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Dissecting the role of Interleukin-23 in the *Tnf*$^{\Delta ARE}$ mouse model of ileitis

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CONTENTS

ABSTRACT

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract with significant morbidity worldwide. CD is generally considered the outcome of a complex interplay between genetic susceptibility, environmental factors, and altered microbiota that ultimately leads to a profound dysregulation of the immune system. Cytokines control multiple aspects of the resulting aberrant immune responses, as their imbalance can impede the resolution of inflammation and perpetuate intestinal pathology. Among them, interleukin-23 (IL-23) has been strongly associated with the course of CD, mostly due to its pro-inflammatory properties. Although population genetics, along with clinical and experimental studies, have attributed a critical role to IL-23 signaling in CD, the mechanism of its action remains poorly understood. Here, using conventional and tissue-specific genetic ablation in distinct immune cell types, we investigate *in vivo* the contribution of IL-23 signaling in the *Tnf*$^{\Delta ARE}$ model of Crohn's-like ileitis. We identify the hematopoietic compartment as the predominant source of IL-23 in the inflamed ileum and show that IL-23 deficiency in bone marrow cells results in attenuated disease. Additionally, we demonstrate that IL-23R-expressing immune cells massively infiltrate the *Tnf*$^{\Delta ARE}$ tissue and provide evidence for a sex-specific pathogenic role of IL-23 signaling in a subset of T-cells. Finally, we exclude a primary pathogenic function of IL-23R in the CD4^{+} T-cell compartment and demonstrate that IL-1 signaling is dispensable for the development of *Tnf*$^{\Delta ARE}$ ileitis.

INTRODUCTION

Inflammatory bowel disease: Crohn's disease

Inflammatory bowel disease (IBD) is a multifactorial chronic inflammatory disorder of the gastrointestinal (GI) tract and a major healthcare burden of significant global morbidity [1]. IBD encompasses two main clinical conditions, namely Crohn's disease (CD) and ulcerative colitis (UC). These two forms of IBD are commonly characterized by aberrant inflammatory processes of unknown origin that result in immune-mediated tissue destruction and compromised function of the intestinal barrier. Concurrently, CD and UC represent independent clinical entities, as they substantially differ in terms of pathophysiology, affected areas of the GI tract, and microscopic features [2]. In CD, inflammatory lesions can occur in any compartment of the alimentary tract, most frequently affecting the distal part of the small intestine (ileum) and the colon. The disease is characterized by a patchy, segmental inflammation pattern that can penetrate transmurally, as well as by aggregation of macrophages that frequently form non-caseating granulomas [3]. Conversely, inflammation in UC is continuous and more superficial; it initiates in the rectum and extends proximally in the colon, while it is restricted to the mucosal and submucosal layers, often leading to abscesses and ulcers [2,4].

IBD patients typically suffer from debilitating clinical symptoms, such as fatigue, impaired appetite, and weight loss, abdominal pain, persistent diarrhea, and rectal bleeding, which often evolve in a relapsing and remitting manner, profoundly affecting patient's quality of life. In particular, 50% of CD patients develop intestinal complications over time (strictures, fistulae, or abscesses), while 21–47% also present with systemic, extraintestinal manifestations (EIMs), including inflammatory disorders of the skin, eye, joints, and respiratory, musculoskeletal and nervous systems [5,6]. Additionally, CD patients may experience vascular or metabolic dysfunction and exhibit an increased risk of colorectal and small-bowel cancer development compared with the general population [7].

Traditionally, CD has been treated with immunosuppressants (such as corticosteroids) or immunomodulators (such as thiopurines) for both induction and maintenance of deep disease remission.[8] Importantly, the introduction of biological agents in the past two decades has transformed the management of CD, and these drugs are now considered the therapy of choice, especially for patients at high risk of disease progression [5]. However, despite these major advances in IBD therapeutics, 'gold standard' therapies are still lacking, as a substantial proportion of patients is unresponsive to initial therapy with these agents or develops resistance over time [9]. Furthermore, attenuation of inflammation often fails to resolve severe intestinal complications, like fibrosis and bowel obstruction. Taken together, these observations underscore the urgent need not only for the

optimization of established treatment options, but also for the deeper comprehension of CD etiopathogenesis that will enable long-term restoration of intestinal homeostasis [10].

Risk factors

Although the precise disease etiology remains incompletely understood, CD is generally considered the outcome of a dysregulated immune response to unknown environmental triggers in genetically susceptible individuals [11]. Consequently, CD has a multifactorial nature driven by the complex and concerted interplay between genetic, immune, and environmental variables.

Environmental factors

Among the factors that positively correlate with CD development, smoking is the best-studied environmental factor, associated with a two-times increase in risk for disease manifestation [2,12]. Other crucial risk factors affect gut microbiota and, in turn, gut immune response, such as antibiotic use and dietary changes [5]. More specifically, antibiotic exposure during childhood and reduced dietary fiber intake constitute two factors that are positively linked with decreased intestinal microbiota diversity and the development of CD [13,14]. Oral contraceptives, aspirin, and non-steroidal anti-inflammatory drugs (NSAIDs) have also been reported to associate with disease onset [15,16], whereas statins and breastfeeding are described, albeit inconsistently, as protective for CD [17,18].

Genetic factors

Apart from the identification of CD-associated environmental factors, significant advances have also been made regarding the delineation of genomic variation that underlies IBD susceptibility. Over the past two decades, the use of genome-wide association studies (GWAS) has enabled the discovery of >200 IBD-related genes, with >140 of them being associated with Crohn's disease [19]. Established risk-conferring variants for CD include genes implicated in pathways related to microbial sensing (e.g. *Nod2, Card9*), microbicidal mechanisms (e.g. *Lrrk2*), epithelial barrier integrity (e.g. *Ptger4*, *Muc2*), autophagy (e.g. *Atg16l1, Irgm*) and adaptive immunity (e.g. *Il23r, Ccr6*) [20,21]. Notably, a number of IBD-protective alleles have also been reported (e.g. *Ptpn22*, *Card9*, *Cul2*, *Il23r*, *Rnf186*), thus demonstrating that IBD genetics and functional genomics could guide the development of novel therapeutic strategies against the disease [21,22].

Mechanisms / Pathophysiology

Intestinal homeostasis

Intestinal homeostasis depends on a delicate equilibrium between the gut microbiome and host immunity. This equilibrium is well orchestrated by the mucus layer and its underlying epithelium, which cooperatively act as physical and biochemical barriers that segregate luminal contents (microbes and food antigens) from the subjacent lamina propria. Defects in epithelial cells and the mucus layer, inflammation of the lamina propria, or dysbiotic events of the microbiota can disrupt this homeostasis and precipitate the inflammatory pathology of CD.

Intestinal barrier

The compromised function of the mucosal barrier is a hallmark of IBD. Barrier disturbances present through various cell types of the intestinal epithelium and affect different levels of its protective mechanisms [23]; unresolved endoplasmic reticulum (ER) stress, defects in autophagy and altered expression of junctional proteins (E-cadherin, β-catenin and claudins) are perturbations frequently detected in the intestinal epithelial cells (IECs) during CD, ultimately leading to increased tissue permeability [24–26]. Moreover, intraepithelial lymphocytes (IELs) undergo functional changes in response to intestinal dysbiosis, displaying either excessive cytotoxic activity or reduced production of anti-inflammatory cytokines [27]. Finally, genetic defects and pathological unfolded protein response (UPR) induce abnormalities in the secretory activity of Paneth cells, further compromising epithelial barrier integrity during CD [28,29].

Immune response

Barrier disruption results in increased tissue exposure to luminal contents and downstream activation of both innate and adaptive immune responses. More specifically, dendritic cells (DCs) and macrophages sense penetrating microbes through Toll-like (TLRs) and NOD-like receptors (NLRs), thereby promoting the development of T_H1 and T_H17 cell subsets [30]. In turn, T_H1 and T_H17 cells secrete multiple pro-inflammatory cytokines (IL-17, IFNγ and TNF) that induce further cytokine production by macrophages, monocytes and endothelial cells, outstripping the immunosuppressive activity of regulatory T-cells (Tregs) [31]. In addition, neutrophils, initially recruited to phagocytose invading microorganisms, gradually accumulate within the gut epithelium and subsequently release inflammatory mediators [2]. Lastly, innate lymphoid cells (ILCs), including natural killer (NK) cells, lymphoid tissue inducer (LTi) cells and γδ IELs, upregulate IL-22, IL-17A and IL-17F in response to

secreted molecules, hence perpetuating the inflammatory and tissue-destructive state of the intestinal milieu [32].

Microbial dysbiosis

Gut dysbiosis, termed as the imbalanced composition of luminal microflora, is commonly observed in IBD, either as a primary or secondary effect of an inappropriate immune response against commensal microbes [33]. In CD specifically, dysbiosis comprises bacterial, viral and fungal alterations both in terms of complexity and stability. With regards to bacterial composition, studies have reported that CD patients harbor an overrepresentation of enterobacteria and decreased representation of the phyla Firmicutes and Bacteroidetes. Abundance alterations in particular taxa have also been described, such as enrichment of the disease-promoting adherent-invasive *Escherichia coli* or reduction of *Faecalibacterium prausnitzii*, a commensal bacterium with anti-inflammatory properties [32,34]. Similarly, fungal and viral studies have identified an expansion of *Candida* spp. and an increase in bacteriophage richness in individuals with CD, thus accentuating the significant contribution of the enteric virome and mycobiome in the dysbiotic environment of IBD [35–37].

Intestinal mesenchymal cells

Intestinal mesenchymal cells (IMCs) are also actively involved in a wide spectrum of pathologic mechanisms that drive intestinal disease [38,39]. Specifically, response against epithelial damage, microbial sensing and immunoregulation constitute some major homeostatic functions of IMCs, which upon deregulation lead to IBD development and fibrosis [40,41]. Recent data from single-cell transcriptomic analyses have further highlighted the crucial role of mesenchymal cells in intestinal pathology by revealing an unprecedented level of IMC heterogeneity. Intriguingly, these studies have identified a distinct inflammation-associated cluster, in both mice and humans with colitis, characterized by a unique inflammatory/fibrotic gene signature [42,43]. Enhanced expression of pro-inflammatory genes has been also detected in an activated fibroblast cluster from CD patients [44,45], whose presence in the intestinal tissue was associated with resistance to anti-TNF therapy [44]. Deeper understanding of the composition and function of IMCs in the context of intestinal pathology will enable the development of new and more targeted therapeutic approaches for the benefit of patients with chronic inflammation.

Crohn's disease mouse models

Our understanding of IBD pathology has been remarkably advanced over the years, owing to the development of numerous animal models of intestinal inflammation. These models, most commonly generated in mice, serve as valuable platforms for the dissection of IBD-underlying mechanisms, as well as for the preclinical evaluation of novel therapeutic concepts. Depending on the method of disease induction, IBD mouse models are classified into chemically induced (DSS, TNBS, oxazolone), adoptive T-cell transfer, infection induced (*Citrobacter rodentium*, *Helicobacter hepaticus*) and genetically engineered models of specific gene deletion or overexpression (*Il-10* $^{-/-}$, *Tnf* $^{\Delta ARE/+}$) [31,46]. The majority of these mice develop colonic inflammation, while a limited number of mouse models display ileal involvement, similar to CD [47]. Additionally, GWAS studies have provided evidence that Crohn's ileitis may, in fact, represent a genetically distinct form of IBD, thus underlining the value of experimental models that reliably recapitulate the pathologic features of the inflamed ileum [48].

Several of the existing ileitis models are developed through genetic disruption of intestinal epithelial cell (IEC) homeostasis. For instance, IEC-specific deficiency of genes related to ER stress (*Xbp1* ^{IECKO}) or cell death signaling pathways (*Casp8* ^{IECKO}, *Fadd* ^{IECKO}, *Setdb1* ^{IECKO}) induces spontaneous inflammation in the distal part of the small intestine and, in some cases, also in the colon [49]. Other highly relevant models of CD-like ileitis are the congenic SAMP1/Yit(Fc) mouse strain, which exhibits inflammation spontaneously without chemical, genetic, or immunologic manipulation, and the genetically engineered *Tnf* $^{\Delta ARE}$ mouse model, which has been generated by our lab and is further discussed in the following section [47,49].

Tnf $^{\Delta ARE}$ mouse model

Tnf $^{\Delta ARE/+}$ mice bear a targeted deletion of the AU-rich elements (ARE) located in the 3'untranslated region (UTR) of the tumor necrosis factor (*Tnf*) gene. This deletion induces an intrinsic defect in the post-transcriptional regulation of *Tnf* mRNA, resulting in its enhanced stabilization and subsequent overexpression [50]. This chronic TNF overproduction, that signals through TNFR1 in mesenchymal cells [51], drives the development of a fully penetrant Crohn's-like IBD pathology, which primarily occurs in the terminal ileum, and occasionally also in the proximal colon. By 8 weeks of age, mouse histopathological features include early villous blunting and segmental ileitis, while by 12 weeks of age there is evident loss of villous architecture, established submucosal and transmural inflammation, and development of granulomas, reminiscent of those seen in patients with severe CD [47,52]. Along with ileitis, *Tnf* $^{\Delta ARE/+}$ mice also present inflammatory polyarthritis and heart valve stenosis, two

common IBD-related comorbidities, which further reflect the strong resemblance of this model with the human condition [50,53].

Interleukin-23 in IBD pathogenesis

IL-23 cytokine

Interleukin-23 (IL-23) is a heterodimeric protein that belongs to the IL-12 cytokine family, itself part of the IL-6 superfamily [54]. It consists of two covalently linked subunits, a unique IL-23p19 (encoded by *Il23a* / *Il23p19* gene) and an IL-12p40 (encoded by *Il12b* / *Il12p40* gene). The latter is commonly shared with the cytokine IL-12. IL-23 exerts its biological functions through engagement with its cognate heterodimeric receptor (IL-23R). More specifically, IL-23p19 and IL-12p40 bind on the IL-23R and IL-12Rβ1 chains, respectively, initiating a signaling cascade that implicates members of the JAK–STAT family [55]. In more detail, upon formation of the ligand-receptor complex, phosphorylation of Janus kinase 2 (JAK2) ensues in parallel with tyrosine kinase 2 (TYK2) and the IL-23R itself, thus creating docking sites for the recruitment of STAT (signal transducer and activator of transcription) proteins [56]. STAT3 predominantly, and to a lesser extent STAT1, STAT4 and STAT5 become subsequently phosphorylated, activate the NF-κB and PI3K-Akt pathways and ultimately induce the transcription of key effector cytokine genes (IL-17A, IL-17F, IL-22 and IFNγ) [57,58]. By contrast, the closely related IL-12 protein, although it similarly signals through JAK2 and TYK2, primarily activates STAT4, mechanistically accounting for the distinct biological effects of the two cytokines [59,60].

IL-23 in intestinal homeostasis

IL-23 is constitutively expressed in the healthy ileum possessing a crucial role in the orchestration of mucosal immune defense [61]. This host-protective function in the intestine has been initially described in a murine model of enteropathogenic *E. coli* infection, where mice deficient for IL-23p19 exhibited increased susceptibility and mortality following challenge with *Citrobacter rodentium* [62]. Additionally, studies using either adoptive transfer, chemically induced or infectious colitis models have shown that genetic deletion of *Il23* or *Il23r* renders mice incapable of repairing barrier damage due to defective wound healing and impaired IL-22-mediated immune responses [63–66]. Also, in the TNBS-induced model, colitis severity was exacerbated upon the absence of IL-23p19, possibly due to loss of counter-regulation of IL-12 and subsequent enhanced production of the cytokine by dendritic cells (DCs) [67]. Consequently, IL-23 significantly contributes to intestinal homeostasis through immunoregulation of the epithelial repair and restitution. Nonetheless, sustained activation of IL-23 signaling for an extended period promotes the chronicity of the associated inflammatory responses, which may eventually turn into a major driver of IBD pathogenesis [68].

IL-23 in IBD

Apart from its immunoregulatory role in the host response against pathogens, IL-23, as a pleiotropic cytokine, also possesses pro-inflammatory functions, which are confoundedly associated with IBD pathogenesis. Initial studies using *Il12p40*-deficient mice or anti-IL12p40 neutralizing antibodies proposed IL-12 as a critical player in intestinal inflammation [69–72]. However, the subsequent discovery that IL-12p40 can also dimerize with IL-23p19 and the following generation of solid experimental data from a plethora of disease models, established IL-23, instead, as the main driver of the pathology [54]. Specifically, disruption of IL-23p19, rather than IL-12p35 (the IL-12-specific subunit), conferred resistance to the development of spontaneous enterocolitis that occurs upon genetic ablation of the anti-inflammatory cytokine IL-10 [73], and diminished colitis induction in lymphocyte-deficient mice challenged with *H. hepaticus*, anti-CD40 antibodies or adoptive T-cell transfer [74–76]. Similar to its ligand, IL-23 receptor was essential for the development of innate cell-driven colitis induced via anti-CD40 treatment [77], as well as for the colitogenic potency of $CD4^{+}CD45RB^{high}$ transferred T-cells to immunodeficient mice [78]. Intriguingly, in a model of intestinal inflammation triggered by barrier disruption ($Nemo^{IECKO}$), the chronicity of the pathology was largely dependent on IL-23, whereas disease initiation was mainly driven by IL-12, suggesting critical and temporally distinct functions of the two cytokines in the course of IBD [79].

Experimental evidence, data from GWAS studies and meta-analyses further highlight the importance of IL-23 in intestinal inflammation. Variants in the *Il23r* locus and SNPs in regions containing the *Jak2*, *Tyk2* and *Stat3* genes have been identified as significant risk factors for the disease [80,81]. Additionally, human CD has been associated with increased expression levels of IL-23 and its related cytokines, and results from patients administered with the approved anti-IL12p40 monoclonal antibody ustekinumab or the recently authorized anti-IL23p19-specific biologic agent risankizumab have shown promising efficacy [82,83]. Concordantly, the genetic susceptibility, along with the abundance of experimental data obtained from murine models and human studies, corroborate a pivotal role for IL-23 signaling in intestinal pathology.

Cellular sources and targets of IL-23 in IBD

In terms of cellular sources and effectors, IL-23 is found at the interface of innate and adaptive immunity. Principal cytokine-producing cells are professional antigen-presenting cells (APCs), mainly DCs and $CD68^{+}$ macrophages [84]. Among them, lamina propria $CD14^{+}$ macrophages expressing IL-23p19 have been described to increase in frequency in the mucosa of CD patients bearing disease refractory to anti-TNF therapy, a result associated with accumulation of apoptosis-resistant

TNFR2$^+$IL-23R$^+$CD4$^+$ T-cells in the gut [85]. Likewise, a single-cell study of CD intestinal biopsies detected an IL-1B$^+$IL-23A$^+$ population of pathogenic macrophages that positively correlated with inflammation and resistance to anti-TNF treatment [44]. IL-23 secretion in the context of IBD has been also observed in a subset of inflammatory monocytes, where IL-10 deficiency enhanced cytokine production through IL-1α/β signaling, ultimately leading to hyperinflammation and anti-TNF non-responsiveness [86]. In contrast to these well-established IL-23-releasing cell types, a study on pediatric IBD identified a subpopulation of CXCR1$^+$CXCR2$^+$ tissue-infiltrating neutrophils as the primary source of the cytokine, thus implicating a previously unappreciated cellular IL-23 producer in the pathology of the inflamed intestine [87].

Regarding cellular responders, T_H17 cells constitute the predominant target of IL-23. The retinoid-related orphan receptor γt (RORγt) transcription factor is the master regulator of this T helper cell subset, which under homeostatic conditions, is present in the intestinal lamina propria constitutively producing IL-17 [9]. However, T_H17 cells are also potent inducers of tissue inflammation and are largely implicated in IBD pathogenesis through the secretion of IL-17 family cytokines (IL-17A, IL-17F and IL-22) [88]. Although their development from naïve CD4$^+$ T-cells is independent of IL-23, the latter is required for the activation and expansion of pathogenic CD4$^+$ lymphocytes upon intestinal inflammation, simultaneously suppressing Treg cell activity [78,89]. Consistently, human studies have shown that the frequency of T_H17 cells is significantly increased in the inflamed mucosa of IBD patients, while IL-23 serum levels positively correlate with reduced Treg/T_H17 cell ratio in peripheral blood mononuclear cells [90,91]. ILCs, another important cell target of IL-23, have been similarly detected to elevate in number in the intestinal tissue of CD patients and to drive an IL-23 dependent intestinal pathology in experimental models of innate colitis [92,93]. Among them, group 3 ILCs (ILC3s) are those that mainly express IL-23R, and deletion of the receptor in this compartment confers protection in mice with anti-CD40-induced disease [77]. Subsets of γδ T-cells and NK cells have been also described as responsive to IL-23, which upon pathogenic insults secrete type 17 signature cytokines, thus serving as additional mediators of local tissue inflammation [94,95].

Taken together, these observations indicate that IL-23 triggers a diverse pro-inflammatory cascade in the intestine, comprising both innate and adaptive pathogenic responses. Nevertheless, despite the great wealth of evidence attributing a crucial role to IL-23 in the development of chronic intestinal inflammation, the exact pathogenetic mechanism of its action remains undefined. In this context, using the *Tnf*$^{\Delta ARE}$ mouse model, along with total and conditional knock-out mice, flow cytometry and molecular biology techniques, we aimed to further dissect the relative contribution of IL-23 in

Crohn's disease ileitis, aspiring to provide valuable insights into the IL-23-dependent mechanisms that underlie the pathology of CD.

MATERIALS & METHODS

Mice

Tnf$^{\Delta ARE/+}$, *Il23r*$^{F/F}$ and *Il23p19*$^{-/-}$ and mice have been previously described [50,66,67]. *CD8αcre* and *CD4cre* mice were obtained from Jackson Laboratory. All mice were bred and maintained on a C57BL/6J background and experiments were performed using littermate control and experimental mice that were co-housed according to gender. Experimental procedures were conducted in the animal facilities of Biomedical Sciences Research Center (BSRC) 'Alexander Fleming' under specific pathogen–free conditions.

All animal studies were authorized by the Institutional Committee of Protocol Evaluation in conjunction with the Veterinary Service Management of the Hellenic Republic Prefecture of Attika according to all current European and national legislation, and performed in accordance with the guidance of the Institutional Animal Care and Use Committee of BSRC 'Alexander Fleming'.

Genotyping

During the second week of age, DNA was extracted from mouse fingers using the phenol/isopropanol/ethanol protocol and subjected to PCR analysis and agarose gel electrophoresis for genotype determination. All experimental samples were re-genotyped after mouse euthanasia for additional validation. PCR reactions were performed on a thermal cycler using the following primers:

Gene	Forward primer	Reverse primer
CD4cre	5'-ATTACCGGTCGATGCAACGAGT-3'	5'-CAGGTATCTCTGACCAGAGTCA-3'
CD8αcre	5'- CAATGGAAGGAAGTCGTGGT-3'	5'-TGGGATTTACAGGGCATACTG-3'
Il23p19	5'-GAACCAAAGGAGGTGGATAGGG-3'	5'-CCATCTTCACACTGGATACGGG-3' 5'-GGAACAAACGGCGGATTGAC-3'
Il23r$^{F/F}$	5'- TCAAAGTTGACTACACTGTAAGGTAGAGGTAGTGG-3'	5'-GGTGGATCTGCAACAAAACGAATCAC-3'

Ileum Single-cell Suspensions

Last 6 cm of small intestine (terminal ileum) from wild type and *Tnf*$^{\Delta ARE/+}$ mice were harvested, flushed with ice-cold PBS, and opened longitudinally after removal of Peyer's patches, fat and adherent connective tissue. Dissected ileums were washed thoroughly with ice-cold PBS and incubated in pre-warmed HBSS (Gibco) containing 5 mM EDTA and 1 mM dithiothreitol (DTT) for 20 minutes at 37°C,

followed by vigorous shaking to achieve epithelium detachment. Tissues were then rinsed with PBS and enzymatically digested for 1 hour at 37°C while shaking in pre-warmed digestion buffer [HBSS, 10% FBS (PAN-Biotech), 10 mM Hepes (Biosera), 300 U/ml Collagenase XI (Sigma), 50 U/ml DNase I (Sigma), 0.08 U/ml Dispase II (Roche)], along with rigorous shaking every 10 minutes. Digested remains were further disaggregated through a 70 µm strainer, washed with PBS/EDTA (2 mM) and pelleted by centrifugation at 400 g for 7 minutes at 4°C. Collected cells were resuspended in PBS/EDTA (2 mM) and ultimately filtered into FACS tubes through mesh to ensure single-cell suspension preparation.

Single-cell Suspensions from Mesenteric Lymph Nodes

Gut-draining mLNs were harvested from *Tnf*$^{\Delta ARE/+}$ and *Il23r*$^{CD8\alpha KO}$ *Tnf*$^{\Delta ARE/+}$ mice and mechanically dissociated with the use of a syringe plunger through a 40 µm strainer. Isolated cells were collected into PBS-containing falcons, pelleted by centrifugation at 400 g for 5 minutes at 4°C and resuspended in PBS/EDTA (2 mM). Digested remains were further disaggregated through a 70 µm strainer and subsequently pelleted as in previous step. Collected cells were resuspended in PBS/EDTA (2 mM) and ultimately filtered into FACS tubes through mesh to ensure single-cell suspension preparation.

Flow Cytometry

Single-cell suspensions of freshly isolated immune cells were pre-incubated with anti-mouse CD16/32 monoclonal antibody (Biolegend, 101302) and counting beads for 15 minutes on ice to block Fc receptors and determine absolute cell numbers, respectively. Cell suspensions were then stained for 20 minutes on ice with combinations of the following surface anti-mouse markers: CD45 AF700 (Biolegend, 103128), CD45 APC-Cy7 (Biolegend, 103116), CD11b APC (Biolegend, 101212), CD11c PE-Cy7 (Biolegend, 117318), CD24 APC-Cy7 (Biolegend, 101840), CD64 BV421 (Biolegend, 139309), Ly6C FITC (BD Biosciences, 553104), Ly6G BV786 (BD Biosciences, 740953), MHCII BV605 (Biolegend, 107639), TcRβ FITC (eBioscience, 11-5961-85), CD4 AF700 (Biolegend, 100536), CD8 APC (Biolegend, 100712) and IL-23R PE (Biolegend, 150903). Cell viability was assessed using Zombie Aqua (Biolegend) and downstream analysis was performed with a FACSCelesta II flow cytometer (BD Biosciences) equipped with FACSDiva software (BD Biosciences). Data were analyzed with FlowJo software (LLC).

Histopathological Analysis

Mouse terminal ileums were dissected, flushed with modified Bouin's fixative (50% ethanol / 5% acetic acid in dH_2O), opened longitudinally and prepared in swiss rolls, as previously described [96]. Tissues were fixed overnight in formalin at 4°C, embedded in paraffin and cut in 4 µm sections using

a SLEE medical microtome. Formalin-fixed paraffin-embedded (FFPE) sections were subsequently deparaffinized, rehydrated (xylene and descending ethanol gradients), mounted on slides and stained with hematoxylin and eosin (H&E) according to standard protocols. Semi-quantitative assessment of intestinal pathology was performed in a blinded manner according to the following scoring system: 0-4 for lamina propria inflammation, 0-4 for submucosal inflammation and 0-2 for muscle layer inflammation. Additionally, an extra score of 0-1 was assigned representing the extension of immune cell infiltration in each of the three layers. Histopathological evaluation was ultimately determined as the summation of individual scores for each sample and histological images were acquired with an Eclipse E800 microscope (Nikon) equipped with a QImaging ExiAqua digital camera using Bioquant Q-capture Pro7 software.

Quantitative Real-Time PCR

Tissue biopsies from mouse terminal ileums were collected, washed with PBS, snapped frozen in liquid nitrogen and stored at -80°C. Intestinal cells were lysed in TRI reagent (MRC) using a pellet pestle cordless motor and RNA extraction was performed according to the manufacturer's protocol. After DNase treatment with RQ1 (Promega, M6101), RNA was quantified by NanoDrop, reverse transcribed using MMLV (Promega, M610A) and further subjected to quantitative real time PCR using SYBR Green (Invitrogen, 11733-046) according to manufacturer's instructions. mRNA expression was calculated relative to *Ywhaz* gene using the ΔΔCt method. Primer sequences are provided below:

Gene	Forward primer	Reverse primer
Ifnγ	5'-GACAATCAGGCCATCAGCAAC-3'	5'-ATTGAATGCTTGGCGCTGGA-3'
Il17a	5'-AACTCCCTTGGCGCAAAAGT-3'	5'-GGCACTGAGCTTCCCAGATC-3'
Tnf	5'-CACGCTCTTCTGTCTACTGA-3'	5'-ATCTGAGTGTGAGGGTCTGG-3'
Gzmb	5'-GGGGCCCACAACATCAAAGA-3'	5'-GCCTTACTCTTCAGCTTTAGCA-3'
Prf	5'-TCTTGGTGGGACTTCAGCTTT-3'	5'-ATTCTGACCGAGTGGCAGTG-3'
Ywhaz	5'-AGAGTCGTACAAAGACAGCAC-3'	5'-GAATGAGGCAGACAAAGGTTG-3'

Bone Marrow Transplantation

For the generation of BM chimeras, 9-week-old wild type and *Il23p19*$^{-/-}$ mice were lethally irradiated with 2 equal doses of 610 rads given 3 hours apart using an MDS Nordion Gammacell 3000 Elan irradiator. After 24 hours, recipient mice were reconstituted with bone marrow cells (2 x 10^6 cells per mouse) obtained from the femora and tibiae of age and sex-matched wild type, *Tnf*$^{\Delta ARE/+}$ or *Il23p19*

$^{-/-}$ *Tnf* $^{\Delta ARE/+}$ donors. Reconstituted mice were supplemented with baytril-treated water for 2 weeks and terminal ileums were collected from all mice 5 months after irradiation for histological analysis.

Anakinra Treatment

Wild type and *Tnf* $^{\Delta ARE/+}$ mice were daily subjected to intraperitoneal injections with the IL-1R antagonist Anakinra (25 mg/kg) or with saline. Body weight was monitored every other day prior to administration. After 38 consecutive days of treatment, mice were euthanized, and terminal ileums were harvested for histological analysis.

ELISA

After euthanasia of vehicle and Anakinra-treated mice, blood was also collected for serum isolation. Blood samples were left at room temperature for 30 minutes, and after consecutive centrifugation steps (3,000 rpm for 10 minutes, 6,000 rpm for 10 minutes and 12,000 rpm for 10 minutes), the clear serum layer was transferred to fresh tube and subsequently used for cytokine measurement. TNF quantification was performed using the mouse TNF Elisa kit, according to manufacturer's instructions (Invitrogen).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0.1 software and reported as mean values ± SEM. Data were normally distributed between and within experimental groups and p-values of less than 0.05 were considered as statistically significant. P-values are depicted as * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$, and ns for non-significant.

RESULTS

Il23p19 deficiency in the hematopoietic compartment mitigates the severity of intestinal inflammation in the *Tnf*$^{\Delta ARE/+}$ ileum

Unpublished data from our lab have shown that genetic deletion of *Il23p19* confers significant alleviation of ileitis in the *Tnf* $^{\Delta ARE/+}$ mouse model. However, the cells that constitute the principal producers of IL-23 in the context of TNF-driven intestinal pathology are uncharacterized. On this notion, we sought to dissect the cellular origin of IL-23 in the *Tnf*$^{\Delta ARE/+}$ model by generating reciprocal BM chimeras. For this purpose, we performed engraftment of BM cells derived from *Tnf* $^{\Delta ARE/+}$ and *Il23p19* $^{-/-}$ *Tnf* $^{\Delta ARE/+}$ mouse donors into lethally irradiated wild type or *Il23p19* $^{-/-}$ recipients. Given the capacity of *Tnf* $^{\Delta ARE/+}$ BM cells to induce IBD [97], the implementation of this system enabled the restriction of IL-23p19 expression in either hematopoietic or radiation-resistant stromal cells in an inflammatory context (Figure 1A). Subsequent histological assessment of intestinal pathology 5 months after BM reconstitution showed that transfer of *Tnf* $^{\Delta ARE/+}$ BM cells into wild type recipients resulted in overt ileitis in female mice (Figure 1B). Unexpectedly, male recipients did not develop full-blown disease and were excluded from downstream analysis, as they could not serve as appropriate controls for the experimental procedure. Regarding female recipients, reconstitution of wild type and *Il23p19* $^{-/-}$ mice with *Tnf* $^{\Delta ARE/+}$ BM did not lead to differential disease modulation, as animals from both groups exhibited marked submucosal thickening and pronounced immune cell infiltration. Contrastingly, wild type and *Il23p19* $^{-/-}$ mice receiving BM cells derived from Il23p19-deficient *Tnf* $^{\Delta ARE/+}$ donors demonstrated reduced inflammatory infiltrates and mitigated intestinal pathology (Figure 1C), overall indicating the hematopoietic compartment as the primary source of pathogenic IL-23 in the *Tnf*$^{\Delta ARE/+}$ iletis mouse model.

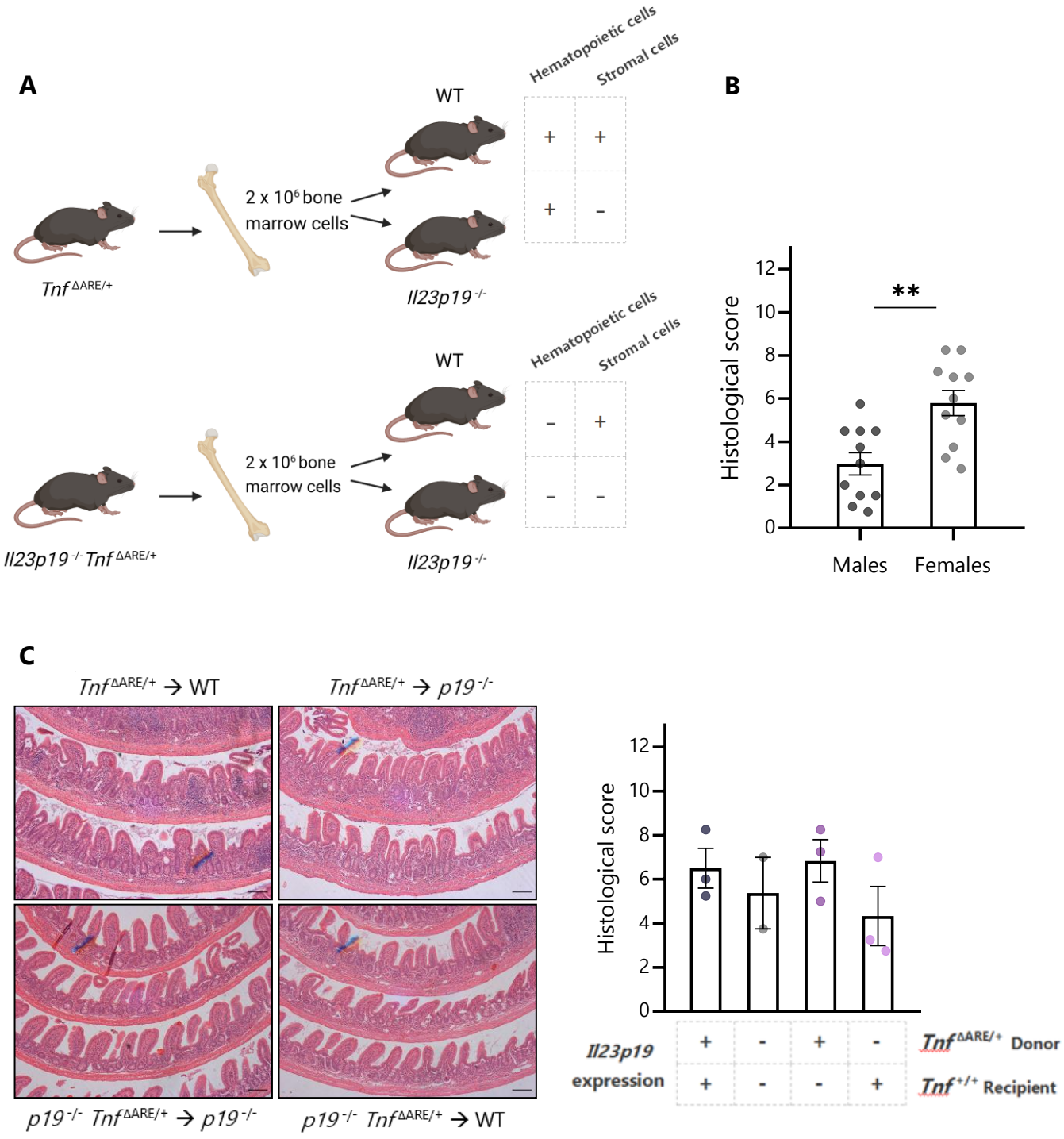
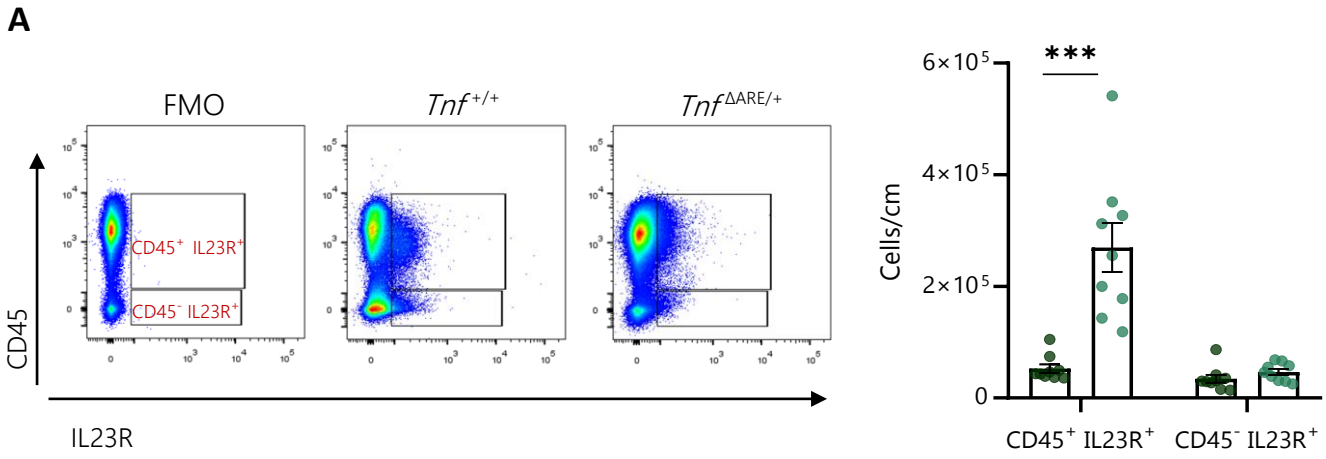


Figure 1: BM chimeras indicate the hematopoietic compartment as the principal source of IL23p19 during intestinal inflammation. (A) Schematic illustration of BM cell transplantation from *Tnf* $^{\Delta ARE/+}$ or *Il23p19* $^{-/-}$ *Tnf* $^{\Delta ARE/+}$ mouse donors into lethally irradiated wild type or *Il23p19* $^{-/-}$ recipients. (B) Terminal ileums of chimeric male and female mice were histologically evaluated 5 months after BM reconstitution (unpaired two-tailed

Student's t-test, ±SEM). (C) Representative images and histological assessment of H&E-stained ileal sections from female WT and *Il23p19* $^{-/-}$ recipient mice (scale bar, 100 µm; one-way ANOVA, Tukey's multiple comparisons test, ±SEM) .

IL23R^{+} immune but not stromal cells increase within the *Tnf*$^{\Delta ARE/+}$ ileum

To gain further insight into the role of IL-23 signaling in CD-like ileitis, we sought to identify the number of IL-23-responsive cells residing within the stromal or hematopoietic compartment altered upon intestinal inflammation. Flow cytometric analysis on IL-23R^{+}-gated cells isolated from the terminal ileum of wild type and *Tnf*$^{\Delta ARE/+}$ mice (Supplemental figure 1) revealed a definite increase of IL-23R-expressing immune cells in the *Tnf*$^{\Delta ARE/+}$ intestinal tissue. On the contrary, the number of IL-23R^{+} stromal cells remained unaltered between the two conditions (Figure 2A). Further analysis of immune cells into myeloid cells and T-lymphocytes demonstrated a marked increase specifically in macrophages, DCs and neutrophils (Figure 2B), as well as CD4^{+} T-cells (Figure 2C), while comparable numbers of monocytes, eosinophils and CD8^{+} T-lymphocytes were detected between *Tnf*$^{\Delta ARE/+}$ mice and controls. Taken together, these data reflect the extensive infiltration of IL23R^{+} immune cells in the terminal ileum of the *Tnf* $^{\Delta ARE/+}$ model and attribute a crucial role to IL-23 signaling in the development of the intestinal pathology.



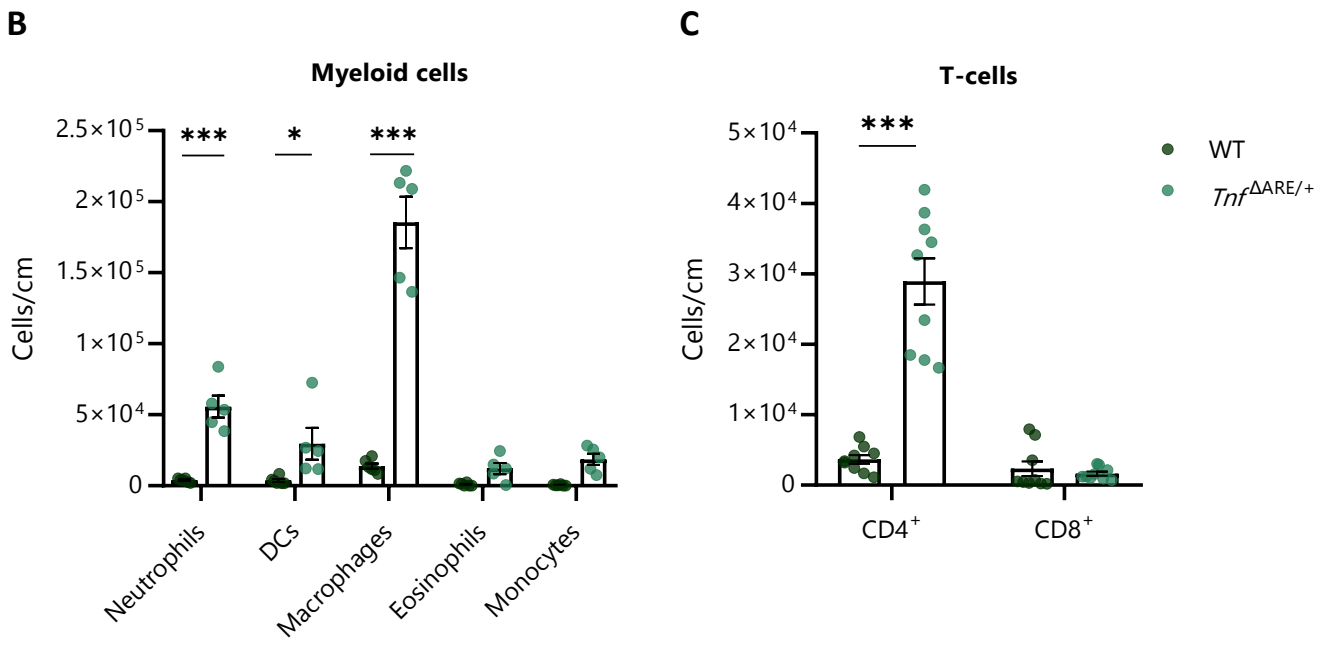


Figure 2: IL23R$^+$ immune cells increase in the *Tnf* $^{\Delta ARE/+}$ ileum. Flow cytometric analysis of IL23R$^+$ stromal cells (A), myeloid cells (B), and T-lymphocytes (C) from the terminal ileum of wild type and *Tnf* $^{\Delta ARE/+}$ mice at 12 weeks of age. Absolute cell number quantification is shown (n = 2–4 mice/genotype, two-way ANOVA, Sidak's multiple comparisons test, ±SEM). Data are representative of three (A and C) or two independent experiments (B).

IL23R deficiency in CD8$^+$ T-cells attenuates intestinal inflammation in *Tnf* $^{\Delta ARE/+}$ mice in a sex-specific manner

Next, focusing on some major immune cell types, we sought to investigate how their responsivensess to IL-23 affects the course of CD. Starting with CD8$^+$ T-cells, which constitute crucial mediators of the *Tnf* $^{\Delta ARE/+}$ pathology [97], we generated mice with CD8-specific IL-23R deletion (*Il23r* $^{CD8\alpha KO}$), and subsequently crossed them with *Tnf* $^{\Delta ARE/+}$ mice to achieve disease induction. To determine Cre specificity in our model, we assessed GFP fluorescence by flow cytometry, as in this construct GFP is co-expressed with Cre recombinase under the control of CD8α-promoter (Supplemental figure 2A). GFP expression in the mesenteric lymph nodes was restricted mainly in the CD8$^+$ T-cell compartment, whereas off-targets were detected only in a small fraction of TcRβ$^-$ cells (Supplemental figure 2B). Regarding ileitis pathology, histological evaluation demonstrated comparable inflammation scores between *Tnf* $^{\Delta ARE/+}$ and *Il23r* $^{CD8\alpha KO}$ *Tnf* $^{\Delta ARE/+}$ mice, initially suggesting that disease development is independent of IL-23 signaling in CD8$^+$ T-cells (Figure 3A). Surprisingly, re-analysis of histological

scores sex-wise revealed that CD8⁺ T-cell-specific ablation of IL-23R significantly attenuated intestinal pathology in *Tnf*$^{\Delta ARE/+}$ female mice. In contrast, no amelioration was observed in their male counterparts (Figure 3B). Conclusively, our data indicate that disruption of IL-23 signaling in CD8⁺ T-cells confers protection against intestinal inflammation in a sex-specific manner.

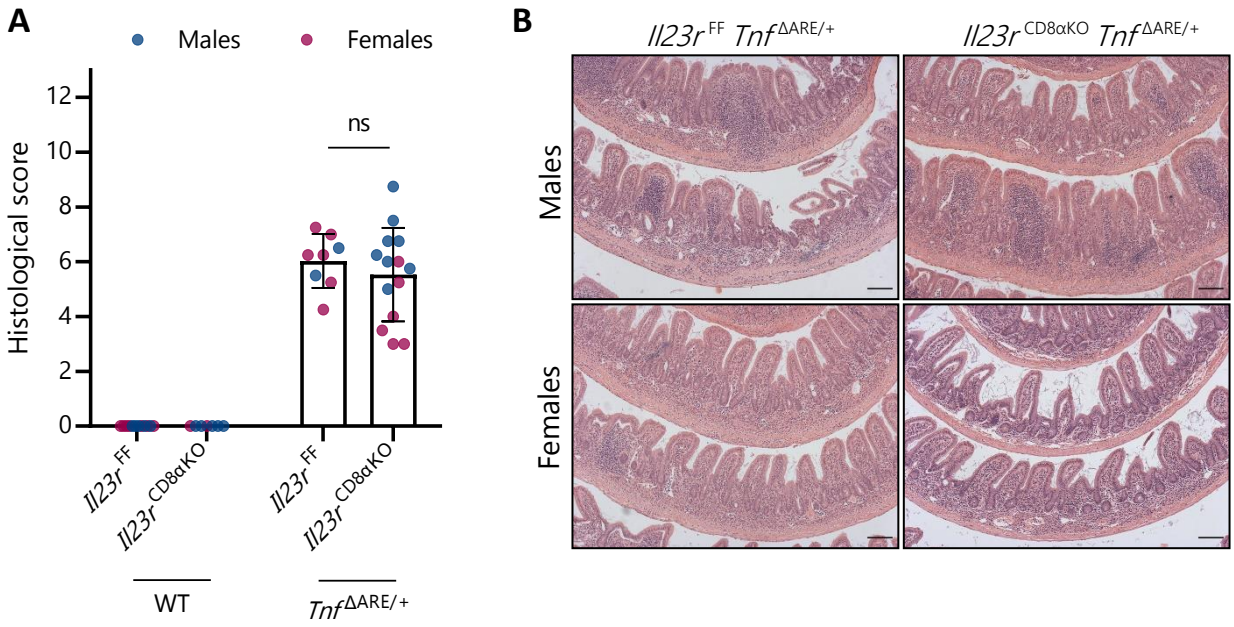
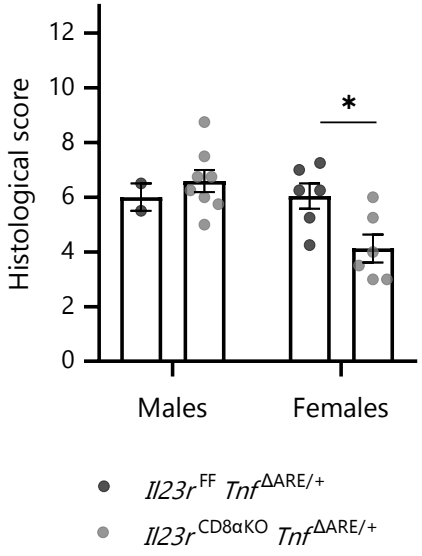


Figure 3: IL23R deletion in CD8⁺ T-cells attenuates intestinal inflammation in female mice. (A) Histopathological score of ileal sections from wild type or *Tnf*$^{\Delta ARE/+}$ *Il23r*^{FF} and *Il23r*$^{CD8\alpha KO}$ mice at 12 weeks of age. (B) Representative H&E images and sex-wise histological analysis of mice presented in A (scale bar, 100 µm; n = 1–5 mice/genotype, two-way ANOVA, Sidak's multiple comparisons test, ±SEM).



CD8⁺ T-cell-specific deletion of IL-23R in the *Tnf*$^{\Delta ARE/+}$ ileum does not alter the expression of IL-23 signaling targets and cytotoxic mediators

In our effort to delineate the mechanism whereby ablation of IL-23R in CD8⁺ T-cells confers protection against TNF-driven ileitis in female mice, we measured the expression of genes encoding for either known targets of IL-23 signaling or cytotoxic mediators. Pooled analysis of male and female *Il23r*^{FF} *Tnf*$^{\Delta ARE/+}$ and *Il23r*$^{CD8\alpha KO}$ *Tnf*$^{\Delta ARE/+}$ mice displayed comparable mRNA levels between the two genotypes for several pro-inflammatory cytokines (TNF, IFNγ, IL-17), as well as for the cytotoxic proteins granzyme B and perforin. Interestingly, re-analysis of expression data sex-wise yielded similar results, as no significant alterations were detected in mRNA levels between male and female animals (Figure 4). These results suggest that the sex-specific effect seen upon deletion of IL-23R in *Tnf*$^{\Delta ARE/+}$ CD8⁺ T-cells is potentially regulated by non-classical mediators of IL-23 signaling.

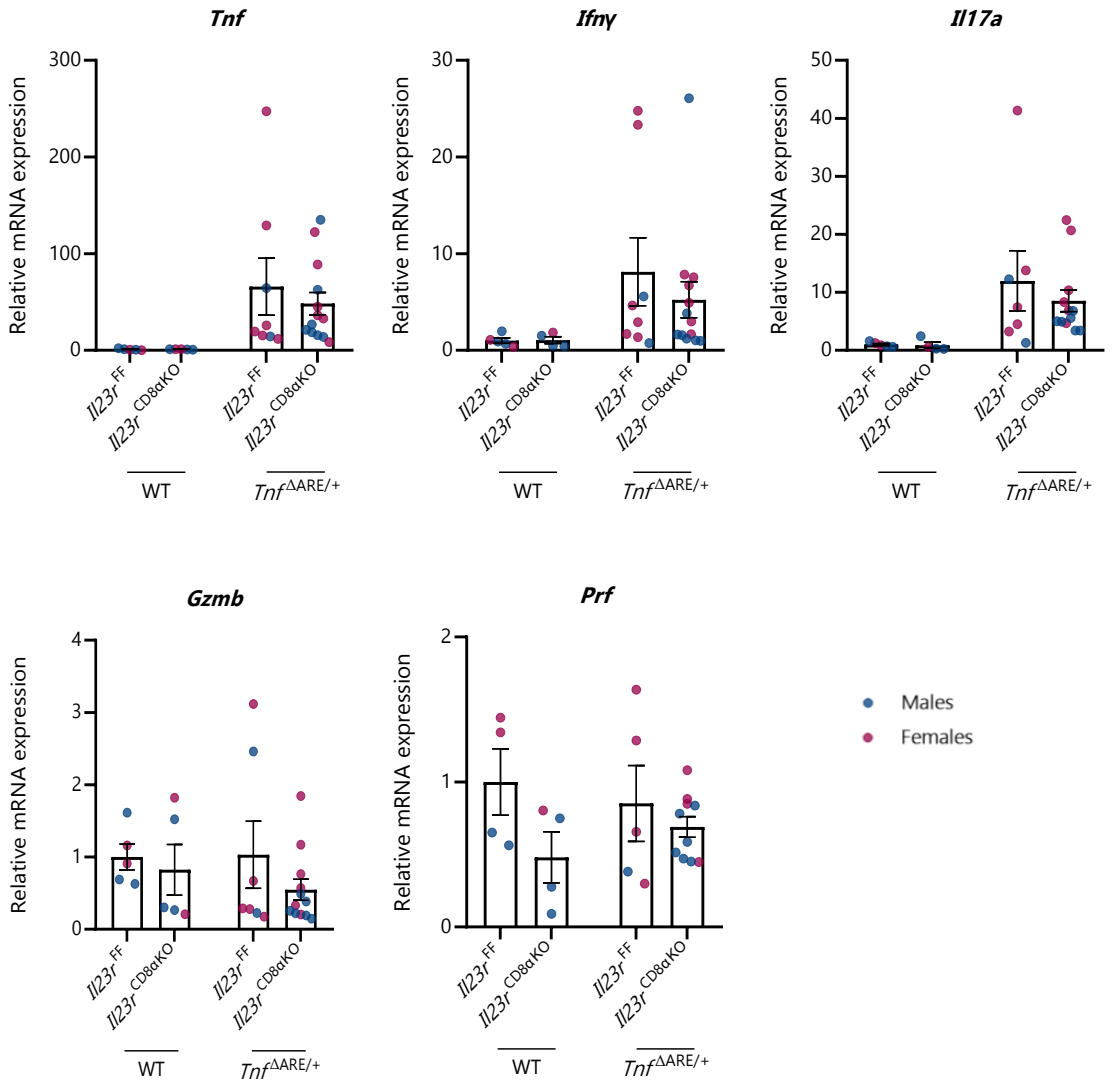


Figure 4: CD8⁺ T-cell-specific deletion of IL-23R in the *Tnf*$^{\Delta ARE/+}$ ileum does not alter the expression of IL-23 signaling targets and cytotoxic mediators. RT-PCR performed in intestinal cells isolated from the terminal ileum of either wild type or *Tnf*$^{\Delta ARE/+}$ *Il23r*^{FF} and *Il23r*$^{CD8\alpha KO}$ mice at 12 weeks of age (two-way ANOVA, Sidak's multiple comparisons test, ±SEM).

CD4⁺ T-cell-specific deletion of IL-23R tends to mitigate the intestinal pathology of *Tnf*$^{\Delta ARE/+}$ female mice

Having identified a pathogenic role for IL-23 in CD8⁺ T-cells during CD-like ileitis, we sought to extend our *in vivo* studies to other immune cell types that may contribute to intestinal pathology through IL-23 signaling. In particular, we focused our interest on CD4⁺ T-cells, which constitute a well-established cellular target of IL-23 in IBD. In this context, we generated mice bearing CD4-specific deletion of IL-23R (*Il23r*^{CD4KO}), crossed them with *Tnf*$^{\Delta ARE/+}$ mice to achieve disease induction, and eventually assessed their ileitis pathology at 12 weeks of age. It should be noted that the CD4-Cre line targeted both CD4⁺ and CD8⁺ T-cells, as CD4 is expressed early during T-lymphocyte development, at the double CD4⁺CD8⁺ thymocytes [98]. Also, our study was restricted to female animals, as male *Il23r*^{FF} *Tnf*$^{\Delta ARE/+}$ controls were not generated, thus impeding the conduction of sex-wise analysis. The subsequent histological assessment of female *Il23r*^{CD4KO} *Tnf*$^{\Delta ARE/+}$ mice revealed a tendency for lower inflammation scores in their intestinal tissues (Figure 5). Considering the dual targeting of CD4⁺ and CD8⁺ T-cells, we can assume that the histology result partially reflects the protective effect seen upon CD8⁺ T-cell-specific deficiency of IL-23R, and potentially excludes a primary pathogenic function of IL23R in CD4⁺ T-cells. An increase in the number of mice and inclusion of both sexes in future experiments will delineate the CD4⁺ T-cell-specific role of IL-23 signaling in the context of intestinal inflammation.

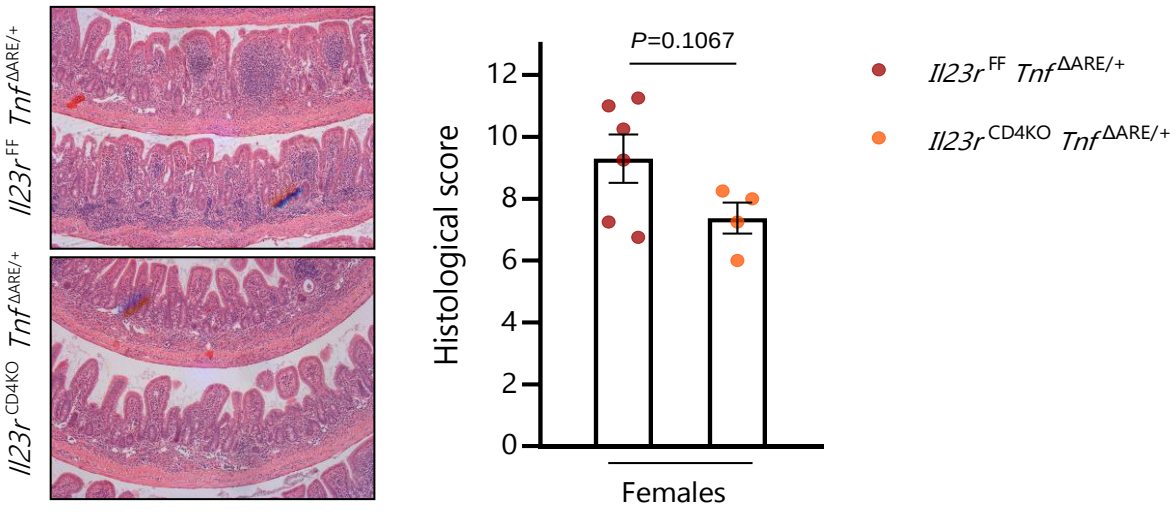
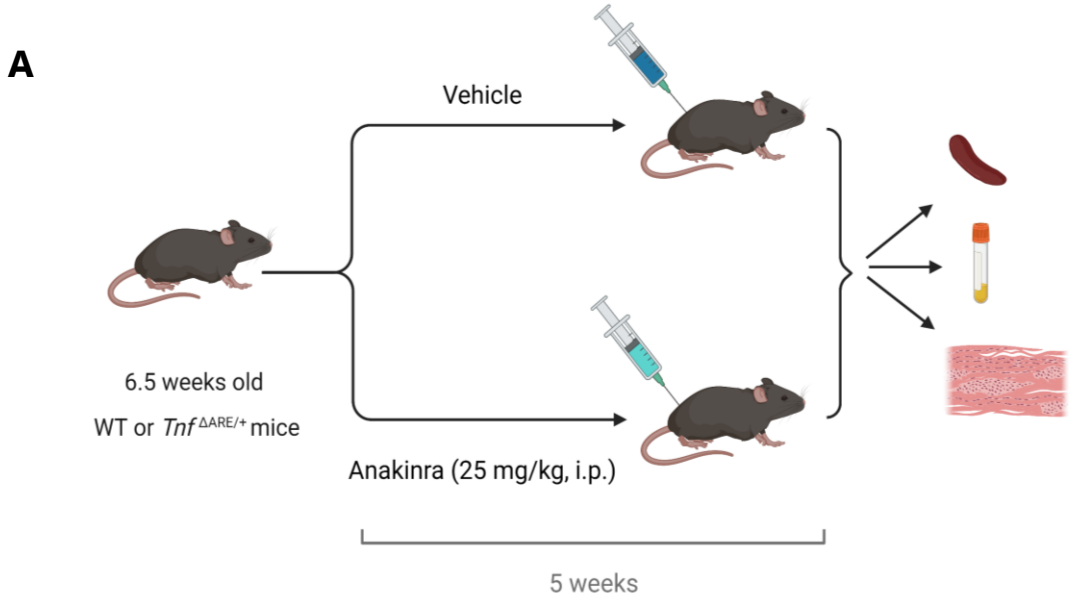


Figure 5: *Tnf* $^{\Delta ARE/+}$ female mice exhibit reduced ileal inflammation upon deletion of IL-23R in CD4^{+} T-cells. Representative images and histological assessment of H&E-stained ileal sections from female *Tnf*$^{\Delta ARE/+}$ mice at 12 weeks of age (scale bar, 100 μm; unpaired two-tailed Student's t-test, ±SEM) .

IL-1R signaling is dispensable for the development of ileitis pathology in the *Tnf*$^{\Delta ARE/+}$ mouse model

We next questioned whether other known pro-inflammatory cytokines contribute to the development of the *Tnf*$^{\Delta ARE/+}$ intestinal pathology. We particularly focused on IL-1, as increased levels of this cytokine have been reported in the inflamed mucosa of both IBD animal models and patients [99–101], while IL-1-dependent mechanisms have been also associated with non-responsiveness to anti-TNF therapies [102]. To this end, we addressed the role of IL-1 in our model by applying pharmacological inhibition of its cognate receptor. For this purpose, we treated wild type and *Tnf* $^{\Delta ARE/+}$ mice prophylactically with the IL-1R antagonist Anakinra or with saline in the case of experimental controls, in a long-term dosage scheme, as previously described [103]. Upon a 5-week treatment protocol (daily administration-see methods and Fig 6A) the mice were euthanized, spleens and sera were collected, and terminal ileums were harvested for histological evaluation (Figure 6A). As depicted in figures 6B-D, no significant differences were detected in inflammation score, spleen to body weight ratio and TNF serum levels between saline and Anakinra-treated *Tnf* $^{\Delta ARE/+}$ mice. Consequently, these results suggest that ileitis pathology in our model develops independently of IL-1R signaling.



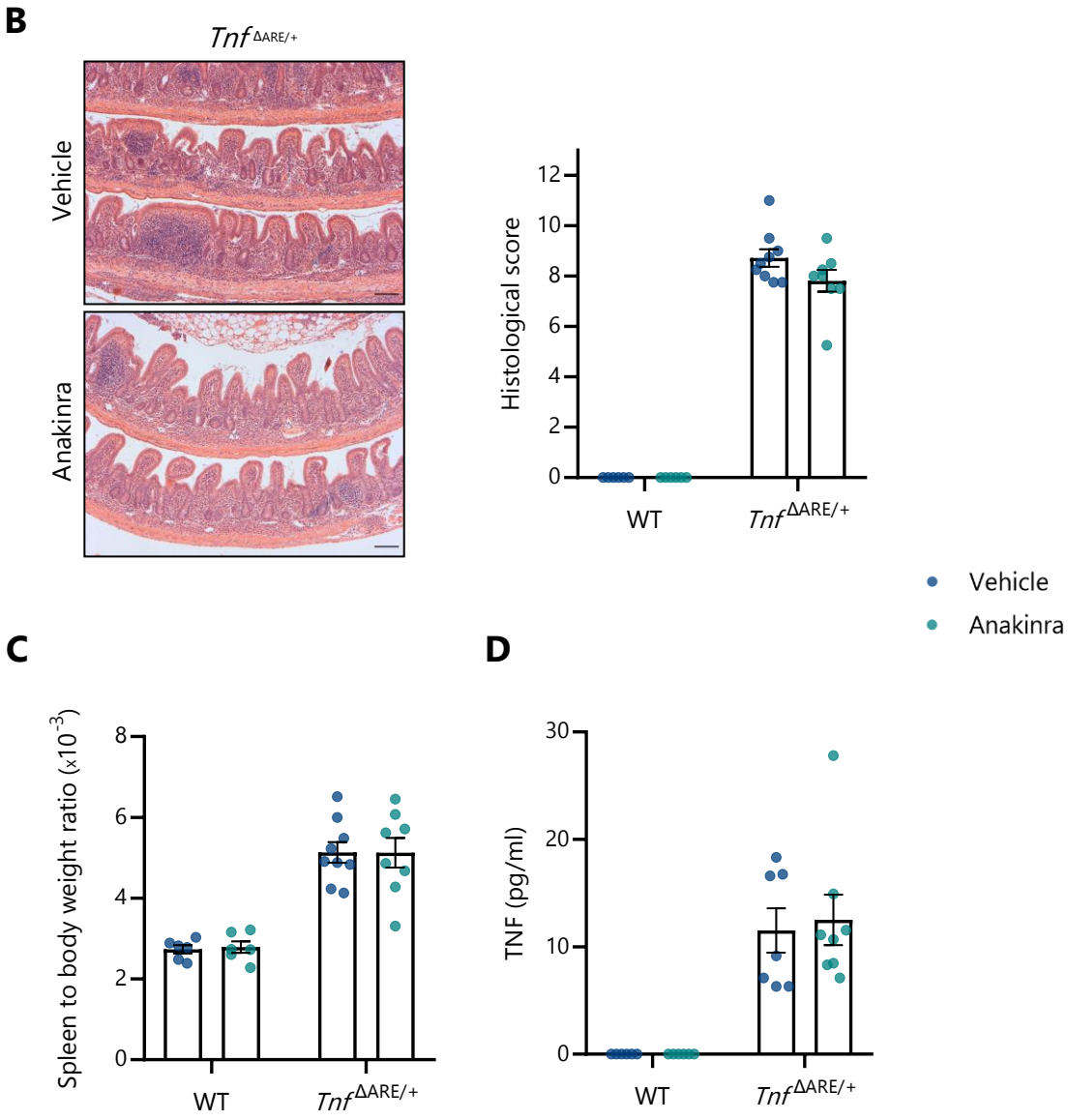


Figure 6: IL-1R blockade does not affect ileitis pathology in the *Tnf*$^{\Delta ARE/+}$ mouse model. (A) Experimental layout for the treatment of wild type and *Tnf*$^{\Delta ARE/+}$ mice with the IL-1R antagonist Anakinra. (B) Representative images and histological evaluation of H&E-stained ileal sections from Anakinra or saline-treated *Tnf*$^{\Delta ARE/+}$ mice at 12 weeks of age (scale bar, 100 µm;). (C) Assessment of spleen to body weight ratio and (D) TNF protein levels as determined by ELISA in sera from Anakinra or saline-treated wild type and *Tnf*$^{\Delta ARE/+}$ mice at 12 weeks of age (two-way ANOVA, Sidak's multiple comparisons test, ±SEM in B, C and D).

DISCUSSION

Converging evidence from experimental murine models, clinical studies, and population genetics have corroborated a pivotal role for IL-23 in regulating intestinal inflammation [9]. Ustekinumab, a therapeutic antibody targeting the common subunit of IL-12 and IL-23, has been widely used as a therapeutic approach against CD [2]. Additionally, the monoclonal antibody risankizumab, was recently licensed as the first IL23p19-specific biologic agent authorized for the treatment of moderately to severely active CD (URL: https://www.clinicaltrials.gov, Unique identifier: NCT03105128). Despite the successful application of these therapies, the relative importance of the diverse immunological pathways downstream of IL-23 in disease propagation remains controversial. In this context, using the *Tnf*$^{\Delta ARE/+}$ mouse model of Crohn's-like ileitis, we attempted to characterize the cellular producers and responders of IL-23 and dissect the potential cell-specific effects of its signaling in IBD pathology.

Starting with the generation of reciprocal BM chimeras, we identified that the predominant source of IL-23 in *Tnf*$^{\Delta ARE/+}$ mice resides within the hematopoietic compartment. This result is in line with existing murine and human studies, where macrophages and DCs appear as the primary producers of the cytokine [104]. Given that male mice were not included in our analysis due to technical reasons, we plan to repeat this experimental procedure with inclusion of both sexes, in order to establish the bone marrow as the main cytokine source in our model. We additionally showed that diverse immune cell types of the innate and adaptive system, but not stromal cells, increase in number during ileitis in response to IL-23. More specifically, macrophages, DCs, neutrophils, as well as CD4$^+$ T-lymphocytes expressing the IL-23 receptor exhibited increased frequencies in the inflamed ileum of *Tnf*$^{\Delta ARE/+}$ mice. These results demonstrate that IL-23 exerts its pathogenic activity through various immune cell types and substantiate the crucial role of its signaling in intestinal disease.

To deepen our understanding regarding the contribution of immune IL-23 cellular targets in CD, we generated mice conditionally deleted for *Il23r*, specifically in the CD8$^+$ T-cell compartment. A recent study on UC described a population of colonic IL-26$^+$ IL-23R-expressing CD8$^+$ T-cells that was associated with potentially protective functions during acute inflammation [105]. In our model, the number of CD8$^+$ IL-23R$^+$ T-lymphocytes was unaltered, as demonstrated by the flow cytometric analysis results. However, unpublished lab data have provided us with strong indications for a CD8$^+$ T-cell-specific function of IL-23 signaling in the *Tnf*$^{\Delta ARE/+}$ model. Specifically, we have detected the restoration in the number of infiltrating CD8$^+$ T-cells upon *Il12p40* and *Il23p19* deficiency, and a shift in their metabolic profile upon IL-23 stimulation.Moreover, the results from the CD8-Cre line revealed that deletion of IL-23R in CD8$^+$ T-cells conferred significant attenuation of ileitis pathology in female,

but not in male *Tnf* $^{\Delta ARE/+}$ mice. Strikingly, this sex-specific effect was not induced by classical mediators of IL-23 signaling or known cytotoxic effectors, as suggested by their expression levels in the inflamed ileum. Such sex-specific differences in the CD8$^+$ T-cell activation have been previously reported in respone to infection and were mechanistically attributed to differential sensitivity to IL-12 [106]. Notably, restrospective studies on the effectiveness of ustekinumab in IBD patients have demonstrated that the male sex was a predictor of non-response to therapy with this biologic agent [107,108]. Collectively, these findings advocate for a pathogenic sex-specific role of IL-23 signaling in the CD8$^+$ T-cell compartment, which constitutes a previously unappreciated IL-23 cellular target in CD.

The protective effect seen upon CD8$^+$ T-cell-specific ablation of IL-23R was partially detected also in the CD4-Cre mouse line, where *Il23r* ^{CD4KO} *Tnf* $^{\Delta ARE/+}$ female mice exhibited a trend for ameliorated pathology compared to their *Tnf* $^{\Delta ARE/+}$ counterparts. We hypothesize that this trend is induced by the simultaneous deletion of the receptor in CD8$^+$ T-lymphocytes, rather than CD4$^+$ T-cells, given that a potentially protective role of IL-23R deficiency in both cell types would lead to greater disease attenuation than that observed upon IL-23R deletion in CD4$^+$ or CD8$^+$ T-cells alone. These results tend to exclude a primary pathogenic function of IL23R in *Tnf* $^{\Delta ARE/+}$ CD4$^+$ T-cells, as seen in other models [104,109]; nevertheless, an increase in the number of experimental animals, as well as inclusion of both sexes, will enable us to deduce safer conclusions and better adress the sex-specific function of IL-23 signaling, as identified when the CD8-Cre mouse line was employed.

Apart from IL-23, IL-1 signaling has been also associated with CD pathology. Studies on various mouse models have described both pro-inflammatory and protective functions of the cytokine in the context of intestinal inflammation [110,111]. However, in our model, the role of IL-1 has not been elucidated, although evidence from previous studies indicate a potential contribution to disease. Specifically, our lab has reported that *Tnf* $^{\Delta ARE/+}$ mice exhibit significantly ameliorated intestinal pathology upon genetic deletion of Myd88 [112], a common mediator of TLR and IL-1R signaling [113]. Considering that the development of ileitis in *Tnf* $^{\Delta ARE}$ model is largely dependent on the microbiota [112], the pathogenic function of Myd88 was attributed to TLRs, without excluding a potential contribution from IL-1 signaling, as well. To delineate this effect, we administered mice with the recombinant human IL-1R antagonist Anakinra. Our findings showed that blockade of IL-1 signaling had neither detectable anti-inflammatory effect in intestinal pathology nor any alteration of disease severity, indicating that ileitis development is independent of IL-1 in the *Tnf* $^{\Delta ARE/+}$ model. Inclusion of genetic models in future experiments will help us yield more straight-forward results regarding the role of IL-1 signaling in the *Tnf* $^{\Delta ARE/+}$ pathology.

Collectively, our data propose that IL-1 signaling is dispensable for the development of ileitis in the *Tnf* $^{\Delta ARE/+}$ model. With regards to IL-23, we show that it is predominantly produced by the hematopoietic compartment and that deletion of IL-23p19 in bone marrow cells results in reduced pathology. We additionally demonstrate that IL-23R-expressing immune cells massively infiltrate the *Tnf* $^{\Delta ARE/+}$ ileum and we provide evidence for a disease-promoting sex-specific role of IL-23 signaling in CD8^{+} T-cells. Future RNA sequencing experiments will enable us to identify differences in the transcriptional profiles of male and female-derived *Tnf* $^{\Delta ARE/+}$ CD8^{+} T-cells and further dissect the mechanism of action of IL-23 in this cellular compartment. Finally, the characterization of ileitis pathology upon myeloid or stromal cell-specific IL-23R deficiency will allow us to gain valuable insights into the relative contribution of this cell type and ultimately decipher the complexity of IL-23 signaling in the pathology of the inflamed ileum.

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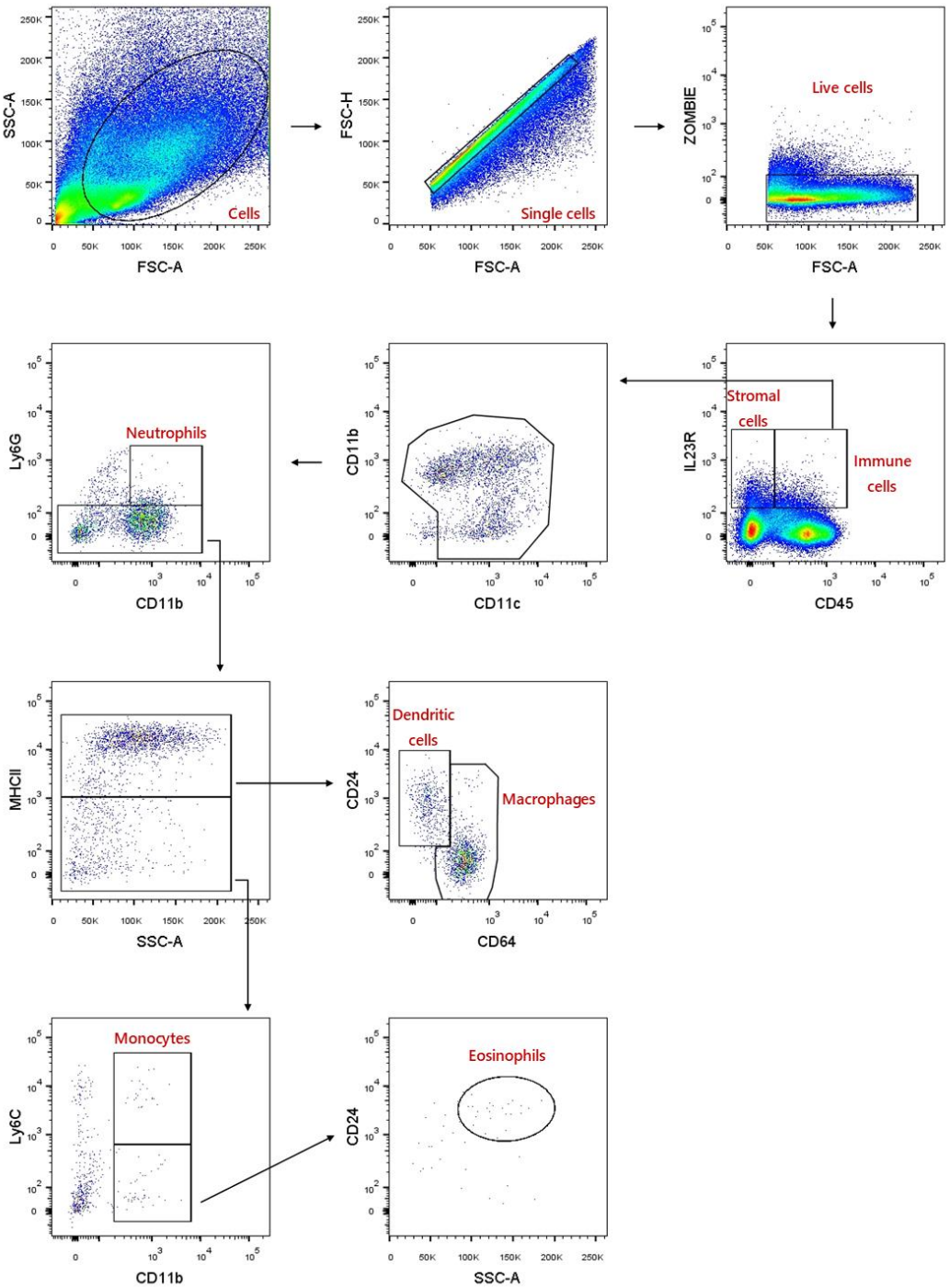
SUPPLEMENTAL MATERIAL

A



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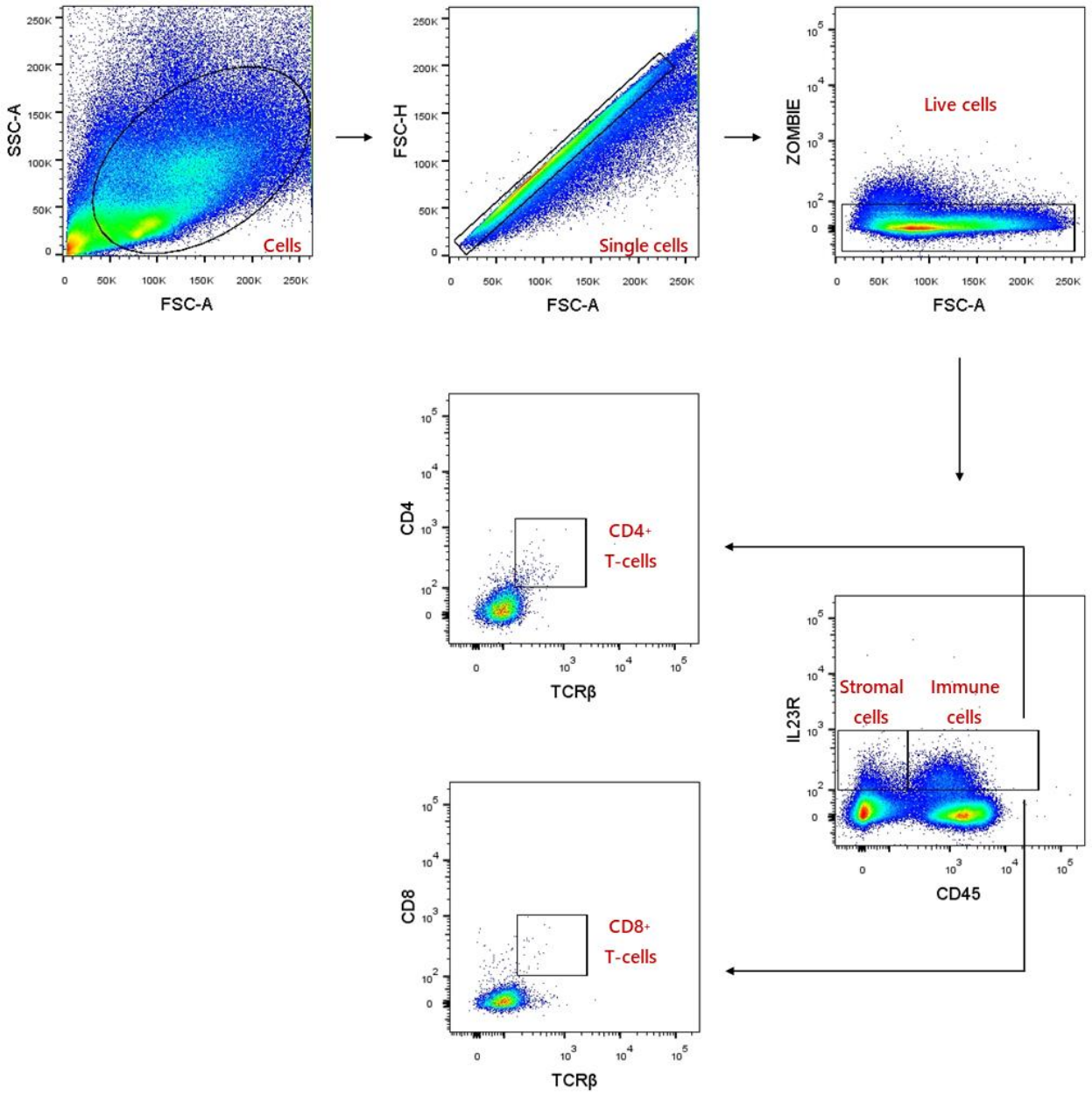
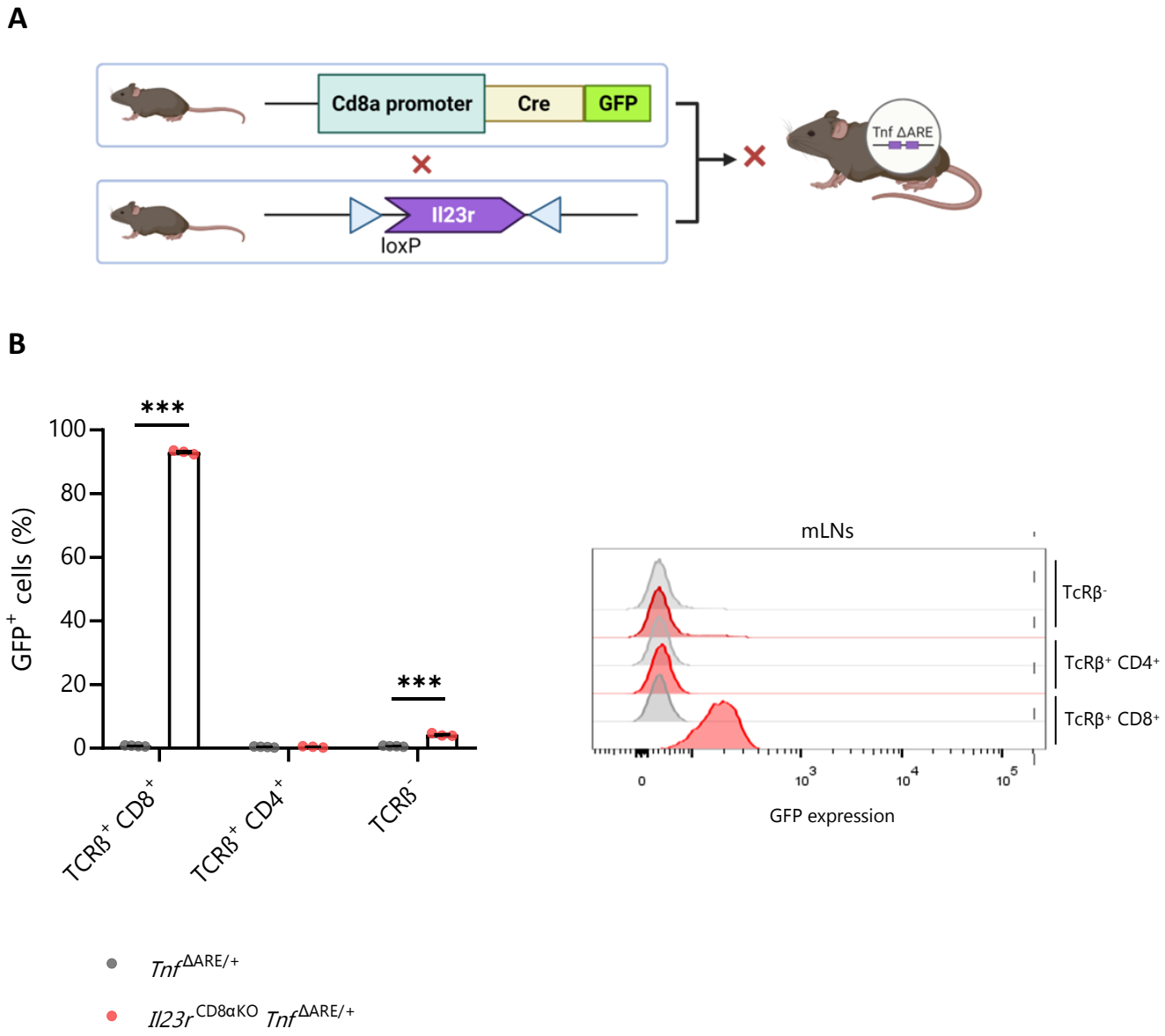


Supplemental figure 1: Gating strategy for IL-23R^{+} intestinal myeloid cells (A) and lymphocytes (B) isolated from terminal ileums of wild type and *Tnf*$^{\Delta ARE/+}$ mice. Representative FACS plots for the indicated cell surface markers.



Supplemental figure 2: Generation and tissue specificity of CD8-specific *Il23r*-deficient *Tnf* $^{\Delta ARE/+}$ mice. (A) Schematic illustration of the generation of CD8-specific IL23R-deficient *Tnf* $^{\Delta ARE/+}$ mice. (B) Tissue specificity of Cre recombinase as determined by flow cytometric analysis of immune cells isolated from mesenteric lymph nodes (mLNs) of *Tnf* $^{\Delta ARE/+}$ and *Il23r* $^{CD8\alpha KO}$ *Tnf* $^{\Delta ARE/+}$ mice based on their GFP expression.