# INVESTIGATING THE ROLE OF 5-HT AND MCH IN THE REGULATION OF HUMAN IMMUNE RESPONSE

by

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## ABSTRACT

The involvement of neurotransmitters and neuropeptides as mediators in the dysregulation of the immune response leading to conditions such as chronic inflammation, autoimmune diseases and haematological cancers has been reported. Indeed, different types of innate and adaptive immune cells have been shown to express neurotransmitter/neuropeptide receptors on their surface membrane and some are even able to produce the neural factors under different conditions. This project was designed to investigate the role of the monoamine serotonin (5-HT) and also that of the orexigenic neuropeptide melanin-concentrating hormone (MCH) in human immune cell functions, specifically T lymphocytes, one of the key players in adaptive immune and inflammatory responses. To investigate the effects of agonist and antagonists targeting 5-HT receptors on the CD4+ and CD8+ T cell response to stimulus, human peripheral blood mononuclear cells were stimulated to induce proliferation in a serum-free environment. It was observed that inhibition of 5-HT synthesis enzyme tryptophan hydroxylase 1 (TPH1) with para-chlorophenylalanin (pCPA; 0.1-1mM) suppressed proliferation of both CD4 and CD8 T lymphocytes with significant inhibition observed at 1mM ($p<0.01$), yet somehow cell survival was maintained in stimulated cultures. However, the addition of 0.1mM 5-HTP was not able to reverse the suppressive effect of pCPA. Next, we demonstrated that exogenous 5-HT (10nM – 10μM) has no impact on neither CD4 nor CD8 T cell proliferation. Stimulated PBMCs when treated with 5-HT (10nM-10μM) has the same cell survival compared with control. Treating the PBMCs with the 5-HT2A/2C receptor antagonist, ritanserin or the 5-HT7 receptor antagonist SB285719 had no effect on proliferation.

We then assessed MCHR1 receptor expression in immune cell lines Jurkat and K562 using Western blot. Both cells showed an MCHR1 protein band at 55kDa comparable to that in the

neuroblastoma cell line SH-SY5Y. In normal human PBMC, we saw a two-fold increase in MCHR1 receptor expression in activated CD3 T lymphocytes while CD19 B lymphocytes exhibited a two-fold decrease ($n=3$, $p<0.01$) in expression level upon stimulation as detected by flow cytometer. The differential expression was significantly observed in naïve CD8 T lymphocytes ($n=5$, $p<0.05$) where MCHR1 has upregulated in the $CD69^+$ population. MCH-treated, isolated naïve T cell subsets had a non-significant increase in percentage of proliferated cells which was not seen in PBMC culture. However, MCH (100nM) failed to induce ERK1/2 signalling protein phosphorylation in Jurkat cells endogenously expressing MCHR1. Immunohistochemistry analysis on inflamed colonic tissue sections obtained from four IBD patients (3 Crohn's, 1 ulcerative colitis) were compared with non-inflamed control tissue, and results showed there to be MCHR1 immunoreactivity on colonic epithelial cells as well as on some mucosal infiltrating immune cells. A few of those cells were $CD11c^+$ while $CD14^+$ cells do not co-express MCHR1. Using the pfaffl method, real time RT-qPCR displayed elevated pMCH gene transcripts but not MCHR1 and MCHR2 in IBD colon tissue samples. Overall the results indicate that 5-HT appears not to have a significant influence in regulating the activity of T lymphocytes while MCH displays a possibly under-appreciated role in specific subsets of immune cells which could be related to the pathogenesis of inflammatory bowel diseases.

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## ABBREVIATIONS

| | |
|---|---|
| 5-HT | Serotonin |
| APC | Antigen presenting cell |
| CD | Cluster of differentiation |
| DAG | Diacylglycerol |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DC | Dendritic cell |
| DMSO | Dimethyl sulfoxide |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylenediaminetetraacetic acid |
| ERK | Extracellular signal-regulated kinases |
| FBS | Feotal bovine serum |
| GPCR | G-protein coupled receptor |
| IBD | Inflammatory bowel diseases |
| IBS | Irritable bowel syndrome |
| IDO | Indoleamine 2,3-dioxygenase |
| IFNγ | Interferon-gamma |
| IL | Interleukin |
| $IP_3$ | Inositol trisphosphate |
| LPS | Lipopolysaccharide |
| LTA | Lipoteichoic acid |
| MAPK | Mitogen-activated protein kinases |
| MCH | Melanin concentrating hormone |

| | |
|---|---|
| MCHR | Melanin concentrating hormone receptor |
| MHC | Major histocompatibility complex |
| NADPH | Reduced nicotinamide adenine dinucleotide phosphate |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NK cells | Natural killer cells |
| NLR | Nucleotide-binding oligomerization domain (NOD)-like receptors |
| OCTN | Organic cation transporter |
| PAMPs | Pathogen-associated molecular pattern molecules |
| PBS | Phosphate-buffered saline |
| pERK | Phosphorylated ERK |
| PIP2 | Phosphatidylinositol 4,5-bisphosphate |
| PKA | Protein kinase A |
| PLC | Phospholipase C |
| PMA | Phorbol 12-myristate 13-acetate |
| PRR | Pattern recognition receptors |
| RIPA | Radioimmunoprecipitation assay |
| RT-qPCR | Reverse transcription-quantitative polymerase chain reaction |
| SDS-PAGE | Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis |
| SERT | Serotonin transporter |
| TEMED | Tetramethylethylenediamine |
| TLR | Toll-like receptor |
| TNFa | Tumor necrosis factor alpha |

# CHAPTER 1 Introduction

## 1.1 Immune system

Our immune system is a complex yet orchestrated system designed to protect us from harmful microorganisms and tissue injury while at the same time creating tolerance towards self-antigens. It comprises of two main divisions; the innate and adaptive immune systems. These two divisions work together where each has strength that covers the weaknesses of the other and vice versa. A disruption to those balances due to genetic or environmental factors could be one of the leading factors in an overactive or aberrant immune response causing inflammation or autoimmunity.

### 1.1.1 Innate immunity

The innate immune system consists of structural and cellular components that act as the primary line of defence against invading pathogens and forms a link with the adaptive immune system. Part of the innate immune system components are the physical barriers that function to prevent the physical movement of pathogens into our body and also create an inhospitable environment to prevent the growth of different types of pathogen. Skin, epithelial glands, mucus-secreting goblet cells, cilia, stomach acid, tears and saliva are all the different forms of physical barriers that create an impermeable or hostile barrier against pathogen in the environment. Should the physical barrier be breached due to an injury, this protective mechanism is further strengthened with the help of the cellular components of the innate immune system including neutrophils, basophils, eosinophils, macrophages, mast cells and natural killer (NK) cells. The cellular response will induce a process known as inflammation to remove the pathogen from our tissues.

Mast cells, alongside granulocytes within the infected tissue will begin to secrete mediators including cytokines that cause vasodilation and promote the inflammatory process. The released cytokines begin recruiting phagocytic macrophages and other granulocytes such as neutrophils to the site of infection. Engulfed pathogens will be broken down inside neutrophils that eventually leads to apoptosis of the cells, creating cellular debris. Other than engulfing bacteria, macrophages also begin to engulf cellular debris thus removing any remnants of infection. NK cells are not directly involved in the inflammation process but have the ability to destroy infected cells or cancer cells non-specifically, and are therefore considered members of the innate immune system (Capra et al. 1999; Griebel et al. 2011).

Innate immune cells are able to recognise pathogens based on their highly conserved structural molecules termed pathogen-associated molecular patterns (PAMPs). Some examples of PAMPs are flagella of motile parasites, viral genetic components or bacterial lipoteichoic acid (LTA) and lipopolysaccharide (LPS). Macrophages and other phagocytes like neutrophils possess a number of receptors known as pattern recognition receptors (PRRs) that are not specific to any single pathogen. Instead, they recognise many pathogens that share specific components that are crucial to their structure and survival, or in some cases, host proteins such as components of the complement system and antibodies that are capable of binding the pathogen (Mogensen, 2009). There are several types of PRRs including CD14 (a surface marker for monocytes), mannose receptors, complement receptors, and the most important, the toll-like receptors (TLRs). There are 11 known TLRs in humans and they are located both at the cellular membrane and the endosomal membrane thus protecting us from extracellular pathogens like bacteria and intracellular viruses. Other intracellular PRRs include nucleotide-binding oligomerization domain-like receptors (NLRs) and retinoic acid-inducible gene-I-like (RIG-I-like) receptors. The latter can specifically recognize viral RNA and induce downstream

signalling that impacts the transcription of cytokines and inflammatory markers, such as interferons (IFN), within the nucleus which have long term effects on how we fight viral infection (Trinchieri & Sher, 2007). In summary, while the innate immune response is not specific, it has developed a way to recognise crucial components that are shared across many pathogens and is therefore able to mount an immediate response. However, it does not have the ability to develop memory.

### 1.1.2 Adaptive immunity

The adaptive immune response, on the other hand, is highly specific for singular components of each pathogen and can develop immunologic memory. Unlike innate immunity, the response may take days or weeks to develop as it requires priming after initial exposure. However, a much more rapid and stronger response will be generated upon subsequent exposure to the same antigen due to circulating memory cells. Adaptive immune responses can be divided into two divisions; 1) humoral or antibody-mediated immunity which predominantly involves B lymphocytes, and 2) cell-mediated immunity which primarily involves helper and cytotoxic T lymphocytes. These two divisions interact with each other to work concomitantly in removing pathogens (Zhu et al. 2010).

All pathogenic antigens that eventually make their way into our body must be presented to T lymphocytes, a member of the adaptive immunity. Antigen-presenting cells (APCs) such as dendritic cells (DCs), macrophages and B lymphocytes, have the capability to engulf and present the antigen peptide fragment on a special protein complex known as the major histocompatibility complex (MHC) found on the cell membrane. These APCs then migrate from the site of infection to the lymph nodes where they present the antigen peptide to

circulating naïve T lymphocytes. There are also secondary lymphoid organ resident DCs that sample antigen from the lymph or blood (Akira et al., 2006). $CD4^+$ helper T cells are able to recognize the MHC class II complex while $CD8^+$ cytotoxic T cells recognize the MCH class I complex. The engagement of the T cell receptor (TCR)/CD3 complex on T lymphocytes with antigen-bound MHC complex triggers the activation of naïve $CD3^+$ T cells which results in proliferation and differentiation into effector and memory subsets of $CD4^+$ and $CD8^+$ T lymphocytes (ref). However, a full activation requires co-stimulation of CD28 on T cells with B7/CD80 on APC. Without the co-stimulation, the T cells will remain in a prolonged state of inactivation known as anergy (Greenfield et al. 1998).

Once activated, T cells begin to synthesize and secretes interleukin-2 (IL-2), which acts as an autocrine cytokine to stimulate clonal expansion and depending on the cytokine milieu of the environment, differentiate into specific effector subsets (Capra et al. 1999). $CD4^+$ helper T (Th) cells can be classified into Th1, Th2 and Th17, each producing their own set of pro-inflammatory cytokines and chemokines resulting in the induction of appropriate humoral and inflammatory response. The IFN-γ secreting Th1 cells is vital in eliminating intracellular pathogens by activating mononuclear phagocytes to promote innate inflammatory immune responses and is associated with organ-specific autoimmunity (Capra et al. 1999). Th2 is mainly active in its response towards extracellular parasites involving key cytokines that include the interleukins IL4, IL5 and IL13 as well as involved in the aetiology of antibody-mediated allergic diseases (Weaver et al. 2006).

Another CD4+ T cell lineage is the pro-inflammatory Th17 that is characterised by its effector cytokines produced; IL17A, IL17F and IL6, and is implicated in the pathogenesis of several autoimmune diseases. It was discovered that IL23 induces IL17 secretion from this particular $CD4^+$ subset forming a critical link between IL23 and Th17 differentiation (Zhu et al. 2010).

Meanwhile, a distinct $CD4^+$ subset that stably expresses the IL2 receptor component CD25 and transcription factor FOXP3 is linked to the maintenance of immune homeostasis and regulating self-tolerance involving anti-inflammatory TGF-β and IL-10. These cells are known as regulatory T (Tregs) cells (Chatila, 2005). Several autoimmune and inflammatory diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) have been associated with the dysregulation of Th17/Tregs balance that resulted in maintenance of inflammation (Noack et al. 2014).

### 1.1.3 Identifying naïve and memory T cells

In this study, it is important to be able to distinguish the different subsets and phenotypes of T lymphocytes that express the neurotransmitter and neuropeptide receptors of interest in order to understand its possible immunomodulatory role in immune responses. Therefore, flow cytometry will be utilised in order to either isolate or identify T cell subsets based on their surface markers in addition to their intracellular cytokine profile. The first marker used to distinguish between naïve and antigen-experienced memory T cells was the CD45 isoform RA for naïve and isoform RO for memory. In 1999, Federico Sallusto expanded this view and managed to identify subsets of memory T cells namely central memory (TCM) and effector memory (TEM) by including CCR7, a chemokine receptor crucial for T cell homing ability to central lymphoid organs (Sallusto et al. 1999). Naïve T cells are positive for both CD45RA and CCR7. Upon antigen encounter and proliferation, CD45RA expression is lost and the cells that are now $CD45RO^+CCR7^+$ are identified as TCM. TCMs are characterised by high proliferative potential but require longer time to display effector functions, unlike $CD45RO^+CCR7^-$ TEM which show a very rapid cytokine production after restimulation. TEM also have shorter

telomeres than TCM and therefore have a lower proliferative potential. Due to the loss of CCR7 they also loses the capability to home to secondary lymphoid organs but preferentially home to inflamed tissue instead (Sallusto et al. 2004). Additionally, within the CD8 T cell compartment, there are memory T cells that have either retained or reacquired the expression of CD45RA and it is known that these cells have a high cytotoxic potential. These cells are $CD45RA^{+}CCR7^{-}$ and are known as terminally differentiated CD8+ effector memory T (TEMRA) cells (Sallusto et al. 1999, 2004). Another important cell surface molecule is CD69, which is upregulated upon TCR-mediated activation and thus denotes the early activated state of T lymphocytes (Testi et al. 1994). This project will mainly focused on the naïve cell population.

### 1.1.4 Mucosal immune system

Perhaps the most fascinating aspect of our immune system is the mucosal immunity particularly of the gastrointestinal (GI) tract. What is interesting is that the intestine host hundreds of enteric commensal bacterial species that contributes to nutrient provision, energy metabolism and immune system development (Farthing. 2004; Bäckhed et al. 2005).

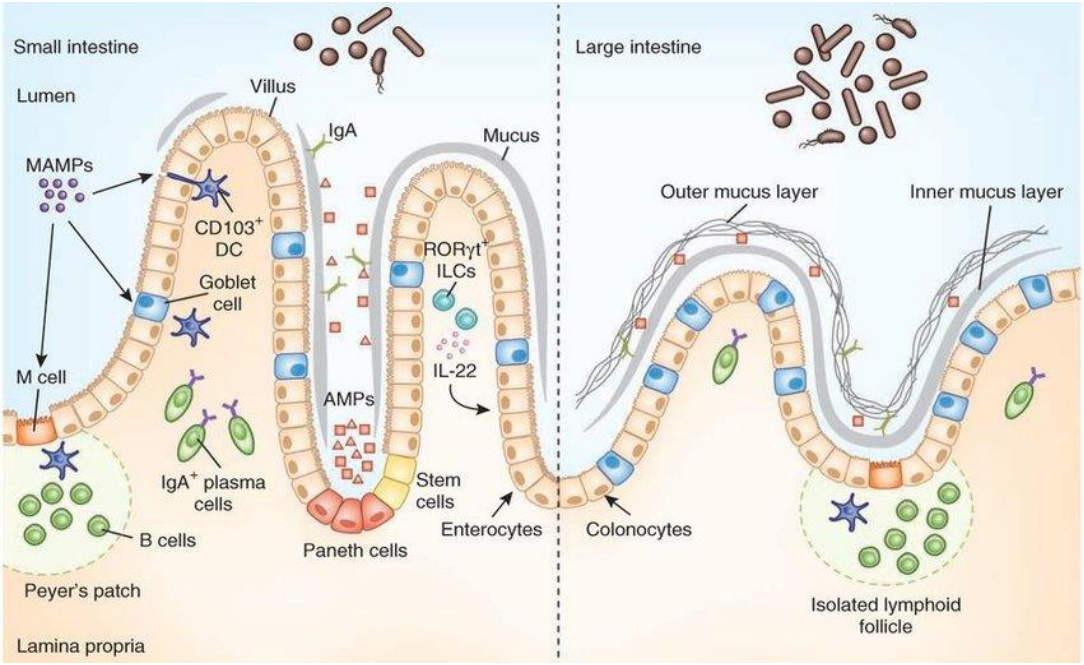



**Figure 1.1 Components of intestinal epithelial barrier which is compose of a single layer of enterocytes (in the small intestine) and colonocytes (in the large intestine) and it is the role of the immune system to protect the integrity of this barrier**. Interspersed between the epithelial cells are specialised cells such as goblet cells that produce mucins to form a dense inner mucus layer and a loose outer mucus layer, whereas Paneth cells enriched in the bases of small intestinal crypts produce antimicrobial peptides (AMP). Microfold (M) cells and dendritic cells (DC) mediate antigen sampling across the intestinal barrier. IgA, immunoglobulin A; ILC, innate lymphoid cell; IL-22, interleukin-22; MAMP, microbe-associated molecular pattern. Adapted from Brown et al. 2013

The intestinal mucosa is covered with crypts and villi made up of intestinal epithelial cells (IEC) and underlying tissues that host the body's largest population of immune cells. The IEC layer is structurally composed of different types of specialized cells such as enterocytes, goblet cells, Paneth cells, M cells and intestinal stem cells that performs an overall function of protecting and maintaining the integrity of intestinal epithelium against microorganisms (Sartor & Wu, 2017). Goblet cells and Paneth cells secrete glycosylated mucin and defensins that make up the protective mucus layer with anti-bacterial properties covering the epithelial surface (McGuckin et al. 2011). The mucus layer and IEC together forms an important barrier against pathogenic invasion.

Underneath the epithelial layer contain scatters of lymphoid tissue structures known as Peyer's patches. These are key sites in coordinating immune responses to pathogens whilst promoting tolerance to harmless microbes. Within the Peyer's patches are specialized phagocytic cells called M cells that facilitate the process of antigen sampling and immune surveillance by DCs, a process that is essential in maintaining intestinal immune homeostasis (Jang et al. 2004). Antigen sampling typically results in tolerogenic activation where the immune system promotes homeostasis mainly by inducing IL-10 secretion by Tregs and IgA antibody production by B cells (Coombes at al. 2008; Cerutti et al. 2011). During an immune response towards microbes, PAMPs can be recognised by various PRRs and TLRs on innate immune cells which subsequently activate intestinal DCs and macrophages to produce pro-inflammatory cytokines, chemokines and anti-bacterial peptides (Akira et al. 2006). This activation results in direct elimination of pathogens and adaptive immune response stimulation. Antigens presented by these cells can activate naïve $CD4^+$ T cells and modulate polarisation to effector subtypes including Th1, Th2 and Th17 which coordinate an escalated immune response involving their own inflammatory molecules and neutrophils recruitment while the generation of Tregs is crucial in preventing pathological immune responses (Kim et al. 2017). The immune response will eventually return to normal homeostatic conditions once bacterial invasion is repulsed and epithelial integrity is restored by replacing damaged cells with new ones from the intestinal crypts (Garrett et al. 2010).

### 1.2 Inflammatory bowel disease (IBD)

Many misunderstood that inflammatory bowel disease (IBD) is an autoimmune disease where the immune cells attack otherwise non-immunogenic self-cells leading to inflamed intestines.

But more recent studies suggest that rather than attacking the cell lining of the intestinal tissues, the immune cells are actually targeting the normal, non-pathogenic microbiota residing in the gut leading to inflammatory injuries (Ni et al. 2017). To maintain a normal homeostasis within the gut, the neuroimmune crosstalk and the control mechanism of the immune system on the gut microbiota needs to be consistently regulated in order to mount an appropriate immune response of tolerance or defense (Bernardazzi et al. 2016). An inappropriate immune response coupled with predisposing genetic and environmental factors contributes to the disruption of the homeostasis condition, leading to symptoms typically associated with IBD. The two major types of IBD are ulcerative colitis (UC) and Crohn's disease (CD), mainly differentiated by the area affected by inflammation. A chronic IBD pursues a protracted, relapsing and remitting course. There are two types of chronic IBD, the one that involves the colon and another that involves the whole gastrointestinal (GI) tract. Common signs and symptom associated with IBD can include, but not always, diarrhoea, rectal bleeding, abdominal pain, fever, weight loss, vomiting, cramps, and muscle spasms (Kim et al. 2017).

A recent review reported that Europeans and North Americans showed the highest prevalence of IBD since 1990 excluding those of paediatric-onset. Meanwhile, there is a rising incidence among developing countries in Asia, Africa and South America most often contributed to partial understanding of the clinical and endoscopic characteristics of IBD leading to a delayed or misdiagnosis (Lee, 2016). Although therapeutic interventions help reduce the incidence in developed countries, the burden remains high due to increasing number of reported IBD cases worldwide (Ng et al. 2017). Therefore, the need for new therapeutic agents is imperative in the health-care system management of this complex disease.

UC is a chronic relapsing IBD intermittent with periods of remission that can progress to colorectal cancer. Endoscopic findings showed continuous inflamed superficial lesion that

progress from distal to proximal colon with characteristic pseudopolyps derived from unusual cell regeneration due to the disease. Mucosal atrophy can be seen in some cases as a result of chronic inflammation and luminal narrowing (Lee, 2016). About 20-40% of UC patients presented with proctitis, characterised by inflamed proximal to rectal sigmoid area up to descending colon while 30-40% of UC patients exhibit a more extensive colitis where the inflammation spread as far as the transverse colon. Other than cancer, more severe cases are associated with spontaneous bleeding and ruptured bowel (da Silva et al. 2014).

CD on the other hand is mainly characterised by skipped lesions with cobblestone appearance restricted to ileocecal area. However, a small percentage of Crohn's patients present with skipped lesions in the colon area. This is known as Crohn's colitis. Relatively more patients are diagnosed with CD compared to UC each year (Ng et al. 2017). Although the pathophysiology of CD is still poorly understood, CD is often associated with characteristics such as the presence of discontinuous, longitudinal ulcers with transmural inflammation involving deep tissues of the intestinal layer. These clinical features can lead to much more severe complications such as stenosis, abscess formation, fistula, perforation, cancer and granuloma (Lee et al. 2016).

### 1.2.1 Protective barrier

IBD is a chronic inflammatory disorder of the intestine with unknown aetiology. However, it is clear that disruption in the homeostatic environment maintained by several cellular and non-cellular components within the gut including the enteric nervous system could eventually lead to an uncontrolled immune response associated with IBD (Brown et al., 2013). An abnormality of the intestinal mucus layer, for example, can results in dysbiosis of commensal flora and increase susceptibility to intestinal inflammation. Depletion or dysfunction of secretory cells

such as goblet cells and Paneth cells are seen in IBD patients and are thought to be involved in the early development of IBD (Kaser et al. 2010). On the other hand, UC patients have a lower level of lecithin in the mucus compared to healthy colons and managed to achieve clinical remission when taking oral lecithin during a clinical trial (Stremmel et al. 2005). The importance of a normal functioning mucus layer as a protective structure was substantiated in animal studies where mice lacking in a gene encoding mucus component develop spontaneous colitis (Van der Sluis et al. 2006). To add, compromised intestinal integrity caused by single nucleotide polymorphism of the cellular organic cation transporter (OCTN) is associated with CD susceptibility while animal models with reduced barrier function develop an IBD-like enteritis (Peltekova et al. 2004; Sartor, 2006). Therefore, environment or genetic factors that cause aberrant mucus production and reduced epithelial integrity can increase one's risk of developing IBD.

### 1.2.2 Intestinal microbiota

As described previously, commensal bacterial also play a crucial role in preserving intestinal homeostasis. Genetic factors and environmental triggers such as diet, stress, smoking, infections and prolonged use of antibiotics can contribute to a dysbiosis condition that could lead to an inflammatory response in the bowel (Kim et al. 2017). Among the many species of resident gut micobiota, non pathogenic *E.coli* strains were shown to induce pro-inflammatory cytokines release which cause ulceration and epithelial injury seen in both UC and CD (Bäckhed et al. 2005). Recently, researchers have identified a more pathogenic strain known as adherent-invasive *E. coli* (AIEC) in patients with CD which induce higher TNF-α release from macrophages but less IL-8 from IECs (Sasaki et al. 2007). It was found that the severity of the

disease correlates with the diversity and number of commensal bacteria in the intestine. However, efforts to restore to normal microbiota composition via methods such as fecal microbial transplantation (FMT) and probiotics supplements in IBD patients still require extensive research to validate its efficacy (Matsuoka et al. 2015).

### 1.2.3 Innate and adaptive immune response

Perhaps the most researched factor of IBD pathogenesis is the innate and adaptive immune response in the intestinal mucosa. Intestinal innate immune cells are constantly in contact with the gut's microorganisms via immune receptors-PAMPs interaction, which determines the appropriate adaptive immune response. Failure in controlling microorganisms within the gut via detection and elimination processes increase one's risk of developing IBD. Impaired TLR signalling, for example, is associated with a weakened intestinal integrity and inappropriate mucosal repair mechanism in colitis (Podolsky et al. 2009) while genetic polymorphisms or lack of PRRs resulted in an abnormal, uncontrolled adaptive immune response mechanism (Kim et al. 2017).

Apart from that, elevated levels of pro-inflammatory cytokines from prolonged inappropriate cellular activation against commensal microorganisms is considered as one of the main causes of pathogenesis of IBD. Excessive Th1 activity and cytokines production such as IFN-$\gamma$ and TNF-$\alpha$, has been observed in the mucosa of CD patients (Franchimont et al. 2004) while elevated Th2-derived cytokines is predominant in the inflamed region of UC patients (Huang et al. 2016). Increased TNF$\alpha$ is associated with local inflammation induced by angiogenesis, Paneth cell necrosis, impaired barrier functions from proteases and inflammatory immune response activity (Neurath, 2014). Therefore, therapies targeting inflammatory cytokines and

cellular signalling pathways have been developed and used in managing inflammation, inducing remission and mucosal healing. Experimental studies revealed that the development of colitis in dextran sulphate sodium (DSS)-induced mice is attenuated with anti-TNF monoclonal antibodies and that several anti-TNF agents have shown efficacy and are approved for CD patients (Kojouharoff et al. 1997; Evans et al. 2012). Since various key cytokines affiliated with IBD development depend on Janus kinase (JAK)-signalling pathways, targeting this pathway is also thought to have potential in alleviating moderate-to-severe IBD. Clinical trials involving UC patients receiving tofacitinib, a JAK1/JAK3 inhibitor, showed promising results (Sandborn et al. 2012) and is now approved for moderate-to-severe UC that is intolerant to tumor necrosis factor (TNF) inhibitors (Agrawal et al., 2020)

Other important cytokines involved in inflammatory development of IBD are Th17-related such as IL-17A, IL-22, and IL-23 in addition to Th17 chemoattractant CCL20. These cytokines were reported to be elevated in the inflamed mucosa of CD patients and are related to the severity of epithelial damage due to neutrophils infiltration and fibroblast secretions (Kim et al. 2017). Interestingly, Th17 is also thought to play an anti-inflammatory role via IL-22 release, which promotes epithelial regeneration and anti-microbial peptides production in the mucus to overcome damages incurred by Th1 cytokines in CD. However, drug therapies involving anti-IL17 to treat CD seems to be unsuccesful (Bilsborough et al. 2016). Furthermore, a disorder in the dynamic balance between Th17 and Tregs is also implicated in the aggravation of inflammation in CD patients (Huang et al. 2016). On that note, a decrease in Tregs cell population was observed in UC patients and IL-10 deficient mice develop spontaneous T cell-dependent colitis, indicating the importance of Tregs in suppressing cell-mediated inflammatory condition (Huibregtse et al. 2007). This collective evidence of complex cytokine

and cellular interactions in IBD pathogenesis is still being explored in attempts to develop potential drug targets for IBD therapy.

### 1.3 Neuro-immune interaction

Mostly synthesized in neurons, neurotransmitter release is brought by electrical changes within the neuron upon stimuli and exert chemical changes within its target cell when binding to its cognate receptors. Today, clinical and scientific research suggest a much broader spectrum of neurotransmitter function. Not only do they play a critical role in the communication between neurons and target organs/tissues, there is also evidence that they play a regulatory role on the immune system. It has been discovered that immune cells express neurotransmitter receptors and not only that; they also have the capacity to produce neurotransmitters when activated. The binding of neurotransmitters to receptors on the immune cells could alter their functional responses such as activation, proliferation, cytokine secretions, migration and cytotoxic activity (Franco et al. 2007). Given their wide range of immune regulatory functions, it is of equal importance to understand their influence in the pathophysiology of immune-mediated diseases such autoimmunity and chronic inflammation.

#### 1.3.1 Neurotransmitters and immune cells

For many years, researchers have been trying to understand the relationship between neurotransmitters and the immune system, which led to the discovery of several types of neurotransmitters that are able to regulate immune cell functions in an autocrine and paracrine manner. Immune cells were shown to increase expression of neurotransmitter system protein

such as receptors as well as synthesizing enzyme of serotonin when activated, indicating a possible role of 5-HT in immune response (Chen et al. 2015). Meanwhile, noradrenaline which is a neurotransmitter in the sympathetic nervous system has been shown to possess anti-inflammatory properties. One study revealed that the anti-inflammatory response comes from its ability to suppress the pro-inflammatory cytokine IL-1, IL-6 and TNF-α from intestinal lymphocytes via β1–adrenoceptor stimulation (Takayanagi et al. 2012). Another neurotransmitter, acetylcholine, seems to have immunosuppressive effect as well. It was demonstrated that acetylcholine reduces endotoxin-induced inflammation via the "cholinergic anti-inflammatory pathway". Stimulation of the vagus nerve in this pathway resulted in fewer circulating pro-inflammatory mediators produced by macrophages via cholinergic activity on nicotinic receptors expressed on these cells (Borovikova et al. 2000). Glutamate has also been proposed as an immunomodulator by having a role in immune activation and inflammation. Animal models of multiple sclerosis display attenuated neuroinflammatory responses when treated with glutamate receptor agonists. This protective effect was thought to be via upregulated activity of regulatory T cells (Tregs) and reduced $CD4^+$ T cell differentiation to pro-inflammatory Th17 cells. It was suggested that this response was mediated through the activation of metabotropic glutamate receptor-4 (mGluR4) expressed on dendritic cells and thus implying a protective role of glutamate in neuroinflammatory diseases (Fallarino et al. 2010). Another well documented immunomodulatory role of neurotransmitters is the action of dopamine on immune cells. Multiple studies revealed that dendritic cells, monocytes/macrophages and T cells express dopamine receptors and produce the neurotransmitter under specific stimulation (Prado et al. 2013). Pharmacological evidence provide insights of the different impacts of dopamine on T cell regulation. For example, a study of dopamine stimulation on D1 receptors expressed on $CD4^+$ T cells showed decreased

suppressive activity of Tregs (Cosentino et al. 2007) while another displays potentiated $CD4^+$ T cells protective response towards infections (Nakano et al. 2009). Other immune cells that are also regulated by dopamine include B cells, NK cells, neutrophils and monocytes indicating its diverse role as a mediator of inflammatory responses and possibly autoimmune disease (Pacheco et al. 2014). This evidence all points to the argument of whether neurotransmitters are beneficial to the cells and ultimately to one's physiological state of health. The answer might lie in the state of the target immune cells that could have an altered response mechanism such as in blood cancers or autoimmune T cells.

### 1.3.2 Neuropeptides and immune cells

Apart from neurotransmitters, small peptides produced and released by neurons known as neuropeptides also have the capacity to act as signalling molecules in the central and peripheral nervous system. There is also increasing evidence that suggests their influence on immune functions in healthy and diseased conditions. Some neuropeptides, as well as hormones, have been recognised as endogenous anti-inflammatory agents that are produced during the inflammatory response in order to maintain a steady state immunity and even participate in suppressing autoreactive effector T cells (Bernardazzi et al. 2016). Other than neurotransmitters, innate and adaptive immune cells can also become alternative sources of neuropeptides and modulate the expression of their cognate receptors during an immune response. The close proximity between neuronal nerve endings and immune cells indicate that our immune system is in constant bidirectional interaction with the neuroendocrine system to coordinate a variety of responses towards stimuli such as stress, infections or tissue injuries. The information sent from the immune system can in turn stimulate the nervous system to

subsequently affect our behaviour such as movement, sleep pattern and eating habits (Souza-Moreira et al. 2011).

Several neuropeptides such as urocortin (UCN), cortistatin (CST), ghrelin (GHR) and adrenomedullin (AM) are produced by the myeloid cell lineage while vasoactive intestinal peptide (VIP) is produced by adaptive immune cells such as $CD4^+$ and $CD8^+$ T lymphocytes (Delgado et al. 2004; Kubo et al. 1998). Upon release, they can act in an autocrine or paracrine manner when bound to their G-protein-coupled receptors (GPCRs) expressed on different immune cells. The receptor binding causes the activation of the cAMP/PKA pathway in the immune cells leading to the downregulation of transduction signalling associated with inflammatory mediators' production (Gonzalez-Rey et al. 2007). For example, VIP and GHR can exert therapeutic effects on animal models with inflammatory or autoimmune conditions by lowering the production of inflammatory cytokines from Th1 or Th2 while enhancing IL-10/TGF-β secretion. In addition, these neuropeptides can also modulate activity of macrophages including activation, phagocytosis and migration thus affecting the innate immune response against pathogens (Delgado et al. 1999; Tümer et al. 2007). However, there were conflicting arguments of whether VIP is pro- or anti-inflammatory based on experimental findings. Delgado and colleagues concluded that the opposing immunomodulatory effects of VIP is based on the G-proteins associated with VIP receptors that are expressed on the cells (Delgado et al. 2004).

Considerable evidence also indicates that neuropeptides such as VIP, UCN and AM are capable of modulating cytokine secretion and/or number of T lymphocytes in inflamed sites possibly by reducing inflammatory cytokine release or impairing specific subset differentiation/migration. Several research groups that have been working on animal models with inflammatory or autoimmune conditions such as experimental arthritis, Crohn's disease,

colitis and experimental autoimmune encephalomyelitis have managed to show a downregulation in inflammatory response when treated with neuropeptides (Delgado et al. 2001; Abad et al. 2003; Gonzalez-Rey et al. 2006). Taken together, these published data provide encouraging insights towards alternative therapeutic approaches targeting neuropeptide G-protein-coupled receptors involved in destructive inflammatory responses.

## 1.4 G protein-coupled receptors (GPCRs)

### 1.4.1 Structural overview

G protein-coupled receptors comprise of the largest known class of membrane receptors and are involved in a plethora of physiological functions when activated, mediating the responses to signals such as hormones and neurotransmitters. Over 800 human GPCRs had been revealed using the human genome analysis and the majority of these receptors are still functionally uncharacterised. These receptors are termed as orphan GPCRs where the cognate ligand is yet to be discovered. Interestingly about half of all known drugs work through GPCRs. Phylogenetic analyses grouped the GPCRs into 5 main families with sub-branches: glutamate (Class C), rhodopsin (Class A), adhesion, frizzled/taste2, and secretin (Fredriksson et al. 2003).

GPCRs are also known as 7-transmembrane (7-TM) receptors as they are composed of a single polypeptide consisting of the extracellular N-terminus, seven hydrophobic transmembrane domains (TM1-TM7) and an intracellular C-terminus end. The transmembrane domains share similar amino acid sequence and are linked by intracellular and extracellular loops (Figure 1.2). The differences between GPCRs can be observed mainly in the amino acid sequence of the N-terminus, the intracellular loop linking TM5 and TM6, and also the amino acid sequence of the C-terminus. The amino acid sequence of the N-terminus varies in length, with monoamine and

peptide receptors being relatively short; about 10-15 amino acids while glycoprotein and glutamate family receptors are much longer, between 400-600 amino acids (Kobilka 2007).

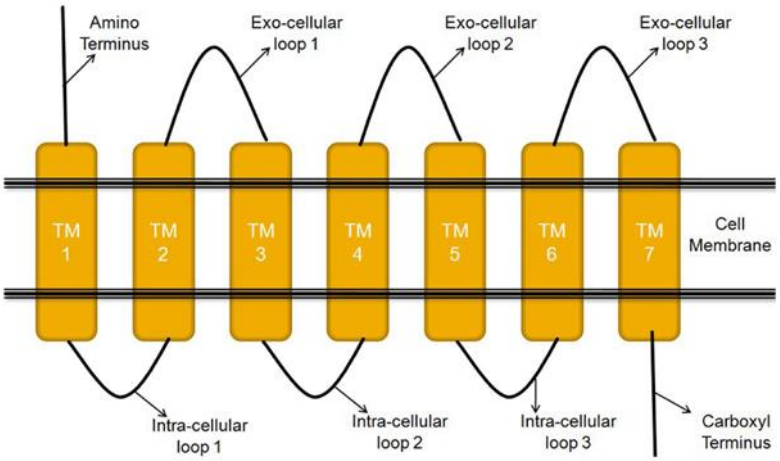



**Figure 1.2** Common structure of GPCRs (Cobanoglu et al. 2010)

The ligands of the receptor however, vary greatly in structure: from small ions to large peptides. The size of the ligand becomes one of the determining factors in predicting its binding sites. For example, the TM segments are the binding sites of mainly small organic agonists while the N-terminus together with a portion of the exocellular loops compose the binding sites for larger peptides with several exceptions such as GPCRs with the large N-terminus being the main binding site for peptides such as glycoprotein hormone (Ji et al. 1998).

### 1.4.2 GPCR signalling pathways

As the name implies, GPCRs have G proteins coupled to the receptors in the plasma membrane of the cell. Many different types of ligand can activate GPCRs such as fatty acids, proteins, peptides and amino acids. When a ligand binds to a GPCR, the G protein attached to the

cytoplasmic side of the plasma membrane is responsible for relaying the information to downstream signalling pathways within the cell. They could be coupled to enzymes or ion channels in the plasma membrane and each type of G protein is specific for different signalling pathways. G proteins have three subunits: an α, β and a γ subunit (Figure 1.3). When the G protein is in an inactive state, the α subunit has a bound guanisine-diphosphate (GDP). The binding of the ligand to the GPCR initiates a conformational change in the G protein. This stimulates the α subunit to exchange its bound GDP for a GTP, changing it into an active state. The activated G protein then dissociates into the α subunit, and a β–γ complex. (Leurs et al. 2005)

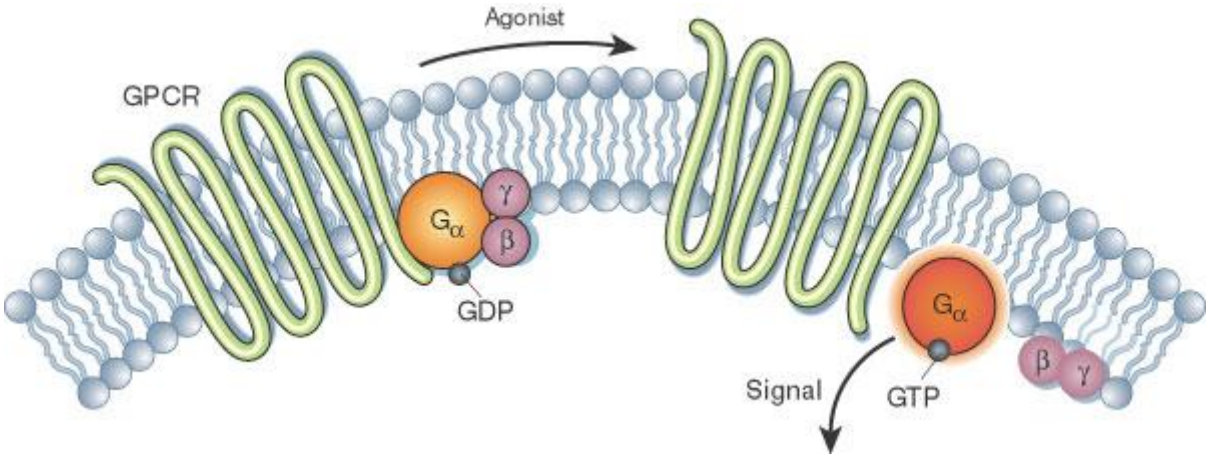



**Figure 1.3** Schematic diagram of G protein subunit dissociation upon agonist binding. (Li et al. 2002)

The actual target of the activated subunit depends on the G protein that is activated (Figure 1.4). In the cAMP signalling pathway, the G protein involved is the stimulatory protein call Gs. The activated α subunit of Gs binds to adenylyl cyclase, an enzyme that converts ATP into cAMP which can then serve directly as a signalling molecule, or it can act indirectly through activation of proteins within the cell. For example, the cAMP molecule can bind to the regulatory subunits

of protein kinase A (PKA) which allows the catalytic subunits of PKA to dissociate and PKA can then phosphorylate intracellular targets (Neves et al. 2002).

On the other hand, another G protein known as Gi protein is identified by its ability to inhibit adenylate cyclase which leads to a decrease in cAMP formation. A wide variety of hormones and neurotransmitters such as adrenaline, acetylcholine, dopamine and 5-HT utilise the Gi signalling pathway and the signal activated by these ligands can be blocked by pertussin toxin that prevents the interaction between the Gα subunit and the receptor (Dorsam et al. 2007). Interestingly, the Gβγ in this pathway can also induce signalling cascades by activating a group of molecules known as mitogen-activated protein kinases or MAPKs. Studies showed that different cells respond to stimuli differently depending on the type of MAPKs signalling pathway being activated (Garrigton et al. 1999). The activation of MAPKs is induced by phosphorylation catalysed by active MAPK kinases (MKKs) which themselves were phosphorylated and activated by MKK kinases (MKKKs). Together, these three members of kinases form a three-kinase module in the protein kinase cascade (Widmann et al. 1999).

Since many hormones and neurotransmitters rely on the cAMP signalling pathway, the response of the cell will depend on the cell type itself. An increase in cAMP signal in adipocytes will cause a very different response than an increase in cAMP in a renal cell or a hepatocyte (Cerione et al. 1985). A homeostatic cell function must have the ability to terminate the signalling pathway after it has accomplished its task. To do so, the cAMP must be broken down using the enzyme cAMP phosphodiesterase. The catalytic subunit of PKA then reassociates with the regulatory subunits. In order for the G protein to become inactivated, the α subunit must hydrolyse its bound GTP back into GDP catalysed by GTPase. The α subunit then reassociates

with the beta-gamma complex, and the G protein is once again back in an inactive state (Li et al. 2002)

G proteins can also initiate another common signalling pathway that utilizes intracellular calcium as a second messenger and is called Gq. In this particular pathway, the activated $\alpha$ subunit activates phospholipase C (PLC), which is an enzyme that acts on the molecule phosphatidylinosinositol 4,5-biphosphote, PIP2. PLC cleaves PIP2 into inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG). IP3 is a small water soluble molecule that is released into the cytosol and travels to the endoplasmic reticulum (ER) that stores a large amount of calcium in the lumen. IP3 binds to a ligand-gated calcium channel in the membrane of the ER allowing calcium to flow into the cytosol. At the same time, DAG is migrating through the plasma membrane to activate PKC which requires calcium for full activation. Once activated, PKC phosphorylates a number of intracellular targets thus transmitting the initial signal of the protein binding to its receptor. In order to terminate this signal, calcium is sequestered back in the ER and PIP2 is reformed (Mizuno et al. 2009). The $\alpha$ subunit of the G protein hydrolyses its bound GTP into GDP and the G protein reassociates. This restores the resting state of the cell.

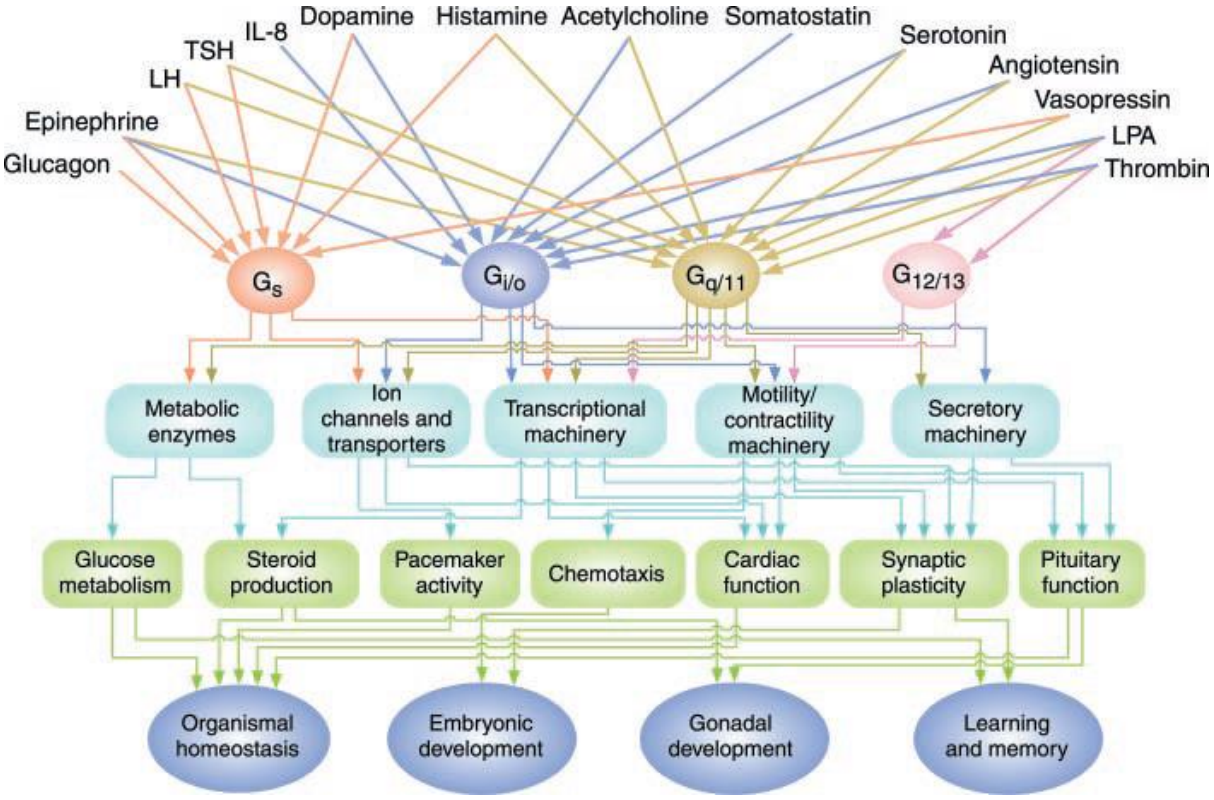



**Figure 1.4** Schematic representation of systemic functions regulated by different G protein signalling pathways. (Neves et al. 2002)

## 1.5 5-hydroxytryptamine (5-HT) and its system proteins

### 1.5.1 Discovery of 5-HT

5-HT was discovered in the 1930s as a substance with the ability to induce smooth muscle contraction found in the gastrointestinal enterochromaffin (EC) cells (Göthert, 2013). This neurotransmitter was also found to have vasoconstriction properties and was extracted from the platelet fraction of the blood (Rapport, 1949). Since the molecules were extracted from serum and possess the capacity to regulate smooth muscle tone, the name serotonin was established from “ser” for serum and “tonin” for the ability to induce muscle tone. Following multiple

structural analyses and chemical syntheses later, the knowledge of the pharmacological effects of 5-HT has greatly improved to provide evidence that 5-HT is involved in a wide range of physiological functions and is associated with many functions including mood disorders (Arreola et al. 2015)

### 1.5.2 5-HT biosynthesis and metabolism

5-HT is synthesised from L-tryptophan which is an essential amino acid. Since humans do not possess the enzymes needed to synthesize tryptophan, deficiency of tryptophan will also results in deficiency of serotonin. The committed and rate-limiting step in serotonin synthesis is catalysed by tryptophan hydroxylase (TPH), a tetrahydrobiopterin (BH4)-dependent and an iron-dependant enzyme. The $Fe^{2+}$ in the active site is required in this process to assist binding of the BH4 in the active site and allowing the reaction to take place (Wang et al, 2002). TPH exists in two isoforms, TPH1 and TPH2. TPH2 is exclusively expressed in nerve cells to produce 5-HT in the brain and the central nervous system as a neurotransmitter that plays an important role in the regulation of a variety of physiologic states such as sleep, food intake, mood and body temperature (Walther et al, 2003). TPH1 on the other hand predominantly synthesises peripheral 5-HT.

Both forms of TPH hydroxylate L-tryptophan to produce 5-hydroxytryptophan (5-HTP). Once 5-HTP is generated, its $\alpha$ carbon will be decarboxylated by L-aromatic amino acid decarboxylase (AAAD), removing the carboxyl group resulting in 5-HT production (Figure 1.5). AAAD is also involved in the synthesis of dopamine from L-dopa. 5-HT can be further process into melatonin using a different set of enzymes. Cells that express TPH and AAAD are cells that are capable of synthesizing 5-HT such as in the raphe nucleus in the brain, intestinal

cells, platelets, endothelial cells. And since 5-HT can be used to synthesize melatonin, the pineal gland also has the capability to synthesize the enzymes. Once 5-HT is released into the bloodstream, it will be actively taken up and stored in platelet's granules at millimolar concentrations (McNicol et al. 1999).

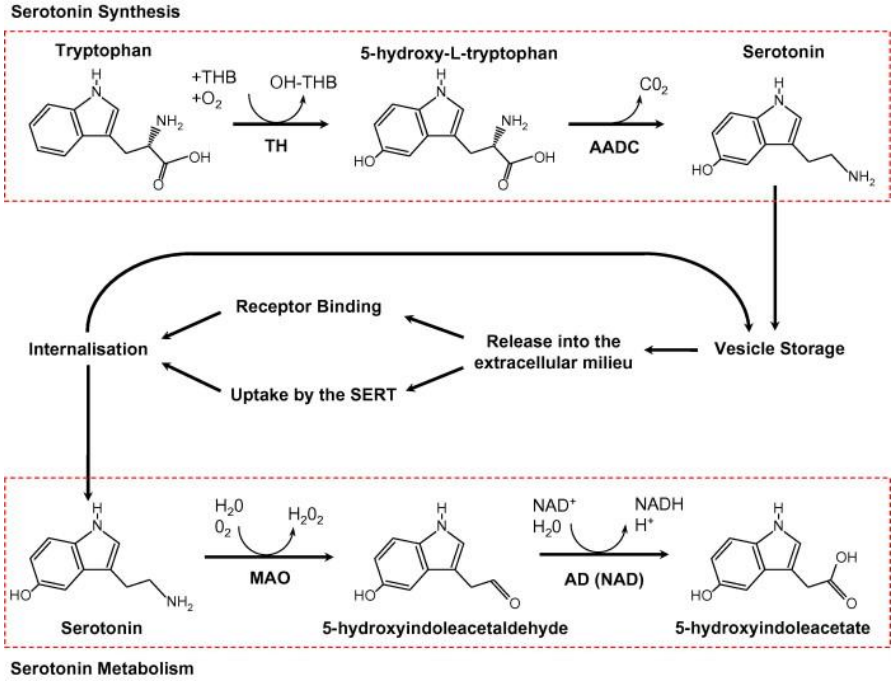



**Figure 1.5** An overview of serotonin synthesis and metabolism with the enzymes involved. (Ruddel et al. 2008)

Peripheral serotonin is proven to be involved in a myriad of regulatory functions of homeostasis, cardiovascular, cell growth and endocrine (Berger et al. 2009). About 95% of the human body's total serotonin is produced in the gut, specifically by enterochromaffin (EC) cells lining the intestinal tract, catalysed by TPH1. Therefore, in addition to its regulatory functions, 5-HT also plays a vital role in gut physiology like secretions, sensations and motility. Dysregulation of 5-HT has been associated with diarrhoea and also constipation. A clinical study done by Minderhoud and colleagues (2007) reported an upregulation of TPH mRNA in Crohn's patients exhibiting irritable bowel syndrome (IBS)-like symptoms, suggesting its role in intestinal function. Further, suggestive evidence from a systematic review reported the

development of chronic diarrhoea and intestinal bowel disease (IBD) were observed in patients on SSRI treatment drugs that selectively inhibit the reuptake of serotonin from its environment and used primarily to treat depression (Mikocka-Walus et al, 2006). Thus, disrupting the balance of 5-HT in its microenvironment can have a physiological impact on the target organ.

Serotonin transport into cells and catabolism are critical processes in maintaining the balance of plasma 5-HT levels. Proteins called serotonin transporters, SERT, mediate the transport of 5-HT into the cell. SERT is also known as *solute carrier family 6, member 4* (SLC6A4), a member of the neurotransmitter family with 12 transmembrane domains. To be able to function, SERT must depend on $Na^+/Cl^-$ transport although the exact mechanism is unknown and exist as a dimer, which relies on several posttranslational modifications (Kocabas et al. 2003). Meanwhile, the breakdown of serotonin can be described using several different pathways depending on the catalytic enzymes involved. One pathway utilizes two enzymatic breakdown steps which results in the production of melatonin as mentioned previously. A second pathway generates formyl-5-hydroxykynurenamine using the enzyme indoleamine 2,3-dioxygenase (IDO) while a third pathway uses amine N-methyltransferase (INMT) to transform 5-HT into N-methylserotonin. In the fourth major pathway, 5-HT is catabolised into 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase A/B (MAO-A or MAO-B) in which the combined use of MAO inhibitors with selective serotonin reuptake inhibitors (SSRIs) in depression therapy can lead to a condition known as serotonin syndrome (Squires et al. 2007)

### 1.5.3 5-HT receptors

According to the International Union of Basic and Clinical Pharmacology (IUPHAR: http://www.guidetopharmacology.org/) database, 6 major families and 13 subfamilies of 5-HT receptor are classified as GPCR family class A except for ionotropic 5-$HT_3$ receptors. These diverse forms of 5-HT receptors that are expressed in different tissues suggest multiple functional roles. However, some of these receptors have non-functional isoforms that arise from alternative splicing during gene expression for example 5-$HT_{2A}$ and 5-$HT_{2C}$. As previously described, signal transduction of GPCRs can be applied to explain the signalling cascades of 5-HT receptors when induced by its ligand binding which in turn elicits a response. Activation of the $G_i$ coupled 5-$HT_1$ and 5-$HT_5$ receptors leads to reduced adenylate cyclase while 5-$HT_3$ subtype as well as stimulatory $G_s$ coupled 5-$HT_4$, 5-$HT_6$, 5-$HT_7$ receptor activation leads to an increase in adenylate cyclase, which causes a surge of cAMP production. Meanwhile, the activation of 5-$HT_2$ receptors, which are Gq-coupled receptors, causes an increase in the activity of PKC/PLC thus leading to the stimulation of phosphoinositide hydrolysis (Kroeze & Roth, 1998).

The ligand-gated ion channel 5-$HT_3$ receptors are composed of five subunits clustered together forming a cationic channel that selectively allows cations to pass through upon binding of 5-HT. Each subunit composed of an extracellular N-terminus that acts as a 5-HT binding site, 4 transmembrane domains linked by intracellular and extacellular loops. The pentameric 5-$HT_3$ receptors can exist in functional heteromers consisting of two 5-$HT_{3A}$ and three 5-$HT_{3B}$ subunits. Other subunits encoded on a different chromosome than the previously mentioned have also been identified and named as 5-$HT_{3C}$, 5-$HT_{3D}$ and 5-$HT_{3E}$. Interestingly, only pentamers formed in combination with 5-$HT_{3A}$ subunits can functionally respond to 5-HT (Barrera et al. 2005).

### 1.5.4 The effects of 5-HT on immune cells

5-HT involvement in innate and adaptive immune responses has been discussed for many years although the underlying mechanism is poorly understood. This study is particularly interested in further understanding the influence of 5-HT on normal immune cell function. Numerous studies have shown that soluble factors such as complement, IgE-containing immune complexes, platelet-endothelial interaction and bacterial and parasitic infections which can activate platelets at inflammatory sites can increase plasma serotonin levels. The release of peripheral 5-HT has been ascribed to many immunoregulatory effects such as the ability to stimulate or inhibit inflammation (Mössner et al. 1998; Wagner et al. 2008). In a review article by Mössner & Lesch (1998), they discussed several research findings that supported the idea of a possible neural-immune interaction in inflammation, which includes the presence of functional 5-HT receptors on immune cells and their modulatory effects. However, criteria such as direct association and innervation of serotonergic neurons with immune cells and immune target cells or organs are not applicable. This is supported by a study that reports the denervation of target organ with neurotransmitter-specific nerve fibres does not affect the level of 5-HT at the site Therefore, a direct neural-immune communication is not present but a different link between serotonin and inflammation has been suggested (Roszman et al. 1985). .

Other than platelets, plasma and immune cells such as mast cells, monocytes/macrophages and lymphocytes are other alternative sources of peripheral 5-HT (Finocchiaro et al. 1988; Herr et al. 2017) due to an emerging number of studies that show these immune cells possess the capability to produce 5-HT when stimulated. Evidence suggests that functional TPH1 is expressed and upregulated upon T cell receptor (TCR) activation followed by an increase in 5-HT release that can act in an autocrine/paracrine manner to further facilitate T cell activation (O'Connell et al. 2006; Leon-Ponte et al. 2007). These findings support the study on murine T

lymphocytes which revealed the inhibitory effect of p-chlorophenylalanine (pCPA), a TPH enzyme inhibitor, on T cell proliferation (Young et al. 1995). This shows that 5-HT plays a role in T cell function and disrupting the synthesis and metabolism of endogenous 5-HT can have an impact on the regulation of immune responses.

The immunomodulatory effects of 5-HT seem to be cell-specific and depend on the expression of serotonergic components in immune cells. Consistent with these effects, different 5-HT receptor subtypes have been identified on human and murine immune cells including T lymphocytes, mast cells, dendritic cells and monocytes (Arreola et al. 2015; Herr et al. 2017). Binding of 5-HT to these receptors can influence different immunological responses such as chemotaxis, leukocyte activation, proliferation, cytokine secretion and induction of cell apoptosis (Idzko et al. 2004; Cloëz-Tayarani et al. 2006; Muller et al. 2009). For example, animal studies demonstrate that 5-$HT_{1A}$ signalling can enhance B cell proliferation while 5-$HT_7$ receptor activation can influence naïve T cell activity by inducing phosphorylation of MAPK pathways which can be impaired with selective receptor antagonists (Iken et al. 1995; León-Ponte et al. 2007). Other i*n vitro* studies demonstrate that 5-HT receptor antagonism can impair the activation and proliferation of human T cells by induction of apoptosis via the 5-$HT_{1B}$ receptor (Aune et al. 1994; Yin et al. 2006). In addition, the anti-inflammatory effects of selective 5-HT receptor antagonists in animal models of inflammatory conditions have been documented. Kim et al (2013) provided evidence that 5-$HT_7$ receptor antagonists can ameliorate the severity of TNBS-induced colitis while another group of scientists reported the efficacy of a 5-$HT_3$ receptor antagonist in attenuating 5-fluorouracil-induced intestinal mucositis (Yasuda et al. 2013), suggesting that targeting these receptors can have potential therapeutic effects in inflammatory diseases.

## 1.6 Melanin concentrating hormone (MCH)

### 1.6.1 Discovery of MCH

Melanin concentrating hormone (MCH) is a cyclic orexigenic peptide first identified in the pituitary of teleosts as a skin-paling factor and also in the regulation of pigmentation in fish (Kawauchi, Adachi, et al. 1980). Initially known as melanophore-concentrating hormone, the 17 amino acids neuropeptide was found to have an antagonistic role with another melanotropin, melanocyte-stimulating hormone (α-MSH), acting as a dual hormonal factor that regulates skin pigmentation in lower vertebrates. Although the existence of such melanophore has been known since 1930's, it wasn't until the early 1980's that the study of the peptide's characteristics began to take place when a group of researchers successfully isolated the hormone from fish (Kawauchi, Kawazoe et al. 1983).

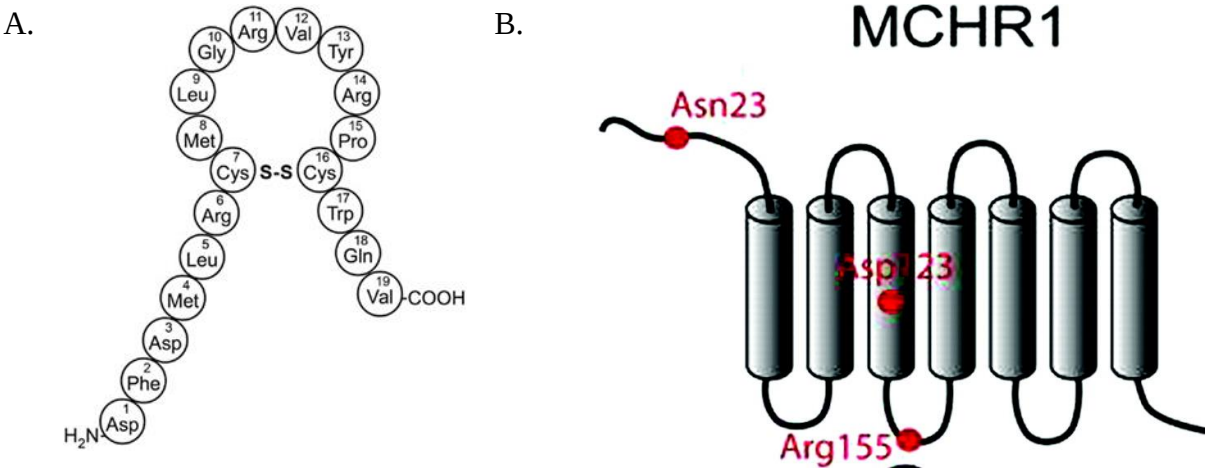



**Figure 1.6** Structure of A) mammalian MCH cyclic peptide and B) MCHR1 with locations of amino acid residues necessary for glycosylation and/or activity of MCHR1 depicted in red. (Adapted from Macneil, 2013; Pissios et al. 2006)

Kawauchi and colleagues succeeded in isolating the hormone from the pituitary gland of chum salmon. Subsequent analysis revealed its 17-amino-acid cyclic structure with one disulphide bond (Takayama et al. 1989), a discovery that helped pave the road to investigating MCH

localization in mammals, structure-activity analysis and functional studies. In 1989, a research group led by Vaughan managed to isolate and establish the MCH-like peptide sequence from rat hypothalamus which displays strong similarity with the fish peptide but with four substitutions and an extended N-terminal by two amino acids (Figure 1.6A). Human MCH is identical to that of rat and mouse, which has 19 amino acids and is mainly produced in the hypothalamus, specifically in the neurons within the lateral hypothalamic area (LHA) of the brain.

Numerous studies later revealed that MCH and its receptor are also detected in peripheral tissues and organs such adipocytes, splenocytes, thymus, skin, intestines, testis and immune cells (Hervieu et al. 1995; Verleat et al. 2002; Hoogduijn et al. 2002). Surprisingly, only the intermediate form of the MCH peptide is produced in rodent's spleen and human thymus (Hervieu et al. 1995). Today, MCH is mainly established as an appetite stimulating hormone, or orexin, in lower vertebrates and mammals.

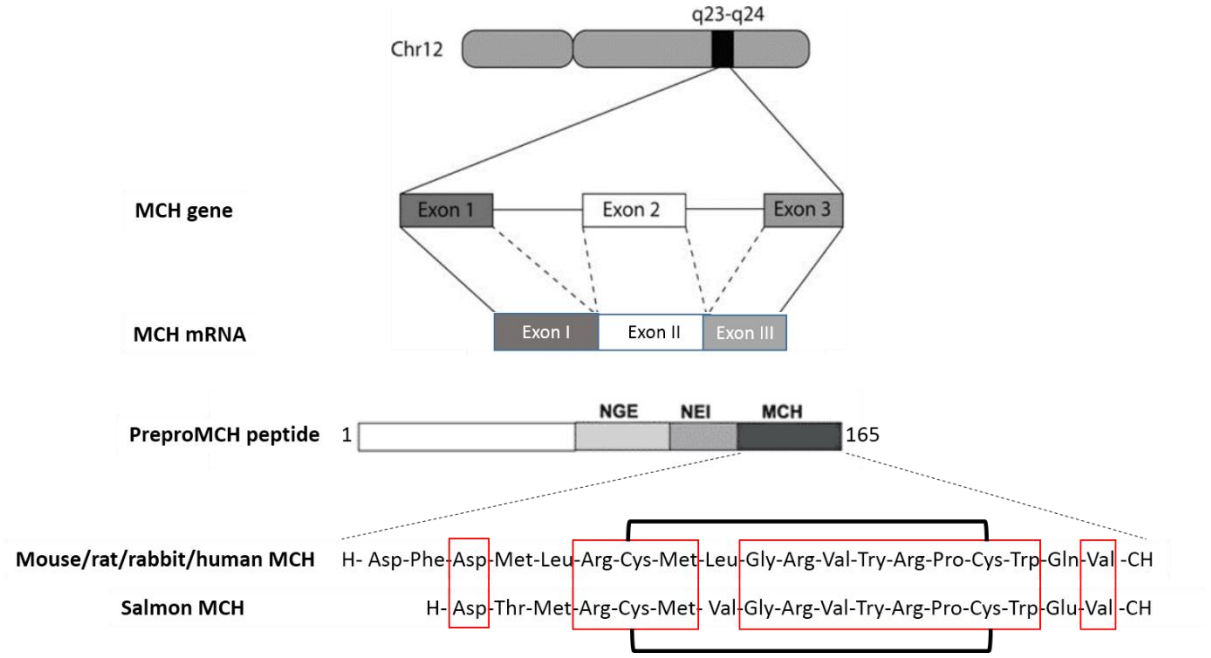



**Figure 1.7** The human MCH gene is located on the long arm of chromosome 12 consisting of three exons and two introns. The preproMCH derived from genes encoded within Exon II and Exon III. Conserved amino acids of MCH between different species in red boxes and disulphide bridge are indicated.

Two MCH genes and their corresponding mRNA were successfully cloned and sequenced from fish, bearing marked difference in terms of intron-exon organisation compared to those of mammals (Nahon, 1994). The highly conserved mammalian MCH gene consists of three exons with two introns (Figure 1.7) while the gene sequenced from fish appears to be intronless. In humans, the single 495 bp gene sequence (*Pmch*), specifically in the second and third exons, encodes for a large molecular weight preprohormone peptide of 165 amino acids in length known as preproMCH (ppMCH). The prohormone also contains an amino acid sequence corresponding to the putative neuropeptide-glycine-glutamic acid (NCE), neuropeptide-glutamic acid-isoleucine (NEI). Viale et al. (1999) demonstrated that neurons producing MCH also co-express prohormone convertases (PCs) which cleave the prohormones to produce multiple peptides. It was concluded that mature cyclic MCH is derived from the C-terminal end of ppMCH via proteolytic processes by multiple PCs. Human MCH peptide shares about 90%

homology with MCH in other mammalian species namely rats, mice, dogs and bovines (Pissios et al. 2006). The mRNA encoding this peptide was detected in various tissues of rats, mice and human centrally and peripherally using approaches like northern blot, RT-PCR and radioimmunoassay. MCH peptide was found in nanomolar concentrations (14-30 nM) in the brain and spleen of rodents and is considered in high concentrations when compared with its affinity to its putative receptors (Huang et al. 1999). Meanwhile human plasma contain picomolar quantities of MCH and this level seems to increase during fasting which also correlates with fat mass (Gavrila et al. 2005). The same experiments both similarly reported that MCH levels are not affected by leptin.

### 1.6.2 MCH receptors and receptor signalling

The activity of MCH is mediated by two GPCR receptors; MCHR1 and MCHR2. Contemporaneous efforts by several research groups in 1999 led to the identification of MCHR1 which was previously known as orphan GPCR, SLC-1/GPR24 (Bächner et al. 1999; Chambers et al. 1999; Lembo et al. 1999). The consensus 353 amino acid sequence shares more than 90% identity between humans, rats and mice (Pissios et al. 2003). The receptor is widely distributed in the brain and matches the distribution of MCH, consistent with reports of MCH biological functions in modulating stress response, anxiety and learning (Chung et al. 2011). Roy et al. (2006) suggested that the anxiolytic-like behaviour displayed by mice lacking MCHR1 could be due to its modulatory impact on serotonin levels in prefrontal cortex that is highly associated with emotional and anxiety responses. However, the inconsistencies in anxiolytic effect of MCH deficiency led to a meta-analysis study on the impact of MCH signalling on metabolism and behaviour. It was found MCH signalling deficiency in transgenic

mouse models led to an overall suppressed anxiolytic and anxiogenic effects (Takase et al. 2014). MCHR1 are also moderately expressed in brain regions implicated in regulating feeding behaviour (Marsh et al. 2002). Peripherally, MCHR1 expression was detected in tissues such as hepatocytes, adipose tissue, intestine and PBMCs (Hill et al. 2001). Meanwhile, low levels of endogenous MCHR1 expression have also been detected in cell lines originating from tissues such as skin, adipose and nervous tissues as well as pancreatic and adrenal cells (Eberle et al. 2010).

The seven transmembrane GPCR comprises of three N-glycosylation sites in the N-terminal and several phosphorylation sites within the intracellular loops. Figure 1.6B shows the common structure of MCHR1 with three amino acid residues crucial for the receptor's binding and functionality. Analysis showed that an aspartic acid ($Asp^{123}$) in the third transmembrane domain is important for MCH binding (Macdonald et al. 2000), while N-linked glycosylation at asparagine residue ($Asn^{23}$) in the N-terminal is required for cell membrane localization (Saito et al. 2003). The same group also later suggested that mutation of an arginine residue ($Arg^{155}$) in the intracellular loop could leads to loss of signal transduction (Saito et al. 2005).

Studies using transfected cell lines revealed that MCHR1 binds to multiple G proteins, which can activate various signalling pathways when bound to its cognate ligand MCH. Through coupling with $G_i$, $G_o$ and $G_q$ proteins, receptor activation leads to an increase in intracellular $Ca^{2+}$ through IP3 stimulation, inhibits forskolin-induced cAMP formation from ATP and increases in phosphorylated extracellular signal-regulated kinases (ERKs) via different protein kinases activities (Hawes et al. 2000). The latter signalling activity was observed in stably transfected cells where it was postulated that the signalling pathway is mediated through both $G_i$ and $G_q$ proteins. Interestingly, MCHR1-transfected HEK293 cells treated with a combination of MCH and forskolin significantly potentiates MAPK phosphorylation compared with MCH

treatment alone, a signal that can be reduced via PKA inhibition. Similar signal transduction can also be detected in rat brain, suggesting a complex interaction involving activation of $G_s$-coupled β-adrenergic receptors (Pissios et al. 2003). On the other hand, studies on signal transduction of MCHR1 endogenously expressed on mammalian cells are critically lacking.

Soon after the identification and characterization of MCHR1 was established, a second MCH receptor was identified. The second receptor known as MCHR2 was first identified in human using genomic sequence search. It has a relatively low homology (~35% amino acid identity) with MCHR1 and its expression pattern is similar with that of MCHR1 but less abundant and not as widely distributed (An et al. 2001; Rodriguez et al. 2001). In human, microarray analysis demonstrated that MCHR2 is present in bone marrow, thymus and lymphocytes with weak expression detected in other peripheral tissues such as pituitary, pancreas and adipocytes (Hill et al. 2001). Functional MCHR2 is present in fish and plays a role in regulating skin pigmentation while its functions in mammals and primates are not completely understood but are thought to possibly have similar biological roles with MCHR1 (Kawauchi et al. 2004). However, MCHR2 expression is notably absent in rodents which contributes to the lack of functional characterization study using animal models. The signalling mechanism is mainly acquired from transfected mammalian cells and it was demonstrated that MCHR2 is mainly coupled to $G_q$ proteins that lead to increase in intracellular $Ca^{2+}$ levels (An et al. 2001). It was hypothesized that the major sequence difference within the intracellular loops necessary for G-protein binding accounts for the difference observed in signal transduction mechanism (Presse et al. 2013). According to a recent discovery, MCHR2 gene duplication may have an influence in the pathogenesis of alopecia areata, an autoimmune disorder (Fischer et al. 2017).

### 1.6.3 Understanding functional roles of MCH

The functional role of MCH as a regulator in appetite, body weight and energy expenditure is by far the most well documented. But other *in vitro* and *in vivo* studies have also revealed its role in central and peripheral functions such as affective behaviour, reproduction, insulin release, fat deposit and immune response (Roy et al. 2007; Gonzales et al. 1997; Tadayyon et al. 2000; Abbott et al. 2003; Lakaye et al. 2009). Taken together, these discoveries further expand the knowledge of the biological functions of MCH in higher mammals other than skin pigmentation regulation as observed in lower vertebrates. However, the exact regulatory mechanism remains poorly understood. Although using transgenic or gene knockout animal models helps in understanding MCH functional roles, discrepancies between genetic and pharmacological studies have been reported. For example, acute central injection of MCH increases food intake in rats (Qu et al. 1996) and this is supported by another observation made by a different group where ppMCH-deficient mice have reduced food intake activity (Shimada et al. 1998). However, a study done by Marsh et al. (2002) reported that mice lacking MCHR1 receptors exhibit excessive eating habits, contradicting previous results, but at the same time, showing hyperactivity and having lean body mass. Similar conflicting results have been observed previously on studies investigating another orexigenic peptide, neuropeptide Y (NPY). The MCHR1 receptor knockout mice seems to be mildly obese (Marsh et al. 1998) while a different research group reported significant suppression of diet-induced weight gain in animals treated with selective receptor antagonist (Ishihara et al. 2006). It was hypothesized that different experimental parameters in each study conducted contributed to the conflicting results observed.

Regardless, pharmacological studies involving receptor antagonists help further strengthen our understanding of the physiological functions of the endogenous MCH system. Small molecule

MCHR1 antagonists show potential as novel therapeutic agents apart from providing a useful tool in studying MCH signalling. In view of the role of MCH in energy homeostasis, several MCHR1 antagonists have been recognised as useful agents in treating obesity. Several studies have consistently shown the efficacy of MCHR1 antagonists in reducing eating behaviour and decreasing body weight of high caloric diet-induced obese animals (Borowsky et al. 2002; Takekawa et al. 2002; Mashiko et al. 2005; Ito et al. 2010). In addition to the modulatory role of central MCH in feeding behaviour and body weight, peripheral MCH system activation was thought to also play a supporting role by modulating insulin and leptin release. These downstream effects help us understand the functional role of peripheral MCHR1 that is known to be expressed on peripheral tissues such as liver, pancreas, adipose tissue and intestines (Bradley et al. 2000; Tadayyon et al. 2000).

Interestingly, Borowsky and colleagues also reported that selective MCHR1 antagonism also has anxiolytic and antidepressant effects. Both acute and chronic depression models displayed reduced stress response behaviour when treated with different anti-MCHR1 with the same efficacy of a clinical antidepressant indicating an MCH influence in regulating stress-like behaviours (Borowsky et al. 2002). The exact antidepressant activity of MCHR1 antagonist is not yet fully elucidated but it has been suggested that the mechanisms involved are distinct from the other FDA-approved antidepressants (Chung et al. 2011). In addition, the non-specific interaction between MCHR1 antagonist and 5-HT receptors may also contribute to the antidepressant effects (Chaki et al. 2005). Meanwhile, pharmacological studies on animals postulated that the anxiolytic-like behaviour displayed by mice chronically administered with MCH might relate to the activation of the HPA axis associated with emotional and anxiety responses. Pretreatment with MCHR1 antagonists seems to be able to reverse the increased ACTH and corticosterone levels within the HPA axis by MCH (Smith et al. 2006). This and the

distribution pattern of MCHR1 in the limbic system (Saito et al. 2001) substantiates the hypothesis of an MCH regulatory role in emotional state.

### 1.6.4 MCH and the immune system

What is most interesting regarding this hormone, and is part of the focus of our study, is its role in the immune system. The majority of immunoregulatory effects of MCH and its receptor antagonists were determined mainly in animal studies but also a few in human immune system. These studies provide compelling evidence that MCH can directly modulate immune response and inflammatory reactions. Intial findings made by Verleat et al. (2002) revealed ppMCH mRNA expression, albeit low, in human tonsilar and peripheral blood immune cells as well as murine splenocytes and thymocytes but so far, details on the immunoregulatory influence of this hormone are scarce. However, this does not undermine the potentially significant roles it may have in immune functions. *In vitro* proliferation assays on human peripheral blood mononuclear cells (PBMCs) exhibit significant inhibition when treated with MCH, an effect not seen when treated with the other putative peptides encoded by ppMCH, when given under the same experimental condition. An increase in intracellular $Ca^{2+}$ and cAMP levels were also observed, presumably via MCHR1 coupling. Years later, another group corroberated the anti-proliferative effect of MCH adding that the MCH-mediated inhibitory action is reversible with IL-2 but not other stimulatory cytokines (Coumans et al. 2007). However, the exact mechanism mediated by MCH remains unknown but is believed to be involved in regulating the secretion of IL-2. To add, human Th2 cells have the capacity to release MCH prohormone in parallel with its regulatory cytokines when activated *in vitro*, proposing the assumption that MCH might involved in allergic response. This phenomenon was not observed in IFN-γ secreting Th1 cells

and it is worth noting that other $CD4^+$ subsets were not investigated in their experiment (Sandig et al. 2007). Meanwhile, MCH can also regulate migration and phagocytic functions of monocytes which are crucial in defense against pathogens, in addition to decreasing IL-10 secretion in LPS-induced monocytes (Karagiannis et al. 2013; Ziogas et al. 2014)

Based on this evidence, it is proposed that activation signals via MCHR1 play an important role in MCH immunomodulatory effects since rodents, which are mostly used in these studies, do not possess functional MCHR2. Additionally, a large-scale high-throughput gene expression analysis detected low levels of MCHR1 mRNA in several human and mouse immune cell lines including leukemic cell lines Jurkat and K562 as well as murine monocytic RAW264.7 (Su et al. 2002; Karagiannis et al. 2013) with supporting data on protein expression (Verleat et al. 2002). However, MCH receptor signalling analysis rarely uses endogenously expressing MCHR1 cell lines.

A research group in Harvard provided evidence on the possible role of MCHR1 activity in modulating inflammatory response. Using two different mouse models representing Crohn's disease and UC, Kokkotou and her team discovered that MCH has a pro-inflammatory role in the development of experimental colitis. Mice pretreated with anti-MCH antibodies exhibit less inflammatory response and mucosal injury when chemically induced to develop colitis. Interestingly, MCH-deficient mice also exhibit less intestinal inflammation compared with those of the wild-type (Kokkotou et al. 2008; Kokkotou et al. 2009). It was subsequently identified that MCH targets the intestinal mucosal cells which helps mediate the development of inflammation possibly by modulating inflammatory cytokine secretion from intestinal immune cells. MCH can stimulate IL-8 and MIP1-β release from colonocytes and inhibit LPS-stimulated IL-10 and TNF-α secretion from monocytes. Moreover, pro-inflammatory

molecules such as IL-1β and bacterial toxin can upregulate MCHR1 expression in colonocytes and monocytes which can be inhibited by regulatory cytokine IL-10 (Karagiannis et al. 2013; Ziogas et al. 2014). Putting together these findings help establish a novel immunomodulatory role of MCH on immune cells specifically in the development of IBD. Therefore, targeting this system might hold potential therapeutic benefits for inflammatory and autoimmune conditions.

### 1.7 Aims

This research project aimed to investigate whether the 5-HT and MCHR1 systems, both mediated via GPCR receptors, have a modulatory role on human lymphocytes in normal physiological state. The first portion of this study focused on the effect of pharmacological agents targeting 5-HT receptors and its metabolic pathway on the function and survival of healthy human peripheral T lymphocytes *in vitro* using flow cytometry. The second portion built on technical expertise and utilised similar approaches to study the role of the orexigenic peptide MCH upon immune cell function. In addition to functional studies, MCHR1 expression on different subsets of immune cells was investigated as well as to identify the changes in the level of expression in resting and activated states. Subsequently, the expression of MCHR1 by human colon tissue sections from patients with IBD was assessed using RT-qPCR and immunohistochemistry techniques in an effort to identify the expression of MCHR1 on gut tissues and infiltrating immune cells. The results assist in exploring the potential benefits of targeting the 5-HT and MCHR1 systems to treat inflammatory disease.

# CHAPTER 2 Materials And Methods

## 2.1 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

All procedures in preparing immune cells for culture were done in a Class II, type A safety laminar flow hood to maintain sterility. This included using sterile pipette tips, serological pipettes with filter, centrifuge tubes and culture plates.

Ethics approval (ERN 09-1002R) was obtained from the University of Birmingham central ethics committee to collect blood samples from healthy donors to be used in this project. Fresh blood from healthy donors was collected into 9 ml vacutainers containing heparin (BD, cat. 367874) and transferred into sterile 50 ml centrifuge tubes to be mixed with an equal volume of pre-warmed serum-free RPMI-1640 with sodium bicarbonate and L-glutamine containing 1% penicillin/streptomycin (all from Sigma, UK). In a different centrifuge tube, 20 ml of the diluted blood was carefully layered onto 20 ml of Ficoll-Paque$^{TM}$ PLUS (GE Healthcare, cat. 17-1440-03) to avoid the blood getting mixed with the Ficoll. The tubes were then centrifuged at 400 x g for 30 minutes with acceleration set at 6 and brake on 0 to separate the PBMCs from whole blood.

After centrifugation, a buffy coat containing the PBMCs appeared between the Ficoll and the plasma layers. Using a sterile pasteur pipette, the buffy coat was carefully aspirated and transferred into a new centrifuge tube to be washed by adding serum-free RPMI and centrifuged at 400 x g for 8 minutes followed by another spin at 300 x g for 8 minutes to remove platelets. During the washing steps, acceleration and brake were set at maximum speed.

Washed PBMCs were resuspended in complete RPMI (RPMI-1640 containing 1% penicillin/streptomycin and 10% heat-inactivated-fetal calf serum)and cells were counted using

a haemocytometer. Cells were resuspended in complete RPMI at the required concentration for different assays.

### 2.2 Cell activation

PBMCs obtained from healthy donors were seeded in a sterile round bottom 96-well plate in the presence either with 1 μg/ml phytohaemagglutinin-M (PHA-M; Roche, cat. 11-082-132-001), SAC or Pansorbin, a protein from *Staphylococcus aureus* at 1:10,000 dilution (Merck Millipore, cat. 507858) in combination with 20 U/ml of Interleukin-2 (IL-2; PeproTech, cat. 200-02), or 100 ng/ml of lipopolysaccharides from *Escherichia coli* (LPS; Sigma, cat. L4391) to stimulate T cells, B cell or monocytes, respectively. Cell cultures were incubated at 37°C for 24 hours. Following incubation, cells were washed and labelled with antibodies (refer Section 2.6 for protocol) for flow cytometric analysis.

### 2.3 Cell proliferation dye labelling

To prepare for the proliferation assay, isolated PBMCs were washed with protein-free PBS and resuspended at $20x10^6$ cells/mL in PBS. An equal volume of 20μM cell proliferation dye eFluor™ 450 (eBioscience, cat. 65-0842-85) diluted in sterile 1xPBS was added to the cells and incubated in the dark for 10 minutes at 37°C. This was then followed by the addition of four to five times volume of cold complete RPMI and 5 minutes incubation on ice to quench the labelling. Cells were then washed with complete RPMI three times before being counted and resuspended in complete RPMI to be seeded in 96-well round bottom culture plates at $2x10^5$ cells/well.

## 2.4 Isolation of immune cell subsets by cell sorter

In some experiments, eFluor™450-stained PBMCs were labeled with a fluorophore-conjugated antibody mix against human CD4, CD8, CD45RA and CCR7 (refer to Table 2.1 for antibodies used) to isolate purified $CD4^+$ and $CD8^+$ lymphocytes. Single-colour stained controls were prepared using 10,000 PBMCs using the same antibodies listed in Table 2.1. The $120x10^6$ cell suspension-antibodies mix in sterile PBS supplemented with 1% FBS were subjected to a MoFlo cell-sorter (Beckman Coulter Instruments, CA). Prior to sorting, the instrument was compensated using the single-stained controls followed by the setting of population gating using the MoFlo cell-sorter Summit software to isolate selected subsets into a sterile FACS tube filled with 300 μl of 20% FBS in RPMI-1640 (credit to Matt MacKenzie of Tech Hub, Institute of Biomedical Research, University of Birmingham). Sorted cells were kept on ice and immediately centrifuged and resuspended in complete RPMI to be used in proliferation assays. The isolated subsets were: $CD45RA^+CCR7^+$ naive, $CD45RA^-CCR7^+$ central memory, $CD45RA^-CC7^-$ effector memory and $CD45RA^+CCR7^-$ effector cells.

**Table 2.1** Antibody mix used in cell preparation for flourescense-activated cell sorting (FACS).

| Surface marker | Conjugate | Clone | Company/ Catalog no. | Dilution |
|---|---|---|---|---|
| CD4 | PE-Cy7 | SK3 | eBioscience 25-0047-42 | 1/80 |
| CD8a | APC | OKT8 | eBioscience 17-0086-41 | 1/400 |
| CD45RA | PE-CF594 | 5H9 | BD Biosciences 565419 | 1/400 |
| CCR7/CD197 | FITC | 3D12 | eBioscience 11-1979-42 | 1/20 |

## 2.5 In vitro proliferation assay

### 2.5.1 5-HT on T cell proliferation

Cells pre-labelled with the proliferation dye were washed three times with complete RPMI and resuspended in serum-free RPMI-1640 containing 1% HEPES buffer, 1000 U/L penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 1% non-essential amino acids and 1% ITS+3 liquid media supplement (all Sigma-Aldrich, U.K.). Cells were seeded in round-bottom 96-well plate at 0.2 $x10^6$ cells per well in a final volume of 200 μL, in triplicates. T cell activation was achieved by adding mitogen phytohaemagglutnin-M (PHA-M, Roche, cat. 11-082-132-001) to a final concentration of 1 μg/ml in the presence or absence of 1mM p-chlorophenylalanin (PCPA, Sigma, cat. C3635). In some experiments, cells were cultured with the addition of 10nM – 3 μM of 5-HT (Sigma, cat. H9523) or 0.1 mM 5-HTP (Sigma, cat. H9772) with or without selective receptor antagonists in a final volume of 200 μL per well. Non-stimulated controls containing serum-free media were run in parallel. Cultures were incubated at 37°C in 5% $CO_2$ for 72 hours. Culture conditions for T cell proliferation assay using serum-free media had been optimised prior to the assay.

### 2.5.2 MCH on T cell proliferation

Pre-labelled PBMCs were cultured in complete RPMI in a 96-well round bottom plate at 0.2$x10^6$ cells per well in triplicates with or without 100nM MCHR1 agonist (Tocris, cat. 3806) or 300nM selective MCHR1 agonist, Ala17-MCH (Tocris, cat. 3434). For PBMC culture, cells were stimulated with 1 μg/ml PHA for three days.

For isolated T cell subsets obtained from FACS (refer Section 2.4), 0.3-0.5x$10^6$ cells were seeded in flat-bottom 96-well plate pre-coated with 2 μg/ml anti-CD3 antibody and co-cultured with 2 μg/ml soluble anti-CD28 antibody with or without 1μM MCH receptor agonist. To pre-coat the plate, 50 μl of anti-human CD3 antibody (eBioscience, cat. 16-0037-85) in sterile PBS as added into each well while PBS only was used in wells for unstimulated cells. The plate containing the antibody was incubated at 37°C in 95% $O_2$ for 1- 3 hours before being washed with sterile PBS twice to remove any unbound antibodies. Isolated immune cells suspended in 50 μl of complete RPMI (0.3-0.5x$10^5$ cells per 50 μl) were added into the wells together with 50 μl of soluble anti-human CD28 antibody (eBioscience, cat. 16-0289-85) diluted in complete RPMI to reach a final concentration of 2 μg/ml. To test the effect of MCH agonist on cell proliferation, the appropriate amount of agonist was added into respective wells to a final volume of 200 μl per well and were incubated at 37°C for 3-4 days.

### 2.5.3 T cell proliferation analysis by flow cytometry

At the end of each culture, PBMCs were washed and stained with fixable viability dye eFluor™ 780 (eBioscience™, cat. 65-0865-14) according to the manufacturer's instructions followed by cell surface markers labelling with a combination of FITC-anti-CD3, Pe-Cy7-antiCD4 and APC-anti-CD8α (all eBioscience) for 20 min at 4°C in PBS/2% BSA (Sigma-Aldrich, UK). Cell surface markers labelling was not required for sorted cells.

Using a CyAn three-laser, nine-colour flow cytometer with Summit software (Beckman-Coulter, UK), cell viability dye can be detected on 780/60 band pass filter equivalent to APC channel. Proliferation dye on live cells can be detected on a 450/50 band pass filter or a Violet

1 channel. Each daughter cell will contain equal amount of the dye from parent cells and therefore can be seen as having half the dye's fluorescent intensity. The addition of CountBright™ absolute counting beads (ThermoFisher, cat. C36950) enables calculation of the absolute cell numbers of proliferated cells that were gated on cells with lower level of positive eFluor™450 staining compared to unstimulated cells.

### 2.6 Immunolabeling for flow cytometry

#### 2.6.1 Immunolabeling for cell surface markers

Cells were washed with FACS buffer (2% FBS in PBS) to remove any stimulus and the culture medium. A mixture of fluorochrome-conjugated antibodies targeting lymphocyte surface markers was prepared in FACS buffer using the following dilutions: Anti-human CD3-Brilliant Violet 510, CD4/-CD8-PE-Cy7, CD45RO/CD45RA-PE-TexasRed and CCR7-FITC/Alexa488 (refer to Table 2.2 for dilutions used). After washing, cells were resuspended and incubated in 50 μl of the antibody mixture for 20 minutes at 4°C protected from light. This was followed by another washing step before the fixing and permeabilizing step for intracellular cytokine staining (Refer to section 2.6.2 for fixation and permeabilisation method). Fixed cells were ready to be analysed on CyAn ADP flow cytometer (Beckman Coulter) by resuspending in 200 μl of FACS buffer.

Spectral compensations were run in parallel by preparing compensation beads (eBioscience, cat. 01-1111-41) stained with the conjugated-antibodies used in the assay with each antibodies was separately incubated with a drop of the compensation beads, by applying the same immunolabeling protocol minus the fixation step.

**Table 2.2** Antibodies used for surface markers in flow cytometry.

| Surface/ Intracellular marker | Conjugate | Clone | Company/ Catalog no. | Dilution |
|---|---|---|---|---|
| CD3 | BrilliantViolet510 | OKT3 | BioLegend 317332 | 1/100 |
| CD4 | PE-Cy7 | SK3 | eBioscience 25-0047-42 | 1/80 |
| CD8 | PE-Cy7 | RPA-T8 | eBioscience 25-0088-42 | 1/80 |
| CD45RA | PE-CF594 | 5H9 | BDBiosciences 565419 | 1/400 |
| CD45RO | PE TexasRed | UCHL1 | BDBiosciences 555493 | 1/400 |
| CCR7/CD197 | FITC | 3D12 | eBioscience 11-1979-42 | 1/80 |

### 2.6.2 Immunolabeling of MCHR1 receptors on human immune cells

To assess the expression of MCHR1 receptors on resting and activated human immune cells, PBMCs were incubated with or without PHA (5 μg/ml) in complete RPMI for 24 hours at 37°C in a round bottom 96-well plate. Following incubation, cells were stained with anti-human CD3-FITC (eBioscience, cat. 11-0039-42) for T cells, anti-CD14-PE/Dazzle™ 594 (BioLegend, cat. 325633) for monocytes and anti-CD19-PE-Cy7 (eBioscience, cat. 25-0199-14) for B cells using the protocol as described in section 2.6.1 with the addition of 10% human Fc receptor blocker (Miltenyi, cat. 130-059-901) to minimize nonspecific staining.

For T lymphocyte phenotyping, T cell subsets surface markers antibodies (refer to Table 2.2) were used as appropriate. MCHR1 antibody used in this experiment targets intracellular C-terminus and therefore requires the fix and permeabilization steps. Cells were fixed and permeabilized to allow for unconjugated rabbit polyclonal anti-MCHR1 antibody (Antibodies-online, cat. ABIN953350) to bind. Once permeabilized, cells were incubated with 2 μg/ml of 10% goat serum (Sigma, cat. I9140) for 10 minutes before adding the primary antibody diluted

in 1X Fix/Perm buffer to a final concentration of 1.5 μg/ml for 30 minutes. The goat serum was used as a blocking buffer for the secondary antibody. A matched isotype control was done in parallel to determine any background staining even in the presence of an Fc receptor blocker. After being washed twice to remove any unbound antibodies, cells were incubated with goat anti-rabbit AlexaFluor® 647-conjugated secondary antibody at 1 μg/ml for 20 minutes before being washed and resuspended in FACS buffer for analysis. All staining steps following fix and permeabilisation were performed at room temperature. Tables 2.2 and Table 2.3 lists the antibodies used in this protocol.

**Table 2.3** Antibodies used in flow cytometry for MCHR1 expression on immune cells.

| **Surface/ Intracellular marker** | **Conjugate** | **Company/ Catalog no.** | **Dilution** |
|---|---|---|---|
| CD3 | FITC | eBioscience, cat. 11-0039-42 | 1/100 |
| CD14 | PE/Dazzle™ 594 | BioLegend, cat. 325633 | 1/100 |
| CD19 | PE-Cy7 | eBioscience, cat. 25-0199-14 | 1/40 |
| MCHR1 | unconjugated | Antibodies-online, cat. ABIN953350 | Final concentration= 1.5 μg/ml |
| Rabbit IgG polyclonal Isotype control | unconjugated | Abcam ab37415 | Final concentration= 1.5 μg/ml |
| Goat anti-rabbit IgG | AlexaFluor® 647 | LifeTechnologies A21244 | 1/1000 |

## 2.7 Maintenance of cell culture

All cell lines used in this project were grown and maintained at 37°C and 95% $O_2$ in designated culture medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Both K562 (ATCC® CCL-243, School of Cancer Science, University of Birmingham) and Jurkat cells are suspension cells and were maintained in complete RPMI by subculturing every 2 or 3 days at 0.5 to 2 x $10^6$ viable cells/ml in T-25 culture flasks (Corning®).

Adherent HEK293 cells were grown in T-75 Corning® flasks in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, cat. D6429) with L-glutamine and 4500 mg/L glucose while the neuroblastoma cell line SH-SY5Y was maintained in DMEM/F12 culture media (Life Technologies, cat. 21041-025). Both cell lines were subcultured when they reached 90% confluency using 0.25% trypsin/0.02% EDTA at 37°C.

## 2.8 Protein expression analysis

### 2.8.1 Sample preparation for Western blot

Whole cell lysate from adherent cells was prepared by removing the media from the culture flask and washing the cells twice with 5 ml PBS. Cells were removed from the flask by using a cell scraper in the presence of PBS. The scraped cells were transferred into a centrifuge tube and resuspended in PBS to be centrifuged at 400 x g for 5 minutes to form a cell pellet. Cells cultured in suspension were immediately transferred into a centrifuge tube and washed with PBS to remove culture medium. After centrifugation, the supernatant was aspirated and discarded, and the cell pellet lysed by sonication or stored at -80°C until further use.

To sonicate, cell pellets were resuspended in 100 µl per $10^7$ cells of ice cold 25 mM Tris buffer pH 7.4 in a 1.5 ml eppendorf tube and were sonicated on ice for 5 seconds for three times each, or until cell homogenization was achieved, with a few seconds interval between each sonication. Cell lysis by sonication disrupted the cell membranes and released intracellular proteins into the solution.

### 2.8.2 Measuring protein concentration by Bradford protein assay

The protein concentration in the cell lysate was determined using the Bradford assay. The Bradford reagent (Sigma, cat. B6916) was brought to room temperature prior to use. If proteins are present, they will form a complex with the Brilliant Blue G dye in the reagent which causes a visible color change from light brown to blue that can be measured using the Eppendorf BioPhotometer plus ™ spectrophotometer. The instrument was calibrated using Bovine Serum Albumin (BSA, Sigma, cat. A2153) dissolved in 25 mM Tris pH 7.4 with a concentration range of 0.1-1.4 mg/ml. The increase in absorption at 595nm wavelength is proportional to the amount of protein-dye complex.

A small volume of cell lysate was diluted in an appropriate amount of 25 mM Tris pH 7.4 buffer and 20 µL of this solution was added to 1 ml of Bradford reagent in disposable polystyrene cuvettes (Fisherbrand, cat. FB55147). Solutions were mixed and allowed to stand at room temperature for 5 minutes before taking absorption measurements. A blank sample was prepared by adding 20 µl of Tris buffer to the reagent in a cuvette. The protein concentration calculated based on the shift of absorption at 595 nm, was recorded and used to calculate the amount of protein loading for western blot.

### 2.8.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

Qualitative and quantitative approaches were used to detect the presence and level of protein of interest in the cell lysate. To use the cell lysate in gel electrophoresis, 50 µl of the cell lysate and 50 µL of 2x RIPA buffer (Cell Signaling Technology, cat. 9806) containing 1mM phenylmethyl sulfonyl fluoride (PMSF, ThermoFischer, cat. 36978), a serine protease inhibitor, were mixed in a 1.5 ml eppendorf tube. The mixture was resuspended with 100 µL of dissolved 2X urea sample buffer (USB) containing 10% (v/v) β-mercaptoethanol. Samples were heated to 37$^{o}$C for 10 minutes immediately prior to loading on a gel.

SDS-PAGE was utilised to separate the protein of interest from the sample lysate and a western blot was performed to visualise the protein with the aid of a chemiluminescent detection kit. A 12% Tris-Glycine-SDS running gel with a 4% stacking gel on top was used because the sizes of the proteins of interest throughout this study were between 10-60kDa. A pre-determined amount of protein of not more than 30 µg from the sample-USB mix, prepared as described previously, was carefully loaded into each well to avoid spillage into the adjacent well. A protein ladder containing pre-stained protein of different molecular weight (ThermoFisher, cat. 26619) was loaded into one well for each gel used in electrophoresis. In a tank filled with electrophoresis buffer, the electrophoresis was run at a constant current of 35 mA per gel for approximately 1 hour and 30 minutes.

Once the proteins from the sample were separated during the electrophoresis, the gel was removed and laid on top of a PVDF blotting membrane (GE Healthcare, cat. 10600023). The PVDF membrane was wetted in 100% methanol and soaked in transfer buffer containing 20% (v/v) methanol for 5 minutes before being used. The gel-PVDF layers were then laid in between

blotting papers pre-soaked in transfer buffer before the blotting process was performed at 1.2 $mA/cm^2$ of a gel (constant current) for 1 hour.

Once the transfer was complete, the membrane blot was blocked in 10% (w/v) non-fat milk in 1X wash buffer, known as blocking buffer, for 2 hours at room temperature on a rotary table. The membrane was then incubated overnight at 4°C with an unconjugated primary antibody in 5% blocking buffer at a predetermined dilution factor.

Following primary antibody incubation, the membrane was washed four times for 5 minutes each in wash buffer before being incubated for 1 hour at room temperature with HRP-conjugated secondary antibody raised against the host species of the primary antibody. After washing the membrane with wash buffer as previously described, a 1:1 ratio of reagent A and B from the EZ-ECL chemiluminescence detection kit for HRP (Biological Industries, cat. 20-500-120) mixture was made and let stand at room temperature for 5 minutes before being added to the membrane and left for another 5 minutes.

Qualitative detection of the protein of interest was achieved by exposing the membrane to Amersham Hyperfilm ECL (GE HEalthcare, cat. 28906839) and developed in ECOMAX X-ray processor (Protex, Germany). For quantitative detection, the membrane treated with the chemiluminescence kit solution was directly placed in the ChemiDoc MP system imager (Bio-Rad) to obtain images and analysed using Image Lab 5.2.1 software (Bio-Rad).

## 2.9 Gene expression analysis

### 2.9.1 Total RNA extraction

The total cellular RNA was extracted following the manufacturer's instruction using an RNeasy® Mini Kit (Qiagen®, cat. 04053228006114) with on-column DNAse digestion to obtain better purity. Fresh or frozen cell pellets containing approximately or less than $5x10^6$ cells were lysed by pipetting in 350 µl of buffer RLT containing 2% (v/v) 2M DTT. Once lysed, an equal volume of 70% molecular biology grade ethanol was mixed with the lysate to precipitate the DNA and RNA. The whole mixture was loaded into the provided RNeasy spin column and washed by centrifugation at 10,000 x g for 15 seconds with buffer RW1. An on-column DNAse digestion was performed by adding 80 µl of DNase solution in buffer RDD (Qiagen, cat. 78254) directly to the top of the spin-column and left at room temperature for 15 minutes. After two washes with buffer RPE, RNA which was retained in the column was eluted with nuclease-free water and ready to be used for downstream application.

### 2.9.2 RNA purity check

Total RNA concentration from the RNA extraction was quantified using a Thermo Scientific™ NanoDrop 2000 spectrophotometer by measuring the optical density (OD) at 230 nm, 260 nm and 280 nm wavelength. The $OD_{260/280}$ ratio was used as an indicator for RNA purity. A ratio of 1.8 – 2.0 was deemed acceptable for gene expression measurement as this represents a relatively low degree of protein contamination.

### 2.9.3 Reverse transcription

Following RNA extraction, 1 μg of RNA was used as a template to make complementary DNA (cDNA) using the SuperScript Double-Stranded cDNA Synthesis System kit (Invitrogen, cat. 11917010) containing 5x First-Strand Reaction Buffer, 0.1 M DTT, 10 mM dNTP mix and SuperScript® II RT (200U/μL). In a 0.2 ml RNase-Dnase-free microtube, 2 μl of 10 mM dNTP mix and 1 μl of Oligo(dT) 12-18 primer (Life Technologies, cat. 58862) were added to the RNA and topped with nuclease-free water to 24 μl total volume before being subjected to heat treatment for 5 minutes at 65°C in the waterbath. The mixture was then immediately cooled on ice before the addition of 8 μl of 5X First Strand Buffer, 4 μl of 0.1M DTT, 2 μl of RNase Out (Invitrogen, cat. 10000840) and 2 μl of the SuperScript® II reagent. The synthesis of the cDNA was performed in the MJ Research Tetrad PTC-225 Thermal Cycler using the following settings; 42°C for 50 minutes, 72°C for 15 minutes and 4°C on hold. The newly synthesized cDNA could be used immediately or stored in -20°C until further use.

### 2.9.4 Multiplex quantitative Polymerase Chain Reaction (qPCR)

Gene expression of MCH, MCHR1 and MCHR2 from human colon tissue samples was investigated by analyzing cDNA derived from the extracted total RNA normalised to endogenous β-actin (ACTB). Samples used in this experiment were "normal" samples, which are from the tissue sections taken a few centimeters away from a colon tumour, and IBD samples, which are from the inflamed area of the colon from patients with Crohn's Disease or ulcerative colitis. All samples collected from consented patients were supplied by Human Biomaterials Resource Center (HBRC; Ref: 15-237). A multiplex real-time quantitative

polymerase chain reaction (RT-qPCR) was performed to compare the mRNA expression levels in "normal" and inflamed colonic mucosa from patients with IBD.

Using a 96-well qPCR microplate (Life Technologies, cat. 4306737), a final volume of 20 μl reaction mixture consisting of 10 μl of Taqman MasterMix (Applied Biosystem, cat. AB4369016), 1 μl of primer/probe mix for each gene of interest (GOI) and 1 μl of the housekeeping gene β-actin (ACTB) mixed in 16 μl of nuclease-free water was added to each well containing 4 μl of cDNA template. The cDNA sample was the product of the reverse transcription reaction from 1 μg of the total RNA template.

The PCR was carried out using Applied Biosystems®7500 Real-Time PCR Systems with the following temperature profile: 50°C for 2 min followed by 95°C for 10 min and 40 cycles of amplification (95°C for 15 sec and 60°C for 60 sec). Pre-mixed primer/probe preparations were purchased from ThermoFischer Scientific and were designed to recognize sequences specific for the GOI: human prepromelanin concentrating hormone (PMCH, assay ID. Hs0104242_g1), MCH-R1 (assay ID. Hs00538798_m1), and MCH-R2 (assay ID. Hs00261272_m1). The GOI probes were dual-labeled containing 5' FAM reporter dye and 3' end MGB NFQ quencher while the probe for housekeeping gene ACTB (assay ID. Hs99999903_m1) uses VIC reporter dye. All probes spans exons. Housekeeping genes were primer-limited. The mastermix used contained the dye ROX which provided fluorescence normalisation to take account of pipetting errors and instrument variation.

## 2.10 Immunohistochemistry

To assess the expression of MCHR1 on infiltrating immune cells in human colonic mucosa and to characterise the immune cell phenotype expressing the hormone receptor, paraffin embedded human colon tissue sections of 4 μm thick on slides provided by the Human Biomaterials Resource Centre (HBRC), University of Birmingham, were used. The slides were rehydrated in two changes of xylene, followed by 100% ethanol twice, 90% ethanol, 70% ethanol and deionized water for 10 minutes each. Heat-induced antigen retrieval was performed in 10 mM citric acid pH 6.0 for 10 minutes in a microwave on a high heat setting and briefly cooled under running tap water before being treated with 0.3% hydrogen peroxide for 30 minutes to block endogenous hydrogen peroxidase activity. Sections were then permeabilized in 0.3% Triton-X100 (Sigma, cat. X100) in 1xPBS for 10 minutes and were subsequently blocked with 2.5% normal horse serum (Vector Labs, cat. S-2012) for 20 minutes at room temperature prior to primary antibody staining. A hydrophobic barrier was drawn around the tissue to localise and allow minimum usage of antibody solutions to the sections. Rabbit polyclonal antibody against human MCHR1 C-terminus (1:500 dilution) was prepared in blocking buffer composed of 2.5% normal horse serum and 10% human Fc receptor blocker to a final antibody concentration of 1 μg/ml. Negative control staining was a matched isotype control with the same final concentration as the primary antibody. Sections were incubated in a dark, humidified chamber at 4°C overnight.

The subsequent staining processes were all performed in a humidified chamber at room temperature. Following overnight incubation, sections were subjected to three washes in permeabilizing buffer for 5 minutes each and incubated in biotinylated goat anti-rabbit secondary antibody (Vector Labs, cat. BA-1000, 1:500 dilution) for 30 minutes. Meanwhile, an

avidin-biotin peroxidase complex from VECTASTAIN® Elite ABC-Peroxidase Kit (Vector Labs, cat. PK-6100) was prepared as per the manufacturer's instruction and allowed to stand at room temperature for 30 minutes. After removing the secondary antibody solution, a few drops of the ABC solution were added to the sections and allowed to sit for another 30 minutes before washing the slides in permeabilizing buffer as described earlier. This was used to amplify the chromogen intensity when reacted with the horseradish peroxidase (HRP) complex bound to the biotinylated secondary antibody. The chromogen used, Vector® DAB (3,3-diaminobenzidine) substrate kit (Vector Labs, cat. SK-4100), turned brown in the presence of HRP enzyme. Incubation in DAB reagent was stopped once the brown color was observed on the sections stained with primary antibody. Deionised water was used to wash away the reagent before proceeding to mounting or subsequent immunolabelling with a different primary antibody.

For multiplex immunohistochemistry, stained sections were treated with 3% $H_2O_2$ for 5 minutes at room temperature to inactivate excess peroxidase following the first chromogen reaction. Sections were blocked again with 2.5% normal horse serum followed by primary antibody or matched isotype incubation. In this experiment, mouse monoclonal antibodies against human CD11c (Abcam, cat. Ab218434, 1:200 dilution) and CD14 (Abcam, cat. Ab182032, 1:500 dilution) were prepared as described previously and used at a pre-determined final concentration (refer Table 2.4) for 1 hour at room temperature. Similar subsequent staining procedures were repeated as the first primary staining described above using biotinylated horse anti mouse secondary antibody (Vector Labs, cat. BA-2000, 1:500 dilution). A second peroxidase substrate producing a high purple color contrast, the Vector® VIP substrate kit (Vector Labs, cat. SK-4600) was used to detect the CD antigen when reacting with the HRP-

conjugated avidin-biotin-antibody complex. Sections were incubated with VIP substrate until optimal color develops followed with wash steps in deionised water for 5 minutes.

Methyl green counterstain was used to label the nuclei by immersing the slides in methyl green solution for 5 minutes at room temperature. The counterstain was made by dissolving the 0.5% methyl green solution in 0.1M sodium acetate buffer pH4.2 at 1 in 500 dilution factor. This process was followed by a rapid succession of dehydration steps by dipping the slides in 95% ethanol, two changes of 100% ethanol and clearing in xylene before mounting using resinous DPX mounting medium (Sigma, cat. 06522).

A batch run of automated image scanning and acquisition at up to 40x high-magnification to view the protein expression on whole slides and selected regions of interest was obtained using the Vectra® automated quantitative pathology imaging system (Perkin Elmer) powered by inForm® software. The software also enables users to create simulated fluorescent images from brightfield IHC images. The inForm® software allows spectral unmixing to be able to have a separate view of each antigen staining represented by different colors.

**Table 2.4** Antibodies used in immunohistochemistry on paraffin-embedded human colon tissues.

| **Primary antibody** | **Isotype** | **Company/ Catalog no.** | **Final concentration** |
|---|---|---|---|
| Anti-CD11c | Mouse monoclonal IgG2b | Abcam Ab218434 | 1 μg/ml |
| Anti-CD14 | Mouse monoclonal IgG1 | Abcam Ab182032 | 2 μg/ml |
| MCHR1 | Rabbit polyclonal | Antibodies-online ABIN953350 | 1 μg/ml |
| **Isotype control** | **Clone** | **Company/ Catalog no.** | **Final concentration** |
| Mouse monoclonal IgG2b | eBMG2b | eBioscience 14-4732-85 | 1 μg/ml |
| Mouse monoclonal IgG1 | MG1-45 | BioLegend 401403 | 2 μg/ml |
| Rabbit IgG polyclonal | | Abcam ab37415 | 1 μg/ml |
| **Secondary Antibody** | **Isotype** | **Company/ Catalog no.** | **Final concentration** |
| Biotinylated Horse anti-Mouse | IgG (H+L) | Vector Labs BA-2000 | 1 μg/ml |
| Biotinylated Goat anti-Rabbit | IgG (H+L) | Vector Labs BA-1000 | 1 μg/ml |

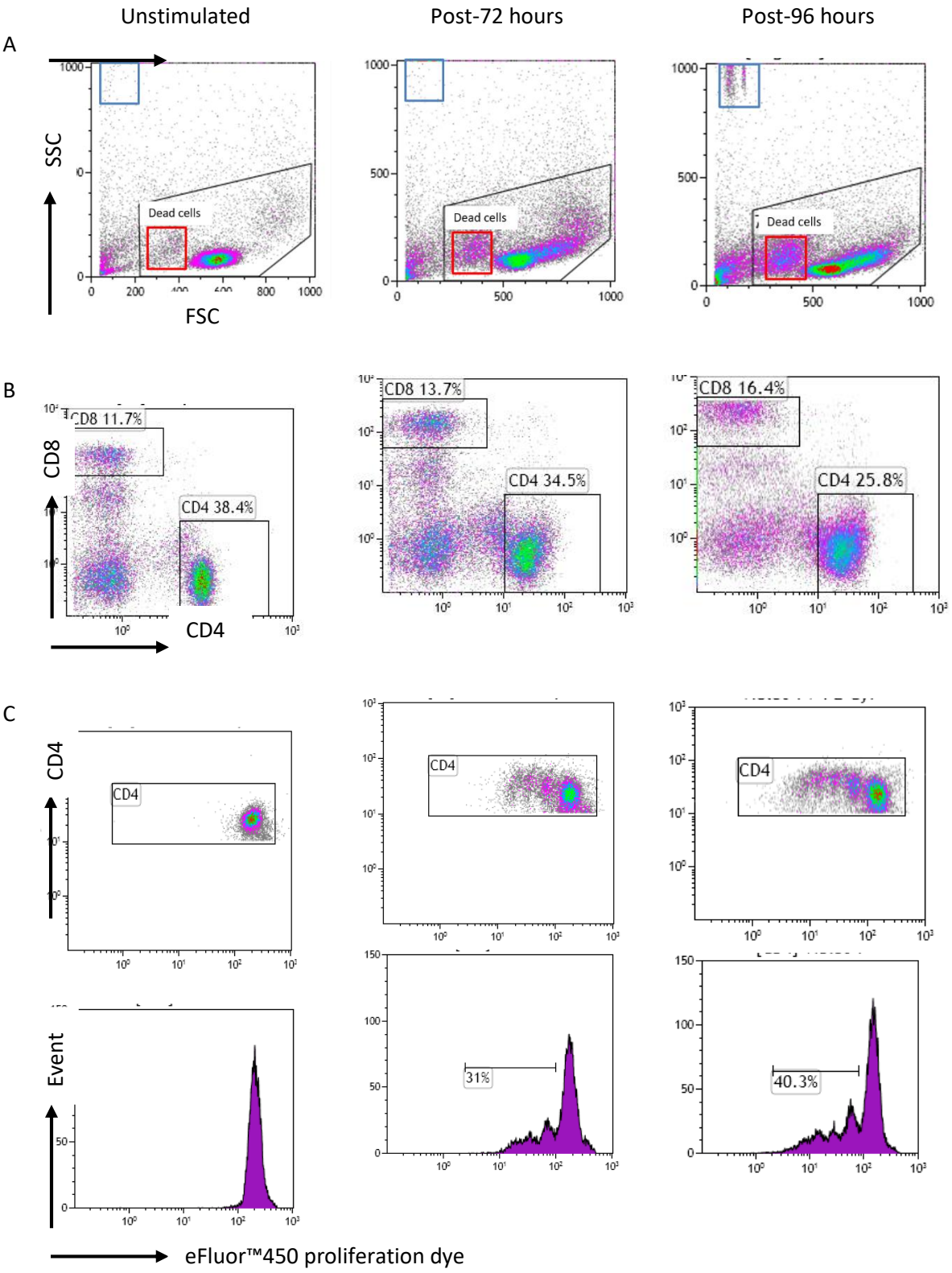
### 2.11 Statistical analysis

Data are represented as mean ± standard error of the mean. Where appropriate, data were analysed by using unpaired t-test or one-way analysis of variance (ANOVA), followed by Dunnett's or Tukey's multiple comparison post hoc tests using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA).

# CHAPTER 3: Results on the functional response of 5-HT in human T lymphocytes

## 3.1 Optimisation of proliferation assay

To study the effect of 5-HT on the proliferation of normal human T lymphocytes, a flow cytometer was used with the aid of counting beads to quantify the number of proliferated daughter cells that have been labelled with the eFlour™450 proliferation dye, as described in Section 2.3. PBMCs from healthy volunteers were cultured at $0.2x10^6$ cells per well with different concentrations of 5-HT in a serum-free condition. To replace the nutrients required for cell growth that are usually obtained from the serum, we substituted the 10% heat-inactivated foetal calf serum with a solution of non-essential amino acids in addition to liquid media supplement containing a mixture of insulin, transferrin and sodium selenite which have been developed for serum-free cell culture application. This supplementary combination can help maintain cells *in vitro* when no serum is added. We worked to observe the proliferation rate for every 24 hours for 4 days and also monitor the generation of dead cells at the end of each incubation period. This procedure enabled us to determine the optimised method to perform the functional assay on human PBMCs in a serum-free environment.


Unstimulated
Post-72 hours
Post-96 hours
A
SSC
FSC
Dead cells
Dead cells
Dead cells
B
CD8
CD4
CD8 11.7%
CD4 38.4%
CD8 13.7%
CD4 34.5%
CD8 16.4%
CD4 25.8%
C
CD4
CD4
CD4
CD4
Event
31%
40.3%
eFluor™450 proliferation dye

Continue

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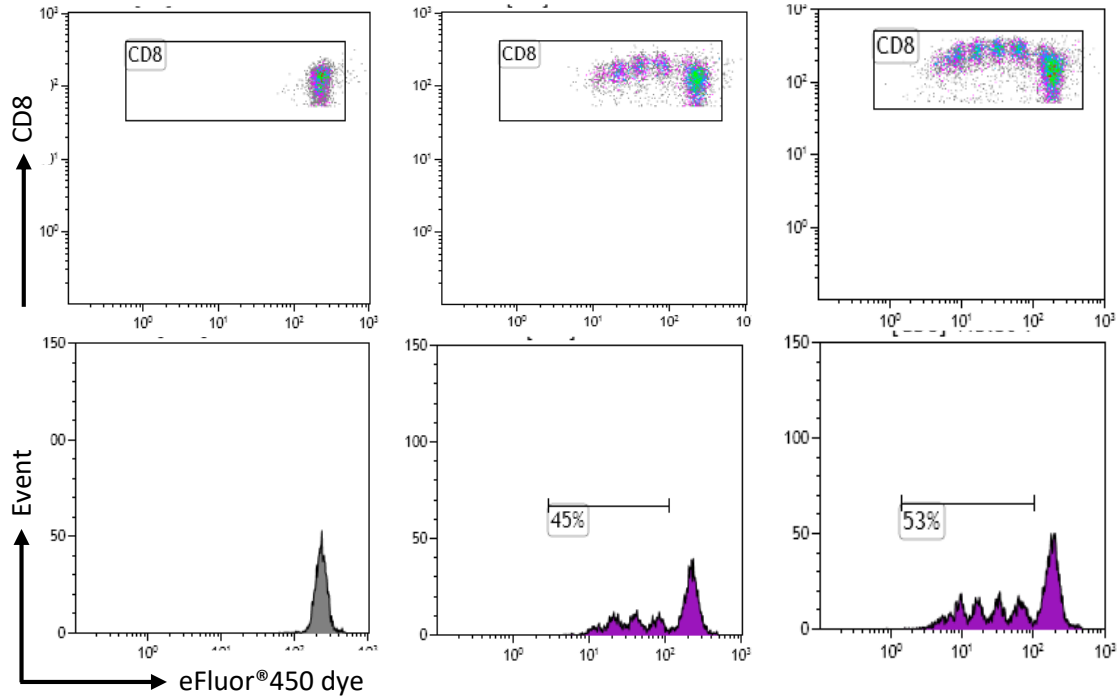



**Figure 3.1. T cell proliferation gating strategy using eFluor™450 proliferation dye and PHA stimulation**. Representative plot of cell proliferation post-72 and -96 hours showing **A** live and dead (red box) cells gated on the basis of their FSC/SSC with counting beads (blue box). **B** After excluding the dead cells, CD4 and CD8 T cells were selected from live cell population based on $CD4^+$ and CD8+ positive cells. **C** and **D** The percentage of proliferated CD4+ and CD8+ T cells, respectively, were selected against that of unstimulated cells based on the fluorescence intensity of the proliferation dye. All gates were identical in all samples within each experiment.

Following the end of each incubation period, cells were washed and immunolabelled with cell viability dye and resuspended in buffer solution prior to flow cytometry analysis. Figure 3.1 represented the gating strategy to measure proliferated cells within $CD4^+$ and $CD8^+$ T cell population after 3 and 4 days of incubation. Data plots at day 1 and day 2 post stimulation were not included as there were minimum or no proliferation observed. Histograms depict the mean fluorescence intensity (MFI) of the proliferation dye gated on $CD4^+$ and $CD8^+$ T lymphocytes with each peak representing each group of daughter cells generated as the cells proliferate. From

the figure above, we can clearly see that $CD8^+$ T lymphocytes (Figure 3.1D) have a higher rate of proliferation compared to $CD4^+$ T cells (Figure 3.1C) within the same duration of incubation. The number of proliferated cells obtained from the histograms were then used to plot against the concentration of PHA used as shown in Figure 3.2.

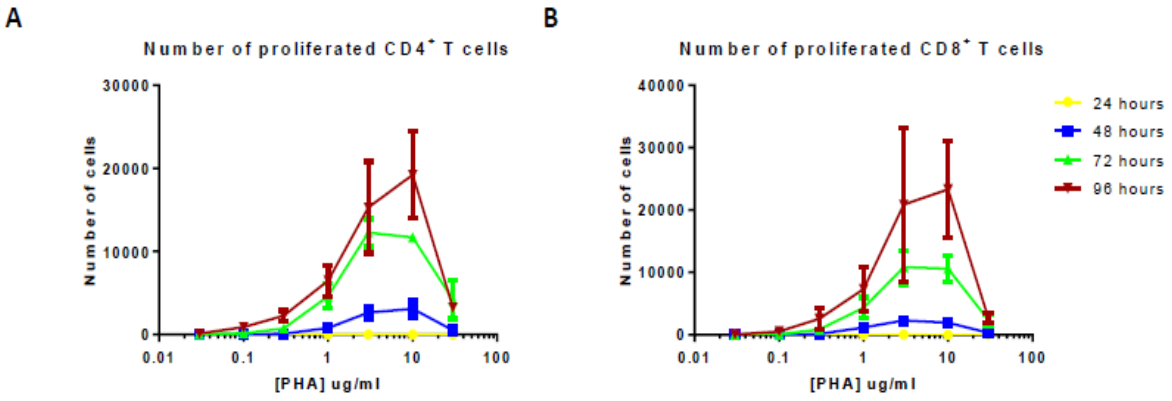



**Figure 3.2 The optimisation of serum-free proliferation assay.** Sub-optimal conditions for T cells proliferation in vitro were determined by culturing eFluor™450- labelled PBMC at $0.2x10^6$ cells per well with different concentrations of PHA. Number of proliferated **A)** CD4+ and **B)** CD8+ T cells at 24, 48, 72 and 96 hours were determined by measuring eFluor®450-positive cells gated on live CD4+ and CD8+ T cells on flow cytometer. Values shown are calculated mean±S.E.M. from three independent experiments.

Figure 3.2 showed both $CD4^+$ and $CD8^+$ T cells have a similar response to mitogenic stimulation in which case both subtypes showed a higher rate of proliferation when stimulated with 5-10 μg/ml PHA while a higher concentration of 50 μg/ml PHA deemed unsuitable to elicit a functional response *in vitro*, as shown by the low number of proliferated cells in the plot above. A slower rate of proliferation was observed in culture stimulated with 1 μg/ml PHA by the end of 72 hours compared to 96 hours incubation period. A marked increase in the number of proliferated cells was detected after 72 hours of incubation when compared to 48 hours and the trend continued until 96 hours except for the cell culture treated with 50 μg/ml of PHA. However, using PHA stimulation at 5 μg/ml and higher also produced a higher percentage of

dead or dying cells at the end of the incubation period as detected by flow cytometer using cell viability dye as represented in Figure 3.1A (in red box). From these data, we were able to design the suboptimal culturing condition comparable to that of serum-supplemented media that enabled us to observe any modulatory effects of neurotransmitters or neuropeptides on T lymphocytes functional responses *in vitro*.

### 3.2 Adverse effect of 5-HT depletion on T lymphocytes.

The biosynthesis of 5-HT is a multistep process which involves several enzymes beginning with catalysing tryptophan to 5-hydoxy-L-tryptophan. One of the enzymes in the biosynthesis pathway is the rate-limiting tryptophan hydroxylase (TPH) which catalyses the hydroxylation of tryptophan to form the 5-HT precursor, 5-hydroxytryptophan or better known as 5-HTP. Here we aimed to evaluate the role of 5-HT in CD4 and CD8 T cell proliferation by interfering with the endogenous 5-HT biosynthesis pathway. The first assay was to inhibit the activity of TPH-1 in immune cells by treating PBMCs with the TPH inhibitor, p-chlorophenylalanin (pCPA). PBMCs stained with proliferation dye were plated and stimulated with 1 ug/ml PHA in the presence of different concentrations of pCPA over the course of 3 days. Referring to Figure 3.3, the results showed that T cells are still able to respond to mitogen stimulus in the presence of up to 10μM PCPA, comparable to control group while 1 mM pCPA can be seen to significantly inhibit proliferation ($p<0.01$) in both T cell subtypes. Interestingly, the suppressive effect is more prominent in CD4+ T cells than CD8+ cells.

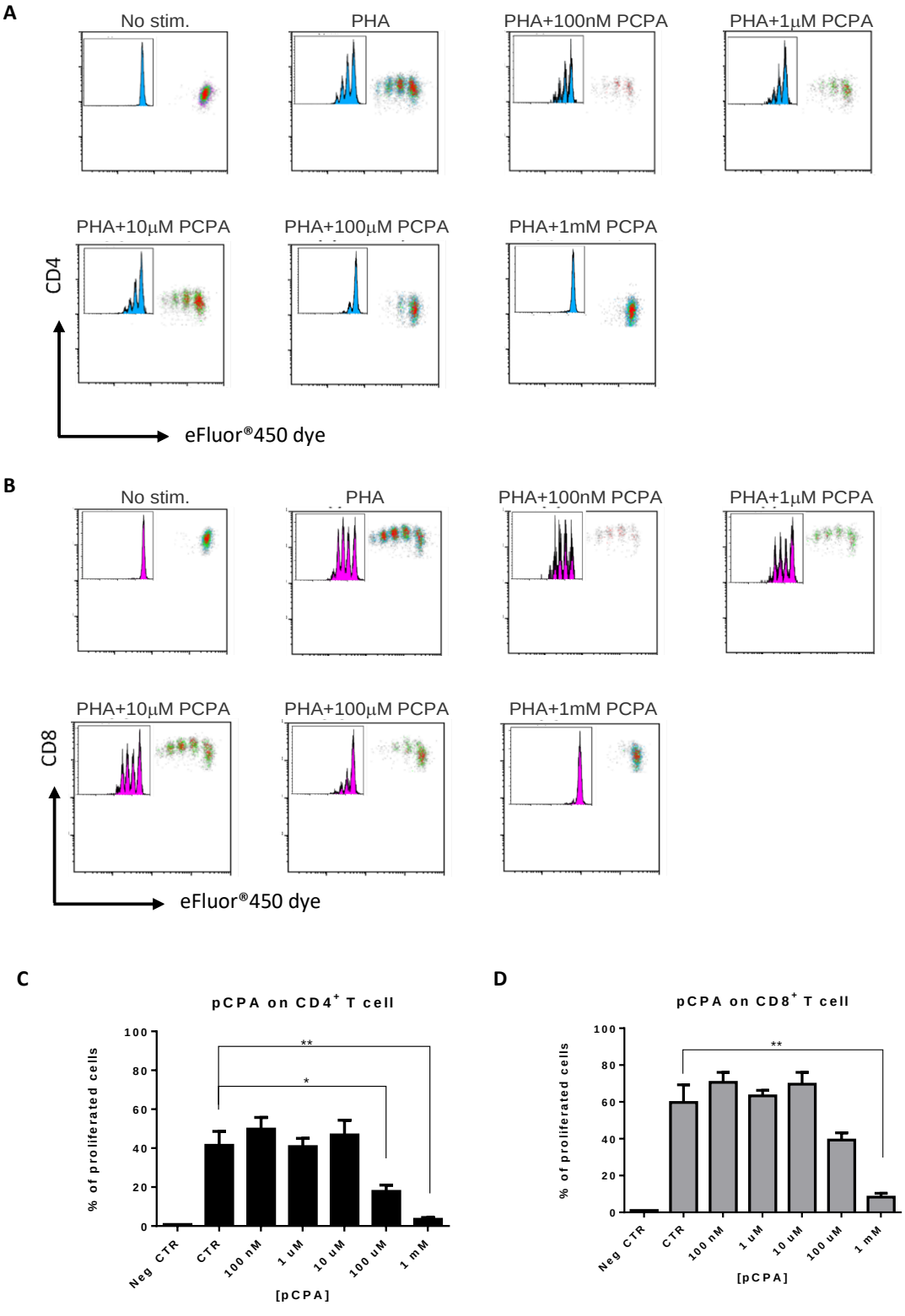



**Figure 3.3 Effect of pCPA on the proliferation of CD3+ T cells from normal PBMCs**. **A** and **B** correspond to $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells respectively. Cells were stimulated with PHA during the course of 3 days. Representative dot plots showing cell divisions by dilution of the violet dye. Inserts with histograms showing the number of cell divisions indicated by the number of peaks observed. Data are also represented in bar charts (**C,D**). Values shown are calculated mean±S.E.M., *p<0.05, **p<0.01 (n=3; One-way ANOVA with Dunnett's multiple comparison test).

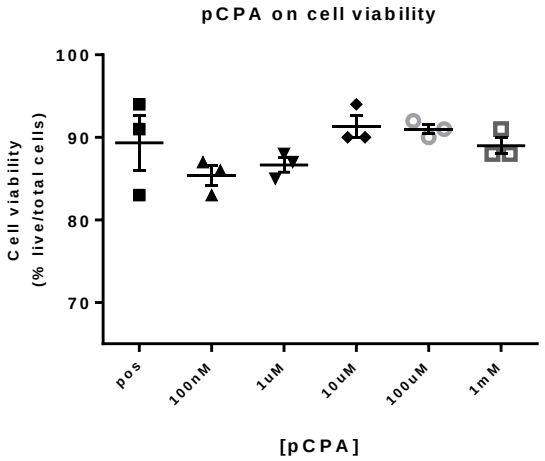



**Figure 3.4 pCPA impact on cell viability.** The effect of increasing pCPA concentrations on cell viability was measured by the percentage of viable stimulated cells over total cells treated with pCPA. PBMCs were stimulated with PHA (1 μg/ml) for three days in the presence of increasing concentration of pCPA. The number of viable cells were analysed by flow cytometer using cell viability dye. Values shown are calculated mean ± S.E.M. (n=3). Pos=PHA-stimulated cells.

Apart from the proliferative response, cell viability of lymphocytes after the end of the incubation period was also assessed. It seems that increasing pCPA concentration does not affect cell viability as the percentage of live cells in culture was similar to that of the control group (Figure 3.4). Therefore, it can be hinted that the inhibitory effect of pCPA on T cell proliferation is not due to the induction of cell apoptosis.

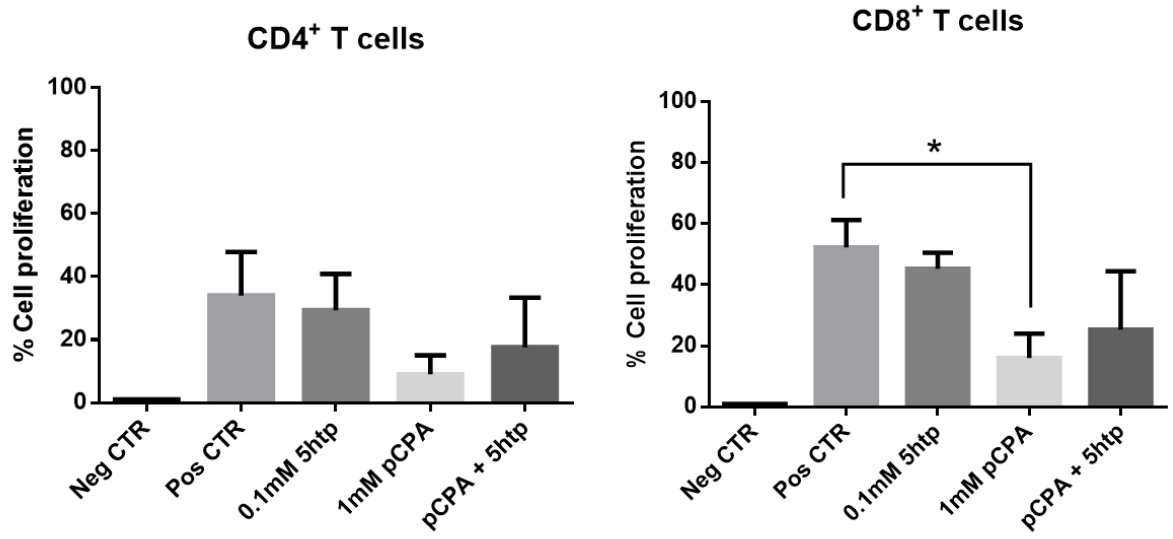



**Figure 3.5 Effect of 5-HTP on the proliferation of pCPA-treated PBMCs** PHA-stimulated PBMCs were cocultured with pCPA and/or serotonin precursor 5-HTP for 72 hours. Data shown are the percentage of proliferated T cells against total T cells. Values shown are calculated mean±S.E.M (n=3) performed in triplicates. Neg CTR= non-stimulated control; Pos CTR= PHA-stimulated control. *P<0.05 (one-way ANOVA with Tukey's test)

We then continue with the next assay to which exogenous 5-HTP (0.1mM) was applied to culture containing 1 mM pCPA to see if it can reverse the suppressive effect of pCPA on cell proliferative response. Based on the percentage of proliferated cells in Figure 3.5, a mixed response were observed between different biological repeats but overall, 5-HTP addition was not able to rescue either CD4 or CD8 T cell proliferation that was suppressed by pCPA. Even though it can be deduced that human T lymphocytes possess the capability to produce 5-HT and depleting intracellular 5-HT storage can affect T cell proliferation negatively, particularly observed here in the CD8 T cell population ($p<0.05$) the modulatory effects reported so far are all non-statistically significant.

### 3.3 Effect of 5-HT on T cell viability

Following the optimisation of cell culture conditions, another proliferation assay was used to assess the effect of 5-HT by stimulating normal PBMCs with 1 µg/ml PHA in serum-free media for 72 hours in the presence of different concentrations of 5-HT. Aliquots of concentrated 5-HT in water were diluted in serum-free media and added into the culture to a final concentration range of 10nM – 10µM. At the end of the incubation period, cells were labelled with Fixable Viability Dye (FVD) eFluor™ 780 prior to cellular surface marker immunolabelling procedure to exclude dead cells. Using flow cytometer, we were able to exclude FVD-positive cells detected with 780/60 band pas filter and measure cell proliferation within live cell population.

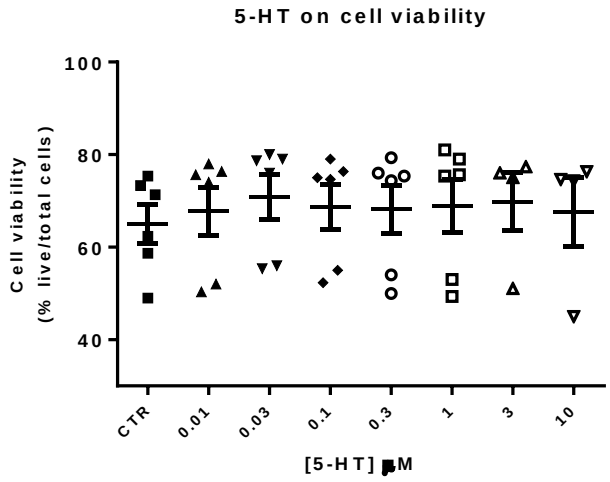



**Figure 3.6 5-HT effect on cell viability.** PBMCs were stimulated with PHA (1 µg/ml) for three days in the presence of increasing concentration of 5-HT. The number of viable cells were analysed by flow cytometer using cell viability dye. Data shown are percentage of live PBMCs 72-hours post-stimulation treated with increasing concentrations of 5-HT compared with control (CTR). Values shown are mean ± S.E.M (n=6; one-way ANOVA with Dunnett's multiple comparison test).

After 3 days incubation, there doesn't seem to be much change in percentage of live cell in 5-HT-treated cultures compared with control (Figure 3.6). Based on the plot in Figure 3.6, 5-HT is able to maintain cell survival independent of the concentration used although not to a significant level. Starting off with the same number of cells per well, all cultures containing 5-HT have less cell death by the end of the 72 hours incubation period, indicating that the cells have a higher survival rate in the presence of 5-HT even at saturating concentration.

### 3.4 Effect of 5-HT on$CD4^+$ T cell proliferation

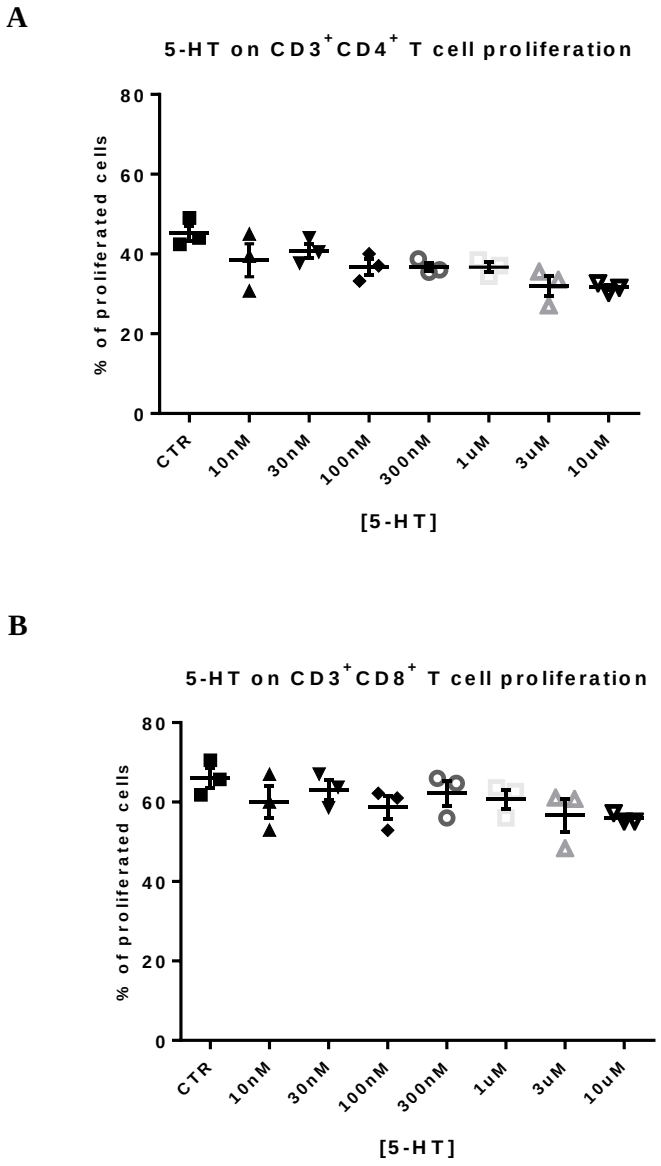



**Figure 3.7 Effect of 5-HT on T cell proliferation**. PHA-stimulated A) CD4+ and B) CD8+ T cells were tested for proliferation response to increasing concentration of 5-HT (CTR= no 5-HT). The results are expressed as percentage of proliferated cells.Values shown are mean ± S.E.M (n=3; One-way ANOVA with Dunnett's multiple comparison test).

The measurement of proliferated cells using flow cytometer showed that a range of 5-HT concentrations (10nM-10μM) had no significant stimulatory effect on the proliferation of $CD4^+$ T cells but the proliferation rate slightly decreased at increasing concentration (Figure 3.7A). Based on the viability assay mentioned above (refer Figure 3.6), we know that the inhibitory effects observed are not due to cell death but rather a modulatory effect of 5-HT possibly via receptor activation. Similar trend was also observed in the proliferation of $CD8^+$ T cell population (Figure 3.7B).

### 3.5 Selective inhibition of 5-HT receptors have no impact on T cell proliferation.

Several reports have pointed out that activated lymphocytes display an upregulation of 5-HT receptors in addition to having the mechanism to produce and store 5-HT (Ahern, 2011). Meanwhile, mature dendritic cells (DCs) possess the ability to uptake and deliver 5-HT to naïve T cells and thereby are able to modulate T cell responses as a co-stimulation. In addition to that, dendritic cells also express mRNA for several 5-HT receptor subtypes. As described earlier in Chapter 1 Section 1.1.2, DCs play a crucial role in providing the link between the innate and adaptive immune responses. One of the aims of this research is to further explore the role of serotonin system in regulating these immune responses. Despite the evidence showing the immunoregulatory role of the serotonergic system, most of the studies were done either using rodents splenic lymphocytes or immune cell lines. Thus the influence of serotonin in native human peripheral lymphocytes remains poorly understood.

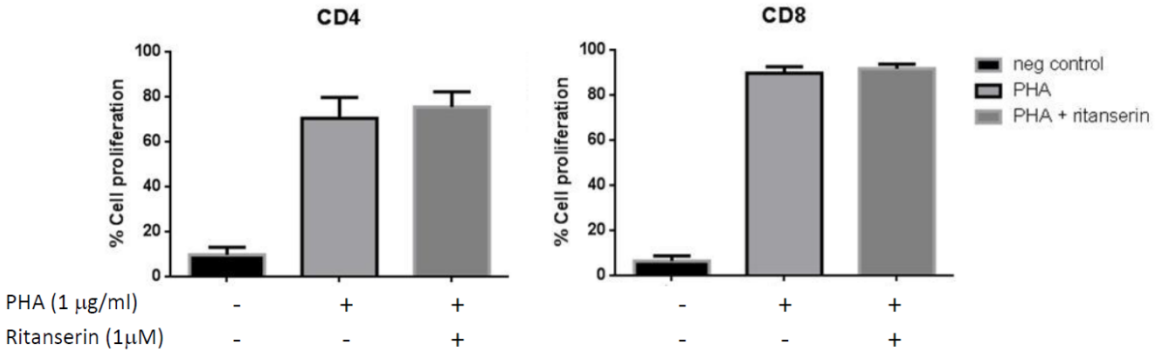



**Figure 3.8 Effect of 5-HT$_{2A/2C}$ receptor antagonist on T cell proliferation.** PHA-stimulated PBMCs were pre-treated with 5-HT2 antagonist, ritanserin, before being stimulated with PHA for 72 hours. Data shown are the percentage of proliferated T cells against total T cells. Values shown are calculated mean±S.E.M (n=3) performed in triplicates.

To explore the possible modulatory function of serotonin in human lymphocytes, we began with pre-treating PBMCs with a selective 5-HT$_{2A/2C}$ receptor antagonist (Figure 3.8) prior to PHA stimulation. The purpose of this assay was to investigate the role of 5-HT$_{2A/2C}$ receptors in the regulation of human lymphocytes proliferation. The result showed that 5-HT$_{2A/2C}$ receptor blockade does not have any effect on either $CD4^+$ or $CD8^+$ T cells proliferation.

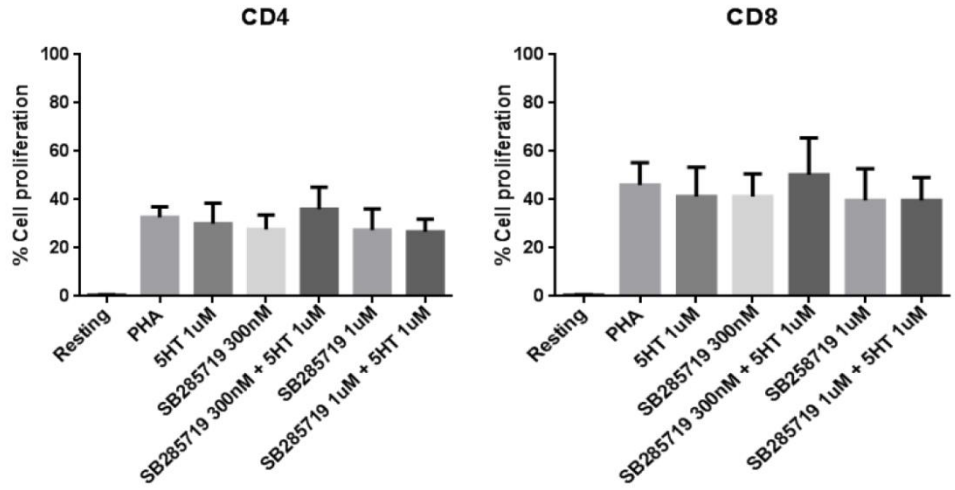



**Figure 3.9 Effect of 5-HT$_7$ receptor antagonist on T cell proliferation.** PHA-stimulated PBMCs were pre-treated with 5-HT$_7$ antagonist, SB285719, before being stimulated with PHA for 72 hours with or without the presence of 5-HT. Data shown are the percentage of proliferated T cells against total T cells. Values shown are calculated mean±S.E.M (n=3; One-way ANOVA with Tukey's test).

We then further continued to investigate the regulatory role of another serotonin receptor subtype, 5-HT$_7$ receptors, on T cell proliferation. We tested the possible role of the 5-HT$_7$ receptor by inhibiting the receptor using SB285719, a selective 5-HT$_7$ receptor antagonist, at two concentrations. The results shown in Figure 3.9 indicate that the antagonist alone at 300nM and 1μM concentration did not inhibit cell proliferation relative to PHA control group. Co-treating the cells with 1μM 5-HT and antagonist did not show any significant changes to the cell proliferation. Treating the cells with 1μM 5-HT alone also did not change the T cell proliferative response. This indicate that exogenous 5-HT at 1μM and 5-HT$_7$ receptor antagonism have no effect on T cell proliferation.

### 3.6 Discussion.

Immune cells are known to possess the serotonergic system components involved in 5-HT synthesis (Young et al. 1995), storage and reuptake (O'Connell et al. 2006) in addition to expressing several of the different receptor subtype classes such as 5-HT1B, 5-HT2A and 5-HT7 (Idzko et al. 2004; Leon-Ponte et al. 2007; Müller et al. 2009). Most of the body's serotonin is produced peripherally and is transported throughout the circulation via platelets, 5-HT is involved in many physiological functions such as the regulation of vascular tone, intestinal motility and immune cell response. Many studies have been done to determine the relationship between the serotonergic system and the immune system in the maintenance of immunity. However, one of the issues recognised in an experiment involving cell culture is the presence of 5-HT in the serum commonly used in maintaining cell culture condition (Ahern, 2011). One previous study detected up to almost 300nM of 5-HT in the foetal calf serum used (Chen et al. 2015) and the concentration can vary between batches (Mothersill et al. 2010). This may be the cause of the variability in the results observed between studies as the 5-HT concentration detected is sufficient to activate different 5-HT receptor subtypes. To address this issue, this research designed a serum-free cell culture protocol to eliminate the possibility of underlying differences to 5-HT responses and to use more carefully controlled chemical components in the culture environment. To my knowledge, this is the first study to use this serum-free formulation in investigating the role of 5-HT on human native immune cells. From the observation I can report that the serum-free cell culture media formulation used throughout this study is compatible in supporting the growth of isolated human PBMC continuously for up to 72 hours as indicated in the flow cytometry analysis in Figure 3.1 without any additional supplement in between incubation periods.

One of the aims of this study is to understand the role of 5-HT in the regulation of immune cell function. T lymphocytes are one of the crucial components of cell-mediated immune responses involved in inflammation. A normal and balanced inflammatory response is important in defending against microbe infections and in tissue repair. Thus it is equally important to understand the regulatory factors that might be involved in maintaining this immune homeostatic balance.

This study is focused on the T lymphocytes response to 5-HT and agents targeting the 5-HT system protein in an effort to understand the influence of the serotonergic system on cell-mediated immune responses involving $CD3^+$ T lymphocytes. It has been described that T cells express several of the serotonergic components such as SERT, TPH and serotonin receptor subtypes 1A, 1B, 2A, 2C, 3A and 7 (reviewed by Herr et al. 2017). In this study, we reported that hampering endogenous 5-HT synthesis via THP1 can significantly decrease T cell proliferation. This result is consistent with a similar study conducted by Aune and colleagues (1994) which revealed similar inhibitory effect of pCPA on murine T lymphocytes. They showed that the addition of growth cytokine IL-2 anda lower concentration of 5-HTP (30μM) can reverse the inhibitory effect of pCPA. It was discovered that pCPA-induced inhibition of proliferation might have been resulted by the reduction in IL-2 receptor which was able to be restored by 5-HT supplement (Young et al. 1995). However, in this study, adding the TPH substrate 5-HTP (100μM) was not able to reverse the inhibitory effect pCPA on T cell proliferation.

To add, findings from previous studies also suggested that T lymphocytes express TPH1 and MOA-A mRNA (O'Connell et al. 2006; Leon-ponte et al. 2007) and these gene transcripts are increased when the lymphocytes are activated particularly in the CD8 T cell subsets. This

observation was further supported by the detection of a higher levels of 5-HT released from stimulated CD8 T cells suggesting that most 5-HT synthesised was from the CD8 T subset. This might explain why CD8 T cells were more sensitive to the inhibitory effect of pCPA than CD4 T cells (Figure 3.5) which implies a possible significant regulatory function of 5-HT in cytotoxic T cell activity. In contrast, a study using a genetically modified animal model posed a different hypothesis in regards to the intrinsic role of 5-HT in immune cells. Viral hepatitis mice which lack the ability to synthesise 5-HT presented an exacerbated liver injury due to reduced microcirculation and delayed CD8 T cell recruitment to the site of inflammation. However, the effector functions of CD4 and CD8 lymphocytes remain unchanged (Lang et al. 2008). These data suggested that the modulatory influence of 5-HT could be attributed to vasculature control and not a direct effect on immune cell responses.

However, gene expression analysis showed both CD4 and CD8 lymphocytes possess the capability to metabolise synthesised 5-HT based on the increased expression of the 5-HT metabolising enzyme MAO-A in parallel with the elevated TPH1 expression. Apart from that, T cells also efficiently store 5-HT in cytosolic vesicles and this is supported by the detection of the vesicular monoamine transporter 1 (VMAT1) mRNA in the cells (Chen et al. 2015). It was also postulated that T cells can take up exogenous 5-HT up to micromolar concentration possibly via dopamine transporter DAT and not SERT (Leon-Ponte et al. 2007). Taking all these data together with the results from this report, it is possible that CD4 and CD8 T cells have similar ability in regulating 5-HT concentrations within its microenvironment and is not affected by the changes in extracellular 5-HT.

Taking into account the data provided from murine studies that the synthesised and released 5-HT can act on 5-HT receptors expressed on T cells in an autocrine manner, we aimed to investigate whether the same could be observed in human immune cells via specific 5-HT

receptors. Although elevated 5-$HT_{2A}$ mRNA transcripts were detected in activated murine T cells, the same could not be concluded in human cells. From the result provided, antagonising 5-$HT_{2A/2C}$ did not produce any differential proliferative response to mitogenic stimulation leading to the theory that human T cells might not express functional 5-$HT_2$ receptor subtypes. However, the theory could not be confirmed in this study. Next, there was no significant change in the percentage of proliferated cells when PBMCs were preincubated with a selective 5-$HT_7$ receptor antagonist treated with 1 μM 5-HT. Leon-ponte and coworkers (2007) characterised functional 5-$HT_7$ receptor expression in naïve T cells extracted from mouse spleen. In vivo pCPA treatment leads to an inhibition of isolated naïve T cell proliferation which was restored with supplement of 5-HT or 5-$HT_7$ receptor agonist AS19. Exogenous 5-HT caused phosphorylation of IκBα, a negative regulator subunit for the translocation of NF-kB, and also ERK1/2 thereby regulating IL-2 receptor expression in isolated naïve T lymphocytes. Pretreating the cells with 5-$HT_7$ receptor antagonist seems to block the 5-HT-induced phosphorylation, suggesting a likely role of 5-HT as a co-factor to trigger early cell activation and proliferation via 5-$HT_7$ receptor. Since this study used human primary immune cells, it is possible that there is a difference in the expression of functional 5-HT receptor subtypes on human mononuclear immune cells compared to murine.

Apart from that, exogenous 5-HT does not have an impact on the percentage of viable cells at any concentration tested *in vitro*. In contrast, it was reported that 5-HT can improve cell survival possibly due to the anti-apoptotic response mediated by 5-$HT_{1A}$ receptor activation. RT-PCR analysis showed the presence of 5-$HT_{1A}$ receptor mRNA in human (Aune et al. 1993) and murine T cells (Abdouh et al. 2004) and it was demonstrated that 5-$HT_{1A}$ receptor activation by 5-HT and its selective receptor agonist R-DPAT on mitogen-stimulated lymphocytes leads to a decrease in cell apoptosis and an increased percentage of lymphocytes in S phase transition

of cell cycle. These effects were supported by 5-HT-induced inhibition of DNA soluble content as well as increases in nuclear levels of NF-kB that regulates cell cycle progression and apoptosis. It was also demonstrated that promotion of cell viability by 5-HT can be reversed by a selective 5-$HT_{1A}$ receptor antagonist WAY-100635 (Abdouh et al. 2004). However, these observations were put into question as there were conflicting reports regarding the expression of functional 5-$HT_{1A}$ receptor in T cells (Stefuji et al. 2000; Leon-ponte et al. 2006).

To conclude, our data show that the proliferation of human peripheral T lymphocytes can be inhibited by interfering with the synthesis of endogenous 5-HT but could not be rescued by adding the substrate for the synthesizing enzyme nor exogenous 5-HT itself. Furthermore, neither 5-HT receptor antagonist tested in the experiment produced any significant chages in T cells functional response. By using an optimised serum-free culture environment to enable us to control the amount of exogenous 5-HT added, there seem to be no significant impact of 5-HT or its agent targeting 5-HT system protein on human lymphocytes nor was there significant difference in functional response between T cell subsets. Therefore we did not continue to pursue the investigation further and instead decided to move on to the next chapter of investigating the effect of the neuropeptide MCH on immune cell function to improve our understanding of the role of neuropeptides in the regulation of the adaptive immune response and their involvement in the pathogenesis of inflammatory disorders.

# CHAPTER 4: Studies on MCHR1

The focus of this chapter of research is to investigate the influence of the orexigenic neuropeptide MCH and its receptor activation on lymphocytes. MCH, which is a peptide predominantly expressed in the lateral hypothalamic area of the brain is also peripherally expressed in tissues such as spleen, thymus and immune cells. Although many studies have proved that human and rodent lymphoid tissues and immune cells express mRNA transcripts for its prehormone peptide pMCH and its receptors, there has been very little demonstration of MCH receptor expression at the protein level and receptor activity when activated by its ligand, particularly in human primary immune cells. In recent years, several research works involving animal models with induced inflammatory conditions interestingly pointed towards the immune modulatory function of MCH that can potentially be targeted in managing inflammatory conditions.

As discussed earlier in Chapter 1.6.2, MCH receptors exist in two isoforms named MCHR1 and MCHR2. Both are expressed in human but only the MCHR1 isoform can be found in rodents. Since previous studies have demonstrated the immunomodulatory impact of MCH in rodents, we can safely conclude that the modulatory effect is mediated by MCHR1 receptor activation. One of the challenges faced in investigating the presence of surface membrane MCH receptor protein is the lack of highly specific antibodies, particularly ones that are raised against the extracellular N-terminus region of MCHR1. Since the working antibodies that are rabbit polyclonal IgG directed against the intracellular C-terminus region (amino acids 396-442) of the human MCHR1, the initial experiments faced a serious issue of high non-specific binding. Therefore, each immunoassay performed in this study has been carefully optimised to minimise the issue of non-specificity of the selected antibodies.

### 4.1 MCHR1 are expressed on human mononuclear immune cells

To begin with, Western blot was performed to detect MCHR1 expression qualitatively on several cell lines that has been reported to endogenously express the G-protein coupled receptor of interest. As shown in Figure 4.1A, using Hypoxanthine-guanine phosphoribosyltransferase (HPRT) as the loading control, MCHR1 protein at 55kDa can be seen in both neuronal (HEK293 and SH-SY5Y) and immune (Jurkat and K562) cells lysates while RBC lysate was used as a negative control. Therefore, throughout this research, either of these cell lines were used as positive control for MCHR1 expression. Meanwhile, preliminary immunoassay also demonstrated the relatively low expression of MCHR1 protein in resting and PHA-stimulated PBMCs. Figure 4.1B showed the expression of MCHR1 between resting and activated state of PBMCs isolated from three different samples obtained from healthy volunteers. The blot shows that all three samples have MCHR1-positive bands before and after a 24-hour mitogen stimulation. Following this, we continue to investigate the expression profile of MCHR1 receptors on different types of mononuclear immune cells by using flow cytometer.

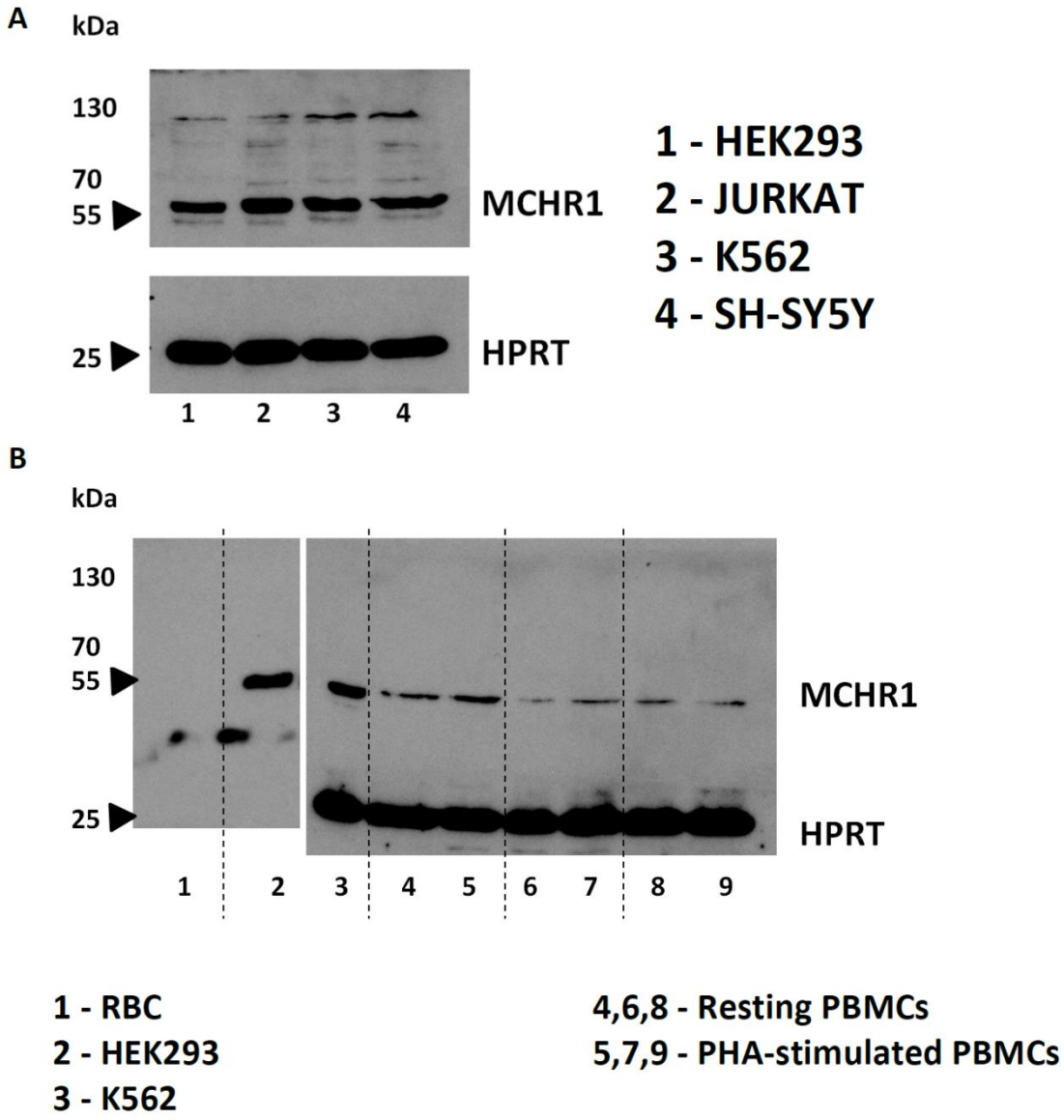



**Figure 4.1 MCHR1 protein expression on different cell lines and normal PBMCs.** A representative Western blot of native MCHR1 expression on (A) immune and neuronal cell lines. (B) RBC lysate acts as negative control and HEK293 and K562 cell lysates as positive control. PBMCs from three donors were treated or left untreated with PHA (5 μg/ml) for 24 hours and the expression of MCHR1 was observed on immunoblot with HPRT as loading control.

Isolated PBMCs from healthy volunteers were prepared as described in Section 2.1 and were stimulated with different stimulus depending on the target cells of interest. After 24-hour stimulation, the cells were immunolabeled and run on flow cytometer for analysis. T cells, B cells and monocytes were gated from live cells based on CD3, CD19 and CD14 markers, respectively. Resting and activated lymphocytes were determined by the expression of the early activation marker CD69, since these cells were only stimulated for 24 hours. Cells incubated with only a secondary antibody were used to discriminate a false positive signal due to non-specific binding. Figure 4.2 showed the MFI of the hormone receptor on three different state of activated lymphocytes based on the CD69 vs surface marker gating. Here (Figure 4.2), I showcase the MCHR1 expression based on the MFI of the signal by comparing between resting $CD69^-$ population with the activated $CD69^-$ and $CD69^+$ populations of $CD3^+$ T and $CD19^+$ B lymphocytes in addition to resting and stimulated live $CD14^+$ monocytes.

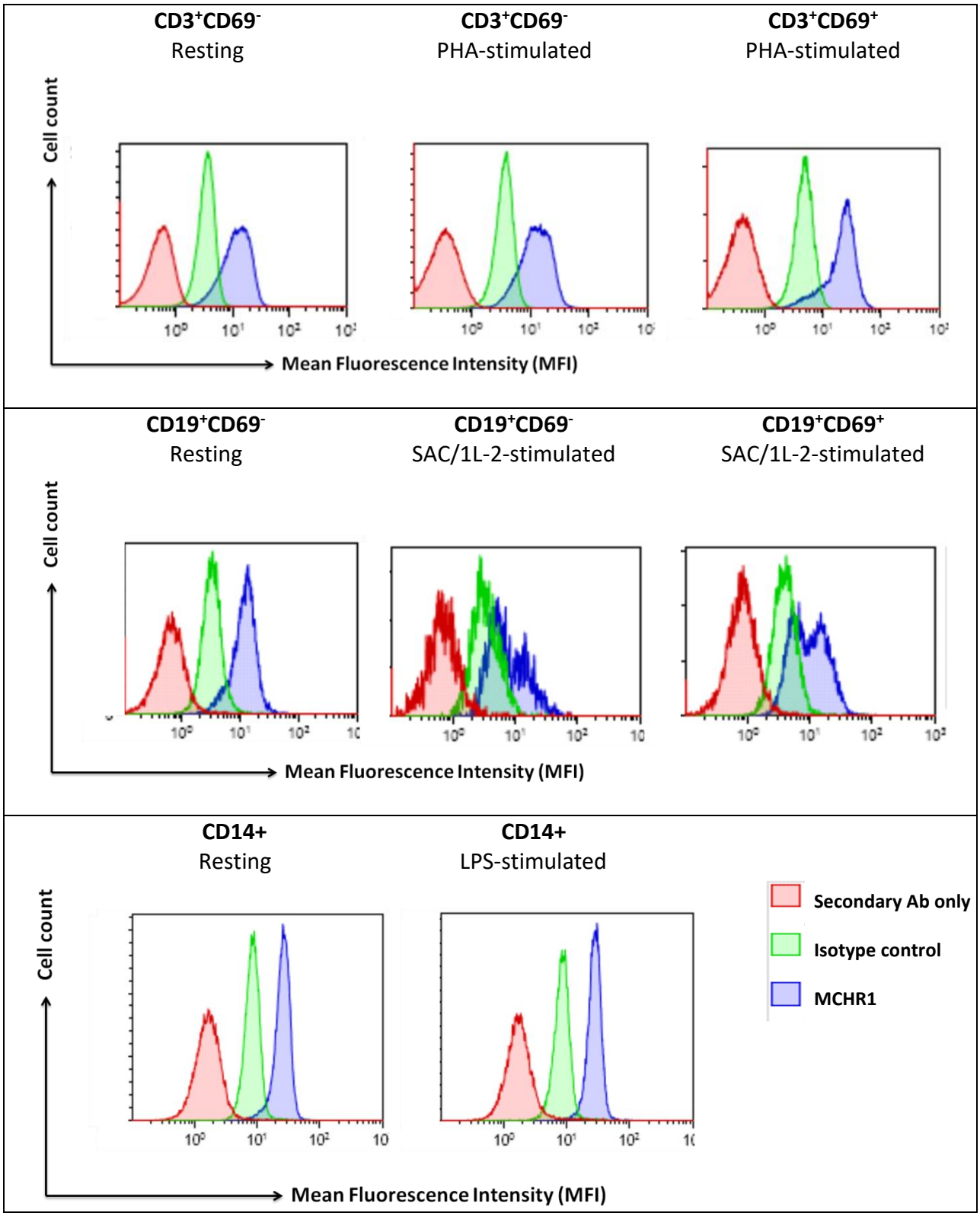



**Figure 4.2 MCHR1 receptor expression immune cells.** Differential expression of MCHR1 receptors on T cells, B cells and monocytes in resting and stimulated conditions. T cells were stimulated with PHA (5 μg/ml), B cells with SAC (1:10,000) and IL-2 (20U/ml), and monocytes with LPS (100 ng/ml) for 24 hours. Rabbit polyclonal antibody was used as isotype control. Histograms are representative examples of three independent experiments.

The MFI represented in bar chart (Figure 4.3) interestingly showed a marked increase of MCHR1 expression in the activated $CD3^+CD69^+$ T lymphocytes population (Figure 4.3A) while there is a significant decrease ($p<0.01$) in receptor expression in both $CD69^-$ and $CD69^+$ populations of activated $CD19^+$ B cells (Figure 4.3B). Meanwhile, MCHR1 in $CD14^+$ cells showed no change in expression between resting and activated state (Figure 4.3C).

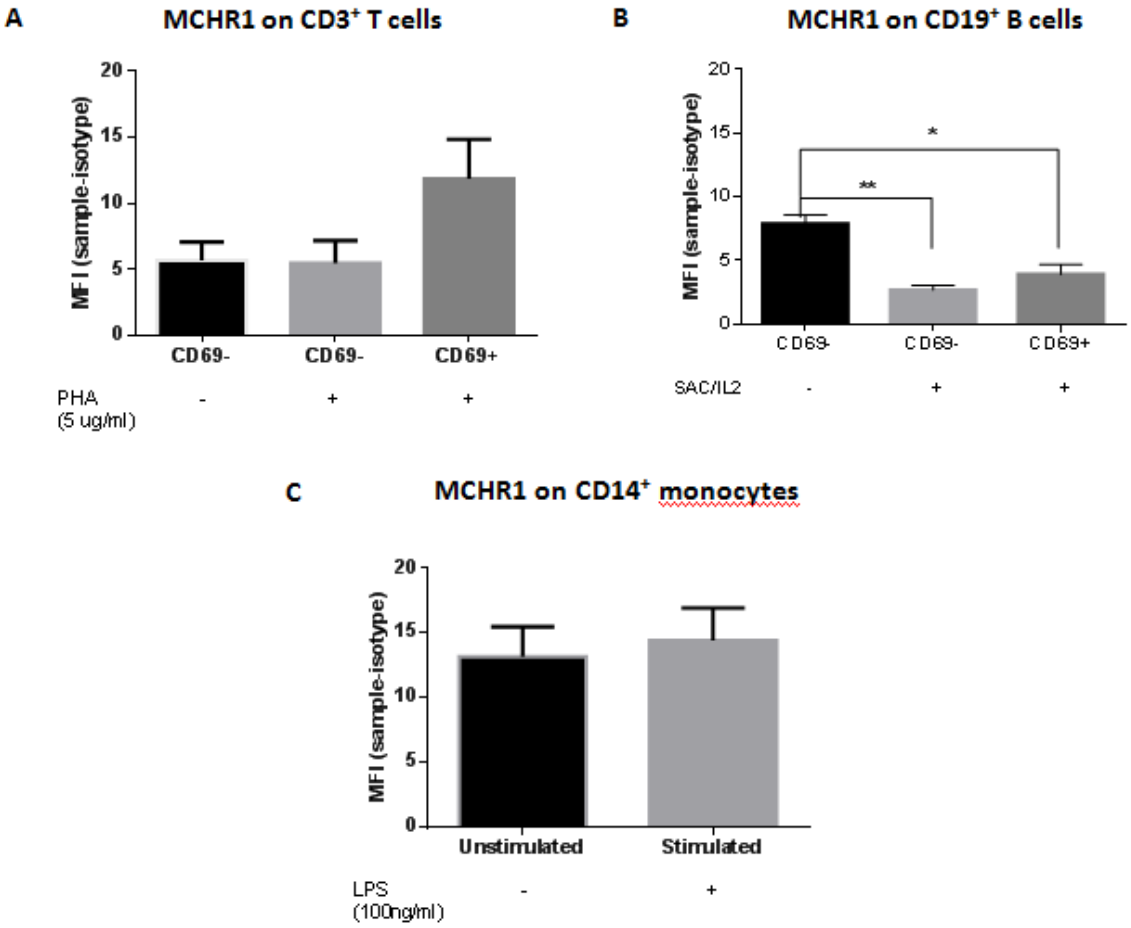



**Figure 4.3 Differential MCHR1 receptor expression on resting and activated immune cells.** Cells were stimulated with either **A)** PHA (5µg/ml), **B)** SAC (1:10,000)/IL-2 (20 U/ml) or **C)** LPS (100 ng/ml) for 24 hours. CD69 marker was used to indicate activated state of **A)** T and **B)** B lymphocytes. Data represent mean ± S.E.M. from three independent experiments. Statistical comparison was performed using one-way ANOVA with Tukey's test. *P<0.05, **P<0.01 compared to resting $CD69^-$ cells for lymphocytes, while **C)** monocytes compared with unstimulated cells (Student's t-test).

### 4.2 MCHR1 receptors are upregulated on activated T lymphocytes

Within the $CD3^+$ T lymphocytes, it was observed that there is a considerable difference in MCHR1 MFI between $CD4^+$ and $CD8^+$ cells. Furthermore, after 24 hour stimulation with a mitogen, there is a significant upregulation of MCHR1 by almost three fold in peripheral $CD8^+$ cytotoxic T cells ($p<0.01$) compared with the $CD4^+$ helper T cells population. I then continued to further analyse the MCHR1 protein expression in different T cell subsets based on phenotypic markers CD45RA and CCR7. Using flow cytometer, I categorised the T cells into naïve and memory subsets to analyse the differential expression of MCHR1 in the resting and activated state. As described by Sallusto et al. (1999), naïve cells can be identified by the presence of the extracellular marker CD45RA, which is a long chain protein with intrinsic tyrosine phosphatase activity. When a naïve T cell encounters and is primed by a foreign antigen, it then differentiates into an antigen-specific effector cells or memory phenotype. A memory phenotype loses the CD45RA expression and instead expresses the short chain CD45RO isoform and thus can be recognised as $CD45RO^+$ or $CD45RA^-$. To further differentiate between an effector and central memory subsets, the homing receptor CCR7 is one of the surface marker candidates that is expressed on the central but not on effector memory subsets as explained in Chapter 1 Section 1.1.3. However, for this study, I only report on the MCHR1 receptor expression in naïve ($CD45RA^+CCR7^+$) and effector memory ($CD45RA^-CCR7^-$) T cells as the other two subsets have barely detectable MCHR1 levels at rest with little to no change in receptor expression upon activation (data not shown).

As presented in Figure 4.4, both $CD4^+$ helper and $CD8^+$ cytotoxic T cells have a measurable level of MCHR1 expression at rest and the MFI level changes upon stimulation, most notably observed in the $CD69^+$ population of activated $CD8^+$ T lymphocytes, with an

almost two-fold increase in MFI. Although activated $CD4^+CD69^+$ T cells also showed an upregulation in MCHR1 expression, was not as statistically significant.

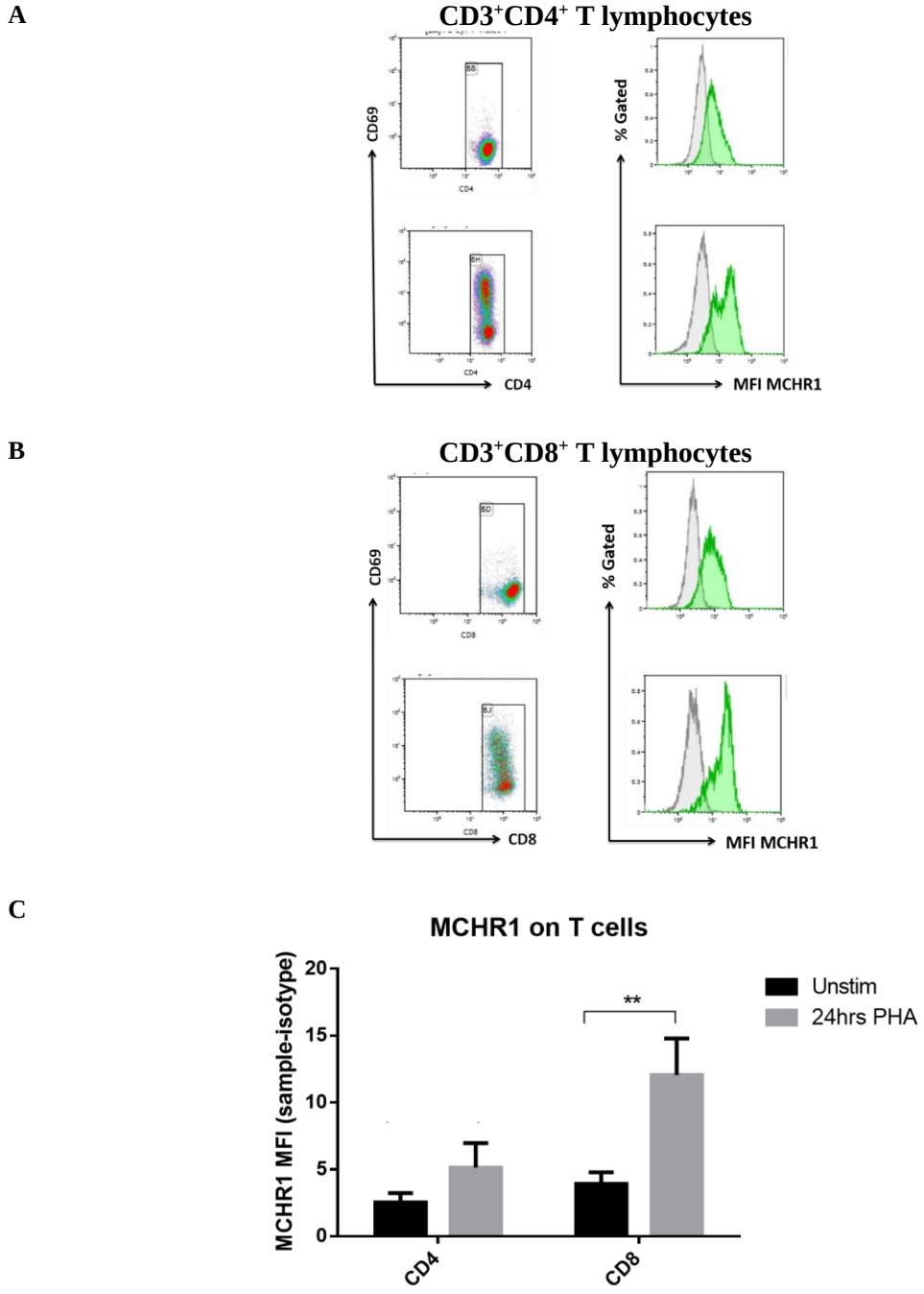



**Figure 4.4 MCHR1 is upregulated in activated T lymphocytes**. Representative flow cytometry data showed a positive shift in MFI values of MCHR1 on mitogen-stimulated **A)** CD4 and **B)** CD8 T lymphocytes after 24 hours using CD69 as activation marker. Cells immunolabeled with primary anti-human MCHR1 antibody (C-term; green histogram) followed by APC-conjugated secondary antibody were compared against matched isotype control (grey histogram). **C)** Quantitative analysis of MCHR1 APC fluorescence intensity on stimulated $CD4^+$ and $CD8^+$ T cells. **, $p<0.01$ (Student's t-test); mean±S.E.M., $n=5$.

Interestingly, in Figure 4.5 it can be seen that activated naïve $CD8^+CD69^+$ T cells produced a significant upregulation of MCHR1 ($p<0.01$) upon mitogenic stimulation while $CD4^+$ T cells were not significantly altered. And it is also worth noting that there is a slight decrease in MCHR1 expression within the $CD69^-$ population of stimulated cells when compared to its resting state. No significant changes in MFI values were observed in the effector memory subsets of either $CD4^+$ or $CD8^+$ population.

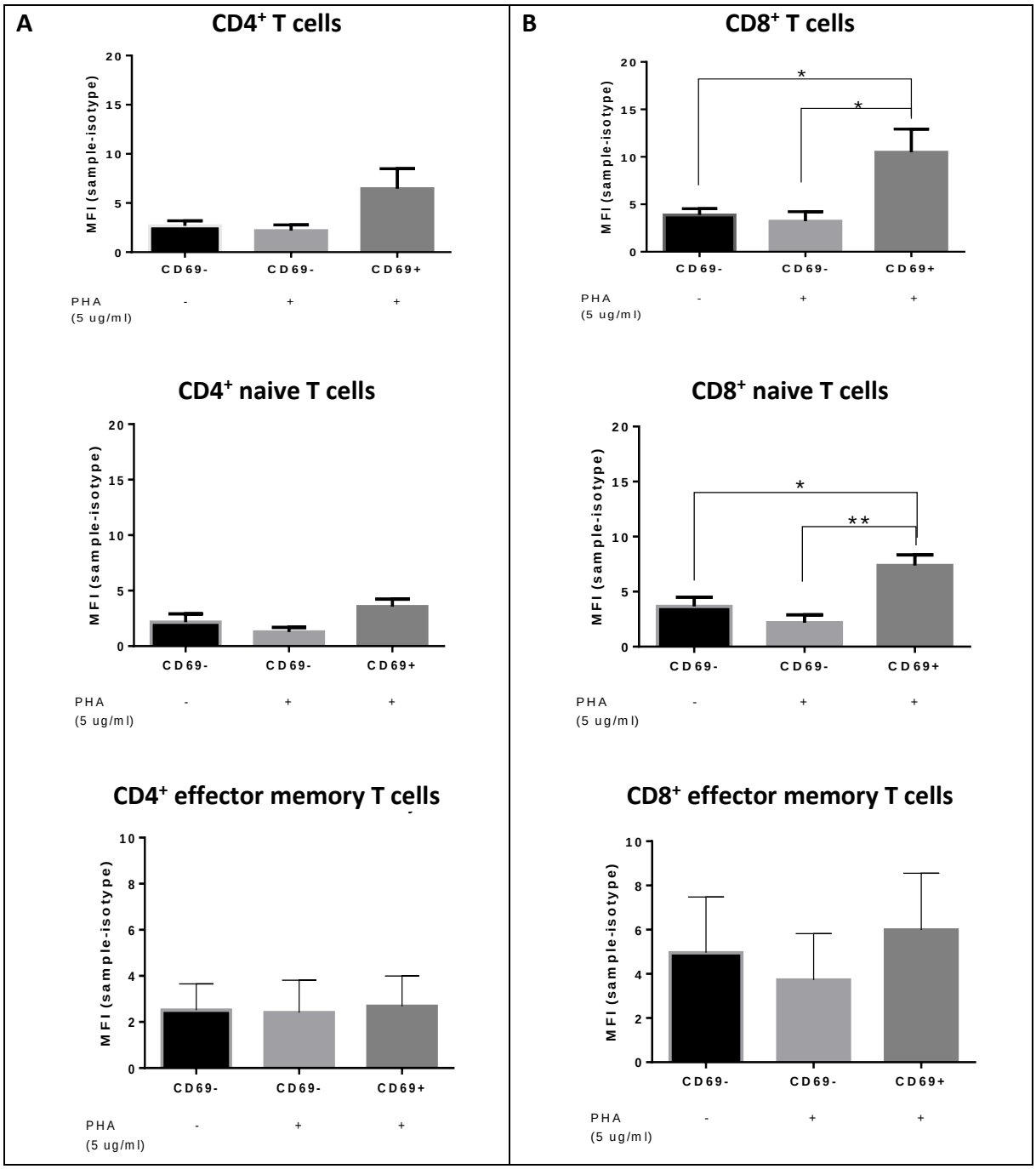



**Figure 4.5 Naive T cells express higher MCHR1 receptors when stimulated.** Flow cytometric analysis showed an increase in expression of MCHR1 on activated A) $CD4^{+}CD69^{+}$ naive T cells and B) $CD8^{+}CD69^{+}$ when compared with resting and stimulated $CD69^{-}$ cell populations. Data shown are represented as MFI values of MCHR1 antibody minus the MFI of the isotype control antibody. Bar graphs shown are calculated mean±S.E.M (n=7). *P<0.05, **P<0.01 (one-way ANOVA with Tukey's test)

### 4.3 Naïve T lymphocytes express functional MCHR1

Based on the western blot and flow cytometer analysis presented above which proved that human immune cells express MCHR1 and is upregulated upon stimulation, I decided to assess the functional activity of the MCHR1 receptor by analysing the effect of MCH on protein kinase phosphorylation and cell proliferation. Since it has been established that MCH receptors are coupled to multiple G protein, the cell receptor activation by exogenous MCH was tested by measuring phosphorylated 42- and 44-kDa protein kinases (pERK1/2) activity using Western blot. For this assay, Jurkat cells were treated with either PMA or 100nM MCH for the duration as indicated in Figure 4.6 before being washed and lysed. Preparation of cell lysates for quantification analysis using ChemiDoc MP system Imager (BioRad) were as described in Chapter 2.9.3 with primary antibodies against pERK1/2 (phospho-p44/42 MAPK; Cell Signaling, cat. no 9101S), total ERK1/2 (p44/42 MAPK; Cell Signaling, cat. no 4695S), HPRT (Abcam, cat. no ab10479) and HRP-conjugated antibody raised against the host species of the primary antibodies used.

As shown in Figure 4.6A, maximal ERK1/2 phosphorylation was clearly observed at 5 minutes of stimulation and persisted for at least 30 minutes when stimulated by direct PKC activation using PMA. However, ERK1/2 phosphorylation mediated by G-protein coupled MCHR1 receptor activation with 100nM MCH was not observed. The pERK bands showed in Figure 4.6B were only visible when the membrane blot was over exposed, as proven by the saturation of the loading control HPRT. This implies that the Jurkat cell line might endogenously express non-functional MCHR1 receptors.

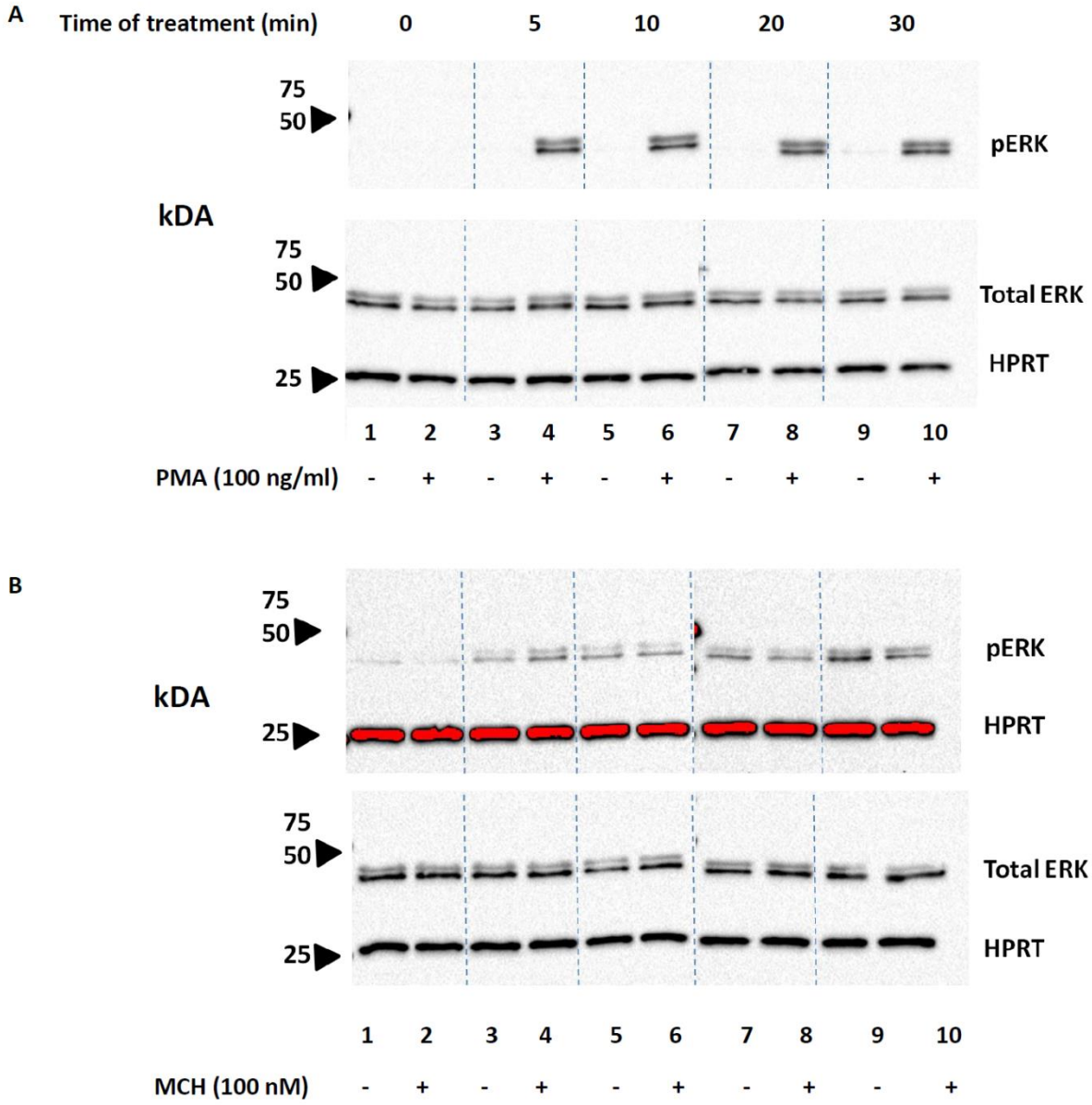
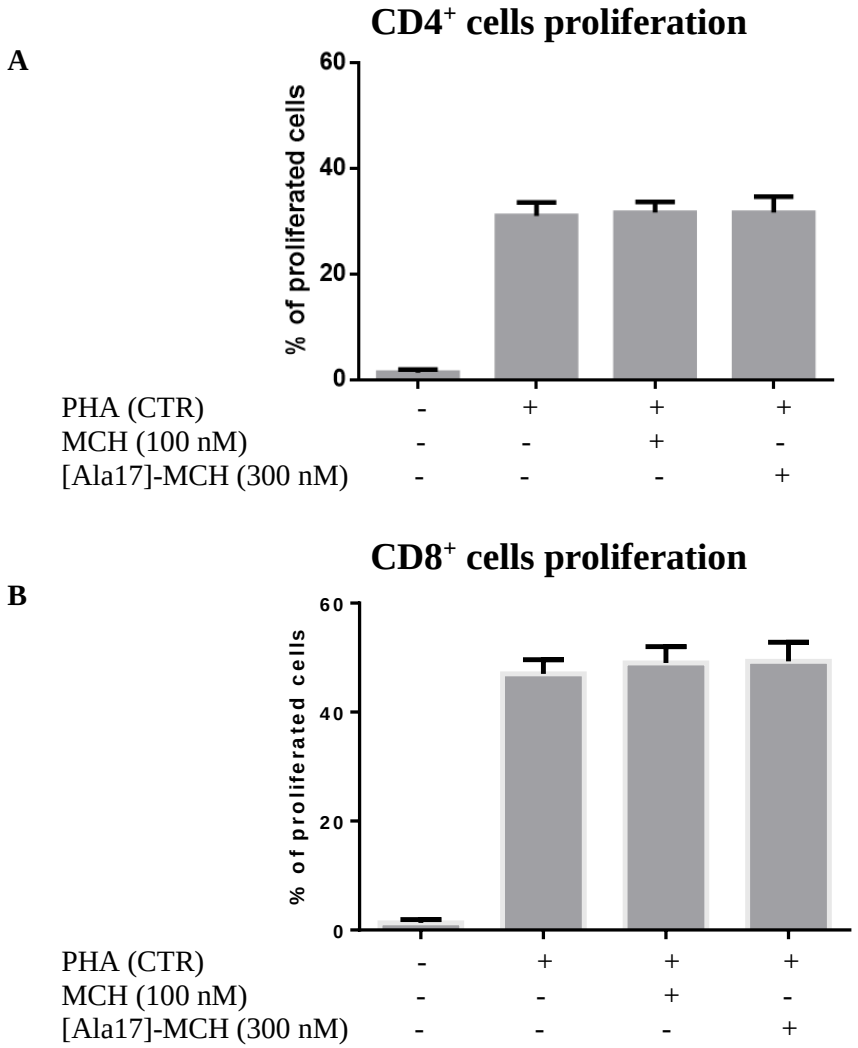



**Figure 4.6** A) **MCH on MAPK (ERK) signalling pathway.** Jurkat cells were stimulated with PMA (100 ng/ml) as positive control and B) with 100 nM MCH (lane 2,4,6,8,10) or left untreated (lane 1,3,5,7,9) for 0, 5, 10, 20 and 30 minutes. The phosphorylation of ERK was determined by immunoblotting with antibody to pERK. Expression was compared to total ERK and normalized to loading control, HPRT. Blots are representative of three independent experiments.

Following the results of MCHR1 expression in immune cells, proliferation assays were set up to assess the functionality of the neuropeptide receptors expressed. PBMCs pre-stained with proliferation dye eFluor™450 which were stimulated with PHA and either treated with 100nM MCH (Tocris Bioscience, cat. no. 3806) or with the potent and selective MCHR1 receptor

agonist [Ala17]-MCH (300nM; Tocris Bioscience, cat. no. 3434) for a 3 days. After incubation, cells were washed and immunostained with anti-human CD4 and anti-human CD8 fluorescent antibodies. The number of proliferated cells within the $CD4^+$ and $CD8^+$ gated populations were analysed and measured by flow cytometer and the result is presented in Figure 4.7. From this study, both agonists do not have any modulatory effect on either $CD4^+$ or $CD8^+$ T cells proliferation at the single concentration tested.



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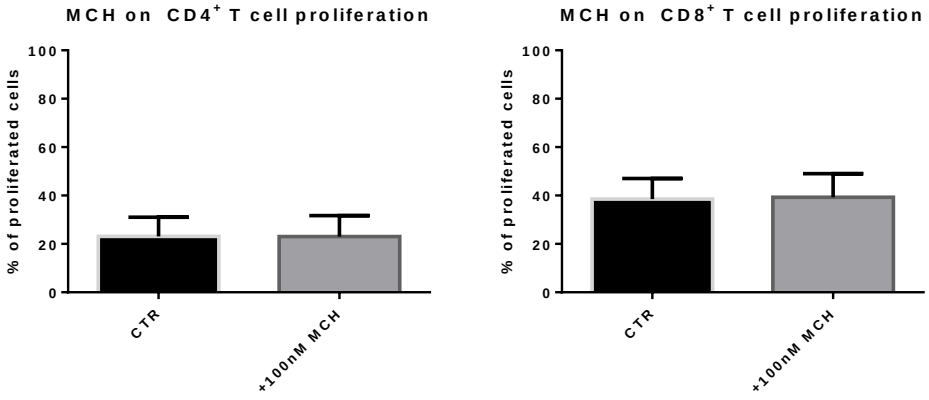


**Figure 4.7 MCH on PBMC proliferation.** PBMCs from healthy donors were stimulated with PHA (5 μg/ml) for 72 hours in the presence of MCHR1 receptor agonist MCH or [Ala$^{17}$]-MCH. Results are presented as the percentage of proliferated CD4+ and CD8+ T cells over total live cells between MCH and Ala-17-treated cells with untreated control group (CTR). Data represent mean±S.E.M (Student's t-test) from two independent experiments.

Another proliferation assay was set up but using isolated naïve T lymphocytes (>90% purity) since this subset showed marked elevation of MCHR1 expression when stimulated. Interestingly, MCH (1μM) has a stimulatory effect on cell division (Figure 4.8). For this assay, immunolabelled $CD3^+CD4^+CD45RA^+CCR7^+$ and $CD3^+CD8^+CD45RA^+CCR7^+$ naïve T cells were isolated from healthy donor's peripheral blood using a Mo-Flo Astrios cell sorter (Beckman Coulter). The cells were then stimulated by direct TCR crosslinking with soluble anti-CD3 in conjunction with co-stimulatory anti-CD28 antibody coated beads and were incubated for 3-4 days before being subjected to flow cytometer analysis. The MCH-treated cells showed a non-significant higher percentage of proliferated cells compared to the non-treated controls (see Figure 4.8). This indicates that mature MCH peptide does not significantly influence human $CD4^+$ and $CD8^+$ naïve T cells functional response to activation stimulus.

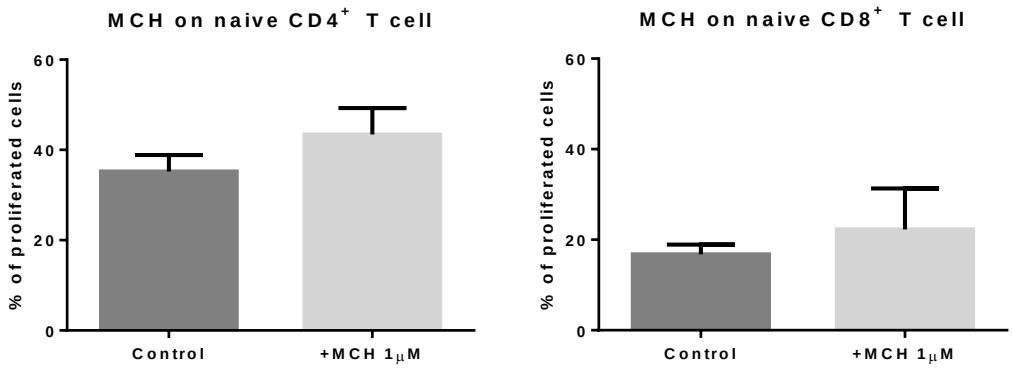


**Figure 4.8 MCH effect on naïve T cells proliferation**. Isolated naïve $CD4^+$ and $CD8^+$ T lymphocytes from healthy donors PBMC were activated with anti-CD3/28 beads with or without MCH (1 μM). Cells were cultured for 3-4 days. Data represent mean±S.E.M (Student's t-test) from three independent experiments.

However, the activity of direct receptor-ligand interaction could not be determined due to the lack of positive control expressing functional receptor. Further studies on human immune cells are needed to support this finding which includes, and not limited to, developing a positive control cell line that is either transiently or stably expressing functional MCHR1 receptor, treatment of target with selective receptor antagonists and measurement of intracellular activity such as cAMP level and calcium mobilisation to correlate the receptor expression with proliferative activity on primary cells.

## 4.4 Discussion

It has been demonstrated that MCH system proteins are expressed in immune cells and are also implicated in diseases where innate and adaptive immune responses are involved (Coumans et al. 2007; Karagiannis et al. 2013; Ziogas et al. 2014). However their exact functions and regulation in the immune system remain elusive. In this research, we were able to detect MCHR1 receptors in human CD3 T lymphocytes, CD19 B lymphocytes and CD14 monocytes in addition to several neuronal and immune cell lines using a rabbit polyclonal antibody raised against the C-terminus of the MCHR1 receptor validated for flow cytometer. The result from this study supports similar findings from previous reports with additional data on MCHR2 receptors which were not detected in any of those cells (Verleat et al. 2002). After 24 hour stimulation, only $CD3^+$ T cells exhibit increased MCHR1 expression while MCHR1 is decreased in $CD19^+$ cells and remains unchanged in the LPS-treated $CD14^+$ population. It is unknown whether human monocytes express functional MCHR1 as most monocyte functional responses in relation to MCH were observed from murine cells. Mouse monocytic cell line RAW264.7 showed elevated MCHR1 immunoreactivity when stimulated with LPS and it was demonstrated *in vitro* that MCH enhanced phagocytosis in a concentration dependant manner that was able to potentiate chemokinesis but not chemotaxis (Karagiannis et al. 2013).

To date, most of the data on the expression profile of MCH receptors in human immune cells were at mRNA transcript level using tools like RT-PCR and microarray analysis with confounding data. One of the earliest studies detected low levels of MCHR2 in lymphocytes, macrophages and spleen while MCHR1 transcripts were absent in the RT-PCR analysis (Hill et al. 2001). In contrast, Verleat et al (2002) were able to detect MCHR1 and not MCHR2 in tonsil, PBMC and granulocytes. Within the same year, another group demonstrated using

microarray analysis that both MCHR1 and MCHR2 transcripts are present in CD4, CD8, CD14, CD19 and CD56 cells as well as in bone marrow, lymph nodes and thymus (Su et al. 2002). Information on the exact role of MCH on lymphocytes still remain scarce but accumulating evidence has suggested that MCH receptor interaction with its ligand can modulate inflammatory responses that involve immune cells. However, these collective evidence were mostly obtained from animal studies and the functional significance of MCH immunomodulatory role in human remains to be seen.

The current study was also able to provide the expression profile of MCHR1 receptor protein in different subsets of lymphocytes with flow cytometer. It is interesting to note that MCHR1 is slightly more expressed in naïve lymphocytes than effector memory subsets. Furthermore, MCHR1 protein expression is barely detectable or remain unchanged in activated effector and memory subsets while naïve cells display an elevation in MCHR1 expression upon stimulation, most significantly in CD8 T cells. The increase in MCHR1 is in parallel with the increase of early activation marker CD69 suggesting that the neuropeptide might be involved in the induction and cytotoxicity of CD8 T cells. On a similar note, another neuropeptide, alpha-melanocyte stimulating hormone (α-MSH), was reported to have similar immunoregulatory influence on CD8 T cells. Alpha-MSH via melanocortin-1 receptor activation attenuates contact allergy responses through enhanced cytolytic activity of CD8 T cells which leads to cell apoptosis thus reducing the allergic reaction (Loser et al. 2010). In a like manner, MCH seems to promote cell proliferation of CD8 T cells. It is known that MCH and α-MSH functions antagonistically in most tissues. Nonetheless, both neuropeptides can co-express and act agonistically for example in pigment cells (Hintermann et al. 2001). Meanwhile, MCHR1 elevation in naïve CD4 T cells is not statistically significant. Despite that, MCHR1 is

upregulated in CD4 T cells within the lamina propria from IBD patients indicating a probable different peripheral role of MCH on T lymphocytes at different localisation (Bai et al. 2012).

The application of MCH (1μM) on isolated naïve T cell cultures managed to slightly enhance proliferation but the modulation effect was not observed in PBMC culture. In contrast, Coumans and colleagues (2007) indicated that a low concentration of MCH (100nM) had the ability to inhibit the proliferation of PBMC and suggested that the inhibitory effect was related to the decreased secretion of growth-promoting IL2 secretion. The inhibitory effect of MCH on PBMC proliferation was similarly reported by Verleat et al. (2002) which was attributed to the increased intracellular cAMP and calcium mobilisation in MCH-induced cells. One of the limitations is the lack of working antibodies targeting the extracellular region of MCHR1 making the process of detecting membrane receptor expression in primary cells difficult. Furthermore, without the appropriate ligand-binding or signalling assays, it was not possible to determine whether an MCH stimulatory effect on naïve T cell subsets was due to the putative receptor activation by its ligand.

Alternatively, studies on understanding the MCH-mediated receptor signalling pathway can be performed using cellular models related to the species and target cells of interest. However, mammalian cell lines that endogenously express MCHR1 remain poorly explored except in a neuroblastoma cell line such as human Kelly cell, SH-SY5Y and IMR32. Both mRNA transcript and protein are present in Kelly neuroblastoma cell and SH-SY5Y which responded to MCH via $G_{i/o}$ protein (Schlumberger et al. 2002; Cotta-Grand et al. 2009). Activation of the ERK1/2 signalling pathway via MCH binding to MCHR1 receptor induced neurite development in SH-SY5Y cells and the phosphorylation could be seen to be partially blocked by pertussin toxin (PTX) which suggested that endogenously expressed MCHR1 is coupled to $G\alpha_{i/o}$ protein (Cotta-Grand et al. 2009). In contrast, a response to MCH stimulation

was only readily detectable when IMR32 cells were transfected with $G\alpha_{16}$ protein (Fry et al. 2006). In immune cell lines, human transcriptome analysis revealed that cell lines K562 and Jurkat have MCHR1 mRNA but it is still unknown whether these cells express the functional receptor protein (Su et al. 2002). In this research, we were unable to detect phosphorylation of ER1/2 protein with MCH stimulation although the protein level detected in Jurkat is comparable to that of human neuronal cell SH-SY5Y. The absence of signalling pathway activity in immune cell lines warrants additional investigation on the regulation of MCHR1 expression that involve analysis of post-translational modification of MCHR1 receptor in a natural expression system.

In summary, this study was able to provide evidence of MCHR1 protein expression in different immune cell subsets, specifically in naïve CD8 lymphocytes. T lymphocytes are majorly involved in inflammatory and autoimmune diseases by releasing pro-inflammatory cytokines that can initiate and maintain inflammatory processes. Accumulating evidence pointed towards the involvement of neuropeptides in the pathogenesis of inflammatory diseases which includes regulation of lymphocyte subsets. There were even suggestions to target neuropeptide receptors as new therapeutic approach in inflammatory disorders. It is clear that the immunological impact of the MCH system is relatively new thus this project aim was to explore the peripheral function of MCH particularly in different subsets of immune cells. However, the lack of a suitable cellular model to study the pharmacology and signalling of MCHR1 hampers the effort to analyse and understand further the possible role of MCH in modulating lymphocytes functional response in inflammatory conditions.

## Chapter 5 MCH and its receptors in human colon tissues.

In light of the discoveries of MCH involvement in the pathogenesis of inflammatory bowel diseases (Kokkotou et al. 2008), the exact role of the MCH system in gastrointestinal physiology remains unknown. As discussed in Chapter 1 Section 1.6.4, it was implied that MCH can mediate inflammation pathogenesis in murine experimental colitis by modulating colonic immune cell inflammatory cytokines secretion. Therefore, this chapter attempts to observe whether infiltrating immune cells within human colonic mucosa express MCHR1 using immunohistochemistry (IHC). RT-qPCR analysis was also performed to assess the level of pMCH, MCHR1 and MCHR2 genes in non-inflamed and inflamed colon tissue samples. Colonic tissue samples were obtained from uninvolved mucosa of 5 colorectal patients (normal) while inflamed mucosa were from 3 patients with Crohn's disease (CD) and 1 patient with ulcerative colitis (UC) and each sample was verified by a pathologist. Tissues were fixed and embedded in paraffin wax to make 4mm-thick sections mounted on glass slides for immunohistochemistry analysis of MCHR1 expression in colonic immune cells while flash frozen tissue samples were processed for RT-qPCR analysis to determine mRNA expression of pMCH, MCHR1 and MCHR2 genes.

### 5.1 RT-qPCR analysis of human colon tissues

Here we investigate the gene expression of mRNA for MCH and both of its receptor subtypes from IBD patients and compared to samples from uninvolved area of colorectal cancer patients. Multiplex RT-qPCR analysis was performed where each target gene of interest was normalised to the housekeeping gene beta-actin (ACTB) and the result is presented as the relative gene expression with standard error bar. Due to the differences in the primer

efficiencies, the pfaffl method was used to calculate the ratio of gene expression. Variations of control and Crohn's groups were assessed by variance-test and data were plotted and analysed using GraphPad PRISM software. Data from UC patient was not included in the statistical analysis as it was only n=1.

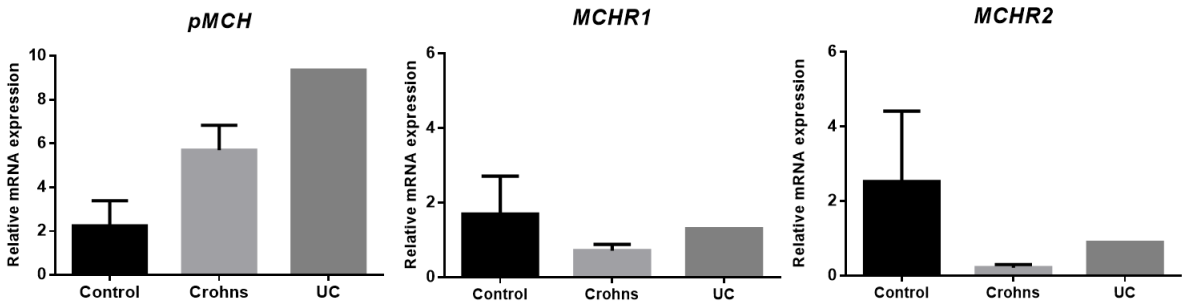


**Figure 5.1 Gene expression ratio of pMCH, MCHR1 and MCHR2** (normalised to beta-actin) from colonic tissues of non-IBD control (n=5), Crohn's (n=3) and ulcerative colitis (n=1) patients. Data represent mean±S.E.M.

Referring to Figure 5.1, both MCHR1 and MCHR2 transcripts are relatively lower in IBD samples than control group. Although pMCH mRNA seems to be relatively higher in IBD samples, these data were only obtained from 3 CD patients and 1 UC patient and further work is required to confirm these preliminary findings.

### 5.2 MCHR1 receptors are expressed on CD11c$^{+}$ cells in human colonic mucosa.

We then characterised the expression of MCHR1 on subsets of gut residence macrophage cells expressing CD11c or CD14 cell markers. A multiplex chromogen-based IHC protocol was optimised and used to stain each paraffin-embedded tissue sample with primary antibodies against MCHR1 C-terminus (rabbit polyclonal IgG) and immune markers CD11c or CD14 (mouse monoclonal IgG) with biotinylated secondary antibodies. Expression of MCHR1 and cell markers were detected using either DAB or VIP substrate kit (Vector Labs) respectively with a peroxidase reaction to detect antibody labelling. In some sections, matched primary antibody isotype were used to replace primary antibodies to act as negative control. All samples were counterstained with methyl green and scanned under Vectra® automated quantitative pathology imaging system (Perkin Elmer) with the support of PerkinElmer's Phenochart™ whole slide viewer to allow navigation across the slide and annotate areas of interest for multispectral acquisition. The integrated InForm analysis software enables separation of overlapping markers in the multiplex IHC and is able to render chromogen view into "false" immunofluorescence images as shown in the all figures below.

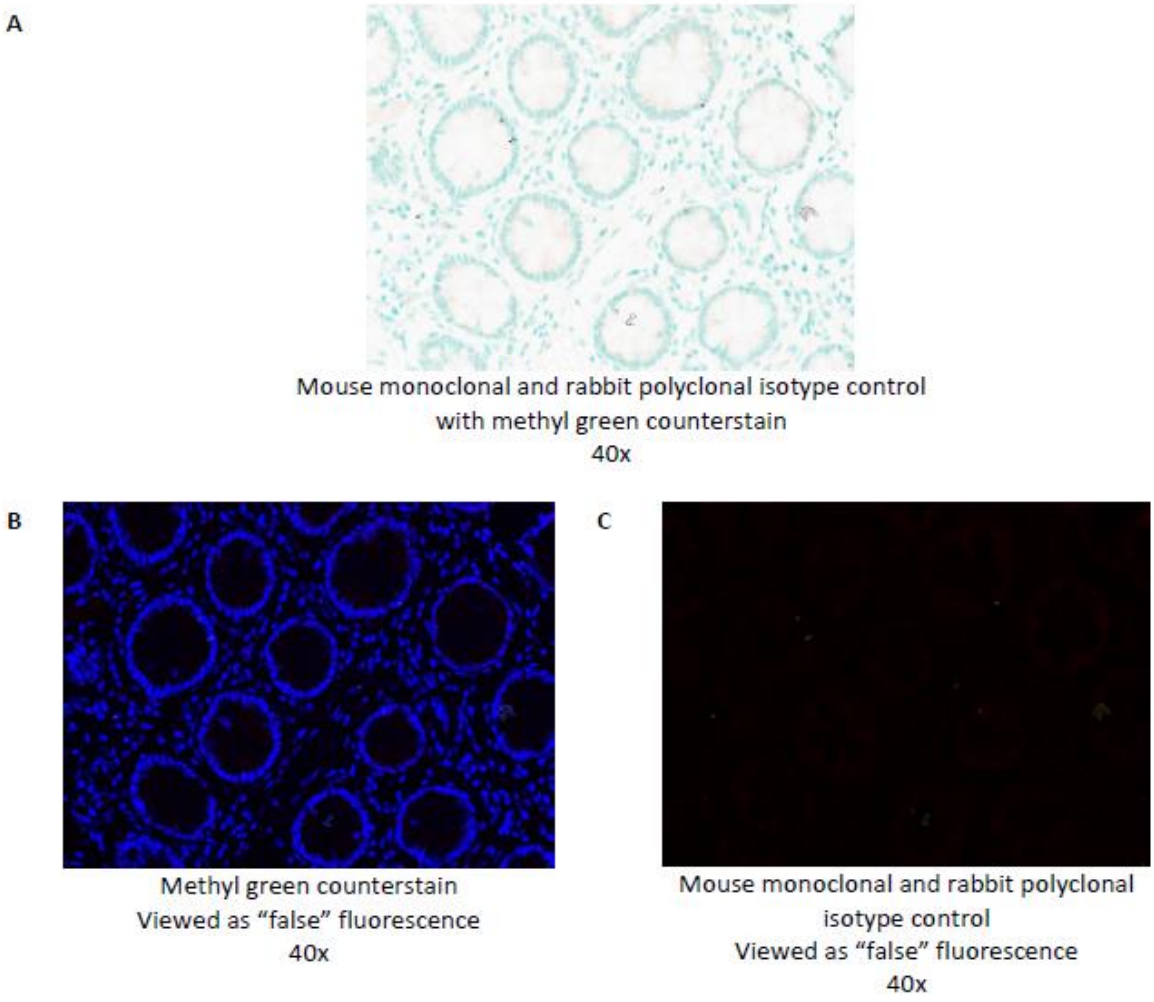


**Figure 5.2 Immunocytochemistry isotype control**. Tissue sections of paraffin-embed "normal" human colon were double stained with mouse monoclonal and rabbit polyclonal antibodies with specific anti-host HRP-conjugated secondary and treated with chromogen VIP and DAB peroxidase substrate followed by methyl green counterstain. Simulated fluorescent images of "false" fluorescence of the counterstain (A) and isotype controls (B) were made to enhance the contrast of normal staining for better view. No background signal was observed with isotype controls using the optimised staining protocol. Figure is a representative for five independent experiments. (Blue = Methyl green-positive nuclei)

Figure 5.2 is a representative of negative control viewed under IHC stains (Figure 5.2A) and immunofluorescent images (Figure 5.2B and C). No background signal was detected as this multiplex IHC protocol has been carefully optimised for the specific primary antibodies used. However, in an effort to minimise non-specific binding of antibodies and isotype background staining, relatively weak signals for MCHR1 and cell markers were produced. Therefore, with the aid of the software provided, the images were viewed as immunofluorescent to allow better discrimination between DAB and VIP signals. The following images in this chapter will be represented by IHC stained colonic mucosa up to the highest magnification of 40x which are then showed alongside "false" immunofluorescent images.

Non-inflamed colon tissue sections ("normal" control) can be characterised by an intact epithelial layer with regularly shaped and uniformly distributed crypts within the lamina propria of the mucosa with minimal cell infiltration (Figure 5.3 and 5.4). Gut-associated lymphoid tissue (GALT) can also be seen in Figure 5.3B showing aggregates of lymphoid cells. Altered morphology of inflamed tract in CD samples showcase the discontinuous segments of the epithelial layer with transmural inflammation. Enlarged mucosa and submucosa layers can also be seen together with the characteristic noncaseating granuloma with irregular crypts shapes and sizes in the lamina propria region (Figure 5.5 and 5.6). Meanwhile, inflamed tissues from UC patients display dense cellular infiltrates with loss of crypts density, branching of crypts and separation from the muscularis mucosae (Figure 5.7 and 5.8).

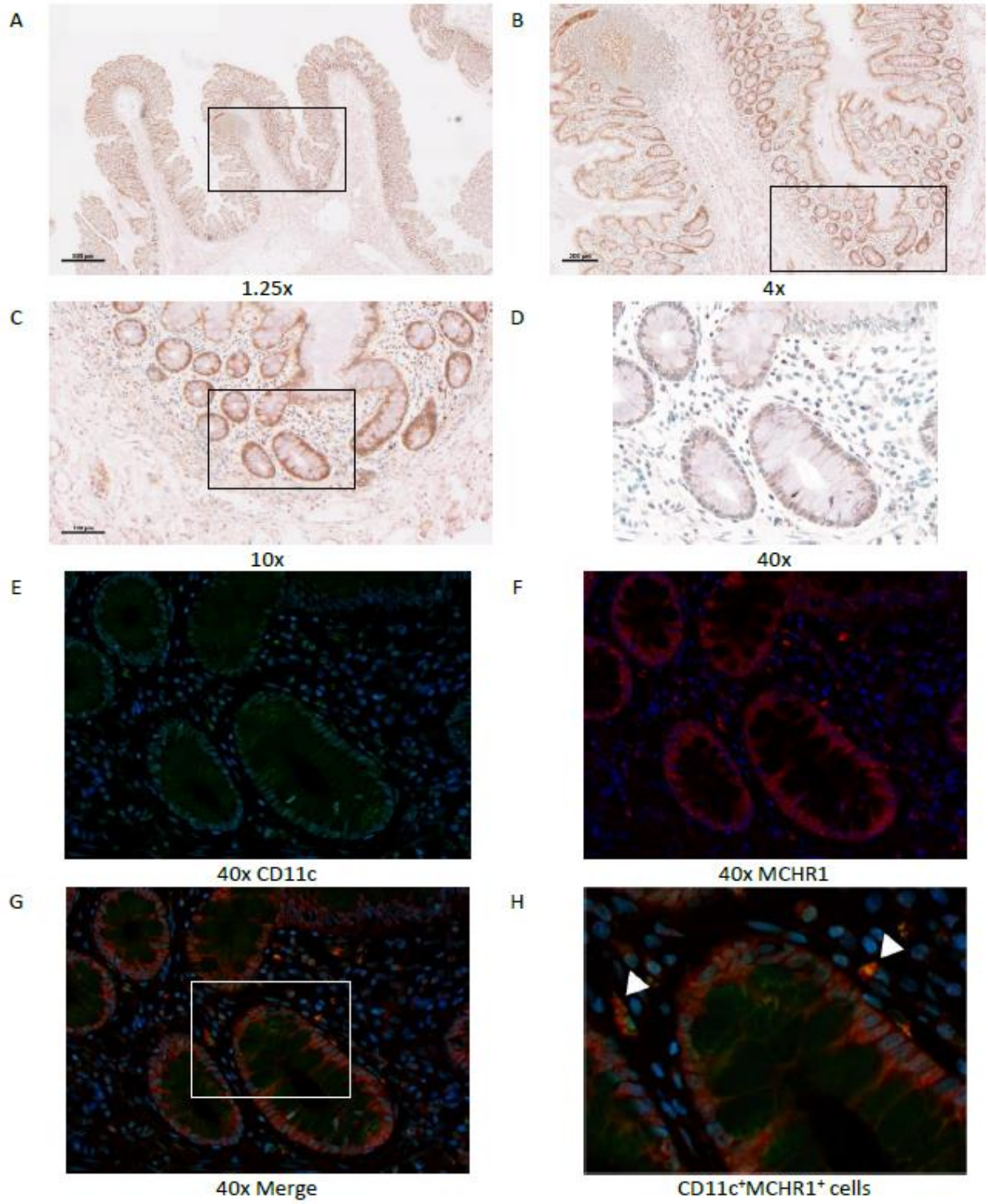


**Figure 5.3 MCHR1 expression on infiltrating CD11c$^+$ cells in "normal" human colon by immunocytochemistry**. Scanned images of human colon tissue sections double-stained with VIP and DAB peroxidase substrate with methyl green counterstain at A) 1.25x, B) 4x, C) 10x and D) 40x magnification. (E-G) Simulated fluorescent images of "false" fluorescence enables view of separate (E,F) and merged (G) images of individual staining. White arrows indicate double positive cells (H). Figure is a representative for five independent experiments. Green = VIP-positive CD11c, Red= DAB-positive MCHR1, Blue = Methyl green-positive nuclei

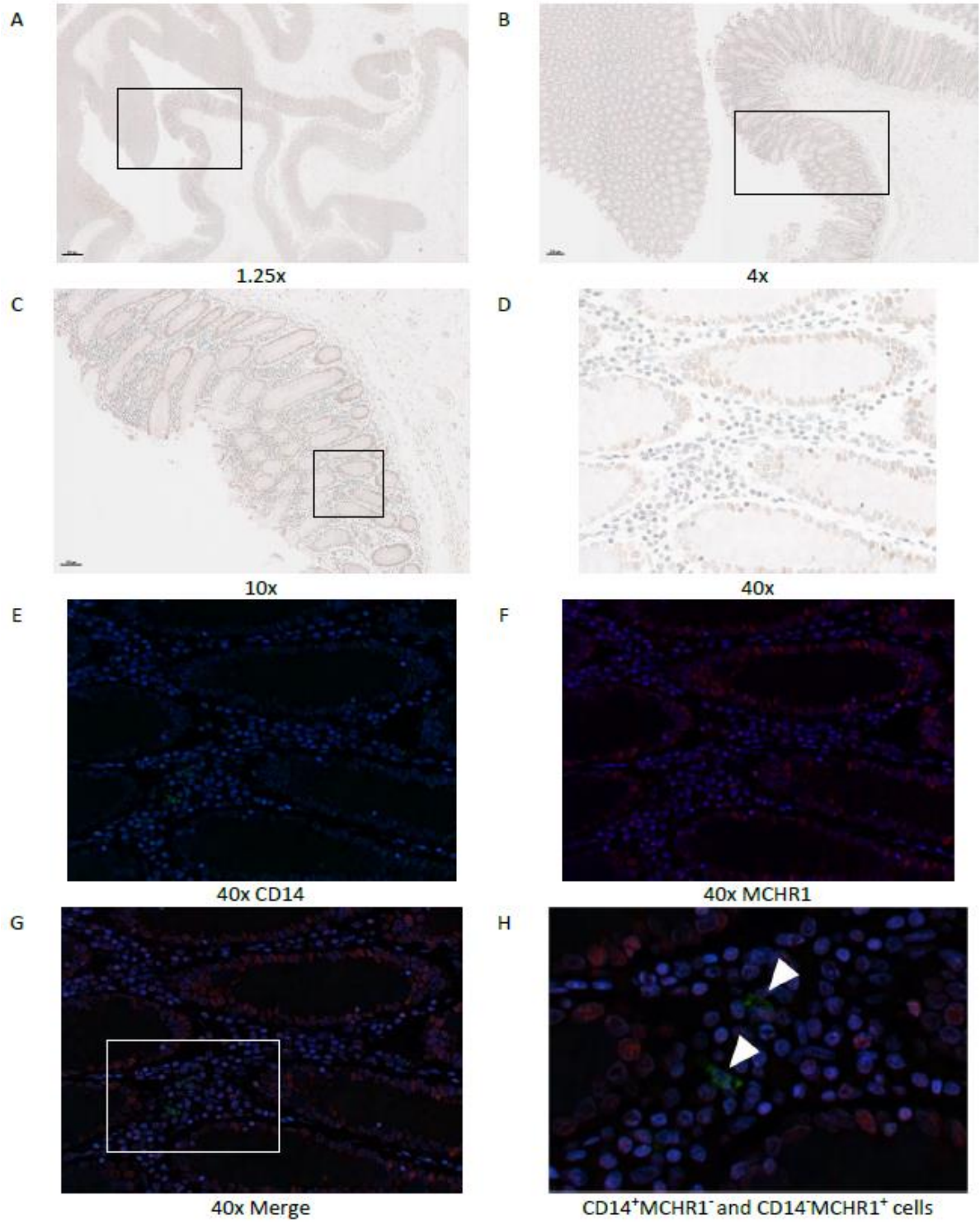


**Figure 5.4 MCHR1 expression on infiltrating CD14$^+$ cells in "normal" human colon by immunocytochemistry**. Scanned images of human colon tissue sections double-stained with VIP and DAB peroxidase substrate with methyl green counterstain at A) 1.25x, B) 4x, C) 10x and D) 40x magnification. (E-G) Simulated fluorescent images of "false" fluorescence enables view of separate (E,F) and merged (G) images of individual staining. White arrows indicate CD14+MCHR1- cells (H). Figure is a representative for five independent experiments. Green = VIP-positive CD14, Red= DAB-positive MCHR1, Blue = Methyl green-positive nuclei.

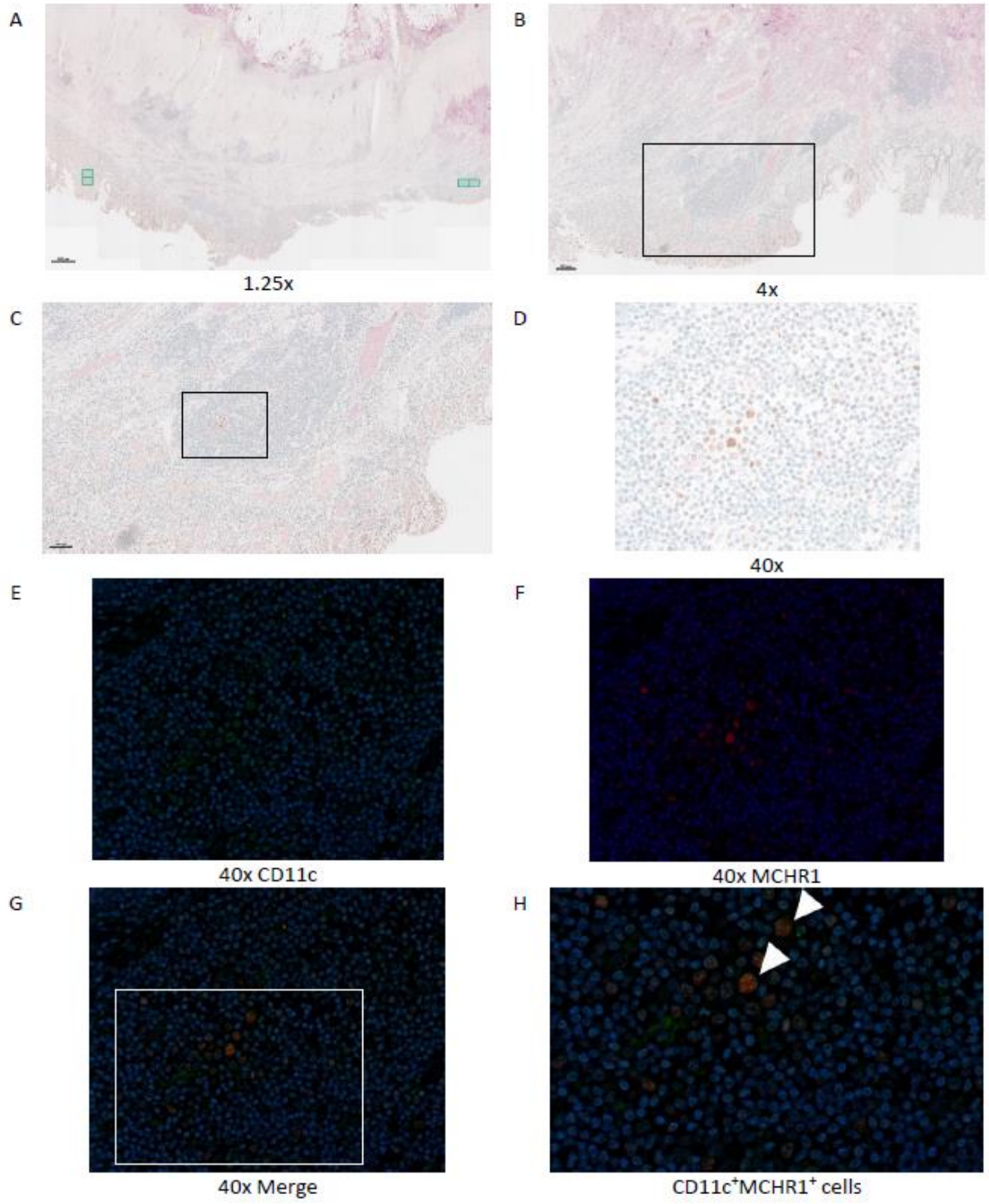


**Figure 5.5 MCHR1 expression on infiltrating CD11c$^+$ cells in human colon with Crohn's disease by immunocytochemistry.** Scanned images of human colon tissue sections double-stained with VIP and DAB peroxidase substrate with methyl green counterstain at A) 1.25x, B) 4x, C) 10x and D) 40x magnification. (E-G) Simulated fluorescent images of "false" fluorescence enables view of separate (E,F) and merged (G) images of individual staining. White arrows indicate CD14+MCHR1- cells (H). Figure is a representative for five independent experiments. Green = VIP-positive CD11c, Red= DAB-positive MCHR1, Blue = Methyl green-positive nuclei.

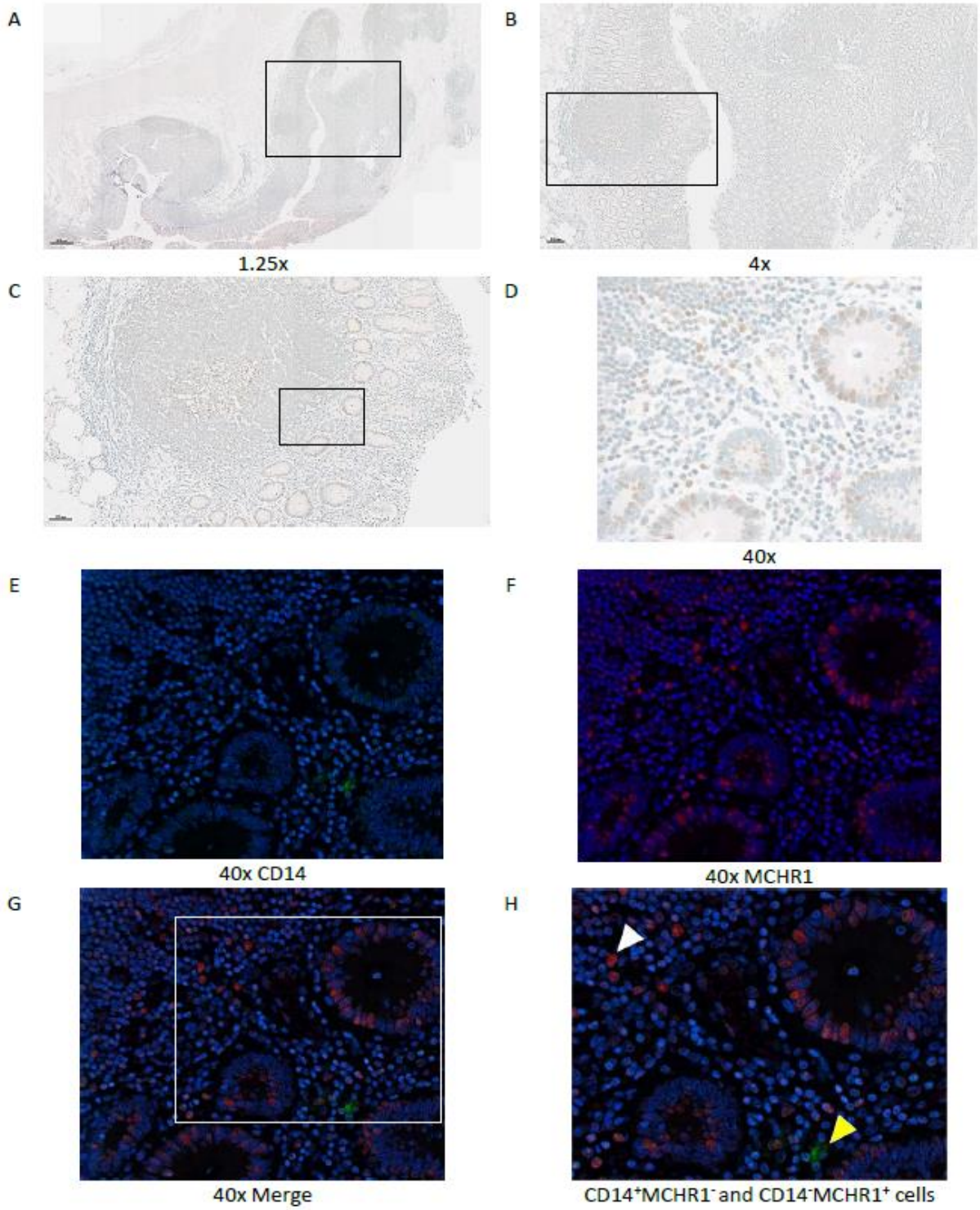


**Figure 5.6 MCHR1 expression on infiltrating CD14$^+$ cells in human colon with Crohn's disease by immunocytochemistry.** Scanned images of human colon tissue sections double-stained with VIP and DAB peroxidase substrate with methyl green counterstain at A) 1.25x, B) 4x, C) 10x and D) 40x magnification. (E-G) Simulated fluorescent images of "false" colour enables view of separate (E,F) and merged (G) images of individual staining. White arrows indicate CD14$^-$MCHR1$^+$ cells and yellow arrow indicate CD14$^+$MCHR1$^-$ cells (H). Figure is a representative for five independent experiments. Green = VIP-positive CD14, Red= DAB-positive MCHR1, Blue = Methyl green-positive nuclei.

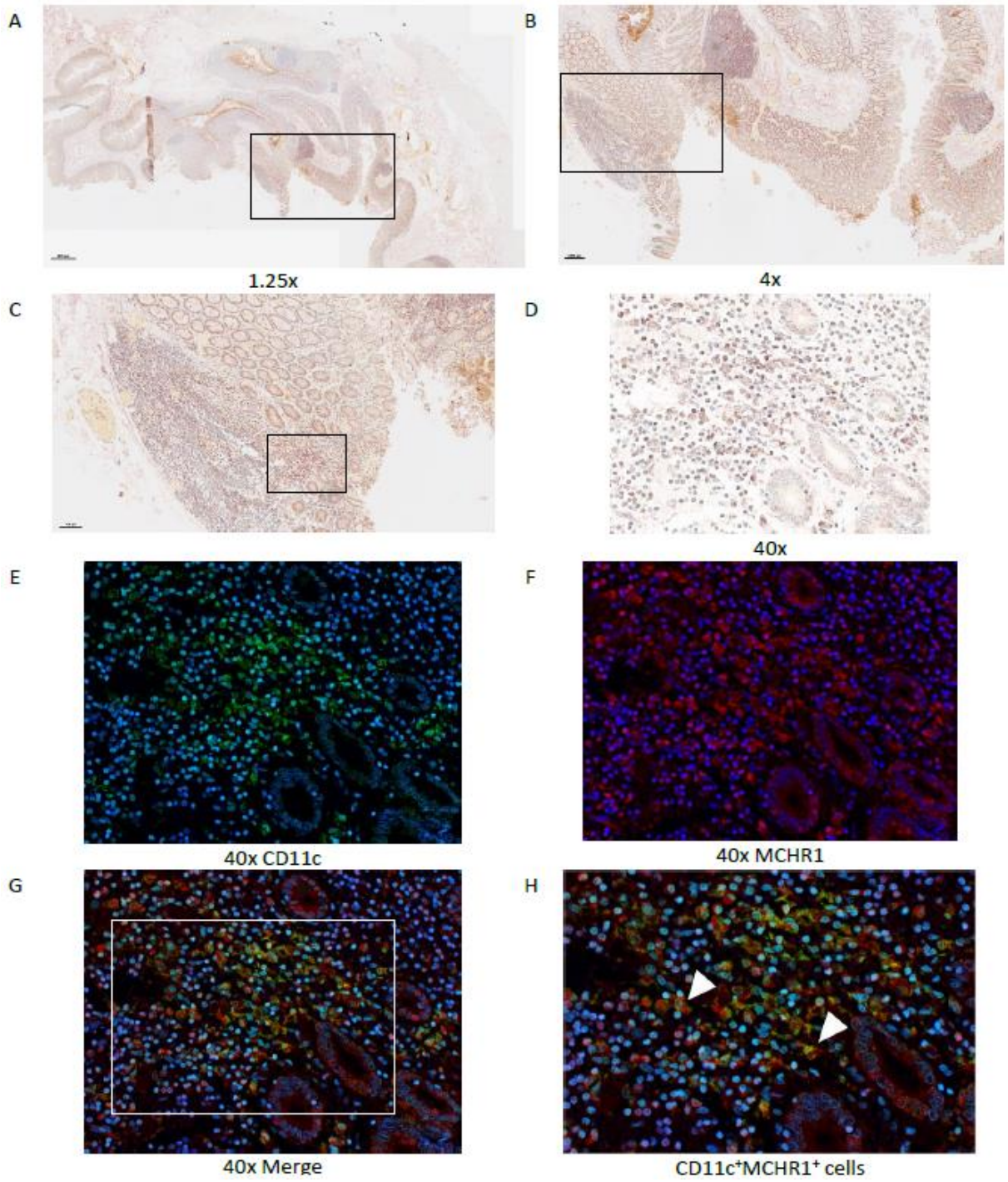


**Figure 5.7 MCHR1 expression on infiltrating CD11c$^+$ cells in human colon with ulcerative colitis (UC) by immunocytochemistry**. Scanned images of human colon tissue sections double-stained with VIP and DAB peroxidase substrate with methyl green counterstain at A) 1.25x, B) 4x, C) 10x and D) 40x magnification. (E-G) Simulated fluorescent images of "false" colour enables view of separate (E,F) and merged (G) images of individual staining. White arrows indicate double positive cells (H). Figure is a representative for five independent experiments. Green = VIP-positive CD11c, Red= DAB-positive MCHR1, Blue = Methyl green-positive nuclei.

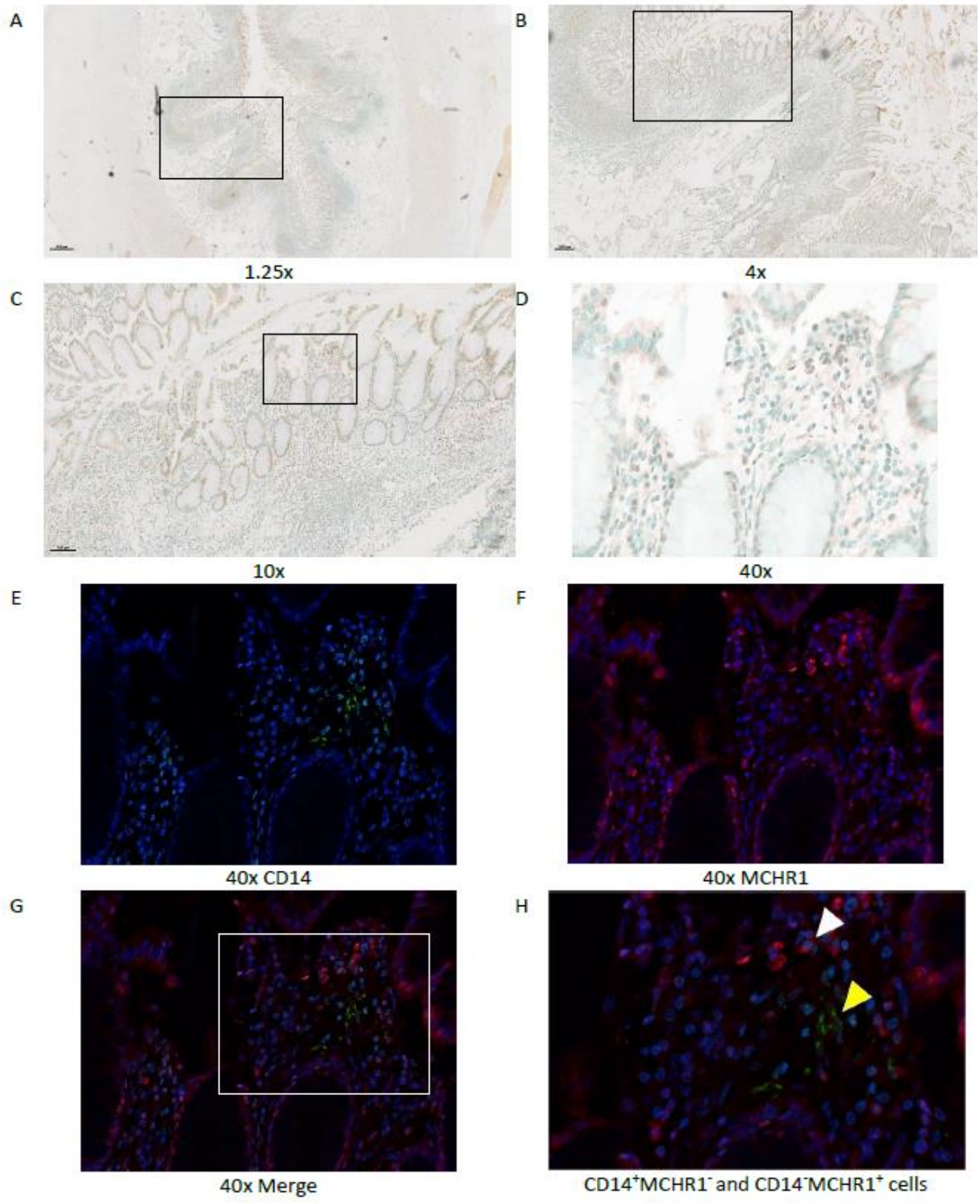


**Figure 5.8 MCHR1 expression on infiltrating CD14$^+$ cells in human colon with ulcerative colitis (UC) by immunocytochemistry.** Scanned images of human colon tissue sections double-stained with VIP and DAB peroxidase substrate with methyl green counterstain at A) 1.25x, B) 4x, C) 10x and D) 40x magnification. (E-G) Simulated fluorescent images of "false" colour enables view of separate (E,F) and merged (G) images of individual staining. White arrows indicate CD14$^-$MCHR1$^+$ cells and yellow arrow indicate CD14$^+$MCHR1$^-$ cells (H). Figure is a representative for five independent experiments. Green = VIP-positive CD14, Red= DAB-positive MCHR1, Blue = Methyl green-positive nuclei.

MCHR1 immunoreactivity in human colonic epithelial cells denotes the expression of MCHR1. Apart from the epithelial layer, DAB-positive cells can also be seen within the mucosal layer as well as the submucosal lymphoid tissues. At this point, however, it is hard to determine whether the receptors are upregulated in inflamed tissues as previously reported. This study focused more on the receptor expression in infiltrating immune cells in the lamina propria to illustrate the potential involvement of the MCH system in regulating the immune cells in inflammatory responses. Looking at the "false" immunofluorescent images from all representatives, the overlay of the combined images showed that some colonic $CD11c^+$ cells co-expressed MCHR1 but not $CD14^+$ cells. Qualitatively, $CD11c^+$ cell density was also increased in UC samples (Figure 5.7) but not in CD (Figure 5. 5) and normal (Figure 5.3). Unfortunately, quantitative evaluation on cells expressing MCHR1 in colonic mucosa could not be performed due to logistic issues thus it was not possible to do a comparative study on immune cells expressing MCHR1 between types of IBD. There are also $MCHR1^+$ cells that are neither $CD11c^+$ nor $CD14^+$ particularly within the lymphoid tissues which indicates the existence of different gut resident immune cell phenotypes that express MCHR1 in the colonic mucosa.

### 5.3 Discussion

It has been established that central MCH has a predominant role in regulating energy balance and appetite and MCHergic neurons are located in the hypothalamus. To date, there are also suggestions of a peripheral role of MCH and its receptors based on transcripts and protein detection in peripheral tissues in human, mouse and rat. Additionally, it was also postulated that MCH has a possible regulatory role in the immune system given that there is emerging evidence on the involvement of MCH in the pathophysiology of intestinal inflammation. Analysis of mRNA expression of the neuropeptide precursor found that the gene transcripts of

MCH prohormone and receptors are increased in stimulated cells and that several cells express functional MCH receptors which can activate a signalling pathway when it binds to its ligand or selective agonists (Verleat et al. 2002). Previous studies have revealed this hypothalamic neuropeptide and its putative receptors are present in the gut such as in the enterochromaffin cells, duodenum and the colon (Hervieu and Nahon, 1995; Kokkotou et al. 2008). Consistent with previous findings, this study reports that human colonic epithelial cells express MCHR1 based on the immunoreactivity observed in IHC analysis while real time RT-PCR analysis indicated that inflamed human colonic tissue expresses higher pMCH than control though not at a statistically significant level. Similar to a previous study, Kokkotou et al. (2008) also demonstrated a higher level of pMCH transcripts in human colitis along with the expression of MCHR1 and MCHR2 receptor. In contrast, gene transcripts for both MCH receptors in this study were detected at relatively low levels and were not upregulated in IBD samples despite the histological evidence of MCHR1 presence. One possible reason that could contribute to these discrepancies is the low efficiency of RT-PCR in which cell lines that endogenously expressed MCHR1 were used as positive control. RT-PCR performance could be improved by using MCHR1-transfected cellular models with higher protein expression for a more robust primer efficiency determination.

One of the questions raised was whether the presence of MCH in inflamed colonic mucosa have a correlation with the inflammatory pathogenesis itself. Murine models of experimental colitis managed to provide the links between MCH and inflammatory responses which can regulate the outcome of the severity of tissue damage when induced. It was first demonstrated that mice deficient in MCH developed attenuated colonic inflammation comparable to those treated with anti-MCH antibody which points to the influence of MCH in the inflammatory processes. It was supported by the discovery that MCHR1 receptor activation

with MCH leads to increased IL-8, which is a strong neutrophil chemoattractant, in HT-29 colonocytes (Kokkotou et al. 2008). Taken together, it can be implied that MCH can regulate immune response indirectly by acting on colonocytes. It was also later discovered that MCH can inhibit IL-10 secretion from stimulated monocytes but an MCHR1 antagonist failed to provide protection against piroxicam-induced colitis in IL-10 deficient mice (Ziogas et al. 2014). IL-10 is a regulatory cytokine that can inhibit pro-inflammatory cytokines TNF-a, IL-1beta, IL-6 and IL-8 which are found elevated in blood serum of IBD patients (Szkaradkiewicz et al. 2009). Together with our data on MCHR1 expression in peripheral CD14 monocytes (refer Chapter 4.1), the pro-inflammatory effect of MCH on colonic mucosa could derive from MCHR1 activation from a different source and not directly on intestinal monocytes. To add to these observations, our IHC sections showed that intestinal $CD14^+$ cells do not express MCHR1 (Figure 5.4, Figure 5.6, Figure 5.8) but there are also the presence of $MCHR1^+$ infiltrating cells as well as within lymphoid tissue that are neither CD11c+ nor $CD14^+$.

The maintenance of intestinal immune homeostasis is largely dependent on the regulatory function of residential macrophages. There are different subsets of intestinal macrophages which can be characterised based on the expression of CD11c together with chemokine receptors CCR2 and CX3CR1. It was described that $CD11c^{high}CCR2^+CX3CR1^+$ monocyte-like cells are expanded in inflamed colon of IBD patients most notably in UC (Bernado et al. 2018). Qualitatively, this study also provided evidence of a more dense infiltration of $CD11^+$ cells in UC tissue sections, and it was interesting to note that these cells co-expressed MCHR1 although the functional analysis and quantitative analysis of receptor expression on specific colonic immune cell subsets are not yet available at this point. $CD11c^{high}$ cells in IBD colon was characterised as monocyte-like due to the shared chemokine receptor expression with circulating peripheral CD14 monocytes and preferentially producing pro-

inflammatory IL-1beta. On the contrary, tissue resident macrophages identified as $CD11^{-}CCR2^{-}CX3CR1^{-}$ predominantly produce anti-inflammatory IL-10 (Bernardo et al. 2018). To relate, MCH-KO mice infected with *Salmonella enterica* display a more severe inflammation with elevated pro-inflammatory cytokines levels of IL-1beta along with TNF-α, IFN-γ in the intestine suggesting an anti-inflammatory influence of MCH. On another note, IHC analysis also showed MCHR1-positive cells that might also contribute to the regulation of inflammatory response in the gut. However, the expression pattern of MCHR1 on gut resident immune cells need to be investigated further to characterise its role in the immune activities in control and inflamed condition in IBD. To add, other than in inflamed mucosa of the intestinal tract, gene expression profiling by Affymetrix platform also exhibited pMCH upregulation in tumour infiltrating CD4 and CD8 lymphocytes in follicular lymphoma but not in peripheral lymphocytes. The altered gene expression was implied to potentially impaired cellular migration to the tumour microenvironment leading to an affected overall survival and time of transformation of patients with follicular lymphoma (Kiaii et al. 2013). Taken together, MCH seems to have a pro-inflammatory influence on immune cells via its putative receptor and it is worth to investigate the degree of impact in modulating the immune response in the pathogenesis of inflammatory diseases. Future studies can also include investigations on the post-translation of the MCHR1 receptor protein which is important in cell membrane trafficking of GPCRs to determine its functionality. The knowledge obtained could be useful in finding novel targets in modulating the pro-inflammatory influence of MCH system in diseases.

## Chapter 6 General Discussion and Conclusion

The relationship between neurotransmitters and neuropeptides and the immune system involves complex bidirectional interactions of primary and accessory cells with the neuronal components. Close proximity between neurons and immune cells is one of the sources of neuropeptide release in response to inflammatory stimuli. Neuroendocrine cells have the capacity to recognise cytokines released by immune cells and in return produce neurotransmitters and neuropeptides that are able to bind to their receptors expressed in immune cells which in turn can lead to modulation of functional responses. Vice versa, immune cells also possess the capacity to produce and release the neuropeptide themselves in response to stimuli which can regulate the activity of neuronal groups in the nervous system to trigger immunomodulatory responses. One prime example to this relationship is the antinociceptive anti-inflammatory effect of opioids during neurogenic inflammation (Sacerdote, P. 2006).

Both serotonin and melanin concentrating hormone are both neuronal agents that have been implicated in the regulation of immune response. Convincing evidence gathered from animal studies as well as human cells and tissues leads to the reason for developing this research. Furthermore, inflammatory pathogenesis that primarily involves both innate and adaptive immune responses have become viable targets in therapeutic strategies for chronic inflammatory diseases and in enhancing protective immune responses towards infections and tumorogenesis. Therefore, it is of great interest to investigate the influence of 5-HT and MCH have in immunity.

To conclude the first part of this work demonstrated that serotonin has minimal to no direct impact on the functional response of T lymphocytes. Perhaps the study on the impact of serotonin on immune response is better diverted to its accessory roles which can indirectly

affect immune responses. To continue, the second part of this study reports for the first time the expression of the neuropeptide receptor MCHR1 in different phenotypes of T cells and that it is upregulated when activated with a significant increase observed in naïve CD8 T lymphocytes. MCH has a slight stimulatory effect on naïve T cell proliferation but not on endogenously expressed MCHR1 on the Jurkat cell line. This preliminary result points towards a possible role of the MCH system in regulating cellular initiation and differentiation processes when encountering antigen. However, data from functional responses is still debatable and more appropriate functional assays are required to confirm this finding and whether the data are reproducible.

In the last part of this research, IHC staining on human colon tissue samples depicted that MCHR1 receptors are expressed in the epithelial of colonic mucosa. Some infiltrating cells in the lamina propria are also positive for MCHR1 and are co-expressed in few $CD11c^+$ cells but such is not the case in $CD14^+$ cells. Real time RT-PCR analysis showed upregulated ppMCH mRNA in inflamed tissues but it is unknown if it is causative or effects of inflammatory response in IBD pathogenesis. The mRNA for MCH receptors however were detected at low levels with no differential expression between the control and inflamed state. The overall results from this study are mainly descriptive. It is obvious that the data obtained are at a preliminary stage and that more molecular and pharmacological studies are required to further understand the role of neuropeptides on specific immune cell subsets in normal physiological functions as well as in diseased states. The knowledge could potentially contribute to the process of developing new therapeutic targets in inflammatory disease.

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