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INVITED REVIEW

Epigenomic regulation of macrophage polarization: Where do the nuclear receptors belong?

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Summary

Our laboratory has a long-standing research interest in understanding how lipid-activated transcription factors, nuclear hormone receptors, contribute to dendritic cell and macrophage gene expression regulation, subtype specification, and responses to a changing extra and intracellular milieu. This journey in the last more than two decades took us from identifying target genes for various RXR heterodimers to systematically mapping nuclear receptor-mediated pathways in dendritic cells to identifying hierarchies of transcription factors in alternative polarization in macrophages to broaden the role of nuclear receptors beyond strictly ligand-regulated gene expression. We detail here the milestones of the road traveled and draw conclusions regarding the unexpectedly broad role of nuclear hormone receptors as epigenomic components of dendritic cell and macrophage gene regulation as we are getting ready for the next challenges.

KEYWORDS
EGR2, epigenome, IL-4, macrophage, nuclear receptors, polarization, PPARγ, STAT6, transcriptional regulation

1 | INTRODUCTION

Macrophages and dendritic cells are essential cellular components of the innate immune system. Despite sharing common functional characteristics, including their phagocytic, antigen-presenting, and cytokine-producing capacities or their ability to respond rapidly to the changing microenvironment, they still have a well-defined and cell-type-specific role in the body under various physiological and pathological conditions. Macrophages are critical contributors in maintaining normal tissue homeostasis, the defense against various pathogens, from bacteria to multicellular parasites, and important regulators of inflammation. Dendritic cells are critical in initiating and regulating pathogen-specific adaptive immune responses and contribute to the development of immunologic memory and tolerance.[1–3]

Macrophages are found in almost all tissues, and although they have common functions, including phagocytotic and antimicrobial capacities, their homeostatic function strongly depends on the tissue microenvironment. Among others, the different macrophage subtypes participate in development, metabolism, wound healing, tissue remodeling, and angiogenesis.[1,4,5] Besides, their maladaptive or altered functional properties also contribute to the development and progression of different chronic inflammatory disorders and cancers.[5–7] This remarkable phenotypic heterogeneity and plasticity of macrophages are tightly determined by their origin and molecular microenvironment. Based on the origin of tissue-resident macrophages, yolk sack macrophages, fetal liver monocytes, and bone marrow monocytes-derived macrophages can be distinguished. In adult tissues in the steady-state, microglia in the brain, alveolar macrophages in the lung, Hofbauer cell in the placenta, and Kupffer cells in the liver are of embryonic origin,

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while both embryonic and bone marrow monocytes-derived macrophages are present alongside one another in the intestine.[8–10] The bone marrow monocyte-derived macrophages also play a critical role in the different organs of the body after tissue injury or infections.[11,12] The macrophage microenvironment consists of a wide range of metabolites, nutrients, immunomodulatory factors, and pathogen-derived molecules, determining various phenotypic and functional features of the distinct macrophage subsets under different circumstances. The two endpoints of functional macrophage polarization induced by the microenvironmental activating signals are the Th1-type cytokine interferon-gamma (IFNγ) or gram-negative bacterial cell wall component lipopolysaccharide (LPS)-dependent classical and the Th2-type cytokines interleukin-4 (IL-4) and IL-13-dependent alternative macrophage polarization. These polarization states have well-distinguishable gene expression profiles and functional characteristics. The classically polarized macrophages have anti-bacterial capacities, while the alternatively polarized macrophages contribute to the protection against nematode infections and tissue regeneration.[6,7,13,14] Nevertheless, the complex in vivo molecular milieu, often simultaneously containing different pathogen-derived molecules, cytokines, and metabolites, can result in various transient macrophage polarization forms associated with unique functional characteristics under normal and pathological conditions (Figure 1.).[15–17] The superfamily of nuclear hormone receptors, in particular, the retinoid X receptor (RXR) heterodimers such as peroxisome proliferator activated receptor gamma (PPARγ), retinoic acid receptor (RAR), vitamin D receptor (VDR), and liver X receptor (LXR), have been implicated at multiple levels of gene regulation in macrophages and dendritic cells, including linage determination to epigenomic bookmarking to conventional ligand activation. Here, we will summarize our contributions to this field.

Dendritic cells were discovered by Steinman and Cohn 50years ago and their findings opened up an entire new field linking the until disparate innate and adaptive immunity fields in a highly mechanistic manner.[18] This generated a fury of investigations using multiple models. The human monocyte-derived cells generated by GM-CSF and IL-4 from CD14+ monocytes became the standard approach for studying human DCs and for harnessing their therapeutic potential in the early 2000s.[19,20] It has been suggested that these cells (MCs = monocyte-derived cells) represent the in vitro counterparts of CD14+ CD11c + inflammatory cells generated by immune response.[21] Human conventional dendritic cells however are generated in vitro from CD34+ cord blood HSPCs containing CD115 expressing progenitors and their differentiation is driven by Flt3L.[22] Therefore, MCs should not be considered conventional DCs. Our work in this domain has focused on MCs, which could be considered immature monocyte-derived dendritic cells. We have carried out systematic work on profiling the transcriptional landscapes and changes in MCs upon the activation of the RAR, VDR, PPARγ, and LXR. This led to the identification of new targets and established a role for these receptors in subtype specification including tissue specificity and specialized immune function (i.e RAR and PPARγ regulated CD1D expression and iNKT cell generation) summarized in a review.[23] By now, most of the human and mouse DC subtypes have been identified and their origins clarified using careful profiling and linage tracing,[18] presenting new opportunities to revisit the role of some of these nuclear hormone receptors for their roles in differentiation as well as in functional specification.

2 | THE TRANSCRIPTIONAL BASES OF MACROPHAGE AND DENDRITIC CELL RESPONSE TO THE IMMUNOMODULATORY AND ACTIVATING SIGNALS

The phenotypic and functional features of macrophages and dendritic cells are tightly controlled at the transcriptional level by the microenvironmental milieu. Several mechanistic studies demonstrated that the macrophage or dendritic cell subtype-specific and the extrinsic or intrinsic activation signals-promoted transcriptional programs are based on the complex collaborative interactions between the cis-regulatory elements, including promoters and enhancers, and trans-acting DNA-binding transcription factors (TFs).[24,25] In general, cis-regulatory elements contain multiple, distinct transcription

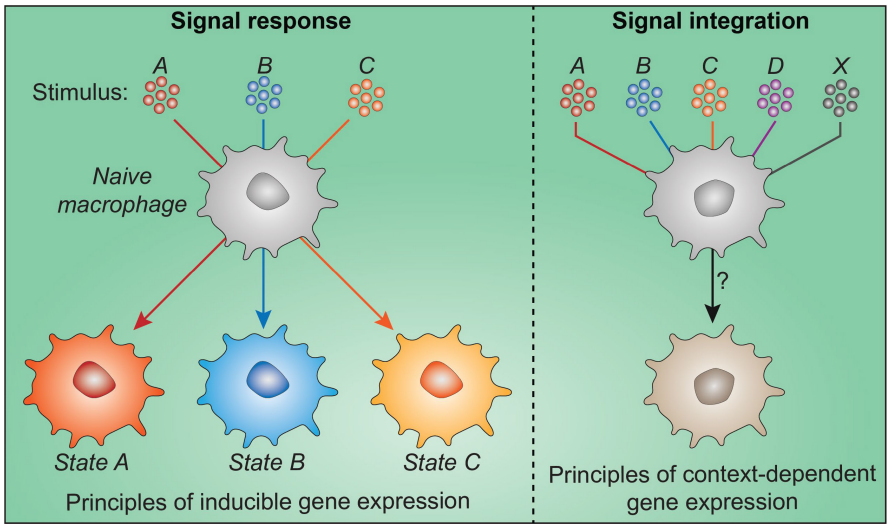

FIGURE 1 General scheme of signal response and integration in macrophages.

factor-binding DNA motifs and have characteristic, partially overlapping histone modification patterns. Promoters are marked by a high level of H3K4 trimethylation, while enhancers are associated with H3K4 mono- and dimethylation instead. Additionally, both cis-regulatory elements exhibit high levels of H3K27 acetylation in the activated state and H3K27 trimethylation in the repressed state.[26–28] The TFs-orchestrated transcriptional regulation shows a multi-layered organization with at least three distinct levels, including (i) the determination of the available enhancer platform by lineage-determining transcription factors (LDTFs), (ii) the development of a primary transcriptional response to microenvironmental signals and tissue demand by signal-dependent transcription factors (SDTFs), and (iii) the formation of a secondary or long-term transcriptional response to microenvironmental changes by SDTFs-regulated TFs or TF networks.[29–31] The core enhancer repertoire in the macrophages and dendritic cells is tightly determined by the cooperative binding of the general cell type-specific LDTFs, including ETS domain, transcription factor PU.1, activator protein 1 (AP1), and CCAAT-enhancer-binding proteins (CEBPs) in macrophages or PU.1 and CEBPβ in dendritic cells.[30,32–34] It has also been described that additional, specific transcription factors, such as GATA binding protein 6 (GATA6) in peritoneal macrophages or Spalt-like transcription factor 1 (SALL1) in microglial cells, can also act as LDTFs, determining the tissue-resident macrophage subtype-specific enhancer sets.[35,36] The available enhancer repertoires serve as a binding platform for the environmental signals-activated SDTFs. Based on the invoked immunological consequences, the SDTFs and their activating signals can be divided into a minimum of two distinct groups. On the one hand, the homeostatic tissue and immunomodulatory signals-activated SDTFs, including immunomodulatory cytokines-activated signal transducer and activator of transcription (STAT) family members or the different hormones and lipids-activated nuclear hormone receptors, contribute to the development of a tissue-specific macrophage and dendritic cell phenotype and modulate their response to danger signals. On the other hand, the pathogen-derived or endogenous danger signals and inflammatory cytokines-activated SDTFs, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and AP-1 TF complexes, orchestrate the macrophage and dendritic cell activation.[25,29] The microenvironmental signals-activated long-term transcriptional programs are provided by a wide range of SDTFs-induced transcription factors, among others, interferon regulatory factor (IRF), basic helix–loop–helix (BHLH), and early growth response factor (EGR) family members.[31,37–40]

3 | NUCLEAR HORMONE RECEPTORS HETERODIMERIZING WITH THE RETINOID X RECEPTOR AS MULTIFACETED HORMONE AND LIPID-SENSING TRANSCRIPTION FACTORS

The nuclear hormone receptor superfamily includes 48 members in humans and 49 in mice, respectively. Based on their ligands and mode of action, the nuclear receptors can be divided into steroid and nonsteroid receptors.[41] The latter includes the RXR heterodimerizing nuclear hormone receptors. This subgroup of the nuclear hormone receptors is quite heterogenous, containing both classical endocrine and adopted orphan receptors. The classical endocrine receptors are activated by high-affinity ligands such as thyroid hormone for thyroid hormone receptor (TR), all-trans-retinoic acid for RARs, and 1α,25-dihydroxy vitamin D_3 for VDR. In contrast, the adopted orphan receptors usually sense various lipid metabolites, including oxysterols for LXRs, fatty acids for PPARs, and bile acids for farnesoid X receptor (FXR), with low affinity.[23,41,42] In the pre-epigenomic era, several general principles for a mode of action of RXR heterodimers were described using combined molecular biological, pharmacological, and genetic approaches. First, RXR heterodimers are bound to the DNA containing their directly repeated response elements with half-site sequence AGGTCA or a variant of it in the nucleus independently from the presence of ligands. Second, RXR heterodimers interact with corepressor complexes and act as transcriptional repressors without an appropriate ligand. Third, the ligand binding induces corepressor-coactivator complex exchange leading to transcription activation.[23] Besides, the phenomenon termed transrepression was also identified. In this case, the ligand-bound nuclear receptor heterodimer modulates transcription of a target gene without direct DNA binding interfering with the activity of other transcription factors through protein–protein interactions and post-translational modifications.[43] However, in the post-epigenomic era, the study of the transcription factor binding and function at the whole-genome level by many next-generation sequencing (NGS)-based methods allowed expanding and clarifying our knowledge about the activities of RXR heterodimers. Among others, these approaches significantly contributed to identifying new functional properties for the RXRs and their heterodimerizing partners, such as ligand-independent bookmarking or cell subtype-specific LDTF activities.[44–46] In this review, we aimed to focus on the complex role of a retinoid X receptor heterodimerizing nuclear hormone receptors in macrophage and dendritic cell biology, especially in the context of our work in this field over the past two decades.

4 | INTEGRATED NGS-BASED EPIGENOMIC AND TRANSCRIPTOMIC APPROACHES TO BETTER UNDERSTAND THE REGULATORY FUNCTION OF RXR HETERODIMERS IN MACROPHAGES

Several studies demonstrated by the early 2010s that the lipid-sensing nuclear receptors can form a bridge between metabolism and innate immune system by directly activating the genome and regulating macrophage functions from oxLDL uptake to inflammatory response[47,48] (Figure 2). However, the technologies available until then were biased and limited to discover ligand-regulated events and did not allow studying the regulatory mechanism of RXR heterodimers at the whole genome level and in an unbiased manner.

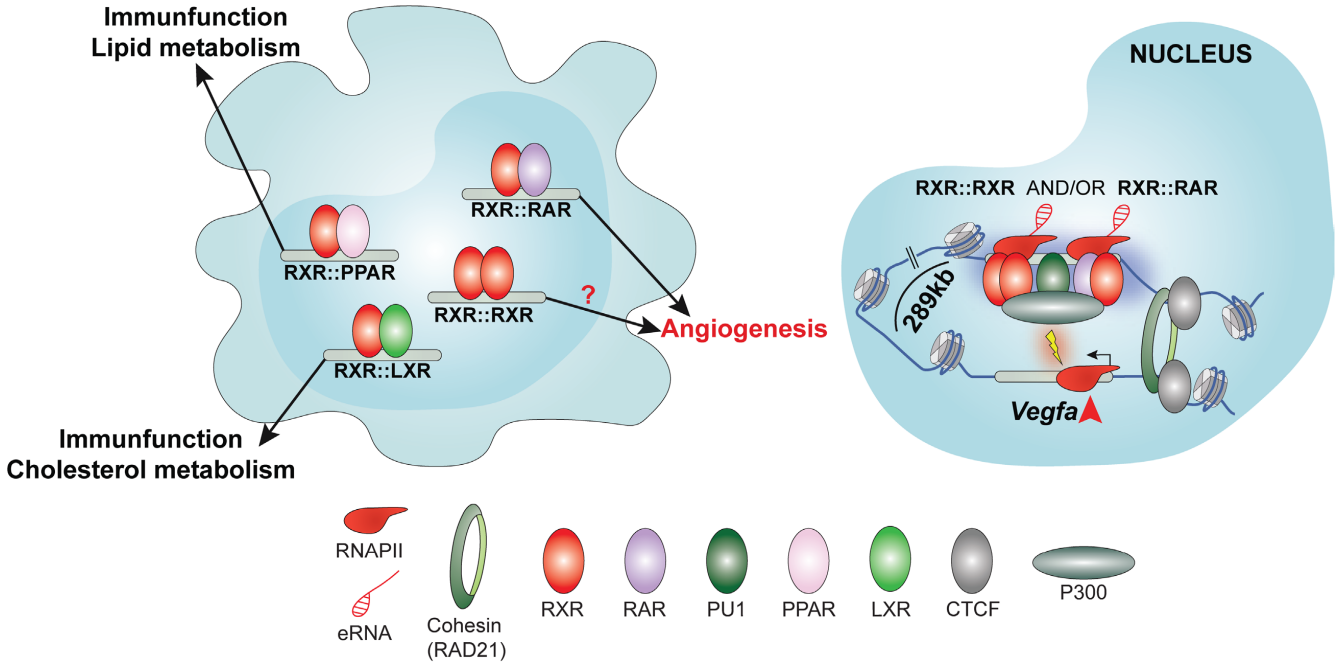

FIGURE 2 The classical ligand-dependent regulatory role of RXR/PPARγ, RXR/LXR, RXR/RAR heterodimers, and RXR/RXR homodimers in macrophage biology and the proposed RXR-mediated enhancer promoter interaction of the Vegfa gene.

The advancement of NGS-based transcriptomic and epigenomic technologies has fundamentally changed the opportunities for studying transcriptional regulation enabling a detailed understanding of the already-known nuclear receptor-mediated regulatory mechanisms and identification of novel functions genome-wide. To take advantage of this technological breakthrough and better understand the regulatory role of RXR heterodimers in the macrophages, first we studied RXR signaling in murine bone marrow-derived macrophages combining chromatin immunoprecipitation sequencing (ChIP-seq), global run-on sequencing (GRO-seq), and RNA sequencing (RNA-seq) methods. By combining these methods, we developed a pipeline by which we called (i) directly regulated genes by utilizing a time course of nascent RNA production (GRO-Seq), (ii) active enhancers by the changing enhancer RNA (GRO-Seq) and active histone marks, (iii) RXR binding sites by ChIP-seq, and (iv) co-activator binding by p300 recruitment in the proximity of the regulated genes.[49] This rigorously applied comprehensive genome-wide approach provided a large amount of information about liganded RXR-mediated transcriptional changes, active binding sites, and cistromic interactions in the context of the 3D genome structure once validated using 3C-Q-RT-PCR. Our results confirmed many elements of our prior knowledge about RXRs and their heterodimeric partners, including nuclear localization and DNA binding capacity of RXRs in the unliganded state or their binding to receptor-specific hormone response elements as NR half sites, DR1, and DR4 motifs. It also established that the RXR cistrome in macrophages is not regulated by exogenously added synthetic ligands to any significant degree. To provide genome-wide evidence for the biological relevance of ligand-dependent transcriptional activation, we identified 387 liganded RXR-activated enhancers characterized by RXR and PU.1 binding, as well as ligand-induced P300 recruitment and enhancer RNA expression. These RXR ligand-activated genomic elements are linked to 226 genes, such as previously identified RXR target genes Abca1, Angptl4, or Tgm2, validating the approach and allowed identifications of novel ones such as Vegfa[49] (Figure 2). Vegfa is a particularly notable example because the identified complex enhancer is almost 300 kilobase downstream of the transcription start site and thus we were able to call it only using the rigorous criteria detailed above. However, our approach could identify more than 5000 RXR-bound genomic regions without any evidence for ligand-mediated effects, nearly half of which are transcriptionally inactive.[49] Intriguingly, the ligand-insensitive fraction of LXRα and β cistromes could also be identified in non-polarized murine immortalized BMDMs indicating that this phenomenon is not restricted to the RXR cistrome.[50] These findings suggested that RXRs and their heterodimeric partners may also have an important ligand-insensitive or non-conventional transcriptional regulatory role in the macrophages. We could confirm the existence of this mechanism in two independent studies in recent years. On the one hand, we identified the elevated expression of several prometastatic genes in lung-derived RXR-deficient myeloid cells associated with their enhanced cancer cell migration and invasion-promoting activities in vitro and increased lung metastasis formation in vivo. The affected prometastatic genes were mainly insensitive to synthetic ligand activation. Still, they are associated with RXR, nuclear receptor corepressor (NCoR), and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) co-bound enhancers suggesting that RXRs regulate their expression through direct ligand-independent transcriptional repression.[51] On the other hand, by studying the potential interactions between the alternative polarizing signal IL-4-activated STAT6 TF and the RXR signaling pathways

in human monocyte-derived differentiating macrophages, we could identify an atypical so-called potentiating effect of ligand-activated RXRs on the transcriptional regulation of IL-4 inducible genes. The IL-4-activated STAT6 cistrome showed extensive overlap with RXR-bound genomic sites following short-term (30 min) IL-4 polarization without influencing the RXR occupancies at the overlapping regulatory regions. The synthetic RXR agonist (LG268) had distinct activities at the selected IL-4-activated and STAT6/RXR co-bound enhancers modulating the IL-4 responsiveness of their target genes regulated by these genomic elements. Based on the role of the RXR ligand, we could divide into three groups the selected IL-4-activated enhancers and genes, including RXR activation independent, synergistically activated, and liganded RXR potentiated categories. In the case of the latter group, the genes and enhancers were insensitive to RXR ligand activation in the non-polarized macrophages. Still, the applied synthetic RXR agonist further increased their IL-4-dependent induction.[52] These findings raised the possibility that the liganded RXR plays a much more complex role in transcriptional regulation than we previously thought, especially in the case of a complex microenvironmental milieu where endogenous RXR ligands may be present. These new mechanisms represent unique opportunities for modulating inflammatory gene regulation.

5 | IL-4-ORCHESTRATED STAT6, EGR2, AND PPARγ CONTAINING TF NETWORK IN THE DETERMINATION OF THE ALTERNATIVE MACROPHAGE POLARIZATION SPECIFIC EPIGENETIC AND TRANSCRIPTIONAL PROGRAM

After spending years to characterize primarily nuclear receptor-mediated events in human monocyte-derived immature dendritic-like cells and mouse bone marrow-derived macrophages, we came to the realization that the origin and state of the observed cell has an oversized and non-negligible influence on nuclear receptor activity both ligand dependent and also ligand independent and thus, we decided to focus on and exhaustively characterize a particular polarization pathway, alternative polarization, and examine and position RXR heterodimer and in particular PPARγ/RXR signaling in its context and hierarchy.

Th2-type immunomodulatory cytokine IL-4 can turn on a specific transcriptional program through the direct activation of its SDTF STAT6 in human and mouse macrophages resulting in their alternative polarization. This macrophage phenotype is characterized by a partially overlapping gene expression signature between mice and humans. Despite the differences in the IL-4-STAT6 signaling pathway-dependent gene expression programs, human and mouse alternatively polarized macrophages participate in the protection against nematode infections and have high tissue regenerative capacities.[15,53,54] The alternatively polarized macrophages are also observed in Th2-type allergic airway inflammation and asthma, breast cancer, fibrotic processes, and the chronic phase of different bacterial infections.[55] Although many features of direct STAT6-mediated transcription activation, including its target genes and its binding DNA motif, were already known in the pre-epigenomic era, the complex NGS-based methodology we developed for mapping nuclear receptor signaling in our prior works contributed to a better understanding of the IL-4-STAT6 signaling pathway-activated transcriptional program in macrophages. Applying comprehensive and integrated ChIP-seq, GRO-seq, and assay for transposase accessible chromatin sequencing (ATAC-seq) methods, we and others have made several important contributions in the context of direct STAT6-regulated epigenetic and transcriptional events. First, STAT6 binds to more than 20,000 genomic regions with specific dynamics. Second, it rapidly activates thousands of constitutively H3K4 mono and demethylated and PU.1-bound enhancers. Third, it can also bind to enhancers lacking LDTF binding and enhancer-specific histone marks in nonpolarized macrophages, leading to the latent or de novo enhancer formation and activation. Forth, it can also act as a direct transcriptional repressor associating with reduced RNA Polymerase II binding and H3K27 acetylation as well as attenuated eRNA expression. These STAT6-repressed enhancers are also characterized by diminished PU.1 and co-activator P300 binding and decreased chromatin accessibility following short-term IL-4 exposure.[56,57] Intriguingly, it has recently been demonstrated that many direct IL-4-STAT6 target genes show distinct IL-4 inducibility in different cell cycle phases of murine bone marrow-derived macrophages, raising the possibility that the STAT6 binding or its transcriptional activator capacity is also regulated in a cell cycle-dependent manner at different enhancer sets.[58]

In addition to the direct transcriptional regulator activity of STAT6, it has also been demonstrated in the last two decades that additional transcription factors downstream from STAT6 are also required for proper alternative macrophage polarization, including IRF4, cMYC, KLF4, BHLH40, and PPARγ.[59–66] However, the full complement of this TF network and the relationship including the hierarchy between the individual TFs were not fully understood. To identify the key regulators during the transition between the early direct STAT6-dependent and the late stable alternative macrophage polarization-specific transcriptional and epigenetic events, we further studied the early and late enhancer activation following IL-4 polarization in murine bone marrow-derived macrophages. Our P300 and H3K27 acetylation-specific ChIP-seq-based approach could identify three IL-4-activated enhancer clusters with distinct dynamics, including "early transient," "early sustained," and "late" enhancers. As expected, STAT6 was responsible for the early activation at the "early transient" and "early sustained" enhancer sets. Besides, we could identify the EGR2 transcription factor as a new regulator of the late alternative macrophage polarization, directly and indirectly controlling the activation of "early sustained" and "late" enhancers following 24 hours length IL-4 exposure. The direct EGR2-dependent enhancer activation is associated with elevated chromatin openness and chromatin remodeling factor BRM binding, as well as increased co-activator P300 and BRD4 bindings. The indirect EGR2-mediated effects on the enhancer activity are probably caused by the activity

of an alternative macrophage polarization-specific TF network containing the previously described KLF4, BHLH40, or PPARγ. Overall, the consequence of the direct and indirect regulatory roles of EGR2 is the altered expression of 77% of IL-4-induced and 64% of IL-4-repressed genes in the EGR2-deficient macrophages indicating that EGR2 is essential for the late stable epigenetic program of alternative macrophage polarization[37] (Figure 3). This was confirmed in a recent publication describing the role of natural genetic variations in the alternative macrophage polarization program also confirmed the contribution of EGR2 to the IL-4-induced gene expression and enhancer activation in murine bone marrow-derived macrophages.[67] A potential EGR2 autoregulatory loop can provide stability to the epigenomic state and gene expression. This is a particularly compelling example of how a transient epigenomic signal (STAT6 binding to chromatin) can be converted to a stable epigenome (EGR2 and downstream TF cistromes) providing transcriptional memory.

As noted above, the lipid-sensing nuclear hormone receptor PPARγ is one of the best-known alternative macrophage polarization-specific TFs. Its induction by IL-4 in macrophages was described very early in 1999, and since then, it has been confirmed several times in different macrophage subtypes and species by us and others.[60,68–70] However, the role of PPARγ during alternative macrophage polarization was quite controversial for a long time. It has been previously described that PPARγ is required for the proper IL-4-mediated alternative polarization, and the myeloid cell-specific disruption of PPARγ sensitizes these mice to the development of diet-induced obesity, insulin resistance, and glucose intolerance.[60] Despite these facts, the activation of PPARγ by the synthetic agonist rosiglitazone (RSG) could not be linked to the induction of alternative polarization-specific gene signature,[70] raising the possibility of a minimum of two plausible explanations, including (i) the role of endogenous PPARγ ligands or (ii) ligand-independent action of PPARγ in this process. According to the first hypothesis, IL-4 can induce many enzymes with endogenous PPARγ ligand-producing potential in murine and human macrophages, including 13-HETE and 15-HODE-producing ALOX15, lysophosphatidic acid-producing ENPP2, and serotonin metabolites-producing MAOA.[68,69,71,72] The second hypothesis was also supported by the fact that PPARγ heterodimeric partner RXR also has a significant genome-bound, ligand-insensitive, and transcriptionally inactive fraction in nonpolarized murine macrophages.[49] To systematically evaluate the synthetic and/or endogenous ligand sensitivity of the PPARγ/RXR heterodimers at the whole genome level in long- and short-term polarization experiments using murine bone marrow-derived macrophages, we performed ChIP-seq and GRO-seq analyses in these systems in the absence or presence of synthetic PPARγ agonist and antagonist. Using this approach, we could draw conclusions about the PPARγ and RXR cistromes and their ligand sensitivity. On the one hand, IL-4 exposure can cause a large expansion of PPARγ cistrome (binding) at the whole genome level associated with the redistribution of RXR cistrome in both polarization systems with minimal loss of binding. On the other hand, the formation of polarization-induced PPARγ/RXR heterodimers is endogenous ligand-independent. Thirdly, the limited number of PPARγ/RXR-bound enhancers annotated to the classical PPARγ target genes, such as *Angptl4* or *Fabp4*, can be influenced by synthetic PPARγ and RXR agonists, PPARγ antagonist. Overall, these findings indicate that

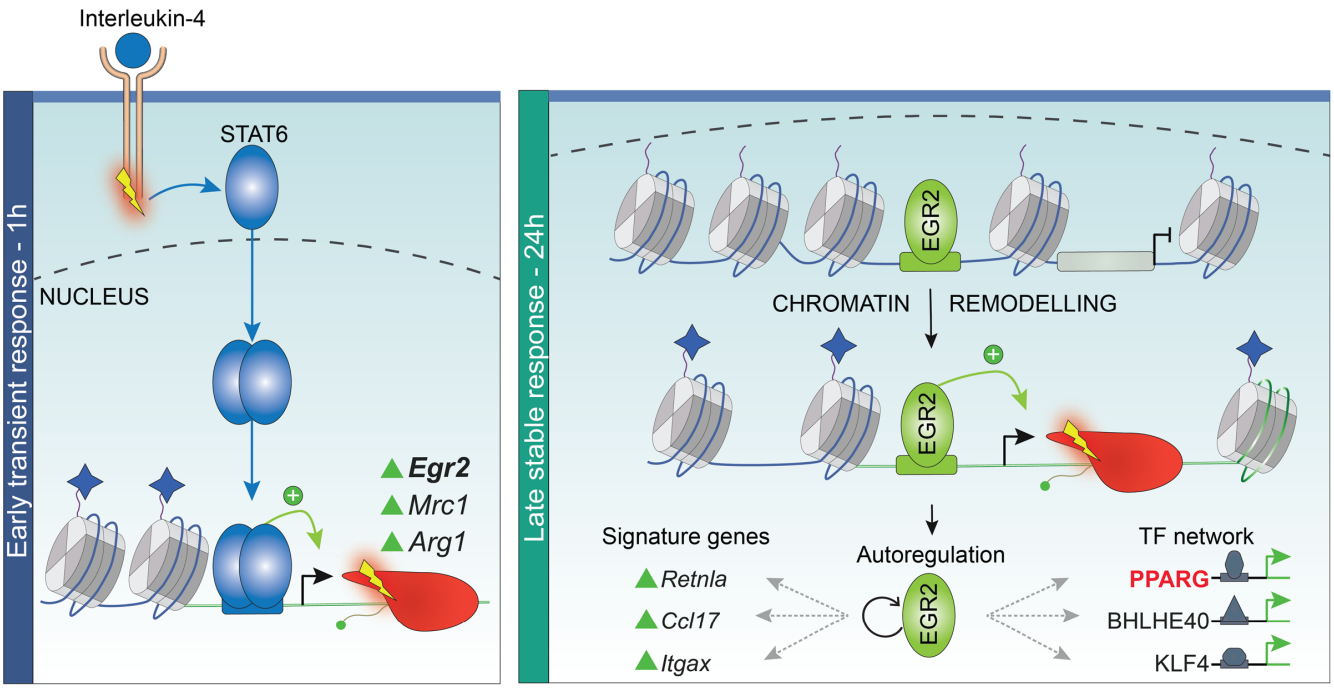

FIGURE 3 The STAT6-EGR2 transcription factor axis-induced PPARγ expression during IL-4-mediated alternative macrophage polarization in murine BMDMs.

alternative macrophage polarization is associated with the PPARγ/RXR heterodimer cistromic expansion at the whole genome level. Still, PPARγ activation by endogenous and synthetic ligands is negligible in volume and probably does not contribute to alternative macrophage polarization per se.[44,73] The further study of ligand insensitive PPARγ/RXR heterodimers-bound enhancers in wild-type and PPARγ deficient alternatively polarized murine macrophages by ATAC-seq, as well as P300 and RAD21-specific ChIP-seq demonstrated these distal regulatory regions are associated with IL-4-induced P300 and RAD21 binding as well as chromatin accessibility in a PPARγ dependent manner. These observations indicate that most PPARγ/RXR heterodimers in the alternatively polarized macrophages act as epigenetic bookmarking factors contributing to the entrenchment of the alternative phenotype by keeping chromatin open and/or allow additional factors to act(-Figure 4.) rather than the simple sensors of the extracellular or intracellular lipid microenvironment.[44]

6 | IL-4-INDUCED TF NETWORK SUPPORTS THE UNIQUE RESPONSIVENESS OF ALTERNATIVELY POLARIZED MACROPHAGES TO THEIR SURROUNDING MOLECULAR MILIEU

Once we mapped the transcriptional cascade supporting alternative polarization and positioned PPARγ/RXR signaling in it we extended our investigations to signal interactions with the alternative polarization and nuclear receptor pathways.

The immunomodulatory signals and exogenous pathogen-derived or endogenous danger signals may be present in the environment of macrophages simultaneously or consecutively. In general, the complex interactions between individual signals can be antagonistic, synergistic, or result in the de novo expression of a specific gene set leading to the development of more specialized macrophage phenotypes and functions aggravating or alleviating the disease outcome.

Transcriptional memory-like features associated with the first-stimulus epigenetic remodeling and metabolic changes are well-known in the macrophages following repeated exposure to the same or different stimuli. Transcriptional memory can result in attenuated (such as LPS tolerance) or increased (such as trained innate immunity) responsiveness to the second stimulus.[74,75] To investigate whether the suspected bookmarking role of PPARγ/RXR heterodimers can influence transcription memory in alternatively polarized macrophages, we applied IL-4 re-stimulation-based experimental system. Surprisingly, we identified an extracellular matrix-associated gene set that is de novo induced by the second IL-4 stimuli. These memory-like features were completely PPARγ-dependent showing a novel epigenomic role of the ligand insensitive PPARγ/RXR heterodimers[44] (Figure 4).

It has been demonstrated previously that Th1-type cytokine IFNγ can reinforce the Toll-like receptor (TLR) ligands-induced inflammatory response in macrophages at multiple levels. IFNγ and TLR ligand co-stimulation results in the so-called super-induction of many canonical inflammatory genes for which STAT1 and IRF1 transcription factors are responsible.[76] Additionally, IFNγ-directed repression of feedback inhibitory and metabolic components of TLR responses are regulated by STAT3, further enhancing the inflammatory response in macrophages.[77] Finally, IFNγ can also impede and reverse TLR ligands-induced macrophage tolerance, leading to exacerbated inflammatory phenotype in autoimmune diseases.[78,79]

The alternative macrophage polarization signal IL-4 may be present simultaneously with the opposing signal IFNγ and different pathogen-derived or endogenous danger signals at the same time in the macrophage microenvironment in various pathological conditions, including helminth-bacterial or helminth-viral coinfections, cancers, or exacerbated Th2-type airway inflammation and asthma.[80–86] The mutual antagonistic effects have long been a known feature of IL-4 and Th1-type cytokines or TLR ligands, and recent studies explored the molecular and epigenetic bases of these interactions. It has been described that IFNγ could repress the basal homeostatic expression of many alternative macrophage polarization-associated genes through the inactivation and disassembly of MAF transcription factor binding enhancers.[87] Nevertheless, the co-stimulation of murine bone marrow-derived macrophages by IL-4 and IFNγ showed that IFNγ-activated enhancers associated with STAT1 and IRF binding are highly resistant to IL-4-dependent inhibition, but the AP-1 and C/EBPβ-bound regulatory regions are more sensitive the inhibitory effects of IL-4.[64] To investigate whether the attenuated IFNγ responsiveness in alternatively polarized macrophages shows a progressive nature, we restimulated the macrophages two, three, and four times with IL-4 followed by IFNγ exposure. We applied washout after each stimulation and rested the cells for 24 hours before the following stimulation. We reported that the attenuated response of the selected genes, including *Ccl5*, *Irg1*, and *Irf8*, to IFNγ in the alternatively polarized macrophages proved quite progressive, resulting in almost complete desensitization after the fourth IL-4 restimulation and thus IFNγ resistance.[44] It has been recently published that macrophage responsiveness to IFNγ markedly reduced in S and G2/M cell cycle phases, while IL-4 induces the cell cycle phase distribution toward the G2/M phase resulting in the subpopulation-specific reduced IFNγ responsiveness in the IL-4 polarized macrophage populations.[58] Besides, we observed that the direct transcriptional repressor activity of the STAT6 transcription factor also affects the specific subset of the inflammatory enhancers attenuating both their basal activity and TLR their inducibility by TLR ligand LPS. This partially reduced inflammatory responsiveness results in blunted NLRP3 inflammasome activation, IL-1β production, and pyroptosis in IL-4-primed and LPS-activate murine bone marrow-derived macrophages[57] (Figure 5). Intriguingly, the IL-4-enhanced inflammatory responsiveness of certain genes was also detected in macrophages suggesting that the interactions between IL-4-STAT6 and TLR signaling pathways are not restricted to antagonism.[57,88,89] Based on these observations, we could identify a specific gene set containing

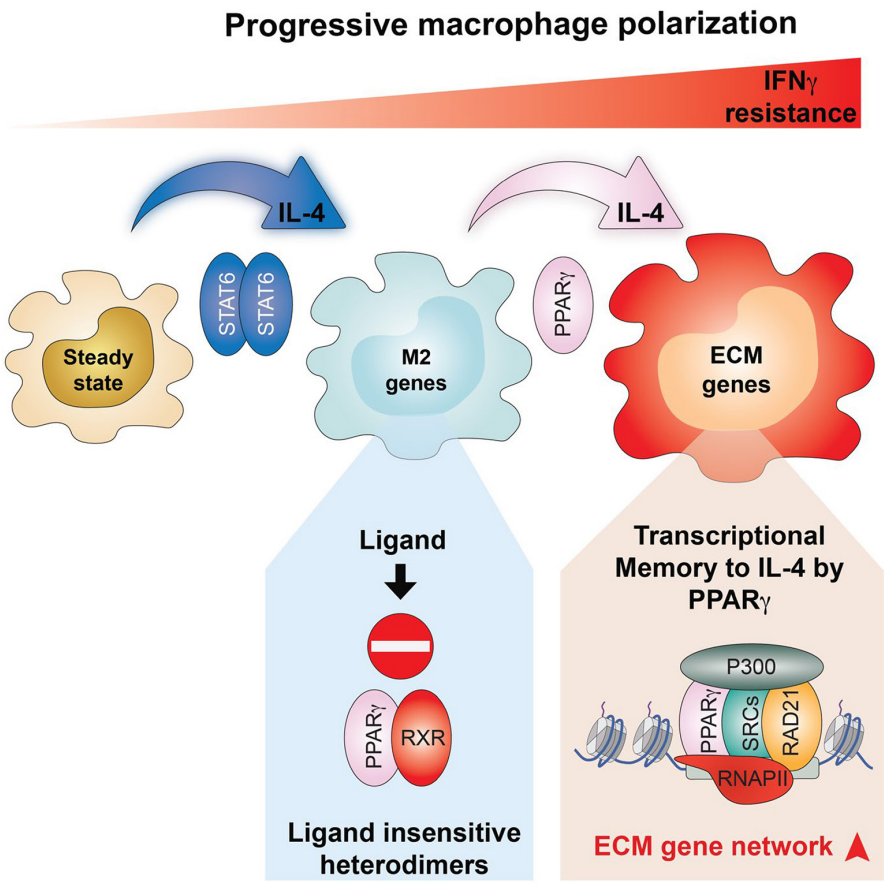

FIGURE 4 The ligand-independent epigenomic ratchet activity of PPARγ leads to transcriptional memory and enhanced gene-specific responsiveness to IL-4 re-stimulation in alternatively polarized macrophages.

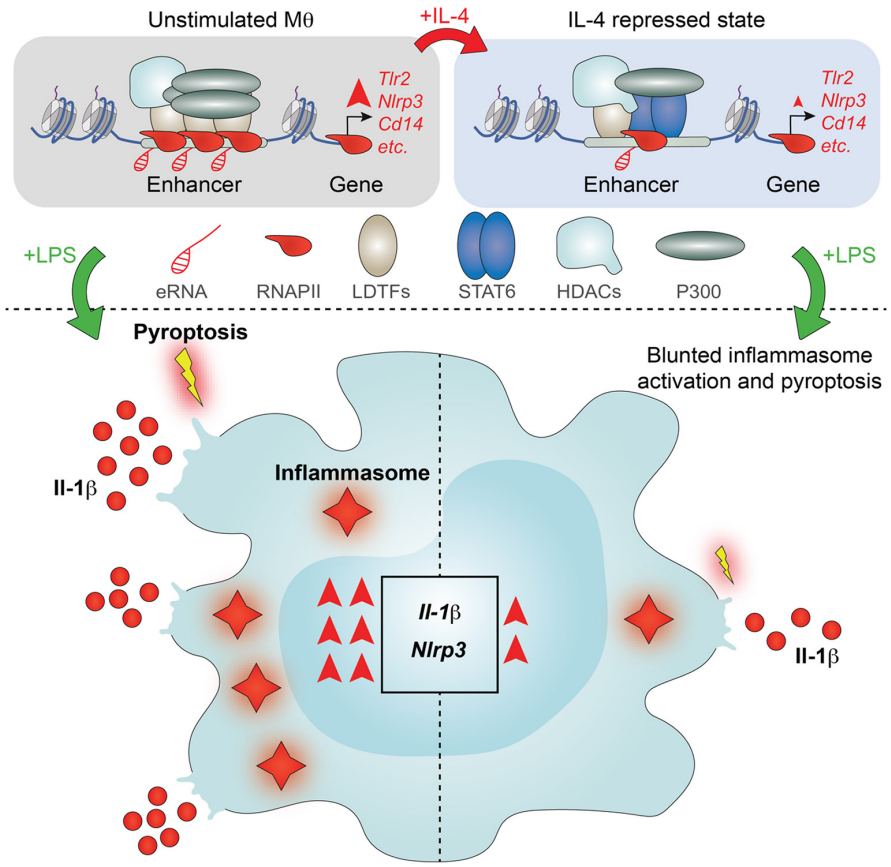

FIGURE 5 The direct transcriptional repressor activity of STAT6 at a specific subset of inflammatory enhancers limits the inflammatory responsiveness in the alternatively polarized macrophages resulting in blunted inflammasome activation and pyroptosis.

more than 1300 genes showing elevated LPS responsiveness, which we termed extended synergy, in IL-4-primed murine bone marrow-derived macrophages compared to the nonpolarized counterparts. Our systematic analyses resulted in the following important findings about extended synergism: (i) "de novo" and "enhanced" NF-KB-p65 binding and synergistic activation are detected at the distal regulatory regions annotated to the synergistically activated genes in IL-4-primed, and LPS-activated macrophages, (ii) both elevated NF-kB-p65 binding and synergistically activated gene expression is completely STAT6 dependent, (iii) the synergistic enhancer activation is associated with increased chromatin openness and BRD4 binding, (iv) EGR2 is an important but not exclusive regulatory factor of this process, (v) the genetic variance can influence the extended synergism, (vi) the extended synergism is observable in different murine tissue-resident and human monocyte-derived macrophages, and (vii) this phenomenon is present in alveolar macs during allergic airway inflammation leading to enhanced LPS-induced inflammation[90] (Figure 6).

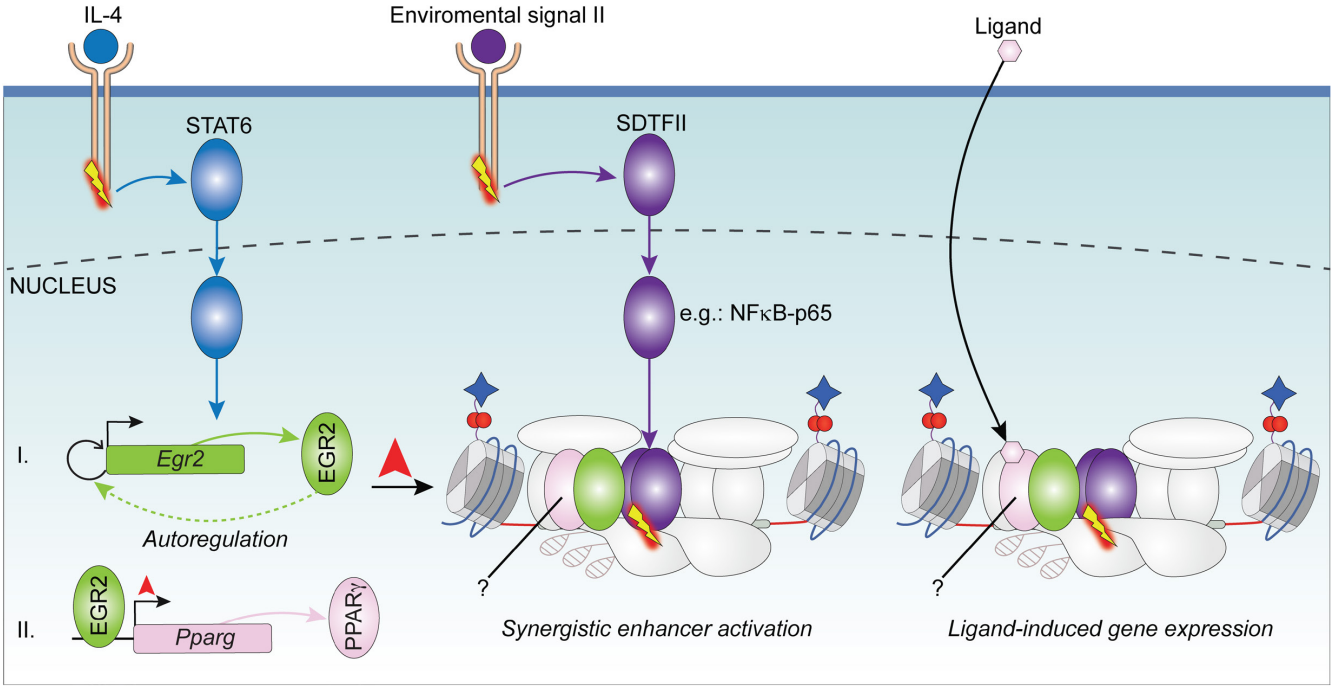

FIGURE 6 The IL-4-induced EGR2 and PPARγ transcription factors contribute to the gene subset-specific enhanced responsiveness to various environmental signals, including pathogen-derived molecules and nuclear receptor ligands, in the alternatively polarized macrophages.

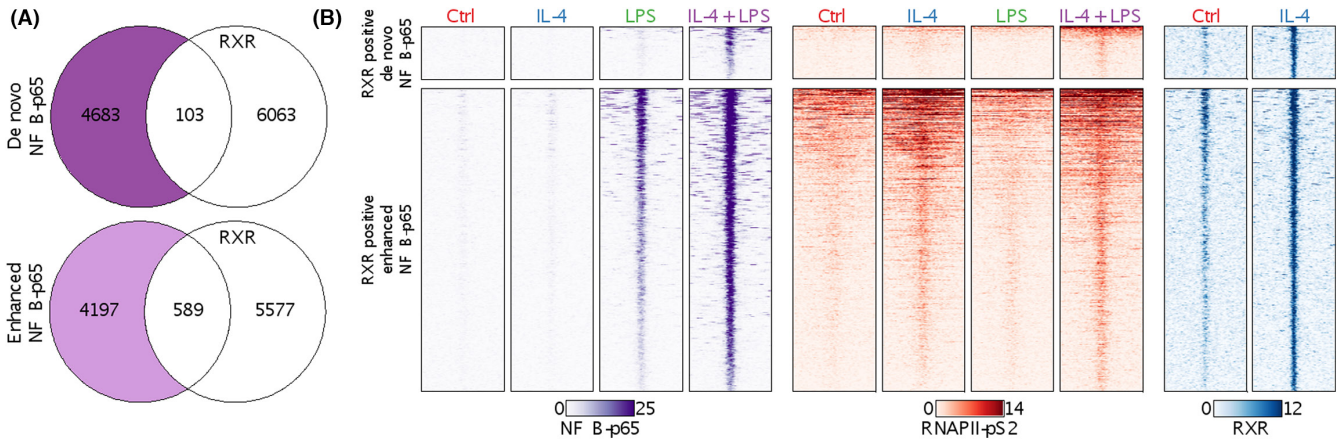

FIGURE 7 The potential connection between the phenomenon of extended synergism between IL4 and TLR signaling and the RXR signaling pathway in the alternatively polarized macrophages. (A) Partial overlap is observed between the LPS-activated de novo/enhanced NF-kB-p65 binding and the RXR cistrome at the synergistically activated enhancers in the alternatively polarized macrophages. Venn diagrams showing the overlap of RXR peaks with de novo and *enhanced* NFkB-p65 genomic regions, (B) Read distribution plot visualization of NF-kB-p65 (purple), RNAPII-pS2 (red), and RXR (blue) binding at the de novo/enhanced NF-kB-p65 and RXR co-binding-associated synergistically activated enhancers (+/−2.5 kb from peak summits).

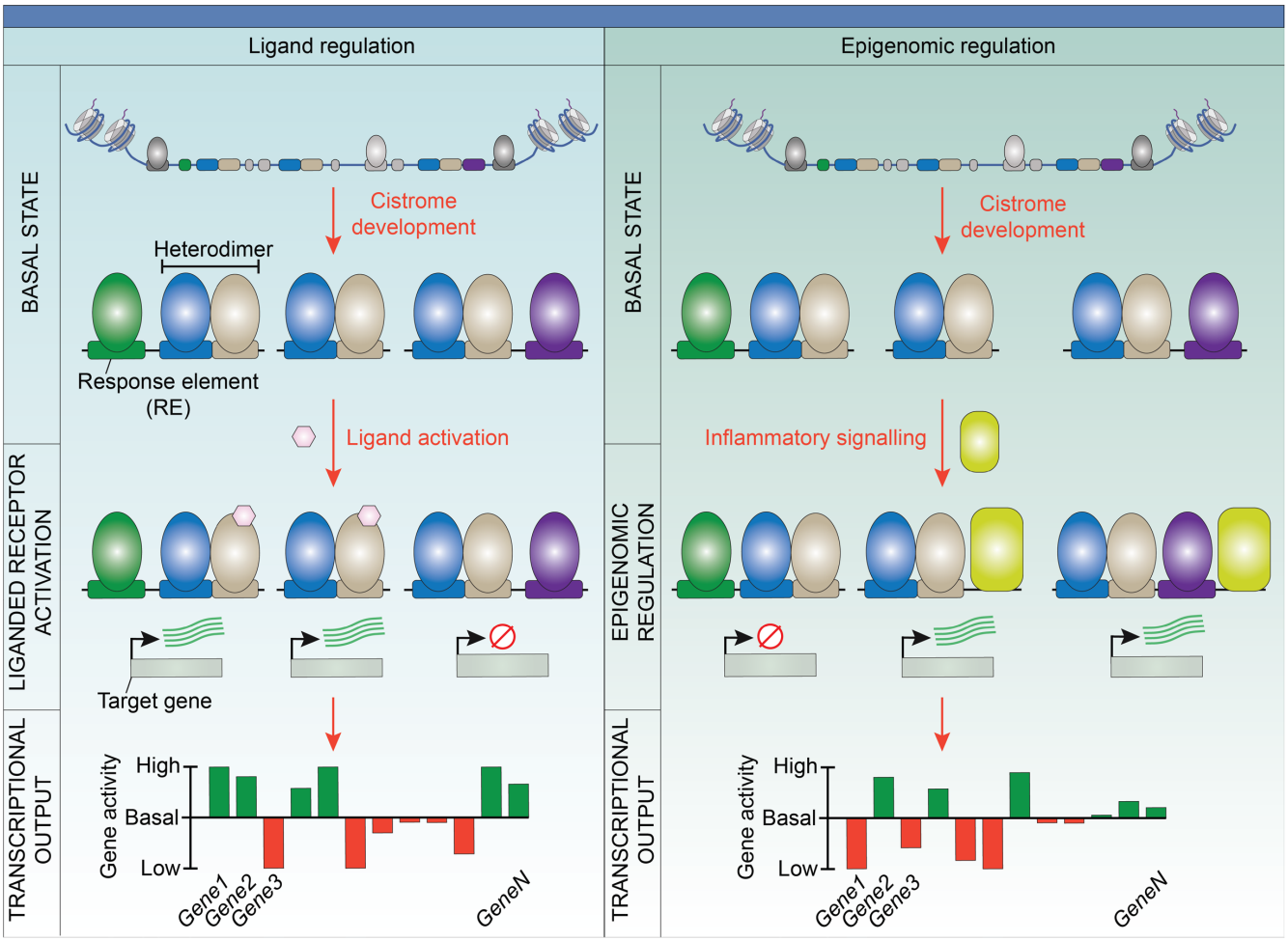

FIGURE 8 Both ligand-dependent and ligand-insensitive epigenomic regulatory roles of nuclear hormone receptors modulate the macrophage responsiveness to various microenvironmental signals at the transcriptional level.

RXRs and their heterodimerization partners can play diverse functions in macrophages, directly or indirectly modulating the inflammatory response. Including but not limited to, LXR agonists can inhibit the inflammatory gene expression through direct repression or transrepression,[43,91] the liganded PPARγ also has an anti-inflammatory effect,[43] while RXR ligand activation leads to the elevated expression of Ccl9 and Ccl6 in macrophages and RXRα deficiency in myeloid cells leads to the lower susceptibility to sepsis in murine CLP model.[92] In addition, many nuclear hormone receptors play in subset-specific LDTF functions in different tissue-resident macrophages, including PPARγ in alveolar macrophages,[93] LXRs in splenic marginal zone macrophages, and Kupffer cells,[45,94] and RXRs in large peritoneal macrophages,[46] potentially also influencing the inflammatory responsiveness of the given macrophage subtypes. However, it is completely unknown whether RXR heterodimerizing nuclear hormone receptors can influence the complex interactions between different immunomodulatory signals and inflammatory signals detailed above. To evaluate the possibility of the modulatory action of RXR heterodimers on extended synergism, we determined the overlap between the RXR cistrome-derived from IL-4-polarized murine bone marrow-derived macrophages and the synergistically activated "de novo" or "enhanced" NFkB-p65 binding associated enhancers. As shown in Figure 7 A and B, we could identify nearly 700 synergistically activated enhancers having RXR binding in the IL-4 polarized macrophages. These findings raised the possibility that RXRs or their heterodimerization partners can influence the synergistic enhancer activation at these sites. Nevertheless, our hypotheses about the role of RXR heterodimers in the complex immunomodulatory cytokine-TLR ligand interactions need further experimental confirmation.

7 | CONCLUSION

Macrophages and dendritic cells are present in almost all tissues in various physiological and pathological conditions associated with the complex microenvironmental milieu. The SDTFs play an important role in creating connections between the continuously changing microenvironment and the cellular response through the transcriptional regulation of several 100–1000s of genes. The activation of the immunomodulatory and danger signal-responsive SDTFs in different combinations leads to a broad spectrum of functional states of macrophage and dendritic cell activation,

often determining the disease onset, progression and outcome. For all these reasons, it is essential to learn about the transcription programs activated by different SDTFs and their combinations. Initially, the lipid-sensing nuclear receptors were recognized in macrophages and dendritic cells as simply forming a bridge between metabolism and inflammatory processes through the ligand-dependent transcriptional activator and repressor activities. However, various in vivo and global NGS-based approaches made it possible to significantly broaden our understanding by identifying novel non-conventional nuclear hormone receptor-mediated epigenomic regulatory functions (Figure 8). In the last decade, it was discovered that nuclear hormone receptors could act as LDTF in various tissue-resident macrophage subsets. We also uncovered their ligand-dependent gene-specific potentiating effect to another signal, their ligand-insensitive repressor, or bookmarking activities. These new developments shed a different light on this group of transcription factors, suggesting that they are much more than mere mediators of lipid signaling. They apparently have epigenomic roles serving processes as signal amplification, inhibition and transcriptional memory. It also questions how these disctinct functions developed during evolution. Which was first, the liganded activity or the epigenomic one? Thus, there are still many open questions waiting to be answered. Additional work is needed in the near future in the nuclear hormone receptor field, including investigating the molecular background and ligand sensitivity of their LDTF activities and the extent of their described non-conventional activities, determining their regulatory role in complex molecular microenvironment-activated transcriptional programs relative to their described conventional ligand-dependent and non-conventional activities. Deciphering these enigmas preferably at the single cell level, can contribute to developing better targeted therapies for diseases associated with altered macrophage and dendritic cell functions.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the NCBI GEO at http://doi.org/10.1093/nar/gky157, reference number GSE110465 and at http://doi.org/10.1016/j.immuni.2022.10.004, reference number GSE181223.

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