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Macrophages in wound healing: activation and plasticity

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- 1 **Running Title:** Wound healing macrophages
- 2 **Keywords:** Macrophage, wound healing, M2 macrophage, resolving macrophage, Th2 cytokine,
- 3 apoptotic cell

4 **Abstract**

5 Macrophages are critically involved in wound healing, from dampening inflammation to clearing
6 cell debris and coordinating tissue repair. Within the wound, the complexity of macrophage
7 function is increasingly recognized, with adverse outcomes when macrophages are
8 inappropriately activated, such as in fibrosis or chronic non-healing wounds. Recent advances in
9 *in vivo* and translational wound models, macrophage-specific deletions, and new technologies to
10 distinguish macrophage subsets, have uncovered the vast spectrum of macrophage activation and
11 effector functions. Here, we summarize the main players in wound healing macrophage
12 activation and function, including cytokines, apoptotic cells, nucleotides and mechanical stimuli.
13 We highlight recent studies demonstrating cooperation between these factors for optimal wound
14 healing. Next, we describe recent technologies such as cell tracking and single cell RNA-seq,
15 which have uncovered remarkable plasticity and heterogeneity in blood-derived or tissue-
16 resident macrophages and discuss the implications for wound healing. Lastly, we evaluate
17 macrophage dysfunction in aberrant wound healing that occurs in aging, diabetes and fibrosis. A
18 better understanding of the longevity and plasticity of wound healing macrophages, and
19 identification of unique macrophage subsets or specific effector molecules in wound healing,
20 would shed light on the therapeutic potential of manipulating macrophage function for optimal
21 wound healing.

22 **Introduction**

23 Wound healing, which is the repair and restoration of tissue to homeostasis following
24 injury caused by infection or mechanical trauma, occurs in three main stages: coagulation and
25 inflammation; resolution of inflammation; and tissue vascularization and regeneration. These
26 stages are common to all wounds, although the cell-types and secreted factors vary depending on
27 the organ (e.g. skin, lung, liver, brain) and type of injury (e.g. burn, pathogen). In all wounds,
28 macrophages are critical players, from contributing to the inflammation necessary to kill
29 potential pathogens, to resolving inflammation once the pathogens are cleared, and initiating and
30 maintaining tissue remodeling and regeneration.¹ Based on these diverse contributions to wound
31 healing, macrophages are broadly grouped into three activation types: first, the inflammatory
32 macrophage for pathogen phagocytosis and killing; second, the resolving macrophage that
33 removes dead cells and dampens inflammation; third, the tissue remodeling macrophage that
34 instructs tissue repair. It is important to recognize that these activation phenotypes do not
35 represent distinct macrophage subsets but likely a continuum of macrophage activation that
36 changes according to cell ontogeny and environmental stimuli.²

37 This review will focus on the macrophages that contribute to the final stages of wound
38 healing, specifically resolution of inflammation mediated by anti-inflammatory macrophages,
39 and tissue repair, mediated by T helper type 2 (Th2) cytokine-activated ‘M2’ macrophages, also
40 known as alternatively activated macrophages. We will first describe recently discovered
41 pathways influencing the activation and function of these cells. Next, we will highlight the
42 important influence of ontogeny and plasticity on the development of these wound healing
43 macrophage subsets. Lastly, we will discuss how dysfunctional activation of these cells can
44 contribute to disease. A better understanding of these wound healing macrophage subsets from

45 activation to effector molecules, and whether they can be altered to improve wound healing,
46 would have significant therapeutic benefit especially since deficient wound healing can be fatal.

47 **Wound healing macrophage activation and function**

48 Numerous soluble and cellular signals instruct macrophage activation for the final stages
49 of wound healing: tissue remodeling and resolution of inflammation. These include: Th2
50 cytokines that mediate a tissue remodeling ‘M2’ program, and apoptotic cells that induce an anti-
51 inflammatory macrophage phenotype. We will summarize the main features of these activation
52 programs and how they act together to promote optimal wound healing. We will also discuss
53 recently discovered factors that influence these macrophage activation programs.

54 **Th2 Cytokines:** M2 macrophages are important players in tissue repair.¹ M2 macrophages are
55 activated by Th2 cytokines, such as IL-4 and IL-13, that are highly produced in allergic
56 inflammation and helminth infection. For this reason, significant functional information on M2
57 macrophages has been acquired from helminth infection and allergy studies.³ These studies have
58 provided insight into the M2-mediated effector pathways for wound healing. Indeed, helminths
59 are macroscopic organisms that cause tissue injury, and the tissue remodeling that occurs in
60 allergic responses shares similarities with the tissue repair stage in wound healing. In these
61 models of tissue injury and inflammation, the Th2 cytokine response is mediated in a two-step
62 process, as summarized in Figure 1. First, an insult to the barrier causes epithelial cells to release
63 alarmins, including thymic stromal lymphopietin (TSLP), IL-25 and IL-33.³ These in turn
64 activate Th2 cytokine-producing innate cells, such as group 2 innate lymphoid cells, mast cells,
65 basophils and eosinophils. The critical importance of M2 macrophages in mediating wound
66 healing is demonstrated in numerous *in vitro* and *in vivo* studies. For instance, IL-4/IL-13-
67 activated human THP-1 cells induced proliferation, collagen synthesis and α -smooth muscle

68 actin (α -SMA) expression by human dermal fibroblasts (HDFs), in a co-culture assay.⁴ These
69 M2-differentiated THP-1 cells also increased dermal fibroblast expression of α -SMA, a feature
70 of myofibroblasts, indicating that fibroblasts were differentiated into myofibroblasts.⁴ Further,
71 abrogation of IL-4R α signaling in macrophages impaired wound repair in *in vivo* models of
72 wound healing by skin punch biopsy, chemical-induced injury or invasive helminth infection-
73 induced injury.⁵⁻⁷ Mechanistically, M2 macrophages initiate wound repair through numerous
74 pathways including growth factors and matrix metalloproteinases (MMP), summarized in Figure
75 2 and in recent reviews.¹ In addition, M2 macrophage-derived arginase 1 (*Arg1*) and RELM α
76 (*Retnla*) are downstream effectors of wound healing in the skin and following helminth
77 infection.^{3, 8} The mechanism by which macrophage-derived arginase promotes skin wound
78 healing is likely two-fold: dampening inflammation, and promoting matrix deposition through its
79 metabolism of L-Arginine. RELM α 's effector function in wound healing has recently been
80 explored.⁹ RELM α activates the enzyme lysyl hydrolase 2 (*Plod2*), which mediates optimal
81 collagen cross-linking.^{5, 10} Additionally, macrophage-derived RELM α was implicated in
82 dampening lung inflammation and promoting tissue repair in a model of helminth infection-
83 induced lung injury with rodent hookworm *Nippostrongylus brasiliensis*.¹¹⁻¹³ In this model, gene
84 expression analysis of RELM α -treated lung macrophages revealed that RELM α promoted
85 expression of genes involved in extracellular matrix remodeling: matrix metalloproteinase 9
86 (*Mmp9*), integrin beta 1 (*Itgb1*), and junctional adhesion molecule A (*F11r*).

87 IL-4R α signaling also stimulates tissue-resident macrophage proliferation, which can
88 have the beneficial outcome of expanding and activating the effector macrophage population for
89 wound healing.^{6, 14} Here, additional signals from the tissue environment promoted the IL-4-
90 induced wound healing capacity of M2 macrophages. Specifically, the surfactant protein A (SP-

91 A) produced in the lung acted through the receptor myosin 18A (Myo18A) to enhance M2
92 macrophage activation and lung wound healing following *N. brasiliensis* infection-induced
93 injury. Interestingly, in the peritoneal cavity, resident macrophages did not respond to SP-A, but
94 instead were activated by the complement protein C1q, which is structurally homologous to SP-
95 A. The stimulatory effect of C1q on M2 macrophages, likely through Myo18a, was observed in
96 several tissues including the peritoneal cavity, liver, spleen and adipose tissue. Functionally, C1q
97 promoted M2 macrophage-mediated liver repair following infection with *Listeria*
98 *monocytogenes*. Whether C1q affects resident tissue macrophage populations, or instead
99 activates monocytes or macrophages recruited to the injury site from the blood or peritoneal
100 cavity is unclear. Another study utilizing carbon tetrachloride (CCL₄) treatment as a model of
101 liver injury demonstrated that peritoneal macrophages could cross the mesothelium and penetrate
102 into the injured liver tissue. These macrophages, originating from the peritoneal cavity,
103 expressed M2 markers Arginase and RELM α , and exhibited a reparative function when recruited
104 to the liver.¹⁵ While tissue-resident macrophages are likely more rapid responders to injury,
105 monocytes recruited from the blood can also differentiate into M2 macrophages and contribute to
106 tissue repair following *Schistosoma mansoni* infection-induced liver injury.¹⁶ This process was
107 impaired in vitamin A deficient mice suggesting that dietary components are important for
108 optimal M2 macrophage activation.

109 M2 macrophage-mediated killing of large extracellular helminths exhibits common
110 features of tissue repair. In *H. polygyrus* infection M2 macrophages are recruited to the helminth,
111 and produce factors to trap and kill the pathogen.¹⁷ The contributing factors that immobilize the
112 worm include wound healing factors such as arginase 1. Understanding the activation pathway
113 and effector molecules of M2 macrophages that kill *H. polygyrus* infection may therefore

114 provide insight into new wound healing mechanisms. Mechanistically, recruitment and killing of
115 *H.polygyrus* by M2 macrophages involved recognition of helminth antigen-antibody immune
116 complexes and the production of CXCR2 ligands.¹⁸ Mice deficient in Fc γ R signaling and
117 activation-induced cytidine deaminase (AID), which contributes to antibody maturation and class
118 switching, exhibited impaired worm killing but also increased intestinal lesions suggesting
119 defective wound repair. Further, at later timepoints, *Fcrg*^{-/-} and *Aid*^{-/-} mice showed severe
120 peritonitis that might be attributed to defective lesion repair resulting in increased bacterial
121 translocation. Investigation of the mechanism of wound repair in this helminth infection model
122 also revealed the importance of an additional cell-type: the myofibroblast, which was activated
123 CXCL2 and CXCL3, via CXCR2, and helminth antigens via dectin-2, to mediate wound closure
124 potentially through expression of α -SMA. Of translational significance, CXCL3 from human
125 monocyte-derived macrophages induced by *Ascaris suum*, the pig helminth closely related to the
126 human parasite *Ascaris lumbricoides*, up-regulated wound healing by human myofibroblasts.
127 These data indicate that crosstalk between M2 macrophages and other cell-types is necessary for
128 effective wound healing.

129 **Apoptotic cells:** A critical step to wound healing is the clearance of apoptotic cells resulting from
130 the inflammatory environment. This process is mediated by resolving, or resolution,
131 macrophages. Resolving macrophages sense and phagocytose phosphatidylserine (PtdSer)-
132 exposed apoptotic cells in a process called efferocytosis.¹⁹ Effective efferocytosis is dependent on
133 the receptor tyrosine kinases Axl and Mertk. In addition to clearing dead cells, resolving
134 macrophages contribute to wound healing and tissue homeostasis by producing anti-
135 inflammatory molecules such as IL-10, and tissue remodeling growth factors such as TGF- β .
136 Th2 cytokines and apoptotic cell engulfment were originally considered distinct signals, which

137 activated M2 or resolving macrophages respectively, however, recent studies have uncovered
138 synergism of these signals for optimal wound healing. Indeed, co-treatment of bone marrow-
139 derived macrophages with apoptotic neutrophils and Th2 cytokines induced maximal expression
140 of wound healing genes *Retnla* (RELM α), *Chil3* (Ym1), *Ear2* (Eosinophil associated,
141 ribonuclease A family, member 2), and *Fnl1* (Fibronectin 1).¹⁹ The essential function of apoptotic
142 cell recognition for optimal M2 activation was demonstrated utilizing macrophages deficient in
143 *Axl* and *Mertk* in *N. brasiliensis* infection as an *in vivo* model of M2 macrophage-dependent
144 wound healing.¹⁹ Critically, *Axl* and *Mertk* functional effects on tissue repair were not restricted
145 to the lung, as these proteins were required for upregulation of the anti-inflammatory and wound
146 healing genes in macrophages in the damaged intestine and peritoneal cavity. It has previously
147 been shown that SP-A induces efferocytosis while C1q activates MERTK expression²⁰, Thus, in
148 addition to M2 activation, SP-A and C1q might promote apoptotic cell sensing.²¹ Together, these
149 studies implicate an inter-dependent, positive feedback loop whereby apoptotic cells and Th2
150 cytokines act together to promote macrophage-mediated wound healing.

151 **Nucleotides:** Macrophage recognition of nucleotides, such as adenosine triphosphate (ATP) and
152 their metabolites (e.g. adenosine), by P2 and P1 purinergic receptors respectively, influences
153 their activation and recruitment.^{22, 23} For instance, ATP released by apoptotic cells promotes
154 macrophage recruitment and efferocytosis.²² In a mouse model of traumatic brain injury,
155 microglia chemotaxis towards the localized injury was dependent on extracellular ATP
156 activation of the P2 receptor P2Y₁₂.²⁴ The P1 purinergic receptors that recognize adenosine also
157 influence macrophage activation. Specifically, activation of the P1 receptors A_{2A} and A_{2B}
158 promotes M2 macrophage activation,²³ while the A₃ receptor signaling induces an anti-
159 inflammatory response.²⁵ Consistent with a tissue protective role for the P1 adenosine receptors,

160 A2A receptor-deficient mice exhibit extensive tissue damage and inflammation following
161 concanavalin A or CCl₄-induced liver injury as well as in response to endotoxic shock.²⁶ One
162 potential mechanism of A2A-mediated tissue protection may be through promoting macrophage
163 production of IL-10.²³ The A2B receptor, which is expressed on macrophages but also non-
164 immune cells such as epithelial cells, also promoted M2 macrophage activation, and was tissue
165 protective in helminth infection and endotoxin-induced lung injury.^{27, 28}

166 MicroRNAs (miRs) also play an important role in macrophage-mediated wound healing
167 by regulating efferocytosis-mediated suppression of the innate immune response. miR21 was
168 upregulated in macrophages after efficient efferocytosis where it suppressed pro-inflammatory
169 TNF α , and induced anti-inflammatory IL-10.²⁹ The mechanism of action of miR21 included
170 silencing of the signaling molecules PTEN, which contributes to NF- κ B-induced inflammation
171 and TNF α expression, and PDC4, which suppresses IL-10 expression. Together, these studies
172 identify nucleotides as additional factors that regulate macrophage activation in injury and
173 inflammation. Regulation of these signals in macrophages may be novel targets to promote
174 wound healing.

175 ***Mechanical stimulus:*** In addition to soluble factors or cells, tissue structure or physical cues in
176 the extracellular matrix during wound healing also affects macrophage activation and behavior.³⁰
177 For instance, macrophage elongation caused by migration in the fibrous tissue of a wound, rather
178 than in healthy tissue or the vasculature, likely provides different mechanical cues. Recent
179 studies suggest that these mechanical stimuli regulate macrophage polarization.³¹ In bone
180 marrow-derived macrophage shape studies, M1 macrophages, activated by IFN γ and LPS, were
181 flattened and round, while Th2 cytokine-activated M2 macrophages were elongated.³¹
182 Conversely, manipulating macrophage cell shape by micropatterning altered expression of

183 macrophage phenotype markers. Cell elongation increased expression of M2 markers arginase-1,
184 CD206, Ym1, characteristic of wound healing macrophages. Moreover, elongation augmented
185 arginase-1 expression induced by IL-4/IL-13 but decreased inducible nitric oxide synthase
186 (iNOS) expression caused by IFN γ /LPS, suggesting that cell elongation preferentially skews
187 macrophages towards an M2 phenotype. The effect of cell elongation on macrophage
188 polarization was mediated by actin and actin-associated contractility because pharmacological
189 inhibition of actin and the actin signaling pathway abrogated up-regulation of arginase-1
190 expression by elongation but not by cytokine stimulation. Hence, polarization induced by
191 changes in the extracellular matrix structure may promote wound healing through activation of
192 genes required for tissue repair. In summary, we have highlighted in this section the complexity
193 of factors that promote wound healing macrophages and have discussed main downstream
194 macrophage effector pathways, outlined in Figure 1.

195 **Wound healing macrophage heterogeneity and plasticity**

196 We have summarized thus far multiple studies mapping the wound healing program of
197 macrophages. Overall, there is a clear consensus that Th2 cytokines and apoptotic cells are key
198 drivers of wound healing macrophages, but activation can be enhanced or modulated by other
199 factors. Two active areas of research remain, of relevance to selective treatment strategies to
200 target wound healing macrophages. First, investigation of the degree of heterogeneity in the
201 wound healing macrophage subsets would allow for the optimal wound healing profile be
202 selected and expanded. Second, evaluating the plasticity of the wound healing macrophage
203 phenotype would guide therapeutic strategies to promote wound healing function. Specifically,
204 are all macrophages able to differentiate into wound healing macrophages, are they long-lived,
205 and can they change their phenotype dependent on the microenvironment? Recent research

206 technologies including macrophage lineage tracking and single cell profiling have made it
207 possible to start addressing these questions.

208 ***Wound healing macrophage heterogeneity:*** While original macrophage studies grouped these
209 cell-types into distinct non-overlapping subsets such as M1, M2 and regulatory macrophages, it
210 is increasingly apparent that macrophage activation represents a spectrum of phenotypes
211 dependent on the tissue microenvironment and cell lineage.³² Two main lineages of macrophages
212 exist: tissue-resident macrophages originating from the embryonic precursors, and monocyte-
213 derived macrophages originating from the bone marrow. The importance of these distinct
214 macrophage lineages in wound healing is still undefined, however, tissue-resident macrophages
215 are likely the first responders to wounds. Following injury, tissue-resident macrophages express
216 adhesion molecules that recruit and guide multiple cell-types.³³ Further, tissue-resident
217 macrophages can replicate to increase their numbers, are highly M2 polarized in response to IL-
218 4, and orchestrate the wound healing stages.³⁴

219 Tissue-resident macrophages are long-lived and have the capacity for self-renewal.
220 Indeed, fate-mapping and parabiosis studies revealed that tissue macrophages such as Kupffer
221 cells, microglia, alveolar, and peritoneal macrophages are established prenatally and can be
222 maintained independently of replenishment by blood monocytes under homeostatic conditions.³⁵
223 In contrast, in the intestine, skin and heart, macrophages are replenished in the steady state by
224 monocytes. The half-life of intestinal and dermal macrophages was estimated to range from 4 to
225 6 weeks, while those of the heart ranged from 8 to 12 weeks.³⁶ In contrast to self-renewing
226 tissue-resident macrophages, monocytes recruited to inflamed tissues cannot maintain
227 themselves and die once inflammation resolves.³⁵ Given the longevity of tissue-resident

228 macrophages, they may constitute a better long-term target for wound healing compared to
229 monocyte-derived macrophages.

230 Tissue-resident macrophage development is governed by distinct groups of transcription
231 factors. For instance, GATA6 drives peritoneal macrophage differentiation, while PPAR γ is
232 involved in alveolar macrophage differentiation.³⁷ With the advent of new technologies such as
233 single cell RNA-seq (SC-RNA Seq), a more comprehensive identification of tissue macrophage
234 subsets has been possible. In particular, the transcription factor zinc finger E-box binding
235 homeobox 2 (ZEB2) was identified as a key determinant of tissue-specific macrophages in
236 diverse organs including the liver, lung, spleen, brain, and colon.³⁸ In ZEB2 deficient mice, there
237 was alteration of different tissue-specific macrophage markers, highlighting the technical
238 difficulty of identifying macrophages within tissues if using limited markers such as CD64 and
239 F4/80.

240 To this end, a study using Single Cell Recognition, a reference-based computational tool
241 that enables unbiased annotation of SC-RNA Seq, investigated macrophage heterogeneity in lung
242 fibrosis.³⁹ They characterized three distinct groups of macrophages, including alveolar
243 macrophages (C1), interstitial macrophages (C3), and an intermediate cluster of cells (C2) in the
244 lung. Both C2 and C3 were highly enriched in bleomycin-induced fibrosis, and C2 had high
245 expression of genes from both C1 and C3, indicating that C2 is a transitional state between C1
246 and C3 in lung fibrosis. CX3CR1 was expressed in both C2 and C3, and these CX3CR1-lineage
247 cells (CLCs) were increased after injury. MHC II expression was decreased, but expression of
248 SiglecF, an alveolar macrophage marker, was increased in these cells, indicating that CLCs
249 transit towards an alveolar macrophage identity. CLCs were in direct contact with fibroblasts
250 expressing PDGF receptors. The ligand, PDGF, was induced in activated MHC II^{high} alveolar

251 macrophages after injury, and conditioned media from MHC II^{high} alveolar macrophages
252 mediated gap closure by fibroblasts better than media from MHC^{low} alveolar macrophages. Last,
253 deprivation of CLCs attenuated bleomycin-induced lung fibrosis, implying that CLCs provide
254 trophic support for fibroblasts in the wound site.

255 Diverse macrophage populations were also identified by PrimeFlow, a flow-based
256 technique which allows RNA cellular profiling of markers that may not have been detectable by
257 antibodies.⁴⁰ PrimeFlow analyses of *in vitro* and *in vivo* M2-activated macrophages revealed
258 differential expression of the canonical M2 markers *Arg1* and *Retnla*, with comparable
259 frequencies of single and double positive subsets.⁴⁰ Since both these genes contribute to wound
260 healing, it is likely that targeting the double positive population would be of greatest therapeutic
261 benefit. Heterogeneity in M2 macrophage subsets was also observed in the human peritoneum.
262 Patients with peritoneal fibrosis caused by peritoneal dialysis exhibited heterogenous M2
263 macrophage populations with differential expression of CD163 and CD206.⁴¹ The functional
264 relevance of these subsets in peritoneal fibrosis is unclear, however, high CD163 but not CD206
265 expression correlated with active peritonitis, and CD163-sorted macrophages produced
266 chemokine CCL18 and promoted fibroblast proliferation.⁴¹ More research is needed to determine
267 to what extent functional heterogeneity is influenced by macrophage origin versus subtle
268 differences in the microenvironmental cues.

269 ***Wound healing macrophage plasticity:*** As highlighted previously, macrophages can respond to
270 a plethora of external cues leading to a wide spectrum of activation phenotypes. Whether these
271 represent terminally differentiated cells, or whether these cells are plastic and can adapt their
272 function according to new environmental cues is less clear. Additionally, transcriptional studies
273 have suggested that the epigenetic landscape may govern macrophage polarization and

274 plasticity.³⁷ Within the ever-changing microenvironment of the wound, plasticity and longevity
275 of the macrophages would be attractive features for therapeutic targeting allowing them to adapt
276 their function to address the immediate needs within the wound. The monocyte/macrophage
277 lineage is general recognized as a highly plastic lineage.² Indeed, more specific research
278 investigating macrophage plasticity between M1 and M2 macrophages does support a certain
279 degree of plasticity in M2 macrophages. M2 macrophages that were differentiated following
280 chronic helminth exposure could respond to M1 activating signals such as LPS and IFN γ .⁴² In an
281 *in vivo* co-infection model, peritoneal M2 macrophages activated by *H. polygyrus* infection were
282 able to change to antimicrobial nitric oxide-producing M1 macrophages capable of killing an
283 attenuated *Salmonella* strain when challenged intraperitoneally.⁴³ In contrast, *Salmonella*-
284 induced M1 macrophages could not be repolarized by IL-4 treatment, indicating that M1
285 macrophage activation has a more restrictive effect on plasticity than M2 macrophage
286 activation.⁴³ These studies show that M2 macrophages have some ability to alter their phenotype
287 in response to different stimuli. It can be inferred from these studies that wound healing M2
288 macrophage subsets may be equally plastic, however, further mechanistic investigation in wound
289 healing models is needed.

290 As mentioned above, pro-inflammatory activated M1 macrophages kill pathogens in
291 wounded tissues, on the other hand, M2 macrophages dampen these inflammatory responses, and
292 these sequential steps are required for wound healing. AMP-activated protein kinase $\alpha 1$ subunit,
293 a catalytic domain (AMPK $\alpha 1$) was proven to modulate M1/M2 macrophage polarization.⁴⁴
294 Following cardiotoxin injection, AMPK $\alpha 1$ activity was increased in the macrophages recruited
295 to the regenerating muscle. In both whole body and macrophage-specific *Ampka1*^{-/-} mice, there
296 were more necrotic myofibers than regenerating myofibers following cardiotoxin injection,

297 suggesting that AMPK α 1 promotes muscle regeneration. *Ampk α 1*^{-/-} macrophages exhibit
298 preferential expression of M1 macrophage markers (e.g. iNOS) over M2 markers (e.g. CD206,
299 CD163, and TGF β). Since AMPK senses the cellular energy level as ratios of ADP:ATP and
300 AMP:ATP, this data supports the concept that energy sensing and metabolic activity dictates
301 macrophage activation.⁴⁵ Indeed, M2 macrophages exhibit high oxygen consumption rate, that is
302 impaired in *Ampk α 1*^{-/-} mice. AICAR, a pharmacological AMPK activator, dampened M1
303 activation and enhanced M2 activation in wild-type but not *Ampk α 1*^{-/-} mice.⁴⁴ Therefore,
304 pharmacologically targeting metabolic pathways in wounds may influence macrophage plasticity
305 and promote a wound healing macrophage subset.

306 **Macrophage function in wound healing disorders**

307 In the previous sections, we have provided evidence supporting the role of macrophages
308 in the wound healing process. Consistent with their importance in wound healing, impaired or
309 aberrant macrophages are key features in dysfunctional wounds. In this section, we will describe
310 wound healing disorders that are mediated by macrophage dysfunction. These include aging and
311 diabetes, which are associated with deficient wound healing macrophage activation. On the
312 flipside, excessive wound healing macrophage activation is also detrimental. This is apparent in
313 fibrotic disorders, which is mediated by uncontrolled M2 macrophage activation.

314 **Aging:** Aging can result in immune dysfunction, including immunosenescence, defined as age-
315 related impairments of the immune system,⁴⁶ and ‘inflamm-aging’, defined as association of
316 advanced age with chronic low-grade inflammation.⁴⁷ Both these age-related immune disorders
317 affect the wound healing process because they can result in impaired pathogen killing, or
318 conversely, the inappropriate control of inflammation. For instance, monocyte-derived
319 macrophages from elderly subjects produce less TNF α and IL-6 in response to *Streptococcus*

320 *pneumoniae*, resulting in hampered bacterial killing compared to young subjects.⁴⁸ Microarray
321 analysis of LPS-stimulated macrophages revealed reduced immune response and signal
322 transduction genes in older mice.⁴⁹ The deficient activation of macrophages from aged mice
323 could be attributed to decreased levels of p38 and c-Jun N terminal kinase, which are important
324 for pro-inflammatory gene expression.⁵⁰ In addition, A20 that inhibits NF- κ B and MAPK
325 signaling pathways was elevated in the lung and alveolar macrophages of aged mice, which
326 caused reduced cytokine responses to bacteria.⁵¹ Stimulation of alveolar macrophages with
327 TNF α increased A20 levels, implying the role of TNF α in inflamm-aging.

328 Conversely, levels of pro-resolving mediators, such as lipoxins, protectins, maresins, and
329 the D- or E-series resolvins, are impaired in aged mice. Specifically, aged mice exhibit altered
330 lipid biosynthesis dynamics with lower level of specialized pro-resolving mediators (SPMs) but
331 higher levels of pro-inflammatory lipid mediators.⁵² The phagocytic ability of peritoneal
332 macrophages is also decreased in aged mice, potentially due to exposure to elevated levels of IL-
333 10 in the peritoneal cavity.⁴⁶ Of significance to the wound healing process, peritoneal
334 macrophages from aged mice are less able to phagocytose necrotic cells.⁵³ In response to certain
335 stimuli, however, macrophages from aged mice remained plastic and were able to respond to
336 factors in their microenvironment, suggesting that macrophage dysfunction can be reversed.
337 Indeed, aged macrophages responded to *in vitro* treatment with IFN γ as effectively as young
338 macrophages, underscoring the importance of the microenvironment over intrinsic defects.⁵⁴
339 Additionally, deficiencies in macrophage polarization in aged mice could be restored with
340 exercise and diet changes.^{51, 55}

341 **Diabetes:** Deficient wound healing is also a severe and potentially fatal consequence of diabetes.
342 The mechanisms by which diabetics suffer from unhealed chronic wounds are multi-factorial but

343 do involve dysfunctional macrophage responses. In particular, macrophage activation via the
344 nuclear receptor PPAR γ is impaired in diabetes.⁵⁶ PPAR γ activation promotes wound healing by
345 decreasing the expression of pro-inflammatory cytokines and increasing wound healing genes.⁵⁶
346 The induction of PPAR γ activity also leads to granulation tissue formation, angiogenesis, and
347 collagen deposition that are key for wound repair. In the diabetic wound, the decreased PPAR γ
348 activity was caused by sustained production of IL-1 β leading to inflammasome activation.⁵⁶ This
349 could be reversed by treatment of the wound with PPAR γ agonists as a promising treatment
350 strategy to promote wound healing macrophages.

351 **Fibrosis:** Optimal wound healing is dependent on a highly regulated M2 macrophage response.
352 While a deficient M2 macrophage response leads to impaired wound closure, excessive M2
353 macrophage activation causes scarred or fibrotic tissue.⁵⁷ In particular, the Th2 cytokine IL-13
354 can drive pathologic fibrosis through excessive M2 macrophage activation. In models of
355 helminth-induced fibrosis, the IL-13 driven inflammation and fibrosis were ameliorated with
356 depletion of CD11b⁺ macrophages.⁵⁷ Additionally, in bleomycin-induced pulmonary fibrosis, the
357 signaling molecule IRAK-M was shown to enhance IL-13 production and fibrosis as *IRAK-M*^{-/-}
358 mice had reduced bleomycin-induced collagen accumulation in the lung.⁵⁸ When lung fibroblasts
359 were co-cultured with macrophages from bleomycin-treated *IRAK-M*^{-/-} mice, collagen and α -
360 SMA expression was reduced compared to wild type mice, suggesting that M2 macrophages
361 were the downstream effectors of IRAK-M signaling in promoting pathologic fibrosis.

362 Mincle, a C-type lectin expressed on macrophages, was also identified as a mediator of
363 fibrosis.⁵⁹ A high-fat diet increased Mincle expression in macrophages in the crown-like
364 structures (CLS) of the epididymal fat, which are a characteristic structure in obese adipose
365 tissue. Mincle was preferentially expressed by CD11b⁺F4/80^{lo} rather than CD11b⁺F4/80^{hi} cells.

366 These Mincle-expressing macrophages had higher CD11c and lower CD206 expression in line
367 with previous data showing that it is classically activated macrophages that express Mincle.⁵⁹
368 *Mincle*^{-/-} mice were protected from hepatic steatosis and insulin resistance, associated with
369 reduced interstitial fibrosis in epididymal fat tissue, α -SMA⁺ cells and myofibroblasts.

370 The beneficial or pathologic effect of M2 macrophages in fibrosis may be critically
371 dependent on the timing. Using a model of liver fibrosis and spontaneous recovery, where mice
372 were treated with CCL₄ for several weeks then left untreated for the liver to recover, Weng *et al.*
373 investigated the function of M2 macrophages.⁷ Intriguingly, macrophage-specific IL-4R α ^{-/-} mice
374 were protected from liver fibrosis progression during CCL₄ treatment, but had delayed fibrosis
375 reversal during the recovery phase. The phase-specific function of M2 macrophages was
376 confirmed with an anti-sense IL-4R α nucleotide at different timepoints, where it was shown that
377 early activation of M2 macrophages promotes fibrosis, while at later timepoints, M2
378 macrophages speed up fibrosis reversal. These studies highlight the importance of tightly
379 controlling macrophage activation to promote wound healing while circumventing pathologic
380 fibrosis, summarized in Figure 2.

381 **Conclusion**

382 Macrophages are critical participants in the wound healing process and provide a useful
383 therapeutic target for wound healing disorders. Indeed, dysfunctional macrophages are key
384 features of delayed wound healing in aging and diabetes, or excessive wound healing in fibrosis.
385 There is increasing evidence that macrophages are long-lived and plastic and can change their
386 phenotype depending on external stimuli. Therefore, it may be possible to skew their function
387 within the aberrant wound for improved outcomes. While both activating factors and
388 downstream effectors of wound healing macrophages are well-defined, challenges to

389 macrophage-specific wound healing strategies remain. These include the extensive spectrum and
390 heterogeneity of the macrophage subsets, and the lack of understanding of the individual cues
391 that can control this heterogeneity. New technologies targeting individual macrophage lineages
392 and macrophage-derived molecules, as well phenotyping these subsets at the single cell level,
393 provide the promising prospect that identification of an optimal wound healing macrophage
394 program of therapeutic potential will soon be possible.

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538

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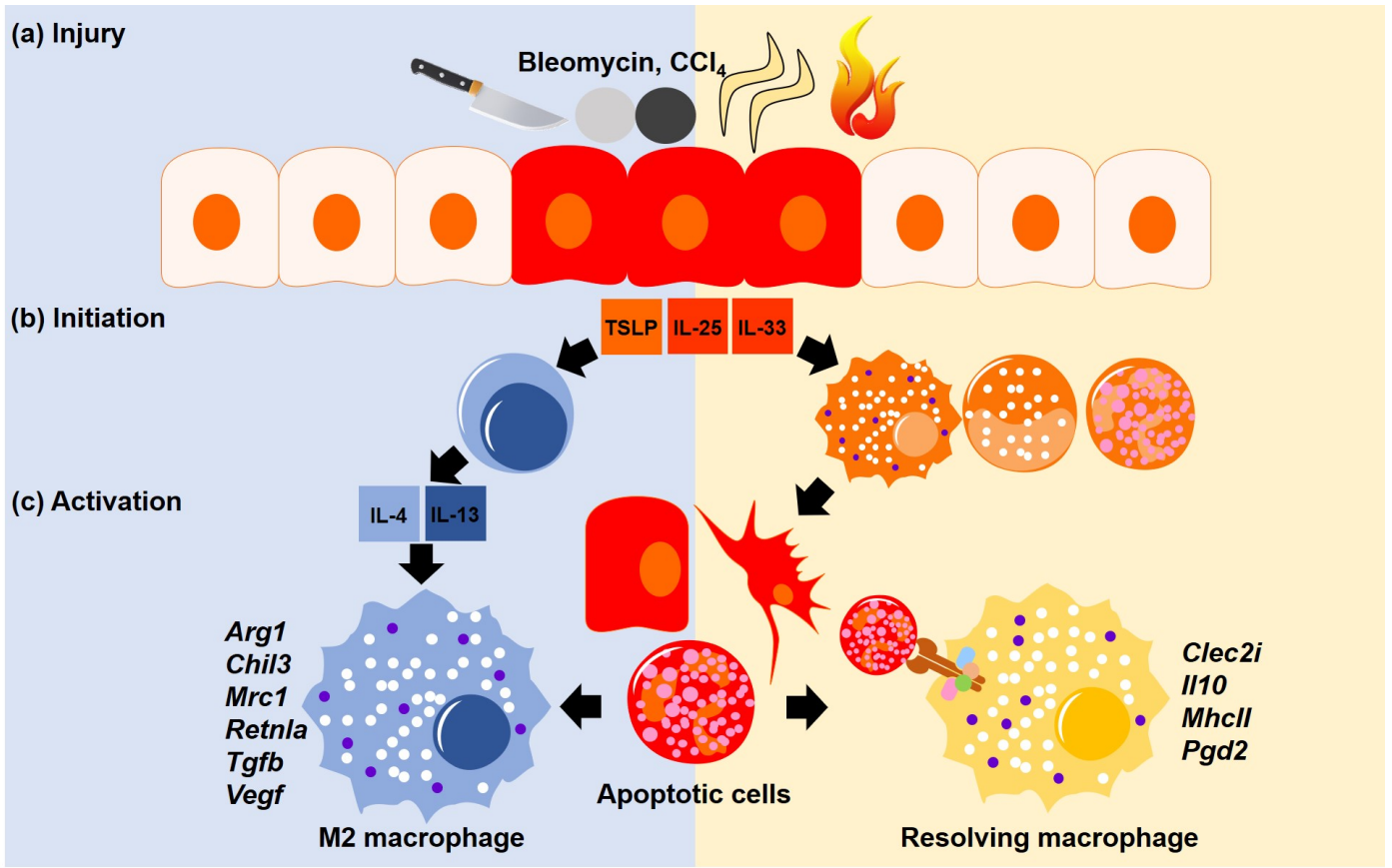
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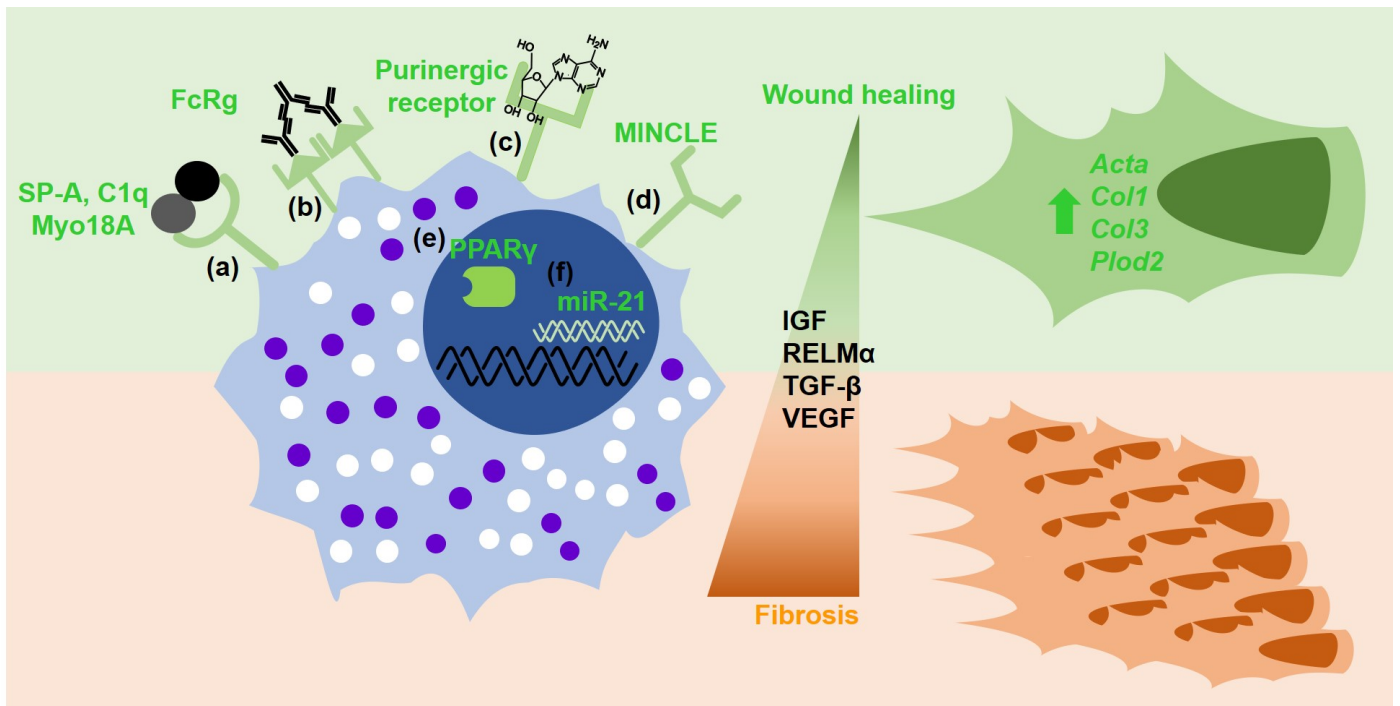
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561 **Figure Legends:**

562 **Figure 1. Wound healing macrophage activation.** (a) Injury by cuts, chemicals (CCl₄ and
563 bleomycin), helminths or burn injury causes a breach in barrier. (b) The wound healing response
564 is initiated by dying cells which release cytokines (TSLP, IL-25, and IL-33) that activate Th2
565 cytokine (IL-4/IL-13) producing cells (blue). Innate cells such as neutrophils (orange) are also
566 recruited to kill invading pathogens, and apoptose once the challenge is resolved. (c) M2
567 macrophages (left) are activated by the Th2 cytokines. Equally important is the activation of
568 resolving macrophages (right) which are activated by phagocytosis of the apoptotic cells
569 resulting from the inflammation. Rather than distinct subsets, both M2 and resolving
570 macrophages represent a continuum of macrophage activation that are influenced by both Th2
571 cytokines and apoptotic cells.

572 **Figure 2. Macrophage enhancers and effectors in wound healing and fibrosis.** Wound
573 healing macrophage activation is enhanced by the following surface markers: (a) signaling
574 through the Myo18A receptor; (b) Fc γ R-mediated signaling by immune complexes; (c) ATP or
575 adenosine binding to purinergic receptors; (d) Mincle surface expression; and intracellular
576 factor; (e) nuclear receptor PPAR γ ; (f) micro RNA 21. These enhance macrophage effector
577 function to promote wound healing (green), but if excessive, can lead to fibrosis (brown).