Original Article
Experimental Allergy and Immunology

Oral myeloid cells uptake allergoids coupled to mannan driving Th1/Treg responses upon sublingual delivery in mice

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Abstract
Background: Polymerized allergoids coupled to nonoxidized mannan (PM-allergoids) may represent novel vaccines targeting dendritic cells (DCs). PM-allergoids are better captured by DCs than native allergens and favor Th1/Treg cell responses upon subcutaneous injection. Herein we have studied in mice the in vivo immunogenicity of PM-allergoids administered sublingually in comparison with native allergens.

Methods: Three immunization protocols (4-8 weeks long) were used in Balb/c mice. Serum antibody levels were tested by ELISA. Cell responses (proliferation, cytokines, and Tregs) were assayed by flow cytometry in spleen and lymph nodes (LNs). Allergen uptake was measured by flow cytometry in myeloid sublingual cells.

Results: A quick antibody response and higher IgG2a/IgE ratio were observed with PM-allergoids. Moreover, stronger specific proliferative responses were seen in both submandibular LNs and spleen cells assayed in vitro. This was accompanied by a higher IFNγ/IL-4 ratio with a quick IL-10 production by submandibular LN cells. An increase in CD4+CD25highFOXP3+ Treg cells was detected in LNs and spleen of mice treated with PM-allergoids. These allergoids were better captured than native allergens by antigen-presenting (CD45+MHC-II+) cells obtained from the sublingual mucosa, including DCs (CD11b+) and macrophages (CD64+). Importantly, all the differential effects induced by PM-allergoids were abolished when using oxidized instead of nonoxidized PM-allergoids.

Conclusion: Our results demonstrate for the first time that PM-allergoids administered through the sublingual route promote the generation of Th1 and FOXP3+ Treg cells in a greater extent than native allergens by mechanisms that might well involve their better uptake by oral antigen-presenting cells.

Keywords: allergoid, glycoconjugate, immunotherapy, mannan, sublingual

Abbreviations: DC, dendritic cell; FOXP3, forkhead box P3; LN, lymph node; N, native allergen; PBS, phosphate-buffered saline; PE, phycoerythrin; PM-OX, oxidized form of PM; PM, polymerized allergoid coupled to nonoxidized mannan; SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; Treg, regulatory T.

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Allergy. 2018;73:875–884. wileyonlinelibrary.com/journal/all
Sublingual allergen-specific immunotherapy (SLIT) has become an alternative to subcutaneous allergen-specific immunotherapy (SCIT) for treating respiratory allergies. Safety is the main advantage for SLIT, given that systemic allergic reactions are very rare; yet, oral side effects are frequent and invariably occur in more than 50% of the patients receiving SLIT. Although mild and self-limited, these aspects make compliance more difficult to achieve, especially in children. Different meta-analysis seems to indicate that SLIT has lower clinical efficacy than SCIT, but these data are only based on indirect evidence and not on head-to-head comparisons.

The immunologic mechanisms underlying the action of SLIT are less well established than SCIT, but similar immunomodulatory pathways have been described. In this regard, both therapies are thought to modulate the allergen-specific T-cell and B-cell responses similarly, including the generation of both regulatory T (Treg) and B (Breg) cells. Allergen uptake by oral mucosal dendritic cells (DCs) is thought to be a critical step for initiating the immune response in SLIT, a step requiring higher allergen concentration and dose frequency for SLIT than SCIT. To improve allergen uptake by oral DCs, some experimental allergen formulations have been assayed, such as conjugation with cholera toxin, binding to mucoadhesive agents, or using nanoparticles.

We previously reported that polymerized allergoids coupled to nonoxidized mannan derived from *Saccharomyces cerevisiae* (PM-allergoids) are better captured than native allergens by human and canine DCs. PM-allergoids are taken up by DCs very rapidly through a receptor-mediated mechanism involving C-type lectin receptors. Besides their better uptake, these glycoconjugates are rendered hypoallergenic and able to activate DCs to promote the induction of functional FOXP3+ Treg cells, thus with improved features for allergen immunotherapy. Therefore, we were prompted to test the immunogenic properties of PM-allergoids in comparison with native allergens in mice that were treated sublingually with each allergen preparation.

Here we show that mice immunized sublingually with PM-allergoids derived from *Phleum pratense* pollen allergens produce an immune response, both humoral and cellular, which is earlier and stronger than that obtained with the native allergens (ie, unmodified, mannan-free). Such a response is a Th1-biased, reflected by higher IgG2a/IgE and IFNγ/IL-4 ratios, and shows a quick IL-10 response and Treg cell induction. Moreover, PM-allergoids are better captured than native allergens by antigen-presenting (CD45+MHC-II+) oral cells, including DCs (CD11b+) and macrophages (CD64+).

**METHODS**

**2.1 | Mice**

BALB/c female mice of 6-8 weeks of age were obtained from Charles Rives, Germany. Animal experiments were approved by the Ethics Committee of Hospital Clinico San Carlos (Madrid, Spain) and performed in accordance with the Spanish national and international/EU legislation regulated by D.C.86/609/CEE; RD 1201/2005.

**2.2 | Allergen preparations**

Grass pollen (*Phleum pratense*) allergens and mannan (*Saccharomyces cerevisiae*) were obtained and purified as described. Unmodified native allergens (N) were coupled to nonoxidized mannan (1:0.5 ratio) exactly as described to render polymerized allergoid-mannan complexes (PM). For control purposes, PM-allergoids were further oxidized (PM-OX) with sodium periodate as described.

**2.3 | Immunization protocols**

Mice (groups of 6 animals) were immunized sublingually with each allergen preparation (N, PM, or PM-OX) using three different protocols. The immunogens were as follows: N (native allergen); PM (PM-allergoids); PM-OX (PM-allergoids further oxidized); PBS (phosphate-buffered saline as a negative control). After the last immunization, all animals were killed. Blood was collected for the measurement of specific antibodies in serum. Spleen and submandibular LN cells were isolated and cultured in vitro for assessing cytokines production and cell proliferation. In some experiments, FOXP3+ cells were determined in freshly isolated spleen and submandibular LNs.
protocols (Figure 1A): Protocol 1: 4 sublingual immunizations at weekly intervals (4 doses/4 weeks); Protocol 2: 9 sublingual immunizations every 4 days (9 doses/6 weeks); Protocol 3: 13 sublingual immunizations every 4 days (13 doses/8 weeks). Immunizations were performed administering 5 μL of the different allergen preparations (200 μg in protein) under the tongue using a calibrated micropipette. To prevent swallowing during the treatment, the sublingual administration was performed under anesthesia (ketamine, 100 mg kg⁻¹ and xylacine, 5 mg kg⁻¹) to ensure proper delivery. A control group (PBS) treated with the same volume of phosphate-buffered saline solution (PBS) was always included. Seven days after the last dose, blood, spleens, and submandibular lymph nodes (LNs) were obtained for further assays (Figure 1B).

### 2.4 | Antibody measurement

Specific antibodies against the native allergens (N) were measured by ELISA in each mouse serum. IgG1, IgG2a, and IgE antibodies were determined using appropriate anti-mouse isotype labeled antibodies (BD Pharmingen).

### 2.5 | Cell proliferation and cytokine measurement

Spleens and submandibular LNs were isolated aseptically, minced, and filtered through sterile steel filters. Erythrocyte-free cells were stained with 2 μM carboxyfluorescein succinimidyl ester as described. The cell suspensions were cultured into 24-well plates at a concentration of 1 × 10⁶ cells/well with or without (control) stimulation with 50 μg/mL of native (N) allergens. IFN-γ, IL-4, and IL-10 were measured in 48-h culture supernatants using CBA Cytometric kit (Beckton Dickinson). Proliferation was measured on an FC-500 flow cytometer (Beckman Coulter) and cytokines in a Gallios flow cytometer (Beckman Coulter). The data were analyzed using the FLOWJO V.10 software.

### 2.6 | Treg cells measurement

For analysis of FOXP3 expression in freshly isolated mouse splenocytes and submandibular LN cells, these were first subjected to surface staining with anti-mouse CD4-PerCP and CD25-PE antibodies (BioLegend). After fixation and permeabilization, cells were stained with anti-mouse FOXP3-Alexa Fluor 488 (BioLegend), according to manufacturer’s recommendations. The corresponding isotype controls were included in each staining. Flow cytometric analysis was performed with a FACS Calibur cytometer (Beckton Dickinson) and Weasel v2.5 software.

### 2.7 | Isolation of antigen-presenting cells from the sublingual mucosa

Antigen-presenting cells were obtained following the protocol described by Tanaka et al.²⁵ Briefly, the mouse sublingual mucosa tissues were excised by trimming off the roots of the tongue and cut into fragments. Tissues were treated with 0.25% trypsin and 1 mM EDTA for 1 hour at 37°C with shaking. Next, they were incubated (1 hour at 37°C with shaking) with 1 mg/mL collagenase (Wako Pure Chemical) and 0.1 mg/mL DNase I (Sigma-Aldrich) in RPMI medium containing 10% fetal bovine serum. Cells were then filtered, using a 70-mm cell strainer. After washing, cells were purified by density gradient centrifugation with Ficoll Histopaque (VWR) following a standard protocol.

CD45 population was isolated with anti-CD45-coated magnetic beads according to the supplier’s protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells obtained were characterized phenotypically by flow cytometry on an FC-500 Beckman Coulter using FLOWJO V.10 software. Briefly, 10⁵ cells were stained (30 minutes at 4°C) with anti-CD45 (mAb clone 104), anti-MHC class-II (mAb clone M5/114.15.2), anti-CD207 (mAb clone 4C7), anti-CD11b (mAb clone M1/70), and anti-CD64 (mAb clone X54-5/7.1) (Biolegend). The corresponding isotype controls were included in each staining.

### 2.8 | Allergen uptake by sublingual antigen-presenting cells

N, PM, and PM-OX allergen preparations were equally labeled with Alexa 488 maleimide (Thermo Fisher Scientific) and allergen uptake performed as described.¹⁶ Briefly, cells (3 × 10⁵) were incubated (30 minutes at 37°C) with 100 μg/mL of each labeled preparation in RPMI. Incubation was stopped by adding cold PBS. Fluorescence was analyzed by flow cytometry (FC-500 Beckman Coulter) using FLOWJO V.10 software. Allergen uptake was assessed in the whole CD45⁺MHC-II⁺ cell population, and in CD11b or CD64 cell subsets.

### 2.9 | Statistics

Data are expressed as mean ± SEM of the indicated parameters. Statistical differences were determined with the paired or unpaired Student’s t test. Significance was defined as *P < .05, **P < .01, and ***P < .001.

### 3 | RESULTS

#### 3.1 | Sublingual immunization with PM-allergoids induces a quicker and stronger antibody response with a higher IgG2a/IgE ratio than native allergens

Three different protocols were used to immunize mice by the sublingual route differing in duration, frequency, and number of doses administered (Figure 1A). The immune responses were analyzed as indicated in Figure 1B.

Antibody serum levels to native *Phleum pratense* pollen extract were measured in sublingually immunized mouse sera 1 week after the last dose. As shown in Figure 2A, mice immunized with PM-allergoids had a significant higher IgG1 and IgG2a levels than those immunized with native allergens using the shorter protocol (Protocol 1). This was also the case for IgG2a when using the intermediate protocol (Protocol 2). By contrast, using the longest protocol (Protocol 3), both IgG1- and IgE-specific antibody levels were significantly
higher in mice with the native allergens than in those with PM-allergoids (Figure 2A). When considering the IgG2a/IgE ratio of these responses, this was always higher in mice immunized with PM-allergoids than in those with the native allergen reaching to significance for Protocol 2 and Protocol 3 (Figure 2B). As it is shown in this figure, the differences in IgG2a/IgE ratio between PM-allergoids and native allergens were not observed when mice were sublingually immunized with the oxidized form (PM-OX).

3.2 | Sublingual immunization with PM-allergoids produces a higher antigen-specific proliferative cell response in submandibular LNs and spleen than native allergens, with a higher ratio of IFNγ/IL-4 production and a quicker IL-10 response

The in vitro proliferative activity of submandibular LN and spleen cells in response to native Phleum pratense pollen allergens was assessed in mice immunized sublingually with each allergen preparation. As shown in Figure 3 (Figure S1 for Protocol 2), the lymphoproliferative response in mice immunized with PM-allergoids (but not with PM-OX) was always significantly higher than that obtained when immunizing with native allergens. Remarkably, in the spleen, a proliferative response above the nonimmunized control mice was only seen when using PM-allergoids (Figure 3B).

Figure 4 shows the in vitro cytokine production by submandibular LN and spleen cells in response to native Phleum pratense pollen allergens in immunized mice. As shown, a significantly higher IFNγ production was always seen in submandibular LN cell cultures derived from mice immunized with PM-allergoids (Figure 4A). When testing the spleen cells from the same mice, such an increase was observed using the longest immunization Protocol 3 (Figure 4B) and also with the intermediate Protocol 2 (Figure S1). This was accompanied by a lower IL-4 production, and therefore, a significant higher IFNγ/IL-4 ratio was seen in mice treated with PM-allergoids using

**FIGURE 2** Serum antibody response in mice after sublingual immunization with Phleum pratense pollen allergens. The immunogens were as follows: N (native allergen); PM (PM-allergoids); PM-OX (PM-allergoids further oxidized); PBS (phosphate-buffered saline as a negative control). (A) Serum levels of IgG1, IgG2a, and IgE were measured by ELISA against native allergens (Phleum pratense) in mice immunized with different protocols. (B) IgG2a/IgE ratios of antibody levels from each individual mouse immunized with different protocols. Results are the mean ± SEM of 6 mice. Statistical differences were analyzed with Student’s t test. *P < .05, **P < .01, ***P < .001.
Protocol 3 (Figure 4B). This ratio was always higher in submandibular LNs from mice immunized with PM-allergoids as well (Figure 4A). Regarding IL-10 production, it was significantly higher when testing submandibular LN cells derived from mice immunized with PM-allergoids using the shorter Protocol 1 (Figure 4A). Once again, the above differences on cytokine production obtained when immunizing mice with PM-allergoids were completely lost if using PM-OX instead (Figure 4).

3.3 | Sublingual immunization with PM-allergoids increases FOXP3⁺ Treg cells in submandibular LNs and spleen

The frequency of Treg cells (CD4⁺CD25⁺highFOXP3⁺) was assessed in freshly isolated submandibular LNs and spleen from mice immunized sublingually with all the allergen preparations. Using the shorter Protocol 1, the percentage of FOXP3⁺ Treg cells was significantly increased in submandibular LNs from mice immunized with PM-allergoids, as compared with native allergens (Figure 5A). This increase was not seen in the spleen of the very same mice except when using the longest Protocol 3 (Figure 5B). By contrast, no increase in the FOXP3⁺ Treg cell populations was observed when instead of PM, its oxidized form (PM-OX) was used (Figure 5). Using Protocol 2, the spleen of mice immunized with PM-OX also showed a decrease in FOXP3⁺ Treg cells, although without significant differences between N and PM-immunized mice (Figure S1).

3.4 | PM-allergoids are better captured than native allergens by sublingual antigen-presenting (CD45⁺MHC-II⁺) cells, including CD11b⁺ DCs and CD64⁺ macrophages

The foregoing results indicated a superior FOXP3⁺ Treg cell induction in mice immunized sublingually with PM-allergoids than with native (N) allergens. As such an induction has been reported to be dependent on allergen uptake by CD11b⁺ cells present on the sublingual mucosa, we were prompted to assess whether PM-allergoids were better captured by these cells. For this purpose, fluorescent-labeled allergen preparations were incubated with CD45⁺ cells isolated from the sublingual mucosa as described in Materials and Methods. About 40% of these cells were identified as antigen-presenting cells (CD45⁺MHC-II⁺) as the main cell population (ca. 75%), followed by myeloid (CD11b⁺) DCs (ca. 15%) and Langerhans cells (CD207⁺) as a minor cell population (ca. 1-5%) (Figure 6A). Figure 6B shows that PM-allergoids were captured much more efficiently by CD45⁺MHC-II⁺ cells than native allergens (up to 5-fold in some experiments) and this uptake was completely abolished when PM-allergoids were further oxidized (PM-OX). As shown in Figure 6C, around 80% of CD11b⁺ and 35% of CD64⁺ cells were able to internalize PM-allergoids. In contrast, less than 40% of CD11b⁺ DCs or 10% of CD64⁺ cells were able to internalize the native allergen (Figure 6C).

4 | DISCUSSION

In the study performed herein, we have compared the immune response obtained by the sublingual administration of novel PM-allergoids derived from Phleum pratense pollen, with that achieved by the conventional native (unmodified) allergens used in SLIT. PM-allergoids are the result of coupling polymerized allergoids to nonoxidized mannan from Saccharomyces cerevisiae in such a way that the mannan structure is duly preserved. Our results indicate that PM-allergoids induce a faster and stronger immune response than that obtained with the native allergen, both at the cellular and humoral levels. It is noteworthy to mention that every response assessed was always against the native allergens, irrespectively of the allergen preparation used for immunization. The immune response obtained with PM-allergoids is achieved in spite of their high molecular weight, early suggested to be a serious impediment
FIGURE 4  Cytokine production in submandibular LNs and spleen from mice after sublingual immunization with Phleum pratense pollen allergens. The immunogens were as follows: N (native allergen); PM (PM-allergoids); PM-OX (PM-allergoids further oxidized); PBS (phosphate-buffered saline as a negative control). Cytokines were measured in the culture supernatant by flow cytometry (CBA) in response to the native allergens (*Phleum pratense*) after 48-h culture. The results obtained with submandibular LN (A) or spleen (B) cells, from mice immunized with different protocols, are the mean ± SEM of 6 mice per group. N.d. (nondetermined). Statistical differences were analyzed with Student’s t test. *P < .05, **P < .01, ***P < .001
for the immunization through the sublingual route. Supporting this route also for PM-allergoids is the response obtained in submandibular LNs, the primary draining lymph nodes of sublingual tissue. In this sense, besides antigen penetration across the sublingual epithelial barrier, an active capture by intraepithelial DCs or through the ductal epithelial cells has been described, even for particulate antigens, silica beads, or microbes. Bigger structures than PM-allergoids, like particles of different sizes or whole cell bacteria, have also been successfully used for immunization through the sublingual route.

The results also show that the main immunomodulatory features of PM-allergoids are greatly dependent on keeping the structural integrity of mannan. We previously demonstrated that PM-allergoids are much better captured than native allergens by human and canine DCs and that this property is lost upon mannan oxidation. Here we show that this is also the case in mice when assessing antigen-presenting cells (CD45⁺MHC-II⁺) from the sublingual mucosa. Among them, CD64⁺ cells were the most abundant (around 75%), CD64, one of the most specific macrophage markers, has been shown useful in discriminating macrophages from conventional CD11b⁺ DCs. The latter (CD11b⁺ DCs) were about 15% of total CD45⁺MHC-II⁺ cells, whereas CD207⁺ Langerhans cells was a minor population (less than 5%) in our cell sample. These percentages fit well with those described by Tanaka et al in sublingual and lingual tissues when using a similar protocol for isolating antigen-presenting cells. Interestingly, most of CD11b⁺ DCs were able to capture PM-allergoids, but only a minor part of CD64⁺ macrophages did so. Both cell subsets showed a quite similar increase in their capacity to uptake PM-allergoids in comparison with native allergens. Thus, our results suggest that, likely due to a better uptake, both myeloid cells might be involved in the superior response of mice immunized with PM-allergoids than native allergens, including the induction of Treg cells (see below).

Several mechanisms have been proposed for allergen uptake by DCs in SLIT, including the capture through specific IgE bound to FcεRI on oral Langerhans cells, a major DC population within the oral mucosa in human beings. In the case of PM-allergoids, however, their low IgE binding reactivity would make this mechanism unlikely. Nevertheless, other IgE-independent mechanisms for allergen uptake have been described further, like CD207 (Langerin), a putative mannose receptor, or CD209 (DC-SIGN). Previously, we have shown that the enhanced uptake of PM-allergoids by human
monocyte-derived DCs is mediated by different C-type lectin receptors binding mannose residues, such as CD206 (mannose receptor) and CD209 (DC-SIGN). These receptors have been described in oral DCs as well, including the CD11b+ cells involved in tolerance induction during SLIT. Interestingly, IL-4 may increase the expression of the mannose receptor on myeloid cells, a fact already reported in DCs from allergy patients, thus opening the possibility of a better uptake of PM-allergoids in these patients. A relevant point of the present study is that the cells used for allergen uptake were resident cells obtained directly from the oral mucosa in a steady-state situation. Thus, it appears that PM-allergoids are better uptaken by either resident DCs or monocyte-derived DCs.

DCs are the main initiators of immune responses with an important role in the polarization of naive T cells. It is widely accepted that the efficiency of allergen-specific T-cell responses during allergen immunotherapy correlates with the capability of this therapy for skewing DCs. We have shown that PM-allergoids promote the generation of Th1 and Treg cells in vitro, but also in vivo upon subcutaneous immunization in mice. Here we show that similar responses might be induced by the sublingual route, which is evidenced by higher IFNγ/IL-4 and IgG2a/IgE production ratios, together with an increase in CD4+CD25+FOXP3+ Treg cells and a quick IL-10 response in submandibular LNs. Treg cells were also increased in the spleen of mice treated with PM-allergoids when using the longest immunization protocol. In this sense, the sublingual route seems less effective than the subcutaneous to induce these cells systemically (ie, in the spleen), as by s.c. injection, the increase in Treg cells was readily detectable in the spleen after just 3 doses/4 weeks with the same PM-allergoids used here. The preferential regional induction of Treg cells by the sublingual administration fits with the description of an increase in Treg cells in the sublingual mucosa in patients receiving SLIT. These cells may migrate from regional lymphoid organs or induced within the oral mucosa through local DC-T cell interaction. Interestingly, an increase in Treg cells in submandibular LNs following SLIT has been described with antigen formulations that presumably facilitates the allergen capture by oral DCs. In this sense, antigen-specific FOXP3+ Treg cells have been shown to be induced in submandibular LNs following antigen transportation by conventional DCs (CD11b+) present in the oral mucosa. The fact that PM-allergoids are better captured by CD11b+ cells, as we show here, may point to the same direction. In this regard, PM-allergoids, besides facilitating allergen uptake by DCs, enhance Treg cell induction through an increase in PD-L1 in DCs, a mechanism recently described for a successful induction of antigen-specific Treg cells derived from naive T cells in draining LNs.

It has been suggested that an ideal allergen vaccine would target DCs, yet not be recognized by mast cell surface IgE. Mastocytes are abundant in the sublingual mucosa, and their activation through IgE is thought to account for the frequent local side effects during SLIT. In this sense, PM-allergoids have the advantage over the native allergens that show a very limited reactivity with specific IgE and very low ability to activate skin mastocytes or blood basophils from...
sensitized patients.\textsuperscript{16} In addition, PM-allergoids may not directly activate human basophils as, unlike DCs, they do not express DC-SIGN and mannose receptors.\textsuperscript{43} Taken together, polymerized allergoids coupled to nonoxidized mannan may be suitable candidates for the development of improved SLIT vaccines targeting DCs.

ACKNOWLEDGMENTS

This work was supported by grants IDI-20110410 and IDI-20141131 from the Centre for the Development of Industrial Technology (CDTI—MINECO, Spain) to INMUNOTEK, S.L., Spain. I.S., J.L.-R, and A.I.M are recipients of Torres Quevedo grants (PTQ-12-05785, PTQ-12-05786, PTQ-12-05787) from MINECO, Spain. O.P. is a Ramon y Cajal Scholar funded by MINECO and the European Social Fund. A.A. and C.B.-V. are recipients of UCM and FPU predoctoral fellowships from UCM and MECD, respectively.

CONFLICT OF INTEREST

J.L.S. is the founder of INMUNOTEK S.L., and shareholder together with M.C. and E.F-C. INMUNOTEK S.L. has submitted a patent related to the vaccine described herein (WO/2014/162036). O.P. has received grants from INMUNOTEK S.L. I.S., J.L.-R., J.T.T., C.D.R., B.C., and A.I.M are employees of INMUNOTEK S.L. The rest of authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

JLS, OP, EFC, and MC conceived and designed the experiments; IS, JL-R, MV, JIT, AA, CB-V, and CMD-R performed the experiments; JLS, OP, IS, JL-R, and CB-V wrote the study and analyzed the data; JLS, OP, AIM, and BC contributed with reagents, materials, and analysis tools. All the authors revised and contributed to the manuscript.

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