



Published in final edited form as:

*J Immunol.* 2008 June 01; 180(11): 7648–7654.

## Suppressive Oligodeoxynucleotides Inhibit Silica-Induced Pulmonary Inflammation

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### Abstract

Inhalation of silica-containing dust particles induces silicosis, an inflammatory disease of the lungs characterized by the infiltration of macrophages and neutrophils into the lungs and the production of proinflammatory cytokines, chemokines, and reactive oxygen species (ROS). Synthetic oligodeoxynucleotides (ODN) expressing “immunosuppressive motifs” were recently shown to block pathologic inflammatory reactions in murine models of autoimmune disease. Based on those findings, the potential of suppressive ODN to prevent acute murine silicosis was examined. In vitro studies indicate that suppressive ODN blunt silica-induced macrophage toxicity. This effect was associated with a reduction in ROS production and p47phox expression (a subunit of NADPH oxidase key to ROS generation). In vivo studies show that pretreatment with suppressive (but not control) ODN reduces silica-dependent pulmonary inflammation, as manifest by fewer infiltrating cells, less cytokine/chemokine production, and lower levels of ROS ( $p < 0.01$  for all parameters). Treatment with suppressive ODN also reduced disease severity and improved the survival ( $p < 0.05$ ) of mice exposed to silica.

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The inhalation of dust containing crystalline silica particles causes silicosis, an incurable lung disease that progresses even after dust exposure ceases. The World Health Organization estimates that over a million US workers are exposed to silica dust annually, and that thousands worldwide die each year from silicosis (1, 2). The pulmonary inflammation caused by silica inhalation is characterized by a cellular infiltrate and the accumulation of chemokines, cytokines (including TNF- $\alpha$ , IL-1, and IL-6), and ROS in bronchoalveolar lavage (BAL)<sup>2</sup> fluid (3–7).

Macrophages are the predominant immune cell type present in alveolar spaces where they play an important role in the lung pathology associated with silica inhalation (3). The uptake of silica particles by macrophages triggers the production of reactive oxygen species (ROS; including H<sub>2</sub>O<sub>2</sub>) via the oxidative stress pathway, which in turn contributes to pulmonary damage and macrophage death (8–11). The dominant pathway by which silica-stimulated

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#### Disclosures

The authors have no financial conflict of interest.

<sup>2</sup>Abbreviations used in this paper: BAL, bronchoalveolar lavage; ODN, oligodeoxynucleotide; ROS, reactive oxygen species; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; LDH, lactate dehydrogenase; phox, phagocytic oxidase.

macrophages produce H<sub>2</sub>O<sub>2</sub> involves the activation of NADPH oxidase (3, 10–12). In this pathway, cytoplasmic p47 phagocytic oxidase (p47phox) interacts with p67<sup>phox</sup> to form an active gp91 enzyme complex, with changes in ROS production correlating closely with levels of p47phox expression (12–14).

One potential strategy for limiting the production of proinflammatory cytokines and ROS after silica exposure involves treatment with “suppressive” oligonucleotides (ODN). Suppressive ODN express motifs based on the repetitive TTAGGG hexamers present at high frequency in the telomeric ends of self DNA (15). Previous studies showed that these motifs (released by injured host cells) block Th1 and proinflammatory cytokine production in vitro and down-modulate over-exuberant/pathologic immune responses in vivo (such as those found in septic shock and autoimmune diseases) (15–20).

To explore whether suppressive ODN might be of benefit in treating the pulmonary damage caused by silica inhalation, a well established murine model of acute silicosis was used (5, 21, 22). Although human disease typically progresses over many years (even decades), pulmonary inflammation in this model system develops rapidly and resolves over the course of several weeks. Nevertheless, this murine model shares important characteristics with human silicosis, including acute neutrophilic extravasation, increased protein in BAL, and progressive fibrosis (21, 23, 24). Results from this study establish the utility of suppressive ODN in the prevention of acute pulmonary inflammation, and identify the cell types and mechanism associated with this protection.

## Materials and Methods

### ODNs and reagents

Phosphorothioate ODNs were synthesized at the Center for Biologics Evaluation and Research core facility. The following ODNs were used: suppressive ODN, TTAGGGTTAGGGTTAGGGTTAGGG; and control ODN, TTCAAATTCAAATTCAAATTCAA. ODN contained <0.1 units of endotoxin/mg, as assessed by a *Limulus* amoebocyte cell lysate assay. Silica particles were obtained from U.S. Silica and were sterilized at 200°C for 2 h to inactivate any contaminating endotoxin (25).

### In vitro studies

The mouse macrophage-like cell line RAW264.7 and the murine bronchial cell line MM14.Lu were purchased from American Type Cell Culture and maintained in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES, 1.0 mM sodium pyruvate, nonessential amino acids, and 0.0035% 2-ME. Peritoneal cells were isolated from mice injected i.p. with 3% thioglycolate, as previously described (20). Single-cell suspensions were allowed to attach to the plate over 24 h and were then cultured with 20–40 µg/cm<sup>2</sup> of silica in 6- or 96-well plates. Phosphorothioate ODNs were added to culture 1 h before the introduction of silica.

## In vivo studies

The 10-wk-old female BALB/c mice were obtained from the National Cancer Institute. Mice were anesthetized using a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). Silicosis was induced by the intratracheal instillation of 2.5 mg of sterilized silica particles (MIN-U-SIL 5; mean particle size 1.7  $\mu$ m) in 100 liters of sterile saline as previously described (26). Some mice were treated by i.p. injection of 300  $\mu$ g of suppressive or control ODN 24 and 3 h before silica administration. BAL fluid was collected from anesthetized mice by delivering and then removing 0.8 ml of PBS into the lungs 5 times, using a 22-gauge catheter (26). Cell differentials (200 cells counted) were performed on cytocentrifuge preparations of BAL after methanol fixation and staining with Diff-Quick (Dade Behring). All studies were approved by the Center for Biologics Evaluation and Research Animal Care and Use Committee.

## Cytokine ELISA

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

## Cell viability assays

Silica-mediated cytotoxicity was assessed using both MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and LDH (lactate dehydrogenase) assays. Cells were seeded in 96-well plates at a density of 10,000 cells/well and allowed to adhere for 24 h. The cultures were then exposed to silica particles  $\pm$  3  $\mu$ M of suppressive or control ODN for 24 h. Culture supernatants were collected and analyzed using an LDH release kit (Roche). Briefly, the kit's catalyst and dye solution were mixed and added to the culture supernatants for 30' at room temperature. The reaction was stopped, and LDH concentration quantified colorimetrically by comparison to a standard curve.

An MTT assay was performed on cells that had been cultured with silica  $\pm$  ODN as described above. Briefly, 100  $\mu$ l of media containing MTT (Sigma-Aldrich; 0.5 mg/ml) was added to the adherent cells for 2 h. Non-internalized MTT was then washed away, and the cells lysed by the addition of 50  $\mu$ l DMSO. This released the MTT internalized by viable cells. MTT concentration was measured colorimetrically, and cell viability determined as the OD at 570 nm of treated/untreated cultures.

## Detection of ROS

A total of 100,000 cells were cultured with 40  $\mu$ g/cm<sup>2</sup> of silica particles  $\pm$  3 M of ODN in 96-well plates for 24 h in medium lacking phenol red (which interferes with the hydrogen peroxide colorimetric assay). Culture media was collected after 24 h, and H<sub>2</sub>O<sub>2</sub> levels quantified using an Fe<sup>3+</sup>-xylenol orange reaction kit (BioAssay Systems), as recommended

by the manufacturer. Duplicate assays in which supernatants were treated for 5' with 0.5 U/well of murine liver-derived catalase (Sigma-Aldrich) were run to insure that the activity measured reflected H<sub>2</sub>O<sub>2</sub> concentration.

### Western blots

Cells were cultured with 40  $\mu\text{g}/\text{cm}^2$  of silica particles  $\pm$  3 M of ODN for 24 h, and then lysed in cold buffer containing 137 mM sodium chloride, 20 mM Tris, 1 mM EDTA, 50 mM sodium fluoride, 1% Triton X, and pro-tease inhibitor. Protein concentrations were determined using a BCA Protein Assay kit (Pierce), and 10  $\mu\text{g}$  of whole cell extract then boiled for 5 min in sample buffer. The boiled samples were run on 4–12% gradient SDS-PAGE and transferred onto a PVDF membrane. Immunoblots were probed with Ab specific to p47phox (Upstate Biotechnology), followed by HRP-conjugated secondary Ab (Amersham Biosciences). Signals were visualized by ECL system using an ECL kit (Amersham Biosciences). Blots were reprobed with anti- $\beta$ -actin Ab (Sigma-Aldrich) to normalize for protein loading.

### Statistical analysis

Statistical analyses was performed using MedCalc, version 9.3.7.0 (Med-Calc Software) and S-Plus (Version 7; Insightful). Differences between groups were assessed using a one-way ANOVA with Bonferroni post-hoc test control for type I error. Differences in survival were determined using the log rank test of Kaplan-Meier. Analysis of weight loss was performed using mixed effects longitudinal regression models (28). All tests were two-sided; probability values  $<0.05$  were considered significant. All values are expressed as means  $\pm$  SE unless otherwise noted.

## Results

### Suppressive ODN reduce the inflammatory response of MM14.Lu lung cells cultured with silica

The murine MM14.Lu cell line provides a useful in vitro model for studying the effect of silica exposure on pulmonary cells. MM14.Lu cells incubated with 40  $\mu\text{g}/\text{cm}^2$  of silica respond by producing the proinflammatory chemokine KC (Fig. 1). The inclusion of suppressive ODN during culture abrogated this silica-induced KC production in a dose-dependent manner ( $p < 0.01$ ), whereas control ODN had no effect (Fig. 1). Of note, a similar down-regulation in IL-6 production by silica-stimulated MM14.Lu cells treated with suppressive ODN was also observed (data not shown).

### Suppressive ODN reduce the production of ROS and improve the viability of macrophages cultured with silica

Macrophages contribute to the lung pathology induced by silica inhalation (3). Macrophages exposed to silica produce ROS (including H<sub>2</sub>O<sub>2</sub>) via a p47phox-dependent pathway (8–14, 29–31). Consistent with those findings, peritoneal macrophages and the RAW264.7 macrophage cell line exposed to silica particles in vitro responded by up-regulating p47phox expression and producing large amounts of H<sub>2</sub>O<sub>2</sub> (Figs. 2 and 3;  $p < 0.05$ ). The elevation in

both H<sub>2</sub>O<sub>2</sub> secretion and p47phox expression were significantly blunted by treatment with suppressive (but not control) ODN ( $p < 0.05$ ).

Silica exposure can also result in macrophage death (32, 33). The viability of RAW264.7 cells and freshly isolated peritoneal macrophages was monitored by both MTT and LDH release assays. As expected, macrophage viability was significantly reduced by culture with 10–40  $\mu\text{g}/\text{cm}^2$  of silica for 24 h (Fig. 4; data not shown). Treating the silica-exposed macrophages with suppressive (but not control) ODN significantly improved their viability, consistent with suppressive ODN preventing silica-dependent inflammatory damage (Fig. 4;  $p < 0.05$ ).

### Suppressive ODN inhibit silica-induced pulmonary inflammation in vivo

The ability of suppressive ODN to prevent pulmonary inflammation was examined in a murine model of acute silicosis. As previously described (26), silicosis was induced by instilling 2.5 mg of silica via an intratracheal catheter into the lungs of normal BALB/c mice. The resultant inflammatory response peaked 3 days later, and was characterized by a pulmonary infiltrate dominated by neutrophils and the production of inflammatory cytokines and chemokines (26, 34, 35). To assess whether treatment with suppressive ODN reduced this inflammation, two doses of suppressive ODN were administered one day before silica installation (this treatment regimen was selected on the basis of successful preliminary experiments).

BAL fluid was collected from mice on days 1, 3, 5, 7, and 14. As seen in Fig. 5A, silica instillation resulted in a significant increase in BAL cellularity by day 3 (rising from  $1.3 \times 10^5$  cells/ml in controls to  $14.6 \times 10^5$  cells/ml in mice treated with silica;  $p < 0.01$ ). This pulmonary infiltrate consisted primarily of neutrophils ( $79.4 \pm 4.9\%$ ) with some macrophages ( $20.2 \pm 4.6\%$ ), whereas BAL from normal mice was composed almost exclusively of macrophages ( $96.0 \pm 1.0\%$ ; Fig. 5, A and B). Among mice pretreated with suppressive ODN, the silica-induced increase in BAL cellularity and associated neutrophil accumulation were significantly reduced at peak, and resolved more rapidly ( $p < 0.01$ ; Fig. 5, B and C, and data not shown). No such beneficial effects were observed when mice were pretreated with control ODN, establishing the specificity of these outcomes.

Consistent with previous studies (34, 36), silica instillation also triggered a significant increase in the level of KC in BAL ( $p < 0.01$ ; Fig. 6). The production of this inflammatory chemokine was reduced by nearly 50% in mice pretreated with suppressive (but not control) ODN (Fig. 6;  $p < 0.01$ ). The silica-induced production of pulmonary IL-12 was similarly reduced by suppressive ODN treatment (data not shown).

It was difficult to detect H<sub>2</sub>O<sub>2</sub> in BAL (which is diluted upon collection), but consistent with the ability of silica to trigger ROS production by macrophages in vitro, H<sub>2</sub>O<sub>2</sub> levels were significantly elevated in the serum of mice 3 days post-silica instillation (Fig. 7;  $p < 0.01$ ). Treating these mice with suppressive ODN resulted in a significant reduction in systemic H<sub>2</sub>O<sub>2</sub> levels (Fig. 7;  $p < 0.01$ ).

### Suppressive ODN reduce the pathology induced by in vivo silica exposure

The inflammatory response induced by instilling silica into the lungs of normal BALB/c mice had a systemic impact on animal health, as evidenced by significant weight loss (>12%) that peaked on day 3 and persisted for greater than 2 wk (Fig. 8; data not shown). Both the magnitude and duration of this weight loss were significantly reduced among mice pretreated with suppressive ODN (Fig. 8;  $p < 0.0008$ , mixed effects longitudinal regression analysis). This effect was sequence specific, as control ODN had no significant effect on silica-induced weight loss ( $p = 0.37$ ).

The pulmonary inflammation induced by silica instillation caused appreciable mortality. Approximately 25% of the mice treated with silica alone and 40% of the animals treated with silica plus control ODN died by day 7 (Fig. 9). Histologic analysis of BAL from these animals showed massive inflammatory cell infiltration and alveolar hemorrhage, both of which are consistent with acute silicosis as the cause of death. Of note, the modest (albeit not statistically significant) effect of control ODN on weight loss was primarily attributable to the death of severely ill animals. Consistent with the beneficial impact of suppressive ODN on silica-induced pathology, there were no deaths among the silica-instilled mice treated with suppressive ODN ( $p < 0.05$ ).

### Discussion

Exposure to silica-containing dust can lead to acute and chronic pulmonary diseases, including silicosis and lung cancer (37). Evidence suggests that silica-dependent lung damage is mediated by the production of ROS by macrophages in situ (3, 38, 39). Current studies demonstrate that suppressive ODN block the production of ROS by silica-activated macrophages, and reduce the pulmonary damage characteristic of acute silicosis.

Macrophages are the predominant immune cell present in alveoli and are rapidly activated by silica particles in situ (neutrophil infiltration is a subsequent event) (3, 8, 40). Crystalline silica triggers a respiratory burst in phagocytes characterized by the early production of superoxide ( $O_2^-$ ), followed by  $H_2O_2$  release via phagocytic oxidase (phox), NADPH oxidase, and mitochondrial oxidase production, all of which contribute to cell death (3, 11, 32). This study examined the effect of suppressive ODN on RAW264.7 cells and freshly isolated murine macrophages exposed to silica. Consistent with previous reports, macrophages cultured with silica produced large amounts of  $H_2O_2$  (Fig. 2). We also established that exogenously administered  $H_2O_2$  significantly reduced the viability of RAW264.7 cells (data not shown). Current studies show that macrophages treated with suppressive ODN are protected from  $H_2O_2$  and silica-induced toxicity in vitro (Fig. 2; data not shown), whereas mice treated with suppressive ODN are resistant to the mortality and morbidity mediated by silica instillation (Figs. 8 and 9).

ROS are directly toxic to lung cells. By promoting the production of inflammatory cytokines and chemokines via the NF- $\kappa$ B pathway, ROS also facilitate the infiltration of neutrophils into the lungs (26, 41–44). p47phox (10) and mitochondrial pathway (45) play a critical role in mediating silica-induced ROS production (14, 46). Since suppressive ODN maintained

mitochondrial viability assessed by MTT and LDH in non-phagocytized macrophage cells, RAW 264.7 (Fig. 4), silica-induced ROS might be derived mainly by p47phox.

Current results show that suppressive ODN inhibit this activity thereby attenuating H<sub>2</sub>O<sub>2</sub> generation (Figs. 2, 3, and 7). As a consequence, suppressive ODN significantly reduce both the cellular toxicity and pulmonary inflammation induced by silica exposure, improving survival and reducing morbidity in silica-challenged mice (Figs. 8 and 9). Other groups have shown that the up-regulation of p47phox contributes to the inflammatory pathology observed in other disease states, including cancer, and cardiovascular and renal disease (47–50). Thus, the therapeutic utility of suppressive ODN may be considerable if they can reduce p47phox expression in these other inflammatory conditions.

Hydrogen peroxide was used as a marker for ROS generation since i) silica-activated macrophages produce large amounts of H<sub>2</sub>O<sub>2</sub> via the ROS pathway and ii) H<sub>2</sub>O<sub>2</sub> levels correlate with intracellular oxidative stress and tissue pathology in acute silicosis (3, 11, 51). Guided by results from previous studies (15, 17, 52), the effect of suppressive ODN on cytokine and chemokine production was also examined. As expected, suppressive ODN inhibited the silica-induced production of IL-6, IL-12, and KC in vitro and in the lungs (Figs. 1 and 6; data not shown). These effects were dose dependent and sequence specific (as control ODN had no effect).

Preliminary studies were conducted to identify a treatment regimen that optimized the effectiveness of suppressive ODN in acute murine silicosis. Those studies established that a single dose of suppressive ODN reduced lung inflammation, weight loss, and improved the survival of mice challenged intratracheally with 0.25–2.5 mg of silica, but that two doses of suppressive ODN were more effective. Preliminary studies also established that the timing of suppressive ODN administration was relevant; optimal protection was observed when suppressive ODN were delivered shortly before exposure to the inflammatory stimulus (consistent with studies in which suppressive ODN mediated protection against toxic shock) (20).

Human silicosis typically arises following prolonged exposure to low levels of silica, and manifests as a chronic and slowly progressive disease. The murine model used in the current work used a high dose of silica that caused rapid and severe pulmonary inflammation. Although demonstrating that suppressive ODN can be used to control silica induced pulmonary inflammation, further studies are planned to assess the effect of site, dose, and duration of ODN administration on the fibrosis and tumor development associated with long-term low-level silica exposure.

## Acknowledgments

We thank Mr. Octavio Quinones for his support in the statistical analysis of this work. The assertions herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the National Cancer Institute at large.

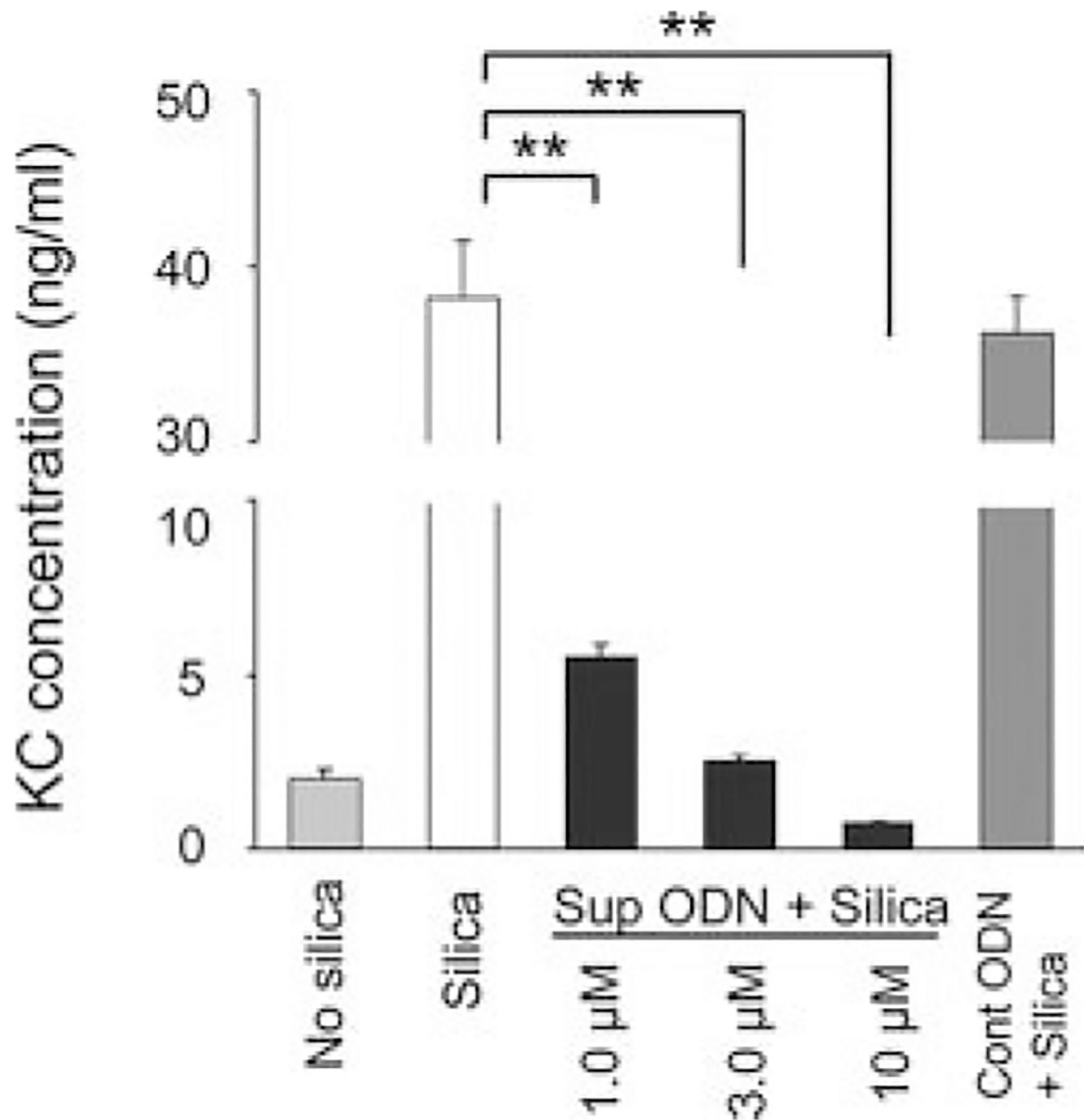
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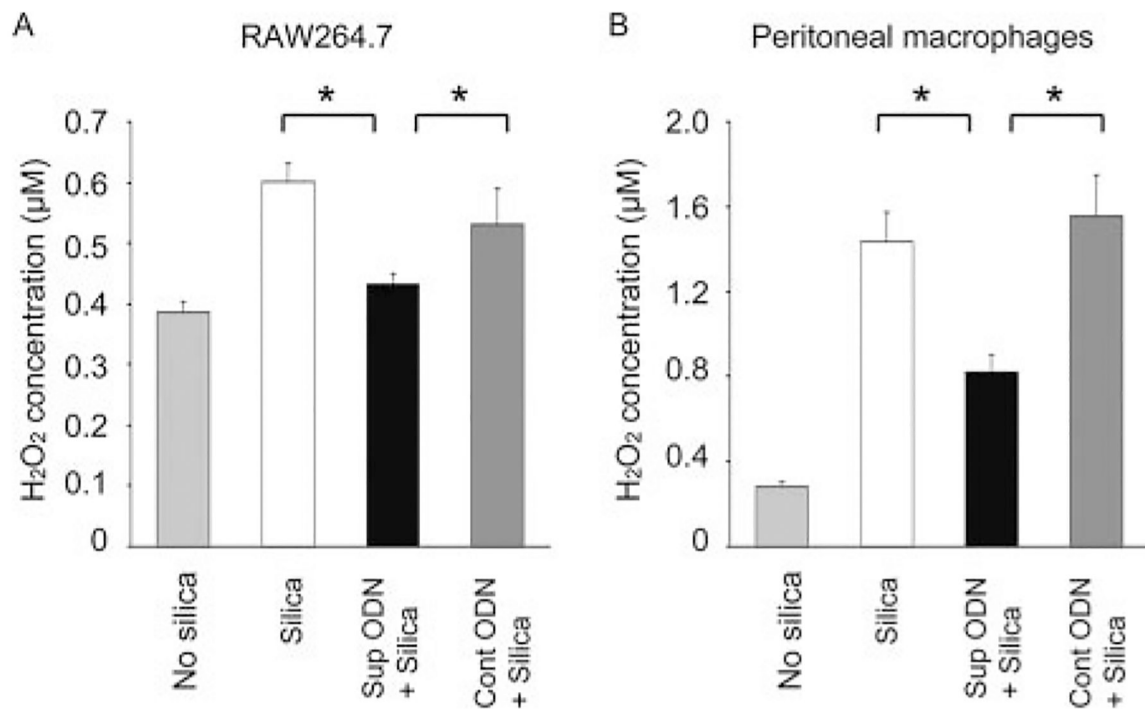
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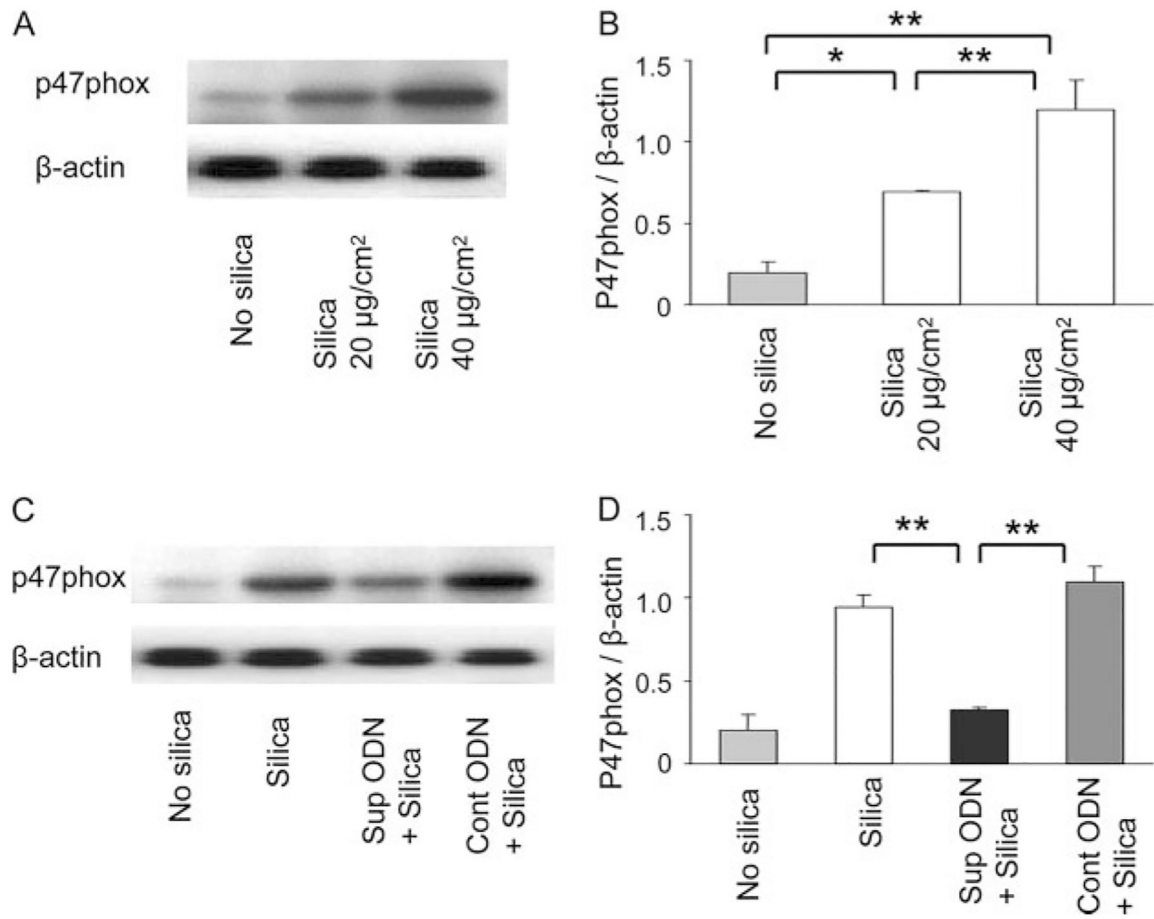
**FIGURE 1.**

Effect of suppressive ODN on silica-induced cytokine and chemokine production by MM14.Lu cells. MM14.Lu cells were cultured with  $40 \mu\text{g}/\text{cm}^2$  silica for 24 h with or without 3 M ODN (unless otherwise noted). Supernatants were collected and assayed for KC concentration. Results represent the mean  $\pm$  SE of three independent experiments. \*\*,  $p < 0.01$ .

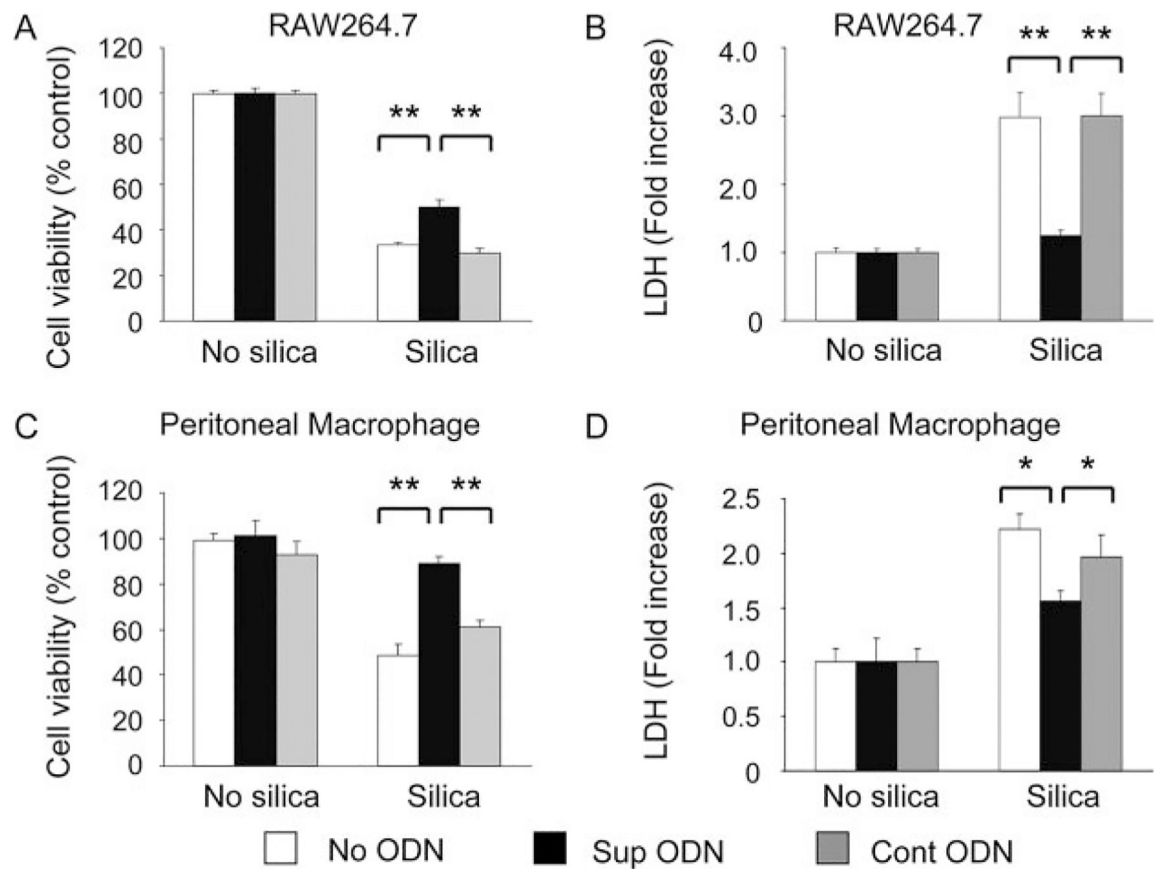


**FIGURE 2.**

Effect of suppressive ODN on silica-induced H<sub>2</sub>O<sub>2</sub> production. RAW264.7 cells (A) or freshly isolated peritoneal macrophages (B) were cultured with 40 μg/cm<sup>2</sup> silica for 24 h with or without 3 μM ODN. Cell viability and H<sub>2</sub>O<sub>2</sub> levels were measured using an Fe<sup>3+</sup>-xylenol orange based assay. To facilitate comparisons between experiments, H<sub>2</sub>O<sub>2</sub> concentration was standardized as a function of viable cell number/culture. To insure that H<sub>2</sub>O<sub>2</sub> concentration was monitored, background activity in catalase-treated cultures was subtracted. Results represent the mean ± SE H<sub>2</sub>O<sub>2</sub> concentration from three independent experiments. \*, *p* < 0.05.

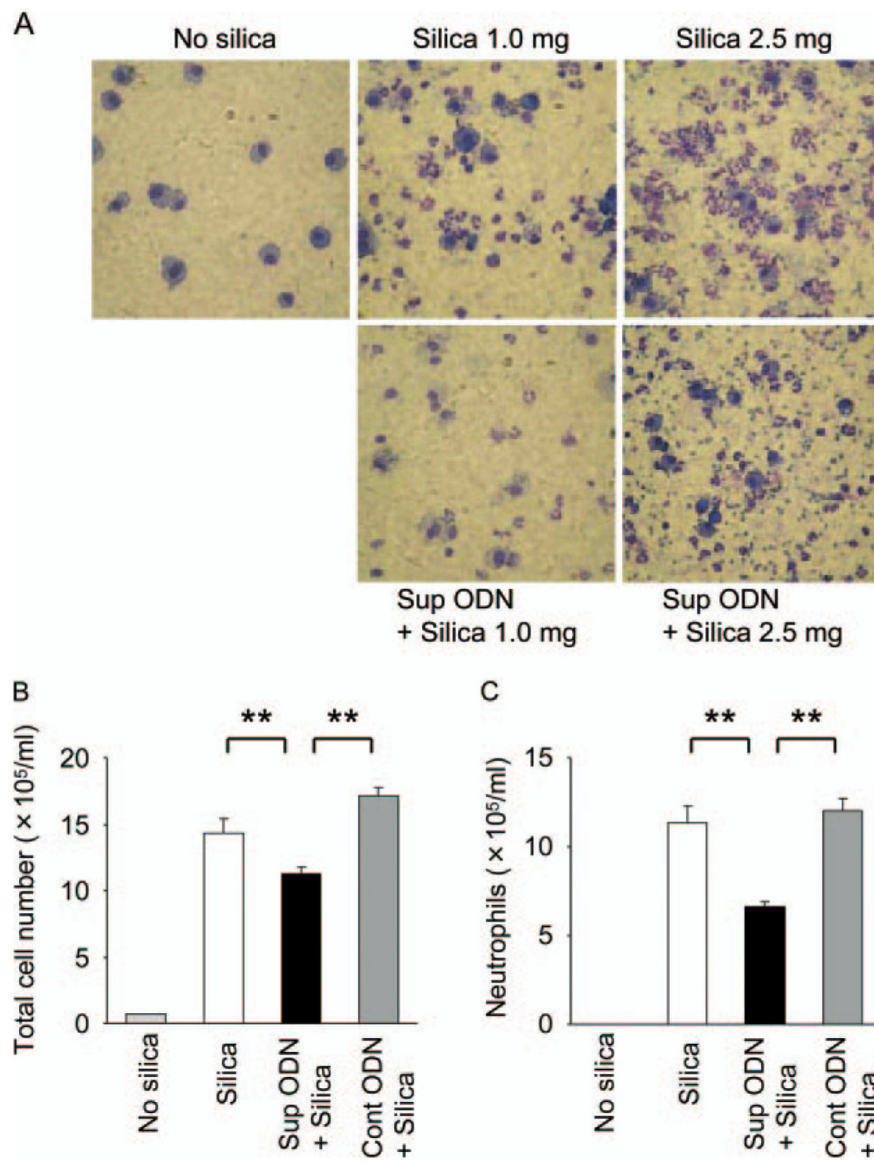
**FIGURE 3.**

Effect of suppressive ODN on silica-dependent p47phox induction. RAW264.7 cells were incubated with 20–40  $\mu\text{g}/\text{cm}^2$  of silica  $\pm$  3 M ODN for 24 h. *A* and *C*, Cell lysates were analyzed by Western blot using anti-p47phox and anti- $\beta$ -actin Abs. *B* and *D*, Densitometric analysis of band intensity representing the mean  $\pm$  SE of three independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .



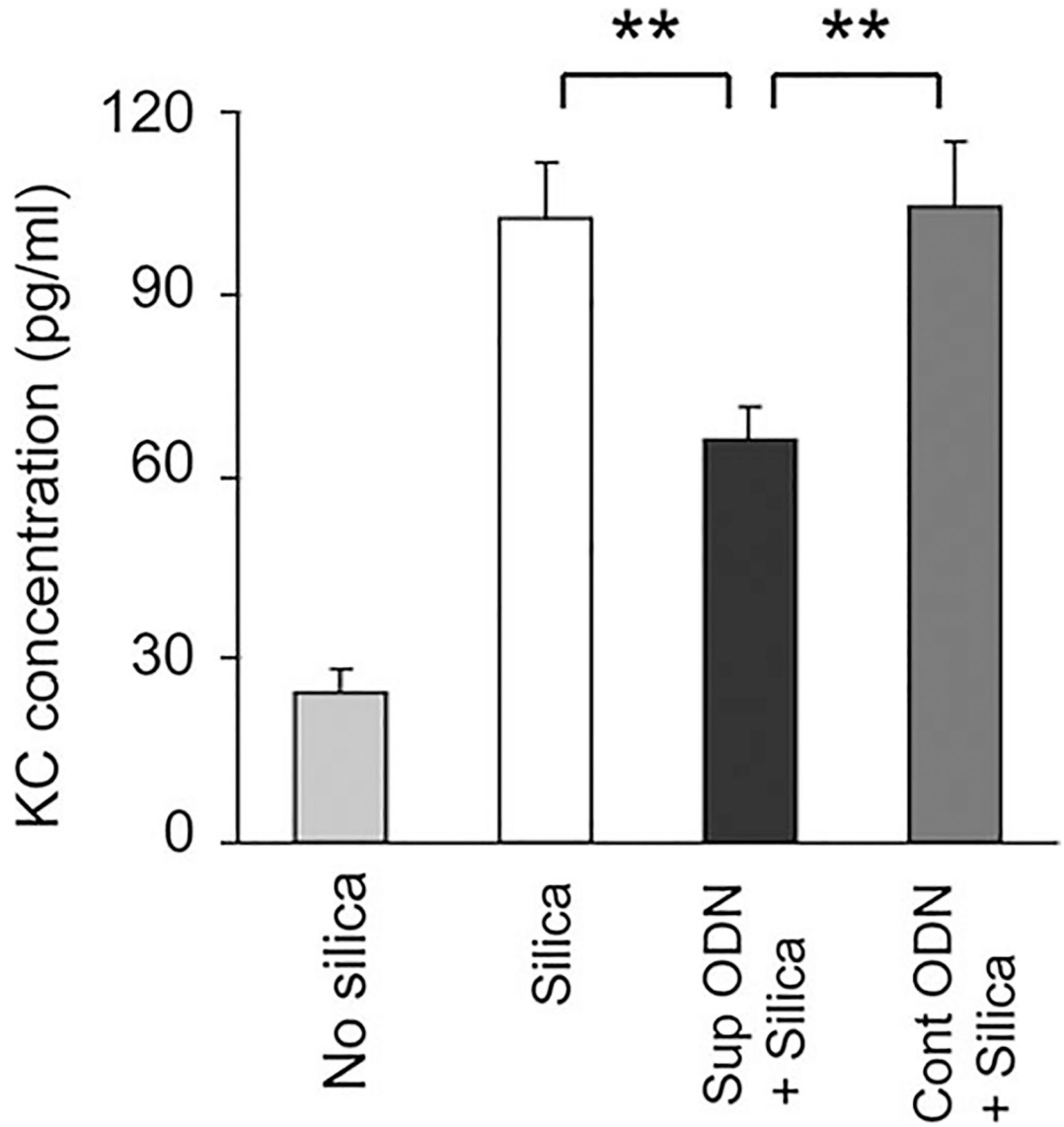
**FIGURE 4.**

Effect of suppressive ODN on silica-mediated macrophage death. Silica-mediated cellular toxicity was assessed using MTT and LDH releases assays. RAW264.7 cells (A and B) or freshly isolated peritoneal macrophages (C and D) were cultured with 40 g/cm<sup>2</sup> silica ± 3 M ODN for 24 h. Results show the mean percent viability (A and C) and fold increase over background LDH levels (B and D) ± SE from three independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .



**FIGURE 5.**

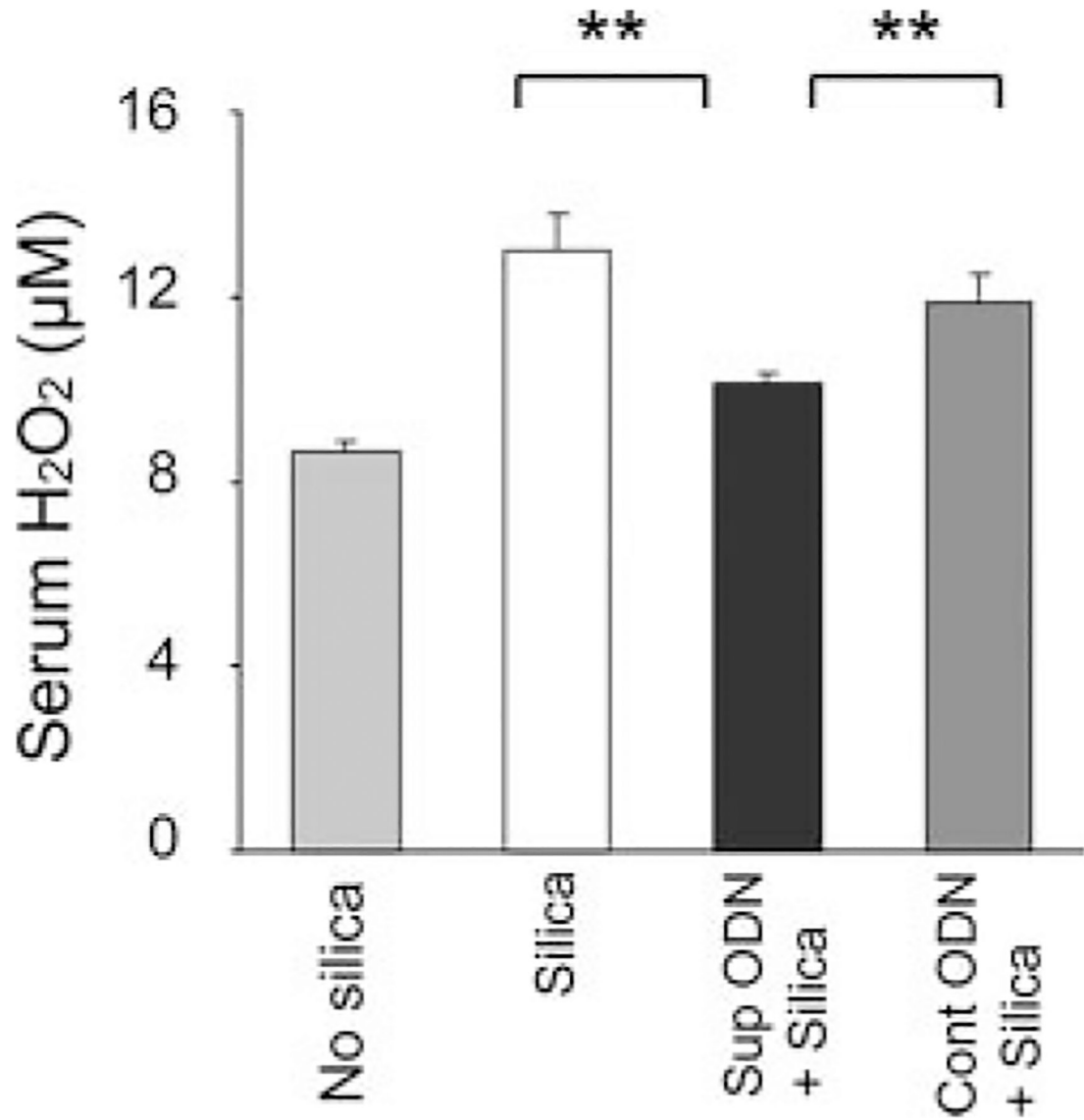
Effect of suppressive ODN on silica-induced pulmonary inflammation. Mice were injected i.p. with 300  $\mu$ g of ODN 24 and 3 h before intratracheal instillation of 1 or 2.5 mg of silica particles. *A*, Photomicroscopic images of bronchoalveolar lavage cells collected 3 days after silica instillation and stained with Diff-Quick (original magnification  $\times 600$ ). Total cellularity (*B*) and neutrophil content of BAL fluid (*C*) 3 days after mice were exposed to 2.5 mg of silica particles. Results represent the mean  $\pm$  SE of two to three independent experiments involving a total six to nine mice/group. \*\*,  $p < 0.01$ .



**FIGURE 6.**

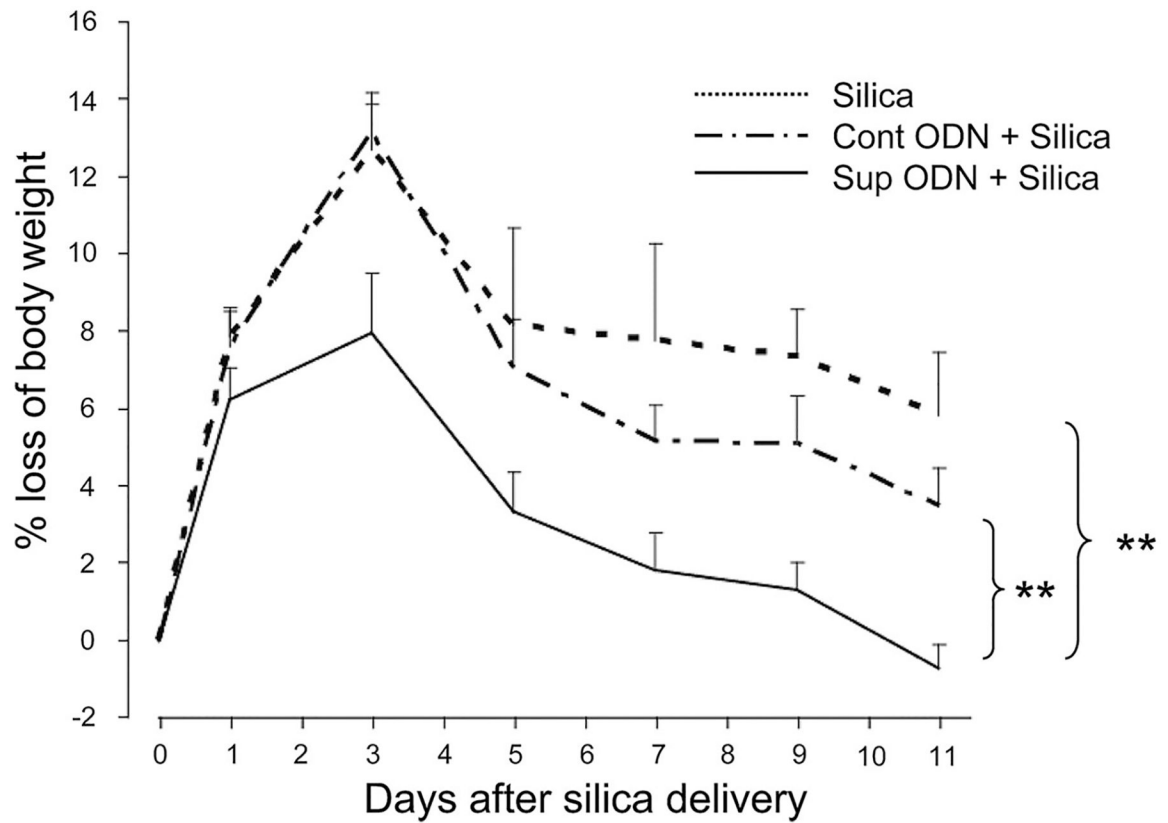
Effect of suppressive ODN on silica-induced KC production in the lungs. Mice were treated as described in Fig. 5. The concentration of KC in BAL collected 3 days after silica exposure was determined by ELISA. Results represent the mean  $\pm$  SE from two to three independent experiments involving a total five to eight mice/group. \*\*,  $p < 0.01$ .





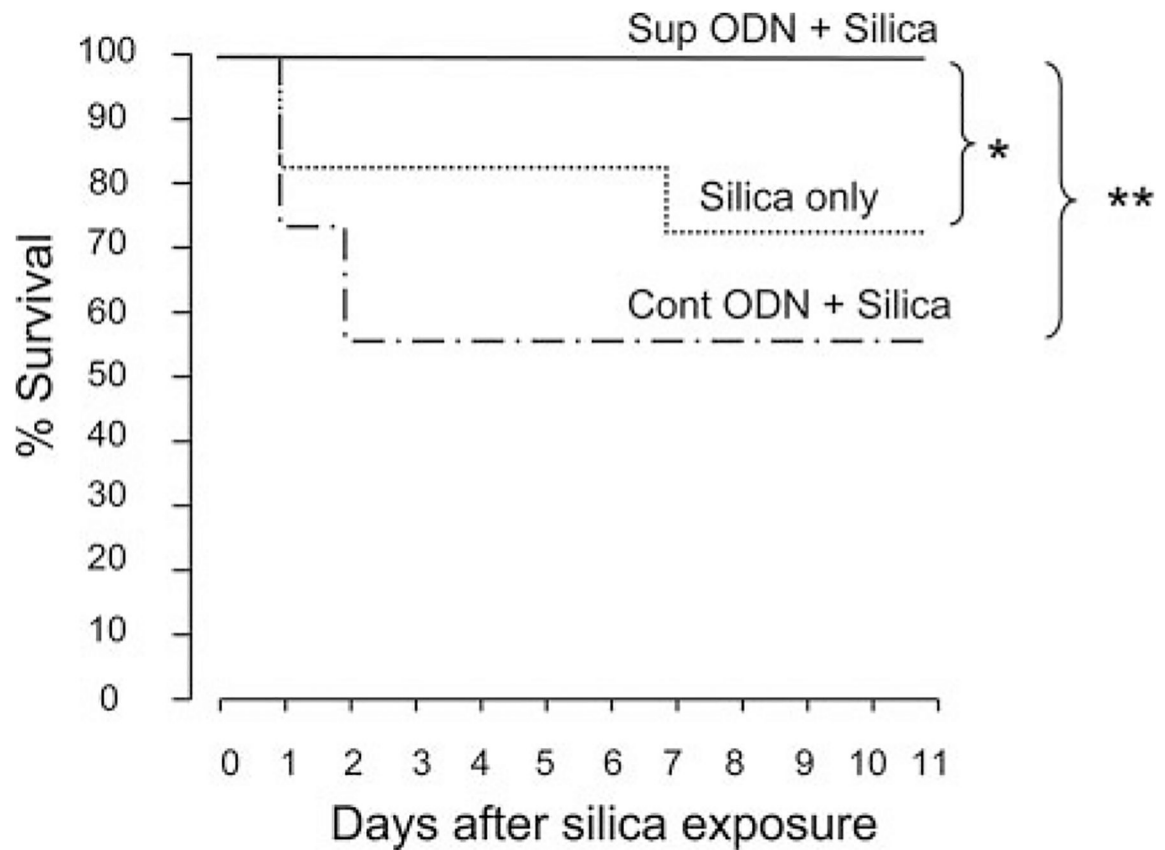
**FIGURE 7.**

Effect of Suppressive ODN on H<sub>2</sub>O<sub>2</sub> production in vivo. BALB/c mice were treated as described in Fig. 5. H<sub>2</sub>O<sub>2</sub> levels were measured using a Fe<sup>3+</sup>-xylenol orange-based assay. To insure that H<sub>2</sub>O<sub>2</sub> concentration was monitored, background activity in catalase-treated samples was subtracted. Results represent the mean serum H<sub>2</sub>O<sub>2</sub> concentration ± SE from two to three independent experiments involving a total 5–10 mice/group. \*\*, *p* < 0.01.



**FIGURE 8.**

Effect of suppressive ODN on silica-induced weight loss. BALB/c mice ( $n = 10/\text{group}$ ) were treated as described in Fig. 5. The mean percent change in body weight  $\pm$  SE was determined for each animal at each time point. Statistic significance was analyzed by longitudinal regression analysis. \*\*,  $p < 0.01$ .



**FIGURE 9.**

Effect of suppressive ODN on silica-induced mortality. BALB/c mice were treated as described in Fig. 5. Survival curves were analyzed by Kaplan-Meier statistics using the log rank test. Data from two independent experiments involving 10–17 mice/group were combined to generate the survival curves. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .