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Positive regulation of immune cell function and inflammatory responses by phosphatase PAC-1

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Mitogen-activated protein kinases facilitate many cellular processes and are essential for immune cell function. Their activity is controlled by kinases and dual-specificity phosphatases. A comprehensive microarray analysis of human leukocytes identified *DUSP2* (encoding the phosphatase PAC-1) as one of the most highly induced transcripts in activated immune cells. We generated *Dusp2*$^{-/-}$ mice and found considerably reduced inflammatory responses in the 'K/BxN' model of rheumatoid arthritis. PAC-1 deficiency led to increased activity of Jun kinase (Jnk) but unexpected impairment of the activity of extracellular signal–regulated kinase (Erk) and the kinase p38, reduced activity of the transcription factor Elk1 and a complex of mobilized transcription factor NFAT and the AP-1 transcription factor and decreased effector immune cell function. Thus, PAC-1 is a key positive regulator of inflammatory cell signaling and effector functions, mediated through Jnk and Erk mitogen-activated protein kinase crosstalk.

The mitogen-activated protein kinases (MAPKs) are fundamental regulators of immune cell function, including differentiation, apoptosis, chemoattraction, lymphocyte development and inflammatory mediator production[1–3]. There are four main classes of mammalian MAPKs: extracellular signal–related kinases, consisting of the classic Erk1 and Erk2, p38 proteins (p38α, p38β, p38γ and p38δ) and Jun N-terminal kinases (Jnk1, Jnk2 and Jnk3). These are all activated by dual phosphorylation of the threonine and tyrosine residues in a conserved 'TXY' motif (where X is E for Erk, G for p38, or P for Jnk). Larger MAPK molecules (Erk3, Erk5, Erk6 and Erk7) have also been identified. A general feature of MAPK pathways is a three-tiered kinase cascade consisting of a MAPK, MAPKK (MEK) and a MAPKKK[2] and, along with their activators and scaffolds, these form a finely tuned network to tightly control the extent of MAPK activation. Activated MAPKs shuttle into the nucleus and initiate the rapid transcription of immediate-early genes through the phosphorylation of pre-existing transcription factors, particularly Elk1 or ATF-2 (ref. 2). MAPKs also control inflammatory cytokine mRNA stability through *cis*-acting sequences within 5′ or 3′ untranslated regions; the best characterized regulatory elements are the adenosine-uridine–rich elements[4]. Pharmacological inhibition of p38 and Erk results in decreased production of inflammatory mediators[1,5] and, despite their broad expression profile, MAPKs have attracted considerable interest as targets for the development of new treatments for inflammatory diseases.

Dual-specificity phosphatases (DUSPs) that regulate MAPK activity through TXY-motif dephosphorylation represent particularly important negative regulators[6–9]. In addition to their dephosphorylating capacity, DUSPs serve to anchor or shuttle MAPKs and control their subcellular localization[10,11]. At least ten mammalian DUSPs have been identified, and these constitute three subfamilies based on sequence similarity, substrate specificity and subcellular localization[6,12]. DUSP1, DUSP2, DUSP4 and DUSP5 localize to the nucleus and are induced by growth or stress signals. DUSP6, DUSP7 and DUSP9 are cytoplasmic and 'preferentially' recognize Erk1 and Erk2 *in vitro*, whereas DUSP8, DUSP16 and DUSP10 'preferentially' recognize Jnk and p38 (refs. 6,12,13). At present the *in vivo* physiological functions of the many DUSPs are mostly unknown. Deletion of DUSP1 (also called MAPK phosphatase 1 (MKP1)) initially yielded no obvious abnormal phenotype, with normal development and MAPK activity in fibroblasts[14], yet increased p38 activity is apparent in DUSP1-deficient alveolar macrophages[15]. DUSP1 reportedly suppresses endotoxic shock through feedback control of p38 and Jnk activity[16,17]. DUSP9 (also called MKP4) is required for placental development yet seems not to influence detectable differences in MAPK phosphorylation[18], whereas DUSP10 (also called MKP5) is an important regulator of innate and adaptive immune responses mediated by attenuation of Jnk activity[7].

Of the DUSPs, DUSP2 (also called phosphatase of activated cells 1 (PAC-1)), has been closely associated with immune cells. The gene

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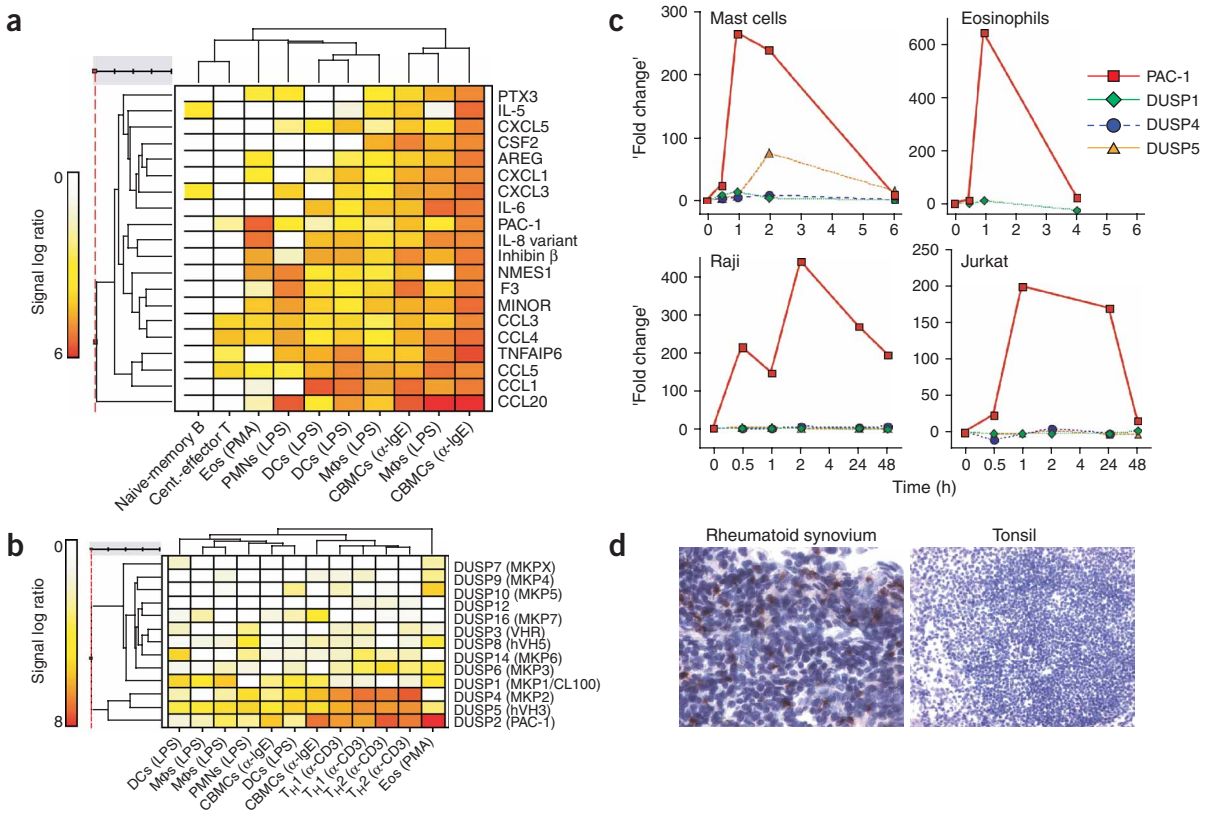

Figure 1 *DUSP2* is the most highly induced DUSP transcript in activated human leukocytes, and PAC-1 protein associates with inflammatory tissue. (**a**) Heat map representation of the 20 most highly regulated genes (products encoded, right margin) after FcεRI-dependent activation of two independent cultures of human cord blood–derived mast cells, using microarray gene profiling. 'Data mining' of these genes was then done across a range of primary human activated leukocytes. (**b**) Heat map representation and hierarchical clustering of transcript regulation of known DUSPs after activation of primary human leukocytes. B, B cells; Cent., central; T, T cells; Eos, eosinophils; PMNs, polymorphonuclear cells; DCs, dendrtitic cells; MΦs, macrophages; CBMCs, cord blood–derived mast cells; T_H1, T helper type 1 cells; T_H2, T helper type 2 cells; PMA, phorbol-12-myristate-13-acetate; α-, antibody to. (**c**) Quantitative PCR of *DUSP2* mRNA compared with the mRNA of other nuclear DUSPs after FcεRI crosslinking of human cord blood–derived mast cells, peripheral blood eosinophils activated with phorbol-12-myristate-13-acetate, Raji B cells activated with anti-IgM, and Jurkat T cells activated with anti-CD3. The 'fold change' is calculated relative to that of unstimulated cells. (**d**) Immunohistochemical staining of PAC-1 protein in human rheumatoid arthritis synovium and tonsil with polyclonal anti-PAC-1. Original magnification, ×400. Data are representative of two to three independent experiments.

encoding PAC-1 was originally cloned from human T cells as an immediate-early gene[19] and it is highly induced in T cells and B cells after activation[19,20]. PAC-1 expression has been mostly associated with immune cells and tissues, such as spleen and thymus[9,19]. Additionally, PAC-1 is not detected in quiescent cells[20] and functions as a MAPK phosphatase *in vitro*, with selectivity for p38 and Erk[8,9,21]. Notably, MAPKs themselves can regulate transcription of *Dusp2* (ref. 20). Moreover, the proapoptotic factor p53 activates transcription of *Dusp2*, suggesting PAC-1 is required for p53-mediated apoptosis[22].

Here we show that PAC-1 has an unexpected positive function in the induction and perpetuation of certain inflammatory responses. We identify PAC-1 as one of the most highly induced transcripts in activated immune effector cells and one of the most abundant and regulated DUSPs in many activated leukocytes. Unlike expression of the other DUSPs and the MAPKs, PAC-1 expression was also specific to leukocytes. PAC-1-deficient mice were mostly protected in the 'K/BxN' animal model of inflammatory arthritis. This protection was because the important effector immune cells in this model, such as mast cells and macrophages, had impaired effector responses such as inflammatory mediator production and survival with PAC-1 deficiency. PAC-1-deficient macrophages and mast cells showed decreased phosphorylation of Erk and p38 and, ultimately, decreased transcriptional activation of transcription factors NFAT–AP-1 and Elk1. Finally, Jnk activity was increased in PAC-1-deficient macrophages and mast cells, demonstrating PAC-1 acts mainly as a negative regulator of Jnk *in vivo*. The decreased Erk in PAC-1-deficient cells could be restored completely with a specific inhibitor of Jnk, identifying negative crosstalk between Jnk and Erk. These results indicate complex positive and negative regulation of MAPK pathways in immune cells by regulatory factors such as PAC-1 that ultimately results in positive regulation of effector immune cell function.

RESULTS

Induction of *DUSP2* in activated leukocytes

A comprehensive analysis of gene expression in numerous subsets of human leukocytes using Affymetrix GeneChip microarrays identified *DUSP2* as one of the genes most highly induced after activation of human mast cells (**Fig. 1a**). Other effector leukocytes such as eosinophils, T helper type 1 and type 2 cells, neutrophils and macrophages also showed considerable induction of *DUSP2* after activation (**Fig. 1a** and **Supplementary Fig. 1** online). In most leukocytes, *DUSP2* was expressed more abundantly than the other DUSP genes, including

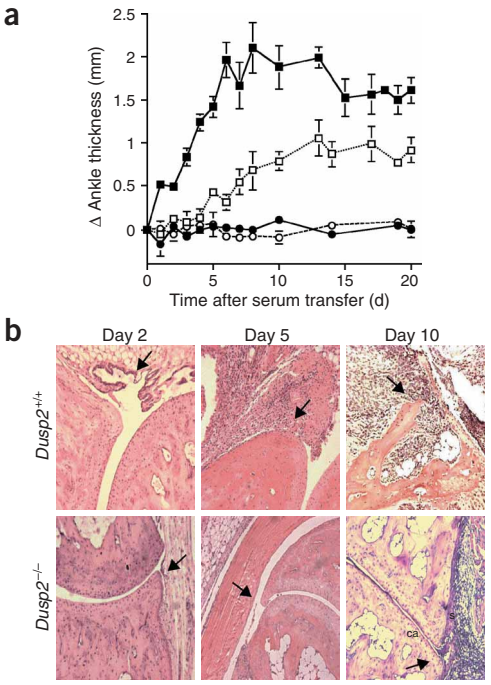

Figure 2 Targeted deletion of mouse *Dusp2* results in protection of *Dusp2*$^{-/-}$ mice in the K/BxN model of rheumatoid arthritis. (**a**) Change in ankle thickness in *Dusp2*$^{-/-}$ mice (open squares) and wild-type littermate control mice (filled squares) given arthritogenic K/BxN serum (150 μl) on days 0 and 2. Circles indicate mice injected with nonarthritic control serum (filled, wild-type; *Dusp2*$^{-/-}$, white). Error bars indicate s.e.m. of 12 mice. (**b**) Ankle sections from *Dusp2*$^{+/+}$ and *Dusp2*$^{-/-}$ mice at early and late stages of disease. Arrows indicate junctions between cartilage and synovium within the joint. Original magnification, ×400. Representative sections are selected from individual mice of groups of seven to nine mice.

DUSP1 and *DUSP10* (**Fig. 1b**), that have regulatory functions in immune responses[7,16]. Notably, hierarchical clustering showed a strong association among the nuclear DUSP genes (*DUSP1*, *DUSP2*, *DUSP4* and *DUSP5*), which in contrast to the cytoplasmic DUSP genes, were the most strongly regulated in activated immune cells (**Fig. 1b**). Quantitative RT-PCR confirmed that *DUSP2* was the main nuclear DUSP gene expressed and regulated in activated human mast cells, eosinophils, B cells and T cells (**Fig. 1c**). Mouse mast cells also had high induction of *Dusp2* after activation, and PAC-1 protein was rapidly induced in activated mouse macrophages (**Supplementary Figs. 1–3** online). GeneChip analysis of mouse RNA also showed that expression of *Dusp2*, in contrast to that of other DUSP and MAPK genes, was restricted to hematopoietic cells (**Supplementary Fig. 1** online). Human PAC-1 protein expression, assessed using commercial polyclonal antisera, showed only sparse expression in lymphoid tissue but an association with certain inflammatory responses, particularly immune cells infiltrating human rheumatoid synovium (**Fig. 1d**). Thus, PAC-1 may serve as a chief regulator of MAPK activity, specifically in immune effector cells associated with inflammatory reactions.

Protection of *Dusp2*$^{-/-}$ mice from arthritis

The exclusive transcriptional regulation of *Dusp2* in activated leukocytes, coupled with the essential function of MAPK pathways for immune cell function[1], prompted us to examine the physiological function of PAC-1 in immune responses through *in vivo* loss of function. *Dusp2*$^{-/-}$ mice, generated by gene targeting (**Supplementary Fig. 4** online), developed and aged normally and showed no abnormalities in basic immune features, including lymphoid tissue development, blood and bone marrow lymphocyte or granulocyte subset numbers (data not shown), or immunopathology. Hence, like DUSP10 (ref. 7), PAC-1 seemed to have no obvious function in immune cell development. We next assessed *Dusp2*$^{-/-}$ mice in the K/BxN model of inflammatory arthritis[23]. Disease pathogenesis in this model is dependent on effector leukocytes, particularly mast cells, neutrophils and macrophages[24–26], after the transfer of autoantibodies to glucose-6-phosphate isomerase contained in the serum[27]. Control littermate mice injected with arthritogenic K/BxN serum developed a peripheral inflammatory arthritis within 2 d (**Fig. 2** and **Supplementary Fig. 4** online), with many of the features of human rheumatoid arthritis; notably, this included an inflammatory cell infiltrate consisting mainly of neutrophils, pannus formation and eventual joint destruction (**Fig. 2b**). *Dusp2*$^{-/-}$ littermates were protected, particularly during the first 5 d after serum transfer (**Fig. 2** and **Supplementary Fig. 4** online), and thereafter showed greatly diminished clinical and histological features of arthritis (**Fig. 2b** and **Supplementary Fig. 4** online). Thus, PAC-1 serves a critical and unexpected positive function in promoting inflammatory responses *in vivo*.

Dusp2$^{-/-}$ cells produce fewer inflammatory mediators

We next explored the effect of PAC-1 on gene expression in relevant immune effector cells (**Fig. 3**). Comparison of gene expression by GeneChip analysis of lipopolysaccharide (LPS)–stimulated *Dusp2*$^{+/+}$ and *Dusp2*$^{-/-}$ mouse macrophages elicited by thioglycollate demonstrated considerably reduced expression of transcripts for proinflammatory mediators and cytokines in *Dusp2*$^{-/-}$ (**Fig. 3a**), several of which have been linked to inflammatory diseases, particularly rheumatoid arthritis. These included transcripts encoding interleukin 6 (IL-6), IL-12α, cyclooxygenase 2, IL-1β and inflammatory chemokines (**Fig. 3a**). A similar picture emerged from analysis of bone marrow–derived mast cells (BMMCs) activated with the immunoglobulin E (IgE) receptor FcεRI (**Fig. 3g**). Other proteins reduced by PAC-1 deficiency included chemoattractant receptors (such as C5aR), matrix metalloproteinases, adhesion molecules and apoptosis-related factors (**Fig. 3a**,**g**). Notably, none of the other DUSPs were upregulated in the absence of PAC-1 (**Supplementary Fig. 3** online), ruling out functional compensation as an explanation for the *Dusp2*$^{-/-}$ phenotype. *Dusp2*$^{-/-}$ macrophages had decreased expression of the mRNA (data not shown) and protein of inflammatory mediators, including tumor necrosis factor (TNF) and IL-6 (**Fig. 3b**,**c**) and had reduced numbers of IL-12-producing cells as well as reduced IL-12 per cell (**Fig. 3f**). There was also considerably reduced production of prostaglandin E_2 and nitric oxide after stimulation of PAC-1-deficient macrophages (**Fig. 3d**,**e**). Despite normal functional and phenotypic development of mast cells and similar rates of degranulation (**Supplementary Fig. 5** online), *Tnf* mRNA was reduced in *Dusp2*$^{-/-}$ FcεRI-activated BMMCs (**Fig. 3h**), as was IL-6 protein (**Fig. 3i**). Hence, PAC-1 seemed to control mediator release in mast cells at the level of gene expression. Analysis of the long-term viability of *Dusp2*$^{+/+}$ and *Dusp2*$^{-/-}$ BMMCs in culture showed that *Dusp2*$^{-/-}$ mast cells had reduced survival (**Fig. 3j**) that coincided with increased apoptosis of *Dusp2*$^{-/-}$ BMMCs (**Fig. 3k**). Hence, PAC-1 has profound positive control over two important immune cell functions, inflammatory mediator synthesis and cell growth and/or survival, at least in mast cells.

Phosphatase-dependent 'rescue' of cytokine production

To confirm the positive effect of PAC-1 on inflammatory mediator production, we transduced immature *Dusp2*$^{+/+}$ macrophages with bicistronic retroviruses encoding green fluorescent protein (GFP) and either *Dusp2* or a 'phosphatase-dead' mutant[28] (*Dusp2*^{C257S}). Notably, there was always less expression of ectopic wild-type *Dusp2* than of the *Dusp2*^{C257S} mutant in the packaging cell line Plat-E[29] (data not shown) and in 8-day-old retrovirally transduced bone marrow–derived macrophages (BMDMs; **Fig. 4a**), despite similar expression of GFP, suggesting that *Dusp2*^{C257S} has enhanced stability due to the disruption of a MAPK-DUSP negative feedback loop[30]. Whereas constitutive overexpression of wild-type *Dusp2* in *Dusp2*$^{+/+}$ BMDMs resulted in an increase in the number of IL-12-producing cells as well as enhanced IL-12 expression per cell (**Fig. 4a**), enforced expression of the *Dusp2*^{C257S} mutant not only failed to promote cytokine production but also acted as a dominant negative regulator of cytokine expression (**Fig. 4a**), indicating that the phosphatase activity of PAC-1 is required for the promotion of cytokine production. We obtained a similar result with TNF and IL-6 (data not shown). Notably, ectopic expression of *Dusp2* in *Dusp2*$^{+/+}$ or *Dusp2*$^{-/-}$ BMMCs resulted in enhancement or restoration, respectively, of TNF and IL-4 production (**Fig. 4b**). This finding was highly influenced by PAC-1 protein abundance, indicating that appropriate physiological outcomes during an inflammatory response could be shaped by tight control of PAC-1 expression. Introduction of the *Dusp2*^{C257S} mutant failed to restore cytokine production (**Fig. 4b**), confirming the requirement for the phosphatase activity of PAC-1 for the positive regulation of effector immune cell function.

Regulation of MAPK activity in *Dusp2*$^{-/-}$ cells

Studies using ectopic *Dusp2* expression in T cells and fibroblasts have shown PAC-1 is a MAPK-specific phosphatase with selectivity for p38

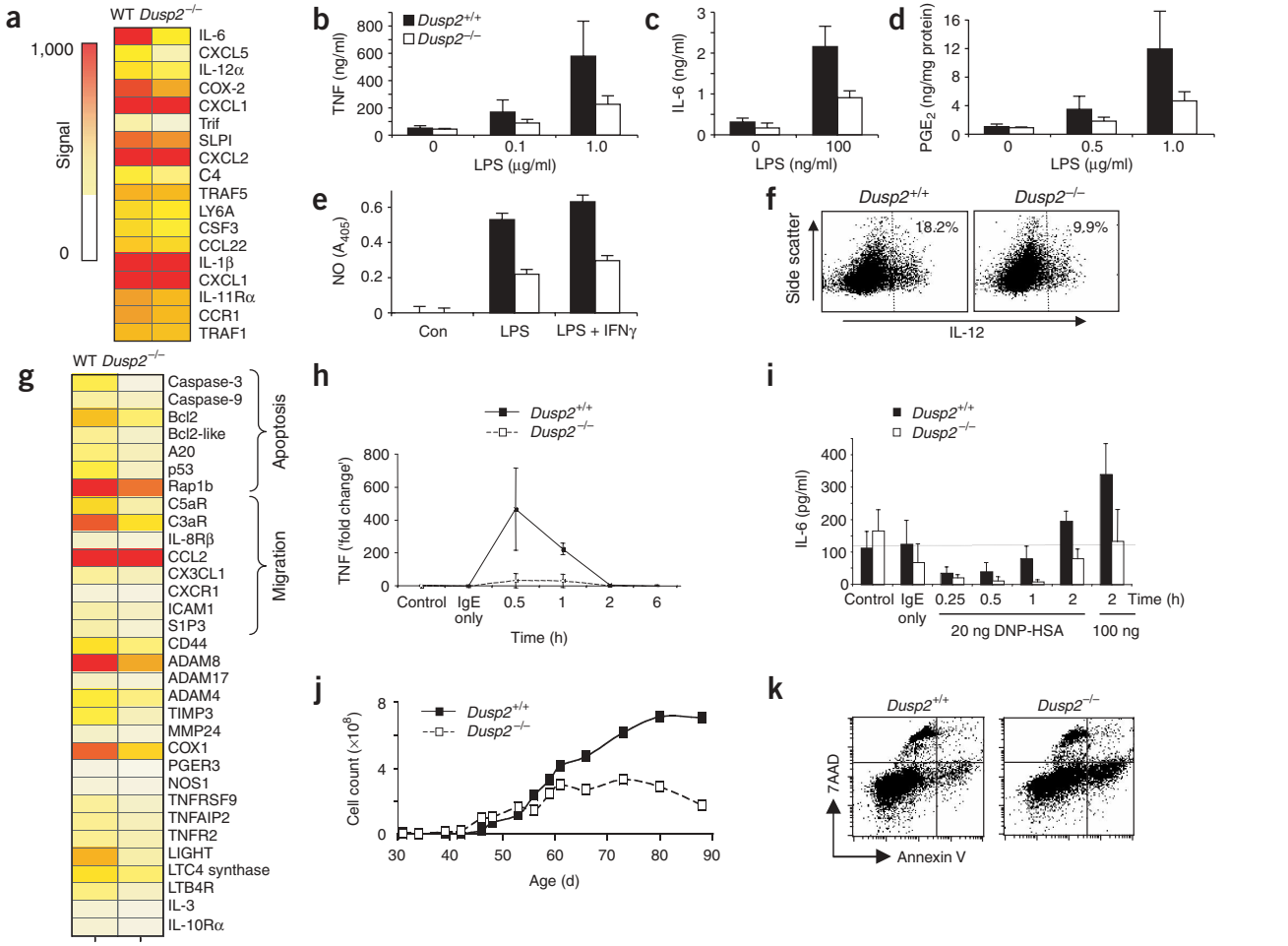

Figure 3 *Dusp2*$^{-/-}$ macrophages and mast cells are compromised in their production of proinflammatory mediators. (**a**) Transcriptional profiling of *Dusp2*$^{+/+}$ (WT) and *Dusp2*$^{-/-}$ LPS-stimulated macrophages at 6 h, showing inflammation-related genes (products, right margin). (**b**,**c**) ELISA of mean production of TNF (**b**) and IL-6 (**c**) by four independent macrophage cultures stimulated for 6 h with LPS. (**d**,**e**) Production of prostaglandin E_2 (PGE_2) in macrophages stimulated with various concentrations of LPS (**d**) and nitric oxide (NO) production in macrophages with no stimulation (Con) or after stimulation with 1 μg/ml of LPS only or in combination with 10 ng/ml of IFN-γ (**e**). Results are representative of four independent experiments done in triplicate. (**f**) IL-12p40 production after LPS stimulation, measured by intracellular cytokine staining of BMDMs. Numbers in dot plots indicate percent IL-12$^+$ cells. Data are representative of three independent cultures of macrophages. (**g**) Transcriptional profiling of *Dusp2*$^{+/+}$ (WT) and *Dusp2*$^{-/-}$ FcεRI-stimulated mast cells at 2 h. (**h**,**i**) Production of *Tnf* mRNA (**h**) and IL-6 protein (**i**) by FcεRI-stimulated BMMCs. Either 20 ng/ml or 100 ng/ml of DNP–heat-stable antigen (DNP-HSA) was used. Control, cells sensitized with IgE for 18 h without antigen crosslinking. Error bars are s.e.m. of pooled results of three to four independent mast cell cultures. (**j**) Reduced cell numbers in long-term culture of *Dusp2*$^{-/-}$ BMMCs. (**k**) Increased cell death of *Dusp2*$^{-/-}$ BMMCs, as assessed by annexin V and 7-amino-actinomycin D (7-AAD) staining. Data (**j**,**k**) are representative of three independent cultures of mast cells.

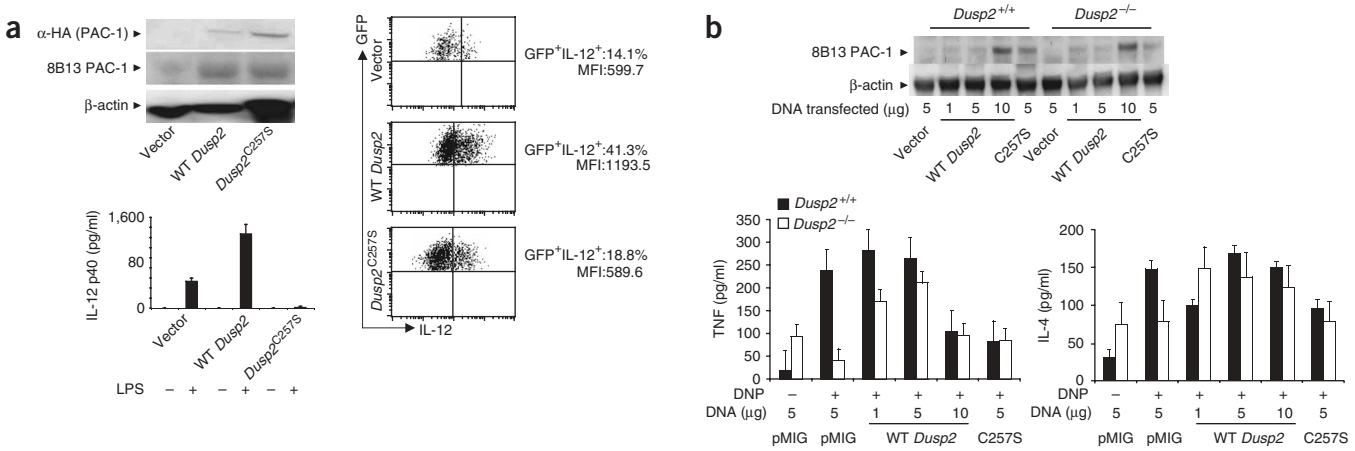

Figure 4 Re-expression of *Dusp2* enhances or restores cytokine production in a phosphatase activity–dependent way. (**a**) Retroviral transduction efficiency and protein expression in 'day-8' BMDMs. Naive *Dusp2*$^{+/+}$ bone marrow cells were transduced with bicistronic retroviruses encoding GFP only (Vector; control) or GFP plus wild-type *Dusp2* (WT *Dusp2*) or 'phosphatase-dead' *Dusp2*^{C257S} (*Dusp2*^{C257S}) and were differentiated to maturity with colony-stimulating factor 1. Protein expression was analyzed by immunoblot (top left) with anti-hemagglutinin (α-HA) and anti-PAC-1 (monoclonal antibody 8B13). IL-12 production after LPS stimulation was measured by intracellular cytokine staining (right) or ELISA of supernatants (bottom left) of mature *Dusp2*$^{+/+}$ BMDMs. Only GFP$^+$ cells are presented, and gates are set on GFP$^+$ and the corresponding nonstimulated cells. Numbers at right indicate percent GFP$^+$IL-12$^+$ cells and mean fluorescence intensity (MFI) for IL-12. Results are representative of three independent transduction experiments in triplicate. (**b**) Top, PAC-1 protein expression in *Dusp2*$^{+/+}$ and *Dusp2*$^{-/-}$ BMMCs transfected with various concentrations (below lanes) of C-terminal triple-hemagglutinin-tagged *Dusp2* plasmids, as measured by immunoblot with anti-PAC-1 (monoclonal antibody 8B13). β-actin is used to confirm equal loading throughout. Bottom, production of TNF (left) and IL-4 (right) by FcεRI-stimulated *Dusp2*$^{+/+}$ and *Dusp2*$^{-/-}$ BMMCs transfected with various concentrations (below bars) of wild-type *Dusp2* (WT *Dusp2*) or *Dusp2*^{C257S} (C257S) expression vectors. Results are representative of two independent transfection experiments in two independent BMMC cultures.

and Erk *in vitro*[8,9,19,21], albeit with less efficacy than other DUSPs such as MKP2 (ref. 8). Hence, to further investigate this unexpected positive function for PAC-1 in immune cell responses, we assessed the phosphorylation status and kinase activity of p38 and Erk in *Dusp2*$^{+/+}$ and *Dusp2*$^{-/-}$ macrophages and mast cells. The activation of Erk and p38 was decreased in PAC-1-deficient mast cells and macrophages. Immunoblot analysis of lysates prepared from BMMCs showed substantial reduction of both phosphorylated Erk and phosphorylated p38 in *Dusp2*$^{-/-}$ cells after FcεRI activation (**Fig. 5a**). Macrophages (both thioglycollate elicited and BMDMs) showed reduced and more transient phosphorylation of p38 but little effect on global phosphorylated Erk with PAC-1 deficiency after Toll-like receptor 4 activation (**Fig. 5b** and data not shown). Despite the reported low specificity of PAC-1 for Jnk[8,31], the third MAPK subclass,

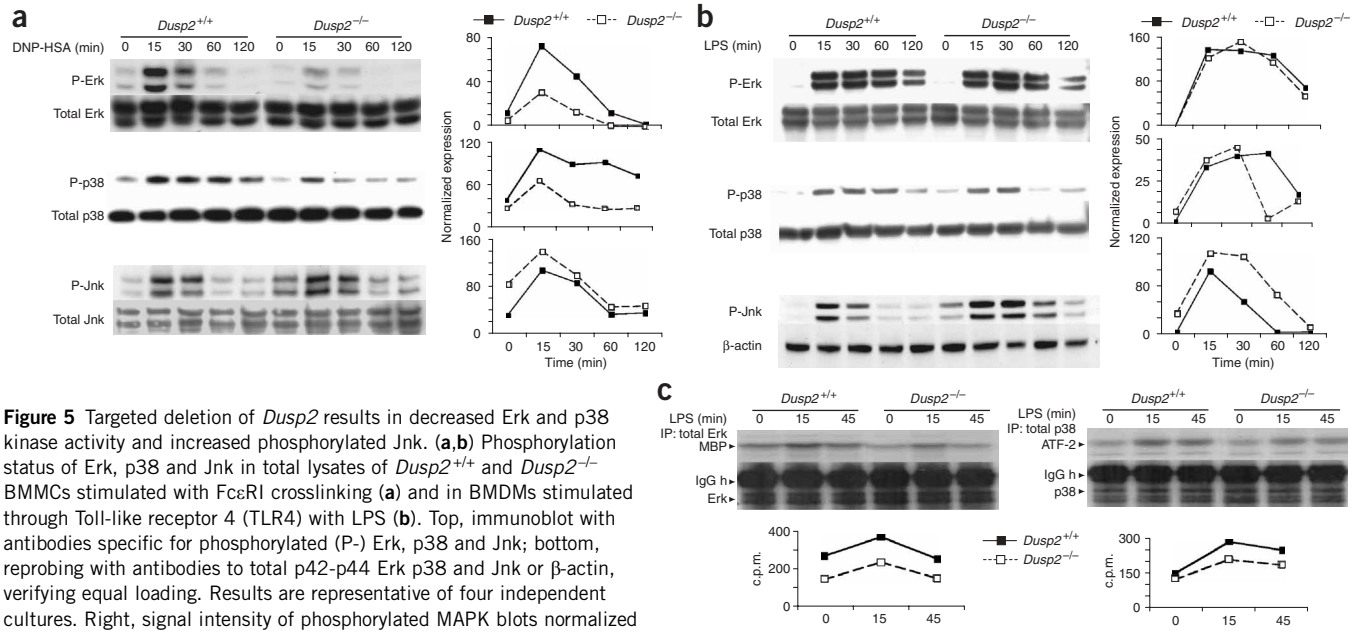

Figure 5 Targeted deletion of *Dusp2* results in decreased Erk and p38 kinase activity and increased phosphorylated Jnk. (**a**,**b**) Phosphorylation status of Erk, p38 and Jnk in total lysates of *Dusp2*$^{+/+}$ and *Dusp2*$^{-/-}$ BMMCs stimulated with FcεRI crosslinking (**a**) and in BMDMs stimulated through Toll-like receptor 4 (TLR4) with LPS (**b**). Top, immunoblot with antibodies specific for phosphorylated (P-) Erk, p38 and Jnk; bottom, reprobing with antibodies to total p42-p44 Erk p38 and Jnk or β-actin, verifying equal loading. Results are representative of four independent cultures. Right, signal intensity of phosphorylated MAPK blots normalized to the signal of total MAPK and plotted against time. (**c**) *In vitro* kinase activity of immunoprecipitated (IP) Erk and p38 in Toll-like receptor 4–activated *Dusp2*$^{+/+}$ and *Dusp2*$^{-/-}$ BMDMs with myelin basic protein (MBP) and ATF-2, respectively, as substrates. Results are representative of two independent experiments. IgG h, IgG heavy chain.

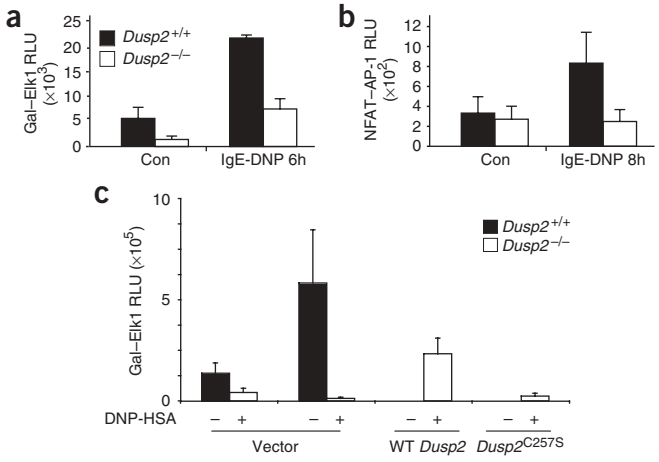

Figure 6 Reduced transcriptional Elk1 and NFAT–AP-1 activity in *Dusp2*$^{-/-}$ BMMCs. (**a**,**b**) Reduced transcriptional Elk1 activity (**a**) and NFAT–AP-1 activity (**b**) in *Dusp2*$^{-/-}$ BMMCs, as measured by standardized luciferase activity. Gal–Elk1 or NFAT–AP-1 reporters were transfected into *Dusp2*$^{+/+}$ and *Dusp2*$^{-/-}$ BMMCs and were activated for 6 h (Elk1) or 8 h (NFAT–AP-1) by FcεRI crosslinking. RLU, relative luciferase activity. (**c**) Elk1 activity after introduction of *Dusp2* or *Dusp2*^{C257S} into *Dusp2*$^{+/+}$ and *Dusp2*$^{-/-}$ BMMCs. BMMCs were transfected with Gal–Elk1 reporter constructs plus empty vector alone (left), full-length wild-type *Dusp2* (middle) or *Dusp2*^{C257S} (right). Error bars indicate s.d. of triplicate samples. Similar results were obtained in five independent experiments.

phosphorylated Jnk was considerably increased in the absence of PAC-1 after Toll-like receptor 4 stimulation of macrophages (**Fig. 5b**) and FcεRI crosslinking of BMMCs (**Fig. 5a**). *In vitro* kinase assays of Erk and p38 MAPKs, purified regardless of their phosphorylation status from *Dusp2*$^{+/+}$ and *Dusp2*$^{-/-}$ BMDMs, showed a reduction in the kinase activity of Erk in *Dusp2*$^{-/-}$ macrophages, as assessed by its ability to phosphorylate myelin basic protein (**Fig. 5c**) or its physiological substrate, the Ets domain transcription factor Elk1 (data not shown). The *in vitro* kinase activity of p38 was also reduced with PAC-1 deficiency (**Fig. 5c**). These differences in global phosphorylated MAPK and the *in vitro* kinase assays suggest that distinct subcellular pools of Erk and p38 are decreased with PAC-1 deficiency in macrophages. Similar activation of nuclear transcription factor NF-κB was evident in *Dusp2*$^{+/+}$ and *Dusp2*$^{-/-}$ BMDMs (**Supplementary Fig. 6** online), demonstrating that this important signaling pathway for proinflammatory mediator induction was not affected by PAC-1 deficiency.

Reduced Elk1 and AP-1 activity in *Dusp2*$^{-/-}$ cells

Nuclear MAPKs contribute to immune cell effector function in inflammatory responses in a pleiotropic way whereby they directly activate transcription factors, remodel chromatin, expose NF-κB-binding sites, regulate nuclear export and stabilize cytokine transcripts[1,5,32]. As PAC-1 is a nuclear DUSP[21], it is likely PAC-1 deficiency specifically impairs nuclear MAPK-dependent events such as transcription factor activity. Thus, we assessed the transcriptional activity of two main nuclear targets of MAPK pathways, the NFAT–AP-1 complex and Elk1, using a Gal4–Elk1 luciferase reporter system[33]. In *Dusp2*$^{-/-}$ BMMCs, there was a substantial reduction in basal and FcεRI-induced Elk1 activity (**Fig. 6a**). Complete and partial inhibition of Gal–Elk1 reporter activity was achieved by MEK and p38 inhibition, respectively (**Supplementary Fig. 7** online), confirming that this transcriptional reporter assay reflected nuclear MAPK activity. Notably, reintroduction of *Dusp2* into *Dusp2*$^{-/-}$ BMMCs increased Elk1 reporter activity, but reintroduction of *Dusp2*^{C257S} did not (**Fig. 6**) confirming the idea that PAC-1 and its phosphatase catalytic site are required for activation of Elk1. NFAT–AP-1 activity was also decreased in *Dusp2*$^{-/-}$ BMMCs (**Fig. 6b**). That effect most likely reflected a reduced contribution of MAPK to the NFAT–AP-1 complex[34] rather than a failure to mobilize cytoplasmic NFAT, as judged by the normal kinetics of NFAT dephosphorylation in *Dusp2*$^{-/-}$ BMMCs (**Supplementary Fig. 6** online). Indeed, GeneChip analysis of activated *Dusp2*$^{-/-}$ macrophages and mast cells showed a significant reduction in expression of *Junb* ($P = 0.04$), an Elk1 target gene encoding a component of AP-1 (ref. 35; **Supplementary Fig. 6** online). Both MAPK pathway inhibitors also blocked TNF and IL-4 production in *Dusp2*$^{+/+}$ BMMCs but did not further reduce cytokine production in *Dusp2*$^{-/-}$ BMMCs (**Supplementary Fig. 7** online), linking the observed reduction in MAPK activation and

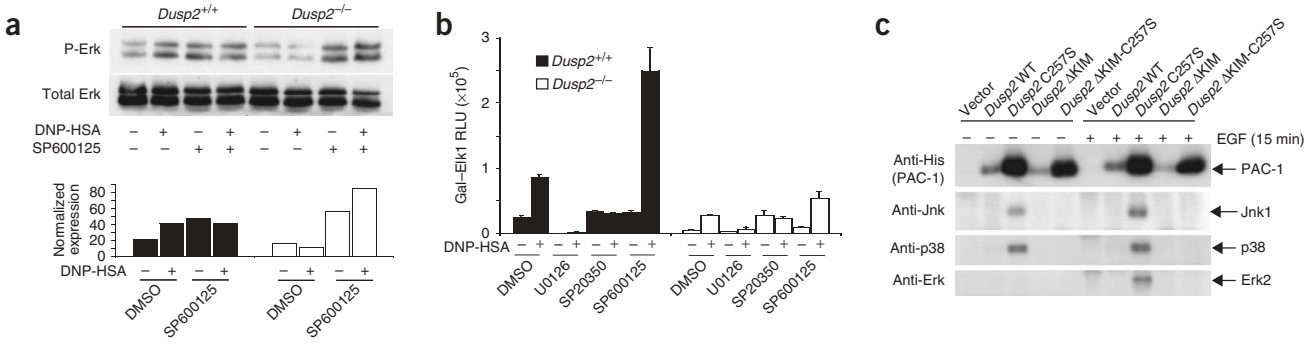

Figure 7 Inhibition of Jnk can 'rescue' the reduced phosphorylated Erk in *Dusp2*$^{-/-}$ BMMCs and increase Elk1 transcriptional activity. (**a**) Immunoblot of BMMCs incubated with 50 μM SP600125 for 30 min before FcεRI crosslinking for 15 min. DMSO, dimethyl sulfoxide. Bottom, signal intensity of phosphorylated Erk normalized to the signal of total Erk. (**b**) Gal–Elk1 activity in *Dusp2*$^{+/+}$ and *Dusp2*$^{-/-}$ BMMCs treated with 50 μM U0126, SB203850 and SP600125 to inhibit MEK1-MEK2, p38 and Jnk, respectively, before FcεRI crosslinking. Results are representative of two independent experiments. (**c**) 'Phosphatase-dead' *Dusp2*^{C257S} traps Jnk-1, p38 and Erk in a kinase-interactive motif–dependent way. Phoenix-eco cells were transiently transfected with expression vectors encoding various *Dusp2* constructs (above lanes) or empty pMIG vector (Vector). At 24 h after transfection, cells were 'starved' in growth medium containing 2% FCS for 18 h and then were stimulated with 100 ng/ml of human recombinant EGF (+) or were left untreated (–). Cells were lysed and C-terminally histidine-tagged PAC-1 proteins were immunoprecipitated with monoclonal anti-histidine, then immune complexes were analyzed by immunoblot (antibodies, left margin). ΔKIM, mutants with the kinase-interactive motif sequence altered to LLAAAAA.

Elk1 and NFAT–AP-1 reporter activity with impaired cytokine production in PAC-1-deficient cells. Therefore, PAC-1 positively modulates important nuclear MAPK-dependent events required for the transcription of target genes needed for immune cell effector function.

PAC-1 regulates Jnk: evidence for Erk-Jnk crosstalk

To understand how Erk and p38 activity was reduced in the absence of a MAPK phosphatase, we investigated whether the considerable increase in Jnk phosphorylation in PAC-1-deficient cells resulted in negative crosstalk between Jnk and Erk, as suggested before[36]. We specifically inhibited Jnk activity with the Jnk inhibitor SP600125 in PAC-1-deficient mast cells and found that the decreased Erk activity noted in PAC-1-deficient mast cells could be completely restored through Jnk inhibition (**Fig. 7a**). Notably, in wild-type cells, Jnk inhibition positively regulated basal Erk phosphorylation and considerably increased Elk1 transcriptional activity after activation (**Fig. 7b**). We next examined the physical interaction of PAC-1 with the MAPKs. PAC-1 immunoprecipitated together with Jnk1 but not with Jnk2 in both stimulated and unstimulated cells. This was dependent on the kinase-interactive motif LLRRRAR found in the N-terminal MAPK-binding domain of PAC-1 (refs. 12,37), as mutants with the kinase-interactive motif sequence altered to LLAAAAA did not interact with Jnk (**Fig. 7c**). Furthermore, PAC-1 also interacted with p38 in unstimulated and stimulated cells and with Erk2 only in stimulated cells. The kinase-interactive motif of PAC-1 protein was required for these interactions (**Fig. 7c**), strongly suggesting the physiological effectors of PAC-1 are found in the MAPK family.

DISCUSSION

DUSPs dephosporylate MAPKs and thus have been regarded as negative regulators of MAPK signaling and cellular responses. Here we have shown that PAC-1 deficiency resulted in an increase in Jnk activity but an unexpected reduction in Erk and p38 activity. That 'translated' into substantial defects in MAPK-dependent proinflammatory gene expression in mast cells and macrophages, resulting in protection from inflammatory arthritis. Those findings underlie the complex interactions between MAPKs and other pathway members and demonstrate that an individual DUSP can regulate immunological responses in a positive way.

DUSP2 was one of the most highly induced genes and indeed the most highly induced DUSP after cellular activation of many human leukocyte types. PAC-1 expression, unlike that of other DUSP family members, was restricted to immune cells and was particularly associated with immune cells infiltrating inflammatory lesions. That indicates an important and unique function for PAC-1 in the regulation of MAPK signaling for immune cell effector function. The reversible activation of MAPKs in the presence of persistent activating stimuli suggests that protein phosphatases are as important as kinases for the control of MAPKs. It is likely that multiple DUSPs act cooperatively to control the spatio-temporal activity of MAPKs required for gene expression. Determining the tissue expression patterns of DUSPs and the control of their activity by either transcription or oxidation of the catalytic cysteine[34], coupled with identification of the subcellular locations of the individual DUSP family members, will be key in understanding the biological outcome of signaling through different MAPK pathways. For PAC-1, the very early and robust transcription in immune cells indicates PAC-1 is a chief regulator of nuclear MAPK events after receptor activation that is specific for immune responses.

Deficiency in PAC-1 had a profound effect on the function of activated mast cells and macrophages. The most obvious and quantifiable effects were reduced production of proinflammatory mediators. The expression of inflammatory cytokines, chemokines and other mediators was much lower in *Dusp2*$^{-/-}$ cells after activation through Toll-like receptor 4 or Fcε receptor crosslinking. That phenotype was not simply an artifact of PAC-1 deficiency, as overexpression of PAC-1 in cells enhanced inflammatory mediator production, and replacement of PAC-1 in PAC-1-deficient cells restored cytokine production. The reduced production of inflammatory cytokines in cells from *Dusp2*$^{-/-}$ mice was consistent with the protection we noted *in vivo* in the K/BxN model of arthritis. Many of the inflammatory mediators affected by PAC-1 deficiency have been linked to the pathogenesis of inflammatory arthritis, including TNF, various chemokines, C5aR and IL-6 (refs. 25,38,39). The other important effect we noted in PAC-1-deficient mast cells was reduced survival. Survival of mast cells has been linked mainly to IL-3 produced in an autocrine way independently of FcεRI signals[40]. Reduced mast cell survival in IL-3-dependent cultures in the absence of PAC-1 is consistent with the prosurvival effects of Erk[41] and the contrasting proapoptotic functions for Jnk[42,43] in regulating the death inducer Bim in mast cells. Mast cells are essential for disease pathogenesis in the K/BxN arthritis model[24]; hence, the profound effect of PAC-1 deficiency on mast cell function may be one reason we noted an obvious abnormal phenotype in this model. Because there was high induction of PAC-1 in many leukocyte types after cellular activation, it will be useful to assess whether PAC-1 has a similar proinflammatory function in neutrophils, T cells and B cells as well as in other inflammatory models.

We have provided evidence at many levels for the negative effects of PAC-1 deficiency on Erk and p38 MAPK signaling; decreased Erk and p38 phosphorylation also resulted in reduced kinase activity and, ultimately, in decreased Elk1 and NFAT–AP-1 transcriptional activity. The reduced production of proinflammatory mediators by mast cells and macrophages was due to the unexpected impairment of Erk and p38 phosphorylation. We initially considered that PAC-1 may serve additional functions in immune cells beyond MAPK regulation and might act on other substrates to positively control immune responses. However, mutation of the phosphatase catalytic site demonstrated the phosphatase-dependent nature of PAC-1 function. Moreover, studies using the kinase-interactive motif mutant suggested the importance of MAPK binding for PAC-1 function and that indeed PAC-1 did interact directly with Jnk, p38 and Erk. Despite the reportedly low phosphatase activity of PAC-1 for Jnk *in vitro*[8], PAC-1-deficient cells had increased and sustained Jnk activity, suggesting that *in vivo*, PAC-1 acts as a phosphatase of Jnk. Blockade of Jnk with a specific Jnk inhibitor completely reversed the reduced Erk activity in PAC-1-deficient cells, suggesting that crosstalk between these MAPKs is essential for the positive regulation of immune responses by PAC-1. In wild-type cells, Jnk inhibition also substantially increased Elk1 transcriptional activity in mast cells, emphasizing that this crosstalk is a chief contributor to cellular function. There are precedents for the involvement of MAPK crosstalk in the control of cellular responses[33,36]. Transforming growth factor-β, for example, inhibits inflammatory cytokine production through MAPK crosstalk, specifically Erk-dependent inhibition of p38 (ref. 44).

Mice deficient in DUSP10 (MKP5), a Jnk-dephosphorylating DUSP, have an expected increase in Jnk activity in T cells and no effect on p38, despite *in vitro* evidence for dephosphorylation of p38 by DUSP10 (MKP5)[7]. That coincides with enhanced cytokine production but reduced T cell proliferation[7]. DUSP1 (MKP1)–deficient mice have prolonged activity of both Jnk and p38 and no change in

Erk activity, resulting in alterations in the pattern of innate immune cell responses and, ultimately, in increased sensitization to endotoxin-induced sepsis[16]. In contrast, the increased Jnk activity but reduced Erk and p38 activity in PAC-1-deficient cells 'translated' into substantial defects in the expression of many MAPK-dependent proinflammatory genes and protection from inflammatory arthritis. That suggests that Erk and p38 are more important than Jnk in the control of inflammatory cytokine gene expression. We conclude that PAC-1 has a positive regulatory effect on immune cell functions that is mediated through crosstalk between Jnk and Erk. This previously unknown positive regulatory function of PAC-1 and also of another DUSP, JSP-1, which has demonstrated positive regulatory functions dependent on the phosphatase catalytic center[45,46], shows that DUSPs have the ability to influence biological outcomes in both a positive and a negative way.

In conclusion, PAC-1 fulfills a nonredundant function *in vivo* as a positive regulator of MAPK-dependent immune cell functions. PAC-1 transcription and expression is likely to be an important mechanism regulating leukocyte signaling, immune cell effector function and inflammatory responses. Because of its restricted expression pattern in immune cells, inhibition of the PAC-1 phosphatase function could be a useful means for manipulating MAPK activity specifically in immune cells, in particular for the treatment of inflammatory diseases.

METHODS

DNA microarray analysis of primary human leukocytes. Pure populations of primary human leukocytes were isolated and were given appropriate stimuli to examine gene expression after activation or differentiation, using Affymetrix GeneChip arrays as described[47]. Detailed information regarding isolation protocols, stimulus and time of activation, and microarray data sets is available (http://linkage.garvan.unsw.edu.au/public/microarrays/). The procurement of all human cells and tissues was approved by and was in accordance with the human research ethics committee of St. Vincent's Hospital (Sydney, Australia).

Generation of *Dusp2*$^{-/-}$ mice. The coding region of *Dusp2* (1.9 kilobases) was targeted by homologous recombination to produce deletion of the entire gene. A 6.3-kilobase *Xba*I restriction fragment flanking the 5′ coding region of the mouse *Dusp2* and a 2.3-kilobase *Hin*dIII fragment encompassing part of the *Dusp2* 3′ untranslated region and a sequence further downstream were cloned into the plasmid vector pGKNeo[48]. *Not*I-linearized pPAC1 plasmid was transfected by electroporation into W9 embryonal stem cells[49]. G418-resistant embryonic stem cell colonies were grown as described[49] and two independent embryonic stem cell clones (104 and 106), each with a targeted *Dusp2* allele, were further expanded and were microinjected into C57BL/6 blastocysts. Chimeric offspring were used for the generation of mice homozygous for the targeted null allele (*Dusp2*$^{-/-}$). Mice were backcrossed ten generations onto a C57BL/6 background and littermate control mice were used for experiments described. All experiments were approved by and were done in accordance with the animal research ethics committee of St. Vincent's Hospital and the Garvan Institute (Sydney, Australia).

Quantitative PCR. Mast cells were derived from human cord blood using an established method[50]. Mononuclear leukocytes were isolated from cord blood with a Ficoll density gradient and were matured over 10 weeks with 100 ng/ml of stem cell factor, 10 ng/ml of IL-10 and 5 ng/ml of IL-6. Cells were primed for 18 h with 4 μg/ml of human IgE antibody to dinitrophenol (anti-DNP) and then were activated for various times with 5 μg/ml of mouse anti-human IgE. Peripheral blood eosinophils were purified with Percoll and magnetic cell sorting (MACS; Miltenyi) with anti-CD16 for removal of contaminating neutrophils and were stimulated with 50 ng/ml of phorbol-12-myristate-13-acetate for various times. The human RAJI B cell line was stimulated for various times with 10 μg/ml of anti-IgM (Jackson) and the Jurkat T cell line, with 2 μg/ml of anti-CD3 (BD Pharmingen). Mouse thioglycollate macrophages and mouse BMMCs were isolated and stimulated as described (**Supplementary Methods** online). TRIzol reagent was used for the extraction of RNA from all cell types examined and, after synthesis of cDNA with the Reverse-IT RTase Blend Kit (ABgene), the Light Cycler-FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals) was used for real-time PCR, according to the manufacturer's specifications. The value obtained for each gene was normalized to that of the 'housekeeping' gene encoding glyceraldehyde phosphate dehydrogenase before the 'fold change' was calculated using crossing point values. Primers were as follows: h*DUSP2* forward 5′-CGG GAAAGACCGAAAGGAAG-3′, and reverse, 5′-GCCTCCTCGCAGGAAG TACA-3′; h*DUSP1* forward, 5′-CTGCCTTGATCAACGTCTCA-3′, and reverse, 5′-ACCCTTCCTCCAGCATTCTT-3′; h*DUSP4* forward, 5′-tacaagtgcatcccagtg-ga-3′, and reverse, 5′-CCCGTTTCTTCATCATCAGG-3′; h*DUSP5* forward, 5′-CAAATGGATCCCTGTGGAAG-3′, and reverse, 5′-GAGACCATGCTCCTC CTCTG-3′; and m*Tnf* forward, 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, and reverse, 5′-TGGGAGTAGACAAGGTACAACCC-3′.

K/BxN rheumatoid arthritis model. Blood was collected from 60-day-old K/BxN mice; serum was separated from blood and was frozen at –80 °C. Larger pools of serum were prepared for *in vivo* experiments and enzyme-linked immunosorbent assays (ELISAs) were done to confirm the concentration of glucose-6-phosphate isomerase arthritogenic immunoglobulin autoantibody. Arthritis was induced in recipient mice by intraperitoneal injection of 150 μl of serum on days 0 and 2, and disease progress was monitored for 25 d. Ankle thickness was measured with a caliper, with the change ankle thickness being defined as the difference from the measurement on day 0.

***Dusp2*$^{+/+}$ and *Dusp2*$^{-/-}$ macrophage and mast cell GeneChip arrays.** Thioglycollate-elicited peritoneal macrophages were stimulated for 6 h with 100 ng/ml of LPS and BMMCs were sensitized for 18 h with anti-DNP IgE (100 ng/ml) and were stimulated for 2 h with 20 ng/ml of DNP–human serum albumin. Total RNA was extracted with TRIzol and was reversed-transcribed with a poly(T) primer containing a T7 RNA polymerase promoter (5′-GGC CAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)-3′; Geneworks). Then, cRNA was transcribed from cDNA and was biotinylated with the BioArray HighYield RNA Transcript Labeling System (Enzo Diagnostics). Samples were hybridized to single Affymetrix mouse U74A (macrophages) or 430A (mast cells) microarrays and fluorescent signals were measured on the arrays with an Agilent GeneArray Laser Scanner. Gene transcript abundance was determined and was 'scaled' to 150 with algorithms in the MicroArray Analysis Suite Software 5.0 (Affymetrix). Two GeneChip analyses of independent mouse BMMC cultures were done. The signal data of the '.CEL' files for the four Affymetrix 430A chips (two *Dusp2*$^{+/+}$ and two *Dusp2*$^{-/-}$) were normalized using the RMA method[51]. The 'ebayes' function implemented in version 1.3.12 of the Limma package (2003) of Bioconductor was used to analyze the data, and *P* values were adjusted for multiple testing. After conversion from logged values to the original intensities, the data were filtered to remove probe sets with mean nonlogged intensities below 100. Transcripts with *P* values of less than 0.05 were considered different from *Dusp2*$^{+/+}$. Spotfire software was used for analysis and visualization of data.

The β-hexosaminidase assay. Supernatants from activated BMMCs were incubated for 2 h at 37 °C with 5 mM pNAG (*p*-nitrophenyl-*n*-acetyl-β-*d*-glucosaminidec; Sigma) in 0.05 M sodium citrate, pH 4.5. Reactions were stopped by the addition of 0.2 M glycine, pH 10.6, and the absorbance of samples at 405 nm was determined, where percent β-hexosaminidase release = absorbance of supernatant / (absorbance supernatant + absorbance lysate).

Cytokine ELISA. The concentration of TNF, IL-6 and IL-4 was measured in cell-free culture supernatants by ELISA (BD PharMingen) according to the manufacturer's instructions.

Retroviral infection of BMDMs. The retroviral packaging cell line Platinum-E (Plat-E) and the vector pMX have been described[29]. A human cDNA clone of *Dusp2* and the 'phosphatase-dead' mutant *Dusp2*^{C257S} were subcloned in-frame with an N-terminal triple-hemagglutinin epitope tag into the pMX vector containing an independent ribosomal entry signal, allowing expression of GFP. Plat-E cells were cultured in DMEM (4,500 mg/ml of glucose; Sigma), 10% FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 2 mM glutamine.

Virus was collected 48 and 72 h later after calcium phosphate transfection and was concentrated and processed as described[52]. Isolated bone marrow cells were cultured in suspension for 1 d, then were collected and resuspended in retrovirus BMDM medium (10% L929 supernatant supplemented with 10 ng/ml of IL-3, 50 ng/ml of colony-stimulating factor 1 and 8 μg/ml of polybrene). Cells were distributed at a density of 3×10^6 to 4×10^6 cells/1.5 ml per well of a nonadherent six-well plate (Evergreen Scientific). For spin-culture infection, plates were centrifuged for 90 min at 805*g* at 32 °C. Then, 1.5 ml medium (30% L929) supplemented with IL-3 and colony-stimulating factor 1 was added. On day 5, infected suspension cells were collected and were seeded on tissue culture dishes in BMDM medium (20% L929), which was 'exchanged' 1 d later. Experiments were done on day 8, and infection efficiency was in the range of 15–25%. Cells were stimulated for 4 h with LPS (1 μg/ml) with brefeldin A (10 μg/ml) for blockade of Golgi function, and intracellular cytokines were analyzed by flow cytometry of GFP^+ cells with anti-TNF, anti-IL-12 and anti-IL-6 (BD Pharmingen).

Transient transfection and luciferase assays in BMMCs. For the measurement of transcriptional Elk1 activity, 4×10^6 BMMCs were transfected with 1.75 μg pG5E4D38–Luc, 1.75 μg pSG-Gal–Elk1 and 1 μg pCMVβ (Clonetech) with solution T and an Amaxa 'nucleofection' device (set to the program T-016). For the measurement of transcriptional NFAT–AP-1 activity, BMMCs were transfected with 1 μg pCMVβ and 3.5 μg of the NFAT reporter plasmid pNFAT-Luc as described above. This NFAT reporter carries three tandem copies of the distal NFAT–AP-1 composite element of the human *IL2* promoter[53], which were cloned upstream of the minimal *IL2* promoter (from position –89 to position +51) and the luciferase reporter gene. Thus, this reporter is transactivated only if both NFAT and AP-1 are present in the nucleus and are assembled into a ternary complex. After transfection, BMMCs were plated in 3 ml culture medium and were allowed to recover for 24 h and then were sensitized for 18 h with 100 ng/ml of anti-DNP IgE. Subsequently, cells were split and were stimulated for 6 h (Elk1) or 8 h (NFAT–AP-1) with 20 ng/ml of DNP–heat-stable antigen or were left untreated. After being stimulated, cells were lysed in luciferase lysis buffer (Promega) and raw luciferase activity was determined with a luciferase activity kit (Promega). Relative luciferase units were calculated by division of luciferase activity with the corresponding relative β-galactosidase activity.

Immunoblot. Cells were lysed in ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, 1.5 mM $MgCl_2$, 1mM EGTA, 10 mM pyrophosphate, 100 mM NaF, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, 1 mM phenylmethylsulfonylfluoride, 200 μM Na_3VO_4, 20 μM MG132, 1 μM dithiothreitol. Lysates were centrifuged at 9,447*g* and protein concentration was assessed by detergent-compatible protein assay (BioRad); 10–15 μg protein was boiled in sample buffer (10% SDS, 30% glycerol, 0.75 M Tris, pH 6.8, 15% β-mercaptoethanol and bromophenol blue), was separated by 12% SDS-PAGE and was transferred to a nitrocellulose membrane. Membranes were blocked for 1 h at 25 °C with 5% skim milk powder in Tris-buffered saline–Tween (0.01%). Membranes were blotted according to the manufacturers' instructions with anti-phosphorylated Erk and anti-phosphorylated p38 (Cell Signaling Technology); anti-phosphorylated Jnk (Santa Cruz); antibodies to total Erk, total p38, total Jnk and NFATc1 (Santa Cruz); anti-hemagglutinin (Babco); and anti-histidine (Serotec).

***In vitro* kinase assays.** BMDMs were stimulated as described (**Supplementary Methods** online) and cells were lysed in 250 μl lysis buffer (20 mM HEPES, pH 7.5, 75 mM NaCl, 0.05% Triton, 2.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM $NaVO_4$, 0.2 mM EDTA, 50 mM β-glycerophosphate, 10 mM NaF, 1 mM benzamidine and protease inhibitors). Equal amounts of protein were then immunoprecipitated with antibodies to total Erk and p38 that cross-react with the unphosphorylated (inactive) form of MAPK and were used for kinase activity assays. Enzymes were resuspended in kinase assay buffer (20 mM HEPES, pH 7.5, 2 mM dithiothreitol, 20 mM $MgCl_2$, 0.1 mM Na_3VO_4, 20 mM β-glycerophosphate, 20 mM pNPP and 80 μM ATP) and were incubated for 60 min at 30 °C with 10 mg/ml of myelin basic protein (for Erk) and 1.5 mg/ml of ATF-2 (for p38) and 10 μCi [^{32}P]ATP. Reactions were stopped by the addition of sample buffer and heating for 5 min at 95 °C. Supernatants were analyzed by SDS-PAGE followed by autoradiography for 24 h.

Accession codes. GEO database: human leukocyte microarray data, GSE3982; $Dusp2^{+/+}$ and $Dusp2^{-/-}$ macrophages and mast cell microarrays, GEO4014.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (See the *Nature Immunology* website for details).

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