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ORIGINAL ARTICLE

The synergy in cytokine production through MyD88-TRIF pathways is co-ordinated with ERK phosphorylation in macrophages

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Although specific single Toll-like receptor (TLR) ligands are known to drive the development of Th1 or Th2 immunity, the outcome of different combinations of TLR ligands on innate immunity is not well defined. Spatiotemporal dynamics are critical in determining the specificity of the immune response, but the mechanisms underlying combinatorial TLR stimulation remain unclear. Here, we tested pairwise combinations of TLR ligands separated by different time intervals for their effect on cytokine production in macrophages. We observed that stimulation via a combination of MyD88- and TRIF-utilizing adaptors leads to a highly synergistic cytokine response. On a timescale of 4–24 h, macrophages pretreated with poly(I:C) (TLR3 ligand) are cross-primed to a second stimulation with R848 (TLR7 ligand) and vice versa, and each condition exhibits different optimal time windows of synergistic response for each cytokine. We show that the synergy resulting from combinatorial stimuli (poly(I:C) and R848 is also regulated by the order and dosage of the TLR agonists. Secondary response genes, which depend on new protein synthesis for transcription, show greater synergy than primary response genes, and such enhancement is abolished when new protein synthesis is inhibited. Synergistic cytokine production appears concordant with sustained ERK phosphorylation, suggesting that the *de novo* factors act via inhibition of ERK dephosphorylation, for example, by the downregulation of dual specificity phosphatase 6. Taken together, our findings illustrate a checkpoint in the innate immune system, where the synchronization of timing of both MyD88 and TRIF pathways is required for a maximal cytokine response and potential memory effect in macrophages.
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The innate immune system possesses a range of receptors, which detect conserved microbial ligands called pathogen-associated molecular patterns (PAMPs).[1] In the mouse and human Toll-like receptor (TLR) system, a total of 13 different receptors are known, each of which recognizes distinct bacterial or viral PAMP such as single-stranded and double-stranded RNA (TLR7 and TLR3, respectively).[2] In spite of the recognition of a diverse range of PAMP, TLR signaling converges on only two adaptors, MyD88 and TRIF. All TLRs utilize MyD88 with the exception of TLR3, which depends exclusively on TRIF. TLR4 is unique as it signals via both MyD88 and TRIF.

Although TLRs make use of a shared set of signal transduction molecules downstream of these two adaptors, the biological effect of each TLR agonist can differ considerably. For example, *in vitro* stimulation of dendritic cells (DCs) with TLR5 agonist, flagellin, induces Th1 responses, whereas TLR2 agonist, Pam_3CSK_4, induces a Th2 response.[3] Further complexity occurs *in vivo* when immune cells encounter pathogens bearing multiple TLR ligands. The importance of this complexity is reflected in several studies,[4–10] which have noted that certain pairwise combinations of TLR ligands induce much higher cytokine production when administered simultaneously rather than individually, a phenomenon described as synergy. Most of these studies have focused on the synergistic induction of interleukin (IL)-12p70 by DC, which acts on adaptive immunity to drive Th1 responses.[4–8] However, the effect of combinatorial PAMP stimulation on macrophages, which are normally the first cells to encounter pathogens in host tissues and exert their

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effect on innate rather than adaptive immunity,[9,10] remains relatively unexamined.

Although synergistic combinations of TLR agonists are known, the underlying logic governing TLR–TLR interaction and the resulting non-additive responses to complex stimuli remains unclear. The presence of multiple receptors and adaptors make it possible for crosstalk and nonlinear responses to occur, thus maintaining a more finely controlled, rapid and lower-energy system.[11] Both empirical and *in silico* data suggest that the temporal aspects of immune signaling can have profound effects on biological outcomes.[12,13] In adaptive immunity, the second response to a pathogen is much higher due to immunological memory of the first infection. However, this occurs on the timescale of several days to weeks, whereas innate immunity responds within hours. Our preliminary data showed that certain combinations of TLR ligands led to an enhanced cytokine response. We hypothesized that cells of the innate immune system could show either (1) an enhanced response to a second stimulation with a different TLR ligand (cross-priming) or (2) a reduced response to a second stimulation with the same TLR ligand (tolerance or antagonism), demonstrating a 'memory' of the first stimulation. Therefore, we examined the effect of dosage and temporal intervals between two TLR agonists known to induce synergistic cytokine production: poly(I:C) (TLR3 ligand) and R848 (TLR7 ligand) in macrophages. Our results show that appropriate timing and dosage of TLR agonists are critical parameters regulating the synergistic production of different cytokines, with variable optimal time windows for different classes of cytokines. In addition, we showed that while poly(I:C) dose-dependently enhances synergistic responses, R848 exhibits a threshold effect, suggesting two different mechanisms by which each signal contributes to synergy. We also demonstrate that synergy is dependent on new protein synthesis and is associated with enhanced and sustained ERK phosphorylation, which may be caused by the downregulation of dual specificity phosphatase 6 (DUSP6).

RESULTS

Combinatorial stimulation with poly(I:C) and R848 induces highest synergy in cytokine production

As a preliminary study, we stimulated RAW264.7 cells with pairwise combinations of TLR ligands for up to 24 h and measured a panel of 18 cytokines via a multiplex cytokine assay. We found that out of the

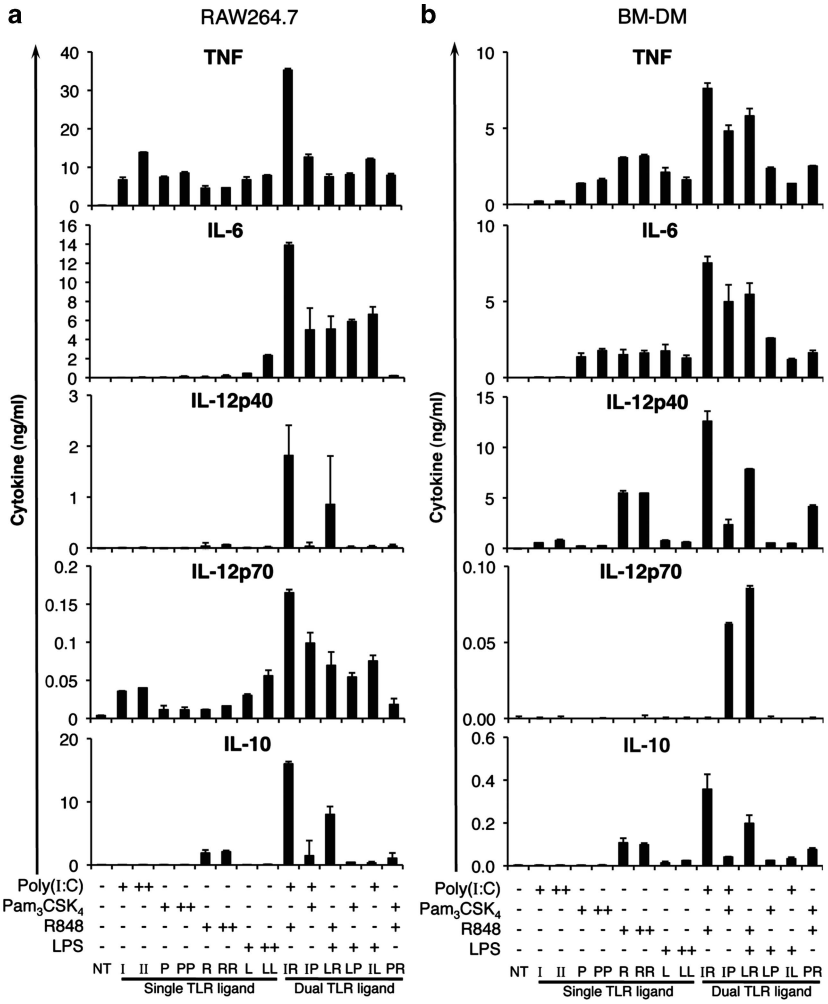


Figure 1 Multiplex cytokine assay of cells stimulated with various TLR ligand combinations. (**a**) RAW264.7 cells and (**b**) bone marrow-derived macrophages derived from BALB/c mice were stimulated with the indicated combinations of TLR ligands and cell culture supernatants were harvested after 24 h. The dosages of the TLR ligands were as follows: poly(I:C) 10 μg ml^{-1}, Pam_3CSK_4 10 ng ml^{-1}, R848 25 ng ml^{-1} and LPS 10 ng ml^{-1}. '++' Indicates a double dose of the same ligand. The profiles of a panel of five cytokines (TNF, IL-6, IL-12p40, IL-12p70 and IL-10) were analyzed by a multiplex cytokine bead array (Panomics) according to the manufacturer's instructions.

a

Ligand		Dose 1	2	3	4	5	6
Poly(I:C) (μg/ml)	I	10	20	40	80	160	320
LPS (ng/ml)	L	0.4	0.8	1.6	5	10	50
R848 (ng/ml)	R	1.25	2.5	5	10	20	40
Pam_3CSK_4 (ng/ml)	P	10	20	40	80	160	320

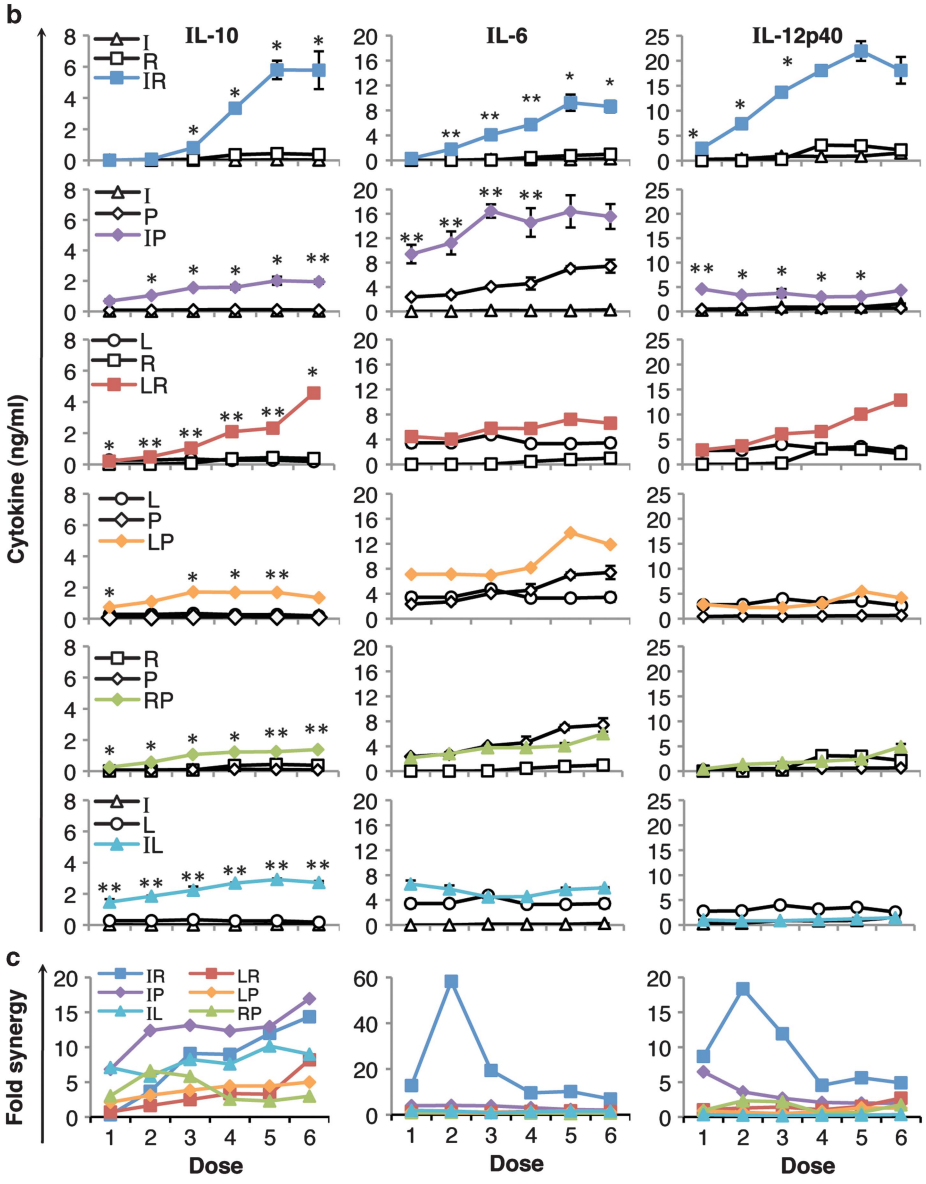
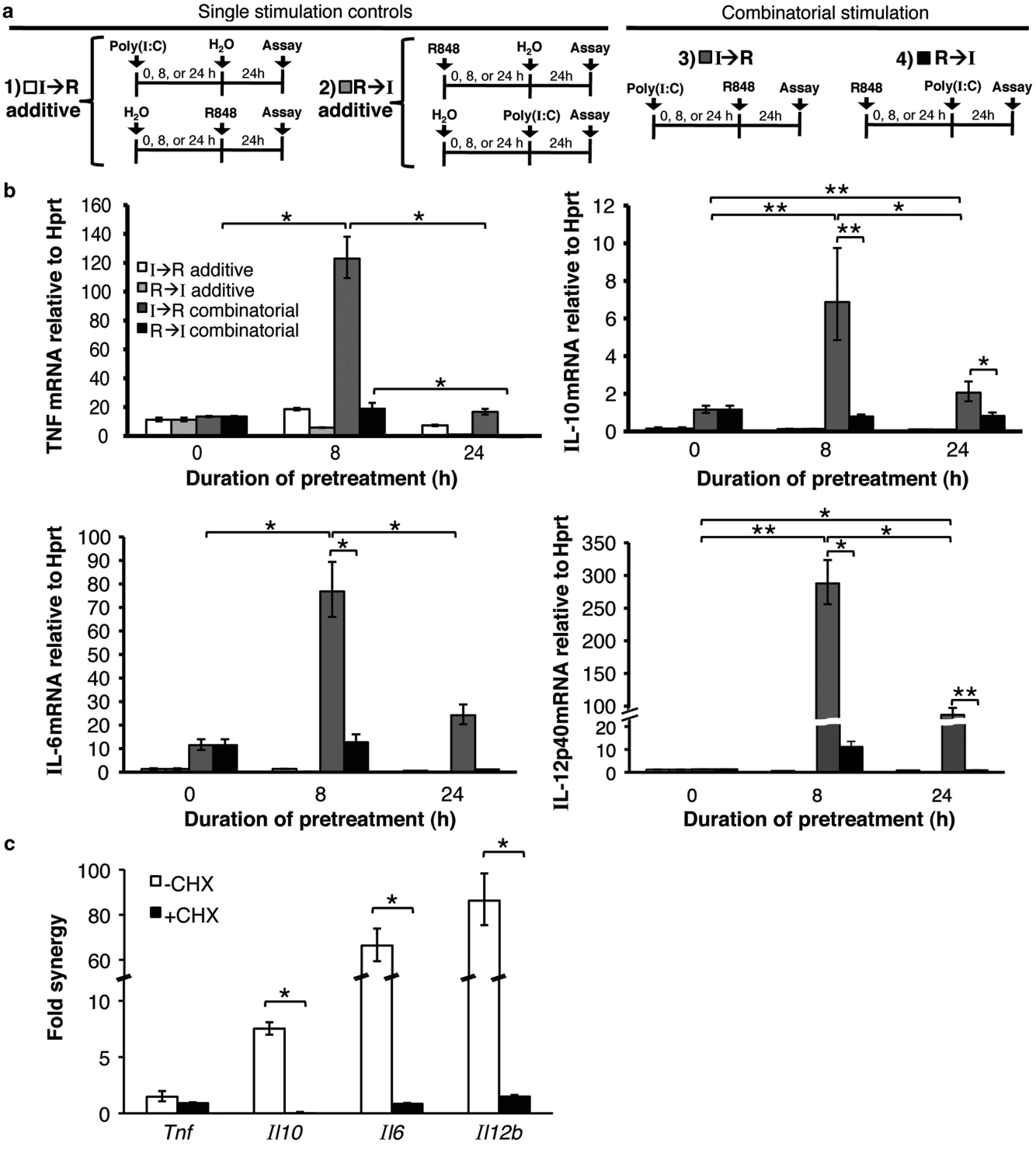


Figure 2 Combinatorial ligand stimulation of TRIF and MyD88 shows the greatest synergy in mouse macrophages. BALB/c bone marrow-derived macrophages (BM-DM) were stimulated with the indicated combinations and doses of TLR ligands as indicated in (**a**) for 24 h and cytokine concentration in cell-free supernatants were measured by ELISA. (**b**) Cytokine production and (**c**) fold synergy of IL-10, IL-6 and IL-12p40. Single TLR ligand stimulated values are indicated with open symbols and black lines: poly(I:C) (triangle), R848 (square), Pam_3CSK_4 (diamond) and LPS (circle). Combinatorial TLR ligand-stimulated values are indicated by filled and coloured symbols: poly(I:C) and R848 (blue square), poly(I:C) and Pam_3CSK_4 (purple diamond), LPS and R848 (red square), LPS and Pam_3CSK_4 (orange diamond), R848 and Pam_3CSK_4 (green diamond), or poly(I:C) and LPS (cyan triangle). Data shown are representative of three independent experiments. Student's *t-test* was performed to compare double TLR ligand stimulation (coloured lines) with the sum of single stimulations (white symbols, black lines) $^*P<0.05$, $^{**}P<0.01$.

18 cytokines measured, only tumor necrosis factor (TNF), IL-6, IL-10 and IL-12p40 were synergistically upregulated (Figure 1a). Based on this initial study, these four highly upregulated cytokines and IL-12p70 were further examined in BALB/c-derived bone-marrow macrophages (BM-DMs; Figure 1b). We observed comparable profiles of cytokine synergy in both the RAW 264.7 cell line and primary BM-DM cells derived from BALB/c mice, under similar conditions of TLR ligand stimulation (Figures 1a and b). Poly(I:C) was used as a TLR3 ligand, which signals exclusively via TRIF. Pam_3CSK_4 (TLR2 ligand) and R848 (TLR7 ligand) were used as MyD88-exclusive ligands, which signal via

the cell surface and endosomal compartments, respectively. Lipopolysaccharide (LPS) was included as a positive control that activates both the TRIF and MyD88 pathways. We found that simultaneous stimulation with two TLR ligands induced higher cytokines than double dose of a single TLR ligand (Figure 1, $++$) if the TLR ligands collectively activated both MyD88 and TRIF pathways.

In order to dissect the logic governing synergistic cytokine production in response to dual TLR ligand stimulation, we challenged BALB/c BM-DM with different pairwise combinations of MyD88- and TRIF-utilizing TLR ligands at increasing doses as indicated in Figure 2a, and cytokine production was measured 24 h later (Figure 2b). Using these different TLR ligands, we then tested the hypothesis that induction via MyD88 and TRIF combinations (poly(I:C) + R848/Pam_3CSK_4) would lead to synergistic cytokine production due to an interaction between the two pathways, whereas combinations of ligands inducing MyD88-MyD88



(R848 + Pam_3CSK_4) and TRIF-TRIF (poly(I:C) + LPS) would not. We observed that only low to moderate levels of both pro- and anti-inflammatory cytokines were produced when the BM-DM were challenged with single TLR ligands (Figure 2b, blank symbols), even at increasing doses. When challenged simultaneously with two TLR ligands, there was no significant increase in cytokine production if the two ligands activated the same signaling pathways (Figure 2b, green, R848 + Pam_3CSK_4; both engage only MyD88). However, when challenged with the combination of MyD88- and TRIF-utilizing ligands (Figure 2b, blue, poly(I:C) + R848; or purple, poly(I:C) + Pam_3CSK_4), the production of pro- and anti-inflammatory cytokines was much greater (Figure 2b, filled symbols) than that observed for single ligand stimulation (Figure 2b, open symbols). This was especially profound in the case where poly(I:C) (TRIF only) was paired with R848 or Pam_3CSK_4 (MyD88 only; Figure 2b, blue and purple, respectively). In an intermediate condition, where cells were challenged with LPS (MyD88 and TRIF ligand), further addition of MyD88-utilizing ligands, R848 or Pam_3CSK_4 (Figure 2b, LPS + R848, red; LPS + Pam_3CSK_4, orange), caused no synergy in the production of IL-6 and IL-12p40 (Figure 2b, red and green, respectively), although there was still synergistic production of IL-10.

To quantify the synergistic effect, the cytokine production under combinatorial stimulation was divided by the sum of the respective single stimulations to give a value we termed 'fold synergy' (Figure 2c). For the proinflammatory cytokines, IL-6 and IL-12p40, the levels produced by combinatorial stimulation is much greater than the single stimulated cells, with up to 20- to 60-fold synergy at low doses. However, as the dose increases, single stimulated cells also began to increase cytokine production, whereas the double stimulated cells approached saturation levels of cytokine production, causing the calculated fold synergy to drop. Interestingly, the anti-inflammatory cytokine, IL-10, shows a reverse trend, with synergy increasing as the dose increases (Figure 2c). This indicates that synergy affects different classes of cytokines in opposing ways, and that there is a negative feedback regulation limiting the amount of proinflammatory cytokines produced. Importantly, these patterns were reproduced in C57BL/6-derived primary macrophages (Supplementary Figure 1), showing that this was a general rather than strain-specific phenomenon.

Appropriate timing and order of TLR stimulation influence the optimal synergy

Following from the demonstration that the combination of different doses of poly(I:C) and R848 leads to synergy, we further investigated the kinetic effects with these two ligands. During the course of infection, macrophages may encounter different TLR ligands in a sequential manner. Thus, we tested the effect of both poly(I:C) followed by R848 [I→R combinatorial] and R848 followed by poly(I:C) [R→I combinatorial], measuring mRNA expression of TNF, IL-10, IL-6 and IL-12p40. Briefly, cells were pretreated with the first TLR ligand for 0, 8 or 24 h and then collected 8 h after stimulation with the second TLR ligand. To discriminate additive effects from potential synergy or antagonism, stimulations with single TLR agonists were included as controls (Figure 3a). The synergy of all cytokine mRNA was sensitive to the duration of pre-stimulation, with synergy increasing from 0 to 8 h and declining by 24 h (Figure 3b). Interestingly, the effect of the order of stimulation also strongly influenced the response where [I→R combinatorial, dark grey] led to much higher expression of cytokine mRNA than [R→I combinatorial, black] at the same time points. Importantly, our data show that priming by poly(I:C) is crucial for optimal synergy in cytokine gene expression in the macrophages, possibly via the action of synergy factor(s).

De novo protein synthesis is required for synergy effect on secondary response genes

To examine whether the 'synergy factor' is a newly synthesized or pre-existing protein, we used cycloheximide (CHX) to inhibit *de novo* protein synthesis in BM-DM and measured its effect on the cytokine transcripts. For IL-6 and IL-12p40, CHX treatment abrogated the synergy (Figure 3c), indicating a strong requirement for the newly synthesized protein. For TNF, there was only a low synergy of ~1.5-fold observed at the transcriptional level, which was slightly reduced by CHX treatment. It appears that synergy for the expression of secondary response genes (*Il6* and *Il12b*) mainly occurs at the transcriptional level and is highly dependent on new protein synthesis. In contrast, the synergy of primary response genes (*Tnf* and *Il10*) more likely occurs at the post-transcriptional level, and may not require new protein synthesis. Our observation is consistent with previous reports that LPS induction of secondary response genes but not primary response genes requires new protein synthesis.[14]

Both timing and dose of stimulation affects cytokine protein production

Next, we extended our study to the protein level using both BM-DM and RAW cells, as there is no apparent difference in the cytokine expression profiles for the primary cells and cell line (Figures 1a and b and Supplementary Figure 2). Briefly, BM-DM were stimulated with

Figure 3 Synergy in mRNA expression is dependent on *de novo* protein synthesis. (**a**) Graphical summary of experimental design. Bone marrow-derived macrophages (BM-DMs) were treated with either poly(I:C) followed by R848 [I→R combinatorial, dark grey] or R848 followed by poly(I:C) [R→I combinatorial, black]. Assuming a purely additive effect of two TLR stimulations, the single stimulation control for I→R [I→R additive, white] was taken to be the sum of cytokines produced by cells stimulated first with poly(I:C) for 0, 8 or 24 h followed by a mock stimulation with water for 8 h, and cells which were mock stimulated first with water for 0, 8 or 24 h followed by R848 stimulation for 8 h. Similarly, the single stimulation control for R→I [R→I additive, light grey] was the sum of cytokines produced by cells stimulated with R848 for 0, 8 or 24 h followed by water for 8 h and cells stimulated with water for 0, 4, 8, 16 or 24 h followed by poly(I:C) for 8 h. (**b**) BM-DM were pretreated with one TLR ligand for 0, 8 or 24 h, followed by a second TLR ligand for another 8 h. 0 h pretreatment denotes simultaneous stimulation. [I→R] (dark grey) symbolizes poly(I:C) as the first TLR ligand, followed by R848 as the second TLR ligand. [R→I] (black) indicates the same stimulations in reverse order. The single stimulation controls are computed for [I→R] (white) and [R→I] (light grey) as described in **a**. IL-10, TNF, IL-6 and IL-12p40 mRNA were measured by real-time PCR using Taqman probes specific to each cytokine. Similar results were obtained in two other independent experiments. Data represent means ± s.d. of triplicate measurements in a representative experiment. Student's *t*-tests were performed from the data of all three independent experiments (* = $P<0.05$, ** = $P<0.01$). (**c**) BM-DM were pretreated with poly(I:C) for 8 h, followed by R848 for another 4 h in the absence (white bars) or presence (black bars) of 10 μg ml^{-1} CHX for 15 min. Fold synergy was calculated by dividing the experimental group by the control group data. Similar results were obtained in two other independent experiments. Data represent means ± s.d. of triplicate measurements in a representative experiment. Student's *t*-tests were performed from the data of all three independent experiments (* = $P<0.05$).

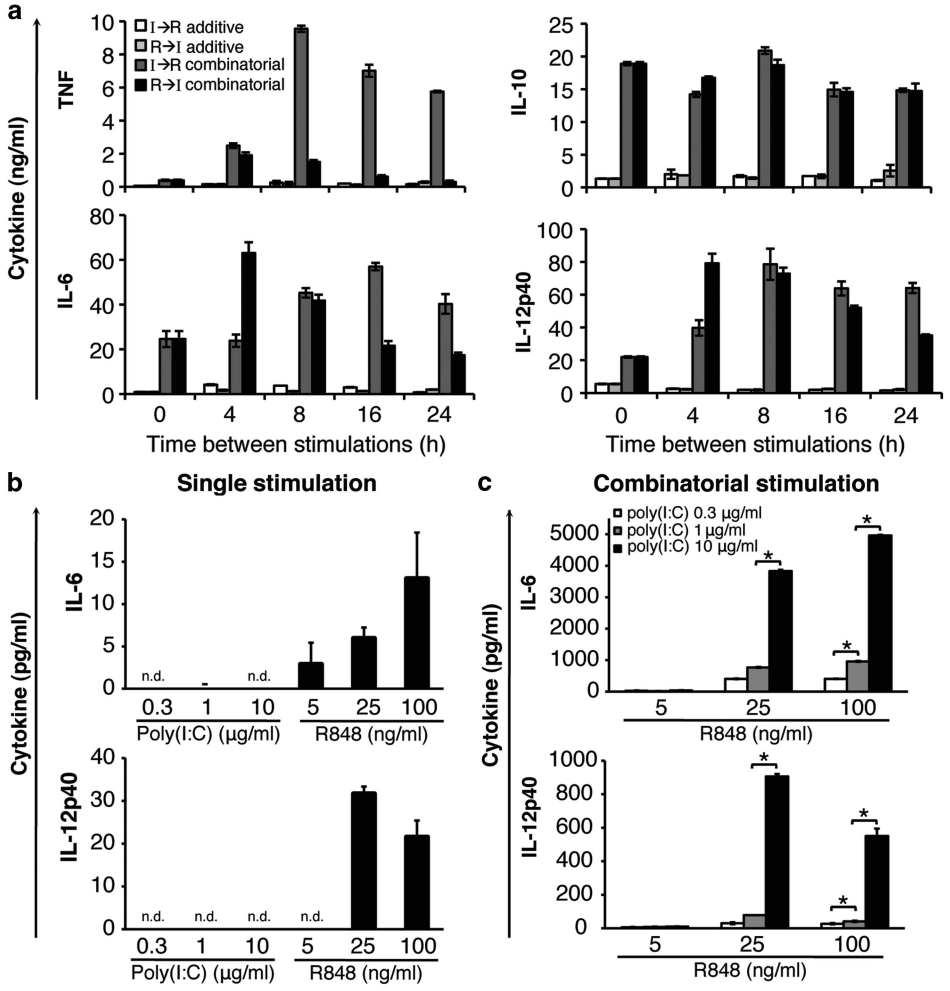


Figure 4 Time and dosage of TLR ligands induce synergy in cytokine production. (**a**) Pattern of cytokine protein production induced by different time intervals between poly(I:C) and R848 stimulation. Bone marrow-derived macrophages were pretreated with one TLR agonist for 0, 4, 8, 16 or 24 h (0 h pre-stimulation denotes simultaneous stimulation), followed by a second TLR agonist for 24 h. [I→R combinatorial] (dark grey) symbolizes 10 μg ml^{-1} of poly(I:C) as the first TLR agonist, followed by 25 ng ml^{-1} of R848 as second TLR agonist. [R→I combinatorial] (black) indicates the same stimulations in reverse order. The hypothetical additive effects are computed for [I→R additive] (white) and [R→I additive] (light grey). IL-10, TNF, IL-6 and IL-12p40 in cell-free supernatants were measured by ELISA. Data are means ± s.d. of triplicate measurements in a representative experiment. Similar results were obtained in two other independent experiments. (**b**) In RAW264.7 cells, stimulation with single TLR agonists results in low levels of cytokine production. (**c**) Poly(I:C) dose-dependently enhances R848-induced IL-6 production when both TLR ligands were applied simultaneously to RAW264.7 cells (upper panel). Stimulation with poly(I:C) for 8 h before R848 also shows dose-dependent effect on IL-12p40 production (lower panel). Cell-free supernatants were collected 24 h after the second stimulation. IL-6 and IL-12p40 concentrations were measured by ELISA. ND describes 'not detectable' within the limits of assay (lower limit of detection, 5 ng ml^{-1}). Data represent means ± s.d. of triplicate measurements in a representative experiment. Similar results were obtained in two other independent experiments. Student's *t*-tests were performed from the data of all three independent experiments (*$P<0.05$).

poly(I:C) for 0–24 h followed by R848 for 24 h [I→R] or R848 for 0–24 h followed by poly(I:C) [R→I]. At these time points, combinatorial cells were still healthy, showing a level of apoptosis similar to that of untreated cells (Supplementary Figures 2a and b). Similar to our previous findings, stimulation with single TLR ligands led to only a low level of response (Figure 4a, white and light gray bars). However, double TLR ligand stimulation led to a high and synergistic response for all cytokines tested and at all time intervals, as indicated by the difference between the white and dark grey bars or the light grey and black bars (Figure 4a). This enhancement of cytokine production was also seen by intracellular cytokine staining for IL-6 and IL-12p40 (Supplementary Figure 2c) Interestingly, each of the cytokines showed differential sensitivity to the order and timing of the second TLR stimulation. First, the anti-inflammatory cytokine, IL-10, was produced at a high and synergistic level (Figure 4a) regardless of the timing of the second TLR stimulation. In stark contrast, proinflammatory TNF was produced at a much higher level under [I→R] stimulation than [R→I] when the time interval between stimulations was 8 h or more. At 8 h interval, the difference in the order of stimulation was the most apparent, where [R→I] showed 9.5 ng ml^{-1} TNF production, whereas [I→R] only showed six times less TNF production (1.5 ng ml^{-1}, Figure 4a). This suggests that there are different mechanisms of synergy depending on the order of stimulation, where the synergy is greatest for R848-primed cells at 4 h after first stimulation and was sustained over longer time. In contrast, poly(I:C)-pretreated cells remain highly cross-primed to second stimulation with R848 from 8 to 24 h after pre-stimulation. The [R→I] stimulation induced a pattern of production of IL-6 and IL-12p40 similar to TNF, where 4 h pre-stimulation with R848 followed by poly(I:C) led to the highest cytokine production (Figure 4a).

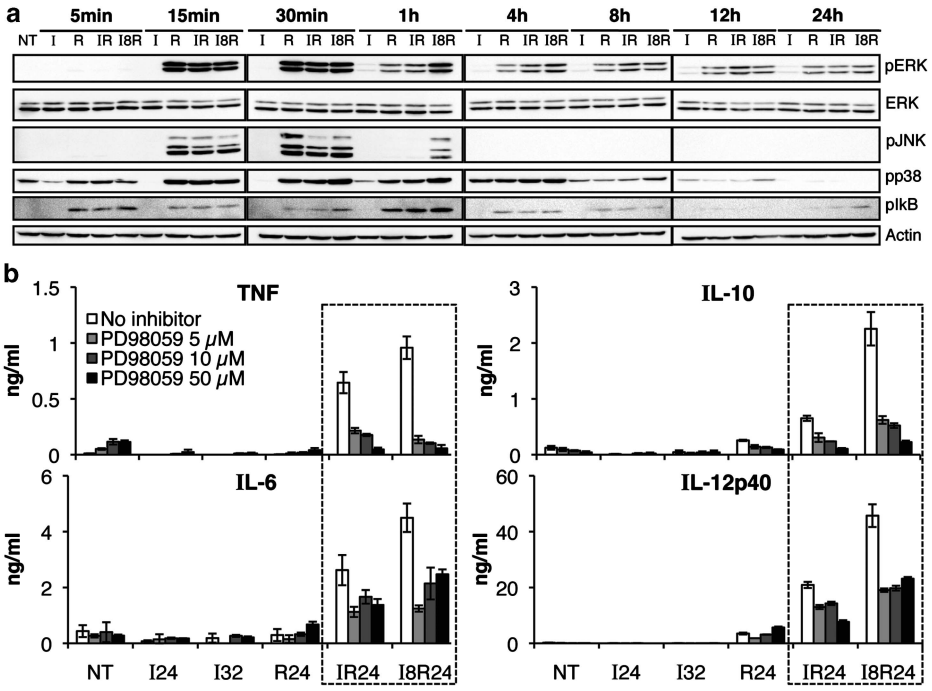


Figure 5 Synergy in cytokine expression is associated with sustained ERK phosphorylation. (**a**) Bone marrow-derived macrophages were treated with either 10 μg ml^{-1} poly(I:C) or 25 ng ml^{-1} R848 alone, poly(I:C) and R848 together simultaneously (IR) or pretreated with poly(I:C) for 8 h followed by a second stimulation of R848 (I8R). After second PAMP stimulation, cells were harvested at the indicated time points. Cell lysates were resolved on SDS-polyacrylamide gel electrophoresis gel, transferred to poly(vinylidene fluoride) membrane and probed with anti-phospho-ERK, anti-total-ERK, anti-phospho-p38, anti-phospho-JNK, anti-phospho-IkB and anti-actin. (**b**) ERK inhibition reduces but does not completely abolish synergistic cytokine production (boxed). Cells were left untreated (NT) or stimulated with 10 μg ml^{-1} poly(I:C) for 24 h (I24) or 32 h (I32), 25 ng ml^{-1} R848 for 24 h (R24), poly(I:C) and R848 simultaneously for 24 h (IR24), or pretreated with poly(I:C) for 8 h followed by R848 for 24 h (I8R24), in the presence or absence of increasing doses ERK inhibitor, PD98059 (5 μM, 10 μM or 50 μM). TNF, IL-10, IL-6 and IL-12p40 in cell-free supernatants were measured by ELISA.

Longer intervals (8–24 h) between the first and second stimulations remained synergistic, but the fold synergy declines as the interval increases. This suggests that priming with poly(I:C) induced a factor which is maximally active 4 h after R848 stimulation. In contrast, longer pre-stimulation with poly(I:C) from 8 to 16 h led to the maximal production of IL-6 and IL-12p40 under [I→R] stimulation. Thus, poly(I:C) may be acting via a mechanism that is most active 8–16 h after poly(I:C) stimulation. Taken together, our results show that crosstalk between signaling pathways induced by poly(I:C) and R848 must be synchronized for maximal cytokine production, with different time windows for different classes of cytokines.

Poly(I:C) strongly enhances R848-induced cytokine production although it is a weak inducer by itself

The requirement for signaling synchronization prompted us to examine the respective roles of poly(I:C) and R848 in signaling crosstalk. Similar to others,[4–6] we observed that R848 alone induced a basal level of IL-6 and IL-12p40 production, whereas poly(I:C) alone induced a low or undetectable amount of cytokines (Figure 4b). However, we observed that poly(I:C) strongly enhanced R848-induced cytokine production at the mRNA level (Figure 3). To substantiate this finding, we tested whether poly(I:C) and R848 would contribute to the synergy in a dose-dependent manner by using three doses each of poly(I:C) (0.3, 1 and 10 μg ml^{-1}) followed by R848 (5, 25 and 100 ng ml^{-1}; Figure 4c). By testing all nine possible combinations of poly(I:C) and R848 with various doses each, and measuring IL-6 and IL-12p40 production 24 h after the second TLR stimulation, we observed no significant synergy at 5 ng ml^{-1} of R848, even when the dose of poly(I:C) was as high as 10 μg ml^{-1}. Increasing the dosage of R848 to 25 ng ml^{-1} led to significant synergy, but no further enhancement was observed at 100 ng ml^{-1} (Figure 4c). Taken together, these results further support the notion that R848 alone is not able to induce a high level of cytokine production even at increasing dosage, although it contributes a basal level of induction. In contrast, under 25 and 100 ng ml^{-1} of R848, poly(I:C) enhanced IL-6 production dose dependently (Figure 4c), although on its own, poly(I:C) did not induce detectable amounts of IL-6 (Figure 4b). These data suggest that poly(I:C) contributes to synergy via induction of 'synergy factors' rather than a direct induction of IL-6. Similar observations were made for IL-12p40 (Figure 4c, lower panel). Collectively, these results support our observation that R848 stimulates basal levels of cytokine production, whereas poly(I:C) dose-dependently induces 'synergy factors', which seemed to greatly enhance the R848-induced cytokine production. Although blocking the type I interferon receptor slightly reduced the amount of cytokines produced (Supplementary Figure 3a), suggesting that the 'synergy' factor might be a type I interferon, addition of exogenous IFN-β failed to induce high levels of cytokine synergy compared with poly(I:C) pretreatment for 8 h (I8R) (Supplementary Figure 3b). The priming effect of poly(I:C) is robust as a low concentration (0.3 μg ml^{-1}) was sufficient to synergize with R848 for effective cytokine induction. Our results also underscore the concept that the innate immune system elicits a *memory* response after the first encounter with a PAMP, and it is able to adjust the level of synergy according to the dose and timing/order of infection.

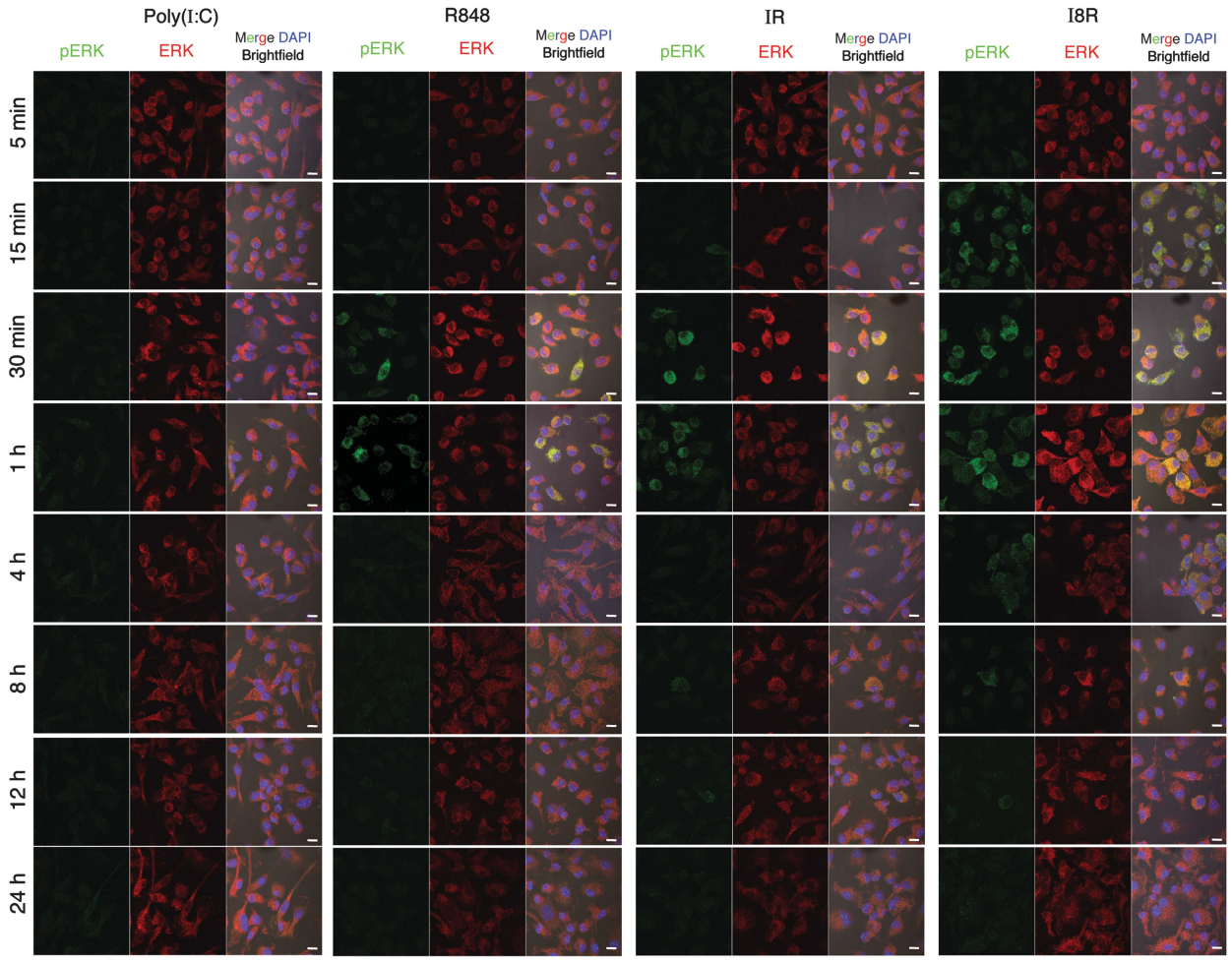


Figure 6 Phospho-ERK is localized to the cytoplasm following TLR stimulation. Bone marrow-derived macrophages were treated with either 10 μg ml^{-1} poly(I:C) or 25 ng ml^{-1} R848 alone, poly(I:C) and R848 together simultaneously (IR) or poly(I:C) for 8 h followed by R848. Cells were fixed at the indicated time points with 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 PBS and stained overnight at 4 °C, for phospho-ERK and total ERK, using antibodies raised in rabbit and mouse, respectively. Cells were washed in PBS and then stained with goat anti-rabbit Alexa-488 and donkey anti-mouse Alexa-546 for 1 h at room temperature. Nuclei are stained (blue) with 4′-6-diamidino-2-phenylindole. Images were taken on LSM510 confocal microscope (Zeiss). Scale bar indicated is 10 μm.

Synergy is associated with sustained cytoplasmic ERK phosphorylation

Next, we investigated the involvement of signaling molecules in the synergy resulting from combinatorial TLR stimulation of BM-DM. Single TLR stimulation is known to result in transient mitogen-activated protein kinase (MAPK) activation, upstream of cytokine expression. In human DCs, synergistic induction of IL-12p70 is known to be associated with sustained c-Jun phosphorylation.[4,15] Thus, we measured the phosphorylation of three key components of the MAPK pathway, extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK), as well as phosphorylated IκBα, in macrophages over time. We found that while single stimulations with either poly(I:C) or R848 led to similar profiles of p38, JNK and IκBα phosphorylation, simultaneous stimulation with both poly(I:C) and R848 (IR) enhanced ERK phosphorylation, which was sustained up to 8 h (Figure 5a). This result suggests that strong and sustained ERK phosphorylation might have a role in enhancing cytokine production observed when poly(I:C) and R848 are simultaneously encountered by the macrophages. Interestingly, this effect is even more pronounced when cells were pretreated with poly(I:C) for 8 h (Figure 5a, I8R). In this experiment, cells were either pretreated with or without poly(I:C) for the first 8 h, then stimulated with R848 and collected at the indicated time points. Our data show that although there is no direct phosphorylation caused by poly(I:C) from 4 h onwards, poly(I:C) pre-stimulation led to a much stronger phosphorylation of ERK upon second challenge with R848 (Figure 5a).

To confirm the role of sustained ERK signaling in synergy, we investigated the effect of PD98059, a chemical inhibitor of MEKs upstream of ERK. We found that inhibition of ERK reduced the production of all cytokines tested (Figure 5b), showing that sustained ERK phosphorylation is a possible mechanism of cytokine synergy.

Localization of ERK in synergy

ERK signaling regulates a vast number of cellular processes, from development to proliferation and apoptosis, sometimes in opposing ways. Other than the strength and duration of the ERK signal, specificity in response might also be determined by the subcellular localization of activated ERK, which determines substrate specificity. Cytoplasmic ERK is known to be associated with the cytoskeleton and activates various substrates such as Raf-1[16] and epidermal growth factor receptor[17], whereas nuclear-localized ERK activates transcription factors such as Elk-1[18] and c-Jun.[19] Thus, we sought to

determine the subcellular localization of phosphorylated ERK under dual TLR ligand stimulation. Consistent with the western blot data, single stimulation with either poly(I:C) or R848 led to a transient phosphorylation of ERK with a peak of 30–60 min, whereas dual TLR ligand stimulation (IR or I8R) led to sustained ERK phosphorylation up to 12 h post-stimulation (Figure 6). In either single or dual TLR stimulation, phosphorylated ERK was cytoplasmically distributed, suggesting that synergy might be mediated by a cytoplasmic target of ERK.

To further investigate the mechanism by which phosphorylated ERK was being retained in the cytoplasm, we examined the mRNA levels of DUSPs by real-time PCR. DUSPs are involved in the negative regulation of MAPK signaling, acting by dephosphorylating and thus inactivating their substrates.[20] We hypothesized that if sustained ERK signaling is the cause of synergy, we would see a reduction of DUSP under synergistic conditions. ERK is known to be a substrate of DUSPs 1–9 and 14. The mRNAs of DUSPs 3, 4, 5, 7, 8, 9 and 14 were not detected by reverse transcription PCR in untreated or stimulated samples. We were, however, able to detect transcripts of DUSP1, 2 and 6 (Figure 7). Interestingly, DUSP2 is known to be localized to the nucleus[21], whereas DUSP6 is localized in the cytoplasm.[22] Our data show that although DUSP1 and DUSP2 are upregulated upon synergistic stimulation, DUSP6 is downregulated (Figure 7). This suggests that the sustained ERK signaling responsible for synergistic cytokine production is plausibly due to a downregulation of DUSP6.

DISCUSSION

We have shown that innate immune response to complex stimuli is determined not only by the combination of TLR ligands, but also by the timing, order and dosage of TLR agonists. The requirement for optimal timing and order indicates that signaling pathways elicited by the two TLR agonists (double-stranded RNA poly(I:C) and single-stranded RNA R848) must be synchronized for optimal induction of the respective cytokines. Our results recapitulate the concept that the immune system is able to adjust the level of synergy according to the dose and timing/order of infection. Therefore, it is important to consider the context of infection when studying immune-signaling crosstalk. In addition, we have narrowed down the range of possible synergy factors to newly synthesized protein(s), which appear(s) to sustain ERK phosphorylation and cytoplasmic localization, facilitating future identification and characterization.

Synergistic cytokine induction has been extensively studied in DC, with a focus on IL-12p70 production and subsequent Th1 adaptive immune responses.[4–8] In contrast, macrophages are known to produce much lower levels of IL-12p70 in a non-synergistic manner.[15] This is unsurprising, given that macrophages function primarily in innate rather than adaptive immunity. Indeed, DC and macrophages are known to produce different cytokine profiles in response to the same pathogen, with DC secreting higher amounts of IL-12p70 in response to *Mycobacterium tuberculosis*, compared with that of macrophages.[23] We have shown, however, that macrophages are able to synergistically secrete high amounts of IL-12p40, one of the subunits of IL-12p70. Nevertheless under the same conditions, only low amounts of IL-12p70 and IL-23 were secreted (Supplementary Figure 4), raising the possibility that macrophages produce large amounts of IL-12p40 as a monomer or homodimer (p80). In this form, IL-12p40 can act as a macrophage chemoattractant, and it has shown to be involved in the recruitment of macrophages to the lung in response to Sendai virus infection in mice, conferring resistance to lethality.[24] It is noteworthy that Sendai virus is a single-stranded RNA virus, which undergoes a double-stranded RNA phase during genomic replication in the host cell, and thus contains both TLR3 and TLR7 ligands similar to those used in our study. Therefore, synergistic levels of production of IL-12p40 by macrophages may promote innate immunity by increasing the

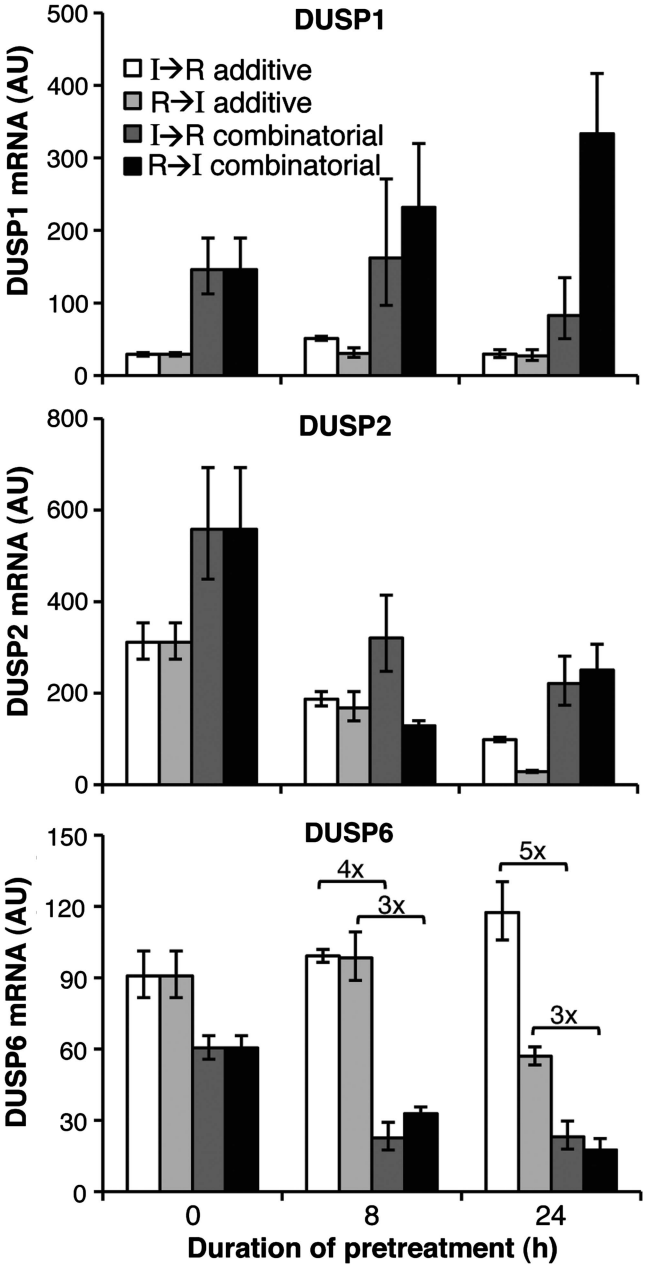


Figure 7 DUSP 1, 2 and 6 mRNA expression under synergistic conditions. Bone-marrow derived macrophages were pretreated with a first TLR ligand for 0, 8 or 24 h, followed by a second TLR ligand for another 8 h. 0 h pre-stimulation denotes simultaneous stimulation. [I→R combinatorial, dark grey] symbolizes poly(I:C) as the first TLR ligand, followed by R848 as second TLR ligand. [R→I combinatorial, black] indicates the same stimulations in reverse order. The hypothetical additive effects were computed for [I→R additive, white] and [R→I additive, light grey]. DUSP1, DUSP2 and DUSP6 mRNAs were measured by real-time PCR in the SYBR green format using primers specific to each mRNA. Data represent means ± s.d. of a representative experiment. The fold downregulation of DUSP6 mRNA for each of the combinatorial stimulations compared with their respective controls are as labeled.

number of immune cells at the site of infection, rather than combining with IL-12p35 to form IL-12p70 and drive Th1 responses. These cell type-specific differences may be attributed to differential usage of TLRs,[23] or differential chromatin status (the IL-6 promoter is constitutively open in fibroblasts but closed in macrophages),[25] or cell type-specific synergy factors.

Although there are differences in IL-12p70 secretion, both macrophages and DC synergistically produce IL-6 and TNF in response to combinatorial stimulation by poly(I:C) and R848.[4] This is consistent with the idea that the activation of both MyD88- and TRIF-mediated pathways is required for synergy.[8,9] From our data, poly(I:C) was unable to induce IL-6 or IL-12p40 production (Figure 4c) and yet it boosted the fold synergy of these two cytokines in a dose-dependent manner. It is likely that R848 elicits a basal level of cytokine induction (Figure 4c), which was enhanced by poly(I:C) signaling through a protein synthesis-dependent mechanism (Figure 3c). This possibility is supported by our study of signaling molecules, which shows that poly(I:C) is a weaker inducer than R848 alone, but has a modulating effect when simultaneously administered with R848, prolonging and enhancing the ERK phosphorylation signal (Figures 5 and 6). However, these results do not rule out the possible involvement of other signaling pathways such as the interferon regulatory factors. An alternative explanation could be that poly(I:C) causes synergy with R848 through chromatin modifications, a mechanism that has been demonstrated in other studies.[26–29] Taken together, our results show that a first encounter with poly(I:C) seems to imprint a memory effect, which cross-primes the macrophages for a more rapid, higher and sustained immune response to the second PAMP challenge with R848.

Previous microarray studies have found that DUSP genes are synergistically upregulated under combinatorial TLR stimulation.[4,30] Our study is the first to implicate the downregulation DUSP proteins in the regulation of synergistic cytokine production, linking DUSP6 downregulation to the sustained phosphorylation of ERK. Regulation of the phosphoproteome is a key mechanism regulating innate immunity,[31] and our findings suggest that dephosphorylation events are just as important as kinase activity in modulating the magnitude of the response.

Our results showed that secondary response genes such as *Il6* and *Il12b* elicited a greater extent of synergy (70- to 90-fold) at the mRNA level than primary response genes such as *Tnf* (1- to 2-fold; Figure 3b, white bars) in response to poly(I:C) and R848. Our observation is consistent with previous reports that secondary response gene (for example, *Il6*, *Il12b*) production in response to LPS strictly requires both MyD88 and TRIF signaling, whereas primary response genes (for example, *Tnf*, *Il10*) still have residual production in the absence of either MyD88 or TRIF, and this is probably regulated by a newly synthesized protein.[14] Interestingly, we found that the fold synergy of secondary response genes is highly dependent on new protein synthesis (Figure 3c), whereas the primary response genes are not affected. Considering the importance of *trans*-regulatory elements[13] and chromosome remodeling[14,25,32] in gene regulation, the newly synthesized proteins are plausibly enhancer-binding proteins or proteins which may influence chromatin remodeling. Identification of these 'synergy-inducing proteins' is warranted in future studies to further understand the mechanism of signaling crosstalk and regulation of secondary response genes in *innate immune memory*.

METHODS

Materials

Media and supplements were purchased from Gibco, Life Technologies (Carlsbad, CA, USA). Fetal bovine serum was purchased from Hyclone, Thermofischer Scientific (Waltham, MA, USA). Recombinant mouse macrophage colony stimulating factor (M-CSF) was purchased from Ebioscience (San Diego, CA, USA). TLR ligands R848 and low-molecular-weight poly(I:C) were from Invivogen (San Diego, CA, USA), Pam_3CSK_4 from Calbiochem, EMD Biochemicals (Darmstadt, Germany) and *Escherichia coli* 055:B55 LPS from Sigma-Aldrich (St Louis, MO, USA). Maxisorp microtitre 96-well plates were obtained from Nunc (Roskilde, Denmark). Rabbit monoclonal antibodies against phospho-p38 (#4511), phospho-ERK1/2 (#4370) and phospho-JNK (#4668) were obtained from Cell Signaling Technologies (Boston, MA, USA). Rabbit monoclonal antibody to β-actin and goat polyclonal horse radish peroxidase (HRP)-conjugated secondary antibodies to rabbit IgG were from Sigma-Aldrich. Alexa Fluor-488 secondary goat anti-rabbit (H + L) highly cross-absorbed was from Molecular Probes, Invitrogen (Carlsbad, CA, USA).

Animals

Mouse BM-DM were prepared as described previously.[33] Briefly, femurs were isolated from 8-week-old female BALB/c mice, and the resulting bone-marrow cells were resuspended in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin and 100 U ml^{-1} recombinant M-CSF at a density of 4×10^6 cells per ml. On day 3 post harvest, additional M-CSF media was supplied and cells were cultured for another 4 days before plating. Experiments on mice were performed according to the guidelines and regulations of the Institutional Animal Care and Use Committee (IACUC protocol number: 049-11).

Cell culture

Primary BM-DM and mouse macrophage cell line RAW 264.7 were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Cells were stimulated 24 h after plating with 1 ml of fresh medium per well with the respective TLR ligands. At indicated time intervals, the second PAMP or pyrogen-free water (mock) was applied to the cells as second stimulation. The final concentrations of TLR ligands in the culture were 25 ng ml^{-1} of R848, 10 μg ml^{-1} of poly(I:C), 10 ng ml^{-1} Pam_3CSK_4 or 10 ng ml^{-1} *E. coli* 055:B55 LPS.

CHX treatment

BM-DM at a density of 1×10^6 cells per ml in 0.5 ml was plated per well into 24-well plates. At 24 h after plating, the cells were pre-treated with 10 μg ml^{-1} of CHX (Sigma, St Louis, MO, USA) for 15 min, and then 2 μl of poly(I:C) at 2.5 μg ml^{-1} or pyrogen-free water was applied as first stimulation. This was followed 8 h later by a second stimulation with 2 μl of R848 at 6.25 ng ml^{-1} or pyrogen-free water.

Measurement of cytokine production

Culture supernatants were collected at 24 h after the second stimulation and stored at −80 °C. The levels of IL-12p40, IL-6, IL-10, TNF (BD Biosciences Inc.) were measured according to the manufacturer's instructions. For the multiplex cytokine assay, a luminex kit (Panomics/Affymetrix, Freemont, CA, USA) was used according to the manufacturer's instructions. The lower limit of detection was 2.5 pg ml^{-1} for all cytokines tested. Student's *t*-tests were performed from the data of three independent experiments and $P < 0.05$ were considered significant. For CHX treatment, BM-DM were pretreated with 10 mg ml^{-1} of CHX from Sigma-Aldrich for 15 min before stimulation.

Real-time PCR

Cells were harvested with Trizol (Invitrogen) and frozen at −80 °C until mRNA extraction. cDNAs were synthesized with Superscript III reverse transcriptase (Invitrogen). All real-time PCR were carried out with Light cycler 480 (Roche, Penzburg, Germany). Lightcycler 480 probes master mix (Roche) were used for the Taqman assays, and pre-designed TaqMan Gene Expression Assays (Life Technologies) Mm00446190_m1, Mm00434174_m1, Mm01290062_m1 Mm00446968_m1 were used for the detection of *Il6*, *Il12b*, *Il10, Tnf and Hprt* mRNA, respectively. The PCR cycles constituted 1 cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s. The mRNA levels were normalized to that of *Hprt* and expressed as relative amount

to *Hprt*. DUSP1, DUSP2 and DUSP6 mRNAs were detected with SYBR Green method by using Lightcycler 480 SYBR Green I Master Mix (Roche), and the mRNA levels were normalized to that of β-actin. Forward primer: 5′-CACCCGCGAGCACAGCTTCT-3′ and reverse primer: 5′-TGCTCTGG GCCTCGTCACCC-3′ was used for detection of β-actin mRNA. Forward primer 5′-TTCGCTTTCAACGCCGGCCA-3′ and reverse primer 5′-AGCTC AGGGCAGGAAGCCGA-3′ was used for DUSP1 mRNA detection. Forward primer 5′-CCGTGTGCTTCTTGCGAGGC-3′ and reverse primer 5′-TTG GGGCAGCTGGCAGAGACA-3′ was used for DUSP2 mRNA detection. Forward primer 5′-ACCGCTTTACCAGGCGCTGC-3′ and reverse primer 5′-TCGCAGTGCAGGGCGAACTC-3′ was used for DUSP6 mRNA detection. All primers were designed to amplify intron spanning cDNA products, thereby excluding the amplification of genomic DNA. The PCR reaction conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 62 °C for 10 s and 72 °C for 15 s.

Western blot analysis

Cells were lysed in RIPA buffer in the presence of protease and phosphatase inhibitor cocktail from Roche. A measure of 10 μg total protein was separated on 10% SDS-polyacrylamide gel electrophoresis and electrotransferred to a poly(vinylidene fluoride) membrane. Transblots were washed three times in Tris buffered saline with Tween-20 (TBST) (50 mM Tris-base, 150 mM NaCl, 0.01% Tween-20, pH 7.6) before blocking with 5% skimmed milk in TBST for 1 h. Primary antibodies diluted in 5% bovine serum albumin TBST and incubated with the membranes overnight at 4 °C with gentle agitation. Following incubation with primary antibodies, membranes were washed three times with TBST. Protein detection was carried out by incubating blots with respective HRP-conjugated secondary antibodies (1:5000) for 1 h. Molecular weights were calibrated in proportion to the running distance of Precision Plus dual colour protein standards from Bio-Rad (Richmond, CA, USA). The immunodetected bands were visualized on Kodak film using an ECL system from Pierce, Thermofischer Scientific.

Immunofluorescence microscopy

BALB/c derived BM-DM were cultured on glass coverslips in 24-well tissue culture plates overnight, followed by stimulation with poly(I:C) or R848 or both. After the indicated time points, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline for 15 min, then permeabilized with 5% bovine serum albumin/phosphate-buffered saline with 0.1% Triton X-100 for 15 min. Cells were then stained with rabbit anti-phospho-ERK (1:250) and mouse anti-total ERK (1:250) followed by Alexa Fluor-488 secondary goat anti-rabbit (1:500) and Alexa Fluor-546 secondary donkey anti-mouse (1:500). Nuclei were stained with 4′-6-diamidino-2-phenylindole in ProLong Gold antifade reagent. Images were obtained on a Carl Zeiss LSM 510 Meta microscope.

1 Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S. Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host Microbe* 2008; **3:** 352–363.
2 Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; **124:** 783–801.
3 Agrawal S, Agrawal A, Doughty B, Gerwitz A, Blenis J, Van Dyke T *et al.* Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J Immunol* 2003; **171:** 4984–4989.
4 Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* 2005; **6:** 769–776.
5 Theiner G, Rössner S, Dalpke A, Bode K, Berger T, Gessner A *et al.* TLR9 cooperates with TLR4 to increase IL-12 release by murine dendritic cells. *Mol Immunol* 2008; **45:** 244–252.
6 Zhu Q, Egelston C, Vivekanandhan A, Uematsu S, Akira S, Klinman DM *et al.* Toll-like receptor ligands synergize through distinct dendritic cell pathways to induce T cell responses: implications for vaccines. *Proc Natl Acad Sci USA* 2008; **105:** 16260–16265.
7 Bohnenkamp HR, Papazisis KT, Burchell JM, Taylor-Papadimitriou J. Synergism of Toll-like receptor-induced interleukin-12p70 secretion by monocyte-derived dendritic cells is mediated through p38 MAPK and lowers the threshold of T-helper cell type 1 responses. *Cell Immunol* 2007; **247:** 72–84.
8 Krummen M, Balkow S, Shen L, Heinz S, Loquai C, Probst HC *et al.* Release of IL-12 by dendritic cells activated by TLR ligation is dependent on MyD88 signaling, whereas TRIF signaling is indispensable for TLR synergy. *J Leukoc Biol* 2010; **88:** 189–199.
9 Bagchi A, Herrup EA, Warren HS, Trigilio J, Shin HS, Valentine C *et al.* MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. *J Immunol* 2007; **178:** 1164–1171.
10 De Nardo D, De Nardo CM, Nguyen T, Hamilton JA, Scholz GM. Signaling crosstalk during sequential TLR4 and TLR9 activation amplifies the inflammatory response of mouse macrophages. *J Immunol* 2009; **183:** 8110–8118.
11 Macagno A, Napolitani G, Lanzavecchia A, Sallusto F. Duration, combination and timing: the signal integration model of dendritic cell activation. *Trends Immunol* 2007; **28:** 227–233.
12 Alon U. Network motifs: theory and experimental approaches. *Nat Rev Genet* 2007; **8:** 450–461.
13 Litvak V, Ramsey SA, Rust AG, Zak DE, Kennedy KA, Lampano AE *et al.* Function of C/EBPdelta in a regulatory circuit that discriminates between transient and persistent TLR4-induced signals. *Nat Immunol* 2009; **10:** 437–443.
14 Kayama H, Ramirez-Carrozzi VR, Yamamoto M, Mizutani T, Kuwata H, Iba H *et al.* Class-specific regulation of pro-inflammatory genes by MyD88 pathways and IkappaB-zeta. *J Biol Chem* 2008; **283:** 12468–12477.
15 Mäkelä SM, Strengell M, Pietilä TE, Osterlund P, Julkunen I. Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells. *J Leukoc Biol* 2009; **85:** 664–672.
16 Anderson NG, Li P, Marsden LA, Williams N, Roberts TM, Sturgill TW. Raf-1 is a potential substrate for mitogen-activated protein kinase *in vivo*. *Biochem J* 1991; **277** (Pt 2), 573–576.
17 Northwood IC, Gonzalez FA, Wartmann M, Raden DL, Davis RJ. Isolation and characterization of two growth factor-stimulated protein kinases that phosphorylate the epidermal growth factor receptor at threonine 669. *J Biol Chem* 1991; **266:** 15266–15276.
18 Marais R, Wynne J, Treisman R. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* 1993; **73:** 381–393.
19 Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E, Woodgett JR. Phosphorylation of c-jun mediated by MAP kinases. *Nature* 1991; **353:** 670–674.
20 Lang R, Hammer M, Mages J. DUSP meet immunology: dual specificity MAPK phosphatases in control of the inflammatory response. *J Immunol* 2006; **177:** 7497–7504.
21 Rohan PJ, Davis P, Moskaluk CA, Kearns M, Krutzsch H, Siebenlist U *et al.* PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase. *Science* 1993; **259:** 1763–1766.
22 Mourey RJ, Vega QC, Campbell JS, Wenderoth MP, Hauschka SD, Krebs EG *et al.* A novel cytoplasmic dual specificity protein tyrosine phosphatase implicated in muscle and neuronal differentiation. *J Biol Chem* 1996; **271:** 3795–3802.
23 Pompei L, Jang S, Zamlynny B, Ravikumar S, McBride A, Hickman SP *et al.* Disparity in IL-12 release in dendritic cells and macrophages in response to Mycobacterium tuberculosis is due to use of distinct TLRs. *J Immunol* 2007; **178:** 5192–5199.
24 Gunsten S, Mikols CL, Grayson MH, Schwendener RA, Agapov E, Tidwell RM *et al.* IL-12 p80-dependent macrophage recruitment primes the host for increased survival following a lethal respiratory viral infection. *Immunology* 2009; **126:** 500–513.
25 Ramirez-Carrozzi VR, Braas D, Bhatt DM, Cheng CS, Hong C, Doty KR *et al.* A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. *Cell* 2009; **138:** 114–128.
26 Foster SL, Hargreaves DC, Medzhitov R. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* 2007; **447:** 972–978.
27 Park SH, Park-Min KH, Chen J, Hu X, Ivashkiv LB. Tumor necrosis factor induces GSK3 kinase-mediated cross-tolerance to endotoxin in macrophages. *Nat Immunol* 2011; **12:** 607–615.
28 Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S *et al.* Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci USA* 2012; **109:** 17537–17542.
29 Quintin J, Saeed S, Martens JH, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C *et al.* Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe* 2012; **12:** 223–232.
30 Tross D, Petrenko L, Klaschik S, Zhu Q, Klinman DM. Global changes in gene expression and synergistic interactions induced by TLR9 and TLR3. *Mol Immunol* 2009; **46:** 2557–2564.
31 Weintz G, Olsen JV, Frühauf K, Niedzielska M, Amit I, Jantsch J *et al.* The phosphoproteome of toll-like receptor-activated macrophages. *Molecular systems biology* 2010; **6:** 371.
32 Ramirez-Carrozzi VR, Nazarian AA, Li CC, Gore SL, Sridharan R, Imbalzano AN *et al.* Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. *Genes Dev* 2006; **20:** 282–296.
33 Zhang X, Goncalves R, Mosser DM. The isolation and characterization of murine macrophages. *Curr Protoc Immunol* 2008, Chapter 14 (Unit 14), 11.

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