Suppressive Oligodeoxynucleotides Protect Mice from Lethal Endotoxic Shock[1]

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Endotoxic shock is a life-threatening condition caused by exposure to bacterial LPS. LPS triggers the release of acute phase, proinflammatory, and Th1 cytokines that facilitate the development of endotoxic shock. Synthetic oligodeoxynucleotides (ODN) expressing suppressive TTAGGG motifs effectively down-regulate the production of proinflammatory and Th1 cytokines elicited by a variety of immune stimuli. The current results demonstrate that suppressive ODN protect mice from LPS-induced endotoxic shock. Underlying this protective effect is the ability of suppressive ODN to bind to and prevent the phosphorylation of STAT1 and STAT4, thereby blocking the signaling cascade mediated by LPS-induced IFN-β and IL-12. These findings suggest that suppressive ODN might be of use in the treatment of endotoxic shock. *The Journal of Immunology,* **2005, 174: 4579–4583.**

Septic shock is a common cause of mortality in intensive care units despite the availability of antibiotics to treat infection and vasopressors to support tissue perfusion (1, 2). Bacterial LPS has been identified as a major cause of septic shock (3). LPS binds to TLR4 expressed on macrophages and monocytes, triggering the rapid release of cytokines, including TNF-α, IL-12, IL-18, IFN-β, IL-6, and IL-1β (3–5). These factors initiate a secondary immunostimulatory cascade that includes the up-regulation of NO synthase, reactive oxygen species, macrophage migration inhibitory factor, high mobility group box 1, and IFN-γ through STAT1 and STAT4 phosphorylation (6–11). Agents capable of blocking this immunostimulatory cascade might be useful in the prevention/treatment of endotoxic shock.

Several recent reports indicate that suppressive oligonucleotides (ODN)[3] are capable of blocking the deleterious production of Th1 and proinflammatory cytokines (12–19). One class of suppressive ODN is characterized by the presence of methylated CG or unmethylated GC sequences that selectively block CpG-induced immune activation (20–24). The second class of suppressive ODN contains repetitive TTAGGG motifs patterned after those present in mammalian telomeres (12). Previous studies established that telomeric DNA and suppressive ODN containing TTAGGG motifs down-regulate the production of proinflammatory and Th1 cytokines (12–15). Although initially identified by their ability to block CpG-induced immune activation, this class of suppressive ODN (typified by ODN A151) was subsequently shown to block multiple forms of immune stimulation (14–19) and to be effective in the prevention/treatment of pathologic autoimmune responses (13, 15, 25).

The current findings indicate that suppressive TTAGGG ODN also inhibit LPS-mediated endotoxic shock. This effect is mediated by the binding of suppressive ODN to STAT1 and STAT4, preventing the phosphorylation of these molecules and thus inhibiting the signal transduction cascade initiated by the immunomodulatory cytokines released by LPS exposure.

Materials and Methods

LPS shock model

Eight-week-old female BALB/c mice were obtained from the National Cancer Institute). The mice were injected i.p. with 300 μg of ODN (a dose previously found to prevent the development of autoimmune disease) and/or challenged i.p. with 50–200 μg of LPS (*Escherichia coli* serotype O55:B5; Sigma-Aldrich) in 200 μl of PBS. All studies were approved by the Center for Biologics Evaluation and Research animal care and use committee.

Oligodeoxynucleotides and reagents

Phosphorothioate ODNs and biotin- and FITC-labeled ODNs were synthesized at the Center for Biologics Evaluation and Research core facility. The following ODNs were used: suppressive ODN_{A151} (TTAGGGTTAGGGTTAGGGTTAGGG), control ODN_{C151} (TTCAAATTCAAATTCAAATTCAAA), immunostimulatory ODN_{1555} (GCTAGACGTTAGCGT), and suppressive ODN_{2010} (GCGGCGGGCGGCGCGCGCCC). Murine anti-IL-12 Ab was purchased from R&D Systems. Anti-IFN-β Ab was purchased from Yamasa. Nonspecific control Ab was purchased from BD Pharmingen.

Cell preparation

Peritoneal cells were isolated from mice injected i.p. with thioglycolate. These cells were cultured in RPMI 1640 plus 5% FCS for 4 h, the nonadherent cells were removed, and peritoneal macrophages in the adherent monolayer were used for further study (26). NK cells were purified from BALB/c splenocytes by positive selection using anti-DX5 microbeads (Miltenyi Biotec). The cells were cultured with 50 ng/ml recombinant murine IL-2 (R&D Systems) for 6–10 days. The resulting cell population contained >90% $DX5^+$ cells, as measured by flow cytometry.

Cytokine ELISAs

Cytokine levels in culture supernatants were measured by ELISA, as previously described (27). Paired TNF-α-, IL-12-, IL-6-, and IFN-γ-specific mAbs were purchased from BD Pharmingen. Ninety-six-well Immulon H2B plates (Thermo LabSystems) were coated with first-stage, cytokine-specific Abs, then blocked with PBS/1% BSA. Culture supernatants were added, and bound cytokine was detected by the addition of biotin-labeled secondary Ab, followed by phosphatase-conjugated avidin and a phosphatase-specific colorimetric substrate (Pierce). Standard curves were generated using recombinant cytokines purchased from R&D Systems. The

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[3] Abbreviations used in this paper: ODN, oligodeoxynucleotide; IRF, IFN regulatory factor.

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concentrations of IL-18 were determined using a mouse IL-18 ELISA kit (MBL). All assays were performed in triplicate.

Ligand binding studies

Spleen cells were incubated for 3 h with 5 μM biotinylated ODN before lysis and subjected to microcentrifugation to remove nuclear debris. Alternatively, clarified cellular lysates were incubated with 1 μM biotinylated ODN and 5 μM free ODN for 1 h. Twenty-five microliters of avidin-coated agarose (Sigma-Aldrich) was added to 200 μl of lysate and rotated for 30 min at 4°C. Pellets were washed four times in lysis buffer, boiled for 5 min, and then analyzed by Western blotting (28).

Western blots

Stimulated cells were lysed in cold lysis buffer containing protease and phosphatase inhibitors. This solution was boiled for 5 min, size-separated on a 4–12% gradient SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane. Immunoblots were probed with Abs specific to phospho-STAT1 (Tyr^{701}), phospho-JNK, phospho-p38, phospho-ERK1/2, total Iκ-Bα (New England Biolabs), phospho-STAT4 (Tyr^{693}), and IFN regulatory factor 3 (IRF-3; Zymed Laboratories), followed by HRP-coupled donkey secondary Abs. Signals were visualized by autoradiography using the LumiGLO detection kit (New England Biolabs). Blots were then stripped and reprobed with specific Abs to STAT1, JNK, p38, ERK1/2 (New England Biolabs), and STAT4 (Santa Cruz Biotechnology).

Confocal microscopy

Cells were incubated with FITC-labeled ODN for 60 min at 37°C. Cells were washed, fixed, permeabilized, and stained with rabbit anti-STAT1 Ab, followed by Cy3-labeled anti-rabbit Ab. The subcellular localization of Cy3 and FITC signals was analyzed by laser scanning microscope (LSM5 Pascal; Carl Zeiss).

Statistical analysis

Differences in survival were determined using the generalized Wilcoxon test of Kaplan-Meier plots. Other data were analyzed using Student's *t* test.

Results

Suppressive ODN A151 protects mice from LPS-induced endotoxic shock

BALB/c mice challenged with 200 μg (10 mg/kg) of *E. coli* LPS uniformly succumb to endotoxic shock within 2–3 days (Fig. 1*A*). Experiments were conducted to examine the abilities of the two different classes of suppressive ODN described in the literature to prevent this LPS-induced shock. Survival was significantly improved by treating mice with the broadly suppressive class of ODN (typified by ODN A151) (12) 3 h before LPS challenge ($p < .001$; Fig. 1*A*). In contrast, animals treated with the other class of suppressive ODN (known to selectively block CpG-induced immune activation, typified by ODN 2010) (21) were not protected (Fig. 1*B*). Treatment with control ODN also had no impact on survival (Fig. 1*A*). In this model system, administration of immunostimulatory CpG ODN reduced survival (Fig. 1*C*), a finding consistent with the ability of CpG DNA to synergistically enhance LPS-induced cytokine production (29, 30).

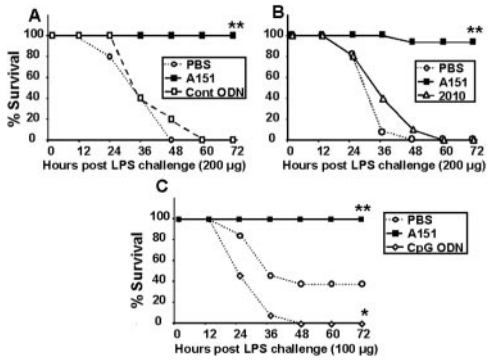

FIGURE 1. Suppressive ODN protect mice from LPS-induced endotoxic shock. BALB/c mice were injected i.p. with PBS, 300 μg of suppressive ODNs A151 or 2010, or 50 μg of CpG ODN. The animals were challenged 3 h later with 200 μg (*A* and *B*) or 100 μg (*C*) of *E. coli* LPS. Survival curves were analyzed by the Kaplan-Meier method. Results from two to four independent experiments involving a total 10–16 mice/group were combined, because control animals behaved similarly in all experiments. *, $p < 0.05$; **, $p < 0.001$ (compared with PBS treatment group by the generalized Wilcoxon's test).

Kinetic studies showed that ODN A151 provided optimal protection (100% survival; $p < 0.01$) when administered shortly before LPS challenge. Delaying ODN delivery until 1 h after challenge resulted in the survival of only 20% of challenged mice, although the duration of survival was significantly prolonged ($p < .05$; Fig. 2).

Suppressive ODN A151 does not affect LPS-induced cytokine production or cell signaling

The interaction of LPS with its cognate ligand (TLR4) leads to MAPK activation and NF-κB signaling through a MyD88-dependent pathway and IRF-3 activation through a MyD88-independent pathway (5). There was no difference in the rapidity or magnitude of phosphorylation of representative elements of these signaling cascades when peritoneal macrophages were stimulated in vitro with LPS with or without suppressive ODN (Fig. 3).

As a consequence of activating these signaling pathways, LPS induces the rapid release of proinflammatory cytokines, including TNF-α, IL-12, IL-6, and IL-18 (Figs. 4 and 5*A*). Treatment with suppressive ODN had no effect on the production of these cytokines (Figs. 4 and 5*A*). Similarly, in vitro cytokine production by LPS-stimulated murine spleen cells was not affected by suppressive ODN (data not shown).

Suppressive ODN A151 inhibits secondary IFN-γ production

Previous studies established that the cytokines and chemokines induced directly by LPS modulate the subsequent production of additional immunomodulatory factors. For example, LPS directly induces the secretion of IL-12 and IFN-β, with serum levels of these cytokines peaking by 3 h (Fig. 5*A* and data not shown). These, in turn, stimulate the production of IFN-γ through a STAT4-dependent pathway, with IFN-γ levels peaking at 6–9 h (Fig. 5*A*) (11, 31). As expected based on this model of cascading immune stimulation, the production of IFN-γ after LPS stimulation is inhibited by neutralizing Abs against IL-12 and IFN-β (Fig. 5*B*).

Although IFN-γ is not directly induced by LPS, it contributes to the development of endotoxic shock and serves as a marker for the immunostimulatory cascade central to the pathogenesis of this disease (32). It is therefore relevant that suppressive ODN significantly reduced IFN-γ production in LPS-challenged mice (Fig. 5*A*;

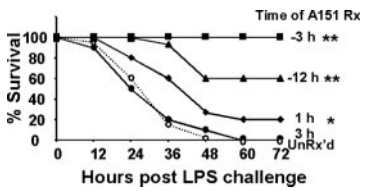

FIGURE 2. Reduction of LPS-induced endotoxic shock by suppressive ODN A151 is time dependent. Mice were injected with 300 μg of suppressive ODN from 12 h before to 3 h after challenge with 200 μg of LPS. Results from two to four independent experiments involving a total 10–20 mice/group were combined, because control animals behaved similarly in all experiments. *, $p < 0.05$; **, $p < 0.001$ (compared with no treatment group by the generalized Wilcoxon's test).

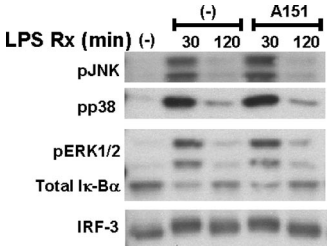

FIGURE 3. Suppressive ODN do not alter LPS-induced signaling. Peritoneal macrophages were incubated with 3 μM suppressive ODN for 30 min, then treated with 10 ng/ml LPS for the indicated time. Cell lysates were analyzed by direct Western blotting using specific Abs to phosphorylated JNK, p38, and ERK and total Iκ-Bα and IRF-3. All experiments were repeated three times with similar results.

$p < 0.001$). Similarly, LPS-dependent IFN-γ production in vitro was significantly down-regulated by inclusion of suppressive ODN (Fig. 5*B*). These effects were cytokine specific, because suppressive ODN had no effect on the production of other factors, such as TNF-α (Fig. 5*B*).

Suppressive ODN A151 blocks STAT1 and STAT4 phosphorylation

Based on the observation that suppressive ODN have no effect on initial LPS-induced immune activation, but instead inhibit the subsequent up-regulation of IFN-γ, the effect of suppressive ODN on the proteins that regulate IFN-γ activation was examined. As shown in Fig. 6, both STAT1 and STAT4 are phosphorylated after LPS-mediated stimulation of macrophages. Of considerable interest, this phosphorylation was significantly reduced by the addition of suppressive ODN. A similar effect was observed when macrophages were stimulated with IFN-β (rather than LPS), and when NK cells were stimulated with IL-12 (Fig. 7). These effects were dependent on the concentration of suppressive ODN added (Figs. 6 and 7) and were motif specific (with control ODN having no effect on STAT phosphorylation).

Suppressive ODN A151 binds to STAT1 and STAT4

To clarify the mechanism by which suppressive ODN A151 blocks STAT phosphorylation, ligand binding studies were performed. The results show that this suppressive ODN selectively binds to STAT1 and STAT4. This interaction is highly specific, because A151 did not bind to JNK or other NF-κB- and MAPK-related molecules, nor did control ODN bind to STAT1 or STAT4 (Fig. 8*A* and data not shown). Competitive inhibition studies further documented the specificity of suppressive ODN for STAT1 and STAT4. Unlabeled A151 blocked the binding of biotinylated A151 to these molecules, whereas control ODN had no effect (Fig. 8*B*).

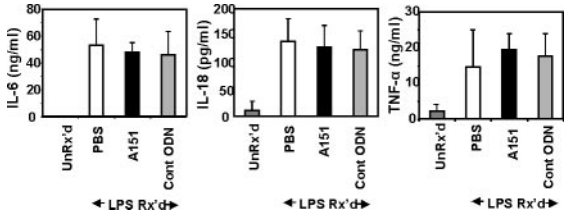

FIGURE 4. Suppressive ODN do not alter LPS-induced proinflammatory cytokine production. BALB/c mice ($n = 5$/group) were injected with PBS or 300 μg of ODN and challenged 3 h later with 50 μg of LPS. Serum levels of TNF-α, IL-6, and IL-18 were measured after 90 min. The experiment was repeated three times with similar results.

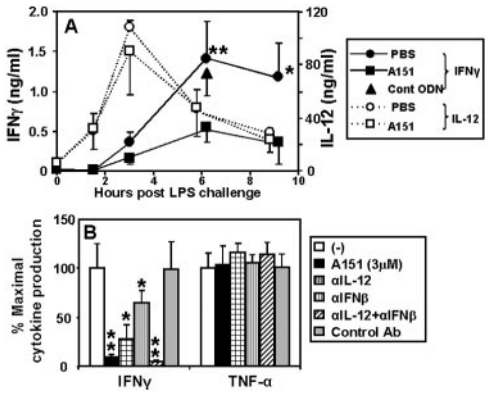

FIGURE 5. Suppressive ODN A151 inhibits LPS-mediated IFN-γ production. *A*, BALB/c mice were treated as described in Fig. 4, and serum IFN-γ and IL-12 levels were monitored for 9 h. Data represent results from two or three independent experiments involving a total of 4 to 12 mice per group. *B*, Spleen cells were incubated with A151 or Abs against IL-12 and/or IFN-β, then stimulated with LPS. The amounts of IFN-γ and TNF-α present in 16-h culture supernatants were determined by ELISA. Results represent the mean ± SD of three independent experiments. All experiments were repeated three times with similar results. *, $p < 0.05$; **, $p < 0.001$ (compared with PBS treatment (−) group).

Confocal microscopy was used to monitor the interaction between suppressive ODN A151 and STAT molecules. In the absence of A151, STAT1 and STAT4 (red) distributed throughout the cytoplasm, where they did not colocalize with control ODN. Of considerable interest, when administered to cells treated with A151, the location of these STAT molecules shifted, such that they colocalized with the suppressive ODN (yellow).

Discussion

This work is the first to demonstrate that synthetic ODN patterned after the suppressive TTAGGG motifs present at high frequency in mammalian telomeres reduce mortality from LPS-induced endotoxic shock. This form of therapy was maximally effective when initiated shortly before LPS challenge, but it prolonged survival

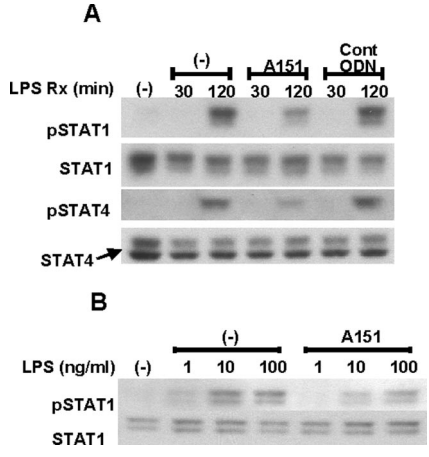

FIGURE 6. Suppressive ODN A151 inhibits LPS-induced STAT1 and STAT4 phosphorylation. Peritoneal macrophages were incubated with 3 μM ODN for 30 min, then treated with 10 ng/ml LPS for the indicated times (*A*) or for 120 min (*B*). Cell lysates were analyzed by Western blot using anti-phosphorylated STAT1 (pSTAT1) and anti-STAT1 Abs, and anti-phosphorylated STAT4 (pSTAT4) and anti-STAT4 Abs. The experiments were repeated three times with similar results.

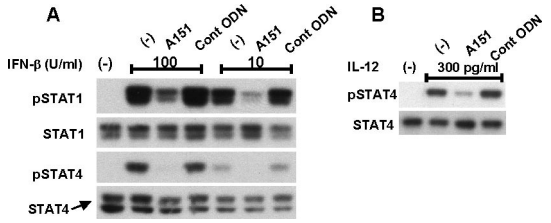

FIGURE 7. Effect of suppressive ODN A151 on STAT1 and STAT4 phosphorylation. Peritoneal macrophages (*A*) or NK cells (*B*) were incubated with 3 μM ODN for 30 min, then stimulated with the indicated dose of IFN-β (*A*) or IL-12 (*B*) for 20 min. The effect on STAT1 and STAT4 phosphorylation was determined by Western blot analysis of cell lysates. The experiments were repeated three times with similar results.

even when initiated after LPS exposure (Figs. 1 and 2). Suppressive ODN A151 bound to and prevented the phosphorylation of STAT1 and STAT4, thereby blocking the downstream immunostimulatory cascade elicited by the cytokines induced by LPS (Figs. 6–9).

The first suppressive ODN identified were composed of poly-GC-rich sequences that competitively inhibit the interaction of CpG ODN with TLR9 (20). Although inactive in the current experiments, such ODN are capable of inhibiting the immune activation triggered by CpG DNA (20–24). The current study used a second, more broadly active class of suppressive ODN containing repetitive TTAGGG motifs like those found at high frequency in mammalian telomeres. This class of ODN, typified by ODN A151, form G tetrads through Hoogstein binding of poly-G runs (12, 16–19). Current results indicate that this class of suppressive ODN directly binds to and prevents the phosphorylation of STAT1 and STAT4, thereby blocking the signaling pathway that drives the production of IFN-γ (Figs. 5–9). This finding confirms and extends the observations of Jing et al. (33), who recently reported that G-rich ODN could block the phosphorylation of STAT1 induced by IFN-γ in a cancer cell line. Confocal analysis indicates that suppressive ODN induce the migration of STAT1 and STAT4 to vesicles, where they colocalize with A151 (but not control ODN; Fig. 9). Additional findings showed that the activity of suppressive ODN was sequence specific and dose dependent (Figs. 6–9).

The interaction of LPS with TLR4 on macrophages and monocytes directly triggers the production of various proinflammatory and Th1 cytokines (3, 4). These cytokines, in turn, trigger the release of additional immunomodulatory factors that together contribute to the development of endotoxic shock (34–39). Initial studies showed that suppressive ODN did not inhibit LPS-induced cell activation or reduce LPS-dependent production of IL-6, IL-12,

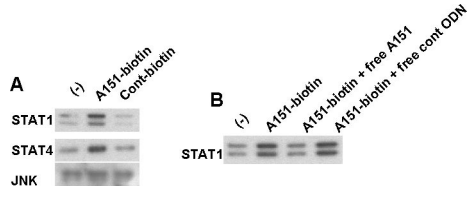

FIGURE 8. Suppressive ODN bind to STAT1 and STAT4. *A*, Spleen cells were incubated with 5 μM biotin-labeled ODN for 3 h at 37°C. The ODN plus bound proteins were isolated from cell lysates using avidin-coated agarose, then analyzed by immunoblotting with anti-STAT1, anti-STAT4, and anti-JNK Abs. *B*, Spleen cell lysates were incubated with 1 μM biotinylated A151 plus 5 μM unlabeled ODN at 4°C for 1 h. The biotinylated ODN was precipitated, and the associated proteins were analyzed by immunoblotting with anti-STAT1 Ab. The experiments were repeated three times with similar results.

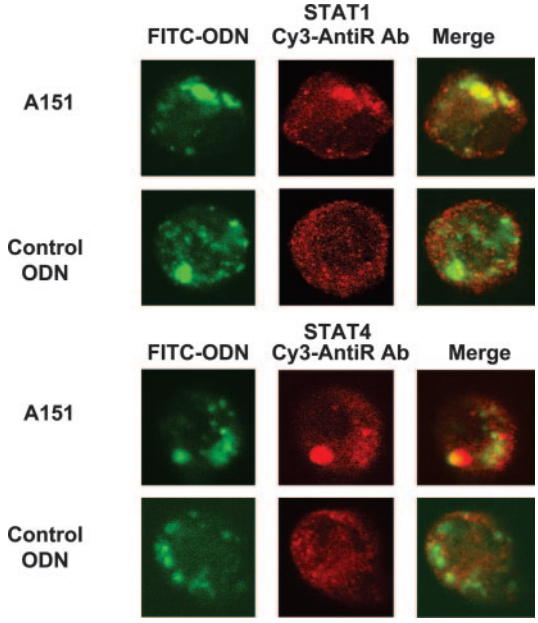

FIGURE 9. Colocalization of suppressive ODN A151 with STAT1 and STAT4 in vesicles. Peritoneal macrophages were incubated with 1 μM FITC-labeled ODN for 60 min, then fixed, permeabilized, and stained with rabbit anti-STAT1 Ab, followed by Cy3-labeled anti-rabbit Ab. The cells were examined with confocal microscopy. The *left panels* show FITC-labeled ODN (green). The *middle panels* show STAT1 and STAT4 (red). Merged panels (*right*) show that suppressive ODN colocalize with STAT1 and STAT4 (yellow).

TNF-α, or IL-18 (Figs. 3–5). Additional experiments showed that suppressive ODN did not induce the production of factors capable of down-regulating inflammatory responses, such as IL-10, TGF-β, PGE_2, or inhibitory molecules such as cytokine-inducible Src homology 2 containing protein/suppressor of cytokine signaling, IL-1R-associated kinase-M, A20, or Src homology 2 domain-containing phosphatase 1/2 (data not shown). Rather, suppressive ODN acted at a subsequent stage of LPS-initiated immune activation: they blocked STAT1 and STAT4 phosphorylation triggered by the cytokines initially elicited by LPS (such as IL-12 and IFN-β). This effect was not limited to the down-regulation of IFN-γ; suppressive ODN also blocked the production of IFN-inducible protein-10, another gene regulated through the IFN-β/STAT1 pathway (data not shown). Studies involving STAT1, STAT4, and Tyk2 knockout mice (TyK2 is a JAK that participates in the IFN-β- and IL-12-mediated signaling cascade) demonstrate that these pathways contribute to LPS-induced endotoxic shock (26, 40). Thus, interventions capable of targeting these signaling pathways should be of therapeutic benefit.

Previous reports demonstrated that suppressive ODN of the A151 class can down-regulate Th1 responses induced by a variety of immune stimuli (12–19). The current results establish that these ODN dampen Th1 signaling by binding to and preventing the phosphorylation of STAT1 and STAT4, thereby reducing the production of IFN-γ, a cytokine critical to the maintenance of Th1 immunity. Suppressive ODN could therefore be of use in the treatment of diseases mediated by pathologic Th1 immune responses. Consistent with this possibility, studies in relevant animal models indicate that suppressive ODN not only improve survival after LPS-induced endotoxic shock, but are also useful in the prevention/treatment of autoimmune diseases ranging from arthritis to systemic lupus erythematosus (13, 15, 25, 41, 42). These encouraging results support the continued development of this class of suppressive ODN.

Disclosures

The authors have no financial conflict of interest.

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