

Bovine β -lactoglobulin-induced passive systemic anaphylaxis model using humanized NOG hIL-3/hGM-CSF transgenic mice

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Abstract

Food allergy is a common disease caused by intake of allergen-containing foods, such as milk, eggs, peanuts and wheat. Systemic anaphylaxis is a severe hypersensitive allergic reaction resulting from degranulation of mast cells or basophils after cross-linking of surface high-affinity IgE receptors (Fc ϵ -RI) with allergen-specific IgE and allergens. In this study, we developed a novel human mast cell/basophil-engrafted mouse model that recapitulates systemic anaphylaxis triggered by β -lactoglobulin (BLG), a major allergen found in cow's milk. Human CD34⁺ hematopoietic stem cells were transferred into NOG (non-Tg) or NOG hIL-3/hGM-CSF transgenic (Tg) mice. After 14–16 weeks, bovine BLG-specific human IgE was intravenously injected into humanized mice, followed by intravenous or oral bovine BLG exposure 1 day later. Body temperature in Tg, but not in non-Tg, mice gradually decreased within 10 min, and 80% of Tg mice died within 1 h by intravenous BLG exposure. Serum histamine levels and anaphylaxis scores in Tg mice were markedly increased compared to non-Tg mice. Furthermore, these allergic symptoms were significantly inhibited by epinephrine treatment of the Tg mice. Therefore, the current NOG hIL-3/hGM-CSF Tg mouse model may be useful for development of novel anaphylaxis drugs for treatment of food allergies and for safety assessment of low-allergenicity extensively hydrolyzed cow's milk whey protein-based infant formulas.

Keywords: basophil, humanized mice, mast cell, milk allergy, PSA model

Introduction

Food allergy is a common allergic disease caused by intake of allergens present in foods, such as milk, egg, peanuts and wheat. Allergen exposure triggers a remarkable acute inflammatory response called anaphylaxis, which is mediated by allergen-specific IgE antibodies. IgE receptor (Fc ϵ -RI)-expressing mast cells and basophils play a crucial role in systemic anaphylaxis and release inflammatory mediators, such as histamine and leukotrienes, from intracellular granules upon cross-linking of Fc ϵ -RI and IgE/allergen complexes (1, 2). Since there are no specific drugs or curative therapies for food allergies, adequate animal models are needed. Animal models of food allergy are widely used to investigate the molecular and cellular mechanisms of immune cell activation, and can be used to explore the use of drug candidates to suppress the symptoms of anaphylaxis (3–5). Rodent models are mostly used to mimic human diseases, including allergies,

and can be used to investigate the effects of therapeutic drugs for preclinical studies. However, rodent models do not always fully recapitulate human diseases because of species differences. Most therapeutic drugs identified through research and development have no effect in clinical trials even when they were found to be highly effective in preclinical rodent models (6, 7). Therefore, it is important to develop mouse models in which functional human immune cells can be engrafted (8). Humanized mice reconstituted with human hematopoietic stem cells (HSCs) are unique tools to investigate immune diseases mediated by human immune cells and can be used to test novel therapeutic drugs. However, it is difficult to generate human allergy models using conventional humanized mice because human myeloid lineage cells, such as macrophages, mast cells and granulocytes, develop poorly in these mice (8). To solve this issue, transgenic (Tg) mice with

several human cytokine genes have been established (9–11). NOD.Cg-*Prkdc^{scid}Il2rg^{tm1Wjl}* Tg (CMV-IL3, CSF2, KITLG) (NSG-SGM3) mice, in which human IL-3, human granulocyte-macrophage colony-stimulating factor (GM-CSF) and human stem cell factor (SCF) are systemically expressed, show human IgE-mediated passive systemic anaphylaxis (PSA) responses *in vivo*. FcεRI-expressing human mast cells differentiated in these models by transferring human bone, liver and thymus (BLT) (5). Previously, we developed immunodeficient NOG mice containing human IL-3 and GM-CSF genes (hIL-3/hGM-CSF Tg). These genes enabled differentiation of functional FcεRI-expressing human mast cells, which successfully provoked human IgE-mediated passive cutaneous anaphylaxis (PCA) (12). In the current study, we developed a novel human mast cell/basophil-engrafted NOG hIL-3/hGM-CSF Tg mouse model that recapitulates systemic anaphylaxis triggered by β-lactoglobulin (BLG), the major allergen found in the whey fraction of cow's milk. Furthermore, we demonstrated that the adrenaline agent epinephrine could be used to suppress anaphylaxis symptoms in this model.

Methods

Mice

NOG (NOD/Shi-*scid*-IL2rg^{null}, formal name, NOD.Cg-*Prkdc^{scid}Il2rg^{tm1Sug}*/Jic) mice and NOG hIL-3/hGM-CSF Tg [formal name, NOD.Cg-*Prkdc^{scid}Il2rg^{tm1Sug}* Tg (SRa-IL3, CSF2)/Jic] mice were generated as previously described (12, 13). All mice were bred and maintained under specific pathogen-free conditions in the animal facility at the Central Institute for Experimental Animals (CIEA). Six- to 12-week-old mice were used as the recipients for human HSC transplantation.

Development of humanized mice

Human CD34⁺ HSCs-transferred humanized mice were developed according to a previous study (14). Briefly, cryopreserved human umbilical cord blood-derived CD34⁺ cells (StemExpress LLC., Folsom, CA, USA) were thawed in a 37°C incubator and prepared for transplantation according to the manufacturer's instructions. Then, 3–5 × 10⁴ HSCs were intravenously transplanted into NOG or NOG hIL-3/hGM-CSF Tg mice, which were subjected to 1.5 Gy whole-body X-ray irradiation (MBR-1520R-4, Hitachi Power Solutions Co., Ltd., Ibaraki, Japan) 1 day before transplantation. After 8–10 weeks of HSC transplantation, the chimerism of human leukocytes [the frequency of human CD45⁺ cells in whole peripheral blood mononuclear cells (PBMCs)] was determined by flow cytometry. Mice with engrafted human CD45⁺ cells with >25% chimerism in whole PBMCs were used for PSA induction assays (Fig. 1).

Induction of PSA

An anti-BLG chimeric human IgE monoclonal antibody (clone 8F7.1) (15) was used for passive sensitization of humanized mice. Humanized NOG or NOG hIL-3/hGM-CSF Tg mice were intravenously injected with 2 μg anti-BLG IgE antibody 16–18 weeks after transplantation. One day later, 15 mg or 500 μg BLG (Sigma-Aldrich, St. Louis, MO, USA) was orally or intravenously

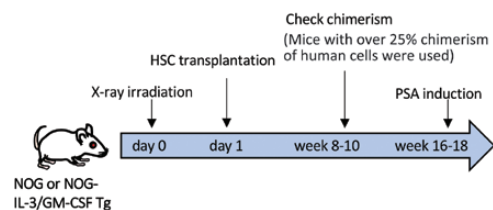


Fig. 1. Schema of passive systemic anaphylaxis (PSA) induction using NOG or NOG hIL-3/hGM-CSF transgenic (Tg) mice.

administered, respectively. The rectal body temperature was measured every 10 min after BLG exposure and the anaphylaxis score was determined 30 min after induction as previously described (16). For suppression of PSA, IgE-sensitized humanized hIL-3/hGM-CSF Tg mice were intraperitoneally treated with 10 μg epinephrine (Bosmin, Daiichi-Sankyo, Tokyo, Japan) 5–10 seconds after BLG challenge.

Enzyme-linked immunosorbent assay

Serum was collected from humanized NOG hIL-3/hGM-CSF Tg mice with or without anaphylaxis, following 20–60 min of BLG oral or intravenous challenge. An anaphylaxis response was defined as a >5% reduction in body temperature. BLG and human IL-3 levels in serum samples from responder and non-responder mice were quantified using a BLG-specific enzyme-linked immunosorbent assay (ELISA) kit (Bertin Pharma, Montigny le Bretonneux, France) and a human IL-3 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA).

Multiplex bead-based cytokine immunoassay

The BD Cytometric Bead Array Human Cytokine or Chemokine Kit (BD Biosciences, San José, CA, USA) was used to quantify human IFNγ, IL-1β, IL-6, IL-8, IL-10, IL-17, TNFα, MIP1α and MCP-1 levels, as well as mouse IFNγ, IL-1β, IL-6, TNFα and MCP-1 levels, in mouse plasma in accordance with the manufacturer's instructions. Data were acquired using a BD FACS Canto flow cytometry system (BD Biosciences) and analyzed using BD FCAP Array v. 3.0 software (BD Biosciences). A standard curve was generated using a five-parameter logistic equation.

Flow cytometric analysis

Following removal of red blood cells using BD Pharm-Lyse (BD Biosciences), PBMCs or splenocytes were isolated and stained with appropriate fluorescent anti-human monoclonal antibodies for 20 min in the dark. Chimerism of human cells in PBMC fractions from humanized mice was determined using anti-human CD45 antibodies (BioLegend). Human mast cells in splenocytes of humanized mice were defined as CD45⁺FcεRI⁺c-kit⁺ cells. To this end, anti-human FcεRI and c-kit antibodies (BioLegend) were used. The frequency of human CD63⁺ mast cells was measured by staining with anti-human CD63 antibody (BioLegend).

Histamine quantification

Serum samples were collected from anti-BLG IgE-sensitized humanized hIL-3/hGM-CSF Tg or non-Tg NOG

mice 30 min after BLG exposure. Serum histamine levels were measured using a Histamine EIA kit (Bertin Pharma, Bordeaux, France) according to the manufacturer's instructions.

Immunohistochemistry

The small intestine and spleen were isolated from humanized NOG hIL-3/hGM-CSF Tg mice. Tissues were fixed overnight in 10% buffered formalin (Wako, Osaka, Japan) and then embedded in paraffin. Tissue sections (3 μ m thick) were placed on aminosilane-coated glass slides (Muto Pure Chemicals, Tokyo, Japan). Slides were immunostained with anti-human CD3 T cell marker (clone PS1, Nichirei Bioscience, Tokyo, Japan), anti-human mast cell tryptase mast cell marker (clone 10D11, Leica Biosystems, Melbourne, Australia) and anti-human CD68 macrophage marker (clone PG-M1, DAKO Cytomation, Glostrup, Denmark) antibodies. Detection and color development, including hematoxylin counterstaining, were performed using the Bond Polymer Refine Detection

system and Leica Bond-Max automatic immunostainer (Leica Biosystems).

Ethics

All animal experiments were approved by the Institutional Animal Care and Use Committee of CIEA (Certification Number: 17026) and were performed in accordance with the guidelines of CIEA. All experiments using human cells were approved by the institutional ethics committee of CIEA (Certification Number: 08-11) and were performed in accordance with the guidelines of CIEA.

Statistical analysis

Data are presented as mean $\pm$ SE of the mean unless otherwise noted. Statistical significance was determined using two-tailed Student's *t*-tests by Excel (Microsoft, Redmond, WA, USA) or Prism 6 (GraphPad, San Diego, CA, USA) software. Significant differences were identified as **P* < 0.05, ***P* < 0.01, ****P* < 0.005, or *****P* < 0.001.

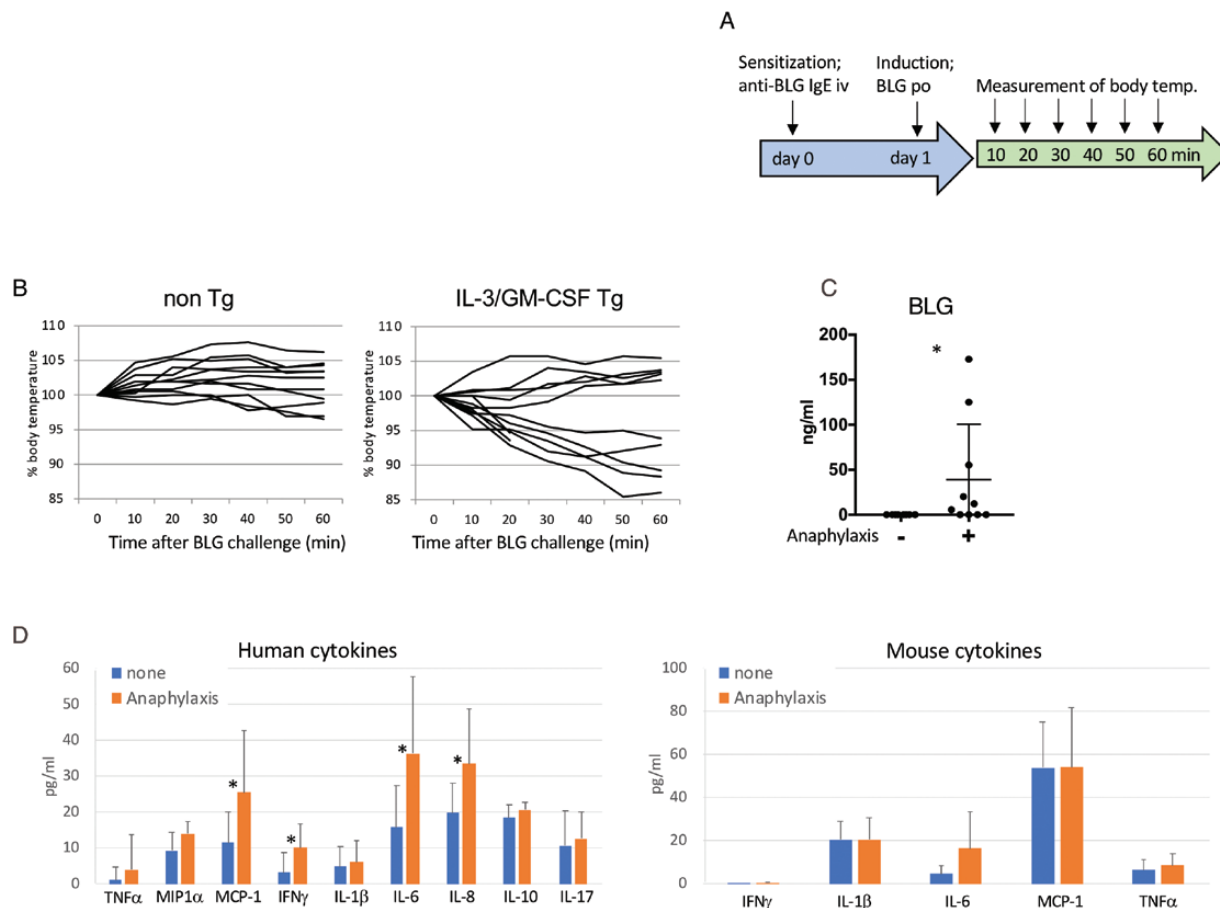
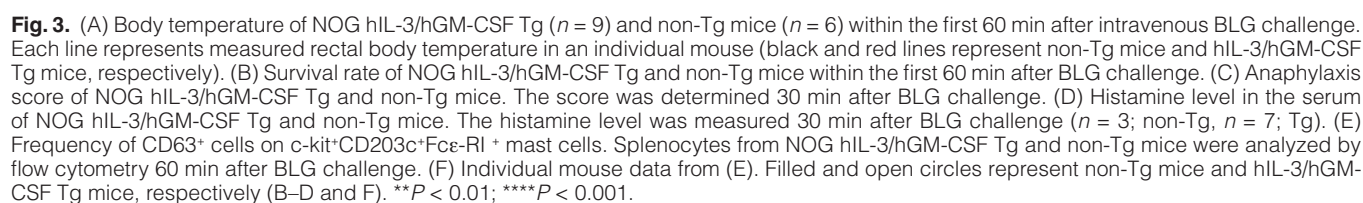


Fig. 2. (A) Procedure for the analysis of humanized mice following induction of anaphylaxis. Humanized mice were intravenously sensitized with anti- β -lactoglobulin (BLG) human IgE and subsequently orally challenged with BLG. (B) Change in body temperature of anti-BLG human IgE-sensitized humanized NOG hIL-3/hGM-CSF Tg ($n = 12$) and non-Tg mice ($n = 11$) within the first 60 min after oral BLG challenge. Each line represents measured rectal body temperature in an individual mouse. (C) Quantification of BLG in serum samples from non-responder (–, $n = 8$) and responder (+, $n = 10$) anaphylactic NOG hIL-3/hGM-CSF Tg mice. (D) Quantification of human (TNF α , MIP1 α , MCP-1, IFN γ , IL-1 β , IL-6, IL-8, IL-10 and IL-17) and mouse (IFN γ , IL-1 β , IL-6, MCP-1 and TNF α) inflammatory cytokines in serum samples from non-responder ($n = 8$) and responder ($n = 10$) anaphylactic NOG hIL-3/hGM-CSF Tg mice.



NOG hIL-3/hGM-CSF Tg mice (12). Histological analysis also revealed that infiltration of human T cells, mast cells and macrophages in the small intestine and spleen did not differ between responder and non-responder mice (Supplementary Figure S2). These findings implied that intestinal inflammation is not essential for enhancement of permeability during anaphylaxis induction by oral challenge with BLG. Although the mechanisms underlying differences between responders and non-responders should be further clarified, it might be difficult to establish a stable PSA model by oral administration of allergens to humanized mice. A previous report demonstrated that IL-9-knockout mice failed to develop oral antigen-induced anaphylaxis. Notably, IL-9 overexpression enhanced this anaphylaxis by increasing intestinal permeability via mast cell activation (22). We anticipate that NOG hIL-3/hGM-CSF Tg mice expressing the human IL-9 gene may serve as a better model for oral induction of PSA in humanized mice.

Intravenous challenge PSA in humanized NOG hIL-3/hGM-CSF Tg mice

Several researchers have demonstrated anaphylaxis by intravenous administration of antigens/allergens to human IgE-sensitized humanized mice (5, 23–25). Since oral challenges were partially successful, we attempted to induce PSA by intravenous injection of BLG after sensitization with anti-BLG human IgE. We observed a higher level of BLG in mice

after intravenous BLG inoculation (Supplementary Figure S3), compared with oral BLG administration. Eight of 9 anti-BLG human IgE-sensitized Tg mice exhibited a decrease in body temperature and died within 60 min after BLG challenge, whereas no effect was observed in non-Tg mice (Fig. 3A and B). In addition, anaphylaxis scores (see the “Methods” section) were significantly higher in Tg mice compared to non-Tg mice (Fig. 3C). Consistent with these results, serum histamine levels were also significantly increased in Tg mice (Fig. 3D). We further tested the level of surface CD63 expression, which is a specific marker on activated and degranulated mast cells upon FcεRI cross-linking (26). CD63 was highly expressed on splenic c-kit⁺CD203c⁺FcεRI⁺ mast cells from Tg mice but not from non-Tg mice (Fig. 3E and F). These results demonstrated that PSA responses were stably elicited in our anti-BLG human IgE-sensitized humanized NOG hIL-3/hGM-CSF Tg mice by intravenous BLG injection.

SCF is a critical growth factor that promotes mast cell differentiation, proliferation, and survival. Isolated mouse c-kit⁺ mast cells exhibited significant proliferation and differentiation within *in vitro* cultures that contained both IL-3 and SCF, but not in cultures that contained IL-3 alone (27). However, long-term exposure to SCF reduced the degranulation and activation capacities of mast cells. This phenomenon was caused by downregulation of Hck or other calcium influx signaling (28). In a human study concerning the roles of SCF and IL-3 in mast cell development, Dahlin *et al.* found that

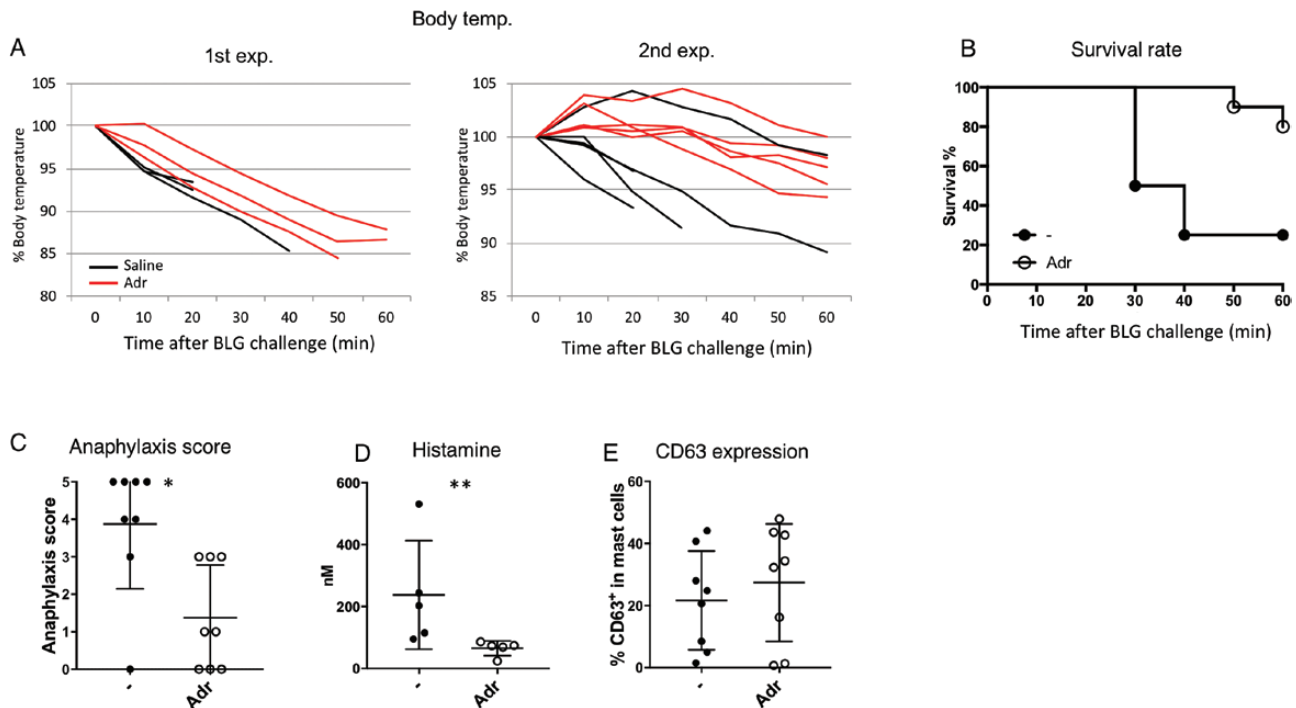


Fig. 4. (A) Body temperature of anaphylaxis-induced NOG hIL-3/hGM-CSF Tg mice with ($n = 8$) or without ($n = 8$) epinephrine treatment. Each line represents measured rectal body temperature in an individual mouse (black and red lines represent saline and epinephrine treatment, respectively (Adrenaline: Adr)). (B) Survival rate of NOG hIL-3/hGM-CSF Tg mice with or without epinephrine treatment within the first 60 min after BLG challenge. (C) Anaphylaxis score of NOG hIL-3/hGM-CSF Tg mice with or without epinephrine treatment. (D) Histamine level in the serum of NOG hIL-3/hGM-CSF Tg mice with ($n = 5$) and without ($n = 5$) epinephrine treatment. (E) Frequency of CD63⁺ cells on splenic c-kit⁺CD203c⁺FcεRI⁺ mast cells from NOG hIL-3/hGM-CSF Tg mice with and without epinephrine treatment. Filled and open circles represent non- and epinephrine-treated NOG hIL-3/hGM-CSF Tg mice, respectively (B–E). * $P < 0.05$; ** $P < 0.01$.

circulating mast cell progenitors were able to proliferate and mature without KIT signaling. Moreover, IL-3 alone promoted the survival of mast cell progenitors, whereas SCF alone did not (29). These reports suggest that SCF is dispensable for mast cell differentiation and activation. The findings are consistent with our PSA model, in which SCF-independent human mast cells caused severe anaphylaxis in mice.

Suppression of PSA by epinephrine in humanized NOG hIL-3/hGM-CSF Tg mice

The adrenaline agent, epinephrine, is widely used as the primary therapy for anaphylactic patients. Epinephrine efficiently decreases the risk of death from anaphylaxis (30). To investigate whether our PSA model was suitable as a therapeutic intervention model, we treated anaphylactic anti-BLG human IgE-sensitized hIL-3/hGM-CSF Tg mice with epinephrine. After intravenous injection of BLG into Tg mice, 2 independent experiments showed that epinephrine, but not saline, treatment partially abrogated body temperature decrement (Fig. 4A). Furthermore, the survival rates in Tg mice were recovered by epinephrine treatment (Fig. 4B). Finally, anaphylaxis scores and serum histamine levels were significantly lower in epinephrine-treated Tg mice (Fig. 4C and D), which is in line with effects observed in epinephrine-treated anaphylactic patients (1). Surprisingly, CD63 expression levels on splenic mast cells remained unaltered upon epinephrine treatment (Fig. 4E). This discrepancy needs more investigation, but we speculate that this is due to the different *in vivo* half-life of CD63 and histamine (15 h for CD63 versus a few minutes for histamine) (31, 32). Since we analyzed CD63 expression 60 min after BLG challenge and epinephrine treatment, it is possible that this time point was too early to detect any possible change in surface CD63 expression. Epinephrine treatment suppresses the production of inflammatory mediators from mast cells and basophils by inducing upregulation of cyclic AMP (33), which results in inhibition of airway swelling and hypotension, and in a decrease in mortality. Collectively, our anti-BLG human IgE-sensitized humanized NOG hIL-3/hGM-CSF Tg mouse model recapitulated the effect of epinephrine in anaphylaxis and may be useful as a preclinical therapeutic model. In addition, our humanized PSA model could possibly facilitate *in vivo* evaluation of low-allergenicity infant formulas, such as extensively hydrolyzed cow's milk whey protein-based formulas (34), as part of allergenicity safety assessments.

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Figure S1

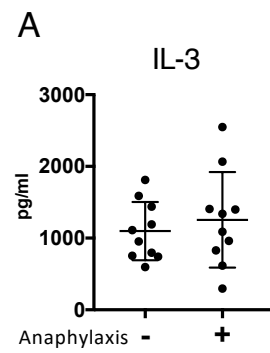


Figure S2

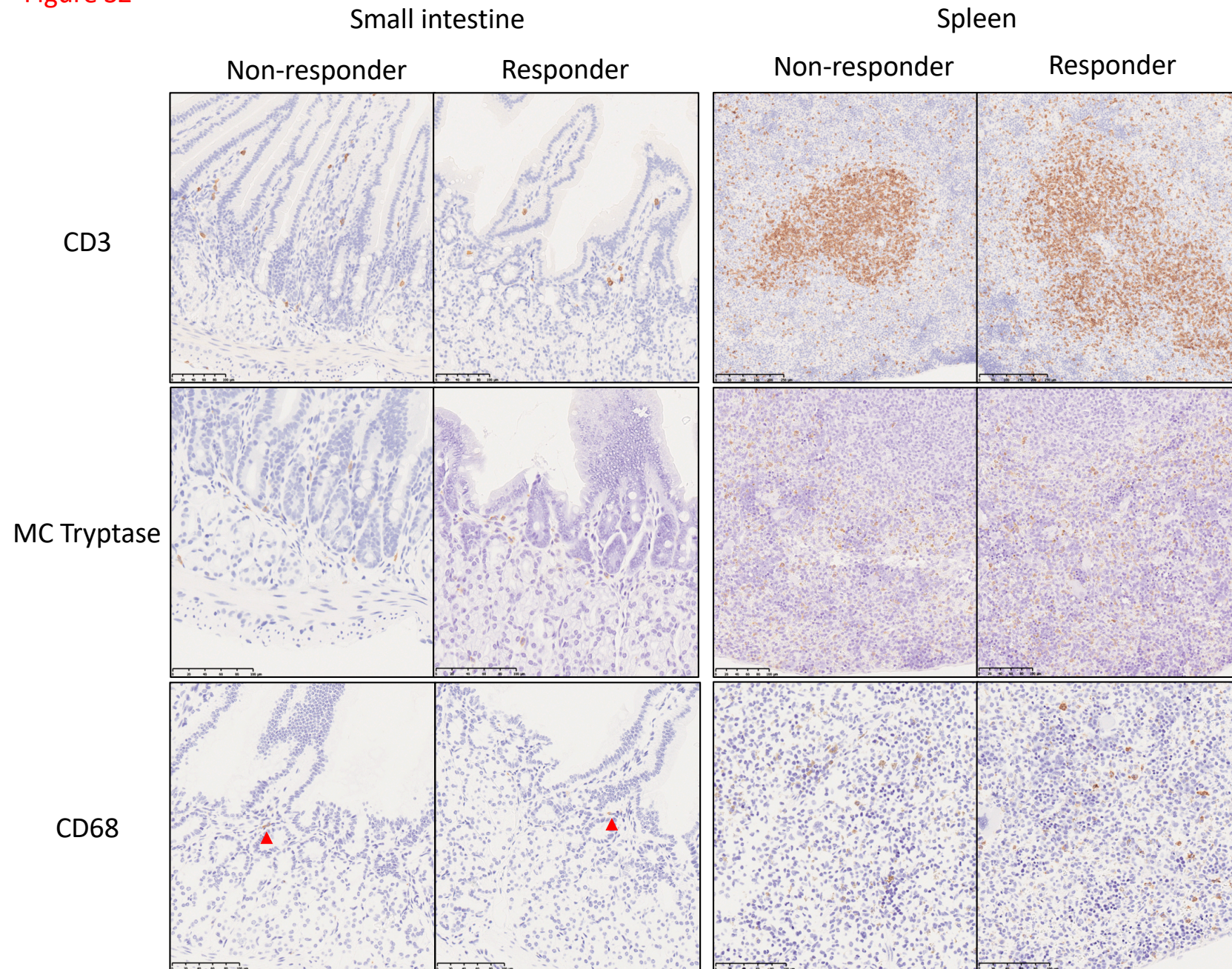


Figure S3

