# Regulation of the IL-10-driven macrophage phenotype under incoherent stimuli

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## Abstract

Macrophages are ubiquitous innate immune cells that play a central role in health and disease by functionally "polarizing" to distinct phenotypes, which are broadly divided into classical inflammatory responses (M1) and alternative responses (M2) that promote immune suppression and wound healing. Although macrophages are attractive therapeutic targets, incomplete understanding of polarization limits clinical manipulation. While individual stimuli, pathways, and genes involved in polarization have been identified, how macrophages evaluate complex *in vivo* milieus comprising multiple divergent stimuli remains poorly understood. Here, we used combinations of "incoherent" stimuli – those that individually promote distinct macrophage phenotypes – to elucidate how the immunosuppressive, IL-10-driven macrophage phenotype is induced, maintained, and modulated under such combinatorial stimuli. The IL-10-induced immunosuppressive phenotype was largely dominant but required sustained IL-10 signaling to maintain this phenotype. Our data also implicate the intracellular protein, BCL3, as a key mediator of the IL-10-driven phenotype. IL-12 did not directly impact polarization of IL-10-treated macrophages, but IFNγ disrupted a positive feedback loop that may reinforce the IL-10-driven phenotype *in vivo*. This novel combinatorial perturbation approach thus generated new insights into macrophage decision making and local immune network function.

## Keywords

interleukin-10 (IL-10); macrophages; macrophage polarization; tumor-associated macrophages (TAM)

## Introduction

Macrophages are ubiquitous cells of the innate immune system that play central roles in both host defense and disease. A central feature of macrophage biology is the capacity to

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functionally "polarize" to various phenotypes in response to diverse environmental stimuli.[1–5] Although these phenotypes span a continuum of states, macrophage heterogeneity may be broadly divided into classic inflammatory responses (M1), such as those mounted to combat microbial infections, and alternative responses (M2), with the latter type mediating killing of parasites, activation of Th2 helper T cells, and immuno-suppression and tissue remodeling during wound healing.[6] In many chronic diseases, macrophage-mediated responses become dysfunctional and promote disease, as is perhaps best illustrated in cancer.

Many types of cancer involve dysfunctional macrophage responses, which drive tissue remodeling, tumor proliferation, invasion, and metastasis.[3, 7–9] However, macrophages may also limit cancer growth and promote clearance of established tumors. Tumor infiltrating macrophages (TIM), tumor-associated macrophages (TAM, which are also present at sites away from the tumor bed, such as the peritoneum), and related myeloid-derived suppressor cells (MDSC) help to establish and propagate the dysfunctional immunological microenvironment at tumor sites and proximal sites of peripheral immune function including lymph nodes[10–13], where other immune cell types such as T cells and dendritic cells are converted to phenotypes that promote tumor survival. Dysfunctional local immune networks are generally considered to be one of the most significant barriers to immunotherapies for cancer, including both adoptive T cell-based therapies (such as those in which T cells are engineered to express chimeric antigen receptors)[14, 15] and induced immunity through cancer vaccines[16]. For these reasons, macrophages are attractive targets for therapeutic intervention.[17–19] The promise of such a strategy was highlighted by a recent report that shifting macrophage phenotype was sufficient to provide benefits in a pre-clinical model of cancer treatment.[20] However, despite the importance of macrophage polarization to a broad range of diseases, a mechanistic and systems-level understanding of these phenomena is generally lacking.[4, 21, 22]

To date, investigations into macrophage functional polarization generally involve stimulating cells with one or more microenvironmental stimuli known to promote a particular functional phenotype.[1, 3, 23–27] Such cues are predominantly cytokines or chemokines but may also be metabolites or physical conditions such as hypoxia.[3] An important limitation of these approaches is that nearly all natural microenvironments *in vivo* are characterized by multiple stimuli that, taken individually, would promote polarization into distinct macrophage phenotypes. Therefore, in this study, we investigated how macrophages resolve physiologically and therapeutically relevant combinations of "incoherent" stimuli – those that individually promote divergent phenotypes – at both phenomenological and mechanistic levels. In particular, we investigated how the IL-10-driven macrophage phenotype is modulated by co-exposure to stimuli that may be considered incoherent with IL-10. IL-10 directs macrophage polarization to an immunosuppressive phenotype that has been likened to that observed in TAM.[1, 21, 23, 28, 29] Moreover, IL-10 is prevalent in both tumors and other immunosuppressive environments established by MDSC and regulatory T cells (Treg).[30, 31] Although stimuli such as IL-4 or IL-13 drive polarization to a distinct M2 phenotype[1], here we use "M2" as a shorthand refer to the IL-10-induced macrophage phenotype that is the focus of this study. We identified key molecular mechanisms by which the IL-10-driven macrophage phenotype is induced, maintained, and modulated under incoherent stimuli.

Together, these findings provide new insights into macrophage decision making and interactions within local immune networks.

## Materials and methods

### Cell culture

RAW264.7 cells (RAW cells) were a gift from David Segal, Experimental Immunology Branch, NIH (Bethesda, MD). RAW cells were cultured in complete DMEM medium (DMEM + 10% heat-inactivated FBS + 4 mM L-glutamine) on tissue culture treated dishes. Cells were passaged via incubating in PBS-EDTA (0.05 M EDTA) for 5 min followed by gentle cell scraping. M-CSF-containing L929-conditioned medium was made by culturing L929 cells (ATCC) in complete RPMI (RPMI + 10% heat-inactivated FBS + 4 mM L-glutamine) for 7 days. Bone marrow-derived macrophage (BMDM) differentiation medium comprised 10% L929-conditioned medium added to complete RPMI. To generate BMDM, 4–8 week old C57BL/6 male mice (Jackson Labs) were sacrificed and bone marrow cells in the femur were harvested and cultured in differentiation medium.[32, 33] Medium was replaced with fresh differentiation medium every 3 days of culture. At day 7, BMDM were harvested and characterized by flow cytometry to confirm differentiation using antibodies against surface antigens F4/80 (BD bioscience) and CD11b (BD bioscience) (Fig. S1). Where indicated, cells were activated with 100 ng/mL E. Coli 055:B5 LPS (Sigma-Aldrich). Cytokine treatments (IL-10, IL-12p70, IFN$\gamma$) were conducting using recombinant cytokines purchased from R&D Systems.

### Real-time quantitative PCR

RNA was isolated from cells (Omega Bio-Tek) and reverse transcribed into cDNA (Quanta Bioscience). Real-time quantitative PCR (qPCR) was conducted using SYBR green super mix (Bio-Rad). Approximately 10 ng of mRNA template were loaded per run. Primer concentrations were 200 nM for both forward and reverse primers, and primer sequences are presented in Table S1. Cycling conditions were as follows: 95 °C 5 min, 40 cycles (95 °C for 30 s, 55 °C for 30 s). qPCR data were analyzed using normalized Ct analysis[34], for which GAPDH was selected as an internal reference "housekeeping" gene. Experiments were conducted in biological triplicate and, unless otherwise stated, were normalized to samples from cells treated with LPS only. Error bars represent one standard deviation. Statistical analyses were conducted using two-tailed Student's t-tests.

### Western blots

Western blots were performed on whole cell lysates. RIPA buffer was used for protein extraction followed by centrifugation at 4 °C for 20 min to pellet cell debris. Protein lysate was boiled at 95 °C for 5 min in Laemmli buffer. Protein concentration was quantified by Bradford assay (Thermo Scientific), and 30 μg per sample was loaded and run in pre-cast gels (Bio-Rad). Samples were transferred to PVDF membranes overnight at 50 V. Antibodies used for Western blot analyses were: anti-SOCS1 (Abcam), anti-SOCS3 (Abcam), anti-pSTAT3 (Abcam), anti-pSTAT4 (Y693) (Abcam) anti-STAT3 (Cell Signaling), anti-STAT4 (Cell Signaling), anti-GAPDH (Abcam), anti-rabbit HRP-conjugated secondary antibody (Invitrogen). Transferred membranes were blocked using 5 % BSA in

TBST for 2 h followed by primary antibody labeling in 5% BSA in TBST overnight. Membranes that were incubated with primary antibodies overnight were washed with TBST three times followed by secondary antibody staining in 5% BSA in TBST for 1 h. Membranes were treated with ECL solution (Bio-Rad) for 5 min and then exposed on films (GE).

### Flow cytometry

**Surface staining**: cells were incubated in PBS-EDTA followed by gentle scraping to suspend, pelleted, and re-suspended in flow buffer (0.1 % BSA + PBS). Cells were re-pelleted, suspended in 50 ul of flow buffer and incubated in fixing buffer (1 % paraformaldehyde in PBS) for 30 min at 4 °C before washing 2 times in flow buffer. Normal mouse serum (Thermo Scientific) was used to block non-specific binding. Primary antibodies were added to the mouse serum-containing cell samples and incubated for 1 h at 4 °C. Cells were washed with flow buffer three times and stained with secondary antibodies for 30 min at 4 °C before washing 2 times with flow buffer and collecting for flow analysis. Positive signal were determined using isotype control samples to determine negative gating. **Intracellular staining**: Cells were harvested, washed and fixed as for surface staining. Cells were then suspended in permeabilization wash buffer (0.5 % saponin and 0.2 % BSA in PBS). Normal mouse serum was used to block both surface and intracellular non-specific binding. Primary conjugated antibodies were added to mouse serum-containing cell samples and incubated for 1 h at 4 °C before washing 2 times with flow buffer and collecting for flow analysis. Antibodies used for flow cytometry analyses were: surface antigens F4/80 (BD bioscience) and CD11b (BD bioscience) and intracellular antigen TNFα (BD bioscience). Samples were analyzed on an LSR II (BD), and mean fluorescent intensity (MFI) was calculated using FlowJo software (Treestar) and used to quantify relative protein expression levels. Statistical analyses were conducted using two-tailed Student's t-tests.

## Results

### Resolution of incoherent stimulation by IL-10 and IL-12

To begin investigating how IL-10 drives macrophage phenotype in the presence of incoherent stimuli, we first examined a "competition" between the cytokines IL-10 and IL-12. IL-12 is one of the few cues that has been reported to drive phenotypic conversion of TAM, TIM, and MDSC *in vivo*, promoting expression of M1-associated genes and suppressing M2-associated genes.[35–37] Some of these effects may be driven indirectly, since IL-12 induces IFNγ production in multiple cell types that may appear at the tumor site or in proximal lymph nodes, including NK cells, type I T helper (Th1) cells and dendritic cells[38, 39], and IFNγ promotes expression of M1-associated genes[1, 21, 40]. Nonetheless, IL-12 may also act upon macrophages directly and has been reported to drive phenotypic conversion of TAM and TIM *in vitro* (Fig. S2).[35, 41] However, the specific mechanisms by which IL-12 might induce phenotypic conversion, especially in the presence of immunosuppressive signals such as IL-10, are not known.

The downstream pathways through which the IL-10 and IL-12 receptors signal exhibit several potential modalities for cross-talk and competition, via the suppressors of cytokine

signaling proteins, SOCS1 and SOCS3 (Fig. 1a). IL-10 induces signaling via the transcription factor Signal Transducer and Activator of Transcription 3 (STAT3), which drives the production of SOCS1 and SOCS3.[42, 43] IL-12 induces signaling via STAT4, which promotes the production of SOCS1.[44] SOCS1 inhibits signaling downstream of both IL-10 and IL-12 receptors[45], while SOCS3 inhibits signaling downstream from IL-12 but not IL-10[43]. Therefore, SOCS1 and SOCS3 may mediate asymmetric feedback regulation of signaling pathways that regulate macrophage phenotype determination. This network has also been reported to exhibit positive feedback in response to IL-10 or IL-12, via increased IL-10 secretion[46] and increased expression of the IL-12 receptor[47], respectively. Notably, this qualitative conceptual model (Fig. 1a) cannot predict the outcome of a competition between incoherent stimuli IL-10 and IL-12.

To experimentally investigate how macrophages resolve an IL-10/IL-12 competition, murine RAW264.7 (RAW) macrophages were pre-treated with IL-10, IL-12, or both at biologically-relevant doses[35, 39, 41] for 12 h, and then cells were "activated" with lipopolysaccharide (LPS). LPS-induced Toll-like Receptor 4 (TLR4) signaling generally promotes macrophage M1 responses and can also be induced via endogenous signals, for example in necrotic tissue.[48, 49] However, in the presence of IL-10, TLR4 signaling enhances expression of M2-associated genes that are not induced by LPS or IL-10 alone.[50] In this experiment, gene expression was profiled 3 h post-LPS activation to focus on early phase responses (Figs. 1b, S3). In general, pre-treatment with IL-10 suppressed some M1-associated gene expression and enhanced M2-associated expression of IL-10. Inclusion of IL-12 in the pre-treatment shifted expression patterns somewhat, and several genes (e.g., IL-4, IDO, and IFNγ) were moderately up-regulated by IL-12 pretreatment. However, in general, IL-12 neither promoted M1 genes nor suppressed M2 genes. To investigate whether this dominance was an artifact of the RAW cell line, we compared responses of RAW cells to those of primary bone marrow derived macrophages (BMDM) following IL-10/IL-12 pre-treatment, focusing on the most dynamically-regulated genes (TNF α and IL-10). Both cell types exhibited similar patterns of TNFα gene and protein expression in response to IL-10 dose, which was irrespective of IL-12 co-treatment (Figs. 1c–d, S4). In BMDM, IL-10 expression was moderately suppressed by IL-12 pre-treatment, although this effect was not observed in RAW cells. Because TNFα expression is regulated post-transcriptionally by mRNA stabilization[51, 52], we also investigated whether TNF α protein levels correlated with mRNA levels. TNF α protein expression was assessed by intracellular flow cytometry in order to capture patterns evident at this early time point (3 h post-LPS activation), at which time secreted protein levels are expected to be low[53, 54]. Intracellular TNFα protein levels largely mirrored the pattern observed at the mRNA level. Controls in which cells were treated with IL-10/IL-12 without LPS activation exhibited similar patterns (Fig. S3b). To investigate whether this apparent dominance of IL-10 was limited to the early response, an additional IL-10/IL-12 competition was conducted in which gene expression was profiled at 3, 6, and 12 h after LPS activation (Fig. S5). Overall, macrophages exposed to IL-10 and IL-12 simultaneously again exhibited gene expression patterns similar to those induced by IL-10 alone.

Having characterized the IL-10/IL-12 competition at a phenomenological level, we next investigated potential molecular mechanisms underlying the dominant role of IL-10 in

determining macrophage phenotype in this system. We hypothesized that IL-10 may exert its dominant effect via either "upstream" or "downstream" mechanisms, or both. An "upstream" mechanism would block the effects of IL-12 by blocking signaling via its receptor (e.g., induction of SOCS3 expression) (Fig. 1a). A "downstream" mechanism would comprise IL-10 signaling-induced expression of a regulator of transcription or translation that suppresses the expression of M1 genes, such that expression of this regulator is not sensitive to IL-12-induced signaling.

To first investigate upstream mechanisms, we examined the involvement of SOCS proteins, given their potential roles discussed above (Fig. 1a). SOCS1, which blocks both STAT3 and STAT4 phosphorylation[18, 55], was expressed at a basal level that did not vary substantially (at either protein or mRNA levels) following treatment with various combinations of IL-10, IL-12, and LPS (Fig. 2a, b). In contrast, SOCS3 mRNA and protein expression increased proportionally with IL-10 treatment (Fig. 2a, b). Elevated SOCS3 expression did not require LPS activation, suggesting that this mechanism could confer blockade of IL-12-mediated STAT4 phosphorylation prior to activation with LPS. To investigate this possibility, STAT4 phosphorylation and SOCS3 expression were monitored over time during an IL-10/IL-12 competition in the absence of LPS (Fig. 2c–e). At both low and high doses of IL-10 (2, 10 ng/mL, respectively), IL-12-mediated STAT4 phosphorylation was impaired for times greater than ~30 minutes, which correlated with the elevated SOCS3 gene and protein expression observed beginning 30–60 minutes post-cytokine treatment (Fig. 2d–f) and is consistent with known mechanisms of SOCS3[43]. However, high IL-10 doses impaired STAT4 phosphorylation at time points preceding elevated SOCS3 expression (e.g., 5 minutes post-cytokine treatment), and this effect was not observed for low IL-10 doses. Together, these results indicate that an "upstream" mechanism mediated by IL-10-induced SOCS3 expression may explain long-term blockade of IL-12-mediated signaling, and additional upstream mechanisms may exist (see Discussion).

Since these upstream mechanisms would not explain why IL-10 induced a dose-dependent suppression of M1 genes and induction of M2-associated IL-10 (Fig. 1), we next investigated whether potential "downstream" regulators could explain these observations. Note that such a proposed IL-10-induced regulator need not necessarily promote the expression of IL-10, since IL-10-induced STAT3 phosphorylation already drives expression of IL-10 and other M2 genes[56]. One candidate regulator is cyclic AMP-responsive element-binding protein (CREB), which indirectly induces M2 genes and suppresses M1 genes. CREB induces the expression CEBPβ, which drives expression of M2 genes (e.g., IL-10). CREB also drives the expression of a negative regulator of M1 gene expression termed dual specificity protein phosphatase 1 (DUSP1).[4, 57, 58] An additional candidate regulator is BCL3, since expression of this protein is induced by STAT3 phosphorylation, and BCL3 blocks NF-κB (p50/p65)-mediated induction of TNFα gene expression.[59, 60] A final candidate examined is interferon regulatory factor (IRF5), which is a transcriptional activator for multiple inflammatory cytokines including TNFα, IL-12 and IFNγ, and IRF5 also suppresses expression of anti-inflammatory cytokines including IL-10.[4, 61]

We investigated whether the expression of these candidate genes fit the pattern predicted for a downstream regulator: dose-dependence on IL-10 and independence of IL-12 treatment.

Of all candidates examined, only BCL3 and DUSP1 expression fit this pattern (Figs. 2g, S6). However, DUSP1 induction was dependent on activation of cells with LPS, while BCL3 induction did not require LPS activation, suggesting that only BCL3 might mediate the dominant effects of IL-10 prior to activation by LPS. Note that BCL3 gene expression was enhanced about 4-fold by LPS activation, but the pattern of dose-responsiveness to IL-10 was unaltered by LPS activation (Figs. 2g, S6). Thus, BCL3 may play an important role in the induction of the IL-10-driven macrophage phenotype both before and after these cells become activated.

## Phenotypic reversion upon IL-10 removal

We next investigated how the IL-10-driven macrophage phenotype may be sustained or altered following initial functional commitment. In prior work, when TIM or TAM were removed from tumor-bearing mice and cultured *in vitro,* addition of IL-12 to the *in vitro* culture suppressed M2-associated expression (e.g., IL-10 secretion by TAM) and enhanced M1-associated expression (e.g., TNF α secretion by both TIM and TAM).[35] In such experiments, removal of macrophages from an animal may reduce the concentration of cytokines to which these cells are exposed, potentially rendering them more directly responsive to IL-12. By analogy, we next investigated whether removing extracellular IL-10 from cultures of macrophages previously driven to M2 in our distinct *in vitro* model would alter phenotype and responsiveness to IL-12. RAW cells and BMDM were exposed to IL-10 for 12 h, then exogenous IL-10 was removed from the system by replacement with fresh medium (+/− IL-12), and 12 h later, cells were activated with LPS (Figs. 3a, S7). Notably, removal of IL-10 was generally sufficient to drive reversion of both BMDM and RAW cells to a more M1-like phenotype; addition of IL-12 induced no significant changes in RAW cell phenotype, and with the exception of IL-12/IL-23p40, IL-1β and IL-23p19, BMDM gene expression was also largely unaltered by IL-12 addition. Interestingly, IL-12 treatment resulted in a modest decrease in IL-23p19 gene expression in BMDM, via unknown mechanisms. Thus, sustaining the IL-10-induced M2 phenotype required sustained IL-10-induced signaling, reversion to a phenotype that could be driven to M1 upon LPS activation was spontaneous upon IL-10 removal, and neither of these processes was directly impacted by IL-12.

We next investigated whether this spontaneous phenotypic reversion was reflected in the expression of the key regulator BCL3. We hypothesized that if BCL3 is a central regulator of IL-10-induced polarization, then since removal of IL-10 was sufficient to restore M1-like responses to LPS (Fig. 3a), then BCL3 expression would be expected to decrease following removal of IL-10 from the environment. As predicted, when macrophages were pre-treated with IL-10 for 12 h, and then IL-10 was removed from the culture medium, BCL3 gene expression dropped to baseline within 3 h (Fig. 3b). This pattern is thus consistent with our proposed model in which BCL3 plays a central role in mediating the dose-dependent and reversible effects of IL-10-induced suppression of M1-associated gene expression.

## Resolution of incoherent stimulation by IL-10 and IFNγ

Since IL-12 did not directly modulate IL-10-induced macrophage polarization in our model, we next investigated a competition between IL-10 and the potent pro-M1 stimulus IFNγ. As

noted above, IL-12-induced production of IFNγ by neighboring immune cells may mediate indirect effects of IL-12 on macrophages *in vivo*.[38, 39] Previously, the primary insight into this interaction was that when macrophages were pre-polarized to M1 via exposure to IFNγ during differentiation from primary monocytes, subsequent exposure to IL-10 + LPS did not result in induction of M2 genes associated with immunosuppression.[62] Furthermore, this IFN γ-induced pre-polarization prevented IL-10-mediated inhibition of the expression of M1-associated genes and proteins. How macrophages would resolve *simultaneous* exposure to this set of incoherent stimuli, however, was not clear. To investigate, RAW cells and BMDM were treated with combinations of IL-10 and IFNγ, followed by activation with LPS (Figs. 4, S8, S9). Interestingly, co-stimulation with IFNγ did not block IL-10-mediated suppression of TNFα gene expression, although IFNγ did suppress IL-10-induced expression of IL-10. These effects were similar in RAW cells and BMDM (Fig. 4b, S9). In addition, IFNγ induced the expression of iNOS and IL-12/IL-23p40 even in the presence of IL-10 (Figs. 4a, S8), indicating that IFNγ promoted some M1-associated gene expression even in IL-10-rich environments.

We next investigated potential mechanisms by which macrophages may resolve incoherent stimulation by IL-10 and IFNγ. Because IFNγ failed to reverse IL-10-mediated suppression of TNFα expression following activation with LPS, we hypothesized the absence of any "upstream" mechanism (e.g., induction of SOCS1 expression) that would block signaling downstream of the IL-10 receptor. To investigate, we examined SOCS1 expression and STAT3 phosphorylation following treatment with IL-10 and/or IFNγ (Fig. 5). Although IFNγ treatment enhanced expression of SOCS1 mRNA after LPS-mediated activation, IFNγ treatment alone did not drive increased production of SOCS1 protein, nor did IFNγ treatment substantially impair IL-10-mediated phosphorylation of STAT3. These observations support the lack of an upstream mechanism in the absence of LPS, and thus downstream regulator(s) may mediate the resolution of incoherent stimulation by IL-10 and IFNγ (see Discussion).

## Discussion

This investigation revealed several important features of macrophage responses to complex stimuli. First, direct competition between IL-12 and IL-10 yielded a general dominance of the IL-10-driven M2 phenotype (Figs. 1, S2, S3, S4). Moreover, removal of IL-10 was sufficient to drive reversion of cells to a phenotype resembling M1 after activation with LPS (Fig. 3a). While IL-10-induced expression of SOCS3 may confer long-term non-responsiveness to IL-12, IL-10-mediated reduction in IL-12-induced STAT4 phosphorylation was sometimes apparent as early as 5 minutes post cytokine treatment (Fig. 4). It is possible that the IL-10 and IL-12 receptors compete for downstream signaling molecules, such as Tyrosine kinase 2 (TyK2), which is involved in both pathways.[63–65] We also present evidence that BCL3 is a central "downstream" mediator of IL-10-induced suppression of the M1 phenotype[59], and BCL3 expression increased upon exposure to IL-10 and decreased upon IL-10 removal (Figs. 2g, 3b, S6). Together, these results suggest that BCL3 may be an attractive therapeutic target for disrupting at least some features of the dominant IL-10-induced immunosuppressive phenotype. These results also suggest that comparing our observations with a direct IL-10/IL-12 competition in TIM and TAM, which are directly

responsive to IL-12 *in vitro*[35, 41], may elucidate whether IL-10 exerts a similarly dominant influence in these macrophages.

In a distinct pairing of incoherent stimuli, IFNγ suppressed IL-10-induced expression of IL-10 (Fig. 4, S8, S9), which could disrupt an important positive feedback loop within immunosuppressive networks[46]. Since IFNγ production is induced by IL-12 *in vivo*[38, 39], it may thereby mediate a key indirect effect of IL-12 on macrophage polarization, even in the presence of IL-10 (Fig. 6a). Although IFNγ also drove the expression of M1-associated genes iNOS and IL-12/IL-23p40 in the presence of IL-10, some M1 genes (including TNFα) were suppressed (Figs. 4, S8), suggesting that co-exposure to this pairing of incoherent stimuli partially mutes both IFNγ and IL-10-induced macrophage phenotypes. Our data did not support the existence of an upstream mechanism by which IFNγ induces expression of SOCS1 to inhibit IL-10-mediated signaling (Fig. 5), and thus downstream regulator(s) may mediate resolution of co-stimulation by IL-10 and IFNγ. One candidate regulator is glycogen synthase kinase 3 (GSK3), which is activated by dephosphorylation in response to IFNγ treatment.[66, 67] GSK3 blocks LPS-induced expression of IL-10 by impairing the MAPK-mediated phosphorylation of CREB downstream from TLR4 (Fig. S10).[58, 67, 68] Since GSK3 does not block TLR4-mediated activation of NF-κB, this mechanism would also explain why IFNγ does not render cells non-responsive to LPS[40]. Ultimately, such models of core regulatory networks can also motivate the investigation of clinically relevant strategies. For example, if we speculate (or determine) that the core IFNγ/IL-10 regulatory network proposed here (Fig. 6b) applies to TAM and TIM in vivo, it would be informative to investigate whether IL-10 receptor blockade can boost the efficacy of macrophage-targeted drugs. For example, in a model of glioblastoma multiforme (GBM), BLZ945 (a colony stimulating factor-1 receptor inhibitor) drove suppression of M2-associated genes but not expression of M1-associated genes, despite elevated IFNγ at the tumor[69]. Our model suggests that sustained IL-10 signaling could explain this effect, and perhaps blockade of this pathway could promote enhanced M1-associated phenotypes.

This investigation employed a novel approach to studying macrophage decision-making by applying "incoherent" stimuli to illuminate dominant features of cellular regulation. This strategy has previously proven informative for elucidating networks regulating processes such as apoptosis/survival[70], stem cell differentiation[71], T cell lineage commitment[72], and B cell fate outcome[73]. An important feature of these investigations, including ours, is that qualitative descriptions of cellular networks are insufficient to predict the outcomes of such inherently quantitative experimental investigations. Indeed many related cells types including macrophages of different types, MDSC, monocytes and dendritic cells likely share similar intracellular signaling topologies. Thus, experimental interrogation by incoherent stimuli could reveal whether similar patterns of dominance are present in related cells, perhaps most importantly in TIM, TAM, and the distinct IL-4/IL-13 induced M2 phenotypes[1]. In general, the degree to which conclusions reached using pairwise combinatorial stimuli hold as environmental complexity increases must also be evaluated. Ultimately, such an approach may identify additional experimentally testable hypotheses and help to bridge the divide between *in vitro* experiments and complex environments *in vivo*.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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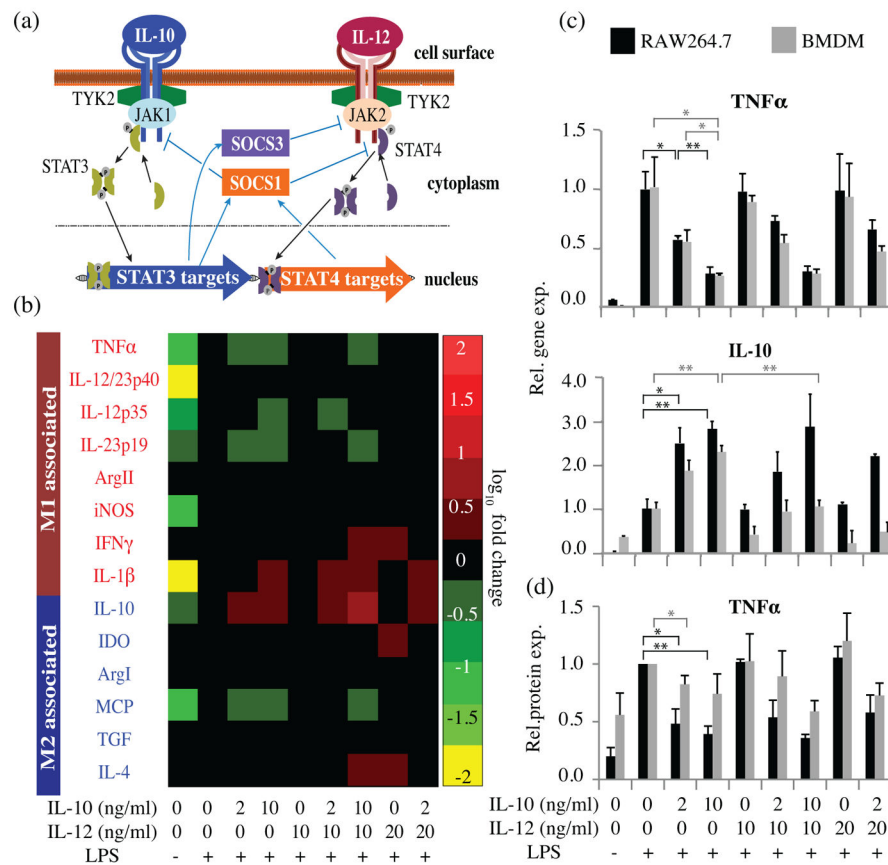
**Figure 1. Dose dependent responses to incoherent stimuli IL-10 and IL-12**
**(a)** Conceptual model of potential motifs of cross-talk and competition in the IL-10/IL-12 signaling network in macrophages. **(b)** RAW cells were treated with combinations of IL-10 and IL-12 for 12 h, activated with LPS for 3 h, and profiled by qPCR. Red text: genes typically annotated as M1, blue text: genes typically annotated as M2. Heat map colors indicate up-regulation or down-regulation vs. the LPS-only control as per the scale bar. All data, including non-responsive genes not shown here, are shown with error bars in Figure S3. **(c)** Comparative gene expression responses of RAW cells and BMDM to IL-10/IL-12 competition. Error bars represent +/−1 standard deviation, from biological triplicates. **(d)** Comparative intracellular TNFα protein expression by RAW cells and BMDM following IL-10/IL-12 competition, quantified by flow cytometry. Error bars represent +/−1 standard deviation, from three independent experiments. For additional statistical analysis of panels C–D, see Figure S4. (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001)
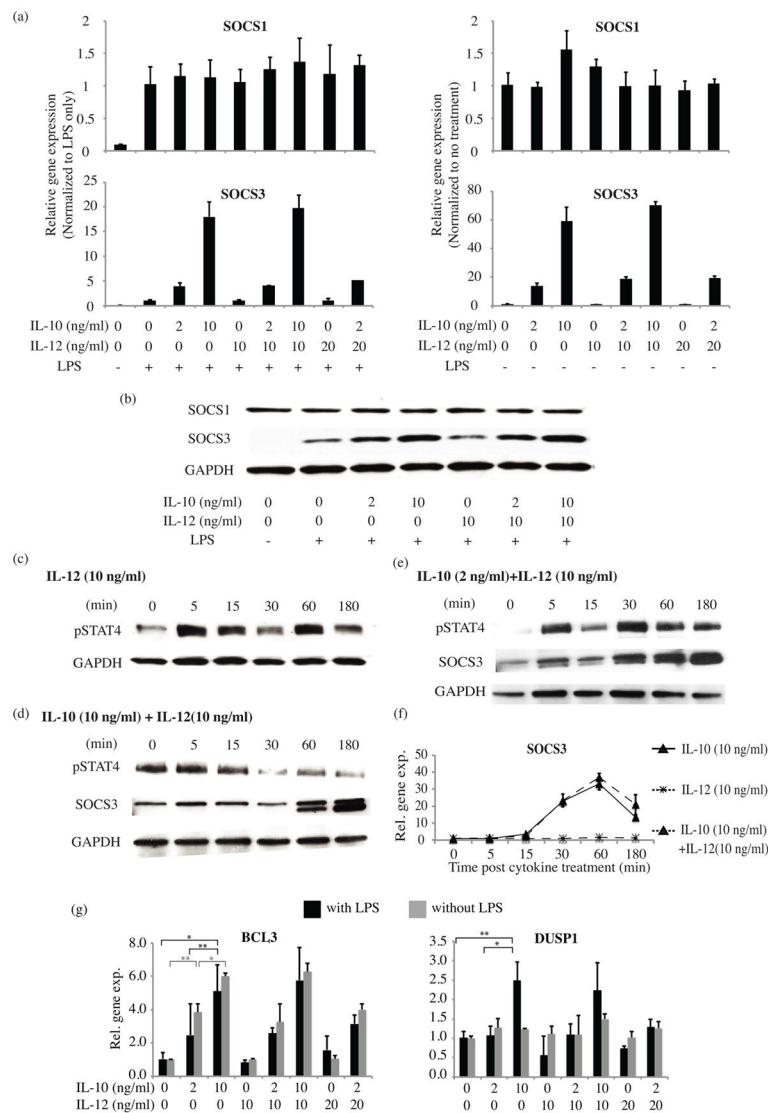
**Figure 2. Potential regulation of IL-10-dominated polarization**
**(a)** SOCS1 and SOCS3 gene expression in response to 12 h of IL-10 and IL-12 treatment (+/− subsequent activation by LPS for 3 h). Experiments in panel (a) were conducted in biological triplicate in RAW cells; the left panel was normalized to the LPS-only control, and the right panel was normalized to the "no treatment" control. **(b)** SOCS1 and SOCS3 protein expression in response to IL-10/IL-12 treatment followed by 3 h activation with LPS **(c)** pSTAT4 protein expression post IL-12 exposure **(d, e)** pSTAT4 and SOCS3 protein expression after IL-10/IL-12 treatment **(f)** SOCS3 gene expression following IL-10/IL-12 treatment. Experiments in panel (f) were conducted in biological triplicate in RAW cells and normalized to the "no treatment" control. **(g)** RAW cells were treated with IL-10 and IL-12 for 15 h (+/− activation with LPS for the final 3 h) and harvested for qPCR analysis. Each data series was normalized to its own "no cytokine" control. For additional statistical analysis, see Figure S6. (*p   0.05, **p   0.01, ***p   0.001)
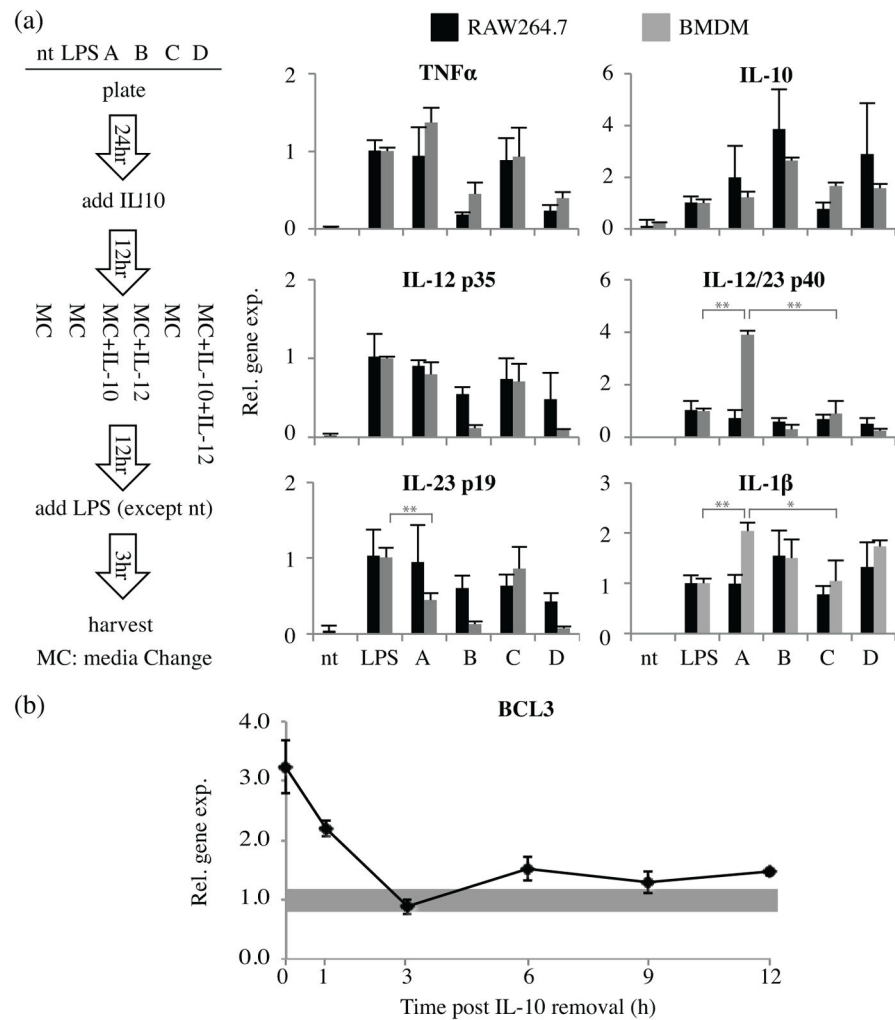
**Figure 3. Maintenance of macrophage phenotype via sustained IL-10 exposure**
**(a)** RAW cells (black) and BMDM (grey) were exposed to the various sequential treatment conditions described in the schematic at left. Cells were harvested 3 h after LPS activation for qPCR analysis. Error bars represent +/−1 standard deviation, from biological triplicates. (MC: Media change, *p 0.05, **p 0.01, ***p 0.001) **(b)** BCL3 gene expression was measured at various time points after IL-10 removal. Data were normalized to a no treatment control, shown by a grey bar spanning +/−1 standard deviation, from biological triplicates. (*p 0.05, **p 0.01, ***p 0.001)
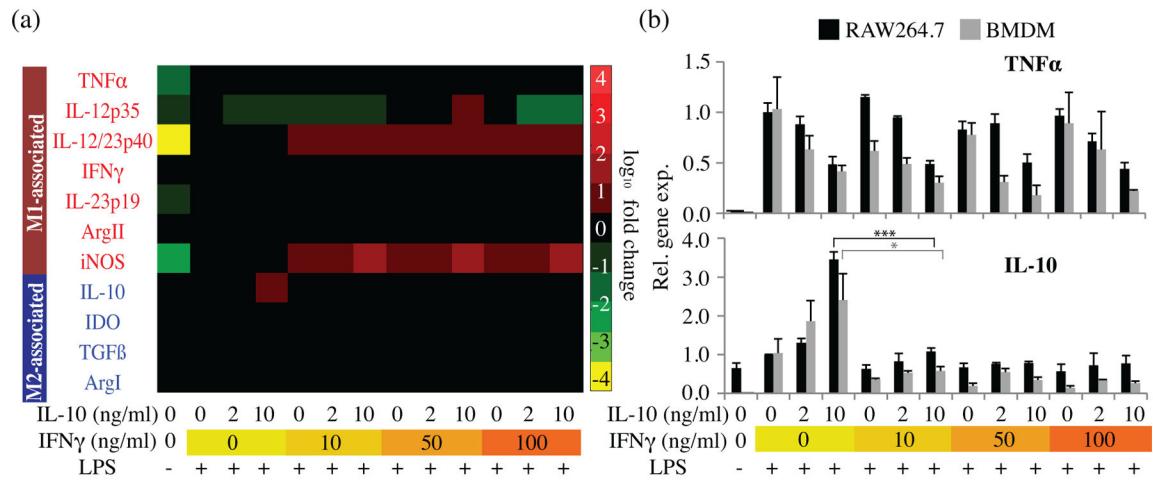
**Figure 4. Macrophage polarization in response to incoherent stimuli IL-10 and IFNγ**

**(a)** RAW cells were treated with combinations of IL-10 and IFNγ for 12 h, activated with LPS for 3 h, and profiled by qPCR. Heat map is formatted as in Figure 1, and all data, including non-responsive genes not shown here, are reported with error bars in Figure S8. **(b)** Comparative gene expression for RAW cells and BMDM treated as in (a). Error bars represent +/−1 standard deviation, from biological triplicates. For additional statistical analyses, see Figure S9. (*p    0.05, **p    0.01, ***p    0.001)
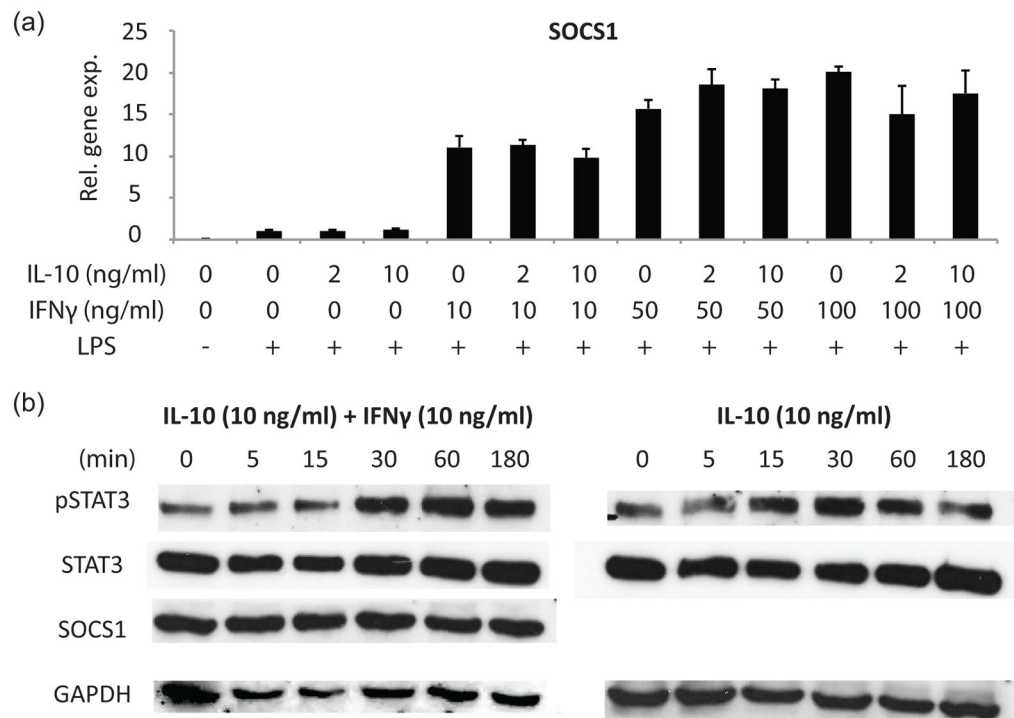
**Figure 5. Evaluating upstream regulation of the competition between incoherent stimuli IL-10 and IFNγ**

**(a)** SOCS1 gene expression after 12 hours treatment with IL-10 and IFNγ followed by 3 hours of activation with LPS. Experiments were conduced in biological triplicate in RAW cells and normalized to the LPS only control. **(b)** SOCS1 protein expression and STAT3 phosphorylation following treatment with IL-10 and IFNγ without LPS activation.
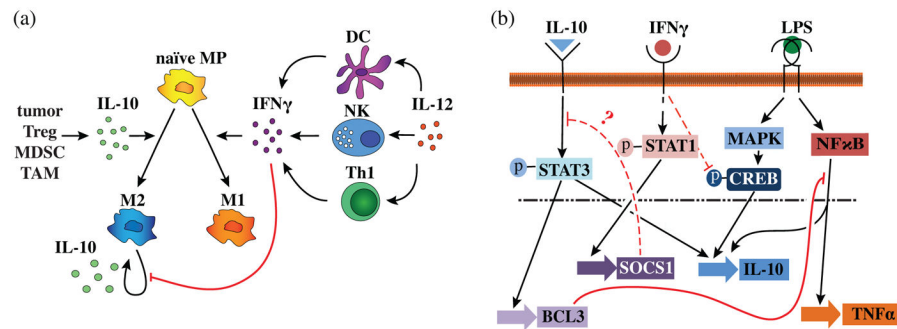
**Figure 6. Resolution of incoherent stimuli by multiscale networks**
**(a)** Proposed multicellular network model by which IL-12 may indirectly promote an M1-like phenotype. **(b)** Proposed core intracellular regulatory network mediating resolution of IL-10/IFN γ-induced signaling. Dotted lines indicate potential mechanisms requiring further investigation (see Fig. S10 for additional detail).