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1 **Inflammation and skeletal muscle regeneration: leave it to the macrophages!**

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7
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10
11 **Abstract**

12 Inflammation is usually considered as harmful; however, it is also necessary for tissue recovery
13 after injury. Macrophages exert immune and non-immune functions all throughout this process.
14 During skeletal muscle regeneration, they mount an inflammatory response while exerting trophic
15 roles on muscle and mesenchymal stem cells. Pro-inflammatory macrophages shift to being anti-
16 inflammatory, triggering the resolution of inflammation. Studies have highlighted that during this
17 shift, a crosstalk ensues, integrating cues for resolution, efferocytosis, cellular metabolism and
18 signaling pathways. During the restorative phase, macrophages dampen inflammation while
19 promoting stem cell differentiation, angiogenesis, and matrix remodeling. Since blunting the
20 inflammatory phase can be detrimental for muscle regeneration, we suggest that rather than
21 fighting inflammation, it should be allowed to operate and resolve, thus allowing for tissue
22 recovery.

23 **What does "inflammation" mean?**

24 Inflammation is a process that is usually considered as being negative and which must be fought.
25 Indeed, inflammation in higher eukaryotes is associated with numerous diseases, including
26 chronic diseases and cancer. However, the word "inflammation" also encompasses the immune
27 system's acute response occurring after a single episode of infection or following tissue injury. The
28 inflammatory response, which is the response to tissue to a damage, is also "inflammation".
29 Tissue injury occurring in a sterile environment does not require the mounting of an acquired
30 immune response and includes two main phases: i) the pro-inflammatory phase, where leukocytes
31 infiltrate the injured area to exert pro-inflammatory functions, followed by ii) the anti-inflammatory
32 or restorative phase, during which the tissue, according to the properties of its parenchymal cells,
33 heals (skin), scars (heart) or regenerates (skeletal muscle). Therefore, depending on both the
34 tissue and the type of injury (traumatic, toxic, ischemic...), specific characteristics to the
35 inflammatory response have been observed (reviewed in [1]). However, in any case, the resolution
36 of inflammation, *i.e.*, the transition from the inflammatory to the anti-inflammatory phase, is
37 absolutely required for tissue recovery. This process is mainly controlled by macrophages, which
38 can be considered as the main orchestrators of the inflammatory response after sterile injury [1].
39 Recent advances have been made in the understanding of the roles of macrophages during the
40 inflammatory response and of their skewing at the time of resolution of inflammation. Here, the
41 roles and regulation of macrophages in the post-injury inflammatory response are presented in the
42 context of skeletal muscle regeneration, representing an excellent paradigm of complete tissue
43 repair. In this scenario, hampering the inflammatory shift of macrophages, or triggering resolution
44 too early have both been shown to be detrimental for muscle recovery [2, 3]. Recent evidence
45 using mouse models and human samples support the hypothesis that inflammation should not be
46 counteracted, but rather, managed in a timely way to ensure optimal skeletal muscle regeneration.

48 **The inflammatory response after tissue damage**

49 Tissue damage immediately triggers the inflammatory response: it begins with the vascular phase,
50 including vasodilatation, increased blood flow, increased vascular permeability, and leading to the
51 formation of an edema at the injury site. Subsequently, the cellular phase ensues, where
52 leukocytes extravasate from blood, cross the endothelium and traffic towards the injured area [4].
53 In the absence of any immune challenge, immune functions are mainly focused on the recruitment
54 of cells that will clear tissue debris. The first to arrive on site are neutrophils that mount the pro-
55 inflammatory response to attract monocytes before dying in a couple of days (e.g. mice, humans,
56 zebrafish) [4]. Closely following neutrophils, macrophages participate in the pro-inflammatory
57 phase. They clear the surrounding debris through efferocytosis, which skews their phenotype (see
58 below). Then, they are actively involved in the restorative phase of the inflammatory response
59 during which the tissue recovers and returns back to homeostasis [5]. In addition, macrophages
60 exert a variety of non-immune functions by developing close interactions with their neighboring
61 cells and impacting their behavior.

63 **Sequential and pleiotropic macrophage functions during skeletal muscle regeneration**

64 Post-injury skeletal muscle regeneration in vertebrates is an excellent paradigm for studying the
65 inflammatory response. Injured myofibers, which contain post-mitotic nuclei, are destroyed. The
66 injury disrupts the quiescence niche of **muscle stem cells** (MuSCs; satellite cells) (see glossary),
67 which become activated and enter into an expansion phase while committing to the myogenic
68 program [6, 7]. Most myogenic cells further engage into the terminal myogenic differentiation and
69 eventually fuse to form new functional myofibers while a subset self-renews to return to
70 quiescence [6, 7] (Key Figure, Figure 1).

71 The widely used toxic muscle injury mouse models (e.g., injection of cardiotoxin or BaCl₂) allow to
72 follow the timing of the biological events occurring during skeletal muscle regeneration including
73 myogenesis, angiogenesis, inflammation and matrix remodeling [6, 7]. The absolute requirement
74 of monocyte-derived macrophages in skeletal muscle regeneration has been shown in myeloid
75 depletion studies (**clodronate**, **CD11b-DTR mouse**) [8] or the use of chemokine/chemokine

76 receptor deficient mice, namely **Ccl2** and **Ccr2** knockout (KO) animals [9, 10]; in these mice,
77 severe impairment of skeletal muscle regeneration is observed [8-10]. Of note, the requirement of
78 circulating monocytes in skeletal muscle regeneration applies to toxic muscle injury. Other kinds of
79 injury (*e.g.* traumatic or exercise-induced) may elicit different macrophage populations, such as
80 resident macrophages. However, while tissue resident macrophages (RTMs) (**Box1**) have been
81 described in several tissues, to our knowledge, they have not yet been characterized in skeletal
82 muscle.

83 Supporting roles for macrophages during skeletal muscle regeneration were originally suggested
84 by pioneer studies [11], and were later confirmed by lineage tracing, tissue-specific gene deletion
85 and co-culture assays of macrophages with MuSCs in both mice and humans. Studies pinpointed
86 a variety of roles for macrophages and revealed opposite functions for **Ly6C^{pos}** (positive)
87 macrophages freshly arrived at the injury site and **Ly6C^{neg}** (negative) macrophages present in the
88 later phases of skeletal muscle regeneration (Key Figure, Figure 1). Approaches included cell
89 tracing using *Cx3cr1^{GFP}* mice, depletion of monocyte entry into the injured muscle using *Ccr2* KO
90 mice, as well as coculture assays of sorted macrophages and MuSCs from regenerating muscle,
91 and purified human cells showed that recruited **Ly6C^{pos}** monocytes/macrophages could exhibit a
92 pro-inflammatory profile (see below), stimulate MuSC proliferation, and prevent premature
93 myogenic cell **fusion** [8, 12, 13]; in addition, these myeloid cells could regulate the number of
94 **fibro-adipo-progenitors** (FAPs) by inducing their apoptosis [14, 15]. After the tissue debris are
95 cleansed by phagocytosis, macrophages proliferate and these numerous cells exhibit an anti-
96 inflammatory/restorative profile (see below) [8, 16, 17]. Depletion of these intramuscular
97 macrophages (in the *CD11-DTR* mouse) leads to the formation of smaller myofibers, suggesting
98 these cells bear trophic functions [8]. The restorative phase is characterized by myogenesis,
99 growth of new myofibers, angiogenesis and matrix remodeling. Restorative macrophages
100 stimulate all these processes by exerting pro-differentiating and pro-fusogenic effects on MuSCs
101 [12, 18, 19], pro-angiogenic action on endothelial cells [20], and stimulating functions on FAPs [14,
102 15]. Furthermore, cell interactions of macrophages with surrounding cells can reciprocally impact

103 their phenotype, as exemplified by an observed delay in the macrophage phenotype transition
104 (assessed by the late appearance of Ly6C^{pos} macrophages) in the regenerating muscle of mice
105 that were irradiated or infected with a pathogen prior to the induction of muscle damage [21, 22].

106 Other than muscle, in most murine tissues studied so far after injury, a similar dichotomy between
107 Ly6C^{pos} pro- and Ly6C^{neg} anti-inflammatory macrophages has been identified, namely the heart
108 (e.g. after infarction) [23], liver (e.g. after toxic injury) [24], brain (e.g. after ischemia) [25] or skin
109 (e.g. after wound induction) [26]. New markers, notably those identified to characterize RTMs (**Box**
110 **1**), have emerged in mice in the context of post-injury tissue repair and skeletal muscle
111 regeneration [27]. Because pro- and anti-inflammatory macrophages often exert opposite
112 functions, their orchestration in time and space is required to ensure the coordination of the
113 biological processes necessary for tissue repair.

114

115 **Tracking Ly6C^{pos} inflammatory monocytes to Ly6C^{neg} restorative macrophages**

116 In mouse, Ly6C^{pos}Ccr2^{pos}**Cx3cr1**^{lo} circulating monocytes enter damaged tissues while
117 Ly6C^{neg}Ccr2^{neg}Cx3cr1^{hi} monocytes patrol to control vessel wall integrity [28]. In all organs studied
118 so far, only Ly6C^{pos} monocytes extravasate into injured tissues. In skeletal muscle, entry of
119 Ly6C^{pos} monocytes mainly occurs through the Ccl2-Ccr2 axis [9, 10], although the **complement**
120 **protein (C3a)** has also been implicated [29]. As a consequence, the resolution of inflammation is
121 not performed by a secondary recruitment of Ly6C^{neg} monocytes, but through the shift of
122 inflammatory Ly6C^{pos} macrophages into Ly6C^{neg} anti-inflammatory/restorative cells within the
123 tissue, as shown using the Ccr2 KO mouse (i.e. no appearance of Ly6C^{neg} macrophages in the
124 regenerating muscle) [9, 15, 30]; engulfed fluorescent beads from circulating Ly6C^{neg} monocytes
125 (no appearance of bead fluorescent Ly6C^{neg} macrophages in the regenerating muscle) [8]; and the
126 Ly6C^{neg} monocyte deficient mouse (*Nr4a1* KO) (no alteration in the kinetics of muscle
127 regeneration) [31].

128 Kinetics of macrophage gene profiling performed during skeletal muscle regeneration in mouse
129 after cardiotoxin injury served to conclude that the arbitrary phenotypic nomenclature for
130 macrophages M1/M2 (**Box 2**) was not strictly applicable to Ly6C^{pos} and Ly6C^{neg} cells *in vivo*, as
131 these cell subsets do not differentially express the canonical M1 and M2 gene profiles [16, 32, 33].
132 Although not M1, Ly6C^{pos} macrophages do express an inflammatory profile, which may be
133 muscle-specific or may reflect the signature of a sterile tissue injury [16, 32], which is yet to be
134 determined. The Ly6C^{pos} macrophage signature differs following tissue injury relative to infection.
135 For instance, macrophages do not release Reactive Oxygen Species (ROS) nor express an anti-
136 oxidant response after skeletal muscle toxic-induced injury [34]. It would be reasonable to assume
137 that after sterile tissue injury, the pro-inflammatory phase is different in nature, exhibits a lower
138 intensity, and is shorter than after a (more harmful) immune challenge. Specifically, the
139 inflammatory profile of Ly6C^{pos} monocytes/macrophages includes the expression of acute phase
140 proteins, inflammatory markers, and iron-scavenging molecules (Lipocalin-2, Haptoglobin) [31]
141 protecting against iron-mediated toxicity -- a hallmark of inflammatory macrophages [35]. Later on,
142 macrophages lacking ferroportin impede skeletal muscle regeneration, preventing myogenesis
143 and inducing adipogenesis in FAPs [36]. Thus, the transition from an iron-retention to an iron-
144 donation state is essential for skeletal muscle recovery, as shown in other tissues in mouse [35].
145 Ly6C^{neg} macrophages exhibit a restorative phenotype with a high secretory phenotype of
146 molecules involved in intercellular communications and extracellular matrix remodeling (*e.g.*
147 cytokine, chemokines, proteoglycans, matricellular proteins, assembly proteins). They are not
148 canonical M2 cells since IL-4 signaling has not been demonstrated during skeletal muscle
149 regeneration [16, 32, 37]; this would constitute a difference in Ly6C^{neg} macrophages and M2
150 macrophages during skin wound healing [38]. However, while most studies have used C57/Bl6
151 mice, recent work used DBA2/J mice to show an IL-4 signature in restorative macrophages during
152 post-cardiotoxin skeletal muscle regeneration [39]; this finding underscores the importance of
153 thoroughly examining genetic backgrounds when investigating the inflammatory response.

Mechanisms of the resolution of inflammation

The resolution of inflammation is a crucial process to avoid uncontrolled inflammation and to allow tissue return to homeostasis. It must be tightly controlled and it is regulated by a variety of mechanisms that may partially overlap to ensure the efficacy of resolution. Recent studies have evidenced how these mechanisms are intertwined (Key Figure, Figure 1).

Intrinsic mechanisms

In vitro investigations first identified that after a pro-inflammatory stimulus (bacteria or cytokine), macrophages acquire a pro-inflammatory phenotype, and then rewire into anti-inflammatory cells without any other intervention. This was shown for instance for Secretory Leukocyte Protease Inhibitor (SLPI), which is secreted late after exposure to a pro-inflammatory cue [40] and which is now identified as a mediator of resolution of inflammation in mice [4]. The mechanisms for the regulation of the early or delayed expression of pro- and anti-inflammatory molecules appears to include post-transcriptional modifications controlling mRNA stability and/or translation (adenine- and uridine-rich element (ARE)-mediated and micro RNA (miRNA)-mediated post-transcriptional control) and were mainly addressed *in vitro* [reviewed in 41].

Mediators of the resolution inflammation

Mediators of the resolution of inflammation (*e.g.* **Annexin A1** [AnxA1], SLPI, Glucocorticoid-induced leucine zipper [GILZ], developmental endothelial locus-1 [DEL-1]) promote the shift in macrophage status from pro- to anti-inflammatory [4]. Lipids, notably Specialized Proresolving Mediators (SPMs) also appear at the onset of the resolution of inflammation, due to the rewiring of lipid metabolism from the pro-inflammatory leukotrienes and prostaglandins, to lipoxins, resolvins, protectins, and maresins [42]. Kinetics of lipidome coupled with transcriptomic analysis of macrophage subsets identified a temporal shift of lipid expression from Ly6C^{pos} macrophages at day 1 post-injury, to Ly6C^{neg} cells at day 4 post-injury in regenerating skeletal muscle after

cardiotoxin injury in mice[17]. This shift is marked by a transition from arachidonic acid-derived pro-inflammatory mediators towards SPMs and the increased expression of the master regulator of lipid metabolism Peroxisome Proliferator-Activated Receptor (PPAR) γ [17, 19]. To better understand the highly dynamic nature of macrophages during this shift, kinetic analyses should be a requirement; this can also enable modeling predictions on putative macrophage functions during skeletal muscle regeneration [16, 17, 32].

Macrophage metabolism and inflammatory status

Macrophage metabolism has been widely investigated in recent years and metabolic programs are known to control macrophage inflammatory status, as described above for lipids. However, although our understanding of metabolic control of macrophages is growing based on *in vitro* studies, there is still fragmented knowledge on how macrophage metabolism is controlled *in vivo* [43, 44]. Longitudinal analyses of gene profiling have shown an increase in glutamine metabolism (involved in the activation of the TCA cycle and oxidative phosphorylation) during the late steps of post-toxic skeletal muscle regeneration in mice, associated with restorative Ly6C^{neg} macrophages [16]. Of note, in this model, pathways linked to oxidative metabolism are upregulated between days 1 and 2 after injury in both macrophage subsets, while their inflammatory status shifts from day 2, suggesting that specific metabolic changes precede, and therefore may control, the inflammatory status transition of macrophages [16].

Efferocytosis links metabolic rewiring with the inflammatory shift

An essential process in the resolution of inflammation is efferocytosis, *i.e.* phagocytosis of apoptotic and necrotic cells, that was demonstrated 30 years ago [45]. Efferocytosis induces the resolution of inflammation by rewiring macrophage inflammatory status as well as by ‘cleansing’ the damaged tissue area to allow repair/healing. *In vitro* experiments in mouse and human macrophages have shown that recognition and engulfment of dead cells can trigger the

transcription of anti-inflammatory effectors such as Transforming Growth Factor (TGF) β , IL-10 [46, 47] and SPMs, concomitant with the downregulation of inflammatory cytokines [48]. Recent findings in mice defined a link between efferocytosis and metabolism, needed for the resolution of inflammation *in vivo*. For instance, to resolve inflammation during periodontitis or peritonitis in mice, the nuclear receptors **LXRs** were reported to trigger the expression of the cholesterol transporter, TGF β , as well as SPMs upon efferocytosis in macrophages (induced by apoptotic cell-induced exposure of phosphatidyl serine or via treatment with the mediator of resolution of inflammation DEL-1) [49, 50]. Biological processes in mitochondria are also involved in efferocytosis. Specifically, The mitochondrial membrane protein UnCoupling Protein (UCP)2 is required for phagocytosis, since loss of *Ucp2* reduces mouse macrophage phagocytic capacity, while phagocytosing macrophages increase their expression of *Ucp2* [51]. Efferocytosis also requires mitochondrial fission, mediated by the protein Drp1, which induces the release of calcium from the endoplasmic reticulum, in turn required for the formation of the **phagosome** [52]. Recently, an elegant murine study linked efferocytosis and mitochondria to the control of IL-10 expression in macrophages, using a combination of gene silencing, metabolomic profiling, measurement of mitochondrial activity and pharmacological approaches *in vitro* and *in vivo* in mouse. In brief, efferocytosis was associated with fatty acid oxidation, activation of the electron transport chain, leading to the production of NAD⁺. In turn, NAD⁺ triggered DNA binding of the transcription factor Pbx-1 in a **sirtuin** (SIRT)1-dependent manner to drive IL-10 expression and resolve inflammation after myocardial infarction [53]. The authors assumed that ingestion of dead cell metabolites via efferocytosis impacted the metabolic flux and mitochondrial functions of macrophages, since phagocytosis of inert material (*e.g.* beads) did not trigger mitochondrial changes, nor IL-10 expression [51, 53].

The resolution of inflammation during skeletal muscle regeneration

Several molecular pathways control the resolution of inflammation during skeletal muscle regeneration *in vivo* in the cardiotoxin-induced muscle injury in mouse. Investigations of the role of

234 macrophages *in vivo* have used: i) tissue specific deletion with Lysozyme M-Cre (LysM-Cre)
235 mouse strain, that specifically targets floxed genes in myeloid cells [54] and ii) bone-marrow
236 transplantation. Analysis of macrophage subsets can include flow cytometry analysis (expression
237 of Ly6C, CD206, etc.) and the expression of inflammatory genes by cell sorted Ly6C^{pos/neg}
238 macrophage subsets. Insulin Growth Factor (IGF)-1, a well-known regulator of myogenesis, exerts
239 autocrine action on pro-inflammatory macrophages to induce their conversion into restorative
240 macrophages [55]. MKP-1 (mitogen-activated protein kinase [MAPK] phosphatase-1) also controls
241 the macrophage phenotypic transition by restricting p38 MAPK activation in order to prevent the
242 shift from occurring too early [3]. Recently, the heme-binding transcriptional repressor Bach1 was
243 shown to regulate distal regulatory elements in a series of inflammatory genes from muscle
244 macrophages. In brief, silencing *Bach1* or its target heme oxygenase-1 accelerated or delayed the
245 macrophage phenotype shift, respectively, impairing muscle regeneration in both cases [56].
246 Furthermore, using mice mutated in two CREB-binding sites in the *Cebpb* promoter, a CREB-
247 C/EBP β cascade was shown to be required in macrophages that acquired a restorative phenotype
248 after muscle injury [57]; this deletion blocked downstream induction of M2-specific *Msr1*, *Il10*,
249 *Il13ra*, and *Arg-1* (Arginase-1) genes (the latter significant for metabolism); these findings
250 supported the concept that genes controlling metabolic pathways could be involved in the
251 macrophage inflammatory shift [58]. In that context, activation of the metabolic regulator **AMP-**
252 **activated protein kinase** (AMPK α 1) was necessary to induce a macrophage inflammatory shift.
253 Among other activities, AMPK is involved in mitochondria biogenesis and function, notably through
254 the NAD-SIRT1 pathway [59], as mentioned, important for controlling IL-10 synthesis. LysM-Cre
255 *Ampka1^{fl/fl}* macrophages did not acquire the restorative phenotype and functions *in vitro* and *in*
256 *vivo*, leading to a defect in skeletal muscle regeneration [60]. *In vitro*, pro-inflammatory
257 macrophages having ingested dead MuSCs decreased the expression/secretion of pro-
258 inflammatory effectors (TNF α , iNOS, Ccl3) and increased that of anti-inflammatory molecules
259 (TGF β , CD163, CD206) [8, 60]. Ampk-deficient macrophages did not rewire their inflammatory
260 program and biological functions (stimulation of myogenesis) upon efferocytosis compared to

261 wildtype (WT) controls in this model [60]. Moreover, the mediator of resolution of inflammation
262 *AnxA1*, also known to increase efferocytosis [4], is a potent activator of AMPK in macrophages,
263 through its FPR2/AXL receptor, and can promote the resolution of inflammation[61]. Another
264 example is that of the scavenger receptor SRB1; mice lacking this receptor in hematopoietic cells
265 (transplantation of *Srb1* KO bone marrow) exhibit an impairment in skeletal muscle regeneration,
266 associated with a defect in acquiring the restorative phenotype in macrophages relative to WT
267 controls [62].

268 These studies have defined certain links between specific signaling pathways with efferocytosis,
269 and/or metabolic regulation; thus, it is reasonable to speculate that given the importance of the
270 resolution of inflammation, several molecular pathways are likely to converge to cooperatively
271 achieve specific changes in macrophage transcriptional programs. Of note, *AnxA1* is mostly
272 expressed by neutrophils and Ly6C^{pos} macrophages at early time points after toxin-induced
273 muscle injury; this suggests that initiating cues are present for the resolution of inflammation as
274 early as the beginning of the inflammatory response, supporting the concept of "the beginning
275 programs the end" [63].

277 **From mice to men - the timing of the resolution of inflammation for muscle recovery**

278 Timing matters

279 As mentioned above, timing is important for the resolution of inflammation. Blocking any of the
280 above described molecular pathways leads to defects in the dynamics of macrophage shift, which
281 is deleterious for skeletal muscle regeneration in mice [55, 57, 60, 64]. A disrupted inflammatory
282 environment due to chronic infection by *Toxoplasma gondii* can impair skeletal muscle
283 regeneration due to altered resolution by macrophages, indicating that a preexisting inflammatory
284 environment in the tissue can alter reparative immunity and the quality of tissue regeneration. [22].
285 Specifically, shortening the inflammatory phase *via* early inhibition of pro-inflammatory cues (IFN γ)
286 or *via* early addition of anti-inflammatory effectors (IL-10) can similarly prevent skeletal muscle

287 regeneration in the mouse cardiotoxin injury model [3, 65]. Moreover, altering COX2 activity (by
288 providing Non Steroid Anti-Inflammatory Drug [NSAID]) in this model has been found to modify
289 macrophage lipid profiles and prevent the appearance of restorative macrophages in regenerating
290 muscle [17]. Therefore, we propose that after sterile tissue injury, the inflammatory phase must
291 occur completely before the resolution of inflammation can ensue, and initiate the restorative
292 phase. Testing this hypothesis still requires extensive and robust testing.

293 294 Management of the resolution of inflammation in skeletal muscle injuries

295 One of the most popular treatments following skeletal muscle injury is applying ice, or cooling the
296 injured area. Icing is integrated in the RICE (for Rest, Icing, Compression, Elevation) protocol, for
297 which not a single, randomized clinical trial has proven effectiveness [66] (Box 3). Nevertheless,
298 this treatment is widely used with the aim of stopping bleeding and reducing pain, and with the
299 idea that edema and inflammation should be reduced, according to the long-held view that
300 inflammation is detrimental for tissue repair. However, experiments in rodents have shown that
301 icing or cooling is detrimental for skeletal muscle regeneration [67-71]. Cooling induces a
302 decrease of both neutrophil and macrophage numbers and a delay in the kinetics of the
303 inflammatory profile of macrophages in the regenerating muscle; consequently, the size of the
304 regenerating myofibers is smaller than in untreated animals, and interstitial fibrosis ensues, a
305 negative outcome for proper muscle function [67, 69-71]. In humans, recent studies have either
306 reported no effects, or detrimental outcomes of cooling after injury on muscle function recovery
307 (measurement of force and fatigue) after exercise-induced muscle damage [68, 72-74].
308 Noteworthy, the variety of protocols and the lack of histological/cellular investigations in human
309 renders these conclusions hardly definitive. Moreover, the use of anti-inflammatory drugs such as
310 NSAIDs following muscle injury have led to conflicting results in humans, and have also shown
311 detrimental outcomes in animal models with muscle injury [2]. Therefore, in practice, we support
312 the recommendation made by Maria Urso: "The opinion here is that the therapy should not be to

obliterate the inflammatory response, but instead, to restore the normal regulation of inflammatory processes" [2].

Concluding remarks

Data collection from the fields of molecular and cell biology, immunology, and physiology are allowing us to increase and integrate our knowledge of macrophage biology during the inflammatory response. High throughput technologies coupled to the isolation of cell subpopulations at different time points after injury have established kinetics of macrophage profiles and functions during skeletal muscle regeneration. These reveal how macrophages are dynamic during the inflammatory response and how they control the sequential phases of tissue recovery by permanently adapting their gene expression programs, particularly at the time of the resolution of inflammation. Now that a bigger picture of macrophage biology during the post-injury inflammatory response has emerged, further challenges include digging into specific details to answer many pending questions (see Outstanding questions box).

Single cell analysis might allow to decipher the molecular mechanisms driving macrophage biology for each tissue and each type of injury, as well as to identify commonalities and specificities of the inflammatory response among different organs. Kinetic analyses should decipher the interactome of macrophages with their neighboring cells, as this landscape constantly evolves during the inflammatory response. Single cell studies should also identify new markers for macrophage subsets to refine the characterization of these cell subpopulations.

Finally, the extent and type of muscle injury are important parameters to consider. Skeletal muscle injury models in rodents are usually severe (about 100% of injured muscle fibers). However, it is not known whether milder injuries give rise to similar inflammatory responses. One hypothesis posits that in human muscle injuries that lead to over 50% of immediate loss of muscle force (or incomplete force recovery, 1 week post exercise), the inflammatory response is likely to occur as described above [12, 75]. However, for moderate or mild injuries, evidence for an acute

339 inflammatory response is either controversial or lacking, respectively [75]. In that context, it is
340 (speculatively) suggested that RTMs may play a role in modulating inflammation according to
341 degree of injury. In resting skeletal muscle, RTMs are mainly located in the **perimysium and**
342 **epimysium** [76]; and furthermore, upon severe (toxic) injury in mice, they have been reported to
343 participate in mounting an inflammatory response by recruiting monocytes to damaged muscle
344 [77]. Thus, a careful description of RTMs and their putative participation in different stages of the
345 inflammatory response should help provide a more complete picture of macrophage biology during
346 skeletal muscle regeneration, and will undoubtedly represent an interesting area of further
347 investigation.

348

Box 1 - Tissue resident macrophages

Until a decade ago, macrophages present in tissues were generally believed to come from circulating monocytes. A first series of investigations in mouse showed that Langerhans cells in skin, and microglia in the brain, were not from bone marrow origin [78-80]. Subsequent work using lineage tracing genetic tools (*e.g.* *Cx3cr1*^{Cre-ERT2};GFP mice) described variations in the origin of resident macrophages according to tissue [81]. In mice, resident tissue macrophages (RTMs) are established during development in the brain, liver and spleen, and are maintained independently from the bone marrow during adulthood, while progressively being replaced by monocytes after birth in the gut and heart [82]. During development, RTMs may originate from: i) primitive hematopoiesis from the yolk sac (E7.0); ii) from transit definitive hematopoiesis giving rise to erythro-myeloid precursors (E8.0) from yolk sac and then from fetal liver, and iii) from definitive hematopoiesis (E10.5) that will establish bone marrow hematopoiesis [82]. A series of genetic tools have revealed the kinetics of RTMs in a variety of tissues. One of the latest models developed was the *Ms4a3*^{Cre-ERT2} fate-mapping mouse model that distinguishes monocyte- versus embryonic-derived RTMs [83]. In this model, monocyte contribution to RTM homeostasis differed depending on the type of injury: upon injury depleting RTMs (*e.g.* thioglycollate-induced peritonitis), monocytes replaced RTMs, in contrast to injury that did not deplete RTMs (*e.g.* LPS-induced peritonitis) [83]. Similar results were observed for kidney RTMs [84].

On the one hand, this raises the question of 'seed and soil': local determinants defining specific niches may be responsible for the local programming of macrophages entering that location, recently shown for cavity resident mouse macrophages [85]. On the other hand, Ly6C^{pos} derived monocytes can give rise to "*bona fide*" RTMs exhibiting specific tissue locations in mouse; indeed, two monocyte-derived RTM subpopulations can coexist across tissues with distinct functional profiles and distinct locations (near nerves or blood vessels) [86].

Deciphering the respective contributions of RTMs (whether they are embryonic- or bone marrow-derived) as well as monocyte-derived macrophages in tissue homeostasis and in response to injury is highly challenging; indeed, these cells are highly plastic and constantly carry a dialogue

with their environment. Thus, new definitions of subsets, along with a better specification and combined use of several markers (e.g. MHCII, LYVE1, TIMD4, CD206, CCR2) can help highlight the respective functional contribution of such macrophage subsets in tissue healing and homeostasis maintenance. Even identifying small RTM subsets can allow an investigation of their function, including heart protection after infarction [87], or supporting kidney repair after macrophage phenotype reprogramming [84].

Box 2 - the M1/M2 paradigm

Stemming from earlier *in vitro* studies made with human macrophages, the arbitrary M1/M2 paradigm spread all over the field. With this type of nomenclature, M1 macrophages are considered classically activated macrophages that are activated by LPS in the presence or absence of IFN γ [88]. M2 macrophages are considered IL-4-induced alternatively activated macrophages [88]. Because a variety of anti-inflammatory cues induced overlapping gene expression *in vitro*, M2 macrophages (with or without the M2a, b, c declination), were presumably all anti-inflammatory [88]. Being easy-to-use, the M1/M2 terminology was widely adopted, naming pro-inflammatory and anti-inflammatory macrophages, respectively, despite its lack of accuracy on the inflammatory status of cells, particularly *in vivo* (see below). *In vitro*, this dichotomy was proven to be false, where a plethora of 29 different well-defined activating cues gave rise to 29 different inflammatory status in human macrophages, all different from the M1 and M2 status [89]. Therefore, several voices have called to abandon the M1/M2 designation, and instead, use more specific names to characterize macrophage inflammatory status [5, 90].

Box 3. Clinician's Corner box

- After severe muscle injury, inflammatory cells recruited into the injured area exert beneficial effects on myogenesis (expansion of muscle stem cells) and fibrosis (limitation on the number of fibroblastic cells).
- Anti-inflammatory treatments (icing, corticoids, NSAIDs) should be avoided during the few days after a severe skeletal muscle injury. All these treatments prevent the entry of monocytes/macrophages in the regenerating area, extend the inflammatory phase and delay the resolution of inflammation. Hence the regeneration and functional recovery are delayed.

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610

611 Key Figure, Figure 1

612 **Sequential roles of macrophages during the inflammatory response in mouse skeletal**
613 **muscle regeneration.** Upon muscle injury, neutrophils and Ly6C^{pos} monocytes extravasate from
614 circulation and secrete Specialized Proresolving Mediators (SPMs) (e.g., AnxA1) while muscle
615 stem cells (MuSCs) exit from quiescence. Neutrophils undergo apoptosis in the next 2 days while
616 monocytes become Ly6C^{pos} inflammatory macrophages [6, 7]. They stimulate MuSC proliferation
617 and induce apoptosis of some Fibro-Adipogenic Precursors (FAPs)/fibroblasts [6, 7]. The
618 Integration of SPM cues together with efferocytosis initiate the resolution of inflammation, which is
619 further supported by the rewiring of cell metabolism in pro-inflammatory macrophages [6, 7]. This
620 shifts the expression of the inflammatory program and allows the acquisition of the anti-
621 inflammatory/restorative phenotype by macrophages (among which the loss of Ly6C expression)
622 [6, 7]. Ly6C^{neg} macrophages proliferate and dampen inflammation while stimulating MuSCs,
623 endothelial and fibroblastic cells to promote myogenesis, angiogenesis and matrix remodeling,
624 respectively. For illustration purposes, representative micrographs on the right show transversal
625 hematoxylin/eosin stained histological sections of mouse skeletal muscle at various times after
626 toxic (cardiotoxin) injury [60]. At day (D)1, myofibers are necrotic and immune cells invade the
627 injured area. At D2, macrophages start to phagocytose myofiber debris. At D4, new myofibers are
628 visible, surrounded by numerous macrophages. At D8, regenerating myofibers are growing and
629 the number of inflammatory cells is reduced. At D14, the muscle exhibits its previous appearance,
630 with nuclei in a central localization in myofibers [6, 7]. The fine coordination in time and space of
631 these biological processes is required for a timely resolution of inflammation that ensures the
632 recovery of the tissue and the return to homeostasis.

634 Glossary

- 635
- 636 • **AMP-activated protein kinase:** Heterotrimeric complex monitoring cellular energy status by
637 sensing ATP/ADP/AMP concentrations. AMPK is activated (phosphorylated) by metabolic
638 stress that inhibits mitochondrial ATP production or accelerates ATP consumption. Once

639 activated, AMPK restores cellular energy balance by switching on catabolic, while switching off
640 anabolic pathways.

- 641 • **Annexin-A1:** abundant in myeloid cells; has profound effects on several phases of the
642 resolution of inflammation: inhibition of neutrophil recruitment, induction of neutrophil apoptosis,
643 enhancement of monocyte recruitment and efferocytosis, macrophage reprogramming
- 644 • **Ccl2/Ccr2 axis:** Ccl2 chemokine (MCP-1), released by damaged tissues, binds to its receptor
645 Ccr2 expressed on circulating Ly6C^{pos} monocytes. The Ccl2/Ccr2 axis is the main entry route of
646 monocytes from blood to an injured tissue.
- 647 • **CD11b-DTR mouse:** transgenic mouse in which the receptor for the Diphtheria Toxin (DTR) is
648 expressed under the control of the myeloid *CD11b* promoter. Only monocytes and
649 macrophages are targeted in this mouse, depending on the toxin injection site.
- 650 • **Clodronate:** A single intravenous injection of liposome-encapsulated clodronate is engulfed by
651 monocytes, killed by clodronate, leading to a transient (about 12 h) monocyte depletion in
652 circulation.
- 653 • **Complement protein (C3a):** The complement system contributes to both innate and adaptive
654 immune responses. Complement activation triggers the generation of cleavage products,
655 among which, C3a and C5a are anaphylatoxins. Complement proteins can play a role in tissue
656 repair.
- 657 • **Cx3cr1:** Receptor of Cx3cl1 chemokine (fractalkine). The Cx3cr1^{GFP} knock-in mouse is useful
658 to trace monocytes and monocyte-derived macrophages expressing the GFP protein.
- 659 • **Epimysium, perimysium:** Each single myofiber is surrounded by a specific extracellular
660 matrix, the basal lamina. Between fibers, the interstitial space is filled with collagenous
661 extracellular matrix (endomysium). In skeletal muscle, myofibers are grouped to form bundles
662 or fascicles. Each fascicle is surrounded by a thicker sheath of connective tissue (perimysium).
663 The whole muscle is wrapped by the epimysium (or fascia), a thick collagenous connective
664 tissue.
- 665 • **Fibro-adipo-progenitors:** Interstitial stem cells in mouse skeletal muscle capable of self-
666 renewal and differentiation into fibroblasts, adipocytes and osteoblasts; present similarities with
667 mesenchymal stem cells.
- 668 • **(Myogenic cell) Fusion:** Once committed into the terminal myogenic differentiation, MuSCs
669 eventually fuse with each other to form myotubes (in vitro and in vivo), and with preexisting
670 myofibers (in vivo) to rebuild damaged myofibers and recover muscle integrity.
- 671 • **LXRs:** Liver X Receptors belong to the superfamily of nuclear receptors (which includes PPARs
672 and RXRs). They play important roles in regulating lipid and glucose homeostasis.
- 673 • **Ly6C:** expressed by a subset of circulating monocytes also bearing Ccr2. They arise from bone
674 marrow progenitors and give rise to Ly6C^{neg} monocytes that patrol within vessels. Ly6C^{pos}

675 monocytes enter injured tissues through Ccl2/Ccr2 and become pro-inflammatory Ly6C^{pos}
676 macrophages. At resolution of inflammation, they lose Ly6C expression. Depending on the
677 tissue, resident macrophages may be either Ly6C^{pos} or Ly6C^{neg} or both.

- 678 • **Muscle stem cells (satellite cells):** Laying along the myofibers in a quiescent state, they exit
679 quiescence upon muscle injury to perform the adult myogenesis program; monopotent adult
680 stem cells capable of both self-renewal and differentiation to form new functional myofibers and
681 repair damaged muscle.
- 682 • **Phagosome:** intracellular vesicle formed by invagination of the plasma membrane with its
683 associated lipids and proteins during phagocytosis (of microbes or dead cells). It fuses with
684 lysosomes to form phagolysosomes to degrade phagosome content by lysosomal enzymes.
- 685 • **Sirtuins:** belong to class III histone deacetylase family of enzymes; involved in regulating
686 cellular metabolism: they are NAD⁺ sensors requiring NAD⁺ to function. SIRT1 can contribute to
687 inflammation by altering histones and transcription factors such as NFκB and AP1; important
688 enzymes at the crossroads of metabolism and inflammation.

689

690

Inflammatory phase:

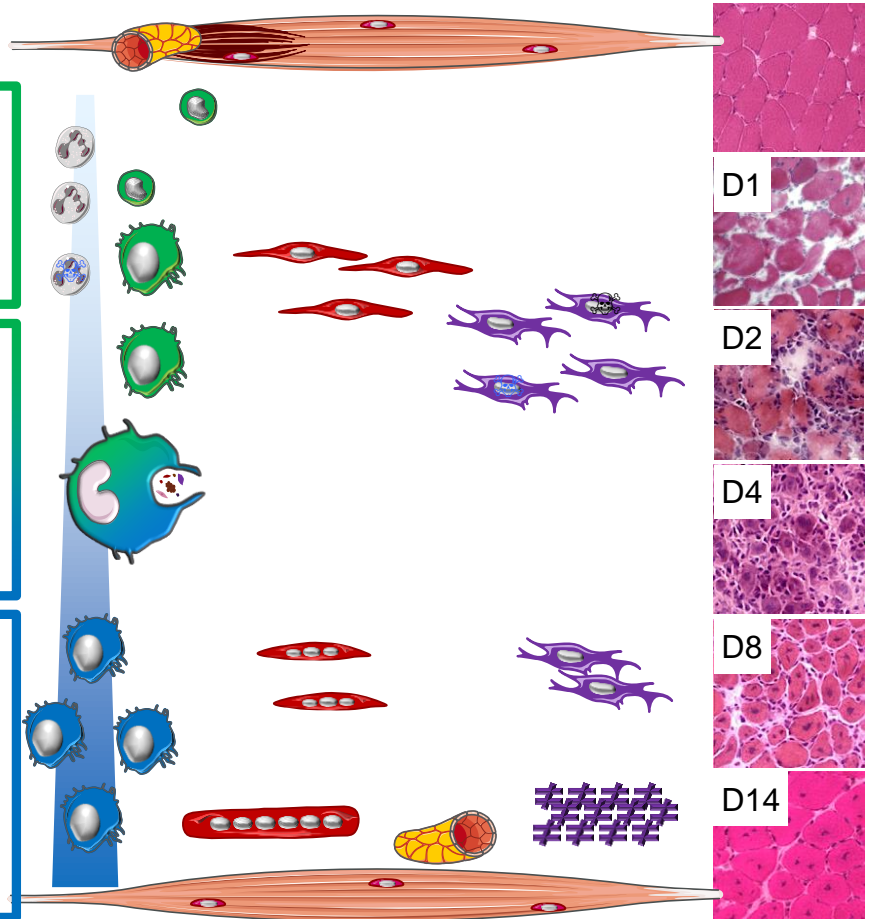
- Increased inflammation
- Muscle stem cell proliferation
- FAP/fibroblast apoptosis

Resolution of inflammation:

- Integration of SPM cues
- Efferocytosis
- Metabolic rewiring
- Inflammation reprogramming
- Proliferation

Restorative phase:

- Extinguished inflammation
- Muscle stem cell differentiation
- Angiogenesis
- Fibroblast matrix production and matrix remodeling



Key



Neutrophils



LyC6^{pos} monocytes



LyC6^{pos} macrophages



LyC6^{neg} macrophages



SPM concentration



Myofiber with muscle stem cells (quiescent)



Muscle stem cells (proliferating)



Muscle stem cells (differentiating)



FAPs/fibroblasts – extracellular matrix



Endothelial cells – vessels