Description of characteristic genes and potential transcription factors of *Il22ra2*-expressing cells

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Abstract

The immune system is an essential modulator of processes that fight and prevent infections, subvert changes in cells that may lead to malignancies, and promote tissue regeneration after injury. Adversely, immune responses can become dysregulated and cause chronic inflammation leading to ongoing tissue damage at barrier sites. Tight regulation of immune components involved in these inflammatory pathways is critical in preventing the deleterious effects of excessive inflammation.

IL-22BP is an important modulator of the adverse effects that arise from excessive and uncontrolled IL-22 signaling. This soluble receptor has so far been reported to be produced by dendritic cells, eosinophils, macrophages, and $CD4^+$ T cells, the latter of which has been found to induce a pathogenic outcome in Inflammatory Bowel Disease (IBD). To date, not much is known about the specific $CD4^+$ T helper cell subsets that express the gene for IL-22BP (*Il22ra2*).

The focus of this project was to investigate the transcriptional profile and characteristic gene markers of cells expressing *Il22ra2*. For this purpose, a reporter mouse was generated enabling the identification and isolation of *Il22ra2*-expressing cells which has identified novel cellular sources of the soluble receptor that have not yet been described in the literature. Single cell sequencing techniques have revealed that *Il22ra2*$^+$ cells are a heterogeneous population made up of $Foxp3^+$ T cells, T_h1-like cell types, NK cells, and macrophage populations. Mouse models of inflammation, including colitis, cancer, and infection (e.g. malaria) have shown that frequency levels of *Il22ra2* expression change significantly in $CD4^+$ T cells. Lastly, sequencing of *Il22ra2*$^+$ cells under different inflammatory conditions has identified common transcription factors that may be responsible for the regulation of the receptor, namely, *E2f2, E2f8, Mxd3, Mybl2, Pycard, and Hmgb2*.

Zusammenfassung

Die wichtigste Aufgabe des Immunsystems ist es, Prozesse zu modulieren, welche Infektionen bekämpfen, bösartige Veränderungen in Zellen verhindern, und die Geweberegeneration nach Verletzungen fördern. Nachteilig ist jedoch, dass eine dysregulierte Immunantwort chronische Entzündungen verursachen kann, die zu anhaltenden Gewebeschäden unter Anderem an Barrierestellen führen. Eine strenge Regulierung aller Immunkomponenten, die an diesen Entzündungswegen beteiligt sind, ist entscheidend, um die schädlichen Auswirkungen einer übermäßigen Entzündung zu verhindern.

IL-22BP ist ein wichtiger Modulator der nachteiligen Auswirkungen, die sich aus einer übermäßigen und unkontrollierten Produktion von IL-22 ergeben. Bisher wurde berichtet, dass dieser lösliche Rezeptor von dendritischen Zellen, Eosinophilen, Makrophagen und $CD4^+$ T-Zellen produziert wird, wobei sich herausgestellt hat, dass letztere bei chronisch entzündlichen Darmerkrankungen (CED) eine pathogene Wirkung haben. Bislang ist jedoch nicht viel über die spezifischen $CD4^+$ T-Helferzell-Untergruppen bekannt, die das Gen für IL-22BP (*Il22ra2*) exprimieren.

Der Schwerpunkt dieses Projekts lag auf der Untersuchung des Transkriptionsprofils und der charakteristischen Genmarker von Zellen, die *Il22ra2* exprimieren. Zu diesem Zweck wurde eine Reportermaus erzeugt, die die Identifizierung und Isolierung von *Il22ra2*-exprimierenden Zellen ermöglichte, wodurch neue zelluläre Quellen des löslichen Rezeptors identifiziert werden konnten, welche in der Literatur bisher noch nicht beschrieben wurden. Einzelzellsequenzierungstechniken haben gezeigt, dass *Il22ra2*-positive Zellen eine heterogene Population sind, die sich aus $Foxp3^+$-T-Zellen, Th1-ähnlichen Zelltypen, NK-Zellen, und Makrophagenpopulationen zusammensetzt. Mausmodelle für Entzündungen, einschließlich Kolitis, Krebs und Infektionen (z. B. Malaria) haben gezeigt, dass sich die *Il22ra2*-Expression in $CD4^+$ T-Zellen signifikant während dieser Krankheiten verändert. Schließlich wurden bei der Sequenzierung von *Il22ra2*-positiven Zellen unter verschiedenen Entzündungsbedingungen gemeinsame Transkriptionsfaktoren identifiziert, die für die Regulierung des Rezeptors verantwortlich sein könnten, nämlich *E2f2*, *E2f8*, *Mxd3*, *Mybl2*, *Pycard,* und *Hmgb2*.

Contents

Introduction

The health of a host relies strongly upon the maintenance of a stable environment, also referred to as homeostasis [1]. An important aspect of this process involves complex interactions between the immune system and tissues to sustain this mode of equilibrium after changes occur within the host. The immune system is an essential modulator of processes that fight and prevent infections, subvert changes in cells that may lead to malignancies, and promote tissue regeneration after injury [2].

Contrarily, immune responses can become dysregulated and cause chronic inflammation leading to ongoing tissue damage at barrier sites. Dysregulated immune responses have been described to promote the development of immune-mediated inflammatory diseases (IMIDs) [3]. Chronic inflammatory diseases such as inflammatory bowel disease (IBD), rheumatoid arthritis, and multiple sclerosis (MS) are described as IMIDs. These diseases share common inflammatory pathways and are characterized by chronic inflammation resulting in non-healing tissue damage [4]. The cause of such damage is strongly attributed to an aberrant immune response, although the exact etiopathogenesis of each of these diseases is unknown [3,4]. On the other hand, malaria is an infectious disease in which the launch of a strong inflammatory response is crucial to combatting the infection, but can also be detrimental to the host if not efficiently controlled [5-7].

Tight regulation of immune components involved in these inflammatory pathways is critical in preventing the deleterious effects of excessive inflammation. Interleukin-22 (IL-22) is one such immune component that is crucial for promoting inflammation and tissue regeneration at epithelial barriers but may have adverse pro-inflammatory and hyper-proliferative effects when left uncontrolled [8]. The soluble inhibitor of IL-22, most often referred to as IL-22 binding protein (IL-22BP), has been identified as an essential regulator of the effects of IL-22. The IL-22/IL-22BP axis has many implications at epithelial barriers, particularly for maintaining homeostasis at these sites [9].

This thesis aims to use a novel reporter mouse for identifying cells that express the gene for IL-22BP (*Il22ra2)*. The reporter mouse has been utilized to aid studies focused on the role of the receptor in inflammatory mouse models of IBD, colitis-associated colorectal cancer (CAC), and infection (e.g. malaria).

Particularly, a focus has been placed on *Il22ra2*-expressing $CD4^+$ T helper cell subsets and their contribution to different inflammatory settings. Therefore, sequencing experiments were carried out to define characteristic genes, the most important of which is the potential transcription factors that may regulate the expression of *Il22ra2* in $CD4^+$ T helper cells. Ultimately, defining how *Il22ra2*-expressing cells are transcriptionally regulated could aid in identifying ways to promote its protective effects or prevent its inhibitory effects in aberrant inflammatory conditions.

Background

Inflammatory bowel disease

Inflammatory bowel disease (IBD) describes a group of diseases characterized by chronic inflammation of the gastrointestinal tract. The most well-described diseases within this group comprise Crohn's disease and ulcerative colitis. IBD is described as a multifactorial disease affecting 6.8 million people around the world, with the highest incidences being in developed countries [10,11].

IBD is characterized by uncurbed inflammation and tissue damage in the gastrointestinal tract and is often associated with diarrhea, abdominal pain, fatigue, weight loss, and extra-intestinal symptoms [12]. Histological characteristics vary between the two major representatives of IBD. Crohn's disease is characterized by focal and transmural inflammation, which can occur anywhere along the gastrointestinal tract and may lead to complications such as abscesses and stenosis [13,14]. Contrastingly, ulcerative colitis is defined as a mucosal inflammation that solely affects the rectum and the colon. Inflammation and disease severity is often constant throughout the colon [15]. Importantly, patients suffering from chronic inflammation have an increased risk of developing dysplasia, which may ultimately develop into colon cancer [16]. Extra-intestinal manifestations may also occur, as many IBD patients have been reported to also suffer from PSC, arthritis, and skin-related diseases.

Although the etiology is unclear, key factors that determine an individual's risk of developing IBD include genetics, dietary habits, microbiota composition, and dysregulated immune responses to external threats [17]. To date, more than 150 susceptibility loci have been identified to be associated with IBD development [18]. Recently, incidences are rising globally, seemingly due to epidemiological changes in diet and lifestyle choices. In particular, the host´s microbiota composition is very impressionable and can be altered through minimal changes in diet, smoking habits, antibiotic intake, or substance abuse [19-21].

Interestingly, genome-wide association studies (GWAS) studies have identified characteristic genes for many immune components that are strongly associated with IBD, particularly components of cytokine networks related to T helper cells [22]. In general, an imbalance of cluster of differentiation 4^+ ($CD4^+$) T helper cell subsets has

been attributed to driving IBD development and affecting the severity of inflammation [23].

Treatment of IBD

Essentially, treatment plans for patients with IBD aim to reduce inflammation and promote mucosal healing. Currently, corticosteroids, thiopurines, and biologicals, such as antibodies directed against tumor necrosis factor-alpha (TNFα), IL-12p40, or the α4 β7 integrin, are used to treat IBD patients [24,25]. However, significant side effects and non-responsiveness to such regimens call for another course of action with an even more targeted approach [26]. Understanding the underlying immune mechanisms behind IBD development is critical for developing new therapeutic strategies. For instance, specific aspects of the immune system may be targeted to prevent dysregulated and pathological inflammatory responses, or a more customized approach may be taken to alter the host´s microbiota to a less colitogenic composition. Examples of recently developed immune-based treatments include the targeting of pro- or anti-inflammatory pathways [24], inhibition of integrins [25] and other molecules which modulate the migration of leukocytes into the inflamed intestinal tissue [27-29], mesenchymal stem cells [30,31] and engineered regulatory T cells [32].

The intestinal microbiota plays an important role in IBD. Remarkably, analysis of stool from IBD patients shows a distinct decrease in two major phyla that are known to dominate the gut microbiota in healthy individuals, namely *Firmicutes* and *Bacteroides*. Instead, increases in other less abundant phyla, such as *Proteobacteria* and *Actinobacteria* are found in IBD patients [33]. Such alterations have been proven to be unfavorable for the host, such that changes in the microbiota compositions can result in dysbiosis leading to an aberrant immune response. In addition, distinct microbiota signatures have been associated with reduced patient responsiveness to current therapy regimens [34]. One method to restore gut flora homeostasis is through fecal microbiota transplantation, which has been tested in clinical trials, but has not been approved as a therapeutic option. This method describes the transfer of healthy donor microbiota to affected patients to introduce a more diverse and less colitogenic microbiota composition in the recipient with minimal manipulation [35]. Alternatively, supplementation with probiotics has been approved for the treatment of ulcerative

colitis. Examples include oral intake of *Escherichia coli* Nissle and VSL#3, a probiotic mixture made up of eight bacterial strains [36,37]. Another, more recent method of altering the gut microbiota is through the use of bacteriophages, which is being tested in preclinical models and can selectively target disease-contributing pathobionts known to exacerbate intestinal inflammation in IBD patients [38].

Finally, surgical removal of damaged portions or the entire colon may be applied as a last resort [39]. IBD is a lifelong disease, and although relapses and inflammation flares may occur, patients will remain in remission for most of their lives if they are continuously treated and responsive to medication. Most patients lead similar lifestyles to before their diagnosis and have average life expectancies, although the quality of life may be reduced [40].

Colitis-associated colorectal cancer

Colorectal cancer (CRC) is listed as the fourth most deadly cancer in the world [41] and the incidence of this cancer entity is rising rapidly, especially in developing countries worldwide [42]. CRC can be split into multiple categories. For example, it may arise due to genetic predispositions or sporadically, but can also occur as a complication of chronic IBD-associated inflammation, otherwise known as colitis-associated colorectal cancer (CAC). Specifically, chronic inflammation can result in mutations that can alter the molecular makeup of epithelial cells, which then become hyper-proliferative and affect the future progeny of these cells. Patients with chronically active IBD in the colon have a significantly increased risk of developing CAC than the general population. Furthermore, these patients have a seemingly worse prognosis than those that develop sporadic CRC [43]. This development is mostly attributed to unresolved chronic inflammation or particularly aggressive inflammation that affects most of the colon in these patients [43].

Studies have shown that the genetic mechanisms leading to the development of sporadic CRC and CAC are very similar and arise due to chromosome instabilities and mutations of key tumor suppressor genes. However, remarkable histological differences have been well-defined and aid in identifying whether the patient has developed CRC as a result of chronic colitis [43]. For example, CRC is often associated with adenomatous polyps, whereas CAC is mostly identified by dysplasia of cells.

Chronic inflammation and dysregulated wound healing mechanisms initiated and maintained by excessive IL-22 signaling can have detrimental effects on the host and may ultimately lead to malignancy if not mediated by such factors as IL-22BP. Investigating the regulation of both components of this axis can prove beneficial when attempting to reduce malignant outcomes due to excessive inflammation associated with IBD.

Immune responses in IBD

An immune response in a host consists of the recognition and elimination of a threat. Whether this threat originated externally or arises within the host, the immune response aims to eradicate it as fast, and with as little damage to the surrounding tissue, as possible. The activities of the immune system are carefully orchestrated by the many different types of leukocytes that work together to manage infections and inflammation. The two arms of the immune system, made up of either innate or adaptive immune cells, are described to have specific roles and protective mechanisms within the gastrointestinal tract. The interplay between the mucosal immune system in the gut, epithelial barrier, and microbiota has particular consequences on the development of IBD. Specifically, numerous innate and adaptive lymphocyte populations which already reside within the lamina propria and between epithelial cells keep the intestinal microbiota under constant surveillance and have been reported to contribute to IBD pathogenesis, when left unchecked [44].

Innate immune cells

The innate immune response is mediated through cells of myeloid origin which possess innate recognition receptors, known as pattern recognition receptors (PRRs), Although responses via innate cells are fast, the limited repertoire of receptors expressed on the surface of these cells is mostly non-specific. Generally, cells belonging to this arm of the immune system are regarded as sensory cells that act to amplify the overall immune response. Innate cell activation results in the direct destruction of invading pathogens or the release of alarm signals, in the form of cytokines and chemokines, which alert other immune cells to take action and propagate further defense mechanisms.

Macrophages

Macrophages are termed ‘scavenger’ cells that have the general purpose of removing remnants of dead cells and invading pathogens. Macrophages are derived from inflammatory monocytes which migrate from the bone marrow and become resident in almost all tissue types. They are particularly important for regulating tissue repair and fibrosis after tissue injury. This cell type is known for its phagocytic function, which it utilizes to engulf and kill pathogens rapidly after encountering them. Another essential role of macrophages is to propagate further immune responses through the release of cytokines and chemokines to recruit other cell types to aid in removing threats to the host. Macrophages are heterogeneous cells that are highly adaptable and can take on distinct functions in response to environmental cues [45]. Specifically, they can differentiate into either pro-inflammatory or anti-inflammatory subsets during inflammatory conditions, which can result in different outcomes in the tissue affected [46]. In the context of IBD, this cell type is crucial for the resolution of inflammation, as they dampen inflammatory responses from effector T cells and promote repair of the epithelial barrier [47].

Neutrophils

Neutrophils belong to a group of cells referred to as granulocytes. This cell type is relatively short-lived after encountering inflammatory signals and is known to contribute to tissue injury as it amplifies the inflammatory response. Phagocytic neutrophils are recruited from the bone marrow to sites of inflammation, and increase in number when a threat is recognized by other cells of the immune system. A major effector function of neutrophils is that they release granules comprising degradative enzymes and toxic proteins that can destroy microbial threats. Additionally, this cell undergoes a unique form of apoptosis used as a method of killing extracellular parasites by forming neutrophil extracellular traps (NETs) [48]. Although neutrophils are critical for the resolution of inflammation, infiltration of this cell type in the epithelial barrier is a characteristic feature of IBD, and excessive release of reactive oxygen species (ROS) by these cells results in epithelial barrier damage [49].

Eosinophils

Eosinophils are another type of granulocyte that are important contributors to destroying parasitic threats and are active in allergic inflammatory reactions. This cell type also has granules comprising of cytotoxic enzymes and proteins, and like

neutrophils, are also reported to cause tissue damage when activated. Up-regulation in eosinophils is linked to T_h2 cell-mediated responses, where the increased production of IL-5 induces eosinophilopoiesis and activation [50]. Generally, eosinophils exert inflammatory and pro-fibrotic functions in the context of IBD [51].

Natural Killer (NK) cells

NK cells are cytotoxic immune cells of the innate branch which are important sensors of virus-infected and tumor cells. Although they share cytotoxic effector functions with T cells and produce some of the same cytokines, NK cells lack the antigen-specific rectors of adaptive immune cells. Instead, they recognize altered or 'non-self' cells through germline-encoded activating and inhibitory receptors. Significantly, inhibitory receptors that recognize MHC I molecules function to prevent NK cells from killing a normal host cell. Characteristically, NK cells are larger than T and B cells and have cytotoxic cytoplasmic granules. These granules contain granzymes and perforin proteins that create pores and induce apoptosis in target cells [52]. Additionally, distinct functional subsets of NK cells that exert different functions exist, particularly in the intestinal mucosa [53]. Although NK cells are generally increased in the inflamed mucosa of IBD patients, this cell type can have varying effects on the pathogenesis of IBD, which is dependent on the conditions within the host [54].

Innate lymphoid cells (ILC)

The ILC family comprises of NK cells, Group 1 ILC (ILC1), ILC2, and ILC3 cells. This group of cells is defined by their lack of antigen-specific receptors and are important mediators of inflammatory responses at barrier tissues, as they rapidly respond to host and microbial cues. Strikingly, members of the ILC family resemble T helper cell subsets as they are regulated by the same promoting transcription factors and have similar cytokine production profiles. Specifically, the ILC1 subset is regulated by T-bet and releases interferon gamma (IFNγ) and TNFα to provide protection against intracellular pathogens. ILC2s are regulated by GATA3 and produce IL-4, IL-5, and IL-13. Lastly, ILC3s resemble T_h17 cells, in that they express RAR-related orphan receptor gamma (thymus-specific isoform) (RORγt) and produce IL-17 and IL-22 to combat extracellular pathogens. Just like T helper responses, the cytokine production from these cells must be efficiently regulated to prevent aberrant and excessive inflammatory responses [55].

Dendritic cells

Dendritic cells are key cells active in both the innate and adaptive immune systems that enable crosstalk between the two. Although dendritic cells can phagocytose, they mainly function as professional antigen-presenting cells whose purpose is to activate the rest of the immune system. These cells are equipped with the ability to activate B and T cells, the two major cell types that contribute to adaptive immunity. Specifically, in the intestinal tract, dendritic cells sample the intestinal lumen and take up bacterial antigens, which they then transport across the epithelium, ending up in either Peyer's patches or mesenteric lymph nodes. Both these areas are rich in immune cells, and antigen presentation here induces differentiation of B cells and activation of T cells [56].

Adaptive immune cells

In contrast to the innate response, the adaptive immune response is defined as highly specific. Adaptive immune cells rely on interactions with innate immune cells to launch an appropriate immune response. Although an adaptive response may take longer to initiate any effector functions, it does confer long-lasting immunity against the specific pathogen that initiated the immune response [57].

A major cell type that makes up part of the adaptive branch of the immune response is a T cell. T cells carry out a cell-mediated response which is based on their ability to distinguish between host and non-host cells. T cells recognize foreign entities when the antigens are presented by the host's own cells via major histocompatibility complex (MHC) proteins and can be distinguished by their cell surface proteins, cluster of differentiation 4 (CD4), or cluster of differentiation 8 (CD8). $CD8^+$ T cells are cytotoxic and can release granzymes and directly lyse target cells after being activated by MHC I molecules. $CD4^+$ T cells are helper cells that aid in the activation of other cells, produce cytokines that recruit other cells to the site of injury, and may also have cytotoxic activities. $CD4^+$ T helper cells are specially equipped to mediate reactions to inflammation as these cells can differentiate into different subsets. These assorted subsets are capable of eradicating a whole range of threats posed by different classes of pathogens or tissue changes [57].

After primary development in the thymus, naïve cells are recruited to the peripheral tissue via the vascular and lymphatic networks until they come across an appropriate antigen and become activated. T helper cells increase their metabolic activity by

binding of the antigen/MHC II complex to the T cell receptor (TCR) with the help of co-stimulatory molecules, such as CD28, to enhance receptor signals enabling the induction of transcription factors. Upon activation, T cells differentiate into distinct effector T cell subsets that elicit a certain type of immune response and undergo rapid cell division to form a large repertoire of cells. These different types of subsets are conventionally defined by the cytokines they produce and the master transcription factors that regulate gene expression [57]. Essentially, $CD4^+$ T cells are considered to promote intestinal inflammation and many subsets are considered major contributors to the development of IBD [58].

$CD4^+$ T helper cell subsets

T_h1 cells are typically defined by the master transcription factor T-bet and their production of IFNγ. Differentiation of this cell type is dependent on stimulation via fate specifying cytokines, such as members of the interferon family and IL-12. Once activated, downstream signaling via Signal Transducer and Activator of Transcription (STAT) 1 or STAT4, induce expression of *Ifng*. This cell subset is suited to combatting intracellular pathogens, such as certain bacteria, viruses, and protozoan. Specifically, T_h1 cells recognize antigens on macrophages and promote the microbial killing capacity of these cells [57,59]. For the most part, T_h1 cells are reported to promote the development of IBD, mostly due to the release of IFNγ under inflammatory circumstances [58,59].

Differentiation of naïve T cells into T_h2 cells is initiated after these cells encounter IL-4. Activation results in signaling via STAT6, which induces the master transcription factor, GATA3. T_h2 cells are characterized by their production of IL-4, IL-5, and IL-13. These cytokines are particularly important in enhancing mucosal barrier immunity by recruiting eosinophils and mast cells to contain infections caused by extracellular parasites, such as helminths. Both T_h1 and T_h2 subsets have a positive feedback loop with their cytokine expression, which is a mechanism used to program the development of the cell to a specific subset type and to maintain the relative cytokine expression profile [57]. T_h2 cells are historically associated with ulcerative colitis, and the high production levels of IL-13 have been shown to destabilize epithelial tight junctions, promote apoptosis and encourage tissue fibrosis [58,59].

The development of T_h17 cells occurs when there are copious amounts of IL-6 and Transforming growth factor beta (TGFβ) in the environment of differentiating cells. Activation of these cells promotes up-regulation of the master transcription factor, RORγt, and induces the expression of the IL-23R. The expansion of these cells and determination of their cellular state requires further signaling via IL-23. T_h17 cells are critical mediators of immune responses at barrier sites and in protecting the host against pathogens through the induction of antimicrobial peptide production from epithelial cells. Specifically, the mode of action of T_h17 cells involves the recruitment of neutrophils to epithelial barriers to combat extracellular bacteria and fungi infections [57]. During inflammation in the gut, there is a great up-regulation in IL-17-expressing $CD4^+$ T cells, although their role in IBD pathogenesis can be either protective or detrimental [59].

The cytokine IL-22 was originally identified to be produced by T_h1 and T_h17 cells. However, it can also be produced by a specific subset defined by the production of this very cytokine, the T_h22 cell subset. The development and transcriptional regulation of IL-22-producing cells are discussed in more detail in the next section.

Regulatory T cells differ in that they suppress T cell responses instead of activating them. Recently, two different kinds of regulatory T cell types have been defined, Forkhead box $p3^+$ (Foxp3) Tregs and Type 1 regulatory (T_r1) cells. $Foxp3^+$ Tregs may become activated in different locations. Natural Tregs (nTregs) refer to these cells that have developed in the thymus and induced Tregs (iTregs) develop after antigen recognition in peripheral host tissues [60]. Differentiation of naïve T cells into $Foxp3^+$ T regs requires the presence of TGFβ but also requires the cytokine IL-6 and other inflammatory cytokines to be absent. The resulting regulatory T cells are immunomodulatory and are defined by their expression of the master transcription factor, Foxp3, the surface marker, CD25, and the production of IL-10 and TGFβ [57]. On the contrary, T_r1 cells are defined as IL-10-producing cells that are not regulated by the transcription factor, Foxp3 [61]. IL-10 is a common cytokine for both these subsets and is commonly associated with suppressing other T cell subsets from differentiating and producing cytokines [62]. Regulatory T cell subsets are integral to limiting the immune response to prevent excessive damage, and for resolving inflammation. In general, depletion of such regulatory cells results in an uncontrolled

inflammatory response that results in damage to the epithelial barrier in mouse models of intestinal inflammation and in IBD patients [63].

$CD4^+$ T helper cell subsets have many remarkable traits that make them well-suited to induce and alleviate inflammatory responses at epithelial barrier sites. Encountered antigens and the surrounding cytokine milieu determine the specific cytokines produced by T helper cells and determine whether they adapt their cell profiles to promote or reduce inflammation. For instance, specific T helper cell subsets can cross-regulate and inhibit the differentiation and cytokine production of other T cell subsets [57]. Yet another exceptional characteristic harbored by these cells is that T helper cells can be commensal specific, in that specific antigens induce differentiation into a specific subset [64]. Also, T cell subsets have recently been identified to be plastic and can adapt their cytokine expression to suit the surroundings [59]. All these characteristics are pivotal for promoting host defense and tissue repair.

NKT cells

NKT cells are characterized by their recognition of lipids presented by CD1d molecules, their possession of TCR machinery, and their innate-like properties. NKT cells can be either $NK1.1^+$ or $NK1.1^-$ and can also differ in the presence or absence of CD4 and CD8 surface molecules. Compared to other adaptive cell types, these cells are recognized to respond rapidly to antigenic stimulation. Specifically, all invariant NKT (iNKT) cells express the transcription factor promyelocytic leukemia zinc finger (*PLZF*). Functionally distinct subsets of these iNKT cells exist and resemble T helper cells, in that they are regulated by the same transcription factors and produce the same key cytokines as T helper cells. These subsets are known as iNKT1, iNKT2, and iNKT17 [65].

B cells

B cells have distinct antigen receptors. The B cell receptor is formed by the same genes that encode antibodies. Antibodies and B cell receptors directly recognize the epitopes of native antigens in the serum or extracellular spaces. B cells can act as antigen-presenting cells but mostly require help from $CD4^+$ T helper cells to elicit an appropriate immune response. T cells regulate B cell responses to most antigens.

In intestinal tissues, B cells migrate out of the mesenteric lymph node, differentiate into plasma cells and begin to produce immunoglobulin (Ig) A, which is transported across

the epithelial layer via transcytosis. IgA is an important component of the mucosal immune system, as it induces antibody-dependent cell-mediated cytotoxicity (ADCC) and promotes the degranulation of granulocytes, all contributing to the removal of pathogenic threats [57].

To conclude, different cell types have distinct functions which may vary according to the site of inflammation, the degree of inflammation, and the overall signals being received by the cell. Specifically, cytokines are the key orchestrators of communication between these cell types and work to ensure tissue maintenance and protection from excessive tissue damage. Within the gastrointestinal tract, the immune response to intestinal microbiota can be a promoter of inflammation. Dysregulation of the physiological mechanisms, which have evolved to maintain mucosal homeostasis may induce and promote pathological inflammation which can in turn promote the development of IBD.

Malaria

Malaria is an acute febrile disease that is potentially life-threatening and occurs primarily in tropical and subtropical countries. Geographically, malaria is an endemic disease that currently affects 84 countries around the world (World malaria report 2022, WHO). It is a preventable and curable disease, and progress has been made in eradicating infections in some parts of the world, however, it remains prevalent in developing countries. Globally, 247 million cases were reported in 2021, of which 619,000 deaths were a consequence of the infection (World malaria report 2022, WHO). Although the trend in incidence was decreasing before 2019, this increased again by 5% in 2020 due to disruptions in health services caused by the COVID-19 pandemic [66]. The incidence and burden of malaria are highest in African countries, where nearly 80% of malaria-related deaths occur in children under 5 years of age (World malaria report 2022, World Health Organization).

The disease is caused by obligate intracellular parasites of the genus *Plasmodium* and is transferred to humans through bites of female *Anopheles* mosquitos [67]. It is possible for humans to become infected by five different strains of *Plasmodium*, including *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. [68,69]. Of the five species, the most lethal is infection due to *P. falciparum*, which is associated with

severe symptoms, complications in pregnancy, and ultimately, most malaria-related deaths [70].

Prevention of infection

To prevent the spread of infection, many efforts have been devoted to vector control. Examples of this method include the use of insecticide-treated nets or indoor residual spraying [68,69]. However, this is becoming less efficient over time because of the emergence of insecticide resistance in the *Anopheles* mosquitos, and changes in the timely feeding behavior of these mosquitos, in which people are being bitten earlier in the day [71].

Another tactic for reducing infections in populations at risk is through prophylactic anti-malaria drugs. These include administration of medications such as chloroquine, atovaquone-proguanil, doxycycline, mefloquine or primaquine [72]. The administration of these drugs is dependent on which *Plasmodium spp.* is endemic in the region and whether resistance is suspected [69].

Lastly, the first effective malaria vaccine, the RTS, S/AS01 (MosquirixTM), has been approved as a preventative measure for children at risk. As of 2021, has been provided to millions of children in Africa as part of a program by the WHO (WHO). This vaccine targets the pre-erythrocytic stage of the *P. falciparum* life cycle [73,74]. It encompasses virus-like particles that express the *P. falciparum* circumsporozoite protein regions and a hepatitis B surface antigen, which is administered with the adjuvant, AS01e [73,74]. According to a report describing the efficiency of the vaccine after one year of the program, children between 6 weeks and 17 months received four doses in total, which resulted in a 30% reduction of severe disease related to infection (WHO)[75].

Symptoms, diagnostics, and treatment

Different *Plasmodium* spp. are associated with varying symptoms and course of disease. The severity of illness is also dependent on host immune factors. Some infected individuals may present as asymptomatic, but others could develop severe disease. Common symptoms include moderate fever, chills, muscle aches, vomiting, and diarrhea, and can rapidly progress to high fever and exhaustion. Complications most often occur in populations at risk [69]. Mostly, malaria-related complications are a severe threat to children under 5, pregnant women, and co-infection with HIV [69,76].

Severe malarial symptoms include anemia, cerebral malaria, and multi-organ damage that is often fatal [69].

When a patient presents with these symptoms, diagnostic verification of infection is most often determined through detection of the parasite or specific antigens in the blood. The most common and cheapest technique for diagnosis is through a blood smear and microscopic detection of parasites in the blood [69]. Alternatively, rapid diagnostic tests that detect malarial antigens in a blood sample, such as histidine-rich protein-2, can also be used [77]. Lastly, PCR techniques can also be used, but are expensive and impractical, as such tests require a lab set-up [78]. Mostly, PCRs are used to screen for polymorphisms in certain anti-malaria drug resistance genes, such as *Pfk13*, *Pfcrt*, *Pfmdr1, Pfdhfr,* and *Pfdhps* [79]. Polymorphisms in these genes were originally associated with resistance to chloroquine but have also been shown to enhance resistance to artemisinin-based combination therapies (ACT) [80,81]. The gold standard treatment for malaria at this present time is the administration of ACTs, which comprise of a combination of two drugs, including an artemisinin derivative and a quinine derivative [69].

Plasmodium life cycle

Plasmodium parasites are obligate, unicellular eukaryotes [67]. *Plasmodium spp.* have a complex developmental and reproductive system that requires cyclical infection of human and female *Anopheles* mosquito hosts [69]. Infected mosquitos transmit the parasites to humans during a blood meal, in which they inject the sporozoite form of the *Plasmodium* into the dermis. These motile sporozoites can move through the lymphatic system and initially infect hepatocytes. This stage of infection is asymptomatic in human hosts, due to the immunotolerogenic environment of the liver that the parasite takes advantage of [82,83]. Within the hepatocytes, thousands of merozoites are generated from a single sporozoite, which are then released into the bloodstream. Merozoites are able to infect red blood cells to either replicate or sexually mature into male or female gametocytes. The blood stage of infection is where the human host exhibits clinical symptoms from the infection [82]. If the host is bitten again at this stage, the gametocytes within the blood stream are taken up and travel to the stomach of the mosquito. The interaction between the male and female gametocytes in the stomach of the mosquito results in zygotes, which then become motile ookinetes,

then develop into oocysts in the gut, and finally, release sporozoites via their salivary glands [69,82,84,85].

Malaria mouse models

Mouse models of malaria have been crucial in revealing many aspects of parasite development and host immunity to infections. They continue to be essential in guiding the development of efficient vaccines and understanding mechanisms of immune cell-mediated protection [82]. *Plasmodium berghei* ANKA, *Plasmodium yoelii* NL, and *Plasmodium chabaudi* are the three main rodent-specific strains that are commonly used for *in vivo* experiments [86,87]. Each strain can be utilized to study different stages of the disease. For example, non-lethal mouse strains such as *Plasmodium yoelii NL* and *Plasmodium chabaudi* can mimic all stages of the infection that occur in humans, including clearance of parasites. For a more severe course of infection, *Plasmodium berghei* ANKA is often used to recapitulate cerebral malaria, in which mice can develop ataxia, convulsions, coma, and ultimately, death [86,87].

Immune response

Plasmodium spp. have developed many features that facilitate successful infection and avoidance of immune clearance by the host. Specifically, *Plasmodium spp.* exhibit an array of antigens at different stages of their developmental cycle, polymorphisms of certain genes that help evade responses in already immune individuals, and exploitation of immune-privileged sites such as the liver and red blood cells [88,89]. Hence, why finding suitable drug targets or immune therapies continue to be challenging in this field.

Nevertheless, host immune components are critical in identifying the parasite, launching an offensive response, and eliminating the infection. Immune responses are stage-specific, and both cellular and humoral arms of the immune system are important for resolving infection [82]. However, it is important to note that immune cells may also contribute to immunopathology as a result of the infection.

T cell immunity to malaria

During the pre-erythrocytic stage of infection, both $CD4^+$ T cells and $CD8^+$ T cells are important for combatting infected hepatocytes, before dissemination into the bloodstream [82,90,91]. Initially, antibody responses choreographed by $CD4^+$ T cells can

prevent sporozoite infection of hepatocytes [82,92]. Next, cytotoxic $CD8^+$ T cells that detect parasitic antigens in infected hepatocytes are capable of directly eliminating these cells, and therefore, preventing the development of parasites to the blood stage and subsequent clinical manifestations [82,93]. Contrastingly, parasite-specific $CD8^+$ T cells can also act pathogenically and are speculated to be the cause of cerebral malaria through the excessive release of perforin, granzyme B and IFNγ in humans and mice [94,95].

In the blood stage, merozoites take advantage of the lack of MHC molecules in red blood cells and use this stage to copiously replicate and generate thousands of progenies [96]. The host response is led through the indirect targeting of infected red blood cells by $CD4^+$ T cells and γδ T cells [82]. Generally, within $CD4^+$ T cell subsets, T_h1, T_r1, and Treg cells have the most impact on regulating the infection. T_h1 cells, for example, release IFNγ, which is a crucial cytokine involved in the clearance of parasites [97]. In general, regulatory $CD4^+$ T cell subsets producing IL-10 are important for suppressing inflammatory cells and preventing excessive pro-inflammatory cytokine production that could lead to severe pathology in the host.

B cell immunity to malaria

$CD4^+$ T helper cells assist B cells in generating antigen-specific antibodies against the invading *Plasmodium* parasites [98]. Effectively, these antibodies can bind to antigens on the surface of *Plasmodium* cells and directly prevent the invasion of hepatocytes and red blood cells. Furthermore, the attachment of these antibodies can initiate complement-associated lysis of the parasite [99]. Unfortunately, plasma levels of pathogen-specific antibodies have been found to be short-lived [98] [100]. However, memory B cells that can confer immune protection against re-infection remain. On the other hand, evasion mechanisms developed by the parasite, such as antigenic variation and polymorphisms of surface proteins might render these cells ineffective in future infections [100].

The cytokine IL-22

The cytokine IL-22 is famous for its pleiotropic qualities and is considered a critical mediator of inflammation and maintenance of epithelial integrity at barrier sites. IL-22 was initially introduced in the year 2000 and was originally named IL-10-related T cell-derived inducible factor (IL-TIF) [101,102]. It was identified as a new member of the IL-10 superfamily due to its structural similarities and use of common receptors to other cytokines within the group [103]. The human *IL22* gene is found on chromosome 12 and shares 79% homology with the mouse *Il22* gene found on chromosome 10 [104].

IL-22 is mainly produced by cells of lymphoid origin in the lung, liver, kidneys, thymus, skin, pancreas, skin, and intestine [8]. Predominant producers of IL-22 include different T cell subsets, ILCs [105], NK cells [106], and neutrophils. Significantly, the different cellular sources of IL-22 are disease- and organ-dependent [107,108]. IL-22 signals through a heterodimeric signaling complex comprising of the IL-22 receptor α1 (IL-22RA1) and IL-10 receptor β2 (IL-10R2) membrane-bound subunits [102,109]. IL-22 initially binds to IL-22RA1 which subsequently enables secondary binding of the IL-10R2 subunit and initiates signaling via the Janus kinase (JAK)/STAT pathway. STAT1, 3, and 5 have all been described to be activated by IL-22 signaling, although STAT3 is primarily phosphorylated. Additionally, IL-22 can also activate mitogen-activated protein kinase (MAPK), Akt, and p38 pathways [102,110].

The specificity of IL-22 signaling is attributed to the binding of its membrane-bound receptor, IL-22RA1. The IL-22RA1 is explicitly expressed on non-hematopoietic cells [111], although exceptions have been identified in a dysregulated disease state where the membrane-bound receptor is expressed on lymphoid cells in lymphoma patients [107,112,113]. IL-22 is an essential modulator of inflammatory responses at barrier sites. Signaling in epithelial cells stimulates the production of antimicrobial peptides, lipocalin, and mucin [114]. Additionally, it promotes survival and proliferation within these cells [115]. Therefore, IL-22 is considered to play an active role in mediating epithelial integrity, a balanced microbiota, and a mounting immune response against external threats [116].

IL-22 is a highly clinically relevant cytokine involved in a multitude of inflammatory diseases including IBD, psoriasis, rheumatoid arthritis, cancer, and infections [117]. Although it is generally considered to be protective in nature during acute inflammatory settings, the outcome of excessive production of this cytokine during chronic settings often becomes detrimental to the host. Particularly, overt tissue damage and hyper-proliferation of cells can result in unresolved inflammation and tumor growth. Speculations of why the actions of this cytokine may become dysregulated include aberrant control of IL-22 signaling and perturbations to host microbial compositions. The source of IL-22 is also an important factor in the function of IL-22 in a particular disease context.

Induction of IL-22 can be implemented by various transcription factors, cytokines, and receptors. Positive regulation of IL-22 is exerted via the cytokine IL-23 and specific transcription factors. IL-23 promotes IL-22 production in both innate and adaptive immune cells that possess the IL-23 receptor on their surface. The aryl hydrocarbon receptor (AhR) is the most common transcription factor that induces the production of IL-22 in T_h17, T_h22, $\gamma\delta$ T cells, and ILC3s [118-121]. Additionally, RORγt and Notch are also known to initiate IL-22 transcription [120].

On the other hand, the cytokine TGFβ can both positively and negatively regulate IL-22. Interestingly, this cytokine is particularly important for Treg and T_h17 differentiation, which, in the latter, is important for promoting IL-17A production. However, it has been described to inhibit T_h22 differentiation and IL-22 production in T_h17 cells [122,123]. Conversely, there is strong evidence that TGFβ induces IL-22 production *in vitro* [124] and can also induce the co-production of IL-22 and IL-17A in T_h17 cells *in vivo* [125].

Lastly, the soluble decoy receptor, IL-22BP, is an essential modulator of IL-22 signaling that appears to have a substantial effect on the overall action of IL-22 during steady state and inflammatory conditions. The ratio between IL-22 and IL-22BP is a major factor that comes into play in many disease outcomes. Similar to the divergent effects of IL-22 in different disease contexts, IL-22BP has also been described to have both protective and detrimental effects during inflammatory processes. The effects of this soluble endogenous receptor have not yet been adequately investigated and remain largely unknown for most IL-22-related diseases.

The soluble receptor IL-22BP

IL-22BP is the soluble endogenous inhibitor of IL-22 with a binding affinity of up to 1000-fold higher than the membrane-bound IL-22RA1 [126,127]. IL-22BP was initially identified to be a unique receptor for IL-22 belonging to the class II cytokine receptor family by three independent research groups in 2001 [109,128,129].

The *IL22RA2* gene is encoded by a separate gene to *IL22RA1* and *IL10R2*. It is found on chromosome 6 in humans and chromosome 10 in mice. Sequence homology of the mouse gene compared to the human has been calculated at 67.1% [130] and in both species is flanked by *IL20RA* and *IFNGR1*. Interestingly, these flanking genes are both paralogues of *IL22RA2* (in reference to the ENSEMBL genome browser).

IL-22RA2 has 34% amino acid identity with the extracellular domain of the IL-22RA1 subunit but does not encode a transmembrane or cytoplasmic domain [128]. Predictably, the membrane-bound and the soluble receptor have overlapping binding domains for IL-22 [131]. The superior binding affinity of IL-22BP ensures the inhibition of interactions between IL-22 and its membrane-bound receptor, preventing further downstream signaling in responsive cells.

Lastly, three different isoforms of this gene are known in humans. Within these splice variants, only IL-22BPi2 and IL-22BPi3 are secreted. Although IL-22BPi2 is more efficient in inhibiting IL-22 signaling and subsequent gene induction, IL-22BPi3 is more abundantly expressed during steady state. Additionally, IL-22BPi2 is the only isoform that increases in abundance when myeloid cells are stimulated with toll-like receptor 2 (TLR2) or retinoic acid. Contrastingly, IL-22BPi1 is not secreted, and therefore, is unable to inhibit IL-22 signaling [132]. In comparison, there is only one isoform found in mice, and its sequence is homologous to that of IL22BPi2 [133].

IL-22BP is found to be most highly expressed in tissues of the breast, lung, liver, spleen, and colon [9,128]. Known cellular sources of IL-22BP include dendritic cells, $CD4^+$ T cells, eosinophils, and macrophages [9,134-137].

Unfortunately, technical limitations have prevented the extensive study of IL-22BP and elucidation of regulatory mechanisms that impact its role in models of inflammation. So far, studies of IL-22BP have been reliant on RT-PCR quantification and western blot techniques. To date, reliable staining of murine IL-22BP protein using commercially available antibodies could not be established in the host lab. Only human IL-22BP antibodies have proven reliable so far in experiments carried out for this project. Hence, a reporter mouse was generated for the purpose of this project, proving to be a major tool that has enabled detailed analysis of this receptor in *in vivo* models.

IL-22BP in the gastrointestinal tract

To date, studies focusing on the impact of IL-22BP in disease contexts often comment on the balance of the IL-22/IL-22BP axis and how inflammation in the host can lead to a skewing of this axis in a certain direction. Initially, it was described that IL-22 and IL-22BP have inverse expression patterns during tissue damage in the intestine: Under homeostatic conditions, the gene for IL-22BP is expressed at high levels, while the gene for IL-22 is hardly expressed. Upon tissue damage, a drastic up-regulation of IL-22 gene expression occurs, while IL-22BP gene expression is down-regulated. This was exemplified in the gastrointestinal tract of mice during the course of the dextran sulfate sodium (DSS)-induced colitis model [9,138,139]. Specifically, Sugimoto *et al.* were able to show that injecting vectors containing IL-22BP cDNA into regions of the colon resulted in a local up-regulation of IL-22BP, which was a cause for down-regulation in phosphorylated STAT3 and a significant reduction in goblet cells and mucin production within that region [138]. More recently, however, it is understood that both IL-22 and IL-22BP can be up-regulated simultaneously under certain inflammatory conditions [135,136]. Here, the relative cellular sources add a level of complexity to understanding how the expression of these two entities is regulated.

A major cellular source of IL-22BP in both mice and humans is dendritic cells, which express the gene for the soluble receptor at higher levels when immature, compared to lower levels when they become activated [9,134,140,141]. One of the first studies investigating the role of dendritic cell-derived IL-22BP reported that the sensing of tissue damage results in the down-regulation of IL-22BP in $CD11c^+$ dendritic cells via the release of IL-18 by the NLRP3/6 inflammasomes [9]. Another study further

described this phenomenon, in which IL-22BP production in a $CD103^+$ $CD11b^+$ conventional dendritic cell population can be up-regulated by retinoic acid. When this subset of dendritic cells matures, for example, via stimulation with antigens, IL-22BP production is significantly reduced [134].

After initial studies described these dendritic cells to be important in counteracting excessive IL-22 signaling, other research groups found similar IL-22BP-producing dendritic cell populations in specific niches, where they have a specialized role. For example, one study describes a $CD11b^+$ $CD8\alpha^-$ dendritic cell subset that highly expresses the gene for IL-22BP and is important for blocking the effects of IL-22 in the sub-epithelial domes of the Peyer's patches under homeostatic conditions. Specifically, IL-22BP gene expression in this region of the Peyer's patches is incredibly important as ablation results in enhanced IL-22-related effects, such as increased production of mucin and antimicrobial peptides, ultimately ensuing in reduced uptake and sampling of bacterial antigens [142]. More recently, a transcriptionally distinct subset of $CD11c^+$ dendritic cells located in the cryptopatches and isolated lymphoid follicles of the gut has been found to produce IL-22BP during steady state. This distinct dendritic cell population is stimulated to produce IL-22BP via lymphotoxin-β receptor signaling coming from the surrounding $CCR6^+$ ILC3s. In addition, it was suggested that the presence of the lymphotoxin-β receptor is important for controlling the expression of lipid transporters, as ablation from these specific dendritic cells resulted in reduced lipid transporter expression on epithelial cells and diminished body fat [143].

The beneficial effects of dendritic cell-derived IL-22BP have also been examined in tumor progression. Specifically, Huber *et al.* were the first to highlight the crucial function of dendritic cell-derived IL-22BP in blocking the excessive tissue regenerative effects of IL-22 signaling. The authors were able to illustrate the essential role of IL-22BP in controlling tumorigenesis in a spontaneous adenomatous polyposis coli ($APC^{min/+}$) model of tumorigenesis and a colitis-associated colorectal cancer model. Here, it was shown that when IL-22BP was ablated from the system, uncontrolled IL-22 signaling led to increased tumor development, as mice became more susceptible to tumorigenesis [9]. In a follow-up study by the same group, further investigations in tumors isolated from colorectal cancer patients indicated that *IL22RA2* expression levels are reduced in tumor tissues and that low levels of IL-22BP are associated with

shorter survival times. This study furthermore corroborates the finding that IL-22BP produced by dendritic cells is regulated by lymphotoxin-β signaling. Specifically, signaling via the non-canonical NFκB pathway was shown to be necessary for IL-22BP production in colon tumors and cultured human dendritic cells [144]. Whereas up-regulation of IL-22BP is important for controlling aberrant IL-22-signaling leading to tumorigenesis, the availability of IL-22 is critical during early exposure of gastrointestinal stem cells to carcinogens to ensure sufficient DNA damage repair. Hence, *Il22ra2* expression is down-regulated upon DNA damage ensuring high levels of bioactive IL-22 [145].

Other studies exploring the role of IL-22BP in cancer have further reiterated the protective effects of this protein and provide evidence for its potential as a target for gene therapies. For example, Zhang and colleagues were able to demonstrate that delivering IL-22BP mRNA attached to cationic liposomes to C26 tumor cells promoted apoptosis and restricted angiogenesis and growth within these tumors [146].

The inverse relationship between IL-22 and IL-22BP identified in previous studies does not seem to be applicable in all inflammatory conditions. For instance, studies carried out in IBD patients have indicated that both IL-22 [139,147] and IL-22BP [135,136] are significantly up-regulated in inflamed intestinal biopsies. Interestingly, Martin *et al.* attribute eosinophils as being one abundant source of IL-22BP in colonic tissues isolated from IBD patients. Furthermore, Pelczar *et al.* provide evidence that $CD4^+$ T cell-derived IL-22BP contributes to the development of IBD. Both research groups recognize that excessive IL-22BP production in these cell types has a pathogenic role in colitis development, in that it blocks the protective effects of IL-22 in rodents and humans. To go into further detail, both groups also emphasize that dendritic cell-derived IL-22BP is not a major contributor to colitis development in this case. In the gut mucosa of healthy controls, Crohn's disease, and ulcerative colitis patients, it was found that eosinophils greatly outnumber the amount of conventional dendritic cells using immunofluorescence analyses [136]. Similarly, the isolation of dendritic cells from Crohn's disease and ulcerative colitis patient biopsies indicated no significant difference in *IL22RA2* expression levels when compared to dendritic cells isolated from healthy controls. However, isolated $CD4^+$ T cells from the same patients showed a 10- to 100-fold up-regulation of *IL22RA2* expression compared to $CD4^+$ T cells from

healthy controls. Additionally, IL-22BP was found to be correlated to patient responsiveness to anti-TNFα therapy, although the mechanisms attributing to this effect have not yet been elucidated [135]. Future investigations focusing on the underlying transcription factors that are involved in regulating the expression of IL-22BP under such disease contexts will provide insight into the characteristics of these pathogenic T cell subsets.

IL-22BP in the liver

Although IL-22 is considered to be a protective entity in many diseases in the liver, IL-22BP has also been found to play a crucial role in blocking the detrimental pro-inflammatory effects of IL-22 that may arise in chronic inflammatory conditions. A good example of the critical implications of the IL-22/IL-22BP axis in the liver was provided quite recently by Schwarzkopf *et al*. In this study, a phenomenon was described in which IL-22 can be protective at certain stages of liver cirrhosis, at which the inhibition of IL-22 signaling via IL-22BP may be detrimental to the host due to impairment of liver synthesis capacity. However, in more advanced stages, IL-22BP is critical for blocking the adverse pro-inflammatory effects of IL-22 in patients with severe liver disease. In patients with acute-on-chronic liver failure, ratios of IL-22BP/IL-22 were reported to be lower. Thus, the amount of IL-22BP is insufficient, and therefore, cannot block the detrimental effects of IL-22 in these patients, which in turn, impacts mortality [148]. A similar down-regulated expression pattern was seen in patients with alcoholic hepatitis, where low ratios of IL-22BP/IL-22 were shown to be associated with an increased one-year mortality rate [149]. In concurrence with human studies, IL-22BP was found to be vital in mouse models of ischemia reperfusion and acetaminophen-induced liver injury, as it controls IL-22-induced recruitment of proinflammatory cells. In this study, the authors saw that IL-22BP deficiency resulted in an increased susceptibility to liver damage as a result of dysregulated IL-22 signaling [150].

Interestingly, the protective effects exerted by IL-22BP in the liver diseases listed previously are inconsequential as soon as an infection is implicated. A study carried out on patients with *Schistosomiasis* infections highlights the protective effects exerted by elevated *IL22* expression levels, resulting in decreased hepatic fibrosis and portal hypertension. Contrastingly, *IL22RA2* expression is down-regulated in the blood of

patients chronically infected with *Schistosoma japonicum*. Moreover, single nucleotide polymorphisms (SNPs) associated with IL22RA2 were equally found to be correlated with severe fibrosis, which were described to correlate to the same ailment as in hepatitis C patients [151]. An older study also demonstrated that *IL22RA2* expression was up-regulated in *Schistosoma mansoni*, *Mycobacterium tuberculosis,* and *Toxoplasma gondii* infections of the liver, although whether this up-regulated expression pattern ensued a pathogenic or protective effect was not investigated [152].

IL-22BP in the skin

Studies carried out in patients with psoriasis and mouse models of skin inflammation have indicated that the IL-22/IL-22BP ratio is particularly important in maintaining homeostasis in the skin. Particularly, overproduction of IL-22 was found to be detrimental in this organ [153] [154]. In psoriatic skin lesions, *IL22* expression was greatly up-regulated, whereas, in contrast, only a moderate up-regulation of *IL22RA2* was seen in healthy regions. Thus, the IL-22/IL-22BP ratio in the skin is skewed to a greater expression of IL-22, indicating a deficiency in IL-22BP and limited control of the adverse effects of IL-22 [155]. Furthermore, ablation of IL-22BP was shown to cause increased epidermal thickness and infiltration of inflammatory cells, partially dependent on more bioactive IL-22 being available in rodent models of imiquimod-induced psoriasis [155,156].

Other studies corroborate that IL-22 is significantly up-regulated when comparing skin biopsies from psoriatic patients and healthy controls. In these biopsies, however, IL-22BP was significantly down-regulated [141,157]. Specifically, epidermal keratinocytes are the main source of IL-22BP in the skin under homeostatic conditions, and development of psoriatic lesions leads to a distinct down-regulation of its expression within these cells. IL-22BP is critical in blocking excessive inflammation and keratinocyte alterations caused by IL-22 in psoriatic mice [157]. In another study in which the focus was placed on the regulation of dendritic cell-derived IL-22BP, prostaglandin E2 was identified as a prominent suppressor of IL-22BP production in immature monocyte-derived dendritic cells *in vitro*. Notably, stimulation of these cells with IL-6 was found to greatly up-regulate IL-22BP expression [141]. Lastly, a very recent study focusing on another chronic inflammatory disease of the skin, namely

Prurigo nodularis, attributes inflammation in lesional skin to be a result of excessive T_h22-related cytokine gene expression. Interestingly, expression levels of *IL22RA1* and *IL22RA2* were also significantly elevated in these lesions [158].

IL-22BP in the lung

One of the first studies to be carried out about IL-22BP in the inflamed lung found the protein to be produced by macrophages, alveolar epithelial cells, and neutrophils [159]. Since this initial study, IL-22BP has mostly been explored in the context of lung infections. In this organ, IL-22BP exerts a pathogenic role in the majority of studies, as IL-22 is a critical component in protecting epithelial integrity and preventing systemic translocation of microbes [160-163]. Specifically, IL-22BP-deficient mice were utilized to show that absence of this soluble receptor rendered mice less susceptible to lung infections. In a model of bacterial superinfection, IL-22BP deficiency resulted in decreased bacterial load and an increased survival outcome attributed to better preserved epithelial integrity [161]. A further study focused on the beneficial effects of IL-22 during influenza infection, in which the authors described IL-22 to be particularly essential for tight junction formation, resulting in less fluid leakage in the lung [162]. Furthermore, increased IL-22 production in these receptor-deficient mice infected with *Streptococcus pneumoniae* resulted in a down-regulation of oxidative phosphorylation (OXPHOS) genes and an up-regulation of glycolysis in macrophages [160]. Lastly, expression and protein levels of IL-22, IL-22RA1, and IL-22BP have been reported to be up-regulated in patients and mice with chronic obstructive pulmonary disease (COPD), although the mechanisms as to how these entities contribute to pathogenesis remain to be elucidated [164].

IL-22BP in the kidney

Not much is known about the effects of IL-22BP in the kidneys to date, however, two independent studies on lupus nephritis found elevated levels of IL-22BP in the urine of patients with active renal disease [165,166]. Specifically, patients who responded to treatment had reduced IL-22BP levels after six months, but the same was not seen in non-responding patients [165].

IL-22BP in the brain

MS is an inflammatory neurodegenerative disease associated with dysregulated immune reactions, in which the effects of IL-22BP appear to be quite strongly related to pathogenesis. Linkage assessment in congenic rat lines enabled the identification of *Il22ra2* as a risk gene for MS in 2010 and was then verified continuously in humans and many experimental autoimmune encephalomyelitis (EAE) experiments in rodents. Initially, IL-22 produced by T_h17 cells was found to compromise tight junctions in the blood-brain barrier and contribute to pathogenesis in the brain [167,168]. Similarly, pathogenic properties of IL-22BP are evident in that elevated levels of *Il22ra2* expression were seen in congenic rat lines that were more susceptible to EAE. To reiterate this finding, the same study showed that *Il22ra2* expression was reduced in a newly generated rat line with reduced susceptibility to EAE. Specifically, *Il22ra2* expression was reduced in activated macrophages and splenocytes in these less susceptible rats [169].

Similarly, elevated expression levels of both *IL22* and *IL22RA2* were also found in studies of multiple sclerosis patients [170]. Furthermore, *IL22RA2*-related SNPs were identified as MS risk variants, providing more evidence of the pathogenic properties of this receptor in the brain [171-174]. Specifically, one study attributed a particular SNP responsible for the low secretion of all three isoforms of IL-22BP, increasing the risk of developing MS [175]. Although IL-22 has been shown to have detrimental effects, it also appears to be crucial in preventing damage to brain tissue caused by other inflammatory cytokines. A study by Lindahl *et al.* reported the importance of IL-22 in inhibiting IFNγ-derived damage and suggests how IL-22BP-mediated inhibition of IL-22 may lead to increased neuroinflammation in the brain of rodents [172]. The adverse effects of IL-22BP were also seen in a study where IL-22BP-deficient mice displayed a reduced susceptibility to severe EAE and less immune infiltration compared to their wild type counterparts [176].

IL-22BP in the blood

Although the functions of IL-22BP in the circulatory system have not been sufficiently studied, there are many reports providing evidence of the important role of IL-22 in the blood, especially during blood infections. Many of these reports focus on the effects of

IL-22 in combatting *Plasmodium* infections that result in the development of malaria. One such report studying different SNPs of IL22 identified in West African patients, indicated that possession of certain SNPs can cause a patient to be either more susceptible or more resistant to *Plasmodium falciparum* infection [177]. In a similar study instigating IL-22 as an important factor involved in the pathogenesis of cerebral malaria, *IL22*-associated SNPs identified in children predisposed them to developing this complication. However, *IL22RA2*-associated SNPs identified in the same study did not seem to have an impact on cerebral malaria susceptibility in these children [178].

With regards to mouse studies, only a very early study on sepsis has reported the direct effects of IL-22BP production in the blood. In this study, blocking IL-22 with recombinant IL-22BP was deemed protective, in that administration of IL-22BP-Fc resulted in enhanced recruitment of neutrophils and phagocytes, leading to an attenuated bacterial load in mice with septic peritonitis [179].

Impact of IL-22BP on inflammatory diseases

In summary, literature has provided copious evidence that IL-22BP has an important role during homeostasis and in many inflammatory settings affecting different organ systems. To date, this receptor has not been investigated as well as its cytokine counterpart, IL-22. Interestingly, many studies on IL-22 omit the role of this endogenous receptor completely.

The main cellular source of IL-22BP focused on throughout this collection of reports is dendritic cells. More recently, $CD4^+$ T cells [135] and eosinophils [136] have also been shown to be significant producers of IL-22BP in the context of IBD. Unfortunately, an unbiased approach, such as newly developed sequencing techniques have never before been utilized to identify unknown cell types that may be important sources of IL-22BP during the steady state and inflammatory conditions. This is mostly due to the lack of tools, as it has so far been difficult to isolate a pure population of IL-22BP-producing cells from mice. Specifically, there is an absence of reliable mouse antibodies to identify these cells using flow cytometry. Therefore, there is still a vast amount that is unknown about other sources of IL-22BP. Specifically, these sources may be important in chronic inflammatory disorders such as IBD, which will be addressed during this thesis using the new *Il22ra2*^{eGFP} reporter mouse that has been established. Principally, $CD4^+$ T helper cell subsets that produce IL-22BP are of particular interest as they have been demonstrated to be the cause of detrimental effects seen in IBD. Lastly, the new reporter mouse and the accompanying total knockout mouse for the IL-22BP gene are utilized to examine the role of this soluble receptor in a mouse model of malaria. Revelations from these studies will elucidate the impact of IL-22BP in this model and will help identify common characteristics of *Il22ra2*-expressing $CD4^+$ T cells in different inflammatory models.

Aims

IL-22BP is an important modulator of the adverse effects that arise from excessive and uncontrolled IL-22 signaling. This soluble receptor has so far been reported to be produced by dendritic cells, eosinophils, monocytes/macrophages, and $CD4^+$ T cells, the latter of which has been found to induce a pathogenic outcome in IBD. To date, not much is known about the specific $CD4^+$ T helper cell subsets that express the gene for IL-22BP (*Il22ra2*). Additionally, there have not yet been any studies describing the impact of IL-22BP on the infectious setting of Malaria. Defining how *Il22ra2*-expressing cells are transcriptionally defined and regulated could aid in identifying ways to promote its protective effects or prevent its inhibitory effects in aberrant inflammatory conditions.

So far, technical limitations, such as the absence of reliable flow cytometry antibodies and low IL-22BP-producing cell numbers, have prevented studies from deciphering the sources of IL-22BP and their regulation. For this purpose, a reporter mouse was generated enabling the identification and isolation of *Il22ra2*-expressing cells via eGFP labeling. Steady state analyses and inflammatory *in vivo* models were utilized to investigate the transcriptional profile and gene markers of cells expressing *Il22ra2*. Specifically, the *Il22ra2*^{eGFP} mouse was used to determine the predominant cell types that express *Il22ra2*. A particular focus was placed on identifying $CD4^+$ T helper cell subsets and their transcriptional profile. The use of the *Il22ra2*^{eGFP} reporter mouse enabled comparisons of *Il22ra2*-expressing cell types during homeostasis and inflammation, allowing the identification of characteristic genes and potential transcription factors that may be important for regulating *Il22ra2* expression. The following aims were investigated within this project:

1. To analyze the heterogeneity of *Il22ra2*-expressing cells during homeostasis
2. To identify cell types that are capable of expressing *Il22ra2* during homeostasis and in inflammatory disease models
3. To identify potential transcription factors that could regulate *Il22ra2* expression during homeostasis and in inflammatory disease models

Materials

Table 1: General lab equipment for processing and data analysis

Device	Company
Nano-drop	Thermo Fisher Scientific
Precellys 24 Homogenizer	Bertin Instruments
UV Trans-illuminator	Bio-Rad
Centrifuge 5810/5427R	Eppendorf
Thermomixer comfort	Eppendorf
Hertherm incubator	Thermo Fisher Scientific
Hera Safe Clean Bench	Hanau
C100 Thermal Cycler	Bio-Rad
StepOne Plus Real-Time PCR system	Applied Biosystems
Bioanalyzer	Agilent
Qubit	Thermo Fisher Scientific
Vortex genie 2	Scientific Industries
Waterbath WNB	Memmert
Neubauer chamber (0.0025mm^2)	Superior Marienfeld
Microtome	SLEE
Gavage needle	Th. Geyer
Coloview system (endoscope)	Karl Storz
LSR II Fortessa flow cytometer	BD Biosciences
FACSAria Fusion Cell Sorter	BD Biosciences
Rhapsody Single-cell analysis system	BD Biosciences
Chromium controller	10x Genomics

Table 2: Microscopes

Microscope	Use	Objective
Leica Light Microscope	For counting cell numbers	Hi Plan I, 20x/0.30 Ph1
Zeiss Axio Vert.A1	For taking histological pictures	Inverse FL-LED, W-Pl 10x/23 br foc

Reagents

Table 3: General lab reagents

Reagent	Company
Forene (Isofluran)	Abbvie
Distilled water	B. Braun
Roti-Histofix Paraformaldehyde (4%)	Carl-Roth
Ethanol	Th. Geyer
Azoxymethane (AOM)	Sigma-Aldrich
Dextran sulfate sodium (DSS)	MP Biomedicals
Trypan blue (0.4% L)	Sigma-Aldrich
Wright-Giemsa Staining Solution	Sigma-Aldrich
RPMI media	Thermo Fisher Scientific
1x Dulbecco's Phosphate Buffered Saline (PBS)	PAA
Fetal Bovine Serum	Sigma-Aldrich
Staphylococcal enterotoxin B from *Staphylococcus aureus* (SEB)	Sigma-Aldrich
Formaldehyde solution	Sigma-Aldrich
Nonidet P40 (NP40)	Sigma-Aldrich
Mouse IL-10 secretion assay - detection kit (PE)	Miltenyi

Table 4: Reagents for genotyping

Reagent	Company
10x Dream Taq green buffer	Thermo Fisher Scientific
dNTP mix	Fermentas
Dream Taq DNA polymerase	Thermo Fisher Scientific
Agarose LE	Biozym
Ethidium bromide (0.07%)	AppliChem PanReac
GeneRuler 100bp plus DNA ladder	Thermo Fisher Scientific
Quick-load 1kb DNA ladder	New England BioLabs
6x DNA gel loading dye	Thermo Fisher Scientific
Phusion high-fidelity DNA polymerase	Thermo Fisher Scientific
Proteinase K enzyme	Roche

Table 5: Buffers for genotyping

Buffer	Components
Proteinase K buffer	12.1g Tris,10ml 0.5M EDTA, 11.7g NaCl, 5ml SDS (from20% Stock), fill up with distilled H2O to final volume of 1L
TBE buffer	108 g Tris, 55 g Boric acid, 40 ml 0.5 M EDTA, fill up with distilled H2O to a final volume of 1L

Table 6: Reagents for Immunohistochemistry

Reagent	Company
Xylolersatz medium (Xylene)	DiaTec
Lithium carbonate	Roth
Mayer's hemalum solution (Hematoxylin)	Merck
Eosin Y	Leica

Table 7: Reagents for cell isolation

Reagent	Company
Dithioerytheriol (DTT)	AppliChem PanReac
10x Hank's balanced salt solution (HBSS)	Thermo Fisher Scientific
Collagenase IV (100U), from Clostridium hystolyticum	Sigma-Aldrich
DNase	BD Bioscience
Percoll	GE Healthcare
Pancoll	Pan-Biotech

Table 8: Buffers for cell isolation

Buffer for cell isolation	Components
1x PBS	137 mM NaCl, 2,7 mM KCl, 100 mM Na_2HPO_4, 2 mM KH_2PO_4, adjusted to pH 7,4 with either HCl or NaOH
Wash buffer	1x PBS, 1% FBS
MACS buffer	1x PBS, 1%FBS, 0.5% EDTA
DTE solution	50ml 10x HEPES (23,8 g HEPES (100 mM final), 21 g sodium bicarbonate (250 mM final), dH2O to 1 liter, adjust pH to 7.2 with HCl), 50ml 10x HBSS, 50ml 10% FBS, 1mM DTT, 350ml distilled H_2O

Collagenase buffer	500ml RPMI, 100 U/ml collagenase, 1000 U/ml DNase, 10% FBS, 100x HGPG (59,6 g HEPES, 14,6g L-glutamine, 1x106 U penicillin, 1 g streptomycin, 2,5 mg gentamicin, RPMI to 500 ml, adjust pH to 7,5 using HCl), 1mM $MgCl_2$, 1mM $CaCl_2$
10x ACK lysis buffer	20.05 g NH4Cl, 2.5 g KHCD3, 0.093 g EDTA, ad 250 ml distilled H2O

Table 9: Reagents for RNA extraction, cDNA synthesis, and Real-time PCR

Reagent	Company
2-proponal (Isopropanol)	Th-Geyer
Chloroform	JT Baker
High Capacity cDNA reverse transcription kit	Applied Biosystem
Dynabeads mRNA direct kit	Invitrogen
TaqMan fast advanced master kit	Thermo Fisher Scientific
PeqGold TriFast (Trizol reagent)	PeqLab
DEPC Water	Carl Roth
RNeasy plus micro kit	Qiagen
Big C lysing matrix (microbeads suitable for RNA)	MP Biomedical
MicroAMP 96-well reaction plate	Applied Biosystems
ß-mercaptoethanol	Gibco

Table 10: Reagents for flow cytometry

Reagent	Company
FACS flow solution	BD Bioscience
FACS clean solution	BD Bioscience
FACS rinse solution	BD Bioscience

Table 11: Reagents for MACS isolation

Reagent	Company
Biotin anti-mouse CD25	Biolegend
Biotin anti-mouse CD44	Biolegend
Biotin anti-mouse CD3	Biolegend
CD4 microbeads, mouse	Miltenyi Biotech
Click’s medium	Irvine Scientific

L-Glutamine	Invitrogen
Penicillin/Streptomycin, 10,000 units/ml	Invitrogen
Streptavidin microbeads	Miltenyi Biotech

Table 12: Reagents for 10x single cell sequencing

Reagent	Company
Chromium single cell 3' GEM module	10x genomics
Chromium single cell 3' Library module	10x genomics
Chromium single cell 3' v3 gel beads	10x genomics
Dynabeads MyOne SILANE	Invitrogen
Chromium partitioning oil	10x genomics
Chromium recovery agent	10x genomics
Chromium Chip B and gaskets	10x genomics
Chromium i7 sample index plate	10x genomics
10% Tween 20	Bio-Rad
SPRIselect reagent kit	Beckman Coulter
Buffer EB	Qiagen

Table 13: Reagents for BD rhapsody

Reagent	Company
BD Rhapsody Cartridge Reagent Kit	BD Bioscience
BD Rhapsody Cartidge Kit	BD Bioscience
BD Rhapsody cDNA kit	BD Bioscience
BD Rhapsody Targeted mRNA + Abseq amplification kit	BD Bioscience
AMPure XP magnetic beads	Beckman Coulter
1x RBC Lysis buffer	Thermo Fisher Scientific
Calcein AM cell permeant dye	Thermo Fisher Scientific
Propidium iodide	Thermo Fisher Scientific

Table 14: Primers

Gene	Sequence	WT	Mutant
Foxp3^{mRFP}	1. CAA AAC CAA GAA AAG GTG GGC	692	470
	2. GGA ATG CTC GTC AAG AAG ACA GG		
	3. CAT CTT GGA GAG TCG GTG TG		
Il17a^{Katushka}	1. CAC CAG CGC TGT GTC AAT	370	300
	2. ACA AAC ACG AAG CAG TTT GG		
	3. ACC GGC CTT ATT CCA AGC		
Il22^{sgBFP}	1. GTG CTC AGC AAG CAA ATG TC	700	619
	2. TAC GCT TGA GGA GAG CCA		
	3. AAT GAT GGA CGT TAG CTT		
	4. CCC GAC CAC ATG GGT TGA A		
Rag1$^{-/-}$	1. GAG GTT CCG CTA CGA CTC TG	474	530
	2. CCG GAC AAG TTT TTC ATC GT		
	3. TGG ATG TGG AAT GTG TGC GAG		
Il22ra2^{eGFP}	1. GCCTCAGACCAGTTCATGGA	629	733
	2. CAGGGTTCGTTAGTCGGAAGG		
	3. GCTGAACTTGTGGCCGTTTA		
Il22ra2$^{-/-}$	1. GTC TGT CCT AGC TTC CTC ACT G	624	447
	2. GGG GAC TTT GAC CAT GCA TC		
	3. CTA AGC AAG TGG CTG CCA GC		
High Fidelity Taq Polymerase PCR Primers	1. CTGTATGAGAGGAGAGCCTCACAGGG 2. GCTGAACTTGTGGCCGTTTA 3. GGGCTTCTTCTGTCAAACATGGGC	5103	5294

Table 15: Taqman probes

Taqman probes	Assay ID
Hprt1	Mm03024075_m1
Il22ra2	Mm01192969_m1
Il20ra	Mm00555504_m1

Ifngr1	Mm00599890_m1

Table 16: Mouse antibodies

Mouse Antigen	Clone	Staining	Dilution	Fluorochrome	Company
CD45	30-F11	Extracellular	1:600	Pe-Cy7	Biolegend
CD45	30-F11	Extracellular	1:800	BV 785	Biolegend
CD3	17A2	Extracellular	1:200	BV650	Biolegend
CD4	RM4-5	Extracellular	1:800	Pacific blue	Biolegend
CD4	GK1.5	Extracellular	1:800	APC	Biolegend
CD11c	N418	Extracellular	1:600	APC/Cy7	Biolegend
CD11c	N418	Extracellular	1:400	AF700	Biolegend
CD8a	53-6.7	Extracellular	1:600	AF700	Biolegend
I-A I-E (MHC II)	M5/114.15.2	Extracellular	1:400	AF700	Biolegend
CD19	6D5	Extracellular	1:400	AF700	Biolegend
CD44	IM7	Extracellular	1:400	APC/Cy7	Biolegend
CD62L	MEL-14	Extracellular	1:400	BV510	Biolegend
CD45RB	C363-16A	Extracellular	1:600	AF647	Biolegend
CD25	PC61	Extracellular	1:400	BV650	Biolegend
NK1.1	PK136	Extracellular	1:600	BV421	Biolegend
Siglec-F	E50-2440	Extracellular	1:200	PE	Biolegend

Table 17: Human antibodies

Human Antigen	Clone	Staining	Dilution	Fluorochrome	Company
CD45	HI30	Extracellular	1:800	FITC	Biolegend
CD3	UCHT1	Extracellular	1:100	BUV 737	Biolegend
CD4	OKT4	Extracellular	1:400	Pe-Cy7	Biolegend
Foxp3	259D	Intracellular	1:100	Pacific blue	Biolegend
IFNγ	4S.B3	Intracellular	1:100	BV785	Biolegend
IL-10	JES3-19F1	Intracellular	1:100	PE	Biolegend
IL-22BP	875504	Intracellular	1:100	APC	Biolegend
Isotype	LHD1315041	-	1:100	APC	Biolegend

Table 18: Viability antibodies

Viability staining	Dilution	Company
Pacific Orange Succinimidyl Ester	1:1000	Life Technologies
Zombie UV	1:200	Biolegend

Methods

Animals

All mouse lines used within this project have either been bought from Jackson laboratories/NCBI (*Rag1*$^{-/-}$, *Il22ra2*$^{-/-}$, C57BL/6, *Foxp3*^{mRFP}, *Il17a*^{FP365}, *Il22*^{sgBFP}) or generated in our facility in Hamburg (*Il22ra2*^{eGFP}, vector generated by Cyagen) by Dr. Irm Borgmeyer. Purchased mouse lines have been re-derived into colonies by embryo transfer and kept in specific pathogen-free (SPF) conditions in the animal facilities of the University Medical Center Hamburg-Eppendorf. To investigate the effects of different microbiota compositions, *Rag1*$^{-/-}$ and *Nlrp6*$^{-/-}$ mice were imported from facilities at Yale University, USA, and bred in IVC cages. All experiments were carried out on age-matched and sex-matched groups of mice between 8-24 weeks of age. Mice were euthanized as soon as they lost more than 20% of their original body weight, or when their overall condition worsened. All animals were cared for in accordance with the Institutional Review Board "Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz" (Hamburg, Germany). Ethical animal protocol number G17/13.

Genotyping

All genetically modified mice were genotyped using conventional PCR reactions to determine suitability for experiments. To extract genomic DNA, tail biopsies taken from 2-week-old mice were digested in a Proteinase K lysing buffer at 55 °C overnight. Enzymatic action was stopped by incubation at 95 °C for 15 minutes, after which the solution was diluted with water. Conventional PCR techniques were used to selectively amplify the gene of interest using the reagents in table 4 and primers in table 15. A common PCR master mix containing the following amounts of each reagent was used for all reactions:

Table 19: Conventional PCR master mix

Reagent	1x [µL]
10x MM	1,5
dNTPs	0,3
DreamTaq	0,11
Primer1 (1:10)	0,45
Primer2 (1:10)	0,45
H_2O	10,19
Sample DNA	2

For validation of the eGFP insertion in the *Il22ra2*^{eGFP} mouse, a specific high fidelity

Taq polymerase was used to amplify PCR products of more than 3000bp. The following master mix was used for this reaction:

Table 20: High fidelity Taq polymerase PCR master mix

PCR master mix	1x [µL]
2x Phusion MM	25
DMSO	1.5
Forward Primer (1:10)	5
Reverse Primer (1:10)	5
H_2O	12.5
Sample DNA	1 (or 200 ng)

All PCR products were stained with 0.07% ethidium bromide and separated by size on a 2% agarose gel by electrophoresis. Amplicon bands were visualized with a UV trans-illuminator.

Mouse disease models

Inflammation was induced in mice through four different models. All reagents used for these experiments are listed in table 3.

T cell transfer colitis model

The spleen and lymph nodes of 8-12-week-old reporter mice were extracted and $CD4^+$ T cells were enriched using magnetic activated cell sorting (MACS) columns. Next, these cells were sorted using FACS to obtain a pure population of $CD4^+$ $CD25^-$ $CD45RB^{high}$ cells. A 100µl aliquot containing 200,000 of these cells was intraperitoneally injected into each *Rag1*$^{-/-}$ recipient mouse. Colitis development and disease severity were determined through monitoring of weight loss and endoscopic procedures. Mice were sacrificed after they had reached a score of 5 or more for cell isolation.

AOM/DSS tumor model

An initial injection of azoxymethane (AOM) at a concentration of 7.5mg/kg body weight was administered intraperitoneally to reporter mice at 8-12 weeks of age. After 5 days, mice were fed with 2.5 % dextran sodium sulfate (DSS) via their drinking water for another 5 days, before being given autoclaved water for the following 16 days. This cycle was repeated two more times before the mice were sacrificed. Tumor

development and disease severity were determined by monitoring weight loss and endoscopic procedures. Tumor development was scored on day 21 of each cycle.

Plasmodium berghei ANKA infection model

A stock of frozen *Plasmodium berghei ANKA-infected* red blood cells (iRBCs) was obtained from the AG Jacobs group in the Bernhard Nocht Institute for Tropical Medicine. Initially, wild type donor mice were infected with 100µl of 1 x 10^6 frozen iRBCS diluted in 1x PBS each. After 5-7 days, donor mice that displayed symptoms of malaria infection and had parasitemia levels between 1-10% were sacrificed and fresh blood was isolated. The fresh blood containing parasitized RBCs was diluted in 1x PBS to a concentration of 1 x 10^6 iRBCs/ml. Next, experimental mice were intraperitoneally injected with 100µl of fresh blood and incubated for up to 7 days. Mice were monitored throughout the course of infection, in which weight loss, parasitemia, and cerebral malaria scores were recorded. Cerebral symptoms were determined according to the following scoring system [180]:

1. no symptoms
2. ruffled fur, decreased activity
3. uncontrolled, irregular movement
4. apathetic, not able to grab grid, not reacting to touching, no movement, coma
5. death

If a mouse was recorded to have a weight loss of more than 20% or displayed a CM score equal to or higher than 3, it was immediately euthanized according to the animal protocol TVA 13/17.

Cell isolation protocols

Spleen

The spleen was extracted from each mouse and stored in RPMI media with 1% FBS. The organ was smashed through a 100µm filter into a single cell suspension and washed with 1x PBS 1%FBS. After centrifugation, cells were re-suspended in 1ml of 1x ACK lysis buffer for 5 minutes. Cells were washed and stained with antibodies in preparation for flow cytometry.

Colon

Colon samples were cut longitudinally and washed with 1x PBS before being stored in

RPMI media with 1% FBS. Intraepithelial lymphocytes were isolated through incubation with a DTE solution containing 1mM DTT for 20 min at 37 °C (table 8). The samples were then cut and incubated in collagenase buffer containing collagenase (1mg/ml) and DNase I (10 U/ml) for 45 mins at 37 °C (table 8). Next, the remaining tissue was homogenized through a 100μm filter and re-suspended in 1x PBS, 1% FBS. Next, these cells were further enriched for leukocytes using a Percoll gradient (40%:67%) and slow centrifugation. The resulting interphase was extracted, washed, and stained with antibodies in preparation for flow cytometry.

Lymph nodes

Inguinal, axillary, brachial, superficial cervical, and mesenteric lymph nodes were identified and extracted from the mice. All fat was removed, and lymph nodes were stored together in RPMI media with 1% FBS. Total lymph nodes were smashed through a 100μm filter into a single cell suspension and washed with 1x PBS 1%FBS. Cells were then stained with antibodies in preparation for flow cytometry.

Blood

Around 200μl of blood was extracted from the inferior vena cava of each mouse using a syringe. The blood sample was stored in a 1.5ml Eppendorf tube with 4μl of EDTA to prevent clotting. Red blood cells were lysed using 1x ACK lysis buffer and the cells were washed and centrifuged down. Afterward, the supernatant was removed, and the cells were stained with antibodies in preparation for flow cytometry.

IL-10 secretion assay

After the isolation of cells from each organ, samples were re-suspended in 90μl of cold medium with 10μl of the IL-10 catch reagent and incubated on ice for 10 minutes. Next, the samples were diluted with 10ml of warm medium and incubated for 45 minutes at 37 °C at a slow continuous rotation. Lastly, cells were washed and stained with 10μl of the IL-10 detection antibody attached to a PE fluorophore, along with other extracellular markers in preparation for flow cytometry.

Endoscopic procedure

Colitis scores and tumor development were determined using the Coloview system (Karl Storz) on disease-relevant days in a blinded fashion. For this purpose, mice were

anesthetized with Isofluran until they were no longer mobile. Colitis scoring was based on a murine endoscopic index of colitis severity (MEICS). In this scoring system, mice are given a score from 0-3 for each specific criterion: stool consistency, vascular pattern, translucency, fibrin development, and granularity of the colon. Tumor development was graded from 1-5, based on the size of each tumor. The total tumor score was calculated by adding up the scores from each tumor that was visible during the colonoscopy. Both scoring criteria were previously described by Becker *et al*. in 2006 [181].

MACS of mouse CD45RB^{high} cells

For MACS cell sorting methods, all steps of the protocol were performed under a sterile hood. The spleen and lymph nodes of 8-12-week-old reporter mice were extracted and filtered through a strainer to obtain a single cell suspension. For the acquisition of $CD4^+$ T cells, cells were incubated with $CD4^+$ microbeads (1:10) for 30 minutes at 4 °C and run through new MACS columns attached to a magnet. The $CD4^-$ cells were collected in the flow through, and the rest of the $CD4^+$ T cells were flushed through the MACS column after removal from the magnetic stand. These $CD4^+$ T cells were then further sorted using FACS to obtain a pure population of $CD4^+$ $CD25^-$ $CD45RB^{high}$ cells.

RNA isolation using Trizol

RNA was extracted from samples that had been snap-frozen with dry ice. Samples were placed in RNase-free tubes with microbeads and PeqGold TriFast trizol reagent, then homogenized for 2 min. Before centrifugation, chloroform was added for purification. The aqueous phase was transferred to a clean tube with 100% isopropanol for precipitation and incubated at -20 °C overnight. On the next day, samples were centrifuged and washed in 70% ethanol, before the RNA pellet could be dissolved in DEPC-H_2O using a Thermomixer (5 min, 60 °C).

RNA isolation using Qiagen RNeasy kit

RNA was extracted from FACS-sorted cells. Cells were collected in RNase-free tubes with RLT buffer and β-mercaptoethanol, where they were directly lysed. Lysed cell contents were run through a gDNA eliminator column and centrifuged. After dilution with 70% ethanol in a 1:1 ratio, the sample was run through an RNA extraction column. The columns were washed a few times with RPE buffer and RNA was eluted from the

column using DEPC-H_2O in preparation for cDNA synthesis or bulk sequencing.

cDNA synthesis

cDNA was synthesized from isolated RNA using the high-capacity reverse transcriptase cDNA synthesis kit, following the manufacturer's instructions. All samples were synthesized at a concentration of 1μg/ml.

Real-time PCR

Quantification of gene expression by real-time PCR (RT-PCR) was performed using the TaqMan fast advanced master mix on the StepOne Plus system. Probes of the genes of interest were purchased from Applied Biosystems, as listed in table16. The cycle of threshold (C_t) was measured by the fit-point method and the relative expression of a gene was normalized to *Hprt1* using the 2-ΔΔCt method.

Histology

Colon tissue samples that had been kept in 4% PFA (Histofix) were embedded in paraffin and cut into sections of 4μm. Hematoxylin and Eosin (H & E) staining was carried out to allow visualization of tissue structure and morphological changes. Initially, cut sections were deparaffinized in xylene and then rehydrated in descending concentrations of ethanol (96 % > 80 % > 70 %). After washing with water, the cut sections were stained in hematoxylin, which stains nuclei structures. After washing again, slides were dipped briefly in a saturated lithium carbonate solution for faster blueing, before being counter-stained with eosin, which stains membrane structures and other proteins within the cytoplasm. Finally, cut sections were dehydrated in ascending concentrations (70 % > 80 % > 96 %) of ethanol and fixed again using xylene. Cover slides were glued on top to prevent the cuts from drying out.

Single cell RNA sequencing

A similar analysis strategy was applied to both BD Rhapsody and 10X Genomics data sets. The BD Rhapsody data sets were aligned to the reference genome GRCm38-PhiX-gencodevM19-20181206.tar.gz using the Seven Bridges platform. The 10x Genomics datasets were aligned to the same reference genome using Cellranger (10x Genomics, version 3.0.1). A standard preprocessing pipeline using Scanpy was performed by filtering out cells with less than 30 genes expressed. In pre-processing, no additional filtering was used. ScTransform was used to normalize the data using 2000 genes and without regressing out any variable. Both technologies were combined using Seurat FindIntegrationAnchors and IntegrateData using reciprocal principal component analysis (PCA) on two batches named Rhapsody and 10X. Data was scaled and subsequently, Harmonypy was performed to integrate single batches using sigma = 0.07, theta = 1.1, nclust = 20, which were hand-selected by manual inspection of Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) plots. Scanpy was used for further analysis. UMAP plots were computed with n_neighbors = 100, cosine distance metric, and on the first 12 principal components. A resolution of 0.62 was hand-picked by inspecting marker gene tables for various resolutions. T-test was used for marker gene detection. Two clusters were identified based on S-score and marker genes as proliferating cells and were removed for downstream analysis. Nuclear gene fraction to allow the detection of dead cells was computed based on nucleoplasmatic genes. Scrublet was used for doublet detection and cells with a doublet probability above 0.1 were removed. After filtering, UMAP was re-computed using PAGA positions as initial positions, and clustering was done with a resolution of 0.6.

10x Genomics

Cells from the spleen of *Il22ra2*^{eGFP} and *Foxp3*^{mRFP} mice were collected in an RNase-free tube containing 1x PBS + 0.04% BSA. Barcoding of cells, cDNA synthesis and library preparation were all carried out following the manufacturer's instructions (Chromium Single Cell 3' Reagent Kits v3 User Guide). Samples were sent to Novogene for sequencing using the Novaseq6000 with an S4 Flowcell.

BD Rhapsody

Cells from the spleen of *Il22ra2*^{eGFP} and *Foxp3*^{mRFP} mice were collected in an RNase-free tube containing sample buffer. Labeling of cells, cDNA synthesis, and library preparation were all carried out following the manufacturer's instructions (BD Rhapsody Single-Cell Analysis System Instrument User Guide). Samples were sent to Novogene for sequencing using the Novaseq6000 with an S4 Flowcell.

Bulk RNA sequencing

Cells from the spleen of *Il22ra2*^{eGFP} mice were collected in an RNase-free tube containing RLT buffer and β-mercaptoethanol. After extraction of RNA using the Qiagen RNeasy kit, samples were sent to Novogene for sequencing. Raw read files were trimmed for low quality bases using Trimmomatic (version 0.33), followed by alignment to the mouse genome (draft GRCm38.84, downloaded 2016-06-02) using STAR (version 2.5). A standard RNAseq pipeline was used for data analysis of bulk-sorted cell populations. Briefly, mitochondrial genes were excluded from the analysis as well as genes that fail to have over 5 counts in more than 4 samples. Data normalization and differential gene expression (DEG) analysis was performed in R environment using the DESeq2 package. The default normalization method of RNA counts in the DESeq2 package is based on "Relative Log Expression". Sample level quality control was checked on count data, to see that the Log2-transformed normalized count was used to assess the similarity between samples using principal component analysis (PCA) and hierarchical clustering. Generally, we focused on PC1 and PC2 (showing the largest amount of variation in the data) and plotted them against each other to see if there are any outliers. Accordingly, outlier samples were removed before further analysis. After DEG analysis, cutoffs were specified for further filtering: $|Log2FC| > 1$ and $(Padj) < 0.05$ for up-regulated genes and $|Log2FC| < -1$ and $(Padj) < 0.05$ for down-regulated genes to identify qualified DEG for analysis.

Statistical analysis

Statistical analysis was carried out on flow cytometry and real-time PCR data using the GraphPad Prism 7.0 software. The non-parametric Mann-Whitney U, Kruskal-Wallis, Gehan-Breslow-Wilcoxon, and 2-way ANOVA tests were used accordingly, where a p-value of <0.05 was considered significant. Data alignment, integration, and processing of single cell sequencing and bulk RNA sequencing data was carried out by Dr. Babett Steglich, Dr. Can Ergen-Behr, and Dr. Yogesh Kumar using R Studio software and associated packages.

Data acquisition and software

Table 21: Software

Software	Company
FACS Diva Software v.6.1.3	BD Bioscience
Flow jo_V10	FlowJo LLC
GraphPad Prism 7.0	Graphpad Software Inc.
Microsoft office package	Microsoft
EndNote X9	Clarivate Analytics
Graphics	Apple
R studio	R Studio, Inc.
Seven bridges	Seven Bridges Genomics

Results

Validation of the newly generated *Il22ra2*^{eGFP} reporter mouse

From experiences within the host lab, commercially available antibodies for murine IL-22BP seem to be unreliable and inefficient in detecting the soluble receptor for flow cytometry purposes. Due to these technical limitations, a new reporter mouse was generated to enable detection and extraction of cells expressing the gene coding for IL-22BP, *Il22ra2*. For this purpose, an enhanced green fluorescent protein (eGFP) reporter gene was attached to the end of exon 5 in the *Il22ra2* gene. Specifically, mouse genomic fragments containing homology arms spanning from the intronic regions (after exon 3) to the untranslated regions (after exon 5), were amplified from a bacterial artificial chromosome (BAC) clone using high fidelity Taq polymerase. These were assembled into the targeting vector along with T2A, eGFP, flippase and neomycin resistance gene components to enable selection of cells with the targeted insertion (**Figure 1a**).

To validate the targeted insertion, these chimeric mice were genotyped for the possession of the targeted eGFP-containing gene using primers that detected the homology arms and additional gene components. A high-fidelity polymerase chain reaction (PCR) enabled the detection of a band in the size of 5294 base pairs and confirmed the insertion of the reporter gene in these mice (**Figure 1b**). Here, gel electrophoresis indicated the presence of the targeted insertion (red arrow) in the transgenic mouse sample. The absence of this band was demonstrated in a wild type control sample. For further validation, a pure population of $CD4^+$ T cells that either expressed eGFP or were negative for the reporter protein were isolated using fluorescence-activated cell sorting (FACS) (**Figure 1c**) and quantified for *Il22ra2* expression using Real Time-PCR (RT-PCR) (**Figure 1d**). After normalization to a housekeeping gene, *Hprt1*, it was observed that the $eGFP^+$ cells did indeed express higher levels of *Il22ra2* than the $eGFP^-$ cells, indicating that $eGFP^+$ cells are enriched for *Il22ra2*.

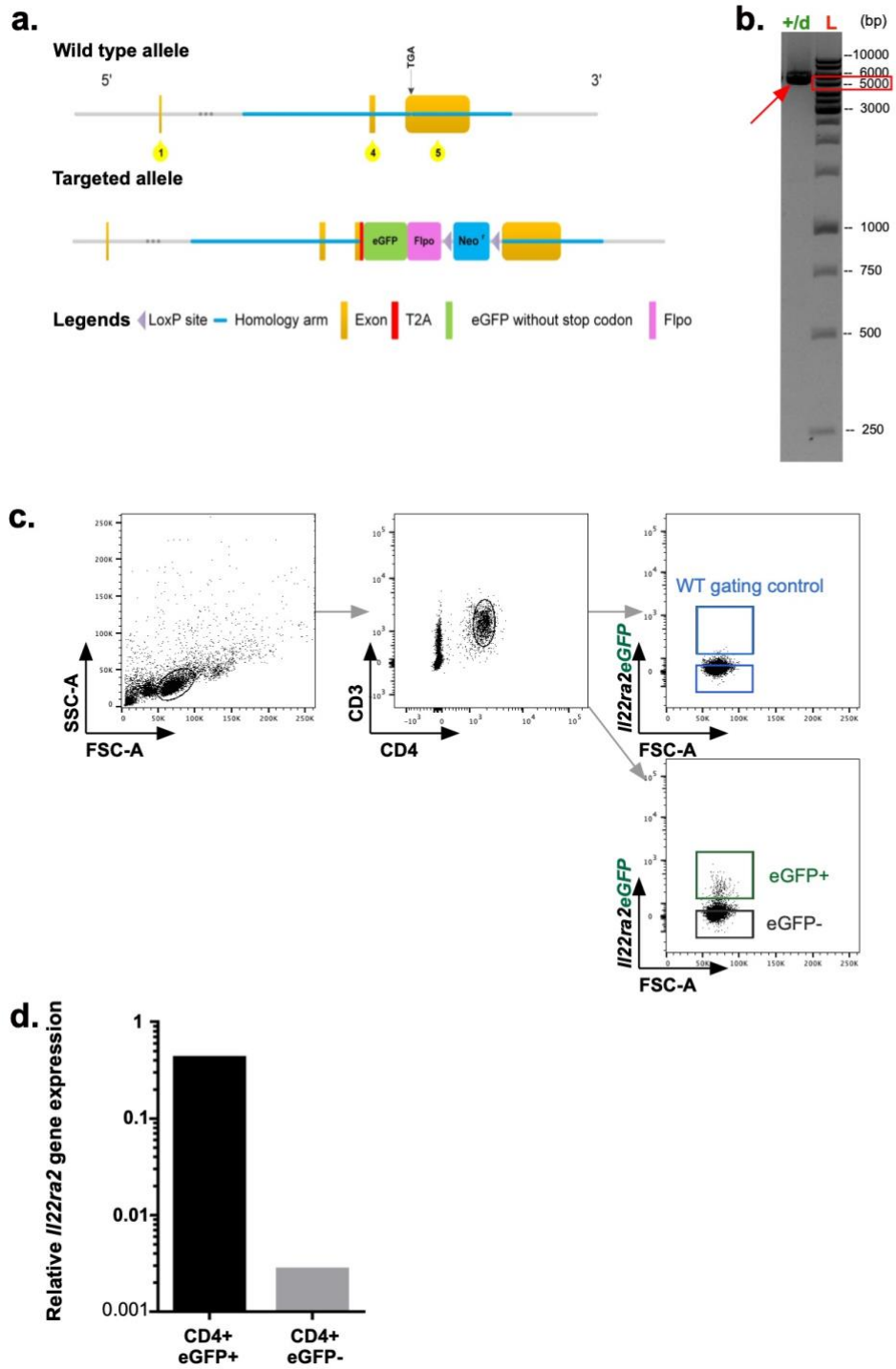


Figure 1: Generation and validation of the *Il22ra2*^{eGFP} reporter mouse **a)** Targeting vector construct with T2A, eGFP, Flippase, and Neomycin-resistance elements (generated by Cyagen). The construct was inserted in the target gene at the end of exon 5, just before the untranslated region. **b)** Phusion high-fidelity DNA polymerase PCR run alongside GeneRuler 1kb DNA ladder (reference) on a 1% agarose gel. The 5000bp maker on the gene ladder is marked by a red box. The 5294bp band representing successful insertion of the T2A and eGFP is indicated with a red arrow (L = Ladder, +/d = heterozygous DNA sample **c)** Gating strategy for isolation of CD4$^+$ T cells with or without eGFP marker **d)** RT-PCR of *Il22ra2* gene expression (normalized to *Hprt*) from sorted cells.

Next, experiments were carried out to make sure that the targeted insertion did not shift or cause changes to the gene expression patterns of flanking genes (**Figure 2a**). Quantification of *Il22ra2*, *Ifngr1,* and *Il20ra* gene expression levels was carried out on total spleen tissue extracted from the newly generated reporter mice and C57BL/6J wild type mice as a control. Quantitative RT-PCR analysis shows that there were no significant changes observed in the expression patterns of any of these genes (**Figure 2b**). As well as this, general cell population frequencies such as lymphocytes (initial lymphocyte gate in flow cytometry determined through the forward scatter and sideward scatter), $CD45^+$ cells, $CD4^+$ T cells, and $Siglec^+$ cells (eosinophils) were investigated to ensure that there were no abnormalities in the new mouse. As seen in **Figure 2c-f**, there were no significant differences between the *Il22ra2*^{eGFP} mouse and wild type mice in any of these populations in the organs investigated.

Taken together, validation experiments confirmed that a working *Il22ra2* reporter mouse has been generated and that the inserted targeting vector did not affect the gene expression or the cellular distribution in the mice. Furthermore, the reporter mouse was used in all future *in vivo* experiments as a tool for identifying *Il22ra2*-expressing cells for the purpose of studying aspects of the three aims that were mentioned above.

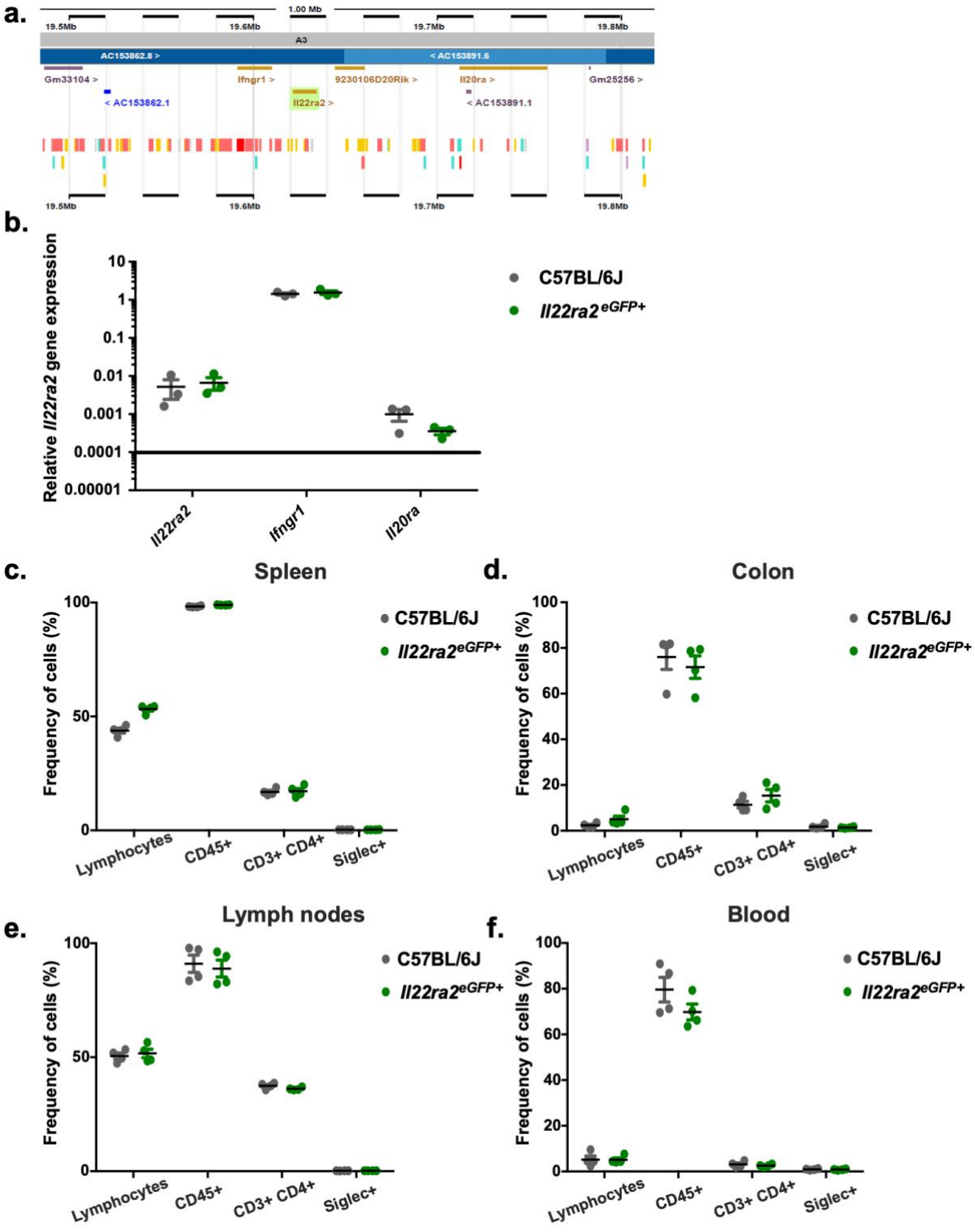


Figure 2: Targeted insertion did not disrupt flanking genes or general cellular compositions a) Gene location map of murine *Il22ra2* (highlighted in green) and flanking genes on chromosome 10. Information on map is adapted from the ENSEMBL website **b)** RT-PCR of *Il22ra2* and flanking genes *Ifngr1* and *Il20ra* (all normalized to *Hprt*) extracted from total spleen tissue of C57BL/6J and *Il22ra2*^{eGFP} mice (2-way ANOVA). Flow cytometry analysis (**c**-**f**) of total lymphocytes, CD45^{+} cells, CD4^{+} T cells, and Siglec^{+} cells (eosinophils) from **c)** spleen, **d)** colon, **e)** lymph nodes and **f)** blood of C57BL/6J and *Il22ra2*^{eGFP} mice (2-way ANOVA)**.** Significance tests indicate that comparisons were all non-significant. Bars indicate standard error.

$CD3^-$ $NK1.1^+$ NK, $CD3^+$ $NK1.1^+$, $CD8^+$ and $CD19^+$ cells are novel *Il22ra2*-expressing cell types

Previous publications have elucidated dendritic cells, eosinophils, and $CD4^+$ T cells to be significant *Il22ra2*-expressing cells [9,134-136]. As part of the first aim within this project, the *Il22ra2*^{eGFP} reporter mice were utilized to investigate the proportions of each of these *Il22ra2*-expressing cell types in the spleen, lymph nodes, colon, and blood of steady state mice. Using flow cytometry, the proportion of each contributing source of *Il22ra2* was quantified after gating on the total $CD45^+$ *Il22ra2*$^{eGFP+}$, followed by gating on the respective markers for each specific cell type (**Figure 3a**). Alongside these previously described cell types, it was discovered that other cellular sources of *Il22ra2* may have a considerable role during the steady state, namely $CD3^-$ $NK1.1^+$ cells (otherwise referred to as NK cells), $CD3^+$ $NK1.1^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells (**Figure 3a-e**).

To date, *Il22ra2*-expressing $CD11c^+$ dendritic cells have been studied most extensively and have been shown to have significant consequences on disease outcomes with regards to blocking IL-22-signaling [9,136,144]. Among the identified cell populations expressing *Il22ra2*, it was observed that $CD11c^+$ cells do indeed makeup one of the major cell types expressing the soluble receptor in all four organs. In the colon, lymph nodes, and blood, $CD11c^+$ makeup around 35% of the total *Il22ra2*-expressing cells (**Figure 3c-e**), whereas, in the spleen, they make up the second most predominant cell type, after NK cells, at nearly 20% (**Figure 3b**).

Moreover, $CD4^+$ T cells were observed to make up a substantial proportion of *Il22ra2*$^-$ expressing cells in the lymph nodes at a frequency of 20%, making it the third most predominant cell subset in this organ (**Figure 3c**). The frequencies of *Il22ra2*-expressing $CD4^+$ T cells in the spleen and colon lie at around 5% and between 1-2% in the blood of steady state mice (**Figure 3b, d, and e**). $CD4^+$ T cells have been identified as a pathogenic source of IL-22BP in colitis [135], hence, it is interesting to see that they can be identified as a source of the receptor in many organs during steady state. Further experiments investigating how proportions of these cell types differ in animal models of disease will be discussed later on and will provide insight into which cell types play a significant role under inflammatory conditions.

In line with a previous publication stating the importance of IL-22BP-producing eosinophils in colitis development [136], this data set verifies that Siglec$^+$ cells are capable of expressing *Il22ra2* in the colon, and in other organs. In this data set, Siglec$^+$ cells are the second most abundant cell type expressing *Il22ra2* in the colon at a frequency of around 10% (**Figure 3d**). In the three other organs, eosinophils contribute to *Il22ra2* expression at a frequency of around 5% (**Figure 3b, 3c, and 3e**).

This data set also describes novel cellular sources that are capable of expressing *Il22ra2* that have not yet been investigated in either steady state or disease conditions. Strikingly, NK cells seem to make up a large proportion of *Il22ra2*-expressing cells, particularly in secondary lymphoid organs. In the spleen and lymph nodes, the proportion of *Il22ra2*-expressing NK cells appears to be similar to, if not larger than the proportion that makes up the CD11c$^+$ cells, at 40% (**Figure 3b**) and 30% (**Figure 3c**), respectively. CD3$^+$ NK1.1$^+$ T cells, on the other hand, appear to only form a smaller proportion of *Il22ra2*-expressing cells during homeostasis in most organs. However, CD3$^+$ NK1.1$^+$ T cells make up the second most abundant *Il22ra2*-expressing cells in the blood at a frequency of 20% (**Figure 3e**). Lastly, CD8$^+$ T cells and CD19$^+$ B cells are also shown to express *Il22ra2* in all four organs at a frequency of less than 10%, although they are significantly lower than other cell types in most organs (**Figure 3b-e**).

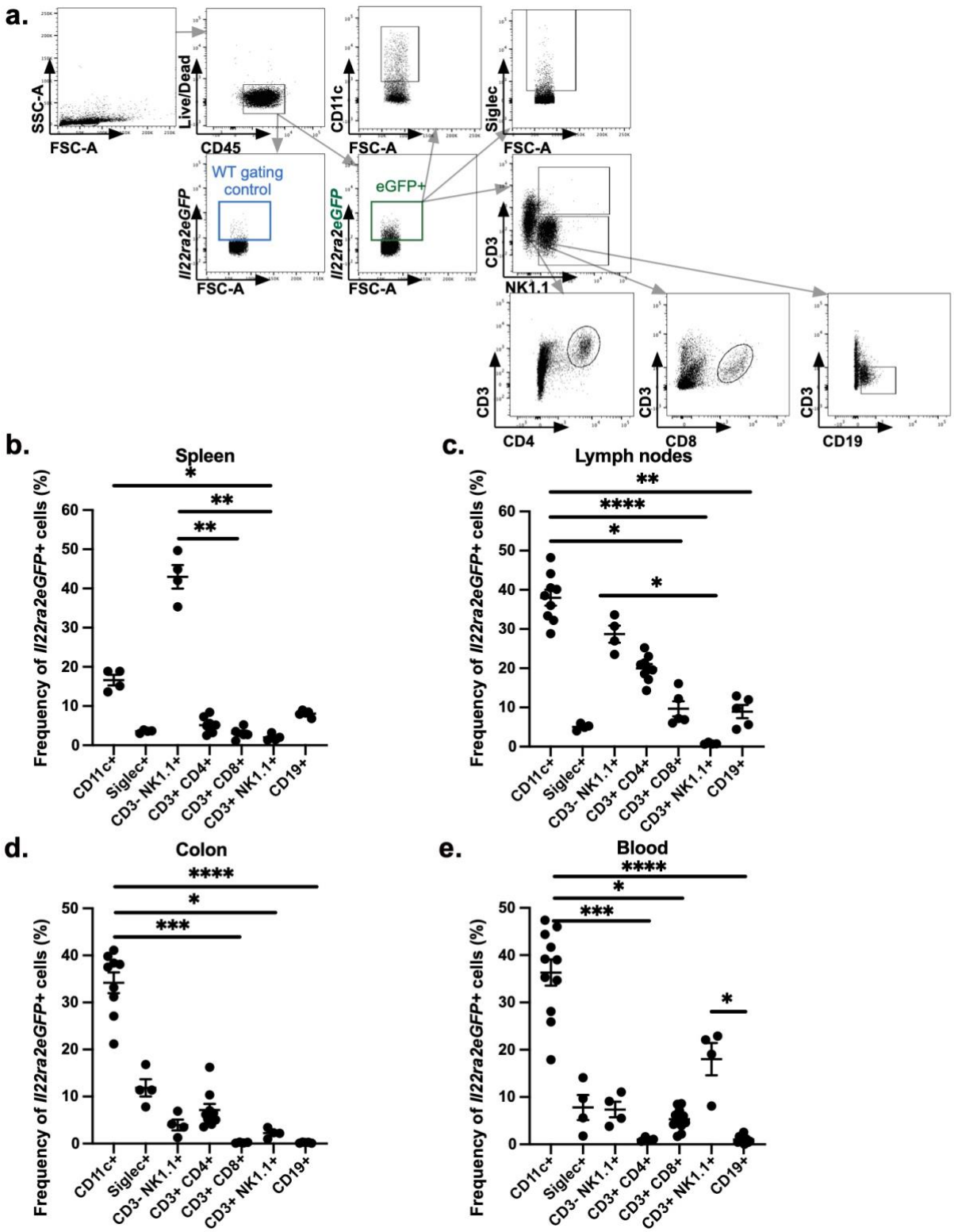


Figure 3: Relative contribution of CD45$^+$ cells that express *Il22ra2* during steady state a) Representative gating strategy depicting cell types that express *Il22ra2*^{eGFP} from total CD45$^+$ cells in the spleen. Frequency of cells expressing *Il22ra2*^{eGFP} in **b)** spleen, **c)** lymph nodes, **d)** colon and **e)** blood (Kruskal-Wallis Test). Significance is marked with p = <0.05 (*), p = <0.0005 (***) or p = <0.0001 (****), otherwise comparison is non-significant. Bars indicate standard error.

Il22ra2 expression in T helper cell subsets under homeostatic conditions

The effects of cytokines produced by $CD4^+$ T cells are often described to be microbiota-dependent, and slight alterations in microbiota compositions have been shown to have dramatic consequences on the host's response [19]. This is particularly true for the cytokine IL-22 [108], and therefore, may also have an impact on its receptor counterpart, IL-22BP. Il22ra2 expression has never been studied regarding microbiota dependency, so an initial investigation was carried out into whether expression levels differ between three different microbiota compositions. Indeed, *Il22ra2* expression appears to be microbiota dependent, as expression levels were different between the cells extracted from mice with different microbiota compositions (**Figure 4a**). Furthermore, *Il22ra2* expression levels were investigated in naïve and effector memory cell populations, sorted by the CD44 and CD62L (L-selectin) cellular markers.

Specifically, quantification of gene expression through RT-PCR indicated that germ-free mice had the highest amount of *Il22ra2* expression in the $CD44^+$ memory T cell compartment, followed by expression levels in cells extracted from *Nlrp6*$^{-/-}$ mice, which are known to host a dysbiotic microbiota composition [182]. When analyzing the $CD44^+$ $CD62L^-$ effector memory T cell groups, cells originating from germ-free mice had significantly higher expression of *Il22ra2* than cells from both other microbiota compositions. Similarly, within the $CD44^+$ $CD62L^+$ central memory cell groups, cells originating from specific pathogen-free (SPF) mice had significantly lower *Il22ra2* expression than cells originating from germ-free mice.

Moreover, the highest *Il22ra2* expression levels were reported in $CD44^+$ $CD62L^+$ central memory and $CD44^+$ $CD62L^-$ effector memory T cells, and the lowest gene expression levels were observed in $CD44^-$ $CD62L^+$ naïve T cells (**Figure 4a**). Interpretation of the data indicates that there are no differences between the different T cell types extracted from SPF and *Nlrp6*$^{-/-}$ mice, although there were significant differences between naïve and memory compartments in cells extracted from germ-free hosts.

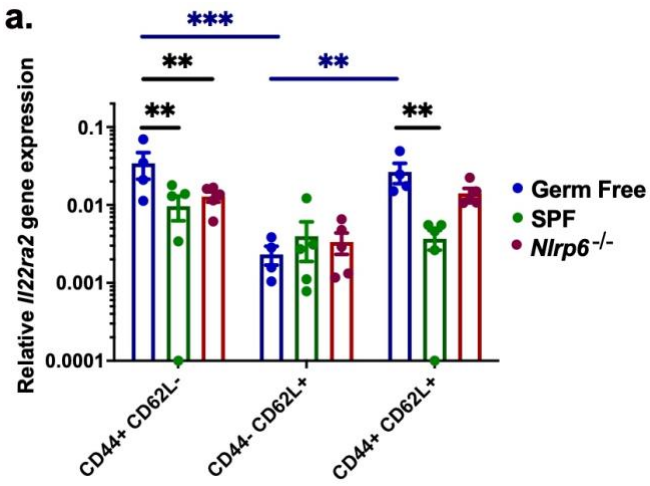


Figure 4: *Il22ra2* expression is dependent on host microbiota composition and is highest in CD44$^+$ effector cells a) RT-PCR of *Il22ra2* gene expression (normalized to *Hprt*) from sorted cells. Naïve (CD44$^-$ CD62L$^{+)}$, central memory (CD44$^+$ CD62L$^+$), and effector memory (CD44$^+$ CD62L$^-$) T cells were extracted from mice hosting different microbiota compositions: Germ-free, SPF, and *Nlrp6*$^{-/-}$ (2-way ANOVA). Significance is marked with p = <0.05 (*) or p = <0.005 (**), otherwise comparison is non-significant. Bars indicate standard error.

After discovering that *Il22ra2* is predominantly expressed by memory T cells, a particular focus was placed on learning about the different CD4$^+$ T helper cell subsets that are responsible for expressing *Il22ra2* and learning how these cells are transcriptionally regulated. Although CD4$^+$ T cells are not the predominant *Il22ra2*-expressing source in the colon (**Figure 3d**), the study by Pelczar *et al.* raised important questions as to which specific T helper cell subset may be responsible for pathogenic IL-22BP production during colitis. So far, this has not been addressed in humans, and previous investigations in mouse models of colitis have indicated that common conventional CD4$^+$ T helper cell (T$_h$) subsets were not responsible for expression of *Il22ra2* [135].

The aim of the next figure was to identify whether specific CD4$^+$ T helper cell subsets are capable of expressing the receptor using the *Il22ra2*^{eGFP} reporter as a tool. Previous investigations carried out on Foxp3$^+$ T regs and IL-17A$^+$ CD4$^+$ T cells using RT-PCR techniques showed that these two subsets were not responsible for expressing *Il22ra2* during colitis [135]. However, this remained to be verified on a pure population of *Il22ra2*$^+$ CD4$^+$ T cells, and a major hypothesis throughout this project was to determine whether known T cell subsets, such as T$_h$17 cells, T$_h$22 cells or regulatory *Foxp3*$^+$ T cells can express the receptor. Initial experiments using the *Il22ra2*^{eGFP} reporter mouse were carried out to determine whether these T helper cell subsets were capable of expressing *Il22ra2* during homeostasis. For this purpose, reporters for

Foxp3^{mRFP}, *Il17a*^{FP365} and *Il22*^{sgBFP}, which were readily available in the lab [125], were incorporated with the *Il22ra2*^{eGFP} reporter mouse. In addition to this, an IL-10 secretion assay was used to determine whether IL-10-producing CD4$^+$ T cells could also express *Il22ra2* (**Figure 5a**). During homeostasis, it was observed that *Il22ra2* is expressed by nearly 2% of CD4$^+$ T cells in the spleen and is produced by around 3% of CD4$^+$ T cells in the colon. In the lymph nodes, this frequency is significantly reduced, where only 0.2% of CD4$^+$ T cells express *Il22ra2* (**Figure 5b**).

Furthermore, different T helper cell subsets were investigated for *Il22ra2* expression in the same organs (**Figure 5c**). In general, it appears that the only T helper cell subset seen to co-express *Il22ra2* in all three organs are *Foxp3*$^{mRFP+}$ Tregs at around 5% in the spleen and lymph nodes, and close to 20% in the colon. This subset is significantly higher in the colon compared to the other two organs investigated. The frequency of *Il17a*$^{FP365+}$ and *Il22*$^{sgBFP+}$ in the spleen and lymph nodes are negligible and are significantly lower than *Foxp3*$^{mRFP+}$ T cells and *Foxp3*$^{mRFP-}$ IL-10$^+$ T cells in most organs. Nevertheless, a frequency of 5% and 3% are observed in the colon, respectively. Within the colon, *Foxp3*$^{mRFP+}$ IL-10$^+$ and *Foxp3*$^{mRFP-}$ IL-10$^+$ T cells are shown to express *Il22ra2* at considerable frequencies of 3% and 15%, respectively. Interestingly, *Foxp3*$^{mRFP-}$ IL-10$^+$ T cells are significantly higher in the colon than the other organs investigated.

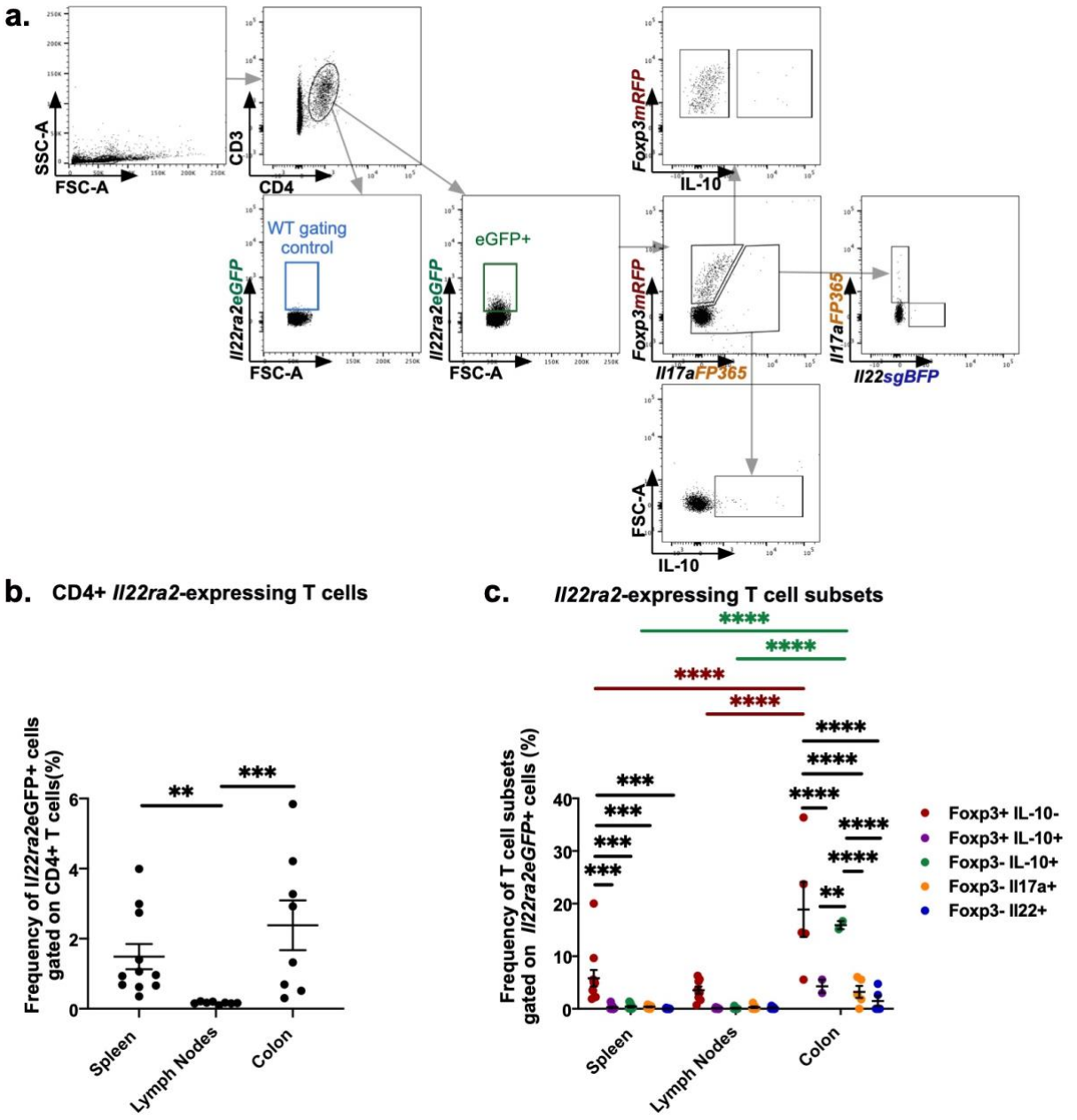


Figure 5: *Il22ra2* expression in T helper cell subsets under homeostatic conditions **a)** Gating strategy for quantification of total CD4^{+} *Il22ra2*$^{eGFP+}$ populations and *Il22ra2*$^{eGFP+}$ T cell subsets from *Il22ra2*^{eGFP}*Foxp3*^{mRFP}*Il17a*^{FP365}*Il22*^{sgBFP} reporter mice. **b)** Frequency of CD4^{+} cells that express *Il22ra2* extracted from spleen, lymph nodes and colon (Kruskal-Wallis test). **c)** Frequency of CD4^{+} *Il22ra2*$^{eGFP+}$ T helper cell subsets extracted from spleen, lymph nodes and colon (2-way ANOVA). Significance is marked with p = <0.05 (*),or p = <0.005 (**), p = <0.0005 (***) or p = <0.0001 (****), otherwise comparison is non-significant. Bars indicate standard error.

Il22ra2 can be expressed by heterogeneous CD11c^{+} and CD4^{+} populations

Previous experiments have indicated that *Il22ra2* can be expressed in many different cell types, including newly identified sources such as CD3^{-} NK1.1^{+} NK cells, CD3^{+} NK1.1^{+} T cells, CD8^{+} T cells and CD19^{+} B cells (**Figure 3**). In addition to this, the expression of *Il22ra2* in CD4^{+} T helper cells was verified (**Figure 3**) and Foxp3^{+} Tregs have been identified as cells that can co-express *Il22ra2* during homeostasis in mice (**Figure 5c**). Unfortunately, the approach of using flow cytometry only allows detection of a limited number of markers at a time. This means that one can only investigate cell subsets that can be identified by available reporter mice or antibodies, leaving a significant percentage of *Il22ra2*$^{+}$ cells undefined.

In order to characterize the undefined portions of the remaining *Il22ra2*-producing CD4^{+} T cells, a single cell sequencing approach was utilized. This particular technique allows identification of different cellular subsets that express *Il22ra2*, with the additional advantage of looking into the heterogeneity and transcriptional profiles of these cells. To this end, pure populations of CD4^{+} T cells and CD11c^{+} cell populations expressing *Il22ra2* were isolated in order to study the second aim of this project. Both cell types have been identified to express *Il22ra2*, but not much has been revealed as to whether these might be heterogeneous populations and which subsets of these general cell types are responsible for expressing *Il22ra2*. In addition, description of the transcriptional profile of each of these populations may provide insight into the function of specific subsets.

The experimental set-up was designed to isolate a pure population of CD11c^{+} and CD4^{+} cells that either expressed *Il22ra2*, or were negative for the reporter gene, from the spleen of *Il22ra2*^{eGFP} reporter mice during steady state (**Figure 6a**). Specifically, CD11c^{+} cells were gated for prior to CD4^{+} T cells, as certain dendritic cell populations are known to express CD4 [183], and are therefore less likely to interfere with differential gene expression in sorted T cells (**Figure 6b**). To remove sequencing biases and batch effects, these two sorted populations were subsequently pooled together in equal proportions before being barcoded and processed further. As a control, a pure population of *Foxp3*$^{mRFP+}$ CD4^{+} T cells was also collected from *Foxp3*^{mRFP} mice, as

previous experiments have indicated that some Foxp3^{+} Tregs can co-express *Il22ra2* during homeostasis (**Figure 5c**).

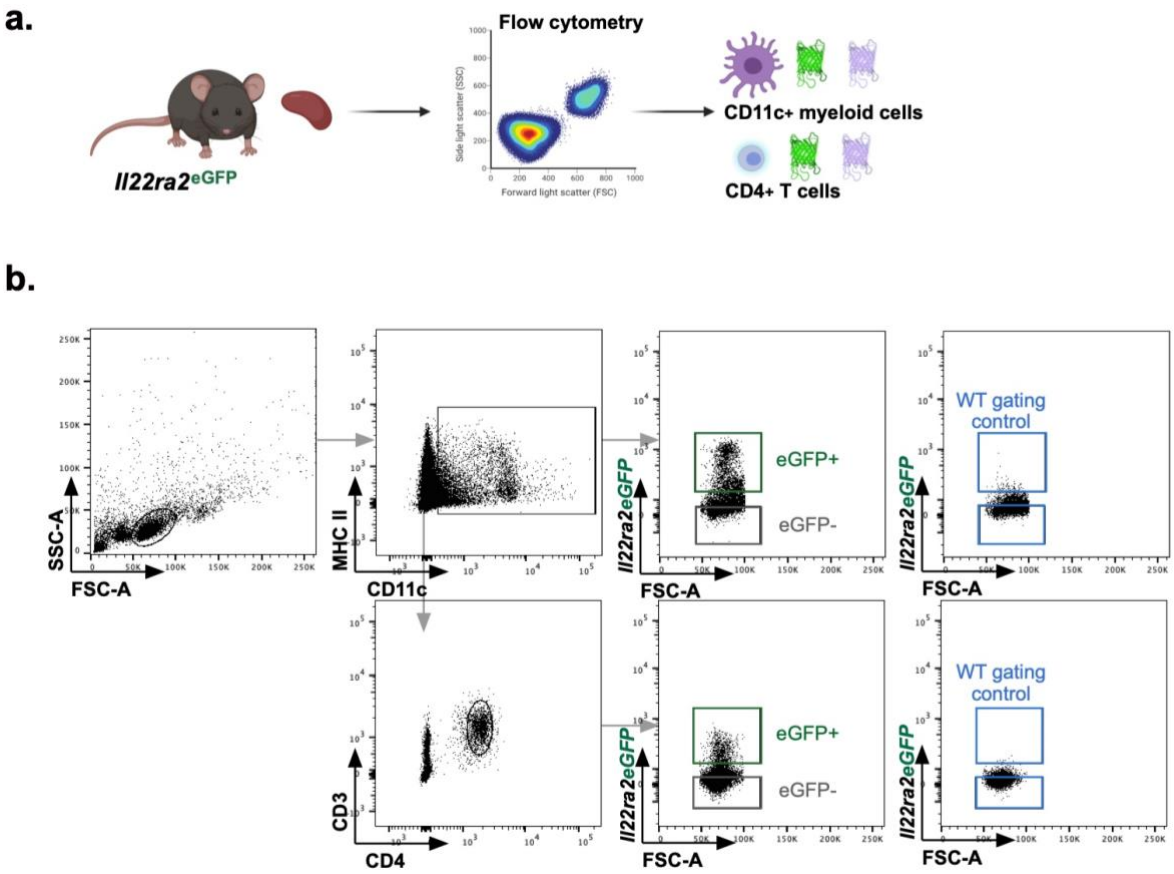


Figure 6: Isolation of CD11c^{+} *Il22ra2*$^{eGFP+}$ and CD4^{+} *Il22ra2*$^{eGFP+}$ cells from *Il22ra2*^{eGFP} reporter mice
a) Experimental scheme for isolation of *Il22ra2*$^{eGFP+}$ cell populations. Spleens of *Il22ra2*^{eGFP} reporter mice were extracted, homogenized to a single cell suspension, and pooled together. Cells were run through the FACS and pure populations of MHC II^{+} CD11c^{+} and CD3^{+} CD4^{+} that were either *Il22ra2*$^{eGFP+}$ or *Il22ra2*$^{eGFP-}$ were collected. RNA was extracted from the sorted cell samples using 10x and BD Rhapsody protocols and libraries were sequenced. **b)** Gating strategy for sorting of cell samples.

After isolation of the samples, single cells were separated into droplets and barcoded individually, allowing for identification of the specific genes that come from each barcoded cell. Readouts from the three samples were integrated and visually represented by a UMAP, which describes how the cells compare and overlap in relation to each other (**Figure 7a**).

As $CD11c^+$ cells and $CD4^+$ T cell samples were pooled together for sequencing, the gene expression patterns of *Itgam* and *Cd4*, among others, were used to distinguish between myeloid and T helper cell sources of *Il22ra2* (**Figure 7b and 7c**). In accordance with the defined samples that were isolated through FACS, gene expression maps show that the labelled $Il22ra2^+$ sample does indeed represent cells that express *Il22ra2* (**Figure 7d**). In addition to this, the control sample containing $Foxp3^{mRFP+}$ cells that was sorted using FACS is similarly defined by *Foxp3* gene expression (**Figure 7e**).

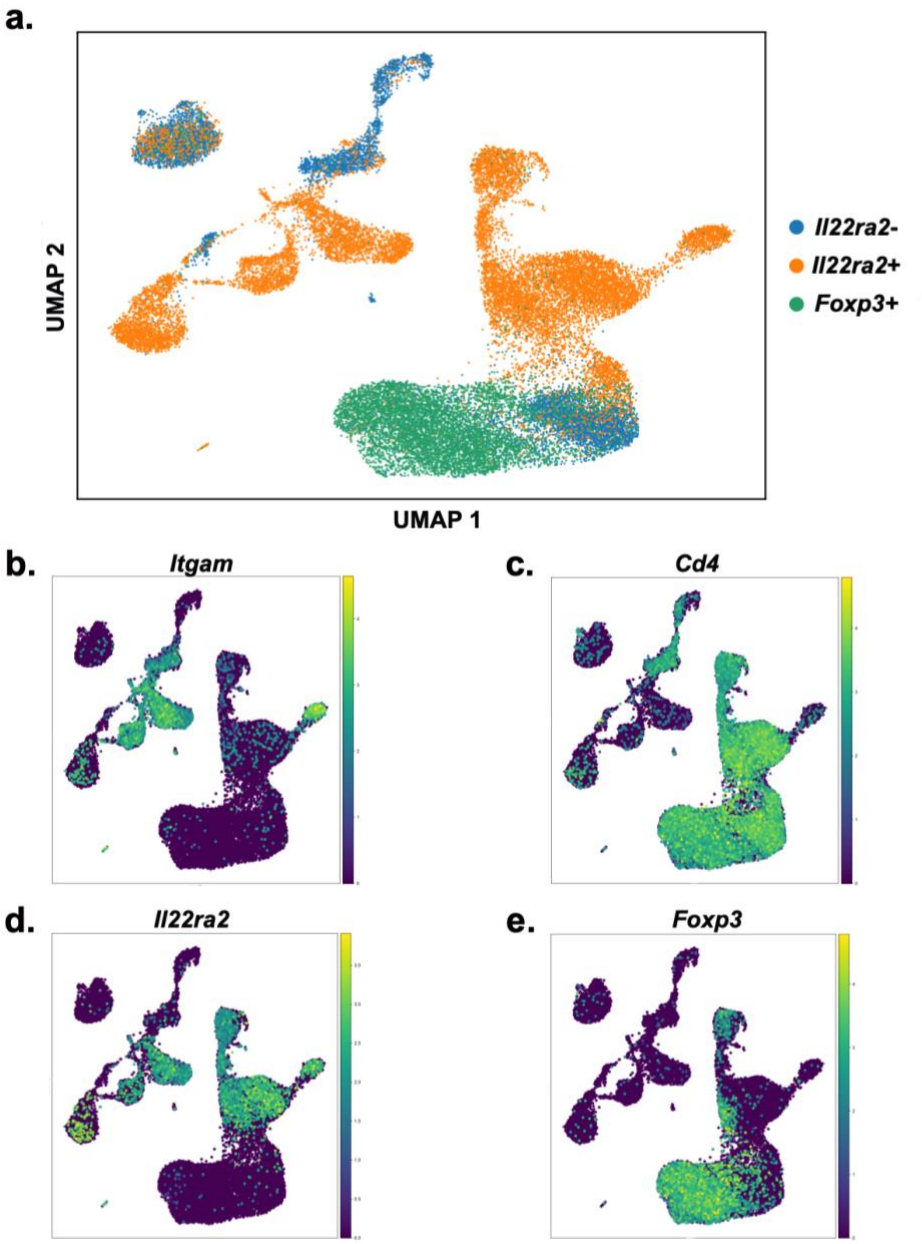


Figure 7: *Il22ra2* can be expressed by heterogeneous CD11c$^+$ and CD4$^+$ populations a) UMAP clustering of *Il22ra2*$^-$, *Il22ra2*$^+$, and *Foxp3*$^+$ cells. UMAP showing distribution of **b)** *Itgam*, **c)** *Cd4*, **d)** *Il22ra2* and **e)** *Foxp3* gene expression.

Next, the sequenced samples were run through the Seurat algorithm and separated into clusters based on the similarity of gene expression levels. This resulted in the separation of twelve different clusters defining specific *Il22ra2*$^{+}$, *Il22ra2*$^{-}$ and *Foxp3*$^{+}$ cell populations (**Figure 8a**). Analysis of the top fifty highest differentially expressed genes in each cluster enabled identification of signature marker genes that were used to define each cell type (**Figure 8b**). An extended list entailing all top fifty genes for each cluster has been included in the appendix of this thesis. After close inspection of each cluster, it can be inferred that both CD11c^{+} and CD4^{+} cells that express *Il22ra2*, are made up of heterogeneous populations (**Figure 8**).

In summary, myeloid cells that express *Il22ra2* include two different clusters of macrophages, as well as activated and conventional dendritic cells. Interestingly, the majority of the myeloid-derived *Il22ra2*$^{+}$ cells cluster as macrophages (cluster 5 and 8), with only a few *Il22ra2*$^{+}$ cells appearing in the dendritic cell clusters (cluster 7 and 10). Both macrophage clusters have similar defining gene signatures, such as *Lyz2*, *Csf1r*, *Cebpb*, *Clec4a3* (**Figure 8b**). According to the algorithms used to generate this data set, the difference between the two macrophage clusters is the expression of Ly6c. The expression of this gene is indicative of its proinflammatory and phagocytic capabilities. As well as this, expression of *Malat1* [184] and *Ace* [185] further define cluster 8 as an M1 macrophage cluster (**Figure 8b**). The Ly6c^{-} macrophages in cluster 5 are most likely anti-inflammatory and contribute to wound healing [186]. Specifically, the gene *Cd68* is often associated with tissue residency in the liver, lung and spleen [187] (**Figure 8b**). Surprisingly, the number of *Il22ra2*$^{+}$ cells displayed in the two dendritic cell clusters is less than expected. Closer observations of these two clusters depict that considerably more *Il22ra2*$^{+}$ cells are seen in the conventional dendritic cell cluster (**Figure 7a and 7d**). The difference between the dendritic cell clusters lies in the activation status of the cells. Typical dendritic cell genes include *Cd74*, *Itgax* and MHC class II-associated genes. Activation markers include *Relb* [188] and *Cd83* [189] (**Figure 8b**).

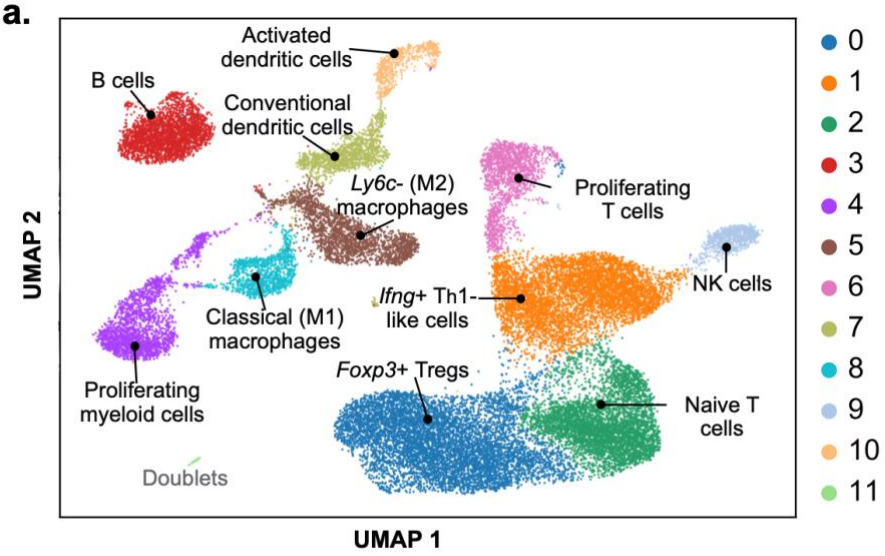
Additionally, a cluster comprising of NK cells (cluster 9) is also described. This population clusters closer to the CD4^{+} T cells rather than innate cells, although the majority of these cells do not express typical T cell genes (data not shown) (**Figure 8a**). Typical cytotoxic genes, which are also known to be characteristic NK cell genes,

including *Nkg7*, *Klrd1* and granzyme genes, are shared between cluster 9 and cluster 1 [190,191] (**Figure 8b**). This population was defined according to the signature NK cell transcription factors *Eomes* [192,193].

Besides this, $CD4^+$ T cells can be separated into naïve T cells (cluster 2) and *Ifng*$^+$ T_h1-like cells (cluster 1). Cluster 2 can be described by *Lef1* and *Il7r* (**Figure 8a**). Cluster 1 is an interesting cluster as it has typical cytotoxic genes and T cell genes: *Ifng*, *Gzma*, *Nkg7* and *Klrg1*. Interesting genes that show up describing *Il22ra2*-expressing T cells throughout sequencing data sets include *Ahnak*, *Lgals1*, *Hmgb2* and *Il18r1* (**Figure 8b**).

Additionally, a few *Il22ra2*-expressing cells overlap with cells in cluster 0, representing $Foxp3^+$ Tregs. Typical Treg genes include *Izumo1r, Foxp3*, *Il2ra*, *Ctla4* and *Cd27* (**Figure 8b**) [194-196]. Also, a significant cluster that is defined by B cell genes (cluster 3), includes a few *Il22ra2*-expressing cells, although the majority of this cluster comprises of *Il22ra2*$^-$ cells (**Figure 8a**). Cells in clusters 4 and 6 consist of predominantly *Il22ra2*-expressing cells and are described to be highly proliferative in nature. Cluster 11 is disregarded as it is made up of doublets and does not provide valuable information on characteristics of the cells.

a.



b.

Cluster 0	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
Foxp3+ T cells	*Ifng*+ Th1-like cells	Naive T cells	B cells	Proliferating myeloid cells	*Ly6c*- (M2) macrophages
Rac2	*Ahnak*	*Lef1*	*Cd74*	*Tmbsb4x*	*Lyz2*
Izumo1r	*Lgals1*	*Gimap3*	*Cd79a*	*Lyz2*	*Cybb*
Ikzf2	*Nkg7*	*Il7r*	*Igkc*	*Ccl5*	*Csf1r*
Gimap3	*Ccl5*	*Cd3e*	*Cd79b*	*Tyrobp*	*Apoe*
Foxp3	*Cd48*	*Lck*	*Cd19*	*Ftl1*	*Cebpb*
Cd3e	*Klrg1*	*Itk*	*Cd22*	*S100a6*	*Clec4a3*
Il2ra	*Hmgb2*	*Ccr7*	*Ly6d*	*Apoe*	*Cd68*
Ctla4	*Il18r1*	*Foxo1*	*Ebf1*	*Gzma*	*Ceacam1*
Sell	*Ifng*	*Foxp1*	*Pax5*	*Cebpb*	*Cx3cr1*
Cd27	*Gzma*	*Ly6c1*	*Pou2af1*	*Hmgb2*	*Ly6e*

Cluster 6	Cluster 7	Cluster 8	Cluster 9	Cluster 10	Cluster 11
Proliferating T cells	Conventional dendritic cells	Classical (M1) macrophages	NK cells	Activated dendritic cells	Doublets
Hmgb2	*Cd74*	*Lyz2*	*Gzma*	*Ccr7*	*Ccl6*
Mki67	*Itgax*	*Malat1*	*Nkg7*	*Cd74*	*Junb*
Lgals1	*Ciita*	*Tmsb4x*	*Klrd1*	*Icosl*	*Fcer1g*
Birc5	*Ffar2*	*Cebpb*	*Ahnak*	*Relb*	*Ccl3*
Ccna2	*Jun*	*Ace*	*Gzmb*	*Id2*	*Ccl9*
Itgb7	*Adam23*	*Csf1r*	*Il2rb*	*Cd83*	*Gata2*
Hmgb1	*Fcer1g*	*Clec4a3*	*Eomes*	*Birc3*	*Csf1*
Ms4a4b	*Ptprc*	*Ccl5*	*Lgals1*	*H2.Aa*	*Cd7*
Anxa2	*Flt3*	*Ftl1*	*Itga2*	*Adam23*	*Cxcr2*
Cd48	*Icosl*	*Ifngr1*	*Irf8*	*Flt3*	*Cd9*

Figure 8: Description of *Il22ra2*$^-$, *Il22ra2*$^+$ and *Foxp3*$^+$ clusters a) UMAP showing distribution of 12 different clusters. **b)** Descriptive genes used to define each cluster. Only select genes in the top 50 differentially expressed genes were listed here, as they best represented the cell type described (Top 50 genes in each cluster are listed in Table 22 in the appendix).

Il22ra2-expressing T cells co-express *Foxp3*, *Ifng* and *Tbx21*

Closer inspection of representative T helper cell genes in **Figure 9** have enabled better description of *Il22ra2*-expressing clusters. As mentioned above, *Foxp3* gene expression defines cluster 0, the *Foxp3*$^{+}$ Treg cluster (**Figure 9a**). However, only a minor proportion of *Foxp3*-expressing cells in cluster 0 exhibit *Il10* gene expression, another hallmark gene of suppressive *Foxp3*$^{+}$ Tregs. Strikingly, a small portion of cells on the left side of cluster 1, which was defined as an *Ifng*$^{+}$ T_h1-like population, also highly expressed *Foxp3*. The *Il22ra2*-expressing *Foxp3*$^{+}$ T cells in cluster 1, do not show any *Il10* co-expression. Interestingly, there are certain cells on the left side of cluster 1 that simultaneously express *Foxp3, Il22ra2* and *Ifng* (**Figure 9b**). After referring to the existing literature on IL-22BP, these cells have never been described before. When concentrating on the cells on the right side of cluster 1, it is observed that these cells highly express characteristic T_h1 genes such as *Tbx21* and *Ifng*.

On another note, cluster 9, which is clearly defined by markers for NK cells, is similarly shown to highly express *Tbx21* and *Ifng* expression (**Figure 9b**). Interestingly, *Gata3*, a defining transcription factor for T_h2 cells, is also highly expressed in this cluster (**Figure 9c**). However, it appears that the expression of *Gata3* is a defining feature of the T cells in this data set. Other defining features of T_h2 cells, such as expression of the cytokines *Il4* and *Il13*, are not expressed in any of the cell types visualized in this UMAP.

Lastly, gene expression patterns of *Rorc*, *Il17a* and *Il22* were very low or non-existing, in all three samples that were sequenced (**Figure 9d**). These genes are characteristic of T_h17 cells, indicating that this cell subset is not a source of *Il22ra2* expression during homeostasis.

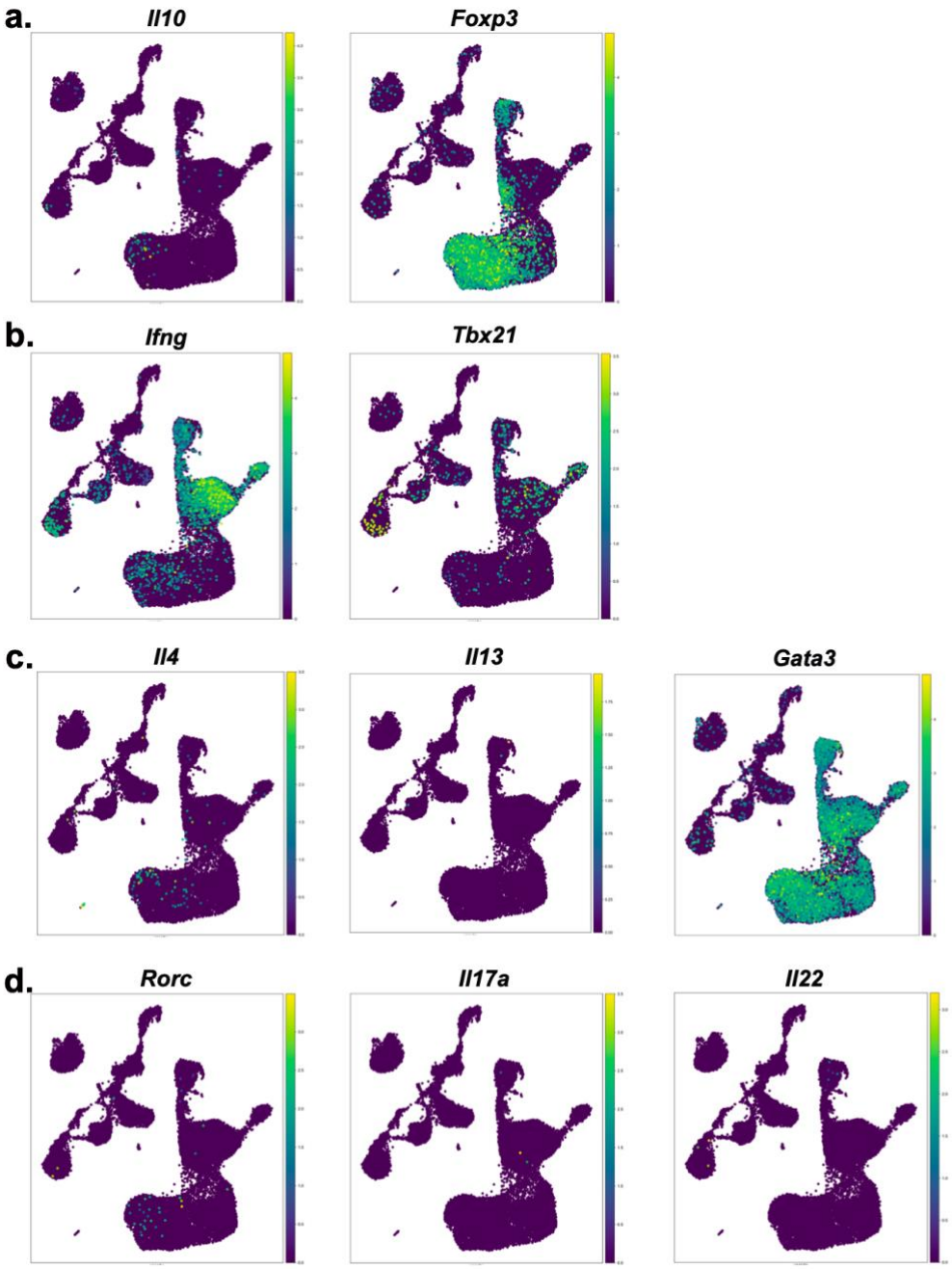


Figure 9: Gene distribution describing specific T helper cell subsets in *Il22ra2*$^{-}$, *Il22ra2*$^{+}$ and *Foxp3*$^{+}$ clusters Distribution of characteristic **a)** Foxp3^{+} Treg, **b)** T_h1, **c)** T_h2 and **d)** T_h17 genes.

Description of IL-22BP$^+$ T helper cells in human peripheral blood mononuclear cells (PBMCs)

Previous studies have provided evidence that CD4$^+$ T cells extracted from colonic biopsies of IBD patients and healthy controls can produce IL-22BP. In addition, it has been suggested that high expression levels of *IL22RA2* in these biopsies could be used as a diagnostic feature to determine how a patient may respond to anti-TNFα therapy and determine their chance of survival if they further develop colon cancer [135,144]. As obtaining colonic biopsies is invasive to the patient, and cell isolation processes are time-consuming and not always successful, an alternative analysis on PBMCs may be able to provide information on a patient's IL-22BP production levels. To date, IL-22BP production from CD4$^+$ T cells has not yet been studied in peripheral blood. To investigate whether analysis of PBMCs is plausible for studying IL-22BP in CD4$^+$ T cells, blood samples from healthy donors and IBD patients were collected. Specifically, blood samples from IBD patients were taken at a time point where an acute inflammation was endoscopically diagnosed.

For this analysis, a density gradient was used to isolate PBMCs, which were then stimulated overnight using the super antigen Staphylococcal enterotoxin B (SEB) (**Figure 10a**). These stimulated cells were then extracellularly stained with fluorophore-bound antibodies for IL-22BP, FOXP3, IL-10 and IFNγ (**Figure 10b**). These cytokines were stained for as previous single cell sequencing data from mouse *Il22ra2*-expressing CD4$^+$ T cells indicated that Foxp3$^+$ Tregs and *Ifng*-expressing cells can co-express *Il22ra2* (**Figure 9a and 9b**).

Interestingly, IL-22BP-producing CD4$^+$ T cells could indeed be extracted from PBMCs of both IBD patients and healthy controls, verified by the use of a negative isotype control (**Figure 10b**). Furthermore, in the limited number of samples that were obtained, production of IL-22BP by CD4$^+$ T cells appears to be higher in IBD patients, although this is only by trend. Specifically, the frequency of cells producing IL-22BP in healthy donors averages at around 2%, whereas in IBD patients, the average frequency lies at 4% (**Figure 10c**).

Although co-production of FOXP3, IL-10 and IFNγ could be detected in IL-22BP-producing $CD4^+$ T cells, a significant difference in the frequency of these subsets between the IBD patients and healthy donor groups was not observed. The most abundant cell population represented in $IL\text{-}22BP^+$ T cells in this data set are $FOXP3^+$ $IL\text{-}10^-$ T cells, at around 7%, and $FOXP3^-$ $IFN\gamma^+$ T cells at around 5% (**Figure 10d**). Notably, these $FOXP3^+$, $IL\text{-}10^+$, and $IFN\gamma^+$ subsets only define around 20% of the total $CD4^+$ $IL\text{-}22BP^+$ T cells, but the remaining population of cells remain undefined.

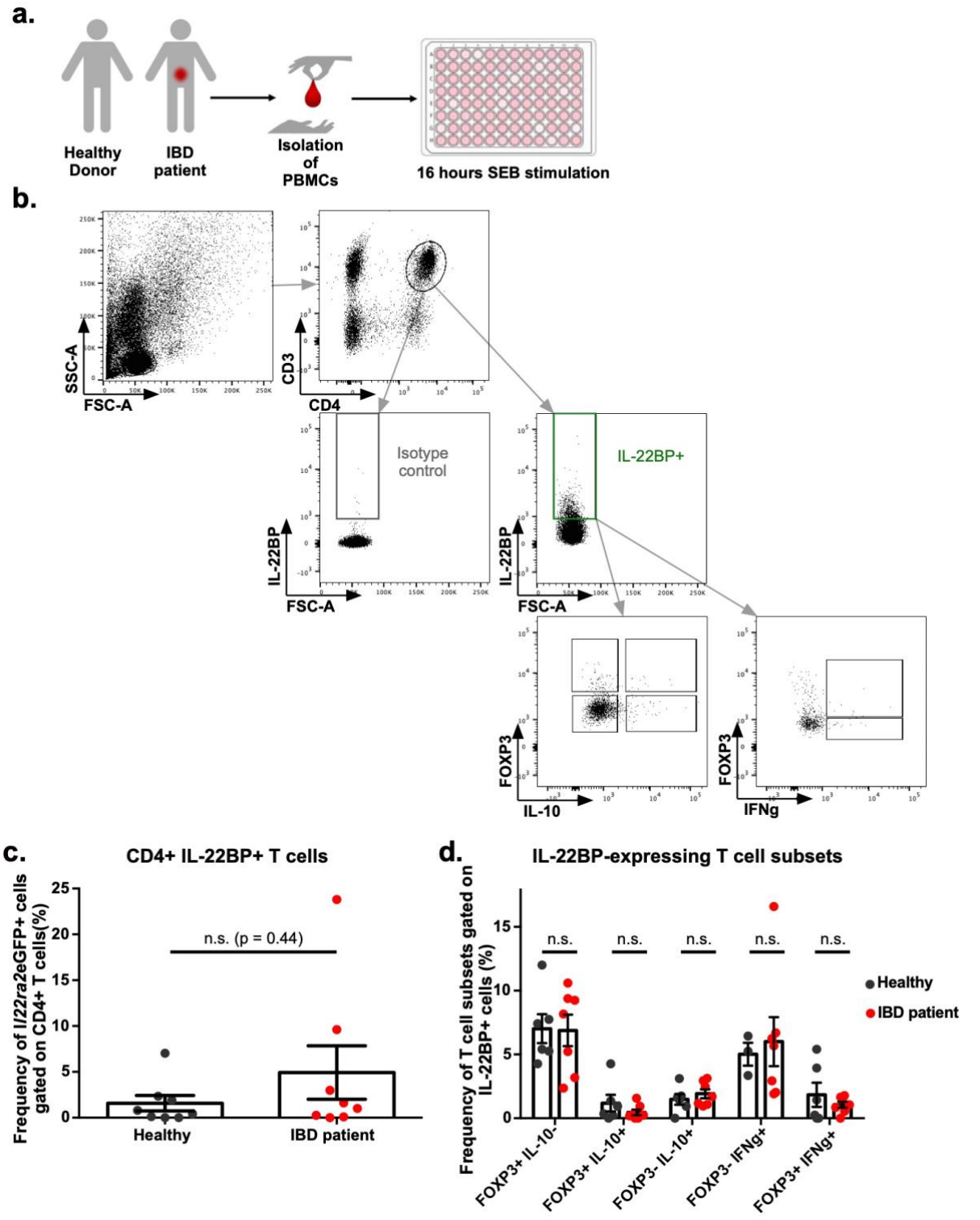


Figure 10: IL-22BP production by T helper cell subsets in human PBMCs a) Experimental scheme for isolation and stimulation of PBMCs from healthy donors and IBD patients. Healthy donor samples were chosen at random and were not age or sex-matched to the IBD patients. **b)** Gating strategy indicating how $CD4^+$ $IL\text{-}22BP^+$ T cells were identified and quantified using flow cytometry. Grey box highlights isotype control and green box highlights $IL\text{-}22BP^+$ cells detected by fluorescent antibody **c)** Frequency of total $CD4^+$ cells that produce IL-22BP extracted from PBMCs of healthy donors and IBD patients (Mann-Whitney Test). **d)** Frequency of $CD4^+$ T helper cell subsets gated on $IL\text{-}22BP^+$ cells extracted from PBMCs of healthy donors and IBD patients (2-way ANOVA). Significance tests indicate that comparisons were all non-significant. Bars indicate standard error.

Analysis of *Il22ra2* expression in different conventional T helper cell subsets in the T cell transfer colitis model

Previous experiments helped identify specific T helper cell subsets that are capable of expressing *Il22ra2* during homeostasis, such as Foxp3^{+} Tregs. Hereafter, experiments were carried out to understand whether the proportions of different cellular sources of *Il22ra2* change in intestinal disease models. Specifically, a particular focus was placed on characterizing CD4^{+} T helper cell subsets that co-express *Il22ra2*, as this may aid in deciphering why these cells act in a pathogenic manner under inflammatory settings in the gastrointestinal tract.

The T cell transfer model of colitis was the main model previously used to show that depletion of IL-22BP from CD4^{+} T cells led to an ameliorated development of disease, compared to wild type counterparts. For this reason, the same model was used in the new reporter mice to identify which CD4^{+} T cell subsets may be responsible for the pathogenic characteristics of *Il22ra2*-expressing cells. For this purpose, magnetic activated cell sorting (MACS) isolated CD4^{+} T cells from *Il22ra2*^{eGFP}*Foxp3*^{mRFP}*Il17a*^{FP365}*Il22*^{sgBFP} mice were sorted for a pure population of CD45RB^{high}CD25^{-} cells. These cells were injected intraperitoneally into *Rag1*$^{-/-}$ recipient mice and incubated for two weeks (**Figure 11a**). Mice were observed for colitis development via endoscopic colitis score [181] (**Figure 11b and 11d**), weight loss tracking (**Figure 11c**) and observation of histopathological changes at the peak of disease after 14 days (**Figure 11e**). Here, mice lost around 10% of their total weight at the start of the experiment (**Figure 11c**) and developed a colitis with an average score of 7 at the time point where they were sacrificed (**Figure 11b**). Specifically, stool consistency was less solid (*), the surrounding organs were no longer visible, indicating thickening of the colon, and the colon wall became granular (x) (**Figure 11d**). Histological examination indicated a disruption of the intestinal crypts and increased cellular infiltration (**Figure 11e**).

First, RNA was extracted from total colon tissue, where inflammation is microscopically and macroscopically visible, to determine whether an up-regulation of *Il22ra2* expression can be seen using RT-PCR techniques. Although the difference in *Il22ra2* gene expression between steady state and inflamed colonic tissue was not significant,

a slight trend showing the up-regulation of *Il22ra2* in the inflamed tissue can be detected (**Figure 11f**).

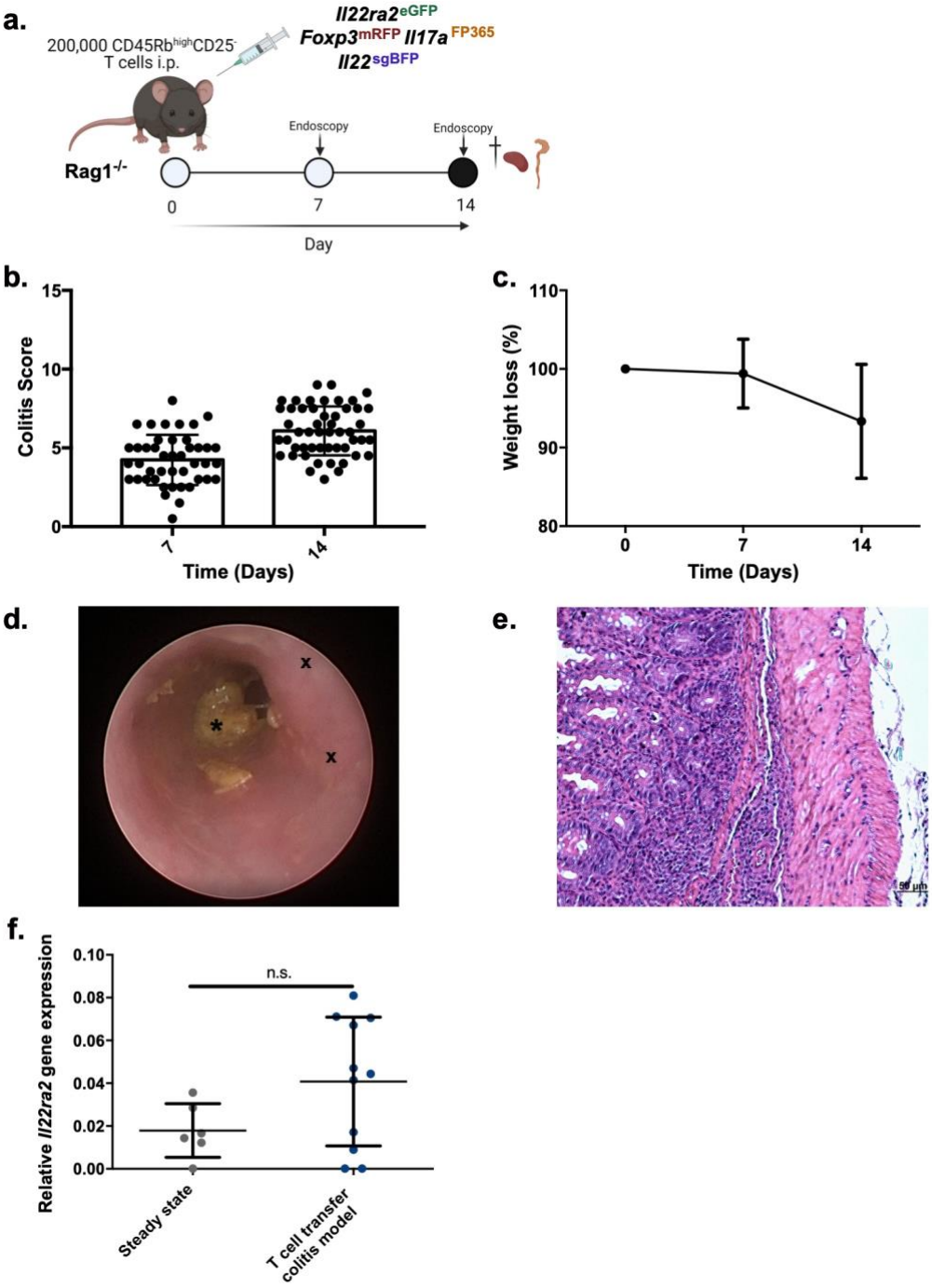


Figure 11: Colitis development in T cell transfer model of colitis **a)** Experimental scheme showing transfer of 200,000 $CD4^+$ $CD25^-$ $CD45RB^{high}$ cells extracted from secondary lymphoid organs of *Il22ra2*^{eGFP}*Foxp3*^{mRFP}*Il17a*^{FP365}*Il22*^{sgBFP} reporter mice into each *Rag1*$^{-/-}$ recipient mouse. Colitis development and disease severity were determined through monitoring of the weight loss and endoscopic procedures before euthanization of mice on day 14. The spleen, colon and lymph nodes were extracted from each mouse and processed for cellular analysis. **b)** Colitis score of recipient mice over the course of disease. **c)** Weight loss of recipient mice over the course of disease. **d)** Representative endoscopic picture indicating stool inconsistency (*) and granularity (x). Thickening of the colon wall can also be observed throughout this section of the colon. **e)** Representative histological specimen. Scale bar indicates 50μm. **f)** RT-PCR of *Il22ra2* (normalized to *Hprt*) extracted from total colon tissue of *Rag1*$^{-/-}$ recipient mouse that had developed colitis (Mann-Whitney Test). Significance tests indicate that comparisons were all non-significant. Bars indicate standard error.

Next, cellular analysis (**Figure 12a**) at the time of euthanization indicated a remarkable increase in CD4^{+} T cells expressing *Il22ra2* in organs extracted from recipient *Rag1*$^{-/-}$ mice that had developed colitis, when compared to frequencies in organs extracted from *Il22ra2*^{eGFP}*Foxp3*^{mRFP}*Il17a*^{FP365}*Il22*^{sgBFP} mice during homeostasis (**Figure 12b**). Whereas the frequencies of CD4^{+} T cells that express *Il22ra2* average at 2% or below in all three organs during steady state, in this model, these cells were drastically up-regulated to above 50% in the spleen and lymph nodes, and around 30% in the colon (**Figure 12b**). Also, the frequency of CD4^{+} T cells in the secondary lymphoid organs was significantly higher than the colon in this model. Surprisingly, *Foxp3*$^{mRFP+}$ T cells displayed a significant down-regulation of *Il22ra2* co-expression frequency in all organs. Specifically, frequencies of *Foxp3*$^{mRFP+}$ T cells co-expressing *Il22ra2* were down-regulated from close to 5% in the spleen and lymph nodes to less than 0.5% (**Figure 12c and 12d**). In the colon, this down-regulation was even more dramatic, as 20% of all CD4^{+} *Il22ra2*$^{eGFP+}$ cells were *Foxp3*$^{mRFP+}$ during steady state, and again less than 0.5% in this colitis model (**Figure 12e**). There were no significant changes in the frequency of *Il17a*$^{FP365+}$ and *Il22*^{sgBFP} co-expressing T cell subsets in any of the organs analyzed in this model (**Figure 12c-e**).

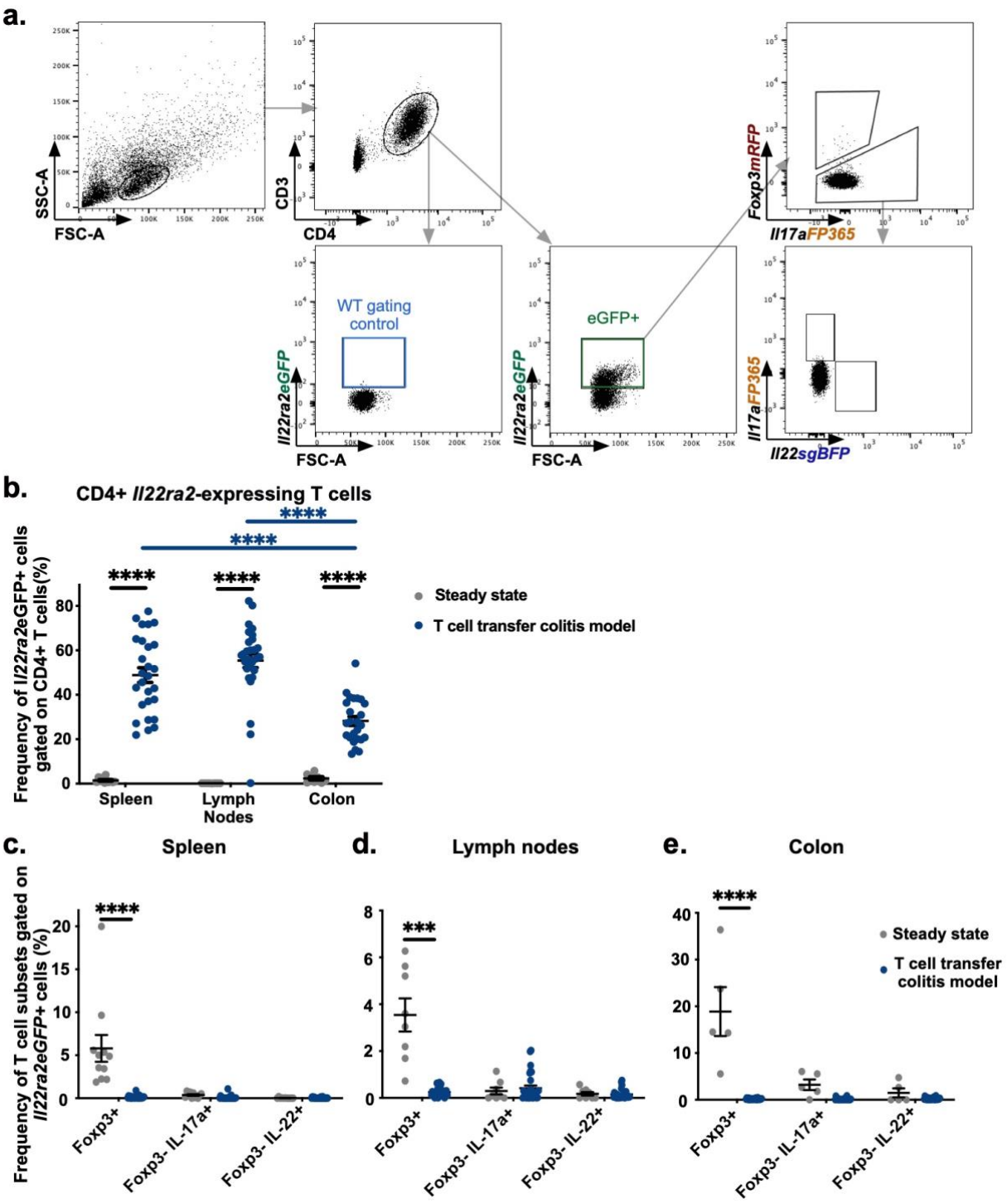


Figure 12: ***Il22ra2 expression* in CD4⁺ T cells is greatly up-regulated in the T cell transfer colitis model a)** Gating strategy for quantification of total CD4⁺ *Il22ra2*$^{eGFP+}$ populations and *Il22ra2*$^{eGFP+}$ T cell subsets. **b)** Frequency of CD4⁺ cells that express *Il22ra2* extracted from spleen, lymph nodes and colon injection (2-way ANOVA). Frequency of CD4⁺ *Il22ra2*$^{eGFP+}$ T helper cell subsets extracted from **c)** spleen, **d)** lymph nodes and **e)** colon (2-way ANOVA). Significance is marked with p = <0.0005 (***) or p = <0.0001 (****), otherwise comparison is non-significant. Bars indicate standard error.

Analysis of *Il22ra2* expression in a CAC model

The protective effects of IL-22BP have been demonstrated in the context of tumorigenesis in the colon in both human studies and mouse CAC models [9,144]. Such studies have provided evidence that dendritic cell-derived IL-22BP is important for modulating excessive and uncontrolled IL-22 signaling within this chronic inflammatory setting that may lead to tumor development. From previous experiments carried out during the course of this project, it has become evident that other cellular sources may be involved in the inflammatory processes occurring in this disease setting. For this reason, *Il22ra2*$^{eGFP+}$ reporter mice were utilized in a CAC model to provide insight into the *Il22ra2* expression patterns in the different relevant cellular sources identified during the steady state.

The experimental set-up for these experiments consisted of an initial intraperitoneal injection of AOM into reporter mice, followed by three subsequent feeding cycles of 2.5% DSS for five days, and normal drinking water for 16 days (**Figure 13a**). Endoscopic analysis indicated the growth of tumors (#) in these mice after three cycles of DSS feeding [181] (**Figure 13b**). Mice were sacrificed on day 68 and were analyzed at a histological and cellular level (**Figure 13c, Figure 14 and Figure 15**). Histological analysis of colonic tumors at the point of euthanasia indicated polyp formation with inflammatory infiltrates and features of neoplasia (**Figure 13c**).

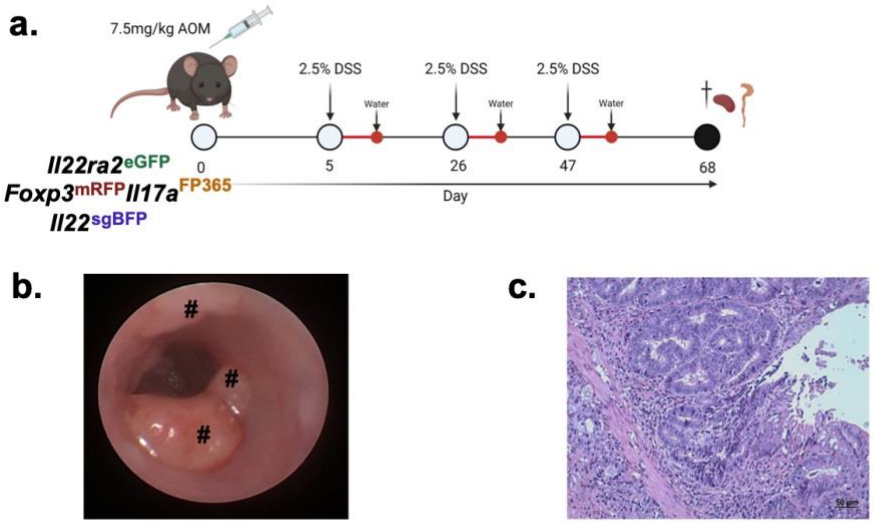


Figure 13: Tumor development in the AOM/DSS Tumor model a) Experimental scheme showing an initial intraperitoneal administration of AOM to *Il22ra2*^{eGFP}*Foxp3*^{mRFP}*Il17a*^{FP365}*Il22*^{sgBFP} reporter mice at a concentration of 7.5mg/kg body weight. After 5 days, mice were fed with 2.5 % DSS via their drinking water for another 5 days, before being given autoclaved water for the following 16 days. This cycle was repeated two more times before the mice were sacrificed. The spleen, lymph nodes, colon and tumors (pooled form several mice for each experiment) were extracted from each mouse and processed for cellular analysis on day 68. Tumor development and disease severity was determined by endoscopic procedures. **b)** Representative endoscopic picture indicating tumor development (#). **c)** Representative histological specimen. Scale bar indicates 50μm.

As this particular inflammatory model was not specifically driven by T cells, unlike the T cell transfer colitis model, different cellular sources of *Il22ra2* expression (**Figure 14a**), as well as specific CD4$^+$ T cell subsets, could be studied (**Figure 15a**).

An intriguing pattern that is worth mentioning is the consistent and significant down-regulation in the proportion of CD11c$^+$ cells in organs extracted from tumor-bearing mice, compared to steady state counterparts. For example, the frequency of CD11c$^+$ cells in the spleen is close to 20% during homeostasis but is only around 15% in tumor-bearing mice (**Figure 14b**). The down-regulation of *Il22ra2* expression in this particular cell type is even more apparent in the lymph nodes and colon, where frequencies close to 40% are drastically reduced to around 10% (**Figure 14c and 14d**). Another interesting finding in this data set is that the frequency of *Il22ra2*-expressing CD3$^-$ NK1.1$^+$ (otherwise referred to as NK cells) is significantly reduced in proportion, from 40% to around 12% in the spleen, whereas the opposite is seen in this cell type in the colon. In this particular organ, only 7% of *Il22ra2*-expressing CD3$^-$ NK1.1$^+$ cells are present in steady state, whereas this doubles in frequency during colitis-associated inflammation (**Figure 14b and 14d**).

In the colon, the greatest differences in *Il22ra2*-expressing cellular sources were observed compared to the steady state condition (**Figure 14d**). It is here that a significant up-regulation of $CD4^+$ T cell-derived *Il22ra2* expression is seen, where the frequency jumps from 5% during the steady state to around 20% in tumor-bearing mice.

Lastly, out of all the cell types analyzed in this model, $CD3^-$ $NK1.1^+$ cells are the predominant cell type expressing *Il22ra2* in the draining lymph nodes and colon (**Figure 14c and 14d**). Another interesting finding in this model, is that the frequency of $CD4^+$ T cells expressing *Il22ra2* is significantly higher than the frequency of $CD11c^+$ cells in both these organs. This is the first time that a higher frequency of $CD4^+$ T cell-derived *Il22ra2* expression has been seen in any data set in terms of frequency (**Figure 14c and 14d**).

A caveat of studying the tumors that develop in this model is that the frequencies of cells extracted from tumor tissues have to be statistically compared to those of the colon, as these samples would not exist during the steady state (**Figure 14e**). Another issue is that very few cells can be extracted from each tumor growth at a given time point. For this reason, tumors from each experiment consisting of around ten mice were pooled together, and $CD3^-$ $NK1.1^+$ and $CD3^+$ $NK1.1^+$ T cells were only studied in one experiment out of three. There were no significant differences in frequencies between the tumor tissue and non-tumor tissue in this model.

In conclusion, it appears that the cellular sources making up the largest frequency of *Il22ra2*$^+$ cells in the colon are $CD4^+$ T cells and $CD3^-$ $NK1.1^+$ cells in this model. Although IL-22BP derived from $CD11c^+$ cells has been proven to be critical in controlling IL-22 signaling and tumor development [9], it does not seem to be the cellular source expressing the highest frequency of *Il22ra2*$^+$ cells in this model, and are even significantly down-regulated in most organs, when compared to steady state (**Figure 14b-d**).

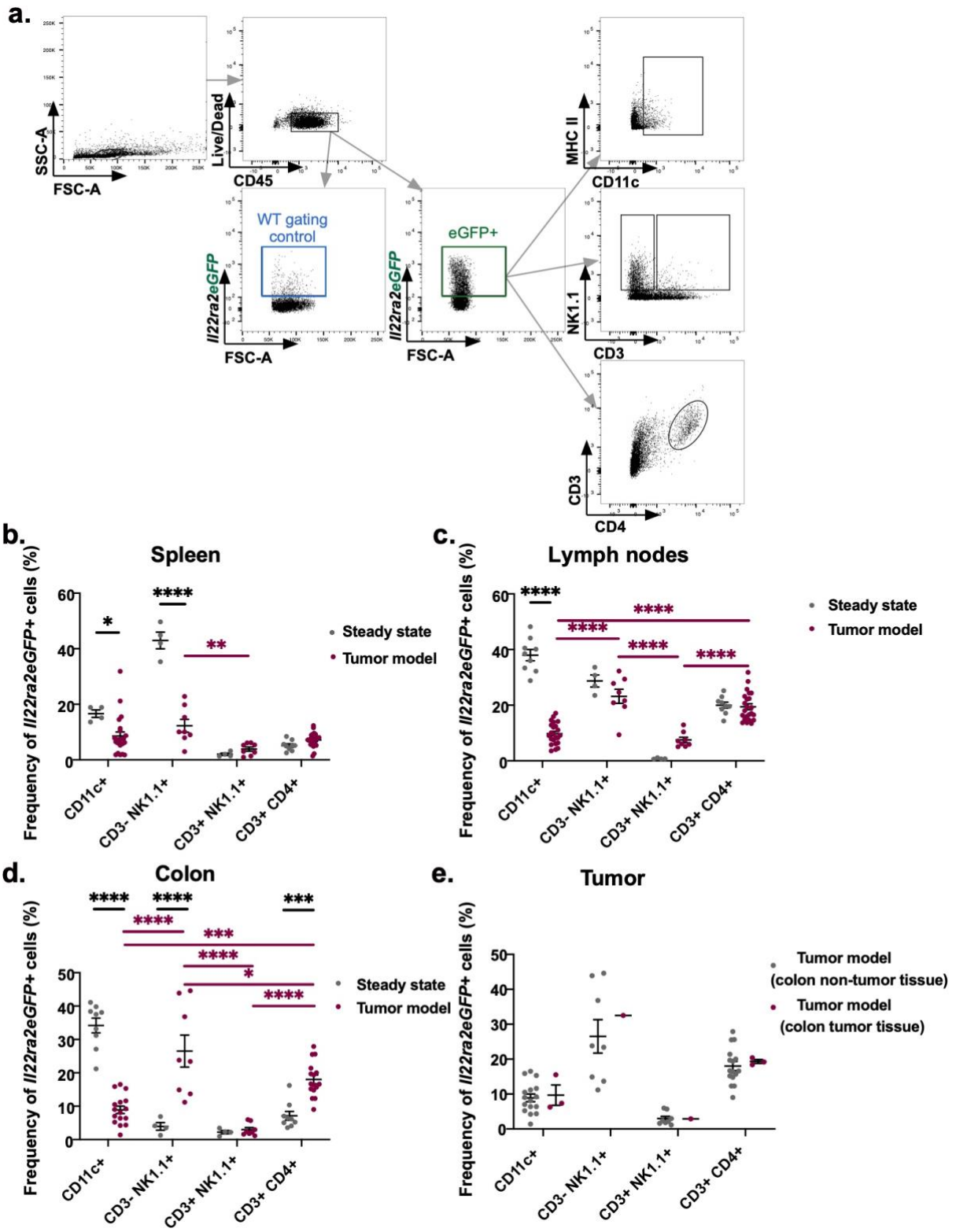


Figure 14*: Il22ra2* derived from CD4⁺ T cells and NK cells may play a role in the AOM/DSS tumor model a) Gating strategy for quantification of total *Il22ra2*$^{eGFP+}$ populations. Frequency of cell types that express *Il22ra2* extracted from **b)** spleen, **c)** lymph nodes, **d)** colon and **e)** tumors (2-way ANOVA). Significance is marked with p = <0.05 (*), p = <0.005 (**), p = <0.0005 (***) or p = <0.0001 (****), otherwise comparison is non-significant. Bars indicate standard error.

Next, specific $CD4^+$ T helper cell subsets were further analyzed in this mouse CAC model. Here, the frequency of *Foxp3*$^{mRFP+}$ T cells co-expressing *Il22ra2* remained similar to those displayed during the steady state, however, a significant increase from 3% to 8% was observed in this subset in the lymph node samples (**Figure 15c**). The frequencies of *Foxp3*$^{mRFP+}$ T cells were, again, significantly higher than *Il17a*$^{FP365+}$ and *Il22*^{sgBFP} co-expressing T cell subsets in this model in all organs except for in tumor tissues (**Figure 15b-e**). Differences in frequencies of *Il17a*$^{FP365+}$ and *Il22*^{sgBFP} $CD4^+$ T cells were negligible in all organs (**Figure 15b-e**). Once again, there were no significant differences in frequencies between the tumor tissue and non-tumor tissue in this model (**Figure 15e**).

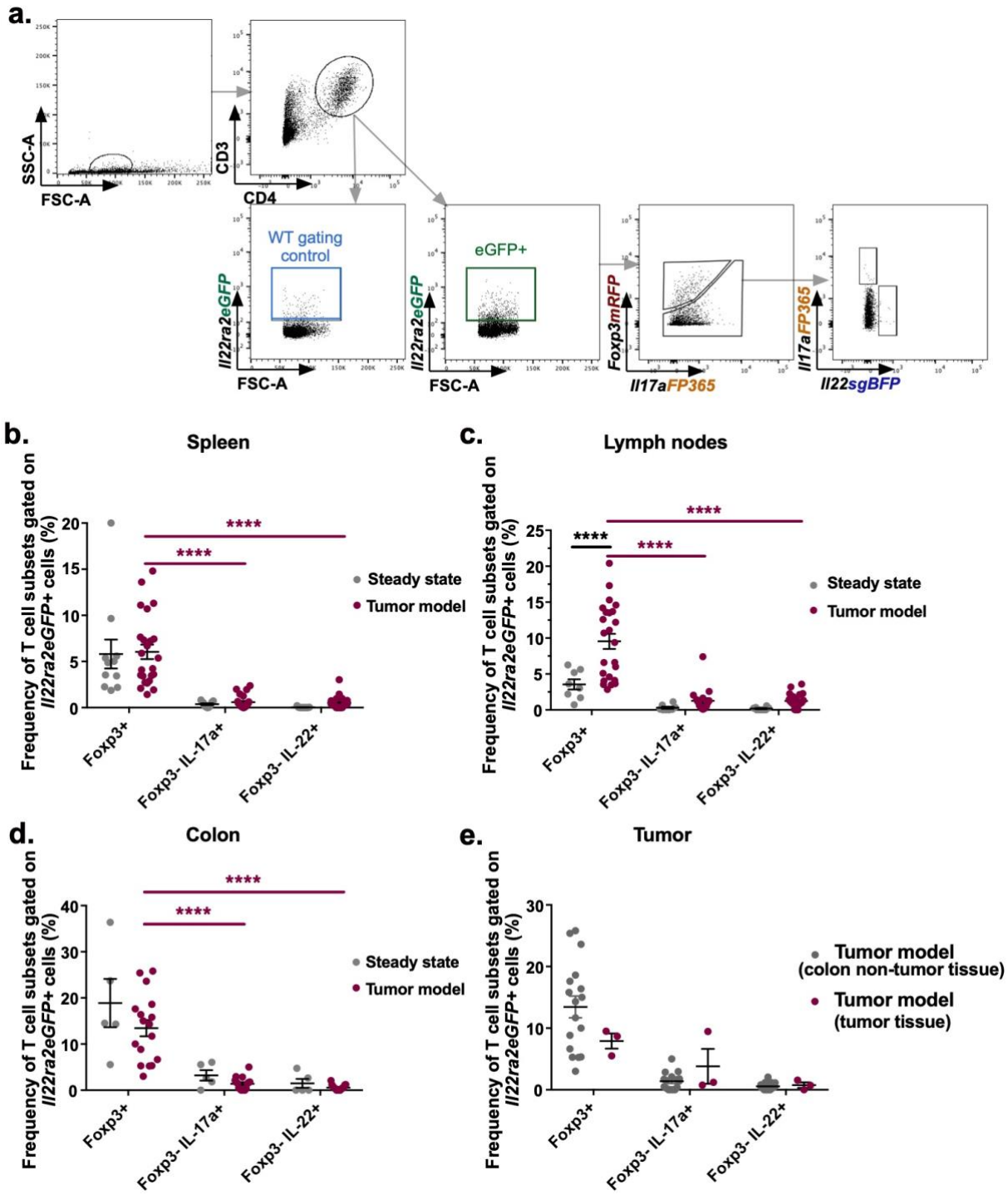


Figure 15: Frequency of *Il22ra2* derived from specific CD4⁺ T subsets in the AOM/DSS tumor model a) Gating strategy for quantification of *Il22ra2*$^{eGFP+}$ T cell subsets. Frequency of CD4⁺ *Il22ra2*$^{eGFP+}$ T helper cell subsets extracted from **b)** spleen, **c)** lymph nodes, **d)** colon and **e)** tumors (2-way ANOVA). Significance is marked with p = <0.0001 (****), otherwise comparison is non-significant. Bars indicate standard error.

Different cell types are responsible for *Il22ra2* expression in the *Plasmodium berghei ANKA* infection model

In addition to investigating the cellular sources and transcriptional regulation of *Il22ra2*-expressing cells in mouse models of colitis, it is important to identify whether the same cell types and transcriptional networks can govern *Il22ra2* expression in other organs, and in a different inflammatory setting, such as infection. From previous experiments carried out on human PBMCs, high frequencies of IL-22BP-producing $CD4^+$ T cells were observed, particularly in PBMCs isolated from IBD patients (**Figure 10**). Further experiments in mice proved that identifying and obtaining *Il22ra2*-expressing $CD4^+$ T cells in mouse blood was also possible (**Figure 3e**), with the added advantage that the mice did not have to be sacrificed for the purpose of cell acquisition. The following experiments investigated the distribution of *Il22ra2*-expressing cells in inflammatory circumstances that directly affect the blood. To this end, the mouse model of malaria infection, initiated through the parasite *Plasmodium berghei* ANKA, was applied to the *Il22ra2*^{eGFP} reporter mouse.

Known cellular sources of *Il22ra2* were investigated for changes in gene expression patterns over the course of the disease; before infection (Day 0), at a time point when parasitemia can be observed in blood smears (after Day 4), and when mice show signs of cerebral malaria and must be euthanized (Day 6) (**Figure 16a**). Isolation of cells from organs that were affected by *Plasmodium berghei* ANKA infection revealed specific trends of *Il22ra2* expression in these cell types (**Figure 16b**).

In this model, the spleen and liver are directly affected by the parasites circulating in the blood [197]. When analyzing expression patterns over the course of the disease in the spleen, a significant down-regulation of *Il22ra2*-expression frequency was observed in $CD11c^+$ cells, where the average frequency of cells dropped significantly from over 30% to 10% (**Figure 16c**). Again, a decreasing frequency of $CD11c^+$ cells expressing *Il22ra2* was seen in the blood, although this particular cell type was observed to be slightly up-regulated on Day 4 and then down-regulated again on Day 6 (**Figure 16e**). A similar trend in expression levels was seen in the liver, but in this case, there was only a significant up-regulation on Day 4, but not a noteworthy down-regulation on Day 6 (**Figure 16d**).

Interestingly, the frequency of $CD4^+$ T cells expressing *Il22ra2* was observed to be on an upward trend in the spleen and liver, and even significantly up-regulated in the blood of infected mice (**Figure 16e**). The frequency of this cell type in the blood was dramatically increased from 1% to nearly 20% on Day 6.

Similarly, the frequency of $CD8^+$ T cells expressing *Il22ra2* was also increased when comparing steady state to Day 6 of infection (**Figure 16c-e**). Once again, this up-regulation was strongest in the blood, where total $CD8^+$ *Il22ra2*$^+$ T cells increased remarkably in frequency, from less than 1% to 10% (**Figure 16e**). Lastly, the frequencies of $CD19^+$ B cells expressing *Il22ra2* showed no significant changes in the different organs analyzed.

In conclusion, this time course experiment has provided an insight into how different cellular sources react to mouse malaria infection, indicating that $CD11c^+$ cell expression of *Il22ra2* is significantly down-regulated, whereas expression from $CD4^+$ T cell is up-regulated. However, other cell types that have previously been identified to be major cellular sources of *Il22ra2*, such NK cells, macrophage populations, as well as distinct $CD4^+$ T helper cell subsets, were not considered in this analysis. These cell types should also be investigated in the future to provide more insight into the impact of *Il22ra2*-expressing cells in this model.

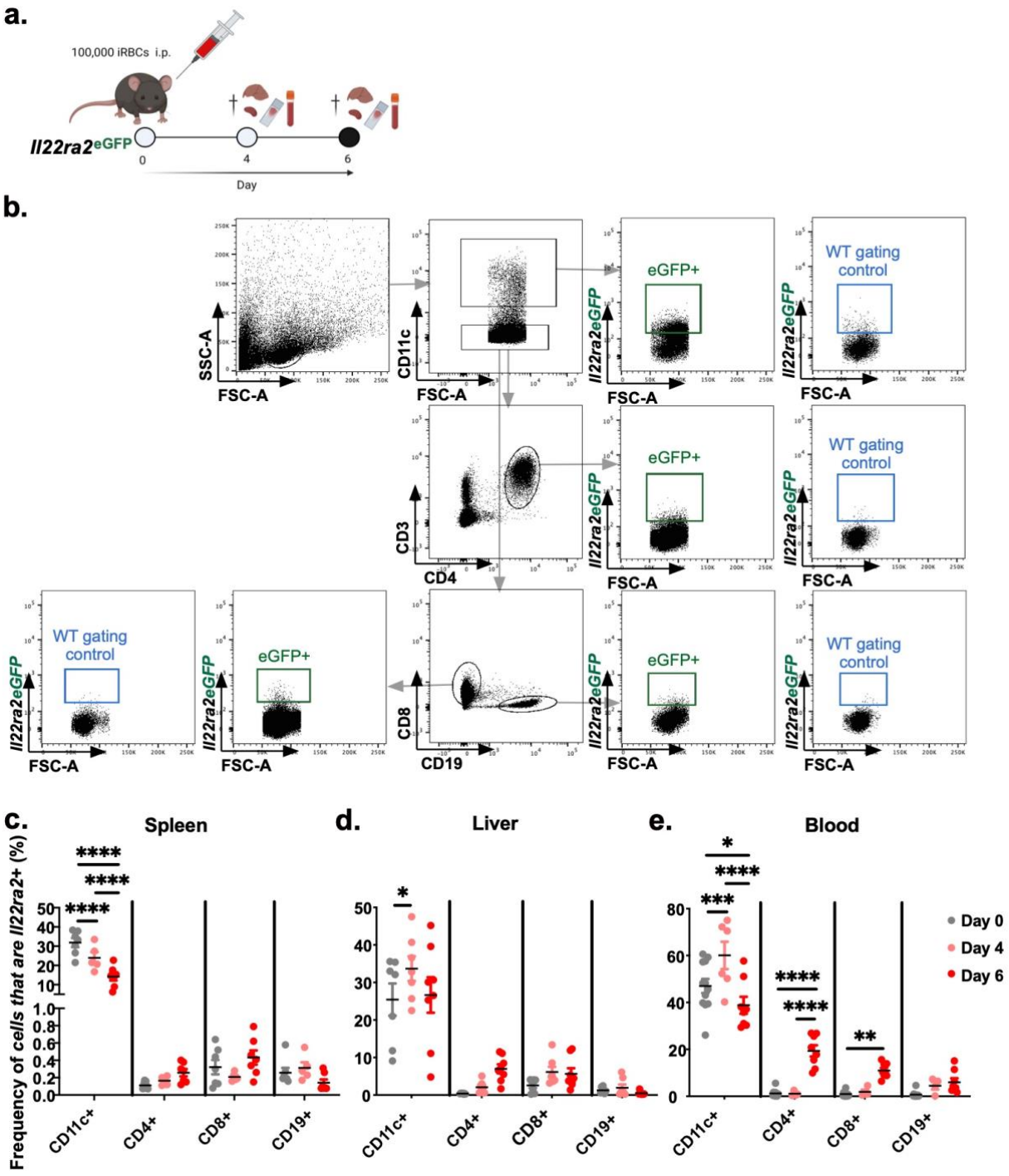


Figure 16: Frequency of *Il22ra2* expression changes in different cell types during *Plasmodium berghei* ANKA infection a) Experimental scheme showing infection of *Il22ra2*^{eGFP} reporter mice with 100,000 iRBCs and course of disease progressing for 6 days. Mice were sacrificed on Day 0, 4 and 6 for cellular analysis purposes. Successful infection was determined through blood smears via quantification of parasitemia and spleen, liver and blood were extracted from mice at time point of euthanization. **b)** Gating strategy for quantification of total *Il22ra2*$^{eGFP+}$ populations. Frequency of cell types that express *Il22ra2* extracted from **c)** spleen, **d)** liver and **e)** blood (2-way ANOVA). Significance tests compared quantified values at Day 0 to Day 4 and Day 6 for each cell type in each organ. Significance is marked with p = <0.05 (*), p = <0.005 (**), p = <0.0005 (***) or p = <0.0001 (****), otherwise comparison is non-significant. Bars indicate standard error.

Depletion of *Il22ra2* does not result in significant phenotypical changes in the *Plasmodium berghei* ANKA infection model

Next, the *Plasmodium berghei* ANKA infection model was applied to *Il22ra2*$^{-/-}$ mice to analyze whether the relative expression of the *Il22ra2* gene has an impact on malaria disease progression, as this has never been investigated before. To this end, *Il22ra2*-deficient and -sufficient littermate mice were infected with 100,000 fresh infected red blood cells (iRBCs), intraperitoneally (**Figure 17a**). Littermate mice are defined as mice born in the same litter but with differing genotypes and are used to prevent confounding factors such as microbiota, from influencing disease development. The mice were surveilled over the course of the disease for 7 days before being euthanized.

In general, weight loss curves indicated that both groups lost weight within the experimental course and developed some cerebral symptoms, indicating that the infection procedure was successful. Specifically, in this experimental set-up, nearly 50% of wild type mice were euthanized due to their symptoms on Day 6. On the other hand, most of the *Il22ra2*-deficient mice survived until the experimental end-point on Day 7. However, the difference between the two groups is statistically insignificant (**Figure 17b**). Additionally, wild-type mice had higher cerebral malaria scores, both on Day 6 and Day 7 (**Figure 17c**) and a higher parasitemia at the peak of disease by trend (**Figure 17e**), although both these parameters did not reach statistical significance. Interestingly, a significant difference could be observed in the weight loss of the two groups on Day 6, where wild type mice lost more weight than the *Il22ra2*-deficient mice (**Figure 17d**). To conclude, although *Il22ra2*-deficient mice presented a slight delay in development of malaria-related symptoms and prolonged survival rates compared to their wild type counterparts, there does not seem to be major differences in the course of infection dependent on *Il22ra2* expression.

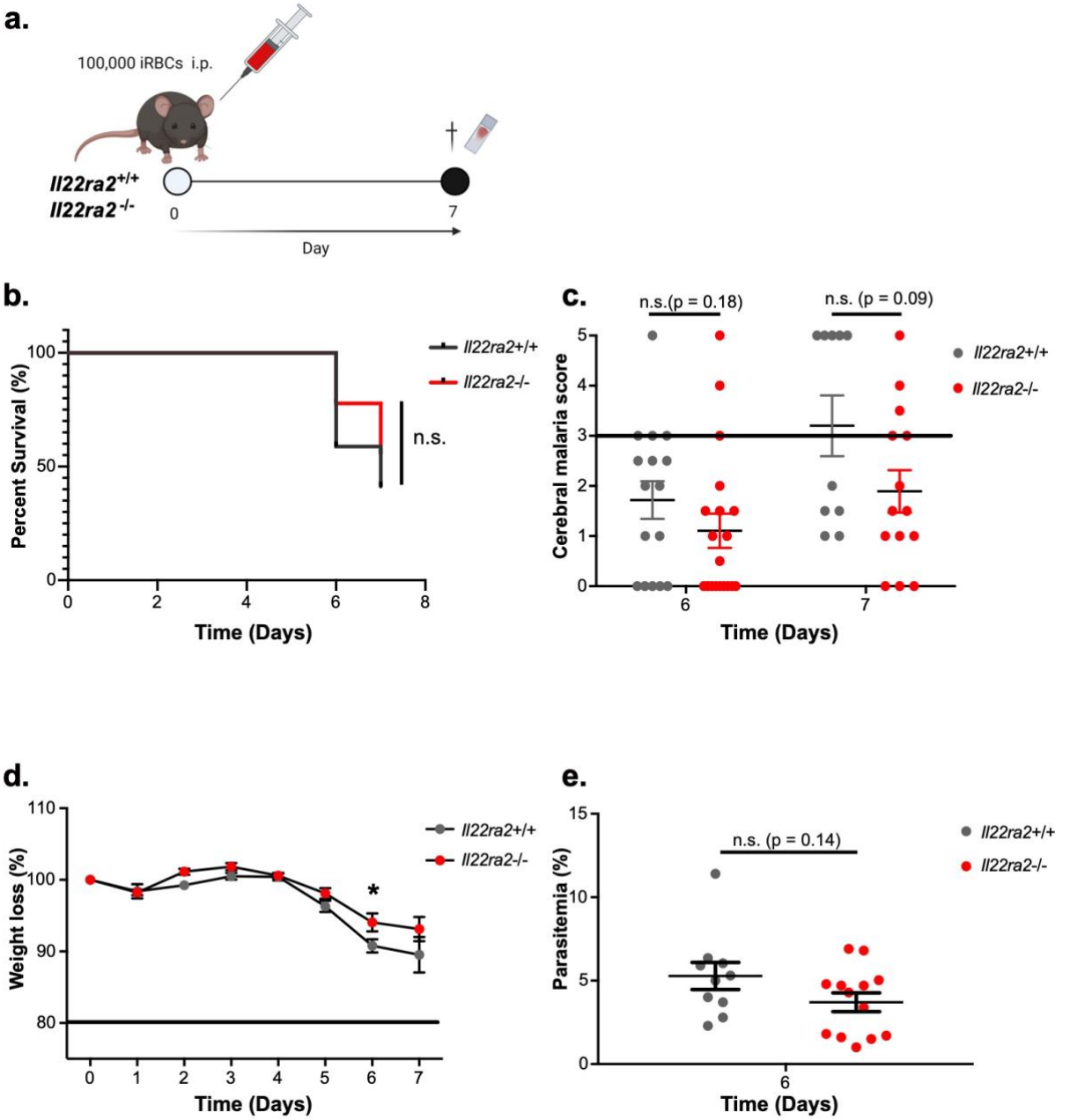


Figure 17: Disease development is comparable between *Il22ra2*-deficient and wild type mice in the *Plasmodium berghei* ANKA infection model **a)** Experimental scheme showing infection of *Il22ra2*$^{+/+}$ and *Il22ra2*$^{-/-}$ with 100,000 iRBCs and course of disease progressing for 7 days. Mice were sacrificed if they developed severe malaria-related symptoms or otherwise on Day 7 after infection. Successful infection was determined through blood smears via quantification of parasitemia. **b)** Survival curve of *Il22ra2*$^{+/+}$ and *Il22ra2*$^{-/-}$ mice after infection (Gehan-Breslow-Wilcoxon test). **c)** Cerebral malaria score of *Il22ra2*$^{+/+}$ and *Il22ra2*$^{-/-}$ determined on Day 6 and Day 7. Mice were sacrificed if they received a score of >3 (2-way ANOVA). **d)** Weight loss of *Il22ra2*$^{+/+}$ and *Il22ra2*$^{-/-}$ mice over the course of infection. **e)** Quantification of parasitemia in *Il22ra2*$^{+/+}$ and *Il22ra2*$^{-/-}$ mice on Day 6 (Mann-Whitney test). Significance for difference in survival curves was determined using the. Significance is marked with p = <0.05 (*), otherwise comparison is non-significant. Bars indicate standard error.

Transcriptional profile of *Il22ra2*-expressing T cells during steady state

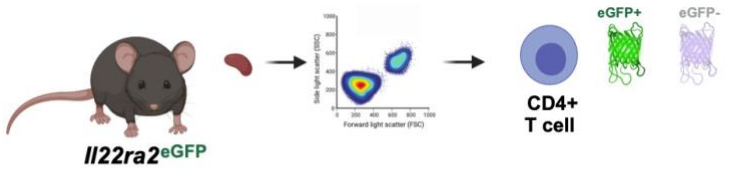
The last aim of this project was to identify the transcriptional regulation of CD4$^+$ T cell-derived *Il22ra2* expression. For this purpose, bulk RNA sequencing techniques and biostatistical analysis were used to identify genes that were significantly up-regulated in CD4$^+$ *Il22ra2*$^{eGFP+}$ samples compared to CD4$^+$ *Il22ra2*$^{eGFP-}$ samples extracted from the spleen and lymph nodes of steady state *Il22ra2*^{eGFP} reporter mice (**Figure 18a**). These CD4$^+$ cells were isolated through FACS using the same gating strategy as **Figure 6b**.

Differentially regulated genes depicted in a volcano plot provide insight into which genes are up-regulated or down-regulated in CD4$^+$ *Il22ra2*$^{eGFP+}$ cells (**Figure 18b**). Complete lists containing all differentially expressed genes have been included in the appendix of this thesis. Fundamentally, this plot validates the use of the reporter mouse for sorting CD4$^+$ *Il22ra2*$^{eGFP+}$ cells, as these GFP$^+$ samples did indeed have significantly higher *Il22ra2* gene expression, compared to CD4$^+$ *Il22ra2*$^{eGFP-}$ samples. The genes *Lgals1* and *Lgals3*, encoding galectins, and *Anxa1* and *Anxa6*, encoding annexins, were the most significantly up-regulated genes in this analysis. Significant T cell-related genes that were up-regulated include *Itga2, Il18r1, Gzma*, and *Klra4*. The most significantly down-regulated genes include *Ephx1,* an epoxide hydrolase, and *Izumo1r*, a gene often associated with Tregs [194] [196]. The transcription repressor gene, *Plagl1*, is included in the list of the most significantly down-regulated genes. In addition, *Cd83*, an immunosuppressive molecule when expressed on T cells [198], and the co-stimulatory molecule, *Cd27,* which enhances T cell activation and promotes survival of these cells [199], were also included in this list.

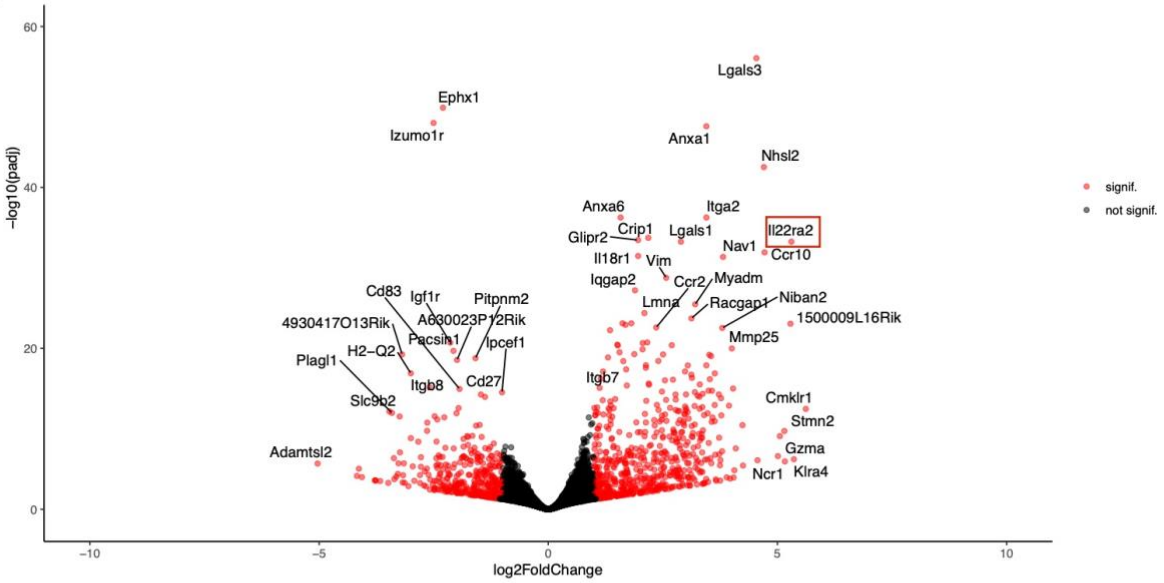
Furthermore, gene ontology analysis of this data set provided insight as to which specific biological processes these genes regulate. Down-regulated genes were shown to be related to promoting immunoglobulin production and T cell-mediated toxicity (**Figure 18c**). Interestingly, the most significantly up-regulated genes in this data set all indicate that *Il22ra2*$^+$ CD4$^+$ T cells highly express cell cycle genes and are metabolically active (**Figure 18d**).

Next, a list of potential transcription factors whose gene expression levels were significantly up-regulated were identified within this data set. Many IFNγ-inducible and cell cycle regulating (E2f family) transcription factors are up-regulated. It is noteworthy that some common T cell-related transcription factors are also up-regulated, including *Rxra*, *Rora* and *Foxm1* (**Figure 18e**).

a.



b.



c.

Down-regulated biological processes
positive regulation of peptidyl-tyrosine phosphorylation
regulation of immunoglobulin production
response to thyroid hormone
positive regulation of leukocyte mediated cytotoxicity
positive regulation of T cell mediated cytotoxicity
positive regulation of protein tyrosine kinase activity
regulation of cartilage development
positive regulation of lymphocyte proliferation
regulation of calcium ion-dependent exocytosis
positive regulation of natural killer cell mediated cytotoxicity

d.

Up-regulated biological processes
mitotic sister chromatid segregation
mitotic cell cycle phase transition
mitotic spindle organization
DNA metabolic process
microtubule cytoskeleton organization involved in mitosis
DNA replication
metaphase plate congression
G1/S transition of mitotic cell cycle
mitotic nuclear division
sister chromatid segregation

e. Up-regulated transcription factors

Flna	Hlf	Klf12
E2f2	E2f1	Mybl1
Rxra	Fancd2	Ifi205
Mxd3	Runx3	Ifi207
E2f8	**Pycard**	Ifi204
Cmklr1	Cdkn2a	Msc
E2f7	Nfam1	Ifi211
Rora	Foxm1	Zeb2
Runx2	**Hmgb2**	Trp73
Mybl2	Ar	

Figure 18: Transcriptional profile of *Il22ra2*-expressing T cells during steady state

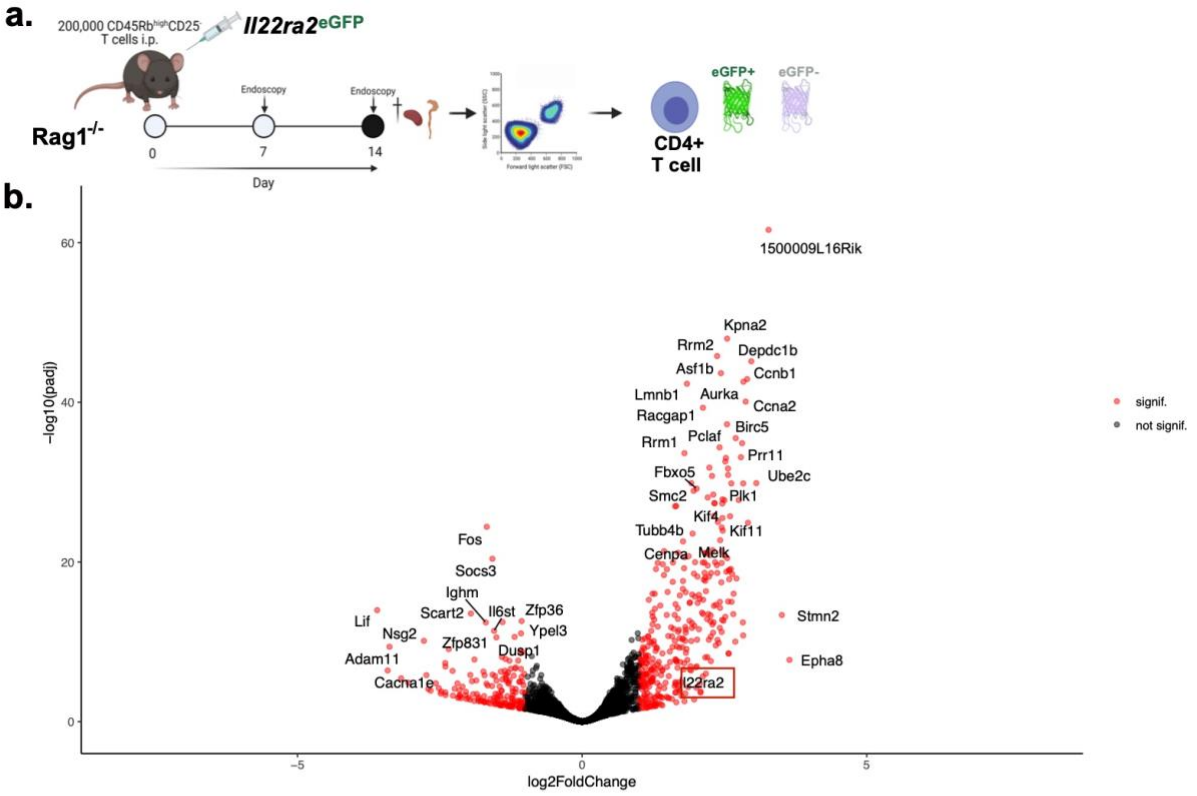
a) Experimental scheme showing extraction of a pure population of CD4$^+$ *Il22ra2*$^{eGFP+}$ and CD4$^+$ *Il22ra2*$^{eGFP-}$ T cells from the spleens of *Il22ra2*^{eGFP} reporter mice during steady state. Cells were lysed and RNA was extracted before sequencing. **b)** Volcano plot comparing CD4$^+$ *Il22ra2*$^{eGFP-}$ (left side) to CD4$^+$ *Il22ra2*$^{eGFP+}$ (right side) samples indicating significantly down-regulated (left side) and up-regulated (right side) differentially expressed genes (|Log2FC| > 1 and (Padj) < 0.05 for up-regulated genes and |Log2FC| < -1 and (Padj) < 0.05 for down-regulated genes). The gene *Il22ra2* is highlighted in a red box. **c)** Gene ontology describing the biological processes related to down-regulated differentially expressed genes. **d)** Gene ontology describing the biological processes related to up-regulated differentially expressed genes. **e)** List of all up-regulated transcription factors in *Il22ra2*$^+$ cells. Genes highlighted in bold red are transcription factors that are found in the list of up-regulated genes in cells extracted during homeostasis and in disease models.

Transcriptional profile of *Il22ra2*-expressing T cells during T cell transfer colitis

Next, the T cell transfer colitis model was chosen to study the transcriptional regulation of *Il22ra2*$^{+}$ T helper cells exposed to inflammatory conditions. This model was specifically chosen as *Il22ra2* expression is considerably up-regulated in CD4^{+} T cells of patients suffering from IBD, and because this model was previously shown to promote a pathogenic effect of CD4^{+} T cell derived-IL-22BP production [135]. Additionally, this model was observed to induce the highest frequency of CD4^{+} *Il22ra2*-expressing cells out of all the models investigated within this project (**Figure 12b**). For this purpose, CD4^{+} *Il22ra2*$^{eGFP-}$ and CD4^{+} *Il22ra2*$^{eGFP+}$ cells were isolated from the spleen of mice that had developed a colitis score of 7 or higher and compared for significant gene differences (**Figure 19a**). These cells were isolated through FACS using the same gating strategy as **Figure 12a**.

The volcano plot depicting differentially expressed genes shows a significant down-regulation of genes that are associated with Tregs, namely *Izumo1r* and *Lif*, and genes that are related to T_h17, such as *Il21*, *Il23r* and *Il17rb* (**Figure 19b**). On the right side of the volcano plot, the significantly up-regulated genes in the CD4^{+} *Il22ra2*$^{eGFP+}$ samples are predominantly associated with cell cycle genes. These include *Ccnb1*, *Ccna2*, *Aurka*, *Plk1*, *Pclaf*, *Kif11*, *Kif4*, *Melk* and *Fbxo5*. Following on, there is also an up-regulation of DNA synthesis genes (*Rrm1 and Rrm2*) and histone-related genes (*Asf1b, Cenpa*), indicating that these cells are in the process of replicating [200,201]. Lastly, *Il22ra2* is once again significantly up-regulated in CD4^{+} *Il22ra2*$^{eGFP+}$ when compared to CD4^{+} *Il22ra2*$^{eGFP-}$ cells.

The gene ontology analyses of these samples define the down-regulated genes to be involved in biological processes related to tyrosine phosphorylation of STAT proteins and cell chemotaxis (**Figure 19c**). As already described from the specific genes in the volcano plot, the significantly up-regulated genes are associated with cell replication and DNA repair (**Figure 19d**). Finally, the transcription factors that may potentially induce *Il22ra2*-expression under T cell transfer colitis conditions were analyzed (**Figure 19e**). Many genes were found to be involved in DNA repair, including *Nucks1*, *Rad21*, *Foxm1*, *E2f2*, *E2f7*, *E2f8*, and *Hmgb2*. Also, transcriptional repressors such as *Msc*, *Mxd3*, *E2f7*, *E2f8* and *Ezh2* were identified.



c.

Down-regulated biological processes
positive regulation of tyrosine phosphorylation of STAT protein
regulation of tyrosine phosphorylation of STAT protein
cellular response to cytokine stimulus
cell chemotaxis
calcium-mediated signaling
cytokine-mediated signaling pathway
positive regulation of peptidyl-tyrosine phosphorylation
ruffle organization
myeloid leukocyte differentiation
negative regulation of cell adhesion

d.

Up-regulated biological processes
microtubule cytoskeleton organization involved in mitosis
mitotic spindle organization
mitotic sister chromatid segregation
DNA metabolic process
sister chromatid segregation
mitotic cell cycle phase transition
mitotic nuclear division
DNA repair
DNA replication
positive regulation of cell cycle process

e. Up-regulated transcription factors

Msc	**E2f2**
Mxd3	Mybl1
Foxm1	Nucks1
E2f8	Rad21
Mybl2	Ezh2
Hmgb2	**Pycard**
Cmklr1	Vax2
Cdkn2a	Anxa4
E2f7	

Figure 19: Transcriptional profile of *Il22ra2*-expressing T cells during T cell transfer colitis a) Experimental scheme showing extraction of CD4$^+$ *Il22ra2*$^{eGFP-}$ and CD4$^+$ *Il22ra2*$^{eGFP+}$ T cell populations from the spleens of *Rag1*$^{-/-}$ mice that had previously received CD4$^+$ CD25$^-$ CD45RB^{high} cells from *Il22ra2*^{eGFP} reporter mice and developed colitis. **b)** Volcano plot comparing CD4$^+$ *Il22ra2*$^{eGFP-}$ cells (left side) to CD4$^+$ *Il22ra2*$^{eGFP+}$ cells (right side) indicating significantly down-regulated (left side) and up-regulated (right side) differentially expressed genes (|Log2FC| > 1 and (Padj) < 0.05 for up-regulated genes and |Log2FC| < -1 and (Padj) < 0.05 for down-regulated genes). The gene *Il22ra2* is highlighted in a red box. **c)** Gene ontology describing the biological processes related to down-regulated differentially expressed genes. **d)** Gene ontology describing the biological processes related to up-regulated differentially expressed genes. **e)** List of all up-regulated transcription factors in *Il22ra2*$^+$ cells. Genes highlighted in bold red are transcription factors that are found in the list of up-regulated genes in cells extracted during homeostasis and in disease models.

Transcriptional profile of *Il22ra2*-expressing T cells during *Plasmodium berghei* ANKA infection

Lastly, the *Plasmodium berghei* ANKA infection model was also utilized as a means of studying *Il22ra2*-expressing CD4$^+$ T cells. Previous experiments have revealed an up-regulation in the frequency of CD4$^+$ T cells that express *Il22ra2* over the course of infection, where this was even significant in the blood (**Figure 16c-e**). Comparisons between the characteristic genes and transcription factors up-regulated in this data set with those of cells extracted from the steady state and T cell transfer colitis model, will aid in identifying potential genes responsible for regulating the expression of *Il22ra2* in CD4$^+$ T cells.

For this experimental set-up, CD4$^+$ *Il22ra2*$^{eGFP-}$ and CD4$^+$ *Il22ra2*$^{eGFP+}$ cells were extracted from the spleen of *Il22ra2*^{eGFP} reporter mice on day 6 post *Plasmodium berghei* ANKA infection (**Figure 20a**). Reporter negative and positive CD4$^+$ T cells were isolated through FACS using the same gating strategy as **Figure 6b**. Biostatistical analysis comparing these two samples revealed differentially expressed genes that were plotted in a volcano plot (**Figure 20b**), in a similar feat to the steady state and T cell transfer colitis sequencing data set.

The down-regulated genes depicted on the left side of the volcano plot show many transcriptional repressors, such as *Bcl6*, *Id3* and *Ikzf4*, as well as neuron-related genes, including *Gpm6b*, *Nsg2*, *Ntrk3* and *Tox2* (**Figure 20b**). Many genes related to receptor tyrosine kinases are also listed. Interestingly, three different Toll-like receptor genes are included in this list of down-regulated genes, including *Tlr1*, *Tlr7* and *Tlr12*. Similar to the previous data set, genes that are associated with Tregs were also listed, including *Foxp3*, *Ikzf4* and *Izumo1r*. Particularly, *Izumo1r*, which is associated with T cell anergy and is highly expressed in Foxp3$^+$ Tregs [194] [196], is a common gene down-regulated in all three data sets (**Figure 18b, 19b and 20b**).

The characteristically up-regulated genes visualized in this volcano plot are related to lymphocyte migration, for example, *Itga2*, *Ly6g*, *Ccr5* and *Cxcr6*, and indicate that CD4^{+} *Il22ra2*$^{eGFP+}$ cells may have cytotoxic functions, indicated with genes such as *Gzmb*, *Nkg7* and *Ifng*. Once again, *Il22ra2* and *Ifng* are revealed to be co-expressed on the same cell, even under inflammatory conditions. Next, many genes related to apoptosis are also up-regulated, however, there is disparity on whether these CD4^{+} *Il22ra2*$^{eGFP+}$ cells are more pro- or anti-apoptotic. For example, *Gzmb*, *Lgals1* and *Lgals7* promote apoptosis, whereas *Anxa1*, *Lgals3* and *Birc5* are anti-apoptotic. Another interesting observation is that the cytotoxic gene *Gzmb* is up-regulated, but genes associated with inhibition of this protein, namely, *Serpinb9* and *Serpinb9b*, are also significantly up-regulated.

It is noteworthy that all three bulk sequencing data sets have common significantly up-regulated genes. These include the galectin *Lgals1*, a strong inducer of T cell apoptosis [202,203], and *1500009L16Rik*, a gene found to be overexpressed in colon cancer [204].

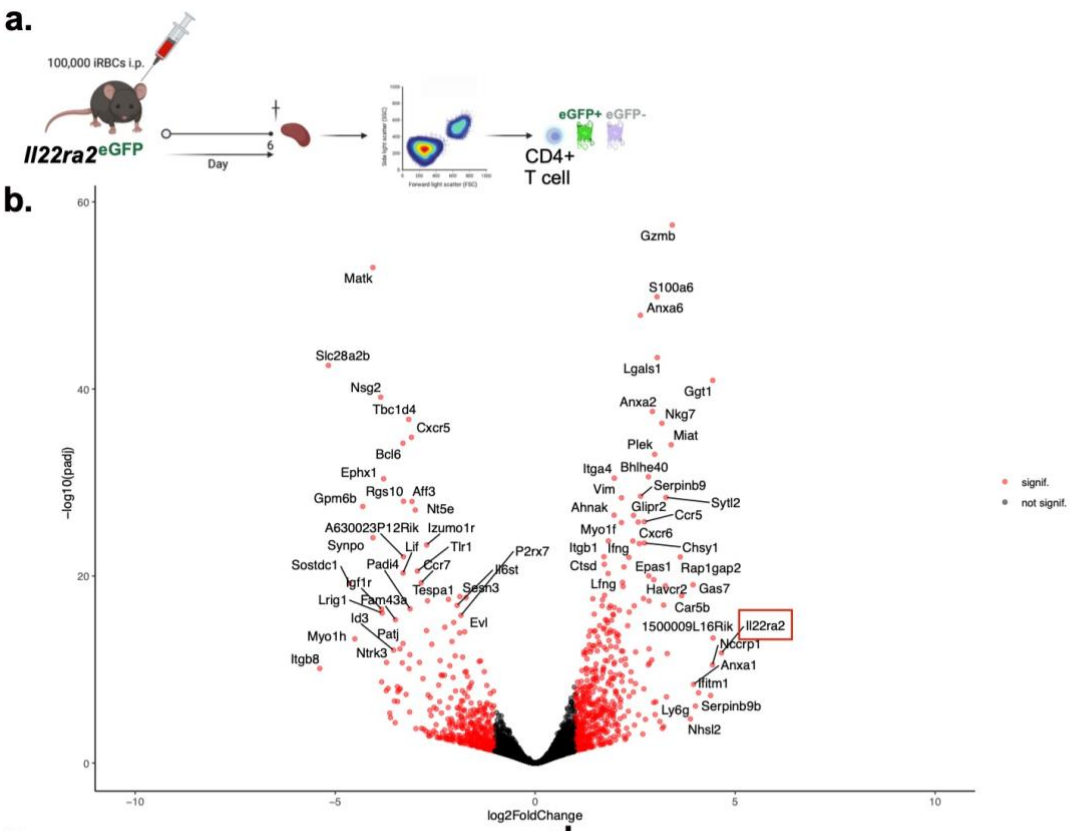
Other common genes in cells extracted from the steady state and this model include the annexins *Anxa1*, *Anxa6*, and the galectin *Lgals3*. Also, the genes *Itga2*, *Glipr2*, *Crip1*, *Vim* and *Il18r1* are common between these two data sets (**Figure 18b and 20b**). Once again, up-regulation of *Il22ra2* is visible and verifies the use of the reporter mouse (**Figure 20b**).

The gene ontogeny lists summarizing the biological processes that are down-regulated in this data set are related to B cell proliferation and dendritic cell maintenance and migration (**Figure 20c**). On the other hand, genes that are up-regulated are associated with cell replication, congruent to what was observed in CD4^{+} *Il22ra2*$^{eGFP+}$ cells extracted from the T cell transfer colitis setting (**Figure 19d and 20d**).

Within the list of up-regulated transcription factors in **Figure 20e**, there are many immune response related genes. These include *Havcr2*, *Bhlhe40*, *Prdm1*, *Tbx21*, *Id2*, *Il10* and *Rxra*. T-bet^{+} cells (the protein associated with *Tbx21* expression) and *Il10* are particularly known to be up-regulated in the mouse malaria model [205]. Again, more cell cycle genes are included in this list, indicating once more, that these CD4^{+} *Il22ra2*$^{eGFP+}$ cells are highly replicative. Also, Kruppel-like family member genes, such as *Klf2*, *Klf6* and *Klf11* are included in this list and are known to play a role in

lymphocyte differentiation and trafficking [206]. Common transcription factor genes that were also seen in the steady state data set include the cell cycle genes *Runx2*, *Runx3* and *Rxra*. Only the gene *Rad21* is common between this data set and the T cell transfer colitis data set, which is known to be involved in DNA replication and repair [207].

Finally, the genes highlighted in red throughout the last three figures are potential transcription factors that are repeatedly included in the lists of up-regulated genes in cells extracted from mice during homeostasis and in disease models (**Figure 18e, 19e and 20e**). The transcription factors, *E2f2, E2f8, Mxd3, Mybl2, Pycard and Hmgb2* are therefore promising targets and will be further studied as candidate genes for the regulation of *Il22ra2* expression.



c.

Down-regulated biological processes
regulation of B cell proliferation
regulation of B cell apoptotic process
positive regulation of B cell proliferation
regulation of B cell receptor signaling pathway
dendritic spine organization
dendritic cell chemotaxis
B cell activation
dendritic cell migration
dendritic spine maintenance
negative regulation of B cell apoptotic process

d.

Up-regulated biological processes
mitotic sister chromatid segregation
microtubule cytoskeleton organization involved in mitosis
mitotic spindle organization
regulation of cell cycle process
mitotic nuclear division
positive regulation of cell cycle process
mitotic cytokinesis
mitotic cell cycle phase transition
sister chromatid segregation
mitotic chromosome condensation

e. Up-regulated transcription factors

Havcr2	**E2f8**	Runx3
Epas1	**Mybl2**	Klf11
Bhlhe40	Sub1	Hnrnpab
Adam8	Flna	Rad21
Prdm1	**Mxd3**	**Pycard**
Hmgb2	**E2f2**	Nod1
Runx2	Klf2	Fanca
Tbx21	Rxra	Pim1
Id2	Itgb2	
Il10	Klf6	

Figure 20: Transcriptional profile of *Il22ra2*-expressing T cells during *Plasmodium berghei* ANKA infection **a)** Experimental scheme showing extraction of CD4$^+$ *Il22ra2*$^{eGFP-}$ and CD4$^+$ *Il22ra2*$^{eGFP+}$ T cell populations from the spleens of *Il22ra2*^{eGFP} reporter mice after 6 days of *Plasmodium berghei* ANKA infection. Differentially expressed genes identified from the samples extracted from mice that had developed malaria infection were compared to the samples extracted from steady state mice. **b)** Volcano plot comparing CD4$^+$ *Il22ra2*$^{eGFP-}$ cells (left side) to CD4$^+$ *Il22ra2*$^{eGFP+}$ cells (right side) indicating significantly down-regulated (left side) and up-regulated (right side) differentially expressed genes (|Log2FC| > 1 and (Padj) < 0.05 for up-regulated genes and |Log2FC| < -1 and (Padj) < 0.05 for down-regulated genes). The gene *Il22ra2* is highlighted in a red box. **c)** Gene ontology describing the biological processes related to down-regulated differentially expressed genes. **d)** Gene ontology describing the biological processes related to up-regulated differentially expressed genes. **e)** List of all up-regulated transcription factors in *Il22ra2*$^+$ cells. Genes highlighted in bold red are transcription factors that are found in the list of up-regulated genes in cells extracted during homeostasis and in disease models.

Discussion

Experimental use of the *Il22ra2*^{eGFP} reporter mouse

So far, studies quantifying levels of IL-22BP have mostly been reliant on readouts of *Il22ra2* mRNA expression and detection of IL-22BP protein via western blot techniques. To date, a reliable staining of murine IL-22BP protein using commercially available antibodies could not be established in the host lab. Only human IL-22BP antibodies have proven reliable so far in experiments carried out for this project. For the purpose of this project, the *Il22ra2*^{eGFP} reporter mouse was generated and validated for gene expression (**Figure 1**). Indeed, it was found that the eGFP expression correlates well with the *Il22ra2* mRNA expression. Although this mouse doesn't provide information on the translation of the mRNA, or the folding and biological activity of the protein, the detection of eGFP using fluorescent detection techniques provides a good indication of its relative expression. Specifically, this mouse was designed using the T2A peptide. From the literature, it has been shown that this method of gene manipulation ensures that the eGFP does not interfere with the activities of IL-22BP, as it is transcribed and translated separately to the *Il22ra2* gene it follows on from [208]. However, IL-22BP protein activity in this reporter mouse remains to be validated. As it has proven difficult to detect mouse IL-22BP in western blots, an alternative method can be utilized instead. One possibility was described by Sugimoto *et al.*, where immunoblot techniques were used for detection of STAT3 phosphorylation and were able to provide information on the activity of the soluble receptor indirectly. To describe the method in more detail, the authors showed that application of IL-22BP resulted in down-regulation of local STAT activity required for IL-22 signaling. In other words, the expression levels of STAT3 in the mouse are a good indication of the level of IL-22 inhibition [138]. Therefore, detection of STAT3 expression levels of in the *Il22ra2*^{eGFP} reporter mouse and comparisons to wild type mice can also provide information on whether the level of IL-22 inhibition has been altered.

The *Il22ra2*^{eGFP} reporter mouse was an asset in investigating the three main aims of this project. Working with the reporter mouse was advantageous as it allowed the use of flow cytometry techniques and enabled isolation of a pure population of *Il22ra2*-expressing cells, allowing identification of different cellular sources of the receptor and

exploration of the heterogeneity of such cells. The supervised approach of using flow cytometry means that one can only investigate cell subsets that can be identified by available reporter mice or antibodies, leaving a significant percentage of *Il22ra2*$^{+}$ cells undefined. This caveat was overcome in certain experiments through the use of RNA sequencing techniques as an unsupervised approach of analyzing these cells. Explicitly, focus was placed on learning more about the different CD4^{+} T cell subsets that express *Il22ra2*, with the aim of describing the heterogeneity and transcriptional profiles of these cells (**Figure 6-9**). As CD11c^{+} dendritic cells are known as one of the predominant producers of IL-22BP [9,134,143], the experiment was designed to also include all *Il22ra2*$^{eGFP+}$ CD11c^{+} cells as a positive control to compare gene signatures to *Il22ra2*$^{eGFP+}$ CD4^{+} T cells. Consequently, the sole use of the CD11c^{+} marker resulted in not only dendritic cell populations being included in this sample, but also many macrophage populations. Additionally, preliminary data indicated that *Foxp3*$^{+}$ cells were capable of co-expressing *Il22ra2*, so these cells were also analyzed in this experiment at a single cell level. In hindsight, this experiment could have been designed in an alternative way, which may have provided more information on *Il22ra2*-expressing cell types. For one, how the cells were sorted was biased and confined to previous knowledge from literature. An alternative experimental set-up including all CD45^{+} *Il22ra2*$^{eGFP+}$ cells would have allowed for identification of all immune cells that have the ability of expressing the receptor. However, this would have led to a lower number of *Il22ra2*$^{eGFP+}$ CD4^{+} T cells in total, which was the focus of the experiment carried out in this project.

Lastly, future experiments utilizing immunofluorescence imaging of tissue from the *Il22ra2*^{eGFP} reporter mouse and other fluorescent reporters, such as *Il22*^{sgBFP}, would provide insight into the spatial-temporal relationship between the receptor and its cytokine. Analysis of tissue from mice during steady state and inflammatory models would enable visualization of which cellular sources come into play under each of these conditions.

Cellular sources of *Il22ra2*

From previous literature, it is known that dendritic cells, eosinophils, and $CD4^+$ T cells are the most relevant sources of *Il22ra2* during inflammatory conditions [9,134-137,143]. For this reason, most gating strategies used for analyzing experiments in this project include markers for these cell types. This has been both beneficial and limiting to the purpose of this project. For one, markers such as CD11c and CD4 have resulted in the inclusion of NK and B cells in sorted cell populations. On the other hand, by using such markers, it has resulted in a biased analysis of cells that express *Il22ra2*, where it would have been wiser to sort all $CD45^+$ lymphocytes, which may have led to the identification of other cell types not listed here. Nevertheless, the experiments carried out during the course of this project have revealed some novel cellular sources of *Il22ra2* expression that have not yet been studied. These include $CD11c^+$ macrophages, $CD8^+$ T cells, $CD19^+$ B cells, $CD3^-$ $NK1.1^+$ NK cells, $CD3^+$ $NK1.1^+$ T cells, and *Ifng*$^+$ T_h1-like cells. This section of the discussion will examine cellular sources of *Il22ra2* that have recently been identified in sequencing databases and as part of this project.

Sequencing Databases

To begin with, a detailed search was carried out on available sequencing databases such as the Single Cell Expression Atlas, Human Cell Atlas and the Immunological Genome Project. Although limited, these databases have revealed some information on *Il22ra2* expression from experiments carried out in other laboratories, which may verify what has been revealed within this project. It is important to mention that, although these cell types are listed to express *Il22ra2* in these databases, they haven't been explicitly mentioned in publications, or referred to as significant cellular sources of the receptor, thus far. Nevertheless, in the Single Cell Expression Atlas database, mouse data from different sequencing experiments show highest expression of *Il22ra2* in lymphoid and myeloid progenitor cells, granulocytes, and monocytes (Link to source in table 22). In the Immunological Genome Project database, mouse data from RNAseq show expression of *Il22ra2* in neutrophils, lymph node endothelial cells, thymic epithelial cells, and hematopoietic stem cells (Link to source in table 22). Interestingly, microarray data from the same database show the highest expression of *Il22ra2* in epidermal dendritic cells, $CD8^+$ dendritic cells from the spleen, and also in

naïve $CD8^+$ T cells from the spleen (Link in table 22). A significant difference depicted in the published data compared to the experiments carried out during this project is that the sequenced cells originated from 6-week-old mice, whereas the cells sequenced in this project were always extracted from mice sacrificed after 8 weeks of age. Preliminary experiments carried out in the Huber lab have revealed that *Il22ra2* expression increases with age in mice (data not shown), suggesting that cells originating from 6-week-old mice may not have expressed the soluble receptor at the time of sequencing. One major caveat of referring to these online databases is that not all cell types are listed. For example, only naïve $CD4^+$ T cells were sequenced in the RNAseq data set, whereas data gathered from this project indicate that *Il22ra2* is not abundantly expressed in naïve cells, but rather in effector $CD4^+$ T cells. In both RNAseq and microarray data, expression of *Il22ra2* in all the aforementioned cell types are considered to be in the low range.

Interestingly, expression of *IL22RA2* in PBMCs visualized in the Human Cell Atlas database confirms some of the findings obtained during this project. For example, receptor expression was highest in NK memory cells, $CD8^+$ T cells, resting Treg cells, and eosinophils.

Unfortunately, all the available databases analyzed do not show significant expression of *Il22ra2* in $CD4^+$ T cells, although this was clearly observed in the RNAseq data in **Figures 6-9** and **Figures 18-20** as part of this project. To elaborate, none of these data sets specifically focused on *Il22ra2* in $CD4^+$ T cells, suggesting that either *Il22ra2* is lowly expressed and therefore could not be detected, or that only very few $CD4^+$ T cells express *Il22ra2* in the specific settings and locations of cell extraction in these experiments.

Furthermore, certain cell types identified in these databases, such as $CD8^+$ cells, neutrophils and progenitor cells, could be of great interest to study during inflammatory conditions. In conclusion, data from available databases have confirmed some of the cellular sources that have been identified within this project. The other sources listed here should be studied in future experiments.

Innate immune cells

Macrophages

The single cell sequencing experiment revealed an interesting finding concerning the CD11c$^+$ cells that were sorted, namely, that the majority of the myeloid-derived *Il22ra2*$^+$ cells cluster as macrophages, with only a few *Il22ra2*$^+$ cells appearing in the dendritic cell clusters (**Figure 8**). Although monocytes and macrophages have been identified as sources of *Il22ra2* expression in previous reports [134,137,142,159,169], this was a surprising outcome, as most reports define dendritic cells as an important source of *Il22ra2* expression during homeostasis and inflammatory conditions [9,134,143,144]. As it is now obvious that CD11c is not a specific enough marker for defining conventional dendritic cells, especially with regards to *Il22ra2* expression in the spleen, future experiments should include more markers to distinguish between these cells and macrophages. A good example would be to determine expression of the *Zbtb46* gene to discriminate between them, as this transcription factor is only expressed by conventional dendritic cells, but not by other myeloid cell populations [143,209]. Scrutiny of the sequencing data described in **Figure 8** revealed that there is an up-regulation of this particular gene in the conventional dendritic cell cluster, but not in either of the macrophage clusters (data not shown).

There are two different macrophage populations (cluster 5 and 8) that are both very high in *Il22ra2* expression. They are defined as either classical M1 macrophages with *Ly6c* gene expression, indicating that they are inflammatory macrophages, or Ly6c$^-$ M2 macrophages, otherwise defined as non-classical/resident macrophages [210]. Other than that, both clusters have similar defining gene signatures, such as *Lyz2*, *Csf1r*, *Cebpb*, *Clec4a3*, all of which are characteristic macrophage genes [211-213]. In conclusion, it was shown here that macrophages are more abundant *Il22ra2* expressing cells than dendritic cells when sorted for via CD11c.

NK cells

During this project, NK cells have been revealed as a novel cellular source of *Il22ra2*. Early experiments showed that CD3$^-$ NK1.1$^+$ NK cells express *Il22ra2* in many organs and are one of the major sources of *Il22ra2* in the spleen and lymph nodes of steady state mice (**Figure 3**). Analysis using single cell sequencing techniques further confirmed this cell type as a source of the receptor (**Figure 8**). What is intriguing about this data set, is that the NK cell cluster is located next to CD4$^+$ T cell populations in the

UMAP. One argument for why these NK cells may cluster close to T cells, specifically the *Ifng*$^{+}$ T_h1-like cluster, could be due to the expression of cytotoxic genes and NK-related transcription factors. For example, the high expression of genes such as *Gzma*, *Gzmb*, *Nkg7*, *Klrd1*, *Eomes*, and *Itga2* clearly define this cell type (**Figure 8b**). Furthermore, this cluster is similarly shown to highly express *Tbx21* and *Ifng* (**Figure 9b**). Again, this cell type was not specifically sorted for but ended up being collected and sequenced. This may have been due to the CD11c^{+} gate, as NK cells have been reported to express this marker [214,215]. Confirmation that this cell cluster was not actually an NKT cell cluster, but indeed NK cell cluster in this data set was determined through the lack of significant *Cd3e* gene expression levels (data not shown). More evidence that this cluster is not made up of NKT cells is through the lack of typical transcription factors defining NKT cells, such as *Plzf*.

Adaptive immune cells

CD3^{+} *NK1.1*$^{+}$ *T cells*

Following on from the description of *Il22ra2* in the previous cell type, it remains to be confirmed whether the CD3^{+} NK1.1^{+} T cells that express *Il22ra2* are bonafide NKT cells. This is due to a lack of specific antibodies in the flow cytometry analysis carried out during the previous experiments. In order to verify that these are indeed NKT cells, antibodies detecting a PBS57-loaded CD1d-tetramer (which engages with the NKT cell TCR) are required to verify this [216]. Nevertheless, the only inflammatory model this cell type was included in the analysis of, was the mouse CAC model, and here, only a slight up-regulation of this cell type was seen in the lymph nodes (**Figure 14c**).

CD19^{+} *B cells*

B cells have, thus far, never been reported to express *Il22ra2*, but were clearly shown to express the receptor in the data accumulated in this project. Specifically, the single cell sequencing data show B cells that were sorted for as *Il22ra2*$^{eGFP+}$ and were confirmed to have *Il22ra2* mRNA expression. Furthermore, the *Il22ra2*$^{eGFP+}$ B cells were defined by classical B cell gene markers and transcription factors such as *Bhlhe41*, *Ebf1*, *Pax5* and *Pou2af1* [217-219] (**Figure 8b**). Surprisingly, these cells were not a target of cell sorting but may have been collected during the sorting process due to the presence of either CD11c or MHC II on the cells, as some B cells in the spleen are known to express these markers [220].

On the other hand, the ontogeny data describing differentially expressed genes in the *Plasmodium berghei* ANKA model show that down-regulated genes are collectively associated with B cell activation and proliferation (**Figure 20c**). This was also verified in the flow cytometry data, as the frequency of $CD19^+$ B cells expressing *Il22ra2* was down-regulated by trend, in the spleen and liver of infected mice (**Figure 16c and 16d**).

$CD8^+$ T cells

This cell type was only studied through flow cytometry during steady state and in the *Plasmodium berghei* ANKA infection model, where expression of the receptor was shown to be significantly up-regulated in $CD8^+$ T cells in the blood of infected mice (**Figure 16e**). As mentioned before, data gathered from available databases report that $CD8^+$ cells, whether they are T cells or dendritic cells, express *Il22ra2*, providing more evidence that they may be a relevant source of the receptor. Unfortunately, the experimental design of the single cell sequencing means that $CD8^+$ T cells expressing *Il22ra2* were not sorted for. Thus, including all $CD45^+$ *Il22ra2*$^{eGFP+}$ cells would have probably included such cells and enabled further analysis of both $CD8^+$ dendritic cells and T cells. $CD8^+$ cells are worth investigating in other models of inflammation to see if they have a significant impact on pathogenesis.

$CD4^+$ T cells

Next, descriptive experiments utilizing the *Il22ra2*^{eGFP} reporter mouse aimed at determining whether *Il22ra2* could be expressed by known $CD4^+$ T cell subsets, as part of the first and second aims to define the cellular sources of *Il22ra2* and define whether they are a heterogeneous population. Experiments carried out during this project have identified that this receptor can indeed be expressed by a heterogeneous population of $CD4^+$ T cells. Specifically, flow cytometry and single cell sequencing techniques have shown co-expression of either *Ifng* or *Foxp3* with *Il22ra2* in distinct $CD4^+$ T cell populations. Furthermore, both these subsets were also verified to produce IL-22BP in PBMCs extracted from human blood samples of both healthy donors and IBD patients (**Figure 10d**).

$Foxp3^+$ T cells

Explicitly, attempts were made to understand the implication of *Foxp3* co-expression in *Il22ra2*$^+$ T cells during this project. The *Il22ra2*^{eGFP}*Foxp3*^{mRFP}*Il17a*^{FP365}*Il22*^{sgBFP} reporter mouse used here enabled identification of *Il22ra2* and *Foxp3* co-expressing

cells, in steady state and inflammatory models. It must be noted, however, that this particular subset was challenging to compare within the different mouse models. To begin with, co-expression of *Il22ra2* and *Foxp3* was easily detectable in all organs analyzed during homeostatic conditions. However, the use of the T cell transfer colitis model provided with some caveats. The extraction of donor $CD4^+$ T cells in this model calls for depletion of $CD25^+$ cells before transfer to recipient mice. This means that the majority of precursor Tregs [221] are not transferred with the $CD45RB^{high}$ cells, and therefore, should not have a substantial impact on disease development. For this reason, it became obvious that the down-regulation of *Foxp3*$^+$ *Il22ra2*$^+$ T cells was mostly due to this reason, and therefore, frequencies of this subset should not be compared to frequencies from steady state cells (**Figure 12c-e**). On the other hand, it must be argued that in the other two bulk sequencing data sets describing $CD4^+$ *Il22ra2*$^+$ T cells extracted from steady state and *Plasmodium berghei* ANKA infected mice, the *Foxp3* gene or genes that are associated with Tregs, such as *Ikzf4*, *Izumo1r* and *Lif*, are consistently down-regulated (**Figure 19b and 20b**) [194,222,223]. Lastly, and contrastingly to other inflammatory mouse models, analysis of organs extracted from the CAC model revealed a significant up-regulation of *Foxp3*$^+$ *Il22ra2*$^+$ T cells in the draining lymph nodes of mice that had developed tumors compared to those extracted from steady state mice (**Figure 15c**).

Ifng$^+$ *T*$_h$*1-like cells*

A major $CD4^+$ T cell source of *Il22ra2* described in the single cell sequencing data is an *Ifng*-expressing cell type, which has characteristic gene expression of T_h1 cells. The cluster containing these cells is significantly high in both *Il22ra2* expression and *Ifng* expression, as well as in the transcription factor defining T_h1 cells, *Tbx21* (**Figure 8** and **9b**). The gene expression signature of this *Il22ra2*/*Ifng*-expressing cell type provides evidence that these cells have a cytotoxic function in which they induce expression of genes commonly expressed by T_h1 and NK cells. Unfortunately, a multiple reporter mouse enabling simultaneous detection of *Il22ra2* and *Ifng* was not available at the time these inflammatory mouse models were carried out, therefore, the co-expression of these two genes remains to be elucidated under inflammatory conditions. Bulk sequencing techniques analyzing $CD4^+$ *Il22ra2*$^{eGFP+}$ T cell samples from two inflammatory models revealed co-expression of *Il22ra2* and *Ifng* in cells extracted from *Plasmodium berghei* ANKA infected mice (**Figure 20b**).

T_h17/T_h22 cells are not a cellular source of Il22ra2 under homeostatic or inflammatory conditions

The results of the single cell sequencing experiment give a strong indication that there is co-expression of *Il22ra2* and *Ifng* by the same CD4$^+$ T cell type during steady state, and that this should be further explored in *in vivo* mouse models of inflammation. This was an unexpected outcome, as original hypotheses revolved around the idea that these *Il22ra2*-expressing cells may be pathogenic effector cells, hence the reason why *Il22ra2*^{eGFP} mice were originally crossed to *Il17a* and *Il22* reporters, known cytokines of effector T cell subsets with potential pathogenic qualities. To go further, it was even speculated whether the cytokine and the receptor could be expressed on the same cell and could therefore self-regulate excessive cytokine expression. Although co-expression of *Il22* and *Il22ra2* by the same cell type does not seem likely to happen, plasticity of a CD4$^+$ T cell that may, at one time express *Il22*, and then switch to expression of *Il22ra2*, could still be a possibility. To determine whether this would be the case, 'Fate' reporter mice, such as those used previously for *Il17a*, would have to be generated [224]. Although there is some co-expression of *Il22ra2* with *Il17a* and *Il22*, these relative T helper cell subsets do not seem to be responsible for abundant *Il22ra2* expression, as depicted in single cell sequencing data (**Figure 9d**) and *in vivo* experiments (**Figure 5c, 12c-e and 15b-e**). Future experiments should incorporate a reporter mouse that has both *Il22ra2*^{eGFP} and a coloured reporter for *Ifng*, such as the *Ifng*^{Katushka} [225].

Transcriptional profile of *Il22ra2*-expressing CD4$^+$ T cells

The use of single cell and bulk RNA sequencing techniques have been essential in studying *Il22ra2*-expressing CD4$^+$ T cells. For one, these techniques have verified the use of the *Il22ra2*^{eGFP} reporter mouse, as all sequencing data sets show that *Il22ra2* was one of the most significantly up-regulated genes in *Il22ra2*$^{eGFP+}$ samples. Alongside the *Il22ra2* gene, comparative analysis of CD4$^+$ *Il22ra2*$^{eGFP+}$ T cell samples against CD4$^+$ *Il22ra2*$^{eGFP-}$ T cell samples in all bulk sequencing data sets enabled identification of characteristic genes and candidate transcription factors that describe *Il22ra2*-expressing CD4$^+$ T cells best. Significant features of genes that were consistently up-regulated in both homeostatic and inflammatory mouse model data sets will be discussed in this section.

Lgals1

The *Lgals1* gene encodes for Galectin 1, a carbohydrate binding protein that is involved in a multitude of cell functions, including proliferation, migration, immune responses and apoptosis [226]. Of note, this protein has immunomodulatory functions, in that it has inhibitory effects on neutrophil and T cell migration, as well as being a strong inducer of T cell apoptosis [202,203]. Explicitly, studies have discovered that Galectin 1 only induces apoptosis in pro-inflammatory T cell subsets such as T_h1 and T_h17, but naïve, T_h2 and Tregs are not affected by this protein [227]. Nevertheless, a recent publication described an up-regulation of *Lgals1* in *Plasmodium*-specific TCR transgenic $CD4^+$ T cells and found this gene to be implicated in promoting T_h1 differentiation [228]. Lastly, Galectin 1 is up-regulated in tumors and secretion of this protein aids immune evasion tactics and inhibits T cell migration into the tumor [229].

1500009L16Rik

Although 'Rik' genes are annotated so due to the absence of an official name and data on its function, this particular gene has since been defined as C12orf75 or Overexpressed in Colon Carcinoma 1 protein (OCC-1) [204]. This long non-coding RNA was initially identified in colorectal cancer and was described to have a tumor-suppressive role [230]. Not much is known about this gene, but recent publications have identified that OCC-1 has functions in regulating cell cycle phases [230] and can promote Wnt signaling [231].

E2f family genes

Members of the E2f family of transcription factors are critical coordinators of the cell cycle. This gene family is encoded by eight genes, in which some members are associated with activating transcription, whereas others are associated with repressing transcription [232]. Within the up-regulated gene lists in all three bulk sequencing data sets, the genes, *E2f2*, a transcriptional activator, and *E2f8*, an atypical repressor were commonly present [233,234]. Both *E2f2* and *E2f8* were found to be involved in DNA repair [235,236]. Other E2f family genes up-regulated in these lists include *E2f1* and *E2f7*, which are described to be a transcriptional activator and repressor, respectively. E2f family member proteins come into action at different times during the cell cycle. For example, production of the activator protein, *E2f2*, is highest during the G1-S transition phase, whereas production of the atypical repressor protein, *E2f8*, peaks late

in the S phase [233]. E2f family proteins are crucial components of a transcriptional complex. This complex is made up of cyclin-dependent kinase (CDK) proteins, Retinoblastoma (RB) proteins, and E2f proteins, and is essential for the regulation and coordination of the cell cycle during cellular replication[233]. This complex is encoded by genes that are all driven by Myc [237]. Specifically, E2f transcriptional activity is modulated through the direct binding of RB proteins, which are in turn modulated by CDK proteins. Inhibition of E2f transcriptional activities occurs through binding of RB proteins, which when phosphorylated by CDK proteins, results in the release of E2f proteins, allowing transcriptional activation of target genes [233]. E2f family members do not only play a role in the cell cycle, but are also implicated in tumor development, apoptosis and angiogenesis [233,238].

Mxd3

The MAX dimerization protein 3 (MXD3) encoded by the gene, *Mxd3*, is a transcriptional repressor that antagonizes *Myc* transcriptional activity [239]. This gene is a member of the MXD family of basic-helix-loop-helix-leucine-zipper (bHLHZ) transcription factors, which interacts with the MYC/MAX/MAD transcriptional network and is an essential regulator of the cell cycle and cell proliferation [240,241]. Specifically, MXD3 is an atypical MXD protein, as it is up-regulated during S phase in proliferating cells, whereas other family members are linked to cell cycle exit and cell differentiation [242]. *Mxd3* has been found to be over-expressed in a variety of different cancers and is associated with a poor prognosis [240,241].

Mybl2

This gene encodes the MYB proto-oncogene like 2 (MYBL2) protein, which is a member of the MYB transcription factor family and is yet another important cell cycle regulator. Expression of the *Mybl2* gene is controlled by E2F proteins as part of the DREAM multiprotein complex [243]. High expression levels of *Mybl2* have been observed in many different cancer types and is commonly associated with a poor outcome [244].

Pycard

The gene, *Pycard*, encodes the apoptosis-associated speck-like protein containing CARD (ASC), an adaptor protein that is a major constituent of inflammasomes. Essential features of this protein that ensure its function are the pyrin and caspase recruitment domain (CARD). Once this adaptor protein assembles these domains within inflammasomes such as NLRP2, NLRP3, NLRP6 and AIM2, CARD domains of pro-caspase 1 can aggregate and induce proteolytic cleavage of itself to generate active caspase 1. The active form can then cleave the pro-inflammatory cytokines pro-IL-18 or pro-IL-1ß, which exist in an immature form until cleaved and secreted [57]. ASC is an essential modulator of apoptosis and inflammation [245].

The contribution of *Pycard* on *Il22ra2* expression has already been alluded to in the publication by Huber *et al.*, where it was shown that maturation of dendritic cells, promoted by IL-18 secreted from NLRP inflammasomes that had sensed microbial ligands, results in down-regulation of *Il22ra2* [9]. Experiments examining ASC deficiency in mice revealed that there was no such down-regulation of *Il22ra2* when ASC is absent. With reference to the data obtained during this project, it could be hypothesized that $CD4^+$ T cells encourage the down-regulation of *Il22ra2* expression in dendritic cells through *Pycard* up-regulation, while simultaneously up-regulating *Il22ra2* expression themselves. Such expression patterns are exemplified in the mouse CAC model and *Plasmodium berghei* ANKA model, where the frequencies of *Il22ra2* expression are down-regulated in dendritic cells and up-regulated in $CD4^+$ T cells.

Hmgb2

The *Hmgb2* gene encodes a chromatin-associated protein, High Mobility Group Box 2. Involved in modulating transcription, cell proliferation and is implicated in tumorigenesis. Specifically, this protein has been shown to bend DNA into circles, with the purpose of enhancing transcription as it enables genomic components to move closer together. *Hmgb2* has pro-inflammatory characteristics and is also known to have antimicrobial features in gastrointestinal epithelial cells [246].

Homeostatic and inflammatory model data sets

In the next section, important genes identifying each bulk sequencing data set and similarities between data sets are discussed.

Steady state

The most highly up-regulated genes from the bulk sequencing data include galectins (*Lgals3* and *Lgals1*) and annexins (*Anxa1* and *Anxa6*) (**Figure 18b**), both of which are proteins involved in modulating inflammatory and apoptotic processes [247]. Specifically, galectins were also highly up-regulated in the proliferating T cell, NK cell and *Ifng*$^{+}$ T$_h$1-like clusters in **Figure 8b**. Both sequencing data sets (**Figure 8b and 18b**) reported up-regulation of significant T cell-related genes, including *Itga2*, *Il18r1*, *Gzma*, and *Klra4*. Specifically, it is worth mentioning that some common T cell-related transcription factors that are important for cell cycle regulation are also up-regulated in this bulk sequencing data set, namely, *Rxra*, *Rora,* and *Foxm1*. Interestingly, the correlation between *Il22ra2* and *Ifng* is referred to again in the list of potential transcription factors, where many IFNγ-inducible transcription factors are up-regulated (**Figure 18e**). Collectively, the signature genes describing *Il22ra2*$^{eGFP+}$ cells all indicate towards this cell type being highly proliferative, metabolically active, and cytotoxic in nature.

T cell transfer colitis model

This model was specifically chosen for bulk sequencing analysis due to considerably up-regulated *IL22RA2* expression in CD4^{+} T cells of patients suffering from IBD, and because this model was previously shown to promote a pathogenic effect of CD4^{+} T cell derived-IL-22BP production [135]. Additionally, it was observed to induce the highest frequency of CD4^{+} *Il22ra2*-expressing cells out of all the models investigated within this project. Elucidating the characteristic gene expression features of these CD4^{+} *Il22ra2*$^{eGFP+}$ T cells in this model will enable identification of novel target genes that could potentially be targeted to ameliorate inflammation in IBD.

To begin with, the highest differentially down-regulated genes identified in the samples originating from the T cell transfer colitis model are associated with Tregs (**Figure 19b**). This makes sense as the extraction of donor CD4^{+} T cells in this model calls for depletion of CD25^{+} cells before transfer to recipient mice. This means that the majority of precursor Tregs [221] are not transferred with the CD45RB^{high} cells, and therefore,

should not have a substantial impact on disease development. As previously discussed, this T helper cell subset is not greatly involved in this model [135] and mouse colitis experiments carried out during this project verified this by revealing that *Il22ra2*$^{+}$ CD4^{+} T cells co-expressing *Foxp3* are down-regulated when compared to steady state population frequencies (**Figure 12c-e**).

Another set of genes that are down-regulated are related to T_h17, such as *Il21*, *Il23r* and *Il17rb* (**Figure 19b**). This is quite intriguing as T_h17-related genes have often been implicated in the pathogenesis of this model [248,249]. These results provide yet more evidence that *Il22ra2*$^{+}$ CD4^{+} T cells do not belong to the T_h17 cell subset.

Next, the CD4^{+} *Il22ra2*$^{eGFP+}$ samples extracted from this model are observed to be highly proliferative, as cell cycle and DNA repair genes are abundantly expressed (**Figure 19b**). This is re-iterated in the gene ontogeny description (**Figure 19d**) and once again noted in the list of potential transcription factors, such as *Nucks1*, *Rad21*, *Foxm1*, *E2f2*, *E2f7*, *E2f8*, and *Hmgb2* (**Figure 19e**). All genes listed are related to DNA repair. It could be speculated that such genes are promoted in cells extracted from the area of inflammation, as the host cells are working fast to repair the damage and are prone to mutagenesis under such conditions [250]. Also, these cells are described to be highly replicative, and high proliferation rates are associated with increased errors in DNA replication [233].

Plasmodium berghei ANKA infection model

This particular disease model was chosen for bulk sequencing as an alternative model of inflammation in which CD4^{+} T cells that express *Il22ra2* are also up-regulated. It is quite intriguing that among CD4^{+} *Il22ra2*$^{eGFP+}$ T cells, certain transcriptional repressor genes were down-regulated compared to CD4^{+} *Il22ra2*$^{eGFP-}$ T cells (**Figure 20b**). For example, the gene *Bcl6*, which represses the development of T_h1, T_h2 and T_h17 cells, and is a good indication that such effector cell types are probably important players in the immune response against the pathogenic threat. Once again, Treg-related genes are down-regulated in this data set. Specifically, the repressive genes *Foxp3* and *Ikzf4,* are important for the inhibitory activity of Tregs, specifically in suppressing IFNγ expression [222,251]. The down-regulation of such repressive genes conforms with what is known about this model, as the pro-inflammatory cytokine, IFNγ, plays a particularly important role in the pathogenesis of malaria infection [97]. In this data set,

expression of the gene, *Ifng*, is observed to be strongly up-regulated in $CD4^+$ *Il22ra2*$^{eGFP+}$ T cells extracted from this model (**Figure 20b**). Just like in the single cell sequencing data, these *Il22ra2*$^+$ *Ifng*$^+$ cells seem to have cytotoxic functions, evident through the significant up-regulation of granzyme and NK genes (**Figure 8b and 20b**).

The presence of the master transcription factor of IFNγ-producing T_h1 cells, *Tbx21,* and the anti-inflammatory cytokine, *Il10*, in the list of up-regulated transcription factors is quite intriguing as it corroborates what is already known about the polyfunctionality of $CD4^+$ effector T cells in this infection model. From literature, it has been reported that these cells display plasticity and can release a mixture of cytokines commonly associated with T_h1, T_{fh} and T_r1 cells [82]. Furthermore, the expression of the transcription factors *Tbx21* and *Prdm1*, and the cytokines *Ifng* and *Il10*, is characteristic of T_r1 cells [205]. Up-regulation of these distinct genes provides an indication that *Il22ra2* expression by T_r1 cells may have an implication on disease pathogenesis in this model.

Il22ra2 expression in plasmodial pathogenesis

So far, not much has been elucidated about IL-22BP and its effect on malaria pathogenesis. Only human polymorphism studies from patients infected with malaria have revealed associations of IL-22 with cerebral malaria, but there are no associations related to IL-22BP [178]. Furthermore, there are some mouse studies reporting an overall protective role of IL-22 [252-254], but no such studies exist describing the role of IL-22BP in a mouse model of malaria infection.

When focusing on the role of IL-22 in malaria mouse models, one study reported that $CD8^+$ T cell-derived IL-22 was found to be protective against liver damage in *Plasmodium chabaudi*-infected mice [253]. Another study showed that γδ T cell-derived IL-22 was critical in *Plasmodium berghei* ANKA-infected mice, and that IL-22 deficiency resulted in a more severe disease outcome, earlier appearance of cerebral symptoms and lower parasitemia [254]. The data from this project showed that, although there were no significant differences between wild type and *Il22ra2*-deficient mice in terms of disease outcome, the majority of *Il22ra2*-deficient mice had a delayed development of cerebral symptoms and weight loss, as well as prolonged survival rates by trend (**Figure 17**). One could speculate that this may be due to the deficiency in the receptor enabling more availability of IL-22, and therefore an ameliorated disease

progression. Another point worth mentioning from the previous publication, is that *Plasmodium berghei* ANKA infected mice exhibited higher levels of IFNγ [254]. It could be speculated that the cells producing these high levels of IFNγ may also be high in *Il22ra2* expression production, due to the significant co-expression of *Il22ra2* and *Ifng* seen in the sequencing data sets generated during the course of this project (**Figure 9b and 20b**).

In addition to investigating the cellular sources and transcriptional regulation of *Il22ra2*-expressing cells in mouse models of gastrointestinal inflammation, focuses were placed on identifying whether the same cell types and transcriptional networks could govern *Il22ra2* expression in other organs and in a different inflammatory setting. Hence why *Il22ra2* expression was also studied in this model. Flow cytometry analysis revealed a few interesting features of certain cell types in this disease model. For instance, from the time course experiment, it was revealed that $CD11c^+$ cell expression of *Il22ra2* is strongly down-regulated from Day 0 to Day 6 of infection, whereas expression by $CD4^+$ T cell is up-regulated in this time (**Figure 16c-e).** From this data, it can be speculated that specific cellular sources of *Il22ra2*, such as $CD4^+$ or $CD8^+$ T cells, could be responsible for blocking the protective effects of IL-22 in this model, as these cell types were up-regulated over the course of infection.

Unfortunately, the cell-to-cell interaction of sources of *Il22* and *Il22ra2* expression has not yet been studied in the *Plasmodium berghei* ANKA infection model, however, literature [254] and the results from this project provide strong evidence that studying both the cytokine and its antagonist together in this model could reveal interesting information on how this axis contributes to pathogenesis in malaria.

Lastly, the findings of these experiments could be transferable to human studies, as PBMC samples from malaria patients could provide information on which cell types are prominent producers of IL-22BP during infection, just like it was done for the IBD studies (**Figure 10**).

Il22ra2 expression derived from CD11c$^+$ cells limits the pathogenic effects of IL-22, whilst CD4$^+$ T cell-derived *Il22ra2* may block the protective effects of IL-22

Similar to IL-22, there seems to be a dual role of IL-22BP in inflammatory conditions. Within the gastrointestinal tract the divergent roles of this protein could be attributed to different cellular sources, such as dendritic cells, eosinophils and CD4$^+$ T cells [9,135,136,144].

So far, the data acquired during the course of this project indicate that, in conditions where IL-22 is inadequately controlled, the frequency of *Il22ra2*-expressing CD11c$^+$ cells is down-regulated, whereas the frequency of *Il22ra2*-expressing CD4$^+$ T cells are up-regulated when compared to steady state. The best evidence for this is seen in the CAC model (**Figure 14b-d**) and the *Plasmodium berghei* ANKA infection model (**Figure 16c-e**). When referring to the expression pattern of *Il22ra2*-expressing CD11c$^+$ cells, this data is compliant with the 'dogma' of IL-22 biology, in that *Il22* and *Il22ra2* have inverse expression patterns, and *Il22ra2* is down-regulated during inflammation in order to allow *Il22* to exert its protective effects. However, this inverse expression pattern rule is not followed by *Il22ra2*-expressing CD4$^+$ T cells in this case, where the frequency of this cell type is conversely up-regulated during inflammation, and hence results in a pathogenic role of CD4$^+$ T cell-derived *Il22ra2* expression.

To argue further, the high frequency of immature dendritic cells expressing *Il22ra2* during steady state being down-regulated in mature dendritic cells during inflammatory conditions has already been explained by Huber *et al*. and Martin *et al.* [9,134]. Specifically, CD11c$^+$ dendritic cells mature when they come into contact with LPS as a result of inflammation and reduce expression of *Il22ra2* modulated via IL-18 [9,134]. On the other end of the argument, from data in **Figure 4a**, it was shown that only CD44$^+$ effector T cells express *Il22ra2*, not naïve T cells. So, these cells must be activated by signals from the surrounding cytokine milieu, which happens during inflammatory conditions, in order for effector T cells to release relevant cytokines for acting against and resolving the cause of inflammation. In other words, this could mean that during the steady state, *Il22ra2* is expressed predominantly by CD11c$^+$ cells in a protective manner that prevents the deleterious effects of IL-22. However, once there is inflammation and tissue destruction, *Il22* expression must be up-regulated in order to mediate the threat, and the receptor is therefore down-regulated. It could be speculated that the amount of *Il22ra2* expression by CD11c$^+$ dendritic cells would be

excessive under inflammatory conditions and may overpower the protective effects exerted by IL-22. Therefore, *Il22ra2* expression in effector $CD4^+$ T cells may be induced with the intention of mediating IL-22 signaling slowly and in a controlled manner, although these cells may become dysregulated under excessive inflammatory conditions.

Microbiota-dependent effects of *Il22ra2* expression

The impact of the microbial milieu on the development of the host immune system and the progression of immune-related disease has long been of scientific interest. For one, the composition of the microbiota is easily altered via changes to lifestyle, diet, and substance intake, which ultimately has implications on the host tissue and immune response [19-21]. Also, studies have shown that distinct microbial components can instigate specific pro-inflammatory or regulatory outcomes. Examples of this effect include monocolonization of germ-free mice with Segmented Filamentous Bacteria to encourage T_h17 cell differentiation [255], or colonization with *Helicobacter hepaticus* to skew T cell differentiation towards a T_{reg} phenotype [64,256]. Unfortunately, constant exposure to specific microbial entities may result in dysregulated cytokine expression and uncontrolled inflammation [108].

The specific microbial composition harbored by the host can impact susceptibility of developing inflammation in the gastrointestinal tract and has been correlated with therapy response in IBD patients [257-259]. Strikingly, studies have shown that manipulation of the microbiota to a more complex microbiota via fecal microbiota transplantation can reduce inflammation and relieve symptoms in some IBD patients [260]. However, this is still discussed controversially and multi-center trials confirming this are missing. Of note, it has been observed that microbiota signatures can partially predict therapy responses [257-259], although this has not been replicated in multi-center prospective trials yet.

To date, there are many studies discussing the effects of certain microbial entities or components on IL-22 [108], which seems to be a very important aspect to consider, not only in inflammatory diseases of the gastrointestinal tract, but also extra-intestinally when studying this cytokine. However, most reports fail to consider the effects that the microbiota may have on *Il22ra2* expression, and on the other hand, how the expression of *Il22ra2* may impact the microbiota composition harbored by the host. So far, only

one study by Jinnohara *et al.* has shown that there may not be an impact of the microbiome on expression levels of *Il22ra2*, as RT-PCR readouts of this gene in the Peyer's patches were comparable between germ-free and SPF mice [142]. This is surprising as receptor-deficient mice had significant differences in expression levels of mucin, antimicrobial peptide, and fucosylation genes compared to wild type counterparts, all of which are important entities that usually impact the overall microbial composition of the host [142,261]. Contrastingly, a major finding established in **Figure 4a** of this project shows that there are significant differences in *Il22ra2* expression in colonic tissues of mice housed under different conditions, indicating that *Il22ra2* expression may be influenced by the microbiota composition. It was also revealed that *Il22ra2* expression levels differ in memory and effector T cells. The highest *Il22ra2* expression levels were reported in $CD44^+$ $CD62L^+$ central memory and $CD44^+$ $CD62L^-$ effector memory T cells, and the lowest gene expression levels were observed in $CD44^-$ $CD62L^+$ naïve T cells (**Figure 4a**). In $CD44^+$ $CD62L^+$ central memory T cells and $CD44^+$ $CD62L^-$ effector memory T cells, significant differences were seen in *Il22ra2* expression patterns in mice harboring different microbial compositions. Here, germ-free mice had significantly higher expression of *Il22ra2* compared to mice harboring SPF microbiota in both cell types, and even mice with *Nlrp6*$^{-/-}$ microbiota in $CD44^+$ $CD62L^-$ cells.

Unfortunately, the microbial composition harbored by the host is a confounding factor that was not fully addressed during the course of this project. In reference to the data in **Figure 4a**, different compositions would most likely change the relative frequencies of *Il22ra2* expression in each of the cell types investigated. Mice hosting both low and high-complexity flora are used within the Hamburg animal facilities, and differences have been observed in the development of colitis within these mice. The *Il22ra2*^{eGFP} reporter mice used for most experiments were housed under a Hamburg SPF microflora. Only mice harboring this flora were used to negate the possible effects of the composition on the frequency of *Il22ra2* expression.

Another aspect that was not considered during experiments is that microbiota differences in specific regions of intestines could make a difference in cytokine production levels. Jinnohara *et al.* have provided evidence that different regions of mouse intestines have varying *Il22ra2* expression patterns. They showed that the

Peyer's patches have the highest levels of *Il22ra2* expression in the gastrointestinal tract of mice [142]. Throughout all the experiments carried out during this project, efforts were made to consistently exhume the same parts of the organs for flow cytometry, RNA isolation and histology.

In vitro systems studying *Il22ra2* expression in different cell types

As the reporter mouse has been established and novel cell types expressing *Il22ra2* have been described, efforts should now be placed on establishing an *in vitro* system. Such a system will provide a platform to study these cell types in a more controlled environment, where one can determine the influences of certain inflammatory cues on the expression of *Il22ra2*.

So far, the only successful *in vitro* method used to study *IL22RA2* utilized monocytes. In a report by Martin *et al.*, *IL22RA2* expression was highly induced in human monocytes cultured with GM-CSF, IL-4, and a retinoic acid receptor agonist [134]. As a focus was placed on defining $CD4^+$ T cell subsets that can express *Il22ra2* in this project, initial attempts were made to differentiate naïve cells and determine receptor expression patterns (data not shown). A caveat in wanting to study these cells using classical *in vitro* differentiation techniques is the use of naïve T cells for most T helper cell conditions. Initial experiments in this project demonstrated that effector T cells had the highest frequency of *Il22ra2* expression *in vivo*, whereas the frequency of naïve T cells expressing *Il22ra2* were much lower (**Figure 4a**). To follow up, attempts were made to extract *Il22ra2*$^+$ $CD44^+$ effector T cells from reporter mice, but unfortunately, these cells could not sustain expression of eGFP after 5 days in culture (data not shown).

Validation of transcriptional candidates

The use of sequencing techniques has enabled identification of potential transcription factors that may modulate *Il22ra2* expression in certain cell populations. Future experiments planned for this project include using a luciferase assay to probe potential transcription factors to determine whether they have a role in promoting *Il22ra2* expression. For this purpose, the promoter region of the *Il22ra2* gene has been cloned and will be tested to determine whether expression of the gene can be promoted via select transcription factors such as *E2f2, E2f8, Mxd3, Mybl2, Pycard and Hmgb2*.

Conclusion

Data obtained during the course of this project has revealed many new features of *Il22ra2*-expressing cells. Preliminary experiments utilizing the *Il22ra2*^{eGFP} reporter mouse have provided insight into novel cellular sources that express *Il22ra2*, which have not yet been described in the literature. As various CD4$^+$ T cell subsets are known to promote different outcomes of disease, this project aimed to explore the heterogeneity of *Il22ra2*-expressing T cells and identify potential transcription factors that may regulate its expression in inflammatory conditions using RNA sequencing techniques.

Results from this project have revealed that CD4$^+$ *Il22ra2*$^+$ T cells are a heterogeneous population made up of proliferative and T$_h$1-like cell types with cytotoxic qualities. On the other hand, the data acquired during this project suggests that CD11c$^+$ *Il22ra2*$^+$ cells are mostly macrophages during steady state, and not dendritic cells as originally thought. Mouse models of inflammation, including colitis, cancer and infection (e.g. malaria) have shown that frequency levels of *Il22ra2* expression change in these CD11c$^+$ and CD4$^+$ cellular sources, however, the implications of these changes have not yet been elucidated. Lastly, a co-expression of *Il22ra2* and *Ifng* in single cell sequencing and bulk sequencing data sets places a focus on T$_h$1 cells and prompts further studies into the implications of *Il22ra2* expression from this T cell subset. Common transcription factors between the three bulk sequencing data sets have revealed candidate genes that may be responsible for the regulation of *Il22ra2* expression. These include *E2f2, E2f8, Mxd3, Mybl2, Pycard and Hmgb2*.

Further studies are required to verify characteristic gene expression patterns of these novel cellular sources of *Il22ra2* expression, and to examine the relevancy of the potential transcription factors identified. Defining how these *Il22ra2*-expressing cells are transcriptionally regulated could aid in identifying ways to promote its protective effects or prevent its inhibitory effects in aberrant inflammatory conditions.

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Appendix

List of Abbreviations

°C	Degree celcius
ACK	Ammonium-Chloride-Potassium
ACT	Artemisinin-based combination therapy
ADCC	Antibody-dependent cell-mediated cytotoxicity
AhR	Aryl hydrocarbon receptor
AOM	Azoxymethane
APC	Adenomatous polyposis coli
BAC	bacterial artificial chromosome
CAC	Colitis-associated colorectal cancer
CD	Cluster of differentiation
cDNA	Complementary DNA
COPD	Chronic obstructive pulmonary disease
CRC	Colorectal cancer
DEG	Differential gene expression
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate
DSS	Dextran sulfate sodium
DTE	Dithioerythritol
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
FACS	Fluorescence-activated cell sorting
Foxp3	Forkhead box p3
FP365	Fluorescent protein 365
GWAS	Genome-wide association studies
IBD	Inflammatory bowel disease
IFNg	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-12p40	Interleukin 12 subunit p40

IL-22	Interleukin 22
IL-22BP	Interleukin 22 binding protein
IL-22RA1	IL-22 receptor a1
IL22RA2	IL-22 receptor subunit alpha 2
IL-TIF	IL-10-related T cell-derived inducible factor
ILC	Innate lymphoid cells
IMID	Immune-mediated inflammatory disease
iNKT	Invariant NKT cells
iRBC	Infected red blood cells
iTreg	Induced Treg
JAK	Janus kinase
MACS	Magnetic activated cell sorting
MAPK	MAPK
MEICS	Murine endoscopic index of colitis severity
MHC	Major histocompatibility complex
mRFP	Monomeric red fluorescent protein
mRNA	Messenger RNA
MS	Multiple sclerosis
n.s.	Non-significant
NET	Neutrophil extracellular trap
NK	Natural killer
NLRP	Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain
NP40	Nonidet P40
nTreg	Natural Treg
OXPHOS	Oxidative phosphorylation
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffered Saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PLZF	Promyelotic leukemia zinc finger
PRR	Pathogen recognition receptor
RAG	Recombination activating gene
RNA	Ribonucleic acid
RORgt	RAR-related orphan receptor gamma (thymus-specific isoform)

ROS	Reactive oxygen species
RT-PCR	Real-time PCR
SEB	Staphylococcal enterotoxin B
sgBFP	SuperGlo blue fluorescent protein
SNP	Single nucleotide polymorphism
SPF	Specific pathogen-free
STAT	Signal Transducer and Activator of Transcription
TCR	T cell receptor
TGFb	Transforming growth factor beta
T_h	T helper cell
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-alpha
T_r1	Type 1 regulatory
Treg	Regulatory T cell
UMAP	Uniform Manifold Approximation and Projection for Dimension Reduction
UV	Ultraviolet
µ	Micro
WHO	World health organization

(most genes have been omitted here for practical purposes)

List of top 50 genes

Table 22: Top 50 genes in each cluster in Figure 8b

	0	1	2	3	4	5	6	7	8	9	10	11
1	Rac2	Ahnak	Lef1	Cd74	Tmsb4x	Fcer1g	Hmgb2	Cd74	Lyz2	Gzma	Marcks	Ccl6
2	Izumo1r	Itgb1	Dgka	Cd79a	Tpt1	Cst3	Mki67	Itgax	Malat1	Ccl5	Marcksl1	Alox5ap
3	Ikzf2	Lgals1	Actn1	Igkc	Tmsb10	Lyz2	H2afz	H2.Eb1	H2-D1	Fcer1g	Etv3	Junb
4	Gimap3	Ms4a4b	Igfbp4	Ighm	Lyz2	Cybb	Lmnb1	H2.Ab1	Tmsb4x	Nkg7	Ccr7	Lilr4b
5	Selenow	Nkg7	Gimap3	Ighd	Rplp1	Ifitm3	Lgals1	Gm2a	H2-K1	Klrk1	Basp1	Fcer1g
6	Pfn1	Ccl5	Il7r	Cd79b	Fau	Ctss	Birc5	H2.Aa	Apoe	Klrd1	Cd74	Ccl3
7	Pabpc1	AW112010	Ablim1	Ms4a1	Ccl5	Ctsb	Crip1	Rgs2	Tpt1	Ncr1	Icosl	Ccl9
8	Ptma	Id2	Nsg2	H2.Ab1	Rpsa	Csf1r	Ccna2	Marcks	Cybb	Klre1	Relb	Ifitm1
9	Sf3b1	Cd48	Als2cl	H2.Aa	Ubb	Apoe	Hmgn2	Ccnd1	Sat1	Klrb1c	Anxa3	Ier2
10	Samhd1	Lck	Gm20186	H2.Eb1	H2-D1	Ifitm2	Nucks1	Plbd1	Cst3	AW112010	Cacnb3	Hdc
11	Set	Gm42418	Gimap6	Cd19	Crip1	Ace	Itgb1	Pmaip1	Tmsb10	Ahnak	Fscn1	Csf2rb
12	Rplp2	Hopx	Cd3e	H2.DMb2	Sh3bgrl3	Napsa	Lbr	Pip4k2a	Fcer1g	Klrc2	Plxnc1	Itm2b
13	Foxp3	Crip1	Bcl11b	Iglc3	Rplp0	Cebpb	Cdca8	Ciita	Cebpb	Ctsw	Dnase1l3	Ccl4
14	Pim1	Anxa6	Atp1b3	Iglc2	Cst3	Ms4a6c	Hmgb1	Cbfa2t3	Psap	Klra8	Apol7c	Cd63
15	X1.Sep	Fau	Lck	Fcmr	Rplp2	Clec4a3	Emp3	Ffar2	Grk3	Klra9	Cxcl16	Fxyd5
16	Cd3e	Itgb7	Ets1	Cd22	Tyrobp	Ear2	Itgb7	H2.DMb2	Csf1r	Klra7	Lsp1	Jun
17	Rhoh	Lat	Rflnb	Bank1	Ftl1	Lst1	Dek	Cyp4f16	Fau	Gzmb	Mxd1	Nedd4
18	Il2ra	Gm9844	Arl4c	Ly6d	S100a6	Ftl1	Clic1	Jun	Pou2f2	Klra4	Id2	Cyp4f18
19	Pnrc1	Il18rap	Itk	Mef2c	S100a4	Fcgr4	H2afx	Dnase1l3	Tmcc1	Ctsd	Cd83	Gata2
20	Rack1	Cd3e	Ccr7	Fcer2a	Apoe	Adgre4	Nusap1	Nav1	Rpsa	Atp1b1	Pfkfb3	Csf1
21	Rgs10	Cx3cr1	Ms4a4b	H2.Ob	Ifitm3	Gpx1	Cenpa	Csf2ra	Rplp1	Il2rb	Ctsh	Cd7
22	Ctla4	Klrg1	Pced1b	Ebf1	H2-K1	Cd300a	Cenpe	Egr1	Tyrobp	Prf1	Icam1	Hacd4
23	Serbp1	Atp1b3	Npc2	Ly6e	Hist1h2ap	Gngt2	Kif11	Filip1l	Napsa	Itgam	Bmp2k	Cxcr2
24	Inpp4b	Cd52	Gm2682	Pax5	S100a10	Cyba	Ezh2	Adam23	Zeb2	Il18rap	Mreg	Grina
25	Rplp0	Ctsd	Dapl1	Cd37	Vim	Cd300e	H2afv	Ctss	Ace	Ccl3	Birc2	Ms4a2
26	Sell	Hmgb2	Atp1b1	Fcrla	Fcer1g	Ifngr1	Cks1b	Nrros	Son	Id2	Aebp2	Egr1
27	Ptprcap	Arpc1b	Foxo1	Cd55	Gzma	Cd300c2	Cdk1	Fcer1g	Ubb	Ly6c2	Rogdi	Ftl1
28	X7.Sep	Itgal	Foxp1	Ly86	Uba52	Fgr	Anp32e	Fgr	Ifitm3	Klri2	Fam49a	Neat1
29	Cd3g	Il18r1	Fam78a	H3f3a	Gngt2	Clec4a1	Ncapd2	Ier5	Clec4a3	Cma1	H2.Aa	Emilin2
30	Ift80	Emp3	Emb	Cd24a	Hist1h2ae	Cd68	Ccnb2	Fbrsl1	Lrp1	Eomes	Ccdc88a	Cyp11a1
31	Ptp4a2	Eef2	Ly6c1	Ralgps2	Ppia	Alox5ap	Hsp90b1	Grk3	Stap1	Lgals1	H2.Eb1	H3f3b
32	Sf1	Laptm5	Grap2	Blk	Lst1	Grk3	Incenp	I830077J02R	Ccl5	Chsy1	Rgs1	Cd9
33	X6.Sep	Bin2	Pabpc1	Cr2	Hist1h1b	Ceacam1	Kif15	Ctsh	Adgre4	Gm42418	Pmaip1	Cd200r3
34	Pdcd4	Arl4c	Pdk1	Bcl11a	Gm42418	Itgal	Cks2	Alox5ap	Ftl1	Ccl4	Apobec3	Cpa3
35	Cd3d	Hcst	Pik3ip1	Irf8	Serf2	Atp1a1	Esyt1	Ptprc	Itgal	Klrb1f	Cd63	Gsr
36	Ptprc	Ifng	Ikzf1	Btla	Gpx1	Hfe	Kif23	Dock10	Spn	Ms4a4b	Clic4	Lat2
37	Prrc2c	Itgb2	Macf1	Apobec3	Cebpb	Fau	Hnrnpa2b1	Man2b1	Adgre5	Itgb2	Nfkbia	Hgf
38	Ikzf4	Il2rb	Rapgef6	Ciita	Ubc	Itgb2	Cdc25b	Plxnc1	Ptpre	Itga2	Rnf19b	Ier3
39	X9.Sep	Cd4	Il6ra	Blnk	S100a11	Cd9	Ms4a4b	Pld4	Gpr141	Irf8	Adam23	Cdh1
40	Peli1	Ccr2	Eef1b2	Filip1l	Plac8	Lyn	Anxa2	Btg2	Ctsb	Klrc1	Gpr132	Emb
41	Sec61g	Maf	Inpp4b	Nap1l1	Ifitm2	Adgre5	Cdca3	Dpp4	Stk38	Klrb1a	Rassf3	Csf2rb2
42	Ppia	Atp2a3	Cd3g	Pou2af1	Stmn1	Csf2ra	Dnmt1	Mdh2	Rplp2	Anxa2	Cflar	Lgals3
43	Ppp1r18	Lsp1	Cd3d	Mzb1	Sub1	Cx3cr1	Gm10282	Otulinl	Tln1	Txk	Adam8	Igf1r
44	Cd27	Ccnd3	Cd4	Icosl	Txn1	Ccl6	Ncapg	Rgs1	Rbpms	Fgl2	Cbfa2t3	Aldoa
45	Ctse	Cxcr6	Npm1	Lmo2	Ptma	Mpeg1	Cd48	Rnf149	Pid1	Arsb	Pim1	Ctsd
46	Psmb8	Gtf2i	Pik3cd	Foxp1	Trbc2	Msrb1	Fbxo5	Icosl	Cd300a	Itga4	Arl5c	Ncf1
47	Sdf4	Gzma	Add3	Pxk	Slfn2	Nfam1	Cbx3	Prkcb	Neat1	Gm19585	Fbrsl1	Mpc2
48	Prkar1a	Capn2	Peli1	Ptpn6	Srgn	Ly6e	Mad2l1	Ly86	Ifngr1	Efhd2	Dusp5	Fcer1a
49	Resf1	Flna	Ms4a6b	Fcrl1	Hmgb2	Cyp4f18	Hmmr	Nr4a1	Ubc	Ccr5	Gtf2a1	Gstm1
50	Cd2	Klf2	Pitpnc1	Gga2	Uqcrh	Cd300ld	Cenpf	Lyst	Ssh2	Camk2n1	Flt3	Nfkbia

Links to sequencing databases

Table 23: Sequencing databases

Single cell expression atlas	**https://www.ebi.ac.uk/gxa/genes/ensmusg00000039760?bs=%7B%22mus%20musculus%22%3A%5B%22CELL_TYPE%22%5D%7D&ds=%7B%22kingdom%22%3A%5B%22animals%22%5D%7D#baseline.)**
Imm gen	**http://rstats.immgen.org/Skyline/skyline.html (search for *Il22ra2*)**
Human cell atlas	**http://immunecellatlas.net/ICA_Skyline.php?gene=OR1J4&celltype=all&organ=Blood&datatype=rnaseq&scale=Local (search for *IL22RA2*)**

List of Tables

List of Figures

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Publications:

1. **Sabihi M**, Böttcher M, Pelczar P, Huber S. *Microbiota-Dependent Effects of IL-22.* Cells. **2020** Sep 29;9(10):2205.

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Declarations

Statement under oath

I hereby declare upon oath that I have written the present dissertation independently and have not used further resources and aids than those stated.

Hamburg, April 2023

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hamburg, April 2023