

# TPL2-mediated activation of ERK1 and ERK2 regulates the processing of pre-TNF $\alpha$ in LPS-stimulated macrophages

Simon Rousseau<sup>1,\*</sup>, Matoula Papoutsopoulou<sup>2</sup>, Antony Symons<sup>2</sup>, Dorthe Cook<sup>3</sup>, John M. Lucocq<sup>4</sup>, Alan R. Prescott<sup>4</sup>, Anne O'Garra<sup>3</sup>, Steven C. Ley<sup>2</sup> and Philip Cohen<sup>1,\*</sup>

<sup>1</sup>MRC Protein Phosphorylation Unit, College of Life Sciences, University of Dundee, Dow Street, Dundee, DD1 5EH, UK

<sup>2</sup>Division of Immune Cell Biology, National Institute for Medical Research, Mill Hill, London, NW7 1AA, UK

<sup>3</sup>Division of Immunoregulation, National Institute for Medical Research, Mill Hill, London, NW7 1AA, UK

<sup>4</sup>Division of Cell Biology and Immunology, College of Life Sciences, University of Dundee, Dow Street, Dundee, DD1 5EH, UK

\*Authors for correspondence (e-mails: s.rousseau@dundee.ac.uk; p.cohen@dundee.ac.uk)

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## Summary

Activation of the TPL2-MKK1/2-ERK1/2 signalling pathway is essential for lipopolysaccharide (LPS)-stimulated production of TNF $\alpha$  in macrophages. Here, we demonstrate that, unexpectedly, TPL2-deficient or MKK1-inhibited macrophages produce near normal levels of pre-TNF $\alpha$  when TLR2, TLR4 and TLR6 are activated by their respective agonists, but fail to secrete TNF $\alpha$ . We show that LPS stimulates the appearance of pre-TNF $\alpha$  at the cell surface and that this is prevented by inhibition of MAPK kinases 1 and 2 (MKK1/2) or in TPL2-deficient macrophages. However, the transport of pre-TNF $\alpha$  from the Golgi to the plasma membrane is

unaffected by inhibition of the TPL2-MKK1/2-ERK1/2 pathway. Finally, we show that TACE, the protease that cleaves pre-TNF $\alpha$  to secreted TNF $\alpha$ , is phosphorylated by ERK1 and ERK2 (ERK1/2) at Thr735 in LPS-stimulated macrophages. Therefore, although TACE activity per se is not required for the LPS-stimulated cell surface expression of pre-TNF $\alpha$ , the phosphorylation of this protease might contribute to, or be required for, the cell surface expression of the pre-TNF $\alpha$ -TACE complex.

Key words: COT, TNF, MAP kinase, TACE, TLR

## Introduction

Tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) is a key player in inflammatory disorders (Choy and Panayi, 2001; Russo and Polosa, 2005), and agents that bind to and inactivate TNF $\alpha$  in the circulation have been approved for the treatment of these diseases. These drugs have had a major impact on the treatment of rheumatoid arthritis (RA) but they are expensive, have to be injected and are only partially effective (only have the desired effect in 50% of patients) (Feldmann et al., 2005). For these reasons, there is undiminished interest in identifying novel, orally active compounds that block the production of TNF $\alpha$ .

Macrophages are a major site of TNF $\alpha$  synthesis. Upon stimulation by bacterial or viral pathogens, Toll-like receptors (TLRs) are engaged, triggering the activation of numerous signalling programmes that lead to the secretion of TNF $\alpha$  into the circulation (Janssens and Beyaert, 2003). For example, the engagement of TLR4 by bacterial lipopolysaccharide (LPS) triggers activation of TPL2 (also called COT or MAP3K8), which activates the mitogen-activated protein kinase kinases MKK1 (MAP2K1) and MKK2 (MAP2K2), which in turn switch on the extracellular signal regulated kinases ERK1 and ERK2 (ERK1/2) (Dumitru et al., 2000; Eliopoulos et al., 2003). The analysis of TPL2-deficient murine macrophages has established that this protein kinase is required for the LPS-stimulated activation of ERK1/2 and is not required for other signalling pathways (Dumitru et al., 2000). Moreover, retroviral transduction of the wild-type, but not a catalytically inactive mutant of, TPL2 into TPL2-deficient cells rescued both the activation of ERK1/2 and the secretion of

TNF $\alpha$  (Robinson et al., 2007). It has been reported that TPL2 regulates TNF $\alpha$  production at a post-transcriptional level by promoting the export of *Tnfa* mRNA from the nucleus (Dumitru et al., 2000).

Here, we found that, unexpectedly, the TPL2 signalling pathway is crucial for the processing of pre-TNF $\alpha$  to the secreted form of TNF $\alpha$ .

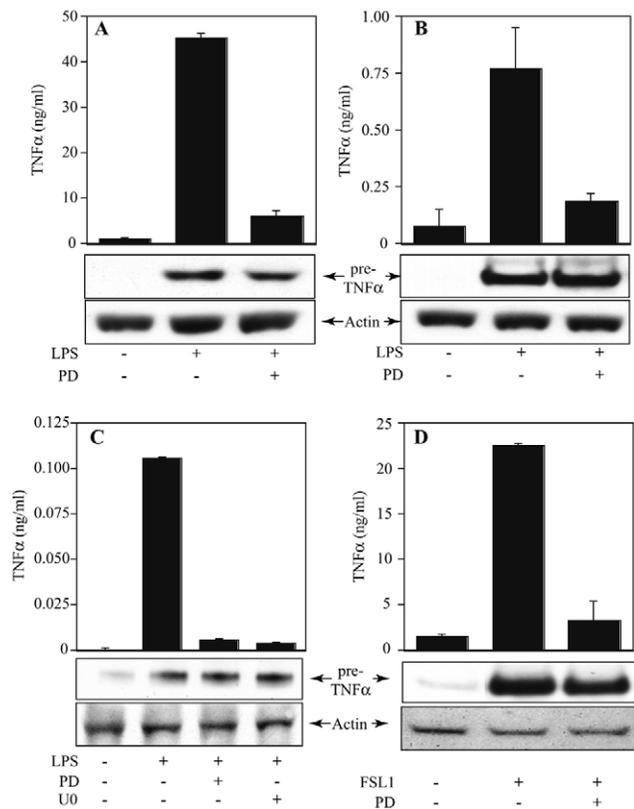
## Results and Discussion

ERK1/2 regulate TNF $\alpha$  secretion with minimal effect on pre-TNF $\alpha$  production

ERK1/2 are the only known substrates of their activators MKK1/2. We therefore assessed the role of ERK1/2 in TNF $\alpha$  production by using two structurally distinct inhibitors of MKK1, namely PD 184352 (Davies et al., 2000; Sebolt-Leopold et al., 1999) and U0126 (Davies et al., 2000; Favata et al., 1998). These compounds are both specific MKK1 inhibitors and do not affect the 70 other protein kinases that we have tested in vitro (Bain et al., 2007). In cells, PD 184352 also inhibits the MKK5-catalysed activation of ERK5, although at concentrations 10- to 20-fold higher than those required to suppress the activation of ERK1/2 (Mody et al., 2001). By contrast, U0126 suppresses the activation of ERK1/2 and ERK5 at similar concentrations (Kamakura et al., 1999). The concentration of PD 184352 used here was sufficient to prevent the activation of ERK1/2, but not ERK5. In the experiments described below, the efficacy of the inhibitors to block the activation of ERK1 and ERK2 was checked in each experiment (results not shown).

We initially studied the amount of TNF $\alpha$  secreted into the cell culture medium following a 4-hour exposure of murine RAW264.7 (hereafter called RAW) macrophages to LPS, and found that inhibiting the activation of ERK1/2 caused >80% inhibition (Fig. 1A). TNF $\alpha$  is synthesised as a 26-kDa precursor, termed pre-TNF $\alpha$ , which is then cleaved to produce the mature secreted 17 kDa form, termed TNF $\alpha$ . To determine whether ERK1/2 regulates the synthesis and/or processing of pre-TNF $\alpha$ , we subjected cell lysates to immunoblotting. Surprisingly, inhibiting the activation of ERK1/2 reduced LPS-induced production of pre-TNF $\alpha$  only slightly (Fig. 1A) and, consistent with this finding, there was only a small, transient reduction in the level of mRNA encoding pre-TNF $\alpha$  (results not shown). These findings implied that ERK1/2 play an important role in regulating the maturation of pre-TNF $\alpha$  prior to TNF $\alpha$  secretion.

To establish the physiological relevance of this data, we next determined the effect of MKK1/2 inhibition on TNF $\alpha$  production by primary bone-marrow-derived murine macrophages (BMDM) (Fig. 1B) and human monocytes/macrophages (Fig. 1C).



**Fig. 1.** Effect of protein kinase inhibitors on TLR-induced production of intracellular and extracellular TNF $\alpha$  in murine macrophages. (A) RAW cells were pre-treated for 60 minutes with or without 2  $\mu$ M PD 184352 (PD) and then either left untreated or were stimulated for 4 hours with 100 ng/ml LPS. The cell culture supernatants were collected and the levels of TNF $\alpha$  analysed using the beadlyte system (histogram). The cell lysates (30  $\mu$ g protein) were subjected to SDS-PAGE and, after transfer to nitrocellulose, the membrane was probed with a murine anti-TNF $\alpha$ -specific antibody or an anti-actin antibody (lower panel). (B) As in A, except that BMDM were used instead of RAW cells. (C) As in A, except that primary human monocytes-macrophages were used instead of RAW cells and 10  $\mu$ M U0126 (U0) was used as an additional MKK1 inhibitor. (D) As in A, except that cells were stimulated for 4 hours with 100 ng/ml FSL1. The results are shown as mean  $\pm$  s.e.m. for three (A,B) or two (C,D) separate experiments.

Consistent with the RAW cell experiments, inhibiting the activation of ERK1/2 had little effect on induction of pre-TNF $\alpha$  by LPS in primary macrophages, although secretion of TNF $\alpha$  was reduced by >90% (Fig. 1). Moreover, a similar effect was observed when RAW macrophages were treated with a synthetic lipoprotein (FSL1) that mimics the effect of bacteria to activate TLR2 and TLR6 (Fig. 1D).

### Regulation of TNF $\alpha$ synthesis in TPL2-deficient macrophages

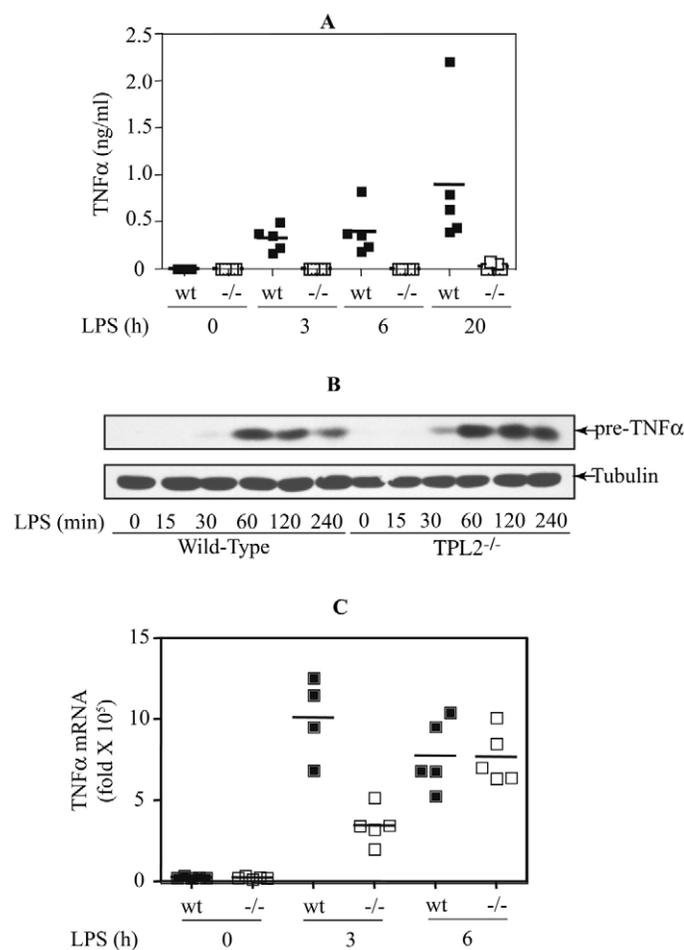
To investigate further the role of ERK1/2 in the regulation of TNF $\alpha$  production, we generated BMDM from TPL2-deficient mice, which are unable to activate MKK1/2 and hence ERK1/2 in response to LPS (see Introduction). As observed previously (Dumitru et al., 2000) and consistent with the effects of PD 184352 (Fig. 1), LPS-induced TNF $\alpha$  secretion was abolished in BMDM from TPL2-deficient mice but not from control wild-type littermates (Fig. 2A). Moreover, as found for PD-184352-treated wild-type cells, the LPS-induced synthesis of pre-TNF $\alpha$  was similar in TPL2-deficient macrophages and those from littermate controls (Fig. 2B). Consistently, TPL2 deficiency did not affect the level of mRNA encoding pre-TNF $\alpha$  induced after 6 hours of LPS stimulation, although a transient inhibition was observed at earlier times (Fig. 2C). These data again suggested a major role for the TPL2-MKK1/2-ERK1/2 pathway in controlling the maturation of pre-TNF $\alpha$  to TNF $\alpha$ , although an additional, more minor, contribution of this pathway to the production of pre-TNF $\alpha$  at the level of transcription/translation could also be observed.

### Preventing the activation of ERK1/2 blocks the expression of pre-TNF $\alpha$ at the cell surface

The processing of pre-TNF $\alpha$  is thought to involve its movement from the Golgi complex to the cell surface and its cleavage by the TNF $\alpha$ -converting enzyme (TACE), also called a disintegrin and metalloprotease 17 (ADAM17) (Moss et al., 1997). To address the mechanism by which ERK1 and/or ERK2 regulate the processing of pre-TNF $\alpha$ , we examined the intracellular localisation of TNF $\alpha$  by immunogold electron microscopy. These studies revealed that, following LPS-stimulation, TNF $\alpha$  was present in the Golgi apparatus, the endocytic pathway, the secretory pathway and the plasma membrane (Fig. 3A-C), as expected from earlier studies (Murray et al., 2005). Quantitation of the data showed that blocking the activation of ERK1/2 with PD 184352 had no significant effect on the intracellular localisation of TNF $\alpha$  (Fig. 3D), implying that ERK1/2 do not regulate movement of TNF $\alpha$  via the endocytic or secretory pathways. Consistent with this conclusion, we were unable to see any effect of LPS on the level of expression of proteins known to be involved in vesicle fusion, such as VAMP3, syntaxin 4 and RAB11 (Murray et al., 2005) (results not shown).

The experiments described above suggested that the regulatory step(s) controlled by ERK1 and/or ERK2 might occur at the cell surface and we therefore examined the cell surface expression of pre-TNF $\alpha$  in intact cells by FACS analysis. These studies showed that 90 minutes of LPS-stimulation induced a transient expression of pre-TNF $\alpha$  at the cell surface in wild-type, but not in TPL2-deficient, macrophages (Fig. 4A). This localisation was transient and had disappeared after 5 hours (results not shown), presumably because pre-TNF $\alpha$  was progressively cleaved to TNF $\alpha$  during this period.

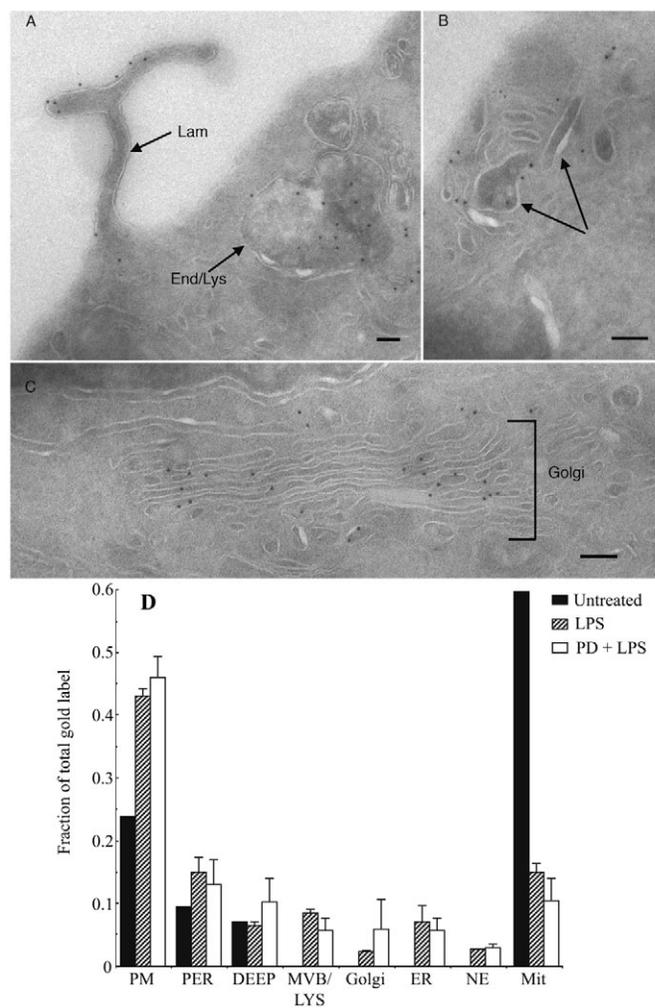
As a control, cells were pre-treated with brefeldin to prevent transport from the Golgi and permeabilised to allow for



**Fig. 2.** The TPL2 pathway regulates TNF $\alpha$  production mainly at the post-translational level. (A) BMDM from wild-type (wt, black squares) and TPL2-deficient (-/-, white squares) mice were either left untreated or were stimulated with 10 ng/ml LPS for the times (hours) indicated. The levels of TNF $\alpha$  in the cell culture supernatant were analysed by standard ELISA. Each square represents a determination using a different mouse. (B) BMDM were lysed in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 100 nM okadaic acid, 2 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, a mixture of protease inhibitors and 1% Nonidet-P 40. Lysates from wild-type and TPL2-deficient mice were either left untreated or were stimulated with 100 ng/ml LPS for the times (minutes) indicated, and were immunoblotted for pre-TNF $\alpha$  as in Fig. 1 or for tubulin. (C) The experiment was carried out as in A except that total RNA was purified from the lysates, reverse transcribed and the amount determined by real-time quantitative PCR.

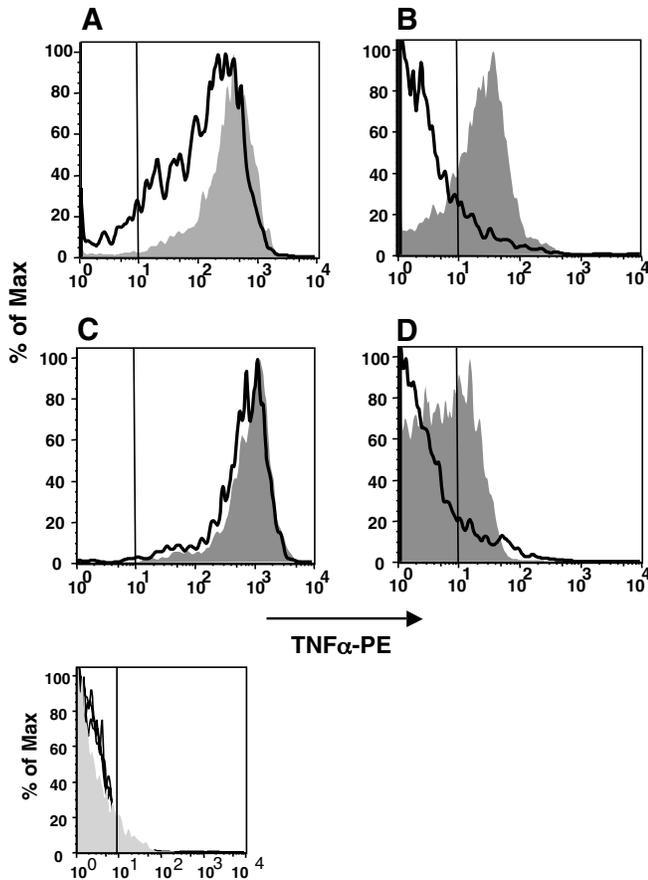
intracellular staining of TNF $\alpha$ . Consistent with the immunoblotting results (Fig. 2), the expression of pre-TNF $\alpha$  was similar in LPS-stimulated macrophages from TPL2-deficient and wild-type mice (Fig. 4B).

We next carried out immunofluorescence staining in the presence of an inhibitor of TACE to allow pre-TNF $\alpha$  to accumulate to levels that can be visualised easily (Murray et al., 2005). Under these conditions, pre-TNF $\alpha$  could be observed at the surface of non-permeabilised cells that had been stimulated with LPS for 4 hours (Fig. 5B), but not in unstimulated cells (Fig. 5A). Moreover, this LPS-induced accumulation of pre-TNF $\alpha$  at the cell surface was prevented by inhibition of MKK1/2 (Fig. 5C), consistent with the results obtained by FACS analysis of wild-type and TPL2-deficient macrophages (Fig. 4B). By contrast, the LPS-induced TNF $\alpha$  signal



**Fig. 3.** LPS-induced pre-TNF $\alpha$  intracellular distribution is not affected by preventing ERK1/2 activation. (A-D) RAW cells were pre-treated for 60 minutes with 10  $\mu$ M of the TACE inhibitor TAPI-1, with or without 2  $\mu$ M PD 184352, and then either left untreated or stimulated for 4 hours with 100 ng/ml LPS. The medium was removed, the cells fixed, and thawed cryosections prepared as described in Materials and Methods. The sections were labelled with antibodies against TNF $\alpha$  followed by species-specific antibodies and protein-A gold. Micrographs in A-C are taken from the most intensely labelled cells to illustrate the structure of the labelled compartments detected by quantitative methods (D). (A) Labelling is located over lamellipodia-like (Lam) structures and endosome/lysosome profiles (End/Lys); (B) labelling is concentrated in putative peripheral endosomes with pleiomorphic tubulovesicular morphology (arrows); (C) labelling is found over a stack of Golgi cisternae (Golgi). (D) For RAW cells treated with LPS, or PD 184352 plus LPS, values are means of percentage gold counts from two EM grids  $\pm$  range. PM, plasma membrane; PER, peripherally located tubulo-vesicular structures; DEEP, tubulo-vesicular structures located deep in the cell; MVB/LYS, multi-vesicular bodies; Lys, endolysosomes with heterogeneous content; ER, endoplasmic reticulum; NE, nuclear envelope; MIT, mitochondria. For unbiased counting methods and further details on compartment identification refer to Watt et al. (Watt et al., 2002). Scale bars: 100 nm.

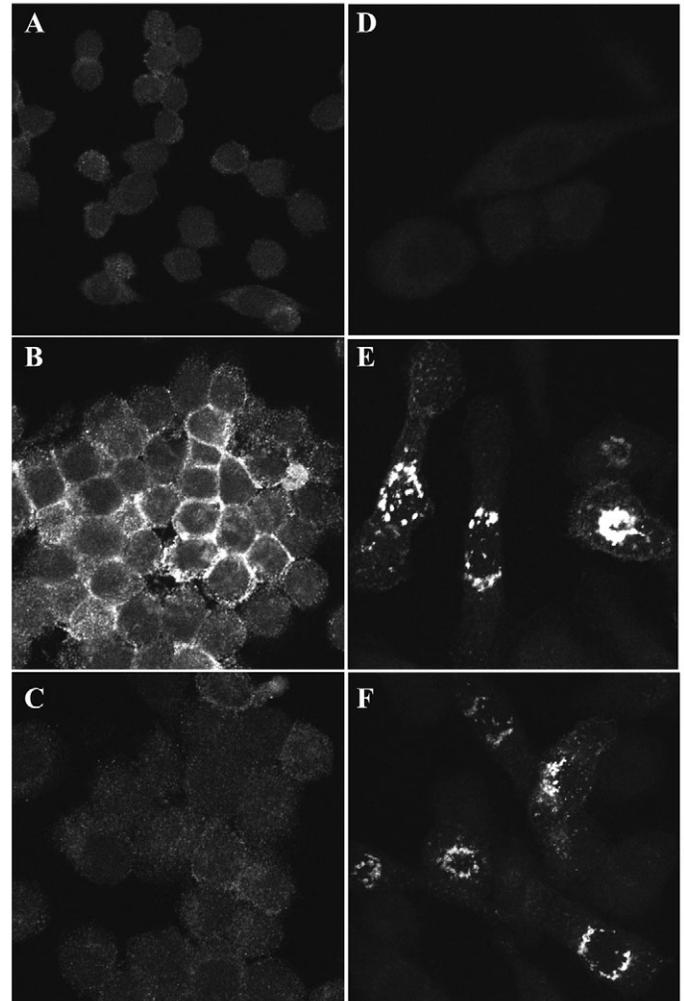
in permeabilised cells was detected in the absence (Fig. 5E) or presence of PD 184352 (Fig. 5F), and was observed mainly in vesicles that co-localise with the Golgi and peripheral endosomes. This was consistent with the electron microscopy data presented earlier.



**Fig. 4.** Genetic ablation of TPL2 blocks pre-TNF $\alpha$  expression at the cell surface. BMDMs were stimulated with LPS (1  $\mu$ g/ml) for (A,B) 1.5 or (C,D) 3 hours in (A,C) the presence or (B,D) absence of Brefeldin A. Permeabilised (A,C) and intact (B,D) cells were stained with PE-conjugated anti-TNF $\alpha$  and APC-conjugated anti-F4/80. Histograms are gated on F4/80<sup>+</sup> cells. Grey histograms denote wild-type cells and solid lines denote TPL2-deficient cells. Unstimulated cells (solid lines) and isotype-control staining (grey histogram) are shown in the bottom panel.

#### Phosphorylation of TACE at Thr735 is abolished in PD-184352-treated RAW cells or in TPL2-deficient macrophages

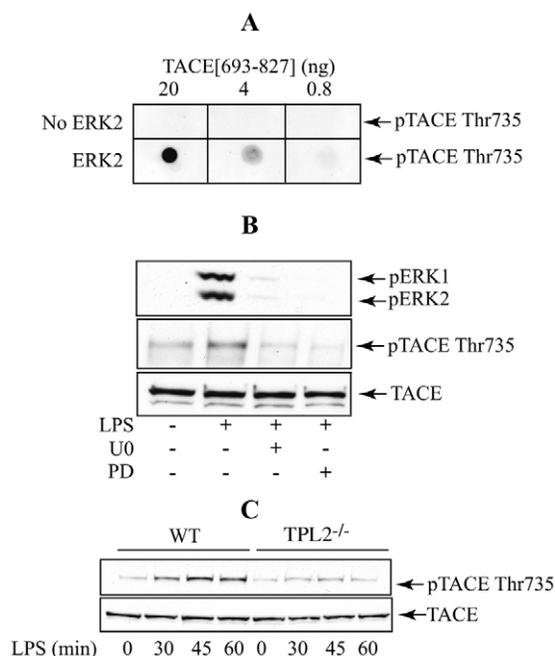
The results presented thus far indicated that the control of pre-TNF $\alpha$  processing by the TPL2-MKK1/2-ERK1/2 pathway did not involve the secretory or endocytic pathways, but instead involved increased expression of pre-TNF $\alpha$  at the cell surface. This suggested that ERK1/2 might regulate the assembly of a protein complex needed for the cell surface expression and cleavage of pre-TNF $\alpha$ . Although the results presented in Fig. 5 demonstrated that the surface expression of pre-TNF $\alpha$  was not dependent on the catalytic activity of TACE (Figs 3 and 5), such a complex would nevertheless be expected to include TACE. It is therefore possible that a covalent modification of TACE, such as phosphorylation, is required for the ERK1/2-stimulated cell surface expression and processing of pre-TNF $\alpha$ . A further reason for considering this possibility was that phorbol esters and growth factors have been shown to stimulate the phosphorylation of wild-type TACE, as well as TACE-dependent shedding of the TRKA (NTRK1) receptor (Diaz-Rodriguez et al., 2002). Moreover, the phorbol-ester-induced shedding of the TRKA receptor is suppressed by the MKK1 inhibitor U0126 (Diaz-Rodriguez et al., 2002; Soond et al., 2005),



**Fig. 5.** Inhibition of ERK1/2 prevents translocation of pre-TNF $\alpha$  to the cell membrane. RAW cells were plated on Labtek chambers and then pre-treated for 60 minutes with 10  $\mu$ M of the TACE inhibitor TAPI-1 (A-F), with (C,F) or without (A,B,D,E) 2  $\mu$ M PD 184352, and were then either left untreated (A,D) or were stimulated for 4 hours with 100 ng/ml LPS (B,C,E,F). Cells were either left unpermeabilised (A-C) or were permeabilised with 0.1% (w/v) saponin (D-F). TNF $\alpha$  was visualised using an antibody coupled to Alexa-Fluor-488-conjugated anti-sheep IgG by confocal microscopy at 600-fold magnification. Similar results were obtained in three independent experiments.

and the phorbol ester-stimulated phosphorylation of TACE did not occur if Thr735 was mutated to Ala.

Thr735 is located in the C-terminal intracellular tail of TACE and lies in a Pro-Xaa-Thr-Pro sequence (where Xaa is any amino acid residue), which is a consensus for phosphorylation by ERK1/2. We therefore raised a phospho-specific antibody that recognises TACE phosphorylated at Thr735 and used it to show that ERK2 phosphorylated the recombinant C-terminal intracellular tail of TACE at Thr735 *in vitro* (Fig. 6A). In cells, LPS induced the phosphorylation of a protein with a similar molecular mass to TACE (95 kDa), which was recognised by the anti-phospho-Thr735 antibody. Phosphorylation was largely suppressed if the RAW cells were pre-incubated with either PD 184352 or U0126 (Fig. 6B). LPS stimulated the phosphorylation of the same protein in BMDM from wild-type mice, but not in BMDM from TPL2-deficient mice (Fig. 6C). The identity of this



**Fig. 6.** TACE phosphorylation at Thr735 is mediated by the TPL2-MKK1/2-ERK1/2 pathway. (A) The C-terminal tail of TACE [TACE(693-827)] was expressed in bacteria as a GST-fusion, purified and phosphorylated with or without ERK2, as described (Morton et al., 2006). The indicated amounts (ng) of TACE(693-827) were spotted onto nitrocellulose and probed with an antibody that recognises TACE phosphorylated at Thr735 (pTACE Thr735). (B) RAW cells were pre-treated for 60 minutes without (-) or with (+) 10  $\mu$ M U0126 (U0) or 2  $\mu$ M PD 184352 (PD) and then either left untreated or were stimulated for 45 minutes with 100 ng/ml LPS. Immunoblotting was carried out as in Fig. 1 using an antibody that recognises ERK1/2 phosphorylated at the TEY motif (pERK), one that recognises TACE phosphorylated at Thr735 or an antibody that recognises the phosphorylated and unphosphorylated forms of TACE equally well (TACE). (C) BMDM from wild-type (WT) or TPL2-deficient (TPL2<sup>-/-</sup>) mice were either left untreated or were stimulated with 100 ng/ml LPS for the times (minutes) indicated. Immunoblotting was performed as in B.

band as phosphorylated TACE was established by showing that the phospho-protein could be immunoprecipitated with a TACE antibody and that the phospho-protein band was no longer observed after siRNA 'knock-down' of TACE in EGF-stimulated HeLa cells (results not shown).

The results described above demonstrated that TACE is a physiological substrate for ERK1/2 in LPS-stimulated macrophages and further suggest that TACE phosphorylation at Thr735 might be a pre-requisite for the LPS-stimulated cell surface expression and cleavage of pre-TNF $\alpha$ . However, to establish whether this hypothesis is correct will require the analysis of mice that express TACE(T735A) instead of the wild-type protein, and the generation of these animals has recently been initiated in our laboratory.

The essential role of TPL2 in TNF $\alpha$  biosynthesis (Dumitru et al., 2000) has prompted interest in targeting this protein kinase to develop an anti-inflammatory drug. TPL2 would appear to be an attractive drug target because its inhibition should prevent the activation of ERK1/2 by LPS, TNF $\alpha$  and IL-1 $\beta$ , without affecting the activation of ERK1/2 by other agonists, such as growth factors, which activate this pathway via RAF and not TPL2. Membrane-inserted TNF $\alpha$  is biologically active (Kriegler et al., 1988) and might be able to partially (Alexopoulou et al., 2006) or weakly (Ruuls et

al., 2001) support pro-inflammatory activity in vivo. Therefore, our observation that pre-TNF $\alpha$  is not expressed at the cell surface if the activation of ERK1/2 is blocked is important, because it implies that pre-TNF $\alpha$  produced when TPL2 is inhibited is unlikely to retain significant pro-inflammatory activity.

## Materials and Methods

### Materials

The MKK1/2 inhibitor U0126 (Davies et al., 2000) and TACE inhibitor TAPI-1 were purchased from Calbiochem; Brefeldin A and *Escherichia coli* LPS from Sigma or Alexis Biochemicals; and unmethylated CpG dinucleotides (ODN1826) and FSL1 from Invivogen. PD 184352 was synthesised by an improved method (Shapiro and Marquez, 2005). Sources of other materials are detailed elsewhere (Rousseau et al., 2002).

### Cell culture, transfection and cell lysis

Murine bone-marrow-derived macrophages (BMDM) and murine RAW264.7 cells were prepared and maintained as described (Rousseau et al., 2002). TPL2 (*Map3k8*)-deficient mice were provided by Philip Tschlis (Tufts-New England Medical Center, Boston, USA) (Dumitru et al., 2000). Human elutriated monocytes were from Advanced Biotechnologies, and were cultured in DMEM supplemented with 20% heat-inactivated FBS and 10% heat-inactivated human AB-positive serum. Human monocytes were cultured for 1 week and then re-fed with fresh media until a macrophage monolayer had formed. The cells were lysed and extracts prepared as described (Rousseau et al., 2006; Rousseau et al., 2002), unless stated otherwise.

### Quantitation of TNF

For wild-type and TPL2 (*Map3k8*)-deficient cells, TNF $\alpha$  in the cell culture medium and *Tnfa* mRNA in cell extracts were quantified by ELISA (Papoutsopoulou et al., 2006). For other cells, TNF $\alpha$  was quantified after a tenfold dilution in fresh culture media, using the Beadlyte Mouse Multi-Cytokine Beadmaster kit and appropriate Beadlyte Beads for TNF $\alpha$  (Upstate). This coupled antibody-based system allows the simultaneous quantification of multiple cytokines. The results were quantified on the Bio-plex system (Bio-Rad).

### Antibodies and immunoblotting

Anti-TNF $\alpha$  antibodies were purchased from R&D Systems and immunoblotting was carried out using the ECL detection system (GE Healthcare). An antibody was raised against the phosphopeptide CFPAPQT(P)PGRL [where T(P) is phospho-threonine], corresponding to residues 730 to 739 of human TACE (same sequence in mice) plus an N-terminal cysteine residue, and was affinity purified on antigen-Sepharose as described (Rousseau et al., 2002). The anti-mouse TACE was from Spring Biosciences and anti-actin from SantaCruz Biotechnologies.

### Electronic microscopy

RAW macrophages culture medium was removed and the cells fixed with 8% PFA in 0.2 M Pipes pH 7.2. After pelleting, cryoprotection (2.3 M sucrose/PBS) and freezing in liquid nitrogen, thawed frozen sections were prepared and picked up on droplets of methyl cellulose/sucrose. Ultra-thin sections were stained using goat antibodies followed by goat anti-rabbit polyclonal antibodies and 11 nm protein-A gold prepared as described (Lucocq, 1993). Labelled sections were contrasted using methylcellulose/uranyl acetate and examined in a JEOL 12EX transmission electron microscope. Quantification of gold label was carried out by the scanning method detailed elsewhere (Lucocq et al., 2004).

### FACS analysis

BMDMs were plated in their growth media supplemented with 1% FCS and incubated overnight before stimulation with 1  $\mu$ g/ml LPS (Alexis) in the presence (intracellular staining) or absence (extracellular staining) of 1  $\mu$ g/ml Brefeldin A. Cells were washed once with ice-cold PBS, incubated for 10 minutes in 0.3 ml PBS/2.5 mM EDTA and blocked for 10 minutes on ice with 1  $\mu$ g/ml 24G2 antibody (BD Pharmingen). For extracellular staining, macrophages were stained for 30 minutes on ice with a mix of F4/80-APC (BD Pharmingen) and TNF $\alpha$ -PE (BD Pharmingen), both at 1  $\mu$ g/ml in PBS/Sodium Azide/1% BSA. For intracellular staining, macrophages were first stained as above with F4/80-APC only and the cells fixed for 20 minutes on ice in 2% paraformaldehyde/PBS. They were then permeabilised for 4 minutes at room temperature in 0.1% Nonidet P40 before staining for 30 minutes on ice with 1  $\mu$ g/ml TNF $\alpha$ -PE. In both staining procedures, 1  $\mu$ g/ml rat IgG1-PE (BD Pharmingen) was used as an isotype control. Cells were analysed by flow cytometry on a FACSCalibur (BD) using FlowJo software (TreeStar).

### Immunofluorescence

RAW cells were fixed in 3.7% (v/v) formaldehyde, permeabilised (when indicated) with 0.1% (w/v) saponin in phosphate-buffered saline pH 7.4 and stained with anti-TNF $\alpha$  antibody (R&D systems). The cells were mounted using ProLong antifade kit

(Molecular Probes) and the images were collected on a confocal microscope (LSM 510 META; Carl Zeiss MicroImaging).

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