



Egg yolk augments type 2 immunity by activating innate cells

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Abstract

Purpose Egg yolk (EY) may play a role during the sensitizing phase of egg allergy by exerting intestinal type 2-biasing effects. We aimed to identify the mechanism and role of EY in the induction of allergy to egg white (EW).

Methods BALB/c mice were exposed intragastrically to EW, EY, or the mixture of EW:EY. In addition in vitro experiments were conducted with intestinal epithelial cells (IECs), dendritic cells (DCs), and T cells from naïve mice. Inflammatory and type 2 responses were evaluated.

Results Administration of EW upregulated duodenal expression of factors that influence epithelial barrier integrity and function, such as *Muc2* and *Cldn2*, type 2-promoting epithelial cytokines *Il33* and *Il25*, DC genes *Irf4* and *Tnfrsf4*, and Th2-cytokines *Il14* and *Il13*. EW:EY further increased the expression of *Il25* and *Tslp* in the duodenum, *Il33* and *Tslp* in the jejunum, and the proportion of lamina propria group 2 innate immune cells (ILC2s) over EW alone. Moreover, it distinctively enhanced the expression of *Irf4* and *Cd1d1* in the Peyer's patches (PPs), and of *Il6*, *Il33*, *Gata3*, and *Il13*, both in PPs and mesenteric lymph nodes. In co-cultures of DCs and T cells, EW:EY induced a higher expression of *Gata3*, *Il4*, and *Il13*, secretion of IL-13 and expansion of CD4⁺ T cells expressing ST2, the IL-33 receptor, than EW or EY added individually.

Conclusion Co-administration of EY may promote sensitization to EW through activation of innate immune cells, such as IECs, DCs and ILC2s, that are central to the progress of allergies.

Keywords Egg allergy · Egg white · Egg yolk · Dendritic cells · Group 2 innate immune cells · Intestinal epithelial cells

Introduction

Even though food allergy is a relevant health issue because of its increasing prevalence, not much is known on the molecular mechanisms implicated in the sensing of food antigens and the triggering of allergic reactions bypassing the default tolerance state. The biological functionality of proteins, in particular proteolytic activity, is recognized as an important feature, but other biochemical properties can favour the detection of antigens by cells of the innate immune system, such as intestinal epithelial (IECs) or

dendritic cells (DCs), to initiate adaptive Th2 immunity and subsequent allergic reaction [1]. Furthermore, in addition to the intrinsic characteristics of food proteins, accompanying molecules from the food matrix alter their allergenic potential by affecting the way in which proteins are degraded during digestion, absorbed through the gastrointestinal tract, recognized at the cellular level and presented to the immune system [2]. Among them, dietary lipids modify the immunostimulating properties of proteins, exert pro-inflammatory or anti-inflammatory functions on cells of the innate immunity and influence adaptive immunity [3].

Upon contact with immune stimuli, IECs release the epithelium-derived cytokines IL-33, IL-25, and TSLP that regulate type 2 responses at the intestinal level [4]. On the one hand, they contribute to expand group 2 innate lymphoid cells (ILC2s), while upregulating the costimulatory molecule OX40L on DCs [5] and, on the other hand, they amplify allergic inflammation through the expansion and maintenance of Th2 memory cells [6]. ILC2s rapidly respond to these cytokines by producing factors, such as

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IL-4, IL-5 and IL-13, shared by Th2 cells, through which they directly modulate T cell function and condition DCs to induce Th2 cell activation [7]. Concomitantly, dietary lipids can exert intrinsic adjuvant activity through recognition by CD1d, a lipid-binding molecule found on the surface of IECs and DCs, responsible for the presentation of lipid antigens to invariant natural killer T (iNKT) cells, which act as amplifiers of specific Th2 responses [8].

Egg, the second most frequent cause of allergic reactions in children, contains several allergenic proteins mainly in the egg white (EW) [2]. However, egg yolk (EY), which comprises 65% lipids in dry matter, may play a role during the sensitizing phase of egg allergy by exerting intestinal type 2-biasing effects [9]. We aimed to identify the mechanism and role of EY in the induction of allergy to EW by conducting in vivo experiments in BALB/c mice. Using a murine model of acute feeding of EW, EY and their mixture, we assessed the integrity and function of the intestinal barrier by studying the expression of the genes encoding for IL-22, Muc-2, and Claudin-2 (*Il22*, *Muc2*, and *Cldn2*, respectively). The production of epithelial cytokine-related Th2-biasing proteins was examined using the expression levels of IL-33, IL-25, and TSLP genes (*Il33*, *Il25*, and *Tslp*, respectively). To estimate the development and functional specialization of DCs, we explored the genes encoding for OX40L, Jagged2, IRF4, IRF8, and CD1d (*Tnfrsf4*, *Jagged2*, *Irf4*, *Irf8*, and *Cd1d1*, respectively). Finally, the proportion of lamina propria ILC2s was analysed and the induction of effector T cell responses was evaluated through the relative expression of genes encoding for pro-inflammatory (IL-6), Th2 (IL-4, IL-13) and Th17 (IL-17) cytokines (*Il6*, *Il4*, *Il13*, and *Il17*, respectively), as well as for the transcription factors GATA-3 and ROR γ t (*Gata3* and *Rorc*, respectively). These studies were complemented with in vitro assays with IECs, bone marrow (BM)-derived DCs, and CD4⁺ T cells. As a result, we found that EY may promote sensitization to EW through activation of innate immune cells, such as IECs, DCs, and ILC2s.

Materials and methods

Samples

EW and EY were carefully separated from fresh eggs and lyophilized. Protein content, lipid composition, and the absence of cross-contamination were examined as in [9]. The lipopolysaccharide (LPS) content was below 0.4 EU mg⁻¹ in all samples (Pierce LAL Chromogenic Endotoxin Quantitation Kit, Thermo Scientific, Waltham, USA). Lyophilized EW and EY were mixed (EW:EY) in a proportion similar to that in whole egg (approximately 1:2 in dry matter) [10].

Experiments in mice

Female 6 week-old BALB/c mice (purchased from Charles River Laboratories, Saint Germain sur l'Arbresle, France) were administered by intragastric gavage: PBS (0.2 mL), cholera toxin (10 µg; CT, List Biologicals, Campbell, USA), EW (50 mg), EY (100 mg) or EW:EY (50:100 mg) (all dispersed in 0.2 mL PBS) daily for 4 consecutive days. On the fourth day, mice were given a second dose 2 h after the first one and sacrificed by CO₂ inhalation 2 h later. In parallel experiments, mice received intraperitoneally PBS or recombinant murine IL-33 (2 µg; carrier-free, R&D Systems, Minneapolis, Canada) daily for 4 consecutive days and sacrificed 24 h after the last dose.

Duodenum, jejunum, ileum, Peyer's patches (PPs) and mesenteric lymph nodes (MLNs) were individually collected for gene expression analyses. Lamina propria cells were isolated by incubation with HBSS/EDTA (2 mM EDTA, 10 mM HEPES, Biowest SAS, Nuaille, France) and digested in RPMI containing 25 µg mL⁻¹ Liberase TM and 10 U mL⁻¹ DNase I (both from Roche, Sigma-Aldrich; St. Louis, MO, USA) for 45 min (37 °C and 250 rpm). ILC2s from the lamina propria were analysed by flow cytometry.

Isolation and culture of intestinal epithelial cells

IECs from the small intestine of naïve BALB/c mice were isolated as described [11] except that HBSS containing 2% glucose and 500 µg mL⁻¹ Liberase DL (Roche, Sigma-Aldrich) were used for digestion. Isolated IECs (1 × 10⁶ cells mL⁻¹) were cultured in DMEM High Glucose, supplemented with 2.5% FBS, 1% L-glutamine, 10 mM HEPES, 1% penicillin/streptomycin (Biowest, SAS), 0.25% insulin-transferrin-selenium 100x (ITS-A, Thermo Fisher Scientific, Waltham, MA, USA), and 20 ng mL⁻¹ epidermal growth factor (Sigma-Aldrich). IECs were stimulated with DMEM (as negative control), EW (50 µg mL⁻¹), EY (100 µg mL⁻¹), EW:EY (50:100 µg mL⁻¹), or IL-33 (0.1 µg mL⁻¹; Peprotech, London, UK), LPS (0.1 µg mL⁻¹; 055:B5 from *Escherichia coli*, Sigma-Aldrich), or CT (1 µg mL⁻¹) as positive controls. After 24 h, supernatants were collected and used for conditioning BM-DCs, and IECs were preserved for gene expression analyses.

Conditioning of bone-marrow-derived dendritic cells

BM cells were isolated from femurs of naïve BALB/c mice and cultured in RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin (all from Biowest SAS), containing 20 ng mL⁻¹ GM-CSF (Peprotech).

After 10 days, BM-DCs were incubated (1×10^6 cells mL^{-1}) with the supernatants collected from IEC cultures or directly with the stimuli, as indicated above, for 24 h. Before and after stimulation, BM-DCs were analysed by flow cytometry. Following culture, BM-DCs were preserved for gene expression analyses.

Co-culture of dendritic cells and T cells

Splenic CD4^+ T cell from naïve mice were isolated using EasySep negative selection kit (StemCell Technologies, Vancouver, Canada) and labelled with carboxyfluorescein succinimidyl ester (CFSE; CellTrace CFSE cell proliferation kit, Life Technologies, Carlsbad, CA, USA). BM-DCs (1×10^5 cells mL^{-1}) were incubated with CD4^+ T cells (1×10^6 cells mL^{-1}) and stimulated with RPMI (as negative control), EW (50 $\mu\text{g mL}^{-1}$), EY (100 $\mu\text{g mL}^{-1}$), EW:EY (50:100 $\mu\text{g mL}^{-1}$), or IL-33 (0.1 $\mu\text{g mL}^{-1}$), LPS (0.1 $\mu\text{g mL}^{-1}$), or CT (1 $\mu\text{g mL}^{-1}$) as positive controls. After 3 days, anti-CD3 (3 $\mu\text{g mL}^{-1}$; eBioscience, San Diego, CA, USA) was added and the co-culture was maintained for 3 additional days. IL-13 production was determined in the supernatants by ELISA using a commercial kit from eBioscience, cell proliferation was assessed by flow cytometry, and cells were also used for gene expression analyses.

Gene expression analyses

Total RNA extraction from tissues and cells, and qPCR assays were performed as described [12]. Primer pairs and thermal cycling conditions are included in Supplementary Table 1. Relative gene expression was calculated by the $2^{-\Delta\Delta\text{CT}}$ method [13], normalizing data to the expression of the *Actb* gene (encoding for β -actin).

Flow cytometry analyses

Isolated and cultured cells were recovered in PBS containing 2% fetal bovine serum and 1 mM EDTA. Samples were stained with the antibodies listed in Supplementary Table 2. Live cells were determined with LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific). Cells were acquired with a Gallios flow cytometer (Beckman Coulter, Krefeld, Germany) and analyses were performed with FlowJo software (version 7.6.5, Ashland, OR, USA).

Statistical analyses

Results are presented as means $\pm$ SEM. Differences between a control and an experimental group were assessed by the unpaired two-tailed Student's *t* test and differences among three or more groups were determined by one-way ANOVA, followed by Tukey post-hoc test, except for gene expression

data, which were evaluated by Mann Whitney *U* test. $p < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism v6 (GraphPad Software Inc., San Diego, USA).

Results

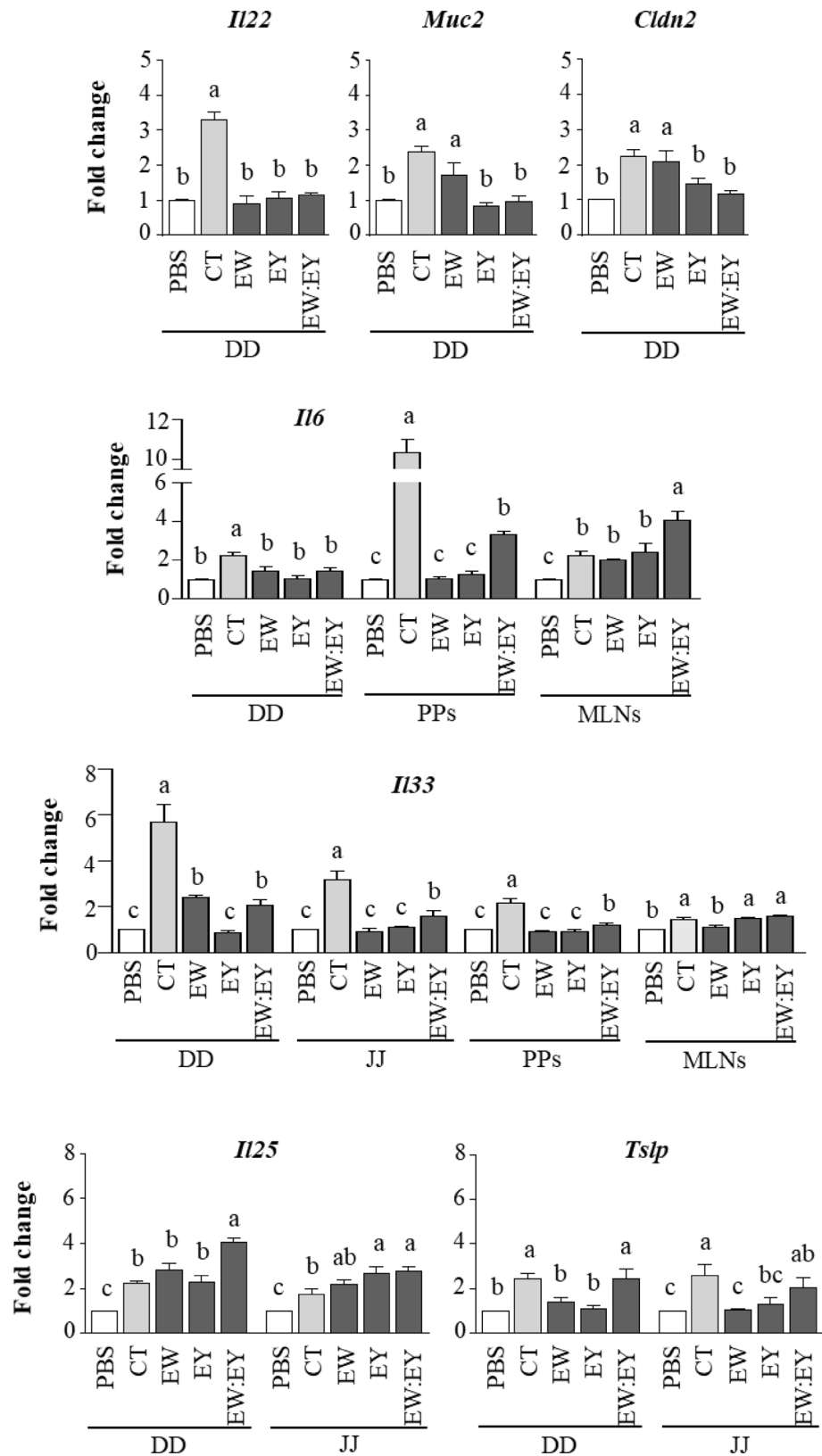
Egg yolk acts as an adjuvant in vivo increasing type 2 responses to egg white

The in vivo effects of EW and EY were investigated by conducting experiments in mice that were given 4 daily doses of EW, EY, or the mixture of EW:EY, plus a boost administration 2 h after the last dose. CT, a Th2-adjuvant broadly used to induce food allergy in mouse models [14], was similarly administered as a control. In view of the importance of intestinal permeability and failure to maintain homeostasis in the development of food allergy, and the potential role of food lipids in these processes [3], we investigated the expression of factors that influence epithelial barrier integrity and function, such as *Il22*, *Muc2*, and *Cldn2* (Fig. 1). The expression of these genes, as well as that of the pro-inflammatory mediator *Il6*, was increased in the duodenum of mice administered CT. EW also enhanced the expression of *Muc2* and *Cldn2*, albeit not that of *Il22* or *Il6*, in the duodenum of mice, whereas EY did not affect *Il22*, *Muc2*, *Cldn2* or *Il6* expression. Furthermore, administration of the mixture EW:EY abolished the effects of EW on the regulation of *Muc2* and *Cldn2* (Fig. 1).

Because sensitization by the oral route has been proposed to be initiated by the intestinal release of cytokines that expand ILC2s at mucosal surfaces and enable DCs to promote Th2 responses [4], we then assessed the gene expression of epithelium-associated *Il33*, *Il25*, and *Tslp* (Fig. 1). CT upregulated the expression of *Il33*, *Il25*, and *Tslp* in the duodenum and jejunum. Gavage with EW enhanced the expression of *Il33* in the duodenum and *Il25* in the duodenum and jejunum. EY also upregulated *Il25* in the duodenum and jejunum to a level similar to that induced by EW. However, the combination of EW:EY increased the expression of *Il33*, *Il25*, and *Tslp* at both levels, leading to a higher expression of *Il25* and *Tslp* in the duodenum, and of *Il33* and *Tslp* in the jejunum than EW alone (Fig. 1). No changes in the expression of *Il33*, *Il25*, or *Tslp* were found in the ileum as a result of the administration of CT or the egg fractions (not shown). Noteworthy, the proportion of ILC2s, which are GATA3-dependent and produce IL-4, IL-5 and IL-13 [15], was enhanced in the lamina propria of mice administered CT, EY, and EW:EY (Fig. 2 and gating strategy in Supplementary Fig. 1a).

Also in agreement with the upregulation of type 2-inducing epithelial cytokines, administration of CT enhanced, in

Fig. 1 Relative gene expression of *Il22*, *Muc2*, *Cldn2*, *Il6*, *Il33*, *Il25*, and *Tslp*, determined in different tissues (*DD* duodenum, *JJ* jejunum, *PPs* Peyer's Patches, *MLNs* mesenteric lymph nodes) of mice administered intragastrically: PBS, cholera toxin (CT), egg white (EW), egg yolk (EY), and a mixture 1:2 (w:w) egg white: egg yolk (EW:EY), for 4 consecutive days. Gene expression was normalized to the reference gene *Actb* and compared with mice administered PBS. Data are expressed as means $\pm$ SEM ($n=6$). Different letters indicate statistically significant differences ($p<0.05$) among different mouse groups



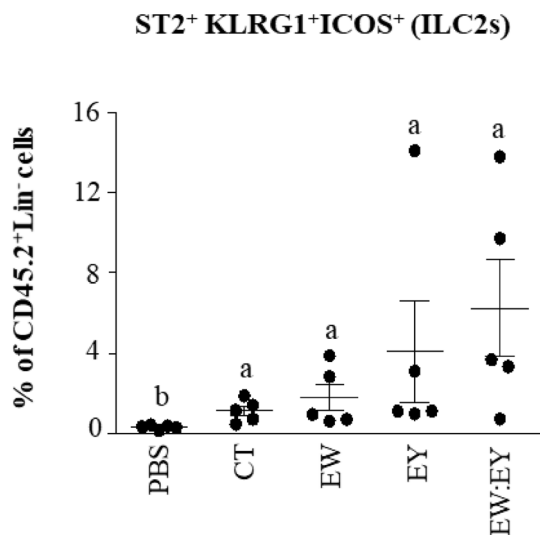


Fig. 2 Group 2 innate lymphoid cells (ILC2s) in the lamina propria of mice administered intragastrically: PBS, cholera toxin (CT), egg white (EW), egg yolk (EY), and a mixture 1:2 (w:w) egg white: egg yolk (EW:EY), for 4 consecutive days. ILC2s were defined as ST2⁺KLRG1⁺ICOS⁺ within CD45.2⁺ Lineage⁻ (CD3⁻, CD45R⁻, CD11b⁻, TER-119⁻, Ly-G6⁻, and CD19⁻) cells. Data are expressed as means $\pm$ SEM ($n=5$). Different letters indicate statistically significant differences ($p < 0.05$) among different mouse groups

non-lymphoid and lymphoid intestinal tissues, the expression of DC genes critical for Th2 differentiation, such as *Irf4* (encoding for interferon regulatory factor 4 -IRF4-, that determines DC development and functional specialization; [16], *Tnfsf4* (that encodes for OX40L) [14], and the Notch ligand *Jagged2* [17] (Fig. 3). Moreover, since it has been proposed that modulation of IRF4 in DCs promotes Th2 responses through IL-33 production [16], we examined *Il33* expression in intestinal lymphoid tissues and found that it was upregulated in the PPs and MLNs of CT-administered mice (Fig. 1). Accordingly, *Gata3* (the Th2 master transcription factor), and the Th2 cytokines *Il4* and *Il13* were overexpressed in all the tissues examined, except for *Il4* and *Il13* in the MLNs (Fig. 3). Likewise, expression of *Rorc* and *Il17a*, corresponding to the key transcription factor and hallmark cytokine of Th17 cells, was increased (Fig. 3), reinforcing the concept that the adjuvant activities of CT go beyond Th2 stimulation, also activating Th17 responses [14].

EW and the mixture EW:EY also upregulated *Irf4* and *Tnfsf4* in the duodenum, and EW:EY distinctively enhanced the expression of *Irf4* in the jejunum and PPs; although administration of EW, EY, or their mixture did not induce the expression of DC Th2-polarizing genes in the MLNs (Fig. 3). Interestingly, EY, alone or in combination with EW, increased, in the duodenum, jejunum, and PPs, the expression of *Cd1d1* (Fig. 3), which encodes for CD1d, a lipid receptor placed on the surface of IECs and DCs that contributes to food allergy via activation of iNKT cells [18, 19].

Administration of EW and EW:EY enhanced the expression of *Il14* and *Il13* in the duodenum and jejunum, and that of *Gata3*, *Rorc* and *Il17a* in the jejunum, with duodenal expression of *Il4* being augmented in mice administered EW: EY over those administered EW and EY separately (Fig. 3). As compared with mice given EW, the intestinal expression of *Gata3*, *Il13*, *Rorc*, and *Il17* was not affected in mice treated with the mixture EW:EY. However, EW:EY promoted the expression of *Il6*, *Il33*, *Gata3*, and *Il13*, both in the PPs and MLNs, and that of *Il4* in the MLNs, in comparison with EW only, which points at a type 2-adjuvant effect of the joint administration (Figs. 1, 3).

The mixture of egg white and egg yolk enhances type 2 responses in intestinal epithelial cells and dendritic cells, and induces the expansion of ST2-expressing T cells in vitro

With the aim of investigating the factors that drive the promotion of type 2 immunity by EW:EY, we conducted in vitro experiments with BM-DCs conditioned with the supernatants of IECs cultured with different stimuli. IECs were isolated from the small intestine of mice and incubated with EW, EY or their mixture. In addition to CT, LPS, recognized for its role on type 1 and also type 2 immunity [20], was also used as a stimulant. As shown in Fig. 4, incubation with CT upregulated the expression of *Il33* and *Tslp* in IECs, but LPS did not show any effect on the expression of epithelial cytokines. EW, as well as the combination EW:EY, also increased the expression of *Il33*, albeit to a lower extent than CT. IL-33, however, could not be detected by ELISA at the protein level (not shown). Of note, the mixture EW:EY remarkably upregulated *Cd1d1* on IECs (Fig. 4).

We next conditioned BM-DCs with the supernatants of IECs cultured with the stimuli, as well as with IL-33. In agreement with the results obtained in vivo, BM-DCs conditioned with the supernatants of IECs stimulated with CT overexpressed *Irf4*, *Tnfsf4* and *Jagged2* (Fig. 5). The supernatants of LPS-cultured IECs enhanced in BM-DCs the expression of both type 1- and type 2-driving factors, such as *Irf8* [21] and *Irf4*, but not of *Jagged2* (Fig. 5). LPS also upregulated *Tnfsf4*, recognized as a molecule that enables DCs to polarize Th2 responses, but can also induce Th1 responses in the presence of IL-12 [22], and the expression of *Il12p40* was, in fact, increased by LPS (not shown). Conversely, the supernatants of IECs pulsed with EW, EY, or their mixture did not change the expression of genes involved in the Th2-priming properties of BM-DCs and, surprisingly, IL-33 did not have any effect either; although EY, EW:EY, IL-33, CT, and, particularly, LPS upregulated the expression of *Il33* (Fig. 5). On the other hand, the supernatants of IECs cultured with EY and EW:EY induced the expression of *Cd1d1* in BM-DCs, as did those of IECs

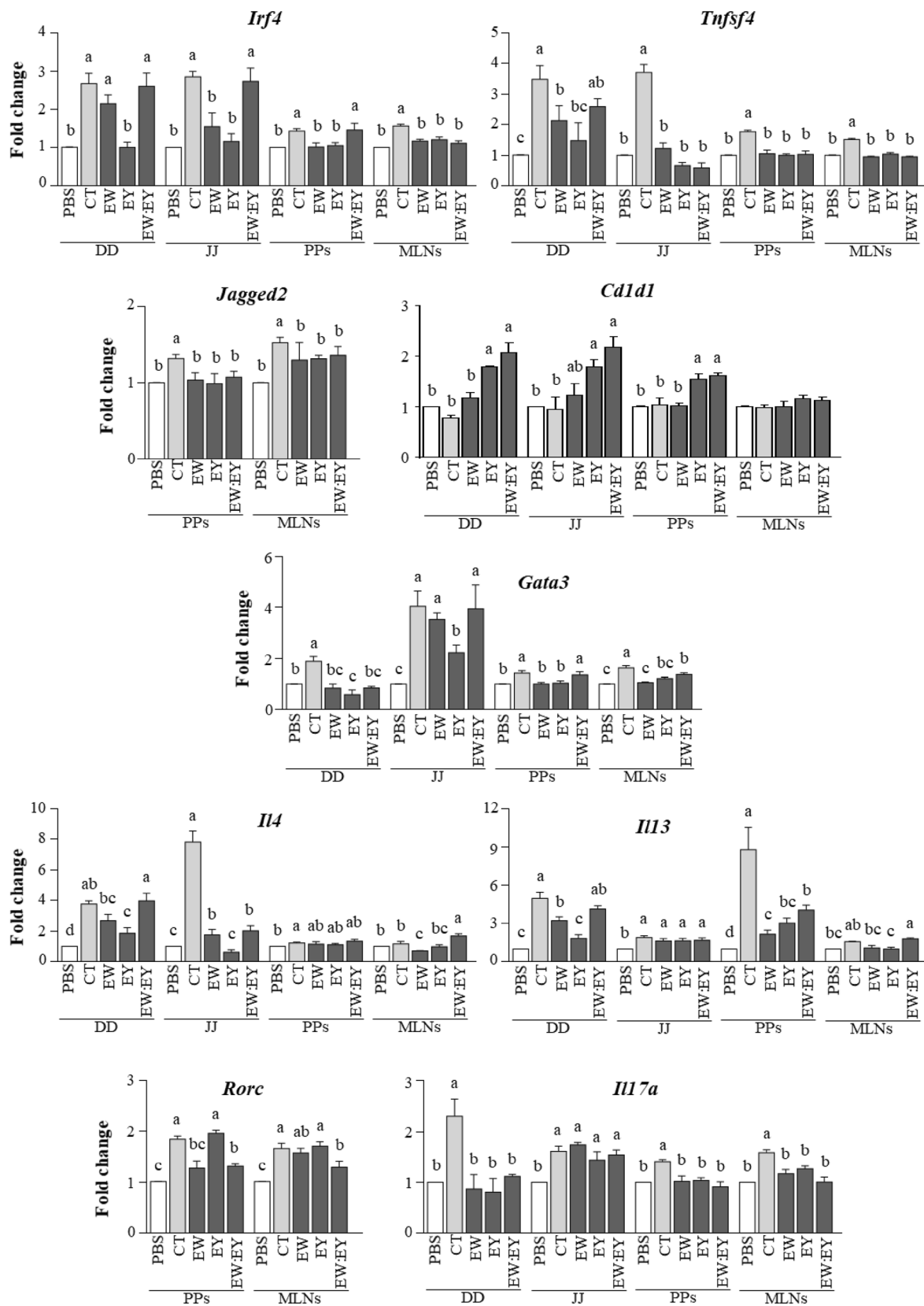


Fig. 3 Relative gene expression of *Irf4*, *Tnfsf4*, *Jagged2*, *Cd1d1*, *Gata3*, *Il4*, *Il13*, *Rorc*, and *Il17a*, determined in different tissues (*DD* duodenum, *JJ* jejunum, *PPs* Peyer's Patches, *MLNs* mesenteric lymph nodes) of mice administered intragastrically: PBS, cholera toxin (CT), egg white (EW), egg yolk (EY), and a mixture 1:2 (w:w) egg white: egg yolk (EW:EY), for 4 consecutive days. Gene expression was normalized to the reference gene *Actb* and compared with mice administered PBS. Data are expressed as means $\pm$ SEM ($n=6$). Different letters indicate statistically significant differences ($p<0.05$) among different mouse groups

stimulated with CT and LPS (Fig. 5). Direct culture of BM-DCs with the stimuli yielded similar results, precluding a significant effect of soluble factors released by IECs on this system (not shown).

In a previous study, we found that, even if EW:EY did not change in BM-DCs the expression of surface molecules involved in interactions with T cells (such as CD80, CD86 or OX40L), it induced proliferation of Th2 cells in co-cultures of BM-DCs and CD4⁺ T cells from EW-sensitized mice [9]. In order to assess whether EW:EY could modulate DCs to induce a Th2 cell response on CD4⁺ T cells from naïve mice, BM-DCs were co-cultured with CFSE-labelled CD4⁺ T cells in the presence of the stimuli (Fig. 6). CT, and, particularly, IL-33 significantly enhanced the expression of *Gata3*, *Il4*, and *Il13* in responder T cells (Fig. 6a). Co-culture of BM-DCs and CD4⁺ T cells with IL-33 also increased IL-13 secretion (Fig. 6b). Regarding the egg fractions, the mixture EW:EY induced a higher expression of *Gata3*, *Il4*, and *Il13*, and higher IL-13 secretion than EW or EY added individually (Fig. 6a, b). Moreover, IL-33 and EW:EY, but not CT or LPS or the separated egg fractions, selectively promoted the expansion of activated (CD69⁺) CD4⁺ T cells expressing the IL-33 receptor (ST2) (Fig. 6c and gating strategy in Supplementary Fig. 1b), thus showing that the combination of EW and EY conditioned DCs to induce Th2 cytokine expression and expansion in CD4⁺ T cells of naïve mice.

With the aim to clarify the role of IL-33, we performed in vivo experiments consisting in the intraperitoneal administration of IL-33 to mice for 4 days. The results showed that it deeply stimulated the expression of *Il33*, *Il25*, *Tslp*, and *Il6* at the intestinal level, and of Th2-polarizing factors of DCs (*Irf4* and *Tnfsf4*) and Th2 cytokines (*Il4* and *Il13*) in duodenum, jejunum, PPs and MLNs (Supplementary Fig. 2).

Discussion

From a mechanistic point of view, it is not fully clear how innocuous foods trigger or amplify allergic reactions. There is a close relationship between allergens and lipids, which can behave as adjuvants changing the immunomodulating properties of proteins [3]. In this study we show that EW exerts local type 2-promoting effects in the small intestine

and that activation of innate immune responses by orally gavaged EY could contribute to sensitization to co-administered EW.

It is recognized that factors that compromise the barrier function of the intestinal mucosa play a role in the triggering of food allergy. At the duodenal level, administration of CT enhanced the expression of *Il22*, *Muc2*, *Cldn2*, and *Il6*, which were previously found to be overexpressed in the small intestine of mice sensitized to EW with the aid of CT [23]. IL-22 favours epithelial integrity but also produces inflammatory effects and it is associated with the severity of allergic diseases [24]. IL-22 promotes hypersecretion of mucus, whose major constituent is *Muc2*, and modifies intestinal permeability through the induction of *Claudin-2*, a tight junction protein that decreases transepithelial resistance and forms pores for cations [25, 26]. Noteworthy, EW increased the expression of *Muc2* and *Cldn2* without modifying that of *Il22*, likely because type 2-promoting and Th2 cytokines, such as IL-33, IL-4 and IL-13, whose expression was increased by EW administration, cause goblet cell hyperplasia and mucus production, and also impair the intestinal tight junction barrier through the increase of pore-forming *Cldn2* expression, but also through additional mechanisms that enhance the flux of macromolecules [27–30]. Thus, disruption of the intestinal barrier by EW may favour its access to immune cells, playing a role in the initiation of sensitization. EY in the EW:EY mixture, however, attenuated the effects of EW on *Muc2* and *Cldn2* expression, likely because polyunsaturated chain fatty acids, which amount, approximately, 40–45% of the fatty acid composition of EY lipids [10], improve intestinal barrier resistance and reduce the effects of IL-4 on intestinal permeability [31].

Intragastric administration of EW:EY to mice enhanced *Il33*, *Il25*, and *Tslp* expression, either in the duodenum and/or the jejunum, as compared with EW alone. Furthermore, expression of *Il25* and *Tslp* in the duodenum and jejunum of mice administered EW:EY was comparable or even higher than in intestinal tissues of mice administered the potent type 2-inducing adjuvant CT. In murine models of gastrointestinal peanut allergy in which CT is used as an adjuvant, levels of IL-33, IL-25, and TSLP are increased, in parallel with the expansion of ILC2s in the small intestine and MLNs [5].

ILC2s, innate cells that depend on GATA3 for their development and function, constitute an early source of Th2 cytokines in response to both IL-33 and IL-25 [15]. IL-4, IL-5, and IL-13 produced by these cells play a role in promoting food allergy by the disruption of Treg cell induction and the enhancement of Th2 cell differentiation and mast cell activation [32]. Our results show that both CT and EY stimulated the development of ILC2s in the lamina propria of mice and that EY amplified the effect of EW. In this respect, it should be noted that, while IL-33, IL-25, and

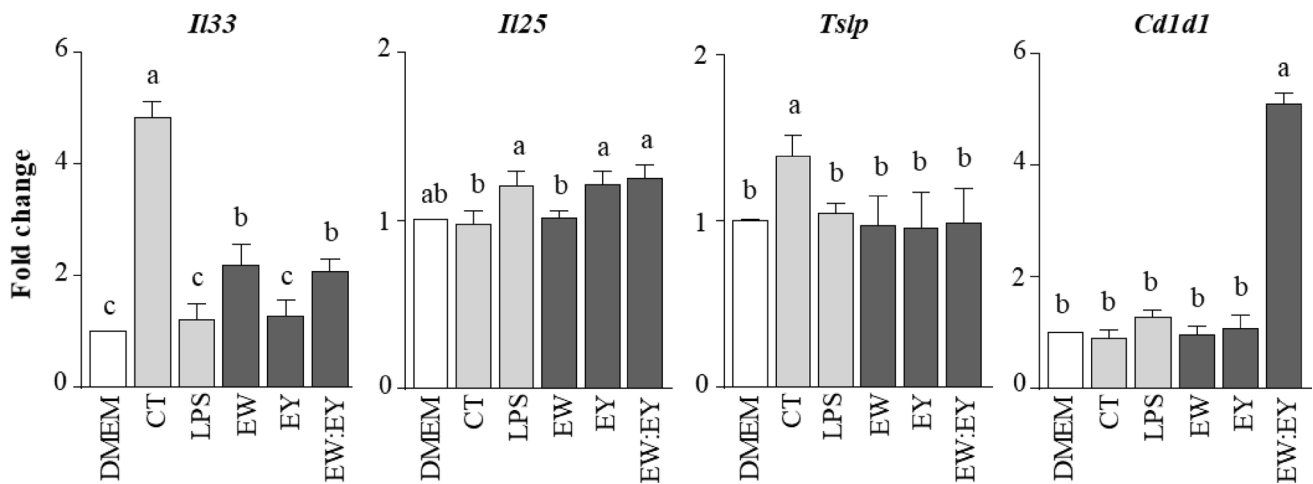


Fig. 4 Relative gene expression of *Il33*, *Il25*, *Tslp*, and *Cd1d1* in intestinal epithelial cells from naïve mice cultured with: DMEM, cholera toxin (CT), lipopolysaccharide (LPS), egg white (EW), egg yolk (EY), and a mixture 1:2 (w:w) egg white: egg yolk (EW:EY), for 24 h. Gene expression was normalized to the reference gene *Actb* and

compared with cells cultured in DMEM. Data from a representative experiment, expressed as means $\pm$ SEM ($n=3$) are shown. Different letters indicate statistically significant differences ($p < 0.05$) among different stimuli

TSLP presumably released by EW:EY on contact with IECs may have modulated ILC2s, a direct effect of EY lipids cannot be discarded, as multiple lipid mediators have been found to drive activation of ILC2s [33]. Nevertheless, information on the effect of dietary lipids on ILC2s is scarce. It has been reported that co-administration of EW and medium-chain triglycerides induces allergy to EW, characterized by high specific antibody levels, increased numbers of lamina propria Th2 cells, mast cells, and DCs, but not ILC2s; even if medium size triglycerides stimulate the jejunum expression of IL-25, IL-33, and TSLP, and allergy can be prevented or suppressed by the inhibition of any or the three epithelial cytokines, respectively [34, 35].

Despite the role of ILC2-produced cytokines in Th2 immunity, ILC2s have been found dispensable in the initiation of food allergy, with antigen-induced CD4⁺ Th2 cells being either sufficient or essential ILC2 co-operators [36, 37]. In this respect, several studies have shown the importance of the expression of OX40L on DCs that depend on the transcription factor IRF4 for the expansion and survival of Th2 cells [4]. Indeed, CT, orally fed to mice, induces DC migration, maturation and up-regulation of genes that prime for Th2 responses, such as *Tnfsf4* and *Jagged2*, in the MLNs [5, 14, 38]. Accordingly, in our study, CT enhanced the expression of *Irf4*, *Tnfsf4*, and *Jagged2* in the PPs and MLNs of mice in vivo, as well as in vitro in BM-DCs. EW, as well as EW:EY, also upregulated *Irf4* and *Tnfsf4* in the duodenum, probably through IL-33, IL-25, and TSLP produced by IECs. These cytokines activate Th2-inducing DCs and drive OX40L expression [5, 22] and, in fact, we found that intraperitoneal administration of IL-33 to mice upregulated *Irf4* and *Tnfsf4* expression in non-lymphoid and lymphoid

intestinal tissues, in parallel with significantly enhanced Th2 responses. Furthermore, expression of *Irf4* was enhanced in the jejunum and PPs of EW:EY administered mice. Notably, delivery of antigens to PPs is believed to increase their ability to induce Th2 immunity as compared with epithelial absorption [39].

However, we did not observe upregulation of co-stimulatory molecules in BM-DCs stimulated with EW:EY or with supernatants of EW:EY-conditioned IECs, nor augmented expression of *Irf4*. Unexpectedly, incubation of BM-DCs with IL-33 did not upregulate *Irf4*, *Tnfsf4*, or *Jagged2* either. There are conflicting reports on the Th2-skewing factors activated by IL-33 on BM-DCs in vitro, which indicate either enhanced [40, 41] or unchanged [42] *Tnfsf4* expression, despite it is documented that intraperitoneal administered IL-33 increases *Tnfsf4* expression in the small intestine and MLNs [5], in agreement with our own results.

Although the major sources of IL-33 are endothelial and epithelial cells [27], IL-33 itself and Toll-like receptor (TLR) ligands, such as LPS, induce expression and production of IL-33 by DCs, expanding type 2 inflammation, an effect that can be further amplified by certain lipids [41, 43]. This agrees with our results showing upregulation of *Il33* in BM-DCs under the influence of the supernatants of IECs cultured with IL-33 and, mainly, with LPS. Moreover, it has been reported that signals that augment *Irf4* expression in DCs (which is regulated by NF- κ B downstream of TLRs) skew the ensuing T cell response towards Th2 differentiation, without affecting the surface expression of DC co-stimulatory molecules, such as CD80, CD86 or OX40L, through the activation of the transcription of *Il33* [16]. *Il33* was overexpressed in BM-DCs conditioned with the

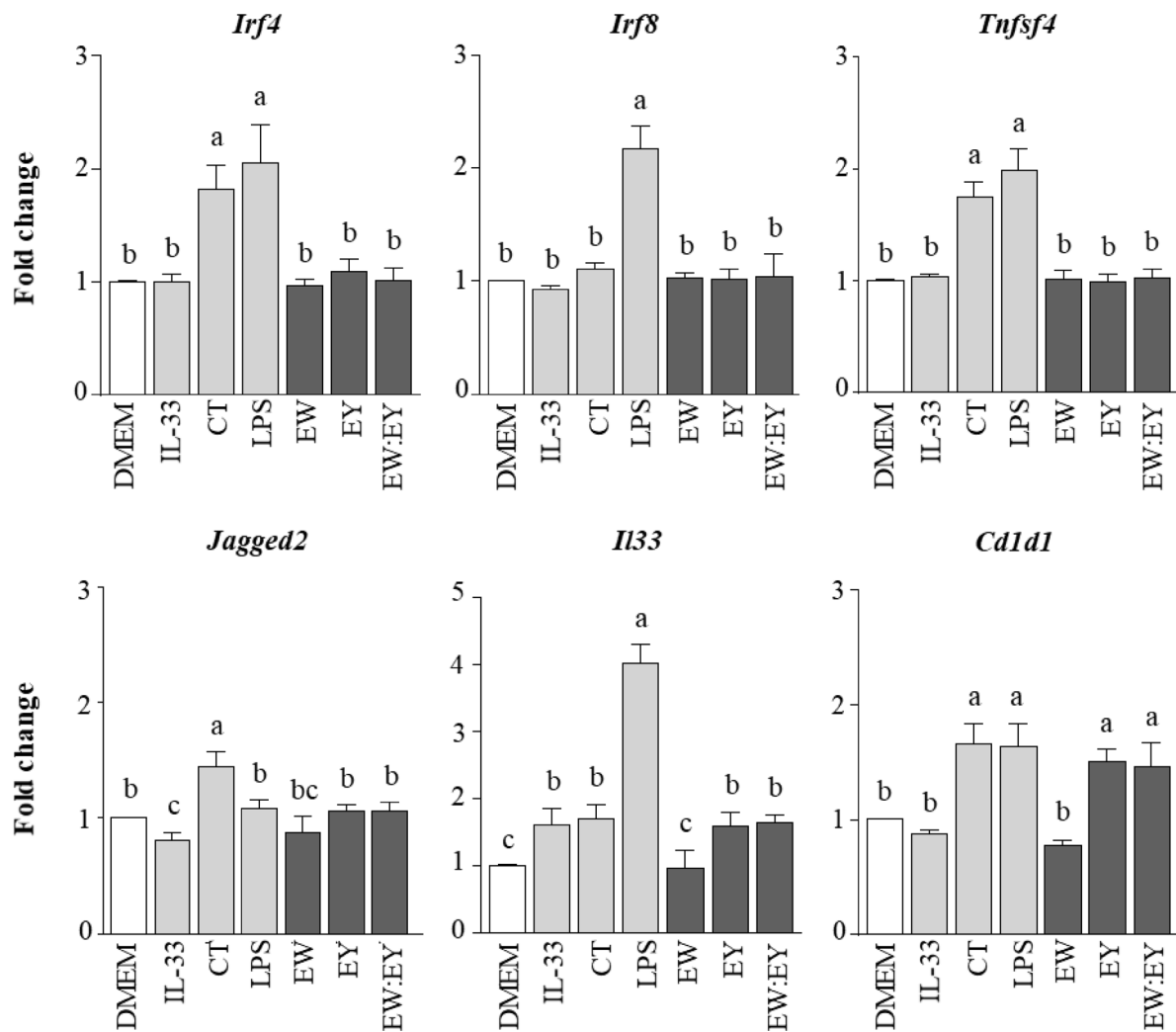


Fig. 5 Relative gene expression of *Irf4*, *Irf8*, *Tnfsf4*, *Jagged2*, *Il33*, and *Cd1d1* in bone marrow-derived dendritic cells from naïve mice conditioned with the supernatants of intestinal epithelial cells from naïve mice previously cultured with: DMEM, IL-33, cholera toxin (CT), lipopolysaccharide (LPS), egg white (EW), egg yolk (EY), and a mixture 1:2 (w:w) egg white: egg yolk (EW:EY), for 24 h. Gene

expression was normalized to the reference gene *Actb* and compared with cells cultured in DMEM. Data from a representative experiment, expressed as means $\pm$ SEM ($n=3$) are shown. Different letters indicate statistically significant differences ($p<0.05$) among different stimuli

supernatants of IECs stimulated with EW:EY (as well as with EW:EY itself, not shown), and an enhanced expression of *Il33* was found in the PPs and MLNs of mice administered EW:EY, suggesting that DCs were a source of IL-33 under the stimulus of EW:EY.

Noteworthy, on co-cultures of BM-DCs and CD4⁺ T cells, EW:EY behaved as IL-33, in that it promoted *Gata3*, *Il4*, and *Il13* expression and IL-13 production, as did CT and LPS, but, in contrast to CT and LPS, it also induced the expansion of T cells expressing ST2, the IL-33 receptor. This suggests that, by means of IL-33 production, EW:EY may be implicated, on the one hand, in the production of Th2 cytokines by naïve T cells and, thus, in the initiation of Th2 responses and, on the other hand, in the proliferation

of existing Th2 cells, as these, over naïve cells or Th1 cells, preferentially present the IL-33 receptor [44]. Indeed, IL-33, through DCs, has been reported to be involved both in the polarization of naïve T cells into atypical Th2 cells, which produce IL-5 and IL-13, but not IL-4 [28, 40, 42]; and in cytokine production by already polarized Th2 cells [27, 45]. The observation that IL-33 and EW:EY enhanced *Il4* expression and expansion of ST2⁺ cells from CD4⁺ T cells, that may contain antigen experienced cells as well as naïve cells, implies that proliferation of existing Th2 cells is taking place simultaneously to Th2 differentiation. In contrast, in vivo, administration of IL-33 clearly induces *Il4* expression in multiple tissues [27], as found in the present work, although likely at the expense of cells of the innate immune

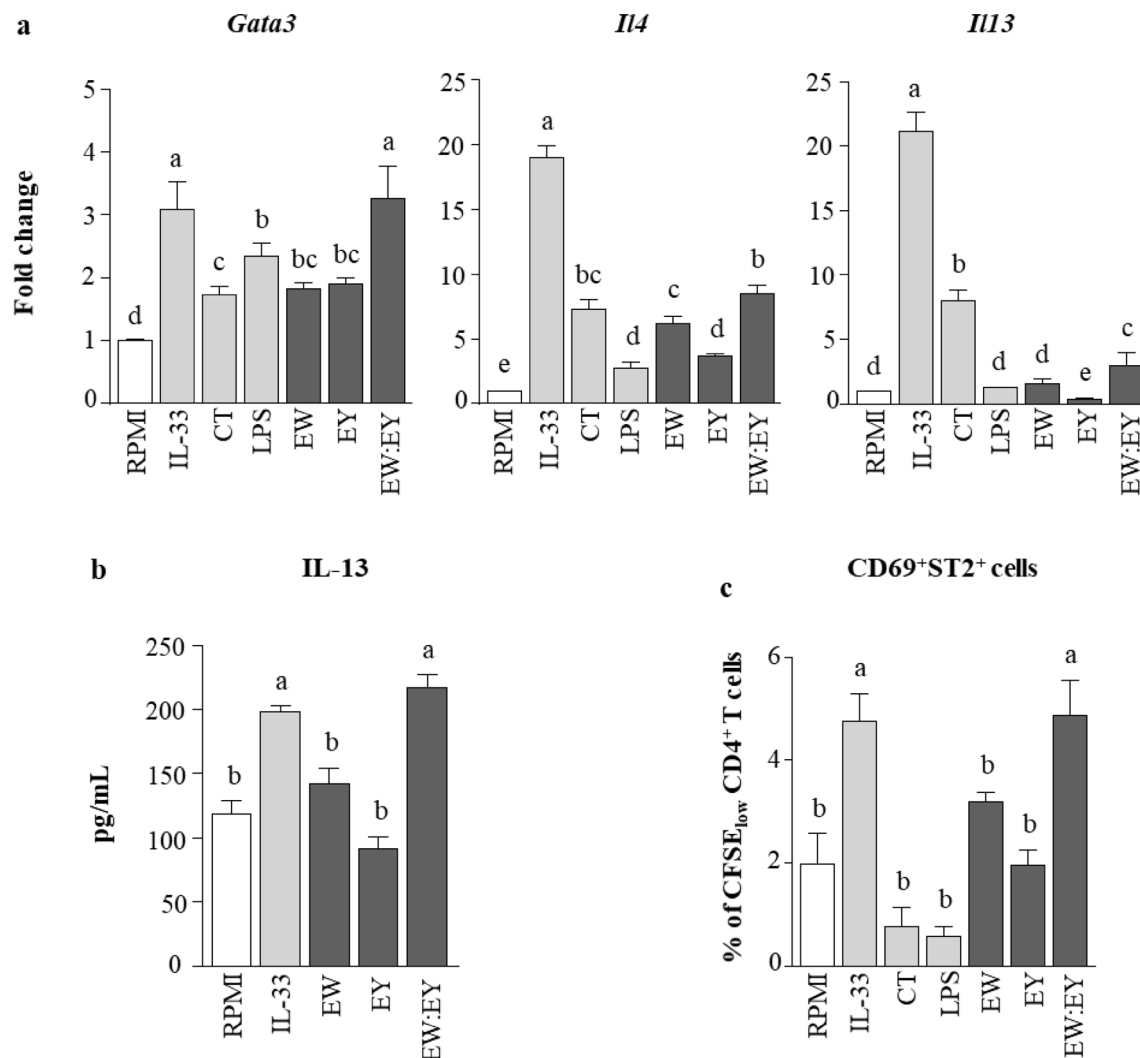


Fig. 6 Relative gene expression of *Gata3*, *Il4*, and *Il13* (**a**), concentration of IL-13 (**b**), and percentage of activated (CD69⁺) ST2⁺ cells within CD4⁺ cells (**c**) in cocultures of bone marrow-derived dendritic cells and CD4⁺ cells from naïve mice with: RPMI, IL-33, cholera toxin (CT), lipopolysaccharide (LPS), egg white (EW), egg yolk (EY), and a mixture 1:3 (w:w) egg white: egg yolk (EW:EY), for

3 days, followed by stimulation with anti-CD3 for 3 additional days. Gene expression was normalized to the reference gene *Actb* and compared with cells cultured in RPMI. Data from a representative experiment, expressed as means $\pm$ SEM ($n=2$) are shown. Different letters indicate statistically significant differences ($p < 0.05$) among different stimuli

system [46]. The reason why LPS and CT did not stimulate expansion of ST2⁺ CD4⁺ T cells, despite enhancing *Irf4* and *Il33* expression deserves further investigation.

ST2, associated with Th2 functions, is known to be expressed mainly in ILC2s, mast cells, Th2, and regulatory T cells, but it is also expressed in iNKT cells, which respond to IL-33, as well as to IL-25, by in vitro expansion and functional activation, and recognize lipid structures that are presented by CD1d molecules on IECs and DCs [47, 48]. Among iNKT cells, which mature into three different subsets based upon expression of transcription factors and cytokines, iNKT2 cells mirror Th2 cells in expression of GATA3 and IL-4 and IL-13 production [48]. Oral gavage of lipid antigens stimulates iNKT2 cells in the MLNs of BALB/c mice

to locally release IL-4 and to promote the differentiation of Th2 cells, showing that intestinal lipids provide signals to iNKT cells through DCs trafficking to MLNs [49]. Therefore, it can be speculated that EW:EY could cause proliferation and Th2 cytokine expression by iNKT cells, as much as it promoted overexpression of *Il33* and *Cd1d1* in different tissues in vivo, as well as in BM-DCs in vitro, in addition, or as a previous step to Th2 cell expansion. Indeed, it was found that human monocyte-derived DCs upregulate CD1d expression following exposure to polar lipids (including phospholipids and glycolipids), glycerolipids and free fatty acids, resulting in iNKT activation in vitro [50].

The aforementioned results show that EW works as a type 2 immune stimulus at the intestinal level that alters intestinal

barrier function and activates secretion of Th2-skewing cytokines from IECs, features that might underlie its potential as a food allergen. Furthermore, co-administration of EY may promote sensitization to EW through activation of innate immune cells, such as IECs, DCs, ILC2s, and, possibly, iNKT cells, to upregulate lipid presenting molecules (CD1d), type 2-promoting cytokines (IL-33), Th2 lineage determining factors (GATA3, IRF4), and, eventually, Th2 cytokines (IL-4, IL-13) that are central to the progress of food allergies.

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Compliance with ethical standards

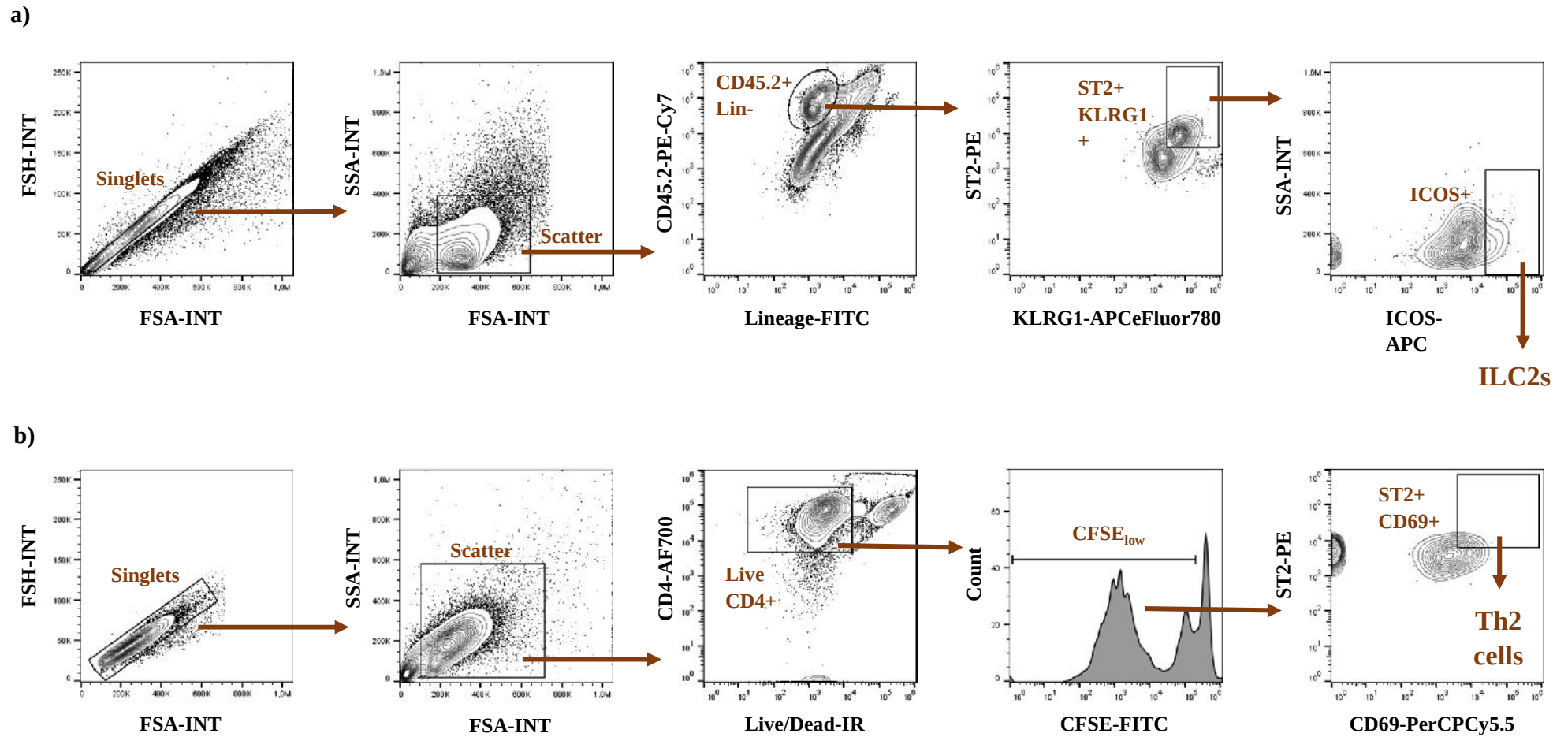
Conflict of interest The authors declare no conflict of interest.

Research involving animals All protocols followed the European legislation (Directive 2010/63/EU) and were approved by Comunidad de Madrid (Ref PROEX 089/15).

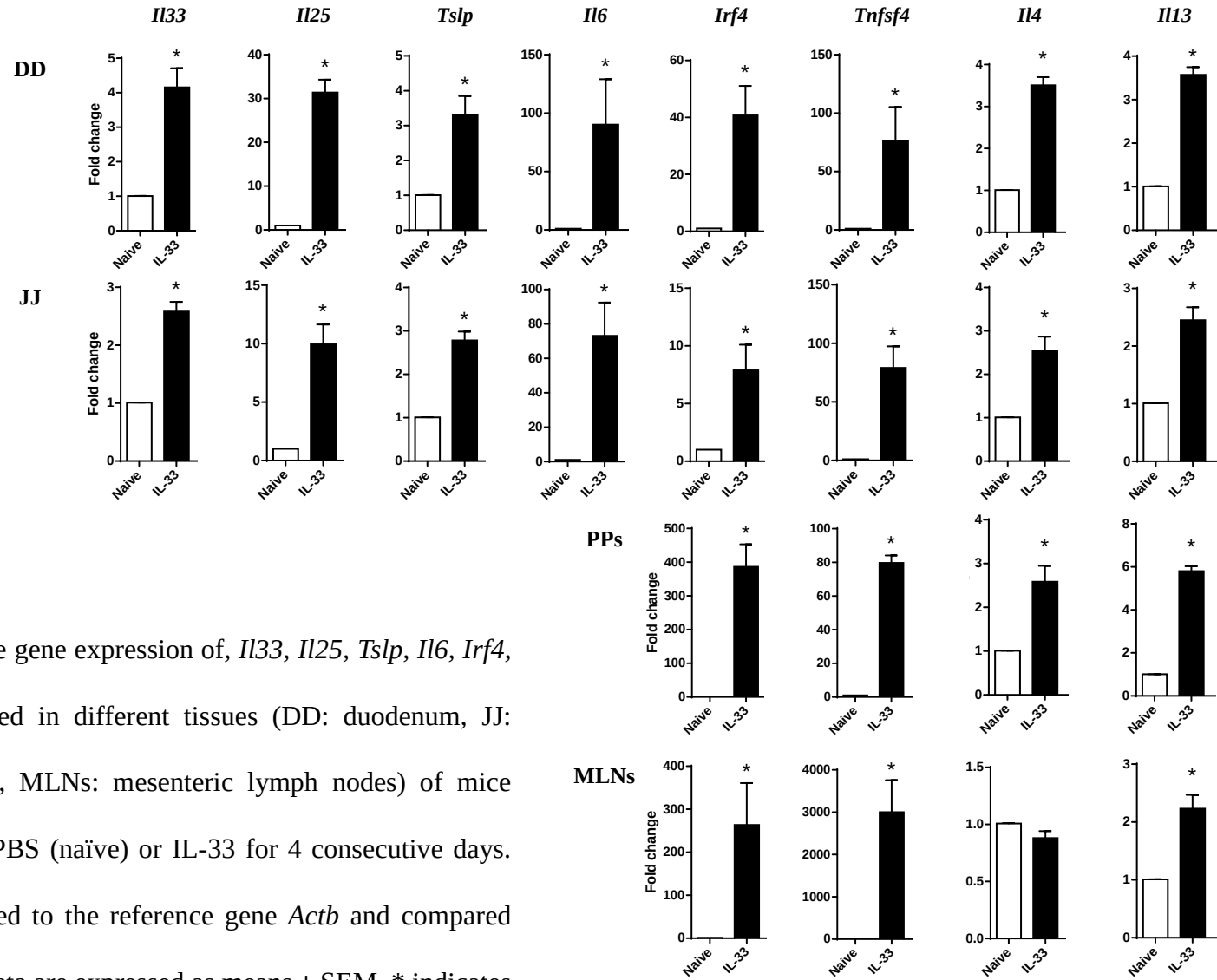
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Supplementary Fig. 1. Gating strategy for the analysis of (a) group 2 innate immune cells (ILC2s) and (b) proliferating CD4⁺ Th2 cells by flow cytometry.



Supplementary Fig. 2. Relative gene expression of, *Il33*, *Il25*, *Tslp*, *Il6*, *Irf4*, *Tnfsf4*, *Il4*, and *Il13* determined in different tissues (DD: duodenum, JJ: jejunum, PPs: Peyer's Patches, MLNs: mesenteric lymph nodes) of mice administered intraperitoneally PBS (naïve) or IL-33 for 4 consecutive days. Gene expression was normalized to the reference gene *Actb* and compared with mice administered PBS. Data are expressed as means $\pm$ SEM. * indicates statistically significant differences ($p < 0.05$).

Gene	Primer pairs	Protein product	Reference	Cycling conditions
<i>Actb</i>	<i>fw</i> 5' AGCTGCGTTTTACACCCTTT 3' <i>rv</i> 5' AAGCCATGCCAATGTTGTCT 3'	β -actin	[1]	<i>Pre-Incubation</i> 2 min 50°C <i>Incubation</i> 10 min 95°C 40 cycles: <i>Denaturation</i> 15 s 95°C <i>Annealing/Extension</i> 60 s 60°C
<i>Cd1d1</i>	<i>fw</i> 5' GAATGACACCTGCCCCCTATT 3' <i>rv</i> 5' ACCCACACAGGTTTTGGGTA 3'	CD1d	[2]	
<i>Gata3</i>	<i>fw</i> 5' CCTTAAACTCTTGGCGTCC 3' <i>rv</i> 5' AGACACATGTCATCCCTGAG 3'	GATA3	[3]	
<i>Il17a</i>	<i>fw</i> 5' TGCCTGTGGCACTGAAGTAG 3' <i>rv</i> 5' TTCATGGCTGCAGTGAAG 3'	IL-17	[4]	
<i>Il25</i>	<i>fw</i> 5' ACAGGGACTTGAATCGGGTC 3' <i>rv</i> 5' TGGTAAAGTGGGACGGAGTTG 3'	IL-25	[5]	
<i>Il33</i>	<i>fw</i> 5' ATTTCCCCGGCAAAGTTCAG 3' <i>rv</i> 5' AACGGAGTCTCATGCAGTAGA 3'	IL-33	[5]	
<i>Irf4</i>	<i>fw</i> 5' TCCTCGTCCCTTGCTGAAAC 3' <i>rv</i> 5' GGGCTTTGGGGCTTCTAGTT 3'	IRF4	This work	
<i>Irf8</i>	<i>fw</i> 5' ACCGGCGGCAGGATGT 3' <i>rv</i> 5' ACAGCGTAACCTCGTCTTCC 3'	IRF8	This work	
<i>Jagged2</i>	<i>fw</i> 5' GGCAAAGAATGCAAAGAAGC 3' <i>rv</i> 5' GCTCAGCATTGATGCAGGTA 3'	Jagged2	[6]	
<i>Muc2</i>	<i>fw</i> 5' GCTGACGAGTGGTTGGTGAATG 3' <i>rv</i> 5' ATGAGGTGGCAGACAGGAGAC 3'	Muc2	[7]	
<i>Rorc</i>	<i>fw</i> 5' TCACCTGTGAGGGGTGCAAG 3' <i>rv</i> 5' GTTCGGTCAATGGGGCAGTT 3'	ROR- γ	[8]	
<i>Tslp</i>	<i>fw</i> 5' AGGCTACCCTGAAACTGAGA 3' <i>rv</i> 5' GGAGATTGCATGAAGGAATAC 3'	TSLP	[9]	
<i>Actb</i>	<i>fw</i> 5' AGCTGCGTTTTACACCCTTT 3' <i>rv</i> 5' AAGCCATGCCAATGTTGTCT 3'	β -actin	[1]	<i>Pre-Incubation</i> 2 min 50°C <i>Incubation</i> 10 min 95°C 40 cycles: <i>Denaturation</i> 15 s 95°C <i>Annealing/Extension</i> 45 s 58°C + 15 s 60°C
<i>Cldn2</i>	<i>fw</i> 5' GTCATCGCCCATCAGAAGAT 3' <i>rv</i> 5' ACTGTTGGACAGGGAACCCAG 3'	Claudin-2	[10]	
<i>Il4</i>	<i>fw</i> 5' CCTCACAGCAACGAAGAACA 3' <i>rv</i> 5' ATCGAAAAGCCCCGAAAGAGT 3'	IL-4	[11]	
<i>Il6</i>	<i>fw</i> 5' TTCCATCCAGTTGCCTTCTTG 3' <i>rv</i> 5' GGGAGTGGTATCCTCTGTGAAGTC 3'	IL-6	[12]	
<i>Il13</i>	<i>fw</i> 5' CATGGCCTCTGTAACCGCAA 3' <i>rv</i> 5' CCTCATTAGAAGGGGCCGTG 3'	IL-13	This work	
<i>Il22</i>	<i>fw</i> 5' CATGCAGGAGGTGGTACCTT 3' <i>rv</i> 5' CAGACGCAAGCATTCTCAG 3'	IL-22	[13]	
<i>Actb</i>	<i>fw</i> 5' AGCTGCGTTTTACACCCTTT 3' <i>rv</i> 5' AAGCCATGCCAATGTTGTCT 3'	β -actin	[1]	<i>Pre-Incubation</i> 2 min 50°C <i>Incubation</i> 10 min 95°C 40 cycles: <i>Denaturation</i> 15 s 95°C <i>Annealing/Extension</i> 30 s 56°C + 30 s 58°C
<i>Tnfsf4</i>	<i>fw</i> 5' GGGATGCTTCTGTGCTTCATCT 3' <i>rv</i> 5' TTTGGATTGGAGGGTCCTTTG 3'	OX40L	[14]	

Supplementary Table 1. Primer pair sequences and conditions used for the analyses of gene expression.

fw, forward; *rv*, reverse

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Supplementary Table 2. Antibodies used for the analysis of T cell and ILC2 subsets by flow cytometry (all from eBioscience, San Diego, CA, USA).

Population	Antibody	Clone	Fluorophore
	CD16/CD32	2.4G2	
CD4 ⁺ T cells	CD4	GK1.5	Alexa Fluor 700
	CD69	H1.2F3	PerCP-Cy5.5
	CD183 (CXCR3)	CXCR3-173	APC
	ST2 (IL-33 receptor)	RMST2-2	PE
ILC2	CD45.2	104	PE-Cy7
	CD3	17A2	FITC
	CD45R (B220)	RA3-6B2	
	Hematopoietic lineage	CD11b	
		TER-119	
		Ly-G6 (Gr-1)	
		CD19	
	ST2 (IL-33 receptor)	RMST2-2	PE
	KLRG1	2F1	APC eFluor 780
	CD278 (ICOS)	C398.4A	APC