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# Fungal pattern receptors down-regulate the inflammatory response by a cross-inhibitory mechanism independent of interleukin-10 production

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## Summary

**Cyclic AMP regulatory element binding protein and signal transducer and activator of transcription 3 (STAT3) may control inflammation by several mechanisms, one of the best characterized is the induction of the expression of the anti-inflammatory cytokine interleukin-10 (IL-10). STAT3 also down-regulates the production of pro-inflammatory cytokines induced by immunoreceptor tyrosine-based activation motif (ITAM)-coupled receptors, a mechanism termed cross-inhibition. Because signalling via ITAM-dependent mechanisms is a hallmark of fungal pattern receptors, STAT3 activation might be involved in the cross-inhibition associated with invasive fungal infections. The fungal surrogate zymosan produced the phosphorylation of Y705-STAT3 and the expression of *Ifnb1* and *Socs3*, but did not induce the interferon (IFN)-signature cytokines *Cxcl9* and *Cxcl10* in bone marrow-derived dendritic cells. Unlike lipopolysaccharide (LPS), zymosan induced IL-10 and phosphorylated Y705-STAT3 to a similar extent in *Irf3* and *Ifnar1* knockout and wild-type mice. Human dendritic cells showed similar results, although the induction of *IFNB1* was less prominent. These results indicate that LPS and zymosan activate STAT3 through different routes. Whereas type I IFN is the main effector of LPS effect, the mechanism involved in Y705-STAT3 phosphorylation by zymosan is more complex, cannot be associated with type I IFN, IL-6 or granulocyte–macrophage colony-stimulating factor, and seems dependent on several factors given that it was partially inhibited by the platelet-activating factor antagonist WEB2086 and high concentrations of COX inhibitors, p38 mitogen-activate protein kinase inhibitors, and blockade of tumour necrosis factor-α function. Altogether, these results indicate that fungal pattern receptors share with other ITAM-coupled receptors the capacity to produce cross-inhibition through a mechanism involving STAT3 and induction of SOCS3 and IL-10, but that cannot be explained through type I IFN signalling.**

**Keywords:** cytokines; dendritic cells; fungal infection; inflammation.

Abbreviations: BMDC, bone-marrow-derived dendritic cells; CBP, CREB-binding protein; CRE, cAMP regulatory element; CREB, CRE-binding protein; DC, human monocyte-derived dendritic cells; EP, E-type prostanoid receptor; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; IFNAR1, IFN-α and IFN-β receptor subunit 1; IL-10, interleukin-10; IRF, IFN regulatory factor; ITAM, immunoreceptor tyrosine-based activation motif; JAK, Janus kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MSK, mitogen- and stress-activated kinases; PAF, platelet-activating factor; $PGE_2$, prostaglandin $E_2$; SOCS, suppressors of cytokine signalling; STAT, signal transducer and activator of transcription; TIR, Toll–interleukin receptor; TLR, Toll-like receptor; TNF-α, tumour necrosis factor-α; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adapter-inducing IFN-β; WT, wild-type


 

## Introduction

The main function of interleukin-10 (IL-10) is its capacity to block cytokine production from T helper type 1 cells. Macrophages and dendritic cells (DC) are robust producers of IL-10 and the balance among IL-10, IL-12 p70 and IL-23 released from DC is a critical determinant of the polarization of the immune response. Renewed interest in the role of IL-10 emerged from the study of the mechanisms of defence against invasive mycoses, a growing risk in patients with a compromised immune system. The commensal *Candida* is the most prevalent infectious fungus, but *Cryptococcus*, *Aspergillus* and *Pneumocystis* are also common opportunistic pathogens. The first line of defence against fungi is the innate immune system, which is mainly composed of phagocytic cells endowed with a set of pattern recognition receptors. A prominent production of IL-10 accompanied by a low production of IL-12 p70 characterizes the response to fungal patterns.[1,2] Given that an unbalanced production of IL-10 may also contribute to the evasion by microbes of the immune response in other prevalent infections, e.g. *Mycobacterium tuberculosis*,[3] the analysis of the mechanisms of IL-10 regulation is of clinical relevance.

At the transcriptional level, several scenarios of *IL10* regulation have been disclosed. The one centred on the transcription factor cAMP regulatory element binding protein (CREB) and its co-activators CREB-binding protein (CBP) and CREB-regulated transcription co-activator is widely accepted in the case of the fungal mimic zymosan and in many cases lipopolysaccharide (LPS),[4–7] whereas other reports have focused on the role of signal transducer and activator of transcription 3 (STAT3) and an autocrine cycle dependent on interferon-$\beta$ (IFN-$\beta$) and IFN regulatory factor 3 (IRF3) downstream of the Toll–interleukin receptor (TIR)-domain-containing adapter-inducing IFN-$\beta$ (TRIF)/TRIF-related adaptor molecule (TRAM) branch of the LPS/Toll-like receptor 4 (TLR4) system.[8,9] The study of the response to fungal patterns has been facilitated by the use of the fungal surrogate zymosan, a cell-wall extract from *Saccharomyces cerevisiae* that contains a $\beta$-glucan component, recognized by the C-type lectin receptor dectin-1, and a mannose-based component that mainly signals through TLR2 and the C-type lectin receptor dectin-2.[10–13] Although most views agreed on the lack of a significant induction of type I IFN upon fungal challenge, this issue should be revisited on the basis of recent findings. In fact, *Ifnar1*$^{-/-}$ mice were reported to have an improved survival in response to *Candida* infection,[14] and studies of humans with gain-of-function dominant mutations of *STAT1* have shown an increased risk of invasive fungal infection.[15] On the other hand, type I IFN was associated with host defence response against *Candida*[16] and another study unveiled a unique mechanism of induction of IFN-$\beta$ in mice by *Candida* through IRF5 activation downstream of dectin-1 and the adaptor protein caspase recruitment domain-containing protein 9,[17] which might depend on IκB kinase-$\beta$ activity.[18,19] This mechanism was found in DC but not in macrophages, indicating that there may be cell-dependency and, probably also species-dependency. Another study has reported IFN-$\beta$ induction via tumour necrosis factor-$\alpha$ (TNF-$\alpha$) and IRF1, so disclosing new mechanisms of regulation.[20,21] Given the strong induction of TNF-$\alpha$ elicited by zymosan and the large list of secondary mediators that are released concomitantly and that can activate STAT3 – e.g. IL-6, IL-12, IL-23, granulocyte–macrophage colony-stimulating factor (GM-CSF), chemokines, prostaglandin $E_2$ ($PGE_2$) and platelet-activating factor (PAF)[22–26] – it seems necessary to address the role of STAT3 in shaping the inflammatory response to fungal patterns. Our studies using mouse bone-marrow-derived DC (BMDC) and human monocyte-derived DC have shown: (i) the activation of STAT3 by zymosan through a mechanism independent of type I IFN, (ii) the lack of involvement of the Janus kinase (JAK)/STAT3 system on the production of IL-10 elicited by zymosan, and (iii) a cross-inhibitory effect elicited by zymosan receptors on type I IFN responses.

## Materials and methods

### *Reagents and cells*

Zymosan, mannan from *Saccharomyces cerevisiae*, and the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 were obtained from Sigma Chemical Co. (St Louis, MO). The E-type prostanoid receptor 2 (EP2) receptor antagonist PF-04418948 and the EP4 receptor antagonist L-161982 were from Cayman Chemical Industries (Ann Arbor, MI). Mannose-depleted zymosan was obtained by boiling zymosan in 10 M NaOH. Interleukin-10 was assayed with Biotrack ELISA systems from Amersham Biosciences (Chalfont St Giles, UK). The cell-permeable STAT3 inhibitor peptide was from Millipore (Billerica, MA) (ref: 573096). Ruxolitinib and tofacitinib were from InvivoGen (San Diego, CA). Anti-IL-10 antibody (AF-217-NA) and anti-IL-6 (AF-206-NA) were from R&D Systems (Minneapolis, MN). The 12/15-lipoxygenase inhibitor ethyl 3,4-dihydroxybenzylidenecyanoacetate was from Santa Cruz Biotechnology, Inc. (Dallas, TX). The separation of monocytes and their differentiation into DC has been reported previously.[27] Briefly, monocytes were cultured in the presence of GM-CSF (800 U/ml) and IL-4 (500 U/mL) for 5 days, in RPMI-1640 medium supplemented with 4 mM L-glutamine and 10% fetal bovine serum. Differentiation into DC was addressed by flow cytometry of CD40, CD80, CD83, CD86, CD11b and CD11c. *Irf3*$^{-/-}$ mice were obtained by Dr Castrillo through a collaboration with Drs G. Cheng (University of California, Los Angeles) and T. Taniguchi (University of Tokyo).[28] *Ifnar1*$^{-/-}$ mice were kindly

provided by Dr C. Ardavín (Centro Nacional de Biotecnología, Madrid, Spain) with permission from Dr U. Kalinke (Centre for Experimental and Clinical Infection Research, Hannover, Germany).[29] *Irf5*$^{-/-}$ mice were obtained from the Wellcome Trust Sanger Institute Mouse Genetics Project (Sanger MGP). Bone marrow from the femora and tibiae of C57BL/6 mice was used to generate BMDC by culture in the presence of murine GM-CSF for 5 days. Purity of BMDC was confirmed by flow cytometry, which showed 85% cells to be positive for the integrin CD11c. Before carrying out the experiments, BMDC were maintained for 3 hr in the absence of GM-CSF to avoid the activation of STAT3 elicited by GM-CSF. The population of adherent cells, presumably composed of bone marrow macrophages and termed GM-CSF macrophages, was used in some experiments. *Candida albicans* was provided by Dr Jesús Pla, from Universidad Complutense de Madrid.

### *Ethics*

The study was approved by the Bioethical Committee of the Spanish Council of Research before starting, and so meets the standards of the Declaration of Helsinki in its revised version of 1975 and its amendments of 1983, 1989 and 1996. The written informed consent of all healthy donor participants was obtained at Centro de Hemoterapia y Hemodonación de Castilla y León Biobank. The researchers received the samples anonymously. The animal experiments were carried out with the permission of the local authority and conform to institutional standards.

### *Immunoblots and chromatin immunoprecipitation assays*

Proteins were separated by electrophoresis in SDS–PAGE and transferred to nitrocellulose membranes. The membranes were used for immunodetection of P-Y705-STAT3 (#9131), STAT3 (#9132), P-Y701-STAT1 (#9171S) and STAT1 (#9172) with reagents from Cell Signaling Technology (Beverly, MA). Anti-COX-2 antibody (sc-1745) and anti-IRF1 antibody (sc-497 and sc-13041) were from Santa Cruz Biotechnology, Inc. Anti-IRF3 antibody (catalogue no. 39033) was from Active Motif (Carlsbad, CA). Anti-IRF5 antibody (ab2932) was from abcam plc (Cambridge, UK). Quantification of the blots was carried out using BioRad Quantity One gel imaging software (Bio-Rad, Hercules, CA). Chromatin immunoprecipitation assays were carried out as described previously in DC fixed with 1% formaldehyde.[4] Chromatin sonication was carried out using a Bioruptor device. Cross-links were reversed by heating at 67°C, and the DNA bound to the beads was isolated by extraction with phenol/chloroform/isoamylalcohol. PCR were carried out with primers designed from the *IL10* promoter.

### *Real time RT-PCR of IL10, IL12A, IL23A, SOCS3, CXCL9, CXCL10, CSF1, CSF2, CSF3, IFNB1* and *GAPDH*

Purified RNA was used for reverse transcription reactions. The resulting cDNA was amplified in a PTC-200 apparatus equipped with a Chromo4 detector (Bio-Rad) using SYBR Green I mix containing Hot Start polymerase (Thermo Fisher Scientific, Waltham, MA). The sets of primers for PCR are shown in the Supplementary material (Table S1). *GAPDH* was used as a housekeeping gene to assess the relative abundance of the different mRNA using the comparative $C_T$ (threshold cycle) method.

## Results

### Effect of *Irf3* deletion on the production of IL-10

Given that the production of IL-10 in the LPS/TLR4 route depends on the induction of IFN-$\beta$ and the responses to the fungal mimic zymosan are elicited by C-type lectin and TLR2 receptors, our first hypothesis was that IL-10 production should display mechanistic differences in both cases given the central role of the TRIF/TRAM/IRF3/IFN-$\beta$ pathway in LPS/TLR4 signalling. In keeping with these views, a reduced production of IL-10 was observed in BMDC of *Irf3*$^{-/-}$ mice stimulated with LPS, whereas the response to 0·1 mg/ml zymosan was similar in both groups and significantly increased in *Irf3*$^{-/-}$ mice stimulated with 1 mg/ml. Zymosan was a stronger stimulus than LPS because concentrations of IL-10 as high as 7·5 ng/ml were observed, whereas LPS only induced concentrations of ~ 0·5 ng/ml (Fig. 1a). *Il10* mRNA expression in response to LPS showed a significant decrease in *Irf3*$^{-/-}$ mice compared with wild-type (WT) animals. In contrast, the response to zymosan showed a tendency to be higher in the knockout mice, although it did not reach statistical significance (Fig. 1b, c). The WT mice showed a strong induction of *Ifnb1* mRNA in response to 0·1 mg/ml zymosan, which was even higher than that elicited by LPS, as well as induction of *Il23a* mRNA (Fig. 1d). Deletion of *Irf3* abrogated the expression of *Ifnb1* in response to LPS, and reduced significantly the response to zymosan (Fig. 1d), which might be explained by a remaining IRF5-dependent induction. The contribution of type I IFN signalling to the overall effect of zymosan was assessed by assaying *Cxcl9* and *Socs3* mRNA. Zymosan was a weak inducer of *Cxcl9* but induced *Socs3* expression at a level similar to that elicited by LPS (Fig. 1e), suggesting that Socs proteins could be involved in the cross-inhibition of type I IFN signalling.[30–32] Tyrosine phosphorylation of STAT1 and STAT3 was detected in both *Irf3*$^{-/-}$ and WT mice (Fig. 1f, g). STAT1 phosphorylation was robust at 3 hr


 

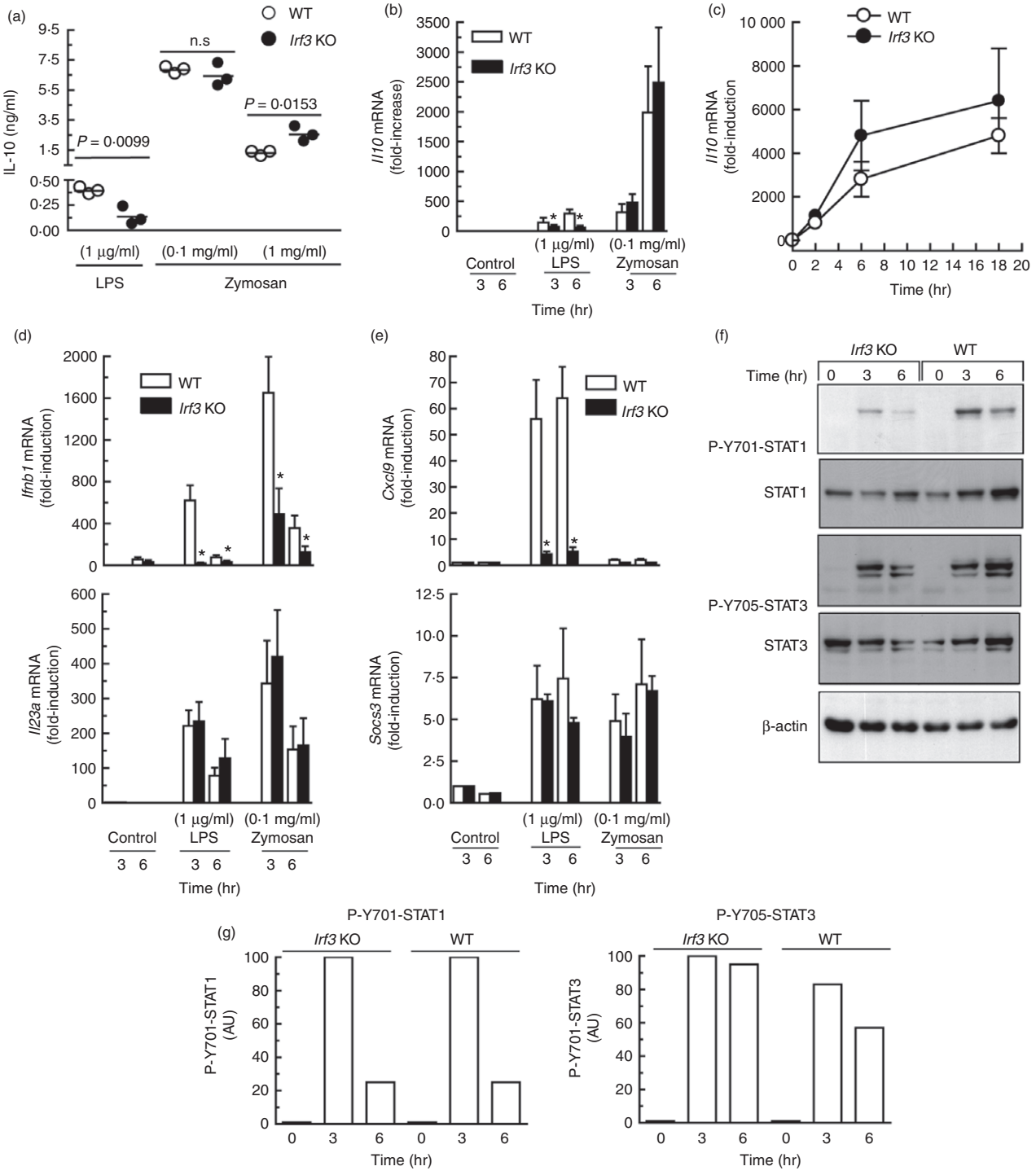


**Figure 1.** Effect of *Irf3* deletion on cytokine production by mouse bone-marrow-derived dendritic cells (BMDC). (a) BMDC were incubated for 18 hr in the presence of either zymosan or lipopolysaccharide (LPS) at the concentrations indicated and the supernatants were collected for the assay of interleukin-10 (IL-10) protein. (b) Three independent pools of BMDC obtained from three wild-type (WT) and three *Irf3*$^{-/-}$ mice were stimulated for 3 and 6 hr and collected for the assay of *Il10* mRNA. (c) Time–course of *Il10* mRNA induction in response to 0·1 mg/ml zymosan in *Irf3*$^{-/-}$ and WT mice. (d, e) *Ifnb1*, *Il23a*, *Cxcl9* and *Socs3* mRNA expression in response to LPS and zymosan in the same pools of animals studied in (b). (f) Effect of *Irf3* deletion on Y701-STAT1 and Y705-STAT3 phosphorylation in BMDC stimulated with 0·1 mg/ml zymosan. (g) Densitometric scanning of the blots. This experiment was carried out using BMDC from an individual animal for each condition. Data in (a) and (c) represent mean ± SEM of three mice individually studied. n.s., not significant. AU, arbitrary units.

and decreased at 6 hr; it was difficult to disclose whether it was more robust in either the knockout or the WT animals after normalization of the blots (Fig. 1g). Taken together, these data indicate that IL-10 protein production and Y705-STAT3 phosphorylation in response to zymosan show a limited dependence on IRF3 and that zymosan may induce *Ifnb1* mRNA expression in the absence of IRF3.

### IL-10 production in response to zymosan and *Candida* is independent of the IFN-α/β receptor (IFNAR)

The BMDC of both *Ifnar1*$^{-/-}$ and WT mice produced similar amounts of IL-10 in response to zymosan (Fig. 2a). In contrast, the response to LPS was significantly decreased (Fig. 2b). An explanation for the lower levels of IL-10 protein assayed in this set of experiments compared with the *Irf3*$^{-/-}$ mice can be the different background of the animals, although in both cases mice were of the C57BL/6 strain. Mannose-depleted zymosan induced a low amount of IL-10 protein and heat-killed *Candida* released IL-10 to the same extent in both WT and knockout mice (Fig. 2c). Consistent with the results of the IL-10 protein assays, the *Ifnar1*$^{-/-}$ mice showed a reduced induction of *Il10* mRNA in response to LPS, but no differences could be detected in response to zymosan (Fig. 2d). The expression of *Ifnb1* mRNA showed similar values in both WT and *Ifnar1*$^{-/-}$ mice (Fig. 2e), the levels at 2 hr being somewhat higher than at 4 hr in LPS-treated BMDC, suggesting a rapid, and perhaps transient, *trans*-activation of *Ifnb1* consistent with a robust induction of IFN-β protein (Fig. 2f).

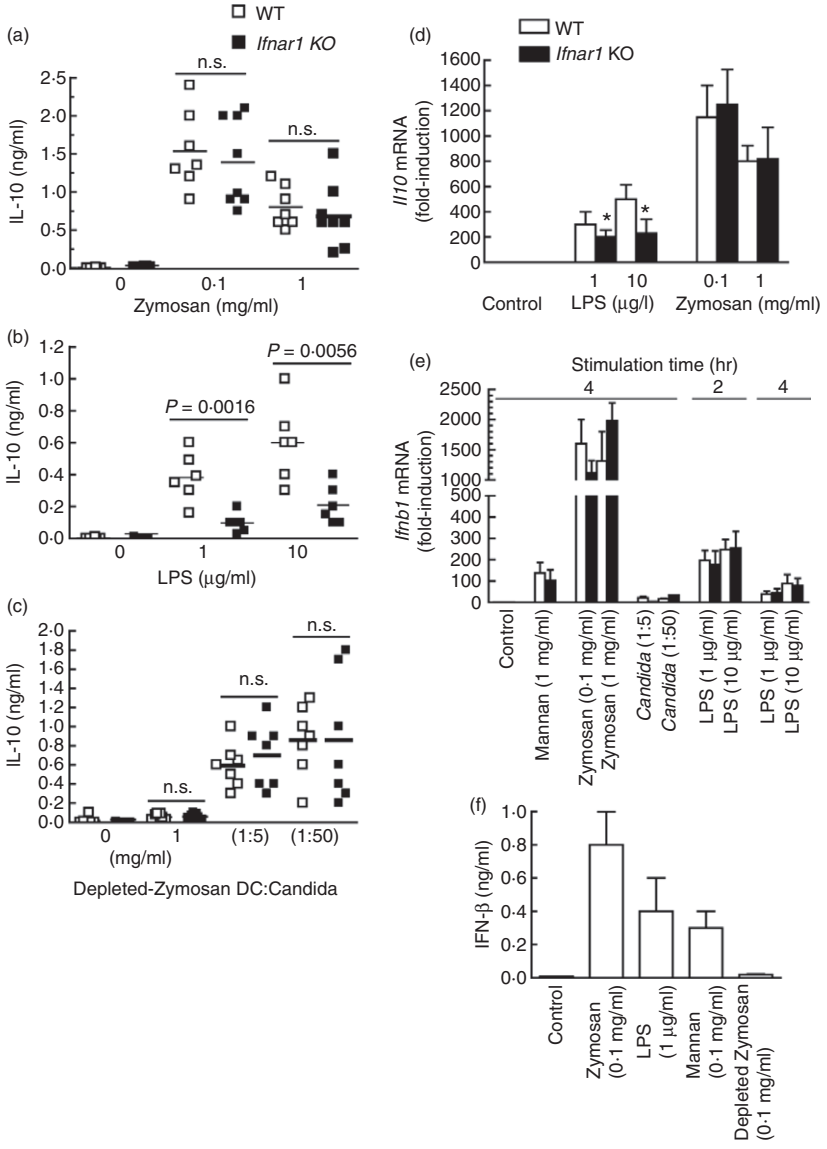


**Figure 2.** Production of interleukin-10 (IL-10) by wild-type (WT) and *Ifnar1*$^{-/-}$ mice. (a–c) Bone-marrow-derived dendritic cells (BMDC) were incubated in the presence of different stimuli and the IL-10 protein was assayed in the supernatants after 18 hr of stimulation. Experiments with *Candida albicans* were carried out with heat-killed *Candida* at two different ratios of DC : *Candida* as indicated. (d) *Il10* mRNA induction in samples collected after 4 hr of stimulation. (e) *Ifnb1* mRNA induction in samples collected at the times indicated. (f) Production of interferon-β (IFN-β) induced by different stimuli after 18 hr of incubation. Data in (d) represent mean ± SEM of six animals. Data in (e) represent mean ± SEM of three animals at 2 hr and nine animals at 4 hr. Data in (f) represent mean ± SEM of three mice.


 

These results show a limited role of type I IFN signalling on the production of IL-10 elicited by zymosan and *Candida*, agree with earlier reports showing a limited effect of mannose-depleted zymosan on IL-10 production, and point to the mannose-based component of zymosan as the main pathogen-associated molecular patterns involved in the production of IL-10.

### Functional consequences of the phosphorylation of Y705-STAT3 by zymosan components and comparison with the human system

Given that STAT3 activity seemed independent of type I IFN signalling, its possible role in the development of cross-inhibition came into prominence. Y705-STAT3 phosphorylation by LPS was clearly observed at 3 hr and reached a maximum between 6 and 18 hr. Zymosan showed a similar effect, although it was not detected at 3 hr (Fig. 3a). This time–course was consistent with the involvement of STAT3 in the induction of *Il10* by LPS, as Y705-STAT3 phosphorylation preceded the expression of *Il10* mRNA (Fig. 3b). In keeping with previous reports, zymosan was a weak stimulus of *Il12a* mRNA expression[33,34] and a strong inducer of *Il23a* and *Ifnb1* mRNA (Fig. 3b). P-Y705-STAT3 was observed to a similar extent in both WT and *Ifnar1*$^{-/-}$ mice in response to zymosan and *Candida* (Fig. 3c). Human DC showed a time–course of Y705-STAT3 phosphorylation (Fig. 4a) and a pattern of cytokine induction similar to those observed in mouse BMDC (Fig. 4b), with the exception of a tendency of *IL10* mRNA to decrease at 18 hr and a lower induction of *IFNB1* mRNA. In contrast, human macrophages showed a somewhat higher induction (Fig. 4c). To address the mechanism underlying STAT3 activation in human DC, the effects of the different components of zymosan and some cytokines were assayed. Zymosan, mannan and high concentrations of IL-10 were robust inducers, whereas mannose-depleted zymosan was a weak stimulus (Fig. 5a). Only mannan activated the phosphorylation of Y701-STAT1 robustly (Fig. 5b). Zymosan was more active than mannan and depleted-zymosan to induce *IL10* and *IL23A* mRNA (Fig. 5c), whereas the

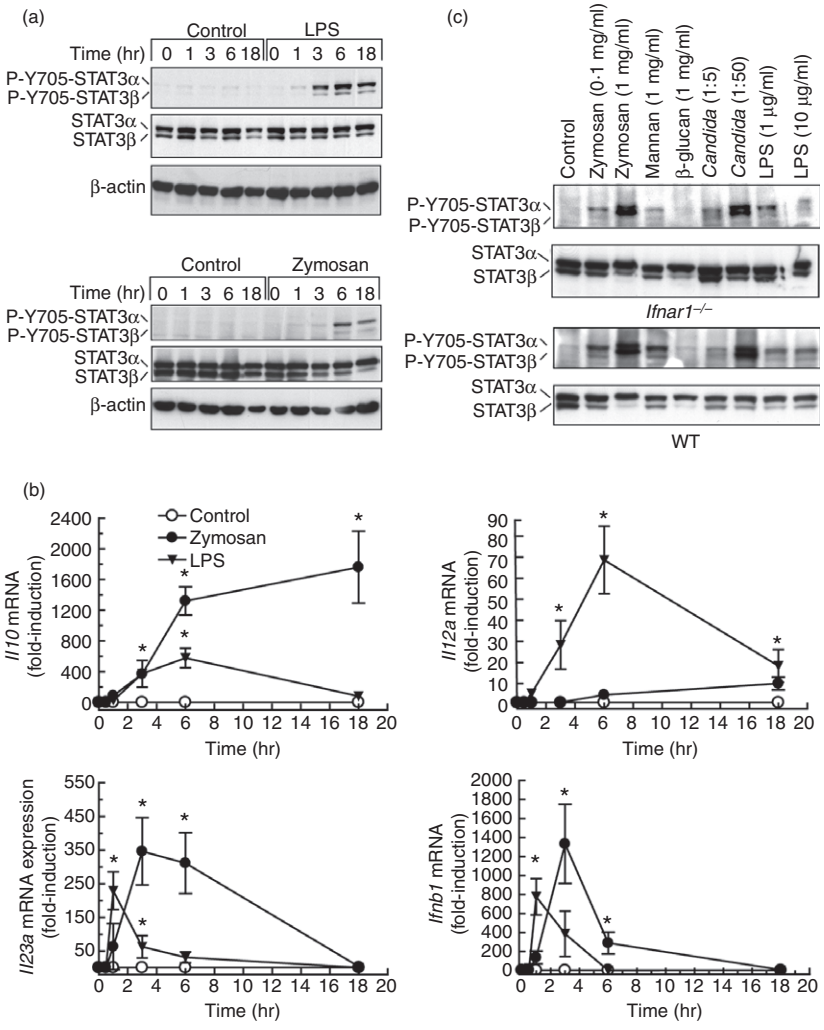


**Figure 3.** Y705-STAT3 phosphorylation and cytokine expression in response to different stimuli in bone-marrow-derived dendritic cells (BMDC). (a) Time–course of Y705-STAT3 phosphorylation in BMDC of mice stimulated with 10 μg/ml lipopolysaccharide (LPS) and 0·1 mg/ml zymosan. (b) Induction of *Il10*, *Il12a*, *Il23a* and *Ifnb1* mRNA in response to zymosan and LPS. (c) Effect of different stimuli on Y705-STAT3 phosphorylation in cells from *Ifnar1*$^{-/-}$ and wild-type (WT) mice stimulated for 6 hr. Data in (b) represent mean ± SEM of three different experiments. *$P < 0{\cdot}05$.

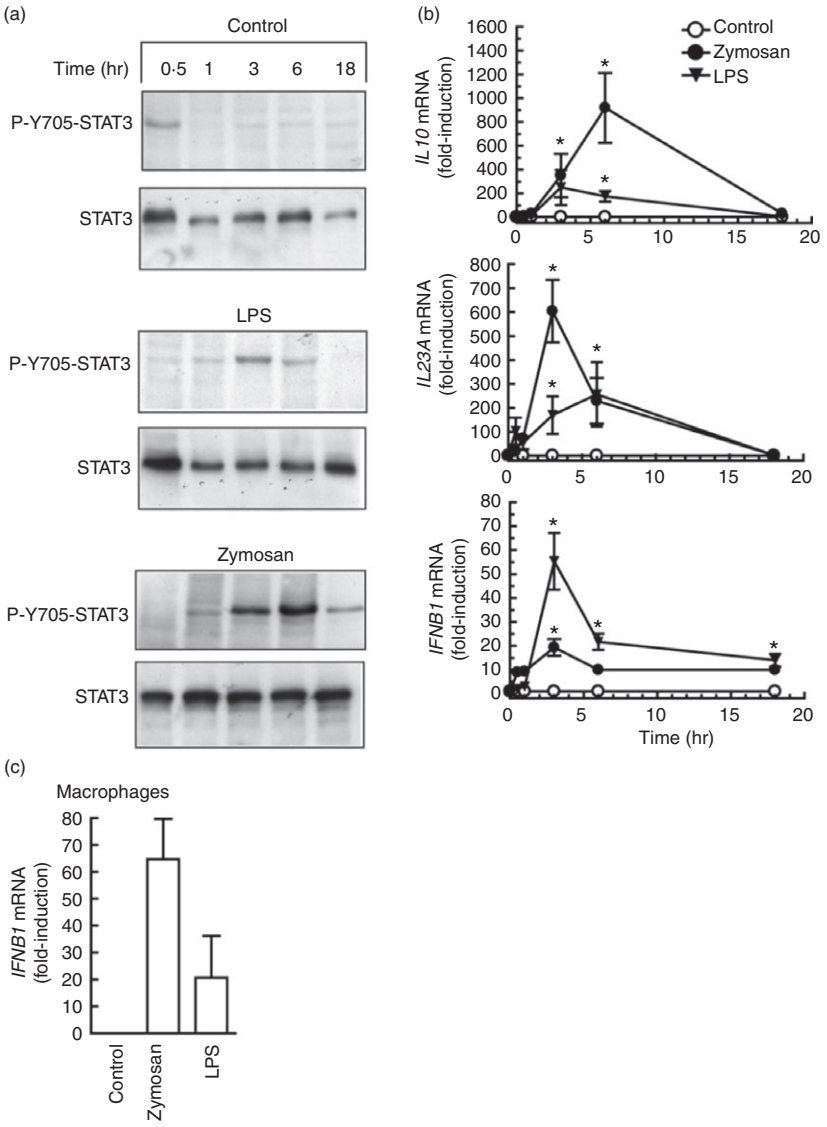


**Figure 4.** Y705-STAT3 phosphorylation and cytokine expression induction in human dendritic cells (DC). (a) Time–course of Y705-STAT3 phosphorylation. (b) Induction of *IL10*, *IL23A*, and *IFNB1* mRNA in response to 0·1 mg/ml zymosan and 10 μg/ml lipopolysaccharide (LPS). (c) Effect of zymosan and LPS on *IFNB1* mRNA induction in human macrophages stimulated for 6 hr with 1 mg/ml zymosan and 10 μg/ml LPS. Data in (b) and (c) represent mean ± SEM of three different experiments. $*P < 0{\cdot}05$ compared with controls.

IFN-$\beta$-inducible genes *CXCL9* and *CXCL10* showed reduced expression compared with the effect of IFN-$\beta$ (Fig. 5d). Interleukin-10 induced its own mRNA to a low extent at a concentration of 10 ng/ml, which seems to be close to the maximum concentration that could be reached under physiological conditions. The selectivity of this effect being confirmed by the inhibition with anti-IL-10 antibody (Fig. 5e). Consistent with the lack of expression of its cognate receptor, IL-23, which signals through a receptor coupled to the JAK/STAT system in some types of lymphoid cells,[35] did not induce the expression of *IL10* mRNA. These findings show limited evidence for type I IFN responses in the phosphorylation of Y705-STAT3 and agree with an actual inhibition of type I IFN responses by the ITAM-dependent receptors dectin-1 and dectin-2. Overall, the results observed in mice can be translated into humans with only a few cautions.

### Mechanism of STAT3 activation in human DC

Several secondary mediators induced by zymosan may phosphorylate STAT3 at Y705 by JAK- and Src-dependent routes.[22,36] In keeping with the limited effect of IL-10 on STAT3 activation, a neutralizing anti-IL-10 antibody reduced Y705-STAT3 phosphorylation only slightly, even when combined with an anti-IL-6 neutralizing antibody (Fig. 6a). The JAK inhibitors ruxolitinib and tofacitinib blocked the effect of zymosan. Ruxolitinib showing a robust effect at a lower concentration (Fig. 6b). STAT3 showed a mobility shift in response to zymosan that was observed in the absence of Y705-STAT phosphorylation and could be explained by other post-translational modifications, e.g. acetylation of lysine residues.[37,38] Notably, the JAK inhibitors did not affect the expression of *IL10* mRNA in response to zymosan, whereas they inhibited the response to LPS and enhanced the *IL23A* mRNA


 

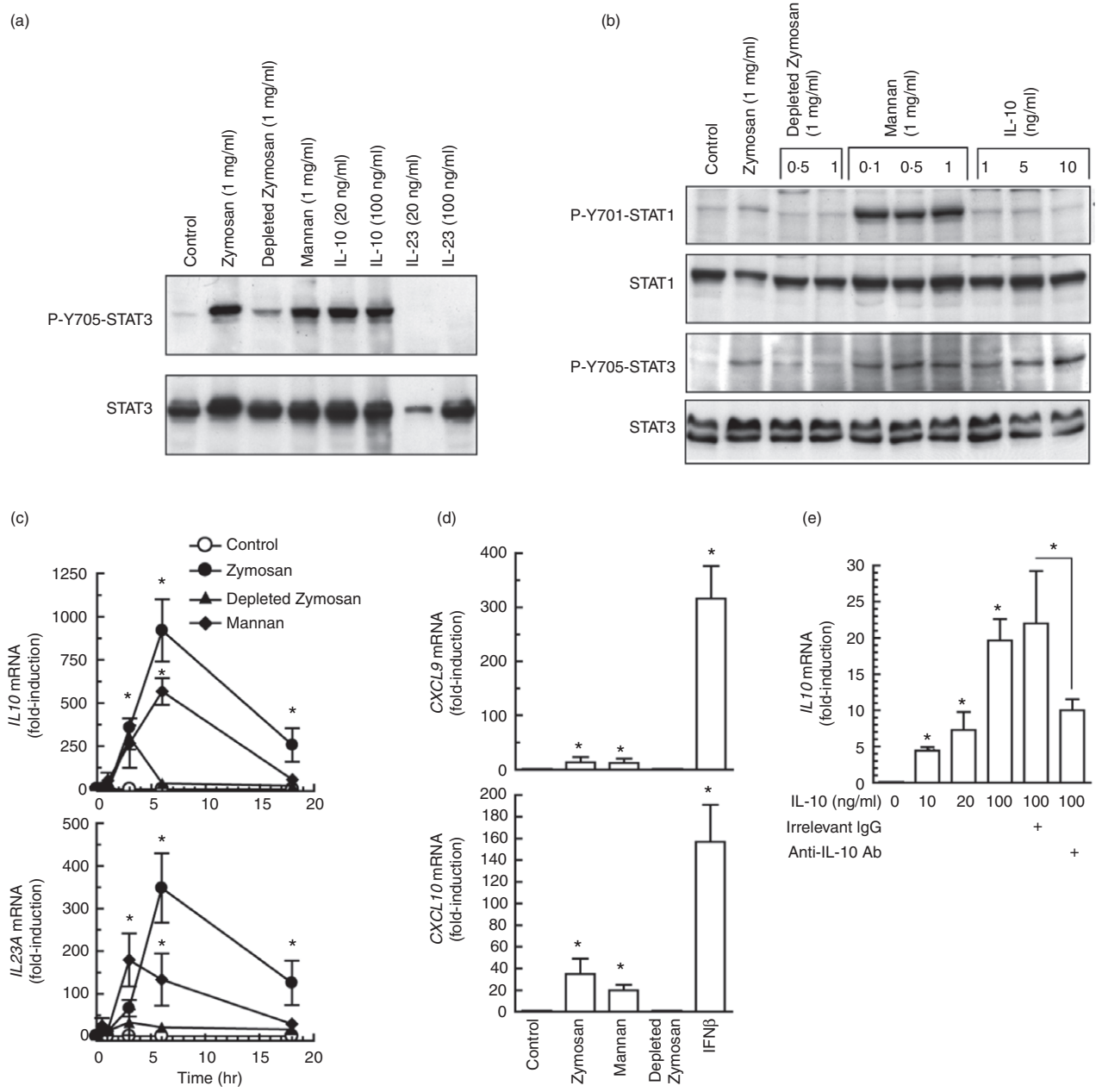


**Figure 5.** Effect of the different components of zymosan on Y705-STAT3 and Y701-STAT1 phosphorylation, and on cytokine induction. (a, b) Human dendritic cells (DC) were stimulated for 6 hr with different stimuli and cell lysates were used for the assay of Y705-STAT3 and Y701-STAT1 phosphorylation. (c, d) Effect of zymosan, mannan and mannose-depleted zymosan at the concentration of 1 mg/ml on the induction of the mRNA of *IL10*, *IL23A*, *CXCL9* and *CXCL10*. Interferon-$\beta$ (IFN-$\beta$) was used as a positive control at the concentration of 1000 U/ml. (e) Effect of interleukin-10 (IL-10) and control irrelevant antibody on the effect of exogenous IL-10 on *IL10* mRNA induction expression. Antibodies were used at the concentration of 10 μg/ml. Data represent mean ± SEM of four different experiments. $^*P < 0{\cdot}05$ as compared to controls.

induction in response to both stimuli (Fig. 6c, d), suggesting that the JAK-activity-dependent down-regulation of *IL23A* expression could be explained by a direct blockade of IL-10 signalling, given that the expression of *IL10* mRNA was not affected. The possible involvement of $PGE_2$ was assessed because it is an autocrine effector of IL-10 production and may influence CREB[4] and STAT3[24] activation. Although a direct effect of $PGE_2$ on P-Y705-STAT3 was not observed, $PGE_2$ receptor antagonists showed a slight inhibitory effect on zymosan and mannan responses (Fig. 7a). Given the prominent role of COX-2 in the autocrine production of $PGE_2$ and in STAT3 activation,[39] its expression was addressed. Zymosan and mannan were robust inducers, whereas mannose-depleted zymosan was inactive (Fig. 7b). These results disclose some commonality of the mechanisms involved

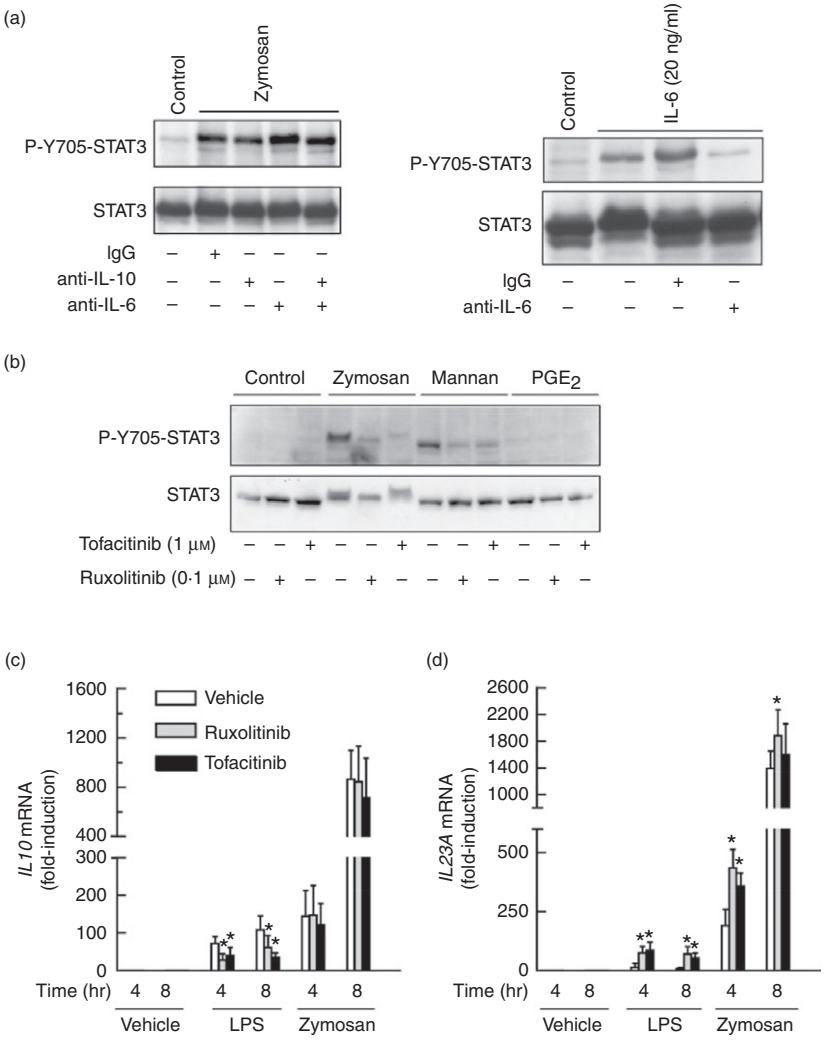


**Figure 6.** Effect of anti-interleukin-10 (IL-10) and anti-IL-6 antibody on Y705-STAT3 phosphorylation in response to zymosan. (a) Human dendritic cells (DC) were incubated for 30 min with 10 μg/ml of the different antibodies before the addition of 1 mg/ml zymosan. Cell lysates were collected at 6 hr to assay Y705-STAT3 phosphorylation. To confirm the blocking activity of anti-IL-6 antibody, DC were stimulated with 20 ng/ml of IL-6 for 1 hr and after this time, lysates were collected to assay Y705-STAT3 (right panel). (b) Effect of ruxolitinib and tofacitinib on Y705-STAT3 phosphorylation elicited by 1 mg/ml zymosan and mannan, and 1 μM prostaglandin $E_2$ ($PGE_2$). (c, d) Effect of ruxolitinib and tofacitinib on *IL10* and *IL23A* mRNA expression. Data represent mean ± SEM of three independent experiments. $^*P < 0{\cdot}05$.

in the induction of both *PTGS2*/*COX2* and *IL10*. Stimulation with PAF did not induce Y705-STAT3 phosphorylation, even when combined with $PGE_2$, but similar to the effect of COX inhibitors, the PAF receptor antagonist showed some inhibitory effect that was enhanced in some cases by the combined addition of the COX inhibitors sc-560 and sc-236 (Fig. 7c–e). This effect is consistent with the expression of functional PAF receptors in DC and its cooperation with the signalling routes elicited by other agonists.[40–42] Densitometric analysis of the inhibition of P-Y705-STAT3 phosphorylation in three independent experiments showed a 38% inhibition (Fig. 7e). Given that the transcriptome induced by fungal patterns disclosed that genes displaying growth factor activity are induced to a maximal extent[43] and GM-CSF (encoded by the gene *CSF2*) is a strong activator of STAT3, we addressed the mRNA expression of *CSF1*, *CSF2* and *CSF3*. As shown in Fig. 7f, *CSF2* mRNA showed maximal increase at 4 hr, making it likely its involvement as an autocrine activator of Y705-STAT3 phosphorylation. However, stimulation with zymosan in the presence of a blocking antibody of GM-CSF did not influence the expression of *IL10* mRNA (Fig. 7d). The effect of GM-CSF could also be ruled out by the absence of Y694-STAT5 phosphorylation, a definite marker of GM-CSF signalling (not shown). Given that a portion of the cross-inhibition elicited by ITAM-containing receptors has been associated with a signalling cascade dependent on p38 MAPK and mitogen- and stress-activated kinases 1/2 (MSK1/2), which induces Y705-STAT3 phosphorylation and *Il10* expression,[30,31] the effect of the p38 MAPK inhibitor SB203580 was addressed. SB203580 showed 30% inhibition of Y705-STAT3 phosphorylation in a densitometric scanning (Fig. 8a), whereas it inhibited by ~ 80% *IL10* and *IL23A* mRNA expression, suggesting that its overall effect might depend on several targets (Fig. 8b). The inhibitor of Src kinases PP2 showed a tendency to enhance *IL10* mRNA expression and did not influence Y705-STAT3 phosphorylation (Fig. 8b, c). The Millipore STAT3 inhibitor did not elicit any significant effect (Fig. 8b). Together, these results show the involvement of JAK in the activation of STAT3 elicited by zymosan. This


 

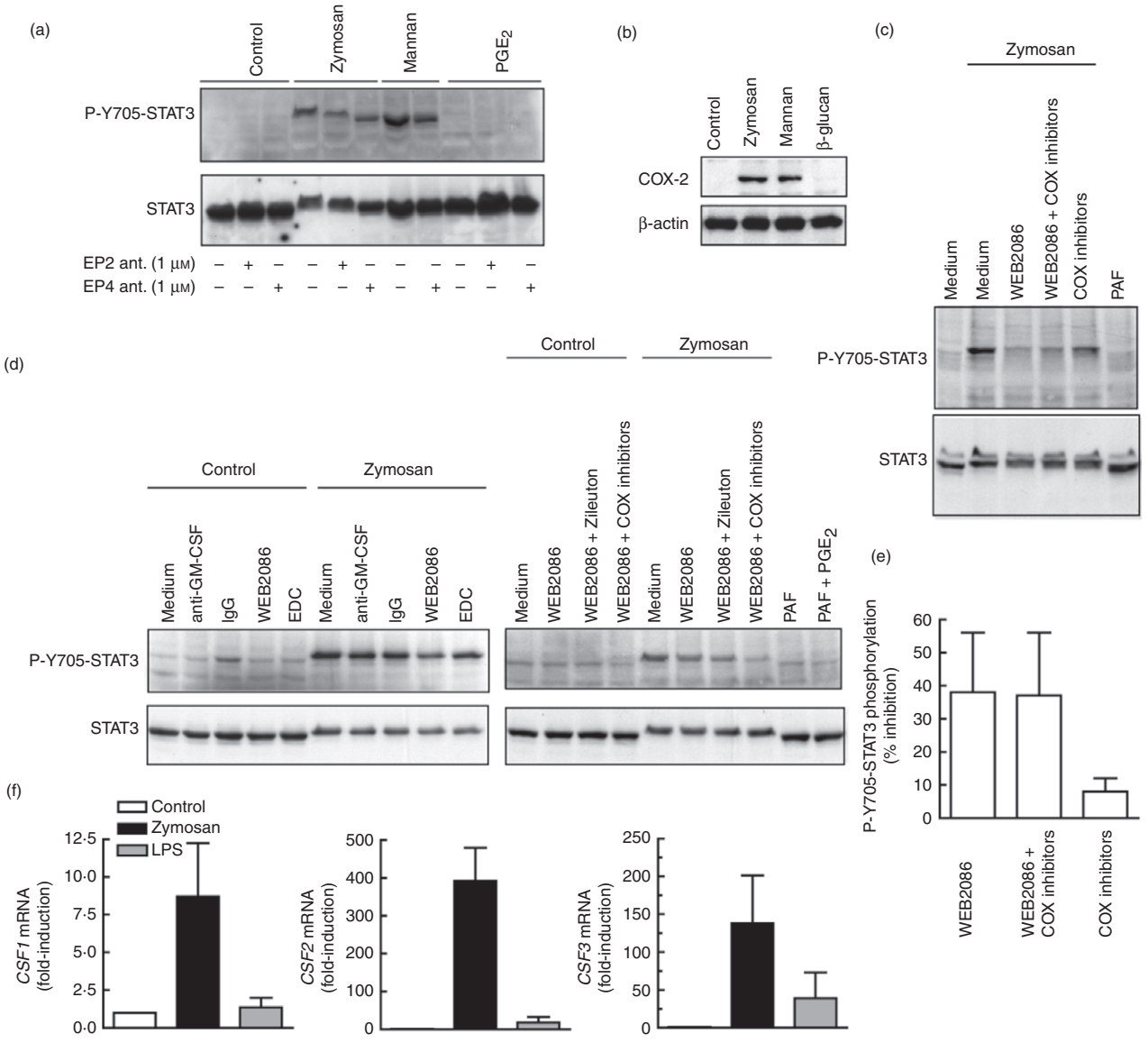


**Figure 7.** Analysis of the effect of lipid mediators and granulocyte–macrophage colony-stimulating factor (GM-CSF) on Y705-STAT3 phosphorylation in human dendritic cells (DC). (a) Effect of the antagonists of E prostanoid receptor 2, PF-04418948 and EP4, L-161982, and exogenous prostaglandin $E_2$ ($PGE_2$) (1 μM) on Y705-STAT3 phosphorylation. (b) Effect of the different components of zymosan on COX2 induction. (c–e) The effect of the blockade of endogenous mediators on Y705-STAT3 was assessed with anti-GM-CSF and IgG control antibody (10 μg/ml), the platelet-activating factor (PAF) receptor antagonist WEB2086 (30 μM), the COX-1 inhibitor sc-560 (50 nM), the COX-2 inhibitor sc-236 (50 nM), the 5-lipoxygenase inhibitor zileuton (10 μM), and the 12/15-lipoxygenase inhibitor ethyl 3,4-dihydroxybenzylidenecyanoacetate (3 μM). PAF was used at a concentration of 1 μM. A typical experiment of Y705-STAT3 phosphorylation inhibition by WEB2086 is shown in (c), together with the densitometric analysis of a set of three independent experiments in (e). (f) Induction of *CSF1*, *CSF2* and *CSF3* mRNA in response to zymosan and lipopolysaccharide (LPS). Results show mean ± SEM of three independent experiments. $^*P < 0{\cdot}05$ as compared to controls.

activation has a minimal effect on *IL10* expression, but may contribute to diminish the pro-inflammatory effects associated with *IL23A* induction expression. In addition, our data rule out the involvement of some purported effectors on the induction of Y705-STAT3 phosphorylation and suggest the contribution of several mechanisms involving the lipid mediators PAF and $PGE_2$, as well as p38 MAPK.

### IRF5 displays different effects in the induction of IL-10 elicited by LPS and zymosan

Because the effect of IRF3 may be countered by IRF5, which uncouples the binding of RNA polymerase II to the *Il10* promoter, the effect of IRF5 deletion was addressed.[44] The initial hypothesis was that the effect of IRF5 might prevail in the absence of IRF3. Experiments

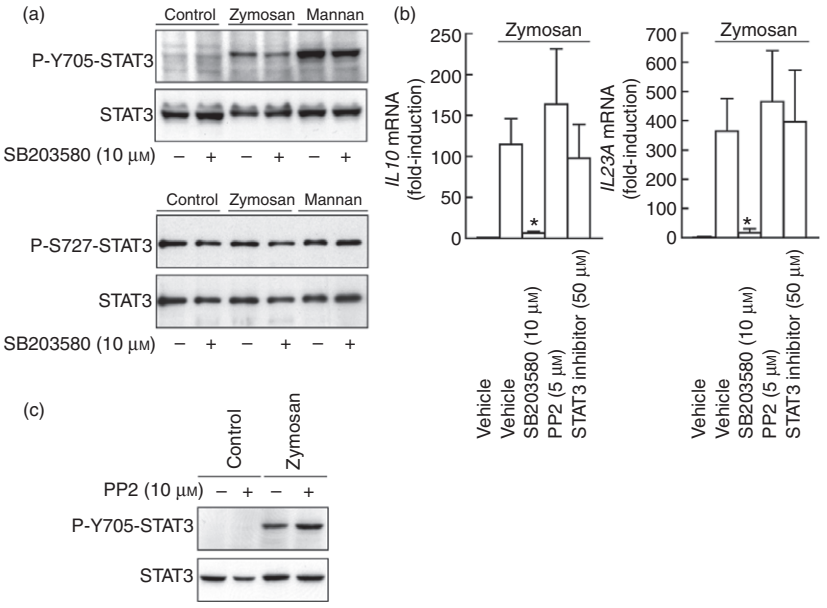


**Figure 8.** Effect of kinase and signal transducer and activator of transcription 3 (STAT3) inhibitors. (a) Effect of p38 mitogen-activated protein kinase (MAPK) inhibition on P-Y705-STAT3 and P-Ser727-STAT3 phosphorylation. (b) Effect of the p38 MAPK inhibitor SB203580, the Src kinase inhibitor PP2, and the STAT3 inhibitor on *IL10* and *IL23A* induction. (c) Effect of Src kinase inhibitor PP2 on P-Y705-STAT3 phosphorylation. Results show mean ± SEM of three independent experiments. $*P < 0{\cdot}05$.

disclosed that *Irf5*$^{-/-}$ mice showed a significant reduction of *Ifnb1* and *Il12a* mRNA in response to zymosan compared with WT mice, whereas no significant changes were observed in *Il10* and *Il23a* mRNA (Fig. 9a). The assay of IL-10 protein did not show differences in response to zymosan, mannan and *Candida* conidia between *Irf5*$^{-/-}$ and WT mice (Fig. 9b), whereas the production of IL-10 and the expression of *Il10* and *Ifnb1* mRNA in response to LPS was reduced in the *Irf5*$^{-/-}$ animals (Fig. 9c). Of note, GM-CSF macrophages from *Irf5*$^{-/-}$ mice showed a reduced expression of *Ifnb1* and *Il10* mRNA in response to zymosan (Fig. 9d). No evidence of type I IFN cytokine signature was observed, as judged from the absence of *Cxcl9* and *Cxcl10* induction in response to zymosan (data not shown). The assay of transcription factor binding to the *IL10* promoter showed a significant binding of P-Y705-STAT3 to the most proximal IFN-γ-activated (GAS)-site (Fig. 10a). This was not observed in response to *β*-glucan and agrees with the limited extent of Y705-STAT3 phosphorylation elicited by this stimulus. To confirm the specificity of the results, a region in the proximal promoter of *IL23A* lacking GAAA core sequences showed no binding when the assay was carried out with the set of primers indicated in the Supplementary material (Table S1). Unexpectedly, the effect of mannan was less prominent than that elicited by zymosan, although it was a robust activator of Y705-STAT3 phosphorylation. A likely explanation could be that mannan also activates STAT1 and this might compete for binding at the same site. Binding of IRF3 and IRF5 to the IRF site increased significantly in response to both mannan and zymosan (Fig. 10b, c), the response to mannan being more robust. The binding of IRF1 was addressed because it is involved in the autocrine loop started by TNF-α that activates type I IFN-response genes. However, binding of IRF1 occurred to a low extent (Fig. 10d) and blockade of TNF-α with the soluble TNF receptor etanercept showed some degree of inhibition of Y705-STAT3 phosphorylation (Fig. 10e). Taken together, these data indicate that induction of *Ifnb1* and *Il12a* by zymosan in BMDC depends on IRF5, whereas the induction of *Il10* and *Il23*a mRNA is not affected. Conversely, the production of IL-10 protein by LPS was significantly reduced in *Irf5*$^{-/-}$ mice. IRF5 seems also involved in the induction of *Ifnb1* and *Il10* mRNA in GM-CSF macrophages. Given that IRF5 can bind to the *IL10* promoter, it is possible that their functions might be exerted through either a direct effect at the promoter level or indirectly through *Ifnb1* induction.

## Discussion

Our data underscore the distinct signalling routes involved in the expression of IL-10 triggered by bacterial and fungal patterns, disclose some mechanisms whereby ITAM-associated receptors interfere with the function of the IFN-α/*β* receptor, and provide some clues about the mechanism of activation of STAT3 by the secondary mediators induced by fungal patterns. The genetic approach using *Irf3*$^{-/-}$, *Irf5*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice proved that the IFN-*β*-dependent autocrine cycle observed in the LPS/TLR4 route is not involved in the production of IL-10 elicited by both *Candida* and zymosan. Given that zymosan is a strong inducer of *Ifnb1* expression, there is not a clear reason to explain the differences between both models. The induction of *Ifnb1* expression by zymosan has been associated with the presence of yeast nucleic acid and engagement of TLR7 and TLR9.[45] However, zymosan did not induce the expression


 

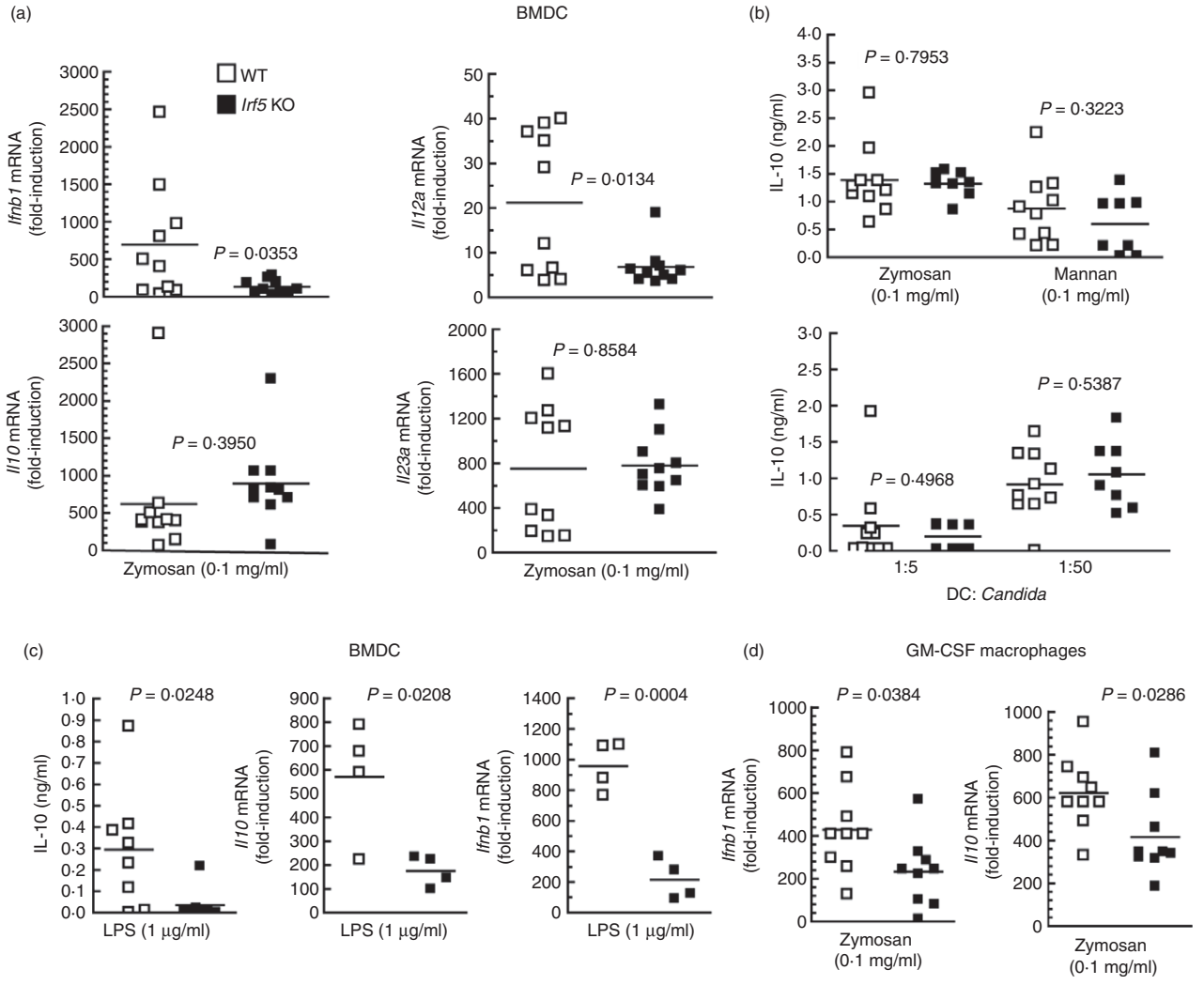


**Figure 9.** Effect of *Irf5* deletion on interleukin-10 (IL-10) production by mouse bone-marrow-derived dendritic cells (BMDC) and granulocyte–macrophage colony-stimulating factor (GM-CSF) macrophages. (a–c) Bone marrow cells were cultured in medium supplemented with murine GM-CSF. On day 5, the floating cells (BMDC) were collected and plated before stimulation with the indicated stimuli. (d) Adherent cells (GM-CSF macrophages) were processed separately and stimulated with 0·1 mg/ml zymosan under the same conditions as BMDC. The stimulation was maintained for 4 hr for collection of RNA or 18 hr for the assay of IL-10 protein.

of *CXCL9* and *CXCL10* mRNA and blunted the induction of those cytokines by LPS.[31] These results can be explained by the induction of SOCS1 and SOCS3, and the robust activation of STAT3, which suppresses STAT1 function by allowing the formation of STAT1/STAT3 heterodimers.[46] These results agree with reports on the transcriptome of macrophages stimulated with *Candida*, where *Il10*, *Il23a* and *Socs3* mRNA were expressed to a large extent in the absence of an IFN-cytokine signature.[43] Together, our data do not support the involvement of STAT3 in the induction of *IL10* expression by fungal patterns, and the low production of IL-10 elicited by mannose-depleted zymosan agrees with the involvement of receptors that recognize mannose-based patterns.[7,13,47] Because STAT3 activation did not occur immediately after stimulation, the identification of secondary mediators other than type I IFN was necessary. Interleukin-10 could be a possible effector, but a central role was ruled out in view of the high concentration of the cytokine required to induce P-Y705-STAT3 and the limited effect of a blocking antibody. A similar reasoning was assigned to IL-6. The involvement of $PGE_2$ and PAF was also considered. Although these mediators may be necessary for the activation of STAT3, they are not sufficient in view of our inability to show Y705-STAT3 phosphorylation in response to these agonists. A possible explanation might be that they are produced at different times after zymosan challenge and this makes it difficult to reproduce the conditions for Y705-STAT3 phosphorylation to occur. Although GM-CSF is a component of the

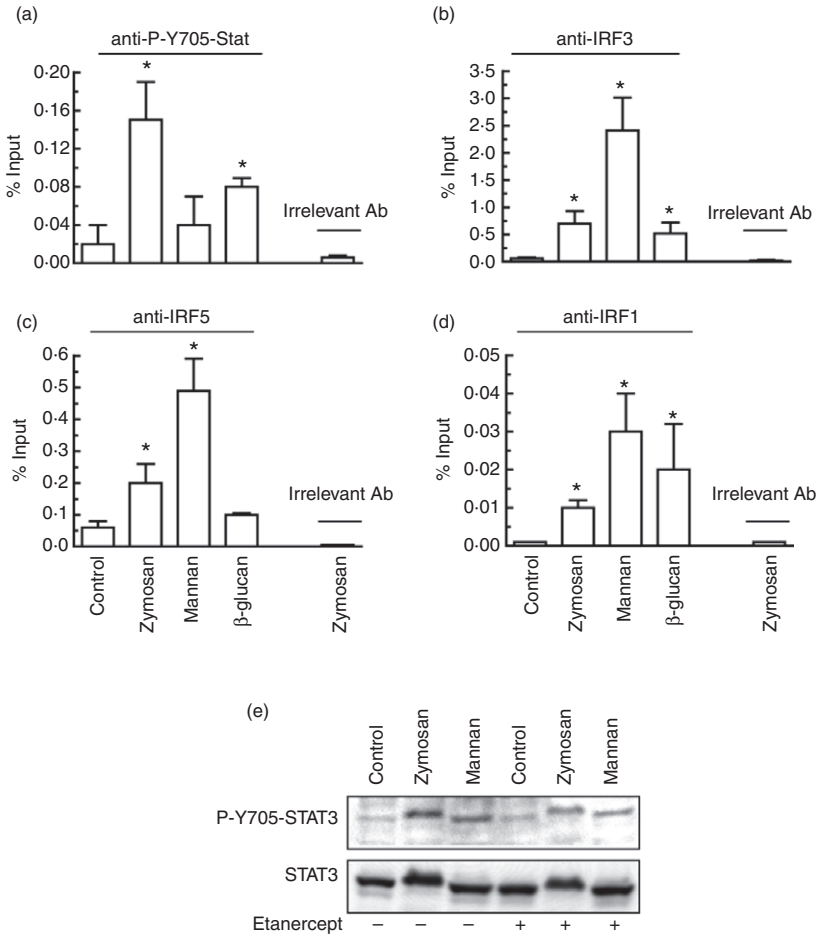


**Figure 10.** Binding of P-Y705-STAT3 and interferon regulatory factors 1/3/5 (IRF1/3/5) to the proximal GAS site of the human *IL10* promoter. (a–d) Binding of P-Y705-STAT3 was studied at 6 hr after addition of the stimuli. Binding of the different IRF was studied at 4 hr after addition of the stimuli. The selection of times was carried out according to the time–course of Y705-STAT3 phosphorylation and with reports on optimal binding of IRF. The specificity of the response was confirmed by the absence of antibody binding to a region in the proximal promoter of *IL23A* lacking GAAA core sequences and the results with irrelevant antibody. (e) Effect on Y705-STAT3 phosphorylation of the blockade of tumour necrosis factor-α (TNF-α) action with 1 μg/ml etanercept. Results represent mean ± SEM of three to five independent experiments. *$P < 0{\cdot}05$ compared with controls.

cytokine-signature of zymosan, the effect of both exogenous and endogenous GM-CSF was discarded using distinct approaches. Consistent with the notion that JAK inhibition blunts the IFN-$\beta$ feedback loop and enhances the production of pro-inflammatory cytokines,[47] JAK inhibitors enhanced *IL23A* mRNA expression, although they did not influence *IL10* induction in response to zymosan. The involvement of Src kinases was ruled out because of the limited effect of the Src inhibitor PP2. Taking into account the most recent notions highlighting the central role of the energetic metabolism in the innate immune response, the chance that STAT3 phosphorylation might be the result of pyruvate kinase M2 activity is a challenging hypothesis.[48] Likewise, recent research has disclosed the role of mTORC1 complex as a central element of the late phase of the TNF-α response by regulating downstream genes that inhibit inflammatory signalling and nuclear factor-κB activation. This route has a rigorous sensitivity to the metabolic state and oxygen levels and depends on IL-10 induction and a delayed activation of STAT3.[49]

Strong activation of ITAM-containing receptors by high-avidity cross-linking has been found to suppress IFNAR signalling via protein kinase C-mediated recruitment of SHP2 and the induction of SOCS3 and repressors of gene transcription such as HES1 and STAT3.[30] On this basis, Y705-STAT3 phosphorylation might depend on a network of secondary mediators, rather than on a direct activation of JAK. For instance, the phosphorylation of Y705-STAT3 and the production of IL-10 by FcγR and integrins have been related to the activation of MSK, which are downstream from p38 MAPK.[7,50] However, our experiments showed that whereas p38 MAPK inhibition was associated with a strong down-regulation of *IL10* mRNA, only a modest inhibition of Y705-STAT3 phosphorylation was observed.

Binding of IRF3 to the seven GAS-sites detected in the −2000/+2000 nucleotides of the transcription start site in the *Il10* promoter has been associated with *Il10* transcription.[51] Given the down-regulatory effect of IRF5 on *Il10* expression ascertained in some systems,[44] we posited that IRF3 and IRF5 may compete for binding to the GAS-sites and exert opposing effects on transcription. On this basis, IRF3 deletion might leave unrestrained the effect of IRF5 and enhance the expression of phenotypic markers of M1 macrophages.[52] Taken collectively, experiments showed that the function of IRF5 is complex and may be cell- and stimulus-dependent. A recent report showed that


 

IRF5 is necessary for IL-10 production in response to TLR7 and TLR9 stimulation in mouse GM-CSF macrophages and DC, whereas the response to TLR2 and TLR4 ligands did not reach statistical significance, although it was lower in *Irf5*$^{-/-}$ mice.[53] In summary, we observed a robust activation of STAT3 and production of IL-10 in human and mouse DC stimulated with zymosan that depends on its mannose-based component. The effect on STAT3 phosphorylation is explained by an interplay of secondary mediators, which includes lipids, TNF-α and p38 MAPK-dependent reactions, but is independent on a feed-forward mechanism involving IRF3 and type I IFN signalling. These results highlight the different transcriptional programmes involved in IL-10 production by bacterial and fungal pathogen-associated molecular patterns and disclose a mechanism whereby activation of ITAM-containing receptors by fungal pathogen-associated molecular patterns may suppress IFNAR signalling.

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## Disclosures

The authors declare that they have no conflict of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Primers used for chromatin immunoprecipitation and real time RT-PCR in human and murine dendritic cells.


 