Lipid-mediated ex vivo gene transfer of viral interleukin 10 in rat lung allotransplantation

Hideki Itano, MD^a
 Bassem N. Mora, MD^a
 Wanjiang Zhang, MD^a
 Jon H. Ritter, MD^b
 Timothy J. McCarthy, PhD^c
 Nelson S. Yew, PhD^d
 T. Mohanakumar, PhD^a
 G. Alexander Patterson, MD^a

Background: Recent studies suggest that viral interleukin 10 suppresses alloimmune response in transplantation and that cationic lipids are one of the most promising nonviral vehicles for gene therapy. The aim of this study was to examine the effect of ex vivo lipid-mediated viral *IL10* gene transfer into rat lung allografts on subsequent rejection.

Methods: Male F344 rats (RT11vl) underwent left lung transplantation with allografts from Brown Norway rats (RT1n). Allografts were transvascularily transfected 15 minutes after harvest with 5 mL of 1:20-diluted (group 1, n = 7) or 1:40-diluted (group 2, n = 6) GL67-pCMVieIL-10 complex. Group 3 (n = 7), serving as the control group, received 1:40-diluted GL67-pCF1-chloramphenicol acetyltransferase complex. All allografts were preserved for 3 hours at 10°C before transplantation. In all groups recipients were killed on postoperative day 5. Transgene expression of viral interleukin 10 was assessed by means of both reverse transcriptase–polymerase chain reaction and immunohistochemistry. Histologic rejection score, allograft gas exchange, exhaled nitric oxide level, and allograft cytokine mRNA expression were also assessed.

Results: Dose-dependent transgene expression of viral interleukin 10 was detected by means of both reverse transcriptase–polymerase chain reaction and immunohistochemistry. Allograft gas exchange (Pao₂) in groups 1 (114.06 ± 61.1 mm Hg) and 2 (108.58 ± 35.7 mm Hg) was significantly better than that in group 3 (66.4 ± 8.22 mm Hg; *P* = .020 and *P* = .023, respectively). The vascular rejection score in group 1 was significantly lower than that in group 3 (*P* = .032, Kruskal-Wallis test). Exhaled nitric oxide levels in group 2 (5.150 ± 6.38 ppb) were significantly lower than those in group 3 (13.517 ± 10.4 ppb; *P* = .039). Allograft interleukin 2 mRNA expression levels in group 1 (1.123 ± 0.23 relative units) were significantly lower than those in group 3 (1.753 ± 0.71 relative units; *P* = .038 vs group 3).

Conclusions: Lipid-mediated ex vivo viral *IL10* gene transfer into rat lung allografts improved graft gas exchange, reduced histologic rejection scores, downregulated graft interleukin 2 mRNA expression, and reduced exhaled nitric oxide levels by postoperative day 5. These results suggest a therapeutic potential of graft viral *IL10* gene transfer as an effective immunosuppressive strategy against lung allograft rejection.

From the Division of Cardiothoracic Surgery,^a Department of Pathology,^b Department of Radiology,^c Washington University School of Medicine, St Louis, Mo, and the Genzyme Corporation,^d Framingham, Mass.

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Address for reprints: G. Alexander Patterson, MD, 3108 Queeny Tower, One Barnes-Jewish Hospital Plaza, St. Louis, MO 63110 (E-mail: kellym@msnotes.wustl.edu).

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Lung transplantation has evolved into an effective therapeutic option in the management of patients with end-stage pulmonary diseases.¹ However, allograft rejection remains a serious impediment to the clinical success of lung transplantation. Gene transfer, the temporary introduction of genes to organs, which results in transient gene expression and production of a functional gene product to modify tissue responses, might be an effective strategy to decrease transplant rejection. Ex vivo gene delivery to grafts during cold storage before implantation might offer a specific opportunity for achieving graft-targeted, sustained, endogenous production of therapeutic gene products, while at the same time minimizing possible unwanted exposure to the transgene product of nontargeted organs. Gene transfer of immunosuppressive cytokine genes to the allograft may be a viable local immunosuppressive strategy by markedly impairing effective antigen presentation, reducing or eliminating immunogenicity, and preventing rejection or prolonging graft survival while avoiding the systemic toxicity of conventional immunosuppression.

Interleukin 10 (IL-10) is mainly produced by macrophages and T_H2 cells. It inhibits the synthesis of cytokines by T_H1 cells activated by monocyte/macrophage antigen-presenting cells.² It has been shown that IL-10 inhibits monocyte/macrophage-dependent T-cell activation and antigen presentation,^{3,4} as well as alloreactivity in vivo.⁵ Because of these effects, it has been suggested that IL-10 might be useful in the prevention of transplant rejection. Stimulatory effects of IL-10, however, have also been observed on both T- and B-cell differentiation, including enhancing major histocompatibility complex (MHC) class II expression on B cells. Viral IL-10 (vIL-10), a product encoded by the Epstein-Barr virus BCRF1 open-reading frame, is homologous to cellular IL-10, especially in the coding region of the mature protein sequence.⁶ It shares many biologic properties with cellular IL-10, including cytokine synthesis inhibitory activity and down-regulation of class II MHC expression on monocytes. vIL-10, however, does not possess such costimulatory activities of cellular IL-10,⁷ which suggests that vIL-10 may be a more potent immunosuppressant molecule.

In the current study we examined the influence of lipid-mediated viral *IL10* gene transfer and overexpression within lung allografts on subsequent rejection in an MHC-mismatched strain combination.

Material and Methods

Plasmids

The plasmids pCMVieIL-10 and pCF1-chloramphenicol acetyltransferase (CAT) were kindly provided by Genzyme Corporation (Framingham, Mass). pCMVieIL-10 was constructed to encode the immunosuppressive cytokine vIL-10 under the control of the human cytomegalovirus immediate early promoter in the sense ori-

entation. pCF1-CAT consists of the human cytomegalovirus immediate early gene promoter-enhancer CAT cDNA, as previously described.⁸ The sequence of vIL-10 was confirmed by DNA sequencing before use with the 377 DNA Sequencer (PE Applied Biosynthesis, Foster City, Calif). The bioactivity of pCMVieIL-10 was confirmed by the in vitro functional assay, as reported previously,⁹ on the basis of the ability of IL-10 to inhibit the synthesis of interferon (IFN) γ by phytohemagglutinin antigen P-stimulated human peripheral blood mononuclear cells.⁶

Liposomal Vector

Genzyme lipid 67 (Genzyme Corporation) is an amphiphile consisting of a hydrophobic lipid anchor group (cholesterol) linked to a positively charged spermine head group in a T-shaped configuration. Lipid 67/dioleoylphosphatidylethanolamine in a molar ratio of 1:2 was supplied as dried films and kept at 80°C, as previously described.⁸ The dried lipid films were hydrated with sterile 4°C water, treated in a vortex, and kept at 4°C for 10 minutes. This homogeneous suspension was mixed with an equal volume of plasmid DNA and kept at 30°C for 15 minutes. The final concentrations were 1 mmol/L for the cationic lipid mixture and 4 mmol/L for the plasmid DNA. Immediately before the injection to the allografts, the complex solution was diluted into 5 mL of saline solution in a 1:20 or 1:40 ratio.

Animals

Male inbred Brown Norway and F344 rats, weighing 250 to 320 g, were obtained from Harlan Sprague-Dawley (Indianapolis, Ind). All animal procedures were approved by the Animal Study Committee of Washington University. Animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996.

Study Design, Gene Transfer, and Transplantation

Animals were divided into 3 groups. Transfection was accomplished with 5 mL of 1:20-diluted (group 1, n = 7) or 1:40-diluted (group 2, n = 6) GL67-pCMVieIL-10 complex. Group 3 (n = 7), serving as the control group, received 1:40-diluted GL67-pCF1-CAT complex. Donor rat pulmonary arteries were flushed with 20 mL of cold (4°C) saline solution after systemic heparinization. Heart-lung blocks were immediately excised en bloc and kept at 10°C. The GL67-plasmid complex diluted in 5 mL of saline solution was injected into the allograft pulmonary vein retrograde through a 20-cm polyethylene tube (internal diameter 0.38 mm), with a pressure of 20 cm H₂O for 10 minutes. After storage in saline solution at 10°C for 3 hours, the grafts were flushed with cold (4°C) saline solution to eliminate the remaining liposome-DNA complexes from the pulmonary vasculature. Left lung allografts were then orthotopically transplanted by means of a modification of the previously described cuff technique.¹⁰

Assessment

Recipient animals underwent median sternotomy on postoperative day 5. After crossclamping of the right main bronchus and pulmonary artery, allografts were ventilated with 100% oxygen (1.5 mL of tidal volume at a rate of 100 breaths/min). Peak airway pres-

tures were measured, and exhaled gas was collected for 3 minutes. After 5 minutes of graft ventilation, arterial blood samples were obtained for blood gas analysis. Recipients were then killed, and left lung allografts were immediately flushed with 20 mL of cold saline solution. The lower half of the lung graft was frozen in liquid nitrogen and stored at -80°C for reverse transcriptase–polymerase chain reaction (RT-PCR) assessment. The upper half of the graft was fixed with Histochoice fixative (AMRESCO, Solon, Ohio) flushed through the trachea with 20 cm H_2O for histologic assessment. These sections were stained with hematoxylin and eosin and examined by a pathologist (J.H.R.) blinded to the tissue group. The sections were graded separately for both acute vascular rejection and airway rejection on the basis of the revised working formulation, as described previously.¹¹

RNA Isolation and cDNA Synthesis

Total lung RNA was prepared by means of guanidine isothiocyanate extraction with RNeasy Mini Kit (QIAGEN Inc, Valencia, Calif), according to the manufacturer's instructions. Extracted RNA was quantified by a spectrophotometer (Spectronic Genesys 5; Spectronic Instruments, Inc, Rochester, NY). Each microgram of extracted RNA was reverse transcribed at room temperature for 10 minutes, 42°C for 15 minutes, 99°C for 5 minutes, and 5°C for 5 minutes in a total of 20 μL of reaction mixture: 5 mmol/L MgCl_2 ; 50 mmol/L KCl; 10 mmol/L Tris-HCl (pH 8.3); 1 mmol/L of each deoxynucleotide triphosphate, 1 μL of RNase inhibitor, 2.5 U/ μL cloned murine leukemia virus reverse transcriptase, and 2.5 $\mu\text{mol/L}$ oligo d(T)₁₆ (Perkin Elmer, Foster City, Calif).

Semiquantitative PCR

Reverse-transcribed cDNA was amplified by PCR with 0.01 to 0.03 U/ μL Hot StarTaq Polymerase (QIAGEN Inc, Valencia, Calif) on a Mastercycler gradient (Eppendorf Scientific, Hamburg, Germany) in a 22- μL reaction (2.0 μL of RT product, 1.5 mmol/L MgCl_2 , 200 $\mu\text{mol/L}$ of each deoxynucleotide triphosphate, and 0.3 to 0.5 $\mu\text{mol/L}$ of each primer, PCR buffer [QIAGEN Inc, Valencia, Calif]), including KCl, Tris-HCl, and $(\text{NH}_4)_2\text{SO}_4$, after the first step of 95°C for 17 minutes, 25 to 35 cycles at 95°C for 30 seconds, 58°C (annealing temperature varying for different primers) for 30 seconds, and 72°C for 1 minute, followed by a final incubation at 72°C for 10 minutes. The sense and antisense sequence of primer pairs was designed for glyceraldehyde-3-phosphate dehydrogenase, vIL-10, IL-2, tumor necrosis factor α , inducible nitric oxide synthase (iNOS), IFN- γ , and endogenous IL-10.

PCR cycles and annealing temperature were calibrated for each primer pair. The glyceraldehyde-3-phosphate dehydrogenase bands were used to calibrate PCR input at unsaturated amplification for semiquantification. PCR products were electrophoresed on a 0.8% agarose gel and visualized by means of ethidium bromide staining. The gel images were stored by using the Gel Documentation System Image Store 7500 (version 7.12; UVP Inc, Upland, Calif) with a UV transilluminator. The average optical density of bands was quantitated by using the GelBase/GelBlot-Pro software (version 3.10; Syngene/Synoptics Ltd, Frederick, Md). Normal left lung samples of Brown Norway rats ($n = 6$) were also assayed in the same way as samples from the other groups for comparison.

Immunohistochemistry for vIL-10

Paraffin-embedded lung allograft sections were mounted on charged glass slides. After deparaffinization and hydration in graded ethanol, antigen retrieval was performed by means of steam treatment in Target Retrieval Solution (DAKO, Carpinteria, Calif) for 35 minutes at 95°C . Blocking steps included Fc Receptor Blocker (Accurate Chemical & Scientific Corp, Westbury, NY) and Super Block Blocking Buffer (PIERCE, Rockford, Ill) supplemented with 10% normal goat serum and 1% bovine serum albumin. All washes consisted of TBS (100 mmol/L Tris and 500 mmol/L NaCl, pH 7.4) supplemented with 0.1% saponin and 0.2% Triton X-100, by using the R&D Systems (Minneapolis, Minn) permeabilization protocol for cytokines. The immunohistochemistry procedure was enhanced with the Tyramide Signal Amplification kit (TSA-Indirect; NEN Life Science Products, Boston, Mass), according to the manufacturer's instructions. All steps were performed at room temperature. In brief, the slides were incubated overnight with biotinylated rat antiviral IL-10 mAb (1:25; Pharmingen, San Diego, Calif). After quenching endogenous peroxidase with 3% H_2O_2 /phosphate-buffered saline solution for 15 minutes, the slides were incubated with streptavidin-horse-radish peroxidase for 30 minutes, biotinyl tyramide solution for 25 minutes, and streptavidin-alkaline phosphatase for 30 minutes. Color development was performed with BCIP/NBT substrate (Vector Laboratories, Inc, Burlingame, Calif), including levamisole, 5 mmol/L. Slides were counterstained with nuclear fast red and permanently mounted with Cytoseal 60 (Stephens Scientific, Kalamazoo, Mich).

Exhaled Nitric Oxide Analysis

Nitric oxide (NO) levels in allograft exhaled gas were measured with a Sievers 280 NO analyzer (Sievers Instruments, Inc, Boulder, Colo) within 30 minutes of collection. Emissions from electronically excited nitrogen dioxide were detected in a gas-phase chemiluminescent reaction between NO and ozone. The sensitivity of the measurement is less than 1 ppb. At every measurement, it was confirmed that the baseline NO level of 100% oxygen was 0 ppb. Exhaled NO from normal left lungs of Brown Norway rats ($n = 5$) was also measured in the same way as that for the other study groups.

Statistical Analysis

Data were analyzed by means of 1-way analysis of variance and the Fisher post hoc multiple comparison test. Data not normally distributed were transformed with a square root or logarithmic transformation before performing the analysis of variance. Nonparametric data were analyzed with the Kruskal-Wallis test and the Mann-Whitney test with Bonferroni correction. All data are expressed as means $\pm$ SD.

Results

Semiquantitative RT-PCR for Transgene vIL-10

Semiquantitative RT-PCR for vIL-10 was performed to confirm allograft pCMVieIL-10 transfection at transcriptional levels. vIL-10 transgene mRNA expression in lung grafts was detected in all samples of groups 1 and 2. No vIL-10 mRNA was detected in group 3 (Figure 1). vIL-10 transgene mRNA

Figure 1. Semiquantitative RT-PCR analysis of vIL-10 mRNA transgene expression in rat lung allografts. The peak areas of bands were expressed as a relative ratio of vIL-10/glyceraldehyde-3-phosphate dehydrogenase (*GA3PDH*). The gel image shows vIL-10 bands from groups 1 and 2 but not from group 3. The relative ratio of vIL-10/glyceraldehyde-3-phosphate dehydrogenase in group 1 was significantly higher than that in group 2 ($P = .0050$ vs group 2). Values are means $\pm$ SD of 6 or 7 animals per group. PC, Positive control.

expression (mean $\pm$ SD) in group 1 (2.33 ± 1.44 relative units) was significantly higher than that in group 2 (0.637 ± 0.74 relative units; $P = .005$). These results confirmed the presence of transgene vIL-10 expression in all lung allografts of groups 1 and 2 in a dose-dependent fashion.

Immunohistochemistry for Transgene vIL-10

Immunohistochemistry for vIL-10 was performed with sections prepared from paraffin-embedded tissues to confirm allograft pCMViewIL-10 transfection at translational levels. There was strong cytoplasmic staining in the majority of bronchial epithelial cells. No nuclear staining was observed. Positive staining in alveolar epithelial cells was also observed in both flattened type I cells and hub-like type II cells. The staining was more focal and was seen in approximately 25% of the alveolar epithelial cells. Alveolar macrophages also showed positivity in the cytoplasm. No staining was observed in endothelial cells, stromal cells, or lymphocytes within the perivascular cuffs (Figure 2). These

findings demonstrate vIL-10 transfection to specific types of cells and local production of vIL-10 transgene protein within allografts. The staining was specific to transgene vIL-10 because we did not see any staining in group 3.

Histologic Rejection Score

The vascular rejection score in group 1 was significantly lower than that in group 3 ($P = .032$, Kruskal-Wallis test). There were no significant differences between groups in histologic airway rejection grading (Figure 3). These results suggest that allograft vIL-10 overexpression could lead to major immunoalteration to the point at which histologic differences can be seen and lung allograft rejection is reduced.

Lung Graft Function

Pao₂ (mean $\pm$ SD) levels in arterial blood obtained 5 minutes after right hilar crossclamping in groups 1 (114.06 ± 61.1 mm Hg) and 2 (108.58 ± 35.7 mm Hg) were significantly higher than those in group 3 (66.40 ± 8.22 mm Hg;

Figure 2. Immunohistochemistry for transgene vIL-10. Transgene vIL-10 protein was detected by means of immunohistochemistry in bronchial endothelial cells, alveolar epithelial cells, and alveolar macrophages. (Original magnifications: A, 200 $\times$; B, 200 $\times$; C, 400 $\times$; D, 400 $\times$.)

$P = .020$ and $P = .023$, respectively, vs group 3; Figure 4). There were no significant differences between groups in Paco_2 levels ($P = .82$) and peak airway pressures ($P = .64$). These data show that graft vIL-10 overexpression significantly improved graft lung function, suggesting the alleviation of allograft rejection.

Exhaled NO Levels

Exhaled NO levels (mean $\pm$ SD) in group 2 (5.150 ± 6.38 ppb) were significantly lower than those in group 3 (13.517 ± 10.4 ppb; $P = .039$ vs group 3), whereas exhaled NO levels from normal left lungs of Brown Norway rats (0.00 ± 0.00 ppb) were significantly lower than those in groups 1 and 3 ($P < .005$, respectively). There were no significant differences between groups 1 and 3 ($P = .49$, Figure 5). These results indicate that local vIL-10 overexpression with only low-dose pDNA-lipid complex significantly suppresses

exhaled NO levels. The results suggest that exhaled NO levels in normal lungs are low (and not detectable in this model) and that the upregulated exhaled NO levels in allograft rejection can be remarkably suppressed by graft vIL-10 overexpression.

Semiquantitative RT-PCR for Allograft Cytokine Expression

Allograft IL-2 mRNA expression levels (mean $\pm$ SD) in group 1 (1.123 ± 0.23 relative units) were significantly lower than those in group 3 (1.753 ± 0.71 relative units; $P = .038$ vs group 3), whereas IL-2 expression levels in group 2 (1.181 ± 0.74 relative units) were also lower than those in group 3, trending toward the significant level ($P = .068$ vs group 3). Normal lung IL-2 expression levels (0.666 ± 0.23 relative units) were significantly lower than those in group 3 ($P = .0014$ vs group 3, Figure 6). These results suggest that

Figure 3. Histologic rejection score. The sections were graded separately for both acute vascular rejection (*A-score*) and airway rejection (*B-score*). The acute vascular rejection score in group 1 was significantly lower than that in group 3 ($P = .042$ vs group 3, Kruskal-Wallis test). There were no significant differences (NS) in airway rejection scores. Bars show median values.

IL-2 mRNA expression levels are low in normal lungs and significantly upregulated during allograft rejection by day 5. Furthermore, our data indicated that allograft IL-2 upregulation is significantly reduced by local vIL-10 overexpression.

Normal lung mRNA expression of tumor necrosis factor α , iNOS, IFN- γ , and endogenous IL-10 was also significantly lower than that in other groups ($P < .05$, respectively), although no significant differences were observed between groups 1 and 3 or groups 2 and 3 (Figure 7). These findings suggest that mRNA expression of these cytokines was minimal in normal lungs and remarkably upregulated during allograft rejection by postoperative day 5. However, graft vIL-10 overexpression in this model did not lead to any significant change in graft cytokine levels.

Figure 4. Arterial blood oxygen tension (PaO₂). PaO₂ obtained 5 minutes after right hilar crossclamping is shown. Each PaO₂ in groups 1 (114.06 ± 61.05 mm Hg) and 2 (108.58 ± 35.7 mm Hg) was significantly higher than that in group 3 (66.4 ± 8.22 mm Hg; $P = .020$ and $P = .023$ vs group 3). Values are means $\pm$ SD of 6 or 7 animals per group.

Figure 5. Exhaled NO levels from rat lung allografts. Exhaled NO levels in group 2 (5.150 ± 6.38 ppb) were significantly lower than those in group 3 (13.517 ± 10.4 ppb; $P = .020$ vs group 3). Exhaled NO levels in normal lungs (NL; 0.00 ± 0.0 ppb) were significantly lower than those in groups 1 and 3 ($*P < .005$, respectively). Values are means $\pm$ SD of 5 to 7 animals per group.

Discussion

The present results demonstrated that lipid-mediated ex vivo viral *IL10* gene transfer into rat lung allografts improved graft gas exchange, reduced histologic rejection scores, downregulated graft IL-2 mRNA expression, and reduced exhaled NO levels on post-transplantation day 5 in a model of BN-to-F344 lung allotransplantation. Several other studies have indicated that local vIL-10 production can suppress

Figure 6. Semiquantitative RT-PCR analysis of IL-2 mRNA expression in rat lung allografts. The peak areas of the bands were expressed as the relative ratio of IL-2/glyceraldehyde-3-phosphate dehydrogenase (*GA3PDH*). IL-2 mRNA expression in group 1 and in normal lungs was significantly lower than that in group 3 ($P = .039$ and $P = .0014$ vs group 3). IL-2 mRNA expression in group 2 was lower than that in group 3, trending toward significance ($P = .068$ vs group 3). Values are means $\pm$ SD of 6 or 7 animals per group.

immune reactivity in response to alloantigen. Although some reports indicated detrimental effects¹² or ineffectiveness¹³ of cellular IL-10, which possesses immunostimulatory activities on allograft rejection, retroviral or lipid-mediated gene transduction of vIL-10 prolonged graft survival when injected directly into cardiac allografts.^{14,15} Viral *IL10* gene transfer also decreased allogeneic lymphocyte proliferative response to cultured human islets.¹⁶ Consistent with these observations, the present data seem to show the significant efficacy of graft ex vivo viral *IL10* gene transfer as a strategy for local lung-graft alloimmune suppression.

A diverse range of delivery vehicles is currently being assessed to facilitate gene transduction. These include recombinant replication-deficient viral vectors and cationic lipids, and both delivery systems possess certain advantages and disadvantages. Although adenovirus-mediated gene transfer is significantly more efficient than that afforded by cationic lipids,⁸ the recombinant viruses can be prone to inflammation and inactivation by the host immune sys-

tem.¹⁷ Cationic lipids, one of the most promising nonviral vehicles,¹⁸ are less immunostimulatory. They are invariably composed of a hydrophobic lipid anchor group capable of forming liposomes and interacting with membranes linked to a positively charged head group that interacts with and perhaps condenses the pDNA. DNA-cationic lipid complexes also possess dose-dependent toxicity, especially when directly delivered to the lungs, which can be attributed to the detergent-like activity of the cationic lipid component¹⁹ or the immunostimulatory unmethylated CpG sequences harbored in plasmid DNA.²⁰ Cationic lipids facilitate gene transfer through still poorly understood processes. This involves formation of a positively charged DNA-cationic lipid complex, adherence to the negatively charged cell membrane, endocytosis of the complex, release of DNA from the endosome into the cytoplasm, and translocation of DNA into the nucleus, leading to eventual gene expression.²¹ Optimized formulation of GL67 has been shown to be highly efficient and relatively less toxic for gene transfer

Figure 7. RT-PCR analysis for mRNA expression of tumor necrosis factor (*TNF*) α , iNOS, IFN- γ , and endogenous IL-10 in rat lung allografts. Normal lung (NL) mRNA expression in these cytokines was significantly lower than that in other groups (* $P < .05$, respectively). There were no significant differences between groups 1, 2, and 3. Values are means $\pm$ SD of 4 to 7 animals per group. *GA3PDH*, Glyceraldehyde-3-phosphate dehydrogenase.

to the lung and 100 times more active than the early generation cationic lipid.⁸ Our prior study of naked viral *IL10* gene transfer with a BN-to-Lewis lung allograft rejection model could not exert substantial reduction of rejection, except for decreased graft expression of iNOS mRNA and suppression of exhaled NO₉. The present GL67-mediated vIL-10 transfection attenuated allograft rejection, which may be attributable to the enhanced transfection efficiency of GL67 in comparison with DNA administered alone.²²

In the current study the ex vivo GL67-mediated approach was used for a graft-targeted viral *IL10* gene transfer. Our prior work on this method of gene transfer has suggested that gene transfer at low temperatures during graft storage yielded consistent and reproducible transgene expression, even with short storage times.²³ With storage at 10°C for 1 hour, transgene CAT expression became detectable 4 to 6 hours after implantation, and significant levels were observed for at least 28 days. We and others have shown that

lung allografts are rejected rapidly, between 4 and 6 days after transplantation in such an RT1-incompatible donor-recipient combination as BN to F344.^{24,25} Thus, we expected that fulminant rejection would be present in control recipients if animals were killed on postoperative day 5 and that transgene expression might be achieved before the time of alloimmune reaction. Another study in our laboratory using the same approach also suggested that the optimum concentration of lipid-DNA complex is 100 or 200 μ mol/L DNA for transfection without pulmonary toxicity.²⁶ On the basis of these findings, we used the current concentration of lipid 67-DNA complex, storage temperature, and time.

In the current study allograft vIL-10 overexpression significantly suppressed intra-graft IL-2 mRNA upregulation. In the rejection process IL-2, produced by activated T cells, promotes proliferation of T cells, leading to destruction of the allograft. Substantial evidence both from experimental and clinical studies has shown that IL-2 upregulation is

associated with allograft rejection. Studies evaluating IL-2 mRNA from biopsy specimens in clinical cardiac transplants have demonstrated a strong association between IL-2 expression and histologic rejection.^{27,28} A number of immunosuppressive treatments, resulting in decreased production of IL-2 by graft-infiltrating cells, also allow prolongation of organ allografts.²⁹ It has been reported that IL-10 directly inhibits human T-cell proliferation by specifically inhibiting IL-2 production at the transcriptional level when these cells are activated in an antigen-presenting, cell-independent manner.³⁰ This is a direct effect of IL-10 on T cells mediated by their constitutively expressed IL-10 receptor.³¹ Florquin and colleagues³² have also shown that endogenous IL-10 induced by staphylococcal enterotoxin B in CD4⁺ cells plays a cell-dependent immunoregulatory role in a model of T cell-dependent shock by downregulating IL-2 and IFN- γ production. Put into the context of these studies, the present findings of significant suppression of intragraft IL-2 expression by vIL-10 suggest that reduced rejection can at least in part be attributed to the IL-2-suppressive effect of vIL-10.

The present results demonstrated the upregulation of exhaled NO levels in rejected allografts. We also observed suppression of exhaled NO levels with low-dose pDNA-lipid complex but without suppression of iNOS mRNA on histologic allograft rejection. High-dose pDNA-lipid complex did not suppress exhaled NO levels but reduced the histologic allograft rejection. The exhaled NO level has become increasingly recognized as a noninvasive marker of acute lung allograft rejection.³³ Upregulation of iNOS mRNA expression and increased production of NOS and NO during allograft rejection have been also demonstrated in many experimental and clinical transplantations,^{25,34} whereas increased immunosuppressive therapy has been able to restore these to normal levels.³⁵ Our prior study of naked viral *IL10* gene transfer with a BN-to-Lewis lung allograft rejection model demonstrated a direct correlation between high exhaled NO levels from rejected lungs and iNOS mRNA upregulation.⁹ In the current study, however, the effects of vIL-10 on allograft iNOS expression levels were not clear, and there was no positive correlation between these 2 parameters on postoperative day 5, despite significantly suppressed exhaled NO levels with low-dose pDNA-lipid complex. These results may be attributed to the proinflammatory properties of liposome GL67/unmethylated pDNA complex, which can promote proinflammatory cytokine expression in a dose-dependent fashion,²⁰ including IFN- γ . Given that exhaled NO levels reflect both iNOS upregulation and the net allograft rejection, as shown in above-mentioned studies, the present data may still support the notion that the vIL-10 immunosuppressive activity parallels iNOS/exhaled NO levels in acute lung rejection, whereas its suppressive effect on iNOS expression was

obscured by proinflammatory properties of pDNA-lipid complex in a dose-dependent fashion.

This study has a number of potential limitations. Results might be different with alternate doses of plasmid or lipid vector. However, as noted above, these doses were selected after careful review of our own preliminary data and data from other reports. The ex vivo route of administration has proven effective, especially when transgene expression is not required at the time of transplantation. In addition, it is recognized that no matter how effective, gene transfer results in only temporary transgene expression. In this study the 5-day kill time was selected because in this major antigenic mismatch combination fulminant rejection with graft loss in untreated animals is known to occur 5 days after transplantation. Our hope was to affect this fulminant rejection. However, the endobronchial route of transfection provides the possibility of repeat administration in an effort to have a lasting effect on graft rejection.

In conclusion, the present study demonstrated that lipid-mediated ex vivo viral *IL10* gene transfer into rat lung allografts improved graft gas exchange, reduced histologic rejection score, downregulated graft IL-2 mRNA expression, and reduced exhaled NO levels by postoperative day 5 in a model of rat lung allotransplantation. These data strongly support the notion that lipid-mediated graft viral *IL10* gene transfer may be an effective immunosuppressive strategy that may overcome acute lung allograft rejection.

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