Interleukin-33 orchestrates an immune network to counteract severe acute colitis

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Zusammenfassung

Chronisch-entzündliche Darmerkrankungen (CED) manifestieren sich durch Schleimhautschäden und Ulzerationen im Darmtrakt und gelten als Risikofaktoren für die Entstehung von Darmkrebs. Unter CED leiden weltweit rund 6.8 Millionen Menschen, wobei die Prävalenz stetig zunimmt. Trotz umfangreicher Forschungen zur Pathogenese von CED ist die genaue Ätiologie noch unbekannt und dementsprechend konnte bislang keine kurative Therapie entwickelt werden. Aufgrund dieser Tatsache konzentrieren sich die aktuellen Therapien hauptsächlich auf die Linderung und Unterdrückung klinischer Symptome wie anhaltender Durchfall, starke Bauchschmerzen und Gewichtsverlust. Obwohl konventionelle Therapien bemerkenswerte Fortschritte erzielt haben, zeigt nur weniger als die Hälfte der Patienten ein effizientes Ansprechen auf die Medikamente. Um die Lebensqualität der Patienten zu verbessern und unerwünschte Komplikationen wie Operationen oder sogar die Entstehung von Darmkrebs zu vermeiden, ist es von wesentlicher Bedeutung, neue therapeutische Ansätze zu finden.

In jüngster Zeit zeigte sich, dass Interleukin-33 (IL-33) und dessen Rezeptor ST2 wichtige Modulatoren bei entzündlichen Erkrankungen darstellen. Insbesondere bei CED haben mehrere Studien den IL-33/ST2 Signalweg als möglichen therapeutischen Ansatz hervorgehoben. Bisherige Studien führten jedoch nicht zu eindeutigen Ergebnissen zur Funktion von IL-33 bei Darmentzündungen. Daher wurde die Rolle von IL-33 während einer akuten Kolitis und dessen Potenzial als neuartiges Therapeutikum in dieser Arbeit eingehend untersucht. Interessanterweise ist in CED Patienten die Expression von IL-33, jedoch nicht die seines Rezeptors ST2, im entzündeten Darmgewebe signifikant erhöht, im Vergleich zu nicht entzündetem Gewebe. Auch Mäuse, denen mit Dextransulfat-Natrium eine akute Kolitis induziert wurde, zeigten einen Anstieg der IL-33-Expression im Dickdarm im Vergleich zu gesunden Kontrollmäusen. ST2-defiziente Mäuse zeigten im Vergleich zu Wildtyp-Mäusen eine verstärkte Pathologie im Darm, was auf eine protektive Funktion von IL-33 während einer Kolitis hinweist. Darüber hinaus führte die exogene IL-33-Behandlung zu einer starken Linderung der Darmentzündung durch eine erhöhte Integrität der Schleimhautbarriere. Im Rahmen dieser Arbeit wurden regulatorische T Zellen (Tregs), Eosinophile und angeborene lymphoide Typ-2 Zellen (ILC2s) als zentrale Ziele des IL-33/ST2 Signalweges identifiziert. Umfangreiche immunologische Analysen zeigten weiterhin, dass ILC2s

eine wesentliche Funktion zur Aufrechterhaltung der Schleimhautbarriere ausübten. Zudem bekräftigten unsere Ergebnisse, dass Tregs und Eosinophile notwendig sind, um gemeinsam mit ILC2s zur Eindämmung von Darmentzündungen beizutragen.

Zusammenfassend unterstreicht diese Studie die Schutzwirkung einer exogenen IL-33-Behandlung auf die Darmbarriere. Durch die IL-33-vermittelte Expansion und Förderung der Funktion von ILC2s, Tregs und Eosinophilen werden intestinale Entzündungen reduziert.

Summary

Inflammatory bowel disease (IBD) is mainly characterized by mucosal damage and ulceration, which are both considered to be high-risk conditions for the development of colorectal cancer. Globally, around 6.8 million people are suffering from IBD, with increasing prevalence. Despite extensive research investigating the pathogenesis of IBD, the precise etiology is still unknown and no curative therapy has been developed so far. Due to this fact, current therapies mainly focus on alleviating and suppressing clinical manifestations like recurring diarrhea, excessive abdominal pain and pronounced body weight loss. Even though drugs for the treatment of IBD have reached remarkable progress, only less than half of the patients show an efficient response. To improve the patients' quality of life and to avoid unwanted complications like surgery or even the development of bowel cancer, it is of essential importance to develop new therapeutic approaches. Recently, interleukin-33 (IL-33) and its receptor ST2 have emerged as important modulators in inflammatory disorders. Especially in the context of intestinal inflammation, several studies have emphasized the IL-33/ST2 signaling pathway as a key target for therapeutic strategies. Nevertheless, findings obtained so far did not achieve conclusive results about the IL-33 function in the intestine. Therefore, we investigated the role of IL-33 during intestinal inflammation and further elucidated its potential as a novel therapeutic drug. Interestingly, the expression of IL-33, but not its receptor ST2, was significantly enhanced in inflamed intestinal lesions of IBD patients compared to non-inflamed tissues. Well in line, mice with Dextran Sulfate Sodium (DSS)-induced colitis exhibited a strong increase of IL-33 expression in the colon compared to healthy control mice. Mice deficient for ST2 displayed an aggravated colon pathology compared to wild type mice, suggesting a favorable function of IL-33 during colitis. Indeed, exogenous IL-33 treatment resulted in a tremendous amelioration of intestinal inflammation by enhancing the mucosal barrier integrity. We identified regulatory T cells (Tregs), eosinophils and type 2 innate lymphoid cells (ILC2s) as central targets of IL-33. Subsequent analyses further emphasized ILC2s as essential players to maintain the mucosal barrier integrity and supporting but necessary functions of Tregs and eosinophils to restrain intestinal inflammation. In summary, this study highlights the potential of exogenous IL-33 treatment to promote tissue protection during colitis by orchestrating ILC2, Treg and eosinophil function.

1 Introduction

1.1 The immune system

The immune system is a complex network consisting of different soluble as well as cellular components to defend and protect the body from pathogens, including bacteria, viruses, parasites and fungi but also from abnormal cells of the host to prevent the development of cancer. A fine-tuned balance between these components is essential to achieve an efficient immune response and to prevent an excessive immune reaction that could further result in tissue damage or autoimmunity. Immune cells arise from hematopoietic stem cells and differentiate in the bone marrow or the thymus into common myeloid or lymphoid progenitor cells. While myeloid stem cells give rise to innate immune cells, lymphoid progenitors develop into mature lymphocytes, necessary for the adaptive immune response. Furthermore, lymphoid progenitors can also develop into innate lymphoid cells, which comprise functions of both innate and adaptive immune responses [1, 2].

1.1.1 The innate immune system

The innate immune response consists of an evolutionary conserved defense system to secure the survival of the host and is shared between plants, invertebrates and mammals [3]. As a first line of defense, the innate immune system is triggered rapidly within minutes upon pathogen encounter via several mechanisms. These mechanisms include physical barriers such as epithelial cell layers, expressing tight cell-cell contacts to prevent pathogen invasion [1], but also other non-specific host defenses, which are based on germline-encoded receptors, the so-called pattern recognition receptors (PRRs). These PRRs are invariant receptors, which are able to recognize molecules expressed by pathogens (pathogen-associated molecular pattern, PAMPs) or molecules released by damaged cells upon inflammation or infection (damage-associated molecular pattern, DAMPs) [4]. PRRs are mainly expressed by hematopoietic cells such as neutrophils, macrophages, eosinophils, basophils, dendritic cells (DCs), mast cells and natural killer (NK) cells, but also by non-hematopoietic cells like fibroblasts, endothelial and epithelial cells of the skin, respiratory and gastrointestinal tract [5]. Upon pathogen encounter, innate immune cells are activated via PRR recognition to initiate the secretion of soluble proteins and bioactive molecules like cytokines and chemokines. Subsequently, innate immune cells are further recruited to the site of inflammation to carry out distinct

signaling pathways, leading to the elimination of pathogens and further initiation of the adaptive immune response [6]. Thus, innate immune cells, such as macrophages, neutrophils, NK cells, DCs, eosinophils as well as ILC2s comprise a broad range of immune functions. Since the present study will have a closer look at eosinophils and ILC2s, these cells will be described in more detail.

1.1.1.1 Eosinophils

Eosinophils develop in the bone marrow from multipotent hematopoietic stem cells and were first discovered in 1846 [7]. Arising from the myeloid lineage, eosinophils are considered to belong to the granulocyte family and are mainly recognized for their cytotoxic effector functions [8].

Eosinophils are known to circulate at relatively low levels in the bloodstream but are primarily known to be tissue-dwelling cells and reside in the respiratory and gastrointestinal tract as well as in the ovary, spleen and lymph nodes. Under inflammatory conditions, eosinophils are recruited from the bone marrow to accumulate at the site of infection [9]. Eosinophils contain numerous intracellular granules with toxic proteins and other inflammatory mediators such as major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO). Upon activation in response to inflammatory stimuli, eosinophils can immediately undergo a degranulation process to release proteins into the extracellular matrix. These proteins are cationic and can cause epithelial cell damage by binding to negatively charged cell membranes, resulting in the disorder of the lipid bilayer or modulation of different enzyme activity within the tissue [10]. Due to their destructive potential against pathogens but also against the neighboring host tissues, eosinophils have been proposed to be end-stage cells during innate immunity [11]. Over the last decades, eosinophils were mainly studied in the context of allergic diseases and parasite infection. However, recent studies have been recognizing eosinophils to play multiple roles, e.g. in tissue repair, fibrosis and in the modulation of the adaptive immune response [8, 12]. Apart from toxic proteins, eosinophil granules can also harbor a variety of cytokines, chemokines and growth factors such as IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemoattractant C-C motif chemokine ligand 11 (CCL11, eotaxin), but also IL-4, IL-13 and IL-25. Depending on the secretory product, eosinophils can affect the immune microenvironment as well as immune function, including their own chemotaxis and survival within tissues [8]. Besides the

intracellular granules, eosinophils express a variety of surface receptors and molecular markers, including PRRs and major histocompatibility complex (MHC) class II, each with specific immunomodulatory functions. PRRs enable the activation of eosinophils upon alarmin or DAMP signals to enhance their survival and mediate their migration to areas of tissue injury or necrosis [13]. Through the expression of MHC class II eosinophils harbor an antigen-presenting cell (APC) function and are therefore able to stimulate antigen-specific T cell proliferation to promote the activation of adaptive immune responses [14, 15].

Even though, the underlying immunomodulatory mechanisms are not completely clear, there is evidence that eosinophils play an essential role in maintaining tissue and immune homeostasis, since previous studies could show that depletion of eosinophils in mice resulted in an enhanced apoptosis of plasma cells in the bone marrow, which are essential for long-term immune protection [16].

1.1.1.2 Type 2 innate lymphoid cells

Type 2 innate lymphoid cells (ILC2s) belong to the innate lymphoid cells (ILCs), which were originally described in 2006 as non-B and non-T cells. These ILCs require the transcriptional repressor inhibitor of DNA-binding 2 (ID2) for their development and rely on the signaling through the interleukin-7 receptor subunit-α (IL-7Rα) for their maintenance [17]. ILC2s were characterized as IL-13 producing cells of lymphoid origin and dependent on the expression of IL-25 [18]. The discovery of this innate cell type was further confirmed by independent studies, resulting in different names, including natural helper cells, nuocytes and innate type 2 helper cells [19-21]. Since 2013 and due to Spits *et al.*, these cells are universally recognized as type 2 innate lymphoid cells [22].

ILC2s are present at various mucosal tissue sites such as the lung, small intestine, colon and mesenteric lymph nodes but also in the spleen, bone marrow and liver [23, 24]. The development of ILC2s is dependent on the transcription factors retinoic acid receptor-related orphan receptor α (RORα) and GATA binding protein 3 (GATA3), as it was shown that deletion of GATA3 or RORα *in vivo* in mice prevented the development of ILC2 progenitor cells in the bone marrow [25, 26]. Further studies could show that ILC2s were able to proliferate upon IL-25 stimulation in RAG1$^{-/-}$, IL-4$^{-/-}$, IL-5$^{-/-}$, IL-9$^{-/-}$ and IL-13$^{-/-}$ mice, demonstrating that ILC2 development is independent of T and B cells and type 2 cytokines [18].

Innate lymphoid cells, including ILC2s, share the transcriptional and functional

phenotype of both cytotoxic $CD8^+$ T cells and $CD4^+$ T helper cells but lack the expression of antigen-specific receptors [22]. It was shown that ILC2s respond specifically to the epithelial cell-derived cytokines IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) to produce IL-4, IL-5, IL-9, IL-13 and epidermal growth factor receptor (EGFR) ligand amphiregulin (AREG) [27, 28]. Subsequent studies could reveal that even though ILC2s seem to be homogenous, they can be classified into two major groups based on their responsiveness to IL-25 and IL-33. IL-25 responsive ILC2s, which were mainly described during helminth infection, are referred to as inflammatory ILC2s. IL-33-responsive ILC2s are present in the steady state and are considered to be natural ILC2s and characterized to express high levels of the IL-33 receptor, serum Stimulation-2 (ST2) [29, 30]. Upon activation, ILC2s release a variety of cytokines and are able to mediate type 2 immune responses by shaping both innate as well as adaptive immunity long before traditional type 2 helper T (T_H2) cells arrive at the site of inflammation. Consequently, ILC2 functions are tightly regulated. It has been shown that both type I and type II interferons (IFN-β and IFN-γ) are able to negatively regulate ILC2 function by suppressing ILC2 proliferation and cytokine production in mice [31, 32]. These findings suggest ILC2s to be highly reactive and early effectors during the innate immune response. However, the tissue-specific gene expression patterns as well as the biological function of ILC2s depending on their location is still under investigation.

1.1.2 The adaptive immune system

Compared to the innate immune system, the adaptive immune system is comprised of a smaller number of cells due to their specificity for individual pathogens. Thus, the adaptive immune cells require the encounter with antigens to proliferate and attain sufficient numbers to mount an effective reaction against pathogens [2]. A key feature of the adaptive immune response is the ability to produce long-lived cells, commonly referred to as memory T and B cells. These memory cells persist in a dormant state and are able to re-express their effector functions rapidly upon second pathogen encounter.

The adaptive immune response is driven by T and B cells. Their activation is initiated by the antigen-specific receptor expression on their surfaces. The antigen-specific receptor, including T cell receptor (TCR) and immunoglobulin (B cell antigen receptor, Ig), are assembled via a repertoire of a few hundred germ-line-encoded

gene elements, which enables the formation of millions of different antigen receptors, each with a potentially unique specificity [1]. Both T and B cells arise from hematopoietic stem cells in the bone marrow. However, while mature B cells also develop in the bone marrow, T cell progenitors migrate to the thymus for further maturation into $CD4^+$ and $CD8^+$ T cells. Upon antigen encounter by antigen-presenting cells (APCs), T and B cells are primed and activated, respectively, resulting in their differentiation and homing to the site of inflammation to elicit their distinct immunomodulatory functions (Figure 1) [2].

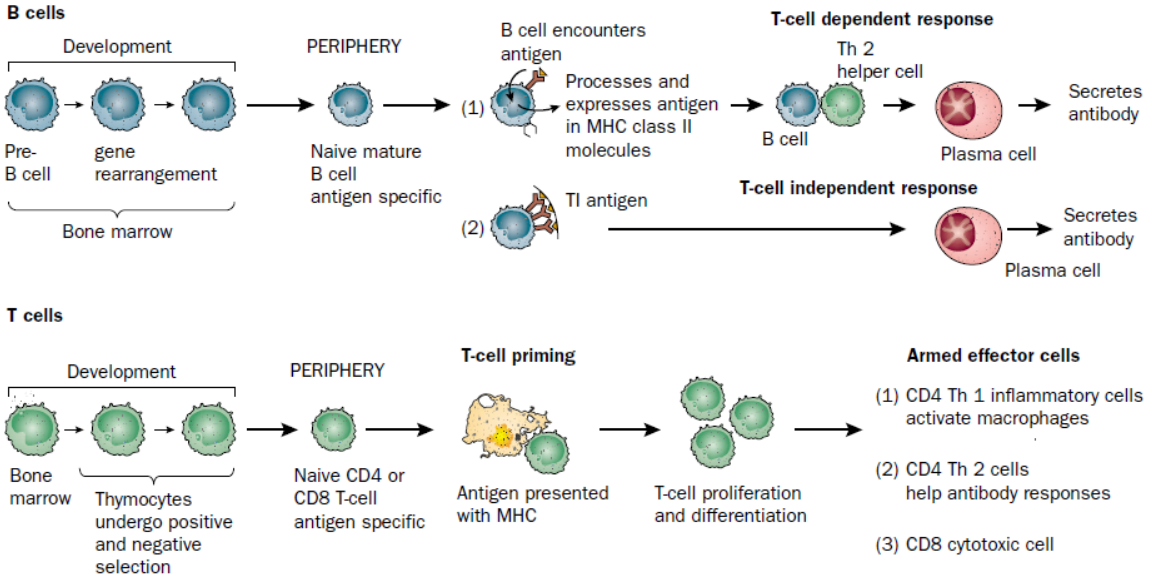


Figure 1: The development and activation of T and B cell to elicit specific immune responses.
Both T and B cells arise from hematopoietic stem cells in the bone marrow, but while mature B cells also develop in the bone marrow, T cell progenitors migrate to the thymus for maturation. Naïve mature B and T cells, including $CD4^+$ and $CD8^+$ T cells, circulate in the periphery, until antigen encounter leads to their activation and priming. B cell activation can result in a T cell dependent manner, where B cells act as antigen-presenting cells in order to drive T cell responses to promote their own proliferation and differentiation, but also in a T cell independent manner, where B cells secrete antibodies and differentiate directly into plasma cells. Naïve T cell activation results in the differentiation of distinct T helper cells as well as the development of cytotoxic $CD8^+$ T cells [2].

B cells are able to elicit their immune response in either a T cell dependent or a T cell independent manner. By processing and presenting antigens in association with MHC class II molecules, B cells are able to promote $CD4^+$ T cell responses. Another aspect of B cells is their T cell independent ability to secrete antibodies, commonly referred to as immunoglobulins (Igs). These antibodies circulate in the periphery and migrate into different organs to bind specifically to the foreign antigen that in turns stimulated their production. Binding of antibodies to their antigens inhibits the antigens ability to bind to host cell receptors and subsequently, inactivates foreign molecules such as viruses and microbial toxins (Figure 1) [2, 33].

T cells express a wide range of unique TCRs, which can bind specifically to foreign peptides. APCs, including B cells, macrophages, fibroblasts and epithelial cells but most commonly DCs, are able to process antigens and display their specific antigen peptides bound to MHC molecules on their surface. Upon recognition of these specific antigen peptides by TCRs, T cells undergo a priming process to elicit their immunomodulatory capacity and differentiate into effector T cells. These effector T cells are divided into two major groups, the $CD4^+$ T helper cells and the cytotoxic $CD8^+$ T cells [34].

Cytotoxic $CD8^+$ T cells are primarily involved in the elimination of pathogen-infected cells, but also tumor cells. The killing process is initiated after APCs phagocyte the foreign pathogen and present the MHC class I-embedded antigen on their surface. Upon recognition via the complementary TCR, the $CD8^+$ T cells are activated to proliferate according to clonal selection. Resulting $CD8^+$ T cells are then able to identify non-APCs displaying the same MHC class I antigen, including viral proteins and tumor cells. Consequent release of degradative enzymes, including perforin and granzymes, induces the apoptosis of the infected cells [35].

The central role of $CD4^+$ T helper cells is establishing and maximizing the immune response in an indirect manner. Naïve $CD4^+$ T helper cells recognize antigens loaded on MHC class II molecules via their TCR, resulting in their activation and differentiation into distinct subsets (Figure 2) [36].

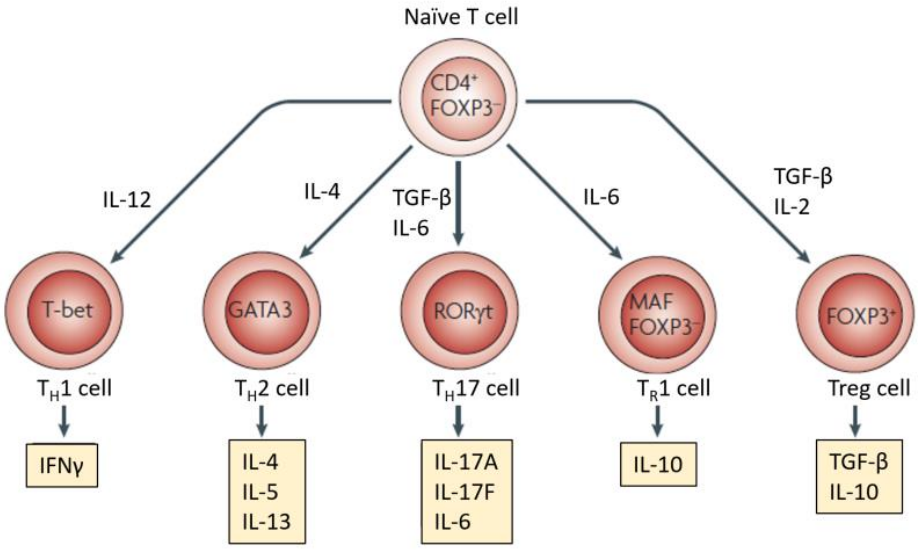


Figure 2: $CD4^+$ T helper cell subset differentiation.
Activation of the innate immune response upon pathogen encounter further induces the priming of naïve $CD4^+$ T cells. Specific cytokines favor the development of distinct $CD4^+$ T helper cell subsets, including T_H1, T_H2, T_H17, T_R1 and Tregs, each with a specific effector function. T_H1 cells are induced by the cytokine IL-12, express the T-box transcription factor (T-bet) and secrete IFN-γ. T_H2 cells differentiate in the presence of IL-4, express the transcription factor GATA-binding protein 3 (GATA3) and release IL-4, IL-5 and IL-13. T_H17 cells respond to

TGF-β and IL-6, express retinoid acid receptor-related orphan receptor gamma t (RORγt) and secrete the cytokines IL-17 and IL-6. T_R1 cells express the transcription factor proto-oncogene c-Maf (MAF), are activated by IL-6 and release IL-10. Treg cells are induced by TGF-β and IL-2, express the transcription factor forkhead box P3 (FoxP3) and secrete TGF-β and IL-10 (adapted from [36]).

The cytokine stimuli to promote $CD4^+$ T helper cell differentiation is mainly initiated by APCs but also by other members of the innate immune system, which is then commonly referred to as bystander activation. Type 1 helper (T_H1) cells express the T-box transcription factor (T-bet) and differentiate in response to IL-12. T_H1 cells are considered to act primarily against intracellular pathogens by secreting IFNγ to activate macrophages and NK cells. Type 2 helper (T_H2) cells are induced in the presence of IL-4 and IL-2. The major regulator of T_H2 differentiation is the transcription factor GATA-binding protein 3 (GATA3). The release of T_H2 signature cytokines IL-4, IL-5 and IL-13 is involved in the production of immunoglobulin E (IgE) by B cells, but also in the development and recruitment of mast cells and eosinophils, both demonstrated to be effective in the clearance of parasites. Type 17 helper (T_H17) cell differentiation is dependent on the transcription factor retinoid acid receptor-related orphan receptor gamma t (RORγt) and stimulated by TGF-β and IL-6 [37]. The T_H17 response is characterized by the production of IL-17A and IL-17F, which are able to induce the production of granulocyte-colony stimulating factor (G-CSF) and chemokines by stroma cells, resulting in the recruitment and maturation of neutrophils from the bone marrow to the site of inflammation [38]. Type 1 regulatory T (T_R1) cell differentiation is induced by IL-6 and regulated by the expression of the transcription factor c-MAF to produce IL-10. Regulatory T cell (Tregs) differentiation is stimulated by the cytokines TGF-β and IL-2 and regulated by the transcription factor forkhead box P3 (FoxP3) to produce TGF-β and IL-10. Both T_R1 cells and Tregs play an important role in the maintenance of immunologic tolerance to self and foreign antigens. However, the role of T_R1 cells is considered to be limited to local immune microenvironments, where specific antigens exist, whereas Tregs exert their regulatory immune functions throughout the whole body. As indicated by the name, Tregs are in charge of regulating and suppressing potentially deleterious activities of the aforementioned T helper cells to contribute to tissue homeostasis [37, 39].

1.1.2.1 Regulatory T cells

Regulatory T cells were first described in 1995 by Sakaguchi *et al.* as $CD4^+$ $CD25^+$ T cells, which exhibited a suppressive capacity [40]. Subsequent studies further

characterized the transcription factor FoxP3 to be essential for Treg development, function and homeostasis. Depending on their origin, $CD4^+$ Tregs are classified into two groups, natural Tregs (nTregs) and induced Tregs (iTregs). While nTregs develop and mature in the thymus, iTregs develop in the periphery from conventional naïve $CD4^+$ T cells. Another aspect of Treg function is their contribution to maintain self-tolerance, required for the prevention of autoimmune reactions. The underlying suppressive mechanisms consist of inhibitory cytokines, cytolysis, metabolic disruption and modulation of APC function. Tregs can secrete the inhibitory cytokines IL-10 and TGF-β, but also induce cytolysis by releasing granzyme A in humans and granzyme B in mice. Metabolic disruption of effector T cells is mediated by the high expression of CD25 on Tregs, through which Tregs are able to block and deprive local IL-2 [41]. Subsequent absence of the survival signal IL-2, results in a controlled cell death, also known as apoptosis. Apart from CD25, Tregs also express surface molecules such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and the lymphocyte activation gene 3 (LAG-3). By interacting with CD80/CD86 and MHC class II molecules presented on APCs, Tregs are able to enhance APC functions to suppress target cells. Accordingly, a dysregulation of Tregs in numbers and functions can therefore result in the development of autoimmune diseases [42].

1.1.3 The intestinal immune system

The human gastrointestinal tract reaches a surface of 400 m^2 and is continually exposed to 10^{14} microorganisms of at least 1000 bacterial species, particularly in the colon, where bacterial concentrations are highest [43]. Mucosal surfaces, such as the intestinal epithelium, are therefore uniquely adapted to prevent the invasion of foreign microorganisms, but are at the same time also able to discriminate harmful from harmless antigens. Most bacteria along the intestinal tract display beneficial functions, including vitamin production, absorption of ions (e.g. calcium, magnesium and iron), protection against pathogens, enhancement of the immune system as well as the fermentation of non-digestible foods to short chain fatty acids (SCFA) and other metabolites [43]. Therefore, it is not surprising, that the gastrointestinal tract comprises the largest compartment of the immune system, commonly referred to as the gut-associated lymphoid tissue (GALT). Accordingly, the majority of immunological processes take place in the intestinal mucosa, which is divided into

different segments, including the mucus layer, the epithelium and the underlying lamina propria (Figure 3).

The mucus layer extents up to 150 µm from the epithelial cell surface and contains antimicrobial peptides (AMPs), which are secreted by intestinal epithelial cells (IECs). These peptides consist of defensins, cathelicidins and C-type lectins and are able to eliminate bacteria directly through enzymatic lysis of the bacterial cell wall or by disrupting the inner wall of the bacteria. Furthermore, AMPs also include lipocalin 1, a protein with the ability to deprive bacteria of essential heavy metals like iron to limit their growth. Apart from this intracellular killing process, AMPs are also able to target intracellular components to damage organelles causing DNA fragmentation or to inhibit their enzyme activity [44]. Another component of the mucus layer are mucin glycoproteins. They are secreted by goblet cells and important for tissue protection [36]. Accordingly, studies could demonstrate that transgenic mice, deficient for the mucin glycoprotein Muc2, develop spontaneous intestinal inflammation due to the direct exposure to commensal bacteria [45]. Additional function of goblet cells is their ability to form goblet cell-associated antigen passages (GAPs) to transfer luminal substances to APCs in the underlying lamina propria in a controlled manner to induce the innate and adaptive immune responses in the GALT [46].

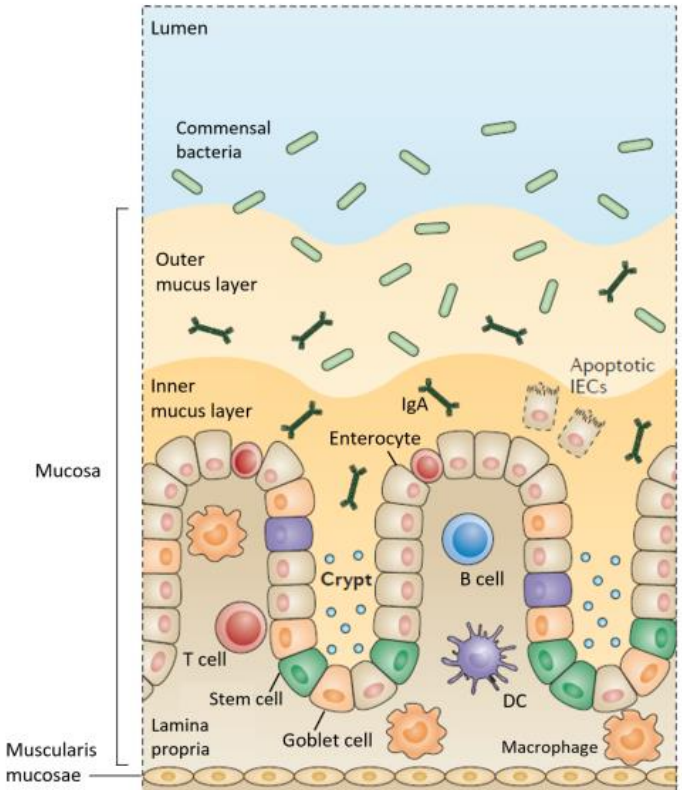


Figure 3: Conceptual structure of the intestinal mucosa.
The intestinal mucosa is divided into different layers, including the outer mucus layer, the inner mucus layer and the epithelium. Commensal bacteria are abundant in the outer mucus layer, but are unable to penetrate the inner mucus layer. The epithelial cells like enterocytes and goblet cells secrete antimicrobial peptides to support bacteria elimination. Goblet cells produce mucin glycoproteins that assemble an extensive and thick layer of protective mucus. The underlying lamina propria contains most of the intestinal immune cells, like T and B cells, macrophages and DCs. Differentiation of B cells into plasma cells, which can secrete IgA, further limits the numbers of penetrating bacteria (adapted from [47]).

The epithelium is essential for the maintenance of gut homeostasis. It is composed of a single cell layer and forms a physical barrier against commensal bacteria. Depending on the anatomical region of the gastrointestinal tract, including the small and the large intestine, the composition and function of the epithelium differ to adapt to the acquired immune response. The lamina propria harbors most of the intestinal immune cells from both the innate and adaptive immune system, including macrophages, DCs, ILC2s and eosinophils, but also T and B cells to regulate an appropriate immune reaction [47]. A unique feature of the intestinal immune system is the ability to process antigens from digested food or commensal bacteria without mounting an inflammatory response to maintain intestinal homeostasis [48]. This feature is mainly performed by the innate immune system through the induction of systemic immune tolerance and IgA secretion. During this process, APCs such as macrophages and DCs are critical for the decision between tolerance to self-antigens and inducing immunity to non-self-antigens [49]. APCs are preferentially localized beneath the epithelial monolayer to survey the luminal environment and to act immediately upon antigen encounter. At steady state, these tissue resident APCs continuously sample luminal antigens to shape an appropriate immune homeostasis. While macrophages are able to induce the clearance of apoptotic or senescent cells, DCs are considered to be tolerogenic through the induction of immune suppressing FoxP3$^+$ Tregs [50, 51]. In particular, mucosal tolerance is mainly induced by intestinal CD103$^+$ DCs, which are located throughout the lamina propria. These CD103$^+$ DCs induce Treg differentiation by producing retinoic acid in the presence of TGF-β, resulting in the recruitment of Tregs to intestinal tissues [52]. Another pivotal aspect to maintain intestinal homeostasis is the secretion of immunoglobulin A (IgA) antibodies by plasma cells. IgA antibodies display 4 to 8 antigen-binding sites, making them strongly effective against several bacterial antigens through the antibody-dependent cell-mediated cytotoxicity (ADCC) reaction. Therefore, IgA antibodies are able to prevent microbes from binding to IECs, suppress the pathogen growth and virulence and neutralize their toxins [53].

If the innate immune response is not sufficient to mount an effective response against the pathogenic stimuli, adaptive immune cells are recruited to the GALT primarily via APCs. Depending on the cytokine milieu and the dose of antigen presentation, the adaptive immune response can be protective and tolerogenic but can also create an inflammatory environment [54]. Upon pathogen encounter, APCs in the peripheral tissues load the antigen-specific peptides onto MHC class I and II

molecules. These antigen-loaded APCs migrate to the mesenteric lymph nodes, Peyer's Patches or isolated lymphoid follicles via the intestinal lymphatics. Once arrived, these APCs further support the generation of antigen-responding T cells to induce their priming. Activated T cells acquire a gut-homing phenotype to migrate back to the lamina propria via the blood system to initiate the adaptive immune response. Nevertheless, a fine-tuned balance between the inductions of T helper cell responses is needed to prevent the establishment of specific T helper cell-driven diseases [55]. Hence, a tight regulation of the GALT is necessary to maintain immune balance.

Collectively, the intestinal immune system consists of several physiological but also immunomodulatory functions to maintain tissue homeostasis while constantly being exposed to environmental microorganisms at steady state, but can also elicit an efficient immune reaction upon pathogen encounter without resulting in an excessive response to self-antigens. However, failure to regulate these functions can lead to severe pathological outcomes like inflammatory bowel diseases.

1.2 Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) describe disorders that involve chronic inflammation of the digestive tract. Two major conditions of IBD are Ulcerative colitis (UC) and Crohn's disease (CD) and both tend to arise in early adulthood [56]. In 2017, there were around 6.8 million people worldwide suffering from IBD, with a prevalence of 843 per 100.000 people. Interestingly, IBD has been considered as a disease of high-income nations; however, the epidemiology pattern has shifted over the last years with growing burdens and prevalence in newly industrialized countries in South America, Eastern Europe, Asia and Africa [57].

Even though CD and UC are both characterized by inflammatory processes within the intestinal tract, there are different manifestations of clinical features, including abdominal pain, rectal bleeding, fever, diarrhea and body weight loss. Furthermore, disease relapsing occurs in a random way and is still unpredictable. Since this persistent inflammatory activity negatively affects the patients' quality of life, therapeutic strategies aim to induce and maintain an enduring remission and to prevent disease progression.

Clinical manifestations of inflammation in patients suffering from CD can occur anywhere in the alimentary tract, from the mouth to the anus, exhibiting patchy, transmural inflammation of the mucosa. Consequent complications include the

development of strictures, abscesses and fistulae. In contrast, UC is a non-transmural inflammatory disease, exhibiting continuous, more superficial inflammation of the mucosa and submucosa and is restricted to the colon (Figure 4) [56]. Furthermore, it has been shown that UC patients exhibited an increased risk to develop colorectal cancer, which is estimated to be 2 % after 10 years, 8 % after 20 years and 18 % after 30 years of disease [58].

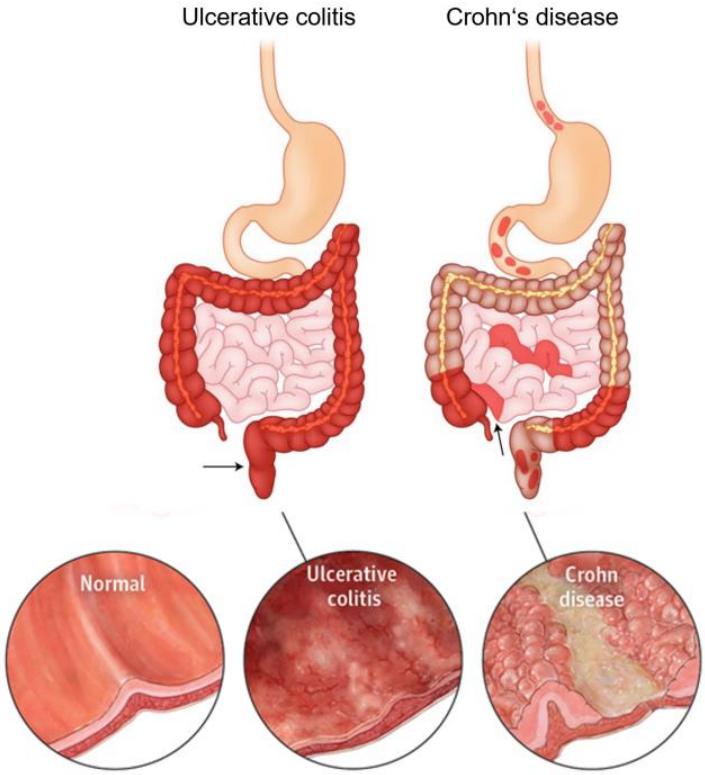


Figure 4: Clinical features of Crohn's disease and Ulcerative colitis.
Intestinal inflammation during Ulcerative colitis usually affects only the inner layer of the bowel wall and is mostly found in the rectum (arrow), spreading over the whole colon. In contrast, the inflammation during Crohn's disease has a segmented pattern all over the gastrointestinal tract, including all layers of the bowel wall and mostly affects the terminal ileum (arrow) (adapted from [56, 59]).

1.2.1 Etiology of inflammatory bowel disease

Accelerating incidences of IBD have been reported over the last years, resulting in extensive investigations in both human and animal IBD studies. However, the precise etiology of IBD is still unknown. So far, an imbalance of the complex interaction between genetic predisposition, environmental triggers, alteration in the gut microbiome composition as well as immunological factors has been considered to cause IBD [60] (Figure 5).

The influence of genetic predisposition to promote the development of IBD was confirmed by epidemiological studies. Genome-wide meta-analyses could reveal

several distinct loci, containing genetic evidence for an association with UC and CD, some unique and limited to one disease and some which are shared by both [61, 62]. In 2001, DNA analysis and sequencing resulted in the identification of the first association between the pathogenesis of IBD and the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) gene. A frameshift mutation in the NOD2 gene was reported to increase the risk of CD [63]. Subsequent studies further identified other genes with a susceptibility to IBD like the IL-23 receptor and caspase recruitment domain-containing protein 9 (CARD9) [64, 65]. Additional family studies further provided the evidence that genetic predisposition influences the development of IBD. Globally, up to 23 % of IBD patients reported to have a first-degree relative with the same disease [66]. However, the precise function of each gene regarding IBD is still unclear, since other studies could show that people with similar genotypes did not develop intestinal disorders, indicating the importance of other factors in IBD [67].

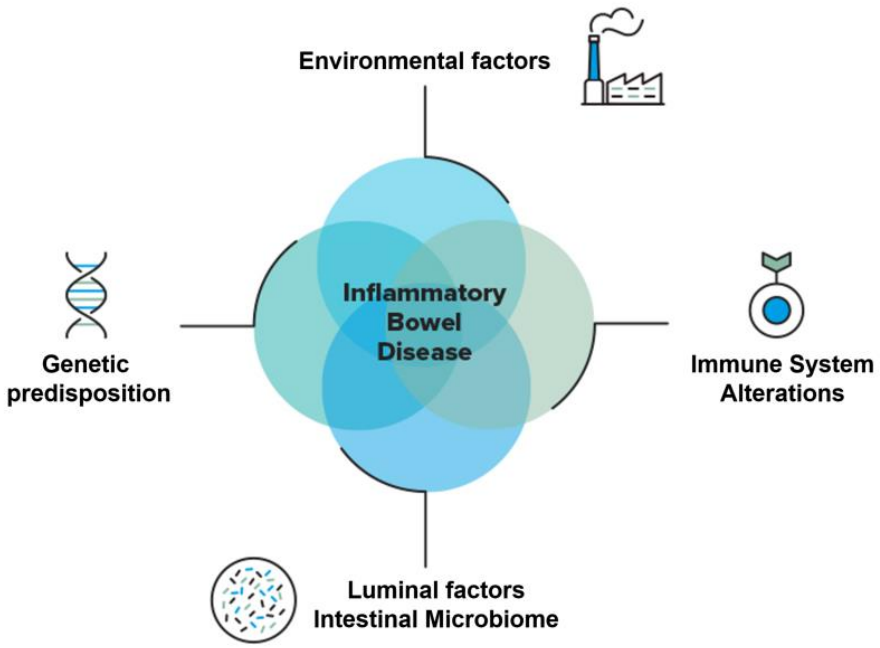


Figure 5: Interaction between different factors contributing to inflammatory bowel disease.
An Imbalance in the complex interaction between various factors, including genetic predisposition, environmental factors, luminal microbiome and alterations in the immune system, contributes to the establishment of inflammatory bowel disease (adapted from [68]).

As aforementioned, IBD is considered as a disease of high-income nations. Therefore, it has been suggested that environmental factors, including nutrition, antibiotics and sanitation, could be presumably involved in the establishment of IBD. However, no causative connections between these factors and IBD have been made so far [69]. Luminal factors from the intestinal microbiome are also proposed

to play a role during the development of IBD. Studies could show that treatment with broad-spectrum antibiotics and probiotics was able to improve clinical symptoms of IBD patients. This suggestion was further confirmed by experimental models of colitis, as rats and mice were not able to develop spontaneous colitis in a germ-free environment [70].

Currently, it is suggested that these genetic and environmental factors are the initial triggers for the establishment of IBD by impairing the intestinal barrier function (Figure 6) [71].

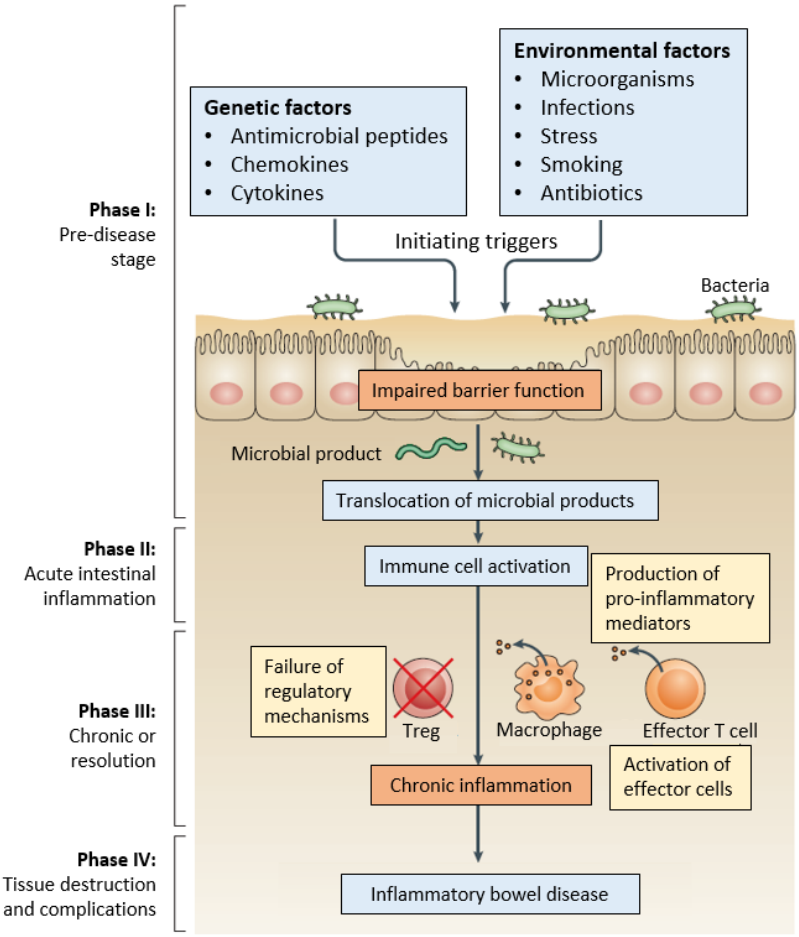


Figure 6: Conceptual structure for the pathogenesis of inflammatory bowel disease.
Several genetic as well as environmental factors, including antimicrobial peptides, chemokines, cytokines, microorganisms, infections, stress, smoking and antibiotics, are initial triggers to impair epithelial barrier function. Subsequent translocation of microbial products from the lumen into the lamina propria, results in an acute intestinal inflammation with immune cell activation and cytokine production. If the acute inflammation is not resolved by the anti-inflammatory immune suppression, chronic inflammation, including tissue destruction and complications such as fibrosis, abscess, fistula and cancer can occur (adapted from [71]).

Disruption of the intestinal barrier results in the translocation of commensal microorganisms into the bowel wall. Consequently, innate immune cells are recruited, resulting in their accumulation and the production of different cytokines and chemokines. Persistent microbial stimuli further lead to the alteration of the

cytokine milieu into a pro-inflammatory environment, which is first induced by innate immune cells but can be perpetuated by adaptive immune cells. IL-1 cytokines, including IL-1β and IL-18, have been demonstrated to be increased in the mucosa of UC and CD patients. Both IL-1β and IL-18 were shown to promote inflammation via the enhancement of T_H1 responses [72, 73]. Furthermore, T_H17 cells are considered to play a central role in the induction and maintenance of intestinal inflammation. However, their precise contribution is still under debate, since secreted cytokines, including IL-17A and IL-17F, comprise both pro-inflammatory as well as protective effects. IL-17 can amplify the induction of other pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), IL-6 and IL-1β, but is also important for neutrophil recruitment and induction of antimicrobial peptides [74]. IL-6 exerts its pro-inflammatory effects through the signal transducer and activator of transcriptions-3 (STAT3). STAT3 in turns induces anti-apoptotic factors like Bcl-2 and Bcl-xl to promote T cell resistance against apoptosis. The uncontrolled T cell expansion further contributes to the perpetuation of chronic inflammatory conditions [75]. Another key cytokine to drive IBD pathogenesis is TNF-α. Studies could show that TNF-α was able to induce the expression of aforementioned pro-inflammatory cytokines like IL-1β and IL-6 to promote disease progression. Moreover, TNF-α levels in the serum of IBD patients positively correlated with clinical severity [76]. Persistent release of pro-inflammatory cytokines further leads to an inappropriate activation of the adaptive immune response. Tregs are known to play a crucial role to preserve immune homeostasis and to regulate inflammatory responses to foreign and non-pathogenic antigens. Consequently, defective Treg functions due to the uncontrolled and excessive release of pro-inflammatory cytokines can result in chronic inflammatory disorders. Indeed, ablation of Treg function through mutations of CD25 and IL-10, both shown to be involved in Treg differentiation, resulted in an increased susceptibility to intestinal inflammation. In line, impairment of TGF-β signaling in Tregs also increased colitis progression [77].

Finally, these uncontrolled processes further promote the impairment of intestinal homeostasis by disrupting the intestinal barrier function. Persistent translocation of microbial products induce the excessive activation of immune cells and therefore maintain the pro-inflammatory environment. Together with a dysfunction in regulatory mechanisms, these processes lead to the perpetuation of chronic gut inflammation and favors the establishment of IBD (Figure 6) [78, 79].

1.2.2 Current therapy of inflammatory bowel disease

The diagnosis of UC and CD is often delayed despite their prominent manifestation, including abdominal pain, diarrhea, bloody stools and weight loss. Nevertheless, several therapeutic approaches were developed over the last years to treat IBD. To alleviate moderate to severe flare-up symptoms of UC patients, aminosalicylates are most commonly used in combination with steroids. This therapy generally consists of sulfasalazine, containing 5-aminosalicylic acid (5-ASA) linked to sulfapyridine by an azo bond. The disadvantages of this drug preparation are its inability to maintain remission and the increasing side effects such as headache and nausea, which makes it unsuitable for long-term therapies. In contrast, the immunosuppressant azathioprine requires several weeks to show a therapeutic effect, making it suitable for long-term treatment but ineffective during the acute phase [80, 81]. However, the efficacy of the 5-ASA medication is less striking in patients suffering from CD compared to UC patients. Therefore, glucocorticoid properties of hydrocortisone and prednisolone have been preferentially used for the treatment of both UC and CD patients. These corticosteroids are able to induce and maintain remission of IBD [82]. Nevertheless, these current drugs aim to induce and maintain the patients in remission but fail to modify or reverse the underlying pathogenic mechanisms.

Hence, mucosal immune cell recruitment and priming together with their mediators have emerged as an important area for therapeutic strategies. In particular, several studies have identified elevated production of different pro-inflammatory cytokines in patients suffering from IBD [83-85]. Experimental models of IBD in mice displayed additional evidence of a regulatory role for cytokines. Previous studies could show that mice deficient for the IL-2 or IL-10 cytokine spontaneously developed intestinal inflammation in a T-cell-dependent manner [86, 87]. Conversely, treatment with recombinant anti-inflammatory cytokines, including IL-10 and IL-22 was able to alleviate experimental colitis [88, 89]. Furthermore, the pro-inflammatory cytokines IL-6 and TNF-α have been demonstrated to be important mediators of intestinal inflammation, as both have been shown to be augmented in patients with active IBD compared to patients in remission or healthy controls [90, 91]. Well in line, suppression of the cytokine signaling with antibodies against TNF-α or IL-6 resulted in the reduction of intestinal inflammation in experimental mouse models of IBD [92, 93]. Human pilot trials using an antibody against IL-6R (tocilizumab) or an IL-6

blocking antibody to treat CD patients could report clinical efficacy [94]. However, in some patients IL-6 signaling blockade resulted in unwanted complications like abscess formation and intestinal perforation [95]. Clinical studies using neutralizing antibodies against TNF-α (infliximab, adalimumab and certolizumab) have shown to be effective in both CD and UC and are therefore currently used for IBD therapy [96]. Nevertheless, there are still many patients, who fail to respond to anti-TNF-α therapy or display a reduced clinical response over time [97].

1.2.3 Mouse models of inflammatory bowel disease

The limitations of human studies, including ethical principles, and the remarkable anatomical and physiological similarity between humans and animals, resulted in the development of various animal models to investigate the pathogenesis and etiology of human IBD. Even though these animal models do not summarize all features of human IBD, they still provide indispensable insights into the histopathological as well as the morphological changes and underlying factors, which are associated with the pathogenesis of IBD.

1.2.3.1 Dextran sulfate sodium-induced colitis

The Dextran sulfate sodium (DSS)-induced colitis model was initially reported in 1990 by Okayasu *et al.* [98] and represents the most widely established experimental model of intestinal inflammation. DSS is a negatively charged sulfated polysaccharide, which is soluble in water and has a highly variable molecular weight ranging from 5 to 1400 kDa [99]. Administration of DSS via the drinking water induces intestinal inflammation, primarily initiated by the disruption of the epithelial barrier and consequent translocation of luminal bacteria and associated antigens into the underlying lamina propria. The dissemination of pro-inflammatory contents leads to intestinal inflammation, mediated by the activation of the innate immune response [100]. Mice given DSS exhibit a pronounced body weight loss, altered stool consistency leading to bloody diarrhea and mucosal ulceration. During DSS-induced acute colitis, first symptoms of pathology are mucin and goblet cell depletion, epithelial disruption and ulceration as well as the infiltration of granulocytes into the lamina propria and submucosa. During DSS-induced chronic colitis, additional changes occur, including crypt architecture disarray and widening of the gap between the base of the crypt [101]. Moreover, prolonged application of DSS can result in metaplasia of the rectal mucosa and in combination with a pro-carcinogenic compound it can lead to a carcinogenic activity in the colon, such as

colitis-associated colorectal cancer (CAC) [102]. The effectiveness of intestinal inflammation can vary, depending on the DSS concentration, the duration and frequency of DSS administration as well as the age and genetic background of the experimental mice. Another aspect regarding the DSS effectiveness includes the microbial environment, whether the animals are germ-free or specific pathogen-free [99].

1.2.3.2 HA-specific T cell transfer colitis model

To analyze inflammation in the intestine in a T-cell-driven manner, the adoptive transfer of specific T cells into transgenic mice can be utilized. The first T cell transfer colitis model was described by Powrie *et al.* in 1994, when transfer of naïve $CD4^+CD45RB^{high}$ T cells into immunodeficient (SCID) mice or recombination activating gene 1 knock out (*RAG1*$^{-/-}$) mice resulted in the induction of intestinal inflammation [103].

In our experiments, we used the transfer of hemagglutinin (HA)-specific T cells into VILLIN-HA transgenic mice. VILLIN-HA mice specifically express the A/PR8/34 HA from influenza A under the control of the villin promoter in intestinal epithelial cells and were first described in 2005 [104]. TCR-HA x FoxP3eGFP mice express an α/β-TCR, which recognizes the MHC class II restricted epitope of HA, and the enhanced green fluorescent protein (eGFP) under the control of the FoxP3 promoter. Thus, binding of these HA-specific T cells to the HA epitope in intestinal epithelial cells of VILLIN-HA mice results in their activation and the promotion of inflammatory responses. In particular, adoptive transfer with sufficient numbers of HA-specific $CD4^+FoxP3^-$ T cells via i.v. administration into VILLIN-HA transgenic mice induces the development of acute colonic inflammation, indicated by the body weight loss, crypt loss and increased immune cell infiltration into the lamina propria [105].

1.3 IL-33/ST2 expression and signaling pathway

Interleukin 33 (IL-33) was first described in 2005 as a novel member of the IL-1 cytokine family 1 [106]. Subsequent studies discovered that IL-33 is encoded by a gene located on chromosome 9 in humans and chromosome 19 in mice [107]. The human gene product is a 30 kD protein and contains the 12 β sheet and a tetrahedron structure, which is characteristic for all members of the IL-1 cytokine family [108]. Sequencing analysis revealed a 52% conformity of human and mice IL-33 at amino acid level. In addition, it was shown that recombinant murine IL-33

was able to activate mouse lymphoid cells as efficiently as recombinant human IL-33 [107].

The IL-33 protein is composed of two evolutionary conserved domains, the nuclear domain, comprising the chromatin-binding motif and the IL-1-like cytokine domain, containing the cleavage site for caspases. Previous studies could show that the nuclear domain is essential for IL-33 function and regulation *in vivo* as specific deletion of this domain resulted in a systemic non-resolving lethal inflammation caused by a constitutive secretion of IL-33 into the circulation [109]. The nuclear and the IL-1-like cytokine domain are separated by a highly divergent linker region, the central domain, which holds the cleavage sites for inflammatory proteases (Figure 7) [107].

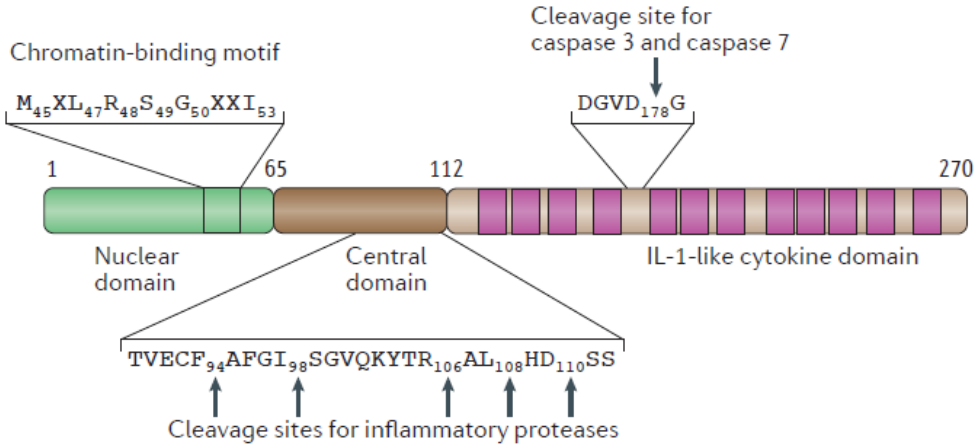


Figure 7: The interleukin-33 protein.
The interleukin-33 gene is located on chromosome 9 in humans and on chromosome 19 in mice. The gene consists of three different domains, including the nuclear domain, responsible for the chromatin binding, the central domain, containing the cleavage sites for inflammatory proteases and the IL-1-like cytokine domain, containing the cleavage site for caspases [107].

In contrast to conventional inducible cytokines, IL-33 is constitutively expressed at high levels in the nuclei of different cells, including fibroblast-like, endothelial and epithelial cells. At steady state, IL-33 acts as a nuclear transcription factor and is not actively secreted by cells [110]. Nuclear IL-33 (full-length) binds to heterochromatin and regulates different gene expressions. By interacting with histones H2A and H2B, IL-33 can activate histone deacetylase-3 (HDAC) activity, influencing gene expression by remodeling chromatin structures [111, 112]. Furthermore, IL-33 is able to bind to the N-terminal domain of the p65 subunit of the nuclear factor κB (NF-κB), driving the suppression of NF-κB-regulated gene expression, which are necessary for pro-inflammatory signaling [113]. However, it has been suggested that

the primary role of nuclear full-length IL-33 is to act as a regulator of its biological more active form (mature IL-33), preventing unwanted cytokine activity during tissue homeostasis [114].

The receptor for IL-33 was already described in 1988 as a gene, which was strongly induced during the initiation of cell proliferation of quiescent BALB/c-3T3 cells and was therefore termed serum stimulation 2 (ST2) [115]. Subsequent studies reported that the ST2 gene encoded a protein with high similarities in the sequences of the members from the IL-1 receptor family and is therefore also known as IL-1RL1 [116]. The ST2 gene is located on chromosome 2 in humans and on chromosome 1 in mice [117]. The encoded receptor exists in two splicing variants, the soluble ST2 (sST2) and the membrane-bound ST2 (ST2L) [118]. Since sST2 acts as a decoy receptor, it regulates IL-33 signaling by binding to secreted IL-33, inhibiting its interaction with ST2L. Apart from sST2, IL-33 can be further regulated by the phosphorylation and ubiquitylation of ST2L, leading to the internalization and degradation of the membrane-bound receptor. Accordingly, IL-33 is able to activate its cytokine signaling cascade by binding to its membrane-bound receptor ST2L. Subsequent conformational change allows ST2 to interact with the co-receptor IL-1 receptor accessory protein (IL-1RAcP). Consequent formation of a heterodimer leads to the dimerization of the Toll/IL-1 receptor (TIR) domain, which further leads to the recruitment of the myeloid differentiation primary response protein 88 (MyD88). Consecutive activation of the kinases IL-1R-associated kinase 1 (IRAK1), IRAK4 and TNF receptor associated factor 6 (TRAF6) results in the activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinases, such as extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinases (JNK) and promotes the transcription of different genes (Figure 8) [119].

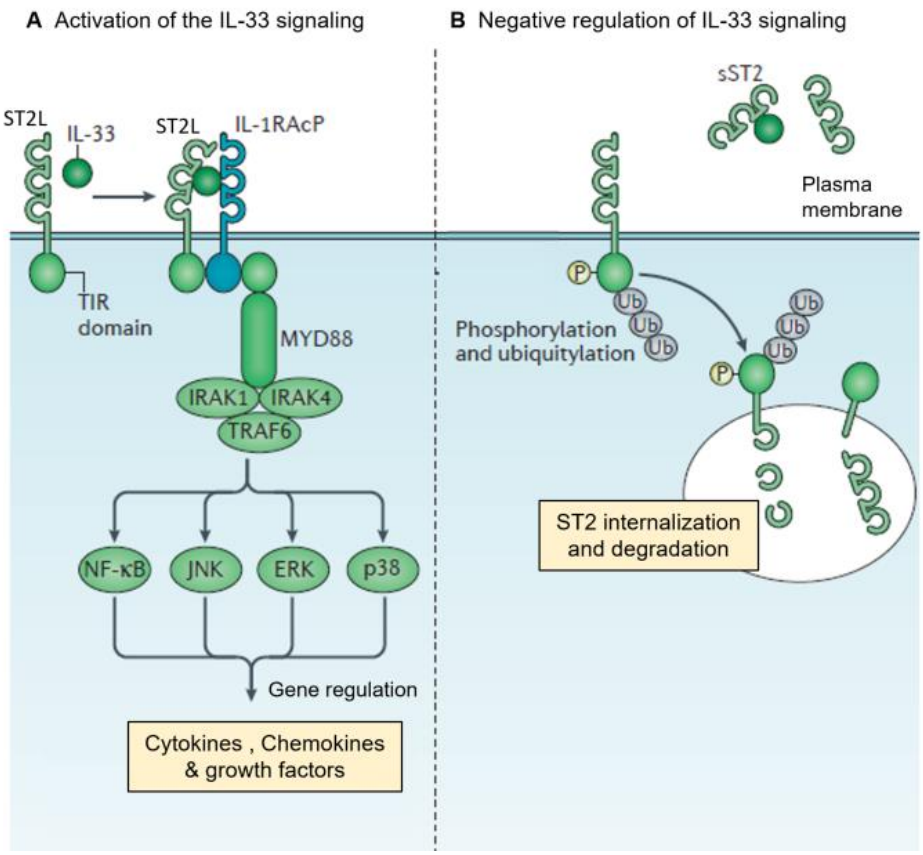


Figure 8: Activation and regulation of IL-33 signaling.
A. Interleukin-33 (IL-33) exerts its signaling by binding to its membrane-bound receptor serum stimulation-2 (ST2L). Subsequent conformational change enables the interaction of ST2L with the IL-1 receptor accessory protein (IL-1RA$_C$P), resulting in the dimerization of the Toll/IL-1 receptor (TIR) domain and the recruitment of myeloid differentiation primary response protein 88 (MyD88). Consequent activation of the kinases IL-1R-associated kinase 1 (IRAK1), IRAK4 and TNF receptor associated factor 6 (TRAF6) further leads to the activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinases (ERK, p38 and JNK), which drives the promotion of different gene transcriptions, such as cytokines, chemokines and growth factors. **B.** IL-33 is regulated by the soluble form of ST2 (sST2). sST2 acts as a decoy receptor and binds to secreted IL-33, preventing its ability to interact with ST2L. In addition, phosphorylation and ubiquitylation of ST2L leads to the internalization and degradation of the receptor and inhibits the interaction with IL-33 (adapted from [107]).

1.3.1 IL-33/ST2 expression in the intestinal epithelium and during IBD

In the gastrointestinal tract, IL-33 is expressed by non-hematopoietic cells, including adipocytes, fibroblast-like, endothelial and epithelial cells [120]. However, it has been shown that upon activation some hematopoietic cells such as DCs and macrophages are also able to express IL-33 at low quantities [121, 122]. IL-33 has been demonstrated to be constitutively expressed at high levels in endothelial and epithelial cells of the intestine. Upon cell injury or death, these cells release IL-33 as a full-length product into the extracellular environment to function as an endogenous danger signal or alarmin. In response to inflammatory stimuli, immune cells like mast cells and neutrophils secrete proteases to cleave full-length IL-33 to its more active

mature form. Subsequent mature (cleaved) IL-33 is then able to promote immune processes by binding to its receptor (Figure 9) [107].

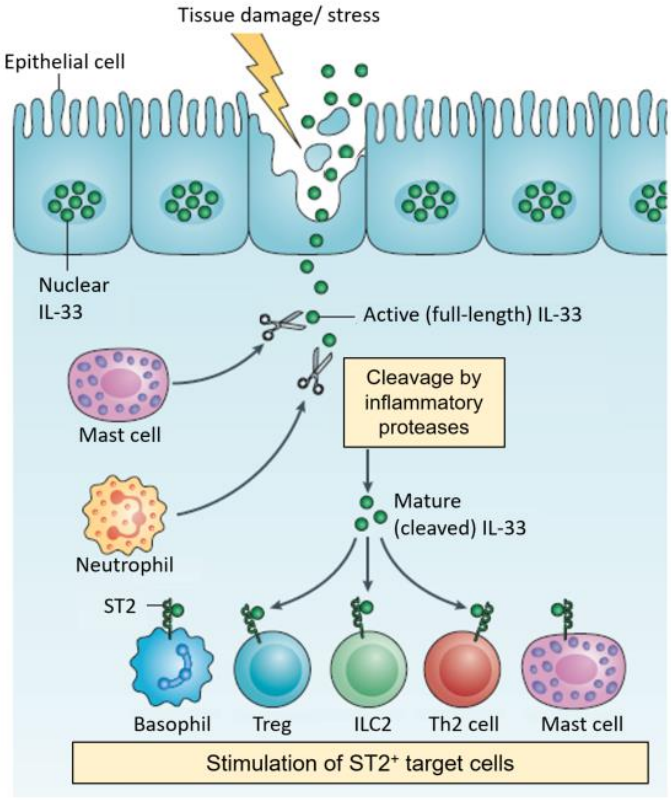


Figure 9: IL-33 activity upon tissue injury. Full-length IL-33 is released from the nucleus of epithelial cells upon tissue damage or stress and functions as an alarmin. Immune cells, including mast cells and neutrophils, produce inflammatory proteases to cleave full-length IL-33 to mature IL-33. Cleaved (mature) IL-33 possesses a strong cytokine activity and represents the major bioactive form of IL-33. Thus, mature IL-33 is able to promote different immune responses by interacting with $ST2^+$ target cells (adapted from [123]).

Since ST2L is expressed on the surface of a variety of immune cells, including T and B cells, but also type 2 innate lymphoid cells, macrophages, DCs and eosinophils, IL-33 is able to exerts its signaling through several immune cells to enhance their functions [119].

As aforementioned, IBD is primarily caused by a dysregulated immune response due to commensal gut bacteria encounter, resulting in the production of pro-inflammatory cytokines. The secretion of these cytokines and concomitantly, the release of chemokines leads to the attraction and accumulation of different immune cells to further impair the barrier integrity. Consequent disruption of the gut mucosa promotes the release of IL-33 as an alarmin [124].

Consistently, some studies could demonstrate that mucosal IL-33 expression was upregulated in patients with active IBD compared to patients in remission or healthy controls [125, 126]. Hence, the investigation of IL-33 function has emerged as a critical modulator during intestinal inflammation. So far, several studies using different animal models of colitis to elucidate the role of IL-33 have yielded conflicting results. The impact of IL-33 in the intestine can be detrimental or protective and seems to be dependent on different parameters, including the colitis

model and the genetic background of the mice, and whether the results were obtained during acute or chronic inflammation.

IL-33 is able to stimulate both innate and adaptive immune cells via the ST2 receptor, supporting their proliferation and survival as well as directing their migration capacity. Results obtained from Duan *et al.* demonstrated that DCs support Treg expansion in the lamina propria via the secretion of IL-2 upon IL-33 stimulation [127]. IL-33 can further stimulate IL-2 production by mast cells to mediate Treg enhancement [128]. Accordingly, Schiering *et al.* could show that IL-33 promoted the development of Tregs through the enhancement of transforming growth factor-β1 (TGF-β1) [129]. Other studies proposed that IL-33 treatment shifted excessive T_H1-directed cytokine responses during colitis toward a T_H2-like immune reaction to reduce intestinal inflammation [130]. Well in line, Monticelli *et al.* could demonstrate that IL-33 was able to protect and restore intestinal tissue homeostasis by promoting AREG-producing ILC2s [27]. Subsequent studies further revealed the ability of activated ILC2s to promote the polarization of M2 macrophages in the intestine [131]. Apart from these wound healing capacities of IL-33 activated ILC2s, they have also been shown to mediate intestinal inflammation by promoting the secretion of pro-inflammatory cytokines [132]. Additionally, it has also been demonstrated that IL-33 can mount an eosinophilic infiltration into the lamina propria, to restrain intestinal parasite infection but can also contribute to intestinal inflammation by enhancing the chemoattractant receptor-homologous molecule 2 (CRTH2) [133].

Even though these observations are controversial regarding the function of IL-33 in the intestine, they strongly suggest that IL-33 is implicated in the maintenance of the intestinal barrier function. Thus, IL-33 seems to play an important role in the development of inflammatory conditions in the intestine. Nevertheless, the diverse functions of IL-33 in the intestine due to the variety of several $ST2^+$ target cells are poorly understood and therefore need further investigation.

1.4 Aim of this study

In the last decades, extensive investigations have been done to understand the mechanisms and pathways involved in human IBD. Several studies have identified numerous potential targets; however, translating results from the bench to the bedside is still challenging. To prevent various complications and side effects, it is of essential importance to find improved therapeutic targets to counteract IBD development and disease progression.

IL-33 is a member of the IL-1 cytokine family and constitutively expressed at high levels in the nucleus of intestinal epithelial cells. Upon tissue injury or stress, mediated by the disruption of the epithelial barrier, endogenous IL-33 is released as an alarmin to promote inflammatory immune responses. However, previous findings about a host-protective or host-pathogenic role of IL-33 during intestinal inflammation remain controversial.
Therefore, the aim of the present study was to investigate the interplay of IL-33 and $ST2^+$ immune cells in more detail during intestinal inflammation to find new therapeutic approaches.

To analyze the function of IL-33 during IBD, two independent mouse models of acute colitis were used, including the chemical-induced mouse model of DSS colitis and a T cell transfer colitis model. Mice suffering from acute colitis were treated exogenously with recombinant IL-33 to elucidate its potential as a therapeutic drug. Due to the variety of $ST2^+$ target cells, we determined the impact of IL-33 on several immune cells from the innate as well as the adaptive immune system. After identifying regulatory T cells (Tregs), eosinophils and type 2 innate lymphoid cells (ILC2s) as key target cells, we investigated the IL-33-mediated contribution of these cells to intestinal inflammation.

Results obtained in this study will support the relevance of the IL-33 signaling during intestinal inflammation, highlighting its potential as a therapeutic drug to restrain DSS-induced acute colitis.

2 Material & Methods

2.1 Materials

2.1.1 Human samples

Mucosal biopsies were acquired from macroscopically inflamed or non-inflamed tissues of 13 IBD (3 MC and 10 UC) patients undergoing colonoscopy. Informed consent was obtained from all patients and ethical approval was provided by the Faculty of the University of Duisburg-Essen (15-6183-BO). Biopsy specimens were kindly provided by Prof. Dr. Jost Langhorst (Department of Internal and Integrative Medicine, Klinikum Bamberg, Chair for Integrative Medicine, University of Duisburg-Essen, Bamberg, Germany).

2.1.2 Experimental animals

Husbandry

All transgenic as well as wild type mice were bred and housed in the animal facility of the University Hospital Essen under specific pathogen-free conditions. Female and male mice used for the experiments were at least ≥ 8 weeks old and kept in a room with controlled temperature (~23 °C) and under 12 hour light and dark cycle. Animals were all on BALB/c background and housed in individually ventilated (IVC2) cages, receiving water and food *ad libitum*. All experiments were performed in accordance with the guidelines of the German Animal Protection Law and approved by the State Agency for Nature, Environment and Consumer Protection (LANUV), North Rhine-Westphalia, Germany.

2.1.2.1 BALB/c mice

Adult (6-8 week old) BALB/c wild type (WT) mice were purchased from ENVIGO (Horst, Netherlands).

2.1.2.2 DEREG/c mice

DEREG/c mice express a fusion protein, the simian diphtheria toxin (DT) receptor together with the enhanced green fluorescent protein (eGFP), under the control of the endogenous FoxP3 promoter. Transgenic DEREG/c mice were bred with BALB/c wild type mice to generate transgenic and wild type littermates. Administration of DT results in the ablation of $CD4^+$ $FoxP3^+$ Tregs with no effect on other effector T cells [134].

2.1.2.3 RAG2-Knockout (*RAG2*$^{-/-}$) mice

RAG2$^{-/-}$ mice exhibit a disruption of the recombination activation gene 2 (RAG2) resulting in a total inability to initiate V(D)J rearrangement and thereby fail to generate mature T and B lymphocytes [135]. Due to homozygous breeding, wild type mice from ENVIGO were co-housed for at least two weeks prior to experiment beginning and used as wild type control groups.

2.1.2.4 ST2-Knockout (*ST2*$^{-/-}$) mice

ST2$^{-/-}$ mice do not express the ST2 receptor for IL-33 [136]. These mice were bred homozygous to generate complete knockout mice. BALB/c wild type mice from ENVIGO were used as wild type control groups and co-housed with *ST2*$^{-/-}$ mice for at least two weeks prior to experiment onset.

2.1.2.5 TCR-HA x FoxP3eGFP mice

TCR-HA x FoxP3eGFP transgenic mice express an alpha/beta T cell receptor (α/β-TCR), specific for the HA epitope of influenza virus presented through the MHC class II (I-Ed) [137]. Furthermore, these mice co-express FoxP3, the transcription factor of regulatory T cells, and eGFP under the control of the endogenous promoter.

2.1.2.6 VILLIN-HA mice

VILLIN-HA transgenic mice exhibit the expression of hemagglutinin (HA) of influenza virus A/PR8/34 exclusively under the villin promotor, which is active in enterocytes of the intestinal epithelium [104].

2.1.3 Antibodies for flow cytometry

Table 1: Antibodies for flow cytometry

Epitope	Fluorochrome	Clone	Manufacturer
CD11b	APC	M1/70	BD Biosciences, Heidelberg, Germany
CD11c	APC	HL3	BD Biosciences, Heidelberg, Germany
CD127	PE	A7R34	BD Biosciences, Heidelberg, Germany
CD19	APC	1D3	BD Biosciences, Heidelberg, Germany
CD25	V450	PC61	BD Biosciences, Heidelberg, Germany

CD3	AF647	145-2C11	BioLegend, San Diego, USA
CD4	PB	RM4-5	BD Biosciences, Heidelberg, Germany
CD45	PerCP-Cy5	30-F11	BD Biosciences, Heidelberg, Germany
CD49b	APC	DX5	BioLegend, San Diego, USA
F4/80	FITC	BM8	Thermo Fisher Scientific, Braunschweig, Germany
FoxP3	FITC	FJK-16s	BD Biosciences, Heidelberg, Germany
Gr-1	APC	RB6-8C5	BD Biosciences, Heidelberg, Germany
ICOS (CD278)	BV510	C398.4A	BioLegend, San Diego, USA
Ly6G	PB	1A8	BD Biosciences, Heidelberg, Germany
SiglecF	PE	E50-2440	BD Biosciences, Heidelberg, Germany
ST2	PE-Cy7	RMST2-2	BD Biosciences, Heidelberg, Germany

2.1.4 Enzymes and nucleic acids

Table 2: Enzymes and nucleic acids

Enzymes / nucleic acids	Manufacturer
Gene Ruler 100 bp ladder Plus	Thermo Fisher Scientific, Braunschweig, Germany
GoTaq Hot Start Polymerase	Promega, Mannheim, Germany
GoTaq 5x flexi reaction buffer	Promega, Mannheim, Germany
Collagenase Type IV	Merck, Darmstadt, Germany
Desoxyribonuclease I (DNase I)	Merck, Darmstadt, Germany
Maxima SYBR Green/ROX qPCR Master Mix (2x)	Thermo Fisher Scientific, Braunschweig, Germany
Midori Green Advance	Nippon Genetics Europe, Düren, Germany
M-MLV RT (H-) Point Mutant	Promega, Mannheim, Germany

Oligo dT Primer	Invitrogen, Karlsruhe, Germany
Proteinase K	Merck, Darmstadt, Germany
Random Hexamer Primer	Invitrogen, Karlsruhe, Germany

2.1.5 Cytokines and antibodies for *in vivo* treatment

Table 3: Cytokines and antibodies for *in vivo* treatment

Cytokine/ antibody	Manufacturer
Recombinant mouse IL-33 (carrier-free)	BioLegend, San Diego, USA
*InVivo*MAb anti-mouse IL-5 (αIL-5)	BioXCell, Lebanon, USA

2.1.6 Primers

Table 4: Primer pair sequences for mouse genotyping PCR

Transgene	Sequence 5' → 3'	Annealing Temperature T^A (°C)
Foxp3eGFP	CGGCAAGCTGACCCTGAAGT GGATGTTGCCGTCCTCCTTG	58
Il33	GAGATGGCGCAACGCAATTAATG TGTGACTCACTCTGTGCAGAAGTGC	55-65
St2	GAGATGGCGCAACGCAATTAATG ATTTAAGGGAGGAGGTGAGGAACC	68
Rag2	GCTATTCGGCTATGACTGGG GAAGGCGATAGAAGGCGATG ATGTCCCTGCAGATGGTAACA GCCTTTGTATGAGCAAGTAGC	57
VILLIN HA	GCCTTAAGCCGGCTGTGATAGCA CTGGAAGCAGTGGGTCGCATTCT	55,6

VillinCre	ACGACCAAGTGACAGCAATG CTCGACCAGTTTAGTTACCC	60
TCR HA	CCTGAACTGGGGATTCTACTCTTCC AGTCAGCTTATTATTGCCTCCACTC	58

Table 5: Primer pair sequences for RT-qPCR - human

Target gene	Sequence 5' → 3'	Annealing Temperature T^A (°C)
Il33	GGAAGAACACAGCAAGCAAAGCCT TAAGGCCAGAGCGGAGCTTCATAA	58
Rps9	CGCAGGCGCAGACGGTGGAAGC CGAAGGGTCTCCGCGGGGTCACAT	58
sSt2	GAAAAAACGCAAACCTAACT TCAGAAACACTCCTTACTTG	50
St2l	AGGCTTTTCTCTGTTTCCAGTAATCGG GGCCTCAATCCAGAACATTTTTAGGATGATAAC	60

Table 6: Primer pair sequences for RT-qPCR - mouse

Target gene	Sequence 5' → 3'	Annealing Temperature T^A (°C)
Il-33	CTACTGCATGAGACTCCGTTCTG AGAATCCCGTGGATAGGCAGAG	58
Muc2	GCTGACGAGTGGTTGGTGAATG GATGAGGTGGCAGACAGGAGAC	58

Retnlb	GAACGCGCAATGCTCCTTTGAG AGCCACAAGCACATCCAGTGAC	58
Rps9	CTGGACGAGGGCAAGATGAAGC TGACGTTGGCGGATGAGCACA	58
Spdef	CACGTTGGATGAGCACTCGCTA AGCCACTTCTGCACGTTACCAG	58
sSt2	CGTGGGTCGTCTGCAGAAAT GCTCTCTGAGGTAGGGTCCA	55
St2l	TGTGAGCCGTGTGAGTTTGAGTGT TGGAGCAGCAGGCATGAGGAAGC	55

2.1.7 Buffer and media

Table 7: Buffer and media

Buffer/ media	Compound
ACK lysis buffer (pH 7.2-7.4)	Aqua dest. + 8,30 g/l Ammonium chloride + 1 g/l Monopotassium phosphate + 0,1 mM EDTA
Elution buffer	Aqua dest. + 100 mM Glycin, pH 3
IMDM complete media	IMDM with GlutaMax™ I and 25 mM HEPES + 10 % (v/v) FCS + 100 µg/ml Streptomycin + 100 U/ml Penicillin + 25 µM β-Mercaptoethanol
FACS buffer	PBS buffer + 2 % (v/v) FCS + 2 mM EDTA
Neutralization buffer	Aqua dest.

	+ 1 M Tris, pH 7,5
PBS buffer	Aqua dest. + 8 g/l Sodium chloride + 2 g/l Potassium chloride + 1,44 g/l Disodium phosphate + 0,2 g/l Potassium phosphate
PBS/EDTA buffer	PBS buffer + 3 mM EDTA
RPMI 1640 media	RPMI with GlutaMax™ I and 25 mM HEPES
RPMI/FCS media	RPMI with GlutaMax™ I and 25 mM HEPES + 20 % (v/v) FCS
RPMI/FCS/EGTA/$MgCl_2$ media	RPMI with GlutaMax™ I and 25 mM HEPES + 1 % (v/v) FCS + 1 mM EGTA + 1,5 mM $MgCl_2$
Tail buffer	Aqua dest. + 100 mM Tris HCL + 200 mM NaCl + 5 mM EDTA + 0,2 % SDS
TE buffer	Aqua dest. + 10 mM Tris HCL + 1 mM EDTA
TBE buffer	Aqua dest. + 89 mM Tris + 89 mM Boric acid + 2,53 mM EDTA

2.1.8 Chemicals

Table 8: Chemicals

Chemicals	Manufacturer
LE Agarose	Biozyme Scientific GmbH, Oldendorf, Germany

AutoMACS Pro Washing Solution	Miltenyi Biotec, Bergisch Gladbach, Germany
AutoMACS Running Buffer	Miltenyi Biotec, Bergisch Gladbach, Germany
Boric acid	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Collagenase IV	Sigma-Aldrich, St. Louis, USA
Dextran sulfate sodium – MW, 36-50 kDa (DSS)	MP Biomedicals, Eschwege, Germany
Diphtheria toxin	Merck, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethyleneglycoltetraacetic acid (EGTA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
EX-CELL® 610-HSF Serum-Free medium for hybridoma cells	Merck, Darmstadt, Germany
Fetal calf serum (FCS)	Biochrom GmbH, Heidelberg, Germany
Fixable viability dye eFluor780 (FvD)	BD Biosciences, Heidelberg, Germany
Heparin-sodium-25000-ratiopharm©	Merckle GmbH, Blaubeuren, Germany
HEPES 1 M	Gibco, Life Technologies, Carlsbad, Germany
Immobilized Protein G	Thermo Scientific, Darmstadt, Germany
Magnesium chloride ($MgCl_2$)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Monopotassium phosphate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Paraformaldehyde (PFA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Penicillin	Sigma-Aldrich, St. Louis, USA
Potassium chloride	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Potassium phosphate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Protein G IgG Binding Buffer	Thermo Scientific, Darmstadt, Germany
RLT buffer	Qiagen, Hilden, Germany

Streptomycin	Sigma-Aldrich, St. Louis, USA
Tris	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Typan blue	Sigma-Aldrich, St. Louis, USA
β-Mercaptoethanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

2.1.9 Consumables

Table 9: Consumables

Consumables	Manufacturer
Culture plate (6-, 12-, 24-, 96-well)	Greiner BioOne, Frickenhausen, Germany
Cell strainer (40, 70, 100 µm)	Falcon, Durham, USA
Disposable syringe (2, 10 ml)	BD Biosciences, Heidelberg, Germany
Micro tube (1.5, 2 ml)	Sarstedt, Nümbrecht, Germany
Needle	BD Biosciences, Heidelberg, Germany
Petri dish (92 x 16 mm)	Sarstedt, Nümbrecht, Germany
Pipettes (5, 10, 25 ml)	Greiner BioOne, Frickenhausen, Germany
Pipette tips (10, 200, 1000 µl)	Biozym, Thermo Fisher Scientific, Sarstedt, Germany
Poly-Prep chromatography column	BioRad, Düsseldorf, Germany
Tubes (15, 50 ml)	Greiner BioOne, Frickenhausen, Germany
Round bottom plates (96-well)	Greiner BioOne, Frickenhausen, Germany
Corning® Roller Bottles	Merck, Darmstadt, Germany
Slide-A-Lyzer Dialysis cassette	Thermo Scientific, Darmstadt, Germany
Syringe (1 ml)	BD Biosciences, Heidelberg, Germany

2.1.10 Kits

Table 10: Kits

Kits	Manufacturer
Alexa Fluor 467-Protein Labeling Kit	Invitrogen, Karlsruhe, Germany
$CD4^+$ T cell Isolation Kit, mouse	Miltenyi Biotec, Bergisch Gladbach, Germany
FoxP3 Staining Kit	eBioscience, San Diego, USA
Luminex Assay Mouse premixed Multi-Analyte Kit	R&D Systems, Minneapolis, USA
RNeasy Fibrous Tissue Mini Kit	Qiagen, Hilden, Germany

2.1.11 Equipment

Table 11: Equipment

Equipment	Manufacturer
7500 Fast Real-Time PCR System	Thermo Scientific, Darmstadt, Germany
autoMACS® Pro Separator	Miltenyi Biotec, Bergisch Gladbach, Germany
BD FACS Aria II cell sorter	BD Biosciences, Heidelberg, Germany
Binokular Axiovert Z1	Carl Zeiss Microscopy GmbH, Jena, Germany
Centrifuge MULTIFUGE 3SR+	Thermo Fisher Scientific, Waltham, USA
Centrifuge 5417R	Eppendorf AG, Hamburg, Germany
Flow cytometry BD LSR II	BD Biosciences, Heidelberg, Germany
GelDoc station	INTAS®, Göttingen, Germany
Electrophoresis Apparatus Horizon 11.14	Analytik Jena, Jena, Germany
Heracell 150i CO_2 Incubator	Thermo Scientific, Darmstadt, Germany
Luminex MAGPIX	Luminex Corportation, Austin, Texas, USA

NanoDrop 1000 Photometer	Peqlab, Erlangen, Germany
Neubauer counting chamber (0,0025 mm^2)	Superior, Marienfeld, Germany
Thermocycler T3000	Biometra, Göttingen, Germany
Work Bench Msc Advantage	Thermo Scientific, Darmstadt, Germany
Vortexer D-6013	Neo Lab. Heidelberg, Germany
Water bath	GFL, Burgwedel, Germany

2.1.12 Software

Table 12: Software

Software	Manufacturer
7500 Fast System Software	Thermo Scientific, Darmstadt, Germany
BD FACSDiva™ Software	BD Biosciences, Heidelberg, Germany
GraphPad Prism 7	GraphPad Software, La Jolla, USA
Luminex xPONENT Software	Luminex Corporation, Austin, USA
Microsoft Office 2016	Microsoft Corporation, Redmond, USA

2.2 Methods

2.2.1 Animal procedures

2.2.1.1 DSS-induced acute colitis

The DSS-induced acute colitis model was established by applying the respective amount (3 % – 4 %) of DSS within the drinking water for six consecutive days, followed by one day of normal drinking water. Mice were monitored daily for signs of sickness. The body weight was determined. Disease activity index, including body weight changes, rectal bleeding and the consistency of the feces, was scored as seen in table 13.

Table 13: Disease activity index

Score	0	1	2	3	4
Body weight loss relative to the initial weight	0 %	1-5 %	6-10 %	11-20 %	>20 %
Rectal bleeding	no blood	-	blood visible	-	blood around anus
Feces consistency	normal	soft stool	very loose stool	-	diarrhea

2.2.1.2 Adoptive transfer of T cells into VILLIN-HA transgenic mice

To induce acute colonic inflammation in VILLIN-HA transgenic mice, HA-specific T cells were FACS-sorted from the spleen of TCR-HA x FoxP3eGFP mice and adoptively transferred intravenously (i.v.) into VILLIN-HA transgenic mice (see chapter 1.2.2.2). 3 x 10^6 TCR-HA$^+$CD4$^+$FoxP3$^-$ cells were transferred per mouse. Mice were monitored daily for signs of sickness.

2.2.1.3 IL-33 treatment

Recombinant mouse IL-33 (rmIL-33) was administered intraperitoneally (i.p.) at 1 µg per mouse in a final volume of 200 µl PBS. If not mentioned otherwise, mice were treated at day 0, 2 and 5 after onset of DSS-induced acute colitis.

2.2.1.4 Eosinophil reduction

To decrease the amount of eosinophils *in vivo*, mice were injected i.p. daily with a monoclonal antibody (mAb) against mouse IL-5 (αIL-5) during DSS-induced colitis at a concentration of 200 µg per mouse in a final volume of 200 µl PBS [138].

2.2.1.5 Depletion of regulatory T cells

To deplete Tregs *in vivo,* DEREG/c transgenic mice were injected i.p. with 750 ng diphtheria toxin per mouse at day 0, 2 and 5 of DSS colitis in a final volume of 200 µl PBS [134].

2.2.1.6 Colonic biopsies

After isolating the colon, both length and weight were determined. Colonic biopsies of approximately 15 mg were taken from the rectal part prior to further processing the colon, e.g. to isolate lamina propria lymphocytes (LPLs).

To investigate the cytokine secretion from the colonic tissue, biopsies were cultivated in 300 µl IMDM complete medium for six hours. Subsequent centrifugation at 14.000 x g for 10 minutes separated the colonic tissue from the supernatant containing the released cytokines. Levels of cytokines were then quantified via Luminex technology (see chapter 2.2.3.6).

To identify the expression level of different genes via RT-qPCR, colonic biopsies were frozen in 300 µl RLT buffer at -20 °C for RNA isolation (see chapter 2.2.2.3).

2.2.1.7 Histopathological analysis

To identify signs of inflammation within the colonic tissue, histopathological analysis was performed. After isolating the colon, feces were flushed out with ice-cold PBS. The clean tissue was then embedded as a swiss roll in a tissue cassette between two filter papers and fixed in 4 % formaldehyde solution. For sectioning, the tissue was placed in paraffin and trimmed into slices with a thickness of about 4 – 5 µm, followed by staining with hematoxylin and eosin (H&E) and analysed at different magnifications. The severity of inflammation was determined using different parameters (table 14), which were scored individually from 0 to 3, where 0 means no pathology and 3 represents the most severe pathology.

Sectioning, staining and scoring of the pathology of the colon were performed by Robert Klopfleisch, Freie Universität Berlin, Institute of Veterinary Pathology, Germany, in a blinded manner.

Table 14: Histopathological parameters of intestinal inflammation

Scoring	Parameter
0 - 3	Infiltration of inflammatory cells in the *lamina propria* and *tela submucosa*
0 - 3	Epithelial defects
0 - 3	Goblet cell depletion
0 - 3	Granulocyte infiltration
0 - 3	Crypt abscess
0 - 3	Hyperplasia
0 - 3	Ulceration

2.2.1.8 Periodic Acid-Schiff staining to detect goblet cells

The periodic acid-schiff (PAS) staining is a histochemical technique to demonstrate the presence of carbohydrates and carbohydrate compounds, including polysaccharides, mucin, glycogen and fungal wall components. The PAS staining consists of an oxidative process, where polysaccharides react with the periodic acid, which in turn results in the production of an oxidized compound, called aldehyde. Subsequent fixation with the colorless Schiff reagent results in the coloration of aldehydes and detected as red or magenta. In this study, the PAS staining is used to visualize the polysaccharides in goblet cells located in the colon. Staining of the colonic sections was performed by Robert Klopfleisch, Freie Universität Berlin, Institute of Veterinary Pathology, Germany.

2.2.2 Molecular procedures

2.2.2.1 Genotyping of transgenic mice

To identify mice, which carry a WT and/or transgenic allele of the gene of interest, a polymerase chain reaction (PCR) was performed using tail biopsies. These tail biopsies from mice with an age of at least four weeks were digested in 90 µl tail-buffer, containing 10 µl Proteinase K (10 mg/ml), at 56 °C for at least three hours or overnight. Subsequently, samples were heat-inactivated for five minutes at 95 °C

using a Thermocycler and centrifuged at 6.000 x g for 5 minutes. The obtained supernatant, containing the genomic DNA, was diluted 1:50 in sterile H_2O to perform a PCR. The PCR was conducted by adding 1 µl of the diluted DNA to 19 µl of master mix (table 15). Depending on the target genes to determine the genotype of the mice, different primer pairs were used (table 4). Samples were run on a Thermocycler T300 with the corresponding program (table 16).

Table 15: Master mix for mouse genotyping PCR

Volume or concentration	Components
1x	Green GoTaq Flexi Buffer (5x)
1,5 mM	$MgCl_2$
1 mM	dNTPs
5 µM	forward primer
5 µM	reverse primer
0,5 U	GoTaq Hot Start Polymerase
Ad 20 µl	H_2O sterile

Table 16: PCR program for mouse genotyping PCR

Transgene			35 cycles		
St2	Temperature (°C)	94	94	68	72
	Time (mm:ss)	02:00	00:30	01:00	05:00

Trans-gene			10 cycles (decrease 1°C/ cycle)			30 cycles			
Il33	Temp (°C)	94	94	65	72	94	55	72	72
	Time (mm:ss)	05:00	00:15	00:30	00:40	00:15	00:30	00:40	05:00

Transgene			34 cycles			
Rag2	Temperature (°C)	94	94	57	72	72
	Time (mm:ss)	05:00	10:00	01:00	02:00	05:00

Transgene			10 cycles			27 cycles		
Cre, VILLIN-HA, TCR-HA, Foxp3eGFP	Temp (°C)	95	95	T^A	72	95	T^A	72
	Time (mm:ss)	10:00	00:30	00:30	01:30	00:15	00:45	00:45

2.2.2.2 Agarose gel electrophoresis

The amplification products of the PCR were analysed by agarose gel electrophoresis using a 1 % agarose gel in TBE buffer containing 0.005 % Midori Green DNA stain to visualize the DNA under an ultraviolet illuminator. The imaging was performed using the gel documentation system GelDoc Station.

2.2.2.3 RNA isolation

RNA was purified from colonic biopsies using the RNeasy Fibrous Tissue Mini Kit according to the manufacturer's instructions. In brief, the colonic tissue was lysed in a guanidine-isothiocyanate buffer and treated with proteinase K to efficiently release all RNA from tissue sections. After DNA digestion, followed by washing and centrifugation steps, the RNA was eluted in RNase-free water and stored at -80 °C for further analysis. RNA concentration was quantified using a NanoDrop spectrophotometer.

2.2.2.4 Synthesis of complementary DNA

1 – 2 µg of RNA was used to synthesize complementary DNA (cDNA). For primer annealing 0,5 µl of oligo(DT) and 0,5 µl of random primer were added to 13 µl of RNA and incubated in a Thermocycler T3000 for 10 minutes at 70 °C. After cooling down the samples on ice for at least one minute to ensure single-stranded RNA and efficient target annealing, the residual reaction components (table 17) were added to start the DNA polymerization at 42 °C for 60 minutes. The resulting cDNA synthesis was completed after enzyme heat-inactivation at 95 °C for five minutes (table 18).

Table 17: Master mix for cDNA synthesis per sample

Volume or concentration	Components
1 µl 10 mM	dNTPs
4 µl	M-MLV RT 5x Reaction Buffer
1 µl	M-MLV RT (H-) Point Mutant

Table 18: Program for cDNA synthesis

Step	1	2	3
Temperature (°C)	70	42	95
Time (mm:ss)	10:00	60:00	05:00

2.2.2.5 Semi-quantitative PCR

To validate the amount of cDNA and to ensure the concentration is equal throughout the analysed samples, a semi-quantitative PCR was performed prior to the RT-qPCR. The concentration was determined through the expression of the housekeeping gene ribosomal protein s9 (RPS9) by using the corresponding RPS9 primers (table 5 and 6), the PCR reaction mix described in table 15 and the PCR program in table 19. After analyzing the products via agarose gel electrophoresis (see chapter 2.2.2.2), the cDNAs were equalized to the same concentration.

Table 19: Program for semi-quantitative PCR

Target gene			10 cycles			27 cycles		
Rps9	Temp (°C)	95	95	T^A	72	95	T^A	72
	Time (mm:ss)	10:00	00:30	00:30	01:30	00:15	00:45	00:45

2.2.2.6 Real-Time quantitative PCR

To quantify the relative amount of specific genes the Real-Time quantitative PCR was performed using the MAXIMA SYBR Green Master Mix and the 7500 Fast Real-Time PCR system according to the manufacturer's recommendations. The composition for the RT-qPCR master mix is shown in table 20 and the program in table 21. A standard curve was generated to determine the quantity of each gene, which was then normalized to the housekeeping gene RPS9 as followed:

$$Relative\ expression = \frac{mean\ quantity\ of\ target\ gene}{mean\ quantity\ of\ RPS9\ gene}$$

To define the degree of quantity change between different groups and treatments, the fold change was calculated as followed:

$$Fold\ change = \frac{relative\ expression\ of\ each\ sample}{relative\ expression\ of\ controls}$$

Table 20: Master mix for RT-qPCR

Volume or concentration	Components
~ 20 ng	cDNA template
1x	MAXIMA Fast SYBR Green Master Mix
50 – 900 nM	Forward primer
50 – 900 nM	Reverse primer
Ad 20 µl	H_2O sterile

Table 21: Program for RT-qPCR

		40 – 45 cycles			melting curve			
Temperature (°C)	95	95	T_a	72	95	60	95	60
Time (mm:ss)	10:00	00:15	00:30	00:30	00:15	01:00	00:15	00:15

2.2.2.7 Purification of HA-specific (6.5) monoclonal antibody

To induce intestinal inflammation in VILLIN-HA transgenic mice, HA-specific T cells are required (see chapter 1.2.2.2). To be able to identify and sort these cells from TCR-HA x FoxP3eGFP mice, a monoclonal antibody (6.5 mAb), which is able to bind to the HA-specific TCR of $CD4^+$ T cells, was generated.

The hybridoma technology was used to purify the antibody from the supernatant of the 6.5 hybridoma cell culture (see chapter 2.2.3.1). The supernatant was diluted in a ratio of 1:1 with Protein G IgG Binding Buffer and transferred into a Poly-Prep chromatography column containing the immobilized Protein G to purify the antibody. After a washing step with 15 ml Protein G Binding Buffer, the antibody was extracted using an elution buffer and five fractions were collected, each in 100 µl neutralization buffer to counteract the physical instability of the antibody. In order to pool the three fractions with the highest amount of mAb, the protein concentration was determined at 280 nm using the spectrophotometer NanoDrop. To further purify the 6.5 mAb, the sample was loaded into a Slide-A-Lyzer Dialysis cassette and dialyzed in PBS

for 24 hours. Subsequent coupling with fluorochrome Alexa Fluor 467 using the Alexa Fluor 467 Protein Labeling Kit was performed according to the manufacturer's recommendations and enabled the 6.5 mAb detection via flow cytometry.

2.2.3 Cell biology procedures

2.2.3.1 Cell culture of 6.5 hybridoma cells

The hybridoma technology was used to produce the 6.5 mAb against the HA-specific TCR. Therefore, 6.5 hybridoma cells were cultured in IMDMc for 5 – 8 days at 37 °C and 5 % CO_2 to obtain a cell density of at least 1 x 10^8 cells. Cells were then split equally into two roller bottles, each containing 500 ml EX-CELL 610 – HSF serum-free media for hybridoma cells and cultured rotating for 10 rpm at 37 °C and 5 % CO_2 for 10 - 14 days. The viability was monitored from day 10 on via Trypan Blue staining (see chapter 2.2.3.3) until the percentage of viable cells was below 20, as the antigen-specific hybridoma cells produce high titers of antibodies under stress situation or cell death. The supernatant containing the secreted antibody was collected after a centrifugation at 300 x g for 10 minutes, followed by a second centrifugation at 1000 x g for 15 minutes and stored at 4 °C for further antibody purification (see chapter 2.2.2.7).

2.2.3.2 Preparation of single cell suspension

Single cell suspensions were generated from different organs (lamina propria and spleen) for further analyses. Prior to the isolation of these organs as well as the collection of blood through puncturing the heart, mice were sacrificed by carbon monoxide asphyxiation.

2.2.3.2.1 Lamina propria lymphocytes

To obtain lymphocytes from the lamina propria (LPLs), the colon was isolated and flushed with ice-cold PBS to clear the tissue from the feces. After removing remains of fat and connective tissue, the colon was cut into small pieces of about 1 cm length. Afterwards, the colon pieces were washed twice in 40 ml PBS supplemented with EDTA at 37 °C and 5 % CO_2 for 10 minutes under constant stirring to remove epithelial cells. Subsequent incubation of the colon pieces twice with 20 ml RPMI media supplemented with FCS, EGTA and $MgCl_2$ at 37 °C and 5 % CO_2 for 15 minutes under permanent stirring ensured the complete removal of EDTA prior to the digestion as EDTA inactivates the collagenase activity. After transferring the tissue pieces into a 50 ml tube containing 10 ml RPMI media supplemented with

FCS, EGTA and $MgCl_2$, samples were mixed for 15 seconds on a vortexer and washed in a 70 µm cell strainer with PBS. Samples were homogenized and digested constantly stirring in 30 ml RPMI media containing 20 % FCS and supplemented with collagenase IV (100 U/ml) for one hour at 37 °C and 5 % CO_2. During this incubation step, the cell suspension was raised up and down in a syringe ten times to further dissociate the tissue. This dissociation was repeated at the end of the incubation and the cell suspension was filtered through a 40 µm cell strainer, followed by a centrifugation at 300 x g for 10 minutes and a second centrifugation at 450 x g for five minutes. Cells were resuspended in IMDMc, filtered through a 30 µm filter to eliminate tissue remains and stored at 4 °C.

2.2.3.2.2 Spleen

Spleens were passed through a 70 µm cell strainer using a syringe stamp to disrupt the pulp and release the splenocytes. This process was done in erythrocyte lysis buffer (ACK) to exclude erythrocytes. After a washing step with FACS buffer to stop the lysis and a centrifugation at 300 x g for 10 minutes, cells were resuspended in IMDMc and stored at 4 °C until further investigation.

2.2.3.3 Cell counting

The absolute number of cells within a single cell suspension was quantified using a hemocytometer, the Neubauer chamber. Hence, the cell suspension was diluted and stained with Trypan Blue and 10 µl were applied into the counting chamber. Viable intact cells do not take up the dye, whereas dead or damaged cells will be stained and appear blue under the microscope.

2.2.3.4 Flow cytometry

Flow cytometry is a method to detect and analyze single cells in a cell suspension. This technique allows individual cells to pass through a laser beam, resulting in scattered light, which can provide information about the size and the granularity of the cells. Moreover, fluorochrome-conjugated antibodies can be used to label proteins on the surface of the cells, intracellular or even in soluble form. Emitted light from the excited fluorochrome can be detected through different filters and correlates with the expression of the specific molecule.

Flow cytometric analysis was performed using the Flow cytometer BD LSR II. For the adoptive T cell transfer colitis model, cells were analysed and sorted on a FACS Aria II. All data were visualized and analysed with the FACS DIVA software (8.0.1).

Staining of surface proteins

To analyze the expression of surface proteins, single cell suspensions were seeded in a 96-well round bottom plate and centrifuged for five minutes at 300 x g. Cells were then resuspended with FACS buffer supplemented with the fluorochrome-labelled antibodies of interest as well as a fixable viability dye to distinguish viable from dead cells. After an incubation of 10 minutes at 4 °C in the dark, cells were washed with FACS buffer and centrifuge for five minutes at 300 x g. Subsequent resuspension in FACS buffer enabled the analysis by a flow cytometer.

Intracellular staining of FoxP3

To stain the intranuclear transcription factor FoxP3, the FoxP3 Staining Kit was used according to the manufacturer's recommendations. In brief, after the surface staining, cells were incubated with a fixation and permeabilization buffer for one hour at 4 °C in the dark, followed by a washing step with the provided washing buffer and a centrifugation for five minutes at 300 x g. Cells were then stained with a permeabilization buffer containing the antibody against FoxP3 for 30 minutes at 4°C in the dark. After incubation, cells were washed, centrifuged and resuspended in FACS buffer for flow cytometry analysis.

2.2.3.5 Magnetic activated cell sorting

Magnetic activated cell sorting (MACS) is a method to enrich or deplete specific immune cell populations. $CD4^+$ cells were enriched using the $CD4^+$ T cell Isolation Kit according to the manufacturer's recommendations. In brief, cell suspensions were incubated with biotin-coupled antibodies binding to all non-target cells for 10 minutes at 4 °C, followed by five minutes incubation with anti-biotin MicroBeads. Subsequent separation via a MACS column was performed using the magnetic field of an autoMACS instrument. Due to the depletion program, non-target cells were bound, whereas $CD4^+$ target cells were untouched and passed through the column for further analysis.

2.2.3.6 Luminex technology

Cytokine secretion by colonic explants were quantified by Luminex technology using a Kit from R&D systems and performed according to the manufacturer's recommendations. In brief, this technology is based on a dual-laser flow system, which involves the incubation with color-coded beads coated with cytokine-specific antibodies. Subsequent incubation with a mixture of biotinylated detection

antibodies and a streptavidin-phycoerythrin (PE) reporter enables the quantification of secreted cytokines via two different lasers. One laser is able to determine the bead region and the corresponding assigned cytokine, whereas the other laser is able to determine the magnitude of the PE-derived signal and therefore provide information about the amount of the cytokine. The assay was performed on a Luminex 200 system and analyzed with the Luminex xPONENT software.

2.2.4 Statistics

Normality of results was tested using D'Agostino and Pearson and Shapiro-Wilk normality test. Statistical analyses were calculated using Mann-Whitney test, Wilcoxon matched-pairs signed rank test, one-way or two-way ANOVA, followed by Tukey's or Dunn's multiple comparison test or Sidak post-test. All analyses were performed using GraphPad Prism software version 7.03 (GraphPad Software, La Jolla, USA). Statistical significance were indicated with $*P < 0.05$; $**P < 0.01$ and $*** P < 0.001$.

3 Results

3.1 Impact of IL-33 signaling on intestinal inflammation

Inflammatory bowel diseases, including Crohn's disease and Ulcerative colitis, are disorders of the gastrointestinal tract, which are caused by a dysregulated immune response, and result in excessive induction of pro-inflammatory cytokines.

Recently, IL-33 has emerged as a critical modulator of the immune cell response upon tissue injury at mucosal surfaces; however, whether IL-33 exerts a host-protective or host-pathogenic function remains to be clarified. Thus, defining the mechanisms of IL-33 signaling in the intestine, specifically regarding its interaction with $ST2^+$ responding cells and their contribution to intestinal inflammation still needs further research.

3.1.1 Upregulation of *Il33* expression in the colonic tissues of IBD patients

To investigate the function of IL-33 at mucosal surfaces during intestinal inflammation, mRNA levels of *Il33* and its receptor *St2*, including the soluble (*sSt2*) and the membrane-bound form (*St2l)*, were measured in colonic biopsies of IBD patients, including both CD and UC patients, using RT-qPCR. Interestingly, there was a significant upregulation of *Il33* expression in colonic biopsies of inflamed tissues compared to non-inflamed control tissues (Figure 10A). In contrast, the expression of both the soluble form (*sSt2*) and membrane-bound (*St2l*) form of *St2* were not altered due to inflammation (Figure 10B).

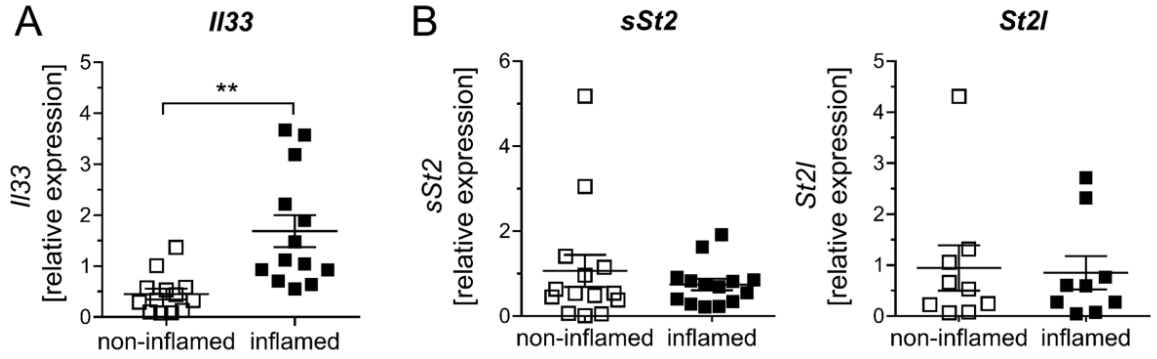


Figure 10: Expression of *Il33*, *sSt2* and *St2l* in colonic biopsies of IBD patients.
(**A**, **B**) The mRNA levels of *Il-33*, *sSt2* and *St2l* were determined in colonic biopsies of IBD patients using RT-qPCR (n = 9-14). Biopsies were differentiated into non-inflamed and inflamed tissues from the same patient. All data are presented as mean ± SEM. Statistical analyses were performed using Wilcoxon matched-pairs signed rank test. $^{**}P < 0.01$.

3.1.2 Upregulation of *Il33* expression in murine DSS-induced colitis

To study the role of IL-33 during acute intestinal inflammation in more detail, the murine DSS-induced colitis model was used. BALB/c were administered with 4 % of DSS in the drinking water for six consecutive days, followed by one day of normal drinking water (Figure 11A) and compared to healthy BALB/c mice (ctrl). Mice administered with DSS started to develop severe intestinal inflammation after four days. Severity of DSS-induced inflammation increased over time and was strongest at day seven, as indicated by the pronounced body weight loss (Figure 11B) and a high disease activity index (Figure 11C).

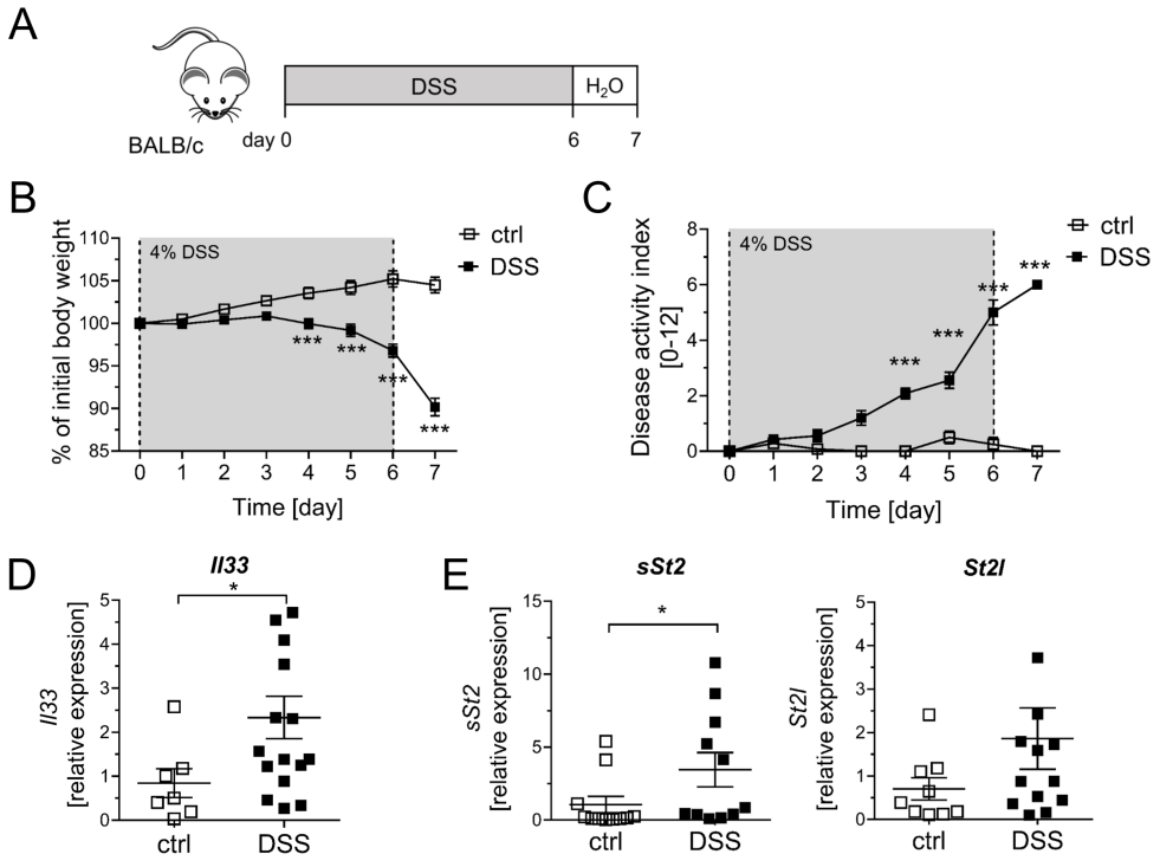


Figure 11: Expression of *Il33*, *sSt2* and *St2l* in colonic biopsies after induction of DSS colitis.
(**A**) To induce acute intestinal inflammation, BALB/c mice were given 4 % of DSS via the drinking water for six consecutive days, followed by one day of normal drinking water (n = 7-15 mice per group). (**B**) The body weight changes relatively to the initial weight as well as (**C**) the disease activity index were monitored daily. (**D, E**) mRNA expression of *Il33, sSt2* and *St2l* were quantified via RT-qPCR at day seven. All data are presented as mean ± SEM. Statistical analyses were performed using two-way ANOVA, followed by Sidak post-test (B, C) or Wilcoxon matched-pairs signed rank test (D, E). **P* < 0.05; ****P* < 0.001.

Interestingly, results obtained from DSS-treated mice were consistent with results from IBD patients. Compared to healthy control mice, DSS-treated mice displayed a significant upregulation of *Il33* expression in colonic biopsies (Figure 11D), which was accompanied by a slight increase of *sSt2* and *St2l* transcripts (Figure 11E).

In line with the mRNA analysis, secretion of the endogenous IL-33 cytokine in the supernatant of colonic explants was also significantly upregulated in DSS-treated mice compared to healthy controls (Figure 12A). Moreover, elevated levels of IL-33 positively correlated with the severity of intestinal inflammation, indicated by the disease activity index (Figure 12B). As expected, the development of severe intestinal inflammation due to DSS treatment, involved a significant increase of pro-inflammatory cytokines and chemokines, such as TNF-α, IL-6 and CXCL1/KC (Figure 12C).

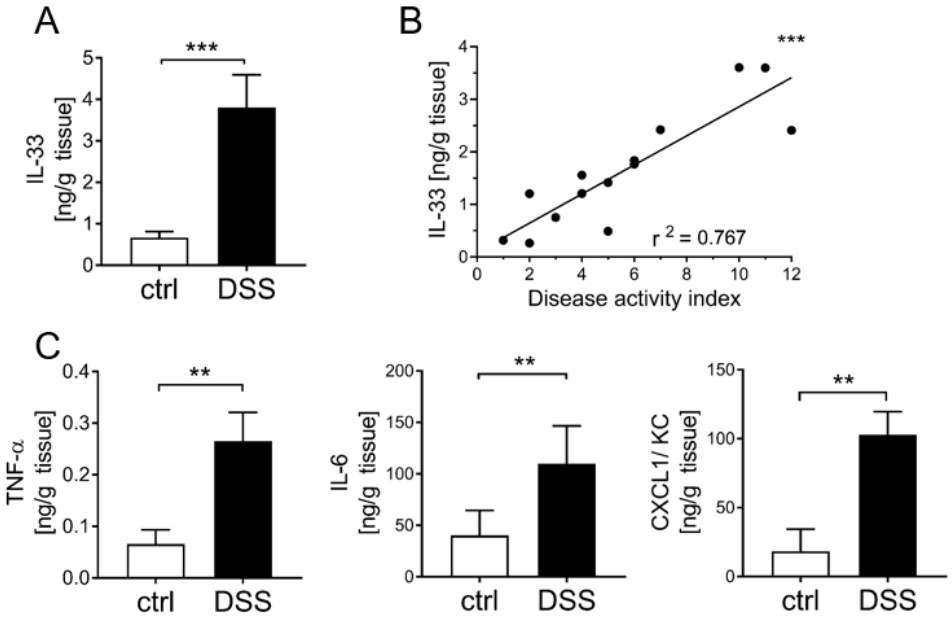


Figure 12: Elevated levels of pro-inflammatory cytokines and chemokines IL-33, TNF-α, IL-6 and CXCL1/KC in mucosal biopsies of DSS mice.

DSS colitis was induced in BALB/c mice by administration of 4 % of DSS via the drinking water for six consecutive days, followed by one day of normal drinking water. (**A, C**) Secretion of pro-inflammatory cytokines and chemokines, including IL-33, TNF-α, IL-6 and CXCL1/KC from the supernatant of colonic explants in DSS-treated mice compared to healthy control mice was quantified at day seven using Luminex technology (n = 7-15 mice per group) (B) IL-33 secretion was correlated with the disease activity index. All data are presented as mean ± SEM. Statistical analyses were performed using Mann-Whitney test (A, C) or the Pearson correlation coefficient (B). ***P* < 0.01; ****P* < 0.001.

3.1.3 ST2 expression is mandatory for the protection of mice from severe DSS-induced intestinal inflammation

To further investigate the role of IL-33 during acute colitis, we induced acute DSS colitis in ST2 deficient (*ST2*$^{-/-}$) mice and compared them to DSS-treated wild type (WT) BALB/c mice (Figure 13A). Interestingly, *ST2*$^{-/-}$ mice developed a more severe intestinal inflammation (*ST2*$^{-/-}$/DSS) compared to WT mice (WT/DSS), indicated by strong body weight loss (Figure 13B) and a significant higher disease activity index (Figure 13C). Consistently, the colon length shortening, which is characteristic for intestinal inflammation, was more pronounced in *ST2*$^{-/-}$ mice compared to wild type mice after DSS treatment (Figure 13D).

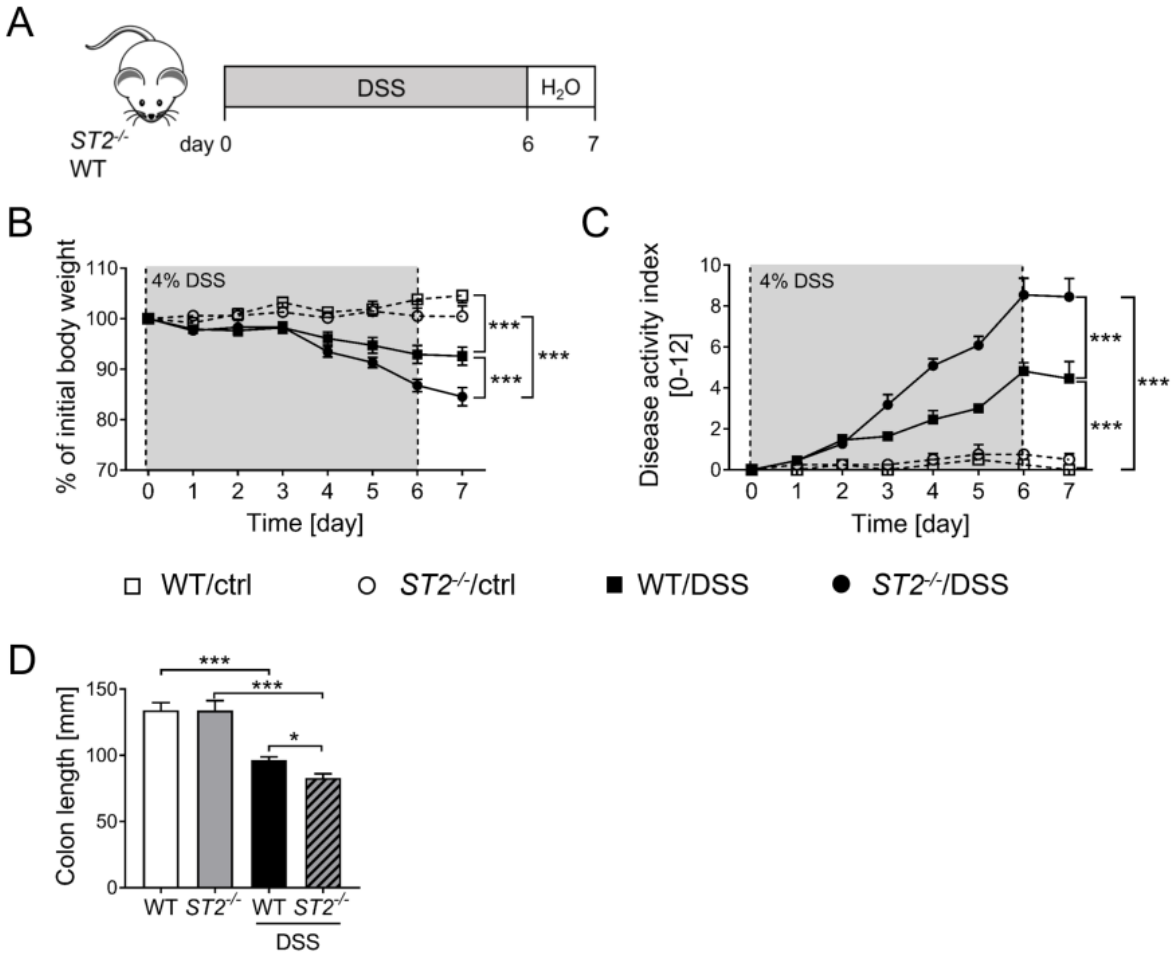
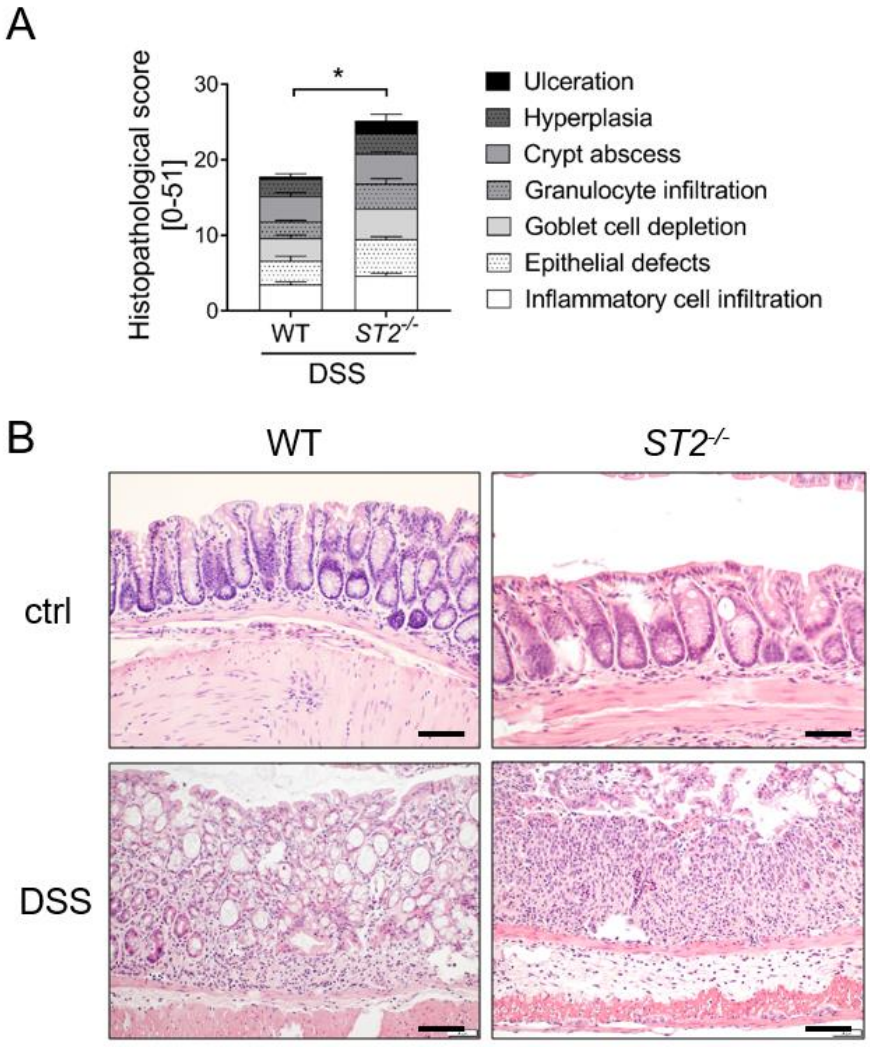


Figure 13: DSS-induced colitis is aggravated in *ST2*$^{-/-}$ mice.
(**A**) To induce intestinal inflammation in ST2 deficient (*ST2*$^{-/-}$) mice and wild type (WT) mice, 4 % of DSS was administered via the drinking water for six consecutive days, followed by one day of normal drinking water (n = 10-14 mice per group). (**B**) The body weight changes relative to the initial body weight and (**C**) the disease activity index were monitored daily. (**C**) The colon length was determined at day seven. All data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA (D) or two-way ANOVA (B, C), each followed by Tukey's multiple comparison test. **P* < 0.05; ****P* < 0.001.

The development of a more severe colitis was further confirmed by higher histopathological scores with increased disruption of the mucosal epithelial layer in *ST2*$^{-/-}$ mice in comparison to WT mice (Figure 14A, B). Well in line, DSS-treated *ST2*$^{-/-}$ mice exhibited an augmented pro-inflammatory cytokine and chemokine profile compared to DSS-WT mice with higher levels of pro-inflammatory cytokines and chemokines IL-33, IL-6, CXCL1/KC and in tendencies, more TNF-α (Figure 14C).

Collectively, these results demonstrate that the murine DSS-induced colitis model could mirror the inflammatory condition in human IBD tissues with a significant increase of endogenous IL-33. Strikingly, the absence of the IL-33/ST2 signaling pathway aggravated the disease severity in *ST2*$^{-/-}$ mice suggesting a host-protective function for IL-33 during DSS-induced acute colitis.



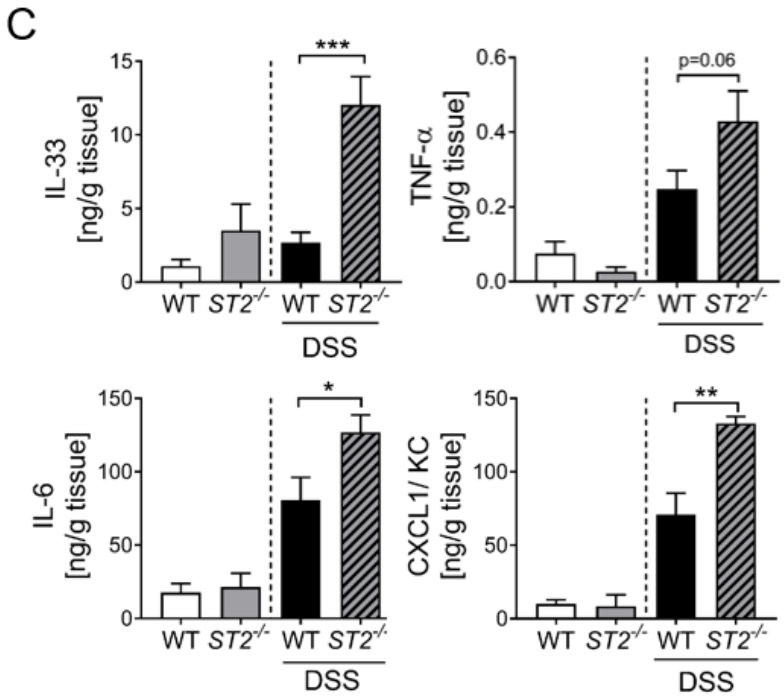


Figure 14: Increased colonic inflammation in *ST2*$^{-/-}$ mice was accompanied by higher levels of pro-inflammatory cytokines and chemokines.
To induce intestinal inflammation in ST2 deficient (*ST2*$^{-/-}$) mice and wild type (WT) mice, 4 % of DSS was administered via the drinking water for six consecutive days, followed by one day of normal drinking water. (**A**) Histopathological scores, including inflammatory cell infiltration, epithelial defects, goblet cell depletion, granulocyte infiltration, as well as crypt abscess, hyperplasia and ulceration were determined at day seven (n = 6 mice per group) and (**B**) representative hematoxylin and eosin (H&E) staining of colonic tissues from *ST2*$^{-/-}$ mice as well as WT mice were prepared. Scale bars represent 100 µm. (**C**) Secretion of pro-inflammatory cytokines and chemokines, including IL-33, TNF-α, IL-6 and CXCL1/KC by *ST2*$^{-/-}$ DSS-treated mice and WT DSS-treated mice as well as control mice were determined in the supernatants of colonic explants and quantified via Luminex technology (n = 10-14 mice per group). All data are presented as mean ± SEM. Statistical analyses were performed using Mann-Whitney test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

3.2 Exogenous IL-33 treatment as a therapeutic approach to prevent DSS-induced colitis

To further elucidate the function of the IL-33/ST2 signaling pathway in intestinal inflammation, BALB/c wild type mice were treated i.p. with recombinant IL-33 at day 0, 2 and 5 during DSS-induced acute colitis. DSS mice treated with PBS at the indicated time points served as internal controls (Figure 15A). Healthy control mice treated with IL-33 (IL-33/ctrl) showed no alterations in body weight or disease activity index compared to PBS-treated control mice (PBS/ctrl) (Figure 15B, C). Remarkably, during DSS-induced intestinal inflammation, the treatment with IL-33 had a tremendous impact on disease outcome. After IL-33 application, DSS mice exhibited a significant improved body weight at day six, which was even more pronounced at day seven when disease pathology was most severe in PBS-treated DSS (PBS/DSS) mice (Figure 15B). After five days, PBS/DSS mice started to

develop severe intestinal inflammation, indicated by the presence of body weight loss, diarrhea and rectal bleeding, which is summarized in the disease activity index. In contrast, the disease activity index remained low in IL-33/DSS mice and was strongly decreased at day seven compared to PBS/DSS mice (Figure 15C). Accordingly, shortening of the colon was not detectable in IL-33/DSS mice but was significantly induced in PBS/DSS mice (Figure 15D).

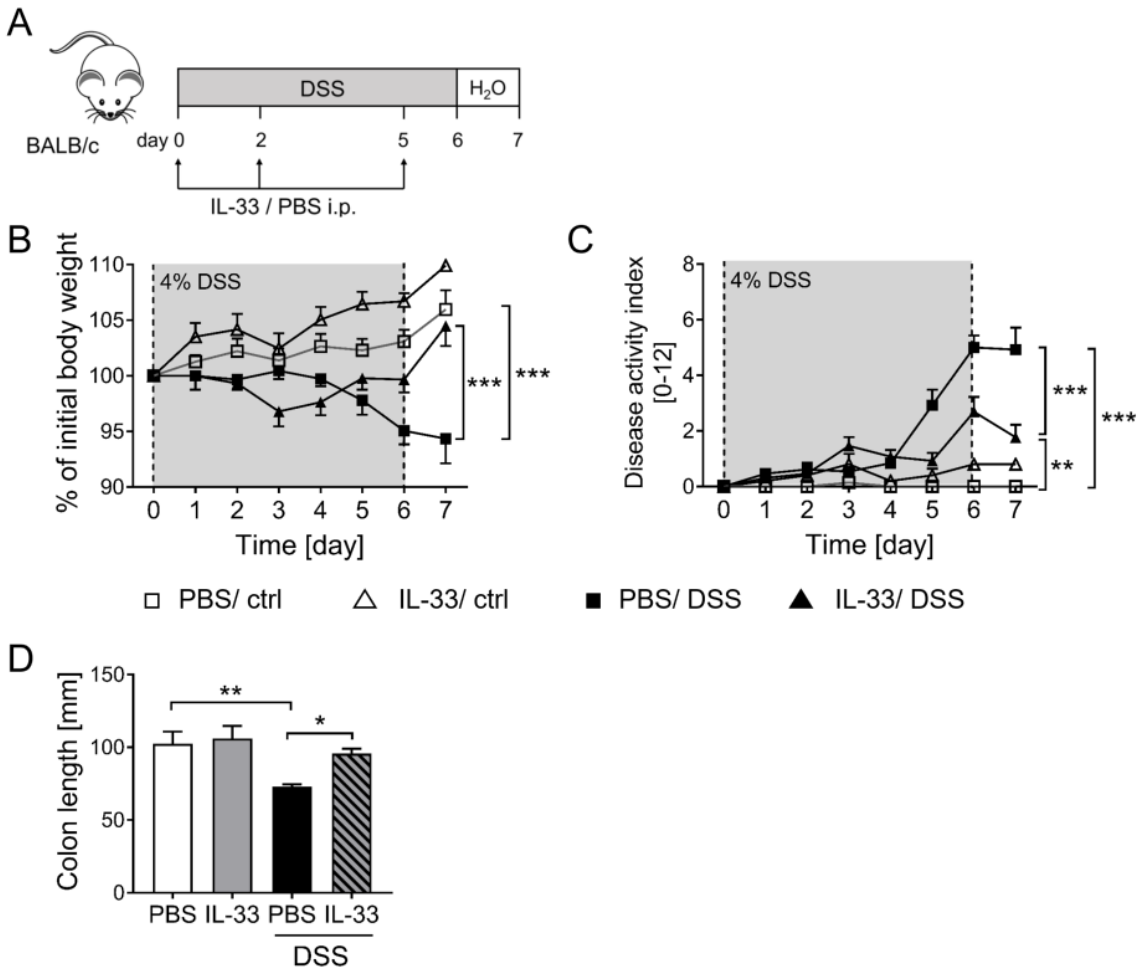
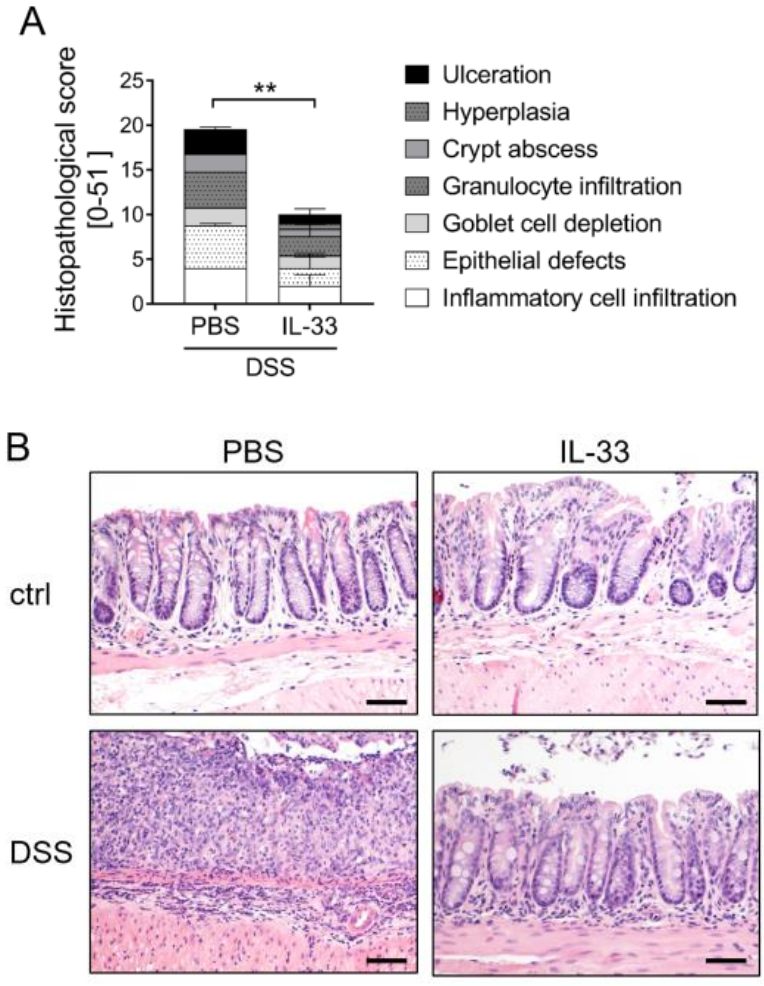


Figure 15: Exogenous IL-33 treatment ameliorates DSS-induced acute colitis.
(**A**) To induce intestinal inflammation, BALB/c mice were given 4 % of DSS via the drinking water for six consecutive days, followed by one day of normal drinking water. Mice were additionally treated i.p. with 1 µg of recombinant IL-33 or with 200 µl PBS per mouse on day 0, 2 and 5 (n = 7-13 mice per group). (**B**) The body weight changes relative to the initial weight and (**D**) the disease activity index were monitored daily. (**D**) Colon length was determined at day seven. All data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA (D) or two-way ANOVA (B, C), each followed by Tukey's multiple comparison test. *P < 0.05; **P < 0.01; *** P < 0.001.

Amelioration of intestinal inflammation in BALB/c mice due to exogenous IL-33 treatment was further confirmed by histopathological analyses. IL-33/DSS mice displayed a significant reduced score of inflammatory parameters, including inflammatory cell infiltration, epithelial defects, goblet cell depletion, granulocyte

infiltration as well as crypt abscess, hyperplasia and ulceration (Figure 16A). Consistently, disruption of the colonic epithelial layer was less prominent in IL-33-treated DSS mice compared to PBS-treated DSS mice (Figure 16B). Pro-inflammatory cytokine and chemokine secretion in the supernatants of colonic explants, including IL-33, TNF-α, IL-6 and CXCL1/KC, was significantly enhanced in DSS mice compared to healthy control mice. Strikingly, this increase was completely abrogated upon IL-33 treatment and comparable to the cytokine and chemokine levels of healthy control mice (Figure 16C). Furthermore, IL-33-treated mice showed a significant upregulation of type 2 cytokines IL-5 and IL-13, which was independent of DSS treatment (Figure 16D). Taken together, these findings demonstrate that treatment with exogenous IL-33 resulted in a strong amelioration of DSS-induced intestinal inflammation, suggesting IL-33 as a potential therapeutic drug to restrain colitis.



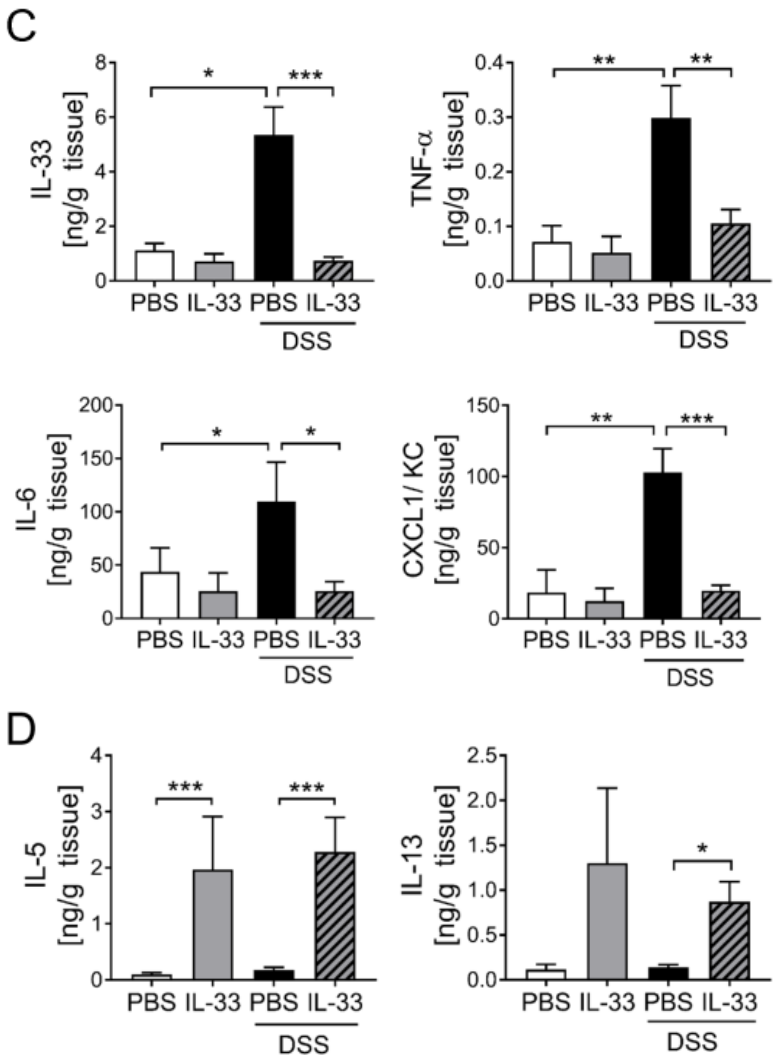


Figure 16: IL-33 treatment improves the colon pathology and reduces pro-inflammatory colonic cytokine secretions in DSS mice.
To induce DSS colitis in BALB/c mice, 4 % of DSS was administered via the drinking water for six consecutive days, followed by one day of normal drinking water. Mice were additionally treated i.p. with 1 µg of recombinant IL-33 or with 200 µl PBS per mouse on day 0, 2 and 5. (**A**) Histopathology, including inflammatory cell infiltration, epithelial defects, goblet cell depletion, granulocyte infiltration, as well as crypt abscess, hyperplasia and ulceration was scored in the colon at day seven (n = 5-13 mice per group) and (**B**) representative hematoxylin and eosin (H&E) staining of colon sections were prepared. Scale bars represent 100 µm. (**C-D**) Secretion of cytokines and chemokines, including IL-33, TNF-α, IL-6, CXCL1/KC, IL-5 and IL-13 was determined in the supernatants of colonic explants and quantified via Luminex technology. All data are presented as mean ± SEM. Statistical analyses were performed using Mann-Whitney test (A) or one-way ANOVA, followed by Tukey's multiple comparison test (C, D). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

3.2.1 Protective effects of IL-33 on intestinal inflammation are not limited to DSS-induced colitis

DSS treatment induces intestinal inflammation by compromising the mucosal barrier function. This promotes the translocation of commensal bacteria from the gut lumen to the lamina propria, resulting in an excessive pro-inflammatory immune response initiated by innate immune cells. Hence, the acute DSS colitis model is particularly

useful to study the contribution of the innate immune system to the development of intestinal inflammation. To address the question, whether the IL-33-mediated protection is limited to the DSS model, we also analyzed the impact of IL-33 administration in a T cell transfer colitis model. Here, HA-specific (TCR-HA$^+$) CD4$^+$FoxP3$^-$ T cells were adoptively transferred into VILLIN-HA transgenic mice at day 0 and mice were subsequently treated with recombinant IL-33 or PBS at day 0, 2 and 5 (Figure 17A). Upon T cell transfer, VILLIN-HA mice developed intestinal inflammation, demonstrated by a prominent body weight loss, formation of disease activity and a significant shortening of the colon length (Figure 17B, C, D). In contrast, IL-33 treatment was able to improve disease manifestations, since IL-33-treated VILLIN-HA mice showed no alterations in the body weight or in the colon length after HA-specific T cell transfer (Figure 17B, D).

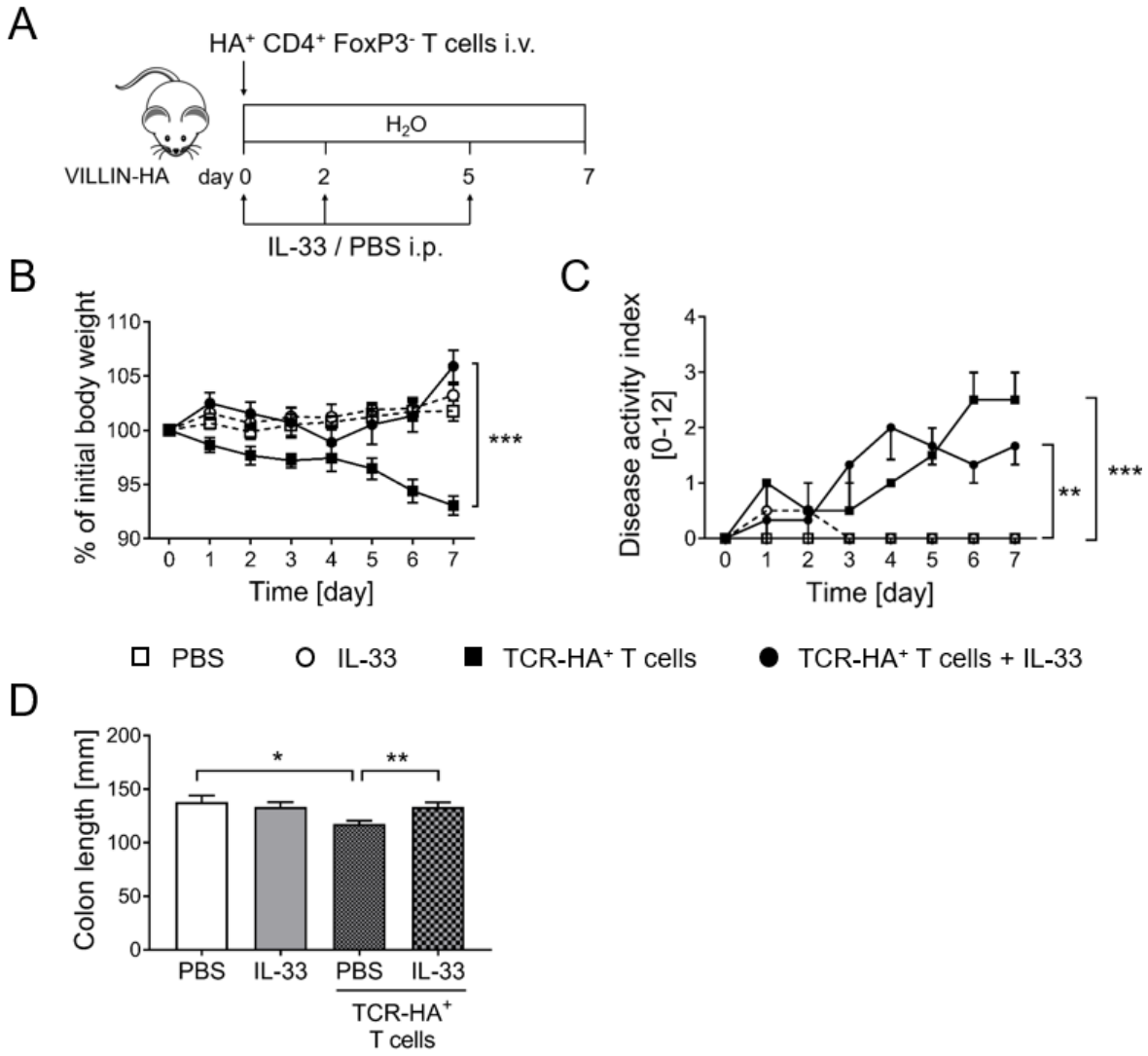
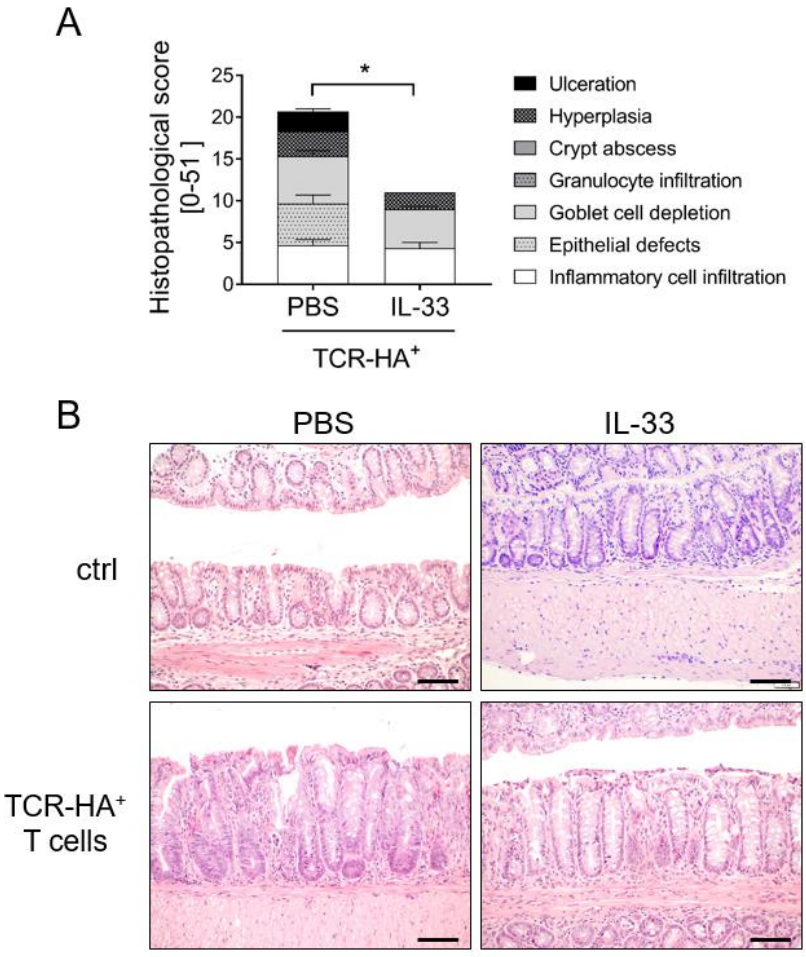


Figure 17: Exogenous IL-33 treatment protected mice from intestinal inflammation induced by the adoptive transfer of HA-specific T cells into VILLIN-HA mice.
(**A**) To induce intestinal inflammation, HA-specific (TCR-HA$^+$) CD4$^+$FoxP3$^-$ T cells were FACS-sorted from the spleens of TCR-HA x FoxP3eGFP mice and 3 x 10^6 cells per mouse were transferred i.v. into VILLIN-HA transgenic mice at day 0. Mice were additionally treated with 1 µg of recombinant IL-33 or 200 µl PBS per mouse

at day 0, 2 and 5 (n = 8-9 mice per group). (**B**) The body weight changes relative to the initial weight and (**C**) disease activity index were monitored daily. (**D**) Colon length was measured at day seven. All data are presented as mean ± SEM. Statistical analyses were performed using one-way (D) or two-way (B, C) ANOVA, each followed by Tukey's multiple comparison test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Accordingly, the histopathological score as well as the disruption of the epithelial integrity due to inflammation was significantly reduced upon IL-33 treatment. Interestingly, VILLIN-HA mice transferred with TCR-HA$^+$ T cells and treated with exogenous IL-33 showed no signs of epithelial defects, granulocyte infiltration, crypt abscesses or ulceration at all, in comparison to PBS-treated VILLIN-HA mice transferred with HA-specific T cells (Figure 18A, B). Pro-inflammatory cytokine secretion of TNF-α and IL-6 in the supernatants of colonic explants was slightly increased upon adoptive transfer of HA-specific T cells into VILLIN-HA mice, but was significantly reduced when mice were additionally treated with exogenous IL-33 (Figure 18C). Conversely, IL-33 treatment resulted in an upregulation of type 2 cytokines, including IL-5 and IL-13 (Figure 18D).



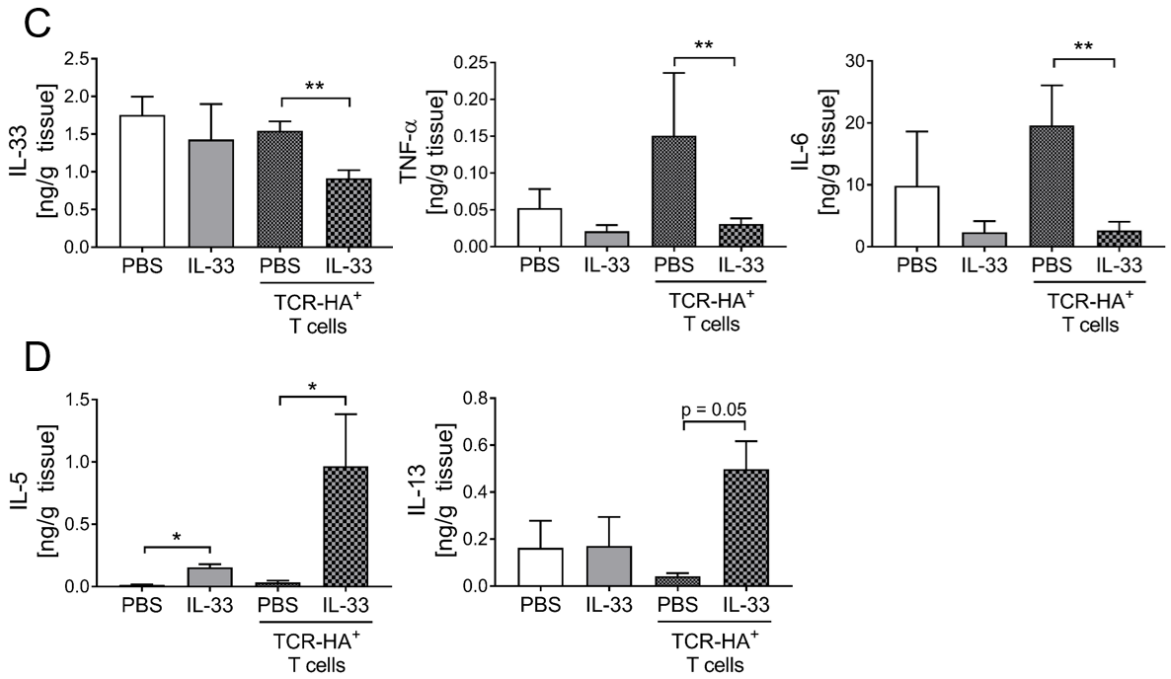


Figure 18: Exogenous IL-33 treatment improves the colon pathology and reduces pro-inflammatory colonic cytokine secretion in VILLIN-HA T cell transfer colitis.
Intestinal inflammation was induced by transferring 3 x 10^6 HA-specific (TCR-HA$^+$) T cells into VILLIN-HA mice. Mice were additionally treated with 1 µg of recombinant IL-33 or with 200 µl PBS per mouse at day 0, 2 and 5. (**A**) Histopathological score, including inflammatory cell infiltration, epithelial defects, goblet cell depletion, granulocyte infiltration, as well as crypt abscess, hyperplasia and ulceration was determined at day seven (n = 3 mice per group) and (**B**) representative hematoxylin and eosin (H&E) staining of colonic sections were prepared. Scale bars represent 100 µm. (**C**) Secretion of pro-inflammatory cytokines, including IL-33, TNF-α and IL-6 and type 2 cytokines IL-5 and IL-13, was determined in the supernatants of colonic explants and quantified via Luminex technology at day seven (n = 8-9 mice per group). All data are presented as mean ± SEM. Statistical analyses were performed using Mann-Whitney test (A) or one-way ANOVA, followed by Tukey's multiple comparison test (C, D). *P < 0.05; **P < 0.01.

Collectively, these results confirmed that the IL-33-mediated protection against intestinal inflammation was not limited to the DSS-induced colitis model but was also prominent in a T cell-driven colitis model.

3.3 Impact of IL-33 on innate and adaptive immune cells during DSS-induced colitis

Since ST2, the receptor for IL-33, is expressed on a variety of immune cells, including cells from both the innate as well as the adaptive immune response, IL-33 is able to promote distinct immunomodulatory functions. Upon tissue injury due to DSS treatment, innate immune cells are recruited to the colon [139]. Accordingly, we could observe a moderate increase in the frequencies of macrophages, NK cells, and DCs in the colonic lamina propria in mice with DSS-induced colitis (Figure 19A; C, D). Neutrophils and eosinophils were significantly expanded in the colon of DSS-treated mice compared to healthy control mice (Figure 19B, E). No alteration was

observed in the frequency of colonic ILC2s in DSS-treated mice (Figure 19F). Interestingly, IL-33 treatment caused a strong increase in the frequencies of both eosinophils and ILC2s compared to PBS-treated DSS mice (Figure 19E, F).

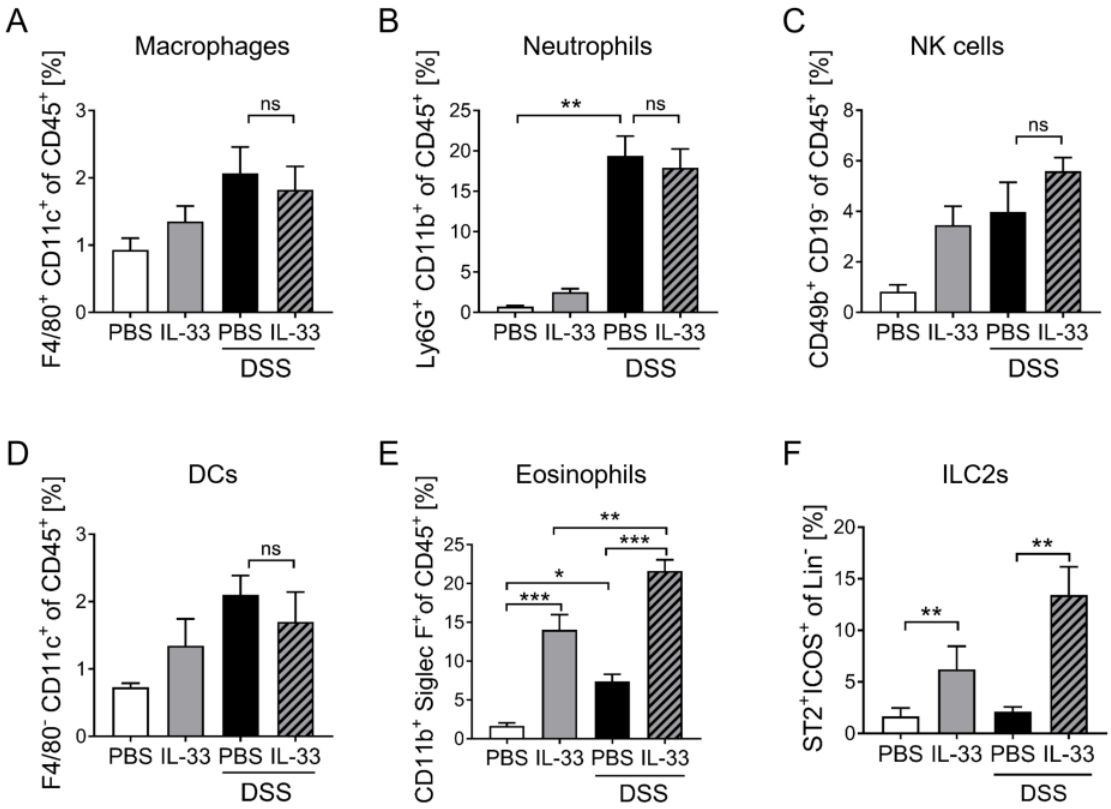


Figure 19: Exogenous IL-33 treatment enhances the frequencies of colonic eosinophils and ILC2s during DSS-induced colitis.
DSS colitis was induced in BALB/c mice by administering 4 % of DSS via the drinking water for six consecutive days, followed by one day of normal drinking water. Mice were additionally treated with 1 µg of recombinant IL-33 or 200 µl of PBS per mouse at day 0, 2 and 5. Lymphocytes were isolated from the colonic lamina propria at day seven (n = 4-11 mice per group). All frequencies were analyzed via flow cytometry. (**A**) Macrophages were defined as $F4/80^+CD11c^+CD45^+$, (**B**) neutrophils were defined as $Ly6G^+CD11b^+CD45^+$, (**C**) NK cells were defined as $CD49b^+CD19^-CD45^+$, (**D**) DCs were defined as $F4/80^-CD11c^+CD45^+$, (**E**) eosinophils were defined as $CD11b^+SiglecF^+CD45^+$ and (**F**) type 2 innate lymphoid cells (ILC2s) were defined as $ST2^+ICOS^+Lineage^-$ cells. All data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Dunn's multiple comparison test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

The colonic lamina propria harbors immune cells from both the innate as well as the adaptive immune response. Therefore, we assessed the frequencies of B and T cells during DSS-induced colitis and further analyzed, whether these cell were affected by IL-33 treatment. No alterations were observed in the frequencies of B cells and $CD8^+$ T cells in DSS-treated mice compared to healthy control mice, independently of PBS or IL-33 treatment (Figure 20A, B). Even though the overall frequencies of $CD4^+$ T cells showed no significant changes (Figure 20C), the frequencies of Tregs were slightly increased upon DSS-induced colitis, which was even more prominent after IL-33 treatment (Figure 20D).

These results demonstrated that treatment with IL-33 during DSS-induced colitis had a strong impact on three distinct cell populations, namely Tregs, eosinophils and ILC2s, suggesting a crucial role of these cells for the IL-33-mediated tissue protection.

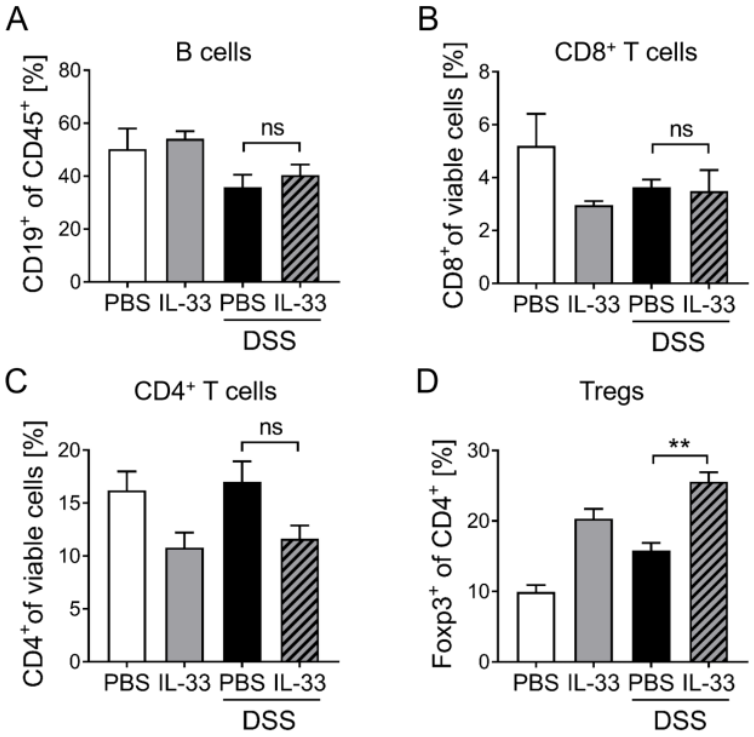


Figure 20: Exogenous IL-33 treatment induces the expansion of regulatory T cells in the colon of DSS mice.
To induce DSS colitis in BALB/c mice, 4 % of DSS was administered via the drinking water for six consecutive days, followed by one day of normal drinking water. Mice were additionally treated with 1 µg of recombinant IL-33 or 200 µl of PBS per mouse at day 0, 2 and 5. Lymphocytes were isolated from the colonic lamina propria at day seven and analyzed via flow cytometry (n = 4-11 mice per group). (**A**) B cells were defined as $CD19^+CD45^+$, (**B**) $CD8^+$ T cells were defined as viable $CD8^+$ cells, (**C**) $CD4^+$ T cells were defined as viable $CD4^+$ cells and (**D**) regulatory T cells (Tregs) were defined as $FoxP3^+CD4^+$ T cells. All data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Dunn's multiple comparison test. $^{**}P < 0.01$.

3.3.1 IL-33-mediated amelioration of DSS-induced colitis is partially dependent on Tregs

Since IL-33-mediated protection against DSS-induced colitis was accompanied by a pronounced enhancement of different immune cells, we further assessed the contribution of each cell type to further understand the function of IL-33. First, we used DEREG/c mice to analyze the impact of Treg expansion upon IL-33 treatment in DSS-induced colitis. DEREG/c mice express the diphtheria toxin (DT) receptor together with a green fluorescence protein (GFP) under the control of the FoxP3 promoter, which allows the specific depletion of $FoxP3^+$ Tregs by DT injection, but also enables the tracking of $FoxP3^+$ Tregs via the GFP signal. Accordingly,

DEREG/c mice were injected with DT at day 0, 2 and 5 and additionally treated with PBS or recombinant IL-33 at day 1, 3 and 6 (Figure 21A). The depletion of FoxP3$^+$ Tregs via DT application was confirmed in the blood. The amount of GFP$^+$ Tregs was efficiently reduced by DT injection from day four onwards, independent of additional IL-33 treatment (Figure 21B).

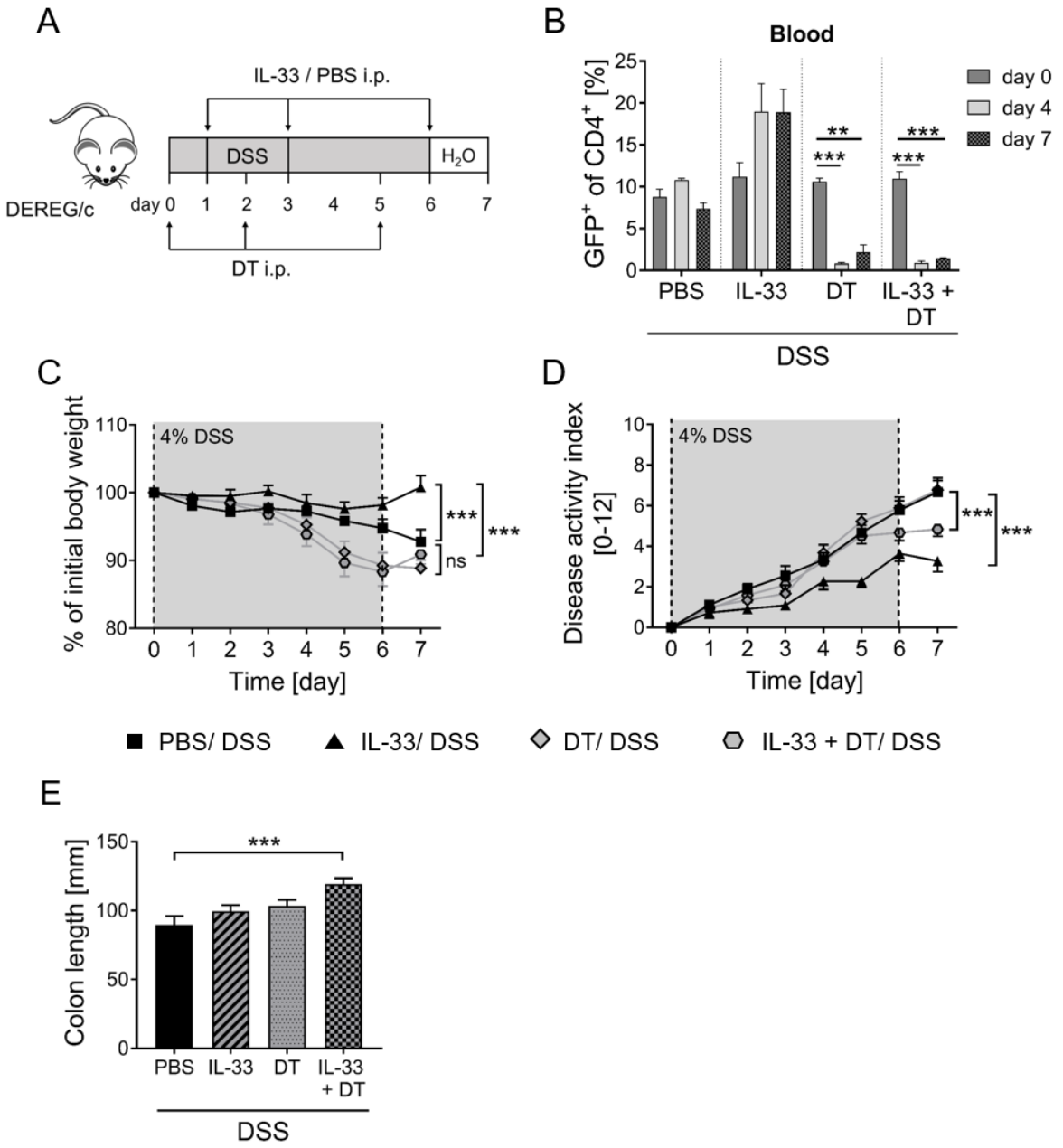


Figure 21: Treg depletion impedes IL-33-mediated protection against DSS-induced colitis.
(**A**) To induce intestinal inflammation, DEREG/c mice were treated with 4 % of DSS in the drinking water for six consecutive days, followed by one day of normal drinking water (n = 5-14 mice per group). To deplete GFP$^+$ Tregs, mice were injected i.p. with 750 ng diphtheria toxin (DT) per mouse at day 0, 2 and 5. DEREG/c mice were additionally treated i.p. either with 200 µl of PBS or with 1 µg of recombinant IL-33 at day 1, 3 and 6. (**B**) Frequencies of GFP$^+$ Tregs were analyzed in the blood at day 0, 4 and 7 by flow cytometry. (**C**) Body weight changes relative to the initial weight and (**D**) disease activity index were monitored daily. (**E**) Colon length was measured at day seven. All data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA (E) or two-way ANOVA (C, D), followed by Tukey's multiple comparison test. $^{**}P < 0.01$; $^{***}P < 0.001$.

Treg-depleted DSS-treated (DT/DSS) mice exhibited a pronounced body weight loss and disease acitvity index, which was slightly but not significantly stronger in comparison to PBS-treated DSS (PBS/DSS) mice (Figure 21C, D). Interestingly, aggravated intestinal inflammation in Treg-depleted mice was not ameliorated upon IL-33 treatment (IL-33 + DT/ DSS) in terms of the body weight, but showed a significant improvement in the disease activity index as well as a higher colon length compared to PBS-treated Treg-deficient DSS (DT/DSS) mice (Figure 21D, E). However, this improved disease outcome due to IL-33 treatment was not as prominent as in Treg-sufficient DSS (IL-33/ DSS) mice. Accordingly, secretion of IL-33, TNF-α and IL-6, was reduced in both Treg-depleted as well as Treg-sufficient DSS-treated mice upon IL-33 treatment (Figure 22A). In contrast, the upregulation of type 2 cytokines, such as IL-5 and IL-13, due to IL-33 application was abrogated upon Treg depletion (Figure 22B).

Collectively, these results could show that Tregs play an important role for the IL-33-mediated tissue protection during DSS-induced colitis.

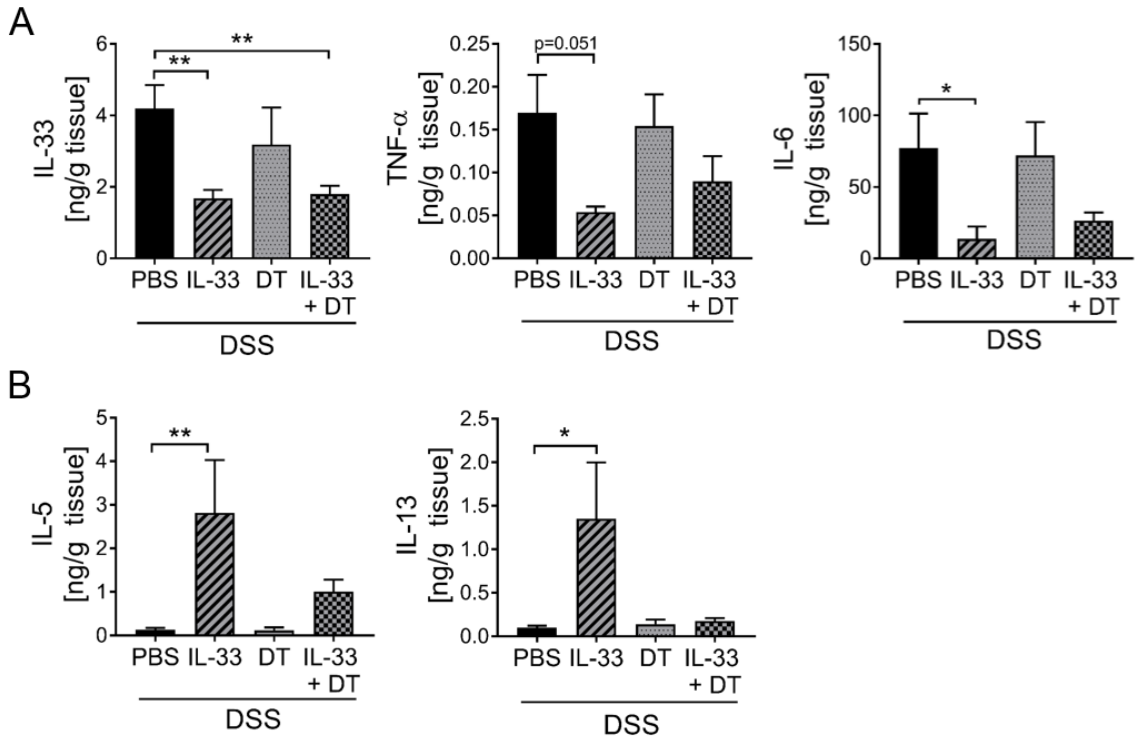


Figure 22: Pro-inflammatory cytokines were reduced upon IL-33 treatment in both Treg-deficient and Treg-sufficient DSS mice.

To induce intestinal inflammation, DEREG/c mice were treated with 4 % of DSS in the drinking water for six consecutive days, followed by one day of normal drinking water (n = 5-14 mice per group). To deplete GFP$^+$ Tregs, mice were injected i.p. with 750 ng diphtheria toxin (DT) per mouse at day 0, 2 and 5. DEREG/c mice were additionally treated i.p. either with 200 µl of PBS or with 1 µg of recombinant IL-33 at day 1, 3 and 6. (**A-B**) Secretion of pro-inflammatory cytokines IL-33, TNF-α and IL-6 and type 2 cytokines IL-5 and IL-13 in the supernatants of colonic explants were quantified using Luminex technology at day seven (n = 5-14 mice per

group). All data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Dunn's multiple comparison test. *P < 0.05; **P < 0.01.

3.3.2 IL-33 treatment promotes innate immune cells to counteract DSS-induced colitis

As the IL-33-mediated amelioration of DSS-induced intestinal inflammation seemed to be only partially dependent on Tregs, we further addressed the overall importance of adaptive immune cells using *RAG2*$^{-/-}$ mice. *RAG2*$^{-/-}$ mice lack mature T and B cells, which allows the analysis of IL-33 signaling exclusively on innate immune cells during colitis. Thus, DSS colitis was induced in *RAG2*$^{-/-}$ mice and mice were additionally treated with PBS or recombinant IL-33 (Figure 23A).

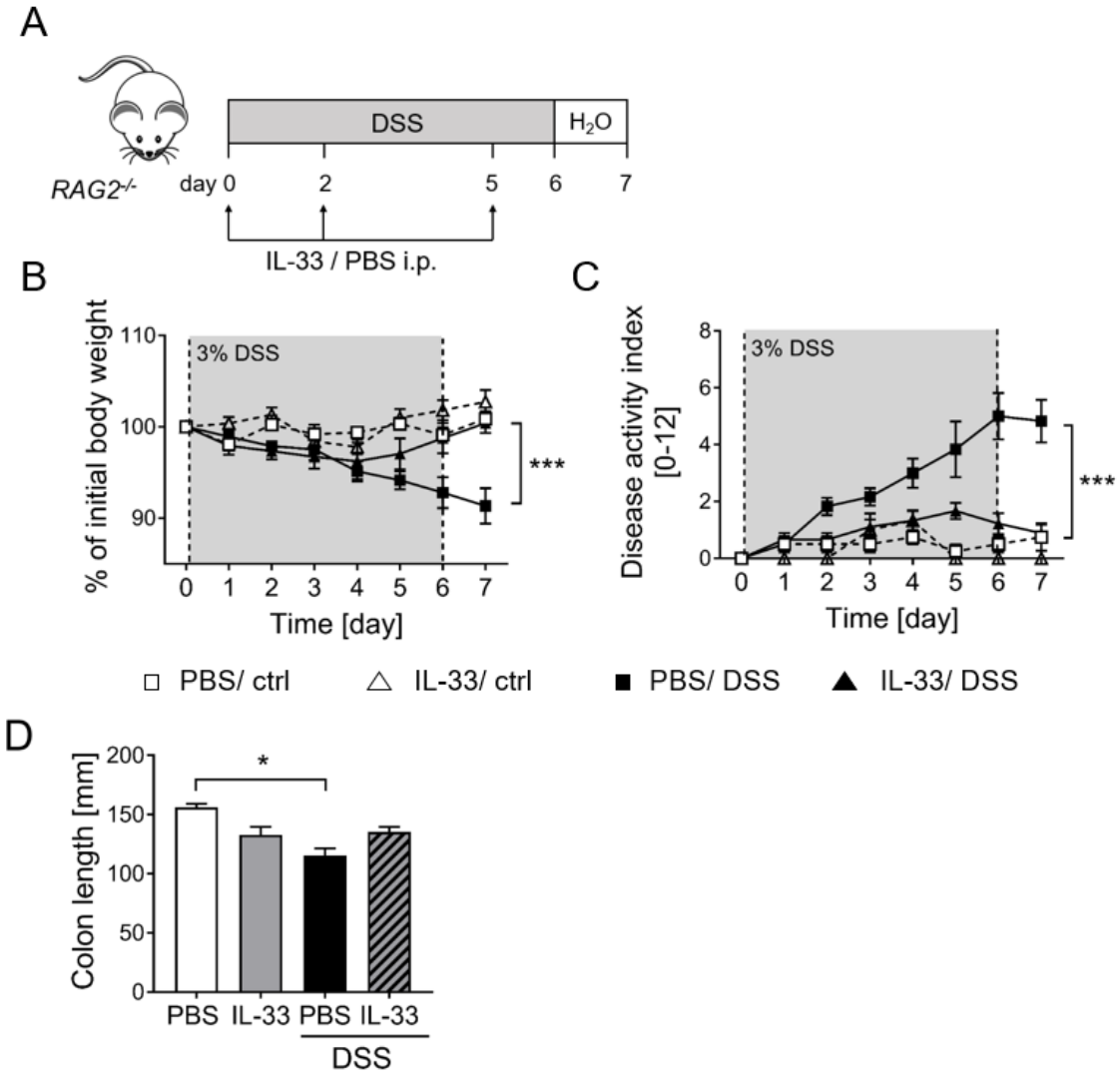
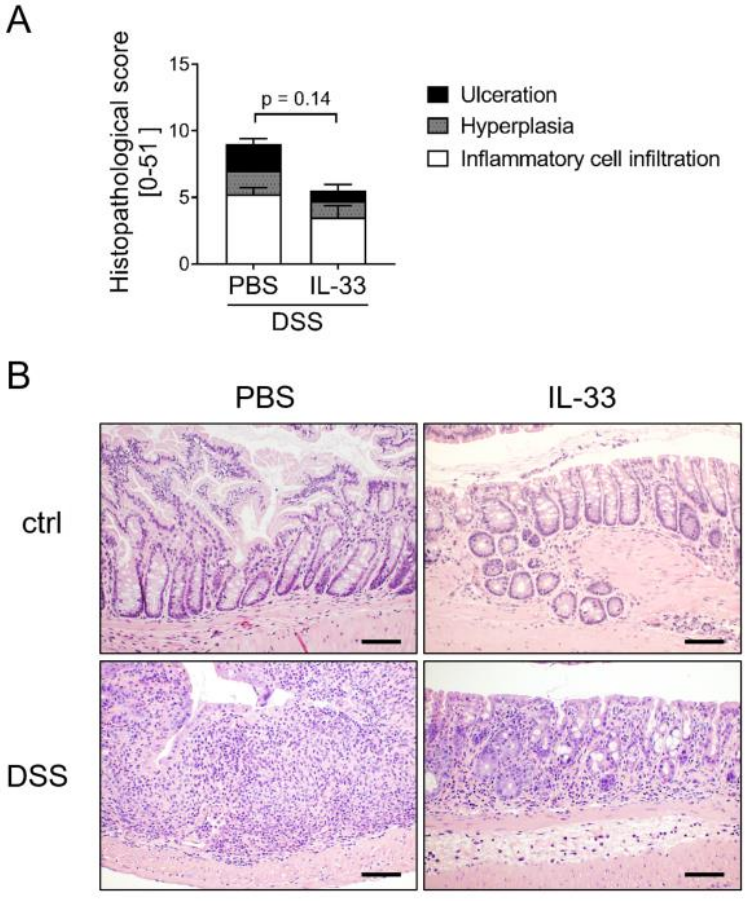


Figure 23: Exogenous IL-33 protects *RAG2*$^{-/-}$ mice from DSS-induced colitis.
(**A**) To induce intestinal inflammation *RAG2*$^{-/-}$ mice were given 3 % of DSS in the drinking water for six consecutive days, followed by one day of normal drinking water. Mice were additionally treated i.p. with 200 µl of PBS or with 1 µg of recombinant IL-33 per mouse at day 0, 2 and 5 (n = 3-9 mice per group). (**B**) Body weight changes relative to the initial weight and (**C**) disease activity index were monitored daily. All data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Dunn's multiple

comparison test (D) or two-way ANOVA, followed by Tukey's multiple comparison test (B, C). *$P < 0.05$; ***$P < 0.001$.

DSS treatment induced the development of severe intestinal inflammation in PBS-treated DSS *RAG2*$^{-/-}$ (PBS/DSS) mice, indicated by a pronounced body weight loss and a significant increase of the disease activity index (Figure 23B, C). Surprisingly, treatment with recombinant IL-33 in *RAG2*$^{-/-}$ DSS mice (IL-33/DSS) resulted in a similar reduced disease progression comparable to IL-33 treatment in BALB/c wild type DSS mice. After IL-33 application, DSS-treated *RAG2*$^{-/-}$ mice were not affected by body weight loss and intestinal inflammation, illustrated by the disease activity index (Figure 23B, C). Furthermore, shortening of the colon was not as prominent in IL-33/DSS-treated mice as in PBS/DSS-treated mice (Figure 23D). The histopathological manifestations of DSS-induced colitis in *RAG2*$^{-/-}$ mice were limited to inflammatory cell infiltration, hyperplasia and ulceration. However, consistent with the reduction in body weight loss and disease activity index, *RAG2*$^{-/-}$ mice treated with IL-33 exhibited a lower histopathological score as well as a reduced disruption of the colonic epithelial layer compared to PBS-treated DSS mice (Figure 24A, B).



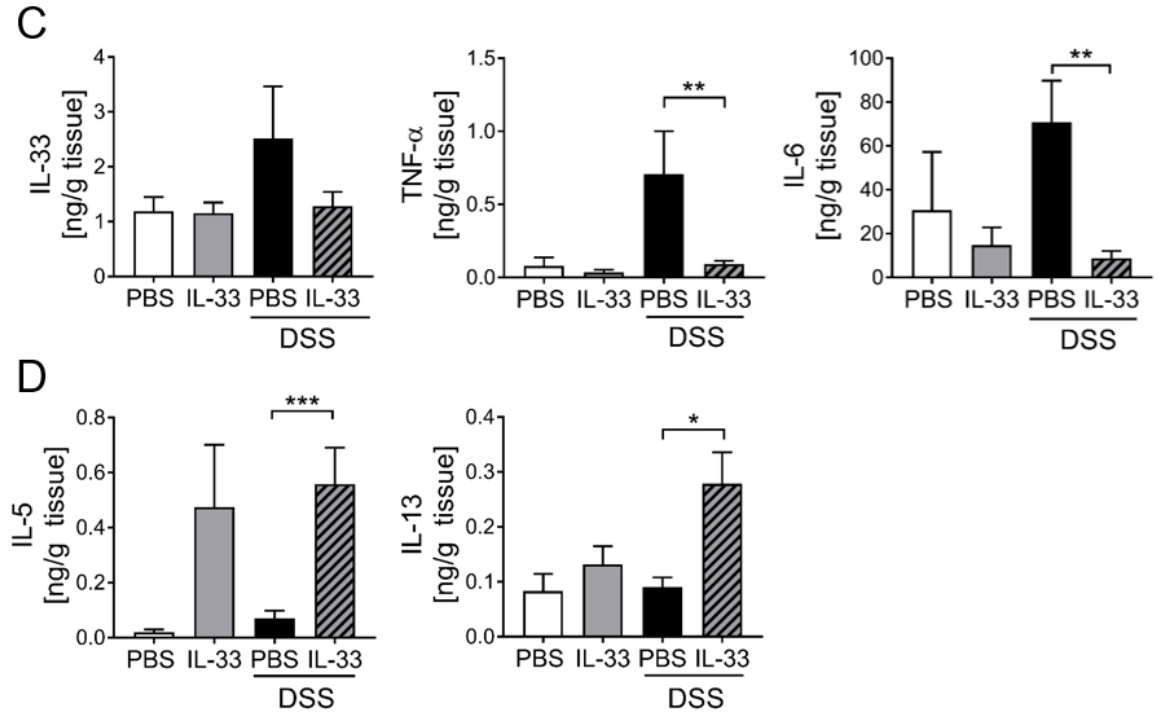


Figure 24: IL-33-treated *RAG2*$^{-/-}$ mice display reduced histopathological score and pro-inflammatory cytokines.
Intestinal inflammation was induced in *RAG2*$^{-/-}$ mice by administering 4 % of DSS via the drinking water for six consecutive days, followed by one day of normal drinking water. Mice were additionally treated i.p. with 200 µl PBS or with 1 µg of recombinant IL-33 per mouse at day 0, 2 and 5. (**A**) Histopathological score, including inflammatory cell infiltration, hyperplasia and ulceration was determined at day seven (n = 4 mice per group) and (**B**) representative hematoxylin and eosin (H&E) staining of colonic sections were prepared. Scale bars represent 100 µm. (**C, D**) Secretion of cytokines IL-33, TNF-α, IL-6, IL-5 and IL-13 in the supernatants of colonic explants were quantified at day seven via Luminex technology (n = 3-9 mice per group). Data are presented as mean ± SEM. Statistical analyses were performed using Mann-Whitney test (A) or one-way ANOVA, followed by Dunn's multiple comparison test (C, D). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Well in line, the production of pro-inflammatory cytokines, including IL-33, TNF-α and IL-6 was enhanced due to DSS treatment in *RAG2*$^{-/-}$ mice, but the upregulation was abrogated upon IL-33 application (Figure 24C). As observed in BALB/c wild type mice, IL-33 also caused an increase of the type 2 cytokines IL-5 and IL-13 in the colon of *RAG2*$^{-/-}$ mice (Figure 24D). Independent of the absence of the adaptive immune cells in *RAG2*$^{-/-}$ mice, IL-33 administration was able to promote a significant increase of the frequencies of both eosinophils and ILC2s (Figure 25C, D). Thus, given the ameliorated disease outcome in *RAG*$^{-/-}$ mice, we further addressed the contributing role of eosinophils and ILC2s to counteract DSS-induced colitis upon IL-33 administration.

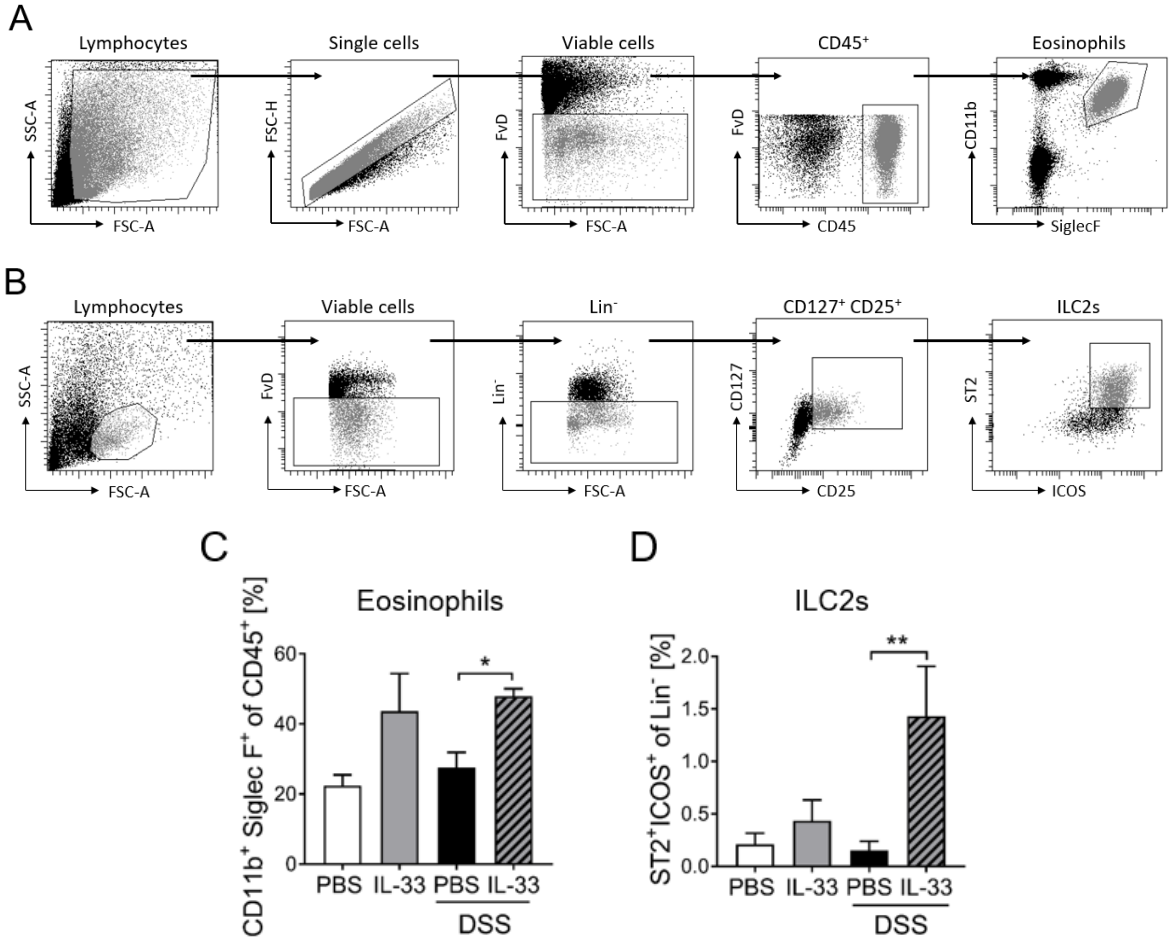


Figure 25: Exogenous IL-33 application leads to the expansion of innate immune cells during DSS-induced colitis in *RAG2*$^{-/-}$ mice.
Intestinal inflammation was induced in *RAG2*$^{-/-}$ mice by applying 4 % of DSS via the drinking water for six consecutive days, followed by one day of normal drinking water. Mice were additionally treated i.p. with 200 µl PBS or with 1 µg of recombinant IL-33 per mouse at day 0, 2 and 5. Immune cells were isolated from the colonic lamina propria at day seven. Representative gating strategy of (**A**) CD11b^{+}SiglecF^{+}CD45^{+} eosinophils and (**B**) ST2^{+}ICOS^{+}lineage^{-} ILC2s. (**C-D**) The frequencies of eosinophils and ILC2s were determined using flow cytometry. All data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Dunn's multiple comparison test. *P < 0.05; **P < 0.01.

3.3.3 Reduction of eosinophils partially attenuates the beneficial effect of IL-33 on DSS colitis

IL-33 application resulted in a strong increase in the frequencies of colonic eosinophils (Figure 25C). Thus, we further analyzed the involvement of these cells during DSS-induced colitis and IL-33 treatment by reducing the number of eosinophils via anti-IL-5 antibody (Ab) administration in BALB/c wild type mice (Figure 26A). Daily administration of anti-IL-5 Ab efficiently reduced the frequencies of eosinophils in the colonic lamina propria of DSS mice, independent of additional IL-33 treatment (Figure 26B). Mice treated with anti-IL-5 Ab (αIL-5/DSS) showed a reduced DSS-induced intestinal inflammation compared to PBS-treated DSS

(PBS/DSS) mice, indicated by the moderate body weight loss and the lower disease activity index (Figure 26C, D). Additional IL-33 application (IL-33+αIL-5/DSS) was further able to alleviate intestinal inflammation slightly but not significantly compared to αIL-5/DSS mice. Moreover, this alleviation was not as prominent as in DSS mice treated with IL-33 only (IL-33/DSS), which is also indicated by the shorter colon length (Figure 26E).

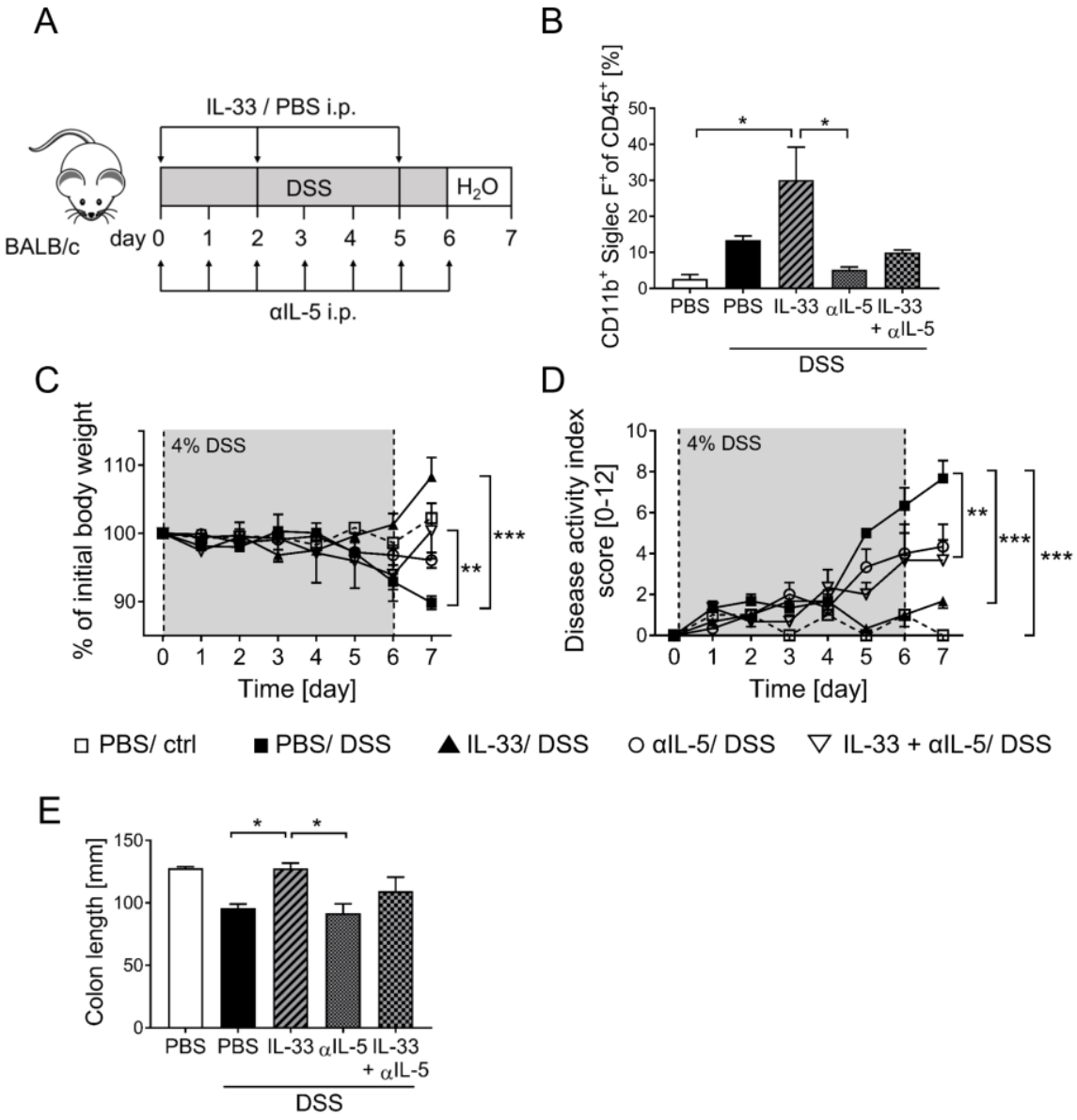


Figure 26: Eosinophil reduction influences IL-33 protective effect on DSS-induced colitis.
(**A**) To induce intestinal inflammation, BALB/c mice were treated with 4 % of DSS via the drinking water for six consecutive days, followed by one day of normal drinking water. Mice were treated i.p. either with 200 µl of PBS or with 1 µg of IL-33 per mouse at day 0, 2 and 5, with or without additional daily injections of 200 µg anti-IL-5 Ab (αIL-5) per mouse (n = 3 mice per group). (**B**) Colonic frequencies of $CD11b^+SiglecF^+CD45^+$ eosinophils were determined via flow cytometry at day seven. (**C**) Body weight changes relative to the initial weight and (**D**) disease activity index were monitored daily. (**E**) Colon length was measured at day seven. All data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Dunns' multiple comparison test (B, E) or two-way ANOVA, followed by Tukey's multiple comparison test (C, D). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Surprisingly, the strong upregulation of pro-inflammatory cytokine secretion from colonic explants after DSS treatment, including IL-33, TNF-α and IL-6, was decreased upon anti-IL-5 Ab treatment, independently of additional IL-33 application and comparable to mice, which were only treated with IL-33 (Figure 27A). Similar to the results obtained upon Treg-depletion in DEREG/c mice, eosinophil reduction completely diminished the upregulation of type 2 cytokines, IL-5 and IL-13, after IL-33 administration (Figure 27B).

Taken together, these findings showed that IL-33-mediated amelioration of DSS-induced colitis was slightly but not completely abrogated upon additional anti-IL-5 Ab treatment, suggesting a supportive but not essential role of eosinophils during IL-33-mediated tissue protection.

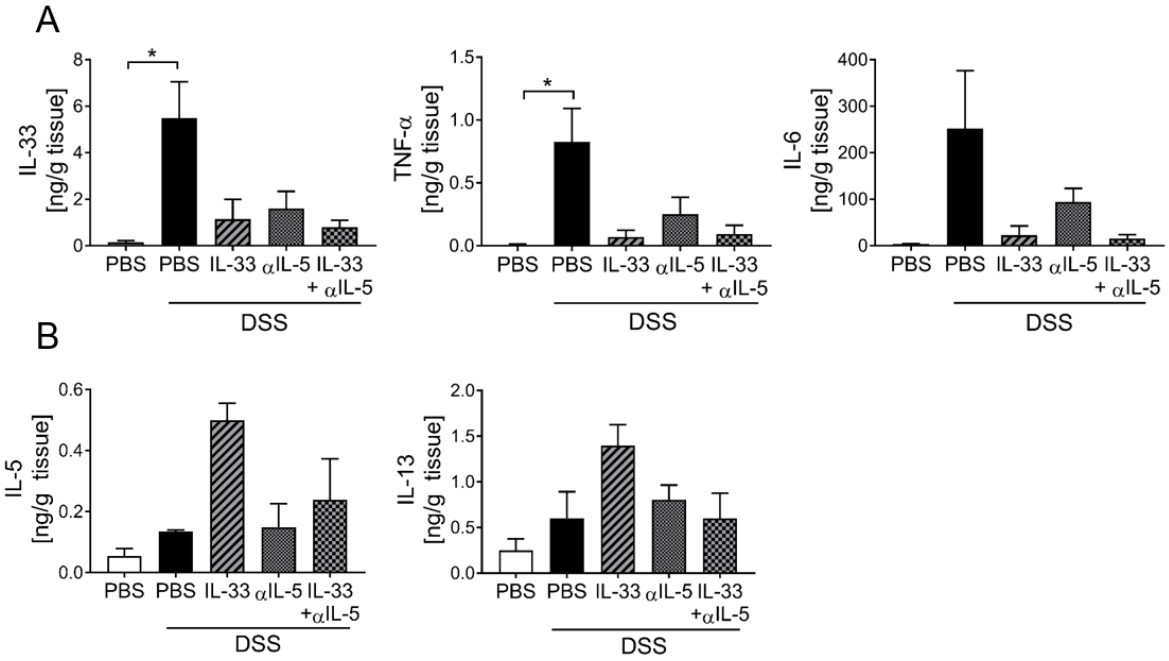


Figure 27: Secretion of colonic cytokines upon exogenous IL-33 and anti-IL-5 Ab treatment in DSS mice. To induce intestinal inflammation in BALB/c mice, 4 % of DSS was administered via the drinking water for six consecutive days, followed by one day of normal drinking water. Mice were treated i.p. either with 200 µl PBS or with 1 µg of IL-33 per mouse at day 0, 2 and 5, with or without additional daily injections of 200 µg anti-IL-5 Ab (αIL-5) per mouse (n = 3 mice per group). (**A-B**) Production of pro-inflammatory cytokines, including IL-33, TNF-α and IL-6 and type 2 cytokines IL-5 and IL-13 from the supernatants of colonic explants were quantified via Luminex technology at day seven. All data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Dunns' multiple comparison test. **P* < 0.05.

3.4 ILC2 transfer protects mice from severe acute intestinal inflammation

Besides eosinophils and Tregs, IL-33 application strongly expanded ILC2s in both BALB/c mice and *RAG2*$^{-/-}$ mice (Figure 19F, 25D). To further address the impact of ILC2s on DSS-induced intestinal inflammation in more detail, we expanded ILC2s *in vivo* in donor BALB/c mice by applying recombinant IL-33 (Figure 28A). After *in*

vivo expansion, ILC2s from the spleens of donor mice were sorted via flow cytometry and adoptively transferred into BALB/c recipient mice at day 0, right before DSS treatment. The adoptive transfer of ILC2s in DSS-treated mice at day 0 was compared with injection of recombinant IL-33 or PBS at day 0, 2 and 5 (Figure 28B). Interestingly, ILC2-pre-transferred DSS mice exhibited an ameliorated disease outcome, indicated by the reduced body weight loss as well as decreased disease activity index in comparison to PBS-treated DSS mice (Figure 28C, D). However, this amelioration was not as pronounced as in IL-33-treated DSS mice, which was further indicated by the colon length shortening (Figure 28E).

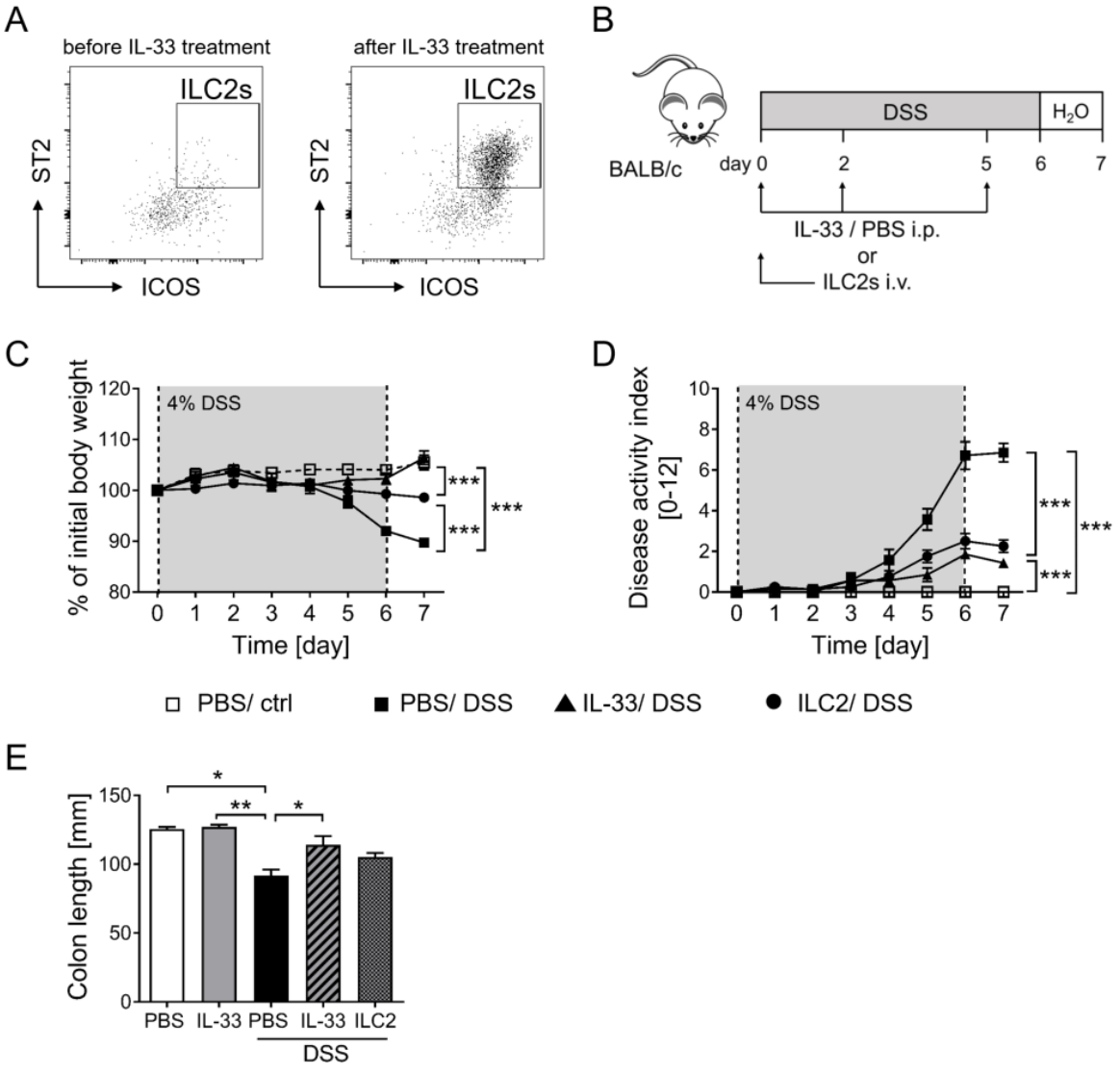


Figure 28: ILC2 transfer reduced DSS-induced intestinal inflammation.
ILC2s were expanded *in vivo* in BALB/c donor mice by injecting recombinant IL-33 at day -7, -5, -3 and -1 prior to the adoptive transfer. (**A**) Representative dot plots show the expansion of viable $ST2^+ICOS^+$ $lineage^-$ ILC2s in the spleen of donor mice after IL-33 administration. (**B**) To induce intestinal inflammation, BALB/c mice were treated with 4 % of DSS via the drinking water for six consecutive days, followed by one day of normal drinking water. Mice were either adoptively transferred i.v. with 2 x 10^5 ILC2s at day 0 or injected i.p. with 1 µg of recombinant IL-33 or 200 µl of PBS per mouse at day 0, 2 and 5 (n = 4-7 mice per group). (**C**) Body weight changes relative to the initial weight and (**D**) disease activity index were monitored daily. (**E**) Colon length was

determined at day seven. All data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Dunn's multiple comparison test (E) or two-way ANOVA, followed by Tukey's multiple comparison test (C, D). *P < 0.05; **P < 0.01; ***P < 0.001.

Consistently, the DSS-induced secretion of pro-inflammatory cytokines and chemokines, including IL-33, TNF-α, IL-6 and CXCL-1/KC in the supernatants of colonic explants was reduced in both ILC2-pre-transferred and IL-33-treated mice compared to PBS-treated DSS mice (Figure 29).

Taken together, these results demonstrated that the adoptive transfer of activated ILC2s was sufficient to ameliorate DSS-induced intestinal inflammation, strongly suggesting ILC2s to be essential players during IL-33-mediated alleviation of colitis.

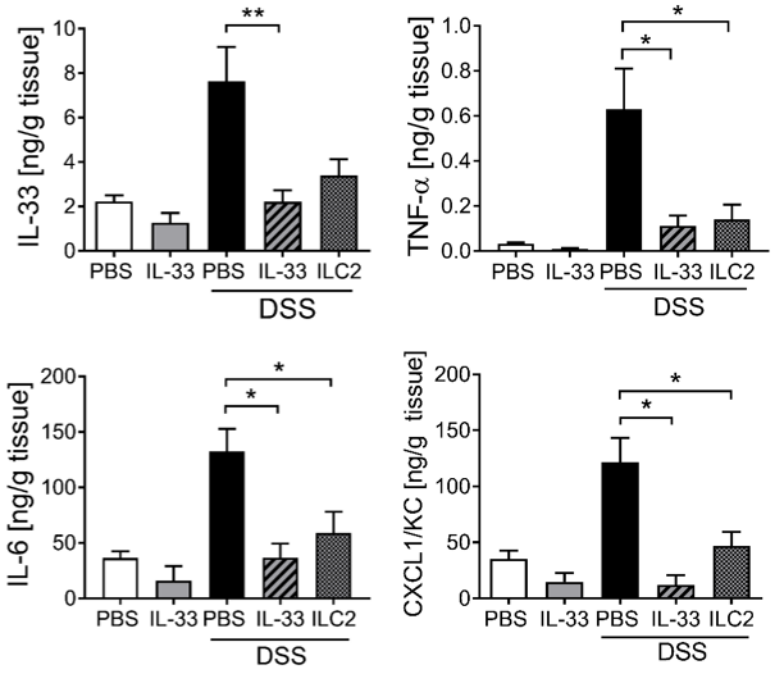
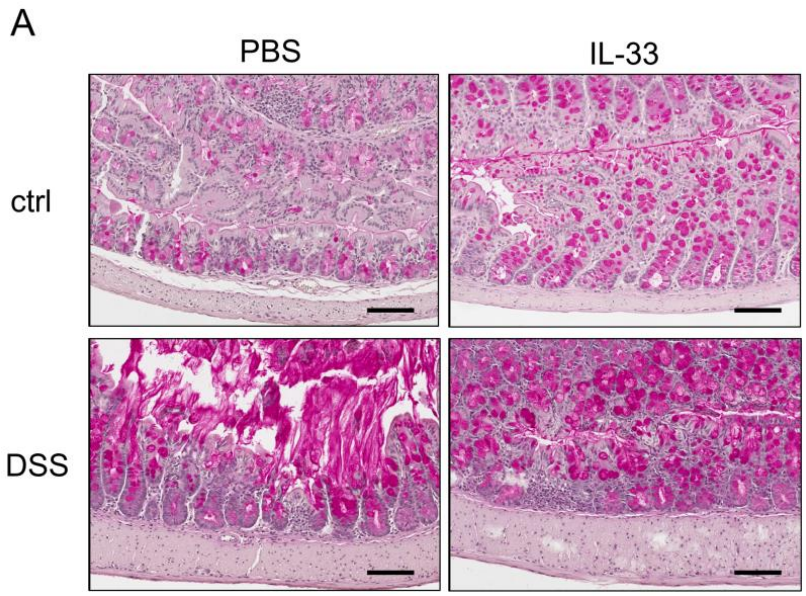


Figure 29: ILC2 transfer reduced DSS-induced pro-inflammatory cytokines and chemokines in the colon. To induce intestinal inflammation, BALB/c mice were treated with 4 % of DSS via the drinking water for six consecutive days, followed by one day of normal drinking water. Mice were either adoptively transferred i.v. with 2 x 10^5 ILC2s at day 0 or injected i.p. with 1 µg of recombinant IL-33 or 200 µl of PBS per mouse at day 0, 2 and 5 (n = 4-7 mice per group). Secretion of pro-inflammatory cytokines and chemokines, including IL-33, TNF-α, IL-6 and CXCL1/KC in the supernatants of colonic explants was quantified via Luminex technology at day seven. All data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Dunns' multiple comparison test. *P < 0.05; **P < 0.01.

3.4.1 ILC2s promote their protective function through the induction of intestinal goblet cell differentiation

IL-33 activates ILC2s to produce IL-13 and thereby induces the differentiation of goblet cells [140]. Accordingly, we could show that IL-33 treatment *in vivo* in healthy BALB/c (ctrl/IL-33) mice resulted in an expansion of goblet cells (Figure 30A). Goblet cells belong to the first line of defense of the intestinal immune system during inflammatory conditions. Thus, we could detect a moderate enhancement of goblet cell expansion upon DSS-induced colitis (DSS/PBS). However, this increase of goblet cells was even stronger upon additional IL-33 treatment (DSS/IL-33) (Figure 30A). To determine whether IL-33 treatment maintains the barrier function via goblet cells, we further analyzed the role of goblet cells in more detail. Indeed, DSS mice treated with recombinant IL-33 exhibited elevated levels of the primary mucin *Muc2* (Figure 30B) and significantly higher expression of *SAM* (*sterile alpha motif*) *pointed domain epithelia specific transcription factor (Spdef)* and *Resistin-like-beta* (*Retnlb*), transcription marker and secretory product of goblet cells, respectively, in the colon (Figure 30C). More importantly, transfer of activated ILC2s to DSS mice was sufficient to display a phenotype comparable to IL-33-treated DSS-treated mice, since both showed an upregulation of colonic *Muc2*, *Spdef* and *Retnlb* expression. These results further support the idea of a reinforced goblet cell function due to the indirect impact of IL-33 on ILC2s.



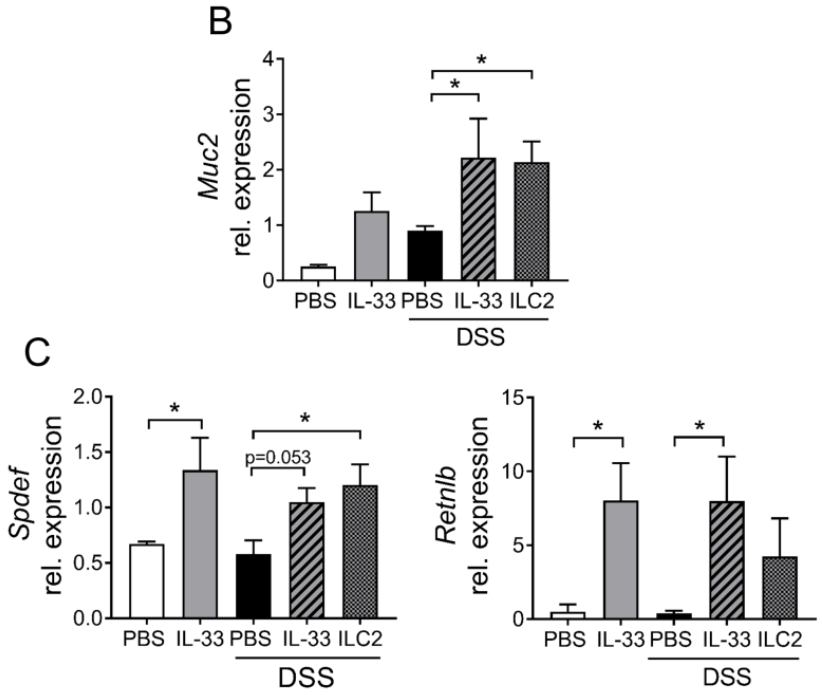


Figure 30: Exogenous IL-33 treatment or adoptive transfer of ILC2s increases goblet cell function.
To induce intestinal inflammation, BALB/c mice were treated with 4 % of DSS via the drinking water for six consecutive days, followed by one day of normal drinking water. Mice were either adoptively transferred i.v. with 2 x 10^5 ILC2s at day 0 or injected i.p. with 1 µg of recombinant IL-33 or 200 µl of PBS per mouse at day 0, 2 and 5 (n = 4-7 mice per group). (**A**) Representative histological pictures of periodic acid Schiff (PAS) staining to visualize goblet cells in the colonic lamina propria were prepared. Scale bars represent 100 µm. (**B-C**) Colonic mRNA expression of *Muc2*, *Spdef* and *Retnlb* were assessed by RT-qPCR. All data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Dunns' multiple comparison test. **P* < 0.05.

Overall, the results presented here further endorse the IL-33-mediated amelioration of severe acute colitis by enhancing the epithelial barrier integrity. We conclude that exogenous IL-33 administration orchestrates an immune network, consisting of ILC2s in the center and supportive but necessary functions of Tregs and eosinophils to restrain DSS-induced intestinal inflammation.

4 Discussion

Inflammatory bowel diseases are characterized by mucosal damage and ulceration [57]. However, since the precise etiology of IBD is still incompletely understood, there is currently no causative cure. Due to this fact, most of the available drugs only reduce and suppress the symptoms to remain the patient in remission [141]. Thus, it is of essential importance to find new therapeutic approaches to improve the patients' quality of life and to prevent unwanted complications.

Current research consider an imbalance of cytokine production and concomitant T cell dysfunction as key factors in the pathogenesis of intestinal inflammation [69]. In particular, several studies in IBD patients or experimental animal models of intestinal inflammation could identify elevated levels of specific inflammatory cytokines, emphasizing their crucial role as immune regulators at mucosal surfaces [83-87].

In this study, expression of IL-33 was found to be significantly increased in colonic biopsies of both IBD patients as well as mice with intestinal inflammation (Figure 10, 11). Moreover, increase of IL-33 secretion positively correlated with disease activity index in mice. Since IL-33 is considered to act as an alarmin upon tissue damage or stress at mucosal barrier site, elevated levels of IL-33 could be an indicator of inflammation [106]. Several studies have therefore proposed a unique and essential role for IL-33 during the development of intestinal inflammation [126, 142]. Nevertheless, it is not clear whether this increase of IL-33 is a consequence of inflammation, to support the pro-inflammatory environment or whether this increase is specifically induced in response to the inflammation to promote mucosal protection. Further understanding the function of IL-33 and its interaction with $ST2^+$ immune cells in more detail, might reveal new options for IL-33 as a therapeutic target.

In accordance with our results, active lesions of IBD patients exhibited increased levels of IL-33 compared to healthy control tissues. These studies further proposed that IL-33 could be a potential biomarker for IBD [143, 144]. In contrast, serum levels of IL-33 were shown to be decreased in IBD patients compared to healthy individuals, indicating additional roles of IL-33 during IBD [145]. However, subsequent studies investigating the precise function of IL-33 in the intestine did not result in conclusive evidences but rather unveiled a dichotomous function of IL-33 regarding a host-protective or host-pathogenic effect.

Thus, we further analyzed the role of IL-33 during intestinal inflammation in more detail. Interestingly, in the absence of IL-33 signaling, *ST2*$^{-/-}$ mice developed a significantly stronger DSS-induced colitis compared to wild type mice, which points towards a favorable function of IL-33 during intestinal inflammation (Figure 13, 14). To further support this suggestion, we could observe that mice treated with exogenous IL-33 exhibited a tremendous amelioration of intestinal inflammation compared to PBS-treated mice in two independent colitis models, the DSS-induced colitis model and the T cell transfer colitis model (Figure 15, 17). Surprisingly, the strong increase of endogenous colonic IL-33 was completely absent when mice were additionally administered with exogenous IL-33 (Figure 16). Our results highlight the critical difference between the presence of endogenous and exogenous IL-33 and may further explain the controversial observations regarding its function in the intestine in former studies. In line with this assumption, a previous study revealed a difference between full-length IL-33 and mature IL-33. While full-length IL-33 exists in the nucleus of epithelial cells and has been shown to accumulate upon intestinal inflammation, mature IL-33 is processed upon cleavage by different caspases and is present in the extracellular space. The same study could demonstrate that over-expression of full-length IL-33 by intestinal epithelial cells did not result in inflammation, but was able to induce gene expressions, which are associated with an increased T_H2 immune response, including *Gata3*, *Il4* and *Il13*. Hence, it seems like the mature (cleaved) form rather than the nuclear (full-length) form of IL-33 is able to mediate the inflammatory impact on intestinal epithelial cells [114]. Well in line, Ali *et al.* proposed that full-length IL-33, but not mature IL-33, is able to sequester NF-$_K$B. Thus, IL-33 impairs NF-$_K$B–triggered TNF-α expression to reduce pro-inflammatory signaling [113]. These results emphasize the capacity of full-length IL-33 to behave as a transcriptional repressor to decrease inflammation. From these findings, we assume that exogenous recombinant IL-33 resembles the full-length form of IL-33 and therefore its application reduces the expression of pro-inflammatory cytokines and increases the expression of type 2 cytokines. Well in line, we could observe significant reduction of TNF-α, IL-6 and IL-33 secretion in the colon after exogenous IL-33 treatment of DSS mice, while IL-5 and IL-13 levels were significantly elevated (Figure 16, 22, 24). Consequent suppression of the pro-inflammatory status within the lamina propria further leads to the reduction of excessive immune responses, whereas enhancement of type 2 cytokines further promotes tissue healing. While secreted IL-33 by colonic explants seems to be the

cleaved mature form of IL-33, which is released from the nucleus of epithelial cells as a pro-inflammatory mediator, exogenous treatment with recombinant IL-33 seems to protect the mucosal barrier integrity by inhibiting the secretion of its mature form.

A hallmark of IBD is the excessive activation of intestinal immune cells [56]. Hence, we further addressed the effect of IL-33 on $ST2^+$ immune cells during DSS-induced colitis. By analyzing cells from both the innate and adaptive immune response, we could show that IL-33 application significantly increased the frequencies of Tregs, eosinophils and ILC2s in the lamina propria (Figure 19, 20). This increase was independent but more pronounced upon DSS treatment, suggesting that these cells trigger the IL-33-mediated protective effect.

Tregs have been considered to be important players in the pathogenesis of intestinal inflammation, since their frequencies were reduced in peripheral blood of IBD patients [146, 147]. Moreover, dysfunction of Tregs has been shown to be the major cause for the perpetuation of IBD [69]. Hence, we had expected that Treg ablation resulted in a stronger intestinal inflammation. Indeed, we could see a slight increase in the severity of inflammation in Treg-depleted DSS mice compared to Treg-sufficient mice. But more importantly, exogenous IL-33 treatment was able to reduce intestinal inflammation even in the absence of Tregs. As this IL-33-mediated reduction of disease severity was still not as prominent as in Treg-sufficient DSS, we further suggest a supportive but also necessary function of Tregs for the IL-33-driven tissue protection. While Treg-depleted DSS mice displayed a similar reduction of pro-inflammatory cytokines compared to Treg-sufficient DSS mice upon IL-33 treatment, the upregulation of type 2 cytokines was completely diminished (Figure 22). These findings further indicate a dependent or at least supportive function of Tregs to other immune cells to produce IL-5 and IL-13. IL-13 has been previously described to promote the regeneration of the intestinal epithelium [148]. Hence, the reduction of IL-13 expression in the colonic tissue could be one reason for a less pronounced amelioration of colitis upon IL-33 treatment in Treg-depleted DSS mice. Well in line, results obtained by Duan *et al.* also emphasized that Tregs are required for IL-33 function, as their depletion via anti-CD25 application significantly abrogated the indirect impact of IL-33 on DCs to alleviate a TNBS-induced intestinal inflammation [127]. Furthermore, Tregs are considered to control the development of various autoimmune diseases such as rheumatoid arthritis,

Kawasaki disease and systemic lupus erythematosus [149-151]. Nevertheless, their frequency as well as their functional capacities and concomitantly, their precise roles and molecular mechanisms have not been fully defined yet [152]. Even though Treg deficiency has been demonstrated to result in lethal autoimmunity and intestinal inflammation [153, 154], our results proved that IL-33 was able to reduce the inflammation in the absence of Tregs. This observation propose the ability of IL-33 to orchestrate other immune cells and cytokines apart from Tregs to restrain intestinal inflammation.

Therefore, we further addressed the overall importance of the adaptive immune cells using *RAG2*$^{-/-}$ mice. Surprisingly, we could observe that treatment with IL-33 significantly improved the DSS-induced intestinal inflammation in *RAG2*$^{-/-}$ mice (Figure 23, 24), whereas a study conducted by Qiu *et al.* showed contrary results. In their study treatment with IL-33 in *RAG2*$^{-/-}$ mice resulted in a less favorable outcome of DSS-induced colitis compared to PBS-treated *RAG2*$^{-/-}$, indicated by a higher histopathological score in the colon [155]. These conflicting results could be due to the fact that first, they used C57BL/6 mice, which are more susceptible to DSS compared to BALB/c mice and second, they performed daily injections of IL-33. Thus, we cannot rule out a dose-dependent function of IL-33 application during intestinal inflammation.

Nevertheless, our *RAG2*$^{-/-}$ model could show significant reductions of pro-inflammatory cytokines, which was accompanied by an upregulation of type 2 cytokines after IL-33 treatment (Figure 25A, B). Independent of the adaptive immune cells, IL-33 application induced the expansion of both eosinophils and ILC2s (Figure 25C), indicating that these cell types, or at least one of them, could be the key driver for the IL-33-mediated tissue protection.

Interestingly, mice suffered less from DSS-induced intestinal inflammation upon eosinophil reduction via anti-IL-5 Ab application, which was slightly but not strongly improved upon additional IL-33 application (Figure 26C). Since eosinophils contain granules filled with inflammatory mediators and factors, they are mainly considered as pro-inflammatory cells in the context of IBD [156]. Thus, it is not surprising that reduction of eosinophils alleviated DSS-induced colitis in our experiments, which was consistent with previous studies [157]. Moreover, several studies have addressed eosinophil-associated gastrointestinal disorders comprising eosinophilic esophagitis, eosinophilic gastritis and eosinophilic enteritis, all involving an

abnormal eosinophil accumulation in the intestinal tract [158, 159]. Surprisingly, in our study the ameliorating effect of IL-33 treatment on intestinal inflammation was decreased upon eosinophil reduction, providing a favorable function of eosinophils upon IL-33 stimulation (Figure 26, 27). In line with our assumption, Lampinen *et al.* could observe that the number of activated eosinophils was significantly higher in the colonic lamina propria of IBD patients in remission compared to patients with active IBD, emphasizing their involvement during tissue repair processes [160]. Subsequent studies by Yousefi *et al.* demonstrated that the infiltration of eosinophils into the intestinal tract upon microbial sepsis was associated with a protection against severe inflammation [161]. Their results suggested that eosinophils might build up a second physical barrier to limit bacterial invasion and decrease inflammatory stimuli, which would further explain the attenuated IL-33-mediated protection against intestinal inflammation upon eosinophil reduction in our study. Similar to the results obtained by our experiments using Treg-depleted DEREG/c mice, reduction of eosinophils abrogated IL-33-mediated upregulation of type 2 cytokines IL-5 and IL-13. These findings suggest that IL-33-driven amelioration of DSS-induced colitis partially requires the presence of eosinophils, emphasizing a tissue-protective function of eosinophils during intestinal inflammation. Consistent with our suggestion, Kobayashi *et al.* observed that overexpression of IL-5 in transgenic mice increased the infiltration of eosinophils into the intestinal mucosa. This infiltration was further accompanied by less pronounced pathologic features of DSS-induced colitis as well as an increase of IL-13 compared to wild type mice [162]. Moreover, Jung *et al.* proposed that the intestinal mucus layer maintenance was dependent on eosinophil presence in the lamina propria, as mice deficient for eosinophils exhibited a significant reduction of mucus-secreting goblet cells [163]. Accordingly, we conclude that the IL-33 activation of eosinophils induces their production of IL-13 to promote the regeneration of the intestinal epithelium. Even though this process seems to be a necessary aspect of the IL-33-mediated mucosal protection, we cannot confirm eosinophils to be the major players to counteract intestinal inflammation, since we could still observe an ameliorated effect of IL-33 administration on DSS colitis in the absence of eosinophils. To further elucidate the specific role of eosinophils upon IL-33 treatment, additional experiments are required. Reduction of eosinophils via anti-IL-5 application in *RAG2*$^{-/-}$ mice or in Treg-depleted DEREG/c mice would rule out the impact of adaptive immune cells and Tregs on the function of eosinophils.

At last, the contribution of ILC2s to the IL-33-mediated protection against DSS-induced colitis was analyzed. Interestingly, we could show that mice pre-transferred with activated ILC2s exhibited less signs of intestinal inflammation (Figure 28C, D). These results emphasize intestinal ILC2s to play a central role during the IL-33-driven tissue protection. ILC2s has been extensively investigated in the context of respiratory allergic inflammation and identified to play a key role in the initiation and orchestration of inflammation in the lung [164]. In contrast to the high amounts ILC2s in the lung, ILC2s are only present at low frequencies in the entire gastrointestinal tract at steady state. However, well in line with our findings, previous studies observed that ILC2 numbers were strongly increased upon IL-33 stimulation in the lung, gastrointestinal tract, adipose tissue and skin [165-168]. However, the functions of ILC2s seem to be diverse regarding the regulation of pro-inflammatory and tissue-protective responses at intestinal barrier sites. Some studies even highlighted positive correlations of ILC2 frequencies and disease severity using different models of intestinal inflammation. They could demonstrate that both impairment of ILC2 development or suppression of ILC2 function resulted in an improved disease outcome [132, 155]. In contrast to these studies and in line with our observations, other studies have proposed a tissue-protective capacity of gut-associated ILC2s by contributing to the maintenance of epithelial integrity. Results obtained from Monticelli *et al.* revealed that the IL-33-driven expansion of ILC2s protected mice from colitis by promoting the expression of growth factors essential for tissue protection and the restoration of intestinal homeostasis [27]. Subsequent studies by Satoh-Takayama *et al.* demonstrated that IL-33 activation of ILC2s resulted in an enhanced production of IgA antibodies to eliminate commensal bacterial burden in the intestine [169]. In our experiments, we could further show a significant reduction of pro-inflammatory cytokines in ILC2-pre-transferred DSS-treated mice, which was even comparable to IL-33-treated DSS mice (Figure 29). This similar cytokine phenotype underlines the essential role of ILC2s for the IL-33-mediated amelioration of DSS-induced colitis.

As our histological analyses demonstrated a reduced disruption of the epithelial layer, we assume that the amelioration of DSS-induced colitis was due to an increased intestinal barrier function. Thus, we investigated the impact of IL-33 treatment and ILC2 transfer on goblet cell expression. Indeed, we could observe that mice treated with IL-33 displayed increased expression of goblet cell differentiation markers as well as increased expression of their primary mucin

product. Since goblet cells are considered to be essential for the epithelial barrier integrity by producing the intestinal mucus layer, they are referred to as the first line of defense [36, 46]. Consequently, thickening of the mucus layer is an effective mechanism to increase epithelial barrier function. Interestingly, mice pre-transferred with activated ILC2 exhibited a similar phenotype to IL-33-treated DSS mice (Figure 30). Both results propose an indirect impact of IL-33 treatment to induce intestinal goblet cell differentiation via the expansion of IL-13 producing ILC2s. Accordingly, previous studies could identify ILC2s as the primary source of the type 2 cytokines IL-5 and IL-13, as mice deficient for ILC2s failed to increase the expression both cytokines upon IL-33 stimulation [19, 119]. Well in line, using *in vitro* experiments, Waddell *et al.* could show that intestinal goblet cell proliferation were mainly mediated by the IL-13 cytokine [140]. In this context, the increase of IL-13 expression seems to be of central importance during intestinal inflammation, as it was shown before that IL-13 was able to impair the secretion of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 [170]. However, our observed ILC2-mediated tissue protection seems not to be restricted to IL-13 production. You *et al.* demonstrated that the transfer of pre-activated ILC2s was able to repair epithelial damage during colitis and restore the body weight of *RAG1*$^{-/-}$ mice by promoting the polarization of M2 macrophages in the gut [131]. Furthermore, Monticelli *et al.* could show the IL-33-driven expansion of ILC2 resulted in an increased production of amphiregulin to limit intestinal inflammation and decreased disease severity [27]. In addition to our results, these findings further emphasize diverse capacities of ILC2s to promote tissue protection. Nevertheless, in our experiments, the ameliorating impact of pre-activated ILC2 transfer was not as pronounced as IL-33 treatment. These findings indicate a central contribution for ILC2s to restrain intestinal inflammation, but also support the collaborative roles of other factors or immune cells like Tregs and eosinophils. To proof this assumption, *RAG2*$^{-/-}$ common γ chain-double-deficient (*RAG2*$^{-/-}\gamma_C^{-}$) mice could be analyzed in DSS-induced colitis. These mice lack the expression of T, B and NK cells but also innate lymphoid cells, including ILC2s [171]. If ILC2s are really essential for the IL-33 mediated tissue protection, these mice should not benefit from IL-33 treatment during DSS colitis and develop a stronger intestinal inflammation than IL-33-treated wild type mice.

Nevertheless, with its diverse functions, IL-33 could be the connecting mediator between different ST2^{+} cells to orchestrate a network of immune cells to counteract intestinal inflammation. Indeed, several studies could already demonstrate that IL-

33 administration induced Treg numbers and functions indirectly via the promotion of ILC2s. A study conducted by Nascimento *et al.* observed that the IL-33-mediated activation of ILC2s resulted in the polarization of M2 macrophages and subsequently induced the expansion of Tregs via the production of IL-10 [172]. Moreover, Halim *et al.* further proposed that ILC2s are even required for Treg response to IL-33, since ablation of ILC2 function was able to inhibit the expansion of Tregs upon IL-33 treatment [173]. In contrast, results obtained from Neill *et al.* suggested an unknown mechanism of T cells to mediate the prolonged ILC2 expansion and survival, as increased numbers of ILC2 were not maintained over time in the absence of T cells [20]. However, these results further indicate not only a crosslink between ILC2s and Tregs but also highlight a promoting capacity of ILC2s to induce Tregs expansion indirectly upon IL-33 stimulation. Notably, the IL-33-mediated amelioration of intestinal inflammation seems not to be restricted to interaction of ILC2s and Tregs, as we could also observe an IL-33-driven protection in the absence of T cells using *RAG2*$^{-/-}$ mice. The improved disease outcome in *RAG2*$^{-/-}$ mice was accompanied by a significant increase of both ILC2 and eosinophils, emphasizing a collaborative interaction of these cells. As previously mentioned, ILC2s have been proofed to be the major source of the type 2 cytokine IL-5 [19, 119], which is an important mediator involved in the proliferation, cell survival and maturation as well as the effector functions of eosinophils [174]. Hence, it is not surprising that ILC2s are crucial regulators of eosinophil differentiation but also promote their expansion upon IL-33 stimulation [175, 176]. Furthermore, recent studies could demonstrate that eosinophils from the lamina propria were able to induce the differentiation of naïve CD4^{+} T cells into Tregs via the expression of TGF-β and retinoic acid, further suggesting a protective collaboration of ILC2s, Tregs and eosinophils against intestinal inflammation [177].

Consistent with this suggestion, we could observe that the upregulation of the ILC2 signature cytokine IL-13 was abrogated upon Treg depletion and eosinophil reduction. These results indicate that Treg as well as eosinophil ablation compromised ILC2 function. However, we cannot confirm that this compromise is caused directly or indirectly via the stimulation of other immune cells or cytokines, since we still observed an upregulation of type 2 cytokines in *RAG2*$^{-/-}$ mice. Therefore, additional experiments are needed to analyze ILC2 function in more detail. One method could involve the activation and expansion of ILC2s *in vivo* in the absence of Tregs or eosinophils, using DEREG/c mice or anti-IL-5 application,

respectively. Subsequent isolation and transfer of these ILC2s into wild type mice might reveal alterations in ILC2 functions during intestinal inflammation.

As aforementioned, there is still no curative therapy for IBD. Even though ongoing research have already reached remarkable progress in therapeutic strategies, these conventional therapies mainly focus on the improvement of clinical manifestations. Therefore, more recent studies highlight mucosal healing as a key parameter in the management of intestinal inflammation [178, 179]. Mucosal healing primarily requires the coordinated activity of intestinal epithelial cells and goblet cells to improve the intestinal barrier function [180]. Well in line, our results could show that the IL-33-driven activation of ILC2s was able to increase the barrier integrity via goblet cell expansion and mucin layer thickening.

Importantly, improved barrier function and mucosal healing were associated with more effective disease control as well as lower rates of hospitalization and surgery, resulting in the patients' improved quality of live, which is the essential goal of every therapeutic approach [181].

The study presented here identified the ability of IL-33 to orchestrate an immune network, consisting of ILC2s as central players and collaborative functions of both Tregs and eosinophils to promote mucosal barrier integrity and counteract intestinal inflammation. The central role of ILC2s could be due to the fact that these cells constitutively express the IL-33 receptor ST2, allowing them to act immediately upon IL-33 stimuli, whereas only a subpopulation of Tregs and eosinophils express ST2. However, given the diverse function of each immune cell that is affected by IL-33 administration, especially during intestinal inflammation, further investigations are necessary to identify the underlying mechanisms of ILC2-mediated mucosal protection.

Taken together, these results highlight the potential of exogenous IL-33 treatment as a therapeutic drug during acute colitis and its crucial role for ILC2 activation to ameliorate intestinal inflammation.

5 Literature

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6 Index

6.1 List of abbreviations

5-ASA	5-aminosalicylic acid
ADCC	Antibody-dependent cell-mediated cytotoxicity
AMPs	Antimicrobial peptides
APC	Allophycocyanin
APC	Antigen-presenting cell
AF	Alexa Fluor
Bcl-2	B cell lymphoma-2
Bcl-xl	B cell lymphoma-extra large
BSA	Bovine serum albumin
BV	Brilliant violet
CD	Cluster of differentiation
CD	Crohn's disease
cDNA	complementary deoxyribonucleic acid
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXCR	C-X-C chemokine receptor
DAI	Disease activity index
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
DC	Dendritic cell
Dnase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
DSS	Dextran sodium sulfate
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid

ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box 3
g	Gravity
G-CSF	Granulocyte-colony stimulating factor
GALT	Gut-associated lymphoid tissue
GAPs	Goblet cell-associated antigen passages
GFP	Green fluorescence protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
H&E	Hematoxylin & Eosin staining
HA	Hemagglutinin (of influenza virus)
i.p.	intraperitoneal
i.v.	intravenous
IBD	Inflammatory bowel disease
ICOS	Inducible T cell costimulator
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IgA	Immunoglobulin A
IL	Interleukin
IL-1RA$_C$P	Interleukin-1 receptor accessory protein
IRAK	Interleukin-1 receptor-associated kinase
IRF4	Interferon regulatory factor 4
IMDM	Iscove's modified dulbecco‘s medium
JNK	c-Jun N-terminal kinase

KC	Keratinocyte chemoattractant
KO	Knock out
Lag-3	Lymphocyte activation gene 3
LPL	Lamina propria lymphocyte
mAb	monoclonal antibody
MACS	Magnetic activated cell sorting
MgCl2	Magnesium chloride
MHC	Major histocompability complex
mLN	Mesenteric lymph node
mRNA	messenger ribonucleic acid
Muc	Mucin glycoprotein
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor κB
NK	Natural killer cell
NOD2	Nucleotide-binding oligomerisation protein 2
PAMP	Pathogen-associated molcecular pattern
PAS	Periodic acid schiff
PB	Pacific blue
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cyanine 7
PerCP	Peridinin chlorophyll
PRR	Pattern recognition receptor
qPCR	quantitative polymerase chain reaction
RAG	Recombinant-activating gene
Retnlb	Resistin-like-beta

RPMI	Roswell Park Memorial Institute
RPS9	Ribosomal protein 9
SCFA	Short chain fatty acids
SCID	Severe combined immunodeficient
SEM	Standard error of mean
Spdef	SAM (sterile alpha motif) pointed domain epithelia specific transcription factor
ST2	Serum stimulation-2
STAT3	Signal transducer and activator of transcription 3
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
T_H	T helper cell
TIR	Toll/Interleukin-1 receptor
TNBS	Trinitrobenzenesulfonic acid
TNF-α	Tumor necrosis factor-alpha
TR1	Type 1 regulatory T cell
TRAF6	Tumor necrosis factor receptor-associated factor 6
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis

6.2 List of figures

6.3 List of tables

7 Appendix

7.1 Acknowledgements

Die Danksagung ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

7.2 Curriculum Vita

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

7.3 Declarations

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. (2) d) + f) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbstständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegebenen Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Essen, den_____ _____

Nhi Ngo Thi Phuong

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. (2) e) + g) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät/ Fachbereich abgelehnt worden ist.

Essen, den_____ _____

Nhi Ngo Thi Phuong

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. (2) g) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Interleukin-33 orchestrates an immune network to counteract severe acute colitis“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Nhi Ngo Thi Phuong befürworte.

Essen, den_____ _____

Prof. Dr. Astrid Westendorf