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Regulation of Macrophage Polarization and Wound Healing

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Abbreviations and Acronyms

$A_{2A}R$ = adenosine A_{2A} receptor

FIZZ1 = found in inflammatory zone 1

IFN-γ = interferon gamma

IL = interleukin

IL-4Rα = interleukin-4 receptor alpha

IRF5 = interferon regulatory factor 5

Jmjd3 = Jumonji domain containing-3

LPS = lipopolysaccharide

MR = mannose receptor

M1 Mϕ = classically activated Mϕ

M2 Mϕ = alternatively activated Mϕ

Mϕ = macrophage

(continued)

Background: Macrophages (Mϕs) participate in wound healing by coordinating inflammatory and angiogenic processes. Mϕs respond to environmental cues by adopting either "classically" activated (M1) proinflammatory or "alternatively" activated (M2a, M2b, M2c, M2d) wound healing phenotypes.
The Problem: Mϕ polarization is essential for wound healing and aberrations in this process are linked to several pathologies. It is important to elucidate molecular mechanisms underlying Mϕ polarization.
Basic/Clinical Science Advances: Mϕs are categorized as proinflammatory (M1) or anti-inflammatory/wound healing (M2). M1 Mϕs are observed in initial tissue damage responses, are induced by exogenous pathogen-associated molecular patterns or endogenous damage-associated molecular patterns, and exhibit increased phagocytosis and pro-inflammatory cytokine production, facilitating innate immunity and wound debridement. M2 Mϕs predominate later in repair, express vascular endothelial growth factor, transforming growth factor beta, and interleukin 10 (IL-10), are activated by varied stimuli, assist in the resolution of inflammation, and promote tissue formation and remodeling. Recent work has characterized a novel "M2d" phenotype resulting from adenosine-dependent "switching" of M1 Mϕs that exhibits a pattern of marker expression that is distinct from canonical IL-4/IL-13–dependent M2a Mϕs. Recent studies have demonstrated important roles for specific transcriptional elements in M1 and M2a Mϕ polarization, notably members of the interferon regulatory factor family interferon regulatory factor 5 (IRF5) and IRF4, respectively. The role of these IRFs in M2d polarization and wound healing remains to be determined.
Clinical Care Relevance: Knowledge of microenvironmental signals and molecular mechanisms that mediate Mϕ polarization should permit their manipulation to regulate important physiological processes and resolve pathological conditions.
Conclusion: Proper Mϕ polarization is essential to effective wound healing, and distinct phenotypes, such as the angiogenic M2d Mϕ, may be of critical importance to this process. The IRF5 transcription factor has been shown to play a key role in M1 Mϕ activation and the Jumonji domain containing-3-IRF4 pathway has been implicated in M2 Mϕ activation.

BACKGROUND

As immune effector cells, the role of macrophages (Mϕs) in inflammation and host defense is well characterized. Additionally, Mϕs are integral in the promotion of proper wound healing as well as the resolution of inflammation in response to pathogenic challenge or tissue damage. These diverse physiological functions stem from the remarkable plasticity of Mϕs, which allows these

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cells to dramatically change their form and function in response to local environmental signals.[1–3] Unstimulated Mϕs are typically quiescent; stimulation of these cells, however, results in the development of markedly polarized phenotypes in response to molecular cues residing in the local microenvironment. Current classification of Mϕs recognizes polarization into two distinct phenotypes, termed "classically" activated (M1) or "alternatively" activated (M2).[2] M1 Mϕs are induced by recognition of pathogen-associated molecular patterns, such as bacterial lipopolysaccharides (LPS) and peptidoglycan, or damage-associated molecular patterns, such as released intracellular proteins and nucleic acids, as well as stimulation by the T-cell–secreted cytokine interferon gamma (IFN-γ). M1 Mϕs represent a proinflammatory phenotype, exhibiting increased phagocytic and antigen processing activity as well as increased production of proinflammatory cytokines (*e.g.,* interleukin 1 [IL-1], IL-6, IL-12, and tumor necrosis factor alpha [TNF-α]) and oxidative metabolites (*e.g.,* nitric oxide and superoxide) to promote host defense and removal of damaged tissue. In contrast, M2 Mϕs are induced by a variety of stimuli (*e.g.,* IL-4/IL-13, glucocorticoids) and represent a phenotype that is potentially important in the promotion of wound healing and tissue remodeling as well as the resolution of inflammation.[1–3]

CLINICAL PROBLEM ADDRESSED

The remarkable plasticity of Mϕs has important implications for clinical science. Proper Mϕ polarization is necessary in several important physiological processes including, but not limited to, wound healing, immune response, and nerve/muscle regeneration.[1–5] Thus, it is not surprising that aberrations in Mϕ polarization are associated with some of the pathology observed in defective wound healing, diabetes, muscular dystrophy, fibroproliferative diseases such as rheumatoid arthritis and liver and lung fibrosis, as well as tumor progression.[1–4,6–8] Elucidating the specific microenvironmental signals that contribute to Mϕ polarization could potentially lead to methods for the pharmacological manipulation of Mϕ phenotypes to promote favorable processes (*e.g.,* wound healing) or inhibit pathologic processes (*e.g.,* fibroproliferative diseases and tumor growth).

RELEVANT BASIC SCIENCE CONTEXT

One of the hallmarks of Mϕs is their remarkable plasticity, that is, the ability to alter their phenotype in response to different environmental stimuli. Two major categories of Mϕs, those exhibiting proinflammatory and anti-inflammatory/wound healing phenotypes, are currently recognized and are termed M1 and M2, respectively.[2] Considerable research has sought to both identify the wide variety of signals that induce these phenotypes as well as characterize the molecular profiles of M1 and M2 Mϕs, as outlined in Fig. 1.[1–3] However, as our knowledge of Mϕ polarization becomes more complex, it has emerged that there is a broader set of signals that induce distinct Mϕ phenotypes than the traditional M1/M2 classification accommodates. For instance, although IL-4/IL-13 signaling through the IL-4 receptor-α (IL-4Rα) represents the prototypical M2 Mϕ activation pathway, recent research has demonstrated the presence of Mϕs exhibiting M2-like characteristics even in the absence of this signaling.[9] In addition, M2 Mϕs induced by IL-4, although exhibiting reduced phagocytic activity, show markedly increased secretion of proinflammatory cytokines in response

Abbreviations and Acronyms *(continued)*

NF-κB = nuclear factor-κB

TGF-β = transforming growth factor beta

TLR = Toll-like receptor

TNF-α = tumor necrosis factor alpha

VEGF = vascular endothelial growth factor

Ym1 = eosinophil chemotactic factor L

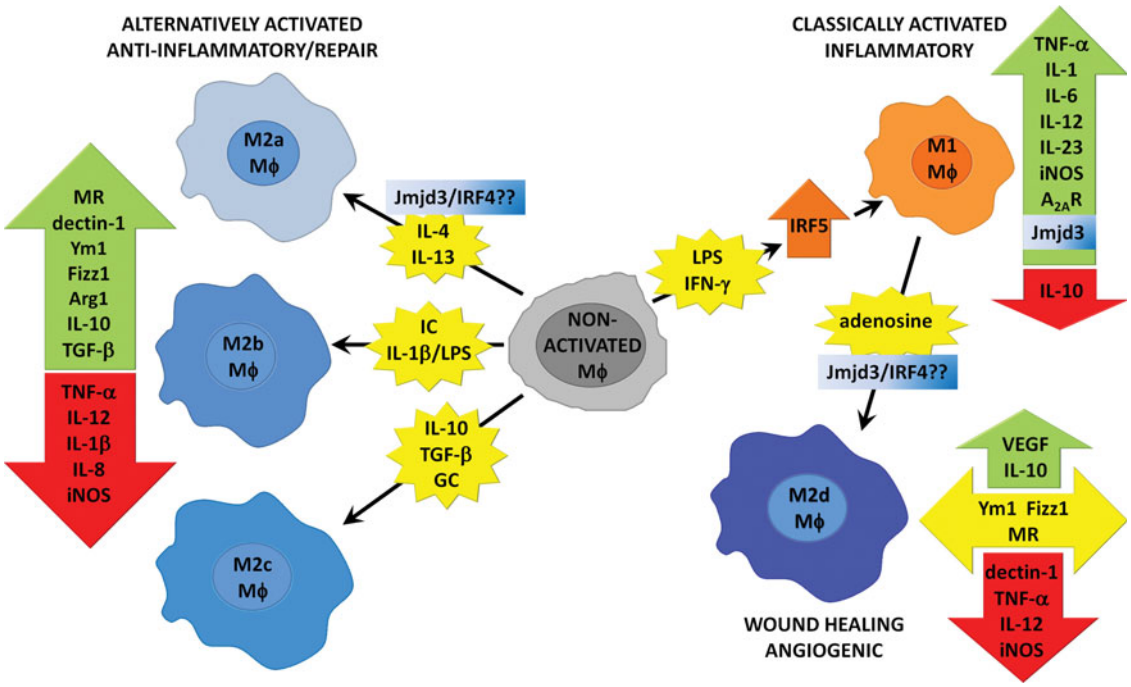

Figure 1. Pathways of Mϕ polarization. Nonactivated Mϕs are polarized into distinct phenotypes by specific inducing agents and display typical changes in gene expression. Note that not all inducing agents are included and expression profiles for different M2 Mϕ subtypes can differ based on the nature of induction.

to LPS challenge.[10] Finally, we recently defined a subtype of M2-like Mϕs induced by costimulation of Mϕs with Toll-like receptor (TLR) and adenosine A_{2A} receptor (A_{2A}R) agonists that display a distinct molecular signature. A_{2A}R stimulation by adenosine in the presence of TLR agonists switches Mϕs from a M1 phenotype into an angiogenic M2-like phenotype, which we have termed "M2d."[11] The discovery of this and other novel Mϕ activation states underscores the importance of local extracellular signals in determining Mϕ function. Thus, a more complete understanding of the spatiotemporal changes in signaling molecules during wound healing and their effect on Mϕ function may allow for the enhancement of this critical process.

TARGET ARTICLES

1. Lucas T, Waisman A, Ranjan R, Roes J, Krieg T, Muller W, Roers A, and Eming SA: Differential roles of macrophages in diverse phases of skin repair. J Immunol 2010; **184:** 3964–3977.

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EXPERIMENTAL MODEL OR MATERIAL: ADVANTAGES AND LIMITATIONS

Studies of the influence of Mϕ polarization on wound healing have traditionally utilized *in vitro* culture of Mϕs and *in vivo* models of wound healing in mice as experimental models. *In vitro* culture represents a good model for the identification of Mϕ activation stimuli as well as elucidation of the molecular profiles of resultant Mϕ populations, because of the ability to precisely control the extracellular milieu. However, the lack of other cell types that would normally be present, as well as other physiological limitations, could obscure important *in vivo* changes that may occur in the local environment that could affect Mϕ polarization. *In vivo* models of wound healing certainly address some of these concerns and the use of mice allows for genetic manipulation to identify proteins and

other factors that may contribute to Mϕ polarization, but important limitations remain. In particular, significant species-specific differences in Mϕ protein expression have been demonstrated, indicating the need for further elucidation of M1/M2 Mϕ polarization in the human system.[1]

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

Mϕs are a population of immune cells that orchestrate a diverse array of functions including inflammation, tissue repair, and immune responses. This functional diversity is achieved by the remarkable heterogeneity of Mϕs, which have the capacity to dramatically change their phenotype as a result of differentiated plasticity as well as local environmental cues. Mϕs are generally classified as either classically (M1) or alternatively (M2) activated. M1 Mϕs have a proinflammatory phenotype exhibiting increased phagocytic activity and secretion of proinflammatory cytokines that aid in the removal of pathogens and abnormal or damaged tissues. M2 Mϕs have a polar opposite phenotype exhibiting high levels of anti-inflammatory cytokines and fibrogenic and angiogenic factors that serve to resolve inflammation and promote wound healing.[1–3] Both M1 and M2 Mϕs express distinct molecular markers as outlined in Fig. 1; however, further characterization of each phenotype has begun to demonstrate marked variability in Mϕ molecular profiles as well as the activating agents that induce them.[1–3,11] Thus, M2 Mϕs are now classified in three distinct subgroups, termed M2a, M2b, and M2c, based upon the inducing agent and molecular marker expression (Fig. 1).[1,2] In this classification, the M2a subtype represents the prototypical IL-4/IL-13–dependent, IL-4Rα–dependent M2 phenotype.[1] Our laboratory has characterized an additional M2 subtype, which, unlike previously described M2 Mϕs, involves "switching" from an inflammatory M1 into an angiogenic M2 phenotype. This subtype, which we termed M2d, is induced by $A_{2A}R$ signaling pursuant to initial stimulation by TLR agonists and is marked by decreased proinflammatory cytokine release concurrent with upregulation of traditional M2 cytokines such as IL-10 and the potent angiogenic molecule vascular endothelial growth factor (VEGF). We have recently characterized the phenotypic characteristics of this M2d Mϕ population in comparison to M2a Mϕs. M2d Mϕs express high levels of IL-10 and VEGF and low levels of TNF-α and IL-12 and do not show increased expression of eosinophil chemotactic factor L (Ym1), found in inflammatory zone 1 (FIZZ1), mannose receptor (MR), or dectin.[11]

Efforts to establish a more complete picture of Mϕ phenotypes have recently uncovered two transcriptional regulators that appear central to M1 and M2 Mϕ polarization. These regulators, interferon regulatory factor 5 (IRF5) and IRF4, are members of the interferon regulatory factor family and have been recently reported to play important roles in M1 and M2 Mϕ polarization, respectively. Krausgruber *et al.* demonstrated that IRF5 expression drives M1 polarization in Mϕs, with IRF5 directly activating transcription of several M1 proinflammatory cytokines, such as IL-12 and IL-23, while repressing transcription of the anti-inflammatory cytokine IL-10, an established M2 cytokine. IRF5 is found at high levels in M1 Mϕs, and its expression is induced by IFN-γ, LPS, or GM-CSF. Knockdown of IRF5 severely impairs the expression of inflammatory cytokines.[12] IRF5 represents a potentially important clinical target, as genetic polymorphisms in IRF5 have been linked to several autoimmune diseases, including rheumatoid arthritis and multiple sclerosis.[13,14] Likewise, Satoh *et al.* have implicated IRF4 as a crucial mediator of M2 Mϕ polarization. IRF4 inhibits TLR signaling by interacting with MyD88, but the molecular mechanisms of M2 Mϕ induction by IRF4 remain unknown.[15,16] An upstream effector of IRF4-induced M2 polarization, the histone demethylase Jumonji domain containing-3 (Jmjd3), was also identified in this study. Jmjd3 and IRF4 are critically involved in the IL-4–dependent induction of a subset of genes expressed by M2a Mϕs (arginase 1, Ym1, FIZZ1, and MR), whereas regulation of iNOS is independent of this Jmjd3/IRF4 pathway.[16] The roles of IRF4 and IRF5 in the adenosine-dependent induction of the M2d phenotype are not yet known; however, studies from our lab clearly indicate that this induction is independent of IL-4/IL-13 and STAT6, and switching to the M2d phenotype occurs unimpeded in Mϕs from IL-4Rα knockout mice. Interestingly, Jmjd3 expression is induced in Mϕs by TLR signaling via nuclear factor-κB (NF-κB), and induction of the angiogenic M2d phenotype also requires TLR stimulation and NF-κB signaling.[17,18]

Wound healing is a complex and dynamic process, requiring the coordination of many different signals and cell types to promote effective scar tissue formation. The functional plasticity of Mϕs in response to spatiotemporal changes in environmental signals underlies their ability to participate in diverse aspects of wound repair. Wound Mϕs in the early stage of repair are more M1-like, when clearance of foreign/damaged matter is required,

but M2 Mϕs predominate in later stages of repair in response to the need for new tissue formation.[9] Lucas *et al.* have recently demonstrated the essential role of Mϕs in the early and middle phases of wound repair, characterized by inflammation and granulation tissue formation, respectively. In these studies, transgenic mice expressing the human diphtheria toxin receptor under the control of the CD11b promoter (CD11b-DTR mice) were used to enable selective depletion of Mϕs from skin wounds at different phases of repair by administration of diphtheria toxin. Wounds depleted of Mϕs during either the early or middle stages of wound repair demonstrate markedly attenuated wound repair. Loss of Mϕs in the early stage impairs induction of granulation tissue formation, myofibroblast differentiation, and angiogenesis. Interestingly, these changes correlate with the impairment of M2 Mϕ polarization, as evidenced by lack of Ym1/FIZZ1 expression and reduced secretion of transforming growth factor beta (TGF-β) and VEGF. Depletion of Mϕs during the middle stage of wound repair resulted in similar pathologic alterations, with wounds displaying immature granulation tissue and impaired angiogenesis because of endothelial cell apoptosis. Likewise, this impaired healing was associated with the lack of the M2 cytokines TGF-β and VEGF, suggesting a role for M2 Mϕ polarization in this process.[19] These studies again strongly confirm the classical observations of Leibovich and Ross (1975) concerning the roles of Mϕs in wound repair.[20] With regard to Mϕ polarization, however, it should be noted that this study used prototypical M2a markers to identify polarized Mϕ phenotypes. Additional studies to analyze the presence and role of the M2d Mϕ population are required. The M2d population expresses elevated levels of A_{2A}Rs and A_{2B}Rs, but does not express elevated MR, Ym1, FIZZ1, or dectin. These studies are currently in progress.

TAKE-HOME MESSAGES

Basic science advances

- Local environmental cues influence the phagocytic and secretory behavior of Mφs to promote development of either an inflammatory Mφs phenotype (M1) or an anti-inflammatory/wound healing phenotype (M2).
- M2a Mφs are induced by IL-4Rα–dependent activation and express MR, Ym1, and FIZZ1; M2d Mφs are induced by TLR/adenosine A_{2A} receptor-dependent activation, in an IL-4Rα-independent manner, and do not express elevated MR, Ym1, and FIZZ1.
- M2 Mφs play an essential role in early and middle stages of wound repair. Depletion of Mφs during healing attenuates TGF-β and VEGF signaling and delays the formation and maturation of new tissue. Further studies of the roles of M2a and M2d Mφs are required.
- IRFs play critical roles in the polarization of Mφs. IRF5 promotes polarization of Mφs into the M1, proinflammatory phenotype, whereas IRF4 influences M2a Mφ polarization in response to transcriptional regulation by Jmjd3 demethylation.

Clinical science advances

- Characterization of local environmental signals and subsequently induced Mφ subpopulations that regulate inflammatory and tissue repair phases of wound healing provides insight into potential mechanisms for therapeutic modulation. Therapeutic modulation of Mφ polarization presents novel opportunities for the treatment of conditions whose pathogenesis is linked to aberrant Mφ activation.
- The identification of adenosine "switching" of Mφs from an M1 to M2d phenotype provides a novel paradigm for analysis of Mφ polarization, underscoring the importance of signaling crosstalk in the complex processes of wound repair and disease pathogenesis.
- The identification of the roles of IRF4 and IRF5 in M1/M2 polarization of Mφs provides novel insights that should prove valuable for development of selective therapeutic modulation.

INNOVATION

Our discovery of the novel M2d Mϕ subtype coupled with recent discoveries elucidating the essential role of Mϕs in the early and middle stages of wound repair suggests an important role for adenosine-mediated "switching" in the regulation of angiogenesis during the repair process. Moreover, the identification of "adenosine switching" of Mϕs highlights the importance of crosstalk among several signaling pathways in mediating Mϕ polarization. The extracellular milieu of wounds represents a "primordial soup" of signaling cues, and it is quite likely that several stimuli act on Mϕs at any given time. Thus, it becomes important to characterize the spatial and temporal changes in the wound environment and determine the differential contributions of the stimuli present to Mϕ polarization. This could potentially enable the artificial manipulation of Mϕs polarization to enhance normal physiological processes, such as wound repair, while combating pathological processes resulting from dysregulation of Mϕ function. Our experimental model, along with recent knowledge of the central roles of IRF4 and IRF5 in Mϕ polarization outlined above, presents a promising approach for analyzing relative contributions of disparate signals on Mϕ polarization.

CAUTION, CRITICAL REMARKS, AND RECOMMENDATIONS

Although significant progress has been made identifying factors underlying Mϕs polarization, much work remains. It is important to remember that, despite traditional classification of Mϕs as classically (M1) or alternatively (M2) activated, many novel phenotypes that do not fit the canonical molecular profile of these two groups have been identified. As our identification of the M2d phenotype has demonstrated, Mϕ polarization can be influenced by the concurrent stimulation of several different signaling pathways. The extracellular milieu in a healing wound comprises different palettes of signaling cues based upon spatial location within the wound, temporal location during the repair process, and the organism's surrounding environment. The study of Mϕ polarization at the transcriptional level, as observed with IRF4 and IRF5, should provide ways to observe the interplay of various signaling pathways on Mϕ polarization. Additional benefits may result from the fact that factors regulating transcription appear to influence clusters of genes expressed during polarization, potentially providing insight into the varied molecular profiles observed during polarization.

FUTURE DEVELOPMENTS OF INTEREST

Recent research has detailed the critical role of M2 Mϕs in the early stages of wound healing. The discovery of the ability of adenosine signaling in the presence of TLR stimulation to induce phenotypic switching from M1 into M2d Mϕs, coupled with evidence documenting impaired healing and, most notably, crippled angiogenesis due to decreased VEGF signaling in the absence of Mϕs, underscores the importance of evaluating the effect of TLR/A_{2A}R costimulation of Mϕs on wound repair. Additionally, evaluation of the intracellular signaling pathways underlying M1 to M2 "switching" could aid in the development of therapeutics to regulate this switch, thereby allowing for the enrichment of certain Mϕ populations to promote enhancement of selective physiological processes to aid in the treatment of infection and disease.

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DISCLAIMER

The contents of this article are solely the responsibility of the author and do not necessarily represent the official views of the NIGMS or NIH.

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