Supplemental Data

Histamine-releasing factor as a food allergy amplifier

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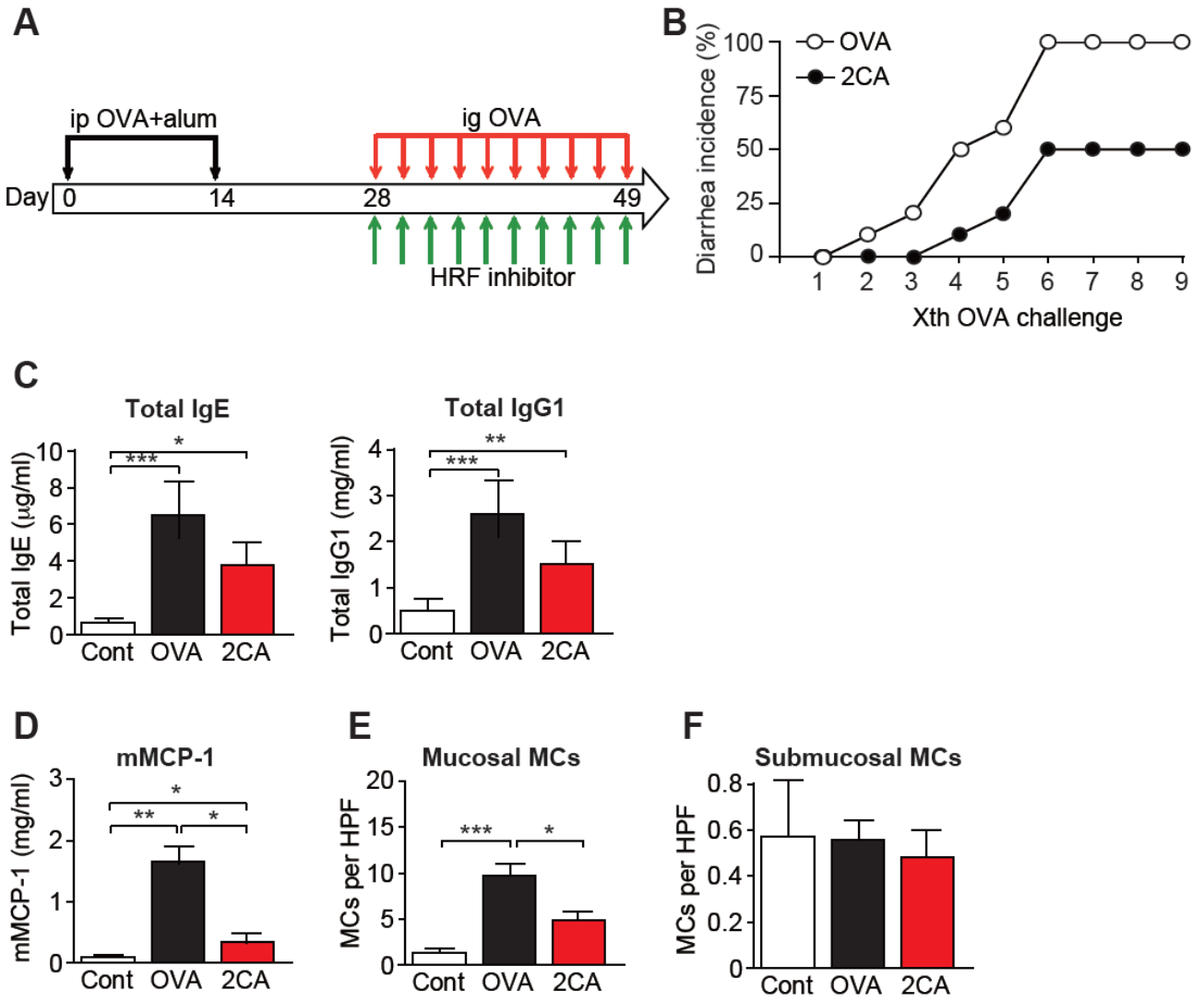

Figure S1. HRF-2CA inhibits the development of diarrhea and intestinal mucosal mastocytosis. Mice were immunized and i.g. challenged with OVA as described in the Fig. 1 legend. Mice were pretreated with 100 μg of HRF-2CA (2CA) or PBS (OVA) before OVA gavages. (**A**) Procedure scheme. (**B**) The occurrence of diarrhea. Log-rank test: p = 0.0499 (PBS n = 10 *vs.* HRF-2CA n = 10; pooled data of two independent experiments). (**C**) Total IgE and IgG1, and (**D**) mMCP-1 concentrations in sera were measured by ELISA. (**E,F**) Sections of jejunum were stained with chloroacetate esterase to quantify mucosal (**E**) and submucosal (**F**) mast cells. *, **, ***: p<0.05, p<0.01, p<0.001 by ANOVA with Tukey's multiple comparison.

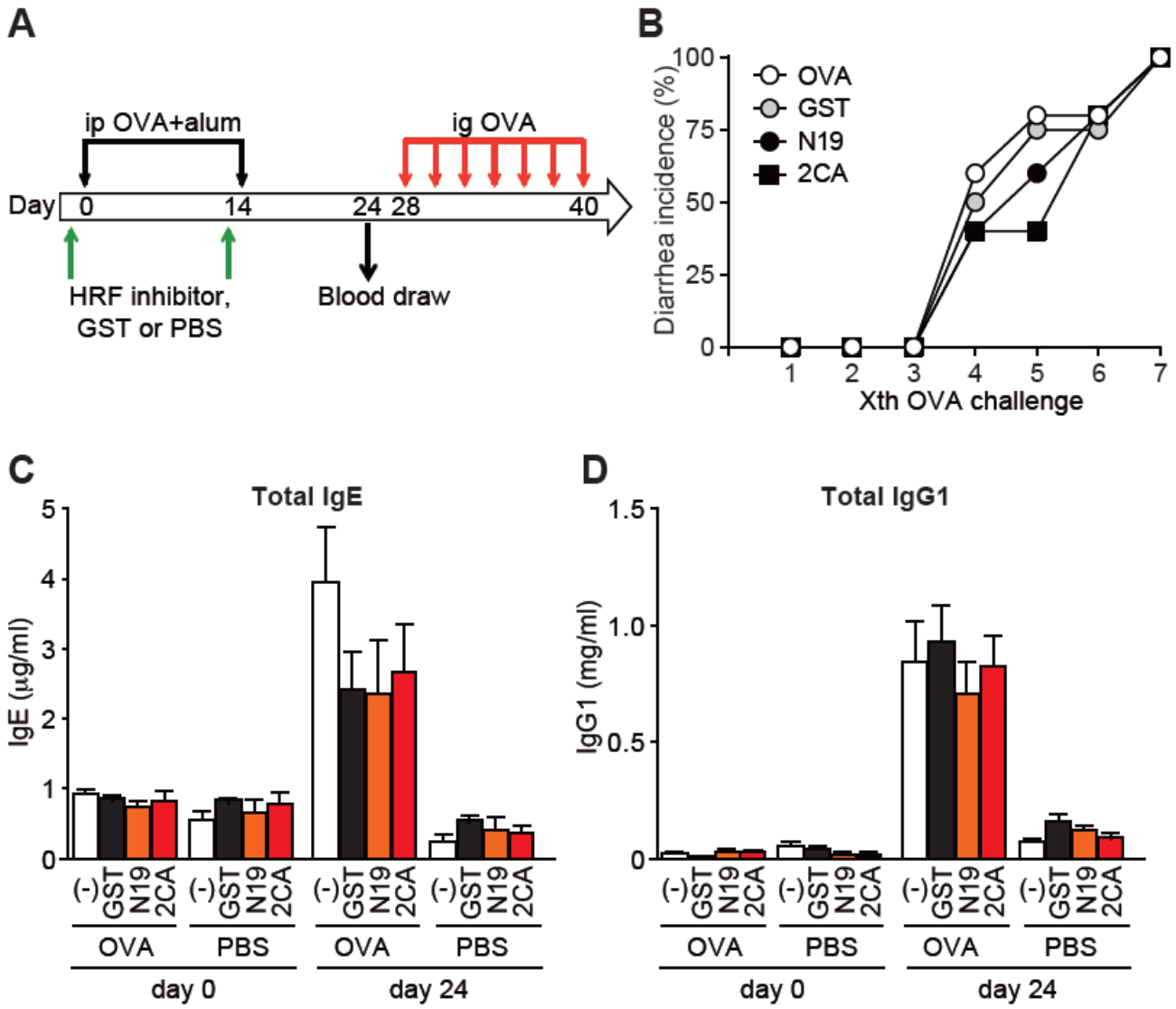

Figure S2. HRF inhibitors do not affect the sensitization phase of murine food allergy. Mice were i.g. pretreated with 100 μg of GST, GST-N19, HRF-2CA or PBS (OVA) one day before i.p. immunization with OVA plus alum on days 0 and 14. From day 28, mice were i.g. challenged with OVA three times a week. (**A**) Procedure scheme. (**B**) The development of diarrhea was monitored after OVA challenge. (n = 5 for OVA, n = 4 for GST, n = 5 for N19, n = 5 for 2CA, and n = 3 each for unsensitized pretreated groups (not shown)). (**C,D**) Total IgE and IgG1 were measured by ELISA.

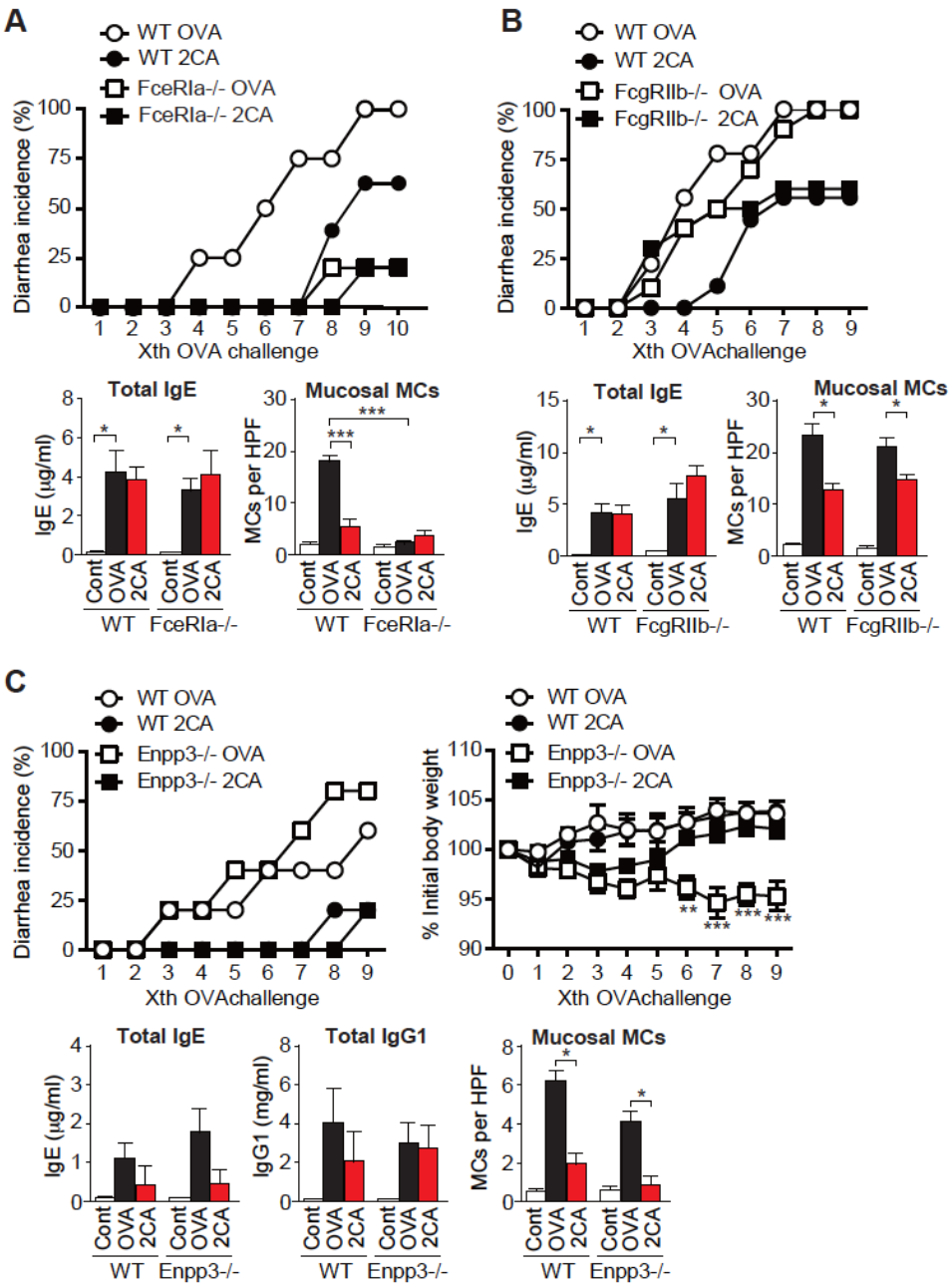

Figure S3. HRF-2CA suppresses allergic diarrhea via FcεRI. WT and mutant mice were subjected to food allergy experiments as described in the Fig. 1 legend. *FceRIa*$^{-/-}$ (n = 4-5 each for group with sensitization and n = 3 each for unsensitized group (Cont; no diarrhea was observed))(**A**), *FcgRIIB*$^{-/-}$ (n = 9-10 each for groups with sensitization and n = 4-5 each for unsensitized groups (Cont; no diarrhea was observed); pooled data of two independent experiments) (**B**), and *Enpp3*$^{-/-}$ (n = 9-10 each for groups with sensitization and n = 7 each for unsensitized groups (Cont; no diarrhea was observed); pooled data of two independent experiments) (**C**) mice along with WT mice were used. *, ***: $p<0.05$, $p<0.001$ by Student's t-test.

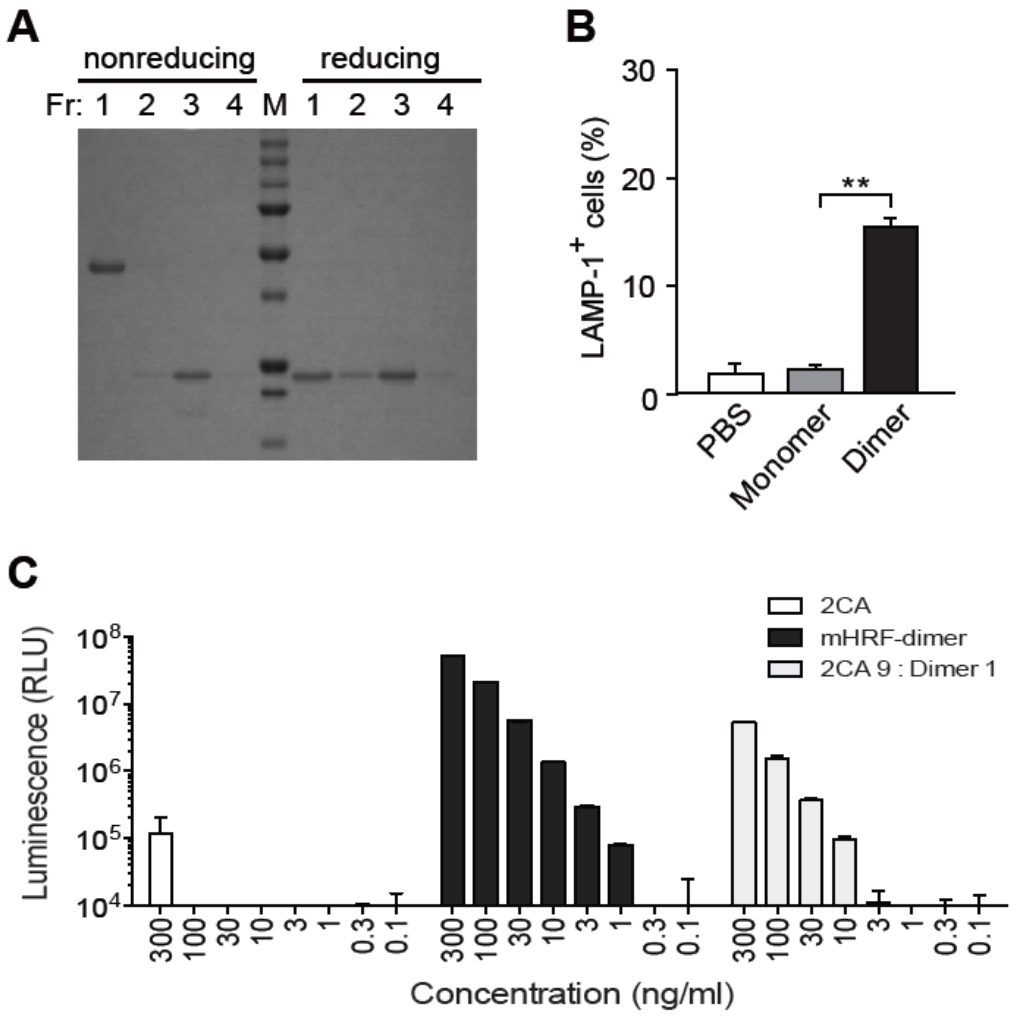

Figure S4. HRF dimer, but not monomer can activate mast cells. (**A**) Purified dimeric and monomeric HRF expressed in *E. coli*. Recombinant HRF-His_6 was purified first by Histrap HP and then Sephacryl S-200 HR16/60. Two peaks were observed by Sephacryl size fractionation. The first (Fr1) and second (Fr3) peaks contained dimer and monomer, respectively, as shown by SDS-PAGE. Fr1 and Fr3 were used for mast cell stimulation. (**B**) Cells were released with 10 mM EDTA from small intestines of OVA-sensitized/OVA-challenged mice, and mononuclear cells were selected. The cells were incubated for 15 min at 37°C with mHRF monomer or dimer (100 μg/ml), or PBS, and Kit^+ mast cell activation ($LAMP\text{-}1^+$) was measured by flow cytometry (n = 3). (**C**) Control experiments indicate an excellent specificity of the assay for HRF multimers. HRF-2CA (2CA) was used as a monomer control (n = 2 for each concentration). Note that total concentrations of the 1:9 mixture of dimer:monomer gave almost the same luminescence values as did ten-fold less concentrations of dimer.

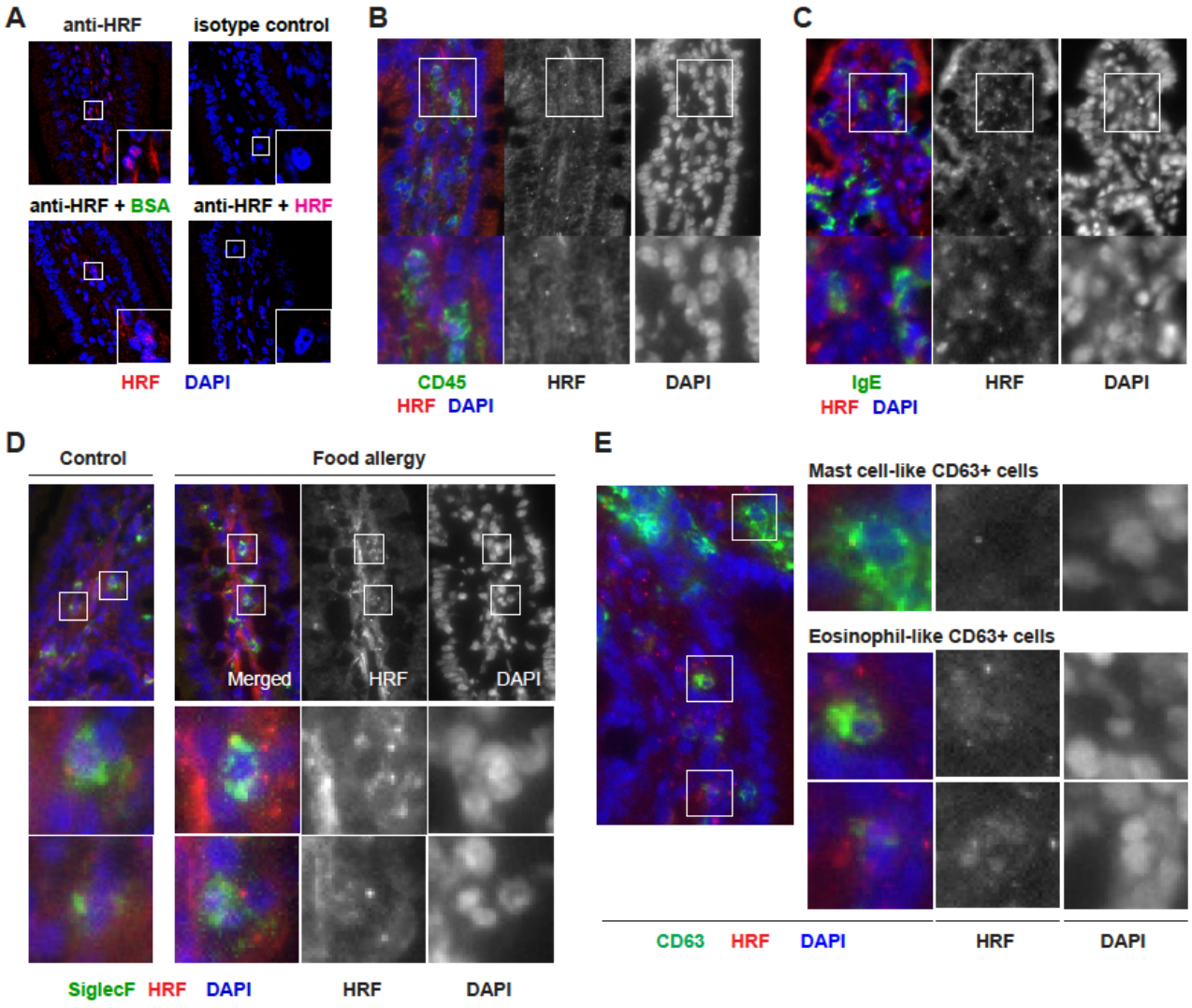

Figure S5. Localization of HRF in the jejunum. (**A**) Jejunum from diarrheal mice was stained with indicated antibodies preincubated with or without recombinant HRF or BSA. Bound antibody was detected by Alexa Fluor 647-conjugated anti-rabbit antibody. Fluorescence was observed by confocal laser microscopy. (**B-E**) Jejunum from diarrheal mice were co-stained with anti-HRF and anti-CD45 (**B**), anti-IgE (**C**), anti-Siglec F (**D**), or anti-CD63 (**E**) antibodies. Fluorescence signal was detected and separated from autofluorescence by Nuance Multispectral Imaging System (PerkinElmer).

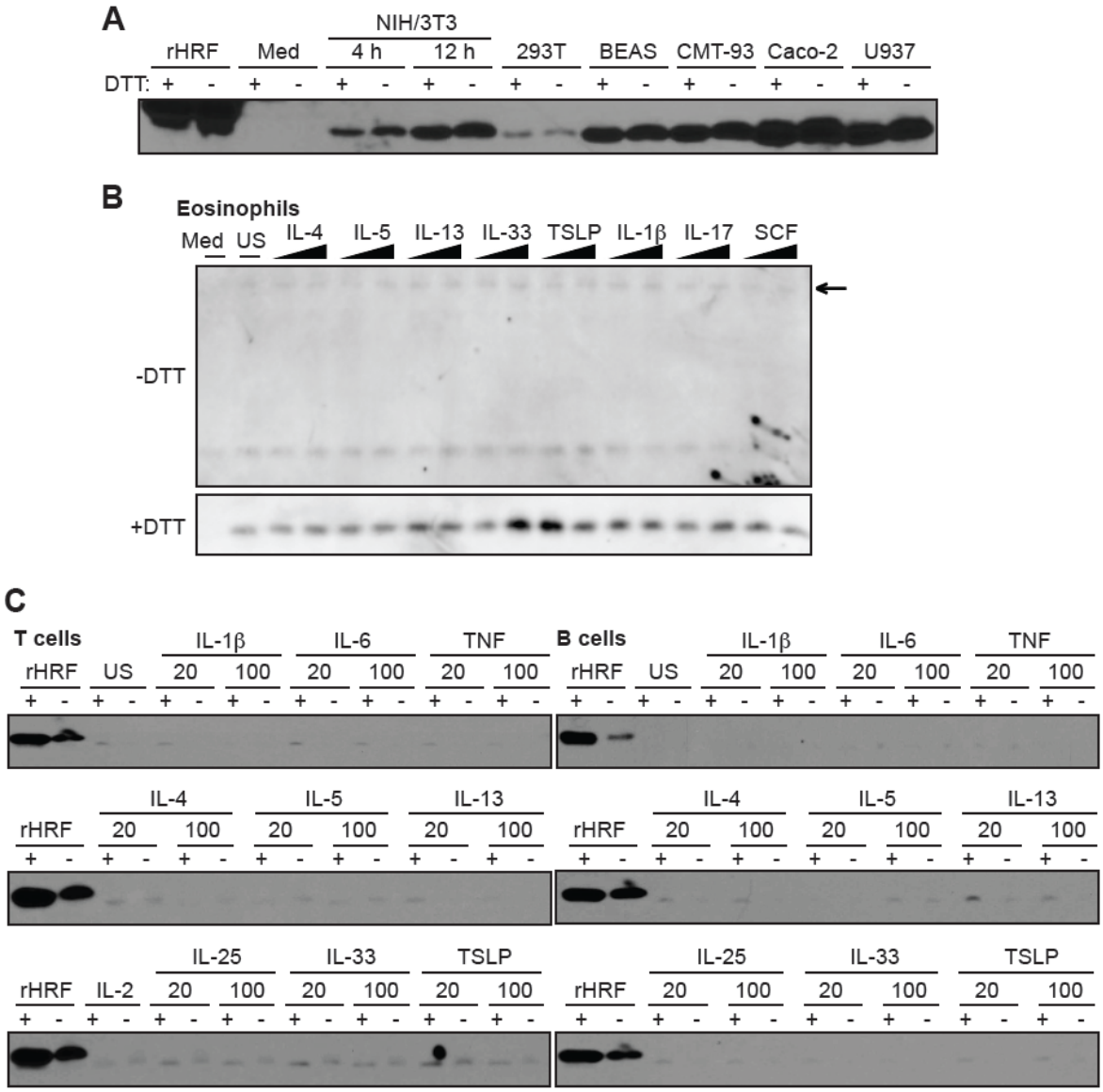

Figure S6. HRF is secreted from various cells. (**A**) Various cells were incubated overnight except for the NIH/3T3 cells, which were cultured for 4 or 12 h after confluency was reached. Culture supernatants were treated with DTT or not before run on an SDS gel. Western blot analysis was done with anti-HRF mAb to detect HRF monomer except for panel **B**, where both HRF dimers and monomers are shown in a non-reducing gel. (**B**) Bone marrow-derived eosinophils (>95% pure) were kept unstimulated (US) or stimulated overnight with the indicated cytokines (20 or 100 ng/ml). (**C**) Splenic T and B cells were stimulated overnight by the indicated cytokines (ng/ml). rHRF, recombinant HRF; Med, medium alone. Arrow indicates the position of HRF dimer.

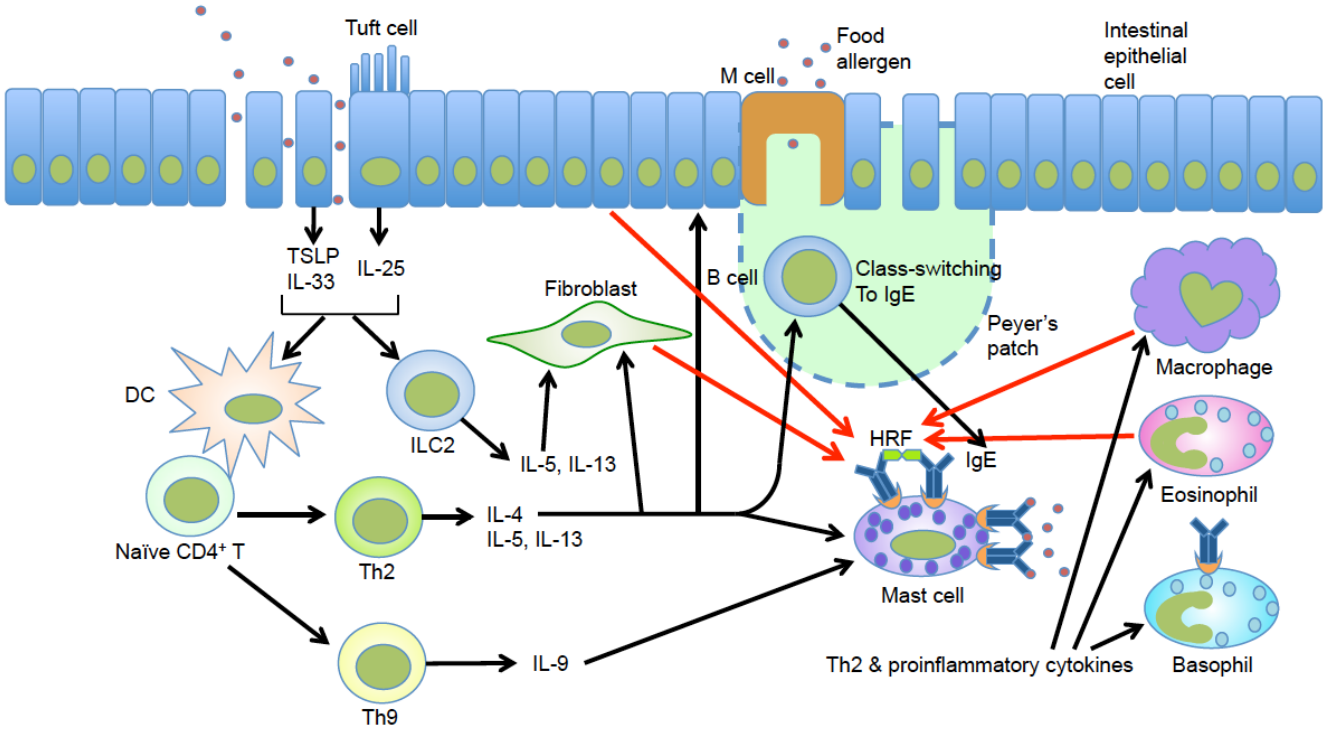

Figure S7. HRF amplifies intestinal allergic inflammation. Epithelial damage or inflammation in the gut promotes increased entry of food allergens and secretion of the epithelial-derived cytokines TSLP, IL-25 and IL-33. These cytokines induce a Th2-skewed immune response. TSLP can enhance OX40L expression in dendritic cells, which induce Th2 cell differentiation of naïve $CD4^+$ T cells. IL-25 secreted by tuft cells may help the expansion of type 2 innate lymphoid cells (ILC2). Th2 cells along with ILC2 cells promote the Th2 cell-mediated immune response, which includes IgE class switch recombination in B cells, eosinophil accumulation, and mastocytosis. IL-9 promotes the expansion of IL-9-producing mucosal mast cells (MMC9) as an important component of food allergy-associated inflammation. In this study, HRF dimer/multimers secreted from several types of cell amplify intestinal inflammation by activating IgE-bound mast cells synergistically with antigen via the FcεRI. This is likely due to increased HRF secretion by several types of cell in response to Th2, proinflammatory and even epithelial-derived cytokines.

Supplemental Methods

Enzyme-linked immunosolvent assays (ELISAs)

ELISA kits for human total IgE, IgG and mMCP-1 were purchased from eBioscience. Mouse total IgE, IgG1 and IgG2b (not a) levels were similarly analyzed using the following antibodies to capture and to detect the antibodies: purified rat anti-mouse IgE (BD Biosciences, Cat 553413) and biotin-conjugated rat anti-mouse IgE (BD Biosciences, Cat 553419); purified rat anti-mouse IgG1 (BD Biosciences, Cat 553445) and biotin-conjugated rat anti-mouse IgG1 (BD Biosciences, Cat 553441); purified rat anti-mouse IgG2b (BD Biosciences, Cat 553396) and biotin-conjugated rat anti-mouse IgG2b (BD Biosciences, Cat 553393). 96-well ELISA plates were coated overnight with capturing antibodies (each at 1 μg/ml in 0.1 M carbonate buffer [pH 9.5]). The plates were washed and blocked with 10% FCS. Next, diluted plasma or sera were incubated in the coated plates, after which bound immunoglobulins were detected by incubation with biotinylated detection antibodies followed with HRP-conjugated streptavidin (BD Biosciences). Color was developed using TMB substrate (Biolegend), and absorbance at 450 nm was measured and corrected with absorbance at 570 nm.

HRF was measured using anti-TPT1/TCTP antibody (Novus Biologicals, Cat# H00007178-M06, clone 2A3) for capturing and anti-TPT1/TCTP antibody (self-biotinylated mAb, Novus Biologicals, Cat# H00007178-M03, clone 2C4) and streptavidin-β-Gal conjugate (Roche, Cat# 11112481001) for detection. After incubation with streptavidin-β-Gal conjugate and washing, ELISA wells were incubated with 0.2 mM 4-Methylumbelliferyl-β-D-galactopyranoside (4-MU-Gal, Sigma-Aldrich, Cat# M1633) for 1 h at 37°C. Fluorescence was measured at excitation of 365 nm and emission of 445 nm. HRF-reactive IgE was measured using in-house ELISAs: ELISA wells were coated with 10 μg/ml recombinant human (or mouse) HRF-His_6 in 0.1 M sodium carbonate buffer (pH 9.5) for overnight at room temperature (RT). After washings, the wells were blocked with ImmunoBlock (DS Pharma Biomedical, Japan, Cat# CTKN001) for 2 h. The wells were washed, and incubated with 1 μg/ml biotin anti-human IgE (anti-mouse IgE) for 1 h at RT. Then the wells were washed and incubated with streptavidin-β-Gal conjugate, followed washings and incubation with 0.2 mM 4-MU-Gal for 1 or 2 h. Fluorescence was measured at excitation of 365 nm and emission of 445 nm. HRF-reactive IgG was similarly analyzed except for the use of biotin anti-human IgGs or biotin anti-mouse IgGs instead of anti-human IgE. Biotin anti-human IgE, IgG1 and IgG4 antibodies were also purchased from BD Biosciences for HRF-reactive Ig ELISA (Cat 555858, 555869 and 555882, respectively).

HRF-reactive IgE increase index

HRF-reactive IgE increase ratio was calculated by the formula: (HRF-reactive IgE at 12 months – HRF-reactive IgE at 1 week) divided by (HRF-reactive IgE before OIT). Increase index was further calculated by the formula: 2 divided by $\{1 + e^{-(\text{HRF-reactive IgE increase ratio})}\}$ -1. This index was compared between patients with no decrease in threshold and patients with severe decrease in threshold (**Figure 6H**).