

TAp73 is required for macrophage-mediated innate immunity and the resolution of inflammatory responses

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The multiple isoforms of p73, a member of the p53 family, share the ability to modulate p53 activities but also have unique properties, leading to a complex and poorly understood functional network. *In vivo*, p73 isoforms have been implicated in tumor suppression (TAp73^{-/-} mice), DNA damage (Δ Np73^{-/-} mice) and development (p73^{-/-} mice). In this study, we investigated whether TAp73 contributes to innate immunity and septic shock. In response to a lethal lipopolysaccharide (LPS) challenge, TAp73^{-/-} mice showed higher blood levels of proinflammatory cytokines and greater mortality than their wild-type littermates. *In vitro*, TAp73^{-/-} macrophages exhibited elevated production of tumor necrosis factor alpha, interleukin-6 and macrophage inflammatory protein-2 as well as prolonged survival, decreased phagocytosis and increased major histocompatibility complex class II expression. Mice depleted of endogenous macrophages and reconstituted with TAp73^{-/-} macrophages showed increased sensitivity to LPS challenge. These results suggest that macrophage polarization is altered in the absence of TAp73 such that maintenance of the M1 effector phenotype is prolonged at the expense of the M2 phenotype, thus impairing resolution of the inflammatory response. Our data indicate that TAp73 has a role in macrophage polarization and innate immunity, enhancing the action field of this important regulatory molecule.

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P73 is a member of the p53 family,¹ in which all members are transcribed from two distinct promoters and undergo alternative splicing, giving rise to numerous isoforms whose individual functions have yet to be completely elucidated.² In the case of p73, the two major isoform families are the TAp73 proteins and the Δ Np73 proteins (lacking the N-terminal domain). P73 isoforms have been implicated in processes as varied as tumor suppression,^{3,4} apoptosis,⁵ control of genomic stability,^{6–8} DNA damage repair,⁹ development¹⁰ and reproduction.¹¹ However, the sheer number and inter-regulation of p73 isoforms results in a complex network in which it is difficult to dissect the functional contributions of individual isoforms. Using mouse models in which all p73 isoforms are deleted (p73^{-/-} mice),¹⁰ or only TAp73 isoforms (TAp73^{-/-} mice),³ or only Δ Np73 isoforms (Δ Np73^{-/-} mice),⁹ the involvement of various p73 proteins in tumorigenesis, development and inflammatory responses has been demonstrated. In this study, we focused on elucidating the role of TAp73 in inflammatory responses and innate immunity.

Cells mediating innate immunity, particularly macrophages and dendritic cells (DCs), express members of the Toll-like

receptor (TLR) family,¹² whose ligands are often pathogen components. For example, TLR4 binds to lipopolysaccharide (LPS), which is an integral cell wall component of gram-negative bacteria. LPS treatment of an animal provokes innate immune cells to release copious amounts of proinflammatory cytokines, resulting in a life-threatening condition called septic shock.¹³ The essential role of the LPS–TLR4 cascade in LPS-induced septic shock was established by studies of mice carrying spontaneous, induced or targeted mutations in genes contributing to this signaling pathway. At the molecular level, TLR4 engagement in innate immune cells leads to the activation of MAPKs and I κ B kinases, and thus NF κ B activation.¹⁴ At the cellular level, the binding of LPS to TLR4 on resting macrophages first induces STAT-dependent activation and maturation of these cells into M1 effector macrophages, which show enhanced proinflammatory cytokine release.¹⁵ Later in the inflammatory response, some of these M1 macrophages differentiate in a STAT3/6-dependent manner into regulatory M2 macrophages, which exhibit immunosuppressive properties. Moreover, specific Th2 cytokines as interleukin-4 (IL-4) are also known to induce

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Abbreviations: LPS, lipopolysaccharide; TNF α , tumor necrosis factor alpha; IL, interleukin; MIP, macrophage inflammatory protein; MHC, major histocompatibility complex; TLR, Toll-like receptor; i.p., intraperitoneal; EPM, elicited peritoneal macrophage; IFN, interferon; p53RE, p53-responsive element; CXCL, chemokine; DC, dendritic cell; MEF, mouse embryonic fibroblast; UTR, untranslated region

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M1-to-M2 transition.¹⁶ M2 cells produce only low levels of inflammatory cytokines, begin to secrete anti-inflammatory cytokines, and acquire increased capacity to phagocytize apoptotic cells.¹⁷ Owing to their central role in innate immunity, the hyperactivation of macrophages, and/or their extended maintenance as M1 cells, causes sustained inflammation that can disrupt the functions of vital organs.¹⁸

The results of this study show that the responses of TAp73^{-/-} macrophages to LPS treatment are deregulated, resulting in the increased proinflammatory cytokine production characteristic of M1 macrophages. TAp73^{-/-} macrophages also show partially impaired phagocytic ability and increased expression of major histocompatibility complex class II (MHCII). Finally, TAp73^{-/-} macrophages exhibit increased resistance to apoptotic death that is associated with abnormal expression of antiapoptotic genes. These data probably explain why TAp73-deficient mice show heightened sensitivity to LPS-induced septic shock, and indicate that TAp73 acts as a suppressor of the innate immune response by modulating macrophage polarization.

Results

TAp73-deficient mice are hypersensitive to LPS-induced inflammation and septic shock. According to above statements and previous studies,¹⁹ we hypothesized that an absence of TAp73 in mice might lead to accelerated tissue damage, and thus increased sensitivity to LPS-induced septic shock. To test this idea, we intraperitoneally (i.p.)-injected wild-type (WT) and TAp73^{-/-} mice with a lethal dose of LPS (25 mg/kg) and monitored the survival. A significant increase in lethality was observed in the absence of TAp73 (Figure 1a). Interestingly, TAp73^{-/-} mice also showed accelerated tissue damage compared with LPS-treated WT mice, with lesions appearing within 6 h of LPS injection (Supplementary Figure S1). This survival defect was specifically related to the loss of TAp73 isoforms, as the survival of LPS-treated Δ Np73-deficient mice was comparable to that of LPS-treated WT mice (Supplementary Figure S2). Moreover, we showed that while p53 was not modulated in TAp73-deficient cells, TAp73 was rapidly phosphorylated, suggesting a induction of its activity (Supplementary Figure S3A and S3B). These data suggested that TAp73 might have a modulatory role in the innate immune response.

The hypersensitivity of TAp73^{-/-} mice to LPS-induced septic shock correlates with increased production of proinflammatory cytokines. Hypersensitivity to LPS-induced septic shock is often a consequence of high levels of inflammatory cytokines in the blood and tissues of the affected animals.²⁰ We therefore analyzed proinflammatory cytokine levels in the serum of LPS-treated WT and TAp73^{-/-} mice. By 6 h post-LPS injection, elevated levels of tumor necrosis factor alpha (TNF α), IL1- β , macrophage inflammatory protein-2 (MIP-2) and IL-12p70 were observed in TAp73^{-/-} serum compared with WT controls (Figure 1b), in line with our hypothesis.

Macrophages are the major producers of proinflammatory cytokines during innate responses. To determine whether macrophages were the source of the elevated cytokines in

LPS-treated TAp73^{-/-} mice, we i.p. injected thioglycollate into WT and TAp73^{-/-} mice to elicit peritoneal macrophages and cultured these cells *in vitro* in the presence of LPS to stimulate cytokine production. Indeed, levels of TNF α , IL1- β and IL-12p70 were increased in the culture supernatants of LPS-treated TAp73^{-/-} elicited peritoneal macrophages (EPMs) compared with LPS-treated WT EPMs (Figure 1c). Similar results were obtained when we compared cytokines in culture supernatants of LPS-treated mouse embryonic fibroblasts (MEFs) isolated from WT and TAp73^{-/-} mice (Figures 1d and e). Thus, loss of TAp73 enhances proinflammatory cytokine production by macrophages in response to LPS stimulation both *in vivo* and *in vitro*. Interestingly, the same phenotypic features have been observed in LPS-treated p53^{-/-} mice,¹⁹ highlighting the importance of p53 family members in inflammation.

Loss of TAp73 skews macrophage polarization towards the M1 phenotype. Vigorous production of proinflammatory cytokines is a hallmark of classically activated macrophages, also known as effector or M1 macrophages. Excessive or prolonged activation of the molecular program that drives M1 polarization is deleterious for the host and correlates with increased severity of septic shock.²¹ Interferon gamma (IFN γ) is a key cytokine involved in the M1 program, inducing macrophage activation characterized by increased MHCII expression and elevated inflammatory mediator production.²² We found that IFN γ levels were increased compared with controls both in the serum of TAp73^{-/-} mice at 6 h post-LPS treatment (Figure 2a, left) and in the culture supernatants of LPS-treated TAp73^{-/-} EPMs (Figure 2a, right). In addition, although similar total EPM numbers (as determined by F4/80⁺ staining) were recovered from thioglycollate-injected WT and TAp73^{-/-} mice (Supplementary Figures S4 A and B), a greater percentage of macrophages from TAp73^{-/-} mice (30%) were positive for MHCII compared with macrophages from WT mice (16%), representing an 1.83-fold increase (Figure 2b, left). Moreover, the level of MHCII expression in TAp73^{-/-} F4/80⁺ MHCII⁺ macrophages was 1.9-fold higher than in WT F4/80⁺ MHCII⁺ macrophages, as determined by mean fluorescence intensity (Figure 2b, right). This difference in MHCII expression was confirmed *in vitro* when WT and TAp73^{-/-} EPMs were treated with LPS (Figures 2c and d and Supplementary Figures S4 C and D). Surprisingly, TAp73^{-/-} EPMs were also less able than WT EPMs to phagocytize apoptotic thymocytes following an LPS/IFN γ challenge (Figure 2e). Taken together, these data suggest that an absence of TAp73 promotes the polarization of macrophages towards the M1 phenotype.

Loss of TAp73 modifies gene expression patterns in LPS-treated macrophages. TAp73 is a powerful transcription factor and drives the expression of a wide variety of genes with some related to immune cell responses/abilities. We used cDNA microarray analysis to evaluate the effect of TAp73 loss on macrophage gene expression by treating WT and TAp73^{-/-} EPMs with LPS and isolating RNA at various time points thereafter. LPS is known to profoundly modulate the transcriptional profile of macrophages in a reproducible

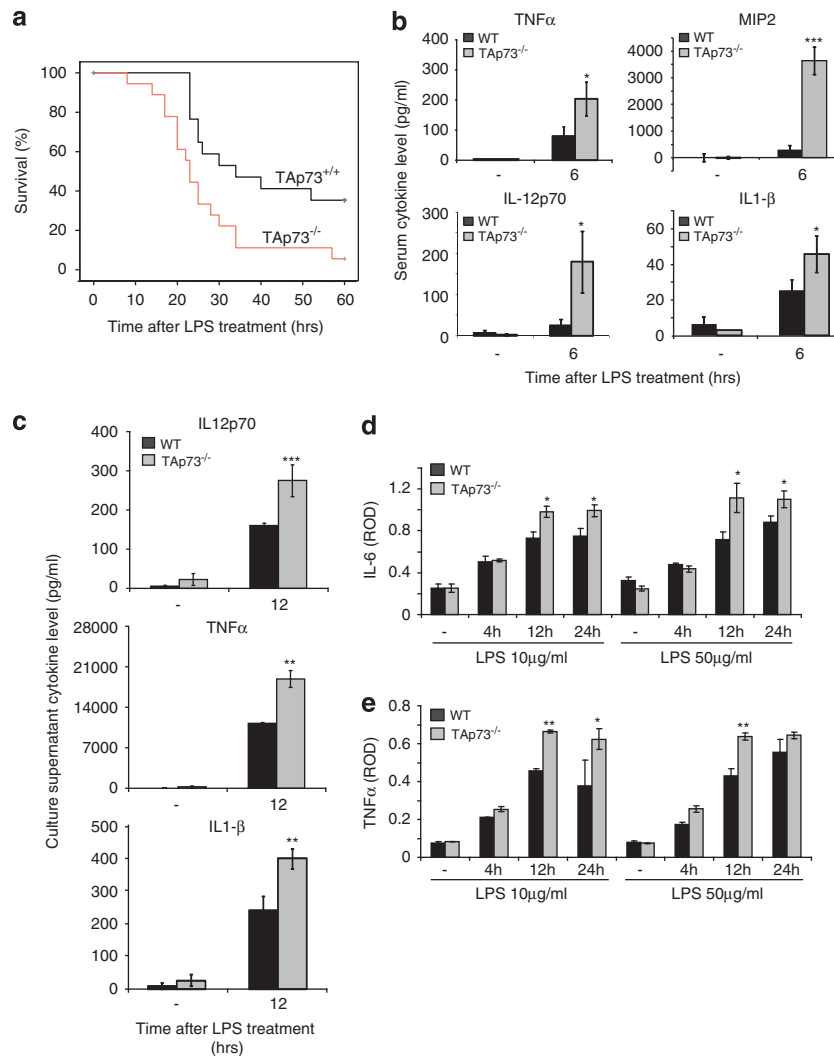


Figure 1 Increased production of inflammatory cytokines and enhanced septic shock in TAp73^{-/-} mice. **(a)** Hypersensitivity to LPS-induced septic shock. LPS (25 mg/kg) was administered to WT and TAp73^{-/-} male mice (10–14-week-old; $n = 18$ /group) and survival was monitored for 60 h. Results shown are data pooled from two independent experiments. $P < 0.007$ (total $n = 18$ /group; log-rank test). **(b)** Increased serum TNFα, MIP-2, IL-12p70 and IL1-β. WT and TAp73^{-/-} mice were treated with LPS (25 mg/kg) for 6 h and the indicated serum cytokines were measured by ELISA. Data are the mean \pm S.D. ($n = 3$) and are representative of two independent experiments. **(c)** Increased EPM cytokines. EPMs from WT or TAp73^{-/-} mice were stimulated *in vitro* with LPS (1 μg/ml) for 12 h, and IL-12p70, TNFα and IL1-β in culture supernatants were measured by ELISA. **(d and e)** Increased MEF cytokines. WT and TAp73^{-/-} MEFs were stimulated *in vitro* with the indicated doses of LPS for the indicated times, and IL-6 **(d)** and TNFα **(e)** in culture supernatants were measured by ELISA (ROD; relative optical density). For B–E, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's *t*-test)

way, allowing us to validate our model and establish lists of genes that are differentially regulated in the absence of TAp73. We observed such changes to the expression of genes involved in macrophage differentiation and activation (Figure 3a), in survival or apoptosis (Supplementary Figure S5), as well as in immune responses and in metabolism (Supplementary Figure S6). In particular, expression levels of the proapoptotic genes *Sparc*, *Bim* and *Ankrd1* were all decreased in TAp73^{-/-} EPMs, whereas genes involved in survival and proliferation, including *Pim1*, *Api6* and *Pik3ap1*, were all upregulated in these cells (Supplementary Figure S5). Expression levels of the macrophage chemokines *c10* and *MCP3* were also upregulated, but genes involved in or regulated by phagocytosis, such as *MFG-E8*, *ligp1*, *Mgat5* and *p57* were variably affected by loss of TAp73 (Figure 3a,

Supplementary Figures S5 and S6). These data are in line with our earlier results (Figure 2e) and confirm a recent report that MFG-E8 is a direct target of p73.²³ Notably, the expression of *GM-CSF*, which promotes M1 macrophage polarization,¹⁷ was increased in LPS-treated TAp73^{-/-} EPMs (Supplementary Figure S5). Finally, the expression patterns of genes involved in macrophage metabolism were greatly modified in the absence of TAp73 (Supplementary Figure S6). Although little is known regarding macrophages metabolism/activity, it is actually an expanding field in which fatty acid and polyamines metabolism seems crucially involved.²⁴

Next, we validated our cDNA microarray results using real-time PCR analysis of selected genes. Gene expression patterns were altered compared with WT controls not only in

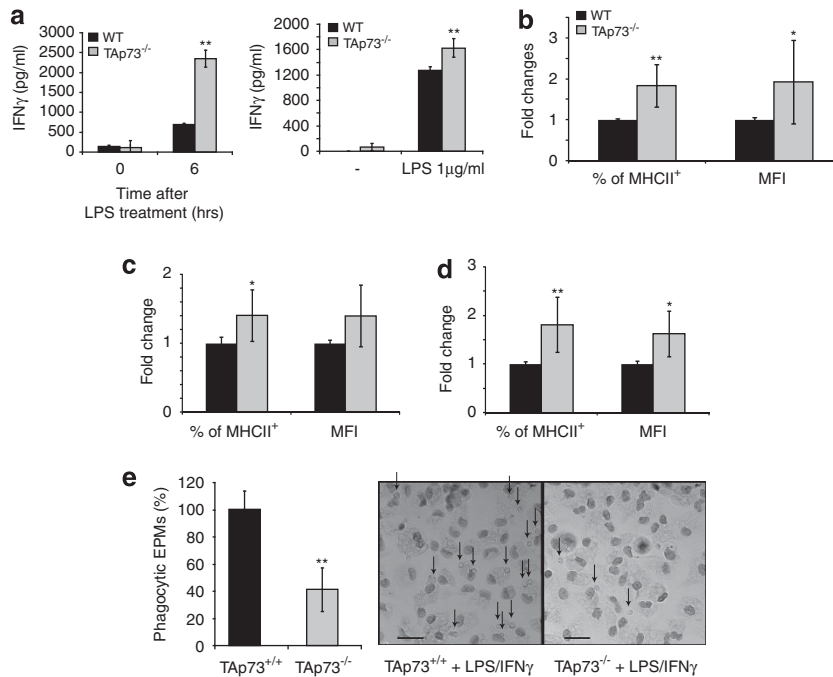


Figure 2 TAp73 deficiency promotes M1 polarization of macrophages. **(a)** Increased IFN γ . IFN γ levels in WT and TAp73 $^{-/-}$ mouse serum samples (left) and EPM culture supernatants (right) were determined as for Figures 1b and c, respectively. **(b)** Increased MHCII. EPMs were recovered from WT ($n = 6$) and TAp73 $^{-/-}$ ($n = 7$) mice at 96 h postthioglycollate injection. Total cells from peritoneal washes were gated on F4/80 $^{+}$ macrophages and analyzed by flow cytometry for expression of surface MHCII. Fold change in (left) the percentage of MHCII $^{+}$ macrophages present in the F4/80 $^{+}$ population, and (right) mean fluorescence intensity attributable to MHCII in F4/80 $^{+}$ /MHCII $^{+}$ macrophages. **(c and d)** Confirmation of MHCII increase *in vitro*. WT and TAp73 $^{-/-}$ EPMs were cultured *in vitro* for 24 h and then left untreated **(c)** or treated with LPS (1 μ g/ml) for 24 h **(d)**. The fold change in the percentage of MHCII $^{+}$ macrophages present (left), and the mean fluorescence intensity (MFI) of MHCII in F4/80 $^{+}$ /MHCII $^{+}$ macrophages (right) are shown. **(e)** Decreased percentage of phagocytotic EPMs. WT or TAp73 $^{-/-}$ EPMs were incubated for 2 h with apoptotic thymocytes in the presence of 10 ng/ml LPS plus 100 nM IFN γ . Macrophages that had phagocytized apoptotic cells were quantified and the data expressed as a phagocytic index (see Materials and Methods). Left: results shown are the mean phagocytic index \pm S.E.M. ($n = 3$). Right: representative micrographs of WT and TAp73 $^{-/-}$ EPMs that had bound to or phagocytized apoptotic cells (arrows). Scale bar = 20 μ m. For **(a–e)**, data shown are pooled from two independent experiments. * $P < 0.05$; ** $P < 0.01$; (Student's *t*-test)

TAp73 $^{-/-}$ EPMs treated with LPS *in vitro* (Figure 3b and Supplementary Figure S7A) but also for the lung (Figure 3c) and liver (Figure 3d and Supplementary Figure S7B) tissues isolated from LPS-injected TAp73 $^{-/-}$ mice. Among the genes with the most significantly modified profiles (Figure 3a, Supplementary Figures S5 and S6, column 8 and 10, respectively) were several containing putative p53-responsive elements (p53RE).²⁵ By using chromatin immunoprecipitation assay we validated for some candidates that TAp73 was physically interacting with their p53REs (Figure 3a, column 9). These results suggest that an absence of TAp73 promotes changes in gene expression patterns that skew the polarization of macrophages responding to stimuli that activate innate immunity.

TAp73 $^{-/-}$ macrophages show impaired induction of the IFN- β pathway. To determine whether a specific pathway was involved in the gene expression modifications we observed in the absence of TAp73, we analyzed our cDNA microarray results in depth. In response to LPS exposure, WT macrophages activate the transcription of a large number of proinflammatory genes through signaling pathways downstream of TLR4. Many of these genes are expressed sequentially, with early gene products driving the secretion of soluble factors that activate the transcription of genes

expressed later in the response. We found that many of the genes whose expression patterns were altered in the absence of TAp73 were active components of the IFN- β pathway, a cytokine synthesized immediately after LPS stimulation.²⁶ We found that levels of IFN β mRNA were lower in the lung (Figure 3c1) and liver (Figure 3d1) tissues isolated from LPS-injected TAp73 $^{-/-}$ mice compared with tissues from LPS-injected WT mice. This difference reached a maximum at ~ 1.5 h post-LPS, correlating with the established timing of IFN β induction. TAp73 $^{-/-}$ EPMs treated with LPS *in vitro* also showed a decrease in IFN β mRNA expression compared with LPS-treated WT EPMs (Figure 3b1).

We then investigated whether TAp73 could directly regulate IFN β transcription. We transfected the RAW264.7 murine macrophage cell line with a vector expressing a luciferase reporter gene driven by the IFN β promoter region. As shown in Figure 3e, overexpression of the α isoform of TAp73 (TAp73 α) alone was able to enhance IFN β expression. However, TAp73 α -expressing RAW264.7 cells treated with LPS showed a much greater induction of IFN β . These results imply that a link exists between the LPS signaling pathway and TAp73 that promotes IFN β production. Nevertheless, extensive analysis of the IFN β promoter region did not reveal the presence of a p53RE, suggesting that the effect of TAp73 α on IFN β transcription is indirect.

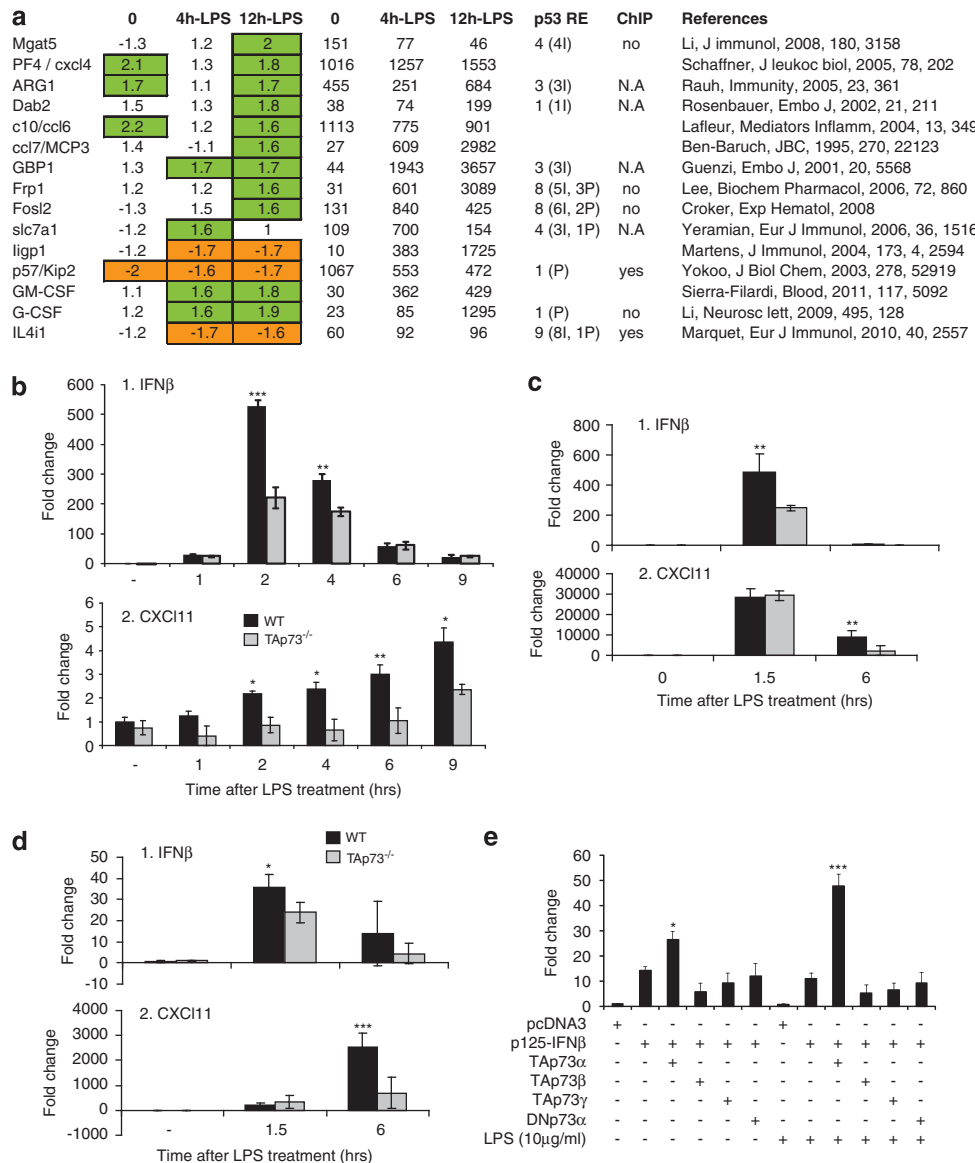


Figure 3 Loss of TAp73 modulates gene expression profiles. **(a)** Expression of key genes involved in macrophage activity/activation. Fold change represents the ratio of the hybridization intensity of a given gene in TAp73^{-/-} EPMs compared with WT EPMs. Data are shown for the 'no treatment' condition (0), and for LPS treatment for 4 h (4 h-LPS) or 12 h (12 h-LPS). Columns 5–7 are showing raw value data from microarray analysis. Only genes for which a statistical difference of $P < 0.01$ was obtained are shown. The p53RE column contains results obtained from the p53 Family Target Genes Database, and p53RE present in an intron (I), the promoter region (P) or in the 3' untranslated region (U). Column 9 shows ChIP results done on WT EPMs after 12 h of LPS treatment. **(b)** WT or TAp73^{-/-} EPMs were treated for increasing period of time from 1–9 h with LPS (10 μ g/ml) and levels of (1) IFN β and (2) CXCL-11 mRNAs were determined by real-time PCR. Data shown are the mean fold change \pm S.D. in mRNA expression after LPS treatment. Results are one trial representative of three independent experiments. **(c)** Lung tissue. WT and TAp73^{-/-} mice were treated with LPS (25 mg/kg) for 1.5 or 6 h. Lung tissues were recovered and levels of (1) IFN β and (2) CXCL-11 mRNAs were determined as in **(b)**. Data are the mean fold change \pm S.D. in mRNA level ($n = 3$ mice/group). Results are representative of two independent experiments. **(d)** Liver tissue. Levels of (1) IFN β , and (2) CXCL-11 mRNAs in liver tissues from the mice in **(c)** were analyzed as in **(c)**. **(e)** TAp73 promotes IFN β transcription. RAW267.4 cells were co-transfected with a vector containing the p125 luciferase reporter gene driven by the IFN β promoter, either alone (p125-IFN β) or with vectors encoding the indicated p73 isoforms. These cells were left untreated (-LPS) or treated for 24 h with LPS (10 μ g/ml). Luc values were determined as described in Materials and methods. Data shown are mean \pm S.D. ($n = 3$) and are representative of three independent experiments. For **(a–e)**, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's *t*-test)

To confirm that the observed decrease in IFN β production associated with loss of TAp73 was sufficient to modify IFN β -dependent events in the LPS-induced inflammatory cascade, we revisited our mRNA induction data and focused on the expression of chemokine (CXCL)-11 (also known as I-TAC), which is known to be an IFN β -dependent gene.²⁷ Compared with controls, significantly less

CXCL-11 mRNA was expressed in LPS-treated TAp73^{-/-} EPMs (Figure 3b2 and Supplementary Figure S6), as well as in the lung (Figure 3c2) and liver (Figure 3d2) tissues from LPS-injected TAp73^{-/-} mice. These results further implicate TAp73 in the regulation of IFN β pathway, and are consistent with TAp73's link to M1/M2 polarization.²⁸

TAp73-deficient macrophages are resistant to apoptosis induced by LPS. Survival pathways and apoptotic pathways are often coupled to the same receptors, allowing an immune cell to control its lifespan and restrict the extent of its activation such that damage to self tissues is limited.²⁹ Several studies have reported that macrophage survival is closely linked its M1/M2 phenotype. Unlike DCs, macrophages do not die immediately after activation with LPS but become refractory to a subsequent LPS challenge and more susceptible to death induced by other means.³⁰ Following previous hypothesis, macrophages that exhibit prolonged survival are most probably those that have been activated during an LPS-induced cascade, being a marker of their functional status in innate response.³¹ Accordingly, these cells should show decreased proapoptotic gene expression and increased survival gene expression. On the basis of our earlier results, TAp73^{-/-} macrophages are hypersensitive to LPS treatment and should show even lower levels of proapoptotic gene expression and higher levels of survival gene expression, a hypothesis confirmed by our data in Supplementary Figure S5. In addition, when we treated WT and TAp73^{-/-} EPMs *in vitro* with various doses of LPS, the TAp73^{-/-} EPMs were less sensitive to LPS-induced death as judged by their lower levels of caspase-3 activity (Figure 4a). The percentage of PI/AnnexinV-positive cells was also reduced among LPS-treated TAp73^{-/-} EPMs compared with WT controls (Supplementary Figure S8A). These data confirm that loss of TAp73 decreases sensitivity to LPS-induced cell death. We then treated WT and TAp73^{-/-} EPMs with various other death-inducing agents and showed that the absence of TAp73 protected the cells against death induced by IFN γ and/or LPS as well as by staurosporine, but not by transforming growth factor beta or etoposide (Figure 4b and Supplementary Figure S8B). Resistance of TAp73^{-/-} EPMs to staurosporine-induced cell death confirms the role of TAp73 in the intrinsic pathway of the apoptotic process.³² Such a regulatory role could explain why loss of TAp73 prolongs the M1 program in macrophages of mice subjected to agents inducing septic shock.

TAp73 is crucial for the regulation of macrophage responses to LPS *in vivo*. Our results above implicated TAp73 as an important regulator of TLR4-dependent macrophage responses in innate immunity. We therefore asked whether the hypersensitivity to septic shock observed in TAp73^{-/-} mice was due to the abnormal behavior of their macrophages. To explore this hypothesis, we devised a strategy to replace the endogenous macrophages of BALB/c mice with WT or TAp73^{-/-} EPMs. We reconstituted WT and TAp73^{-/-} EPMs in allogeneic BALB/c mice that had been depleted of macrophages by gadolinium chloride (GdCl₃) treatment.¹⁵ Then, we determined the effects of LPS treatment on recipients reconstituted with WT or TAp73^{-/-} EPMs. Mice reconstituted with TAp73^{-/-} EPMs started to die within 11 h of LPS challenge (Figure 4c). By 24 h post-LPS, >90% of mice that had received TAp73^{-/-} EPMs had died, whereas only 50% of mice that had received WT EPMs had succumbed. By the end of the experiment, 100% of mice that had received TAp73^{-/-} EPMs had died, whereas 25%

of mice that had received WT EPMs were still alive. This accelerated LPS-induced death of mice reconstituted with TAp73^{-/-} EPMs is consistent with deregulated macrophage function.

When we measured the levels of various cytokines in serum samples from recipients reconstituted with WT or TAp73^{-/-} EPMs at 6 h post-LPS, we saw a 10-fold increase in serum TNF α in mice that had received TAp73^{-/-} EPMs compared with those reconstituted with WT EPMs (Figure 4d). Interestingly, the serum TNF α level in recipients reconstituted with WT EPMs was comparable to that in WT mice at 6 h post-LPS challenge (Figure 1b). These results confirm that mice possessing TAp73^{-/-} macrophages produce more TNF α , rendering the animals more susceptible to LPS-induced septic shock than mice possessing WT macrophages. Thus, the hypersensitivity of TAp73^{-/-} mice to LPS-induced septic shock is due to dysregulation of the macrophage-mediated innate immune response.

Discussion

Little is known about the function of p73 in the immune system. Indeed, of all p53 family members, only p53 has been clearly shown to be involved in regulating innate immunity.¹⁹ CEP-1, the *Caenorhabditis elegans* homolog of p53, has been implicated in an ancient innate immune mechanism that combats bacterial pathogens,³³ and p53^{-/-} mice show uncontrolled increased inflammation.³⁴ A function for p73 in regulating inflammation has been hinted at by the phenotypes of mice functionally deficient for all p73 isoforms, as these animals exhibit chronic infections and persistent inflammation.¹⁰ In humans, several recent reports have linked p73 expression to inflammatory diseases such as otitis media, gastritis and chronic rhinosinusitis.^{35,36} However, definitive evidence as to which p73 isoforms are involved and their specific roles has been lacking.

Our examination of TAp73^{-/-} mice has delineated a role for at least the TAp73 isoform in innate immunity. TAp73^{-/-} mice exhibit heightened responses to LPS stimulation compared with TAp73^{+/+} mice, producing greater amounts of proinflammatory cytokines such as TNF α , IL-6 and MIP-2. Importantly, our *in vitro* work confirmed that both inflammatory TAp73^{-/-} cells (thioglycollate-EPMs) and primary TAp73^{-/-} cells (MEFs) demonstrate the same abnormalities: an enhanced response to LPS and increased production of proinflammatory cytokines. We believe that it is the impaired macrophage polarization that occurs in TAp73^{-/-} mice that is responsible for the increased morbidity of these mutants upon LPS exposure.

Macrophages are routinely described as either M1 effectors (classically activated) or M2 regulators (alternatively activated) (for a review, see Murray *et al.*³⁷). M1 macrophages have potent microbicidal properties and promote strong IL-12-mediated Th1 responses, whereas M2 macrophages have a role much later in infection and help to resolve inflammation by promoting endocytic clearance, synthesizing trophic factors, and producing fewer proinflammatory cytokines while ramping up anti-inflammatory cytokines. However, it remains unclear whether these M1 and M2 subtypes are truly representative of the tissue macrophages that are present in

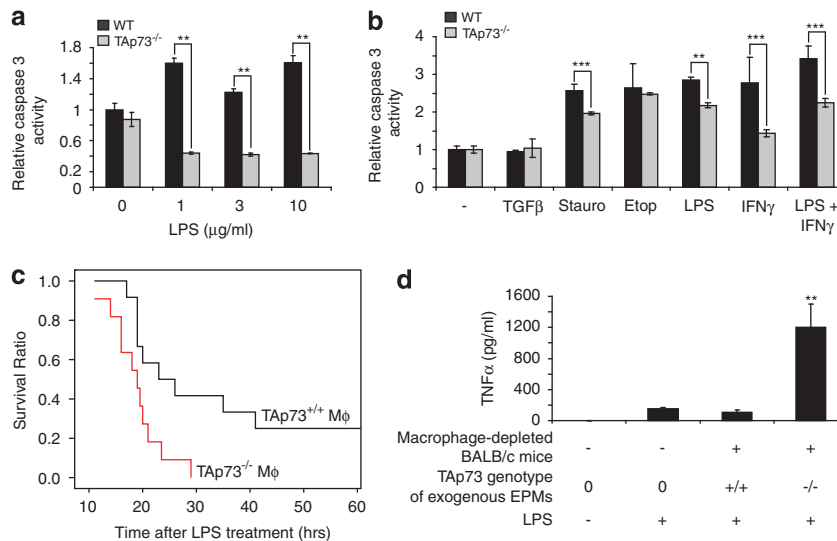


Figure 4 Loss of TAp73 protects EPMs from some apoptotic stimuli and is responsible for the hypersensitivity of TAp73^{-/-} mice to LPS-induced septic shock. (a) LPS-induced apoptosis. WT and TAp73^{-/-} EPMs were treated *in vitro* for 12 h with the indicated doses of LPS. Caspase-3 activity was determined as described in Materials and methods. (b) Other apoptotic stimuli. WT and TAp73^{-/-} EPMs were treated with the indicated apoptotic stimuli (see Materials and methods) for 48 h, and caspase-3 activity was determined as for (a). Stauro, staurosporine; Etop, etoposide. (c) Survival after macrophage reconstitution (see Materials and methods). $P < 0.01$ (log-rank test). (d) Increased serum TNFα determined by ELISA (see Materials and methods). For (a–d), data shown are the mean \pm S.D. (a, b and d) and are percentage survival (c) and are pooled results from two experiments. For (a and b), each involves EPMs from two mice/genotype (Student's *t*-test), for (c) involving three WT and three TAp73^{-/-} EPMs/experiments injected in two BALB/c mice each (log-rank test), and for (d) $n = 6$ per group (Student *t*-test). ** $P < 0.01$, *** $P < 0.001$.

whole animals and participate in processes related to homeostasis, infection, and tissue repair. It is also unknown how plasticity in gene expression patterns regulates macrophage functions *in vivo*. In our study, we investigated whether TAp73^{-/-} macrophages could carry out normal M1/M2 polarization and found that a loss of TAp73 led to increased production of proinflammatory cytokines, particularly the M1-polarizing cytokine IFNγ. In addition, compared with WT EPMs, TAp73^{-/-} EPMs showed both enhanced basal expression of MHCII and a greater increase in MHCII following LPS exposure. A high level of MHCII expression is one of the criteria used to determine M1 polarization, because antigen presentation is enhanced when the M1 subtype predominates. Finally, TAp73^{-/-} EPMs could not phagocytize apoptotic thymocytes to the same extent as WT EPMs, an important observation as bacterial clearance is a key component of sepsis resolution and is driven mainly by M2 macrophages.³⁸

Our transcriptomic analyses of TAp73^{-/-} macrophages also provide evidence that TAp73 is a key factor in macrophage polarization. In the absence of TAp73, macrophages showed decreased expression of many phagocytosis-related genes, including *MFG-E8*, which was recently identified as a direct target of p73.²³ Our results further revealed that TAp73^{-/-} macrophages had a reduced ability to produce IFNβ in response to LPS treatment, and enhanced IFNβ production has been correlated with M2 polarization.²⁸ In addition, we noted significant differences in macrophage survival and death after LPS challenge in the absence of TAp73. Although the mechanisms driving macrophage death during early acute inflammation have not been fully elucidated, apoptosis clearly occurs. A recent study has shown that decreased macrophage apoptosis results in enhanced inflammation, whereas increased macrophage apoptosis

contributes to the resolution of inflammation.³⁹ Our results are consistent with this line of evidence, in that we demonstrated that TAp73^{-/-} macrophages show decreased apoptotic death, and TAp73^{-/-} mice exhibit greater sensitivity to an LPS challenge and succumb faster to rampant sepsis. Finally, our injections of TAp73^{-/-} or TAp73^{+/+} macrophages into macrophage-depleted recipients confirmed that loss of TAp73 in macrophages reduces the survival of animals challenged with LPS. Finally our transcriptomic analysis did not show any modulation of other characterized p73-related gene that could have been implicated in M1/M2 balance as IL4Ra for the IL4 pathway, a well-described process known to impact on M1/M2 balance, in favor of M2 type. Interestingly, we found IL4-related genes such as IL411, a gene reported as expressed by M2 macrophages and whose expression is decreased in TAp73-deficient macrophages, reinforcing the hypothesis that TAp73 is implicated in M1/M2 macrophages switch. Taken together, our data show that M1/M2 macrophage polarization is compromised in the absence of TAp73 such that M1 cells predominate and that the resulting lack of M2 macrophages prevents the resolution of inflammation and increases the likelihood that the mutant mice will die of septic shock.

It is now generally accepted that chronic inflammation creates conditions facilitating tumorigenesis. Regarding our present data, it can be supposed that the development of cancer in mice deficient for p53 or TAp73 may not be due solely to a lack of p53-mediated control of genomic stability. Indeed, our work leads to hypothesize that the loss of normal M1/M2 macrophage polarization and the establishment of chronic proinflammatory conditions, through TAp73 loss, may be implicated in the establishment of a tumorigenic context. Such study is actually under investigation. In addition, because a variety of chronic inflammatory diseases, including

rheumatoid arthritis, atherosclerosis, diabetes and obesity, are regulated by macrophage-mediated innate immunity, it would be interesting to examine TAp73's contribution to these disorders.

Materials and Methods

Animals and *in vivo* LPS challenge. Male BALB/c mice (6–8 weeks old, weighing 16–20 g) were obtained from Charles River (L'Arbresle, France). TAp73^{+/+} and TAp73^{-/-} mice have been described previously.³ All mice were maintained at U1068 animal facility under pathogen-free conditions according to the Guidelines for the Care and Use of Laboratory Animals prepared by the US National Academy of Sciences and published by the US National Institutes of Health (publication 86–23; revised 1985). Male mice (10–14 weeks old) were i.p. injected with a single lethal dose of LPS (25 mg/kg; Sigma-Aldrich, Lyon, France).

Elicitation of peritoneal macrophages and EPM culture conditions. Male mice (12 weeks old) were i.p. injected with 1 ml 10% thioglycollate broth (T9032-550G; Sigma-Aldrich) and peritoneal macrophages were recovered 4 days later as previously described.⁴⁰ EPMs obtained from TAp73^{+/+} and TAp73^{-/-} mice, and the murine macrophage cell line RAW264.7 (from ATCC, Molsheim, France) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) at 37 °C in a humidified atmosphere containing 5% CO₂. Primary MEFs obtained from TAp73^{+/+} and TAp73^{-/-} mice were cultured in DMEM medium containing 10% FBS and antibiotics as above.

Phagocytosis. Freshly collected EPMs from WT or TAp73^{-/-} mice were plated at 2.5×10^4 cells/well on a 16-well chamber slide and cultured for 6 h until the cells became firmly adherent. To create targets for apoptosis, freshly collected WT thymocytes (10^7 cells/ml) were cultured for 12 h in RPMI containing 10% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, followed by irradiation (2 Gy). This procedure caused more than 50% of the thymocytes to undergo apoptosis, as judged by PI/Annexin-V analyses. The macrophage cultures were preincubated with LPS (10 ng/ml) plus IFN γ (100 nM) for 12 h and washed twice in PBS, after which the apoptotic thymocytes were added at a ratio of 4 : 1 (apoptotic cells/macrophages) for 2 h. Cultures were washed three times in PBS to remove loosely adherent thymocytes, fixed in methanol for 10 min, stained with hematoxylin/eosin, and covered in mounting medium. Phagocytosis was evaluated by counting 200–300 macrophages per well. Results were expressed as percentage of EPMs containing at least one ingested thymocyte (percent phagocytic). Values obtained for WT EPMs were referred as 100 percent.

Adoptive transfer of macrophages. Age- and sex- matched cohorts of BALB/c mice were i.v. injected with GdCl₃ (Sigma-Aldrich; 10 mg/kg body weight) to eliminate endogenous macrophages *in vivo*. At 24 h post-GdCl₃ injection, the macrophage-depleted mice were i.v. injected with TAp73^{+/+} or TAp73^{-/-} EPMs (1×10^7) resuspended in 100 µl pyrogen-free PBS. After another 24 h, the mice were i.p. injected with PBS or a lethal dose of LPS (25 mg/kg; Sigma-Aldrich) and mouse survival was monitored for 72 h. Serum level of TNF α was monitored in other set of mice killed 6 h after LPS injection.

Histopathology. Hematoxylin and eosin staining was used to visualize tissues, which were isolated and incubated in 4% formaldehyde fixative.

Real-time PCR. RNA was extracted from whole tissues, EPMs or RAW264.7 cells using Trizol Reagent (Life Technologies, Saint Aubain, France). Total RNA (1 µg) was reverse-transcribed with the Improm-II Reverse Transcription System (Promega, Charbonnières, France), and 5 µl cDNA was subjected to real-time PCR using specific SYBR green dye (Ozyme, Saint Quentin Yvelines, France) and a Lightcycler 2.0 PCR system (Roche Diagnostic, Meylan, France). PCR primers were acquired mainly from primer bank (<http://pga.mgh.harvard.edu/primerbank/>) (see Supplementary Information for sequences). Fold induction of gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Values for genes of interest were normalized to Tata box-binding protein expression values.

Luciferase reporter assay. RAW264.7 cells were plated at 5×10^4 cells/well in 48-well culture dishes. Using Fugene (Roche Diagnostics), cells were transfected with pcDNA3 (empty vector control), or p125-luc vector driven by the

IFN β promoter region (p125-IFN β); or co-transfected with p125-IFN β plus pcDNA3 plasmids expressing mouse TAp73 α , TAp73 β , TAp73 γ , DNp73 α , or p53. pEGFP was used as a transfection control. Luciferase activity was detected by the Luciferase Assay System (Promega).

Statistics. Most data are presented as the mean \pm S.D., and values of $P < 0.05$ were considered statistically significant as determined by the Student's *t*-test. Survival curves were estimated with univariate analyses according to the Kaplan–Meier method, and significance was determined using the log-rank (Mantel–Cox) test. Statistical analysis of the mean intensity fluorescence curve was performed using the Kolmogorov–Smirnov test.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

RT, GM, TWM and JLI designed the research; RT, VS, LP, AKT, JN and MW performed the research; RT, LP, VS, SV, MW, PB, GM and JLI analyzed the data; and RT wrote the paper.

- Yang A, Kaghad M, Caput D, McKeon F. On the shoulders of giants: p63, p73 and the rise of p53. *Trends genet* 2002; **18**: 90–95.
- Melino G, Lu X, Gasco M, Crook T, Knight RA. Functional regulation of p73 and p63: development and cancer. *Trends Biochem Sci* 2003; **28**: 663–670.
- Tomasini R, Tsuchihara K, Wilhelm M, Fujitani M, Rufini A, Cheung CC *et al*. TAp73 knockout shows genomic instability with infertility and tumor suppressor functions. *Genes Dev* 2008; **19**: 2677–2691.
- Flores ER, Sengupta S, Miller JB, Newman JJ, Bronson R, Crowley D *et al*. Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. *Cancer Cell* 2005; **4**: 363–373.
- Pietsch MC, Skyes SM, McMahon SB, Murphy ME. The p53 family and programmed cell death. *Oncogene* 2008; **27**: 6505–6521.
- Talos F, Nemajerova A, Flores ER, Petrenko O, Moll UM. p73 suppresses polyploidy and aneuploidy in the absence of functional p53. *Mol Cell* 2007; **4**: 647–659.
- Tomasini R, Mak TW, Melino G. The impact of p53 and p73 on aneuploidy and cancer. *Trends Cell Biol* 2008; **5**: 244–252.
- Tomasini R, Tsuchihara K, Tsuda C, Lau SK, Wilhelm M, Rufini A *et al*. TAp73 regulates the spindle assembly checkpoint by modulating BubR1 activity. *Proc Natl Acad Sci USA* 2009; **3**: 797–802.
- Wilhelm MT, Rufini A, Wetzel MK, Tsuchihara K, Inoue S, Tomasini R *et al*. Isoform-specific p73 knockout mice reveal a novel role for deltaNp73 in the DNA damage response pathway. *Genes Dev* 2010; **24**: 549–560.
- Yang A, Walker N, Bronson R, Kaghad M, Oosterwegel M, Bonnin J *et al*. p73-deficient mice have neurological, phenomonal and inflammatory defects but lack spontaneous tumours. *Nature* 2000; **404**: 99–103.
- Levine AJ, Tomasini R, McKeon FD, Mak TW, Melino G. The p53 family: guardians of maternal reproduction. *Nat Rev Mol Cell Biol* 2011; **12**: 259–265.
- Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol* 2005; **1**: 1–14.
- Lin WJ, Yeh WC. Implication of Toll-like receptor and tumor necrosis factor alpha signaling in septic shock. *Shock* 2005; **3**: 206–209.
- Kawai T, Akira S. TLR signaling. *Cell Death Differ* 2005; **13**: 816–825.
- Kong XN, Yan HX, Chen L, Dong LW, Yang W, Liu Q *et al*. LPS-induced down-regulation of signal regulatory protein [alpha]22. contributes to innate immune activation in macrophages. *J Exp Med* 2007; **11**: 2719–2731.
- Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci* 2008; **13**: 453–461.
- Leidi M, Gotti E, Bologna L, Miranda E, Rimoldi M, Sica A *et al*. M2 macrophages phagocytose rituximab-opsonized leukemic targets more efficiently than m1 cells *in vitro*. *J Immunol* 2009; **182**: 4415–4422.
- Sakamori R, Takehara T, Ohnishi C, Tatsumi T, Ohkawa K, Takeda K *et al*. Signal transducer and activator of transcription 3 signaling within hepatocytes attenuates

- systemic inflammatory response and lethality in septic mice. *Hepatology* 2007; **46**: 1564–1573.
19. Komarova EA, Krivokrysenko V, Wang K, Neznanov N, Chernov MV, Komarov PG *et al*. p53 is a suppressor of inflammatory response in mice. *FASEB J* 2005; **19**: 1030–1032.
20. Sekine H, Mimura J, Oshima M, Okawa H, Kanno J, Igarashi K *et al*. Hypersensitivity of aryl hydrocarbon receptor-deficient mice to lipopolysaccharide-induced septic shock. *Mol Cell Biol* 2009; **29**: 6391–6400.
21. Mehta A, Brewington R, Chatterji M, Zoubine M, Kinasevitz GT, Peer GT *et al*. Infection-induced modulation of m1 and m2 phenotypes in circulating monocytes: role in immune monitoring and early prognosis of sepsis. *Shock* 2004; **22**: 423–430.
22. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 2004; **75**: 163–189.
23. Yang CP, Hayashida T, Forster N, Li C, Shen D, Maheswaran S *et al*. The integrin α v β 3 ligand MFG-E8 is a p63/p73 target gene in triple negative breast cancers but exhibits suppressive functions in ER+ and erbB2+ breast cancers. *Cancer Res* 2011; **71**: 937–945.
24. Desvergne B. PPAR δ /p53: the lobbyist switching macrophage allegiance in favor of metabolism. *Cell Metab* 2008; **7**: 467–469.
25. Sbisà E, Catalano D, Grillo G, Licciulli F, Turi A, Liuni S *et al*. p53FamTaG: a database resource of human p53, p63 and p73 direct target genes combining in silico prediction and microarray data. *BMC Bioinform* 2007; **8**(Suppl 1): S20.
26. Toshchakov V, Jones BW, Perera PY, Thomas K, Cody MJ, Zhang S *et al*. TLR4, but not TLR2, mediates IFN- β -induced STAT1 α /STAT1 β -dependent gene expression in macrophages. *Nat Immunol* 2002; **3**: 392–398.
27. Coelho LF, Magno de Freitas Almeida G, Mennechet FJ, Blangy A, Interferon- α Uzé G. and β differentially regulate osteoclastogenesis: role of differential induction of chemokine CXCL11 expression. *Proc Natl Acad Sci USA* 2005; **102**: 11917–11922.
28. Wong SC, Puaux AL, Chittezhath M, Shalova I, Kajiji TS, Wang X *et al*. Macrophage polarization to a unique phenotype driven by B cells. *Eur J Immunol* 2010; **40**: 2296–2307.
29. Park JM, Greten FR, Wong A, Westrick RJ, Arthur JS, Otsu K *et al*. Signaling pathways and genes that inhibit pathogen-induced macrophage apoptosis—CREB and NF- κ B as key regulators. *Immunity* 2005; **23**: 319–329.
30. Medzhitov R. Origin and physiological roles of inflammation. *Nature* 2008; **454**: 428–435.
31. De las Heras B, Hortelano S, Giron N, Bermejo P, Rodriguez B, Bosca L. Kaurane diterpenes protect against apoptosis and inhibition of phagocytosis in activated macrophages. *Br J Pharmacol* 2007; **2**: 249–255.
32. Seitz SJ, Schleithoff ES, Koch A, Schuster A, Teufel A, Staib F *et al*. Chemotherapy-induced apoptosis in hepatocellular carcinoma involves the p53 family and is mediated via the extrinsic and the intrinsic pathway. *Int J Cancer* 2010; **26**: 2049–2066.
33. Fuhrman LE, Goel AK, Smith J, Shianna KV, Aballay A. Nucleolar proteins suppress *Caenorhabditis elegans* innate immunity by inhibiting p53/CEP-1. *PLoS Genet* 2009; **5**: e1000657.
34. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS *et al*. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 1992; **356**: 215–221.
35. Rye MS, Bhutta MF, Cheeseman MT, Burgner D, Blackwell JM, Brown SD *et al*. Unraveling the genetics of otitis media: from mouse to human and back again. *Mamm Genome* 2010; **22**: 66–82.
36. Carrasco G, Diaz J, Valbuena JR, Ibanez P, Rodriguez P, Araya G *et al*. Overexpression of p73 as a tissue marker for high-risk gastritis. *Clin Cancer Res* 2010; **16**: 3253–3259.
37. Murray PJ, Wynn TA. Obstacles and opportunities for understanding macrophage polarization. *J Leukoc Biol* 2001; **89**: 557–563.
38. Belikoff BG, Hatfield S, Georgiev P, Ohta A, Lukashev D, Buras JA *et al*. A2B adenosine receptor blockade enhances macrophage-mediated bacterial phagocytosis and improves polymicrobial sepsis survival in mice. *J Immunol* 2011; **186**: 2444–2453.
39. Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol* 2010; **10**: 36–46.
40. Terenzi F, Diaz-Guerra MJ, Casado M, Hortelano S, Leoni S, Bosca L. Bacterial lipopeptides induce nitric oxide synthase and promote apoptosis through nitric oxide-independent pathways in rat macrophages. *J Biol Chem* 1995; **270**: 6017–6021.

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