than T-state. Ingenuity Pathway Analysis (IPA) using secretion data predicted positive outcomes in wound healing and angiogenesis for T-state PolyHb made with a 30:1 glutaraldehyde:Hb polymerization ratio. When tested *in vivo* in diabetic mouse wounds, T-state PolyHb resulted in the most improved wound healing response, as evidenced by greatest epidermal thickness and vascular endothelial CD31 staining.

For the Hb-Hp studies, unexpectedly, Hp treatment decreased a majority of inflammatory factors; Hb increased many; and Hb-Hp had intermediate trends; indicating that Hp attenuated overall inflammation to the greatest extent. From this data, IPA software identified High motility group box 1 (HMGB1) as a key canonical pathway— strongly downregulated from Hp, strongly upregulated from Hb, and slightly activated from Hb-Hp. HMGB1 measurements in macrophage supernatants confirmed this trend.

Taken together, our studies further the characterization and investigation of Hb-based therapies on macrophages in highly-inflammatory, wound healing relevant applications. The effects of PolyHb on macrophages depended upon polymerization ratio and state, and T-state PolyHb yielded secretion profiles that were most beneficial in angiogenesis and wound healing. In addition, Hp —and not Hb-Hp, which is known to be superior in non-inflammatory conditions—reduced inflammation in LPS-stimulated macrophages, and HMGB1 signaling was also implicated. Future work includes thorough analysis of the HO-1 pathway over time, by varying LPS and treatment concentrations, and measuring resulting expression of HMGB1, HO-1, IL-10, and other inflammatory factors. Future studies will also consider the oxygen delivery capability of these Hb-based therapies, particularly in hypoxic conditions that are more representative of the chronic wound environment.

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TABLE OF CONTENTS

ABSTRACT OF THE DISSERTATION ii

ACKNOWLEDGEMENTS iv

TABLE OF CONTENTS vii

LIST OF TABLES xi

LIST OF ILLUSTRATIONS xii

CHAPTER 1: INTRODUCTION 1

1.1 Clinical and economic significance of chronic wounds 1

1.2. The wound healing process and chronic vs. acute wounds 2

1.3. The general role of macrophages in wound healing 3

1.4 Origins of skin macrophages 5

1.4.1 Dermal macrophages and skin appendages 5

1.4.2 Dermal macrophages and wound healing 7

1.4.3 Monocyte recruitment and differentiation in wound healing 8

1.5 Macrophage phenotypes 11

1.5.1 Pro-inflammatory, pro-wound healing and pro-resolving macrophages 13

1.5.2 M1/M2 macrophage spectrum 14

1.5.3 Macrophage standardization efforts 18

1.6. Human vs. murine models 19

1.7. Macrophage phenotypes during acute wound healing 21

1.8. Macrophage dysregulation and chronic wounds 23

1.9 Experimental therapies and wound macrophages 27

1.9.1 Endogenous M1 macrophage attenuation *27*
1.9.2 Exogenous M2 macrophage supplementation *28*
1.9.3 Endogenous macrophage modulation/M2 phenotype promotion *30*
1.9.3.1 Mesenchymal stromal cells *31*
1.9.3.2 Growth factors *33*
1.9.3.3 Immunomodulatory biomaterials *35*
1.9.3.4 Heme oxygenase-1 induction *38*
1.9.3.5 Oxygen therapy *40*
1.10. Conclusions and future directions 40
1.11 Abbreviations 42
1.12 References 42
Chapter 2: Macrophage Modulation by Polymerized Hemoglobins: Potential as a Wound Healing Therapy 52
2.1 Introduction 52
2.2. Materials and methods 55
2.2.1 PolyHb synthesis *55*
2.2.2 Monocyte isolation and macrophage differentiation *56*
2.2.3 Cell culture *56*
2.2.4 Metabolic activity measurement *56*
2.2.5 Multiplex immunoassay *57*
2.2.6 Principal Component Analysis (PCA) *57*
2.2.7 Ingenuity Pathway Analysis (IPA) *57*
2.2.8 In vivo wound healing studies *58*
2.2.9 Histological staining *59*
2.2.10 Statistics *59*
2.3 Results and discussion 60

2.3.1 Effect of Hb/PolyHb on cellular metabolic activity of macrophages *60*
2.3.2 Net effect of Hb/PolyHb on secretion of inflammatory factors from macrophages *61*
2.3.3 Principal Component Analysis on treatment secretion profiles *64*
2.3.4 Significant effects of Hb/PolyHb on secretion of key inflammatory factors from macrophages *66*
2.3.5 Ingenuity Pathway Analysis (IPA) modeling – biological disease and function predictions *66*
2.3.6 Effect of Hb/PolyHb in in vivo murine wounds *69*
2.4 Conclusions 72
2.5 Supporting information 73
2.6 References 76
Chapter 3: Anti-inflammatory Effects of Haptoglobin on LPS-stimulated Macrophages: Role of HMGB1 Signaling and Implications in Chronic Wound Healing 80
3.1 Introduction 80
3.2 Materials and Methods 82
3.2.1 Reagents *82*
3.2.2 Monocyte isolation and macrophage differentiation *83*
3.2.3 Cell culture and metabolic activity measurement *83*
3.2.4 Multiplex immunoassay and net secretion scoring *84*
3.2.5 HMGB1 ELISA *84*
3.2.6 Ingenuity Pathway Analysis *84*
3.2.7 Wound healing scoring *85*
3.2.8 Statistics *85*
3.3 Results 86
3.3.1 Effect of treatments on cellular metabolic activity *86*
3.3.2 Overall inflammatory secretion profiles *87*
3.3.3 Most significant secretion trends *87*
3.3.4 Ingenuity Pathway Analysis and HMGB1 signaling *90*
3.3.5 Prediction of wound healing effects *91*

3.4 Discussion 92

3.5 Abbreviations 98

3.6 References 99

CHAPTER 4: CONCLUSION 103

4.1 Key Findings 103

4.1.1 Polymerizing Hb affects metabolic activity and secretion profile of macrophages 103

4.1.2 T-state PolyHb yields secretion profile that is most favorable for angiogenesis and wound healing 104

4.1.3 Hp, and not Hb-Hp complexes, attenuate inflammation to the greatest extent in LPS-stimulated macrophages 105

4.1.4 HMGB1 signaling is implicated in interactions between macrophages and Hp/Hb/Hb-Hp 105

4.2 Limitations and challenges 106

4.2.1 Primary cell culture 106

4.2.2 Haptoglobin protein source 106

4.2.3 Challenges in macrophage and chronic wound healing fields 107

4.3 Future directions 107

4.3.1 Expand macrophage characterization 107

4.3.2 Wound healing studies in humanized mice 108

4.3.3 Oxygen uptake studies 109

4.4 References 110

LIST OF TABLES

Table 1.1: Guide to Discussed Macrophage Questions/Controversies 5

Table 1.2: Macrophage Phenotypes and Characteristics..... 15

Table 1.3: Experimental Approaches to Modulate Macrophages in Wound Healing 28

Table 1.4: Frequently-used Abbreviations..... 42

LIST OF ILLUSTRATIONS

Figure 1.1: Monocyte-Macrophage Recruitment and Differentiation in Wounds 11

Figure 1.2: The Role of Macrophage Phenotypes in Wound Healing 13

Figure 2.1: Cellular Metabolic Activity vs. Hb/PolyHb Concentration..... 61

Figure 2.2: Net Analysis of Macrophage Secretion Profiles..... 63

Figure 2.3: Significant Inflammatory Factor Trends Due to Hb/PolyHb Treatment 67

Figure 2.4: IPA Modeling of Inflammatory Factor Data to Predict Biological Outcomes 68

Figure 2.5: Effect of Hb/PolyHbs (H/R:30/T:30) on Wound Closure *In Vivo*, as Compared to Vehicle-treated Controls (C)..... 70

Figure 2.6: Histological Sections from Day 35 Mice Stained with H&E..... 71

Figure 2.7: CD31 Staining on Day 35 Histological Sections to Indicate Blood Vessel Formation 72

S.I. Figure 2.1: Attached Cell Number vs. Hb/PolyHb Concentration 73

S.I. Figure 2.2: Morphology and Intracellular ROS for Macrophages Treated with Increasing Concentrations of Hb 74

S.I. Figure 2.3: PCA Details for Treatment Secretion Profiles. 75

S.I. Figure 2.4: Measurement of Intracellular ROS Following Hb/PolyHb Treatment 76

Figure 3.1: Metabolic Activity of Macrophages Following Hp/Hb/Hb-Hp Treatments..... 86

Figure 3.2: Inflammatory Secretion Profiles of Macrophages Treated with Hb/Hp/Hb-Hp 88

Figure 3.3: Significant Trends for Specific Factors from the Multiplex Immunoassay of Macrophages Treated with Hb/Hp/Hb-Hp..... 89

Figure 3.4: High motility group box 1 (HMGB1) IPA Predictions and ELISA Measurements from Hb/Hp /Hb-Hp Treated Macrophages..... 90

Figure 3.5: Predicted Implications for Hb/Hp/Hb-Hp in Chronic Wound Healing Based on Macrophage Secretion Profile..... 92

Figure 3.6: Diagram Summarizing Hypothesized Interactions between Hb/Hp/Hb-Hp Treatments, LPS, HMGB1, and Inflammatory Factors in the *in vitro* Macrophage Culture System 96

CHAPTER 1: INTRODUCTION

Note: This chapter is reproduced from the following publication written by **Paulina Krzyszczyk** (1):

Paulina Krzyszczyk, Rene Schloss, Andre Palmer, François Berthiaume. "The role of macrophages in acute and chronic wound healing and interventions to promote pro-wound healing phenotypes". *Front Physiol* **9**, 419 (2018).

1.1 Clinical and economic significance of chronic wounds

Following surgical incisions and minor lacerations, diabetic, venous and pressure ulcers are the most common wounds on a global scale (2, 3). Whereas a majority of surgical incisions and lacerations are categorized as acute wounds and often heal with minimal complications, ulcers are chronic wounds that resist healing and require expensive treatments. Furthermore, as surgical wounds become less of a problem due to the advances of minimally invasive surgery, chronic wounds are on the rise, as they often occur in growing populations, such as the elderly, obese and diabetic. In recent years, there were approximately 4.5, 9.7, and 10 million pressure, venous and diabetic ulcer wound patients globally (2, 3). The numbers of pressure and venous ulcers are rising at rates of 6-7% annually, and growth is even larger for diabetic ulcers (9%) due to the increased incidence of diabetes in the developed world. Unfortunately, the staggering number of chronic, non-healing wounds is growing much faster than the emergence of new, effective therapies.

Standard wound care involves patient and wound assessments, offloading, debridement of necrotic and infected tissue, treatment with antibiotics, and regular wound dressing changes (4, 5). Advanced therapies are available for wounds that do not improve after several weeks of standard care. These include negative pressure wound therapy, topically applied platelet-derived growth factor (Regranex), acellular extracellular matrices (Integra, Matristem, Theraskin), and

bioengineered cell-containing therapies (Apligraf, Dermagraft), to name a few. Other possible treatments include hyperbaric or topical oxygen treatment in order to restore oxygen to the wound. In the case of wounds in which infection and severe tissue damage cannot be controlled, the effects of which may otherwise be life-threatening, amputation is performed. In fact, two thirds of all lower-limb amputations are due to diabetic ulcers (6). Since many chronic wounds do not improve with standard care, treatment quickly becomes expensive with the introduction of advanced therapies, and sometimes amputation.

With so many people suffering from chronic wounds, and so many failed attempts at treating them, it is not surprising that wound care costs are also enormous. In the United States, over $25 billion dollars are spent annually on chronic wound care (6). In England, costs for pressure ulcer treatment can reach up to 6500 pounds per patient (greater than $8,000 U.S. dollars) (7). Similarly, in the United States, the average cost of Medicare spending on pressure and arterial ulcers in 2014 was $3696 and $9015 per patient, respectively—the two most expensive of all types of wounds included in the study (8). Furthermore, each amputation procedure can cost well over $35,000 (6, 9, 10). Due to the increasing prevalence of diabetes in the U.S., the total cost of diabetic ulcer care has also drastically increased in the past 20 years (8). There is an urgent need to understand the pathophysiology of non-healing wounds in order to develop effective therapies that restore their ability to resolve and heal.

1.2. The wound healing process and chronic vs. acute wounds

Chronic wounds fail to heal, despite the use of current therapies, because they are stalled in the early inflammatory state within the wound healing stages (11). In contrast, acute wounds progress through this process in a timely manner as they heal.

The wound healing process is composed of three overlapping phases: inflammation, proliferation and remodeling (12-14). After skin injury occurs, platelets are activated at the site of blood vessel rupture and promote clot formation to stop blood loss. Platelets also release factors that attract immune cells from the circulation into the wound. This marks the beginning of the

inflammatory phase. Polymorphonuclear neutrophils are first to arrive, followed by monocytes that quickly differentiate into macrophages (15). Neutrophils produce high levels of reactive oxygen species (ROS), proteases and pro-inflammatory cytokines to sanitize the wound. When this process is complete, neutrophils apoptose and become phagocytosed by the newly arrived macrophages. Macrophages will also phagocytose bacteria and debris in order to clean the wound (4). During this time, the wound is sterilized and prepared for tissue regrowth, which occurs in the proliferative phase (13). As the name indicates, wound cells proliferate and migrate during this phase, in order to regenerate the lost tissue. This includes endothelial cells, fibroblasts and keratinocytes. A preliminary, vascularized extracellular matrix (ECM), called the granulation tissue (GT), is laid down and keratinocytes migrate upon it to close the wound. During remodeling, the final phase of wound healing, ECM within the granulation tissue matures and increases in mechanical strength (5). Wound healing is complete following apoptosis of myofibroblasts and vascular cells, leaving behind a collagen-rich scar (11).

In chronic wounds, the proliferative and remodeling stages do not readily occur (11). The wound remains in the inflammatory phase, which does not favor tissue regeneration, and therefore, the wound cannot heal (4). Targeting and correcting cellular and molecular causes of prolonged inflammation in chronic wounds may be an effective method to return them to healing states.

1.3. The general role of macrophages in wound healing

There is considerable evidence that macrophages are key regulators of the wound healing process, during which they take on distinct roles to ensure proper healing. It is well-established that the phenotype of macrophages evolves with the stages of wound healing (16, 17). Initially, pro-inflammatory macrophages, traditionally referred to as “M1” macrophages, infiltrate after injury in order to clean the wound of bacteria, foreign debris and dead cells. In acute wounds, as the tissue begins to repair, the overall macrophage population transitions to one that promotes anti-inflammatory effects (traditionally and collectively referred to as “M2” macrophages), and

the migration and proliferation of fibroblasts, keratinocytes and endothelial cells to restore the dermis, epidermis and vasculature, respectively. This process will eventually close the wound and produce a scar. Macrophages also play particularly important roles in vascularization, by positioning themselves nearby newly forming blood vessels and aiding in their stabilization and fusion (18, 19). In the beginning of the final remodeling phase, macrophages release matrix metalloproteinases (MMPs) to breakdown the provisional extracellular matrix, and then apoptose so that the skin can mature to its original, non-wounded state (20). In chronic wounds, pro-inflammatory macrophages persist without transitioning to anti-inflammatory phenotypes, which is believed to contribute to the impairment in tissue repair (11, 21).

Macrophage phenotype readily changes based on spatiotemporal cues during wound healing, and several different subsets of macrophages, beyond the limited confines of simply *M1* and *M2* (22), have been defined depending on their cell surface markers, cytokine/growth factor/chemokine production, and function. **The goal of this review** is to highlight the importance of macrophages as they pertain to acute and chronic wound healing. The physiology of monocyte recruitment, macrophage differentiation and their roles in wound healing are also discussed. Evidence towards a stalled pro-inflammatory macrophage phenotype in chronic wounds is also presented. Lastly, examples are provided of several different approaches that have been taken towards attenuating pro-inflammatory (M1-like) macrophages and promoting anti-inflammatory (M2-like) macrophages in order to heal chronic wounds. It is important to note that, due to the complexity of macrophages, there are several unanswered questions and controversial topics within the field. These are discussed throughout the text, and are also summarized in Table 1.1.

Guide to Discussed Macrophage Questions/Controversies	
Topic	**Questions/Controversy in Literature**
Dermal Macrophages	· What is the contribution of tissue-resident, dermal macrophages to wound healing?
Monocyte Recruitment/ Macrophage Differentiation	· Are monocytes pre-programmed to becoming a specific macrophage phenotype prior to entering the wound and accordingly recruited when needed? · Or, does the wound microenvironment dictate monocyte differentiation/macrophage fate?
In Vitro vs. *In Vivo* Macrophages	· Do the phenotypes that are defined based on *in vitro* studies translate into *in vivo* wound macrophages?
M1/M2 Macrophages	· Do macrophages possess distinct phenotypes with unique functions or do their characteristics form a spectrum? · Can all macrophages transition from one phenotype to another? · Can wound macrophages proliferate *in situ* or are they replenished by newly-infiltrated monocytes?
Human vs. Murine Models	· How translatable are results obtained from murine models to human chronic wounds?
Macrophages and Wound Healing	· Which macrophage phenotypes/characteristics are required, and at what time, to result in effective wound healing?
Targeting Macrophages to Promote Wound Healing	· Are M2-like macrophages the answer to promoting wound healing in all situations? If so, which specific phenotypes/characteristics? · What is the ideal treatment time for chronic wounds in order to promote desired wound macrophages and wound healing?

Table 1.1: Guide to Discussed Macrophage Questions/Controversies

1.4 Origins of skin macrophages

Skin macrophages are derived from two different sources: 1) a resident macrophage population established before birth and 2) circulating monocytes that are recruited to areas of injury and differentiate into macrophages (20, 23) . The first type consists of a self-renewing pool of cells derived from the embryonic yolk sack. These cells, called dermal macrophages, are permanent residents in healthy adult skin, often found in nearby skin appendages. In contrast, during injury, bone marrow-derived monocytes are recruited to the skin, locally differentiate into macrophages and play key roles in wound healing, as discussed previously (20, 23).

1.4.1 Dermal macrophages and skin appendages

There are several types of well-studied tissue-resident macrophages throughout the body, which play important roles in their respective organs. A few examples are microglia in the brain, Kupffer cells in the liver, and alveolar macrophages in the lungs (24). Their general roles include debris clearance (e.g. surfactant in alveolar macrophages and red blood cells in Kupffer cells), initiation of the inflammatory response and the return to homeostasis. Due to these general

functions of tissue-resident macrophages, it is not surprising that tissue-resident macrophages in skin (dermal macrophages) contribute to the maintenance and renewal of skin appendages during homeostasis, and wound healing.

Dermal macrophages are located in close proximity to hair follicles, in the surrounding connective tissue sheath and help regulate the hair growth cycle (25-27). One of the activities of macrophages during hair growth is phagocytosis of collagen, to allow for matrix remodeling (28). In a murine model, Castellana *et al.* 2014 found that apoptosis of skin-resident macrophages activated epithelial hair follicle stem cells, which contribute to hair regeneration (25). Macrophage-specific Wnt-signaling was shown to be central to this process; when it was inhibited, hair follicle growth was delayed. Apoptosis of macrophages occurred immediately prior to hair follicles' transition from telogen to anagen—the hair cycle's resting and growth phases, respectively. Although the study did not use a wound healing model, the results have potential implications in regenerating hair in healing skin. In contrast, Osaka *et al.* 2007 used a wound model (full-thickness murine wounds) to study signaling pathways and macrophage activation during subsequent hair growth (29). They found that apoptosis signal-regulating kinase 1 (ASK1) is important for efficient hair regrowth; ASK1-deficient mice exhibited delayed hair regeneration following wounding. ASK1 has previously been shown to be increased in the epithelial layer of wound peripheries in rats (30). ASK1-deficient mice also had dysregulated macrophage function; less macrophages were recruited to the wound site and several chemotactic and activating factors (IL-1β, TNF-α) were downregulated (29). When bone-marrow derived, cytokine-stimulated macrophages were introduced to the wounds via intracutaneous injection in both ASK1+ and ASK1- mice, hair growth was stimulated.

These studies highlight the importance of dermal macrophages in hair growth, which can have many implications in the development of future therapies that promote wound healing along with hair regeneration. Although there is more research linking macrophages to hair follicles rather than sweat or sebaceous glands, there is still evidence that macrophages can also respond to

cues in the microenvironment created by these appendages. For example, the type of lipids produced by sebocytes can impact whether local macrophages take on pro- or anti-inflammatory characteristics, which could potentially impede or promote healing in that area (31). Overall, appendage regeneration remains one of the biggest challenges in wound repair (32). Large wounds that are able to heal have a lack of hair and are unable to produce sweat and oil, which leads to cosmetic deficiencies and discomfort to patients. In general, the relationship between macrophages and skin appendages warrants attention, specifically in the context of wound healing.

1.4.2 Dermal macrophages and wound healing

A proposed role for tissue-resident macrophages during injury is that they serve as early indicators of injury or invading pathogens. Some of these macrophages express CD4 and are located near capillaries (23). They are first-responders to injury by recognizing damage-associated molecular patterns (DAMPs; *e.g.* free heme, ATP) and releasing hydrogen peroxide, which initiates a powerful pro-inflammatory cascade (33). In the case of infection, tissue-resident macrophages recognize pathogen-associated molecular patterns (PAMPs; *e.g.* lipopolysachharide, LPS). Responses to DAMPs and PAMPs result in the recruitment of neutrophils to help fight early infection (23, 33). Monocyte-derived macrophages are also recruited to the wounded area to further amplify the inflammatory response (24). Although tissue-resident macrophages aid in the recruitment of immune cells, they are not the only cells in the wound (*e.g.* platelets) that produce chemokines and signals that have this effect. In general, dermal macrophages can be identified by several surface markers, such as CD64+, MERTK+ and CCR2-/low. They are also highly phagocytic and have a slow turnover (23). Near the end of wound healing, during resolution, dermal macrophages self-renew and clear apoptotic cells as the tissue returns to homeostasis (24).

In addition to macrophages, there are also dendritic cells in the skin that are derived from monocytes (e.g. Langerhans cells). These cells share many surface markers with macrophages, including MHCII, F4/80, CD14 and IL-10, which can make it difficult to distinguish them from

each other (23, 33). Some even consider Langerhans cells as a type of tissue-resident macrophage, as they have a similar gene expression profile (24, 33-35), and interestingly, a correlation between healing diabetic foot ulcers and increased numbers of Langerhans cells has been reported (35, 36). The specific role of Langerhans cells in wound healing—particularly chronic—has yet to be defined, however, they do repopulate the epidermis during re-epithelialization in acute wound models (36).

1.4.3 Monocyte recruitment and differentiation in wound healing

Whereas dermal macrophages initiate the local inflammatory response and have relatively short-term effects, monocyte-derived macrophages are systemically recruited approximately 24 hours post-wounding (in mice) in order to heighten the inflammatory response and protect the tissue from further damage (33, 37). Monocyte-derived macrophages are initially recruited to the wound by signals from damaged tissue via DAMPs or PAMPs (15, 18, 38). For example, lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is a PAMP that macrophages recognize via binding with toll-like receptor 4 (39). This signaling pathway activates the transcription factor, NF-κB, which leads to expression of pro-inflammatory genes. Extracellular DNA, RNA and ATP, released due to cell death, are examples of DAMPs that signal immune cells and attract them to injury sites (40). Monocytes can also be recruited to the wound by chemokines and cytokines downstream of DAMPs/PAMPs, such as IL-1, IL-6, TNF-α and CCL2 (MCP-1), although in mice, CCL3(MIP-1α) and CCL4(MIP-1β) play this role (41).

Multiple monocyte types, categorized as pro-inflammatory and anti-inflammatory, are attracted to the wound site (18). The former, sometimes defined as “classical” monocytes, are derived from the bone marrow and spleen, increase in concentration in the bloodstream following injury, and are $CD14^{+}CD16^{-}$ (human) or $Ly6C^{+/high}$ (mice) (18, 42). Surface cell adhesion molecules, such as the $\alpha_4\beta_1$ integrin and CD62L, are responsible for recruiting these cells from the circulation to the blood vessel wall. When there is no injury, pro-inflammatory monocytes do

not tightly adhere. However, in the vicinity of the wound, the local presence of inflammatory chemokines and cytokines, such as CCL2(MCP-1), TNF-α, and IFN-γ, promotes expression of cell adhesion molecules. This facilitates the firm adhesion of pro-inflammatory monocytes to the endothelium and subsequent translocation into the tissue space. In addition to extravasation, another mechanism of monocyte recruitment to wounds is by entering through micro-hemorrhages in damaged blood vessels (33, 43). With a half-life of only 20 hours (in mice), the numbers of pro-inflammatory monocytes fluctuate with the supply of new cells recruited from the bone marrow and circulation, but reach a peak approximately 48 hours after injury (18, 44). The second type of recruited monocytes consists of anti-inflammatory monocytes, which have a longer half-life (>2 days, in mice). Human markers include $CD14^{low/-}CD16^{+}$ and for mice, $Ly6^{-/low}$. These cells attach to the blood vessel wall via $\alpha_L\beta_2$ integrin (LFA-1) and L-selection (CD62L). The expression of CD62L enables anti-inflammatory monocytes to crawl on the endothelium even during homeostasis, so that they are nearby to aid in tissue and vascular repair when needed (18, 42, 45). This suggests that, in addition to tissue-resident macrophages, "resident" monocytes may exist as well. Interestingly, pro- and anti-inflammatory monocytes in mice are attracted to areas of inflammation via different signals: CCR2 vs. CX3CR1-dependent pathways, respectively (18, 37).

Others have used a different nomenclature to group human monocytes into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{dim}CD16^{++}$) and non-classical ($CD14^{++}$, $CD16^{+}$) phenotypes. The classical phenotype is analogous to the pro-inflammatory phenotype previously described, whereas the non-classical phenotype is analogous to anti-inflammatory monocytes (42). Each subset exhibits a different morphology following tissue culture, with classical being the largest and roundest, and non-classical being the smallest and having poor attachment. They each have distinct secretomes and respond to different stimuli to varying degrees. For example, classical and intermediate monocytes readily respond to bacteria-associated signals, whereas non-classical monocytes are much less responsive (46). All monocyte subsets are capable of differentiating into

M1 and M2 macrophages *in vitro*, however M1 macrophages derived from classical monocytes are the most phagocytic and hence, the "most M1-like" (42). Interestingly, non-classical monocytes can differentiate into macrophages even in the absence of differentiation media. This may support one of the proposed models that monocytes themselves transition from classical to non-classical, before differentiating into macrophages (18, 42). So, in addition to the existence of several monocyte phenotypes, each possesses varying potentials to differentiate into different macrophage phenotypes as shown through these *in vitro* studies. This adds further complexity in understanding monocyte recruitment/macrophage differentiation in *in vivo* wound healing, where this process is also not entirely clear.

In humans, at homeostasis, approximately 85% of blood monocytes are classical, 5% are intermediate and 10% are non-classical (37). In inflammatory conditions, classical monocytes differentiate into M1-like macrophages whereas non-classical monocytes aid in tissue repair and differentiate into M2-like macrophages (Figure 1.1, Process 1). Accordingly, classical monocytes are recruited to wounds to a higher extent following injury compared to non-classical monocytes. There is also evidence that classical monocytes are recruited to the skin for their pro-inflammatory effects, can become non-classical monocytes and eventually differentiate into M2-like macrophages (18, 47). With several different monocyte and macrophage phenotypes, the recruitment and differentiation processes are complex, especially within dynamic wound microenvironments. It is not surprising that several models of monocyte recruitment and macrophage differentiation during injury have arisen. Although not exhaustive, a few widely discussed models are depicted in Figure 1.1.

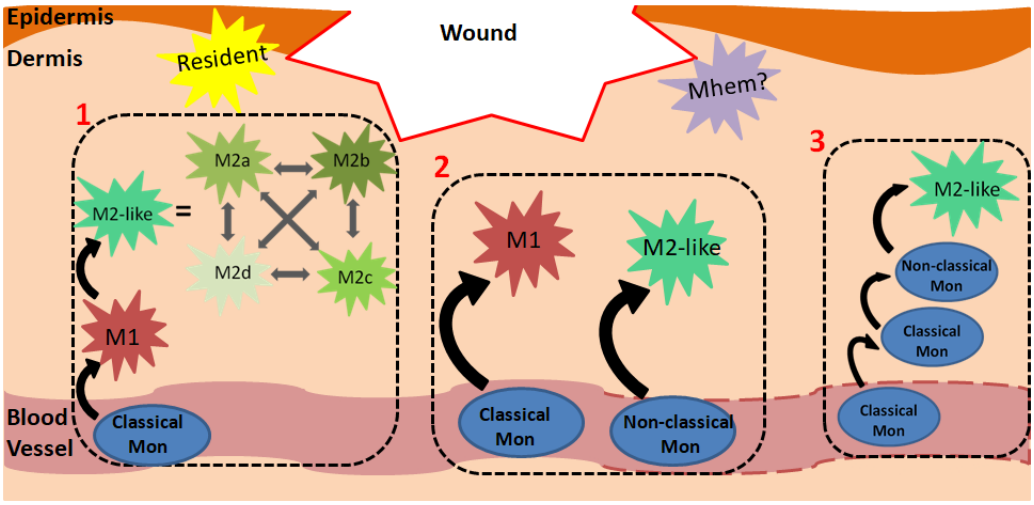

Figure 1.1: Monocyte-Macrophage Recruitment and Differentiation in Wounds

The mechanism of monocyte recruitment and macrophage differentiation during dermal wound healing can vary depending on spatiotemporal cues. A few models are presented: 1) Classical monocytes in the circulation are primed to differentiate into M1 macrophages following extravasation. In the wound microenvironment, they respond to spatiotemporal cues and can differentiate into any of the M2-like phenotypes, which can transdifferentiate into one another. For brevity, M2a, b, c and d phenotypes are also categorized as M2-like in the remaining processes. 2) Classical monocytes can differentiate into M1 macrophages in the wound. In contrast to the first model, in this panel, macrophages retain the M1 phenotype without further differentiating to M2-like macrophages. Similarly, non-classical monocytes are primed to differentiate into M2-like macrophages and can retain this phenotype. This panel suggests that the final macrophage phenotype is predetermined by the starting monocyte phenotype, and an M1/M2 transition does not occur. 3) This model shows that classical monocytes, rather than macrophages, can also persist in the wound environment for several days, and at a later time, differentiate into non-classical monocytes and then M2-like macrophages. Dashes on the blood vessel indicate that monocytes can exit damaged vasculature via micro-hemorrhages. The yellow star-shape represents resident macrophages, which are established during embryonic development. The purple star-shape represents a possible Mhem phenotype in wounds (analogous to that found in atherosclerotic plaques) which breakdowns hemoglobin and releases anti-inflammatory factors.

1.5 Macrophage phenotypes

General markers for wound macrophages include $CD14^+$, $FXIIIA^+$, F4/80 (in mice), CD68 (macrosialin) and lysozyme M (LYZ2). Macrophages can also be identified by their relatively strong autofluorescence, which differentiates them from similar $CD14^+$ monocyte-derived dendritic cells (23, 48). In general, primary macrophages have limited proliferative capabilities *in vitro*, although there is evidence that dermal macrophages can self-renew *in vivo* (24). In contrast, it is not clear whether monocyte-derived macrophages proliferate *in vivo*, or if

they are simply recruited to the site of injury as needed, and apoptose following healing (37, 49). Furthermore, their proliferative capability may depend on the particular microenvironment or stage of healing (37, 50).

Two categories of macrophages have been traditionally defined—classically activated, *M1 macrophages* and alternatively activated, *M2 macrophages*. M1 macrophages are typically associated with pro-inflammatory events, whereas M2 macrophages are recognized as anti-inflammatory and pro-regenerative. However, accumulating data suggest that this bipolar M1/M2 definition is grossly oversimplified. It is important to note that M1 and M2 macrophages are not distinct categories, however they form a spectrum in which cells possess varying degrees of M1- or M2-like qualities (22). In support of this view, *in vivo* studies suggest that a heterogeneous population of macrophages exists, with each cell exhibiting a variety of M1 and M2 characteristics (18). Some have even described macrophage activation as a "color wheel", with classically-activated, wound healing and regulatory macrophages as the primary colors, and the secondary colors representing intermediate macrophage phenotypes (17). As a result, many additional subpopulations of macrophage phenotypes have been described and defined in the literature.

Before discussing the specifics of M1- and M2-like macrophages, a different categorization will be presented—one that separates macrophage phenotype based on their role within the wound healing process. In regards to this review, this categorization is more relevant and intuitive, although it is not as widely accepted as the M1/M2 spectrum. Discrete M1/M2 phenotypes are useful *in vitro* when the stimulating molecule is known and experimentally introduced to the system, however this nomenclature is less applicable when discussing *in vivo* macrophages in a wound healing context (51). All of the macrophages associated with wound healing across both *in vitro* and *in vivo* classification systems are presented in Figure 1.2, along with their respective roles.

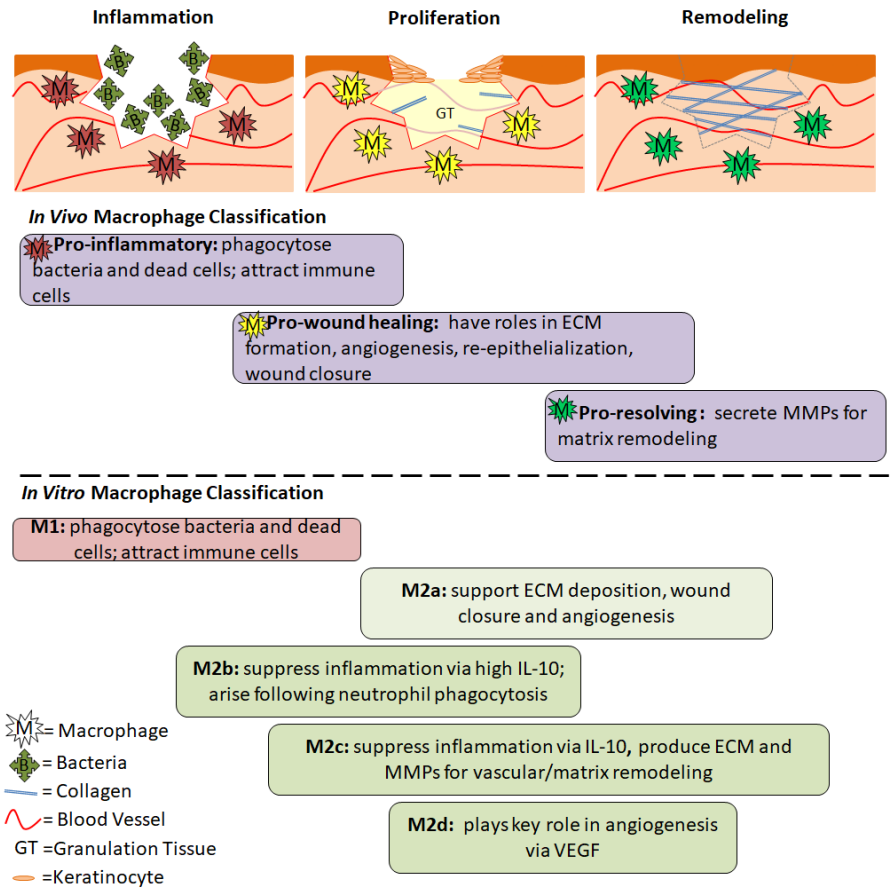

Figure 1.2: The Role of Macrophage Phenotypes in Wound Healing

Acute wounds progress through the phases of inflammation, proliferation and remodeling as they heal. In **inflammation,** pro-inflammatory macrophages are present. Their role is to phagocytose dead cells and bacteria and prepare the wound for healing. In **proliferation**, pro-wound healing macrophages are present. They secrete factors that aid in angiogenesis, formation of granulation tissue, collagen deposition and reepithelialization. In **remodeling**, pro-resolving macrophages aid in breakdown of the provisional granulation tissue to allow for maturation of collagen and strengthening of the newly regenerated skin.

Below the diagrams are the general roles and timing of different macrophage phenotypes during the wound healing process. Differences between *in vivo* and *in vitro* classifications are separated by the dashed line, however similar roles can be seen between many of the phenotypes. The timing is an estimate based on the role of each phenotype, and has not been experimentally confirmed.

1.5.1 Pro-inflammatory, pro-wound healing and pro-resolving macrophages

In agreement with the phases of wound healing, pro-inflammatory macrophages are present shortly after a wound is formed, followed by pro-wound healing macrophages that support cellular growth and proliferation, and finally pro-resolving macrophages that drastically

down-regulate the immune response and aid in collagen reorganization and maturation (20, 49). Pro-inflammatory macrophages produce nitric oxide, ROS, IL-1, IL-6 and TNF-α. They also secrete MMP-2 and MMP-9 in order to break down the extracellular matrix and make room for infiltrating inflammatory cells (49). Pro-wound healing macrophages produce elevated levels of growth factors such as PDGF, insulin-like growth factor 1 (IGF-1), VEGF and TGF-β1 (20, 49), which aid in cellular proliferation, granulation tissue formation and angiogenesis. They also produce tissue inhibitor of metalloproteinases 1 (TIMP1) in order to counteract MMPs and allow for ECM formation (49). Pro-resolving macrophages (aka regulatory macrophages) suppress inflammation via upregulation of IL-10. They also express arginase 1, resistin-like molecule-α (RELMα) programmed death ligand 2 (PDL2) and TGF-β1. MMPs (some evidence pointing towards MMP-12 and MMP-13 specifically) are produced to remodel and strengthen the ECM (20, 21). The function of pro-resolving macrophages is to restore homeostasis while minimizing fibrosis via apoptosis of myofibroblasts, suppression of T cell proliferation and a return to balanced MMP/TIMP levels (49). Just as wound healing phases overlap, these different macrophages also share some characteristics with one another. This is especially true for pro-wound healing macrophages which fall between the early and late phases of wound healing, and therefore exhibit characteristics similar to both pro-inflammatory and pro-resolving macrophages.

1.5.2 M1/M2 macrophage spectrum

Analogous to pro-inflammatory macrophages, M1 macrophages dominate during the pro-inflammatory phase of wound healing, and through their highly phagocytic behavior, serve the role of sanitizing the wound and clearing it of dead tissue. M1 macrophages also activate other immune cells during the early phase of the wound healing process. *In vitro,* M1 macrophages are stimulated by intracellular proteins and nucleic acids released from lysed cells (*e.g.* IFN- γ), and bacterial components, such as LPS and peptidoglycan (16). M1 macrophages express CD86, and produce high levels of reactive oxygen species (ROS) and pro-inflammatory cytokines, such as interleukins 1 and 6 (IL-1, IL-6), TNF- α, and IFN-γ.

Traditionally defined *M2 macrophages* serve a regenerative role. M2 macrophages are stimulated by IL-4 and IL-13 and express high levels of the mannose receptor (CD206), dectin, interleukin 10 (IL-10) and transforming growth factor β (TGF-β). They produce low levels of pro-inflammatory cytokines such as TNF-α, IL-12 and CXCL8(IL-8) (16). It has also been found that interferon regulator factors (IRF4/IRF5) are transcriptional regulators that play a role in differential signaling seen between M2 and M1 macrophages, respectively.

M2 macrophages have been divided into different subtypes according to differential expression of surface markers. These subtypes have been traditionally used and identified *in vitro* to study M2-like macrophages with different characteristics. In *in vivo* studies, this nomenclature is not as widely used to identify macrophages, potentially due to the heterogeneous populations present, which are generated from a variety of stimuli within wounds (51). Table 1.2 identifies the different names and markers for each macrophage phenotype. The table is not comprehensive, and it is important to note that marker expression for each phenotype can vary from study to study, hence adding to the complexity and difficulty of defining macrophages.

Phenotype	Other Nomenclature	Activation Molecule	Markers	Other Notes
M1	classically activated; pro-inflammatory	--	Surface: CD86, CD68, CD80, MHC-II Secreted: TNF-α, IL-6, IL-12, IL-1β	abundant and persistent in chronic wounds activated *in vitro* by LPS, peptidoglycans and pro-inflammatory cytokines
M2	all M2-phenotypes collectively: alternatively activated; anti-inflammatory	--	--	--
M2a	alternatively activated; wound healing	M(IL-4)	Surface: CD206, arginase (mice), Ym1 (mice) CD163, MHC-II, CD209 Secreted: TGF-β, IL-10, IL-1RA	aid in ECM formation, angiogenesis
M2b	type 2; regulatory	M(Ic)	Surface: CD86, MHC-II Secreted: TNF-α, IL-1, IL-6, IL-10	similar to M1 macrophages, but dampen inflammation
M2c	deactivated; pro-resolving?	M(IL-10), M(GC), M(GC+TGF-β)	Surface: CD86, CD163, CD206 Secreted: IL-10, CD206, TGF-β, MMP-9	involved in vascular and matrix remodelling some shared characteristics with Mhem
M2d	--	--	Secreted: VEGF, IL-10, IL-12, TNF-α, TGF-β	pro-angiogenic activated *in vitro* by stimulating adensine and toll-like receptors
Mhem	HA-Mac; Heme-directed macrophage	M(Hb)	Surface: CD163, CD206 Secreted: IL-10 Internal: HMOX-1 gene, activating transcription factor (ATF)	found near hemmorrhaged vessels in atherosclerotic plaques anti-inflammatory effects
M4	CXCL4 derived macrophage	--	Surface: CD206, CD86, Lack CD163 Secreted: IL-6, TNF-α	associated with atherosclerosis in human models M1-like low phagocytosis
Mox	Oxidised phospholipid derived macrophages	--	Surface: ↓arginase-1 Secreted: ↓MCP-1, ↓TNF-α Internal: HMOX-1 gene, HO-1, sulfiridoxin 1, theoredoxin reductase-1	associated with atherosclerosis in murine models low phagocytosis antioxidant properties
TAMs	tumor-associated macrophages	--	Surface: CD163, CD206, CD204 Secreted: IL-10, MIF, CXCL12, VEGF, IL-6, IL-23, TGF-β Internal: HIF-1α	located nearby tumors promote angiogenesis and cell proliferation M2-like

Table 1.2: Macrophage Phenotypes and Characteristics

The previously described M2 macrophages, also known as wound healing macrophages, align with what is now defined as the *M2a subset* (18). M2a macrophages are stimulated by IL-4/IL-13 and exhibit IL-4 receptor α (IL-4Rα) signaling (16). CD206 is a distinguishing surface marker and they produce high levels of arginase-1 (in mice), PDGF-BB, IGF-1 and several chemokines (CCL17, CCL18, CCL22) (18). M2a macrophages produce collagen precursors and factors that stimulate fibroblasts. Thus, M2a macrophages play a key role in ECM formation, which is required during the proliferative phase of wound healing. They also secrete high levels of PDGF, which is involved in angiogenesis (52).

M2b macrophages, which express CD86, CD68 and MHCII, are stimulated by immune complexes and TLF/Il-1 agonists (18, 21). They are also known as type 2 macrophages. M2b macrophages suppress inflammation by increasing IL-10 production, although they also secrete IL-6, IL-β and TNF, and express high levels of iNOS. M2b macrophages also produce several different MMPs. *In vitro*, macrophages take on an M2b phenotype following phagocytosis of apoptotic neutrophils (53).

M2c macrophages are stimulated by glucocorticoids, IL-10 and TGF-β (54, 55). They express CD206 and MERTK. M2c macrophages produce high levels of IL-10, MMP-9, IL-1β and TGF-β, and low levels of IL-12. M2c macrophages also express CD163, which is the hemoglobin receptor. This is important to note, as there exists another macrophage phenotype, called Mhem, that is similarly characterized by high CD163 expression and IL-10 production, albeit stimulated by hemoglobin and typically identified in atherosclerotic plaques (56). These shared features may indicate that different stimuli can elicit the same, or very similar, macrophage phenotypes that are nevertheless referred to by different names (M2c vs. Mhem). Furthermore, due to their production of ECM and MMPs and hence, their matrix remodeling capability, M2c macrophages may be analogous to the aforementioned pro-resolving macrophages. M2c macrophages are also sometimes referred to as deactivated macrophages as

they can arise from M1 macrophages that have "deactivated" the M1 gene profile to become M2c macrophages. M2 macrophages can shift between *a, b* and *c* phenotypes (18).

In contrast to *M2a* macrophages, *M2d* macrophages do not have either elevated mannose receptor (CD206) or dectin-1 expression (16). M2d macrophages arise from stimulation by IL-6 and adenosine. They produce high levels of vascular endothelial growth factor (VEGF) as well as IL-10 and TGF-β. They also down-regulate pro-inflammatory TNF-α and IL-12. M2d macrophages are activated by concurrent stimulation of toll-like receptor (via IL-6) and adenosine A_{2A} receptors (16, 54).

Several other macrophage types have been defined, however, they tend to be associated with specific diseases, such as atherosclerosis or cancer (57, 58). For example, Mox, M4 and Mhem arise from macrophages stimulated by oxidated phospholipids, CXCL4 and hemoglobin-haptoglobin complexes, respectively. Although these phenotypes are not typically associated with chronic wounds, it is possible that some wound macrophages have some shared characteristics, especially in regards to Mhem, as hemoglobin-haptoglobin receptor (CD163) expression and cellular regulation of iron are associated with wound healing (41, 59, 60). There also exist cancer-specific macrophages, called tumor-associated macrophages (TAMs), which support tumors by stimulating angiogenesis, aiding in metastasis and inhibiting T-cell anti-tumor responses (61). They can differentiate from resident progenitor cells, but are more often derived from recruited monocytes from the blood stream. TAMs are more similar to M2 macrophages, as they produce anti-inflammatory cytokines and promote proliferation and growth to support the tumor microenvironment. These additional, disease-specific macrophages underline the unique plasticity and range of phenotypes and functions that macrophages possess and can exhibit in different microenvironments.

Overall, macrophage nomenclature within this vast spectrum is not yet agreed upon and it is unclear whether the phenotypes are distinct, or even applicable to *in vivo* wound healing (22). It is important to remember that the macrophage population during wound healing is complex;

wound macrophages can take on a different phenotype depending on several factors, such as the anatomical location of the wound (foot, lower back), the specific region within the wound (center/edge), the environment (moist, dry) and whether or not the wound is infected (16). Unsurprisingly, it is still unclear the exact signals and differentiation cascade required to produce a specific macrophage phenotype (M2a vs M2b vs M2c, etc.; see Figure 1.1). Adding further complexity to this question is the fact that these phenotypes exist on a spectrum, and macrophages can easily transition from M1-like to M2-like (and M2a-like, M2b-like etc.). Furthermore, wound macrophage populations are heterogenous, as it is possible for pro- and anti-inflammatory cytokines to be present simultaneously (22, 51). Interestingly, although differentiation of M2a macrophages is stimulated by IL-4 and IL-13 *in vitro*, these cytokines are not present in healing murine wounds that contain M2-like macrophages (62), further underlining the disconnect between *in vitro* and *in vivo* models. Although the defined macrophage definitions are useful *in vitro*, they must be regarded with caution when considering macrophage phenotypes in the *in vivo* wound healing process.

1.5.3 Macrophage standardization efforts

Murray *et al*. 2014 acknowledge the complexity of the current macrophage nomenclature and provide suggestions for improvement moving forward (63). The authors met to attempt to set a foundation towards consolidating and standardizing the wealth of macrophage activation terms and methods that have arisen throughout the years.

Their recommendations include:

1. differentiating murine or human bone marrow/peripheral blood monocytes with CSF-1 or GM-CSF to generate macrophages, and using post-differentiation stimuli IFN-γ and IL-4 to obtain M1 and M2 macrophages, respectively;

2. reporting defined metrics such as tissue culture conditions, media, time, etc., to characterize *in vitro* macrophage cultures;

3. defining the activator for *in vitro* macrophages using the following notation: M(LPS), M(IFN-γ), M (IL-10), etc. and referencing a provided spectrum of M1/M2-like characteristics;
4. the avoidance of the term "regulatory macrophages", as well as the use of GM-CSF to create M1 macrophages and CSF for M2 macrophages; and
5. use of a combination of markers (cytokines, chemokines, scavenger receptors and more) to describe macrophage state.

The authors not only discussed how to define *in vitro* macrophages, but also macrophages isolated from *in vivo* models. A main point includes encouraging scientists to detail the isolation process in publications. Researchers should also characterize *ex vivo* macrophages and attempt to fit them within the *in vitro* macrophage spectrum defined in the article, in a manner similar to that depicted in Figure 1.2. They also acknowledge the differences between interspecies macrophages, and suggest thorough side-by-side comparisons in order to glean information about human macrophage behavior. With more characterization and understanding, scientists will begin to bridge the gap between macrophages from different sources and species.

These guidelines were a vital starting point to tackling the complex challenge of streamlining macrophage nomenclature and research/reporting practices. These standards should be broadly distributed, and scientists should regularly meet to update them. As a result, understanding of macrophage function and behavior will improve across the entire field. This may prime faster advancement in the development of therapies that target macrophages, within chronic wound healing applications and many others.

1.6. Human vs. murine models

Mice are commonly-used animal models for wound healing studies due to their affordability and ease-of-use, however, it is important to acknowledge differences between human and murine skin anatomies, wound healing processes, and immune systems (and hence, macrophage behaviors) (50). In terms of anatomy, mice have more densely-packed hair follicles

and thinner epidermal and dermal layers compared to human skin (64). It is also generally believed that murine skin heals by contraction—that is, the edges of the wound pull in towards each other, like a drawstring bag, in order to quickly close. In contrast, human skin heals by re-epithelialization, during which keratinocytes crawl over the granulation tissue in order to close the wound. This assumption has recently been revisited, to argue that mice heal both by contraction and re-epithelialization, making them better models for wound healing than previously assumed (65).

Diabetic mice are used as *in vivo* chronic wound models, as they exhibit delayed wound healing. Mice are either bred to contain a genetic mutation which results in a diabetes-like phenotype, or it is induced via chemical means, for example, injection with streptozocin (66). Diabetic mouse wounds share several key characteristics in common with chronic wounds in diabetic patients (67). These include decreased nerve count, angiogenesis, granulation tissue formation and collagen content compared to acute wounds. They both contain higher levels of MMPs and lower levels of TGF-β1, IGF-1, and PDGF. More is actually known about diabetic mouse wounds compared to human diabetic ulcers, due to an increased number of studies and an increased ability to probe and measure tissue characteristics (particularly *ex vivo*). So, whereas there are several studies showing decreased VEGF, FGF and KGF in diabetic mouse wounds, in human diabetic ulcers, there is both an incomplete panel of measured cytokines and growth factors, as well as less significant trends due to large biological variability.

One discrepancy between murine and human macrophages is their expression of inducible nitric oxide synthase (iNOS) (68). Mouse macrophages readily express iNOS in response to LPS or IFN-γ, and for this reason, it is recognized as an M1 marker in mice. Human macrophages, however, do not over-express iNOS in response to these same stimuli. General markers to identify murine and human macrophages differ as well. In humans, they are CD14 and CD33, and in mice, they are F4/80 and CD11b. Other murine-specific M2 markers include Ym1, FIZZ1 and arginase-1 (54). Human and mouse macrophages also express different FcR and IgG

receptors, which play a bigger role in the immune system as a whole, by linking the adaptive and innate immune systems (68).

The function of specific receptors can also differ between species (68). For example, CD163 is a common M2-like macrophage marker that functions as the hemoglobin-haptoglobin receptor (69). In humans, the binding of hemoglobin and haptoglobin significantly increases endocytosis of hemoglobin and activation of downstream signaling pathways. In mice however, haptoglobin does not promote binding of hemoglobin to CD163. Although this may seem insignificant, it is just one specific example of how human and murine macrophages have different mechanisms and behaviors. To overcome these discrepancies, there is a need to conduct thorough, side-by-side experiments (*e.g.* single-cell and bioinformatics approaches) using monocytes and macrophages from different species and sources (50). Through these efforts, well-informed comparisons can be made across models while taking advantage of their other benefits (affordability, ease-of-use, etc.).

In addition to specific differences between human and murine skin and macrophages, on a whole, it is important to remember that, although diabetic mice are slower to heal than wild type mice, they do eventually heal. Diabetic mice are not an ideal model for non-healing, chronic wound studies, but they do have many fundamental similarities on the tissue and cellular levels, making them a widely-accepted model in current wound healing research (66).

1.7. Macrophage phenotypes during acute wound healing

Except for fetal wounds, which have the capacity to regenerate in the absence of inflammatory response, macrophages are vital for successful adult wound healing (70, 71). Studies have shown that the depletion of macrophages in wounded mice results in delayed re-epithelialization, reduced collagen formation and impaired angiogenesis (72, 73). These effects occurred along with increased levels of TNF-α and decreased VEGF and TGF-β1. Furthermore, in the absence of macrophages, there was a prolonged neutrophil presence and reduced wound contraction (73).

Although the general importance of macrophages in wound healing is known, there is still much to learn about the details regarding timing, relative proportion and specific role of each phenotype. Mirza & Koh, 2011 isolated macrophages during the wound healing process in mice at Days 5, 10 and 20 post-injury in order to study the temporal phenotype change (74). In wild type mice, pro-inflammatory macrophages were detected on Day 5. These macrophages expressed high levels of IL-1β, MMP-9 and nitric oxide synthase (NOS). By Day 10, the expression of these pro-inflammatory factors decreased, concurrent with an increase in expression of anti-inflammatory markers CD206 and CD36 and growth factors IGF-1, TGF-β and VEGF. Non-diabetic mice had efficient wound repair, achieving wound closure after 20 days, at which elevated expression remained for CD206, CD36 and TGF-β, but not for IGF-1 or VEGF.

Evans *et al.* 2013 used an acute wound model in humans to better understand the pro- to anti- inflammatory macrophage transition in blisters (41). Wounds were chemically induced by application of cantharidin, a topical treatment for warts. Blister fluid was collected 16 and 40 hours after injury, to represent the inflammatory and resolving phases of wound healing, respectively. Cell counts from the fluid yielded more monocytes/macrophages at the 40 hour time point compared to 16 hours. Furthermore, the proportion of CD163+ macrophages increased over 10 fold at the later time point (3.4% vs. 47.6%), indicating that CD163 is strongly associated with the resolution phase of healing. Amounts of inflammatory mediators were also measured in the wound fluid. At the 16 hour "inflammatory" time point, there were significantly higher levels of CCL2(MCP-1), CXCL8(IL-8), TNF-α, CCL3(MIP-1α), CCL4(MIP-1β) and CCL11(eotaxin). At the 40 hour "resolution" time point, there was significantly more macrophage-derived chemokine (MDC) and TGF-β in the wound fluid. Interestingly, this study reported undetectable levels of IL-10 at either time point, which is surprising, as it is recognized as a cytokine produced at high levels by anti-inflammatory macrophages.

1.8. Macrophage dysregulation and chronic wounds

When macrophages become dysregulated, several wound healing complications can arise, such as the formation of chronic wounds or excessive scarring (20).

Macrophages in chronic wounds have a reduced capability to phagocytose dead neutrophils, which, as a result, accumulate and promote a strong inflammatory environment. Diabetic patients have macrophages with reduced apoptotic clearance activity because of the effects of hyperglycemia and advanced glycation endproducts (21, 75). The act of neutrophil clearance by macrophages can induce the phenotypic switch of M1 macrophages to M2b, and lead to the resolution of inflammation (21, 53). This is one of many reasons as to why chronic wounds may have an abundance of M1 macrophages.

Significantly higher numbers of macrophages are found in the peripheries of venous and diabetic ulcers compared to acute wounds (76). In this study, CD68 was used as a general marker to detect macrophages (although other studies define it as an M1 marker, this study did not make that clear). In acute wounds, the number of macrophages was highest at the first time point, and decreased as healing progressed. In contrast, venous and diabetic ulcers had the highest number of macrophages compared to acute wounds. The results of this study also suggested that macrophages are not the only immune cell that is dysregulated in chronic wound healing, as lower numbers of T-cells and higher numbers of B-cells were also observed. Another study also detected high levels of CD68 macrophages in the dermis and wound edges in chronic leg ulcers (77). CD16 and CD35 were also measured and defined as “activation-associated markers”, with positive staining denoting the presence of mature macrophages (rather than monocytes) in inflammatory environments. Most of the wounds studied had low expression of these markers (<12%), and the few areas that were positive were near the vasculature, suggesting that other microenvironments in the wound suppress or prevent macrophage activation. Although this study provided information about macrophage presence and marker expression in chronic wounds, particularly in different locations of a single wound, a low patient number (12 patients) was

evaluated and results were not compared relative to patients with acute wounds. These early studies provided important histological data on macrophages in human wounds, but did not explicitly discriminate between pro- or anti-inflammatory phenotypes, nor did they measure cytokines or growth factors in the wound environment.

Most *in vivo* studies, especially in humans, do not tend to study macrophages directly via detection of cell markers, but rather indirectly through the cytokines and proteins present in the wound tissue or fluid. Macrophages are major producers of cytokines and growth factors during wound healing, so, based on the identities and amounts measured, a determination can be made on whether the local macrophage population is more pro- or anti-inflammatory (M1/M2-like).

Accordingly, studies show that chronic wound fluid contains high levels of pro-inflammatory cytokines, particularly TNF-α and IL-1β, which were measured to be 100-fold higher compared to acute wounds fluids (mastectomy drain fluids) (78). IL-6 was also elevated, but only 2-4 fold. In contrast, mastectomy fluid had the highest levels IL-1β and IL-6 on Day 1 post-surgery and thereby steadily decreased to Day 7. Interestingly, TNF-α levels remained constant during this time period. This is in agreement with observations by Wallace & Stacey, who also observed higher levels of total TNF-α in chronic wounds (79). Interestingly, the amount of bioactive TNF-α in both healing and non-healing wounds was not significantly different. The amount of bioactive TNF-α did not change as acute wounds closed, suggesting that the regulation of other cytokines may be more important in progressing wound healing. Chronic wound fluid also contains high levels of MMPs, specifically MMP-2 and MMP-9 (80). MMPs degrade proteins and extracellular matrix and are not favorable for extended periods, as they do not support tissue regrowth in the proliferative phase of wound healing. Macrophages produce MMPs, so they may be responsible for maintaining elevated levels in chronic wounds (81). Specifically, human blood monocytes are stimulated to produce MMP-2 and MMP-9 in the presence of pro-inflammatory signals, such as LPS, IFN-γ, IL-1β and TNF-α (81, 82).

Furthermore, the proteases degrade and decrease the bioactivity of growth factors that may be present, hence cancelling out their pro-healing benefits as inflammation prevails (78).

Since there are no non-healing animal models of chronic wounds, diabetic mice are often used as they exhibit delayed wound healing and share several characteristics with human chronic wounds (66, 67). Studies investigating wound macrophages show that their function is not properly regulated in diabetic versus wild type mice, with a prolonged M1 macrophage presence and hence, inefficient transition to the M2 phenotype (74). Mirza & Koh found that, although macrophages from non-diabetic mouse wounds had transitioned from a pro- to anti-inflammatory phenotype by Day 10, macrophages from diabetic mice retained pro-inflammatory characteristics. This included two-fold higher levels of pro-inflammatory factors IL-1β and IFN-γ, and approximately two-fold lower anti-inflammatory IL-10 in the general wound environment. More specifically, isolated macrophages from Day 10 wounds in diabetic versus non-diabetic mice had significantly higher mRNA expression of IL-1β and MMP-9 and significantly lower expression of CD206 and CD36. At the same time, they have reduced growth factor production (IGF-1, TGF-β1, VEGF and IL-10). In non-diabetic wounds, these factors are already present and contributing to key events in wound healing such as cell proliferation and migration, ECM formation and angiogenesis. Most of the aforementioned cytokine and growth factor trends were retained until Day 20, which is in stark contrast to non-diabetic wounds, which had already healed by this point and long-completed the M1-to-M2 transition. Also interesting to note, is the fact that diabetic wounds contained fewer mature macrophages (more Ly6C expression v. F4/80) even at Day 10, suggesting that the monocyte-to-macrophage transition is impaired and may contribute to delayed wound healing. Overall, diabetic and non-diabetic wound macrophages only started to exhibit significantly different cytokine/growth factor differences by Day 10; at Day 5, they had similar levels. This suggests that between Day 5 and Day 10, non-diabetic mouse wounds are transitioning to the proliferative phase, in accordance with the M1 to M2-like macrophage phenotype change. Diabetic mouse wounds remain highly inflammatory, guided by

persistent pro-inflammatory macrophages. Overall, this study provided key evidence of delayed macrophage phenotype transition concurrent with delayed wound healing in diabetic mice.

Other studies have shown prolonged presence of other pro-inflammatory cytokines in diabetic mouse wounds, much longer than seen in wild type mice. One study compared the expression of IL-1β and TNF-α in three different mouse strains: Balb/c, C57BLKS and db/db (83). No IL-1β was detected after Day 7 in the first two strains, whereas high levels persisted into Day 13 in diabetic mouse wounds. Similarly, TNF-α was detected at the highest levels in db/db mouse wounds at Day 13, and was completely absent or present at very low levels in the wild type groups at the same time. The diabetic group also retained elevated levels of MIP-2 and CCL2(MCP-1) mRNA and protein into Day 13, whereas both strains of wild type mice had stopped producing those factors by Day 13 or even earlier. MIP-2 and CCL2(MCP-1) are chemoattractants, so their presence continually attracted more macrophages, which was detrimental to healing, as the macrophages that were recruited maintained an M1 phenotype. Again, the prolonged presence of pro-inflammatory/M1 macrophages is a hallmark of delayed wound healing in diabetic mice.

Differential iron regulation by macrophages is another factor that can promote M1/M2 phenotypes (60). M1-like cells store the majority of the iron intracellularly as ferritin, whereas, M2-like macrophages release it to the extracellular environment via the transmembrane channel, ferroportin. Sindrilaru *et al*. 2011 identified the role of high intracellular iron stores in maintaining M1 macrophages in chronic wounds, particularly chronic venous ulcers (59). The source of iron was hemoglobin from erythrocytes that escape from damaged blood vessels and enter the wound environment. In a corresponding wounded murine model with iron delivered intravenously, wound macrophages produced high levels of TNF and hydroxyl radical, and a senescence program was induced in nearby fibroblasts. As a result, wound closure was delayed.

The prolonged presence of the M1 phenotype is not the only macrophage-related problem that can contribute to wound healing disruption. In fact, if M2-like macrophages remain for too

long, there may be excessive collagen formation, resulting in scarring (15, 20). It is interesting to note that fetal wound healing is scarless, with virtually no infiltrating macrophages, and many have attempted to mimic this model to improve wound healing outcomes in adults (71). These examples suggest that an overabundance or prolonged presence of macrophages, regardless of the phenotype, can lead to wound healing complications.

1.9 Experimental therapies and wound macrophages

Based on the role played by the different types of macrophages in the wound healing response, it has been hypothesized that interventions that dampen the M1 macrophage phenotype and promote M2-like characteristics may help the healing of chronic wounds. Some have even delivered exogenous macrophages as cell therapies for chronic wounds. A few of these approaches are highlighted below and summarized in Table 1.3. Note that the table focuses on key *in vivo* studies, whereas the text in the following subsections includes both *in vivo* and *in vitro* results.

1.9.1 Endogenous M1 macrophage attenuation

Goren *et al.* 2007 aimed to silence M1 macrophages in obese/obese (ob/ob) mouse wounds (84). These animals have diabetes and hence, exhibit impaired wound healing. In the study, anti-TNF-α or anti-F4/80 antibodies were systemically administered beginning seven days post-wounding, concurrent with the end of the inflammatory phase. These treatments resulted in wound closure and re-epithelialization while control wounds treated with a non-specific antibody remained unhealed with scabs. There were fewer total macrophages and decreased levels of TNF-α, IL-1β and CCL2(MCP-1) proteins in wounds with anti-TNF-α and anti-F4/80. Furthermore, a greater proportion of wound macrophages were apoptotic compared to control groups. Overall, anti-TNFα and anti-F4/80 therapies reduced the impact of M1 macrophages, and accelerated the healing of diabetic wounds. It is noteworthy that by choosing Day 7 post-injury as the time point to begin treatment, necessary early inflammatory events in M1 macrophages were not disrupted. Treatment timing was strategically chosen to rescue the wound healing response during late-stage

Method	Wound Model	Treatment Details	Conclusion
Endogenous M1 attenuation			
Monoclonal antibodies: ·anti-TNF-α ·anti-F4/80 ·control: non-specific, rat IgG	·ob/ob mice ·Full-thickness excisional wounds (5 mm diameter)	·Systemic Administration · 1 µg/g body weight ·Day 7, 9 and 11 post-wounding (End of inflammatory phase)	· TNF-α and F4/80 antibodies effectively target and kill pro-inflammatory wound macrophages, resulting in accelerated healing
Exogenous M2 supplementation			
Injection of *in vitro* polarized: ·M2a macrophages (by IL-4) ·M2c macrophages (by-IL-10) ·control: non-polarized macrophages (M0) ·control: saline	·db/db mice ·full-thickness excisional wounds (4 mm diameter)	·intradermal injection (0.5 x 10^6 cells) ·Day 1 and 3 post-wounding	·*In vitro* -polarized M2 macrophage supplementation immediately after wounding did not accelerate healing
Ulcers treated with: ·macrophages from blood of young, healthy donors, stimulated by hypo-osmotic shock (n=72 ulcer patients) · conventional wound care (n=127)	· human pressure ulcers in elderly patients · range of sizes (not indicated)	· intradermal injections near ulcer periphery and topically on ulcer · 0.05 mL/injection; 0.5-1 cm between injections along periphery · 2 x 10^6 cells/mL · majority of ulcers treated 1 time; rare case of reinjection ccurred 2 months after initial	· Injection of blood-derived macrophages to pressure ulcers resulted in healing of 27% of those treated vs. 6% in controls
Ulcers treated with: ·macrophages from blood of young, healthy donors, stimulated by hypo-osmotic shock (n=141 ulcers) · conventional wound care (n=75)	· human pressure ulcers (in legs) · human diabetic ulcers · large range in wound sizes; average approximately 30 cm^2	· intradermal injections near ulcer periphery and topically on ulcer · 0.1 mL/injection; 1 cm between injections along periphery · 15-40 mL total depending on size · 2-4 x 10^6 cells/mL · re-injection depending on wound condition approximately 4 weeks after initial treatment	· Injection of blood-derived macrophages lead to healing of a majority (69.5%) of pressure and diabetic ulcers compared to only 13.3% healed with standard treatment
Endogenous Macrophage Modulation/M2 Promotion			
MSCs			
Conditioned media from: · bone-marrow derived MSCS · control: fibroblasts	· healthy mice (Balb/C) · full-thickness excisional wounds (6 mm diameter)	· 100 µL total administered · subcutaneous (80 µL) and topical injections (20 µL)	· MSC-conditioned media resulted in increased numbers of macrophages and endothelial progenitor cells in the wound. Wound closure was significantly accelerated.
· human gingiva-derived MSCs (in PBS) · control: PBS	· healthy mice (C57BL/6J) · full-thickness excisional wounds (6 mm diameter)	· intravenous injection (2 x 10^6 cells) · one time, on Day 1 after wounding	· Wound closure with MSC treatement was significantly accelerated. This occurred with a decrease in TNF-α and IL-6 and an increase in IL-10 and arginase-1
Autologous bone-marrow derived: · MSCs · mononuclear cells · control: saline and standard care	· human diabetic ulcers · average size approximately 4 cm^2	· intramuscular injection · 20 separate sites all on Day 1 · many cells used (exact number not clear)	· Ulcers treated with MSCs had accelerated healing compared to MNCs. Patients in this group also had better outcomes in terms of time to painless-walking, transcutanoeus oxygen pressure and blood vessel formation
Growth Factors			
· PDGF-BB · control: collagen-vehicle · control: non-irradiated	· healthy rats (Sprague-Dawley) · linear surgical incisions (6cm long) · irradiated (whole-body or topically) to depress wound healing	· topical (2 µg and 10 µg/wound)	· Wounds treated with PDGF had higher cellularity scores and breaking strength. Effect of PDGF-BB was only seen in rats containing wound macrophages (topical irradiation vs. whole-body irradiation)
· recombinant human GM-CSF	· human chronic venous leg ulcers · range of sizes (not indicated)	· intradermal injection at 4 corners of wound · 150 µg	· GM-CSF causes wound macrophages to increase VEGF production, which results in improved vascularization in wounds
Biomaterials and macrophages			
PEG-RGD hydrogels of varying stiffnesses: · 130 kDa · 240 kDa · 840 kDa	· healthy mice (C57BL/6)	· subcutaneous implantation · 5mm diameter hydrogels	· Macrophage infiltration was the greatest in the stiffest hydrogels (840 kDa). Generally, stiffer hydrogels resulted in more severe foreign body responses.
HO-1 expression			
· Hemin (in diabetic rats) · controls: vehicle (in diabetic rats) non-diabetic rats	·diabetic rats (streptozotocin-induced) ·full-thickness excisional wounds	· topical 10% hemin ointment · daily	· HO-1 was induced in wounds of diabetic rats receiving hemin treatment. These wounds healed significantly faster than vehicle controls, at rates similar to non-diabetic rats. Hemin treatment led to decreased levels of TNF-α and IL-6 in wound tissue
· Hemin injection · topical povidone-iodine (positive control) · Saline injection	· healthy rats (Wistar) · full-thickness excisional wounds (2 x 2 cm^2)	· hemin solution (diluted in saline) · intraperitoneal injection (30 mg/kg)	· Hemin treatment increased wound closure and collagen synthesis. mRNA of pro-inflammatory cytokines ICAM-1 and TNF-α were decreased whereas anti-inflammatory IL-10 was increased. In some cases, the effect of hemin was greater than the positive conrol.
Oxygen Therapy			
Hyperbaric Oxygen (HBO) Therapy · controls: normoxia hyperoxia increased pressure only	· *in vitro* human macrophage culture · cells stimulated with LPS, THA, IL-1β or TNF-α	· cells cultured in HBO, normoxia, hyperoxia or increased pressure for 90 min, 3 hours or 12 hours	· Short-term hyperbaric oxygen therapy (both increased pressure *and* oxygenation) has immunosuppressive effects on macrophages

Table 1.3: Experimental Approaches to Modulate Macrophages in Wound Healing

inflammation, during which the macrophage population should begin transitioning to M2.

1.9.2 Exogenous M2 macrophage supplementation

Since the appearance of M2 macrophages correlates with a desirable progression in the wound healing response, direct addition of isolated M2 macrophages has been attempted to stimulate healing. However, as reported by Jetten *et al.* 2014, who used macrophages that were

polarized into M2a and M2c phenotypes *in vitro* and then injected them into mouse wounds, this approach did not accelerate healing in wild type mice and even delayed healing in diabetic mice (85). The M2 macrophages were introduced to the wounds during early inflammation (post-injury Days 1 and 3), and they continued to express M2 markers 15 days post-wounding. The lack of improvement in wound healing may be attributed to the timing of the treatment, which may have disrupted the function of M1 macrophages at a stage when they are presumably still needed. This study exemplifies the need to have an adequately-timed therapeutic approach.

In contrast, in Israel, treatment of chronic ulcers with blood-derived macrophages is an approved procedure, and it has been used successfully in over 1000 patients (86). Danon *et al.* 1997, treated pressure ulcers in elderly patients with macrophages derived from blood units of young, healthy donors (87). The macrophage isolation method is completely sterile, using a closed system of interconnected bags containing the various reagents needed for the process. In order to stimulate isolated macrophages to produce factors beneficial for wound healing, they were activated by hypo-osmotic shock for 45 seconds (86). Related studies characterized these cells by measuring mRNA expression in over 72 genes (88). The results revealed that expression of several genes related to wound healing (IL-1, IL-6, TGF-β, FGF-8, TNF receptors, VEGF and GM-CSF, to name a few) dramatically increased due to hypo-osmotic shock. Protein measurements revealed that hypo-osmotic shock could increase production up to 123- and 175-fold, in the case of IL-1 and IL-6, respectively, although donor-to-donor variability does exist. Hence, although this study did not utilize a traditional M2a/b/c/d-polarization method, macrophages were stimulated to be more anti-inflammatory via hypo-osmotic shock prior to wound application. However, the induced cell population was not completely characterized, particularly on the protein level.

In the clinical study, patients' ulcers were injected with the isolated macrophages near the wound periphery (87). A portion of the cell suspension was also deposited on top of the wound, which was then covered with dressings. Macrophage treatment was performed a single time in

most patients, unless they still exhibited delayed healing about one month later, in which case a second treatment was performed. The effects of the treated ulcers were compared with other patient ulcers at the same hospital treated with conventional methods, including debridement, antibiotics and wound dressings. Results revealed that 27% of ulcers treated with macrophages healed, whereas only 6% of controls did, and that there were no adverse reactions to treatment.

The same group later published results of a more comprehensive study, including randomization of patients between macrophage-treated and standard-of-care groups (89). In addition to providing more data, including healing time, etiology and size of the wounds in this study, subsets of patients with diabetic ulcers were also included and analyzed separately. The overall results for all ulcers demonstrated improved statistics compared to the previous study: 69% of macrophage-treated patients healed in an average of 86.7 days, whereas control groups had only 13.3% full-closure wounds in 117.7 days. Similarly, in the diabetes groups, 65.5% of wounds with the macrophage treatment and only 15.4% of controls healed. Again, wounds in the treatment group healed in a faster time compared to controls.

These studies provided an interesting strategy of using exogenous macrophages from healthy individuals, stimulated by hypo-osmotic shock, without the use of LPS, IFN-γ, or any other stimulus, to aid in the healing of chronic ulcers. The success of the treatments in both pressure and diabetic ulcer patients is promising, however more work must be done to determine the reason why some wounds do not respond to treatment, and investigate ways to improve these outcomes. Additionally, isolation and purification of macrophage populations was not extensive in these studies, and therefore cell types other than monocytes/macrophages may be contributing to this therapy.

1.9.3 Endogenous macrophage modulation/M2 phenotype promotion

Several different approaches have been taken to modulate wound macrophages with the goal of promoting M2-like characteristics, which often simultaneously attenuate M1 characteristics. Although not a comprehensive list, methods using mesenchymal stromal cells

(MSCs), growth factors, biomaterials, heme oxygenase-1 (HO-1) induction and oxygen therapy are discussed.

1.9.3.1 Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) secrete many growth factors that are required for wound healing, and have therefore been explored as cell therapies. Their use in animal and human studies has been successful, resulting in accelerated wound closure and more mature angiogenesis and granulation tissue (90). Evidence shows that MSCs and their secreted products affect a variety of skin and immune cells. Of particular interest to this review are MSC interactions with macrophages. MSCs have such powerful modulating effects on macrophages, that some have defined yet another phenotype of macrophages based on this interaction (91). These MSC-educated macrophages exhibit M2-like characteristics (IL-10 high, IL-12 low, IL-6 high, TNF-α low) and hence possess a secretome that can have powerful benefits in wound healing.

One of the mechanisms of MSC action on wounds is via recruitment of macrophages. Chen *et al*. 2008 used MSC-conditioned media *in vitro* and found that it accelerated migration of macrophages, in addition to keratinocytes and endothelial cells (92). In a murine excisional wound model, subcutaneous injection and topical application of the MSC-conditioned media also led to increased presence of macrophages and endothelial progenitor cells. Macrophage recruitment by MSCs may be attributed to high levels of secreted chemoattractants CCL3(MIP-1α), MIP-2 and CCL12(MCP-5).

MSCs also secrete an important regulator, prostaglandin E-2 (PGE-2), that has a direct effect on macrophages, by reprogramming them to up-regulate the M2-like marker, IL-10 (93, 94). In a murine sepsis model, Németh *et al*. 2009 showed that systemic MSC administration reduced mortality and improved organ function, but only in the presence of macrophages (93). When macrophages were depleted, the benefits of the MSC-treatment were eliminated. In response to the MSC-treatment, extracted lung macrophages produced significantly higher IL-10

(an M2-like marker) compared to those from control groups. As a result, neutrophil tissue infiltration was decreased, which has a protective effect due to lower levels of local oxidative tissue damage. The group also performed *in vitro* studies to determine the molecular interaction between MSCs and macrophages that leads to IL-10 upregulation. Results suggested that PGE-2 from MSCs stimulates macrophages to produce IL-10. Similar findings were confirmed *in vitro* by Barminko *et al*. 2014, showing that MSCs, via PGE-2, reduced TNF-α and increased IL-10 secretion from macrophages, hence attenuating M1, and promoting M2, characteristics (94). Although these studies were not performed in a chronic wound model, they reveal relevant interactions between MSCs and macrophages, which may partially explain the success of MSC therapy in wound healing studies.

In a wound healing context, MSCs have a similar effect on macrophages. Zhang *et al*. 2010 studied the effect of human gingival-derived MSCs on macrophage phenotype and found that *in vitro*, they increased expression of M2-like markers IL-10, IL-6 and CD206, decreased expression of TNF-α (M1-like marker) and decreased induction of Th-17 cell expansion (95). In an *in vivo* murine wound healing model, systemically-administered MSCs accumulated near the wound area, and induced M2 characteristics in surrounding macrophages, such as increased IL-10 and decreased TNF-α and IL-6. Wound healing was accelerated with MSC-treatment.

Several clinical studies have also investigated the potential of MSCs as a treatment for chronic wounds. As discussed in the previous section, exogenous monocyte/macrophage cell therapies have also been tested in human wounds (87, 89). One interesting clinical study compared the effects of MSCs versus mononuclear cells (MNCs) in diabetic ulcers (96). Both cell sources were autologous and bone-marrow derived. MSCs were expanded *in vitro*, whereas the mononuclear fraction—containing a variety of hematopoietic cell types including monocytes—was isolated from bone marrow aspirate. Prior to administration to ulcers, MSCs and MNCs were analyzed for levels of angiogenic factors. Interestingly, MSCs produced significantly higher levels of VEGF, FGF-2 and angiopoeitin-1 than MNCs in both hypoxic and normoxic conditions.

In the clinical study, ulcers treated with MSCs healed significantly faster, and were fully closed 4 weeks earlier than treatment with MNCs. The MSC-group also had the best outcomes in terms of pain-free walking time, transcutaneous oxygen pressure, and blood vessel formation, followed by the MNC-group and, lastly, the saline controls. These results showed that MSCs had more potent effects on diabetic wounds compared to MNCs. This suggests that MSCs, rather than a monocyte-based treatment, may have more potent effects in a wound environment. Another possibility is that the MNC group may have performed better if it was stimulated, for example by hypo-osmotic shock, or pre-polarized into an M2-like phenotype via biochemical stimulation. Regardless, MSCs are known to have powerful modulating effects on macrophages, therefore this approach may be better-suited for wound healing compared to monocyte/macrophage supplementation, as suggested by these results.

In developing new therapies, it is pertinent to consider the special characteristics of chronic wound environments, such as low oxygen tension, and how they may affect macrophage function. Through *in vitro* studies, Faulknor *et al*. 2017 demonstrated that a hypoxic environment lessened macrophage plasticity in response to MSCs (97). Macrophages cultured in normoxic conditions (20% O_2) with MSCs produced high levels of the M2 marker, IL-10, however, in hypoxic conditions (1% O_2), secretion was significantly lower. As macrophages possess a high degree of phenotypic plasticity, they react not only to the treatments that are introduced, but also to the existing microenvironment, which may affect their ability to respond to treatment. This is an important consideration that emerging chronic wound therapies should address.

1.9.3.2 Growth factors

Cell therapies provide wounds with numerous cytokines and growth factors, which in sum affect local cells and enhance the coordinated wound healing process. Another approach to treating chronic wounds is through the application of a single growth factor, which can elicit cellular responses. As advertised on their website, Regranex is the, “first and only FDA-approved recombinant platelet-derived growth factor (PDGF) therapy for diabetic neuropathic ulcers” (98).

The mechanism of action involves macrophages as a key player. During hemostasis, PDGF recruits macrophages to the wound in order to initiate inflammation. In the next phase, macrophages are stimulated by Regranex to produce more PDGF, as well as TGF-β, to stimulate extracellular tissue formation by fibroblasts. An early study on surgical incisions in rats investigated the mechanism of PDGF therapy by studying its effects in rats receiving either surface irradiation or total body irradiation (99). Whereas surface-irradiated rats retain bone marrow elements and wound monocytes/macrophages, total body irradiated rats are depleted of them. Hence, this approach was used to determine the importance of macrophages in the efficacy of PDGF in wound healing. The results revealed that PDGF therapy was ineffective in aiding wound healing in rats depleted of monocytes/macrophages (total body irradiation). In contrast, in rats that were surface-irradiated, macrophages were able to migrate into the wounds and PDGF treatment successfully aided healing. The number of wound fibroblasts increased, as well as wound strength, presumably by the formation of more collagen. Interestingly, in wounds that contained fibroblasts but no macrophages (surface-irradiated rats), PDGF did not stimulate collagen synthesis. This suggests that macrophages are the first-responders to PDGF treatment, and in response, they must activate fibroblasts, via TGF-β, to proliferate and synthesize collagen, which contributes to granulation tissue formation and wound closure. Overall, wound macrophages are a vital part in the mechanism of action of Regranex.

Interestingly, granulocyte macrophage-colony stimulating factor (GM-CSF) has also shown benefits in chronic wound healing (100), despite the fact that it is used *in vitro* to promote the M1 phenotype. In cell culture, GM-CSF induces macrophages to produce more pro-inflammatory factors (TNF, IL-23) and less anti-inflammatory factors (IL-10) compared to baseline levels, however, LPS and IFN-γ are often used as more potent M1-stimuli that activate different signaling pathways (101). In contrast, *in vivo* effects of GM-CSF, particularly in a chronic wound healing environment, can promote healing. Cianfarani *et al*. 2006 demonstrated that GM-CSF injections to non-healing venous leg ulcers induced VEGF transcription in the

wound bed, primarily within macrophages (102). As PDGF, in the previous example, stimulates macrophages to produce TGF-β, GM-CSF stimulates macrophages to produce VEGF. This finding was corroborated with *in vitro* results showing the same effect of GM-CSF on VEGF production in a differentiated monocytic cell line but not in keratinocytes. In patient ulcers, increased VEGF transcription by GM-CSF lead to improved vascularization and healing. GM-CSF also acts on other skin cells, which further explains its pro-wound healing effects. In addition to macrophages, GM-CSF also has chemotactic effects on fibroblasts, endothelial cells and keratinocytes. Accordingly, formation of granulation tissue, blood vessels and the epidermal layer are improved with exogenous GM-CSF (103).

A potential explanation of the pro-wound healing effect of GM-CSF *in vivo* versus its perceived pro-inflammatory role *in vitro* is that, within the complex chronic wound environment, which contains several interacting cell types and signaling pathways, an intermediate macrophage phenotype is formed. A combination of M1-like and M2-like factors, such as IFN-γ, IL-6 and TGF-β are increased upon upregulation of GM-CSF *in vivo*, all of which have distinct roles in the wound healing process (103). Interestingly, evidence shows that GM-CSF is more effective in accelerating chronic wound healing rather than acute (104). This discrepancy further underlines the complex role of macrophages within the intricate, multi-cellular wound healing environment.

Many other growth factors (*i.e.* VEGF, FGF, EGF) have shown potential in aiding in wound healing (104), however less work has been published showing their direct effect on macrophages, as they primarily act on other cells such as endothelial cells, fibroblasts and keratinocytes. Regardless, FGF and EGF are approved wound care therapies in Japan and Cuba, respectively (4).

1.9.3.3 Immunomodulatory biomaterials

Any material that comes in contact with the body has the potential to elicit an immune response and affect surrounding cells and tissue. The body's response to the material is not always harmful, and can be tuned to promote healing if the material possesses the right

characteristics. Immunomodulatory materials are being developed with the goal of limiting negative reactions to implants and instead, promoting their integration into the body (38). General approaches when designing immunomodulatory materials include 1) carefully selecting physical properties, 2) altering chemistry, 3) incorporating therapeutic molecules for controlled release and, 4) combination with cell therapies. As the last two points were discussed in previous sections, the following discussion is focused on physical and chemical properties of biomaterials that modulate macrophage behavior. The discussion includes examples of both currently used wound healing materials, and those under development for potential future applications.

One chemical approach involves modifying native extracellular matrix molecules. Hyaluronan (HA), a glycosaminoglycan (GAG), is one such ECM component that can cause macrophages to take on pro- or anti-inflammatory characteristics depending on certain chemical modifications (38, 105). For example, sulfated GAGs can bind and interact with growth factors and cytokines, thereby preventing them from affecting macrophage behavior. Kajahn *et al*. 2012 tested the *in vitro* response of monocytes to biomaterials composed of collagen and HA, or sulfated HA derivatives (made by simultaneously degrading and sulfating native HA), within an inflammatory environment created by exogenous IL-6, IFN-γ and MCP-1 (106). In the presence of collagen with highly-sulfated HA derivatives, monocytes resisted an M1 phenotype transition (via lower levels of IL-1β, CXCL8(IL-8), IL-12 and TNF-α), and instead differentiated into M2-like macrophages with increased IL-10 production and CD163 expression. Other experimental conditions, including collagen only, collagen + non-sulfated HA, and collagen + lowly-sulfated HA derivatives, promoted macrophages with more M1 characteristics. The results of this study are interesting in regards to wound healing, as several wound-care products are based on ECM proteins.

Chitosan is another material that is in found in several FDA-approved wound products (107). It is known for its antimicrobial effects and also acts on skin cells to aid in wound healing. Researchers have also investigated its effect on macrophages (108). In response to culturing with

chitosan, macrophages increased production of TGF-β1, which stimulates ECM formation. In contrast, chitosan did not stimulate direct ECM formation by fibroblasts. This result highlights the importance of macrophage subset modulation as they produce many growth factors that can affect other local wound cells. Additionally, chitosan also stimulated macrophages to produce high levels of PDGF, which is important in angiogenesis. Other studies have shown that chitosan promotes nitric oxide production and chemotaxis in macrophages (109). It is believed that the cellular interaction occurs via N-acetylglucosamine on chitosan and corresponding receptors on macrophages.

Physical cues on biomaterials can also affect macrophages by causing them to take on rounded versus elongated shapes, which are likely to exhibit M1or M2-like characteristics, respectively (110). One approach to achieving M2-like macrophages on biomaterials is by micropatterning ECM molecules or integrins that promote cell attachment and spreading (110, 111). Modifications like these can also alter the stiffness of the cell/biomaterial interface. Blakney *et al*. 2012 investigated the effect of hydrogel stiffness and macrophage adhesion in an *in vivo,* subcutaneous implantation murine study (112). All hydrogels (130kPa, 240 kPa and 840kPa moduli) were composed of polyethylene glycol and RGD, to allow for cell attachment. Hydrogels were implanted into mice, and 28 days later, were removed for histological analysis. Staining with a macrophage-specific cell-surface marker, Mac3 (CD107b), revealed that the softest hydrogels had significantly lower macrophage infiltration compared to the other two groups. These results suggest that stiffness of wound care products may be important in directing macrophage fate, and overall wound healing success.

In some cases, a combination of chemical and physical cues and an understanding of which has the greater effect, can further promote differentiation of the desired macrophage phenotype (110, 111). This can be optimized by intentional selection of material properties to achieve successful immunomodulatory biomaterials. Surprisingly, many studies in the literature seem to consider immunomodulatory properties of wound dressings and therapies as an

afterthought with their experimental treatment or product. Recognizing the importance of macrophages in the wound healing process, it is pertinent to ensure that a material that is introduced to chronic wounds does not further promote a pro-inflammatory environment, but rather attenuates M1 macrophages and promotes the transition to M2-like phenotypes. Moving forward, immunomodulatory properties of materials should be a key design factor for new wound healing therapies.

1.9.3.4 Heme oxygenase-1 induction

Heme oxygenase-1 (HO-1) is an inducible enzyme that catalyzes heme breakdown and releases anti-inflammatory factors. When hemoglobin is endocytosed by macrophages, the HO-1 pathway breaks it down into iron, carbon monoxide, and bilirubin, which is later converted to biliverdin.

The three products of HO-1 activity can individually affect wound healing responses. Carbon monoxide and bilirubin can exert anti-inflammatory properties to help wound healing (113, 114) however, differential regulation of the iron product can promote M1- or M2-like macrophages (60). M1-like cells store the majority of the iron intracellularly as ferritin, whereas, M2-like macrophages release it to the extracellular environment via the transmembrane channel, ferroportin. Likewise, M2-like macrophages express higher levels of ferroportin compared to M1. M2-like cells have a higher number of hemoglobin-binding receptors (specifically CD163). Thus, the HO-1 signaling pathway is more active. Perhaps the downstream effects of this process, such as higher HO-1 activity, carbon monoxide and bilirubin production, and iron release in M2 macrophages contributes to their pro-regenerative properties.

The HO-1 pathway is important in wound healing, as it plays roles in angiogenesis and re-epithelialization (115). Mice with inhibited or deleted HO-1 exhibit delayed wound healing, and diabetic mice inherently have lower levels of HO-1, which may partially explain their challenges with wound healing. Restoring HO-1 expression in wild type and diabetic mice resulted in improved and accelerated wound healing, which suggests an important role of

hemoglobin breakdown in the wound healing process. HO-1 is also expressed in fibroblasts and keratinocytes, which underlines its role in dermal wound healing (116).

During acute wound healing, HO-1 protein was expressed at high levels in a murine model three days after creation of full-thickness excisional wounds, before returning to basal levels (117). HO-1 mRNA levels continued to be high until Day 7. Macrophages and proliferating keratinocytes along the wound edge were the primary cell types that overexpressed HO-1. Interestingly, it was also found that patients with psoriatic skin constitutively overexpress HO-1, as well as HO-2. *In vitro* studies found that reactive oxygen species, rather than growth factors or cytokines (KGF, EGF, TNF-α), directly stimulated HO-1 expression.

HO-1 has also been targeted in models of delayed wound healing. In a wounded diabetic rat model, HO-1 expression was induced using topical, 10% hemin ointment (118). Wound TNF-α and IL-6 levels, as measured by Western blot, were significantly decreased compared to rats treated with vehicle controls. VEGF and intercellular adhesion molecule (ICAM) serum levels were increased, and accordingly, so was blood vessel density. Induction of HO-1 in diabetic rats brought levels of several measured biomolecules, as well as wound healing rates, back to those seen in non-diabetic controls. Even in non-diabetic rats, studies have shown that hemin accelerates healing, concurrently with decreased levels of pro-inflammatory proteins ICAM-1 and TNF-α and increased IL-10 (119). The involvement of these prototypical M1/M2 markers suggests the involvement of macrophages in the enhancement of wound healing.

In fact, there is evidence that HO-1 expression in macrophages promotes an M2-like phenotype (Mhem) (120). Several animal studies in different disease models, have induced HO-1 expression and measured subsequent macrophage markers. Resulting M2-like markers include arginase-1, mannose receptor, and CD163, among others. HO-1 has demonstrated potential as a method to promote M2-like characteristics in macrophages to aid in healing, however, as with many macrophage-targeted therapies, timing must be well-suited in order to successfully resolve inflammation (116).

Hemoglobin-based substances (polymerized hemoglobin, PEG-encapsulated hemoglobin, etc.) may be interesting approaches to activate the HO-1 pathway, while simultaneously delivering oxygen (121, 122). This method would elicit anti-inflammatory effects from local macrophages, and restore oxygen levels, thereby targeting two major deficiencies of chronic wounds with one therapy.

1.9.3.5 Oxygen therapy

Chronic wounds are hypoxic, as blood flow and, hence, oxygen delivery to the tissues are disrupted (123). Direct delivery of oxygen to skin wounds, such as by exposing patients to 100% oxygen at 2-3 atm pressure in hyperbaric chambers, has been shown to enhance wound healing. The effect of increasing oxygen levels in the wound are multifaceted, but evidence suggests that one of the targets may be the wound macrophages. One study investigated the direct effects of hyperbaric oxygen and hyperoxia (without increased pressure) on the cytokine profiles of cultured macrophages (124). Hyperbaric oxygen dampened IL-1β and TNF-α secretion by approximately 40%, while hyperoxia alone had no effect. However, when hyperbaric oxygen exposure exceeded 6 hours, an increase, rather than a decrease, in the production of these pro-inflammatory mediators was observed. This study did not investigate any M2-like macrophage functional parameters.

1.10. Conclusions and future directions

It is clear that macrophages play an important role in wound healing, and that anti-inflammatory, M2-like phenotypes are desirable for efficient healing. Questions remain regarding the details behind monocyte recruitment and macrophage differentiation, specifically whether monocytes are predestined to become one particular phenotype (M1/M2-like) or if macrophages themselves change from M1 to M2 phenotypes (or vice versa) within the tissue (Figure 1.1) (20). More thorough histological studies on *in vivo* wound environments (both acute and chronic) would lead to a better understanding of macrophage phenotypes and their spatiotemporal and functional contributions during healing. This information could help identify macrophage

phenotypes needed to promote healing in chronic wounds. Another challenge in this field is that the definition of each macrophage sub-phenotype is neither clear, nor agreed upon. There are also inconsistencies between *in vitro* and *in vivo* macrophage phenotypes, especially in chronic wound models, which further confuse this area of research. There is a need for a more thorough characterization of macrophage phenotypes and a definition of their respective roles (Table 1.2 and Figure 1.2). Novel technologies and tools that can quickly and thoroughly define macrophage phenotypes, even within heterogeneous populations, would advance research (50, 125). In the midst of this work, it is also important to recognize differences between murine and human wound healing processes and roles of immune cells (64).

Current experimental therapies that are being investigated for their potential to promote wound healing macrophages include mesenchymal stem cells, growth factors, biomaterials and more (Table 1.3). Up-and-coming methods to control macrophage fate include microRNA therapies to affect macrophage transcriptome and function (126). Delivery time for novel therapies, in regards to current macrophage phenotype and the needs of the particular wound, should not be overlooked, as it can make the difference between an effective and an ineffective therapy. Another question is whether or not directly promoting M2-like phenotypes is entirely necessary, or, is it possible that by only attenuating M1 macrophages, the wound environment will be reprogrammed to successfully heal? Furthermore, is targeting macrophages alone enough to promote healing, within the complex, multi-cellular chronic wound environment? Hence, an effective treatment may need to address multiple deficiencies of chronic wounds. As macrophages are involved in all phases of wound healing, and their dysregulation in chronic wounds leads to a stalled and heightened inflammatory state, an improved understanding of these key regulators will ultimately lead to advancements in wound healing therapies.

1.11 Abbreviations

Frequently-used Abbreviations	
DAMPs	damage/danger-associated molecular patterns
ECM	extracellular matrix
EGF	epidermal growth factor
FGF	fibroblast growth factor
GC	gluococorticoid
GM-CSF	granulocyte-macrophage colony-stimulating factor
GT	granulation tissue
HA (in text)	hyaluronan
HA (in table)	hemorrhage-associated
HO-1	heme oxygenase-1
IFN-γ	interferon-gamma
IGF-1	insulin-like growth factor 1
IL-(1β, 6, 10, etc.)	interleukins
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
MCP-1/5	monocyte chemoattractant protein-1/5 (CCL2/CCL12)
MHCII	major histocompatibility complex class II
MIP-1α/β	macrophage inflammatory protein-1 alpha/beta (CCL3/CCL4)
MNCs	mononuclear cells
MMPs	matrix metalloproteinases
MSCs	mesenchymal stromal cells
NF-κβ	nuclear factor kappa beta
PAMPs	pathogen-associated molecular patterns
PDGF	platelet-derived growth factor
PGE-2	prostaglandin E-2
ROS	reactive oxygen species
TAMs	tumor-associated macrophages
TGF-β1	transforming growth factor beta 1
TNF-α	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor

Table 1.4: Frequently-used Abbreviations

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CHAPTER 2: MACROPHAGE MODULATION BY POLYMERIZED HEMOGLOBINS: POTENTIAL AS A WOUND HEALING THERAPY

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2.1 Introduction

Chronic wounds are a major healthcare problem in the United States, affecting 6.5 million people (1). A wound is *chronic*, if it remains open for greater than one month and does not show signs of healing (2); in many cases such wounds remain open for longer than 12 months (3). Such wounds are usually located in areas with impaired blood flow, such as extremities (legs/feet) in the case of diabetic, venous, and arterial ulcers, or underneath bony surfaces for pressure ulcers, which are common in spinal cord injured patients. Although the underlying etiology of chronic wounds may be different, they share several characteristics, such as being stuck in a pro-inflammatory state, having poor vascularization and low oxygen levels, and resisting regeneration of dermal and epidermal skin layers, which also makes them prone to infection (3).

Typical treatment approaches include wound debridement, delivery of antibiotics, and periodic wound dressing changes. If wounds do not respond, more advanced therapies are used. One example is oxygen delivery-based approaches, such as hyperbaric oxygen therapy (HBOT) or topical oxygen therapy (TOT). HBOT involves a pressurized chamber at 2 times atmospheric pressure, containing 100% oxygen (4). As patients lay within the chamber, increased levels of

oxygen enter their lungs and blood plasma, thus increasing pO_2 within the wound. In addition to increased oxygen transport, other reported benefits of HBOT include reduced wound edema, stimulation of progenitor stem cells and angiogenesis, as well as improved fibroblast function (5). TOT involves direct delivery of oxygen gas to the wound surface via a pump, either at normobaric or pressurized conditions. (6). Both HBOT and TOT have been reported to accelerate healing of chronic ulcers in specific instances (4-6). Downsides of these approaches are that the patient has limited mobility, they are time-consuming, and require additional, specialized machinery that is costly and not readily available. An alternative approach would be to use an oxygen carrier to enhance oxygen delivery locally even under normal atmospheric conditions.

Hemoglobin (Hb) is the protein in our red blood cells responsible for binding and delivering oxygen throughout the body (7). It achieves this by changing structure in response to the partial pressure of oxygen (pO_2)—a relaxed state (R-state) when pO_2 is high and Hb is saturated with 4 oxygen molecules (O_2) bound, and a tense state (T-state) when pO_2 is low and no oxygen is bound. Hb can readily change from the R- to T-state depending on the surrounding pO_2, and as a result, oxygen is delivered throughout the body. To exert a level of control of oxygen delivery, Hb can be chemically crosslinked into the R- or T-states (8). This has been achieved in the development of hemoglobin-based oxygen carriers (HBOCs), particularly polymerized hemoglobins (PolyHb). PolyHbs can also have a range of molecular weights, depending on the extent of cross-linking, which are much greater than native Hb (9). HBOCs have been traditionally studied as alternatives to blood transfusions, as they have extended shelf-life and there is no need to match red blood cell-type (7, 10). More recently, their oxygenation potential has been studied in other applications such as islet transplantation (11), and treatment-targeting of tumors (12).

Hb-based therapies have also been investigated in wound healing applications. For example, Plock *et al.* intravenously delivered Hb vesicles to mice with ischemic skin flaps (13). As a result, oxygenation, tissue survival, and healing of the skin flap edges were increased.

Furthermore, Hb vesicle injection resulted in higher capillary counts and endothelial nitric oxide synthase (eNOS) expression. Another Hb-based therapy, called Granulox (Sastomed GmbH, Georgsmarienhütte, Germany), is approved for treatment in Europe for surgical wounds and diabetic, venous, and arterial ulcers (14). In human chronic wounds, Granulox increased oxygen levels (15), and reduced wound exudate, wound size, and pain levels (16).

In these examples, the regenerative effects of Hb-based therapies have been attributed to oxygen delivery, but we aim to investigate their effects on macrophages, which play important roles in wound healing. Chronic wounds are characterized by a highly inflammatory environment that is dominated by pro-inflammatory, M1 macrophages (17). These cells have high levels of damaging reactive oxygen species (ROS), and secrete pro-inflammatory cytokines and chemokines, such as tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), and interferon-γ (IFN-γ), to name a few (18). In healing wounds, the macrophage population transitions to an anti-inflammatory M2 phenotype, which secretes factors such as IL-10, vascular endothelial growth factor (VEGF), and others that reduce inflammation and promote vascularization and regeneration (19). In chronic wounds, the M1-M2 macrophage phenotype transition does not occur and healing is stalled. Therefore, it is desirable in wound healing for the M1 phenotype to be attenuated, while the M2 phenotype is promoted.

There is evidence that Hb can interact with macrophages in order to elicit a M2-like phenotype via the heme oxygenase 1 (HO-1) pathway (20-22). For example, in atherosclerosis, in areas of intraplaque hemorrhage, red blood cells are ruptured and iron is released from Hb (23, 24). In these high-iron areas, a unique macrophage phenotype was identified, with higher expression of M2 markers CD206 and CD163, and lower expression of the M1 marker TNF-α (25). Hb also forms tight complexes with the plasma protein haptoglobin. The complexes are then internalized by the monocyte/macrophage specific receptor CD163. Intracellular breakdown of heme activates the HO-1 pathway, resulting in downstream upregulation of the anti-inflammatory cytokine, IL-10 (25-27). Since Hb can interact with macrophages and alter their secretion profile,

further investigation into Hb/macrophage and PolyHb/macrophage effects are warranted when studying their potential as chronic wound healing therapies. The current study aims to characterize the inflammatory secretion profile of macrophages treated with Hb and PolyHbs, in order to determine which type may be the most beneficial for wound healing.

Here, we studied the effect of Hb and PolyHbs on macrophage phenotype in a pro-inflammatory, *in vitro* environment that mimics chronic wounds. For PolyHbs, both R- and T-state forms were tested, including two polymerization molar ratios. We hypothesized that PolyHbs would be less toxic to macrophages, as the chemical crosslinks hinder the release of the toxic heme group, which leads to oxidative damage. This was verified experimentally, and several key proteins secreted by macrophages were identified that exhibited significant differences between Hb and PolyHb treatments. Furthermore, T-state PolyHb exhibited the most potential in stimulating wound healing and angiogenesis.

2.2. Materials and methods

2.2.1 PolyHb synthesis

PolyHb was synthesized as described in *Zhang et al.* (8). Briefly, human red blood cells were lysed by exposure to hypotonic conditions (28, 29). To remove cell debris, the lysate was passed through a glass wool column and then further purified using a 3-step tangential flow filtration process. Purified Hb was diluted in phosphate buffer solution (PBS). R-state PolyHb was synthesized by reacting glutaraldehyde with completely oxygenated Hb for 2 hours at 37°C. T-state PolyHb was synthesized by reacting glutaraldehyde with completely deoxygenated Hb. Two different polymerization molar ratios were used to synthesize R- and T-state PolyHbs – 30:1 and 35:1 (glutaraldehyde:Hb molar ratio), denoted as R:30, R:35, T:30, and T:35. After 2 hours, $NaBH_4$ was added to quench the polymerization reaction. Following polymerization, PolyHb was subjected to diafiltration to remove unpolymerized Hb and other small molecules from the solution. PolyHb was concentrated to 100 mg/mL in a modified Ringer's lactate buffer, sterile filtered, and stored at -80°C until needed.

2.2.2 Monocyte isolation and macrophage differentiation

Human blood/buffy coat donations were purchased from the New York Blood Center (New York City, NY). Primary monocytes were isolated using Ficoll-Paque density gradient centrifugation and CD14+ magnetic bead separation (Miltenyi, Bergisch Gladbach, Germany), in a process similar to that reported in Faulknor *et al.* (30). CD14+ cells were cultured at 5 x 10^5 cells/mL with 5 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems, Inc., Minneapolis, MN) for 7 days at 37°C and 5% CO_2 to differentiate them into M1 macrophages. Complete media for all monocyte/macrophage cell culture was Advanced RPMI 1640 (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 4 mM L-glutamine.

2.2.3 Cell culture

Macrophages were cultured in complete media in a 37°C, 5% CO_2 incubator, at 5 x 10^4 cells/well in plastic 24 well-plates, and allowed to attach for 24 hours. Then, they were activated with 1 μg/mL lipopolysaccharide (LPS) from *E. Coli* (Sigma Aldrich, Saint Louis, MO) to induce inflammation. Simultaneously, macrophages were treated with 0.2, 2.0, or 20.0 mg/mL of Hb (H) or PolyHb (T:30, T:35, R:30 or R:35) for 48 hours at which point supernatants were collected and stored at -80°C until use in the multiplex immunoassay.

2.2.4 Metabolic activity measurement

Following supernatant collection, Alamar Blue Cell Viability Reagent (Life Technologies Corporation, Carlsbad, CA) was mixed 1:10 with media containing 1 μg/mL LPS. 500 μL was added to each well to measure net cellular metabolic activity. The assay is a fluorescent-based detection method, wherein resazurin is reduced and converted to a fluorescent compound, resorufin, by living cells. After 6 hours, fluorescent measurements (excitation 535nm; emission 595 nm) were made on a DTX 880 Multimode Detector plate reader with Multimode Detection Software (Beckman Coulter, Brea, CA).

2.2.5 Multiplex immunoassay

A panel of 27 cytokines, chemokines, and growth factors related to inflammation were measured using a Bio-Plex Pro Human Cytokine 27-plex Assay (BIO-RAD, Hercules, CA). All measured factors are listed on the heatmap in Figure 2.2A. The assay was carried out according to the manufacturer's instructions. Measurement was performed on media controls and 2.0 mg/mL Hb/PolyHb samples on a Bio-Plex 200 System (BIO-RAD, Hercules, CA). Results are represented as fold changes. Raw secretion concentrations were normalized to media baseline values. Then, fold change was determined by taking the $\log_2$ of the media-normalized value.

2.2.6 Principal Component Analysis (PCA)

Matlab (Mathworks, Natick, MA) was used to run PCA on the secretion profile datasets. The code produced a clustergram for the dataset, and also determined the co-variance of each treatment pair. Principal component scores based on the weights of 27 inflammatory factors were generated. Principal component values (PC1, PC2, PC3) for each treatment were plotted against each other.

2.2.7 Ingenuity Pathway Analysis (IPA)

IPA version 01-13 (Qiagen, Venlo, Netherlands) was used to make predictions of biological/wound healing outcomes of Hb/PolyHb treatments from the secretion datasets. Average fold changes from three experiments for each condition were ran in an expression core analysis with no mutations, including direct and indirect relationships, interaction and causal networks, all node types and data sources, experimentally observed and high (predicted) confidence, restricted to fibroblast and macrophage cell lines and primary human cells including endothelial cells, keratinocytes, fibroblasts, and immune cells (dendritic cells, granulocytes, mononuclear leukocytes, peripheral blood leukocytes). z-scores assigned a value to the predicted up/down regulation of vascular and wound healing events from the treatment compared to media baseline conditions. z-scores represent the predicted activity of biological events using the expression patterns of the downstream factors, based on relationships published in the literature.

2.2.8 In vivo wound healing studies

All animal protocols were approved by Rutgers University Institutional Animal Care and Use Committee. 10-week old, male, genetically diabetic mice— BKS.Cg-Dock7m +/+ Leprdb/J (The Jackson Laboratory, Bar Harbor, ME) — were used. Animal handling, wounding procedure, and histological analyses were performed as previously described (31-33). On the day prior to surgery (Day -1) mice were anesthetized using inhaled isoflurane (Henry Schein Animal Health, Melville, NY), delivered via a nose cone. The backs were shaved using clippers to remove the majority of the hair where the wounds would be administered. Residual hair was chemically removed by topical application of Nair™ (Church & Dwight Co. Inc, NJ) for 60 seconds and then cleaned with a paper towel and warm water. Mice were caged individually from this point on. On the day of wounding (Day 0), mice were again anesthetized. The wound area was cleaned three times with alternating betadine antiseptic surgical scrub (Avrio Health L.P., Stamford, CT) and 70% ethanol. A 1cm x 1cm template was traced on the mouse skin to demarcate the edges of the wound to be excised. Using autoclave-sterilized tweezers in one hand, the center of the traced region was lifted, and then cut through the center with surgical scissors. Then, the remainder of the skin was removed by cutting along the edge of the traced region. An image was taken of the wound with a ruler to serve as reference so that the initial wound size could be determined.

Mouse wounds were topically treated with either human Hb, human T-state PolyHb (30:1), human R-state PolyHb (30:1) or Ringer's Lactate (vehicle control). 20 mg of each treatment (200 μL of 100 mg/mL solution) was applied on Day 0. Tegaderm™ (3M, Saint Paul, MN) dressing was sutured over the wound in order to hold the treatment in place. Following surgery and treatment on Day 0, mice were injected subcutaneously with analgesic (buprenorphine, 0.05 mg/kg) and returned to their cages. Mice received an additional 10 mg (100 μL of 100 mg/mL) of treatment delivered every 7 days for 4 weeks.

Images were taken of the wound with a ruler for reference on Days 0, 3, 7 and once weekly until all wounds closed. Then, they were analyzed in Image J (NIH, Bethesda, MD). The wound area was measured by tracing the wound edges with the polygon tool and converting the area from pixels^2 to cm^2. "100% closed wounds" were defined as those that had no visible open skin or scab. During the course of healing, percent wound closure (W_p) was normalized to wound size on Day 0 of each individual wound and defined by:

$$W_p = \left(1 - {}^{W_x}/_{W_0}\right) * 100.$$

where W_x is the wound area on Day x and W_0 is the initial area on Day 0.

2.2.9 Histological staining

On Day 35, mice were sacrificed and wound/scar tissues were harvested. Tissues were fixed in 10% formalin for 24 hours and then stored in ethanol at 4°C. Tissues were then paraffin-embedded, sectioned (5 μm), and stained with hematoxylin and eosin (H&E). Immunohistochemical staining of CD31 was performed as described in *Kumar et al.* (34). A primary rabbit polyclonal anti-CD31 antibody (Abcam, Cambridge, MA; 1:200) was used, followed by secondary antibody (Biotinylated Goat Anti-Rabbit,1:200; Vector Laboratories, Burlingame, CA).

4x images of sections were taken with a brightfield microscope with an Infinity 3 color camera and Infinity Analyze Software Version 6.5.2 (Lumenera, Ontario, Canada). ImageJ software (NIH, Bethesda, MD) was used to analyze images. Epidermal thickness was measured in three locations per slice (center, left, and right edges). CD31 density was determined by measuring dermal area in the image view, and then counting positively stained areas.

2.2.10 Statistics

GraphPad Prism 8.1.1 (330) (GraphPad Software, San Diego, CA) was used for all statistical analyses, as well as to generate all plots other than those in Figure 2.2. One-way ANOVA with Tukey's *post-hoc* analysis was used to identify significant trends in all analyses, except for

metabolic activity (Figure 2.1) and inflammatory secretion (Figure 2.3) analyses, in which two-way ANOVA was used. *, +, and # are used to identify significance between groups, which are specified in the figure legends. Increasing *, +, or # indicate increasing level of significance. For example, * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$, and **** denotes $p<0.0001$. The same holds true for increasing numbers of + and # symbols.

2.3 Results and discussion

2.3.1 Effect of Hb/PolyHb on cellular metabolic activity of macrophages

First, we tested a range of concentrations of Hb/PolyHb on macrophages in order to determine doses at which net cellular metabolic activity/viable cell number is not negatively affected. M1 macrophages seeded 24 hours prior were stimulated with 1 μg/mL of LPS. At the same time, 0.2, 2.0, or 20.0 mg/mL of Hb/PolyHb treatments were added. PolyHb treatments included both the T-state and R-state polymers, at polymerization molar ratios of 30:1 and 35:1. After 48 hours of incubation, supernatants were collected and net metabolic activity per well was measured. Results are shown in Figure 2.1, and are normalized to media baseline measurements.

At increasing concentrations of Hb, net metabolic activity decreased. This was significant at 20 mg/mL, compared to media baseline and lower Hb concentrations (0.2 and 2.0 mg/mL). In contrast, net metabolic activity per well remained close to baseline conditions for macrophages treated with any type and concentration of PolyHb (Figure 2.1). The decrease in net metabolic activity at 20 mg/mL of Hb may be a reflection of a decrease in attached cell number (S.I. Figure 2.1, Section 2.5). Additional morphological characterization and ROS level measurements of macrophages treated with increasing concentrations of Hb were also performed and confirmed that 20 mg/mL Hb is damaging to macrophages (S.I. Figure 2.2, Section 2.5). As a whole, these results suggest that 0.2-2.0 mg/mL of Hb is a safe concentration range for macrophages. Furthermore, this range extends to 20 mg/mL for PolyHbs, which were less toxic to macrophages at this higher dose compared to unmodified Hb.

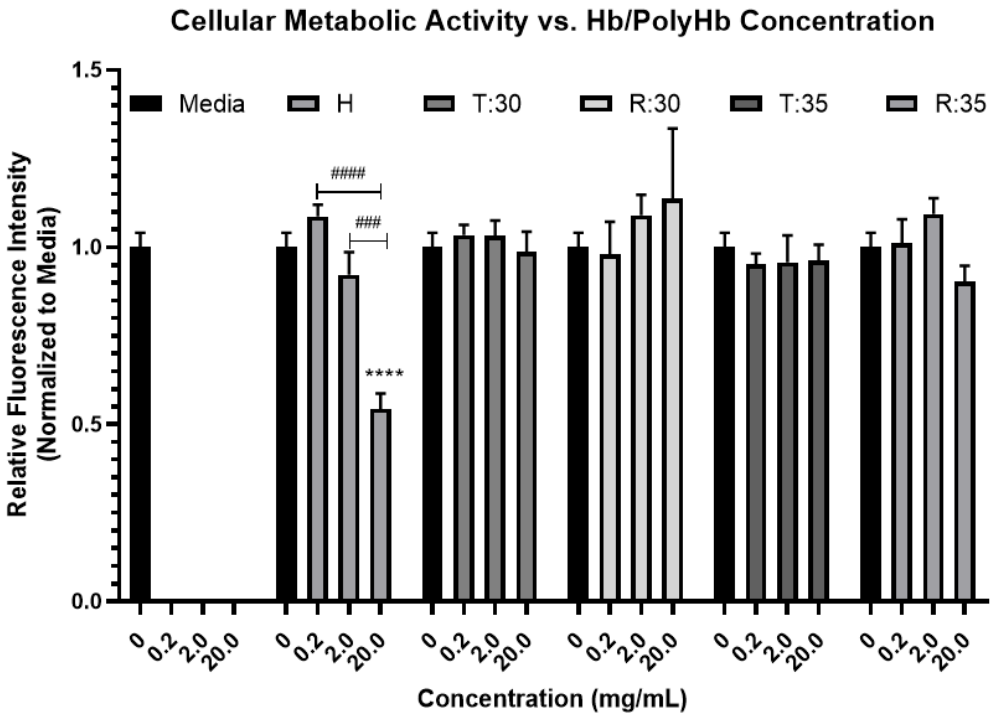


Figure 2.1: Cellular Metabolic Activity vs. Hb/PolyHb Concentration

Metabolic activity of macrophages cultured in media with or without 0.2, 2 or 20 mg/mL Hb, T:30 PolyHb, R:30 PolyHb, T:35 PolyHb or R:35 PolyHb. Results were obtained using the Alamar blue assay, and relative fluorescence intensity (RFI) was measured on a plate reader. Results are grouped by treatment type, across all 3 concentrations. Black bars represent values for the media group (controls). * indicates significance compared to media baseline. # indicates significance between bracketed groups.

One possible reason for less toxicity of PolyHbs versus Hb at high concentrations may be that the damaging heme group is less readily released from PolyHbs due to chemical crosslinking. In unmodified Hb, the heme group is easily released from the protein, and causes oxidative damage, which can ultimately lead to apoptosis of cells (35). Based on these results, further experiments were carried out at 2.0 mg/mL of Hb/PolyHb or lower, within the safe range for both Hb and PolyHbs, thus allowing for easy comparison between groups.

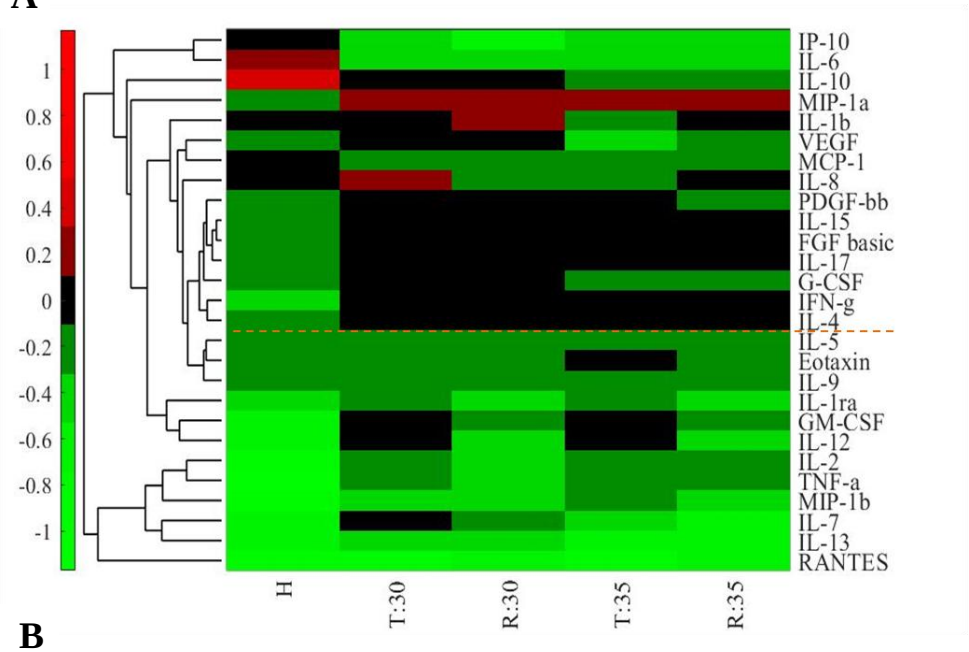
2.3.2 Net effect of Hb/PolyHb on secretion of inflammatory factors from macrophages

In order to characterize macrophage phenotype in response to Hb/PolyHb treatment, we measured inflammatory protein secretion using a 27-plex immunoassay. Figure 2.2A provides a

heatmap of the secretion results for each treatment and protein. Results were normalized to media conditions and represented as fold changes above or below (red/green, respectively) media levels. As a whole, a majority of factors (21/27) decreased with Hb treatment, whereas trends with PolyHbs were more variable. For about half of the factors (15/27; from IP-10 to IL-4), trends for Hb were generally different than for PolyHbs. For example, for IL-6, treatment with Hb resulted in an increase in secretion, whereas treatment with all PolyHbs led to a decrease. Similarly, for MIP-1α, the Hb group exhibited a decrease in secretion whereas all PolyHbs exhibited an increase. Several other factors (IL-15, IL-17, IFN-γ, and IL-4) decreased with Hb treatment, and remained close to baseline with PolyHbs. In the second half of the clustergram (IL-5 – RANTES), Hb treatment had similar results to PolyHbs. For example, IL-5 and IL-9 decreased in all Hb/PolyHb groups. From IL-1ra to RANTES (9/27 factors), Hb and R-state PolyHb groups had similar, decreasing trends, and T-state PolyHb groups were slightly higher. These trends generally hold true regardless of polymerization molar ratio of T-or R-state PolyHbs. Overall, Hb treatment decreased a majority of the factors, and had similar trends to PolyHbs for about half of the measured factors, but displayed more similarity to R-state PolyHb group trends.

In order to further compare the effects of Hb/PolyHb treatments on macrophage secretion, correlation values between groups were calculated. The first column of Figure 2.2B lists correlation of each PolyHb versus Hb group, in order of highest to lowest correlation. Hb was not closely correlated with any of the PolyHbs, but was better correlated with the R-state PolyHbs (0.50 – 0.51) than the T-state PolyHbs (0.27 – 0.34). These correlation values were much lower compared to values in the remaining columns (0.72-0.84), which compare PolyHbs to one another. This indicates that PolyHb secretion profiles are generally more similar to one another than to Hb. This is not surprising, as PolyHbs are more physically similar to each other;

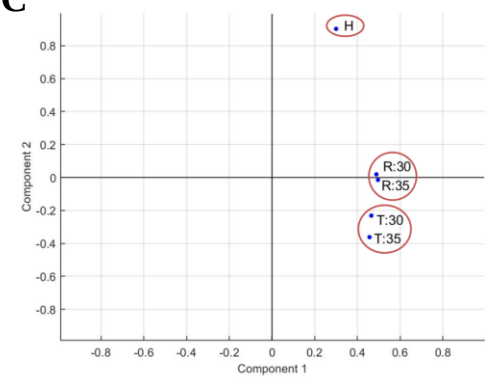
A



B

	Correlation with H	Correlation with R:35	Correlation with R:30	Correlation with T:30	Correlation with T:35
H	1.00				
R:35	0.51	1.00			
R:30	0.50	0.84	1.00		
T:30	0.34	0.75	0.80	1.00	
T:35	0.27	0.82	0.72	0.75	1.00

C



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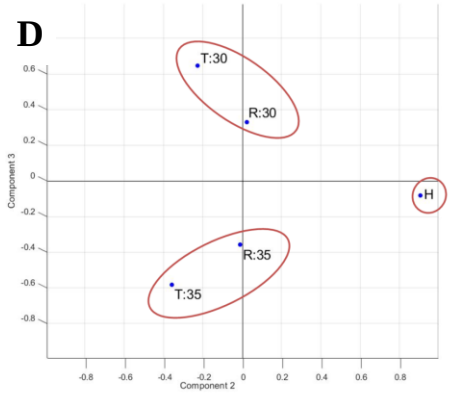


Figure 2.2: Net Analysis of Macrophage Secretion Profiles

Analysis of secretion profiles measured via multi-plex immunoassay of macrophages treated with 2 mg/mL media, Hb, T-state PolyHb (30:1), R-state PolyHb (30:1), T-state PolyHb (35:1) and R-state PolyHb (35:1). (A) Clustergram showing differences in secretion of cytokines/chemokines/growth factors from macrophages treated with Hb/PolyHbs. All results are represented as average fold changes (FCs) for each respective factor normalized to the media group measurement. Shades of red indicate an increase in secretion, black indicates a negligible change in secretion, while shades of green indicate a decrease in secretion. Color brightness

indicates the relative fold change, as shown by the scale bar. The orange dashed line between IL-4 and IL-5 indicates two groups – above the line, the H group generally has opposite trends to PolyHbs and below the line, H and PolyHb groups have similar trends. (B) Table with correlation values between treatment groups. Groups are listed in order of similarity to the H group in the first column. A value of +1 is exactly correlated, a value of zero is not correlated, and a value of -1 is oppositely correlated. (C) PC1 and PC2 values plotted for each treatment. (D) PC2 and PC3 values plotted for each treatment.

regardless of polymerization in the R or T quaternary state, since PolyHbs have undergone chemical crosslinking procedures, unlike Hb. As a result, PolyHbs have higher molecular weights, as multiple Hbs are bound together. These physical and chemical changes may cause PolyHbs to have more similar interactions with macrophages than unmodified Hb, which is evident through comparison of the resulting secretion profiles.

2.3.3 Principal Component Analysis on treatment secretion profiles

PCA was used to more systematically separate treatment groups based on their secretion trends. This method identifies which factor(s) contribute the most in generating different responses among experimental groups. With this data, the first 4 PCs accounted for 97.8% of the variance of the dataset (S.I. Figure 2.3A, Section 2.5). Each PC is based on a linear combination of the inflammatory factor data, each assigned a different weight/score (S.I. Figure 2.3B, Section 2.5). In Figure 2.2C, PC1 and PC2 values are plotted for each treatment. PC1 accounts for 71.9% of the variance of the dataset and PC2 accounts for 16.2%. Along PC1, H (PC1=0.30) slightly separates from PolyHbs, all of which have similaS.I. Figure 2.2, Section 2.5 values (PC1= 0.46-0.50). MIP-1α and IP-10 are both factors that have high scores for PC1 (absolute values above 2) and their trends are opposite for Hb vs PolyHbs, suggesting that they contribute to this observed slight separation along PC1. In contrast, RANTES and IL-13 are the top two scoring factors for PC1, and all treatment groups have similar trends, leading to less separation along PC1. Separation is seen to a greater extent along PC2; H has a high positive value (PC2=0.9), both R:30 and R:35 have values close to zero, and T:30 and T:35 fall between –0.2 and -0.4, generally clustering by state of Hb/PolyHb (unmodified, R-state PolyHb, or T-state PolyHb). IL-2 and GM-

CSF are in the top 8 cytokines that contribute most to the total PC2 value, and in the heatmap, exhibit similar trends within R-state PolyHb, regardless of polymerization molar ratio, and within T-state PolyHb, regardless of polymerization molar ratio. These factors may contribute to the separation seen between groups along PC2, and may be key macrophage markers that react differently to PolyHb quaternary state, possibly due to differences in oxygenation or binding with macrophage receptors.

Figure 2.2D plots PC2 against PC3 values (accounts for 5.8% variance). Along PC3, treatments separate by polymerization molar ratio. T:30 and R:30 have PC3 values greater than 0.3. In contrast, T:35 and R:35 have PC3 values less than -0.3. H is in-between, with a PC3 value close to zero. VEGF and MIP-1β are two top scoring contributors to PC3. As seen in the heatmap, VEGF has similar trends when treated with PolyHbs at constant polymerization molar ratios, regardless of the quaternary state. MIP-1β also has similar values between T:30 and R:30. As they are more consistently regulated between polymerization molar ratio, rather than PolyHb quaternary state, VEGF and MIP-1β secretion in response to Hb-based therapies may be more affected by molecular weight than oxygenation.

Taken together, PC1 represents the most variability of the dataset, but only separates Hb/PolyHbs slightly. PC2 contributes to the second most variability in the dataset, and the values cluster by quaternary state, whereas PC3, contributing to less variance, separates by polymerization molar ratio. As discussed in the clustergram results, this supports the observation that the most obvious distinction in the data set is between Hb and PolyHbs as a whole (consistent with PC1). Next, it is more apparent that differences in macrophage secretion result from PolyHb quaternary state (PC2), rather than the polymerization molar ratio (PC3). The PC separation trends, as well as the percentage of variance explained by each one, provide an overarching commentary for the dataset.

2.3.4 Significant effects of Hb/PolyHb on secretion of key inflammatory factors from macrophages

Although the overall trends for the dataset were identified in Figure 2.2, there were also several key, significant trends for specific inflammatory factors (Figure 2.3). These trends are divided into 3 groups. Figure 2.3A shows results for factors in which Hb treatment results in lower secretion compared to PolyHbs. For TNF-α and IL-2, the H group resulted in levels that were significantly lower than treatment with any other PolyHb. For GM-CSF and IL-12, H treatment still resulted in the lowest secretion, but it was only significantly lower than T-state groups, and not the R-state groups. This supports the observation that Hb has more similar macrophage secretion trends to R-state PolyHbs than T-state PolyHbs. In the second group of factors, in Figure 2.3B (IL-6 and IL-10), PolyHbs have significantly lower secretion than Hb. These cytokines contribute to the observation that PolyHbs generally separate from Hb. Lastly, in Figure 2.3C, Hb and PolyHbs act similarly for RANTES and IL-13, by decreasing secretion compared to media. This suggests that, for these particular factors, chemical crosslinking does not affect secretion levels. Perhaps Hb and PolyHbs are similar enough in chemistry and physical properties to result in similar secretion trends for pathways in which IL-13 and RANTES secretion are implicated. Overall, Figure 2.3 identifies inflammatory factors with significant trends that support conclusions made from the clustergram and PCA analysis presented in Figure 2.2.

2.3.5 Ingenuity Pathway Analysis (IPA) modeling – biological disease and function predictions

Next, we used IPA, which is based on published trends on protein interactions and pathways, to interpret the secretion results and make predictions on the effects of Hb/PolyHbs within a biological/wound healing context. Interesting trends were identified in vascular and wound healing categories, and are shown in Figure 2.4. For vascular trends (Figure 2.4A), T-state PolyHb is the only treatment that has a predicted increase for *migration of endothelial cells*, *cell*

movement of microvascular endothelial cells, and *tubulation of vascular endothelial cells*. The remaining treatments predict decreases for these processes, indicating that T-state PolyHb may

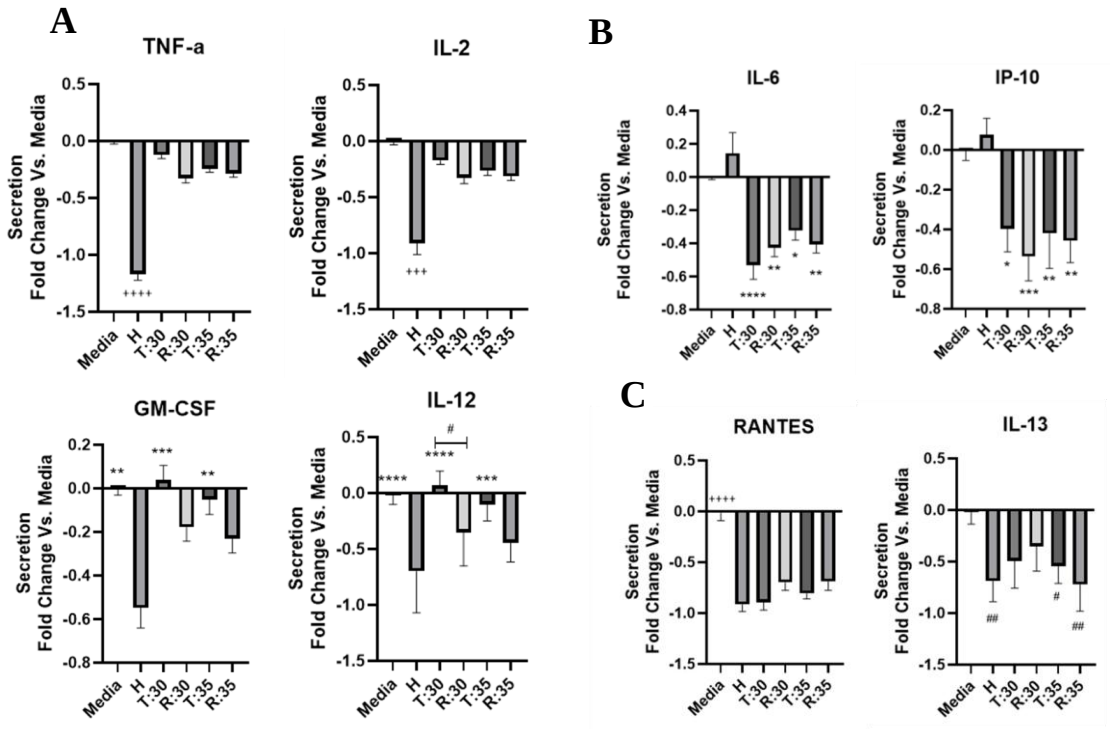


Figure 2.3: Significant Inflammatory Factor Trends Due to Hb/PolyHb Treatment

Key inflammatory factors revealed several significant trends when macrophages were treated with 2 mg/mL H, T:30, R:30, T:35, or R:35. (A) Four factors –TNF-α, IL-2, GM-CSF, and IL-12 – in which several groups (media and PolyHbs) are significantly higher than H. + denote significant difference between all other groups. * denote significance compared to H. # indicates significance between bracketed groups. (B) Secretion levels for IL-6 and IL-10, in which values for H remain close to media baseline, but remaining PolyHb groups are significantly lower than H. * denote significance compared to H. (C) Secretion levels for RANTES and IL-13, in which several Hb/PolyHb groups are significantly lower than media. + denote significance between all other groups. # denote significance compared to media.

promote vascularization, which is necessary for wound healing. These predictions are specifically based on increases in secretion of PDGF, VEGF, IL-8, and GM-CSF in the T:30 group, as seen in the heatmap for the factors that are implicated in *migration of endothelial cells*.

Wound healing related predictions for T:30 compared to other experimental groups include the most *migration of cells*, *cell viability*, and *growth of connective tissue* (Figure 4AB). These predictions are based on a majority of the cytokines measured in the dataset; for example,

for *migration of cells*, the prediction was based on 25/27 of the cytokines measured. A decrease in a majority of the inflammatory factors due to Hb treatment led to a strong predicted decrease in *migration of cells*. Increases for some of these factors in the T:30 group led to a less extreme prediction for *migration of cells*. In addition to favorable IPA predictions for T:30, this group also resulted in the lowest levels of intracellular ROS in macrophages, which would be desirable for lowering inflammation in chronic wounds (S.I. Figure 2.4, Section 2.5). To follow up on these predictions, the next step was to test the Hb/PolyHb treatments in an *in vivo* wound healing model, with our hypothesis being that T-state PolyHb would lead to faster wound closure and more angiogenesis compared to other experimental groups.

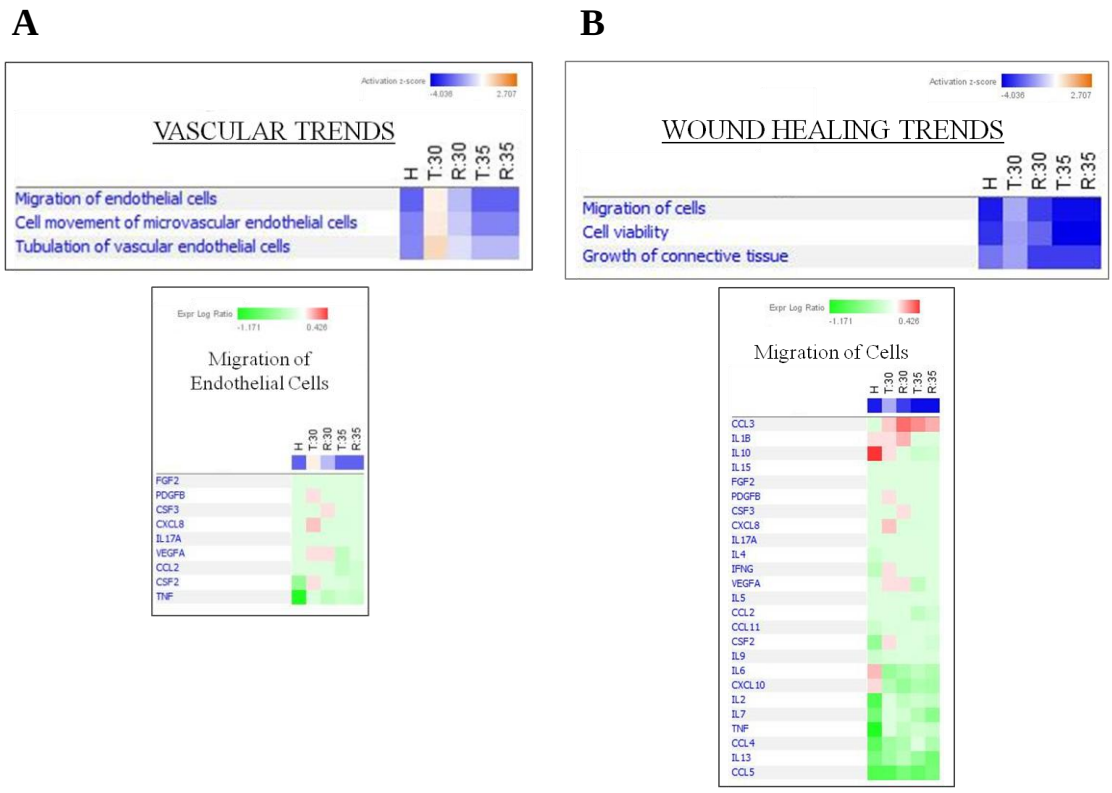


Figure 2.4: IPA Modeling of Inflammatory Factor Data to Predict Biological Outcomes
(A) Predictions related to vascularization and (B) wound healing for each Hb/PolyHb treatment are shown on the orange and blue heatmaps. Shades of orange indicate a predicted increase of the function above media baseline and shades of blue indicate a predicted decrease. The brightness of the blue/orange shade depends on the relative z-score of the prediction. These predictions (specifically for *migration of endothelial cells* and *migration of cells*) are based on the factors listed in the red and green heatmaps. Shades of red indicate an increase in secretion of the factor compared to media baseline and shades of green indicate a predicted decrease.

2.3.6 Effect of Hb/PolyHb in in vivo murine wounds

The *in vivo* wound healing study investigated the effect of different Hb-based formulations (non-polymerized H, R:30, and T:30) versus vehicle controls (Ringer's lactate) on diabetic mouse wounds. Excisional wounds were made on the backs of mice, 200 μL of 100 mg/mL of Hb/PolyHb solution was topically applied, and the wound was covered with Tegaderm™ wound dressing. Treatment was reapplied once a week for four weeks. Images of the wound area were taken on Days 0, 3, 7, and then weekly until Day 35 until all wounds were closed.

T-state trends towards faster closure of wounds compared to R-state, H, and control groups throughout the entire 35 days of the study (Figure 2.5). This is significant on Day 21, when T-state treated wounds are significantly smaller than all other groups. Throughout the study, H and R-state mice exhibited slower healing, with similar wound closure curves. Figure 2.6 shows Day 35, H&E stained, histological sections of uninjured skin, and wound areas that had received the various treatments. In the uninjured skin, nicely formed, mature epidermal, dermal, fat, and muscle layers are identified. In the wounded sections, the layers are more difficult to separate, as they are newly regenerated. The epidermal layer is blueish/purple, and has less folds and surface area than the uninjured epidermis. The neodermis is compact, with newly-formed extracellular matrix (pink) and infiltrated cells (blue dots) that have filled the wound bed. No appendages (hair follicles, sebaceous glands) are present in the neodermis. Qualitatively, the epidermis of R- and T-state PolyHb treated mice appears thicker than control and Hb-treated mice. Epidermal thickness was measured and quantified in ImageJ, and normalized to uninjured skin. Treatment with R- or T-state PolyHbs resulted in the highest epidermal thicknesses, both significant compared to uninjured skin. The T-state PolyHb group was also significantly higher than the control group. H and C groups had lower, but similar, epidermal thicknesses, between 1.5-2 times higher than uninjured skin.

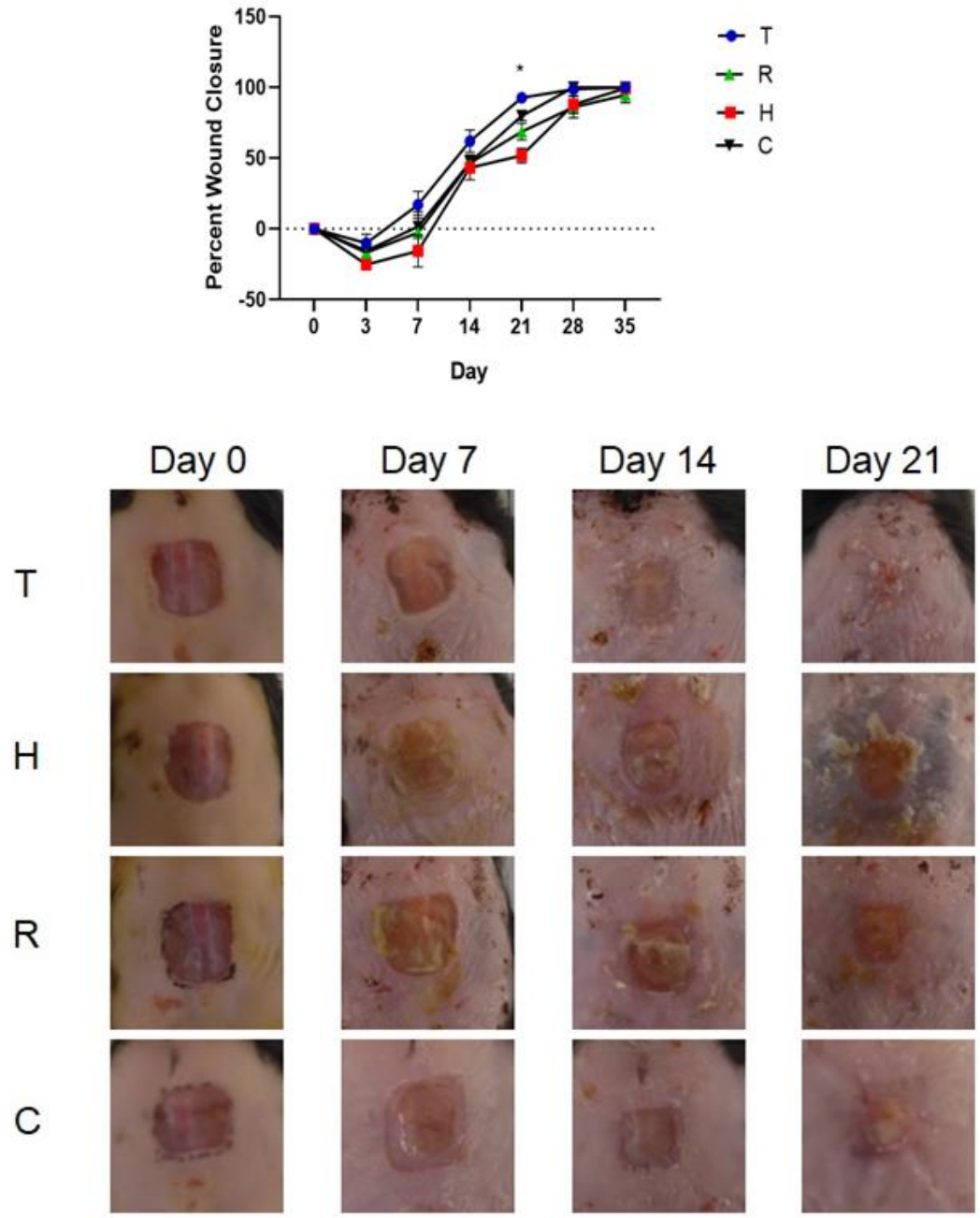


Figure 2.5: Effect of Hb/PolyHbs (H/R:30/T:30) on Wound Closure *In Vivo*, as Compared to Vehicle-treated Controls (C)

Percent wound closure as a function of time, representing wound closure rate for each treatment. * denotes significantly higher wound closure of T versus all other groups on Day 21. The images below are representative of wound size for each treatment group on Days 0, 7, 14, and 21.

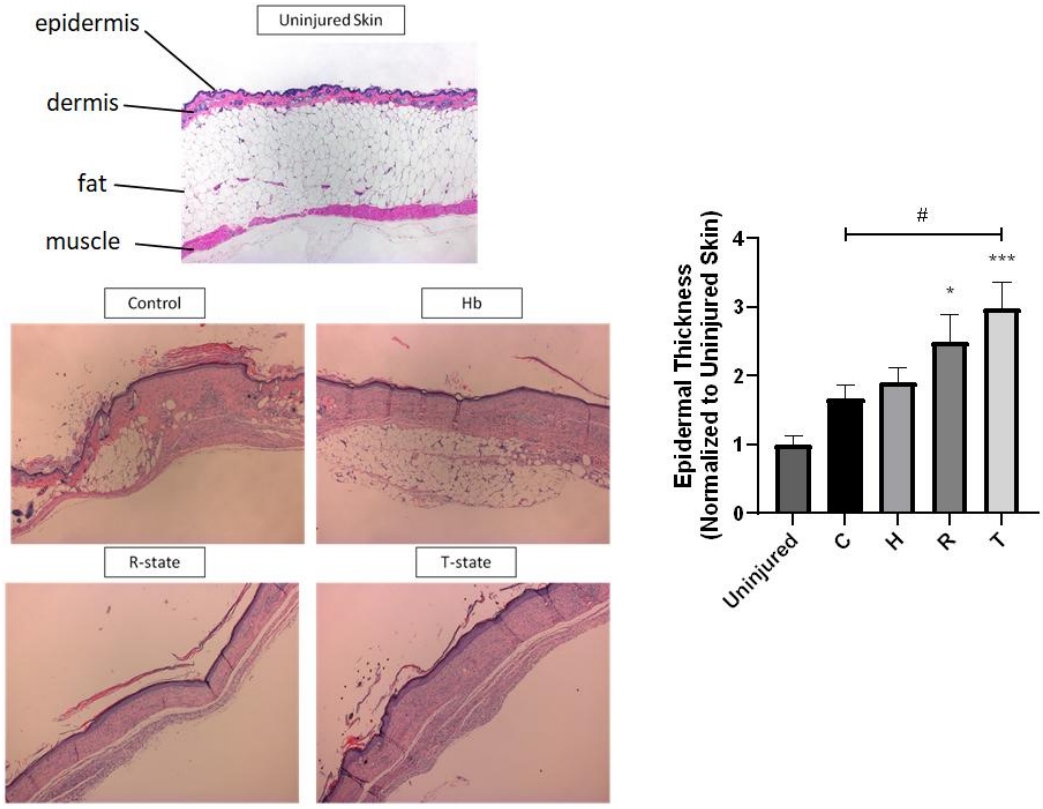


Figure 2.6: Histological Sections from Day 35 Mice Stained with H&E
Epidermal thickness of skin was measured in ImageJ. Results were normalized to the epidermal thickness of uninjured skin. * denote significance versus uninjured skin. # denote significance between groups indicated.

CD31 staining was also performed on Day 35 histological sections (Figure 2.7). CD31 is a marker for endothelial cells, indicating blood vessel formation. Qualitatively, non-polymerized Hb and R-state PolyHb treatment groups CD31 staining appeared wider than clusters observed in groups treated with T-state PolyHb. Quantitatively, mice treated with T-state PolyHb had significantly higher CD31 density than control, Hb, and R-state PolyHb groups; on average, T-state groups had approximately 2 times higher CD31+ density than other groups. As predicted in IPA, T-state PolyHb also exhibited the most benefits *in vivo*, in terms of angiogenesis and wound healing (CD31 density and epidermal thickness). This may be attributed to increased levels of PDGF, VEGF, IL-8, and GM-CSF detected from T-state PolyHb treated macrophages in the *in vitro* studies.

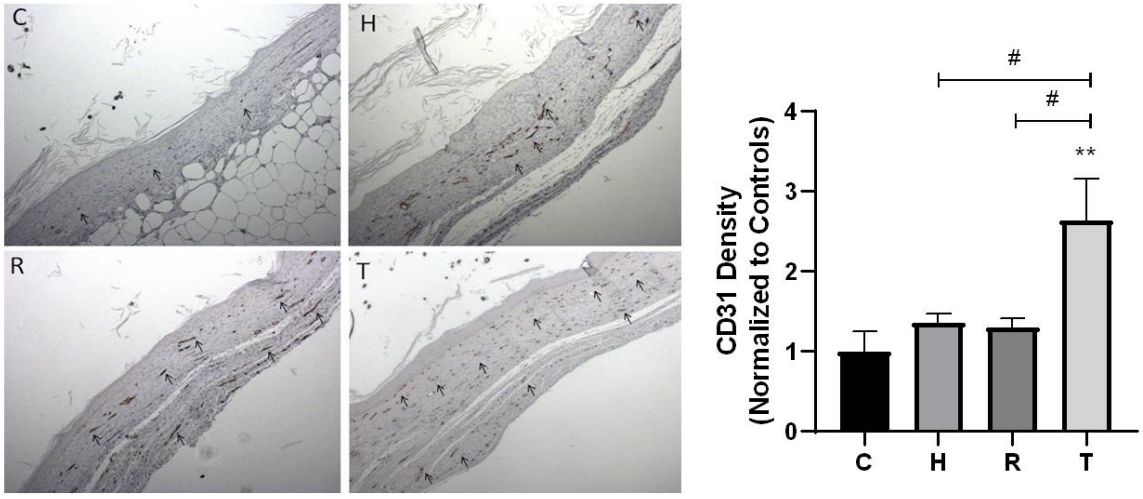


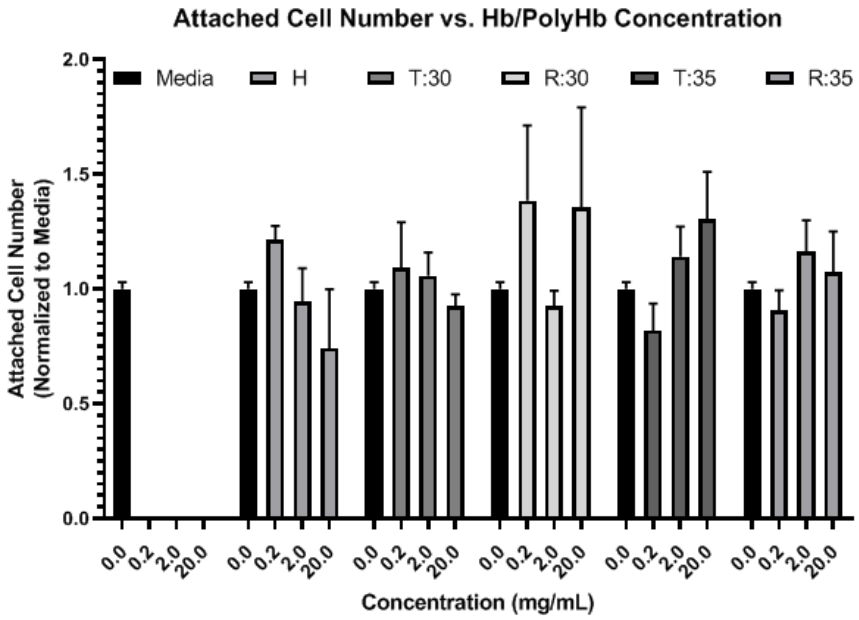
Figure 2.7: CD31 Staining on Day 35 Histological Sections to Indicate Blood Vessel Formation
Arrows point to positive staining (red/brown areas). Results were quantified in the graph. * denote significance versus controls (C). # denote significance between groups indicated.

2.4 Conclusions

These studies lay the groundwork for investigation of the effects of PolyHbs in inflammatory conditions, particularly in relation to macrophages and chronic wound healing. Overall, Hb is toxic to macrophages at concentrations > 20 mg/mL, unlike PolyHbs. Hb reduced secretion of a majority of proteins on an inflammatory panel, whereas the effect of PolyHbs was less drastic, and more comparable to one another across quaternary states and polymerization molar ratios. The inflammatory secretion trends of Hb exhibited more similar trends to R-state PolyHb rather than to T-state PolyHb. IPA analysis identified T-state PolyHb as having the secretion profile most likely to stimulate angiogenesis and wound healing. This was confirmed in an *in vivo* study, where CD31 density and epidermal thickness was the highest in mouse wounds topically treated with T-state PolyHb. Future work should include incorporation of PolyHbs into a wound dressing material, such as a hydrogel, in order to test therapeutic effects in a clinically-relevant delivery system. This work showed that PolyHbs are less toxic to macrophages than Hb,

and that chemical modifications of Hb can affect inflammatory macrophage secretion, which can have an ultimate effect on wound healing.

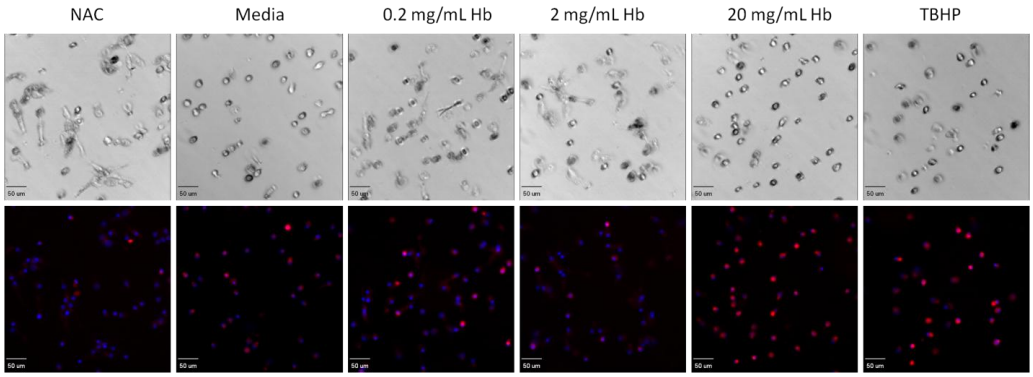
2.5 Supporting information



S.I. Figure 2.1: Attached Cell Number vs. Hb/PolyHb Concentration

Attached cell number for cells treated with 0.2, 2.0, or 20 mg/mL of Hb/PolyHbs (T:30, T:35, R:30, R:35). Following 48 hours of incubation, cells were fixed in 10% paraformaldehyde for 10 minutes, and then stored in 1% paraformaldehyde/PBS at 4°C until cell staining. Prior to adding the stain, fixed cells were washed 3 x 5 minutes with PBS. Hoechst 33342 (Life Technologies, Carlsbad, CA) was added at a ratio of 1:10000 in PBS to cells for 1 minute. Cells were washed with PBS 3 x 5 minutes and then imaged on a fluorescence microscope (Olympus, Shinjuku, Japan) using a 4x objective. Three images were taken (center, left, and right) in each well/experimental condition. Slidebook software (Intelligent Imaging Innovations, Denver, CO) was used to image wells and ImageJ (US National Institutes of Health, Bethesda, MD) was used to count cells per image. Results are normalized to media baseline levels. No significance between groups is detected, due to large error bars and variability with this measurement technique; however, 20mg/mL Hb treatment does have the lowest cell number on average across all groups.

A



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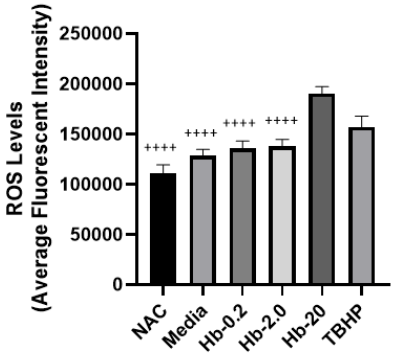
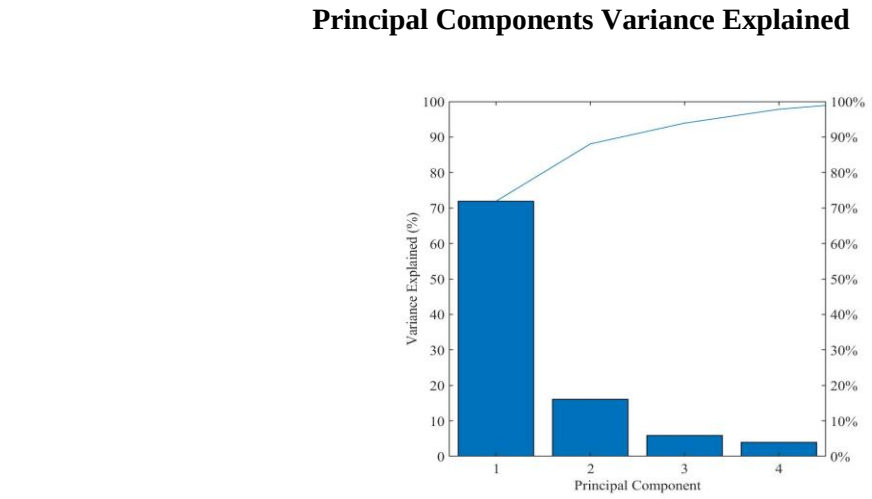


S.I. Figure 2.2: Morphology and Intracellular ROS for Macrophages Treated with Increasing Concentrations of Hb

Macrophages were cultured in media, or increasing concentrations of Hb (0.2, 2.0, and 20.0 mg/mL). 5 μM CellRox Red dye was added for 30 minutes to detect intracellular ROS (Red) and Hoechst 33342 (1:10000 in PBS) to stain nuclei (blue). 5 mM N-acetyl cysteine (NAC) and 300 μM TBHP were used as negative and positive ROS controls, respectively. (A) The first row contains 20x DIC images to show the general morphology of cells with treatments. NAC, 0.2 mg/mL Hb, and 2.0 mg/mL Hb show elongated cells, whereas 20 mg/mL Hb and TBHP groups have rounded cells. The second row contains fluorescent images showing intracellular ROS levels (red). All groups have low levels of red intensity/ROS, except for 20 mg/mL Hb and TBHP. (B) Quantification of ROS levels, by averaging red fluorescent value per cell across images. NAC, media, 0.2 mg/mL Hb, and 2.0 mg/mL Hb have significantly lower levels of ROS than 20.0 mg/mL Hb, which has similar ROS levels as the positive ROS control, TBHP.

A



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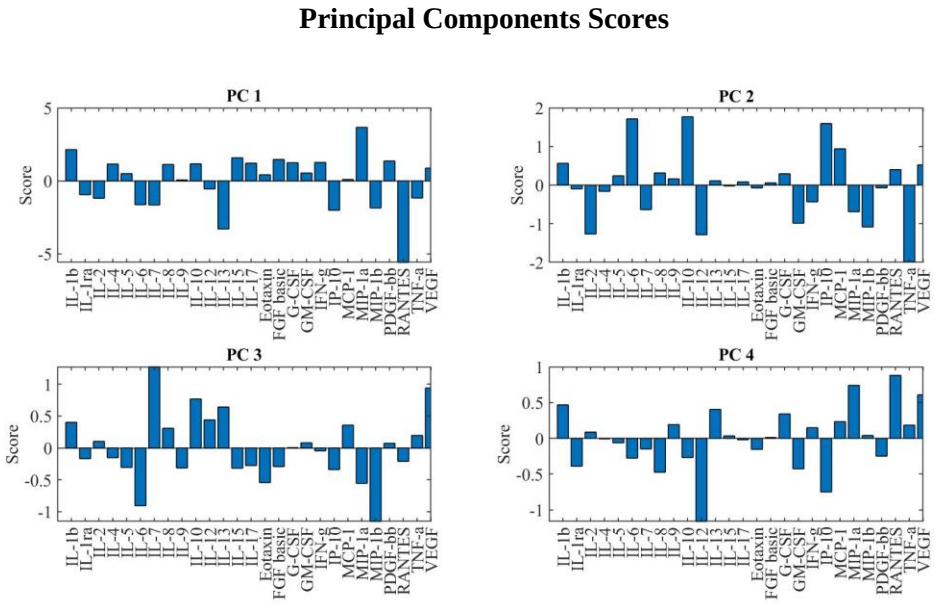
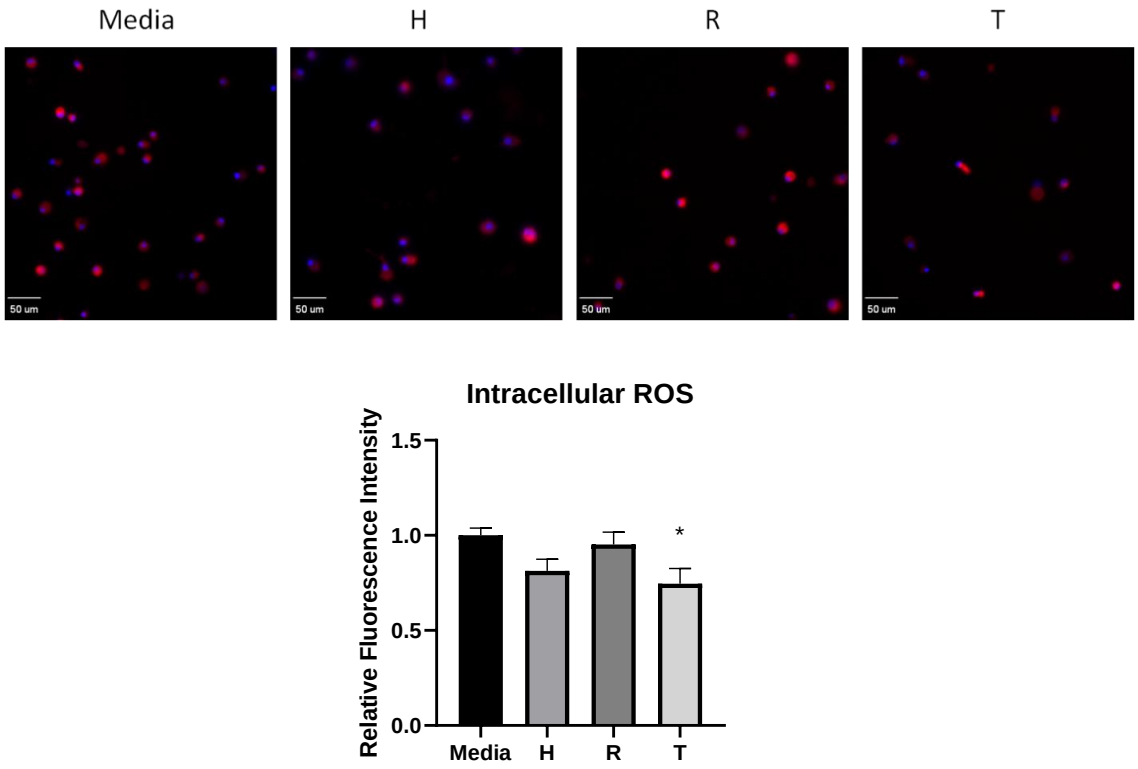


S.I. Figure 2.3: PCA Details for Treatment Secretion Profiles.

(A) Variance of dataset explained for top 4 PCs identified. (B) Scores/weights for each protein in the dataset, in determining values for each PC for each treatment.



S.I. Figure 2.4: Measurement of Intracellular ROS Following Hb/PolyHb Treatment

In this experiment, macrophages were treated with 0.2 mg/mL of Hb, R:30, or T:30. 5 μM CellRox Red dye was added for 30 minutes to detect intracellular ROS (red) and Hoechst 33342 (1:10000 in PBS) to stain nuclei (blue). Representative 10x fluorescent images of macrophages taken are shown. Results are quantified in the plot below, showing the average intracellular ROS/fluorescence intensity for each group. * indicates that T-state PolyHb has significantly lower ROS than media baseline.

2.6 References

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CHAPTER 3: ANTI-INFLAMMATORY EFFECTS OF HAPTOGLOBIN ON LPS-STIMULATED MACROPHAGES: ROLE OF HMGB1 SIGNALING AND IMPLICATIONS IN CHRONIC WOUND HEALING

Note: This chapter is reproduced from the following publication written by **Paulina Krzyszczyk**: **Paulina Krzyszczyk**, Yixin Meng, Maurice D. O'Reggio, Kishan Patel, Ivan Susin Pires, Martin L. Yarmush, Rene S. Schloss, Andre F. Palmer, François Berthiaume. "Anti-inflammatory effects of haptoglobin on LPS-stimulated macrophages: role of HMGB1 signaling and implications in chronic wound healing". *Wound Rep and Regen*, Submitted (2019).

3.1 Introduction

Chronic wounds are stalled in the first stage of wound healing – inflammation – and have difficulty progressing to the final two stages of proliferation and remodeling, when tissue regeneration and wound closure occur (1). A major driver of inflammation in chronic wounds is the prolonged presence of pro-inflammatory macrophages, generally referred to as M1 macrophages (2). M1 macrophages phagocytose dead cells and bacteria, produce high levels of reactive oxygen species (ROS), and recruit other immune cells, such as neutrophils, to the wound (3). Stimulation with pro-inflammatory cytokines or bacterial components promotes the M1 macrophage phenotype, which secretes tumor necrosis factor α (TNF-α), interleukin 8 (IL-8), IL-6, and IL-1β, to name a few (4). In healing wounds, macrophage populations transition to having an M2-like anti-inflammatory phenotype, while the M1 phenotype is attenuated. M2 macrophages produce increased levels of growth factors, such as vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) to support angiogenesis, cell proliferation, and tissue regeneration (3, 5). An increase in interleukin 10 (IL-10) is also typically associated with the resolution of inflammation, and accumulation of M2 macrophages. The prolonged presence of

M1 macrophages, and a delayed switch to the M2 phenotype, are associated with chronic wound conditions. Therefore, therapeutic methods that promote the transition from M1 to M2 macrophages have been attempted to stimulate healing of chronic wounds (2).

There is a body of research that has investigated the potential of hemoglobin (Hb) and haptoglobin (Hp) complexes (Hb-Hp) in eliciting anti-inflammatory macrophages, via the monocyte/macrophage specific receptor CD163 and heme oxygenase-1 pathway (HO-1) (6-10). Hb-Hp bind to CD163 on monocytes and macrophages with high affinity and are endocytosed. Intracellularly, the HO-1 enzyme converts heme into Fe^{2+} (less reactive form of iron), carbon monoxide and biliverdin/bilirubin (6, 11, 12). Carbon monoxide and bilirubin have been shown to have anti-inflammatory effects, and a strong upregulation of IL-10 is also associated with this pathway (7, 13, 14). This pathway is thought to be active *in vivo* in regions of vascular hemorrhage in atherosclerotic plaques, where high levels of iron were concurrent with M2 macrophage markers, such as high CD206, CD163, and low levels of the M1 marker, TNF-α (8, 15).

The Hb-Hp/CD163/HO-1/IL-10 pathway may also have potential benefits in wound healing. Expression of CD163 is desirable as it is upregulated as inflammation is resolved, and wounds begin to enter proliferation (6, 7). Furthermore, deletion or inhibition of HO-1 results in delayed wound healing (16). Diabetic mice also have lower HO-1 expression than non-diabetic mice, which may partially explain their delayed healing. Lastly, IL-10 is a potent anti-inflammatory cytokine, and is associated with M2-like macrophages that support wound healing activities (4, 17). Therefore, promotion of the Hb-Hp/CD163/HO-1/IL-10 pathway may have potential benefits in wound healing.

In the current study, our goal was to characterize and predict the therapeutic potential of Hb-Hp complexes on macrophages within an inflammatory, chronic wound healing context, by measuring their resulting cytokine/chemokine/growth factor secretion profiles. To create a highly-inflammatory *in vitro* system, macrophages were stimulated with lipopolysaccharide

(LPS; 1μg/mL). Stimulation with LPS is known to increase several pro-inflammatory factors, such as interleukin-8 (IL-8), interferon gamma (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF), to name a few (18). Additionally, chronic wounds are often infected, making LPS an appropriate inflammatory stimulus for this model. As controls, Hp and Hb were also delivered individually, in addition to Hb-Hp complexes. The Hb-Hp group was predicted to elicit the strongest anti-inflammatory effects, since the complex has the highest affinity with the CD163 receptor compared to the individual proteins (6, 11, 12). Furthermore, the individual proteins are usually not reported to have benefits, especially in comparison to Hb-Hp (7, 8). Few studies have reported results of this pathway with highly inflammatory, LPS-stimulated macrophages, and those that did, did not thoroughly report results with Hb only or Hp only controls. Furthermore, few studies report cytokine secretion results beyond IL-10. We expanded this characterization as there are many more pro- and anti-inflammatory factors that affect biological outcomes, specifically in regards to wound healing.

Surprisingly, we found that Hp alone was the strongest inhibitor of inflammatory mediator secretion, followed by Hb-Hp. Hb alone significantly increased secretion of many pro-inflammatory factors. Hb-Hp treatment displayed trends similar to Hb for some factors, but also trends similar to Hp for others, including IL-10. Based on current literature, we hypothesize that Hp alone serves to sequester free HMBG1 that is generated by macrophages stimulated with LPS (19-21). As a whole, this work demonstrates the potential of Hp in counteracting inflammatory signaling in macrophages, not only in response to Hb, but in response to LPS as well. Hp treatment may also lead to a macrophage phenotype that is beneficial in a chronic wound healing application.

3.2 Materials and Methods

3.2.1 Reagents

Low endotoxin level Hp (mixed phenotype) purified from human plasma was purchased from Athens Research Technology (Athens, GA). LPS from *E. Coli* was purchased from Sigma

Aldrich (Saint Louis, MO). Human Hb dissolved in Ringer's Lactate was provided by Dr. Andre Palmer's lab, following hypotonic lysis of red blood cells and subsequent purification using tangential flow filtration and 0.2μm filters, in a process similar to that reported in Elmer *et al.* (22).

3.2.2 Monocyte isolation and macrophage differentiation

Human blood donations/buffy coats were received from New York Blood Center (New York City, NY). Primary monocytes were isolated using Ficoll-Paque density gradient centrifugation and $CD14^+$ magnetic bead separation (Miltenyi, Bergisch Gladbach, Germany) according to manufacturer's protocol, in a process similar to that reported in Faulknor *et al.*(23). $CD14^+$ cells were cultured at 37°C at 5 x 10^5 cells/mL with 5ng/mL GM-CSF (R&D Systems, Inc., Minneapolis, MN) for 7 days in complete media – Advanced RPMI 1640 (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum, 1% penicillin-streptomycin (P/S) and 4mM L-glutamine. After 7 days, cells that attached (macrophages) were trypsinized for 15 minutes, frozen down with 1% dimethyl sulfoxide (DMSO), and stored in liquid nitrogen until use for experiments.

3.2.3 Cell culture and metabolic activity measurement

Macrophages were cultured in complete media at 1 x 10^5 cells/well in black, glass-bottom 24-well plates (Cellvis, Sunnydale, CA) and allowed to attach for 24 hours. Then, cells were activated with 1μg/mL of LPS in serum-free media (Advanced RPMI 1640, 1% P/S, 4mM L-glutamine). Concurrently, 0.2 mg/mL of Hp, Hb, or Hb-Hp was added to cells. After 24 hours, supernatants were collected and stored at -80°C until analysis for secreted factors. Alamar Blue Cell Viability Reagent (Life Technologies Corporation, Carlsbad, CA) for measuring cellular metabolic activity was added to media with 1μg/mL LPS at a 1:10 ratio. Once an hour up to 4 hours, fluorescence measurements (excitation 535nm; emission 595 nm) were performed on a DTX 880 Multimode Detector plate reader with Multimode Detection Software (Beckman Coulter, Brea, CA).

3.2.4 Multiplex immunoassay and net secretion scoring

Cellular supernatants were used in the Bio-Plex Pro Human Cytokine 27-plex Assay (BIO-RAD, Hercules, CA)—a magnetic-bead based immunoassay that detects 27 cytokines, chemokines, and growth factors related to inflammation. The list of all 27 factors can be found on the heatmap in Figure 3.2. The assay was carried out according to the manufacturer's instructions, with addition of 0.05% bovine serum albumin to serum-free supernatants. Results were obtained using a Bio-Plex 200 System (BIO-RAD, Hercules, CA). Results are represented as fold changes. Raw secretion concentrations were normalized to media baseline values. Then, fold change was determined by taking the $\log_2$ of the media-normalized value.

Net secretion scoring was determined by summing the fold change values across all factors for each treatment, and rounding to the nearest whole number. Media baseline is represented as a score of zero.

3.2.5 HMGB1 ELISA

HMGB1 levels in supernatants were measured used an HMGB1 ELISA (Biomatik Corporation, Ontario, Canada). The procedure was performed following manufacturer's protocol, and absorbance at 450nm was measured using a DTX 880 Multimode Detector plate reader with Multimode Detection Software (Beckman Coulter, Brea, CA).

3.2.6 Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA; Qiagen, Venlo, Netherlands) version 01-13 was used to interpret secretion profile results. Average fold changes from three experiments for each condition were ran in an expression core analysis with no mutations, including direct and indirect relationships, interaction and causal networks, all node types and data sources, experimentally observed and high (predicted) confidence, restricted to macrophage cell lines and primary human cells within epidermis, dermis, and skin organs, including endothelial cells, keratinocytes, fibroblasts, macrophages, mononuclear leukocytes, and peripheral blood mononuclear cells. z-

scores assigned a value to the predicted up/down regulation of HMGB1 from the treatment compared to media baseline conditions. z-scores represent the predicted activation state of upstream regulators using the expression patterns of the downstream factors, based on relationships published in the literature.

3.2.7 Wound healing scoring

Pro-wound healing trends were identified from a paper by Ligi *et al*. that found significant differences for cytokine/chemokine/growth factor levels between human chronic venous ulcers that were in the inflammatory state versus those showing signs of healing in the proliferative state (24). In that study, the same multiplex immunoassay from BIO-RAD was used to perform measurements in chronic wound fluid. Nine significant pro-wound healing trends were identified in the paper. The trends observed with the Hp /Hb/Hb-Hp treatment secretion profiles were compared to these pro-wound healing trends for each of the 9 factors and incorporated into a scoring system to predict which secretion profile is most likely to promote healing.

Raw secretion data from the current study was normalized to media baseline values. Then, fold change was determined by taking the $\log_2$ of the media-normalized value. If the experimental trend was consistent with the pro-wound healing trend for a specific factor and had an absolute value fold change of 0.1 to 1, +1 point was awarded. If the trend was consistent and had an absolute value fold change of >1, +2 points were awarded. If the trends were inconsistent (*i.e.* experimental trend increased a factor whereas the pro-wound healing trend decreased that factor), points were subtracted rather than added. If the fold change value was between >-0.1 and <0.1, no points were awarded.

3.2.8 Statistics

GraphPad Prism Version 8.1.1 (330) (GraphPad Software, San Diego, CA) was used to generate plots and perform statistical analyses. All numerical results are presented as means ± standard error of the mean (SEM). Statistical analysis of three or more independent experiments were assessed. Raw secretion data was normalized to media baseline values. Then, fold change was

determined by taking the $\log_2$ of the media-normalized value. Positive values represent upregulation and negative values represent downregulation, with zero representing media baseline levels.

One-way ANOVA was used for all datasets except for the multiplex data, in which two-way ANOVA was used. Tukey's *post-hoc* analysis was used to identify groups that displayed significant trends. * denote significance versus media groups. + denotes significance versus Hp group. # denotes significance versus groups indicated by brackets. Increasing numbers of symbols indicate increasing levels of significance. For example, * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$, and **** denotes $p<0.0001$. The same holds true for increasing numbers of + and # symbols.

3.3 Results

3.3.1 Effect of treatments on cellular metabolic activity

In order to assess the general effect of Hb/Hp/Hb-Hp treatments on macrophages and to provide more context for functional characterization, metabolic activity per well was measured (Figure 3.1). This assay was performed on macrophages after 24 hours of exposure to 1μg/mL of LPS, together with 0.2mg/mL Hp, Hb, or Hb-Hp complexes (1:1 mass ratio of Hb:Hp). Results were compared to media controls. No difference was detected among groups, indicating that net metabolic function of macrophages per well was unaffected by the treatments. For this reason, measured secretion profiles were directly compared among groups, in all subsequent studies.

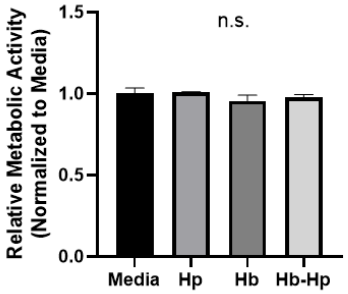

Figure 3.1: Metabolic Activity of Macrophages Following Hp/Hb/Hb-Hp Treatments

Macrophages were treated with or without 0.2mg/mL of Hp, Hb, or Hb-Hp complexes, concurrently with 1μg/mL of LPS. After 24 hours of treatment, metabolic activity was measured using the Alamar Blue Assay. Fluorescence was measured after 2 hours on a plate reader and results were normalized to the media group. n.s. = no significance between all groups.

3.3.2 Overall inflammatory secretion profiles

A multiplex, bead-based immunoassay, of 27 different factors related to inflammation was used to characterize the supernatants of treated macrophages (Figure 3.2A). The heatmap shows levels for each factor in each treatment group normalized to the respective baseline level measured in the media control. Upregulation is indicated by shades of red and downregulation by shades of green. Most measured factors were downregulated by Hp treatment. In stark contrast, Hb-treated groups had a strong upregulation in several factors. Results for Hb-Hp were intermediate – there were several factors that were upregulated, downregulated, or remained close to baseline. The numbered linear scale depicts the net secretion score across all measured factors for each treatment, taken by summing the fold change values for each measured factor (Figure 3.2B). Hp resulted in a net secretion score of -6, reflecting a downregulation of many factors compared to media baseline. Hb was on the opposite extreme, strongly upregulating many of the measured factors, yielding a net secretion score of +15. Hb-Hp also had a positive net secretion score of +10.

3.3.3 Most significant secretion trends

Measurements for individual factors that exhibited significant changes among treatment groups are shown in Figure 3.3. In general, several factors were downregulated by Hp treatment, and strongly upregulated by Hb treatment. Hb-Hp treatment also lead to upregulation, which was less than that of Hb alone, but not significantly different. Factors demonstrating these trends were GM-CSF, IL-6, TNF-α, and IL-2 (Figure 3.3A). For all of these factors, resulting levels from Hb and Hb-Hp treatments were significantly higher than media baseline and Hp groups. Hp decreased secretion of these factors below media, significantly for IL-6. In this group of factors, there was no significant difference between Hb and Hb-Hp. For MIP-1β (macrophage

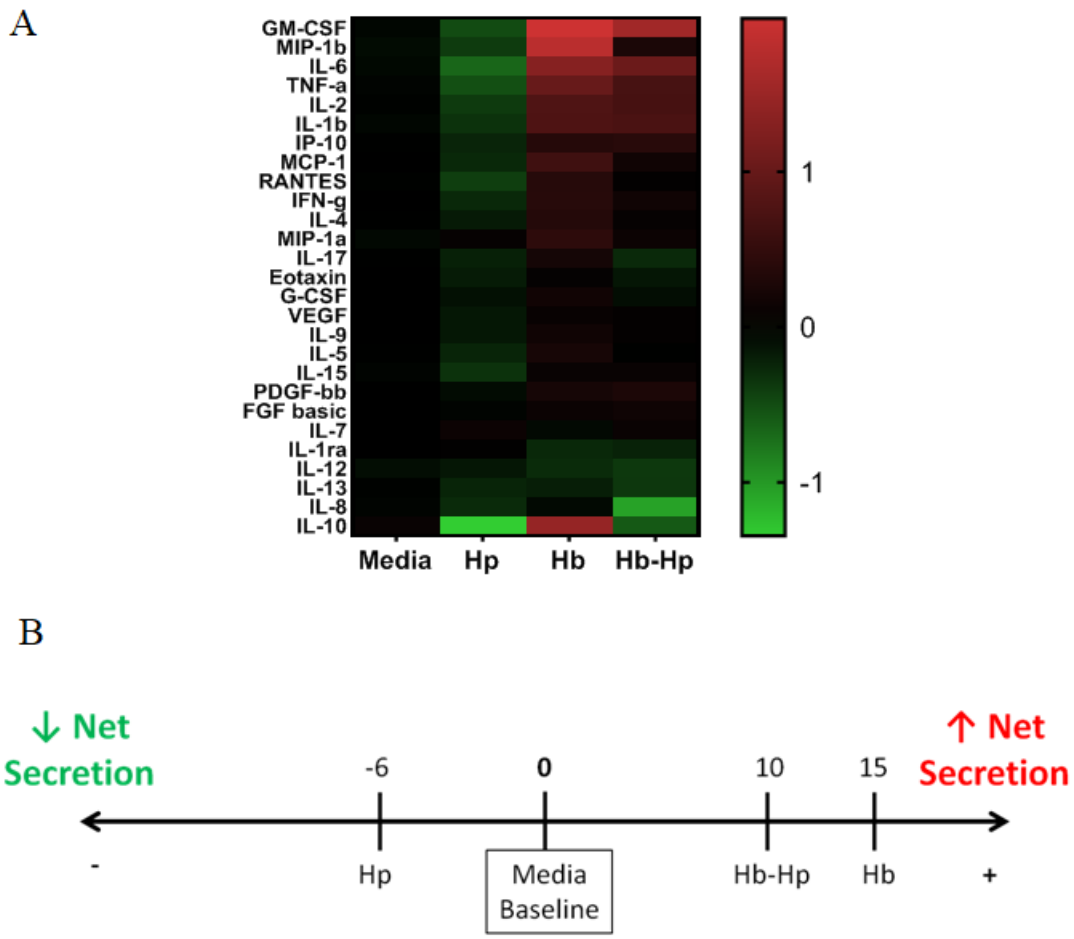

Figure 3.2: Inflammatory Secretion Profiles of Macrophages Treated with Hb/Hp/Hb-Hp

Macrophages were treated with 0.2mg/mL of Hp, Hb or Hb-Hp, concurrently with 1μg/mL of LPS. After 24 hours of treatment, supernatants were collected. Cytokine/chemokine/ growth factor secretion was measured using a BIO-RAD multiplex assay. (A) Heatmap of secretion profile of macrophage supernatants. For each respective factor, results are represented as fold change above (red; positive values) or below (green; negative values) media baseline (black; value of 0). (B) Net secretion scoring based off of secretion profile. Fold change values across all secreted factors were summed and rounded to yield a final score. Scores are depicted on the number line, with media baseline (zero) at the center.

inflammatory protein 1β; aka CCL4), IL-10, and IL-8 (aka CXCL8), Hb and Hb-Hp had significantly different levels (Figure 3.3B). For these three factors, Hb-Hp had significantly lower secretion compared to Hb. For MIP-1β, Hb-Hp resulted in levels close to baseline, with Hb significantly higher, and Hp slightly lower (but not significantly). IL-10 had striking trends as a result of treatments. Hp significantly decreased IL-10 levels compared to media baseline ($p<0.0001$). Hb had significantly higher IL-10 compared to media and Hp groups. Hb-Hp

decreased IL-10 compared to baseline, significantly lower than Hb ($p<0.0001$), as well as slightly higher than Hp alone ($p<0.05$). For IL-8, Hb-Hp resulted in significantly lower values compared to all other groups. The remaining groups had IL-8 levels close to media baseline, and were not significantly different from each other.

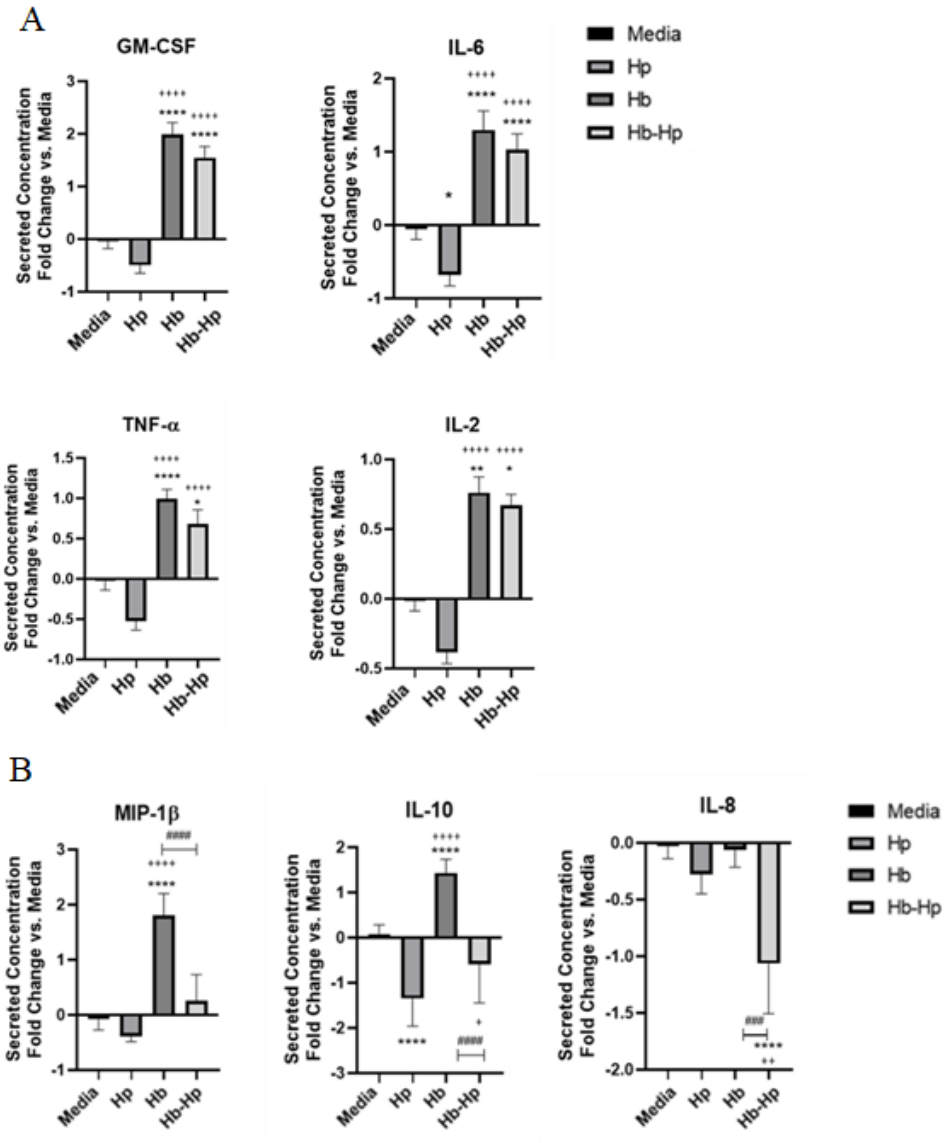

Figure 3.3: Significant Trends for Specific Factors from the Multiplex Immunoassay of Macrophages Treated with Hb/Hp/Hb-Hp

Values are shown as average fold change compared to media baseline (0). Error bars represent SEM. * denotes significance compared to media and + denotes significance compared to Hp group. # denotes significance between groups in associated brackets. (A) Secretion results for a group of 4 factors that have similar trends: GM-CSF, IL-6, TNF-α, and IL-2. Here, Hp has secretion below media baseline (significant for IL-6). Hb and Hb-Hp have secretion higher than baseline, with Hb typically higher than Hb-Hp. Hb and Hb-Hp are all significantly higher than

both Media and Hp groups for all factors. (B) Secretion results for a group of 3 factors, MIP-1β, IL-10, and IL-8, in which levels resulting from Hb-Hp are significantly different than Hb treatment.

3.3.4 Ingenuity Pathway Analysis and HMGB1 signaling

Multiplex secretion data was input into IPA in order to identify canonical pathways that may be responsible for the observed secretion trends. The HMGB1 pathway was identified as likely downregulated by Hp treatment (z-score=-3.606) and upregulated with Hb treatment (z-score= 1.941) (Figure 3.4A). Slight activation of HMGB1 was predicted with Hb-Hp treatment (z-score= 0.832). The list of factors that are implicated in HMGB1 that this prediction was based on are listed in the chart, along with their trends. Of the measured factors, 14 out of 27 contributed to this prediction. To verify this prediction, HMGB1 levels from the supernatants were measured by ELISA (Figure 3.4B). IPA-predicted trends matched experimentally measured trends. More specifically, Hp had the lowest HMGB1 levels, below media baseline and other groups. Hb resulted in significantly higher HMGB1 levels than Hp. Hb-Hp resulted in intermediate levels, significantly higher than Hp alone, but still less than Hb on average.

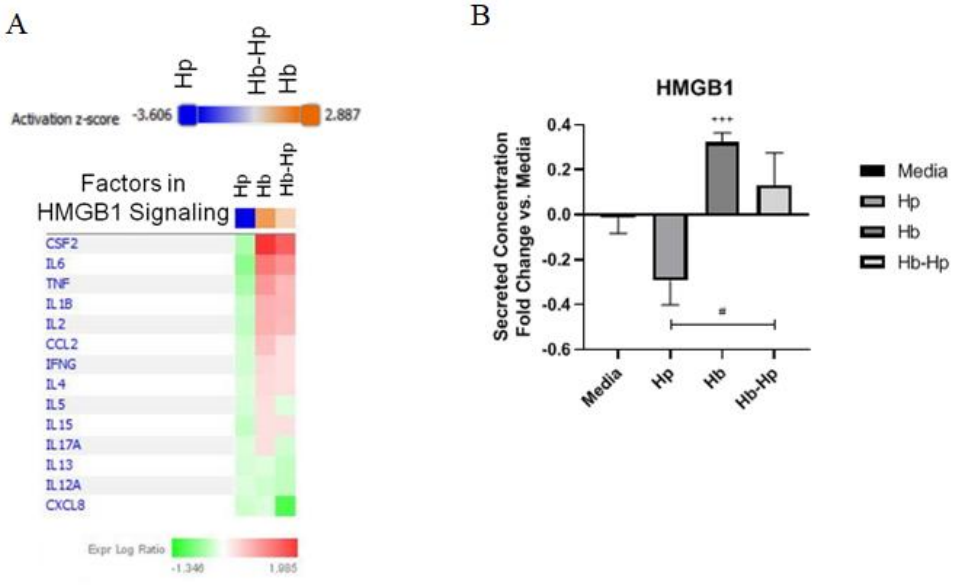

Figure 3.4: High motility group box 1 (HMGB1) IPA Predictions and ELISA Measurements from Hb/Hp /Hb-Hp Treated Macrophages

Secretion profile results were analyzed using Ingenuity Pathway Analysis (IPA), which identified the HMGB1 pathway to be strongly up/down regulated by these treatments. (A) A list of the factors in the secretion profile dataset involved in HMGB1 signaling is shown in the heatmap. The treatments are listed in each column. For each factor, the intensity of red indicates how much it is upregulated due to each treatment, compared to media baseline. Shades of green indicate downregulation. IPA integrates this information to predict whether HMGB1 signaling, as a whole, is likely to be up- (orange) or down- (blue) regulated, and assigns a z-score to represent the confidence of this prediction. Hp group is predicted to be strongly downregulated, whereas Hb is predicted to be strongly upregulated, and slight activation for Hb-Hp. (B) Results from HMGB1 ELISA measured from supernatants from previous studies treated with LPS and 0.2mg/mL of Hp, Hb, or Hb-Hp complexes. Results are normalized to media baseline, and presented as fold changes. Error bars represent SEM. + denotes significance versus Hp. # denotes significance between indicated groups.

3.3.5 Prediction of wound healing effects

We wanted to interpret the inflammatory secretion results within a chronic wound healing context in order to assess which treatment may yield a macrophage secretion profile that can promote healing. Ligi *et al*. used the same BIO-RAD assay to measure inflammatory factors in the wound fluid of human venous ulcers.(24) Samples were taken from wounds in both the inflammatory stage, and granulating/proliferation stage of wound healing. Factor levels were compared between wounds in the two stages, and 9 significant trends were identified. These trends are listed in the table in Figure 3.5. To compare the Hp/Hb/Hb-Hp treatments to these pro wound-healing trends, a scoring system was developed, based on experimental trends compared to the media group. Points were awarded to treatments that followed the pro-wound healing trend for the specific factor, and deducted if the trend was not consistent. The total wound healing score was determined by tallying up the number of points awarded to each treatment. Total wound healing scores are shown on the number line. Hp had the highest score of +5, exhibiting the most pro-wound healing trends. Hb-Hp had a slightly lower score, of +3. Hb had a net negative score of -1, indicating that its secretion profile had the fewest pro-wound healing trends, as observed in *Ligi et al.* This comparison of our experimental data with published pro-wound healing secretion trends in chronic wounds predicts that Hp treatment is likely to be more effective in promoting wound healing than Hb-Hp or Hb treatments.

Pro-healing Trend	Hp	Hb	Hb-Hp
↓ IL-1β	1	-1	-1
↓ IL-12	1	1	1
↓ IL-10	2	-2	1
↓ IL-8	1	0	2
↓ GM-CSF	1	-2	-2
↓ VEGF	1	0	0
↑ IP-10	-1	1	1
↑ RANTES	-1	1	0
↑ PDGF	0	1	1
Total Score	**5**	**-1**	**3**

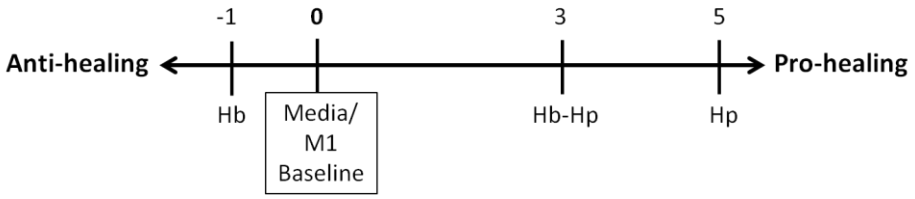

Figure 3.5: Predicted Implications for Hb/Hp/Hb-Hp in Chronic Wound Healing Based on Macrophage Secretion Profile

Wound healing scoring based off of secretion profile measured from the multiplex immunoassay, compared to data from healing chronic wound fluid in Ligi *et al.* (24). The authors also used the same BIO-RAD Multiplex Assay to perform measurements of the same 27 inflammatory factors. Significant pro-wound healing trends for 9 factors were identified as human venous ulcers began healing. The resulting secretion profiles/fold changes from Hp, Hb, or Hb-Hp in the current study were compared to media baseline to determine the experimental trend. The treatment trends were then compared to the pro-wound healing trends from Ligi *et al*. If the experimental trend was consistent with the pro-wound healing trend and had an absolute value fold change of 0.1 to 1, +1 point was awarded. If the trend was consistent and had an absolute value fold change of >1, +2 points were awarded. If the trends were inconsistent, points were subtracted rather than added. If the fold change value was between >-0.1 and <0.1, no points were awarded. The table shows the score for each treatment for each pro-wound healing trend, and the number line shows the total score for each treatment.

3.4 Discussion

Hb and Hp form complexes (Hb-Hp) that modulate macrophage behavior and promote the M2-like phenotype, by activating the HO-1 pathway and upregulating IL-10. Previous studies that have observed this behavior have focused on *in vitro* cell culture systems that are not highly inflammatory, and therefore do not contain LPS (7, 10, 15). We were specifically interested in determining if Hb-Hp treatment could yield an anti-inflammatory secretion profile in macrophages that could counteract the inflammatory environment encountered in chronic wounds, in order to promote healing. Hence, as an inflammatory stimulus for our *in vitro* cell

culture, we used LPS to mimic bacterial infection and inflammation that is commonly found in chronic wounds. We wanted to determine if Hb-Hp could promote anti-inflammatory macrophages in this highly inflammatory, LPS-stimulated environment, and compare the results to macrophages treated only with Hb or Hp.

The measured secretion profile of macrophages included 27 factors related to inflammation. Compared to media baseline levels, Hp reduced secretion of many of the inflammatory mediators (*e.g.* IL-6, TNF-α, IL-1β). Hb alone had the opposite effect, and significantly increased many factors (*e.g.* GM-CSF, IL-6, TNF-α, IL-2). Hb-Hp treatment decreased some factors (*e.g.* IL-8, IL-17, IL-13), and increased others (*e.g.* GM-CSF, IL-6), although to a lesser extent than Hb alone. Overall, Hp reduced inflammatory secretion, Hb increased it, and Hb-Hp resulted in intermediate effects.

Surprisingly, the anti-inflammatory IL-10 did not increase for macrophages treated with Hb-Hp, in contrast to what other studies have reported (7-9, 15). In fact, treatment with Hb-Hp decreased IL-10 secretion compared to baseline, whereas Hp decreased IL-10 levels even more (Figure 3.3B). The distinction between previously published systems and the current study is the inclusion of LPS as a pro-inflammatory stimulus. LPS is a potent stimulator of many cytokines in human macrophages, including iNOS, TNF, IL-1β, IL-10, and others (17, 18, 25). The fact that LPS strongly upregulates IL-10—the prototypical M2 marker—is counterintuitive, because LPS is recognized as an M1 inducer. Our observation of decreased IL-10, along with numerous pro-inflammatory cytokines has been reported a few times in similar systems using LPS, and Hp/Hb-based treatments. For example, Arredouani *et al*. used LPS-stimulated human macrophages, treated them with Hp at increasing concentrations, and measured cytokine levels 72 hours later (26). A significant decrease in IL-10 was observed, beginning at 250μg/mL Hp. At the same time, several other pro-inflammatory factors, such as TNF-α and IL-12p70, were also decreased with Hp treatment. Another example of decreased IL-10 secretion, in conjunction with a decrease in other pro-inflammatory factors from macrophages in an LPS-stimulated system, was in Roach *et*

al. (27). Macrophages were pre-incubated with a Hb-based oxygen carrier (HBOC) called Polyheme to induce HO-1, followed by LPS treatment (27). Pre-incubation with HBOC, followed by LPS stimulation, resulted in decreased levels of IL-10, as well as TNF-α, MCP-1, and IL-6. Similar to these two studies, treatment with Hp in our LPS-stimulated system, resulted in a decrease in IL-10 along with many other pro-inflammatory cytokines.

One possible explanation to the observed decrease, rather than increase, in IL-10 may be due to a self-regulated negative feedback loop that results in signaling that ultimately inhibits p38 phosphorylation and decreases IL-10 production (25, 28). LPS-stimulation in these studies may initially drastically increase IL-10 levels, which activates the negative feedback loop with Hp and Hb-Hp treatment. In contrast, a self-regulated positive feedback loop also exists that affects the extracellular signal-regulated kinase (ERK) pathways, and results in IL-10 upregulation (25). These positive and negative IL-10 feedback loops, implicated with ERK and p38 signaling, respectively, may be key in explaining different trends observed between previous literature and this study, which specifically used an LPS-stimulated system. This warrants further investigation, as well as extending observation time, as IL-10 levels can rise and fall depending on the specific timepoint assayed (29).

We also used IPA to identify any canonical pathways that could explain the observed secretion profiles, and identified HMGB1 as a pathway with strong predictions that Hp treatment would downregulate the pathway, while Hb treatment would upregulate it (Figure 3.4A). It also predicted that Hb-Hp treatment would only slightly increase HMGB1 compared to the media baseline. Consistent with these predictions, HMGB1 secretion levels were decreased by Hp in LPS-stimulated macrophages. Hb had the highest levels of HMGB1, and Hb-Hp was in-between, both significantly different than Hp alone. Thus, each experimental treatment yielded different inflammatory secretion profiles/trends that closely correlated with different HMGB1 levels.

Recently, a link between Hp, HMGB1, and HO-1 has been made in the literature, which helps explain the findings in the current study. Yang *et al*. inadvertently discovered that free Hp

has the potential to bind HMGB1 via CD163 and counteract its inflammatory effects (20, 21). They had intended to use Hp -conjugated beads to remove extracellular Hb from the blood of septic rats, however, found that significant amounts of HMGB1 was also captured. Further experiments demonstrated the link between HMGB1-Hp, CD163, HO-1, and IL-10. HMGB1-Hp also led to a decrease in pro-inflammatory factors such as TNF-α and IL-8 in human macrophages, similar to trends seen in the current study with Hp treatment.

The overall trends and results seen between LPS/treatment, HMGB1, and inflammatory secretion outcome are summarized in Figure 3.6. *In vitro* macrophages were stimulated with LPS, leading to high HMGB1 production (19). Hp alone is free to bind to HMGB1 and reduce its pro-inflammatory effects. Hb does not bind with HMGB1 to sequester it. In fact, Hb amplifies the pro-inflammatory effects of LPS, so HMGB1 levels are further increased, leading to heightened levels of pro-inflammatory factors (20). In the Hb-Hp group, these complexes have extremely high affinity (K_d on the order of 10^{-15} M) (20). In theory, there is no free Hp for HMGB1-Hp complexes to form, so there is nothing to counteract the additional LPS/HMGB1 challenge, and overall pro-inflammatory factor levels remain high, although to a lesser extent than Hb alone. It is noteworthy that Hb-Hp mixtures were formed in a 1:1 mass ratio. Since Hb and Hp bind at a 1:1 molar ratio, using a mass ratio is likely to yield excess Hb, which may explain why the secretion profile of Hb-Hp was more similar to Hb, rather than Hp. Moving forward, Hb-Hp complexes will be formed at a 1:1 molar ratio, followed by chromatographic separation to remove any non-complexed Hb and Hp. Furthermore, comparison of activation of HO-1 in the system is pertinent, as both Hb-Hp complexes, and HMBG1-Hp have been reported to upregulate the pathway (8, 9, 15, 20, 21). Thorough dose response of LPS, Hp, and Hb-Hp, and measurement of HO-1 activation could lead to better understanding of these trends.

Beyond the HMGB1 and general inflammatory secretion trends, our goal was to understand the impact of Hp/Hb/Hb-Hp treatment on chronic wound healing. Ligi *et al.* took wound fluid samples from human pressure ulcers that were either in the inflammatory or the

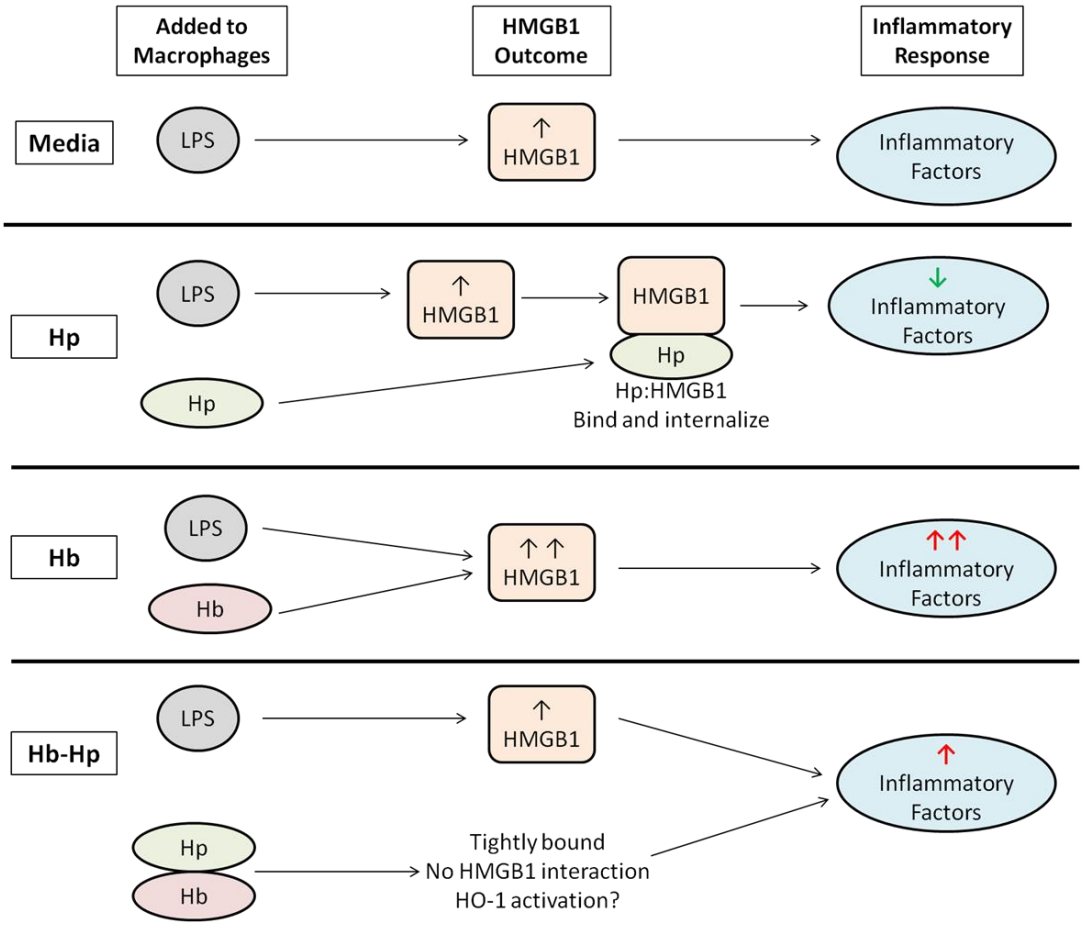

Figure 3.6: Diagram Summarizing Hypothesized Interactions between Hb/Hp/Hb-Hp Treatments, LPS, HMGB1, and Inflammatory Factors in the *in vitro* Macrophage Culture System

All four experimental groups included culturing macrophages in the presence of 1μg/mL of LPS. In media alone, LPS leads to an increase in HMGB1 production, which activates several inflammatory factors. In the second row, the Hp treatment is able to bind free HMGB1 and internalize it, thereby leading to a decrease in the level of inflammatory factors. The Hb group, along with LPS, both increase HMGB1, leading to a strong, pro-inflammatory effect. In the last panel, macrophages are treated with LPS and Hb-Hp complexes, which bind tightly. As usual, LPS leads to the increase in HMGB1. As Hb and Hp are bound very tightly, they do not dissociate, and as a complex, Hb-Hp does not bind HMGB1. Therefore, there is an intermediate net inflammatory result, as the HMGB1 challenge is not resolved.

granulating phases of wound healing (24). They used the same 27-plex immunoassay in the current study to identify significant trends in changes of cytokine levels as healing progressed. The significant pro-wound healing trends observed include decreased IL-1β, IL-12, IL-10, IL-8, GM-CSF, and VEGF, and increased IP-10, RANTES, and PDGF. We compared the secretion

profiles of our study to those, as these significant trends have desirable effects in healing (Figure 3.5). Points were awarded if the treatment (Hp, Hb, or Hb-Hp) followed the pro-wound healing trend, subtracted if it did not, and summed across all factors to yield a final wound healing score. The secretion profile from Hp alone resulted in the most pro-wound healing potential. Hb-Hp has less pro-wound healing trends, and Hb is not predicted to support wound healing. An interesting note is that Ligi *et al.* measured a significant decrease in IL-10 levels between inflammatory and granulating wounds (24). Thus, IL-10 increase does not necessarily correlate with better healing, as is often assumed with its designation as an M2 marker. A decrease in IL-10 is observed in the Hp group (as well as Hb-Hp) of the current study, suggesting that this may be a beneficial wound healing therapy.

Overall, decreased HMGB1 levels, as seen with the Hp group, may also be desirable in a wound healing context. HMGB1 is a damage-associated molecular pattern (DAMP) (30, 31). It can act intracellularly as a transcription factor, but it is also released extracellularly during injury. Downstream HMGB1 signaling activates nuclear factor-κB (NF-κB), increasing the secretion of pro-inflammatory factors, such as TNF-α, IL-8, MCP-1, and others. Higher levels of HMGB1 are also found in individuals with type 2 diabetes (32), and therefore may play a role in the development of diabetic ulcers. In wound healing specifically, lower HMGB1 results in minimal scarring, higher wound breaking strength and wound collagen content (30, 31). Therefore, Hp treatment, which decreases HMGB1 and other pro-inflammatory cytokines in an LPS-stimulated system, may yield benefits in chronic wound healing.

The next steps for this work would be to test these results in a more relevant chronic wound healing system, either using *ex vivo* chronic wound macrophages, or *in vivo*, preferably within a humanized mouse model. The immune systems of wildtype mice and humans have several fundamental differences, including those that affect monocyte/macrophage receptors, such as CD163 (33, 34). In humans, the binding of Hb-Hp to CD163 significantly increases endocytosis and activation of downstream signaling pathways, but in mice, Hp does not promote

binding of Hb to CD163, and Hb can effectively bind to the receptor on its own. There are also concerns about the relevance of testing Hb-based products in mice, as they produce ascorbic acid to counteract pro-inflammatory effects (35). Humans do not have this ability, so results in mice using Hb in wound healing may not be reflective of the human condition (36, 37). Alternative animals that do not produce ascorbic acid, such as guinea pigs, may be more appropriate for *in vivo* testing of Hb/Hp-based products (36, 38).

Another interesting avenue for investigation is the effect and potency of different forms of Hp on the observed anti-inflammatory effects. Human Hp has 3 phenotypes: Hp1-1, Hp2-1, and Hp2-2. The difference in the α subunit of Hp2-1 and Hp2-2 allows for the formation of different sized polymers, and therefore the overall molecular weight can be quite different (89-900 kDa) (21, 39, 40). As Hp phenotype can serve as a predictor for diabetic vascular complications (40), it may be interesting to investigate the abilities of each to bind HMGB1 or Hb, and compare resulting HO-1 activation and inflammatory secretion.

In conclusion, this study revealed that in an inflammatory environment typical of chronic wounds, Hp treatment attenuated pro-inflammatory factor production to the greatest extent with predicted benefits in wound healing. Hp also decreased HMGB1 levels, as predicted by IPA software analysis and measured experimentally via ELISA. A recent, and relatively unknown discovery by Yang *et al.*, found that in addition to binding Hb with high affinity, free Hp also binds to HMGB1 with high, but lesser affinity (20, 32). This binding interaction and sequestration of HMGB1 may explain the decrease in associated pro-inflammatory signaling in LPS-stimulated macrophages with Hp treatment. Taken as a whole, these results suggest that Hp on its own can have anti-inflammatory effects on macrophages by affecting HMGB1 signaling, which may have potential in chronic wound healing applications.

3.5 Abbreviations

CCL2 = see MCP-1; CCL4 = see MIP-1β; CSF2 = see GM-CSF; CXCL8 = IL-8; DMSO = dimethylsulfoxide; FGF = fibroblast growth factor; G-CSF = granulocyte-colony stimulating

factor; GM-CSF = granulocyte-macrophage colony-stimulating factor, aka CSF2; Hp = haptoglobin; HBOC = hemoglobin-based oxygen carrier; Hb = hemoglobin; Hb-Hp = hemoglobin-haptoglobin complex; HO-1 = heme oxygenase 1; HMGB1 = high motility group box 1; IFN-g = interferon-γ; IL = interleukin; IPA = Ingenuity Pathway Analysis; LPS = lipopolysaccharide; MCP-1 = monocyte chemoattractant protein-1, aka CCL2; MIP-1β = macrophage inflammatory protein 1β, aka CCL4; NF-κB = nuclear factor-κB; PDGF = platelet-derived growth factor; P/S = penicillin-streptomycin; ROS = reactive oxygen species; SEM = standard error of the mean; TNF-α = tumor necrosis factor-α; VEGF = vascular endothelial growth factor

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CHAPTER 4: CONCLUSION

4.1 Key Findings

The aim of this dissertation work was to investigate the potential of Hb-based complexes for use in chronic wound healing applications. Hb-based therapies have the ability to deliver oxygen and modulate macrophage phenotype. Two types of Hb-based treatments were tested: PolyHbs and Hb-Hp complexes. PolyHbs specifically allow for tuning of oxygen delivery due to polymerization in their oxygenated/deoxygenated (R/T) quaternary states. The molecular weight of resulting PolyHbs can also be controlled by changing the ratio of Hb:glutaraldehyde (cross-linking reagent). Hb-Hp complexes were studied due to their ability to activate the HO-1 pathway in macrophages, which highly upregulates IL-10, a typical M2 macrophage marker (1-4). To study this interaction in a highly inflammatory environment analogous to chronic wounds, LPS was used to stimulate *in vitro* M1 macrophages. The inclusion of LPS-stimulation, Hp and Hb controls, as well as analysis of an inflammatory panel of 27 secreted factors within the experimental design, allowed for thorough comparison of groups and novel conclusions regarding the involvement of Hp in the HMGB1 signaling pathway. The following sections summarize the 4 key conclusions from these studies:

4.1.1 Polymerizing Hb affects metabolic activity and secretion profile of macrophages

Treating macrophages with increasing concentrations of Hb and PolyHbs (0.2, 2, and 20 mg/mL) for 48 hours revealed that 20 mg/mL Hb impaired macrophage viability and function, detected by a significant decrease in net cellular metabolic activity. Net metabolic activity of macrophages treated with 20 mg/mL R and T-state PolyHbs (both 1:30 and 1:35 polymerization ratios) was not affected, suggesting that PolyHbs are less toxic than Hb. When the inflammatory secretion profile was measured from macrophages treated with 2 mg/mL of Hb or PolyHbs, the secretion levels for a majority of the factors decreased (21/27). This was the most drastic result; PolyHbs decreased secretion for fewer factors and to a lesser extent. For example, Hb had significantly lower levels than all other groups for TNF-α and IL-2. The effect of PolyHb

treatment was generally similar to one-another, although Hb yielded more similar secretion results to R-state versus T-state PolyHbs. For example, GM-CSF and IL-12 levels for Hb-treated macrophages were significantly lower than T-state groups, but not R-state. Between treatment groups, the difference between PolyHb secretion versus Hb was the most apparent, and was captured by the most dominant principal component in PCA (accounting for 71.9% of data variance). The second and third principal components, which represented 16.2% and 5.8% variance, clustered groups by polymerization state, and by polymerization ratio, respectively. These results show that PolyHbs have differing effects on macrophages than unmodified Hb, measured by differences in metabolic activity and inflammatory secretion.

4.1.2 T-state PolyHb yields secretion profile that is most favorable for angiogenesis and wound healing

PCA results from macrophage secretion data indicated that differences between polymerization states were more apparent than differences between polymerization ratio. This result was supported following IPA analysis, which identified beneficial trends in T-state treatment (1:30), when interpreted in an angiogenesis and wound healing context. The prediction for T-state 1:30 group indicated an increase in *migration of endothelial cells*, *cell movement of microvascular endothelial cells*, and *tubulation of vascular endothelial cells*, compared to other Hbs/PolyHbs. Specific factors contributing to these predictions were PDGF, IL-8, VEGF, and GM-CSF, which were highest for the T-state 1:30 group. T-state 1:30 also had predictions for the most *migration of cells*, *cell viability*, and *growth of connective tissue* versus other experimental groups. When tested in *in vivo* diabetic mouse wounds, T-state 1:30 treatment resulted in the thickest epidermal tissue, as well as the highest vascular endothelial CD31 staining density. Overall, T-state 1:30 PolyHb was predicted to have the most beneficial trends for angiogenesis and wound healing through IPA analysis, which was confirmed with *in vivo* results.

4.1.3 Hp, and not Hb-Hp complexes, attenuate inflammation to the greatest extent in LPS-stimulated macrophages

Hb-Hp complexes are reported to have anti-inflammatory effects due to their high affinity with CD163 receptors on macrophages, which upon endocytosis, activates the HO-1 pathway and IL-10 production (1, 3, 5, 6). Most of the previous literature observed this in non-inflammatory systems; however, within our highly-inflammatory system stimulated with LPS, we unexpectedly observed a large decrease in inflammatory secretion in the Hp group. This was seen in factors such as GM-CSF, IL-6, IL-10, and TNF-α. At the same time, Hb treatment resulted in significantly higher secretion of many inflammatory factors, including the aforementioned ones. Hb-Hp group yielded secretion levels only slightly lower than Hb alone. Overall, Hp resulted in much lower net inflammatory secretion (score of -6) compared to Hb and Hb-Hp groups (scores of 15, and 10, respectively).

4.1.4 HMGB1 signaling is implicated in interactions between macrophages and Hp/Hb/Hb-Hp

Secretion results were analyzed using IPA, which identified HMGB1 as a canonical pathway that may be affected by the treatments (14/27 measured inflammatory factors implicated in the pathway). IPA predicted a strong decrease in HMGB1 for Hp treatment, a strong increase for Hb treatment, and a slight increase in Hb-Hp treatment. These predictions were confirmed by measurement of HMGB1 in the supernatants of treated macrophages. Our hypothesis is that free Hp binds HMGB1 found at high levels in our *in vitro* system, specifically due to LPS stimulation. This supports recent findings in the literature that Hp can bind HMGB1 and CD163, which activates HO-1, and decreases inflammatory secretion (7, 8). This reveals potential benefits of free Hp over Hb-Hp complexes: Hp can sequester HMGB1 to fight this inflammatory challenge, whereas Hb-Hp complexes are tightly bound and cannot decrease HMGB1 levels.

4.2 Limitations and challenges

4.2.1 Primary cell culture

The use of primary human cells is one of the strengths of this research, but also doubles as a limitation. The use of primary human cells, rather than a cell line, makes this work more translatable, as primary cells behave more similarly to those found *in vivo*. Furthermore, cell lines are immortalized to promote proliferation and expansion in cell culture systems. As a result, they can express different signaling pathways and receptors than they normally would. Of relevance to this project is that, out of 8 murine and human monocyte/macrophage cell lines, only one is known to express CD163 (SU-DHL-1) (9). Since the basis of this project heavily relies on the Hb-Hp interaction with CD163, experiments had to be performed using primary cells, which come with several challenges. These include a long isolation process – 8 hours to isolate CD14+ monocytes from blood donations, followed by 7 days for macrophage differentiation. At the end of this, many cells fail to attach and differentiate into macrophages, with some donors having better results than others. These differences may be due to donor-specific factors, such as age or state of health at time of donation. Currently, there is no way to detect whether a donor's cells will have adequate attachment before the differentiation process is complete. Due to these difficulties, some donors do not have a high enough cell number to use in subsequent studies, as they do not proliferate in culture. Therefore, the usable cell number is capped at a much lower number than that of cell-lines. So, care must be made in planning out studies and number of experimental groups. Probing for different proteins or markers in follow-up studies is also limited by the remaining cell number for the donor of interest.

4.2.2 Haptoglobin protein source

In addition to challenges with the cell source, protein source must also be carefully selected. Proteins should be pure, with low or negligible levels of endotoxin, since macrophages are highly sensitive to any inflammatory signal. In fact, it has been shown that even low levels of endotoxin (0.2-2 ng/mL LPS) can affect the secretion of cytokines from monocytes, such as IL-8,

IL-6, TNF-α and IL-1β (10). Early studies in this project used Hp that was not highly purified. When compared to the low-endotoxin Hp, the original Hp drastically promoted secretion of inflammatory proteins from macrophages; 1000 fold higher in the case of IL-10. This masked the specific effect of Hb-Hp interactions that we were interested in studying. Another consideration is the phenotype of Hp used in experiments. In these studies, mixed phenotypes were used (Hp 1-1, 2-2 and 2-1), however, there is evidence that each phenotype has varying binding affinity with Hb and CD163 (11). Specifically, Hp 1-1 has the highest affinity and therefore the highest anti-oxidant capability. Future work should move towards using pure rather than mixed Hp phenotypes in studies in order to standardize the experimental treatment.

4.2.3 Challenges in macrophage and chronic wound healing fields

More generally, the limitations of this research project lie in the fundamental complexity of this field of work. Many questions remain unanswered about even the physiology of chronic wounds—the recruitment and differentiation of monocytes and macrophages, characterization of the different phenotypes, and their specific roles in the healing process (12). More research and information in this area are needed to advance the development of effective therapies. Table 1.1 lists some of the challenging questions that remain to be answered in this area of research.

4.3 Future directions

Future work on this project will work towards addressing some of the larger questions in Table 1.1, particularly macrophage phenotype characterization and use of a relevant *in vivo* wound model.

4.3.1 Expand macrophage characterization

The studies in this thesis include a thorough characterization of secreted inflammatory cytokines, chemokines and growth factors from macrophages in response to Hb-based complexes. Although secreted factors are indicators of the resulting macrophage phenotype, characterization should be further expanded to include gene and cell-surface receptor expression using polymerase chain reaction (PCR) and flow cytometry, respectively. Additional M1/M2 markers could be

simultaneously measured (CD68, CD86, CD206, etc; see Table 1.2), to give a better sense of the specific macrophage phenotype that results from PolyHb or Hb-Hp treatments.

Of particular interest to the Hb-Hp project is the expression of HMOX-1 (HO-1 gene) and CD163 surface receptor. More thorough dose responses of LPS and Hp/Hb/Hb-Hp concentrations should be performed and assayed over time to observe how HMGB1, CD163, HO-1, and IL-10 are affected. We are particularly interested in comparing the ability of Hp-HMGB1 to activate HO-1 with that of Hb-Hp at various concentrations, and also validating Hp results using an anti-HMGB1 neutralizing antibody.

4.3.2 Wound healing studies in humanized mice

Mice are commonly-used animal models for wound healing studies due to their affordability and ease-of-use, however, it is important to acknowledge differences between human and murine skin anatomies, wound healing processes, and immune systems (and hence, macrophage behaviors) (13). The immune system, in particular, has several fundamental differences between humans and mice (14), For example, a majority of human blood (~60% of total cells) is composed of granulocytes and monocytes (15). In contrast, these cells make up less than 25% of the composition of murine blood, which is dominated by B- and T-cells (~75%). Furthermore, monocyte/macrophage markers and receptors differ between mice and humans (15). In particular, the CD163 receptor, which is central to our motivation for studying the effects of Hb complexes on macrophages, differs between human and mice (16). In humans, the binding of Hb and Hp to CD163 significantly increases endocytosis of Hb and activation of downstream signaling pathways. In mice however, Hp does not promote binding of Hb to the receptor.

Future work should include an *in vivo* model in which the immune system is more translatable to humans. A suitable *in vivo* model for this purpose may be humanized mice, which are becoming more advanced, and therefore more prevalent, in immunology and inflammatory research (17). Humanized mice are typically immunodeficient mice that are transplanted with human hematopoietic stem and progenitor cells (14). Although overall, resulting mice have more

human-like immune systems over non-humanized mice, particularly macrophage survival and function are defective. Human macrophages are present in low numbers, exhibit an immature phenotype and do not function properly. To address this, a mouse strain, called MI(S)TRG, has been developed to contain higher numbers of macrophages that behave similarly to those in humans (14). These mice are created by knocking-in genes for 4 human proteins to replace the mouse analogs: M-CSF, GM-CSF, IL-3, thrombopoietin. The mice also contain a transgene for human SIRP-1α. Results with MI(S)TRG are highly promising, as resulting monocytes exhibit similar diversity as those in humans. They also successfully invaded a tumor model and exhibited M2-like macrophage characteristics. These and other humanized mice with knock-in human genes are commercially available through The Jackson Laboratory. The use of these mice would provide a more relevant model for studying the effect of Hb-based complexes on wound macrophages *in vivo*.

4.3.3 Oxygen uptake studies

Although the current thesis focuses on cell-signaling and protein production due to Hb's interaction with macrophages, oxygen delivery is another important component that should be examined for experimental chronic wound healing treatments. PolyHbs have the ability to tune oxygen delivery whether in their T-state or R-state, and depending on the extent of polymerization (18). Since T-state PolyHbs release oxygen more readily than R-state PolyHbs, particularly at low oxygen tensions (18), we predict that T-state-treated cells would have higher oxygen consumption rates. Investigation of the direct effect of oxygen delivery to macrophages by PolyHbs would provide insight on whether this component of the treatment has a significant effect on cells, or if macrophages are more affected by direct Hb/macrophage interactions and signaling. Oxygen consumption could be tested not only in macrophages, but also in other skin cells, such as fibroblasts, keratinocytes and endothelial cells. This would reveal interesting information into whether oxygen delivery by PolyHbs has a greater effect on a specific cell type.

The Agilent MitoXpress Intra Intracellular Oxygen Assay is one assay that could be used to measure intracellular oxygen. It is compatible with a 96 well-plate/plate reader format, which would allow for concurrent measurements in many different conditions (19). The assay is based on an oxygen-sensitive, fluorescent, nanoparticle probe that enters cells, where oxygen quenches the emitted fluorescent signal. The resulting measurement is proportional to the amount of cellular oxygen uptake.

Another method that could be used to measure oxygen consumption rate of *in vitro* cells is through respirometry (20). This assay measures respiratory activity in mitochondria, which are key organelles involved in oxygen uptake by cells. Cells are permeabilized so that only the mitochondrial membrane is left intact, which allows for their direct interaction with the test substance. One system, called the Oroboros Oxygraph-2k, contains a closed chamber that monitors oxygen concentration in the culture media, as well as oxygen consumption by mitochondria, over time (21). PolyHbs can be injected into the chamber, and resulting oxygen consumption rate of macrophage mitochondria can be measured. Respirometry should be a secondary approach to the Agilent intracellular oxygen assay, as it requires expensive, specialized equipment, limited sample throughput, and permeabilization of cells.

Another addition to these studies could be the use of a hypoxic chamber to create a better *in vitro* chronic wound model, since chronic wounds have decreased oxygen levels (22, 23). The oxygen consumption rate of PolyHb-treated cells cultured in normoxia (21% O_2; standard biological incubators) could be compared with those cultured in hypoxia (1% O_2). The use of commercially-available incubators that allow for a wide range of oxygen control (1-14%) may lead to interesting results between PolyHbs.

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