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Immunomodulation via Novel Use of TLR4 by the Filarial Nematode Phosphorylcholine-Containing Secreted Product, ES-62¹

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Filarial nematodes, parasites of vertebrates, including humans, secrete immunomodulatory molecules into the host environment. We have previously demonstrated that one such molecule, the phosphorylcholine-containing glycoprotein ES-62, acts to bias the immune response toward an anti-inflammatory/Th2 phenotype that is conducive to both worm survival and host health. For example, although ES-62 initially induces macrophages to produce low levels of IL-12 and TNF- α , exposure to the parasite product ultimately renders the cells unable to produce these cytokines in response to classic stimulators such as LPS/IFN- γ . We have investigated the possibility that a TLR is involved in the recognition of ES-62 by target cells, because phosphorylcholine, a common pathogen-associated molecular pattern, appears to be responsible for many of the immunomodulatory properties of ES-62. We now demonstrate that ES-62-mediated, low level IL-12 and TNF- α production by macrophages and dendritic cells is abrogated in MyD88 and TLR4, but not TLR2, knockout, mice implicating TLR4 in the recognition of ES-62 by these cells and MyD88 in the transduction of the resulting intracellular signals. We also show that ES-62 inhibits IL-12 induction by TLR ligands other than LPS, bacterial lipopeptide (TLR2) and CpG (TLR9), via this TLR4-dependent pathway. Surprisingly, macrophages and dendritic cells from LPS-unresponsive, TLR4-mutant C3H/HeJ mice respond normally to ES-62. This is the first report to demonstrate that modulation of cytokine responses by a pathogen product can be abrogated in cells derived from TLR4 knockout, but not C3H/HeJ mice, suggesting the existence of a novel mechanism of TLR4-mediated immunomodulation. *The Journal of Immunology*, 2005, 174: 284–293.

Characterization of the TLRs, the family of pattern recognition receptors with homology to *Drosophila* Toll, has rapidly advanced our understanding of the relative specificity of pathogen recognition by the innate immune system (reviewed in Ref. 1). TLRs, which are members of the Toll/IL-1R (TIR)³ superfamily, comprise extracellular leucine-rich repeats, a transmembrane region, and an intracellular region containing a conserved intracellular TIR domain. They mediate responses to a diverse range of pathogen products as well as several host pro-

teins, e.g., heat shock proteins (HSP) and components of the extracellular matrix (reviewed in Ref. 1).

TLR4, the most widely studied TLR, is a critical member of the receptor complex responsible for the recognition of and response to bacterial LPS. TLR4 also mediates the response to taxol (a product of the Pacific yew, *Taxus brevifolia*, which exhibits potent antitumor activity in humans), *Chlamydia pneumoniae* HSP60, the fusion protein of respiratory syncytial virus and the envelope protein of mouse mammary tumor virus, as well as host-derived HSPs (HSP60 and HSP70), surfactant protein A, and extracellular matrix components (e.g., extra domain A of fibronectin, oligosaccharides of hyaluronan, soluble heparan sulfate, and fibrinogen) (reviewed in Refs. 1 and 2).

Intracellular signaling is initiated by the association, via their TIR domains, of TLRs with adaptor proteins, such as MyD88, TIR-containing adaptor protein/MyD88 adaptor-like (TIRAP/Mal), TIR-containing adaptor inducing IFN- β /TIR-containing adaptor molecule-1 (TRIF/TICAM-1), TRIF-related adaptor molecule (TRAM), and sterile α and HEAT-armadillo motifs (SARM) (reviewed in Ref. 3). Different downstream events are triggered by association of the various adaptors, e.g., TLR4-mediated NF- κ B activation occurs via MyD88-dependent and -independent mechanisms, whereas IRF3 activation is mediated by TRIF (4).

Using ES-62 from the rodent filarial nematode *Acanthocheilonema viteae*, we have previously characterized, both in vitro and in vivo, the immunomodulatory properties of this major secreted (ES) product (5–12). ES products are postulated to be responsible for the immunomodulatory activities of filarial parasites, thereby promoting their survival within mammalian hosts (reviewed in Ref. 13). Consistent with this, ES-62 inhibits the ability of B and

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³ Abbreviations used in this paper: TIR, Toll/IL-1R; BLP, bacterial lipopeptide Pam₃CSK₄; DC, dendritic cell; bmDC, bone marrow-derived DC; bmM, bone marrow-derived macrophage; HPRF, hypoxanthine-guanine phosphoribosyltransferase; HSP, heat shock protein; KO, knockout; LBP, LPS-binding protein; LDL, low density lipoprotein; ox-PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine; PC, phosphorylcholine; TRIF, TIR-containing adaptor inducing IFN- β ; MD-2, myeloid differentiation-2.

T lymphocytes to respond to ligation of their Ag receptors by rendering cells hyporesponsive to stimulation (reviewed in Ref. 14). It can also bias the immune response toward a Th2 phenotype, thereby preventing the induction of Th1-mediated pathology, which would be deleterious to both host and parasite (9–11).

Although able to induce IL-10 secretion from B1 cells (15), the Th2-biasing effects of ES-62 appear to be due mainly to its modulation of APC function. ES-62 drives the differentiation of dendritic cells (DCs) to a DC2 phenotype (10) and also suppresses LPS-mediated induction of the Th1-inducing cytokine IL-12 by macrophages (11). Furthermore, it suppresses the LPS-mediated induction of the proinflammatory cytokines IL-6 and TNF- α (11). Interestingly, although ES-62 suppresses LPS-mediated cytokine induction, treatment of macrophages and DCs with ES-62 alone initially and transiently induces low levels of these cytokines (11, 16). The precise significance of this low level cytokine induction is not known but it is made use of in the present investigation.

Many of the effects of ES-62 have been attributed to the presence of phosphorylcholine (PC) moieties covalently attached to the carbohydrate of this glycoprotein (5, 7, 9). However, the receptors/signaling molecules used by ES-62/PC to modulate the activity of macrophages and DCs have not been identified. Using knockout mice, we now show that the effects of ES-62 on IL-12 and TNF- α production by macrophages and DCs are mediated via a TLR4-MyD88 pathway. However, unlike LPS, ES-62 does not require fully functional TLR4, because macrophages and DCs from TLR4-mutant C3H/HeJ mice respond normally to the parasite product.

Materials and Methods

Preparation of ES-62 and PC-OVA

ES-62 was prepared from 500 ml of spent culture medium (endotoxin-free RPMI 1640 (Invitrogen Life Technologies) with added endotoxin-free glutamine (2 mM), endotoxin-free penicillin (100 U/ml), and endotoxin-free streptomycin (100 μ g/ml)) of adult *A. viteae*. To remove larval forms (microfilariae) released by the adult female worms, the medium was passed through a 0.22- μ m pore size filter (Sigma-Aldrich). It was then transferred to a stirred cell ultrafiltration unit containing a YM10 membrane (Amicon). After reducing the volume of the sample to 5–10 ml and transferring the holding medium to endotoxin-free PBS, pH 7.2 (Cambrex Bioscience), it was further concentrated to 200–500 μ l using Centricon microconcentrators with a 30-kDa cutoff membrane (Amicon). The sample was then applied to a 30 \times 1-cm Superose 6 column (HR 10/30; BD Biosciences), fitted to an isocratic FPLC system (BD Biosciences), previously equilibrated with endotoxin-free PBS, pH 7.2, at room temperature. The column was eluted at a flow rate of 0.5 ml/min and monitored for absorbance at 280 nm. More than 95% of the protein eluted as a single peak, and this was purified ES-62. The purity and identity of each batch were confirmed by a combination of SDS-PAGE and Western blotting, the latter using a rabbit antiserum specific for ES-62.

Diazoniumphenyl-PC was prepared by dissolving 1 mmol of aminophenyl-PC (Toronto Research Chemicals) in 3 ml of 1 N HCl and adding 1 mmol of sodium nitrite. Diazoniumphenyl-PC (125 μ mol) was coupled to 1.5 μ mol of OVA by incubation in 5 ml of 0.1 M borate buffer with 0.15 M sodium chloride, pH 9.0, for 12 h at 4°C, followed by dialysis against 10 mM phosphate buffer, pH 7.2. The absence of endotoxin from the ES-62, PC-OVA, and OVA samples was confirmed using an Endosafe kit (Charles River Laboratories).

Cell culture

Macrophages and DCs were prepared from 6- to 8-wk-old, male, wild-type BALB/c and C57BL/6, TLR2 knockout (KO), TLR4 KO, MyD88 KO, C3H/HeN, and C3H/HeJ mice. C3H/HeN and C3H/HeJ mice were purchased from Harlan Olac. Wild-type C57BL/6, TLR 2, TLR4, and MyD88 KO mice (all on a C57BL/6 background) were maintained at the Universities of Manchester and Cambridge and were gifts from Prof. S. Akira (University of Osaka, Osaka, Japan). ST2 KO mice were maintained at the University of Glasgow and were gifts from Dr. A. McKenzie (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.).

Resident peritoneal macrophages were removed by peritoneal washing, enriched by plastic adherence for 2 h, and rested overnight before stimu-

lation. Bone marrow-derived macrophages (bmMs) and dendritic cells (bmDCs) were prepared by 6-day culture of bone marrow cells from femurs. The bmMs were derived by culture in full DMEM (2 mM glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 10% FCS) supplemented with 5% horse serum and 20% L929 cell line-conditioned medium (contains CSF-1). The bmDCs were derived by culture in full RPMI 1640 (2 mM glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 10% FCS) supplemented with 10% GM-CSF-transfected X63 myeloma cell line-conditioned medium and 50 μ M 2-ME.

Priming and surgical implantation of osmotic minipumps

Alzet Osmotic MiniPumps (model 2002; Charles River Laboratories) were primed, according to the manufacturer's instructions, with ES-62 or PBS and implanted s.c. on the backs of male 6-wk-old BALB/c mice as described previously (11). All animals were housed individually and were killed on day 14 by a rising concentration of CO₂. Resident peritoneal macrophages and bmDCs were prepared as described above.

TaqMan real-time PCR

TaqMan real-time RT-PCR was performed as described previously (11). The probes used were 5'-FAM (reporter) and 3' TAMRA (quencher): murine TLR4: probe, 5'-FAM-GCCAAATTTTGTCTCCACAGCCACCA-TAMRA-3'; forward primer, 5'-AGGAAGTTTCTCTGGACTAACCAAGTTAGTA-3'; reverse primer, 5'-AAATTGTGAGCCACATTGAGTTTC-3'; murine CD14: probe, 5'-FAM-CCCGACCCTCCAAGTTTATAGCGCTG-TAMRA-3'; forward primer, 5'-AGCCCTCTGTCCCTCAAGT-3'; reverse primer, 5'-TCTCCATCCCCGCGTTAC-3'; and murine myeloid differentiation-2 (MD-2): probe, 5'-FAM-CGAAGCGTAAGGAAGTTCTGTGCATG-TAMRA-3'; forward primer, 5'-CCTATTCATCAGTGTCACCTCCATAGA-3'; reverse primer, 5'-CTGCAAAAAGAATAGTCATCATGT-3'. PCRs were performed in triplicate in an ABI PRISM 7700 sequence detector (Applied Biosystems). Data analysis was performed using Applied Biosystems Sequence Detection software, and samples were normalized by the reference reporter hypoxanthine-guanine phosphoribosyltransferase (HPRT).

LPS-binding protein (LBP)/LPS interaction assay

The Endoblock LBP ELISA test kit (HyCult Biotechnology) was used according to the manufacturer's instructions to investigate whether ES-62 disrupts the interaction between LPS and LBP. Briefly, binding of biotinylated LPS to precaptured human LBP was assessed using streptavidin peroxidase, followed by tetramethylbenzidine. ES-62 was preincubated with either LBP or biotinylated LPS for 30 min. Polymyxin B, which competes with LBP for binding to LPS, was used as a positive control by 30-min preincubation with biotinylated LPS.

ELISA

IL-12 p40 and TNF- α ELISAs were performed according to the manufacturer's instructions using an Ab pair and a mono/poly ELISA kit, respectively (both from BD Pharmingen).

Flow cytometry

The protocol for staining cells for analysis by flow cytometry was described previously (10). Cells were stained with Abs specific for CD11c in conjunction with Abs specific for the TLR4/MD-2 complex (eBioscience), MHC class II, B7.1 (CD80), B7.2 (CD86), CD54, and CD40, along with the relevant isotype controls (BD Pharmingen). Flow cytometry was conducted using a FACSCalibur immunocytometry system (BD Pharmingen).

Statistics

Statistical significance of ELISA data was calculated using Student's *t* test.

Results

ES-62 does not alter the availability of components of the LPS receptor complex

Although ES-62 induces low levels of proinflammatory cytokines such as IL-12 and TNF- α from macrophages and DCs, it renders such cells refractory to rechallenge with LPS (10, 11, 16). Homologous and heterodesensitization of LPS responses have been reported to be due to down-regulation of various components of the LPS receptor complex and signaling cascades (17–20). We have previously shown that macrophages and DCs from mice implanted s.c.

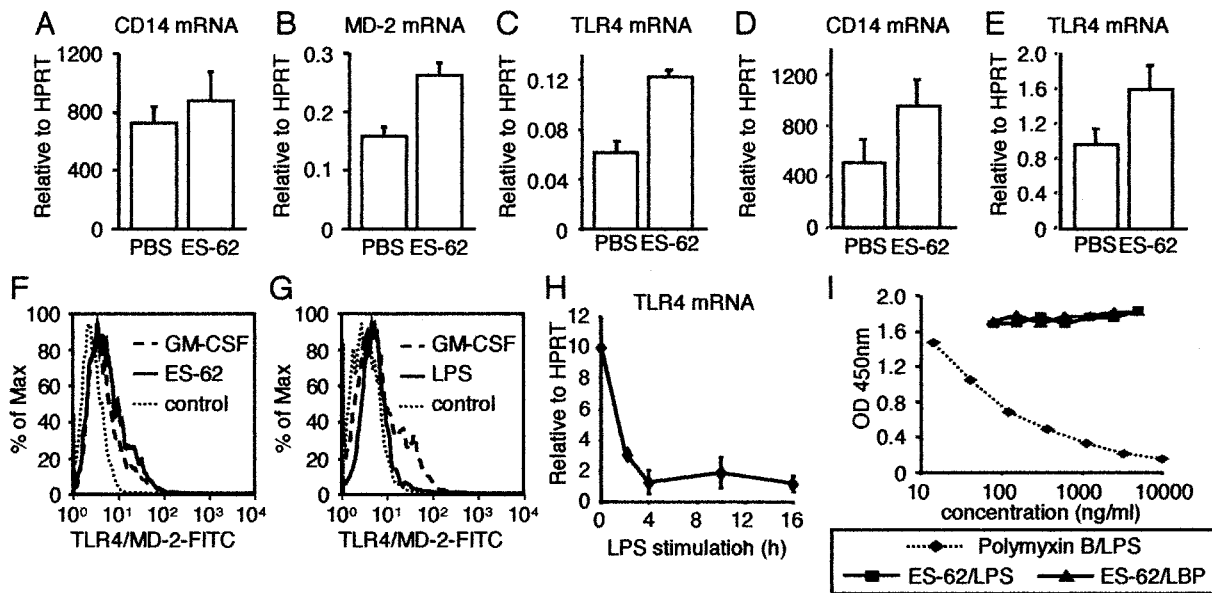


FIGURE 1. Availability of LPS receptor complex components after exposure to ES-62 in vivo. *A–E*, Mice were exposed to PBS or ES-62 (0.05 $\mu\text{g}/\text{h}$ in *A–C* or 0.2 $\mu\text{g}/\text{h}$ in *D* and *E*) by constant release from osmotic pumps for 2 wk. CD14, MD-2, and TLR4 mRNA levels in peritoneal macrophages (*A–C*) and bmDCs (*D* and *E*) were assessed by TaqMan real-time RT-PCR (expressed relative to HPRT mRNA). *F* and *G*, bmDCs were treated with either 2 $\mu\text{g}/\text{ml}$ ES-62 (*F*) or 1 $\mu\text{g}/\text{ml}$ *E. coli* LPS (*G*) for 24 h before analysis of TLR4/MD-2 surface expression by flow cytometry. *H*, TLR4 mRNA levels in bmDCs after 1 $\mu\text{g}/\text{ml}$ *E. coli* LPS treatment were assessed by TaqMan real-time RT-PCR (expressed relative to HPRT mRNA). *I*, Binding of biotinylated LPS to precaptured human LBP was assessed. ES-62 was preincubated with either LBP or biotinylated LPS for 30 min. Polymyxin B, which competes with LBP for binding to LPS, was used as a positive control by 30-min preincubation with biotinylated LPS.

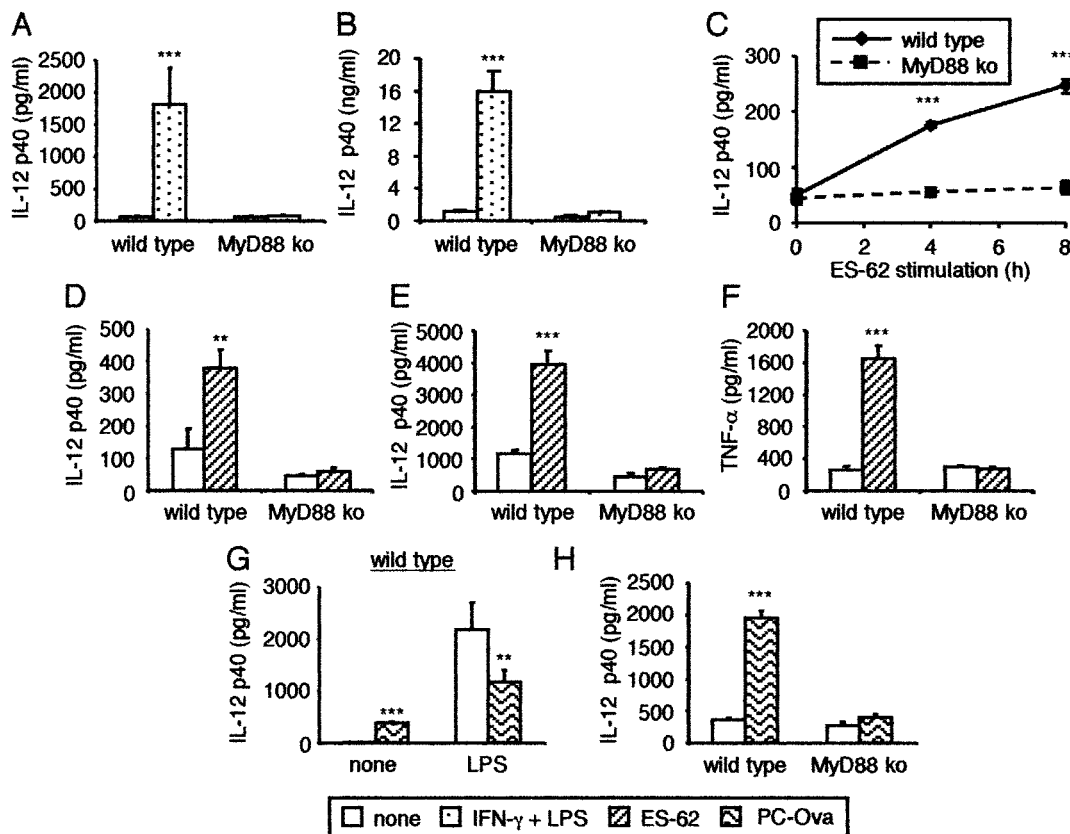


FIGURE 2. ES-62 and PC-OVA treatment of macrophages and DCs from MyD88 KO mice. Peritoneal macrophages (*A* and *D*), bmMs (*C*) and bmDCs (*B*, *E*, and *F*) from wild-type and MyD88 KO mice were treated with 100 U/ml IFN- γ and 100 ng/ml *S. minnesota* LPS (*A*), 1 $\mu\text{g}/\text{ml}$ *E. coli* LPS (*B*), or 2 $\mu\text{g}/\text{ml}$ ES-62 (*C–F*) for 24 h or the times indicated (*C*). *G*, bmDCs from wild-type mice were pretreated with 20 $\mu\text{g}/\text{ml}$ PC-OVA for 18 h before stimulation with 1 $\mu\text{g}/\text{ml}$ *E. coli* LPS. *H*, bmDCs from wild-type and MyD88 KO mice were treated with 20 $\mu\text{g}/\text{ml}$ PC-OVA for 24 h. IL-12 p40 and TNF- α in culture supernatants were measured by ELISA. Data are presented as the mean \pm SD and are representative of three experiments. **, $p < 0.01$; ***, $p < 0.001$.

with osmotic pumps that release ES-62 at a constant rate for 2 wk to give a serum concentration equivalent to that found during natural infection are hyporesponsive to *ex vivo* stimulation with LPS compared with macrophages from mice exposed to PBS-releasing pumps (11). We therefore assessed whether ES-62 exposure by release from osmotic pumps alters the expression of LPS receptor complex components by macrophages and DCs. Peritoneal macrophages from mice exposed to ES-62-releasing osmotic pumps did not show reduced levels of CD14, MD-2, or TLR4 mRNA (Fig. 1, A–C). The bmDCs derived from control and ES-62-treated mice expressed similar levels of CD14 and TLR4 mRNA (Fig. 1, D and E). Flow cytometry data confirmed that ES-62 does not alter the surface expression of TLR4-MD-2 (Fig. 1F). In contrast, we have found that LPS treatment down-regulates both mRNA and surface expression levels of TLR4-MD-2 (Fig. 1, G and H).

Finally, to determine whether ES-62 interferes with LPS binding to APCs, we used an LBP ELISA kit, which detects binding of biotinylated LPS to LBP, to assess the ability of ES-62 to prevent binding of LPS to LBP, which is required to disaggregate LPS and enable its presentation to the LPS receptor complex. Polymyxin B, which competes with LBP for binding to LPS, was used as a positive control. At concentrations $\leq 5 \mu\text{g/ml}$, ES-62 did not prevent LPS binding to LBP after preincubation with either LPS or LBP (Fig. 1I), indicating that ES-62 does not disrupt the LPS-LBP interaction by binding to either LPS or LBP.

Because ES-62 does not appear to disrupt the detection of LPS, it seems likely that the nematode product achieves its effects by modulating intracellular signaling pathways. As a first step, this would involve its recognition by target cells; hence, this was investigated.

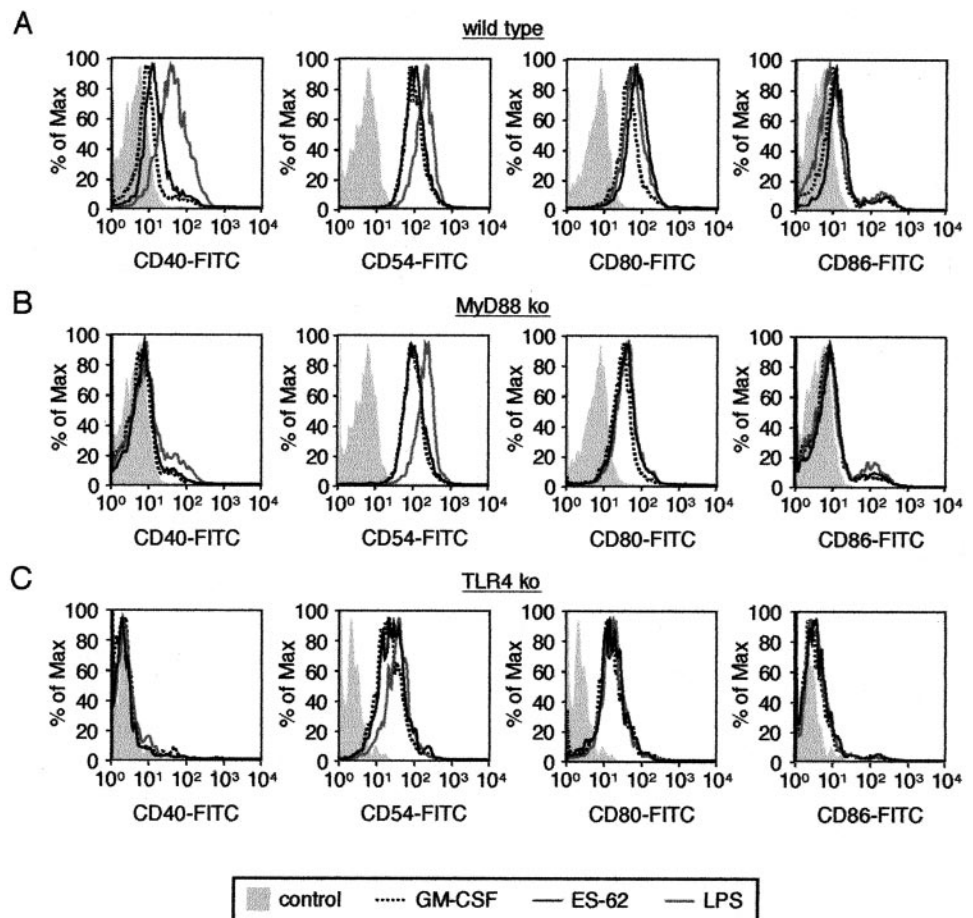
ES-62 signals in an MyD88-dependent manner

The PC moiety of ES-62 appears to be responsible, at least in part, for the immunomodulatory effects of ES-62 (9, 16). Because PC is a molecular pattern common to a variety of pathogen products, it was considered possible that ES-62 signals via a TLR. To address this possibility, we examined whether the ES-62 responses of macrophages and DCs were dependent on MyD88, a key TLR adaptor molecule (reviewed in Ref. 1), using wild-type and MyD88 KO mice (Fig. 2).

In agreement with previously published data (21, 22), LPS-induced secretion of IL-12 p40 and TNF- α from macrophages and bmDCs was essentially completely ablated in MyD88 KO mice compared with wild-type C57BL/6 mice (Fig. 2, A and B, and results not shown). These findings are consistent with LPS-induced proinflammatory cytokine production being TLR4 and MyD88 dependent (21–25). We now show that low level induction of these cytokines by ES-62 is also ablated in MyD88 KO mice (Fig. 2, C–F). Furthermore, with respect to the PC moiety of ES-62, OVA-conjugated PC (PC-OVA) also promotes the modulation of cytokine induction (Fig. 2, G and H), and as with the nematode product, this was shown to be absent in the MyD88 KO mouse.

In addition to inducing cytokine release from bmDCs, ES-62 treatment is known to result in the generation of bmDCs of a rather immature phenotype relative to those exposed to LPS (10). Thus, consistent with this, in bmDCs from wild-type C57BL/6 mice, maturation with LPS resulted in a strong up-regulation of CD40 and CD54 expression and a weak up-regulation of CD80 and CD86 relative to bmDCs cultured with GM-CSF alone (Fig. 3A).

FIGURE 3. Costimulatory molecule expression on MyD88 and TLR4 KO DCs. The bmDCs from wild-type (A), MyD88 KO (B), and TLR4 KO (C) mice were matured with 2 $\mu\text{g/ml}$ ES-62 or 1 $\mu\text{g/ml}$ *E. coli* LPS for 24 h. The CD11c⁺ population was subsequently analyzed for costimulatory molecule expression by flow cytometry.



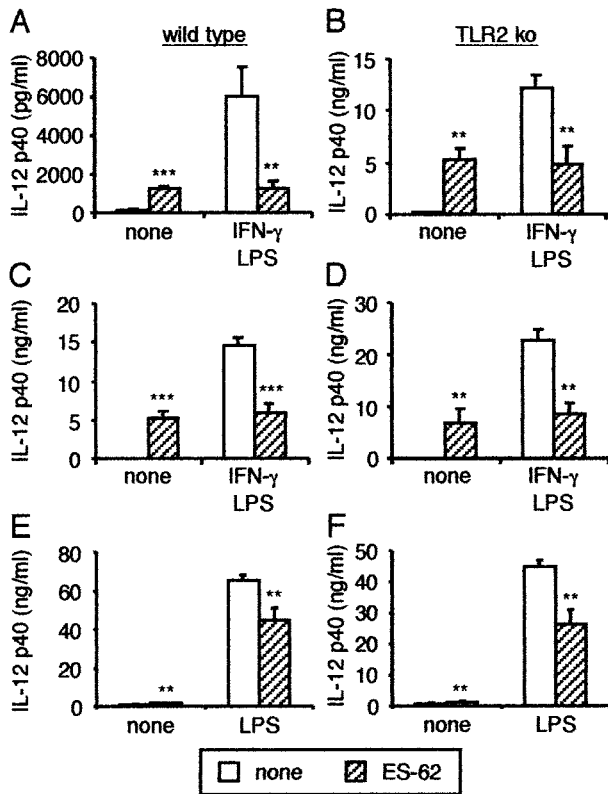


FIGURE 4. ES-62 treatment of macrophages and DCs from TLR2 KO mice. Peritoneal macrophages (A and B), bmMs (C and D), and bmDCs (E and F) from wild-type (A, C, and E) and TLR2 KO (B, D, and F) mice were pretreated with 2 $\mu\text{g/ml}$ ES-62 for 18 h before stimulation with 100 U/ml IFN- γ and 100 ng/ml *S. mimesota* LPS (A–D) or 1 $\mu\text{g/ml}$ *E. coli* LPS (E and F) for 24 h. IL-12 p40 in culture supernatants was measured by ELISA. Data are presented as the mean \pm SD and are representative of three experiments. **, $p < 0.01$; ***, $p < 0.001$.

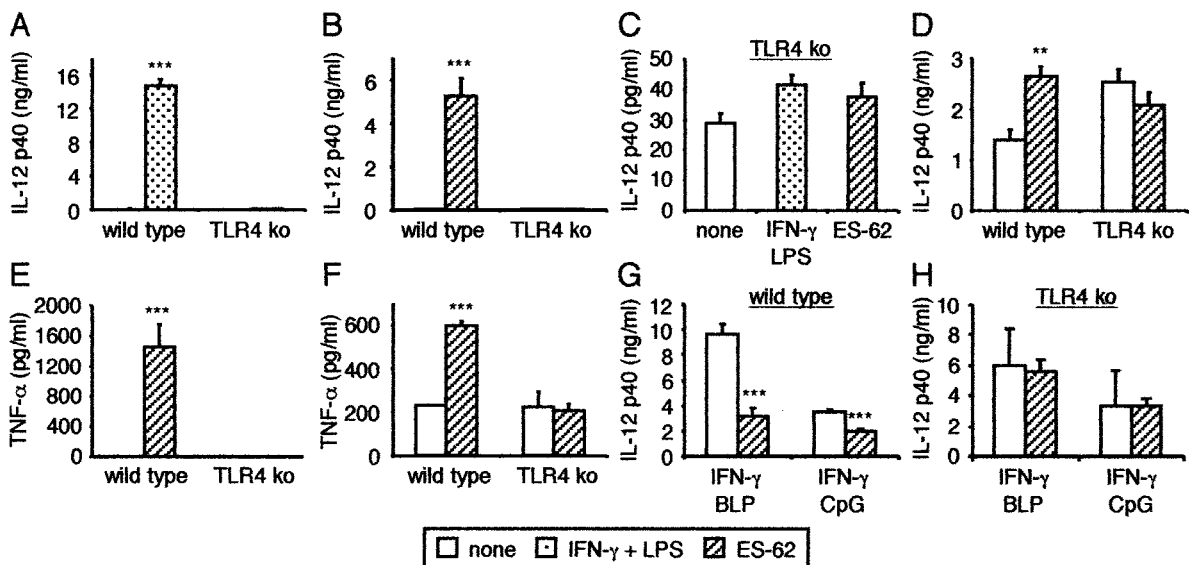


FIGURE 5. ES-62 treatment of macrophages and DCs from TLR4 KO mice. The bmMs (A–C and E) and bmDCs (D and F) from wild-type and TLR4 KO mice were treated with 100 U/ml IFN- γ plus 100 ng/ml *S. mimesota* LPS (A and C) or 2 $\mu\text{g/ml}$ ES-62 (B–F) for 24 h. C, Enlarged version of the TLR4 KO data from A and B. G and H, bmMs were pretreated with 2 $\mu\text{g/ml}$ ES-62 for 18 h before stimulation with 100 U/ml IFN- γ and 10 ng/ml BLP or 0.1 μM CpG for 24 h. IL-12 p40 and TNF- α in culture supernatants were measured by ELISA. Data are presented as the mean \pm SD and are representative of three experiments. **, $p < 0.01$; ***, $p < 0.001$.

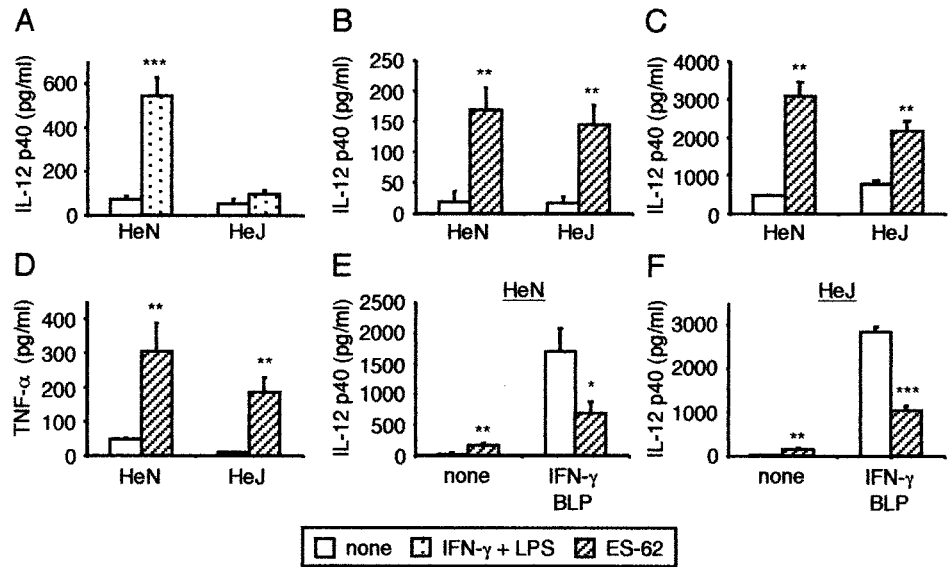
In contrast, although ES-62 induced CD80 up-regulation to a similar extent as LPS, it resulted in only marginal increases in the expression of CD40, CD54, and CD86 by CD11c⁺ bmDCs relative to the levels observed with GM-CSF alone (Fig. 3A). Investigation of the role of MyD88 in determining costimulatory molecule expression and DC phenotype indicated that although the expression of CD40 after exposure to either LPS or ES-62 was dramatically reduced, the defect in MyD88 signaling had little effect on the level of CD54, CD80, or CD86 expression (Fig. 3B). These results are therefore consistent with previous studies indicating that some LPS responses, particularly those relating to the induction of costimulatory molecules, can be MyD88 independent (22). Although this is also true of ES-62, the cytokine and costimulatory data taken together indicate that other immunomodulatory effects of ES-62 are MyD88 dependent and therefore are likely to implicate a role for one or more TLRs in ES-62 signaling.

Modulation of cytokine responses by ES-62 is abrogated in TLR4, but not TLR2, mice

To further investigate the possibility that ES-62 acts via one or more TLRs, we examined the modulation of cytokine production in macrophages and bmDCs from TLR2 and TLR4 KO mice. Both low level induction of IL-12 p40 and TNF- α as well as suppression of LPS-induced IL-12 p40 and TNF- α production were intact in peritoneal macrophages, bmMs, and bmDCs from TLR2 KO mice (Fig. 4 and results not shown). Similarly, the phenotypes of bmDCs induced in response to LPS or ES-62 were essentially identical in wild-type and TLR2 KO mice (results not shown). These data indicate that TLR2 is not required for modulation of cytokine production by ES-62. In contrast, ES-62 failed to stimulate low level IL-12 or TNF- α production by macrophages and bmDCs from LPS-unresponsive, TLR4 KO mice (Fig. 5, A–F). Likewise LPS- and ES-62-mediated modulation of the bmDC phenotype was lost in TLR4 KO mice (Fig. 3C).

Because TLR4 KO cells fail to respond to LPS, it was not possible to determine whether TLR4-mediated signals are responsible for the suppression of the LPS response by ES-62 observed in wild-type cells. Thus, to determine whether ES-62 acts simply by

FIGURE 6. ES-62 treatment of macrophages and DCs from TLR4-mutant C3H/HeJ mice. The bmMs (A and B), bmDCs (C), and peritoneal macrophages (D) from C3H/HeN and C3H/HeJ mice were treated with 100 U/ml IFN- γ and 100 ng/ml *S. minnesota* LPS (A) or 2 μ g/ml ES-62 (B–D) for 24 h. E and F, bmMs from HeN and HeJ mice were pretreated with 2 μ g/ml ES-62 before stimulation with 100 U/ml IFN- γ and 10 ng/ml BLP for 24 h. IL-12 p40 and TNF- α in culture supernatants were measured by ELISA. Data are presented as the mean \pm SD and are representative of three experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.



subverting LPS-TLR4 signaling or can signal via TLR4 to disrupt the action of other TLR ligands, we pretreated macrophages with ES-62 for 18 h before stimulation with IFN- γ and either the synthetic version of bacterial lipopeptide Pam₃CSK₄ (BLP; a TLR2 ligand) or CpG (a TLR9 ligand). ES-62 pretreatment suppressed the induction of IL-12 p40 and TNF- α by IFN- γ plus BLP and IFN- γ plus CpG in macrophages from wild-type, but not TLR4 KO, mice (Fig. 5, G and H, and data not shown). These data therefore indicate that TLR4 is required to mediate the modulation of IL-12 and TNF- α production by ES-62, and that, via TLR4, ES-62 alters the responses of macrophages and DCs to signals through multiple TLRs.

ES-62 is functional in macrophages and DCs from TLR4 mutant C3H/HeJ mice

The C3H/HeJ mouse strain has a point mutation in the TIR domain of TLR4 (23, 25). These mice express TLR4, but fail to produce cytokines in response to LPS. The Pro⁷¹²His mutation has been suggested to prevent the recruitment of MyD88 to TLR4 and thus to disrupt downstream signaling (26). Surprisingly, although the

LPS response was lost (Fig. 6A), ES-62-induced low level IL-12 p40 and TNF- α production was intact in macrophages and DCs from HeJ mice (Fig. 6, B–D, and data not shown). Furthermore, the ES-62-mediated suppression of IFN- γ plus BLP-induced IL-12 was also intact in cells from this strain (Fig. 6, E and F).

Interestingly, some of the effects of ES-62 (and LPS) on the bmDC phenotype (e.g., the up-regulation of CD40 expression on CD11c⁺ cells) are broadly abrogated in C3H/HeJ cells (Fig. 7). In contrast, the other ES-62- and LPS-mediated effects, such as up-regulation of CD54, CD80, and CD86, were largely unaffected by the mutation. It should be noted that ES-62 reproducibly induces a greater degree of maturation, in terms of up-regulation of costimulatory molecule expression, of bmDC derived from C3H/HeN mice than that observed with either C57BL/6 (Fig. 3) or BALB/c (10, 16).

ES-62 does not modulate non-MyD88-dependent signals

Because ES-62 suppresses signals from various TLRs, it is likely that it targets a common component of TLR pathways, such as one of the adaptor molecules. Consistent with this, neither ES-62 nor

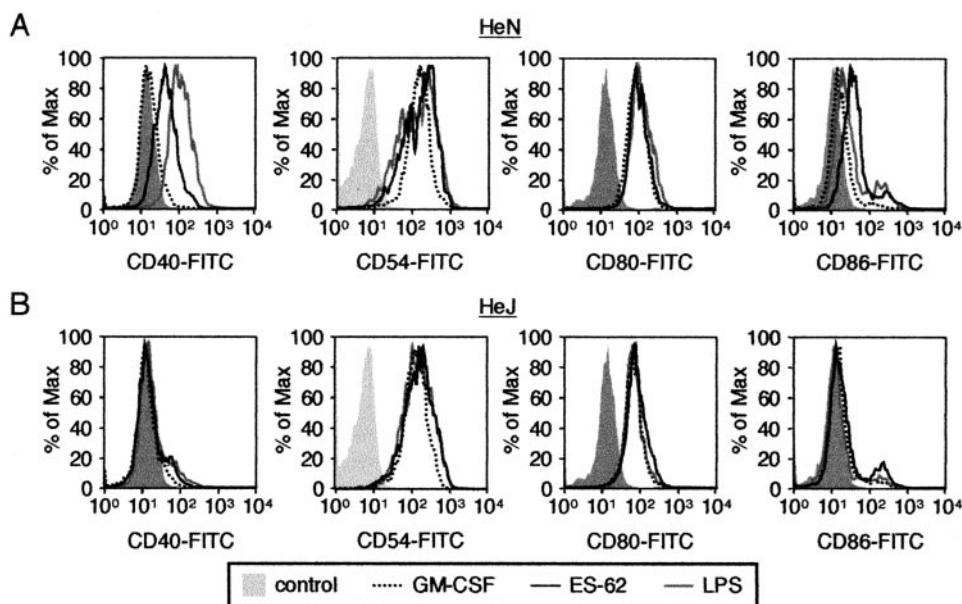


FIGURE 7. Costimulatory molecule expression on C3H/HeJ DCs. The bmDCs from C3H/HeN (A) and C3H/HeJ (B) mice were matured with 2 μ g/ml ES-62 or 1 μ g/ml *E. coli* LPS for 24 h. The CD11c⁺ population was subsequently analyzed for costimulatory molecule expression by flow cytometry.

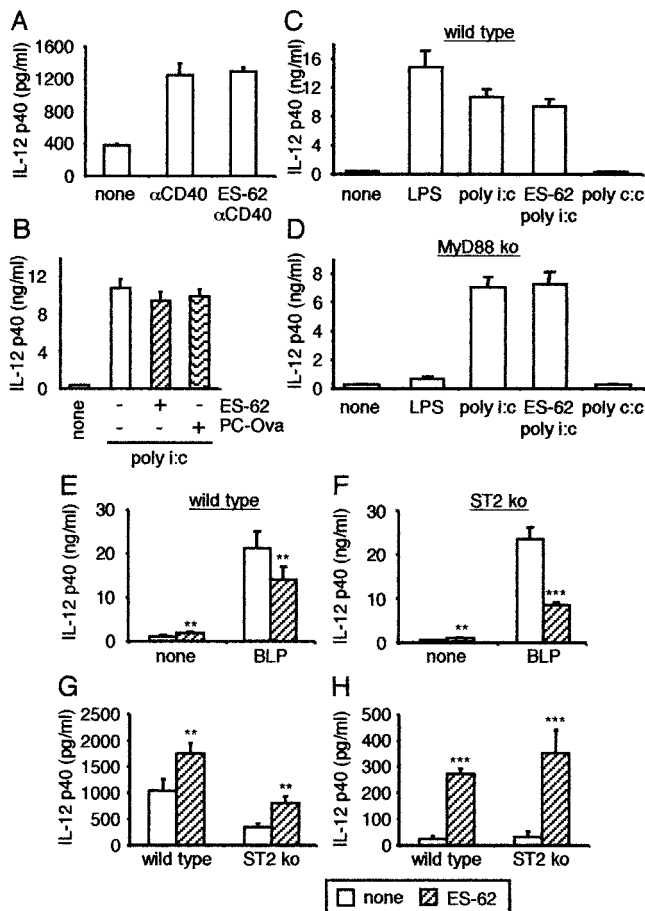


FIGURE 8. ES-62 treatment of macrophages and DCs from ST2 KO mice. *A* and *B*, bmDCs from wild-type C57BL/6 mice were treated with 2 μ g/ml ES-62 or 20 μ g/ml PC-OVA for 18 h before stimulation with 10 μ g/ml anti-CD40 (*A*) or 100 μ g/ml poly(I:C) (*B*). *C* and *D*, Wild-type (*C*) and MyD88 KO (*D*) bmDCs were treated with 2 μ g/ml ES-62 for 18 h before stimulation with 100 μ g/ml poly(I:C) or with 1 μ g/ml *E. coli* LPS and 100 μ g/ml poly(C:C) as controls. *E* and *F*, bmDCs from wild-type (*E*) and ST2 KO (*F*) mice were treated with 2 μ g/ml ES-62 for 18 h before stimulation with 10 ng/ml BLP. *G*, An enlarged version of the ES-62-only data from *E* and *F*. *H*, bmMs from wild-type and ST2 KO mice were treated with 2 μ g/ml ES-62 for 24 h. IL-12 p40 in culture supernatants were measured by ELISA. Data are presented as the mean \pm SD and are representative of three experiments. **, $p < 0.01$; ***, $p < 0.001$.

PC-OVA suppressed the induction of IL-12 after ligation of the non-TLR receptor CD40 using an anti-CD40 Ab (Fig. 8*A* and data not shown). Furthermore, neither ES-62 nor PC-OVA was capable of suppressing IL-12 produced after stimulation with the TLR3 ligand poly(I:C) (Fig. 8, *B–D*). Although the TLR adaptor molecule MyD88 was originally thought to be required for cytokine induction via TLR3, MyD88 does not appear to be recruited to TLR3, and its role in transducing TLR3 signals remains controversial (reviewed in Ref. 3). In our hands, poly(I:C) (and not control poly(C:C)) is capable of strongly inducing IL-12 production by bmDCs from both wild-type and MyD88 KO mice (Fig. 8, *C* and *D*), suggesting that MyD88-independent signals are responsible for IL-12 production. The inability of ES-62 to suppress anti-CD40 and poly(I:C) signals suggests that ES-62 may target MyD88 to modulate cytokine induction triggered via MyD88-dependent TLRs, but not MyD88-independent receptors.

Suppression of cytokine production by ES-62 is not mediated by the TIR family member ST2

A recent report demonstrated that signaling from TLR4, but not TLR3, can be negatively regulated by the membrane-bound form of the IL-1R-related TIR family member ST2 (27). ST2 disables LPS signaling by sequestering the MyD88 and Mal adaptors, thereby preventing their recruitment to TLR4. To determine whether ES-62 could be acting via ST2, we examined the modulation of cytokine production by ES-62 in bmMs and bmDCs from ST2 KO mice. Both low level IL-12 production and inhibition of LPS- or BLP-induced IL-12 were intact in ST2 KO macrophages and DCs (Fig. 8, *E–H*, and data not shown), indicating that ES-62 signals are not transduced by ST2.

Taken together, these data suggest that the use of TLR4 by ES-62 occurs by a novel mechanism, which requires TLR4 to be present, but not fully functional. Suppression of TLR signaling may involve sequestration of adaptor molecules, but this is not mediated by ST2.

Discussion

We have previously demonstrated the capacity of the filarial nematode secreted product ES-62 to modulate the activation of macrophages and DCs, thereby biasing the subsequent immune response to a Th2/anti-inflammatory phenotype (10, 11). Thus, ES-62 induces the development of DCs that are capable of driving Th2 responses (10) and inhibits the Th1/inflammatory response of macrophages and DCs to treatment with bacterial LPS (11). Interestingly, however, ES-62 induces low level induction of IL-12 and TNF- α in vitro, which appears at the outset of its exposure to macrophages, before the appearance of the inhibitory effect on Th1/proinflammatory cytokines (this study and Ref. 11). This ability of ES-62 to induce weak IL-12 and TNF- α responses in vitro could be considered atypical, because the majority of our data indicates a dominant anti-inflammatory/Th2-promoting capacity (28). However, we have also previously shown that exposure of naive murine spleen cells to ES-62 results in IFN- γ production, whereas this is reduced and compensated for by the production of IL-4 if the cells under study are obtained from mice that had been exposed to ES-62 (9, 29). Consistent with these findings, a recent paper showed proinflammatory/Th1, but not Th2, cytokine production during early in vitro responses to infection with the human filarial nematode *Brugia malayi*, an infection that is normally considered to be associated with a Th2 response (30). Thus, there seems to be a pattern emerging that filarial nematodes and in particular their immunomodulatory molecules, such as ES-62, may generate some Th1/proinflammatory activity before settling down to a Th2/anti-inflammatory phenotype.

Although the precise role of the low level cytokine induction by macrophages and DCs exposed to ES-62 and its relationship to the suppressive effect are as yet unclear, it is our belief that they are likely to be related. We propose that ES-62 exposure may trigger abortive signaling, which initially results in the production of low levels of cytokine, but subsequently renders cells hyporesponsive to further stimulation. At present, the precise molecular mechanisms underlying this desensitization are not clear, but we have previously shown that ES-62 treatment of macrophages suppresses the LPS-induced activation of p38 and JNK MAPKs (31, 32), which have been widely established to be required for proinflammatory cytokine induction. In addition, and in a manner reminiscent of that observed with ES-62-mediated desensitization of BCR signaling (33), we have reported that ES-62 induces activation of an ERK-dependent negative feedback inhibition mechanism that suppresses IL-12 production (32).

We now show that ES-62 does not suppress the response of macrophages and DCs to LPS by either down-regulating the expression of components of the LPS receptor complex (TLR4, CD14, and MD-2) or disrupting the interaction between LPS and LBP, which is required to disaggregate LPS and enable its presentation to the LPS receptor complex (Fig. 1). Moreover, we have demonstrated that ES-62 suppresses the responses of macrophages and DCs to other TLR ligands, such as BLP (TLR2) and CpG (TLR9) (Figs. 5 and 6). It therefore seems likely that ES-62 does indeed achieve its effects via modulation of intracellular signaling, rather than by altering receptor availability.

Many of the immunomodulatory effects of ES-62 can be attributed to the presence of PC moieties attached to this glycoprotein. Indeed, the inhibition of LPS-induced proinflammatory cytokine production by macrophages and DCs can be mimicked by treatment with PC alone or PC conjugated to denatured BSA or OVA (Fig. 2) (16). PC is a molecular pattern associated with pathogen products of a diverse range of organisms, including bacteria, fungi, and protozoa, as well as filarial and gastrointestinal nematodes (reviewed in Ref. 34). It enables detection of pathogens by the host, but can also function to promote pathogen survival via modulation of host immune responses. PC is also an important component of a variety of mammalian glycoproteins, phospholipids, and glycolipids with roles in the immune response and disease induction, including platelet-activating factor (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine) and sphingosylphosphorylcholine (35–38). Furthermore, PC-containing constituents of low density lipoproteins (LDLs) have been implicated in the development of atherosclerosis and are expressed by apoptotic and necrotic cells (39). PC therefore represents a candidate TLR ligand for the recognition of both pathogen- and host-associated molecules. Thus, ES-62, as a pathogen PC-containing molecule, could arguably be predicted to interact with a TLR.

Consistent with this proposal, we have now shown, using knockout mice, that ES-62 mediates at least some of its immunomodulatory effects via a TLR4 pathway, because ES-62-induced low level cytokine induction was absent in macrophages and DCs from TLR4 KO mice (Fig. 5). Similarly, inhibition of signals through TLR2 (BLP) and TLR9 (CpG) was absent in TLR4 KO macrophages/DCs (Fig. 5). Furthermore, the signaling triggered by ES-62 or PC-OVA to induce low levels of cytokine occurs via recruitment of the adaptor MyD88 (Fig. 2). We were unable to determine whether the inhibition induced by ES-62 is also MyD88 dependent, because it is not possible to examine LPS, BLP, and CpG responses in MyD88 KO mice. Nevertheless, the ES-62-mediated suppression of the cytokine responses to a variety of TLR ligands suggests that ES-62 might operate by preventing the association of a component common to TLRs. For example, ES-62 could act by suppressing the recruitment of the MyD88 adaptor to TLRs. Consistent with this, neither ES-62 nor PC-OVA appears capable of suppressing the response of macrophages and DCs to a non-TLR receptor (CD40) or the TLR3 ligand poly(I:C), which in our hands is MyD88 independent (Fig. 8).

At first sight, the effect of ES-62 is reminiscent of the LPS tolerance phenomenon. LPS preincubation is widely known to induce both homotolerance to subsequent LPS stimulation and heterotolerance to stimulation with other TLR ligands, such as BLP and CpG (40, 41). The mechanism implicated for LPS tolerance involves impaired recruitment of MyD88 to TLR4 and subsequent activation of IRAK-1 (42, 43). Brint et al. (27) recently showed that the membrane-bound form of the TIR family member ST2 inhibits TLR4, but not TLR3, signaling by sequestering MyD88 and another adaptor molecule, Mal. However, as we have now shown that the effects of ES-62 are ST2 independent, these data

suggest that ES-62 and LPS act via distinct mechanisms. Moreover, LPS has been reported to result in down-regulation of TLR4, and although controversial, this has been suggested to contribute to LPS tolerance (20). Our data support the proposal that LPS does indeed down-regulate TLR4 mRNA and surface expression. However, we found that, by contrast, ES-62 does not reduce TLR4 expression (Fig. 1). Moreover, preliminary analysis of intracellular signaling pathways (p38 MAPK, PI3K-Akt, and NF- κ B) triggered by LPS and ES-62 in macrophages and DCs also indicates differences in the mechanisms used by ES-62 and LPS (H. S. Goodridge, W. Harnett, and M. M. Harnett, unpublished observations). Taken together, these data strongly suggest that ES-62 induces desensitization of TLR-mediated proinflammatory cytokine production by a mechanism distinct from that of LPS tolerance.

Other PC-containing molecules, including phospholipid oxidation products of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (ox-PAPC) contained in LDL fractions, are known to achieve their effects via TLR4 (44–46). Miller et al. (44) showed that minimally modified (mildly oxidized) LDL requires TLR4/MD-2 to induce F-actin polymerization and macrophage spreading. Furthermore, Walton et al. (45) showed that TLR4 is required for ox-PAPC-mediated induction of IL-8 production by endothelial cells, along with a 37-kDa GPI-anchored protein, which is not CD14. Although oxidation of the phospholipids/fatty acids appears to be critical for these responses, it has been proposed that such oxidation serves to alter presentation of the polar head groups resulting in their receptor recognition (44).

In another study Walton et al. (46) proposed that ox-PAPC disrupts LPS activation of endothelial cells by preventing the transmigration of TLR4 and MD-2 to caveolae and causing the redistribution of caveolin-1 from the cell surface to the internal membrane. Like ES-62, ox-PAPC also inhibited the effects of TLR2 (*Mycobacterium tuberculosis* lipoprotein) as well as TLR4 ligands on macrophages. However, it did not affect TNF- α - or IL-1 β -induced IL-8 production. Similarly, we found that although ES-62 treatment triggers IL-8 production by neutrophils, it does not inhibit IL-18-induced IL-8 production (S. Culshaw, B. Leung, I. McInnes, M. M. Harnett, and W. Harnett, unpublished observations).

Direct binding of ligands to their TLRs has been demonstrated for some pathogen products (47–50). However, it is not currently known whether any of the PC-containing compounds interact directly with TLR4. Walton et al. (45) showed that scavenger receptor A, lectin-like oxidized LDL receptor-1, and CD36 are not responsible for ox-PAPC-mediated induction of IL-8 production by endothelial cells. Similarly, scavenger receptor A does not appear to be responsible for mediating the immunomodulatory effects of ES-62 (S. Gordon and W. Harnett, unpublished observations). The Asp²⁹⁹Gly TLR4 polymorphism in humans has been shown to be associated with a reduced risk of atherosclerosis (51). One potential mechanism for this could be a reduced response to ox-PAPC due to defective recognition by the mutant TLR4. Thus, Asp²⁹⁹, which is located in the ectodomain of TLR4, may be a critical residue for the response to PC-containing molecules such as ox-PAPC and ES-62. Interestingly, mutation of this residue does not affect the recognition of *Escherichia coli* LPS by monocytes (52).

Our observation that ES-62 is functional in macrophages and DCs from TLR4 mutant C3H/HeJ mice (Fig. 6) demonstrates a novel mechanism for pathogen use of TLR4. In contrast to the recognition of and response to LPS, ES-62 does not require TLR4 to be fully functional. Similarly, a recent paper showed that recombinant heat shock fusion proteins activated C3H/HeJ DCs almost as well as DCs from wild-type mice, whereas heat shock

fusion proteins did not activate DCs from B10/ScNcr mice, which lack the TLR4 gene (53).

The Pro⁷¹²His mutation found in TLR4 in C3H/HeJ mice was originally suggested to lead to hyporesponsiveness to LPS as a result of uncoupling from MyD88, based on the finding that MyD88 fails to associate with human TLR2 bearing an analogous Pro⁷¹²His mutation (54). However, several reports (55–57) show that TLR4 bearing the Pro⁷¹²His mutation can still recruit MyD88 and Mal, and that the mutation may, instead, result in the inability of the receptor to homodimerize. This proposal may therefore suggest that in cells bearing the Pro⁷¹²His mutation, ES-62, unlike LPS, might be able to either signal via TLR4 monomers or, alternatively, drive TLR4 homodimerization or heterodimerization, the latter with an as yet unidentified coreceptor. Our data showing that MyD88 is involved (Fig. 2) might implicate another TLR. However, data from TLR2 and TLR6 KO studies indicate that neither of these TLRs is involved with respect to ES-62 (Fig. 4) (H. Goodridge, F.-Y. Liew, S. Akira, O. Takeuchi, M. M. Harnett, and W. Harnett, unpublished observations). Whatever the mechanism, these data indicate that the use of C3H/HeJ mice to probe for TLR4 dependence of responses should be treated with caution in the absence of corroborating data from TLR4 KO mice.

Interestingly, unlike with ES-62, fully functional TLR4 does appear to be required for the minimally modified LDL response (44). It is possible, therefore, that the PC component of ES-62 is required for recognition by TLR4, but that another component of the ES-62 glycoprotein is additionally responsible for coreceptor recruitment and/or signal transduction. Indeed, although free PC or PC conjugated to denatured BSA/OVA can mimic many of the effects of ES-62, preliminary data indicate that denaturation of this glycoprotein is sufficient to partially disrupt its function (our unpublished observations), perhaps implicating a role for the protein component in at least some of its immunomodulatory properties.

In summary, ES-62 acts via a TLR4-dependent pathway to disrupt macrophage and DC responses to ligands for this (LPS) and other (BLP and CpG) TLRs. It operates by a novel mechanism, which requires TLR4 to be present, but not fully functional, possibly involving collaboration with another TLR and impaired recruitment of MyD88. This study along with further characterization of the composition of the ES-62 receptor complex will advance our understanding of innate sensing of and immune modulation by pathogens.

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