

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Immunotherapy with oligomannose-coated liposomes ameliorates allergic symptoms in a murine food allergy model

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Abstract

Background: Allergen-specific immunotherapy has been anticipated to be a disease-modifying therapy for food allergies. We previously reported that CD8⁺ regulatory T cells may prevent antigen-sensitized mice from developing allergic diarrhea. Because oligomannose-coated liposomes (OML) have been shown to induce MHC class I-restricted CD8⁺ T cell responses, we analyzed the adjuvant activities of OML for inducing regulatory CD8⁺ T cells and mucosal tolerogenic responses in allergen-sensitized mice.

Methods: The BALB/c mice that were previously sensitized to ovalbumin (OVA) were intranasally immunized with OVA-encased in OML (OVA-OML) or OVA-encased in non-coated liposomes (OVA-NL). We assessed allergic diarrhea induced by oral OVA administration, OVA-specific immunoglobulin production, and cytokine production in the intestines and mesenteric lymph nodes (MLNs).

Results: Intranasal immunization with OVA-OML, but not OVA-NL, suppressed the development of allergic diarrhea. This was associated with *in vitro* Ag-induced IL-10 production and the *in vivo* expansion of CD8⁺ CD28⁻ and CD4⁺ CD25⁺ Foxp3⁺ T cell populations among mesenteric lymph node mononuclear cells, and was significantly ablated by anti-SIGNR1 or anti-CR3 mAbs. Up-regulation of serum OVA-specific IgE was suppressed, whereas OVA-specific IgG1, IgG2a, and soluble IgA production were enhanced by intranasal administration of OVA-OML. Adoptive transfer of CD8⁺ CD28⁻ T cells but not CD28⁺ CD8⁺ T cells from the MLNs of OVA-OML-treated mice ameliorated the development of diarrhea.

Conclusion: These results suggest that intranasal immunization with Ag-encased OML may be an effective immunotherapy for food allergies, as it induces a subset of regulatory CD8⁺ T cells as well as CD4⁺ CD25⁺ Foxp3⁺ T cell and modulates humoral immune responses in allergen-sensitized mice.

Food allergy is often associated with aberrant Th2-type immune responses and the breakdown of oral tolerance to food antigens (Ags). Recently, a number of immunotherapeutic approaches have been reported that focused on the induction of oral tolerance (1, 2). Classical allergen-specific immunotherapy for food allergy by delivering Ag via the subcutaneous route can result in severe adverse reactions. Thus, appropriate Ag delivery routes and systems are needed to improve Ag targeting to the mucosal immune system.

The reduction of food allergy symptoms by immunotherapy or by outgrowing them has been hypothesized to be mediated by the induction of regulatory T cells, as well as by a shift from a Th2 to a Th1 response, and/or by the balance between allergen-specific IgE and IgG antibodies, which may regulate mast cell and basophil activities (3, 4). We recently demonstrated that inducible CD8⁺ T cells may prevent Ag-sensitized mice from developing allergic diarrhea (5). Thus, manipulating CD8⁺ regulatory T cells

is anticipated to be a novel therapeutic strategy for food allergy.

Presentation of exogenous Ags on MHC class I molecules, termed cross-presentation, is essential for the induction of CD8⁺ T-cell responses (6–8). Several studies have demonstrated that Ag mannosylation or mannosylated Ag-delivery systems, such as mannosylated-liposomes, could enhance not only MHC class II-, but also MHC class I-restricted Ag presentation and T cell stimulation by targeting mannose receptors on APCs (9, 10). Recently, oral delivery of highly mannosylated Ag has been shown to selectively target dendritic cells in the lamina propria via specific ICAM-3 grabbing non-integrin-related 1 (SIGNR1) and, thereby, induce the generation of CD4⁺ type 1 regulatory T (Tr1)-like cells that expressed IL-10 and interferon- γ (11). However, incorporating new glycosylation sites by genetic engineering or by direct attachment of mannose residues to non-glycosylated Ags may compromise their inherent immunogenicity.

Kojima et al. (12) reported that liposomes coated with synthetic neo-glycolipids comprised of mannotriose and dipalmitoylphosphatidylcholine (oligomannose-coated liposomes; OML) induced a Th1-like immune response with cytotoxic T cells specific for Ags encased in the liposomes following subcutaneous or intraperitoneal administration (12, 13). Intranasal delivery of OML was shown to induce both mucosal and systemic immune responses (14). SIGNR1 acts as a receptor for the recognition of OML (15). These results led us to hypothesize that OML could be used as a mucosal adjuvant to induce regulatory CD8⁺ T cells, Tr1-type immunity, and mucosal immune responses.

The aim of this study was to determine whether intranasal administration of OML could induce mucosal tolerogenic responses in mice that had been previously sensitized to ovalbumin (OVA), a model food Ag. Our results indicate that intranasal administration of OML induces regulatory CD8⁺ T cells and Ag-specific secretory IgA in localized tissues of OVA-sensitized mice and, thereby, ameliorates the development of allergic diarrhea.

Materials and methods

Food allergy animal models

The BALB/c mice were bred under standard pathogen-free conditions. All animal experiments were performed in accordance with institutional guidelines as approved by the Animal Care Review Board of the University of Fukui. Six-week-old female mice were sensitized to OVA or ovomucoid (OM; Sigma-Aldrich Co., St. Louis, MO, USA) by intraperitoneal injection of 100 μ g of OVA or OM in alum (Imject Alum; Thermo Scientific, Rockford, IL, USA) on days –35 and –21. Beginning on day 0, the sensitized mice received challenges by oral gavage of 20 mg OVA or OM dissolved in 0.2 ml PBS every other day for up to six doses. Before each intragastric challenge, mice were deprived of food for 2 h. Diarrhea was assessed visually and body temperature was monitored for up to 1 h following intragastric challenge.

OVA-OML and treatments

Ovalbumin-encased in oligomannose-coated liposomes (OVA-OML) and control OVA-encased in non-coated naked liposomes (OVA-NL) were purchased from Bio Med Core Inc. (Yokohama, Japan). The OML were comprised of dipalmitoylphosphatidylcholine: cholesterol: mannotriose-dipalmitoylphosphatidylethanolamine (10 : 10 : 1) with a particle size of 1 μ m (13). Ovalbumin-encased in oligomannose-coated liposomes, OVA-NL, or OVA PBS solution (20 μ l/dose each), containing 0.2 μ g OVA/dose, was administered into the left side of the nose of sensitized mice by intranasal instillation over 5 min for up to five doses from days –14 to –10. Anti-SIGNR1 mAb (ER-TR9; AbD Serotec, Oxford, UK), or anti-complement receptor 3 (CR3) mAb (M1/70; AbD Serotec), or control rat Ig (2 μ g/dose) was intranasally administered 5 min before each intranasal instillation of OVA-OML (Data S1).

Adoptive transfer of primed CD8⁺ T cells

Total CD8⁺ T cells, and CD28⁺ and CD28[–] CD8⁺ T cell subsets were purified from mesenteric lymph nodes (MLNs) of OVA-sensitized and challenged mice using MACS CD8⁺ T cell Isolation Kits (Milteni Biotec GmbH, Bergish Gladbach, Germany) and a FACS Aria III (BD Biosciences, Franklin Lakes, NJ, USA). A total of 0.8×10^6 CD8⁺ T cells, CD28⁺ CD8⁺ T cells, or CD28[–] CD8⁺ T cells per mouse were adoptively transferred into OVA-sensitized mice by intravenous injection on day –1.

Monoclonal antibodies and flowcytometry

Anti-mouse CD3, CD4, CD8, CD25, CD28, CD103, CD122, and CTLA-4 mAbs were purchased from BD Biosciences. Cells were stained using standard procedures and analyzed with a FACSCalibur (BD Biosciences).

Cell culture

Twenty-four hours after the last OVA challenge, mononuclear cells from MLNs were isolated. Cells (1×10^5 /well) were cultured either with medium alone (RPMI 1640 supplemented with 50 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2×10^{-5} M 2-mercaptoethanol, and 10% heat inactivated fetal calf serum (Sigma-Aldrich Co.) or with OVA (1 mg/ml) for 72 h. Supernatants were collected for cytokine measurements.

Cytokine and OVA-specific antibody measurements

The IL-4, IL-10, and IFN- γ in the culture supernatants were measured by a two-site sandwich ELISA, as described previously (5). Serum levels of OVA-specific IgG1, IgG2a, and IgE and the concentration of OVA-specific secretory IgA in intestinal lavage fluids, obtained by washing 10 cm of intestine with 1 ml of PBS, were determined by ELISAs, as described previously (Data S1) (5).

Real-time polymerase chain reaction

Total RNA was isolated from samples of the jejunum and mRNA levels were quantified as described previously (Data S1) (5). Results were expressed as relative units, which were calculated by the comparative C_t method.

Statistical analysis

Results are given as means $\pm$ standard errors of the mean. Comparisons of two groups used unpaired Student's t -tests, unless an F -test showed that the variances were significantly

different. When variances were significantly different, Welch's test was used. Comparisons of the occurrences of diarrhea were made by Kaplan–Meier survival analysis. A P -value < 0.05 was considered statistically significant.

Results

Nasal immunization with OVA-OML alleviates allergic diarrhea

As previously described (5, 16, 17), the OVA- and OM-sensitized mice developed allergic diarrhea accompanied by

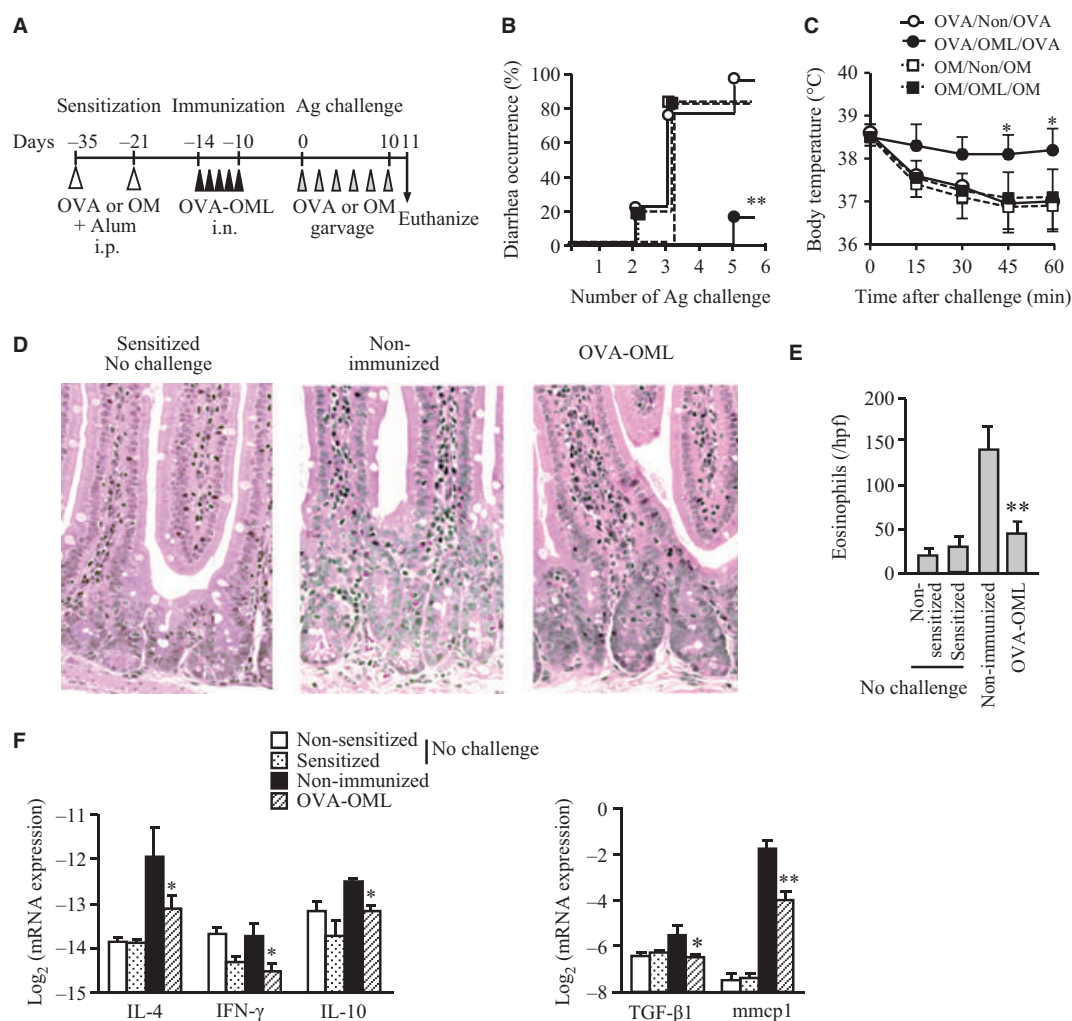


Figure 1 Intranasal immunization with OVA-OML ameliorates the development of allergic diarrhea. (A) Experimental protocol. (B) Diarrhea occurrence following repetitive challenges and (C) body temperature changes after the last challenge in non-immunized, OVA-sensitized mice (open circles), non-immunized, OM-sensitized mice (open squares), OVA-OML-immunized, OVA-sensitized mice (closed circles), or OVA-OML-immunized, OM-sensitized (closed squares); $n = 12/\text{group}$. (D) Jejunum of negative control non-challenged, positive control non-immunized or OVA-OML immunized OVA-sensitized mice. (E) Numbers of eosinophils and (F) cytokine

and mmcp1 mRNA expression in the jejunum of positive control non-immunized mice (filled bars) and OVA-OML immunized mice (hatched bars) after the last challenge. Results for non-sensitized mice (open bars) and sensitized mice (dotted bars) without challenges are negative controls. Results are means $\pm$ SE ($n = 6$) and are representative of three independent experiments. Kaplan–Meier survival analysis, unpaired Student's t -tests, and Welch's tests were used for statistical analysis. OVA, ovalbumin; OM, ovomucoid; OML, oligomannose-coated liposomes. * $P < 0.05$, ** $P < 0.01$.

hypothermia after repetitive intragastric OVA and OM challenges, respectively (Fig. 1B,C). Intranasal instillation of OVA-OML inhibited the development of allergic diarrhea and hypothermia in OVA-sensitized and challenged mice, but not in OM-sensitized and challenged mice, indicating that the effect of immunotherapy with OML is Ag-specific.

In the jejunum of OVA-sensitized mice, intragastric OVA challenges increased the infiltration of eosinophils and the mRNA expression of IL-4, IFN- γ , IL-10, and TGF- β with a Th2 dominant pattern and increased mucosal mast cell protease-1 (mmcp1) mRNA expression (Fig. 1D–F). Immunotherapy with intranasal instillation of OVA-OML significantly suppressed the accumulation of eosinophils and the mRNA up-regulations of mmcp1 and IL-4, whereas it marginally reduced the mRNA expression of IFN- γ , IL-10, and TGF β 1 (Data S1 and Fig. S1).

Oligomannose residues are essential for the adjuvant effects of OML

We next asked whether the oligomannose residues on liposomes were critical for the suppressive effects of intranasal instillation of OVA-OML. Ovalbumin-sensitized mice were intranasally immunized with OVA entrapped in carbohydrate-uncoated, bare liposomes (OVA-NL) or OVA alone.

Intranasal instillation of OVA-NL or OVA alone did not inhibit the development of allergic symptoms, the accumulation of eosinophils, and the increases in IL-4 and mmcp1 mRNA expression in the jejunum (Fig. 2).

Intranasal immunization with OVA-OML modulates Ag-specific immunoglobulin production.

Before OVA challenges there were no significant differences in the serum levels of OVA-specific IgE, IgG1 and IgG2a Abs between OVA-OML-treated mice and untreated mice (Fig. 3). After repetitive challenges, the serum OVA-specific IgE levels of OVA-OML-treated mice were lower than those of untreated mice, whereas the serum levels of OVA-specific IgG1 and IgG2a and the concentration of secretory OVA-specific IgA in the intestinal lavage fluids of OVA-OML-treated mice were higher than those of untreated mice.

OVA-OML treatment modifies the phenotype and function of MLN cells

Since MLNs play critical roles in the development of food allergy and oral tolerance (17, 18), we analyzed *in vitro* OVA-induced cytokine production by MNL mononuclear

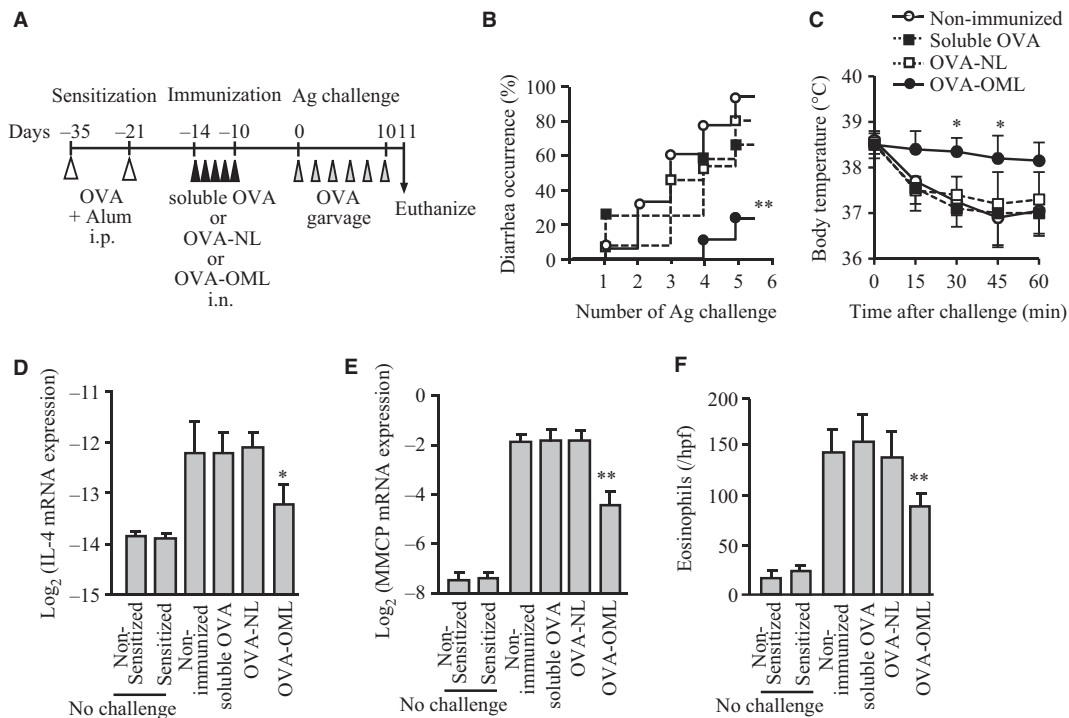


Figure 2 Intranasal immunization with OVA-NL or OVA alone fails to suppress the development of allergic diarrhea and hypothermia. (A) Experimental protocol. (B) Diarrhea occurrence following repetitive challenges and (C) body temperature changes after the last challenge in non-immunized mice (open circles) or in mice immunized with OVA alone (closed squares), OVA-NL (open squares), or OVA-OML (closed circles); $n = 12/\text{group}$. (D) IL-4, (E) mmcp1 mRNA expression, and (F) numbers of infiltrated eosinophils in the

jejunum of non-immunized mice or mice immunized with OVA alone, OVA-NL, or OVA-OML after challenges. Results for non-sensitized or sensitized mice without challenges are negative controls. Results are means $\pm$ SE ($n = 6$) and are representative of three independent experiments. Kaplan–Meier survival analysis, unpaired Student's t -tests and Welch's tests were used for statistical analysis. OVA, ovalbumin; OML, oligomannose-coated liposomes; NL, non-coated liposomes. * $P < 0.05$, ** $P < 0.01$.

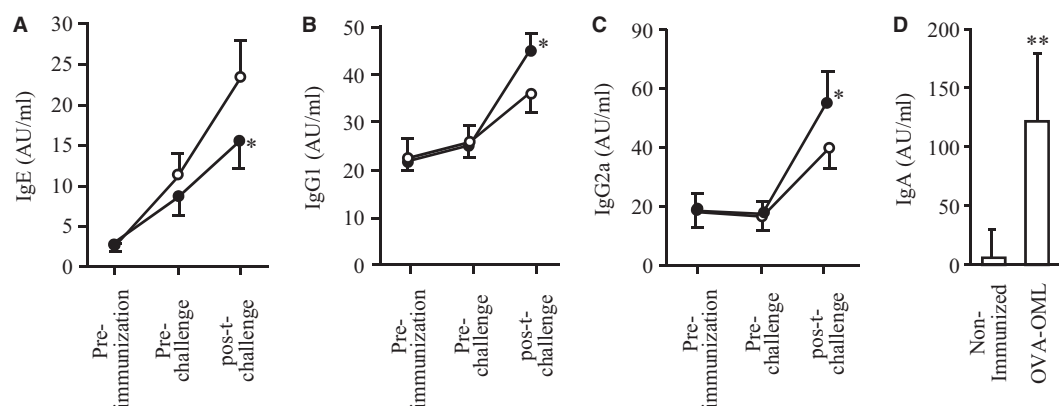


Figure 3 Intranasal immunization with OVA-OML modulates Ag-specific immunoglobulin production. Serum OVA-specific (A) IgE, (B) IgG1 and (C) IgG2a concentrations of non-immunized (open circles) or OVA-OML-immunized mice (closed circles) were determined at pre-immunization, and at pre- and post-challenges. (D) OVA-specific

secretory IgA in the intestinal lavage fluid was determined after challenges. Results are means $\pm$ SE ($n = 6$) and are representative of three independent experiments. Unpaired Student's *t*-tests and Welch's tests were used for statistical analysis. OVA, ovalbumin; OML, oligomannose-coated liposomes. * $P < 0.05$.

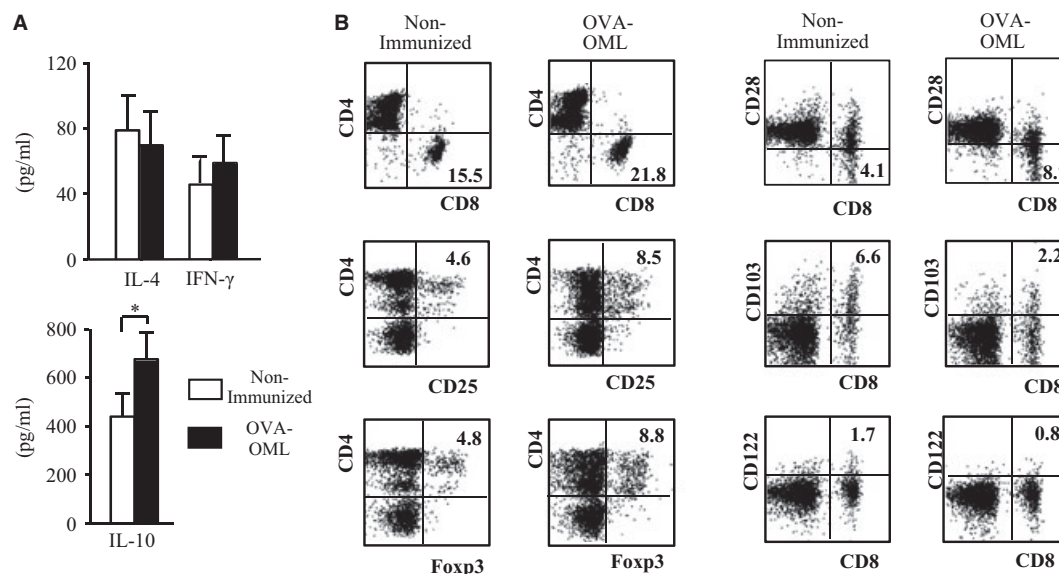


Figure 4 Intranasal immunization with OVA-OML enhances OVA-induced IL-10 production by MLN mononuclear cells *in vitro* and alters MLN T cell populations. (A) *In vitro* OVA-induced IL-4, IFN- γ , and IL-10 production by MLN mononuclear cells purified from non-immunized (open bars) and OVA-OML-immunized (filled bars) mice after challenges. Results are means $\pm$ SE ($n = 6$) and are representative of three independent experiments. Unpaired Student's *t*-tests

were used for statistical analysis. * $P < 0.05$ (B) Results for cell surface phenotypes of MLN T cells from non-immunized and OVA-OML-immunized mice after challenges were obtained by gating on CD3⁺ cells. Indicated values are the percentages of each subset among MLN T cells. Results of one representative experiment of six are shown. OVA, ovalbumin; OML, oligomannose-coated liposomes; MLN, mesenteric lymph node.

cells purified from the mice after repetitive OVA challenges. Intranasal immunization with OVA-OML significantly increased the *in vitro* OVA-induced IL-10 production by MLN mononuclear cells, but did not significantly affect OVA-induced IL-4 and IFN- γ production (Fig. 4A).

To assess the expansion of regulatory T cell populations in MLNs after intranasal immunization with OVA-OML, we analyzed MLN T cell subsets. As shown in Fig 4B, the frequencies of CD4⁺ Foxp3⁺ T cells, and CD8⁺ CD28⁻ T cells, but not that of CD8⁺ CD122⁺ T cells, or

CD8⁺ CD103⁺ T cells among MLN T cells significantly increased in the intranasally-immunized mice compared with non-immunized mice.

Adoptive transfer of MLN CD8⁺ T cells alleviates allergic diarrhea.

To determine whether the MLN CD8⁺ T cells functioned as regulatory T cells *in vivo* to inhibit the development of allergic diarrhea, mononuclear cells or CD8⁺ T cells that were

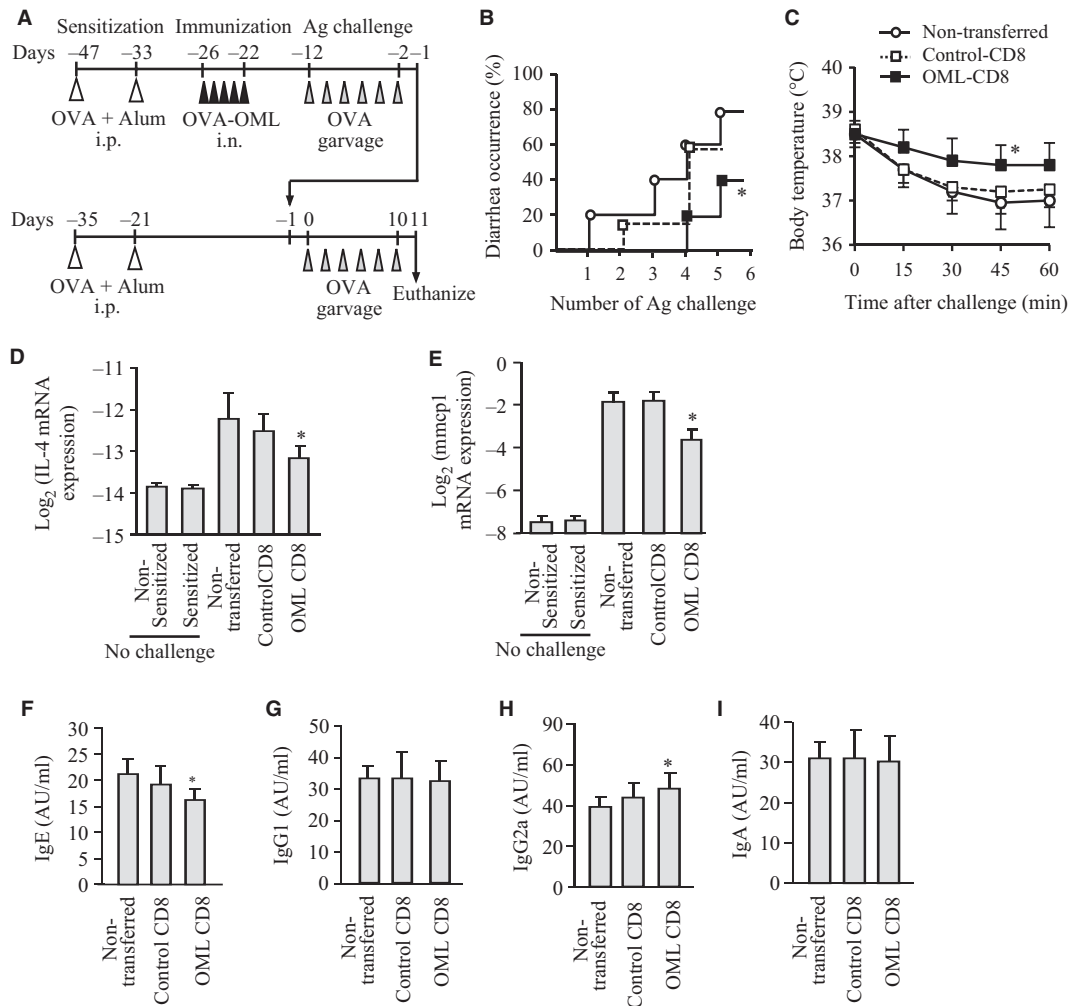


Figure 5 Adoptive transfer of MLN CD8⁺ T cells from OVA-OML-treated mice ameliorates allergic diarrhea. (A) Experimental protocol. (B) Diarrhea occurrence following repetitive challenges and (C) body temperature change after the last challenge in non-transferred mice (open circles), mice with CD8⁺ T cells transferred from non-immunized controls (open squares), and OVA-OML immunized mice (closed squares); $n = 12/\text{group}$. (D) IL-4 and (E) mmcp1 mRNA expression in the jejunum, serum OVA-specific (F) IgE, (G) IgG1, and (H) IgG2a concentrations, and (I) OVA-specific secretory

IgA in the intestinal lavage fluid from non-transferred mice or from mice with MLN CD8⁺ T cells transferred from non-immunized or OVA-OML immunized mice. mRNA expression for non-sensitized and sensitized mice without challenges are negative controls. Results are means $\pm$ SE ($n = 6$) and are representative of three independent experiments. Kaplan–Meier survival analysis, unpaired Student's t -tests, and Welch's tests were used for statistical analysis. OVA, ovalbumin; OML, oligomannose-coated liposomes; MLN, mesenteric lymph node. * $P < 0.05$.

purified from OVA-OML-treated or untreated mice after repetitive OVA challenges were adoptively transferred to other OVA-sensitized mice. As shown in Fig. 5, adoptive transfer of CD8⁺ T cells from OVA-OML-treated mice, but not from untreated mice, significantly inhibited the development of diarrhea and hypothermia. These cells also abrogated the up-regulation of mmcp1 and IL-4 mRNA expression in the intestine, although to a lesser extent. Adoptive transfer of CD8⁺ T cells from OVA-OML treated mice slightly, but significantly, suppressed the up-regulation of serum levels of OVA-specific IgE and enhanced serum OVA-specific IgG2a levels, but did not affect OVA-specific secretory IgA levels in intestinal lavage fluids (Fig. 5).

To characterize the phenotype of CD8⁺ T cells with these suppressive effects, CD28[−] CD8⁺ T cells were purified from MLNs of OVA-OML-treated mice using fluorescence-activated cell sorting. CD28[−] CD8⁺ T cells were responsible for the suppressive effects on the allergic symptoms *in vivo*, whereas CD28⁺ CD8⁺ T cells had little, if any, effect (Fig. 6A–C).

SINGNR1 and CR3 are necessary for the therapeutic effects of OVA-OML

Since CR3 is known to cooperatively act with SINGNR1 as a receptor for recognition and uptake of OMLs (15), we

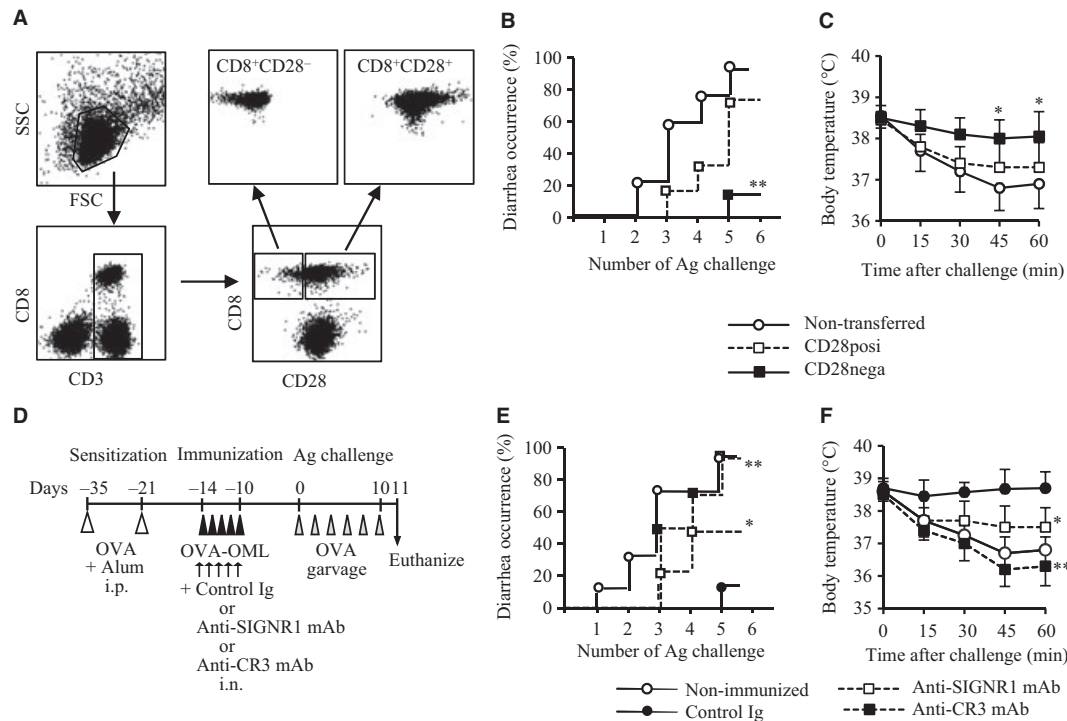


Figure 6 Adoptive transfer of MLN CD28⁻ CD8⁺ T cells but not CD28⁺ CD8⁺ T cells ameliorates allergic diarrhea. (A) CD28⁻ CD8⁺ T cells and CD28⁺ CD8⁺ T cells were purified from MLNs mononuclear cells. (B) Diarrhea occurrence following repetitive challenges and (C) body temperature change after the last challenge in non-transferred mice (open circles), or in mice with CD28⁻ CD8⁺ T cells (closed squares) or CD28⁺ CD8⁺ T cells (open squares; 0.8×10^6 /mouse) transferred from OVA-OML immunized mice; $n = 8$ /group).

administered anti-SIGNR1 mAb or anti-CR3 mAb to OVA-sensitized mice before intranasal instillation of OVA-OML. The suppressive effects of intranasal instillation of OVA-OML were significantly ablated by anti-SIGNR1 mAb or anti-CR3 mAb (Fig. 6D–F).

Discussion

In the present study, we examined a novel therapeutic approach for food allergy using intranasal immunization of Ag entrapped in OML. The mechanisms of intranasal immunotherapy with OML appeared to be due (1) to the induction of regulatory CD8⁺ T cells, primarily among the CD28⁻ CD8⁺ T cell population, as well as CD4⁺ CD25⁺ Foxp3⁺ T cells and (2) to the modulation of humoral immune responses, including enhanced Ag-specific IgGs and secretory IgA production and suppressed up-regulation of Ag-specific IgE. SIGNR1 and CR3 are involved in the therapeutic effects of OVA-OML.

Various phenotypes of regulatory CD8⁺ T cells have been identified in different experimental systems (19, 20). In an experimental inflammatory bowel disease model, naturally occurring CD8⁺ CD28⁻ CD122⁻ regulatory T cells inhibited

(D) Experimental protocol for anti-SIGNR1 and anti-CR3 mAb treatments (E) Diarrhea occurrence following repetitive challenges and (F) body temperature change after the last challenge in non-immunized mice (open circles), or in mice pre-treated with control Ig (closed circles), anti-SIGNR1 mAb (open squares), or anti-CD11b mAb (closed squares); $n = 12$ /group. Kaplan–Meier survival analysis was used for statistical analysis. OVA, ovalbumin; OML, oligomannose-coated liposomes; MLN, mesenteric lymph node. * $P < 0.05$.

IFN- γ production by colitogenic CD4⁺ T cells and prevented colitis. (21). Intraperitoneal application of zwitterionic capsular polysaccharides of commensal bacteria increased CD8⁺ CD28⁻ T cells, which exhibited immunosuppressive properties on CD4⁺ T cells (22). The expanded CD8⁺ CD28⁻ T cell population found in the MLNs of OVA-OML immunized mice displayed a more robust regulatory function than did the CD8⁺ CD28⁺ T cell population *in vivo*, suggesting that the therapeutic effects of OML instillation could be attributed, in part, to the induction of CD8⁺ CD28⁻ regulatory T cells.

Treatment of peanut Ag-sensitized mice with a Chinese herbal medicine preparation (FAHF-2) prevented oral Ag-challenge-induced anaphylaxis (23). The inhibitory effect of FAHF-2 was mediated by increased IFN- γ production by CD8⁺ T cells. By comparison, we recently demonstrated that the IL-10-producing capability of CD8⁺ T cells and IL-10 expression by MLNs were associated with alleviating allergic diarrhea (5). IL-10 and TGF- β play essential roles in the suppressive activities of CD8⁺ CD28⁻ regulatory T cells (21, 22). Takayama et al. (24) reported that IL-10-producing CD4⁺ CD25⁺ Foxp3⁺ T cells in Peyer's patches inhibited the development of allergic diarrhea. Intranasal immunization with

OVA-OML increased the percentages of CD4⁺ CD25⁺ Foxp3⁺ T cells and CD28⁻ CD8⁺ T cells, and enhanced Ag-induced IL-10 production by MLN cells *in vitro*. IL-10 rather than IFN- γ production by MLNs may be involved in the therapeutic effects of OVA-OML immunization.

It is well known that the production of IgG1 and IgE is regulated by a Th2 response, whereas IgG2a production is regulated by a Th1 response (25). Immunotherapy with OVA-OML enhanced the up-regulation of both OVA-specific IgG2a and IgG1, indicating no appreciable shift from Th2- to Th1-dominant humoral responses. IL-21R^{-/-} mice exhibit impaired Ag-specific IgG1 production and augmented Ag-specific IgE production (26). Although IL-21 was not detected in the supernatants of OVA-stimulated MLN mononuclear cells (data not shown), IL-21 might explain the discordant IgE and IgG1 responses in OVA-OML-treated mice. Schmitz et al. (27) reported that immunotherapy with recombinant cat allergen displayed on virus-like particles induced allergen-specific IgG1 production and abolished an IgE memory response in allergen-sensitized mice and that the allergen-specific IgG antibodies alleviated allergic symptoms in Fc γ RIIb-dependent and independent manners. The enhanced OVA-specific IgG1 and IgG2a production might also account for the therapeutic effects of OVA-OML immunotherapy.

Actively tolerized mice were found to have higher fecal Ag-specific IgA titers than anaphylactic mice (28). Adoptive transfer of CD8⁺ T cells from OVA-OML immunized mice failed to induce OVA-specific secretory IgA production and tended to exhibit weaker inhibitory effects on the development of food allergy than OVA-OML immunization itself (Figs 2 and 5). Although this might have been due to the small number of transferred CD8⁺ T cells, secretory IgA may also play a protective role against the development of food allergy by preventing the uptake of food Ag with intact epitopes from mucosal surfaces.

In summary, our results demonstrate that Ag entrapped in OML has potential uses for treating established allergies and at least two different mechanisms may be involved: induction

of regulatory T cells and modulation of humoral immunity. It is difficult to determine the relative contributions of CD8⁺ CD28⁻ and CD4⁺ CD25⁺ Foxp3⁺ T cells and humoral immunity to OML-induced suppression of food allergic symptoms. Multiple mechanisms may act synergistically to suppress allergic symptoms. Because OML are comprised of innocuous materials, are ubiquitously distributed throughout the body (29), they could be useful as an immunotherapy adjuvant and Ag delivery system for food allergy.

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Author contribution

AK and HS contributed equally to this work. Each named author in this report contributed to its content either through being integral to the experimental planning and/or its implementation at the bench.

Conflict of interest

The authors declare that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods and their results.

Figure S1. The inhibitory effects of OVA-OML immunization depend on the doses and frequency of intranasal instillation.

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Supplemental material

Methods

Subcutaneous or intraperitoneal immunization with OVA-OML

On day -14, 100 μ l of OVA-OML solution containing 1 μ g of OVA was injected either subcutaneously or intraperitoneally. Beginning on day 0, the sensitized mice received challenges by oral gavage of 20 mg OVA dissolved in 0.2 ml PBS every other day for up to 6 doses.

Cytokine measurements

IL-21 in the culture supernatants were measured by a two-site sandwich ELISA, using the protocols recommended by the antibody suppliers (R&D systems Inc. Minneapolis, MN).

OVA-specific antibody measurements

Biotin-conjugated anti-mouse IgG1, IgG2a, IgE, and IgA monoclonal antibodies (BD Biosciences) were used as the detection antibodies. Standard curves were derived using hyperimmune anti-OVA sera. Results were expressed as arbitrary antibody units relative

to the standard. Results were expressed as arbitrary antibody units relative to the standard.

Real-time polymerase chain reaction

Total RNA was isolated from samples of the jejunum with an RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), and was reverse transcribed with Superscript III (Invitrogen, Carlsbad, CA). mRNA levels were quantified by real-time quantitative PCR with an ABI PRISM7000 Sequence Detector (Applied Biosystems Japan Ltd., Tokyo Japan) using TaqMan Pre-Developed Assay Reagents according to the manufacturer's instructions. PCR amplification of the housekeeping gene GAPDH was performed for each sample for normalization between samples. Results were expressed as relative units, which were calculated by the comparative Ct method; the Ct for GAPDH was subtracted from the Ct for the target amplicon, and then the normalized amount of target was calculated using the equation $2^{-\Delta Ct}$.

Results and Discussion

Nasal immunization with OVA-OML alleviates allergic diarrhea.

Five times instillation of 5 μ l/ dose or twice instillation of 20 μ l/ dose of OVA-OML

also inhibited the development of allergic diarrhea and hypothermia in OVA-sensitized and challenged mice to a lesser extent (Figure S). Thus, the inhibitory effects of intranasal immunization with OVA-OML are dependent on the dose and administration frequency of OVA-OML.

Subcutaneous immunization with a synthetic peptide encapsulated in OML induced peptide-specific, MHC class I-restricted CD8⁺ cytotoxic lymphocytes (29). However, neither subcutaneous nor intraperitoneal immunization with OVA-OML could inhibit the development of allergic diarrhea in sensitized mice (data not shown), indicating that the OVA-OML administration route was important for these inhibitory effects. Intranasal instillation of OVA-OML did not increase the percentages of perforin and CD95 ligand expressing CD8⁺ T cells (data not shown). Thus, typical CD8⁺ T cells' cytotoxic activity may not be essential for inhibiting allergic diarrhea.

Sublingual immunization with OVA-OML showed a tendency to alleviate allergic diarrhea, which did not reach a statistical significance (data not shown). OML might be susceptible to gastrointestinal digestion. Awake mice immediately swallowed OVA-OML, resulting in the disruption of its adjuvant activity. Since patients are voluntarily able to keep OVA-OML in the sublingual cavity for a certain period of time, sublingual immunization with OML would be practically more applicable and tolerable

than intranasal immunization from the point of view of comorbidities and adverse effects.

Figure S1. The inhibitory effects of OVA-OML immunization depend on the doses and frequency of intranasal instillation. (A) Experimental protocol. (B) Diarrhea occurrence following repetitive challenges and (C) body temperature changes after the last challenge in non-immunized mice (open circles) or in mice immunized with OVA-OML; 20 μ l/dose X twice (closed squares), 5 μ l/dose X 5 times (open squares), or 20 μ l/dose X 5 times (closed circles); n = 12/group. (D) IL-4, (E) mmcp1 mRNA expression, and (F) numbers of infiltrated eosinophils in the jejunum of non-immunized mice or mice immunized with OVA alone, OVA-NL, or OVA-OML after challenges. Results for non-sensitized or sensitized mice without challenges are negative controls. Results are means $\pm$ SE (n = 6) and are representative of 3 independent experiments. Kaplan-Meier survival analysis, unpaired Student's t-tests and Welch's tests were used for statistical analysis. * p < 0.05, ** p < 0.01

Figure S1

