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

Original Articles

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Short title: Immunotherapy with oligomannose-coated liposomes

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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: 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 $CD8^+CD28^+$ 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.

Key Words: food allergy; liposome; mouse model; oligomannose; regulatory T cells

Word count: 2484

Introduction

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 manonnosylated 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 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

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) by intraperitoneal injection of 100 μg of OVA or OM in alum (Imject Alum, Thermo Scientific, Rockford, IL) 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 6 doses. Before each intragastric challenge, mice were deprived of food for 2 hours. Diarrhea was assessed visually and body temperature was monitored for up to 1 hour following intragastric challenge.

OVA-OML and treatments

OVA-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 μm (13). OVA-OML, OVA-NL, or OVA PBS solution (20 μl/dose each), containing 0.2 μg OVA/dose, was administrated into the left side of the nose of sensitized mice by intranasal instillation over 5 minutes for up to 5 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 μg/dose) was intranasally administrated 5 minutes before each intranasal instillation of OVA-OML.

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 FACSCanto II (BD Biosciences). 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 x 10^5/well) were cultured either with medium alone (RPMI 1640 supplemented with 50 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 x 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

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 (5).

147 **Real-time polymerase chain reaction**

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

151

152 **Statistical analysis**

153 Results are given as means ± standard errors of the mean. Comparisons of 2 groups
154 used unpaired Student's *t*-tests, unless an *F*-test showed that the variances were
155 significantly different. When variances were significantly different, Welch's test was
156 used. Comparisons of the occurrences of diarrhea were made by Kaplan-Meier survival
157 analysis. A p-value < 0.05 was considered statistically significant.

158

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 hypothermia after repetitive intragastric OVA and OM challenges, respectively (Fig. 1B and 1C). 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 effects 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, while it marginally reduced the mRNA expression of IFN-γ, IL-10, and TGFβ1.

Oligomannose residues are essential for the adjuvant effects of OML.

We next asked whether the oligomannose residues on liposomes were critical for the

177 suppressive effects of intranasal instillation of OVA-OML. OVA-sensitized mice were
178 intranasally immunized with OVA entrapped in carbohydrate-uncoated, bare liposomes
179 (OVA-NL) or OVA alone. Intranasal instillation of OVA-NL or OVA alone did not
180 inhibit the development of allergic symptoms, the accumulation of eosinophils, and the
181 increases in IL-4 and mmcp1 mRNA expression in the jejunum (Fig. 2).
182
183 **Intranasal immunization with OVA-OML modulates Ag-specific immunoglobulin**
184 **production**.
185 Before OVA challenges there were no significant differences in the serum levels of
186 OVA-specific IgE, IgG1 and IgG2a Abs between OVA-OML-treated mice and untreated
187 mice (Fig. 3). After repetitive challenges, the serum OVA-specific IgE levels of
188 OVA-OML-treated mice were lower than those of untreated mice, whereas the serum
189 levels of OVA-specific IgG1 and IgG2a and the concentration of secretory
190 OVA-specific IgA in the intestinal lavage fluids of OVA-OML-treated mice were higher
191 than those of untreated mice.
192
193 **OVA-OML treatment modifies the phenotype and function of MLN cells**
194 Because 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 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 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

213 also abrogated the up-regulation of mmcp1 and IL-4 mRNA expression in the intestine,
214 although to a lesser extent. Adoptive transfer of $CD8^+$ T cells from OVA-OML treated
215 mice slightly, but significantly, suppressed the up-regulation of serum levels of
216 OVA-specific IgE and enhanced serum OVA-specific IgG2a levels, but did not affect
217 OVA-specific secretory IgA levels in intestinal lavage fluids (Figure 5).

218 To characterize the phenotype of $CD8^+$ T cells with these suppressive effects,
219 $CD28^-CD8^+$ T cells were purified from MLNs of OVA-OML-treated mice using
220 fluorescence-activated cell sorting. $CD28^-CD8^+$ T cells were responsible for the
221 suppressive effects on the allergic symptoms *in vivo*, whereas $CD28^+CD8^+$ T cells had
222 little, if any, effect (Figure 6A-C).

223

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

225 Since CR3 is known to cooperatively act with SIGNR1 as a receptor for recognition
226 and uptake of OMLs (15), we administered anti-SIGNR1 mAb or anti-CR3 mAb to
227 OVA-sensitized mice before intranasal instillation of OVA-OML. The suppressive
228 effects of intranasal instillation of OVA-OML were significantly ablated by
229 anti-SIGNR1 mAb or anti-CR3 mAb (Figure 6D-F).

230

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 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

249 attributed, in part, to the induction of $CD8^+CD28^-$ regulatory T cells.

250 Treatment of peanut Ag-sensitized mice with a Chinese herbal medicine preparation
251 (FAHF-2) prevented oral Ag-challenge-induced anaphylaxis (23). The inhibitory effect
252 of FAHF-2 was mediated by increased IFN-γ production by $CD8^+$ T cells. By
253 comparison, we recently demonstrated that the IL-10-producing capability of $CD8^+$ T
254 cells and IL-10 expression by MLNs were associated with alleviating allergic diarrhea
255 (5). IL-10 and TGF-β play essential roles in the suppressive activities of $CD8^+CD28^-$
256 regulatory T cells (21, 22). Takayama et al. (24) reported that IL-10-producing
257 $CD4^+CD25^+Foxp3^+$ T cells in Peyer's patches inhibited the development of allergic
258 diarrhea. Intranasal immunization with OVA-OML increased the percentages of
259 $CD4^+CD25^+Foxp3^+$ T cells and $CD28^-CD8^+$ T cells, and enhanced Ag-induced IL-10
260 production by MLN cells *in vitro*. IL-10 rather than IFN-γ production by MLNs may be
261 involved in the therapeutic effects of OVA-OML immunization.

262 It is well known that the production of IgG1 and IgE is regulated by a Th2 response,
263 whereas IgG2a production is regulated by a Th1 response (25). Immunotherapy with
264 OVA-OML enhanced the up-regulation of both OVA-specific IG2a and IgG1,
265 indicating no appreciable shift from Th2- to Th1-dominant humoral responses.
266 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 FcγRIIb-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 (Fig. 2 and Fig. 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 suppress of 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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Conflict of interest: The authors declare that they have no conflict of interest.

Author contribution: A.K. and H.S. 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.

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391 **Figure legends**

392 **Figure 1.** Intranasal immunization with OVA-OML ameliorates the development of
393 allergic diarrhea. (A) Experimental protocol. (B) Diarrhea occurrence following
394 repetitive challenges and (C) body temperature changes after the last challenge in
395 non-immunized, OVA-sensitized mice (open circles), non-immunized, OM-sensitized
396 mice (open squares), OVA-OML-immunized, OVA-sensitized mice (closed circles), or
397 OVA-OML-immunized, OM-sensitized (closed squares); n = 12/group. (D) Jejunum
398 of negative control non-challenged, positive control non-immunized or OVA-OML
399 immunized OVA-sensitized mice. (E) Numbers of eosinophils and (F) cytokine and
400 mmcp1 mRNA expression in the jejunum of positive control non-immunized mice
401 (filled bars) and OVA-OML immunized mice (hatched bars) after the last challenge.
402 Results for non-sensitized mice (open bars) and sensitized mice (dotted bars) without
403 challenges are negative controls. Results are means ± SE (n = 6) and are representative
404 of 3 independent experiments. Kaplan-Meier survival analysis, unpaired Student's
405 *t*-tests, and Welch's tests were used for statistical analysis. *$p < 0.05$, **$p < 0.01$

406

407 **Figure 2.** Intranasal immunization with OVA-NL or OVA alone fails to suppress the
408 development of allergic diarrhea and hypothermia. (A) Experimental protocol. (B)

409 Diarrhea occurrence following repetitive challenges and (C) body temperature changes
410 after the last challenge in non-immunized mice (open circles) or in mice immunized
411 with OVA alone (closed squares), OVA-NL (open squares), or OVA-OML (closed
412 circles); n = 12/group. (D) IL-4, (E) mmcp1 mRNA expression, and (F) numbers of
413 infiltrated eosinophils in the jejunum of non-immunized mice or mice immunized with
414 OVA alone, OVA-NL, or OVA-OML after challenges. Results for non-sensitized or
415 sensitized mice without challenges are negative controls. Results are means ± SE (n = 6)
416 and are representative of 3 independent experiments. Kaplan-Meier survival analysis,
417 unpaired Student's *t*-tests and Welch's tests were used for statistical analysis. *$p < 0.05$,
418 **$p < 0.01$
419
420 **Figure 3.** Intranasal immunization with OVA-OML modulates Ag-specific
421 immunoglobulin production. Serum OVA-specific (A) IgE, (B) IgG1 and (C) IgG2a
422 concentrations of non-immunized (open circles) or OVA-OML-immunized mice (closed
423 circles) were determined at pre-immunization, and at pre- and post-challenges. (D)
424 OVA-specific secretory IgA in the intestinal lavage fluid was determined after
425 challenges. Results are means ± SE (n = 6) and are representative of 3 independent
426 experiments. Unpaired Student's *t*-tests and Welch's tests were used for statistical

analysis. *$p < 0.05$

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 ± SE (n = 6) and are representative of 3 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 6 are shown.

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

445 squares); n = 12/group. (D) IL-4 and (E) mmcp1 mRNA expression in the jejunum,
446 serum OVA-specific (F) IgE, (G) IgG1, and (H) IgG2a concentrations, and (I)
447 OVA-specific secretory IgA in the intestinal lavage fluid from non-transferred mice or
448 from mice with MLN $CD8^+$ T cells transferred from non-immunized or OVA-OML
449 immunized mice. mRNA expression for non-sensitized and sensitized mice without
450 challenges are negative controls. Results are means ± SE (n = 6) and are representative
451 of 3 independent experiments. Kaplan-Meier survival analysis, unpaired Student's
452 *t*-tests, and Welch's tests were used for statistical analysis. * $p< 0.05$
453
454 **Figure 6.** Adoptive transfer of MLN $CD28^-CD8^+$ T cells but not $CD28^+CD8^+$ T cells
455 ameliorates allergic diarrhea. (A) $CD28^-CD8^+$ T cells and $CD28^+CD8^+$ T cells were
456 purified from MLNs mononuclear cells. (B) Diarrhea occurrence following repetitive
457 challenges and (C) body temperature change after the last challenge in non-transferred
458 mice (open circles), or in mice with $CD28^-CD8^+$ T cells (closed squares) or $CD28^+CD8^+$
459 T cells (open squares) (0.8×10^6/mouse) transferred from OVA-OML immunized mice;
460 n = 8/group). (D) Experimental protocol for anti-SIGNR1 and anti-CR3 mAb treatments
461 (E) Diarrhea occurrence following repetitive challenges and (F) body temperature
462 change after the last challenge in non-immunized mice (open circles), or in mice

463 pre-treated with control Ig (closed circles), anti-SIGNR1 mAb (open squares), or
464 anti-CD11b mAb (closed squares); n = 12/group. Kaplan-Meier survival analysis was
465 used for statistical analysis. * $p < 0.05$

Figure 1

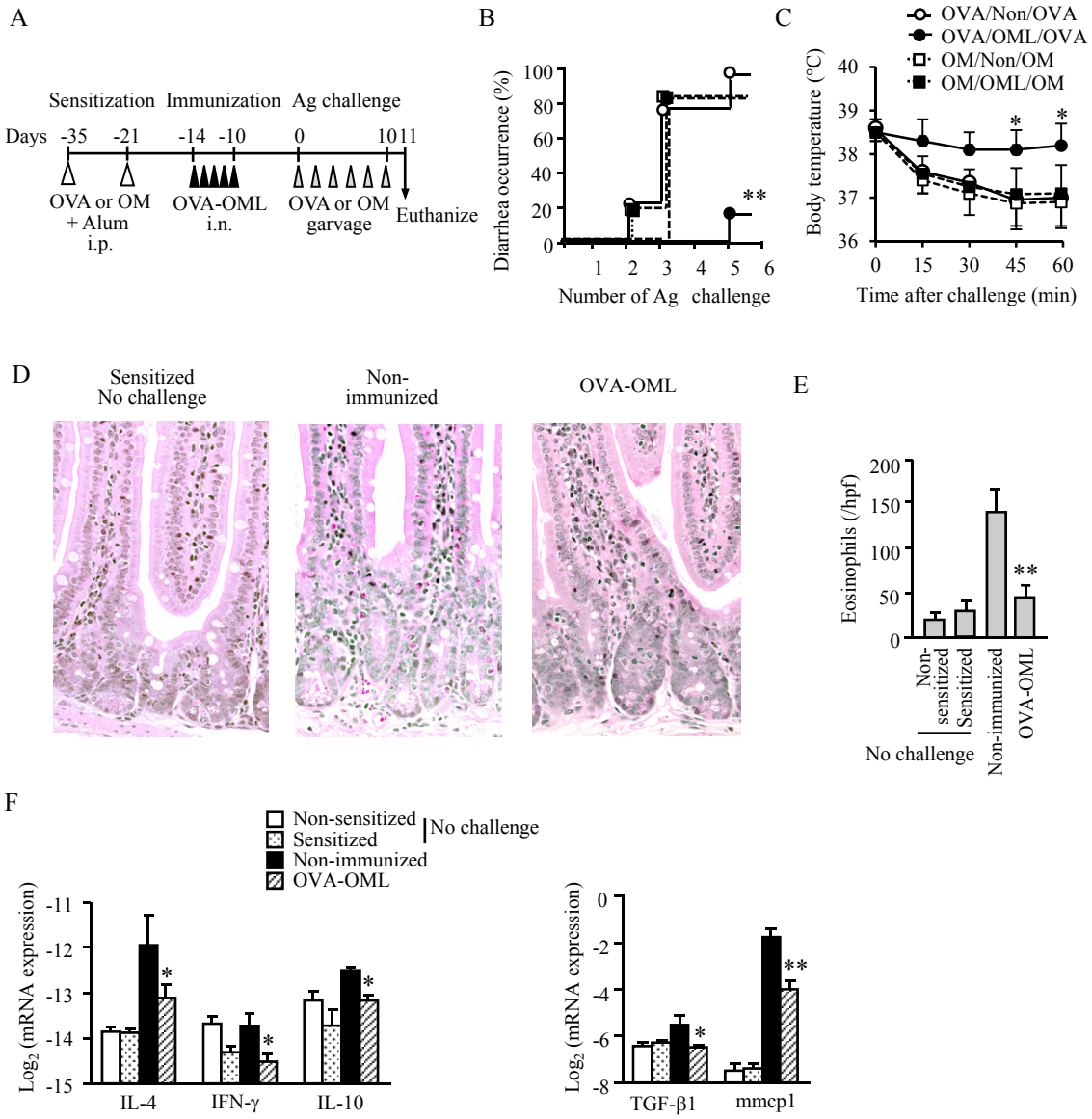

A
Sensitization
Immunization
Ag challenge
Days -35 -21 -14 -10 0 10 11
OVA or OM + Alum i.p.
OVA-OML i.n.
OVA or OM garvage
Euthanize
B
Diarrhea occurrence (%)
Number of Ag challenge
C
OVA/Non/OVA
OVA/OML/OVA
OM/Non/OM
OM/OML/OM
Body temperature (°C)
Time after challenge (min)
D
Sensitized No challenge
Non-immunized
OVA-OML
E
Eosinophils (/hpf)
Non-sensitized
Sensitized
Non-immunized
OVA-OML
No challenge
F
Non-sensitized
Sensitized
Non-immunized
OVA-OML
No challenge
Log2 (mRNA expression)
IL-4
IFN-γ
IL-10
TGF-β1
mmcp1

Figure 2

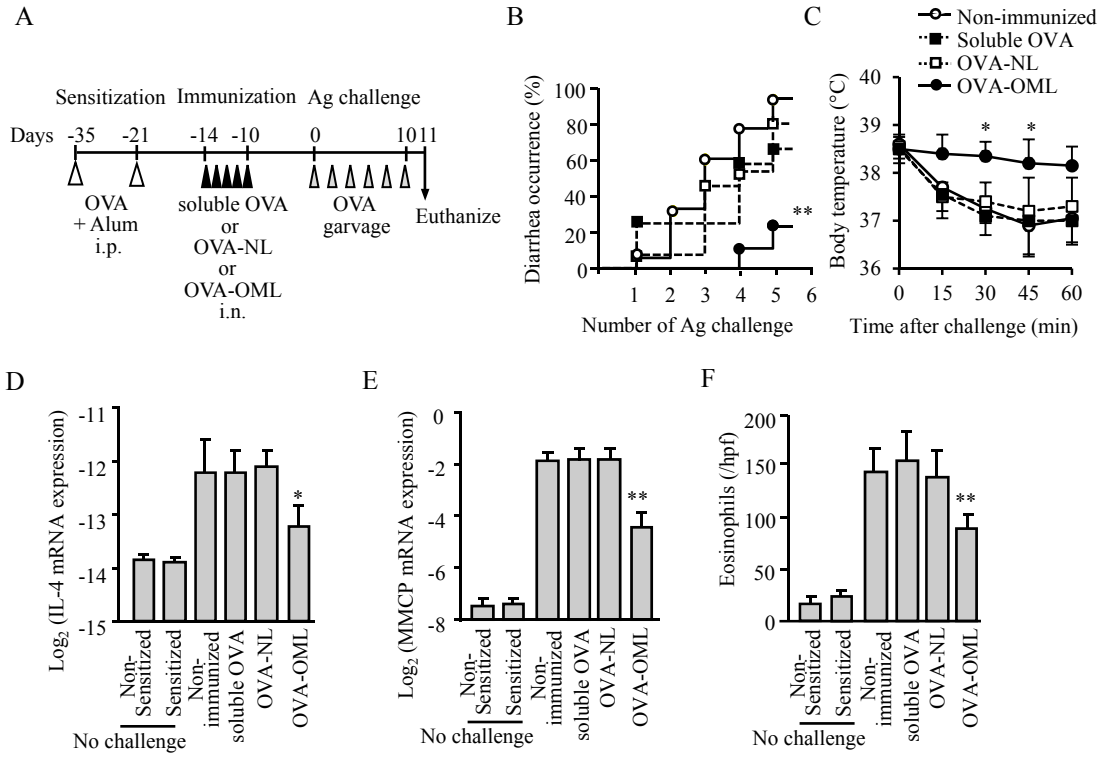

A
Sensitization
Immunization
Ag challenge
Days -35 -21 -14 -10 0 10 11
OVA + Alum i.p.
soluble OVA or OVA-NL or OVA-OML i.n.
OVA garvage
Euthanize
B
Diarrhea occurrence (%)
Number of Ag challenge
C
Non-immunized
Soluble OVA
OVA-NL
OVA-OML
Body temperature (°C)
Time after challenge (min)
D
Log2 (IL-4 mRNA expression)
E
Log2 (MMCP mRNA expression)
F
Eosinophils (/hpf)
Non-Sensitized
Sensitized
Non-immunized
soluble OVA
OVA-NL
OVA-OML
No challenge

Figure 3

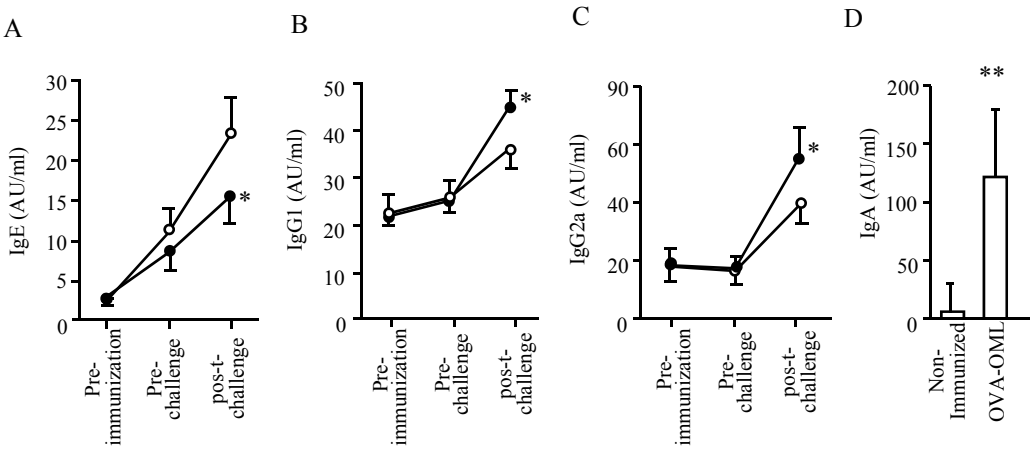

A
B
C
D
IgE (AU/ml)
IgG1 (AU/ml)
IgG2a (AU/ml)
IgA (AU/ml)
Pre-immunization
Pre-challenge
pos-t-challenge
Non-Immunized
OVA-OML
*
**

Figure 4

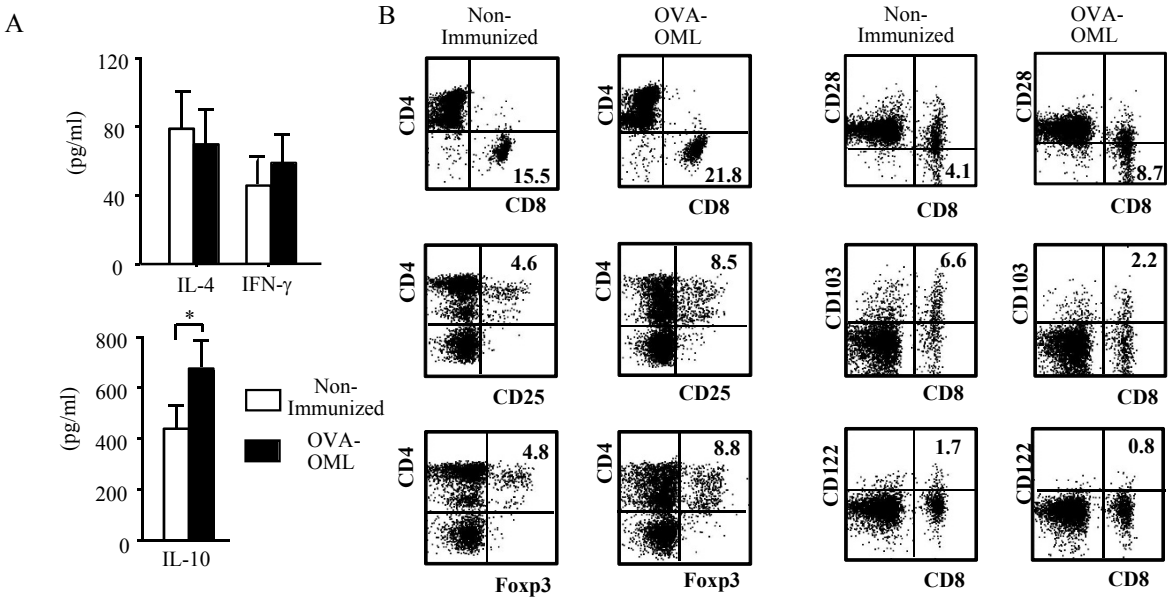

A
120
80
40
0
(pg/ml)
IL-4
IFN-γ
*
800
600
400
200
0
(pg/ml)
IL-10
Non-Immunized
OVA-OML
B
Non-Immunized
OVA-OML
Non-Immunized
OVA-OML
CD4
CD8
15.5
21.8
CD28
4.1
8.7
4.6
8.5
CD25
CD103
6.6
2.2
4.8
8.8
Foxp3
CD122
1.7
0.8

Figure 5

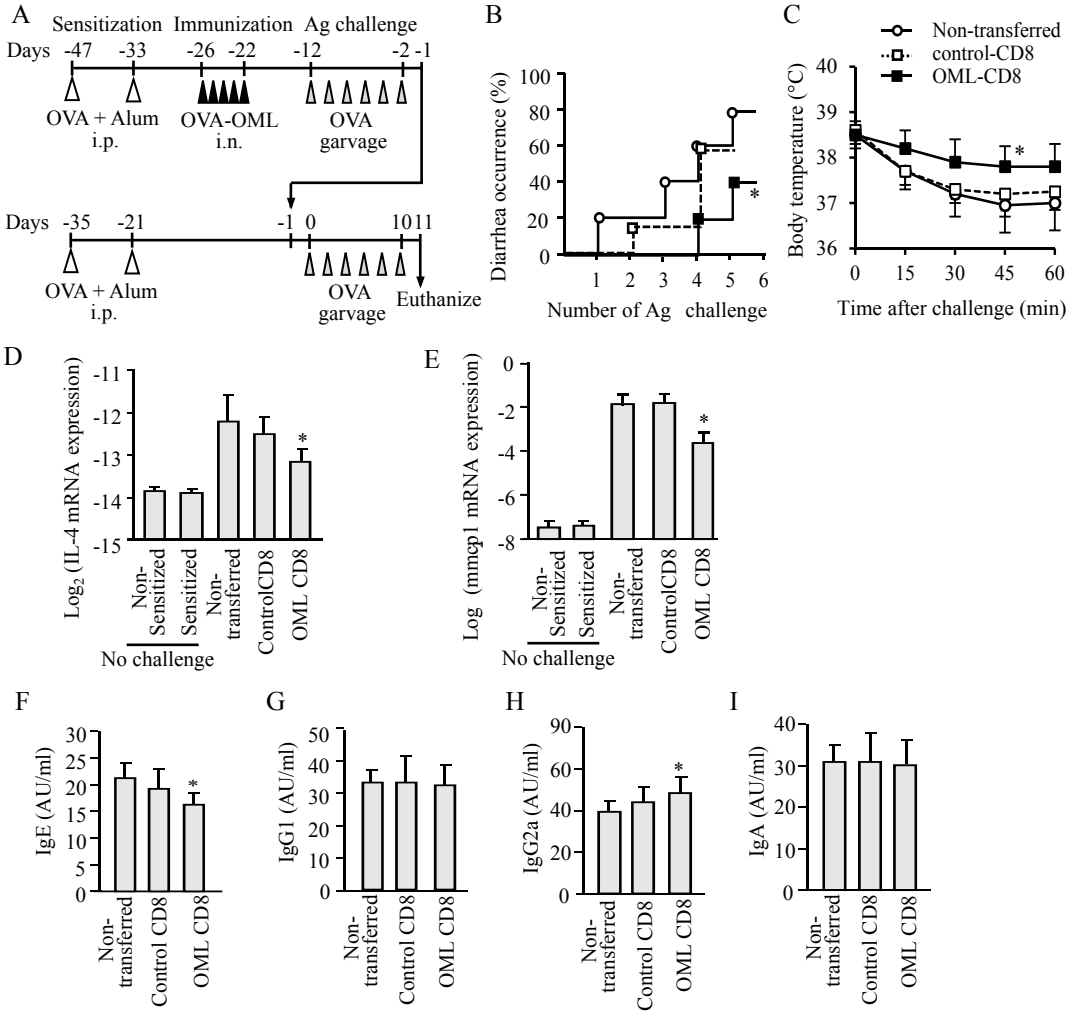
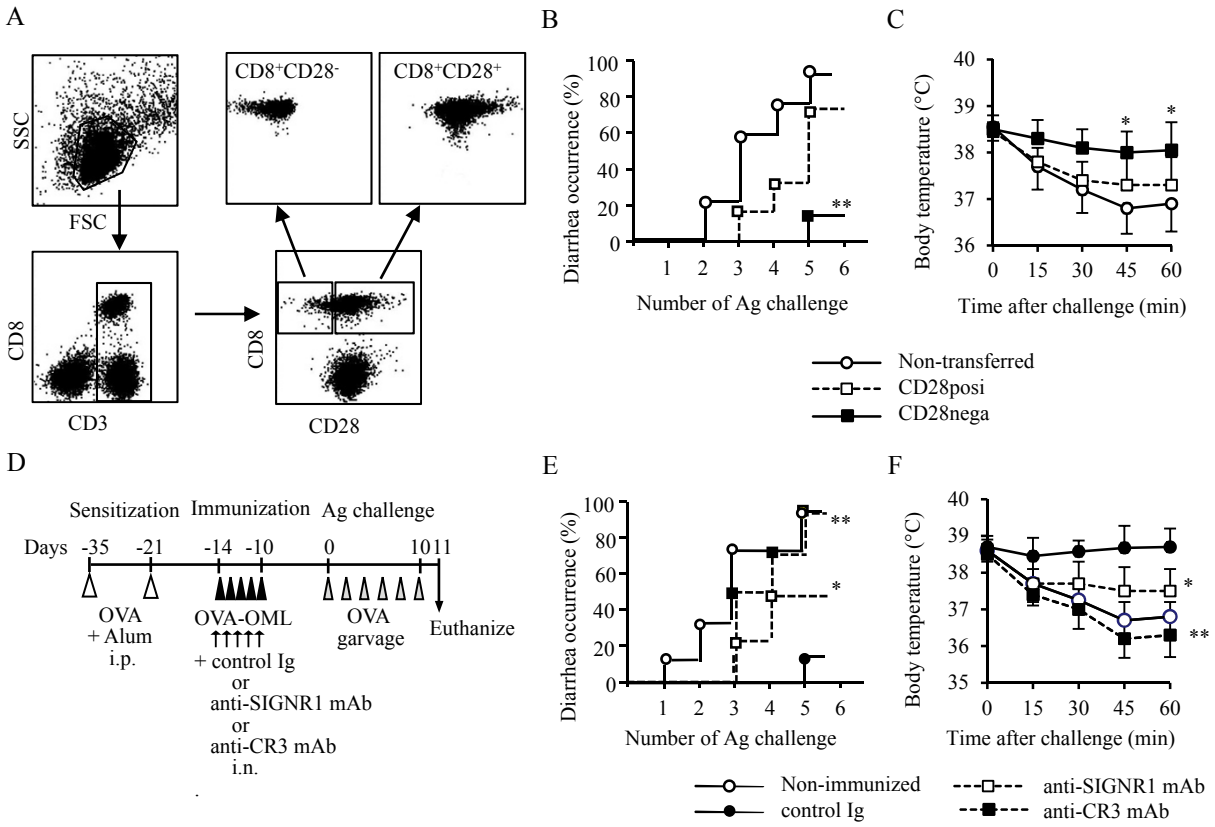

A
Sensitization
Immunization
Ag challenge
Days -47 -33 -26 -22 -12 -2 -1
OVA + Alum i.p.
OVA-OML i.n.
OVA garvage
Days -35 -21 -1 0 10 11
OVA + Alum i.p.
OVA garvage
Euthanize
B
Diarrhea occurrence (%)
Number of Ag challenge
C
Non-transferred
control-CD8
OML-CD8
Body temperature (°C)
Time after challenge (min)
D
Log2 (IL-4 mRNA expression)
Non-Sensitized
Sensitized
Non-transferred
ControlCD8
OML CD8
No challenge
E
Log (mmcp1 mRNA expression)
Non-Sensitized
Sensitized
Non-transferred
ControlCD8
OML CD8
No challenge
F
IgE (AU/ml)
Non-transferred
Control CD8
OML CD8
G
IgG1 (AU/ml)
Non-transferred
Control CD8
OML CD8
H
IgG2a (AU/ml)
Non-transferred
Control CD8
OML CD8
I
IgA (AU/ml)
Non-transferred
Control CD8
OML CD8

Figure 6


A
SSC
FSC
CD3
CD8
CD8+CD28-
CD8+CD28+
CD8
CD28
B
Diarrhea occurrence (%)
Number of Ag challenge
C
Body temperature (°C)
Time after challenge (min)
Non-transferred
CD28posi
CD28nega
D
Sensitization
Immunization
Ag challenge
Days -35 -21 -14 -10 0 10 11
OVA
+ Alum
i.p.
OVA-OML
+ control Ig
or
anti-SIGNR1 mAb
or
anti-CR3 mAb
i.n.
OVA
garvage
Euthanize
E
Diarrhea occurrence (%)
Number of Ag challenge
F
Body temperature (°C)
Time after challenge (min)
Non-immunized
control Ig
anti-SIGNR1 mAb
anti-CR3 mAb