CCR8-CCL1 axis leads to eosinophil migration in experimental murine allergic enteritis

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A mi familia

To my family

meiner Familie

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Other publications based on the result obtained in this project are currently in preparation.

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1. Introduction

1.1. Food allergy

Food allergies are defined as “an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food” (Boyce et al., 2010; Tordesillas et al., 2017) . The prevalence of food allergies has increased in the past decade. Epidemiologic studies involving controlled food challenges for the diagnosis of food allergies indicated that between 1 % to 10.8 % of the population have immune-mediated non-toxic food hypersensitivity (Rona et al., 2007; Loh and Tang, 2018; Osborne et al., 2011; Strachan, 1989). Food allergies have been recognized as an important public health problem, in particular due to the risk of fatal anaphylaxis (Boyce et al., 2010; Lieberman and Sicherer, 2011).There are several postulates that explain the increased prevalence of food allergy: e.g. a reduced microbial burden during childhood as consequence of a westernized lifestyle and diet, reduced risk of infectious diseases (the hygiene hypothesis), the use of antibiotics in early childhood, and altered gut microbiome in the consequence (Strachan, 1989; Droste et al., 2000; Romagnani, 2004). Factors related to the allergens itself are their physical, biological and immunological properties in addition to timing and route of sensitization. Individual genetic factors also influence on the onset of allergy (Cochrane et al., 2009; Loh and Tang, 2018; Strachan, 1989; Droste et al., 2000; Brown et al., 2011; Romagnani, 2004; Sicherer and Sampson, 2018; Hong et al., 2015).

Despite the increasing prevalence, no curative treatment has been established for food allergies so far. The recommended management of food allergies for patients with the risk of anaphylaxis consist in identification of the food allergen responsible of the reaction, avoidance of the offending food and treatment with epinephrine, antihistamines and corticosteroids to control severe reactions after accidental consumption (Bischoff and Crowe, 2004; Boyce et al., 2010; Burks et al., 2018; Sicherer and Sampson, 2010). These conditions significantly affect the quality of life of the patients (Boyce et al., 2010; Burks et al., 2018; Sicherer and Sampson, 2010; Primeau et al., 2000).

Several studies have aimed to elucidate the cellular and molecular mechanisms of food allergies by investigating association of the skin barrier dysfunction with food allergen sensitization, the development of natural and therapeutically induced

tolerance, the role of microbiota, the role of inflammatory and regulatory immune cells in development of inflammation and tolerance, or biochemical and immunological properties of food allergens, as well as cytokines and chemokines involved in maturation, activation and migration of the immune cells. Such studies are crucial to establish a preventive and therapeutic treatment of food allergies (Sicherer and Sampson, 2018; Huang et al., 2018; Bischoff and Crowe, 2005; Berin and Mayer, 2009; Yu et al., 2016). While there is no stablished treatment to food allergies beside the complete avoidance of the elicited food, several clinical trials have been performed, including a phase III clinical trial using oral immunotherapy, that has shown promising results by increasing the dose of peanuts protein ingested by the patients involved in the clinical trial (Vickery et al., 2018; *Peanut Allergy Oral Immunotherapy Study of AR101 for Desensitization in Children and Adults (PALISADE) - Full Text View - ClinicalTrials.gov;* Varshney et al., 2011)

1.2. Classification of food allergies

Food allergies are classified into a) IgE-mediated and b) non-IgE mediated (T-cell mediated) allergies, depending on immunologic pathways in the pathogenesis (Wang and Sampson, 2011; Cianferoni and Spergel, 2009; Patel and Volcheck, 2015; Sicherer and Sampson, 2006).

1.2.1. IgE-mediated food allergies

IgE-mediated allergy (also referred as type I allergy) is elicited rapidly and are characterized for an onset of acute symptoms that can appear from minutes up to 2 hours after the ingestion of the offending food. This reaction is reproducible upon a re-exposure to elicited food and can be diagnosed by the detection of IgE (Burks et al., 2012; Tordesillas et al., 2017; Cianferoni and Spergel, 2009). The symptoms typically involve the skin, the respiratory tract, gastrointestinal tract and/or the cardiovascular system.

After the sensitization phase inducing IgE production, food allergen binds to IgE associated with FcεRI on the surface of mast cells and basophils upon a re-exposure to the offending food. The FcεRI engagement activates mast cells and basophils, and triggers the release of granules containing anaphylactic and inflammatory mediators like histamine, tryptase and chymase. Mast cells and basophils also produce chemokines, leukotrienes and prostaglandins. The mediators released from granules

and de novo synthesized lipid products elicit a range of physiological reactions as vasodilatation, mucus secretion, smoot muscle contraction, increased vascular permeability (Fig. 1) (Burks et al., 2012; Renz et al., 2018; Morita et al., 2013).

The cardiovascular symptoms are the most severe manifestations of a systemic reaction (Patel and Volcheck, 2015; Renz et al., 2018; Ho et al., 2014). Its severity could vary from pruritus alone, oral allergy syndrome to anaphylactic shock (Patel and Volcheck, 2015; Muluk and Cingi, 2018). The most prevalent symptoms are cutaneous manifestations (up to 20% of the cases), which include urticaria, angioedema, flushing or pruritus (Ho et al., 2014; Patel and Volcheck, 2015). The respiratory symptoms include sneezing, congestion, wheezing, rhinorrhea and laryngeal edema (Patel and Volcheck, 2015). Asthma on the other hand is an uncommon phenotype of food allergies. Gastrointestinal symptoms such as nausea, vomiting, abdominal pain, throat discomfort, tongue itchiness, swelling of the lips and diarrhea could be clinical manifestation of IgE-mediated food allergy, while bloody stools, malabsorption, and constipation are usually not symptoms related to IgE-mediated food allergy, but more non-IgE mediated allergy (addressed in the next section) (Ho et al., 2014; Patel and Volcheck, 2015; Cianferoni and Spergel, 2009; Muluk and Cingi, 2018).

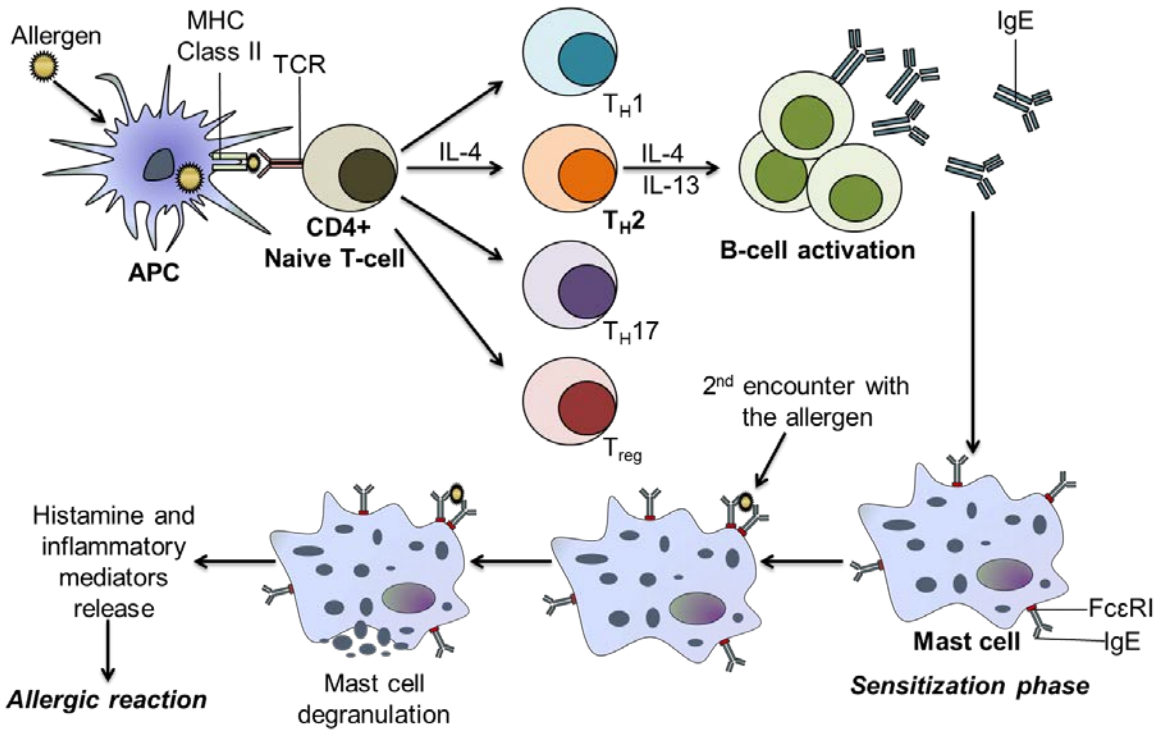

Figure 1: Basic mechanism of type I allergy. Upon the first contact with an allergen antigen presenting cells (APC) capture, process and present it as a complex of antigenic

peptides with MHC Class II molecule for $CD4^+$ T-cell activation and differentiation. Native $CD4^+$ T-cells tend to differentiate into Th2 cells in allergic status. IL-4 and IL-13 secreted by Th2cells lead to t IgE production by B cells. IgE binds to the IgE high affinity receptor (FcεRI) on the surface of mast cells and basophils. FcεRI engagement with IgE and allergens induce degranulation and activation of mast cells and basophils, which lead to allergic reaction.

1.2.2. Non-IgE-mediated food allergies

In contrast to IgE-mediated food allergies, non-IgE-mediated food allergies have been less studied. One of the main reasons for the lack of knowledge about non-IgE-mediated allergies is the difficulty in access to the gut-associated lymphoid tissue (GALT), responsible tissues for induction and development of immune cell responses in the gastrointestinal tract (Nowak-Węgrzyn et al., 2015; Ruffner and Spergel, 2016). Most of non-IgE-mediated allergies are diagnosed based on the clinical history and empiric food avoidance, while a endoscopy and/or biopsy are rarely performed (Ruffner and Spergel, 2016; Nowak-Węgrzyn et al., 2015).

Non-IgE-mediated food allergies are more heterogeneous than IgE-mediated food allergies. It is postulated that T-cell mediated responses are involved in the progression of the pathology (Kim and Burks, 2015), although the exact mechanism how non-IgE-mediated food allergies take place is poorly understood. The spectrum of non-IgE-mediated food allergies includes food protein-induced enterocolitis syndrome (FPIES), food protein-induced allergic proctocolitis (FPIAP), food protein induced enteropathy, and allergic enteritis (Boyce et al., 2010; Nowak-Węgrzyn et al., 2015; Morita et al., 2013). Such allergies are characterized by chronic inflammatory processes mainly in the intestines that generate clinical symptoms including abdominal pain, diarrhea and blood stool several hours after the exposition to the offending food (Nowak-Węgrzyn et al., 2015; Morita et al., 2013; Renz et al., 2018). Intestinal inflammation in non-IgE-mediated food allergies are characterized by edema, flattened villi, and accumulation of leukocytes e.g. eosinophils, mast cells and lymphocytes (Caubet and Nowak-Węgrzyn, 2011; Caubet et al., 2017; Chung et al., 1999).

Importantly, patients with non-IgE mediated food allergies often develop IgE, although IgE is not detectable in the initial stage of disease. In such cases, the reaction is characterized as a mixture of IgE and T-cell mediated reactions (Burks et al., 2012; Kim and Burks, 2015).

1.3. Allergic enteritis

Allergic enteritis (AE) is basically classified as a non-IgE-mediated food allergy. This disease is predominantly observed in small children, but has been reported also in adults (Tan and Smith, 2014; Michelet et al., 2017; Czerwionka-Szaflarska et al., 2017). The pathomechanism of allergic enteritis is not well known in comparison with other clinical phenotypes of food allergies (Prussin, 2014). The initial stimulus leading to the development of allergic enteritis remains to be elucidated, but it is postulated that delayed Th2 cell mediated-responses are involved in the development of the inflammation (Pineton de Chambrun et al., 2015; Caldwell et al., 2014).

Patients with allergic enteritis frequently develop IgE to the offending food, despite to be classified as a non-IgE-mediated food allergy (Ruffner et al., 2013; Caubet et al., 2014; Katz et al., 2011; Hwang et al., 2009; Ishige et al., 2015). Caubet *et al* reported that 24% of the subjects evaluated in their study with allergic enteritis confirmed by controlled food challenge developed specific IgE to the elicitor food (Caubet et al., 2014). AE has been reported to be commonly outgrown (Ruffner et al., 2013; Caubet et al., 2014; Katz et al., 2011), but it seems that there is a role of IgE in the persistence of AE. Patients showing detectable IgE to the offending food in the initial diagnosis or during the follow up tend to experience a prolonged course of the disease. In some cases, it was reported that the disease turned into a IgE-mediated allergy over time (Nowak-Wegrzyn et al., 2003; Sicherer, 2005; Caubet et al., 2014; Ishige et al., 2015). In the same study, Caubet *et al* reported that 41% of patients with specific IgE against cow´s milk never became tolerant during the study (Caubet et al., 2014). These studies have suggested that the presence of detectable levels of specific IgE is a poor prognostic factor of allergic enteritis (Savage and Johns, 2015; Nowak-Wegrzyn et al., 2003; Nowak-Węgrzyn et al., 2015; Czerwionka-Szaflarska et al., 2017).

The gastrointestinal symptoms of allergic enteritis are nonspecific, resulting in thet fact that a broad differential diagnoses including diagnostic approaches for allergic diseases is necessary to rule out other gastrointestinal diseases (Czerwionka-Szaflarska et al., 2017; Pineton de Chambrun et al., 2015; Bischoff, 2010) (Ruffner and Spergel, 2016; Fleischer and Atkins, 2009). Biopsies of patients with allergic enteritis, have shown infiltration of inflammatory cells (e.g. mast cells, eosinophils, neutrophils, and T cells) in the lamina propria, disruption of intestinal villi, edema, and

presence of goblet cells (Rothenberg, 2004; Czerwionka-Szaflarska et al., 2017) in the intestine.

1.4. Gut-associated lymphoid tissue (GALT)

The gastrointestinal tract is the largest surface in the human body. To achieve protection from pathogens and immune tolerance to food derived components, the gastrointestinal tract is equipped with gut-associated lymphoid tissue (GALT) that is the largest reservoir of immune cells in the body (Bischoff, 2010; Sicherer and Sampson, 2010; Berin and Sampson, 2013). GALT comprise a collection of inductive sites like Peyer´s patches, appendix, isolated lymphoid follicles (ILFs). In these sites the antigen specific cellular and humoral response are first generated. The inductive sites are situated throughout the gastrointestinal tract and in combination with effector sites like mesenteric lymph nodes (MLNs) and lamina propria conform a immunological barrier (Donaldson et al., 2015; Paul, 2008; Pabst and Mowat, 2012; Berin and Sampson, 2013).

GALT is able to distinguish between innocuous food antigens and pathogenic microorganisms and generate the correspondent response, having an important role in the development of tolerance or the pathogenesis of food allergies (Vighi et al., 2008; Berin and Sampson, 2013). Studies have highlighted that MLNs are required to the development of tolerance. Through surgical or immunological ablation of the MLN the oral tolerance was abolished (Spahn et al., 2002; Worbs et al., 2006). Nakajima-Adachi *et al.* showed that Peyer´s patches and MLN, but not ILFs, are important in the development of allergic intestinal inflammation as consequence of their role in the generation and maintenance of IL-4 producing $CD4^+$ T-cells, although MLN plays a more central role in the development, compared to Peyer´s patches(Nakajima-Adachi et al., 2014).

The lamina propria contributes in the induction of inflammation and tolerance, as it harbors large populations of activated T-cells, and Treg constitute more of 30% of $CD4^+$ T-cells in the colonic lamina propria and about 20% in the small intestine lamina propria (Tanoue et al., 2016; Hadis et al., 2011). It is important for the expansion of B cells and their terminal differentiation to antibody-secreting plasma cells. Also, it is the site of antigen uptake and loading of the migratory dendritic cells that encounter naïve T-cells in the MLNs (Pabst and Mowat, 2012; Brandtzaeg et al.,

2007; Berin and Mayer, 2009). The lamina propria has also a crucial role in the development of inflammatory responses due the high number of immune cells that contains, as eosinophils, T-cell, neutrophils, macrophages and plasma cells (Montalban-Arques et al., 2018; Egan et al., 2011; Schröder-Braunstein et al., 2014).

1.5. Immune cells involved in development of allergic inflammation

As described above, there is a variety of immune cells involved in development and persistence of food allergies: T-cells, B cells, mast cells, eosinophils, neutrophils, macrophages and innate lymphoid cells (ILCs) (Zundler and Neurath, 2017; Montalban-Arques et al., 2018).

1.5.1. T-cells

T-cells are derived from HSCs that undergo differentiation in the thymus, giving origin of its name thymus-derived (T) lymphocytes, and migrate to the peripheral lymphoid tissue (Yang et al., 2010; Paul, 2008).

T-cells can be classified upon TCR that are expressed on their surface. The majority of T cells express a TCR that consists of α and β chains, while a minor group express that conformed by γ and δ chains (Rothenberg, 2011; Chien et al., 2014). T-cells expressing α/β TCR are divided into two subtypes $CD4^+$ and $CD8^+$ T-cells, classified on base of receptor expressed on the cell surface (Paul, 2008). These cells also differ on the interaction with MHC class I ($CD8^+$ T-cells) and class II ($CD4^+$ T-cells) molecules on the surface of APCs (Paul, 2008; Lustgarten et al., 1991).

$CD4^+$ T cells, also known as T-helper cells (Th) carry out various functions including activation of B cells, cytotoxic T cells and other nonimmune cells (Zhu and Paul, 2008; Luckheeram et al., 2012). CD4+ T-cells can polarize into different effector subsets: T helper type 1 (Th1) cells secreting mainly interleukin 2 (IL-2), tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ), Th2 cells secreting mainly IL-4, IL-5 and IL-13 (Zhu and Paul, 2008), Th9 cells secreting IL-9 (Goswami, 2017; Veldhoen et al, 2008), Th17 cells secreting IL-17A (Ouyang et al., 2008), or Th22 cells secreting IL-22 (Plank et al., 2017).

In the development of allergic disease, Th2 cells play a crucial role as depicted in figure 1. Beyer *et al* reported in the gastrointestinal system an enhanced production of Th2 cytokines in duodenal biopsies of cow´s milk food allergic patients, as well as

a high T-cell $CD4^+$ proliferation in the lamina propria (Beyer et al., 2002). Another study showed an increased number of T-cells, IL-4 expressing cells, IgE-bearing cells and eosinophils in the duodenal biopsies of patients with food-related gastrointestinal symptoms with negative in skin prick testing and serum specific IgE to the offending food, suggesting a possible role of $CD4^+$ T-cells and local of IgE in the pathology (Lin et al., 2002).

In addition to effector T-cells, $CD4^+$ T-cells differentiate into Tregs. Tregs are a heterogeneous population of T cells with immunoregulatory activities that are of importance in the development and maintenance of tolerance to self and foreign antigens and avoiding an excessive immune response to pathogens (Paul, 2008; Palomares, 2013; Luckheeram et al., 2012). Tregs exists as $CD4^+$ $CD25^+$ forkhead box protein 3 (Foxp3) natural thymus derived subset and as peripheral induced Treg cells (Luckheeram et al., 2012; Workman et al., 2009). Tregs are able to suppress sensitization and effector phases of allergic reactions through different mechanisms, e.g. by producing immune suppressive cytokines such as IL-10 and TGF-β and releasing cytolytic components such as granzyme (Arce-Sillas et al., 2016). IL-10 and TGF-β inhibit activation of effector T cells, mast cells and granulocytes, and thereby suppress development and aggravation of allergy (Zhang et al., 2014; Taylor et al., 2006; Shachar and Karin, 2013)

1.5.2. B cells

B cells in mammalians derive from lymphoid progenitor cells in the bone marrow. B cells are present in the follicular areas in the lymphoid tissues and represent the 5 to 25% of all human and murine blood cells (Ruiz et al., 1999). B cells play a role in the humoral immunity because its principal function is production of immunoglobulins (alternatively called as antibody) (LeBien and Tedder, 2008; Hoffman et al., 2016). B cells express immunoglobulin on their surface as B-cell receptor (BCR) binding to antigen. Upon stimulation with BCR, CD40 and cytokine receptor(s), B cell undergo immunoglobulin class switch (Kondo, 2010; Paul, 2008; Janeway, 2001; Zou et al., 2017).

There are five immunoglobulin classes (isotypes) i.e. IgM, IgD, IgG, IgA and IgE. IgM is the first isotype expressed in the development of B cells and initially dominates the humoral immune response (Ohta and Flajnik, 2006; Schroeder and Cavacini, 2010).

IgG is the predominant isotype in the body (approximately 70-75% of the total Ig) and has the longest serum half-life of all immunoglobulin classes (Schroeder and Cavacini, 2010; Paul, 2008). IgA is the dominant immunoglobulin class in the mucosal secretions as well as in breast milk and colostrum (Woof and Mestecky, 2005). IgE is involved in defense against parasitic infections but also triggers type I allergy as described above (Schroeder and Cavacini, 2010; Zou et al., 2017).

IgG is further divided into subclasses: IgG1, IgG2, IgG3, IgG4 in human, whereas IgG1, IgG2a, IgG2b and IgG3 in mice (Vidarsson et al., 2014; Michaelsen et al., 2004). Among the subclasses, IgG1 is the most prevalent (60 – 75 % of serum IgG). IL-4 induces class switch to IgG1 and IgE in both human and mice, but higher amount of this cytokine is required for IgE production (Lebman and Coffman, 1988; Moon et al., 1989). IL-13 also induces class switch to IgE in human and mice (Vicario et al., 2010; Takhar et al., 2007). IFN-γ induces class switch to IgG2 and IgG2a in human and mice, respectively, and inhibits class switch to IgG1 and IgE (Bossie and Vitetta, 1991; Paul, 2008). IL-10 induce class switch to IgG4 in human and IgG3 in mice (Paul, 2008; Schroeder and Cavacini, 2010).

1.5.3. Mast cells.

Mast cells are long-lived granulated sentinel cells (Bonnekoh et al., 2018; Dawicki and Marshall, 2007; Wouters et al., 2016). A primary role of mast cells is in innate immune responses to pathogens (specially parasites and bacteria) by releasing a broad spectrum of mediators and enhancing the earliest processes in the development of immune responses (Dawicki and Marshall, 2007). Nevertheless mast cells are well recognized as the key cells in induction of type I allergy (Bonnekoh et al., 2018; Bischoff et al., 2000).

Mast cells express the IgE receptor, FcεRI on their cell surface. The association of allergens with IgE binding to FcεRI leads to the release of granules. This process of degranulation in mast cells is called “the early phase reaction”. Granules contain various proteases (e.g. tryptase and chymase), which directly damage the tissues and trigger inflammation (Galli and Tsai, 2012; Janeway, 2001; Brown et al., 2008). Upon FcεRI engagement, mast cells also induce de novo synthesis of cysteinyl leukotrienes, and gene expression of cytokines and chemokines (Laidlaw and Boyce, 2012; Peters-Golden et al., 2006; Nakajima et al., 2002).

Mast cells are capable of producing various types of cytokines and chemokines. Such cytokines include TNF-α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-11, IL-12, IL-13, IL-16, IL-33, IFN-γ and TGF-β1 (Mukai et al., 2018). Chemokines are low-molecular-weight proteins that induce migration of immune cells, also known as chemotaxis (Moser and Willimann, 2004; Graves and Jiang, 1995), whereas chemokines include CC chemokine ligand (CCL)1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL9, CCL11, CCL17, CCL20, CCL22, CXC chemokine ligand (CXCL)2, and CXCL8 (Mukai et al., 2018). Depending on the cytokine milieu, types of stimulus, their localization in the tissues, and many other factors, mast cells alter production profiles of cytokines and chemokines (Galli et al., 2008). These cytokines and chemokines drive Th2 immune responses and leukocyte trafficking into the inflammatory sites. This infiltration of leukocytes into an area of inflammation is the late phase reaction. This late phase reaction in type I allergy is different from those in type IV allergy (T-cell mediated allergy) (Stone et al., 2010; Galli et al., 2008).

Mast cells distribute in most tissues but are particularly abundant in body barriers with a potential infection like the skin, airways and the gastrointestinal tract (Dawicki and Marshall, 2007; Wouters et al., 2016). In the intestine, MCs comprise 2-3% of the mononuclear cells in the lamina propria and sub-mucosa, but can be also found in the intraepithelial, smooth muscle and serosal layers of the intestine, in general near blood vessels, nerves and lymphatic tissue (Bischoff et al., 2000; Bischoff et al., 1996; Wouters et al., 2016). The intestinal mast cells regulate the epithelial permeability, secretion, peristalsis, nociception, angiogenesis, fibrosis and tissue repair, bacterial defense, chemotaxis as well as innate and adaptive immunity (Bischoff, 2007).

1.5.4. Eosinophils

Eosinophils are granulocytes produced in the bone marrow and circulate in the peripheral blood in low levels (1-3% of white blood cells) (Possa et al., 2013; Bischoff and Ulmer, 2008; Jung and Rothenberg, 2014). Most eosinophils generated under healthy conditions home to all the segments of the gastrointestinal tract lamina propria (except the esophagus) which constitutively contain eosinophils in contrast to other mucosa (Jung and Rothenberg, 2014). Their role is mainly involved in the maintenance of homeostasis with the gut microbiota and defense against parasites (Jung and Rothenberg, 2014).

Activated eosinophils release cytotoxic granule proteins such as eosinophil cationic protein (ECP), major basic protein (MBP) and eosinophil peroxidase (EPO) that initiate, escalate and maintain local inflammatory responses; those response are strong weapons of the cells in defense against parasites (Fulkerson and Rothenberg, 2013; Woodruff et al., 2011).

Numerous inflammatory mediators are associated to the eosinophil accumulation in the inflammatory site like cytokines IL-1, IL-3, IL-4, IL-5, IL-13 and GM-CSF and chemokines CCL3 (macrophage inflammatory protein 1 α), CCL5 (RANTES), CCL7 (monocyte chemoattractant protein 3: MCP-3), CCL13 (MCP-4), as well as CCL11 (eotaxin 1), CCL24 (eotaxin 2) and CCL25 (eotaxin 3) (Teixeira et al., 1995; Rothenberg, 1998; Rothenberg, 1999; Jacobsen et al., 2012). Among eosinophil chemoattractants, IL-5 is considered to have the most potent eosinophil specific regulatory properties (Powell et al., 2010; Sanderson, 1992). IL-5 promotes eosinophil proliferation and maturation in the bone marrow, as well as their release into the circulation (Collins et al., 2017; Clutterbuck et al., 1989; Yamaguchi et al., 1988; Shalit et al., 1995). IL-5 also inhibits the apoptosis of eosinophils, enhances the survival, stimulates eosinophil degranulation and primes their response to chemokines important in the mucosal recruitment (Collins et al., 2017; Powell et al., 2010; Powell et al., 2010; Tai et al., 1991; Kita et al., 1992).

In addition to IL-5, CCL11 is an important selective eosinophil chemoattractant, promoting the recruitment of eosinophils in the gastrointestinal tract in healthy individuals and is expressed constitutively in the intestinal lamina propria (Powell et al., 2010; Matthews et al., 1998; Mishra et al., 1999). Studies using mouse models have demonstrated that the absence of CCL11 or its receptor CCR3, abolish eosinophil trafficking to the intestinal tract (Matthews et al., 1998; Humbles et al., 2002; Hogan et al., 2001). Abundance of CCL11 n the broncho-alveolar lavage correlates with an increased eosinophilia in the inflammatory site in both humans and rodents with allergic airway inflammation (Jose, 1994; Lamkhioued et al., 1997). In intestinal inflammatory pathologies like ulcerative colitis, Crohn´s disease in humans or experimental colitis models in mice, CCL11 expression in the inflamed intestinal tissue was reported to correlate with an increased eosinophilia (Vieira et al., 2009; Garcia-Zepeda et al., 1996).

1.5.5. Neutrophils

Neutrophils are short lived polymorphonuclear leukocytes that play a major role in acute inflammatory response to infection or injury (Kim and Luster, 2015). During infection neutrophils can recognize, phagocyte and kill pathogens by producing reactive oxygen species (ROS) with antimicrobial potential and releasing lytic enzymes such as myeloperoxidase, cathepsin-G, elastase, lysozyme and defensins from their granules, having as well as an important role in acute inflammation (Wéra et al., 2016; Kolaczkowska and Kubes, 2013; Barker and Reisfeld, 1993).

Most of the studies regarding to the role of neutrophils in allergic inflammation have been performed in asthma. There is growing evidence that neutrophils play an important role in pathogenesis of both allergic and non-allergic asthma, given the correlation of airway neutrophilia with severity of airway obstruction in asthmatic patients (Agrawal and Shao, 2010). Neutrophils are postulated to promote more-severe airway inflammation by mediating direct tissue injury or by releasing pro-inflammatory mediators (Oliveira et al., 2016; Lacy, 2006). The presence of neutrophils in biopsies from patients with allergic enteritis has also been reported (Wéra et al., 2016; Kristjánsson et al., 2004)

CXCL8, a ligand of CXCR1 and CXCR2, stands out as the most likely chemokine for neutrophil trafficking (Sawant et al., 2015; Nasser et al., 2009). Increased CXCL8 expression in sputum of both non-allergic and allergic patients with asthma has been observed (Gras et al., 2010; Daldegan et al., 2005). IL-17A, which is largely produced by Th17 cells, alone or in combination with other cytokines, induced CXCL8 production from airway epithelial cells and airway smooth muscle cells (Bullens et al., 2006; Honda et al., 2016). In addition, CXCL1 a ligand for CXCR2 and CXR1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL7 ligands for CXCR2 are known to recruit neutrophils in intestinal tissues (Zimmerman et al., 2008).

1.5.6. Macrophages

Macrophages (Mϕ) are myeloid immune cells specialized in the neutralization and phagocytosis of cellular debris and potentially hazardous agents like pathogens (Varol et al., 2015). In a simplified way Mϕ can be classified as M1 and M2 as

suggested by Mills et al in 2000, mirroring the Th1/Th2 paradigm due observations made in mice (Mills et al., 2000).

In inflammatory status, Mϕ have three major functions: a) antigen presentation, b) phagocytosis and c) either aggravation of inflammation or induction of immunomodulation through the production of cytokines and growth factors (Fujiwara and Kobayashi, 2005), depending on tissues, cytokine milieu, and other factors such as microbiome (Wynn and Barron, 2010; Mosser and Edwards, 2008). Mϕ can play a role in the different phases of inflammation as initiation, maintenance and resolution (Oishi and Manabe, 2018).

When Mϕ are exposed to inflammatory stimuli, these are able to secrete multiple cytokines including TNF-α, IFN-γ, IL-1, IL-6, IL-8, and IL-12, as well as chemokines, leukotrienes, prostaglandins and complement (Arango Duque and Descoteaux, 2014; Fujiwara and Kobayashi, 2005). Those factors are able to increase the vascular permeability and recruitment of inflammatory cells, and produce systemic effects as fever and the production of inflammatory response proteins (Arango Duque and Descoteaux, 2014). Intestinal Mϕ seems also to have a role in the development of tolerance. In a mouse model, intestinal Mϕ expressed high levels of IL-10; that seems to be important for the expansion of Tregs in the lamina propria (Hadis et al., 2011). The role of macrophage in AE has not been identified.

1.5.7. Innate lymphoid cells (ILCs)

Innate lymphoid cells (ILCs) are a family of heterogeneous immune cells derived from a common bone marrow-derived lymphoid precursor. ILCs share similarities with $CD4^+$ T-cell phenotype and functions. However, ILCs lack of rearranged antigen receptors (TCR and BCR), myeloid cell markers and lineage markers (Lin^-), but express the IL-2 receptor (CD25) and the IL-7 receptor (IL-7R; CD127) (Geremia and Arancibia-Cárcamo, 2017; Spits and Cupedo, 2012; Artis and Spits, 2015).

ILCs express and developmentally depend on transcription factors Id2, the common γ-chain, GATA3 and PLZF, that control their cytokine profile which could be comparable to their corresponding adaptive counterparts (Artis and Spits, 2015; Klose and Artis, 2016). There are three main subsets that have been described reflecting the functional characteristics of the Th subsets. ILC1 that like Th1 cells express the transcription factor T-bet and are characterized by the secretion of IFN-γ

and TNF-α and are involved in the response against intracellular pathogens. ILC2 express GATA3^{hi} as do Th2 cells, secrete IL-4, IL-5, IL-9 and IL-13, and are involved in the control of helminth infections and development of allergic asthma and atopic dermatitis (McKenzie, 2014; Hurrell et al., 2018; van Rijt et al., 2016). ILC3 express RORγt and correspond to Th17 cells and secrete IL-17, IL-22, GM-CSH and are part of the protective response against pathogens in the mucosa (Artis and Spits, 2015; Klose et al., 2014; Hoyler et al., 2012; Klose and Artis, 2016; Melo-Gonzalez and Hepworth, 2017).

Recently, Krämer *et al* showed the ILC distribution in the intestinal tract. ILC1 are the major fraction in the upper gastrointestinal tract, while ILC3 are the predominant population in ileum and colon that correlates with a higher distal expression of IL-7. The presence of ILC2 in the intestine has been shown (Krämer et al., 2017). However, their role in the development of allergic enteritis is not known.

1.6. Chemokine and Chemokine receptors

In allergy and other inflammatory conditions immune cells as T-cells, B cells, monocytes, neutrophils, eosinophils and basophils migrate to the inflammatory site due a common process controlled by chemokines (Castan et al., 2017; Kaplan, 2001; Dembic, 2015). Chemokines function as G protein-coupled chemotactic factors which also activate the cells with which they interact and their effect on the immune cell migration is initiated by the binding to its receptor (Kaplan, 2001; Dembic, 2015), after that the chemokine receptor facilitates the migration to the site of highest chemokine concentration even at fast blood flow, allowing the diapedesis from the blood vessels and the final infiltration in the inflammatory site (Dembic, 2015).

A broad group of chemokines as CCL1, CCL7, CCL13, CCL11, CCL17, CCL22, CCL24, CCL26, are highly expressed in eosinophil, basophils and Th2 lymphocytes, three cell types mainly involve in the development of allergic inflammation (Garcia et al., 2005). Human and murine studies on asthma have shown that the role of that chemokines and their receptors including CCR3, CCR4 and CCR8 are potentially important in the development of allergic inflammation (Garcia et al., 2005; Castan et al., 2017; Hernández-Ruiz and Zlotnik, 2017; Lee et al., 2000; Legler and Thelen, 2016; Romagnani, 2002).

In food allergy, despite the mechanisms are not very well understood and there are some contradictory results, there is a general agreement that the chemokines are crucial for the recruitment of immune cells to the intestine (Castan et al., 2017; Hernández-Ruiz and Zlotnik, 2017). Taking this in consideration, chemokines and G-protein coupled receptors are an interesting target for therapeutic approaches, being reported that 30% of all available medicines exert their function by these receptors (Drews, 2000).

Taking in consideration that 1) chemokines and chemokine receptors are an attractive target in drug development for the treatment of inflammatory diseases. 2) As shown by targeted deletions in mice most of chemokines/chemokine receptors are not essential for life with the exception of CXCL12 knock-out mice that is fatal due of multiple organ failure (Castan et al., 2017). 3) The CCL1/CCR8 axis is not very well understood as well as its role in allergic enteritis despite that CCL1 was one of the first chemokines described (Burd et al., 1987; Miller et al., 1989; Miller and Krangel, 1992). CCR8KO mice were used in an AE model to evaluate the role of CCR8 in the development of allergic enteritis.

1.7. Mouse models of food allergies

Animal models are a useful tool to study allergic diseases *in vivo*, helping to understand the mechanism and pathogenesis of food allergies, and to assess anti-allergic effect of new therapeutics pre-clinically, although differences with the human system cannot be ignored (Bischoff and Crowe, 2005; van Gramberg et al., 2013; Liu et al., 2016; Fritsché, 2003).

Ideally, murine models of food allergy should mirror pathology in human. However, feeding allergenic diet alone induces little or no immune response, because mice tend to develop oral tolerance more strongly than humans (Chehade and Mayer, 2005). Use of transgenic mice expressing monoclonal TCR specific for ovalbumin (OVA, an egg white allergen) has been used to induce allergic reaction and inflammation by feeding allergenic diet alone. However, such gene modified mice expressed only monoclonal TCR and do not reflect allergic status in human with allergen-specific polyclonal T cells. For this reason adjuvants like aluminum hydroxide (alum) or cholera toxin (CT) are frequently used to induce Th2 immune responses including IgE production, and to accelerate the hypersensitivity reaction

(Kanagaratham et al., 2018; Smit et al., 2015; Gonipeta et al., 2015). Alum is administered systemically by intraperitoneal injection and boost adaptive responses though activating inflammatory DCs via inflammasome activation (Kool et al., 2008; Petrovsky, 2015; He et al., 2015). The CT, a well-known mucosal adjuvant, is administered intragastrically and induces innate immune changes that generate allergen specific responses from T and B cells (Snider et al., 1994; Tamura et al., 1994; Hörnquist and Lycke, 1993; Marinaro et al., 1995). The disadvantage in the use of CT are induction of CT specific IgE responses (Marinaro et al., 1995; Snider et al., 1994; Glenn et al., 1998). Therefore in many studies, Alum is used as an adjuvant (Lexmond et al., 2017; Kim et al., 2016; Shin et al., 2015; Jin et al., 2017; Hiraide et al., 2017)Taking this in consideration previously our group developed a mouse model of allergic enteritis to study the pathomechanism of the disease (Burggraf et al., 2011).

2. Aim

Allergic enteritis (AE) is one clinical phenotype of food allergies. The pathological mechanism for AE is not well known, which is partly due to difficulty in the access to inflammatory tissues in AE patients. The ultimate objective of this study is to elucidate cellular and molecular mechanism for the development of AE.

In the previous study, our group developed a mouse model of AE (Burggraf et al., 2011). Upon sensitization with allergen and challenge by feeding an allergenic diet, this model exhibits clinical symptoms (e.g. weight loss and soft stools) and inflammation in small intestines, and those are similar to pathological features observed in AE patients. Increased numbers of mast cells in basal area and accumulation of eosinophils in submucosa were observed in inflammatory sites of AE mice. In addition, DNA microarray analysis showed upregulated gene expressions of CC chemokine ligands CCL1 and CCL8 and its receptor CCR8, in the jejunum of AE mice (Fig. 2 and table 1, unpublished data). Previous studies by other groups showed that mast cells are the source of CCL1 to recruit eosinophils at inflammatory sites in murine models of allergic asthma (Gonzalo et al., 2007). Marked inflammatory infiltration by leukocytes is a characteristic feature of allergic diseases (Toda et al., 2007; Galli et al., 2008; Pawankar et al., 2011). Elucidation of the mechanisms for leukocyte trafficking into inflammatory sites is an important factor for target identification and establishment of novel anti-inflammatory strategies for treatment of such diseases. Leukocyte trafficking is controlled by tissue-specific expression of chemokines and chemokine receptor expression on the leukocyte surface.

Mast cells play a crucial role in IgE-mediated type I allergy. However, a role of mast cells in pathophysiology of AE is not known. Increased mast cells in biopsy of patients with AE have been observed (Czerwionka-Szaflarska et al., 2017; Rothenberg, 2004). FcεRI engagement by IgE and allergen induces strong activation of mast cells (Bax et al., 2012; Shiraishi et al., 2013; Galli and Tsai, 2012). AE is initiated by non-IgE-mediated mechanisms, but become persistent when patients developed IgE antibodies.

Taking together, the following hypotheses were set up:

(i) An axis of CCR8 and CCL1/CCL8 is involved in the development of AE

(ii) Mast cells produce CCL1 and CCL8 in the intestinal tissue that recruits eosinophils to the inflammatory site

(iii) IgE is involved in the exacerbation of AE.

To evaluate the hypothesis, the following experimental aims were set up:

1) To investigate an involvement of CCL1 and CCR8 in the development of AE.
2) To investigate a role of mast cells in the development of AE.
3) To investigate a role of IgE in the exacerbation of AE.

Collectively, this study would allow us to elucidate molecular and cellular mechanisms of AE, and to establish a novel strategy for treatment of such allergic disease.

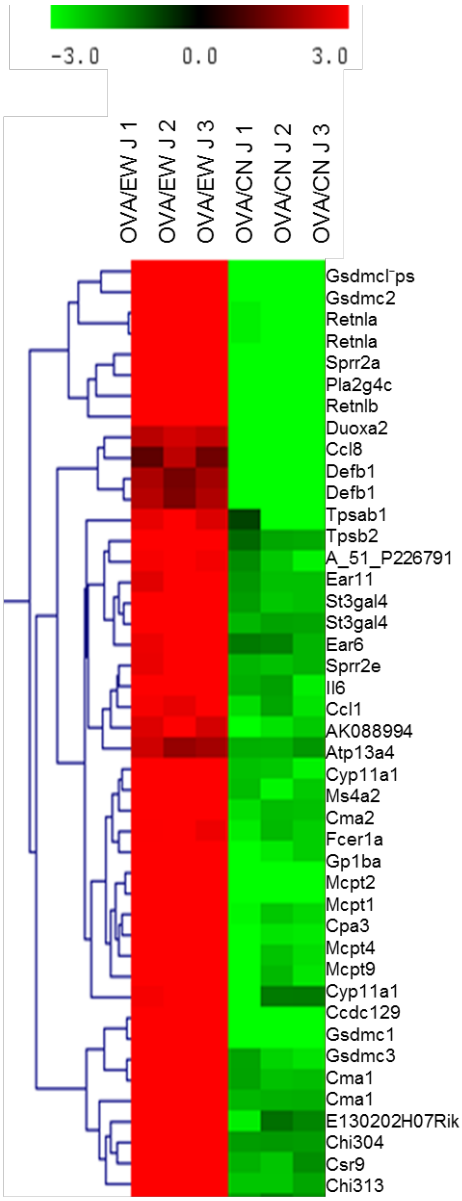

Figure 2: Microarray DNA analysis from the jejunum of mice with AE. BALB/c mice (n=3/group) were i.p. sensitized with OVA plus ALUM twice at a two-week's interval. Two weeks after the last sensitization, the mice were fed EW-diet (EW) or casein diet (CN) for 3 days. The jejunums (J) were harvested from the mice and the levels of gene expression in the tissues were assessed by microarray analysis (Data was provided by Dr. Toda). Green color indicates downregulated genes and red upregulated genes.

Table 1. Microarray analysis - significant upregulated genes in mice with AE.

Symbol	Gene name/description	Genbank Accession	Fold Change
Gsdmcl-ps	Adult male testis cDNA, RIKEN full-length enriched library	AK016931	100.00
Gsdmc2	Gasdermin C2 (Gsdmc2)	NM_177912	100.00
Retnla	Resistin like alpha (Retnla)	NM_020509	93.30
Retnla	Resistin like alpha (Retnla)	NM_020509	93.56
Sprr2a	Small proline-rich protein 2A (Sprr2a)	NM_011468	100.00
Pla2g4c	Phospholipase A2, group IVC (cytosolic, calcium-independent) (Pla2g4c)	NM_001004762	100.00
Pla2g4c	Cell embryo 1 cell cDNA, RIKEN full-length enriched library, clone:I0C0013F18 product:weakly similar to Cytosolic phospholipase A2 gamma [Homo sapiens], full insert sequence	AK145339	100.00
Retnlb	Resistin like beta (Retnlb)	NM_023881	100.00
Duoxa2	Dual oxidase maturation factor 2 (Duoxa2)	NM_025777	100.00
Ccl8	Chemokine (C-C motif) ligand 8 (Ccl8)	NM_021443	47.91
Defb1	Defensin beta 1 (Defb1)	NM_007843	46.27
Defb1	Defensin beta 1 (Defb1)	NM_007843	43.24
Tpsab1	Tryptase alpha/beta 1 (Tpsab1)	NM_031187	30.24
A_51_P226791	Unknown		40.26
Ear11	Eosinophil-associated, ribonuclease A family, member 11 (Ear11)	NM_053113	42.53
St3gal4	ST3 beta-galactoside alpha-2,3-sialyltransferase 4 (St3gal4)	NM_009178	50.32
Ear6	Eosinophil-associated, ribonuclease A family, member 6 (Ear6)	NM_053111	34.43
Sprr2e	Small proline-rich protein 2E (Sprr2e)	NM_011471	37.49
Il6	Interleukin 6 (Il6)	NM_031168	47.57
Ccl1	Chemokine (C-C motif) ligand 1 (Ccl1)	NM_011329	41.66
AK088994	2 days neonate thymus thymic cells cDNA, RIKEN full-length enriched library, clone:E430034M04 product:unclassifiable, full insert sequence	AK088994	45.86
Atp13a4	ATPase type 13A4 (Atp13a4)	NM_172613	15.50
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1 (Cyp11a1), nuclear gene encoding mitochondrial protein	NM_019779	75.19

Ms4a2	Membrane-spanning 4-domains, subfamily A, member 2 (Ms4a2), mRNA	NM_013516	68.02
Cma2	Chymase 2, mast cell (Cma2)	NM_010779	69.30
Fcer1a	Fc receptor, IgE, high affinity I, alpha polypeptide (Fcer1a)	NM_010184	46.80
Gp1ba	Glycoprotein 1b, alpha polypeptide (Gp1ba)	NM_010326	74.26
Mcpt2	Mast cell protease 2 (Mcpt2)	NM_008571	98.50
Mcpt1	Mast cell protease 1 (Mcpt1)	NM_008570	92.52
Cpa3	Carboxypeptidase A3, mast cell (Cpa3)	NM_007753	73.56
Mcpt4	Mast cell protease 4 (Mcpt4)	NM_010779	81.99
Mcpt9	Mast cell protease 9 (Mcpt9)	NM_010782	78.25
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1 (Cyp11a1), nuclear gene encoding mitochondrial protein	NM_019779	70.00
Ccdc129	Coiled-coil domain containing 129 (Ccdc129)	AK085190	30.60
Gsdmc1	Gasdermin C1 (Gsdmc1)	NM_031378	100.00
Gsdmc3	Gasdermin C3 (Gsdmc3)	NM_183194	100.00
Cma1	Chymase 1, mast cell (Cma1)	NM_010780	97.46
Cma1	Chymase 1, mast cell (Cma1)	NM_010780	95.63
E130202H07Rik	Mus musculus 0 day neonate eyeball cDNA, RIKEN full-length enriched library, clone:E130202H07 product:unclassifiable, full insert sequence	AK053684	98.82
Chi3l4	Chitinase 3-like 4 (Chi3l4)	NM_145126	88.60
Cst9	Cystatin 9 (Cst9)	NM_009979	97.84
Chi3l3	Chitinase 3-like 3 (Chi3l3)	NM_009892	89.13

3. Materials and Methods

3.1. Materials

3.1.1. Equipment

Device	Model	Company
Flow cytometry	BD FACS LSRII SORP	BD Biosciences, Heidelberg, Germany
Luna™ Automated Cell Counter	L10001	Logos biosystems, Gyunggi-do, South Korea
Centrifuges	Micro Centrifuge 100Vac	Carl Roth, Karlsruhe, Germany
	Centrifuge 5417 R	Eppendorf, Hamburg, Germany
	Centrifuge 5415 R	Eppendorf, Hamburg, Germany
	Megafuge 1.0R	Heraeus, Hanau, Germany
	Multifuge 1S-R	Heraeus, Hanau, Germany
Vortex	REAX 2000	Heildolph Instruments, Schwalbach, Germany
ELISA reader	SpectraMax Plus 340	Molecular Devices, Munich, Germany
CO_2 cell incubator	BBD 6220 CU	Heraeus, Hanau, Germany
Laminar air flow	SterilGARD III	Labotect, Rosdorf, Germany
Magnetic stirrer	MR3001	Heidolph Instruments, Schwalbach, Germany
Orbital incubator	S5150	Fisher Scientific, Darmstadt, Germany
Balance	EK-300i	A&D, Tokyo, Japan
Microprobe thermometer	BAT-12	Physitemp Instruments LLC, New Jersey, USA

3.1.2. Solutions, buffers preparation

Solution / Buffer	Composition	Usage
Phosphate buffered saline (PBS)	1.5 mM potassium di-hydrogen phosphate (KH_2PO_4) 0.8 mM disodium phosphate Na_2HPO_4 137 mM sodium chloride (NaCl) 3 mM KCl diluted in distillated H_2O, pH 7.1	ELISA, cell culture, Lamina propria dissociation
Coating buffer	16 mM sodium carbonate (Na_2CO_3) 34 mM sodium hydrogen carbonate ($NaHCO_3$) in 1 L distillated H_2O, pH 9.6	ELISA
Washing buffer (PBS-T) for ELISA	0.05 % Tween® 20 diluted in PBS	ELISA
Blocking buffer (PBS-10%FCS)	10 % FCS diluted in PBS	ELISA
FACS Buffer	1 % BSA 20 mM EDTA 0.03 % sodium azide (NaN_3) diluted in PBS	FACS
Fixation solution (FACS-1%PFA)	1 % Paraformaldehyde (PFA) diluted in PBS and filtrated by 0.22 µm membrane	FACS

MACS buffer	5 % bovine serum albumin (BSA) 2 mM ethylenediaminetetraacetic acid (EDTA) diluted in PBS	T-cell isolation
HEPES solution	1 M 2-[4-(2-hydroxyethyl)- piperazin-1-yl]ethane sulphonic acid, pH 7.9	Lamina propria dissociation
0.5 M EDTA	186.1 g disodium EDTA diluted in 1 L distillated H2O. pH 7.5	Lamina propria dissociation
Penicillin/ streptomycin/ L-glutamine	3.14 g/L penicillin G potassium salt 5 g/L streptomycin sulphate 15 g/L L-glutamine	Cell culture
Trypan Blue solution	4% Trypan Blue diluted in PBS	Cell counting

3.1.3. Media and media composition

Media	Company or Composition	Usage
T-cell assay media	500 ml RPMI 1640 media 0.5 % (w/v) Streptomycin 0.3 % (w/v) Penicillin 1.5 % (w/v) L-Glutamin 5 % FCS 1.75 µl 2-mercaptoethanol	T- cell assay

Wash media	500 ml RPMI 1640 media 0.5 % (w/v) Streptomycin 0.3 % (w/v) Penicillin 1.5 % (w/v) L-Glutamin 1.75 µl 2-mercaptoethanol	T- cell assay
Roswell Park Memorial Institute (RPMI) 1640 media	Gibco, ThermoFisher Scientific, Darmstadt, Germany	T- cell assay

3.1.4. Chemicals

Chemical	Producer	Usage
Imject® Alum (aluminium hydroxide and magnesium hydroxide)	ThermoFisher Scientific, Darmstadt, Germany	Mice sensitization
Lysing Buffer (red blood cell lysis buffer)	BD Biosciences Pharm Lyse™, Heidelberg, DE	Cell culture
Paraformaldehyde (PFA)	Sigma-Aldrich, Munich, Germany	FACS
Potassium chloride (KCl)	Sigma-Aldrich, Munich, Germany	ELISA, cell culture, Lamina propria dissociation
Histofix (phosphate buffered, 4%)	Carl Roth, Karlsruhe, Germany	Histology
Sodium azide (NaN_3)	Sigma-Aldrich, Munich, Germany	FACS

Sodium carbonate (Na_2CO_3)	Sigma-Aldrich, Munich, Germany	ELISA
Sodium chloride (NaCl)	Sigma-Aldrich, Munich, Germany	ELISA, cell culture, Lamina propria dissociation
Sodium hydrogen carbonate ($NaHCO_3$)	Sigma-Aldrich, Munich, Germany	ELISA
Sodium di-hydrogen phosphate (NaH_2PO_4)	Sigma-Aldrich, Munich, Germany	ELISA, cell culture, Lamina propria dissociation
Di-Sodium hydrogen phosphate (Na_2HPO_4)	Sigma-Aldrich, Munich, Germany	ELISA, cell culture, Lamina propria dissociation
Sulfuric acid (H_2SO_4) 5N	Merck, Darmstadt, Germany	ELISA
Trypan Blue	Sigma-Aldrich, Munich, Germany	Cell counting
Tween® 20	Sigma-Aldrich, Munich, Germany	ELISA
Hanks' Balanced Salt Solution (HBSS)	ThermoFisher Scientific, Darmstadt, Germany	Lamina propria dissociation
Dithiothreitol (DTT)	Molekula, Munich, Germany	Lamina propria dissociation
Bicinchoninic acid assay (BCA)	ThermoFisher Scientific, Darmstadt, Germany	Protein concentration

Fixable Viability Dye eFluor™ 450	ThermoFisher Scientific, Darmstadt, Germany	FACS
EDTA 0.5 mM	Media kitchen PEI	Lamina propria dissociation
HEPES	Media kitchen PEI	Lamina propria dissociation
2-mercaptoethanol	Sigma-Aldrich, Munich, Germany	Cell culture
FoxP3/Transcription factor staining set	ThermoFisher, Bonn, Germany	FACS
3, 3', 5, 5'-tetramethylbenzidine (TMB)	Carl Roth, Karlsruhe, Germany	ELISA

3.1.5. Protein and enzymes

Name	**Producer**	**Usage**
Protease inhibitor cocktail set 1	Merck, Darmstadt, Germany	Intestinal homogenates
Streptavidin HRP	BD Biosciences, Heidelberg, Germany	ELISA
Collagenase D from *Clostridium histolyticum*	Sigma-Aldrich, Munich, Germany	Lamina propria dissociation
Dispase II protease	Sigma-Aldrich, Munich, Germany	Lamina propria dissociation
DNase I grade II, from bovine pancreas	Sigma-Aldrich, Munich, Germany	Lamina propria dissociation

Albumin (OVA), from chicken egg grade V	Sigma-Aldrich, Munich, Germany	Mice sensitization
Bovine serum albumin (BSA)	Sigma-Aldrich, Munich, Germany	FACS, lamina propria dissociation

3.1.6. ELISA antibodies

Name	Clone	Company
Purified Anti-mouse IgE	R35-72	BD Biosciences, Heidelberg, Germany
Biotin Anti-mouse IgE	R35-118	BD Biosciences, Heidelberg, Germany
Anti-mouse IL-4 Purified	11B11	ThermoFisher Scientific, Darmstadt, Germany
Anti-mouse IL-4 biotin	BVD6-24G2	ThermoFisher Scientific, Darmstadt, Germany
Anti-human/mouse IL-5 purified	TRFK5	ThermoFisher Scientific, Darmstadt, Germany
Anti-mouse IL-5 biotin	TRFK4	ThermoFisher Scientific, Darmstadt, Germany
Anti-mouse IFN_{γ} purified	XMG1.2	ThermoFisher Scientific, Darmstadt, Germany
Biotin Anti-mouse IFN_{γ}	R4-6A2	ThermoFisher Scientific, Darmstadt, Germany
Anti-mouse IgG1 ($_{\gamma}$1) horseradish peroxidase (HRP) conjugate		ThermoFisher Scientific, Darmstadt, Germany

Anti-mouse IgG2a horseradish peroxidase (HRP) conjugate	ThermoFisher Scientific, Darmstadt, Germany
Murine Eotaxin (CCL11) Standard ABTS ELISA Development Kit	Peprotech, Hamburg, Germany
Mouse CCL1/TCA-3 DuoSet ELISA	R&D systems, Wiesbaden, Germany
Mouse CCL8/MCP-2 DuoSet ELISA	R&D systems, Wiesbaden, Germany
MCPT1 Mouse Uncoated ELISA Kit	ThermoFisher Scientific, Darmstadt, Germany
Ready set GO! Anti-mouse IL-13	ThermoFisher Scientific, Darmstadt, Germany
Ready set GO! Anti-mouse IL-33	ThermoFisher Scientific, Darmstadt, Germany
Murine IL-10 Standard ABTS ELISA Development Kit	Peprotech, Hamburg, Germany

3.1.7. FACS antibodies

Name	Clone	Company
FITC anti-mouse CD45	30-F11	Biolegend, Fell, Germany
PE Rat IgG2b, κ Isotype Ctrl	RTK4530	Biolegend, Fell, Germany
PE- anti-mouse CD198 (CCR8)	SA214G2	Biolegend, Fell, Germany
Purified anti-mouse CD16/CD32	93	ThermoFisher Scientific, Darmstadt, Germany

PE anti-mouse Ly6G (Gr1)	RB6-8C5	ThermoFisher Scientific, Darmstadt, Germany
eFluor 660 anti-mouse CD170 (Siglec F)	1RNM44N	ThermoFisher Scientific, Darmstadt, Germany
PE-Cyanine5 anti-mouse CD11b	M1/70	ThermoFisher Scientific, Darmstadt, Germany
PE anti-mouse CD4 PE	GK1.5	ThermoFisher Scientific, Darmstadt, Germany
APC anti-mouse CD25 APC	PC61.5	ThermoFisher Scientific, Darmstadt, Germany
PE-Cyanine5 anti-mouse/rat FoxP3	FJK-16s	ThermoFisher Scientific, Darmstadt, Germany

3.1.8. Software

Name	Company
BD FACSDiva™ Software, Version 6.1.3	BD Biosciences, Heidelberg, DE
SoftMax® Pro Software, Version 5.2 rev C	Molecular Devices, Munich, Germany
FlowJo V10.0.8r1	FlowJo, LLC, Oregon, US
GraphPad Prism 7	GraphPad Software, La Jolla, US

3.2. Methods

3.2.1. Animals

Animal experiments were performed in compliance with national law and approved by local authority (Regierungspräsidium Darmstadt, Germany, license number: F107/1020) following the guidelines of the Paul-Ehrlich-Institut. Mice on BALB/c

background were bred and maintained under pathogen free conditions in the animal facility of the Paul-Ehrlich-Institut.

3.2.1.1. Mouse strains

BALB/c wild type (WT) mice were purchased from Charles River Laboratories (Kisslegg), and maintained in the breeding facility of the Paul-Ehrlich-Institut.

CCR8KO mice (C.129P2-Ccr8^{tm1Yiw}) on BALB/c background were generated by the replacement of S*ph*I-BglII fragment (1.5 kbp) containing the exon-2, that contains the translation initiation site, by a neomycin resistance gene (Neo) to disrupt the Ccr8 gene, generating the absence of the receptor (Yabe et al., 2015). This mouse strain was provided by Prof. Yoichiro Iwakura (The University of Tokyo, Japan).

KIT$^{w\text{-}sh/w\text{-}sh}$ mice carry spontaneous mutations at both alleles of the dominant white spotting (W) locus, generating a marked reduction in c-kit signaling, that is traduced in a mast cell deficiency (Grimbaldeston et al., 2005; Wolters et al., 2005; Lyon and Glenister, 1982). This mouse strain was provided by Dr. Michael Stassen, University Medical Center of the Johannes Gutenberg-University Mainz, Germany

Cre-mediated mast cell eradication (CreMaster) mice (C.129P2-Cpa3$^{tm3(icre)Hrr}$) were generated using a Knock-in strategy to induce the Cre recombinase expression, deleting 28 nucleotides of the first exon of the *Cpa3* promoter by homologous recombination in ES cells (Feyerabend et al., 2011). This promoter encodes for the mast cell associated protease CPA3. The heterozygous mice (Cpa3$^{Cre/+}$) show an almost complete lack of mast cells, probably due an Cre-induced genotoxicity, but a normal immune system (Feyerabend et al., 2011; Galli et al., 2015). This mouse strain was provided by Dr. Hans-Reimer Rodewald, Division of Cellular Immunology, German Cancer Research Center (DKFZ**),** Germany.

IgE knock-in mice (C.Ighg1$^{tm1.1Pyu}$) on BALB/c background were created by replacement of exons encoding for the soluble part of the constant region of the murine IgG1 with the IgE counterpart. The homozygous IgE knock-in mice (IgE$^{ki/ki}$, referred here as IgEki) display a 10-fold increase of total serum IgE and complete absence of IgG1 (Lubben et al., 2013). This mouse strain was provided by Dr. Philipp Yu, Institute of Immunology, Philipps University of Marburg, Germany.

The gene modified mice and KIT$^{w\text{-}sh/w\text{-}sh}$ mice were bred in the mouse breeding facility of the Paul-Ehrlich- Institut in compliance with national law and approved by local authority (Regierungspräsidium Darmstadt, Germany). For some experiments (e.g. preparation of antigen presenting cells for T cell assay), BALB/c wild type (WT) mice were purchased from Charles River Laboratories (Kisslegg).

3.2.2. Induction of allergic enteritis

Mice (female, 6 to 8 weeks old) were sensitized by an intraperitoneal injection with 10 µg of ovalbumin (OVA, grade V, Sigma-Aldrich, Munich, Germany) and 1 mg of ALUM (ThermoFisher Scientific, Darmstadt, Germany) in 500 µl of PBS, or treated only with PBS or Alum twice at a two-week interval as described previously (Burggraf et al., 2011). Alum was used as adjuvant to trigger strong Th2 response and IgE antibodies against OVA (Korsholm et al., 2010; Brewer et al., 1999).

Two weeks after the second sensitization, the mice were fed an Egg white diet (EW-diet) containing high amount of OVA for 7 days (Fig. 3A). The EW diet is a pellet-based diet containing 100% EW as source of 20% of protein prepared at ssniff Spezialdiäten GmbH (Soest, Germany) as described in table 2.

Table 2. EW-diet composition

20.00%	Dried egg white
48.45%	Corn starch
9.00%	Alfa starch
5.00%	Sugar
5.00%	Cellulose
6.00%	Soy oil
5.00%	AIN 93M Min Mix
1.30%	AIN 93M Vit mix
0.25%	Choline chlorid

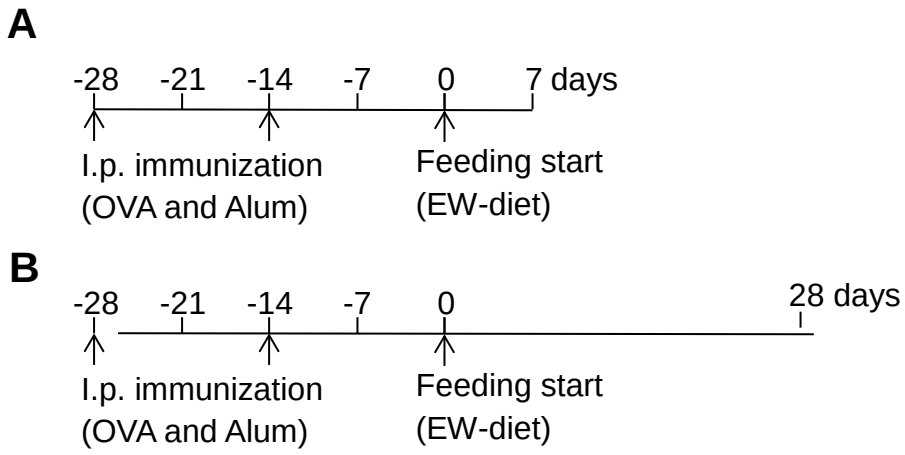

Figure 3: Immunization schedule. Mice were i.p. sensitized with OVA plus ALUM twice at a two-week's interval. Two weeks after the last sensitization, mice were fed EW diet for (A) seven days or (B) 4 weeks.

3.2.3. Blood harvest

Blood was harvested from the submandibular facial vein before or 7 days after the start of EW-diet. In the harvest, mouse was hold by the scruff of the neck, trying to keep the mouse relaxed as possible. Then with a lancet a puncture was performed in the back of the jaw of the mouse, slightly behind the hinge of the jawbones, toward the ear. There a small vascular bundle is located where orbital veins, submandibular vein and other veins draining the facial region join to form the beginning of the jugular vein. The blood was the collected in tubes containing a separation gel for collection of serum and whole blood (Golde et al., 2005).

3.2.4. Mouse euthanasia

After 7 days of EW-diet feeding, it was necessary to euthanize the mice in order to harvest several organs like spleens, MLNs and small intestines for immune assay. The euthanasia was performed using a CO_2 chamber.

3.2.5. Measuring serum levels of OVA-specific antibodies

Unless indicated otherwise, all dilutions following the coating step were done with 10% FCS in PBS and all washings between the incubation steps with PBS-T (PBS, 0.05% Tween 20) at room temperature. The volumes used were: 50 µl of antigen solution (OVA), primary antibody or secondary antibody; 100 µl of blocking buffer, 200 µl of PBS-T, 50 µl of samples or standards, 100 µl of TMB substrate and 50 µl of stop solution.

The serum levels of OVA-specific IgE, IgG1 and IgG2a antibodies were measured by ELISA as reported previously (Burggraf et al., 2011). Antibodies used for ELISA are listed in the section 3.1.6. Microtiter plates (Sarstedt, Nümbrecht, Germany) were coated with OVA in 50 mM sodium carbonate buffer (pH 9.6) at 4°C overnight. Plates were washed 3 times with PBS tween-20 1% (PBS-T). After blocking with 10% FCS in PBS at 4°C overnight, serum samples and standard were applied to the wells and incubated for two hours at room temperature (RT). Serum samples were diluted in blocking buffer in the following ratio: $1:10^5$ for IgG1, $1:10^2$ for IgG2a and IgE, except for samples from IgEki mice that were diluted $1:10^4$ for IgE detection. Following monoclonal OVA-specific IgG1 and IgG2a antibodies were used as standard: IgG1 antibody (clone: OVA-14), and IgE antibody (clone. E-G5).

After incubation with serum samples and standard with serial dilution 1:2 from 200 ng/ml for IgG1 and IgG2a and 1000 ng/ml for IgE, plates were washed with PBS-T 3 times, and applied with detection antibodies. IgE binding was detected by biotin-conjugated rat anti-mouse IgE antibodies and HRPO-conjugated streptavidin. For detection of IgG1 and IgG2a antibodies, HRPO-conjugated goat anti-mouse IgG1 and HRPO-conjugated rabbit anti-mouse IgG2a antibodies were used. After 1 hour of incubation at RT, plates were washed 3 times with PBS-T and 3, 3', 5, 5'-tetramethylbenzidine (TMB) was used as streptavidin-HRP substrate. 1N sulfuric acid (H_2SO_4) was used as stop solution. The colorimetric change was measured by an ExpectraMax Plus ELISA reader.

3.2.6. Intestinal homogenate preparation

After harvest of small intestines, 15 cm of the jejunum (9.5 cm distal from the duodenum) were longitudinally separated. Tissue was rinsed with cold PBS to eliminate the feces. Peyer´s patches were removed. The jejunum was cut in 0.5 cm pieces, transferred to Eppendorf tubes and immediately placed into liquid nitrogen to freeze the samples. The samples were stored at -80°C until use.

The frozen tissue was minced to a fine powder using a cold mortar and pistil. The powder of tissues was then transferred to an Eppendorf tube and suspended in 300 µl of ice-cold PBS with protease inhibitors (Merck, Darmstadt, Germany) using a vortex. The samples were centrifuged at 12.000 g for 20 min and supernatant was collected. The protein content was determined using the bicinchoninic acid (BCA)

assay for the colorimetric detection and quantitation of total protein. Samples were adjusted to a concentration of 5 mg/ml in PBS and applied for ELISA assays to determine the concentrations of chemokines, cytokines and mMCP1 in the intestinal tissue homogenates.

3.2.7. Determination of chemokine concentrations of CCL1, CCL8 and CCL11 in intestinal homogenates by ELISA

The concentrations of CCL1 and CCL8 in intestinal tissue homogenates were determined using a R&D ELISA Duoset kit, whereas the concentrations of CCL11 were determined using a Preprotech kit following the manufacturer indications with slightly modifications. Briefly, microtiter plates were coated with CCL1 or CCL8 capture antibody in a dilution of 1:180 and CCL11 in a ratio 1:200 in PBS and incubated overnight at 4°C. The plates were washed 3 times with PBS-T and blocked with PBS-10% FCS at 4°C overnight. Plates were then washed 3 times with PBS-T. Standards with known concentration and the intestinal tissue homogenates were added to the wells and incubated at 4°C overnight. Plates were washed 3 times with PBS-T and detection antibodies were added to the plates in the same ratio than the capture antibodies to CCL1 and CCL8 in a ratio of 1:180, and in a ratio of 1:100 in PBS-T and incubated for 2 hours at RT. Then the plates were washed 3 times with PBS-T and Streptavidin-HRP was added to each well and incubated at RT for 30 min. Plates were washed 3 times with PBS-T and TMB was used as streptavidin-HRP substrate. The colorimetric change was measured by an ExpectraMax Plus ELISA reader.

3.2.8. Determination of cytokines in intestinal samples by ELISA

Unless indicated otherwise, all dilutions following the coating step were done with 10% FCS in PBS and all washings between the incubation steps with PBS-T (PBS, 0.05% Tween 20) at room temperature. The volumes used were: 50 µl of antigen solution (OVA), primary antibody or secondary antibody; 100 µl of blocking buffer, 200 µl of PBS-T, 50 µl of samples or standards, 100 µl of TMB substrate and 50 µl of stop solution.

IL-4, IL-5, IL-6, IL-10, IL-13, IL-33 and IFN-γ were detected in the intestinal tissue homogenates by ELISA. For detection of IL-10, IL-13 and IL-33, commercially available kits were used following instructions by manufacturers. For detection of IL-

4, IL-5, IL-6, or IFN-γ plates were coated with the capture antibody at a dilution of 1:10^3 in coating buffer and incubated overnight at 4°C. The plates were washed 3 times with PBS-T and blocked with PBS-10% FCS at 4°C overnight. After washing with PBS-T three times, standards with known concentrations and intestinal tissue homogenates were incubated in the wells overnight at 4°C. After washing with PBS-T, detection antibodies were incubated in PBS-10%FCS in the wells for 2 hours at RT. The plates were washed 3 times with PBS-T and Streptavidin-HRP in in PBS-10%FCS was added to each well and incubated at RT for 30 min. Plates were washed 3 times with PBS-T and TMB was applied as streptavidin-HRP substrate. 1N H_2SO_4 was used as stop solution. The colorimetric change was measured by an ExpectraMax Plus ELISA reader.

3.2.9. Determination of mMCP1 concentration in serum and intestinal samples

Mouse mast cell protease 1 (mMCP1) was detected in sera and intestinal tissue homogenates by ELISA using a commercially available kit. The capture antibody was added in each well in a dilution 1:250 in 1X Coating buffer and incubated at 4°C overnight. The plate was washed 3 times with PBS-T and blocked using the 1X/ELISA/ELISPOT diluent at 4°C overnight. After washing the plates, the standard and the diluted samples (1:10 for intestine homogenates and 1:10^3 for sera) were added and incubated at 4°C overnight. After washing with PBS-T 3 times, detection antibody in a dilution 1:250 in 1X/ELISA/ELISPOT were applied to the wells, and incubated 1 hour at RT. The plates were washed with PBS-T and the streptavidin-HRP (1:250) in 1X/ELISA/ELISPOT was incubated 30 min at RT. The plates were then washed 3 times with PBS-T and TMB was applied as substrate. The colorimetric change was measured by an ExpectraMax Plus ELISA reader.

3.2.10. Histology of jejuna samples

Longitudinal sections of intestinal tissue (2 cm) were taken from the jejunum. The tissues were fixed in 4% formalin and embedded in paraffin. Sections (5 µm thick) were prepared and stained with hematoxylin and eosin (H&E) for morphologic analysis and detection of eosinophils. Toluidine blue staining was used for detection of mast cells. Additionally, Eosinophil-Mast Cell Stain Kit (Teomics, Houston, USA) was used to specifically visualize eosinophils. To visualize neutrophils, tissues were incubated with anti-Ly6G mAb (clone 1A8, Biolegend) in combination with Alexa 488

conjugated rabbit anti-rat IgG antibodies after blocking with rat IgG. Normal rat IgG2a (Biolegend) was used as an isotype control. To detect CCR8 and CD68, frozen tissues were blocked with goat sera, and stained with goat anti-mouse CCR8 polyclonal antibodies (Abcam, Tokyo, Japan) and rat anti-mouse CD68 monoclonal antibody (BioLegend, Tokyo, Japan). As isotype controls, normal Goat IgG antibodies (PM094 MBL) and Rat IgG2a antibodies (BioLegend) were used. After incubating with the primary antibodies or isotype controls, the tissues were treated with Alexa Fluor 647-conjugated donkey anti-goat IgG H&L Abs pre-adsorbed (Life technologies, Tokyo, Japan) and Alexa Fluor 488-conjugated donkey anti-rat IgG H&L Abs pre-adsorbed (Life technologies). The histology staining and the criteria to assess inflammation grade was performed by the collaborator partners Irene Gonzalez-Menendez, Manuela Martella and Leticia Quintanilla-Martinez at the Institute of Pathology and Neuropathology, Eberhard Karls University of Tübingen and Comprehensive Cancer Center, University Hospital Tübingen. Histological and immunohistochemistry was performed by the collaborator partners Yoichiro Kato and Masaharu Ohbayashi at the Department of Pathology, Tokyo Women's Medical University, Tokyo, Japan.

Inflammation levels in the tissues were assessed in a blind manner by pathologists in University Hospital Tübingen with a score as describe in table 3. The total histological score represents the sum of the inflammation grade and villi score. Score ranges from 0 to 6 (total score = I + V +E).

Table 3. Histological score system	
Inflammation grade	0- Rare granulocytes, isolated cells detected in the mucosa of villi and between the crypts. 1- Few granulocytes scattered in the mucosa. 2- A solitary group of less than 10 granulocytes 3- 2-3 groups of up to 10 granulocytes 4- More than 3 groups of granulocytes and larger than 10 cells.
Villi	0- Normal 1- Shorter villi – diffused and homogenous mild atrophy of the villi due to intestinal dilation with obvious central lumen
Edema	0- Not present 1- Diffused edema in the lamina propria

3.2.11. Analysis of eosinophil and neutrophil frequency in intestinal lamina propria cells

Intestinal lamina propria cells were prepared according to a protocol described by Weigmann *et al.* with slight modifications (Weigmann et al., 2007). Small intestines were harvested from OVA-sensitized or non-sensitized mice on day 7 of EW-diet.

After removal of Peyer´s patches, intestines were cut into 4-5 cm pieces, washed with cold PBS to eliminate the feces, and opened longitudinally. To remove the mucous the tissues were then cut into 1 cm pieces, and treated with HBSS (ThermoFisher containing 5 mM DTT (Molekula, Munich, Germany) at 37°C for 20 min at a ratio of 1.0 g of intestinal sample per 20 ml solution (1.0 g/20ml). The tissue was put on a 100 µm cell strainer to remove the solution. To remove epithelial cells from the tissues, these were incubated in 30 ml of a pre-digestion solution, i.e. HBSS containing 5 mM EDTA and 10 mM HEPES at 37°C for 20 min. The tissue was then put on a 100 µm cell strainer to remove the pre-digestion buffer, and re-suspended in 20 ml of fresh buffer at 37°C for 20 min. The remaining pieces were separated using a 100 µm cell strainer and washed with HBSS containing 10 mM HEPES at 37°C for 10 min to remove the remaining EDTA of the sample. After the removal of epithelial

cells, the digestion was performed in 7.5 ml of PBS containing 500 µg/ml Collagenase D, 500 µg/ml DNase I and 0.5 U/ml Dispase II for 20 min at 37°C. The tissue suspensions were then applied on a 40 µm cell strainer. The suspension though the strainer was kept on ice and the remaining tissue was subject again to a second digestion.

The suspensions was collected and centrifuged at 350 g for 10 min. After washing with cold PBS twice, the cells were treated with anti-CD16/CD32 mAb and Fixable Viability Dye eFluor 450, and stained with FITC-conjugated anti-CD45 mAb and eFluor 660-conjugated anti-CD170 (SiglecF) mAb to identify eosinophils, or with FITC-conjugated anti-CD45 mAb, PE-Cy5-conjugated anti-CD11b mAb and PE-conjugated anti-Ly6G mAb to identify neutrophils for FACS analysis. Antibodies used in FACS analysis are listed in section 3.1.7.

3.2.12. Preparation for single cell suspension of spleen and MLNs

Spleens and MLNs were isolated from mice and disaggregated mechanically using a syringe plunger and a 70 µm cell strainer in a Petri dish containing cold PBS. The cell suspension was transferred to a 15 ml Falcon tube and centrifuged at 350 g at 4°C for 10 min. To eliminate erythrocytes, the cell suspensions from the spleens were re-suspended in 2 ml of red blood cell lysing buffer (BD Biosciences Pharm Lyse™) and incubated at RT for 2 min. The cells were then washed twice with 10 ml of wash media (described in section 3.1.3) and centrifuged at 350 g at 4°C for 10 min. Cell concentration was determined using a Luna automated cell counter. These cells were used for FACS analysis or T-cell assay.

3.2.13. T-cell assay

MLNs and spleens were isolated from OVA-sensitized mice on day 7 of EW-diet as described before. Anti-CD3 mAb (200 µl) was added to culture plates at a concentration of 10 µg/ml and incubated overnight at 4°C. The plates were washed 3 times with sterile PBS to remove the non-bound soluble antibody. MLN and spleen cells ($2.0x10^6$ cells/ml) were seeded in the plates and incubated for 72 h at 37°C and 5%CO_2. The concentrations of IL-4 and IL-5 in the culture supernatant were determined by means of ELISA.

3.2.14. Measuring T-cell frequency in spleens and MLNs

MLNs and spleens were harvested from the mice, and single cell suspension was prepared as described in the section of “single cell suspension from Spleen and MLN”. The cell suspension ($1.0x10^6$ cells in 100 ul) was transferred to FACS tubes. For IgG receptor blocking a rat anti-mouse CD16/32 mAb was added at 1µg/ml and incubated on ice for 20 min. After 50 µl of a mixture of fluorescence conjugated antibodies against the surface markers of interest (PE-conjugated anti-mouse CD4 mAb, APC-conjugated anti-mouse CD25 mAb) and the viability dye (fixable viability die eFluor 450) to distinguish dead cells were added and incubated for 30 min on ice.. Samples were washed with 2 ml of FACS buffer 3 times. The cellular pellet was re-suspended in 300 µl of FACS buffer with 0.2% paraformaldehyde. The samples were measured in a BD FACS LSRII SORP and analyzed with FlowJo Engine v3.04910.

In detection of Treg cells, after the final wash in the staining of cell surface molecules, cell permeabilization was performed using fixation/permeabilization buffer in the Anti-mouse/Rat Foxp3 Staining set at 4°C overnight. After the incubation, 2 ml of permeabilization buffer was added and centrifuged at 350 g for 5 min at RT 2 times. Samples were re-suspended in 100 µl of permeabilization buffer containing rat anti-mouse CD16/CD32 at 1 µg/ml and incubated 15 min at RT. PE-Cy5-conjugated anti-mouse Foxp3 antibody was then added and incubated 1 hour at RT. Samples were washed twice with 2 ml of permeabilization buffer and centrifuged at 350 g for 5 min at RT. The cells were re-suspended in 300 µl of FACS buffer and measured in a BD FACS LSRII SORP. The FACS data was analyzed with FlowJo Engine v3.04910.

3.2.15. Statistical analysis

Comparison of mean values between different groups was performed by student t-test in GraphPad Prism 7 (San Diego, USA), or by ANOVA followed by Dunnett's test in IBM SPSS statistics (Chicago, USA). p values < 0.05, and < 0.01 were designated with * and ** respectively, and considered significant.

4. Chapter 1: A role of CCR8 in the development of allergic enteritis

4.1. A short introduction

Our previous study showed that gene expression of CC chemokine receptor (CCR) 8 and its ligand CC chemokine ligand (CCL) 1 and CCL8 was upregulated in inflamed tissues of AE mice (Fig. 2, page 17). Chemokines are a large family of small secreted chemotactic proteins of 8 to 12 kD that induce cell migration in homeostasis and disease condition (Garcia et al., 2005; Kaplan, 2001; Karin, 2018; Lloyd and Rankin, 2003). Chemokines exert their activity by binding to a family of specific 7 transmembrane G-protein-coupled surface receptors and are classified into four groups depending of the spacing of the N-terminal cysteine residues as CXC, CC, C and CX3C (where X is any amino acid) (Griffith et al., 2014; Rossi and Zlotnik, 2000; Arimont et al., 2017).

The selective expression of chemokines and their receptors inducing migration of specific cells in diseases states makes them appealing drug targets. Three chemokine receptors CCR3, CCR4 and CCR8 are preferentially expressed by Th2 cells, mast cells or eosinophils therefore represent interesting therapeutic targets in allergy (Chantry and Burgess, 2002; Owen, 2001; Bonecchi et al., 1998). Among these receptors, the role of CCR8 is less understood than the others.

Several studies have shown that CCR8 is also expressed by Th2 cells, Treg, macrophages, a subset of dendritic cells and endothelial cells, but its expression vary depending on experimental settings and tissues (Zheng et al., 2009; Kremer et al., 2001; Hoshino et al., 2007; Qu et al., 2004; D'Ambrosio et al., 1998; Zingoni et al., 1998; Bünemann et al., 2018). CCR8 is highly expressed in thymus and its expression has also been detected in the spleen, lymph nodes, lung, skin and the brain (Napolitano et al., 1996; Tiffany et al., 1997; Mutalithas et al., 2010; McCully and Moser, 2011). Human CCR8 has four ligands: CCL1, CCL8, CCL16 and CCL18 (Islam et al., 2013; Karin, 2018; Bernardini et al., 1998; Asojo et al., 2003; Howard et al., 2000), whereas murine CCR8 has two ligands CCL1 and CCL8 (Islam et al., 2011; Devi et al., 1995). CCR8 is the only known receptor for both human (I309) CCL1 and murine (TCA3) (Karin, 2018).

CCR8 has been shown to play a role in the pathogenesis of asthma, allergic rhinitis and atopic dermatitis (Gombert et al., 2005; Gonzalo et al., 2007; Islam et al., 2011;

Mutalithas et al., 2010; Buckland et al., 2007; Yabe et al., 2015; Zhang et al., 2007). However, the role of CCR8 in development of AE was not investigated. To evaluate the possible role of CCR8 in the pathogenesis of allergic enteritis CCR8KO mice were used in this study.

4.2. Results

4.2.1. CCR8 and CCL1 expressions are enhanced in AE tissues of WT mice

First, to verify the results of DNA microarray analysis, i.e. increased expression CCR8 and its ligand CCL1, the CCR8 expression in AE tissues of WT mice by immunohistochemically analysis was performed. The analysis showed an increased presence of CCR8 expressing cells in the jejunum of OVA/EW WT mice in comparison with NC/EW WT mice (Fig 4). To detect CCL1 expression in the tissues, ELISA was performed in intestinal homogenates of OVA/EW WT mice, ALUM/EW mice, or non-sensitized and EW-diet-fed (NC/EW) WT mice. Increased concentrations of CCL1 in OVA/EW WT mice were then detected, when compared to those in NC/EW WT mice or OVA/CN WT mice (Fig. 5). The results confirm that CCR8 and CCL1 expression was enhanced in AE tissues of WT mice.

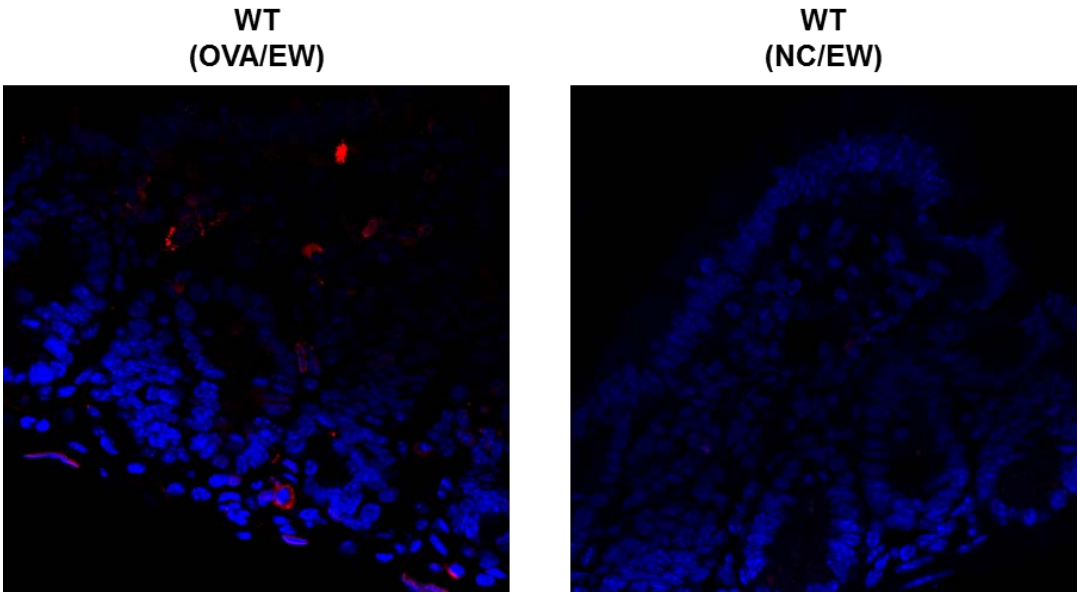

Figure 4: CCR8 expression in the inflamed intestinal tissues of WT mice. WT mice were i.p. sensitized with OVA plus ALUM or non-sensitized and fed EW diet for 7 days. The jejunums were harvested, and stained with anti-CCR8 Abs (red) and propidium Iodide (blue). The data are representative for two independent experiments. OVA/EW: OVA-sensitized and EW-diet fed. NC/EW; non-treated and EW-diet fed.

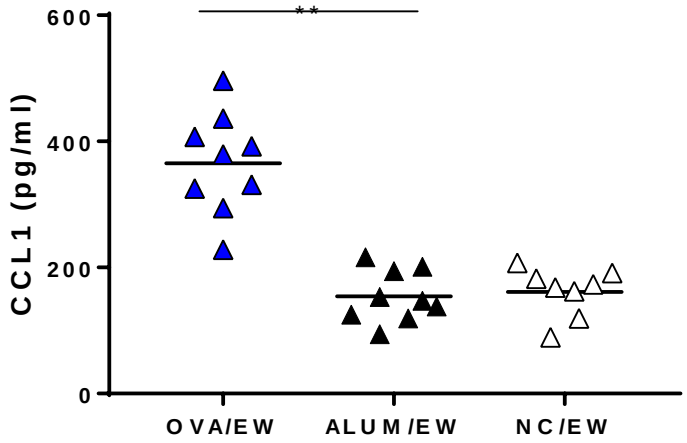

Figure 5: Expression of CCL1 in inflamed tissues of AE mice. BALB/c mice were i.p. sensitized with OVA plus ALUM, treated with ALUM only or non-sensitized twice at a two-week's interval. Two weeks after the last sensitization, the mice were fed EW-diet for 7 days. The jejunums were harvested from mice. The concentrations of CCL1 in homogenates of the tissues were measured by ELISA. Each symbol represents an individual mouse. The data are pooled of two independent experiments. OVA/EW; OVA-sensitized and EW-diet fed, ALUM/EW; ALUM-treated and EW-diet fed, NC/EW; non-treated and EW-diet fed. ** $p<0.01$

4.2.2. CCR8 deficiency did not suppress the development of clinical symptoms

In order to investigate a role of CCR8 in development of AE, WT and CCR8KO mice received sensitization with OVA plus ALUM and fed EW diet. OVA sensitized and EW diet fed (OVA/EW) WT mice presented significantly reduced body weight and temperature, when compared to non-sensitized and EW diet fed (NC/EW) WT mice (Fig. 6 and Fig. 7). OVA-sensitized mice on conventional diet or non-sensitized mice on EW-diet did not develop such symptoms, suggesting that clinical symptoms are induced by challenge with EW-diet in OVA-specific manner. OVA/EW CCR8KO also showed reduced body weight and temperature in comparison to NC/EW WT mice and NC/EW CCR8KO mice. However, the levels of reduction in OVA/EW CCR8KO tended to be lower than those of OVA/EW WT mice. The results suggest that CCR8 is partially involved in the development of clinical symptoms.

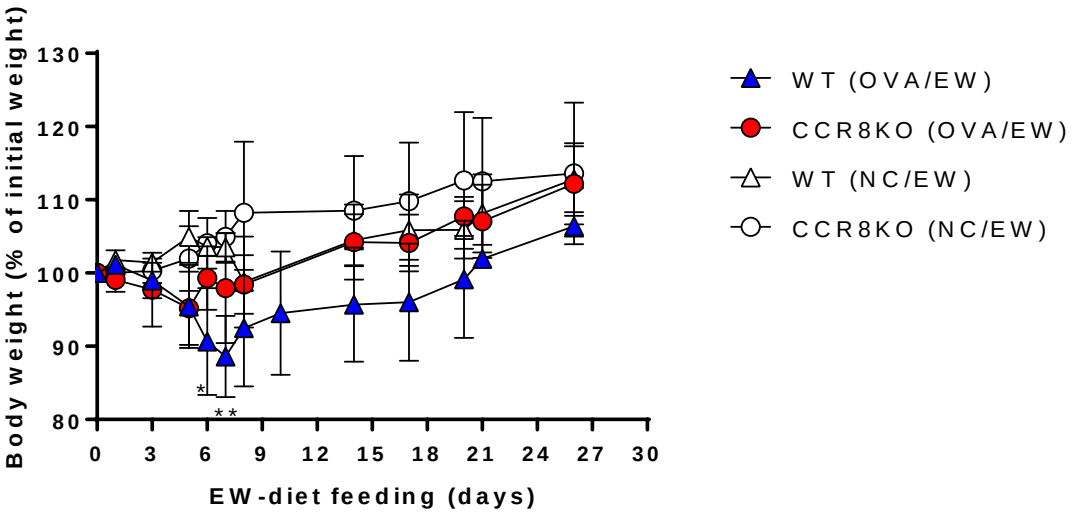

Figure 6: Reduced loss of body weight in CCR8KO mice. WT and CCR8KO mice were i.p. sensitized with OVA plus ALUM, or treated only with PBS, and fed EW-diet for up to 28 days. The body weight was measured and represented as percentage of the initial weight. OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed. * p<0.05, ** p<0.01

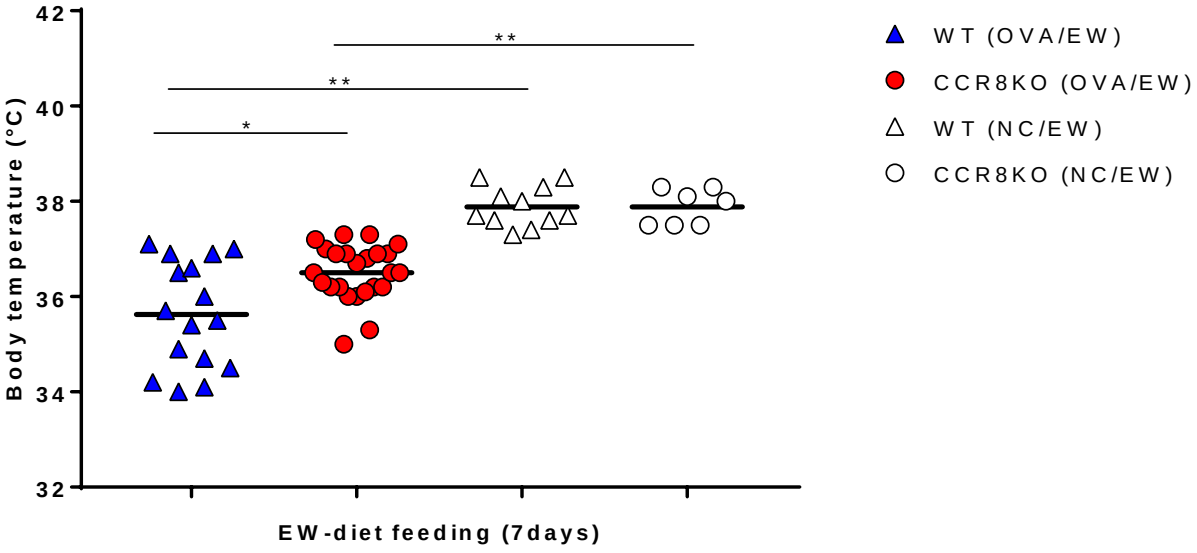

Figure 7: Reduced body temperature drops in CCR8KO mice on 7 days of EW-diet. WT and CCR8KO mice were i.p. sensitized with OVA plus ALUM, or treated only with PBS. Body weight was measured using a microprobe thermometer. OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed. * p<0.05, ** p<0.01

4.2.3. CCR8 deficiency reduced development of AE, but only moderately.

Next, the impact of CCR8 deficiency on AE development by histological analysis was assessed. H&E-stained tissues showed that OVA/EW WT and OVA/EW CCR8KO mice developed inflammation, which is characterized by irregular villi, a thickened

muscular layer, crypt elongation, and accumulation of granulocytes in the lamina propria (Fig. 8). A histology scoring analysis, which is based on grade of accumulation of granulocytes, villi morphology, and presence or absence of edema, indicated that inflammation levels were higher in OVA/EW WT mice, compared to OVA/EW CCR8KO mice (Table 4). Interestingly, the profile of granulocyte accumulation was different in the different groups. OVA/EW WT mice presented intensive infiltration of eosinophils and neutrophils (Fig. 9 and 10), whereas OVA/EW CCR8KO mice showed reduced accumulation of eosinophils (Fig. 9), but increased accumulation of neutrophils (Fig. 10). Morphological changes were not observed in the tissues of non-sensitized mice, NC/EW WT and NC/EW CCR8KO mice (Fig. 8). Animals that received ALUM alone and fed an EW-diet did not develop AE neither (Burggraf et al., 2011).

Table 4. Assessment of inflammation levels in WT and CCR8KO mice.

Group	Inflammation	Villi	Edema	Total
WT (OVA/EW)	$3.75^{**(1),*(2)}$ ± 0.5	0.75 ± 0.5	0.75 ± 0.5	$5.25^{**(1),*(2)}$ ± 1.5
WT (NC/EW)	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
CCR8KO (OVA/EW)	$2.75^{**(3)}$ ± 0.5	0.75 ± 0.5	0.50 ± 0.6	$4.00^{**(3)}$ ± 0.8
CCR8KO (NC/EW)	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0

WT and CCR8KO mice were i.p. sensitized with OVA plus ALUM, or treated only with PBS, and fed EW-diet for 7 days. The jejunums were harvested from the mice, and Inflammation levels in the tissues were assessed. The total histological score represents the sum of the inflammation grade, villi and edema score. Score ranges from 0 to 6 (total score = I + V + E). OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed. * $p<0.05$, ** $p<0.01$. (1) *vs* WT (NC/EW); (2) *vs* CCR8KO (OVA/EW); (3) *vs* CCR8KO (NC/EW).

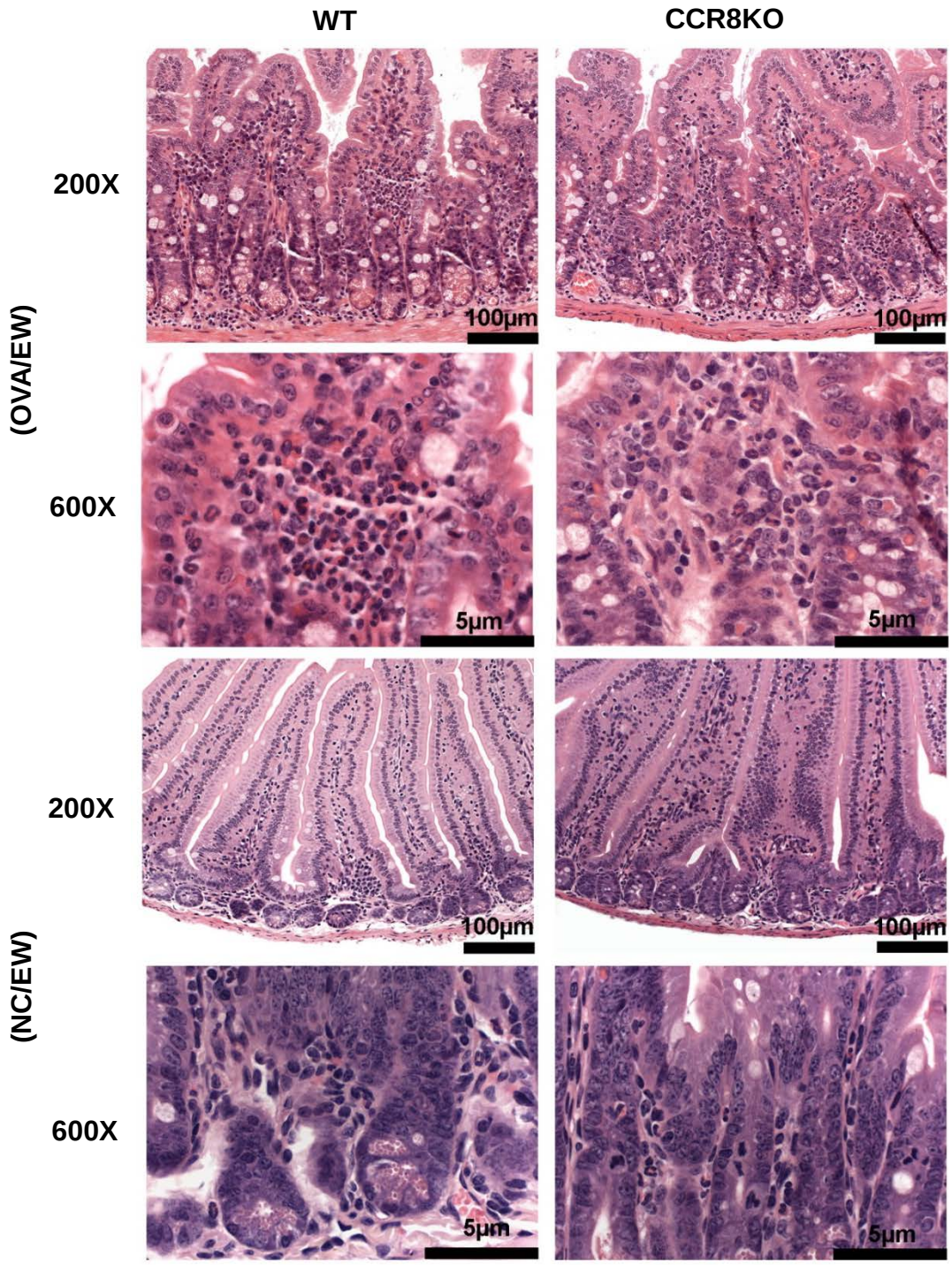

Figure 8: Development of AE in CCR8KO mice. WT and CCR8KO mice were i.p. sensitized with OVA plus ALUM, or treated only with PBS, and fed EW-diet for 7 days. The jejunums were harvested from the mice, and stained with H&E. All images were taken in same magnification. OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed. The data are representative for three independent experiments.

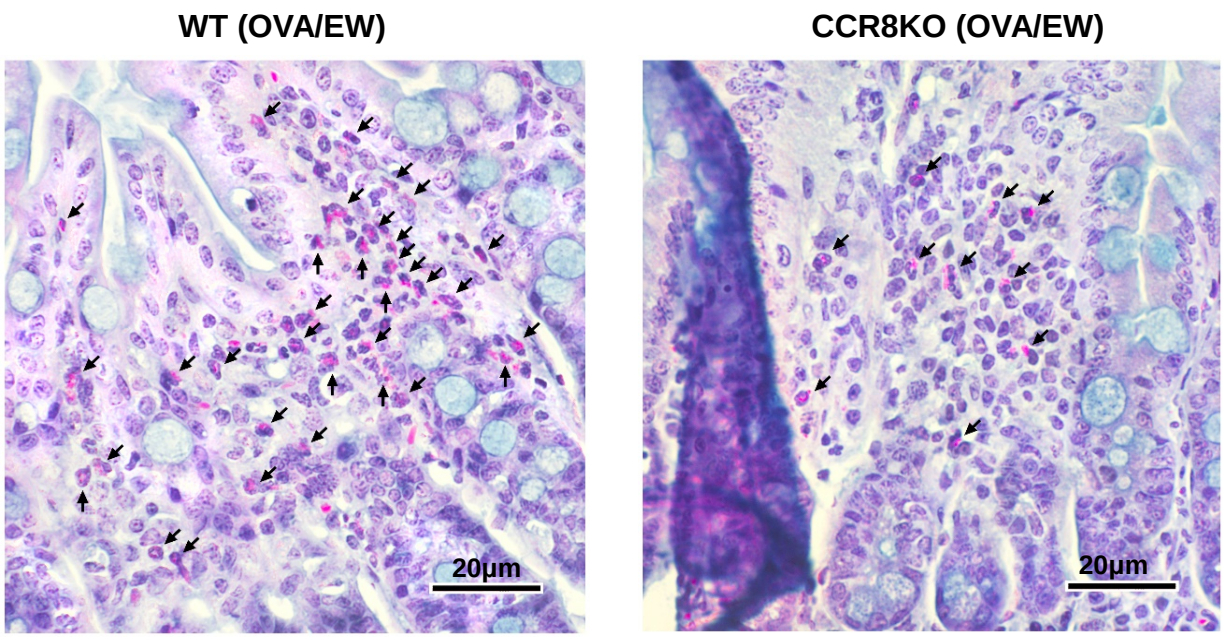

Figure 9: Reduced eosinophil accumulation in CCR8KO mice. WT and CCR8KO mice were i.p. sensitized with OVA plus ALUM, and fed EW-diet for 7 days. The harvested jejunums were stained with vital new red solution to visualize eosinophils. Arrows indicate eosinophils in the inflamed tissues of the WT and CCR8KO mice. The data are representative for two independent experiments.

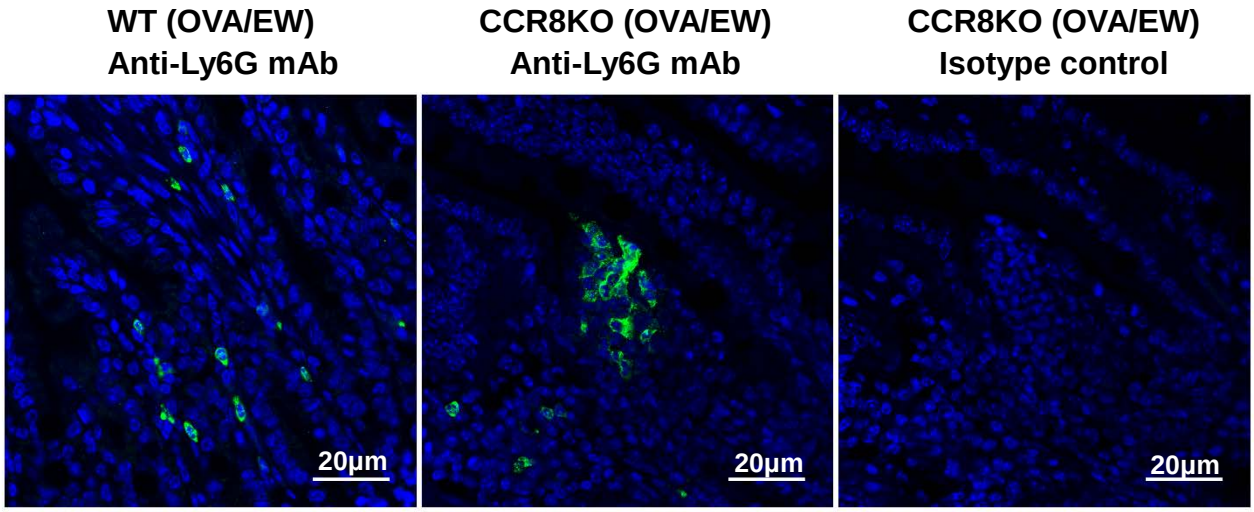

Figure 10: Enhanced neutrophil accumulation in CCR8KO mice. WT and CCR8KO mice were i.p. sensitized with OVA plus ALUM, and fed EW-diet for 7 days. The tissues were stained with anti-Ly6G mAb to visualize neutrophils. The data are representative for two independent experiments.

4.2.4. CCR8 deficiency reduced eosinophil accumulation, but enhanced neutrophil accumulation in AE tissues

To verify the results of the histological analysis, lamina propria cells were isolated from small intestines of WT and CCR8KO mice for FACS analysis. Leukocyte population in the lamina propria cells were comparable between WT and CCR8KO in OVA/EW or NC/EW group. However, a decreased frequency of eosinophils (SiglecF$^+$ CD11b$^+$ cells) and an increased frequency of neutrophils (Ly6G$^+$ CD11b$^+$ SiglecF$^-$ cells) among the lamina propria leukocytes (CD45$^+$ cells) was observed in OVA/EW CCR8KO mice (Fig. 11), compared to that in OVA/EW WT mice. The result suggests the differential involvement of CCR8 in eosinophil and neutrophil migration to the inflamed tissues in AE.

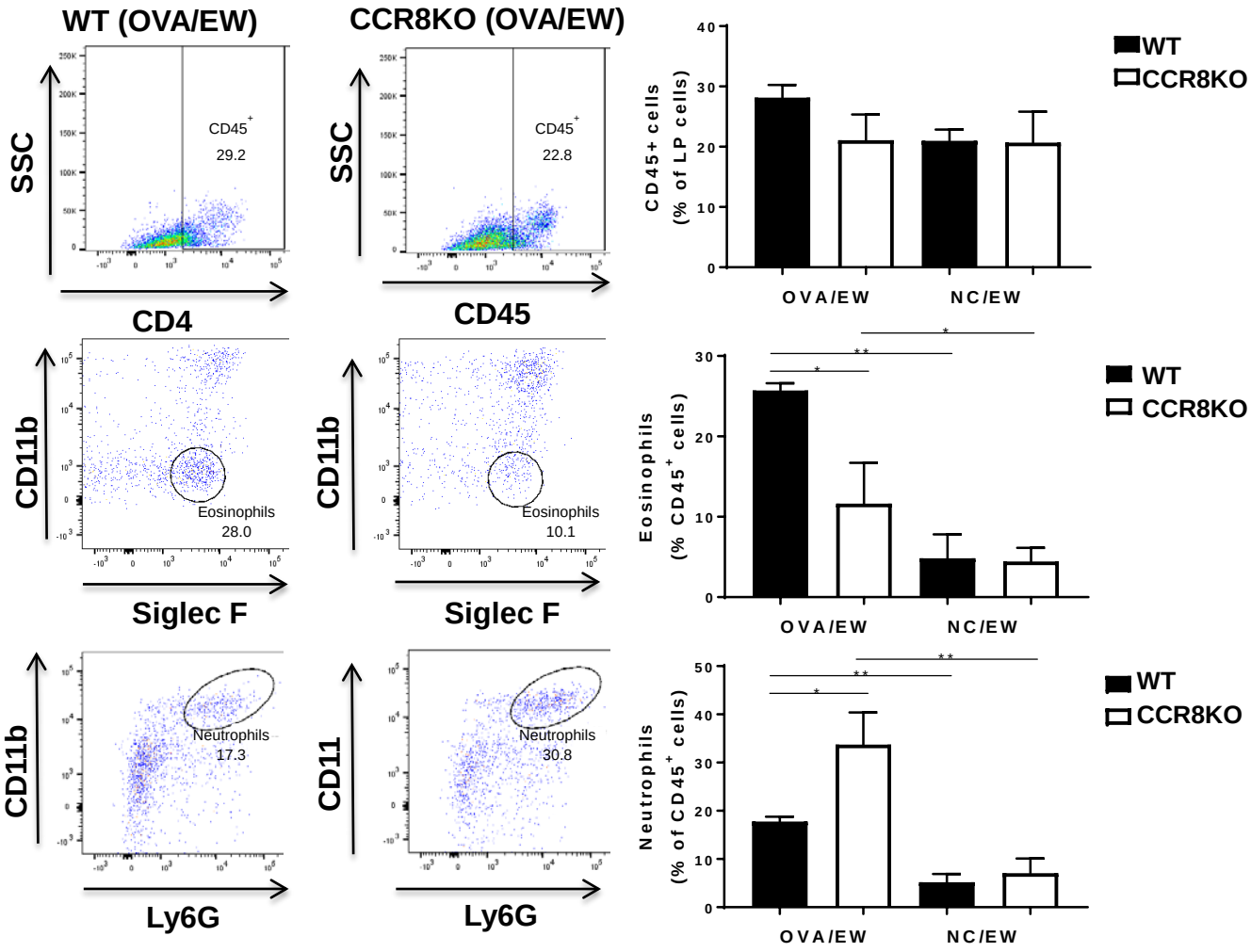

Figure 11: Reduced frequency of eosinophils and increased frequency of neutrophils in the inflamed intestinal tissues of CCR8KO mice. WT and CCR8KO mice (n=4/group) were sensitized with OVA plus ALUM, and fed EW-diet for 7 days. The jejunums were harvested from the mice, and subjected to enzymatic treatment for preparation of lamina propria cells. The frequency of CD45$^+$ cells in lamina propria cells, and the frequency of

eosinophils (SiglecF$^+$ CD11b$^-$ cells) and neutrophils (Ly6G$^+$ CD11b$^+$ SiglecF$^-$ cells) in CD45$^+$ cells population were determined by FACS. The data are representative for three independent experiments. * p<0.05, ** p<0.01

4.2.5. CCR8 deficiency did not affect Th2 immune response in AE mice

A previous study showed reduced eosinophil accumulation in allergen-induced airway inflammation of CCR8KO mice due to defective Th2 immune response (Chensue et al., 2001). To determine whether CCR8 influences the development of adaptive immunity in a murine model of AE, T-cell and antibody responses in WT and CCR8KO mice were assessed. Mesenteric lymph node (MLN)-derived T-cells and splenic T-cells from OVA/EW WT and OVA/EW CCR8KO mice produced similar levels of IL-4, and IL-5, Th2 cytokines that induce IgE production and eosinophil maturation/migration respectively in response to OVA (Fig. 12A-D). In the evaluated setting, the frequencies of CD4$^+$ T-cells in MLNs and spleens were not statistical significant when OVA/EW WT and OVA/EW CCR8KO mice were compared (p=0.689 and p=0.154, respectively) (Fig. 13A). There was also no significant difference in the frequency of T-reg cells (CD4$^+$ CD25$^+$ Fox p 3$^+$ cells) in MLNs and spleens between OVA/EW WT and OVA/EW CCR8KO mice (p=0.079 and p=0.988, respectively), although the frequency was higher in NC/EW WT mice, compared to NC/EW CCR8KO mice (Fig. 13B). In addition, serum levels of OVA-specific IgE, IgG1, and IgG2a antibodies were similar in both groups of mice on day 7 of EW-diet (p=0.149, p=0.146, and p=0.378, respectively), although CCR8KO mice showed lower IgE and IgG2a levels before beginning the EW-diet (Fig. 14A-C). Increased levels of mMCP1, a marker of mast cell activation, were similarly detected in the sera of OVA/EW WT and OVA/EW CCR8KO mice (Fig. 14D). The results suggest that the absence of CCR8 does not influence the development of Th2-mediated immune responses.

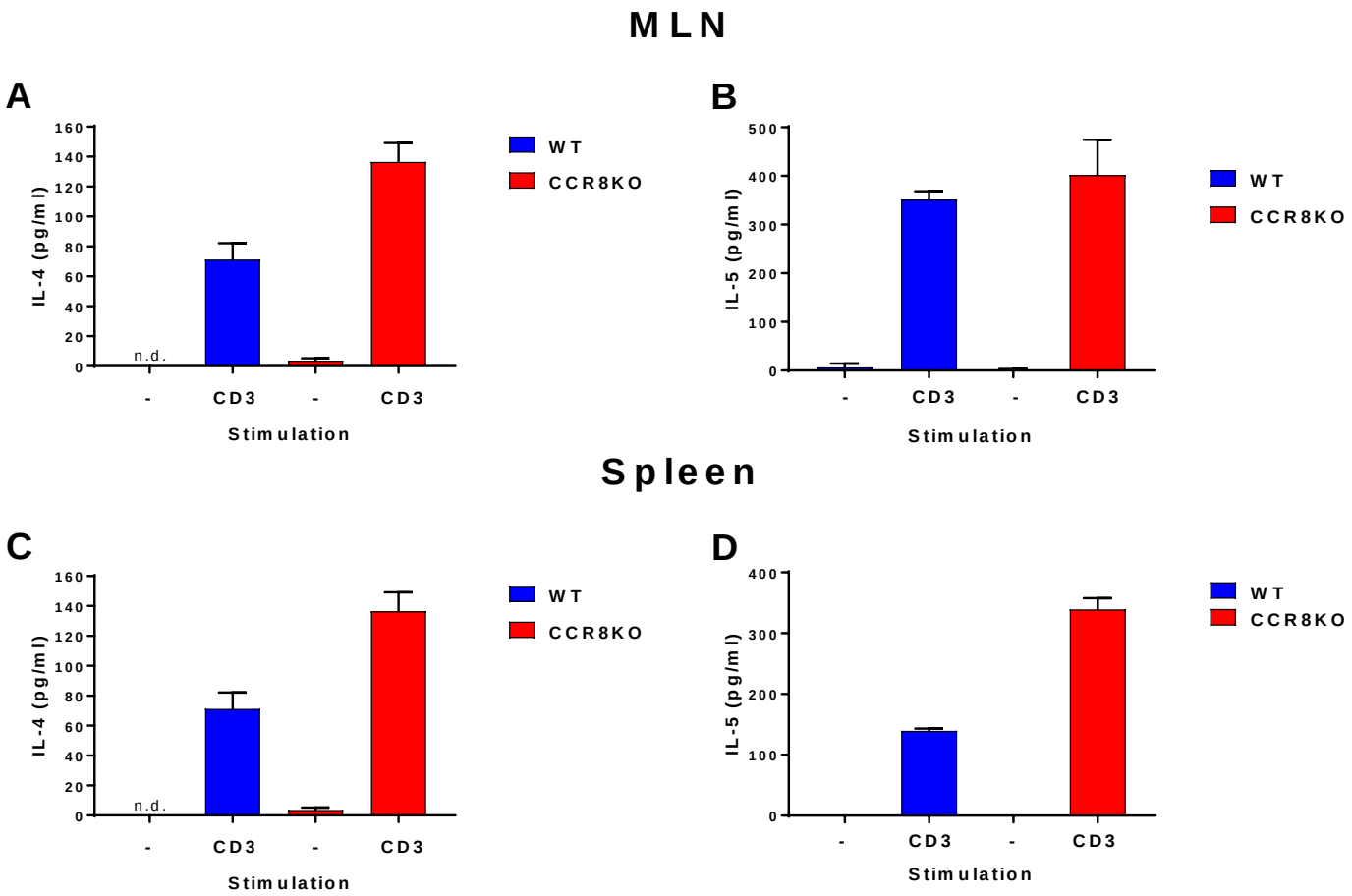

Figure 12: Th2 cytokine production by T-cells CCR8KO mice. WT and CCR8KO mice were sensitized with OVA plus ALUM and fed EW-diet for 7 days. (A) Mesenteric lymph node (MLN) cells and (B) splenocytes derived from the mice were cultured in the wells coated with anti-CD3 mAb. Concentrations of IL-4 and IL-5 in the cell culture supernatants after 72 hrs. of stimulation were measured by ELISA.

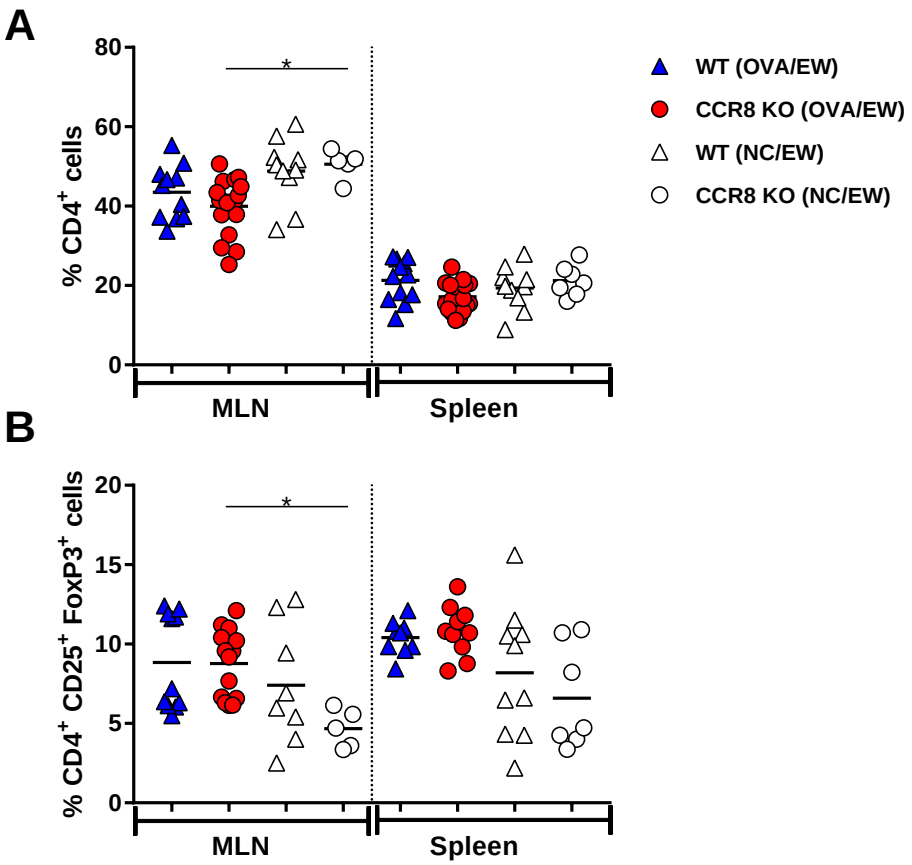

Figure 13: Similar T-cell frequency in WT and CCR8KO mice. WT and CCR8KO mice were sensitized with OVA plus ALUM and fed EW-diet for 7 days. (A) The frequency of $CD4^+$ T-cells and (B) the frequency of Treg ($CD4^+$ $CD25^+$ Fox $p3^+$) cells in Mesenteric lymph node (MLN) and spleen were determined by FACS. The data are pooled of three independent experiments using n=2-4/group. * $p<0.05$.

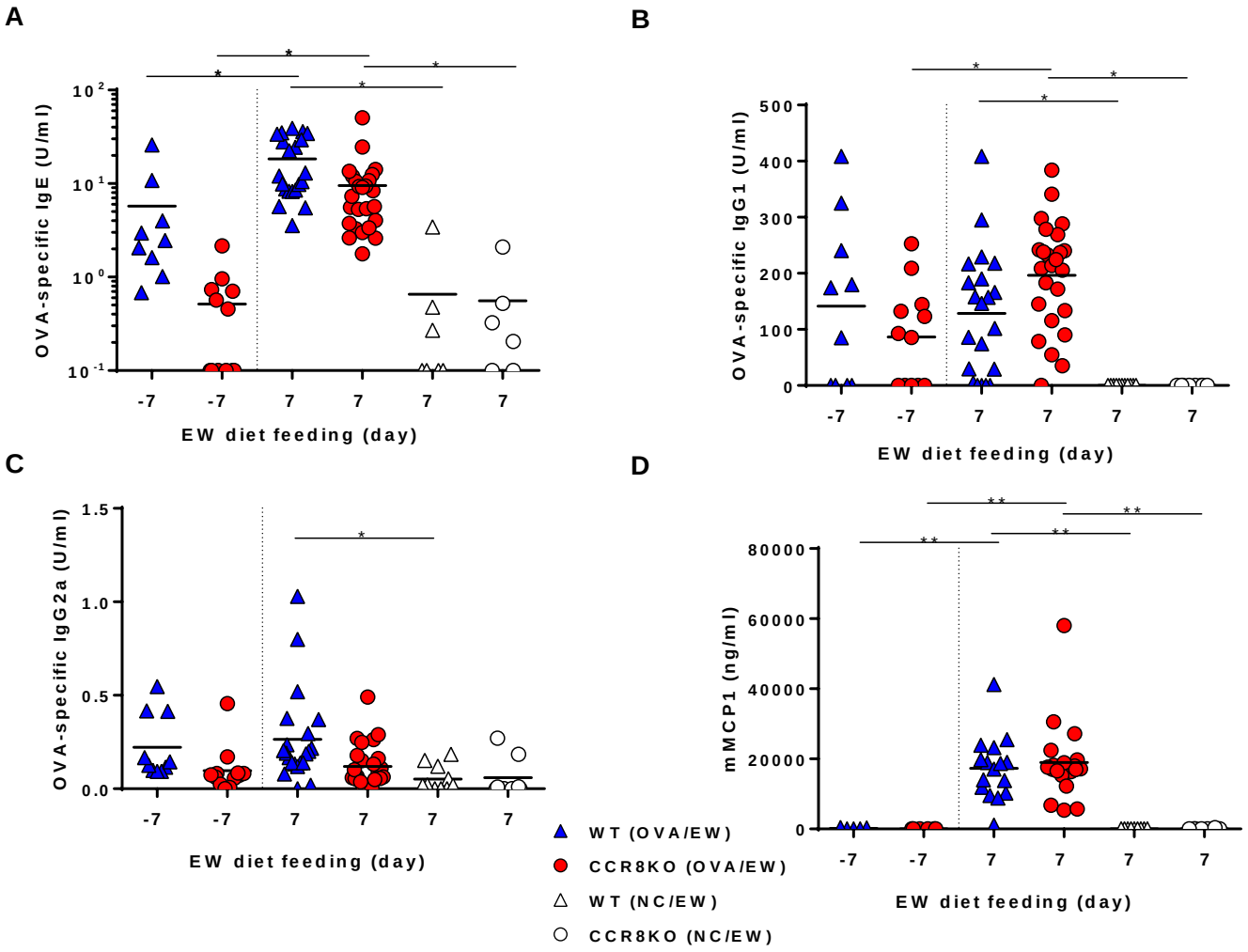

Figure 14: No difference in the levels of OVA-specific antibody responses between WT and CCR8KO mice. WT and CCR8KO mice (n=3-5/group) were i.p. sensitized with OVA plus ALUM, and fed EW-diet for 7 days. The serum levels of (A) IgE, (B) IgG1 and (C) IgG2a Abs specific for OVA and (D) mMCP1 on days -7 and 7 of EW diet were measured by ELISA. Each symbol represents an individual mouse. The data are collected from three independent experiments. * p<0.05, ** p<0.01

4.2.6. CCR8 deficiency reduced CCL11 expression in AE tissues

In addition to IL-5, several chemokines e.g., CCL11, CCL24, and CCL26 directly induce eosinophil migration (Griffith et al., 2014; Ransohoff, 2009; Islam et al., 2011). Among them, enhanced gene expression of CCL11 in the intestinal tissues of OVA/EW WT mice was detected by microarray analysis (see Table 1 from previous study). Therefore, it was assessed whether the deficiency of CCR8 influences protein expression of CCL11 in the intestinal tissues of WT and CCR8KO mice. Notably, CCL11 concentrations were significantly lower in the tissue homogenates of OVA/EW CCR8KO mice, when compared to those in the tissue homogenates of OVA/EW WT

mice (Fig. 15A). These results suggest that CCR8 is involved in CCL11 expression in AE sites.

The protein expression of CCR8 ligands CCL1 and CCL8 (Islam et al., 2011; Devi et al., 1995) was measured by ELISA in the jejuna tissue homogenates (Fig. 15 B-C). The treatment with OVA/EW enhanced the CCL1 protein expression in both WT and CCR8KO mice in comparison with the NC/EW counterparts; while CCL8 expression was not significantly different between the mice and treatments.

Next, in order to analyze the expression of cytokines in the intestinal tissues, the Th2 type associated cytokines IL-5, IL-13, and IL-33 levels in intestinal tissues homogenates of OVA/EW WT and OVA/EW CCR8KO mice were measured. The concentrations of IL-5 were comparable in the homogenates of OVA/EW WT, OVA/EW WT CCR8KO mice and their control NC/EW animals (Fig. 15D). In addition, the concentrations of IL-13 (Fig. 15E) and IL-33 (Fig. 15F) were similar in the homogenates of mice from OVA/EW WT and OVA/EW CCR8KO mice. Furthermore, the concentration of mMCP1 in the intestinal tissue homogenates (Fig. 15G) and the number of intestinal mast cells (Fig. 16) were comparable in OVA/EW WT and OVA/EW CCR8KO mice, suggesting that mast cell activation was similar in the intestinal tissues of both type of mice.

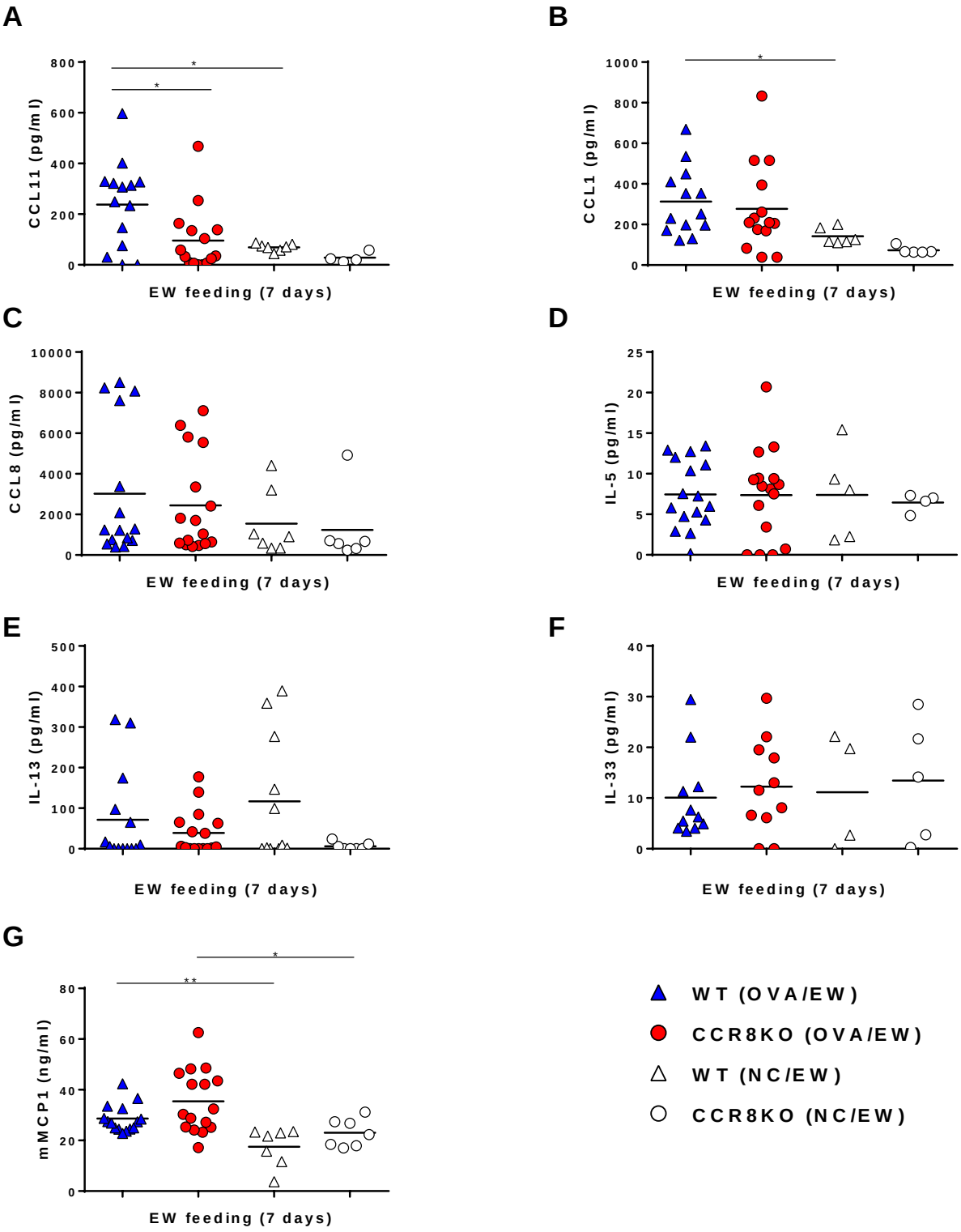

Figure 15: Reduced expression of CCL11 in the inflamed intestinal tissues of CCR8KO mice. WT and CCR8KO mice (n=3-5/group) were i.p. sensitized with OVA plus ALUM, or non-sensitized, and fed EW-diet for 7 days. Small intestines were harvested from the mice. The concentrations of (A) CCL11, (B) IL-5, (C) IL-13, (D) IL-33 and (E) mMCP1 in the intestinal tissue homogenates were measured by ELISA. Each symbol represents an individual mouse. The data are collected from three independent experiments. * $p<0.05$. , ** $p<0.01$.

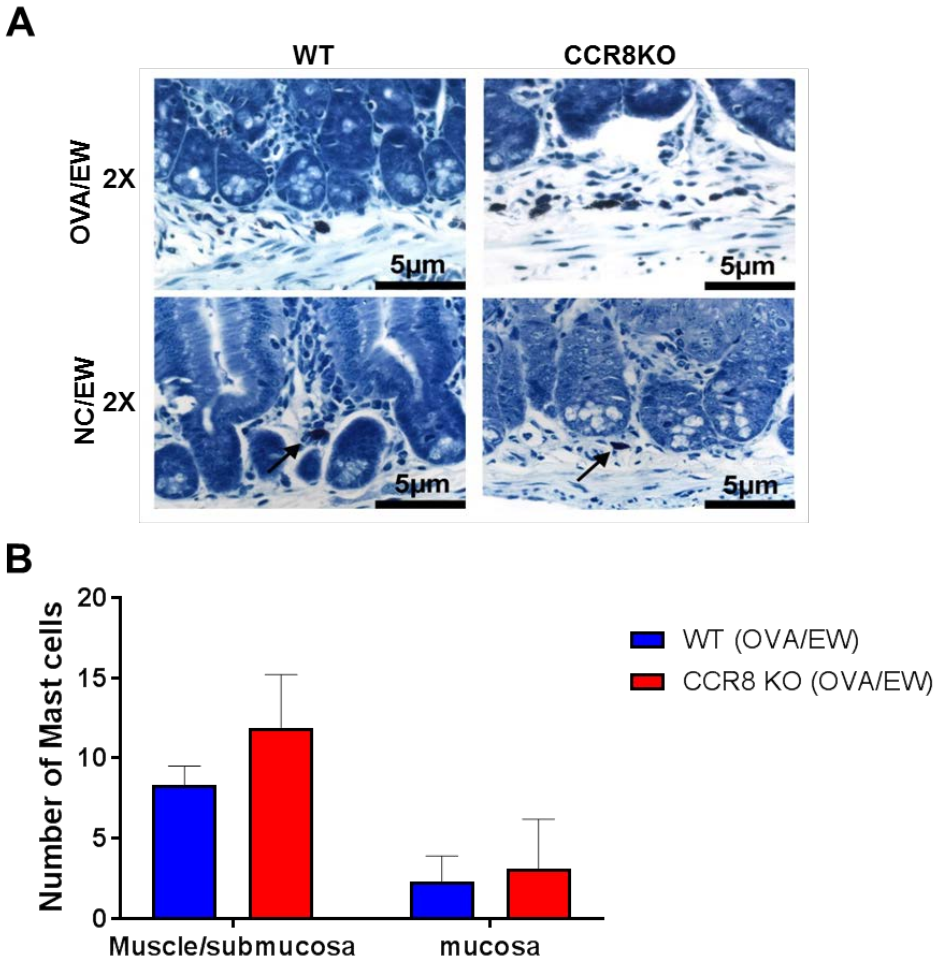

Figure 16: Similar number of mast cells in the inflamed intestinal tissues of WT and CCR8 KO mice. WT mice and CCR8 KO mice (n=3) were i.p. sensitized with OVA plus ALUM, and fed EW diet for 7 days. The jejunums were harvested from the mice, fixed in 4% paraformaldehyde, and embedded in paraffin. The tissues were cut and stained with toluidine blue. The numbers of stained mast cells in the tissues were counted under microscope. (A) Mast cells in jejunum. (B) Number of mast cells in muscle-submucosa and mucosa.

5. Chapter 2: The role of mast cells in the development of AE.

5.1. A short introduction

Mast cells play a crucial role in the development of allergic disease (Bonnekoh et al., 2018; Bischoff et al., 2000). Not only because of the broad range of mediators released from their granules, but also newly synthetized cytokines and chemokines (Burks et al., 2012; Renz et al., 2018; Morita et al., 2013).

In the previous section, it was found that gene expression of CCL1 was up-regulated in the jejunum of AE mice (see Fig. 2). Notably, CCL1 binds only to CCR8 (Karin, 2018). Mast cells have been reported to be the main producers of CCL1 in the lung of patients with allergic asthma and asthmatic mice (Gonzalo et al., 2007). An increased number of mast cells in intestinal tissues of OVA/EW mice was observed (Fig. 16). Taking together, it suggests an involvement of mast cells in the development of AE. To investigate whether and how mast cells are involved in the AE, two different types of mast cell deficient mouse strains i.e. $KIT^{w\text{-}sh/w\text{-}sh}$ mice and CreMaster mice were used.

$KIT^{w\text{-}sh/w\text{-}sh}$ mice carry spontaneous mutations at both alleles of the dominant white spotting (W) locus, generating a marked reduction in c-kit signaling, that is traduced in a mast cell deficiency (Grimbaldeston et al., 2005; Wolters et al., 2005; Lyon and Glenister, 1982). Cre-mediated mast cell eradication (CreMaster) mice (C.129P2-$Cpa3^{tm3(icre)Hrr}$) were generated using a Knock-in strategy to induce the Cre recombinase expression, deleting 28 nucleotides of the first exon of the *Cpa3* promoter by homologous recombination in ES cells (Feyerabend et al., 2011). This promoter encodes for the mast cell associated protease CPA3. The heterozygous mice ($Cpa3^{Cre/+}$) show an almost complete lack of mast cells, probably due an Cre-induced genotoxicity, but a normal immune system (Feyerabend et al., 2011; Galli et al., 2015).

5.2. Results

5.2.1. Mast cell deficiency abolished the development of clinical symptoms

In order to assess the role of mast cells in development of clinical symptoms and inflammation in AE, WT, $KIT^{w\text{-}sh/w\text{-}sh}$, or CreMaster mice received i.p. sensitization with OVA plus ALUM and challenge with EW diet (see immunization schedule in Fig.

3). OVA/EW WT mice exhibited drop in body temperature (Fig 17), weight loss (Fig 18) and ruffled hair (data not shown) during EW-diet for 7 days. In contrast, OVA/EW CreMaster mice and OVA/EW KIT$^{w\text{-}sh/w\text{-}sh}$ mice did not exhibit such clinical symptoms (Fig. 17 and 18). The results suggest that mast cells play an essential role in the development of clinical symptoms in experimental AE.

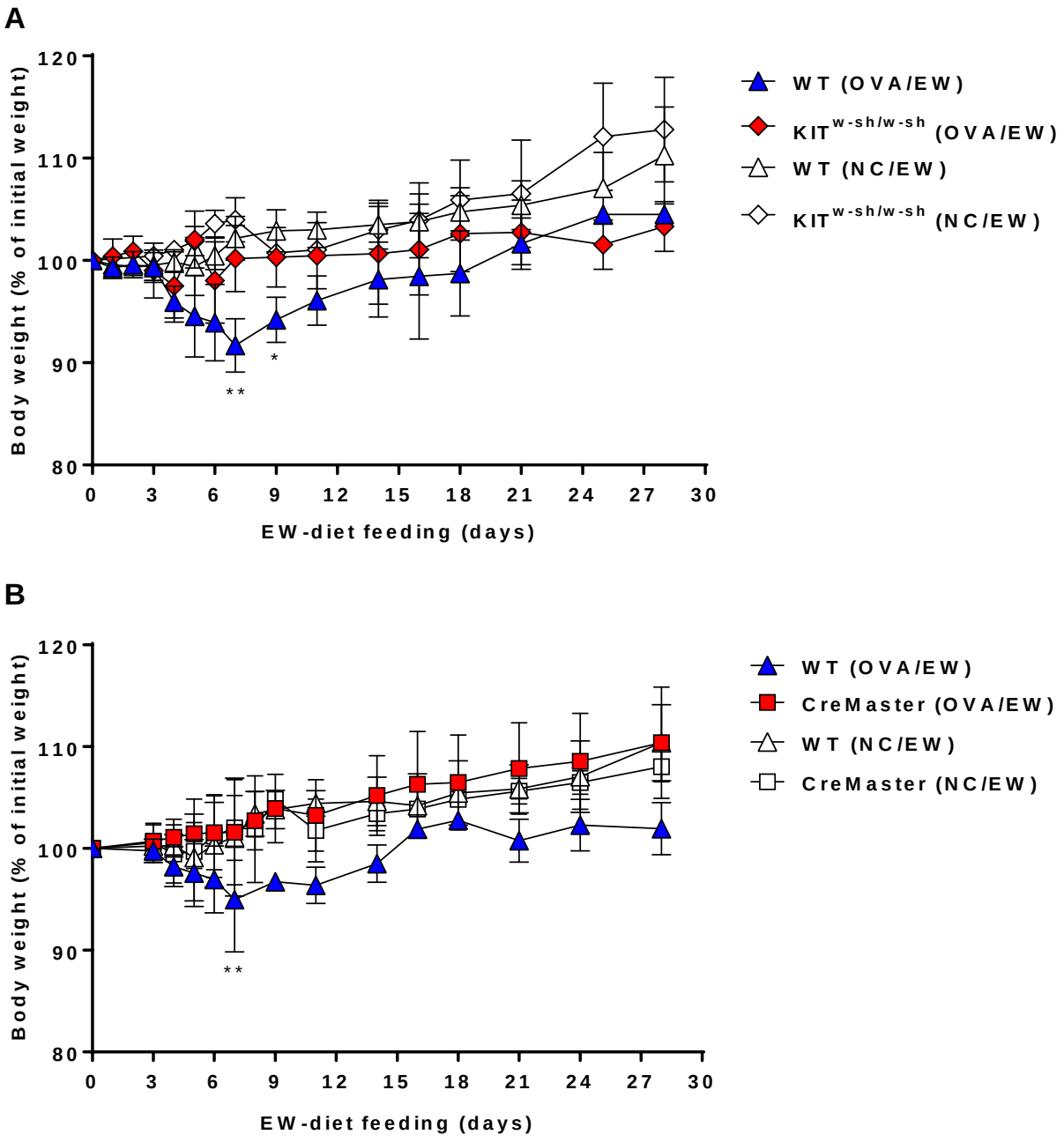

Figure 17: Abolished body weight loss in mast cell deficient mice. WT, KIT$^{w\text{-}sh/w\text{-}sh}$ and CreMaster mice were i.p. sensitized with OVA plus ALUM, or treated only with PBS, and fed EW-diet for up to 28 days. The body weight was measured and represented as percentage of that on day 0 of EW diet. OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed. * $p<0.05$, ** $p<0.01$.

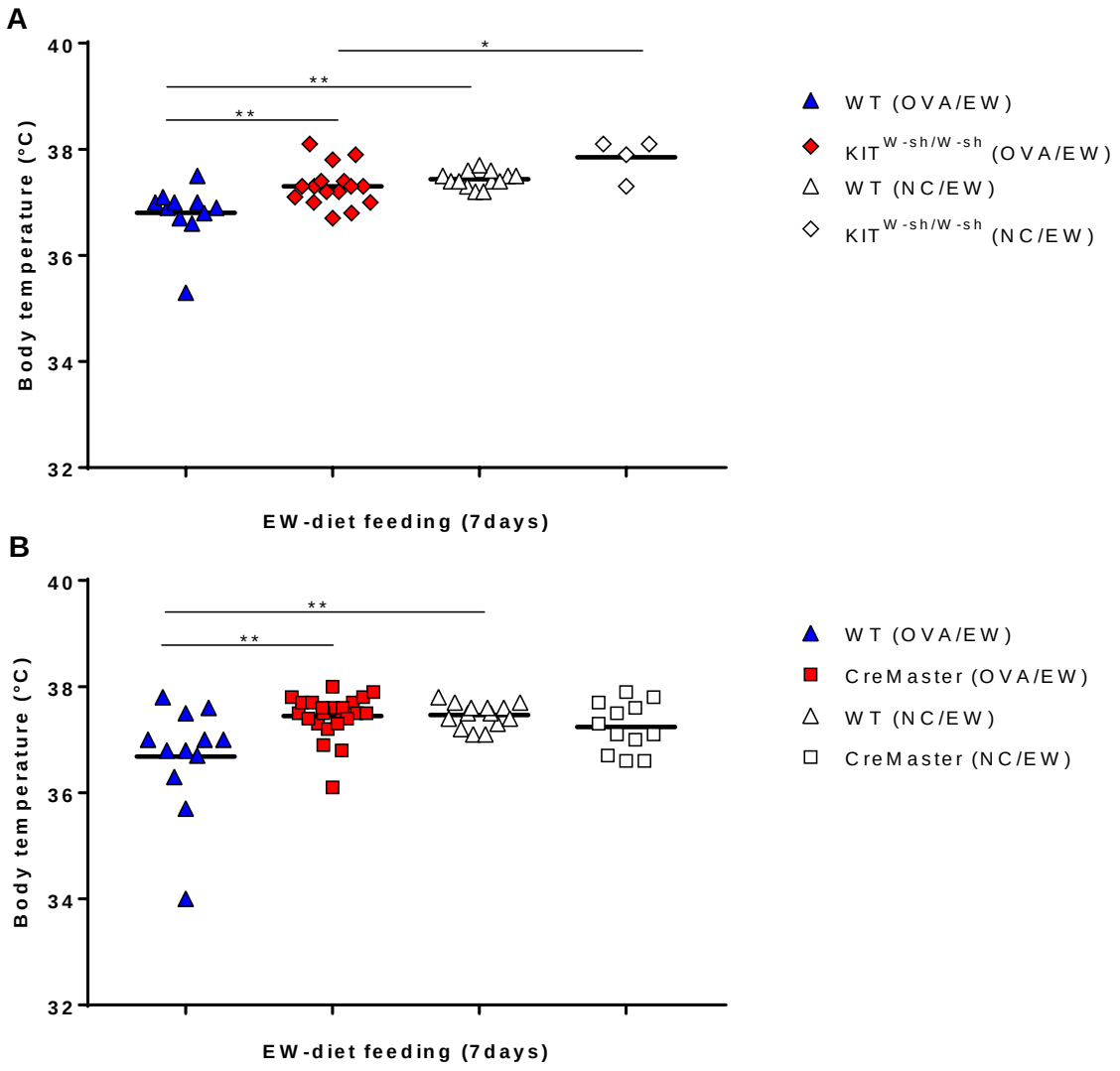

Figure 18: Abolished reduction in body temperatures in mast cell deficient mice. WT, KIT$^{w\text{-}sh/w\text{-}sh}$ and CreMaster mice were i.p. sensitized with OVA plus ALUM, or treated only with PBS on 7 days of EW-diet. Body weight was measured using a microprobe thermometer. OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed. * p<0.05, ** p<0.01

To verify the mast cell deficiency in KIT$^{w\text{-}sh/w\text{-}sh}$ and CreMaster, toluidine blue staining of the jejuna samples from the mice was performed (Fig 19A). The number of mast cells was increased in OVA/EW WT mice in comparison with NC/EW mice, whereas in both OVA/EW and NC/EW groups of KIT$^{w\text{-}sh/w\text{-}sh}$, and CreMaster mice showed nearly no mast cells in the tissues (Fig 19A and Fig 19B). In addition, mMCP-1 a mast cell activation marker, that has been associated to the gastrointestinal manifestations of food allergies in several murine models (Benedé and Berin, 2018; Vaali et al., 2005), was not detectable in in both mast cell deficient mice (Fig. 20).

The results suggest that mast cells are not increased in KIT$^{w\text{-}sh/w\text{-}sh}$, and CreMaster mice by OVA sensitization and EW diet feeding.

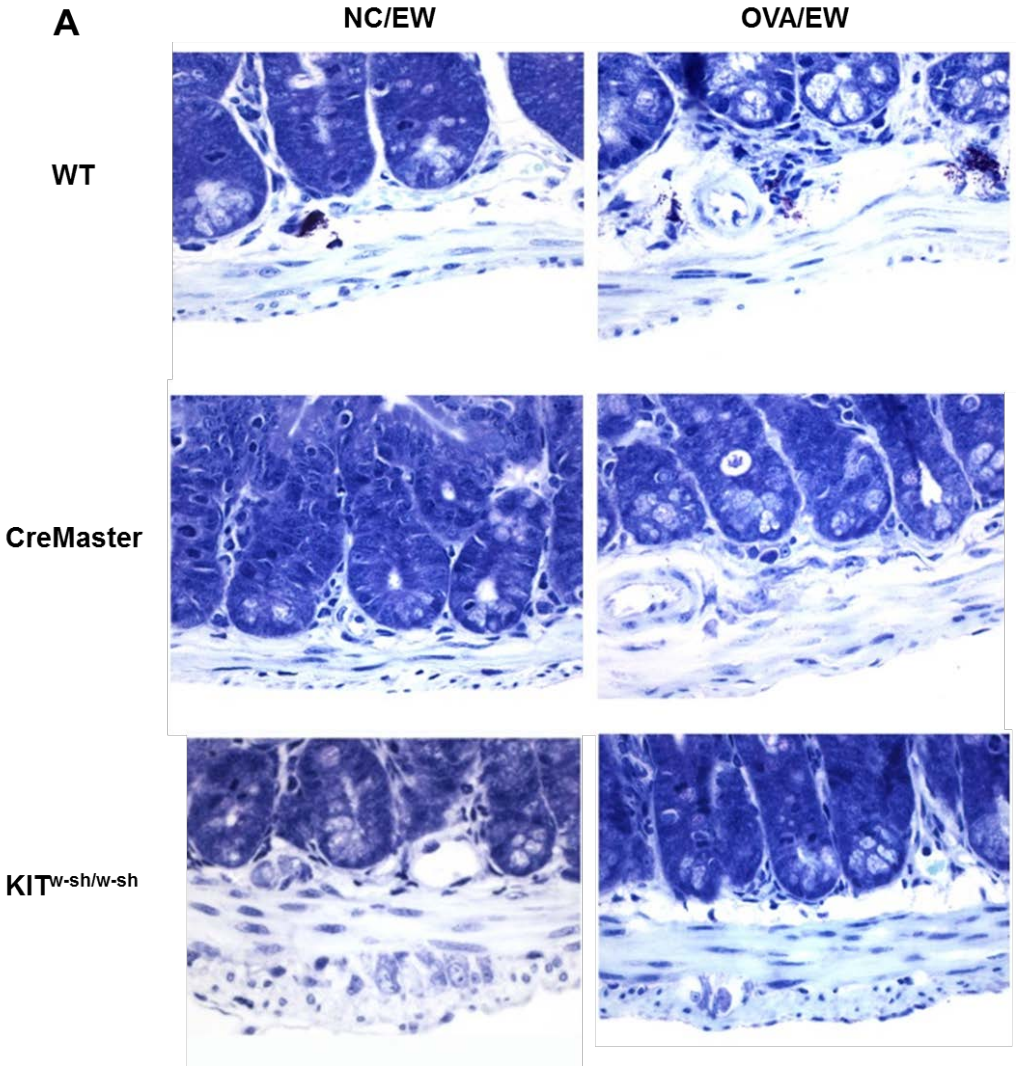

Figure 19: Reduced number of mast cells in mast cell deficient mice. WT, KIT$^{w\text{-}sh/w\text{-}sh}$ and CreMaster mice were i.p. sensitized with OVA plus ALUM, and fed EW diet for 7 days. The jejunums were harvested from the mice, and stained with toluidine blue. The numbers of stained cells in the tissues were counted under microscope. (A) Images of stained jejunum of mice. (B) Number of mast cells in muscle-submucosa and mucosa in the jejunums of mice. OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed.

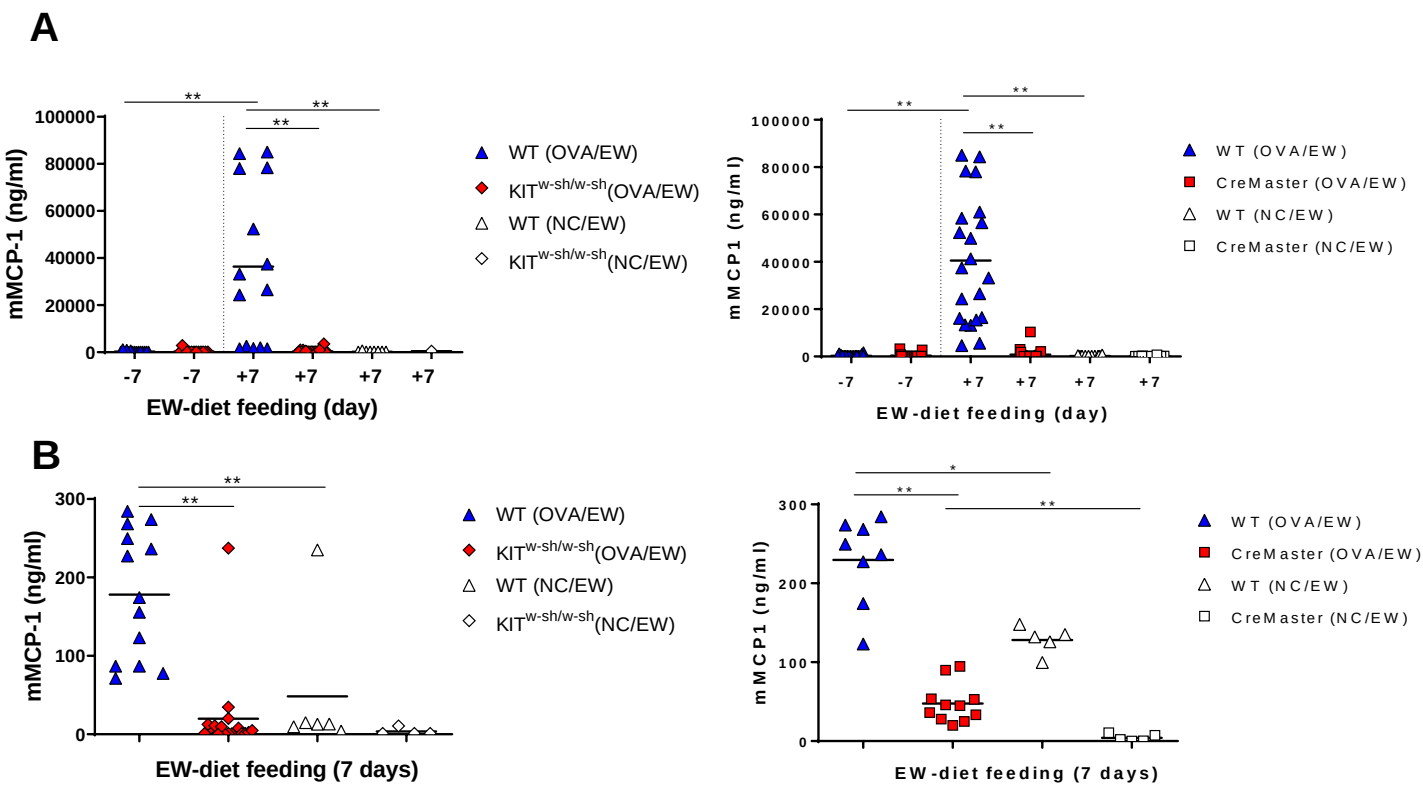

Figure 20: Reduced expression of mMCP1 in mast cell deficient mice. WT, KIT$^{w\text{-}sh/w\text{-}sh}$ and CreMaster mice were i.p. sensitized with OVA plus ALUM, or non-sensitized and fed EW-diet for 7 days. The concentrations mMCP1 in their (A) sera and (B) intestinal tissue homogenates were measured by ELISA. Each symbol represents an individual mouse. OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed. * p<0.05, ** p<0.01

5.2.2. Mast cell deficiency results in reduced development of AE

Next, the development of AE in mast cell deficient mice by histological analysis was assessed. H&E-stained small intestines, in particular jejunums showed that, OVA/EW WT mice and OVA/EW CreMaster mice developed inflammation, which is characterized by irregular villi, crypt elongation, goblet cell hyperplasia and accumulation of granulocytes in the lamina propria (Fig. 21). In contrast, OVA/EW KIT$^{w\text{-}sh/w\text{-}sh}$ mice did not develop inflammatory features in their intestines (Fig. 22). The assessment of the histology scores showed that inflammatory levels of OVA/EW WT mice were significantly higher than those of OVA/EW CreMaster mice, and KIT$^{w\text{-}sh/w\text{-}sh}$ mice. NC/EW mice did not develop intestinal inflammation in all strains. The results suggest that mast cells are dispensable for development of AE.

Table 5. Assessment of inflammation levels in WT and Mast cell deficient mice

Group	Inflammation	Villi	Edema	Total
WT (OVA/EW)	3.75**(1),**(2),**(4) ± 0.5	0.75 ± 0.5	0.75 ± 0.5	5.25**(1),**(2), **(4) ± 1.5
WT (NC/EW)	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
CreMaster (OVA/EW)	2.29**(3) ± 1.0	0.29 ± 0.5	0.71 ± 0.5	3.29**(3) ± 0.8
CreMaster (NC/EW)	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
$KIT^{w\text{-}sh/w\text{-}sh}$ (OVA/EW)	1.75**(5) ± 1.5	0.00 ± 0.0	0.75 ± 0.5	2.50**(5) ± 1.3
$KIT^{w\text{-}sh/w\text{-}sh}$ (NC/EW)	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0

WT, CreMaster and $KIT^{w\text{-}sh/w\text{-}sh}$ mice were i.p. sensitized with OVA plus ALUM, or treated only with PBS, and fed EW-diet for 7 days. The jejunums were harvested from the mice, and inflammation levels in the tissues were assessed. The total histological score represents the sum of the inflammation grade, villi and edema score. The score ranges from 0 to 6 (total score = I + V + E). OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed. * $p<0.05$, ** $p<0.01$. (1) *vs* WT (NC/EW); (2) *vs* CreMaster (OVA/EW); (3) *vs* CreMaster (NC/EW); (4) *vs* $KIT^{w\text{-}sh/w\text{-}sh}$ (OVA/EW); (5) *vs* $KIT^{w\text{-}sh/w\text{-}sh}$ (NC/EW)

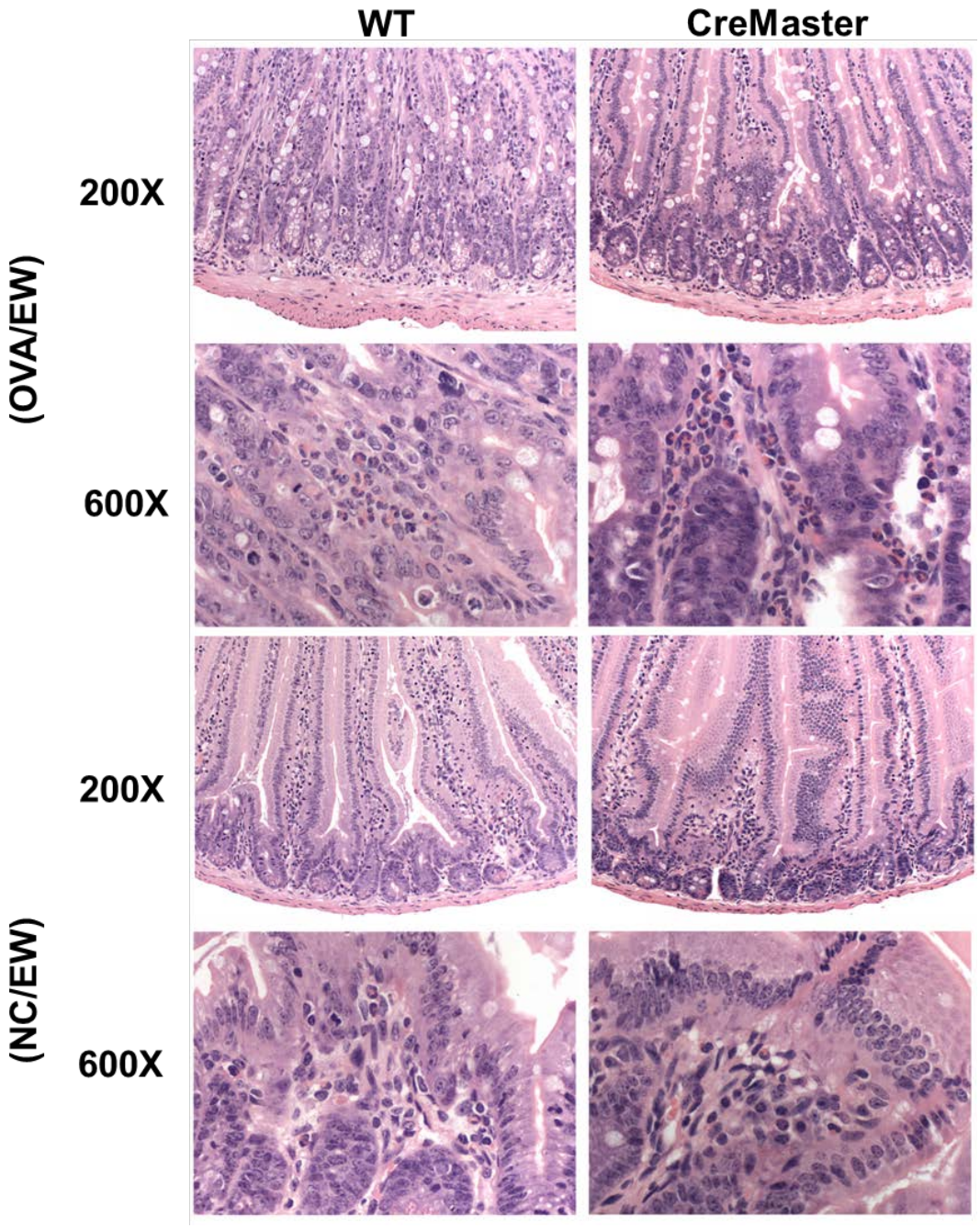

Figure 21: Development of AE in CreMaster mice. WT and CreMaster mice were i.p. sensitized with OVA plus ALUM, or treated only with PBS, and fed EW-diet for 7 days. The jejunums were harvested from the mice, and stained with H&E. All images were taken in the same magnification. OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed. The data are representative for three independent experiments.

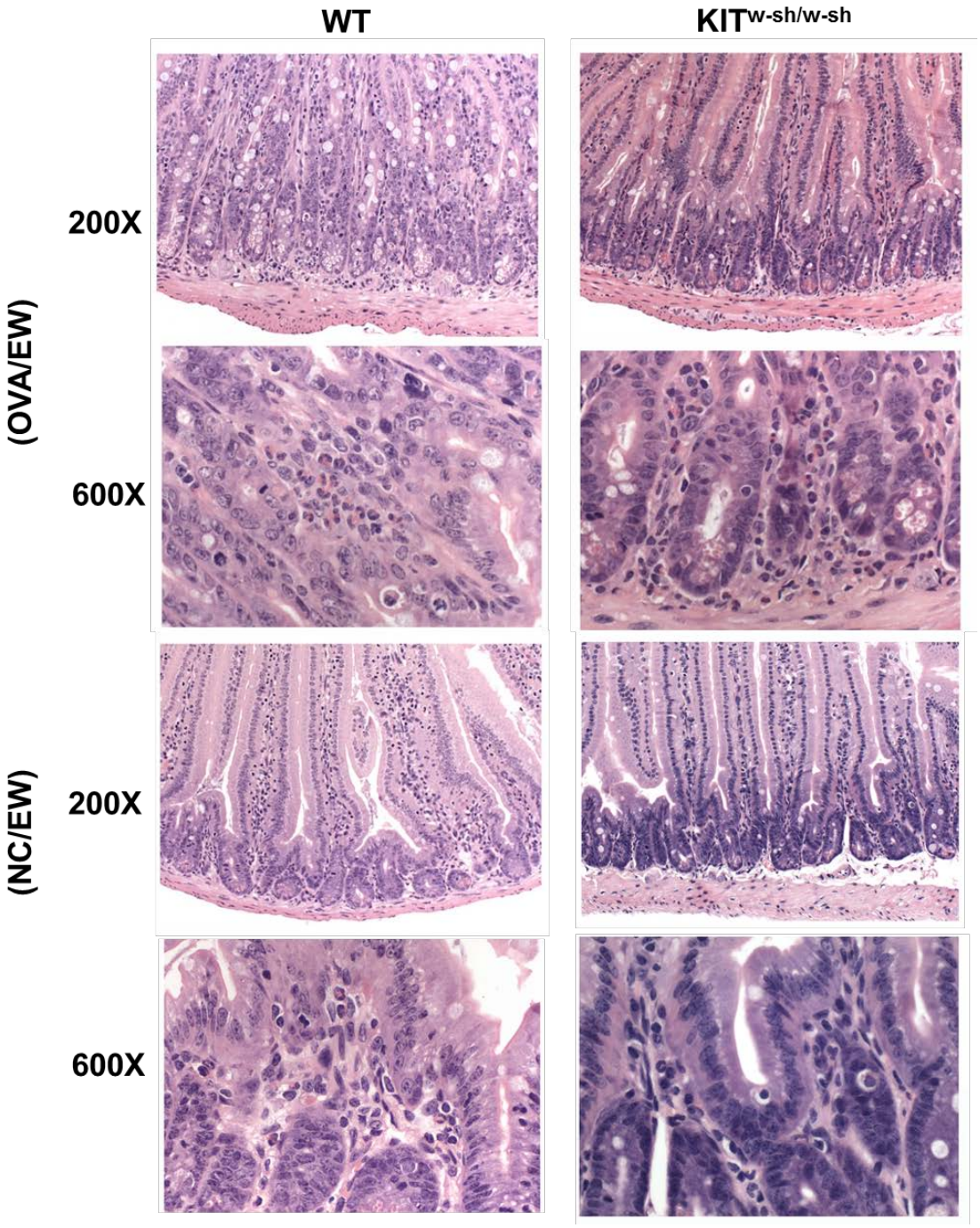

Figure 22: Abolished development of AE in KIT$^{w\text{-}sh/w\text{-}sh}$ mice. WT and KIT$^{w\text{-}sh/w\text{-}sh}$ mice were i.p. sensitized with OVA plus ALUM, or treated only with PBS, and fed EW-diet for 7 days. The jejunums were harvested from the mice, and stained with H&E. All images were taken in the same magnification. OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed. The data are representative for three independent experiments.

5.2.3. Mast cell deficiency reduced accumulation of eosinophils in AE tissues.

To assess whether the mast cell deficiency influences the types of accumulating cells in AE tissues of OVA/EW CreMaster mice and KIT$^{w\text{-}sh/w\text{-}sh}$ mice, FACS analysis of intestinal lamina propria cells was performed. The frequency of granulocytes (CD45^{+})

in intestinal lamina propria was comparable between all mouse strains. In addition, a decreased frequency of eosinophils (SiglecF$^+$ CD11b$^-$ cells) and a similar frequency of neutrophils (Ly6G$^+$ CD11b$^+$ SiglecF$^-$ cells) in the lamina propria leukocytes (CD45$^+$ cells) were observed in OVA/EW CreMaster mice, when compared to those in OVA/EW WT mice (Fig. 23). In case of OVA/EW KIT$^{w\text{-}sh/w\text{-}sh}$ mice, the frequency of both eosinophils and neutrophils were significantly reduced (Fig. 24). The result suggests mast cell play a crucial role in recruitment of eosinophils, but not of neutrophils.

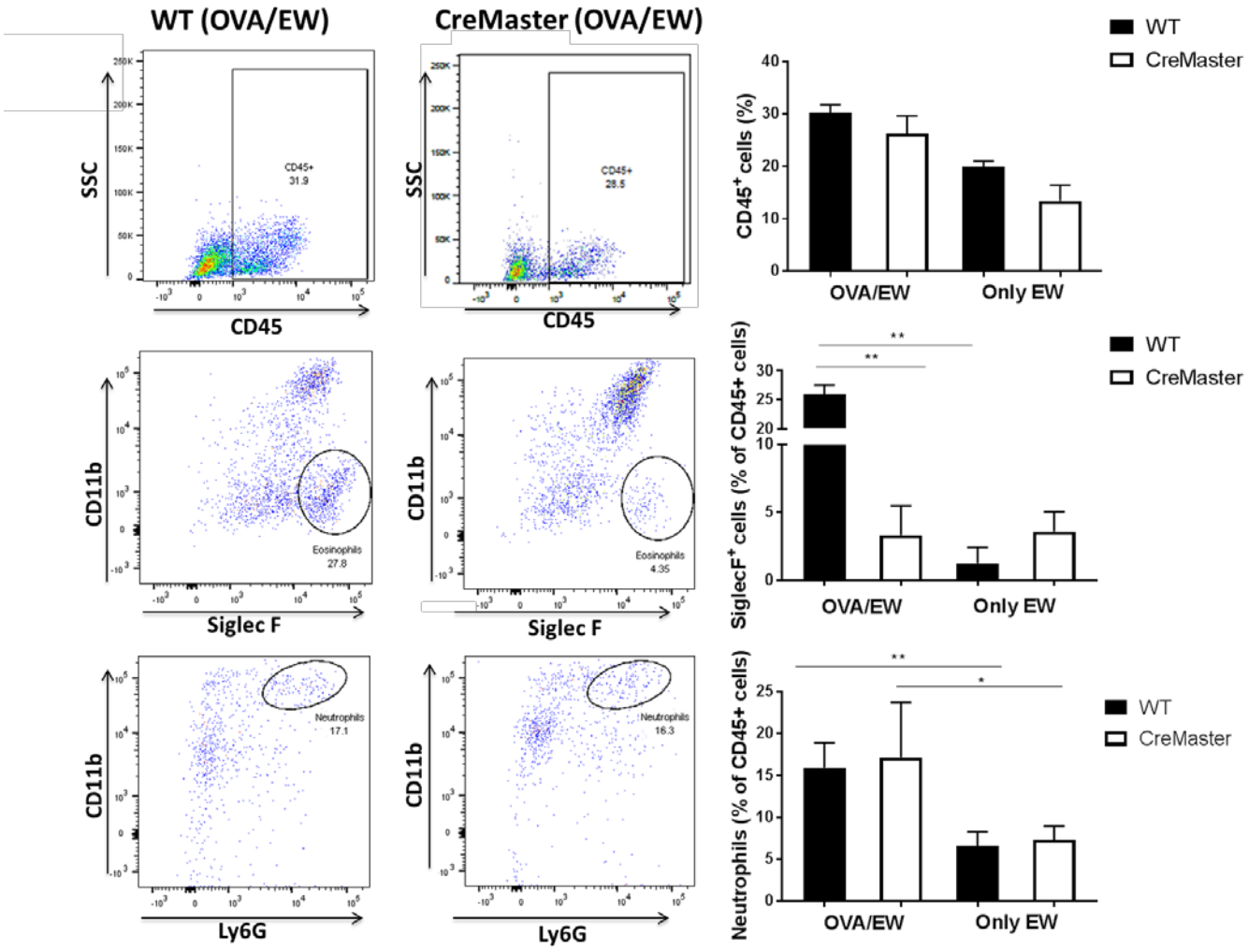

Figure 23: Reduced frequency of eosinophils and similar frequency of neutrophils in the inflamed intestinal tissues of CreMaster mice. WT and CreMaster mice (n=4/group) were sensitized with OVA plus ALUM, and fed EW-diet for 7 days. The jejunums were harvested from the mice, and subjected to enzymatic treatment for preparation of lamina propria cells. The frequency of CD45$^+$ cells in lamina propria cells, and the frequency of eosinophils (SiglecF$^+$ CD11b$^-$ cells) and neutrophils (Ly6G$^+$ CD11b$^+$ SiglecF$^-$ cells) in CD45$^+$ cells population were determined by FACS. The data are representative for three independent experiments. * p<0.05, ** p<0.01

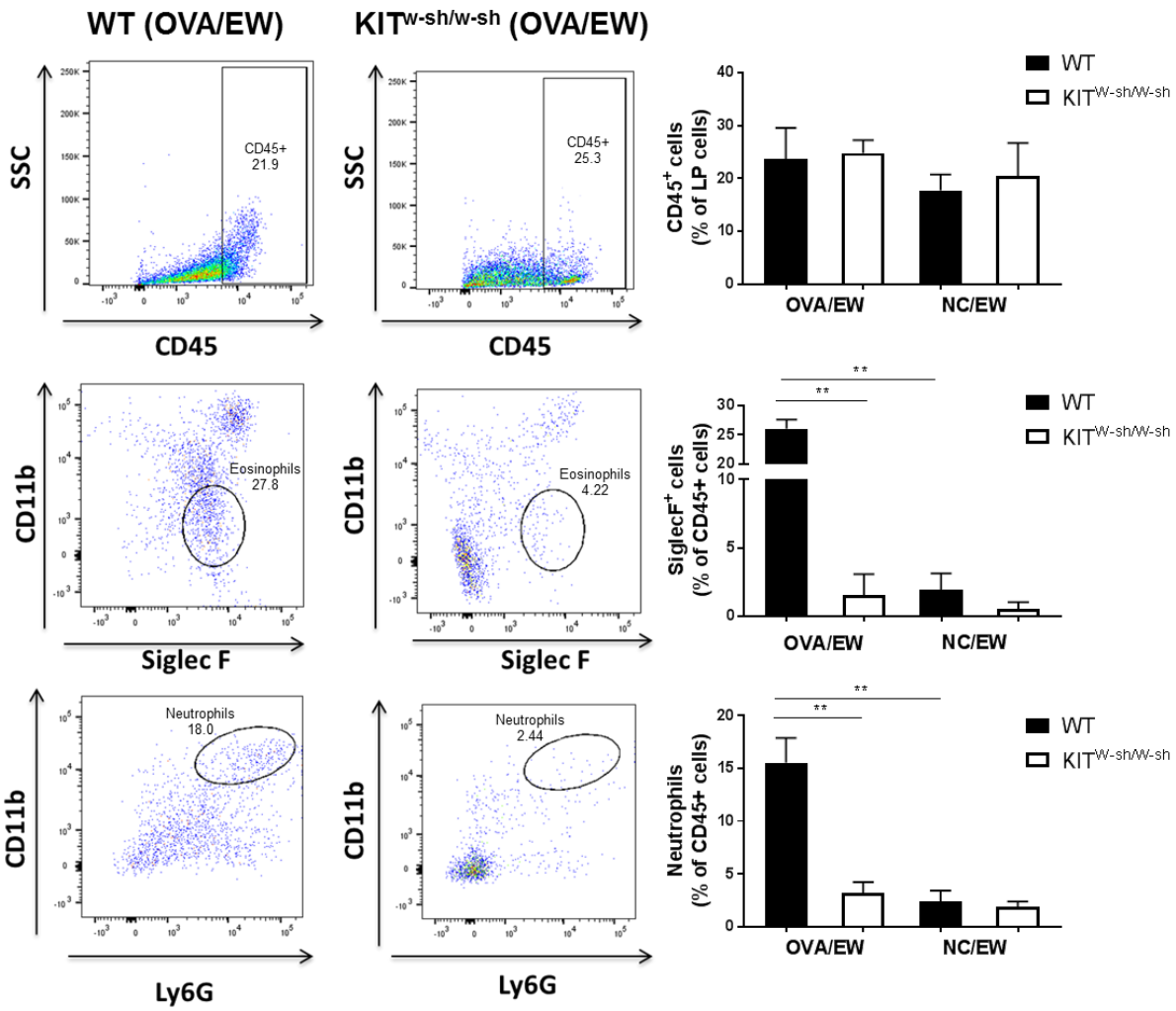

Figure 24: Reduced frequency of eosinophils and neutrophils in the intestinal tissues of KIT$^{w\text{-}sh/w\text{-}sh}$ mice. WT and KIT$^{w\text{-}sh/w\text{-}sh}$ mice (n=4/group) were sensitized with OVA plus ALUM, and fed EW-diet for 7 days. The jejunums were harvested from the mice, and subjected to enzymatic treatment for preparation of lamina propria cells. The frequency of CD45$^+$ cells in lamina propria cells, and the frequency of eosinophils (SiglecF$^+$ CD11b$^-$ cells) and neutrophils (Ly6G$^+$ CD11b$^+$ SiglecF$^-$ cells) in CD45$^+$ cells population were determined by FACS. The data are representative for three independent experiments. * p<0.05, ** p<0.01

5.2.4. Mast cell deficiency affected the frequency of CD4$^+$T cell frequency but not the antibody development.

Next, whether mast cell deficiency has an influence on T cell responses was assessed. The frequencies of CD4$^+$ T-cells in MLNs were enhanced in OVA/EW Cremaster and OVA/EW KIT$^{w\text{-}sh/w\text{-}sh}$ mice in comparison with OVA/EW WT, whereas those in spleens were comparable among the groups (Fig 25). In contrast, the frequency of Treg cells (CD4$^+$ CD25$^+$ Fox p 3$^+$ cells) in spleens was reduced in OVA/EW Cremaster and OVA/EW KIT$^{w\text{-}sh/w\text{-}sh}$ mice, in comparison with OVA/EW WT, whereas frequencies in spleens were comparable among the groups.

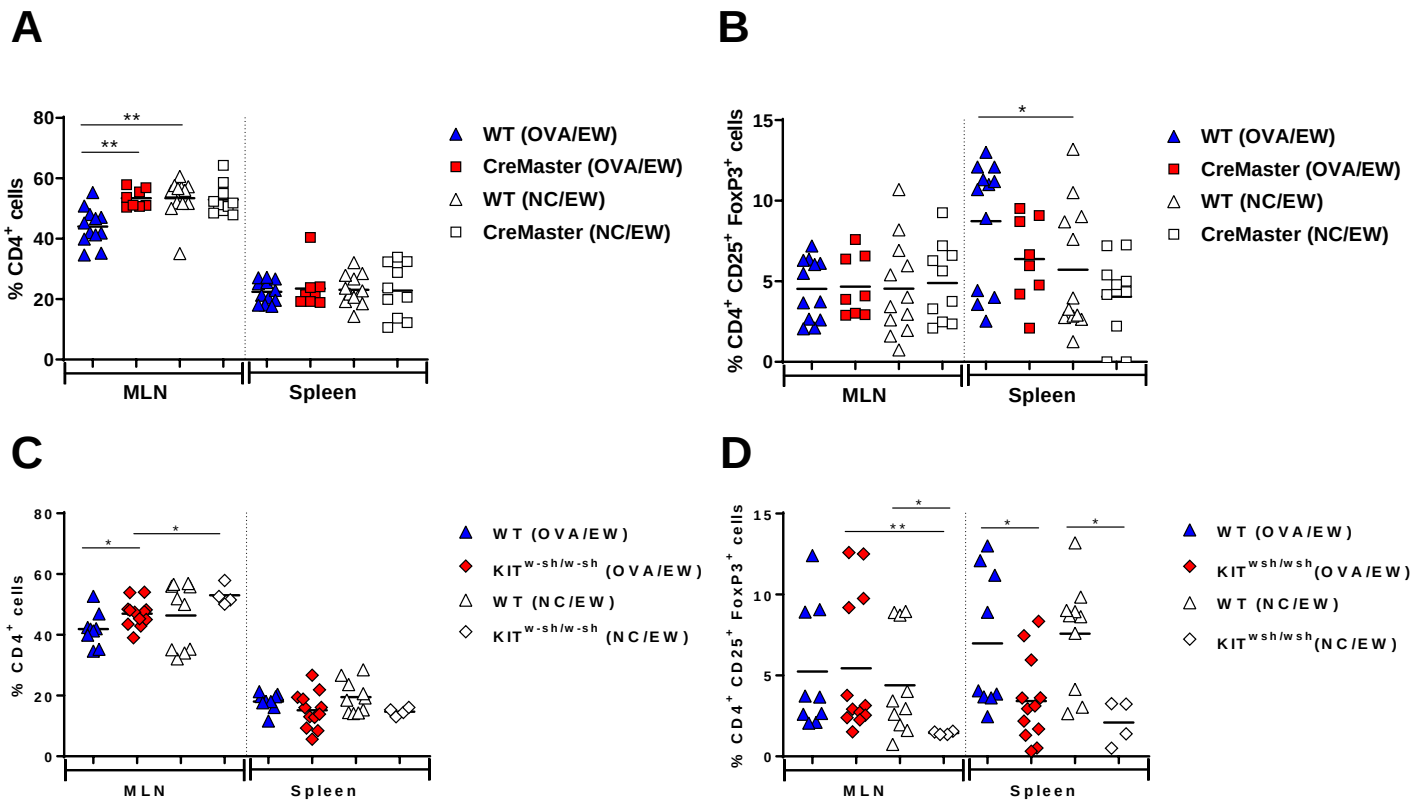

Figure 25: Altered T-cell frequency in in mast cell deficient mice. WT, CreMaster and KITw-sh/w-sh Mice (n=4-5/group) were sensitized with OVA plus ALUM and fed EW-diet for 7 days, and mesentric lymph nodes (MLNs) and spleens were harvested from them. The frequency of $CD4^+$ T-cells in the tissues of (A) CreMaster and (B) $KIT^{w\text{-}sh/w\text{-}sh}$, as well as the frequency of Treg ($CD4^+$ $CD25^+$ Fox $p3^+$) cells in the tissues of (C) CreMaster and (D) $KIT^{w\text{-}sh/w\text{-}sh}$ in were determined by FACS. The data are pooled of three independent experiments using n=2-4/group. * p<0.05, ** p<0.01

One of the most potent chemoattractants for eosinophils is IL-5. Th2 cells are a main IL-5 producing cell type. To see if mast cell deficiency affects the development of Th2 cells, T-cell and antibody responses in the mice were assessed. MLN-derived T-cells from OVA/EW CreMaster mice and OVA/EW $KIT^{w\text{-}sh/w\text{-}sh}$ mice produced IL-4 and IL-5 at similar levels, when compared to those from OVA/EW WT mice (Fig. 26). In addition, there were no statistical differences in serum levels of OVA-specific IgE, IgG1, and IgG2a antibodies between OVA/EW WT mice and OVA/EW CreMaster mice (Fig. 27). Serum levels of OVA-specific IgE and IgG1 antibodies in OVA/EW $KIT^{w\text{-}sh/w\text{-}sh}$ mice tended to be lower than OVA/EW WT mice, although serum levels of OVA-specific IgG2a antibodies were not different between these mice (Fig. 28). IgE and IgG1 are antibodies induced by the Th2 cytokine IL-4, whereas IgG2a is

antibody induced by the Th1 cytokine IFN-γ. These results suggest that (i) WT mice and CreMaster mice developed systemic Th2 cells at similar levels upon OVA sensitization and EW diet feeding, and (ii) the development of systemic Th2 immune responses was partly defective in OVA/EW $KIT^{w\text{-}sh/w\text{-}sh}$ mice, but it does not significantly affect IL-4 and IL-5 production in $CD4^+$ T-cells.

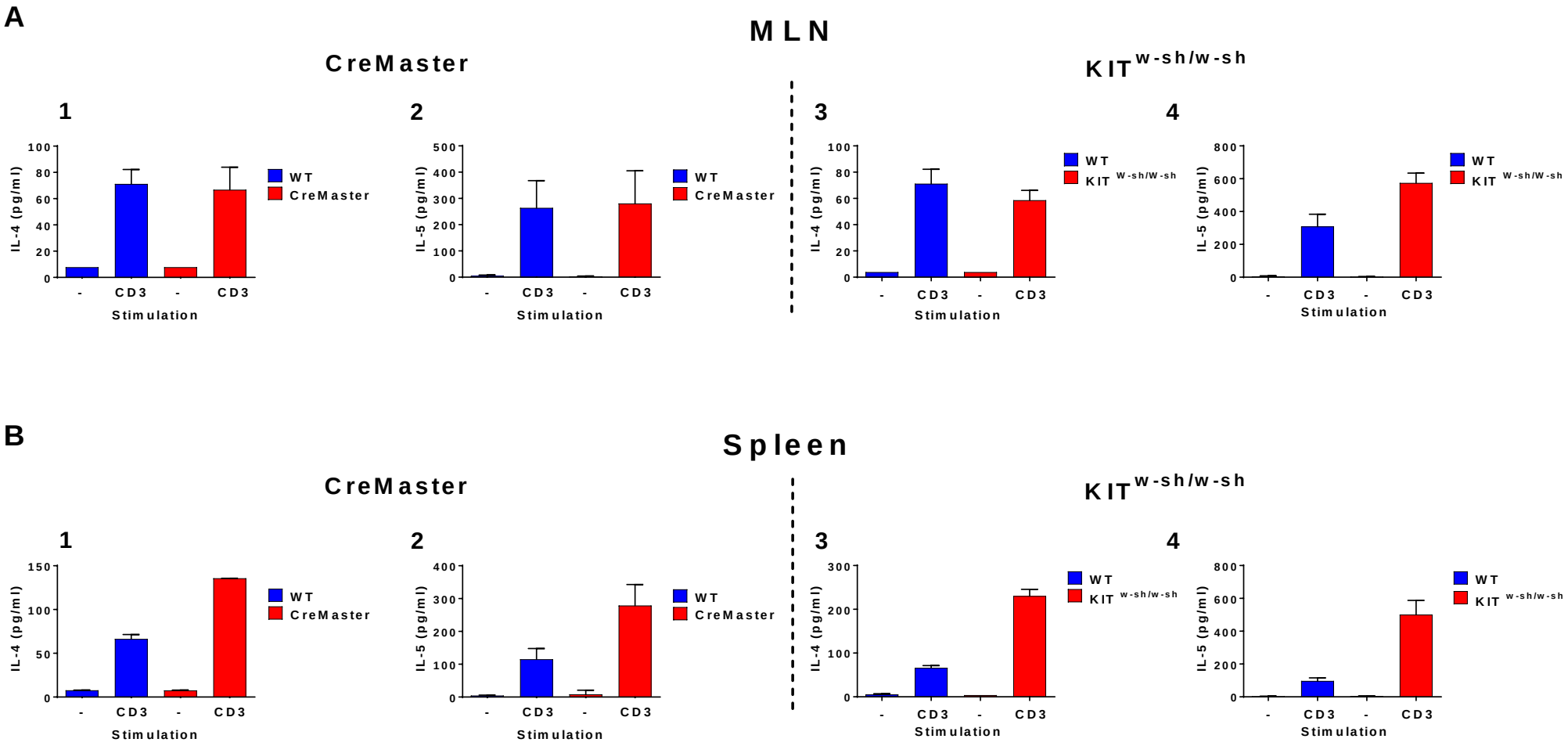

Figure 26: Th2 cytokine production by T-cells in mast cell deficient mice. WT, CreMaster and Kit$^{W\text{-}sh/Ws\text{-}sh}$ mice (n=4-5/group) were sensitized with OVA plus ALUM and fed EW-diet for 7 days. (A) Mesenteric lymph node (MLN) cells and (B) splenocytes derived from CreMaster mice (1-2) and KIT$^{w\text{-}sh/w\text{-}sh}$ mice (3-4) were cultured in wells coated with anti-CD3 mAb. Concentrations of IL-4 and IL5 in the cell culture supernatants 72 hrs. after stimulation were measured by ELISA.

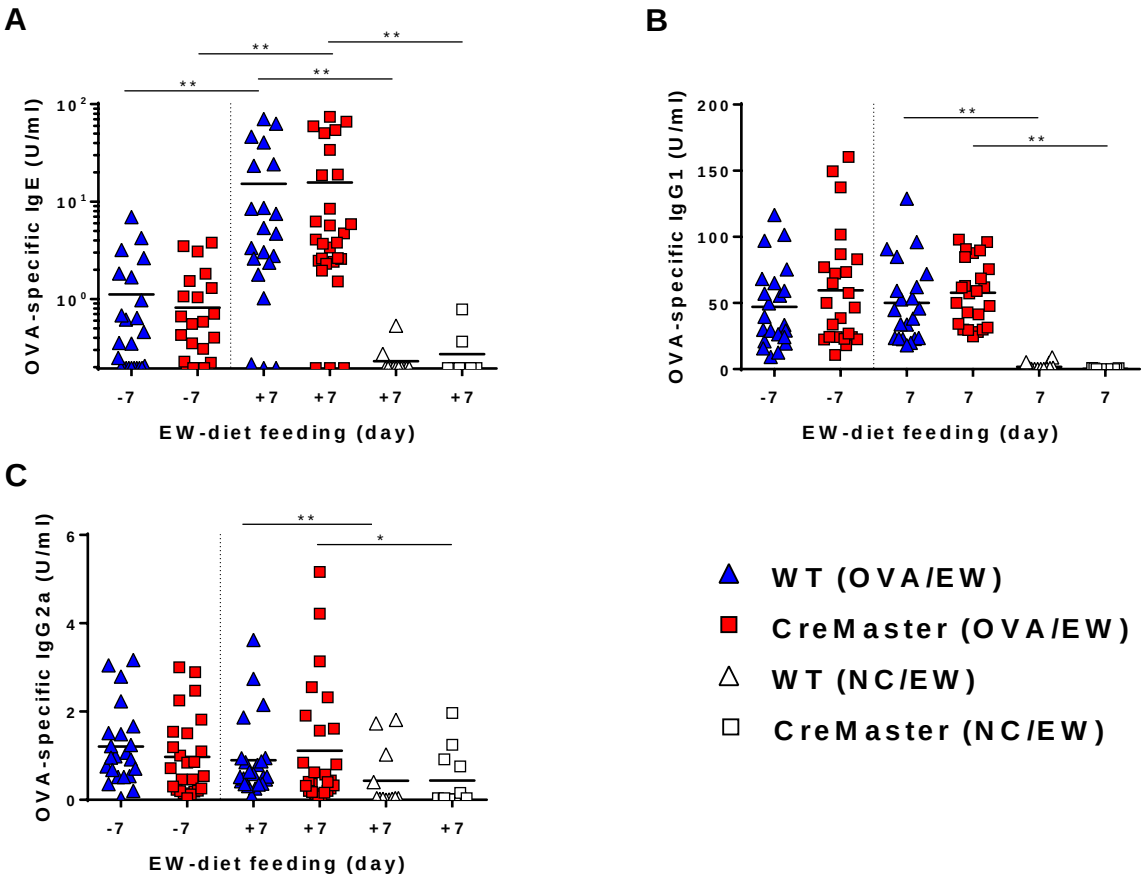

Figure 27: No variation in the levels of OVA-specific antibody production in WT and CreMaster mice. WT and CreMaster mice (n=4-5/group) were i.p. sensitized with OVA plus ALUM, and fed EW-diet for 7 days. The serum levels of (A) IgE, (B) IgG1 and (C) IgG2a Abs specific for OVA on days -7 and 7 of EW-diet were measured by ELISA. Each symbol represents an individual mouse. The data are pooled of three independent experiments. * $p<0.05$, ** $p<0.01$

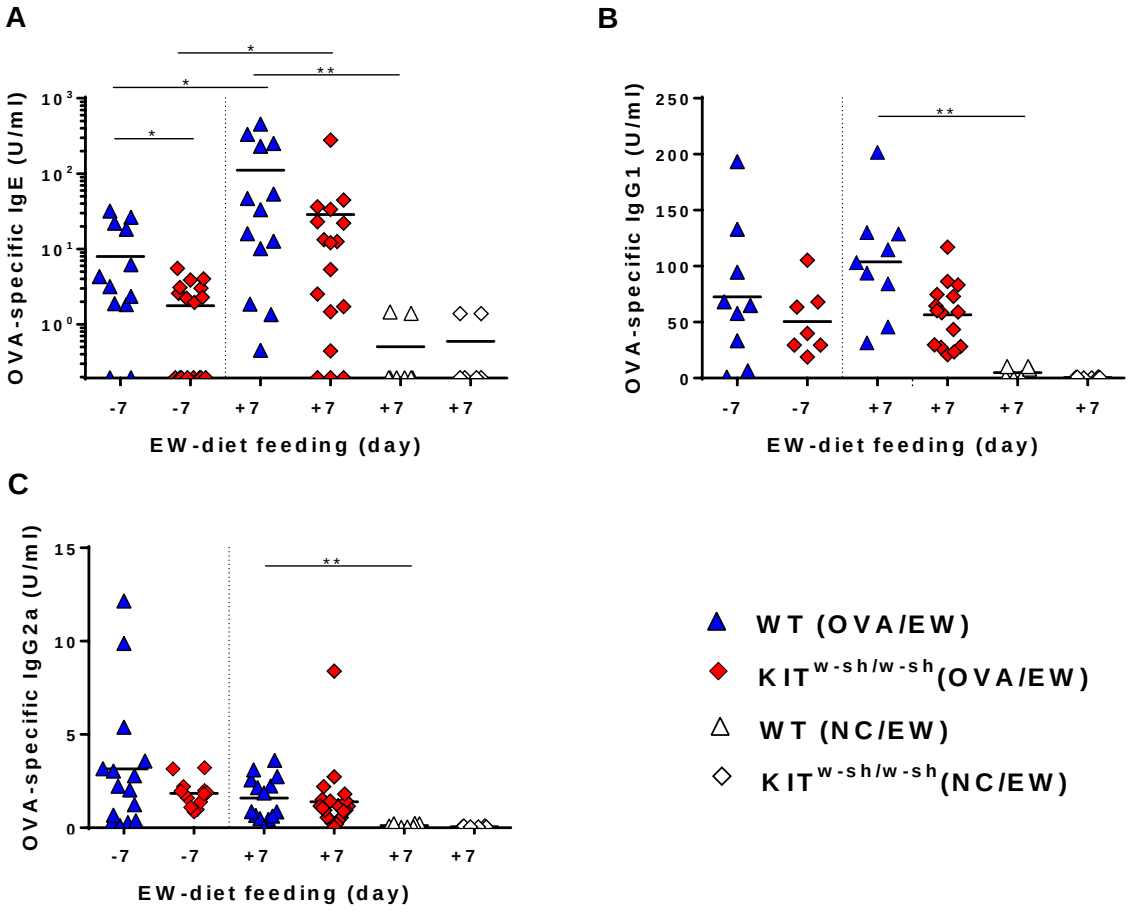

Figure 28: No variation in the levels of OVA-specific antibody production in WT and KIT$^{w\text{-}sh/w\text{-}sh}$ mice. WT and KIT$^{w\text{-}sh/w\text{-}sh}$ mice (n=4-5/group) were i.p. sensitized with OVA plus ALUM, and fed EW-diet for 7 days. The serum levels of (A) IgE, (B) IgG1 and (C) IgG2a Abs specific for OVA on days -7 and 7 of EW-diet were measured by ELISA. Each symbol represents an individual mouse. The data are pooled of three independent experiments. * p<0.05, ** p<0.01

5.2.5. Mast cell deficiency partly reduced CCL11 concentration in AE tissues

FACS analysis showed reduction in the frequency of eosinophils in the AE tissues of OVA/EW mast cell deficient mice (Fig X). To get insight into mechanism for the reduced frequency of eosinophils in mast cell deficient mice, we assessed the protein concentrations of several eosinophil chemoattractants, e.g. CCL11 and IL-5, and other Th2 and inflammatory cytokines (Fig 29 and Fig 30).

CCL11 and IL-5 concentrations in the homogenates of OVA/EW Cremaster mice tended to be lower (Fig 29A) when compared to WT mice. In addition, CCL11 concentrations were significantly reduced in the homogenates of OVA/EW KIT$^{w\text{-}sh/w\text{-}sh}$ mice (Fig 30A). These results suggest that the mast cell deficiency influences, at

least partly the expression of CCL11 in the jejunum of AE mice. In addition, when compared to those in WT mice, (i) the concentrations of Th2 type cytokines (IL-4 and IL-13) were similar (Fig. 29 B, E), and (ii) the concentrations of IFN-g, IL-6 and IL-10 tended to be higher in OVA/EW CreMaster mice (Fig. 29 D, F-G). However, IL-13 concentrations were rather higher, whereas IL-10 concentrations were lower in OVA/EW KIT$^{w\text{-}sh/w\text{-}sh}$ mice (Fig. 30E-F), although the concentrations of other cytokines were similar.

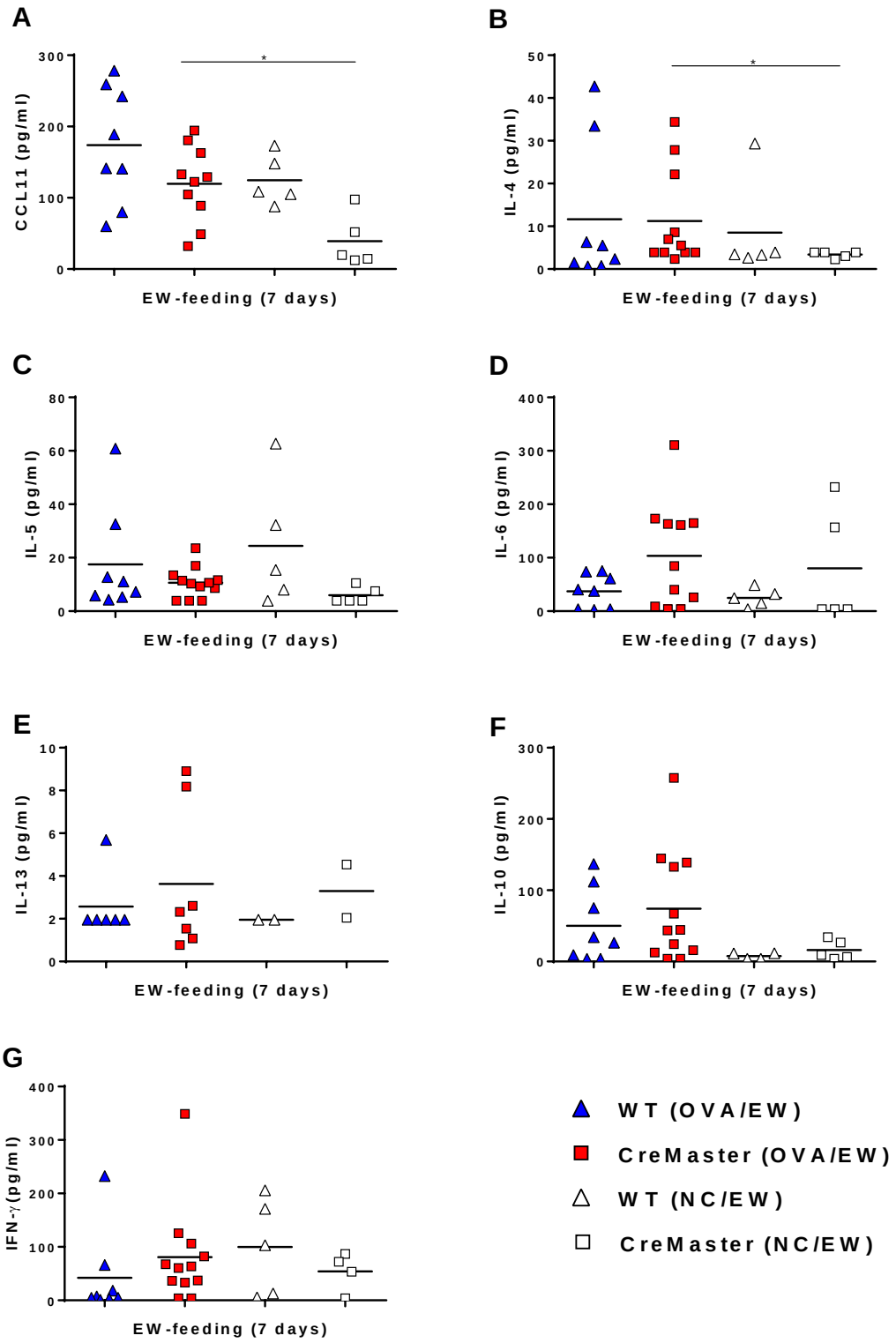

Figure 29: CCL11 expression tends to reduce in the inflamed intestinal tissues of CreMaster mice. WT and CreMaster mice (n=4-5/group) were i.p. sensitized with OVA plus ALUM, or non-sensitized and fed EW-diet for 7 days. Small intestines were harvested from the mice. The concentrations of (A) CCL11, (B) IL-4, (C) IL-5, (D) IL-6, (E) IL-13, (F) IL-10 and (G) IFN-γ in the intestinal tissue homogenates were measured by ELISA. Each symbol represents an individual mouse. The data are pooled of three independent experiments. * $p<0.05$.

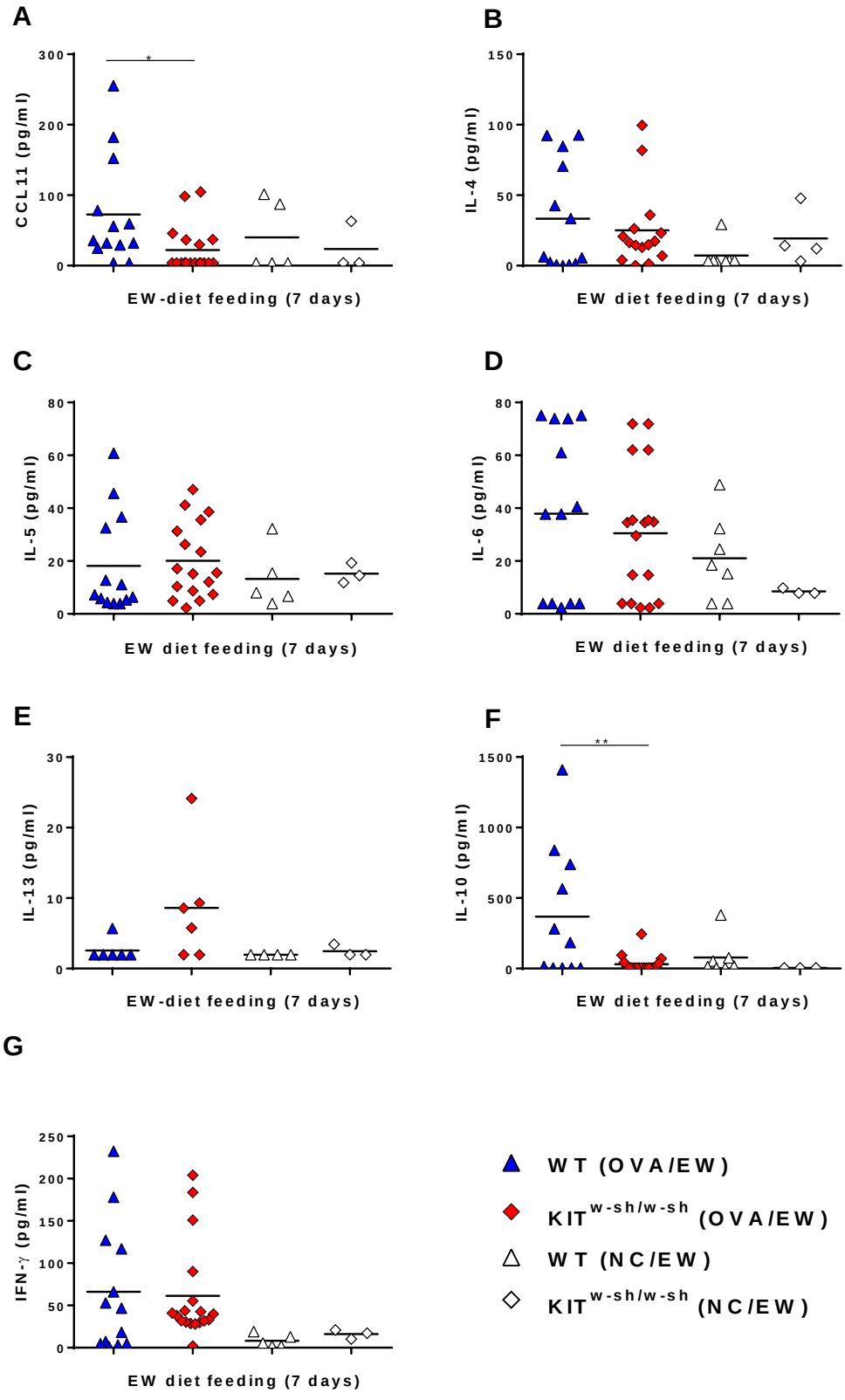

Figure 30: Reduced CCL11 expression but similar cytokine production in the inflamed intestinal tissues of KIT$^{w\text{-}sh/w\text{-}sh}$ mice. WT and KIT$^{w\text{-}sh/w\text{-}sh}$ mice (n=4-5/group) were i.p. sensitized with OVA plus ALUM, or non-sensitized, and fed EW-diet for 7 days. Small intestines were harvested from the mice. The concentrations of (A) CCL11, (B) IL-4, (C) IL-5, (D) IL-6, (E) IL-13, (F) IL-10 and （G）IFN-γ in the intestinal tissue homogenates were

measured by ELISA. Each symbol represents an individual mouse. The data are pooled of three independent experiments. * p<0.05.

5.2.6. Mast cells are the main producer of CCL1 in AE tissues

A previous study showed that mast cells are the main producer of CCL1 (chemokine that specifically recognize CCR8) in human and murine lungs (Gonzalo et al., 2007). To evaluate whether this is the case in AE, the concentrations of CCL1 and CCL8 in the intestinal homogenates of mast cell deficient mice were determined by ELISA. The concentrations of CCL1 in OVA/EW mast cell deficient mice were significantly lower than those in OVA/EW WT mice (Fig. 31). There was nearly no difference in the concentrations of CCL8 between OVA/EW WT mice and mast cell deficient mouse strains. The results suggest that mast cells are a main producer of CCL1, but not CCL8 in the inflamed tissues of AE mice.

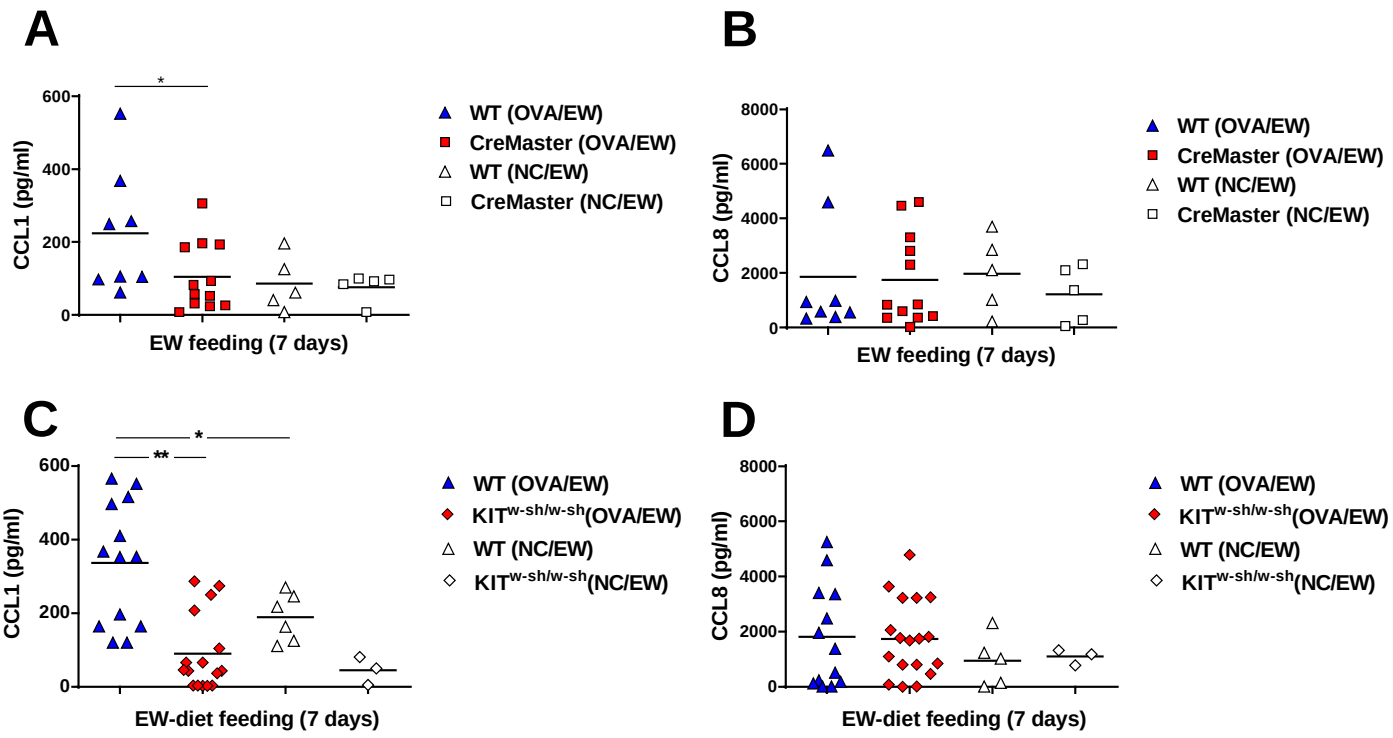

Figure 31: Reduced CCL1 but similar CCL8 protein expression in the jejunum of mast cell deficient mice. WT, CreMaster and KIT$^{w\text{-}sh/w\text{-}sh}$ mice (n=4-5/group) were sensitized with OVA plus ALUM and fed EW-diet for 7 days. the concentration of (A and C) CCL1 and (B and D) CCL8 in homogenates of their intestinal tissues were measured by ELISA * p<0.05., **p<0.01.

6. Chapter 3: The role of IgE in the exacerbation of AE

6.1. A short introduction

The role of IgE in allergic reactions has been extensively studied and it is very well known. As commented before, this roll is mainly as consequence of the IgE production by B cells that is then bond to the FcεRI expressed on the cell surface of mast cells and basophils (Burks et al., 2012; Ho et al., 2014; Laffleur et al., 2017). After exposure with the eliciting allergen, the allergen-FcεRI engagement activates mast cells and basophils. Mast cells and basophils release mediators contained in their granules or newly synthetized mediators that generate physiological reactions like vasodilatation, mucus secretion, smoot muscle contraction, increased vascular permeability, and influx of other inflammatory cells (Burks et al., 2012; Renz et al., 2018; Morita et al., 2013).

While these statements are true in IgE-mediated type I allergy, the role of IgE in the exacerbation of AE is not well understood. Despite AE is described as T-cell mediated allergy, patients with AE often develop IgE during the course of the pathology (Burks et al., 2012; Kim and Burks, 2015; Ruffner et al., 2013; Caubet et al., 2014; Katz et al., 2011; Hwang et al., 2009). It has been reported that AE patients with high levels of specific IgE to the offending food presented a prolonged allergy and even developed a IgE-mediated systemic reaction over the time (Nowak-Wegrzyn et al., 2003; Sicherer, 2005; Caubet et al., 2014). Based on the background the role of IgE in the development of AE was investigated. To this end, IgEki mice were used. The homozygous IgEki mice are able to express 10-fold higher total IgE and a complete absence of IgG1 in comparison with WT, as a consequence of the replacement of exons encoding for the soluble part of the constant region of the murine IgG1 with the IgE counterpart (Lubben et al., 2013).

6.2. Results

6.2.1. OVA-specific antibody responses by IgEki mice

To assess whether allergen-specific IgE production is enhanced in IgEki mice, the levels of OVA specific IgE, IgG1 and IgG2a antibodies in IgEki mice were determined by ELISA. As expected, IgEki mice showed highly enhanced IgE levels after the sensitization and feeding of EW-diet (Fig 32A). In IgEki mice was not observed

production of OVA specific IgG1 as consequence of the genetic modification (Fig 32B). Interestingly, an enhanced production of OVA specific IgG2a antibodies after the sensitization with OVA (-7 days) that was maintained after the EW-diet (+7 days) was observed in IgEki mice (Fig 32C).

The levels of mMCP1 in the mice were also measured. The concentrations of mMCP1 in IgEki mice tended to be higher than those in WT mice, although it was not statistically significant (Fig 32D).

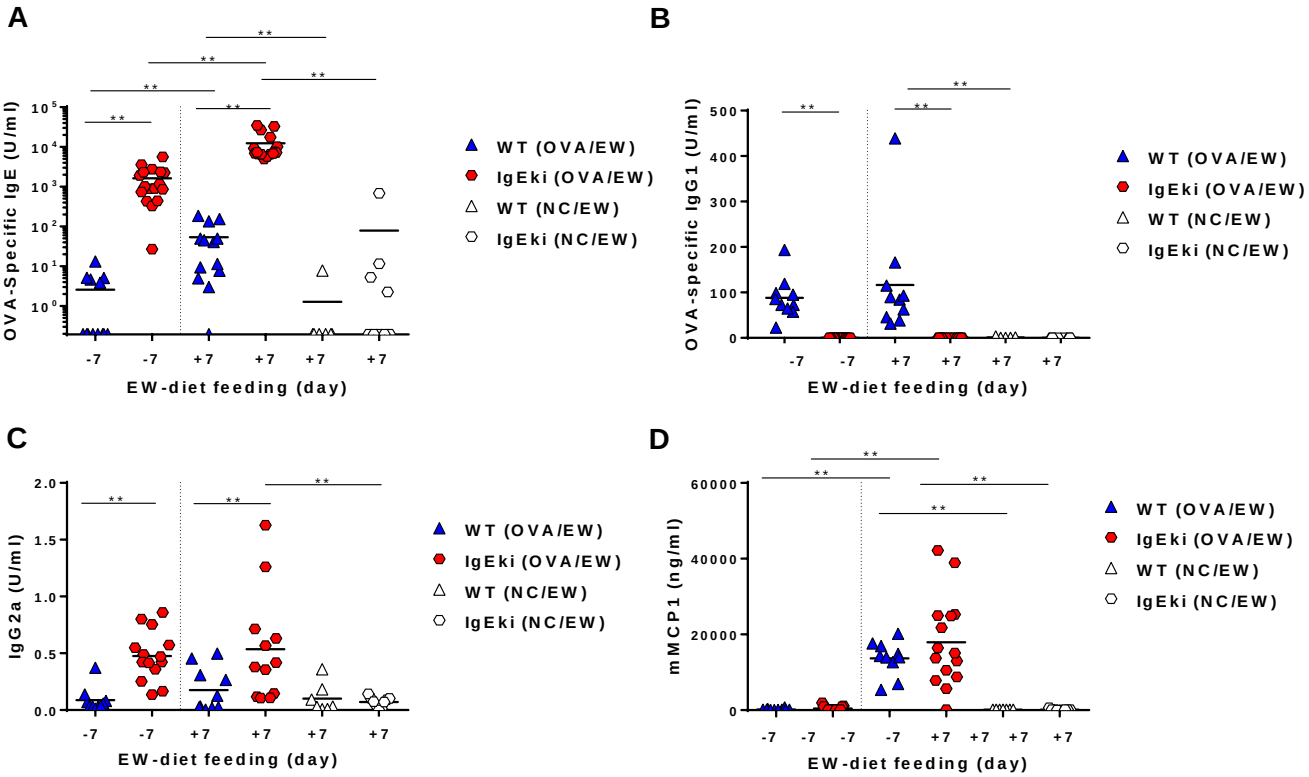

Figure 32: IgEki mice enhanced levels of OVA-specific IgE and IgG2a antibodies but did not produce OVA-specific IgG1 antibodies. WT and IgEki mice ((n=4-5)) were i.p. sensitized with OVA plus ALUM, and fed EW-diet for 7 days. The serum levels of (A) IgE, (B) IgG1 and (C) IgG2a Abs specific for OVA and (D) mMCP1 on days -7 and 7 of EW-diet were measured by ELISA. Each symbol represents an individual mouse. The data are pooled of three independent experiments. * p<0.05, ** p<0.01.

6.2.2. Enhanced IgE expression promoted the development of clinical symptoms

WT and IgEki mice were sensitized with OVA and Alum, and challenged with an EW-diet as described. OVA/EW IgEki mice developed significantly stronger clinical symptoms, i.e. reduction in body weight (Fig. 33) and drop of body temperature (Fig.

34) during 7 days of continues feeding with EW-diet, when compared to OVA/EW WT mice and NC/EW controls.

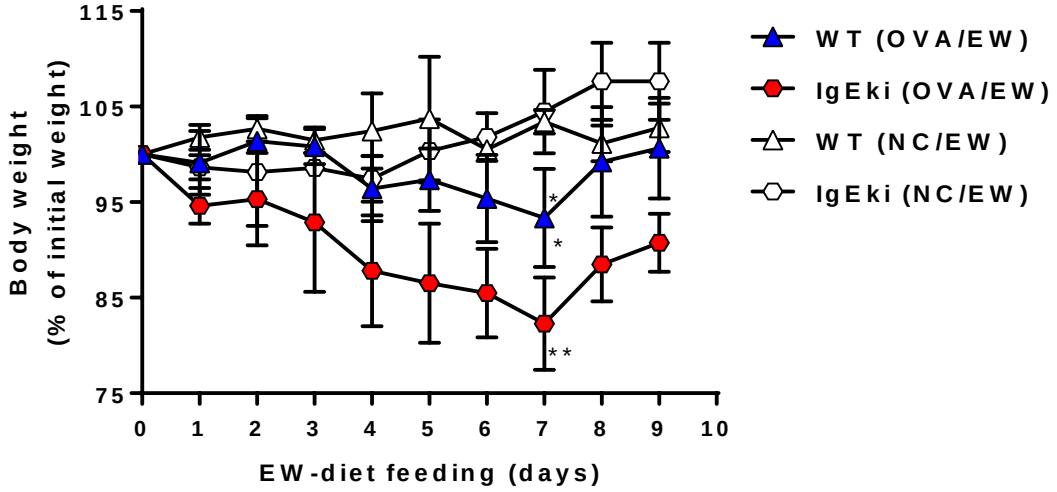

Figure 33: High reduction in body weight of IgEki mice. WT and IgEki mice (n=4-5/group) were i.p. sensitized with OVA plus ALUM, or treated only with PBS, and fed EW-diet for up to 9 days. The body weight was measured daily and presented as percentage of the initial weight. OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed. * p<0.05, ** p<0.01

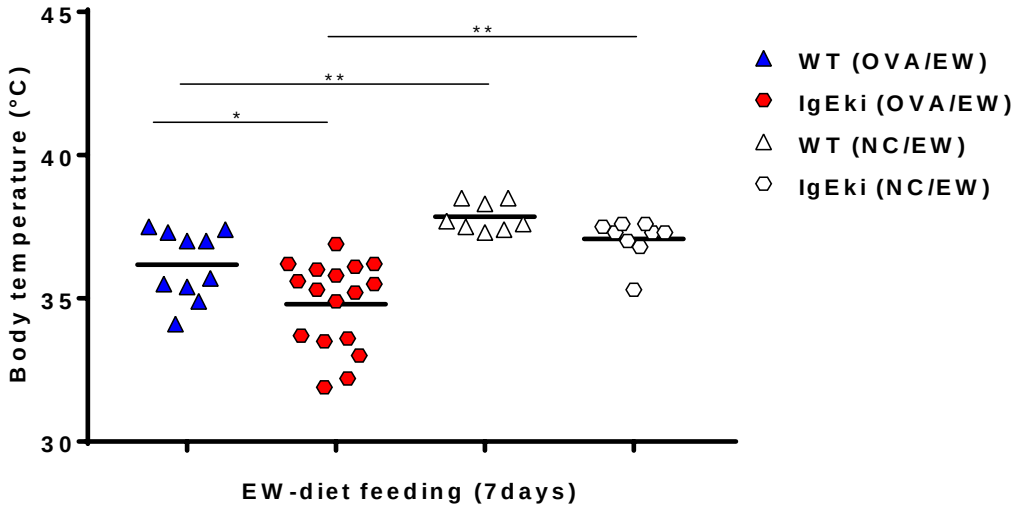

Figure 34: Enhanced body temperature drop in IgEki mice on 7 days of EW-diet. WT and IgEki mice (n=4-5/group) were i.p. sensitized with OVA plus ALUM, or treated only with PBS. Body weight was measured using a microprobe thermometer. OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed. * p<0.05, ** p<0.01

To assess the influence of IgE on the number of mast cells in intestinal tissue, jejunum tissues from the mice were stained with toluidine blue (Fig. 35A). There was no difference in the mast cell number between WT OVA/EW mice and IgEki OVA/EW

mice. In addition, concentrations of mMCP-1 (mast cell activation marker) in the intestinal tissue homogenates were comparable between the mouse strains (Fig. 35B).

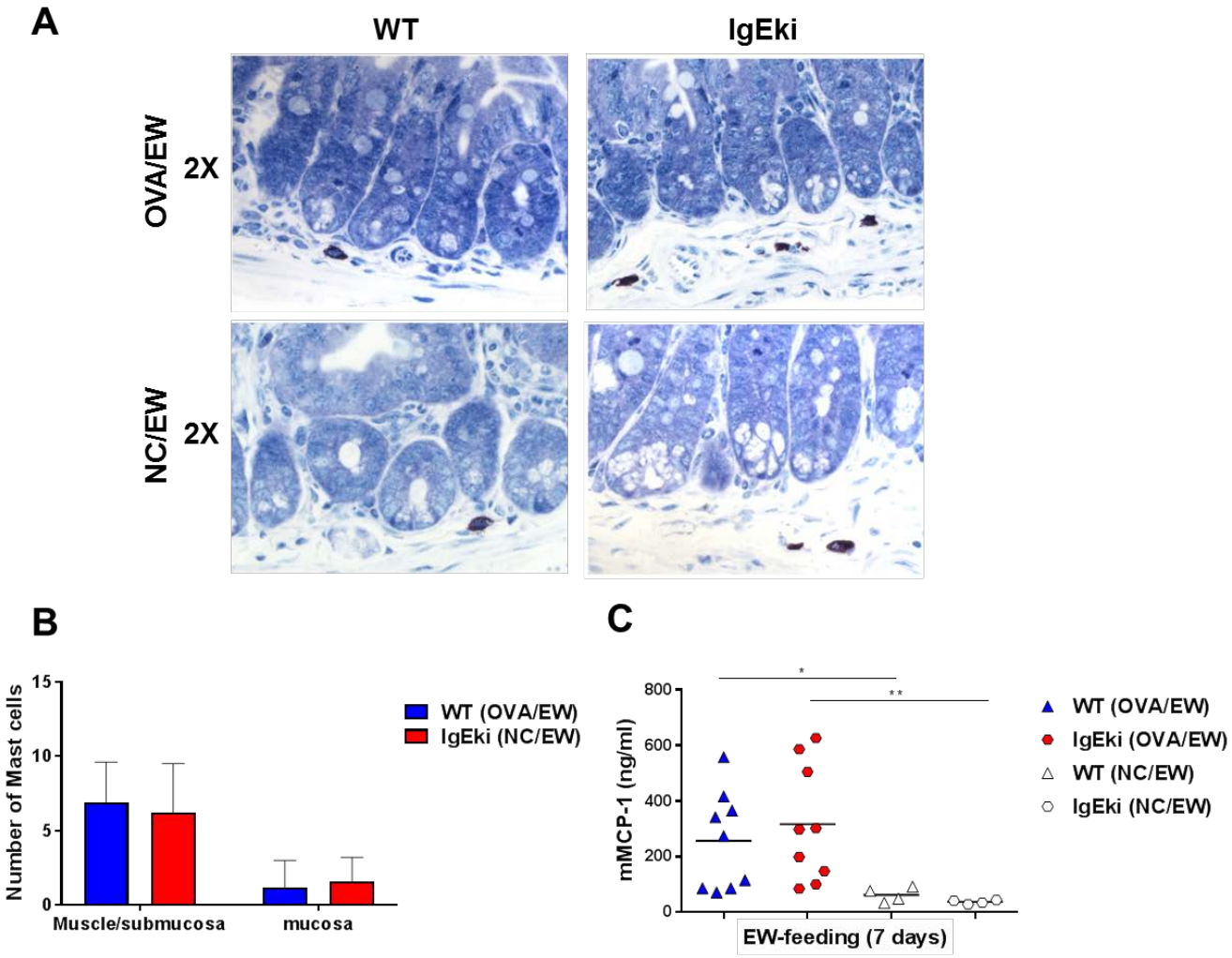

Figure 35: No difference in the number of mast cells in the inflamed intestinal tissues of IgEki and WT mice. WT and IgEki mice were i.p. sensitized with OVA plus ALUM, and fed EW diet for 7 days. The jejunums were harvested from the mice, fixed in 4% paraformaldehyde, and embedded in paraffin. The tissues were cut and stained with toluidine blue. The numbers of stained mast cells in (A) jejunum and (B) in muscle-submucosa and mucosa were counted under the microscope. (C) The concentrations of mMCP-1 in the intestinal homogenates of the mice.

6.2.3. Enhanced IgE expression promoted the development of AE

Next, histological analysis to assess development of AE in IgE Ki mice was performed. OVA/EW IgEki mice developed inflammation in the intestine as a consequence of the treatment (Fig. 36 and table 6). The animals showed dilated intestines and atrophy of the villi with edema in the lamina propria more visibly, when

compared to OVA/EW WT mice. In addition, OVA/EW IgEki mice showed mildly elongated crypts. Their mucosa was lined by abundant hypertrophic goblet cells and Paneth cells were observed in the crypts. In addition, large numbers of neutrophils and eosinophils were infiltrated in the lamina propria of OVA/EW IgEki mice. It's important to notice that a small degree of inflammation was observed in the NC/EW IgEki mice.

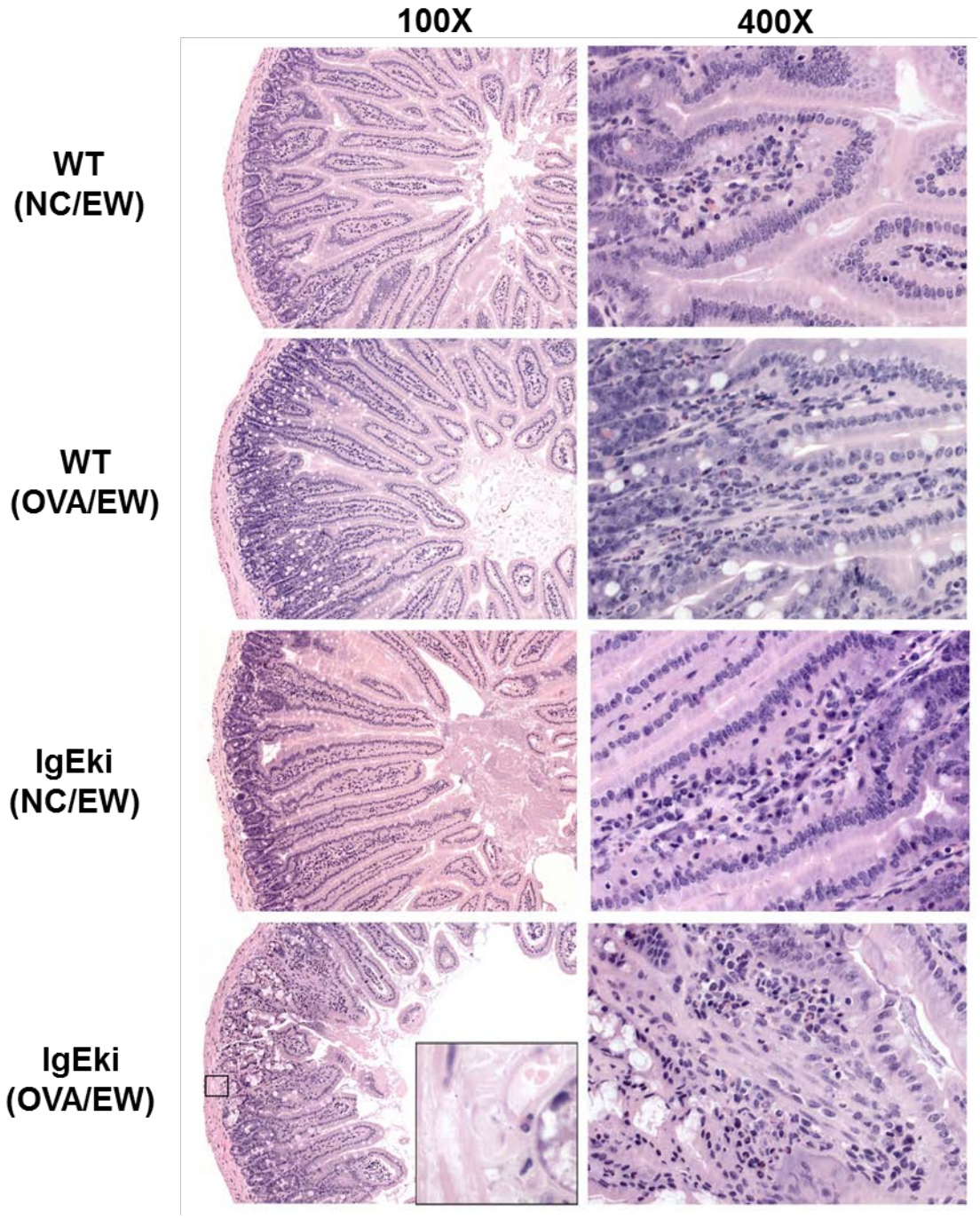

Figure 36: Development of AE in IgEki mice. WT mice and IgEki mice were i.p. sensitized with OVA plus ALUM, or treated only with PBS, and fed EW-diet for 7 days. The jejunums were harvested from the mice, and stained with H&E. All images were taken in same

magnification. OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed. The data are representative for three independent experiments.

Table 6. Assessment of inflammation levels in WT and IgEki mice

Group	Inflammation	Villi	Edema	Total
WT (OVA/EW)	3.75**(1) ± 0.5	0.75 ± 0.5	0.75 ± 0.5	5.25**(1) ± 1.5
WT (NC/EW)	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00* ± 0.0
IgEki (OVA/EW)	4**(3) ± 0.0	0.80 ± 0.5	0.60 ± 0.6	5.40**(3) ± 0.5
IgEki (NC/EW)	1.33*(1) ± 0.6	0.00 ± 0.0	0.00 ± 0.0	1.33**(1) ± 0.5

WT and IgEki mice were i.p. sensitized with OVA plus ALUM, or treated only with PBS, and fed EW-diet for 7 days. The jejunums were harvested from the mice, and Inflammation levels in the tissues were assessed. The total histological score represents the sum of the inflammation grade, villi and edema score. Score ranges from 0 to 6 (total score = I + V + E). OVA/EW; OVA-sensitized and EW-diet fed, NC/EW; non-sensitized and EW-diet fed. * $p<0.05$, ** $p<0.01$. (1) *vs* WT (NC/EW); (2) *vs* IgEki (OVA/EW); (3) *vs* IgEki (NC/EW).

6.2.4. Enhanced IgE expression promoted accumulation of eosinophils and neutrophils in AE tissues

To verify the results of histological analysis, FACS analysis using intestinal lamina propria cells isolated from the mice was performed. Consistent with histological analysis, the frequency of eosinophils ($SiglecF^+$ $CD11b^-$) and neutrophils ($Ly6G^+$ $CD11b^+$) in the lamina propria $CD45^+$ cells were significantly enhanced in IgEki OVA/EW mice, compared to WT OVA/EW mice (Fig. 36). The results suggest an involvement of IgE in the eosinophil and neutrophil migration to the inflamed tissues in AE.

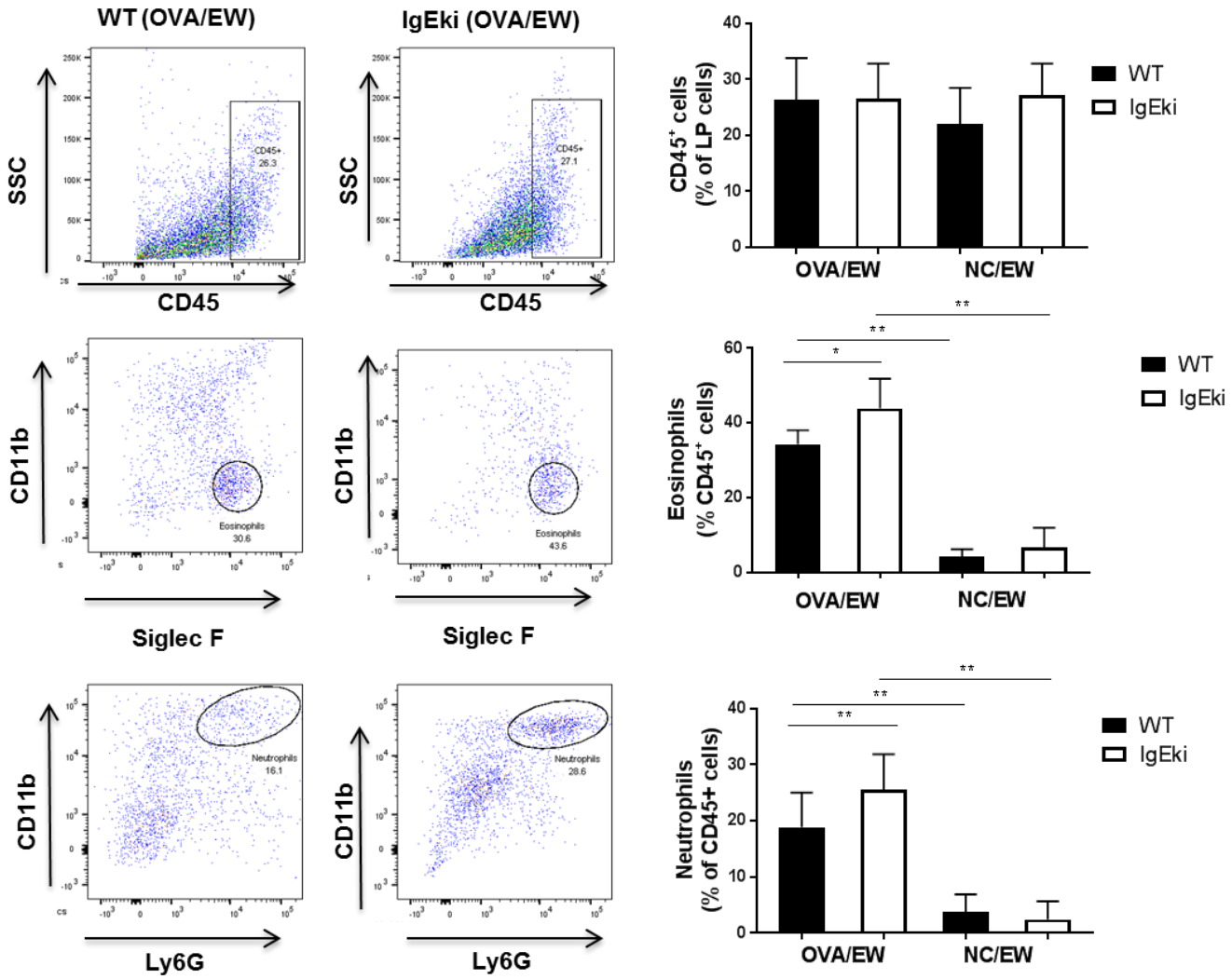

Figure 37: Enhanced frequency of eosinophils and neutrophils in the inflamed intestinal tissues of IgEki mice. WT and IgEki mice (n=4/group) were sensitized with OVA plus ALUM, and fed EW-diet for 7 days. The jejunums were harvested from the mice, and subjected to enzymatic treatment for preparation of lamina propria cells. The frequency of $CD45^+$ cells in lamina propria cells, and the frequency of eosinophils ($SiglecF^+$ $CD11b^-$ cells) and neutrophils ($Ly6G^+$ $CD11b^+$) in $CD45^+$ cells population were determined by FACS. The data are representative for three independent experiments. * p<0.05, ** p<0.01

7. Discussion

7.1. The role of CCR8 in the development of AE.

Eosinophils have long been observed in the inflamed tissues of allergic patients and have been proven to be therapeutic targets in allergic diseases (Davoine and Lacy, 2014; Vieira et al., 2009). Therefore, it is essential to identify which chemokine receptor and its ligands contribute to the migration of eosinophils to sites of allergic inflammation. CCR3/CCL11 and IL-5 receptor/IL-5 are well known to act in eosinophil migration to peripheral tissues, including the gastrointestinal tract (Jung and Rothenberg, 2014; Palmqvist et al., 2007). In the present study, an engagement of CCR8 in eosinophil migration to the inflamed AE tissues was found. However, unlike CCR3 and IL-5R, CCR8 seems to be only indirectly involved in eosinophil migration by inducing CCL11 expression. This finding is consistent with a previous study by Islam *et al* showing that CCL11 expression was reduced in the skin of allergen-sensitized CCR8KO mice (Islam and Luster, 2012). Furthermore, it was found that CCR8 deficiency influences neutrophil migration. CCR8KO mice showed increased neutrophil accumulation and developed AE, although eosinophil accumulation in the intestinal tissues was reduced. There is increasing evidence that neutrophils play an important role in the pathogenesis of allergic inflammation by mediating direct tissue injury or by releasing pro-inflammatory mediators (Tecchio et al., 2014). Increased neutrophil numbers have also been detected in AE patients (Czerwionka-Szaflarska et al., 2017). The potential of CCR8 antagonists has been considered to treat allergic asthma, since several studies have shown a role for CCR8 in the recruitment of Th2 cells and in the development of inflammation in murine models of allergic asthma (Mikhak et al., 2009; Wang et al., 2013; Pease, 2010). However, the results of this project suggest that CCR8 is not a suitable target in AE treatment (Fig. 8-11).

Diverse roles for CCR8 in allergic asthma have been reported so far: e.g., involvement in the development of systemic Th2-type immune response and migration of Th2 cells, or regulatory T-cells, into the inflamed airway tissues (Nguyen et al., 2009; Mutalithas et al., 2010; Bishop and Lloyd, 2003). Using models of *Schistosoma mansoni* soluble egg antigen-induced granuloma formation, as well as OVA and cockroach antigen-induced asthma, Chensue *et al* have shown that

eosinophil recruitment is reduced in CCR8KO mice (Chensue et al., 2001). This reduction in eosinophil recruitment in inflamed airway tissues was explained by a systemic reduction in IL-5 concentration due to defective development of Th2-type immune responses. Islam *et al* have shown that CCR8 recruits IL-5 expressing Th2 cells in atopic dermatitis using CCR8KO mice (Islam et al., 2011). However, in this thesis the IL-5 production in splenic and MLN T-cells from OVA/EW WT and OVA/EW CCR8KO mice were not significantly different. In addition, IL-5 concentrations in intestinal tissue homogenates of both mice were not statistically different. There was no difference in the the frequency of regulatory T-cells in spleens and MLNs of both mice (see fig. 13). These results suggest that a defect in systemic Th2-type immune response or induction of regulatory T-cells is not the main mechanism underlying reduced eosinophil accumulation in the inflamed tissues of CCR8KO mice.

Several studies have shown that histamine released from mast cells induces CCL11 expression in epithelial cells of allergen-challenged skin and lung. However, it is very unlikely that CCR8 is involved in mast cell activation and, subsequently, in CCL11 expression by epithelial cells. This postulation is supported by the fact that the number of mast cells and the concentration of mMCP1, a marker of mast cell activation, showed no statistical difference in intestinal tissues of OVA/EW WT and OVA/EW CCR8KO mice. In addition to epithelial cells, type 2 innate lymphoid cells (ILC2) could be a source of CCL11 (Lee et al., 2015). ILC2 have been associated with the allergic sensitization to foods due to their capacity to produce high amounts of IL-5 and IL-13 in intestinal mucosa (Burton et al., 2018). IL-33 has been reported to induce activation of ILC2 (Burton et al., 2018; Geremia and Arancibia-Cárcamo, 2017). However, it is also unlikely that CCR8 influences the induction or activation of ILC2, since the concentrations of IL-5, IL-13, and IL-33, which are associated with ILC2 activation and function, were not statistically significant between the intestinal tissue homogenates of OVA/EW WT and OVA/EW CCR8KO mice (see Fig. 15). Alternatively, intestinal macrophages could act as CCL11 producing cells. Waddell *et al* showed that F4/80 positive cells are the producer of CCL11 in inflamed colon using a murine model of colitis (Waddell et al., 2011). In this thesis, it was found that OVA/EW WT mice exhibited higher numbers of macrophages (CD68 positive cells) in villi of their small intestines, compared to OVA/EW CCR8KO mice (Fig. S1).

However, immunohistochemically analysis showed that the CD68 positive cells do not express CCR8 (Fig. S2). These results suggest that intestinal macrophages do not have a direct CCR8 engagement in AE. CCL11 producing cells in the inflamed small intestines still need to be identified.

It was unexpected that CCR8 deficiency accelerated neutrophil accumulation in the intestinal tissues (Fig. 10 and Fig. 11). In studies on allergic asthma using CCR8KO mice, such accelerated neutrophil accumulation has not been reported. CCR8 deficiency might enhance expression of neutrophil chemoattractants, such as IL-8, leukotriene B4 (LTB4), and formyl-methionyl-leucyl-phenylalanine (fMLP), in intestinal tissues. The accelerated neutrophil accumulation may also be explained by a consequence of higher growth factor availability due to the reduced accumulation of eosinophils in CCR8KO mice. For instance, granulocyte-macrophage colony-stimulating factor stimulates both eosinophils and neutrophils and enhances survival of these cells (Pitrak, 1997; Esnault and Kelly, 2016). Interestingly, Cheng *et al* have shown that CCL11 expression counter-regulates accumulation of neutrophils in a murine model of endotoxemia. This finding suggests that reduced CCL11 expression could lead to the enhanced neutrophil accumulation in the intestinal tissues of OVA/EW CCR8KO mice (Pitrak, 1997).

OVA/EW CCR8KO mice tended to exhibit lower levels of clinical symptoms, i.e. reduction of body weight and temperature, compared to OVA/EW WT mice (Fig. 6 and Fig. 7). The development of clinical symptoms upon i.p. sensitization with OVA and feeding of the EW diet is induced by FcεRI-engaged mechanism in BALB/c mice (manuscript in preparation, personal communication with Dr. Masako Toda). CCR8KO mice showed lower IgE levels before beginning the EW-diet, which could explain the lower level of clinical symptoms observed.

In summary, in this study was identified a chemokine receptor that leads to eosinophil recruitment in AE sites. To my knowledge, this is the first study to show a contribution of CCR8 in eosinophil recruitment in intestinal tissues. However, the results also suggest a potential involvement of CCR8 in the regulation of neutrophil recruitment in AE tissues (Fig. 38). In a future study, it would be necessary to assess whether CCR8 antagonists enhance neutrophil accumulation in AE.

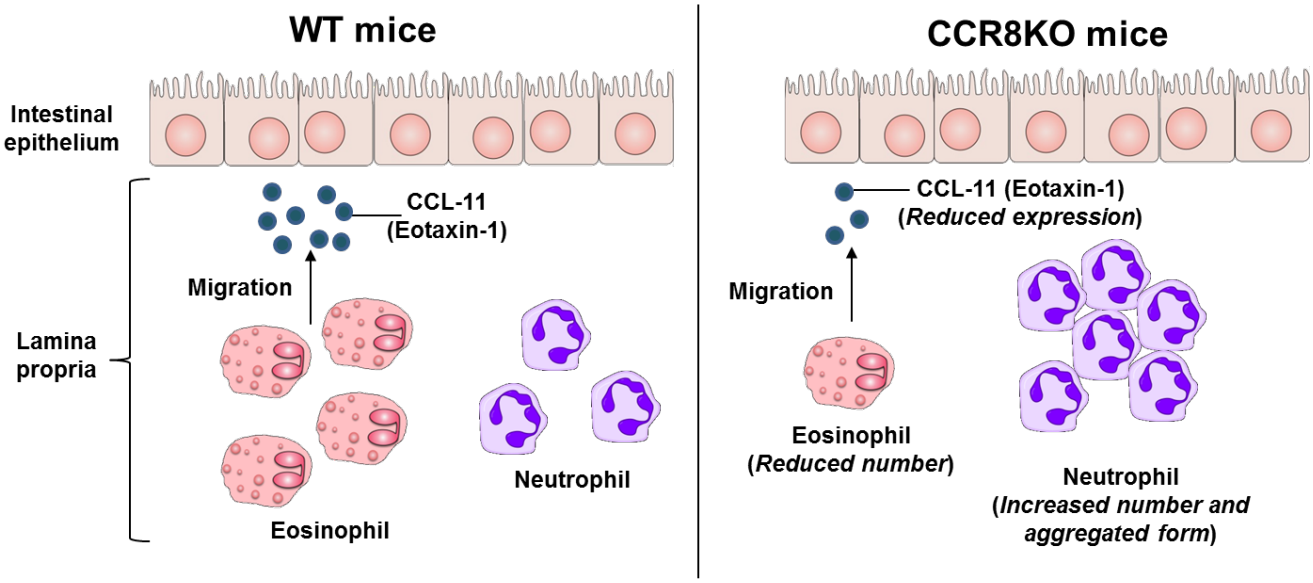

Figure 38: Summary: The role of CCR8 in the development of AE. CCR8 deficiency reduced the number of infiltrating eosinophils and CCL11 expression, but enhanced the number of neutrophils in the AE tissues. The results suggest that CCR8 is involved in eosinophil accumulation in AE tissues by inducing CCL11 expression.

7.2. The role of mast cells in the development of AE

Mast cells are well recognized as a key player in the induction of type I allergy and allergic inflammation including asthma, conjunctivitis and colitis (Jang and Kim, 2015; He, 2004). However, their role in the development of AE remained to be investigated. Mast cells are able to release a broad spectrum of mediators, depending on cytokine milieu, types of stimulus, their localization in the tissues, and many other factors (Mukai et al., 2018; Galli et al., 2008). In this study, it was found that mast cells were involved in CCL1 and CCL11 expression and eosinophil recruitment in AE. To my knowledge, this is the first AE study to use two independent MC-deficient mouse models, showing a consistent result about the role of mast cells in eosinophil recruitment at AE sites.

Several studies showed that CCL1 is the predominant chemokine secreted from IgE-activated human and mouse mast cells (Nakajima et al., 2002; Gonzalo et al., 2007; Gombert et al., 2005; Wu et al., 2013), whereas CCL11 is produced by epithelial cells, endothelial cells, macrophage, or type 2 innate lymphoid cells (Waddell et al., 2011; Rothenberg and Hogan, 2006; Matthews et al., 1998; Diny et al., 2016). In this study was found that CCL1 production is reduced in both $KIT^{w\text{-}sh/w\text{-}sh}$ mice and

CreMaster mice (Fig. 31). The result suggests that mast cells are a main producer of CCL1 in AE tissues.

In the experimental setting of this study, it was not possible to detect expression of CCR8, the receptor for CCL1, by eosinophils in intestinal lamina propria cells (data not shown) by FACS. This observation suggests that mast cell derived CCL1 recruit eosinophils at AE sites indirectly by inducing eosinophil chemoattractant(s), although CCR8 might not be detectable on the cell surface of eosinophils by FACS due to desensitization (i.e. endocytic internalization by ligand binding) upon engagement with CCL1.

Importantly, as reported in the previous section, CCR8 deficiency also reduced CCL11 expression and the number of eosinophils, but not the number of mast cells in intestinal tissues (Fig. 19, Fig. 23-24 and Fig. 29-30). A reduction of CCL11 expression in mast cell deficient mice was also observed. The results suggest that an axis of CCR8 and mast cell derived CCL1 leads to CCL11 expression and subsequent eosinophil recruitment in AE tissues. Das *et al*. reported the migration of eosinophils to the peritoneal cavity in response to CCL11 and proposed an indirect mechanism that involves the resident mast cells, where a reduction of 93% of mast cells resulted in a significant reduction of 52% in the number of CCL11-induced eosinophil accumulation, while no modulation in the neutrophil infiltration was observed (Das et al., 1997; Das et al., 1998). Harris *et al*. reported similar results observing a delayed and reduced eosinophil migration to the peritoneal cavity in mast cell deficient mice (WBB6Fl/J-Kit^{W}/Kit^{W-v} (W/W')), suggesting a role of the mast cells in the eosinophil migration potentially through an amplification of the CCL11 effect (Harris et al., 1997). In a model of asthma using OVA as a model allergen without any adjuvant, Williams *et al.* showed that mast cells are important in the recruitment of eosinophils to the airways, suggesting that mast cells are critical in regulating the eosinophil infiltration during allergic inflammation in mice (Williams and Galli, 2000). In summary, these data suggest that mast cells are involved in recruiting eosinophils in many types of allergic diseases including AE.

It is noteworthy that the development of AE was abolished in $KIT^{w\text{-}sh/w\text{-}sh}$ mice, but not in CreMaster mice. $KIT^{w\text{-}sh/w\text{-}sh}$ mice showed a reduced accumulation of both eosinophils and neutrophils, whereas only the accumulation of eosinophils was reduced in CreMaster mice. The accumulation of eosinophils is a typical change

observed upon microscopically inspection in the majority of patients with AE (Czerwionka-Szaflarska et al., 2017; Yagi et al., 2019). In addition, Salinas *et al.* observed infiltrations with neutrophils and eosinophils in the biopsies of their AE patients (Sierra Salinas et al., 2006). A severity scale system recently proposed by Yagi *et al.* indicates that the presence of neutrophils in the intestinal biopsies is an indicator of a severe course of the disease (grade 3 of 3) (Yagi et al., 2019). These results suggest that (i) eosinophils and neutrophils participate in the development of AE, and (ii) targeting mast cells or eosinophils is not enough for the treatment of allergic enteritis, when neutrophil accumulation is observed at inflammatory sites in AE patients.

Interestingly, it has been reported that the balance between infiltrating cells can change during the course of the pathology. For example in the acute phase of AE there is higher number of mast cells than eosinophils, that inverts over time (Yagi et al., 2019). This suggest that in AE the mast cells play a role in the infiltration of eosinophils to the intestine (Shakoory et al., 2004; Sakamoto et al., 1998).

There are general differences between both mast cell deficient mouse strains. CreMaster shows a virtual lack of mast cells in every tissue and a moderate reduction of basophils, while the $KIT^{w\text{-}sh/w\text{-}sh}$ affects all the cells in the c-Kit lineage (Feyerabend et al., 2011; Galli et al., 2015). These differences may explain the discrepancies observed between CreMaster mice and Kit mutant mice in the AE model: i.e. $KIT^{w-sh/w-sh}$ mice, but not CreMaster mice showed significant reduction in neutrophil accumulation and serum levels of IgE and IgG1 antibodies. As well as WBB6F1-$KIT^{W/W-v}$ mice, $KIT^{W-sh/W-sh}$ mice had been the most commonly used kit mutant mice for studies of mast cell functions *in vivo* (Galli et al., 2015; Michel et al., 2013). However, as commented before mutations of c-Kit also affect additional cells of hematopoietic and nonimmune origin (Grimbaldeston et al., 2005; Nigrovic et al., 2008; Zhou et al., 2007). Michel *et al* showed that the w-sh mutation broadly affects the expression of c-Kit in precursor cells of the myeloid lineage, and accumulate Ly6G-expressing cells resemble granulocytic myeloid-derived suppressor cells (G-MDSC) in spleens (Michel et al., 2013). Transfer of MDSC from naïve w-sh mice into line 1 alveolar cell carcinoma tumor-bearing wild-type littermates leads to enhanced tumor progression, suggesting that MDSC can exert function as suppressor cells (Michel et al., 2013). Such presence of G-MDSC might reduce IgE and IgG1

production and neutrophil accumulation in $KIT^{W-sh/W-sh}$ mice (Zilio and Serafini, 2016; He et al., 2018).

It may be also needed to consider the impact of c kit mutation on ILCs, that can express c-kit in different degrees (Mazzurana et al., 2018; Boyd et al., 2014; Vivier et al., 2018; Hochdörfer et al., 2019). Murine lymphoid tissue-inducer cells (LTi) show a high expression of c-Kit in comparison with ILC3s that have a weak expression of the receptor, while murine ILC1 and ILC2 express c-Kit in some but not all the cell subsets (Vivier et al., 2018; Hochdörfer et al., 2019). Considering the fact that a small number of ILCs can generate a strong response, the c-Kit mutation could also affect the development of ILCs and thereby suppress development of AE in $KIT^{W-sh/W-sh}$ mice in the AE model as observed in a mouse model of multiple sclerosis (Brown and Weinberg, 2018; Russi et al., 2015). In a model of multiple sclerosis, $KIT^{w/Wv}$ mice showed defects in the ILC2 development, as observed by the failure to increase the number of ILC2s in the lymph nodes, spinal cord and brain (Brown and Weinberg, 2018). ILC2s were found to be related to the development of different allergic diseases (Cosmi et al., 2017; Stier and Peebles, 2017; Barlow and McKenzie, 2014). A potential involvement of ILC2 in AE needs to be investigated.

It was striking that both $KIT^{W-sh/W-sh}$ mice and CreMaster mice abolished clinical symptoms: i.e. weight loss and reduced body temperature nearly completely (Fig. 17 and Fig. 18). The results indicated that mast cells play an essential role in the development of clinical symptoms in the AE model. These findings are consistent with previous studies showing that CreMaster or c-Kit deficient mice exhibited neither reduced body temperature nor mortality when subjected to an IgE-dependent model of passive systemic anaphylaxis (PSA) (Ando et al., 1993; Lilla et al., 2011; Mekori and Galli, 1990). It has been reported that the body temperature reduction in murine models of systemic anaphylaxis is controlled by histamine, which is mainly released during mast cell degranulation as a consequence of the crosslinking of the FcεRI on the surface of the mast cells with the IgE (Makabe-Kobayashi et al., 2002; Lundius et al., 2010; Carlos et al., 2006).

In summary, in this study was found that mast cells play a crucial role in development of both clinical symptoms and AE development. Mast cells produce CCL1, and thereby lead to CCL11 expression and subsequent eosinophil recruitment in AE tissues (Fig. 39). The results of this study also showed mast cell deficiency did not

reduce neutrophil accumulation in the development of AE. These findings will have important implications to establish AE treatments that target infiltrating leucocytes in AE tissues.

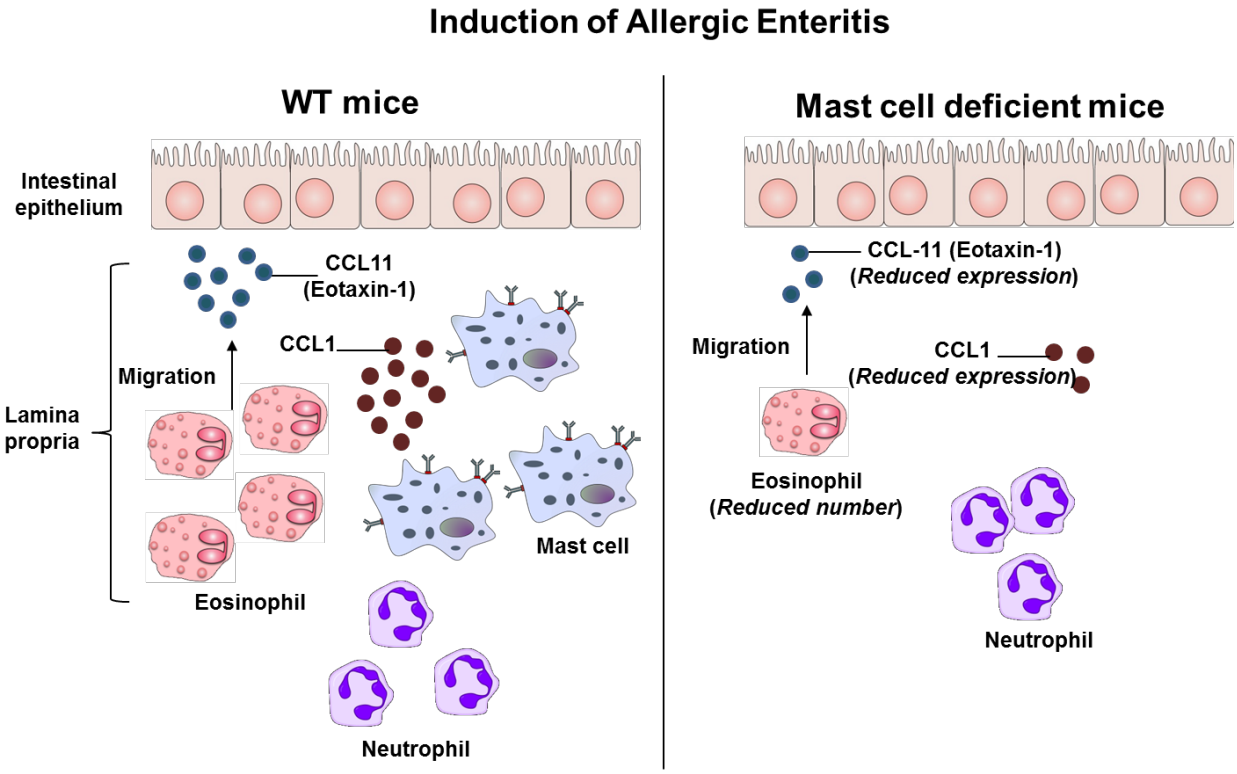

Figure 39: Summary: An important role of mast cells in the development of allergic enteritis. Cremaster mice and $KIT^{w\text{-}sh/w\text{-}sh}$ mice showed reduced eosinophil accumulation and AE levels in their small intestines. The mast cell deficiency also reduced CCL1 expression significantly and CCL11 expression partly in the inflamed tissues. Taken together, these results suggest that mast cells trigger CCL11-inducing recruitment of eosinophils and development of AE by production of CCL1.

7.3. The role of IgE in exacerbation of AE

AE is classified as a non-IgE-mediated food allergy. However, the patients often develop IgE against the offending food. Therefore presence of IgE is considered a poor prognosis factor associated with a prolonged course of the disease (Caubet et al., 2014; Ruffner et al., 2013; Nowak-Węgrzyn et al., 2015; Czerwionka-Szaflarska et al., 2017; Ishige et al., 2015). The role of IgE in AE is not well understood in part due to the difficult access to tissue samples in humans, but also because of the low level of IgE in some patients (Yang et al., 2012; Talay et al., 2012; Hovanec-Burns, 2006; Elkuch et al., 2017).

To overcome this problem and evaluate the possible role of IgE in the development of AE, IgEki mice (overexpressing IgE) were used (Lubben et al., 2013). The results

of this study demonstrated that high levels of allergen-specific IgE enhance the development of clinical symptoms i.e. body weight, temperature, and ruffled fur. In addition, high levels of allergen-specific IgE enhanced development of AE by exacerbating the accumulation of eosinophils as well as neutrophils to the inflammatory site (Fig. 36 and Fig. 37). The cells receiving IgE-mediated stimulation are mast cells. It is well known that mast cells produce a wide range of chemokines including CCL1 upon engagement of FcεRI (Oliveira and Lukacs, 2001; Matsuda et al., 2005; Watanabe et al., 2008).

Therefore, I postulate that high levels of IgE enhance the expression of chemokines produced by mast cells that promote the migration of immune cells to the inflammatory site in AE tissues.

IgE is a survival factor of mast cells. Therefore, it was expected that the number of mast cells were enhanced in IgEki mice. Unexpectedly, histological analysis and measurement of serum mMCP1 showed that the number of mast cells was not statistically different between OVA/EW WT and OVA/EW IgEki mice (Fig. 35). In this study, such mast cell related data using OVA-sensitized mice on day 7 of EW diet was analyzed. However, the small-scale experiment indicated that the number of mast cells is enhanced in IgEki mice when they receive feeding of EW diet more than 14 days (data was taken by Dr. Toda and are not shown in this thesis). The result suggests that IgE could induce persistent AE by enhancing inflammation and the number of mast cells in AE sites. A further study using IgEki mice on long-term allergenic diet would be required to elucidate a role of IgE in persistent AE more in detail.

In summary, it was found that IgE plays a crucial role in the exacerbation of clinical symptoms in AE. IgE promotes migration of eosinophils and neutrophils and may thereby be involved in persistent AE (Fig. 40). Attenuating IgE-mediated signals may be a potential strategy treatment for persistent AE. My study using mast cell deficient mice showed that mast cell-derived CCL1 leads to eosinophil migration into AE tissues. It is likely that FcεRI engagement via IgE-antigen binding promotes CCL1 production in mast cells and enhances eosinophil migration. However, it is still necessary to elucidate how IgE leads to neutrophil migration in AE in the future study.

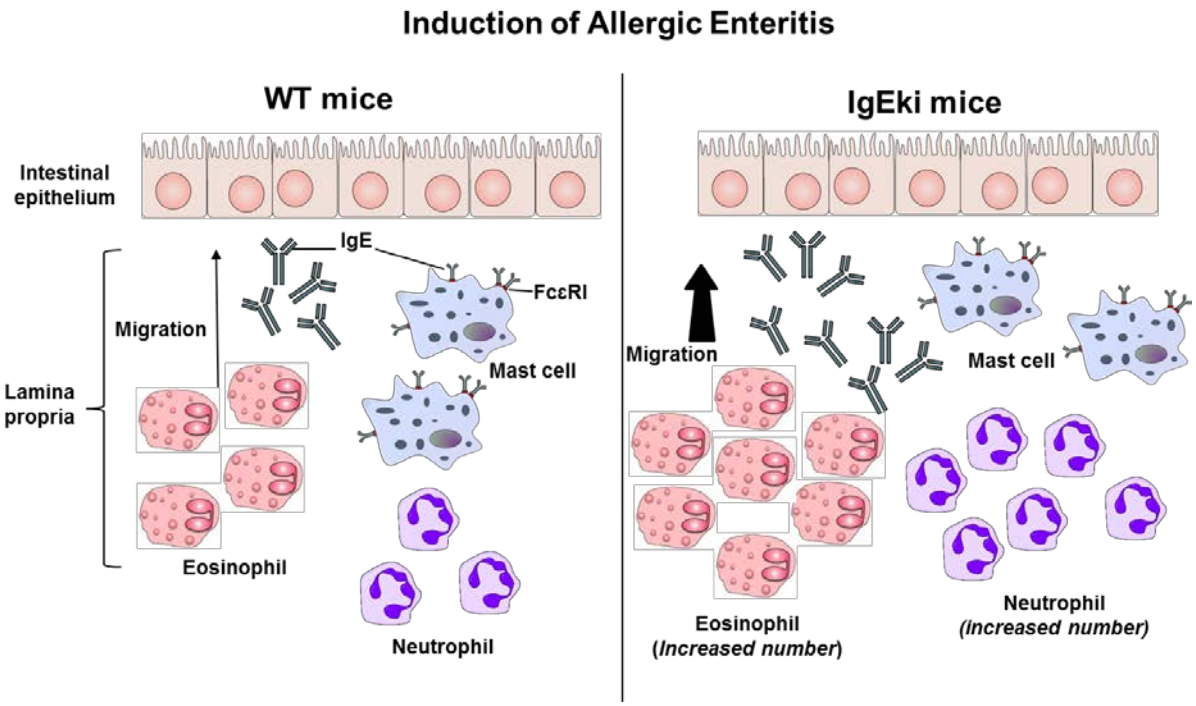

Figure 40: Summary: The role of IgE in the development of AE. To evaluate the possible role of IgE in the development of allergic enteritis, IgEki mice (overexpress IgE and no IgG1) were used. The presence of high levels of allergen-specific IgE enhanced the infiltration of eosinophils as well as neutrophils to the inflammatory site and promotes the development of AE.

7.4. A proposed model: Molecular and cellular mechanisms of allergic enteritis.

Taking together the data generated in this study, the following molecular and cellular mechanisms for the development of AE are proposed. In the initial stage, mast cells in the intestinal lamina propria secrete CCL1 that interacts with the CCR8 on the surface of an immune cell not yet identified. This interaction of CCL1 with CCR8 induces CCL11 secretion that recruits eosinophils to the inflammatory site. I postulate that intestinal macrophages are producers of CCL11 as discussed in section 7.1. This hypothesis needs to be tested in a further study. Neutrophils migrate in a CCR8-CCL1 independent mechanism. IgE is an important factor influencing the severity of the clinical symptoms and AE. The present study has advanced our knowledge of the pathomechanism for AE, and may contribute to establish a novel strategy for the treatment of AE.

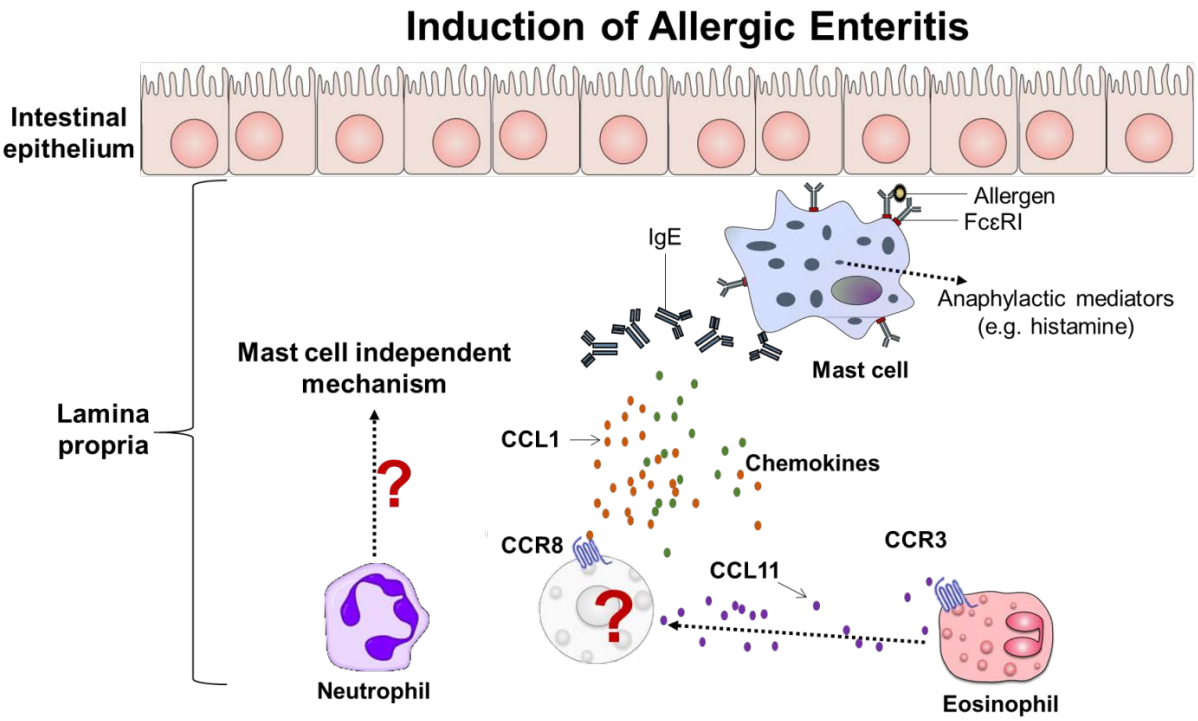

Figure 41: Proposed model: Eosinophils migrate into AE sites by an CCR8-CCL1 axis resulting in an induction of CCL11, whereas neutrophils migrate independent of this axis. IgE enhances both eosinophil and neutrophil migration.

8. Summary

Food allergies are defined as an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food. The prevalence of food allergies has increased in the past decade. Epidemiologic studies involving controlled food challenges for the diagnosis of food allergies indicated that between 1 % to 10.8 % of the population have immune-mediated non-toxic food hypersensitivity.

Despite the increasing prevalence, no curative treatment has been established for food allergies so far except the complete avoidance of the elicited food. To establish safe and effective immunotherapy for food allergies, it is of crucially importance to elucidate pathological mechanism of such diseases.

Food allergies are classified into IgE-mediated and non-IgE mediated (T-cell mediated) allergies, depending on the immunologic pathways and the role of the IgE on the pathogenesis of the disease. Allergic enteritis (AE) is a gastrointestinal form of food allergy. It is classified as non-IgE-mediated food allergy. However, patients with AE often develop IgE and high levels of IgE have been associated with development of persistent AE. The gastrointestinal symptoms of AE are nonspecific, resulting in the fact that a broad differential diagnoses including diagnostic approaches for allergic diseases are necessary to rule out other gastrointestinal pathologies. Biopsies of patients with allergic enteritis have shown infiltration of inflammatory cells (e.g. mast cells, eosinophils, neutrophils, and T cells) in the lamina propria, disruption of intestinal villi, edema, and presence of goblet cells in the intestine.

It is well known that in allergy as well as in other inflammatory conditions immune cells migrate to the inflammatory site due a common process controlled by chemokines. Chemokines are a large family of small secreted chemotactic proteins of 8 to 12 kD that induce cell migration in homeostasis and disease condition. Chemokines exert their activity by binding to a family

of specific 7 transmembrane G-protein-coupled surface receptors and are classified into four groups depending of the spacing of the N-terminal cysteine residues as CXC, CC, C and CX3C (where X is any amino acid). Due to their role in the migration of immune cells chemokines and chemokine receptors have been an attractive target in drug development for the treatment of inflammatory diseases.

In comparison with other phenotypes of food allergy, the pathomechanism of AE is not well characterized. The objective of this study was to elucidate cellular and molecular mechanisms of AE using a murine model. A previous study of our group showed that gene expressions of CC chemokine receptor 8 (CCR8) and its ligand, CC chemokine ligand 1 (CCL1, known as I309 in human and TCA3 in mice) were up-regulated in inflamed tissues of AE mice. CCR8 has been shown to play a role in the pathogenesis of asthma, allergic rhinitis and atopic dermatitis. Mast cells have been suggested to produce CCL1 in respiratory tissues. However, an involvement of CCR8 and its ligands and a role of mast cells in AE remained unclear. Based on this background, the project aimed at elucidating the role of CCR8 and mast cells in the development of AE using CCR8 knock out (KO) mice and two mast cell deficient strains $KIT^{w\text{-}sh/w\text{-}sh}$ and Cre-mediated mast cell eradication (CreMaster). In addition, the possible role of IgE in the exacerbation of AE was investigated using IgE knock-in (IgEki) mice. To induce AE, BALB/c WT mice, mast cell deficient mice, or IgEki mice received intraperitoneal sensitization with ovalbumin (a major egg white allergen) plus Alum as adjuvant and feeding of an egg white diet.

First, the role of CCR8 in the AE development was assessed using CCR8KO mice. Histological analysis revealed that CCR8KO mice developed AE, but exhibited less eosinophil accumulation in the inflamed tissue, when compared to WT mice. FACS analysis showed a decreased frequency of eosinophils but an increased frequency of neutrophils in the inflamed tissues of CCR8 mice. In addition, by means of ELISA, it was found that protein expression of

CCL11, an eosinophil chemoattractant is significantly reduced in their tissues. CCR8KO mice exhibited reduced clinical symptoms as body weight reduction and body temperature drop. CCR8 deficiency did not affect development of OVA-specific T cell and antibody responses. These results suggest that CCR8 induces eosinophil migration and regulates neutrophil migration in AE.

Next, the role of mast cells in the development of AE was assessed using $KIT^{w\text{-}sh/w\text{-}sh}$ and CreMaster mice. $KIT^{w\text{-}sh/w\text{-}sh}$ mice carry spontaneous mutations at both alleles of the dominant white spotting (W) locus, generating a marked reduction in c-kit signaling and therefore a mast cell deficiency. CreMaster mice are deficient of mast cells due to a genotoxicity effect of the Cre insertion in the mast cell protease CPA3 promotor. Histology analysis showed that the development of AE was partly reduced in CreMaster mice, whereas it was abolished in $KIT^{w\text{-}sh/w\text{-}sh}$ mice. FACS analysis showed that the accumulation of eosinophils, but not of neutrophils, was reduced in CreMaster mice, whereas $KIT^{w\text{-}sh/w\text{-}sh}$ mice showed a reduction of both eosinophil and neutrophil accumulation. Importantly, both $KIT^{w\text{-}sh/w\text{-}sh}$ mice and CreMaster mice reduced CCL1 protein expression and tended to reduce CCL11 protein expression in their inflamed tissues. In addition, both mice abolished development of clinical symptoms nearly completely. Mast cell deficiency did not affect development of OVA-specific T cell and antibody responses significantly. It seems that reduction of neutrophil accumulation in $KIT^{w\text{-}sh/w\text{-}sh}$ mice is due to influence of c-kit mutation on other cells than mast cells. These results suggest that mast cells are essential for the development of clinical symptoms, and involved in the eosinophil recruitment and development of AE by producing CCL1.

Finally, the role of IgE in the exacerbation of AE was assessed using IgEki mice. IgEki mice overexpress IgE instead of IgG1 by replacement of exons encoding for the soluble part of the constant region of the murine IgG1 with the IgE counterpart. Histology analysis showed that IgEki mice developed more severe AE than WT mice. FACS analysis showed an enhanced

eosinophil and neutrophil accumulation in the inflamed tissues of IgEki mice. In addition, IgEki mice showed a significantly enhanced development of clinical symptoms. These results suggest that IgE act as an exacerbation factor in the development of clinical symptoms and inflammation in AE.

Collectively, this study demonstrated that (i) the axis CCR8-CCL1 has an indirect involvement in eosinophil recruitment by inducing CCL11 expression, (ii) mast cells are the main CCL1 producer in the inflamed tissues of AE and (iii) high levels of IgE lead to the development of severe clinical symptoms and enhance the development of AE. The potential of CCR8 antagonists has been considered to treat allergic asthma, since several studies have shown a role for CCR8 in the recruitment of Th2 cells and in the development of inflammation in murine models of allergic asthma. However, the results of this study suggest that CCR8 is not a suitable target in AE treatment. In addition, the results suggest that (i) eosinophils and neutrophils contribute to the development of AE, and (ii) targeting mast cells or eosinophils will not be sufficient for the treatment of AE, when neutrophil accumulation is observed at inflammatory sites in AE patients. Mast cells are fully activated upon FcεRI engagement. IgE would promote migration of eosinophils and neutrophils and thereby be involved in persistent AE. Attenuating IgE-mediated signals may be a potential strategy for the treatment of persistent AE. However, it is still necessary to elucidate the mechanism of migration of neutrophils in AE. This study advanced our knowledge of the molecular and cellular mechanisms of AE, and may contribute to the establishment of effective anti-inflammatory strategies in AE treatment.

9. Summary in German

Lebensmittelallergien sind gesundheitsschädigende Auswirkungen spezifischer Immunreaktionen, die infolge der Aufnahme bestimmter Lebensmittelbestandteile auftreten. Die Prävalenz solcher Lebensmittelallergien ist in den letzten Jahren kontinuierlich gestiegen. Aus epidemiologischen Studien, die kontrollierte Nahrungsmittelbelastungstests zur Diagnose von Lebensmittelallergien in Betracht ziehen, geht hervor, dass 1% bis 10.8% der Population von einer immunologisch vermittelten nicht-toxischen Lebensmittelhypersensitivität betroffen sind.

Trotz der steigenden Prävalenz konnte bislang keine Heilbehandlung für Lebensmittelallergien etabliert werden, die Vermeidung des Nahrungsmittels stellt also bis dato die einzige Art der Behandlung dar. Um sichere und effektive Immuntherapien entwickeln zu können, ist die Aufklärung der zugrunde liegenden pathologischen Mechanismen von entscheidender Bedeutung.

Lebensmittelallergien werden entweder als IgE-vermittelt oder als nicht-IgE-vermittelt (T-lymphozytär vermittelt) eingestuft, abhängig von der Immunantwort und der Rolle von IgE Antikörpern in der Pathogenese der Erkrankung. Allergische Enteritis (AE) ist eine gastrointestinale Form der Lebensmittelallergie und gilt als nicht-IgE-vermittelte Lebensmittelallergie. Nichtsdestotrotz entwickeln AE PatientInnen häufig auch IgE, was mit einem verlängerten Krankheitsverlauf einhergeht. Gastrointestinale Symptome von AE sind unspezifisch, dementsprechend sind umfassende Differentialdiagnosen notwendig, um andere Magen-Darm-Erkrankungen auszuschließen zu können. Biopsien von AE-PatientInnen zeigen eine Infiltration von Entzündungszellen (z.B. Mastzellen, Eosinophile, Neutrophile und T-Lymphozyten) in der Lamina Propria, sowie eine Störung der Dünndarmzotten, Ödeme und Vorkommen von Becherzellen im Dünndarm.

Charakteristisch für allergische und andere entzündliche Erkrankungen ist die durch Chemokine kontrollierte Migration von Entzündungszellen hin zum

Entzündungsherd. Sekretierte Chemokine sind als chemotaktische Proteine in der Lage, Vorgänge der Zellmigration sowohl in Homöostase, als auch im Krankheitszustand zu beeinflussen. Diese 8 kDa bis 12 kDa kleinen Proteine entfalten ihre Aktivität durch die Bindung an bestimmte G-Protein-gekoppelte Rezeptoren und werden in vier Gruppen unterteilt, abhängig vom Abstand der N-terminalen Cysteinreste: CXC, CC, C und CX3C, wobei X für jede andere mögliche Aminosäure steht. Aufgrund ihrer Rolle in der Migration von Immunzellen stellen Chemokine und deren Rezeptoren attraktive Zielmoleküle in der Wirkstoffentwicklung für die Behandlung entzündlicher Krankheiten dar.

Im Gegensatz zu anderen klinischen Phänotypen der Lebensmittelallergie ist der Pathomechanismus der AE nicht gut charakterisiert. Ziel dieser Studie war es, die zellulären und molekularen Mechanismen der AE im murinen Modell aufzuklären. Vordaten aus unserer Gruppe zeigen eine Hochregulierung der Genexpression von CC- Chemokin-Rezeptor 8 (CCR8) und seinem Liganden, CC-Chemokin-Ligand 1 (CCL1, auch bekannt als I309 im Menschen und TCA3 in der Maus) in entzündetem Gewebe von AE Mäusen. Es wurde gezeigt, dass CCR8 eine Rolle in der Pathogenese von Asthma, allergischer Rhinitis und atopischer Dermatitis spielt. Die Produktion von CCL1 durch Mastzellen in respiratorischen Geweben war bereits beschrieben, ihre Rolle in der AE jedoch unklar. Vor diesem Hintergrund zielte das Projekt darauf ab, die Rolle von CCR8 und Mastzellen während der Entwicklung von AE unter Zuhilfenahme von CCR8 *knock out* (KO) Mäusen und zwei Mastzell-defizienten Mausstämmen (*KIT*$^{w\text{-}sh/w\text{-}sh}$ und *Cre-mediated mast cell eradication* (CreMaster)) aufzuklären. Des Weiteren wurde die Rolle des IgE während der Exazerbation einer AE mittels IgE *knock-in* (IgEki) Mäusen untersucht. Eine AE wurde durch intraperitoneale Sensibilisierung mit Ovalbumin (einem Hauptallergen im Hühnereiweiß) und Alum als Adjuvans, sowie Fütterung einer Hühnereiweiß-Diät in BALB/c WT, mastzell-defizienten oder IgEki Mäusen ausgelöst.

Zunächst wurde die Rolle von CCR8 in der Entwicklung von AE untersucht. Histologische Untersuchungen zeigten, dass CCR8KO Mäuse AE entwickelten, aber weniger Ansammlungen eosinophiler Granulozyten im entzündeten Gewebe im Vergleich zu WT Mäusen aufwiesen. In FACS Analysen konnte eine verringerte Frequenz von Eosinophilen und eine verstärkte Frequenz an neutrophilen Granulozyten im entzündeten Gewebe von CCR8KO Mäusen gezeigt werden. Zusätzlich wurde in diesen Geweben eine signifikante Reduktion der Proteinexpression von CCL1, einem Chemoattraktor für Eosinophile, mittels ELISA nachgewiesen. Diese Ergebnisse weisen daraufhin hin, dass CCR8 in der AE die Migration von Eosinophilen induziert, sowie die Migration von Neutrophilen reguliert.

Nachfolgend wurde die Rolle von Mastzellen in der Entwicklung von AE unter Zuhilfenahme von $KIT^{w\text{-}sh/w\text{-}sh}$ und CreMaster Mäusen betrachtet. $KIT^{w\text{-}sh/w\text{-}sh}$ Mäuse tragen Spontanmutationen an beiden Allelen des dominanten White Spotting Lokus (W-Lokus), was zu einer ausgeprägten Reduktion des c-kit Signaling führt und dadurch zu einer Mastzelldefizienz. CreMaster Mäuse bilden keine Mastzellen aus aufgrund des genotoxischen Effekts einer Cre Insertion im Promoter der Mastzellprotease CPA3.

In histologischen Untersuchungen konnte gezeigt werden, dass die Ausbildung einer AE in CreMaster Mäusen teilweise reduziert war, während sie in $KIT^{w\text{-}sh/w\text{-}sh}$ Mäusen gänzlich fehlte. Mittels FACS Analysen konnte in CreMaster Mäusen eine reduzierte Akkumulation von Eosinophilen, jedoch nicht von Neutrophilen, demonstriert werden, während $KIT^{w\text{-}sh/w\text{-}sh}$ Mäuse in Bezug auf beide Zellpopulationen eine Reduktion aufwiesen. Wichtig ist anzumerken, dass beide Mausstämme eine reduzierte CCL1 Proteinexpression und eine Tendenz zu verminderter CCL11 Proteinexpression im entzündeten Gewebe aufwiesen. Zusätzlich wurde die Ausprägung klinischer Symptome beinahe vollständig verhindert. Die Defizienz an Mastzellen hatte keinen signifikanten Einfluss auf die Ausprägung OVA-spezifischer T-Lymphozyten und Antikörper. Es ist

naheliegend, dass die reduzierte Akkumulierung von Neutrophilen in KIT$^{w\text{-}sh/w\text{-}sh}$ Mäusen auf die c-kit Mutation in Zellen, die nicht Mastzellen sind, zurückzuführen ist. Aus diesen Ergebnissen kann geschlossen werden, dass Mastzellen essentiell für die Entwicklung klinischer Symptome sind, dass sie an der Rekrutierung von Eosinophilen beteiligt sind und zur Entstehung von AE durch Produktion von CCL1 beitragen.

Schließlich wurde die Beteiligung von IgE an der Exazerbation von AE untersucht. Zu diesem Zwecke wurden IgEki Mäuse verwendet. In diesen Mäusen wird statt des löslichen Teils der konstanten Region von murinem IgG1 jener von IgE überexprimiert. Histologische Untersuchungen belegten einen deutlich schwereren Verlauf der AE in IgEki Mäusen im Vergleich zu WT Mäusen. In FACS Analysen wurden erhöhte Akkumulationen an Eosinophilen und Neutrophilen im entzündeten Gewebe der IgEki Mäuse nachgewiesen. Zusätzlich zeigten IgEki Mäuse signifikant verstärkte klinische Symptome. Diese Ergebnisse belegen, dass IgE als Exazerbationsfaktor in Bezug auf klinische Symptome und Entzündungsreaktionen in AE wirken kann.

Zusammenfassend konnte in dieser Studie gezeigt werden, dass (i) die CCR8-CCL1-Achse indirekt an der Rekrutierung von Eosinophilen über die Induktion einer CCL11 Expression beteiligt ist, (ii) Mastzellen die Hauptproduzenten von CCL1 in entzündeten AE Gewebe sind und (iii) hohe IgE Level zur Ausprägung von schweren klinischen Symptomen führen und die Entwicklung einer AE verstärken können. Das Potenzial von CCR8 Antagonisten wurde bereits zur Behandlung von allergischem Asthma in Erwägung gezogen, da in einigen Studien in murinen Modellen des allergischen Asthmas eine Beteiligung von CCR8 an der Rekrutierung von Th2 Zellen und der Ausbildung einer Entzündungsreaktion gezeigt werden konnte. Die Ergebnisse der vorliegenden Studie legen jedoch nahe, dass CCR8 kein adäquates Ziel in der AE Behandlung ist. Weiterhin lassen die Ergebnisse darauf schließen, dass (i) Eosinophile und Neutrophile an der

Entwicklung einer AE beteiligt sind und (ii) die Ausrichtung der AE Therapie auf Mastzellen oder Eosinophile nicht ausreichend sein wird, sofern auch Akkumulationen von Neutrophilen an Entzündungsherden in AE PatientInnen beobachtet werden. Nach Kreuzvernetzung der FcεRI Rezeptoren sind Mastzellen vollständig aktiviert. IgE würde die Einwanderung von Eosinophilen und Neutrophilen förden und so an einer persistenten AE beteiligt sein. Eine mögliche Therapiestrategie für persistente AE könnte folglich die Abmilderung IgE-vermittelter Signale sein. Dennoch ist es nötig, die Mechanismen der Migration von Neutrophilen in der AE aufzuklären. Diese Arbeit erweitert unser Wissen über die molekularen und zellulären Mechanismen der AE und kann möglicherweise zur Etablierung von effektiven anti-entzündlichen Therapiestrategien der AE beitragen.

10. Supplemental data

10.1. CCR8KO mice exhibited a reduced number of macrophages in the intestine.

Several cell types has been reported to express CCR8; e.g. Th2 cells, Treg, DCs, endothelial cells and macrophages (Zheng et al., 2009; Kremer et al., 2001; Hoshino et al., 2007; Qu et al., 2004; D'Ambrosio et al., 1998; Zingoni et al., 1998; Bünemann et al., 2018). The evaluation of Th2 cells and Treg for possible effect as consequence of the absence of CCR8 was shown in the previous sections. Several studies showed an important role of intestinal macrophages in the development of inflammatory responses (Bain and Mowat, 2014; Grainger et al., 2017; Liu et al., 2018). Taking this in consideration the number of macrophages in the intestinal tissue of OVA/EW WT and OVA/EW CCR8KO mice were evaluated. It was observed that OVA/EW WT mice exhibited higher numbers of macrophages, defined as CD68 positive cells, in the villi of the small intestines when compared to OVA/EW CCR8KO mice (Fig. S1A-B). However, immunohistochemical analysis showed no co-localization of CD68 positive cells and CCR8 (Fig. S2). That suggests that the CD68 positive cells do not express CCR8, what implies that probably intestinal macrophages would not have a direct CCR8 engagement in AE.

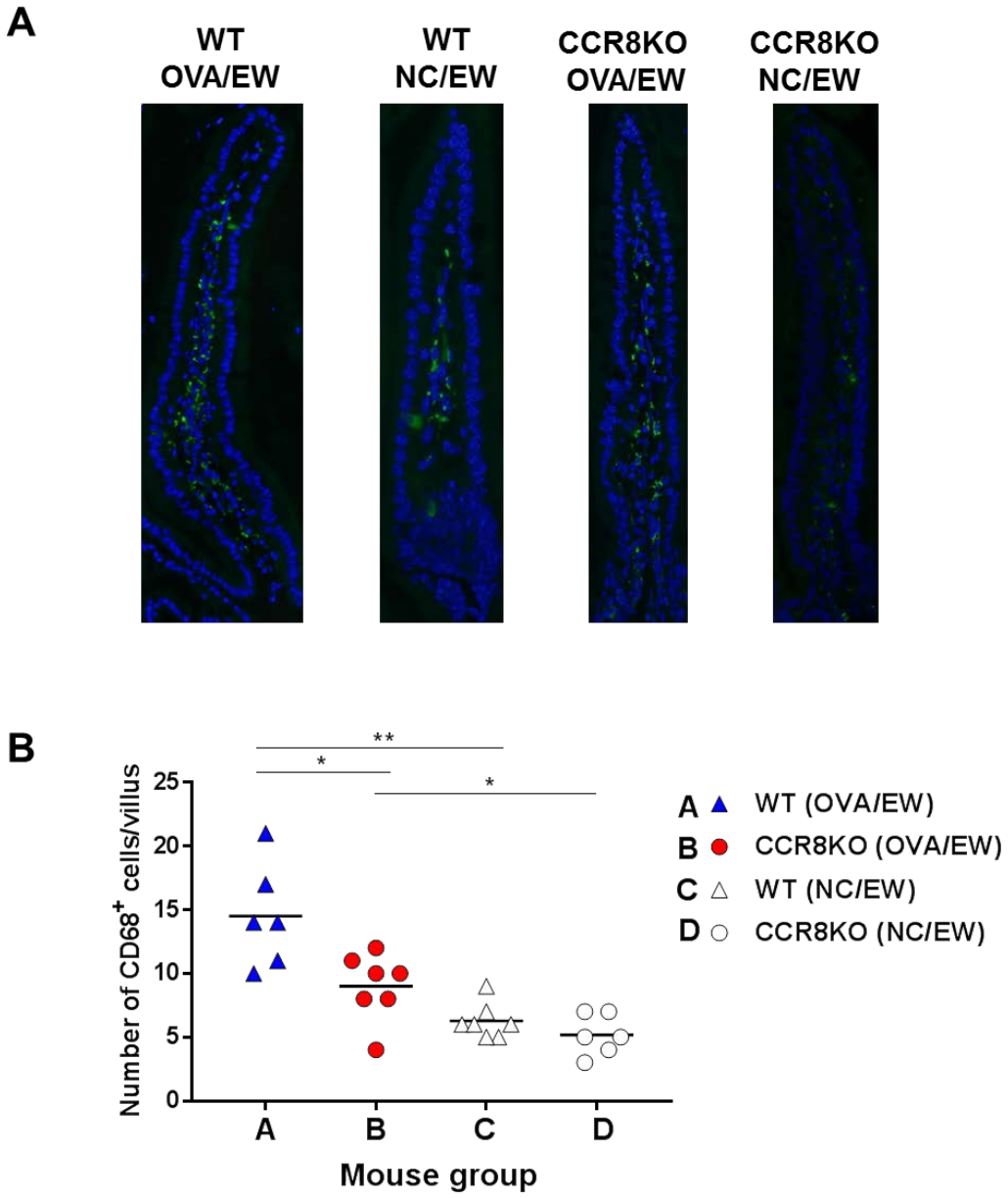

Figure S1: Reduced number of CD68 positive cells in AE tissues of CCR8KO mice. WT and CCR8KO mice were i.p. sensitized with OVA plus ALUM and fed EW-diet for 7 days. As controls, mice fed EW-diet without sensitization. On day 7 of EW diet, jejunums were harvested from the mice. (A) The tissues were stained with anti-CD68 mAb (green) and propidium Iodide (blue). (B) The numbers of CD68 positive cells/villus in the jejunum of the mice were counted. OVA/EW; OVA-sensitized and EW-diet fed. NC/EW; Non-sensitized and EW-diet fed. * P<0.05.

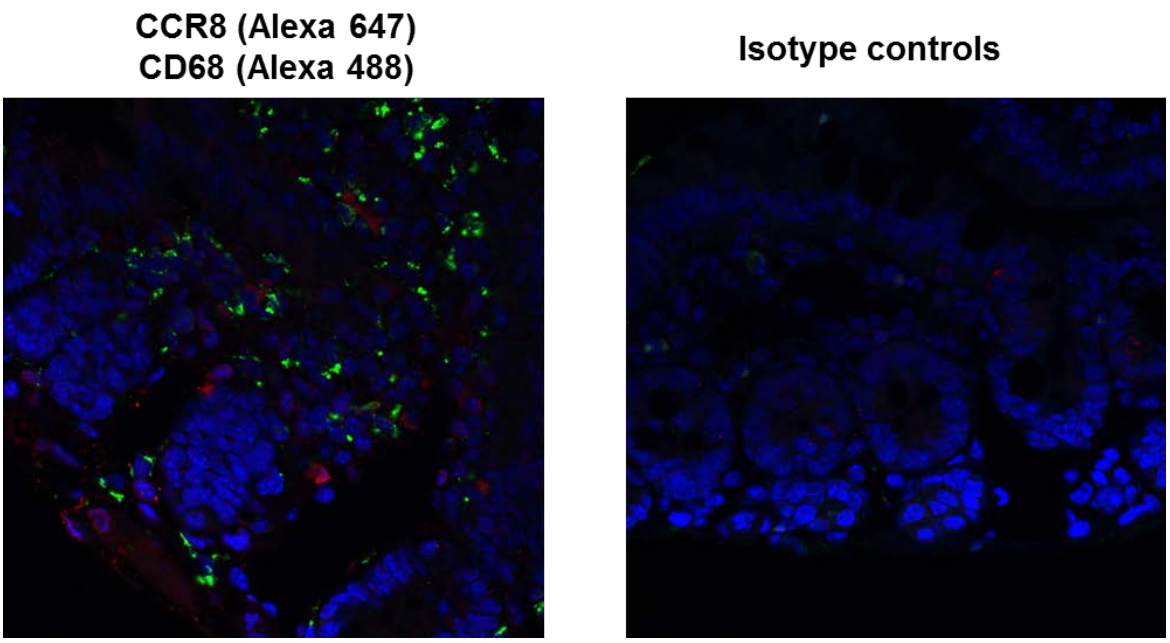

Figure S2: CD68 and CCR8 expression in the inflamed tissues of WT mice. WT mice were i.p. sensitized with OVA plus ALUM and fed EW-diet for 7 days. On day 7 of EW diet, jejunums were harvested from the mice. The tissues were stained with anti-CD68 mAb (green), anti-CCR8-Abs (red), or isotype controls plus propidium Iodide (blue).

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12. Acknowledgements

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Prof. Vieths, I need to thank you so much. Thank you for always be open and available to discussions and hear me when I needed it. Thank you for your always accurate comments and your guidance. Looking back seams so far away that almost 5 years ago I sent to you an email asking if there was an option for a PhD in your laboratory and now it's getting to its end. Thank you very much for the opportunity and the trust.

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EDUCATION

Oct 2015- to date	**PhD Student: Department of Biochemistry, Chemistry & Pharmacy in the Johann Wolfgang Goethe-Universität Frankfurt.** Research done at Paul-Ehrlich-Institut, Langen Germany, under the supervision of Priv. Doz. Masako Toda and Prof. Dr. Stefan Vieths. Thesis "CCR8-CCL1 axis leads to eosinophil migration in experimental murine allergic enteritis"
Feb 2016	**Venezuelan Institute for Scientific Research (Instituto Venezolano de Investigaciones Científicas)** **MSc. In Biology, Mention Immunology** Thesis "Identification of the bioactive compounds with anti-inflammatory activity in the ethanolic extract of *Stevia lucida* leaves".
Oct 2002- Dec 2008	**Universidad Central de Venezuela** **Diploma in Biology (5 years degree) Mention: Cellular biology** Thesis "Preparation and evaluation of allergenic extracts of *Periplaneta americana*, *Blatella germanica* and *Anisakis simplex*, and evaluation of the allergic reaction in children at school age" **(Honored)**

PROFESSIONAL EXPERIENCE

Nov 2011 –Jul 2015	**Research Assistant Associate** Cellular and Molecular Pathology lab Instituto Venezolano de Investigaciones Científicas (IVIC). Caracas, Venezuela
Nov 2008- Nov 2011	**Research Assistant Associate** Physiology and pathology lab (Immunogenetic area) Instituto Venezolano de Investigaciones Científicas (IVIC). Caracas, Venezuela
Mar 2006 - Sept 2008	**Internship** Immunopathology lab *2006-2008* Instituto de Biomedicina. Caracas, Venezuela

PUBLICATIONS

2019 **Blanco-Pérez F**, Kato Y, Gonzalez-Menendez I, Laiño J, Ohbayashi M, Burggraf M, Krause M, Kirberg J, Iwakura Y, Martella M, Quintanilla-Martinez L, Shibata N, Vieths S, Scheurer S, Toda M. **CCR8 leads to eosinophil migration and regulates neutrophil migration in murine allergic enteritis.** Sci Rep. 2019 Jul 3;9(1):9608. doi: 10.1038/s41598-019-45653-7

Blanco-Pérez F, Papp G, GoretzkiA, Moller TM, Anzaghe M, Schülke S. **Adjuvant Allergen Fusion Proteins as Novel Tools for the Treatment of Type I Allergies**. Arch Immunol Ther Exp (Warsz). 2019 Jun 20. doi: 10.1007/s00005-019-00551-8

Blanco-Pérez F, Goretzki A, Wolfheimer S, Schülke S**. The vaccine adjuvant MPLA activates glycolytic metabolism in mouse mDC by a JNK-dependent activation of mTOR-signaling by a JNK-dependent activation of mTOR-signaling**. Molecular Immunology 106 (2019) 159–169

2018 Akcha S, Gómez-Ruiz S, Kellou-Tairi S, Lezama L, **Blanco-Pérez F,** Benali-Baitich O. **Synthesis, characterization, solution equilibria, DFT *study*, DNA binding affinity and cytotoxic properties of a cobalt(II) complex with a 5-pyrazolone ligand.** Inorganica Chimica Acta 482 (2018) 738–748

2017 Laiño J, Wangorsch A, **Blanco F**, Wolfheimer S, Krause M, Flaczyk A, Möller TM, Tsai M, Galli S, Vieths S, Toda M, Scheurer S, Schülke S. **Targeting of Immune Cells by Dual TLR2/7 Ligands Suppresses Features of Allergic Th2 Immune Responses in Mice.** J Immunol Res. 2017;2017:7983217.

2016 Villamizar, José E.; Angarita, Ana Y.; **Blanco, Frank E.;** Taylor, Peter G.; Salazar, Franklin J. **"Synthesis of novel podocarpa-8,11,13-triene-7- and 13-nitriles and evaluation of their anti-inflammatory and cytotoxic activity**" Journal of Chemical Research, Volume 40, Number 8, August 2016, pp. 449-513, pp. 502-505(4)

2008 Franca Puccio, Domenico Cifarelli, **Frank Blanco**, Elianska López, Luís Sarmiento, Rodrigo ordaz, Ivonne Figueroa, Arnaldo Capriles, Isabel Hagel, Maria Cristina di Prisco, Maria Elena Roque, Lisette Machado & Maria Elena Ghezzi. "**Reactividad alérgica a *Anisakis simplex* y su asociación con asma bronquial en niños escolares del estado Nueva Esparta, Venezuela".** Boletín de Malariología y salud ambiental Vol. XLVIII, Nº 2, Agosto-Diciembre, 2008

GRADUATE TRAINING

2018 **MACS Technology Seminar** (3h).
Paul-Ehrlic-Institut (PEI).
Langen, Germany.

2017 **15th EAACI Immunology Winter School "Basic Immunology Research in Allergy"**
European Academy of Allergy and Clinical Immunology (EAACI)
Sierra Neveda, Spain

2016 **Scientific Presentation – Getting it Right** (16 hrs)
Paul-Ehrlich-Institut (PEI)
Langen, Germany

2016 **Introductory Course for Working with Laboratory Animals**
FELASA B accredited category B course 033/11 with a minimum of 40 hours and 50% practical training. Registration No. 033/11/2016/150.
Heidelberg, Germany.

2015 **Scientific writing** (16hrs).
Paul-Ehrlich-Institut (PEI)
Langen, Germany

2015 **Basics in Molecular and Clinical Immunology** (12 hrs).

Paul-Ehrlich-Institut (PEI)
Langen, Germany

2015 **ICGEB Theoretical Course "Mouse Genetics; Models for Human Diseases"** (19 hrs)
International Centre for Genetic Engineering and Biotechnology (ICGEB)
Trieste, Italy

2011 **Lower Saxony International Summer Academy (LISA) "Inflammation, Regeneration and Immunity – Basic Aspects, Novel Approaches and Experimental Models".** (160 hrs)
Hannover Biomedical Research School (HBRS).
Hannover, Germany

2009 **XXIV Updating Theoretical Practical Course on Molecular Genetics.**
Instituto de Diagnóstico y Referencia Epidemiológicos. The American Society for Histocompatibility and Immunogenetics. Fundación Comparte Vida, A.C. Universidad Nacional Autónoma de México. American Board of Histocompatibility and Immunogenetics
Mexico City, Mexico

CONGRESS PRESENTATIONS

2018

BLANCO-PÉREZ F, KATO Y, GONZALEZ-MENENDEZ I, LAIÑO J, OHBAYASHI M, BURGGRAF M, KRAUSE M, KIRBERG J; IWAKURA Y, QUINTANILLA-MARTINEZ, SHIBATA N, VIETHS S, SCHEURER S, TODA M**. CC Chemokine Receptor 8 (CCR8) leads to eosinophil migration and regulates neutrophil migration in murine allergic enteritis** (Poster discussion). FAAM 2018. Copenhagen, Denmark.

BLANCO F, KRAUSE M, LAIÑO J, KIRBERG J, IWAKURA Y, FEYERABEND T, RODEWALD H, GALLI S, VIETHS S, SCHEURER S, TODA M. **Mast cells are involved in an axis CCR8-CCL1 leading to eosinophil migration in experimental allergic enteritis.** (Oral presentation) **.**30. Mainzer Allergie-Workshop. Mainz, Germany

BLANCO F, KRAUSE M, LAIÑO J, KIRBERG J, FEYERABEND T, RODEWALD H, VIETHS S, SCHEURER S, TODA M. **Mast cells induce eosinophil migration in experimental acute allergic enteritis.**(Oral presentation) 12th Annual Scientific Meeting on Biomedical Research of the Paul-Ehrlich-Institut. Ronneburg, Germany

2017 BLANCO F, KRAUSE M, LAIÑO J, KIRBERG J, VIETHS S, SCHEURER S, TODA M. **CC chemokine receptor 8 is engaged in eosinophil migration in experimental allergic enteritis.** (Poster). International Symposium on Molecular Allergology (ISMA 2017). Luxembourg

BLANCO F, BURGGRAF M, KRAUSE M, KIRBERG J, TAUBE C, STASSEN M, VIETHS S, SCHEURER S, TODA M. **A critical role of mast cells in the inflammatory mechanism of experimental allergic enteritis**. (Poster) 15th EAACI Immunology Winter School "Basic Immunology Research in Allergy". Sierra Nevada, Spain

BLANCO F, BURGGRAF M, KRAUSE M, KIRBERG J, TAUBE C, STASSEN M, VIETHS S, SCHEURER S, TODA M. **A critical role of mast cells in the inflammatory mechanism of experimental allergic enteritis**.(Oral presentation). 11th Annual Scientific Meeting on Biomedical Research of the Paul-Ehrlich-Institut. Ronneburg, Germany

2016 BLANCO F, BURGGRAF M, ADACHI-NAKAJIMA H, HACHIMURA S, KOHLHOFER U , KIRBERG J, KIYONO H, VIETHS S, SCHEURER S, TODA M. (2016) **Inflammatory mechanism of experimental allergic Enteritis**. (Poster). 10th Annual Scientific Meeting on Biomedical Research of the Paul-Ehrlich-Institut. Ronneburg, Germany

2014 BLANCO F, SALAZAR F, FRAILE S, MICHELANGELI F, RUIZ M, FERNÁNDEZ A, TAYLOR P. **Posible efecto antiinflamatorio de *Stevia lucida*.** (Oral presentation). ASOVAC 2014. Caracas, Venezuela

BLANCO F, SALAZAR F, FRAILE S, MICHELANGELI F, RUIZ M, FERNÁNDEZ A, TAYLOR P. **Stevia… more than a sweetener? Anti-inflammatory activity of *Stevia lucida.*** (Oral presentation). SILAE 2014. Marsala, Italy.

GÁMEZ C, COLMAN L, CANUDAS N, VILLAMIZAR J.E, TAYLOR P, BLANCO F. **Phototoxicity testing of new aloe-emodin derivatives.**(Poster) SILAE 2014. Marsala, Italy.

2013 BLANCO F, MICHELANGELI F, RUIZ M, FERNÁNDEZ A, GONTO A, FRAILE S, TAYLOR P (2013), **Screening of Venezuelan plants for anti-inflammatory activity. Results from an *in vitro* nitric oxide assay**. 61st International Congress and Annual Meeting of the GA. (Poster) Münster, Germany.

BLANCO F, ESTRADA O, MICHELANGELI F, RUIZ M, FERNÁNDEZ A, TAYLOR P (2013). **Inhibición de la producción de óxido nítrico por extracto etanólico de *Stevia lucida*. ¿Una planta con actividad antiinflamatoria?.** (Poster) SILAE 2013. Puntarenas, Costa Rica.

2009 F BLANCO, F PUCCIO, D CIFFARELLI, E LÓPEZ (2009). **Local allergen extracts preparation and determination of allergic reactivity to cockroaches in Venezuelan scholar children**. (Poster) WAC 2009. Buenos Aires, Argentina.

2008 PUCCIO F, BLANCO F, CIFARELLI D, LOPEZ E, SARMIENTO L, MACHADO L, GHEZZI ME (2008). **Reactividad alérgica frente a cucarachas como factor de riesgo para el desarrollo de enfermedades alérgicas en niños escolares venezolanos. (Allergic reactivity against cockroaches as a risk factor for the development of allergic diseases in Venezuelan scholar children)** (oral presentation) XV Congreso Latinoamericano – XVII Congreso Venezolano de Alergia, Asma e Inmunología. Isla de Margarita. Venezuela

PUCCIO F, BLANCO F, CIFFARELLI D, LOPEZ E, SARMIENTO L, ORDAZ R,

FIGUEROA I, CAPRILES A, MACHADO L, GHEZZI ME (2008). **Asociación entre la reactividad alérgica frente a *Anisakis simplex* y el asma bronquial en un grupo de niños escolares del Estado Nueva Esparta. (Association between allergic reactivity against *Anisakis simplex* and bronchial asthma in a group of scholar children of Nueva Esparta state).**(oral presentation) XV Congreso Latinoamericano – XVII Congreso Venezolano de Alergia, Asma e Inmunología. Isla de Margarita. Venezuela

BLANCO F, PUCCIO F, CIFARELLI D, VASQUEZ N (2008). **Uso de extractos alergénicos de *Periplaneta americana* y *Blatella germanica* para el diagnóstico de alergias. (Use of allergen extracts of *Periplaneta americana* and *Blattella germanica* for the diagnosis of allergies).** (Poster) III Jornadas de Extensión de la Facultad de Ciencias. Caracas, Venezuela.

PUCCIO F, CIFARELLI D, BLANCO F, LOPEZ E, SARMIENTO L, MACHADO L, GHEZZI M, CAPRILES A, ORDAZ R, FIGUEROA I. (2008). **Association between *Anisakis simplex* reactivity and bronquial asthma in Venezuelan scholar children.** (Poster). EAACI 2008. Barcelona, España.

PUCCIO F, LOPEZ E, CIFARELLI D, BLANCO F, SARMIENTO L, CAPRILES A, MACHADO L, GHEZZI M. (2008). **High allergic reactivity in Venezuelan scholar children with coexistence of parasite and infectious diseases**. (Poster) EAACI 2008. Barcelona, España.

2007 PUCCIO F, BLANCO F, LOPEZ E, CIFARELLI D, ORDAZ R, FIGUEROA I, SARMIENTO L, MACHADO L, GHEZZI M, ROMER P, PALENQUE M (2007). **Posible implicación del *Anisakis simplex* en el desarrollo del asma bronquial de niños escolares del estado Nueva Esparta**. **(Possible involvement of *Anisakis simplex* in the development of bronchial asthma in scholar children in Nueva Esparta state).** (Poster). XVIII Congreso Latinoamericano de Parasitología, Isla de Margarita, Venezuela.

PUCCIO F, CIFARELLI D, MACHADO L, GHESSI ME, SARMIENTO L, LOPEZ E, VASQUEZ N, ORDAZ R, BLANCO F (2007). **Allergy in Latin America. A public health problem who´s time has come**. (Poster) World Allergy Congress, Bangkok, Thailand.

BLANCO F, PUCCIO F, CIFARELLI D, LOPEZ E, SARMIENTO L, ORDAZ R, FIGUEROA I, PALENQUE M, MACHADO L, GHEZZI ME. (2007). **Evaluación de la reactividad alérgica frente a *Anisakis simplex* en un grupo de niños escolares atópicos del estado Nueva Esparta.** Assessment of allergic reactivity against *Anisakis simplex* in a group of atopic children in Nueva Esparta state. (Poster)Convención Anual de AsoVAC 57. Táchira, Venezuela.

2002 BLANCO F, CARDENAS J, DUQUE C, GUERRERO M, SANE K. (2002). (Oral

Presentation). **Determinar la letalidad de la hemolinfa producida por la larva de la mariposa *Lonomia achelous* en ratones de la cepa Balb/c y C57BL.(To determine the lethality of the haemolymph produced by larvae of the butterfly *Lonomia achelous* in mice of the strain Balb / c and C57BL).** XXXIV Festival Regional Juvenil de la Ciencia. Caracas, Venezuela.

AWARDS

2014 Granted with the Young researcher award in the session Cell Biology 1 for the oral presentation "Posible efecto antiinflamatorio de *Stevia lucida"*. ASOVAC 2014. Caracas, Venezuela

2009 The best poster 2009 "The annual price for the best annual basic scientific investigation Dr. Jose Maria Vargas and Applied Scientific Investigation Dr. Luis Razetti"10th edition. For the research on the Allergic reactivity to *Anisakis simplex* and its association to bronquial asthma on children at school age in Nueva Esparta State, Venezuela.

2008 Honor award to thesis: "Preparation and evaluation of allergenic extracts of *Periplaneta americana*, *Blatella germanica* and *Anisakis simplex*, and evaluation of the allergic reaction in children at school age"

SCHOLARSHIPS AND GRANTS

2017 EAACI Attendance Grant, to attend to 15th EAACI Immunology Winter School "Basic Immunology Research in Allergy and Clinical Immunology". Sierra Nevada, Spain.

2015 DAAD awarded a scholarship for further study and training. Research Grants - Doctoral Programmes in Germany, 2015/16 (57129429). 4 years PhD scholarship

2011 LISA-DAAD Attendance Grant, to attend to the Lower Saxony International Summer Academy (LISA) "Inflammation, Regeneration and Immunity – Basic Aspects, Novel Approaches and Experimental Models". Hannover, Germany.

2009 Granted by The World Allergy Congress Organization Committee to attend to the World Allergy Congress 2009 in Buenos Aires, Argentina.

MEMBERSHIPS AND ASSOCIATIONS

European Academy of Allergology and Clinical Immunology (EAACI)

Association for the Advancement of University Research (ASOVAC)

Italo-Latin American Society of Ethnomedicine (SILAE)

Deutsche Gesellschaft für Allergologie und Klinische Immunologie e.V.(DGAKI)

LANGUAGES

Spanish (native)
English (fluent)
German (basic)

14. Publication list

The following articles were published during the time of my PhD studies.

Publications related to the work performed in the Paul-Ehrlich-Institut:

Blanco-Pérez F, Kato Y, Gonzalez-Menendez I, Laiño J, Ohbayashi M, Burggraf M, Krause M, Kirberg J, Iwakura Y, Martella M, Quintanilla-Martinez L, Shibata N, Vieths S, Scheurer S, Toda M. **CCR8 leads to eosinophil migration and regulates neutrophil migration in murine allergic enteritis.** Sci Rep. 2019 Jul 3;9(1):9608. doi: 10.1038/s41598-019-45653-7

Blanco-Pérez F, Goretzki A, Wolfheimer S, Schülke S**. The vaccine adjuvant MPLA activates glycolytic metabolism in mouse mDC by a JNK-dependent activation of mTOR-signaling.** Molecular Immunology 106 (2019) 159–169. doi: 10.1016/j.molimm.2018.12.029

Blanco-Pérez F, Papp G, GoretzkiA, Moller TM, Anzaghe M, Schülke S. **Adjuvant Allergen Fusion Proteins as Novel Tools for the Treatment of Type I Allergies**. Arch Immunol Ther Exp (Warsz). 2019 Jun 20. doi: 10.1007/s00005-019-00551-8

Laiño J, Wangorsch A, *Blanco F*, Wolfheimer S, Krause M, Flaczyk M, Möller TM, Tsai M, Galli S, Vieths S, Toda M, Scheurer S, Schülke S. **Targeting of Immune Cells by Dual TLR2/7 Ligands Suppresses Features of Allergic Th2** Immune Responses in Mice. J Immunol Res. 2017;2017:7983217. doi: 10.1155/2017/7983217

Other articles published during this time, but not related to the work performed in the Paul-Ehrlich-Institut:

Akcha S, Gómez-Ruiz S, Kellou-Tairi S, Lezama L, *Blanco-Pérez F*, Benali-Baitich O. **Synthesis, characterization, solution equilibria, DFT *study*, DNA binding affinity and cytotoxic properties of a cobalt(II) complex with a 5-pyrazolone ligand.** Inorganica Chimica Acta 482 (2018) 738–748.

Villamizar, José E.; Angarita, Ana Y.; *Blanco, Frank E*.**;** Taylor, Peter G.; Salazar, Franklin J**. "Synthesis of novel podocarpa-8,11,13-triene-7- and 13-nitriles and evaluation of their anti-inflammatory and cytotoxic activity"** Journal of Chemical Research, Volume 40, Number 8, August 2016, pp. 449-513, pp. 502-505(4).

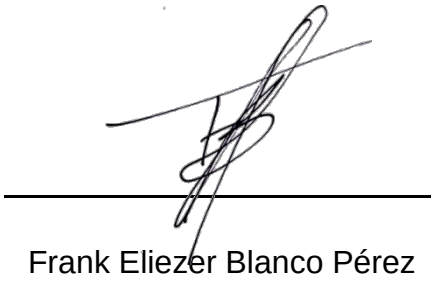
14. Declaration of academic integrity

Except where stated otherwise by reference or acknowledgment, the work presented was generated by me under the supervision of my advisors during my doctoral studies.

Whenever a figure, table or text is identical to a previous publication, it is stated explicitly in the thesis that copyright permission and/or co-author agreement has been obtained.

Parts of this thesis have been previously published in:

Blanco-Pérez F, Kato Y, Gonzalez-Menendez I, Laiño J, Ohbayashi M, Burggraf M, Krause M, Kirberg J, Iwakura Y, Martella M, Quintanilla-Martinez L, Shibata N, Vieths S, Scheurer S, Toda M. **CCR8 leads to eosinophil migration and regulates neutrophil migration in murine allergic enteritis.** Sci Rep. 2019 Jul 3;9(1):9608. doi: 10.1038/s41598-019-45653-7



Frank Eliezer Blanco Pérez