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IKKβ in intestinal epithelial cells regulates allergen-specific IgA and allergic inflammation at distant mucosal sites

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Abstract

Regulation of allergy responses by intestinal epithelial cells remain poorly understood. Using a model of oral allergen sensitization in the presence of cholera toxin as adjuvant and mice with cell-specific deletion of IKKβ in intestinal epithelial cells IECs (IKKβ$^{\Delta IEC}$), we addressed the contribution of IECs to allergic sensitization to ingested antigens and allergic manifestations at distant mucosal site of the airways. Cholera toxin induced higher proinflammatory responses and altered the profile of the gut microbiota in IKKβ$^{\Delta IEC}$ mice. Antigen-specific IgE responses were unaltered in IKKβ$^{\Delta IEC}$ mice, but their IgA Abs, Th1 and Th17 responses were enhanced. Upon nasal antigen challenge, these mice developed lower levels of allergic lung inflammation, which correlated with higher levels of IgA Abs in the airways. The IKKβ$^{\Delta IEC}$ mice also recruited a higher number of gut-sensitized T cells in the airways after nasal antigen challenge and developed airway hyper-responsiveness, which were suppressed by treatment with anti-IL-17A. Fecal microbiota transplant during allergic sensitization reduced Th17 responses in IKKβ$^{\Delta IEC}$ mice, but did not affect IgA Ab responses. In summary, we show that IKKβ in IECs shapes the gut microbiota and immune responses to ingested antigens and influences allergic responses in the airways via regulation of IgA Ab responses.

Introduction

Ingestion of food antigens generally fails to promote brisk immune responses but rather results in a state of immune tolerance. However, aberrant immune responses, including food allergy, can develop in individuals with a genetic predisposition. Clinical manifestations of food allergies include gastrointestinal, systemic (anaphylaxis), cutaneous (eczema) or

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respiratory (asthma) symptoms [1, 2] and are generally regarded as pathologic responses to food antigens mediated by excessive Th2 responses and antigen-specific IgE Ab responses. Past research on allergy and asthma focused on the role of cells and molecules involved in adaptive immunity. More recently, epithelial cells lining the sites of antigen entry and innate immune responses have emerged as important players in these pathologies [1, 3, 4]. Studies in animal models have demonstrated that oral sensitization to allergens primes for adverse inflammatory responses at distant sites of the airways or the skin [5, 6]. However, little is known about the mechanism(s) employed by intestinal epithelial cells (IECs) to shape immune responses to allergens and influence allergic manifestations in distant mucosal sites such as the airways.

The nuclear factor κB (NF-κB) pathway plays an important role in inflammatory responses [7]. Its activation is regulated by the IkB kinase (IKKβ), the catalytic subunit of the IKK complex responsible for NF-κB translocation and transcription [7]. Previous studies have shown that the IKKβ-NF-κB signaling controls a number of biological processes via tissue-specific regulation of inflammatory and anti-inflammatory responses and can mediate both pro- and anti-inflammatory effects [8, 9]. These opposite effects were attributed to the nature of immune cells where the IKKβ-NF-κB signaling was affected [10]. However, both attenuation of chronic and exacerbation of acute gut inflammatory diseases were reported in mice with alteration of IKKβ-dependent NF-κB activation in IECs [11]. Others have shown that inhibition of IKKβ-dependent NF-κB activation limits the production of Th2-inducing cytokine by IECs and thus, impairs the development of protective immunity against the gut-dwelling parasite, *Trichuris*[12].

The large intestine of mammals contains a huge community of commensal bacteria, which contributes to the digestive functions [13], prevent the development of inflammatory bowel diseases [14], and support the maturation of gut immune cells [15, 16]. The gut microbiota can be perturbed by endogenous or exogenous factors and it is now established that microbial dysbiosis is associated with allergy [17, 18], obesity [19], and inflammatory diseases [20, 21]. IECs sense changes in gut microbiota and transplantation of healthy infant gut microbiota could protect mice from developing allergy responses to food antigens [17],

Oral administration of food antigen with cholera toxin as adjuvant in experimental animals is a well-accepted model to study allergic sensitization to food antigens [5, 6, 22]. To address the role of IECs in pathogenic immune responses to ingested food antigens, mice with targeted deletion of IKKβ in IECs ($IKK\beta^{\Delta IEC}$) [23] were orally sensitized to a food antigen in the presence of cholera toxin. We show that a localized impairment of IKKβ in IECs alters the gut microbiota during oral allergic sensitization and regulates the profile of allergic inflammatory responses at the distant airways through IgA Abs and Th17 responses.

Results

IKKβ deletion in IECs enhances gut inflammatory responses to the adjuvant cholera toxin

Sixteen hours after oral cholera toxin treatment, pNF-κB expression was enhanced in IECs of C57BL/6 mice and primarily found in the nucleus. Cholera toxin also increased pNF-κB expression in IECs of $IKK\beta^{\Delta IEC}$ mice, but the localization was primarily cytoplasmic (Fig.

1). Except for TSLP mRNA levels that were lower in the IKKβ$^{\Delta IEC}$ group, we found no difference between control and IKKβ$^{\Delta IEC}$ mice before treatment. Cholera toxin elevated mRNA levels of the pro-inflammatory cytokines in both IKKβ$^{\Delta IEC}$ mice and control C57BL/6 mice, but TNF-α mRNA were increased by ~ 10-fold in IKKβ$^{\Delta IEC}$ mice (Fig.1B). Cholera toxin also significantly increased TGFβ mRNA levels in the gut of IKKβ$^{\Delta IEC}$ mice (Fig. 1B). At the time point examined, cholera toxin treatment did not change TSLP mRNA levels in the gut of control mice, but raised TSLP mRNA levels in IKKβ$^{\Delta IEC}$ mice (Fig. 1B). The enhanced level of cytokine mRNA responses in the gut of IKKβ$^{\Delta IEC}$ mice was associated with higher levels of pSTAT3 responses as determined by western blotting (Fig. 1C) and immuno-histochemistry (Fig. S1).

Loss of IKKβ in IECs favors dysbiosis in cholera toxin–treated mice

Unlike controls, IKKβ$^{\Delta IEC}$ mice showed no signs of intestinal fluid accumulation 16 hrs after treatment with cholera toxin (Fig. S2). Analysis of bacterial 16S rRNA genes in fecal pellets showed no significant difference between the ration of *Bacteroidetes* and *Firmicutes* phyla in controls and IKKβ$^{\Delta IEC}$ mice before cholera toxin treatment (Fig. 2A and Table S1). Four days after oral administration of cholera toxin, a dysbiosis affecting phyla (Fig. 2A), class (Fig. 2B) and species (Fig. 2B) was observed in the fecal pellets of IKKβ$^{\Delta IEC}$ mice (Details in Fig. S3-S5 and Table S1-S6).

Loss of IKKβ in IECs alters antigen-specific CD4^{+} T cell and Ab responses to oral sensitization

Segmented Filamentous Bacteria (SFB) are noncultivable commensals Gram-positive anaerobic bacteria that share strong 16S rRNA similarities with the genus *Clositridium* [24–26] and regulate T helper cytokine responses [27, 28]. Four days after oral administration of cholera toxin, the percentage of SFB was slightly increased in controls, but was significantly elevated in IKKβ$^{\Delta IEC}$ mice (Fig. 3A).

We next examined whether innate cytokine responses and dysbiosis in the gut of IKKβ$^{\Delta IEC}$ mice were associated with changes in allergen-specific T helper cytokine and antibody responses. Mesenteric lymph node (MLN) and spleen T cells from control and IKKβ$^{\Delta IEC}$ mice secreted the same level of IL-4 after *in vitro* restimulation with OVA (data not show). On the other hand, IL-5 and IL-10 secretion by cells from IKKβ$^{\Delta IEC}$ mice was significantly decreased (Fig. 3B), while IL-17 and IFNγ responses were both enhanced (Fig. 3B). Th1-associated IgG2a (IgG2c) responses were significantly enhanced in IKKβ$^{\Delta IEC}$, while IgE and IgG1 Ab responses were unchanged (Fig.3C). In addition, IKKβ$^{\Delta IEC}$ mice developed higher OVA-specific IgA Ab responses than control C57BL/6 mice.

To clarify whether the changes in cytokine and antibody levels were the result of a changed microbiome or were a consequence of lack of IKKβ in epithelial cells, microbiome swap were performed by fecal microbiome transplant 4 days after each oral sensitization (Fig. 4A). The fecal microbiome swap affected antigen-specific T cell cytokine responses by mesenteric lymph node cells as it reduced IL-17A responses of IKKβ$^{\Delta IEC}$ when compared to wild-type mice and eliminated the difference between the levels of IL-5, and IL-10 responses between these mice (Fig. 4B). Interestingly, IKKβ$^{\Delta IEC}$ mice recipient of fecal

microbiome transplant from orally sensitized wild-type mice retained higher levels of serum IgA responses (Fig. 4C). These results suggest that intestinal dysbiosis in IKKβ$^{\Delta IEC}$ mice alters antigen-specific CD4^{+} T cell responses, while IKKβ in intestinal epithelial cells primarily regulates IgA Ab levels.

To further establish how local (gut) *versus* systemic (circulating myeloid cells) alteration of IKKβ affected allergen-specific Ab responses, we analyzed these responses in mice lacking IKKβ in myeloid cells (IKKβ$^{\Delta Mye}$) throughout the body[8]. These mice showed higher pSTAT3 responses in he small intestine than C57BL/6 mice 16 hours after oral administration of cholera toxin and developed higher levels of antigen-specific Th1-associated IgG2a (IgG2c) responses (Fig. S6). The OVA-specific IgE and IgG1 Ab responses were unchanged in IKKβ$^{\Delta Mye}$ mice, while IgA responses were enhanced although not to the same extent as in IKKβ$^{\Delta IEC}$ mice (Fig. S6). Thus, IKKβ signaling is epithelial cells or in circulating myeloid cells regulate Ab responses to ingested allergens.

Alteration of IKK signaling in IECs limits allergic inflammation in the airways

Allergic inflammation can develop in the airways following nasal allergen challenge of mice sensitized by the oral route [5, 6]. Other reports have indicated that intestinal microbes influence susceptibility to food allergy [29] and regulate immunity to respiratory influenza virus infection [30]. Lung inflammation was not seen in naïve (non-sensitized and not challenged), or challenged but not sensitized C57BL/6, IKKβ$^{\Delta Mye}$ and IKKβ$^{\Delta IEC}$ mice. Orally sensitized control C57BL/6 mice developed lung inflammation with cell recruitment in the lung parenchyma and the perialveolar and perivascular space after nasal antigen challenge (Fig. 4A-4B). Interestingly lung inflammation was significantly reduced in IKKβ$^{\Delta IEC}$ mice (Fig. 4A-4B), and in IKKβ$^{\Delta Mye}$ mice (Fig. 4A). Only minimal mucus formation was seen in the lungs of IKKβ$^{\Delta IEC}$ mice after antigen challenge and none in the lungs of IKKβ$^{\Delta Mye}$ mice (Fig. 4C). In contrast with C57B/6 mice or IKKβ$^{\Delta Mye}$ mice, BAL of IKKβ$^{\Delta IEC}$ mice contained higher levels of IgA (Fig. 4D), and their lungs showed more IgA secreting cells (Fig. S7). IgA Abs are believed to suppress allergic inflammation [31]. The presence of IgA in BAL of IKKβ$^{\Delta IEC}$ mice was associated with a reduction of eosinophils and macrophages, but the number of neutrophils was increased (Fig. 4E). On the other hand, other mechanisms are likely involved in the protection of IKKβ$^{\Delta Mye}$ mice, which exhibited overall lower number of cells in the lung after allergen challenges. In this regard, the proportion of F4/80^{+}CD11c^{+} alveolar macrophages after nasal challenge was reduced in IKKβ$^{\Delta IEC}$ when compared to control C57BL/6 mice (20±2.2% versus 28±2.5%) (Fig. S8A). Nasal challenge of orally sensitized control C57BL/6 mice recruited CD11c+F4/80-CD103+ cells in the lungs, an effect significantly reduced in mice with impaired IKKβ (Fig. S8B). We also found a higher frequency of CD3^{+} T cells in the lungs of IKKβ$^{\Delta IEC}$ mice than in control C57BL/6 or IKKβ$^{\Delta Mye}$ mice after nasal challenge of orally sensitized mice (Fig. S8C).

Protection by intestinal epithelial cell IKKβ requires oral sensitization

To further establish the role played by IECs in protection of IKKβ$^{\Delta IEC}$ mice against allergic airway inflammation, mice were sensitized by intraperitoneal injection of OVA plus cholera toxin. Although IgG2a Abs were lower in IKKβ$^{\Delta IEC}$ mice, the rest of Ig isotype and IgG

subclass responses were similar to control C57BL/6 mice (Fig. S9A). No difference was seen in lung inflammation after nasal antigen challenge, although IKKβ$^{\Delta IEC}$ mice exhibited higher mucus production than control mice (Fig. S9B). These results underline the influence of the route of antigen sensitization on Ab responses and airway responses to subsequent antigen exposure or challenge.

Gut-sensitized IL-17A producing CD4$^+$ T cells limit protection by intestinal epithelial cell IKKβ

We next examined airway cytokine responses and lung functions after allergen challenges. IKKβ$^{\Delta IEC}$ and IKKβ$^{\Delta Mye}$ mice exhibited lower lung eotaxin (CCL11) and eosinophil peroxidase (Epx) mRNA responses, but enhanced IFN-γ, IL-17A and MIP2 mRNA responses, with highest levels of response in IKKβ$^{\Delta IEC}$ mice, after nasal antigen challenge (Fig. 6A). Furthermore, low or no IFN-γ and IL-17 responses were measured in BAL of control mice after antigen challenge, while their levels were elevated in IKKβ$^{\Delta IEC}$ mice (Fig. S10). No mice developed signs of airway hyper-responsiveness without prior oral sensitization (Fig.6B) and sensitized IKKβ$^{\Delta Mye}$ mice were completely protected against airway hyper-responsiveness. Upon challenge, IKKβ$^{\Delta IEC}$ mice developed airway hyper-responsiveness responses, which were not statistically different from control C57BL/6 mice (Fig. 6C). Interestingly, nasal treatment with anti-IL-17A mAbs reduced airway hyper-responsiveness in IKKβ$^{\Delta IEC}$ mice (Fig.6D), and the percentage of neutrophils in BALs (Fig. 6E-6F).

Finally, we adoptively transferred MLN cells of orally sensitized control C57BL/6 and IKKβ$^{\Delta IEC}$ mice into naïve CD45.1$^+$ congenic recipient mice (Fig. 6G). Analysis of lung cells after nasal antigen challenge showed a higher number of donor CD3$^+$CD4$^+$CD45.2$^+$ cells in recipients of cells from IKKβ$^{\Delta IEC}$ mice (Fig. 6H). A larger number of these donor CD4$^+$ T cells also expressed α4β7 and CCR9, suggesting they were sensitized in the gut (Fig. 6H).

Discussion

Immune homeostasis is crucial at the mucosal surface of the gastrointestinal tract, which is constantly exposed to ingested antigens and commensal flora. Alteration of this homeostasis can lead to inflammatory bowel disease or food allergy. The higher incidence of asthma in inflammatory bowel diseases patients has suggested that these pathologies could share common etiological factors [32]. Furthermore, growing evidence suggest that gut commensal microbiota can affect distant mucosal sites such as those of the airways, and regulate innate and adaptive immune responses to respiratory virus infection [30, 33] and allergic airway inflammation 34. Using genetically modified mice with cell-specific alteration of IKKβ, we found that depletion of IKKβ-NFκB signaling in IECs alters the microbial community in the gut, reshapes immune responses to food allergens, and regulates allergic responses in the airways via its effect on IgA Ab and Th17 cell responses.

Allergic sensitization to food antigens can be modeled by oral administration of antigen with cholera toxin as adjuvant [5, 6, 22]. Cholera toxin can break oral tolerance and promote adaptive immunity by binding to IECs and stimulating inflammatory cytokines [35, 36]. The

NF-κB pathway can mediate both pro- and anti-inflammatory effects [8, 9] and alteration of the IKKβ-NF-κB in IECs could either attenuate chronic or exacerbated acute gut inflammatory diseases [11]. We clearly show that lack of IKKβ in IECs does not prevent these cells from developing proinflammatory and pSTAT3 responses, but also increased TGFβ mRNA in IKKβ$^{\Delta IEC}$ mice. The latter finding is significant since bone marrow stromal cells were reported to suppress allergic responses via induction of TGFβ [37]. Intestinal bacteria play a role in the maturation of gut immune cells [15, 27, 28] and selected commensal bacteria support allergic responses [38]. The Clostridium-related bacterium SFB promotes Th17 responses [27, 28], but is also known to support IgA Abs [39, 40]. It is important to indicate that immunoglobulin switch to the IgA isotype requires IL-6 and TGFβ [41], which also support the differentiation of Th17 cells [42]. Our results clearly show that alteration of IKKβ signaling in IEC can reorganize the gut microbiota, and increase the proportion of *Clostridium* species and SFB during allergic sensitization in our experimental model. Our findings are consistent with the reported role of SFB and suggest that this bacterium and other *Clostridium sp* helped enhance Th17 and IgA Ab responses in IKKβ$^{\Delta IEC}$ mice.

Nasal exposure to antigen after allergic sensitization promotes asthma-like pathology with increased airway hyper-responsiveness, airway inflammation, eosinophilia, and mucus secretion [2, 43]. Despite similar levels of IgE Ab responses than control wild-type mice, IKKβ$^{\Delta IEC}$ mice only developed limited lung inflammation upon antigen challenge. The fact that only IKKβ$^{\Delta IEC}$ mice showed high level of IgA Abs and IgA secreting cells in the airways, support the notion that IgA Abs suppress allergic inflammation [31], possibly by neutralizing allergens in mucosal tissues.

Consistent with the reported role of alveolar macrophages in asthma-induced inflammation [44], their percentage was lower in IKKβ$^{\Delta IEC}$ mice than in control mice. Airway hyper-responsiveness and eosinophilia in mice were reported to be associated with a high number of CD103^{+} dendritic cells[45]. Our study suggests that these cells also play a role in pathologies associated with priming to food antigens in the GI tract. IL-17A is a mucogenic cytokine [46, 47], which regulates airway inflammation [48] and double negative T cells, producing IFNγ and IL-17A, were reported to be the major responders in the lungs of mice during pulmonary infection with a live *Francisella tularensis* vaccine [49]. We show that CD4^{+} T cells primed in mucosal tissues represent an important fraction of effector cells recruited in the lungs of IKKβ$^{\Delta IEC}$ mice upon antigen challenge and that they limits the protective affect of IgA Abs on allergic airway symptoms in this model via production of IL-17A.

We have shown that IKKβ deficiency in intestinal epithelial cells promote a cascade of events that ultimately protect the airways against the development of allergic inflammation (Fig. 7). Our results suggest that future efforts for controlling allergic responses could include strategies that promote IgA Ab responses and prevent or reduce IL-17 responses.

Materials and Methods

Mice

Mice in which IKKβ-dependent NFκB signaling was selectively eliminated in the intestinal epithelial cells ($IKK\beta^{\Delta IEC}$) or myeloid cells ($IKK\beta^{\Delta mye}$) were generated as previously described [8, 23] and breed in our facility. Control C57BL/6 mice were obtained from the NCI-Frederick, and housed for 3–4 weeks with IKKβ deficient mice. Studies were performed on mice 10–12 weeks of age, in accordance with NIH and OSU IACUC guidelines.

Quantification of mRNA by real-time RT-PCR

Real-time RT-PCR was performed as previously described [50], and mRNA responses were expressed as mRNA relative expression= (1/2^ΔCt)*100*1000) where $\Delta Ct = CP_{unknown} - CP_{\beta\text{-actin}}$.

Histology

Five 5 μm paraffin sections were stained with hematoxylin and eosin, alone or with anti-pSTAT3 (Cell-Signaling), anti-pNF-κB p65 (Santa Cruz Biotech) or subjected to PAS staining.

Immunoblotting

Intestines were lysed and proteins were separated by SDS-PAGE. After transfer, PVDF membranes were probed with anti-pSTAT3 (Cell Signaling) and an HRP-conjugated secondary antibody. The membranes were re-probed with β-actin-specific antibody (Santa Cruz Biotech) and relative ratios of pSTAT3/β-actin were determined using Image J software

Oral treatment, oral sensitization and nasal challenge of mice

For oral treatment, mice were deprived of food for 2 hours and given 250 μl of sodium bicarbonate 30 min before intragastric gavage of 10 μg cholera toxin in 250 μl of PBS. Oral sensitization was performed on days 0 and 7 by intragastric gavage of 250 μl of PBS containing 1 mg of ovalbumin (OVA) and 10 μg cholera toxin as adjuvant. Blood samples were collected on days 7 and 14. Nasal antigen challenges were performed on days 15, 16 and 19. For this purpose, mice were anesthetized by intraperitoneal injection of ketamine/xylazine and administered 200 μg of OVA in PBS. In selected experiments, mice were nasally treated (10 μg/dose) with an anti-IL-17A mAb or isotype control Ab (R&D Systems).

Analysis of gut microbiota

Bacterial tag-encoded FLX amplicon pyrosequencing (Roche Titanium 454 FLX pyrosequencing) was used for detection and identification of the primary populations of microbes in fecal pellet samples. For identification of segmented filamentous bacteria, blast search was performed with the fasta sequences against *Candidatus Arthromitus* (taxid: 49082) genome sequences (*Candidatus Arthromitus* sp. SFB-mouse-Yit and *Candidatus*

Arthromitus sp. SFB-mouse-Japan), using Megablast (optimized for highly similar sequences; ≥ 95 %) and E-values below 1e-15.

Fecal microbiota transplantation

Fecal material for microbiota transfer was prepared by using a modification of a previously described method [51]. Briefly, freshly emitted fecal pellets were homogenized by vortexing in sterile PBS (1 ml per 0.1g of fecal material). After filtration of particulate matter, mice were gavage with 0.2 ml of the suspension.

Antigen-specific $CD4^+$ T cell cytokine responses

Spleens and MLN were collected one week after the last immunization and mononuclear cells were restimulated *in vitro* with OVA (1 mg/ml) as previously reported [52]. After 5 days of culture, levels of Th1 (IFN-γ), Th2 (IL-4, IL-5, IL-10) or Th17 (IL-17A) cytokines in supernatants were determined by ELISA using mAb pairs and cytokine standards (BD Biosciences or R&D Systems).

Antigen-specific Ab responses and total IgA levels

OVA-specific Ab responses were measured by ELISA as previously described [52]. Total IgA levels, were determined by ELISA and IgA standards. The frequency of IgA secreting cells were evaluated by ELISPOT [53].

Analysis of lung functions and airway responses to metacholine challenge

Mechanical properties of the mouse lung were assessed using the forced-oscillation technique [54] and a flexiVent computer controlled piston ventilator (SCIREQ®, Montreal, Canada). Mice were exposed to increasing doses of metacholine (0.1, 1, 10, 20 and 50 mg/ml) in sterile normal saline [54] and total lung resistance were recorded.

Adoptive transfer

Cells ($8x10^6$) from control C57BL/6 (CD45.2) or $IKK\beta^{\Delta IEC}$ (CD45.2) were injected i.v. into congenic CD45.1 mice. Donor and recipient cells were discriminated in tissues of recipient mice by flow cytometry using $CD45.1^-$ and $CD45.2^-$ specific mAbs (BD Biosciences).

Bronchoalevolar lavages

Bronchoalveolar lavage fluids were obtained via cannulation of the exposed trachea, by infusion of 600 μl of sterile PBS through a 22-gauge catheter into the lungs [5].

Analysis of cell populations in lung tissues

Whole-lung tissue was dissociated in 0.5 mg/ml collagenase type V (Sigma) 15 min at 37°C. Single cell preparations were stained with one or a combination of the fluorescent anti-mouse Abs: anti-CD11b, anti-CD11c, anti-F4/80 anti-B220, anti-CD3, anti-CD4, anti-CD8, anti-CD103, anti-α4β7, and anti-CCR9 (BD Biosciences and eBiosciences) and analyzed by flow cytometry (Accuri Cytometers, Inc).

Statistics

Results are expressed as the mean ± 1 SD. Statistical significance was determined by Student's T test or by ANOVA followed by the Fisher Least Significant Difference Test. For analysis of mRNA responses, we used one-way ANOVA, followed by Duncan's Multiple Range Test.. All statistical analyses were performed with the Statistica 9.0 software package (StatSoft, Inc., Tulsa, OK).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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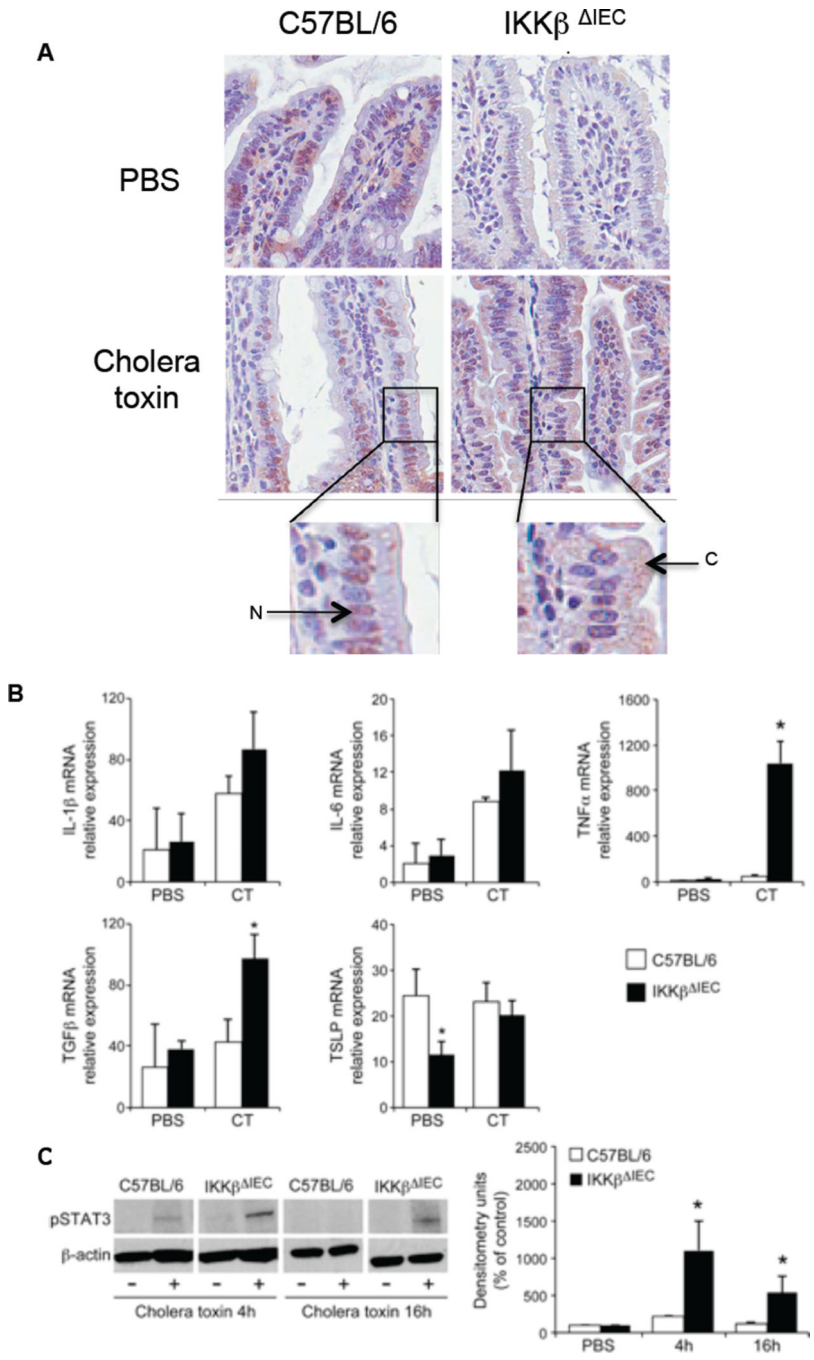


Figure 1. Innate responses to cholera toxin in intestinal tissues of IKKβ$^{\Delta IEC}$ mice

Mice were orally administered cholera toxin (10 μg) by gavage and small intestines were collected 4 or 16 h later. (A) pNFkB expression 16 h after administration of saline (PBS) or cholera toxin. Tissue sections were labeled with anti-pNF-κB p65 Ab and counter-stained with hematoxylin and eosin. Each image (x400) is representative of at least three independent experiments. Higher magnification show pNF-κB p65 expression in the nucleus (N) and cytoplasma (C) of epithelial cells (B) Real-time RT-PCR analysis of cytokine mRNA. Data are expressed as mean relative expression levels ± one SD. (C) Western blot

analysis of phosphoSTAT3 (pSTAT3) expression and quantification of relative pSTAT3 expression as mean densitometry units. Data are from 4 separate experiments (*, $p < 0.05$)

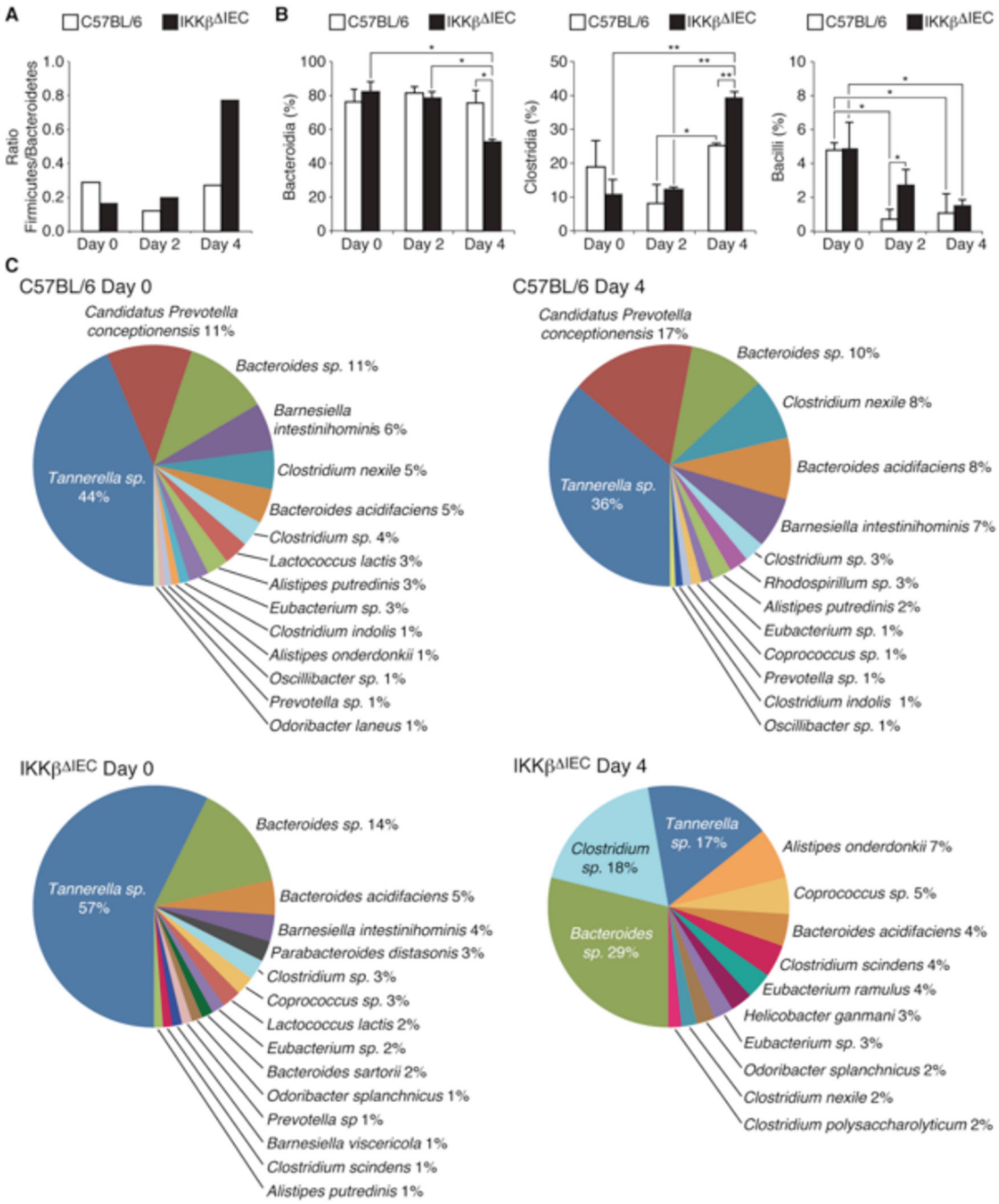


Figure 2. Cholera toxin alters the gut microbiota in IKKβ$^{\Delta IEC}$ mice

Bacterial flora was analyzed in fecal pellets collected before (day 0) and 2 and 4 days after administration of cholera toxin. (A) Ratio of main bacteria Phyla, (B) Percentage of main bacteria genus, (C) Pie-diagrams of the 15–17 main bacteria species, The results in the panel B are expressed as mean percentage ± one SD (*, $p < 0.05$; **, $p < 0.01$). All results were from 3–4 mice per groups

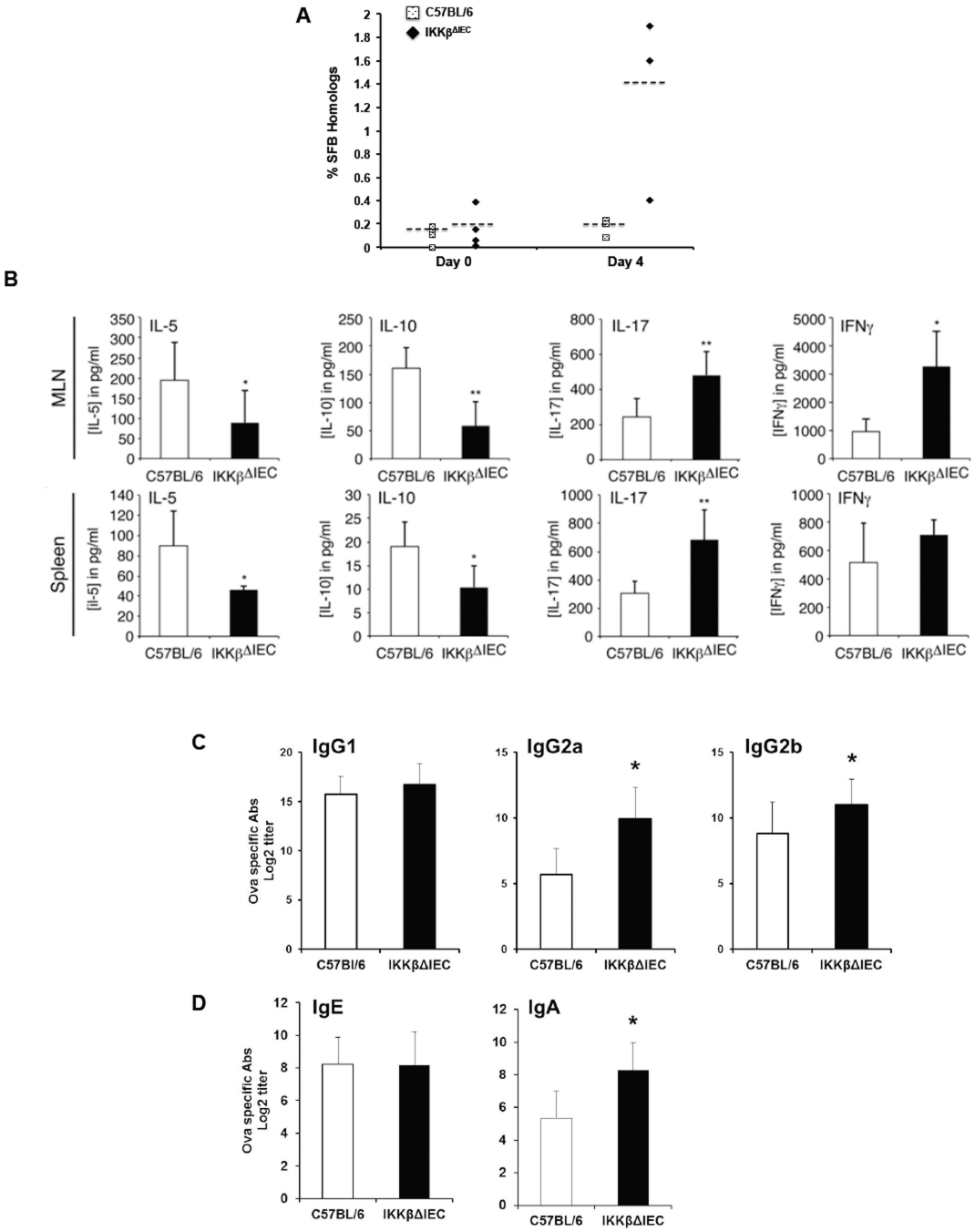


Figure 3. IKKβ-deficiency in intestinal epithelial cells alters the profile of antigen-specific CD4$^+$ T cell and serum Ab responses in orally sensitized mice

(A) Percentage of segmented filamentous bacteria homolog before (day 0) and 4 days after administration of cholera toxin. (B) Cytokine secretion by OVA-specific MLN and spleen T cells were analyzed by ELISA. Results are expressed as mean ± SD of three separate experiments, with 4 mice per group. (*, $p < 0.05$; **, $p < 0.01$ compared to control C57BL/6 mice). (C) OVA-specific IgG subclass and (D) IgE and IgA isotypes. Blood was collected on day 14, and Ab titers were analyzed by ELISA. The results are expressed as the $\log_2$

titers ± one SD and are from three experiments and five mice / group. (*, $p < 0.05$ compared to control C57BL/6 mice).

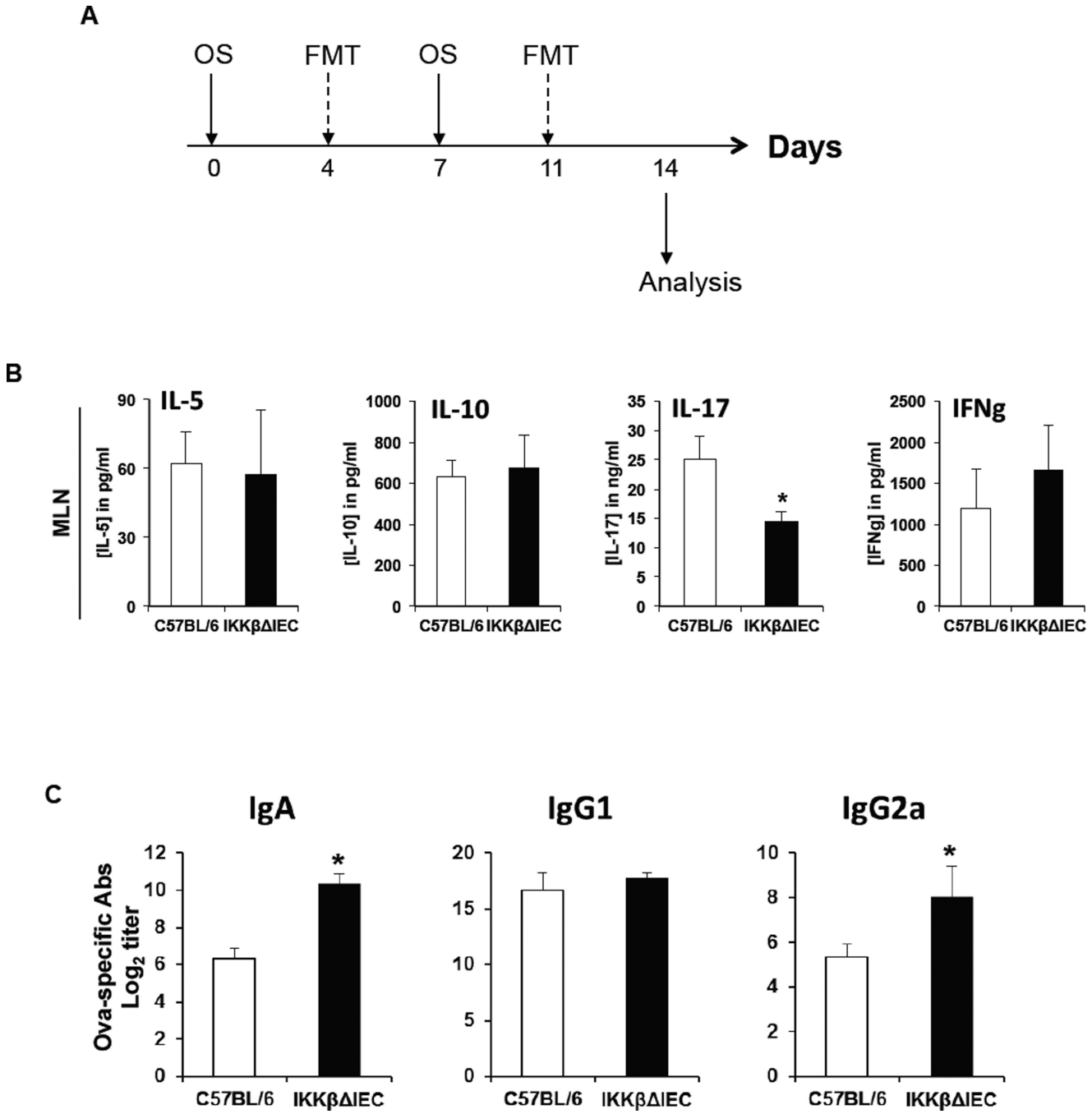


Figure 4. Fecal microbiota transfer modulates antigen-specific CD4$^+$ T cell cytokines, but not serum IgA responses orally sensitized IKKβ$^{\Delta IEC}$ mice

(A) Schedule of oral sensitization (OS) and fecal microbiota transfer (FMT). (B) Cytokine secretion by OVA-specific MLN T cells were analyzed by ELISA. (C) OVA-specific IgG subclass and IgA responses. Blood was collected on day 14, and Ab titers were analyzed by ELISA. The results are expressed as the $\log_2$ titers ± one SD and are from three experiments and five mice / group. (*, $p < 0.05$ compared to control C57BL/6 mice).

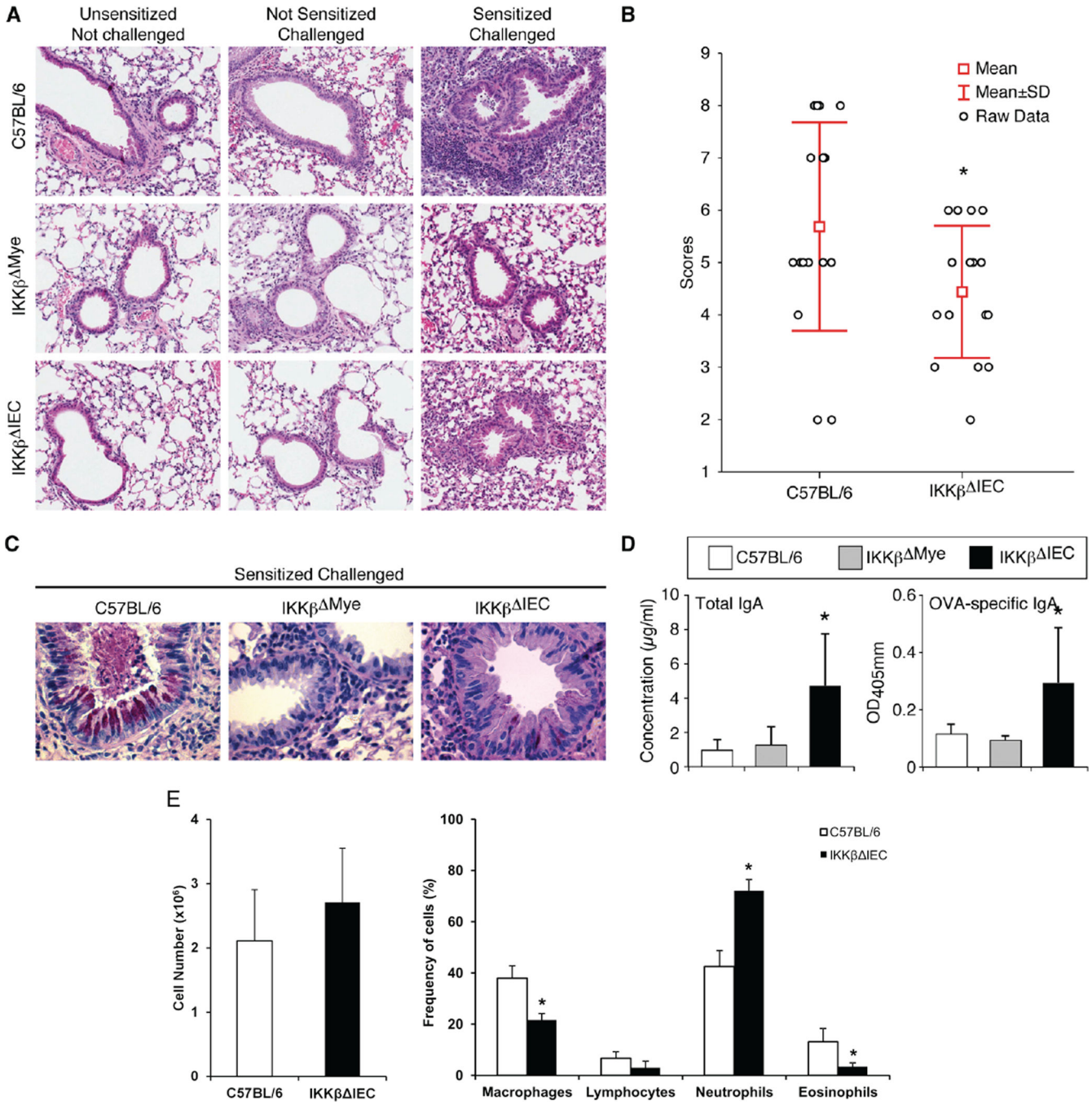
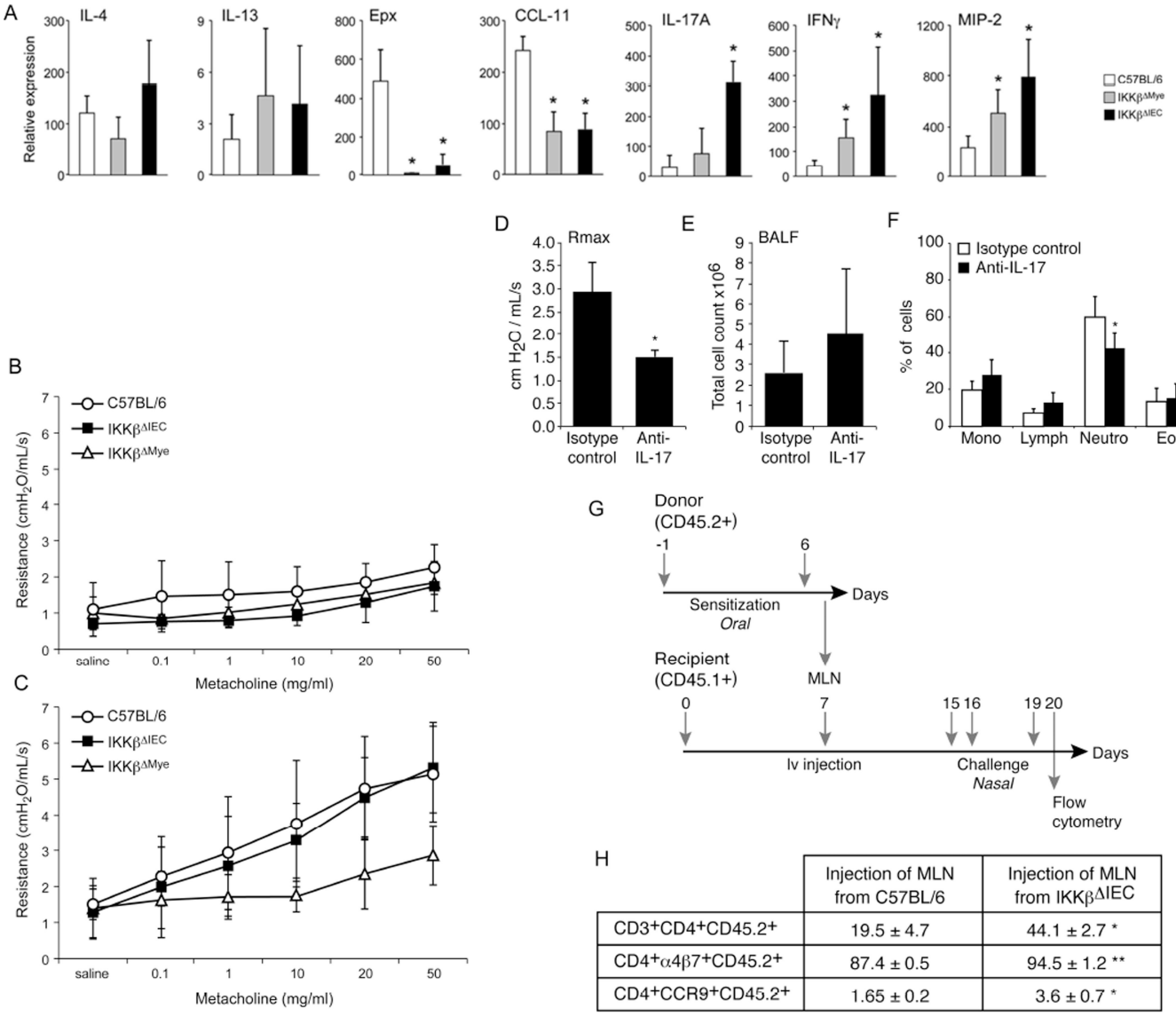


Figure 5. IKKβ-deficiency in intestinal epithelial cells limits lung allergic inflammation in orally sensitized mice

(A) Hematoxylin and eosin staining of lung sections (x40). (B) Inflammation scores in lungs of C57BL/6, and IKKβ$^{\Delta IEC}$ mice. (C) Tissues were stained with PAS and counterstained with hematoxylin and eosin to visualize mucus (x400). Each picture in (A) and (C) is representative of three separate experiments, with 3–4 mice per group. (D). Total and OVA-specific IgA Ab levels, and (E) cell subsets in BAL fluids . The results are expressed as the mean ± one SD and are from three experiments and five mice / group. (*, $p < 0.05$ compared to control C57BL/6 mice).



H

	Injection of MLN from C57BL/6	Injection of MLN from IKKβΔIEC
$CD3^+CD4^+CD45.2^+$	19.5 ± 4.7	44.1 ± 2.7 *
$CD4^+\alpha4\beta7^+CD45.2^+$	87.4 ± 0.5	94.5 ± 1.2 **
$CD4^+CCR9^+CD45.2^+$	1.65 ± 0.2	3.6 ± 0.7 *

Figure 6. Gut-sensitized IL-17A producing $CD4^+$ T cells limit the protective effect of IgA Abs in the airways of $IKK\beta^{\Delta IEC}$ mice

(A-C) Mice were orally sensitized on day 0 and 14 and nasally challenged on days 15, 16, and 19 (A) Cytokines, chemokines and neutrophil peroxidase mRNA responses in lung tissues on day 20. Results are expressed as mean ± SD of three separate experiments, with 4 mice per group. (*, $p < 0.05$ compared to control C57BL/6 mice). (B). Airway hyper-reactivity after nasal challenge of naive mice. (C) Airway hyper-reactivity after nasal challenge (day 20) of orally sensitized mice. (D-F) Responses to anti-IL-17A treatment. (D) Airway hyper-reactivity, (E) Total number of cells and (F) immune cell populations in BAL. Results are expressed as mean ± SD of 5 mice per group. (*, $p< 0.05$). (G-H) Adoptive transfers of MLN. (G) Timeline of adoptive transfer experiments. (H) Frequency of donor (CD45.2) CD4+ T cells expressing α4β7 and CCR9. Results are expressed as the mean ± one SD and are from four separate experiments. (*, $p< 0.05$; **, $p< 0.01$).

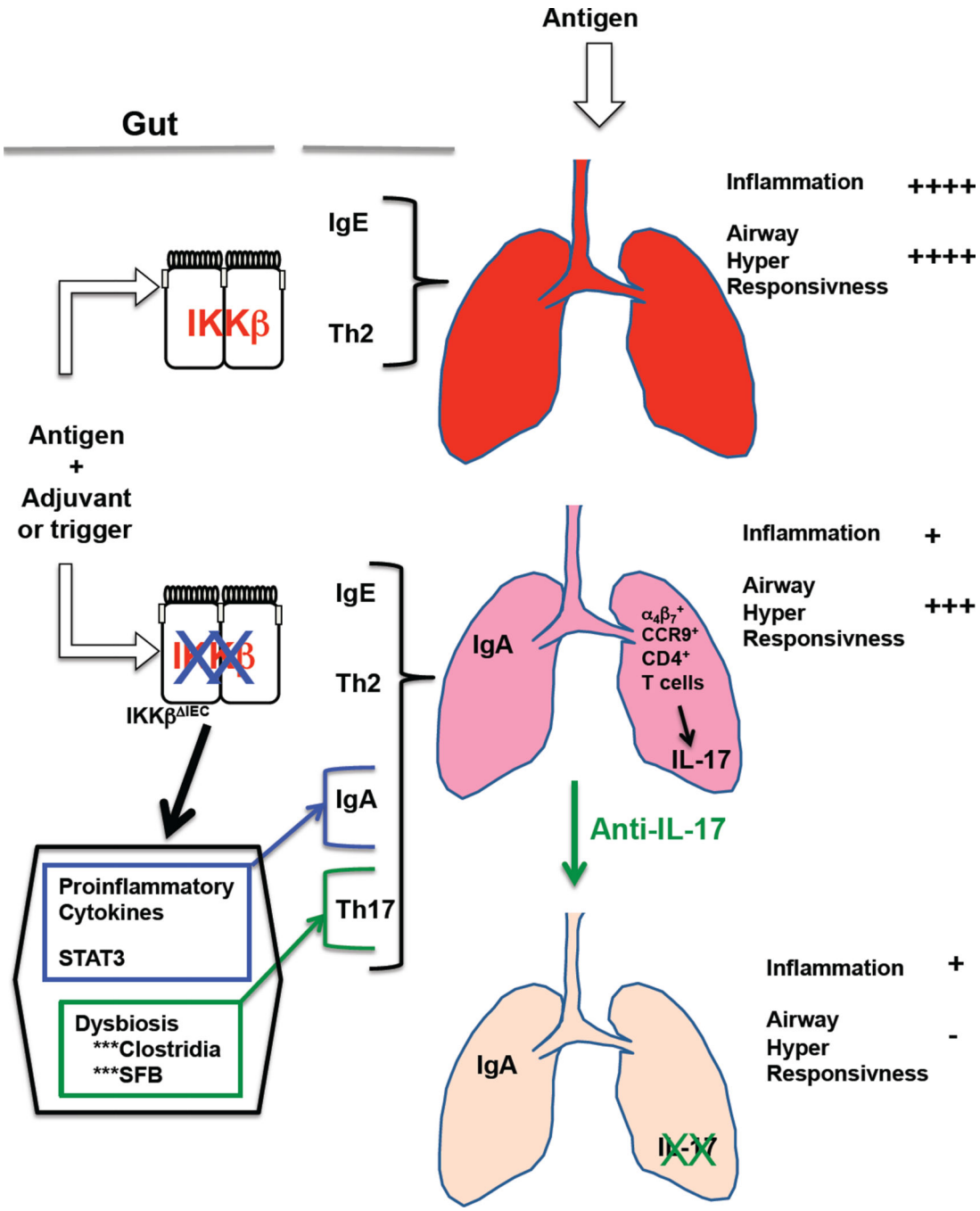


Figure 7. Regulation of pathogenic airway responses to allergens by IKKβ in intestinal epithelial cells

Allergic sensitization in the gut promotes antigen-specific IgE and Th2 responses, which will later induce allergic airway inflammation and airway hyper-responsiveness support in the event of airway exposure to the same antigen. Loss of IKKβ in IEC does not alter immune homeostasis in the gut at the basal level. However, during allergic sensitization, lack of IKKβ signaling in IEC could enhance gut proinflammatory responses and promote dysbiosis. The change in cytokine milieu could support IgA Abs, while alteration of the gut microbiota would support Th17 responses. Subsequent exposure of the airways to allergen

will result in the accumulation of IgA Abs, which will protect against allergic inflammation. $CD4^+$ cells expressing gut the homing receptors $\alpha4\beta7$ and CCR9 and producing IL-17 are also recruited in the airways of $IKK\beta^{\Delta IEC}$ mice upon antigen exposure. These cells more likely support hyper-responsivess responses of the airways to antigen exposure, which could be suppressed by treatment with anti-IL-17A Ab.