

HEALTH AND MEDICINE

Nanoparticle-based thymulin gene therapy therapeutically reverses key pathology of experimental allergic asthma

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Despite long-standing efforts to enhance care for chronic asthma, symptomatic treatments remain the only option to manage this highly prevalent and debilitating disease. We demonstrate that key pathology of allergic asthma can be almost completely resolved in a therapeutic manner by inhaled gene therapy. After the disease was fully and stably established, we treated mice intratracheally with a single dose of thymulin-expressing plasmids delivered via nanoparticles engineered to have a unique ability to penetrate the airway mucus barrier. Twenty days after the treatment, we found that all key pathologic features found in the asthmatic lung, including chronic inflammation, pulmonary fibrosis, and mechanical dysregulation, were normalized. We conducted tissue- and cell-based analyses to confirm that the therapeutic intervention was mediated comprehensively by anti-inflammatory and antifibrotic effects of the therapy. We believe that our findings open a new avenue for clinical development of therapeutically effective gene therapy for chronic asthma.

INTRODUCTION

Asthma is one of the most common chronic pulmonary disorders, affecting more than 330 million people worldwide, but remains incurable despite decades of collective efforts to prevent, diagnose, and treat the disease in a timely manner (1). Current clinical guidelines recommend management of symptoms at best (2). However, advanced standard-of-care palliative options, such as anti-inflammatory agents and long-acting β_2 agonists, cannot effectively control disease progression as a stand-alone therapy, ultimately leading to progressive matrix changes (i.e., remodeling and/or fibrosis), a key histopathologic hallmark of asthma (3, 4). Alternatively, gene therapy is a potential means to intervene or even reverse the development of this life-threatening pathologic feature in asthmatic lungs by providing stable expression of therapeutic mediators (5).

We have demonstrated that a thymic nonapeptide, thymulin or serum thymus factor, is capable of preventing cascades of inflammatory and fibrotic responses in a mouse model of allergic asthma (6). However, the clinical relevance of this finding obviously lies in the ability of thymulin to provide modulatory effects in a therapeutic manner. Thus, we sought to evaluate whether thymulin-based gene therapy provides a clear therapeutic benefit when it is given after a preclinical asthma model is fully established in a sustained manner (Fig. 1A), unlike previous studies where animals were treated concomitantly with or before induction of disease.

Inhalation is the most straightforward method of administration for delivery of therapeutics, including gene therapy, to the lung. However, the mucus gel layer covering the lung airways is a critical barrier that hampers widespread distribution and long-term retention of inhaled therapeutics within the lungs (7, 8). Moreover, the mucus gel is further thickened in muco-obstructive lung diseases, such as asthma, which reinforces the barrier properties (7, 9, 10). We have previously developed a biodegradable nanoparticle-based gene delivery platform capable of efficiently penetrating the airway mucus barrier, thereby providing widespread and robust reporter transgene expression in mouse lungs (11). On the basis of this finding, we hypothesized that plasmids encoding a biologically active thymulin analog locally delivered by this mucus-penetrating particle would therapeutically reverse the lung inflammation and remodeling in a mouse model of chronic allergic asthma.

RESULTS

Physicochemical characterization of biodegradable nanoparticles carrying thymulin-expressing plasmids

The formation of compacted biodegradable nanoparticles carrying thymulin-expressing plasmids was confirmed by transmission electron microscopy. Morphologic examination revealed spherically shaped nanoparticles (Fig. 1B), in agreement with our previous observation with identically prepared nanoparticles but carrying reporter-expressing plasmids (11). The hydrodynamic diameters of the nanoparticles were 52 ± 2.5 nm, with polydispersity index values of ~ 0.22 (Fig. 1C). We also confirmed that nanoparticles exhibited a near-neutral surface charge (i.e., ζ -potential of 0.3 ± 1.9 mV), indicating that surface polyethylene glycol (PEG) corona effectively shielded positive charges originating from the core cationic poly(β -amino ester) (PBAE) polymers. We then conducted a conventional gel electrophoretic retardation assay to determine the plasmid packaging capacity. While free thymulin-expressing plasmids, as expected, drifted away from the well toward the cathode, the identical plasmids

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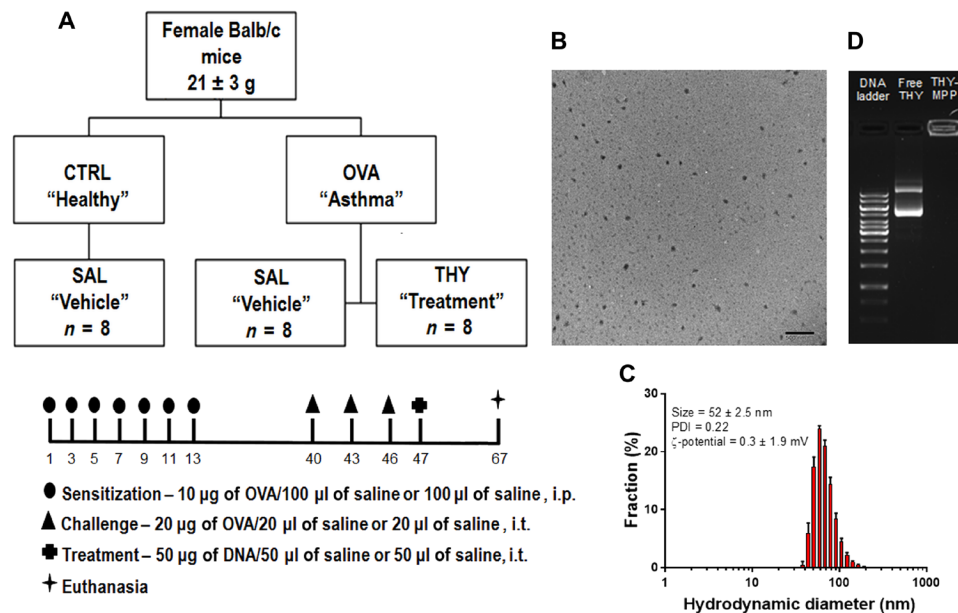


Fig. 1. Experimental design and thymulin-expressing nanoparticle characterization. (A) Experimental animal groups and timeline for “therapeutic” nanoparticle-based thymulin gene therapy study. Mice are intraperitoneally (i.p.) sensitized and intratracheally (i.t.) challenged. CTRL-SAL, mice sensitized and challenged with saline, intratracheally treated with saline on day 47 and sacrificed on day 67 (i.e., healthy animal control); OVA-SAL, mice sensitized and challenged with ovalbumin (OVA), intratracheally treated with saline on day 47 and sacrificed on day 67 (i.e., untreated asthmatic animal control); OVA-THY, mice sensitized and challenged with OVA, intratracheally treated with thymulin-expressing nanoparticles (50 µg of plasmids packaged within 1.2 mg of particle mass per mouse throughout the whole study) on day 47 and sacrificed on day 67. (B) Representative transmission electron micrograph of thymulin-expressing nanoparticles. Scale bar, 300 nm. (C) Physicochemical properties, including the particle diameters and surface charges, and (D) DNA compaction of thymulin-expressing nanoparticles. Free THY, free (i.e., unpackaged) thymulin-expressing plasmids; THY-MPP, thymulin-expressing plasmids packaged into DNA-loaded nanoparticles [i.e., mucus-penetrating particles (MPPs)]; PDI, polydispersity index.

packaged into the nanoparticles were fully retained in the well without a trace amount of migratory population, suggesting an excellent compaction (Fig. 1D).

Pulmonary alterations perpetuate in OVA murine model

It is critical to confirm that key pathophysiologic features are fully established and retained in the model throughout the study to evaluate the therapeutic, rather than prophylactic, effects of an applied therapy. Thus, we sought to confirm that our chronic mouse model of allergic asthma perpetuated pathologically driven morpho-functional alterations over time (a 20-day period) by a first set of comprehensive biochemical and functional analyses. The model was induced in mice by a series of sensitizations and challenges with ovalbumin (OVA) (fig. S1A; OVA group) and compared to healthy control mice that received saline using an identical schedule (CTRL group). Specifically, asthma-associated pathologic features, including asthmatic inflammation, pulmonary fibrosis, and mechanical defect, were analyzed by quantitatively assessing the inflammatory cell influx, collagen fiber content, and airway hyperresponsiveness (AHR), respectively, 1 day after the last OVA challenge (day 47) and 20 days later (day 67).

We found that the total leukocyte and eosinophil counts were more than doubled in the model compared to the healthy mice (i.e., CTRL group) at days 47 and 67 ($P < 0.05$; fig. S1A), and the levels were virtually identical in the model at these two time points. Similarly, collagen fiber content and AHR (as determined by the methacholine challenge test) were both stably elevated in the model at days 47 and 67 relative to the control healthy mice ($P < 0.05$; fig. S1, B and C). These findings suggest that asthma-associated pulmonary fea-

tures persist and do not self-resolve in the model at least over this 20-day period.

Lung histology

We then evaluated whether a single intratracheal dose of nanoparticles carrying thymulin-expressing plasmids (50 µg of plasmids packaged within 1.2 mg of particle mass) provided therapeutic benefits in the allergic asthma model. Lung morphometric analysis of OVA-challenged mice treated with saline (OVA-SAL group) demonstrated a significant increase in the fractional area of alveolar collapse compared to healthy control mice receiving saline as a sham treatment (CTRL-SAL group) ($P < 0.05$; Table 1), in good agreement with previous observations (12). However, the alveolar collapse induced by OVA was significantly reduced in the lungs of mice treated with the thymulin-expressing nanoparticles (i.e., OVA-THY group) to the level comparable with that of healthy mice ($P < 0.05$; Table 1). In addition, the contraction index (CI; degree to which the airway diameter is constricted) was normalized in mice in the OVA-THY group compared to those in the OVA-SAL group which exhibited significantly increased index values compared to healthy mice ($P < 0.05$; Fig. 2A and Table 1).

Another remarkable feature of chronic allergic asthma is mucus accumulation (13). In agreement with a previous study (14), we found a significant increase in the mucus score in the OVA-SAL group ($P < 0.05$), but the value was markedly reduced in the OVA-THY group back to the levels observed with healthy mice ($P < 0.05$; Table 1). We also analyzed two key hallmark readouts of lung remodeling, including collagen fiber and α -smooth muscle actin content; both were significantly increased in the OVA-SAL group compared

Table 1. Lung histology results demonstrating alveolar collapse, the contraction index, mucus score, tissue cellularity, collagen fiber content, and α-smooth muscle actin (n = 8 mice per group).

Groups	Alveolar collapse (%)	Contraction index	Mucus score	Tissue cellularity (%)		Collagen fibers (%)		α-smooth muscle actin (%)	
				MN	PMN	Airways	Alveolar septa	Alveolar duct	Terminal bronchiole
CTRL-SAL	3.3 ± 1.5	2.6 ± 0.07	0.0 ± 0.0	29.6 ± 3.0	1.2 ± 0.6	13.1 ± 4.0	1.7 ± 1.2	1.8 ± 0.4	2.0 ± 1.0
OVA-SAL	25.9 ± 1.9*	3.6 ± 0.2*	2.7 ± 0.6*	23.9 ± 2.3*	12.1 ± 2.0*	36.8 ± 18.4*	5.8 ± 2.8*	8.6 ± 2.8*	10.1 ± 2.2*
OVA-THY	5.0 ± 1.2†	2.3 ± 0.4†	0.6 ± 0.4†	24.3 ± 2.2	2.0 ± 0.7†	13.0 ± 3.2†	2.1 ± 1.6†	4.7 ± 1.9†	5.2 ± 1.6†

CTRL-SAL, mice sensitized and challenged with saline, intratracheally treated with saline on day 47 and sacrificed on day 67; OVA-SAL, mice sensitized and challenged with OVA, intratracheally treated with saline on day 47 and sacrificed on day 67; OVA-THY, mice sensitized and challenged with OVA, intratracheally treated with thymulin-expressing nanoparticles (50 μg of plasmids/50 μl) on day 47 and sacrificed on day 67; MN, mononuclear cells; PMN, polymorphonuclear cells.
*Statistically significant difference (P < 0.05) compared to the CTRL-SAL group. †Statistically significant difference (P < 0.05) compared to the OVA-SAL group.

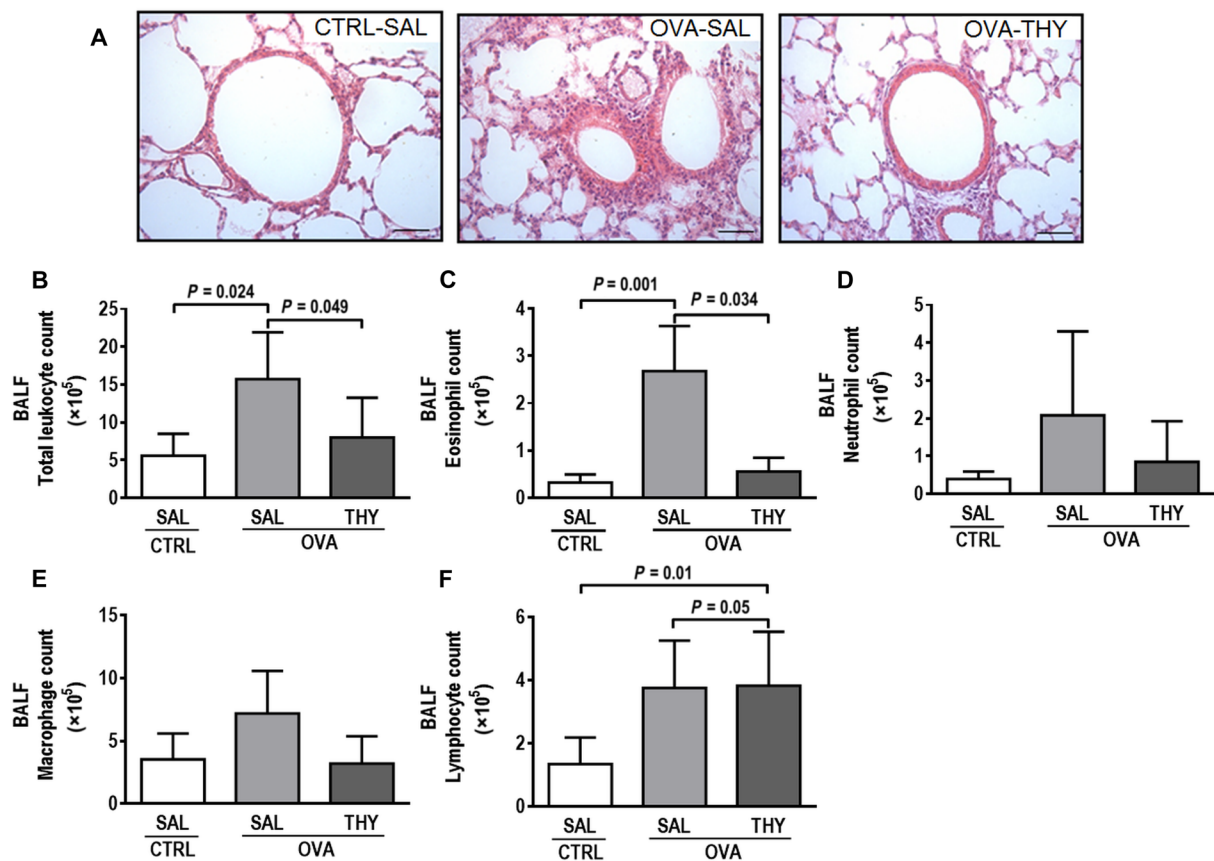


Fig. 2. Quantification of pulmonary cellularity in the bronchoalveolar lavage fluid. (A) Representative photomicrographs of airways in all experimental animal groups histologically stained with hematoxylin and eosin. Scale bars, 200 μm. Cellularity in the BALF: counts of (B) total leukocytes, (C) eosinophils, (D) neutrophils, (E) macrophages, and (F) lymphocytes (n = 6 mice per group).

to healthy mice ($P < 0.05$; Table 1), presumably by the constant pro-inflammatory stimuli inherent to the allergic asthma (15). All these pathologic perturbations were significantly alleviated in the OVA-THY group with the collagen fiber content in both airway and alveolar compartments normalized close to the values observed in the CTRL-SAL group (Table 1).

Mechanism of action in the inflammatory process
Tissue and BALF cellularity

As expected in the model of OVA-induced asthmatic inflammation (16), histological analysis revealed that the number of polymorphonuclear cells in lung tissue was significantly greater in the OVA-SAL group compared to the CTRL-SAL group ($P < 0.05$; Table 1.

However, this pathologically driven immune cell infiltration was markedly diminished in the OVA-THY group toward the level observed with healthy mice ($P < 0.05$; Table 1).

We also quantified the counts of total leukocytes and various immune cells in bronchoalveolar lavage fluid (BALF). There were significant increases in total leukocyte count and eosinophil population, the main cell type involved in the initiation of chronic allergic airway inflammation process, in the OVA-SAL group compared to the healthy CTRL-SAL group ($P < 0.05$; Fig. 2, B and C). However, both readouts were fully normalized in the OVA-THY group (Fig. 2, B and C). Neutrophil and macrophage counts exhibited similar trends to the eosinophil count, but the differences between CTRL-SAL and OVA-SAL groups were not statistically significant (Fig. 2, D and E). Lymphocyte count was elevated in the model relative to the control healthy mice ($P < 0.05$; Fig. 2F), which was not normalized upon the treatment with thymulin-expressing nanoparticles (Fig. 2F).

Immune cell-recruiting chemokines

To complement the cellularity analysis (Fig. 2), we quantified various immune cell-recruiting chemokines present in the BALF harvested from mice in different treatment groups. Specifically, we measured the levels of chemokine C-C motif ligand 11 (CCL11) (i.e., eotaxin-1), C-X-C motif ligand 1 (CXCL1), and CCL5, which drive the recruitment of eosinophils, neutrophils, and macrophages, respectively (17–19). We found that while both CCL11 and CXCL1 were significantly increased in the disease model compared to the healthy control, the levels were completely normalized upon the treatment with a single intratracheal dose of thymulin-expressing nanoparticles ($P < 0.05$; Fig. 3, A and B). The CCL5 levels were virtually identical among different groups (Fig. 3C).

Increase in lymphocyte population in the model is previously reported (14, 16) and somewhat expected from the critical roles of T helper 2 (T_H2) lymphocytes on the pathogenesis of human asthma (20). However, unlike other immune cells, the lymphocyte count remained high even after the treatment (Fig. 2F). To this end, we hypothesized that this phenomenon might reflect a phenotypic shift

from T_H2 toward regulatory T lymphocytes (T_{reg}) upon the treatment, given the well-established immunomodulatory functions of T_{reg} in various immune-perturbed pathological conditions, including asthma (21, 22). We thus quantified the BALF levels of CCL17, one of the key chemoattractants that induce T_{reg} transmigration (23). The CCL17 level was not altered by the OVA-induced asthma per se but significantly elevated when the mice received the thymulin-expressing nanoparticles ($P < 0.05$; Fig. 3D).

Pro- and anti-inflammatory mediators

To further investigate the impact of the nanoparticle-based thymulin gene therapy on asthmatic inflammation observed in the model, we next quantified the main mediators associated with the pro-inflammatory T_H2 response, including interleukin-4 (IL-4) and IL-13, as well as the anti-inflammatory cytokine IL-10 (24, 25) in BALF. Although the differences in the IL-10 levels were not statistically significant between the groups, all the T_H2 mediators were significantly increased in the OVA-SAL group compared to the CTRL-SAL group and were reduced in the OVA-THY group back to normal levels ($P < 0.05$; Fig. 4, A to C).

Remodeling factors

It is well established that vascular endothelial growth factor (VEGF) and transforming growth factor- β (TGF- β) play critical roles in the remodeling observed in the lungs of patients with asthma (26) and in OVA-based allergic asthma models (27). We thus investigated whether a single intratracheal dose of thymulin-expressing nanoparticles was capable of therapeutically modulating these profibrotic mediators. We noticed that the levels of both VEGF and TGF- β were significantly increased in the OVA-SAL group compared to the CTRL-SAL group but reverted to normal levels after the treatment ($P < 0.05$; Fig. 4, E and F).

Lymphocyte mediators and macrophage polarization

We next conducted reverse transcription polymerase chain reaction (RT-PCR) analysis of lymphocytes extracted from BALF to further elucidate their involvement in OVA-induced asthma and thymulin gene therapy. Consistent with our observation with the whole BALF analysis (Fig. 4), the levels of IL-13 in lymphocytes were increased in the model (OVA-SAL group) but fully normalized after the treatment with thymulin-expressing nanoparticles (OVA-THY group) ($P < 0.05$; Fig. 5A). The level of IL-4 in lymphocytes trended in a similar manner, but the difference was not statistically significant (Fig. 5A). Unlike the whole BALF analysis, we found that the level of lymphocytic IL-10 was significantly reduced in the model but normalized by the treatment with thymulin-expressing nanoparticles ($P < 0.05$; Fig. 5A). We also quantified the lymphocytic Foxp3 transcription factor, which is constitutively expressed in T_{reg} (28) and is responsible for suppressing the T_H2 response in allergic asthma (29). Similar to our observation with IL-10, the level of Foxp3 was significantly decreased in the OVA-SAL group, but the level was fully restored after the treatment with thymulin-expressing nanoparticles (OVA-THY group) ($P < 0.05$; Fig. 5A).

Macrophage polarization has been shown to pose a profound impact on the pathogenesis of allergic asthma (30). Specifically, M2 macrophages have increasingly been thought to contribute to the asthmatic T_H2 response (31, 32). We thus assessed the levels of cytokines and/or markers positively correlated with M1 and M2 macrophage phenotypes, including macrophage inflammatory protein 1 (MIP1) and arginase, respectively, in macrophages isolated from the harvested lung tissues via RT-PCR. We found that the MIP1 level, while negligible in the OVA-SAL group similar to healthy mice,

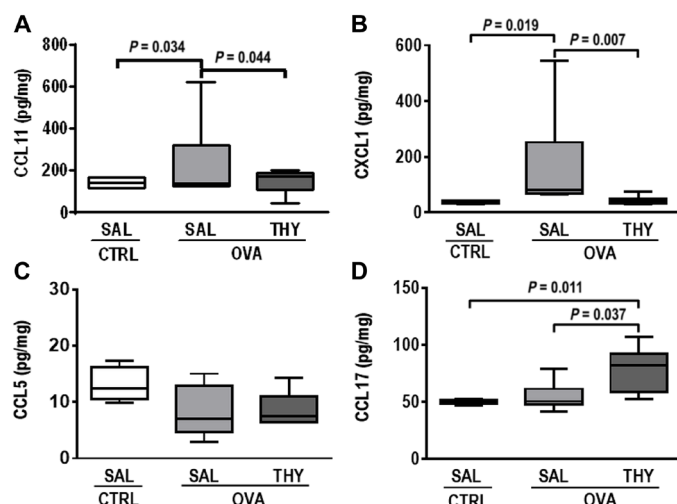


Fig. 3. Quantification of immune cell-recruiting chemokines in the BALF. The levels of (A) CCL11, (B) CXCL1, (C) CCL5, and (D) CCL17 quantified by enzyme-linked immunosorbent assay (ELISA) ($n = 6$ mice per group). CCL11, CXCL1, CCL5, and CCL17 represent specific chemokines that recruit eosinophils, neutrophils, macrophages, and T_{reg} lymphocytes, respectively.

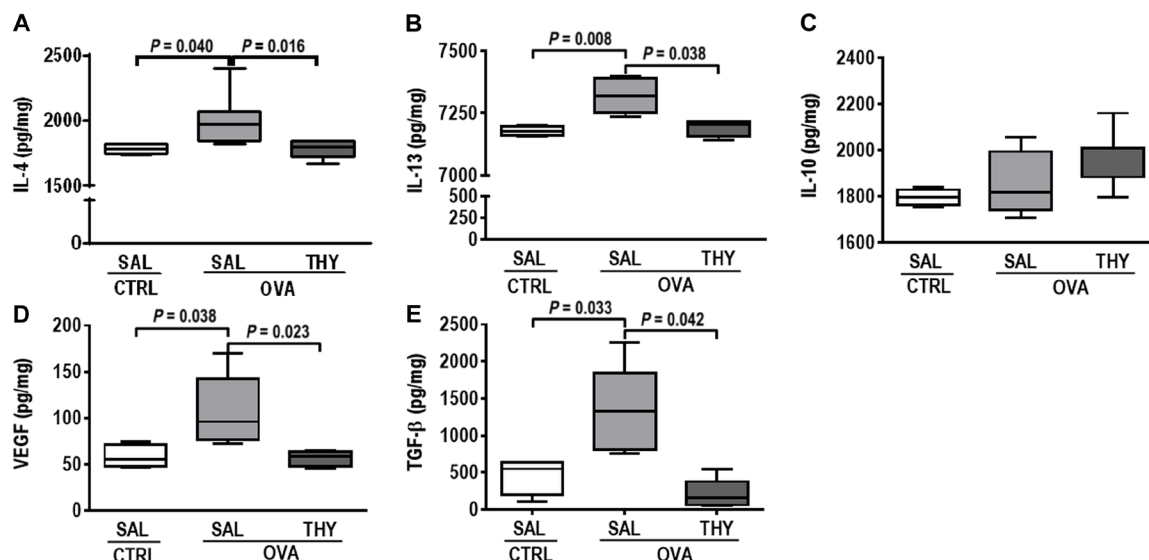


Fig. 4. Quantification of asthma-related mediators in the BALF. The levels of pro-inflammatory T_H2 cytokines, including (A) IL-4 and (B) IL-13, (C) an anti-inflammatory cytokine, IL-10, and profibrotic cytokines, including (D) VEGF and (E) TGF- β , were quantified by ELISA ($n = 6$ mice per group).

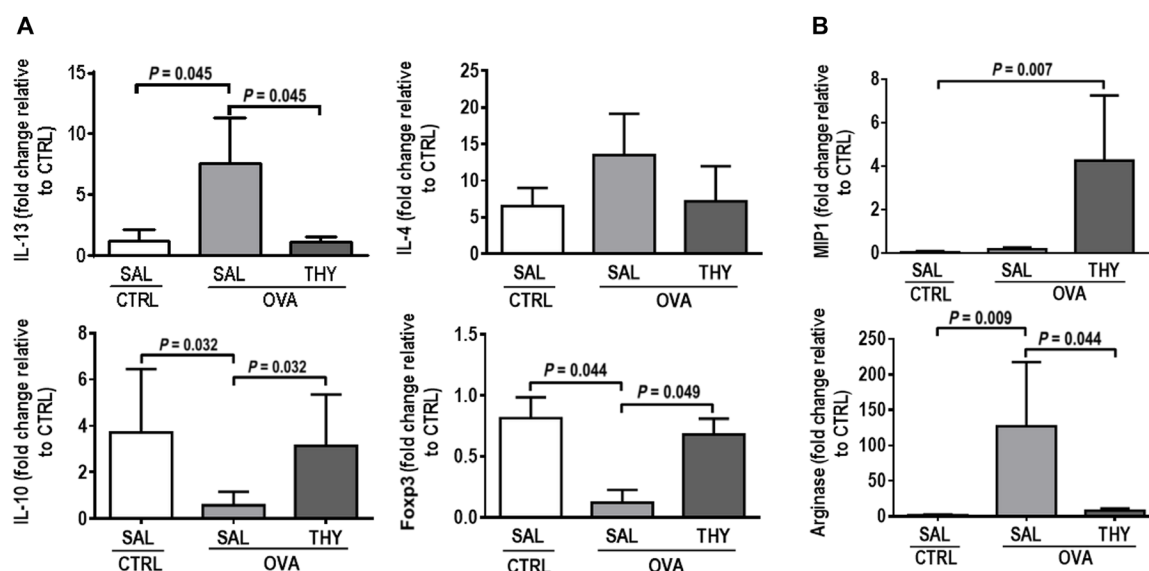


Fig. 5. Quantification of asthma-related mediators produced by pulmonary lymphocytes and macrophages. The levels of (A) IL-13, IL-4, IL-10, and Foxp3 produced by lymphocytes extracted from the BALF and (B) MIP1 and arginase produced by macrophages extracted from the lung tissues were quantified by RT-PCR ($n = 4$ mice per group).

was significantly increased upon the treatment with thymulin-expressing nanoparticles ($P < 0.05$; Fig. 5B). In contrast, the level of arginase significantly increased in the model but was restored to the normal level in the OVA-THY group ($P < 0.05$; Fig. 5B).

Pulmonary function

We next conducted the methacholine challenge test, which is commonly performed in the clinic to evaluate the lung function of patients with asthma (33). Of note, dose-dependent increase in airway resistance and decrease in dynamic compliance (C_{dyn}) are more pronounced in asthmatic lungs undergoing the fibrotic process (34), indicating AHR. We again confirmed that mice in the OVA-SAL

group exhibited a significant increase in airway resistance (Fig. 6A) and a decrease in C_{dyn} (Fig. 6B), primarily at higher doses, compared to the CTRL-SAL group ($P < 0.05$). However, mice in the OVA-THY group that received a single intratracheal dose of thymulin-expressing nanoparticles exhibited a response that was virtually identical to that in healthy mice (CTRL-SAL group) ($P < 0.05$; Fig. 6, A and B).

DISCUSSION

Built on our previous proof-of-concept study in which we demonstrated preventive roles of thymulin gene therapy (6), here, we showed

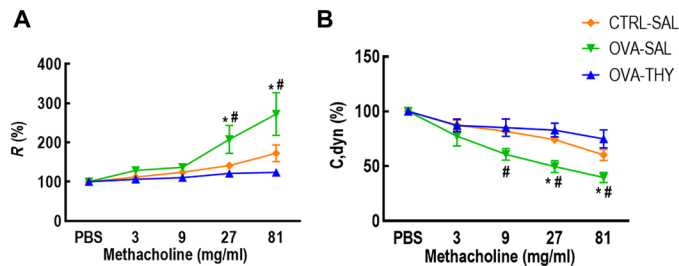


Fig. 6. Measurement of AHR. The changes in (A) airway resistance (R) and (B) dynamic compliance (C, dyn) of the lungs from animals in different groups in response to challenge by incrementing methacholine concentrations ($n=8$ mice per group). *Statistically significant differences ($P < 0.05$) compared to the CTRL-SAL group; #statistically significant differences ($P < 0.05$) compared to the OVA-SAL group.

that chronically established allergic asthma could be cured precisely in a therapeutic manner, which is rare in studies reported thus far. Specifically, we demonstrated that a single intratracheal dose of mucus-penetrating particles carrying thymulin-expressing plasmids normalized all the key pathologic features stably established in asthmatic lungs, including chronic inflammation, pulmonary fibrosis, and mechanical perturbation. We confirmed that all the main characteristics present in both airway and alveolar compartments of the current model (12) were fully retained, when unperturbed, throughout the study period with no self-resolution. These findings highlight that the thymulin-mediated intervention observed in this study was indeed therapeutic rather than preventive.

We tested a novel delivery platform that we had recently shown to provide a markedly greater ability to mediate reporter transgene expression in mouse lungs compared to a clinically tested system (11) that was used in the aforementioned prophylactic study (6). The dose of thymulin-expressing plasmids applied here (50 μ g), despite being half the dose used in the prophylactic study, was sufficient to therapeutically normalize all the monitored pathologic features associated with allergic asthma, presumably because of the superior ability of our nanoparticles to mediate transgene expression in the lung (11). Comprehensive therapeutic effects lasted at least up to 20 days, despite the short-acting nature of the cytomegalovirus (CMV) promoter that controlled thymulin transgene expression in this study (6). However, the current model is limited in that mice are not continuously exposed to allergen as in human asthma. To this end, further validation using a model with repeated allergen challenge is warranted for clinical development of our approach. Encouragingly, we have demonstrated that a single intratracheal dose of our nanoparticles mediates pulmonary reporter transgene expression at least up to 4 months when a eukaryotic promoter is used (11), which underscores the potential for long-term therapeutic benefits.

As expected from the critical roles of eosinophils in mediating allergic inflammation in asthma (35), we found that pulmonary infiltration of eosinophils primarily drove the increase in the overall leukocyte counts in the lungs of the asthma model. However, we found that gene therapy with thymulin-expressing nanoparticles reduced the eosinophil counts back to the normal level, suggesting that the therapy effectively blocked their infiltration into the lungs. This observation is likely due to the reduction in IL-4, IL-13, and VEGF, which facilitates transmigration of circulating eosinophils into the asthmatic lungs (36, 37). Concomitantly, normalization of the CCL11 level by the therapy likely contributed to blockade of eosinophil recruitment, given that the chemokine serves as a primary

chemoattractant to eosinophils (37, 38). It has been previously reported that eosinophil-deficient mice are notably protected from airway collagen deposition and smooth muscle accumulation despite the challenge by OVA (39). In addition, eosinophil count and AHR are directly correlated in asthmatic lungs (40, 41). Thus, thymulin-mediated reduction of eosinophils likely played a key role in therapeutically reverting the fibrotic responses and mechanical dysfunction observed in the OVA-challenged mouse lungs.

The neutrophil counts in the airway secretions from patients with mild and moderate asthma are comparable to those in the secretions from normal individuals; however, it has been previously demonstrated that the count is particularly elevated in the lungs of patients with severe asthma (42, 43) and induces profibrotic epithelial-to-mesenchymal transition in bronchial epithelial cells (44). We found that neutrophil-recruiting CXCL1 and profibrotic mediators were significantly elevated in the model but reduced back to the levels on par with healthy mice upon the treatment with a single dose of thymulin-expressing nanoparticles. Likewise, the neutrophil count trended similarly, although the increase in the count in the model was not statistically significant presumably because of the variation among individual mice. The findings here suggest that our therapeutic approach, upon clinical development, may benefit a broad spectrum of patients with asthma.

Accumulated evidence highlights the role of T_{reg} on maintaining immunologic homeostasis against pathologic immune perturbation, such as asthmatic T_H2 responses (40, 41). In this study, RT-PCR-based analysis of cytokines and a transcription factor produced by the BALF lymphocytes revealed that inhaled thymulin gene therapy mediated a clear phenotypic shift from pathological T_H2 subtype to therapeutic T_{reg} . On the basis of previous reports suggesting the general role of thymulin on intra- and extrathymic T lymphocyte differentiation and maturation (45), we initially speculated that thymulin might have induced differentiation of migratory naïve lymphocytes to T_{reg} in the lung. However, it is more likely that thymulin-expressing nanoparticles induced transmigration of T_{reg} from local and/or systemic lymphoid organs, because the T_{reg} -recruiting CCL17 level was significantly elevated by the treatment. One concern may be a potential susceptibility to infection by pathogens due to the thymulin-mediated domination of T_{reg} . However, T_{reg} response observed in this study is most likely specific to OVA and thus may not affect effector functions against pathogens (46, 47). Furthermore, lymphocytic immune response against inhaled pathogens is primarily mediated by T_H1 lymphocyte (48), which is unlikely perturbed by our therapeutic approach. To the contrary, we found that the inhaled thymulin gene therapy mediated M1 polarization of macrophages, potentially favoring T_H1 response upon pathogen invasion (49).

We found that thymulin gene therapy abrogated the M2-biased macrophage phenotype in the OVA-challenged asthmatic lungs, while the difference in the macrophage count between the induction of disease and therapeutic intervention was not significant. It is well established that lymphocytes function in coordination with other immune cells, including macrophages (50). Thus, thymulin-mediated phenotypic deviation of macrophages from the M2 phenotype may be attributed to a reduction in M2-inducing T_H2 cytokine production and release by lymphocytes. However, thymulin may have directly acted on macrophages, given that macrophages have previously been shown to respond differently to thymulin depending on the pathologic state in vitro (50, 51). In summary, we demonstrate

here that inhaled thymulin gene therapy by mucus-penetrating particles therapeutically reverses key biochemical, histologic, and functional disease phenotypes of allergic asthma via direct and/or indirect modulation of various immune cells, including eosinophils, neutrophils, lymphocytes, and macrophages.

MATERIALS AND METHODS

Experimental design

This animal study was approved by the Animal Ethics Committee of the Health Sciences Centre, Federal University of Rio de Janeiro (Universidade Federal do Rio de Janeiro or UFRJ). To establish a mouse model of allergic asthma, female BALB/c mice (20 to 25 g) were first sensitized by intraperitoneal injection of OVA (10 μ g in 0.1 ml of saline; Sigma-Aldrich, St. Louis, MO, USA) every other day up to seven injections. Forty days after the beginning of sensitization, intratracheal OVA challenges were performed (20 μ g in 20 μ l of saline) three times with 3-day intervals between the applications (12). In parallel, the control mice received saline instead of OVA during both sensitization and challenge. To confirm that a stable disease model was established, BALF and lung tissue were harvested from each animal 1 day (day 47) or 21 days (day 67) after the last OVA (OVA group) or saline (CTRL group) challenge for further analyses.

To assess the therapeutic efficacy of thymulin-expressing nanoparticles, the asthmatic and control animals were established by the aforementioned protocol. Twenty-four hours after the last challenge (day 47), individual animals were randomly assigned to different groups and treated with 50 μ l of nanoparticles (carrying 50 μ g of thymulin-expressing plasmids; OVA-THY group) or saline (CTRL-SAL or OVA-SAL groups) intratracheally using a microsprayer (MicroSprayer Aerosolizer Model IA-1C; Penn-Century). Twenty days after the treatment (day 67), animals were euthanized, and BALF and lung tissues were harvested for further analyses (Fig. 1A).

Preparation and physicochemical characterization of DNA-loaded nanoparticles

Biodegradable cationic polymer, PBAE, and PEG-conjugated PBAE (PEG-PLGA) copolymer were prepared, as described previously (11). Briefly, PBAE polymers were synthesized by a two-step Michael addition, and PEG-PBAE polymers were prepared by chemical conjugation of methoxy PEG succinimidyl succinates (5 kDa; JenKem Technology, Plano, TX, USA) to the terminal primary amine groups at both ends of the PBAE polymers. Thymulin-expressing plasmids (fig. S2), driven by the CMV promoter, were transformed into and propagated in DH5 α -competent *Escherichia coli* cells and subsequently extracted using EndoFree Plasmid Giga Kit (Qiagen, Hilden, Germany). DNA-loaded nanoparticles were formulated by dropwise addition of five equivalent volumes of a thymulin-expressing plasmid solution (0.1 mg/ml) to one equivalent volume of polymer solution containing a mixture of PBAE (12 mg/ml) and PEG-PBAE (63 mg/ml) and subsequently purified/concentrated by centrifugation (11). The resultant nanoparticles were characterized for morphology, hydrodynamic diameter, polydispersity index, and surface charge (i.e., ζ -potential) using a transmission electron microscope (H7600; Hitachi, Japan) or a Zetasizer (Nano ZS90; Malvern Instruments, Southborough, MA, USA). In addition, we conducted a conventional gel electrophoretic migration assay to confirm the compaction of thymulin-expressing plasmids within the nanoparticles.

AHR parameters

At day 47 or 67, AHR was assessed as a change in airway function after the application of aerosolized methacholine in a FinePoint R/C Buxco Platform (Buxco Electronics, Sharon, CT, USA), as described previously (52). Animals were sedated [diazepam (1 mg/kg), intraperitoneally], anesthetized [thiopental sodium (20 mg/kg), intraperitoneally], tracheotomized, and paralyzed [vecuronium bromide (0.005 mg/kg), intravenously] in a sequential manner. Airflow and transpulmonary pressure were recorded using a Buxco Pulmonary Mechanics Processing System (Buxco Electronics, Wilmington, NC, USA). Airway resistance (cm H₂O/ml/s) and lung elastance (cm H₂O/ml) in each breath cycle were calculated with a Buxco system. Analog signals from the computer were digitized using a Buxco analog/digital converter (Buxco Electronics). Mice were allowed to stabilize for 5 min, and increasing concentrations of aerosolized methacholine (3, 9, 27, and 81 mg/ml) were administered for 5 min at each concentration. The outcome measures comprised the mean absolute values of the responses collected during 5 min after each administration of methacholine.

Lung histology

The animals received intravenous heparin (1000 IU) and were then exsanguinated. The lungs were removed, fixed, and embedded in paraffin. Tissue sections (4 μ m) were prepared and stained with hematoxylin and eosin. Morphometric analysis was performed using an integrated eyepiece with a coherent system consisting of a grid with 100 points and 50 lines of known length coupled to a conventional light microscope (Axioplan, Zeiss, Oberkochen, Germany). The volume fraction of the lung occupied by collapsed alveoli (alveoli with rough or plicate walls) or normal pulmonary areas (at a magnification of $\times 200$) and the percentage of mononuclear and polymorphonuclear cells in pulmonary tissue (at a magnification of $\times 1000$) were determined by the point-counting technique across 10 randomly selected and nonoverlapping microscopic fields (53).

To determine the CI, we used a microscope equipped with a specialized eyepiece lens on which parallel horizontal lines are drawn, as previously described (54). Briefly, the number of lines overlaid at least partially with epithelial basal membrane [i.e., number of intercepts (NI)] and the number of line-edge points overlaid within the airway lumen (NP) were counted at a magnification of $\times 400$, and the magnitude of bronchoconstriction (i.e., CI) was computed using the equation, $CI = NI/\sqrt{NP}$. Measurements were performed in five airways from each animal in a blinded manner.

Collagen fiber content was quantified using a digital analysis system and image processing software (Image-Pro Plus 5.1 for Windows; Media Cybernetics, Silver Spring, MD, USA). The presence of mucus-filled cells in each airway was revealed by periodic acid-Schiff staining and scored on a scale of 0 to 4. The average value obtained from 6 to 10 airways was taken as the overall mucus score per mouse.

Strips (2 mm by 2 mm by 10 mm) from the right lung were fixed and embedded in paraffin for immunohistochemical analysis using a monoclonal antibody (Ab) against α -smooth muscle actin (Dako, Carpinteria, CA, USA) at a 1:500 dilution. Using a 121-point grid, the volume proportion of α -smooth muscle actin was calculated as the ratio of points overlaid with actin-stained versus unstained tissues. We note that all the histologic analyses described here were performed in a blinded manner.

Total and differential cell counting

Mice were euthanized with a lethal dose of pentobarbital sodium, and the tracheas were immediately cannulated. Cell influx into the airway lumen and alveolar space was quantified by counting cells recovered from BALF. BALF was obtained by flushing the airways three times with 0.4 ml of 1% phosphate-buffered saline (PBS) solution and retrieving the fluid by gentle aspiration. BALF was then centrifuged (239g, 10 min), and the cell pellet was resuspended in PBS. Total leukocyte values were obtained by diluting the cells in Türk solution followed by counting in a Neubauer chamber using an optical microscope. The differential cell counting of eosinophils, neutrophils, lymphocytes, and macrophages recovered from BALF was conducted by staining cells using a commercial kit (Panótico Rápido LB, Pinhais, RS, Brazil), followed by calculating the percentage of each cell type per 100 cells.

Quantification of cytokines, chemokines, and growth factors

The levels of cytokines, including IL-4 (PeproTech, Rocky Hill, NJ, USA), IL-13 (PeproTech), and IL-10 (PeproTech); chemokines, including CCL11 (PeproTech), CXCL1 (R&D Systems, Minneapolis, MN, USA), CCL5 (R&D Systems), and CCL17 (R&D Systems); and growth factors, including VEGF (PeproTech) and TGF- β (BioLegend, San Diego, CA, USA), in BALF from all animal groups were quantified by enzyme-linked immunosorbent assay (ELISA), as per the manufacturer's protocol.

Analysis of mediators produced by lymphocytes and macrophages

Lymphocytes and macrophages were isolated from BALF and lung tissues, respectively, using magnetic-activated cell sorting. Three mice in each condition were euthanized with an overdose of pentobarbital. Briefly, lung tissues were cut into small pieces and digested by collagenase 1% for 40 min with mild agitation. Proteolysis was halted by Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and the digested tissues were centrifuged at 400g at 4°C for 5 min. The pellet was resuspended in PBS containing biotinylated anti-CD3 Ab (lymphocytes) and anti-CD11b Ab (monocytes) on ice for 30 min. Cells were washed with PBS and then incubated with Dynabeads Biotin Binder (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction for 20 min on ice. Subsequently, cells were isolated by exposure to a magnetic field.

RT-PCR was performed to measure mRNA levels of IL-4, IL-10, IL-13, and Foxp3 in lymphocytes and MIP1 and arginase in macrophages. Cells were lysed for RNA extraction using the ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI, USA) as per the manufacturer's protocols. The total RNA concentration was measured by spectrophotometry using a NanoDrop ND-1000 system. First-strand complementary DNA (cDNA) was synthesized from total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Relative mRNA levels were measured with a BRYT Green detection system (Promega, Fitchburg, WI, USA) using PCR Mastercycler ep Realplex Eppendorf (Eppendorf, Hamburg, Germany). All samples were measured in duplicate. The relative mRNA level of each gene was normalized to that of the housekeeping gene (acidic ribosomal phosphoprotein P0 or 36B4) and presented as the fold change relative to the CTRL-SAL group, using the $2^{-\Delta\Delta Ct}$ method, where $\Delta Ct = Ct$ (target gene) – Ct (housekeeping gene).

Statistical analysis

The sample size calculation for testing the primary hypothesis (thymulin gene therapy reverses allergic asthma) was based on previous measurements made by us (12) and on pilot studies. Accordingly, a sample size of six animals per group (considering one animal as dropout) would provide the appropriate power ($1 - \beta = 0.8$) to identify significant ($\alpha = 0.05$) differences between mice in OVA-SAL group and those treated with thymulin gene therapy (OVA-THY group), taking into account an effect size $d = 1.9$, a two-sided test, and a sample size ratio = 1 (G*Power 3.1.9.2, University of Düsseldorf, Germany).

Statistical analyses were performed using one-way analysis of variance (ANOVA) or ANOVA on ranks followed by the Tukey test or Dunn test, respectively. Repeated-measures ANOVA was used to compare methacholine dose ranges. Parametric data were expressed as mean $\pm$ SD. All tests were performed using GraphPad Prism v6.00 (GraphPad Software, La Jolla, California, USA).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/6/24/eaay7973/DC1>

[View/request a protocol for this paper from Bio-protocol.](#)

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