Research Article

Beneficial Effects of Prebiotic *Saccharomyces cerevisiae* Mannan on Allergic Asthma Mouse Models

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Received 10 February 2017; Revised 31 May 2017; Accepted 4 June 2017; Published 1 August 2017

One of the unmet needs for asthma management is a new therapeutic agent with both anti-inflammatory and anti-smooth muscle (ASM) remodeling effects. The mannose receptor (MR) family plays an important role in allergen uptake and processing of major allergens Der p 1 and Fel d 1. We have previously reported that ASM cells express a mannose receptor (ASM-MR) and that mannan derived from *Saccharomyces cerevisiae* (SC-MN) inhibits mannosyl-rich lysosomal hydrolase-induced bovine ASM cell proliferation. Using a humanized transgenic mouse strain (huASM-MRC2) expressing the human MRC2 receptor in a SM tissue-specific manner, we have demonstrated that ASM hyperplasia/hypertrophy can occur as early as 15 days after allergen challenge in this mouse model and this phenomenon is preventable with SC-MN treatment. This proof-of-concept study would facilitate future development of a potential asthma therapeutic agent with dual function of anti-inflammatory and anti-smooth muscle remodeling effects.

1. Introduction

The currently available asthma therapeutics are effective in controlling inflammation but ineffective in controlling pathological changes of airway smooth muscle (ASM) remodeling that can occur at a young age [1, 2]. Therefore, an anti-inflammatory agent that can successfully inhibit ASM remodeling is extremely desirable to reduce asthma-related morbidity and mortality.

There has been a growing interest in the therapeutic use of living microorganisms (probiotics) for many human diseases, including asthma [3–5]. Similarly, prebiotics that are nonliving and indigestible polysaccharides can have beneficial effects on the host. Multiple mannosyl-rich oligosaccharides are capable of blocking antigen-driven T cell proliferation and antigen uptake and presentation [6, 7]. Acemannan from the Aloe plant induces maturation of dendritic cells [8] and inhibits the proliferative responses of tumor cells by affecting the expression of T lymphocytes [9]. Since the discovery of beer by a Mesopotamian farmer, *Saccharomyces cerevisiae* (Brewer’s or Baker’s yeast) is quantitatively and economically one of the most important groups of microorganisms exploited by man for food and alcoholic beverages. Mannan derived from *S. cerevisiae* (SC-MN), a prebiotic polymer of mannosyl (Man9 and a Gln residue, connected by α linkages), composes 45% of the cell wall of *S. cerevisiae* [10].

The mannose receptor (MR) family is an intricate part of innate immunity and the homeostatic clearance system [11] and plays an important role in uptake and processing of major allergens Der p 1 and Fel d 1, although the binding sites of these antigens differ [12, 13]. We have previously reported that airway smooth muscle cells express a mannosyl receptor (ASM-MR) [14] and SC-MN can inhibit bovine
ASM cell proliferation induced by endogenous mannosyl-rich MR ligands such as lysosomal hydrolases (β-hexosaminidases Hex A and Hex B and β-glucuronidase) [15].

In a murine allergic asthma model, mycobacterial acyl chains and mannose groups of lipopolysaccharides have been shown to suppress allergic disease and increase IL-10 secretion from cervical lymph nodes and splenocytes [16]. We have chosen to investigate the effects of SC-MN because mannose from pathogenic microorganisms is capable of eliciting IL-17 production [17]. However, this is not true of SC-MN [17], making it an appealing therapeutic agent as its beneficial effects could be achieved without IL-17-driven inflammation. We have confirmed this finding in our study as well as documented its lack of ability to induce IL-13 production in the lungs of mice despite a potential concern of its ability to regulate dendritic cell function in favor of Th1/2 polarization [18]. Furthermore, SC-MN had no effect on IL-33 that can stimulate group 2 innate lymphoid cells important in fungal allergy [19].

Here, we present evidence that by cloning of the huASM-MRC2 and development of a humanized transgenic mouse model, overexpression of huASM-MRC2 results in accelerated ASM remodeling and SC-MN can offer dual anti-inflammatory and anti-smooth muscle remodeling benefits.

2. Materials and Methods

2.1. Animals, Cloning huASM-MRC2, and Transgene Construct. All protocols were approved by the University of Tennessee Health Science Center (UTHSC) Institutional Biosafety Committee and Institutional Animal Care and Usage Committee. Wild-type (WT) FVB/NJ and BALB/c were purchased from the Charles River Laboratories or the Jackson Laboratory and housed in a pathogen-free vivarium at the UTHSC. The human ASM-MRC2 was cloned by RT-PCR using primers designed from cDNA of MRC2 and mRNA isolated from human bronchial ASM cells. The human ASM-MR cDNA (5658 bp) is 99% identical to the full-length cDNA of the huASM-MR cDNA (5658 bp) [20].

The transgenic mice (FVB/NJ) were then backcrossed onto an allergy-prone BALB/c strain, and N10 generation mice of ages 6–8 wk were used for the study. Transgene expression was confirmed using qPCR by Transnetyx Inc. (Memphis, TN, USA): target sequence 3′-cagcagagccagtctgtctgctcagtgccagctacacgaggtctacaccatccagggaaactcccacggaaagccg-5′.

For smooth muscle remodeling studies, mice with qPCR signal values between 7.5 and 13.0 were used.

2.2. Allergen Sensitization and Challenge. WT BALB/c or humanized transgenic mice were OVA-immunized on days 0 and 14 with 20 μg OVA (Sigma, St. Louis, MO, USA) in Imject® Alum (Pierce, Rockford, IL, USA) [21]. i.p.; nonimmunized (sensitized) mice received alum only. Increased serum OVA-specific IgE levels on day 1 versus day 21 were confirmed using ELISA (AbD Serotec, Oxford, UK). Responsive mice (sIgE ≥ 500 ng/ml) were pretreated with control saline or SC-MN (Sigma, prepared for patented use for asthma therapeutic, endotoxin level < 2 EU/ml) 30 min before OVA challenge on days 28, 29, and 30 (100 μg OVA i.n. in 25 μl phosphate-buffered saline (PBS)).

2.3. Airway Physiology Measurements. To assess isolated thoracic flow, compartmentalized double chambers (Buxco) were used to exclude nasal airway resistance. Conscious spontaneously breathing animals were retrained in the chambers to analyze thoracic-specific airway resistance (sRaw) in response to aerosolized saline or escalating doses of Mch (2.5–25 mg/ml, 1 ml) for 3 min [22]. Readings were taken averaged for 5 min following each nebulization.

2.4. Liposome Construction. Liposomes were prepared by the evaporation method using different molar ratios of HSPC:Chol:DSPE-PEG2000 (e.g., 50:45:5) (Dr. George C. Wood, Department of Pharmaceutical Sciences, UTHSC). Appropriate quantities of lipids and near-infrared fluorescent dye (DiIC18 = DiR) were dissolved in 1:9 solvent mixtures of chloroform and methanol. The mixture was evaporated in a Rota-evaporator at 40°C under vacuum at 100 rpm overnight. The lipid film was hydrated at 65°C with HEPES buffer containing 0.15 M NaCl and 10 mM EDTA on the Rota-evaporator at 100 rpm for 2 hr. The resulting fluorescent liposomes were large multilamellar vesicles of approximately 0.8–1.0 μm. Depending on the vesicle size required, liposomes were extruded through stacked nucleopore filters for a total of 6–10 passes, producing a narrow particle size distribution of unilamellar liposomes. Targeting of PEG-maleimide liposomes with the cRGD peptide was performed via covalent coupling through thioester bond formation between the maleimide group of the liposomes and cyclic RGD [23].

2.5. Fluorescent Reflectance Imaging (FRI). On day 32, 48 hr after OVA challenge, mice were anesthetized using isoflurane. Fluorescent liposome (–DiR) (800 nm, 90 μg/ml, 25 μl, each nostril, i.n.) or fluorescent cRGD-liposome (–DiR) (80 nm, 20 μg/ml, 200 μl, i.v.) was administered to the mice. The imaging procedures were performed at 2, 6, and 24 hr postadministration of fluorescent liposome with fixed fiducial markers for optical imaging with different dye
concentrations as a standard. The FRI system includes a liquid nitrogen CCD camera (Photometric Chemipro, Roper Scientific, Trenton, NJ) mounted onto a darkbox and a shutter mounted with a filter wheel (Sutter Instruments Co., Lambda 10-2 Optical Filter Changer, Novato, CA) that allows for multiple excitation filters to be controlled through software (Metamorph, Universal Imaging, Downingtown, PA). An excitation filter centered at 710 nm (Chroma Technology Corp., Rockingham, VT) and a 3-cavity narrow bandpass filter (10 nm spectral width) (Andover, Salem, NH) were used to visualize the near-infrared light. The biologic autofluorescence region is approximately 400–600 nm wavelength, and therefore, DiR signals were able to be detected without interference by autofluorescence.

2.6. Bronchoalveolar Lavage (BAL) and Lung Tissue Collection. Bronchoalveolar lavage was performed with 0.5 ml of warm PBS (37°C) under ketamine/xylazine sedation (100 mg/kg each). Cell count was performed by cytospin, and cell-free BAL fluid (BALF) was stored at −80°C. Total lung tissue was homogenized in 1 ml of PBS containing protease inhibitor cocktail (Sigma) on ice. Protein was quantified by using a Bradford Protein Assay kit (Thermo Fisher). Both BAL fluids and lung homogenates were analyzed for cytokines by a Quantikine ELISA kit from R&D systems according to the manufacturer’s instructions. Muc5ac in BAL fluid was analyzed by an ELISA kit (USCN Life Sci. Inc., Houston, TX) according to the manufacturer’s instructions.

2.7. IL-10 ELISPOT Analysis. Naïve mice were fed once with SC-MN (80 mg/kg) or saline, via gavage. Eighteen hours later, BAL cells and unselected splenocytes (1 × 10^5 cells/well of each cell type) were plated onto a Millipore microtiter well plate for IL-10 ELISPOT analysis. Cells were stimulated with or without SC-MN (1 mg/ml) and cultured for 72 hrs at 37°C in CO_2 incubator (5% CO_2/95% air). The IL-10-producing cells were identified following the manufacturer’s instructions (BD Pharmingen) and sent out to BD Biosciences analysis service.

2.8. Histology, Immunohistochemistry, Histochemistry, and Analysis. Mice were sacrificed (cervical dislocation under anesthesia) and perfused with 4% paraformaldehyde. Paraffin-embedded trachea and lung tissue sections were cut at 10 μm for H&E staining, immunohistochemistry was performed for smooth muscle isoactin, and Alcian blue-periodic acid Schiff (PAS) stains were used for mucin-containing goblet cells. Smooth muscle α-isoactin was probed with the α-smooth muscle actin antibody clone 1A4 according to the manufacturer’s instruction using a kit (Sigma). All slides were scanned using the ScanScope® XT at 0.25 μm/pixel resolution (~400x magnification). Trachea cross sections were analyzed for the trachealis muscle area at day 38, 45, or 52. The small airway smooth muscle area was analyzed by selecting 3–5 small airway branches with cross-sectional cut (120–300 μm calibers) at day 45. Data collection and analyses were performed blinded (n=5–6 mice) using the fiduciary marker 200 μm average (i.d.) airway caliber and 31,416 μm² luminal area. Each data point represents an averaged value from one mouse.

2.9. Statistical Analysis. All animal experiments were repeated three times. Data are expressed as mean or median ± 95% CI (for PC200R, provocative methylcholine concentration effecting a 200% increase in airway resistance) or mean ± SD (or SEM) and analyzed by the Kruskal-Wallis test followed by post hoc tests for unpaired data or one-way
analysis of variance followed by Dunnett’s post hoc test using Prism 6 software (GraphPad, San Diego, CA). For FRI data, software (Metamorph, Universal Imaging, Downingtown, PA) was used to quantify the intensity of fluorescence. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Anti-Inflammatory Effects of SC-MN on OVA-Allergic WT BALB/c Mice. For a global view of anti-inflammatory effects of SC-MN, we employed a whole-body imaging system to identify sites of inflammation; fluorescent cyclic arginine-glycine-aspartic acid- (cRGD-) labeled liposomes were used to detect integrin upregulation as a marker of inflammation. Intravenously (i.v.) administered cRGD first homed to the cervical lymph nodes and then to the lungs in the OVA-allergic mice, which was partially mitigated by intranasally (i.n.) administered SC-MN treatment. The upper limit of signals from lymph nodes was 4 times higher than that of the signals in the lungs. Cyclic RGD homing to the cervical lymph nodes and lungs was prominent compared to the control or SC-MN treatment in mice (Figures 1(a), 1(b), 1(c), 1(d), 1(e), and 1(f)), suggesting that SC-MN treatment suppressed inflammation in the draining lymph nodes and lungs. Intranasal pretreatment with SC-MN (1 mg, 30 min before each of three OVA challenges) inhibited allergic airway inflammation allowing i.n. administered liposome to reach the lungs of SC-MN-pretreated allergic mice, compared to the little deposition of liposome in the lungs of control allergic mice (Figures 2(a), 2(b), 2(c), 2(d), 2(e), 2(f), 2(g), and 2(h)). This finding is presumably due to

<table>
<thead>
<tr>
<th>Aerosol challenge</th>
<th>Total cell number/ml</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
<th>Eosinophils</th>
<th>Neutrophils</th>
</tr>
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<tr>
<td>PBS</td>
<td>326,000 ± 6000</td>
<td>245,000</td>
<td>55,000</td>
<td>9780</td>
<td>6520</td>
</tr>
<tr>
<td>OVA</td>
<td>530,000 ± 30,000 ∗</td>
<td>260,000</td>
<td>196,000</td>
<td>27,000</td>
<td>11,000</td>
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<tr>
<td>SC-MN + OVA</td>
<td>242,000 ± 18,000</td>
<td>138,980</td>
<td>96,960</td>
<td>4040</td>
<td>2020</td>
</tr>
</tbody>
</table>

Mice pretreated with SC-MN (i.n.) showed marked reduction in total BAL cell numbers compared to the OVA group. The decrease is also reflected in all four cell types analyzed. Results are mean ± SEM for total cell numbers in BALF and mean differential cell counts (n = 3). ∗P < 0.05, OVA group versus PBS or SC-MN + OVA group.

Table 2: A single oral administration of SC-MN increases IL-10 producing cell numbers in naïve mice.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Gavage feeding</th>
<th>Immunospot</th>
<th>Mean spot sizes</th>
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<tbody>
<tr>
<td>BAL</td>
<td>Saline</td>
<td>163 ± 161</td>
<td>0.0052 ± 0.0022</td>
</tr>
<tr>
<td>BAL</td>
<td>SC-MN</td>
<td>586 ± 211 ∗</td>
<td>0.0060 ± 0.006</td>
</tr>
<tr>
<td>Splenocytes</td>
<td>Saline</td>
<td>35 ± 13</td>
<td>0.0133 ± 0.0064</td>
</tr>
<tr>
<td>Splenocytes</td>
<td>SC-MN</td>
<td>427 ± 221</td>
<td>0.0077 ± 0.0009</td>
</tr>
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</table>

Results are mean ± SEM (n = 3). We observed that administering SC-MN to mice increased the number of IL-10-producing cells in the BAL (∗P < 0.05). A similar trend was seen for splenocytes from SC-MN-treated mice.
mucociliary clearance of the liposome particles in the inflamed airways of untreated allergic mice. Allergic mice pretreated with SC-MN showed marked inhibition of cellular infiltration (Table 1).

Similar to the report on lipoglycan [16], SC-MN can stimulate IL-10 regulatory cytokine as shown in Table 2.

To confirm that SC-MN does not cause potentially harmful effects of IL-17 production [17], T_{h}2 polarization [18], or IL-33 production that can stimulate ILC2 cells [19], we have measured IL-13, IL-17, IL-22, and IL-33 in BALF and supernatants of lung homogenates in naïve BALB/c mice after daily SC-MN (or saline) for 3 consecutive days. There was no increase in any of the abovementioned cytokines in SC-MN-treated naïve BALB/c mouse lung homogenate supernatants: IL-13, 115 ± 82 versus 51 ± 11; IL-17, 125 ± 31 versus 123 ± 28; IL-22, 8 ± 7 versus 8 ± 9; and IL-33, 5792 ± 2012 versus 5553 ± 1257 pg/mg protein (saline versus SC-MN group, resp., n = 7 mice). Similarly, there was no discernable difference in any of these cytokines measured in the BALF of these two groups.

3.2. Effect of SC-MN on Airway Mucin. Mucous hypersecretion from goblet cells is a hallmark of asthma, which can lead to airway obstruction and increased morbidity [24]. The effect of SC-MN on airway neutral mucin was examined by PAS staining in the midlung sections (Figure 3); stored mucin within the epithelium was inhibited by SC-MN treatment (59–81% inhibition, n = 6). Muc5ac protein levels in BALF were significantly elevated in allergic mice that were effectively inhibited by SC-MN treatment (Figure 4).

3.3. Inhibition of AHR by SC-MN (Intranasal) in WT Allergic BALB/c Mice. Based on preliminary dose response studies (6–60 mg/kg), we have selected 45 mg/kg as an optimum efficacious SC-MN dose targeting ASM cells and thoracic sRaw parameter. Mice pretreated with SC-MN (45 mg/kg, i.n., 30 min before OVA challenges) showed significantly blunted AHR, measured by PC200R (provocative Mch concentration effecting a 200% increase in airway resistance), compared to untreated allergic mice (OVA group) (Figure 5).
3.4. Accelerated ASM Remodeling in a huASM-MRC2 Mouse Model. Groneberg et al. have demonstrated airway smooth muscle remodeling in OVA-allergic BALB/c mice using a long-term, labor-intense protocol [24]. We have developed a mouse model to substantially shorten the process from a total of 163 days to 45 days (Figures 6(a) and 6(b)). Trachealis muscle mass in OVA-sensitized and OVA-challenged mice remained unchanged in WT (data not shown) and Tg-negative littermate control groups at day 52 (Figure 6(b)). The transgenic allergic mice showed AHR by a shift to the left of the thoracic airway resistance curve (Mch sensitivity) compared to Tg-negative littermate control allergic mice (Figure 6(c)). At the maximum dose of Mch (25 mg/ml), numerous breaths of Tg+ allergic mice were rejected from data acquisition due to shallow labored breathing.

3.5. Inhibition of Large and Small Airway Smooth Muscle Remodeling and AHR by SC-MN in Smooth Muscle-Specific huASM-MRC2 Transgenic Allergic Mice. The level of β-Hex in the BAL fluid of allergic mice is 3 times higher than that of naïve counterparts (data not shown). To examine the impact of blocking ASM-MR/β-Hex-mediated remodeling using SC-MN, the universal allergen OVA was used to induce general allergic inflammatory states in transgenic mice. SC-MN not only significantly inhibited large and small airway smooth muscle mass (Figures 7 and 8) but also inhibited the increase in the number of small airway smooth muscle nuclei (Figure 8(d)). SC-MN treatment (45 mg/kg) significantly restored the decrease in PC200R seen in transgenic allergic mice (Figure 9).

4. Discussion

In this study, we have demonstrated the efficacy of SC-MN in suppressing inflammation including stored and secreted mucin and airway hyperreactivity in WT BALB/c allergic mice. In addition, our newly developed transgenic huASM-MRC2 mouse model enabled us to demonstrate ASM remodeling in a reasonable experimental time period, compared to other models [25], that was successfully modified by SC-MN. Airway hyperreactivity was inhibited in this model as well. Airway remodeling in this model showed two elements:
an accelerated increase in ASM mass and (2) proliferation of ASM, indicated by ASM nucleus count. Both of these elements were effectively inhibited by SC-MN, consistent with antimitogenic/antiproliferative benefits seen in bovine ASM cells [15].

The mannose receptor blocker, mannan, elicits structure- and source-dependent differential responses in dendritic cells [26]. For instance, the response to monomeric engagement of mannan-MR stimulates IFN-γ and IL-12, aiding host immunity against pathogens and regulating TH2 microenvironment. On the other hand, mycobacterial lipoglycans have lipid appendages tending to aggregate MRs and suppress IFN-γ and IL-12 production, causing immunosuppression in host. The SC-MN preparation used in this study lacks acyl moieties (confirmed by Avanti Polar Lipids Inc. analytical service). Therefore, a higher dose of SC-MN compared to that of lipoglycans [16] was required to exert an anti-inflammatory effect. Regarding the mechanism of anti-inflammation, a single dose of oral SC-MN to naive FVB/NJ mice, followed by in vitro stimulation with SC-MN for 72 hr, resulted in IL-10 production by BAL cells and splenocytes. Sayers et al. also reported a robust induction of IL-10 by lipoglycan from splenocytes and mediastinal lymph nodes from allergic mice using anti-CD3/CD28 stimulation in vitro [16]. However, transgenic overexpression of IL-10 can cause lung fibrosis, suggesting potential harmful effects of excess IL-10 beyond its beneficial immunoregulatory role [27].

There are multiple advantages of SC-MN over mannan from other sources: SC-MN is water-soluble and the molecular mass is relatively small (36 kD) compared to that of MN from Aloe barbadensis (1000 kD) [8]. Additionally, acemannan and MN from many other sources have beta linkages that are likely to be antigenic [28].

Our findings highlight an important role of huASM-MRC2 (a phylogenetically conserved pattern recognition receptor) that is encoded within chromosome17q23.2 near ORMDL3 17q12-21 of which pathogenic variants increase the risk of asthma [29]. Therefore, members of the MR family, endogenous ligands, and receptor blockers can be exploited for the treatment of human asthma and other diseases. Glycoprotein ligands for the MR family members may be distinct with predilection to bind to different domains [30], and specific function of each member may be diverse depending on the type of ligand, cell, and tissue. For instance, complex protease-resistant endogenous glycoproteins bind to MR at the high-affinity binding domains (CTLD4-5) while SC-MN binds through CTLD4–8 [14].

Figure 7: Intranasal mannan blocked the OVA-induced increase in small airway smooth muscle as well as in cellular infiltration, in allergic transgenic mice. OVA-sensitized mice treated with SC-MN prior to OVA or PBS challenge. Representative sections show tissue morphology at day 45, visualized by H&E staining (midlevel left lung). (a) Saline + PBS (control); (b) saline + OVA; (c) SC-MN 45 mg/kg + OVA. Isoactin staining shows organized and thickened ASM in the OVA group (c) compared to the control (d) and SC-MN-treated mice (f). PAS staining shows magenta neutral mucin staining in OVA-sensitized and OVA-challenged mice (h).
been reported as important factors in asthma [32]. Regarding glycoprotein endogenous ligands for MR, β-hexosaminidase levels are significantly elevated in serum of severe asthmatic patients [33]. There is also a growing interest in immunoregulatory capacity of glycans and glycan-binding proteins such as galactins, selectins (C-type lectins, MR is a ligand for L-selectin) [34], and siglecs [35].

In conclusion, the prebiotic mannose receptor blocker SC-MN is a promising agent that can render dual benefits in asthma: anti-inflammatory and anti-smooth muscle remodeling at the level of both large and small airways.

**Abbreviations**

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<tr>
<td>AHR</td>
<td>Airway hyperreactivity</td>
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<tr>
<td>ASM</td>
<td>Airway smooth muscle</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<tr>
<td>β-Hex A and B</td>
<td>β-Hexosaminidase A and B (aka NAGA (N-acetyl-β,D-glucosaminidase), NAG, and NAGase)</td>
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SC-MN: Mannan derived from Saccharomyces

CTLD: C-type (calcium-dependent binding) lectin-like domain
cRGD: Cyclic arginine-glycine-aspartic acid (D)
DiR: DiIC18-indotricarbocyanine iodide-phycocerythrin
DSPE-PEG: 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol
FRI: Fluorescent reflectance imaging
huASM-MRC2: Mannose receptor C-type 2 cloned from human airway smooth muscle cells
MRC1: C-type mannose receptor 1 (CD206, macrophage mannose receptor)
MRC2: C-type mannose receptor 2 (CD280, Endo180, urokinase plasminogen activator receptor-associated protein)
OVA: Ovalbumin
PC200R: Provocative methacholine (methylcholine) concentration effecting a 200% increase in airway resistance
PC: Phosphatidylcholine
SC-MN: Mannan derived from Saccharomyces cerevisiae.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors wish to acknowledge Dr. Erik Bonten, Dr. Alessandra d’Azzo, and St. Jude Children’s Research Hospital Transgenic Core Facility for huASM-MRC2 transgenic mouse development; Dr. George C. Wood (UTHSC, Department of Pharmaceutical Sciences) for the liposome construction; Dr. Elizabeth A. Tolley (UTHSC, Department of Biostatistics and Epidemiology) for the statistical consultation; Histology Digitizing Service Core at UTHSC; and University of Tennessee Research Foundation-Cumberland Pharmaceuticals & Emerging Technologies (D. Betty Lew), NIH R01HL56812 and R41HL090108 (D. Betty Lew), NIH R01HL56812 and R41HL090108 (D. Betty Lew), University of Tennessee Clinical Translational Science Institute (D. Betty Lew).

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