FBXW7α attenuates inflammatory signalling by downregulating C/EBPδ and its target gene Tlr4

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Toll-like receptor 4 (Tlr4) has a pivotal role in innate immune responses, and the transcription factor CCAAT/enhancer binding protein delta (C/EBPδ, Cebpd) is a Tlr4-induced gene. Here we identify a positive feedback loop in which C/EBPδ activates Tlr4 gene expression in macrophages and tumour cells. In addition, we discovered a negative feedback loop whereby the tumour suppressor FBXW7α (FBW7, Cdc4), whose gene expression is inhibited by C/EBPδ, targets C/EBPδ for degradation when C/EBPδ is phosphorylated by GSK-3β. Consequently, FBXW7α suppresses Tlr4 expression and responses to the ligand lipopolysaccharide. FBXW7α depletion alone is sufficient to augment pro-inflammatory signalling in vivo. Moreover, as inflammatory pathways are known to modulate tumour biology, Cebpd null mammary tumours, which have reduced metastatic potential, show altered expression of inflammation-associated genes. Together, these findings reveal a role for C/EBPδ upstream of Tlr4 signalling and uncover a function for FBXW7α as an attenuator of inflammatory signalling.
Hypoxia-induced inflammatory responses to infection are induced in part by Toll-like receptors (TLRs), which belong to the pattern recognition receptor family. To date, 10 human and 12 mouse TLRs are known, each of which binds specific ligands. TLR4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria and signals in combination with other co-receptors to activate the NF-kB transcription factors. TLR4 is involved in diseases such as sepsis and chronic inflammatory disorders. TLR4 signalling in tumour cells is associated with suppression of immune surveillance, proliferation, inflammatory cytokine production and invasive migration. Therefore, understanding the regulation of TLR4 expression and signalling may be important for the management of these conditions.

**C/EBPα is an inflammatory response gene.** C/EBPα amplifies LPS signalling, and it is essential for the expression of many LPS-induced genes and the clearance of Gram-negative bacterial infection. Celpα deficiency partly protects mice from LPS-induced mortality and autoimmune encephalomyelitis, suggesting that C/EBPα has a role in the progression of systemic inflammatory diseases such as sepsis and multiple sclerosis.

We reported that C/EBPα directly inhibits the expression of the F-box and WD repeat domain containing protein 7 alpha (FBXW7) in mammary tumour cells. The Fbxw7 gene encodes three protein isoforms, of which the alpha isoform is the most abundantly expressed. FBXW7 functions as the substrate-recognition subunit of SCF-type ubiquitin ligase complexes. FBXW7 is a mammalian oncoprotein for degradation, including c-myc, cyclin E, mTOR, c-jun and Notch.

The role of FBXW7 in inflammatory signalling has not been reported for mammary tumour cells. FBXW7 depletion increased the levels of C/EBPα expression in macrophages, which augments survival under hypoxia. The role of hypoxia and HIF-1α expression in tumour metastasis is complex and is necessary for cellular adaptation to hypoxia. HIF-1α target genes promote angiogenesis and the metabolic switch to glycolysis, which augments survival under hypoxia. In agreement with the role of hypoxia and HIF-1α in tumour metastasis, loss of Cebpα results in reduced metastatic progression of MMTV-Neu transgenic mice; MMTV-Neu transgenic mouse macrophages/monocytes and neutrophils independent of genotype (mean ± s.e.m. n = 4 mice).

In vivo LPS treatment (6 h) reduced FBXW7 protein levels in WT PECs but not in Cebpα−/− cells, while C/EBPα expression was induced by this treatment in WT PECs (Supplementary Fig. S1b). Furthermore, the higher levels of basal FBXW7α that were observed in Cebpα−/− PECs (Supplementary Fig. S1b, c) were correlated with reduced levels of its targets mTOR and Aurora A and reduced phosphorylation of AKT, S6K1 and GSK-3β (Supplementary Fig. S1c). Taken together, these data show that macrophages express FBXW7α and that FBXW7α expression is downregulated by C/EBPα and LPS.

**Results**

LPS and C/EBPα inhibit FBXW7α expression in macrophages. We previously reported that C/EBPα directly inhibits Fbxw7α gene expression in tumour cells, which in turn augments HIF-1α expression. To investigate this pathway in macrophages, we first analysed FBXW7 isoform expression. Semiquantitative analysis suggested that macrophages and mammary tumours expressed only Fbxw7α mRNA but not Fbxw7β or Fbxw7γ, while all three isoforms were detected in mouse embryonic fibroblasts (Fig. 1a). In mouse primary peritoneal macrophages (PPMs), basal levels of Fbxw7α mRNA were higher in Cebpα−/− (KO) compared with wild-type (WT) macrophages. LPS treatment decreased Fbxw7α transcripts in WT but not in KO macrophages (Fig. 1b). The silencing of CEBPD by RNA interference (RNAi) in U-937 human monocyctic cells increased the basal level of FBXW7 mRNA and abolished its repression upon LPS treatment (Supplementary Fig. S1a). Next, we analysed resident peritoneal exudate cells (PECs), which consisted of ~67 ± 2.7% macrophages/monocytes, 23 ± 1% lymphoid cells and 4.9 ± 0.6% neutrophils independent of genotype (mean ± s.e.m. n = 4 mice). In vivo LPS treatment (6 h) reduced FBXW7α protein levels in WT PECs but not in Cebpα−/− cells, while C/EBPα expression was induced by this treatment in WT PECs (Supplementary Fig. S1b). Furthermore, the higher levels of basal FBXW7α that were observed in Cebpα−/− PECs (Supplementary Fig. S1b, c) were correlated with reduced levels of its targets mTOR and Aurora A and reduced phosphorylation of AKT, S6K1 and GSK-3β (Supplementary Fig. S1c). Taken together, these data show that macrophages express FBXW7α and that FBXW7α expression is downregulated by C/EBPα and LPS.

C/EBPα and FBXW7α control HIF-1α expression in monocytes. LPS and hypoxia cooperatively induce HIF-1α expression in macrophages, and we confirmed these results in ANA-1 mouse macrophages (Supplementary Fig. S1d). Under these conditions, primary human monocytes treated with CEBPD RNAi had increased FBXW7α expression and reduced HIF-1α accumulation (Fig. 1c). As expected, CEBPD depletion also increased FBXW7α mRNA levels (Fig. 1d). Interestingly, FBXW7α depletion increased the levels of C/EBPα and HIF-1α protein (Fig. 1c) and of CEBPD mRNA (Fig. 1d), demonstrating that FBXW7α suppresses C/EBPα expression. In Cebpα−/− PPMs, HIF-1α accumulation could be rescued by knockdown of the elevated FBXW7α (Fig. 1e). These results demonstrate that C/EBPα promotes HIF-1α expression in activated macrophages through the inhibition of FBXW7α expression, as previously reported for mammary tumour cells.

HIF-1α is critical for hypoxia-induced glycolysis in macrophages. Therefore, we examined if reduced HIF-1α expression in Cebpα null PECs affected their glycolytic activity and activation. Under inflammatory conditions (LPS + 1% O2), Cebpα null PECs exhibited reduced hallmarks of the glycolytic switch, such as glucose consumption and lactate generation (Supplementary Fig. S1e). In agreement with these data, ATP production and the survival of Cebpd null peritoneal cells were reduced under these conditions (Supplementary Fig. S1f-g). Furthermore, Cebpd-deficient peritoneal macrophages exhibited limited induction of pro-inflammatory genes, such as Mmp9, Cxcr4, Vegfc and Il6, after stimulation with LPS + 1% O2 (Supplementary Fig. S1h). These genes are known HIF-1 targets. Il6 and Cxcr4 are also direct targets of C/EBPα. Collectively, these findings show that C/EBPα supports HIF-1-mediated inflammatory responses.

Importantly, there was no difference in the number of PECs isolated from Cebpα−/− mice compared with the controls. However, there was a small but significant decrease in the number of PPMs isolated from Cebpα−/− mice after elicitation, and there was a significant decrease in the recruitment of peritoneal cells...
C/EBPδ promotes HIF-1α expression in macrophages through inhibition of FBXW7α. (a) RT-PCR analysis of FBXW7 isoform expression from different sources as follows. (1) primary peritoneal macrophages (PPMs); (2) RAW 264.7 cells; (3) MMTV-Neu mammary tumour tissue; (4) primary mouse embryo fibroblasts. Numbers indicate the position of size markers in base pairs. (b) RT-qPCR analysis of Fbxw7 transcript levels in PPMs from WT and Cebpd−/− mice, cultured +/− LPS (100 ng ml⁻¹, 24 h), compared with WT untreated (n = 4, *P < 0.05; **P < 0.001). (c) Western analysis of nuclear extract (NE) from primary human monocytes nucleofected with siRNA oligos (C, control; D, CEBPD; F, FBXW7) and treated with LPS (100 ng ml⁻¹) and 1%O₂ (16 h) as indicated. SE, short exposure; LE, long exposure. (d) RT-qPCR analysis of FBXW7 and CEBPD transcripts in primary human monocytes as in panel (c) (n = 3, *P < 0.05; **P < 0.001). (e) Western analysis of NE from PPMs nucleofected with siRNA oligos and treated with LPS (100 ng ml⁻¹) and 1% O₂ for 16 h as indicated. SE, short exposure; LE, long exposure. Where applicable, data are mean ± s.e.m., evaluated by two-tailed unequal variance t-test.

FBXW7α targets C/EBPδ for degradation. FBXW7α is not a transcription factor. Therefore, its downregulation of Cebpd mRNA levels must be through indirect mechanisms. As C/EBPδ can activate its own promoter and has a degron-like sequence commonly found in FBXW7 substrates, we investigated whether FBXW7α regulated C/EBPδ expression at the protein level. Pulse-chase analysis (Fig. 2a) and cycloheximide-chase experiments (Supplementary Fig. S3a) showed that the half-life of C/EBPδ protein increased significantly when Fbxw7α was silenced in RAW 264.7 macrophages. Furthermore, inhibition of the proteasome by MG132 increased the basal expression of C/EBPδ and revealed its polyubiquitination, which was significantly reduced upon Fbxw7α silencing (Fig. 2b and Supplementary Fig. S3b). In Fbxw7α-silenced cells, MG132 did not further increase C/EBPδ protein levels. In contrast, ectopic FBXW7x further decreased the half-life of C/EBPδ and increased its polyubiquitination (Supplementary Fig. S3c,d). Co-immunoprecipitation assays showed that ectopic and endogenous C/EBPδ physically interacted with FBXW7α (Supplementary Fig. S3e). These results indicate that FBXW7α is required for the ubiquitination and degradation of C/EBPδ in RAW 264.7 macrophages. Accordingly, C/EBPδ binding to its own promoter increased when Fbxw7α was silenced (Supplementary Fig. S3f). Collectively, these data demonstrate a negative feedback loop from FBXW7α to C/EBPδ.

FBXW7-substrate interaction requires a phospho-degron motif, which is also present in C/EBPδ (Fig. 2c). To investigate the role of this motif, we mutated the potential phospho-acceptor residues serine and threonine to alanine (TTS/AAA). Figure 2d shows that FBXW7x decreased the steady-state levels of the ectopic wild-type C/EBPδ but not the TTS/AAA mutant. Co-immunoprecipitation assays revealed that the degron motif of C/EBPδ was necessary for its interaction with FBXW7α (Fig. 2e). Indeed, FBXW7α mediated the polyubiquitination of WT- but not TTS/AAA-C/EBPδ in vitro (Fig. 2f), and the degron motif was required for polyubiquitination in vivo (Fig. 2g). Next, we generated a TTS/DDD mutation to mimic its phosphorylation, and we confirmed that this protein interacted with FBXW7α (Fig. 2h). The half-life of TTS/DDD-C/EBPδ was significantly reduced compared with the stabilization observed with the TTS/AAA mutation (Fig. 2i). In the presence of MG132, WT- and TTS/DDD-C/EBPδ were expressed at similar steady-state levels; these findings corroborated the notion that the low levels of TTS/DDD-C/EBPδ were due to degradation (Fig. 2j). Collectively, these data show that degron-phosphorylation regulates the stability of C/EBPδ.

GSK-3β regulates C/EBPδ protein stability. The serine/threonine kinase GSK-3β is responsible for the phosphorylation of most FBXW7α substrates. Indeed, phospho-threonine could be detected on WT-C/EBPδ expressed in RAW 264.7 cells, but this upon LPS treatment in vivo (Supplementary Fig. S2a,b). An analysis of baseline myeloid haematopoiesis suggests that myeloid development is normal in Cebpd−/− mice (see Supplementary Note 1 and Supplementary Fig. S2c-j). Thus, we conclude that the functional differences detected in the macrophages from Cebpd−/− mice are not due to developmental defects.
phosphorylation was significantly reduced by the GSK-3β inhibitor CHIR or the TTS/AAA mutation (Fig. 3a). Consistent with this result, the GSK-3β inhibitors CHIR or BIO increased the expression of C/EBPδ in PPMs and RAW 264.7 cells (Fig. 3b).

In contrast, the expression of TTS/DDD-C/EBPδ was not increased by CHIR (Fig. 3c). These results suggest a role for the GSK-3β pathway in the regulation of C/EBPδ expression. Indeed, in vitro kinase assays with recombinant activated GSK-3β confirmed that GSK-3β directly phosphorylated C/EBPδ (Fig. 3d). The TTS/AAA mutation significantly reduced C/EBPδ phosphorylation, and phospho-peptide analysis confirmed that GSK-3β targets T156 of the degron (Fig. 3d and Table 1).

Phosphorylation at S160 was also detected but to a much lesser extent. In addition, T49 was phosphorylated by GSK-3β (Fig. 3e) and in the half-life of C/EBPδ (Fig. 3f). Furthermore,
elevated levels of FBXW7 are suggested to have a role in attenuating pro-inflammatory signalling. To test this hypothesis, we expressed FBXW7α in PPMs to mimic the elevated levels of FBXW7α in Cebpd null cells (Supplementary Fig. S4a). FBXW7α suppressed all tested responses of PPMs to LPS, such as the expression of iNOS, Cebpβ, p65, Notch-intracellular-domain (NICD) and COX-2 and the phosphorylation of ERK1/2 and STAT3 (Fig. 4a). Similar data were obtained with RAW 264.7 macrophages (Supplementary Fig. S4b). Furthermore, the transcript levels of Nos2, Cebpd, Il6, Vegfc and Mmp9 were significantly reduced by ectopic FBXW7α in PPMs (Fig. 4b), as was NO production and the glycolytic switch in response to LPS + 1% O2 (Supplementary Fig. S4c,d). These data are reminiscent of the phenotype of Cebpd null cells, which express elevated levels of FBXW7α. The profound suppression of LPS-responses by ectopic FBXW7α suggested that upstream elements in the LPS signalling pathway were downregulated by FBXW7α. Intracellular LPS signalling is

**Figure 3 | C/EBPβ stability is regulated by GSK-3β phosphorylation.** (a) RAW 264.7 cells were transfected with WT- or TTS/AAA-C/EBPβ expression plasmids and treated ± GSK-3β inhibitor CHIR99021 (5 μM, 2h). Cell extracts were immunoprecipitated with anti-C/EBPβ or IgG (with an aliquot of the indicated extract) and the western analysed with anti-phosphothreonine (pT) antibody. (b) Western analysis of NE from PPMs (left panel) or RAW 264.7 cells (right panel) treated with GSK-3β inhibitors CHIR99021 or BIO (6-bromoindirubin-3’-oxime) (5 μM, 2h). β-catenin, which is known to be targeted for degradation by GSK-3β phosphorylation, served as positive control. (c) Western analysis of RAW 264.7 cells transfected with WT- or TTS/AAA-C/EBPβ expression plasmids and treated ± GSK-3β inhibitor CHIR99021 for 2h. (d) GSK-3β phosphorolysates C/EBPβ in vitro. HEK293T cells were transfected with WT- or TTS/AAA-C/EBPβ expression plasmids. C/EBPβ proteins were immunoprecipitated from cell extracts and in vitro kinase assay reactions were carried out in the presence or absence of GSK-3β. Samples were resolved by SDS–PAGE and subjected to autoradiography (top panel). Total radioactivity (bottom panel) incorporated into the C/EBPβ protein was quantified (n=3). Representative input levels of C/EBPβ are shown by western (inset). (e) RAW 264.7 cells were treated ± LPS (4 h) and cell extracts were immunoprecipitated with anti-C/EBPβ or IgG antibodies and western analysis was carried out with anti-phosphothreonine (pT) antibody. Input (2.5% of the lysate) was analysed as indicated. (f) Western analysis (top panel) of RAW 264.7 cells treated ± LPS (100 ng ml⁻¹, 4 h) followed by CHX for the indicated times, and quantification of C/EBPβ normalized to β-actin signal (bottom graph) compared with respective untreated (n=3, P<0.05; **P<0.001; ****P<0.0001). (g) Western analysis of RAW 264.7 cells transfected with HA-GSK-3β-S9A expression plasmids treated ± LPS (4 h) as indicated. (h) Western analysis of RAW 264.7 cells pretreated with the PI3K/AKT kinase pathway inhibitor LY294002 (10 μM, 1h) followed by LPS (100 ng ml⁻¹, 4 h) as indicated. Where applicable, data are mean ± s.e.m., evaluated by two-tailed unequal variance t-test.

ectopic active GSK-3β-S9A27 (Fig. 3g) or pharmacological inhibition of PI3K/AKT (Fig. 3h) reduced LPS-induced C/EBPβ expression. These data show that LPS activates C/EBPβ expression at least in part by inhibition of the GSK-3β/FBXW7α pathway. **FBXW7α regulates TLR4 expression through C/EBPβ.** As FBXW7α targeted C/EBPβ for degradation, FBXW7α could have a role in attenuating pro-inflammatory signalling. To test this hypothesis, we expressed FBXW7α in PPMs to mimic the elevated levels of FBXW7α in Cebpd null cells (Supplementary Fig. S4a). FBXW7α suppressed all tested responses of PPMs to
initiated by TLR4. Indeed, ectopic FBXW7α reduced expression of TLR4 along with C/EBPβ in PPMs (Fig. 4c), while RNAi against Fbxw7α increased the basal and LPS-induced levels of TLR4 and C/EBPβ (Fig. 4d). In addition, several pro-inflammatory markers, such as NICD, iNOS and p65, were induced by Fbxw7α-silencing alone (Fig. 4d). These data prompted the hypothesis that FBXW7α regulates TLR4 expression through C/EBPβ. The depletion of C/EBPβ prevented the upregulation of TLR4 in response to Fbxw7α silencing (Fig. 4e), demonstrating that C/EBPβ mediates TLR4 upregulation. Even basal expression of TLR4 depended on C/EBPβ. Finally, co-expression of degradation-resistant TTS/AAA-C/EBPβ with FBXW7α rescued TLR4 expression in RAW 264.7 macrophages, demonstrating that FBXW7α downregulates TLR4 through the inhibition of C/EBPβ expression (Fig. 4f).

FBXW7α suppresses inflammatory signalling. Increased basal levels of TLR4 and C/EBPβ protein owing to RNAi against Fbxw7α were also observed in RAW 264.7 macrophages, along with increased transcript levels of the pro-inflammatory genes Cebp, Tlr4, Tnfα, Il6, Nos2 and Mmp9 in PPMs (Supplementary Fig. S5a,b). Taking this approach further, we silenced Fbxw7α in vivo by intraperitoneal injection of small interfering RNA (siRNA). In vivo RNAi can cause non-specific effects that include activation of the immune system28. Indeed, control siRNAs led to a modest increase of C/EBPβ expression in PECs compared with vehicle treatment (Supplementary Fig. S5c). In comparison, two Fbxw7α RNAi oligos caused a greater increase in both C/EBPβ and TLR4 protein levels. Following this pilot experiment, Ctrl1 and Fbxw7α1 siRNA were used for subsequent analyses. Peritoneal cells that were isolated 2 days after the injection of Fbxw7α siRNA exhibited reduced FBXW7α levels and higher basal expression of C/EBPβ, TLR4, NICD, p65 and iNOS protein compared with control siRNA (Fig. 4g). In addition, transcripts for Cebp, Nos2 and Il6 were increased (Supplementary Fig. S5d). More cells were recovered from Fbxw7α-siRNA treated mice, indicating the activation of recruitment pathways (Supplementary Fig. S5e). However, the ratios of different PECs was not altered (Supplementary Fig. S5f). Furthermore, Fbxw7α siRNA resulted in detectable levels of plasma IL-6 in otherwise untreated mice and in increased IL-6 concentrations in LPS-treated mice (Fig. 4h). These data show that endogenous FBXW7α is necessary to prevent pro-inflammatory gene expression. RNAi depletion of FBXW7α in PPMs sensitized the cells, such that 1 ng ml⁻¹ LPS elicited a response that was comparable to 10–100 ng in control cells, as measured by the expression of C/EBPβ and p65 and the phosphorylation of ERK and p38 MAP kinase (Fig. 4i). Note that Fbxw7 RNAi increased the basal TLR4 protein levels to LPS-induced levels at this 4 h time point. Taken together, these data show that FBXW7α attenuates the LPS response through inhibition of C/EBPβ and TLR4 expression and that FBXW7α-depletion alone is sufficient to activate inflammatory signalling.

TLR4 is a direct transcriptional target of C/EBPβ. As C/EBPβ promoted TLR4 protein expression, we next addressed the mechanism underlying this regulation. The loss of C/EBPβ in KO PECs or RNAi-depleted RAW 264.7 macrophages reduced Tlr4 mRNA levels (Fig. 5a). Similarly, overexpression of FBXW7α suppressed Tlr4 mRNA levels (Fig. 5b), which was consistent with the induced Tlr4 mRNA levels upon Fbxw7α silencing (Supplementary Fig. S5b). Interestingly, the expression of Tlr2, Tlr4 and Tlsr 5–9 was also reduced in Cebpd-deficient PPMs, while the expression of Tlr1 and Tlr3 was increased (Supplementary Fig. S5g). These data implicate C/EBPβ in the regulation of most Tlr genes. Because of our aforementioned data, we focused our subsequent analyses on TLR4. Inspection of the Tlr4 promoter sequence revealed putative C/EBPβ-binding sites within 200 bp upstream of the transcription start site (Fig. 5c). Chromatin Immunoprecipitation (ChIP) analysis of PPMs demonstrated the binding of C/EBPβ to the proximal Tlr4 promoter region but not to a distal promoter region, where there were no putative binding sites (Fig. 5c). Consistent with these data, Cebpd RNAi or FBXW7α overexpression both reduced the activity of a Tlr4 promoter-luciferase reporter construct (Fig. 5d). Next, we assessed the effect of TLR4 reconstitution in Cebpd null PPMs (Fig. 5e). Overexpression of TLR429 in WT PPMs had no significant effect on the LPS-induced expression of Nos2 and Il6. In Cebpd-deficient macrophages, however, ectopic TLR4 significantly enhanced LPS-induction of Nos2 and Il6 transcripts (Fig. 5f). C/EBPβ binds the Il6 promoter30 and may regulate the iNOS promoter directly31. Our data show that the impaired LPS response of Il6 and Nos2 in Cebpd null macrophages is in part due to reduced TLR4 levels, and it is less owing to the role of C/EBPβ as a downstream effector of TLR4. The role of basal C/EBPβ expression was further supported by an analysis of early LPS signalling events. The accumulation of p65 and the phosphorylation of ERK, p38 and JNK kinases in response to LPS were attenuated in Cebpd null PPMs within 30–60 min of treatment (Fig. 5g). In summary, these results show that C/EBPβ also functions upstream of LPS signalling through activation of Tlr4 gene expression.

C/EBPβ augments inflammatory signalling in tumours. TLR4 is expressed in both macrophages and tumour cells4. Proteins such as HMGB1 and S100A8 act as ligands that activate TLR4 signalling, and these ligands are important in tissue repair, inflammatory diseases and cancer3,31,32. Inflammation-associated gene expression is strongly correlated with tumour malignancy33. Given that C/EBPβ promotes metastatic progression of MMTV-Neu mammary tumours13, we investigated whether C/EBPβ modulates TLR4 expression in tumour cells. Stable depletion of C/EBPβ in a mouse mammary tumour cell line or in human MCF-7 breast tumour cells reduced TLR4 protein expression and induced FBXW7α levels (Fig. 6a). Analyses of MMTV-Neu tumour tissue confirmed the reduced Tlr4 mRNA, increased Fbxw7 mRNA, and, on average, lower TLR4 protein levels in Cebpd⁻/⁻ tumours compared with WT (Fig. 6b). In addition, iNOS protein expression (Fig. 6b) and Il6, Nos2, Arg1 and Tnfα transcript levels were lower in Cebpd⁻/⁻ tumours (Fig. 6c). Interestingly, the transcript levels of Il10 and Il13, which are expressed in cells including alternatively activated macrophages and T cells, were significantly higher in Cebpd⁻/⁻ tumours (Fig. 6c). The inverse correlation of Cebpd and Il10 expression in vivo is consistent with a previous report on C/EBPβ functions in dendritic cells12.

We also examined the expression of chemokines and chemokine receptors, which have an important role in breast tumour progression and metastasis34. Cebpd KO tumours

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**Table 1** Sites of C/EBPβ phosphorylated by GSK-3β.

| Fractions | Edman degradation | Peptide | Phosphoamino acid |
|-----------|-------------------|---------|-------------------|
| 6-7       | 4                 | LGSIPeTPAMY | Tα9          |
| 68-70     | 7 (1)             | LAAAAPeTPFTSPEPGRGSPGSPL | Tα5 (S)β600 |

To identify the phosphorylation sites, labelled WT- and TSS/AAA-C/EBPβ were subjected to in-gel digestion with pepstatin followed by HPLC analysis. Edman degradation and phosphoamino acid analysis was performed on the indicated fractions.
Figure 4 | FBXW7α suppresses TLR4-mediated LPS responses through C/EBPδ. (a) Western analysis of NE from PPMs transfected with vector or FBXW7α expression constructs and treated with LPS (100 ng ml⁻¹, 16 h) as indicated. (b) RT-qPCR of the indicated mRNA levels in PPMs nucleofected with FBXW7α expression plasmids and treated with LPS (100 ng ml⁻¹, 16 h) as indicated (n = 3, *P < 0.05, **P < 0.001, ****P < 0.0001). (c) Western analysis of PPMs transfected with HA-FBXW7α and treated with LPS (100 ng ml⁻¹, 16 h) as indicated. SE, short exposure; LE, long exposure. (d) Western analysis of proteins from PPMs nucleofected with control or αFbxw7a RNAi: against Fbxw7α or control siRNA. (e) Western analysis of RAW 264.7 macrophages transfected with siRNA oligos against endogenous Cebpδ or Fbxw7α alone or in combination and treated with LPS (100 ng ml⁻¹, 16 h) as indicated. The line separates analysis of cytoplasmic (top) and nuclear (bottom) extracts. (f) Western analysis of RAW 264.7 macrophages transfected with the indicated expression plasmids. (g) Western analysis of PECs isolated from FVB/N mice 48 h after injection of RNAi against Fbxw7α or control siRNA. (h) Plasma IL-6 concentrations from mice injected with RNAi against Fbxw7α or control siRNA for 72 h followed by LPS (40 ng) or vehicle (saline) for 1 h. The horizontal line indicates the median IL-6 concentration (n = 4–5, P values were determined by the Wilcoxon rank-sum test, P < 0.001). (i) Western analysis of PPMs transfected with control or Fbxw7α siRNA oligos and treated with LPS (100 ng ml⁻¹, 4 h) as indicated. SE, short exposure; LE, long exposure. Where applicable, data are mean ± s.e.m., evaluated by two-tailed unequal variance t-test (except panel 4 h).
exhibited significantly reduced expression of the metastasis-promoting gene Cxcr4, which was consistent with our previous report that C/EBPδ directly regulates Cxcr4 in cultured mammary tumour cells. In contrast, C/EBPδ-null tumours exhibited increased expression of Ccl3 and Ccl5, which augment T-cell mediated anti-immune responses (Supplementary Fig. S6). Of these genes, the Ccl3 gene promoter is directly bound by C/EBPδ after LPS induction. Collectively, these data show that the loss of C/EBPδ leads to complex alterations of pro-and anti-inflammatory genes in mammary tumour tissue and that this complexity may be owing to its multifaceted roles in macrophages and mammary epithelial cells.

Macrophages and tumour cells engage in crosstalk, and a metastasis-promoting paracrine loop has been described with breast carcinoma cells producing colony stimulating factor-1 (Csf1) and macrophages expressing epidermal growth factor (EGF). We found that both Csf1 and Egf expressions were significantly reduced in Cebpd null MMTV-Neu mammary tumours (Fig. 6d). Though further analyses will be required to dissect the contribution of different cell types to these observations, the results are likely due to C/EBPδ action in both the immune cells and tumour cells. In summary, these data observations, the results are likely due to C/EBPδ action in both the immune cells and tumour cells. In summary, these data collectively create a largely pro-inflammatory microenvironment in mammary tumours.

**Discussion**

In this study, we identified a positive feedback loop between C/EBPδ and TLR4 and a negative feedback loop between C/EBPδ and FBXW7, which together modulate TLR4 signalling and...
pro-inflammatory gene expression (Fig. 7). Phosphorylation of C/EBPβ by GSK-3β is required for its degradation by FBXW7α. Therefore, inhibition of GSK-3β by LPS stabilizes C/EBPβ. Identification of TLR4 as a direct transcriptional target of C/EBPβ renders C/EBPβ a pro-inflammatory factor upstream of TLR4 in addition to its functions downstream.

Macrophages can be activated by several pathways, and C/EBPβ together with C/EBPδ also participates in Fcγ receptor-mediated inflammatory cytokine and chemokine production and in IgG IC-stimulation of macrophages9. In addition to our data, the regulation of Tlr8 expression by C/EBPδ as well as its binding to the Tlr6 gene promoter have been reported10,38. A critical role of C/EBPβ in LPS responses has previously been shown in vitro and in vivo. Cebpd null mice are hypersensitive to persistent bacterial infection10 and hyposensitive to septic shock after sensitization11. Both phenotypes were attributed to the role of C/EBPδ as an inflammatory response gene and regulator of target genes such as Il6. Furthermore, the role of C/EBPδ in amplifying LPS signalling has been described39. It should be noted that one study reported that C/EBPδ is dispensable for LPS-induced Il6 expression40. It remains to be determined which experimental details are responsible for the difference in results.

Our findings place C/EBPδ upstream of LPS signalling for expression of the TLR4 receptor. The loss of C/EBPδ does not abolish Tlr4 expression entirely, which explains why LPS responses are not completely impaired. Interestingly, our data from reconstituting Cebpd−/− cells with ectopic TLR4 suggest that the precise role of C/EBPδ downstream of TLR signalling should be re-evaluated in light of its role in regulating TLR4 expression. Low dose LPS specifically induces C/EBPδ expression rather than NF-κB41, supporting the notion that C/EBPδ is critical in sensitizing cells to LPS.

Our data show that C/EBPδ promotes macrophage activation in part by augmenting HIF-1α expression. Inflamed tissue is hypoxic and HIF-1 mediates hypoxia adaptation by regulating the transcription of many genes associated with angiogenesis, glycolysis and migration10. C/EBPδ also promotes tumour lymphangiogenesis through HIF-1α42. Our results show that macrophage functions that require HIF-1 are blunted in the absence of C/EBPδ because of Fbxw7α derepression. HIF-1α expression in the myeloid lineage also promotes the differentiation of myeloid-derived tumour suppressor cells, which contribute to tumour progression17,18,43. This mechanism may underlie the pro-metastatic function of C/EBPδ in addition to the role of this pathway in epithelial-derived tumour cells13.

Figure 6 | Schematic describing the feedback loops between TLR4, C/EBPδ and FBXW7α that control LPS signalling. The shaded boxes indicate the elements of this pathway that were identified in this study.
Interestingly, TLR4 is also a target of HIF-1 (ref. 44); hence, C/EBPβ induces TLR4 expression directly under normoxia and also indirectly through HIF-1 induction under hypoxia. This effect provides an additional positive feedback loop because C/EBPβ is a hypoxia-induced gene that is likely downstream of HIF-1 (ref. 13,42). However, this pro-inflammatory loop requires simultaneous inhibition of FBXW7α expression by C/EBPβ, suggesting that FBXW7α serves as an important brake on inflammatory signalling.

In this study, we found that Cebpd-deficient mouse mammary tumour tissues, which exhibit reduced metastatic progression13, express increased FBXW7α and reduced TLR4 levels. The effects of TLR4 signalling on cancer appear complex and may depend not only on the cell type but also on the stage of tumour development57,45–48. In our study, reduced TLR4 expression in Cebpd null tumours correlated with mostly reduced pro-inflammatory and increased anti-inflammatory gene expression. This result may be due to the role of C/EBPβ in mammary tumour cells and in inhibiting immune cells, directly targeting by C/EBPβ, or directed downstream effects. Interestingly, reduced innate immune responses in Cebpd null mice are also consistent with the increased mammary tumour multiplicity in these mice13.

Our data warrant further dissection of the role of C/EBPβ in tumour-associated macrophages and their crosstalk with mammary tumour cells, which will be addressed by conditional gene deletion in future analyses.

From this study, FBXW7α emerged as a potent attenuator of inflammatory signalling. This activity is at least in part due to suppression of C/EBPβ expression at the protein and mRNA levels and is likely to affect not only Cebpd but also other genes/proteins that modulate inflammation. Therefore, our data lay the groundwork for further analyses of FBXW7α functions in the modulation of immune cells. We also suggest that the tumour suppressor activity of FBXW7α14 could be in part due to its role as an attenuator of pro-inflammatory gene expression. According to the ‘1000 Genomes’ catalogue (www.1000genomes.org), the FBXW7 gene harbours several SNPs, some with possibly deleterious effects on function. We suggest that these SNPs be included in genome-wide association studies of inflammatory diseases. Given the role of FBXW7α as a suppressor of inflammatory signalling (as shown in this study) and as a bona fide tumour suppressor14,15, FBXW7α is an unlikely therapeutic target. However, better knowledge of the regulation of its expression and its target proteins may provide new avenues for the management of inflammation-associated diseases.

Mice and isolation of peritoneal cells. Cebpd wild-type and knockout mice19 were of the FVB/N strain background (except for data in Supplementary Fig. 52, which are from 129S1 mice) and derived from heterozygous mates. The MMTV-C-C/EBPα tumour model has been described13,19. The subjects were littermates whenever possible. NCI-Frederick (FNLCR) is accredited by AAALLC International and follows the Public Health Service Policy for the care and use of laboratory animals. All experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee.

For the isolation of PPMs, mice were injected with 3% Brewer thioglycollate medium19 in the peritoneal cavity. Four days later, mice were killed, and 1 ml sterile PBS (Ca2+/Mg2+-free) was injected into the peritoneal cavity. The resulting peritoneal fluid was centrifuged and centrifuged for 10 min at 4 °C. The cell pellet was washed once with PBS. Erythrocytes were lysed with sterile water, and the final pellet was suspended in 1:1 DMEM/F12 medium with 10% foetal bovine serum (FBS). Viability was >95%. Cell preparations were characterized by FACs analysis using antibodies that distinguish macrophages from other hematopoietic cells. On average, 82.5 ± 3.7% (mean ± s.e.m., n = 6) of the cells were Mac-1+ and F4/80− before plating. The isolated cells were plated and allowed to adhere for 2 h. Non-adherent cells were washed off with PBS, and new culture medium was added. Cells were cultured for 24 h before experimental treatments.

Resident PECs were isolated as described above but without prior elicitation by thioglycollate. For details, see Supplementary Methods.

Cell culture. MMTV-Neu and MCF-7 cell lines with stable depletion of C/EBPβ or (or green fluorescent protein (GFP) as a control)52. Cells were selected in G418 and maintained as pools. ANA-1, RAW 264.7 mouse macrophages and HEK293T cells were cultured in DMEM containing 10% FBS, PPMs and PECs were cultured in DMEM containing 10% FBS. The U-937 human monocytic cell line and elutriated primary human monocytes were cultured in RPMI medium containing 5% FBS. The MMTV-Neu mouse mammary tumour cell line (a kind gift of Dr William Muller, McGill University) was cultured in DMEM containing 5% FBS and 1X-MEGS (mammary epithelial cell growth supplement). Unless indicated otherwise, LPS was used at 100 ng ml−1 for 16–24 h.

Peripheral blood-derived monocytes were isolated from healthy donors by counterflow centrifugal elutriation under protocols approved by the Institutional Review Boards of both the National Institute of Allergy and Infectious Diseases and the Department of Transfusion Medicine of the National Institutes of Health after appropriate informed consent.

Transient transfections and RNAi. Cells were transfected by nucleofection using the Amaxa Cell-line Nucleofector Kit V (Cat no. V-1903; Lonza AG). A GFP expression construct was included in all transfections to monitor transfection efficiency. The total amount of DNA in each transfection was kept constant by complementation with vector control DNA. All control samples were transfected with vector only. At 24 h post-transfection, cells were treated as indicated. Cebpd siRNAs were purchased (no. L-003109-00) from Dharmacon (L-003109-00). Fbxw7-specific siRNAs (Silencer predesigned) with the following sequences were used13,51.

Fbxw7 RNAi-1 sense: 5'-GGGCCAGCAGCGGCGGAGGAdTdT-3' and antisense: 5'-UCUCCGCCGCGCCGCGGAdTdT-3'. Fbxw7 RNAi-2 Sense: 5'-GCACAGAAAUACAGAAGCATCT-3' and antisense: 5'-GUUUAUCAGAUUUCCCAGGCT-3'. Fbxw7 RNAi-3 sense: 5'-GUGACAGCAUACAGACACCTC-3' and antisense: 5'-UCUACUCCAAACCCACAGGATC-3'. Fbxw7 RNAi-4 Sense: 5'-GCACAGAAAUACAGAAGCATCT-3' and antisense: 5'-GUUUAUCAGAUUUCCCAGGCT-3'. For silencing Fbxw7 in mouse cells, Fbxw7 RNAi-1 (in vitro and in vivo) and RNAi-2 (in vivo) were used. Fbxw7 RNAi-3 and Fbxw7 RNAi-4 were used at a 1:1 ratio in human cells.

Scrambled siRNA (no. D-009160-01-05, Dharmacon) or EGFP siRNA51 (5'-CAAGCTGACCTGAAATTC-3') were used as controls. For in vivo RNAi, mice were injected in the peritoneum with in vivo-jetPEI (Polyplus) according to the manufacturer’s instructions. For details see Supplementary Methods.

Western analysis and in vitro ubiquitination assay. See Supplementary Methods.

Pulse-chase experiment. RAW264.7 cells were transfected with control or Fbxw7α siRNA oligonucleotides. Two days later, the cells were pre-incubated for 30 min in DMEM without methionine and cysteine, pulsed with Tran35S-label (ICN; 300 μCi ml−1; 1 μCi = 37 kBq) for 20 min, and chased with DMEM/10% FBS plus 20 μM methionine and cysteine for the indicated times. Cells were lysed under denaturing conditions, and proteins were immunoprecipitated with specific antibodies and protein G beads. After SDS-PAGE, the dried gel was processed for phosphorimaging. Signals were quantified by ImageQuant software and plotted using GraphPad Prism 5.

Plasma IL-6 measurement. FVB/N mice were injected intraperitoneally with control or Fbxw7α siRNA (100 μg) using in vivo-jetPEI (Polyplus) according to the

Methods

Reagents and antibodies. LPS (from E. coli, L4524) was purchased from Sigma-Aldrich, St Louis, MO. CHIR99021 and BIO (6-Bromomidurin-3′-oxime) were obtained from Stemgent, San Diego, CA. Antibodies were obtained from the following sources: Cell Signaling Technology (pGSK-3β Ser9, no. 9336; PAK-Ser73, no. 4060; Thr389, no. 9205; pSTAT3, no. 9145; Akt, no. 4691; GSK-3β, no. 9316; PKK1, no. 9202; STAT3, no. 4094; Cleaved Notch-1, no. 2412S; phospho-p44/42 MAPK-Thr202/Tyr204 (Erk1/2), no. 9101; p44/42 MAPK (Erk1/2), no. 9102; p38-Thr180/Tyr182, no. 92158; p38, no. 9212; p44/p42 MAPK/JNK-Thr183/Tyr185, no. 46688; SAPK/JNK, no. 9238; phospho-threonine, no. 93863); Abcam (INOS, no. ab12352; F4/80, no. ab26343-100; Cox-2, no. ab-15191; p65 (RelA); no. ab-16502; FBXW7, ab no. 1229D); BD Pharmingen (CD11b (M170), no. 550993; GR1, no. 553128; CD16/CD32, no. 533142); Novus Biologicals (HIF-1α, no. NB100-449; HIF-1 β, no. NB-100-124); Santa Cruz (actin, sc-1616; Ubiquitin, sc-8017); Calbiochem (mTOR, no. OP97); Orbigen (FBXW7alpha, no. PAB-10563); BD Biosciences (Aurora A, no. 610938); Imgenex (TLR4, no. IMG-578A); Bethyl Laboratories (H2AX, no. A030-083A); Ebscission (B220, no. RA3-682; CD3e, no. 500A-2; and isotype controls); Roche (HA, no. 11867423001; clone3F10); and Rockland ( Tubulin, no. 600-101-800). The mouse monoclonal antibody clone L46-743-92.69 (batch DS9319) against C/EBPβ was provided by BD Biosciences Pharmingen as an outcome of an Antibody Co-development Collaboration with the NCI.

For information on plasmids see Supplementary Methods.
manufacturer's instructions. Three days later, mice were injected with LPS (40 ng) or vehicle (saline) and killed 1 h later to collect hepatopancreatic blood. IL-6 was measured in plasma using a mouse IL-6 single analyte ELISA kit according to the manufacturer's instructions (SA Biosciences, Qiagen, USA, no. SEM03015A).

RNA isolation and quantitative real-time PCR. RNA was isolated using TRIzol (Invitrogen), and cDNA was synthesized with SuperScript reverse transcriptase III (RT) according to the manufacturer's instructions (Invitrogen, CA). PCR was performed using Taqman gene expression primer/probe sets using the 7500 Fast Real-Time PCR instrument (Applied Biosystems). Analysis was performed using the 2^-AUCt method normalized to β-actin. The probe sets (Applied Biosystems) were as follows:

**Cebp**

\[
\text{Mm}0078671_{-1:1}, \text{Fbxw7} 7_{-1:1}, \text{mCsf-1} 7_{-1:1}, \text{Vegfc} 7_{-1:1}, \text{C176} 7_{-1:1}.
\]

**ChIP**

\[
\text{Mm}00442991_{-1:1}, \text{Ccl3} 0_{-1:1}, \text{Ptgds} 0_{-1:1}, \text{Cxr4} 0_{-1:1}, \text{Tlr4} 0_{-1:1}, \text{Zbrk1} 0_{-1:1}.
\]

**Luciferase reporter assay**

RAW 264.7 cells were transfected with TLR4 promoter luciferase reporter constructs\(^2\), renilla luciferase expression plasmids along with pRL-CMV (Promega) and firefly luciferase expression plasmids. luciferase activity was assayed using a luciferase assay kit according to manufacturer's instructions (Promega).

Metabolic measurements. Measurements of lactate, glucose, ATP and NO were as described in Supplementary Methods.

**Statistical analysis.** Unless stated otherwise, quantitative data were analysed by the two-tailed unequal variance t-test and are shown as the mean ± s.e.m. The number of samples (n) refers to biological replicates.

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Author contributions

K.B., E.S., K.D.K., J.R.K. and D.K.M. designed experiments. K.B., S.S., Y.Z., K.D.K. and E.S. wrote the paper. G.S., V.C., and T.R. provided reagents, assistance, and/or advice, and Jiro Wada and Allen Kane for preparation of the figures. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

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