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# Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21

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**The tumor suppressor PDCD4 is a proinflammatory protein that promotes activation of the transcription factor NF-κB and suppresses interleukin 10 (IL-10). Here we found that mice deficient in PDCD4 were protected from lipopolysaccharide (LPS)-induced death. The induction of NF-κB and IL-6 by LPS required PDCD4, whereas LPS enhanced IL-10 induction in cells lacking PDCD4. Treatment of human peripheral blood mononuclear cells with LPS resulted in lower PDCD4 expression, which was due to induction of the microRNA miR-21 via the adaptor MyD88 and NF-κB. Transfection of cells with a miR-21 precursor blocked NF-κB activity and promoted IL-10 production in response to LPS, whereas transfection with antisense oligonucleotides to miR-21 or targeted protection of the miR-21 site in *Pdcd4* mRNA had the opposite effect. Thus, miR-21 regulates PDCD4 expression after LPS stimulation.**

Many negative regulatory control mechanisms exist to limit the toxic effects of lipopolysaccharide (LPS)[1]. These include soluble decoy receptors, such as soluble Toll-like receptor 4 (TLR4)[2], and splice variants of signal-transduction proteins, including MyD88-s[3], IRAK-M[4] and TAG[5], which interfere with signal-transduction pathways. The inhibitor of transcription factor NF-κB α-subunit (IκBα) is promptly resynthesized by NF-κB to block excessive transcription factor activity after treatment with LPS[6]. The production of anti-inflammatory cytokines is also induced by LPS signaling, including interleukin 10 (IL-10), which has paracrine effects on neighboring cells to negatively regulate the action of NF-κB, and proinflammatory cytokines such as IL-6 and IL-12 (ref. 7). Noncoding RNA products known as microRNAs (miRNAs) have also been described, such as miR-146a, which is induced by LPS and negatively targets signaling proteins such as IRAK1 and TRAF6 at the post-transcriptional level[8].

PDCD4 was first described as a protein induced by apoptotic stimuli[9] that acts as a tumor suppressor[10]. It is induced by cytokine treatment, consistent with a predicted NF-κB site in its promoter[11]. PDCD4 has been shown to positively influence tumor necrosis factor–induced activation of NF-κB[12]. It has also been shown to be a translational inhibitor through interaction with members of the eIF4 family of eukaryotic translation-initiation factors[13,14]. Target mRNAs include those encoding IL-10 and IL-4 (ref. 15), whose production is therefore suppressed by PDCD4. PDCD4-deficient mice are resistant to models of inflammatory disease, such as experimental autoimmune encephalomyelitis and streptozotocin-induced type II diabetes. It is likely that the proinflammatory effect of PDCD4 is due to its role in NF-κB function and its ability to suppress IL-10 translation.

PDCD4 is targeted for proteasomal degradation by β-TRCP ubiquitin ligases activated by growth factors during tumor promotion[16,17]. The miRNA miR-21 targets *Pdcd4* mRNA post-transcriptionally, blocking production of PDCD4 protein[18–20]. This miRNA is upregulated in many cancers, including lymphoma, leukemia and solid tumors and therefore has been called an 'oncomiR', which may explain the loss of PDCD4 during neoplastic transformation[21].

Here we examine the role of PDCD4 in the inflammatory response to LPS. Similar to experimental autoimmune encephalomyelitis and type II diabetes[15], we found PDCD4-deficient mice were protected from the lethality of LPS, and we examined its role in LPS signaling. We found that LPS modulated the expression of PDCD4 through the induction of miR-21 and obtained evidence that this modulation regulated NF-κB activity while promoting IL-10 production. Our study identifies miR-21 as a negative regulator of TLR4 signaling through the targeting of PDCD4.

## RESULTS

### PDCD4 is required for the lethality of LPS

To examine the role of PDCD4 in TLR signaling and inflammation, we injected PDCD4-deficient and wild-type control mice with LPS and monitored their survival. PDCD4-deficient mice were less susceptible to LPS, with lower mortality than wild-type mice (**Fig. 1a**). Analysis of circulating cytokine concentrations 24 h after LPS injection showed that

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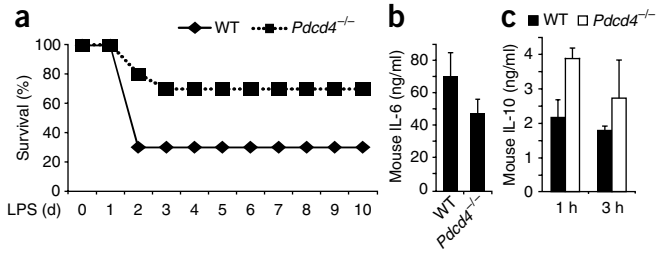


**Figure 1** PDCD4-deficient mice are protected from the lethality of LPS. (**a**) Survival of wild-type (WT) control mice and PDCD4-deficient (*Pdcd4*$^{-/-}$) mice 8–12 weeks of age injected intravenously with LPS, monitored over a period of 10 d; results are plotted as a percentage of total numbers ($n$ = 10 mice per group). (**b**,**c**) Enzyme-linked immunosorbent assay (ELISA) of mouse IL-6 (**b**) and mouse IL-10 (**c**) in blood samples from wild-type and PDCD4-deficient mice ($n$ = 10 (**b**) or 5 (**c**) mice per group), 24 h (**b**) and 1 h and 3 h (**c**) after LPS injection. Data are representative of three (**a**) or two (**b**,**c**) independent experiments.

IL-6 concentrations were lower in PDCD4-deficient mice treated with LPS (**Fig. 1b**), consistent with lower susceptibility. Analysis of cytokine production at earlier times showed striking differences between PDCD4-deficient mice and wild-type mice in terms of production of the anti-inflammatory cytokine IL-10 (**Fig. 1c**). At 1 h after LPS injection, IL-10 serum concentrations in PDCD4-deficient mice were greater than those in wild-type mice, an effect also evident at 3 h after injection. These data indicate that PDCD4 has a proinflammatory role in LPS signaling.

## PDCD4 expression is regulated by TLR4

We investigated whether PDCD4 is a target of TLR signaling. LPS resulted in higher expression of PDCD4 protein in RAW264.7 mouse macrophages, evident at 1 h (**Fig. 2a**); this decreased and was abolished at 24 h. The effect at 24 h was concentration dependent and was evident at a range of LPS concentrations from 0.1 ng/ml to 100 ng/ml (**Fig. 2b**). A profound decrease in *Pdcd4* mRNA in response to LPS was also evident from 4 h (**Fig. 2c**). We also observed the effect on PDCD4 protein in mouse primary bone marrow–derived macrophages (BMDMs; **Fig. 2d**). LPS caused a slight increase 4 h after stimulation, whereas at 8 h and 24 h after LPS, a substantial decrease in PDCD4 was evident. Similarly, treatment of primary BMDMs with other TLR ligands such as $Pam_3CSK_4$ (a TLR2 ligand) and poly(I:C) (a TLR3 ligand) induced PDCD4 protein at earlier times. However, at 24 h after treatment, there was much less PDCD4 (**Fig. 2d**). Notably, in human peripheral blood mononuclear cells (PBMCs), LPS treatment increased the expression of PDCD4 protein at 4 h and 8 h, with a decrease occurring at 24 h (**Fig. 2e**).

## PDCD4 regulates NF-κB and IL-10

To explain the proinflammatory role of PDCD4 in LPS signaling, we examined its ability to affect IL-10 production. Transfection of RAW264.7 cells with small interfering RNA (siRNA) specific for PDCD4 at a concentration of 50 nM and 100 nM decreased endogenous PDCD4 expression, causing a knockdown of 50%, as measured by densitometry scanning (**Fig. 3a**, top). LPS induced a decrease in PDCD4 protein both in cells transfected with control siRNA and in cells treated with PDCD4-specific siRNA. We observed more LPS-induced production of IL-10 in cells transfected with increasing amounts of siRNA specific for PDCD4 (**Fig. 3a**, bottom). A similar increase in IL-10 production was also evident in PDCD4-deficient BMDMs after 4 h of treatment with LPS at concentrations of 10 ng/ml and 100 ng/ml (**Fig. 3b**). PDCD4 is known to inhibit cap-dependent translation of mRNAs with complex 5′ untranslated regions (UTRs), of which *Il10* is an example. To determine if this is the case, we analyzed *Il10* mRNA abundance in PDCD4-deficient BMDMs. Unexpectedly, we found lower *Il10* mRNA expression in PDCD4-deficient BMDMs that produced more IL-10 protein than did wild-type cells (**Fig. 3c**). At 4 h after LPS treatment, PDCD4-deficient BMDMs expressed one-fourth less *Il10* mRNA than did wild-type cells. Other genes that are regulated by eIF4E, which are considered to be sensitive to PDCD4 activity, also show differences in mRNA abundance when translation is modulated[22,23]. To rule out the possibility of transcriptional effects of PDCD4 deficiency on IL-10 expression, we transfected RAW264.7 cells with an *Il10* promoter–luciferase construct alongside siRNA specific for PDCD4. LPS induced the activity of this promoter at a concentration of 100 ng/ml (**Fig. 3d**). However, when PDCD4 expression was knocked down, we detected no substantial difference in *Il10* promoter activity.

We also examined the role of PDCD4 in NF-κB activation by LPS with TLR4-expressing human embryonic kidney cells (HEK293-TLR4 cells) transfected with an NF-κB–luciferase reporter plasmid. Transfection with siRNA specific for PDCD4 at a concentration of 200 nM decreased endogenous PDCD4 in HEK293-TLR4 cells (62% knockdown, as measured by densitometry scanning; **Fig. 3e**, top). The knockdown of endogenous PDCD4 resulted in less activation of NF-κB at all concentrations of LPS tested, with 60–70% inhibition occurring at an siRNA concentration of 200 nM, equivalent to the degree of PDCD4 knockdown (**Fig. 3e**, bottom). We also tested the induction of IL-6, which is NF-κB dependent, in PDCD4-deficient BMDMs (**Fig. 3f**). This response was impaired in PDCD4-deficient cells after 24 h of treatment with LPS at a concentration of 10 ng/ml and 100 ng/ml. We detected significantly less *Il6* mRNA in PDCD4-deficient BMDMs than in wild-type BMDMs after 24 h of LPS treatment (**Fig. 3g**), which indicated this impairment occurs as a result of less NF-κB-induced transcription of *Il6*.

To explain this function of PDCD4, we examined early signaling events in wild-type and PDCD4-deficient BMDMs. Analysis of IκBα protein by immunoblot showed that there was more IκBα degradation at 0.5 h in the PDCD4-deficient cells (**Fig. 3h**, top). Consistent with a positive effect of PDCD4 on NF-κB activation, the

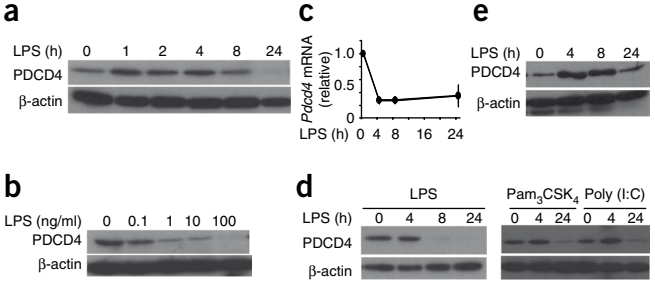


**Figure 2** PDCD4 protein expression is regulated by LPS. (**a**) Immunoblot analysis of PDCD4 in RAW264.7 cells treated for 0–24 h (above lanes) with LPS (100 ng/ml). (**b**) Immunoblot analysis of PDCD4 in RAW264.7 cells treated for 24 h with 0–100 ng/ml (above lanes) of LPS. (**c**) Quantitative RT-PCR analysis of *Pdcd4* mRNA in RAW264.7 cells treated for 0–24 h (horizontal axis) with LPS, presented relative to *Pdcd4* mRNA in untreated cells. (**d**) Immunoblot analysis of PDCD4 in mouse BMDMs treated for 0–24 h (above lanes) with LPS (1 ng/ml), $Pam_3CSK_4$ (100 ng/ml) or poly(I:C) (12.5 μg/ml). (**e**) Immunoblot analysis of PDCD4 in human PBMCs treated for 0–24 h (above lanes) with LPS. β-actin expression serves as a loading control. Data are representative of three independent experiments (**c**; mean ± s.d.) or at least three independent experiments (**a**,**b**,**d**,**e**).

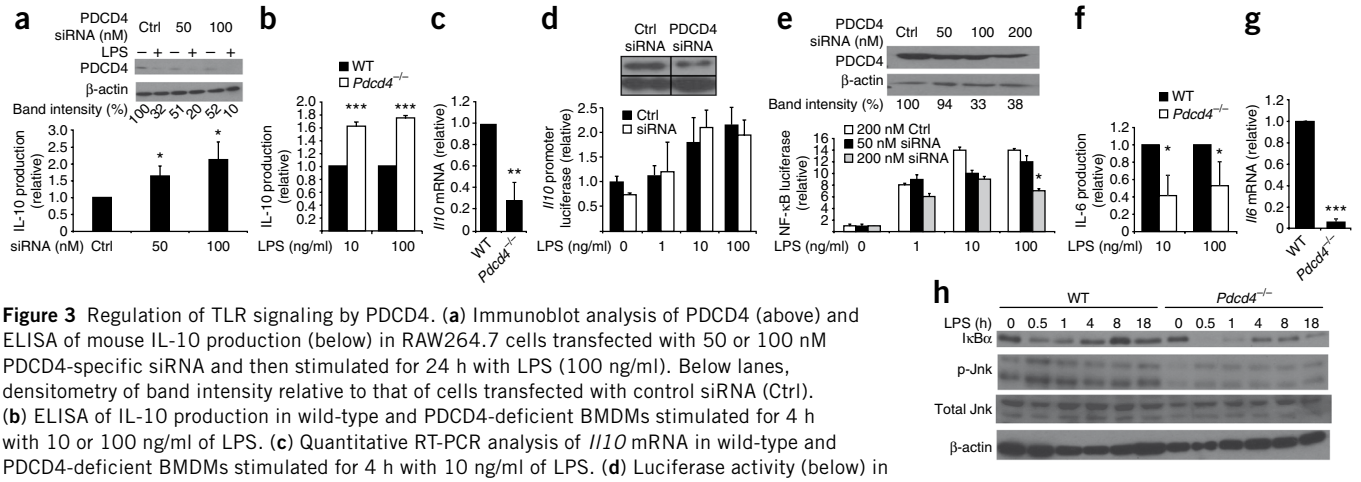


**Figure 3** Regulation of TLR signaling by PDCD4. (**a**) Immunoblot analysis of PDCD4 (above) and ELISA of mouse IL-10 production (below) in RAW264.7 cells transfected with 50 or 100 nM PDCD4-specific siRNA and then stimulated for 24 h with LPS (100 ng/ml). Below lanes, densitometry of band intensity relative to that of cells transfected with control siRNA (Ctrl). (**b**) ELISA of IL-10 production in wild-type and PDCD4-deficient BMDMs stimulated for 4 h with 10 or 100 ng/ml of LPS. (**c**) Quantitative RT-PCR analysis of *Il10* mRNA in wild-type and PDCD4-deficient BMDMs stimulated for 4 h with 10 ng/ml of LPS. (**d**) Luciferase activity (below) in RAW264.7 cells transfected with an *Il10* promoter–luciferase reporter and 100 nM PDCD4-specific siRNA or 100 nM control nontargeting siRNA and then stimulated for 24 h with 0–100 ng/ml of LPS (horizontal axis). Above, immunoblot analysis of PDCD4 in unstimulated RAW264.7 cells transfected with control or PDCD4-specific siRNA. (**e**) Luciferase activity in HEK293-TLR4 transfected with an NF-κB-luciferase reporter and 50 or 200 nM PDCD4-specific siRNA (key) and then stimulated for 24 h with 0–100 ng/ml of LPS (horizontal axis). Top, immunoblot analysis of PDCD4 protein in HEK293-TLR4 transfected with control siRNA or 50 or 200 nM PDCD4-specific siRNA (above lanes); below lanes, densitometry of band intensity relative to that of cells transfected with control siRNA. (**f**) ELISA of IL-6 production in wild-type and PDCD4-deficient BMDMs stimulated for 24 h with 10 or 100 ng/ml of LPS. (**g**) Quantitative RT-PCR analysis of *Il6* mRNA in wild-type and PDCD4-deficient BMDMs stimulated for 24 h with 100 ng/ml of LPS. (**h**) Immunoblot analysis of IκBα protein (top) and Jnk phosphorylation (p-Jnk; middle) in wild-type and PDCD4-deficient BMDMs stimulated for 0–18 h (above lanes) with LPS (10 ng/ml). β-actin serves as a loading control. In siRNA experiments, all cells were transfected with an equal amount of total RNA normalized with negative control siRNA. Results for LPS-treated cells are presented relative to those for wild-type cells or cells transfected with negative control RNA only. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ (two-tailed unpaired *t*-test). Data are representative of three independent experiments (mean and s.d.).



NF-κB-dependent resynthesis of IκBα at later times was markedly impaired in PDCD4-deficient macrophages. These results indicate that the effect of PDCD4 on NF-κB activation is not due to modulation of the IKK complex and is probably indirect and secondary to its primary function as a repressor of translation. PDCD4 is known to affect the activity of the transcription factor AP-1 in tumor progression[24]. Here we analyzed activation of the kinase Jnk by assessing its phosphorylation with an antibody specific for Jnk phosphorylation. Jnk activation was impaired in response to LPS in PDCD4-deficient BMDMs compared with its activation in wild-type cells (**Fig. 3h**, middle). These data confirm that PDCD4 has a proinflammatory role in LPS signaling events.

## Induction of miR-21 by LPS

To explain the lower PDCD4 protein abundance after LPS treatment, we monitored expression of the PDCD4-targeting miRNA miR-21 after LPS treatment. We used the induction of a known LPS-responsive miRNA, miR-146a[8], as a positive control. LPS treatment induced miR-21 expression in RAW264.7 macrophages, which was apparent from 4 h (**Fig. 4a**, left). LPS treatment led to strong induction of both miR-21 and miR-146a at 24 h with similar kinetics in this cell type. The effect of LPS on miR-21 induction after 24 h in RAW264.7 was dose dependent, with 100 ng/ml of LPS being the optimal dose, and again we observed a similar pattern for miR-146a (**Fig. 4b**).

Analysis of the induction of miRNA in primary mouse BMDMs gave a result similar to that obtained with the RAW264.7 cell line (**Fig. 4c**). We found that miR-21 was induced by LPS to an extent similar to that of miR-146a. We also examined the human monocytic cell line THP-1 after 18 h of treatment with LPS (**Fig. 4d**). We found that miR-146a was induced by LPS, consistent with earlier reports[8]; however, we observed no upregulation of miR-21 in this cell line. We also observed upregulation of miR-21 by LPS in human PBMCs (**Fig. 4e**). We found fourfold more miR-21, with a similar effect for miR-146a.

## Induction of miR-21 by LPS requires MyD88 and NF-κB

We examined the induction of miR-21 in immortalized BMDMs deficient in the TLR adaptor proteins MyD88 and TRIF (**Fig. 4f**). LPS caused a tenfold induction of miR-21 in wild-type cells after 18 h. This effect was abolished in the absence of MyD88 and was only slightly impaired in TRIF-deficient BMDMs (**Fig. 4f**, left). We noted a similar dependency on MyD88 for miR-146a; however, in contrast to miR-21 induction, miR-146a induction by LPS also required TRIF (**Fig. 4f**, right). We next examined the role of NF-κB in miR-21 induction by LPS, as the promoter region has a putative NF-κB site located at position −248 (5′-GTGGGAGGTGCCT-3′), as predicted by the Genomatix MatInspector software package. We found induction of miR-21 by LPS in wild-type mouse embryonic fibroblasts, but this was completely abolished in mouse embryonic fibroblasts deficient in the NF-κB subunit p65 and was actually lower than basal expression (**Fig. 4g**, left). We observed a similar dependency on p65 for miR-146a (**Fig. 4g**, right), but to a lesser extent than for miR-21.

## LPS decreases PDCD4 protein via miR-21 induction

Having examined the induction of miR-21 by LPS, we then examined the ability of miR-21 to regulate PDCD4 abundance after TLR4 signaling. We assessed this effect by transfecting RAW264.7 cells with antisense oligonucleotides specific to miR-21 (anti-miR-21) or control antisense RNA (**Fig. 5a**). Treatment with LPS for 24 h resulted in less PDCD4 in cells treated with control antisense RNA. Notably, pretreatment with anti-miR-21 blocked the LPS-induced decrease in PDCD4, particularly at anti-miR-21 concentrations of 12.5 nM and 25 nM, resulting in 1.3-fold and 1.57-fold more PDCD4 protein, respectively, than in control LPS-treated cells, as measured by densitometry scanning. Notably, to verify

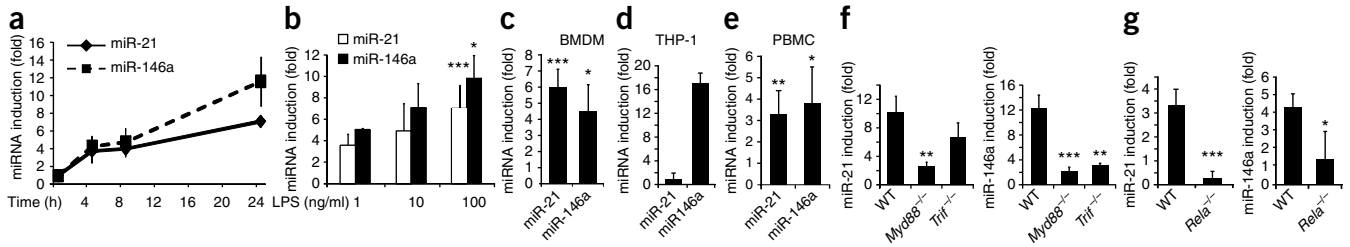


**Figure 4** Induction of miR-21 by LPS treatment in macrophages. (**a**) Time-course analysis of the induction of miR-21 and miR-146a in RAW264.7 cells stimulated for 4–24 h (horizontal axis) with LPS (100 ng/ml). (**b**) Dose-response of the induction of miR-21 and miR-146a in RAW264.7 after treatment for 24 h with 1–100 ng/ml (horizontal axis) of LPS. (**c**–**e**) Induction of miR-21 and miR-146a in mouse BMDMs (**c**), THP-1 cells (**d**) and human PBMCs (**e**) treated for 24 h with LPS (100 ng/ml). (**f**) Induction of miR-21 (left) and miR-146a (right) in immortalized *Myd88*$^{-/-}$, *Trif*$^{-/-}$ or wild-type (C57BL/6) BMDMs stimulated for 18 h with LPS (100 ng/ml). (**g**) Induction of miR-21 (left) and miR-146a (right) in wild-type and p65-deficient (*Rela*$^{-/-}$) mouse embryonic fibroblasts stimulated for 8 h with LPS (100 ng/ml). Results for LPS-treated cells are presented relative to those for untreated cells. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (two-tailed unpaired *t*-test). Data are from at least three independent experiments (mean and s.d.).

this effect of miR-21 was brought about through specific and direct targeting of *Pdcd4* mRNA, we designed a morpholino oligonucleotide specific to the miR-21 site of PDCD4 (morpho-21). This morpho-21 oligonucleotide should target specifically the *Pdcd4* 3′ UTR at the miR-21 site and block the activity of any miRNA at that position. Treatment with LPS again resulted in less PDCD4 protein expression in control cells transfected with the morpholino than in unstimulated cells (**Fig. 5b**). However, transfection of morpho-21 followed by LPS stimulation protected *Pdcd4* protein, particularly at a morpholino oligonucleotide concentration of 5 μM, which resulted in twofold more PDCD4 protein than in control LPS-treated cells, as measured by densitometry scanning. LPS therefore regulates the translation of *Pdcd4* mRNA through the induction of miR-21.

## PDCD4 is regulated by the proteasome

PDCD4 protein has been shown to be targeted for degradation in tumor promotion through the action of ubiquitin ligases and the 26S proteasome[16,17]. We therefore determined if LPS could also target the amount of PDCD4 protein via the proteasome. Pretreatment of RAW264.7 cells with vehicle control and subsequent stimulation with LPS resulted in a slight induction of LPS 1 h after stimulation and a decrease at 6 h after stimulation (**Fig. 5c**). Pretreatment with the proteasome inhibitor MG132 blocked the decrease in PDCD4 at 6 h. However, as prolonged MG132 treatment is toxic to cells, the effect of MG132 on the decrease at 24 h noted before could not be analyzed. However, it was obvious the proteasome did affect the amount of PDCD4 protein at earlier time points after LPS stimulation. To explain this phenomenon, we examined the ability of PDCD4 protein to become ubiquitinated in response to LPS by transfection of HEK293-TLR4 cells with hemagglutinin (HA)-tagged ubiquitin and subsequent LPS stimulation. We immunoprecipitated PDCD4 and subsequently analyzed HA-tagged ubiquitin by immunoblot (**Fig. 5d**). We detected ubiquitination of endogenous PDCD4 at 4 h after LPS treatment in the presence of MG132. Thus, proteasomal degradation activated by LPS can degrade ubiquitinated PDCD4 protein in the cell.

## Regulation of IL-10 production and NF-κB activation by miR-21

To determine if miR-21, through its regulation of PDCD4, affects the production of IL-10, we transfected RAW264.7 cells with the miR-21 precursor pro-miR-21 (**Fig. 6a**). Transfection of pro-miR-21, which will generate mature miR-21, resulted in less PDCD4, particularly at a concentration of 100 nM (**Fig. 6b**). LPS again resulted in less PDCD4 protein (**Fig. 6b**). At the same time, transfection with pro-miR-21 also resulted in more production of IL-10 induced by LPS, with significantly more IL-10 production evident at a pro-miR-21 concentration of 100 nM (**Fig. 6a**, left). Transfection of cells with anti-miR-21, shown above to increase PDCD4 during LPS signaling (**Fig. 5b**), blunted the production of IL-10 by LPS (**Fig. 6a**, middle). To confirm that the effect of miR-21 was brought about specifically through the targeting of PDCD4, we transfected RAW264.7 cells with increasing amounts of morpho-21, shown above to protect PDCD4 from the LPS-induced decrease (**Fig. 5c**). Again we examined the effect of morpho-21 on LPS-induced production of IL-10 and, as with anti-miR-21, found that it significantly inhibited IL-10 production (**Fig. 6a**, right). To confirm that the specificity of the effect of miR-21 on IL-10 production was due to targeting of PDCD4, we transfected BMDMs from wild-type and PDCD4-deficient mice with pro-miR-21 or control RNA and monitored the effect on IL-10 production at 24 h

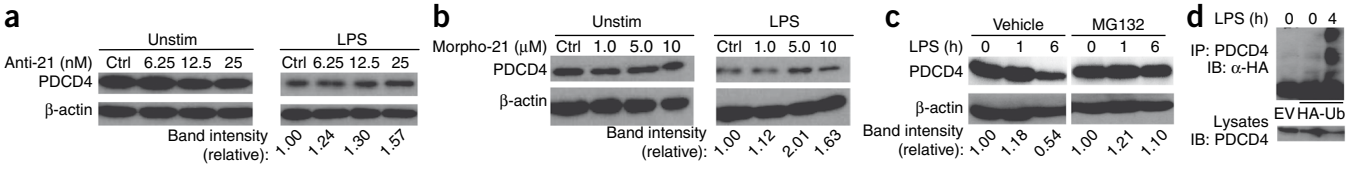


**Figure 5** Regulation of PDCD4 expression in LPS signaling. (**a**) Immunoblot analysis of PDCD4 in RAW264.7 cells transfected with various concentrations of anti-miR-21 (above lanes) and then left untreated (Unstim) or treated for 24 h with LPS (100 ng/ml). (**b**) Immunoblot analysis of PDCD4 in RAW264.7 cells transfected with various concentrations of morpho-21 (above lanes) and then left untreated or treated for 24 h with LPS (100 ng/ml). (**c**) Immunoblot analysis of PDCD4 in RAW264.7 cells pretreated with 1 μM MG132 or vehicle control and then treated for 0–6 h (above lanes) with LPS (100 ng/ml). Below lanes (**a**–**c**), densitometry of band intensity relative to that of cells transfected with control RNA (Ctrl; **a**,**b**) or no LPS (**c**), set as 1. (**d**) Immunoprecipitation (IP; with anti-PDCD4) of endogenous PDCD4 together with overexpressed HA-tagged ubiquitin (HA-Ub) from transfected HEK293-TLR4 cells stimulated with LPS (100 ng/ml) in the presence of MG132 (100 μg/ml), followed by immunoblot analysis (IB) with anti-HA (α-HA). EV, empty vector (control). Bottom, immunoblot analysis of PDCD4 in lysates without immunoprecipitation (loading control). For miRNA-morpholino transfections, all cells were transfected with an equal amount of total RNA normalized with negative control anti-miRNA or a morpholino targeted to a redundant site in the mouse *Pdcd4* 3′ UTR. Data are representative of three independent experiments.

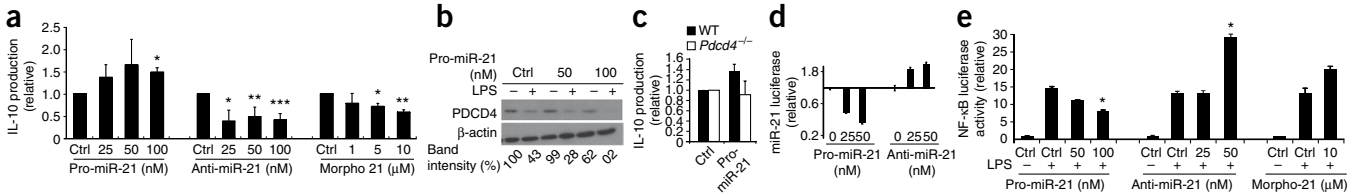


**Figure 6** Regulation of PDCD4 function by miR-21 in TLR signaling (**a**) ELISA of mouse IL-10 in RAW264.7 cells transfected with various doses (horizontal axes) of pro-miR-21 (left), anti-miR-21 (middle) or morpho-21 (right) and then stimulated for 24 h with LPS (100 ng/ml). (**b**) Immunoblot analysis of PDCD4 in RAW264.7 cells transfected with 50 or 100 nM pro-miR-21 (above lanes) and then stimulated for 24 h with LPS (100 ng/ml). (**c**) ELISA of IL-10 production in wild-type C57BL/6 and PDCD4-deficient BMDMs transfected with 100 nM pro-miR-21 and then stimulated for 24 h with LPS (10 ng/ml). (**d**) Luciferase activity in HEK293-TLR4 cells transfected with a miR-21–luciferase reporter and 0, 25 or 50 nM pro-miR-21 (left) or anti-miR-21 (right). (**e**) Luciferase activity in HEK293-TLR4 transfected with an NF-κB luciferase reporter and 50 or 100 nM pro-miR-21 oligonucleotide (left), anti-miR-21 oligonucleotide (middle) or morpho-21 (right) and then stimulated for 24 h with LPS (1 ng/ml). In all RNA-morpholino transfections, cells were transfected with an equal amount of total RNA normalized with negative control pro-miRNA, negative control anti-miRNA or a morpholino targeted to a redundant site in the mouse *Pdcd4* 3′ UTR. In **a**,**d**,**e**, results with LPS are presented relative to those obtained with negative control RNA. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ (two-tailed unpaired *t*-test). Data are representative of three independent experiments (**a**,**d**,**e**; mean and s.d.), three experiments (**b**) or two independent experiments (**c**; mean and s.d. of triplicate samples).

after LPS (**Fig. 6c**). There was slightly more IL-10 production in BMDMs transfected with pro-miR-21 than in control cells. This effect was not present, however, in BMDMs from PDCD4-deficient mice, which indicated that the effect of pro-miR-21 was specifically due to targeting of *Pdcd4* mRNA.

Again we examined the effect of miR-21 on NF-κB activity with HEK293-TLR4 cells transfected with anti-miR-21, as well as those transfected with pro-miR-21 or morpho-21, and measured NF-κB-linked luciferase activity in response to LPS. We assessed the activity of anti-miR-21 and pro-miR-21 in these cells with a miR-21-dependent luciferase construct (which contains a synthetic miR-21 binding site in the 3′ UTR of the luciferase gene). Transfection of cells with pro-miR-21 resulted in less luciferase activity (**Fig. 6d**, left), whereas anti-miR-21 resulted in more miR-21-dependent luciferase activity (**Fig. 6d**, right). Transfection of pro-miR-21 also negatively regulated the activation of NF-κB, with 50% inhibition occurring at a pro-miR-21 concentration of 100 nM (**Fig. 6e**). Notably, we observed the opposite trend when we transfected anti-miR-21 into cells. We observed a doubling in LPS-induced NF-κB activity with transfection of 50 nM anti-miR-21 relative to the activity in cells transfected with a control antisense RNA (**Fig. 6e**, middle). Similarly, transfection of cells with 10 μM morpho-21 resulted in more LPS-induced NF-κB activity than that in cells transfected with control morpholino. Together these results demonstrate that miR-21 affects IL-10 production and also NF-κB activation through its effect on PDCD4 expression.

## DISCUSSION

Here we have found that the control of PDCD4 expression is a key step in the negative regulation of the inflammatory response to LPS, acting as a molecular switch between the proinflammatory (NF-κB) and anti-inflammatory (IL-10) response. This switch is a decrease in PDCD4 protein abundance, which is brought about through the induction of miR-21. This process positively influences IL-10 production while negatively regulating NF-κB activity, presumably to control the LPS response that can be lethal.

Our study has demonstrated upregulation of miR-21 by LPS in many cell types, including macrophages, mouse embryonic fibroblasts and PBMCs. We found that the induction of miR-21 by LPS was dependent on MyD88 and also dependent on NF-κB, consistent with the presence of an NF-κB-binding site in the miR-21 promoter[25,26]. There is upregulation of miR-21 in a variety of disease states. Overexpression of miR-21 has been reported in many types of cancer[27,28]. Also, miR-21 has been reported to be upregulated in many inflamed states, including the inflamed lung in LPS-treated mice[29], allergic airway inflammation[30], osteoarthritis[31], psoriasis and atopic eczema[32], disease-active ulcerative colitis tissue[33], *Helicobacter pylori*–associated gastric cancer[34], cardiac muscle injury[35] and cardiac hypertrophy[36]. Therefore, miR-21 may be an indicator of inflammation and, given its role in tumorigenesis, might be an important link between cancer and inflammation.

Evidence is now emerging indicating that TLR activation affects the expression of many miRNAs. The first LPS-responsive miRNA reported was miR-146a[8], and here we have shown that its induction was similar to that of miR-21 in macrophages. Studies have shown considerable upregulation of miR-155 in macrophages[37] as well as dendritic cells[38], to an extent higher than reported here for miR-21 and miR-146a. Experiments with animals genetically deficient in miR-155 have confirmed its importance in the generation of adaptive immunity[39,40]. Additional TLR-responsive miRNAs include miR-132 (ref. 8), miR-9 (ref. 41), miR-147 (ref. 42) and miR-346 (ref. 43); these are upregulated in various cell types after stimulation with TLR ligands. Some miRNAs have been reported to be downregulated after LPS treatment, including let-7i, which is thought to target TLR4 itself[44], and miR-125b[45]. As many of those miRNAs regulated by TLR signaling are also dysregulated in cancer[27], it is possible that miRNAs form a key link between inflammation and cancer and that the induction of specific miRNAs, including miR-21, by TLRs may be a key step in tumor progression.

PDCD4 has been demonstrated to function as an inhibitor of cap-dependent translation of complex mRNAs through its interaction with the eukaryotic initiation factors eIF4a and eiF4G[13,14]. Gene products inhibited by this mechanism include growth factors and cytokines, including IL-10. Furthermore, PDCD4 has been linked to NF-κB activation by an unknown mechanism[12]. We have demonstrated here that PDCD4 was required for NF-κB activation and attenuated IL-10 production in LPS signaling and was involved in the lethality of LPS. Here we have shown that the suppressive effect of PDCD4 on IL-10 occurred at the translational level, as PDCD4-deficient macrophages had more IL-10 protein yet less *Il10* mRNA, consistent with published reports that IL-10 translation is highly regulated[46–48] and that more eIF4E-directed translation can lead to more mRNA turnover[22,23]. In addition, we have shown that PDCD4 affected NF-κB activation by an undefined mechanism that promotes *Il6* transcription, which may involve a positive effect on Jnk. It is likely that other targets for PDCD4 exist in TLR signaling that are regulated at the translational level.

We have also demonstrated here that modulation of miR-21 had effects opposite to those of PDCD4 in LPS signaling. It attenuated NF-κB activation and promoted IL-10 production in response to LPS. Notably, through the use of target protection of the PDCD4 miR-21 site and through the transfection of PDCD4-deficient BMDMs with miR-21, we have demonstrated that miR-21 has an important role in negatively regulating these processes specifically through the targeting of PDCD4, thereby limiting excessive inflammation.

Highlighting the importance of the removal of PDCD4 for the appropriate development of inflammatory responses was the finding that PDCD4 can also be targeted for proteasomal degradation in LPS signaling. We have demonstrated here that inhibition of the 26S proteasome by MG132 stabilized PDCD4 protein at early time points after LPS stimulation. In addition, we have identified PDCD4 as a key target protein for ubiquitination downstream of TLR signaling. The existence of multiple mechanisms to control PDCD4 protein expression highlights the importance of this molecule in the immune response. Degradation of the pre-existing pool of PDCD4 protein occurred through ubiquitination and the action of the 26S proteasome at early times after LPS treatment (6 h). This occurred in addition to the post-transcriptional silencing of *Pdcd4* mRNA through the action of miR-21 to block the translation of new PDCD4 protein, which, because of the delay in miR-21 induction, had its effect at later times (24 h). As for the induction of PDCD4 protein at earlier time points after LPS treatment (1 h), this was not accompanied by an increase in *Pdcd4* mRNA, which began to decrease 4 h after LPS administration. It is likely that at earlier time points, because of lower basal amounts of miR-21, translation of PDCD4 can occur without halting.

PDCD4 abundance was high initially and facilitated NF-κB activation while suppressing IL-10. LPS decreased PDCD4 via miR-21 to limit NF-κB activity while promoting IL-10 production, thereby providing a negative regulatory loop for TLR4 signaling. The link between miR-21 and PDCD4 and between NF-κB and IL-10 is direct, as shown by use of a morpholino oligonucleotide directed specifically to the miR-21 site of mouse *Pdcd4* mRNA. This stabilized PDCD4 protein and had the opposite effect in terms of enhanced NF-κB and decreased IL-10 relative to that in cells treated with siRNA specific for PDCD4 or PDCD4-deficient cells. It is therefore reasonable to conclude that miR-21 limits PDCD4 in LPS signaling, leading to a decrease in NF-κB and an increase in IL-10, which in turn regulates inflammatory processes induced by LPS. In conclusion, our study has identified an axis involving miR-21 and PDCD4 that provides important new insight into the negative regulation of TLR4 signaling. In terms of the importance of TLR4 in vaccine adjuvancy[49] or for inflammation in processes such as sepsis, rheumatoid arthritis and allergic asthma[50], this finding might present new opportunities for boosting or limiting TLR4 activation therapeutically.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

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### AUTHOR CONTRIBUTIONS

F.J.S. did the functional experiments on PDCD4 and miR-21 and cowrote the manuscript; E.P.-M. did experiments on PDCD4 degradation by the proteasome and experiments on signals in PDCD4-deficient cells; E.J.H. helped with experiments on miR-21; C.M. and J.J.O. provided advice on miRNA profiling experiments; Q.R., D.S.J. and J.Y.C. did the experiments on the lethality of LPS in PDCD4–deficient mice and supplied BMDMs from the mice; and L.A.J.O. directed the work and cowrote the manuscript.

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## ONLINE METHODS

**Reagents.** Ultrapure TLRgrade LPS was from Alexis. $Pam_3CSK_4$ was from Calbiochem and poly(I:C) was from Amersham Biosciences. MG132 was from Calbiochem. SMARTpool siRNAs specific for human and mouse PDCD4 and negative control SMARTpool siRNA were from Dharmacon. Pro-miR-21, anti-miR-21, specific to human and mouse miRNA, and the respective negative control RNAs were from Ambion. Morpholino oligonucleotides with the following sequences were designed in association with GeneTools: PDCD4-miR-21 (morpho-21), 5′-AAGTAGCTTATCAGAACAC CCACAC-3′, and PDCD4-control (morpho-ctrl), 5′-GATCAGGTCCTAAA CATGGCACTTA-3′.

**Cell culture and animal handling.** RAW264.7 and THP-1 cells were from the European Collection of Cell Cultures. HEK293-TLR4 cells (HEK293-TLR4-MD2-CD14) were from Invivogen. Wild-type C57BL/6 mice were housed and maintained at Trinity College, Dublin. Wild-type, MyD88-deficient and TRIF-deficient BMDMs immortalized by retrovirus were provided by D. Golenbock. PDCD4-deficient mice were housed and maintained at the University of Pennsylvania. The p65-deficient and matched wild-type control mouse embryonic fibroblasts were provided by R. Hay. All cells were maintained in either RPMI media or DMEM (Invivogen) supplemented with 10% (vol/vol) FCS and penicillin and streptomycin. For BMDM extraction, animals were killed humanely according to the regulations of the European Union and the Irish Department of Health. Bone marrow was extracted from femurs and tibias and red blood cells were lysed with Red Blood Cell Lysing Buffer (Sigma), and the resulting cells were grown in medium conditioned with macrophage colony-stimulating factor. For analysis of human PBMCs, mononuclear cells were isolated from whole blood with a Ficoll gradient (Sigma) and were grown in RPMI media as described above. For analysis of the lethality of LPS, mice were injected intraperitoneally with LPS at a dose of 30 mg per kg body weight. The survival of mice was monitored for 10 d, after which all surviving mice were killed humanely in accordance with the Animal Research Committee of the University of Pennsylvania.

**RNA extraction and PCR.** Cells were grown to $5 \times 10^6$ to $10 \times 10^6$ cells and RNA was extracted with the RNeasy Kit (Qiagen), modified to obtain small RNA species. For miRNA analysis, individual miRNA TaqMan assays for the endogenous reference RNA RNU6B, miR-21 and miR-146a were done according to the manufacturer's instructions (Applied Biosystems). For analysis of gene expression, cDNA was prepared with the High-Capacity cDNA Archive kit according to manufacturers' instructions (Applied Biosystems), and individual mRNAs were monitored with the following inventoried TaqMan assays (Applied Biosystems): mouse *Gapdh* (glyceraldehyde phosphate dehydrogenase) assay, mouse *Pdcd4* assay (Mm01266062_m1), mouse *Il6* assay (Mm99999064_m1) and mouse *Il10* assay (Mm99999062_m1). The AB7900FAST platform (Applied Biosystems) was used for all PCR, done in triplicate. Changes in expression were calculated by the change in threshold ($\Delta\Delta C_T$) method with RNU6B as the endogenous control for miRNA analysis and *Gapdh* as an endogenous control for gene-expression analysis and were normalized to results obtained with untreated cells.

**Transient transfection.** For transfection of miRNA and morpholino oligonucleotides, $5 \times 10^6$ RAW264.7 cells were transfected with the appropriate RNA oligonucleotides with 2% Lipofectamine 2000 (Invitrogen). For siRNA transfection, $5 \times 10^6$ RAW264.7 cells were transfected with 2% Lipofectamine RNAiMax (Invitrogen). Cells were allowed to recover for 24 h before treatment with LPS for various times. For all RNA transfection, equal total concentrations of RNA were used for each reaction, with negative control RNA molecules used for normalization. For luciferase assays, $5 \times 10^6$ HEK293-TLR4 cells were transfected with endotoxin-free 5× NF-κB reporter luciferase plasmid (a gift from R. Hofmeister) and pRL-TK, the renilla luciferase reporter, with 6% GeneJuice (Novagen). Cells were allowed to recover for 24 h before being transfected with RNA oligonucleotides as described above. Reporter gene activity was measured with the Dual-Luciferase kit (Promega) 18 h after LPS treatment. The pMIR-REPORT miR-21 reporter luciferase plasmid was from A.M. Cheng. The *Il10* promoter luciferase plasmid was a gift from A. Bowie. HA-tagged ubiquitin was a gift from R. Hay.

**ELISA.** Cytokine concentration in supernatants were measured with ELISA DuoSet Development systems according to the manufacturer's instructions (R&D Systems).

**Coimmunoprecipitation of ubiquitinated PDCD4.** HEK293-TLR4 cells ($2.5 \times 10^6$) were grown to 70% confluency and were transfected with 4 μg plasmid encoding HA-tagged ubiquitin with 6% GeneJuice (Novagen). Cells were allowed to recover for 24 h before stimulation for various times with 100 ng/ml of LPS, then were lysed as described[51]. For assistance in the detection of ubiquitinated proteins, proteasomal degradation was prevented by treatment of the cells with MG132 (1 μg/ml) for 4 h before lysis. Anti-PDCD4 (2 μg; 600-401-965; Rockland) was preincubated for 16 h with protein A/G PLUS-Agarose beads (sc_2003; Santa Cruz Biotechnology) and subsequently washed in lysis buffer. PDCD4 was isolated by incubation of beads with cell lysates for 2 h at 4 °C. After beads were washed, immune complexes were eluted with 50 μl Laemmli sample buffer and separated by SDS-PAGE and proteins were detected by immunoblot analysis with anti-HA (HA-101R; Sigma).

**Immunoblot.** Cells ($5 \times 10^5$ to $10 \times 10^5$) were lysed with low-stringency lysis buffer complete with protease inhibitors. Samples loaded with 5× denaturing sample buffer were separated by 12% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane by standard techniques and were subsequently analyzed by immunoblot with the relevant antibodies. Protein abundance was calculated by densitometry scanning of the blot with IMAGE-J software; PDCD4 abundance was normalized to β-actin abundance and is presented relative to results obtained with the control sample (percentage or fraction), set as 1.0. The monoclonal antibody used to detect β-actin was from Sigma (AC-15).

**Statistical tests.** All statistical analyses used the Student's *t*-test, unpaired for normal distributions of at least three independent experiments.

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