

The Role of GGT5⁺ Fibroblasts in Leukocyte Compartmentalisation in the Gut and Synovium

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Abstract

The spatial organisation of leukocytes within tissues is essential for coordinated immune responses. While stromal cells such as fibroblasts are well known to structure leukocyte niches in secondary lymphoid organs (SLOs), their role in organising immune cells within inflamed non-lymphoid tissues remains unclear. Building on mechanisms described in germinal centres (GCs), where follicular dendritic cells (FDCs) restrict B cell movement through the GGT5–GGG–P2RY8 axis, this thesis tests the hypothesis that stromal programs, particularly the induction of GGT5 by fibroblasts, establish perivascular confinement zones that spatially position P2RY8⁺ leukocytes within inflamed synovial and intestinal tissues.

Analysis of publicly available single-cell RNA-sequencing datasets revealed that GGT5, an enzyme shaping local gradients of the inhibitory ligand GGG, is broadly expressed by stromal cells across multiple inflamed tissues. In most tissues, GGT5 expression is restricted to endothelial cells and pericytes, but in RA synovium it expands beyond vascular rings into neighbouring fibroblasts, whereas in gut it is broadly distributed, forming a perivascular gradient associated with vascular activation. TNF α and NOTCH3 ligands induce GGT5 in synovial fibroblasts, while endothelial-cell-derived factors stimulate GGT5 in both synovial and intestinal fibroblasts, suggesting that vascular–stromal communication provides an endothelium-linked cue during inflammation.

Functionally, GGG inhibited CXCL12- and CXCL13-directed migration of human tonsil T follicular helper (Tfh) and GC B cells, and Tfh from inflamed gut, consistent with P2RY8-dependent immobilisation. Pilot live-imaging provides an analysis framework for quantifying chemotactic index and speed under GGG. As inflammation unfolds, leukocytes infiltrate via blood vessels, triggering neighbouring fibroblasts to upregulate GGT5 expression. This facilitates the establishment of a perivascular confinement zone, wherein GGT5⁺ fibroblasts, through the GGT5–GGG–P2RY8 axis, regulate the motility and positioning of P2RY8⁺ leukocytes, contributing to leukocyte compartmentalisation.

Complementary murine colitis and arthritis models probe Ggt5 biology in vivo. However, mice lack P2RY8, limiting direct inference about GGG–P2RY8 signalling. Given that Ggt5 also participates in the leukotriene pathway, in vivo experiments have explored alternative roles for Ggt5 beyond P2RY8 signalling. Together, the data in this thesis supports a model in which endothelial activation and fibroblast GGT5 induction establish perivascular confinement zones that tune leukocyte positioning in IMIDs. These findings motivate the development of tools to map GGG in situ and suggest therapeutic strategies to modulate GGT5 activity to either stabilise protective niches or disperse pathogenic aggregates in RA and IBD.

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Chapter 1: Introduction

1.1 Inflammatory diseases

Immune-mediated inflammatory diseases (IMIDs) arise from dysregulated immune responses that lead to impaired resolution of inflammation [1]. They represent a group of diseases, including rheumatoid arthritis (RA), inflammatory bowel diseases (IBD), psoriasis and systemic lupus erythematosus (SLE), which are characterised by systemic inflammation and multiorgan involvement [2]. The global incidence of IMIDs is increasing [3], contributing to an increasing socio-economic burden [4]. The pathophysiology of IMIDs is not clearly defined, but genetic and environmental factors have been implicated [5-8].

Inflammation is an evolutionarily conserved protective response of vascularised tissues to harmful stimuli. Its purpose is to eliminate harmful agents and to initiate tissue repair, thereby maintaining tissue integrity and homeostasis. Acute inflammation is usually short lived and self-limiting, resolving once the threat is neutralised. It involves timely immune cell recruitment, release of soluble mediators, and restoration of baseline function. Chronic inflammation is a qualitatively different state, where the response becomes persistent and dysregulated, leading to continued immune infiltration, stromal activation, and tissue damage [9]. It is important to note that inflammation is not the same as immune infiltration. Immune

infiltration refers specifically to the presence and accumulation of immune cells in tissue, whereas inflammation encompasses a broader process that includes vascular changes, stromal activation, and soluble mediators. In this thesis, the focus will be on the leukocyte infiltration aspect of inflammation, with stromal and soluble mediator changes considered primarily in relation to their impact on immune cell dynamics. In acute disease settings, immune infiltration is often protective, but in the chronic conditions studied in this thesis, IBD and RA, persistent infiltration and altered stromal responses act as drivers of pathology. Determining whether infiltration is protective or pathological would require access to early disease samples such as acute onset RA or IBD, which is outside the scope of this project.

Current therapeutics offer no cure, where restoring the dysregulated immune system to homeostasis is not possible. Nevertheless, the treatment options have progressed from broad-spectrum immune modulators such as methotrexate and hydroxychloroquine to agents with defined specificity such as monoclonal antibodies and highly targeted pathway inhibitors [10]. Thus, understanding the disease mechanism(s) before designing therapy is crucial in advancing IMIDs therapeutics so that pathogenic pathway(s) can be targeted while side effects can be minimised.

In this project, RA and IBD are used as a model for cross-disease comparison. While RA and IBD manifest in different anatomical locations, the immunological basis for comparing RA and IBD lies in

their shared foundation of immune dysregulation. Both diseases are characterised by aberrant immune responses, involving inflammatory processes that lead to tissue damage. Investigating common immunological pathways may reveal underlying mechanisms contributing to other IMIDs, offering potential insights into targeted therapeutic interventions. In addition, human synovium is a relatively immune-privileged site in health [11], whereas the gut is home to diverse community of microbiota which keeps the gut actively engaged with the immune system [12]. Studying both environments enables the understanding of similarities and differences in immune regulation and activation between tissues.

1.1.1 Leukocyte extravasation cascade

Leukocytes exit the blood circulation through a multistep extravasation process that enables them to migrate from blood vessels into inflamed tissue, in response to infection or injury [13], in an organised and coordinated manner [14]. During this time, leukocytes interact with various cells, such as endothelial cells and later fibroblasts [15]. When regulation of this process fails, immune infiltration can become excessive or prolonged, which is seen in IMIDs like inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) [16, 17].

The cascade proceeds through linked stages: tethering and rolling, chemokine-driven activation, firm adhesion, intravascular

crawling, and finally transmigration across the endothelium [14]. Each stage is dependent on the collaborative actions of the leukocytes, the endothelial cells, and the surrounding stroma and extracellular matrix. This process is driven by local cytokines and chemokines which act on both leukocytes and the endothelium [18].

In the initial tethering and rolling phase, selectins (E-selectin and P-selectin on endothelial cells and L-selectin on leukocytes) create brief, reversible contacts that slow leukocytes as they move along the vessel wall [19, 20]. This rolling motion enables leukocytes to sense and capture endothelial-bound signals. Activation occurs when chemokines engage G protein-coupled receptors (GPCRs) on leukocytes, triggering inside-out signalling that increases integrin affinity [21, 22].

Next, firm adhesion forms through strong binding between leukocyte integrins, like LFA-1 and VLA-4, and immunoglobulin superfamily molecules on endothelial cells, such as ICAM-1, ICAM-2, and VCAM-1 [23, 24]. These adhesive connections hold leukocytes in place under shear flow and are strengthened by inflammatory cytokines like TNF and IL-1 β , which increase endothelial adhesion molecules [25, 26].

Once anchored, leukocytes crawl along the vessel surface to locate suitable sites for transmigration across the endothelium [21, 26]. Transmigration can happen either between endothelial junctions (paracellularly) or directly through the cell body (transcellularly). Molecules like PECAM-1 (CD31), junctional adhesion molecules (JAMs),

and CD99 aid in these transitions, while the basement membrane and surrounding stromal cells provide additional guidance for leukocytes to enter tissue [27, 28].

The perivascular niche, which is made up of pericytes, fibroblasts, and extracellular matrix, plays a key role in this final stage. These cells release chemokines, present adhesion molecules, and remodel the local matrix to guide leukocytes through the vascular barrier [15, 29]. In cases of chronic inflammation, persistent activation of endothelial and stromal cells changes the structure of blood vessels and encourages leukocyte entry, leading to a buildup of leukocytes [30].

Overall, while the extravasation cascade supports normal immune surveillance, dysregulation of any part of this process contributes directly to chronic inflammatory disease.

1.1.2 Inflammatory bowel diseases (IBD)

IBDs are a heterogeneous grouping of chronic inflammatory conditions affecting the gastrointestinal tract, including Crohn's disease (CD) and ulcerative colitis (UC) [31]. IBDs are complex diseases driven by interaction between stromal and resident immune cells [31]. The major cell types that make up the stromal population are fibroblasts, pericytes, lymphatic endothelial cells and vascular endothelial cells [32]. Several studies have characterised the heterogeneity of fibroblasts in the gut [33-36]. In the intestinal crypt axis, fibroblasts are shaped by their positional identity, and they show functional

zonation characterised by distinct phenotype from crypt to the villus [37]. For instance, fibroblasts expressing WNT2B, WNT4 and DKK3 reside near the crypt whereas fibroblasts expressing WNT5A/5B and bone morphogenetic protein (BMP) signalling genes including BMP4 and BMP5 reside near the villus [36]. Crypt-bottom fibroblasts are Pdgfra^{lo} and are situated close to the intestinal stem cells niche. They secrete canonical Wnt ligands (Wnt2 and Wnt2b), Wnt potentiators (Rspo3), and Bmp inhibitors (Grem1) that help support the stem cell niche. On the other hand, crypt-top fibroblasts are Pdgfra^{hi} and they secrete non-canonical Wnt (Wnt5a) and Bmp ligands (Bmp2/3/4/5/7) that induce epithelial differentiation [38]. However, in ulcerative colitis, lamina propria fibroblasts expand and adopt the activated THY1⁺PDPN⁺FAP⁺ phenotype upon exposure to inflammatory stimuli, akin to sublining fibroblasts in RA [36].

Cytokine networks that drive pathogenesis of IBD are well studied [39], but the cellular origins of many cytokines remain unclear. In IBD, it is known that fibroblasts produce cytokines including IL-6 and IL-11 as well as chemokines that recruit monocytes (e.g. CCL2, CCL7), T cells (e.g. CXCL2, CCL19, CCL21, and CXCL12), and neutrophils (e.g. CXCL1, CXCL2 and CXCL8) into the tissue [34, 36, 40]. Intestinal fibroblasts also contribute to the dysregulation of colonic mucosal tolerance. For instance, it is shown that IBD-derived colonic fibroblasts have reduced capacity to induce active regulatory T cells (Tregs). Instead, they generate Tregs with low levels of FoxP3 that

produce less TGF β 1 and no IL-10 [41]. In addition, the expression of immune checkpoint PD-L1 is decreased in CD fibroblasts [42].

Blockade of TNF using monoclonal antibodies is an effective therapeutic approach for IBD, and yet up to 40% of patients with IBD do not respond to anti-TNF therapy, and many patients who initially respond eventually develop therapeutic resistance [43, 44]. Analysis of more than 200 patients with IBD shows that high pretreatment levels of JAK–STAT upstream activator oncostatin M (OSM) and its receptor (OSMR) correlate with histopathological disease severity and are strongly associated with non-response to anti-TNF therapy [45]. In inflamed colon, PDPN⁺ fibroblasts expand, and they express OSMR. These fibroblasts respond to OSM, which in turn produce proinflammatory cytokines including IL-6, leukocyte adhesion factor intercellular adhesion molecule 1 (ICAM-1) and chemokines such as CXCL1, CCL2 and CXCL10 that attract neutrophils, monocytes and T cells [45]. These data suggest that inflammation-associated fibroblasts may play a role in mediating patients' failure to respond to anti-TNF therapy.

To restore the damaged epithelium in IBD, fibroblasts need to migrate, deposit collagen and control rebuilding of epithelial layer [46]. However, colonic lamina propria fibroblasts derived from IBD patients have reduced migratory capacity compared with normal fibroblasts [47]. These fibroblasts not only proliferate faster, but also secrete more collagen compared to control fibroblasts [48]. The fibroblasts located

proximity to epithelial monolayer express sheet collagens (COL4A5, COL4A6), different WNT (WNT5A, WNT5B) and BMP ligands (BMP2 and BMP5), which are essential for epithelial barrier maintenance and tissue repair. Yet, these fibroblasts are diminished in the colon of IBD patients [40].

1.1.3 Rheumatoid arthritis (RA)

RA is an IMID which causes chronic joint inflammation and subsequently results in cartilage and bone erosion if left untreated [16, 49]. It is characterised by autoantibodies against Fc portion of immunoglobulin G (IgG) or also known as rheumatoid factor (RF), and citrullinated proteins, or also known as anti-citrullinated protein antibodies (ACPAs) [49]. However, not all patients with RA develop autoantibodies, and not all individuals with autoantibodies go on to develop RA. In addition, RF is not specific to RA and can be found in other autoimmune diseases and in some healthy individuals. Presence of RF is associated with disease activity [50], whereas presence of ACPAs do not necessarily result in more severe outcomes [51]. Despite treatment advances over the past decades, many patients do not achieve sustained remission [49, 52].

In RA, the site of inflammation is the synovium [53], where it is organised into lining and sublining structures surrounding the joints through cadherin-mediated adhesion [54]. The synovial membrane consists of two distinct mesenchymal structures: epithelial-like lining

layer that lacks tight junctions and underlying sublining layer where the vasculature is located [55, 56]. Lining fibroblasts appear more rounded and compacted [57], and they can be identified by the expression of proteoglycan 4 (PRG4), CD55 and chloride intracellular channel protein 5 (CLIC5) [55, 58]. On the other hand, sublining fibroblasts are loosely packed and have extended processes [57]. They are defined by the expression of THY1 and CD34 [55, 58].

In healthy joints, lining fibroblasts form a one to three cells thick barrier niche with resident macrophages that maintains joint lubrication by secreting proteoglycans such as lubricin and mucin hyaluronan, and aids the clearance of debris from the synovial space [59-61]. However, in RA, these lining fibroblasts produce matrix metalloproteinases (MMPs) that drives cartilage destruction and activate osteoclasts that erode bone [55, 62]. Deeper within the synovial tissue, the sublining layer comprises of fibroblasts, adipocytes, macrophages, blood vessels, lymphatics and nerves [63]. In disease state, sublining layer greatly expands and becomes infiltrated with leukocytes [55, 58, 64]. A particular THY1⁺PDPN⁺FAP⁺ fibroblast population emerges surrounding the blood vessels, which produces pro-inflammatory cytokines and chemokines which promote leukocyte accumulation, including IL-6, CXCL12 and CCL2 [55, 58].

It might appear that the lining and sublining fibroblasts are two distinct populations, expressing different markers and playing different roles. In fact, they exist along a spatial and transcriptional gradient

corresponding to their proximity to the vasculature. In health, the endothelium-derived NOTCH3 signalling drives differentiation of sublining fibroblasts but in RA, NOTCH3 and target genes are significantly upregulated in synovial fibroblasts [56].

Inflammation can recur in the presence of exogenous triggers or spontaneously [65]. Tissues have the capacity to retain memory of previous encounters with infectious agents, injuries and other inflammatory stimuli, and they react more strongly and/or more persistently upon subsequent exposure to the similar insults [66]. RA often involves recurring flare-ups and progressive worsening at specific predilection locations. It has been shown that repeated exposure to inflammatory stimuli leads to enhanced glycolysis, respiration and other metabolic activities in synovial fibroblasts, which subsequently increases their migration, invasiveness and osteoclastogenesis. The metabolic shift also promotes NLRP3 inflammasome activity and prevents activation-induced senescence. In other words, synovial fibroblasts drive the sensitisation of synovial tissues, making them more prone to re-inflammation, a concept that has been termed “stromal memory” [67].

1.2 Lymphoid structures

Lymphoid structures play a crucial role in adaptive immune responses, by facilitating antigen presentation, lymphocyte activation, and interactions among immune cells. These structures are broadly categorised into secondary lymphoid organs (SLOs) and tertiary lymphoid structures (TLSs). SLOs such as lymph nodes, spleen, and mucosa-associated lymphoid tissue (MALT), develop during embryogenesis and persist throughout life, providing a pre-organised framework for immune activation and maintaining immune homeostasis.

In contrast, TLSs are inducible lymphoid structures that form in non-lymphoid tissues in response to sustained immune activation. These ectopic aggregates often exhibit features of SLOs, including B cell follicles, T cell zones, and high endothelial venules (HEVs), yet lack the structural organisation and homeostatic maintenance of canonical lymphoid organs. TLS formation is commonly observed in chronic inflammatory diseases such as Crohn's disease and RA, where it may sustain local immune responses independently of SLOs [68, 69].

A specialised subset of SLOs, mucosa-associated lymphoid tissue (MALT), is strategically positioned at mucosal barriers to regulate immune responses to luminal antigens [70]. MALT encompasses gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), and nasopharynx-associated lymphoid tissue (NALT), each tailored to the immune surveillance needs of its respective

mucosal environment [71]. Within the gastrointestinal tract, GALT consists of Peyer's patches, isolated lymphoid follicles (ILF) and mesenteric lymph nodes (mLNs), which are integral to maintaining immune homeostasis and regulating interactions between the microbiota and the host immune system [72].

Although MALT and TLSs are distinct in their developmental origins, both can be present in mucosal tissues such as the gut. Peyer's patches and ILFs represent MALT, whereas chronic inflammation in conditions such as IBD can drive the formation of ectopic lymphoid aggregates with TLS-like characteristics. These inflammation-induced structures may contribute to local immune regulation but could also exacerbate pathology by sustaining autoreactive lymphocyte activation [73].

1.3 Tertiary lymphoid structures

Tertiary lymphoid structures (TLS), or sometimes also known as ectopic lymphoid structures are organised aggregates of immune cells that develop postnatally in non-lymphoid tissues, and they resemble SLOs in many ways.

SLOs including lymph nodes, spleen, tonsils, Peyer's patches and MALT are strategically situated throughout the body, allowing antigen sampling from different tissues and thereby facilitating the induction of adaptive immune responses. Formation of SLOs during embryogenesis may be useful to help understand the development of TLSs. The seeding and organisation of SLOs are the results of a series

of highly ordered events, involving haematopoietic and stromal cells with critical roles for molecular components including cytokines, chemokines, adhesion molecules, survival and maturation factors [72, 74].

Early during embryogenesis, lymphoid tissue inducer (LTi) cells colonise the lymph node anlagen and these are CD4⁺CD3⁻CD45⁺ innate lymphoid cells that differentiate from foetal liver precursors [75]. LTi cells cluster together and drives the initial steps of SLO formation, where they secrete lymphotoxin $\alpha 1\beta 2$ (LT $\alpha 1\beta 2$ or TNFSF14) and TNF that bind to their respective receptors, LT β R and TNFR1, expressed on stromal cells [76, 77], or more commonly known as mesenchymal lymphoid tissue organiser (LTo) cells. As LTo cells get activated, they enhance the expression of adhesion molecules, vascular cell-adhesion molecule 1 (VCAM1), intercellular adhesion molecule 1 (ICAM1), mucosal addressin cell-adhesion molecule 1 (MAdCAM1), and PNA_d, as well as production of homeostatic chemokines such as C-C motif chemokine ligand 19 (CCL19), CCL21 and CXCL13 [74, 78], resulting in the recruitment of immune cells to the niche [78-80]. The compartmentalisation of lymphocytes is then achieved by the segregated expression of homeostatic chemokines, where CCL19⁺ and/or CCL21⁺ FRCs and CXCL13⁺ FDCs guide the distribution of CCR7⁺ and CXCR5⁺ lymphocytes, forming T cell and B cell zones [81-83] (also discussed in 1.5).

TLSS are not usually present under physiological conditions (except in the gut), but they develop in chronically inflamed environments including autoimmune diseases [84], allograft rejection [85], chronic inflammation [86], and cancer [87, 88]. Despite the anatomical resemblance to SLOs, TLSS in most tissues lack surrounding capsule [89], which allows immune cells to have direct access to local antigens, danger-associated molecular patterns (DAMPs), cytokines and various macromolecules from the inflamed microenvironment. SLO formation follows a well-regulated developmental programme during embryogenesis and early postnatal periods, whereas TLS in adult vascularised tissues are triggered by inflammatory reactions associated with various pathological conditions. Nevertheless, formation of SLOs and TLSS involve the same cytokines of the lymphotoxin and TNF family.

With regards to the upstream initiation of TLSS, it remains uncertain if bona fide LTi cells are essential for priming of the local mesenchymal cells, or whether locally accumulated immune cells can fulfil this role. Supporting the latter hypothesis, various immune cell populations including Th17 cells [90, 91] and innate lymphoid cell type 3 (ILC3) [92], both of which express ROR γ t transcription factor that is also found in classical LTi cells, along with effector CD8⁺ T cells, natural killer cells [93, 94], B cells [95], and M1-polarised macrophages [96] have all been identified as potential surrogate for LTi cells in both murine and human contexts. Unlike the formation of SLOs, the

induction of TLSs may not always require lymphotoxin. For instance, IL-17 produced by CD4⁺ T cells can stimulate murine stromal cells to express CXCL13 and CCL19 in an lymphotoxin-independent way, which are important for the development of induced bronchus-associated lymphoid tissue (iBALT) [97]. In addition, lymphoid aggregates do develop in LTa knockout mice, although these structures lack distinct T and B cell zones and HEVs [98], and thus may not qualify as true TLSs.

Just as surrogate LT_i cells are thought to contribute to TLS formation, it is possible that local stromal and immune cells can act as surrogate LT_o cells. For example, lymphotoxin and TNF signalling can stimulate synovial fibroblasts from RA patients to produce chemokines such as C-X-C motif chemokine 12 (CXCL12), CXCL13, CCL19, and CCL21, and lymphocyte survival factors including BAFF, IL-7, and APRIL [99, 100]. Similarly, adipocytes and vascular smooth muscle cells have also been shown to orchestrate TLS formation by producing chemokines in the creeping fat of Crohn's disease [101] and in atherothrombotic arteries [102], respectively .

Despite unclear mechanisms, several studies show that TLSs mostly occur at perivascular sites [103-106]. In fact, HEV is an important component in TLS. HEVs are present in both TLSs and SLOs, which form a major gateway of entry for circulating immune cells [107]. HEVs can also regulate lymphocyte entry and the type of lymphocytes being recruited through expression of vascular addressins [108]. In non-lymphoid organs, HEVs may precede TLS formation. As they

develop in inflamed tissues, HEVs can promote T cell aggregates formation [109, 110]. There are also studies which show that perivascular fibroblasts have a pivotal role in TLS formation [111-113].

There are some parallels between SLO and TLS including upstream initiation involving lymphotoxin and TNF family as well as downstream chemokines such as CXCL12, CXCL13, CCL19 and CCL21. TLS formation and leukocyte compartmentalisation are dynamic responses to chronic inflammation, which can have both beneficial and detrimental effects. While TLS can enhance immune responses and improve outcomes in cancers, they may also contribute to pathology in certain contexts. Understanding the mechanisms driving leukocyte compartmentalisation is crucial for developing therapeutic strategies aimed at modulating autoimmune responses and harnessing the potential of TLS for targeted immunotherapy.

1.4 Leukocyte compartmentalisation

Leukocytes are professional migratory cells that can navigate through most types of tissues and sense a wide range of directional cues. They are mostly guided by attractive or repulsive environmental cues [114]. Fibroblasts are relatively sessile as compared to other immune cells, which places them in a strategic position to control the positioning and localisation of leukocytes within tissues.

In IMIDs, leukocytes infiltrate the tissues and tend to reside in specific compartments [115]. The infiltration pattern can be classified into three groups. Firstly, the leukocytes are diffused around the tissues and lack organisation. Secondly, the leukocytes form aggregates but lack GC and they usually surround the blood vessels, forming perivascular cuffs or disorganised lymphoid aggregates (LA). For instance, during a neurotropic JHM strain of mouse hepatitis virus (JHMV) infection, the majority of CD4⁺ T cells accumulate in the perivascular space of brains and spinal cords [116]. In experimental allergic encephalomyelitis (EAE), which is an animal model of multiple sclerosis, perivascular cuffing of leukocytes are also observed in the brain [117]. Lastly, the leukocytes are organised into TLS, which contains GCs and resemble those found in the SLOs [118, 119]. TLS has been associated with pathology and therapy responses [68, 69, 120]. Although the mechanism(s) of TLS formation is unclear, they mostly occur at perivascular sites [103-106] and perivascular fibroblasts have a pivotal role in TLS formation [111-113].

1.4.1 G protein-coupled receptors

GPCRs are essential regulators of leukocyte migration, and most leukocytes express more than one GPCR on the surface in order to sense a wide range of chemokines [121]. In fact, GPCRs have been implicated in many diseases, and there are 22 agents in clinical trials targeting chemokine receptors for the treatment of cancer, asthma, RA, chronic obstructive pulmonary disease (COPD) and HIV [122]. The classic example is fingolimod (aka FTY720), which is a first-in-class drug that is approved for treatment of relapsing-remitting multiple sclerosis. Fingolimod is an agonist for sphingosine-1-phosphate receptors (S1PRs), which are mainly expressed on the surface of lymphocytes. After oral administration, fingolimod is phosphorylated by sphingosine kinases to form fingolimod-phosphate, which closely resembles the naturally occurring S1P. Phosphorylated fingolimod then binds to S1PRs on lymphocytes, resulting in internalisation of the receptors. In this way, lymphocytes are prevented from exiting the lymphoid tissues, thereby reducing autoreactive lymphocyte infiltration into the central nervous system and in doing so decreasing their potential to damage the myelin sheath surrounding the axons [123, 124].

In general, different GPCRs have different functions. Many studies on germinal centre dynamics have broadened our knowledge on GPCRs [125-129]. In particular, GPCRs couple to Gai-containing heterotrimeric G-proteins are pro-migratory, i.e. all chemokine

receptors. They transmit signals via Rac which results in cell polarisation and directed migration towards the ligand gradient. On the contrary, GPCRs couple to G₁₃-containing heterotrimeric G-proteins are migration inhibitory, the examples include S1PR2 and P2RY8. The ligands bind to these GPCRs, which activates Rho and subsequently antagonise Rac-mediated migration [130].

1.5 Germinal centre

Germinal centres (GCs) are specialised microenvironments within secondary lymphoid organs that play a crucial role in the adaptive immune response. These structures form in B cell follicles upon exposure to T-dependent antigens and are essential for the production of high-affinity antibodies, memory B cells, and long-lived plasma cells.

1.5.1 T follicular helper cells

T follicular helper (Tfh) cells are essential in a GC reaction as they provide specialised help to B cells. Tfh cell differentiation is a multi-stage process which involves multiple signals, and the process can be heterogeneous. Nonetheless, canonical Tfh cell differentiation process begins with the interaction between a naïve CD4⁺ T cell with a myeloid professional antigen-presenting cells (APCs) such as dendritic cell (DC) outside the follicle [131]. Tfh vs non-Tfh cell differentiation bifurcates early, based on the balance between inductive (BCL-6) and inhibitory signals (B lymphocyte-induced maturation protein-1, BLIMP-1). T cells

commit to the Tfh phenotype by upregulating B cell lymphoma 6 (BCL-6), a central Tfh transcription factor, whereas BLIMP-1 represses BCL6 which turns the T cell differentiation into effector T helper cell programme [132].

T-cell receptor (TCR) signal strength and thus TCR-peptide:MHCII dwell time can bias Tfh cell differentiation, whereby higher-affinity TCR induces BCL6 expression, increases IL-2 and IL-21 production as well as lowering BLIMP-1 expression [133-135]. Strongest TCR signal induces IL-2 production, which suppresses BCL6 and subsequently Tfh development [136], through Stat5 activation and Blimp1 induction [137, 138]. Therefore, strong TCR signal results in IL-2 producing CD4⁺ T cells and predispose them to Tfh commitment, whereas those that do not produce IL-2 but receive IL-2, reinforce their non-Tfh effector commitment [133]. Nonetheless, the relationship between TCR strength and Tfh differentiation can be non-linear [135], depending on the alternative differentiation pathway available such as Th1 or Th2, the cytokine environment and the strength of available costimulatory molecules [139]. In addition to TCR-pMHCII engagement, inducible costimulator (ICOS) provides a critical early signal to induce the transcription factor BCL6, which then induces the expression of CXCR5, a chemokine receptor crucial for Tfh migration into the follicle. Although early Tfh cell differentiation does not require B cells, SLAM-associated protein- and CD40L-mediated signals, B cells are essential for maintaining BCL-6 expression and Tfh cell commitment via

sequential ICOS signals [140]. IL-6 is another factor that helps initiate Tfh cell differentiation by upregulating BCL6 [132].

1.5.2 T cell dynamics

T cell migration dynamics are intricately regulated by a complex interplay between chemokine receptors, their ligands, and the structural organisation of lymphoid tissues. Naïve T cells constantly home to the lymph node via high endothelial venule (HEV) to sample antigens presented by DCs, and this process is mediated by CCR7-CCL19/CCL21 axis [141, 142]. In the early stages of an immune response (day 0-1), activated CD4⁺ T cells rapidly upregulate EBI2 (also known as GPR183), which guides them to the follicle-T zone interface, where they interact with conventional DC2 (cDC2s) and activated B cells to receive signals that promote Tfh cell differentiation [143].

While CXCR5 can be expressed early during CD4⁺ T cell activation, it is not essential for its positioning at the follicle-T zone interface [144]. As the immune response progresses (days 2-3), these T cells continue to receive signals from cDC2s, further driving their Tfh differentiation. By days 4-7, fully differentiated Tfh cells downregulate EBI2 and utilise CXCR5 to migrate into the follicle, where they support the GC response [145]. This dynamic migration pattern ensures that T cells are in the right place at the right time to facilitate GC reactions. GC commitment seems to occur in T cells before B cells [146].

1.5.3 B cell dynamics

Naïve B cells also enter the lymph node via high endothelial venule (HEV), and they rely on CXCR4 and CCR7 in a partially redundant manner [147-150]. These naïve B cells also express CXCR5. After entering the lymphoid tissues, they migrate rapidly into the lymphoid follicles by responding to the CXCL13 gradient made by marginal reticular cells (MRCs) in the subcapsular region of the follicle and FDCs [151-154]. In the follicle, B cells encounter antigens through direct contact with soluble antigens from lymph, interaction with subcapsular sinus macrophages or bind to antigens displayed on FDCs [152].

Early during an immune response (day 0-1) upon antigen encounter, B cells upregulate EBI2 and CCR7 to position themselves along the follicle-T zone interface early during an immune response (day 0-1) to interact with T cells [155-157]. B cells start to express EBI2 at a similar stage of maturation as CXCR5 [129]. Its oxysterol ligand 7 α ,25-dihydroxycholesterol (7 α ,25-HC) is produced from cholesterol by the two enzymes: Ch25h and Cyp7b1 [158], that are mainly expressed by MRCs and CCL19^{lo} TRCs [159], but Cyp7b1 expression in the region is less tightly demarcated [160]. As Ch25h expression is more enriched in the outer follicle and low in the centre, the level of 7 α ,25-HC is higher at the follicle perimeter and low in the follicle centre [158]. During days 2-3, B cells downregulate CCR7 but maintain high EBI2 expression to promote their positioning at outer

follicle and follicle-T zone interface [156], continuing their interaction with T cells. During days 4-7, activated B cells downregulate EBI2 and maintain a low CCR7 expression, but continue to express and utilise CXCR5 to migrate to the centre of the follicle to form GCs, where it overlaps with pre-existing FDC network. In a way, the CXCR5-CXCL13 axis organises follicle, but EBI2-7 α ,25-HC axis further refines the follicular organisation.

1.5.4 Germinal centre organisation

Movement of activated B cells within the GCs require coordinated changes in GPCR functions. GCs are organised into two major zones: the dark zone (DZ) and the light zone (LZ) [161]. GC B cells cycle between the DZ and LZ multiple times, undergoing repeated rounds of proliferation, mutation, and selection. The DZ-LZ polarisation is accompanied by differential distribution of CXCL13, which signals through CXCR5, in the LZ and CXCL12, which signals via CXCR4, in the DZ [125, 162]. GC B cells are guided into the DZ via CXCL12, where they proliferate rapidly and undergo somatic hypermutation (SHM) of their immunoglobulin variable region (IgV) genes [162]. This process generates mutant clones with a broad range of affinities for the antigen. In the LZ, CXCL13 is more abundant and accumulates on the processes of FDCs, which recruit GC B cells into the region [125, 153], where they present acquired antigens to Tfh cells and compete for T cell help. High-affinity B cells receive stronger survival and proliferation signals

such as CD40L [163], while low-affinity or potentially autoreactive B cells undergo apoptosis. This selective pressure leads to the evolution of the antibody repertoire, with the average affinity of GC-derived antibodies increasing over time [164, 165]. Generally, GC B cells as a whole have relatively uniform CXCR5 expression but CXCR4 expression is tightly controlled and is higher on DZ cells [166]. In other words, CXCR4 is a key factor in controlling cell positioning in the DZ vs LZ.

In addition to Gai-mediated migration, GC B cells have higher Gα13 expression, which allows them to utilise this signalling pathway to support migration inhibition. For instance, early GC B cells upregulate S1PR2, which is a Gα13-coupled receptor. Their migration is inhibited in response to its ligand S1P, which helps confine cells to the GC [167]. Another Gα13-coupled receptor P2RY8 that also helps organise GC is discussed in section 1.8.

GC is also the site of immunoglobulin (Ig) class-switching. This process allows B cells to replace the Ig constant region, while maintaining antigen specificity, for the isotype that suits different effector functions [168].

The outcome of the GC reaction is the generation of memory B cells and plasma cells. Early forming memory B cells continue to express CXCR4 and CXCR5, but they downregulate S1PR2 and upregulate EBI2 to promote migration away from GCs [169, 170]. Pre-plasma cells also downregulate S1PR2 and upregulate EBI2 [163], but their early movement out of the follicle might not involve Gai-mediated

mechanism [171]. As B cells differentiate into plasma cells, they downregulate CXCR5 and CCR7, but instead upregulate CXCR4 [172], which promotes their positioning to CXCL12-rich region such as splenic red pulp, lymph node medullary cords and bone marrow [173].

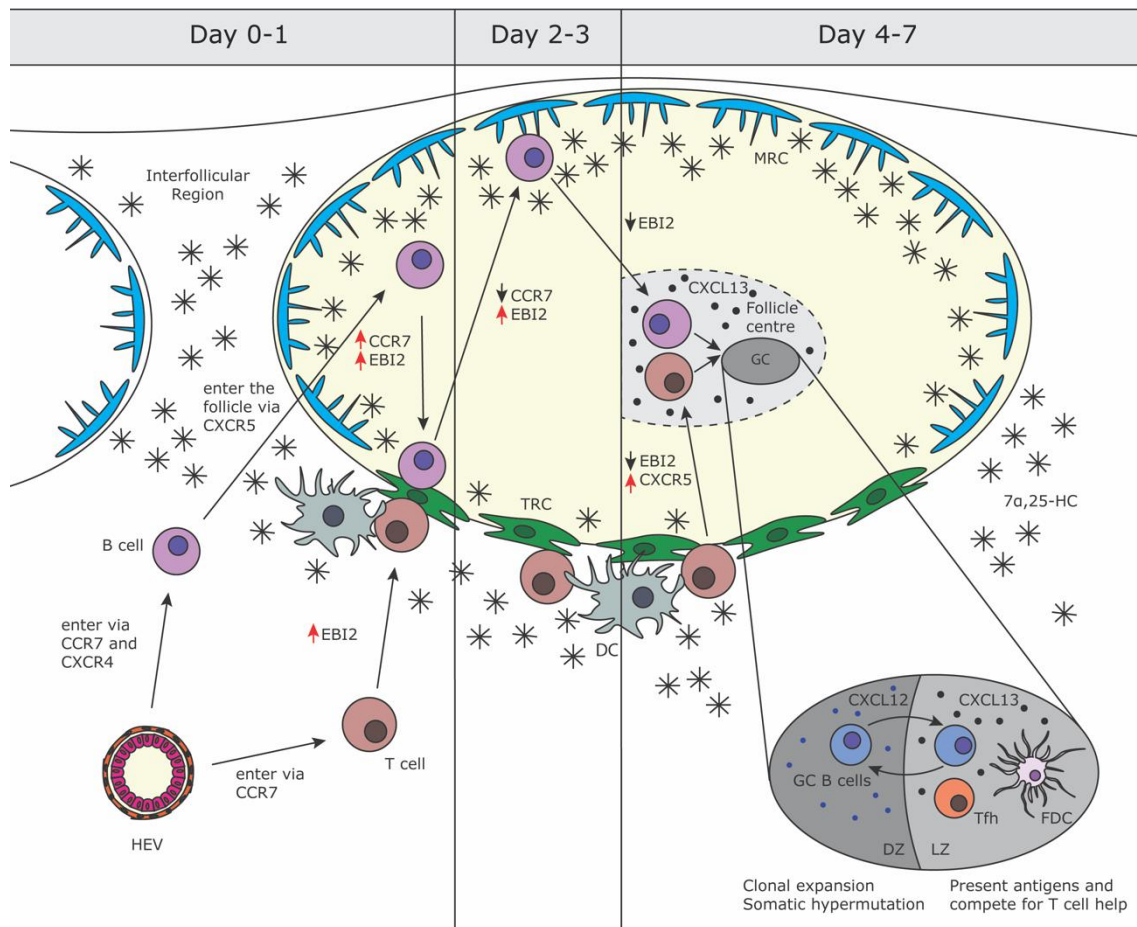


Figure 1.1: Dynamics of T cells and B cells in the formation of GC.

While the processes driving B and T cell differentiation in GCs are well understood, the tissue environment surrounding these cells is crucial for their function. Fibroblasts, which are the most common cells in connective tissues, contribute to the structural framework that supports immune responses in lymphoid organs. Recent advances have revealed that fibroblasts are not simply passive components of tissue architecture, but actively influence immune cell behaviour and tissue responses, particularly in the context of inflammation and disease [174].

1.6 Fibroblast in homeostasis

Fibroblast is derived from the embryonic mesenchyme [175]. It was first described as a distinct cell type by Rudolf Virchow and Mathias Duval in the 19th century [176, 177]. Since then, they were considered featureless cells with a spindle-shaped morphology that only filled up the space between tissues. This view has persisted for many years until recently. The technological advances including single-cell RNA sequencing (scRNA-seq), advanced imaging methods and spatial multi-omics have allowed researchers to study the importance of fibroblast in immunology context.

There is not a consensus in the field regarding the markers identifying a fibroblast. Generally, fibroblast is defined as negative for epithelial, endothelial, and leukocyte markers, and the common positive markers that are used to delineate fibroblast are collagen I α chain (COL1A), podoplanin (PDPN), platelet-derived growth factor receptor alpha (PDGFR α), vimentin, α -smooth muscle actin (α -SMA), and CD90 [178]. However, none of these markers are uniformly or uniquely expressed by the fibroblasts. Unlike CD45 as a universal marker for cells of haematopoietic origin, there is not an exclusive fibroblast marker, which might suggest the fact that fibroblasts are not as universally distributed across the body in the way that leukocytes are, and they can be heterogeneous within or across anatomical regions.

1.6.1 Location matters for fibroblasts

In general, PDGF, TGF β and WNT signalling are essential in regulating fibroblast functions including proliferation, ECM production, contraction and migration [175]. Nonetheless, location is a key factor in determining the functional state of a fibroblast. In fact, fibroblasts isolated from different anatomical locations display distinct and characteristic transcriptional patterns that are maintained across culture passages and Homeobox (HOX) genes, which are established during embryogenesis and maintained into adulthood, are central in directing topographic differentiation and underlie positional memory in fibroblasts [179, 180]. These studies might suggest that fibroblasts at different locations in the body should be categorised as distinct differentiated cell types, but it is debatable whether fibroblast functions exist as distinct subtypes or a spectrum. In fact, it has been shown that adipocytes acquire fibroblast-like phenotype in response to high fat diet, which are characterised by enhanced expression of ECM and suppression of adipocyte programmes [181].

As discussed above, fibroblast heterogeneity, both in health and diseases, is always associated with location in the body. Another example is vitiligo, which is characterised by bilateral symmetric skin depigmentation often in certain anatomic regions of the body such as on the face, neck, hands, and in skin creases [182-184]. In vitiligo-affected skin, dermal fibroblasts are required to recruit and activate CD8⁺ cytotoxic T cells, but anatomically distinct dermal fibroblasts

exhibit intrinsic differences in the level of secreted chemokines, depending on their response towards interferon gamma (IFN γ). Fibroblast subsets that are IFN γ -responsive secrete high levels of chemokines such as CXCL9 and CXCL10, thereby sustaining local recruitment and activation of cytotoxic CD8⁺ T cells. In contrast, fibroblasts that are relatively IFN γ -resistant produce lower amounts of these chemokines and fail to maintain T cell accumulation [185]. In this way, anatomically distinct fibroblast subsets with differential IFN γ responses determine the patterns of vitiligo lesions on the skin.

Although certain features of fibroblasts are imprinted, there is a degree of plasticity in their functional states. In pancreatic ductal adenocarcinoma, fibroblast can swing between iCAF and myCAF states. IL1 activates LIF and downstream JAK/STAT pathway to generate inflammatory phenotype, but TGF β antagonises this process by downregulating IL1R and promote myofibroblast differentiation [186]. In fibrosis, activation of the transcription factor PU.1 promotes pro-fibrotic phenotype in fibroblast whereas TNF-rich and inflammatory conditions interfere with the fibrotic effects of PU.1 [187].

1.6.2 Extracellular matrix homeostasis and mechanical forces

A major shared function of fibroblasts across tissues is producing extracellular matrix (ECM), which mainly comprises of collagen, proteoglycans and glycoproteins [188]. The ECM network serves as a platform providing structural support to neighbouring cells in the tissue

microenvironment [175], and it is also an important conduit system so that small molecules can rapidly diffuse for instance in the lymph node [168]. Fibroblast is also involved in active remodelling and modification of pre-existing ECM through covalent and non-covalent cross-linking, protein glycosylation and controlled proteolysis, which changes ECM composition [189]. Fibroblasts are not invariant across tissues. They can produce distinct ECM with optimised mechanical properties best suited for the respective tissue function, for instance lung respiration and gut motility require ECM with different macromolecule composition and biomechanical properties [190, 191]. Perivascular fibroblasts produce distinct ECM components including *col1a2* and *col5a1* that maintain vascular integrity and stabilisation, without which results in dysmorphic blood vessels with varied diameters [192]. Fibroblasts also exert contractile forces on ECM, resulting in tissue-level mechanical forces and matrix stiffness [193]. It has been shown that ECM biomechanical properties, including stiffness and elasticity, affect various cell behaviours such as stem cell differentiation [194], integrin-mediated cell adhesion [195] and T-cell activation [196].

1.6.3 Secretome

In addition to matrisomes and mechanics, fibroblasts secrete diverse range of cytokines and growth factors. For instance in the lymph node, T-zone reticular cells (TRCs) in the lymphoid organs not only generate migratory cues such as CCL19 and CCL21, but they also

secrete growth factors such as interleukin-7 (IL-7) to sustain T cell homeostasis [197-199]. Mesenchymal reticular cells, on the other hand, cooperate with lymphatic endothelial cells to provide a supportive niche for sinusoidal macrophages via RANK-RANKL axis [200]. In the intestines, small intestinal and colonic fibroblasts express canonical WNT ligands such as WNT2B and WNT signalling potentiators RSPO3 that regulate stem cell renewal and maintenance [201]. In the synovium, synovial lining layer fibroblasts and macrophages form a circuit that supports each other via secretion of growth factors to ensure effective clearance of debris from synovial space and tissue remodelling, but in diseases, they drive cartilage destruction and bone damage [58]. In a way, the specialisation of fibroblast function is intrinsically linked to their anatomical positioning within the body. Fibroblasts in each tissue microenvironment are adapted to support wide range of specialised cells such as tissue-resident stem cells, keratinocytes in skin, epithelial cells in lungs as well as endothelial cells of blood vessels [175]. In fact, fibroblasts have varied gene expression programmes depending on their spatial location [202]. These fibroblasts serve as a molecular landmark with a postcode system of positional identity, providing important niche information.

1.6.4 Progenitor function

Beyond matrisome and secretome, fibroblasts serve as progenitors for specialised mesenchymal cells. Pericytes have been

shown to derive from perivascular fibroblasts using reporter zebrafish strains and time-lapse imaging, where the perivascular fibroblasts gradually lose their *col1a2* expression and upregulate *pdgfrb* while extending pericyte-like cellular processes that wrap around the blood vessel as the embryo develops [192]. Dermal fibroblasts have been shown to be able to differentiate into adipogenic, chondrogenic and osteogenic cells [203]. In fact, fibroblasts can be directly reprogrammed into bone-forming osteoblasts by upregulating specific transcription factors including Runt-related transcription factor 2 (Runx2), and Osterix [204]. In the lymphoid follicles, it has been shown that the follicular dendritic cells (FDCs) are derived from perivascular stromal cells expressing platelet-derived growth factor receptor β (PDGFR β) [112]. Even though these precursor cells are ubiquitous and not limited to lymphoid organs only, further maturation into FDCs still require signalling by lymphotoxin and tumour necrosis factor (TNF) family members [205]. There is another study which suggests that FDCs arise from marginal reticular cells (MRCs) lining the subcapsular sinus, to support rapid B cell expansion and increase space during GC formation [206].

1.6.5 Microenvironmental sensing

Another key role of fibroblast involves microenvironmental sensing. Fibroblasts detect changes in environmental factors such as mechanical forces, oxygen and nutrient levels, which in turns affect the

levels of growth factors they produce for macrophages and other cell types [207]. In the kidney, there are specialised fibroblasts in the peritubular interstitium that sense the level of oxygen and produce erythropoietin as required to maintain a healthy systemic oxygen level. Dysfunction of these fibroblasts results in transdifferentiation into myofibroblasts which contributes to renal fibrosis and anaemia [208]. In the intestines, fibroblasts can sense diet change to modulate intestinal stem cell niche. These specialised fibroblasts surrounding the intestinal crypts increase in abundance upon administration of high fat diet and decrease while fasting. These Lepr⁺ fibroblasts are an important source of insulin-like growth factor 1 (IGF-1) that promotes stem cell proliferation. Without these cells, crypt-villus architecture is compromised, and intestinal regeneration is impaired [209]. In response to bacterial infection in the gut, colonic stromal cells produce CCL2 that recruits inflammatory monocytes which in turns activates the adaptive immunity by producing interleukin-12 (IL-12) [210]. Fibroblasts also help maintain correct cell numbers and ratio in each tissue compartment by sensing tissue density. They detect the change in mechanical force through actin-dependent mechanisms, which then direct macrophage proliferation by adjusting growth factor production. As tissue injury or damage occurs, there is an increased space availability due to cell loss, and this is detected by the fibroblasts, which subsequently increase macrophage numbers by increasing growth factor production [207, 211]. As macrophages clear up the

cellular debris [212], fibroblasts work together with neutrophils to facilitate wound closure by repairing and regenerating the ECM [213].

1.6.6 Participation during immune response

The advancement in technologies has allowed the field of immunology to appreciate the importance of fibroblasts in modulating immune responses and regulation. In the lymph nodes, fibroblasts can transform the environment from a quiescent setting into conditions that are highly conducive for generating adaptive immune responses. There are two main structural elements in lymph nodes: fibroblastic reticular cells (FRCs) that form collagen-based reticular network which supports immune cell interaction, and capsular fibroblasts that build a protective layer around the organ. Upon immunological challenge, lymph nodes can expand more than ten-fold in size within days, while maintaining the structural integrity [214]. Under resting conditions, PDPN activates the actomyosin machinery of FRCs, resulting in collagen contraction and tension within the reticulum. Upon inflammation, accumulating lymphocytes enter and inflate the lymph node, stretching the FRC network and increase tension on the ECM conduit [214]. During this time, activated DCs are also more likely to enter lymph nodes. These DCs express PDPN receptor CLEC-2 that binds to PDPN on FRCs, attenuating the actomyosin contractility. This results in FRCs relaxation and decreased tissue stiffness, which allows lymph node enlargement and FRC network expansion [215]. Such

relaxation only lasts a few days, as DC-mediated relaxation signals fade and FRCs increase their α -smooth muscle actin (α SMA) expression [216]. While intrinsic elasticity of FRCs allow them to accommodate short-term volumetric expansion, sustained strain on the cytoskeleton require network remodelling, where FRCs proliferate and undergo clonal expansion [214].

Interaction between FRCs and platelets via PDPN-CLEC-2 axis is also important in maintaining the high endothelial venules (HEVs) integrity as local release of sphingosine-1-phosphate (S1P) from platelets after PDPN-CLEC-2-driven activation help maintain HEV adherens junctions [217]. The FRCs network also serves as important conduit system that allows the transport of lymph containing antigen and inflammatory mediators [168].

SLOs are distinguished by their intricate cellular organisation, which strategically compartmentalises T cells, B cells, and myeloid cells to orchestrate robust and antigen-specific adaptive immune responses, and this organisation is mainly driven by fibroblasts. TRCs generate CCL19/CCL21 gradients that help locate T cells and DCs into the T cell zone [159, 218]. Under resting condition, the lymphocytes are segregated into well-defined areas, but during an immune response, activated T and B cells interact at the follicle-T-zone interface [219]. Following infection or immunisation, fibroblasts remodel the reticular network and produce migratory cues to facilitate interfollicular B cell–T cell interactions and promoting B cell activation. Within a germinal

centre (GC), B cells migrate between dark zone (DZ) and light zone (LZ) to undergo iterative rounds of immunoglobulin-gene somatic hypermutation and antibody affinity maturation. This cycling is driven by CXCL12 and CXCL13, produced by DZ FDCs and LZ FDCs respectively [125, 220, 221]. In fact, FDCs are instrumental in clustering the B cells within the GCs [222].

Fibroblasts can present antigen to activate an immune response, with the classic example being FDCs, which captures and retains antigens for an extended period of time [223, 224]. During GC reactions, FDCs organise themselves to regulate the antigen dynamics. During the initial flux of antigens, the entire LZ FDC network captures and displays the immune complexes. When different antigens enter during subsequent immunisations, only the peripheral LZ FDCs display new antigens and the central LZ FDC network serves as an antigen reservoir where old and new antigens colocalise, because central LZ FDCs has higher complement receptor CR2 (or CD21) membrane level than peripheral LZ FDCs which strongly improves the immune complexes retention half-life. As multiple waves of antigens enter the system, new antigens partially displace the old ones, maintaining efficient B cell responses [225].

Fibroblasts help shape the microenvironment into favourable conditions for immune responses. For instance, FRCs secrete interleukin-6 (IL-6) to promote CD8⁺ T cell metabolism and survival [226], and IL-15 to promote innate lymphoid cell (ILC) and natural

killer (NK) cell fitness and function [227]. Production of IL-6 and B cell activating factor (BAFF) by FDCs and FRCs respectively also help establish a favourable niche for B cell homeostasis and GC reaction [228, 229]. Once selected for, plasma cells exit the GC and usually home to bone marrow niches, but they can also be guided and retained into the lymph node medulla via CXCL12 produced by medullary FRCs [172]. These reticular cells provide a pro-survival niche for the plasma cells by secreting IL-6, BAFF and APRIL [230, 231]. During viral infection, FRCs respond to type I IFNs by enhancing antigen presentation, increasing CD8⁺ T cell recruitment via type I IFN-induced chemokines such as Cxcl9 and Cxcl10, and more importantly preventing exhaustive CD8⁺ T cells activation [232].

Fibroblasts play a critical role in immune regulation. In the thymus, in addition to self-antigens expressed by medullary epithelial cells, thymic medullary fibroblasts also produce a diverse array of unique self-antigens that shape the T cell repertoire and induce central tolerance [233]. In the lymph node, FRCs can induce antigen-specific CD8⁺ T cell tolerance under both steady-state and inflammatory conditions, by presenting peripheral tissue antigens on major histocompatibility complex-I (MHCI), and thus controlling and deleting self-reactive CD8⁺ T cells [234, 235]. Unlike in the thymus, this presentation of self-antigens in the lymph nodes are independent of autoimmune regulator gene (AIRE) which is not expressed by the fibroblasts, but rather relying on the transcriptional regulator DEAF1

[234, 236]. FRCs can also directly induce antigen-specific tolerance in CD4⁺ T cells by acquiring self-peptide-MHCII complexes from DCs and present them to naïve CD4⁺ T cells, preventing their proliferation and survival [237], or indirectly by presenting self-antigens on MHCII to support homeostatic regulatory T cell (Treg) proliferation [238]. In response to interferon- γ (IFN γ), TNF and direct cell contact with activated T cell, FRCs produce nitric oxide (NO) to control T cell expansion and DC priming capacity [239, 240]. FRCs directly suppress T cell proliferation and skew T cell differentiation away from central memory phenotype via indoleamine-2,3-dioxygenase, adenosine 2A receptor, cyclooxygenase-1 and 2 (COX1 and COX2) and transforming growth factor beta (TGF β) [241]. In the intestines where there are wide ranges of immunogenic stimuli, fibroblasts keep the immune system in a state of tolerance to prevent unnecessary inflammatory responses by expressing programmed death ligand-1 (PD-L1) which limits CD4⁺ T cell proliferation [242, 243].

As discussed above, fibroblasts are crucial for maintaining homeostasis. They ensure the body's balance by regulating the equilibrium between ECM deposition and modifying enzymes, renewing the stem cell niche versus promoting epithelial differentiation, and balancing immune activation with regulation. When this balance is disrupted, diseases can occur.

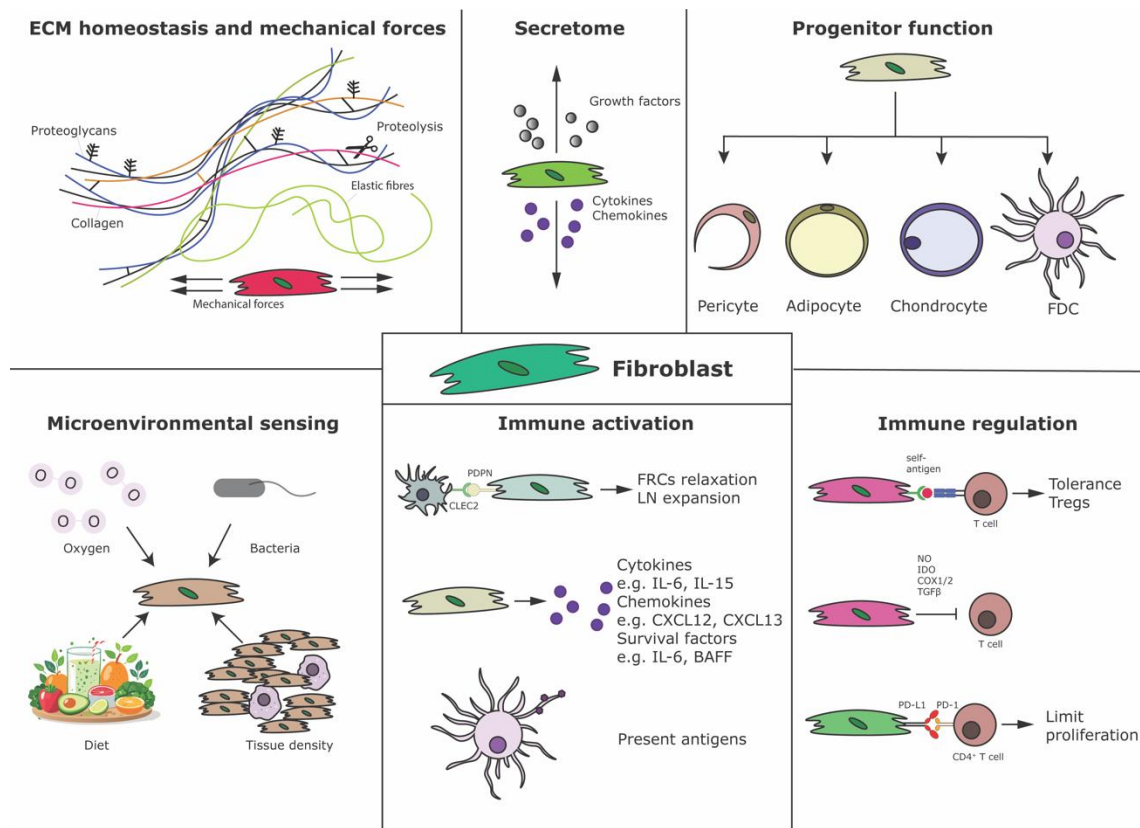


Figure 1.2: Role of fibroblast in biology.

1.7 Fibroblast pathology in inflammatory diseases

Within IMIDs, fibroblasts often acquire pathological phenotypes that contribute towards disease progression, persistence, and recurrence at specific anatomical sites. Although this is problematic, it also presents opportunities for fibroblast-targeting interventions.

Fibroblasts are key contributors and regulators of inflammation [62], through their production of inflammatory cytokines such as IL-6, IL-8, and chemokines such as CCL2, CCL3 and arachidonate metabolites such as prostaglandin E2 (PGE2) [244, 245]. In fact, fibroblasts are the dominant producers of IL-6, and it has been shown that the combination of TNF and IL-17 can synergistically stimulate the fibroblasts to produce IL-6 [246]. When activated, an autocrine feedback loop involving leukemia inhibitory factor receptor (LIFR) and signal transducer and activator of transcription 4 (STAT4) is formed, which drives sustained production of IL-6, IL-8, granulocyte-colony stimulating factor (G-CSF), IL-33, IL-11, IL-1 α , and IL-1 β in fibroblasts [247].

Chronic inflammation has a preference towards certain anatomical sites. For instance, RA occurs solely in the joints and not the gut, whereas IBD affect the gut but not the skin. Although these diseases are classified under the umbrella of IMIDs, recent applications of high-dimensional cellular and transcriptomic analysis have uncovered highly heterogeneous fibroblasts which may contribute to the site specificity of disease pathology [248].

1.7.1 Shared pathological fibroblast phenotypes in IMIDs

Recent research has revealed parallels between RA and IBD, despite their distinct clinical presentations. Evidence suggests shared fibroblast phenotypes in these and other inflammatory conditions. Specifically, CXCL10⁺CCL19⁺ immune-interacting fibroblasts and SPARC⁺COL3A1⁺ vascular-interacting fibroblasts are expanded in RA, IBD, interstitial lung disease, and Sjögren's syndrome [249]. As discussed in section 1.1.2 and 1.1.3, fibroblast can also adopt the THY1⁺PDPN⁺FAP⁺ inflammatory phenotype [36, 55]. The local tissue microenvironment plays a crucial role in determining fibroblast states, with some areas rich in vasculature and others in lymphocytes. This heterogeneity in fibroblast phenotypes across different inflammatory diseases points to common pathogenic mechanisms and suggests potential for developing targeted therapies that could be effective across multiple chronic inflammatory conditions.

Beyond their structural and immune roles, fibroblasts also influence cellular behaviour through signalling pathways. A notable example is the GGT5-GGG-P2RY8 axis, which regulate B cell migration in the GC.

1.8 GGT5-GGG-P2RY8 axis

The Gα13-coupled receptor P2RY8 is frequently mutated in diffuse large B-cell lymphoma (DLBCL), but its functional role was not known [250, 251]. It had been an orphan receptor until recently when S-geranylgeranyl-L-glutathione (GGG) was identified as its potent ligand. It is shown that the GGT5-GGG-P2RY8 axis is involved in organising and controlling GC responses [252].

The inhibitory ligand GGG can bind effectively to P2RY8, down to nanomolar range. When GGG binds to P2RY8, Gα13-mediated activation of Rho is induced, which then leads to inhibition of Rac-mediated migration [130]. The biosynthesis of GGG is not fully understood. It appears that many cell types can produce GGG and it is particularly abundant in the bile. It is possible that GGG is made in most cell types during geranylgeranyl metabolism, but it is only secreted by certain cell types through the transporter ATP-binding cassette subfamily C member 1 (ABCC1) [253]. As GGG is a metabolite, the only method available currently to detect it is by using mass spectrometry, which makes understanding the spatial distribution of GGG in tissues challenging. GGG is metabolised by the enzyme gamma-glutamyltransferase-5 (GGT5) into inactive form, where the γ-glutamyl moiety is cleaved off GGG to form S-geranylgeranyl-L-Cys-Gly.

Within a lymphoid follicle, P2RY8⁺ B cells undergo either random or chemokine-directed migration within the GCs, but when they try to

go beyond, GGG engages P2RY8 and inhibits migration. Within the GCs, the follicular dendritic cell (FDC) network highly expresses GGT5 that inactivates GGG, creating a sharp GGG gradient, with higher concentration in the outer follicle and lower level within the GC. In this way, the B cells are effectively confined within the GCs (Figure 1.3). It has previously been shown that in addition to its sensory role, CCR7 also acts as a generator and modulator of CCL19 gradient to coordinate leukocyte migration [254], but it remains to be studied if P2RY8 can also work as a sink for GGG.

As discussed above, both S1P and GGG, that bind to S1PR2 and P2RY8 respectively, work together to constrain migration of GC B cells and thereby confining them within the GC. S1P gradient does not depend on FDCs whereas GGG gradient depends on FDCs, which may provide a different shape to the gradients. Also, the existence of two inhibitory gradients may allow a sharper boundary and finer level of GC organisation. Nonetheless, biochemical pathways are not solely responsible for cell movement. The stiffness and density of extracellular matrix can affect cell shape, which in turns affect migration [255].

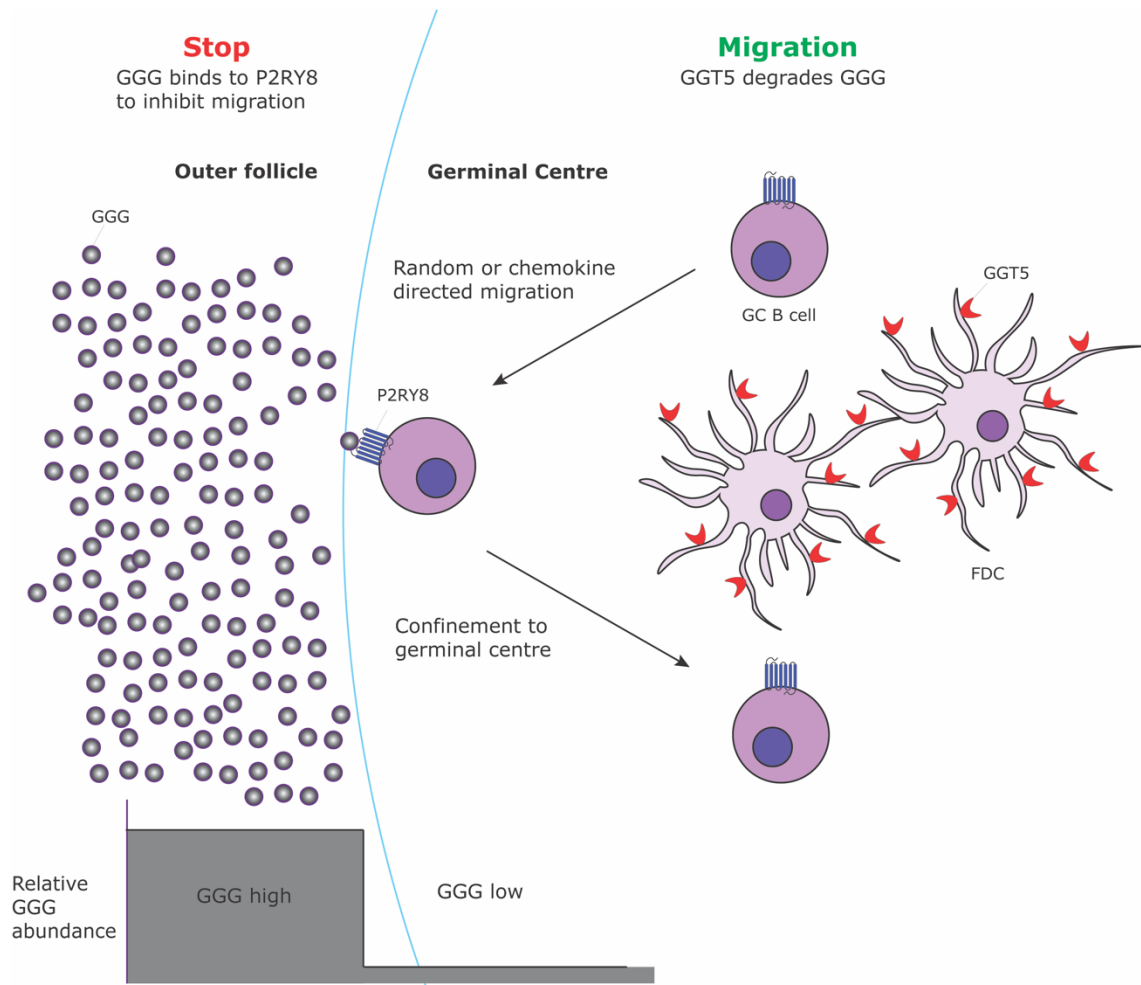


Figure 1.3: GGG is an inhibitory signal that binds to P2RY8 and stops migration. P2RY8⁺ GC B cells migrate either randomly or in a chemokine-directed manner within the GCs, but when they encounter GGG on the outer follicle, migration is inhibited. Within the GCs, GGT5 expressed by the FDCs inactivates GGG, creating a sharp GGG gradient.

1.8.1 Leukotriene pathway

In addition to the GGG-P2RY8 axis, GGT5 is also involved in the leukotrienes pathway [256]. Leukotriene C₄ (LTC₄) is transported out of cells through the same transporter as GGG, which is ABCC1 [257], after which it is converted into leukotriene D₄ (LTD₄) by GGT5 [258, 259]. Both LTC₄ and LTD₄ have well-established receptors: cysteinyl leukotriene receptor 1 (CysLTR1) and CysLTR2, which they bind with high affinity [260]. These cysteinyl leukotrienes have mainly been studied in the context of eosinophils and allergic diseases [261, 262].

Aims and Objectives

Primary hypothesis:

The GGT5–GGG–P2RY8 pathway operates in non-lymphoid tissues and, upon endothelial activation, fibroblasts upregulate GGT5 to shape local inhibitory landscapes that position P2RY8⁺ leukocytes in IMIDs.

Objectives:

- 1) Define the cellular sources and spatial distribution of GGT5 across health and disease in synovium and gut.
- 2) Identify upstream signals that induce GGT5 in fibroblasts (e.g., TNF α , NOTCH3 ligands, endothelial-derived factors) and clarify tissue-specific regulation.
- 3) Test whether GGG restrains migration of Tfh and GC B cells ex vivo (tonsil) and in disease-relevant tissues (inflamed gut), with pre-specified metrics (chemotactic index, speed, arrest coefficient).
- 4) Assess the role of Ggt5 in murine colitis and arthritis while explicitly accounting for the absence of P2RY8 and potential leukotriene-pathway effects.
- 5) Integrate findings into a mechanistic model of a perivascular confinement zone and outline translational implications (e.g., modulating GGT5 activity).

Chapter 2: Materials and Methods

2.1 Experimental Design and Statistical Analysis

All *in vitro* experiments were performed with a minimum of three biological replicates ($n=3$), each with two technical replicates. Biological replicates are independent donors or animals; technical replicates are repeated measurements on the same sample. For each experiment, primary and secondary endpoints were pre-specified. Samples and plates were not blinded during data collection and analysis. Data were tested for normality using the Shapiro–Wilk test when possible; in cases where sample sizes were too small, normality assessment was not performed, and Gaussian distribution was not assumed. Statistical significance was evaluated using two-sided non-parametric tests: the Mann-Whitney U test for comparisons between two groups, and the Friedman test for repeated measures across multiple groups. Post-hoc analysis for the Friedman test was conducted using Dunn’s multiple comparison test. For comparisons involving three or more unmatched groups, Kruskal–Wallis tests with Dunn’s multiple comparison test were used; ANOVA was not performed due to lack of normality or small sample sizes. Multiple testing was controlled using the Holm–Šidák or Benjamini–Hochberg methods where appropriate. Effect sizes (Cohen’s d or rank-biserial r) and exact P values are reported. Outliers were handled by pre-specified rules

(robust Z-score > 3.5) and are indicated in figures if removed. P-values < 0.05 were considered statistically significant.

For in vivo studies, sample sizes were not determined through power analysis as it was a pilot study. Total of 19 mice were used where n=5 for wildtype, n=8 for heterozygous and n=6 for homozygous. Mice were held as littermates in the same cage as wildtype with heterozygous or heterozygous with homozygous to account for potential confounding factors such as litter effects and cage placement. For DSS, the compound was administered in drinking water. For AIA, an independent investigator (Kristina Zec) administered treatments. For histology scoring, the investigators (Mathilde Pohin and Kristina Zec) were blinded from the genotype assignment. Gaussian distribution was not assumed. Statistical significance was evaluated using Kruskal-Wallis tests as there was no matching or pairing. Post-hoc analysis for the Kruskal-Wallis tests was conducted using Dunn's multiple comparison test. Due to small sample sizes ($n \leq 8$ in most groups), normality was not assumed. Non-parametric statistical tests were used for group comparisons.

All statistical analyses were performed using GraphPad Prism version 10.4.1, as well as RStudio Version 2023.06.0+421 or jupyter notebook version 6.4.8. P-values < 0.05 were considered statistically significant. The data are presented as mean $\pm$ standard deviation (SD) unless otherwise stated.

2.2 Cell Lines and Reagents

All primary foetal proximal interphalangeal (PIP) synovial fibroblast cell lines were previously generated in the lab by Ian Reekie, Caio Machado, Rowie Borst and Sarah Davidson. The human embryonic and foetal material was provided by the Joint MRC/Wellcome Trust (grant# MR/X008304/1 and 226202/Z/22/Z) Human Developmental Biology Resource (HDBR, www.hdbbr.org). Ethical approval for the collection and use of human embryonic and foetal tissue was obtained by the HDBR, and informed consent was obtained from donors. The PIP fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) (#41966-029, Gibco) containing 10% heat-inactivated foetal bovine serum (FBS, #10500-064, Gibco), 100 units/mL penicillin and 100 µg/mL streptomycin (#15140-122, Gibco) at 5% CO₂ and 37 °C.

IBD patients were recruited at the John Radcliffe Hospital (Oxford) IBD outpatient clinic and biopsies were collected from terminal ileum, ascending colon, descending colon and/or rectum (colonoscopy) or the descending colon, sigmoid and/or rectum (flexible sigmoidoscopy). All patients and healthy participants gave informed consent and collection was approved by NHS National Research Ethics Service under the research ethics committee reference IBD Cohort 09/H1204/30)/(GI Ethics 16/YH/0247. All primary intestinal fibroblast cell lines were generated by Mathilde Pohin and Matthias Friedrich, and they were cultured in MEM Alpha (#22571-020, Gibco) containing 20% heat-

inactivated FBS, 100 units/mL penicillin and 100 µg/mL streptomycin at 5% CO₂ and 37 °C.

For the stimulation of GGT5 expression, both synovial and intestinal fibroblasts were plated at 50,000 cells per well in a 48-well plate. The number of cells plated was guided by Friedrich et al., who seeded 20,000 fibroblasts per well in a 48-well plate for their *in vitro* assays of fibroblast stimulation [263]. Plated cells were starved overnight in culture medium without FBS prior to stimulation with cytokines or supernatant for 24 hours at 5% CO₂ and 37 °C. After 24 hours, supernatants were aspirated, and cells were lysed in situ using RNA lysis buffer (Buffer RLT) without dithiothreitol (DTT) from RNeasy Micro Kit (#74004, Qiagen).

2.2.1 List of fibroblast donors used in *in vitro* assays

Fibroblast type	Donor number
PIP	16284 18pcw
	15492 17pcw
	17pcw donor 1
	17pcw donor 2
	17pcw donor 3
Intestinal	GI9878 inflamed
	GI17665 inflamed
	GI4528 inflamed

	GI11175 inflamed
	GI5543 inflamed

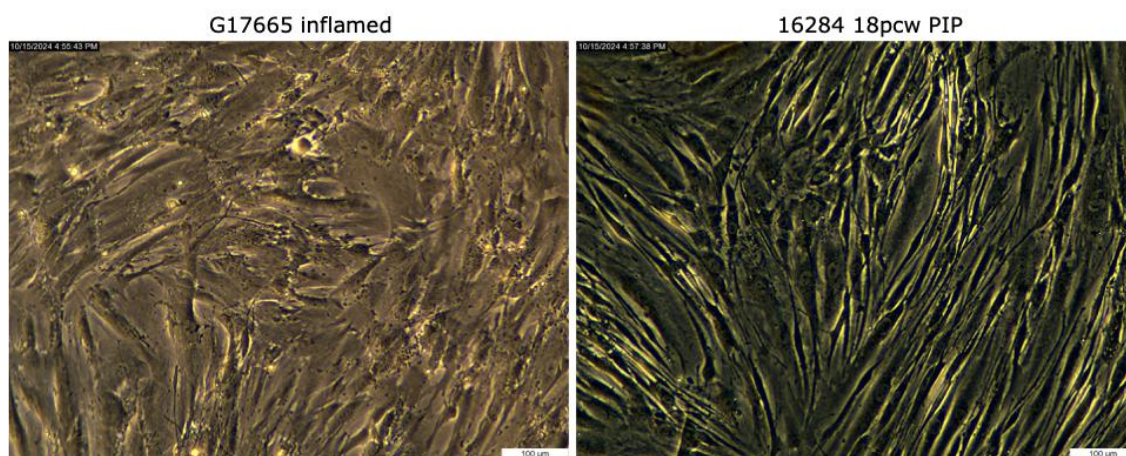


Figure 2.1: Representative images for intestinal and PIP fibroblasts seeded at 50,000 cells per well in a 48-well plate. Images were captured using Leica Microscope Systems with a 10x objective lens (Milton Keynes, UK). Scale bar = 200 μm .

2.2.2 List of recombinant cytokines, proteins and drugs

Reagent	Catalogue no.	Manufacturer	Working concentration
TNF α (human)	10291-TA-050	R&D Systems	1 ng/mL
IL-1 β (human)	201-LB	R&D Systems	20 ng/mL
IFN γ (human)	285-IF-100/CF	R&D Systems	10 ng/mL
DLL4 Fc (human)	10185-D4-050	R&D Systems	5 μ g/mL
Jagged 1 Fc (human)	1277-JG-050	R&D Systems	5 μ g/mL
MK2206	CAY11593	Cayman Chemical	5 μ M
SB202190	S7067	Sigma Aldrich	10 μ M
Fludarabine	3495	Tocris	10 μ M
Stattic	2798	Tocris	0.625 μ M
LDN193189	SML0559-5MG	Sigma-Aldrich	1.25 μ M
SC75741	SML2382-5MG	Sigma Aldrich	10 μ M

MK2206, SB202190 were kindly gifted by Dr. Fabian Fisher.

2.3 NOTCH3 ligands stimulation assay

The assay was done following published protocol by Wei et al [56]. Briefly, the 6-well cell culture plate was coated with recombinant DLL4-Fc (#10185-D4, R&D systems) or JAG1-Fc (#1277-JG, R&D systems)

reconstituted in phosphate-buffered saline (PBS) overnight at 4 °C, at 5 µg/mL. Fibroblasts were then seeded at 3×10^5 cells/well on DLL4-Fc, JAG1-Fc-, or PBS-coated plates for 72 hours, after which the RNA was collected.

For TNF α and DLL4-Fc synergy experiment, the protocol was adapted from a published paper by Zohorsky et al [264]. Firstly, Dynabeads protein G (#10003D, Thermo Fisher) were washed by pipetting up and down a few times with sterile PBS, followed by placing the tube on an Invitrogen™ DynaMag™-2 Magnet (#10723874, Invitrogen) for 2 mins to separate the beads. They were then coated with 2.5 µg/mL of DLL4-Fc for 10 mins at 300 rpm in Eppendorf ThermoMixer® C at room temperature. DLL4-coated beads were then washed with PBS twice to remove loosely bound and unbound DLL4-Fc. After resuspending in serum-free medium, TNF α was added so that the final concentration was 1 ng/mL. Fibroblasts were stimulated with DLL4-coated beads, TNF α or combination of both for 24 hours, after which the RNA was collected.

2.4 Endothelial cell supernatant stimulation assay

The fibroblasts were seeded as described in section 2.2. Pooled primary human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (#CC-2519, Lot: 18TL159195). They were grown in EBM™-2 Endothelial Cell Growth Basal Medium-2 (#CC-3156,

Lonza), supplemented with EGM™-2 Endothelial SingleQuots™ Kit (#CC-4176, Lonza) at 5% CO₂ and 37 °C.

For assay in Figure 4.7, endothelial cells were seeded at 50,000 cells per well in a 48-well plate. Peripheral blood mononuclear cells (PBMCs) were isolated as detailed in section 2.9. The CD3⁺, CD19⁺ and CD14⁺ cells were positively selected according to manufacturer's instruction using Miltenyi Microbeads (CD3: #130-097-043, CD19: #130-050-301, CD14: #130-050-201) and MS columns. Each well contain 5 x 10⁴ cells in 200 µL. The HUVEC and PBMC supernatant were generated for 48 hours, after which they were collected and centrifuged at 800 x g for 5 mins to remove any debris using Eppendorf Centrifuge 5804 R. While the supernatant was being generated, fibroblasts were serum-starved for approximately 48 hours. Prior to stimulation, fibroblasts were pre-treated for 30 minutes with various pathway inhibitors — MK2206 (AKT inhibitor), SB202190 (p38 inhibitor), Fludarabine (STAT1 inhibitor), Stattic (STAT3 inhibitor), LDN-193189 (SMAD inhibitor), and SC75741 (NFκB inhibitor). They were then stimulated with HUVEC and PBMC supernatant, with or without the respective inhibitors. RNA was collected 24 hours after stimulation.

For assay in Figure 4.8, endothelial cells were seeded at 50,000 cells per well in a 48-well plate. The following day, they were activated with 10 ng/mL TNFα and 200 ng/mL IFNγ for 24 hours. This dosing was guided by the protocol described in Jaczewska et al [265]. After

activation, the cytokine-containing medium was aspirated, and the endothelial cells were rinsed twice with PBS. Fresh supplemented EBM-2 medium without hydrocortisone was then added at 200 μ L per well. Hydrocortisone was omitted to minimise the immunosuppressive effect of corticosteroids. The supernatant was collected 48 hours later and centrifuged at 800 $\times$ g for 5 minutes using an Eppendorf Centrifuge 5804 R to remove cellular debris. While the endothelial cell supernatant was being generated, fibroblasts were serum-starved for approximately 48 hours. Prior to stimulation, fibroblasts were pre-treated for 30 minutes with various pathway inhibitors — MK2206 (AKT inhibitor), SB202190 (p38 inhibitor), Fludarabine (STAT1 inhibitor), Stattic (STAT3 inhibitor), LDN-193189 (SMAD inhibitor), and SC75741 (NF κ B inhibitor). They were then stimulated with endothelial cell supernatant, with or without the respective inhibitors. RNA was collected 24 hours after stimulation.

2.5 RNA isolation

Cultured cells were directly lysed on a plate using RNA lysis buffer, followed by RNA isolation using RNeasy Micro Kit with on-column DNase treatment step (#74004, Qiagen). In brief, 300 μ L of Buffer RLT without DTT was added to each well and the suspension was homogenised by vortexing or pipetting. Then, 300 μ L of 70% ethanol was added and mixed by vortexing or pipetting. The lysate was transferred to a RNeasy MinElute spin column (placed into a 2 mL

Collection Tube) and centrifuged at maximum speed ($20,238 \times g$) for about 15 seconds at room temperature using benchtop Eppendorf Centrifuge 5425 R. Flow-through in the collection tube was discarded.

To perform the wash step, 350 μL of Buffer RW1 was added to the spin column and centrifuged at maximum speed ($20,238 \times g$) for about 15 seconds. Flow-through in the collection tube was discarded. DNase I incubation mix was prepared by adding 10 μL of DNase I stock solution to 70 μL of Buffer RDD. The incubation mix was added directly to spin column membrane and incubated at room temperature for 15 minutes. Then, 350 μL of Buffer RW1 was added to the spin column and centrifuged at maximum speed ($20,238 \times g$) for about 15 seconds. Flow-through was discarded. A further 500 μL of Buffer RPE was added and centrifuged at maximum speed ($20,238 \times g$) for about 15 seconds. Flow-through was discarded. As a final wash step, 500 μL of 80% ethanol was added to the spin column and centrifuged at maximum speed ($20,238 \times g$) for 2 minutes. The spin column was transferred to a new collection tube and centrifuged at full speed for 5 minutes to dry the membrane. The collection tube was discarded, and the spin column was transferred to a new 1.5 mL collection tube. Next, 13 μL of nuclease-free water was added directly to the centre of the spin column membrane and centrifuged at full speed for 1 minute to elute the RNA. The spin column was discarded. Purified RNA was immediately used in cDNA synthesis. The above-mentioned DNase I stock solution and

Buffer RPE were reconstituted or prepared as per instructions from Qiagen.

For cells cultured in 6-well or 12-well plates, RNA concentration and purity (260/280 and 260/230 absorbance ratios) were measured using a NanoDrop spectrophotometer, with both ratios exceeding 2. For cells cultured in 48-well plates, RNA concentration was not measured as the yield was below the NanoDrop's detection limit. In these cases, the maximum RNA volume (10 μ L) was used for cDNA synthesis.

2.6 Reverse transcription to complementary DNA

Purified RNA was reversed transcribed into complementary DNA (cDNA) using First Strand cDNA Synthesis Kit (#K1612, ThermoFisher Scientific). The volume needed for normalised amount of RNA sample was added to PCR tube and nuclease-free water was added if necessary to make up the volume to 10 μ L. Each RNA sample was added with 1 μ L of Oligo(dT)₁₈ Primer and span down briefly. A negative control tube was also made by adding 10 μ L of RNA. The PCR tube was placed into PCR Thermal Cycler and incubated at 65°C for 5 minutes. Then, each sample was added with 9 μ L of master mix containing 4 μ L of 5X reaction buffer, 1 μ L of RiboLock RNase Inhibitor, 2 μ L of 10 mM dNTP Mix and 2 μ L of M-MuLV RT. The negative control tube was added with 10 μ L of nuclease-free water instead. The PCR tube containing the reaction mixtures was placed into the PCR Thermal Cycler and

incubated at 37°C for 60 minutes, then the reaction was terminated at 70°C for 5 minutes. The cDNA was stored in -20°C freezer until use.

2.7 Quantitative real-time polymerase chain reaction

2.7.1 Taqman probes

Quantitative real-time PCR (qRT-PCR) was performed using premade, exon spanning TaqMan probes from ThermoFisher Scientific. The target probes were in FAM and as below:

CCL19	Hs00171149
CXCL1	Hs00605382
CXCL2	Hs00601975
CXCL10	Hs00171042
CXCL12	Hs03676656
FAP	Hs00990791
GGT5	Hs00899631
P2RY8	Hs01938524
PDGFRB	Hs01019589
PDGFRA	Hs00998018
PDPN	Hs00366766
PI16	Hs00542137
THY1	Hs00174816
TNF	Hs00174128
TNFRSF1A	Hs01042313
TNFRSF1B	Hs00961750

The housekeeping probes were in VIC PL and as below:

RPLP2 Hs01115128

2.7.2 qRT-PCR cycles

The qRT-PCR reactions were carried out in 384-Well PCR plates (#AB1384W, Thermo Scientific). For each reaction, the cDNA was diluted 1:10 with nuclease-free water and 2.8 μ L of diluted cDNA was added into each well, together with 3 μ L TaqMan™ Gene Expression Master Mix (#4369016, Applied Biosystems), 0.1 μ L of target probe and 0.1 μ L of endogenous control probe, resulting in total volume of 6 μ L per well. Negative control containing only RNA and no template control (NTC) for each probe containing only nuclease-free water without cDNA template were used to monitor for contamination and primer-dimer formation. The plates were sealed with clear StarSeal polyolefin film (#E2796-9793, StarLab) and centrifuged at 4,000 rpm for 1 minute at 4 °C using Eppendorf Centrifuge 5804 R. The PCR cycles were then run on Viia 7 Real-Time PCR System (Applied Biosystems) at standard speed using relative quantitation of Comparative Ct ($\Delta\Delta$ Ct). The cycle steps were as follow: holding at 50°C for 2 minutes and initial denaturation at 95°C for 10 minutes, then 40 cycles of denaturation at 95°C for 15 seconds and extension at 60°C for 60 seconds (+plate read). Each reaction condition was conducted in duplicate wells.

Quantification of gene expression values, relative to housekeeping gene(s) as indicated, was calculated using the $2^{\Delta ct}$ method.

2.8 Multiplexed tissue imaging using CellDIVE

2.8.1 Slide clearing and blocking

Formalin-fixed paraffin embedded (FFPE) human tonsil, synovium and colon slides were baked overnight at 60°C. The next day, the slides were deparaffinised with xylene and rehydrated with 100%, 95%, 70% and 50% ethanol, 5 minutes each step. Then, they were washed in PBS twice and permeabilised in 0.3% Triton X100 for 10 minutes before being washed further in 1x PBS for 5 minutes. Using Biocare NxGen decloaker, antigen retrieval was performed in boiling pH 6 citrate-based antigen unmasking solution (#H3300, Vector), and then pH 9 Tris-based solution, 20 minutes each step. The slides were blocked in 1x PBS solution containing 3% BSA (#A7906, Merck), 10% Donkey serum (#C06SB, Bio-Rad) overnight, then blocked again the next day using 1:100 of FcR Blocking Reagent, human (#130-059-901, Miltenyi) at room temperature for 1 hour. After being washed in 1x PBS for 5 minutes, the slides were stained with DAPI (#D3571, Thermo) and washed again before being coverslipped with mounting media containing 50% glycerol (#G5516, Sigma) and 4% propyl gallate (#2370, Sigma).

2.8.2 Scanplan and background acquisition

All the FFPE human tissue slides were imaged using GE Cell DIVE system [266]. Firstly, a ScanPlan was performed by acquiring at 10x magnification so that regions of interest can be selected. This was then followed by imaging at 20x to acquire background autofluorescence and generate virtual H&E images. This background autofluorescence would be subtracted from the subsequent stained images.

2.8.3 Staining and bleaching

All slides were decoverslipped in 1x PBS prior to staining and bleaching. Each staining round was made up of 3 antibodies prepared in staining buffer (3% BSA in PBS). Unconjugated primary antibodies were used in the first round, which were incubated at 4°C overnight. The next day, the slides were washed thrice in 0.05% PBS-Tween20 (#P9416, Sigma). Secondary antibodies raised in donkey that were conjugated to Alexa Fluor 488, 555 or 647 were then incubated for an hour at room temperature. Directly conjugated antibodies to either of these dyes were used in subsequent staining rounds, and they were incubated at 4°C overnight or 1 hour room temperature. Manually conjugated antibodies were purchased in BSA and azide-free format and conjugated using antibody labelling kit (Invitrogen). The working concentration of most antibodies were 5 µg/mL as guided by both published and unpublished works generated from the CellDIVE facility, most antibodies used in this project had been stained on human tissues

(including synovium, skin, gut and salivary glands) and were published on peer-reviewed journals [249, 263, 267].

Dye inactivation was performed by bleaching the slides with 0.1 M NaHCO₃ pH 10.9-11.3 (#S6297, Sigma) and 3% hydrogen peroxide (#216763, Merck) for 3 rounds (15 minutes each) with a 1 min wash in 1x PBS between. Fresh bleaching solution was prepared each time. The slides were then re-stained with DAPI for 2 minutes before being washed in 1x PBS. The bleached slides were imaged as the new background fluorescence for subsequent subtraction.

DAPI staining was used to assist image registration and alignment. The slides can be multiplexed with panel of 3 antibodies by repeating the staining, bleaching and imaging steps.

2.8.4 List of human antibodies

Sp	Antibody	Catalogue	Clone	Isotype	Stock Conc.	Working Conc.	Fluorochrome
Rb	P2RY8	HPA003631	Polyclonal	IgG	0.2 mg/mL	*2 µg/mL	Primary
Rt	RORgt	14-6988-82	AFKJS-9	IgG2a + k	0.5 mg/mL	5 µg/mL	Primary
Ms	CD45RO	304212	UCHL1	IgG2a, κ	0.2 mg/mL	5 µg/mL	AF488
Rb	CXCR5	ab272936	EPR23463-30	IgG	0.69 mg/mL	5 µg/mL	Conjugated to AF555
Rb	CXCL13	ab270408	EPR23400-92	IgG	0.53 mg/mL	5 µg/mL	Conjugated to AF647
Ms	CD45RA	304114	HI100	IgG2b, κ	0.2 mg/mL	5 µg/mL	AF488
Rb	CD3	ab245731	SP162	IgG	256.42 µg/mL	5 µg/mL	conjugated to AF555
Rb	GGT5	ab283267	EPR24682-102	IgG	0.505mg/mL	**2 µg/mL	conjugated to AF647
Rb	CD20	ab198941	EP459Y	IgG	0.5 mg/mL	5 µg/mL	AF488
Rb	FOXP3	74816SF	D2W8E	IgG	0.71 mg/mL	5 µg/mL	conjugated to AF555
Rb	PD-1	ab220301	NAT105	IgG	0.5 mg/mL	5 µg/mL	AF647
Rb	CD21	ab202692	EP3093	IgG	0.5 mg/mL	5 µg/mL	AF488
Ms	CD8a	372902	C8/144B	IgG1, κ	0.5 mg/mL	5 µg/mL	conjugated to AF555

Rb	T-bet	27112SF	D6N8B	IgG	0.5 mg/mL	5 µg/mL	conjugated to AF647
Rb	CD31	ab215911	JC/70A	IgG	0.5 mg/mL	5 µg/mL	AF488
Rb	CD4	ab280849	EPR6855	IgG1, κ	0.5 mg/mL	5 µg/mL	AF555
Rb	CCR7	ab275165	EPR23192-57	IgG	0.5 mg/mL	5 µg/mL	AF647
Rb	CD146	ab196448	EPR3208	IgG	0.5 mg/mL	5 µg/mL	AF488
Rb	CD19	ab274888	EPR5906	IgG	0.5 mg/mL	5 µg/mL	AF555
Ms	CD8a	372906	C8/144B	IgG1, κ	0.5 mg/mL	5 µg/mL	AF647
Rb	CD11c	ab275738	EP1347Y	IgG	0.5 mg/mL	5 µg/mL	AF488
Rb	LAMP3	ab281573	EPR24265-8	IgG	0.425 mg/mL	5 µg/mL	conjugated to AF555
Rb	CD14	ab226121	EPR3653	IgG	0.65 mg/mL	5 µg/mL	conjugated to AF647

*P2RY8 was used at 2 µg/mL based on previous unpublished validation in the lab.

**GGT5 concentration at 2 µg/mL was validated as shown in Figure 3.1.

Other antibodies were used at 5 µg/mL based on validation from CellDIVE facility and other users.

2.9 Isolation of tonsil leukocytes and PBMCs

Fresh human tonsil tissues were obtained from Oxford Radcliffe Biobank (ORB) through patients undergoing tonsillectomies under the ORB research tissue bank ethics (reference 19/SC/0173). Informed consent was obtained by ORB staffs, which complied with all ethical and regulatory requirements and the samples were anonymised upon collection. These tissues were stored on ice in RPMI medium, and they were processed within 1 hours after surgery.

The processing steps have been adapted from tonsil organoid model [268]. Firstly, the tissue was dissected into smaller pieces before being mashed through a 40 μm or 100 μm strainer to form a cell suspension, which was layered carefully above Lymphoprep (#07851, StemCell) to undergo density gradient centrifugation at 1250 x g for 20 mins (acc 1, dec 1). If granulocytes were required, density gradient centrifugation step was skipped. The leukocytes were washed twice with PBS containing 10% heat-inactivated FBS and 2x penicillin and streptomycin. In some cases when digestion is required, tonsil first underwent mechanical dissociation with scalpels and gentleMACS Dissociator (#130-093-235, Miltenyi) before digestion using purified collagenase CLSPA ((#LS005275, Worthington) for 20-40 minutes.

Blood cones from healthy adult human volunteers were obtained from Oxford NHS Blood and Transplant under NHSBT ethics (reference 11/H0711/7) and processed using Lymphoprep as above. Psoriatic

arthritis (PsA) synovial fluid mononuclear cells (SFMC) were kindly given by Paul Bowness' group. Inflamed gut resection sample was kindly processed and given by Mathilde Pohin.

2.10 Migration assay

2.10.1 Transwell migration assay (Chemotaxis assay)

The migration assay was performed using either PBMCs, tonsil leukocytes or digested inflamed gut cells. If frozen cells were used, they would be thawed and rested at 2×10^6 cells/mL overnight in RPMI-1640 (#11875093, Gibco) containing, 10% heat-inactivated FBS, 1x GlutaMax (#35050061, Gibco), 1 mM sodium pyruvate (#11360-039, Gibco), 1x MEM Non-Essential Amino Acids Solution (#11140-035), 10 mM HEPES buffer (#15630049, Gibco), 100 units/mL penicillin and 100 μ g/mL streptomycin. The leukocytes do not migrate across the transwell filter without being rested overnight.

The migration medium is made up with RPMI-1640 containing 0.5% fatty acid-free bovine serum albumin (#A8806-5G, Sigma Aldrich), 10 mM HEPES, 100 units/mL penicillin and 100 μ g/mL streptomycin. Prior to migration assay, leukocytes were resuspended with migration medium into 10^7 cells/mL and rested in 37 °C water bath for at least 10 minutes.

Recombinant human CXCL12 and CCL19 (Peprotech) were each diluted to a final concentration of 100 ng/mL in migration medium. Pertussis toxin was added at a 1:100 dilution, and GGG was prepared

at varying concentrations in migration medium containing either CXCL12 or CCL19. For each condition, 600 μ L of the respective mixture was added to the lower chamber of a 24-well tissue culture plate. A control condition consisted of migration medium alone in the lower chamber.

Then, 6.5 mm transwell insert with 5.0 μ m pore polycarbonate membrane (#3421, Corning) was placed on top of each well, and 100 μ L of leukocytes (10^6 cells) was added to the transwell insert. The use of 5.0 μ m pore size transwell filter in this assay was guided by published works from He et al. and Lu et al [252, 269]. The cells were allowed to migrate in the incubator for 1.5 hours, after which the migrated cells in the bottom well were collected and counted by flow cytometry. Equal volume of counting beads (#424902, Biolegend) was added to each well before transferring to 96-well plate for staining to account for transfer loss or cell loss during staining steps.

To assess migration, the number of cells that migrated for each well was divided by the number of counting beads to get an absolute number. For data from Cytex Aurora, the number of migrated cells is divided by the volume acquired.

2.10.2 Imaging migration assay

Peripheral CD4⁺ T cells were isolated using RosetteSep™ Human CD4⁺ T Cell Enrichment Cocktail (#15022, StemCell). Briefly, 400 μ L of enrichment cocktail was added into 2 mL of cone blood and left to

incubate at room temperature for 20 mins. Then, the blood was diluted with 2% FBS/2 mM EDTA in PBS before being laid carefully on top of Lymphoprep (#07801, StemCell) in 15 mL Falcon tubes. For density gradient centrifugation, the tubes were centrifugated at 1250 x g for 20 mins (acc 1, dec 1). The opaque interface layer between Lymphoprep and plasma were collected and washed twice with medium, resulting in pure CD4⁺ T cell population. The purity of CD4⁺ T cells was assessed by staining with CD4 and CD8 antibodies, followed by analysis using flow cytometry.

To activate these CD4⁺ T cells, they were added with 25 μ L of ImmunoCult™ Human CD3/CD28 T Cell Activator (#10971, StemCell) per 10⁶ cells and left for 7 days. Every 48 hours, the T cells were split 1:1 with medium supplemented with 100 units/mL of IL-2.

For imaging migration assay, glass bottom μ -slide (#80607, Ibidi) was first coated with 10 μ g/mL ICAM-1 at 37°C for 1 hour. Excess ICAM-1 was removed by washing the slide with room temperature PBS twice. The coated slide was then blocked with sterile 5% BSA in PBS at 37°C for 1 hour. Meanwhile, the T cells were stained with 1:1000 CellTrace Violet (#C34557, Invitrogen) at 37°C for 15 mins. After staining, cells were resuspended into 2 x 10⁶ cells/mL with medium. For this experiment, CXCL12 was diluted to 50 ng/mL and GGG was diluted to 1 and 10 μ M. Both CXCL12 and GGG are made up in heat-inactivated FBS before adding into the μ -slide. The migration of T cells was captured by imaging every second using Zeiss 980 confocal

microscopy for 20 mins, with the chamber set to 37°C and 5% CO₂.

The data was analysed using TrackMate 7 as a Fiji plugin [270].

2.10.3 List of reagents

Reagent	Catalogue no.	Manufacturer
CXCL12 (human)	300-28	Peprtech
CXCL13 (human)	300-47	Peprtech
IL-2	200-02	Peprtech
S-Geranylgeranyl-L-glutathione (GGG)	9004116	Cayman Chemical
Pertussis toxin	3097/50U	R&D Systems
ICAM-1	10346-H02H	SinoBiological

2.11 Flow cytometry

All flow cytometry staining was done in 96-well V bottom plates, and the centrifuge machine (Eppendorf Centrifuge 5804 R) was set to 2-8°C. Flow cytometry staining buffer being used was PBS containing 1% BSA and 2 mM EDTA.

2.11.1 Surface staining

Firstly, cells were centrifuged at 400 x g for 5 mins to get rid of supernatant. The cells were then blocked in 1:100 of FcR blocking reagent (#130-059-901, Miltenyi) and stained with 1:1000 of

LIVE/DEAD™ Fixable Near IR (780) Viability Kit (#L34994, Invitrogen) for 30 minutes on ice (1:2000 of live dead for spectral flow cytometry). Then, the cells were washed with staining buffer, before being stained with 50 µL/well of antibody cocktail containing 1:200 of each human antibody for 30 minutes on ice (working concentration for mouse antibodies are specified below). After incubation, cells were washed twice with staining buffer. The cells were fixed with either 4% paraformaldehyde or FluoroFix buffer (#422101, Biolegend) for 20 minutes at room temperature.

2.11.2 Intracellular staining

When required, intracellular staining was performed after surface staining. The cells were fixed and permeabilised with 100 µL/well of fixation/permeabilization solution (#554714, BD) at 2-8°C for 20 minutes before being washed twice with Perm/Wash buffer. Cells were blocked for 20 min at room temperature with 5% normal donkey serum (Sigma) and 1:100 FcR blocking reagent, stained at room temperature for 1 hour or overnight in the fridge with primary antibodies or conjugated antibodies, washed twice in Perm/Wash buffer and stained for 1 hour at room temperature with secondary antibodies (not required if using conjugated antibodies). The cells were washed twice again before being resuspended in staining buffer.

P2RY8 was stained for using Human Protein Atlas antibody, purchased from Sigma Aldrich (#HPA003631), used at 1:100 dilution

in staining buffer. To control for unspecific staining, purified rabbit polyclonal isotype control antibody (#910801, Biolegend) was used at the same concentration as P2RY8 antibody. No secondary antibody only control was used. The secondary antibody used in intracellular staining was AffiniPure® Fab Fragment Donkey Anti-Rabbit IgG conjugated to AF647 (#711-607-003, Jackson ImmunoResearch).

2.11.3 Flow cytometry

Stained cells were analysed using BD Fortessa X-20 or LSR II or Cytex Aurora (5-laser) spectral flow cytometer. The data was analysed using FlowJo version 10.10.0. Cells were gated sequentially based on forward and side scatter (FSC/SSC) to exclude debris, followed by singlet discrimination and exclusion of dead cells using live/dead staining. Fluorescence minus one (FMO) controls were used to define gating boundaries. Geometric mean fluorescence intensity (gMFI) values were normalised to the corresponding control or baseline sample within each experiment to account for inter-run variability.

2.11.4 List of flow cytometry human antibodies

Species	Isotype	Catalog	Clone	Marker	Fluorochrome	
-	-	L10119	-	LiveDead	Near-IR	1:1000
-	-	L23105	-	LiveDead	Live Dead Blue	1:2000
Ms	562805	562805	G46-6	HLADR	BV421	1:200
Ms	IgG2a, κ	307690	L243	HLADR	Spark UV387	1:200

Ms	IgG2a, κ	348232	IA6-2	IgD	BV605	1:200
Ms	IgG2a, κ	566243	IA6-2 (δ-IA6-2)	IgD	BUV395	1:200
Ms	IgG2a, κ	741400	12G5	CXCR4	BUV563	1:200
Ms	IgG1, κ	302234	HIB19	CD19	BV421	1:200
Ms	IgG1, κ	302219	HIB19	CD19	AF488	1:200
Ms	IgG1, κ	612990	SJ25C1	CD19	BUV615	1:200
Ms	IgG2b, κ	12-0209-42	2H7	CD20	PE	1:200
Ms	IgG1, κ	612834	MI15	CD138	BUV737	1:200
Ms	IgG1, κ	356934	J252D4	CXCR5	BV711	1:200
Rt	IgG2b, κ	741980	RF8B2	CXCR5	BUV805	1:200
Ms	IgG2a, κ	300420	UCHT1	CD3	PE-Cy7	1:200
Ms	IgG2a, κ	300320	HIT3a	CD3	AF488	1:200
Ms	IgG1, κ	300434	UCHT1	CD3	BV421	1:200
Ms	IgG1, κ	304042	HI30	CD45	BV605	1:200
Ms	IgG1, κ	304091	HI30	CD45	Spark Violet 500	1:200
Ms	IgG1, κ	331533	L161	CD1c	BV510	1:200
Ms	IgG1, κ	314524	MHM-88	IgM	BV605	1:200
Ms	IgG2b, κ	317436	OKT4	CD4	BV650	1:200
Ms	IgG2a, κ	353227	G043H7	CCR7	BV711	1:200
Ms	IgG2a, κ	304205	UCHL1	CD45RO	PE	1:200
Ms	IgG2a, κ	304261	UCHL1	CD45RO	BV750	1:200
Ms	IgG1, κ	748281	HP-3G10	CD161	BV786	1:200
Ms	IgG1, κ	323003	W6D3	CD15	FITC	1:200
Ms	IgG2b, κ	304105	HI100	CD45RA	AF488	1:200
Ms	IgG2b, κ	304186	HI100	CD45RA	Spark blue 550	1:200
Ms	IgG1, κ	304829	DREG-56	CD62L	BV785	1:200
Ms	IgG1, κ	570683	DREG-56	CD62L	RB744	1:200
Ms	IgG1, κ	302007	3G8	CD16	PE	1:200

Ms	IgG1, κ	568125	2A3	CD25	RY586	1:200
Ms	IgG1, κ	301340	ICRF44	CD11b	Alexa Fluor 594	1:200
Ms	IgG1, κ	305323	HB15e	CD83	BV421	1:200
Ms	IgG1, κ	305327	HB15e	CD83	PE-Dazzle594	1:200
Ms	IgG2b, κ	305434	IT2.2	CD86	PE-Dazzle594	1:200
Ms	IgG2a, κ	301842	M5E2	CD14	BV510	1:200
Ms	IgG2a, κ	301811	M5E2	CD14	AF488	1:200
Ms	IgG1	H007T02Y05	MEM-15	CD14	NovaFluor™ Yellow 690	1:200
Ms	IgG1, κ	305217	2D10	CD80	PE-Cy7	1:200
Ms	IgG1, κ	392435	301340	CD56	PE-Fire 810	1:200
Ms	IgG1, κ	367403	NAT105	PD-1	PE	1:200
Ms	IgG1, κ	329910	EH12:2H7	PD-1	Alexa Fluor 647	1:200
Ms	IgG1, κ	302855	O323	CD27	Spark-NIR685	1:200
Ms	IgG1, κ	356624	HB-7	CD38	Alexa Fluor 700	1:200
Ms	IgG2b, κ	371509	S-HCL-3	CD11c	APC-Fire 750	1:200
Ms	IgG1, κ	344712	SK1	CD8	PE-Cy7	1:200
Ms	IgG1, κ	344764	SK1	CD8	APC-Fire 810	1:200
Ms	IgG1, κ	558126	I10-1112	CD208	PE	1:200
Rb	IgG	HPA003631	Polyclonal	P2RY8	Secondary AF647	1:200

2.11.5 List of flow cytometry mouse antibodies

T cell panel						
Species	Isotype	Catalog	Clone	Marker	Fluorochrome	Dilution
				Live dead	Near IR	1:1000
Rt	IgG2b, κ	103147	30-F11	CD45	BV711	1:200

Rt	IgG2a, κ	115534	6D5	CD19	PerCP-Cy5.5	
Rt	IgG2b, κ	100229	17A2	CD3	BV650	1:200
Rt	IgG2a, κ	100551	RM4-5	CD4	BV785	1:200
Rt	IgG2a, κ	100743	53-6.7	CD8a	BV605	1:200
Rt	IgG2a, κ	103232	RA3-6B2	B220	AF700	1:200
Rt	IgG2b, κ	145516	L138D7	CXCR5	Pe-Cy7	1:200
Rt	IgG2a, κ	135205	29F.1A12	PD1	PE	1:200
Ms	IgG2a, κ	562894	Q31-378	RORgt	BV421	1:200
Rt	IgG2a, κ	11-5773-82	FJK-16s	FOXP3	FITC	1:200
Rb	IgG	37835	E412K	TBET	AlexaFluor 647	1:200

B cell panel						
Species	Isotype	Catalog	Clone	Marker	Fluorochrome	Dilution
				Live dead	Near IR	1:1000
Rt	IgG2b, κ	103147	30-F11	CD45	BV711	1:200
Rt	IgG2a, κ	115543	6D5	CD19	BV785	1:200
Rt	IgG2b, κ	107622	M5/114.15.2	MHCII (I-A/I-E)	AF700	1:200
Rt	IgG2a, κ	103236	RA3-6B2	B220	PerCP-Cy5.5	1:200
Rt	IgG2b, κ	101245	M1/70	CD11b	BV510	1:200
Rt	IgG2a, κ	564270	5E7	BLIMP1	BV421	1:200
Gt	IgG	1040-02	Polyclonal	IgA	FITC	1:200
Rt	IgG	406609	RMG1-1	IgG1	APC	1:200
Rt	IgG	407110	RMG2a-62	IgG2a		
Rt	IgG1, κ	406712	RMG2b-1	IgG2b		
Rt	IgG2a, κ	405727	11-26c.2a	IgD	BV605	1:200
Rt	IgM, κ	144608	GL7	GL7	PE	1:200

Myeloid panel						
Species	Isotype	Catalog	Clone	Marker	Fluorochrome	Dilution
				Live dead	Near IR	1:1000
Rt	IgG2b, κ	103147	30-F11	CD45	BV711	1:200
Rt	IgG2b, κ	101245	M1/70	CD11b	BV510	1:200
Armenian hamster	IgG	48-0114-82	N418	CD11c	eFluor 450	1:200
Rt	IgG2a, κ	552126	E50-2440	Siglec F	PE	1:200
Rt	IgG2b, κ	107622	M5/114.15.2	MHCII (I-A/I-E)	AF700	1:200
Rt	IgG2c, κ	128018	HK1.4	Ly6C	Pe-Cy7	1:200
Rt	IgG2a, κ	127648	1A8	Ly6G	PE-Dazzle 594	1:200
Rt	IgG2a, κ	123116	BM8	F4/80	APC	1:200
Armenian hamster	IgG	100328	145-2c11	CD3ε	PerCP-Cy5.5	1:200
Rt	IgG2a, κ	103236	RA3-6B2	B220		

2.12 Mice

Ggt5 total knockout mice (C57BL/6J-*Ggt5*^{em1Cys/J}; strain #036530) were obtained from The Jackson Laboratory. These mice carry a CRISPR/Cas9-generated *Ggt5* knock-out allele. The mice were housed in individually ventilated cages under specific pathogen-free (SPF) conditions and 12-hour light cycle. Standard chow and water were provided ad libitum. Environmental enrichment was provided in the form of nesting materials and shelter. Both male and female mice were used in experiments, aged 8-12 weeks at the start of procedures. Mice

were genotyped prior to the start of experiments. Based on genotype, animals were assigned to experimental groups, and cages were then randomly allocated. Investigators were not blinded to group allocation during tissue harvesting, but blinding was maintained during data analysis.

All experimental procedures were approved by the UK Home Office and carried out in accordance with the Animals (Scientific Procedures) Act 1986. Colitis experiments were conducted under project licence PPL PP1160839 and arthritis experiments under PPL PP4834133. All work was performed under personal licence that belonged to me (PIL no: I92316918), Matthias Friedrich (PIL no: IA558C610), and Kristina Zec (PIL no: I19417CCA).

2.12.1 Induction of dextran sulfate sodium (DSS) colitis

Induction of DSS was performed under the supervision of Matthias Friedrich. Colitis was induced by administering 2.5% (w/v) colitis-grade dextran sulfate sodium (DSS; MP Biomedicals, #0216011080) in drinking water for 5 consecutive days. Both male and female mice received DSS treatment. Following DSS exposure, mice were returned to standard drinking water for the remainder of the experiment. Body weight was recorded daily to monitor clinical progression of disease. Mice were euthanised on days 7–9 post-DSS initiation using a rising concentration of CO₂ followed by cervical dislocation. Spleen, mesenteric lymph nodes, and colon were harvested for histology, flow

cytometry, and qRT-PCR analyses. The experiment included *Ggt5* wild-type (WT, n = 5), heterozygous (Het, n = 8), and homozygous knockout (Hom, n = 6) mice. The colon tissues were formalin-fixed, paraffin-embedded (FFPE), sectioned at 5 µm thickness, and stained with H&E by the histology laboratory at the Kennedy Institute.

Experimental comparisons were made between genotypes (*Ggt5* wild-type, heterozygous, and homozygous knockout). No untreated control group was included, as the study focused on genotype-dependent differences in response to DSS-induced colitis.

2.12.2 Induction of antigen-induced arthritis (AIA)

Induction of AIA was performed by Kristina Zec (Udalova group). A modified antigen-induced arthritis (AIA) model was used based on a previously published protocol [271]. Both male and female mice were immunised by two subcutaneous injections of 20 µg methylated bovine serum albumin (mBSA) at 40 mg/ml, emulsified with complete Freund's adjuvant (CFA) at 3.3 mg/ml and PBS to a final volume of 100 µL per mouse. The emulsion was prepared using BTB immunisation kit (BTB Emulsions AB). The injections were performed under isoflurane anaesthesia, one at the base of the tail and the other at the right flank, 50 µL each site. Seven days post-immunisation, joint inflammation was induced via intra-articular injection of 200 µg mBSA (10 µL of a 20 mg/mL solution) into the left knee using a 50 µL Hamilton syringe with Luer tip (Scientific Laboratory Supplies Limited),

under isoflurane anaesthesia. The contralateral knee was injected with 10 µL PBS as an internal control. Knees were harvested seven days after intra-articular injection for histological evaluation. The knee tissues were decalcified, formalin-fixed, paraffin-embedded (FFPE), sectioned at 5 µm thickness, and stained with picosirius red by the histology laboratory at the Kennedy Institute. The experiment included Ggt5 wild-type (WT, n = 3), heterozygous (Het, n = 5), and homozygous knockout (Hom, n = 4) mice.

2.12.3 ARRIVE guidelines

The ARRIVE Essential 10		
Item	Recommendation	Section/line number, or reason for not reporting
Study design	1 For each experiment, provide brief details of study design including: a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. b. The experimental unit (e.g. a single animal, litter, or cage of animals).	a. DSS: Ggt5^{-/-} vs Ggt5^{+/-} vs WT (C57BL/6J) mice. AIA: mBSA-injected knee vs PBS-injected contralateral knee. b. Individual mouse
Sample size	2 a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used. b. Explain how the sample size was decided. Provide details of any <i>a priori</i> sample size calculation, if done.	a. DSS: WT n=5, het n=8 and hom n=6. AIA: WT n=3, het n=5 and hom n=4. b. Based on previous experience and literature to detect expected differences; no formal power calculation was performed.
Inclusion and exclusion criteria	3 a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established <i>a priori</i>. If no criteria were set, state this explicitly. b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no	a. All mice with correct genotype and good health were included. No specific <i>a priori</i> exclusion criteria. b. No animals or data points were excluded from analysis. c. Reported in figure legends and/or results

		exclusions, state so. c. For each analysis, report the exact value of n in each experimental group.	section.
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence. b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	a. Mice were genotyped and then cages randomly assigned to experimental groups. b. Cage assignment was randomised to minimise environmental confounding. No specific order randomisation for measurements.
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	Investigators were aware during tissue harvesting but blinded during data analysis.
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes). b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	a. Body weight loss, histopathology scores, joint inflammation, cytokine expression, and immune cell populations by flow cytometry. b. Body weight loss (DSS) and histological score (AIA).
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used. b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	a. Statistical tests and software (e.g., GraphPad Prism) described in figure legends and Methods. b. Due to small sample sizes ($n \leq 8$ in most groups), normality was not assumed. Non-parametric statistical tests (e.g., Mann-Whitney U test, Kruskal-Wallis test) were used for group comparisons.
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight. b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	a. Mus musculus, C57BL/6J, Ggt5^{-/-}, Ggt5^{+/-} and WT, male and female, aged 8–12 weeks. b. Mice purchased from The Jackson Laboratory; housed under SPF conditions. Genotyped before experiments. No prior procedures.

Experimental procedures	9 For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. What was done, how it was done and what was used. b. When and how often. c. Where (including detail of any acclimatisation periods). d. Why (provide rationale for procedures).	a. DSS in drinking water (2.5%, 5 days); AIA induced via mBSA and CFA immunisation + intra-articular mBSA injection. b. DSS: Days 0–5; tissue collected on days 7–9. AIA: Immunisation day 0, intra-articular injection day 7, tissue collection day 14. c. SPF facility; no specific acclimatisation beyond routine housing before experiments. d. DSS and AIA models were chosen for their relevance in studying colonic and joint inflammation, respectively.
Results	10 For each experiment conducted, including independent replications, report: a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range). b. If applicable, the effect size with a confidence interval.	a. Means $\pm$ SD, reported in Results and figure legends. b. NA

2.13 Transcriptomic analysis codes

All codes were run in RStudio Version 2023.06.0+421 or jupyter notebook version 6.4.8.

2.13.1 Gut Cell Atlas (Figure 3.6) – jupyter notebook

Data is available at <https://www.gutcellatlas.org/#datasets>

Foetal

```
import scanpy as sc
import seaborn as sns
import matplotlib.pyplot as plt

# Set Verdana as the font family
plt.rcParams['font.family'] = 'Verdana'

# load the data
adata = sc.read_h5ad('final_fetal_object_cellxgene.h5ad')

# define gene of interest
gene = 'GGT5'
print(adata.obs.columns)
sc.set_figure_params(dpi=300, dpi_save=300)
sc.pl.dotplot(adata, var_names=[272],
groupby='cell_name_detailed',
dendrogram=False, standard_scale='var',
cmap='viridis', dot_max=0.7, smallest_dot=10,
save='GGT5_foetal_gut_dotplot.png')
```

IBD gut

```
import scanpy as sc
import seaborn as sns
import matplotlib.pyplot as plt

# Set Verdana as the font family
plt.rcParams['font.family'] = 'Verdana'

# load the data
adata = sc.read_h5ad('2_Extended_Pan-
GI_atlas_all_lineages_allgenes_20241119.h5ad')

# define gene of interest
gene = 'GGT5'
```

```

print(adata.obs.columns)
sc.set_figure_params(dpi=300, dpi_save=300)

# Filter for Crohn's disease and ulcerative colitis
adata_ibd =
adata[adata.obs['control_vs_disease'].isin(['crohns_disease',
'ulcerative_colitis'])].copy()

# Create the dotplot with IBD data only
sc.pl.dotplot(adata_ibd, var_names=[272],
groupby='level_3_annot',
dendrogram=False, standard_scale='var',
cmap='viridis', dot_max=0.7, smallest_dot=10,
save='dotplot_GGT5_IBD_gut.png')

```

2.13.2 Foetal finger synovium and RA (Figure 3.7b) – RStudio

Data is available on Synapse (<https://doi.org/10.7303/syn52297840>)

```

#set working directory
setwd("C:/Users/Carl Lee/Documents/Z_interactome_AMP2")

# Load required libraries
library(Seurat)
library(ggplot2)

#custom theme####
my_theme = theme(
  plot.title = element_text(size=25, hjust=0.5, vjust=0.5,
family="Verdana", face="bold"),
  axis.text.x = element_text(size=12, family="Verdana"),
  axis.text.y = element_text(size=12, family="Verdana"),
  axis.title.x = element_text(size=16, family="Verdana"),
  axis.title.y = element_text(size=16, family="Verdana"),
  legend.text = element_text(size=14, family="Verdana"),
  plot.subtitle = element_text(size=12, family="Verdana"),
  plot.caption = element_text(size=12, family="Verdana")
)

#load the RDS object
AMP2 = readRDS("all_cells_raw.rds")
# normalize data
AMP2 = NormalizeData(AMP2, normalization.method =
"LogNormalize", scale.factor = 10000)

#display column names of the metadata
colnames(AMP2@meta.data)

```

```
#create the dotplot
dotplot = DotPlot(AMP2, features = "GGT5", group.by =
"cluster_name") +
  my_theme

#save the plot as PNG
ggsave("GGT5_dotplot.png", dotplot, width = 7, height = 20, dpi
= 300)
```

2.13.3 Pre/Post infliximab (Figure 4.1) - RStudio

The microarray data were deposited at Gene Expression Omnibus (GEO) under the series accession number GSE16879

```
#set working directory
setwd("/Users/zhiyiwong/Documents/Kennedy_Institute_of_Rheumatology/Data_mining_for_GGT5/Human/Arijs_PloS_ONE_61_IBD")

#load necessary packages
library(limma)
library(affy)
library(hgu133plus2.db)
library(AnnotationDbi)
library(dplyr)
library(ggsignif)
library(ggpubr)
library(devEMF)
library(ggplot2)
library(emmeans)

#custom theme####
my_theme = theme(
  plot.title = element_text(size=25, hjust=0.5, vjust=0.5,
family="Verdana", face="bold"),
  axis.text.x = element_text(size=12, family="Verdana"),
  axis.text.y = element_text(size=12, family="Verdana"),
  axis.title.x = element_text(size=16, family="Verdana"),
  axis.title.y = element_text(size=16, family="Verdana"),
  legend.text = element_text(size=14, family="Verdana"),
  plot.subtitle = element_text(size=12, family="Verdana"),
  plot.caption = element_text(size=12, family="Verdana")
)

# Extract the contents of the .tar file
untar("GSE16879_RAW.tar", exdir = "GSE16879_RAW")
```

```

# List the files in the extracted directory to identify the data
file
list.files("GSE16879_RAW")

# Set the directory where your .CEL.gz files are located
data_directory = "GSE16879_RAW"

# List all files in the directory
all_files = list.files(data_directory, full.names = TRUE)

# Filter for .CEL.gz files
cel_files = all_files[grep(".CEL.gz$", all_files)]

# Check if any .CEL.gz files are found
if (length(cel_files) == 0) {
  stop("No .CEL.gz files found in the specified directory.")
} else {
  print(cel_files)
}

# Load the data into R
raw_data = ReadAffy(filenamees = cel_files, compress = "gz")

# Extract expression values
exprs_values = exprs(raw_data)

# Background correction using affy with the "rma" method
bg_corrected_data = bg.correct(raw_data, method = "rma")

# Normalization using rma
normalized_data = rma(bg_corrected_data)

# Summarization
expression_values = exprs(normalized_data)

# Perform quality control (Optional)
# Example of quality control plots
boxplot(expression_values)

#Prepare Sample Information####
# load sample data
ss = read.table("sample_sheet.csv", header=TRUE, sep=',')

# makes sure the group information is a factor
ss$sample_group = factor(ss$sample_group)
ss$UC_CD_pre_post_response = factor(ss$UC_CD_pre_post_response)

#extract the expression matrix
expression_matrix = exprs(normalized_data)

```

```

# Extract probe set IDs from the ExpressionSet object
probe_set_ids = featureNames(normalized_data)
#map probe set IDs to gene symbols
gene_symbols = AnnotationDbi::select(hgu133plus2.db, keys =
probe_set_ids, columns = "SYMBOL", keytype = "PROBEID")

# Replace probe set IDs with gene symbols in the expression
matrix
rownames(expression_matrix) <-
gene_symbols$SYMBOL[match(rownames(expression_matrix),
gene_symbols$PROBEID)]

# Assuming expression_matrix is your expression matrix where
rows represent genes
gene_of_interest <- "GGT5" # Replace with your gene symbol of
interest

# Check if the gene of interest is in the row names of the
expression matrix
gene_in_matrix <- gene_of_interest %in%
rownames(expression_matrix)

if (gene_in_matrix) {
  print(paste("The gene", gene_of_interest, "is present in the
expression matrix."))
} else {
  print(paste("The gene", gene_of_interest, "is not present in
the expression matrix."))
}

# Write expression matrix to CSV file
write.csv(expression_matrix, file = "expression_matrix.csv")

#load expression matrix####
expression_matrix = read.csv(file = "expression_matrix.csv",
header=TRUE, sep=",")

#compare pre-post####
#Parse the data
gene_data = expression_matrix %>% filter(X == "GGT5")
rownames(gene_data) <- gene_data$X
#remove X column
gene_data$X <- NULL
#transpose
gene_data = t(gene_data)
#turn the table into data frame
gene_data = data.frame(gene_data)
# Sort gene_data by patient ID

```

```

gene_data = gene_data[order(row.names(gene_data)), , drop=F]
# Sort ss by patient ID
ss = ss[order(ss$sample), ]
#add a new column call pre_post_infliximab into gene_data table
gene_data$pre_post_infliximab = ss$pre_post_infliximab
#change the column titles
names(gene_data) = c("expression", "pre_post_infliximab")
#modify the order
gene_data$pre_post_infliximab =
factor(gene_data$pre_post_infliximab, levels = c("control",
"pre", "post"))

#anova test
compare_means(expression ~ pre_post_infliximab, data =
gene_data, method = "anova")
my_comparisons <- list( c("control", "pre"), c("control",
"post"), c("pre", "post"))

##combine violin and jitter plot####
ggp_violin_jitter = ggplot(gene_data, aes(x=pre_post_infliximab,
y=log10(expression+0.01), fill=pre_post_infliximab)) +
  geom_violin(width = 0.5 , alpha = 0.7, trim = FALSE ,
show.legend = FALSE) +
  scale_fill_manual(values = c("darkgrey", "darkblue",
"darkgreen")) + # Set fill colors manually
  geom_jitter(width = 0.1 , colour = "black", show.legend =
FALSE) +
  stat_compare_means(comparisons = my_comparisons, label.y =
c(0.88, 0.93, 0.98)) + # Add pairwise comparisons p-value
  stat_compare_means(label.y = 1.005, label.x = 1.35, family =
"Verdana", size = 5) + # Add global p-value
  #label the plot
  labs(x = "Pre/Post Infliximab", y = "Expression (log10)") +
  my_theme
#view the violin plot
ggp_violin_jitter

#save the violin jitter plot
emf("GGT5_pre_post_infliximab.emf", height=6, width=8)
print(ggp_violin_jitter)
dev.off()

#compare pre, post, responder, non-responder####
# load sample data
ss2 = read.table("sample_sheet_pre_post_response.csv",
header=TRUE, sep=',')
# makes sure the group information is a factor

```

```

ss2$pre_post_response = factor(ss2$pre_post_response)

#Parse the data
gene_data = expression_matrix %>% filter(X == "GGT5")
rownames(gene_data) <- gene_data$X
#remove X column
gene_data$X <- NULL
#transpose
gene_data = t(gene_data)
#turn the table into data frame
gene_data = data.frame(gene_data)
# Sort gene_data by patient ID
gene_data = gene_data[order(row.names(gene_data)), , drop=F]
# Sort ss by patient ID
ss2 = ss2[order(ss2$sample), ]
#add a new column call pre_post_response into gene_data table
gene_data$pre_post_response = ss2$pre_post_response
#change the column titles
names(gene_data) = c("expression", "pre_post_response")
#modify the order
gene_data$pre_post_response =
factor(gene_data$pre_post_response, levels = c("control",
"pre_yes", "post_yes", "pre_no", "post_no"))

# Step 1: Create 'time', 'response', and 'group' variables
gene_data <- gene_data %>%
  mutate(
    time = case_when(
      grepl("^pre", pre_post_response) ~ "pre",
      grepl("^post", pre_post_response) ~ "post",
      TRUE ~ NA_character_
    ),
    response = case_when(
      grepl("yes$", pre_post_response) ~ "responder",
      grepl("no$", pre_post_response) ~ "non_responder",
      TRUE ~ NA_character_
    )
  ) %>%
  filter(!is.na(time) & !is.na(response)) %>%
  mutate(
    time = factor(time, levels = c("pre", "post")),
    response = factor(response, levels = c("non_responder",
"responder")),
    group = interaction(time, response, drop = TRUE)
  )

# Step 2: Two-way ANOVA
model <- lm(expression ~ time * response, data = gene_data)

```

```

anova_result <- summary(aov(model))

# Step 3: Post-hoc pairwise comparisons
pairwise <- emmeans(model, pairwise ~ time * response)
pairwise_df <- as.data.frame(pairwise$contrasts)

# Step 4: Prepare comparison table for stat_pvalue_manual
selected_comparisons <- pairwise_df %>%
  filter(contrast %in% selected_contrasts) %>%
  mutate(
    group1 = sub(" -.*", "", contrast),
    group2 = sub(".*- ", "", contrast),
    group1 = gsub(" ", ".", group1),
    group2 = gsub(" ", ".", group2),
    xmin = factor(group1, levels = levels(gene_data$group)),
    xmax = factor(group2, levels = levels(gene_data$group)),
    p.label = as.character(signif(p.value, digits = 3)),
    y.position = seq(
      0.9,
      0.9 + 0.05 * (n() - 1),
      length.out = n()
    )
  )

selected_comparisons <- selected_comparisons %>%
  filter(p.value < 0.05)

# Step 5: Create violin + jitter plot
colors <- c(
  "pre.non_responder" = "cyan",
  "pre.responder" = "darkblue",
  "post.non_responder" = "orange",
  "post.responder" = "red"
)

ggp_violin_jitter <- ggplot(gene_data, aes(x = group, y =
log10(expression + 0.01))) +
  geom_violin(aes(fill = group), width = 0.5, alpha = 0.7, trim
= FALSE, show.legend = FALSE) +
  geom_jitter(aes(fill = group), width = 0.1, colour = "black",
show.legend = FALSE) +
  stat_pvalue_manual(selected_comparisons, label = "p.label",
tip.length = 0.01, hide.ns = TRUE) +
  scale_fill_manual(values = colors) +
  labs(x = "Pre/Post Infliximab Responder vs Non-Responder", y =
"Expression (log10)") +
  my_theme +
  theme(axis.text.x = element_text(angle = 45, hjust = 1))

```

```
# Display the plot
print(ggp_violin_jitter)

#save the violin jitter plot
emf("GGT5_pre_post_responder_non_responder.emf", height=7,
width=8)
print(ggp_violin_jitter)
dev.off()
```

GGT5 was selected a priori based on biological relevance; p-values are uncorrected as only one gene was tested.

2.13.4 Cross tissue Pearson correlation (Figure 4.4) – jupyter notebook

Inflammatory bowel diseases

Bulk RNA sequencing data was deposited at GEO under the series accession number GSE166928

```
import numpy as np
import pandas as pd
import scanpy as sc
import matplotlib.pyplot as plt
from scipy.stats import pearsonr # for correlation test

# load the dataset as a pandas dataframe
df = pd.read_csv("countsmatrix.csv")
df = df.set_index('geneName')
df = df.transpose()

# select the two genes you want to calculate the correlation
coefficient for
gene1 = 'GGT5'
gene2 = 'PDGFRB'

# calculate Pearson correlation coefficient and p-value
r_value, p_value = pearsonr(df[gene1], df[gene2])
print(f"Pearson r = {r_value:.2f}, p-value = {p_value:.3e}")

# create a scatterplot with the two genes
plt.scatter(df[gene1], df[gene2], c='black')

# fit a linear regression line (1st-degree polynomial)
slope, intercept = np.polyfit(df[gene1], df[gene2], 1)
```

```

line = slope * df[gene1] + intercept

# plot the regression line
plt.plot(df[gene1], line, color='red', linestyle='--',
linewidth=2)
# add title with correlation coefficient and p-value
plt.title(f"{gene1} vs {gene2}\nPearson r = {r_value:.2f}, p =
{p_value:.2e}")

# set the x and y axis labels
plt.xlabel(gene1)
plt.ylabel(gene2)

# save the plot as a JPEG image file
plt.savefig('GGT5_PDGFRB.jpeg', format='jpeg', dpi=1200)

# display the plot
plt.show()

```

Rheumatoid arthritis

The RNA-seq data have been deposited in ArrayExpress under Accession code E-MTAB-6141.

```

import numpy as np
import pandas as pd
import scanpy as sc
import matplotlib.pyplot as plt
from scipy.stats import pearsonr # for correlation test

df = pd.read_csv("mat.tsv", sep = '\t')
df = df.set_index('genes')
df = df.transpose()

# select the two genes you want to calculate the correlation
coefficient for
gene1 = 'GGT5'
gene2 = 'ACKR1'

# calculate Pearson correlation coefficient and p-value
r_value, p_value = pearsonr(df[gene1], df[gene2])
print(f"Pearson r = {r_value:.2f}, p-value = {p_value:.3e}")
# create a scatterplot with the two genes
plt.scatter(df[gene1], df[gene2], c='black')
# fit a linear regression line (1st-degree polynomial)
slope, intercept = np.polyfit(df[gene1], df[gene2], 1)
line = slope * df[gene1] + intercept

```

```
# plot the regression line
plt.plot(df[gene1], line, color='red', linestyle='--',
linewidth=2)

# add title with correlation coefficient and p-value
plt.title(f"{gene1} vs {gene2}\nPearson r = {r_value:.2f}, p =
{p_value:.2e}")

# set the x and y axis labels
plt.xlabel(gene1)
plt.ylabel(gene2)

# save the plot as a JPEG image file
plt.savefig('GGT5_ACKR1.jpeg', format='jpeg', dpi=1200)

# display the plot
plt.show()
```

Chapter 3: Characterisation of GGT5 expression in IBD and RA

3.1 Introduction

The mechanisms underlying leukocyte compartmentalisation in non-lymphoid organs during inflammation remain poorly understood, and it is unclear to what extent the principles governing leukocyte migration in SLOs also apply in non-lymphoid organs. To address this gap, this chapter aims to characterise GGT5 expression in inflammatory conditions such as IBD and RA, and also to understand the cell type expressing GGT5 in these conditions.

3.2 Results

Firstly, tonsils are used in this project to validate previously published findings regarding the role of GGT5 in GC organisation [252, 253], before characterising its expression in IBD and RA. To validate the GGT5 antibody, human tonsil tissue was stained with both the anti-GGT5 antibody and a rabbit isotype control. As shown in Figure 3.1, GGT5 staining appears clean and specific, with signal localised to the GCs, consistent with previous reports. A two-point antibody dose titration (2 and 5 $\mu\text{g/mL}$) demonstrated comparable localisation and specificity across concentrations, with no signal observed in matched isotype controls. Based on these results, 2 $\mu\text{g/mL}$ was selected for subsequent experiments as the lowest concentration yielding specific

staining. Co-staining with CD21, a marker of the FDC network, reveals overlapping signal with GGT5, suggesting that GGT5 is expressed by CD21⁺ FDCs within the germinal centres. However, additional donors and further analysis are needed to confirm this observation. CD20 and CD3 were used to mark B cell and T cell zones, respectively (Figure 3.2).

CellDIVE multiplex imaging utilises both DAPI and Cy5 channels to produce virtual H&E (vH&E) images that are similar to the conventional haematoxylin and eosin (H&E) stained sample. In Figure 3.3, the vH&E images show that lymphoid aggregates and TLS are present in IBD (top panel) and RA (bottom panel). The leukocytes form disorganised aggregates in the gut and synovium, and they are only found in certain part of the tissues: lamina propria in the gut and sublining layer in the synovium, as marked by the red circles in Figure 3.3a, c. In some instances, the lymphoid structures can become bigger and more organised (Figure 3.3b and d), for instance TLS is found in IBD (Figure 3.3d).

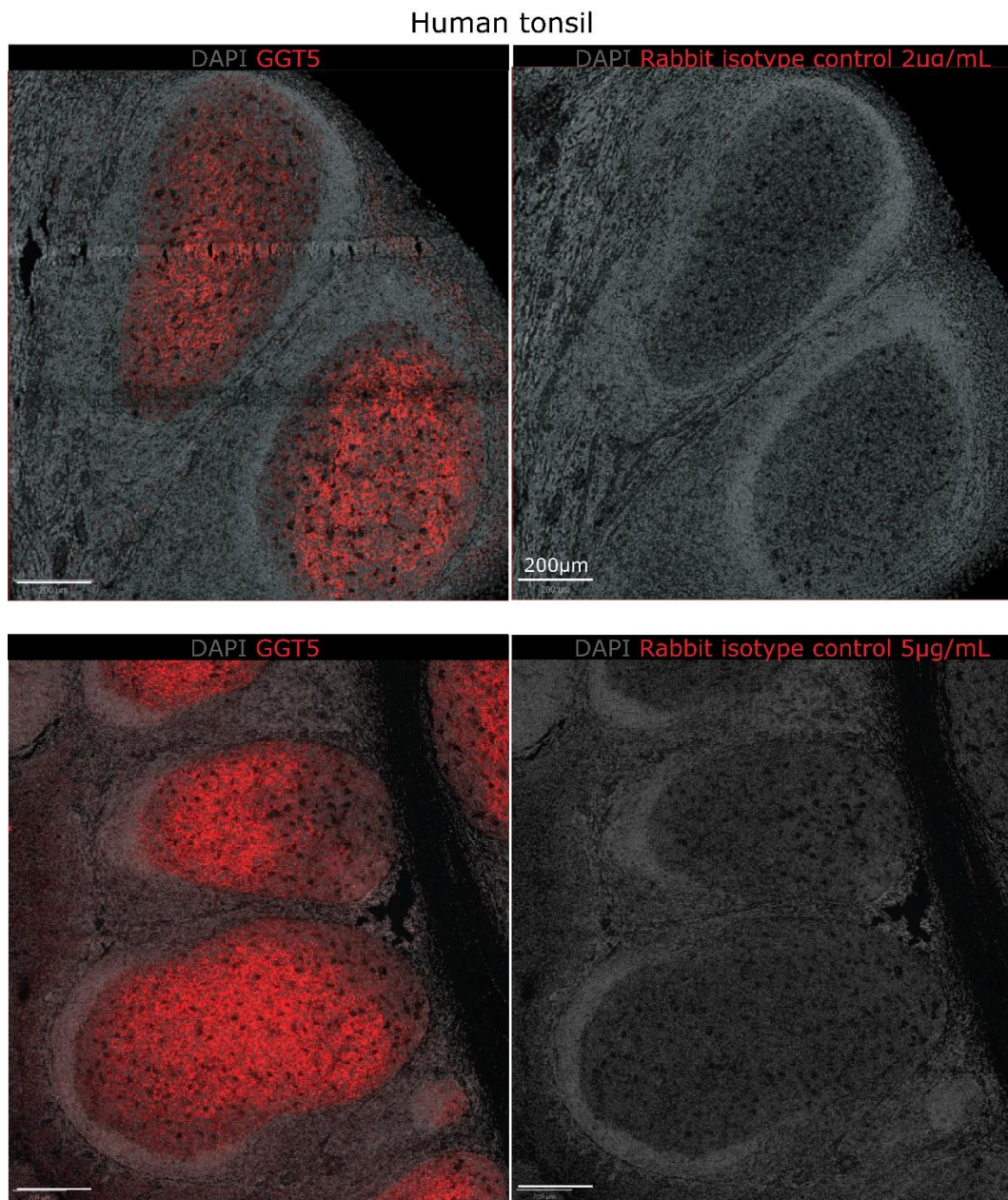


Figure 3.1: Validation of human GGT5 antibody staining in the germinal centres of human tonsil. Tonsil sections were stained with anti-GGT5 antibody at two concentrations to demonstrate dose titration (2 $\mu\text{g/mL}$, top left; 5 $\mu\text{g/mL}$, bottom left) or with rabbit isotype control at the corresponding concentrations (2 $\mu\text{g/mL}$, top right; 5 $\mu\text{g/mL}$, bottom right). Representative image from one tonsil donor ($n = 1$). Scale bar = 200 μm .

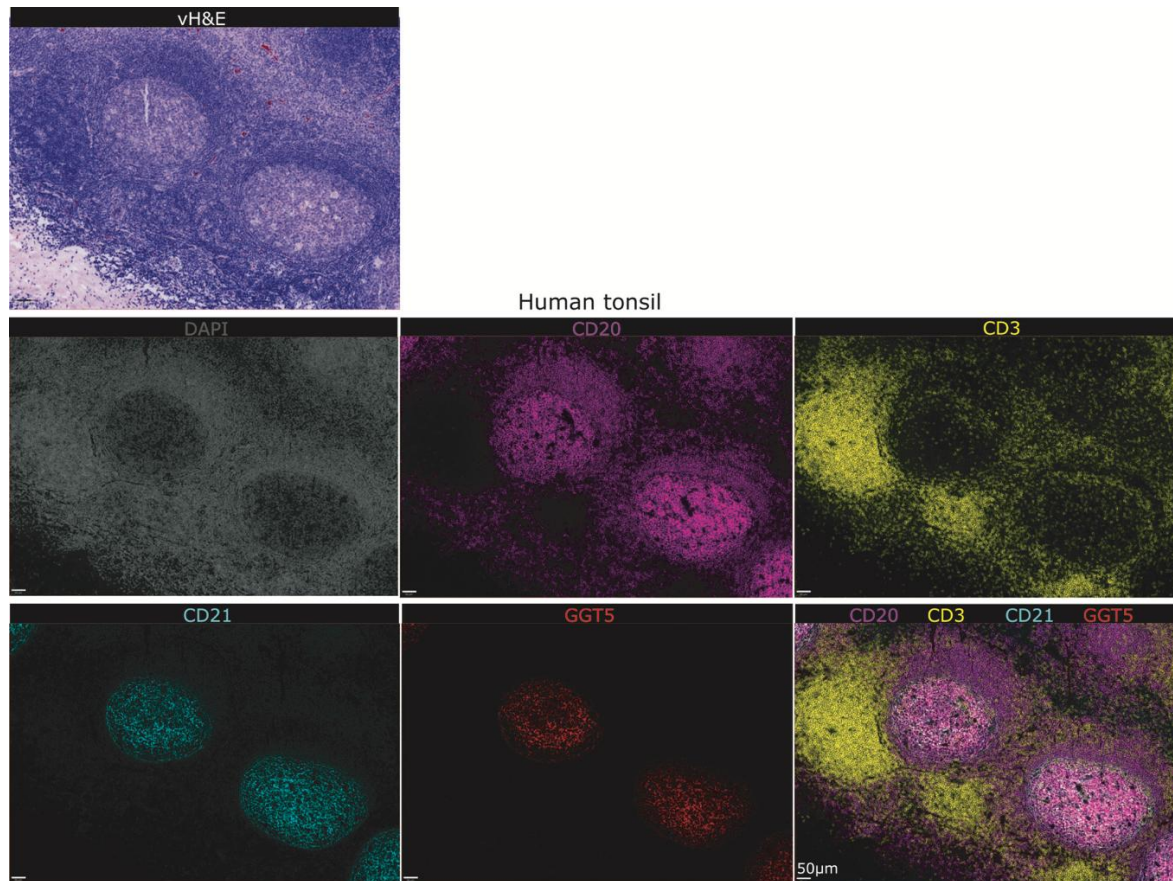
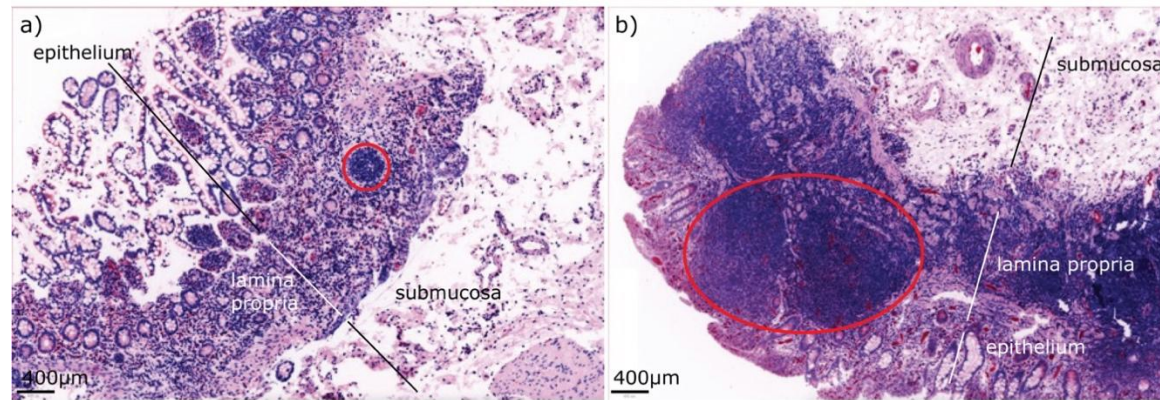


Figure 3.2: Spatial distribution of GGT5 in the germinal centres of human tonsil tissue. Representative image from one tonsil donor (n = 1). CD20 marks B cell zones and germinal centres, CD3 marks T cell zones, and CD21 highlights the follicular dendritic cell (FDC) network. Scale bar = 50 μ m.

Inflammatory bowel diseases



Rheumatoid arthritis

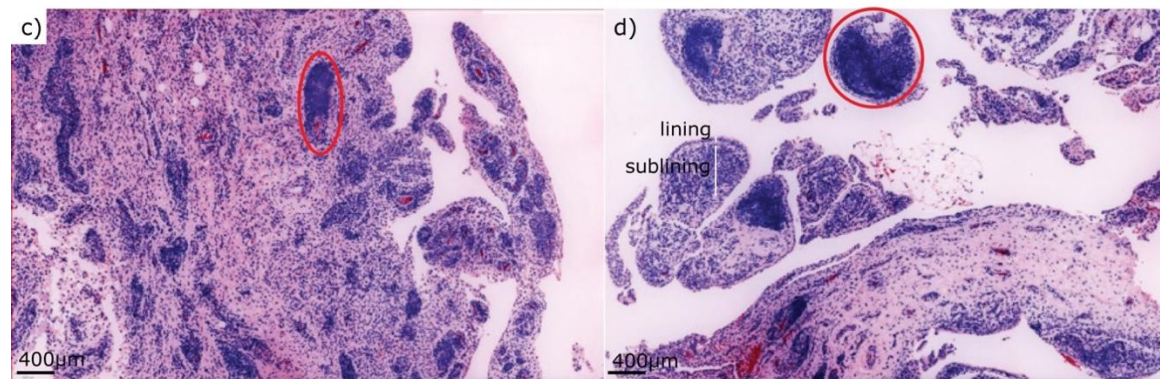


Figure 3.3: Lymphoid structures are present in IBD and RA and range from small cellular aggregates to more organised structures. In intestinal tissue, lymphoid structures are located within the lamina propria (a, b). In synovial tissue, lymphoid structures are observed within the sublining layer of the synovium (c, d). vH&E images from CellDIVE, representative images of n=1 for each tissue. Scale bar = 400 μ m.

As GGT5 is involved in confining B cells to GCs in the SLOs and lymphoid structures including LA and TLS are found in IMIDs, the next logical question is whether GGT5 expression is also found in tissues from IMIDs. Indeed, transcriptomics analysis performed using publicly available dataset shows that GGT5 is expressed in IBD and RA (Figure 3.4).

IMIDs are heterogenous in term of inflammatory infiltrates and histological features, and these patterns have recently been used to stratify diseases by pathotypes in IBD and RA dataset. Amongst these pathotypes, the presence of lymphoid infiltrates and lymphoid structures define specific subsets of patients. These stratified datasets are used to determine if GGT5 was enriched in lymphoid high pathotypes across IBD and RA datasets.

The IBD dataset was derived from Oxford IBD patient discovery cohort, where patients were under different treatment, including aminosaliclates, corticosteroids, anti-TNF and anti-integrin [263]. In the dataset, 'inflammatory fibroblastic/myeloid' is renamed from the M4 and M5 modules in the paper, which are associated with the presence of activated fibroblasts and granulocytes such as neutrophils. 'Lymphoid aggregates' are renamed from M6 module in the paper, showing its association with the presence of lymphoid aggregates in the tissue. There is no significant difference in GGT5 expression across different IBD pathotypes (Figure 3.4a).

The RA dataset was derived from patients in a large cohort of early treatment-naïve patients, namely, the Pathobiology of Early Arthritis Cohort (PEAC) [273]. The pathotypes are not renamed and thus are the same as the transcriptional subgroups in the paper, where there are 'fibroid' or fibroblastic pauci-immune pathotype, 'myeloid' or macrophage-rich diffuse-myeloid pathotype, and 'lymphoid' or lympho-myeloid pathotype that are characterised by lymphocyte and myeloid cell infiltration. There is no significant difference in GGT5 expression across different RA pathotypes (Figure 3.4b).

GGT5 expression shows no significant differences across the RA and IBD pathotypes. Although we initially anticipate higher expression in lymphoid pathotypes, this is not observed, prompting further investigation into the broader functional roles of GGT5. As a first step, we examine its spatial distribution in healthy versus inflamed tissues.

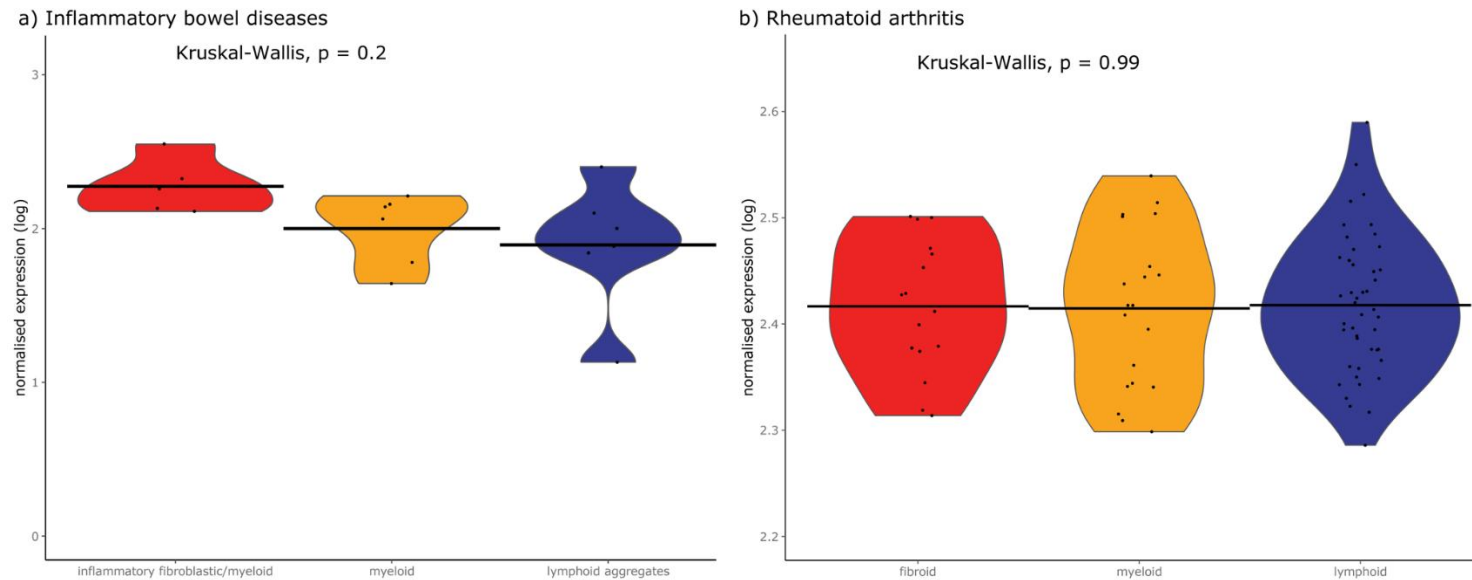


Figure 3.4: GGT5 is expressed in IBD and RA. (a) GGT5 expression in IBD is analysed using published dataset [263] from Oxford IBD patient discovery cohort, where 'inflammatory fibroblastic/myeloid' is M4 and M5 modules associated with presence of activated fibroblasts and neutrophils, and 'lymphoid aggregates' are M6 modules in the paper. (b) GGT5 expression in RA is also analysed using published dataset [273] from the treatment-naïve PEAC cohort.

To understand the spatial distribution of GGT5 in the gut and synovium, tissues are stained and imaged with CellDIVE multiplex imaging. In the healthy synovium (foetal finger), lymphocytes (CD3⁺ T cells, CD20⁺ B cells) are absent in the tissue and GGT5 staining is restricted around the CD31⁺ vessel (Figure 3.5c). In RA synovium, GGT5 staining is still found around the vessels, but GGT5⁺ regions have expanded alongside infiltrates of CD3⁺ and CD20⁺ lymphocytes which are found to cluster around CD31⁺ vessels (Figure 3.5d).

In the gut, CD3⁺ and CD20⁺ lymphocytes infiltrates are present in healthy and inflamed CD tissues (Figure 3.5a). As opposed to the synovium, GGT5 is expressed throughout the lamina propria in both healthy and inflamed gut and not restricted to perivascular region (Figure 3.5b).

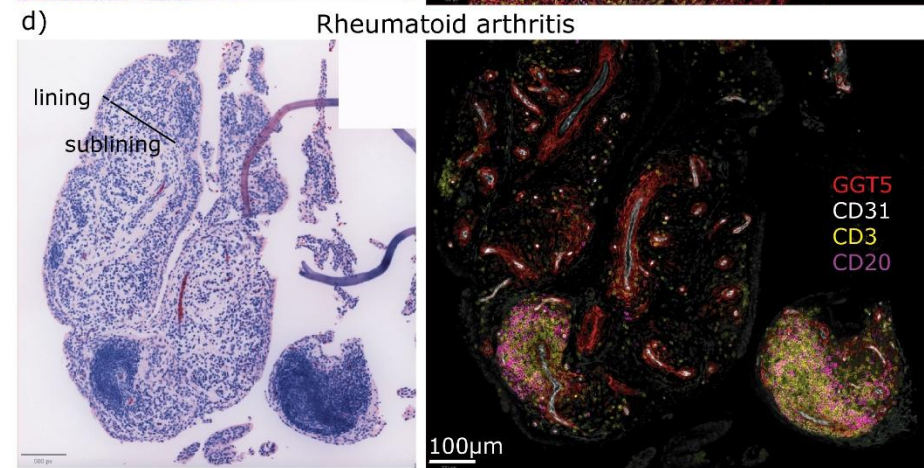
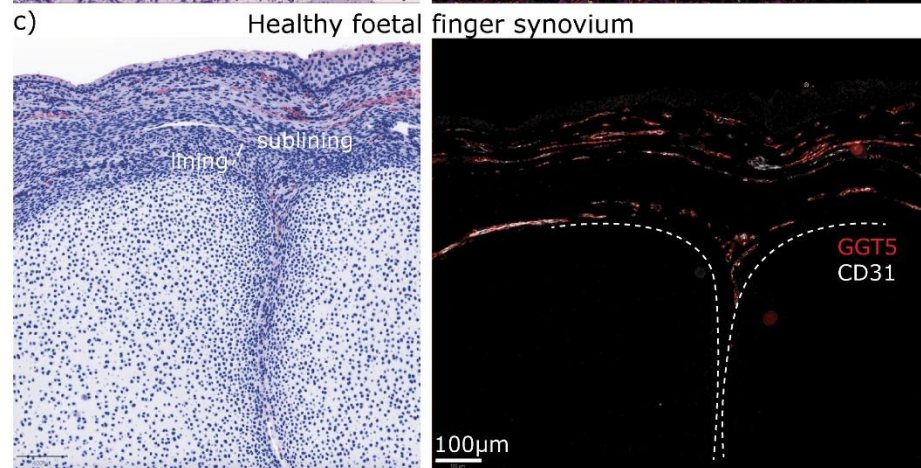
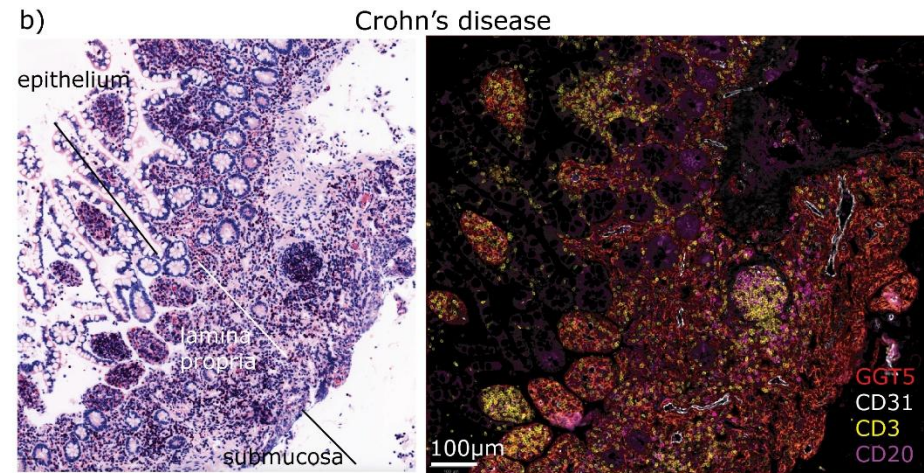
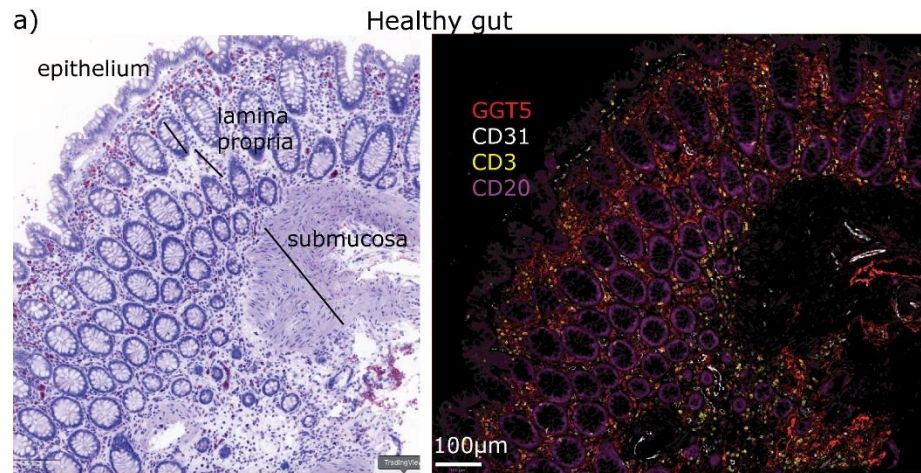


Figure 3.5: GGT5 expression in gut and synovium. vH&E and CellDIVE images showing GGT5 staining in (a) healthy gut and (b) Crohn's disease-affected gut. In gut sections, the epithelium, lamina propria, and submucosa are annotated. Tissues were sectioned in the transverse plane; therefore, intestinal layers do not appear as discrete strata but rather as villi containing goblet cells, with lamina propria located between epithelial structures. vH&E and CellDIVE images showing GGT5 staining in (c) healthy foetal finger synovium and (d) rheumatoid arthritis (RA) synovium, with synovial lining and sublining layers annotated. Images are representative of n = 1 donor per condition. Scale bar = 100 μ m.

Tissue	GGT5 ⁺ fibroblast abundance	Spatial relationship to leukocyte aggregates	Notes
Synovium (foetal)	Low	Lack infiltrating leukocytes	Restricted to the vascular area.
Synovium (RA)	Moderate	Often co-localise with leukocyte aggregates	GGT5 ⁺ regions extend from perivascular zones into areas of leukocyte infiltration.
Gut (normal adjacent)	High	Often co-localise with leukocyte aggregates	Diffuse leukocyte infiltration throughout the tissue; GGT5 ⁺ fibroblasts broadly distributed.
Gut (IBD)	High	Often co-localise with leukocyte aggregates	Marked leukocyte infiltration across the mucosa; widespread GGT5 ⁺ fibroblast signal.

Table 3.1: Qualitative summary of GGT5⁺ fibroblast density and proximity to leukocyte aggregates in human synovium and gut.

GGT5 expression throughout the healthy gut but only localised around blood vessels in the healthy finger synovium could be link to the presence of immune cells in healthy intestinal tissue which is linked to their tolerogenic function towards the intestinal microbiota. The intestinal immune system is mainly composed of innate cells before birth while the recruitment of T and B cells co-occurs with microbial colonisation after birth [263]. Publicly available transcriptomic data of foetal gut prior to adaptive immune cells infiltration is used to compare GGT5 expression in the intestine with the adult one.

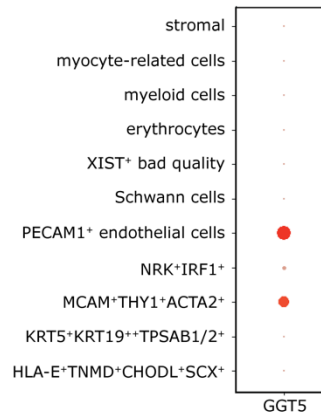
The single-cell transcriptomes of foetal gut are derived from 6-11 weeks post-conception developing human gut [274], and the data shows that GGT5 is mainly expressed by venous endothelial cells and lymphatic endothelial cells in the foetal gut (Figure 3.6a). In IBD affected gastrointestinal tract, GGT5 is expressed by stromal cells including FDC, oesophagus fibroblast, pericyte and rectum fibroblast (Figure 3.6b).

The unpublished single-cell transcriptomes of foetal finger synovium are derived from 8-14 weeks post-conception developing human finger, and the data shows that GGT5 is mainly expressed by PECAM1⁺ endothelial cells and MCAM⁺THY1⁺ACTA2⁺ contractile cells (Figure 3.7a). In RA affected synovium, GGT5 is mainly expressed by NOTCH3⁺ sublining fibroblast. It is also found in some other stromal cells including CD74-hi sublining fibroblast, mural cells,

CXCL12⁺SFRP1⁺ sublining fibroblast and lymphatic cells but to a lesser extent compared to NOTCH3⁺ sublining fibroblast (Figure 3.7b).

Figure 3.6: GGT5 expression in foetal gut vs IBD affected gut. (a) Dotplot showing GGT5 expression in foetal gut is analysed using published foetal dataset from Gut Cell Atlas [274]. (b) Dotplot showing GGT5 expression in IBD affected gut is analysed using data from pan-GI Cell Atlas from Gut Cell Atlas [275], but only including CD and UC data.

a) Foetal finger synovium



b) RA synovium

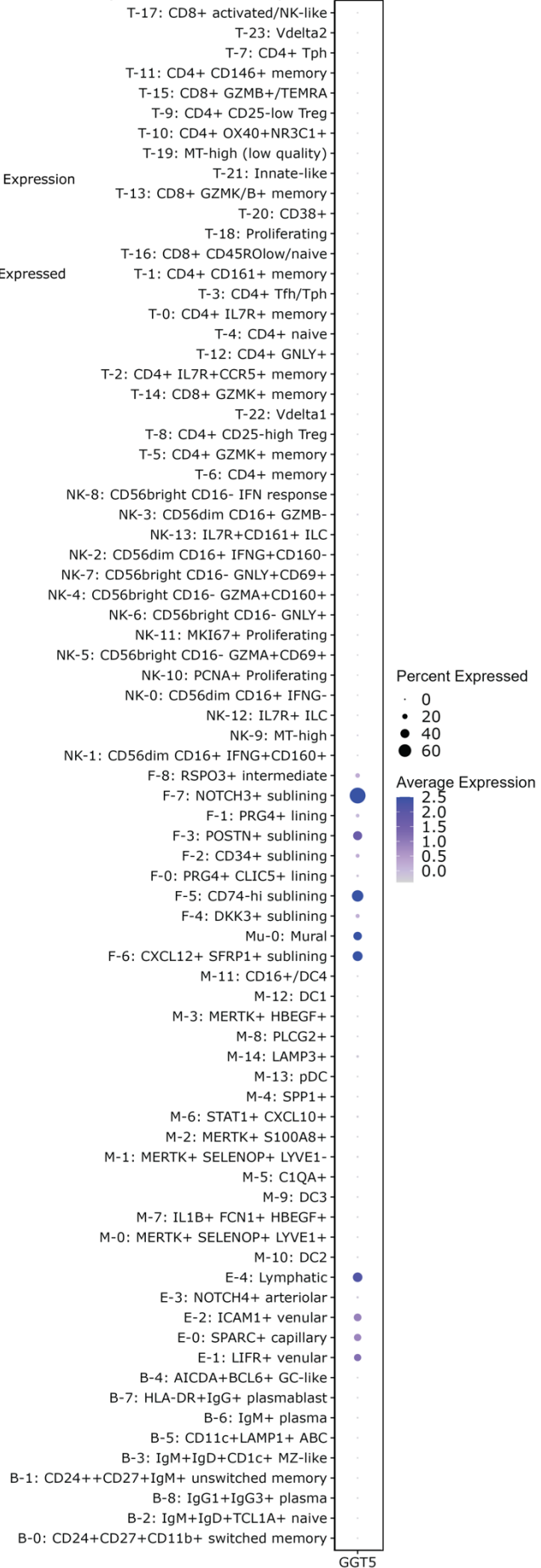


Figure 3.7: GGT5 expression in foetal finger synovium vs RA affected synovium. (a) Dotplot showing GGT5 expression in healthy foetal finger synovium is analysed by Sarah Davidson (Coles/Buckley group) using unpublished data. (b) Dotplot showing GGT5 expression in RA affected synovium is analysed using published dataset [276].

3.3 Discussion

As leukocytes infiltrate inflamed tissues, they typically adopt one of three organisational patterns. These include diffuse interstitial distribution, perivascular clustering, and formation of more complex TLS (section 1.4). While these patterns have been described morphologically, the mechanisms governing their spatial compartmentalisation remain incompletely understood. It has been proposed that infiltrating leukocytes initially disperse randomly within inflamed tissue before gradually accumulating in perivascular regions, and in some cases, developing into TLS [68, 277]. Within this framework, the breakdown of the inhibitory metabolite GGG by the enzyme GGT5 may facilitate leukocyte motility, reducing immobilising signals and thereby enabling their redistribution within the tissue. To test this hypothesis, GGT5 expression was examined across intestinal and synovial tissues, which provide distinct immunological contexts.

Although this model provides a coherent conceptual link between GGT5 activity and leukocyte reorganisation, it remains unclear whether GGT5 is a driver of these patterns or simply a correlate of broader stromal activation. Without direct measurements of GGG concentrations or gradients *in situ*, the inferred relationship between enzyme expression and leukocyte movement remains uncertain. This uncertainty emphasises the need to treat GGT5 not as an isolated determinant but as one component of a complex, multi-layered system governing tissue inflammation.

The gut and synovium represent particularly informative sites for comparison. In health, the synovium is largely immune-privileged, whereas the intestine requires a dense, homeostatic leukocyte network to maintain tolerance to the microbiota. This baseline difference in immune architecture provides a useful contrast to study how GGT5 expression adapts to different tissue needs. Unlike SLOs, such as tonsils, where GGT5 expression is primarily associated with GCs, we observed that in both the gut and synovium, GGT5 is expressed outside of GCs and is frequently localised to stromal compartments, including fibroblasts. This distribution raises the possibility that GGT5's role extends beyond GC organisation to influencing leukocyte positioning in more general tissue niches.

The presence of GGT5 in stromal compartments is particularly important because stromal cells increasingly appear central to inflammatory activity, acting as metabolic, chemotactic, and mechanical organisers. By situating GGT5 within these stromal networks, the data challenge GC-centric interpretations and instead suggest that the enzyme may contribute to a broader stromal programme that defines how permissive or restrictive tissue microenvironments become during inflammation. This interpretation also exposes a potential gap in existing models, which often treat leukocyte organisation as primarily chemokine-driven without integrating metabolic regulation as an equally relevant dimension.

If GGT5 functions predominantly to modulate GGG gradients within highly structured lymphoid niches, it would be expected to be enriched in pathotypes characterised by organised lymphoid aggregates. However, GGT5 expression did not differ significantly across pathotypes in either IBD or RA. This suggests that its role is not confined to supporting TLS or GC architecture but may instead be broadly involved in shaping leukocyte behaviour in inflamed tissues. This is consistent with emerging evidence that stromal cells dynamically regulate leukocyte positioning through metabolic and chemotactic gradients, particularly during chronic inflammation.

The absence of pathotype-specific expression also challenges assumptions that metabolite-modulating enzymes necessarily track with increasing tissue organisation. Instead, the data suggest that GGT5 activity may respond to more fundamental inflammatory cues that are shared across diverse histological patterns. This raises the possibility that metabolic permissiveness for leukocyte movement is a core feature of inflamed tissue, irrespective of whether that tissue progresses toward TLS formation. Disentangling these shared drivers from pathotype-specific processes will be essential for determining which elements of leukocyte organisation are truly dependent on metabolic modulation.

Interestingly, in healthy foetal synovium, GGT5 expression is restricted to vascular rings, whereas in RA, its expression expands into the surrounding region. By contrast, in the gut, GGT5 expression is

widespread under both steady-state and inflamed conditions. This pattern suggests a context-dependent regulation of GGT5, closely associated with leukocyte presence. In the foetal gut, which has minimal microbial colonisation, GGT5 expression is mainly confined to venous and lymphatic endothelial cells, resembling its vascular localisation in foetal synovium. This supports the idea that GGT5 is linked more closely to leukocyte entry and surveillance than to microbial signals per se.

The developmental comparison also highlights a potential temporal dimension to GGT5 regulation. In both foetal tissues, expression patterns appear to mark anatomical gateways for leukocyte entry rather than broader tissue territories. With inflammation in adulthood, the expansion of GGT5 into the interstitium may reflect a shift from a surveillance-oriented system to one adapted for sustained leukocyte entry. This transition underscores that GGT5 expression cannot be interpreted simply as a static feature of tissue type. Instead, it is dynamically shaped by the balance between immune quiescence and inflammatory activity.

This interpretation aligns with previous findings that CD8⁺ T cells actively patrol the endothelium to survey for cognate antigen [278]. In homeostasis, the vascular ring of GGT5 may function to degrade GGG locally, creating permissive zones for leukocyte patrolling across endothelial barriers. In RA, the expansion of the GGT5⁺ domain into the perivascular space may reflect an adaptive response to increased

leukocyte influx, potentially facilitating the formation of perivascular cuffs. Whether this perivascular clustering is protective (serving to spatially contain inflammation) or pathogenic (by sustaining inflammatory foci) remains an open question and warrants functional investigation.

A key limitation in interpreting GGT5 activity is the inability to directly visualise its substrate, GGG. As a glutathione-derived metabolite, GGG detection requires mass spectrometry, and while imaging mass spectrometry has emerged as a promising tool, spatial mapping of GGG within tissue remains unachieved. Previous attempts using matrix-assisted laser desorption ionisation mass spectrometry imaging (MALDI-MSI) have been unsuccessful. Consequently, GGT5 expression currently serves as a surrogate marker, with its presence indicating regions of likely GGG degradation and thus reduced inhibitory signalling.

This reliance on surrogate markers imposes a significant interpretative bottleneck. Without spatial metabolomics, core assumptions about GGG gradients, such as whether they exist at meaningful spatial scales or correlate with leukocyte compartmentalisation, remain untested. This limitation underscores that current interpretations hinge on inferred rather than measured biochemical landscapes. Bridging this gap will require not only improved imaging technologies but also experimental systems capable of perturbing GGG directly to determine its causal role.

In the context of the inhibitory gradient, it is therefore instructive to compare GGT5 to other better-studied regulators of leukocyte positioning. For instance, S1P gradients between tissue and circulation regulate lymphocyte trafficking, and elevated S1P levels in RA synovium have been associated with abnormal lymphocyte retention within inflamed joints. [279]. Adenosine, a metabolite generated from extracellular ATP, acts as a potent immunomodulator, by limiting T cell activation and altering macrophage and fibroblast behaviour [280, 281]. These metabolites offer retention or immobilisation cues at the biochemical level so as to restrict the movement of immune cells forming the inflammatory niche. On the other hand, a degradation of GGG through GGT5 means lifting such immobilising constraints and allowing the formation of permissive niches for immune cells to redistribute. This brings to the forefront questions: Is GGT5 activity opposing the retention effects of molecules such as S1P or adenosine? Or do the retention pathways work in parallel to precisely fine-tune leukocyte positioning? The extent to which these gradients act in an integrated, synergistic, or competitive manner remains unknown, and functional studies will be needed to clarify these interactions.

Comparative analysis with these pathways highlights that metabolic control of leukocyte behaviour is unlikely to be governed by a single dominant gradient. Instead, tissues probably integrate multiple biochemical signals with opposing effects on retention and migration. GGT5-mediated GGG degradation may shift this balance

only modestly, and its effect size could depend heavily on the presence or absence of stronger cues such as S1P. Recognising this interdependency is critical because it suggests that the functional impact of manipulating GGT5 could vary dramatically across tissues or disease states, depending on the metabolic milieu into which the intervention is introduced.

From the translational perspective, GGT5 activity manipulation could offer a strategy for controlling leukocyte dynamics in inflamed tissues. Inhibition of GGT5 could stabilise GGG gradients, strengthening leukocyte retention at the site while suppressing further infiltration. The inverse would promote dispersal or clearance of immune cells from inflammatory foci. Taken together, these observations establish where and under which inflammatory contexts GGT5 is expressed, but do not resolve the upstream cellular or molecular signals responsible for its induction, which are addressed in the following chapter.

Chapter 4: Investigating the signal(s) inducing GGT5 expression in fibroblasts

4.1 Introduction

GGT5 expression appears to follow leukocytes infiltrates in the tissue, suggesting that inflammatory signals may induce GGT5 expression to accommodate leukocyte migration within the tissues. Given that the gut is a reservoir of immune cells even during homeostasis, leukocytes and consequently GGT5 expression are widely found in the gut. Thus, it can be challenging to determine the specific factors inducing GGT5 expression in a tissue with high baseline level. On the contrary, healthy synovial GGT5 expression is limited to the vasculature but expands in RA, offering a model to study how factors are required to upregulate GGT5 expression in this tissue. Identifying the specific signal that induces GGT5 in fibroblast from a vast array of possibilities is challenging. In SLOs, GGT5 expression is limited to GCs suggesting that this niche may contain some potential clues. Considering that tissue inflammation enhances GGT5 expression and cytokines play a key role in driving inflammation, cytokines involved in both GC formation and inflammation can be likely candidates for stimulating GGT5 expression in fibroblast.

A previous study indicates that type 1 TNF receptor is crucial in GC formation [77]. TNF is also a critical signal in driving TLS formation [282-284]. One study demonstrates that overexpressing TNF in a

murine ileitis model using $Tnf^{\Delta ARE}$ mice promotes TLS formation [111-113]. It is hypothesised that GGT5 also plays a significant role in GC development during TLS formation. Furthermore, anti-TNF is the most commonly used biologic treatment in IMIDs including IBD and RA [267], highlighting the important role of TNF in driving both GC formation and inflammation. This raises the question of whether TNF induces GGT5 expression in fibroblasts.

This chapter aims to answer the key question that which factor(s) are responsible for regulating GGT5 expression in fibroblast.

4.2 Results

Anti-TNF therapies are widely used in clinic to treat both IBD and RA patients but only a fraction of patients respond to this therapy. Therefore, several publicly available datasets attempt to identify gene markers predicting response to anti-TNF therapies for both IBD and RA patients are available. These datasets provide an opportunity to study whether TNF is linked to GGT5 expression. Whole-tissue microarray dataset is utilised to study the effect of TNF on GGT5 expression [249]. In total, there were 61 IBD patients (24 UC and 37 CD) and 12 control patients. Endoscopic biopsies were taken before and after intravenous infusion of 5 mg infliximab per kg body weight. For CD, patients' response to infliximab was defined as complete mucosal healing with a decrease of at least 3 points on the histological score [285]. For UC, patients with a decrease to Mayo endoscopic subscore of 0 or 1

together with a decrease to grade 0 or 1 on the histological score were considered responders [286, 287]. Patients who did not achieve this healing were classified as non-responders.

Before infliximab treatment, GGT5 expression is significantly higher than the control group, but the expression significantly decreases after treatment to similar level as control (Figure 4.1a). Pre-infliximab non-responders have a significantly higher GGT5 expression than pre-infliximab responders and control group, but after treatment, GGT5 expression is not significantly different in responders as compared to non-responders. In both responders and non-responders, GGT5 expression is significantly reduced after infliximab infusion suggesting that blocking TNF directly impacts GGT5 expression in the tissue (Figure 4.1b).

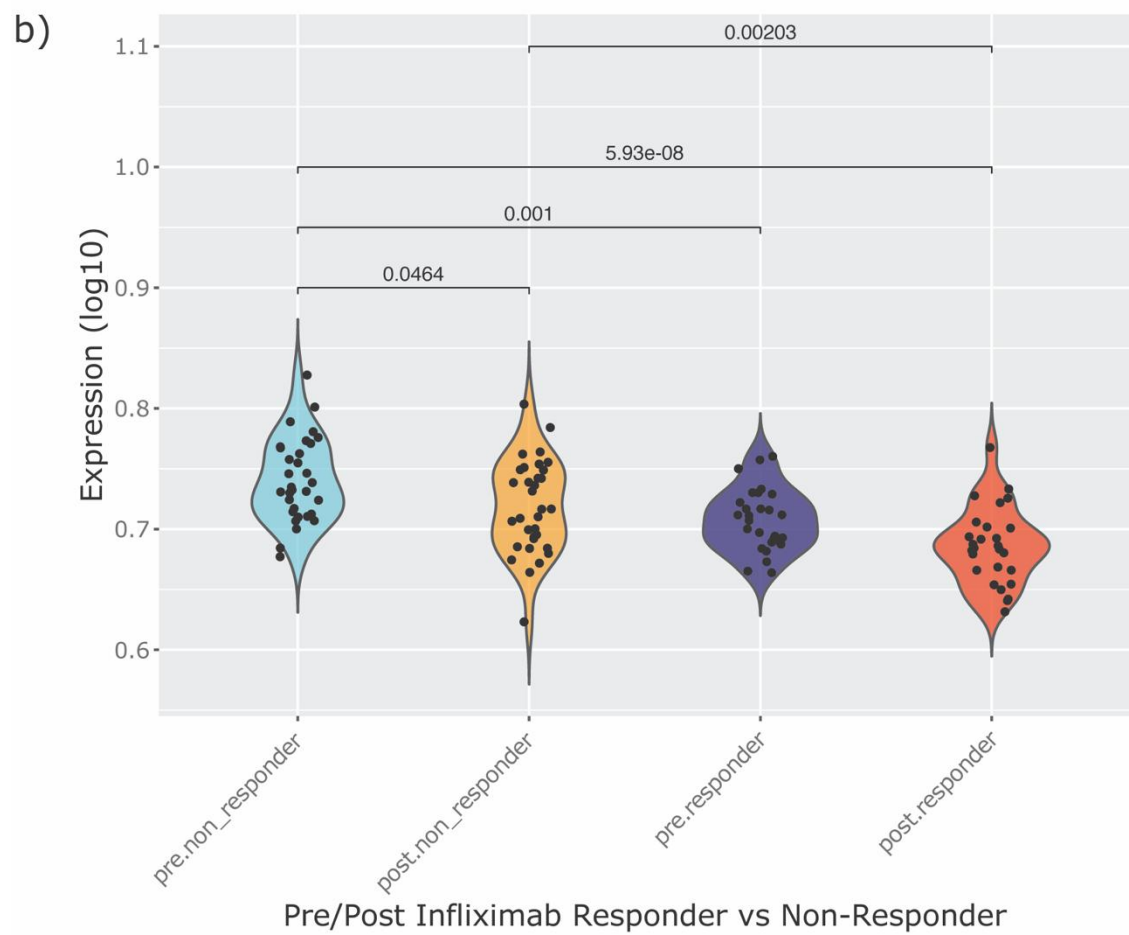
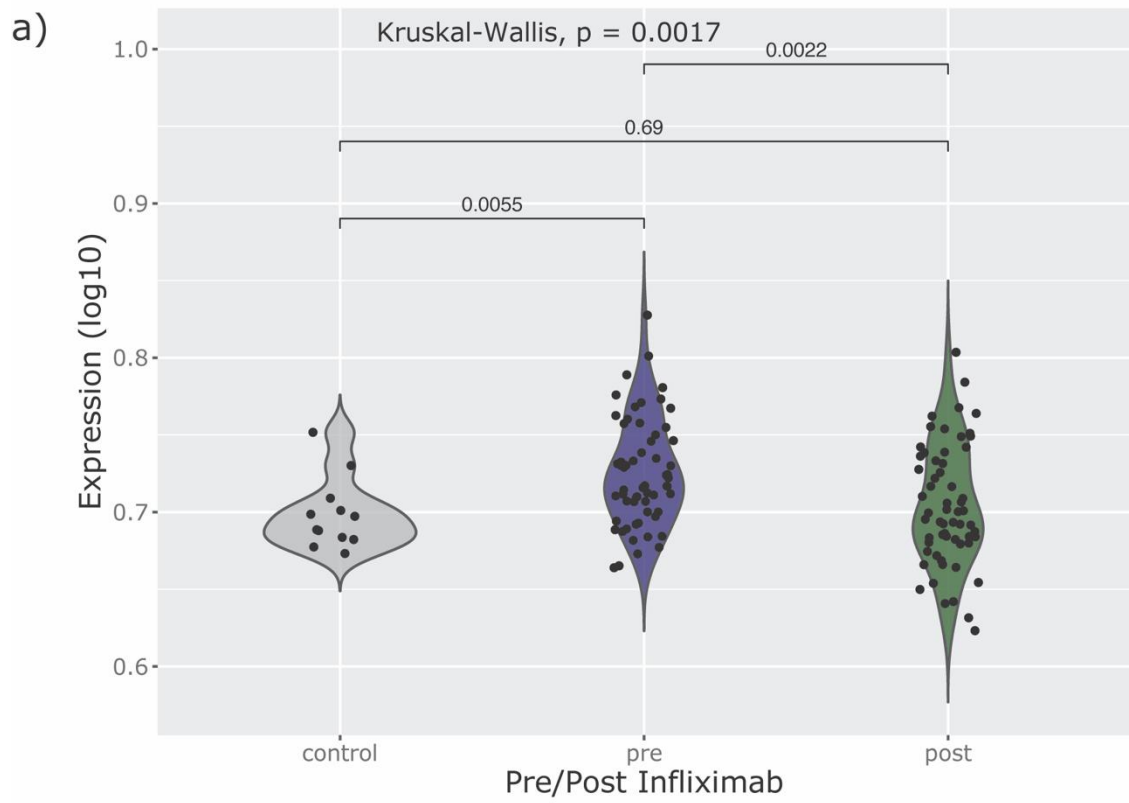


Figure 4.1: GGT5 expression before and after anti-TNF treatment. (a) GGT5 expression pre- and post-infliximab treatment. Statistical analysis was performed using the Kruskal–Wallis test, followed by Dunn’s post-hoc test for multiple comparisons. (b) GGT5 expression before and after infliximab treatment, in responders and non-responders. Statistical analysis was performed using two-way ANOVA to assess the effects of timepoint and treatment response on GGT5. Unadjusted p-values shown for GGT5 expression. The transcriptomic analysis was performed done using publicly available whole tissue microarray data [288]. pre: pre-anti-TNF; post: post-anti-TNF.

To validate the effect of TNF and test if other inflammatory cytokines could induce GGT5 expression, fibroblasts are stimulated with recombinant $\text{TNF}\alpha$, $\text{IL-1}\beta$ and $\text{IFN}\gamma$, as these cytokines are often upregulated in IBD and RA [16, 39]. In this project, primary adult gut-derived fibroblasts are used as a model for intestinal fibroblasts, whereas healthy primary foetal proximal interphalangeal (PIP) fibroblasts are used as a model for synovial fibroblasts. Primary fibroblasts were isolated from the foetal PIP joints due to limited access to healthy adult joint tissue, as such tissue is typically not available from healthy donors for ethical and practical reasons.

Although not statistically significant, intestinal fibroblasts have higher basal GGT5 expression than PIP fibroblasts (Figure 4.2a). Before cytokine stimulation, PIP fibroblasts were treated with TNF dose response to determine the optimal dose for GGT5 stimulation, and GGT5 expression seemed to be ceiling at 1 ng/mL (Figure 4.2b), although the effect was not statistically significant. Thus, 1 ng/mL of recombinant TNF was used to stimulate fibroblasts in subsequent experiments.

In intestinal fibroblasts, $\text{TNF}\alpha$, $\text{IL-1}\beta$ and $\text{IFN}\gamma$ do not significantly induce GGT5 expression (Figure 4.2a) after 24 hours of stimulation. However, $\text{TNF}\alpha$ significantly induces GGT5 in PIP fibroblasts, less so with $\text{IL-1}\beta$ and $\text{IFN}\gamma$ (Figure 4.2b).

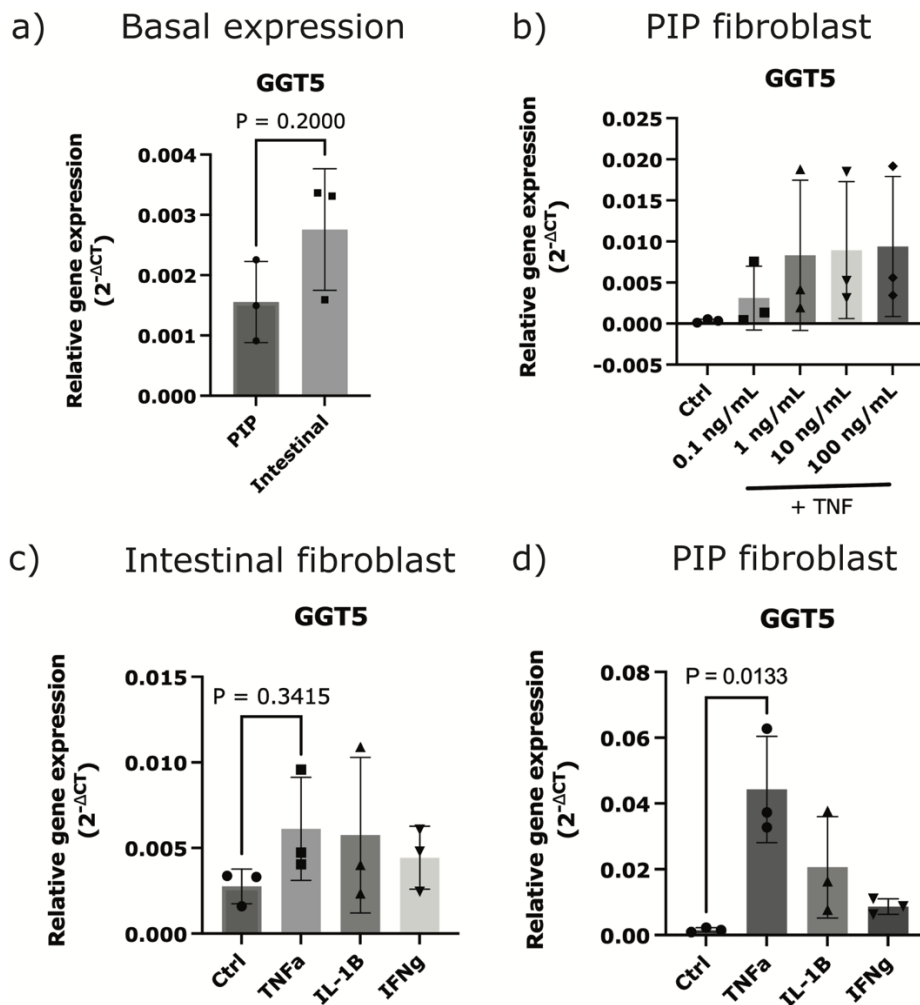


Figure 4.2: Relative GGT5 expression in intestinal and PIP fibroblast after cytokine stimulation. (a) GGT5 expression at baseline pre-stimulation. (b) GGT5 expression in PIP fibroblast after TNF dose response treatment at 0.1 ng/mL, 1 ng/mL, 10 ng/mL and 100 ng/mL. GGT5 expression in (c) intestinal and (d) PIP fibroblasts after 1 ng/mL TNF α , 20 ng/mL IL-1 β and 10 ng/mL IFN γ stimulation for 24 hours. Relative gene expression is displayed as $2^{-\Delta CT}$. Graphs are plotted as means $\pm$ SD, each with n=3 biological replicates; statistical significance was tested using non-parametric Friedman test and Dunn's multiple comparison test.

As TNF selectively stimulates GGT5 expression in synovial fibroblasts but not in intestinal fibroblasts, it suggests the involvement of other more universal factors, or that the expression of GGT5 is already at its highest in the gut. Therefore, there is a need to explore additional signals that may induce GGT5 expression and be shared across different tissue environments.

Data from the cross-tissue single-cell stromal atlas [249] shows that GGT5 is highly expressed in mural, fibroblast and endothelial cell populations, while showing lower expression in glial, epithelial and other leukocyte populations (Figure 4.3a). This aligns with the observation presented in Figure 3.6b and Figure 3.7b.

In the gut affected by CD, GGT5 staining is observed throughout the tissue. Blood vessels are identified by CD31 staining, lymphatic vessels by PDPN staining, and non-leukocytes by vimentin staining (Figure 4.3b). GGT5 co-stains with some but not all vimentin signals, and it is not found in areas stained by CD31 and PDPN.

In the synovium affected by RA, GGT5 expression is localised to the perivascular areas. Blood vessels are marked by CD31 staining, and pericytes are identified by CD146 staining. However, GGT5 does not co-localise with either CD31 or CD146, suggesting that its expression is specific to fibroblasts (Figure 4.3c). Comparing IBD and RA, GGT5 staining is predominantly restricted to the perivascular regions in the synovium, whereas it is more widely distributed in the gut. A limitation of the staining approach is that CD146 may also be

expressed by endothelial cells, while CD31 is not uniformly expressed across all endothelial subsets. Although VWF is a more specific endothelial marker, it does not bleach efficiently with the CellDIVE platform, and therefore CD31 was used instead.

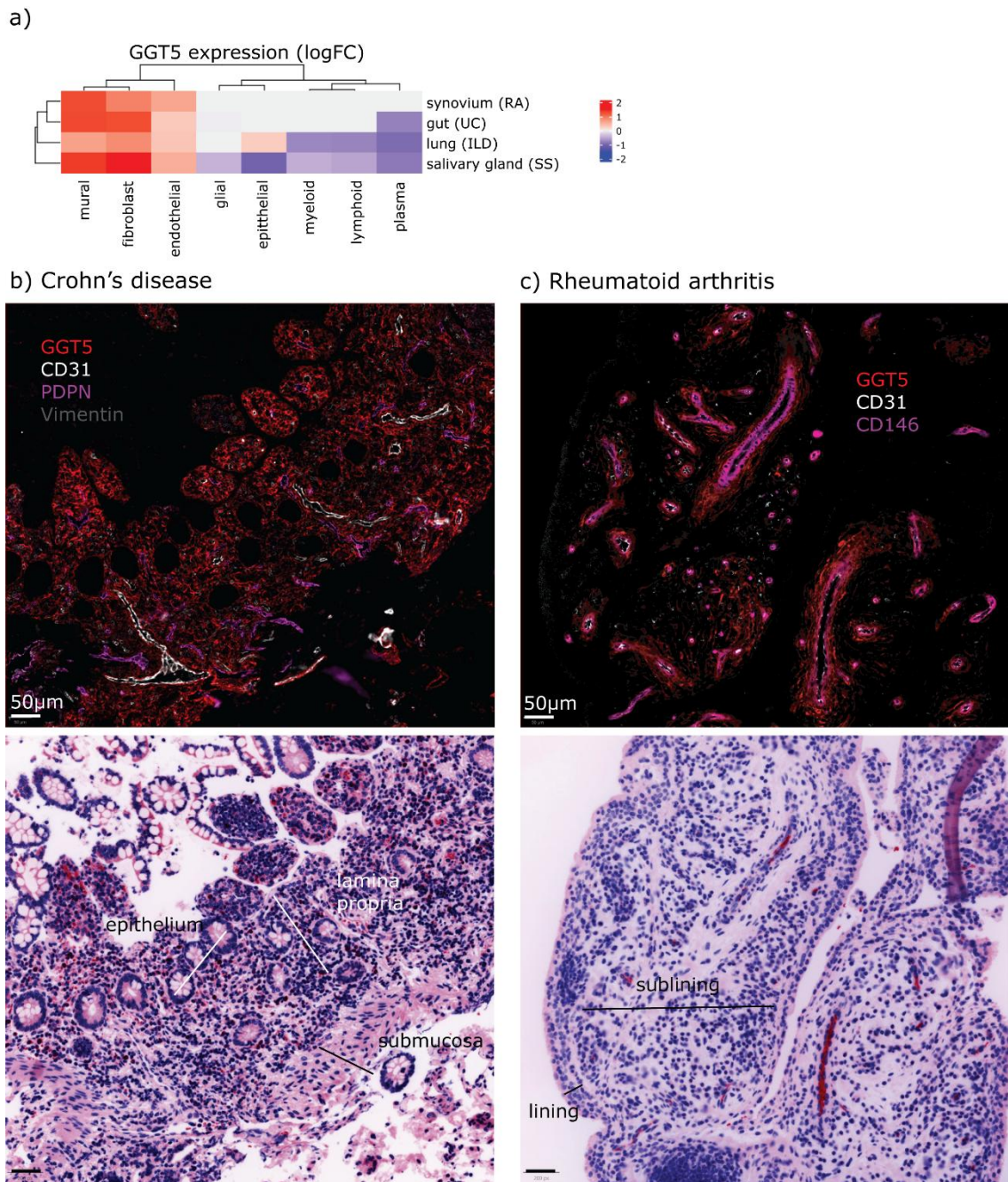


Figure 4.3: GGT5 is expressed in endothelial, mural and fibroblast populations during inflammation. (a) Cross-tissue single-cell stromal atlas shows that GGT5 is highly expressed in endothelial, mural and fibroblast populations across RA, IBD, ILD and SS (Sjögren's syndrome). Data kindly analysed from publicly available data [249] by Ilya Korsunsky. CellDIVE images showing GGT5 staining in (b) CD and

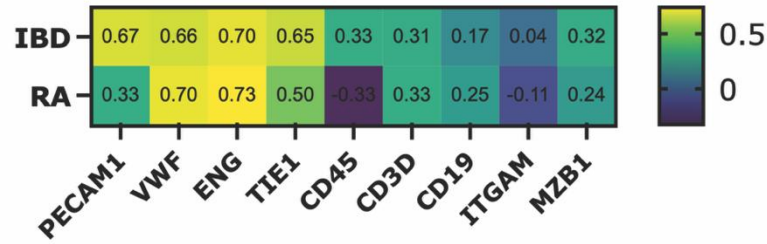
(c) RA affected tissues, together with other vascular and stromal markers, and vH&E showing tissue architecture at the bottom panel. In gut section vH&E, the epithelium, lamina propria, and submucosa are annotated. Tissues were sectioned in the transverse plane; therefore, intestinal layers do not appear as discrete strata but rather as villi containing goblet cells, with lamina propria located between epithelial structures, and a small part of submucosa with less nuclei than lamina propria. In synovium vH&E, lining and sublining layers were also annotated. n=1 donor for each condition. Scale bar = 50 μ m.

To investigate potential signals that might universally stimulate GGT5 expression, publicly available whole-tissue bulk RNA sequencing datasets [263, 273] are analysed using Pearson correlation.

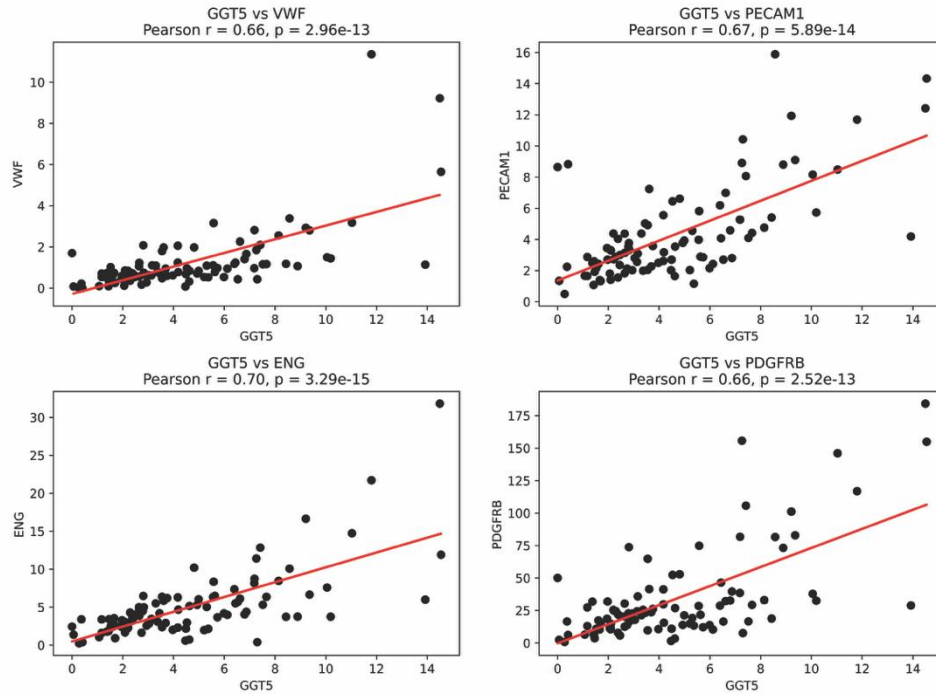
In IBD, vessel markers such as PECAM1 (or CD31), von Willebrand factor (VWF), endoglin (ENG) and tyrosine kinase with immunoglobulin like and EGF like domains 1 (TIE1) show a strong correlation with GGT5, with Pearson correlation coefficients higher than 0.65 (Figure 4.4a, b). PDGFR β also correlates with GGT5, with a coefficient of 0.66 (Figure 4.4b).

In RA, the correlation between PECAM1 and TIE1 with GGT5 is less pronounced compared to IBD. However, VWF and ENG maintain a very strong Pearson correlation, with coefficients higher than 0.7 (Figure 4.4a, c). In addition, GGT5 also shows a high correlation with other vessel-associated genes such as atypical chemokine receptor 1 (ACKR1) and TGFB1 (Figure 4.4c).

a) **Cross tissue GGT5 Pearson correlation**



b) **Inflammatory bowel diseases**



c) **Rheumatoid arthritis**

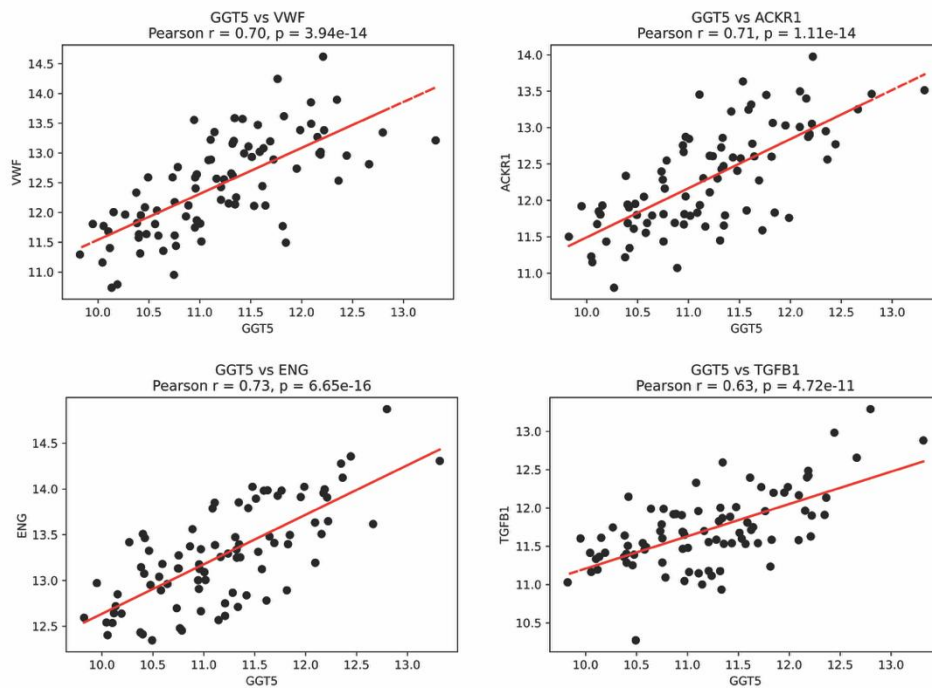
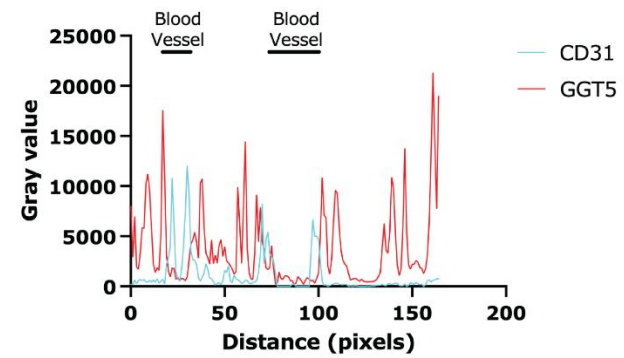
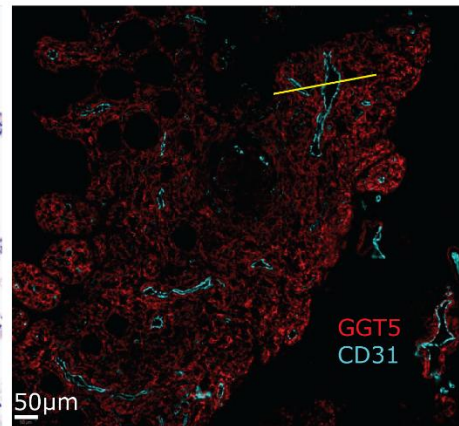
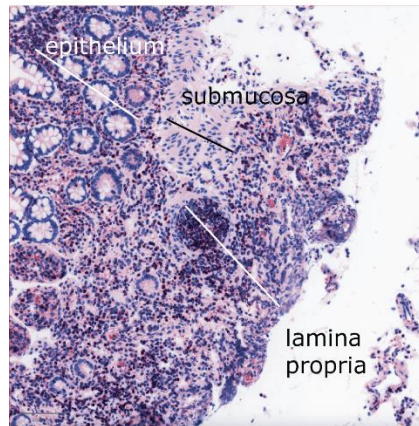


Figure 4.4: Pearson correlation of GGT5 in IBD and RA transcriptomic studies. (a) Correlation of GGT5 against vascular and leukocyte genes in IBD and RA. Examples of highly correlated individual vascular genes in (b) IBD and (c) RA. Data analysed from publicly available whole tissue bulk RNA sequencing data [263, 273].

Spatial analysis of GGT5 expression reveals the most intense staining in the perivascular space, particularly in RA, where blood vessels are marked by CD31 (Figure 4.5b). In IBD, the perivascular pattern is less distinct. However, there appears to be a higher density of blood vessels in the gut. Quantitative analysis will be needed to confirm this observation (Figure 4.5a). A line is drawn across the blood vessels (Figure 4.5 left panel), and the gray values of GGT5 and CD31 are measured using ImageJ (Figure 4.5 right panel). GGT5 staining is the strongest near the blood vessels and shows minimal overlap with CD31 staining, suggesting that GGT5 is either lowly expressed or not expressed by endothelial cells during inflammation.

a)

Inflammatory bowel diseases



b)

Rheumatoid arthritis

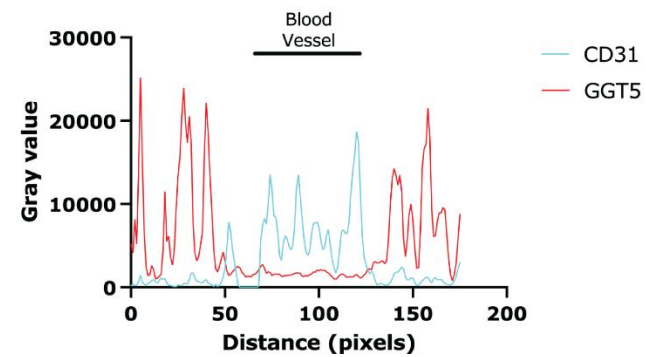
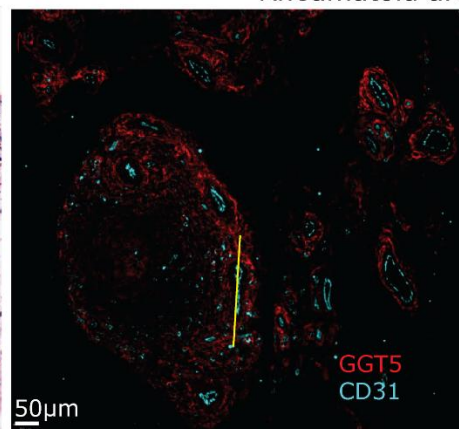
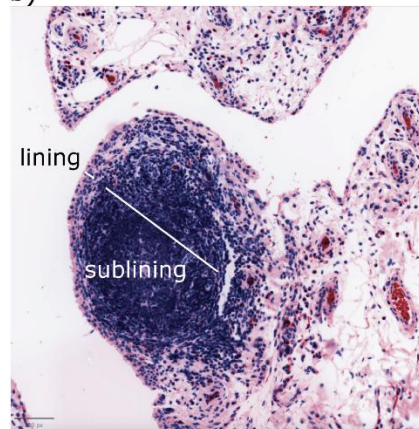


Figure 4.5: GGT5 is most intense around blood vessels in (a) IBD and (b) RA. Left panels show the vH&E, middle panels show the CellDIVE images staining for GGT5 and CD31, and the right panels show the gray values of both markers after a line is drawn across the tissue, as analysed in ImageJ. In gut section vH&E, the epithelium, lamina propria, and submucosa are annotated. Tissues were sectioned in the transverse plane; therefore, intestinal layers do not appear as discrete strata but rather as villi containing goblet cells, with lamina propria located between epithelial structures, and a small part of submucosa with less nuclei than lamina propria. In synovium section v&E, lining and sublining layers were also annotated. n=1 donor for each condition. Scale bar = 50 μ m.

As discussed in section 1.1.3, NOTCH3 signalling plays a crucial role in driving sublining fibroblast identity in the synovium, and it is associated with the vasculature [56]. Besides, GGT5 is mainly expressed by the sublining synovial fibroblast in RA (Figure 3.7b). Given this, NOTCH3 ligands may be responsible for regulating GGT5 expression in fibroblasts. To test this, NOTCH3 ligands JAG1 and DLL4 are coated on a plate, before fibroblasts are seeded on top.

JAG1 and DLL4 do not significantly induce GGT5, NOTCH3 and JAG1 expression in intestinal fibroblasts (Figure 4.6a). This may be due to an insufficient number of intestinal fibroblast donors to capture inter-donor variability or detect subtle effects. However, JAG1 significantly increases GGT5 expression by approximately three-fold in PIP fibroblasts, and it also increases the expressions of both NOTCH3 and JAG1, creating a positive feedback loop (Figure 4.6b).

Given that $\text{TNF}\alpha$ and NOTCH3 ligands stimulate GGT5 expression in PIP fibroblasts, the next question is whether these two signals synergise. When combined, the increase in GGT5 expression is primarily driven by $\text{TNF}\alpha$ (Figure 4.6c), indicating that $\text{TNF}\alpha$ is more potent than DLL4 in stimulating GGT5 expression, and that the two signals do not exhibit synergy.

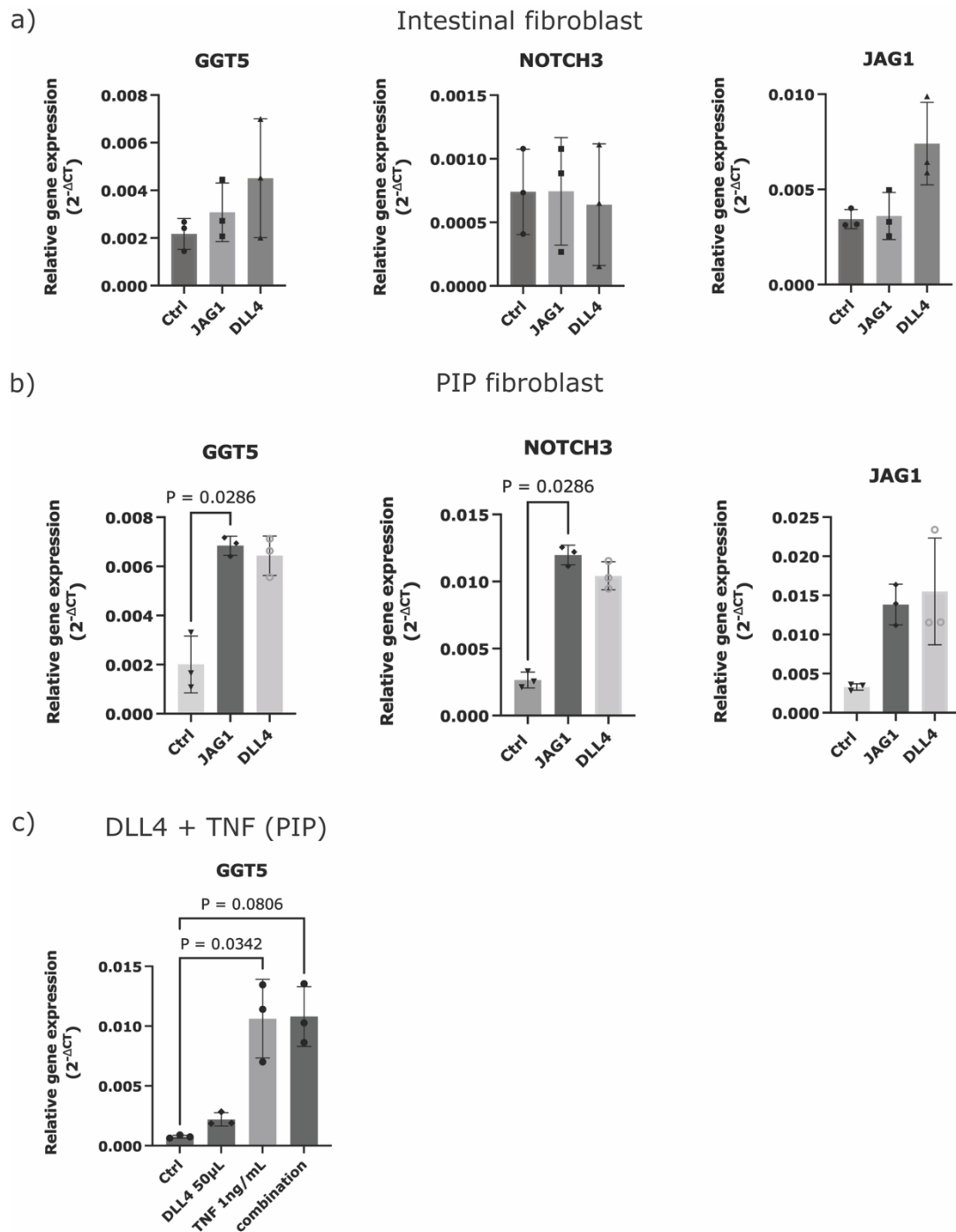


Figure 4.6: NOTCH3 ligands stimulation on intestinal and PIP fibroblasts. Relative GGT5, NOTCH3 and JAG1 expression after JAG1 and DLL4 stimulation in (a) IBD and (b) PIP fibroblasts. (c) Combination of $\text{TNF}\alpha$ and DLL4-coated Protein G Dynabeads treatment on PIP fibroblasts. Relative gene expression is displayed as $2^{-\Delta\text{CT}}$. Graphs are plotted as means $\pm$ SD, each with $n=3$ biological replicates;

statistical significance was tested using non-parametric Friedman test and Dunn's multiple comparison test.

As NOTCH3 ligands do not stimulate GGT5 in intestinal and PIP fibroblasts, there is a need to further understand the effect of vasculature on GGT5 expression.

HUVECs are used as a model for endothelial cells. Supernatant from steady state HUVECs significantly induces GGT5 expression in PIP fibroblasts, whereas supernatant from CD3⁺, CD19⁺, CD14⁺ cells and whole PBMCs do not (Figure 4.7a). The stimulatory effect of steady state HUVEC supernatant is significantly reduced by inhibitors of p38 and NFκB pathway (Figure 4.7b).

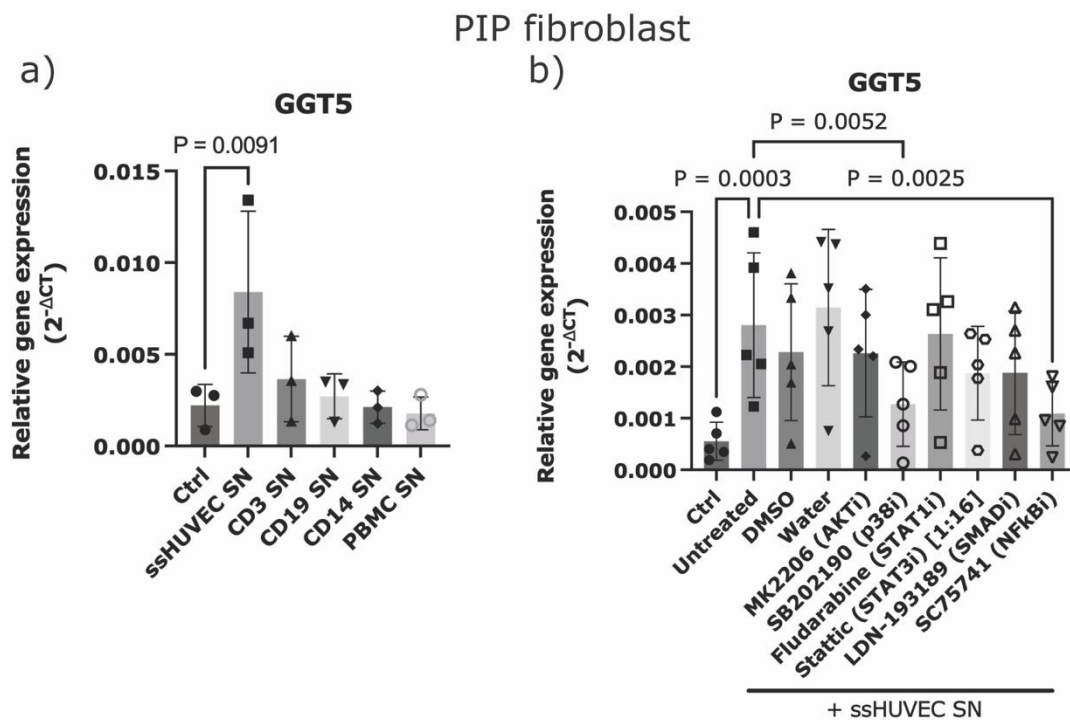


Figure 4.7: Relative GGT5 expression after stimulation from (a) steady state HUVECs, CD3⁺, CD19⁺, CD14⁺ cells and PBMC supernatant. (b) GGT5 expression after steady state HUVEC supernatant stimulation ± different signalling pathway inhibitors. Relative gene expression is

displayed as $2^{-\Delta\text{ct}}$. Graphs are plotted as means $\pm$ SD, each with n=3 or 5 biological replicates; statistical significance was tested using non-parametric Friedman test and Dunn's multiple comparison test.

Now that the effect of HUVEC supernatant on GGT5 expression has been established, the next question is whether there is a difference in GGT5 stimulation between supernatants from steady state and inflammatory signals activated endothelial cells. In these experiments, HUVECs were activated using $\text{TNF}\alpha$ and $\text{IFN}\gamma$.

In intestinal fibroblasts, activated HUVEC supernatant significantly increases GGT5 expression by approximately two-fold compared to the untreated control, with the effect being stronger than that of TNF or steady state HUVEC supernatant (Figure 4.8a). This stimulatory effect is significantly reduced by SC75741, which is a broad $\text{NF-}\kappa\text{B}$ inhibitor that impairs DNA binding of the subunit p65 [289] (Figure 4.8b).

In PIP fibroblasts, GGT5 expression is also significantly increased by approximately ten-fold following stimulation with activated HUVEC supernatant (Figure 4.8c). Similar to intestinal fibroblasts, the stimulatory effect of activated HUVEC supernatant is significantly decreased by SC75741 (Figure 4.8d). On a basal level without any stimulation, intestinal fibroblasts exhibit higher GGT5 expression than PIP fibroblasts, although the difference is not statistically significant (Figure 4.8e).

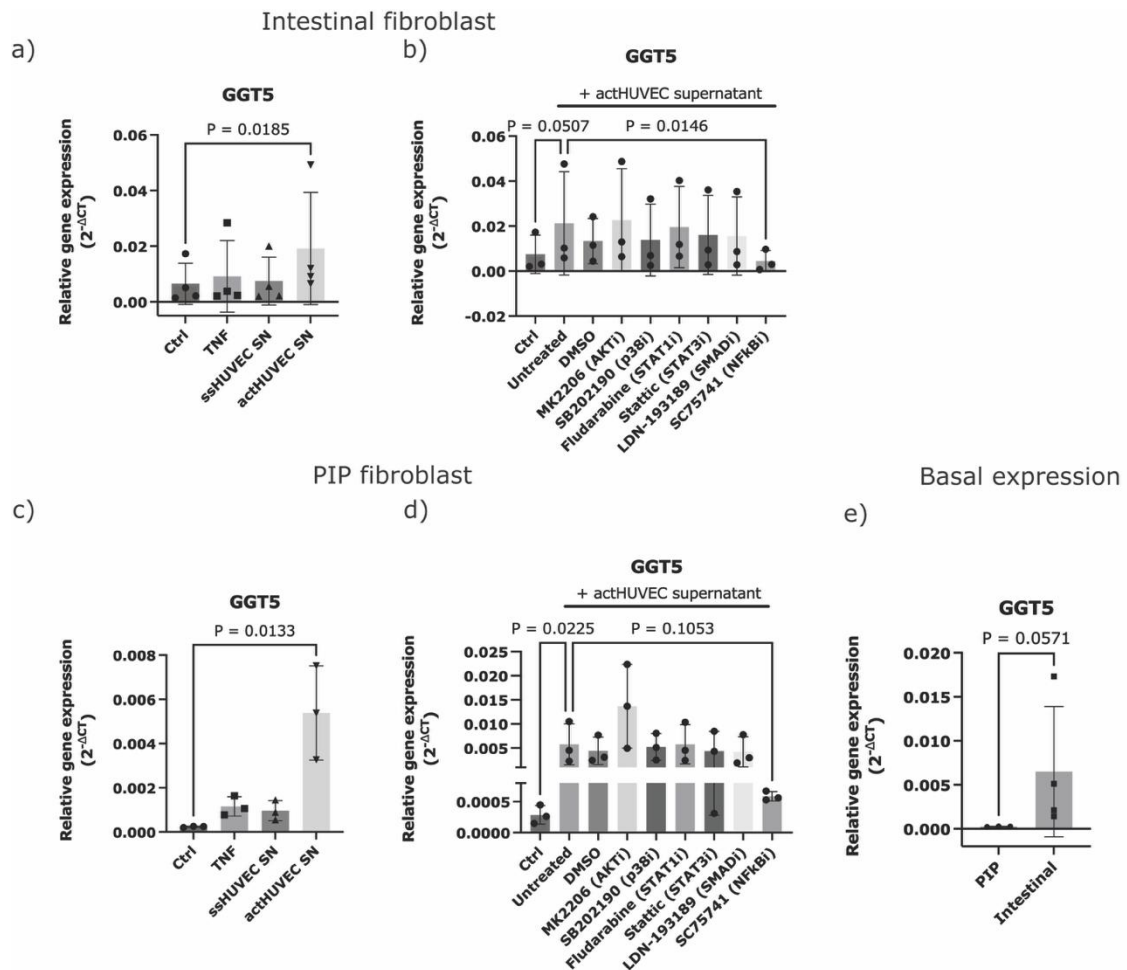


Figure 4.8: Relative GGT5 expression in (a) intestinal and (c) PIP fibroblasts after steady state and activated HUVEC supernatant stimulation. (b) Intestinal and (d) PIP fibroblasts are stimulated with activated HUVEC supernatant with or without different signalling pathway inhibitors. (e) Basal expression of GGT5 in intestinal and PIP fibroblasts. Relative gene expression is displayed as $2^{-\Delta C_t}$. Graphs are plotted as means $\pm$ SD, each with n=3 or 4 biological replicates; statistical significance was tested using non-parametric Friedman test and Dunn's multiple comparison test. Statistical significance for (e) basal expression was tested using non-parametric Mann-Whitney test.

4.3 Discussion

Building on the spatial and contextual patterns of GGT5 expression described in Chapter 3, this chapter focuses on identifying the upstream signals and cellular interactions that drive its induction within fibroblasts during inflammation. Under healthy or non-inflamed conditions, GGT5 expression is typically restricted to areas surrounding blood vessels. However, GGT5 expression expands into the neighbouring fibroblast compartment during IBD and RA. The main aim of this chapter is to determine the factors driving GGT5 upregulation in fibroblasts during inflammation. Importantly, this expansion may reflect multiple non-mutually exclusive mechanisms, including changes in fibroblast activation state, altered cell-cell proximity, or shifts in tissue composition during inflammation.

This shift in localisation raises an important conceptual issue: it remains unclear whether fibroblasts initiate this expansion autonomously in response to inflammatory cues, or whether the change is secondary to vascular or leukocyte-derived alterations within the inflamed niche. Distinguishing whether fibroblasts act as primary responders or as downstream amplifiers of endothelial signalling is crucial for interpreting causality in GGT5 regulation. A further complexity is that spatial expansion alone does not imply transcriptional induction within fibroblasts, as apparent increases could also arise from fibroblast proliferation or selective survival of GGT5⁺ subsets.

Fibroblasts are the most abundant cells in connective tissue, and they are relatively sessile compared to leukocytes. This puts them in a strategic position to regulate and influence leukocyte migration and compartmentalisation. Given that TNF is critical for GC formation in SLOs and that anti-TNF therapies are effective in treating both RA and IBD [290], it is hypothesised that TNF drives GGT5 expression in fibroblasts during inflammation. This is supported by the observation that GGT5 expression decreases following infliximab treatment, regardless of clinical response. However, reduced expression following treatment does not necessarily indicate direct TNF dependency, as anti-TNF therapy induces widespread changes in immune cell infiltration, stromal activation, and vascular remodelling.

However, interpretation of these data is complicated by heterogeneity in anti-TNF responsiveness. As previously described, some patients fail therapy due to anti-drug antibodies that reduce drug bioavailability [291], while others exhibit genuine pharmacodynamic non-response despite therapeutic drug levels [292]. In the Arijs dataset [288], patients with antibodies against infliximab are not excluded, leaving it unclear whether antibody-mediated non-response would affect GGT5 expression post treatment. This introduces uncertainty as to whether observed transcriptomic changes reflect drug action, disease trajectory, or patient stratification effects.

The ambiguity introduced by heterogeneous drug response underscores a broader limitation, where transcriptomic correlates of

therapy cannot be assumed to reflect direct biological regulation. Changes in GGT5 expression may instead indicate shifts in tissue composition, stromal activation states, or leukocyte burden rather than direct TNF dependency. This highlights the importance of integrating single-cell or spatial data to disentangle true regulatory effects from artefacts. Notably, bulk tissue analyses are particularly sensitive to changes in highly abundant stromal populations, which may mask cell-intrinsic regulatory mechanisms.

In contrast to the descriptive analyses in Chapter 3, the experiments below directly test candidate signalling pathways capable of inducing GGT5 expression in fibroblasts.

To validate the role of TNF in GGT5 expression, recombinant cytokines are applied onto PIP and intestinal fibroblasts. TNF α significantly induces GGT5 expression in PIP fibroblasts but has a more limited effect in intestinal fibroblasts. This differential responsiveness suggests that TNF signalling alone is insufficient to explain GGT5 regulation across tissue contexts.

This difference in response may be explained by the higher basal GGT5 expression in intestinal fibroblasts compared to PIP fibroblasts (Figure 4.2c, Figure 4.8e), suggesting that GGT5 expression in intestinal fibroblasts may already be close to their maximum level. However, apparent saturation could alternatively reflect active transcriptional repression, altered signalling thresholds, or prior pathway desensitisation rather than true maximal induction.

A limitation of this study is that TNF receptor levels were not measured in PIP and intestinal fibroblasts, which could have helped clarify whether the observed differences in TNF responsiveness were due to variations in receptor expression. Additionally, including a larger number of intestinal fibroblast donors could have strengthened the analysis and helped account for inter-donor variability. Without these measurements, it is difficult to distinguish between receptor-level effects and downstream signalling divergence.

Although differential basal expression provides one explanation for the variable response, alternative mechanisms remain plausible. For example, intestinal fibroblasts may exist in a refractory or epigenetically primed state due to chronic exposure to microbial and inflammatory signals *in vivo*, resulting in altered chromatin accessibility at the GGT5 locus. Without epigenomic profiling, it is difficult to determine whether TNF non-responsiveness reflects saturation, repression, or lineage-specific regulation. This highlights a key technical limitation of cytokine stimulation assays, which cannot resolve prior *in vivo* conditioning effects.

To search for a global stimulatory factor that increases GGT5 expression rather than one specific to the synovium, an unbiased approach using publicly available whole-tissue bulk RNA sequencing datasets is utilised. Pearson correlation analysis reveals that GGT5 is highly correlated with vascular genes in both inflamed gut and synovium. While suggestive, correlation-based analyses cannot

distinguish direct regulatory relationships from indirect co-expression driven by inflammation severity.

It is worth noting that although GGT5 is associated with GCs in the SLOs, GCs may not be present in all tissue niches in IMIDs. Therefore, its correlation with vasculature is independent of GC presence. Since GGT5 facilitates leukocyte migration by degrading the inhibitory signal GGG, its correlation with blood vessels seems logical as they are the primary entry point for leukocytes during inflammation. Nonetheless, vascular association alone does not establish endothelial control over fibroblast GGT5 expression.

However, correlation with vascular markers does not confirm endothelial regulation. It may instead reflect shared induction by inflammation or spatial co-localisation without direct signalling. Bulk RNA sequencing cannot distinguish whether GGT5 is upregulated in fibroblasts adjacent to vessels, in endothelial cells, or in infiltrating leukocytes. This limitation emphasises the need for model systems or spatial transcriptomic approaches to validate whether vascular association reflects functional signalling or merely anatomical proximity. Thus, conclusions drawn from bulk correlations must be interpreted cautiously.

NOTCH3 signalling has been shown to shape the sublining fibroblast identity in the synovium [56], and to correlate with immune cell infiltration in gastrointestinal cancers [293]. Interestingly, NOTCH3 ligands significantly stimulate GGT5 expression in PIP fibroblasts but

not in intestinal fibroblasts. Specifically, DLL4 and JAG1 not only induce GGT5 expression in PIP fibroblasts, but also increase NOTCH3 receptor and JAG1 expression, creating a positive feedback loop. Such feedback loops may amplify small spatial differences in ligand availability, potentially explaining perivascular confinement of GGT5 expression. These findings align with results published Wei et al., where co-culturing fibroblasts with HUVECs co-culture increases NOTCH3 and JAG1 protein levels. As activation of NOTCH3 signalling requires contact-dependent ligand-receptor engagement, this may explain why the process is slower and more spatially restricted than responses to soluble cytokines. Importantly, NOTCH3 activation has been linked to pro-inflammatory fibroblast states in RA, particularly in sublining perivascular fibroblast populations, raising the possibility that GGT5 is part of a broader NOTCH3-associated gene program involved in organising perivascular immune niches. Whether this induction represents a mechanism for sustaining TLS formation or simply facilitating leukocyte redistribution remains an open question, particularly given that TLS formation is restricted to lamina propria and synovial sublining compartments. Discriminating between these possibilities will require longitudinal and spatially resolved analyses.

The divergence between PIP and intestinal fibroblasts also raises the possibility that NOTCH3-dependent regulation of GGT5 is tissue-restricted rather than universal. This suggests that NOTCH3-driven perivascular fibroblast programmes may be a specific feature of

synovial inflammation rather than a general mechanism across IMIDs. Such context-dependence is important because it challenges the assumption that pathways identified in one tissue translate directly to others, highlighting the need for caution when inferring shared stromal mechanisms across diseases.

The supernatant from endothelial cells is the only signal identified so far that stimulates GGT5 expression in both intestinal and PIP fibroblasts, and the process is NF- κ B pathway dependent. While TNF α and IL-1 β can be excluded as key drivers, many other cytokines and proteins that signal via the NF- κ B pathway remain potential candidates. However, the use of HUVECs as a model for endothelial cells raises the possibility that their secreted factors may not accurately reflect conditions in inflamed gut or synovium due to different tissue microenvironments, highlighting the need for validation using tissue-specific endothelial cells. Interactome analysis of RA and IBD datasets may help to identify candidate soluble mediators. Moreover, microvascular endothelium in synovium and gut exhibits disease-specific features, such as distinct addressin expression and cytokine secretion, which may influence fibroblast responses and GGT5 induction. To further dissect the downstream effects of endothelial-derived stimulation, bulk RNA sequencing could be performed on fibroblasts treated with activated endothelial supernatant. This would enable the identification of the gene programme that is co-regulated

with GGT5, providing insight into the broader transcriptional landscape associated with its induction.

A further challenge lies in determining whether NF- κ B activation is necessary and sufficient for GGT5 induction, or whether it functions as part of a combinatorial signalling network. Endothelial cells release a wide spectrum of alarmins, growth factors, and metabolic mediators during inflammation, many of which could converge on fibroblasts with synergistic effects. Without perturbation studies targeting individual pathways, the signalling specificity behind GGT5 induction cannot be fully resolved.

Several considerations influence interpretation. Firstly, PIP fibroblasts that are derived from healthy foetal fingers originate from a sterile environment and thus are unlikely to have encountered inflammatory cytokines, potentially making them more sensitive to cytokine stimulation. In contrast, intestinal fibroblasts are derived from an inflamed environment, and they exhibit higher basal GGT5 expression. It is also important to note that comparing foetal finger synovium with adult gut tissue may not represent a like-for-like comparison. Nonetheless, these were the most accessible sources of primary fibroblasts for our study. Despite the known phenotypic changes induced by cell culture, GGT5 expression in intestinal fibroblasts remains elevated, raising questions about whether fibroblasts maintain GGT5 expression after interacting with leukocytes in tissues or whether it forms part of a stromal memory, as previously

described [67]. It is also unclear whether GGT5 expression decreases during remission. In addition, the gut harbours a higher baseline level of leukocyte infiltration compared to the synovium, which may further contribute to the elevated basal GGT5 expression observed in gut-derived fibroblasts. These observations suggest that fibroblasts are programmed according to their tissue context, with intestinal fibroblasts existing in a more activated state that is less responsive to further induction by TNF or NOTCH ligands.

Together, these limitations reinforce that fibroblast behaviour cannot be interpreted independently of their developmental origin and environmental history. Tissue conditioning, microbial exposure, and chronic inflammatory activity shape signalling responsiveness in ways that complicate direct comparison across fibroblast types. Future work using adult-matched, inflammation-naïve fibroblasts will be essential for clarifying which observations are intrinsic and which are artefacts of tissue origin or disease state.

While intestinal and PIP fibroblasts serve as useful models, the ideal approach would involve healthy adult synovial and intestinal fibroblasts, though the availability of such tissues is limited. In vitro culture also introduces limitations, as fibroblasts may lose aspects of their *in vivo* phenotype over time, and static culture conditions cannot replicate dynamic endothelial–leukocyte–stromal interactions. Nevertheless, the data suggest that the perivascular expression of GGT5 in steady state conditions is maintained by contact-dependent

NOTCH3 signalling. During inflammation, endothelial cells are triggered by leukocyte entry, which in turns release soluble factors that stimulate neighbouring fibroblasts to upregulate GGT5, as a mean to accommodate the influx of leukocytes into tissues.

These findings underscore the role of perivascular fibroblasts as active organisers of immune infiltration. By integrating signals from endothelial cells and engaging in pathways such as NOTCH3 and NF- κ B, these fibroblasts expand their GGT5 expression to facilitate leukocyte redistribution within inflamed tissues. Their positioning at the vascular interface, coupled with their capacity to acquire specialised phenotypes that produce spatial cues for immune organisation, highlights their contribution to TLS formation and chronic inflammatory pathology [111-113]. Further work is needed to define whether GGT5-mediated changes in fibroblasts simply facilitate leukocyte ingress or also shape the subsequent architecture of immune aggregates, which could have implications for strategies aimed at modulating tissue inflammation.

Together with the spatial framework established in Chapter 3, these findings support a model in which perivascular localisation of GGT5 reflects both anatomical positioning and active regulation by endothelial–fibroblast signalling during inflammation. Its expression is regulated by contact-dependent signals from endothelial cells under steady-state conditions and is further upregulated by endothelial-derived factors during inflammation.

Chapter 5: Investigating the role of GGT5-P2RY8-GGG axis in regulating chemotaxis

5.1 Introduction

The role of GGT5 in leukocyte migration has not been previously studied in IMIDs, but GGT5 itself has been implicated in gastric cancers [294-296]. Gene set enrichment analysis from one study suggests that high expression of GGT5 in stomach adenocarcinoma may be involved in leukocyte transendothelial migration, but no experimental validation has been done [297]. Another study shows that downregulation of GGT5 using shRNA in gastric cancer cell lines decreases their proliferation and migration [294]. Building on the discovery of the GGT5-GGG-P2RY8 axis in SLOs, this chapter examines the extent to which its mechanisms are applicable to non-lymphoid organs, and aims to investigate the functional implications of GGT5-GGG-P2RY8 pathway in IBD and RA.

5.2 Results

Previous study has shown that Tfh and GC B cells are the only populations within SLOs that stop migrating in response to GGG [252]. To validate these findings, tonsil-derived leukocytes are placed on transwell filters with CXCL12, CXCL13 and GGG in bottom chamber to reproduce the reported inhibitory effect of GGG on leukocyte migration

[252, 253]. The migrated cells are then stained and analysed using spectral flow cytometry.

Tonsil leukocytes are initially gated to select live cells, followed by further gating to identify plasma cells, CD45⁺ leukocytes, T cells, and B cells, as shown in Figure 5.1. CD27⁻CD38⁺ B cells, which are reported to exhibit a transitional phenotype [298], are gated as transitional B cells in this analysis (Figure 5.1).

The CD3⁺ T cells are further gated into CD4⁺ and CD8⁺ populations, with CD4⁺ subpopulations subsequently gated as shown in Figure 5.2a.

Although not statistically significant, the migration of PD-1⁺CXCR5⁺ T follicular helper (Tfh) cells are reduced in the presence of GGG alone, even without a chemoattractant (Figure 5.2b). A two-fold increase in Tfh migration towards CXCL12 is observed compared to the control. When GGG is added alongside CXCL12 and CXCL13, Tfh migration decreases (Figure 5.2b). This can be observed more clearly with representative FlowJo pseudocolour plots (Figure 5.2c).

When CXCL12 is added alone, migration increases for CD45RA⁺ naive T cells, CD45RO⁺ memory T cells (Tmem), CD8⁺ T cells, CD62L⁻CCR7⁻ effector memory T cells (Tem), and CD62L⁺CCR7⁺ central memory T cells (Tcm). However, the inhibitory effect of GGG on their migration is not noticeable in these populations (Figure 5.2b).

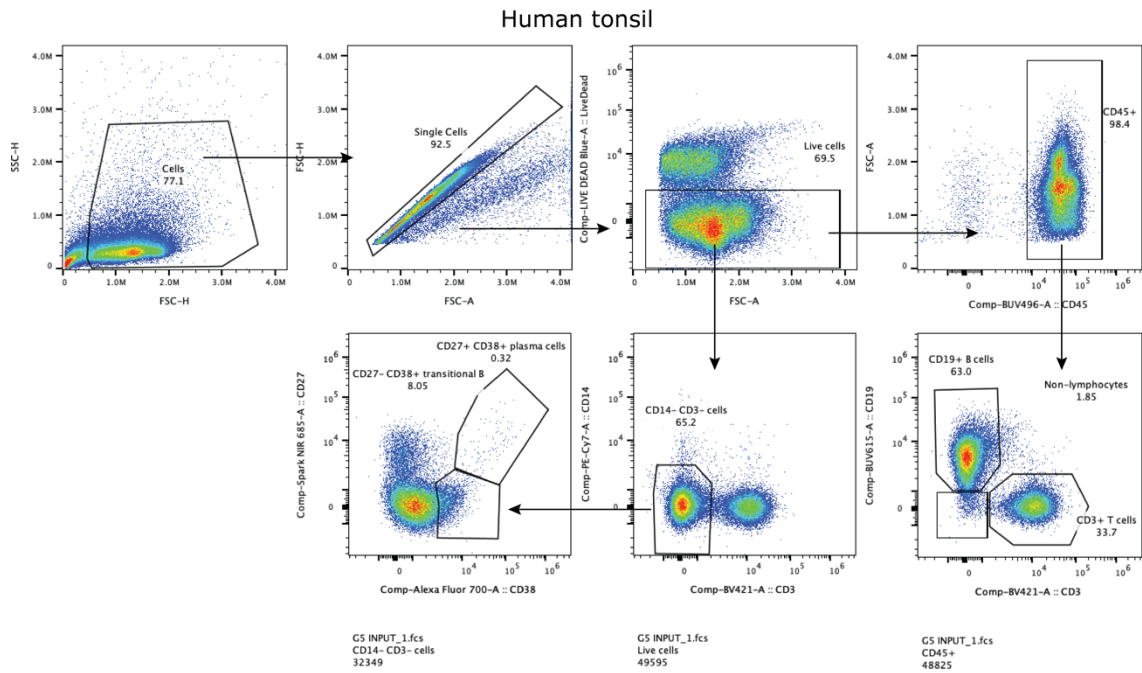
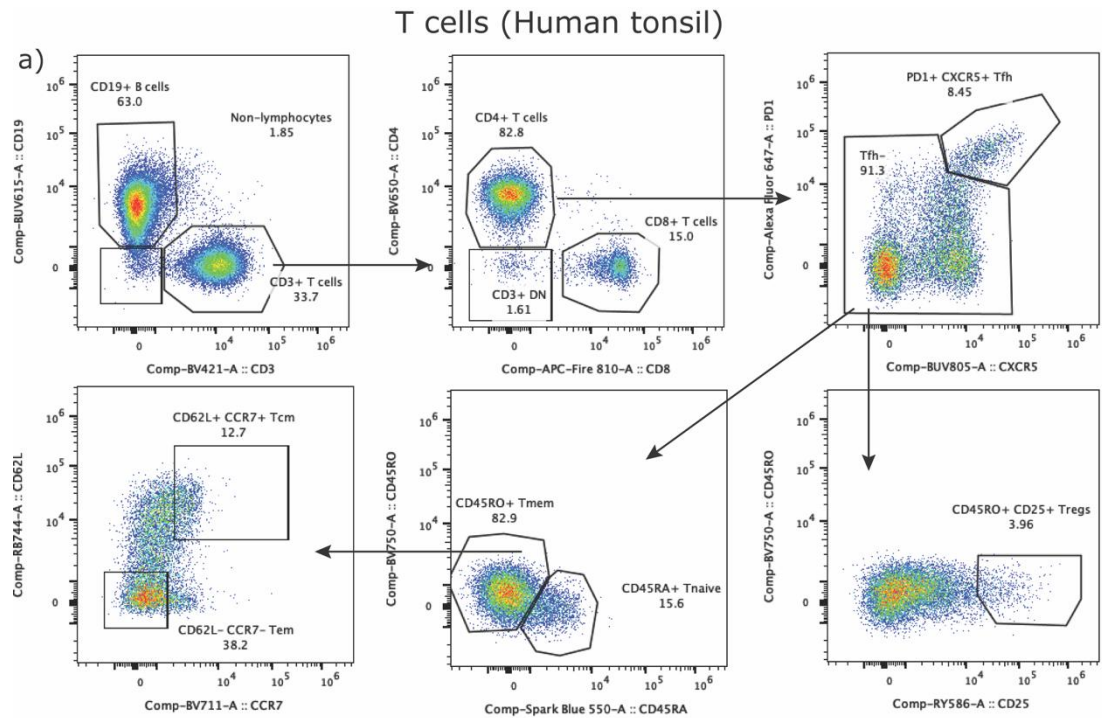


Figure 5.1: Representative gating strategy in human tonsil. Cells were first gated to exclude debris, followed by selection of single cells and live cells (live/dead stain negative). From live cells, CD45⁺ leukocytes were identified and further subdivided into CD19⁺ B cells, CD3⁺ T cells, and non-lymphocyte populations. In parallel, live CD14⁻CD3⁻ cells were gated to identify CD27⁺CD38⁺ plasma cells and CD27⁻CD38⁺ transitional cells.



b) T cell migration

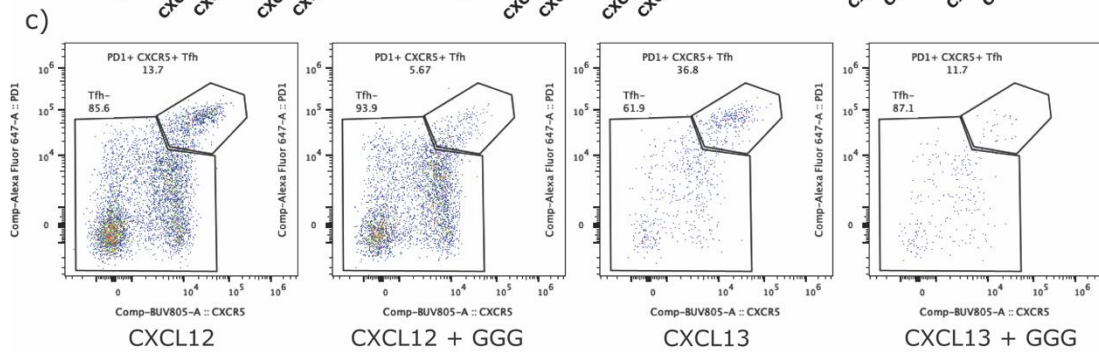
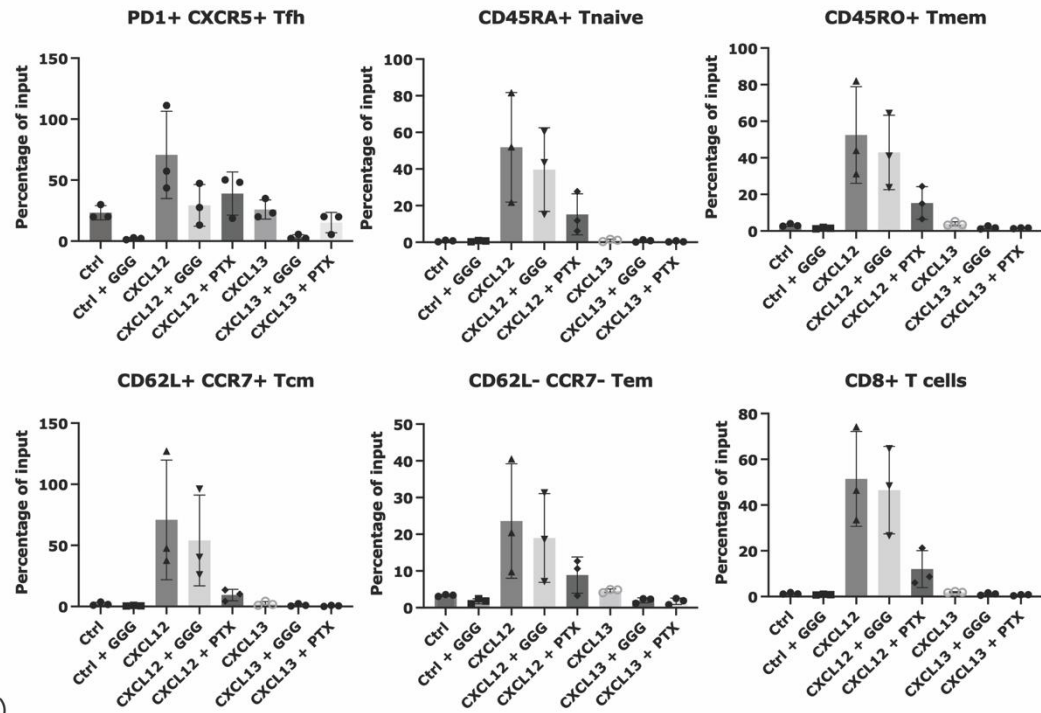


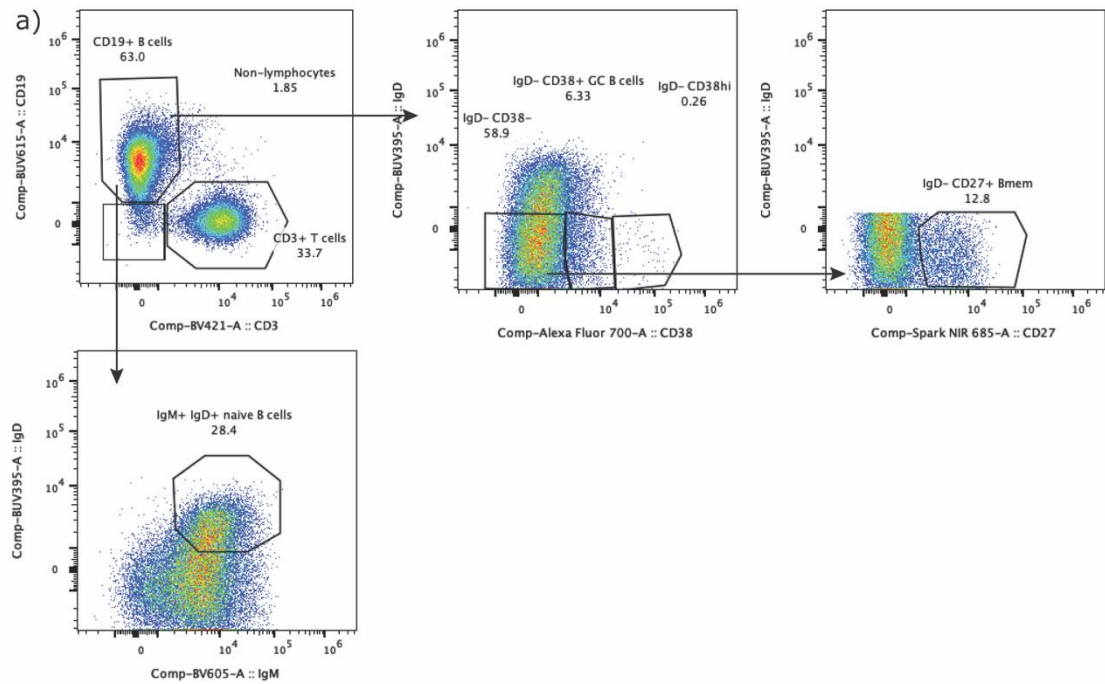
Figure 5.2: Transwell migration of T cells from digested human tonsil cells. T cells were obtained by gating CD3⁺ cells from digested human tonsil samples as shown in Figure 5.1. (a) Representative gating strategy for T cell subpopulations. CD3⁺ T cells were subdivided into CD4⁺ and CD8⁺ populations. Within CD4⁺ T cells, PD-1⁺CXCR5⁺ Tfh cells were identified. From the Tfh⁻ fraction, CD45RO⁺CD25⁺ Tregs) were gated. In parallel, the Tfh⁻ population was further subdivided into CD45RO⁺ Tmem and CD45RA⁺ Tnaive, and CD45RO⁺ Tmem were subsequently gated into CD62L⁺CCR7⁺ Tcm and CD62L⁻CCR7⁻ Tem subsets. (b) Migration of PD-1⁺CXCR5⁺ Tfh cells, CD45RA⁺ Tnaive, CD45RO⁺ Tmem, CD62L⁺CCR7⁺ Tcm, CD62L⁻CCR7⁻ Tem and CD8⁺ T cells towards CXCL12 and CXCL13±GGG, PTX is used as negative control. Graphs are plotted as means ± SD, each with n=3 biological replicates; statistical significance was tested using non-parametric Friedman test and Dunn's multiple comparison test. (c) Representative pseudocolour plot showing percentage of PD-1⁺CXCR5⁺ Tfh migrated towards CXCL12 and CXCL13±GGG.

For CD19⁺ B cells, the subpopulations are further gated as shown in Figure 5.3a. Most B cell subpopulations migrate towards CXCL12. Similar to Tfh cells, IgD⁻CD38⁺ GC B cells show reduced migration with GGG alone. When GGG is combined with a chemoattractant, there is a reduction in GC B cell migration, but this effect is observed only with CXCL13, not with CXCL12 (Figure 5.3b). This is further illustrated in the FlowJo pseudocolor plot (Figure 5.3c).

A reduction in migration is also observed in CD27⁺CD38⁺ plasma cells when GGG is added with CXCL12 and CXCL13, although there is considerable variation between donors.

Non-lymphocytes from tonsils are gated as CD3⁻CD19⁻, as shown in Figure 5.4a. These cells migrate towards CXCL12, with a two-fold increase in the number of migrated cells compared to the control. However, the inhibitory effect of GGG on their migration is minimal (Figure 5.4c).

B cells (Human tonsil)



b) B cell migration

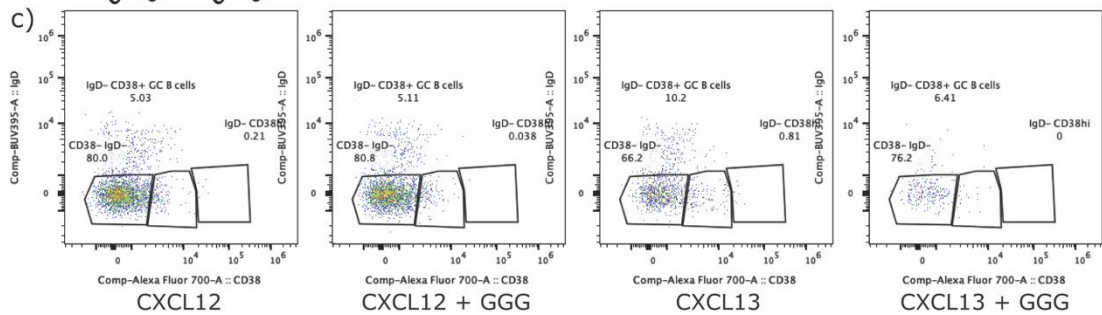
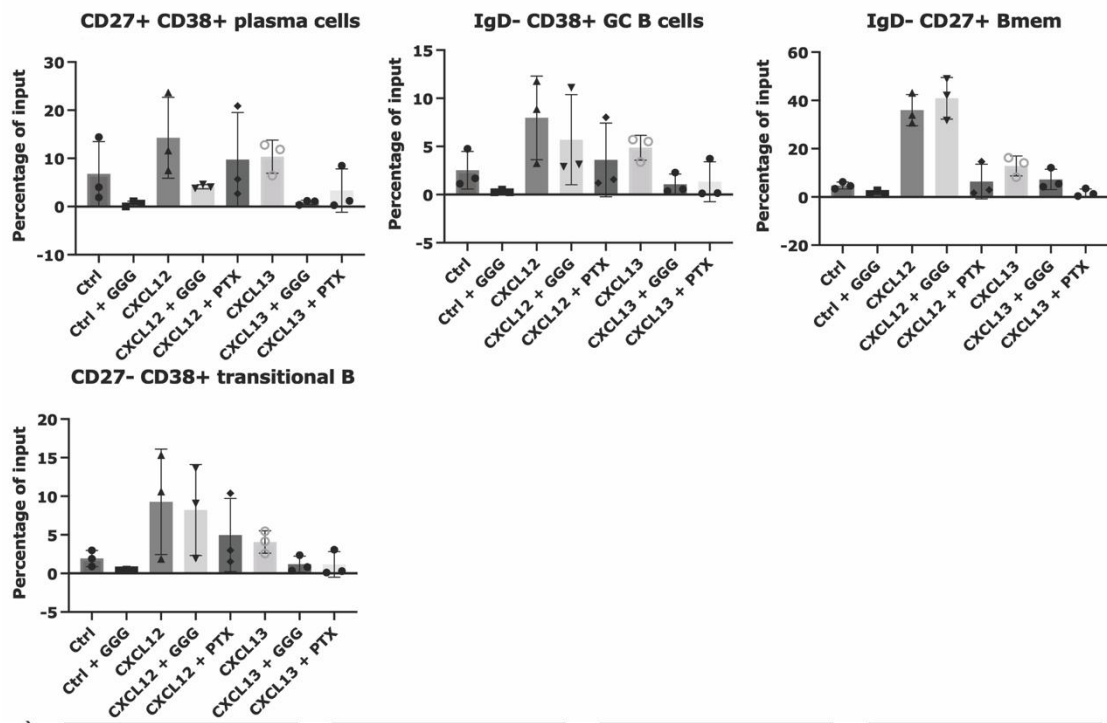


Figure 5.3: Transwell migration of B cells from digested human tonsil cells. B cells were obtained by gating CD19⁺ cells from digested human tonsil samples as shown in Figure 5.1. (a) Representative gating strategy for B cell subpopulations. CD19⁺ B cells were gated to identify IgD⁻CD38⁺ GC B cells, IgD⁻CD38^{hi} cells, and IgD⁻CD38⁻ cells. From the IgD⁻CD38⁻ population, IgD⁻CD27⁺ Bmem were subsequently identified. In parallel, CD19⁺ cells were also gated to identify IgM⁺IgD⁺ naïve B cells. (b) Migration of CD27⁺CD38⁺ plasma cells, IgD⁻CD38⁺ GC B cells, IgD⁻CD27⁺ Bmem and CD27⁻CD38⁺ transitional B cells towards CXCL12 and CXCL13±GGG, PTX is used as negative control. Graphs are plotted as means ± SD, each with n=3 biological replicates; statistical significance was tested using non-parametric Friedman test and Dunn's multiple comparison test. (c) Representative pseudocolour plot showing percentage of IgD⁻CD38⁺ GC B cells migrated towards CXCL12 and CXCL13±GGG.

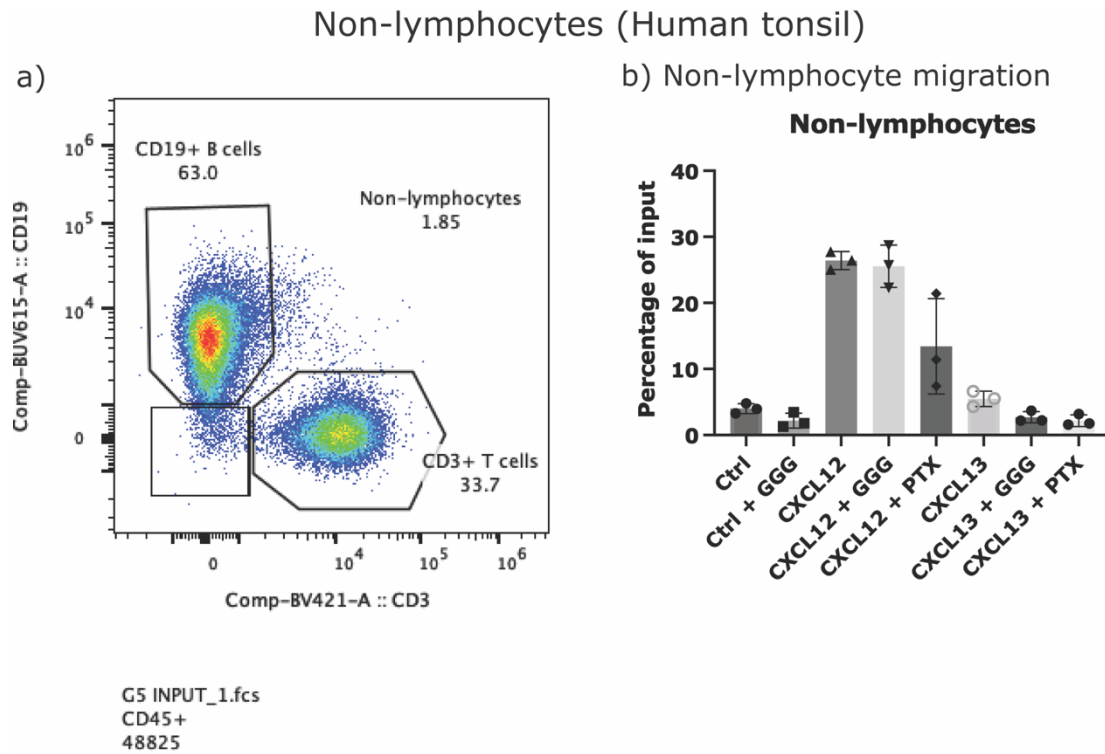


Figure 5.4: Transwell migration of non-lymphocytes from digested human tonsil cells. Non-lymphocytes were obtained by gating CD3⁻CD19⁻ cells from digested human tonsil samples as shown in Figure 5.1. (a) Representative gating strategy for non-lymphocytes. (b) Migration of non-lymphocytes towards CXCL12 and CXCL13±GGG, PTX is used as negative control. Graphs are plotted as means $\pm$ SD, each with n=3 biological replicates; statistical significance was tested using non-parametric Friedman test and Dunn's multiple comparison test.

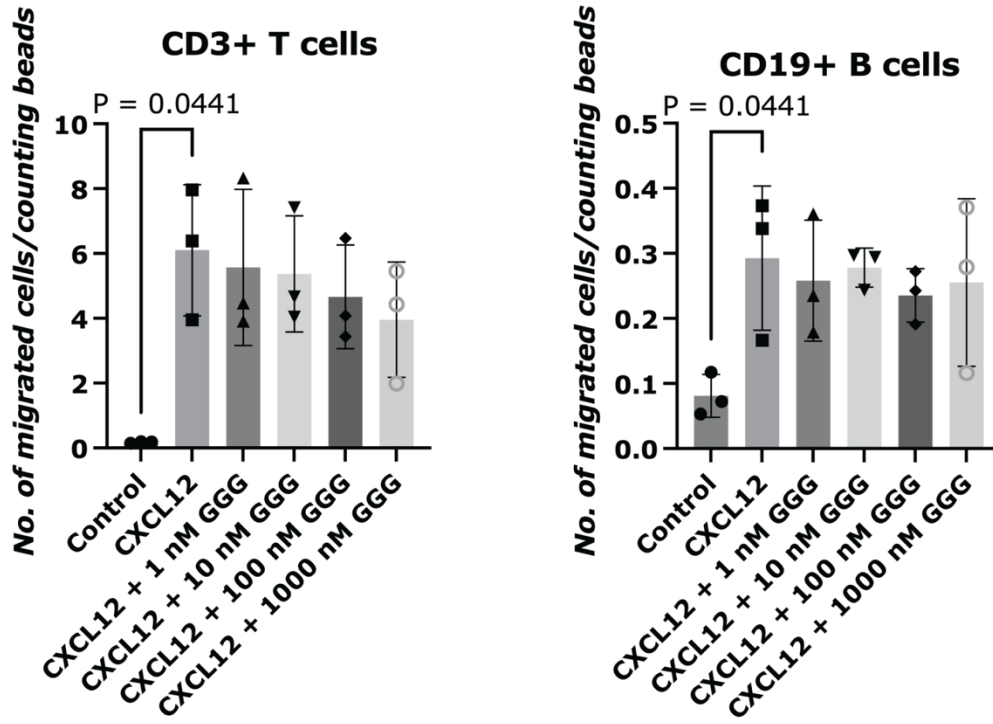
To examine the effect of GGG on leukocyte migration outside SLOs, healthy human PBMCs are placed in transwell filters with CXCL12 and increasing concentrations of GGG. Migrated cells are analysed using conventional flow cytometry, with gating for CD3⁺ T cells and CD19⁺ B cells.

When CXCL12 is present, a significant increase in the number of migrated CD3⁺ T cells and CD19⁺ B cells is observed in the bottom chamber compared to the control without chemoattractant. While a dose-dependent decrease in CD3⁺ T cell migration is noted with increasing concentrations of GGG, the overall effect is not statistically significant. For CD19⁺ B cells, no significant difference in migration is observed when GGG is combined with CXCL12 (Figure 5.5a).

Leukocytes derived from different anatomical locations can exhibit distinct phenotypes. For instance, one study shows that cerebrospinal fluid (CSF) has a unique location-associated composition of leukocytes compared to blood in multiple sclerosis, such that blood shows increased transcriptional diversity within leukocyte subsets, whereas CSF displays increased cell type diversity, including a higher abundance of cytotoxic phenotype T helper cells [299]. Therefore, for disease relevance, human psoriatic arthritis (PsA) SFMCs are obtained from Paul Bowness' group at the Botnar Institute for Musculoskeletal Sciences. Similar to PBMCs, CD3⁺ T cells and CD19⁺ B cells from PsA synovial fluid migrate towards CXCL12 and continue to migrate even in the presence of GGG (Figure 5.5b).

Human PBMC

a)



Human PsA SFMC

b)

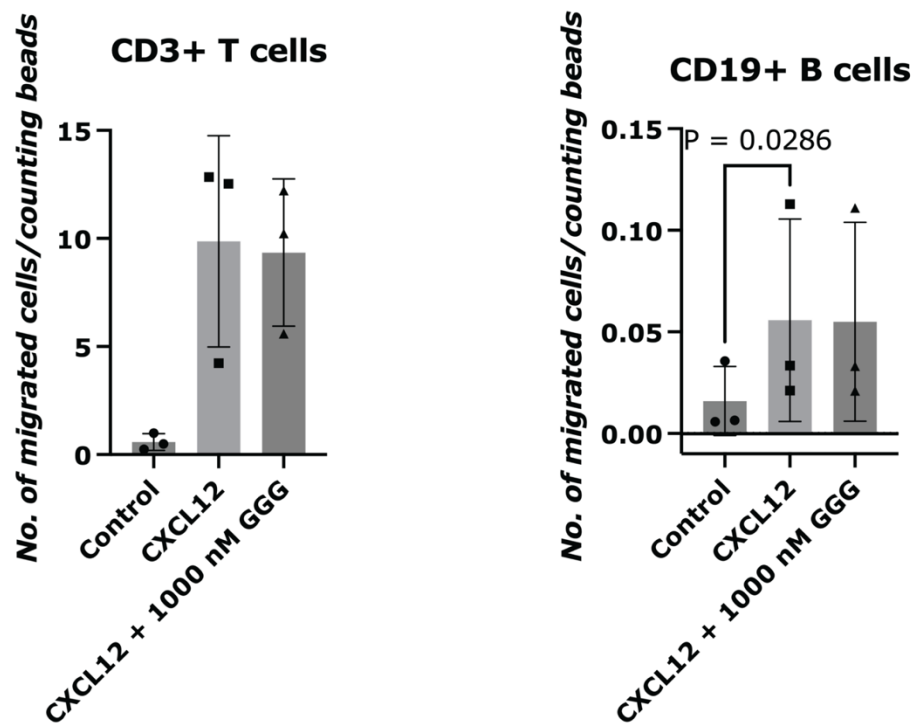


Figure 5.5: Transwell migration assay using healthy human PBMCs and PsA SFMCs. (a) Healthy human PBMCs undergo transwell migration assay with CXCL12 and increasing concentration of GGG and gated for CD3⁺ and CD19⁺ cells. (b) PsA SFMC undergo transwell migration assay with CXCL12 and 1000 nM GGG. Graphs are plotted as means $\pm$ SD, each with n=3 biological replicates; statistical significance is tested using non-parametric Friedman test and Dunn's multiple comparison test.

Since GGG does not inhibit the migration of human PBMCs and SFMCs in the presence of CXCL12, the subsequent step involves investigating the expression dynamics of P2RY8, as its expression levels may provide insight into cellular sensitivity to GGG. To achieve this, CD4⁺ T cells are isolated from healthy human peripheral blood and activated using ImmunoCult Human CD3/CD28 T Cell Activator. P2RY8 level is assessed using flow cytometry, with an isotype control to help in gating. In resting or steady state CD4⁺ T cells, two distinct populations are observed based on FSC-H, with a large proportion of cells expressing P2RY8 (Figure 5.6a).

Upon activation, the isotype control shows a higher AF647 signal compared to the steady state isotype control, suggesting nonspecific background fluorescence. Despite this, the proportion of P2RY8⁺ cells among activated CD4⁺ T cells are reduced (Figure 5.6b). The difference in P2RY8 level between steady and activated states is quantified by normalising the geometric mean fluorescence intensity (gMFI) of P2RY8 to the respective isotype controls. Although not statistically significant, the data suggest a decrease in normalised P2RY8 gMFI in CD4⁺ T cells after activation, and increasing the number of biological donors may help determine whether this trend is consistent. (Figure 5.6c).

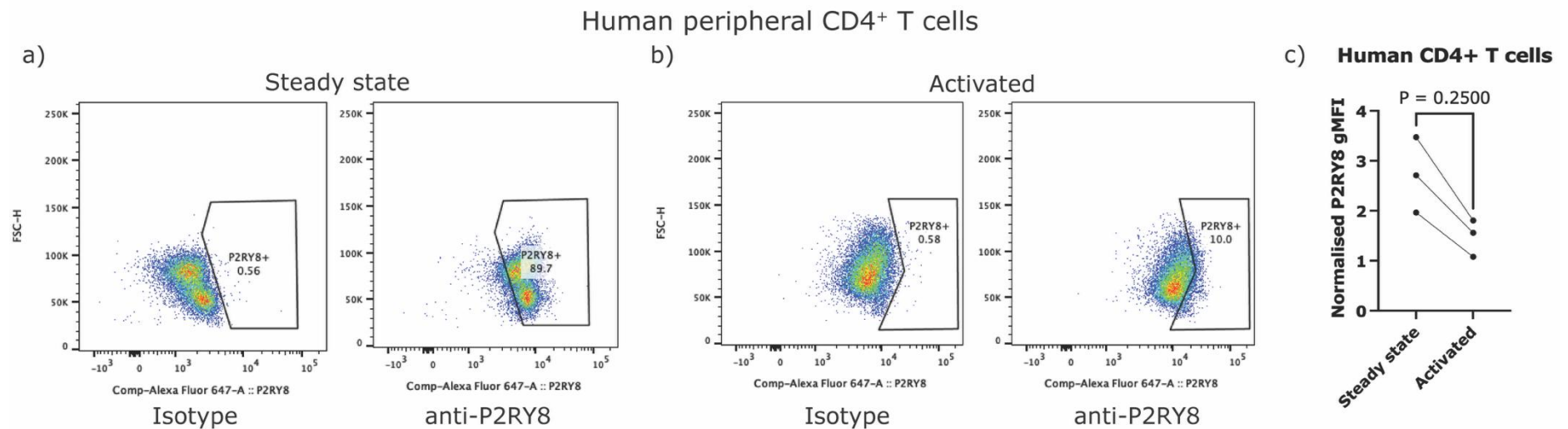


Figure 5.6: Intracellular P2RY8 staining in healthy human peripheral CD4⁺ T cells. Representative image showing P2RY8 gating in (a) steady state and (b) activated CD4⁺ T cells. (c) Comparison of P2RY8 gMFI between steady state and activated CD4⁺ T cells, n=3 biological replicates. Normalised P2RY8 gMFI are generated by dividing P2RY8 gMFI by isotype gMFI. Statistical test is performed using non-parametric Wilcoxon signed-rank test.

As the transwell migration assay only quantifies the number of migrated cells, it does not capture the migratory behaviour of T cells in the presence of GGG. To address this, live migration is tracked using Zeiss 980 confocal microscopy. In this setup, cells are introduced at one end of a channel, while CXCL12 $\pm$ GGG is added at the opposite end (Figure 5.7a). In this pilot experiment, CXCL12 and GGG are diluted in heat-inactivated FBS to increase the medium's density, slowing the diffusion of CXCL12 and GGG to create a pseudo-gradient for activated CD4⁺ T cells.

Under control conditions without CXCL12, some activated CD4⁺ T cells migrate towards FBS, as seen from their tracks. When CXCL12 is added, the cells initially move in the opposite direction before sensing the gradient and migrating towards CXCL12. There is more migration observed in CXCL12 compared to the control condition. When 1 μ M and 10 μ M GGG are added alongside CXCL12, cells still migrate towards the source. However, robust analysis is needed to determine if there is a significant reduction in migration in the presence of GGG.

Pertussis toxin (PTX) catalyses ADP-ribosylation of G-proteins G_i, G_o and G_t at a site that impairs G protein heterotrimer interaction with GPCRs and blocks receptor coupling, thereby blocking downstream G-coupled GPCR signalling [300-302]. Therefore, PTX is used as a negative control. When PTX is added with CXCL12, the tracks appear erratic, and the T cells struggle to migrate effectively towards CXCL12 (Figure 5.7b).

It is important to note that this analysis, performed using TrackMate, is highly preliminary and requires further validation. Therefore, more robust analysis and quantification are needed to draw definitive conclusions.

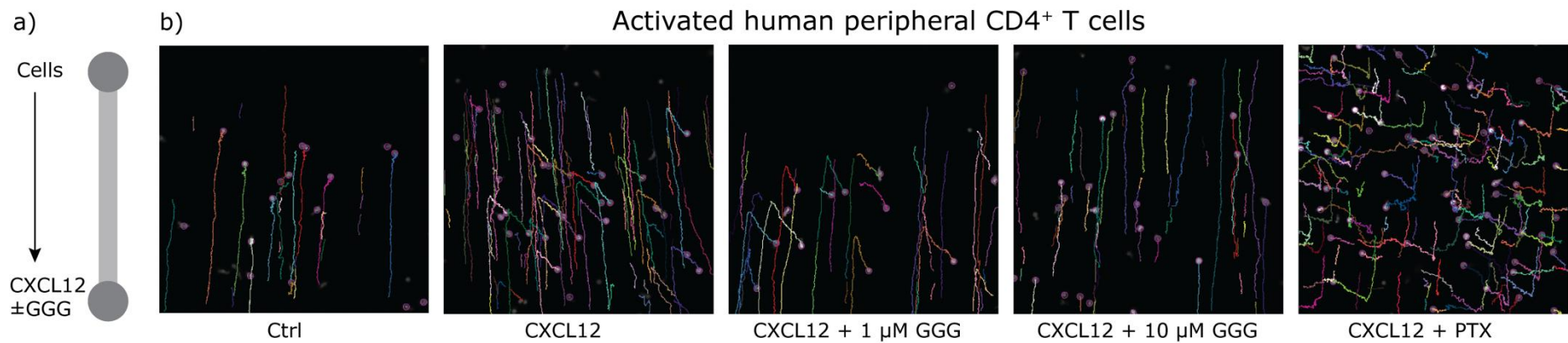


Figure 5.7: Imaging migration assay using activated human peripheral CD4⁺ T cells. (a) Setup of imaging migration assay with CXCL12 $\pm$ GGG added onto one end, and cells added onto the other end of an Ibidi glass bottom μ -slide to create a chemoattractant gradient. (b) Migration of activated T cells in different conditions are imaged every second with a confocal microscope and later tracked using TrackMate [270]. The lines in each condition represent the migration tracks of activated T cells.

As this chapter aims to explore the role of the GGT5-GGG-P2RY8 axis in leukocyte compartmentalisation within IMIDs and leukocytes derived from blood and tissues exhibit distinct phenotypes, it is therefore important to study leukocytes from disease-relevant tissues rather than relying solely on blood or tonsils.

Moving beyond SLOs, leukocytes from inflamed human gut tissues are analysed using a transwell migration assay to evaluate the effect of GGG on cells from non-lymphoid organs. The digested gut cells are first gated to select live cells, followed by additional gating to identify plasma cells, CD45⁺ leukocytes, T cells, and B cells, as illustrated in Figure 5.8. To capture a wider array of leukocyte subpopulations, a broader spectral flow cytometry panel is used in this experiment.

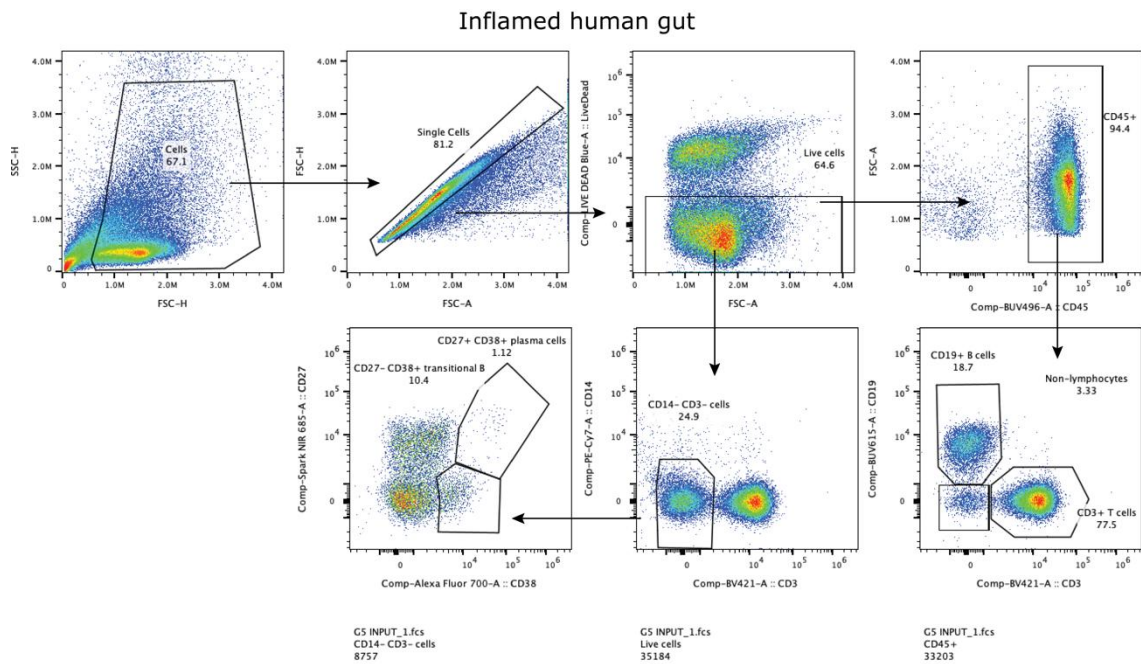


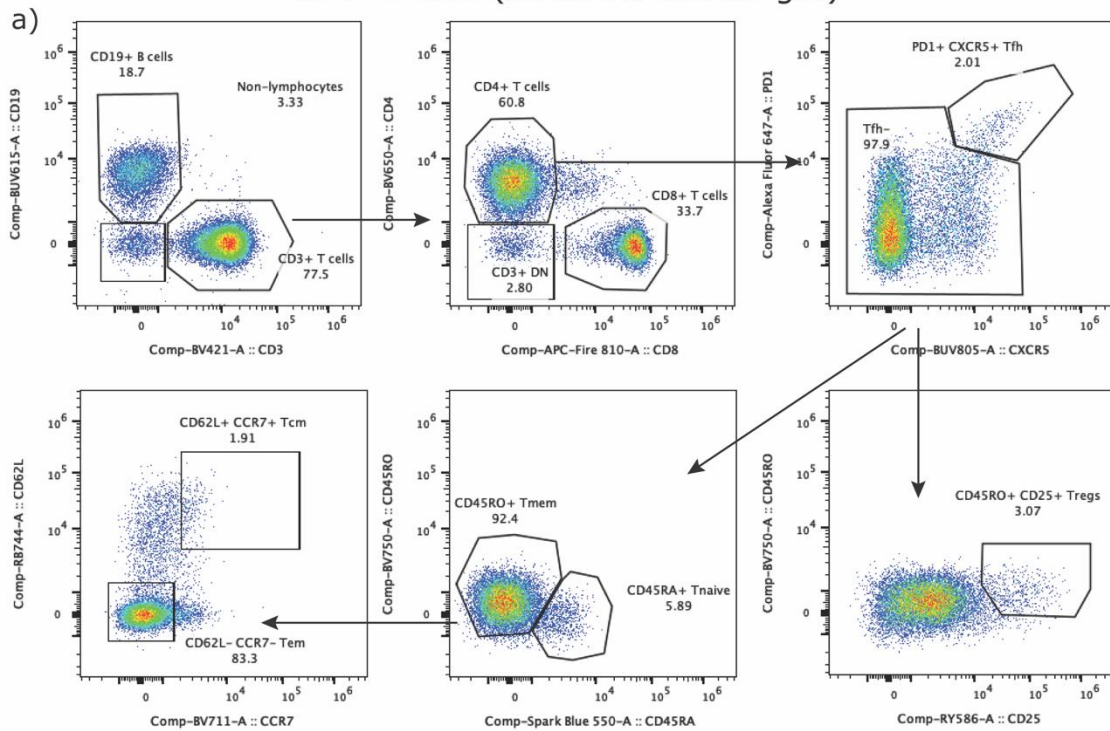
Figure 5.8: Representative gating strategy in inflamed human gut. Cells were first gated to exclude debris, followed by selection of single cells and live cells (live/dead stain negative). From live cells, CD45⁺ leukocytes were identified and further subdivided into CD19⁺ B cells, CD3⁺ T cells, and non-lymphocyte populations. In parallel, live CD14⁻CD3⁻ cells were gated to identify CD27⁺CD38⁺ plasma cells and CD27⁻CD38⁺ transitional cells.

The CD3⁺ T cells are further gated into CD4⁺ and CD8⁺ populations, with CD4⁺ subpopulations subsequently gated as shown in Figure 5.9a.

In preliminary data with the first patient sample, all CD4⁺ T cells migrate towards CXCL12, as indicated by a higher number of cells/ μ L as compared to control (Figure 5.9b). Similar to findings in tonsils, the migration of gut-derived PD-1⁺CXCR5⁺ Tfh cells is reduced in the presence of GGG alone, even without a chemoattractant. Tfh cells show an approximately eight-fold increase in migration towards CXCL12 compared to the control, but their migration is halved when GGG is added. Tfh cells also migrate towards CXCL13, though to a lesser extent than CXCL12, and their migration is similarly reduced in the presence of GGG (Figure 5.9b).

Interestingly, the inhibitory effect of GGG is not observed in other CD4⁺ T cell subpopulations when CXCL12 is present in the bottom chamber. However, CD4⁺CD45RO⁺ Tmem, CD4⁺CD62L⁺CCR7⁺ Tcm, and CD4⁺CD62L⁻CCR7⁻ Tem cells stop migrating towards CXCL13 when GGG is added (Figure 5.9b). Notably, CD45RO⁺CD25⁺ Tregs do not exhibit reduced migration in the presence of GGG with either CXCL12 or CXCL13 as chemoattractant (Figure 5.9b).

CD4⁺ T cells (Inflamed human gut)



b) CD4⁺ T cell migration

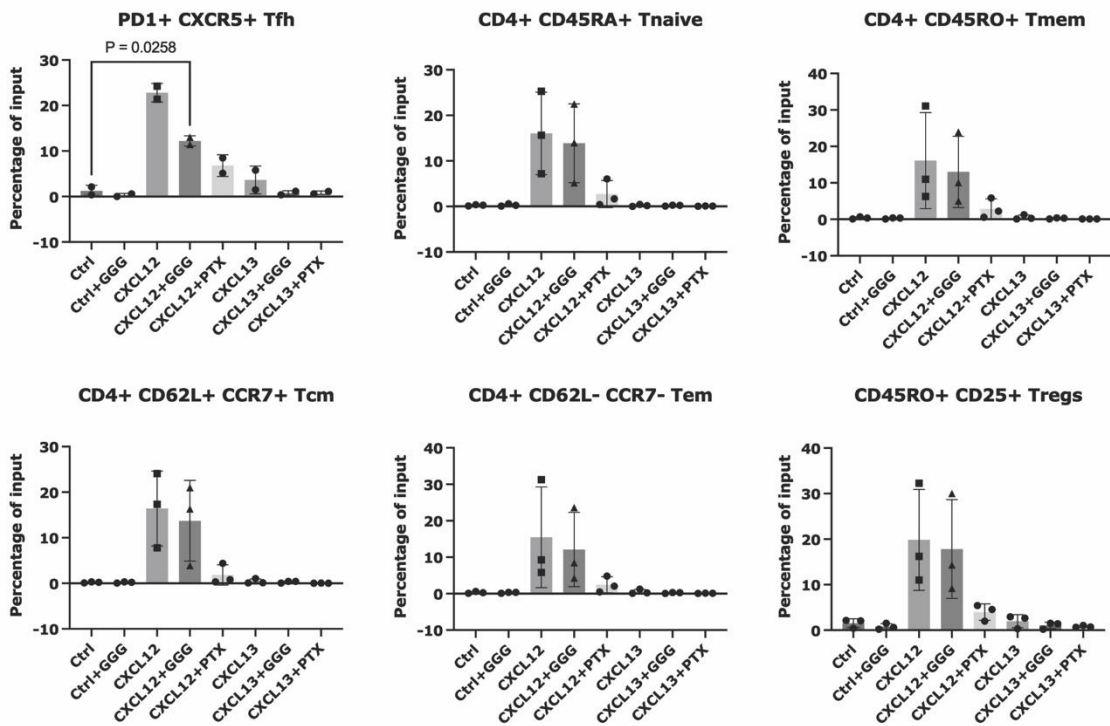
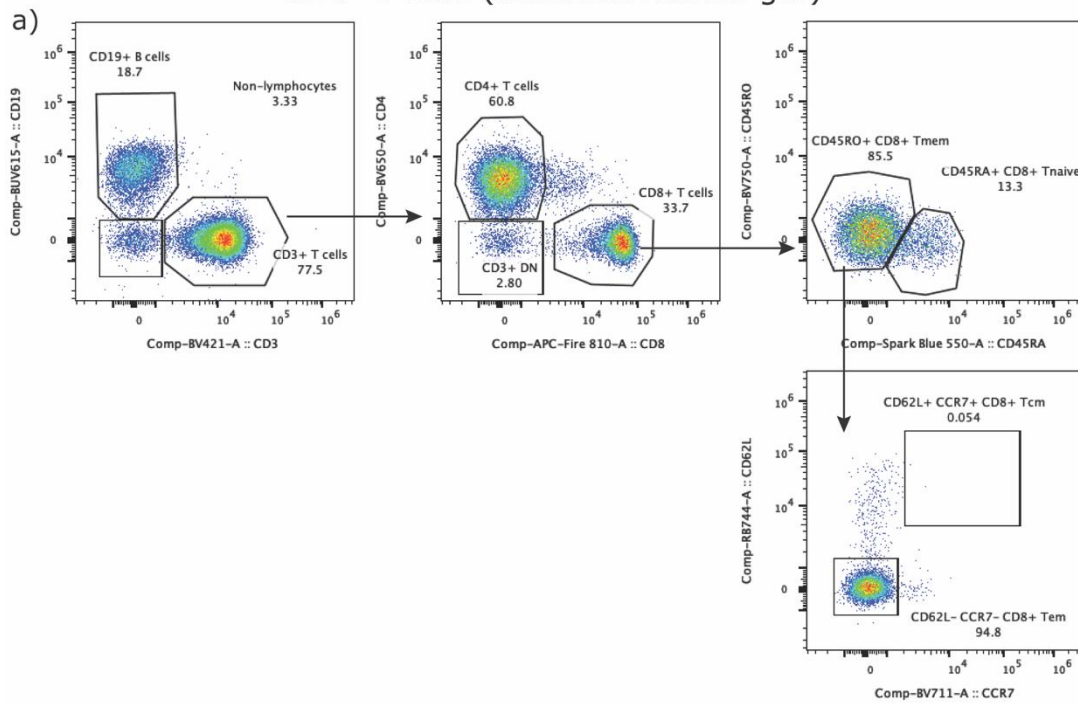


Figure 5.9: Transwell migration assay using CD4⁺ T cells from inflamed human gut. CD4⁺ T cells were obtained by gating CD3⁺ and subsequently CD4⁺ T cells from digested human gut samples as shown in Figure 5.8. (a) Representative gating strategy for CD4⁺ T cell subpopulations. CD3⁺ T cells were subdivided into CD4⁺ and CD8⁺ populations. Within CD4⁺ T cells, PD-1⁺CXCR5⁺ Tfh cells were identified. From the Tfh⁻ fraction, CD45RO⁺CD25⁺ Tregs) were gated. In parallel, the Tfh⁻ population was further subdivided into CD45RO⁺ Tmem and CD45RA⁺ Tnaive, and CD45RO⁺ Tmem were subsequently gated into CD62L⁺CCR7⁺ Tcm and CD62L⁻CCR7⁻ Tem subsets. (b) Migration of PD-1⁺CXCR5⁺ Tfh cells, CD45RA⁺ Tnaive, CD45RO⁺ Tmem, CD62L⁺CCR7⁺ Tcm, CD62L⁻CCR7⁻ Tem and CD45RO⁺CD25⁺ Tregs towards CXCL12 and CXCL13±GGG, PTX is used as negative control. Graphs are plotted as means ± SD, each with n=3 biological replicates; statistical significance was tested using non-parametric Friedman test and Dunn's multiple comparison test.

The CD8⁺ T cells are further gated into subpopulations, as illustrated in Figure 5.10a.

All CD8⁺ T cells migrate towards CXCL12, as indicated by a higher number of cells/ μ L as compared to the control condition (Figure 5.10b). However, the inhibitory effect of GGG is not observed with CXCL12. Interestingly, the migration of CD8⁺CD45RO⁺ Tmem and CD8⁺CD62L⁻CCR7⁻ Tem towards CXCL13 is reduced when GGG is added (Figure 5.10b).

CD8⁺ T cells (Inflamed human gut)



b) CD8⁺ T cell migration

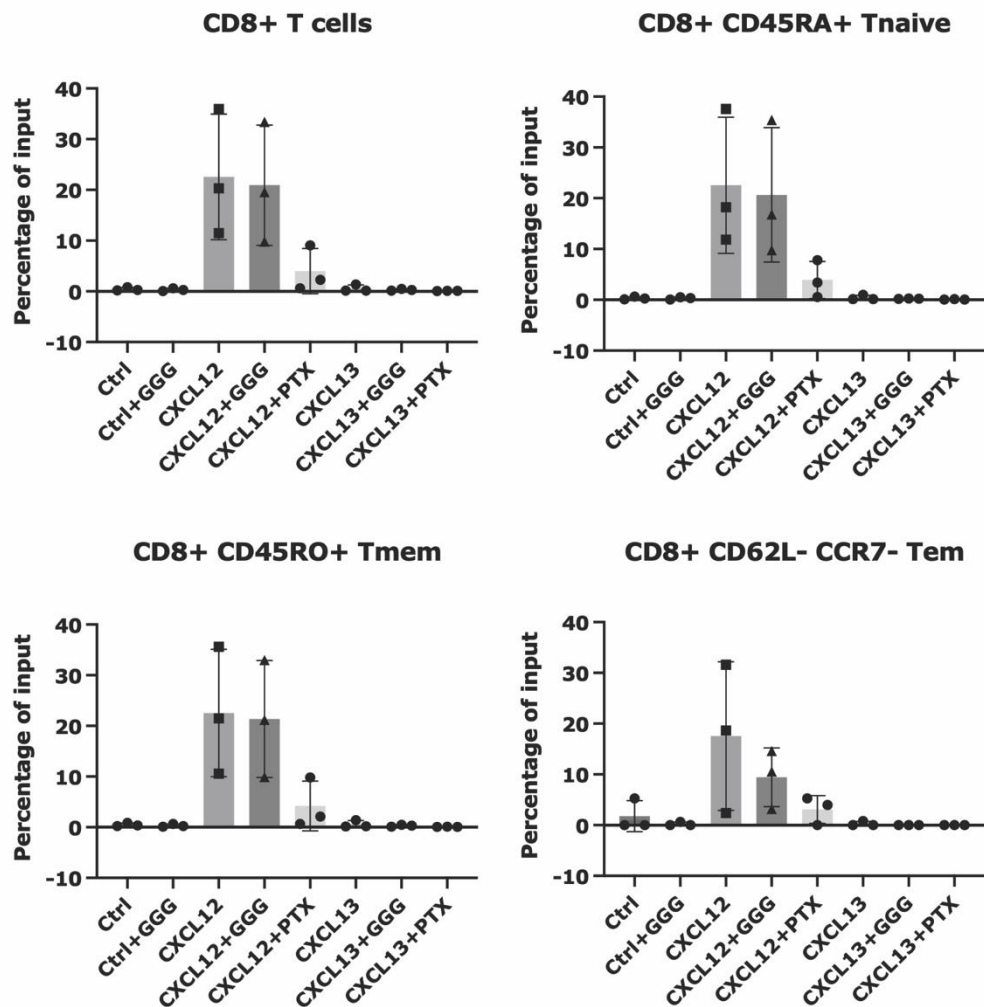


Figure 5.10: Transwell migration assay using CD8⁺ T cells from inflamed human gut. CD8⁺ T cells were obtained by gating CD3⁺ and subsequently CD8⁺ T cells from digested human gut samples as shown in Figure 5.8. (a) Representative gating strategy for CD8⁺ T cell subpopulations. CD3⁺ T cells were subdivided into CD4⁺ and CD8⁺ populations. Within CD8⁺ T cells, CD45RO⁺CD8⁺ Tmem and CD45RA⁺CD8⁺ Tnaive were gated. CD45RO⁺CD8⁺ Tmem were subsequently gated into CD62L⁺CCR7⁺CD8⁺ Tcm and CD62L⁻CCR7⁻CD8⁺ Tem subsets. (b) Migration of CD8⁺ T cells, CD45RA⁺CD8⁺ Tnaive, CD62L⁺CCR7⁺CD8⁺ Tcm and CD62L⁻CCR7⁻CD8⁺ Tem subsets towards CXCL12 and CXCL13±GGG, PTX is used as negative control. Graphs are plotted as means ± SD, each with n=3 biological replicates; statistical significance was tested using non-parametric Friedman test and Dunn's multiple comparison test.

The CD19⁺ B cells are further gated into subpopulations, as shown in Figure 5.11a.

Similar to T cells, all B cells migrate towards CXCL12, with a higher number of cells/ μ L compared to the control. Most B cells also migrate towards CXCL13, with the exception of CD27⁻CD38⁺ transitional B cells. The presence of GGG does not inhibit the migration of IgD⁻CD38⁺ or other B cells towards CXCL12. However, GGG reduces migration towards CXCL13 in IgM⁺IgD⁺ naïve B cells, IgD⁻CD38⁺ B cells, IgD⁻CD27⁺ memory B cells (Bmem), and CD27⁺CD38⁺ plasma cells (Figure 5.11b).

The CD3⁻CD19⁻ non-lymphocytes are gated as shown in Figure 5.12a. Among the non-lymphocytes, CD16⁺CD56⁺ NK cells, CD16^{dim}CD56⁺ NK cells, HLA⁻DR⁺CD11c⁺ DC, and HLA⁻DR⁻CD11c⁺ cells migrate towards CXCL12, as indicated by a higher number of cells/ μ L compared to the control. However, the effect of GGG on their migration is negligible, regardless of the presence of CXCL12 or CXCL13 (Figure 5.12b). Notably, the overall number of non-lymphocytes is too low to draw any meaningful conclusions from this data.

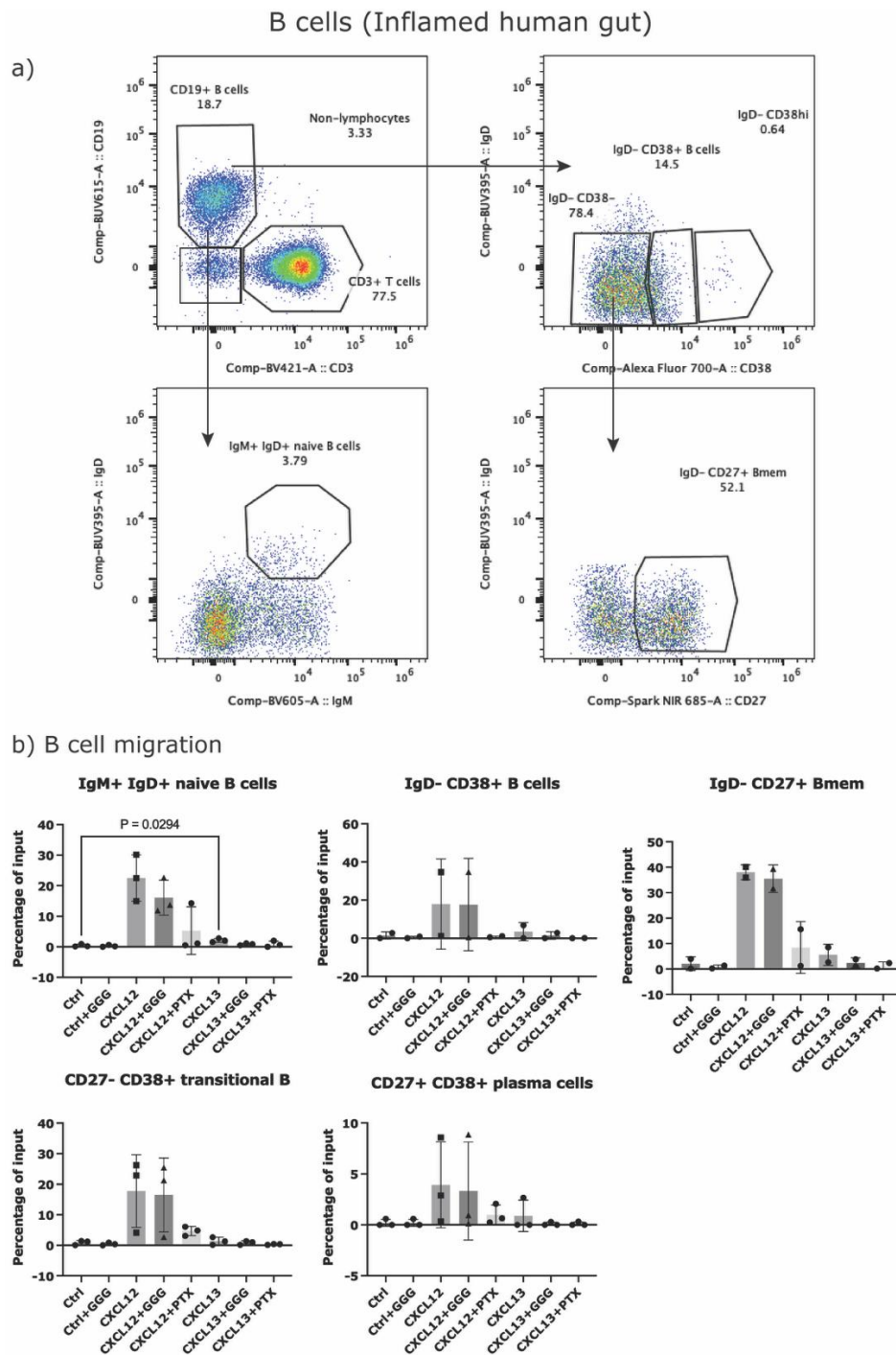
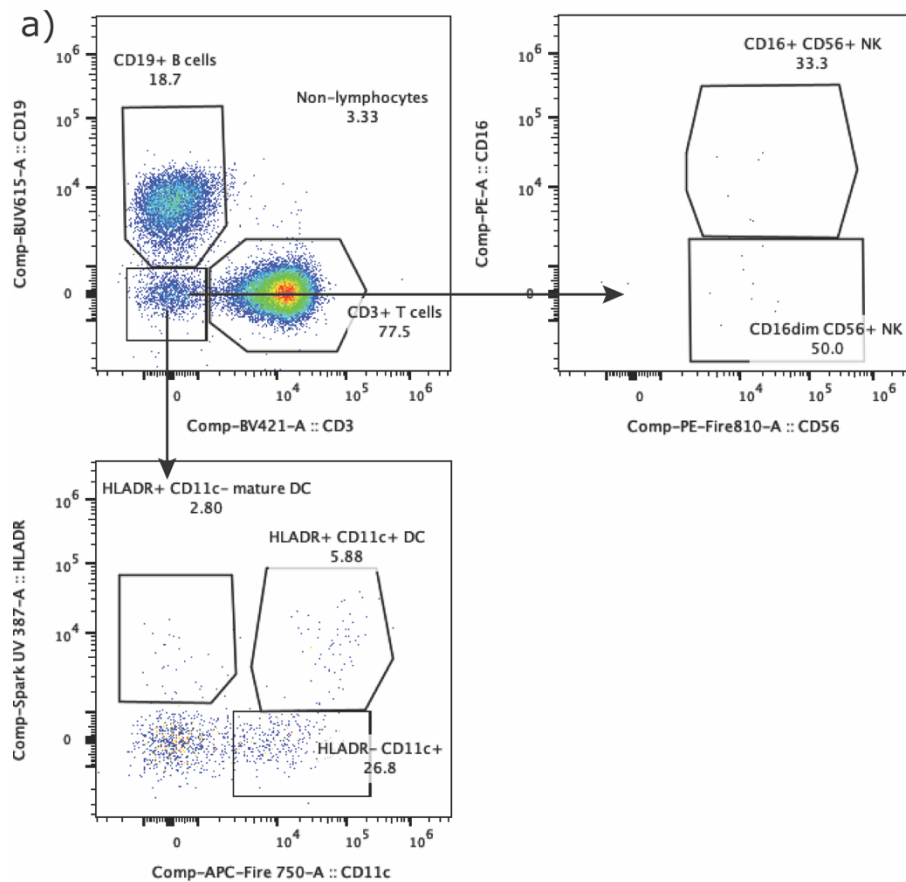


Figure 5.11: Transwell migration assay using B cells from inflamed human gut. B cells were obtained by gating CD19⁺ cells from digested human gut samples as shown in Figure 5.8. (a) Representative gating strategy for B cell subpopulations. CD19⁺ B cells were gated to identify IgD⁻CD38⁺ GC B cells, IgD⁻CD38^{hi} cells, and IgD⁻CD38⁻ cells. From the

IgD⁻CD38⁻ population, IgD⁻CD27⁺ Bmem were subsequently identified. In parallel, CD19⁺ cells were also gated to identify IgM⁺IgD⁺ naïve B cells. (b) Migration of IgM⁺IgD⁺ naïve B cells, IgD⁻CD38⁺ GC B cells, IgD⁻CD27⁺ Bmem, CD27⁻CD38⁺ transitional B cells and CD27⁺CD38⁺ plasma cells towards CXCL12 and CXCL13±GGG, PTX is used as negative control. Graphs are plotted as means ± SD, each with n=3 biological replicates; statistical significance was tested using non-parametric Friedman test and Dunn's multiple comparison test.

Non-lymphocytes (Inflamed human gut)



b) Non-lymphocyte migration

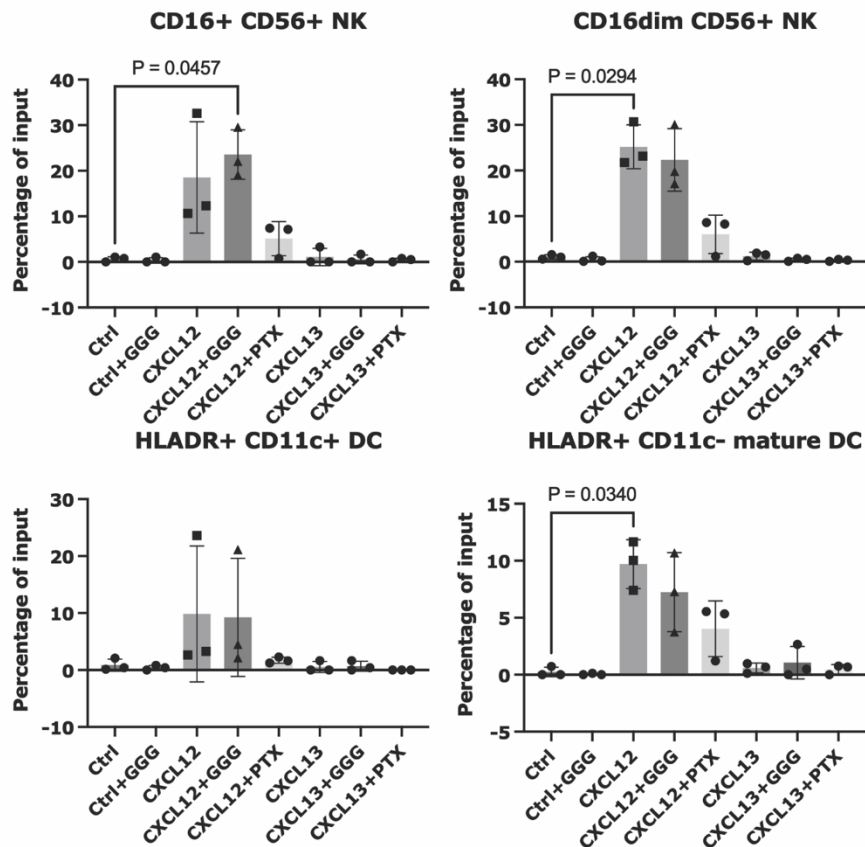


Figure 5.12: Transwell migration assay using non-lymphocytes from inflamed human gut. Non-lymphocytes were obtained by gating CD3⁻CD19⁻ cells from digested human gut samples as shown in Figure 5.8. (a) Representative gating strategy for non-lymphocyte subpopulations. From the CD3⁻CD19⁻ population, CD16⁺CD56⁺ NK cells and CD16^{dim}CD56⁺ NK cells were identified. In parallel, CD3⁻CD19⁻ cells were gated to identify HLA-DR⁺CD11c⁻ mature DCs, HLA-DR⁺CD11c⁺ DCs, and HLA-DR⁻CD11c⁺ cells. (b) Migration of CD16⁺CD56⁺ NK cells, CD16^{dim}CD56⁺ NK cells, HLA-DR⁺CD11c⁺ DCs and HLA-DR⁺CD11c⁻ mature DCs towards CXCL12 and CXCL13±GGG, PTX is used as negative control. Graphs are plotted as means ± SD, each with n=3 biological replicates; statistical significance was tested using non-parametric Friedman test and Dunn's multiple comparison test.

5.3 Discussion

Although the main concepts are borrowed from SLOs, the novelty of this project lies in the fact that it is the first study to investigate the role of the GGT5-GGG-P2RY8 axis in leukocyte compartmentalisation within inflamed non-lymphoid tissues, with a particular focus on its involvement in mechanism underlying perivascular cuffing of lymphocytes.

A broader implication of this is that classical SLO-derived retention cues may be repurposed during inflammation in ways that are context-dependent rather than universally applied across tissues. This highlights that pathways considered 'canonical' in lymphoid biology may behave unpredictably in inflamed non-lymphoid tissues, where stromal composition, extracellular matrix and local metabolite gradients differ substantially from the SLO environment.

One notable observation is that peripheral T and B cells do not respond to inhibitory action of GGG, suggesting that leukocytes exhibit fundamentally different characteristics and behaviours depending on whether they are circulating in the bloodstream or residing within tissues. Furthermore, T and B cells from synovial fluid may not fully represent the dynamics in the tissue, as these cells could have developed mechanisms that allow them to bypass tissue retention signals, thereby making them present in the synovial fluid. Additionally, broad gating for T and B cell populations in PBMCs and SFMCs might not provide the necessary granularity to identify the specific

subpopulations that respond to GGG. This issue could be addressed by using tissue-derived leukocytes in the transwell migration assays, with a broader spectral flow cytometry panel to more precisely identify the populations involved.

Another important consideration is that peripheral blood and synovial fluid both lack the structural and biochemical constraints present in tissues. This means that absence of GGG responsiveness in these compartments cannot be straightforwardly interpreted as a true biological negative. Without tissue-resident cues such as integrin-activating stromal factors, mechanical confinement or altered metabolic states, circulating leukocytes may simply be in the wrong physiological state to display GGG sensitivity, thereby complicating interpretation.

Interestingly, peripheral CD4⁺ T cells downregulate P2RY8 following activation through CD3/CD28. The term "downregulate" is used because P2RY8 staining is performed intracellularly. Thus, even if P2RY8 is internalised after binding GGG, the antibody would still detect it. This observation aligns with the functional roles of activated T cells, which are antigen-trained and therefore need to migrate freely within tissues to execute their functions effectively. In contrast, naïve T cells, which are untrained, must maintain high P2RY8 levels to allow for their restraint via GGG, reducing the risk of unchecked migration.

However, activation *in vitro* does not fully mimic physiological activation within tissues, where co-stimulation, cytokine milieu and

stromal interactions collectively influence receptor expression. Therefore, the observed P2RY8 downregulation may not reflect the full range of *in vivo* states, and more complex activation contexts, for instance antigen-presenting cell co-culture, may be required to accurately infer how P2RY8 behaves during natural T cell activation.

The assumption that receptor expression levels directly correlate with cellular sensitivity to a ligand may not hold true for P2RY8 and GGG. Despite being regarded as fully differentiated, Tfh cells, which are known to have previously encountered antigens presented on MHCII along with co-stimulatory signals such as ICOS-L, remain the only population observed to stop migrating after encountering GGG, even in the presence of strong chemoattractant such as CXCL12 and CXCL13. This behaviour contrasts with the data showing that activated CD4⁺ T cells downregulate P2RY8, which should have reduced their responsiveness to GGG, suggesting that the migratory and retention mechanisms of Tfh cells are uniquely regulated. It is possible that Tfh cells maintain its sensitivity towards GGG, which might be essential for their role in GC organisation. This discrepancy also suggests that P2RY8 expression levels may not directly correlate with GGG activity.

This divergence raises the possibility that post-translational regulation, receptor conformation, or signalling complex composition, rather than expression level alone, may determine GGG sensitivity. Tfh cells may preserve a specific intracellular signalling architecture that prioritises retention cues, reflecting their requirement for strict

confinement within GCs. Testing this would require assessing downstream signalling events following GGG exposure, such as G-protein activity or second messenger changes, rather than relying solely on receptor abundance.

Importantly, P2RY8 levels in tissue have not been investigated in this study, nor has the expression of P2RY8 on Tfh cells in peripheral blood been examined. Therefore, any conclusions regarding the role of P2RY8 in Tfh cell migration or its sensitivity to GGG should be interpreted with caution. Another limitation of the transwell migration assay involves resting the leukocytes overnight in FBS-containing medium, because FBS contains various proteins, including fibronectin, which can bind to integrins and trigger their activation [303, 304]. This causes the cells to be stickier, which introduces a technical confounder. If cells become excessively adherent prior to the assay, reductions in migration may be misinterpreted as biological inhibition rather than artefacts of altered integrin activation. Future experiments may benefit from serum-free resting conditions or defined integrin-neutral media to ensure that observed GGG-mediated inhibition genuinely reflects chemokinetic changes rather than non-specific adhesion effects.

While the transwell migration assay has proven useful for quantifying leukocyte migration, it does not capture the dynamic behaviours of cells during migration. To address this, a confocal microscopy-based imaging migration assay has been set up to track live cell migration and provide further insight into the migratory

characteristics of leukocytes in response to GGG, though more effort is needed to put into optimising the analysis. Once the specific populations responding to GGG are identified through transwell migration assay, these cells can be analysed for migration speed, directionality, and other dynamic behaviours, offering a more comprehensive understanding of their responses to GGG. Alternatively, commercially available live-cell imaging platforms, such as the Incucyte, could be employed to capture these parameters in real time and analysed using built-in software. Moreover, the integration of CellDive multiplexed tissue imaging to stain for leukocytes responding to GGG could be used to assess the spatial distribution of these leukocytes in tissues relative to GGT5, which will be important in validating the results from migration assay.

Combining dynamic migration readouts with high-dimensional spatial imaging will help disentangle whether GGG acts primarily as a stop signal, a retention cue, or a modulator of responsiveness to chemoattractants. These are mechanistically distinct outcomes, and the transwell assay alone cannot distinguish between them. Integrating multiple approaches would therefore strengthen the conclusions and reduce dependence on a single assay system.

In transwell migration assays, it is observed that migration is inhibited by GGG even in the absence of any chemoattractant, which suggests that GGG is able to stop passive migration through gravity.

However, this inhibitory effect is only observed in certain populations, such as Tfh and GC B cells, for reasons that remain unknown.

This selective inhibition suggests that only a subset of leukocytes may possess the molecular machinery necessary to interpret GGG as a stop cue. Identifying these determinants, whether they relate to cytoskeletal regulation, adhesion receptor expression, or unique GPCR signalling modules, will be crucial for understanding why GGG sensitivity appears highly specialised rather than universal.

In both tonsils and inflamed gut samples, CXCL12 consistently attracts more cells across the transwell filter than CXCL13, suggesting that CXCL12 is a stronger chemoattractant. Notably, B cells only respond to GGG in the presence of CXCL13, while their migration remains largely unaffected by GGG in the presence of CXCL12. This raises the possibility that CXCL12's potent chemoattractive force might override the inhibitory effects of GGG. The interplay between chemoattractant and inhibitory signals appears dynamic. CXCL12 effectively overcomes GGG-mediated inhibition, while CXCL13, being a weaker chemoattractant, is unable to sustain leukocyte migration in the presence of GGG. The balance between these signals determines whether leukocytes migrate or remain in place. This dynamic is particularly relevant for immune cell retention within tissues.

This observation highlights that GGG's effects cannot be interpreted in isolation but must be considered within a hierarchy of competing chemokinetic forces. The fact that only weaker chemokines

reveal the inhibitory capacity of GGG indicates that the axis operates within specific chemotactic niches. Understanding this hierarchy is essential for determining whether GGG primarily fine-tunes positioning within microanatomical structures or exerts broader stop signal activity.

It is known that T cell stops migrating in response to agonist MHC-peptide complexes, marking the initial step in immunological synapse formation [305-307]. This delivery of stop signal through TCR is important for selective retention of antigen-specific T cells in the tissues. However, certain dominant chemokines such as CXCL10, CCL19 and CCL21 can override these TCR-mediated stop signals, whereas others cannot [308]. This hierarchical relationship between chemokines and stop signals may explain why CXCL12 overcomes GGG's inhibitory effects. Nevertheless, Tfh cells remain sensitive to GGG, even in the presence of CXCL12. It is possible that cells tune their sensitivity to GGG depending on tissue conditions, or their differentiation states such as naïve versus memory.

If Tfh cells retain GGG sensitivity despite strong competing chemokines, this suggests a distinct regulatory priority for their confinement. This raises an important hypothesis, that GGG-mediated retention may serve as a dominant positioning mechanism for Tfh cells, superseding other migratory cues. Testing this would involve manipulating chemokine receptor signalling strength, either pharmacologically or via genetic perturbation, to determine whether GGG remains the overriding determinant of Tfh positioning.

To obtain statistically robust data, additional donors for tonsil and inflamed gut samples are required. Furthermore, it may be necessary to perform transwell migration assays using chemokines from different hierarchical levels to fully investigate the effects of GGG. Despite the challenges posed by tissue availability and the number of cells that can be obtained, these studies will provide deeper insights into the role of the GGT5-GGG-P2RY8 axis in leukocyte compartmentalisation during inflammation.

Expanding donor diversity will also help determine whether inter-individual variation, such as prior infection history, age, or baseline immune activation, affects GGG responsiveness. Such variability could mask or exaggerate biological effects in small sample sets, and understanding this variation is essential for drawing generalisable conclusions about the GGT5-GGG-P2RY8 axis across human populations.

Chapter 6: Investigating the role of GGT5 in colitis and arthritis models *in vivo*

6.1 Introduction

It remains unclear whether the expansion of GGT5⁺ region in tissues results in a beneficial or harmful effect in the context of IMIDs. While the role of GGT5 has been studied in relation to GC B cells confinement within SLOs, its contribution to IMIDs has yet to be fully explored. To investigate this, *in vivo* colitis and arthritis models were performed using Ggt5 knockout mice, which were generated in University of California, San Francisco and given to The Jackson Laboratory [252, 253]. These mice lack functional Ggt5 protein because a 264 bp segment surrounding the start codon (ATG) in exon 1 was deleted by CRISPR/Cas9, but they are viable, healthy, and fertile.

While mice express Ggt5 and produces the inhibitory signal Ggg, they do not express P2RY8, the identified receptor for GGG in humans, or its murine equivalent has not been identified. Therefore, there is no evidence of GGG-P2RY8 axis in mice.

As discussed in section 1.8.1, GGT5 plays a role in the leukotrienes pathway, where it converts LTC₄ to LTD₄ [256]. One study shows that LTC₄ and LTD₄ stimulate endothelial cells to produce platelet-activating factor, which subsequently induces adhesion of polymorphonuclear leukocytes to the endothelial cells [309]. When interpreting the *in vivo* colitis and arthritis data, it is crucial to account

for the possibility of parallel signalling pathways. For instance, the two pathways (GGG-P2RY8 and LTC4/LTD4-CysLTR1/CysLTR2) may function concurrently, or one pathway may exert a more dominant influence over the other. This interplay could significantly impact leukocyte behaviour and tissue inflammation, further complicating the interpretation of GGT5's role in IMIDs.

6.2 Results

To verify the genotyping performed using mice ear clips, a small section of the colon was used for RNA extraction followed by qRT-PCR analysis. While variations in Ggt5 expression are observed among wild-type mice, heterozygous mice exhibit generally lower Ggt5 expression levels, and no expression is detected in homozygous mice. This confirms the accurate allocation of genotypes (Figure 6.1). We used qRT-PCR rather than tissue staining because reliable anti-Ggt5 antibodies for mouse FFPE sections are not available in the market. Previously published paper [252] demonstrated Ggt5 detection by RNAscope only in fresh-frozen tissue, whereas our tissues were FFPE. Therefore, qRT-PCR provided the most robust validation in our setting.

The dextran sodium sulfate (DSS) colitis model induces colonic inflammation by disrupting the epithelial barrier of the gut, allowing luminal bacteria and antigens to infiltrate the mucosa and trigger an immune response. This results in acute inflammation that mimics aspects of human IBD, including epithelial damage, immune cell infiltration, and cytokine production [310].

After induction of DSS colitis, the mice started to lose weight from about day 5, and by day 7 most of the mice have lost about 20% of weight. The change in weight is not significantly different across different genotypes (Figure 6.2).

These mice do not have significantly different spleen weight (Figure 6.3a), suggesting similar levels of systemic inflammation. The

mice colons of different genotypes were stained with haematoxylin and eosin (H&E) for histological analysis. The scoring was performed by Mathilde Pohin in a blinded manner, evaluating the amount of inflammation, extent of inflammation and amount of crypt damage. The affected areas were also quantified based on the percentage of tissue involvement by the disease process. Amount of regeneration was not included in this scoring system [311].

There is no significant difference in the sum histological scores for proximal/mid colon and distal colon across different genotypes (Figure 6.3b, c), indicating similar degree of inflammation and amount of crypt damage in regions among all mice. The percentage of tissue involvement is also similar across groups. Notably, the distal colon shows higher sum histological scores compared to the proximal/mid colon, which aligns with the fact that DSS-induced pathology is specifically confined to the distal colon where microbial density is greatest [312]. Representative H&E images of the distal colon from wild-type, heterozygous, and homozygous knockout mice are shown in Figure 6.3d.

In the proximal/mid colon, there is no significant difference in either the area or the number of lymphoid aggregates (Figure 6.4a, b). In the distal colon, the area of lymphoid aggregates appears larger in the homozygous colon, but the difference is not statistically significant (Figure 6.4c). Similarly, no significant difference is observed in the number of lymphoid aggregates in the distal colon (Figure 6.4d). The

area of lymphoid aggregates was quantified by first annotating them in QuPath, after which the annotated regions were directly measured.

Given that lymphoid aggregates and TLS are naturally present in the steady-state colon, and this DSS experiment did not include a healthy control, the data should be interpreted with caution. Representative H&E images of the colon from wild-type, heterozygous, and homozygous knockout mice are shown in Figure 6.4e, with lymphoid aggregates highlighted in yellow.

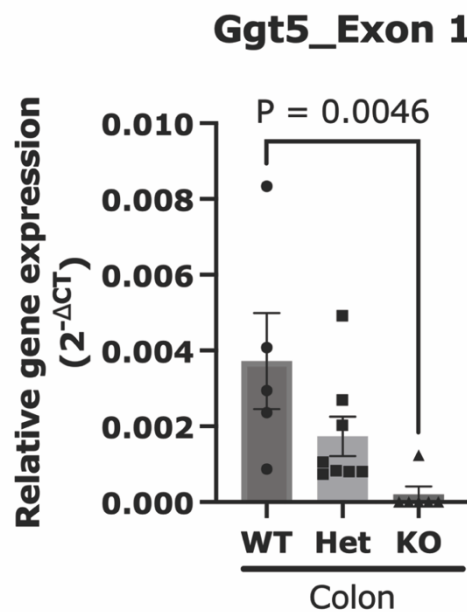


Figure 6.1: Relative expression of Ggt5 in the colon of wildtype, heterozygous and Ggt5 knockout mice. Relative gene expression is displayed as $2^{-\Delta CT}$. Graphs are plotted as means $\pm$ SD; statistical significance is tested using non-parametric Kruskal-Wallis test and Dunn's multiple comparison test.

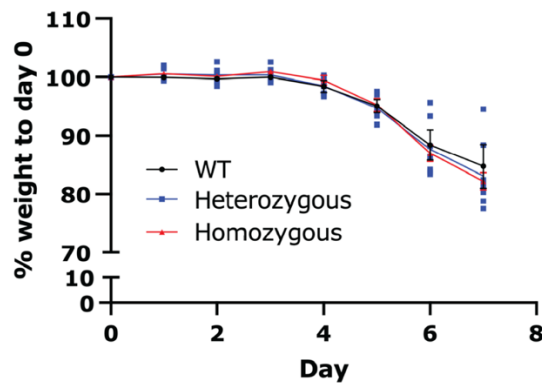


Figure 6.2: The effect of 2.5% DSS colitis on *Ggt5* wildtype, heterozygous and homozygous mice weight. Weight of mice under DSS colitis measured from day 0-7, presented as percentage of change relative to weight on day 0. The experiment included *Ggt5* wild-type (WT, $n = 5$), heterozygous (Het, $n = 8$), and homozygous knockout (Hom, $n = 6$) mice.

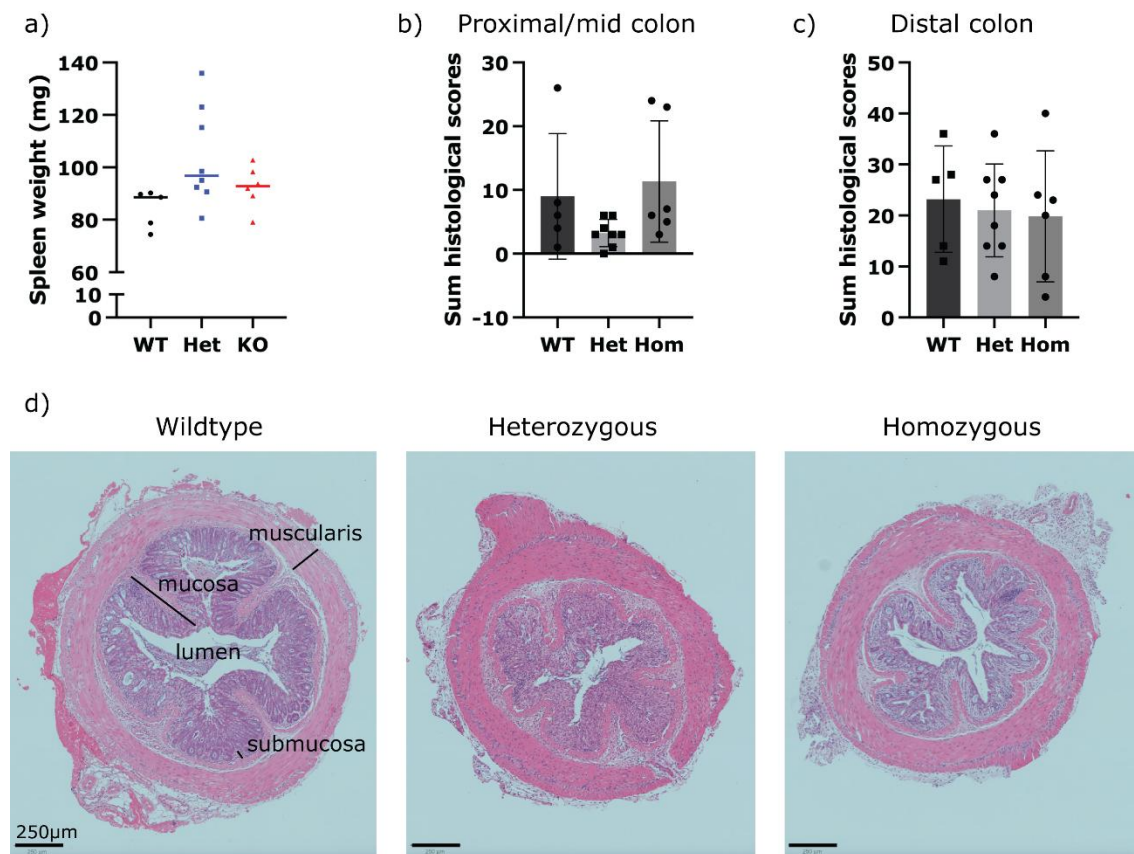
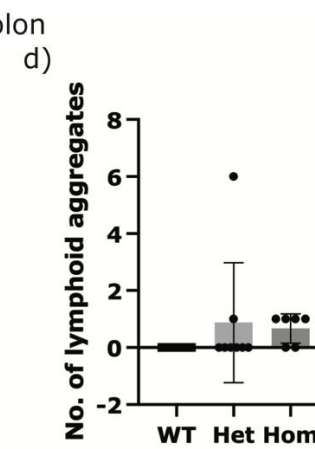
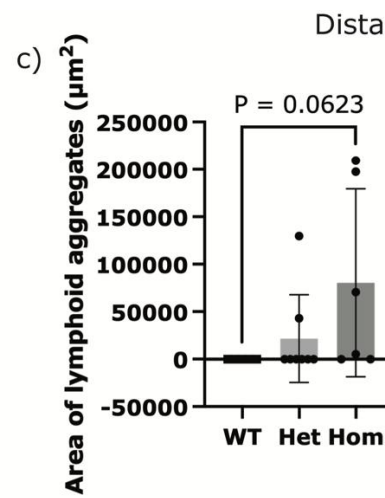
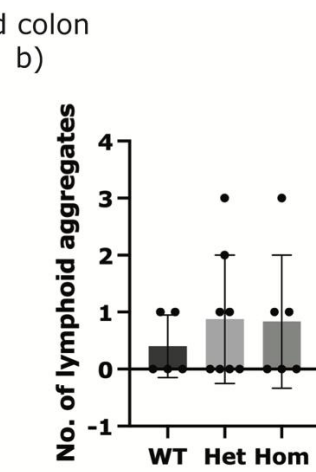
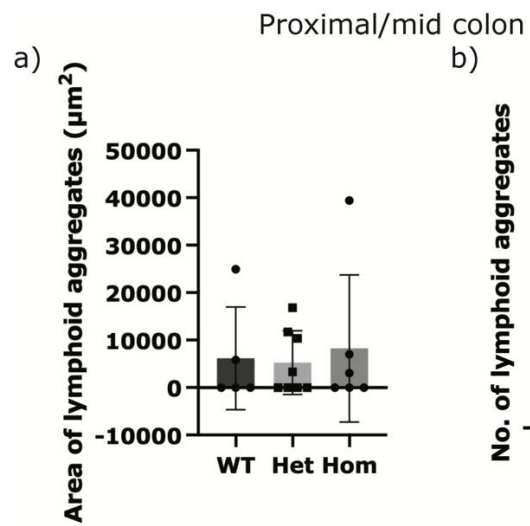


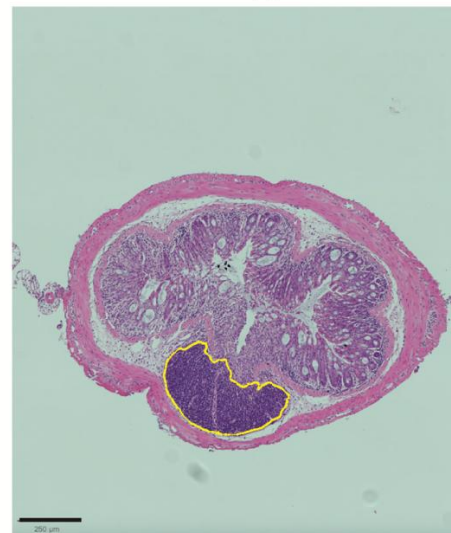
Figure 6.3: Spleen weight and histological scoring on DSS colon of different genotype. The experiment included *Ggt5* wild-type (WT, $n = 5$), heterozygous (Het, $n = 8$), and homozygous knockout (Hom, $n = 6$) mice. (a) Spleen weight of each genotype. Sum histological scores of (b) proximal/mid and (c) distal colon. Scoring was performed by Mathilde Pohin. Graphs are plotted as means $\pm$ SD; statistical significance is tested using non-parametric Kruskal-Wallis test and Dunn's multiple comparison test. (d) Representative H&E images of distal colon from wildtype, heterozygous and homozygous knockout mice. The colon lumen, mucosa (including epithelium and lamina propria), submucosa, and muscularis layers are annotated. Scale bar = 250 μ m.



e) Wildtype



Heterozygous



Homozygous



Figure 6.4: Area and number of lymphoid aggregates in DSS colon of different genotype. (a-b) Proximal/mid colon. (c-d) Distal colon. The experiment included *Ggt5* wild-type (WT, n = 5), heterozygous (Het, n = 8), and homozygous knockout (Hom, n = 6) mice. Graphs are plotted as means $\pm$ SD; statistical significance is tested using non-parametric Kruskal-Wallis test and Dunn's multiple comparison test. (e) Representative H&E images of colon from wildtype, heterozygous and homozygous knockout mice. Area highlighted in yellow shows lymphoid aggregates. Scale bar = 250 μ m.

To further investigate the effects of Ggt5 knockout in a colitis model, a portion of the colon was harvested, digested, and analysed using flow cytometry to analyse leukocyte infiltration.

Normality of the flow cytometry data was assessed using the Shapiro–Wilk test for each cell population across wildtype, heterozygous, and homozygous mice. While some populations fit a normal distribution, others displayed notable deviations from normality ($p < 0.05$). This indicates a violation of this assumption (Table 6.1). Given that parametric tests require normally distributed data and that this condition was not consistently met across all flow cytometry populations, non-parametric statistical tests were selected. This approach ensures a valid comparison between groups without relying on assumptions that may not hold for the dataset.

The gating strategy first involved selecting live Cd45⁺ cells, followed by additional gating to identify various T cell subpopulations, as shown in Figure 6.5a. In homozygous Ggt5 knockout mice, a significant reduction in Rorgt⁺ Th17 cells is observed in the colon compared to wildtype mice (Figure 6.5b). This finding highlights a potential role of Ggt5 in maintaining or recruiting Th17 cells to the inflamed colon. However, no significant difference is observed in other T cell subpopulations across genotypes. It is unclear the effect of Ggt5 knockout may be specific to Th17 cells under these experimental conditions, because the panel is not extensive enough to include other T cell subpopulations such as Tfh.

Further gating is performed to examine Cd11b⁻B220⁺ B cells in the colon, as illustrated in Figure 6.6a. Analysis of B cell subpopulations reveals no significant difference among genotypes (Figure 6.6b). However, there is a slight decrease in the number of IgG⁺ B cells and an increase in the percentage of IgA⁺ B cells in the homozygous Ggt5 knockout mice compared to wildtype controls, suggesting that Ggt5 may influence B cell isotype switching, favouring IgA production while reducing IgG-producing B cells in the colon, potentially impacting mucosal immune responses and barrier function. Notably, the observation that approximately 40% of colonic B cells are IgD⁺ naive cells is unexpected, as naive B cells are typically abundant in the spleen and blood, whereas the gut environment is usually enriched in antigen-experienced B cells. This raises the possibility that the apparent increase may reflect blood contamination during tissue processing rather than a true tissue-resident population.

Similarly, myeloid cells from the colon are gated as shown in Figure 6.7a. Analysis of these populations shows no significant differences among the genotypes (Figure 6.7b), indicating that the absence of functional Ggt5 does not impact the myeloid cell populations within the colon.

	Shapiro-Wilk test p value		
	Wildtype	Heterozygous	Homozygous
Cd3 ⁺ T cells	0.4471	0.0590	0.3188
Cd4 ⁺ T cells	0.4929	0.3757	0.1203
Cd4 ⁺ Foxp3 ⁺ Tregs	0.3674	0.0799	0.6478
Rorgt ⁺ Th17	0.1863	0.0030	0.9361
Cd8 ⁺ T cells	0.5125	0.5121	0.8107
Tbet ⁺ Th1	0.0833	0.0206	0.0014
Cd19 ⁺ B cells	0.4673	0.2482	0.4203
GL7 ⁺ GC B cells	0.3004	0.2972	0.4162
Iga ⁺ B cells	0.9719	0.9583	0.4451
Igd ⁺ naïve B cells	0.8541	0.2776	0.7932
Igg ⁺ B cells	0.5098	0.6357	0.0017
Ly6g ⁺ neutrophils	0.5126	0.0022	0.3446
SiglecF ⁺ eosinophils	0.5754	0.0748	0.4812
Ly6c ⁺ MhcII ⁻ monocytes	0.5467	0.0827	0.0544

MhcII ⁺ Ly6c ⁺ inflammatory monocytes	0.6289	0.0018	0.4879
Cd11b ⁺ Cd11c ⁺ MhcII ⁺ DCs	0.4060	0.0838	0.0812
Macrophages	0.6169	0.0424	0.0740
Cd11b ⁻ Cd11c ⁺ MhCII ⁺ DCs	0.5608	0.0337	0.1620

Table 6.1: Normality test results for T, B, and myeloid cells from mouse colon across wildtype, heterozygous, and homozygous genotypes. Results of the Shapiro–Wilk normality test for T, B, and myeloid cell populations isolated from the colon of wildtype, heterozygous, and homozygous mice. p-values < 0.05 (shown in bold) are considered statistically significant and therefore indicate that the corresponding data do not meet the assumption of a normal distribution.

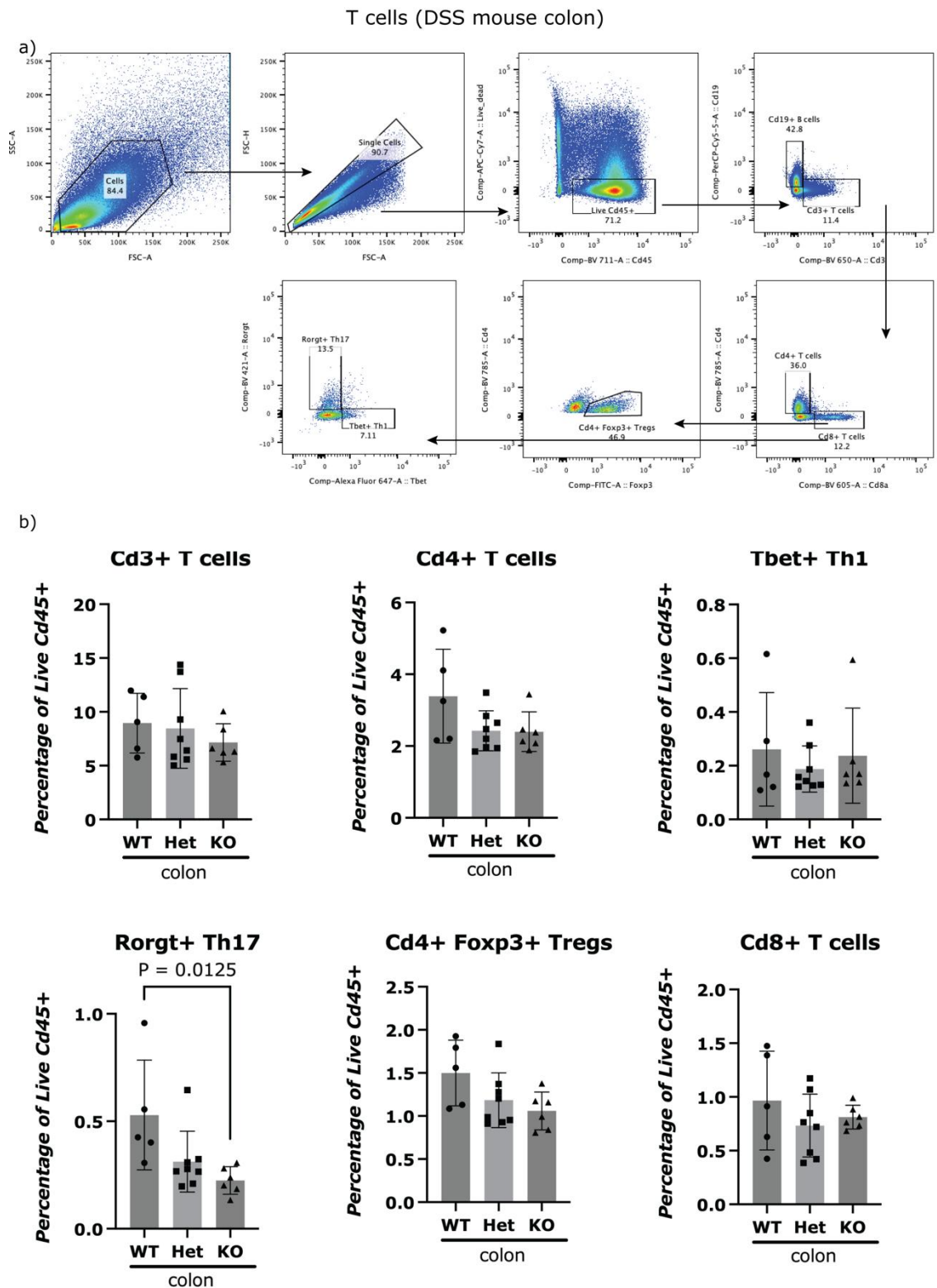
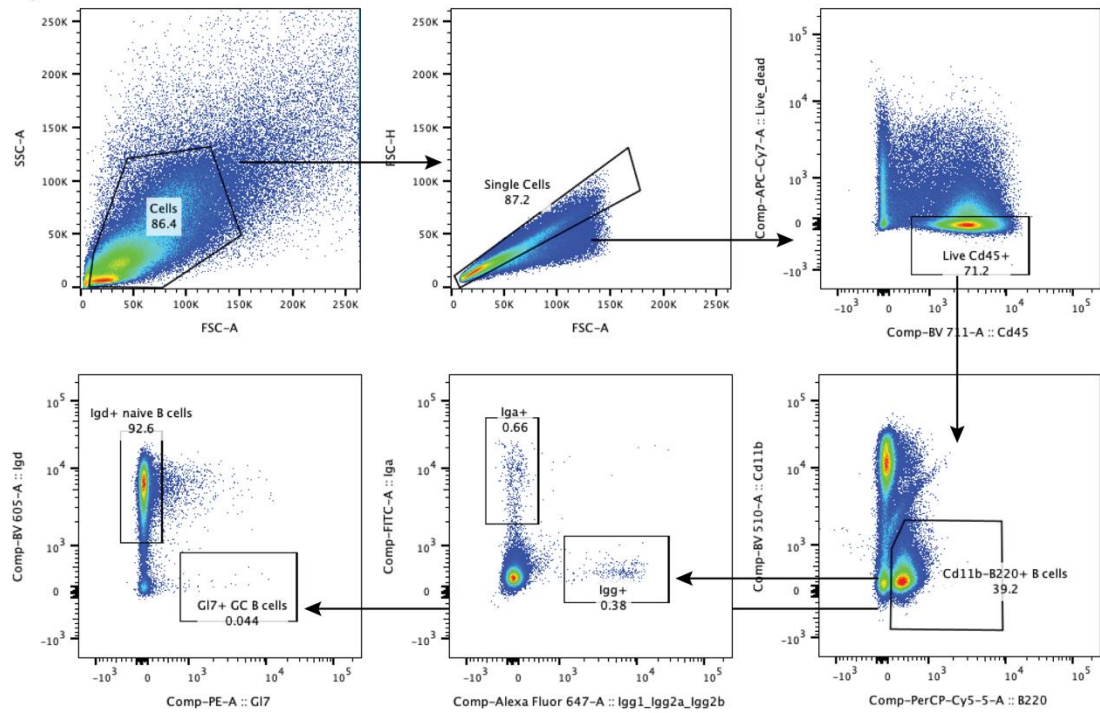


Figure 6.5: The effect of 2.5% DSS colitis on mouse colonic T cells across genotypes. (a) Representative flow cytometry gating for T cells using colon from wildtype mice. Cells were first gated to exclude debris,

followed by selection of single cells and live Cd45⁺ leukocytes (Live/Dead stain-negative, Cd45⁺). From live Cd45⁺ cells, Cd3⁺ T cells and Cd19⁺ B cells were identified. Cd3⁺ T cells were further subdivided into Cd4⁺ and Cd8⁺ populations. Within Cd4⁺ T cells, Foxp3⁺ Tregs were identified; in parallel, Cd4⁺ T cells were gated for Rorγt⁺ Th17 cells and T-bet⁺ Th1 cells. (b) Flow cytometry data for colonic T cell subpopulations (Cd3⁺ T cells, Cd4⁺ T cells, T-bet⁺ Th1 cells, Rorγt⁺ Th17 cells, Cd4⁺Foxp3⁺ Tregs and Cd8⁺ T cells) of different genotypes, presented as percentage of live Cd45⁺ cells. Graphs are plotted as means ± SD; statistical significance is tested using non-parametric Kruskal-Wallis test and Dunn's multiple comparison test. The experiment included *Ggt5* wild-type (WT, n = 5), heterozygous (Het, n = 8), and homozygous knockout (Hom, n = 6) mice.

B cells (DSS mouse colon)

a)



b)

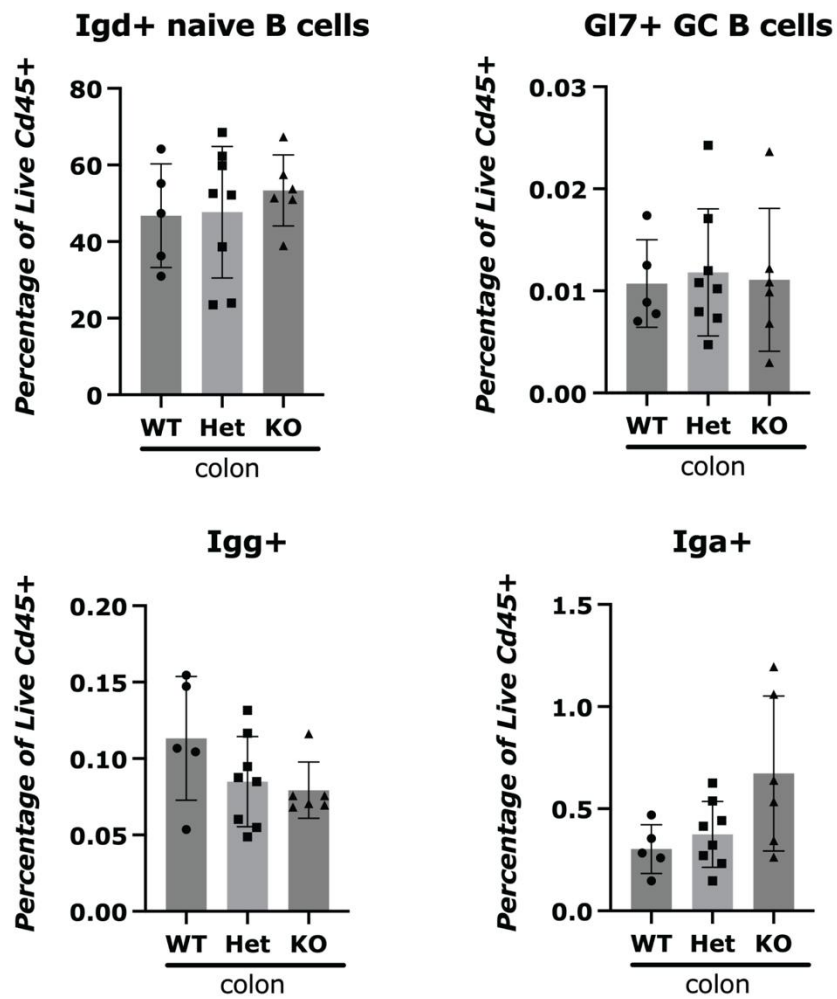


Figure 6.6: The effect of 2.5% DSS colitis on mouse colonic B cells across genotypes. (a) Representative flow cytometry gating for B cells using colon from wildtype mice. Cells were first gated to exclude debris, followed by selection of single cells and live Cd45⁺ leukocytes (Live/Dead stain-negative, Cd45⁺). From live Cd45⁺ cells, Cd11b-B220⁺ B cells were identified. B cells were further gated to identify IgA⁺ and IgG⁺ populations; in parallel, B cells were also gated for IgD⁺ naïve B cells and GL7⁺ GC B cells. (b) Flow cytometry data for colonic B cell subpopulations (IgD⁺ naïve B cells, GL7⁺ GC B cells, IgA⁺ and IgG⁺ populations) of different genotypes, presented as percentage of live Cd45⁺ cells. Graphs are plotted as means $\pm$ SD; statistical significance is tested using non-parametric Kruskal-Wallis test and Dunn's multiple comparison test. The experiment included *Ggt5* wild-type (WT, n = 5), heterozygous (Het, n = 8), and homozygous knockout (Hom, n = 6) mice.

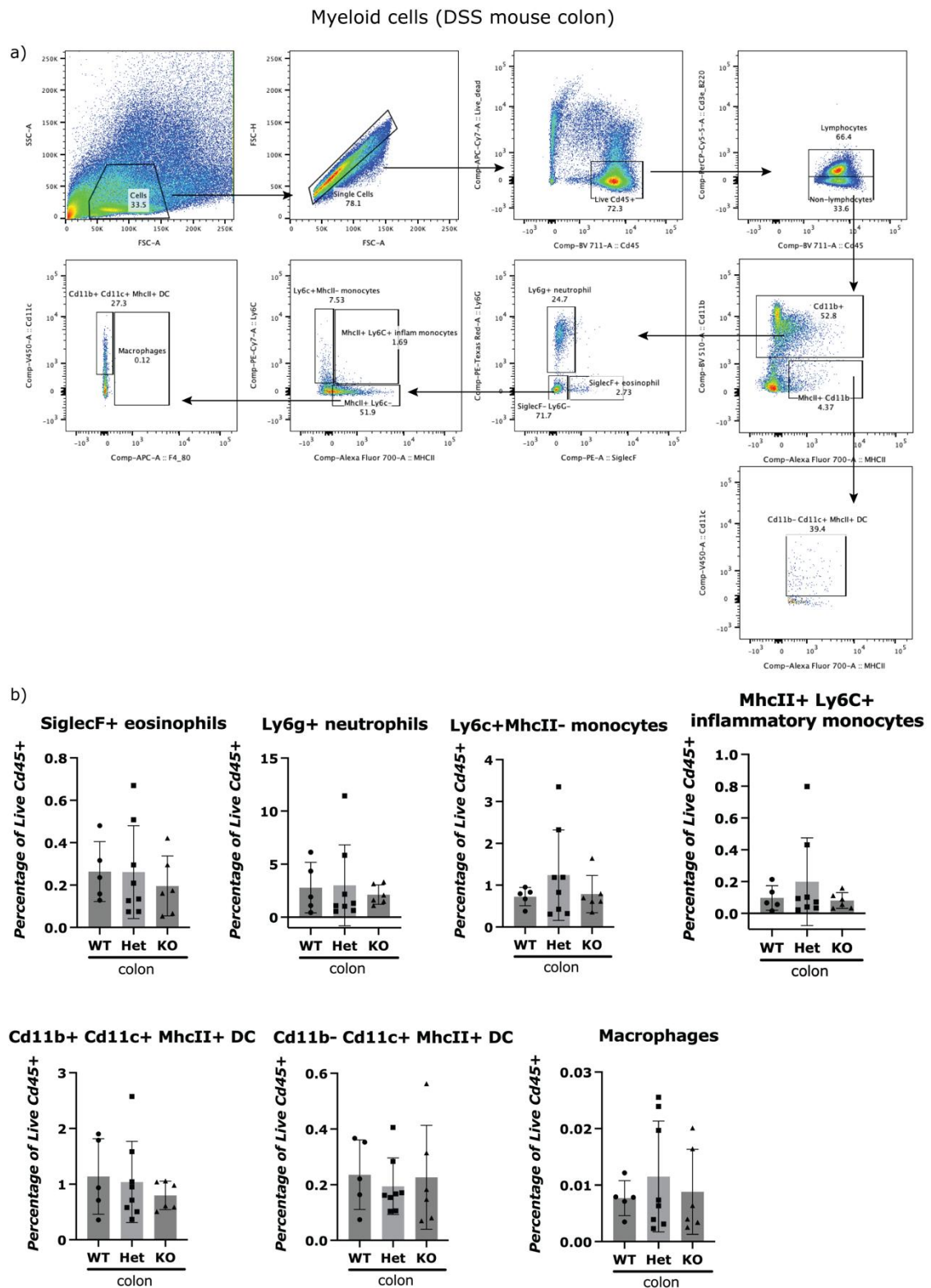


Figure 6.7: The effect of 2.5% DSS colitis on mouse colonic myeloid cells across genotypes. (a) Representative flow cytometry gating for myeloid cells using colon from wildtype mice. Cells were first gated to

exclude debris, followed by selection of single cells and live Cd45⁺ leukocytes (Live/Dead stain-negative, Cd45⁺). From live Cd45⁺ cells, non-lymphocytes were identified by exclusion of Cd3e⁺ and B220⁺ cells. The non-lymphocyte population was then subdivided into Cd11b⁺ and MhcII⁺Cd11b⁻ populations. The MhcII⁺Cd11b⁻ population was gated to identify Cd11b⁻Cd11c⁺MhcII⁺ DCs. Within the Cd11b⁺ fraction, Ly6g⁺ neutrophils and SiglecF⁺ eosinophils were identified. The remaining SiglecF⁻Ly6g⁻ cells were further gated to identify Ly6c⁺MhcII⁻ monocytes, MhcII⁺Ly6c⁺ inflammatory monocytes, and MhcII⁺Ly6c⁻ populations. From the MhcII⁺Ly6c⁻ population, Cd11b⁺Cd11c⁺MhcII⁺ DCs and F4/80⁺ macrophages were identified. (b) Flow cytometry data for colonic myeloid cells (SiglecF⁺ eosinophils, Ly6g⁺ neutrophils, Ly6c⁺MhcII⁻ monocytes, MhcII⁺Ly6c⁺ inflammatory monocytes, Cd11b⁺Cd11c⁺MhcII⁺ DCs, Cd11b⁻Cd11c⁺MhcII⁺ DCs, and F4/80⁺ macrophages) of different genotypes, presented as percentage of live Cd45⁺ cells. Graphs are plotted as means $\pm$ SD; statistical significance is tested using non-parametric Kruskal-Wallis test and Dunn's multiple comparison test. The experiment included *Ggt5* wild-type (WT, n = 5), heterozygous (Het, n = 8), and homozygous knockout (Hom, n = 6) mice.

Next, the immune cell composition of mesenteric lymph nodes (mLNs) draining the colon is also investigated. The gating strategy first involves selecting live Cd45⁺ cells, followed by further gating to identify T cell subpopulations, as shown in Figure 6.8a. In the mLNs of homozygous Ggt5 knockout mice, there is no significant differences observed among T cell subpopulations when compared to wildtype mice (Figure 6.8b). This suggests that Ggt5 knockout may not affect T cell distribution in mLNs.

The Cd11b⁻B220⁺ B cells from mLNs are gated as illustrated in Figure 6.9a. Similarly, no significant differences are detected in the B cell subpopulations across the genotypes. However, a downward trend is observed for Gl7⁺ GC B cells and IgG⁺ B cells in the homozygous Ggt5 knockout mLNs compared to wildtype mice (Figure 6.9b). This suggests a potential reduction in GC activity and class-switched antibody-producing B cells, though the difference is not statistically significant.

Myeloid cells from mLNs are gated as shown in Figure 6.10a. Notably, there is a significant increase in Ly6G⁺ neutrophils in the mLNs of homozygous Ggt5 knockout mice compared to wildtype mice (Figure 6.10b). This finding highlights a specific expansion of neutrophils in mLNs in the absence of functional Ggt5. However, the absolute number of Ly6G⁺ neutrophils is very low, and the biological relevance of this increase should be interpreted with caution. No significant differences

are observed among other myeloid subpopulations across genotypes, suggesting that this effect may be specific to neutrophils.

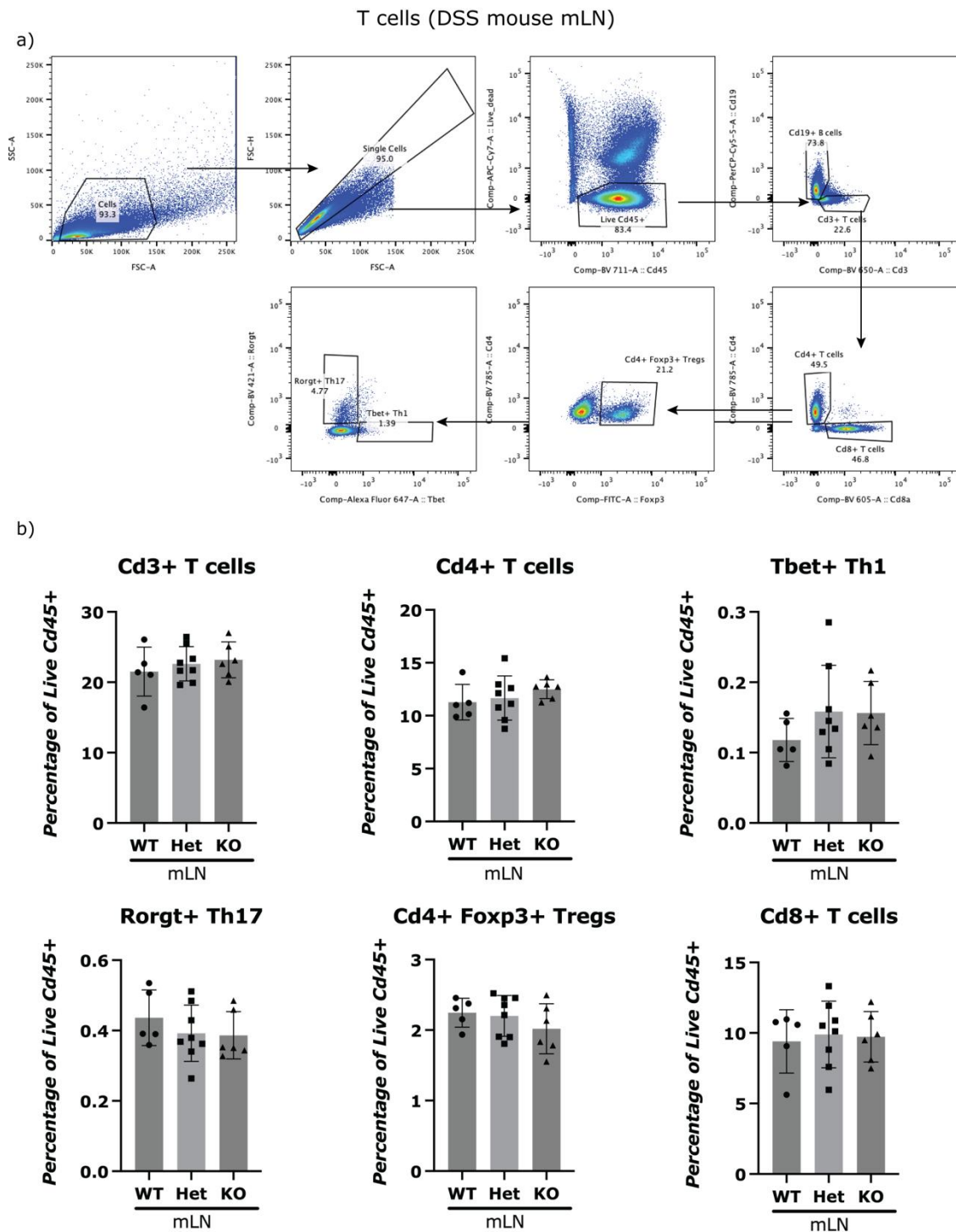
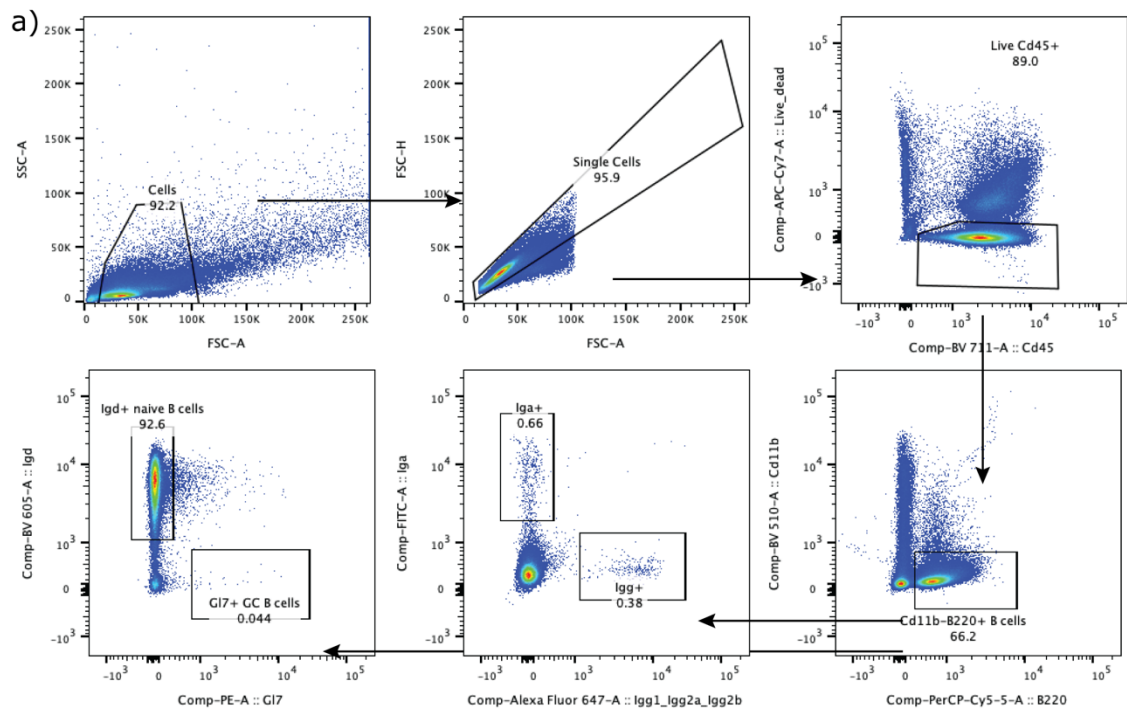


Figure 6.8: The effect of 2.5% DSS colitis on mouse mLN T cells across genotypes. (a) Representative flow cytometry gating for T cells using mLN from wildtype mice. Cells were first gated to exclude debris,

followed by selection of single cells and live Cd45⁺ leukocytes (Live/Dead stain-negative, Cd45⁺). From live Cd45⁺ cells, Cd3⁺ T cells and Cd19⁺ B cells were identified. Cd3⁺ T cells were further subdivided into Cd4⁺ and Cd8⁺ populations. Within Cd4⁺ T cells, Foxp3⁺ Tregs were identified; in parallel, Cd4⁺ T cells were gated for Rorγt⁺ Th17 cells and T-bet⁺ Th1 cells. (b) Flow cytometry data for mLN T cell subpopulations (Cd3⁺ T cells, Cd4⁺ T cells, T-bet⁺ Th1 cells, Rorγt⁺ Th17 cells, Cd4⁺Foxp3⁺ Tregs and Cd8⁺ T cells) of different genotypes, presented as percentage of live Cd45⁺ cells. Graphs are plotted as means ± SD; statistical significance is tested using non-parametric Kruskal-Wallis test and Dunn's multiple comparison test. The experiment included *Ggt5* wild-type (WT, n = 5), heterozygous (Het, n = 8), and homozygous knockout (Hom, n = 6) mice.

B cells (DSS mouse mLN)



b)

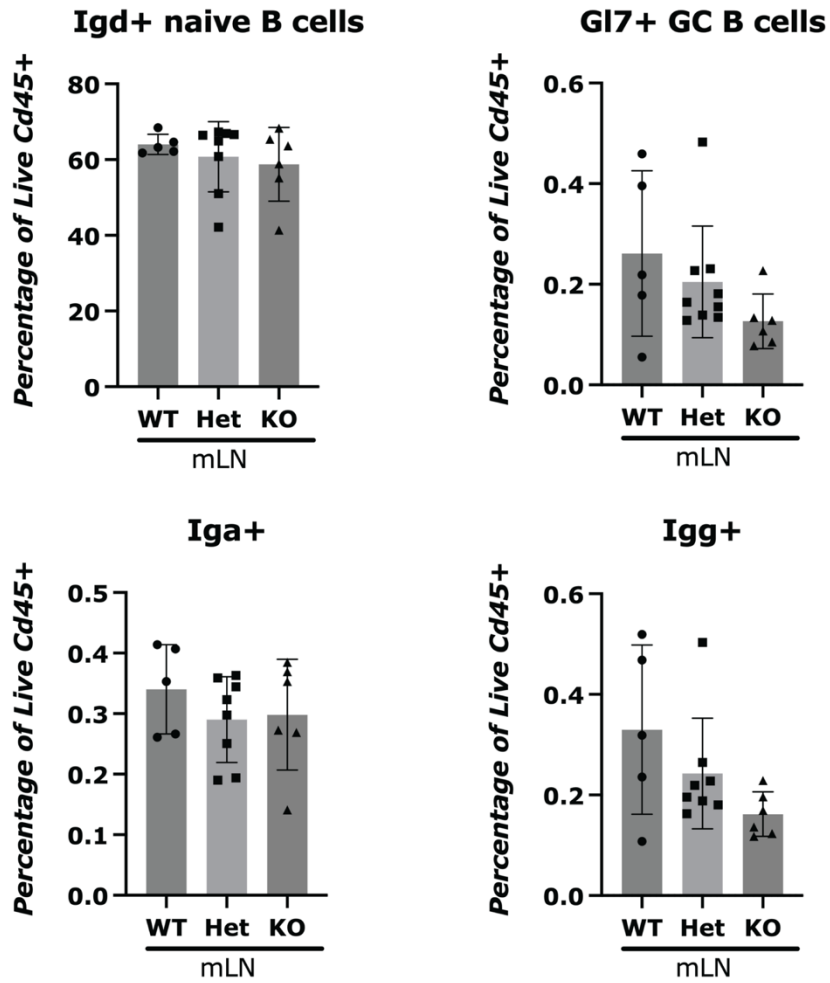


Figure 6.9: The effect of 2.5% DSS colitis on mouse mLN B cells across genotypes. (a) Representative flow cytometry gating for B cells using mLN from wildtype mice. Cells were first gated to exclude debris, followed by selection of single cells and live Cd45⁺ leukocytes (Live/Dead stain-negative, Cd45⁺). From live Cd45⁺ cells, Cd11b-B220⁺ B cells were identified. B cells were further gated to identify IgA⁺ and IgG⁺ populations; in parallel, B cells were also gated for IgD⁺ naïve B cells and GL7⁺ GC B cells. (b) Flow cytometry data for mLN B cell subpopulations (IgD⁺ naïve B cells, GL7⁺ GC B cells, IgA⁺ and IgG⁺ populations) of different genotypes, presented as percentage of live Cd45⁺ cells. Graphs are plotted as means $\pm$ SD; statistical significance is tested using non-parametric Kruskal-Wallis test and Dunn's multiple comparison test. The experiment included *Ggt5* wild-type (WT, n = 5), heterozygous (Het, n = 8), and homozygous knockout (Hom, n = 6) mice.

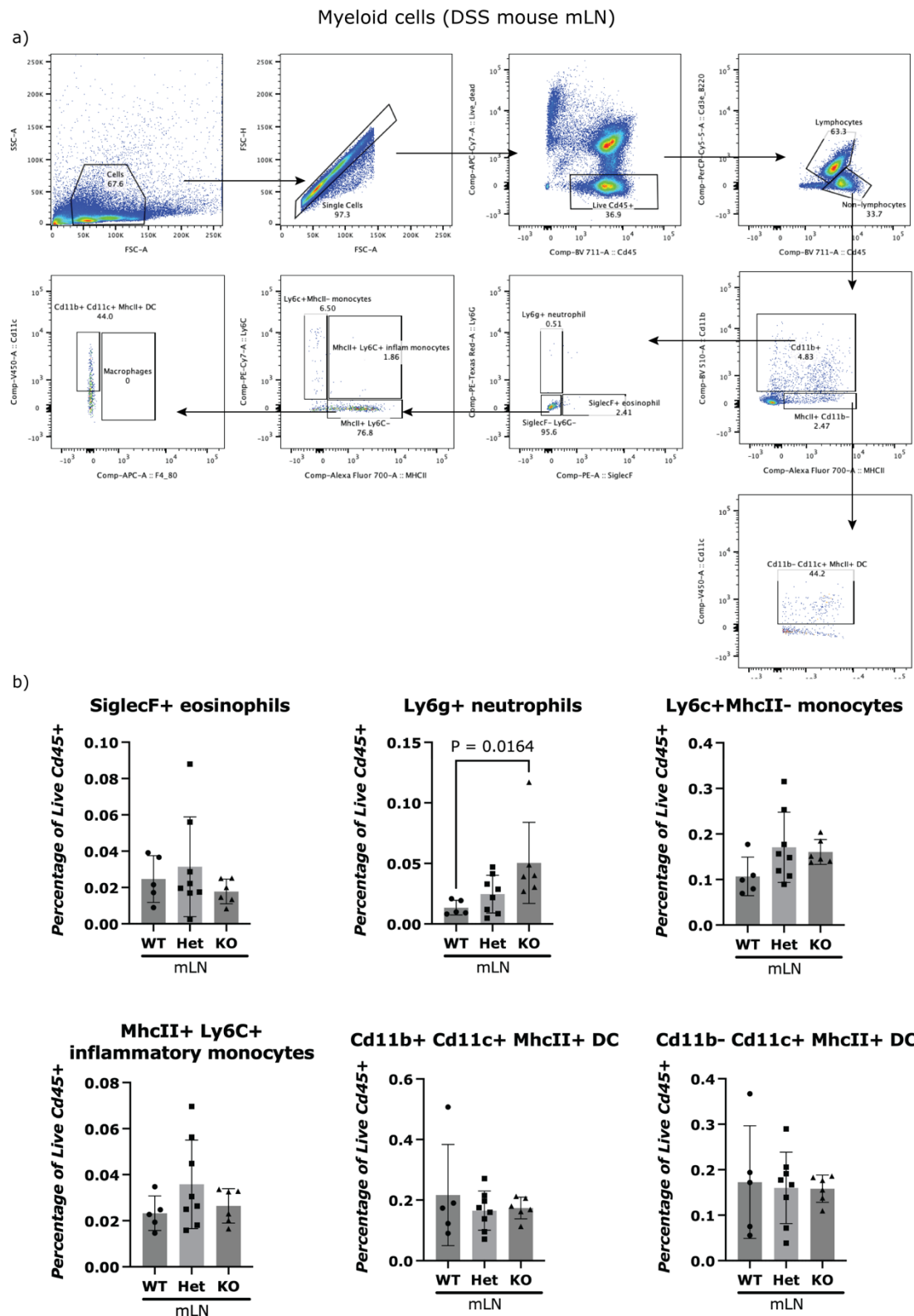


Figure 6.10: The effect of 2.5% DSS colitis on mouse mLN myeloid cells across genotypes. (a) Representative flow cytometry gating for myeloid cells using mLN from wildtype mice. Cells were first gated to

exclude debris, followed by selection of single cells and live Cd45⁺ leukocytes (Live/Dead stain-negative, Cd45⁺). From live Cd45⁺ cells, non-lymphocytes were identified by exclusion of Cd3e⁺ and B220⁺ cells. The non-lymphocyte population was then subdivided into Cd11b⁺ and MhcII⁺Cd11b⁻ populations. The MhcII⁺Cd11b⁻ population was gated to identify Cd11b⁻Cd11c⁺MhcII⁺ DCs. Within the Cd11b⁺ fraction, Ly6g⁺ neutrophils and SiglecF⁺ eosinophils were identified. The remaining SiglecF⁻Ly6g⁻ cells were further gated to identify Ly6c⁺MhcII⁻ monocytes, MhcII⁺Ly6c⁺ inflammatory monocytes, and MhcII⁺Ly6c⁻ populations. From the MhcII⁺Ly6c⁻ population, Cd11b⁺Cd11c⁺MhcII⁺ DCs and F4/80⁺ macrophages were identified. (b) Flow cytometry data for mLN myeloid cells (SiglecF⁺ eosinophils, Ly6g⁺ neutrophils, Ly6c⁺MhcII⁻ monocytes, MhcII⁺Ly6c⁺ inflammatory monocytes, Cd11b⁺Cd11c⁺MhcII⁺ DCs, and Cd11b⁻Cd11c⁺MhcII⁺ DCs) of different genotypes, presented as percentage of live Cd45⁺ cells. Graphs are plotted as means $\pm$ SD; statistical significance is tested using non-parametric Kruskal-Wallis test and Dunn's multiple comparison test. The experiment included *Ggt5* wild-type (WT, n = 5), heterozygous (Het, n = 8), and homozygous knockout (Hom, n = 6) mice.

Given that GGT5 is strongly correlated with vasculature in human IBD and RA, it is important to explore similar pathways in the mouse model. To this end, qPCR analysis was performed to investigate whether Ggt5 knockout affects the expression of key vascular genes in the colon across genotypes. The results show no significant difference in the expression of key vessel-associated genes, including *Pecam1*, *Vcam1*, *Madcam1*, *Icam1* and *Thy1* (Figure 6.11a-e). Similarly, the expression of inflammatory cytokines and chemokines, such *Tnf*, *Ccl19*, *Il6*, *Il1b*, *Ifng*, and *Cxcl9* (Figure 6.12a-f), are not statistically different across genotypes. Given that RNA was extracted from a segment of the colon rather than from specific niches, these findings suggest that Ggt5 knockout has a limited global effect on vascular gene expression and inflammatory mediators in the colon.

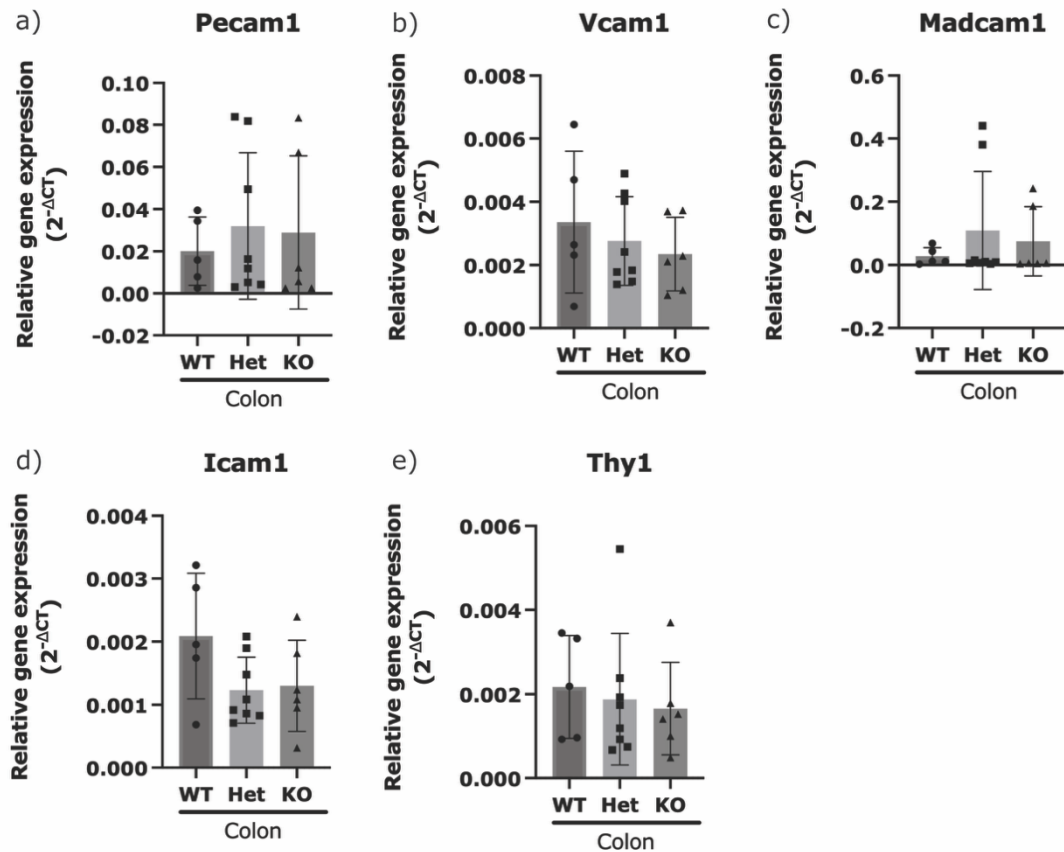


Figure 6.11: Relative gene expressions of blood vessel related genes in colon of wildtype, heterozygous and *Ggt5* knockout mice, including (a) *Pecam1*, (b) *Vcam1*, (c) *Madcam1*, (d) *Icam1* and (e) *Thy1*. Relative gene expression is displayed as 2^{-ΔCt}. Graphs are plotted as means ± SD; statistical significance is tested using non-parametric Kruskal-Wallis test and Dunn's multiple comparison test. The experiment included *Ggt5* wild-type (WT, n = 5), heterozygous (Het, n = 8), and homozygous knockout (Hom, n = 6) mice.

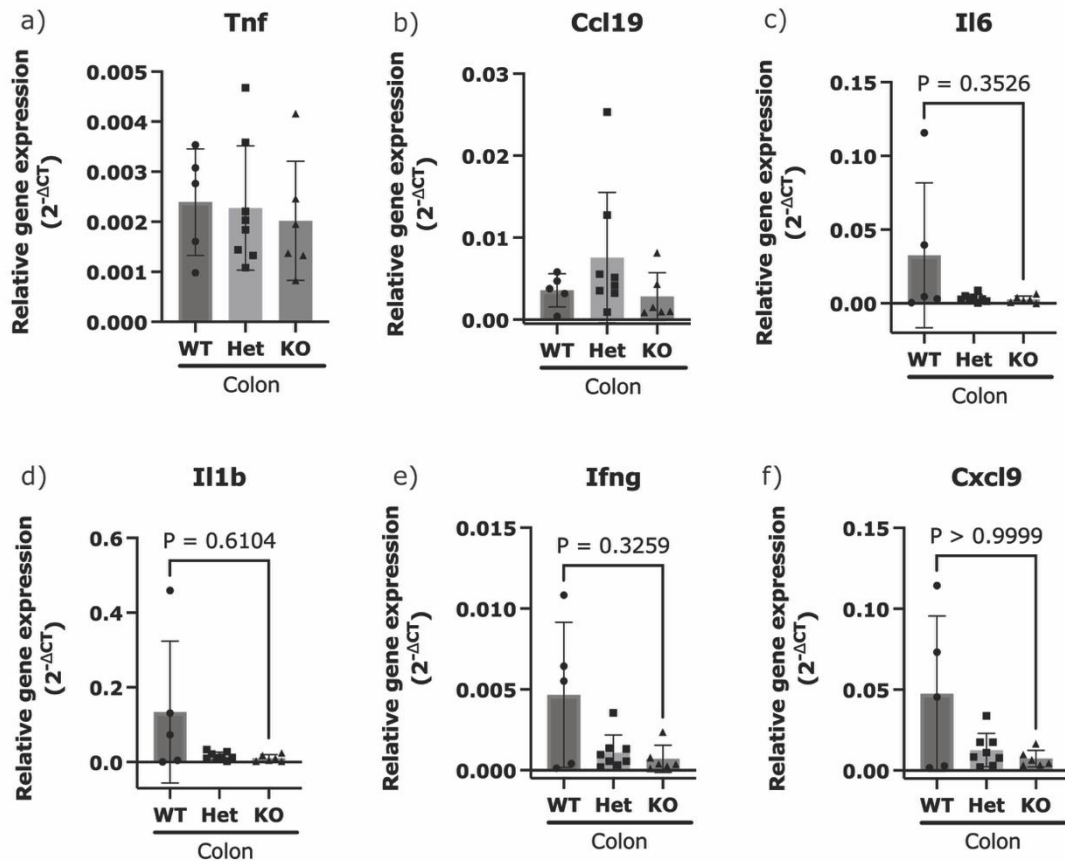


Figure 6.12: Relative gene expressions of inflammatory cytokine and chemokine in colon of wildtype, heterozygous and *Ggt5* knockout mice, including (a) *Tnf*, (b) *Ccl19*, (c) *Il6*, (d) *Il1b*, (e) *Ifng*, and (e) *Cxcl9*. Relative gene expression is displayed as 2^{-ΔCT}. Graphs are plotted as means ± SD; statistical significance is tested using non-parametric Kruskal-Wallis test and Dunn's multiple comparison test. The experiment included *Ggt5* wild-type (WT, n = 5), heterozygous (Het, n = 8), and homozygous knockout (Hom, n = 6) mice.

The antigen-induced arthritis (AIA) is induced by systemic immunisation with an immunogenic antigen, in this case methylated BSA (mBSA) in complete Freund's adjuvant (CFA), and subsequent intra-articular injection of mBSA into the knee joint. This process induces a robust immune response characterised by synovial hyperplasia, immune cell infiltration, and cartilage degradation, mimicking features of human RA [271, 313].

The mice knees of different genotypes were stained with H&E and safranin O for histological analysis. The histological scoring was performed by Kristina Zec in a blinded manner, using criteria that assessed synovial lining hyperplasia, synovial sublining hyperplasia, bone marrow proliferation and cartilage damage following the OARSI grading system. Representative images of the safranin O-stained left knees from wild-type, heterozygous, and homozygous knockout mice are shown in Figure 6.13c.

In this model, it is observed that the left knees display significantly higher histology scores compared to the right knees, indicating the successful induction of AIA (Figure 6.13a and b). However, when comparing the histology scores of the left knees across different genotypes, no significant difference is observed (Figure 6.13b). Additional staining for lymphocyte and myeloid cell markers would provide further information on the inflammatory infiltrates and could help determine whether differences in immune cell composition

exist between genotypes beyond what is captured by histological scoring.

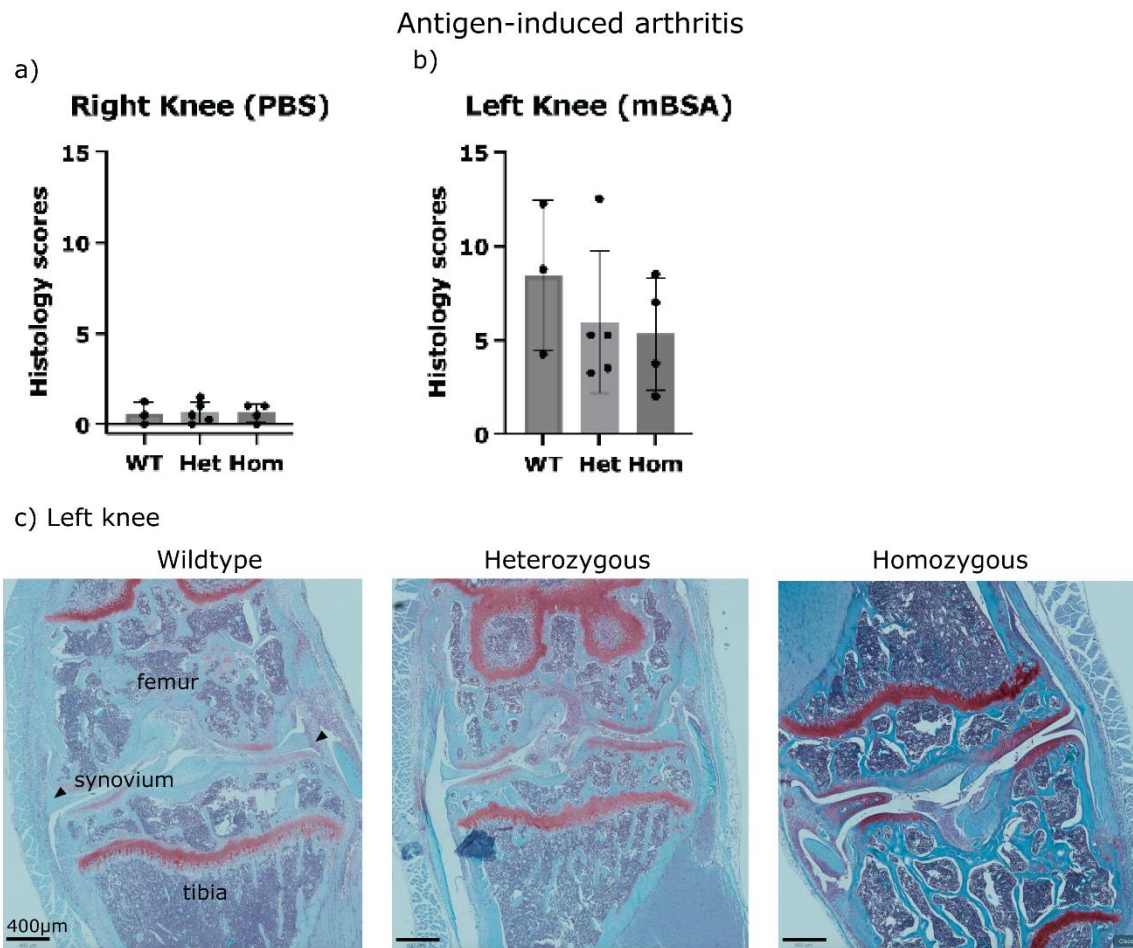


Figure 6.13: Histological scoring on antigen-induced arthritis knees of different genotype. (a) Right knee was injected with PBS as control, and (b) left knee was injected with mBSA. The scoring was performed by Kristina Zec. The experiment included Ggt5 wild-type (WT, $n = 3$), heterozygous (Het, $n = 5$), and homozygous knockout (Hom, $n = 4$) mice. (c) Representative safranin O-stained images of left knees from wildtype, heterozygous and homozygous knockout mice. The femur and tibia are annotated, and the synovium is indicated by black arrows. Scale bar = 400 μm .

6.3 Discussion

Mice produce Ggg as it was first identified in mouse liver extracts, or more specifically in the bile [252]. Unlike human, mice do not express P2RY8, the receptor for the inhibitory signal GGG, and a homologous murine receptor to P2RY8 has not been identified. It is possible that Ggg may be redundant in mice, or that Ggt5 mediates its effects predominantly through the leukotriene pathway. Both GGG and LTC₄ are transported out of cells via ABCC1. However, GGG binds to P2RY8 with 100-fold greater affinity. This creates challenges in using these mouse models, as it becomes difficult to disentangle the roles of GGG and the LTC₄/LTD₄ pathways in disease models. A further complication is that P2RY8 has been evolutionarily lost in rodents yet is otherwise conserved in mammals. This raises questions about whether mice are an appropriate system for modelling GGT5-GGG-P2RY8 biology, since the central receptor is absent. The negative findings in Ggt5 knockout mice could therefore reflect a fundamental species limitation rather than a lack of mechanistic relevance. Another possibility is that compensatory up-regulation of other γ -glutamyl transferases may partially mask the impact of Ggt5 loss, thereby diluting the observable phenotype. Assessing the expression of related enzymes in knockout tissues would help determine whether functional redundancy contributes to the muted outcomes. One possible solution is to engineer mice with humanised P2RY8, or to use adoptive transfer methods using P2RY8-overexpressing lymphocytes. However, it

remains unknown which leukocyte subsets express P2RY8 and drive phenomena such as perivascular cuffing, so any such strategy would require careful cell-type mapping.

From a translational standpoint, targeting GGT5 or P2RY8 therapeutically introduces a distinct set of opportunities and constraints. Modulating GGT5 could alter local GGG availability and influence lymphocyte positioning, but such interventions would inevitably affect leukotriene processing, creating a risk of shifting inflammation through pathways unrelated to GGG. Direct targeting of P2RY8 offers a more specific means of controlling GGG-dependent retention, yet its restricted expression pattern raises the possibility that interventions may disproportionately affect Tfh-mediated GC activity with unpredictable consequences for antibody responses.

An additional layer of complexity lies in the fact that the murine immune system may rely on alternative retention pathways that compensate for the absence of P2RY8 altogether. If true, then even with humanised P2RY8 restoration, the broader signalling architecture in mice may not provide the same regulatory context found in humans. This highlights a conceptual limitation, where reintroducing the human receptor does not necessarily reconstruct the full signalling environment required for accurate modelling.

In the DSS model, mice of different genotype have similar spleen weight, suggesting comparable levels of systemic inflammation. Despite this, there are significantly fewer Rorgt⁺ Th17 cells in the

colons of homozygous mice. It has previously been shown that GGT5 expression is positively correlated with NK cells, macrophages and dendritic cells infiltration in gastric cancer, but negatively correlated with Th17 cell infiltration [295], which aligns with the current DSS model findings. However, the global outcomes of colitis across genotypes does not differ significantly, so it remains unclear if the decrease in the number of Th17 cells in the colon has any biological impact in this mice colitis model. Th17 cells have been suggested to be crucial in TLSs as their cytokines contribute to TLS formation in chronically inflamed tissues [314]. Notably, the lack of Tfh markers in the flow cytometry panel limits the ability to evaluate the presence of Tfh cells in this model. This is particularly important given that Tfh cells are the only population known to be sensitive to the inhibitory signal GGG in human (Figure 5.2b and Figure 5.9b). Without the ability to directly interrogate Tfh populations, the central human-relevant biology of GGG–P2RY8 signalling cannot be assessed, leaving a major gap in interpretation. Adding to this limitation, the experiment lacked healthy control mice, and histological observations may be biased as only a small section of the colon was processed for analysis. To verify these findings and provide a more comprehensive assessment, future experiments should incorporate a broader flow cytometry panel including Tfh markers, as well as collect whole colons for swiss rolls H&E staining.

The absence of clear differences across clinical or histological disease scores also raises the possibility that reductions in Th17 cells represent quantitative variation rather than mechanistic significance. Minor fluctuations in Th17 frequency may not be sufficient to alter overall pathology in an acute DSS model, making it difficult to draw meaningful conclusions about Ggt5 function from these data alone. A model in which Th17 cells have more pronounced pathogenic roles may be required to determine whether Ggt5 influences their behaviour in a functionally relevant way.

The DSS and AIA models may not fully capture the effects of Ggt5 in mice colitis and arthritis, as both are short-term acute models lasting only about 7 days. This limited duration may be insufficient for the recruitment and accumulation of lymphocytes. Optimising timepoints to extend the duration of these models or employing alternative models, such as chronic DSS colitis or *Helicobacter hepaticus*-induced T cell-dependent colitis [315, 316], may provide more robust insights into lymphocyte involvement including Tfh. For arthritis, using a chronic AIA model or collagen-induced arthritis (CIA) model may offer a better understanding of the potential interactions between Ggt5 and lymphocytes [317]. Yet even with chronic models, the absence of P2RY8 in mice will remain a confounding factor, limiting how well such data can be extrapolated to human biology. This underscores the need for either cross-species comparisons (e.g.

animal models that retain P2RY8) or genetic reconstitution of the receptor in mice.

Extending the duration of inflammatory models is also important for assessing lymphocyte spatial organisation, as features such as TLSs, perivascular aggregates and GC-like structures require sustained inflammation to emerge. Short-term models may therefore obscure phenotypes that only become apparent once lymphocytes undergo positioning, selection, or compartmentalisation processes that unfold over longer timeframes.

To further delineate the role of Ggt5 in mice colitis and arthritis, it would be useful to assess key inflammatory markers, specifically markers associated with Th17 responses, such as Il-17A, Il-22 and Rorgt, as well as those linked to lymphoid aggregates including Ccl19, Cxcl13 and Tnf. These data could provide additional mechanistic insights into how Ggt5 deficiency impacts tissue organisation and leukocyte positioning. However, any interpretation must acknowledge that the absence of P2RY8 may shift the balance of leukocyte signalling pathways in a way that does not faithfully recapitulate human immune regulation.

A broader cytokine and chemokine profiling strategy would also help clarify whether Ggt5 loss alters stromal cell states or modifies the broader inflammatory niche. Since Ggt5 acts upstream of multiple lipid mediators, its deletion could reshuffle local signalling networks independently of GGG, meaning that any observed phenotype may

reflect secondary metabolic adaptations rather than direct disruption of the GGG pathway.

Moreover, spatial transcriptomics could be employed to examine Ggt5-dependent changes in cellular composition and gene expression within distinct tissue compartments in colitis and arthritis models. This approach would allow for the identification of region-specific alterations in perivascular, lymphoid aggregates, and inflamed stromal niches, providing a spatially resolved map of transcriptional changes. Alternatively, laser capture microdissection (LCM) followed by bulk RNA-sequencing could also enable a more targeted analysis of specific regions within inflamed tissues, such as perivascular zones or GC-like structures. These techniques would allow for a higher resolution understanding of Ggt5-mediated leukocyte compartmentalisation and its role in shaping inflammatory landscapes in colitis and arthritis models.

Importantly, integrating these spatial methods with lipidomics would enable the direct measurement of Ggg and other Ggt5-derived metabolites *in situ*, clarifying whether biochemical availability aligns with anatomical regions where compartmentalisation is expected to occur. This would provide a mechanistic bridge between enzyme activity, metabolite gradients and cell positioning.

The lack of functional antibodies targeting mouse Ggt5 presents another technical challenge. Visualisation of Ggt5 spatial distribution is limited to methods like RNAScope, which only works with fresh frozen

tissues, presenting another technical hurdle. These technical barriers further complicate efforts to validate the biological relevance of Ggt5, and risk that spatial dynamics are overlooked.

In the absence of protein-level validation tools, functional interpretations become heavily dependent on indirect readouts such as transcript levels or downstream signalling changes. These proxies may not reliably reflect enzymatic activity, especially in inflamed tissues where post-transcriptional regulation is common. Developing alternative detection strategies, such as activity-based probes, could help resolve this gap.

Despite these limitations, the observation that homozygous Ggt5 knockout mice exhibit fewer Gl7⁺ GC B cells and IgG⁺ B cells in the mLNs may indicate impaired GC responses, which are essential for B cell maturation and isotype switching, particularly towards IgG. This finding suggests that Ggt5 may play a role in supporting GC responses or B cell development, even though these differences are not statistically significant (Figure 6.9b). In contrast, Ly6g⁺ neutrophils are significantly increased in the mLNs of homozygous knockout mice, suggesting a potential role for Ggt5 in regulating neutrophil trafficking or retention (Figure 6.10b).

Although these trends provide preliminary clues about Ggt5-associated functions, the variability and lack of statistical significance underscore the need for larger cohorts. Leukocyte dynamics in lymph nodes are highly sensitive to inter-individual variation, and small

sample sizes can easily obscure biologically meaningful differences. Replication across independent experiments would help differentiate true Ggt5-dependent effects from random variation.

In the colon, the slight decrease in IgG⁺ B cells alongside an increase in IgA⁺ B cells in the homozygous Ggt5 knockout mice may suggest a shift in the isotype switching of B cells, potentially favouring IgA production (Figure 6.6b). This shift likely reflects an adaptive response to changes in the gut microenvironment driven by the absence of Ggt5, as IgA plays a critical role in maintaining mucosal immunity and gut homeostasis [318]. Still, without direct evidence linking these findings to P2RY8 signalling, it remains unresolved whether these differences are mechanistically tied to the GGG pathway or represent secondary effects of Ggt5 deletion.

It is also possible that alterations in IgA and IgG distribution result from changes in plasma cell survival or positioning rather than intrinsic differences in class switching. Spatial assessment of plasma cells and B cell maturation zones within the gut would help distinguish between these possibilities.

Taken together, these findings highlight a fundamental limitation of the mouse system for studying GGT5-GGG-P2RY8 biology. The absence of P2RY8 in mice, despite its conservation across most mammals, means that the central signalling axis is missing, and this likely explains why Ggt5 knockout animals do not fully recapitulate expected immune phenotypes. Rather than indicating a lack of

relevance, these results suggest that mouse models are not sufficient on their own to capture the biology of this pathway. Future work may therefore require either humanised mouse models expressing P2RY8, or the use of alternative species such as zebrafish, in which the receptor is retained. This shift in approach would allow the contribution of Ggt5 and GGG–P2RY8 signalling to be examined in a context where the receptor–ligand system is intact.

Ultimately, a multi-species strategy may be necessary to accurately reconstruct the biology of this pathway. Rodent systems may still offer value for studying Ggt5-dependent leukotriene pathways, while species that retain P2RY8 can be used to examine GGG-mediated retention mechanisms. Integrating insights across these complementary systems would provide a more complete understanding than any single model alone.

Chapter 7: Concluding remarks

Perivascular cuffing of leukocytes has long been recognised as a characteristic feature in inflamed tissues, yet the mechanisms underlying this process have remained elusive. The work described in this thesis suggests that GGT5 may be a key player in mediating this phenomenon by acting as a gatekeeper for leukocyte retention within the perivascular niche. This hypothesis is supported by the distinct GGT5 staining patterns observed across tissues. In sterile tissues such as the synovium, GGT5 expression is restricted to the perivascular region. In contrast, in inflamed or immune-rich tissues like the gut, GGT5 expression becomes more widespread, aligning with increased leukocyte infiltration and retention. To further validate this phenotype, it would be valuable to examine additional tissues with varying levels of immune activity. For instance, immune-privileged tissues such as the brain, where the blood-brain barrier limits immune cell entry, could provide insights into the role of GGT5 in perivascular leukocyte dynamics under conditions of minimal immune activity. Conversely, the lungs could serve as an additional model of an immune-rich environment, as they are frequently exposed to live microbes and their by-products. Moreover, the composition of the lung microbiota is distinct from that of the gut, providing a different context to investigate the role of GGT5 in leukocyte compartmentalisation [319].

Unlike TNF, which is central to GC formation and broader tissue inflammation, GGT5 expression appears to be driven by factors

released from endothelial cells. Inflammatory signals and leukocyte infiltration likely activate endothelial cells to release factors that stimulate neighbouring fibroblasts to upregulate GGT5, as a localised response to accommodate the influx of leukocytes. This endothelial-fibroblast interaction likely represents a finely tuned mechanism to balance immune surveillance with the prevention of excessive tissue infiltration.

Our findings show that GGT5 upregulation by HUVEC supernatant is dependent on the NF- κ B pathway, as the stimulative effect of HUVEC supernatant can be reversed by using an NF- κ B inhibitor. To further elucidate this mechanism, tissue-relevant endothelial cells, such as those derived from the gut or synovium, could be cultured and stimulated with inflammatory cytokines to mimic disease conditions. The supernatants from these activated endothelial cells could then be used to stimulate gut and synovial fibroblasts. Alternatively, biopsies from IBD and RA tissues could be digested to generate conditioned medium. This conditioned medium would reflect the inflammatory microenvironment of the tissue, making it more relevant to disease states. The conditioned medium from these biopsies could also be used to stimulate fibroblasts. GGT5 expression in these fibroblasts could subsequently be assessed to validate the findings from endothelial and HUVEC supernatants.

Combining these experiments with interactome analysis tools, such as CellChat [320] or CellPhoneDB [321] using publicly available

IBD and RA single-cell transcriptomic datasets could help identify the specific NF- κ B-dependent factors in the endothelial supernatant driving GGT5 expression. While NF- κ B is known to regulate a broad spectrum of inflammatory mediators, limiting the interaction between endothelial cells and GGT5⁺ fibroblasts in the interactome analysis could help identify the specific factors within this pathway that drive GGT5 upregulation.

To enhance the resolution of these interactions, spatial transcriptomics could be applied to human IBD and RA tissues, allowing for a direct mapping of GGT5⁺ fibroblasts within their respective tissue niches. By integrating spatially resolved gene expression data, key endothelial-derived signals that are in close proximity to GGT5⁺ fibroblasts can be identified, further refining the list of potential factors driving GGT5 upregulation. This would provide a tissue-specific, spatially resolved interactome, enabling a more precise determination of the pathways regulating GGT5 expression in inflamed human tissues. Laser capture microdissection (LCM) followed by bulk RNA-sequencing or proteomics could further refine these findings by isolating GGT5⁺ fibroblast-enriched regions and identifying key molecular drivers.

Once the key factor(s) have been identified, neutralising antibodies targeting these factors could be introduced into the endothelial supernatant to determine whether the stimulatory effect on GGT5 expression can be abrogated. This approach would validate

the causal role of the identified factor(s) in GGT5 modulation and provide deeper insights into the underlying molecular mechanisms.

A critical limitation of the current work is that, while the in vitro findings establish a clear mechanistic link between endothelial activation and fibroblast GGT5 expression, the in vivo evidence remains indirect and descriptive. The reliance on immunostaining and knockout models without functional readouts of leukocyte retention limits the ability to conclusively assign causality. Furthermore, although the NF- κ B pathway has been implicated, the precise molecular intermediates remain undefined, and whether they act through canonical or non-canonical NF- κ B signalling has not been addressed. Future work should therefore incorporate gain- and loss-of-function experiments in human tissue models or organoids to strengthen the mechanistic interpretation. Integration of functional assays (e.g. leukocyte transmigration or adhesion assays) could also clarify how GGT5 directly influences immune cell positioning.

This study also highlights the distinct sensitivity of Tfh cells to the inhibitory signal GGG, suggesting they could play a key role in GGT5-mediated migration. While the role of Tfh in GC reactions within SLOs is well established, it is still unclear whether tissue-resident Tfh interacts with GGT5-expressing fibroblasts during IMIDs. Therefore, there is a need for further investigation into the spatial and functional dynamics of tissue Tfh cells in the context of GGT5-GGG-P2RY8 axis.

Although *in vivo* models such as DSS-induced colitis and AIA were utilised, they did not provide clear insights into functional roles of GGT5. Species-specific differences, particularly the lack of P2RY8 in mice, further limit the direct translation of these findings to human biology. While changes in Th17 cells in the colon, as well as GI7⁺ GC B cells and Igg⁺ B cells in the mLNs of Ggt5 knockout mice suggest a role for GGT5 in immune cell regulation, these findings remain inconclusive. Notably, Tfh cells were not examined in either mouse model, leaving a significant gap in understanding how Ggt5 knockout impacts Tfh cell dynamics during inflammation.

A more critical evaluation of these models reveals that the absence of P2RY8 orthologs in mice substantially weakens the translatability of findings related to the GGT5–GGG–P2RY8 axis. This highlights a broader limitation in immunometabolic research that depends on rodent models for human-specific signalling pathways. The thesis could therefore benefit from acknowledging that the negative or ambiguous *in vivo* findings might not indicate biological irrelevance, but rather model incompatibility. This underscores the need for developing or adopting humanised mouse systems, or *ex vivo* human tissue explant models, to overcome these species barriers.

A deeper understanding will likely require alternative models (as discussed in section 6.3) or new approaches. While widely conserved in vertebrates, P2RY8 lacks a sequence ortholog in rodents [322]. Despite this, it may be possible to investigate the role of GGT5–GGG–

P2RY8 axis in mice colitis and arthritis models by transferring P2RY8-transduced mouse lymphocytes into mice prior to disease induction. This approach would allow for the spatial distribution of P2RY8-transduced lymphocytes to be examined under a microscope in relation to Ggt5 expression in fixed tissues. In addition, live imaging techniques such as intravital microscopy or two-photon microscopy could be employed to track the migration and behaviour of the P2RY8-transduced lymphocytes in real time, providing insights into their interactions within the tissue microenvironment, including their movement, retention, and engagement with other cells in the gut.

In summary, this thesis highlights the emerging role of GGT5 in leukocyte compartmentalisation within inflamed tissues, particularly in the regulation of leukocyte retention in the perivascular niche. The interaction between endothelial cells and GGT5-expressing fibroblasts likely constitutes an important axis, or gatekeeper, to control immune cell localisation in IMIDs. Future research should focus on elucidating the interactions between GGT5⁺ fibroblasts, endothelial cells, and Tfh cells, as these may offer insights into how tissue-specific immune dynamics are regulated during inflammation.

Despite these caveats, this thesis provides one of the first systematic explorations of the GGT5–GGG–P2RY8 axis in inflamed non-lymphoid tissues. By linking endothelial activation, fibroblast reprogramming, and Tfh migration, it advances the conceptual framework for how metabolic and chemotactic gradients cooperate to

shape leukocyte positioning. In doing so, it places GGT5 alongside established regulators such as S1P, but maybe to a different extent to regulate leukocyte compartmentalisation. This also sheds light on the function of fibroblast, where they are not passive scaffolds but also active organisers of immune positioning.

From a critical perspective, a key strength of this thesis lies in the integration of molecular, cellular, and spatial observations to build a coherent model of leukocyte retention. However, the main limitation remains the lack of direct causal evidence linking GGT5 enzymatic activity to in situ immune cell dynamics. Addressing this gap through functional inhibition, rescue experiments, or metabolic tracing would significantly elevate the mechanistic depth. Moreover, future work should include cross-disease comparative analyses to test whether GGT5's regulatory effects are universal across IMIDs or context-specific (e.g., gut vs. synovium vs. skin).

From here, future work should focus on determining whether GGT5 activity is protective or is pathogenic across different IMID contexts, through preferably models that recapitulate the dynamics of human tissue, and whether manipulation of GGT5 could be used therapeutically. Any therapeutic strategy targeting GGT5 or P2RY8 would need to carefully balance potential benefits with risks. On one hand, modulation of this axis could restrain pathological immune infiltration and reduce tissue damage in chronic inflammation. On the other, excessive suppression might compromise protective immune

surveillance or impair germinal centre dynamics, leading to unintended consequences for host defence and tissue homeostasis. This knowledge will assist in determining whether GGT5 would be targeted to decrease immune infiltrates to stabilise them in protective niches or disperse them to resolve chronic inflammatory foci. Thus, the work presented here lays the groundwork for a broader research programme focused on understanding and ultimately, manipulating leukocyte compartmentalisation in human IMIDs.

In the longer term, integrating GGT5 biology into precision immunology frameworks could open avenues for biomarker development and targeted therapies. Combining transcriptomic, metabolomic, and imaging datasets could define GGT5 activity signatures predictive of disease state or therapeutic response. Translational partnerships with clinical consortia studying IBD and RA could enable validation of these findings in patient cohorts, positioning GGT5 as both a mechanistic and diagnostic node in immune regulation.

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