Online data supplement

Immunostimulatory Oligonucleotides Attenuate Airways Remodelling in Allergic Monkeys

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METHODS

Animal Protocol. All monkeys used for these studies were California National Primate Research Center colony-born rhesus macaques (*Macaca mulatta*). Care and housing of animals before, during and after treatment complied with the provisions of the Institute of Laboratory Animal Resources and conforms to practices established by the American Association for Accreditation of Laboratory Animal Care (AAALAC). Prior to sensitization with HDM, male adult monkeys were screened for atopy by skin-prick tests using a panel of one hundred common environmental allergens. All animals selected for the study exhibited positive skin-prick tests for 2 or more allergens and no prior reactivity to HDM. Eight young adult (3-5 years old) rhesus macaques were sensitized and exposed to house dust mite (HDM) allergen (*Dermatophagoides farninae*) as previously described using a combination of subcutaneous (SQ) inoculations along with intramuscular (IM) injections of heat-killed Bordetella pertussis cells, intranasal HDM exposures, and HDM aerosols (E1). The sensitization lasted 11 weeks, after which all eight monkeys demonstrated positive skin tests to HDM. Following the HDM sensitization, each monkey was exposed to HDM aerosol once every two weeks for 20 weeks of exposure, clinical signs of allergic airways disease were confirmed by pulmonary function testing and bronchoalveolar lavage in all animals. Once all 8 monkeys were determined to exhibit clinical and immunologic signs of allergic airways disease, the monkeys were sedated with ketamine and 4 of the eight monkeys were given 3 inhalation exposures (via facemask) of 12.5 mg ISS in 5 ml sterile PBS with each ISS exposure occurring 24h prior to the HDM exposure over 6 weeks. The

oligodeoxynucleotide sequence 5'-TGACTGTGAACGTTCGAGATGA-3' (1018) was utilized for study; this sequence has been previously shown to be an effective immune stimulus for both mice and humans (E2, E3). The remaining 4 monkeys received only sterile PBS (sham treatment). Bi-weekly HDM exposures continued after these 3 ISS treatments for 12 weeks, during which time clinical signs of allergic airways disease were monitored by pulmonary function testing and bronchoalveolar lavage. An additional 4 inhalation exposures of 1.25 mg ISS in 5 ml sterile PBS delivered as described above.

Pulmonary mechanics and airway responsiveness testing. Pulmonary mechanics and airway responsiveness to histamine aerosol were measured as previously described (E1). Briefly, anesthetized and intubated animals were placed in a head-out plethysmograph and airway resistance was measured via transfer impedance during doubling doses of aerosolized histamine (0.0625-32 mg/ml). Histamine challenge was stopped when highest dose was reached or arterial O2 saturation dropped below 75%.

Evaluation of airways remodeling. Two weeks after the last ISS treatment, monkeys were euthanized with an overdose of pentobarbital after being sedated with Telazol (8mg/kg IM) and anesthetized with Diprivan (0.1-0.2 mg/kg/min, IV). The monkeys were then necropsied following exsanguination through the posterior vena cava. Lungs were inflation-fixed as previously described (E1). Five-micron thick paraffin sections were stained with hematoxylin and eosin (H&E) to evaluate basement membrane thickening, with Alcian blue/periodic acid-Schiff (AB/PAS) stain to evaluate acidic and neutral mucosubstances, or with a mast cell tryptase antibody to evaluate the

presence of mast cells. Basement membrane thickness was measured as previously described (E4). Morphometric analysis of mucous cells was performed on tracheal epithelium using procedures described by Hyde and colleagues (E5). All measurements were made using a 20X objective and 5-μm thick paraffin sections. Mucous cells were defined by positive staining with AB/PAS. The mass, as measured by volume (μm^3) of mucous cells per unit area (μm^2) of basement membrane (Vs), was estimated from point and intercept counts with a 90-point cycloid grid by the equation: Vs = (6.6 x Po) / (Ibl x 2), where Po represents the points counted for mucous cells and Ibl represents the number of intercepts of the basal lamina. Mucous cell mass was calculated for each monkey from counts made on at least five fields from trachea and were used to calculate the mean and standard deviation for each group. Determination of significance was based on t-test as $p < 0.05$ (SigmaStat ®, SPSS Science, Chicago, IL) (E6). Qualitative evaluation of mast cell infiltration was determined by a blinded observer. Mast cells were immunohistochemically stained with mast cell tryptase monoclonal antibody AA1 (Novacastra Laboratories, UK) using the ABC immuno-staining system with the nova red peroxidase substrate according to manufacturers instructions (Vector Labs, Burlingame CA). Paraffin sections were ranked, by a blinded observer, according to total mast cells, mast cells associated with smooth muscle and mast cells associated with glands. A ranking of 1 indicates fewest mast cells, while a ranking of 8 indicated the most mast cells. Determination of significance was based on the Mann Whitney test ($p<0.05$, SigmaStat ®) (E6).

E1. Schelegle, E. S., L. J. Gershwin, L. A. Miller, M. V. Fanucchi, L. S. Van Winkle, J. P. Gerriets, W. F. Walby, A. M. Omlor, A. R. Buckpitt, B. K. Tarkington, V. J. Wong, J. P. Joad, K. B. Pinkerton, R. Wu, M. J. Evans, D. M. Hyde, and C. G. Plopper. 2001. Allergic asthma induced in rhesus monkeys by house dust mite (*Dermatophagoides farinae*). *Am J Pathol* 158(1):333-341.

E2. Broide, D., J. Schwarze, H. Tighe, T. Gifford, M. D. Nguyen, S. Malek, J. Van Uden, E. Martin-Orozco, E. W. Gelfand, and E. Raz. 1998. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J Immunol* 161(12):7054-62.

E3. Horner, A. A., J. H. Van Uden, J. M. Zubeldia, D. Broide, and E. Raz. 2001. DNA-based immunotherapeutics for the treatment of allergic disease. *Immunol Rev* 179:102-18.

E4. Evans, M. J., L. S. Van Winkle, M. V. Fanucchi, G. L. Baker, A. E. Murphy, S. J. Nishio, E. S. Schelegle, L. J. Gershwin, P. L. Sannes, and C. G. Plopper. 2002. Fibroblast growth factor-2 in remodeling of the developing basement membrane zone in the trachea of infant rhesus monkeys sensitized and challenged with allergen. *Lab Invest* 82(12):1747-54.

E5. Hyde, D. M., D. J. Magliano, and C. G. Plopper. 1991. Morphometric assessment of pulmonary toxicity in the rodent lung. *Toxicol Pathol* 19(4 Pt 1):428-46.

E6. Glantz, S. A. 1992. Primer of Biostatistics, Third ed. McGraw-Hill, Inc., New York.