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Insight Box

Macrophages are attractive targets for therapeutic intervention given their important role in both advancing and resolving inflammation. We combine experimental evidence with computational modeling to characterize the response of macrophages to combination stimuli. We reveal that co-addition or pre-treatment with pro-inflammatory (M1) stimuli enhances the pro-healing (M2) response over time; in contrast, co-addition or pre-treatment with M2 stimuli diminishes the M1 response. We apply a model selection approach to assess gene regulation topologies of M1-M2 signaling axis, and show that incoherent feed-forward of M1 activation as well as both inhibition and activation of M2 by M1 were required. Our work serves as a basis for understanding the effects of complex signals that may be present in the macrophage microenvironment.

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Regulation of macrophage polarization and plasticity by complex activation signals

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Abstract

Macrophages are versatile cells of the immune system that play an important role in both advancing and resolving inflammation. Macrophage activation has been described as a continuum, and different stimuli lead to M1, M2, or mixed phenotypes. In addition, macrophages expressing markers associated with both M1 and M2 function are observed *in vivo*. Using flow cytometry, we examine how macrophage populations respond to combined M1 and M2 activation signals, presented either simultaneously or sequentially. We demonstrate that macrophages exposed to a combination of LPS, IFN-γ, IL-4, and IL-13 acquire a mixed activation state, with individual cells expressing both M1 marker CD86 and M2 marker CD206 instead of polarizing to discrete phenotypes. Over time, co-stimulated macrophages lose expression of CD86 and display increased expression of CD206. In addition, we find that exposure to LPS/IFN-γ potentiates the subsequent response to IL-4/IL-13, whereas pre-polarization with IL-4/IL-13 inhibits the response to LPS/IFN-γ. Mathematical modeling of candidate regulatory networks indicates that a complex inter-dependence of M1- and M2-associated pathways underlies macrophage activation. Specifically, a mutual inhibition motif was not by itself sufficient to reproduce the temporal marker expression data; incoherent feed-forward of M1 activation as well as both inhibition and activation of M2 by M1 were required. Together these results corroborate a continuum model of macrophage activation and demonstrate that phenotypic markers evolve with time and with exposure to complex signals.

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Introduction

Macrophages are functionally complex cells of the immune system, and participate in both innate and acquired immunity. In addition to their roles as professional phagocytes and antigen-presenting cells, macrophages are sensitive integrators and transducers of biochemical signals with a wide repertoire of responses. A recent study of the macrophage transcriptomic response to a range of stimulatory molecules identified dozens of distinct mRNA coexpression modules.[1] Two particular macrophage response patterns which have been widely recognized are the M1 and M2 programs, named because they are respectively elicited by products of Th1 and Th2 cells.[2,3] The M1 response, also described as classical activation, is typically evoked *in vitro* by treating cells with interferon-γ (IFN-γ) and lipopolysaccharide (LPS), a bacterial cell wall component and TLR4 agonist. The M1 phenotype includes production of inflammatory cytokines including TNF-α and IL-1β.[4] M1 macrophages also undergo a metabolic transition towards glycolysis and secrete free radicals. The M2 response, also known as alternative activation, is evoked by IL-4 and IL-13 treatment and is characterized by increased expression of CD206, scavenger receptors, and—in mice—arginase 1. The M2 response has been further classified as the M2a response after other forms of alternative activation were identified. These stereotyped responses are likely only fully recognized *in vitro* but M1-like and M2-like phenotypes are readily identified in physiological contexts.

M1- and M2-activated macrophages exhibit characteristic transcriptional and secretory profiles. M1 activation is associated with STAT1 and IRF activation, and M2 activation is associated with STAT6 activity.[5] These pathways suppress each other; IL-4-induced STAT6 activation suppresses STAT1-dependent transcription in mouse macrophages,[6] and STAT1 activation suppresses STAT6-dependent transcription.[7] Further, costimulation with IL-4 reduces

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the IFN-γ-dependent surface expression of FcγR on human monocytes.[8] Yet, despite the evidence of mutual repression, markers associated with both M1 and M2 phenotypes have been observed simultaneously on individual cells *in vivo*.[9] This co-expression may reflect simultaneous co-activation of M1 and M2 programs. A similar process is observed in T cells, where differentiation of CD4+ T cells to IFN-γ-secreting Th1 cells or IL-4-secreting Th2 cells in mixed culture conditions yields a tunable continuum of cell fates.[10] Modeling revealed that this outcome was consistent with gene regulation governed by a mutual inhibition, self-activation (MISA) network, a common motif thought to govern alternative fate-decisions in many cell types,[11] including macrophages.[12,13]

M1 and M2 marker coexpression may also indicate that cells are shifting from one phenotype to another as the microenvironment changes. Indeed, macrophages have been shown to exhibit phenotypic plasticity *in vitro*. M1-activated macrophages induced by exposure to bacteria[14] or IFN-γ, alone[15] or in combination with LPS,[16] can be re-polarized to express markers associated with an anti-inflammatory phenotype upon subsequent treatment with IL-4, alone or in combination with IL-13. Similarly, macrophages treated with IL-4 will express inflammatory markers upon subsequent treatment with LPS or IFN-γ.[14] The plasticity of these influential cells has made them an attractive target for immunomodulation; scaffolds and materials that achieve controlled delivery of macrophage-activating agents is an active area of research for treatment of diseases involving macrophage dysregulation (reviewed in Ref. 17). In atherosclerosis, sustained inflammation exacerbates oxidative stress in the plaque.[18] Plaque shoulders, which are prone to rupture, are typically dominated by macrophages expressing markers associated with M1 activation.[9] Reprogramming macrophages toward a M2 phenotype may prevent plaque rupture

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and promote plaque resolution by encouraging matrix deposition.[19,20] In the case of cancer, tumor-associated macrophages (TAMs) are thought to induce anti-inflammatory signaling that helps protect the tumor from immune assault.[21] Recent evidence suggests presenting M1-activating factors to TAMs can help engage the immune system to attack the tumor.[22] An improved understanding of how macrophages respond to stimuli that redirect their phenotype should help develop better therapeutic approaches for these important pathologies.

In this study, we investigated how treatment with mixed M1 and M2 stimuli, either simultaneously or sequentially, regulates macrophage phenotype. We were motivated to understand whether a mixed phenotype represents a superposition of the M1 and M2 phenotypes, a transition between states, or, as some findings have suggested,[23] a unique mixed program. To consider the expression state of individual cells, we used flow cytometry to assay surface expression of M1 marker CD86 and M2 marker CD206. Mouse bone marrow derived macrophages were stimulated with LPS/IFN-γ and/or IL-4/IL-13 at various concentrations over durations of 24-96 hours. Our findings suggest that macrophages adopt a mixed phenotype dependent on the relative strength of stimuli present, and that cells progress towards a M2 phenotype over time. These temporal changes in expression were found to be consistent with a mathematical model comprising a modified MISA network. In addition, reprogramming of macrophages to the opposing phenotype is dependent on the extent of pre-polarization. More specifically, expression of CD206 in response to IL-4/IL-13 is enhanced by pre-polarization towards an M1 phenotype with LPS/IFN-γ. In contrast, expression of CD86 in response to LPS/IFN-γ, particularly at low concentrations, is inhibited by pre-polarization towards an M2 phenotype with IL-4/IL-13. Together, these data provide evidence of a macrophage phenotypic continuum by analysis of phenotypic markers at the single cell level, and suggest that

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macrophage reprogramming by combined activation signals is dependent on initial polarization state and dosage of stimulation.

Results

Co-stimulated macrophages express markers of both M1 and M2 activation

We first sought to establish a concentration range that would yield a submaximal response to facilitate detection of enhancement and repression effects. We exposed mouse bone marrow derived macrophages (BMDM) to varying concentrations of stereotypical M1 (LPS/IFN-γ) or M2 stimuli (IL-4/IL-13). To maximize the sensitivity of our assays, we selected concentrations from 0 to 0.3 ng/ml for LPS/IFN-γ and 0 to 1 ng/ml for IL-4/IL-13 since these ranges did not completely saturate expression of CD86 and CD206. Cells were exposed to stimulus for 48 hours and assayed for expression of M1 marker CD86, a T-cell costimulatory molecule, and M2 marker CD206, a mannose receptor, by flow cytometry. We found that median CD86 labeling intensity increased tenfold as the concentration of LPS/IFN-γ was increased from 0 to 0.3 ng/ml (Fig. S1a and b). Labeling intensity of CD206 increased threefold as IL-4/IL-13 was increased from 0 to 1 ng/ml (Fig. S1c and d). At these concentration ranges, the expression of phenotypic markers was not saturating, so that the expression of markers generally increased with stimulation concentration.

To explore the effect of co-stimulation with M1 and M2 activation signals on macrophages, BMDM were exposed simultaneously to combinations of LPS/IFN-γ and IL-4/IL-13 at concentrations in the determined range for 48 hours. Expression of CD86 and CD206 was analyzed by flow cytometry (Fig. 1a and b). Notably, the population remained single-peaked in plots of CD86 expression vs CD206 expression, and did not show separation into distinct

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subpopulations. Cells generally did not individually commit to exclusive CD86 or CD206 expression. Indeed, CD86 and CD206 expression were only partially inhibited by exposure to their opposing polarization signal. Analysis of CD86 expression in LPS/IFN-γ-stimulated cells (0.3 ng/ml) showed that moderate amounts of co-added IL-4/IL-13 (0.1 ng/ml) in fact increased the median expression of CD86 40% (± 22% SEM, n=3; Fig. 1c). Further increasing the IL-4/IL-13 concentration to 1 ng/ml abrogated this enhancement. Meanwhile, the expression of CD206 in IL-4/IL-13 stimulated cells was not affected by the co-addition of LPS/IFN-γ stimuli at any concentration (Fig. 1d). In sum, these data demonstrate that macrophages exposed to combinations of the activation signals LPS/IFN-γ and IL-4/IL-13 express both CD86 and CD206 at 48 h of stimulation, and repression of the contrasting pathway was only partially observed with these phenotypic markers.

Co-stimulated macrophages progress towards a M2-like phenotype

To examine how macrophage phenotype evolves over time after exposure to stimulus, we exposed BMDM to stereotypical M1, M2, or mixed stimuli, and examined CD206 and CD86 expression at 24 hour intervals for 96 hours (Fig. 2a). In conditions containing LPS/IFN-γ, including the mixed condition, cells began to express more CD206 and less CD86 over time. The IL-4/IL-13-only and no-treatment conditions exhibited a transient increase in CD206 that peaked at 48 hours, and relatively low and stable CD86 expression (Fig. 2b). In cells treated only with LPS/IFN-γ, the expression of CD86 decreased over the course of the 96 hour time period with the greatest decrease occurring between 24 and 48 hours (Fig. 2c). Macrophages exposed to mixed LPS/IFN-γ and IL-4/IL-13 stimulation also displayed a decrease in CD86 expression, which was similar in profile to that of cells stimulated with only LPS/IFN-γ. In contrast, CD206

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expression in response to IL-4/IL-13 increased up to 48 hours after stimulation, and then subsequently decreased after 72 hours, but the levels remain elevated when compared to expression in unstimulated macrophages even at 96 hours (Fig. 2d). The extent of decrease was similar, but occurred at earlier time points, when the stimulus was washed out after 24 hours (Supplementary Fig. S3). Interestingly, mixed stimulation conditions induced a marked increase in CD206 expression, which was significantly enhanced relative to the cells stimulated with only IL-4/IL-13.

Modeling proposes a complex interdependence of M1- and M2-associated pathways

In order to gain further insight into the logic of macrophage activation, we performed mathematical modeling of CD86 and CD206 expression in response to the different co-stimulatory conditions. Our modeling strategy was designed to identify the key features of the regulatory logic linking CD86 and CD206 expression ("outputs") to stimulation by LPS/IFN-γ and/or IL-4/IL-13 ("inputs"). To this end, we analyzed a suite of candidate models and performed model selection based on fitting to the experimental 96-hour timecourse data (Fig. 3). Mathematical descriptions of the models are provided as Supplementary Equations; the parameters are described in Supplementary Table S2 and the best-fit values are given in Supplementary Table S3. Rather than treating signaling and gene regulatory networks in detail (as quantitative parameters remain unknown), the network models comprise a small number of interacting nodes representing inputs, outputs, and M1- and M2-associated pathways. Models of T cell specialization[10,24,25] and fate-decisions in diverse cell types[11] commonly employ a core Mutual Inhibition, Self-Activation (MISA) network motif. We found that the basic MISA motif was insufficient to reproduce the observed temporal expression patterns, including the decrease

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of CD86 expression after 24 hours and the sharp increase of CD206 after 24 hours under co-stimulatory conditions.

We explored a number of additional small-network topologies, consistent with current knowledge of macrophage activation pathways. In the MISA paradigm, costimulus results in a mixed response, in which both markers are expressed simultaneously, albeit at a somewhat reduced level as compared to the strongly polarized case. While the temporal expression of CD86 shows this behavior (dampened, but qualitatively similar kinetics, with addition of IL-4/IL-13), the CD206 kinetics suggests a more complex response to costimulation. We found that successful models (as assessed by either the error or the AICc information criterion) required at least two features extending the core MISA: an incoherent feed-forward loop on the M1 pathway, and a mixed (both inhibiting and activating) character of M2 regulation by the M1-axis (Fig. 3, Supplementary Fig. S3). In the network models, these interactions are mediated by a single additional intracellular regulator (labelled Y). The predictions of the candidate model are presented alongside experimental results in (Fig. 3b). Details of the mathematical modeling can be found in the Supplementary Information.

Multiparametric characterization of macrophage phenotype

To assess whether the expression of CD86 and CD206 are representative of macrophage function, we performed a multiplexed cytokine assay. We found that macrophages stimulated with LPS/IFN-γ alone for 24 hours exhibited the highest secretion of inflammatory cytokines including IL-6, IP-10, MIG, MIP-1a, MIP-2, MIP-1B, RANTES, and TNF-α, which were found at much lower levels in the supernatants of unstimulated cells or cells exposed only to IL-4/IL-13 (Fig. 4a). Cells that were stimulated with mixed LPS/IFN-γ and IL-4/IL-13 secreted somewhat lower levels of inflammatory cytokines, with the greatest proportional decrease observed in G-

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CSF, IL-6, IL-12, IL-15, and TNF-α. Cytokines that were expressed in greater quantity by M1-stimulated cells compared to naïve cells were also expressed by cells exposed to mixed stimuli. This was consistent with a moderate but significant (17% ± 3.5% SEM, n=6) decrease in CD86 expression in cells treated with mixed cytokines compared to the LPS/IFN-γ only condition at 24 hours (Fig. 4C). Examining cytokines typically associated with M2 macrophages, we found that IL-10 was elevated, though not significantly, in the LPS/IFN-γ-only and mixed conditions. TGF-β 1 and 2 expression was similar among all conditions examined. VEGF was strongly suppressed in conditions containing IL-4/IL-13. Indeed, none of the analytes in the ELISA panel were preferentially produced by IL-4/IL-13 treated cells.

Over the course of 96 hours, we observed that most cytokines were either relatively stable or exhibited decay (Supplementary Fig. S2). MCP-1 substantially increased in mixed conditions over this time period, which was not observed in any other condition (Supplementary Fig. S2). In addition, TGF-β increased in IL-4/IL-13-only and, to a lesser extent, mixed conditions, but not in LPS/IFN--only or unstimulated conditions. Together, these data suggest that the time scale of M1 activation is shorter than that of M2 activation, and that cells stimulated with mixed cytokines progress towards a M2-like state. Moreover, expression of some M2 proteins and genes is enhanced under mixed conditions when compared to IL-4/IL-13-only stimulation conditions.

We also examined expression of genes associated with M2 (Fig. 4b) and M1 (Fig. 4c) activation by RT-qPCR at 24 h after stimulation. Transcript expression of *Cd206* as well as *Arg1* showed an increase in expression in mixed cytokine conditions compared to IL-4/IL-13-only conditions (Fig. 4b). However, expression of *Retnla* (Relmα/Fizz1) and *Chi3l3* (Ym1) was

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highest in the IL-4/IL-13-only condition, and co-addition of LPS/IFN-γ inhibited expression of these genes, suggesting that M2 markers may be heterogeneously expressed. Expression of Nos2 was highest in the LPS/IFN-γ only condition. *Tnfa* levels were lower in the LPS/IFN-γ condition compared to the unstimulated and mixed stimulus conditions at the examined timepoint (Fig. 4c), perhaps due to refractory downregulation after stimulation: *Tnfa* is coinduced with genes that degrade *Tnfa* transcripts, leading to a short transcript half-life,[26] and activity at the *Tnfa* promoter stops by 18 hours after stimulation with LPS.[27]

Macrophage state impacts reprogramming by a second activation signal

It is thought that macrophages in wound environments are plastic and can transition from M1-like to M2-like states as signals in their environment change[28] or are presented by therapeutic materials.[17] To investigate this transition *in vitro*, we examined how pre-exposure of macrophages to an inflammatory stimulus influences their response to IL-4 and IL-13. Macrophages were treated with LPS/IFN-γ for 24 hours before IL-4/IL-13 were added for an additional 24 hours. Cells were assayed for CD86 and CD206 expression (Fig. 5a). We found that expression of CD86 in LPS/IFN-γ pre-treated macrophages showed a modest and non-statistically significant increase in response to subsequent addition of IL-4/IL-13 (Fig. 5b). In addition, pre-treatment of cells with LPS/IFN-γ did not block expression of CD206 upon IL-4/IL-13 stimulation, and thus cells pre-polarized to a M1 phenotype were still capable of acquiring characteristics of a M2 phenotype (Fig. 5c). Interestingly, at high concentrations of subsequent IL-4/IL-13 cytokine addition, the extent of CD206 expression was 30% (± 9% SEM, n=5) higher in LPS/IFN-γ pretreated cells when compared to unpretreated cells. These data demonstrate that pre-polarization towards an M1 phenotype with LPS/IFN-γ does not prevent

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subsequent M2 response to IL-4/IL-13, and in fact can enhance the expression of the M2 marker CD206.

Conversely, perturbing M2-like macrophages towards a M1-like phenotype could be therapeutically useful in diseases including cancer, where reprogramming tumor-associated macrophages may help to inhibit tumor growth. To test M2-to-M1 plasticity *in vitro*, we pre-treated macrophages with IL-4/IL-13 for 24 hours, added LPS/IFN-γ for a subsequent 24 hours, and assayed cells for CD86 and CD206 expression (Fig. 5d). We found that at high concentrations of LPS/IFN-γ, pre-treatment with IL-4/IL-13 did not affect CD86 expression when compared to naïve cells, suggesting that M2 pre-polarization does not impact reprogramming towards an M1 phenotype. However, pre-treatment with IL-4/IL-13 inhibited the expression of CD86 upon exposure to low concentrations of LPS/IFN-γ (Fig. 5e). CD206 expression was modestly decreased in response to subsequent addition of LPS/IFN-γ in both the IL-4/IL-13 pre-treated and naïve conditions, although these differences were not statistically different (Fig. 5f). These data suggest that the ability of macrophages to acquire M1-like behavior after entering a M2-like state is dependent on the concentration of subsequent M1 stimulation, and that high concentrations may be necessary for reprogramming than for initial activation of naïve cells.

Discussion

We demonstrate that simultaneous exposure of macrophages to mixed cytokines leads to expression of both CD86 and CD206 in individual cells, which are established respective markers of M1 and M2 activation.[13,29,30] With 48 hours of stimulation, small quantities of co-added IL-4/IL-13 stimuli enhanced CD86 expression in LPS/IFN-γ-stimulated cells, whereas

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greater IL-4/IL-13 concentrations inhibited this enhancement. However, high concentrations of stimulus in the mixed condition induced expression of CD86 equivalent to expression in the LPS/IFN-γ-only condition, and expression of CD206 equivalent to expression in the IL-4/IL-13-only condition. These findings are likely dependent on the time point of observation and the concentrations of simuli, although an increase in M1 markers in response to M2 stimuli has been previously described[31]. Individual cell expressions of CD86 and CD206 expression formed single-peaked, broad distributions, suggesting that individuals do not strongly polarize in mixed cytokine environments. Consistent with this, analysis of the secretome of these populations showed that the presence of IL-4/IL-13 along with LPS/IFN-γ only moderately dampens the level of inflammatory cytokine secretion when compared to LPS/IFN-γ only stimulated cells.

Although both M1 and M2 markers are present upon co-stimulation, their evolution over time is different. In cells stimulated in mixed conditions, the M1 marker CD86 decreases after the first 24 hours and returns almost to basal levels after 96 hours, whereas the M2 marker CD206 continues to increase peaking at 48-72 hours, and remained sustained relative to unstimulated macrophages even after 96 hours. This difference may be indicative of the natural progression of macrophages from inflammatory to anti-inflammatory phenotype during a host response to a wound or infection with pathogen.[28] Unexpectedly, macrophages exposed to mixed conditions had higher levels of CD206 when compared to cells treated with IL-4/IL-13 alone at the longer timepoints, suggesting that presence of an inflammatory stimulus may enhance the long term wound healing response. M2 marker *Arg1* transcripts measured by qRT-PCR were also elevated at each time point in the mixed stimulation condition compared to IL-4/IL-13 only (Supplementary Figure S5).

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Our results suggest that macrophage reprogramming to a contrasting phenotype is dependent on initial polarization state and the strength of the second signal. For cells polarized towards an inflammatory phenotype with LPS/IFN-γ, CD206 expression with IL-4/IL-13 was enhanced compared to naïve cells. In contrast, cells polarized to an anti-inflammatory state with IL-4/IL-13 were more resistant to reprogramming with LPS/IFN-γ towards CD86 expression. This effect was observed specifically at the lower LPS/IFN-γ concentrations; expression of CD86 with high LPS-IFNγ concentrations was not significantly different between pre-polarized and naïve cells. These data suggest the anti-inflammatory phenotype is enhanced by an initial inflammatory signal, and that macrophage progression from M1-like to M2-like phenotypes is favored.

While exploring signaling network topologies that could model macrophage responses, we discovered that the basic MISA motif was insufficient to account for the complex temporal expression patterns of CD86 and CD206, despite suggestions that elements of mutual inhibition and self activation may play a role in macrophage polarization.[12,13] The mathematical models shed light on regulatory interactions which enable macrophages to achieve a spectrum of polarization states, depending quantitatively on microenvironmental cues. The models also suggest a regulatory logic by which individual cells co-stimulated by M1 and M2 signals can achieve transient M1 character followed by progression to a M2 phenotype. Although each node in the small-network models represents the combined action of many species, ‘M1’ and ‘M2’ likely reflect (at least in part) regulation by STAT1 and STAT6, respectively, consistent with their mutual antagonism induced by LPS/IFN-γ and IL-4/IL-13.[6–8,32–34] For construction of a parsimonious model in order to avoid overfitting, a single additional node (‘Y’) was introduced to mediate both the transient nature of CD86 expression, and the mixed inhibiting/activating

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effect of LPS-IFNγ on CD206. As such, 'Y' likely comprises feedback inhibition mechanisms, including those mediated by SOCS and STAT3 (reviewed in 35). Furthermore, 'Y' may reflect regulation by NFκB, which is activated by LPS[36] and inhibited by IL-4.[11] Several studies have suggested a cooperative interaction between NFκB and STAT6 to promote genes downstream of IL-4.[37–40] Incorporating these interactions into the mathematical model enabled us to construct small networks that captured the temporal response to both mixed and polarizing stimuli. A limitation of this approach is that the model is trained on two markers, which captures only some of the changes associated with macrophage activation. A more comprehensive dataset could lead to a more predictive model at the cost of increasing the complexity of the model.

These findings may have implications for therapeutic strategies involving macrophage reprogramming. For modulating the host response to biomaterial implants, delivery of IL-4 and IL-13 has been shown to increase expression of CD206 in surrounding macrophages.[41] Our results suggest that additional delivery of inflammatory cytokines, either concurrently or beforehand, may enhance anti-inflammatory activation and potentially improve the wound healing response. Along these lines, Spiller et. al. recently demonstrated that delivery of IFN-γ increases angiogenesis in response to a decellularized bone implant.[42] For cancer treatment, reprogramming tumor associated macrophages, which are thought to be anti-inflammatory, towards an inflammatory phenotype may require high concentrations of inflammatory stimuli, since low concentrations were not sufficient to induce this transition.

In summary, we find that macrophages exposed to both M1 and M2 activation signals express markers of both phenotypes, but the M1 markers decay over time while the M2 markers remain elevated. The distribution of markers suggest that macrophages do not exist in discrete polarized

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states. In addition, acquisition of the M2 phenotype appears to be enhanced by additional exposure to inflammatory stimulus, suggesting that inflammatory insult potentiates the wound healing response. Together, these results provide a better understanding of macrophage behavior in response to opposing activation signals, which is likely to be involved in the dynamic immune response to pathogens or injury. This improved understanding of macrophage activation will likely help design strategies for treatment of disease in which macrophages are involved.

Methods

Cell isolation and culture

All protocols involving animals were approved by University of California Irvine's Institutional Animal Care and Use Committee, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALACi). Primary bone marrow derived macrophages were obtained by harvesting marrow from femurs of 6-12 week old female C57BL/6 mice, lysing red blood cells with ACK buffer, and then culturing cells for seven days on bacteriological polystyrene plates in DMEM supplemented with 10% FBS, 2% penicillin/streptomycin, 2 mM L-glutamine, and 10% conditioned media from CMG 12-14 cells expressing recombinant mouse M-CSF. Macrophages were treated with the indicated concentrations of LPS (Sigma), IFN-γ, IL-4, or IL-13 (all from Biolegend) for the indicated time. Macrophages were seeded at 3e5 cells/ml, allowed to adhere overnight, and treated with indicated concentrations of IL-4, IL-13, TNF-α, and IFN-γ, and then assayed for flow cytometry or cytokines as described below.

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Flow cytometry

Cells were fixed in 4% formaldehyde and stored at 4 °C until staining with anti-CD86 (clone GL-1, APC conjugate; Tonbo Biosciences) and anti-CD206 (clone C068C2, Alexa 488 conjugate; Biolegend) antibodies or isotype controls. Cells were analyzed on a BD LSR flow cytometer with post-processing in FlowJo (Tree Star). Cell populations were gated on forward and side scatter to select intact single cells. Events were acquired until 10,000 events were collected in a preliminary analysis gate or the sample was exhausted.

Cytokine analysis

Supernatants were collected at 24, 48, 72, and 96 hours after stimulation and analyzed with a Luminex 31-plex mouse cytokine array (Eve Technologies). Hierarchical clustering was performed in R using a complete linkage method and presented with the gplots package.[43]

RT-PCR

For gene expression analysis, cells were lifted from culture plates 24 hours after stimulation, pelleted by centrifugation, and frozen at -80 °C. Cells were lysed and RNA was extracted with the Qiagen RNeasy Mini kit. Reverse transcription was performed with the Qiagen Quantitect Reverse Transcription kit, which uses random priming and includes a DNase treatment. Resulting cDNA was observed to be free of contaminating gDNA by testing with the mVPA1 primer set.[44] qPCR was performed with BioRad SsoFast EvaGreen master mix on a BioRad CFX96 thermocycler using recommended cycling parameters (hot-start activation at 95 °C for 30 s, followed by 40 cycles of 5 s denaturation at 95 °C and 5 s annealing/extension at 55 °C, followed by melt curve collection from 65-95 °C in 0.5 °C increments at 5 s/step). Inhibition was cleared by diluting samples 1:100 in ddH_2O before analysis. Amplification was confirmed to be target-specific with Primer-BLAST[45] and by observing that melt curves had a single peak

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consistent with predicted amplicon melting temperature. Primer sequences and target and amplicon details are presented in Supplementary Table S1. Gene-of-interest expression was determined relative to an ensemble of Hprt, Gapdh, and Ldha expression using the GeNorm method[46] implemented by the package eleven (https://github.com/tdsmith/eleven).

Mathematical modeling

We constructed mathematical models comprising minimal nonlinear Ordinary Differential Equation (ODE) networks. Network nodes included input signals LPS/IFN-γ and IL-4/IL-13, output markers CD86 and CD206, and M1- and M2-associated pathways. Model quality was assessed based on optimization of parameters by fitting to the 96-hour timecourse data (Fig. 2) of four timepoints (24, 48, 72, 96 h) for four different stimulation conditions ({0.3,0}, {0.3,1}, {0,0}, {0,1}). Parameter optimization was performed using the trust-region-reflective algorithm with the MATLAB optimization toolbox. Model selection was performed using the corrected Akaike Information Criterion (AICc).[47] Details of the mathematical models, optimization, and selection protocol can be found in the Supplementary Information.

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Author contributions

T.D.S., M.J.T, E.L.R, and W.F.L. designed the study and interpreted results. T.D.S. and M.J.T performed experiments. T.D.S., M.J.T., E.L.R, and W.F.L. wrote the manuscript.

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Additional information

The authors declare that no competing financial interests exist.

Figure captions

Figure 1. Co-stimulation with LPS/IFN-γ and IL-4/IL-13 leads to expression of both CD86 and CD206.

(a) Schematic illustrating experimental conditions. Macrophages were exposed to LPS/IFN-γ and/or IL-4/IL-13 for 48 hours before analysis.

(b) Density plots of normalized CD206 versus CD86 staining intensity of macrophages subjected to different concentrations of LPS/IFN-γ and/or IL-4/IL-13 (ng/ml) for 48 hours, assessed by flow cytometry. CD86 is normalized to the LPS/IFN-γ-only condition and CD206 is normalized to the IL-4/IL-13-only condition. Representative plots from a single experiment. Median position is indicated by a red dot.

(c) Average median normalized CD86 intensity ± SEM (n = 3) of LPS/IFN-γ treated cells vs. co-added IL-4/IL-13 stimulus, grouped by LPS-IFNγ concentration, normalized per experiment as in B.

(d) Average median CD206 intensity ± SEM (n = 3) of IL-4/IL-13 treated cells vs. co-added LPS/IFN-γ stimulus, grouped by IL-4/IL-13 concentration, normalized per experiment as in B. Asterisk indicates significant difference by two-sided t test, $p < 0.05$.

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Figure 2. Co-stimulated macrophages exhibit decreased CD86 expression and increased CD206 expression over time.

(a) Schematic illustrating experimental conditions. Macrophages were exposed simultaneously to LPS/IFN-γ and/or IL-4/IL-13 for 24, 48, 72, or 96 hours. Each experiment used BMDM isolated from a single mouse.

(b) Expression of CD206 versus CD86 of different stimulation conditions over time. Average of median population location ± SEM (n=5) is shown.

(c) Expression of CD86 staining intensity over time for different stimulation conditions, normalized to the intensity of LPS/IFN-γ condition at 24 hours. Average of median population location ± SEM (n=5) is shown. Asterisk indicates difference vs 24 hours by two-sided t test, $p < 0.05$.

(d) Expression of CD206 staining intensity over time for different stimulation conditions, normalized to the intensity of IL-4/IL-13 condition at 24 hours. Average of median population location ± SEM (n=5) is shown. Asterisk indicates difference vs 24 hours by two-sided t test, $p < 0.05$.

Figure 3. Mathematical modeling of macrophage regulatory logic

(a) A representative set of minimal models for activation of M1 and M2 pathways under costimulation, comprised of a modified MISA (Mutual Inhibition/Self-Activation) network. Y: an unspecified regulator. Dashed line indicates that positive regulation of M2 by Y occurs cooperatively with M2. Models are shown in order of increasing number of parameters. RSS score indicates goodness of fit, and AIC score measures model quality, while penalizing presence of additional parameters. ΔAICc is reported relative to best (lowest) value,

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corresponding to Model 6. In general, successful models (3,5, and 6) incorporated both an incoherent feed-forward loop on M1 and positive regulation of M2, mediated by Y.

(b) Simulated expression of CD86 and CD206 stimulated with LPS/IFN-γ and/or IL-4/IL-13 for 24, 48, 72, or 96 hours. Simulated data are from the model with both the best AICc and RSS score, Model 6, fit to the normalized time course data.

Figure 4. Multiparametric characterization of macrophage phenotype.

(a) Dendrogram and heat map of cytokine release from macrophage cultures exposed to LPS/IFN-γ and/or IL-4/IL-13 for 24 hours. All conditions are normalized to inflammatory stimulus. * indicates difference from LPS/IFN-γ-only condition by two-sided *t* test with Holm correction, $p < 0.05$. Average of 3 experiments.

(b) Mean $\log_2$ transformed fold difference mRNA expression vs. (B) IL-4/IL-13 only or (C) LPS/IFN-γ-only stimulation condition at 24 hours. Missing values indicate amplification below limit of detection. $n = 3$.

Figure 5. Macrophage polarization state influences their plastic response to opposing activation signals.

(a) Schematic illustrating experimental conditions. Macrophages were stimulated for 24 hours with inflammatory stimuli alone before anti-inflammatory stimuli were added for an additional 24 hours, and then assayed.

(b) Expression of CD86 in cells in cells either pre-treated with 1 ng/ml LPS/IFN-γ or untreated for 24 hours and then subsequently treated with IL-4/IL-13 at the indicated dosages. Data are normalized to CD86 expression in 0.3 ng/ml LPS/IFN-γ-only condition, mean ± SEM (n=4).

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(c) Expression of CD206 in the same conditions as B. Data are normalized to CD206 expression in 1 ng/ml IL-4/IL-13-only condition, mean ± SEM (n=4).

(d) Schematic illustrating experimental conditions. Macrophages were stimulated for 24 hours with anti-inflammatory stimuli alone before inflammatory stimuli were added for an additional 24 hours, and then assayed.

(e) Expression of CD86 in cells either pre-treated with 1 ng/ml IL-4 and IL-13 or untreated for 24 hours and then subsequently treated with LPS and IFN-γ at the indicated dosages. Data are normalized to CD86 expression in 0.3 ng/ml LPS/IFN-γ-only condition, mean ± SEM (n=3).

(f) Expression of CD206 in the same conditions as E. Data are normalized to CD206 expression in 1 ng/ml IL-4/IL-13-only condition, mean ± SEM (n=3). Asterisks indicate significant differences compared to unstimulated cells by two-sided t test, $p < 0.05$. Dagger indicates differences between groups with and without pre-treatment by two-sided t test, $p < 0.05$.

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Graphical Abstract (text)

Experimental and computational approaches are used to characterize the macrophage response to opposing activation stimuli.

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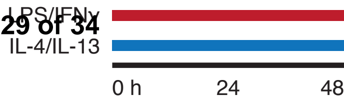

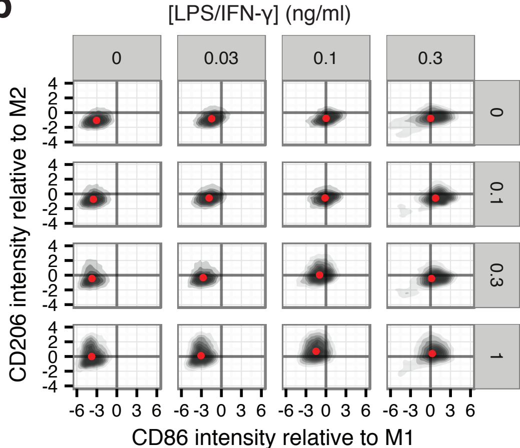

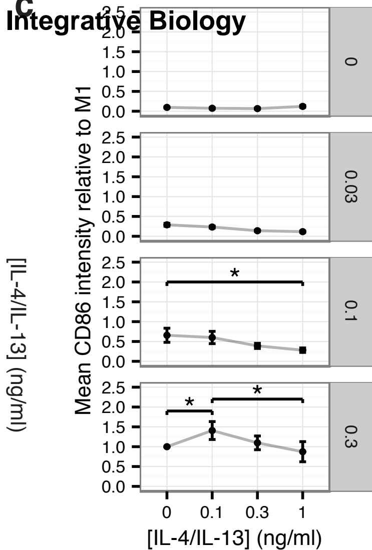

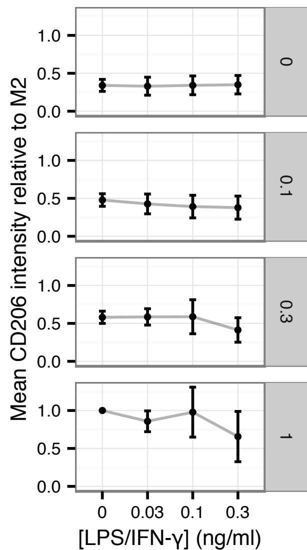

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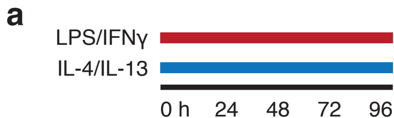

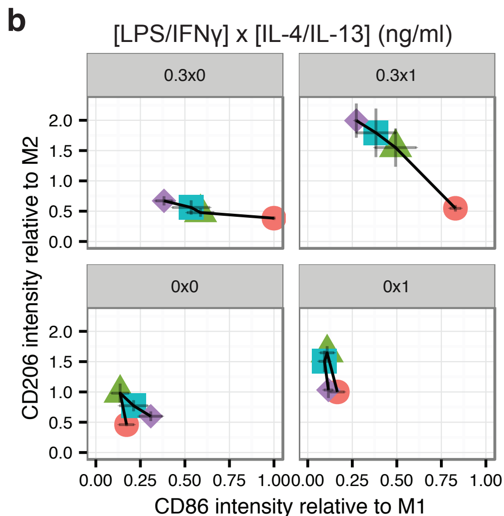

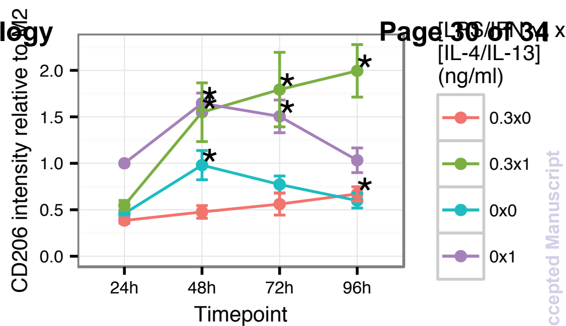

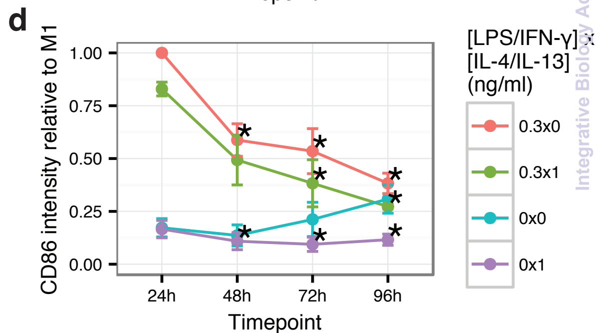

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Model	1	2	3	4	5	6
RSS	0.58	0.53	0.15	0.54	0.11	0.10
AIC	-210.61	-209.11	-244.96	-203.30	-246.78	-248.25
ΔAICc	37.64	39.14	3.29	44.95	1.47	0.00

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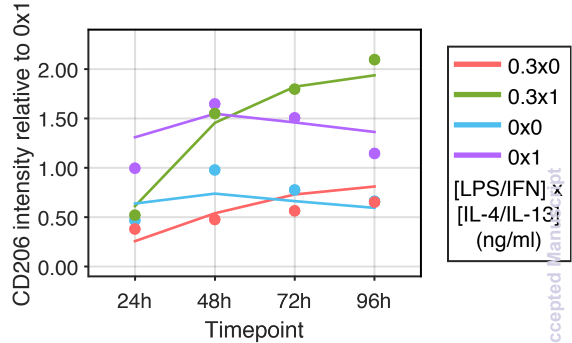

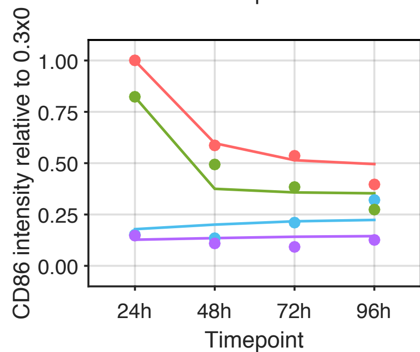

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log2(Fold change vs 0.3x0)

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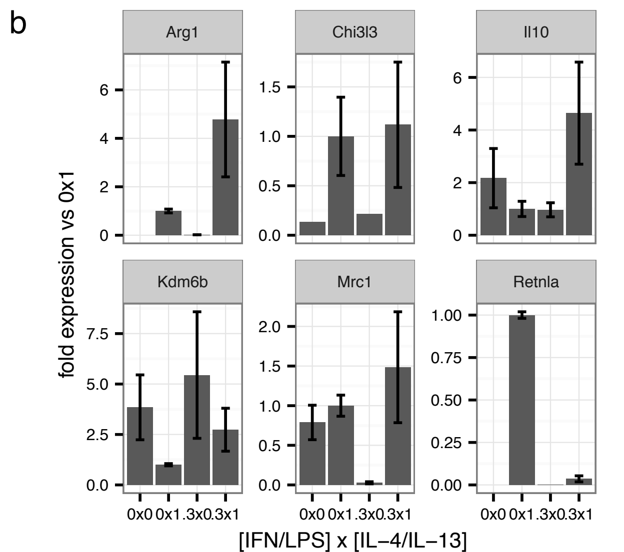
0.3x1
0x0
0x1

[IFN-γ/LPS]x[IL-4/IL-13] (ng/ml)

IFNγ (19) TNFa (77) MIP-2 (73) G-CSF (13) KC (61) IP-10 (57) IL-12 (p40) (45) MIG (74) IL-6 (34) RANTES (75) MIP-1a (64) IL-4 (28) MIP-1B (66) IL-15 (54) IL-1a (21) IL-12 (p70) (47) IL-10 (43) IL-7 (36) IL-1B (25) IL-3 (29) MCP-1 (62) IL-9 (38) IL-5 (30) M-CSF (67) IL-17 (56) LIF (51) IL-2 (26) Eotaxin (14) GM-CSF (15) TGF-B2 (54) TGF-B1 (18) TGF-B3 (36) LIX (53) VEGF (76)

b



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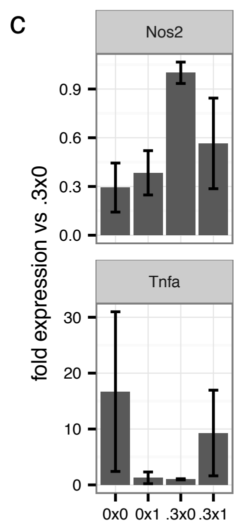

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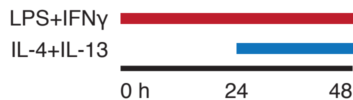

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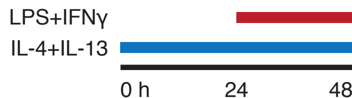

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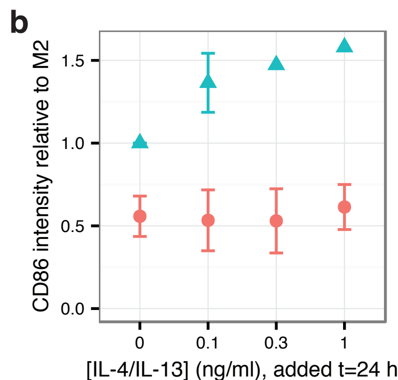

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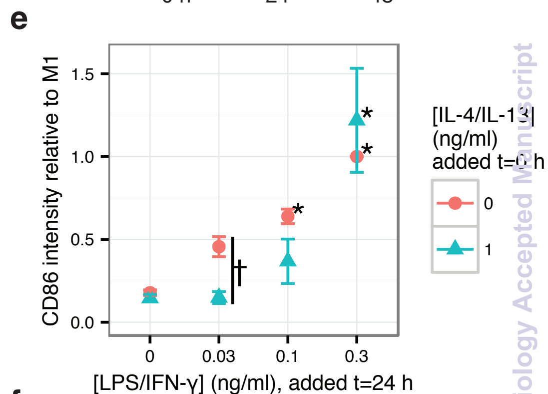

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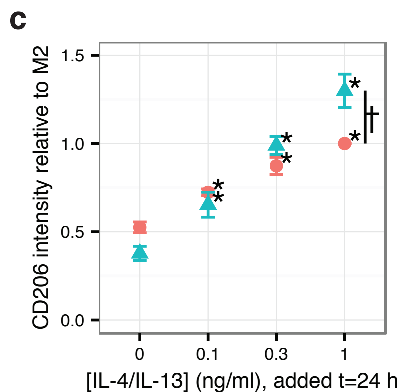

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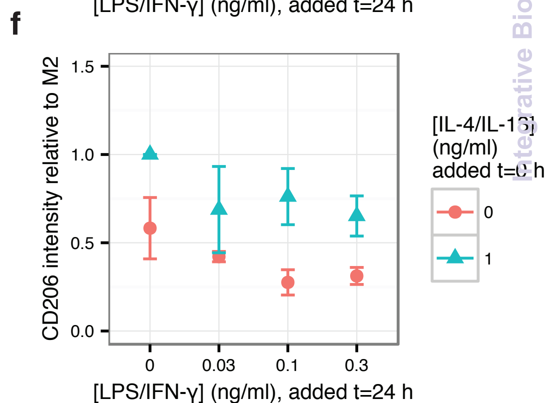

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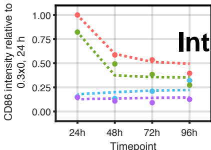

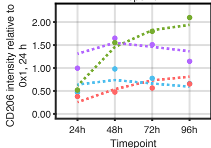

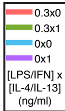

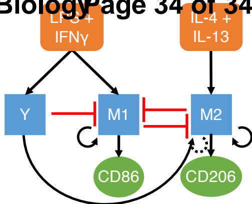

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