Transcriptional regulation of macrophage polarization: enabling diversity with identity

Toby Lawrence and Gioacchino Natoli‡*

Abstract | In terms of both phenotype and function, macrophages have remarkable heterogeneity, which reflects the specialization of tissue-resident macrophages in microenvironments as different as liver, brain and bone. Also, marked changes in the activity and gene expression programmes of macrophages can occur when they come into contact with invading microorganisms or injured tissues. Therefore, the macrophage lineage includes a remarkable diversity of cells with different functions and functional states that are specified by a complex interplay between microenvironmental signals and a hardwired differentiation programme that determines macrophage identity. In this Review, we summarize the current knowledge of transcriptional and chromatin-mediated control of macrophage polarization in physiology and disease.

Mononuclear phagocyte system
(MPS). A group of bone marrow-derived cells with different morphologies (monocytes, macrophages and dendritic cells), which are mainly responsible for phagocytosis, cytokine secretion and antigen presentation.

**Centre d'Immunologie Marseille-Luminy, Parc Scientifique & Technologique de Luminy, Case 906, F-13288 Marseille, France.*
‡Department of Experimental Oncology, European Institute of Oncology (IEO), Via Adamello 16, 20139 Milan, Italy.
e-mails: lawrence@ciml.univ-mrs.fr; gioacchino.natoli@ifom-ieo-campus.it
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The mononuclear phagocyte system (MPS) (FIG. 1) is defined on the basis of ontogeny and phagocytic activity, and includes blood monocytes and the diverse network of tissue macrophages and monocyte-derived dendritic cells (DCs). There is great diversity within each of these subpopulations, and the heterogeneity of monocytes and their relationship to tissue macrophages and DCs is a rapidly evolving field of research[1]. The functional and phenotypic diversity of tissue macrophages has long been appreciated, and it is now clear that this reflects a complex interplay between intrinsic differentiation pathways and environmental inputs received from neighbouring cells[2]. Cells of the MPS have an amazing capacity for functional specialization, as shown by the distinct phenotypes of resident cells in different tissues, including microglial cells of the brain, Langerhans cells in the skin, bone-resorbing osteoclasts in the skeletal system and Kupffer cells in the liver. Recent research has also highlighted the important contribution of monocyte-derived antigen-presenting cells in priming T cell responses and adaptive immunity[3,4] — a property that was originally thought to be exclusive to conventional DCs — thus extending the functions of macrophages to the control of adaptive immune responses.

The MPS is particularly dynamic during inflammation or infection. Under such conditions, blood monocytes are recruited into the tissues, where they differentiate into macrophages[5] or DCs[3]. Depending on the microenvironment, macrophages can acquire distinct functional phenotypes. Two well-established polarized phenotypes are often referred to as classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages). The M1/M2 nomenclature is derived from the cytokines that are associated with these macrophage phenotypes, as these cytokines — namely, interferon-γ (IFNγ) or interleukin-4 (IL-4) and IL-13 — are linked with T helper 1 (T_H1)- and T_H2-type immune responses, respectively[6]. Bacterial lipopolysaccharide (LPS) also promotes the generation of M1 macrophages[6]. LPS induces a strong pro-inflammatory phenotype in macrophages that includes the production of IFNβ and antimicrobial effectors such as nitric oxide. In turn, autocrine IFNβ signalling triggers a macrophage phenotype similar to that induced by IFNγ[7]. Another polarized macrophage phenotype that has received a great deal of attention in the literature is that of tumour-associated macrophages (TAMs)[8]. These are often considered to be synonymous with M2 macrophages. However, although they do have some characteristics of M2 macrophages, TAMs have a transcriptional profile that is quite distinct from those of M1 and M2 macrophages[9,10]. Emerging studies have described polarized macrophage phenotypes in other contexts. However, they represent variations on the same theme, and for the sake of clarity we focus here on the well-characterized examples of M1 and

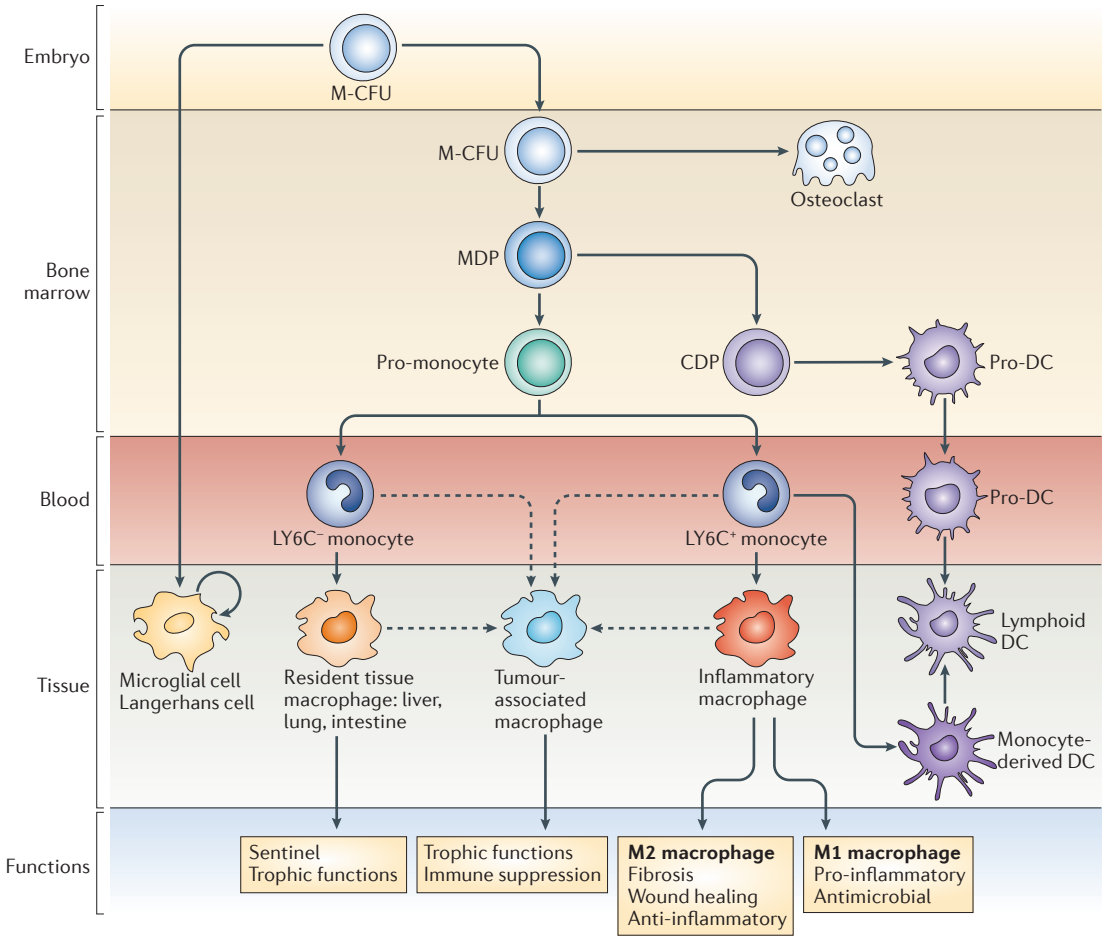

Figure 1 | **The mononuclear phagocyte system.** Myeloid cells are derived from haematopoietic stem cells (myeloid colony-forming units (M-CFU)) in the bone marrow of the mature adult or the yolk sac of the developing embryo. In the bone marrow, monocytes develop from a macrophage colony-stimulating factor 1 (CSF1)-dependent macrophage and dendritic cell (DC) progenitor (MDP), which also gives rise to common DC progenitors (CDPs). Blood monocytes and most tissue macrophages develop from an MDP-derived pro-monocyte precursor. Exceptions include Langerhans cells in the skin and microglial cells in the brain; these tissue-resident macrophage populations are radiation resistant and seem to be maintained through local proliferation, and recent studies indicate that they initially develop from myeloid stem cells (M-CFU) in the yolk sac of the developing embryo. During homeostasis, CSF1 seems to be sufficient for the recruitment and differentiation of most tissue macrophages, which are probably derived from LY6C$^-$ blood monocytes. In response to inflammation, LY6C$^+$ monocytes are recruited from the blood and differentiate into inflammatory macrophages; depending on the inflammatory milieu, these macrophages can polarize into specific phenotypes, such as M1 or M2 macrophages. LY6C$^+$ monocytes also give rise to monocyte-derived DCs in secondary lymphoid organs. Resident tissue macrophages can also become polarized into a specific activation state in the context of inflammation. The origin of tumour-associated macrophages (TAMs) is not clear; they might be derived from recruited LY6C$^+$ or LY6C$^-$ blood monocytes, but tissue-resident macrophages could also contribute to the TAM population.

M1 macrophages
Macrophages with an activation phenotype associated with increased microbicidal activity and antigen-presenting function. M1-type activation is usually modelled *in vitro* by interferon-γ and/or lipopolysaccharide stimulation. In mice, M1-associated markers include interleukin-12, MHC class II molecules and nitric oxide synthase 2 (NOS2); however, human macrophages do not show induction of NOS2 in these conditions, despite having a similar functional phenotype.

M2 macrophages
Macrophages that are associated with parasitic infections and T helper 2-type immune responses. M2-type activation is usually modelled *in vitro* by interleukin-4 (IL-4) and/or IL-13 stimulation. In mice, M2-associated markers include resistin-like-α (also known as FIZZ1), arginase 1, chitinase 3-like 3 (also known as YM1), IL-10 and macrophage mannose receptor 1 (also known as CD206); however, human M2 macrophages do not show induction of resistin-like-α, arginase 1 and chitinase 3-like 3, but instead upregulate indoleamine 2,3-dioxygenase expression. M2 macrophages are associated with anti-inflammatory and homeostatic functions linked to wound healing, fibrosis and tissue repair.

M2 macrophages and TAMs to illustrate the different transcriptional mechanisms associated with macrophage polarization. Most importantly, M1 and M2 phenotypes might not be stably differentiated subsets in the same way as T_H1 and T_H2 cells, for example. At least *in vitro*, LPS-activated macrophages after a few hours become unable to reactivate a large fraction of pro-inflammatory genes following re-stimulation[11]. However, they retain the ability to induce the expression of many other genes, including *Il10*, for example. This altered state of responsiveness to secondary stimulation is commonly referred to as endotoxin tolerance and results in a global and sustained switch of the gene expression programme from a pro-inflammatory M1 signature to an M2-like anti-inflammatory phenotype[12]. Although these data provide proof of principle that macrophages can undergo dynamic transitions between different functional states, the stability of M1 and M2 phenotypes in a physiological setting is still unclear and requires further investigation.

The commonly held view is that macrophage polarization is driven by cues in the tissue microenvironment, which can include cytokines, growth factors and microorganism-associated molecular patterns. These signals are thought to dictate a transcriptional response that shapes the phenotype and function of the macrophages on the basis of the physiological or pathophysiological

context. This model is based on a large number of independent experimental studies; however, the data are still incomplete and far from being systematic, and our knowledge of the mechanistic basis of macrophage diversity in different tissues or in response to changing environments is still very rudimentary. The molecular determinants of macrophage diversity, and specifically the transcription factors that dictate alternative functional outcomes in response to different environmental inputs in different tissues, are to a large extent unknown. In this Review, we highlight recent advances in our understanding of the transcriptional regulation of macrophage polarization and activation in different contexts, including chromatin-mediated regulation of global gene expression and the roles of sequence-specific transcription factors.

A genomic view of macrophage function

Myeloid lineage specification and the earliest stages of myeloid differentiation require the activity of Runt-related transcription factor 1 (RUNX1)[13]. The RUNX1 target gene that is most crucial for myeloid development encodes the ETS family transcription factor PU.1, which must be constantly expressed at high levels to induce and then maintain macrophage differentiation[14–16]. In this section, we describe the essential role of PU.1 in the control of the genomic organization and the transcriptional regulatory repertoire of macrophages.

Pervasive control of the genome by PU.1. Data that have accumulated from chromatin immunoprecipitation followed by high-throughput sequencing (ChIP–seq) in recent years indicate that many transcription factors can bind to thousands of sites in the genome, most often concentrated in 'hotspots' that are associated with multiple transcription factors[17–19]. These hotspots include gene promoters and enhancers, which are *cis*-regulatory elements located at various distances from the gene(s) they regulate. Genomic regions that are active as enhancers in a given cell type can be identified by their association with specific histone modifications, in particular monomethylation of lysine 4 in histone H3 (H3K4me1), although the precise functional effects of such modifications in terms of enhancer activity are still unknown[20,21]. H3K4me1-associated genomic regions are also variably associated with other characteristics, such as H3K27 acetylation (which correlates with enhancer activity)[22,23], hypersensitivity to DNase enzymes (which indicates an increased accessibility of the underlying DNA)[24] and occupancy by transcriptional co-activators such as the histone acetyltransferase p300 (REF. 20). Most importantly, the repertoire of H3K4me1-associated regions (which includes ~35,000–40,000 genomic sites) is highly cell type specific[21]. This indicates that enhancer formation requires cell type-specific transcription factors with distinct binding specificities, possibly functioning in a combinatorial manner.

Recent ChIP–seq studies that mapped the genomic distribution of PU.1 in macrophages showed that PU.1 is associated with nearly all genomic enhancers marked by H3K4me1 (REFS 25,26). Moreover, re-expression of PU.1 in PU.1-deficient myeloid precursors, or even in fibroblasts, led to the local deposition of H3K4me1 and increased accessibility of the underlying DNA at PU.1-bound genomic regions. Taken together, these data indicate that the role of PU.1 in the maintenance of macrophage identity is not executed simply through the transcriptional activation of a few crucial genes encoding essential macrophage regulators (such as the receptor for macrophage colony-stimulating factor 1). Instead, PU.1 seems to function in a genome-wide manner to control the formation and accessibility of the whole complement of macrophage-specific regulatory genomic regions or, in other words, the entire genomic regulatory landscape of macrophages[27].

In B cells, where the concentration of PU.1 is several-fold lower than in macrophages[16], its genomic distribution is completely different and heavily dependent on cooperative interactions with partner transcription factors that are B cell specific[26]. To what extent PU.1 distribution in macrophages requires (or reflects) cooperative binding is still unknown, but an attractive possibility is that the higher concentration of PU.1 in macrophages compared with B cells might relieve dependence on partner transcription factors and/or select for a different subset of binding sites from those occupied in B cells. In any case, these data indicate that the main activity of PU.1 in maintaining macrophage identity might be the selection, from the large number of regulatory regions scattered in the genome, of the specific subset that forms the macrophage-specific repertoire of enhancers. It is highly likely, although not yet experimentally proven, that such an enhancer repertoire will be largely overlapping in different macrophage subtypes, with differences restricted to a small proportion of the macrophage-specific regions.

PU.1 binding is, in principle, sufficient to promote the deposition of H3K4me1 and to create small (150–300 base pair) open regions of accessible DNA that can be bound by other transcription factors, including those activated by external stimuli, such as nuclear factor-κB (NF-κB). Additional transcription factors probably contribute to the determination or regulation of specific subsets of the regulatory elements selected by PU.1. Such transcription factors include those that are known to be relevant for macrophage differentiation, such as IFN regulatory factor 8 (IRF8)[28], or for functional specialization, such as IRF4 and IRF5 (which are involved in M2 and M1 polarization, respectively) and CCAAT/enhancer-binding protein-β (C/EBPβ)[29–31]. However, it is not clear how the master regulator PU.1 interfaces with these other transcription factors at the genomic level.

In summary, the global regulatory landscape controlled by PU.1 represents the general context in which transcription factors regulated by external stimuli operate to modulate macrophage function[32] (FIG. 2).

Global regulation of inducible gene expression. The global landscape determined by PU.1 provides the general regulatory context in which dynamic gene expression changes occur in response to specific environmental cues. The existence of a global regulatory landscape (that

Chromatin immunoprecipitation (ChIP). A powerful method for assessing the physical association of a known nuclear protein with a candidate target locus *in vivo*. Cells are first treated with an agent that crosslinks protein to DNA. The chromatin is then sheared into fragments and the protein is immunoprecipitated. If the candidate target region is co-precipitated (as measured by PCR), the target locus is likely to bind the protein (directly or indirectly) *in vivo*. The immunoprecipitated DNA can also be used for hybridization to high-density microarrays (ChIP–chip) or for high-throughput sequencing (ChIP–seq) to provide a genome-wide view of the binding sites of a specific transcriptional regulator.

Gene promoters
The regulatory regions to which RNA polymerase binds to initiate transcription. Upstream of the RNA polymerase recruitment site (core promoter) is a regulatory promoter where transcription factors bind to control recruitment of the transcriptional machinery.

Enhancers
Control elements located at variable distances from the genes they regulate and to which multiple regulatory proteins bind, thereby influencing gene transcription. *In vitro*, enhancers function in an orientation- and position-independent manner, but it is not clear whether this is also true *in vivo*.

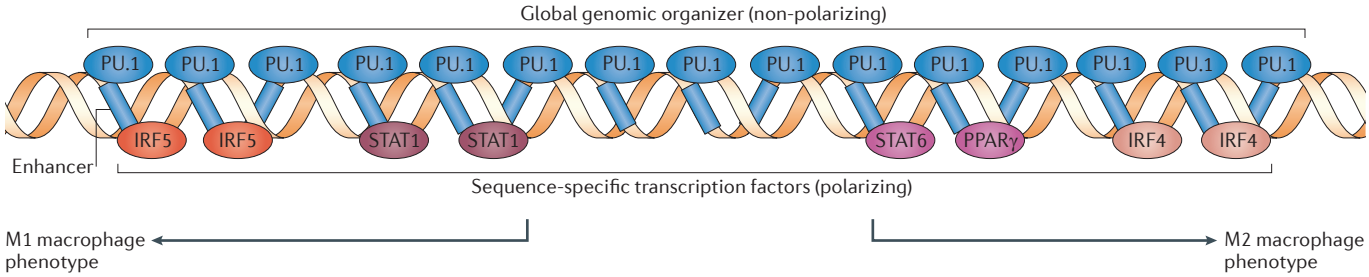

Figure 2 | **Transcriptional control of macrophage identity and polarization.** The scheme depicts the different roles of sequence-specific transcription factors (which determine macrophage polarization) versus non-polarizing transcription factors (which determine macrophage identity through regulation of the global genomic landscape). The M1 macrophage phenotype is controlled by signal transducer and activator of transcription 1 (STAT1) and interferon-regulatory factor 5 (IRF5), whereas STAT6, IRF4 and peroxisome proliferator-activated receptor-γ (PPARγ) regulate M2 macrophage polarization. These factors act on distinct subsets of the available enhancer repertoire. By contrast, the transcription factor PU.1 controls the global macrophage-specific enhancer repertoire, irrespective of polarization.

is, a cell type-specific enhancer repertoire directly controlled by PU.1) is thought to provide the molecular basis for cell type-specific responses to identical stimuli (discussed in REF. 27). Most data in this area have been generated by analyses of the macrophage response to a typical pro-inflammatory stimulus, LPS, which induces classically activated M1 macrophages[6]. However, the general conceptual framework is likely to have broader applicability to other M1-inducing stimuli, such as IFNγ, and also to IL-4 and IL-13 in the case of M2 macrophage polarization. The basic concept is that genes have different activity states and modes of response to stimulation that are predetermined by specific sequence features of their promoters.

The promoters of most primary response genes involved in the initial response to LPS stimulation — such as those encoding tumour necrosis factor (TNF), superoxide dismutase 2 (SOD2) and prostaglandin G/H synthase 2 (PTGS2) — contain CpG islands[33,34], which are regions that are particularly enriched in CpG dinucleotides[35]. CpG islands function as recruitment platforms for constitutively expressed transcription factors such as SP1 (REF. 34) and for proteins that contain the CXXC domain, which directly contacts stretches of unmethylated CpG dinucleotides[36,37]. CXXC domain-containing proteins are components of multimolecular complexes (namely, the SET1 and MLL complexes) that are involved in transcriptional activation and catalyse the deposition of a histone mark associated with active promoters, H3K4me3 (REF. 38). As a result, CpG islands are constitutively associated with RNA polymerase II[34] and have histone modifications indicative of transcriptional activity. In the absence of any stimulation, RNA polymerase II synthesizes long unspliced transcripts starting from CpG island-containing promoters, but it does not produce mature mRNAs[34]. After stimulation with LPS, RNA polymerase II at CpG islands undergoes a specific modification (phosphorylation at serine 2 of its carboxy-terminal domain repeats), and this enables co-transcriptional splicing and therefore productive transcription. A second property of CpG islands is the relative depletion of nucleosomes, which allows unopposed access of the underlying DNA to transcription factors that are activated in response to stimulation[33]. Genome-wide analyses have indicated that CpG islands have a nucleosome-free region that precisely overlaps with the peak of RNA polymerase II occupancy[39]. The model most consistent with these data is that recruitment of RNA polymerase II to a CpG island first causes nucleosome eviction and then creates a barrier to the deposition of new nucleosomes[39]. In addition, the sequence of the CpG island might itself disfavour the deposition of stable nucleosomes[33]. Overall, promoters containing CpG islands can be rapidly activated in response to external stimuli because of the pre-bound RNA polymerase II and the lack of a nucleosomal barrier to the recruitment of transcription factors activated by stimulation (such as NF-κB, AP1 and IRFs).

CpG islands are not present in most secondary response genes, such as those encoding IL-6, nitric oxide synthase 2 (NOS2) and IL-12p40, or in some of the primary response genes (usually those with slower kinetics of activation after LPS stimulation, such as the gene encoding CC-chemokine ligand 5 (CCL5)). These genes have well-assembled nucleosomes at their promoters and require a stimulus-regulated chromatin-remodelling step for the recruitment of some crucial transcription factors (such as NF-κB) and eventually RNA polymerase II[33,34]. Chromatin remodelling is driven in most but not all cases by a dedicated ATP-dependent multimolecular machine — the SWI/SNF complex. Current data support the notion that the SWI/SNF complex is recruited in response to specific transcription factors that have two basic properties: first, they can bind to chromatin that is in a closed configuration (or to sites embedded in nucleosomes); and second, they interact with the SWI/SNF complex, thereby allowing its local recruitment and eventually an increase in the accessibility of the underlying DNA to other transcription factors[33,40]. In LPS-stimulated cells, one such transcription factor that is responsible for chromatin remodelling is IRF3, which is activated by Toll-like receptor 4 (TLR4) signalling through TIR domain-containing adaptor protein inducing IFNβ (TRIF)[33]. A second signal that promotes remodelling might arise directly from the myeloid differentiation primary response protein 88

Primary response genes
Inducible genes whose transcription does not require new protein synthesis.

Secondary response genes
Inducible genes whose transcription requires new protein synthesis (often the synthesis of transcription factors required for the activation of these genes).

SWI/SNF complex
An ATP-dependent chromatin-remodelling protein complex that was initially identified in yeast. Related complexes exist in mammals and are involved in the remodelling of the chromatin of various genes.

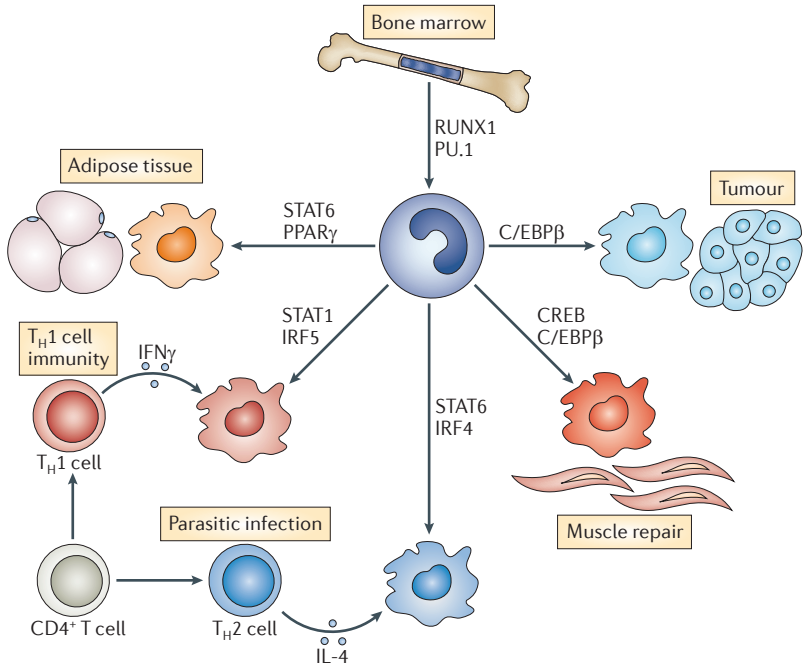

Figure 3 | **Macrophage polarization in physiology and disease.** The figure illustrates different polarized macrophage populations in specific physiological contexts and gives examples of the transcription factors associated with these phenotypes. C/EBPβ, CCAAT/enhancer-binding protein-β; CREB, cAMP-responsive element-binding protein; IFNγ, interferon-γ; IL-4, interleukin-4; IRF, interferon-regulatory factor; PPARγ, peroxisome proliferator-activated receptor-γ; RUNX1, Runt-related transcription factor 1; STAT, signal transducer and activator of transcription; T_H, T helper.

(MYD88)-dependent TLR4 signalling pathway[41]. In the absence of MYD88, some LPS-inducible genes are not activated (despite the lack of substantial signalling defects as a result of compensation through the TRIF pathway), and this correlates with impaired chromatin remodelling and a lack of NF-κB recruitment. However, the identity of the transcription factor(s) controlling remodelling in response to MYD88 is unknown.

Clearly, the lack of a CpG island in a gene promoter generates a constitutive barrier to transcriptional activation that must be overcome by an orchestrated series of events controlled by external stimulation. This has several implications. First, not all stimuli are similarly capable of promoting chromatin remodelling. For example, despite a similar ability to activate pivotal pro-inflammatory transcription factors (such as NF-κB and AP1), TNF and IL-1 receptors and most TLRs are unable to trigger the transcription of a substantial fraction of the pro-inflammatory genes that are activated by TLR4 stimulation[33]. These genes include all direct IRF3 targets (for example, *CCL5* and *IFNB*) and the genes downstream of IFNβ (for example, *NOS2*, *MX1* and *MX2*). This finding can be explained by the lack of IRF3 activation, and thus chromatin remodelling, downstream of these receptors. Therefore, M1 macrophages have a remarkable heterogeneity that reflects the specific stimulus or combination of stimuli that triggered their activation[42]. Second, these additional layers of regulation provide a potential target for pharmacological intervention, as exemplified by the identification of a small anti-inflammatory molecule, iBET, that has some selectivity for slowly activated, secondary response genes in macrophages[43]. iBET prevents interactions between acetylated histones and a specific recognition domain, the bromodomain, that is contained in proteins involved in transcriptional activation. Histone acetylation occurs at both primary response genes containing a CpG island (where it is present in a constitutive manner before stimulation) and at secondary response genes (where it is initially absent and acquired after stimulation). However, histone acetylation might be less relevant for activation in the context of the transcriptionally permissive state of CpG island-containing genes, which might help to explain the selectivity of this drug. Whether the selective targeting of secondary response genes is a limitation or an advantage of this drug is still unclear.

Therefore, specific features of inducible gene promoters, such as their specific combination of transcription factor binding sites and/or the CpG content, impose different requirements for gene activation in response to stimulation[44], thereby providing the possibility of stimulus-specific modulation of macrophage phenotype.

Transcription factors and polarization

By using genome-wide analyses of lineage-specific transcription factors and histone modifications, the recent studies described above have shed new light on the transcriptional mechanisms of macrophage development and activation. The activation of stimulus-specific transcription factors within this macrophage-specific transcriptional landscape is likely to dictate the functional polarization of macrophages through effects on inducible gene promoters with specific features (FIG. 3). In the following sections, we discuss some of the key transcription factors that translate signals in the microenvironment into a polarized macrophage phenotype.

The STAT family. The phenotype that defines classically activated (M1) macrophages is characterized by increased microbicidal activity (including the expression of NOS2); high antigen-presenting activity associated with increased MHC class II expression; and increased production of IL-12 (REF. 2). These characteristics are promoted by IFNγ-mediated Janus kinase–signal transducer and activator of transcription (JAK–STAT) signalling. Stimulation of the IFNγ receptor triggers JAK-mediated tyrosine phosphorylation and subsequent dimerization of STAT1, which binds as a homodimer to *cis* elements known as gamma-activated sequences in the promoters of the genes encoding NOS2, the MHC class II transactivator (CIITA) and IL-12, among others[45]. Studies with macrophages from STAT1-deficient mice clearly show that gene expression induced by both type I IFNs and IFNγ is dependent on STAT1. In addition, STAT1-deficient mice have severe defects in immunity to intracellular bacterial and viral pathogens, which is dependent on the IFN response. LPS-activated macrophages are often classified as M1 macrophages owing to their increased expression of MHC class II molecules, NOS2 and IL-12.

Gamma-activated sequences
DNA binding sites specific for signal transducer and activator of transcription 1 (STAT1) homodimers that mediate the response to interferon-γ.

However, in the LPS response, induction of these genes depends on the autocrine production of IFNβ, which requires TRIF-dependent signalling from TLR4 to IRF3 (REF. 7). In turn, IFNβ signals through the IFNα/β receptor[46]. Although IFNβ also triggers the production of low levels of STAT1 homodimers, the most abundant active STAT complex it induces is the STAT1–STAT2 heterodimer, which recruits IRF9 to form the IFN-stimulated gene factor 3 (ISGF3) complex. ISGF3 binds to distinct *cis* elements known as interferon-stimulated response elements (ISREs), which are also found in M1 signature genes, such as *Nos2*, *Ciita* and *Il12b*. It is clear that STAT1 activity is crucial for M1 macrophage polarization. *In vivo*, this can be driven by IFNγ derived from T cells or innate lymphocytes such as natural killer cells; the crosstalk between lymphocyte-derived IFNγ and macrophages has been shown to be crucial for resistance to infection with intracellular pathogens, including viruses, *Listeria monocytogenes* and *Mycobacterium tuberculosis*[47–49]. The role of type I IFN-mediated signalling and ISGF3 in macrophage polarization *in vivo* is less clear. Indeed, type I IFN can have profound anti-inflammatory activity in certain contexts[50], and IFNα/β receptor signalling in macrophages inhibits resistance to *L. monocytogenes* infection by promoting macrophage apoptosis[51]. This indicates contrasting roles for IFNγ- and IFNα/β-mediated STAT1 activation in macrophage polarization *in vivo* that might diverge at the level of ISGF3 activation.

TAMs are often considered to have an M2-like phenotype. However, although TAMs are generally thought to have low-level expression of M1-associated genes (such as those encoding NOS2, MHC class II molecules and IL-12), they express high levels of IRF3, active STAT1 and ISGF3-regulated genes[10]. IFNγ is an important mediator of tumour immune surveillance, and mice deficient in the IFNγ receptor or STAT1 have increased development of spontaneous tumours after carcinogen exposure[52]. However, the role of STAT1 in TAMs has not yet been addressed. Moreover, although several ISGF3-regulated genes are upregulated in TAMs[9,10], indicating a role for ISGF3 in TAM polarization, the involvement of these genes in TAM phenotype and function has yet to be explored.

The role of IL-4- and IL-13-mediated signalling in M2 macrophage polarization has been well established both *in vitro* and *in vivo*[53]. Mice with a myeloid cell-specific knockout of IL-4 receptor-α (*Il4ra*) were found to lack M2 macrophage development in mouse models of helminth infection and in T_H2 cell-mediated inflammation, where IL-4 has a major role[54,55]. IL-4Rα signals through a JAK–STAT6 pathway[56], and many of the genes associated with mouse M2 macrophages are regulated by STAT6, including arginase 1 (*Arg1*), macrophage mannose receptor 1 (*Mrc1*; also known as *Cd206*), resistin-like-α (*Retnla*; also known as *Fizz1*) and chitinase 3-like 3 (*Chi3l3*; also known as *Ym1*)[53]. There is also a well-established antagonism between STAT6 and STAT1 that has been described for T_H1 and T_H2 cell polarization by IFNγ and IL-4, respectively[57]; a similar mechanism might be important for the exclusivity of M1 and M2 phenotypes in macrophages. Importantly, IL-4 triggers the activation of other signalling proteins, such as phosphoinositide 3-kinase (PI3K), which is also crucial for cell responses to IL-4 (REF. 58). The product of PI3K, phosphatidylinositol-3,4,5-trisphosphate, can be dephosphorylated by the phosphatase SHIP. Thus, as macrophages from *Ship*-knockout mice are prone to M2 polarization[59], PI3K might have an important role in M2 macrophage polarization. The roles of STAT6 and PI3K in inducing the TAM phenotype have not been directly tested; however, there is evidence that IL-4 is an important mediator of TAM polarization in at least some mouse models of cancer[60]. This indicates that the STAT6 and PI3K pathways might also have a role in TAM polarization.

In conclusion, STATs seem to be pivotal factors in M1 and M2 macrophage polarization. STAT1 is an essential mediator of M1 macrophage polarization in the presence of IFNγ, which can be derived from innate lymphocytes or T_H1 cells. By contrast, STAT6 is required to drive M2 macrophage activation during T_H2 cell-mediated immune responses in the presence of IL-4 and/or IL-13. The mutual exclusivity of these signalling pathways might be a crucial factor in M1 versus M2 polarization and a potential tipping point for the modulation of macrophage polarization for therapeutic purposes.

The nuclear receptor PPARγ. M2-like macrophages have also been described in other physiological and pathophysiological contexts besides infection, including wound healing and lipid metabolism, where a role for IL-4 and IL-13 in promoting their differentiation is less clear[61]. Altered macrophage activation in adipose tissue has been linked with decreased insulin sensitivity and diabetes[62]. Adipose tissue macrophages usually have an anti-inflammatory M2-like phenotype, but the inflammatory activation of these macrophages is linked with metabolic dysfunction and disease. Peroxisome proliferator-activated receptor-γ (PPARγ) is a master regulator of lipid metabolism in macrophages and has long been known to inhibit pro-inflammatory gene expression through several mechanisms, including the transrepression of NF-κB[63,64]. More recent studies have shown that PPARγ-deficient macrophages are resistant to M2 polarization and promote insulin resistance[65,66]. It is proposed that obesity and/or inflammatory stress can lead to a switch in the phenotype of adipose tissue macrophages from M2-like to M1, leading to further inflammation and insulin resistance in the absence of PPARγ-mediated pro-inflammatory gene repression. It is not clear exactly how inflammation in adipose tissue leads to insulin resistance and metabolic dysfunction; however, macrophage-derived pro-inflammatory cytokines such as TNF and IL-1β have been shown to be important mediators of obesity-induced insulin resistance.

PPARγ is constitutively expressed by adipose tissue macrophages, but PPARγ expression can also be induced by IL-4 and IL-13 (REF. 67), which indicates that M2 polarization in the context of T_H2 cell responses might also involve PPARγ. Interestingly, a recent study has

Interferon-stimulated response elements (ISREs). Common DNA motifs that are bound by interferon-regulatory factors (IRFs). These elements were initially known as IRF enhancers (IRFEs).

shown a crucial role for STAT6 as a cofactor in PPARγ-mediated gene regulation *in vitro*[68]; therefore, crosstalk between PPARγ and the IL-4–STAT6 axis might coordinately control the M2 phenotype. A second pathway for crosstalk has been described that involves IL-4-mediated stimulation of PPARγ activation through the synthesis of putative endogenous PPARγ ligands[67]. However, the identities of the endogenous ligands for PPARγ are still not clear.

There are no data yet on the role of PPARγ in TAMs. However, it should be noted that synthetic PPARγ ligands have been shown to block the immunosuppressive activity of TAMs *in vitro*[69], again suggesting some discrepancy between the polarization mechanisms of M2 macrophages and TAMs. Ongoing studies on the specific role of PPARγ in TAMs using conditional gene targeting are required to determine the potential contribution of PPARγ to the M2-like TAM phenotype.

The CREB–C/EBP axis. The C/EBP family of bZIP transcription factors has important roles in myeloid development and macrophage activation[31,70]. Both STAT6 and C/EBPβ have recently been shown to be essential for arginase 1 expression in macrophages, but in a stimulus-specific manner owing to the selective use of distinct *cis*-acting elements in the *Arg1* promoter[71]. C/EBPβ was responsible for arginase 1 expression in response to TLR ligands, whereas STAT6 regulated arginase 1 expression in response to IL-4 and/or IL-13. Another recent study showed that C/EBPβ specifically regulates M2-associated genes (including *Arg1*, *Il10* and *Mrc1*) when its expression is induced by another bZIP family transcription factor — cAMP-responsive element-binding protein (CREB)[31]. The requirement for CREB-induced C/EBPβ expression in M2 macrophage polarization was shown by generating mice specifically lacking the CREB binding sites in the *Cebpb* promoter. Although CREB was required for LPS-induced C/EBPβ expression, only M2-associated genes were inhibited in the mutant mice, whereas M1-associated genes (such as *Il12* and *Nos2*) were unaffected. These mice were also defective in muscle repair after injury, which indicates that CREB–C/EBPβ activity is crucial for wound healing, a function that has been attributed to M2-like macrophages[61]. Although CREB-stimulated expression of C/EBPβ seemed not to be required for the expression of pro-inflammatory M1-associated genes, this might reflect functional redundancy between C/EBP family transcription factors[72]. It should be noted that C/EBPβ has previously been shown to be important for pro-inflammatory cytokine expression in macrophages[73], although this might be regulated independently of CREB. Other studies have shown an important anti-inflammatory role for CREB in macrophages in response to LPS, mediated by the p38 mitogen-activated protein kinase (p38; also known as MAPK14), mitogen- and stress-activated kinase 1 (MSK1; also known as RPS6KA5) and MSK2 (also known as RPS6KA4)[74,75]. In this case, CREB-induced expression of IL-10 and dual specificity protein phosphatase 1 (DUSP1) was shown to inhibit the expression of pro-inflammatory genes associated with M1 macrophage activation. These studies indicate that CREB might be a pivotal transcription factor in macrophage polarization by promoting M2-associated genes while repressing M1 activation.

C/EBPβ has also been shown to be required for the development of myeloid-derived suppressor cells (MDSCs) in mice grafted with tumour cells[76]. MDSCs are a heterogeneous population of immature myeloid cells that accumulate mainly in the spleen of tumour-bearing mice[77]. They have profound immunosuppressive activity and are thought to contribute to the evasion of antitumour immunity. Although MDSCs are not macrophages, they might be TAM progenitors, and they have many features of M2 macrophages[77]. The targeted deletion of *Cebpb* in myeloid cells results in T cell-dependent rejection of transplanted tumours[76]. The numbers of MDSCs in the spleens of C/EBPβ-deficient mice are markedly decreased, which indicates that this transcription factor might have a role in MDSC development or survival. The immunosuppressive effector function of MDSCs is attributed, at least in part, to arginase 1 expression (which is also regulated by C/EBPβ in M2 macrophages). Interestingly, both IL-4- and IFNγ-mediated signalling have been implicated in MDSC function, and the co-expression of arginase 1 and NOS2 seems to be required for immunosuppressive activity[78]. This again illustrates the complexity and heterogeneity of myeloid cell phenotype and function in tumours. Although C/EBPβ clearly controls the expansion of myeloid cell (MDSC) populations in tumour-bearing mice, its role in regulating the phenotype of TAMs has not been directly studied; this might be best addressed by the context-specific inhibition of C/EBPβ expression[31].

Interferon regulatory factors. IRFs were originally described as regulators of type I IFN expression and signalling. However, it is now well established that they have additional important functions, including the regulation of macrophage and DC ontogeny[79]. There are nine IRFs in mammals that bind to consensus ISRE sequences overlapping that of ISGF3, but that nevertheless have remarkable specificity in terms of the regulation of gene expression, as shown by the specific phenotypes of mice with different IRF gene knockouts[79]. Recently, IRF4 was shown to specifically regulate M2 macrophage polarization in response to parasites or the fungal cell-wall component chitin[30]. This pathway involves the histone demethylase JMJD3 (also known as KDM6B), which functions by removing an inhibitory histone modification, H3K27me3 (REF. 80). JMJD3 is strongly induced by pro-inflammatory stimuli, although its role in pro-inflammatory gene expression in M1 macrophages is limited to fine-tuning the expression of a few hundred genes (such as *Il12b* and *Ccl5*) in response to LPS[32]. Conversely, the absence of JMJD3 completely blocks the induction of M2 macrophages in mice challenged with helminths or chitin[30], indicating that the role of JMJD3 is greater in M2 than in M1 macrophages. Importantly, although there is some

controversy in this regard[81], M2 macrophage polarization in response to IL-4 seems to be largely independent of JMJD3 (REF. 30). This indicates that the induction of M2 macrophages in response to alternative stimuli follows different routes.

JMJD3 seems to function by controlling expression of the transcription factor IRF4 (REF. 30), which in turn is required for M2 polarization of macrophages *in vitro* and *in vivo*. Although this pathway is strongly supported by genetic data, the molecular and mechanistic details are still not clear. Specifically, it is not known how helminths and chitin signal to JMJD3 and how JMJD3 would be selectively recruited to the *Irf4* gene. However, IL-4 has also been shown to induce IRF4 expression in macrophages[82], which indicates that STAT6- and/or PI3K-mediated IRF4 expression might reinforce the M2 phenotype, although the role of JMJD3 in this context is yet to established.

Another recent study has described a role for IRF5 in the M1 polarization of macrophages[29]. IRF5 has previously been shown to be required for optimal expression of IL-12 and pro-inflammatory cytokines in mice[83]. Granulocyte–macrophage colony-stimulating factor (GM-CSF)-polarized macrophages from human peripheral blood have been used to demonstrate the role of IRF5 in human macrophage polarization[29]. It should be noted that it is not completely clear how these cells fit within the M1/M2 paradigm, which is based on studies in mice. However, GM-CSF-induced human macrophages have an M1-like phenotype, with increased expression of IL-12 and pro-inflammatory cytokines, the ability to activate T_H1 cell immune responses and decreased expression of IL-10 (REF. 29). These characteristics were increased by the overexpression of IRF5 and inhibited by a small interfering RNA (siRNA) targeting endogenous IRF5 expression. IRF5 is directly recruited to gene promoters associated with the M1 phenotype (including *Il12b*), but it represses *Il10*, probably also by binding to an ISRE in the gene promoter. It should be noted that classical M2 macrophage markers in mice (such as resistin-like-α, chitinase 3-like 3 and arginase 1) are not induced in human macrophages even in the presence of IL-4 (REF. 9), so direct comparisons with mouse studies are difficult. However, mouse GM-CSF-induced macrophages have a similar phenotype to those in humans[84], although the authors stopped short of calling them M1 macrophages. An interesting aspect of these studies is the dual function of IRF5 in activating M1 genes while repressing M2 genes by binding to similar *cis*-acting elements in the gene promoters. This indicates that there might be different collaborating transcription factors and/or cofactors for IRF5 at M1 versus M2 gene promoters, and that these factors might eventually direct distinct functional outcomes. Overall, the reciprocal regulation of M1 and M2 genes by the same transcription factors seems to be a common theme in macrophage polarization. This is exemplified by the mutual antagonism of STAT1 and STAT6; the coordinated activation of M2 genes and repression of M1 genes by CREB; and the direct activation and repression of M1 and M2 gene promoters, respectively, by IRF5 (FIG. 4).

The dynamic nature of macrophage polarization

An important aspect of the functional polarization of macrophages is the possibility of a dynamic evolution of the transcriptional output over time. An example of this is the progressive attenuation of pro-inflammatory gene expression in response to long-term exposure to LPS (>16–24 hours), which is known as endotoxin tolerance[12]. Although endotoxin tolerance involves several distinct components, including decreased activity of multiple signalling pathways, one of its most remarkable properties is that it affects different genes in different ways[11]. In general, genes involved in the antimicrobial response, as well as some anti-inflammatory genes (such as *Il10*), are not attenuated: following LPS re-stimulation, they can be induced at levels similar to or higher than those achieved during the primary challenge. Therefore, endotoxin tolerance is a gene-specific response that aims to decrease the risks of excessive inflammation while keeping antimicrobial systems fully active. The tolerization of pro-inflammatory genes requires new protein synthesis, as it probably involves newly synthesized transcriptional repressors. One such factor is ATF3, an ATF/CREB family member that is induced early in the response to LPS and negatively controls the activation of pro-inflammatory genes, such as *Il6* and *Il12* (REF. 85). ATF3 might function through the deposition of repressive histone modifications that enforce the transcriptionally inactive state. However, a global understanding of the evolution of the macrophage gene expression programme during sustained stimulation is still lacking.

Whether a similar phenotypic and functional evolution of macrophages occurs *in vivo* is a controversial issue. In mice, inflammatory macrophages (with an M1 phenotype) are derived from a specific subset of blood monocytes characterized by low-level cell surface expression of CX_3C-chemokine receptor 1 (CX_3CR1; a chemokine receptor that is found on all monocytes) and high-level expression of LY6C and CC-chemokine receptor 2 (CCR2)[2,86]. CCR2 mediates monocyte recruitment from the blood in response to the chemokine CCL2 (also known as MCP1), which is rapidly and transiently expressed by inflamed tissues. In mouse models of inflammation induced by muscle necrosis, $CX_3CR1^{low}LY6C^{+}$ cells are the first to be recruited to the damaged muscle and are then slowly replaced by $CX_3CR1^{hi}LY6C^{-}$ cells that resemble M2 macrophages (in that they express low levels of pro-inflammatory cytokines and high levels of anti-inflammatory molecules that might be involved in promoting repair (such as IL-10 and transforming growth factor-β))[87,88]. In this model of muscle injury and in several others, macrophages are required for the resolution of inflammation and restoration of tissue integrity by removing debris and promoting the proliferation and differentiation of parenchymal cells. Thus, the M1 to M2 switch during the progression of the inflammatory response enables the dual role of macrophages in orchestrating the onset of inflammation and subsequently promoting healing and repair. The controversy refers to the mechanism underlying

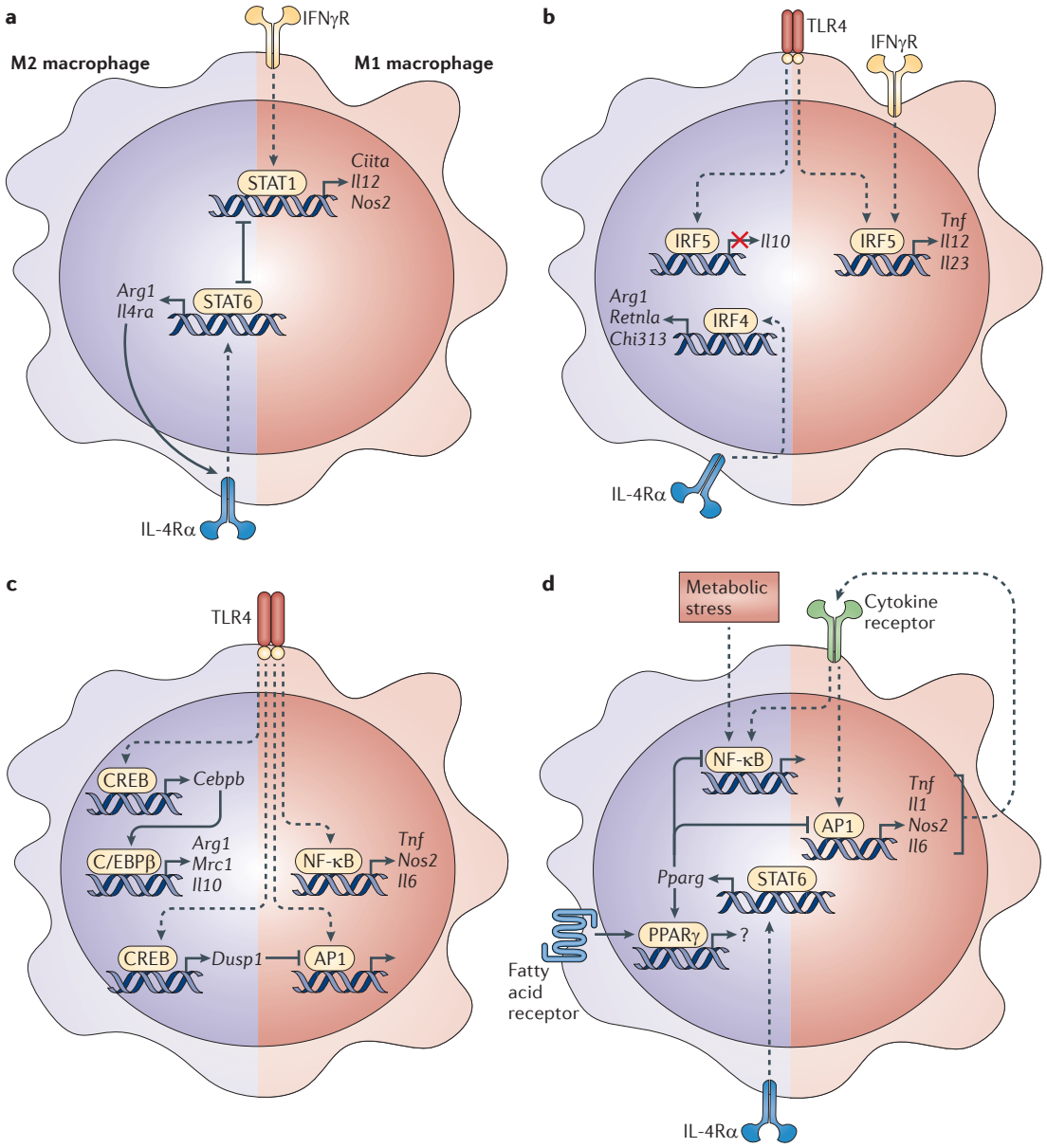

Figure 4 | **Mutual exclusivity and feed-forward signalling maintain macrophage polarization states.** Several different mechanisms for the polarization of macrophage phenotype are illustrated, highlighting the role for feed-forward pathways. These include the induction of interleukin-4 receptor-α (IL-4Rα) expression by signal transducer and activator of transcription 6 (STAT6) (**a**); the increase in interferon-regulatory factor 5 (IRF5) expression mediated by interferon-γ (IFNγ) or granulocyte–macrophage colony-stimulating factor (GM-CSF) and the induction of IRF4 expression by IL-4 (**b**); cAMP-responsive element-binding protein (CREB)-mediated induction of CCAAT/enhancer-binding protein-β (C/EBPβ) (**c**); and STAT6-induced expression of peroxisome proliferator-activated receptor-γ (PPARγ) (**d**). M1 and M2 phenotypes are reinforced by reciprocal regulation of M1 and M2 marker genes by specific transcription factors. Such mechanisms include STAT1-mediated inhibition of STAT6 (**a**); IRF5-mediated IL-10 repression (**b**); CREB-mediated induction of dual specificity protein phosphatase 1 (DUSP1) and subsequent negative regulation of AP1 activation (**c**); and PPARγ-mediated transrepression of nuclear factor-κB (NF-κB) and AP1 (**d**). *Arg1*, arginase 1; *Ciita*, MHC class II transactivator; *Chi3l3*, chitinase 3-like 3 (also known as *Ym1*); *Mrc1*, macrophage mannose receptor 1 (also known as *Cd206*); *Nos2*, nitric oxide synthase 2; *Retnla*, resistin-like-α (also known as *Fizz1*); TLR4, Toll-like receptor 4; *Tnf*, tumour necrosis factor.

this switch. According to one interpretation, the non-inflammatory $CX_3CR1^{hi}LY6C^-$ macrophages in the healing muscle are derived from the original inflammatory macrophage population, which is converted locally to macrophages with an anti-inflammatory profile and the ability to proliferate[88]. Therefore, the evolution of the dominant macrophage population in the damaged tissue would reflect a transcriptional reprogramming of inflammatory macrophages recruited at the onset of inflammation. The alternative interpretation is that the switch in the macrophage population in damaged muscle reflects the sequential recruitment of $CX_3CR1^{low}LY6C^+$

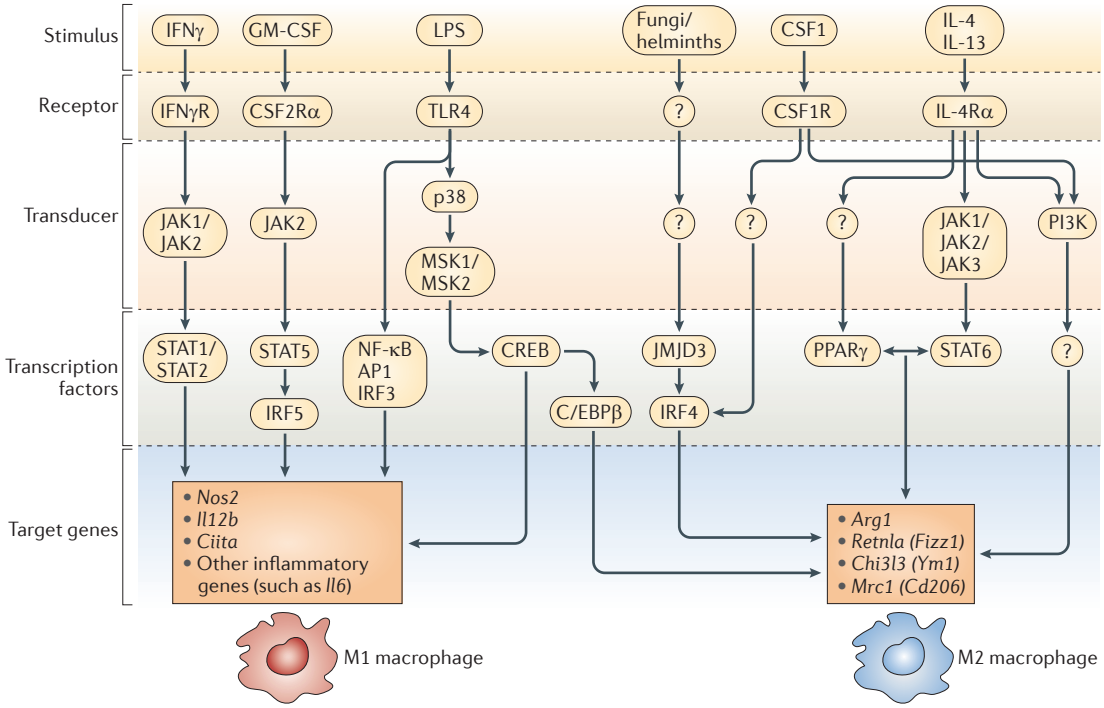

Figure 5 | **Signal transduction pathways to M1 and M2 macrophage polarization.** Signalling pathways implicated in M1 and M2 macrophage polarization are indicated. An initial stimulus leads to the activation of sequence-specific transcription factors that eventually mediate the changes in the transcriptional output. The main genes that are characteristic of either the M1 or the M2 polarized state are also shown. *Arg1*, arginase 1; C/EBPβ, CCAAT/enhancer-binding protein-β; *Chi3l3*, chitinase 3-like 3; *Ciita*, MHC class II transactivator; CREB, cAMP-responsive element-binding protein; CSF, colony-stimulating factor; IFNγ, interferon-γ; IL, interleukin; IRF, interferon-regulatory factor; JAK, Janus kinase; LPS, lipopolysaccharide; *Mrc1*, macrophage mannose receptor 1; MSK, mitogen- and stress-activated kinase; NF-κB, nuclear factor-κB; *Nos2*, nitric oxide synthase 2; PI3K, phosphoinositide 3-kinase; PPARγ, peroxisome proliferator-activated receptor-γ; *Retnla*, resistin-like-α; STAT, signal transducer and activator of transcription; TLR4, Toll-like receptor 4.

and then $CX_3CR1^{hi}LY6C^-$ macrophages from the blood[87]. It is clear that these two interpretations are not mutually exclusive: early recruited macrophages might undergo a reprogramming to $CX_3CR1^{hi}LY6C^-$ M2-type cells similar to those obtained *in vitro* following prolonged exposure to LPS, but the changing microenvironment in the damaged tissue might at later stages promote the preferential recruitment of $CX_3CR1^{hi}LY6C^-$ macrophages from the blood. In any case, addressing the mechanistic bases of these complex population dynamics *in vivo* will be extremely challenging.

Conclusions and future directions

The notion that macrophages phenotypically adapt to the environmental milieu, and that this is associated with radical changes in their transcriptional output, is now strongly rooted in the field and supported by a large amount of data, albeit scattered and not always systematic. At the same time, the molecular determinants that precisely control macrophage plasticity are to a large extent unknown. A major area of research in the future, now made possible by advances in genomic technologies, will be the pairwise definition of the transcriptomes and chromatin landscapes (epigenomes) of macrophages obtained from tissues in both normal and diseased conditions. As macrophages continuously sample the environment and quickly react to it, a major challenge will be the optimization of quick and mild purification procedures that will minimize technical artefacts. Information generated through these approaches will be used to infer pathways and transcription factors that are specifically dedicated to the activation of specialized gene expression programmes with distinct functional outcomes (FIG. 5). The direct therapeutic implications are still difficult to envisage but, in principle, detailed knowledge of the mechanisms that control specific macrophage phenotypes might be used to reprogram their function for therapeutic benefit. For example, reactivating pro-inflammatory genes in TAMs might help to promote tumour rejection. Moreover, reversing the anergic state of LPS-tolerized macrophages in patients recovering from septic shock might help to prevent secondary infections, which still represent a major cause of morbidity and mortality in these patients[12]. In addition to the pro-inflammatory functions of macrophages, the anti-inflammatory properties of M2-like macrophages and their homeostatic functions in wound healing and tissue repair might be harnessed to prevent the tissue injury caused by chronic inflammation. Nevertheless, targeting transcription factors for therapeutic aims is still a largely unexplored and definitely challenging area.

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Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
Toby Lawrence's homepage: http://www.ciml.univ-mrs.fr/science/lab-toby-lawrence/beginners
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