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Doctoral thesis

Involvement of Notch pathway in synovial fibroblasts and hematopoietic cells in experimental models of arthritis

by

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Oslo, June 2023

Katarzyna Justyna Marciniak

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Abbreviations

ACPA	anti-citrullinated protein antibody
ACR	American College of Rheumatology
APC	antigen presenting cell
CAIA	collagen antibody-induced arthritis
CD	cluster of differentiation
CDR	complementarity-determining regions
CFA	complete Freud's adjuvant
CIA	collagen-induced arthritis
COVID-19	Coronavirus disease 2019
CRP	C reactive protein
CTL	cytotoxic T lymphocyte
DAF	decay accelerating factor
DAMP	danger-associated molecular pattern
DLL	Delta-like ligand
DMARD	disease-modifying antirheumatic drug
dsRNA	double-strained RNA
DTH	delayed-type hypersensitivity
DTHA	delayed-type hypersensitivity arthritis
ECM	extracellular matrix
EULAR	European League Against Rheumatism
Fc	fragment crystallizable
FLS	fibroblast-like synoviocyte
HFLS-RA	Human fibroblast-like synoviocytes of RA patients
ICAM1	intercellular adhesion molecule 1
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphatic cell
INF-γ	interferon γ
JAG	Jagged
JAK	Janus kinase
KO	Knockout
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
MAC	membrane attack complex
mBSA	methylated bovine serum albumin
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MPO	myeloperoxidase
MTX	methotrexate
NF-kB	nuclear factor-kappa B
NICD	Notch intracellular domain
NK	natural killer
NRR	negative regulatory region
OPG	osteoprotegerin
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PGR4	proteoglycan 4
PRR	pattern recognition receptor
RA	rheumatoid arthritis
RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
RF	rheumatoid factor
scRNAseq	single cell RNA sequencing
ST2	suppression of tumorigenicity
TCR	T cell receptor
Tfh	follicular helper T cell
TGFβ	transforming growth factor β
Th	helper T cell
THY1	thymus cell antigen 1
TIMP	tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFRSF11	tumor necrosis factor receptor superfamily member 11
TNFSF11	tumor necrosis factor superfamily member 11
Treg	regulatory T cell
VCAM1	vascular cell adhesion molecule 1
VLA4	very late antigen 4

List of Papers

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Paper II
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Front. Immunol. 12:689057. doi: 10.3389/fimmu.2021.689057

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IL-33-deficient rats to study function in model with human-like features
Monika Szymanska, Katarzyna Marciniak, Astrid Haaskjold Lossius, Francesca Gatti, Clara Hammarstrom, Olav Sundnes, Johanna Hol Fosse, Guttorm Haraldsen
Manuscript

Paper not included in the thesis

A novel somatic mutation in GNB2 provides new insights to the pathogenesis of Sturge–Weber syndrome
Roar Fjær, Katarzyna Marciniak *, Olav Sundnes *, Hanne Hjorthaug, Ying Sheng1, Clara Hammarstrom, Jan Cezary Sitek, Magnus Dehli Vigeland, Paul Hoff Backe, Ane-Marte Øye, Johanna Hol Fosse, Tor Espen Stav-Noraas, Yuri Uchiyama, Naomichi Matsumoto, Anne Comi, Jonathan Pevsner, Guttorm Haraldsen * and Kaja Kristine Selmer *
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1 Introduction

This thesis describes elements of the Notch pathway involved in rheumatoid arthritis (RA) and possible therapeutic manipulations of the pathway to attenuate the disease progression studied on animal experimental models and human material.

To understand the topic, the Introduction starts with the description of the immune system and inflammation, followed by a short explanation of autoimmune disorders, with focus on RA. Subsequently, synovial fibroblasts and other synovial cells will be introduced, highlighting their contribution to the disease progression. Lastly the Notch signaling pathway will be explained, as well as a brief description of interleukin 33.

1.1 The immune system

The immune system is a protective machinery evolved in multicellular organisms. It is a collection of cells and molecules, located in the tissue of organs and in the blood. In vertebrates, they are further divided into two main arms of immunity, innate and adaptive immunity, which cooperate to fight the invaders (Figure 1). Once the harmful agent is successfully neutralized and removed, the immune system initiates self-resolving and repairing mechanisms to terminate the reaction. (Kumar, Abbas, Aster, & Perkins, 2018; Murphy & Weaver, 2017). An important function of the immune system is to fight the infection caused by pathogens, yet in other forms of immunity, the reactions can be elicited to the non-infectious agents, like environmental molecules (e.g., allergens), to the neo-antigens of tumors and not the least to host components released during trauma or damage caused by loss of blood supply and necrosis (e.g., myocardial infarction). Therefore, the term “immunity” may be defined as the reaction of the immune system to any molecule that can be recognized as foreign or dangerous. And despite the importance of the immune system action, it may also cause disease development, especially when the response is impaired, or when it fails to tolerate harmless or host molecules.

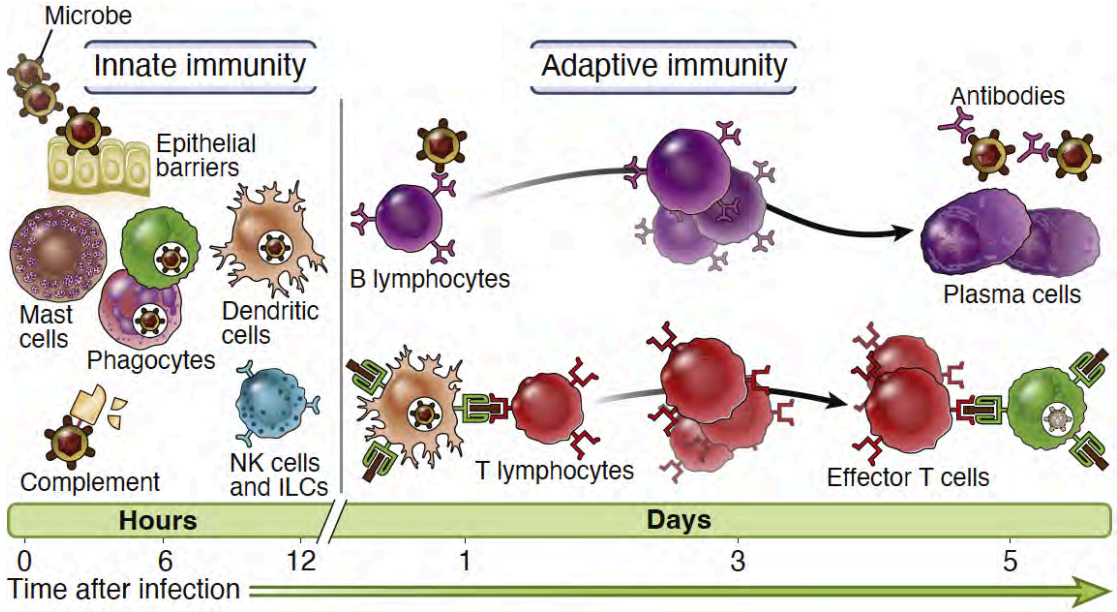

Figure 1. Innate and adaptive immunity (Abbas, Lichtman, Pillai, & Baker, 2020)

The immunity has been conserved among species and is sufficient to fight most of the invaders. The phylogenetically older innate immunity evolved in invertebrates and persists in higher vertebrates. Adaptive immunity, on the other hand, is characteristic only for vertebrates (from jawed vertebrates, like sharks), and developed around 360 million years ago (Abbas et al., 2020; Abbas, Lichtman, Pillai, Baker, & Baker, 2018). Despite conservation of the system, some differences evolved between various species. In this thesis, the description will focus on the human and murine immune system.

1.1.1 The immune system tissues and organs

The first component of the immune system are barriers to block the entry of the pathogens across the epithelial interface of the skin and the mucosa, and respond with the release of mediators that leads to further activation of the immune system (Abbas et al., 2020; Kumar et al., 2018). Other elements of the immune response generally originate from *primary* (generic or central) lymphoid organs where the naïve lymphocytes mature, like bone marrow (B cells) or thymus (T cells), and from *secondary* (peripheral) lymphoid organs, like spleen, lymph nodes, mucosal and cutaneous tissues, where these cells become activated effector or memory cells (Abbas et al., 2020).

1.1.2 Origin of the immune cells

Immune cells are found in various tissues or circulate in the blood. The majority of them are derived from common precursors in the bone marrow, where they - with the exception of T cells - develop to maturity (Figure 2) (Abbas et al., 2020).

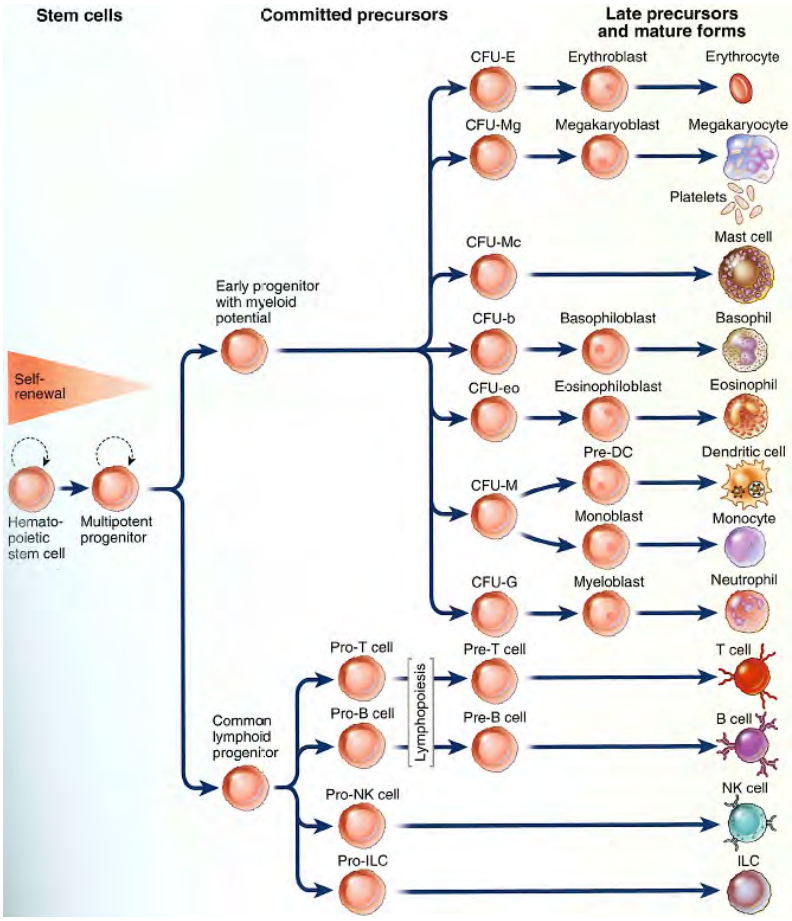

Figure 2. The immune system cells (Abbas et al., 2018)

Other cells, like tissue-resident macrophages, originate from the yolk sack or fetal liver (Figure 3). These cells were seeded into the tissues before birth and are maintained throughout the life. They are independent of macrophages derived from blood circulating monocytes, self-renewing populations of immune cells (Murphy & Weaver, 2017).

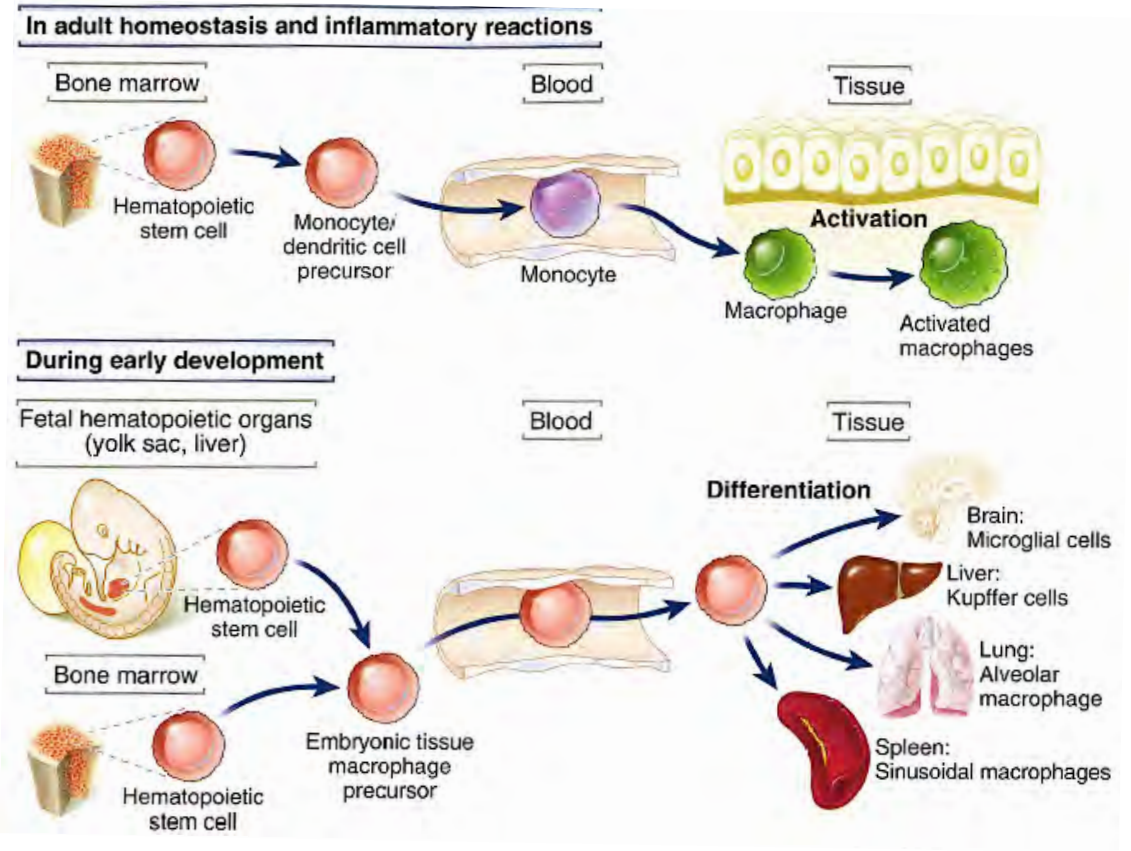

Figure 3. Origin of macrophages (Abbas et al., 2020)

1.1.3 The innate immunity cells

The immune cells belonging to innate immunity are the first line of the defence, and they can be classified as tissue resident cells, such as sentinels (macrophages, dendritic cells, mast cells) or innate lymphatic cells (ILCs), or as blood-derived leukocytes like monocytes, granulocytes (neutrophils, basophils, eosinophils) and natural killer (NK) cells.

One of the main immune cells involved in innate immune responses are macrophages derived from circulating monocytes. They are the main phagocytic cells. Depending on the received signals, monocytes can differentiate into pro- or anti-inflammatory macrophages, type M1 or M2, respectively (Figure 4). This differentiation is rather flexible, and several subtypes were detected, especially for the M2 type. Perhaps accordingly, macrophages have several functions in the tissue: they can phagocyte microbes or dead cells, act as antigen-presenting cells (APCs), secrete pro- and anti-inflammatory cytokines, activate other cells, and contribute to wound healing and even fibrosis.

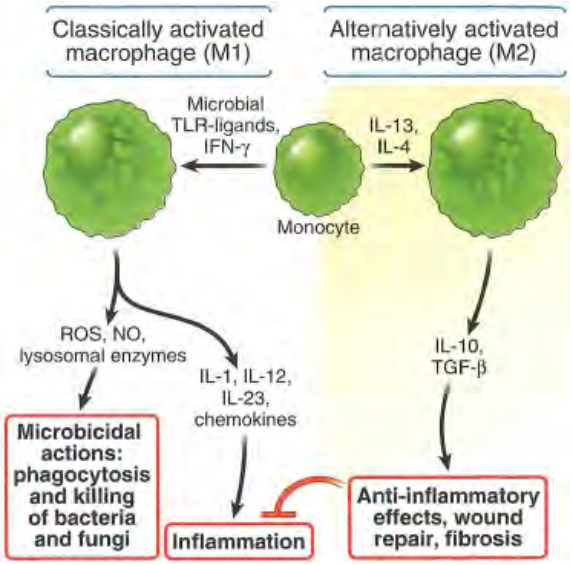

Figure 4. Macrophage polarization (Abbas et al., 2020)

Another abundant cell type is the neutrophil, which also have capability of engulfing and eliminating microbes. They live shorter that macrophages and engage in a "suicidal" action to release nets of DNA that immobilize bacteria.

1.1.4 Receptors of innate immunity

Microbes have specific biochemical features, which are not present in eukaryotes, that result in pathogen-associated molecular patterns (PAMPs), which can be recognized by the innate immune cell receptors (pattern recognition receptor, PRR) and elicit activation (Figure 5). Not only microbes can bind to these receptors and stimulate the response, also signals (molecules) from necrotic cells or injured cells can serve as damage-associated molecular patterns (DAMPs) (Figure 6). Several types of pattern recognition receptor families recognize several classes of microbes PAMPs or DAMPs (Abbas et al., 2020; Kumar et al., 2018). The total number of receptors of different type is above 100 and together they can recognize up to 1000 different molecules. These receptors are expressed by innate immune cells and cannot be modified in the same ways as the receptors of adaptive immunity cells.

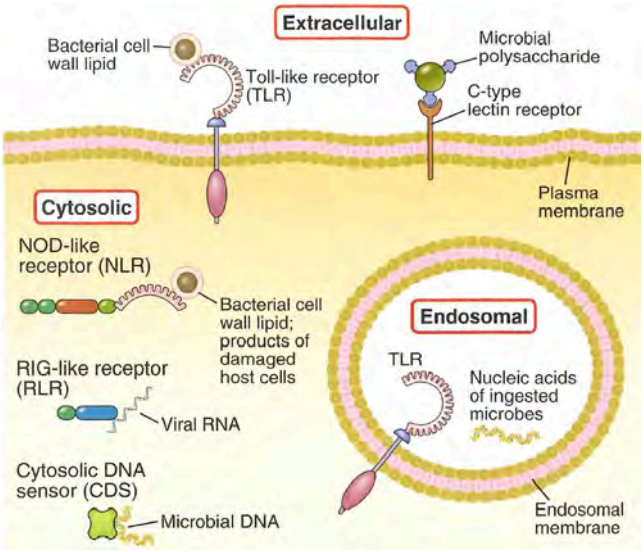

Figure 5. PAMPs and PRRs (Abbas et al., 2020)

1.1.5 The adaptive immunity cells

Adaptive immunity is an acquired response which adopts to the pathogen in efforts to neutralize and eliminate the invader (Kumar et al., 2018). As in the innate immunity, both cells and soluble mediators are involved, but they are less present at the site of the infection, reflecting the complex interplay with several immune cells that must take place in the required microarchitecture of secondary lymphoid organs. Adaptive immunity needs more time to initiate the responses, but once activated, they are very specific and efficient to fight the pathogen.

B and T lymphocytes are mediators of adaptive immunity. Both pass through the time consuming processes of clonal expansion and several differentiation steps in order to be fully activated and ready to respond (Abbas et al., 2020). Naïve lymphocytes circulate between the blood and the secondary lymphoid organs until they encounter their cognate antigen and become active effector cells. In the absence of antigen, they survive in the circulation for several months up to few years. (Abbas et al., 2020; Murphy & Weaver, 2017). Lymphocytes are carrying out two main types of adaptive immunity, the cell-mediated immunity by the effector T cells against intracellular pathogens and the humoral immunity mediated by the extracellular microbe specific antibodies produced by B cells that mature into plasma cells (Abbas et al., 2020).

The effector T cells can be distinguished based on the expression of surface molecules initially identified as cluster of differentiation proteins (CDs) in the early days of leukocyte typing based on monoclonal antibodies (Bernard & Boumsell, 1984). Hence, CD8 positive T cells – also called cytotoxic T lymphocytes (CTL) – are involved in direct elimination of infected cells, while CD4 positive T cells –are also called helper T cells (Th), as they serve other cells, for instance B cells, to produce antibodies, or phagocytes to eliminate microbes. The helper T cells can be further subdivided based on the class of the pathogen they elicited responses to, and for instance we can distinguish Th1, Th2, Th17 or follicular helper (Tfh) cells (Figure 7). CD4 positive T cells secrete proinflammatory cytokines and activate several cell types; for example, Th1 cells produce interferon-γ (IFN-γ) to support proinflammatory macrophages, Th17 cells secrete among other cytokines interleukin (IL) 17, contributing to neutrophil infiltration and Th2 cells secrete IL4, IL5 and IL13, promoting eosinophil recruitment and immunoglobulin class switching. Both Th1 and Th17 cells are involved in defence against microbes but also the damage of autoimmune disorders. On the other hand, Th2 cells, are important in defence against parasites but also aberrantly activated in allergic inflammation. Among CD4 positive cells, there is also special subset, regulatory T lymphocytes (Treg), which function is to limit or even prevent the immune responses. (Abbas et al., 2020; Kumar et al., 2018)

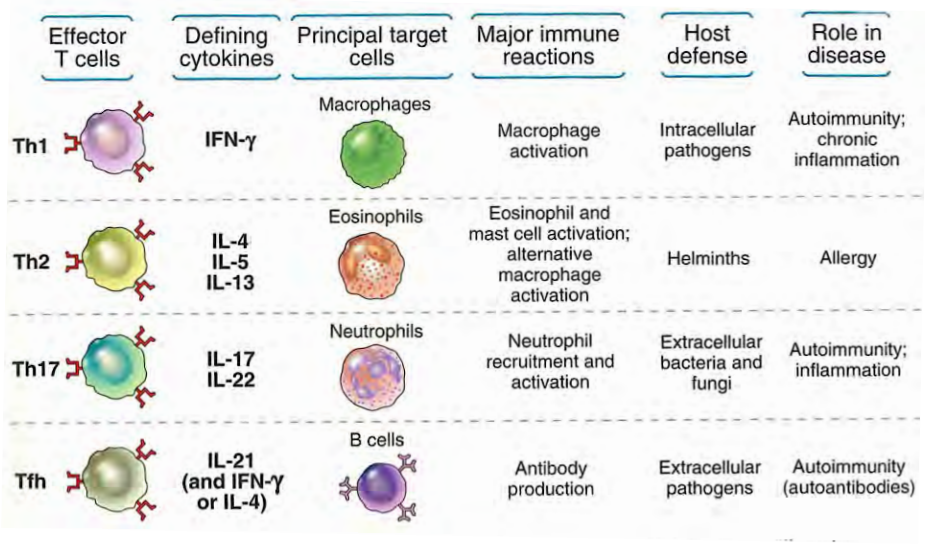

Figure 7. CD4 helper T cells subsets (Abbas et al., 2020)

1.1.6 The T cell receptors

Both B and T cell receptors, as wells as antibodies are highly diverse and variable to fit foreign substances, much wider than innate immune cell receptors. The specific part of the foreign molecule that is recognized by these host molecules is called antigen. it can have pathogen origin, but it might also be a non-infectious molecule. The difference in recognition lies in the size and structure of detected antigen. T cells recognition is limited to peptide fragments of protein antigens, which are processed and displayed by other cells. Antibodies can also recognize three-dimensional structures of proteins, as well as carbohydrates, lipids or nucleic acids (Abbas et al., 2020). There are only two types of receptor backbone T cell receptor (TCR) and in man five classes of antibodies, but somatic hypermutation of their genes allows the adaptive immune system to generate a variability of antigen recognizing sites that can recognize millions of different molecules (Abbas et al., 2020; Kumar et al., 2018).

T cells can recognize processed structures of pathogen or other foreign matter displayed on major histocompatibility complex molecules type I (MHCI, on all host cells) or MHCII (engulfed and displayed) on APCs. In result protecting from intracellular microbes or helping B cells in removal of extracellular pathogens. The TCR recognizing antigens are membrane bound and composed of two subunits (Figure 8).

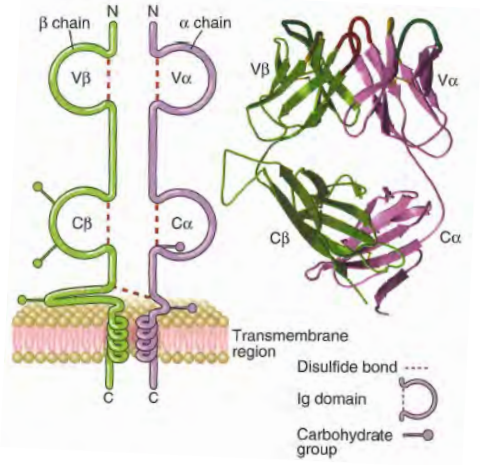

Figure 8. T cell receptor (Abbas et al., 2018)

1.1.7 Antibodies

Antibodies, also called immunoglobulins (Igs), are B cell-derived molecules of adaptive immunity (Figure 9). These proteins bind to the recognized epitope, a dimensional structure on the surface of the pathogen or other molecule. They are designed to block infection, initiate elimination of coated microbes or neutralize toxins produced by pathogens. They work as an immobilizing agent, preventing ability to enter the cell, or as a label leading to recognition by other immune system components (opsonization), like phagocytes, tagging the microbe for the elimination or activation of the complement system. There are five classes of immunoglobulins: IgA, IgD, IgE, IgG, IgM. They differ in structure, localization and type of reaction that they are involved in. Once secreted, they enter the circulation, from where they can translocate to tissue and even to the lumen of mucosal organs. IgA is a mucosal localized, soluble homodimer; IgE is binding parasites or allergens, IgM and IgD are bound to the B cell surface and act as the receptor initiating the process of cell activation (proliferation and differentiation). Finally, IgG is soluble and indeed the most abundant antibody found in the circulation. The general architecture of soluble immunoglobins is composed of two subunits bound together via disulfide bridges. Each subunit contains one heavy and one light chain which are bound also by disulfide bridges and hydrogenic bindings. Both chains have three variable regions called complementarity-determining regions (CDRs) which constitute the recognition site (paratope) of the epitopes. The heavy chain in addition contains constant fragment crystalized (Fc) region, which is the site recognized by Fc receptors on the immune cells (and other cell types) (Abbas et al., 2020; Murphy & Weaver, 2017).

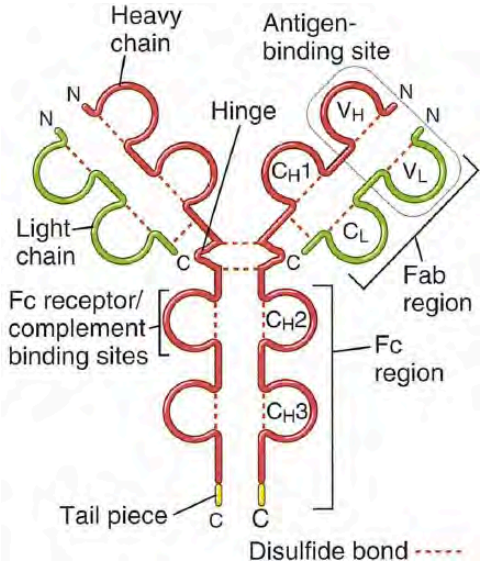

Figure 9. Schematic structure of IgG antibody (Abbas et al., 2018)

1.1.8 Cytokines

Another feature of the immune system are several molecules, among them, soluble proteins called cytokines, important for mediating immune responses. They are a broad category of proteins produced by several cell types in response to activation signals, acting on cells in different manners and serving information to the rest of the environment or even to themselves (autocrine stimulation). More details of main cytokines can be found in Table 1 (Abbas et al., 2020; Fajgenbaum & June, 2020; Kumar et al., 2018).

Mediator	Main Cell Source	Type and Function
Cytokines and growth factors		
Interleukin-1	Macrophages, epithelial cells; pyroptotic cells	Proinflammatory alarmin cytokine; pyrogenic function, macrophage and Th17 cell activation
Interleukin-2	T cells	Effector T-cell and regulatory T-cell growth factor
Interleukin-6	Macrophages, T cells, endothelial cells	Proinflammatory cytokine; pyrogenic function, increased antibody production, induction of acute-phase reactants
Interleukin-9	Th9 cells	Protection from helminth infections, activation of mast cells, association with type I interferon in Covid-19[26]
Interleukin-10	Regulatory T cells, Th9 cells	Antiinflammatory cytokine; inhibition of Th1 cells and cytokine release
Interleukin-12	Dendritic cells, macrophages	Activation of the Th1 pathway; induction of interferon-γ from Th1 cells, CTLs, and NK cells; acting in synergy with interleukin-18
Interleukin-17	Th17 cells, NK cells, group 3 innate lymphoid cells	Promoting neutrophilic inflammation, protection from bacterial and fungal infections
Interleukin-18	Monocytes, macrophages, dendritic cells	Proinflammatory alarmin cytokine; activation of Th1 pathway, acting in synergy with interleukin-12
Interleukin-33	Macrophages, dendritic cells, mast cells, epithelial cells	Proinflammatory alarmin cytokine; amplification of Th1 and Th2 cells, activation of NK cells, CTLs, and mast cells
Interferon-γ	Th1 cells, CTLs, group 1 innate lymphoid cells, and NK cells	Proinflammatory cytokine; activation of macrophages
Tumor necrosis factor	Macrophages, T cells, NK cells, mast cells	Increasing vascular permeability; pyrogenic function
GM-CSF	Th17 cells	Proinflammatory cytokine
VEGF	Macrophages	Angiogenesis
Chemokines		
Interleukin-8 (CXCL8)	Macrophages, epithelial cells	Recruitment of neutrophils
MIG (CXCL9)	Monocytes, endothelial cells, keratinocytes	Interferon-inducible chemokine; recruitment of Th1 cells, NK cells, plasmacytoid dendritic cells
IP-10 (CXCL10)	Monocytes, endothelial cells, keratinocytes	Interferon-inducible chemokine; recruitment of macrophages, Th1 cells, NK cells
MCP-1 (CCL2)	Macrophages, dendritic cells, cardiac myocytes	Recruitment of Th2 cells, monocytes, dendritic cells, basophils
MIP-1α (CCL3)	Monocytes, neutrophils, dendritic cells, NK cells, mast cells	Recruitment of macrophages, Th1 cells, NK cells, eosinophils, dendritic cells; pyrogenic function
MIP-1β (CCL4)	Macrophages, neutrophils, endothelium	Recruitment of macrophages, Th1 cells, NK cells, dendritic cells
BLC (CXCL13)	B cells, follicular dendritic cells	Recruitment of B cells, CD4 T cells, dendritic cells†

Table 1. Cytokines and soluble mediators (Fajgenbaum & June, 2020)

1.1.9 The complement system

The complement system is a cascade of interrelated molecules involved in immune responses (Figure 10). This system is part of innate immunity but also activated by immunoglobulins of adaptive immunity. The main function of the complement is to defend against microbes. There are three activation pathways, called classical, alternative and lectin pathway. The difference is the activation mode of inactive forms of complement proteins, triggered either by the presence of surface-bound antibody (classical pathway, IgM or IgG), microbial surface molecules (lipopolysaccharide (LPS) or endotoxin) and liver-derived mannose-binding lectins that bind microbial carbohydrates (lectin pathway), respectively. All pathways lead to proteolytic activation of downstream elements and form an enzymatic cascade leading to further cleavage of complement proteins. All pathways converge at the activation of C3 convertase which cleaves C3 protein to soluble C3a and C3b. Both parts can participate in further effector functions. For instance, soluble C3a (and downstream C5a) acts as a chemoattractant for leukocytes, whereas C3b binds the surface of microbes and promotes phagocytosis (opsonization) and also by ensuing proteolytic

activity, leads to downstream activation of complement proteins C5-C9 to eventually form membrane attack complex (MAC) and disrupt microbial cell walls (Abbas et al., 2020; Ciurana & Tomás, 1987; Kumar et al., 2018).

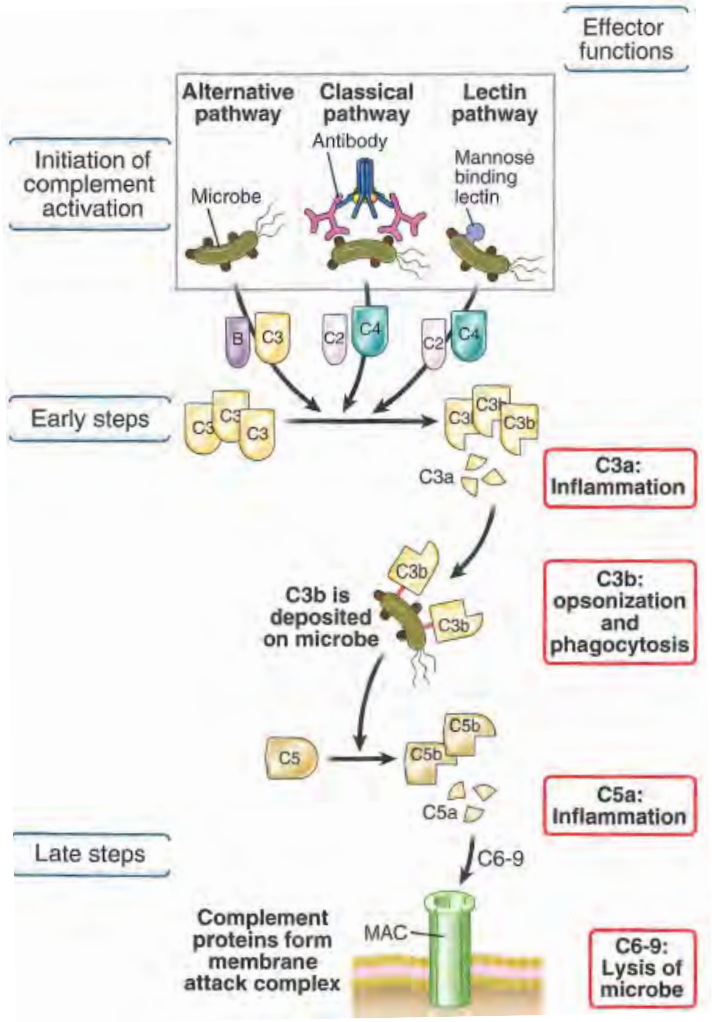

Figure 10. Complement system (Abbas et al., 2020)

1.1.10 The immune system responses

Major types of pathogens are extra- and intra-cellular bacteria (and archaea), viruses, fungi, and parasites. Each has unique ways of invasion, colonize various tissues, and need different mechanisms of elimination. The physical barriers are the first-line defence and if the pathogen penetrates the tissue, the next step is to mount immune responses.

There are three main types of responses (type 1-3) reflecting the properties of pathogen behaviour (Figure 11). They engage the most effective mechanisms of defence, while restraining the remaining types.

Innate immunity responses provide the first line of the defense against infectious pathogens. It is mediated by the cells and proteins constantly present in the body (at the site of contact) and can therefore act upon invaders immediately after the infection occurs. Cells detecting pathogens, secrete distinct cytokines and other stimulating molecules, promoting inflammation (the reaction described in Chapter 1.2) or a response to viral infection. Responses also include the activation of different subsets of innate lymphoid cells (ILCs) which then further promote specific responses, helping

to tailor the type of response best suited to the type of pathogen that was encountered. If these are effective and eliminate invader, then no further responses are needed. However, this type of response is neither antigen-specific nor is it efficient enough to fight all the pathogens, as some of them have developed resistance to innate immunity (Abbas et al., 2020; Kumar et al., 2018; Murphy & Weaver, 2017).

For instance, in the type 1 response, intracellular microbes evoke responses in dendritic cells or macrophages to produce IL12, leading to production of IFN-γ by ILC1 and antigen-activated CD4-positive T cells (Th1). Type 2 responses are induced by parasites, which are too large to be phagocyted, and need a different way of action. Their infection promotes release IL25, IL33 and TSLP from epithelial cells to activate ILC2 cells and their release of IL4, IL5 and IL13, further supported by Th2 cells to activate dendritic cells, mast cells and eosinophil for production of worm coating IgE, eosinophils activation and binding with Fc receptor to IgE, and mucus secretion and peristaltic movement, respectively. These cytokines also shape a polarization of M2 macrophages. Th2 cells may also involve in allergic reactions, where allergens trigger mast cell and eosinophil activation. Finally, the type 3 response involves development of Th17 cells to extracellular bacteria and fungi. Major cytokines released from ILC3s to promote Th17 polarization are IL17 and IL22. Major effector cells are neutrophils and to lesser extent monocytes and inflammation is prominently induced reactions. Th17 produce more IL17 which stimulates other cell type for leukocyte attracting chemokines production, or antimicrobial substances as defensins. IL22 also affects epithelial cell defensin production and tissue surface integrity maintenance (Abbas et al., 2020).

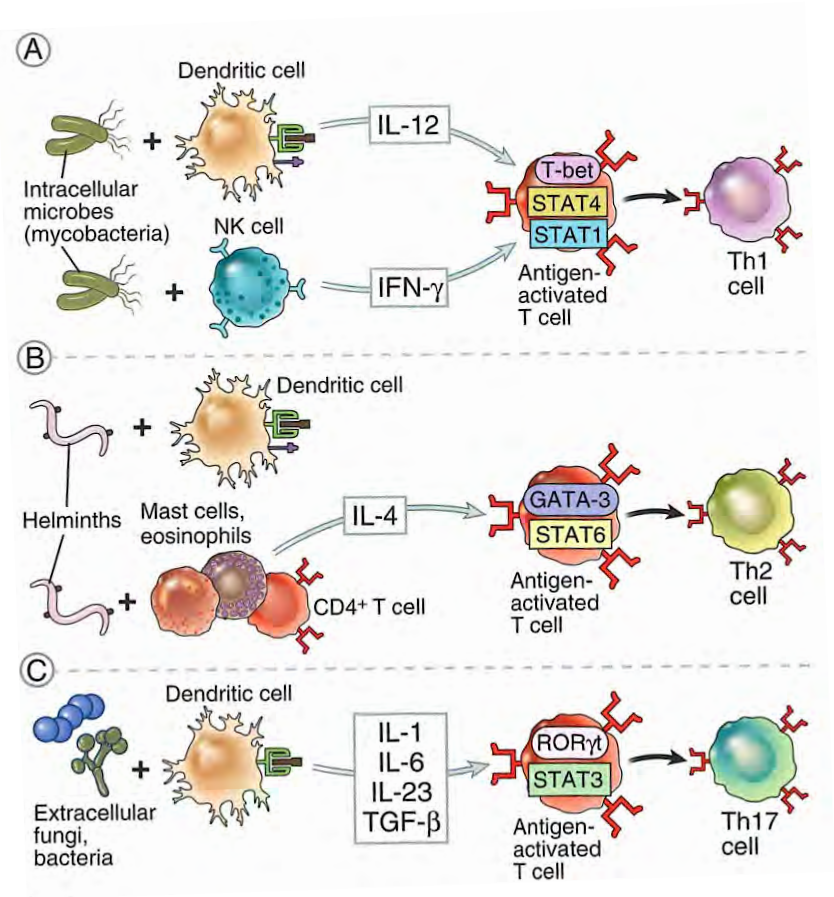

Figure 11. The immune system responses (Abbas et al., 2020)

1.1.11 The immune system memory

Memory against the microbe may mounted, and when the same pathogen occurs again, the adaptive immunity responses will react in more rapid and powerful manner. The memory of adaptive immunity is simply the maintenance cells from the pool of clones which recognize the pathogen, called long-lived memory lymphocytes, and their rapid proliferation (clonal expansion) when the infection recurs. For instance, memory B cells are plasma cells, which migrate to the bone marrow, and are capable to survival for many years, producing antibodies, after the infection was eradicated. Memory T cells are circulating in peripheral blood and can make up to 50% of circulating T cells, accumulated with every infection as the host age (Abbas et al., 2020).

1.2 Inflammation

Inflammation is a physiological body response to adverse stimuli, consisting of trigger removal (in the case of pathogens), tissue repair and return to the state of homeostasis. Acute inflammation, which is a controlled, intermittent and fundamental protective response of the host, however once turned to chronic condition, it may lead tissue damage and inflammatory disorder progression. (Munier, Ottmann, & Perry, 2021). Understanding mechanisms and molecules that are involved in inflammation, especially in persisting inflammation, is important in developing ani-inflammatory drugs and novel therapy strategies (Kumar et al., 2018).

1.2.1 Acute inflammation mechanisms

Clinically, inflammation is characterized by five signs: swelling, redness, warmth, pain, and loss of function (in Latin – *tumor, rubor, calor, dolor, function laesa*), which all, have their reflection in molecular mechanisms (Netea et al., 2017). As a multicomponent reaction, it involves the action of innate immune responses in the vascularized tissue, and if needed, followed by adaptive immunity (Munier et al., 2021).

The first step after microbe entry is activation of sentinel cells like macrophages, dendritic cells, mast cells and other tissue cells which recognize harmful agents (Figure 12). These cells start to secrete proinflammatory cytokines, chemokines and other molecules, which can activate endothelial cells, increase permeability of the vessels, leakage of plasma proteins and recruitment of leukocytes. Once translocated to the site of the inflammation all the components neutralize and fight the invaders. Last step is clearance of the offending agent and termination of the signals (Abbas et al., 2020).

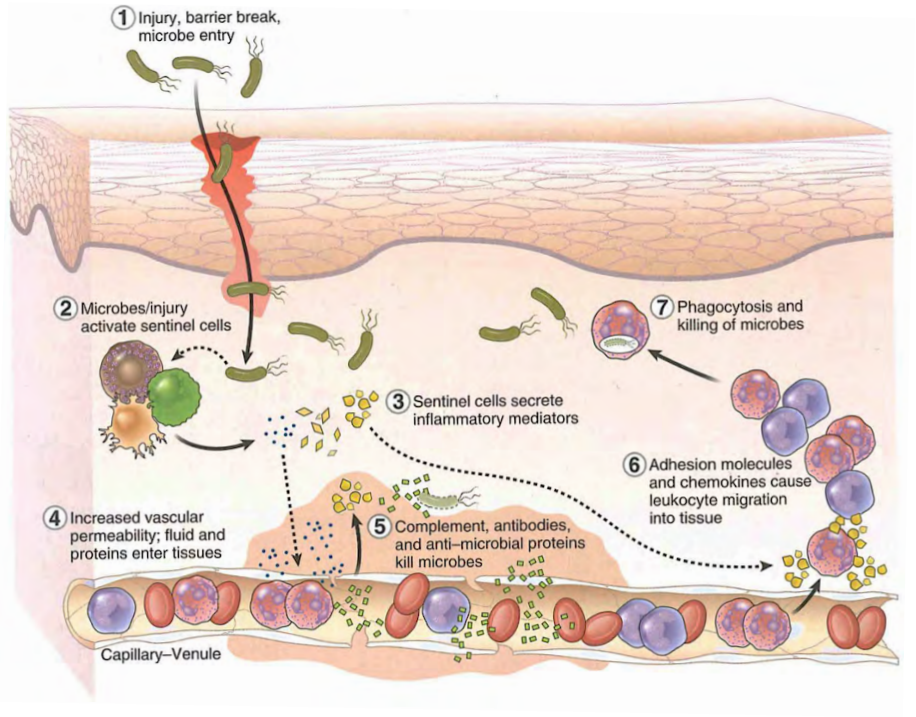

Figure 12. Inflammation (Abbas et al., 2020)

Recruitment of leukocytes is a process where circulating cells roll on the surface of the endothelium due to selectin engagement and facilitated by changes in blood flow and shear forces, until activated by chemokines or other chemoattractant on the endothelial cell surface to engage in firm adhesion of the leukocyte to endothelium and final migration through endothelium into the tissue (Figure 13).

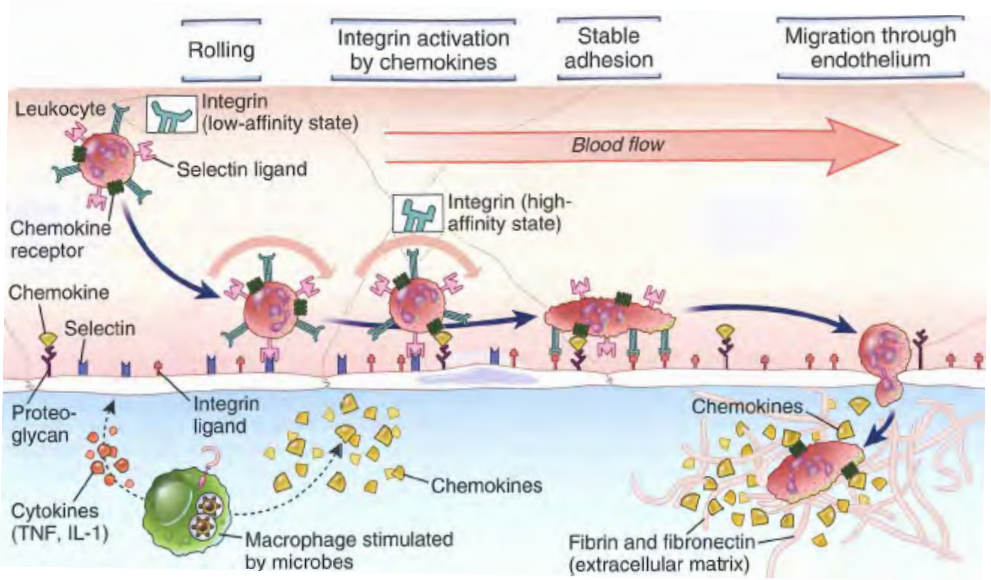

Figure 13. Leukocyte rolling (Abbas et al., 2018)

Inflammation is also associated with acute-phase responses in plasma, due to rapid production of proinflammatory cytokines IL1, IL6 and tumor necrosis factor (TNF) in response to engagement of pathogen recognition receptors (e.g. LPS/endotoxin from

gram-negative bacteria or double-stranded RNA (dsRNA) from RNA-virus). These responses induce systemic reactions, characteristic for severe illnesses, like sepsis or influenza. Clinical and pathogenic changes include rise in body temperature (fever), presence of plasma proteins (C-reactive protein (CRP), fibrinogen, serum amyloid A), elevated numbers of leukocytes in blood, altered blood pressure, rigors, and chills. Acute-phase responses may lead to a cytokine storm seen in sepsis (septic shock) or in coronavirus disease (COVID-19) (Abbas et al., 2020; Kumar et al., 2018).

1.2.2 **Resolution of inflammation and tissue repair**

The natural termination of the acute inflammation is resolution of the proinflammatory signals and tissue repair. However, when the inflammation become chronic, it may lead to more serious tissue damage or development of fibrosis. (Abbas et al., 2020; Kumar et al., 2018; Netea et al., 2017). Inflammation is a powerful process, which cause clearance of invaders, but it is also harmful to the host tissues, by damaging it and toxin insults. Therefore, precise mechanisms resolving the reactions and repair of the tissue are necessary. One of the forms of the control is short half-live of the proinflammatory mediators and activated cells, reducing inflammation signals when the stimulus is removed. In addition, as the reaction evolves, certain stop signals are triggered and actively terminate the process, such as arachidonic acid metabolite production switch from leukotrienes to lipoxins, or release of anti-inflammatory mediators, as IL10 or transforming growth factor β (TGFβ) (Kumar et al., 2018).

Two types of reactions contribute to tissue repair, the regeneration of residual cells and deposition of connective tissue (scar forming or fibrosis). The first reaction replaces damaged parts with components, which enable return to the natural state of the tissue from before the injury. It is due to the cells that survived the injury and have capacity for proliferation, such as cells from the epithelia of the skin or intestine, liver cells and others. The second type of mechanism involves formation of fibrous tissue by fibroblasts, which provides scaffold and structural stability for the tissue incapable of complete restoration but often results in a loss of healthy tissue function. During tissue repair occurs also a close interaction between cells and extracellular matrix (ECM), including new blood vessels formation (angiogenesis). New vessels provide necessary signals and nutrients for the healing processes (Kumar et al., 2018).

1.2.3 **Chronic inflammation mechanisms**

Chronic inflammation is a sustained inflammation lasting for weeks or months, combined with episodes of tissue injury and repair attempts. It can be a continuation of unresolved acute inflammation or can arise independently. It is caused by autoimmune diseases, allergy reactions, prolonged infections, exposure to toxins or transplantation mismatch. In contrast to acute inflammation, it is a result of the activation of mononuclear immune cells, like macrophages, lymphocytes, and plasma cells. Instead of angiogenesis, tissue destruction combined with healing processes are predominant (headed by fibrosis). Macrophages, the dominant cell type contributing to chronic inflammation, produce cytokines and growth factors promoting activation of several cell types to maintain inflammation and tissue injury. They can also display antigens to T lymphocytes contributing to their activation. Other abundant cell types in chronic inflammation are T and B lymphocytes, often involved in persistent and sever inflammation. CD4 positive T cells secrete proinflammatory cytokines, promote activation and recruitment of several cell types. In addition, accumulated mononuclear immune cells cluster together in the tissue and may form tertiary lymphoid organs, a

lymphoid structure resembling lymph node follicle. These structures are often seen in tissues in several disorders, such as in the joint of rheumatoid arthritis patients (Kumar et al., 2018).

1.3 Autoimmune disorders

Autoimmune disorders arise when the immune responses fail to tolerate self-antigens and mount excessive, self-perpetuating reactions, leading to chronic inflammation and tissue damage. Reactions may be mediated by autoantibodies or by self-reactive T cells. The etiology often is not proven in this type of disorders, nevertheless many risk factors have been associated, several genetic, epigenetic, and environmental. Examples of such disorders are multiple sclerosis, rheumatoid arthritis, or systemic lupus erythematosus. Autoimmune disorders can be organ-specific or systemic, damaging particular or several organs. In systemic disorders, autoantibodies complexes act on connective tissue and blood vessels of the affected organ (Kumar et al., 2018).

1.4 Rheumatoid Arthritis

Rheumatoid arthritis is a progressive inflammatory disease, affecting synovial joints (described in Chapter 1.5), leading to their destruction. Patients experience a severe reduction in quality of life due to joint swelling, synovitis and destruction of cartilage and bone. Chronic inflammation also leads to several systemic manifestations, enhancing cardiovascular disease risk. The origin of RA is unclear, however a combination of genetic and epigenetic risk factors with individual environmental exposure, like smoking, microbe infection or other, are major indicators of disease development (Figure 14). Among genetic factors several variants in the MHC class II regions, like HLA-DRB1 alleles predispose RA. Occurrence of autoreactive antibodies is another characteristic, most notably rheumatoid factor (RF) or antibodies against various proteins that had been post-translationally modified, for example by citrullination (anti-citrullinated protein antibody, ACPA), the latter highly specific for RA (ACPA-positive RA) (Deane & El-Gabalawy, 2014). Clinical manifestation may begin several years after the potential triggers occurred. Increasing joint-specific inflammation leads to pre-clinical manifestations and perception by the patient followed by clinical diagnosis and transition to chronic phase (Yau & Holmdahl, 2016).

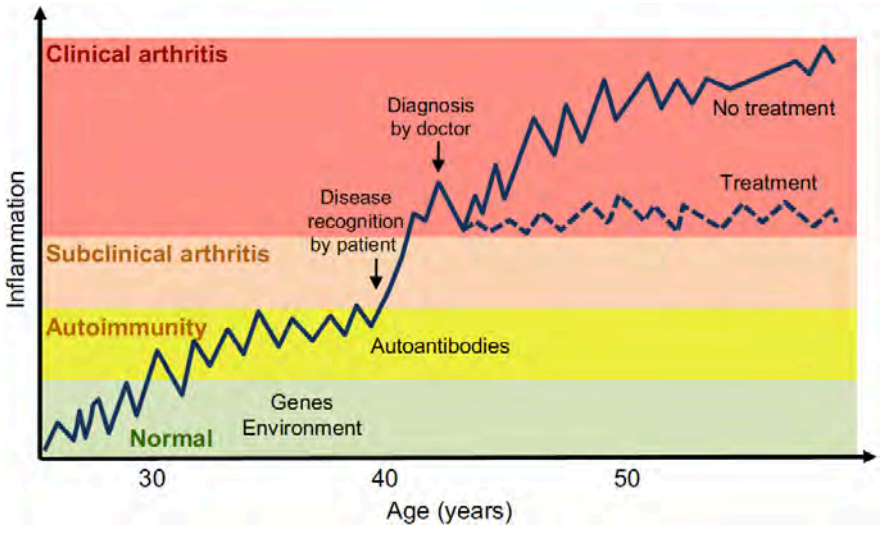

Figure 14. Rheumatoid arthritis stages (Yau & Holmdahl, 2016)

Key features of synovial joint changes in RA are hypertrophy of the synovial lining, mononuclear infiltrates in the sublining with increased vascularity, collagen deposition (fibrobsis) and development of lymphoid follicles (Figure 15). ACPA-positive RA patients tend to have more lymphoid follicles with germinal centres than ACPA-negative, which on the contrary have more fibrosis (Hitchon & El-Gabalawy, 2011).

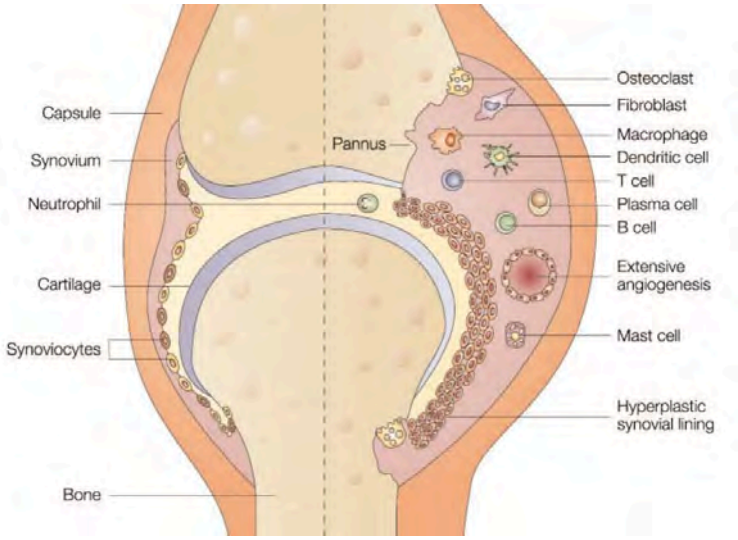

Figure 15. Key features of rheumatoid arthritis (Smolen & Steiner, 2003)

The pathogenesis consists of an abnormal interplay between components of adaptive and innate immune responses (Figure 16). The main observations are initial break of tolerance to self, excessive accumulation of responsive T cells and other newly arrived leukocytes like monocytes in synovium, increased activity, and expansion/-invasiveness of tissue resident cells, such as synoviocytes (macrophages and fibroblasts) and vascular endothelial cells, as well as activation of osteoclasts. Extensive synovial hyperplasia leads to pannus formation. (Alivernini et al., 2019; Scherer, Haupl, & Burmester, 2020). This characteristic, highly destructive tissue contains macrophages, activated osteoclasts and protease-expressing fibroblasts. It is located at the interface between synovium, cartilage, and bone, leading to their erosion and eventually destruction (Hitchon & El-Gabalawy, 2011). The B cell involvement is yet elusive, nevertheless presence of autoantibodies and the fact that B cells were detected in synovial tissues are pinpointing their contribution. (Alivernini et al., 2019; Scherer et al., 2020).

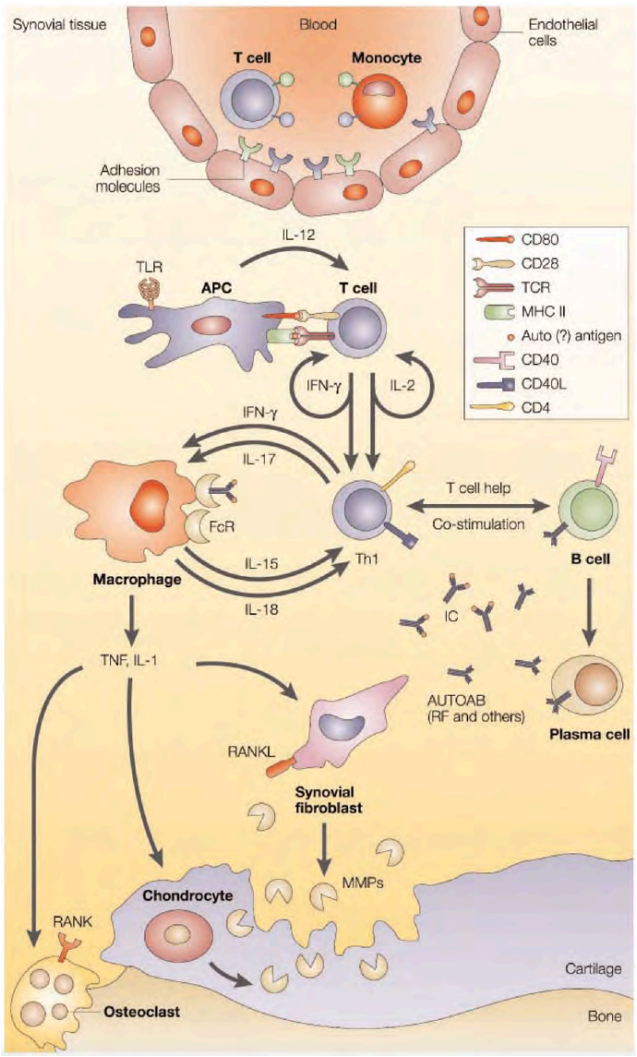

Figure 16. The pathogenesis of RA (Smolen & Steiner, 2003)

Current treat-to-target recommendations of the European League Against Rheumatism (EULAR) are conventional synthetic disease-modifying antirheumatic drugs (DMARDs), like methotrexate (MTX) in combination with short term usage of glucocorticoids, accompanied by pain killers. After few months of evaluation, in case of insufficient performance, further stratification is recommended. Subsequential step is shifting to another groups of drugs, either biological DMARDs (bDMARDs), like TNF inhibitors (adalimumab, certolizumab pegol, etanercept, golimumab, infliximab), anti-T-cell target (abatacept), anti-B-cell agent (rituximab), and IL-6 receptor blocker (tocilizumab), or targeted synthetic DMARDs (tsDMARDs), like Janus kinase (JAK) inhibitors (tofacitinib, barcitinib). If the patient does not prognose well according to the American College of Rheumatology (ACR) classification criteria, and manifest loss of function to the extent that the prothesis is needed, the surgical intervention may be required. In situation when patient reach remission, then the doses of selected treatment are lowered and sustained. Despite several therapy strategies, large number of RA patients (up to 2/3) do not achieve stable remission stage, and moreover current treatments have several side effects. Therefore, further therapeutic options are needed (Donlin et al., 2018; Smolen et al., 2017).

1.5 Synovial joint

Synovial joints are cavitated joints, enabling a wide range of motion (diarthrosis). Hyaline cartilage-covered bones are linked by a joint capsule to embrace a space lined with thin membrane called synovium, structured with ligaments, and filled with lubricant – the synovial fluid – a viscous plasma filtrate enriched with hyaluronic acid, produced by synovial lining fibroblasts (Figure 17).

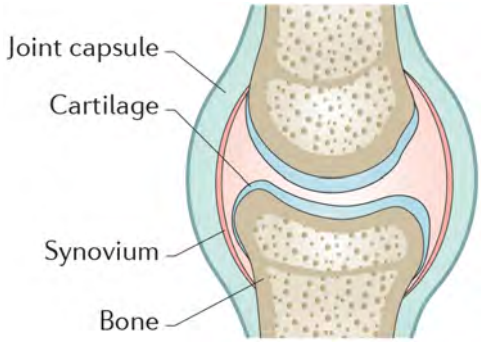

Figure 17. Healthy synovial joint (Smolen et al., 2018)

The majority of the body joints are synovial joints. The cartilage is composed of chondrocytes producing type II collagen, proteoglycans and matrix degrading enzymes (Kumar et al., 2018).

1.5.1 Synovium

Synovium is a thin tissue covering synovial joints. It contains no basement membrane, which improves efficient exchange of nutrients and waste between blood stream and synovial fluid (Kumar et al., 2018). The surface is composed of two layers, a thin lining and a thicker vascularised sublining (Figure 18). The inner lining layer is directly connected to the joint cavity, it contains type A synoviocytes – synovial lining macrophages, and type B synoviocytes – synovial lining fibroblasts. The outer layer, an interstitial sublining contains vasculature and lymphatics, nerve supply and adipocytes, resident cells like dendritic cells, macrophages and their precursors, stromal cells, like synovial fibroblasts and other immune cells from circulation (Iwanaga, Shikichi, Kitamura, Yanase, & Nozawa-Inoue, 2000; Smith, 2011).

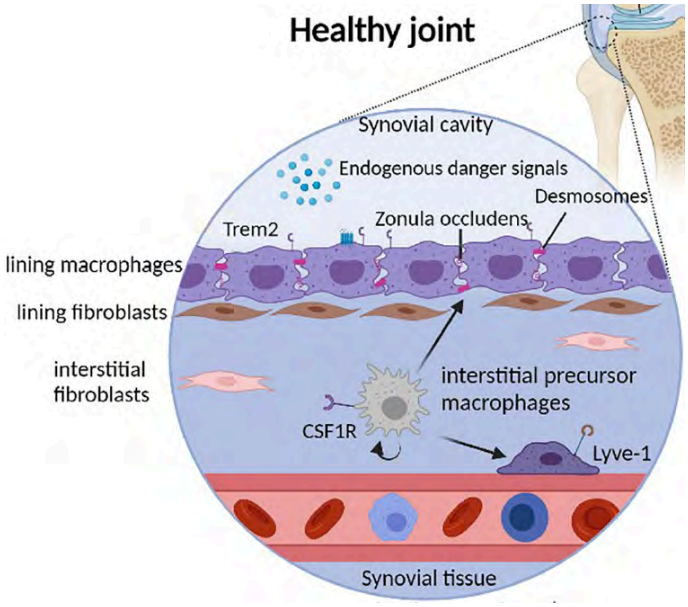

Figure 18. Synovium (Knab, Chambers, & Krönke, 2022)

1.5.2 Synovial fibroblasts

Synovial fibroblasts are distributed in the entire synovium. They are a heterogenous population, with a spectrum of phenotypes related to their location within the tissue. For instance, lining synovial fibroblasts are known to produce high levels of lubricating agents, like hyaluronic acids and lubricin (proteoglycan 4, PRG4) (Figure 19). On the other hand, synovial fibroblasts that agglomerate around vessels in the sublininggexpress high levels of adhesion molecules and some populations further dispersed in the sublining can express MHCII variants. In addition, several markers were described, like CD55 (decay accelerating factor, DAF) is found in high levels in lining fibroblast, whereas CD90 (thymus cell antigen 1, THY1) is more typically found in sublining fibroblasts and CD34 in perivascular subsets.

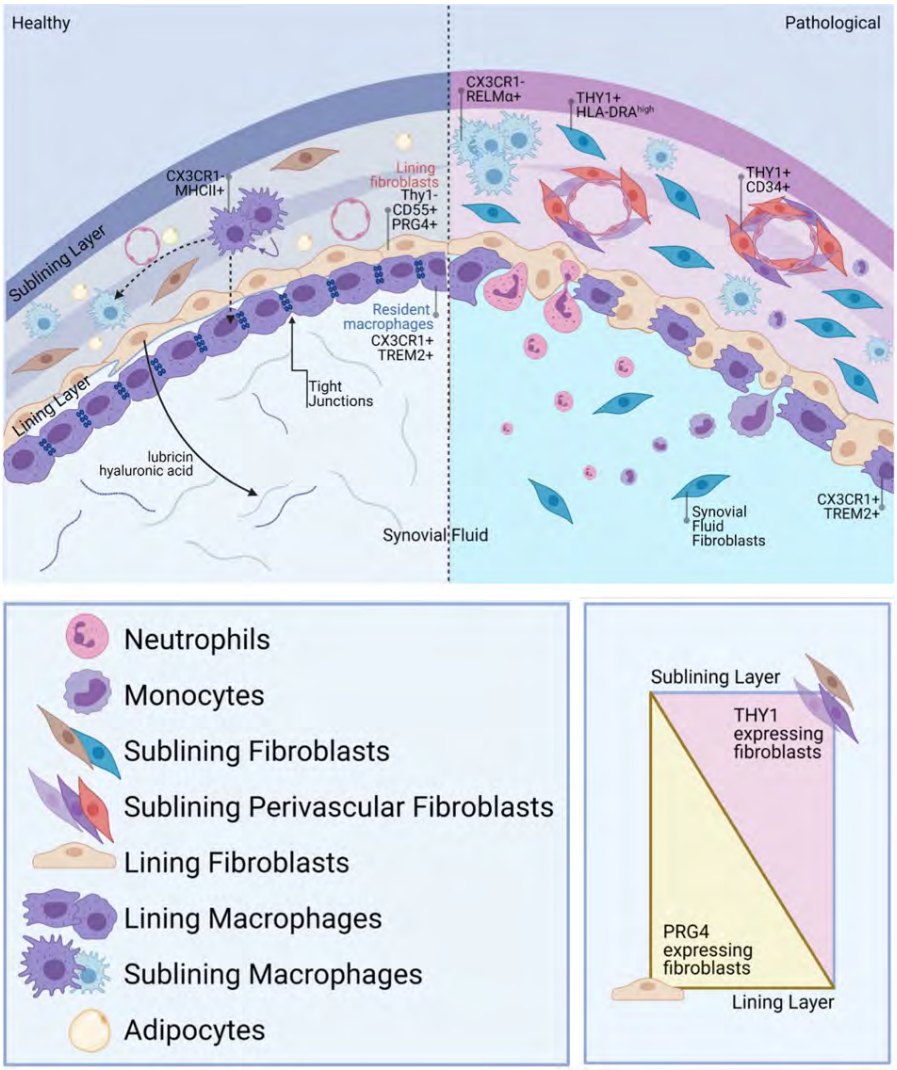

Figure 19. Synovial fibroblasts and synovial macrophages in health and pathological condition (Kemble & Croft, 2021)

Like other fibroblast types, synovial fibroblasts express extracellular matrix components, such as collagens, proteoglycans, fibronectin, and elastin, as well as ECM- degrading enzymes, such as matrix metalloproteinases (MMPs), as well as their inhibitors (e.g. tissue inhibitor of metalloproteinases, TIMPs). In addition, synovial fibroblast may produce RANKL (receptor activator of nuclear factor kappa-B ligand, also known as tumor necrosis factor superfamily member 11, TNFSF11), a major driver of osteoclasts differentiation. Moreover, synovial fibroblasts may serve as a

progenitor cell for other types of mesenchymal cells, like adipocytes, chondrocytes, or osteoblasts. They can also act as a sentinel cell, recognizing danger signals and interact with tissue macrophages or other immune cells. To this end, they express several Toll-like receptors (TLRs) – a class of pattern recognition receptors – especially high levels of TLR3 and TLR4. When responding, they promote inflammation by production of proinflammatory cytokines, chemokines, growth factors and proangiogenic factors. Synovial fibroblasts in RA, express increased levels of IL6, MMP3 and MMP13, which may suggest their contribution in immune responses initiating and/or perpetuating synovitis. In addition, they express adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM1), intercellular cell adhesion molecule (ICAM1), very late antigen 4 (VLA4) also known as integrin α4β1, which may play a role together with other structures in retaining ligand expressing inflammatory cells in the synovium, while other cells lacking the ligand, like neutrophils, may egress to synovial fluids (Hitchon & El-Gabalawy, 2011; Smith, 2011).

All of these characteristics serve to maintain homeostasis in the tissue, and contribute to development of disease if the balance is disturbed (Knab et al., 2022).

1.5.3 Synovial macrophages

Synovial macrophages also constitute heterogenous populations within the synovial tissues (Figure 19). They may be either tissue residents or monocyte-derived. In particular, lining and perivascular synovial macrophages have distinct functions. Lining macrophages express TREM2 and CX3CR1 – markers of high phagocytic activity – and are thought to create tight junctions to form a firm layer in the absence of a basement membrane. On the other hand, perivascular macrophages express chemokines responsible for leukocyte trafficking, like CCL24 and CCL12, and they express markers LYVE1, RELMA, CD163, and CD206/MRC1. The third well-characterized subtype of macrophages are the subintimal pro-inflammatory macrophages positive for CSF1R, MHCII molecules, which may also serve as precursor for other macrophage subpopulations (Figure 20 – no sign of those in figure 20?) (Knab et al., 2022).

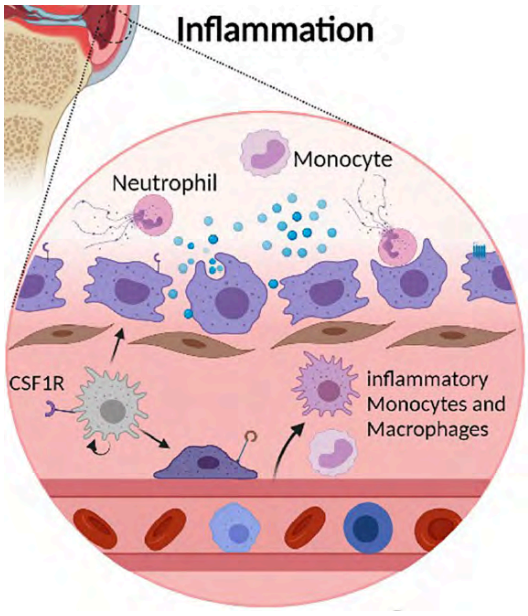

Figure 20. Inflammed synovium (Knab et al., 2022)

1.5.4 Bone and cartilage cells

Even though bone tissue appears static, it is in fact constantly undergoing remodelling processes based on the continuous balanced action of bone cells, among others, osteoblasts and osteoclasts. This turnover is tightly regulated by several cell-cell interactions and signalling molecules. The factors predominantly controlling this activity are RANKL (p.27) produced by fibroblasts and osteoblast, its receptor RANK (transmembrane receptor activator of NF-kB), expressed by osteoclast precursors, as well as osteoprotegerin (OPG, also known as tumor necrosis factor receptor superfamily member 11, TNFRSF11), expressed by stromal cells and osteoblasts (Figure 21). Binding of RANKL induces differentiation of the osteoclast and activation for bone degradation processes. It is an important activator of NF-kB, which promotes survival in osteoclasts. Conversely, soluble OPG acts to neutralize RANKL, blocking ligand access to RANK and as a result inhibiting osteoclast generation. Osteoblasts also produce M-CSF (macrophages colony-stimulating factor) which activates the M-CSF receptor on the osteoclast precursor. The ratio of RANK to OPG is an indicator of either bone resorption or formation (Kumar et al., 2018).

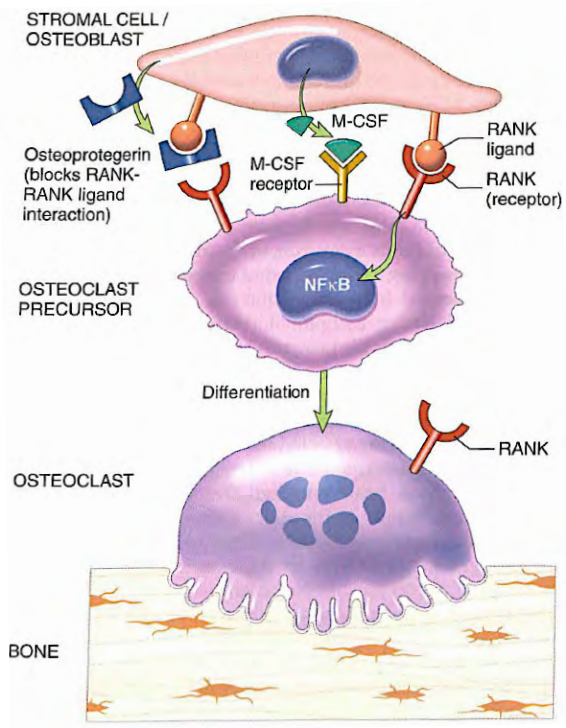

Figure 21. Mechanisms regulating osteoclast formation (He et al., 2020; Kumar et al., 2018)

1.6 Notch signalling pathway

The Notch signalling pathway was discovered in the fruitfly and it an evolutionarily strongly conserved mechanism (extensively reviewed in Zhou et al., 2022). It is known to be involved in several cellular and developmental processes like cell proliferation, apoptosis, migration, tissue homeostasis (Bazzoni & Bentivegna, 2019; Pancewicz, 2020; Siebel & Lendahl, 2017). It has proven action in immune responses and inflammation, reducing the cytokine-mediated histone acetylation at inflammatory enhancers, interfering with PI3K/AKT and NF-kB pathways on several levels (Arasu, Balakrishnan, & Velusamy, 2020) and recruiting inflammatory transcription factors of the AP-1 family. Finally, crosstalk also exists between Notch and the JAK/STAT signalling pathways (Hildebrand et al., 2018; Kamakura et al., 2004).

The Notch signaling pathway in vertebrates is mediated by four receptors Notch1-4 and five ligands Jagged (JAG) 1 or 2, and Delta-like ligand (DLL) 1,3, or 4, both single-pass transmembrane proteins (Figure 22). The receptors share structural similarities that are important for ligand binding, suchas the repetitive EGF-like structures and similarities that are important for receptor activation, such as the negative regulatory region (NRR). Both domains are found in the extracellular domain of the receptor. The Notch intracellular domain (NICD) is composed of an RBPJ association module (RAM) responsible for interactions in the nucleus with transcription factors, a module of ankyrin repeats (ANK) with nuclear localization sequences, and a domain with a proline/glutamic acid/serine/threonine-rich motif (PEST domain) responsible for the stability of the NICD. The ligands also contain EGF-like repeats determining the crosstalk with the receptor.

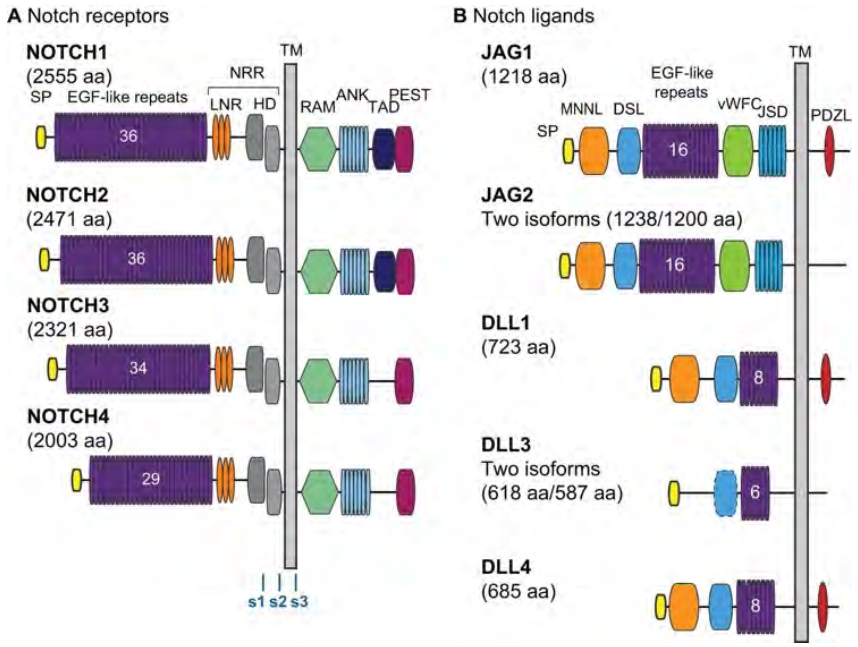

Figure 22. Notch signalling pathway components (Borggrefe & Oswald, 2009)

Upon the ligand binding, receptor cleaving sites are exposed to proteolytic enzymes as ADAM10 or ADAM17, and consecutive cleavage by enzyme, γ-secretase complex (Figure 23). These events lead to release of the cytoplasmic NICD fragment, which translocates to the nucleus, and forms a complex with the transcription factor RBP-Jκ (also known as CSL or CBF1) and becomes the Notch signalling complex. This leads to transcription of several direct and indirect Notch target genes, and action on other pathways, like c-myc and NF-kB (Pancewicz, 2020). The receptors and ligands are widely expressed, yet the expression patterns differ between cell types and tissues. For instance, endothelial cells are known to express NOTCH1, which then has impact on the cell fate and VEGF/Ang-2 mediated angiogenesis (Gao et al., 2012; Gao et al., 2013), as well as inflammation (Poulsen et al., 2018), while pericytes express NOTCH3. As both receptors and ligands are membrane bound, activation of the pathway requires close cell-cell contact between neighbouring cells (canonical pathway or trans interaction), yet Notch signalling also appear to happen between ligands and receptors in one cell (inhibitory non-canonical pathway, or cis interaction). The signals from binding of ligand to receptor are mainly studied for receptor bearing cells, however there are findings suggesting that changes occur also in cells providing

ligand (Bazzoni & Bentivegna, 2019; Pancewicz, 2020). The Notch signalling system was found to modulate immunity. And because of this potential, it become a particularly attractive target for drug development. Several studies showed that inhibition may effectively attenuate inflammation in models of several disorders. One known inhibition strategy is to prevent release of NICD (Figure 23) by targeting the γ-secretase with small chemicals leading to broad (all four receptors) inhibition and gastrointestinal toxicity due to on-target enterocyte trans differentiation. Therefore, more sublime targeting was designed to selectively target specific components of the pathway by means of monoclonal antibodies binding to the extracellular part of the receptor or ligand, preventing the ligand binding and/or receptor activation. To this end, inhibition of NOTCH1 (Choi et al., 2018; Kim et al., 2015), NOTCH3 (Wei et al., 2020) and DLL1 (Sekine et al., 2012) has been found to attenuate experimental arthritis.

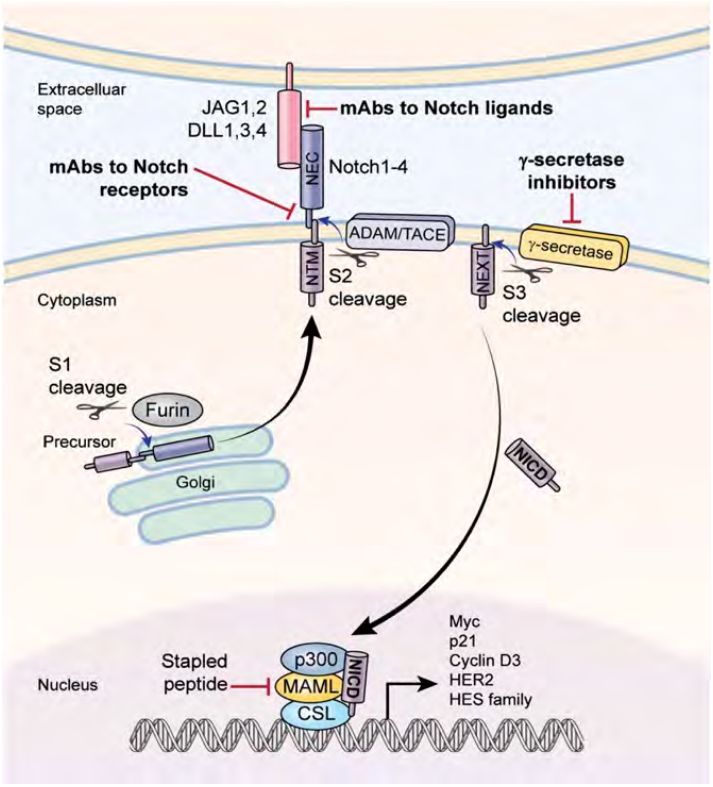

Figure 23. Notch signaling pathway and possible way of targeting pathway (Takebe, Nguyen, & Yang, 2014)

1.7 Interleukin 33

Interleukin 33 (IL33) is a cytokine that belongs to IL1 family. It is an alarmin, released from the cell at the early stage of the inflammatory responses to injury or stress signals. The full-length IL33 consists of two domains joined by a linker region (Figure 24, left panel). A N-terminal nuclear targeting domain has a chromatin-binding motif, the role of which is to retain the molecule in the nucleus. The binding to the chromatin is due to protein-protein interaction with an acidic pocket formed by histones H2A and H2B. A C-terminal domain has an IL1-like cytokine activity, responsible for cytokine-like activity of IL33. The mature form of IL33 has the C-terminal domain only, and it is generated either from alternative splicing transcription or from full-length form, cleaved at the linker region by serine proteases produced by neutrophils or mast cells. The mature form has a significantly higher cytokine activity than the precursor. Despite that,

both forms of IL33 are active in extracellular space, and can bind to its receptor, ST2. Membrane ST2 with bound IL33, interacts with coreceptor IL1RacP, which is shared with other IL1 family members (Figure 24, right panel). Next, MyD88 binds to internal part of the heterodimerized receptor-IL33 complex, followed by IRAK1, IRAK4 and TRAF6. These events mediate signaling of pathways similar to those initiated by IL1 and IL18, and activate transcriptional factors NFkB, p38, JNK and ERK (Cayrol & Girard, 2018; Dwyer, D'Cruz, & Turnquist, 2022).

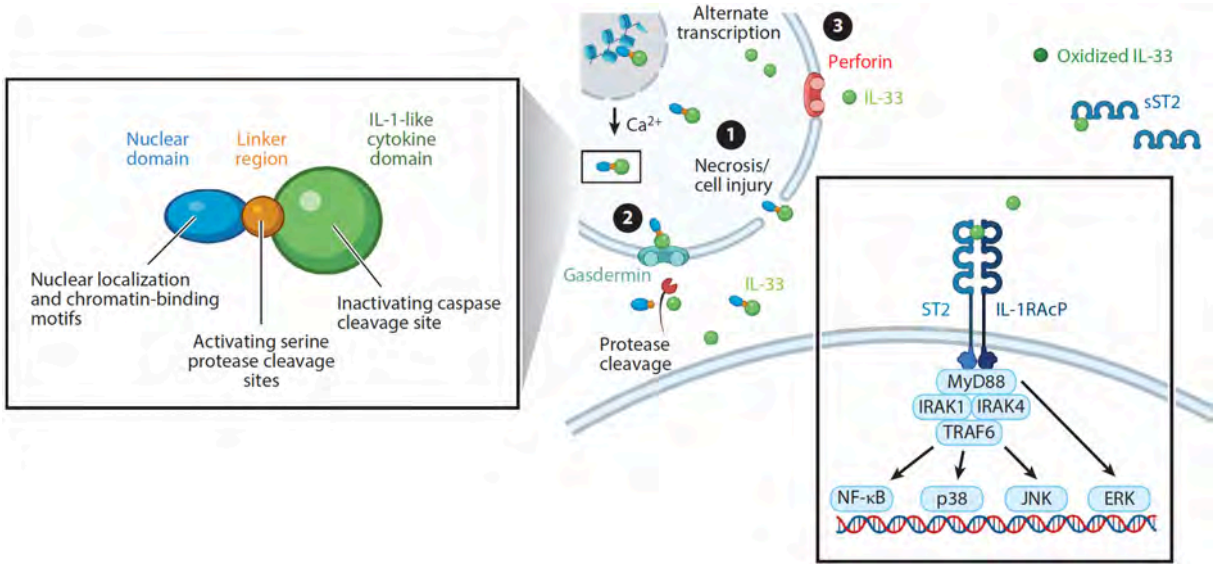

Figure 24. Interleukin 33 structure (left panel) and function (right panel) (Dwyer et al., 2022)

At steady state, IL33 is stored in nucleus in pre-mature form. There is no clear evidence of its action on gene expression. Nevertheless, its extracellular cytokine activity is prominently documented. In contrast to other family members, it is unlikely to be released via the ER-Golgi secretory pathway. Instead, it is released from the cell by several other mechanisms (Figure 24, right panel). First, its pre-mature form located in nucleus, may be secreted from the cell without maturation in the function of an alarmin in response to cell injury or stress signals. The pre-mature form can also translocate to cytoplasm in Ca^{2+}-dependent manner, where it may then be released via gasdermin D. The next possibility, is the secretion of cytoplasmic IL33, generated from alternative splicing and lacking the nuclear domain, in the transmission via perforin-2 mediated pores. The IL33 has strict regulatory mechanisms. First, the nuclear localization is limiting release of the pre-mature form leading to nuclear sequestration. During apoptosis, it may be inactivated by the action of caspase, targeting a cleavage site in the IL1-cytokine domain, leading to its ubiquitination. While IL33 is secreted from the cell, it may be oxidized at cysteine residues, forming disulfide bindings, that cause inactivating structural changes. Finally, a soluble form of ST2 receptor (sST2) has the ability to bind excess extracellular IL33 and therefore prevent binding to membrane ST2, which in consequence, limits transmission of the inflammatory signals (Cayrol & Girard, 2018; Dwyer et al., 2022).

IL33 is expressed at high levels mainly in the nuclei of non-hematopoietic cells in several tissues, such as endothelial cells, epithelial cells, keratinocytes, and stromal cells, as myofibroblasts. (Cayrol & Girard, 2018; Palmer et al., 2009). In contrast to

endothelial and epithelial cells, IL33 is generally not constitutively expressed in fibroblasts in high levels. However, in inflammatory condition, as stimulation with IL1b or TNFa, fibroblasts drastically increase expression and secretion of IL33 (Cayrol & Girard, 2018; Palmer et al., 2009). Moreover, the Notch signaling pathway also, plays a key role in the induction of nuclear IL33, observed in quiescent endothelial cells (Sundlisaeter et al., 2012). The receptor, ST2 is on the other hand, expressed on several immune cells, among others in Th2 cells, mast cells, neutrophils, macrophages, Th1 cells, CD8+ T cells or Treg cells. In addition, non-hematopoietic cells as endothelial cells (Pollheimer et al., 2013) or fibroblasts may also express the receptor(Shakerian et al., 2022).

IL33 has a strong relevance to type 2 immune responses by stimulating proliferation and cytokine production in Th2 cells, ILC2s and mast cells. In type 1 immunity, it activates CD8+ T cells, Th1 cells and NK cells to proliferate and secrete proinflammatory cytokines. IL33 has also immunoregulatory role, maintaining tissue homeostasis, tissue repair and resolution of inflammation by acting on M2 macrophages or Treg cells (Cayrol & Girard, 2018; Dwyer et al., 2022). Recently, IL33 regulatory effect on cellular metabolism (lipid and glucose metabolism) and metabolic checkpoints, like HIF-1a, PPARg, mTOR, AMPK, PI3K-AKT signaling, was revealed. For instance, IL33 contributes to M1 and M2 macrophage polarization and enhancing their metabolic processes, promoting glycolysis in M1, and fatty acid oxidation and oxidative phosphorylation in M2 (Lin et al., 2022).

Due to the role in the inflammation, IL33 involvement in several diseases was demonstrated, including allergies, asthma, atopic dermatitis, infectious diseases, cardiovascular diseases, fibrosis, tumors, and autoimmune disease as rheumatoid arthritis (Cayrol & Girard, 2018). In rheumatoid arthritis, elevated levels of IL33 in serum were associated with deteriorated recovery of the patients. The main source of IL33 in RA appears to be local, from inflamed joints and synovial fluid. TNF-exposed synovial fibroblast cultures obtained from RA patients responded with an increased expression of IL33 and induction of matrix metalloproteases MMP1 and MMP3 production (Cayrol & Girard, 2018; Dinarello, 2019; Dwyer et al., 2022).

Even though IL33 is evolutionary conserved in mammals, the expression of IL33 in several mouse tissues differs from that seen in human. In the mouse, it is not constitutively expressed in vasculature tree, as in human. In human lung, IL33 is highly expressed by lung epithelium, whereas in mice, it is detected in alveolar type II pneumocytes. Species-specific differences may have important impact on therapies that target IL33, especially in situation when results are obtained from mouse models exclusively (Liew, Girard, & Turnquist, 2016).

2 Summary of papers

The thesis is composed of four original papers, concerning rheumatoid arthritis in patients (Paper II) and in experimental models of arthritis (Paper I and III), as well as description of species differences (Paper IV).

2.1 Paper I

In Paper I we have shown selective inhibition of the Notch2, as the most suitable strategy to attenuate mouse arthritis models. We have shown that anti-Notch2 treatment reduces joint swelling and myeloperoxidase (MPO) activity (reflecting leukocyte infiltration), and that it prevents visible signs of cartilage and bone destruction in histology analysis. We have also determined that no gastrointestinal side effects are seen with complete blockage of the pathway with gamma-secretase inhibitors. On this backdrop, we have characterised cell types from synovium which respond to anti-Notch2 treatment by means of single cell RNA sequencing (scRNAseq). The main responders to the treatment were subpopulations of macrophages, osteoclasts and subsets of synovial fibroblasts, all expressing the Notch2 receptor. However, other cell populations also manifested some changes in several transcripts, both expressing and not expressing Notch2 receptor, suggesting that changes might be due to direct and indirect impact of the treatment. These transcripts fall into several pathways that are central to the arthritis development, such as inflammatory responses, cell migration, bone metabolism or programmed cell death, as determined by IPA analysis. More detailed analysis of upstream regulators revealed affected pathways in these main responding cell types, among others NF-kB, STATs, several cytokines and growth factors, Myc, Hif1a, Fap.

2.2 Paper II

Based on findings from Paper I, we proceeded with human material and characterized the response to Notch2 inhibition in human synovial fibroblast, and in mixed synovial cell cultures. Cultures exposed to anti-NRR2 monoclonal antibodies, responded with a transcriptional profile to include genes involved in inflammatory responses, cell migration, and genes involved in bone and cartilage destruction, therefore quite similar to the changes generally observed in the mouse. We found these cultures to contain transcripts encoding both lining fibroblast marker CD55 and sublining marker THY1/CD90. Flow cytometry revealed that all cells in these cultures were CD90 positive and that the majority of these cells also express CD55, therefore similar to those reportedly located in the sublining layer of human and murine synovium. These findings suggest that Notch2 receptor signalling also targets human synovium.

2.3 Paper III

In this paper, we have shown that induction of the delayed-type hypersensitivity arthritis (DTHA) model in mice is not only mediated by CD4+ T cells but also involves B cells defined by detection of increased levels of these cells in draining lymph node, and by increased levels of circulating IgG. In addition, we have shown that anti-inflammatory drugs methotrexate (MTX) and dexamethasone (DEX) differentially suppressed paw swelling and inflammation in mice. These differences were also reflected in differential patterns in Th1, Th17, memory T cells and B cells levels.

2.4 Paper IV

In the final paper of this thesis, we compared the IL33 expression pattern in the rat to that of mouse and man, and we generated with CRISPR/Cas9 technology transgenic

rats that lack functional IL33 (*Il33*$^{-/-}$). There are fundamental differences between murine and human tissue IL33 expression in resting animals and we therefore interspecies differences across several tissues. We found that wild type rat (*WT*) IL33 expression rather resembles man than mouse in several tissues. Next, we compared several parameters between *Il33*$^{-/-}$ rats and *WT* rats. We detected no difference in the body weight or organ morphology. We found slightly elevated numbers of neutrophils, basophils and eosinophils but not of lymphocytes, monocytes. Genome-wide transcriptional profiling of endothelial cells identified 194 differentially expressed genes, among them reflecting pathways or biological processes important for function and activation of endothelial cells, such as the Notch and STAT signaling pathways, response to cAMP, endothelial cell migration and granulocytes chemotaxis. In conclusion, healthy wild type rats resemble man more closely than the laboratory mouse and it deserves further study under angiogenic or inflammatory provocation. *Il33*$^{-/-}$ rats may serve as a suitable model to understand the role of IL33 in man.

3 Aims of the study

The main aim of the thesis is to explore the mechanisms of inflammation with a focus on rheumatoid arthritis and the Notch signaling pathway with the use of animal models and clinical samples. Another aim was to assess a model of IL-33 biology in the rat, based on substantial species differences between mouse and man.

- Assess the efficacy of selective Notch inhibition in experimental models of arthritis, while at the same time avoiding gastrointestinal on-target toxicity (paper I).
- Decipher the effect of Notch2 inhibition at the single cell level by means of single cell RNA-sequencing (paper I)
- Identify the cellular elements in affected joints that express activated Notch receptor (paper I).
- Transcriptionally profile the response to selective Notch2 inhibition in human synovial fibroblast cultures mixed short-term cultures from human RA synovium (paper II).
- Assess the cellular effects and mechanisms of methotrexate treatment in experimental arthritis (paper III)
- Assess the suitability of the laboratory rat as a model for IL33 biology in man (paper IV).

4 Methodological considerations

This section discusses general aspects of the methods used in the thesis, with a brief description of their advantages and limitations. Detailed protocols can be found in the methods section of each paper.

To validate project hypothesis, we designed experiments in different model systems: animal models, cell cultures and human biopsy or surgical resection material. Several molecular biology technics were combined to determine hypotheses at the mRNA or protein level. Experiments were conducted according to approved regulations, based on our previous experience, as well as established and published protocols from high impact factor journals. Suitable statistical analyses were applied.

4.1 Animal experimentation

Several animal models that address arthritis exist. They allow for monitoring in controlled manner the treatment effect in harmonized groups, which is not always possible to achieve in patients. That may help to predict drug candidate efficacy and exclude side effects. Nevertheless, the limitations of animal experiments represent the human condition only to some extent, either due to species differences or to the fact that the model is often a simplified, incomplete course of the disease observed in patients. Despite that, the majority of research findings comes from these types of experiments, allowing for new therapy development that would not be possible to perform in human.

4.1.1 Experimental models of arthritis

There are several models of experimental arthritis that differ in mode of induction and strain susceptibility *(Atkinson & Nansen, 2017)*. In this section the models that will be explained are (i) a commonly used collagen-induced arthritis (CIA) model and models applied in this thesis, (ii) a collagen antibody-induced arthritis (CAIA) and (iii) a delayed-type hypersensitivity arthritis (DTHA). Models of arthritis mimic only some aspects of the RA, because of multi-factorial nature they lack several events, as APC autoantibodies, chronicity and certain mechanisms of adaptive and innate responses. Animal models cannot stand alone, and in the pre-clinical screening of potential therapeutics, it can be advantageous to use a range of different models that can supplement each other in the final evaluation of a drug candidate, while keeping a keen eye on opportunities to compare findings to events in human disease lesions.

4.1.1.1 Collagen-induced arthritis

Collagen-induced arthritis (CIA) is one of the most widely used models. It is induced by immunization with heterologous type II collagen in adjuvant, leading to a breach of tolerance towards self-collagen, activation of collagen II-specific T cells, and generation of collagen II-specific autoantibodies (Brand, Latham, & Rosloniec, 2007). Despite several similarities to RA, it is important to realize that this model shows only a weak to moderate response to anti-TNF-treatment. Indeed, anti-TNFs may not have reached the clinic for RA if preclinical development had been limited to the CIA model (Moore, McCarthy, Parris, & Moore, 2014). CIA is also unsynchronized and time consuming, it requires the use of specific strains (carrying haplotype of MHC: H-2^q or H-2^r) and it shows variable incidence and severity.

4.1.1.2 Collagen antibody-induced arthritis

Collagen antibody-induced arthritis (CAIA) is induced by passive transfer of an antibody cocktail to collagen type II followed by an LPS-induced boost (Khachigian, 2006). Anti-collagen II antibodies coat the cartilage surface, drives immune complexes, Fc receptor and complement activation and leads to innate immune system cells involvement. Initially thought to arise independently of T and B cell, studies nevertheless have shown that adaptive immunity also contributes, as arthritis develops at a reduced rate in T and B cell-deficient mice (RAG2-/-) and is almost absent in mice that lack T-bet (Wang et al., 2006). Onset of CAIA is rapid, development of arthritis is synchronized, incidence is high and strain independent. The major shortcomings of the CAIA model therefore include a less extensive understanding of how adaptive immunity contributes and the high cost of the antibody cocktail.

4.1.1.3 Delayed-type hypersensitivity arthritis

Delayed-type hypersensitivity arthritis (DTHA) is a more recently developed model, in which arthritis is induced in a single paw (Tanaka, Kagari, Doi, & Shimozato, 2007). It is a classical delayed-type hypersensitivity (DTH) reaction (Sido, 2018) with additional administration of anti-collagen II antibody cocktail (identical to the one used in CAIA model), improving severity of joint inflammation and bone degradation (Figure 25). The type II collagen antibody cocktail induction is given between the immunization step with methylated bovine serum albumin (mBSA) in complete Freud's adjuvant (CFA), and the challenge step with local exposure to administrated mBSA into the footpad to boost the response and activate mBSA specific CD4+ T cells. Within 24 hours mice respond to the challenge and develop transient swelling in the challenged, ipsilateral paw, with the strongest swelling sore at day 4-5 after challenge and resolve within 21 days. The contralateral footpad is injected with saline or PBS at the day of challenge and serves as an internal control with no reaction (Atkinson et al., 2012; Schett, Tanaka, & Isaacs, 2021; Tanaka et al., 2007).

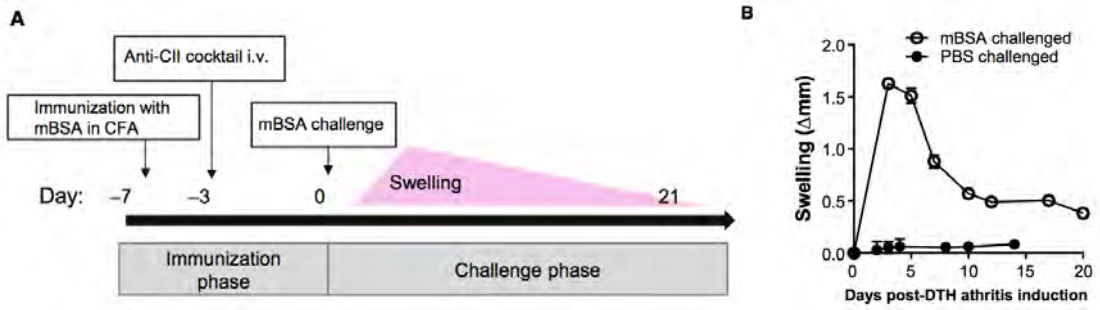

Figure 25. DTHA model. Adopted from (Atkinson & Nansen, 2017).

The disease is characterized by severe paw swelling, infiltration of inflammatory cells, hyperplasia of the synovial membrane, cartilage destruction, and bone erosion. It shares features with both CIA and human RA, it depends on antigen-specific CD4+ T cells for induction, and it can be successfully treated with TNF-blocking biologics and dexamethasone. It has been shown that it is mediated by Th1 and Th17 T cells, but Atkinson et al characterized also involvement of neutrophils, macrophages and osteoclasts (Atkinson, Bleil, et al., 2016; Atkinson, Hoffmann, et al., 2016; Atkinson & Nansen, 2017; Atkinson et al., 2015; Atkinson et al., 2012). The model was successfully implemented in male BALB/c and female C57BL/6 mice, giving rise to the

arthritic phenotype (Atkinson et al., 2012; Schett et al., 2021; Tanaka et al., 2007). It is a robust and reproducible model, increasing successful induction of arthritis in mice, and therefore reducing numbers of used animals. As mentioned, each mouse serves also as an internal control by inducing arthritis only in one hint paw, contralateral paw, allowing paired comparison of joint swelling between that paw and the injected vehicle. The induction of arthritis takes 7 days, and development of highest symptoms (swelling) is within 4 days after starting the reaction. Because the model is self-resolving, there is only one paw affected and the whole procedure is taking approximately 3 weeks, mice experience milder discomfort in comparison to other mouse models of arthritis, like CIA or CAIA. Costs are also reduced, requiring only a small fraction of anti-collagen II cocktail (in comparison to CAIA) and by using commonly available mouse strains *(Atkinson & Nansen, 2017)*.

4.1.2 In vivo imaging of leukocyte recruitment

In vivo imaging system (IVIS) is used for detection of bioluminescent or fluorescent reporters targeting specific biomarkers in living animals. It serves as a diagnostic application in a wide range of animal models. For instance, upon a systemic administration of probe detecting myeloperoxidase (MPO), the enzyme activity can be monitored over a time, reflecting phagocytic activity in inflammatory conditions (Gross et al., 2009). Luminol, a small molecule used as detecting reagent, emits blue bioluminescence at 450 nm that can be detected when exposed to oxidizing agents, products of MPO activity. The signal is then transformed and as assessed in a set region of interest (ROI). The luminol has relatively low toxicity, it is well absorbed and easily excreted by mice, therefore can be used to monitor cell activity and development of inflammation in several animal models, as acute dermatitis, tumors or arthritis.

4.1.3 Generation of transgenic rats

The CRISPR-Cas9 technology has simplified the generation of transgenic animals with specific gene defects including null-mutants (knockout animals) in other species than the mouse. The engineering is done by a single injection of messenger RNA or DNA encoding zinc-finger nucleases into the one-cell embryo from superovulating females, leading to disruption of a target gene locus to introduce site-directed, heritable mutation (Geurts et al., 2009). It then requires implantation of embryos carrying the desired mutation in surrogate females. The CRISPR-Cas9 technology serves as an important strategy to study involvement of the gene of interest in transgenic animal models. There is a risk that deficiency of particular genes may results in the phenotype that has fatal insufficiencies. Therefore, precise experimental design and consideration must be made before implying the technology and after successful generation of transgenic animals, close and careful monitoring of health status is required.

4.2 Tissue and cell suspension processing

Tissue processing is an important procedure that may influence the quality of the experiment. Optimization and understanding of the possible obstacles are essential for successful experimentation. We have used tissues derived from human or animal for different purposes, as a source for cell cultures or for transcriptional analysis (microarray, scRNAseq), immunostaining or immunolabeling (each method explained further). And therefore, proper tissue handling according to the selected method protocol is necessary.

4.2.1 Tissue disaggregation

Disaggregation of the tissue is often pivotal step of tissue processing, for two reasons. First, some analyses require pure, viable single cell suspensions free of doublets or undigested tissue. Second, cells when removed from their natural environmental are exposed to conditions that may negatively affect phenotype and function, due to harsh experimental procedures. Disaggregation of the tissue is typically achieved by mechanical disruption and enzymatic dissociation, where several enzymes can be used, as collagenases or proteinases, trypsin, papain, supplemented with enzymes degrading DNA, as DNase I. Prolonged exposure to mechanical stress and enzymatical treatment may result in pronounced susceptibility to reduced cell viability and stress-related cellular changes. After tissue dissociation, single cells should be handled gently and kept on ice in nutritional medium, to maintain high viability and cell quality.

4.2.2 Magnetic bead-based cell isolation

Dynebeads, the superparamagnetic polymer spheres can be used for selection of cells from heterogenous cell type suspensions. They are coated with antibodies targeting specific marker on the cell surface. There are two approaches that can be implemented; either negative selection/depletion, to remove unwanted cell types, or positive selection where cell of interest is isolated. The method is performed in a magnetic field to separate cells bound to beads from free cells in the supernatant fluids. Separation is fairly efficient and the isolated population if of high purity and viability, and can be used in further downstream analyses, as transcriptional analysis. The disadvantage of the positive selection is that the beads with antibodies may stay bound to the cells even after bead releasing step, and when cells used for the in vitro cell cultures, the beads may be engulfed by the cells affecting their phenotype and function.

4.2.3 Fluorescence-activated cell sorting (FACS)

To separate a particular cell type from heterogenous cell suspension, cell sorting using flow cytometry (FACS) can be applied. It is a process in which cells can be labeled with fluorescent dyes conjugated to antibodies to specific surface molecules. The bound fluorochrome is excited with a laser beam and emitted light is identified by a photomultiplier as the droplet leaves the nozzle. Cells are deflected in an electric field according to fluorescent signal and collected to separate vessels. Cells may also be sorted by other properties, such as size or morphology or by a marker distinguishing dead and life cells. This method enables automated, cost-efficient, high-throughput isolation of cells of interest. The limitation is the requirement of large number of cells as a starting material, and due to a fluid shear forces and pressure created in the nozzle, induction of stress responses and decrease in cell viability (Olsen & Baryawno, 2018).

4.2.4 In vitro cell cultures

In vitro human primary cell cultures serve as simplified model that is the closest you get to in vivo in man. We chose HFLS-RA cell line, which is a primary human cell type obtained from RA patients. These cells are known to be involved in progression and persistence of arthritis in human. Our goal was to determine the changes in behavior and phenotype of HFLS-RA after Notch pathway inhibition. Even more closely resembling the state and environmental seen in the tissue, is another model that we have adopted in this project, mixed cell cultures from disaggregated synovial tissue from RA patient. The superiority of heterogenous cells cultures is that while they retain

the advantage of controlled in vitro conditions, there is the added opportunity to study signals and interactions between several different cell types that may influence the final net effect.

4.3 Transcriptional analysis

Advanced technologies to analyse genome-wide mRNA transcripts (transcriptome) in cells enables researchers to investigate biological processes. Initially developed to hybridize abundant transcripts to single probes (Northern blot) it moved via the polymerase chain reaction (PCR, to also embrace less abundant transcripts) and the solid phase arrays of probes (microarray) to hybridize specific mRNAs from cell cultures or whole tissue. More recently, these methods have developed to the opportunity to assess the transcriptional activity in individual cells isolated from whole tissue. It revolutionized several research areas, as cancer immunobiology, neuroscience, autoimmunity. No surprise that these methods rapidly developed and become the core of analysis in different fields.

4.3.1 Single-cell RNA sequencing (scRNA seq)

The technic gives opportunity to study entire transcriptome on a single cell level from heterogenous population or complex tissue samples. The approach consists of tissue sample preparation and disaggregation steps to isolate/obtain viable single cell suspension, followed by isolation of message RNA from individual cells, reverse transcription and amplification of cDNA, followed by the generation of a sequencing library and analysis of data. Computational algorithms of the sequenced data identify transcripts of individual cells, mapping them into subpopulations (clusters) and revealing heterogeneity of the sample. It gives opportunity to uncover rare cell subsets with limited cell numbers that would be omitted by other technics with lower-resolution level and it give the opportunity to understand how each population – large or small – is acting in the dynamic of multicellularity. Disaggregation of the tissue is an important step (as explained in section above) to provide single cell suspension that also carries the risk of affecting cell viability and inducing stress, with the risk ofchanging transcriptional programs drastically. The main drawbacks are small sample size, the impact of the cell separation procedure, high costs and limited throughput (Cheng et al., 2021).

4.3.2 Genome-wide transcription profiling

Another approach to assess the gene expression in cells, is the hybridization based technology. This transcriptional analysis has a much lower costs than scRNA seq, but the sequenced results are an average of the gene expression in the sample. It is not possible to investigate the contribution of single cells with this method and used on whole tissue RNA there is a substantial loss of significant biological information due to the heterogeneity of sequenced cell populations. Despite this limitation, it remains a relevant and much more sensitive approach to study monocultures with the goal of reaching a mechanistic understanding of cell behaviour It could also serve as a effective diagnostic method for screening changes in the gene expression patterns between patients (Olsen & Baryawno, 2018).

4.3.3 Quantitative reverse transcription PCR (q RT-PCR)

Real-Time quantitative reverse transcription polymerase chain reaction (qRT-PCR) enables reliable detection and measurement of a DNA product proportional to the amount of original RNA template. The total RNA isolated from sample is converted to cDNA with a reverse transcriptase that next is used as a template for qRT-PCR. The

method includes sequence-specific primer pairs to the target gene and intercalating dye that emits the fluorescence signal once bound to dsDNA. Increasing amounts of dsDNA will increase the fluorescence signal that next is detected and quantified. It is an easy and inexpensive method. The drawback is that dyes bind all dsDNA, also non-specific products, which may affect the final result, especially if the primer pairs are poorly designed. It is therefore important to check predicted product size and to select primers bridging exons and therefore avoiding amplification of genomic DNA.

4.4 Immunostaining technics

Several immunochemical technics are developed to detect proteins and antigens in either frozen or fixed tissue samples (immunohistochemistry, IHC) or in cells (immunocytochemistry, ICC). For that purpose, different staining methods may be implemented, roughly divided into immunoenzymatic or immunofluorescence (IF) detection technics. The classical immunoenzymatic protocol uses peroxidase or phosphatase-based reactions, that precipitates a substrate which is visible under a light microscope. Alternatively, antibodies are conjugated to a fluorochrome and can be visualized by excitation with light of optimal wavelength. A hybrid technology is the enzymatic generation of a fluorescent precipitate, such at the Tyramide technology. Unprecedented advantage of immunolabeling methods is the preservation of tissue architecture allowing analysis of cell distribution and possible interactions. Having said that, the visual interpretation of the staining is challenging as the technic resolution, quality of the signal and potential personal bias, may mislead the interpretation of the results. Therefore, it is of essence that the assessment of the results is performed by highly qualified and experienced specialist, to avoid incorrect statements. It is also relevant to confirm the results by other types of technics, as flow cytometry or transcriptional analysis.

4.5 Immunoassays

4.5.1 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) measures the presence and concentration of molecules in a solution using detection antibodies to a specific antigen. In a sandwich ELISA there is also use of a capture antibody in the solid phase. It is highly robust, quick and accurate method commonly used for detection of several biomarkers, as cytokines, from a variety of sample, including serum, plasma or culture medium. To determine the concentration of the measured analyte a standard curve using antigens of a known concentration is necessary. Results are measured by changes in optical density of the solution at specific wavelength, that can be detected as an effect of the colorimetric reaction. In a typical ELISA, first a capture monoclonal antibody is bound to a microtiter plate surface, then a sample with analyte is added, and a target antigen retain bound to the plate. In the next step, an enzyme labelled detection antibody is added and binds the capture antibody-analyte complex –, creating a sandwich of antigen caught between two antibodies. Next, a colorimetric substrate is added and in the result of enzymatic reaction, a colored product is generated. The intensity of the color is proportional to the amount of the detected analyte.

4.5.2 Multiplex assay

Another immunoassay method is multiplex assay, where several analytes can be detected simultaneously from the test sample. It has similar features to a sandwich ELISA, exploiting capture antibodies designed against desire antigens, coupled to a

fluorescently dyed magnetic bead. Next the sample is incubated with a biotinylated detection antibody to create a sandwich complex. The final step is an incubation with a streptavidin-phycoerythrin (SA-PE) conjugate, where SA bind to the biotin on the sandwich complex, and PE serves as a fluorescent reporter. In this method the detection is performed in flow cytometry-based instruments with two lasers, a red laser (635 nm) illuminating the fluorescent dyes from each bead and a green (532 nm) laser exciting PE, to generate a reporter signal. Data is presented as median fluorescence intensity (MFI) and is proportional to the concentration of analytes.

In both methods, the specificity of the antigen -antibody binding is crucial for proper detection of the analyte and for accurate calculation of the analyte concentration. Of equal importance is the correct preparation of the standard curve dilutions.

4.6 Flow cytometry

Flow cytometry is a technique employing fluorescence labelling of specific cell markers that detect and characterise individual cells from heterogenous cell suspensions. It is a fluid-based technology that enables analyses of cell properties as they are passed through a nozzle to generate droplets containing cells. The system is adjusted so that there is a low probability of more than one cell per droplet. Main feature of the technic is analysis of cells, one at the time, that passes through the laser beam, excites the fluorochrome conjugated antibody bound to cell, with a specific wavelength. Next emitted light from excited fluorochrome, passes through appropriate optical filters to the detector. In addition, scattered light is detected and quantified, to serve for additional parameters of measured cell, as cell size (forward scatter, FSC) and granular content (side scatter, SSC). Accurate selection of fluorochrome-conjugated antibodies and adequate optical filters are crucial for successful analysis. In addition, several controls are necessary. First, calibration control is used for setting up the instrument and experiment reproducibility. Another, compensation and gating controls ensure proper signal separation, avoiding signal spillovers. The compensation control is a single fluorochrome stained sample, in our case we used beads. Whereas fluorescence minus one (FMO) control was composed of markers panel used for staining the experimental sample, but with one antibody replaced to isotype-matched irrelevant control antibody that should not bind to the specimen. It is conjugated with the same fluorochrome, as replaced antibody from the panel. Both controls are used to set the gating for the negative and positive populations and spectral overlap. Last, but not least is the biological control, that has no antibody.

4.7 Haematological analysis

To assess blood cells morphology, enumeration and molecular or cytogenic properties, a hematological analysis can be performed. It is a widely used screening method to diagnose and monitor blood condition and disease probability. Results from individuals are compared to a general normal range with upper and lower limits defined for each analyte. There may be other ranges for different genders, age or other conditions as pregnancy. In addition, another species have alternative normal ranges, that differ from human.

4.8 Statistical analysis

Methods of choice and the software used for computations are specified in the methods section of each paper.

4.9 Ethical consideration

This project utilizes samples from several sources, human and animal. To ensure the privacy protection and handling accordingly to regulations, for each subject adequate approvals were obtained and are detailed in the methods section of each paper.

5 Discussion

The main finding of this thesis is the discovery of therapeutic targeting of the Notch2 receptor that may be an attractive drug candidate for rheumatoid arthritis (RA). The effect of the disease progression was investigated in animal models of arthritis and in in vitro human cell cultures.

The Notch signaling pathway was broadly studied and found to be essential in several diseases (Siebel & Lendahl, 2017). Many attempts were made to target this pathway for drug development. Several preclinical trials reported that the pathway modulation is a promising and effective approach, extensively elaborated by Christopoulos et al (Christopoulos et al., 2021). Surprisingly, there is no available drug on the market that would target the pathway. Many clinical trials failed due to severe side effects, in particular in the gastrointestinal system.
In Paper I we have shown using murine models of arthritis that the therapeutic effect of targeting the Notch pathway may be achieved while avoiding the intestine toxicity. The strategy involves selective Notch2 inhibition executed by systemic administration of monoclonal antibody that binds to the negative regulated region (NRR) on extracellular part of the receptor. This binding prevents access to the activating cleaving site on the receptor to the enzyme complex, gamma-secretase, in results suppressing the transduction of the intracellular signal from Notch2 receptor. In response to the treatment, mice manifested decreased paw swelling and reduced inflammation determined by measurement of pro-inflammatory cytokines levels (*Tnf, Il1b, Il6*) in paws, and phagocytic white blood cells activity. We have monitored body weight during the procedure and no fluctuations were observed. To assess probability of the intestinal toxicity we investigated histologically the architecture of the intestinal crypts and the proliferative ability of the crypt base cells, by detecting Ki67-positive proliferating cells. No significant modification of intestine and crypt cells were observed.

We have tested anti-Notch2 effects on two different mouse models, which engaged different immune response mechanisms. Collagen antibody induced arthritis (CAIA) mimics innate responses and delayed-type hypersensitivity arthritis (DTHA) involves adaptive responses with Th1, Th17 and as shown in Paper III, also B cells.

In addition, we examined other selective modulations of the Notch pathway, by the use of monoclonal antibodies targeting NRR of other Notch receptors, Notch1 and Notch3, or targeting structures present on the ligands Dll1, Dll4 or Jag1. None of other targets had the same attenuating effect at the same time with no serious side effects as anti-Notch2. Although inhibition of Notch1 was as effectively reducing paw swelling as Notch2, the former resulted in significant destruction of crypt structure and reduced enterocytes proliferation as well as decreased mouse body weight. These side effects were comparable or even worse than those seen after the pan-inhibition of the Notch pathway, when gamma-secretase inhibitor was used, blocking all four Notch receptors. The reduction of Notch3 was not as strongly attenuating paw swelling and inflammation as in response to Notch2 inhibition, even when antibodies were combined and injected together to seek for synergistic effect (unpublished data). Dll1 and Dll4 demonstrated weak therapeutic effect, and when combined some negative effects on intestine health were observed. Jag1 inhibition instead of attenuating effect, it increased even further the paw swelling (data unpublished). This series of mouse experiments clearly demonstrated that each component of the Notch pathway can manifest a different way of action and has distinct tissue distribution pattern. For

instance, Notch1 seemingly has more important role in the intestine health (van Es et al., 2005) than Notch2 or Notch3. Another remark is that specific ligand-receptor interactions may have different binding affinity and functionality. For instance, generation of marginal zone B cells depends on binding of only DLL1 to Notch2 (Hozumi et al., 2004; Saito et al., 2003). And on the other hand, the same ligand can be shared between receptors, therefore targeting one receptor will not disturb the signal transduction, however when targeting simultaneously several ligands, the signal for several receptors might not be transmitted. That assumption may explain the effect seen in our experiment when we targeted together Dll1 and Dll4 and we have seen reduction in intestine health, probably affecting Notch1 signaling. Or another example from our unpublished data, when we simultaneous administrated anti-NRR1 and anti-NRR2, and as a result, the intestine structure was destroyed after just 4 days of the experiment start with premature death of the animals. These monoclonal antibodies were well characterized and even in Notch2 administration, some changes were seen in lung cells, with no clear impact on animal healthy (Lafkas et al., 2015; Wu et al., 2010). This study in addition to the discovery of Notch2 therapeutic potential, as well addressed and evaluated the toxicity issue after the antibodies administration and should be investigated further.

Concluding first part of the results, in our hands, only anti-Notch2 stood out among other evaluated antibodies, and it led us to pursue further exploration of the mechanistical effects of anti-Notch2 on cellular level.

Next, we have asked which cell types and by which mechanisms they can contribute to attenuation of arthritis in mice? To investigate this, we have performed single cell transcriptional analysis of inflamed synovium from mice either subjected to isotype-matched irrelevant control monoclonal antibody or to anit-NRR2 antibody. We have successfully identified cell types directly responding to the treatment, among them two subsets of synovial macrophages, osteoclasts and synovial (lining) fibroblasts, all expressing Notch2. In these clusters, we have associated differentially expressed genes with pathways and biological processes that are the core events perpetuating pathological changes in the RA. Moreover, we have seen similar changes in the rest of the identified cell types, not as significant as this in the clusters mentioned above. That outcome led us to the conclusion that changes seen in the rest of the cell clusters are most probably the results of indirect effect of Notch2 inhibition. Nevertheless, we were enthusiastically surprised that our treatment strategy may affect several cell types from inflamed synovial tissue environment, the source of the signals perpetuating RA pathology, allowing to target the disease progression at the core.

We are not the first investigators aware of incredible importance of interaction between synovial fibroblast and synovial macrophages. That relation may be a bridge sustaining proinflammatory mechanisms of arthritis but also pro-resolving, depending on which subsets of synovial fibroblasts interact with synovial macrophages. And for instance, when the interaction is between synovial fibroblasts and CD2006/MerTk double negative monocyte-derive macrophages, they are perpetuating proinflammatory phenotypes (Alivernini et al., 2020). When CSF1R expressing macrophages were stimulated with CSF1, IL6 produced by synovial fibroblasts, macrophages have altered proliferation and survival, and were undergoing the metabolic profile rewriting (Saeki & Imai, 2020). Another study showed changes in fibroblasts by signals derived from macrophages. Macrophages expressing HBEGF

and EREG, induced expression of IL6, IL33, EGFR2 in synovial fibroblasts and promoted their invasiveness and pro-inflammatory phenotype. Sekine et al. presented that this mutual dependence might be broken by targeting Notch signalling pathway (Sekine, Nanki, & Yagita, 2014). In the study, they showed that blockage of DLL1 expressed on synovial macrophages, prevents binding to NOTCH2 on synovial fibroblasts, and results in reduced production of IL6, GM-CSF and MMP3, detected in synovial fluid and inflamed paws in mouse arthritis model. Anti-inflammatory responses may be also induced, when resident macrophages (CD206/MerTK), both, Trem2-positive lining macrophages or Lyve1-positive sublining macrophages promote immune-regulatory phenotypes and pro-resolving mechanisms in presence of GAS6 producing synovial sublining fibroblasts (Alivernini et al., 2020).

To further proceed with assessment if Notch2 is a suitable a RA drug candidate, we considered human disease lesions and human models, asking if Notch2 presence occurs on the corresponding cell types in human synovium, and later, if these cells can be as well affected by the anti-NRR2 treatment. For that reason, we have first histologically assessed expression of Notch2 receptor from synovial tissue in RA patients (Paper I). Interestingly, we have detected adequate cell types as these seen in mice with active Notch2 receptor, among them synovial macrophages and synovial lining fibroblasts. Subsequent to this finding, we examine in vitro human synovial cell cultures. In the Paper II we have exposed synovial fibroblasts or synovial mixed cells to anti-NRR2 and assessed the effects. We have performed genome-wide transcriptional analysis on the first model, monocultures of IL1b-stimulated synovial fibroblast derived from RA patients, and we have identified several transcripts, as in murine synovial fibroblasts, affected and belonged to the biological processes critical for RA development. Second, the in vitro model was analyzed for secreted pro-inflammatory markers. We have observed *Il6, MMP1* and *PTX3* significantly changed only after 6 days of culture. Suggesting that indeed we were able to replicate findings from murine system, strengthening our hypothesis.

Of note, characterization of synovial fibroblast may have crucial turning point for more selective treatment of arthritis. As shown, selective inhibition of Notch pathway may influence several phenotypic effects in synovial fibroblast cultures. In order to sustain a proinflammatory environment imitating signals from RA in in vitro cultures, additional stimulation is needed. Jones et al. (Jones et al., 2017) subjected synovial fibroblasts obtained from RA patients to several most essential stimuli known in RA, as cytokines or innate immunity receptor (TLR3) ligand. Among them TNFa, IL1 and Poly(I:C) gave the most similar effects in broad cytokine profiles when comparing RA synovial fluid and activated in vitro synovial fibroblasts. No significant difference in cytokine profiles between these three was detected, therefore we focused on IL1b stimulation in our in vitro cell cultures.

In parallel to experiments involving Notch pathway modulations, we also focused our efforts on another inflammatory mediator (Paper IV). Our understanding of limitations of the murine models led us to proceed with generation of alternative system model, involving another rodent representative, rat. Others have shown that rat is often more closely reflecting human conditions than the mouse system. We have chosen to investigate IL33, which was shown to be a Notch pathway target gene. IL33 has been associated with several diseases. And several reports document that murine distribution of the molecule has completely opposite pattern than human. Therefore,

we have noticed the need to generate rat system model and to open possibilities to study IL33 mechanisms in vivo in still fairly simple model that rodents provide, but much more closely related to the human state.

We have successfully generated healthy rats lacking functional IL33. We have monitored several health status parameters and we bred these rats for several generations with no noticeable health complications. We have assessed that endothelial cell (ECs), that are main source of IL33 in the body, are providing normal vessel structure, determined histologically in several tissues. From genome-wide transcriptional analysis we have observed some relevant changes in dermal ECs. Several genes were impaired and belonged to pathways essential for ECs function and activity. Moreover, we were eager to examine these transgenic rats to inflammatory condition. We have performed provocation with LPS to investigate response differences. Preliminary data reveled some nonsignificant fluctuations in the transgenic rats when compared to wild type (unpublished data), although more experimentation should be done before any final assumption can be stated.

In conclusion, it this thesis we have shown that the knowledge and expertise that I have gained from different subprojects can be versatile and may be implied to study different models. Revealing etiology and pathogenesis of disease are the basis for developing effective treatments and implementing rational preventive measures.

In addition to the work included in this thesis, I have studied a somatic mutation in a neurocutaneous disorder, characterized by vascular malformations, where we have discovered a novel somatic mutation in *GNB2*. Performed functional studies with virus transfected ECs, discovered that this mutation is affecting function of ECs in in vitro cultures, important for new vessels generation as migration and proliferation of ECs. Moreover, we assessed changes in two pathways that may take part in these functions, an already known MAPK pathway was not affected, however the Hippo signaling pathway seemed to be important and involved in the mechanistic modulations.

A lot can elaborate on future perspectives and what could be done next in the project. Some of these ideas were already used in the SPARK and Inven2 project. And another are the basis for two postdoctoral project funding applications. Nevertheless, the knowledge and experience gain during this project opens many possibilities for further career prospects. The mechanisms and models studied here can be implemented in another fields of science. For instance, Notch pathway and IL33 have as well an impact on the brain function. I was briefly studying U87-MG cells, cell line derived from glioblastoma, and exposed it to anti-NRR2 antibody treatment in in vitro cultures. Interestingly, these cells also responded to Notch pathway inhibition in similar way to the synovial fibroblasts by reduction in production of pro-inflammatory cytokines and adhesion molecules expression.

Altogether, this project shows the tremendous need to combine specialists of different educational background and expertise and perform interdisciplinary study. For instance, in neurobiology or cancer research it becomes more and more clear that immunobiologists with bioinformatical computational skills are indispensable experts that would have an significant impact on several issues, that other specialists would not address. We must allow our imagination to suggest that this could also work the other way around.

6 Summary of the thesis

Modern therapeutic approaches increased effectiveness of pharmacological treatment in disorders of chronic inflammation allowing many patients to reach remission, or low disease activity. However, difficult-to-treat disease is not a condition of the past, as reaching the treat-to-target goal is not trivial. Many patients suffering from disorders with chronic inflammation and autoimmune disease, including rheumatoid arthritis respond insufficiently to current therapies. It is therefore not surprising that many investigators have invested strong efforts to predict the efficacy of novel drugs and to identify new targets for future treatments.
Rheumatoid arthritis is a chronic, inflammatory disease caused by the harmful activity of leukocytes, that attack and destroy flexible, synovial joints, which may lead to severe disability if untreated. Patients experience a severe reduction in quality of life and there is an unmet medical need for safer and more effective therapeutic targeting of rheumatoid arthritis. Currently, no approved drug has an efficacy in rheumatoid arthritis that goes beyond achieving low disease activity in 40% and remission in 20% of patients, highlighting the importance of developing novel therapeutic avenues. Importantly, every available drug has a potential for serious side effects, such as infection, reactivation of demyelinating disorders, reactivation of herpes zoster or increasing cholesterol. Therefore, current recommendations do not include combinations of different biological or targeted synthetic disease-modifying anti-rheumatic drugs. This adds up to a situation where about 75% to 80% of patients achieve remission or low disease activity, whereas the remaining patients have no treatment option to reach this level of therapeutic response and stop destruction of joints. These shortcomings of current treatment options in rheumatoid arthritis also resemble those in other disorders of chronic inflammation, probably because the pathophysiology of rheumatoid arthritis overlaps with these inflammatory diseases. In other words, inhibition of novel targets may also be efficient in other disorders of inflammation.

This project was focused on the role of Notch signalling in rheumatoid arthritis and to determine whether Notch inhibition be exploited to treat disorders of chronic inflammation.

The Notch signalling pathway is a highly promising anti-inflammatory drug target as it modulates the inflammatory responses of several disorders of chronic inflammation, including rheumatoid arthritis. However, broad inhibition of Notch signaling or Notch1 inhibition also results in side-effects including gastrointestinal toxicity, and therefore cannot be exploited as a therapeutic modality. On the other hand, selective Notch inhibition appears to globally modulate the inflammatory response, not only interacting with proinflammatory NF-kB signalling but also because it alters the chromatin landscape of inflammatory enhancers. Inhibition of Notch therefore interferes with the downstream effectors of many different cytokine receptor systems, and it has the potential to inhibit leukocyte recruitment and activation irrespective of the provoking agent. Current concerns over gastrointestinal toxicity in response to global Notch inhibition are met by targeting specific receptors that in preclinical, highly reproducible experiments give no detectable side effects, yet an impressively strong anti-inflammatory efficacy.

The recent establishment of single cell genomics and the opportunity to map the efficacy of drug treatment in single cell populations is now expanding the concept of

intelligent drug design. This is because we can identify individual cells, assign them to distinct cell populations and observe directly how they respond to treatment. In other words, mapping the transcriptional activity in single cells while exposing experimental animals to systemic candidate drug treatment gives us the opportunity to observe drug-responsive effects in individual cell populations. In the light of recent technical developments, it becomes clearer that the detailed assessment of the individual cells in a complex tissue is of the utmost importance. The complexity of the articular microenvironment when synovial membrane becomes infiltrated by inflammatory cells represents several different synovial cell populations and even generates aggregates of lymphoid neogenesis.
This project involved the combined efforts of expertise in rheumatology, single cell genomics and experimental pathology. We have discovered that selective inhibition of Notch2 strongly attenuates experimental arthritis while avoiding gastrointestinal toxicity. We have also identified the cellular elements in affected joints that express activated Notch receptor and transcriptionally profile the effect of specific receptor inhibition. We observed that the fraction of modulated transcripts is highest in subsets of macrophages, subsets of fibroblasts, and in bone-degrading osteoclasts. These changes range from reducing transcripts of pro-inflammatory mediators and upregulating anti-inflammatory cues, osteogenesis modulating transcripts, as well as a strong modulation of cellular migration, infiltration, angiogenesis and cell survival. This highly promising preclinical status of Notch2 inhibition has led us to discover activated Notch2 in the nucleus of macrophages and fibroblasts in human synovium. We further evaluated the efficacy of Notch inhibition in human synovial fibroblasts and mixed synovial cell cultures. These approaches revealed that Notch inhibition attenuates the inflammatory phenotype in human ex vivo cultures.

Based on our studies, selective Notch2 inhibition may therefore represent a particularly attractive therapeutic strategy, because it entails the potential to dampen both the production of a range of inflammatory cytokines by synovial fibroblasts and synovial macrophages and other tissue resident cells, as well as their downstream effects in target cells. The anti-inflammatory potential that lies in inhibiting Notch signalling may therefore extend beyond that of any strategy based on targeting an individual cytokine, like tumour necrosis factor (TNF), or even that of targeting the JAK kinases downstream of type I and type II cytokine receptors, because JAK kinases are not downstream of signals from members of the TNF- or IL1-receptor families.

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7 Sammendrag på norsk

Kroniske betennelsessykdommer er kilde til mye lidelse og invaliditet. Mange pasienter kan i dag få god behandling, men det gjelder fortsatt ikke alle. Det er derfor fortsatt behov for nye og bedre legemidler.

Denne avhandlingen beskriver en systematisk tilnærming for en slik utvikling. Vi har tatt utgangspunkt i et signalssystem som kalles Notch fordi man får en effekt mot betennelse om man hemmer denne signalveien i modeller for mange kroniske betennelsessykdommer. Problemet har imidlertid vært at denne behandlingen også gir bivirkning i form av diaré og vekttap.

Vi har derfor i **artikkel I** undersøkt om man kunne få en god effekt mot betennelse om man ikke hemmet hele systemet. Dette systemet består av 4 ligander (DLL1, DLL4, Jagged1 og Jagged2) som alle kan gi signal til 4 reseptorer (Notch1-4). Vi avdekket at det kommer en gunstig effekt av å hemme Notch1, Notch2 og tildels Notch3. Vi får også effekt av å hemme ligandene DLL1 og DLL4, særlig om man hemmer dem samtidig. Vi har brukt dyremodeller for leddgikt, en av de vanligste kroniske betennelsessykdommene til dette formålet. Selv om alle disse tilnærmingen ga effekt mot leddbetennelsen, viste det seg at Notch2 var gunstigst fordi det ikke kunne påvises diaré eller vekttap.

Vi har derfor gått videre med denne kunnskapen og gjort forsøk i mus som avklarer at mange forskjellige celletyper i leddene reagerer gunstig på behandlingen. Ved hjelp av ny teknologi som kan lese genaktiveringen i hver enkelt celle får vi vite hvilken celletype det er og hvordan den reagerer på behandlingen. Det var særlig makrofager, osteoclaster og fibroblaster som responderte på behandling. Dette kan danne grunnlaget for målrettet medisin. Andre celletyper reagerte i mindre grad og noen celletyper var upåvirket, antagelig fordi de ikke har Notch2 reseptoren. Vi undersøkte også leddhinnen hos pasienter med leddgikt og fant at Notch2 reseptoren var aktivert i de samme cellene. Dette kunne vi gjøre fordi vi hadde et antistoff tilgjengelig som binder til en aktiveringsassosiert epitope på halen til den aktiverte reseptoren (NICD2).

Er stort problem særlig innenfor immunologien, er at mus og mennesker kan være ganske forskjellige. Derfor er det viktig å avklare ytterligere om de funn man gjør i mus kan overføres til menneske. I **artikkel II** i denne avhandlingen brukte cellekulturer fra pasienter for å vise at behandlingsprinsippet trolig kan overføres til mennesker. Cellekulturene ble aktivert med et cytokin som gir betennelsesrespons og antistoff mot Notch2 dempet denne responsen. Vi kartla denne responsen med et såkalt mikroarray som gir et bilde av aktiveringen av alle gener i genomet. Responsen var meget lik den vi så i mus. Vi gjorde også lignende forsøk i blandede cellekulturer som ble isolert til korttids primærkulturer og som antas å ha en mer naturtro aktiveringsprofil enn cellelinjer som blir etablert over et lengere tidsperspektiv. Vi konkluderte at Notch2 effekten i mus og menneske er sammenlignbar.

I **artikkel III** har vi forsøkt å avklare virkningsmekanismer for legemiddelet Metotrexat. Dette middelet gis i dag som basisterapi til nesten alle leddgiktspasienter, enda vi vet lite om virkningsmekanismen. Studiens resultater kaster lys over dette.

Den siste studien (**artikkel IV**) befatter seg med et annet legemiddelmål kalt interlevkin 33 (IL33). IL33 er et såkalt cytokin som er med på å drive betennelsesresponsen. Det er gjort omfattende studier av dette cytokinets funksjon i musemodeller, men det har vært forbausende lite interesse for å avklare om disse resultatene ga mening hos menneske. Vi har kartlagt disse forskjellene mer systematisk og sett at mus skiller seg ikke bare fra menneske, men også flere andre pattedyr inkludert rotte. I denførste delen av studien viser vi hvordan rotte ligner mye mer på menneske enn mus når vi undersøker vevsdistribusjonen til IL33. Rotte er som mus generelt et velegnet forsøksdyr pga størrelse, kostnader og mulighet for genetisk manipulasjon. Vi etablerte derfor rotter med null-mutasjon for IL33 ved hjelp av såkalt CRISPR-Cas9 teknologi. Rotten var friske og fertile. Vi isolerte deretter endotelceller fra blodårene, gitt at IL33 uttrykkes sterkt i disse cellene. Vi kartla forskjeller mellom villtype og muterte rotter med et såkalt mikroarray som gir et bilde av aktiveringen av alle gener i genomet. Flere gener viste forskjellig uttrykk og de var relatert til disse cellenes funksjon. Fremtidige studier bør se nærmere på hva som skjer med disse cellene når det kommer til aktivering, enten ved betennelse eller karinnvekst.

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Paper I

Paper II

Paper III

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Activated, Pro-Inflammatory Th1, Th17, and Memory CD4+ T Cells and B Cells Are Involved in Delayed-Type Hypersensitivity Arthritis (DTHA) Inflammation and Paw Swelling in Mice

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Delayed-type hypersensitivity arthritis (DTHA) is a recently established experimental model of rheumatoid arthritis (RA) in mice with pharmacological values. Despite an indispensable role of CD4+ T cells in inducing DTHA, a potential role for CD4+ T cell subsets is lacking. Here we have quantified CD4+ subsets during DTHA development and found that levels of activated, pro-inflammatory Th1, Th17, and memory CD4+ T cells in draining lymph nodes were increased with differential dynamic patterns after DTHA induction. Moreover, according to B-cell depletion experiments, it has been suggested that this cell type is not involved in DTHA. We show that DTHA is associated with increased levels of B cells in draining lymph nodes accompanied by increased levels of circulating IgG. Finally, using the anti-rheumatoid agents, methotrexate (MTX) and the anti-inflammatory drug dexamethasone (DEX), we show that MTX and DEX differentially suppressed DTHA-induced paw swelling and inflammation. The effects of MTX and DEX coincided with differential regulation of levels of Th1, Th17, and memory T cells as well as B cells. Our results implicate Th1, Th17, and memory T cells, together with activated B cells, to be involved and required for DTHA-induced paw swelling and inflammation.

Keywords: CD4+ T cell, methotrexate, rheumatoid arthritis, DTHA mouse model, dexamethasone

INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease elicited by complex interactions between genetic and environmental factors, leading to chronic life-long inflammation of synovial joints (1). Over time this may lead to progressive and severe joint destruction and deformity (2). The hallmark of RA-associated inflammation is the recruitment of a variety of immune cells, including neutrophils, monocytes/macrophages, B lymphocytes (B cells), and CD4+ and CD8+ T lymphocytes (T cells) to the synovial compartment, where pro-inflammatory cytokines and chemokines are produced, together contributing to the pathogenesis of RA (3–5). Although the etiology of the disease remains elusive, aberrant pro-inflammatory CD4+ T cell activity plays a central role in the initiation and perpetuation of RA (6, 7). The two most pronounced CD4+ T cell subsets involved in RA are thought to be CD4+ T helper 1 (Th1) cells and T helper 17 (Th17) cells (8–14). Moreover, memory CD4+ T cells have been found to be enriched in inflamed synovium, assisting B cell activity and Ig production (15, 16). For treating the RA, methotrexate (MTX) is the most versatile drug used for preventing joint damage and glucocorticoids (GCs) for suppressing inflammation. The combination of these two compounds is most frequently used to reduce RA progression (17–20).

In order to understand etiology and pathology of RA, and to explore potential novel therapeutic drugs and strategies, several animal models, which can mimic and resemble that of human RA, have been developed. These include collagen-induced arthritis (CIA), antigen-induced arthritis (AIA), collagen antibody-induced arthritis (CAIA), and delayed-type hypersensitivity arthritis (DTHA) mouse models (21–24). These models all differ in their mode of induction and strain susceptibility to RA development. The DTHA model was initially developed by Tanaka and coworkers and developed further by Atkinson, showing it to mimic several histopathological features of human RA (24, 25). The DTHA model was established in both BALB/c and C57BL/6 mice strains, and exhibits high incidence rate, low variation, and synchronized onset of disease. These characteristics make DTHA model a promising translational murine model with high pharmacological values.

DTHA develops in two phases, the immunization and challenging phase. In the immunization phase, mBSA is injected subcutaneously (s.c.) and mBSA-specific T cells are generated. In the challenge phase, recall responses of the mBSA-specific T cells are induced by injection of mBSA in one of the footpads, contributing to the release of pro-inflammatory cytokines which trigger the recruitment of inflammatory cells such as neutrophils and macrophages at the site of inflammation (26–30). This activity initiates a process that leads to synovial hyperplasia, pannus formation, and destruction of bone and cartilage during disease development. Additionally, an i.p. injection of anti-CII is given to mice between these two mBSA injections to enhance immune response. In the DTHA mouse model, inflammation generally reaches a maximum at 24–48 h after the second mBSA injection, and induction of inflammation and paw swelling relies on CD4+ T cell activity, as antibody-depletion of CD4+ T cells prevents DTHA development (24, 25). The anti-inflammatory subset of CD4+ T cells, regulatory T cell (Treg) has significant influence on DTHA-induced paw swelling since depletion of Treg can exacerbate DHTA severity (31). However, other pro-inflammatory CD4+ T cell subsets, for example, activated, Th1, and memory CD4+ T cells, have not been explored in this model.

In the DTHA model, even though some anti-inflammatory agents such as neutralizing antibodies to TNF-α and IL-17 have been shown effective in reducing inflammation, MTX, has to our knowledge not been explored in the DTHA model (24, 31). MTX interferes with folate-related metabolisms including *de novo* purine and pyrimidine synthesis and promotes production of the anti-inflammatory metabolite adenosine. By this, MTX treatment can inhibit T cell proliferation, induce T cell apoptosis, suppress neutrophil migration, and influence cytokine production (18, 32–39). Moreover, the effect of the synthetic glucocorticoid dexamethasone (DEX) on paw swelling has been shown. However, the effects of DEX on pro-inflammatory cells associated with DTHA have not been investigated (24). Despite this, DEX has been shown to attenuate T cell function either by directly affecting TCR-induced activation or indirectly enhancing PD-1 expression (40, 41).

Here we examined the identity of immune cells involved and compared the effects of MTX and DEX on DTHA-induced inflammation and paw swelling in C57BL/6 mice. Our results demonstrate that Th1, Th17, and memory T cells interplay with B cells in promoting DTHA-induced inflammation and paw swelling.

MATERIALS AND METHODS

Cell Culture

Mouse CD4+ T cells were obtained from splenocytes using Dynabeads untouched CD4+ T cell isolation kit (Thermo Fisher). Mouse purified CD4+ T cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES buffer solution, 1 mM sodium pyruvate, 100 μM MEM non-essential amino acid solution, 50 μM β-mercaptoethanol, 100 U/ml penicillin, 100 ug/ml streptomycin, and 30 U/ml human recombinant IL-2 (all the materials for cell culture are from Sigma-Aldrich). These purified CD4+ T cells were treated with incremental concentrations of MTX (10–1,000 nM; Sigma-Aldrich) overnight followed by stimulation with anti-CD3/CD28 beads (bead:cell = 1:1; Thermo Fisher). Post 3 days incubation, cells were collected for cell activation assay by flow cytometry (see *Flow Cytometry*).

Abbreviations: DTHA, delayed-type hypersensitivity arthritis; H&E, hematoxylin and eosin; IF, immunofluorescence; Th, helper T cell type; IL, interleukin; PBMC, peripheral blood mononuclear cells; RA, rheumatoid arthritis.

Mice and Ethics Statement

Female C57BL/6 mice (8–10 weeks old) were purchased from Charles River and housed in the local animal facility of University of Oslo. Mice were kept under 12-h light/dark cycle, with standard rodent chow *ad libitum* and drinking water. All animal experiment procedures were approved and registered at the National Animal Research Authority (FOTS ID: 5714, 19551, and 13903).

Induction of DTHA

Induction of DTHA is outlined in **Figure 1A**. In brief, mice were immunized intradermally (i.d.) at both sides of the lower back with 50 μg mBSA emulsified in 50 μl CFA (Hooke Laboratories) in each side on day −7 and injected i.p. with anti-CII cocktail (50 mg/kg, MD Bioscience) in 100 μl PBS on day −3. Seven days after initial immunization, mice were challenged with mBSA

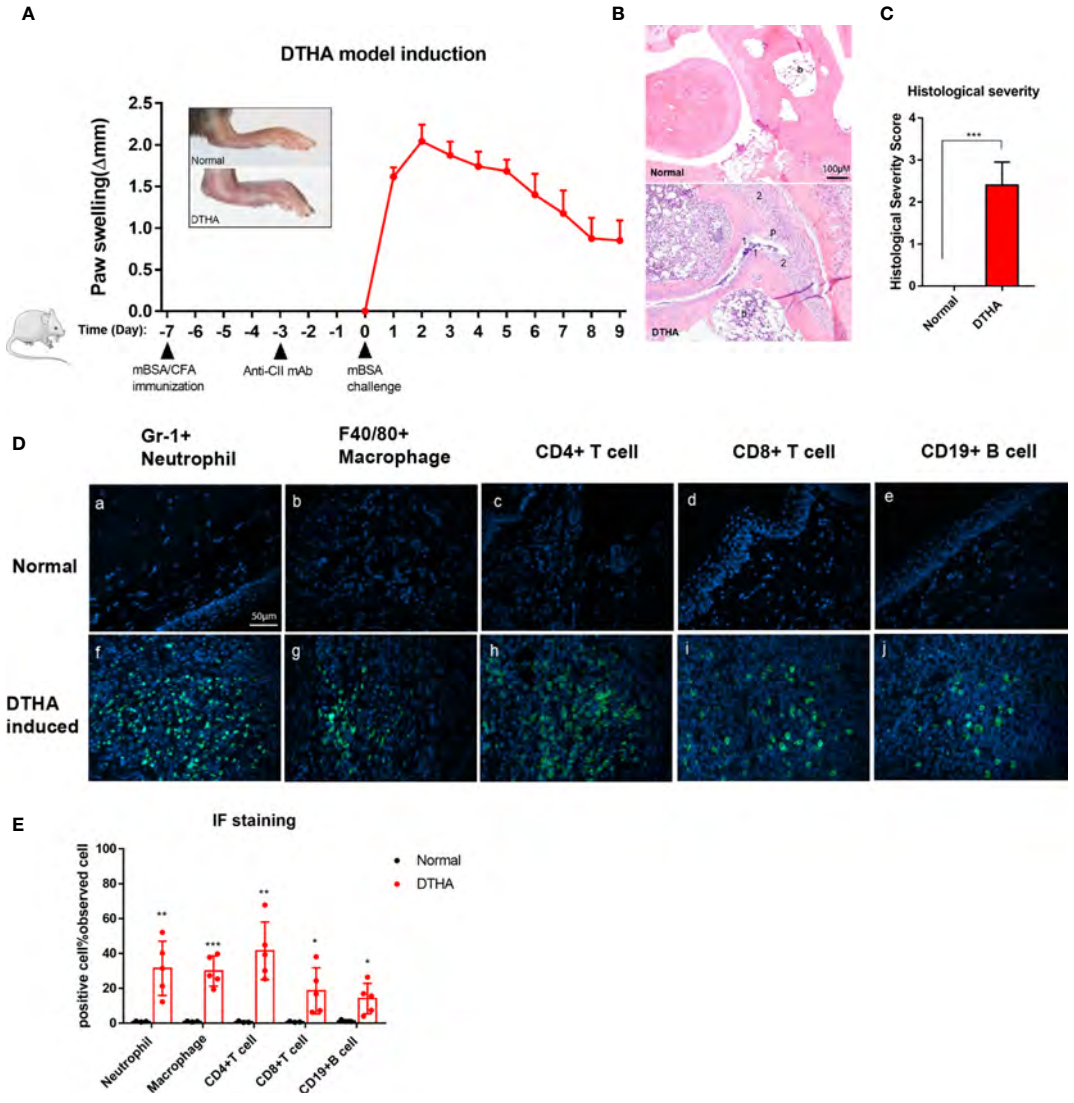

FIGURE 1 | Swelling, inflammatory severity score, and immune cell infiltration of paws induced by DTHA in mice. **(A)** The timeline of DTHA induction in mice by immunization with 100 μg mBSA on day minus 7 (day −7), injection of anti-CII (50 mg/kg) on day −3 and 200 μg mBSA challenge at day 0. Paw swelling was followed between day 0 and 9 post the final 200 ug mBSA challenge. **(B, C)** Representative H&E stained sections (3.5 μM) of the paws **(B)** and histological severity score **(C)** from DTHA-induced and normal mice on day 9. Original magnification is 200×; scale bar, 100 μm; b: bone marrow; p: pannus; 1: destruction of joint cartilage; 2: Inflammation infiltration. Histology scores were calculated by the evaluation scale (see *Materials and Methods*). **(D)** Representative images of immunofluorescence staining for neutrophils (Gr-1+), macrophages (F4/80+), CD4+ T cells (CD4+), CD8+ T cells (CD8+), B cells (CD19+) infiltrating paws from normal (a–e) and DTHA-induced mice (f–j) on day 9. Original magnification, × 400; scale bar, 50 μm. Hoechst 33342 (blue colour) was used for nuclear counterstaining, while corresponding surface marker was shown in green colour. **(E)** Quantification of Gr-1+ neutrophils, F4/80+ macrophages, CD4+ T cells, CD8+ T cells, and CD19+ B cells. Values are expressed as the mean ± SD. All data are representative of two independent experiments with n = 3 in the normal group and n = 5 in DTHA group per experiment. Statistical significance was calculated by Student's *t*-test to measure significant difference between normal group and DTHA group. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). The mouse image is from smart.servier.com.

(5 mg/ml) in 40 µl PBS (Hooke Laboratories) subcutaneously in the left footpad and with vehicle (PBS, Sigma-Aldrich) in the right footpad, which serves as an intra-animal control (day 0). After 3–7 days, acute inflammation and severe arthritis rapidly developed in the mBSA-challenged paw. Mice were terminated on 1 day or 9 days after the mBSA challenge.

MTX and DEX Administration

To determine the efficacy of MTX, mice were given MTX (0.75, 1.5, 3, 6 mg/kg i.p., Sigma-Aldrich) dissolved in 0.5% DMSO-containing PBS or the same volume of 0.5% DMSO-containing PBS (negative control) every other day from 2 weeks before mBSA immunization. In the DEX treatment group, mice were injected (i.p.) daily with 1 mg/kg DEX (Sigma-Aldrich) from the DTHA onset (day 0) to the end of study.

Measurement of Paw Swelling and Histopathology

Clinical signs of arthritis in mice were recorded every day by measurement of paw thickness using a dial thickness gauge (Mitutoyo) after challenge with mBSA. The severity of hind paw swelling was evaluated as the increase in thickness of left inflamed paw (Δthickness = left inflamed paw thickness – right control paw thickness). On the termination day, mBSA-challenged left paws were dissected, fixed in 4% paraformaldehyde (VWR), decalcified in 7% EDTA solution for 2 weeks, and then embedded in paraffin blocks. For general assessment of histopathology, the paraffin-embedded tissues were sectioned (3.5 µm) and stained by hematoxylin and eosin (H&E). The histopathologic score was evaluated by a 1–4 scale: 1, hyperplasia of the synovial membrane and presence of inflammation infiltration; 2, pannus and cartilage erosion; 3, major erosion of cartilage and subchondral bone; and 4, loss of joint integrity and ankyloses (42). For each sample, three joint areas were selected, and the average histopathologic score was used for calculating histological severity score.

Myeloperoxidase Activity Examination by IVIS Imaging

To determine myeloperoxidase (MPO) activity, DTHA mice were injected i.p. with 150 µl/mouse XenoLight RediJect Inflammation probe (Perkin Elmer), a chemiluminescent substrate of MPO, 1 day after mBSA challenge. Luminescence images were captured by SpectrumCT *In Vivo* Imaging System (IVIS; Perkin Elmer) 10 min after injection of the probe. The images were acquired with the following parameters (f/stop = 1; exposure time = 180 s; binning factor = 8). The results were expressed as the intensity of radiance (photons/s/cm^2/sr) using Living Image software (version 4.2; Perkin Elmer).

Assessment of Immune Cell Infiltration in Arthritic Paws by Immunohistochemistry

To detect infiltrated immune cells, paw sections (3.5 µm) were fixed with acetone and denatured with 100, 95, and 75% ethanol for 15 s separately. For antigen retrieval, sections were heated in Tris-EDTA retrieval buffer (10 mM Tris Base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0) in a water bath at 98°C for 20 min. After cooling down at RT for 30 min, sections were incubated with antibodies against F4/80 (clone CI:A3-1; Abcam), GR-1 (clone RB6-8C5; R&D), CD4 (clone 4SM95; Thermo Fisher), CD8 (clone 4SM15; Thermo Fisher), and CD19 (clone 6OMP31; Thermo Fisher) at 4°C overnight for detecting macrophages, neutrophils, CD4+ T cells, CD8+ T cells, and B cells respectively. Next, sections were washed with PBS and incubated with anti-Rat IgG (Alexa Fluor 488 conjugated; Thermo Fisher) secondary antibody. Cell nucleus was counterstained with Hoechst 33342 (Thermo Fisher), and sections were visualized using a fluorescence microscope (Olympus BX61). Quantification of fluorescence intensity was performed by count function in the software of CellSens dimension (Olympus). Briefly, five different regions in arthritis paw from DTHA mice and normal paw from untreated mice with strongest fluorescence intensity were selected for each sample. Then the average value of fluorescence intensity was calculated from these five regions and used for the next statistical analysis.

Flow Cytometry

In order to detect pro-inflammatory cells in DTHA, single-cell suspensions were prepared from draining inguinal lymph node (iLN) and spleen of mice in different groups on the termination day (day 1 or/and day 9). To quantify CD4+ T cells (CD4+), activated CD4+ T cells (CD4+CD69+CD25+), central memory CD4+ T cells (CD4+CD62L+CD44+), effector memory CD4+ T cells (CD4+CD62L−CD44+), Th1 (CD4+ CD183+ CD194−CD196−), Th17 (CD4+CD183− CD194+CD196+), and B cells (B220+CD4−), corresponding antibodies were used for incubation (11, 43, 44). Please find the information of all antibodies for flow cytometry in **Supplementary Table 1**. Post incubation with antibodies, samples were washed and analyzed by FACS Canto II flow cytometer (BD Biosciences). Flow data analyses were done in FlowJo V10 (BD Biosciences).

ELISA Assay for Antibody Determination

Nine days post mBSA challenge, blood samples were collected from DTHA mice treated with PBS or MTX and then centrifuged for 15 min at 2,000 g for plasma preparation. Murine total IgG antibody levels in the plasma were examined by enzyme-linked immunosorbent assays (ELISAs) as per the instructions (Thermo Fisher). Briefly, ELISA plates were coated with purified anti-mouse IgG monoclonal antibody overnight at 4°C, washed three times with wash buffer (PBS containing 0.05% Tween-20 and 1% BSA), and finally blocked with PBS containing 1% BSA overnight at 4°C. Next day, serial dilutions of plasma (diluted at 1:625, 1:2,500, and 1:10000) were added. The plates were detected using horseradish peroxidase-conjugated anti-mouse IgG, followed by TMB substrate. Finally, stop buffer was added, and optical density was measured at 450 nm. Antibody concentration for each sample was calculated by using standard curve.

Statistical Analysis

All significance values between two groups were determined by the unpaired Student's t-test. Comparison between more than

two groups was analyzed with ANOVA with Tukey multiple comparison test. The correlation between two variables was calculated by Pearson correlation test. Data are presented as the mean ± the standard deviation, and all the statistical analyses were performed by GraphPad Prism 9 (GraphPad Software). $P<0.05$ was considered a statistically significant difference, and levels of significance were assigned as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, and $^{****}P \leq 0.0001$.

RESULTS

Establishing the DTHA Mouse Model

The DTHA model in mice was established as described in the *Materials and Methods* and by others and according to the timeline depicted in **Figure 1A** (24, 25). In line with the observations of others, paw swelling was detected and measured 12–24 h post the mBSA challenge denoted day 1 (see *Materials and Methods* and **Figure 1A**). Histopathological examination of tissue sections by H&E staining 9 days post mBSA challenge revealed synovial inflammatory infiltration, pannus formation, and bone/cartilage erosion in DTHA-induced paw (**Figures 1B, C**). In RA, inflammation is associated with the recruitment of a variety of immune cells. Based on this we next qualitatively and quantitatively investigated the identity and levels of infiltrated immune cell composition by immunofluorescence in the paws of DTHA compared to normal unaffected mice, respectively. We observed a significant increase in the levels of GR1+ neutrophils, F4/80+ macrophages, CD4+ T cells, CD8+ T cells, and CD19+ B cells in the paws of DTHA-affected mice compared to normal mice (**Figures 1D, E**).

Levels of Activated CD4+ T Cells in Lymph Nodes and Spleen of DTHA Mice Are Elevated

As mentioned, CD4+ T cells are indispensable for DTHA-induced inflammation and paw swelling (24, 25). Because of this, we measured the level of CD4+ T cells in the spleen and inguinal LN (iLN), the latter as representative paw-draining LN, on day 1 and 9 post DTHA onset. This demonstrated that the proportions of CD4+ T cells in both iLN and spleen were reduced significantly, despite that the absolute numbers increased in iLN and remained the same in spleen (**Figures 2A–F**). In accordance with this result and the fact that the mBSA challenge was expected to induce extensive cell division, we measured the level of activated CD4+ T cells by staining for the CD25 and CD69 cell surface markers (45). This demonstrated that the absolute and relative number of activated CD4+ T cells (CD4+ CD25+CD69+) on day 1 after the mBSA challenge were significantly elevated and declined over the time span investigated (days 1 through 9, **Figures 2G–L**).

Differential Induction of Th1 and Th17 Cells in DTHA Mice

The Th1 and Th17 effector T cells are considered crucial players in the pathogenesis and development of RA (8–14). Flow cytometry analysis was used to quantify Th1 and Th17 cells isolated from iLN of mice challenged with mBSA on days 1 through 9. When comparing to normal mice, sustained elevation of both relative and absolute numbers of Th1 cells (CD4+ CD183+CD194−CD196−) in iLN were observed in the DTHA-induced mice (**Figures 3A–C**). In contrast to Th1 cells, the relative and absolute number of Th17 cells (CD4+CD183−CD194+CD196+) in iLN exhibited a significant increase on day 1, which was transient and had decreased by day 9 (**Figures 3D–F**). We further explored the changes in splenic Th1 and Th17 cells and showed a significant increase in the relative number on day 9 (**Supplementary Figures 1A–D**). Together, these findings imply that levels of Th1 and Th17 cells are differentially upregulated during DHTA development.

Central/Effector Memory CD4 + T Cells Are Increased in DTHA Mice

Levels of memory CD4+ T cells have been reported to be elevated in the inflamed synovium of RA patients and to be essential for the development of the DTH response (15, 16, 46). Memory CD4+ T cells may be subdivided into central and effector memory CD4+ T cells (47). Whereas, CD44 expression is enhanced on all memory T cells, differential expression of CD62L is commonly used to distinguish central memory from effector memory T cells (48, 49). When staining for CD44 and CD62L, we observed a significant long-term increase in both relative and absolute number of both central memory (CD4+CD62L+CD44+) and effector memory (CD4+ CD62L− CD44+) CD4+ T cells isolated from iLN in the DTHA mice (**Figures 4A–E**). This was identical to that of the splenic effector memory but not central memory CD4+ T cells (**Supplementary Figures 2A–E**). However, splenic central memory CD4+ T cells only showed a temporal increase over time.

B Cell Levels Increase in Draining Lymph Node but Not Spleen in DTHA Mice

B cells are instrumental in the pathogenesis of RA as they are activated to produce rheumatoid factor, which, in addition to a number of other autoantibodies, is associated with RA initiation and pathogenesis (50). To investigate a potential role of B cells involvement in the pathogenesis of DTHA, we examined the relative and absolute numbers of B cells (B220+CD4−) in addition to levels of circulating IgG after the mBSA challenge on day 0. We observed a significant twofold increase in the proportion of B cells in iLN, which lasted throughout the study time (days 1 through 9) (**Figures 5A–C**). The rise in B cells coincided with elevated level of IgG in circulation (**Figures 5D, E**). It should be noted that the number of splenic B cells was decreased on day 1 and was restored back to initial levels on day 9 (**Figures 5F–H**).

MTX Prevents DTHA-Associated Paw Swelling and Inflammation

MTX has been demonstrated effective in attenuating inflammation in many experimental arthritis murine models, including the CIA and AIA models (51, 52). Despite this, the effects of MTX on inflammation and paw swelling have not been

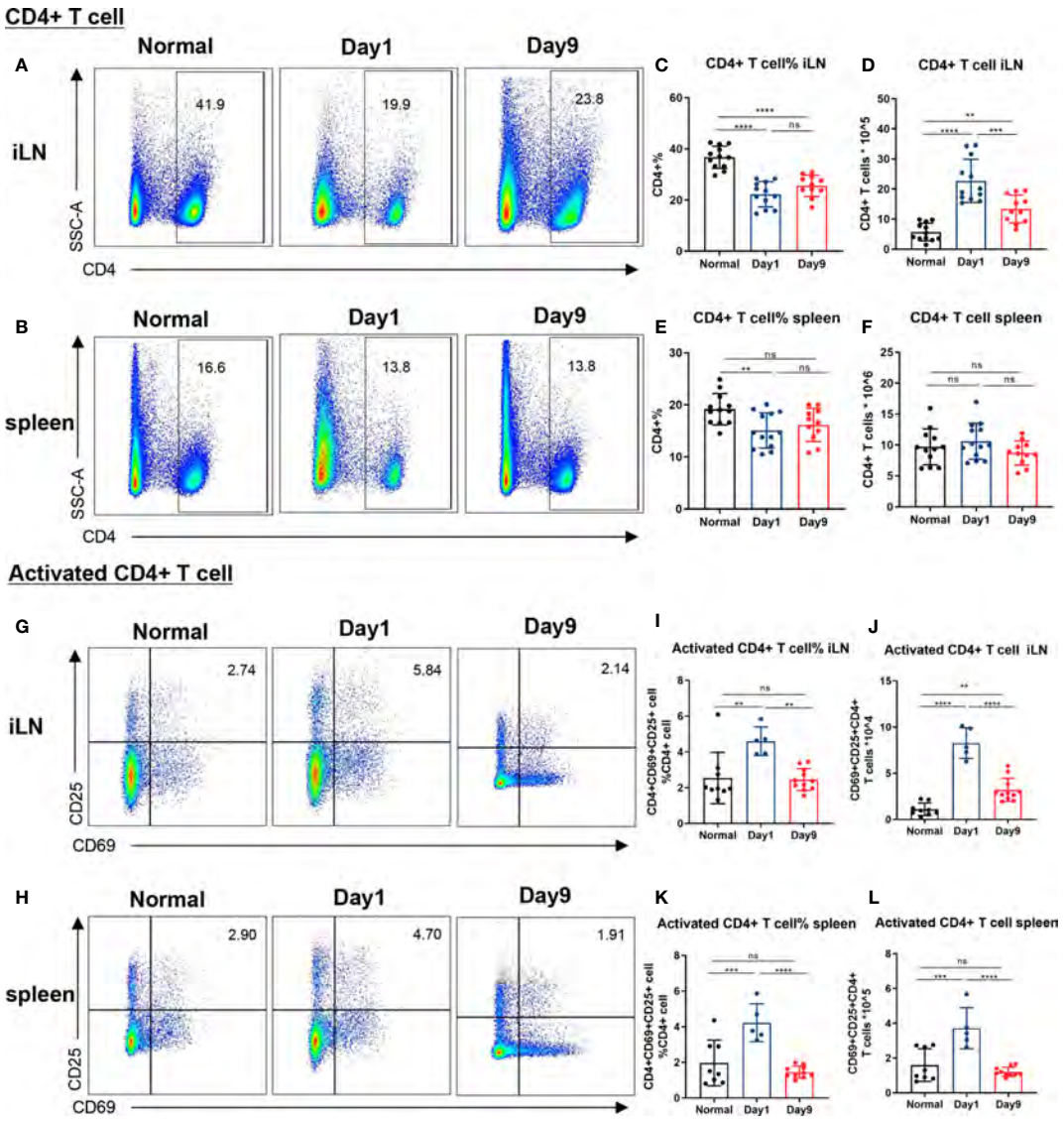

FIGURE 2 | Levels of CD4+ and activated CD4+ T cells in lymph nodes and spleen of DTHA mice. Relative and absolute levels of CD4+ and activated CD4+ T cells isolated from draining inguinal lymph node (iLN) and spleen of normal mice and DTHA-induced mice on day 1 and day 9 post the mBSA challenge. **(A, B)** Representative flow cytometry dot plot of CD4+ T cell from iLN **(A)** and spleen **(B)**. **(C–F)** Quantification of relative and absolute number of CD4+ T cells from iLN **(C, D)** and spleen **(E, F)**. **(G, H)** Representative levels of activated CD4+ T cells (CD4+CD69+CD25+) from iLN **(G)** and spleen **(H)**. **(I–L)** Relative number and absolute number of activated CD4+ T cells from iLN **(I, J)** and spleen **(K, L)** on day 1 and day 9. Data is presented as mean ± SD. All data pooled from two independent experiments (at least five mice per group in each experiment) with the exception of the data of activated CD4+ on day 1, which is from one experiment with five mice. Statistical significance was calculated by ANOVA with Tukey multiple comparison test (**p < 0.01, ***p < 0.001, ****p < 0.0001, ns: p > 0.05).

explored in the DTHA model. To determine the efficacy of MTX in the DTHA model, we initially monitored its effect on paw swelling. Because MTX is a slow-acting regimen, long-time administration before symptom onset was adopted from other inflammatory mice models (51, 53–55). We chose a protocol starting with treatment at day minus 20 of the mBSA challenge (**Figure 6A**). We tested incremental doses of MTX ranging from 0 to 6 mg MTX/kg (**Figure 6B**) and showed that 6 mg MTX/kg was required to significantly delay and reduce paw swelling. The most pronounced decline in paw swelling was apparent during the first 24 h after the mBSA challenge (day 0), where the average paw swelling (Δmm) decreased from 1.84 ± 0.16 to 1.11 ± 0.50 (**Figures 6C, D**). To determine the effect of MTX on the ongoing inflammatory activity caused by DTHA, we next assessed neutrophil infiltration by monitoring myeloperoxidase (MPO) activity. This showed that MPO activity was significantly reduced using 6 mg MTX/kg (**Figures 6E, F**). Finally, the level of luminescence intensity was positively correlated with paw swelling when administrating 6 mg MTX/kg on day minus 20 of the mBSA challenge (**Figure 6G**).

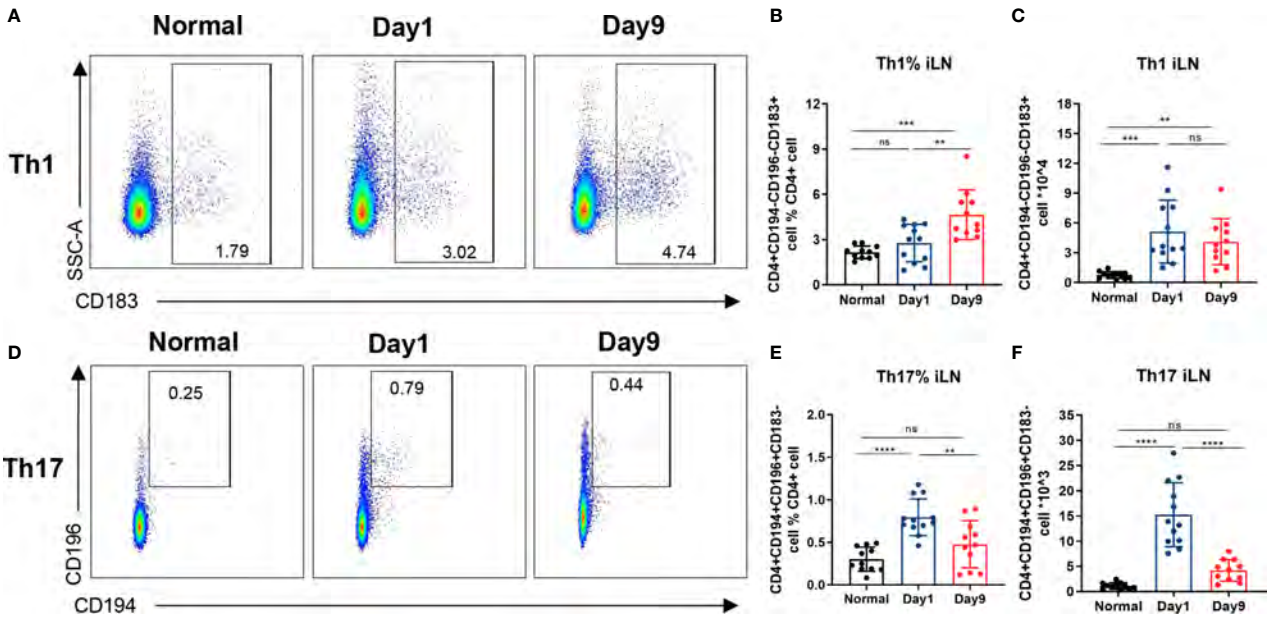

FIGURE 3 | Differential induction of Th1 and Th17 cells in DTHA mice. Relative and absolute levels of Th1 and Th17 cells in draining iLN of normal and DTHA mice on day 1 and day 9 post mBSA challenge analyzed by flow cytometry. **(A)** Representative flow cytometry dot plot illustrating relative population of Th1 cells (CD4+ CD194−CD196−CD183+). **(B, C)** Fraction (%) **(B)** and absolute number **(C)** of Th1 cells on day 1 and day 9 post mBSA challenge. **(D)** Representative flow cytometry dot plot illustrating relative population of Th17 cells (CD4+CD194+CD196+CD183−). **(E, F)** Fraction (%) **(E)** and absolute number **(F)** of Th17 cells on day 1 and day 9 post mBSA challenge. Data is presented as mean ± SD from two independent experiments with at least five mice in each group per experiment. Statistical significance was calculated by ANOVA with Tukey multiple comparison test (**p < 0.01, ***p < 0.001, ****p < 0.0001, ns: p > 0.05).

Differential Effects of MTX and DEX on DTHA Mice

DEX is a potent anti-inflammatory drug used in RA treatment (19, 20, 40, 41). Due to this, we also tested the effects of DEX in the DTHA model and compared the effects to MTX. In contrast to MTX, which had a marked effect on the early stage of DTHA development (between day 0 and 3), DEX exerted a more delayed but sustained effect on paw swelling from days 1 through 9 post the mBSA challenge (**Figure 7A**). It should however be noted that DEX treatment, in contrast to MTX, negatively affected the weight of the mice over time (**Supplementary Figures 3A–C**). This was apparent, despite that the MTX and DEX were administrated from day minus 20 and day 0 or day minus 8, respectively. This discrepancy suggested differential modes of action and prompted us to assess their effects by examining histological severity and inflamed paw cell infiltration. We found that both MTX and DEX ameliorated DTHA-induced histological severity (**Figures 7B, C**). Moreover, both MTX and DEX prevented paw immune cell infiltration, which was associated with reduced levels of neutrophils and CD4+ T cells, whereas macrophages, CD8+ T cells, and CD19+ B cells were not altered significantly (**Figures 7D, E**).

To further examine and compare the effects of MTX and DEX, we monitored the relative numbers of immune cells in iLN on day 9. We found that MTX but not DEX treatment suppressed accumulation of the relative number of activated CD4+ T cells and Th1 cells in the iLN (**Figures 8A–G**). We also noted that the DEX treatment led to notable decrease in the absolute number of CD4+ T cells subsets residing in the iLN (**Figures 8H–N**). This coincided with a marked size reduction of the iLN with decreased cell number after the DEX treatment (**Supplementary Figure 4A**). Such shrinkage was also found for the spleen and coincided with reduced total numbers of splenocytes after the DEX treatment (**Supplementary Figures 5A–G, 4B**). By contrast, MTX did not have any effect on the absolute number of splenic immune cells in general. In fact, the MTX treatment was only associated with a reduction of the relative number of splenic Th1 cells. Together with its effect on iLN Th17 cells, this may indicate that the inhibitory effect of MTX on Th1 occurs through altered differentiation (**Supplementary Figures 5H–N**). In addition to T cells, the effects of MTX and DEX on splenic but not iLN B cell levels were differentiated (**Figures 8M, N** and **Supplementary Figures 5G, N**). To this end, the relative number of splenic B cells were reduced by MTX but not DEX, while the absolute number decreased with DEX (**Supplementary Figures 5G, N**). Finally, we found that 6 mg/kg MTX administration from day minus 20 in contrast to 1 mg/kg DEX exerted no effect on mice weight (**Supplementary Figures 3A–C**). Taken together, we observed that MTX suppressed immune cell numbers in a subset-specific manner, while DEX affected all immune cells investigated.

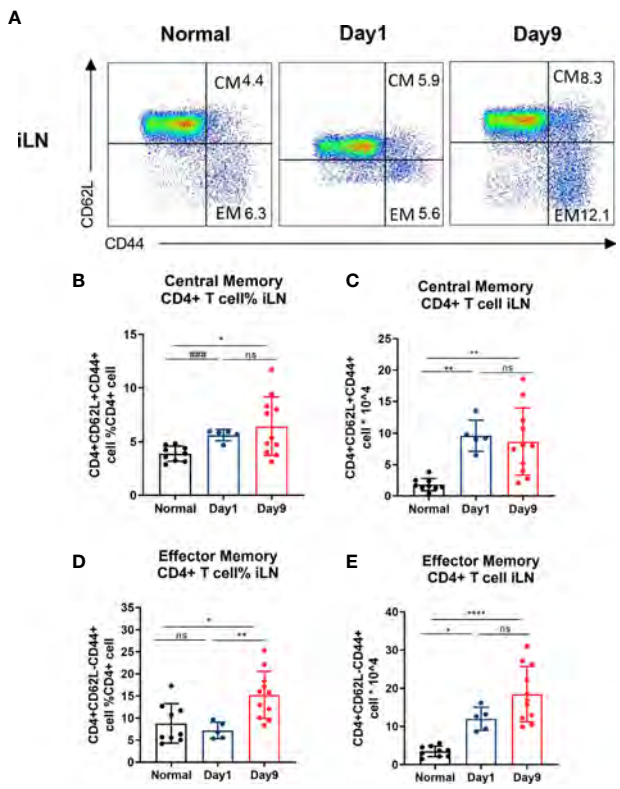

FIGURE 4 | Central/effector memory CD4 + T cells are increased in DTHA mice. Cells were isolated from draining iLN of normal mice and DTHA-induced mice on day 1 and 9 post mBSA challenge and analyzed by flow cytometry. **(A)** Representative flow cytometry dot plot of central/effector memory CD4+ T cells from iLN from normal mice and DTHA-induced mice on day 1 and day 9 post mBSA challenge. **(B, C)** Relative number **(B)** and absolute number **(C)** of central memory CD4+ T cells (CD4+CD62L+CD44+) from iLN in given groups. **(D, E)** Relative number **(D)** and absolute number **(E)** of effector memory CD4+ T cells (CD4+CD62L–CD44+) from iLN are shown. Data is presented as mean ± SD. Data of naïve group and day 9 group are from two independent experiments with at least five mice per group per experiment. Data of day 1 group is from one experiment with five mice. Statistical significance was calculated by ANOVA with Tukey multiple comparison test (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, ns: $p > 0.05$) or Student's *t*-test ($^{###}p<0.001$). CM: central memory CD4+ T cell. EM: effector memory CD4+ T cell.

DISCUSSION

Well-defined preclinical animal models are required to better understand the mechanism of disease pathologies and how to target autoimmunity arthritis (17–20, 42, 43). The DTHA mouse model has recently been established and provides a promising tool to study RA initiation, development, and targeting (20). In this study, paw swelling, inflammation, and levels of inflammatory immune cells associated with DTHA were studied in the presence and absence of the two RA drugs, MTX and DEX.

Consistent with previous studies, DTHA mice exhibited sustained paw swelling and histological manifestations such as synovitis, synovial hyperplasia, and pannus formation, together with infiltration of inflammatory cells (24). The latter included infiltration of neutrophils, macrophages, CD4+ or CD8+ T cells, and B cells at the site of inflammation. These clinical signs are reminiscent of human RA and of the experimental RA mouse models, CIA, AIA, and CAIA model (56–58). It has been shown that paw swelling in the DTHA model depends on the presence and activity of CD4+ T cells and can be augmented by depletion of regulatory T cells (Tregs) (20, 44). Together these findings suggest the mechanism underlying DTHA inflammation requires the presence of CD4+ T cells. We could confirm this in that elevated levels of activated CD4+ T cells were detected in both iLN and spleen on day 1 post the mBSA challenge. Interestingly, the total CD4+ T cells proportion in iLN and spleen of the DTHA mice was significantly reduced rather than increased as compared with normal non-affected mice, although with elevated absolute numbers in iLN. We speculate that this may be a sign of migration of CD4+ T cells to inflamed sites or generation of more proliferated non-CD4+ T cells in these peripheral immune organs.

To investigate this further, the level of CD4+ T cell effector subsets, including Th1 and Th17 cells, were studied. Th17 cells are considered crucial for the development of both RA in human and for the pathophysiological development of CIA in mice (59, 60). We found that Th17 cells were significantly increased in iLN in the DTHA mice. This may be indicative of Th17 cells involvement in DTHA development and is in line with previous studies where neutralizing Th17 cell-specific cytokine IL17 counteracted DTHA-induced inflammation (31). To further study the involvement of CD4+ T cell subsets during DTHA development, levels of Th1 cells were examined. Th1 cells are considered pivotal effector CD4+ T cells involved in RA development in humans and in the DTH response in mice (6). In contrast to Th17 cells, levels of Th1 cells increased in the iLN on day 1, and a significantly twofold elevation was observed on day 9 post the mBSA challenge. Elevated Th1 levels were also observed in the spleen on day 9. The fact that the Th17 cell population was only transiently elevated may suggest that these cells are involved in the early stage of DTHA initiation and development. By contrast, Th1 levels, which remained high throughout the time of observation, may be involved in the regulation of sustained paw swelling. Hence, the distinct dynamics of the Th1 and Th17 cell levels throughout the time of observation (days 1 through day 9) suggest differential roles for these T cells subsets in the initiation and development of DTHA. Moreover, our observation of early elevation of Th17 cells followed by a decline that coincided with a sustained upregulation of Th1 may implicate transdifferentiation of Th17 into Th1 during the first 9 days of DTHA. This suggestion finds support in that Th17 cells display a high degree of context-dependent plasticity and may transdifferentiate into Th1 under certain conditions of autoimmunity including RA (61–64). To what extent this is the case during DTHA development needs further investigation. However, and despite these differences, we suggest that both Th1 cells and Th17 cells are involved and important for DTHA-associated inflammation from day 1 of the mBSA challenge.

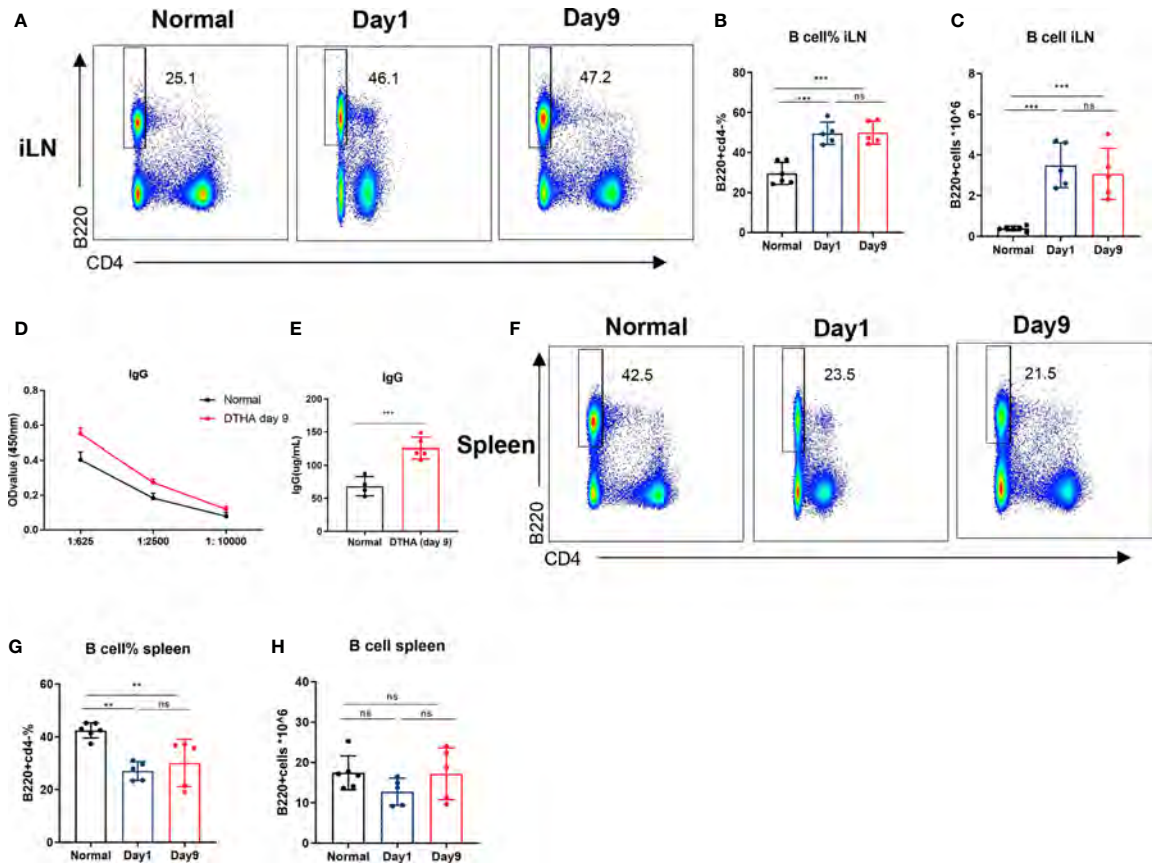

FIGURE 5 | B cell levels increase in draining lymph node but not spleen in DHTA mice. Cells were prepared from the draining iLN and spleen of DTHA-induced and normal mice on day 1 and day 9 and analyzed by flow cytometry. **(A)** Representative flow cytometry dot plot of B cells (B220+CD4–) from iLN DTHA and DTHA mice on day 1 and day 9 post mBSA challenge. **(B, C)** The proportion **(B)** and absolute number **(C)** of B in iLN are shown. **(D)** On day 9, serial dilutions of plasma were analyzed for IgG antibody titers by ELISA from normal mice and DTHA-induced mice. **(E)** The levels of plasma IgG antibodies (prediluted 1:625) determined by ELISA in mice from each group on day 9. **(F)** Representative flow cytometry dot plot of B cells from spleen in normal mice and DTHA mice on day 1 and day 9 post mBSA challenge. **(G, H)** The proportion **(G)** and absolute number **(H)** of B in spleen are shown. All data are presented as mean ± SD and representative of two independent experiments with n = 3–4 in the normal mice group and n = 5 in DTHA group per experiment. Statistical significance was calculated by Student's t-test for the comparison of two mouse groups (***$p < 0.001$). For the comparison of three groups, statistical significance was calculated by ANOVA with Tukey multiple comparison test (**$p < 0.01$, ***$p < 0.001$, ns: $p > 0.05$). ELISA, Enzyme-linked immunosorbent assay.

Elevated levels of memory CD4+ T cells are expressed in the synovial fluid of affected joints in RA patients and are thus suggested to play a role in the pathogenesis of RA (15). We observed a marked and persistent increase in memory T cells in the iLN of DTHA mice, defined as effector and central memory CD4+ T cells, respectively. In the DTH-induced models, memory CD4+ T cells are shown to emerge from antigen-specific T cells within 5–7 days after an initial exposure to the antigen (22, 23, 26, 49). In the present study, the DTHA mice were immunized with mBSA 7 days prior to the mBSA challenge. Consistent with the reports of others, memory CD4+ T cells in the present experimental setup were successfully observed from 7 days post mBSA immunizing event, hence indicating that memory T cells are likely to be involved in the inflammatory response to the mBSA challenge. Recently it has been hypothesized that memory CD4+ T cells are generated from early commitment of Th1 and Th17 cells (65–67). Moreover, it has been reported that peripheral and synovium memory CD4+ T cells express Th1 phenotypic CD183 and IFN-γ, respectively (68, 69). To this end, it should be noted that levels of central/effector memory CD4+ T cells coincided with Th1 cells, supporting a potential association of memory and Th1 cells in the DTHA mouse model. Finally, as mentioned, B cells have been suggested not to be responsible for DTHA development since B cell depletion does not affect DTHA-associated paw swelling (24, 25). However, the B cell-produced autoantibodies have been reported to be associated with increased bone erosion in RA (70, 71). Based on this, we speculated that B cells may play a role in DTHA through promoting bone destruction–related manifestation, which was marked in the DTHA mice, rather than intensifying paw swelling directly.

To substantiate further the role of the pro-inflammatory cells involved in DTHA, we also investigated the effect of the anti-rheumatoid drug MTX and the anti-inflammatory drug DEX on

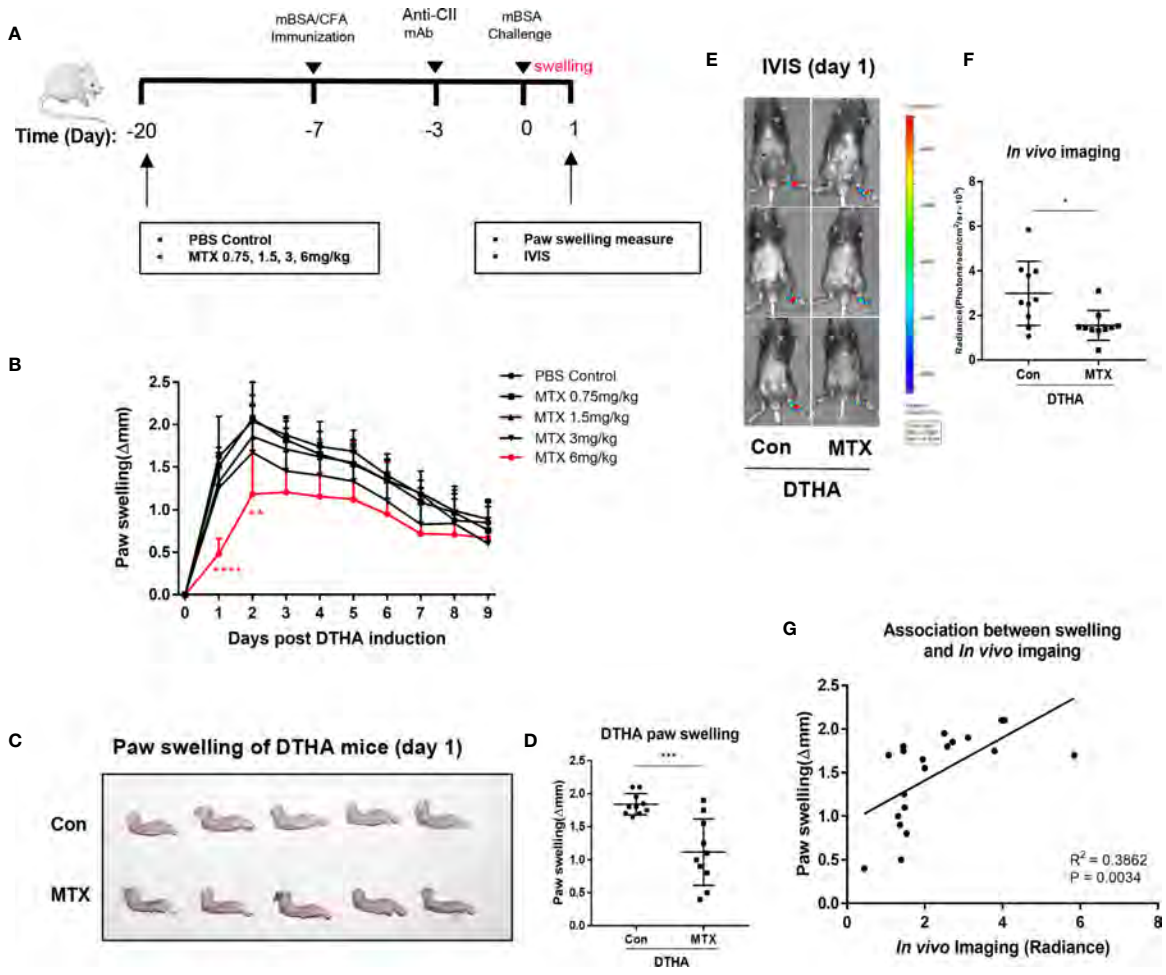

FIGURE 6 | MTX prevents DTHA-associated paw swelling and inflammation. **(A)** MTX treatment regime during DTHA induction is illustrated. **(B)** Effect of different dose of MTX (0–6 mg/kg) on paw swelling were monitored from day 0 to day 9 post DTHA onset. **(C, D)** Representative appearance **(C)** and quantification **(D)** of paw swelling in DTHA mice treated with and without MTX (6 mg/kg). **(E)** Representative bioluminescent images indicting myeloperoxidase (MPO) activity of hind paws from DTHA mice treated with and without 6 mg MTX/kg on day 1. **(F)** Quantification of in *vivo* luminescence imaging for mice treated with and without MTX (6 mg/kg). **(G)** Linear regression analysis of paw swelling and in *vivo* luminescence imaging for MPO activity in DTHA mice treated with and without MTX (6 mg/kg). The data are representative of pooled data from two independent experiments with five mice in each group per experiment, with the exception of the data of 0.75 and 3 mg/kg MTX treatment, which are from one experiment with five mice. Statistical significance was calculated by Student's t-test for the comparison of two mouse groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Stars under the curve in panel B represent significant difference in paw swelling between 6 mg MTX/kg treatment group and PBS-treated control group (**$p < 0.01$, ****$p < 0.0001$). The correlation between paw swelling and imaging was calculated by Pearson correlation test. Con, DTHA Control group. The mouse image is from smart.servier.com.

paw swelling and immune cell infiltration in the swollen paw. As the effects of MTX have not been studied in the DTHA model, we tested its effect by titrating the dose and found that 6 mg MTX/kg was required for successful prevention of DTHA-associated paw swelling and inflammation. We noted that 6 mg MTX/kg is markedly higher compared to the dose of MTX required to dampen inflammation in the CIA and the AIA mice models, which is between 0.75 and 3 mg MTX/kg (51, 52). This may imply that DTHA inflammation is less sensitive to MTX compared to inflammation in the CIA and AIA models. We speculate that this may in part be caused by the anti-CII at day minus 3. Anti-CII has been reported by others to render the inflammatory response less sensitive to MTX treatment (72). Moreover, consistent with a previous study, administration of a low dose (1 mg/kg) of DEX suppressed DTHA-associated paw swelling in a more potent and sustained fashion (24). Specifically, DEX was effective immediately after administration at day 0, whereas MTX had to be administrated at day −20 upon the mBSA challenge. These observations strongly point to differential modes of action of DEX and MTX, which was supported in that the two drugs displayed differential effect on the levels of pro-inflammatory cell in the iLN and spleen. MTX reduced the proportion of activated CD4+ T cells in both DTHA mice and *in vitro* (**Supplementary Figure 6**). Additionally, MTX diminished DTHA-induced increased level of Th1 cells, which is consistent with previous studies showing

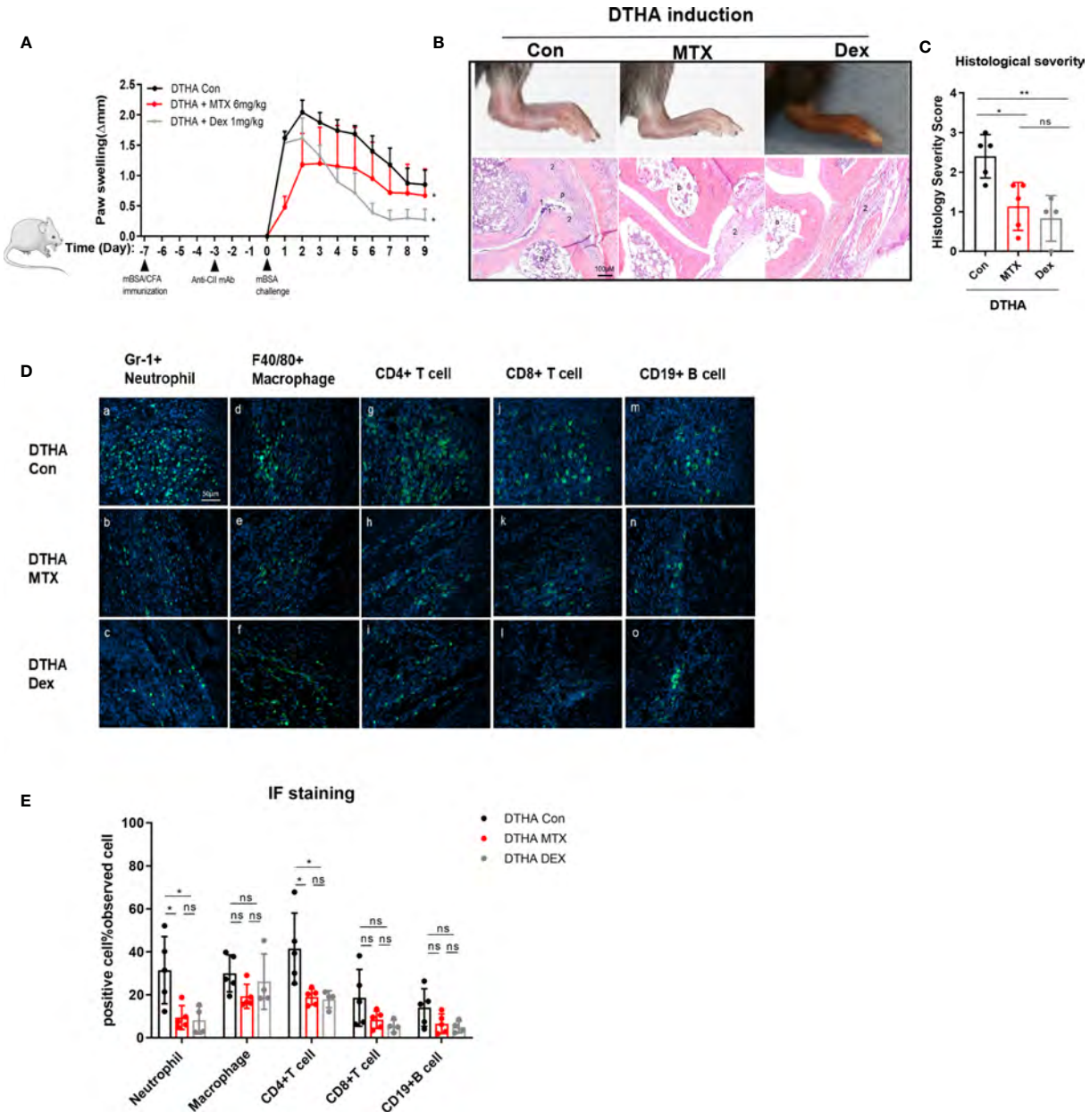

FIGURE 7 | Differential effects of MTX and DEX on DTHA-induced paw swelling but not inflammation. **(A)** Dynamic effects of MTX (6 mg/kg) and DEX (1 mg/kg) on paw swelling from day 0 to day 9 after the mBSA challenge. Changes of paw swelling (Δmm) were used to determine effects of MTX and DEX. (*) significant difference in paw swelling between DTHA mice treated with or without MTX or DEX. Data are representative of two independent experiments (n = 4–6 mice in each group). **(B)** Representative H&E-stained sections (3.5 μM) of the paws were analyzed from one experiment from DTHA mice treated without (n = 5 mice) or with MTX (n = 5 mice) or DEX (n = 4 mice) on day 9. Original magnification is ×200; scale bar, 100 μm; b: bone marrow; p: pannus; 1: destruction of joint cartilage; 2: inflammation infiltration. **(C)** Histology scores were calculated by the evaluation scale (see *Materials and Methods*). **(D)** Representative images of immunofluorescence staining on day 9 for neutrophils (Gr-1+) (a–c), macrophages (F4/80+) (d–f), CD4+ T cells (CD4+) (g–i), CD8+ T cells (CD8+) (j–l), B cells (CD19+) (m–o) of paws from one experiment from DTHA mice treated without (n = 5 mice) or with MTX (n = 5 mice) or DEX (n = 4 mice). Original magnification, ×400; scale bar, 50 μm. Hoechst 33342 (blue color) was used for nuclear counterstaining, while corresponding surface marker was shown in green color. **(E)** Quantification of Gr-1+ neutrophils, F4/80+ macrophages, CD4+ T cells, CD8+ T cells, and CD19+ B cells. Values are expressed as the mean ± SD. Statistical significance in panel A was calculated by Student's *t*-test (*$p < 0.05$) to compare the difference between DTHA control mice and DTHA mice treated with MTX or DEX. Statistical significance in panels C and E was calculated by ANOVA with Tukey multiple comparison test (*$p < 0.05$, **$p < 0.01$, ns: $p > 0.05$). Con, DTHA Control group. The mouse image is from smart.servier.com.

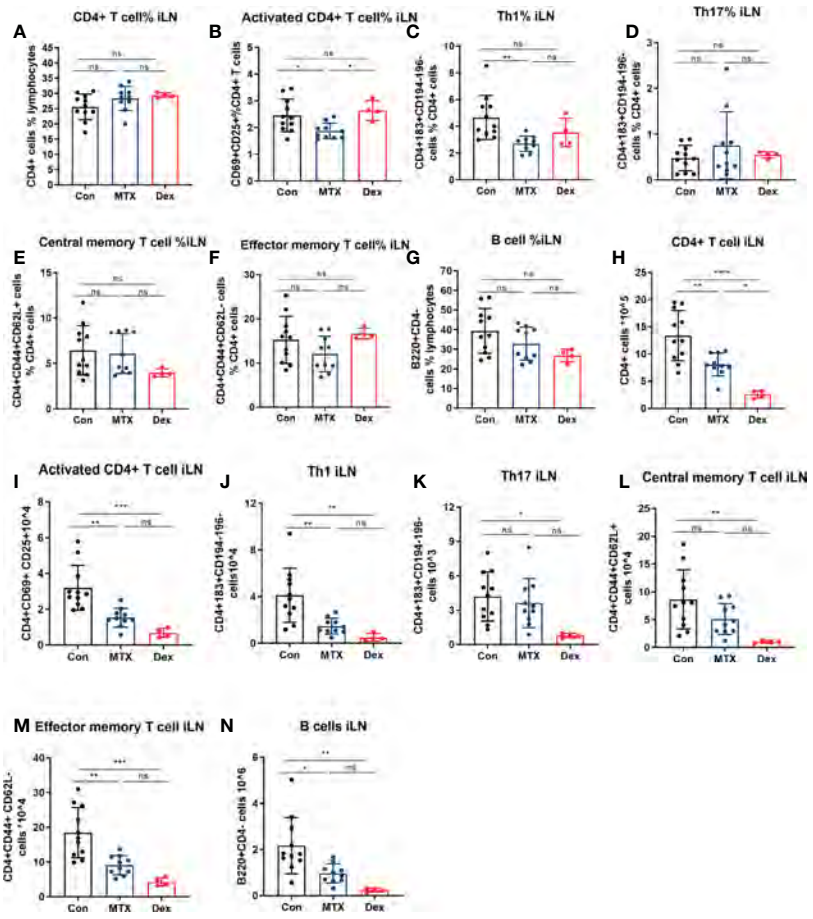

FIGURE 8 | Differential effects of MTX and DEX on levels of DTHA-induced immune cells. Cell suspensions were prepared from the draining iLN of DTHA mice treated without or with MTX or DEX on day 9. CD4+ T cell, activated CD4+ T cell, Th1, Th17, central/effector memory T cell, and B cell were analyzed by flow cytometry. The proportion and absolute number of CD4+ T cell **(A, H)**, activated CD4+ T cell **(B, I)**, Th1 **(C, J)**, Th17 **(D, K)**, central memory CD4+ T cell **(E, L)**, effector memory CD4+ T cell **(F, M)** and B cell **(G, N)** in iLN from mice in given groups are shown.. All data are presented as mean ± SD. The DEX group data is from one experiment. All other data are pooled from two independent experiments with three to six mice in each group per experiment. Statistical significance was calculated by ANOVA with Tukey multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns: $p > 0.05$). Con, DTHA Control group.

that MTX can effectively suppress both Th1 cell population and its cytokine production, such as IFN-γ (73–75). This was in contrast to DEX, which failed to affect any of CD4+ T cell subsets proportion in iLN. Instead, DEX treatment markedly reduced cellularity of both iLN and spleen and thus reduced the absolute number of CD4+ T cell subsets in these immune tissues. Furthermore, DEX but not MTX treatment was associated with weight loss. Based on this, we conclude that DEX exerts a broader inhibitory effect on pro-inflammatory cells compared to MTX.

Results from this study document the dynamics of Th1, Th17, and memory CD4+ T cells, as well as activated B cell-induction during DTHA-induced paw swelling. Furthermore, the two widely prescribed RA drugs, MTX and DEX, revealed differential effects on paw swelling and levels of pro-inflammatory cells, further confirming a vital role for Th1, Th17, and memory T cells, as well as B cells, in contributing to DTHA in mice.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by National Animal Research Authority (Norway).

AUTHOR CONTRIBUTIONS

GL designed, performed, analyzed the experiments and contributed to write the manuscript. SK designed, assisted with the experiments, and contributed to writing the manuscript. SG, KM, and GM helped with experiments that involved mice and

revised the manuscript. PK assisted with the experiments. FG designed the experiments and revised the manuscript. GH revised the manuscript. BS led the project, designed, supervised the experiments and their analyses, and contributed to write the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.689057/full#supplementary-material

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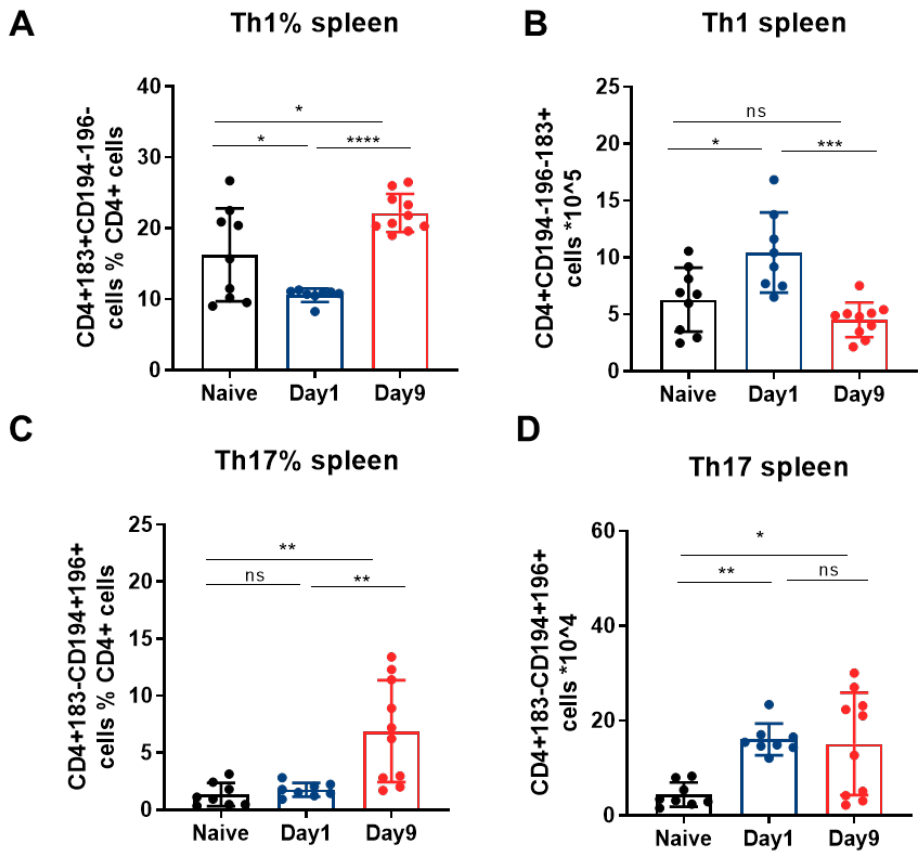
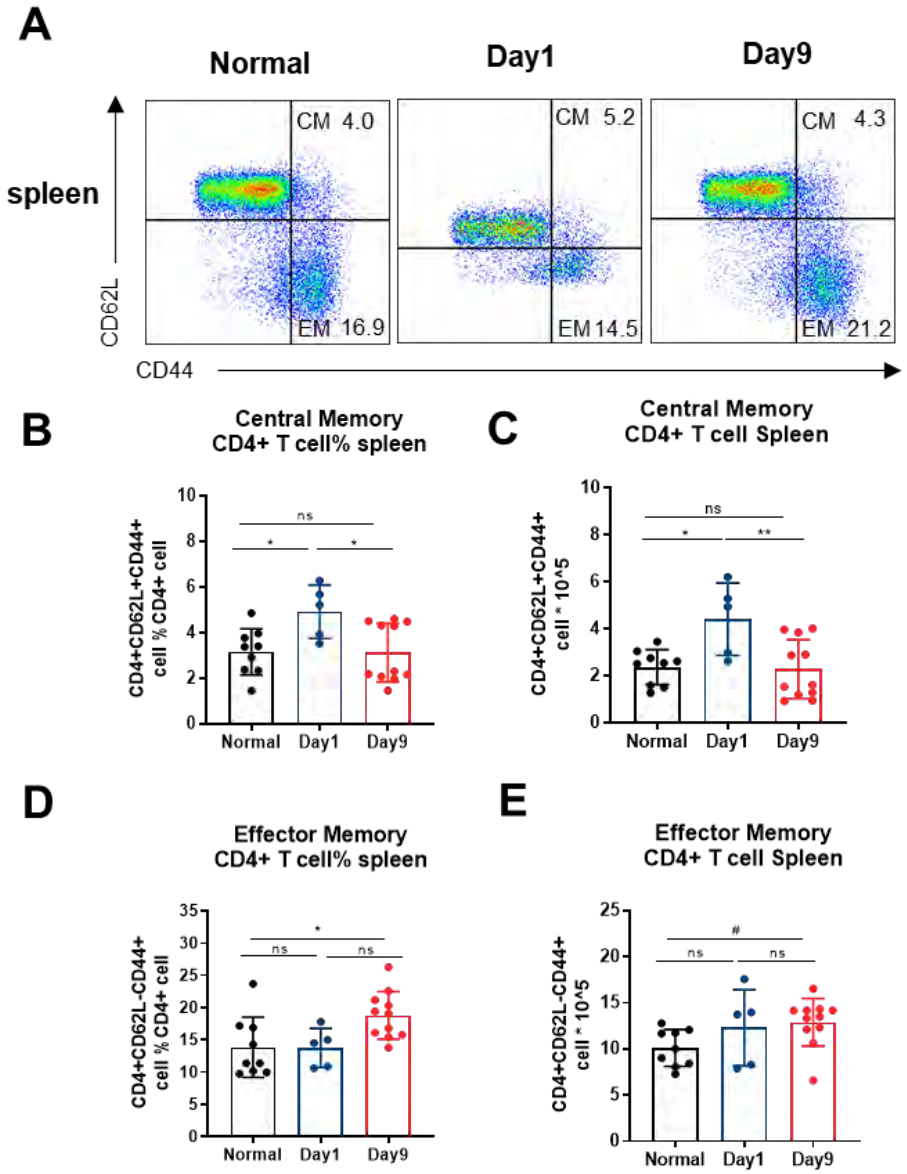
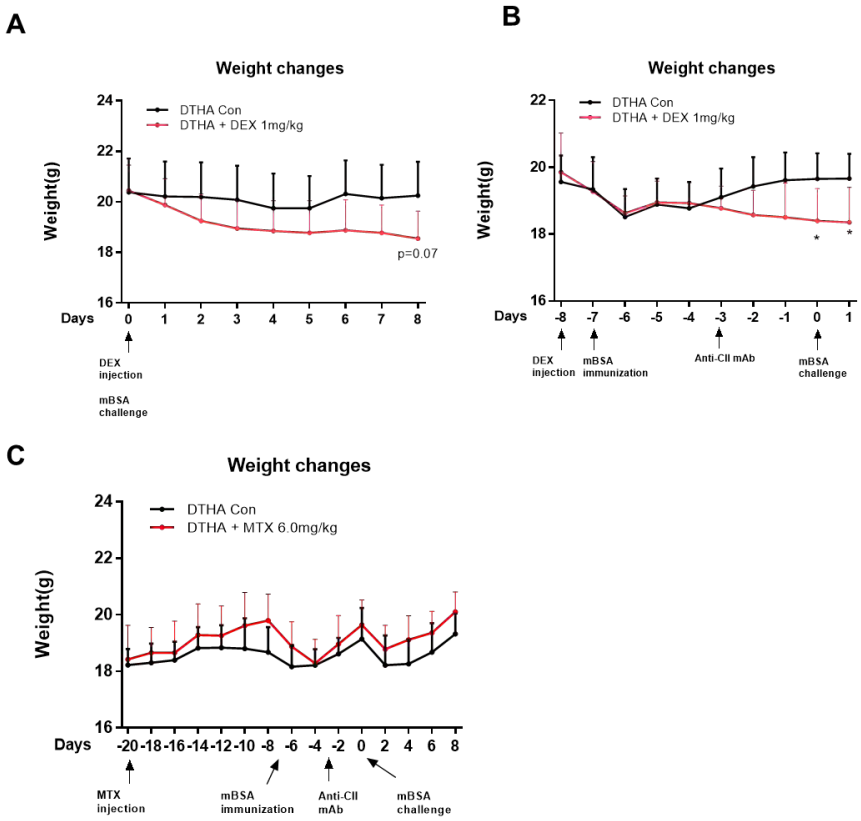
Supplementary Material



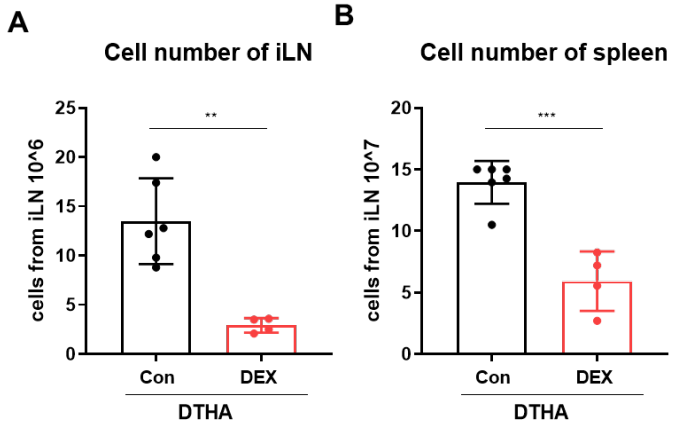
Supplementary figure 1. Th1 and th17 cell levels in spleen. Cell suspensions from the spleen of DTHA induced mice and normal on day 1 and 9 post the mBSA challenge were analysed by flow cytometry. (**A and B**). Fraction (%) number (**A**) and absolute number (**B**) of Th1 cells (CD4+CD194-CD196-CD183+) on day 1 and 9 post mBSA challenge. (**C and D**). Fraction (%) number (**C**) and absolute number (**D**) of Th17 cells (CD4+CD194+CD196+CD183-) on day 1 and 9 post mBSA challenge. Data is presented as mean ± SD from two independent experiments with 4-8 mice in each group per experiment with the exception of the data of day 1, which is from one experiment with 8 mice. Statistical significance was calculated by ANOVA with Tukey multiple comparison test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: p>0.05).



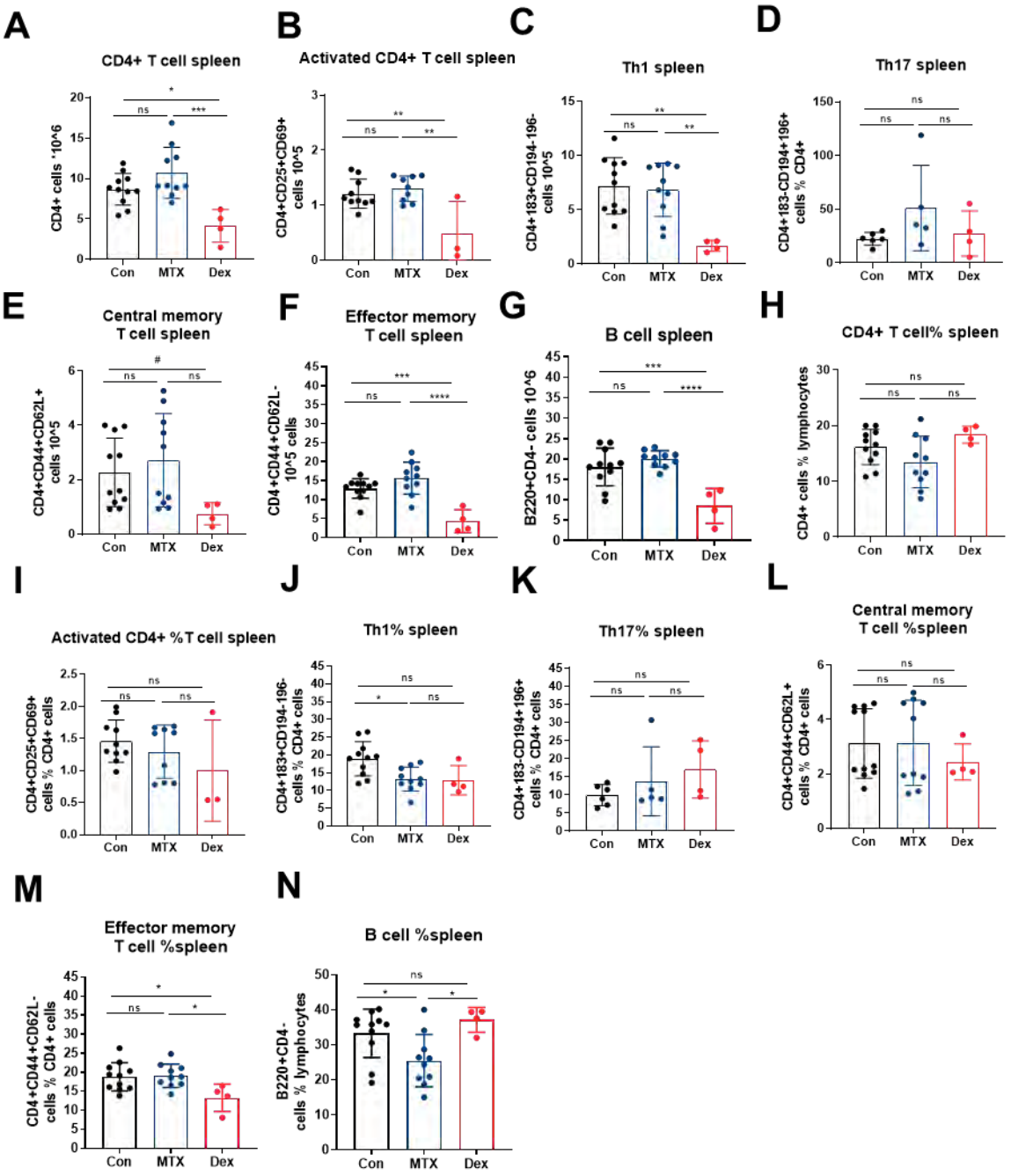
Supplementary figure 2: Splenic central/effector memory CD4 + T cells in DTHA mice. Cell suspension from the spleen of normal mice and DTHA induced mice on day 1 and 9 post mBSA challenge was analysed by flow cytometry. (**A**). Representative flow cytometry dot plot illustrating central/effector memory CD4+ T cells from spleen in normal mice and DTHA induced mice on day 1 and 9 post mBSA challenge. (**B and C**). Relative number (**B**) and absolute number (**C**) of central memory CD4+ T cells (CD4+CD62L+CD44+) from spleen in given groups. (**D and E**). Relative number (**D**) and absolute number (**E**) of effector memory CD4+ T cells (CD4+CD62L-CD44+) from spleen are shown. Data is presented as mean ± SD. Data of normal mice and DTHA induced mice on day 9 are from two independent experiments with at least 5 mice per group per experiment. Data of day 1 group is from one experiment with 5 mice. Statistical significance was calculated by ANOVA with Tukey multiple comparison test (*p<0.05, **p<0.01, ns: p>0.05) or Student's *t*-test (#: p<0.05). CM: central memory CD4+ T cell. EM: effector memory CD4+ T cell.



Supplementary figure 3: Weight changes of mice after treatment of 6mg/kg MTX or 1mg/kg DEX. (A and B). The weight changes of mice with 1mg/kg DEX administrated every day from the day 0 (**A**) or day -8 (**B**) respectively. (**C**) The weight changes of mice treated with 6mg/kg MTX administrated every other day from the day -20. All data are presented as mean ± SD with 4-8 mice in each group. Statistical significance was calculated by Student's *t*-test was for the comparison for DTHA control mice and DTHA mice treated with MTX or DEX. P values are listed under the curve if p value < 0.1. (*p<0.05, ns: p>0.05).

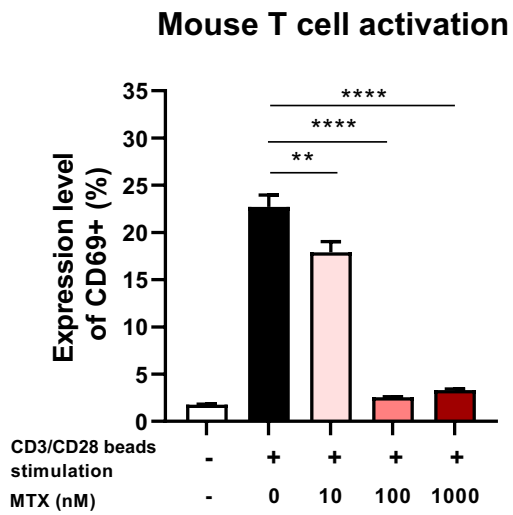


Supplementary figure 4: Decreased cellularity and cell number of iLN and spleen from mice treated with 1mgDEX/kg. (A and B). Single cells were obtained and counted by cell counter on day 9 from iLN (**A**) and spleen (**B**) of DTHA mice administrated with or without 1mgDEX/kg every day from day 0. All data are presented as mean ± SD with 4-6 mice in each group. Statistical significance was calculated by Student's *t*-test was for the comparison for DTHA control mice and DTHA mice treated with DEX. (**p<0.01, ***p<0.001).



Supplementary figure 5: Differential effects of MTX and DEX on levels of DTHA-induced splenic immune cells. Cell suspensions from the spleen of DTHA mice without or with MTX or DEX treatment on day 9. Flow cytometry was used to detect CD4+ T cell, activated CD4+ T cell, Th1, Th17, central/effector memory T cell and B cell. The proportion and absolute number of splenic CD4+ T cell (**A and H**), activated CD4+ T cell (**B and I**), Th1 (**C and J**), Th17 (**D and K**), central memory CD4+ T cell (**E and L**), effector memory CD4+ T cell (**F and M**) and B cell (**G and N**) in spleen from mice in given groups are shown. All data are presented as mean ± SD. DEX group data is from one experiment with 4 mice included. All other data are pooled from two independent experiments with 3-6 mice in each group per experiment. Statistical significance was calculated by ANOVA with Tukey multiple

comparison test (*p<0.05, **p<0.01, ***p<0.001, ***p<0.0001, ns: p>0.05) or Student's *t*-test (#: p<0.05).



Supplementary Figure 6. MTX inhibits mouse CD4+ T cell activation *in vitro*. Mouse CD4+ T cells were treated with MTX (0-1000nM) overnight followed by stimulation with α-CD3/CD28 beads (1 bead/cell) for 72 hours. At 72 hours cell surface expression of CD69 was quantified by flow cytometry. Statistical significance was calculated by ANOVA with Tukey multiple comparison test (**p<0.01, ****p<0.0001).

Antibodies	Clone	Brand
Anti-CD4	RM4-5	BD Biosciences
Anti-CD25	PC61	BD Biosciences
Anti-CD69	H1.2F3	BD Biosciences
Anti-CD62L	MEL-14	BD Biosciences
Anti-CD44	IM7	Invitrogen
Anti-CD183	CXCR3-173	BD Biosciences
Anti-CD194	2G12	BioLegend
Anti-CD196	140706	BD Biosciences
Anti-B220	RA3-6B2	BD Biosciences

Supplementary table 1. Information of antibodies used for flow cytometry analysis.

Paper IV