MEASLES VIRUS-DERIVED PEPTIDE/FOOD ANTGEN ADDUCTS FACILITATE THE ESTABLISHMENT OF ANTIGEN SPECIFIC ORAL TOLERANCE

C. HE1, C.-H. SONG2, L. CHENG2, T. CHEN1, C. LIU1, Z. LIU1, P.-C. YANG2

Allergy is a skewed immune response to common proteins in the body (1). The etiology of allergy is unclear. It is proposed that the breakdown of the oral tolerance plays a role in the pathogenesis of allergy. The present study aims to restore the breached intestinal tolerance with an artificial adduct of a measles virus C protein-derived small peptide (MVCP) and a model antigen, ovalbumin (MOA), and to observe the effect of MOA on inhibition of intestinal allergy in a mouse model. The MOA was formed by the MVCP and ovalbumin. The effect of MOA on regulation of the properties of dendritic cells (DC) and CD4+ T cells was observed with a cell culture model and a mouse model of the gut Th2 pattern inflammation. After treatment with MOA, mouse intestinal DCs showed high levels of aldehyde dehydrogenase (ALDH) activity and expressed transforming growth factor (TGF)-beta; the frequency of Treg in the intestine was also significantly increased. The treatment with MOA efficiently suppressed the antigen-specific Th2 pattern inflammation in the intestine. Administration with the MOA can induce the development of antigen-specific oral tolerance and inhibit the antigen-specific allergic reaction in the intestine. The adduct of MOA has the therapeutic potential for the allergen related immune inflammation.

Key words: food allergy, oral tolerance, regulatory T cell, intestine, measles virus

INTRODUCTION

Allergy is a skewed immune response to common proteins in the body (1). The etiology of allergy is unclear. It is proposed that the breakdown of the oral tolerance plays a role in the pathogenesis of allergy (1). Besides the specific immune therapy (2), no other specific therapeutic remedies of allergic disorders have been reported. Thus, it is necessary to develop novel and effective remedies for the treatment of allergic diseases.

The oral tolerance indicates that the immune system specifically suppresses cellular and/or humoral immune responses to a specific antigen by previous ingestion of the antigen via the oral route (3). Oral tolerance prevents hypersensitivity reactions to protein antigens and bacterial antigens presenting in the digestive tract as well as in other body systems (4). Tolerogenic dendritic cells (DC) and regulatory T cells (Treg) are the important components of oral tolerance. The tolerogenic DCs are featured as expressing high levels of transforming growth factor (TGF)-β, interleukin-10 (IL-10), or aldehyde dehydrogenase (ALDH) activity (5). Tregs include CD4+CD25+Foxp3+ T cells, T helper 3 (Th3) cells and type 1 Tregs. Tregs also express TGF-β or IL-10 (5, 6). The dysfunction of oral tolerance has been noted in the patients with allergy and allergy animal models (7). It is suggested that to promote antigen-specific oral tolerance has the potential to inhibit allergic disorders (8).

It is reported that measles virus (MV) can induce immune suppression from clinical observations (9), such as the inhibition of delayed-type hypersensitivity reactions, suppression of lymphocyte and antigen-presenting cell functions, down-regulation of pro-inflammatory interleukin-12 (IL-12) production and alteration of interferon-alpha/beta signalling pathways (10). Another peculiar feature of MV infection is to confer the body the lifelong tolerance against MV re-infection that also can be achieved by MV vaccination (10). The phenomenon implicates that the immune tolerance is established in the body after MV. Whether some of the MV-derived factors contribute to the establishment of the general immune tolerance is to be further investigated.

The MV P gene codes for three proteins, the protein P, V and C. Comparing with other P gene proteins of MV, the MV C protein is an infectivity factor; it has more active bioactivity (11). The MV C protein reduces viral transcription (12) and inhibits the type I interferon response (13). The C protein, produced by translation of an open reading frame initiated 19 nucleotides downstream of the P/V start codon, colocalizes with the MV RNP in infected cells (14). Since short amino acid sequence can bind large molecular proteins to form adduct that can largely enhance the immunogenicity of the immunogens (15), in the present study, according to the open reading frame concept (16, http://en.wikipedia.org/wiki/Open_reading_frame), we designed 10 short MV peptides (MVCP) based on the gene sequence of the MV C protein. One of them formed an adduct with a model antigen, ovalbumin (OVA). The adduct could induce tolerogenic DCs and antigen-specific Tregs, which showed the capacity to suppress the antigen-specific intestinal Th2 pattern inflammation.
MATERIALS AND METHODS

Mice

The procedures of animal experiments in this study were approved by the Animal Care Committees at McMaster University and Tongji University.

BALB/c mice, 6–8 weeks old, were purchased from Charles River Canada (St. Constant, QC, Canada) and Shanghai Experimental Animal Institute (Shanghai, China). DO11.10 mice were purchased from Jackson Laboratories (Bar Harbor, Maine).

Reagents

Antibodies of CD3 (17A2), CD28 (JJ319), IL-28 (A-3), TGF-β (D-12), Foxp3 (F-9) and the matched isotype IgG of anti-IL-28 (mouse IgG2a) were purchased from Santa Cruz (Santa Cruz, CA). Antibodies of CD11c, CD4 and CD25 were purchased from eBioscience (San Diego, CA). IL-4 ELISA kit (sensitivity = 2 pg) was purchased from R&D Systems (Burlington, ON, Canada). IgE ELISA kit was purchased from AbD Serotec (Raleigh, NC; sensitivity = 8 ng/ml). FITC-labeling kit was purchased from Thermo Scientific (Rockford, IL). The ALDEFLUOR staining kit was purchased from StemCell Technologies (Vancouver, Canada). Measles C protein and antibody, and annexin V (FITC) were purchased from Abcam (Cambridge, MA). The immune cell isolation kits were purchased from Myltenyi Biotech (Cambridge, MA). The Smvp peptides of each ORF were synthesized by GL Biochem (Shanghai, China). SEB was purchased from Sigma Aldrich (Oakville, ON, Canada). The lipopolysaccharide (LPS) levels in all reagents were detected using a limulus assay (Limulus ameocyte lysate QCL 1000, Bio Whittaker, Walkersville, MD). Less than 0.2U LPS/10 µg in all reagents was used in this study.

Flow cytometry

The expressions of cell surface markers and intracellular cytokines were examined by flow cytometry. Cells were collected from the culture and stained with fluorescence-labelled antibodies (or Isotype IgG) (0.5–1 µg/ml) on ice for 30 min. For the intracellular staining, cells were fixed with 1% paraformaldehyde on ice for 30 min and incubated with permeabilization reagents for 30 min on ice. The stained cells were washed and sustained in the buffer and analyzed using a flow cytometer (BD FACSCanto; BD Bioscience, San Jose, CA).

T cell proliferation assay

T cell proliferation was assessed by CFSE (Carboxyfluorescein succinimidyl ester)-dilution assay with flow cytometry. Isolated Th0 (CD3+CD4+CD25–) cells were stained with CFSE and cultured with freshly isolated DCs (T cell:DC = 10:1) in the presence or absence of MOA for 72 hours. Cells were collected at the end of culture and analyzed by flow cytometry to determine the frequency of CFSE+ cells.

Fig. 1. Intestinal dendritic cells’ capture MOA. (A) the bars indicate the OD values that represent the amounts of MOA. (B) MOA and OVA were labeled with biotin and fed BALB/c mice (B1-3, B5); the mice were sacrificed 6 h later. LPMCs were stained with strep-FITC and anti-CD11c, and analyzed by flow cytometry. (B) the zebra plots show CD11c+ DCs in LPMCs. (C) the gated cells in panel B were further analyzed for FITC+ DCs (pointed by arrows); The histograms indicate the frequency of FITC+ DCs in the gated CD11c+ DCs from mice fed with OVA (B1, C1), or fed with MOA (B2, C2); B3 and C3 are negative controls. The data represent 5 experiments.
Development of the intestinal Th2 pattern inflammation mouse model

The procedures were depicted in Fig. 6A. Briefly, mice were immunized with OVA (0.1 mg/mouse) in 0.3 ml alum via i.p. on days 0, 7 and 14. Mice were re-exposed to the specific antigen OVA (1 mg/mouse) on days 28, 30 and 32 via gavage-feeding. Mice were sacrificed on day 33.

Inhibition of Th2 inflammation with MOA

With the same procedures above, a group of mice was treated with MOA by gavage-feeding, 50 µg/mouse, at the same time points in the course of sensitization.

Protein-protein conjugation

With a protein conjugation kit and followed the manufacturer’s instruction MOA was labelled with HRP; the MOA-HRP was then conjugated with OVA. The conjugates were tested with an in-house ELISA. Briefly, anti-OVA antibody (10 µg/ml) was used to coat a 96-well plate; the plate was incubated at 4°C overnight. The conjugates (or BSA, or the whole molecule of MOA, or MOA degraded by gastric fluid; 0.1 ml) was added to the plate at graded concentrations (indicated in Fig. 1A). Tetramethylbenzadine (TMB) (0.1 ml) was then added to the wells; the reaction was stopped by addition of 25 µl/well 4.5N H2SO4. The plate was read with a microplate reader (BioTek, Shanghai, China) at 450 nm. Between all incubation steps the plates were washed three times with TBS.

Isolation of lamina propria mononuclear cells (LPMC)

After sacrifice, mouse small intestine was excised (no Peyer’s patches) and washed with RPMI 1640 medium and cut into 2 mm pieces and then incubated in 0.5 mM EDTA in calcium/magnesium free Hanks’ solution for 20 min at 37°C with shaking to remove mucin. The tissue then was incubated 20 min at 37°C in 20 ml RPMI containing 1 mg/ml collagenase. The tissue was filtered with a 0.1 mm cell strainer first, then 40 µm cell strainer. After washing, cells (up to 2×107) were layered onto a column of Percoll with a 30-70% gradient. Cells were spun at 2200g at room temperature for 20 min. Subsequent cell viability was >90% as determined by trypan blue exclusion.

Intestinal inflammatory cell counts

Numbers of eosinophil, mononuclear cell and mast cell in the intestine were counted with our established procedures (17). Briefly, intestinal segments were fixed with 4% paraformaldehyde or Carnoy solution (for mast cell counting) overnight and processed for paraffin sections. The sections were stained with hematoxylin & eosin or 0.5% toluidine (for mast cell counting) and observed under a light microscope. The slides were coded; observers were not aware of the codes to avoid the observer bias. The numbers of mast cell, eosinophil and mononuclear cells were counted in 20 randomly selected fields (×200) for each sample.

Enzyme-linked immunoassay (ELISA)

Levels of serum specific IgE and IL-4 were measured by ELISA with commercial reagent kits following the manufacturer’s instruction.

Mouse diarrhea record

Diarrhea was recorded from mice after antigen challenge. The water-like stool or loose stool after antigen challenge (0–2 h) was regarded as diarrhea.

Mouse core temperature record

Thirty min after antigen challenge, the core temperature of the mouse was recorded from the rectum with a digital thermometer.

Labeling MVP with FITC or HRP

The MVP or Smvp (or BSA) was labeled with FITC or HRP with the FITC labeling kit or HRP labeling kit following the manufacturer’s instruction.

Statistical analysis

All values were expressed as the means ±S.D. of at least three independent experiments. The values were analyzed using the two-tailed unpaired Student’s t-test when the data consisted of two groups or by ANOVA when three or more groups were compared. P<0.05 was accepted as statistically significant.
**Fig. 3.** MOA induces tolerogenic dendritic cells in the intestine. BALB/c mice were gavage-fed with saline (A1), or OVA (100 µg/mouse; A2), or MOA (100 µg/mouse; A3), daily for 5 days; mice were sacrificed on day 6. LPMCs were isolated and analyzed by flow cytometry. A, the dot plots show the frequency of CD11c+ ALDH+ DCs in LPMCs. A4 is a negative staining control. A5, the bars indicate the summarized data in panel A1-A3. B, the histograms indicate the frequency of TGF-β+ DCs in the gated cells in A1-A4. B5, the bars indicate the summarized data in B1-B3. B4 is a control. The data in bar graphs were presented as mean ± S.D. *, p<0.05, compared with saline group. Each group consisted of 6 mice. The data represent 6 separate experiments.

**RESULTS**

**Intestinal dendritic cells capture luminal MOA**

MVCP was firstly conjugated to OVA to form a complex (MOA) as shown by Fig. 1A. We then observed if the MOA could be absorbed by DCs in vivo. Mice were fed with the MOA or OVA. The mice were sacrificed 6 hours later. As shown by flow cytometry, more than 8% CD11c+ cells were detected in isolated mononuclear cells. 38.5% and 35.4% DCs were also MOA+. The results indicate that these cells can capture MOA in the intestine (Fig. 1B, 1C).

**MOA modulates tolerogenic properties of dendritic cells**

We next observed if MOA could modulate dendritic cells (DC’s) tolerogenic properties. Using ALDH and TGF-β as the tolerogenic markers (5), we stimulated bone marrow-derived DCs (BMDC) with MOA in the culture. The results showed that the exposure to MOA increased the levels of ALDH and TGF-β...
in the BMDCs. Exposure to OVA alone did not alter the expression of either ALDH or TGF-β in BMDCs (Fig. 2).

MOA facilitates the generation of tolerogenic dendritic cells in the intestine

To further verify the ability of MOA in the induction of tolerogenic DCs, BALB/c mice were gavage-fed with OVA or MOA daily for 5 days. After sacrifice, we isolated mononuclear cells from the lamina propria. As analysed by flow cytometry, the frequency of the CD11c+ ALDH+ TGF-β+ DC was higher in mice fed with OVA and was further higher in mice fed with MOA (Fig. 3). The results indicate that the administration with MOA more efficiently promotes the generation of tolerogenic DC in the intestine than OVA does.

MOA increases regulatory T cells generation

Since the administration of MOA induced tolerogenic DCs (Fig. 3), we inferred that the inducible regulatory T cells (Tregs) might be also induced in the intestine. To test the hypothesis, CD4+ T cells were isolated from the lamina propria mononuclear cells of the mice (Fig. 3) and examined by flow cytometry. The results showed that a significant increase in the frequency of Tregs was detected in the intestine of mice treated with MOA (Fig. 4). The induced Tregs had a competent antigen-specific immune suppressor function (Fig. 5). The results indicate that the MOA can promote the generation of Tregs in the mouse intestine, which possibly contributes to the establishment of oral tolerance.

Administration with MOA suppresses food allergen-related Th2 inflammation

It is proposed that the breakdown of oral tolerance plays a role in the Th2 pattern inflammation (18, 19). Tregs are the major cellular component in oral tolerance (6). That administration with MOA-pulsed DCs induced Treg development in the mouse intestine (Fig. 4) implies that MOA may also facilitate the establishment of oral tolerance to inhibit skewed Th2 inflammation in the body. To determine whether this might be the case in our model, we employed a murine model of intestinal Th2 pattern inflammation following the procedures in Fig. 6A. The results showed that mice treated with OVA and alum had the Th2 pattern intestinal inflammation characterized by diarrhea (Fig. 6B), drop the core temperature after antigen challenge (Fig. 6C), and increase in the inflammatory cell extravasation including eosinophils (Fig. 6D), mast cells (Fig. 6E) and mononuclear cells (Fig. 6F) in the intestine, enhancement of antigen specific T cell proliferation (Fig. 7A, 7B), IL-4 (Fig. 7C) and serum antigen specific IgE (Fig. 7D). The sensitized mice received MOA did not show the Th2 pattern inflammation in the intestine; administration with
mutated MOA did not show any inhibitory effect on the Th2 inflammation (Fig. 6, Fig. 7).

**DISCUSSION**

The prevalence of food allergy is as high as 2–8% in the world. The clinical symptoms of food allergy vary from slightly abdominal discomfort to the life threatening anaphylactic shock. Current therapies for food allergy are not satisfactory. The present results shed new light on this field by showing that a MV-derived peptide can form adducts with food antigens. The adducts can induce tolerogenic DCs and antigen-specific Tregs, and suppress the antigen-specific Th2 pattern inflammation in the intestine.

It is reported that the MV virus-specific immunity confers viral clearance and long lasting, or lifelong, protection from clinical MV-reinfection in the body. By exploiting this feature of MV infection, the present study has discovered a novel agent in the induction of immune tolerance. The data link one of the MV-derived peptides to the induction of oral tolerance. A previous study (9) suggests that MV protein induces systemic immune suppression via MV-nucleoprotein and its receptor FcγR on dendritic cells and via the virus envelope glycoproteins and the MV-hemagglutinin cellular receptor, CD46. The present data have revealed another aspect that a MV protein-derived peptide can form adducts with food antigens; the adducts can induce tolerogenic DCs and antigen-specific Tregs in the intestine and facilitate the establishment of oral tolerance. It is noteworthy that the induced immune tolerance is antigen specific. Previous reports also mentioned that MV infection could induce the loss of antigen-specific T cell proliferation and suppress hypersensitivity responses (9); the present data expand the knowledge by showing that the antigen-specific immune tolerance can be induced by the adducts formed by MV protein-derived peptides and food antigens. Upon re-exposure to the specific antigens, the induced antigen-specific Tregs are activated to suppress the antigen-specific Th2 responses.

Activation of dendritic cells is the first step in the initiation of immune responses and oral tolerance. The present data indicate that DC activation can be induced by exposure to MOA in the intestine. Other investigators also report that MV infection activate DCs (20). Since DCs bridge the innate immunity to the adaptive immune responses, the activated DCs are conferred with the ability to modulate the naïve CD4+ T cells' properties. Exposure to MVCP does not inhibit IL-12 production in DCs (21), but increased the production of the tolerogenic molecules, such as TGF-β and ALDH, in DCs. Published data demonstrate that TGF-β and ALDH are important molecules in the induction of immune tolerance (22). As shown by the present data, MOA-primed DCs can dictate naïve CD4+ T cells to become Tregs; the latter can suppress other effector T cell activities. The results are in line with other’s reports that the MV-infected DCs actively inhibit mitogen-driven T-cell expansion in cultures (21); the
phenomenon is similar to the immune regulatory activities of Tregs (6).

In the present study, we observed a peculiar effect of MOA on modulating DC’s properties; after exposure to MOA, DCs induced antigen-specific Tregs. The inducible Tregs could only suppress the antigen-specific effector T cell responses, but could not suppress the effector T cell responses induced by other antigens, such as HRP-induced intestinal Th2 responses. The results indicate that DCs capture and process MOA, produce TGF-β and ALDH to form an environment that benefits the induction of Tregs. Since the MOA contains both MV peptides and a specific antigen; DCs are able to capture the two substances concomitantly. The results implicate that the DCs not only provide a Treg-induction environment, but also transport the specific antigen information to Th0 cells; thus induces the Th0 cells to differentiate into the antigen-specific Tregs. The study also shows that administration with MOA can suppress the antigen-specific Th2 pattern inflammation in the intestine, which implies that the MOA has the therapeutic potential in the treatment of food allergy. The procedures to produce the MOA are standard molecular biology technique, which can be easily produced and translated to the clinical use after carried out the required preclinical tests.

A large number of factors are involved in the pathogenesis of immune diseases, such as nitric oxide are involved in the over production of IgE antibodies (23), a broad array of pro-inflammatory cytokines are also associated with the pathogenesis of immune diseases (24); whether MOA can regulate those skewed cytokine production, further investigations are needed.

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Author’s address: Dr. Ping-Chang Yang, Room T3303, 50 Charlton Ave East, Hamilton, ON, Canada L8N 4A6; E-mail: yangp@mcmaster.ca

Dr. Zhanju Liu, Department of Gastroenterology, The Shanghai Tenth People’s Hospital, Tongji University, Shanghai 200072, China; E-mail: zhanjuliu8@yahoo.com