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Long term effects:

Galectin-1 and specific immunotherapy for allergic responses in the intestine

Running title: Gal-1 enhances specific immunotherapy

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Abstract

Background and aims: Mast cell activation interferes with the effects of allergen-specific immunotherapy (SIT). Galectin-1 (Gal-1) is capable of regulating immune cells' functions. This study tests the hypothesis that administration of Gal-1 promotes and prolongs the efficacy of SIT via suppressing mast cell activation.

Methods: An intestinal allergy mouse model was developed. The co-administration of SIT and Gal-1 on suppression of the allergic responses, prevention of mast cell activation, and generation of antigen-specific regulatory T cells (Treg) in the intestine were observed in sensitized mice.

Results: The coadministration of Gal-1 and SIT markedly suppressed the allergic responses in the mouse intestine vs. the use of either SIT alone or Gal-1 alone. The Gal-1 binds to the IgE/FcεRI complexes on the surface of mast cells to prevent mast cell activation during SIT. Gal-1 promoted the SIT-generated allergen-specific Tregs in the intestine of sensitized mice. Coadministration of Gal-1 and SIT significantly enhanced the efficacy of immunotherapy in suppressing allergic responses in the intestine, which lasted for at least for 12 months.

Conclusions: Long term effects of specific immunotherapy on intestinal allergy can be achieved with Gal-1/SIT therapy by inhibiting mast cell activation and facilitating Treg development.

Keywords: Allergy; Mast cells; Immune regulation; Specific immunotherapy; Galectin-1.

Introduction

The prevalence of allergic diseases, including allergic asthma, allergic rhinitis, food allergy-relevant intestinal inflammation (1), and allergic dermatitis, etc., is more than 20% of the general population worldwide (2, 3). The aberrant T helper (Th) 2 polarization, including profound infiltration of Th2 cells in the local tissue and the Th2 cells producing high amounts of Th2 cytokines such as interleukin (IL) -4, IL-5, and IL-13 (4), is a significant pathological feature of allergy. Th2 cytokines play a critical role in the development of allergic disorders (5), such as IL-4 promoting the IgE isotype switching (6), IL-5 inducing eosinophil infiltration (7) and IL-13, compromising the establishment of immune tolerance (8). However, the regulatory effects on the aberrant Th2 polarization are still unsatisfactory at this point. Immunoglobulin (Ig) E is the major mediator in allergic responses. IgE binds the high affinity receptor, FcεRI, on the surface of mast cells to sensitize them (9). The sensitized mast cells are activated upon re-exposure to specific antigens to release chemical mediators that trigger allergic responses (9). A number of molecules are capable of preventing mast cell activation, such as cromoglicate; this is a mast cell stabilizer which can prevent its degranulation and inhibit allergic responses (10). Although mast cell stabilizers can efficiently inhibit allergic symptoms, the effects only last for a short time. An important

question is how to prolong and permanently maintain the inhibitory effects on the mast cell activation-initiated allergic responses; this will be further investigated.

Immune regulatory cells, including regulatory T cells (Treg) and regulatory B cells (Breg), can suppress other immune cell activities to maintain immune homeostasis in the body (11).

Tregs produce immunosuppressive molecules, such as transforming growth factor (TGF)- β and IL-10, which inhibit skewed immune responses (12), mast cell recruitment, and activation (13). Therefore, to generate Tregs can also inhibit immune disorders (14).

Although it is clear that Tregs can be generated in both humans and animals, to specifically generate the antigen-specific Tregs in an allergic environment still presents challenges (15, 16).

Published data indicate that galectins (Gal) promote the development of Tregs (17). Gal is a family of proteins characterized by their binding specificity for β -galactoside sugars, which can bind to proteins by N-linked or O-linked glycosylation. In total, 15 members have been defined in the Gal family (18). Most have immune regulatory functions (18), such as Gal-3, which facilitates Treg development (17). Gal-1 has immune regulatory ability, though previous studies have shown that Gal-1 can suppress mast cell transmigration in tissue and suppress mast cell degranulation in the context of allergic responses (19, 20). Whether this feature of Gal-1 improves the efficacy of antigen specific immunotherapy (SIT), it has not been investigated.

SIT introduces specific antigens in patients with allergic disorders. The generation of Tregs by SIT may contribute to downregulating expression of IgE, increasing IgG4, and suppressing skewed Th2 polarization (21). Since specific antigens are introduced in the body by SIT, the antigens inevitably bind to the specific IgE/Fc ϵ R1 complexes on the surface of mast cells to activate them (9). The activated mast cells release a number of chemical mediators to

regulate immune responses, such as causing Treg dysfunction (22). Mast cell-derived IL-4 (23) may interfere with the generation of Tregs by inhibiting gene transcription of the forkhead box protein 3 (Foxp3) (24). Thus, blocking mast cell activation during SIT may improve the therapeutic efficacy of SIT by increased generation of Treg. We tested this hypothesis by blocking mast cells with galectin-1 (Gal-1) during SIT in an intestinal allergy mouse model. Our results showed that Gal-1 significantly increased effects of SIT on inhibiting allergic response in the intestine and promoting generation of Tregs. Therapeutic effects lasted at least for 12 months in this study.

Materials and methods

(The materials and methods are presented in the supplemental materials).

Results

Gal-1 inhibits allergic response in the intestine

To test the role of Gal-1 in the inhibition of allergic responses in the intestine, we developed a food allergy mouse model. The mice were treated with Gal-1 or saline before the last challenge with the specific antigen, ovalbumin (OVA; Sigma Aldrich; St. Louis, MO); the mice were then sacrificed, as allergic responses in the intestine were evaluated. The results showed that besides inducing the general allergic responses (Fig. S1A-C in supplemental materials, including the enhancement of serum specific IgE, drop of core temperature, and diarrhea), allergy-relevant responses were induced in the intestine of sensitized mice, including enhancement of the intestinal epithelial short circuit current (Isc), permeability to macromolecular substances, infiltration of mononuclear cells (Fig. 1), and increases in Th2 cytokines in intestinal tissue (Fig. S1D-F), indicating allergic responses are induced in the

intestine. In mice treated with Gal-1, allergic reactions in that area were markedly suppressed (Fig. 1), with results demonstrating that the intestinal allergic system can be suppressed by the administration of Gal-1.

Gal-1 inhibits mast cell activation in the intestine

To understand the mechanisms by which Gal-1 inhibited allergic responses in the intestine, we observed effects of Gal-1 on inhibiting mast cell activation there as well. The administration of Gal-1 significantly reduced mast cell counts in intestinal tissue (Fig. S1G). The sera were collected from mice and analyzed by ELISA. The serum levels of histamine, the marker of mast cell activation, were enhanced in sensitized mice challenged with specific antigens, which were strongly reduced by the treatment with Gal-1 (Fig. 2A).

The data shown in Fig. 2A suggest that Gal-1 prevented the specific antigen, OVA, from binding sensitized mast cells in the intestine of sensitized mice. To test this, we generated bone marrow derived mast cells (BMMCs) to be primed with OVA-specific IgE; the cells were then exposed to OVA or OVA/Gal-1 in culture. As expected, exposure to OVA markedly induced mast cell activation as shown by release of β -hex into the culture supernatant, while exposure to OVA/Gal-1 did not (Fig. 2B). These results imply that Gal-1 may bind to the IgE/Fc ϵ RI complex on the surface of sensitized mast cells to prevent specific antigen OVA from binding to the complexes. We tested this hypothesis by collecting the BMMCs from the culture; the cell extracts of the BMMCs were prepared and analyzed by co-immunoprecipitation. We detected a complex of OVA/IgE/Fc ϵ RI in extracts of mast cells exposed to OVA, but not from those exposed to OVA/Gal-1 (Fig. 2C). Since the mitogen-activation protein kinase (MAPK p38) and nuclear factor (NF)- κ B are involved in mast cell

activation (25), we checked levels of MAPK, I- κ B, and NF- κ B in BMMCs. We found that exposure to a specific antigen, OVA, increased the levels of MAPK and NF- κ B and decreased levels of I- κ B in the sensitized BMMCs, which were abolished by the presence of Gal-1 in the culture (Fig. 2D-F).

Preventing mast cell activation by Gal-1 as required for Tregs in an allergic environment

Since induction of Tregs plays a critical role in the inhibition of allergic responses by specific immunotherapy (SIT) (26), and mast cell activation causes Treg dysfunction (22), we inferred that the prevention of mast cell activation during SIT might be critical to promote therapeutic effects on allergic issues. To this end, we treated OVA-sensitized mice with Gal-1/OVA or OVA alone. The frequency of CD4⁺ CD25⁺ Foxp3⁺ Treg was lower in sensitized mice than in naive control mice. Treating sensitized mice with OVA further reduced Tregs in the intestine and exposure to Gal-1/OVA significantly promoted generation of Tregs in the intestine, while treatment with Gal-1 alone did not (Fig. 3). To test if the antigen-specific Tregs were generated in the mice, we isolated CD4⁺ CD25⁺ CD127⁻ Tregs from lamina propria mononuclear cells (LPMCs) of sensitized mice treated with Gal-1/OVA. Tregs were cultured with dendritic cells in the presence of OVA for 3 days and analyzed by flow cytometry. Exposure to the specific antigen OVA induced Treg proliferation, indicating that the antigen-specific Tregs were generated in sensitized mice by exposure to Gal-1/OVA (Fig. S2).

Moreover, the effects of mast cell activation on the suppression of Treg generation were tested in an in vitro study. Naive CD4⁺ T cells were treated with the Treg-generating condition with or without the presence of the IgE-activated mast cells in the culture. The results showed that the presence of IgE-activated mast cells significantly suppressed

generation of Tregs, which was blocked by the presence of Gal-1 or cromoglicate (a stabilizer of mast cells). Treating CD4⁺ CD25⁻ T cells with OVA (the specific antigen) without the presence of mast cells did not induce Tregs (Fig. 4). Results indicate that mast cell activation interferes with generation of Tregs.

Gal-1 enhances the efficacy of SIT on allergic responses in the intestine

The data reported above suggest that Gal-1 may enhance the efficacy of the traditional SIT on allergic responses. To examine this, we developed a food allergy model with OVA and CT (cholera toxin; Sigma Aldrich) with the established procedures (27). From week 5, the sensitized mice were treated with SIT and/or Gal-1. One week after the treatment, the mice were rechallenged with specific antigen, OVA. The mice were sacrificed next day, and allergic feedback in the intestine was evaluated. The mouse body weight strikingly dropped in the SIT group. Mice in the Gal-1/SIT group gained weight constantly, similar to naive control mice. Mice treated with Gal-1 alone did not drop body weight (Fig. 5A). Heavy infiltration of mononuclear cells, mast cells, and eosinophils in the intestinal mucosa was observed in the SIT group, which was noticeably less in mice treated with the Gal-1/SIT therapy (Fig. 5B-D; Fig. S5F). Serum levels of histamine, the marker of mast cell degranulation, were much higher in the SIT group than the Gal-1/SIT group (Fig. 5E). The intestinal epithelial barrier permeability to dextran in the Gal-1/SIT group was much less than that in the SIT group (Fig. 5F). We treated OVA-sensitized mice with Gal-1/SIT and an anti-CD25 antibody (to inhibit Tregs); the allergic response in the intestine of the mice was similar to that treated with SIT alone (Fig. 5A-F). Treatment with Gal-1/SIT suppressed serum levels of specific IgE, enhanced serum specific IgG4, inhibited serum IL-4, and reduced systemic allergic responses, including core temperature drop, diarrhea, and induced

antigen-specific Tregs (Fig. S4); this was not accomplished by treating mice with SIT alone or Gal-1 alone (Fig. S5).

Gal-1/SIT therapy prolongs the inhibitory effects on allergic responses in the intestine

To determine if the efficacy of Gal-1/SIT on inhibiting allergic responses in the intestine last for longer time periods, we sensitized mice with OVA/CT, and treated them with SIT and/or Gal-1 for two weeks. The mice were maintained in the routine living condition for 12 months. The mice were then challenged with specific antigen OVA via gavage-feeding, and were sacrificed 24 h later. As analyzed with the procedures above, mice treated with SIT alone showed apparent allergic responses in the intestine, high levels of serum histamine, specific IgE and serum IL-4, as well as a lower core temperature, with diarrhea. Mice treated with the Gal-1/SIT therapy showed no apparent allergic reaction in the intestine, no detectable serum IgE, and no systemic allergic response after OVA challenge, while mice treated with Gal-1 had similar results to those treated with SIT alone (Fig. 6; Fig. S6). Specific Tregs were detected in mice treated with Gal-1/SIT therapy (Fig. S7), while depletion of Tregs abolished the effects of immunotherapy (Fig. 6, Fig. S6). Results indicate that compared to the traditional SIT, the Gal-1/SIT therapy can inhibit intestinal allergic responses, which can last for at least 1 year.

Discussion

To achieve long-term therapeutic efficacy on allergic diseases is an essential goal of immunotherapy. The present study reached this goal in an animal model study. By treating sensitized mice with the Gal-1/SIT therapy, the allergic reactions were inhibited. The most significant finding of this study is that the Gal-1/SIT therapy lasted for one year, which was

not achieved with SIT alone. The data show that to inhibit mast cell activation, we must gain favorable results of SIT. In sensitized subjects, mast cells are sensitized by binding IgE antibodies. Re-exposure to specific antigens can activate sensitized mast cells to elicit release of allergic mediators (9). This study showed that re-exposure to the specific antigen OVA, levels of serum histamine, the marker of mast cell activation, were significantly increased. This phenomenon was prevented by administration of Gal-1 with SIT, which had positive therapeutic effects on inhibition of allergic responses in the intestine.

The present data show that the Gal-1/SIT therapy inhibits mast cell activation in mice with intestinal allergy via binding to the FcεRI/IgE complexes on the surface of mast cells, which are the major effector cells in allergic responses. Substances with the capacity to prevent mast cell activation can be medicinal to treat allergic diseases, such as the mast cell stabilizer disodium cromoglycate well inhibits allergic responses (28). Yet, the inhibitory effects of mast cell stabilizers on allergic response are temporary. The present data show that administration of Gal-1 alone also has the effects of inhibiting mast cell activation, which lasted only a short time. However, the combination of the Gal-1 and SIT significantly prolonged the inhibitory time of allergic responses as shown by the present data.

The data show that the Gal-1/SIT therapy generates the antigen-specific Tregs in the allergic environment. The effects of the Gal-1/SIT therapy were abolished by depleting Tregs. To generate antigen-specific Tregs is one approach to restore immune tolerance to alleviate immune disorders (29). Gal-1 is one of the inducers of Treg (30). The present data are in line with the previous studies by showing that administration of Gal-1 promoted the induction of antigen-specific Tregs in the intestine. Without the presence of Gal-1, SIT apparently did not

induce Tregs in the intestinal mucosa of mice with intestinal allergy. This may be attributed to SIT activating the sensitized mast cells to release the allergy relevant mediators for prevention of the induction of Tregs. The inference is supported by the present data, as the serum levels of mast cell-derived mediators were still higher in mice treated with SIT. Other investigators also found that sensitized mast cells were activated to release Th2 cytokines on exposure to specific antigens in the culture (31). The Th2 cytokines, such as IL-4, can increase the expression of GATA3 in CD4⁺ T cells (32); the GATA3 interferes with the activities of Foxp3 (24), and thus interferes with the induction of Tregs.

Results showed that the Gal-1/SIT therapy resulted in inhibitory effects on intestinal allergy for as long as 12 months, which was not achieved by either the SIT therapy alone or the Gal-1 therapy alone; Gal-1 therapy showed an immediate inhibitory effect on the allergic response in the intestine, but its effects only lasted temporarily. The explanation of this phenomenon may be that Gal-1 therapy only temporarily inhibits mast cell activation, but does not contribute to the generation of antigen-specific Tregs. Some reports suggest that Gal-1 induces non-specific Tregs (30, 33), while present data emphasize the administration of Gal-1 and SIT contributing to long-term inhibitory effects on intestinal allergic responses, in which the generation of antigen-specific Tregs plays a crucial role; the therapeutic effects were abolished in depleting Tregs.

It may be argued that reagents used for SIT in allergy clinics are currently specific antigens alone. Although the reported therapeutic efficacy is varied, some encouraging results were reported. For example, Kaufman observed up to 81% improvement in allergic dermatitis symptoms after a two-year SIT (34). Warner et al. observed that SIT resulted in “the greatest

improvement in asthma symptoms” (35). However, unfavorable results were also reported.

Keine-Tebbe et al. did a double-blind random clinical trial of SIT in 450 cases with allergic disorders and found no statistically significant differences in results between the active groups and the placebo group (36). Our studies found that although clinical symptoms of perennial allergic rhinitis were suppressed after treatment with a six-month SIT, symptoms relapsed 1 month after termination (37). Glover et al. found efficacy was “uncertain” in SIT for asthma (38). Galli observed therapeutic that efficacy in the SIT and control group was not different (39).

On the other hand, Gal-1 is known to bind to and induce apoptosis of many cell types, including T cells (40). This raises the question about whether the administration of Gal-1 by gavage could cause cell death in the intestinal tissue and contribute to the findings of the present study? This certainly needs to be further investigated. It would be particularly interesting to examine the impact of Gal-1 released endogenously by intestinal cells on the sensitized mast cells. The present data suggest that endogenous Gal-1 may not have sufficient effects on stabilizing sensitized mast cells in an allergic environment; applying exogenous sources of Gal-1 can better stabilize the sensitized mast cells.

In summary, our results show that the Gal-1/SIT therapy can achieve long term therapeutic effects on allergic responses in the intestine of sensitized mice via inhibiting mast cell activation and generating antigen specific Tregs in the intestine.

Declare of conflict of interest: None to declare.

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Author contribution: LTY, QS, XQL, ZQL, SSQQ, JQL, HJG, LJL, MGL, DBL and LXX performed the experiments, analyzed data and reviewed the manuscript. PCY and ZGL organized the study and supervised the experiments. PCY designed the project and wrote the manuscript.

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Figure legends

Figure 1. Gal-1 inhibits allergic responses in the intestine. Scatter dot plots show levels of short circuit current (I_{sc}) of the jejunal epithelial layer in Ussing chambers (A), permeability to dextran (DX4000) of intestinal epithelial barrier in vivo (B), and counts of mononuclear cells in the jejunal mucosa (C). Mouse treatment is noted on the horizontal axis. Each group consists of 6 mice, and each dot represents individual data in panels A and B (two sample pieces from each mouse were tested). Each dot in panel C indicates data averaged from 20 readouts of each mouse, and D represents photomicrographs of the small intestine ($\times 200$; H&E staining). We also found that $*p < 0.01$ (t test).

Figure 2. Gal-1 prevents specific antigen-induced mast cell activation. A, the serum levels of β -hex in mice ($n = 6$ per group) after treatment was denoted on the horizontal axis. OVA: Mice were treated with OVA. Gal-1/OVA: Mice were treated with Gal-1 and OVA. B, levels of β -hex in the culture supernatant of IgE-primed BMMC after being exposed to OVA or OVA/Gal-1 for 30 min, while C immune blots show complexes of Fc ϵ RI/IgE/OVA/Gal-1 in the extracts of BMMC after treatment of panel B. D-F immune blots show the levels of MAPK p38 (D), I- κ B (E), and NF- κ B (F) in the BMMC extracts. Data of C-F represent 3 independent experiments, and dots in A and B represent individual data. Furthermore, $*p < 0.01$ (t test). [The antibodies used in D-F were purchased from Santa Cruz Biotech (Santa Cruz, CA)].

Figure 3. Gal-1 enhances generation of Treg in the intestine of antigen-sensitized mice. A, CD4⁺ T cells were gated from LPMCs. B, CD4⁺ CD25⁺ Foxp3⁺ Tregs in LPMCs of naïve mice and sensitized mice. C, Tregs in LPMCs of sensitized mice after treatment denoted above each panel. D, the summarized data of Treg in LPMCs of mouse groups listed on the horizontal axis. Again, $*p < 0.01$ (ANOVA), compared with the naïve mouse group ($\#p < 0.01$, with the t test compared to the OVA/Gal-1 group). Each group consisted of six mice.

Figure 4. Assessment of the inhibitory effects of mast cell activation on generation of Treg. Mouse spleen CD4⁺ CD25⁺ T cells were treated (denoted above each panel) in the culture for one week. A-G, the gated dot plots show the frequency of CD4⁺ CD25⁺ Foxp3⁺ Tregs. H, the summarized data of A-G from three independent experiments. As seen, $*p < 0.01$ (ANOVA).

Figure 5. Gal-1 enhances effects of SIT on suppressing allergic responses in the intestine.

The groups of mice are denoted on the horizontal axis. Gal-1: Mice were fed with Gal-1 (2.5 mg/kg) together with SIT at each time point. Ab: Mice were intraperitoneally injected with anti-CD25 (0.25 mg/mouse). A, mouse body weight records (mean $\pm$ SD) in the period of SIT. B-D, counts of mononuclear cells, mast cells, and eosinophils in intestinal tissue sections. Each dot represents an average of 20 light microscope fields ($\times 200$). E, serum histamine levels. F, mouse intestinal epithelial permeability to DX4000 (assessed in vivo). Each group consists of six mice, with * $p < 0.01$ (ANOVA) compared to the naïve group. In addition, # $p < 0.01$ (t test) was compared to the Gal-1 group.

Figure 6. Long-term effects of Gal-1/OVA therapy on suppression of allergic response in the intestine. Bars indicate frequency of mononuclear cells in the jejunal mucosa (A), serum histamine levels (B), and intestinal epithelial barrier permeability (C) of naïve mice and OVA-sensitized mice after treatment on the horizontal axis in week 5, challenged with OVA and sacrificed 12 months later. Each group consisted of six mice, with * $p < 0.01$ (ANOVA) compared to naïve mice. Data bars were presented as mean $\pm$ SD (# $p < 0.01$, t -test was compared to the SIT group alone).





