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Aerosolized Syk Antisense Suppresses Syk Expression, Mediator Release from Macrophages, and Pulmonary Inflammation

Grant R. Stenton,* Moo-Kyung Kim,† Osamu Nohara,* Chin-Fu Chen,‡ Nadir Hirji,* Fiona L. Wills,* Mark Gilchrist* Pyoung-Han Hwang,§ Jong-Gu Park,‖ Warren Finlay,† Richard L. Jones,* A. Dean Befus,2* and Alan D. Schreiber‡

Syk protein tyrosine kinase (PTK) is involved in signaling in leukocytes. In macrophages, Fcγ-receptor cross-linking induces Syk PTK phosphorylation and activation, resulting in Syk-dependent events required for phagocytosis and mediator release. We hypothesized that Syk antisense oligodeoxynucleotides (ASO) delivered by aerosol to rat lungs in vivo would depress Syk PTK expression, mediator release from alveolar macrophages, and Syk-dependent pulmonary inflammation. RT-PCR and RT-in situ PCR demonstrated that aerosolized Syk ASO administration reduced Syk mRNA expression from alveolar macrophages compared with cells isolated from sham-treated rats. Western blot analysis confirmed that Syk PTK expression was reduced after Syk ASO treatment. Compared with sham-treated rats (scrambled oligodeoxynucleotide), Syk ASO treatment suppressed Fcγ-receptor-mediated nitric oxide (86.0 ± 8.3%) and TNF (73.1 ± 3.1%) production by alveolar macrophages stimulated with IgG-anti-IgG complexes. In contrast, Fcγ-receptor-induced IL-1β release was unaffected by Syk ASO treatment. Additionally, Syk ASO suppressed Ag-induced pulmonary inflammation, suggesting that Syk ASO may prove useful as an anti-inflammatory therapy in disorders such as asthma. The Journal of Immunology, 2000, 164: 3790–3797.

Stimulation of macrophage FcγR leads to downstream signaling events, gene transcription, mediator release, and phagocytosis. In macrophages, cross-linking of FcγR also results in the activation of Src and Syk protein tyrosine kinases (PTK).3 These protein tyrosine kinases associate with specific recognition sequences, immunoreceptor tyrosine-based activation motifs, present in the intracellular domains of FcγR (1–5).

In addition to being expressed in macrophages (6, 7), Syk (p72γ) PTK is expressed in eosinophils (8), T cells (9–11), B cells (10, 11), neutrophils (12), and mast cells (13–15). Recently, Matsuda et al. (16) observed that in vitro treatment of human peripheral blood monocytes with Syk antisense oligodeoxynucleotides (ASO) inhibited Syk mRNA and protein expression compared with cells treated with scrambled oligodeoxynucleotides (ODN). This inhibition correlated with the suppression of FcγR-mediated phagocytosis and indicated that Syk PTK plays a critical role in FcγR-mediated cellular signaling and function in monocytes and macrophages. These data suggest that Syk ASO may be effective in vivo in inhibiting Syk PTK expression and cellular function in several cell populations including eosinophils, mast cells, and macrophages.

Alveolar macrophages are the most abundant cell type in the airways and are important in host defense and the inflammatory response. Alveolar macrophages rapidly internalize liposomes and are likely to be exposed to Syk ASO after aerosolization (17).

Therefore, we studied the effects of Syk ASO liposome complexes delivered in vivo by aerosolization on a rat model of pulmonary inflammation. In these studies we also examined the effects of in vivo aerosolized Syk ASO liposome complexes on the expression of Syk PTK and on the function of alveolar macrophages.

Materials and Methods

Reagents

Syk ASO and the scrambled ODN, respectively, were prepared by the Nucleic Acid Facility of the Department of Chemistry at the University of Pennsylvania Cancer Center and the Core DNA Services at University of Calgary. 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) and dioleoylphosphatidyl-ethanol-amine (DOPE) phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). DOTAP liposomal transfection reagent for in vitro studies was purchased from Boehringer Mannheim (Indianapolis, IN). RPMI 1640, Eagle’s MEM (EMEM), FBS, penicillin, and streptomycin were purchased from Life Technologies (Grand Island, NY). Rat IgG2a (κ) and mouse monoclonal anti-rat IgG2a (used to form IgG-anti-IgG complexes) were purchased from Serotec (Toronto, Ontario, Canada). Rabbit anti-murine Syk polyclonal Ab was purchased from Upstate Biotechnology (Lake Placid, NY), and the isotype control, polyclonal rabbit IgG, was purchased from Serotec. F(ab’2) goat anti-rabbit HRP reagent was supplied by The Jackson Laboratory (Bar Harbor, ME), MTT, PMSF, aprotinin, p-tosyl arginine methyl ester, and leupeptin were supplied by Sigma (St. Louis, MO). Rat recombinant TNF and rat IL-1β ELISA kits were purchased from R&D Systems (Minneapolis, MN). Chemiluminescence reagent was purchased from DuPont NEN (Boston, MA).
Animals
Male Sprague Dawley rats were used throughout this study. They were housed in the University of Alberta animal housing facility in filter-top cages to minimize unwanted infection. They were exposed to 12-h light/dark cycles and were given food and water ad libitum. All rats were infected by s.c. injection of 0.5 ml PBS containing 3000 larvae (L3 stage) of *Nippostrongylus brasiliensis* and were studied 4 wk later (18). The worms are expelled by the host within 2–3 wk, and the inflammation in the lungs and the intestine subsides, leaving rats sensitized for re-exposure experiments. This work was approved by the University of Alberta Animal Ethics Committee in accordance with guidelines of the Canadian Council for Animal Care.

Syk ASO and scrambled ODN
A stem-loop rat Syk ASO was designed as described by Matsuda et al. (16) for human Syk ASO. The 60 base ASO consisted of the following sequence: 5′-GCCGGGTGCCCACATGGTATTTGAGTTTGCAATTTGCGAGTATCCCTCCGGCCG-3′. The scrambled ODN, also a 60-base sequence, has the same content of A, T, G, and C, and consisted of the following sequence: 5′-GCCGGGTCCATGATGTACCAGTTAATGTTGCTGATCAGTCCAGTCTGCCCAACC-3′. The stem-loop ODN and the scrambled ODN have three phosphorothioate backbone modifications, which increases their resistance to nuclease degradation. The stem-loop ASO is designed to interact with Syk mRNA at three sites, increasing its efficiency (16).

Liposome preparation
Liposomes were prepared using a method modified from Legendre and Szoka (19). One milligram DOTAP in chloroform and 1 mg DOPE in chloroform were mixed, and the chloroform was evaporated, leaving a film of 1:1 DOTAP:DOPE. Two milliliters of sterile saline were added, resulting in liposome formation composed of equal parts of DOTAP and DOPE. The liposomes varied in size and were dispersed in an ultrasonic bath (for ~1 min) until they were ~200 nm in diameter. The diameters of the liposomes suspended in saline were determined by dynamic light scattering using a Brookhaven particle sizer (Brookhaven Instruments, Holtsville, NY).

Incubation of liposomes with Syk ASO
Because DOTAP:DOPE liposomes are cationic, they form complexes with negatively charged ODN (20). Complexes were formed by incubating a 2:5:1 ratio of the liposomes (1.25 mg) with either Syk ASO (0.5 mg) or the scrambled ODN (0.5 mg) for 45 min at room temperature in sterile saline (final volume, 9 ml). The ratio of liposome:Syk ASO was optimized as described below.

In vitro studies of Syk ASO
To examine rat Syk ASO in vitro, we studied the effects on Syk expression and function in vitro. In a similar manner, the optimum concentration of Syk ASO and scrambled ODN have three phosphorothioate backbone modifications, which increases their resistance to nuclease degradation. The stem-loop ASO is designed to interact with Syk mRNA at three sites, increasing its efficiency (16).

Optimization of aerosol Syk ASO delivery
To confirm that the aerosolized delivery particles to the lower airways, methylene blue was aerosolized. On frozen sections of the areas in and around the alveoli that were stained blue, confirming that the nebulizer delivered dye to the lower airways. In addition, the nebulizer produced methylene blue droplets of 5.1 ± 0.4 μm, which are small enough to enter the lower airways, further validating the aerosolization system. Nebulized Syk ASO/liposome complexes had droplet sizes of 4.1 ± 0.1 μm, similar to those of methylene blue, suggesting that ODN/liposome droplets were small enough to penetrate the lower airways. Particle sizes were measured using Doppler anemometry (Dantec Electronics, Malshaw, NJ) at the exit of the nebulizer.

Optimization of liposome/Syk ASO ratio and concentration
Because i.p. delivery controls the precise dose of Syk ASO administered and because this may not be the case with aerosolization, we used data from i.p. treated rats to optimize liposome/Syk ASO ratio and concentration. We examined the ability of varying liposome/Syk ASO ratios and concentrations to inhibit the manufacturer’s protocols. PCR was modified from purified peritoneal mast cells (22). The optimum ratio and Syk ASO concentration was identified as having the strongest inhibition of IgE-mediated histamine release. Liposome/Syk ASO (2.5:1) delivered i.p. (results not shown) was found to be the optimum ratio, as reported earlier by Zelphati and Szoka (23), for studies of maximal cellular uptake of antisense in vitro. In a similar manner, the optimum concentration of Syk ASO administered i.p. was determined to be 0.25 μg/Syk ASO/animal/day for all experiments using aerosol delivery and maintained the 2.5:1 liposome/Syk ASO ratio.

RNA isolation and solution-phase RT-PCR
Total RNA was isolated from alveolar macrophages and RBL-2H3 using TRIzol reagent (Life Technologies, Burlington, Canada) and quantified using an OD<sub>260</sub>/OD<sub>280</sub> ratio. Frozen at −70°C until RT-PCR analysis of Syk RNA expression. One rat per treatment group provided sufficient alveolar macrophages. After one wash with PBS, the RBL-2H3 cells were incubated as follows: 1.0 ml PAGCM alone (negative control), 1.0 ml PAGCM containing 10 μl calcium ionophore (30 μg/ml) stock (positive control), or 1.0 ml PAGCM containing 10 μl murine anti-rat FcεRI Ab (1 mg/ml) for 30 min at 37°C. The PAGCM containing histamine was removed from the cells and assayed by enzyme immunoassay. Standards were included to establish a dose curve for histamine.

Aerosolized administration of Syk ASO and harvesting of alveolar macrophages
To restrict their movement, animals were confined in plexiglass animal chambers, which were then placed in small plastic boxes with lids for the duration of aerosolization. Nine milliliters of liposome, scrambled ODN/ liposome, or Syk ASO/O liposome was administered by nebulization for ~30 min using a Sidestream nebulizer, model 1200A durable (Medic-Aid, Paghum, U.K.). Twenty-four hours later the procedure was repeated, and another 24 h later the animals were anaesthetized with an i.p. injection of 0.5 ml Rompun (20 mg/ml) and 0.5 ml Ketanest (Ketamine, 100 mg/ml) and then sacrificed by severing of the abdominal aorta. The trachea of each rat was cannulated with polyethylene tubing (1.14-mm diameter; Becton Dickinson, Sparks, MD) attached to a needle, and 5 ml of ice-cold PBS was massaged into the lungs 12 times as described previously (21). The PBS was aspirated into ice-cold polypropylene tubes. The cells were kept on ice until they were washed by centrifugation at 150 × g for 20 min and suspended in PBS, yielding ~95% alveolar macrophages as determined by staining of cytopsins with May-Grünwald-Geimsa stain. Cell viability was >95% as determined by trypan blue exclusion. The isolated macrophages were then studied 1) for RNA isolation and RT-PCR analysis of Syk mRNA expression, 2) by Western blot analysis of Syk protein expression, or 3) by analysis of mediator release.

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arginine methyl ester (serine protease inhibitors), and 5
m
RT-in situ PCR using the following primers: Rat-5’ Syk, 5’-TTGT-
GCAACTACCCCCGG-3’; and Rat-3’ Syk, 5’-ACTTATGATGCCCT-
GCTC-3’. Products were run on a 2% agarose gel and were stained with ethidium bromide, producing a 400-bp Syk product. Controls included his-
tididine decarboxylase primers Rat-5’ histidine decarboxylase (HDC), 5’-
GGAGCCCACTGAATTACCTG-3’; and Rat-3’ HDC, 5’-TGCAGAG-
GACTGTGACGGAA-3’ (460-bp HDC product), for in vivo-treated alveolar macrophages and in vitro-treated RBL-2H3.

RT-in situ PCR

RT-in situ PCR was modified and performed as previously described (24). The macrophages were fixed for 16 h in 10% buffered neutral formalde-
hyde (BDH, Toronto, Ontario, Canada) at 22°C, washed twice with diethyl pyrocarbonate-treated water, and then placed on silane-coated glass slides (Perkin-Elmer, Norwalk, CT), each with three spots for test, positive, and negative controls. The cells were allowed to dry overnight, were digested in 10,000 U/ml pepsin (Boehringer Mannheim, Mannheim, Germany) in 0.01 M HCl for 1 h at 22°C, and then were treated with RNase-free DNaseI (2,000 U/ml; Boehringer Mannheim, Indianapolis, IN) at 37°C for 24 h. The test specimens were treated with a RT solution containing 1 µM ant-
isense primer or 25 µg/ml oligo(dT)12–18 primer (Life Technologies, Bur-
tlington, Canada) with Moloney murine leukemia virus RT (Life Tech-
nologies, Burlington, Canada) for 3 h at 37°C. Amplification of the cDNA was accomplished using a hot start method (24) with a PCR solution containing 4.5 mM MgCl2, 80 µM each of dATP, dCTP, dGTP, and dUTP, 0.8 µM of each primer; 16 µM of digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN), and 120 U/ml Taq polymerase (Life Technologies, Bur-
tlington, Canada) using a GeneAmp In Situ PCR System 1000 (Perkin-
Elmer). Cycling conditions were 5 min at 94°C and 30 cycles of 94°C for 1 min, 64°C (β-actin), 51°C (Syk) for 1 min, and 72°C for 1.5 min. The digoxigenin-11-dUTP-labeled PCR product was detected after incubation with alkaline phosphatase anti-digoxigenin conjugate (Boehringer Mann-
heim, Indianapolis, IN) (3.75 U/ml in 0.1 M Tris-HCl and 0.15 M NaCl (pH 7.5)) for 30 min at 22°C and after development in 4-nitro blue tetra-
zolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mann-
heim, Indianapolis, IN) substrate solution. Test spots with DNase digestion and the RT step showed target mRNA expression in the cytoplasm. Posi-
tive controls without DNase treatment to monitor the length of protease digestion showed nuclear DNA priming, and negative controls, in which the test specimens were treated with DNase and the RT step was eliminated, showed that no priming of genomic DNA was detectable. The primers used for RT-in situ PCR Syk detection were the same as for solution-phase RT-PCR as described in the previous section. β-Actin controls were performed for RT-in situ PCR using the following primers: Rat β-actin 5’ primer, 5’-
GGGGCGGCCCCCAAACCA-3’; and 3’ primer, 5’-GTGTTAAAT-
GTACAACAGTATTG (526-bp β-actin product).

Western blot analysis of Syk protein in RBL-2H3 cells

Cultured RBL-2H3 cells were treated with liposome alone (2 µg), 2 µg of control scrambled ODN and liposome, or Syk ASO and liposome at 1 × 106 cells/ml as described above. Cell lysates were prepared from 5 × 106 cells by boiling in Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM DTT, and 60 mM Tris (pH 6.8)) for 5 min. Protein was loaded from lysates of equal cell numbers, separated by SDS-PAGE (12.5% polyacryl-
amide gel), and transferred to a nitrocellulose membrane in sample buffer (25 mM Tris, 190 mM glycine, and 20% methanol). The membrane was then incubated overnight at 4°C with 1 µg/ml rabbit anti-murine Syk poly-
clonal Ab before incubation (1.5 h at room temperature) with goat anti-
rabbit HRP. Bands on the membrane were visualized with chemilu-
minescence reagent according to the manufacturer’s protocol. After detection of Syk protein, the Abs were removed by incubating the membrane in a strip-
ing buffer containing 100 mM 2-ME, 2% SDS, and 62.5 mM Tris-HCL (pH 6.7) for 30 min at 50°C with occasional agitation. The membrane was then reprobed with an anti-actin Ab (Actin I-19; Santa Cruz Biotechnology, Santa Cruz, CA). Bands on the membrane were visualized with chemilu-
minescence reagent.

Western blot analysis of Syk protein in alveolar macrophages

Alveolar macrophages from sham- and Syk ASO-treated animals were lysed for 1 h at 4°C with 1% Nonidet P-40 in the presence of the following protease inhibitors: 100 µg/ml PMSF, 5 µg/ml aprotinin, 5 µg/ml p-tosyl arginine methyl ester (serine protease inhibitors), and 5 µg/ml of leupeptin (thiol protease inhibitor). Protein extracted from 0.5 × 106 cells was separated on 10% SDS-PAGE and blotted onto Hybond-C Super membrane (Amersham, Oakville, Ontario, Canada). The membrane was blocked for 1.5 h in 3% skim milk and 5% goat serum at room temperature. The membrane was then incubated overnight at 4°C with 1 µg/ml rabbit anti-
murine Syk polyclonal Ab before incubation (1.5 h at room temperature) with goat anti-rabbit HRP. Isotype controls were performed using purified polyclonal rabbit IgG as the primary Ab. Bands on the membrane were visualized with chemiluminescence reagent according to the manufacturer’s protocol.

Release and measurement of NO from alveolar macrophages

Rat alveolar macrophages (2 × 106 cells/well) suspended in complete RPMI 1640 medium (5% FBS) were allowed to rest for 1 h in a humidified incubator (37°C and 5% CO2) before stimulation for 24 h with IgG-anti-
IgG complexes to induce NO production. IgG-anti-IgG complexes were prepared by incubating (37°C for 10 min) 100 µg/ml IgG2a with 20, 50, and 100 µg/ml mouse monoclonal anti-rat IgG2a. These complexes were resuspended and 20 µl was added to cells to make a final volume of 200 µl and final concentrations of 2, 5, and 10 µg/ml mouse anti-rat IgG2a. Because alveolar macrophage NO is rapidly converted to nitrite (NO2−), the Greiss method of NO2− detection was used to determine NO produc-
tion. Cell-free supernatants were mixed with equal volumes of a 1:1 mix-
ture of 1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydro-
chloride dissolved in 2.5% H3PO4 and incubated for 10 min at room temperature (25°C). NO2− concentration was determined at 540 nm with a Molecular Devices Vmax kinetic microplate reader (Molecular Devices, Menlo Park, CA). NaN3O (0.85–100 µM) was used to establish a standard curve for every experiment.

Release and measurement of TNF and IL-1β from alveolar macrophages

Purified alveolar macrophages (2 × 106 cells/well) in complete RPMI 1640 medium were added to each well of a 96-well plate and were allowed to rest for 1 h in a humidified incubator (37°C and 5% CO2). The cells were then stimulated with IgG-anti-IgG complexes for 6 h and 24 h to induce TNF and IL-1β production, respectively, as described for NO release ex-
periments. The bioactivity of TNF was tested for cytotoxicity toward
Effects of Syk ASO in vitro

We first determined the effects of Syk ASO in vitro using the cell line RBL-2H3. Syk ASO substantially reduced the level of Syk mRNA in RBL-2H3 compared with controls (Fig. 1A). In contrast, the expression of HDC mRNA was not affected by Syk ASO treatment (Fig. 1B).

We also examined the effect of Syk ASO on Syk protein expression in RBL-2H3 using Western blot analysis. Cell lysates were probed with anti-Syk Abs to detect Syk protein expression. Syk ASO treatment suppressed Syk protein expression, whereas sham or scrambled ODN treatment had no effect (Fig. 2). In addition, Syk ASO treatment of RBL-2H3 significantly reduced histamine release from 1.9 ± 0.3 ng/10^5 cells to 0.5 ± 0.2 ng/10^5 cells (74% inhibition) after FceRI cross-linking (n = 3; data not shown).

Effects of in vivo Syk ASO treatment on Syk mRNA and Syk protein expression by alveolar macrophages

Treatment of rats in vivo on days 1 and 2 with Syk ASO/liposome complexes inhibited alveolar macrophage Syk mRNA expression, as determined by solution-phase RT-PCR, compared with sham treatment (liposome alone or scrambled ODN/liposome complexes) (Fig. 3A). Syk ASO treatment had no effect on control HDC mRNA expression (Fig. 3B).

Similar results were obtained using RT-in situ PCR to detect Syk mRNA expression (Fig. 4). Fig. 4 demonstrates that the in situ

**FIGURE 2.** Detection of Syk tyrosine kinase expression in RBL-2H3. Western blot RBL-2H3 lysates (5 × 10^6 cells/lane) after treatment on days 1 and 2 with complexes of liposome (lane 1), scrambled ODN/liposome (lane 2), or Syk ASO/liposome (lanes 3 and 4). p72^{Syk}(Syk) was detected using rabbit anti-murine Syk polyclonal Ab. A representative of three independent experiments is shown. The molecular mass markers are shown, and the arrow indicates the position of the 72-kDa Syk protein.

**FIGURE 3.** Effects of in vivo-administered Syk ASO on Syk mRNA levels. A, RT-PCR analysis of Syk mRNA expression by alveolar macrophages after no treatment (lane 6) or aerosolized liposome (lanes 2 and 7), scrambled ODN/liposome complex (lanes 3 and 8), or Syk ASO/liposome complex (lanes 4 and 9) treatment in vivo. Human Syk plasmid positive control (lanes 1 and 10) and DNA ladder (lane 5) are shown. Rats were treated with aerosol preparations on days 1 and 2, and alveolar macrophages were harvested 24 h later. Results are representative of three independent experiments. The Syk product is 400 bp. B, RT-PCR analysis of HDC mRNA expression by alveolar macrophages after aerosolized liposome (lane 1), scrambled ODN/liposome complex (lane 2), or Syk ASO/liposome complex (lane 3) treatment in vivo. DNA ladder is shown in lane 4. Rats were treated with aerosol preparations on days 1 and 2, and alveolar macrophages were harvested 24 h later. Results are representative of three independent experiments. The HDC product is 460 bp.
PCR analysis of Syk (−b) and 13B treatment and PCR steps are performed without the cDNA-forming RT step. Hence, no genomic or cytoplasmic amplification is observed. No DNase digestion step. Therefore, amplification of genomic Syk DNA occurs, and the nuclei are positive for Syk expression.

FIGURE 4. A, RT-in situ PCR analysis of Syk mRNA expression (controls). 1, Test spot. Detection of Syk mRNA in the cytoplasm. Cells undergo DNase digestion to ensure there is no amplification of genomic Syk DNA, hence the nuclei are negative for Syk expression. Reverse transcription (RT) is performed to convert Syk mRNA to Syk cDNA, and the cDNA is amplified by PCR. 2, Positive control. Cells are treated as in 1, except that there is no DNase digestion step. Therefore, amplification of genomic Syk DNA occurs, and the nuclei are positive for Syk expression. 3, Negative control. DNase treatment and PCR steps are performed without the cDNA-forming RT step. Hence, no genomic or cytoplasmic amplification is observed. B, RT-in situ PCR analysis of Syk (1-3) and β-actin (4-6) mRNA expression by alveolar macrophages after aerosolized liposome (1 and 4), scrambled ODN/liposome complex (2 and 5), or Syk ASO/liposome complex treatment (3 and 6). Rats were treated with aerosol preparations on days 1 and 2, and alveolar macrophages (>95% purity) were harvested 24 h later. Syk ASO/liposome complexes inhibited Syk mRNA expression (3) but not β-actin mRNA expression (6). Positive and negative controls, as described for A, were performed for alveolar macrophages from each treatment group (data not shown). Results are representative of two independent experiments.

FIGURE 5. Detection of Syk tyrosine kinase expression. Western blot of alveolar macrophage lysates (>95% purity; 0.5 × 10⁶ cells/ lane) after aerosolized liposome, scrambled ODN/liposome complex (Scr ODN), or Syk ASO/liposome complex treatment. Rats were treated with aerosol preparations on days 1 and 2, and alveolar macrophages were harvested 24 h later. A positive control for Syk (Syk +ve) was performed using purified Syk protein supplied by the manufacturer of the rabbit anti-murine Syk Ab. p72α (Syk) was detected using rabbit anti-murine Syk polyclonal Ab. Isotype control (rabbit IgG) was negative for Syk (data not shown). A representative of three independent experiments is shown. The molecular mass markers are shown, and the arrow indicates the position of the 72-kDa Syk protein.
for 24 h, which resulted in the release of significant levels of NO (measured as [NO$_2^-$]). IgG2a in the absence of anti-IgG2a (sham) failed to induce NO release above spontaneous levels (Fig. 6). When rats were treated with Syk ASO/liposome complexes (compared with control treatment with scrambled ODN/liposome complexes) once daily for 2 days before alveolar macrophage isolation and stimulation (10 µg/ml IgG-anti-IgG2a), NO release was inhibited by 73.1 ± 3.1% when spontaneous NO release was subtracted from FcγR-mediated NO release (Fig. 6; n = 4). There was no significant difference in FcγR-mediated NO release from alveolar macrophages after treatment with scrambled ODN/liposome complex or liposome alone.

**Effects of in vivo Syk ASO treatment on FcγR-mediated TNF and IL-1β release from alveolar macrophages**

The effects of Syk ASO treatment on cytokine release from alveolar macrophages were determined by measuring TNF and IL-1β production (Figs. 7 and 8). After liposome, scrambled ODN/liposome complex, or Syk ASO/liposome complex treatment on days 1 and 2, alveolar macrophages were isolated by BAL and were stimulated with IgG-anti-IgG2a complexes for 6 h (TNF) and 24 h (IL-1β).

Although IgG-anti-IgG2a complexes induced significant TNF and IL-1β release, IgG2a in the absence of anti-IgG2a (Figs. 7 and 8; sham) failed to induce TNF or IL-1β release above spontaneous levels. Syk ASO/liposome complex treatment compared with control treatment with scrambled ODN/liposome complexes significantly inhibited FcγR-mediated (10 µg/ml IgG-anti-IgG2a) TNF release by 86.0 ± 8.3% when the spontaneous TNF release was subtracted from the FcγR-mediated TNF release (Fig. 7; n = 4–5). There was no significant difference in FcγR-mediated TNF release from alveolar macrophages after treatment with scrambled ODN/liposome complexes or liposome alone.

In contrast to the effects on NO and TNF release, in vivo Syk ASO/liposome complex treatment did not significantly inhibit IgG-anti-IgG2a complex-induced IL-1β release compared with treatment with scrambled ODN/liposome complexes or liposome alone (Fig. 8; n = 4).

**Effects of in vivo Syk ASO treatment on pulmonary inflammation**

Using an established model of pulmonary inflammation, we studied the effects of in vivo Syk ASO treatment on pulmonary inflammation as measured by BAL cell number and differential. In these experiments, rats were treated once daily for 2 days with Syk ASO/liposome complexes, scrambled ODN/liposome complexes, or liposome alone before i.v. saline (sham) or Ag challenge (Fig. 9; n = 5). In the liposome treatment group, we observed a significant increase in the total BAL cell number 8 h after Ag challenge (4.3 ± 0.3 × 10^5) compared with 8 h after sham challenge (2.3 ± 0.4 × 10^5). Similarly, the total BAL cell number from scrambled ODN/liposome complex-treated rats 8 h after Ag challenge was increased (4.9 ± 1.0 × 10^5) compared with 8 h after sham challenge (2.2 ± 0.3 × 10^5). By contrast, no significant increase in BAL cell number was observed 8 h after Ag challenge (2.6 ± 0.4 × 10^5) compared with sham challenge (2.0 ± 0.7 × 10^5) in the Syk ASO/liposome complex treatment group.

The proportions of different cell populations in BAL were examined after sham or Ag challenge (8 h). The majority of cells present in all groups were macrophages (range, 91–97%), although...
We observed that in vitro delivery of Syk ASO to the rat mast cell made of DOTAP alone complexed with Syk ASO at a ratio of 2:1. Furthermore, Syk ASO suppression of the CFTR gene has been complexed with DOTAP and successfully transcribed into an in vivo model of systemic anaphylaxis and focused on pulmonary inflammation. It has been established that when rats previously sensitized to *N. brasilensis* are challenged by i.v. injection of Ag isolated from this parasite, anaphylactic shock occurs, one aspect of which is pulmonary inflammation. One method for assessing pulmonary inflammation is to determine the Ag-mediated changes in BAL cell number and cell types found in the BAL. We observed that Syk ASO treatment suppressed the Ag-mediated (8 h) increase in BAL cell number, indicating that Syk ASO has anti-inflammatory properties in the lung. However, the proportions of different cell populations in BAL did not change 8 h after Ag challenge. In future studies, we will examine the effects of Syk antisense on changes in cell populations found in BAL at different times after Ag challenge. We will also determine whether aerosolized Syk ASO selectively inhibits pulmonary inflammation or if it affects other components of the systemic anaphylaxis.

These data extend the observations of Matsuda et al. (16) and pioneer the use of aerosolized Syk ASO in vivo to inhibit Syk mRNA and protein expression as well as the Syk-dependent activation of alveolar macrophages. Syk ASO may prove useful in the

### Table I. Cell composition (%) in BAL fluids from rats 8 h after Ag or saline challenge

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Lip-Sal (%)</th>
<th>Lip-Ag (%)</th>
<th>Scr ODN-Sal (%)</th>
<th>Scr ODN-Ag (%)</th>
<th>Syk ASO-Sal (%)</th>
<th>Syk ASO-Ag (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage</td>
<td>94.2 ± 2.2</td>
<td>94.1 ± 2.8</td>
<td>96.3 ± 1.5</td>
<td>91.0 ± 2.6</td>
<td>96.9 ± 0.5</td>
<td>95.8 ± 1.5</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>0.7 ± 0.4</td>
<td>0.7 ± 0.3</td>
<td>0.6 ± 0.4</td>
<td>1.3 ± 0.6</td>
<td>1.1 ± 0.5</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>1.5 ± 0.6</td>
<td>1.3 ± 1.2</td>
<td>1.3 ± 0.6</td>
<td>2.9 ± 1.6</td>
<td>0.2 ± 0.1</td>
<td>1.1 ± 1.0</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>3.1 ± 2.0</td>
<td>3.9 ± 1.8</td>
<td>0.9 ± 0.4</td>
<td>4.8 ± 2.3</td>
<td>1.2 ± 0.5</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Epithelial</td>
<td>0.5 ± 0.4</td>
<td>0.1 ± 0.1</td>
<td>0.8 ± 0.8</td>
<td>0.1 ± 0.1</td>
<td>0.6 ± 0.4</td>
<td>0.3 ± 0.3</td>
</tr>
</tbody>
</table>

* Rats were treated with aerosolized liposome (Lip), scrambled ODN (Scr ODN), or Syk ASO on days 1 and 2, and 24 h later were challenged with 100 μl saline (Sal) or antigen (Ag) delivered intravenously. Eight hours postchallenge, BAL was performed. The percentages (mean ± SEM) of macrophages, lymphocytes, neutrophils, eosinophils, and epithelial cells recovered from BAL are shown (n = 5).
study of allergic disorders because cells such as eosinophils and mast cells express several Syk-dependent pathways of activation (8, 13–15). Thus, Syk ASO-mediated Syk suppression may prove useful in the development of therapy for inflammatory diseases of the airways, such as asthma and allergic disorders, in addition to being potentially beneficial at other sites of inflammation.

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References


