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Elevated GM-CSF and IL-1β levels compromise the ability of p38 MAPK inhibitors to modulate TNFα levels in the human monocytic/macrophage U937 cell line†

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Rheumatoid arthritis (RA) is a complex, multicellular disease involving a delicate balance between both pro- and anti-inflammatory cytokines which ultimately determines the disease phenotype. The simultaneous presence of multiple signaling molecules, and more specifically their relative levels, potentially influences the efficacy of directed therapies. Using the human U937 monocytic cell line, we generated a self-consistent dataset measuring 50 cytokines and 23 phosphoproteins in the presence of 6 small molecule inhibitors under 15 stimulatory conditions throughout a 24 hour time course. From this dataset, we are able to explore phosphoprotein and cytokine relationships, as well as evaluate the significance of cellular context on the ability of small molecule inhibitors to block inflammatory processes. We show that the ability of a p38 inhibitor to attenuate TNFα production is influenced by local levels of GM-CSF and IL-1β, two cytokines known to be elevated in the joints of RA patients. Within the cell, compensatory mechanisms between signaling pathways are apparent, as selective p38 MAPK inhibition results in the increased phosphorylation of other MAPKs (ERK and JNK) and their downstream substrates (CREB, c-Jun, and ATF-2). Further, we demonstrate that TNFα-neutralizing antibodies have secondary effects on cytokine production, impacting more than just TNFα alone. p38 MAPK inhibition using a small molecule inhibitor also blocks production of anti-inflammatory cytokines including IL-10, IL-1ra and IL-2ra. Collectively, the impact of cell context on TNFα production and unintended blockade of anti-inflammatory cytokines may compromise the efficacy of p38 inhibitors in a clinical setting. The effort described in this work evaluates the effect of inhibitors on multiple endpoints (both intra- and extracellular), under a range of biologically relevant conditions, thus providing a unique means for differentiation of compounds and potential opportunity for improved pharmacological manipulation of disease endpoints in RA.

Introduction

The inability to effectively treat and reverse progression of chronic inflammatory diseases such as rheumatoid arthritis (RA) reflects an incomplete understanding of biological mechanism. Unanticipated findings, including partial or lack of response at the clinical level and loss of efficacy, underscore the need for more in-depth analysis of the multiple factors controlling cell-system inflammatory response in normal and disease states. To this end, we present a multi-dimensional approach to elucidate complexities of cytokine regulation, incorporating measurement of multiple intracellular signals and cytokine release under a range of stimulation conditions, timepoints, and pharmacological interventions. The data obtained provide an expanded means for understanding biological mechanism in inflammation, as well as a direct approach for evaluating drug targets capable of modulating cytokines relevant to RA, with particular focus on p38 MAPK and TNFα.

Rheumatoid arthritis affects ~0.8% of the worldwide population, and while the specific underlying cause of the disease remains under investigation, it is believed to involve both the innate and adaptive immune systems. In RA, the normally hypocellular synovial membrane and associated synovial fluid experience an accumulation and expansion of immune cell types including macrophages, mast cells, neutrophils, CD8+ T-cells, natural killer (NK) cells, NKT cells, B cells and plasma cells.[1] Communication amongst the infiltrating cells *via* cytokines, as well as with the local environment, is essential for disease progression. TNFα

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and IL-1, in particular, are released by macrophages and other cell types at sites of inflammation, and their levels are found elevated in RA synovial fluid.[2,3] The success of biological therapies targeting these cytokines (TNFα: adalimumab, etanercept, infliximab, golimumab; IL-1: anakinra) attests to their critical role in the development and maintenance of RA. These anti-cytokine therapeutics are intended to halt or reverse disease progression rather than merely alleviate symptoms. Combination treatment with both anti-TNFα and anti-IL-1 therapy, however, has proved no more effective than either treatment alone and is in fact associated with a significant increase in opportunistic infections.[4,5] Thus, it remains to be determined how the elevated presence of multiple cytokines affects the inflammatory response, as well as the extent to which it is desirable, necessary or even possible to modulate several cytokines simultaneously. Additional cytokines including, but not limited to, IL-6, IL-8, IL-10, IL-15, IL-17, RANKL and GM-CSF are also involved in the pathogenesis of RA, and many are under investigation as potential points of disease intervention.[6–8]

Although anti-TNFα biologic therapies have revolutionized the treatment of RA, their efficacy is limited and no current diagnostics are available to predict who will respond favorably.[7,9] Based on criteria established by the American College of Rheumatology (ACR), 50–60% of patients achieved an ACR20 (20% improvement) with infliximab treatment (chimeric monoclonal anti-TNFα antibody), up to 75% of patients achieved ACR20 at 3 months following treatment with etanercept (soluble TNF receptor) and 46% of patients achieved ACR20 at 6 months on adalimumab (fully human monoclonal anti-TNFα antibody; www.fda.gov).[7,10] Overall, only 10–40% of patients achieve an improvement of 70% or more with any treatment.[11] Thus, a limited percentage of patients achieve even modest improvement in their disease symptoms using these anti-TNFα therapies and in those who do respond, efficacy is often lost with time. Additionally, these biological therapies require administration *via* injection and are accompanied by significant cost.

Another approach being pursued for the treatment of RA is the targeting of inflammatory cytokines *via* intracellular pathways using orally available, small molecule inhibitors. Several kinases essential for intracellular signal transduction and cytokine production have been considered for the regulation of inflammatory cytokines including TNFα. p38 MAPK has been, by far, the most studied of these kinases in the context of RA and thus has been a target of many pharmaceutical companies who have advanced scores of inhibitors into the clinic.[12–15] p38 MAPK is known to regulate TNFα transcription and mRNA stability *via* MK2, and p38 inhibitors have demonstrated the ability to reduce TNFα levels in animal models.[16–18] However, despite their relative efficacy in regulating TNFα levels, no p38 MAPK inhibitors have yet made it to market, either as a result of efficacy or safety issues.[19–22] In addition to the regulation of TNFα and other inflammatory cytokines, p38 MAPK is also involved in many aspects of normal cellular function in a wide range of cell types.[14] The complexity of cytokine cascades makes it difficult to determine *a priori* if collateral cytokine effects associated with p38 inhibition will synergize or antagonize TNFα production. Relatedly, it also remains to be determined if additional pro- or anti-inflammatory cytokines will be affected following p38 inhibition, as might be predicted based on gene regulation *via* the p38 MAPK pathway, thus influencing disease symptoms.[23] The role of p38 MAPK in TNFα regulation may in fact be inseparable from other house-keeping functions, with its inhibition being the source of unwanted side effects.[24] It also remains to be seen whether inhibition of p38 MAPK using a small molecule inhibitor has the same clinical outcome as targeting circulating TNFα with biologically based therapies.

Multiple other intracellular targets are also being considered for the regulation of RA. IKK2 inhibition blocks NFκB-mediated inflammatory response in human fibroblast-like synoviocytes, chondrocytes and mast cells and protects against bone and cartilage destruction in a rat model of RA, while the IKK inhibitor BMS-345541 blocks both joint inflammation and destruction in collagen-induced arthritis in mice.[25–27] Blocking PI3Kγ has been shown to suppress joint inflammation and damage in mouse models of RA.[28,29] The MEK/ERK pathway plays a central role in a mouse collagen-induced arthritis model of RA, while the MEK inhibitor ARRY-162 is currently in human clinical trials for treatment of RA[30] (www.arraybiopharma.com). JAK-3 inhibitors have been shown to be potent immunosuppressive agents in various murine models and the JAK inhibitors INCB18424 (Incyte Pharma) and CP-690550 (Pfizer) are currently in Phase II/III human trials for RA.[31] The JNK pathway has also been implicated in RA pathology, as JNK2 knockout mice exhibit joint damage and inflammation in a passive murine model of collagen-induced arthritis. Further, JNK is required for metalloproteinase expression and joint destruction in inflammatory arthritis.[32,33] As is the case with p38 MAPK, it is very likely that any pathway inhibition will have multiple downstream consequences both in terms of cytokine release and other cellular functions and thus, consideration will need to be given to the cumulative extent of these effects.

A more thorough understanding of the inflammatory response in its relevant cell types is therefore needed in order to identify effective treatments, understand variations in response rates among patient populations and to better appreciate the consequences associated with any disease intervention. It will also be critical to characterize the role of cellular context in disease modification. In this work, we first sought to identify stimulatory conditions which influence TNFα production in the human U937 monocyte/macrophage cell line and quantitatively assess the global impact of those conditions on intracellular phosphoprotein status and extracellular cytokine release. Small molecule inhibitors targeting multiple different intracellular kinases (under investigation for the regulation of inflammation/TNFα) were then employed under a relevant set of conditions in order to evaluate their effect on 23 intracellular phosphoproteins and the release of 50 cytokines. This combination of extracellular stimuli, small molecule inhibitors and multiple evaluated targets produced a dynamic, self-consistent proteomic dataset that (1) described the influence of cellular context on TNFα production and

multiple secondary endpoints, (2) identified cues that may compromise the ability of p38 inhibition on TNFα production, including intracellular compensatory responses to target inhibition, and (3) quantitatively compared p38 inhibition with TNFα-neutralizing antibodies in terms of effects on global cytokine regulation.

Results

Stimuli influencing TNFα release from U937 cells

In order to better characterize the complex regulation of TNFα production from macrophages in the inflammatory process, we individually treated PMA-differentiated U937 cells with 35 different cytokines implicated in inflammation (see Table 1). The U937 cell line is a well-established model for monocyte/macrophage differentiation and function, thus representing a key cell type in the etiology of RA.[34–37] Cells were treated with each cytokine (100 ng mL^{-1} final) and evaluated at six time points (over a 24 hour period) for their effect on TNFα release. In order to simulate the effect of recruitment or release of additional cytokines during an ongoing inflammatory crisis, each cytokine was also added to the cells in combination with 1 ng mL^{-1} lipopolysaccharide (LPS). LPS is known to trigger an inflammatory response in macrophages including the release of TNFα.[38,39] Cells were incubated with each cytokine +/− LPS for the indicated time, supernatants recovered and TNFα levels evaluated using a Meso-Scale Discovery TNFα assay (Gaithersburg, MD; see Methods). Six cytokines were found to have an influence on TNFα levels in U937 cells (remaining 29 cytokines did not demonstrate an appreciable effect on TNFα and therefore were not evaluated further). IL-1α and IL-1β (without LPS) significantly increase the amount of TNFα released relative to untreated cells, while IFNγ induces a modest increase (Fig. 1A). Combining IL-1α, IL-1β, GM-CSF or IFNγ with LPS increased the amount of TNFα present in the supernatant relative to LPS alone (Fig. 1B). GM-CSF and IFNγ demonstrate the most significant increase, while IL-1α and IL-1β generate a lesser, but consistent, increase in TNFα levels (Fig. 1B). In contrast, IL-6 and IL-10 in combination with LPS significantly reduce the amount of TNFα relative to LPS alone (Fig. 1C). Of note, after two hours of treatment, modest variability in TNFα levels between cytokine treatments exists whereas from 4 to 24 hours significant differences become evident (Fig. 1). These temporal differences potentially reflect transcription/translation, the cumulative buildup of TNFα in the supernatant, the impact of additionally released cytokines, and in some cases, subsequent cellular binding/uptake or proteolysis of secreted cytokines.

Table 1 Cytokines used to treat U937 cells

IL-1α	IL-8	IL-15	IL-33	Insulin	MIP4	RANTES
IL-1β	IL-10	IL-19	BAFF	IFNγ	MIP3α	sRANKL
IL-3	IL-11	IL-20	G-CSF	M-CSF	MIP3β	TNFβ
IL-4	IL-12	IL-22	GM-CSF	MIP1α	MIP5	TWEAK
IL-6	IL-13	IL-31	EGF	MIP1β	Oncostatin M	VEGF

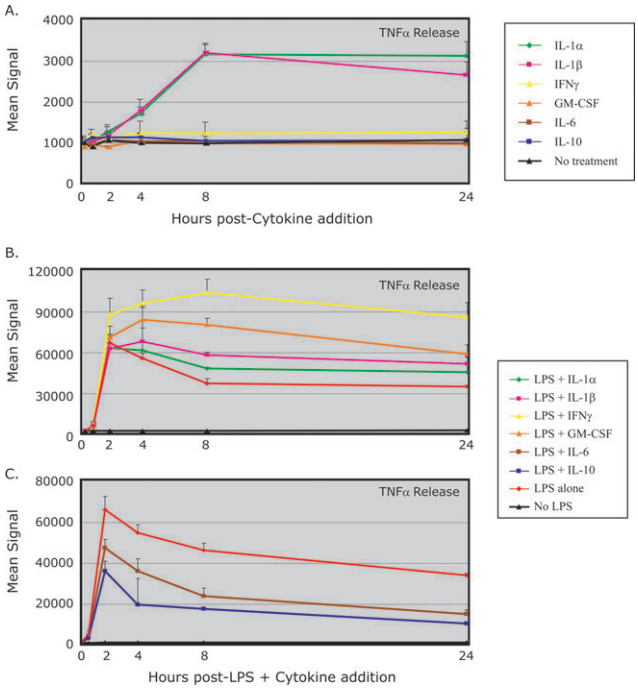

Fig. 1 Time course evaluation of TNFα in U937 cells. PMA-differentiated U937 cells were treated with each cytokine in Table 1, either alone (100 ng mL^{-1} final) or in combination with LPS (1 ng mL^{-1} final). Media alone (no treatment or no LPS) and LPS alone (1 ng mL^{-1}) served as controls. Supernatants were recovered at six timepoints (0, 60, 120, 240, 480 and 1440 min) following treatment with each cytokine +/− LPS and the amount of TNFα determined using an MSD TNFα assay (see Methods). (A) IL-1α (green line), IL-1β (pink line) and IFNγ (yellow line) increase TNFα relative to no cytokine (no treatment; black triangle/line). (B) LPS + IL-1α (green line), LPS + IL-1β (pink line), LPS + GM-CSF (orange line) and LPS + IFNγ (yellow line) increase TNFα relative to LPS alone (red line/diamond). (C) LPS + IL-6 (brown line) and LPS + IL-10 (blue line) decrease TNFα relative to LPS alone (red line).

Evaluating multiple cytokines and phosphoproteins under conditions that influence TNFα

The stimuli which we observed to influence TNFα levels represent ligands for a number of distinct receptors, including IL-1R, GM-CSF/IL-3R, IFNγR, IL-6R, IL-10R and TLR4 (LPS), thus illustrating the complex nature of TNFα regulation. These receptors are linked to multiple distinct intracellular pathways resulting in the activation and release of a wide range of cytokines, which may in turn then influence TNFα and/or the ability to regulate TNFα.[6,40] We therefore sought to evaluate the changes in global cytokine production and dynamics, elicited under those conditions which we identified as influencing TNFα release from U937 cells. Cells were independently treated with the six cytokines +/− LPS (1 ng mL^{-1}) identified in Fig. 1, and the levels of 50 cytokines measured in the supernatant at twelve timepoints spanning 24 hours using a BioPlex/xMAP multiplex cytokine assay (Fig. 2A; Bio-Rad/Luminex). As the stimuli (Cues) are being transmitted *via* intracellular pathways (Signal) before culminating in cytokine release (Response), we also sought to evaluate the phosphorylation status of 17 proteins in lysates

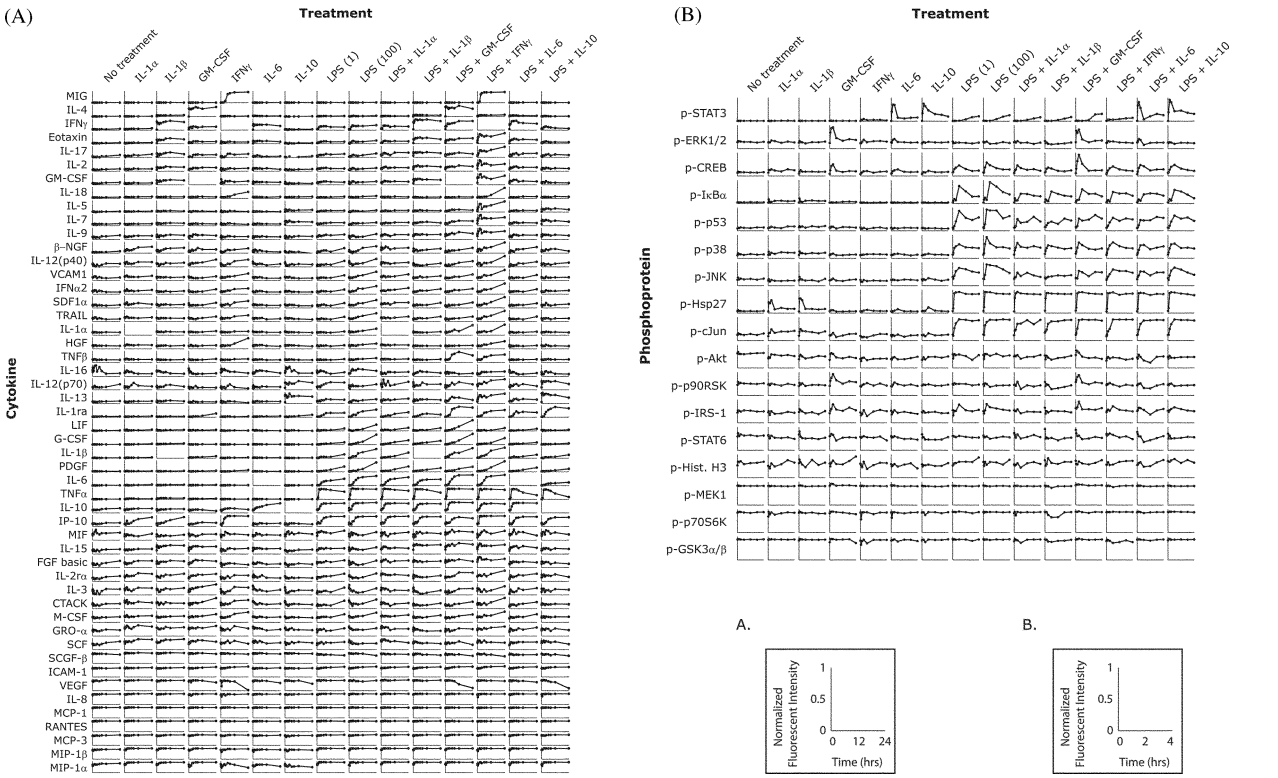

Fig. 2 Cytokine and phosphoprotein profiling. (A) Cytokine levels during a 24 hour timecourse under multiple stimulation conditions. The indicated cytokines were measured (row labels; "Cytokine") from U937 supernatants using a BioPlex/xMAP multiplex cytokine assay (see Methods) following treatment for 0, 5, 15, 30, 60, 120, 240, 360, 480, 720, 960 or 1440 minutes with the indicated stimulus (column labels; "Treatment"). Data are normalized relative to maximum value across all treatments. (B) Evaluation of phosphoprotein response during a timecourse under multiple stimulation conditions. Lysates corresponding to the U937 cells described in (A) were prepared and the indicated phosphoproteins (row labels; "Phosphoprotein") measured using a BioPlex/xMAP multiplex phosphoprotein assay (see Methods; only the first 4 hours of 24 hour timecourse are shown). Data are normalized relative to maximum value across all treatments.

prepared from the same above-described cells (Fig. 2B; BioPlex/xMAP multiplex phosphoprotein assay). Fig. 2A depicts the entire 24 hour timecourse in order to capture the full effect of the cytokines being released, while Fig. 2B shows only the first 4 hours of the 24 hour timecourse in order to focus on phosphorylation events (although the full timecourse for both readouts were analyzed). In both datasets, there are a diverse set of response dynamics.

The cytokine measurements reflect a cumulative assay measuring cytokine accumulation in the media (or consumption). There are some cytokines whose relative production does not change over the timecourse (*e.g.* IL-8, MCP-1, RANTES and MCP-3), cytokines that are rapidly induced following specific stimuli (*e.g.* IL-6, IL-10, and TNFα), as well as cytokines that show gradual increase (*e.g.* G-CSF and PDGF) and late-time uptake or proteolysis (*e.g.* VEGF). Overall, it is also clear that there are distinct responses tied to individual stimuli. LPS (1 and 100 ng mL^{-1}) is by far the most significant stimulus influencing multiple cytokines, while others such as IFNγ show more targeted effects on the release of cytokines such as MIG. Further, the effects of the combination stimuli (LPS + cytokine) are in many cases not simply the sum of the effects of the individual stimuli (*e.g.* IL-1ra with LPS + GM-CSF).

In contrast to the cytokines, the phosphorylation measurements capture snapshots of signal transduction cascades and their intricate regulation. As with the cytokines, a variety of dynamics are apparent, including phosphoproteins that remain relatively static (p-GSK3α/β), those that show transient phosphorylation (p-STAT3) and those that demonstrate sustained activation following stimulation (pHSP27 and p-cJun). Consistent with the role of phosphoproteins in regulating cytokine production, LPS had the most dominant effect on the global phosphoprotein profile. As expected, individual stimuli such as GM-CSF or IL-6 demonstrate more selective effects on individual phosphoproteins such as phospho-ERK and phospho-STAT3, respectively.

Incorporation of inhibitors: effects on cytokine release

Having established the phosphoprotein and cytokine response profile of U937 cells under a range of treatments important for TNFα production, we next sought to evaluate the consequences of p38 MAPK inhibition. Additionally, we compared the effects of p38 MAPK inhibition to multiple other small molecule drug targets which are also under investigation for the regulation of TNFα and/or inflammation. We hypothesized that a broad look at both intra- and extracellular responses, in combination with targeted disruption of signal transduction cascades using specific small molecule inhibitors, would aid in the elucidation of complex regulatory relationships.

Six inhibitors were chosen: a p38 MAPK inhibitor (PHA-00818637), JNK inhibitor (Calbiochem VIII), PI3K inhibitor (LY294002), IKK2 inhibitor (TPCA), MEK inhibitor (CI-1040) and a JAK inhibitor (PF00956980) (see box in Fig. 3A and Methods). Cells were independently treated with each stimulating cytokine +/− LPS (as in Fig. 2) either alone or in combination with a single inhibitor (Fig. 3A). Levels of 50 cytokines were then measured throughout a 24 hour period in order to evaluate their release. To aid in the interpretation of the multivariate data, the time course data are summarized in Fig. 3A using Principal Components Analysis (PCA). PCA is a dimensionality reduction technique that allows for optimal projection of multidimensional data into a lower-dimensional subspace.[41,42] For each measurement, a 1-component PCA model was constructed using the data normalized to the maximum. In order to facilitate interpretation, the matrix was not column centered, so that the loadings are interpreted as the 'average time course' of cytokine production across all the tested conditions. The scores are plotted in the heatmap as multiplicative scaling factors of the average time course. The ability of each PCA model to capture the variation in the data is represented by the R^2 value. Additionally, the heatmap is clustered by cytokine measurement to aid in visualization (see Methods).

It is important to note that in order to observe the effect of an inhibitor on the level of a released cytokine, the initial conditions themselves must elicit or induce that cytokine. We first focus attention to our primary endpoint, TNFα. The ability of the p38 inhibitor to reduce TNFα levels is readily apparent under LPS stimulation (third cytokine down on *y*-axis, lane 7 (p38 MAPKi) of columns H–N). The IKK2 and MEK inhibitors are also able to reduce TNFα levels in the presence of LPS (lanes 5 and 6 of columns H–N, respectively), albeit to a lesser extent than the p38 inhibitor. Interestingly, when TNFα is induced by IL-1β on its own, it is the MEK inhibitor which demonstrates the greatest (relative) reduction (Fig. 3A, column C; re-scaled in Fig. S1, ESI†). Under the same treatment (IL-1β alone), the JNK and PI3K inhibitors *increase* TNFα (relative to the p38 inhibitor and no inhibitor (Fig. S1, ESI†)).

The efficacy of the p38 inhibitor is influenced by the stimulation conditions, as the decrease in TNFα is notably less when IL-1β or GM-CSF is present in combination with LPS (Fig. 3A, columns J and K—lane 7, respectively). Fig. 3B presents the timecourse data for TNFα which underlie the PCA calculations in Fig. 3A. The impact of GM-CSF (and IL-1β) on the ability of the p38 inhibitor (red line) to regulate TNFα is readily apparent.

A similar result is observed in the case of the IL-1 receptor antagonist (IL-1ra), in which both the JAK and IKK2 inhibitors effectively eliminate IL-1ra levels, except under GM-CSF co-stimulation (Fig. 3A, column K). In contrast, levels of some cytokines increase in response to particular inhibitors. This can most readily be seen in the case of the JNK inhibitor, with which a host of cytokines are upregulated (lane 3 in each column). These responses are in some cases stimuli-specific, as illustrated by the significant increase in IL-18 and PDGF levels in the presence of LPS + IFNγ and a JNK or p38 MAPK inhibitor, respectively (column L). Similarly, modest increases in GM-CSF, IFNγ, IL-4, and Eotaxin are observed in the presence of a p38 inhibitor combined with IL-1β (+/− LPS; columns C and J). Also notable is the ability of the MEK inhibitor to reduce basal MIP-1α (seventh cytokine from bottom), and the PI3K inhibitor to reduce the elevated basal level of VEGF (bottom cytokine), again to varying degrees depending on stimulation conditions.

Incorporation of inhibitors: effects on phosphoproteins

It is expected that treatment with an established inhibitor will diminish the activity of a targeted protein and/or its downstream counterparts. What is less obvious is the manner in which other (non-targeted) cellular pathways compensate in response to this inhibition, an effect which is ultimately critical with regard to understanding functional *in-cell* pathway selectivity. Even an exquisitely selective compound may affect multiple pathways by disrupting regulatory interactions. In light of these considerations, we measured the phosphorylation status of 23 proteins using a BioPlex/xMAP phosphoprotein assay in lysates from cells treated with inhibitors as in Fig. 3A (Fig. 4A). Phosphorylation of Hsp27 is eliminated in the presence of the p38 inhibitor PHA-00818637, as would be expected when this kinase–substrate relationship is disrupted (Fig. 4A, lane 7 (p38 MAPK inhibitor) of columns H–N). However, what is not anticipated is the number of additional proteins whose phosphorylation *increases* in the presence of the same p38 inhibitor, despite most of these proteins not being direct canonical constituents of the p38 MAPK pathway. Levels of p-STAT2, p-ERK1/2, p-Akt, p-90RSK, p-JNK, p-GSK3α/β and p-cJun are notably elevated in the presence of the p38 inhibitor. As with the cytokines, the degree of change is considerably influenced by the stimulatory conditions. This is readily apparent when the combination of IFNγ and the p38 inhibitor results in elevated p-STAT2 (relative to no inhibitor; columns E and L, lane 7). Likewise, the combination of GM-CSF (+/− LPS) and a p38 inhibitor results in a significant increase in p-ERK1/2 relative to the inhibitor alone (columns D and K, lane 7). In the case of the JNK inhibitor, increased phosphorylation of ERK1/2, p-STAT6 and p-p70S6K is particularly notable when this inhibitor is present in combination with GM-CSF (column D, third lane from left).

To more fully illustrate the compensatory mechanisms observed in Fig. 4A, timecourses depicting the response of MAPK pathway components in the presence of the p38 inhibitor are shown (Fig. 4B). When U937 cells are treated with LPS + GM-CSF, p-HSP27 is markedly reduced in the presence of the p38 inhibitor, demonstrating efficacy of the inhibitor. However, under the same conditions, phosphorylation of JNK and ERK1/2 increases significantly in response to the same p38 inhibitor (Fig. 4B, p-JNK: green line). In the case of JNK phosphorylation, the degree of upregulation appears proportional to activation of the inhibited pathway, as the presence of LPS results in greater levels of phospho-JNK. Increased phosphorylation of c-JUN and ATF-2, downstream targets of the JNK and p38 pathways, is also evident in the presence of a p38 MAPK inhibitor (Fig. 4B). Compensation

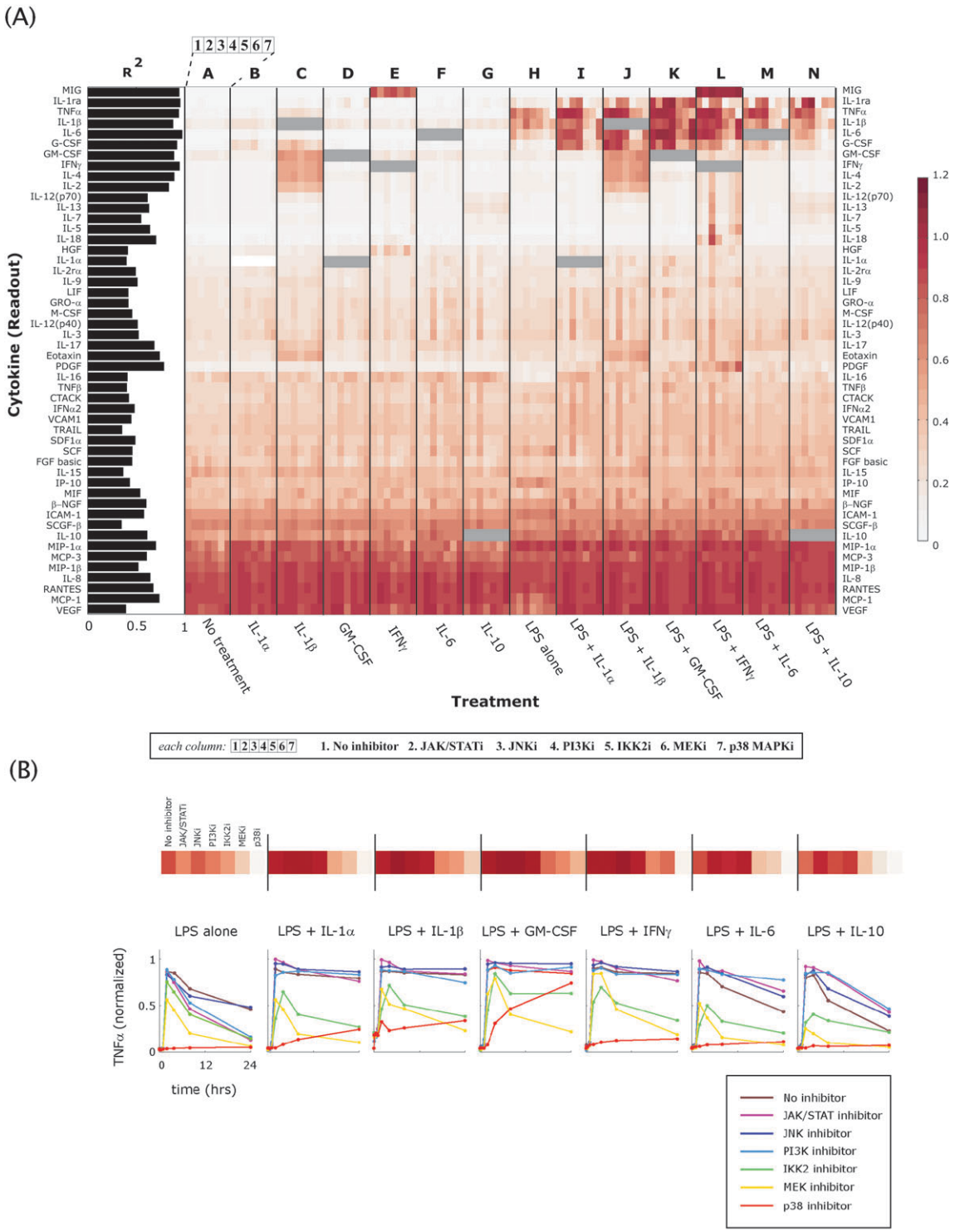

Fig. 3 Cytokine and phosphoprotein profiling with inhibitors. (A) Cytokine levels in the presence of inhibitors. PMA-differentiated U937 cells were incubated with media alone (no inhibitor) or 1 μM of JAK/STATi, JNKi, PI3Ki, IKKi, MEKi or p38 MAPK inhibitor (box at bottom), for 30 minutes prior to each time point (0, 5′, 15′, 30′, 1 h, 2 h, 4 h, 8 h, 24 h) at which time the indicated cytokine +/− LPS was added ("Treatment"). Supernatants were recovered and 50 cytokines evaluated (rows; "Cytokine (Readout)") using a BioPlex/xMAP multiplex cytokine assay. Timecourse data are summarized using a 1-component PCA model (see Methods). (B) Expanded timecourse data for TNFα are shown for cells treated as in (A). Corresponding PCA heatmap is shown above each timecourse.

between the MAPKs is not limited to inhibition of p38 MAPK, as we also observe increases in phosphorylation of p38, JNK and ERK1/2 in the presence of MEK and JNK inhibitors (Fig. 4A and data not shown).

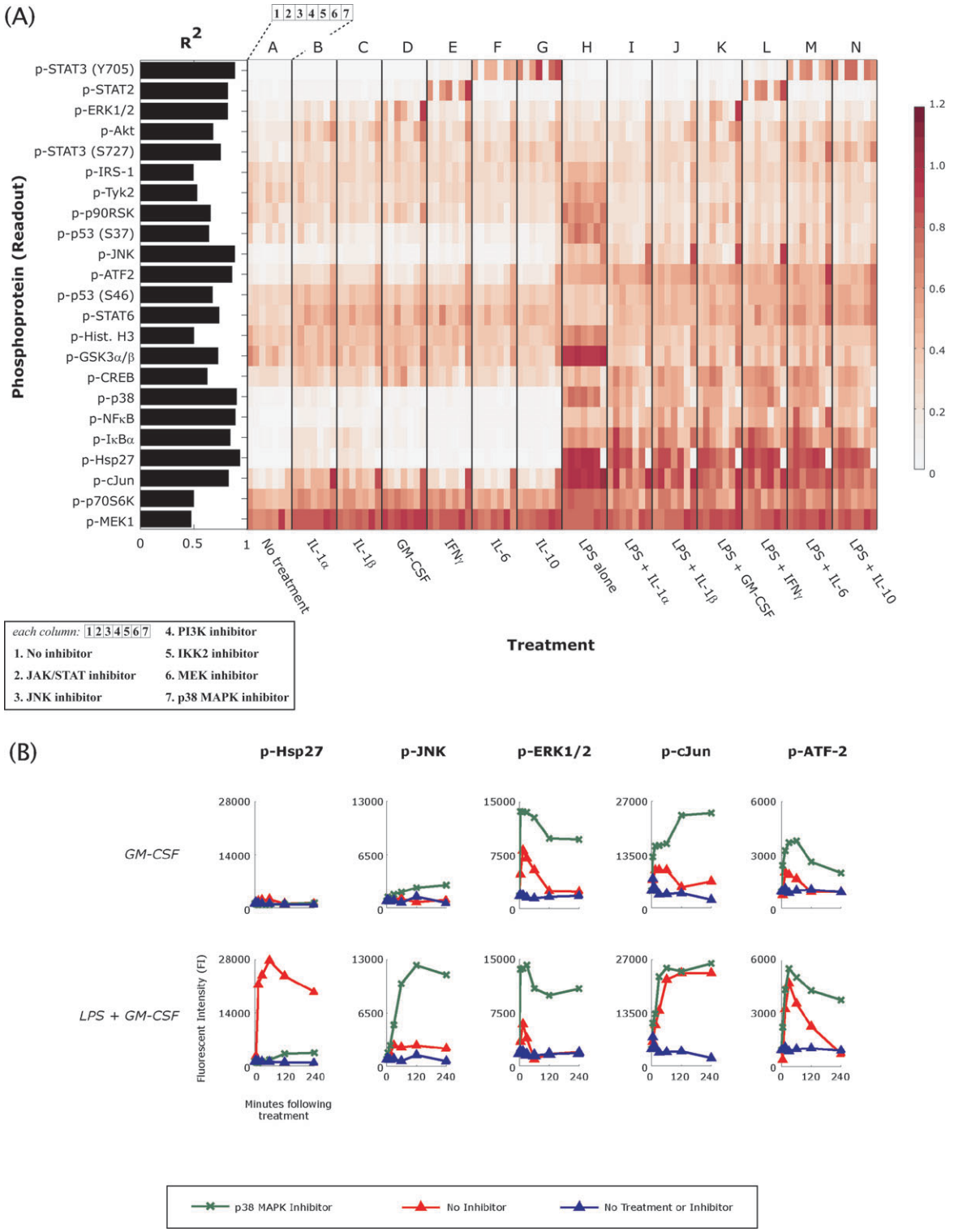

Fig. 4 (A) Evaluation of 23 phosphoproteins during the first 4 hours of a 24 hour time course corresponding to conditions in Fig. 3A are summarized with a 1-component PCA model. The complete dataset is available in ESI.†[84–87] (B) MAPK response to p38 inhibition. U937 cells were treated with GM-CSF (upper) or LPS + GM-CSF (lower), either alone (red line; no inhibitor) or in combination with a p38 MAPK inhibitor (green line) for the indicated period of time. Media alone served as a control (blue line; No Treatment or Inhibitor). Cell lysates were prepared and the indicated phosphoproteins evaluated using a BioPlex/xMAP multiplex phosphoprotein assay.

Cellular context: impacting the efficacy of inhibitors

From our multi-dimensional cytokine profiling, we identified GM-CSF as being able to compromise p38 inhibitor-mediated inhibition of TNFα (Fig. 3A and B). The presence of elevated levels of GM-CSF has been identified in the synovial fluid of RA patients, and indeed, anti-GM-CSF regimens are in

development for the treatment of inflammatory disease.[43–46] To investigate further, we treated cells with LPS + GM-CSF and the indicated inhibitors for a 24 h period and measured TNFα levels at multiple timepoints (Fig. 5A). The combination of GM-CSF with LPS increases the amount of TNFα released relative to LPS alone (Fig. 5A, compare dark green and red lines; see also Fig. 1). As previously observed, the addition of a p38 inhibitor (0.1 μM) reduces TNFα at least 5-fold in the presence of LPS + GM-CSF, but is unable to reduce levels further (Fig. 5A, purple line). In fact, treatment with a range of concentrations of p38 inhibitor up to 10 μM reduced TNFα to the same degree in the presence of LPS + GM-CSF, indicating a maximal effect had been reached (data not shown). However, when the cells are treated with LPS alone, the p38 inhibitor is able to reduce TNFα to negligible levels (Fig. 5A, yellow line). As seen in Fig. 2B, treatment of U937 cells with GM-CSF alone causes a rapid, significant increase in p-ERK1/2 levels (consistent with previous reports in neutrophils), although TNFα levels remain fairly unaffected.[47] We thus treated the cells with a MEK inhibitor in an attempt to block ERK phosphorylation. The addition of a MEK inhibitor to cells treated with LPS + GM-CSF reduced TNFα to a level consistent with that generated by LPS alone (Fig. 5A, orange line). Reasoning that multiple pathways are contributing to TNFα production when LPS and GM-CSF are present, we simultaneously treated the cells with both a p38 inhibitor and a MEK inhibitor (0.1 μM each) in the presence of LPS + GM-CSF. The combination of both inhibitors resulted in reduction of TNFα to background levels (Fig. 5A, light blue line), a feat which could not be achieved by either inhibitor alone. Visual inspection of the cells (remained adherent with no obvious morphological changes), as well as continued phosphoprotein and cytokine response, indicates that the cells are still viable in the presence of dual inhibitors under our experimental conditions.

Similarly, the presence of IL-1β with or without LPS allows the cells to circumvent TNFα inhibition by a p38 inhibitor (Fig. 5B—light blue line; Fig. S2, ESI†). The dynamics of TNFα release observed with LPS + IL-1β are different, however, than those observed with GM-CSF. This may indicate induction and release of a cytokine in response to IL-1β, which then compromises the p38 inhibitor. As demonstrated in Fig. 3A, the MEK inhibitor appeared to be most effective in reducing TNFα levels when the cells are treated with IL-1β alone. We therefore treated the cells with a combination of p38 and MEK inhibitors in the presence of IL-1β +/− LPS, and in both cases TNFα levels were reduced and maintained at background levels (Fig. 5B—purple line; Fig. S2, ESI†).

Using IL-10 (or IL-6) to influence TNFα

Treatment of U937 cells with IL-6 or IL-10 reduces the measurable level of TNFα resulting from exposure of the cells to LPS (Fig. 1C). We wanted to know whether IL-6 and/or IL-10 could be used to modulate TNFα levels when GM-CSF was present, a condition under which a p38 inhibitor alone was not fully effective. Cells were treated with LPS alone (Fig. 5C, red line), LPS + GM-CSF (dark green line), LPS + GM-CSF + IL-6 (purple line) or LPS + GM-CSF + IL-10 (yellow line) for up to 24 hours and TNFα levels evaluated. The addition of IL-10 or IL-6 (100 ng mL^{-1} final) reduced TNFα to a level consistent (equal or below) with that of LPS alone (Fig. 5C). In order to determine whether IL-10 and/or IL-6 could also impact (or complement) the efficacy of an inhibitor with regard to TNFα, cells were treated with LPS + GM-CSF + p38 inhibitor alone (Fig. 5D, light blue line), or in combination with IL-6 (purple line) or IL-10 (yellow line). Addition of IL-10 (or IL-6) is indeed able to significantly reduce the amount of TNFα remaining after p38 inhibitor treatment in the presence of GM-CSF.

Multiple cytokine co-regulation. The extent to which particular cytokines are "hard-wired" to behave similarly may dictate the feasibility of using small molecule inhibitors to achieve desired effects. To explore this idea, we calculated correlations between cytokine profiles across all of our stimulatory conditions in order to gain insight into cytokine–cytokine co-regulation, identifying distinct clusters of cytokines with similar behavior (Fig. 6). Remarkably, there are several cytokines that show exquisitely tight correlation across all stimulation and inhibitory conditions, including cytokines that show a large dynamic range (TNFα and IL-10), those that are constitutively produced (IL-8 and RANTES) and those that are lowly produced (IL-5 and IL-7). For example, there is a block of cytokines comprised of IL-1ra, IL-10, IL-6, TNFα, G-CSF, and IL-1β that are tightly correlated. As this correlation holds across numerous treatment conditions (93 in all) including treatment with multiple small molecule inhibitors, it suggests that these cytokines are co-regulated. The therapeutic implications are that (i) there are groups of cytokines for which it might not be possible to independently manipulate a specific member of the group using small molecule inhibitors and (ii) targeting one member may be sufficient to manipulate the entire group (for better or worse). Minimally, such co-regulation identifies cytokines which should be more carefully monitored when one member of the cluster is targeted and underscores the significance of monitoring multiple endpoints. Further work will be required to determine the hierarchy of cytokine regulation within the cluster. Finally, it is interesting to note a single example of a strong negative correlation (FGF-basic and IL-16).

p38 inhibitor *vs.* TNFα-neutralizing antibody

Anti-TNFα antibodies are currently a significant component in the therapeutic arsenal against rheumatoid arthritis, demonstrating the clinical importance of TNFα signaling in the disease. p38 inhibitors, by contrast, have failed to reach the market despite showing great promise in regulating TNFα *in vitro* and in animal models. Having identified a number of cellular contexts which compromise p38 inhibitor-mediated inhibition of TNFα production, we sought to directly compare a p38 MAPK inhibitor and TNFα-neutralizing antibody with regard to global cytokine production. Cells were stimulated with the indicated treatments either alone or in combination with a p38 inhibitor (PHA00818637; 0.1 μM) or a TNFα-neutralizing antibody (R&D Systems AF-210-NA; 0.02 μg mL^{-1} final). Levels of 50 cytokines were then measured over a 24 hour time course, under 14 stimulation conditions

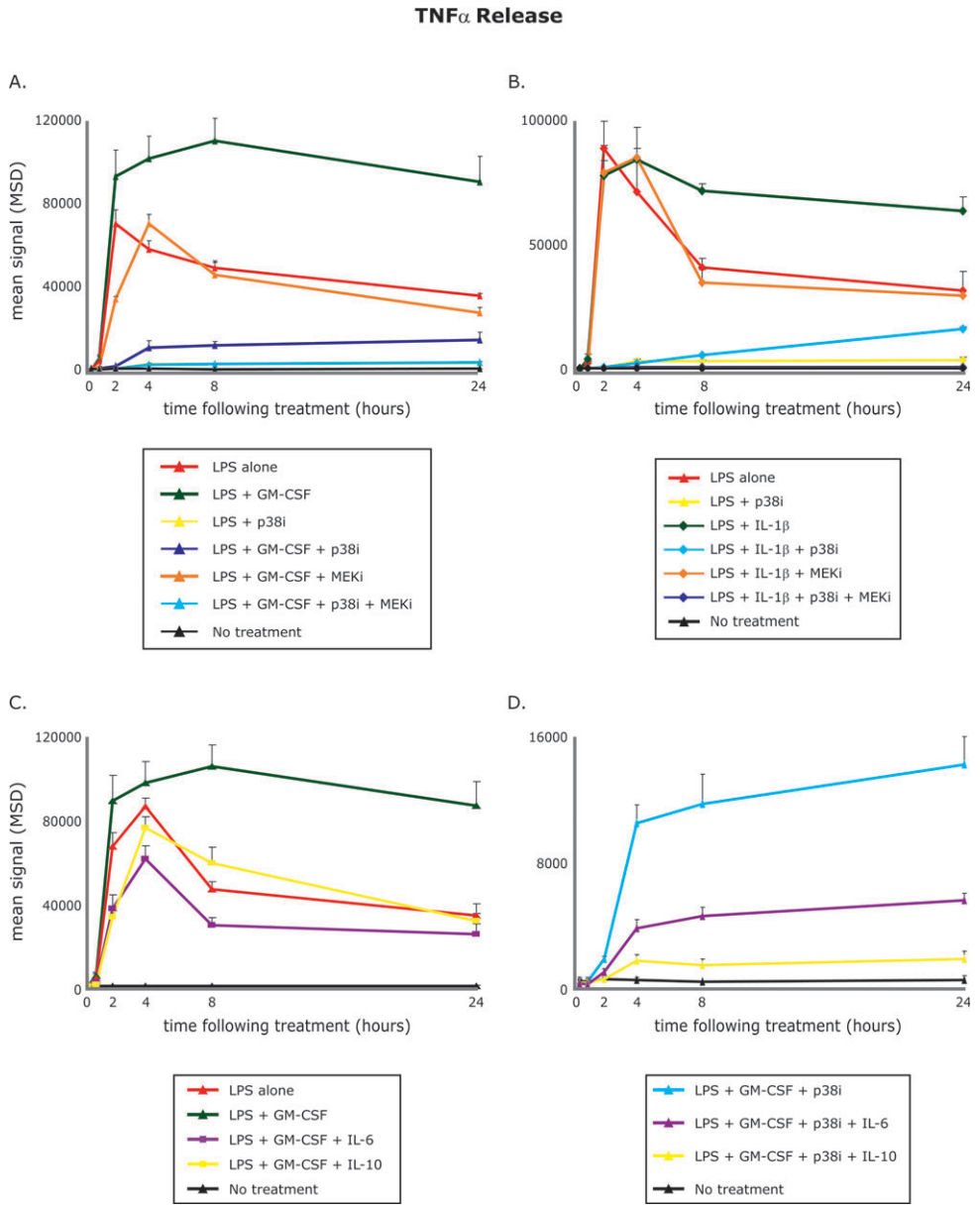

Fig. 5 Impact of cell context on inhibitor efficacy. (A) Effects of GM-CSF on p38 inhibitors. U937 cells were treated with LPS alone (red line), LPS + p38i (yellow line), LPS + GM-CSF (dark green line), or LPS + GM-CSF and either a p38 MAPK inhibitor (purple line), a MEK inhibitor (orange line) or both inhibitors simultaneously (light blue line). Inhibitors were added 30 minutes prior to LPS +/− GM-CSF at *each* timepoint. No treatment (no LPS, GM-CSF or inhibitor) served as a control (black line). Supernatants were recovered for each timepoint and TNFα measured using an MSD TNFα assay. (B) Effects of IL-1β on p38 inhibitors. U937 cells were treated with LPS alone (red line), LPS + p38i (yellow line), LPS + IL-1β (dark green line), or LPS + IL-1β and either a p38 MAPK inhibitor (light blue line), a MEK inhibitor (orange line) or both inhibitors simultaneously (purple line). Remaining details are as in Fig. 5A. (C) TNFα modulation with cytokines. U937 cells were treated with LPS (red line), LPS + GM-CSF (dark green line), LPS + GM-CSF + IL-6 (purple line) or LPS + GM-CSF + IL-10 (yellow line). Media alone (no treatment) served as a control (black line/triangle). Supernatants were recovered at the indicated timepoints following addition of cytokine +/− LPS and TNFα levels determined by MSD TNFα assay. (D) U937 cells were treated with LPS + GM-CSF + p38 MAPK inhibitor alone (light blue line), or in combination with IL-6 (purple line) or IL-10 (yellow line). No LPS, cytokine or inhibitor served as a control (no treatment; black line). Supernatants were recovered and TNFα levels determined using an MSD TNFα assay.

(Fig. 7A; PCA methodology used to depict values as in Fig. 3). As expected, both the p38 inhibitor and anti-TNFα antibody reduced TNFα levels by blocking production or neutralizing the cytokine in the media, respectively (rightmost cytokine). However, the p38 inhibitor also impacted multiple other cytokines in addition to TNFα, as might be expected due to its central signaling role. Most notably, the p38 inhibitor decreased levels of the anti-inflammatory cytokine IL-10 (second cytokine from right), as well as additional anti-inflammatory cytokines including IL-1ra, IL-13 and IL-2ra.

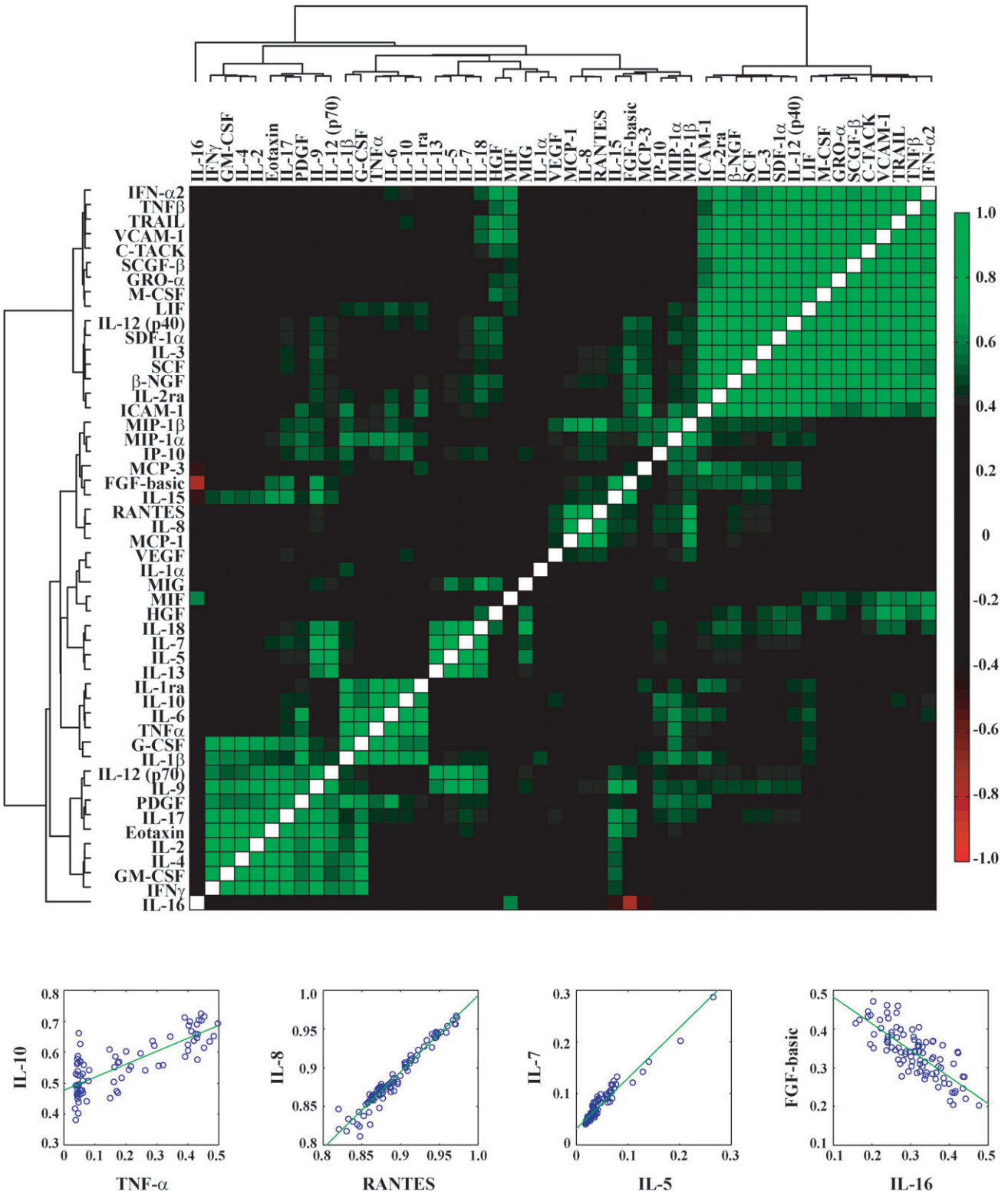

Fig. 6 Cytokine–cytokine correlations. Timecourse cytokine data were normalized to the maximum value of each measurement across all conditions (with/without inhibitors; 93 conditions in total). Average values of each time course were calculated and then pairwise linear correlation coefficients calculated using the 'corr' function in MATLAB R2007b. (Top) Average values from each cytokine time course for each of the 93 conditions were used to calculate pairwise linear correlation coefficients and used for clustering. (Bottom) Sample data plots used to calculate correlation coefficients. IL-10 and TNFα both demonstrate large dynamic signal ranges. IL-8 and RANTES are constitutively produced with minimal range. IL-5 and IL-7 demonstrate low levels and low range. FGF-basic and IL-16 represent the only significant negative correlation.

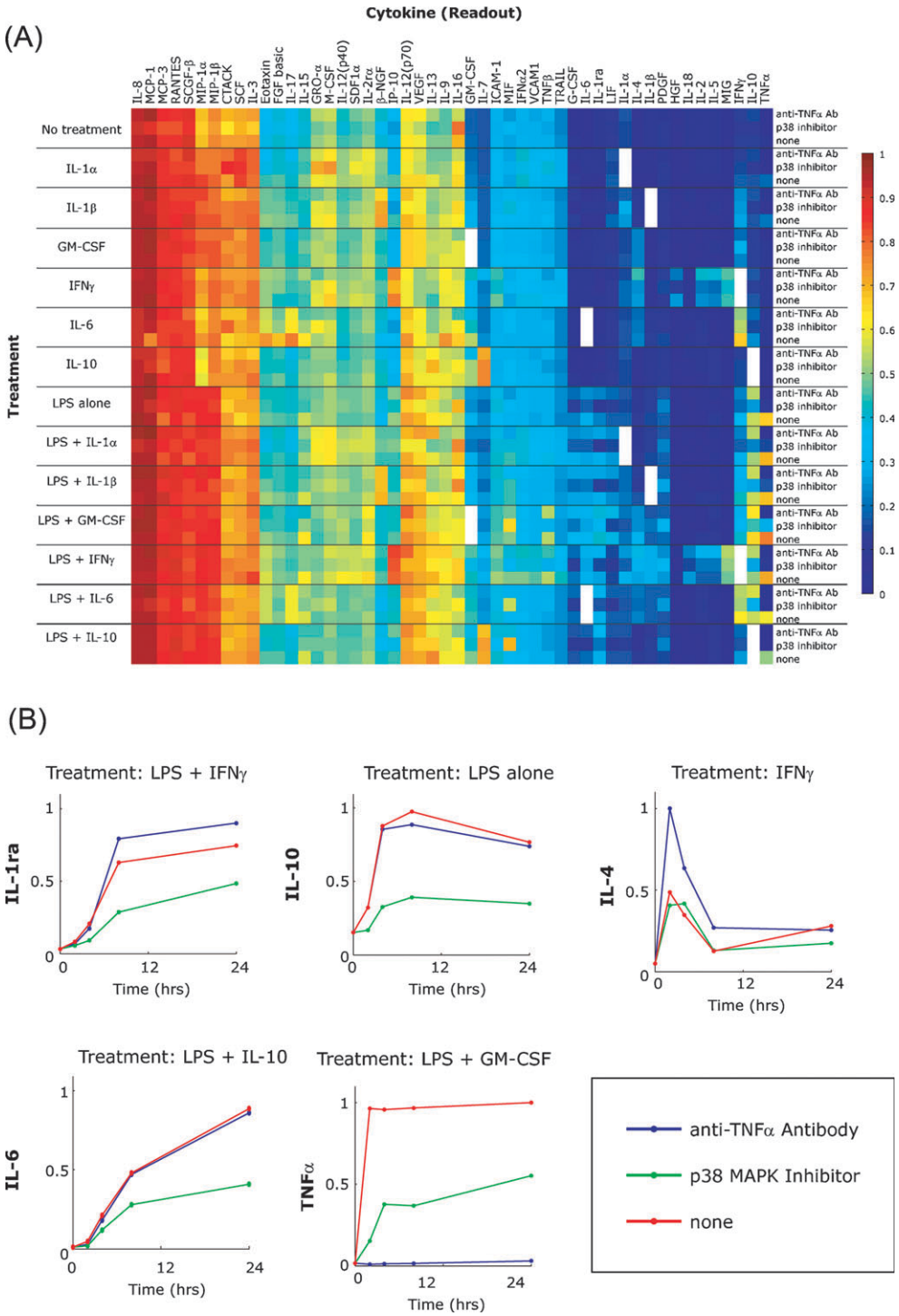

Fig. 7 p38i *vs.* TNFα-neutralizing antibody. (A) U937 cells were treated with cytokine stimuli as indicated (left *y*-axis, treatment) in the presence of (i) a TNFα-neutralizing antibody (anti-TNFα Ab), (ii) a p38 inhibitor or (iii) neither antibody nor inhibitor (none; right *y*-axis). The 50 indicated cytokines were measured in the supernatants over a 24 timecourse using a BioPlex/xMAP multiplex cytokine assay (cytokines, top of graph). For clarity of presentation, the average value of each timecourse is shown in the heatmap, with each cytokine measurement normalized by its maximum value across all treatments. White boxes indicate conditions where the stimulating cytokine was the same as the measured cytokine and could not be evaluated. The complete dataset is available in ESI.† (B) Expanded timecourse data depicting differences between treatment with a p38 inhibitor, anti-TNFα antibody or media alone during a 24 hour period. Treatment conditions are indicated above each graph, cytokine readout is depicted along the *y*-axis and presence of inhibitor (green), antibody (blue) or neither (red) is indicated by line color. Data are normalized to the maximal signal for each condition.

While p38 inhibition may achieve its primary aim of blocking TNFα production, the simultaneous reduction of anti-inflammatory endpoints may effectively undermine its performance in a clinical setting. Further complicating the

cytokine cost–benefit profile, production of MIF, IL-16, M-CSF, CTACK and GROα increased to varying degrees under certain conditions in the presence of the p38 inhibitor (Fig. 7A).

Interestingly, the TNFα-neutralizing antibody does not merely remove TNFα from the supernatant, but also causes additional secondary effects with regard to cytokine regulation. Previous analysis of a more limited set of cytokines using RA synovial membrane cells demonstrated the role of TNFα in regulating multiple downstream cytokines.[48,49] In contrast to the p38 inhibitor, release of IL-10 is unaffected by the TNFα antibody. Additionally, pro-inflammatory cytokines including IP-10 (treatment: IL-1α or IL-1β), IL-15 (treatment: LPS + GM-CSF) and IL-16 (treatment: GM-CSF or IL-6) were reduced in the presence of the anti-TNFα antibody depending on the treatment conditions. GM-CSF (treatment: IL-10) and Eotaxin (treatment: GM-CSF) were also reduced in the presence of the anti-TNFα antibody. On the other hand, IL-13 (treatment: LPS + IL-1α) and IL-12(p70) (treatment: LPS + GM-CSF) are increased with the same antibody. Fig. 7B presents the timecourse data highlighting the differential effects of a p38 inhibitor and anti-TNFα antibody on select endpoints. Presumably, the cumulative effects of modulating these pro- and anti-inflammatory cytokines (and more) using a p38 inhibitor or anti-TNFα antibody ultimately determine the efficacy of the chosen intervention.

Discussion

Establishing a Cue–Signal–Response dataset

Disease intervention occurs at the complicated intersection between stimuli (Cues) representing the cellular context at that moment, activation and compensation of multiple intracellular pathways (Signals), and output from the cell (Response), subsequently resulting in feedback and crosstalk between various cell types. The sum of these influences ultimately determines whether a desired endpoint can be achieved. The synovial fluid of RA patients represents just such a dynamic cytokine milieu which is the result of genetic differences between patients, environmental factors, varying disease states and in some cases, previous exposure to medical treatment. With this in mind, we generated a multi-dimensional dataset describing the phosphoprotein and cytokine response of U937 monocyte/macrophage cells to various disease-relevant stimuli. Macrophages play a central role in the inflammatory process of RA and therefore we chose the U937 cell line for our studies, a well-established model for monocyte/macrophage differentiation and function.[34–37] We have also obtained similar results using peripheral blood mononuclear cells (PBMCs; data not shown), while a more thorough analysis might include the evaluation (and possibly co-culture) of multiple immune cell types from both healthy and diseased individuals. Although the choice of cytokines used to treat the U937 cells was not exhaustive, and the possibility of synergistic effects is real and likely (especially *in vivo*), this work represents a significant effort to identify relevant effector molecules for modulating TNFα production.

The seven stimuli that we focused on (6 cytokines and LPS) are able to modulate multiple distinct pathways, yet all influence TNFα levels, demonstrating the complex nature of cytokine regulation (Fig. S3, ESI†). The ability to simultaneously capture data about multiple cytokines, beyond TNFα alone, provides the opportunity to evaluate additional factors which may be either beneficial (*i.e.* anti-inflammatory) or detrimental (*i.e.* pro-inflammatory) to the disease state. Further, exploring a wide range of points throughout a 24-hour timecourse allows for the integration of multiple mechanisms including transcription, translation, cytokine release, protein degradation, receptor levels and binding of released cytokines (autocrine and paracrine) among other activities. Taken as a whole, the information generated by this Cue–Signal–Response (CSR) dataset has the potential to aid in the identification of unintended side-effects, better understanding of mechanisms of action and recognition of biomarkers, as well as prediction of inhibitor efficacy and patient selection.

Context-dependent behavior

While the notion of context-dependent behavior is well appreciated in biology, it has not yet been strategically incorporated into modern drug discovery. There are many levels at which one needs to consider context-dependence, including differences between normal and disease states, differences in tissue specificity and inter-individual variability, encompassing genetic, environmental or lifestyle differences among others. Further, although monitoring of systemic levels for a particular target (*e.g.* TNFα levels in blood) may serve as a proxy for the efficacy of an inhibitor, it is the local concentration within the diseased tissue such as the synovial joint which is critical in determining how a targeted tissue responds to therapy. At the cellular level, we reconstructed the notion of context through the use of multiple disease-relevant stimulation conditions. This represents a first step towards replicating the complex *in vivo* cellular microenvironment.

The approach presented in this work has enabled us to identify the important roles of GM-CSF and IL-1β in modulating TNFα production, particularly in the context of p38 inhibition. Elevated levels of GM-CSF have been identified in RA synovial fluid and indeed, anti-GM-CSF therapies are being pursued for the treatment of RA providing relevance to our *in vitro* observations.[43,49,50] Additionally, flare-ups of RA have been reported in patients treated with GM-CSF for neutropenia in Felty's syndrome or after chemotherapy.[51,52] A p38 inhibitor alone was unable to eliminate TNFα production in the presence of LPS + GM-CSF (in contrast to cells treated with LPS alone), indicating the potential for GM-CSF to circumvent the efficacy of a p38 inhibitor. Treatment of U937 cells with retinoic acid, which similarly induced phosphorylation of ERK1/2, also circumvented p38 inhibitor-mediated reduction of TNFα levels as well (data not shown), indicating the potential use of an alternative pathway. The relative inhibition achieved by a p38 inhibitor in the presence of GM-CSF is potentially even less significant *in vivo*, as the amount of TNFα induced by LPS in our experimental system may be artificially high. It is also

noteworthy that GM-CSF did not significantly induce TNFα release on its own, indicating that it may act to exacerbate an ongoing inflammatory response, consistent with its reported role in stimulating already-present macrophages and neutrophils to release inflammatory cytokines.[43] When both the p38 MAPK and ERK pathways are simultaneously inhibited, TNFα release is negligible even in the presence of LPS + GM-CSF. The clinical relevance of these results is potentially significant, suggesting it could be valuable to determine if those patients who are non-responsive to p38 inhibitors have elevated GM-CSF in their inflamed joints relative to responders. If so, GM-CSF may represent a potential biomarker which can be used for patient stratification to identify those who are good/bad candidates for p38 inhibitor therapy. Further, a co-drugging regimen of anti-p38 and anti-MEK inhibitors may provide improved disease remedy (or a p38 inhibitor in combination with an anti-GM-CSF antibody). Such combinations would be particularly advantageous if they allowed for lower dosing of each compound with the same or improved overall reduction in TNFα, possibly reducing instances of adverse events or toxicity.

Treatment of cells with IL-1β presented a similar situation, whereby the p38 inhibitor was not able to fully inhibit TNFα. Unlike GM-CSF, however, IL-1β (and IL-1α) is also able to induce TNFα release from U937 cells on its own. Elevated IL-1β levels are detected in the joints of RA patients and anti-IL-1 therapies have in fact been approved for treatment of RA (recombinant IL-1ra; anakinra/Kineret). However, anakinra has not proven to be particularly effective in the treatment of RA (as well as requiring daily subcutaneous injections), while simultaneous treatment with anti-TNF and anakinra has not improved disease outcome relative to either treatment alone.[53,54] To the best of our knowledge, combination of a p38 inhibitor and anti-IL-1 has not been evaluated, however, and may represent another option for simultaneously regulating two (or more) cytokines which are known to be elevated in RA patients. We note that treatment of U937 cells with IL-1β results in the release of GM-CSF, which may in turn influence p38 inhibitor efficacy. Similarly, previous studies demonstrated that anti-TNF antibodies were capable of reducing IL-1, while the reciprocal was not true, indicating a hierarchy of regulation.[48,49] Whether a p38 inhibitor is able to disrupt the interplay between these two cytokines (TNFα and IL-1β) is likely dependent on many factors including their relative levels at the time of therapeutic intervention.

We have also demonstrated that addition of IL-10 (or IL-6) improved (or supplemented) the ability of a p38 inhibitor to reduce TNFα even in the presence of GM-CSF. As with GM-CSF, the levels of IL-10 (or IL-6) within the synovial fluid of RA patients may serve as a biomarker for those who would best respond to a p38 inhibitor. The predictive value of IL-10 levels is however a complicated issue depending on the pathophysiology being investigated, as elevated IL-10 levels have also been associated with tumor occurrence and progression, as well as transplant rejection.[55,56] It should also be kept in mind that IL-10 is believed to play a role in regulating TNF-R levels, and if the manner by which IL-10 is reducing TNFα levels in the cellular environment is *via* increased TNFα uptake, consideration must be given to what effect this may have on the cell.[57] Alternatively, co-administration of recombinant IL-10 with a p38 inhibitor might also be considered, although the cost and logistics of such an approach would need further evaluation. Relatedly, given the apparent role in reducing TNFα levels, it is particularly concerning to see that IL-10 levels are reduced by treatment with a p38 inhibitor. Such an effect has the potential to reduce the efficacy of a p38 inhibitor *in vivo*.

In our hands, addition of IL-6 to U937 cells improved the ability of a p38 inhibitor to reduce TNFα production. IL-6 is a complex cytokine with established pro- and anti-inflammatory behaviors reported.[6,58–60] The recent approval of Tociluzimab (an anti-IL-6R antibody) in Europe for treatment of RA suggests a predominantly pro-inflammatory role, in contrast with our *in vitro* data. Long-term studies will ultimately determine the efficacy of Tociluzimab in the treatment of RA, while additional research would be required to determine if synergy exists between p38 inhibitors and anti-IL6R antibodies in animals or humans.

Measuring multiple endpoints

Although it remains essential to evaluate the efficacy and specificity of an inhibitor using directed *in vitro* assays, the true determination of the value of a compound resides in studying its effect in cells and ultimately the human body. It is increasingly clear that a reductionist view of the kinase-inhibitor–substrate relationship, or even an individual cellular pathway, is inadequate to capture the complex biological response of a cell to an intended therapy. When we blocked the p38 MAPK pathway in U937 cells using a small molecule inhibitor, a compensatory increase in JNK phosphorylation was observed, possibly as a result of disrupting phosphatases downstream of p38, such as MKP-1. The degree of increased JNK phosphorylation was context-dependent: a more significant increase was observed when the p38 pathway is activated. Muniyappa and Das reported similar behavior in A549 cells, primary endothelial cells and MCF7 breast cancer cells using two different p38 inhibitors.[61] We further observed elevated phosphorylation of ERK1/2 kinase in response to the p38 and JNK inhibitors, as well as increased phosphorylation of p38 MAPK in the presence of a JNK inhibitor (Fig. 4A and data not shown), indicating the inter-connectedness of the MAPK pathways. Interestingly, the dynamics of the compensatory responses in most cases was prompt, suggesting release of feedback at the protein, rather than transcriptional level. Such pathway compensation by the cell represents another means by which the efficacy of a kinase inhibitor may be circumvented and should be considered. The identification of such cross-talk between pathways is only possible through monitoring of multiple sentinel molecules within the cellular context.

We further detected increased phosphorylation of c-Jun, ATF-2 and CREB in the presence of the same p38 inhibitor, indicating that the upregulated JNK and/or ERK proteins are functional and activating their signaling cascades. c-Jun and ATF-2 have been identified as oncogenes and their levels associated with a host of cancers, suggesting that their upregulation may warrant consideration when treating with p38 inhibitors.[62–64] Previous studies have hinted at elevated

risk of certain types of cancer (particularly lymphomas) in connection with the use of anti-TNFα therapies, although these observations may be the result of a diminished immune response rather than upregulation of an oncogene.[65–68]

Global cytokine profiling enables one to consider multiple endpoints and lays the foundation for simultaneously evaluating both efficacy and safety outcomes. For example, in addition to TNFα, there are many other cytokines and growth factors which are implicated in RA including VEGF, a key driver of vascularization and angiogenesis and a fundamental component of RA pathology.[69] VEGF is secreted by synoviocytes and infiltrating leukocytes, and is upregulated by the hypoxic conditions in the RA synovium.[70–73] Multiple animal models support the use of angiogenesis inhibitors for the treatment of arthritis, while anti-VEGF therapies including Avastin, "VEGF-Traps" and soluble VEGF-R are being investigated for the treatment of RA in humans.[74–77]

Under our experimental conditions, U937 cells constitutively produce elevated levels of VEGF. However, when IL-10 (or IFNγ) was added to the cells, VEGF levels were reduced (as was FGF-2 basic, to a much lesser degree). Relatedly, IL-10 has been shown to be capable of reducing VEGF levels produced by human A375P melanoma cells and esophageal cancer cells, with a resultant limitation of metastasis and tumor growth.[78,79] When we treated U937 cells with a JAK/STAT inhibitor in combination with IL-10, however, VEGF levels remained elevated—a result consistent with blockage of the IL-10/JAK–STAT signaling cascade (Fig. S4, ESI†). As JAK–STAT inhibitors are currently being pursued for the regulation of TNFα and the treatment of RA, careful consideration should be paid to the effects on VEGF, a factor intimately associated with tumorigenesis. This result highlights the necessity of considering the relevant cytokine context (*i.e.* IL-10/IL-6 levels), the timescale evaluated (VEGF decrease started at 4 hours and continues through 24 hours), and multiple endpoints being evaluated (VEGF, in addition to TNFα and STAT3), before making decisions regarding the benefit of a particular inhibitor.

Comparing an anti-TNFα antibody and a p38 MAPK inhibitor. When one directly compares TNFα-neutralizing antibodies with p38 inhibition, both approaches are effective at reducing local TNFα levels under most conditions. We have shown, however, that TNFα-neutralizing antibodies do more than simply bind free TNFα, but also reduce levels of additional pro-inflammatory cytokines including IP-10, IL-15 and IL-16, presumably as a result of feedback mechanisms. We have also observed similar results, namely up- and down-regulation of additional "non-targeted" cytokines, in response to treatment with various other anti-cytokine antibodies (data not shown). In the presence of IL-10, the TNFα-neutralizing antibody also reduced GM-CSF, thus potentially minimizing another contributor to TNFα production. Similarly, p38 inhibitors also block more than just TNFα alone, although this might be more readily predictable due to the central role of p38 in signaling cascades. Of particular interest is that p38 inhibition also reduced anti-inflammatory cytokines that may play important roles in resolving inflammation. It is conceivable that simultaneously disrupting TNFα and an anti-inflammatory cytokine such as IL-10 will result in a net response that provides no therapeutic benefit. Most importantly, these studies suggest important biomarkers to measure in subsequent clinical trial design.

Connecting Cues, Signals and Responses

Ideally, CSR datasets such as those described in this work can be utilized to draw connections between phosphorylation states and cytokine release under various conditions in order that predictions can be made regarding the best options for disrupting a particular cytokine. Relatedly, the consequences of disrupting a specific intracellular pathway with regard to the cytokines which would be impacted could provide insight into collateral effects. Partial Least-Squares Regression (PLSR) has been used in the past to link signals with responses.[80] We explored PLSR as one such means of providing descriptive insight into the multivariate connection between the measured phosphorylation signals and cytokine responses (Fig. S6, ESI†). In this analysis, it was found that the incorporation of inhibitors into the dataset was critical to disentangle intracellular signaling networks as it provides a means to disrupt correlations that are not causal. However, in most instances the multivariate nature of these correlations reflects the complex nature of cytokine regulation and has made it difficult to translate into clear direction for therapeutic design. Additional data, possibly from treatment with combinations of inhibitors, may provide the necessary information required to more accurately predict outcomes.

Such approaches represent initial attempts at possible methods for analyzing such a rich multi-dimensional phosphoprotein and cytokine dataset and are an area of ongoing research. Linking phosphoprotein and cytokine data further offers a means for bridging small molecule inhibition and biotherapeutics in a rational manner.

Conclusion

In summary, this work describes an approach for investigating the significance of cell context using multiple endpoints in order to evaluate cellular response to putative therapeutic interventions. We have identified disease-relevant extracellular stimuli, including IL-1β, GM-CSF, IL-6 and IL-10, which impact the ability of a p38 inhibitor to regulate TNFα production in the U937 monocyte/macrophage cell line. We have further shown that the same cells compensate for p38 inhibition by upregulating the JNK and MEK/ERK pathways, providing possible means by which to circumvent TNFα inhibition. While some biologic approaches have cast a spotlight on TNFα production as the primary endpoint of interest in regulating RA, anti-TNFα antibodies block additional cytokines in the U937 cell line, many of which are pro-inflammatory. Small molecule p38 inhibitors also blocked multiple cytokines in addition to TNFα, including IL-10, a significant anti-inflammatory cytokine whose reduction may compromise p38 inhibitor efficacy in an *in vivo* or clinical setting. The multi-dimensional profiling that we describe in this work captures scores of complex relationships and we have, no doubt, only scratched the surface of the dataset. Nonetheless, a similar approach is broadly applicable to many

cell types, stimuli and disease indications. Thus, it provides a method to enable enlightened decision-making with regard to therapeutic targets and compound differentiation in early stages of drug discovery, as well as offers potential avenues for improved trial design and/or patient selection.

Methods

Cell culture

The human monocytic U937 cell line (ATCC) was grown in RPMI-1640 supplemented with 10% (final) fetal bovine serum (Gibco; henceforth RPMI). Cells were treated with Phorbol 12-myristate 13-acetate (PMA; Sigma P8139, St. Louis, MO) at 20 ng mL^{-1} (final) for 48 h, media removed and adherent cells scraped from the flask. Cells were washed twice in PBS, resuspended in RPMI, counted using a hemacytometer and aliquoted at 180 000 cells per well in 200 μL volume (final) in 96-well culture plates and allowed to reattach for 24–48 h. Before each timecourse, media was aspirated and replaced with 150–180 μL of fresh RPMI. At each timepoint, 20–50 μL of RPMI containing the appropriate concentration of cytokine (100 ng mL^{-1} final; Peprotech, Rocky Hill, NJ), lipopolysaccharide (LPS; 1 or 100 ng mL^{-1} final; Sigma L3012, St. Louis, MO) or media alone was added (to produce 200 μL final volume). Inhibitors were added in a minimal volume of RPMI thirty minutes prior to addition of cytokine/LPS at each time point. At the conclusion of the time course, supernatants were recovered and lysates prepared in either MSD Lysis Buffer or Bioplex Lysis Buffer as described by the vendors (Meso-Scale Discovery, Gaithersburg, MD; Bio-Rad Corp, Hercules, CA). The chemical name of the p38 MAPK inhibitor (PHA-00818637) is 3-(3-bromo-4-(2,4-difluoro-benzyloxy)-6-methyl-2-oxopyridin-1(2H)-yl)-4-methylbenzamide. The chemical name of the JAK inhibitor (PF-00956980) is ((3R,4R)-4-methyl-3-(methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino)piperidin-1-yl)(pyrrolidin-1-yl)methanone. The remaining inhibitors and their structures are commercially available.

Meso Scale Discovery (MSD)

Assays were performed as described by the vendor (Meso Scale Discovery, Gaithersburg, MD). Briefly, 25 μL of supernatant was aliquoted per well of the assay plate (TNFα plate: K111BHB-1), the plate sealed and shaken at RT for 1–2 h. Supernatants were removed following incubation, plates washed and secondary antibody added followed by shaking at RT for 1 h. Secondary antibody solution was removed, plates washed and Read Buffer T added before being read on a Sector Imager 6000. MSD data in Fig. 1, 5 and Fig. S2 (ESI†) represent an $n = 4$ to 6 per datapoint with standard deviation presented.

BioPlex/xMAP-Luminex

Assays were performed as described for BioPlex technology from Bio-Rad (Hercules, CA). Assay beads were prepared and aliquoted to a 96-well filter membrane plate. 50 μL of supernatant or lysate were mixed with the beads, plate sealed and shaken at RT either overnight (phosphoproteins) or for 2 h (cytokines). Supernatants/lysates were removed by vacuum, beads washed and secondary (detection) antibody added, plate sealed and shaken at RT for one hour. Secondary antibody was removed by vacuum, beads washed and incubated with Streptavidin-PE for 10–20 min before being washed, resuspended in BioPlex Resuspension Buffer and read on a BioPlex 200 System. Phosphoprotein multiplex assays were custom ordered. Cytokines were evaluated using the Human Cytokine Group I and Group II panels. BioPlex data presented in Fig. 2 represent $n = 2$, Fig. 3 $n = 1$ and Fig 7 $n = 2$. Data analysis and graphs were generated using the MatLab-based DataPflex application.[81]

PCA

Principal component analysis (PCA) was conducted in MATLAB R2008a using the 'parafac' function in the *DataRail* toolbox,[82] which is a modified version of the 'parafac' function in the *N-way* toolbox[83] (The MathWorks, Natick, MA, USA). A matrix was constructed for each intracellular phosphoprotein and extracellular cytokine measurement, with each row representing a different stimulation condition and each column a different time point. The data were normalized by the maximum value of each measurement across all conditions (*i.e.* the maximum value in the entire matrix). For visualization, a one-component PCA model was determined for the uncentered data, facilitating the graphical interpretation of the scores and loadings. The PCA scores of the different proteins were hierarchically clustered using the unweighted average Euclidian distance (MATLAB statistics functions 'pdist' and 'linkage'), and the leaves of the cluster tree were ordered using the MATLAB function 'optimalleaforder'. Although uncentered data were preferred for visualization, the lack of centering implies that the first component is strongly influenced by the mean response. Therefore, a one-component PCA model was also determined for each column-centered matrix and used in subsequent PLSR modeling.

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