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Exploiting dynamic enhancer landscapes to decode macrophage and microglia phenotypes in health and disease

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Abstract

The development and functional potential of metazoan cells is dependent on combinatorial roles of transcriptional enhancers and promoters. Macrophages provide exceptionally powerful model systems for investigation of mechanisms underlying the activation of cell-specific enhancers that drive transitions in cell fate and cell state. Here, we review recent advances that have expanded appreciation of the diversity of macrophage phenotypes in health and disease, emphasizing studies of liver, adipose tissue and brain macrophages as paradigms for other tissue macrophages and cell types. Studies of normal tissue resident macrophages and macrophages associated with cirrhosis, obese adipose tissue and neurodegenerative disease illustrate the major roles of tissue environment in remodeling enhancer landscapes to specify the development and functions of distinct macrophage phenotypes. We discuss the utility of quantitative analysis of environment-dependent changes in enhancer activity states as an approach to discovery of regulatory transcription factors and upstream signaling pathways.

Abstract

Troutman et al., review recent advances in transcriptomic and genomic profiling technologies that enable discovery of mechanisms underlying the phenotypic diversity and functions of tissue resident and recruited macrophages in health and disease.

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Declaration of Interests

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Introduction

Macrophages are central players in the immune system whose roles in maintaining homeostasis and tissue repair are dependent on their specialized abilities in pattern recognition, phagocytosis, autophagy and secretion of regulatory chemokines and cytokines. Both resident and recruited macrophages protect the host from pathogen infection and can be involved in chronic inflammation unrelated to infection (Okabe and Medzhitov, 2016; Wynn et al., 2013). Ligation of pattern recognition receptors, such as TLR4 by lipopolysaccharide, activate downstream signaling cascades that cause extensive changes in the epigenetic landscape and gene expression (Glass and Natoli, 2016). Until recently, studying the macrophage-specific gene expression programs involved in such activity at the cellular and molecular levels relied on in-vitro model systems based on generation of primary macrophage-like cells from bone marrow progenitor cells, peritoneal exudates or circulating monocytes (Lawrence and Natoli, 2011). While these model systems remain highly valuable for mechanistic studies, they do not capture the full spectrum of macrophage function and diversity in vivo. Recent advances enabling the tracing, isolation and analysis of single cells are catalyzing rapid progress in defining tissue-specific macrophage cell populations in health and disease (Bandura et al., 2009; Becher et al., 2014; David et al., 2016; Giladi and Amit, 2018; Grindberg et al., 2013). These technologies are providing new insights into the developmental trajectories of macrophages, adding nuance to simplified models of how resident and recruited macrophage phenotypes change during inflammatory events, and raising new questions as to how various macrophage populations acquire tissue-specific identities and functions. Parallel advances in high-throughput genomic assays have also enabled the extension of genome-wide studies of transcriptional enhancers and promoters of resident and recruited macrophage populations in tissues (Gosselin et al., 2014; Gosselin et al., 2017; Lavin et al., 2014; Sakai et al., 2019; Seidman et al., 2020; Soucie et al., 2016). These approaches have been used to infer key transcription factors and upstream regulatory pathways responsible for context-dependent gene expression in vivo. The combination of single-cell isolation and sorting approaches with high-throughput epigenetic assays has allowed for an increasingly fine-grained understanding of the mechanisms underlying macrophage and related myeloid cell diversity in health and disease. Here, we review recent advances in the field, emphasizing studies of liver, adipose tissue and brain macrophages as paradigms for other tissues and cell types.

Background

Lineage tracing studies in mice have demonstrated that during early fetal development, hematopoietic progenitor cells (HPCs) from the yolk sac migrate into tissues and acquire tissue-specific macrophage phenotypes, exemplified by brain microglia and skin Langerhans cells (Ginhoux and Guilliams, 2016; Gomez Perdiguero et al., 2015; McGrath et al., 2015; Schulz et al., 2012) (Figure 1). Transition of hematopoiesis from the yolk sac to the fetal liver results in a second wave of HPC-derived cells entering tissues and differentiating into macrophages, largely replacing yolk sac-derived macrophages in tissues such as liver and lung. Following the establishment of definitive hematopoiesis in the bone marrow, hematopoietic stem cell (HSC)-derived monocytes contribute to and in some cases ultimately replace fetal macrophage populations under homeostatic conditions in tissues

such as the heart (Epelman et al., 2014). Pathological conditions, including trauma, infection and a multitude of chronic diseases, alter the phenotypes of resident macrophages and induce the recruitment of monocyte-derived cells that acquire context-specific macrophage phenotypes (e.g., (Bennett and Bennett, 2020; Lee and Olefsky, 2021; Orme and Basaraba, 2014; Wen et al., 2021; Zaidi et al., 2021)).

Microarray and RNA sequencing analyses of major tissue resident populations of macrophages revealed a high degree of diversity of gene expression superimposed on a core program of macrophage gene expression dedicated to phagocytosis and immune functions (Gautier et al., 2012; Gosselin et al., 2014; Hammond et al., 2019; Hickman et al., 2013; Lavin et al., 2014; Mass et al., 2016). Tissue specific patterns of gene expression are generally linked to corresponding tissue specific developmental and homeostatic functions. For example, microglia monitor synapses and are important sources of growth factors for neurons (Bartels et al., 2020; Hammond et al., 2018; Prinz et al., 2019), while Kupffer cells in the liver play roles in iron homeostasis and are involved in detoxifying gut derived LPS (Krenkel and Tacke, 2017). Recent studies of mouse and human samples support the concept that distinct macrophage phenotypes arise as the consequence of instructive roles of the tissue environment acting on an epigenetic landscape determined by cell of origin (i.e., yolk sac/fetal liver-derived HPCs vs. bone marrow derived HSCs)(Gosselin et al., 2014; Gosselin et al., 2017; Lavin et al., 2014) (Figure 1). The recent application of single cell sequencing and high dimensional flow cytometry has significantly expanded appreciation of the diversity of macrophage phenotypes resident in tissues under homeostatic and disease contexts in both human subjects and mouse models (Bassler et al., 2019; David et al., 2016; Giladi and Amit, 2018; Hammond et al., 2019; Jaitin et al., 2019; Li et al., 2019; Masuda et al., 2019), implying corresponding diversity in instructive niches. While substantial progress has been made in identifying environmental signals, receptors and transcription factors that play essential roles in establishing specific phenotypes, recent studies highlight major knowledge gaps for even the most intensively studied macrophage populations, exemplified by brain microglia, liver Kupffer cells and adipose tissue macrophages. An example is provided by the discovery that the pattern recognition receptor Trem2 is upregulated in each of these cell types in many disease contexts and contributes to their disease-associated phenotypes. Many fundamental questions remain as to the molecular mechanisms by which Trem2 and other signaling pathways determine the identities and functions of resident and recruited macrophages in health and disease. Answering these questions will be a pre-requisite to developing rational methods to target macrophages for therapeutic purposes.

Acquisition of macrophage identity and signal-dependent responses

Peritoneal macrophages, bone marrow derived macrophages and monocyte-derived macrophages can be maintained in tissue culture and retain numerous ‘core’ macrophage functions, including phagocytosis and responses to diverse pathogen and host-derived signals, such as bacterial lipopolysaccharide (LPS) and type I interferons, respectively. Accordingly, these systems have been used extensively to investigate transcriptional mechanisms underlying macrophage identity and function (Lawrence and Natoli, 2011). Cell-specific gene expression is controlled by the concerted actions of promoters and transcriptional enhancers (Furlong and Levine, 2018; Heinz et al., 2013; Levine, 2010).

While promoters are the essential sites of transcriptional initiation of mRNAs, they do not provide sufficient information to direct the diverse range of gene expression required for cellular development and cell-specific responses to internal and external signals. This additional specificity is largely provided by distal enhancers, which are the genomic regions that are the major binding sites of lineage-determining transcription factors. Studies of mouse macrophages in vitro provided evidence that the majority of enhancers are selected by relatively simple combinations of lineage determining transcription factors (LDTFs), including PU.1 and members of the C/EBP, AP-1 and RUNX families (Ghisletti et al., 2010; Heinz et al., 2010) (Figure 2A). These factors collaborate with each other and dozens of other transcription factors to bind to closely spaced DNA recognition motifs at tens of thousands of locations throughout the genome (Link et al., 2018). These binding events are associated with the displacement of nucleosomes and establish ‘primed enhancers’ that provide access to broadly expressed signal-dependent transcription factors (SDTFs), exemplified by nuclear receptors and NFκB (Figure 2B). The mechanisms by which the binding of LDTFs results in nucleosome displacement remain poorly understood, but they presumably require the actions of chromatin remodeling factors. Intriguingly, members of the IRF family of transcription factors provide examples of LDTFs, collaborating TFs and SDTFs. For example, high levels of IRF8 expression are required for the developmental decision of myeloid cells to differentiate into monocytes rather than neutrophils (Murakami et al., 2021). IRF1, IRF2, and IRF4 are constitutively expressed in macrophages and IRF motif mutations reduce the nearby binding of PU.1, C/EBP and AP-1 factors, consistent with required collaborative interactions (Link et al., 2018). IRF3 activity is strongly induced following TLR4 ligation (Takeda and Akira, 2004) and IRF transcription binding motifs are strongly enriched in TLR4-activated enhancers (Ghisletti et al., 2010). The recruitment of signal-dependent factors such as nuclear receptors, NFκB and IRF3 to cell-specific enhancers that are first hierarchically primed by distinct combinations of lineage determining factors thereby results in cell-specific responses to a common signal. An important subset of primed enhancers exhibits a ‘poised-repressed’ state due to recruitment of transcriptional co-repressors (Figure 2A). Examples of poised/repressed enhancers are provided by enhancers occupied by RbpJ or unliganded nuclear receptors that recruit the co-repressor NCoR (Li et al., 2013; Sakai et al., 2019). This ‘collaborative-hierarchical’ model of enhancer selection and function provides a conceptual basis for understanding macrophage diversification in vivo, as discussed further below.

Responses of macrophages to LPS provide one of the major paradigms for investigation of signal dependent gene expression. Ligation of TLR4 by LPS initiates signaling through MyD88- and TRIF-dependent pathways that result in the rapid activation of NFκB, IRF and AP-1 transcription factors (Kawai and Akira, 2011). As alluded to above, these factors primarily bind to a pre-existing cell-specific regulatory landscape (Barish et al., 2010; Fonseca et al., 2019; Ghisletti et al., 2010; Kaikkonen et al., 2013), frequently associated with promoters in which RNA polymerase II is already recruited in a ‘paused’ configuration (Adelman et al., 2009; Gilchrist et al., 2010; Gupte et al., 2013). Three-dimensional chromatin conformation capture assays demonstrated pre-existing connectivity between enhancers and promoters required for maximal responses of LPS-responsive genes (Cuartero et al., 2018; Link et al., 2018), reinforcing the concept of a transcriptional network that is

poised for rapid activation. The immediate consequence of transcription factor binding to enhancers and promoters is the secondary recruitment of an intermediary set of co-activators that play key roles in modifying chromatin and mediating recruitment and/or conversion of paused RNA polymerase to an active elongating form. These include chromatin remodeling complexes (SWI/SNF) (Gatchalian et al., 2020; Lai et al., 2009), complexes with histone acetyl transferase activity (e.g., CBP and p300), histone methyltransferases (e.g., MLL proteins (Kaikkonen et al., 2013)), histone demethylases (e.g., JMJD3 (De Santa et al., 2009)), Bromodomain readers of histone acetylation (e.g., Brd4 (Nicodeme et al., 2010)) and kinase activity (e.g., pTEFb) (Hargreaves et al., 2009). The concerted actions of these complexes at enhancers results in dissociation of the negative elongation factor (NELF) complex release of PolII from its promoter-paused position and rapid induction of gene transcription (Adelman et al., 2009). Many of the key drivers of inflammatory responses to TLR4 ligation in macrophages are associated with dense arrays of enhancers (Hah et al., 2015), referred to as super enhancers (Whyte et al., 2013). Recent studies indicate that LPS treatment of macrophages leads to a substantial increase in the genomic binding sites of Brd4. Myeloid-specific deletion of Brd4 results in a dramatic reorganization of the macrophage super enhancer landscape and reduced activation of a subset of TLR4-responsive genes (Dey et al., 2019). Intriguingly, macrophages compensate for Brd4 deletion by establishing new super enhancers that mitigate the overall impact on LPS-dependent gene expression. The writing, reading and erasing of DNA and histone modifications thus represents an important layer of regulation that is potentially amenable to therapeutic modification (Glass and Natoli, 2016; Seeley and Ghosh, 2017).

Niche-specific education of resident macrophage phenotypes

Macrophages reside in all major tissues, yet each subset adopts a unique transcriptional profile reflecting specialized functions adapted to its niche of residence. These observations prompted questions as to whether it was cell ontogeny or niche-specific instructions that were the key determinants of the terminal transcriptional state. The latter model has been reinforced by various observations. For example, entry of macrophage precursor cells into tissues during embryonic development results in rapid and progressive changes in gene expression, ultimately resolving into tissue-specific gene signatures (Mass et al., 2016). Conversely, removal of macrophages from their native tissue environment results in attenuation of these signatures. For example, transfer of microglia and peritoneal macrophages to an in vitro culture environment was associated with rapid loss of a significant component of each cell’s tissue-specific program of gene expression (Gosselin et al., 2014; Gosselin et al., 2017). A comparison of transcriptomes of human microglia immediately following their transfer to tissue culture environments identified ~2400 genes that were strongly downregulated (Gosselin et al., 2017). Although genes corresponding to core macrophage functions were largely preserved, ~1400 of the downregulated genes correspond to microglia signature genes that are upregulated in primitive mouse macrophages following their entry into the brain during fetal development. In an in vivo demonstration of the role of tissue environment, transfer of mouse peritoneal macrophages to the alveolar air spaces resulted in upregulation of alveolar macrophage specific genes (Lavin et al., 2014). Likewise, in a Kupffer cell ablation system the repopulating monocyte-

derived macrophages adopt a highly convergent gene expression signature (Scott et al., 2016), even though Kupffer cells in healthy animals derive from embryonic precursors.

These findings are consistent with a major role for environment-derived signaling in directing tissue-resident macrophage phenotypic changes and further indicate that macrophage gene expression programs are reversible and require constant reinforcement by such tissue-specific signals. Essential environmental factors have been established for some resident populations. For example, GM-CSF is essential for development of alveolar macrophages (Bonfield et al., 2003), retinoic acid for peritoneal macrophages (Okabe and Medzhitov, 2014), and TGFβ for microglia (Butovsky et al., 2014). However, while necessary, these factors are not sufficient to maintain cell specific phenotypes in vitro. For example, treatment of in vitro microglia with TGFβ only partially activates genes that are highly TGFβ-dependent in vivo, indicating that the in vivo functions of TGFβ are dependent on interactions with other environmental signals (Gosselin et al., 2017).

Under homeostatic conditions, each tissue resident macrophage population exhibits an overlapping but distinct pattern of transcription factor expression, which underlies their tissue specific programs of gene expression overall (Gosselin et al., 2014; Lavin et al., 2014; Mass et al., 2016). The myeloid and B cell lineage determining factor PU.1 is universally expressed, consistent with its requirement for all macrophage populations (Fisher and Scott, 1998). Members of the AP-1, C/EBP, MAF, IRF, RUNX and TFE/MITF family are also broadly expressed across tissue resident populations and function in combination with PU.1 to establish expression of a core set of macrophage genes, including *Csf1r* and genes encoding cytokine receptors, complement and complement receptors, pattern-recognition receptors, phagocytic receptors, and Fc gamma receptors (Mass et al., 2016). ChIP sequencing experiments indicate that the genes encoding the majority of these lineage determining transcription factors reside in the vicinity of super enhancers that are occupied by combinations of each other (Gosselin et al., 2014; Link et al., 2018). For example, the PU.1, C/EBPB and cJun super enhancers are each occupied by PU.1, C/EBPβ and cJun. These relationships are likely to establish a set of self-reinforcing transcriptional networks that result in a stable, environment-independent core macrophage phenotype, exemplified by the combinatorial binding of PU.1, C/EBPβ and cJun at the super enhancer associated with the *Spi1* gene, which encodes PU.1 (Figure 3A).

Diversification of transcription factor expression and function is observed shortly after macrophage progenitor cells enter distinct tissue environments during development, which continues concomitantly with tissue differentiation (Mass et al., 2016; Matcovitch-Natan et al., 2016). Examples of transcription factors that are highly expressed in specific macrophage subsets in comparison to other tissue resident macrophages include SALL1 in microglia, LXRα in Kupffer cells, PPARγ in alveolar macrophages and GATA6 in peritoneal macrophages (Lavin et al., 2014; Mass et al., 2016; Matcovitch-Natan et al., 2016). Consistent with this differential pattern of transcription factor expression, the enhancer landscapes exhibit corresponding cell specific differences, with DNA motif enrichment analysis generally recovering the recognition elements for subset-specific factors. For example, GATA motifs are uniquely recovered from the set of peritoneal macrophage-specific enhancers, PPARγ motifs from alveolar macrophage enhancers, and

LXR motifs from Kupffer cell-specific enhancers (Gosselin et al., 2014; Lavin et al., 2014; Sakai et al., 2019).

In contrast to transcription factors establishing core programs of macrophage gene expression, expression of many of the transcription factors driving tissue-specific macrophage phenotypes require constant environmental input as evidenced by loss of expression when cells are transferred to an in vitro environment. This pattern is exemplified by rapid downregulation of *Gata6* in peritoneal macrophages and *Sall1* in microglia following in vitro culture and is accompanied by a corresponding loss of active enhancers dependent on these factors (Gosselin et al., 2014; Gosselin et al., 2017). Within peritoneal macrophages, *Gata6* expression is controlled by environmentally derived retinoic acid (Okabe and Medzhitov, 2014), which acts on retinoic acid receptors to transcriptionally regulate enhancers controlling *Gata6* expression (Gosselin et al., 2014). In microglia, TGFβ signaling regulates the expression of SMAD proteins that are putative drivers of TGFβ-dependent expression of *Sall1* (Butovsky et al., 2014). These observations suggest a model in which environmental factors regulate the activities of signal dependent TFs to initially induce specific macrophage lineage determining factors, which they subsequently interact with to drive the selection of new enhancers that specify context-specific gene expression.

The loss of tissue-specific macrophage gene expression patterns in vitro represents a significant limitation of in vitro systems to model homeostatic and disease phenotypes. This limitation also applies to macrophages derived from induced pluripotent stem cells (iPSCs) in vitro. For example, generation of microglia-like cells from iPSCs in vitro (Abud et al., 2017; Muffat et al., 2016) resemble primary microglia that have been transferred to a tissue culture environment (Gosselin et al., 2017; Hasselmann et al., 2019). Efforts to restore primary microglia phenotypes in vitro or induce a more in vivo-like phenotype of iPSC-derived microglia in vitro by addition of putative environmental signals, co-culturing with neurons and astrocytes, or incorporating into neuron/astrocyte organoids result in limited transition towards the gene expression pattern observed in microglia in vivo. An important recent advance is the development of systems in which human iPSC-derived HPCs are engrafted into brains of immunodeficient newborn mice in which the mouse gene encoding CSF-1 is replaced by the human CSF-1 gene. The engrafted HPCs differentiate into microglia that morphologically and transcriptionally resemble ex vivo human microglia (Hasselmann et al., 2019; Mancuso et al., 2019; Svoboda et al., 2019), providing a powerful experimental system for investigation of environment-dependent gene expression and human microglia differentiation going forward.

Contribution of cell-ontogeny to macrophage specification

As tissue resident macrophages are derived from progenitor cells distinct from those that give rise to parenchymal cells, a major question is the extent to which ultimate macrophage phenotypes are dependent on their developmental origins. One experimental approach is to deplete the endogenous population of macrophages, creating an empty niche that leads to replacement by circulating HSC-derived cells. This approach has been extensively applied to mouse brain microglia, in which a variety of methods, including diphtheria toxin ablation and CSF1 receptor inhibition, have been used to deplete the resident HPC-derived microglia

populations and characterize the molecular phenotypes of HSC-derived cells that replace them (Bennett et al., 2018; Cronk et al., 2018; Shemer et al., 2018; Xu et al., 2020). In all cases examined, graft-derived cells acquire microglia characteristics, including ramified morphology and expression of numerous microglia-specific genes. However, even after prolonged residence in the CNS, engrafted cells derived from HSCs exhibit hundreds of transcripts that are significantly over or under-expressed in comparison to yolk-sac derived macrophages, indicating a requirement for embryonic origin to acquire a microglia specific transcriptomic signature. Consistent with this, the open chromatin environment as assessed by transposase accessible chromatin (ATAC-seq) exhibits substantial differences (Shemer et al., 2018). Evidence that this distinct regulatory landscape is a determinant of functional potential came with the observation that HSC-derived macrophages engrafted into the brain exhibited an overlapping but distinct response to LPS in comparison to embryonically derived microglia within the identical brain environment (Shemer et al., 2018).

The basis for embryonic origin as a requirement for microglia development remains to be established. A clue is provided by the profound difference in expression of *Sall1*, a transcription factor that is highly expressed in microglia, but not other tissue resident macrophages (Buttgereit et al., 2016; Lavin et al., 2014; Mass et al., 2016). *Sall1* mRNA levels rapidly fall when microglia are transferred to a tissue culture environment (Gosselin et al., 2014; Gosselin et al., 2017), indicating that its expression requires constant brain environmental signals. Conditional deletion of Sall1 results in reduced expression of many microglia-specific genes and upregulation of genes associated with microglia activation, leading to an inflammatory phagocytic phenotype (Buttgereit et al., 2016). Remarkably, *Sall1* is not expressed in HSC-derived cells that engraft the brain after microglia depletion, even after long residence times (Bennett et al., 2018; Cronk et al., 2018; Shemer et al., 2018). Intriguingly, iPSC-derived hematopoietic progenitor cells, which mimic yolk sac derived HPCs, do not express *SALL1* during in vitro microglia differentiation, but upregulate *SALL1* during the process of microglia differentiation that occurs following engraftment into the mouse brain (Hasselmann et al., 2019; Mancuso et al., 2019; Svoboda et al., 2019). Thus, embryonic origin is a prerequisite to *Sall1* expression and microglia identity. The mechanisms enabling *Sall1* to be expressed in yolk sac-derived microglia progenitor cells, but not HSC-derived cells, are unknown. One possible explanation is differential DNA methylation of the Sall1 promoter and/or enhancer elements in the progenitor cell populations, but this remains to be examined.

In the liver, it is possible to rapidly and quantitatively deplete Kupffer cells by selective expression of the diphtheria toxin receptor and administering a single dose of diphtheria toxin (Scott et al., 2016). In contrast to microglia, rapid depletion of embryonically derived Kupffer cells results in repopulation of the empty niche by HSC-derived cells that achieve near complete convergence with the original Kupffer cell pattern of gene expression following two weeks of engraftment (Sakai et al., 2019; Scott et al., 2016). This method thereby provides a powerful model system for investigation of the mechanisms by which macrophage progenitor cells acquire tissue specific macrophage identities. In one study, Kupffer cell loss was found to induce tumor necrosis factor (TNF)- and interleukin-1 (IL-1) receptor-dependent activation of stellate cells and endothelial cells, resulting in the transient production of chemokines and adhesion molecules orchestrating monocyte engraftment

(Bonnardel et al., 2019). Engrafted cells were found to rapidly upregulate Kupffer cell lineage determining factors including LXRα and Id3. Using NicheNet (Browaeys et al., 2020), a computational method evaluating expression of potential ligand/receptor pairs, evidence was obtained supporting a model in which hepatocytes induce Id3 expression and sinusoidal endothelial cells and stellate cells activate LXRα via expression of the Notch ligand DLL4 and BMPs (Bonnardel et al., 2019).

In a complementary series of studies, Kupffer cells were rapidly depleted using an analogous diphtheria toxin model (Sakai et al., 2019) and temporal changes in gene expression in the newly engrafted cells were correlated with quantitative changes in open chromatin as determined by ATAC-seq (Buenrostro et al., 2015) and enhancer activity as assessed by ChIP-seq for histone H3K27 acetylation (Creyghton et al., 2010). Here, the strategy was to use dynamic changes in enhancer landscapes as the basis for inferring transcription factors and upstream regulatory factors responsible for driving Kupffer cell differentiation. The central assumption to this approach is that each enhancer has the potential to serve as a cell-specific signal integration hub based on the combinations of lineage-determining and signal-dependent transcription factor binding sites it contains. Motifs enriched in newly selected enhancers or in enhancers transitioning from a poised to active state would represent binding sites for the responsible transcription factors and suggest possible upstream signaling pathways (Figure 4). Following Kupffer cell depletion, monocytes adhering to the sinusoidal endothelium exhibited rapid changes in histone acetylation at poised enhancers in the vicinity of Kupffer cell lineage determining factors, including LXRα (Sakai et al., 2019). In addition to binding sites for general macrophage lineage-determining factors, these enhancers were enriched for DNA recognition motifs for RBPJ and SMADs, which are downstream of Notch and TGF/BMP signaling pathways, respectively. Consistent with this, the Notch ligand DLL4 and TGFβ were found to be expressed by sinusoidal endothelial cells and to be capable of inducing LXRα expression in monocytes in vitro. The initial induction of Kupffer cell lineage determining factors via activation of a poised enhancer landscape in recruited monocytes was followed by generation of new regions of open chromatin that gained histone acetylation in the vicinity of Kupffer cell identity genes, such as *Clec4f* (Figure 3B). These enhancers had enrichment for recognition motifs of general macrophage lineage determining factors and the RBPJ and SMAD motifs, but in addition also had motifs for Kupffer cell lineage determining factors, including LXR and MAF family members. Motifs for NFκB were also highly enriched, suggesting activation of TLRs by gut derived endotoxins. Kupffer cell deletion of LXRα resulted in loss of a significant fraction of the Kupffer cell specific gene expression as well as corresponding loss of a subset of Kupffer cell-specific regions of open chromatin and histone H3K27ac, consistent with it functioning as a lineage-determining factor that drives enhancer selection in collaboration with core macrophage lineage determining transcription factors, such as PU.1. In concert, these findings provided evidence that the potential of circulating monocytes to acquire the features of resident macrophage populations is encoded in a pre-existing poised enhancer landscape that is capable of being activated by tissue-specific signals (Sakai et al., 2019).

Impact of disease on resident and recruited macrophages

While the diverse functions of resident and recruited macrophages normally play adaptive roles in development, immunity and tissue homeostasis, many of these same functions exert pathogenic effects in a broad spectrum of human diseases (Tabas and Glass, 2013; Wynn et al., 2013). Examples include roles of macrophages in development and clinical complications of atherosclerosis, obesity, nonalcoholic steatohepatitis and Alzheimer's disease. Each of these diseases exhibit both overlapping and distinct features of chronic inflammation, and varying combinations of immune cells that include tissue resident and recruited macrophages. In contrast to acute injury or infection, in which functions of macrophages and other immune cell types contribute to resolution and restoration of tissue homeostasis, many chronic diseases are associated with continuous generation of signals that provoke persistent immune cell inflammatory responses. For example, the development of atherosclerosis is driven by oxidatively modified lipoproteins within the artery wall that are ligands for macrophages and result in foam cell formation (Que et al., 2018). Haploinsufficiency in *Csf1*, the major ligand for the Csf1 receptor outside of the brain, results in near complete resistance to atherosclerosis in mouse models (Rajavashisth et al., 1998; Smith et al., 1995), establishing an essential role for Csf-1-dependent macrophages. Excess nutrient intake resulting in pathologic enlargement and death of adipocytes promotes macrophage accumulation in adipose tissue and the development of insulin resistance (Lee and Olefsky, 2021). Corresponding excess nutrient intake resulting in accumulation of triglyceride in the liver leads to recruitment of monocyte-derived macrophages and initiation of nonalcoholic steatohepatitis (NASH) (Ju and Tacke, 2016). Genetic or pharmacologic reduction of monocyte-derived macrophage accumulation in adipose tissue and liver ameliorates diet induced disease (Huh et al., 2018; Mulder et al., 2017; Parker et al., 2018; Sullivan et al., 2013; Weisberg et al., 2006). Plaques of beta amyloid and tangles of hyperphosphorylated forms of tau in the brain are drivers of altered microglia phenotypes Alzheimer's disease (Wang and Colonna, 2019). In contrast to the examples provided by atherosclerosis, insulin resistance and NASH, AD does not appear to be associated with recruitment of monocyte-derived cells. Genetic evidence (Bellenguez et al., 2020), as described in further detail below, suggests that microglia play essential roles in neuronal dysfunction and ultimate loss in AD.

These examples, as well as many others not discussed, linked tissue macrophages as active participants in many diseases, prompting heightened research directed towards understanding their mechanistic involvement. The concept that macrophages could be functionally classified into dichotomous phenotypes referred to as M1 (classically activated) or M2 (alternatively activated) was initially proposed more than 20 years ago based on differential LPS responses of macrophages derived from mice exhibiting Th1 or Th2 lymphocyte biases (Mills et al., 2000). Macrophages from prototypical Th1 strains (C57BL/6, B10D2) were more easily activated to produce NO with either IFN-gamma or LPS than macrophages from Th2 strains (BALB/c, DBA/2). In contrast, LPS stimulated macrophages from Th2, but not Th1 strains, to increase arginine metabolism to ornithine. This paradigm was subsequently expanded and broadly applied to contrast macrophage phenotypes associated with chronic inflammatory disease (primarily M1) from those

associated with tissue repair and tumor associated macrophages (M2). However, the progressive discovery of signaling molecules recognized by macrophages, the development of improved methods for tracing and isolation of immune cells from tissues and advances in genome-wide transcriptome analysis has generally revealed only limited resemblance of disease associated macrophages to classical M1 or M2 phenotypes (Martinez and Gordon, 2014; Nahrendorf and Swirski, 2016).

DAMs, LAMs and SAMs

The appreciation of myeloid cell heterogeneity under homeostatic and disease conditions has been significantly expanded by high dimensional flow cytometry, and more recently mass cytometry and single cell RNA sequencing (scRNA-seq) methods, which enabled recognition of disease associated macrophage phenotypes that were not readily apparent from RNA-sequencing of bulk populations. Examples include studies of microglia diversity in the brain (Keren-Shaul et al., 2017; Masuda et al., 2019), and myeloid cell diversity in liver (Ramachandran et al., 2019; Seidman et al., 2020), lung (Sajti et al., 2020), and various tumor contexts (Pombo Antunes et al., 2021; Zilionis et al., 2019). Here, we consider disease-associated microglia in the brain, lipid-associated macrophages in adipose tissue and scar-associated macrophages in the liver as examples that illustrate similarities and differences in disease-associated macrophage phenotypes and highlight important knowledge gaps.

Disease associated microglia (DAMs) were identified by comparing scRNA-seq transcriptomes of immune cells in the brains of wild type mice or in a mouse model of Alzheimer's disease characterized by high levels of β amyloid (Keren-Shaul et al., 2017). An analogous microglia phenotype, termed MgND, was discovered through comparative analysis of mouse models of amyotrophic lateral sclerosis, multiple sclerosis and AD (Krasemann et al., 2017). DAMs represented a small fraction of microglia derived from the β amyloid model and exhibited a distinct pattern of gene expression, characterized by increased expression of ~400 genes, including *Trem2*. Histologically, DAMs were localized to regions of amyloid deposition and were of particular interest because loss of function mutations in *TREM2* had previously been genetically linked to increased risk of non-familial forms of AD in humans (Guerreiro et al., 2013; Jonsson et al., 2013). Further, knockout of *Trem2* in the mouse resulted in failure of microglia to cluster around β amyloid plaques and an increase in the spread of amyloid deposits (Jay et al., 2017; Wang et al., 2015). Isolation of microglia from *Trem2* KO mice in the context of the amyloid model suggested that transition of homeostatic microglia to DAM microglia could be separated into an initial *Trem2* independent phase and a subsequent *Trem2*-dependent phase (Keren-Shaul et al., 2017). Based on the increased risk for AD associated with loss of function mutation of *Trem2* and the spread of amyloid plaques in brains of mice and humans with loss of function mutations, the DAM phenotype is considered to exert overall protective functions in AD. However, evaluation of *Trem2* deficiency in the context of the PS19 mouse model of tauopathy yielded a strikingly different result. Crossing *Trem2* KO mice to PS19 mice resulted in significantly reduced microgliosis in brain regions affected by tauopathy, including the hippocampus (Leyns et al., 2017). In addition, gene expression analyses and immunostaining revealed microglial activation was significantly attenuated in *Trem2* KO/

PS19 mice compared to PS19 mice, along with lower levels of inflammatory cytokines and astrogliosis. These findings suggest that the DAM phenotype associated with tauopathy is different than that associated with amyloid, and/or that the consequences of Trem2 signaling are context dependent, being protective in the case of excessive β amyloid and pathogenic in the case of tauopathy. A potential resolution to this seemingly paradoxical result is a recent finding that Trem2 remains protective when both amyloid and tau pathology are present (Lee et al., 2021).

An important discovery linking macrophage accumulation to insulin resistance in the setting of obesity was the finding of increased macrophage gene expression in intact adipose tissue of obese mice in comparison to lean mice as determined by microarray analysis (Weisberg et al., 2003; Xu et al., 2003). This finding led to documentation of monocyte-derived macrophage recruitment into obese adipose tissue and their direct contribution to insulin resistance of adipocytes. A characteristic feature of obese adipose tissue associated with insulin resistance is the presence of ‘crown-like structures’ consisting of monocyte-derived macrophages and other immune cells that surround apoptotic/necrotic adipocytes (Geng et al., 2021). Recent systematic profiling of immune cells in lean and obese adipose tissue by scRNA-seq analysis led to the findings that macrophages within crown like structures expressed high levels of *Trem2* and exhibited numerous cytoplasmic lipid droplets (Jaitin et al., 2019). These so-called lipid associated macrophages (LAMs) depended on Trem2 for localization to necrotic/apoptotic adipocytes. Remarkably, *Trem2* knockout mice exhibited even larger adipocytes than normal and worsened glucose and lipid homeostasis following an obesity inducing diet than wild type mice. Based on these findings, the LAM phenotype is interpreted as protective in the context of obese adipose tissue.

A third example of scRNA-seq leading to identification of a previously unappreciated macrophage disease phenotype resulted from analysis of non-parenchymal cells of the liver in individuals with advanced cirrhosis in comparison to controls (Ramachandran et al., 2019). Cirrhosis was associated with significant changes in gene expression in myeloid cells, endothelial cells and stellate cells. In contrast to mouse models of brain amyloid and obesity, in which disease-associated phenotypes were absent in control animals, the overall population structures of myeloid cells were similar in cirrhotic and control liver samples, but relative proportions exhibited substantial differences. The most significantly altered populations exhibited high levels of expression of *TREM2* and *CD9* and were found to reside on pathological collagen fibers that are characteristic of cirrhosis. These so-called scar-associated macrophages (SAMs) were proposed to be profibrotic based on the ability of SAM conditioned media to promote collagen gene expression in stellate cells in vitro. *Trem2/Cd9* positive myeloid cells were also identified in mouse models of NASH driven by high fat feeding (Daemen et al., 2021; Hou et al., 2021; Remmerie et al., 2020; Seidman et al., 2020; Xiong et al., 2019). In these contexts, loss of *Trem2* was associated with greater body weight and adipose tissue mass, in agreement with studies of LAMs, and increased hepatic triglyceride content (Hou et al., 2021). Whether the Trem2/Cd9 high cells in these mouse models are more closely related to LAMs or SAMs will require further investigation. Molecular and histological analysis suggested that Trem2 signaling in Kupffer cells played a protective role in maintaining mitochondrial function in hepatocytes. Loss of Trem2 has also recently been reported to be associated with compromised recovery from liver

damage (Coelho et al., 2020). Thus, as in the case of DAMs in the brain, Trem2 positive disease-associated macrophages in the liver may play both protective and pathogenic roles dependent on species and/or disease context.

Collectively, the studies of DAMs, LAMs and SAMs provide evidence that Trem2 plays a significant role in sensing disease phenotypes and in driving phenotypic consequences. In each case, the phenotypes of DAMs, LAMs and SAMs bear only limited relationships to classically defined M1 or M2 macrophages. In addition, these do not represent the only disease associated macrophage or microglia phenotypes present in each context. For example, a disease-associated population of microglia distinct from DAMs has been identified that is characterized by lipid accumulation, defective phagocytosis and production of high levels of reactive oxygen species (Marschallinger et al., 2020). As noted above, studies of WT vs *Trem2* KO DAMs in the amyloid model provide evidence for Trem2-dependent and Trem2-independent phases, indicating important contributions of additional sensing and signaling pathways. For example, recent studies indicate that microglia use TAM receptors, which signal through tyrosine kinase activity, to recognize and engulf amyloid (Huang et al., 2021). Of interest, DAMs, LAMs and SAMs are associated with pathogenic features that can be at scales that are much larger than the adjacent microglia or macrophage. Amyloid plaques in the brain, apoptotic adipocytes in adipose tissue and collagen scars in the liver, all exceed the size of substrates amenable to uptake by phagocytosis. This association raises the question of whether an aspect of the Trem2-dependent or Trem2-independent phenotypes of these cells include activation of extracellular degradation mechanisms.

Despite these similarities, each type of disease associated macrophage resides in a distinct environment. Further, DAMs presumably arise exclusively from embryonically derived microglia, whereas LAMs are proposed to be derived from infiltrating monocytes, and SAMs from both resident and recruited macrophages. In concert with these differences, the genes that distinguish DAMs, LAMs and SAMs from the major homeostatic populations of brain, adipose tissue and liver macrophages are largely non-overlapping, despite being at least partially driven by Trem2 signaling. This can potentially be explained at least in part by the divergent regulatory landscapes that Trem2 signaling acts upon within each cell type, analogous to the differential effects of LPS on gene expression of endogenous microglia and engrafted brain macrophages derived from HPCs as discussed above (Shemer et al., 2018). The identification of DAMs, LAMs and SAMs in neurodegenerative disease, obesity and cirrhosis, respectively, suggests that analogous but similarly distinct populations of macrophages will be identified in other disease contexts.

Three major knowledge gaps emerge with respect to Trem2 and disease associated macrophages; 1) What are the mechanisms that induce Trem2 expression? 2) What are the ligands that induce Trem2 signaling? and 3) What are the signaling pathways and transcription factors that drive the downstream responses? (Figure 5A) The topic of Trem2 ligands has been addressed by a recent review (Kober and Brett, 2017). In brief, Trem2 is a pattern recognition receptor that signals primarily through interaction with Dap12 (Tyropb) to activate Syk and PI3K (Deczkowska et al., 2020). A large number of ligands have been proposed, including a variety of lipid species, ApoE, amyloid, and proteoglycans.

Deciphering the most relevant ligands in pathological tissue environments remains a major challenge. Here, we will consider recent findings based on epigenetic analyses that suggest mechanisms leading to *Trem2* expression and downstream transcriptional consequences.

Epigenetic changes associated with a SAM-like phenotype

The observation that Trem2 deficiency leads to a partial acquisition of the DAM phenotype in 5XFAD mouse model of AD indicates a Trem2-independent response of microglia to the accumulation of amyloid (Keren-Shaul et al., 2017). Time course studies were not performed in this model and it is therefore not clear when detection of an altered brain environment occurs. In the case of high fat feeding, significant accumulation of LAMs (defined by Cd9 staining), was not observed until significant obesity had been achieved (greater than 6 weeks) (Jaitin et al., 2019). In a dietary mouse model of NASH, it was possible to trace both the changes in gene expression in resident Kupffer cells and in monocyte-derived cells that localized to the sinusoidal niche (Seidman et al., 2020). These cells acquired a convergent pattern of gene expression that included upregulation of *Trem2*, *Cd9* and other markers of both LAM and SAM phenotypes. Although significant changes in gene expression were observed between 1 and 4 weeks of the NASH-inducing diet, upregulation of Trem2 did not occur in the resident Kupffer cell population until after 10 weeks, ultimately reaching levels of expression >10-fold higher than in resident Kupffer cells in the healthy liver. Collectively, these results from the obesity and NASH models suggest that the upregulation of *Trem2* is driven by responses to emergent tissue pathology (i.e., adipocyte necrosis/apoptosis and liver inflammation, respectively) rather than the pathogenic diets themselves.

Direct comparison of the epigenetic landscapes of resident Kupffer cells of the healthy and NASH livers revealed that more than 4000 regions of open chromatin showed >2-fold increases in H3K27ac, while ~3400 regions exhibited >2-fold decreases (Figure 5B). As expected, putative enhancers exhibiting gained H3K27ac were associated with increases in gene expression associated with the SAM phenotype, whereas enhancers exhibiting loss of H3K27ac primarily occurred at Kupffer cell-specific enhancers and were associated with an attenuation of Kupffer cell identity. An example is provided by the Trem2 gene, which exhibits an open promoter and a putative poised enhancer within intron 1 in homeostatic Kupffer cells (Figure 5C). Following 20 weeks of the NASH-inducing diet, both of these regions exhibit significant increases in H3K27ac, consistent with gene activation. In addition, a region downstream of Trem2 exhibits a NASH-diet specific ATAC-seq peak and H3K27ac, consistent with a NASH-specific enhancer. Of interest, a similar organization of regulatory elements is observed in mouse and human microglia.

Motif enrichment analysis of the ~4000 Kupffer cell genomic regions gaining H3K27ac in the context of the NASH diet yielded highly significant enrichment for ATF3, NFAT and Egr2 motifs, among others (Figure 5B). The NFAT motif is of interest because NFAT transcription factors are regulated by calcium signaling and have been suggested to be downstream of Trem2 based on the use of an NFAT reporter assay (Wang et al., 2015). ATF3 and Egr2 are of interest because their expression in response to the NASH diet begins to increase prior to upregulation of Trem2, suggesting possible roles in its induction. *Atf3* is

one of the most highly induced transcription factors by the NASH diet and ChIP-sequencing experiments suggest it plays a major role in driving the selection and activation of NASH enhancers, in part by repurposing LXRα from a Kupffer cell lineage determining factor to an activator of a subset of genes associated with the SAM phenotype. Notably, both LXR and ATF3 binding are highly induced at Trem2 regulatory elements (Figure 5C). In concert, alterations in the enhancer landscapes of control and NASH Kupffer cells provide evidence that ATF3 and Egr2 function upstream and/or downstream of Trem2 in driving disease associated macrophage phenotypes (Seidman et al., 2020).

Summary and future directions

Advances in single cell sequencing technologies have revealed striking heterogeneity of macrophages in healthy and diseased tissues. The recent ability to quantitatively image hundreds to thousands of specific RNAs at cellular resolution in tissue samples heralds the coming age of spatial transcriptomics, in which gene expression patterns of contiguous cells can be examined in depth (Eng et al., 2019; Moffitt et al., 2018). Although these studies will enable unprecedented analysis of intercellular communication, the extent to which these methods will reveal as yet unappreciated cellular phenotypes remain to be discovered. Regardless, major questions for future studies include defining the functions of specific macrophage subsets, determining the mechanisms responsible for their diversification, and establishing whether these mechanisms provide potential sites of intervention in disease. As elaborated above, recent studies support a model in which hematopoietic progenitor cells and circulating monocytes have the potential to differentiate into tissue distinct macrophage subtypes based on combinations of environment specific signals that act on poised enhancer landscapes. In this model, such signals lead to rapid activation of poised enhancers that directly activate subset-specific lineage determining transcription factors, exemplified by LXRα in Kupffer cells. These factors in turn collaborate with general macrophage lineage-determining transcription factors resulting in the selection of new enhancers that permit further sensing of environmental signals and drive context-specific patterns of gene expression associated with cell fate transitions. Experimental strategies that are based on depletion of resident macrophages followed by repopulation with hematopoietic-derived precursors provide powerful strategies for defining dynamic enhancer landscapes and inferring corresponding transcription factors. A corresponding strategy can also enable inference of transcription factors driving disease associated macrophage phenotypes, representing potentially reversible alterations in cell ‘state’.

In parallel with advances in single cell sequencing, substantial progress is being made in single cell analysis of open chromatin using scATAC-seq (Granja et al., 2021). This methodology has been demonstrated to be a powerful approach for identifying the open chromatin landscapes of distinct cell populations within complex tissues. Motif enrichment analysis of open chromatin within clustered cells enables inference of key lineage determining factors. Further, correlation analysis methods enable in silico estimations of likely enhancer promoter interactions. This methodology thus has substantial promise for advancing mechanistic understanding of myeloid cell heterogeneity. Importantly, recent application of scATAC-seq to human brain samples enabled clustering of brain cell types based on patterns of open chromatin (Corces et al., 2020). Cell-specific patterns of open

chromatin could be assembled from clustered cells, revealing enrichment of AD risk variants in putative microglia-specific regulatory elements. Limitations of scATAC at present include data sparsity and the inability to distinguish between poised and active regulatory landscapes. As illustrated by Kupffer cells in the healthy vs NASH liver, changes in activity states were more numerous than in open chromatin, providing greater power for motif enrichment analysis (Seidman et al., 2020).

The computational advantages of simultaneous measurement of open chromatin and activity as measured by ATAC-seq and ChIP-seq of bulk cell populations are opposed by technical requirements for isolation of a sufficient number of the particular cell type of interest. Improvements in the sensitivities of assays that provide surrogates of enhancer activity, e.g., ‘Cut and run’ assays of histone modifications (Meers et al., 2019) and KAS-seq assays (Wu et al., 2020) of single stranded DNA associated with enhancer and promoter transcription bubbles, suggest that enhancer activity states can be effectively quantified using on the order of 10,000 cells. Cell sorting informed by population-specific markers revealed by scRNA-seq analysis provides the usual starting point for isolation of specific cell types of interest. In addition, recent studies document the power of sorting nuclei according to cell of origin as another starting point for epigenetic analysis (Fullard et al., 2017; Mo et al., 2015). This can be accomplished in a highly cell-specific manner in the mouse through the development of lineage tracing reporters that are localized to the nucleus (Sakai et al., 2019; Seidman et al., 2020). It is also possible to sort nuclei using combinations of cell specific markers that include nuclear envelop proteins (e.g., NeuN in neurons) and cell-specific transcription factors (e.g., PU.1 for microglia). An advantage of this methodology is that it can be applied to freshly frozen human tissues (Corces et al., 2017). This approach was recently used to define enhancer landscapes in microglia, astrocytes, oligodendrocytes and neurons of the human cerebral cortex (Nott et al., 2019). In principle, each distinct cell state should be associated with a corresponding combination of transcription factors, which could be used as markers for nuclear isolation and epigenetic analysis.

As applied to microglia and macrophages isolated from human tissues, definition of dynamic enhancer landscapes in control and diseased tissues has the potential to not only provide insights into mechanisms driving myeloid cell fates and states, but to also enable improved interpretation of natural genetic variation. On the order of 90% of the disease risk alleles identified by Genome Wide Association Studies are typically found to reside in non-coding regions of the genome, implicating effects on regulatory mechanisms. Enhancers have been clearly established to be sites of action of genetic variants, but because enhancers are selected and function in cell-specific manner, interpretation requires atlases of these elements in relevant cell types under healthy and diseased conditions. As an example, risk alleles for Alzheimer’s disease were preferentially enriched in microglia enhancers, whereas risk alleles for psychiatric diseases were preferentially enriched in neurons (Nott et al., 2019). Distinguishing causal variants from passenger alleles in linkage disequilibrium remains a major challenge.

In addition to advances in technology, parallel progress in informatics approaches continue to enable deeper insights to be gleaned through integration of transcriptomic and epigenetic data at multiple scales. The central objective in leveraging dynamic enhancer landscapes

to infer mechanisms driving changes in cell fate or state is to extract motifs that are bound by the corresponding transcription factors and presumably mediate enhancer selection and function. A limitation of these approaches is that motifs are discovered based on an enrichment in target over background sequences and they do not provide insight into the importance of a particular motif in a particular enhancer. The recent development of machine learning approaches that can be trained to recognize active enhancers and are amenable to interpretation are beginning to enable estimation of the importance of specific sequences within individual enhancers at base pair resolution (Libbrecht and Noble, 2015; Zhou and Troyanskaya, 2015). These methods appear promising for development of hypotheses regarding roles of specific transcription factors at subsets of functionally coordinated enhancers, how such transcription factors interact to regulate enhancer function and how these processes are affected by noncoding genetic variants.

Lastly, advances in the ability to reprogram iPSCs into various macrophage and microglia phenotypes are in early stages, but these efforts exhibit substantial potential to enable insights into a broad range of questions in human cells. Recent application of CRISPRi screens to iPSC-derived microglia uncovered genes controlling microglia survival, activation and phagocytosis, including neurodegeneration-associated genes (Dräger et al., 2021). The limitations of in vitro studies can be mitigated by more complex systems, such as engraftment of organoids or humanized mice, as described previously (Hasselmann et al., 2019). For example, by combining transcriptomic and functional analyses with a chimeric AD mouse model, TREM2 deletion from iPSC-derived microglia was found to reduce survival, impair phagocytosis of key substrates including APOE, and inhibit SDF-1α/CXCR4-mediated chemotaxis, culminating in an impaired response to beta-amyloid plaques in vivo (McQuade et al., 2020). Importantly, these approaches also enable epigenetic studies that are very difficult to perform in human subjects.

In conclusion, the expanded recognition of macrophage diversity in normal and diseased tissues has revealed corresponding gaps in knowledge as to underlying mechanisms and cellular functions. The systematic analysis of the dynamic regulatory landscapes of these cells in vivo is now becoming feasible, in concert with the availability of increasingly sophisticated methods of analysis. It should thus be possible in the near future to make rapid progress in defining many of the key transcription factors and upstream signaling pathways that establish specific macrophage phenotypes in health and disease. This knowledge is likely to contribute to the goal of defining pathogenic or protective roles of such pathways and the development of new therapeutic approaches for the broad spectrum of diseases in which macrophages play roles.

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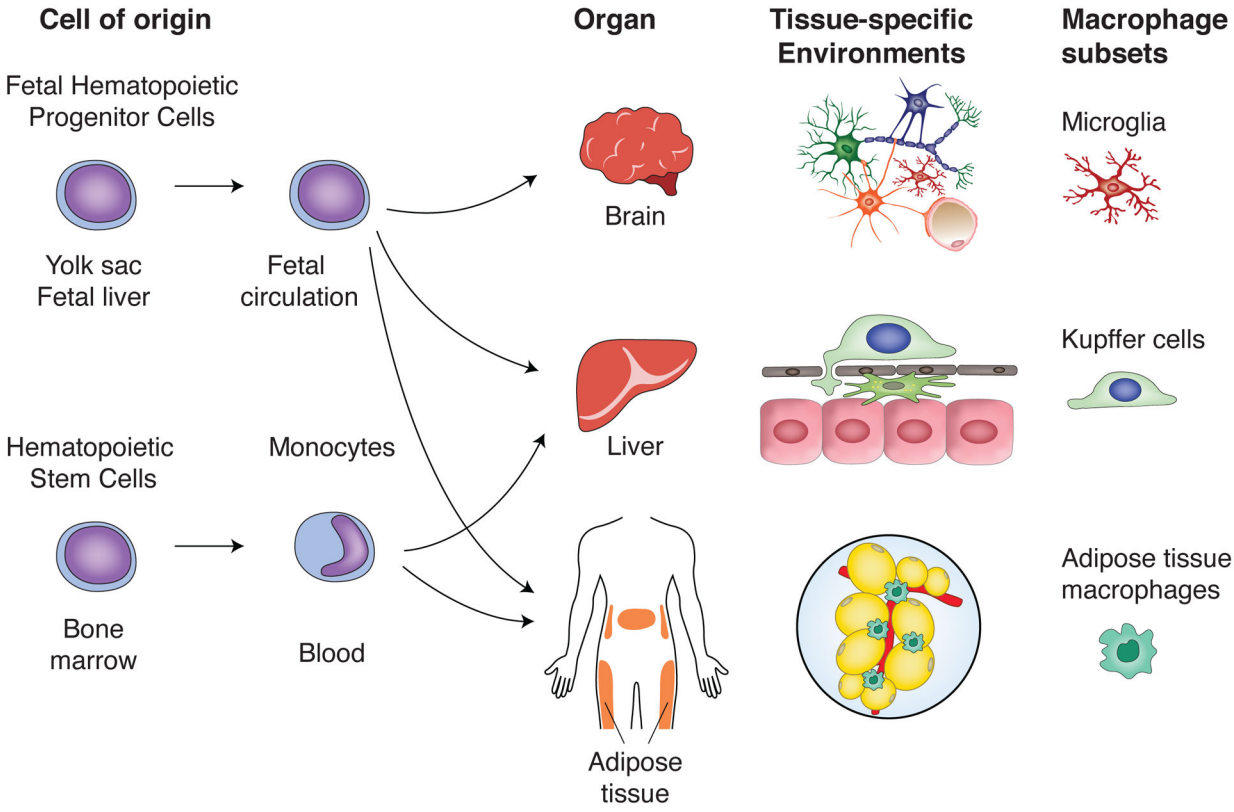

Figure 1. Nature and nurture of tissue resident macrophages.

Yolk sac-derived hematopoietic progenitor cells (HPCs) engraft tissues during early fetal development. This process continues following transition of hematopoiesis from the yolk sac to fetal liver, resulting in all tissues containing embryonically derived resident macrophage populations at the time of birth. Each tissue provides a distinct environmental context that dictates the resulting resident macrophage phenotype, exemplified by brain microglia, liver Kupffer cells and adipose tissue macrophages. Microglia are self-renewing and remain exclusively HPC-derived throughout life in the healthy brain. Resident macrophages are replaced to variable extents in other tissues over time by HSC-derived monocytes that exhibit convergent but non-identical patterns of differentiation with the embryonically derived macrophages.

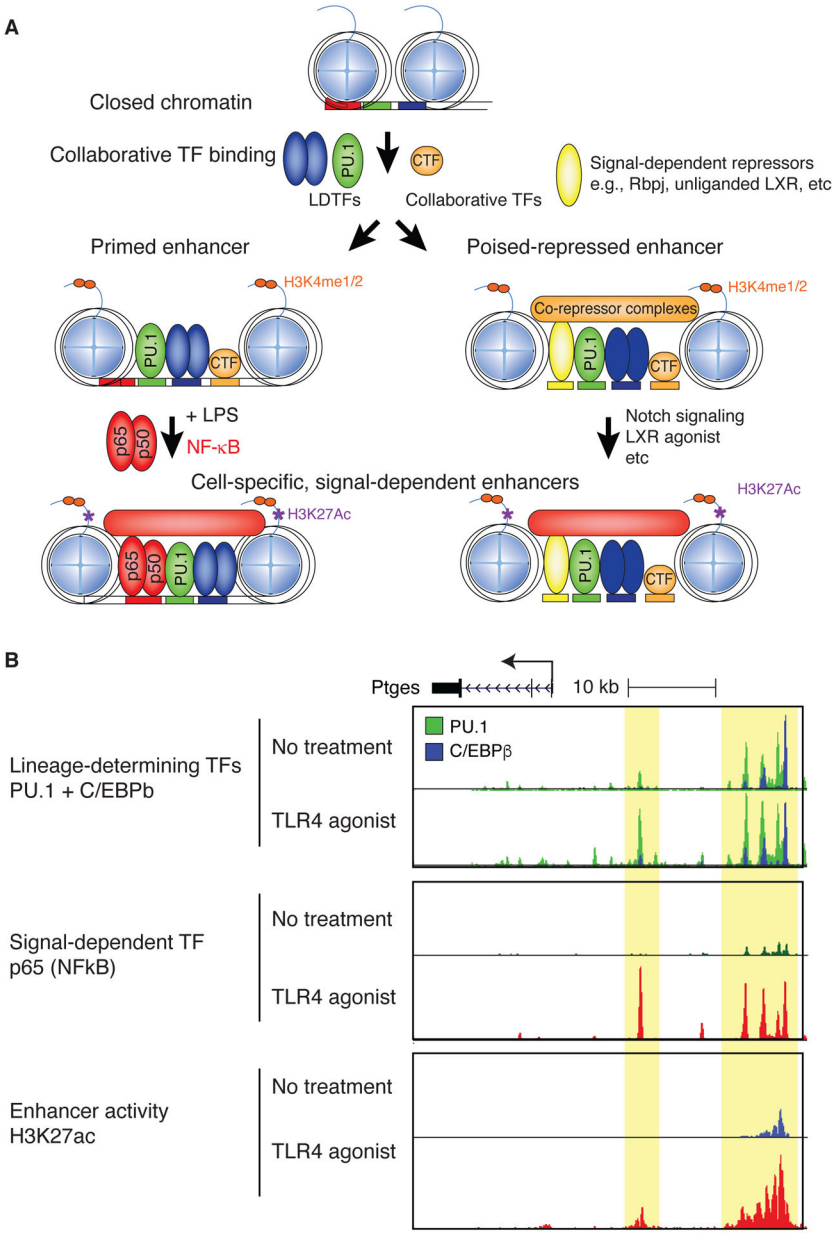

Figure 2. Mechanisms driving the selection and activation of signal-dependent, macrophage-specific enhancers.

Panel A. Combinations of lineage determining transcription factors (LDTFs) with the ability to interact with their DNA recognition motifs in the context of closed chromatin initiate enhancer selection through collaborative binding interactions with each other and dozens of other co-expressed collaborative transcription factors (CTFs). These binding interactions establish regions of open chromatin that enable access to broadly expressed signal-dependent transcription factors (SDTFs) such as NFκB (left branch of pathway). Activation of NFκB by LPS results in its binding to poised enhancers containing κB recognition motifs, resulting in enhancer activation and control of target genes in a macrophage-specific manner. A variation of this pathway involves collaborative interactions with active repressors, which recruit co-repressor complexes that maintain enhancers in a

poised-repressed state (right branch of pathway). Examples include RbpJ in the absence of Notch signaling and unliganded nuclear receptors such as LXRs. Notch signaling, or LXR agonists, convert Notch and LXRs from repressors to activators, respectively, resulting in co-activator recruitment and enhancer activation. Panel B. Example of activation of primed enhancers (Yellow stripes) associated with the *Ptges* gene in bone-marrow derived macrophages. Treatment with a TLR4 agonist leads to p65 (NFκB) binding at genomic locations occupied by PU.1 and C/EBPβ under vehicle treatment conditions. Binding of p65 is associated with gain of H3K27ac and increased expression of Ptges. Data from (Link et al., 2018).

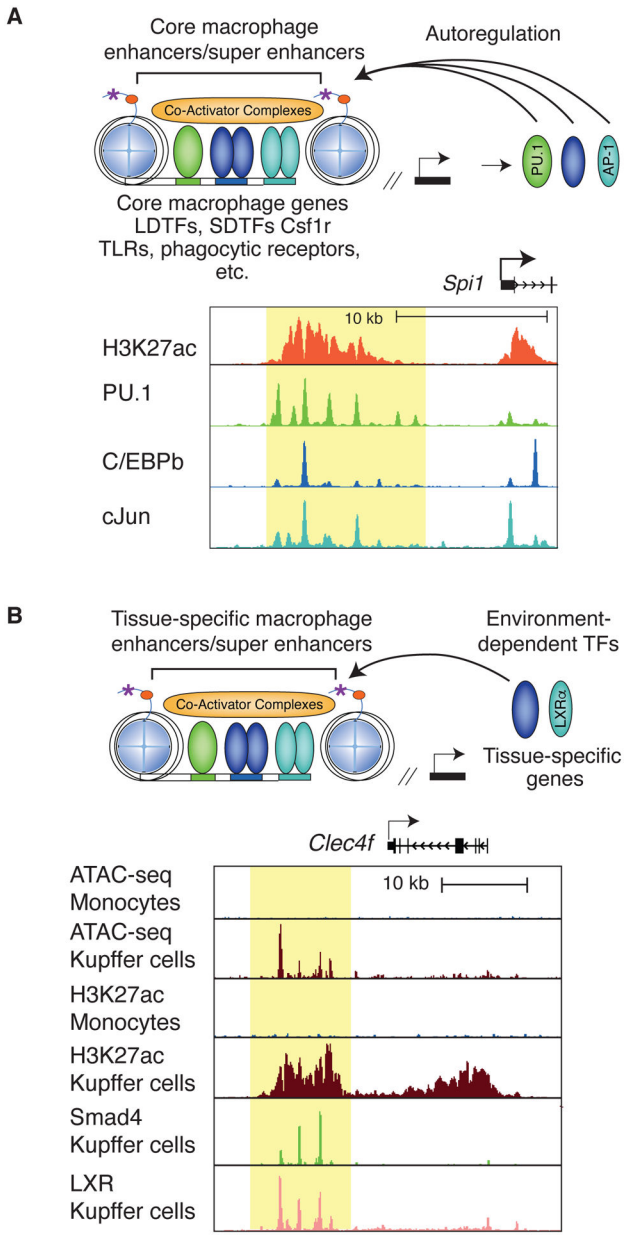

Figure 3. Environment-independent and environment-dependent macrophage enhancers and super enhancers.

Panel A. Genes encoding core macrophage LDTFs are associated with super enhancers in which the component enhancers are bound by combinations of each factor, e.g., PU.1, C/EBPs and AP-1 family members. This property is exemplified by the binding of PU.1, C/EBPβ and cJun at a super enhancer (yellow bar) associated with the Spi1 gene, which encodes PU.1. These relationships establish a mutually reinforcing, environment independent transcriptional network that maintains expression of core macrophage LDTFs and drives expression of core macrophage genes, such as TLRs, phagocytic receptors, etc.

Panel B. Many tissue-specific macrophage enhancers and super enhancers are selected and activated by combinations of transcription factors that are environment-dependent, exemplified by Smad4 and LXRα in Kupffer cells. These factors must be expressed and

active in order for monocytes to acquire a Kupffer cell phenotype following Kupffer cell depletion.

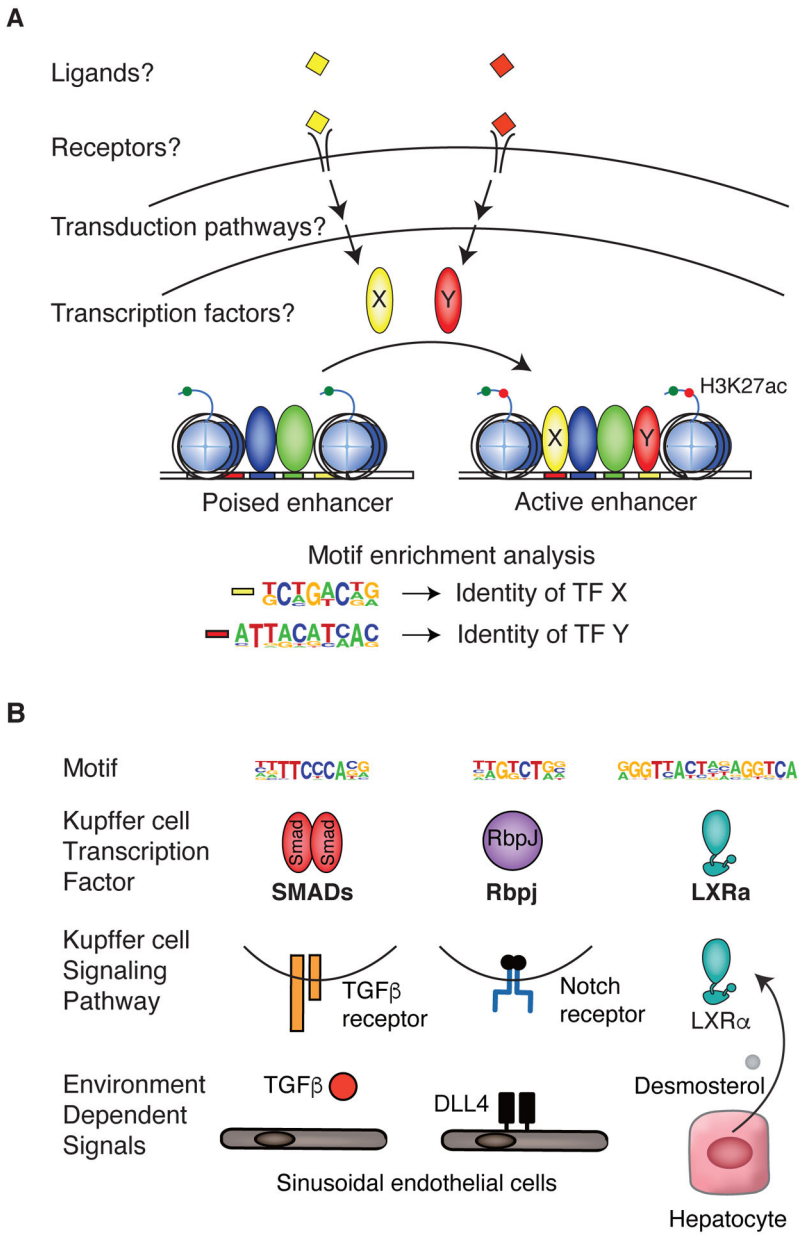

Figure 4. Inference of transcription factors driving changes in macrophage fate and state in response to environmental signals.

Panel A. Transitions in cell fate and state are typically driven by selection and activation of hundreds to thousands of enhancers that in turn regulate gene expression. This dynamic response can be quantitively assessed at a genome-wide level using assays of open chromatin (e.g., through the use of ATAC-seq) and assays that are surrogates of enhancer activity (e.g., ChIP-seq for H3K27ac). Enriched DNA recognition motifs in the set of regulated enhancers represent binding sites for the dominant transcription factors responsible for transitions in enhancer function. The identities of these factors may enable inference of upstream signaling pathways. **Panel B.** Enriched motifs and inferred transcription factors and upstream signaling pathways in enhancers that are activated during monocyte to Kupffer cell differentiation. Motifs for SMADs, RBPJ and LXRs implicate TGFβ-receptor, Notch

and LXRs as signaling pathways important for Kupffer cell differentiation. TGFβ and the Notch ligand DLL4 are potential environmental signals that are provided by sinusoidal endothelial cells. Hepatocytes produce desmosterol that is an activating ligand for LXRs.

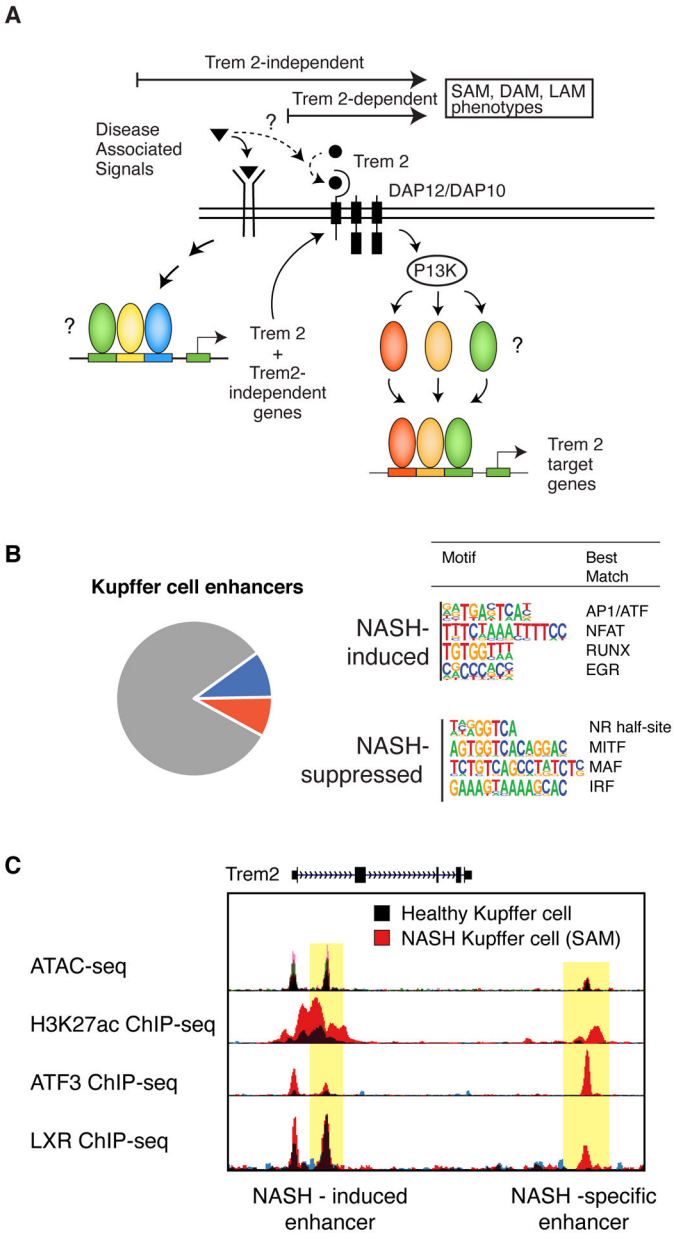

Figure 5. Inference of transcription factors driving changes in macrophage phenotypes in disease.

Panel A. Temporal sequence of events in which resident or recruited macrophages sense disease-associated signals, leading to induction of Trem2 and other Trem2-independent genets. Expression of Trem2 enables additional sensing of disease-associated signals and regulation of downstream gene expression, presumably through Syk/PI3K-dependent mechanisms. Trem2-independent and Trem2-dependent gene expression contribute to generation of disease associated macrophage phenotypes, exemplified by DAMs, LAMs and SAMs. **Panel B.** Inference of transcription factors associated with transition of Kupffer cells to a 'SAM-like' phenotype in a mouse model of NASH. A NASH inducing diet result in gain of H3K27ac at >4000 enhancers and loss of H3K27ac at >3500 enhancers. These changes are associate with upregulation of Trem2 and other LAM/SAM genes and

downregulation of Kupffer cell identity genes. Motifs associated with gained H3K27ac include AP1/ATF, NFAT, RUNX and EGR motifs. **Panel C.** NASH-induced changes in Trem2-associated enhancers. Genome browser tracks for Kupffer cell ATAC-seq, H3K27ac, ATF3 and LXR are illustrated under control and NASH conditions in the vicinity of the Trem2 gene. The NASH diet leads to marked increases in ATF3 and LXR binding that is correlated with selection of a 3' downstream NASH-specific enhancer and increased H3K27ac at the Trem2 promoter, a poised intronic enhancer and the NASH induced 3' enhancer.