

**NOVEL MARKERS FOR HUMAN T
REGULATORY CELLS IN HEALTHY
DONORS AND CANCER PATIENTS**

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List of publications derived from this work

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Elkord, E., Abd Al Samid, M., & Chaudhary, B. (2015). Helios, and not FoxP3, is the marker of activated Tregs expressing GARP/LAP. *Oncotarget*, 6(24), 20026-20036.

Chaudhary, B., Abd Al Samid, M., al-Ramadi, B. K., & Elkord, E. (2014). Phenotypic alterations, clinical impact and therapeutic potential of regulatory T cells in cancer. *Expert Opin Biol Ther*, 14(7), 931-945.

Declaration

This thesis is submitted under the University of Salford rules and regulations for the award of a PhD degree by research. I certify that this report consists of my own original work. All quotations from published sources are acknowledged as such in the text.

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Dedication

This piece of work is dedicated to my wonderful parents; Mr Mohammed Abd Al Samid, the greatest dad ever, and to the best mum in the whole world, Mrs Bushra Abd Al Jabbar. To my wonderful dear husband, Mr Diyar Bazgir. To my lovely brothers (Ahmed & Ali) and sisters (Yasmin, Marwah & Fatimah).

Finally, to my entire family specifically; mother in law (Amira), father in law (Ibrahim) and brother in law (Dana).

Our dream finally comes true.

List of Abbreviations

AIRE: Autoimmune regulator

AP-1: Activator Protein-1

APC: Antigen presenting cells

CD: Cluster of differentiation

CP: Chronic pancreatitis

CRC: Colorectal cancer

CTL: Cytotoxic T lymphocytes

CTLA-4: Cytotoxic T-lymphocyte-associated antigen-4

DCs: Dendritic cells

FoxP3: Forkhead box P3

GARP: Glycoprotein A Repetitions Predominant

GITR: glucocorticoid-induced TNFR family related gene

HDs: healthy donors

IFN- γ : Interferon gamma

ICS: Intracellular cytokine staining

Ig: Immunoglobulin

IL-10: Interleukin-10

IL-2: Interleukin-2

IPEX: Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked

LAP: Latency-associated peptide

LI/CRCs: liver metastases from colorectal cancer

LTBP: latent TGF- β binding protein

MHC: Major Histocompatibility Complex

miRNAs: MicroRNAs

NK: Natural killer

Nrp-1: Neuropilin-1

PBMCs: Peripheral blood mononuclear cells

PC: Pancreatic cancer

pTregs: peripherally-induced T regulatory cells

TCR: T cell receptor

TF: Transcription Factor

TGF- β : transforming growth factor-beta

Th: T helper

TNF- α : Tumour necrosis factor- α

TNFR-II: Tumour necrosis factor receptor-II

Tr1: T regulatory type 1

tTregs: Thymus-derived T regulatory cells

Abstract

CD4⁺CD25⁺FoxP3⁺ T regulatory cells (Tregs) are essential for maintaining self-tolerance and preventing autoimmune diseases. However, FoxP3⁺ Tregs contribute to the progression of cancer and their levels expand in cancer patients compared to healthy donors. Tregs suppress tumour-specific immune responses by accumulating in the peripheral blood and tumour microenvironment. Human Tregs secrete the latent form of transforming growth factor-beta (TGF-β), in which the mature TGF-β protein is bound to latency-associated peptide (LAP) that binds to Glycoprotein A Reiterations Predominant (GARP). Some FoxP3⁺ Tregs express Helios, a member of the Ikaros transcription factor family. The purpose of this study is to identify which of FoxP3^{+/+}-Helios^{+/+} Tregs express GARP and LAP and to investigate if these receptors are vital markers for activated conventional Tregs, and also to examine the different suppressive factors and phenotypes of Tregs. This study compared the levels of Tregs in cancer patients with controls, by measuring the levels of Tregs specific and novel markers. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors (HDs), chronic pancreatitis (CP), malignant pancreatic cancer (PC) and liver metastases from colorectal cancer (LI/CRC) patients. PBMCs were then stained with anti-CD3, anti-CD4, anti-GARP, anti-LAP, anti-Helios, anti-FoxP3, anti-IFN-γ, and anti-IL-10 antibodies. The results demonstrated for the first time that GARP and LAP are mainly expressed on activated CD4⁺FoxP3⁺Helios⁺ Tregs and CD4⁺FoxP3⁻Helios⁺ Tregs for healthy donors and all patient groups. In contrast, CD4⁺FoxP3⁺Helios⁻ and CD4⁺FoxP3⁻Helios⁻ Tregs do not express GARP and LAP. FoxP3⁺Helios⁺ Tregs from cancer

patients showed significantly higher expression of GARP and LAP, compared to healthy donors. Furthermore, there was no increase in the level of FoxP3⁺Helios⁺ Tregs in HDs and PC patients compared to LI/CRC patients. FoxP3⁺Helios⁺GARP⁺LAP⁺ Tregs secrete the IL-10 cytokine but not IFN- γ in comparison with FoxP3⁺Helios⁻ Tregs. This study demonstrated that Helios, and not FoxP3, is the main marker of activated Tregs expressing GARP and LAP.

Chapter 1

Introduction

1.1 Cancer

Cancer requires a succession of genetic changes and epigenetic events to be capable of developing (Nowell, 1976). The epigenetic changes can occur by disease state, the environment, lifestyle, age, and other factors (Deans and Maggert, 2015). The entire process of cancer development is involved in two stages: initiation, which is the genetic mutation in a single cell, and promotion, which are the successive carcinogenic events that complete the neoplastic transformation of initial mutated cell and forms multiplications of tumour cells. A later course of cancer's further stages is the progression: when a malignant tumour starts in any tissue and continues to grow and metastatic spread into surrounding tissues (Pitot, 1993).

Cancer cells are invasive because they can ignore the programmed cell death signals, also known as apoptosis, which is used when the body wants to get rid of the unneeded cells (Curiel et al., 2008). They grow and survive by inducing normal cells to form blood vessels that provide the tumour cells with nutrients and oxygen. In the tumour microenvironment, the cancer cells may be able to influence healthy cells, blood vessels, immune cells, inflammatory cells, fibroblast, lymphocytes, and signalling molecules. Since the cancer cells express both self and tumour-associated antigens, they can escape from the immune system with the help of regulatory immune cells. Although the regulatory immune cells are important to maintain self-tolerance, they are responsible for dampening and inhibiting the anti-tumour immune cells from attacking the cancer cells (Oleinika et al., 2013). These immunosuppressive cells are involved in controlling the occurrence of metastasis. Therefore, it is very important for the clinic to understand the mediators that are involved in these cells, in order to target these cells to promote and inhibit metastatic diseases. Several diseases play

an important etiologic role in cancer occurrence. Many cancer types are also associated with chronic inflammation and by infectious agents such as viruses (Pagano et al., 2004; de Martel and Franceschi, 2009). Patients treated with immunosuppression for tissue transplantation or another medical reason is at significant risk of developing non-Hodgkin's lymphoma (Kinlen, 1985; Curtis et al., 1997; Dantal et al., 1998). Cancer is a genetic disease that occurs from mutations of proto-oncogenes and tumour suppressor genes or polymorphic gene activity governing enzyme systems that either activate or detoxify environmental carcinogens (Pitot, 1993). As a result of errors that occur as cells divide or because of damage to DNA caused by certain environmental exposures include substances, such as radiation (Azzam et al., 2016), the chemicals in tobacco smoke, and ultraviolet rays from the sun (Hitrik et al., 2016; Zhao et al., 2016). Cancer death is increasing widely year after year because of the increasing human populations, pollutions, and other cancer causing atmospheres (Jemal et al., 2011). Referring to several studies, expose to chemicals and radiations, or inherit germline mutations such as BRCA1 arise carcinogenic mutations (Pitot, 1993).

Most cases, early detection of cancer led to better cancer prognosis and more successful treatment. Screening for cancer is a strategy that contributes to morbidity and mortality reduction by both identifying and treating malignancy or by diagnosing invasive diseases at an early stage (Eddy, 1980). Moreover, to establish the basis staging and treatment of cancer, a biopsy of any potential cancer site should be performed in the early process.

Currently, the American society of clinical oncology (ASCO) in the department of adoptive cell immunotherapy has allowed clinicians to genetically reprogram patient's own immune cells to find and attack cancer cells. For example, patients

with acute lymphoblastic leukaemia (ALL), lymphoma and multiple myeloma have been treated with a type of adoptive cell immunotherapy that calls chimeric antigen receptor T cell therapy, that targeting the B cell surface antigen CD19. Luckily, this immunotherapy has led to remarkable results (Geyer and Brentjens, 2016; Mirzaei et al., 2017; Gue et al., 2016). Even though, the immune system is found to play an important role in most cancers, the activation of the immune system can promote metastasis if the inflammation is persuaded (Dahlberg et al., 2015). Therefore, the combination therapy e.g. (suppressing Tregs and enhancing CD8⁺ T cells) is necessary to make a balance in the immune system during the treatment.

1.1.1 Pancreatic malignant cancer

The pancreas function as a digestive organ that secret pancreatic juice, which contains digestive enzymes that enrich the absorption of nutrients and the digestion in the small intestine (Sherwood, 2006) (Figure 1), it consists of both exocrine and endocrine functions. The role of the endocrine is to produce chemicals and hormones, such as insulin, glucagon, somatostatin, and polypeptide to regulate the blood sugar, while exocrine compartment is to produce digestive enzymes such as amylase, protease and lipase (Imai et al., 2013; Hougaard et al., 1986).

Through gene mutations or chronic pancreatic infections, the pancreatic mechanisms can be disturbed, leading to serious pancreatic diseases including different types of pancreatic cancer (Figure 2) (Bloomston et al., 2007). Most pancreatic cancers form in exocrine cells. It is usually hard to diagnose this type of pancreatic cancer early because these tumours do not secrete hormones neither they cause signs or symptoms. For most patients with exocrine pancreatic cancer, current treatments do not cure cancer (Matracci et al., 2015). Endocrine tumours of the pancreas are rare, and it is considered being neuroendocrine neoplasms and classified as APUDomas (an endocrine tumour that arises from APUD cell). Insulinoma is the most characteristic of the pancreatic endocrine tumours, which is distributed evenly throughout the gland (Doppman et al., 1995).

In general, pancreatic cancer is a malignant neoplasm originating from cell overgrowth, and the most common type of pancreatic cancer is the pancreatic ductal adenocarcinoma (PDAC) (Schuller and Al-Wadei, 2012), which in several reports indicated that it affects men more than women (Le et al., 2015). Also, PDAC is usually not discovered until the tumour cells have metastasized;

likewise, this type of cancer is difficult to treat (Hruban et al., 2008). Eventually, the detection and treatment of early non-invasive pancreatic neoplasia have a significant role on pancreatic cancer mortality (Hruban et al., 2008). The most typical lesions of non-invasive pancreatic neoplasia are the pancreatic intraepithelial neoplasia (PanIN) (Hruban et al., 2008). The main non-inherited risk factors that cause PDAC are cigarette smoking, and it believes to be 20% of PDACs (Fuchs et al., 1996). Furthermore, 4% of patients with chronic pancreatitis are also at risk of developing PDAC after 20 years of the disease (Lowenfels et al., 1993). Likewise, approximately 30% of patients with pancreatic cancer have been associated with Diabetes Mellitus (DM) (Chari et al., 2008). Referring to Chari et al., 2008 people with DM are at high risk to develop pancreatic cancer, which suggests that new-onset DM can be cancer-related symptoms that occur shortly before the diagnosis of pancreatic cancer (Chari et al., 2008). Interestingly, a case study demonstrated that insulin-dependent diabetes is more risk of having pancreatic cancer than diabetic patients using metformin (Li et al., 2009). Further studies have proved that obesity (especially at an older age) is also a high-risk factor that is associated with pancreatic cancer development (Li et al., 2009).

Approximately 5% to 10% of individuals with pancreatic cancer can inherit this disease from a close family member (Shi et al., 2009). A study by Murphy et al. identified five mutations in BRCA2 gene sequence that is relatively involved in pancreatic cancer (Murphy et al., 2002). The carrier of BRCA2 mutation gene is an increased risk of PDAC. Additionally, the study outlined that two of five BRCA2 mutation gene carrier report a family history of breast cancer (Murphy et al., 2002). Therefore, so far the most common known mutated gene that associated with PDAC is the BRCA2 gene. Further inherited risk factor that is not

only related to pancreatic cancer but also with a variety of cancers is the Peutz-Jeghers syndrome (PJS), which is an inherited mutation in the STK11/LKB1 gene. Patients with PJS are at very high risk of developing PDAC (Giardiello et al., 2000).

There is considerable evidence of the critical role played by the immune system in controlling the growth of malignant cells. Referring to Yamamoto et al., (2012) patients with pancreatic cancer have a relatively high level of the immunosuppressive Tregs, and it has reported that most patients with pancreatic cancer have immune destruction. Weak anti-tumour immunity leads to immunologic toleration of malignant cells and contribution to the development and progression of malignancies (Abd-Elgaliel et al., 2013). The development of pancreatic cancer could be associated with an unbalanced immune function in the human body (Abd-Elgaliel et al., 2013). However, it is still challenging to fully understand the precise mechanisms by which the immune system modulated in patients with malignant disease.

It is very necessary to identify biomarkers to characterise and evaluate normal biological processes, pathogenic processes, or pharmacologic responses to chemotherapy and radiotherapy (Neoptolemos et al., 2004). However, it has been challengeable to identify diagnostic, prognostic, and predictive biomarkers in pancreatic cancer. To date, the only biomarker that has introduced and approved by FDA is the CA 19-9. This biomarker is recommended to be used in the routine management of pancreatic ductal adenocarcinoma (Winter et al., 2013).

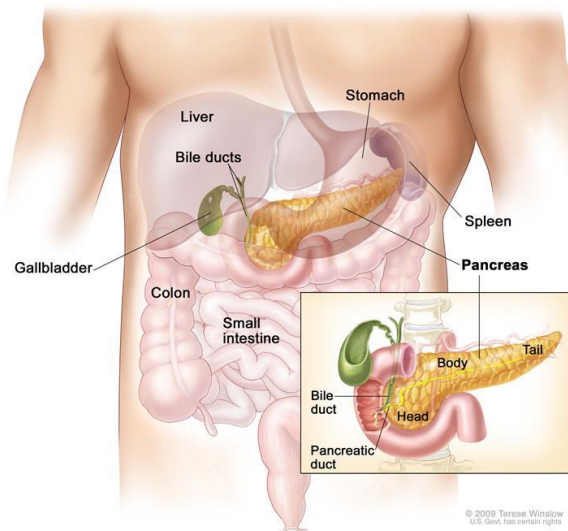


Figure 1: Anatomy of the pancreas.

The location of the pancreas is in the upper abdomen and lies behind the stomach and next to the small intestine. The pancreas consists of three parts; head, body, and tail (Source: National Cancer Institute, 2015).

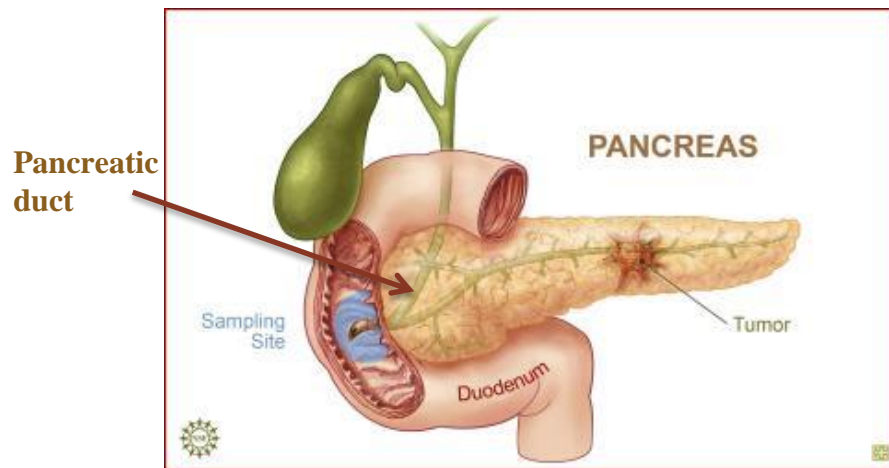


Figure 2: Tumours in pancreas

Tumour cells develop in the pancreas and extend around the pancreas. The majority of pancreatic cancer is the pancreatic ductal adenocarcinoma (Source: Medicine World, 2008).

1.1.2 Liver metastases from colorectal cancer

Colorectal cancer commonly occurs in the large intestine and located in the appendix and rectum (Figure 3) (Deans et al., 1994; Kulke & Mayer, 1999). A study by Foiri et al. demonstrated that 63.6% of obstructive malignancy was found to be in the rectum and 36.3% in the sigmoid/rectosigmoid colon (Foiri et al., 2004). However, cancer in the small intestine does happen, but it is much rarer, and due to the rarity of the small bowel cancer, many patients do not have any symptoms until the late stage of the disease which leads to a delay in diagnosis and therapies (Masselli et al., 2009; Masselli et al., 2013).

Colorectal cancer appears to arise from several genetic changes involved in tumour progression including tumour suppressor genes on chromosome 5, 17, and 18 and K-ras oncogene on chromosome 12 (Vogelstein et al., 1988; Fearon and Vogelstein, 1990; Hamilton, 1993). A molecular study by Jen et al., (1994) reported that when there is a mutation in chromosome 18q (the DCC gene) in patients with stage II colorectal cancer, they strongly develop similarity to that in patients with stage III disease. Accordingly, the patient with stage II colorectal cancer who do not have a mutation in chromosome 18q has a survival rate that is similar to patients with stage I colorectal cancer (Jen et al., 1994). Most colorectal cancer develops as adenomatous polyps which consider placed in the colon and rectal adenocarcinoma (Simon, 2016). Hyperplastic polyps are the most common adenomatous polyp premalignant (Williams et al., 1982), whereas the second type of the polyps is the neoplastic adenoma, which remains to have an invasive malignant potential (Muto et al., 1973).

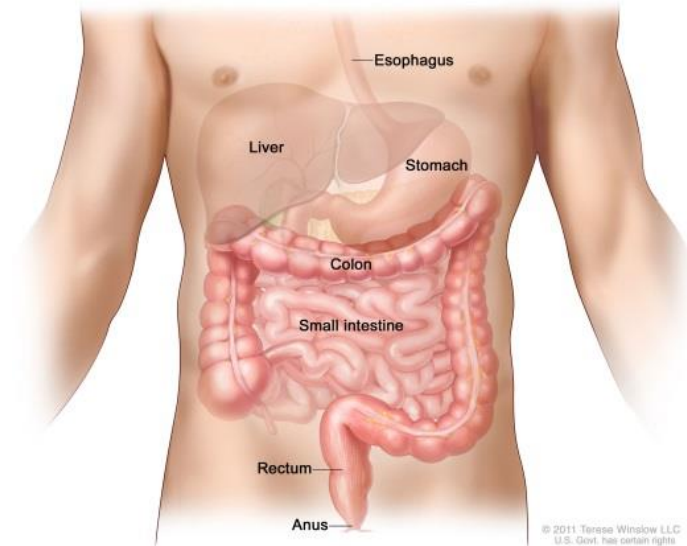


Figure 3: Anatomy of the small and large intestine

The colon and the rectum are parts of the large intestine; it located in the bottom of the body's digestive system (Source: National Cancer Institute, 2015).

Metastases of Colorectal cancer spread to another part of the body through the lymphatic system or the bloodstream. Because blood flows directly from the colorectal to the liver, one of the very commonplaces for colorectal cancer to spread is the liver. When cancer starts in the other parts of the body and spreads to the liver, it is called liver metastasis, and it is the main cause of death in the colorectal cancer patients (de Krijger et al., 2011). The colorectal liver metastasis is stage IV cancer that starts in the intestine and has spread from the rectum or colon to the liver (Figure 4). The symptoms of liver metastases from colorectal cancer are weight loss, fatigue, blood in the stool and extreme stomach-ache. There are several risk factors of the liver metastasis from colorectal cancer. People over the age of 85 consider being at higher risk of developing colorectal cancer (Ng et al., 2016). Furthermore, cigarette smoking, diabetes, colon inflammation, Crohn's disease, obesity, lack of physical activity, heavy alcohol use, eating a lot of red meat and food rich in fat can also cause colorectal cancer (Martinez-Useros and Garcia-Foncillas, 2016; Baena and Salinas, 2015). The genetic mutations appear in patients with liver metastases from colorectal cancer are also studied. Multiple studies have reported that the non-coding RNA, also called microRNAs (miRNAs), expression in the primary tumour can lead to a progression of colorectal cancer and initiation of metastases (Scetter et al., 2012; Li et al., 2011; Vaksman et al., 2011). Depending on the cellular environment in which the miRNAs have expressed, some of the miRNAs acts as either tumour suppressor or oncogenes (Calin et al., 2002). Therefore, an early study by Feiersinger et al., (2016) has analysed three miRNAs (miRNA-21, miRNA-31, and miRNA-373) that accordingly may play a fundamental role in the formation of the liver metastases from colorectal cancer (Asangani et al., 2008; Cottonham et al., 2010; Huang et al., 2008; Cjoi et al., 2000). The results showed that the

expression of all the miRNAs was significantly higher in the primary liver tumour tissue compared to the healthy liver tissues. Additionally, both the miRNA-21 and miRNA-31 were significantly up-regulated in the liver metastases compared to healthy liver tissues (Feiersinger et al., 2016). Liver metastases from colorectal cancer are a serious disease, and the patient has to be treated appropriately. The treatment for advanced colorectal cancer is through chemotherapy, biological therapy, radiotherapy and/or surgery (Gambardella et al., 2016; Sinicrope et al., 2016; Carter et al., 2016; Li et al., 2016).

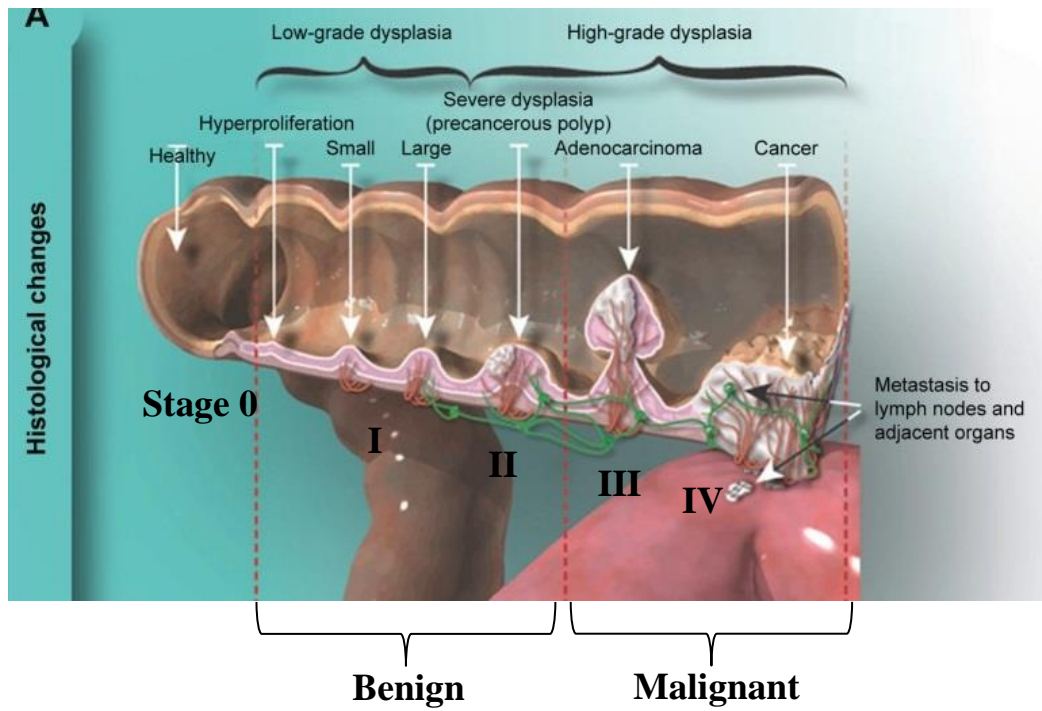


Figure 4: Stages of colon cancer

Colorectal cancer initiates in the colon or rectum. There are four stages of tumour size in colon cancer (Stage: I, II, III, and IV). Early stages of CRC (stage I and II) are a benign tumour that is no tendency to spread or has not spread outside the bowel yet. Adenocarcinoma is late-stage (stage III) of malignant CRC which is uncontrolled proliferation with the invasion of other tissue. In stage IV, the tumour has grown out from the colon wall to the other nearby organs (metastases). The figure is taken from (Simon, 2016).

1.2 Adaptive immune system

The immune system function as a defence mechanism against all types of germs and pathogens such as bacteria, viruses, fungi, and other kinds of organisms that cause diseases. The human immune system is divided into innate and adaptive immunities and is involved in both humoral and cell-mediated immunities (Mosier, 1967). The innate immune system is a non-specific, immediate immune response with no immunological memory. It consists of white blood cells known as leukocytes that differentiate and diverse to five different types that distinguished by their physical and functional characteristics; natural killer cells, basophils, mast cells, eosinophils and the phagocytic cells including macrophages, monocytes, dendritic cells and neutrophils (Abbas, 2009). The adaptive immune system is specific and provides long lasting protection because of their memory immune system, for example, memory B and T lymphocytes (Abbas, 2009) (Figure 5).

Some of the B lymphocytes (B cells) make antibodies against pathogens and other harmful organisms, and they develop into memory B cells to remember the same pathogens for faster antibody production in subsequent infections (Francus et al., 1991). T lymphocytes consist of different subtypes; such as helper, cytotoxic, and regulatory T cells. Helper T cells are $CD4^+$ T cells expressing CD4 glycoprotein, which is a co-receptor, binds to the major histocompatibility complex (MHC) class II that assists the T cell receptor (TCR) in communication with antigen presenting cells (APCs) (Dausset, 1981; Klein, 1979). Memory T cells are derived from normal T cells that have learned how to overcome an invader by remembering the strategy that used to defeat previous infections (Francus et al., 1991). Cytotoxic T lymphocytes (CTL) are known as $CD8^+$ T cells because they express CD8 glycoprotein on their surface. CTL destroy

infected cells or tumour cells by releasing cytotoxins perforin, granzymes and granulysin which enter the cytoplasm of the target cell and activates the caspase cascade which eventually leads to apoptosis (Marzo et al., 2000; Toes et al., 1999; Hung et al., 1998; Ossendorp et al., 1998). T regulatory cells are suppressive cells that maintain tolerance to self-antigens and prevent autoimmune diseases by downregulating effector T cells and shutting down the immune responses (Abbas, 2009; Golovina and Vonderheide, 2010; Zou, 2006; Penhale et al., 1973).

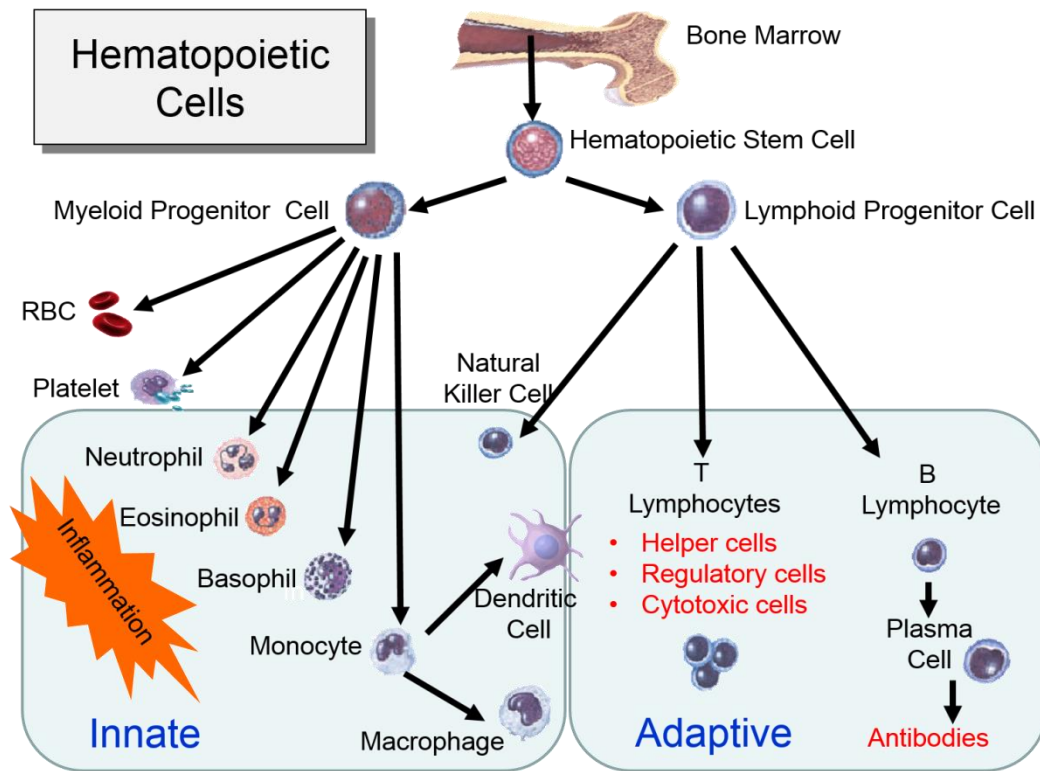


Figure 5: The derivation of the key cells involved in the innate and adaptive immune systems

All blood cells derived from hematopoietic stem cells that reside in the bone marrow. The hematopoietic stem cell divides into myeloid and lymphoid progenitor cells. The myeloid progenitor cell develops into different innate immune cells, which consist of neutrophil, eosinophil, basophil, monocyte, macrophages, dendritic cells, and natural killer (NK) cells. The lymphoid progenitor cell develops into adaptive immune cells, which consist of T and B lymphocytes. Source of image: (Alberts et al., 2002).

T cells need two signals to be activated. The first signal is when their TCR in association with CD3, a protein complex that consist of CD3 γ , CD3 σ , and two of CD3 ϵ chains, generate an activation signal by binding to a peptide antigen that is presented by the MHC class II on the surface of an APC (Ashwell and Klusner, 1990; Call et al., 2004). The second signal is by the co-stimulatory protein CD28, which is a receptor for CD80/CD86 (B7) membrane protein that found on activated APCs (Rudd et al., 2009; Balzano et al., 1992; King et al., 1996). These proteins provide signals for T cell activation, proliferation, differentiation, effector function and survival (Shahinian et al., 1993; King et al., 1996; Linsley and Ledbetter, 1993) (Figure 6). During the activation, T cells start to proliferate and secrete small proteins called cytokines which regulate or assist the activated immune response (Rincon and Flavell, 1994; Kalli et al., 1998; Karin and Ben-Neriah, 2000). Through these cytokines, cells can differentiate into several subtypes such as; T helper 1 (Th1), T helper 2 (Th2), T helper 3 (Th3), T helper 17 (Th17) and Treg cells (Jiang and Chess, 2006) (Figure 7).

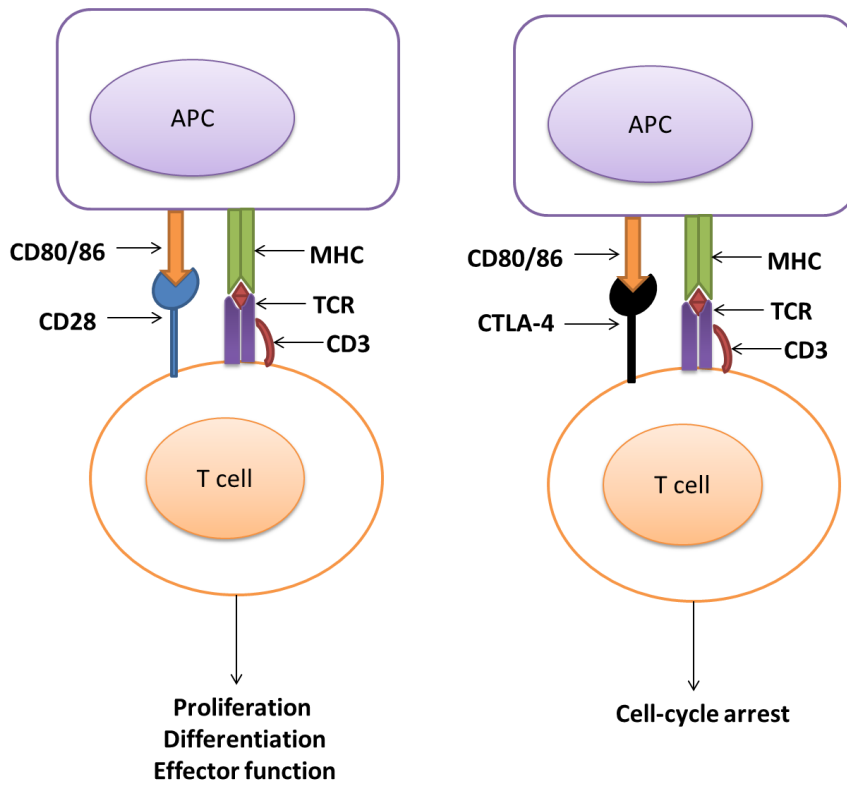


Figure 6: Mechanisms of rested and activated T cells

The first signal of T cell activation occurs when TCR in association with CD3 binds to a peptide antigen that is introduced by MHC class II on the surface of an APC, while the second signal is when CD28, expressed on T cell surface, binds to a CD80/CD86 receptor on the surface of an APC. To downregulate T cells and inhibits T cell activation, CTLA-4 binds to the CD80/CD86 receptor to block the cytokine production and the cell cycle progression (modified from Alegre et al., 2001).

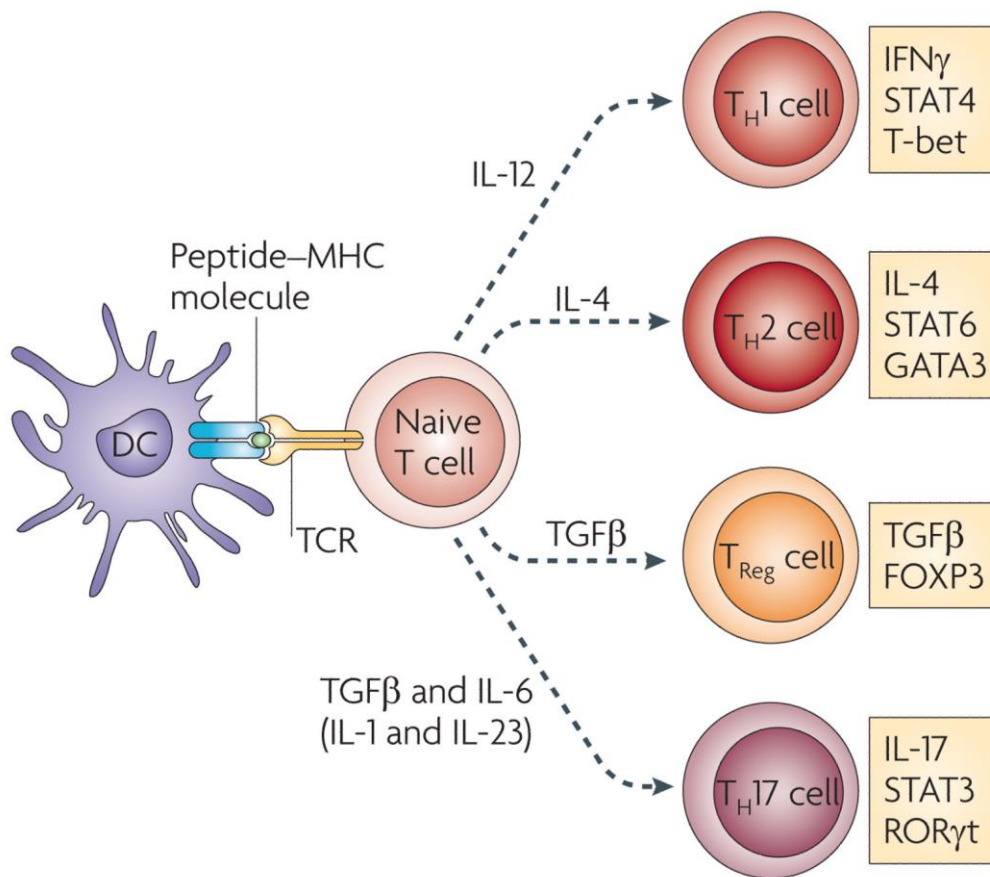


Figure 7: CD4⁺ T cells subsets

The naïve CD4⁺ T cells are activated by antigen presenting cells, such as dendritic cells (DCs). Depending on the various cytokine environment, they polarise into different effector T cell subsets; Th1, Th2, Th17 and Tregs. The differentiation of these effector T cell subsets is controlled by several transcription factors (Jiang and Chess, 2006). In the presence of TGF- β , the naïve CD4⁺ T cells convert to Tregs (Alegre et al., 2001). The figure is taken from (Zou and Restifo, 2010).

1.2.1 Introduction to T regulatory cell subsets

Gershon and Kondo (1970) discovered that lymphocytes could suppress T cell responses in an antigenic-specific manner. The types of T cells that can lead to an antigen-specific tolerance by attenuating T cell activity were named “suppressor cells”, and these cells are known to be a homeostatic immune-regulator (Gershon and Kondo, 1970). Penhale et al., (1973) supported the hypothesis that under normal circumstances the thymus plays a significant role in the prevention of autoimmune reactivity. In the presence of thymus-derived lymphocytes, several cells including B cells are prohibited from reacting against the self-antigens. Tregs are phenotypically distinct from other lymphocyte populations. Although numerous T cell populations have described as possessing suppressive activity, the T cell type that is well known as a regulator cell is the $CD4^{+}FoxP3^{+}CD25^{high}$ suppressive cell (Levings et al., 2001; Kuniyasu et al., 2000).

Different studies established that in the thymus, Tregs are primarily formed by high-avidity or intermediate selection of CD4 single positive thymocytes through MHC class II-dependent TCR interactions (Curotto de Lafaille et al., 2009; Bensinger et al., 2001). However, additional selection may take place, such as selective survival rather than induced differentiation (Van Santen et al., 2004) or the expression of the transcription factor autoimmune regulator (AIRE) in thymic medullary epithelial cells and monocyte-dendritic cells, which function in clone deletions and Tregs selection (Liston et al., 2003; Pitkanen and Peterson et al., 2003; Nomura and Sakaguchi, 2007). Loss of self-tolerance may lead to autoimmunity (Nishikawa and Sakaguchi, 2010). Therefore, Tregs control a range of immune cells that can function against tumour cells, allergens, pathogenic microbes, allogeneic transplant, and the foetus during pregnancy (Baecher-Allen and Anderson, 2006).

These different abilities indicate the various subsets of Tregs. As displayed below in Table 1, there are different Treg subsets with distinctive development, phenotype and functions. The main peripheral subpopulations of FoxP3⁺ Tregs are thymus-derived (tTregs) and periphery induced Tregs (pTregs), while there are other two subsets (Tr1 and Th3) of FoxP3⁻ pTregs. The majority of tTregs express CD4 and the minority of tTregs express CD8 on their surface. pTregs are involved in controlling immune response at the site of inflammation, particularly in the mucosal surfaces in peripheral regions (Yadav et al., 2013). Peripheral naïve CD4⁺CD25⁻FoxP3⁻ cells range from T regulatory 1 (Tr1) cells, which are induced by the anti-inflammatory cytokine IL-10 (Adeegbe and Nishikawa, 2013). Tr1 are IL-10-secreting cells that have the ability to secrete a high level of both IL-10 and TGF- β (Roncarolo et al., 2006). Another study demonstrated that Tr1 might play a role in low-dose oral tolerance (Tsuji et al., 2001). Further work indicated that the TGF- β -dependent T helper 3 (Th3) cells are also induced by low doses of oral antigen in antigen-specific-manners (Table 1) (Weiner et al., 2011).

Table 1: Regulatory T cells subsets and suppressive mechanisms.

Cell type	Origin	Phenotype	Suppressive mechanisms	References
CD4 tTregs	Thymus	CD4 ⁺ , CD25 ⁺ , FoxP3 ⁺ , CD127 ^{-/low} , CTLA-4 ⁺ , LAG-3 ⁺ , GITR ⁺	Cytotoxicity, IL-10, TGF-β, some require contact with other T cells to have effects.	(Sakaguchi, 2004)
CD8 tTregs	Thymus	CD8 ⁺ , CD25 ⁺ , FoxP3 ⁺ , CTLA-4 ⁺ , CD122 ⁺	Some secrete IL-10 or TGF-β, some require contact	(Fontenot et al., 2005a,b)
Induced CD4 pTreg	periphery	CD4 ⁺ , CD25 ⁺ , FoxP3 ⁺ , CTLA-4 ⁺ , GITR ⁺	Some require contact, requires IL-2 & TGF-β	(Apostolou and von Boehmer, 2004)
Tr1	periphery	CD4 ⁺ , CD25 ^{-/low} , FoxP3 ^{-/low}	IL-10	(Groux et al., 1997)
Th3	periphery	CD4 ⁺ , CD25 ⁺ , LAP ⁺ , FoxP3 ⁻	IL-10, TGF-β ^{High}	(Chen et al., 1994; Weiner et al., 2011)

Tregs subsets express a range of different phenotypes and suppressive mechanisms that can be utilised for their identification (modified from Mougiakakos et al., 2010).

1.2.2 The different suppressive mechanisms of T regulatory cells

Sakaguchi and his group in 1995 showed that the transfer of thymic CD25-depleted T cells induced autoimmune disease in thymic nude mice, while adding a small number of CD4⁺CD25⁺ T cells was sufficient to maintain tolerance (Sakaguchi et al., 1995). Accordingly, CD25 is expressed by “suppressive cells” that named as thymus-derived naturally occurring T regulatory cells. CD25 is the alpha chain of the high-affinity interleukin-2 receptor (IL-2R α) that is on the surface of activated cells (Chen et al., 2012). However, even though tTregs do not produce IL-2, they are mainly dependent on IL-2 production by their environment (Allan et al., 2005). The reason for that is IL-2 drives proliferation and clonal expansion of CD4⁺CD25⁺ Tregs (Nelson et al., 2004). To characterise and analyse the homogeneous population of tTregs in mice is usually through CD25 because tTregs expresses high levels of CD25 on its surface compared to other T cell subpopulations. However, it is relatively challenging in humans because according to Baecher-Allan (2004) and others; CD25 provide limited specificity, whose essential expression at variable levels can be noted in approximately 30% of the T cells and is further up-regulated in effector T cells upon stimulation (Baecher-Allan et al., 2004). Conversely, several studies suggest that Tregs proliferate or activate only in the presence of IL-2 (Kuniyasu et al., 2000). Since CD4⁺CD25⁺ Tregs are naturally anergic suppressive T cells (Kuniyasu et al., 2000), they do not proliferate unless IL-2 binds to the CD25 receptor on the surface of Tregs. Tregs were first defined by their high expression level of CD25 receptor and the intracellular transcription factor FoxP3 (Azuma et al., 2003; Baecher-Allan et al., 2004; Sakaguchi et al., 1995; Chen et al., 2012). Accordingly, the effector T cells secrete IL-2 to activate and proliferate. Therefore, since CD4⁺CD127⁻FoxP3⁺ Tregs express a high level of CD25, the

secreted IL-2 is taken up by them, while on the other hand, they suppress the activation and proliferation of the effector T cells (Carbone et al., 2013). In the presence of TGF- β , the majority of peripheral Tregs that generated from naïve CD4⁺FoxP3⁻ T cells will express FoxP3. In addition, IL-2 signals that secreted from the other PBMCs are critical to maintaining stable pTregs and tTregs levels by driving Tregs proliferation. FoxP3⁺ Tregs increase their proliferation level and regulate the production level of FoxP3⁻Helios⁻ T cells and Th cells (Chen et al., 2012; Sakaguchi et al., 1995; Zheng et al., 2004; Carbone et al., 2013). Tregs has been known as naturally anergic cells because of their incapacity to proliferate in response to T cells receptor ligation *in vitro*. However, many recent studies suggesting that a greater proliferative capacity is the thymus-derived CD4⁺CD25⁺ Tregs (Walker, 2004). Apparently, the proliferation level of CD25⁺ T cells is not run by the same process as that of CD25⁻ T cells. It appears that CD25⁺CD4⁺ T cells proliferate in the presence of IL-2 secretion. Blocking of IL-2 receptor on Treg cells leads to a loss of regulatory activity, which verifies that IL-2 is necessary to activate Tregs efficiently.

Genetic mutations can cause severe autoimmune disorders. Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome is a lethal syndrome first described as a unique entity by Powell et al. in (1982). Patients with IPEX syndrome have germline mutations resulting in a FoxP3 gene deletion on the X-chromosome (Bennett et al., 2001; Tanchot et al., 2012). Likewise, loss of function of FoxP3 in mice, either natural or recombinant, results in an analogous immune pathology due to a lack of Tregs (Khatteer et al., 2003). The dominant role of FoxP3 is further verified by the fact that in conventional CD4⁺CD25⁻ T helper cells, the gain of function induced by an

ectopic expression leads to the gaining of a suppressor function and the induction of an incomplete regulatory phenotype in human and mice (Khatteeri et al., 2003).

The FoxP3 gene encodes a transcription factor (TF) of the Forkhead box/winged helix family, and it counts as a master regulator of tTregs development and function. FoxP3 binds to specific regions of DNA to control the activity of genes that are involved in regulating the immune system (Ziegler et al., 2006).

More than one study have suggested that FoxP3 has a dominant role in the human system and it is suitable as bone fide marker of human Tregs (Ocklenburg et al., 2006; Allan et al., 2005; Roncarolo & Gregori, 2008). However, several observations supported the idea that more than FoxP3 is necessary for fully explaining the regulatory phenotype in human. Firstly, without interfering with the expression of effector cytokines such as IL-2 and IFN- γ , TCR stimulation of CD4⁺CD25⁻FoxP3⁻ T cells leads to FoxP3 induction (Gavin et al., 2006; Francois et al., 2009). Secondly, FoxP3 expression by both conventional CD4⁺CD25⁻ Th cells and Th lines and clones does not indeed indicate the acquisition of suppressor function (Francois et al., 2009). Thirdly, recent studies uncovered that demethylation of a conserved FoxP3 intronic region might be a greater marker for suppressor function than the further differential expression of FoxP3 at the protein level or mRNA in Tregs and Th cell clones (Stockis et al., 2009). Fourthly, ectopic expression of FoxP3 in human Th cells does not lead to the establishment of a stable regulatory phenotype (Probst-Kepper et al., 2009). Finally, although the enhancement of FoxP3 expression by TGF- β 1 in activated human CD4⁺CD25⁻ Th cells generates induced Tregs, this phenotype is rapidly lost (Probst-Kepper et al., 2009; Takaki et al., 2008). Altogether these

observations imply that more than FoxP3 is necessary for fully explaining the regulatory phenotype.

In the human system, it shows that FoxP3 is not expressed entirely in Tregs since it has also been found in FoxP3 effector T cells, particularly in conventional CD4⁺ T cells upon activation (Baron et al., 2007). However, FoxP3 expression in activated conventional T cells is only transient, unlike the sustained expression of FoxP3 in naturally occurring human Tregs, which require regulation on an epigenetic level (Baron et al., 2007). This investigation progress has led to a significant number of researchers being interested in investigating the function and the complexity of Tregs in different types of diseases including cancer.

Earlier studies have shown that FoxP3 suppresses the effector functions of T helper cells by undeviatingly hindering the activity of two key transcription factors, NF-AT and NF-kB, which are essential for cytokine gene expression and T cell function. The basal promoter of the FoxP3 gene contains one Activator Protein-1 (AP-1) and six NF-AT binding sites (Mantel et al., 2006). When FoxP3 interacts with NF and NF-kB, it usually represses IL-2, IL-4, and IFN- γ gene transcription (Bettelli et al., 2005). At the same time this complex is involved in the up-regulation of CD25, glucocorticoid-induced TNFR family related gene (GITR), and cytotoxic T-lymphocyte-associated Antigen-4 (CTLA-4) expression (Wu et al., 2006). GITR is a co-stimulatory molecule of T cell subsets, and it is a member of TNFR superfamily that is expressed in different cell types including T cells (Ronchetti et al., 2004). CTLA-4 is a protein receptor that down-regulates the immune system (Walunas et al., 1994). IL-2 has been recognised as the most important T cell growth factor to significantly expand Tregs (Nelson & Willerford., 1998), and also it is essential for their normal activity in vivo (Malek

et al., 2002). However, further investigation showed that CD4⁺CD25⁺ Tregs could expand, even without exogenous IL-2, when DCs are used as APCs (Yamazaki et al., 2013; Sakaguchi, 2004).

TGF- β is a homodimeric protein that is synthesised and secreted by many different cell types, including CD4⁺ Tregs. TGF- β is a cytokine that inhibits the proliferation and the effector function of T cells and the activation of macrophages (Tanchot et al., 2012); these inhibitory actions control the immune and the inflammatory responses (Abbas, 2009). Liu et al., (2007) have shown that proliferation of T cells and uncontrolled inflammation develops in mice when the gene encoding TGF- β is knocked out, or in which signalling is encoding TGF- β blocked. i.e. TGF- β signalling is critical to the thymic development of natural CD4⁺CD25⁺FoxP3⁺ Tregs and IL-2 is a principal driving force promoting the proliferation of natural CD4⁺CD25⁺FoxP3⁺ thymocytes that appear in the absence of TGF- β signalling (Liu and Wrong, 2007).

1.2.3 Thymus-derived naturally occurring T regulatory cells

It is now established that tTregs suppress activation and expansion of cells from adaptive as well as innate immunity, preventing cellular immune responses either by cell contact or soluble factor dependent mechanisms (Chen et al., 2006). During activation, the naïve CD4⁺FoxP3⁻ T cells convert to activated FoxP3⁺ conventional T cells and secrete poly-functional cytokines such as IL-2, IFN- γ and tumour necrosis factor- α (TNF- α) in contrast to tTregs that secrete suppressive cytokines such as IL-10 and TGF- β (Kryczek et al., 2009). Therefore, it was suggested that the best method to identify tTregs is to combine FoxP3⁺ Tregs with these intracellular cytokines staining (Kryczek et al., 2009).

The CD4⁺CD25⁺FoxP3⁺ tTregs possess a potent suppressive activity against both naïve and memory CD4⁺ and CD8⁺ T cell activation, proliferation and function (Levings et al., 2001; Piccirillo and Shevach, 2001). A previous study by Lim et al., (2005) reported that without the need to first suppress Th cells, Tregs could also directly suppress B cell response. FoxP3⁺ tTregs can directly suppress B cell immunoglobulin (Ig) response, partly mediated by TGF- β secretion. This direct suppression of the Ig production by tTregs is followed by inhibition of Ig class switch recombination (Lim et al., 2005; Nakamura et al., 2004).

Furthermore, tTregs that express the membrane-bound TGF- β are also able to directly inhibit the effector function of natural killer cells as well as the function and maturation of DCs (Azuma et al., 2003; Ghiringhelli et al., 2005a; Misra et al., 2004). The mechanism behind T cell suppression, *in vitro*, appears to be IL-10 and TGF- β independent and does not require the presence of APCs but it requires cell contact between Tregs and responder T cells (Tiemessen et al., 2007). On the other hand, to inhibit T cell activation *in vivo*, in the presence of IL-10 and TGF- β , Tregs can form direct interactions with DCs in the lymph nodes. In this manner, this will prevent stable contacts between the APC and responder CD4⁺ T cells (Tiemessen et al., 2007; Yamazaki and Morita, 2013).

1.2.4 Peripherally-induced T regulatory cells

The peripherally induced Tregs (pTregs) are responsible for governing the immune response to a variety of microbial and tissue antigens. They develop outside the thymus in the peripheral lymphoid tissue and progress under sub-immunogenic antigen presentation, during normal homeostasis of the gut, and during chronic inflammation (Curotto de Lafaille et al., 2008). pTregs that arise in the periphery from CD4⁺FoxP3⁻ conventional T cells can generate in vitro (Figure 8) (Schmitt and Williams, 2013; Curotto de Lafaille and Lafaille, 2009). In the presence of different immunosuppressive cytokines including TGF- β , the naïve CD4⁺ T cells converted into peripheral induced CD4⁺FOXP3⁺ Tregs (Vitali et al., 2012). Multiple signalling pathways congregate to influence the efficiency of pTreg generation. Specific TCR affinity and TCR-derived signals, co-stimulatory molecules, and cytokines promote optimal development of pTregs in vivo. Furthermore, high levels of IL-2, IL-10 and/or TGF- β promote the generation of pTregs by creating a decreased aggregate TCR stimulation as compared to conventional T cells (Kretschmer et al., 2005; Gottschalk et al., 2010). An essential regulator of Tregs is the CD28 receptor, a strong and dominant co-stimulatory molecule for T cell activation. A lack of CD28 leads to the development of more rapid and severe autoimmune diseases. Recent studies have documented that CD28 is essential for tTregs development in the thymus and tTregs survival and homeostasis in the periphery (Guo et al., 2008). For the pTregs on the other hand, CD28 co-stimulation required for the generation of pTregs from naïve CD4⁺CD25⁻ T cells through the production of IL-2, where high levels of CD28 co-stimulation suppresses the generation of pTregs from naïve CD4⁺ T cells and promote the expansion of effector T cells (Semple et al., 2011). Conversely, CTLA-4 inhibits T cell activation by blocking cytokine

production and cell cycle progression. Therefore, CTLA-4 co-stimulation is necessary for generating phenotypically and functionally similar adaptive CD4⁺CD25⁺ suppressor cells (Zheng et al., 2006). Peripheral naïve CD4⁺CD25⁻ FoxP3⁻ T cells range from T regulatory 1 (Tr1) and T helper 3 (Th3) cells. Tr1 secrete IL-10 and TGF- β (Groux et al., 1997) and are induced by the anti-inflammatory cytokine IL-10 (Roncarolo et al., 2006) to suppress immune and autoimmune responses (Groux et al., 1997). Another cytokine that identified as one of the important cytokines that promote the generation of Tr1 cells is the Interleukin-27 (IL-27), which is a member of the IL-12 heterodimeric cytokine family and it is also known as effector Th17 suppressor cytokine (Pot et al., 2011). Tolerogenic DCs conditioned in vitro or in vivo by FoxP3⁺ Tregs secrete IL-27 that was initially described as a pro-inflammatory cytokine that induces proliferation of naïve CD4⁺ T cells to induce Th1 cells responses (Takeda et al., 2003; Pflanz et al., 2002; Chen et al., 2000). Furthermore, during Tr1 differentiation, IL-27 induces the expression of Granzyme-B, which is a serine protease that through the Granzyme-B-mediated lysis, in a contact-dependent manner, Tr1 cells suppress the effector T cells responses (Pot et al., 2011) (Figure 9).

Tr1 cells regulate the adaptive immune responses that attack the commensal organisms and promote tolerance in the gut. Tr1 cells modulate immune responses in vivo, to avoid autoimmunity, transplant rejection, and chronic inflammatory diseases (Roncarolo et al., 2006), whereas Th3 cells are in the context of oral tolerance. The TGF- β -dependent Th3 cells are induced by low doses of oral antigen in an antigen-specific-manner. These cells express LAP on their surface (Weiner et al., 2011), and become converted to FoxP3 expressing cells (Bilate and Lafaille, 2012). Several studies have demonstrated that oral

antigens can induce CD4⁺CD25⁺ Tregs (Zhang et al., 2001; Thorstenson et al., 2001). Th3 cells can be distinguished from Th1 and Th2 cells by their secretion of TGF- β (Weiner et al., 2011). Naïve Th3 cells express CD4 and LAP on their surface, and they are generated in the peripheral immune compartment and are activated by TCR signalling in the gut by oral antigens (Figure 10). The activation of these cells in the gut enhances their regulatory properties and initiates a regulatory cascade (Weiner et al., 2011). Furthermore, the secreted TGF- β from Th3 cells maintains thymus-derived naturally occurring CD4⁺CD25⁺FoxP3⁺ Tregs to suppress T helper 1 (Th1) and T helper 2 (Th2) responses. The activated Th3 cells in the gut are able to suppress systemic autoimmune and inflammatory responses. Moreover, the TGF- β secreted from Th3 cells can also affect CD4⁺FoxP3⁻ T cells and converts them to FoxP3⁺CD25⁺LAP⁻ peripheral induced Tregs (Figure 10) (Weiner et al., 2011).

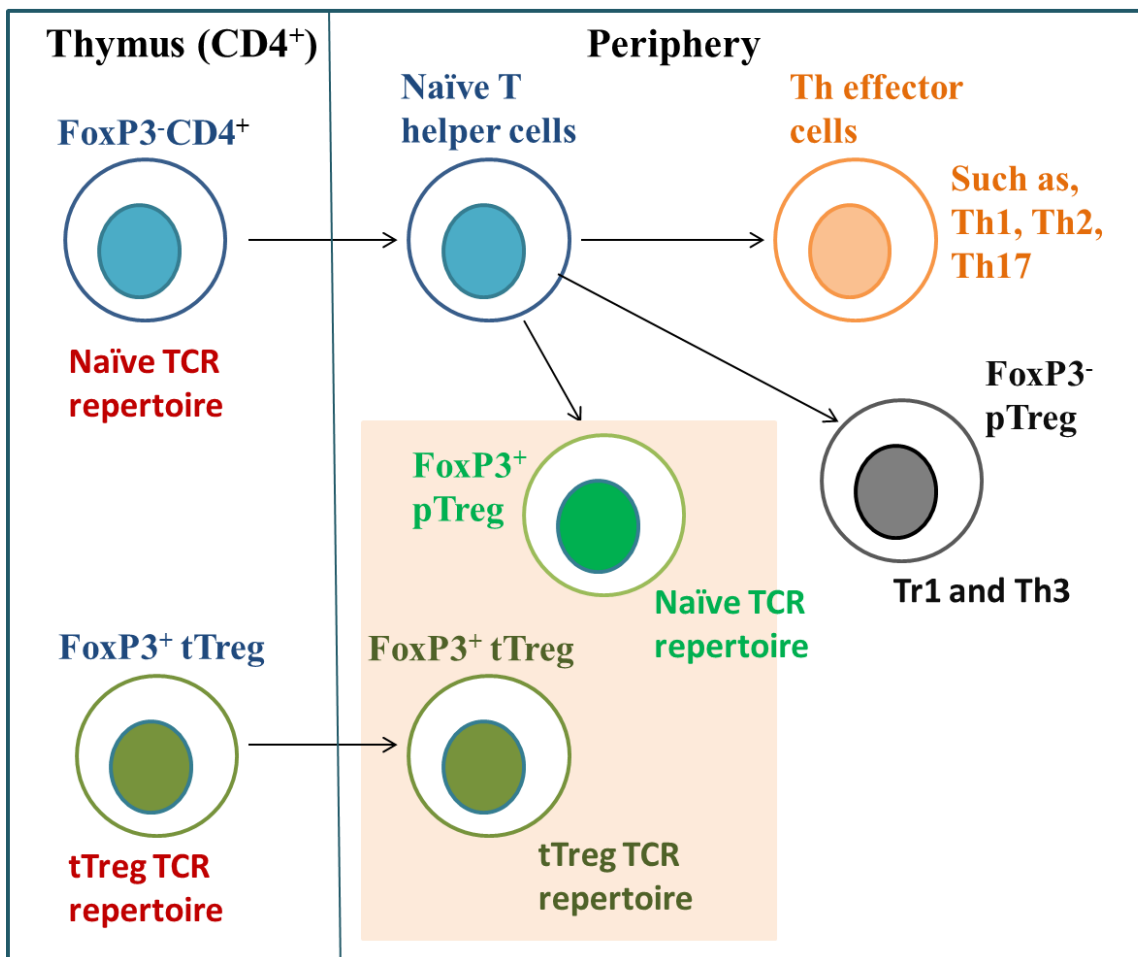


Figure 8: Thymic and Peripheral generation of FoxP3⁺ Tregs

Naïve FoxP3⁻CD4⁺ T cells migrate from thymus to the periphery and become naïve T helper cells that differentiate into T effector cells, such as, T helper 1, T helper 2, and T helper 17. It also differentiates into FoxP3⁻ pTregs, such as Tr1 and Th3, and FoxP3⁺ pTregs. Adaptive FoxP3⁺ Tregs differentiate into secondary lymphoid organs and tissues. Natural FoxP3⁺ Tregs differentiate in the thymus and migrate to peripheral tissue (modified from Curotto de Lafaille & Lafaille, 2009).

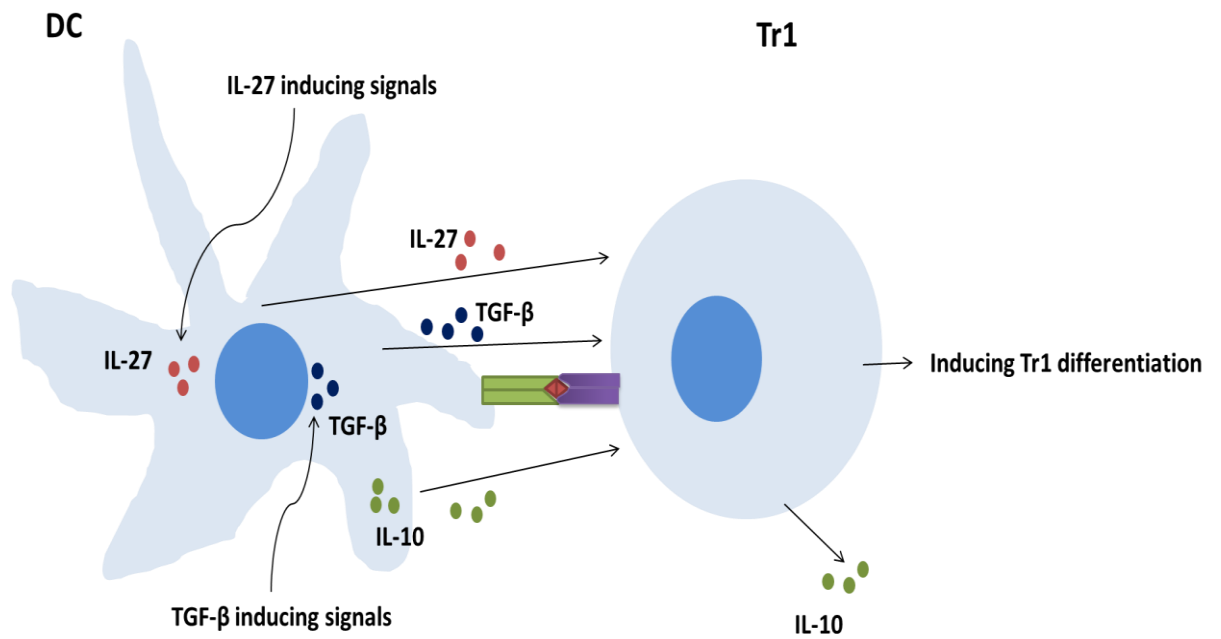


Figure 9: Dendritic cells induce Tr1 differentiation

DC is highly specialised in driving differentiation of Tr1 cells by secreting elevated level of IL-27, IL-10, and TGF- β cytokines (Modified from Kushwah and Hu).

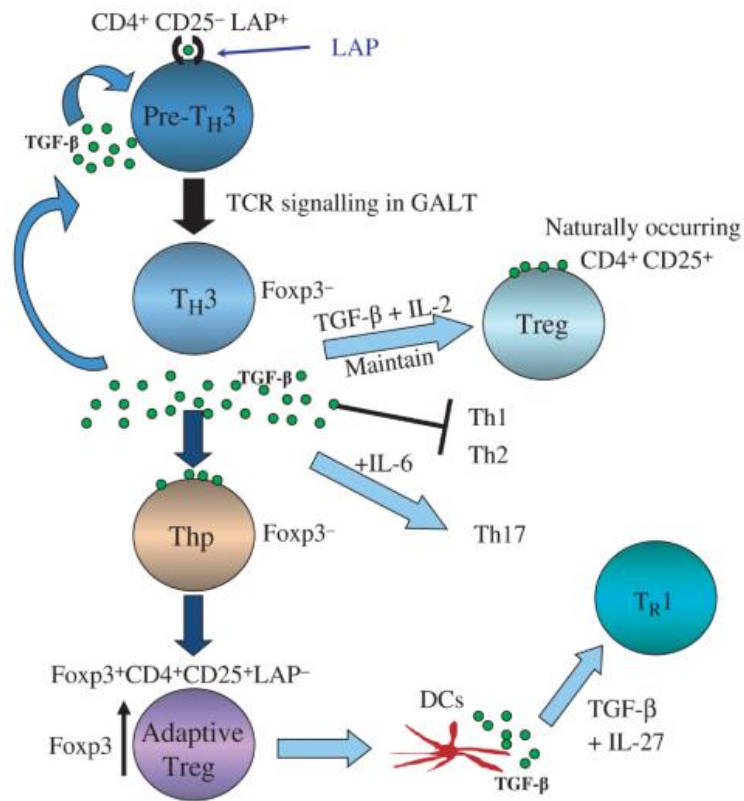


Figure 10: Tregs cascade following induction of Th3 type Tregs by oral antigens or oral anti-CD3

This figure illustrates the development of CD4⁺CD25⁺FoxP3⁺LAP⁺ Th3 cells in the peripheral immune compartment that are activated by TCR signalling in the gut by oral antigen. LAP receptor on the surface of Pre-Th3 cells pick up the TGF-β cytokines that are secreted by Th3 cells. Pre-Th3 cells send signals to stimulate Th3 cells to produce more TGF-β to maintain naturally occurring CD4⁺CD25⁺FoxP3⁺Treg and suppress Th1 and Th2 responses, and in the presence of IL-6, it might induce Th17 responses. TGF-β secreted from Th3 cells can also act on CD4⁺FoxP3⁻ Thp cells and converts them to FoxP3⁺CD25⁺LAP⁻ peripherally induced Tregs. pTregs activate DCs to secrete TGF-β and IL-27 to promote the generation of Tr1 cells (taken from Weiner et al., 2011).

1.2.5 The difficulty in distinguishing tTregs from pTregs

Although numerous of molecules including CD28, TNFR, PD-1, and Toll-like receptors (TLRs) expressed on the surface of Tregs, these markers are not helpful in distinguishing tTregs from pTregs. Based on tTregs and pTregs similar surface markers it is hard to discriminate these two cell populations openly. Therefore, many studies tried to separate tTregs from pTregs based on molecular biology approaches instead. For example, a study by Floess et al. demonstrated that tTregs exhibit a completely demethylated FoxP3 locus, while pTregs despite their high FoxP3 expression display an incomplete DNA demethylation that is associated with unstable regulatory T cell phenotype (Floess et al., 2007). There is lots of lineage plasticity develops in these Treg subpopulations. For that reason, other studies suggesting that understanding the differentiation mechanisms of tTregs and pTregs will determine these cells commitment to lineage and plasticity towards other phenotypes (Komatsu et al., 2009; Povoleri et al., 2013).

Indeed, additional markers are required to determine tTregs from pTregs accurately. Thornton et al., 2010 demonstrated that expression of Helios could differentiate tTregs from pTregs. Accordingly, CD4⁺FoxP3⁺ tTreg cells express a high level of Helios, while the pTregs express a low level of Helios (Thornton et al., 2010). Few studies, on the other end of the spectrum, however, have revealed that Nrp-1 can distinguish tTreg from pTreg. Their studies showed that pTregs produced a low level of Nrp-1 unlike tTregs that expressed a high level of Nrp-1 (Weiss et al., 2012; Yadav et al., 2012). Conversely, a growing body of knowledge is now questioning the reliability of Helios and Nrp-1 as markers that are cable to distinguish these two subpopulations from each other (Szurek et al., 2015). Referring to Szurek et al., 2015, after observing the genetically modified mouse strain that defines tTregs and pTregs formation and analysing the TCR

repertoire of pTreg and tTreg cells, the study demonstrated that Tregs expressed a variable level of Helios and Nrp-1 both in tTreg and pTreg and therefore it could not support the nature differentiation of tTreg and pTreg.

Another finding that supports Szurek studies is by Gottschalk et al., 2012 which also demonstrated that Helios expression could be in pTregs both *in vivo* and *in vitro*. Accordingly, *in vitro*, Helios expression in pTreg was dependent on the presence of APCs.

An additional study using human experiments indicated that ~30–40% of naïve Foxp3⁺ Tregs in the peripheral blood were found to be Helios⁻. This data represent that tTregs could be Helios⁻ and the absent of Helios is not a reliable marker for pTregs (Himmel et al., 2013).

So far, many studies have determined the origin of specific Treg clones by sequencing their TCRs and analysing TCR repertoire by cellular phenotype with a fixed TCRbeta-chain (Lathrop et al., 2008). The high frequency of TCR is derived from CD25⁺CD4⁺ T cells and not from CD25⁻CD4⁺ T cells (Hsieh et al., 2004). Furthermore, FoxP3⁺CD4⁺ pTregs expressed different TCRs than FoxP3⁻CD4⁺ medullary thymocytes and FoxP3⁻CD4⁺ T cells (Pacholczyk et al., 2006).

A further finding that could improve our understanding of tTreg and pTreg is the GARP/LAP expression. Other studies have shown that GARP and LAP are additional markers that could selectively identify activated Tregs with highly potent suppressive function (Wang et al., 2009; Tran et al., 2009). Accordingly, LAP can distinguish activated Tregs from activated FoxP3⁻ and FoxP3⁺ non-Tregs (Tran et al., 2009). Moreover, in the presence of TGF- β , GARP was not induced by activated T cell, and the expression of FoxP3 in conventional T cells did not induce GARP expression but silencing GARP in Tregs reduced their suppressive activity (Wang et al., 2009). Based on these observations this study

has also demonstrated that high GARP expression can distinguish Tregs from Th17 lineage (Wang et al., 2009). Of note, further studies have proved that iTregs are not capable of expressing GARP or LAP (Tran, 2012). A study by Zhou et al. illustrated that forced expression of GARP on all T cells in GARP-transgenic mice caused a decrease in Tregs level in the thymus and periphery (Zhou et al., 2013). The cytokine TGF- β is important for inducing Th17 and pTreg, while the lack of GARP expression on Tregs surface did not compromise their suppressive function. However, IL-1 receptors are better expressed on activated Tregs but not on pTregs (Tran et al., 2009; Mercer et al., 2010). Another interesting study demonstrated that contrary to the human studies, mouse iTreg and pTreg could express GARP (Edward et al., 2013).

1.2.6 CD8⁺ T regulatory cells

There are some studies proves the presence and significance of CD8⁺ Tregs in both rodent transplant recipients and human, likewise in autoimmune disease models. Gershon and Kondo in the 1970s described CD8⁺ Tregs as the first subset of cells capable of inducing immune suppression (Gershon and Kondo, 1970). Previously, there were difficulties to find potential markers to characterise the CD8⁺ T cells, but currently, this concept has re-emerged. The knowledge of understanding the phenotypic and functional characterisation of CD8⁺ Tregs has established, and now it is less challengeable to define potential markers because of the new more accurate machines, developed techniques, and biomarkers (Gilliet and Liu, 2002). So far, the well-known markers that describe CD8⁺ Tregs are CD28⁻, CD45RC^{low} and CTLA-4⁺ (Zhang et al., 2015). However, these

markers indicate only the activation and the memory state and not the regulatory function of CD8⁺ T cells.

An interesting marker for detecting the alloantigen-Induced CD8⁺ Tregs is the CD103 (Uss et al., 2006). Few studies have documented the expression of CD103 on CD4⁺ Tregs, but approximately 4% of circulating CD8⁺ T cells expresses the CD103 (Uss et al., 2006). The $\alpha E\beta 7$ integrin CD103 are expressed in both murine and human CD8⁺ T cells which were found in the intestine, Bronchoalveolar fluid, and allograft tissue (Pauls et al., 2001). Human Tregs can be induced by stimulation with alloantigen in vitro. CD8⁺CD103⁺ T cells secrete IL-10 rather than IFN- γ and maintain their phenotype after re-stimulation with alloantigen. CD8⁺ Tregs directly lysis the effector T cells via perforin and immunosuppressive cytokine secretion (Hu et al., 2004). CD8⁺ Tregs are reported to mediate antigen-specific suppression by producing IL-10 and/ or TGF- β and/or by an immediate inhibitory action on dendritic cells (Chang et al., 2002). Alongside, the secretion of IL-10 and TGF- β , the suppression mechanism of CD8⁺ Treg also includes cell-cell contact. CD8⁺ Tregs target T-helper cells through a Qa-1/peptide-TCR interaction (Kim et al., 2010). Qa-1 is a MHC Class Ib molecule expressed on effector T cells and binds to TCR on CD8⁺ Tregs. Accordingly, the interaction of CD8⁺ Tregs with effector T cells limit the immune responses and a study by Kim et al. reported that impaired expression of Helios by FoxP3⁺CD4⁺ Tregs and Qa-1-restricted CD8⁺ Tregs defect the regulatory activity in mice (Kim et al., 2015). Therefore, Qa-1-restricted CD8⁺ Tregs is a unique regulatory subset that can limit the autoimmune diseases, and in Qa-1 knockout mice, CD8⁺ Tregs will be inactivated, causing autoimmune diseases (Hu et al., 2004). Qa-1 is an interesting molecule because it can bind β -2 globulin, such as classical MHC Class Ia molecule, and can also present a range of peptides (Sarantopoulos et al., 2004).

Referring to the same study, Kim et al., (2015) have further analysed Helios deficiency on CD8⁺ Treg responses. The study reported that Helios-deficient CD8⁺ Tregs failed to suppress the T follicular helper cells, and it is also developed an unstable phenotype during inflammatory responses resulted in reduced FoxP3 expression and increased effector cytokine expression, including IFN- γ , IL-17, and TNF- α .

Another study reported the response of human and mouse CD8⁺CD25⁺FoxP3⁺ Tregs at steady state and during IL-2 therapy (Churlaud et al., 2015). As a result, the study demonstrated that CD8⁺ Tregs express constitutively CD25, and in vitro, they are more sensitive to IL-2 activation than CD8⁺ effector T cells. Likewise, on one end of the spectrum, the result shows that CD8⁺FoxP3⁺CD25⁺ Tregs share the same phenotypic and functional suppressive activity with thymic CD4⁺ Tregs (Churlaud et al., 2015). On the other end, however, there are still differences in the biology of CD8⁺ Tregs and CD4⁺ Tregs. IL-2 cytokine binds to the high affinity trimeric IL-2 receptor (IL-2R) or to the low-affinity dimeric IL-2R. Both CD4⁺ and CD8⁺ Tregs are constitutively expressing the trimeric IL-2R on their surface, but CD8⁺ Tregs express a lower amount of CD25 on its surface compared to CD4⁺ Tregs (Boyman and Sprent, 2012; Churlaud et al., 2015).

Given all these studies, it is interesting to investigate the expression of GARP and LAP not only on CD4⁺FoxP3^{+/-}Helios^{+/-} Tregs but also on CD8⁺FoxP3^{+/-}Helios^{+/-} Tregs.

1.3 Transplantation tolerance and the Immunosuppressive Drugs

Any foreign agents that enter the body will cause a reaction either in the innate and/or adaptive immune system. Transferring cell tissues or organ from one site to another site in the body calls transformation, and in the most cases, transplanted organ (e.g. liver, lung, heart, kidney, or pancreas) from a donor can cause the immune system to develop an elaborate efficient mechanism against the foreign transplanted organ. Immunological rejection happens in all recipients except identical twins because they have the same DNA and their MHC is almost same. The rejection involved in both cell-mediated and antibody-mediated hypersensitive reactions directed against MHC on the foreign graft (LaRosa et al., 2007). There are two types of immunological rejection pathways, direct and indirect pathways. The direct pathways are when the alloreactive T cells such as $CD8^+$ CTL recognise the donor MHC molecule, which expressed on the surface of APCs from the transplanted tissue and destroys the foreign APCs and the transplanted cells (LaRosa et al., 2007). In the indirect pathways, the recipient's APCs introduce the graft cells antigen to the $CD4^+$ T helper cells that secrete $IFN-\gamma$ cytokines for activating the macrophages and destroying the transplanted tissue. Furthermore, the foreign antigen that the host APCs introduce is also able to activate the alloreactive B cells to produce antibodies to work as flags for destruction (LaRosa et al., 2007). Both direct and indirect pathways cause endothelitis (inflammation of the endothelial), cascade process of coagulation and blood vessels inflammation which leads to thrombosis. The cytokines that the alloreactive T cells releases will destroy the other host cells, causing lack of blood flowing to the area, the recipient will get graft ischemia until the graft dies (Novick et al., 1999).

Allograft rejection is the biggest problem that the recipient can face and to prevent this issue the transplant recipients require long-term immunosuppression. However, the consequences of lifelong immunosuppression lead to vulnerable infection, a significant risk of malignancy, and increased mortality and morbidity (Waldmann, 2010). Therefore, the primary goal of transplantation is the induction of tolerance and at the same time avoiding intense long-term immunosuppression to maintain the active immunity against cancers and viral infections (Li and Turka, 2010). Referring to Li and Turka, (2010), FoxP3⁺ Tregs are the key players for the induction of transplantation tolerance. The activation of calcineurin phosphate is critical to the development of Tregs, in contrast to cyclosporine and tacrolimus (FK506), which are immunosuppressive drugs that inhibit calcineurin phosphate activity and leads to a reduction in Tregs generation or Treg-mediated tolerance (Schwaninger et al., 1993). Moreover, these drugs also impact the expansion and survival of FoxP3⁺ Tregs in the periphery by inhibiting IL-2 secretion and NF-AT expression. A study by Kawahara et al. demonstrated that cyclosporine A and FK506 could be used as a treatment for decreasing tumour growth in xenograft-bearing mice. Referring to the same study, bladder cancer lines treated with cyclosporine A and FK506 actively suppressed the tumour cell growth in cancer bladder (Kawahara et al., 2015). However, with the organ transplantation, Tregs are required for tolerance and cyclosporine A and FK506 inhibit the tolerance induction. Therefore, rapamycin is an mTOR-inhibitor, which promotes Tregs induction and Treg-mediated suppression (Battaglia et al., 2005). Furthermore, in the presence of rapamycin, both the apoptosis of effector T cells and the expansion of FoxP3⁺ Tregs increase (Battaglia et al., 2005). Rapamycin and cyclosporine have a unique effect on allograft tolerance (Coenen et al., 2006).

B cells have also been studied for the transplantation tolerance, and several studies demonstrated that the mechanism of B cells plays a prominent role in the induction of B cell tolerance and the antibodies can use as therapies for treating antibody-mediated graft rejection (Nicole and Turgeon, 2010).

Several experiments have confirmed Tregs role in peripheral tolerance and cancer. Achieving balance between transplantation tolerance and cancer is important. Therefore, Tregs therapy in allograft addresses multiple issues such as, the number of Tregs and their efficacy are important to obtain a therapeutic effect (Savage et al., 2013; Hanahan and Weinberg, 2011), the specificity of the antigen is necessary for effective control of rejection, a control of Tregs function and effector T cell/Treg ratios combination is a good strategy to target cancer and target specific Treg subsets to prevent tumour (Elkord et al., 2011).

In conclusion, understanding the immune tolerance will simplify Tregs mechanism and function. Finding appropriate drugs to either enhancing or inhibiting Tregs development will help with the transplantation tolerance and treat cancer cells.

1.4 T regulatory cells in cancer

CD4⁺FoxP3⁺ Tregs control many facets of the immune response reaching from autoimmune disorders to inflammatory diseases and cancer in an attempt to maintain immune homeostasis (Adeegbe and Nishikawa, 2013). Tregs suppressive activity is beneficial by limiting the T cell response against self-antigens to prevent autoimmune diseases and allergies. Considerable progress has been made in understanding the phenotype, function, and mechanism of action of Tregs, and various subclasses of these cells have been defined (Adeegbe and Nishikawa, 2013). However, their inhibitory roles in curtailing immune response against pseudo-self-antigens as in tumours often culminate into negative outcomes (Adeegbe and Nishikawa, 2013). There are plenty of studies highlighting the elevated levels of Tregs in the tumour and/or in the circulation in human cancer patients (Whiteside et al., 2012) and it often correlates with poor antitumour effector response and enhanced tumour immunity (Elkord et al., 2010; Nishikawa and Sakaguchi, 2010). During persistent tumour burden and poor antitumour effector response, an accumulation of Tregs occurs at peripheral sites (spleen, peripheral blood) and in the local tumour microenvironment (Elkord et al., 2010; Nishikawa and Sakaguchi, 2010). Since Tregs increased in the peripheral blood and the tumour tissue of cancer patients, there are several lines of evidence suggesting that these tumour-infiltrating Tregs must play a significant role in dampening anti-tumour immunity (Adeegbe and Nishikawa, 2013).

Tregs in the tumour microenvironment is highly heterogeneous populations that arise through different pathways and mediate immunologic effect through various means including soluble factors and contact-dependent mechanisms (Zou, 2006). The mechanisms mediating Tregs expansion are tumour specific factors that have multiple functions such as; control of inflammation, differentiation, proliferation,

enhance the reaction to autoimmunity, regulate the recruitment or reduced death (Zou et al., 2006). These many factors that tumour cells can secrete are induced Tregs and attract them locally (Zou, 2006). In vitro, there are several soluble or contact-dependent tumour factors contributing to Treg generation including cyclooxygenase-2, CD70, GaII, TGF- β , and yet-to-be-identified factors (Bergman et al., 2007; Yang et al., 2007; Juszczynski et al., 2007; Liu et al., 2007; Li et al., 2007; Curti et al., 2007). These tumour micro-environmental factors are a reason for increased level of Tregs and improved their function. Not to mention, enhanced Tregs proliferation or reduced their apoptosis is an additional mechanism that could increase Tregs in the tumour. For instance, with the contribution of the indoleamine 2,3-dioxygenase enzyme, the plasmacytoid dendritic cells from mouse tumour-draining lymph node can directly activate Tregs (Sharma et al., 2007). Since the suppression by indoleamine 2,3-dioxygenase-activated Tregs requires the PD-1 ligand pathway, Tregs that have PD-1 suppressive mechanism various from Tregs that activates without indoleamine 2,3-dioxygenase (Sharma et al., 2007). Further functions of Tregs that assist tumour cells to survive are that Tregs not only induce macrophages to express inhibitory B7-H4 on its surface, which is a protein that negatively regulates T cell immunity (Sica et al., 2003) but also secretes IL-6 and IL-10 to demonstrate dysfunctional immune cell cross-talk (Kryczek et al., 2006).

There are several possibilities of the composition of FoxP3⁺Tregs within the tumour and/or circulation in human cancer patients; (i), they are tTregs recruited to the tumour site depending on tumour-mediated CCL22 chemokine production and gradient (Curiel et al., 2004). Similarly, another study demonstrated that, in response to TGF- β , tTregs underwent substantial proliferation at the tumour site and drain lymph node (Ghiringhelli et al., 2005b); (ii), they are peripheral

induced Tregs derived from converted CD25⁻ T cells (Liu et al., 2007); and (iii) they are Tr1 cells. Both tTregs and pTregs contribute to tumour tolerance (Zou, 2006), tTregs could increase in the tumour to impede anti-tumour immunity and mediate peripheral self-tolerance, whereas pTregs could increase to dampen ongoing inflammation (Zou, 2006). Tumour Tregs are phenotypically indistinguishable from other Tregs because they likely possess specific characteristics given by the tumour microenvironment. A similar population was lately reported in human cervical cancer (Loddenkemper et al., 2009). However, at this time our understanding and knowledge on these different Tregs subsets are limited, especially in cancer patients. The challenge is to detect the mechanisms and the various markers for these Tregs with the intention of targeting the unbalanced Tregs in cancer patients. Lately, studies suggesting that depending on the tumour microenvironment, there may be several ways to target Tregs in cancer patients. For instance, there have been great strategies to deplete Tregs by targeting the CD25 receptor with monoclonal antibodies (e.g. Daclizumab) or ligand-directed toxins (e.g. Ontak) (Rech and Vonderheide, 2009). Other studies are suggesting that suppression of Tregs function remain by using the anti-CTLA-4 antibodies ipilimumab to activate the effector T cells rather than Tregs modulation (Khan et al., 2011). In this study, investigating GARP/LAP elevated levels on FoxP3⁺ Helios⁺ Tregs subset will give further and wider knowledge on these different Treg subsets, and it will demonstrate further options for disrupting Tregs function.

1.4.1 Depletion of Tregs to improve the treatments of cancer

The type of cancer treatment depends on the kind of cancer that the patients have and how advanced it is because there are many types of cancer treatment. Metastatic cancer may be treated with local therapy (surgery, radiation therapy), systemic therapy (chemotherapy, biological therapy, targeted therapy, hormonal therapy, immunotherapy), or a combination of them. Some people with cancer will have only one treatment, but most people have a combination of treatments. The decision of therapy depends on the type of primary cancer, the size and location, metastatic tumours, the patient's general health and age, and the types of treatment the patient have had in the past.

It has reported that by decreasing Tregs level in cancer patients, the efficacy of therapeutic vaccination for cancer could enhance. The vaccine induces antigen-specific effector cells and Tregs are also antigen-specific cells. For example, a cervical cancer vaccine induces CD4⁺CD25⁺FoxP3⁺ in human, but their function and specificity were not assessed (Welters et al., 2008). To generate antigen-specific effector cells while limiting the generation of antigen-specific Tregs is critical to optimal immunotherapy strategies. Further similar strategies, including CD137 sFv-expression tumour cells (Yang et al., 2007), CD40 agonists plus Toll-like receptor activation (Ahonen et al., 2008), a liposomal vaccine (Chen et al., 2008), and specific dendritic cells (Palucka et al., 2007), have been also tested in several groups.

Cytokines are additional effective therapeutic targets in disease, the importance of cytokines as a critical therapeutic issue has been rapidly growing field with significant pharmaceutical impact (Feldmann, 2008). As cytokines are potent

rate-limiting extracellular molecules, they are excellent targets for the products of the biotechnology industry, such as monoclonal antibodies and antibody-like receptor: Fc fusion proteins. The first big success of anti-cytokine therapy in the form of TNF- α blockade demonstrated in the autoimmune disease Rheumatoid Arthritis (RA). Blocking this single cytokine has marked beneficial effects on all aspects of disease activity and can prevent further joint destruction (Elliott, 1993; Feldmann, 2001). Furthermore, in the control of leukemic cell growth, Smith and Griffin explore the role of cytokines and cytokine receptors (Smith and Griffin, 2008) chronologically. Previous observations and studies emerged assays for T cell growth factors (Gillis et al., 1978), the identification of its receptor, the purification of protein with growth factor activity, and the production of an antibody specific for the receptor (Leonard et al., 1982). These methods helped provide a new framework for lymphocyte growth as a response to antigen-induced growth factor release (Smith et al., 1980). In a mouse sarcoma model, Flt3-L and GM-CSF induced dysfunctional FoxP3⁺ cells (Berhanu et al., 2007). Tregs decrease the advantages of IL-12 gene therapy in a mouse model of hepatocellular carcinoma (Zabala et al., 2007). Systemic IL-2 changes Treg trafficking molecules and may enhance their accumulation in ovarian malignancies in humans (Wei et al., 2007).

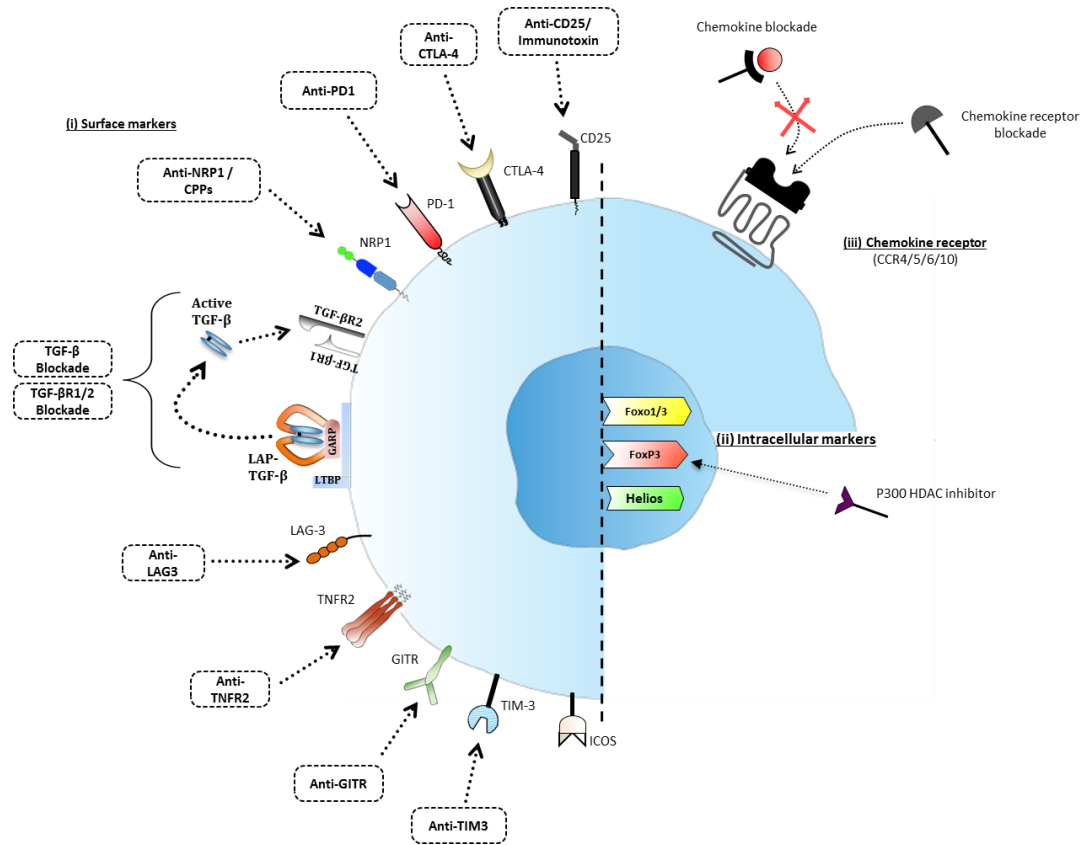
Histone deacetylase inhibitors enhance Treg suppressive functions, and this effect might be enriched with IL-2 (Tao et al., 2007). Many additional unexpected effects on Tregs of drugs used for different indications have been addressed (Ruter et al., 2007). It will be important to review the mechanism of action of many anti-cancer agents, including tumour vaccines, tyrosine kinase inhibitors, and anti-angiogenesis agents, in light of their potential to modulate Treg activity.

Reducing Treg function is a logical therapeutic strategy given the large body of evidence suggesting that elevated Tregs are detrimental in cancer. Tregs arise and function in cancer as depletion; blocking differentiation, trafficking or effector functions; or raising effector cell suppression threshold (Ruter et al., 2007). Depleting Tregs is useful to consider managing Tregs, as strategies aside from elimination are also effective, and depletion might foster rapid Treg regeneration (Colombo and Piconese, 2007). Anti-CD25 antibody remains the prototypical reagent for depleting Tregs in mouse models. Denileukin diftitox is a combination of IL-2 and diphtheria toxin; this active drug is also called (Ontak). The cell dies, when Ontak binds to the IL-2 receptors on the cell surface, diphtheria toxin will release and enter into the cells that express IL-2 receptors. This drug mostly targets leukaemia's and lymphomas cancer cells because some of these cells express these receptors (Litzinger et al., 2007). Also, cyclophosphamide depletes Tregs and improves the efficacy of a dendritic cell vaccine in mouse models for melanoma or colon carcinoma (Liu et al., 2007). Vaccines against FoxP3 improved tumour immunity in a model of renal cell carcinoma (Nair et al., 2007). LMB-2 is another drug that reduces Tregs in cancer patients. It is a CD25-directed *Pseudomonas* immunotoxin (Powell et al., 2007).

It appears that a combination of antitumour vaccination and CTLA-4 blockade, protect the animals from progressive tumour growth, induce the autoimmunity and guide the immune cells to the antigen that the animal was vaccinated against. A study by O'Mahony et al. investigated patients with a cancer vaccine that received the anti-CTLA-4 antibody (Ipilimumab). The purposes of the research were to determine, drug toxicity, tumour response, CD8⁺ CTL response, and a modulation Tregs numbers. In conclusion, the study showed that there was no significant autoimmune toxicity, and Ipilimumab reduced phenotypic Tregs in

blood, but the tumour specific CD8⁺ T cell did not increase, and phenotypic Tregs rebounded quickly, even though their function was not precisely determined (O'Mahony et al., 2007). Furthermore, multiple mechanisms can be used to block Tregs effector functions such as; inhibiting STAT3 reduces Tregs suppressive function (Pallandre et al., 2007), blocking IL-10 receptors, and activating OX40 on tumour Tregs abrogates their suppressive function and amplifies tumour rejection (Piconese et al., 2008).

1.5 Different markers for T regulatory cells



CD25 targeting	Treg depletion Treg re-programming	Anti-CTLA-4	Treg depletion Treg re-programming
Anti-TNFR2	Impaired Treg activity Loss of Treg stability	Anti-PD-1	Treg depletion Impaired Treg activity
TGF-β Blockade	Impaired Treg activity Reduced Treg proliferation and stability	Anti-LAG3	Impaired Treg activity
TGF-βRI/2 Blockade	Impaired Treg activity Reduced Treg proliferation and stability	Anti-TIM3	Impaired Treg activity ? Treg depletion ?
		Anti-NRP1	Impaired Treg activity Treg depletion (CPPs)

Figure 11: T regulatory cell markers.

Tregs express a range of markers that can be utilised for their identification, as summarised in the figure. **(i) Surface markers** are useful for isolating viable cells and also offer the most accessible target for immunotherapies. **(ii) Intracellular markers**, such as FoxP3 and Helios. **(iii) Chemokine receptors** are crucial for Treg migration and can also identify certain Th specific Treg subsets, (Chaudhary et al., 2014).

1.5.1 Neuropilin-1

One of the specific markers for Tregs is the multifunctional transmembrane protein, Neuropilin-1 (Nrp-1/CD304). Nrp-1 together with plexin acts as a co-receptor for Class III semaphotins which is involved in controlling neuronal axon development and in modulating immune functions (Glinka & Prud'homme, 2008). A study by Bruder et al. showed that the expression of Nrp-1 was exclusively upregulated on murine Tregs but down-regulated on other T cell subsets. Nrp-1 expression correlated with FoxP3 expression throughout Treg development from the selection of naïve thymocytes to Treg maturation. Both *in vivo* and *in-vitro* generated Nrp-1 Tregs showed increased immunosuppressive efficacy *in vitro* suppression assays (Bruder et al., 2004). Nrp-1 is also expressed on endothelial and tumour cells, correlating with increased tumour vasculature and progression (Bruder et al., 2004).

Nrp-1 is highly expressed on CD4⁺CD25⁺ tTregs. A study by Yadav et al. showed that Nrp-1 distinguishes tTregs and pTregs among regulatory T cell subsets *in vivo*. Using a combination of novel (MBP)-TCR transgenic mice with defined self-antigen specificity and conventional mouse models, they demonstrated that Nrp-1 is expressed at high levels on tTregs and can be used to separate tTregs from pTregs, by generating pTregs via several mechanisms such as stimulating conventional CD4⁺ cells *in vitro* with TGF- β and CD3/CD28 microbeads or antigen-primed APCs. *In vivo*, transplanting homeostatic conversion of naïve T cells into RAG-deficient mice will generate pTregs. Prolonged *in vivo* sub-immunogenic stimulation of OVA-specific TCR Tg mice with OVA peptide will also generate pTregs due to Nrp-1^{low/-} pTregs (Yadav et al., 2012). Yadav, also reported that Nrp-1⁺CD25⁺ Tregs showed greater efficacy at inhibiting CD4⁺ T cell proliferation, compared with Nrp-1⁻ Tregs. Further

studies by Weiss et al., (2012), demonstrated that *in vitro*, the Nrp-1 expression is driven by TGF- β secretion while IL-6 inhibits TGF- β induced Nrp-1. In conclusion, Nrp-1 is a stable specific marker for murine tTregs, but it is not expressed consistently on human Tregs and cannot consider as a reliable marker of human tTregs.

A study by Elkord and Chaudhary in 2014, reported that Nrp-1 are expressed significantly high on CD3⁺CD4⁺ Tumour infiltrating T regulatory cells (TI Tregs) compared to PBMCs. Referring to Elkord and Chaudhary, Nrp-1 was expressed at a much higher level on CD25⁺FoxP3^{+/-} TI Tregs subsets compared with CD25⁺FoxP3^{+/-} PBMCs. They also examined the co-expression of Nrp-1 and Helios on different CD25^{+/-}FoxP3^{+/-} T cell subsets from PBMC and TI Tregs. The results show that Nrp-1 and Helios were only co-expressed on CD25⁺FoxP3⁺ Tregs from TI Tregs. Furthermore, their results demonstrated that Nrp-1 was not only expressed on Helios⁺FoxP3⁺ and Helios⁻FoxP3⁺ Tregs but also on Helios⁻FoxP3⁻ T cells. In contrast to TI Tregs, there are Tumour infiltrating lymphocytes (TILs) which are immune cells located around the tumour cells. TILs have the capacity to reflect the tumour host interactions more accurately. A study by Gillespie and Russell in 1978 indicated that in animal tumour models, the progressive growing tumours contain weak or non-reactive tumour lymphocytes while the regressing tumours have highly reactive lymphocytes. On the other hand, in human studies, most TILs are immunologically non-reactive (Klein et al., 1977). A high number of TILs are tumour specific CD8⁺ cytotoxic T cells that infiltrate and attack tumours, limiting tumour burden and decreasing tumour growth. Clinically, TILs are used to deplete Tregs to improve the treatment of cancer. In response to tumour-secreted factors, a significant proportion of TILs

population and Tregs, migrate into tumour site to expand in situ via some mechanisms.

1.5.2 GARP and LAP

LAP is a propeptide that is non-covalently associated with the amino-terminal domain of TGF- β , creating a latent complex that can associate with latent TGF- β binding protein (LTBP) to produce a large latent form (Tran et al., 2009). Human Tregs and several other cell types including Th clones secrete latent TGF- β . LAP prevents mature TGF- β from binding to its receptor, and hence from transducing a signal (Stockis et al., 2009a). TGF- β activation occurs when mature TGF- β is cleaved from LAP (Stockis et al., 2009b). Upon TCR stimulation, LAP bind to GARP on the surface of human Tregs, and produce the active TGF- β cytokine (Stockis et al., 2009b). Oida and colleagues (2003) identified a population of CD4⁺CD25⁻LAP⁺ T cells in the spleen (Oida et al., 2003), and another study identified CD4⁺LAP⁺ Tregs in the blood of humans that do not express FoxP3 but express the activation marker CD69, a human transmembrane C-type lectin protein that is expressed on activated NK and T cells (Ghandi et al., 2010). Other studies have found that TGF- β may induce surface LAP expression on CD4⁺ cells independently of FoxP3 induction (Oida and Weiner, 2010b).

The human LAP⁺ Tregs are found to be CD25^{high} and secrete IL-8, IL-9, IL-10, IFN- γ , and TGF- β upon activation. Human LAP⁺ Tregs expresses TGF- β RII and suppresses the effector cells by TGF- β and IL-10-dependent manner (Figure 11 and 12) (Gandhi et al., 2010; Weiner et al., 2011). A study by Chen et al. demonstrated that CD25⁺LAP⁺ T cells suppress myelin oligodendrocyte glycoprotein-specific immune response (TCR transgenic mice and reported that a

large proportion of these mice develop spontaneous isolated optic neuritis), by inducing FoxP3 and by inhibiting IL-17 production (Chen et al., 2008).

GARP (LRRC32) is a transmembrane protein that was observed in a microarray screen for mRNA expressed in activated human CD4⁺CD25⁺ Tregs (Wang et al., 2008). Stockis and colleagues (2009b) examined whether GARP could be a receptor for LAP on the surface of human Tregs by comparing Tregs with Th clones. Resting Tregs expressed on average 100-fold more GARP than in resting Th clones with the results of the highest expressing Th clones close to the results of the lowest expressing Treg clones. GARP was found to be present in stimulated Tregs and not in the stimulated Th clones. Additionally, Stockis and colleagues (2009b) demonstrated that Th clones bear no surface GARP and LAP, while most Tregs bear considerable amounts of both GARP and LAP on their surface. GARP expression correlates with the level of LAP expressed on the cell surface (Stockis et al., 2009b). Currently, a growing body of knowledge suggests that GARP is critical for surface LAP expression, even in the presence of other binding mechanisms. GARP and LAP act as a bridging molecule that attaches to the cell surface (Oida and Weiner, 2010a). Also, GARP is essential for the surface expression of the latent TGF- β complex on activated FoxP3⁺ Tregs, by binding to the complex and functioning as its cell surface receptor; thereby leading to TGF- β release (Stockis et al., 2009a; Tran et al., 2009). The knockdown of GARP with siRNA prevents latent TGF- β from being expressed on activated Tregs (Tran et al., 2009). GARP and LAP recognised on megakaryocytes, platelets, and immature DCs, and they characterise as late stage Treg activation markers.

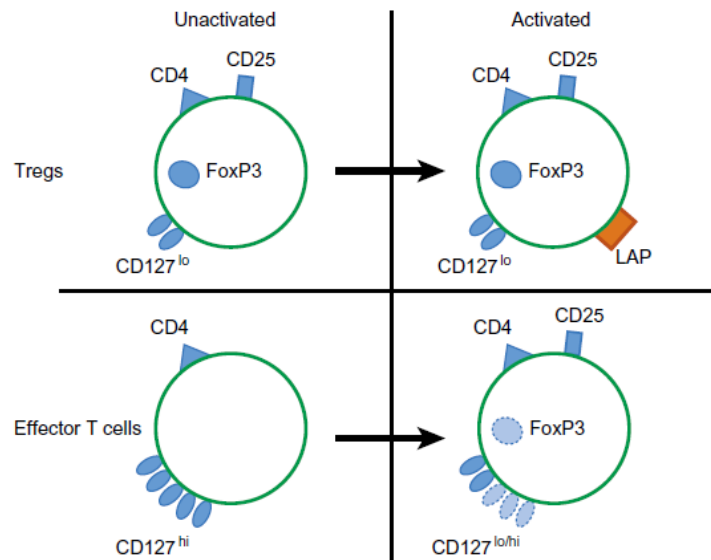


Figure 12: Expression of LAP on activated FoxP3⁺ Tregs.

In the activated settings, current Treg markers such as CD4, CD25 and CD127 do not effectively distinguish activated Tregs from activated effector T cells. LAP provides a surface marker that can precisely discriminate Tregs from effector T cells even after activation, (Lee and Yee, 2012).

1.5.3 Glucocorticoid-induced tumour necrosis factor receptor (GITR)

GITR is also known as (TNFRSF18), cloned from a murine T cell hybridoma (Nocentini et al., 1997). GITR is a member of the TNF-receptor superfamily and has been implied as a costimulatory receptor on T cells. The removal of GITR expressing cells by treatment with anti-GITR monoclonal antibodies developed the organ-specific autoimmune disease (Shimizu et al., 2002).

Tone et al. (2003) reported the cloning of the murine ligand for GITR which have been demonstrated to regulate CD4⁺CD25⁺ Treg function. T regulatory cells express high levels of GITR while resting conventional T cells express low levels that are increased upon activation (Ephrem et al., 2013). Both CTLA-4 and GITR are constitutively expressed on nTregs but also upregulated on CD4⁺ T cells. In vitro the activation of the CD4⁺CD25⁻ T cells is rapidly increased GITR surface expression to levels similar to that on CD4⁺CD25⁺ Tregs. In vitro, the anti-GITR antibodies produced a dramatic functional effect which induced suppression. This effect caused by active signalling into the CD4⁺CD25⁺ Tregs rather than blockade of interaction with the unknown putative GITR ligand. Tone et al. showed that GITR ligand expressed by APC. In vitro, the stimulation of DCs with liposaccharide led to a transient up-regulation of GITR ligand expression. GITR ligand affects Tregs, and GITR signalling has a costimulatory effect on purified conventional CD4⁺CD25⁻ T cells. GITR and CTLA-4 are highly expressed in freshly isolated CD4⁺CD25⁺ T cells (Takahashi et al., 2000).

1.5.4 Identification of T regulatory cells by Helios

Helios is a member of the Ikaros transcription factor family that has been described to be a part of Tregs genetic signature (Hill et al., 2007; Sugimoto et al., 2006). Ikaros proteins were found to be components of highly stable complexes. Helios is a p70 gene that contains four N-terminal zinc finger DNA-binding domains, as well as two C-terminal zinc fingers that show considerable homology to those within Ikaros family proteins (Halm et al., 1998). One study revealed that essentially all thymic Tregs were Helios⁺, but only about 70% of the peripheral Tregs were capable of expressing Helios (Thornton et al., 2010). In vitro and in vivo-generated pTregs failed to express Helios and therefore Thornton et al. (2010) published that Helios expression in murine and human Tregs could distinguish tTregs from pTregs (Thornton et al., 2010). In support of this, several studies in tumour-bearing mice and human cancers have demonstrated the expansion of tumour-infiltrating Tregs on Helios expression. Accordingly, some studies agreed that the Tregs within the tumours are most likely tTregs due to their expression of Helios (Elkord et al., 2011; Redjimi et al., 2012; Wainwright et al., 2011). Another study reported that the majority of tumour-infiltrating Tregs in a murine colon adenocarcinoma expressed low levels of Helios, and based on some additional markers the authors concluded that these were likely to be pTregs (Weiss et al., 2012). However, all these observations only support the possibility that the expression of Helios on tumour-infiltrating Tregs may class as a tTreg marker, but this transcription factor may not necessarily be an indication that they are derivatives of tTregs.

Some studies showed that besides Tregs, Helios is expressed in other T cell subsets, such as T follicular helper cells, Th2 cells, peripherally induced Tregs and activated T cells (Akimova et al., 2011; Serre et al., 2011; Gottschalk et al.,

2012). Therefore, so far the expression of Helios is not a sufficiently reliable marker that distinguishes the origin of tumour-Tregs and it is crucial to understand its full subsets and functions.

Recently, a study by Baine and Basu reported that Helios regulates IL-2 production in Tregs by suppressing IL-2 gene transcription. The results of this study indicated that Helios is necessary for the suppression of IL-2 production in Tregs because Helios binds to the IL-2 promoter to maintain it in a deacetylated state and makes it transcriptionally inactive (Baine et al., 2013). Furthermore, referring to the same study both Helios and FoxP3 cooperate to enforce silencing of IL-2 transcription in Tregs, indicating that Helios regulates FoxP3 binding to the IL-2 promoter. Moreover, loss of Helios in Tregs results in activation of the IL-2 locus; allows Tregs to produce IL-2 upon re-stimulation and loses their anergic phenotype. In the absence of exogenous IL-2, resting Tregs failed to proliferate and died, while knockdown of Helios expression provided Tregs to remain and reproduce (Baine et al., 2013).

A study by Zabransky et al. observed that CD103 and GITR expressed at significant levels on a subset of Helios⁺ Treg cells and Helios⁺ Treg population could be highly improved by FACS sorting using these two markers. The expression of the GITR correlated with Helios expression in unstimulated splenic Treg (Zabransky et al., 2012). CD103 is a α/β integrin associated with gut-homing of lymphocytes (Schon et al., 1999), and preferentially expressed on tumor-infiltrating Treg (Anz et al., 2011) was also relatively up-regulate on Helios⁺ versus Helios⁻ Treg. A study by Feuerer et al. suggested that Helios message correlates with CD103 expression (Feuerer et al., 2010). The combinations of Helios with FoxP3 promoter will upregulate FoxP3 expression

(Getnet et al., 2010). Zabransky showed that in vitro, in the presence of IL-2 and TGF- β , the stimulation of naïve CD4⁺ T cells could lead to the development of both Helios⁺ and Helios⁻ FoxP3⁺ Treg. Further studies, showed that if the global Helios knockout or CD4-targeted Helios knockout, mice showed no overt deficiency in Treg number or function (Thornton et al., 2010). Successful expression of Helios seems to induce apoptosis (Getnet et al., 2010). Zabransky et al. were able to separate a FoxP3 Treg population relatively enriched for Helios expression by classifying unstimulated Treg from the spleens of un-manipulated wild-type mice on CD4, CD25, CD103 and GITR. The results showed an overexpression of CD4⁺CD25⁺CD103⁺GITR⁺ (Helios-enriched) Treg and mediate a significant degree of suppression compared to CD4⁺CD25⁺CD103⁻GITR^{low} Treg. Furthermore, Treg cells in the tumour-infiltrating population dramatically enriched for Helios⁺ cells. However, this study could not improve Helios⁺ Treg from the tumour bed because they found that both Helios⁺ and Helios⁻ Treg expressed similar levels of CD103 and GITR at this location. On the other hand, this study was an excellent agreement with recent studies showing that CD103 markers Treg in a tumour bed (Anz et al., 2011).

1.5.5 Ki-67 antigen marker

To further define Tregs in the tumour microenvironment, T helper cells and Tregs were evaluated of expression Ki-67. The Antigen Ki-67 is a marker for cellular proliferation that is used to identify tumour cells. This antigen identifies the Ki-67 positive tumour cells, and it works directly against the different epitopes of the same proliferation-related antigen. The antigen Ki-67 detects within the nucleus in the interphase (Scholzen & Gerdes et al., 2000). This protein is found to be present when the phases of the cell cycles are activated, and they found to be absent when the cells are not activated. This classification makes the Ki-67 protein as an excellent marker for determining the growth fraction of a tumour cell. With regards to the scientific community, this protein is used as a proliferation marker in both research and diagnosis. A study by Gerdes et al. demonstrated that after stimulating the peripheral blood lymphocytes with phytohaemagglutinin, which is a lectin founds in plants, the expression of the target antigen was recognised by the mouse monoclonal antibody Ki-67, suggesting that Ki-67 recognise the nuclear antigen during the cell proliferation process. This study was generated to nuclear antigen-specific of the Hodgkin lymphoma cell line L428 (Gerdes et al., 1983). Another study by Kryczek et al. stimulated T cells firstly with different extracellular specific antibodies and then with various intracellular antibodies including anti-Ki-67 and anti-FoxP3 antibodies. Their results showed that by 4% to 6% primary FoxP3⁺ T cells expressed Ki-67. However, the results also demonstrated that FoxP3⁻ effector T cells showed significantly higher Ki-67 compared with FoxP3⁺ T cells (Kryczek et al., 2009).

1.6 Flow cytometry

The data analysed on FACSVerse Flow cytometric BD Bioscience, USA with BD FACSuite software. The analyses were performed by gating lymphocytes with six-colour staining, and the acquisition run at 100,000 events.

Flow cytometry is a laser-based technology that simultaneously measures and then analyse multiple physical characteristics of different particles, mainly cells, as they flow in a fluid stream through a beam of light. This technology employed in cell counting, cell sorting, biomarker detection and protein engineering. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence (Barsky et al., 2016).

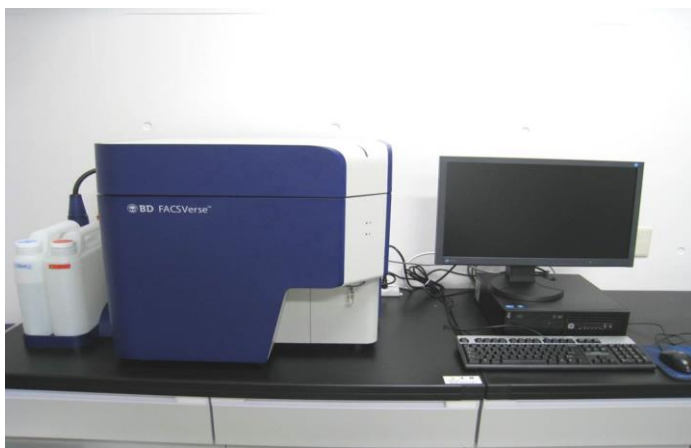


Figure 13 and Figure 14: Images of BD FACSVerse Flow Cytometry machine.

1.6.1 Flow cytometry functional assay

Flow cytometry is a laser-based technique that used to count and analyse the size and shape of cells or particles. It consists of lasers, fluidic system, the optical system, electronics, and a computer. These components work as a backbone for the flow cytometer. The fluid cells in the fluidic system contain liquid stream or sheath fluids that help to carry a line or cells to pass in a single file through the light to the integration point. The fluidic system does also take away the waste. The laser provides a single wavelength that passes through the cells to scan and detects the cells. The optical system consists of several minors that deflect the lights to be detected by certain detectors to convert the analog signal into a digital signal, and there is an amplification system that amplifies that signal, and a computer for analysis of the signal (Shapiro, 2003).

The samples transported to the interrogation point for accurate data collection and it is important that particles or cells are passed to the laser beam one at a time. Most flow cytometers accomplish these by injection the samples that containing cells into a fluid stream of sheath fluid. The flow cytometry works when the beam of light of a single wavelength directed on to a hydrodynamic focus stream of liquid containing a single file of cells achieved by the sheath fluid. The hydrodynamic focusing is when sample stream becomes compress to roughly one cell in diameter. In fact, the flow cytometry can accommodate cells that spend approximately three orders of magnitude in size. In most cases, the cytometer detects cells between 1.0 and 15.0 μm in diameter, but it is possible to detect particles outside this range (Shapiro, 2003).

Many detectors aimed at the point where the stream of the cells passes through the light beam. When the light comes in contact with the cells it is going to get

scattered in various directions. The scattering in the direction of the beam is the forward scattering and the one that is perpendicular to the beam are called side scattering. Each suspended cell that passes through the beam scatters the ray and the fluorescence chemical that is established in the particular or attached to the cells meet the light at a longer wavelength. This combination of scattering and fluorescent light is picked up by the detectors. Each detector analyses the fluctuation in brightness to derive various information about the physical and chemical structure of the cells. The forward scatter correlates to the cell volume, whereas the side scatter correlates to the inner complexity of the particular such as the shape of the nucleus, the amount type of side plasmid granules or membrane roughness (Shapiro, 2003).

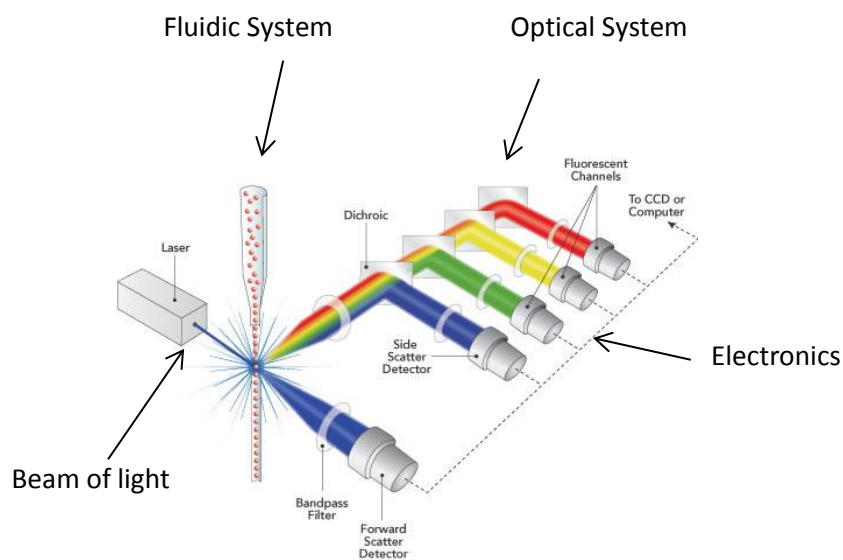


Figure 15: Primary system of flow cytometry

(Source: Semrock, (n.d.))

1.7 Hypothesis

The hypothesis of this research is that the Treg level increases more in cancer patients as compared to patients with chronic pancreatitis and healthy individuals. Also, GARP and LAP are more highly expressed by CD4⁺CD3⁺FoxP3⁺Helios⁺ Tregs as compared to CD4⁺CD3⁺FoxP3⁻Helios⁻ Tregs, speculating that the main key for targeting FoxP3⁺Helios⁺ suppressive Tregs is by the novel markers GARP and LAP.

1.8 Objective

Given the overwhelming evidence, this study is both justified and timely. The aim of this study is to examine the different suppressive factors and phenotypes of T regulatory cells (Tregs). Also, to compare the levels of Tregs in malignant pancreatic cancer and liver metastases from colorectal cancer with controls, by investigating the levels of various Tregs specific and novel markers such as GARP and LAP that are expressed on the surface of activated human FoxP3⁺ Tregs. The purpose of this study is to identify which of FoxP3 (+/-) Helios (+/-) Tregs express GARP and LAP significantly and to investigate if these receptors are vital markers for activated conventional Tregs.

This report will provide an understanding regarding the association of GARP and LAP with FoxP3^{+/-}Helios^{+/-} non-activated and activated human Tregs, and also to investigate if these receptors are key markers for activated conventional T cells. The proposals for achieving these objectives is by examining PBMC samples with intracellular and extracellular staining protocols and then study the fundamentally suppressive cytokines that are secreted by GARP⁺LAP⁺ Tregs.

Chapter 2

Material & Methods

2.1 Blood samples

These studies approved by the UK National Research Ethical Committee, Salford Research Ethics Committee and the Local Research and Development Departments. Written consents obtained from all patients and healthy donors before blood collection. Whole blood samples were collected from patients with chronic pancreatitis, malignant pancreatic cancer and liver metastases from colorectal cancer at the Department of General Surgery at North General Manchester Hospital, UK. Also, blood samples that used as controls were either collected from healthy donors or obtained from UK National Blood Service. The ethics were approved by the University of Salford Research Ethics Committee and Northwest Centre for Research Ethics Committee (NREC), North Manchester General Hospital (NMGH) and Salford Royal Hospital (SRH).

Peripheral blood mononuclear cells (PBMCs) were isolated from 20 ml whole blood samples of healthy donors, chronic pancreatitis, malignant pancreatic cancer and liver metastases from colorectal cancer patients. The samples were collected in 50 ml FalconTM tubes (BD Biosciences, Oxford, UK) containing 200 μ l (1000 IU/ml) heparin.

Table 2 below demonstrate the characteristic features of study subpopulations that are suffering from different cancer types. Whole blood was taken from twenty patients with pancreatic cancer. Their ages were between 47 to 87 years old, and the majority of these patients were males. 75% of PC patients were in stage IV, 20% were in stage II while only 5% was in stage I of tumour malignancy.

Furthermore, whole blood was collected from eleven colorectal cancer patients. Their ages were between 71 to 83 years old, and 73% were male while only 27% were female. Only one was suffering from stage I while five were suffering from stage II and another five were suffering from stage III of tumour malignancy. No one was in stage IV of tumour malignancy.

Whole blood was also taken from nine chronic pancreatitis patients that were used as a control in this study. There were four females and five males, and their ages were between 31 and 84 years old within average of 54 years old.

Table 2: A study of the characteristic features of the subpopulations

	PC	CP		CRC
Number	n=20	n=9		n=11
Age (median)	62 (47-87)*	54 (31-84)*		73(71-83)*
Gender (Male: Female)	13:07	05:04		08:03
TNM stage				
I	0	-		1
II	4	-		5
III	1	-		5
IV	15	-		-
Tumour size (cm)	2.9 (1.9-5.5)*			4.2(1-13)*
Preoperative CA19-9 (0-37 U/ml)	371 (77-1230)*	49		63.9 (1169)*
Preoperative CEA (<2.5 ng/ml)	5 (5-13)*	-		29.5(1-144)*
Tumour site				
Head of pancreas	18	-	Right sided	7
Body of pancreas	0	-	Left sided	3
Tail of pancreas	2	-	Others	1
Histological grade				
Well/moderate	9	-		11
Poor/undifferentiated	11	-		0

PC (pancreatic cancer), CP (chronic pancreatitis), CRC (colorectal) CA19-9 (cancer antigen 19-9), CEA (carcinoembryonic antigen), *Data shown represent median (range).

2.2 Reagents and antibodies

The medium used was Roswell Park Memorial Institute-1640 (Sigma, Dorset, UK).

Pre-warmed 5% complete medium was prepared from 500 ml of RPMI-1640 supplemented with 10% of heat-inactivated foetal calf serum (Thermo Scientific, UK), 1% L-glutamine 200 mM (Sigma-Aldrich, UK) and 1% of antibiotics that are based on 100 µg/ml of streptomycin and 100 U/ml of penicillin, (Live Technologies, UK). A freezing medium that was prepared from 50% foetal calf serum, 40% RPMI-1640 and 10% dimethylsulfoxide (DMSO) (Sigma-Aldrich, UK) was used to freeze the tumour cell lines and the PBMCs,

IgG from human serum (10 µg/ml, Sigma-Aldrich, UK) were used to block the FcR on the surface of the cells to limit the non-specific binding. Normal rat serum (0.5 µg/ml, eBioscience, UK) and mouse serum (Sigma-Aldrich, UK) used for blocking the non-specific binding.

BD Bioscience Fix/Perm buffer was used to fix and permeabilise the cells. The fixation and the permeabilisation technique ensure the antibodies to attach to their antigens by immobilising the antigens; ending up with an authentic more translucent cell. Fix/Perm buffer was prepared by mixing one part of BD Fix/Perm concentrate with three parts of BD Fix/Perm diluent. During staining, cells were washed with permeabilisation BD Bioscience buffer (1 part of 10X permeabilisation buffer with 9 part of Distilled water).

Table 3: Antibodies

Antibodies used for coating and activation:
Anti-human CD3 functional grade purified, clone HIT3a (2 µg/ml, eBioscience, UK)
Anti-human CD28 functional grade purified, clone CD28.2 (2 µg/ml, eBioscience, UK)
The monoclonal antibodies that used for T regulatory cells surface staining:
Mouse anti-human CD4-PerCP-Cy5.5 antibodies (clone RPA-T4, 0.5µg/ml, eBioscience, UK)
Mouse anti-human CD3-APC-H7 antibodies (clone SK7, 0.5µg/ml, BD Bioscience, UK)
Mouse anti-human GARP-APC antibodies (Clone 7B11, 0.5µg/ml, BD Bioscience, UK)
Mouse anti-human LAP-PE antibodies (Clone TW4-2F8, 0.5µg/ml, BD Pharmingen, UK)
The monoclonal antibodies used for T regulatory cells intracellular staining were:
Armenian hamster anti-mouse/human Helios-FITC (2µg/ml, 22F6 Clone, Biolegend, Cambridge, UK)
Rat anti-human FoxP3-PE-Cy7 (clone PCH101, 0.5µg/ml, eBioscience, UK)
Mouse anti-human IL-10-FITC (clone BT-10, 0.5µg/ml, eBioscience, UK)
Mouse anti-human IFN γ -PE-Cy7 (clone 4S.B3, 0.5µg/ml, BD Pharmingen, BD Biosciences, UK)
Rat anti-human IL-2-PE-Cy7 (0.5µg/ml, eBioscience, San Diego, CA)
Rat anti-human IL-10-APC (0.5 µg/ml, BD Pharmingen, BD Biosciences, UK)
Rat anti-human FoxP3-PE (0.5 µg/ml, eBioscience, San Digo, CA)

2.3 Blood isolation

After blood collection from the donors, 20 ml of the blood was slowly layered over 20 ml of Ficoll-Hypaque (Sigma-Aldrich, UK) solution in a universal tube. Ficoll-Hypaque is a hydrophilic polysaccharide which dissolves in solutions, and it is used to separate whole blood components by their variant density. The tube was sealed and centrifuged (3000 RPM, 30 min at room temperature) with no brake. After centrifugation, the plasma was collected and transferred to another universal tube and centrifuged (3000 RPM, 10 min) to remove any cells and platelets, and then was kept in aliquots of 1 ml and stored at -80°C for further analysis. Subsequently, the mononuclear lymphocyte cell layer was transferred to a new universal tube and was washed with RPMI complete medium and centrifuged twice at 1500 RPM for 5 minutes. The cell pellet was re-suspended in 2 ml of RPMI complete medium and counted manually with trypan-blue dye; 1 part of cells was mixed with 1 part of trypan blue and was applied in a Disposal Haemocytometer (C-Chip, DHC-N01, Digital Bio) and placed under a light microscope. Trypan blue is a vital stain that is incorporated in dead cells and introduces a blue colour, while the live cells with intact cell membranes are not coloured. For counting cell numbers it was required to obtain the total number of viable (unstained) cells per ml of the aliquot.

Cells were counted by using the following formula: **PBMC/mL = number of PBMCs counted (average count per 4 squares) X dilution factor X 10⁴ (slide depth and dilution factor).**

Cells were frozen by centrifuging at 1600 RPM in 5 ml total volume of RPMI for 5 minutes. Then the supernatant was removed and re-suspended in cryopreservation medium to aliquot in cryovials and was placed in a freezing

container containing isopropanol (Cryo1C “Mr. Frosty” USA) and placed at -80°C . Twenty-four hours later, the vials placed in liquid nitrogen for long-term storage.

2.4 Extracellular and Intracellular Staining

Characterisation of Tregs that express CD3, CD4, GARP, LAP, FoxP3, and Helios:

For cell activation, non-treated, 24 well plates coated with a combination of 2 µg/ml anti-human CD3 antibodies, 2 µg/ml anti-CD28 antibodies, and 1 ml of bicarbonate buffer (pH 9.6). At 37°C in 5% CO₂ incubator, the plate incubated for 2.5 hours. Meanwhile, PBMC were collected from liquid nitrogen, thawed rapidly in a water bath at 37°C, and then transferred into a universal tube that contained 9 ml of complete medium. The cells were then washed and counted with trypan blue. The coated plate was aspirated and washed twice with sterile phosphate-buffered saline (PBS) buffer. 2 x 10⁶ PBMC were added to each coated well to activate them and another 2 x 10⁶ PBMC added to non-coated well as non-activated cells. Complete medium added, and the plate placed in the incubator for 18-20 hours. 0.5 x 10⁶ cells were taken from both activated and non-activated wells; washed and centrifuged at 1500 RPM for 5 minutes. 1 µg/ml of IgG (100 µg/ml, Sigma) added to each tube, which then incubated at room temperature for 15 minutes. PBMC were stained for extracellular **CD4**, **CD3**, **GARP** and **LAP** markers (1.5 µg/ml was taken from each antibody), and were incubated at 4°C in the dark for 25 minutes. Cells in the FACS tubes were then washed with 2 ml of cold PBS and centrifuged at 1500 RPM for 5 minutes. The supernatant removed carefully, and 0.5 ml of freshly prepared BD Fix/Perm buffer was added, and the tubes were incubated at 4°C in the dark for 45 minutes. The cells were then washed twice with 1 ml permeabilisation buffer, and 2 µg/ml of normal mouse serum (Sigma-Aldrich) and 1 µg/ml of normal rat serum (eBioscience) were added to the cells and incubated for 15 minutes precisely at room temperature for blocking the non-specific binding sites. Without washing cells, 1.5 µl of rat anti-

human **FoxP3-PE-Cy7** and 4 µl of Armenian hamster anti-mouse/ human **Helios-FITC** added to the tubes as intracellular markers for Tregs. The cells were incubated at 4°C in the dark for 30 minutes, which later on was washed twice with permeabilisation buffer. The cells were then suspended in 300 µl of flow cytometry staining buffer and analysed with BD FACSVerse Flow Cytometry machine.

For intracellular cytokines staining:

Cytokines production plays a critical role in the immune system. In response to cellular activation, cytokines consistently and rapidly produced and secrets. For example, the IFN- γ cytokine involved in the induction of many anti-viral proteins and it is produced by many immune cells including NK cells and effector T cells. The intracellular cytokine staining (ICS) is a flow cytometry based assay that is used to detect and investigate the accumulation and production of cytokines within the endoplasmic reticulum after cell stimulation. Although ICS assay requires a decent amount of cells, it allows studying cytokine production more in depth, and it can be used not only on fresh samples but also on cryopreserved cells. ICS assay can also use in combination with surface markers which give an enormous advantage to investigate the different cell types.

The steps of ICS are as follows:

As previously described, 24-well plates were coated with anti-human CD3 and anti-human CD28 functional grade purified antibodies for 2.5 hours in the 37 °C, CO₂ incubator. Cells were then added to the coated wells for activation, after 18-20 hours incubation, 1 µg/ml of the protein transport inhibitor (Golgi Plug) (BD Biosciences) was added to 1 x 10⁶ cells and placed back to the incubator for 4 hours incubation. In response to the antigen stimulation, the activated T cells secrete cytokines. Golgi Plug blocks the intracellular protein transport process and allows the cytokines production level to accumulate, which then increases the ability to detect, measure, and analyse the cytokines with the flow cytometry.

After 4 hours incubation the cells were stained with the same surface antibodies (Mouse anti-human **CD4-PerCP-Cy5.5**, mouse anti-human **CD3-APC-H7**, mouse anti-human **GARP-APC**, mouse anti-human **LAP-PE**), washed with PBS and fixation/ permeabilisation buffer was added for 45 minutes, washed twice with permeabilisation buffer and added the mouse and rat serum and incubated for 15 minutes. For the intracellular staining, after blocking with serum 1.5 µl of **IL-10-FITC**, and 1.5 µl of **IFN γ -PE-Cy7** or 1.5 µl of **IL-2-PE-Cy7** were added and incubated for 30 minutes in 4°C. After the incubation, to keep the cells permeabilised, the cells were washed twice with the permeabilisation buffer and were analysed by flow cytometry.

Characterisation of cytokines secreted from CD3⁺CD4⁺FoxP3⁺Helios⁺ Tregs:

Following the same previous description; coating, activation, and after 18-20 hours incubation, 1 µg/ml of Golgi Plug was added to 1 x 10⁶ cells and incubated for 4 hours. For the surface staining, 0.5 x 10⁶ cells were taken from both activated and non-activated wells; washed and centrifuged at 1500 RPM for 5

minutes. 1 µg/ml of IgG (100 µg/ml, Sigma) added to each tube, which then incubated at room temperature for 15 minutes. Cells were stained with 1.5 µl of **CD3-APC-H7** and **CD4-PerCp-5.5** and were incubated at 4°C in the dark for 25 minutes. Cells in the FACS tubes were then washed with 2 ml of cold PBS and centrifuged at 1500 RPM for 5 minutes. The supernatant then removed, and 0.5 ml of freshly prepared BD Fix/Perm buffer added, and the tubes were incubated at 4°C in the dark for 45 minutes. The cells were then washed twice with 1 ml permeabilisation buffer, and 2 µg/ml of normal mouse serum (Sigma-Aldrich) and 1 µg/ml of normal rat serum (eBioscience) was added to the cells, mixed, and incubated for 15 minutes at room temperature.

For the intracellular staining, without washing the cells, they were stained with 4 µl of **FoxP3-PE** and **Helios-FITC**, and 1.5 µl of **IL-10-APC** and **IFN γ -PE-Cy7** or **IL-2-PE-Cy7** depending on which cytokine being investigated. The cells were incubated at 4°C in the dark for 30 minutes, which later on was washed twice with permeabilisation buffer. The cells were then suspended in 300 µl of flow cytometry staining buffer and analysed with BD FACSVerser Flow Cytometry machine.

2.5 Statistical methods

Comparisons of various lymphocyte subsets were made by using Microsoft Excel, and the correlation analyses were calculated using PRISM 5.0 (GraphPad Software) (Table 4). Paired T-test was used to compare different sub-populations between the same group and unpaired T-test was used to compare different groups between the same sub-populations. P value ≤ 0.05 was considered statistically significant. The data are presented as means \pm SEM.

Likewise, analysis of variance (ANOVA) test was used to determine multiple comparisons. Both unpaired T-test and ANOVA test gave similar results in term of being significant differences or not.

The Mean, Standard Deviation and Standard Error of the Mean (SEM) were calculated with both Normality and Shapiro-Wilk normality test.

Table 4: The scheme that PRISM uses to report the P values

P value	Wording	Summary
< 0.0001	Extremely significant	****
0.0001 to 0.001	Extremely significant	***
0.001 to 0.01	Very significant	**
0.01 to 0.05	Significant	*
≥ 0.05	Not significant	ns

2.6 Gating strategy

The blood samples tested were from twenty healthy donors, twenty pancreatic cancer patients, nine chronic pancreatitis patients, and eleven chronic liver metastatic cancer patients.

Because these four groups are not equally in numbers it is important to use Standard Error of the Mean to show the reliability of the mean. Interpret data when the values are plotted as error bars on a graph.

The gating strategy that was followed is shown in Figure 16. The result shows a summary of CD3⁺CD4⁺ T cells that could express the vital markers for T regulatory cells (FoxP3, Helios, GARP and LAP).

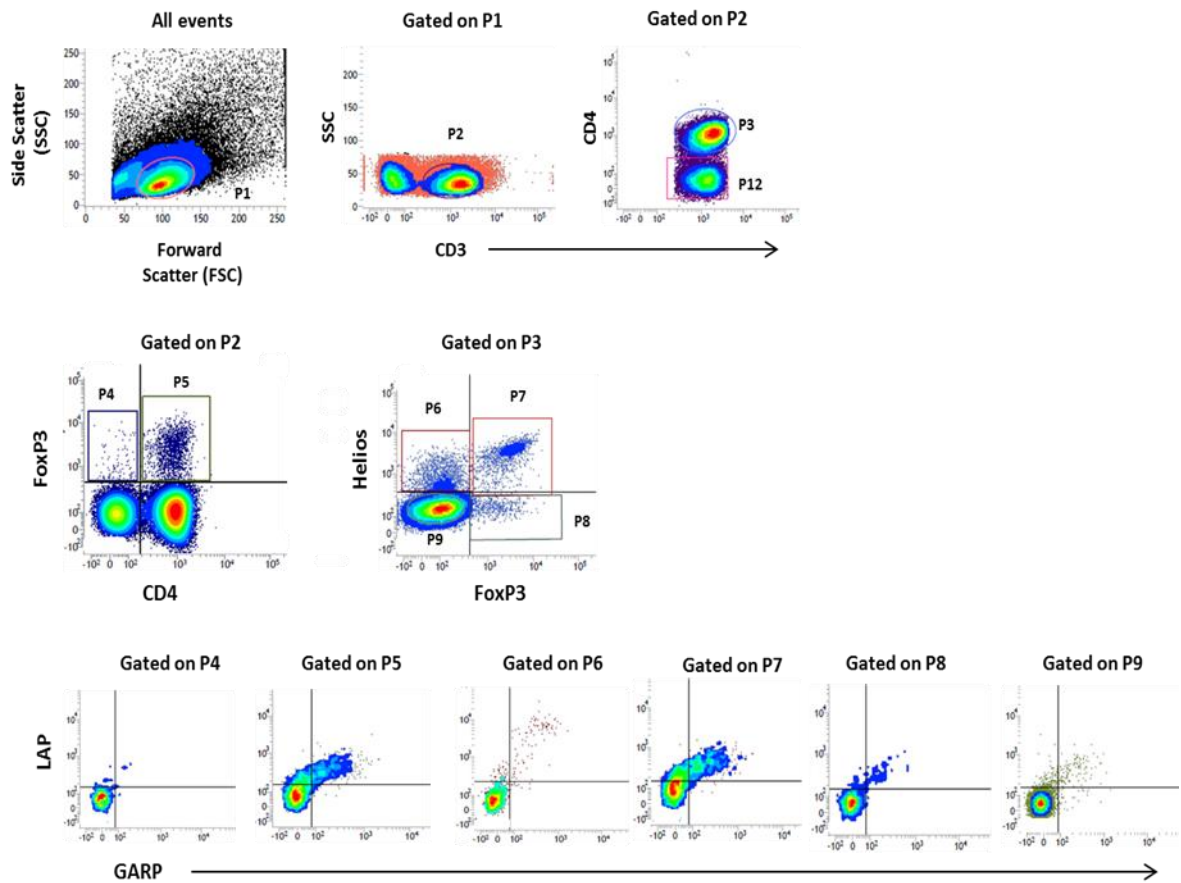


Figure 16: Gating strategy.

A representative example of activated healthy donor flow cytometric plots.

This figure shows the gating strategy to define lymphocytes based on forward and side scatter characteristics (plot 1), $CD3^+$ T cells against side scatter (plot 2), $CD3^+CD4^+$ T cells (plot 3) and $CD3^+CD4^-$ ($CD8^+$) T cells (plot 12) are gated on P2. $CD4^+FoxP3^+$ T cells (plot 4) and $CD4^+FoxP3^+$ Tregs (plot 5) are gated on P2. $FoxP3^-Helios^+$ Tregs (plot 6), $FoxP3^+Helios^+$ Tregs (plot 7), $FoxP3^+Helios^-$ Tregs (plot 8), and $FoxP3^-Helios^-$ T cells (plot 9) are all gated on P3. The rest of the graphs show the expression of $GARP^{+/-}LAP^{+/-}$ for these different subpopulations.

Chapter 3

Results

3.1 Expression of FoxP3, Helios, GARP and LAP in CD3⁺CD4⁺ and CD3⁺CD4⁻ T cells

3.1.1 FoxP3 is expressed mainly on CD3⁺CD4⁺ T cells compared to CD3⁺CD4⁻ (CD8⁺) T cells

Increasing evidence suggesting that CD4⁺ regulatory T cells are critical regulatory cells and the best marker to identify these cells is by the FoxP3 transcription factor. In addition to CD4⁺ Regulatory T cells, CD8⁺ suppressive T cells also function as immune regulator and they supposed to play a regulatory role in autoimmune diseases and transplantation. However, CD8⁺ Tregs are less well characterised than CD4⁺ Tregs. There have been efforts to understand the cellular and the molecular mechanisms of CD8⁺ Tregs, and there have been difficulties in isolating these cells due to the lack of defining markers. Therefore, in this study, the expression levels of CD4 and CD8 were investigated for both rested (Figure 17A & B) and activated (Figure 18A & B) CD3⁺FoxP3⁺ T cells. CD8⁺ T cells are also known as (CD4⁻) T cells. The results indicate that for both rested and activated cells, the subpopulation of FoxP3⁺CD3⁺ T cells that express CD4 on its surface are significantly higher in percentages ($2.53 \pm 0.20\%$) compare to the subpopulation of FoxP3⁺CD3⁺ T cells that express CD8 on its surface ($0.44 \pm 0.15\%$).

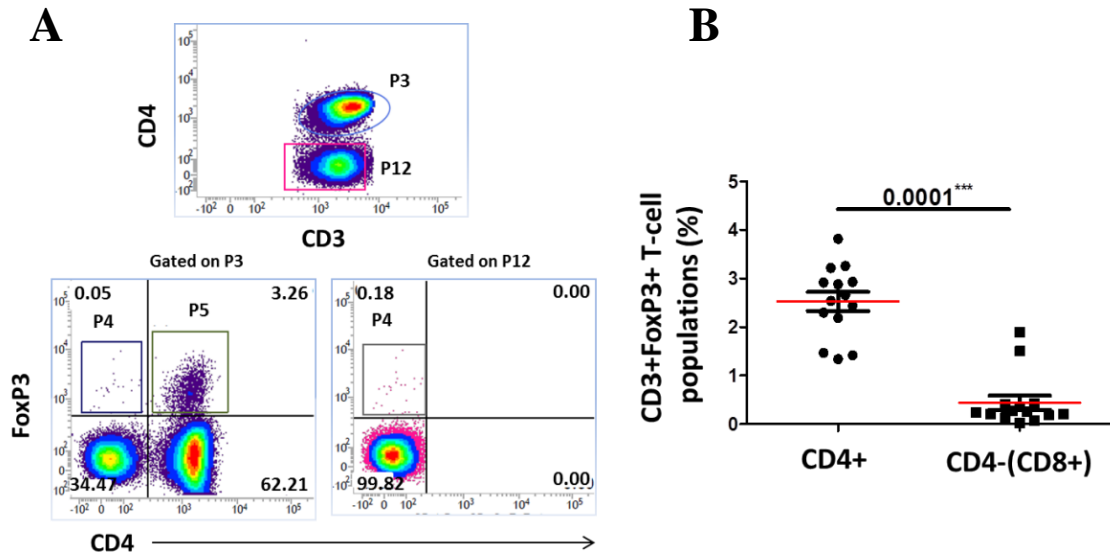


Figure 17: Expression level of CD4 on CD3⁺FoxP3⁺ resting T cells.

Representative flow cytometry plots showing the expression of CD4⁺FoxP3⁺ T cells (P5) gated on P3 and CD4⁻FoxP3⁺ T cells (P4) gated on P3 and P12 (A). Scatter plots show the mean percentages \pm SEM expression of CD4⁺ and/or CD4⁻ CD3⁺FoxP3⁺ T cells. The expression of CD4 on PBMC was analysed from 14 healthy donors. The percentages of CD3⁺FoxP3⁺ Tregs that express CD4 were significantly higher than CD3⁺FoxP3⁺ Tregs that did not express CD4 and expressed CD8 on its surface (B).

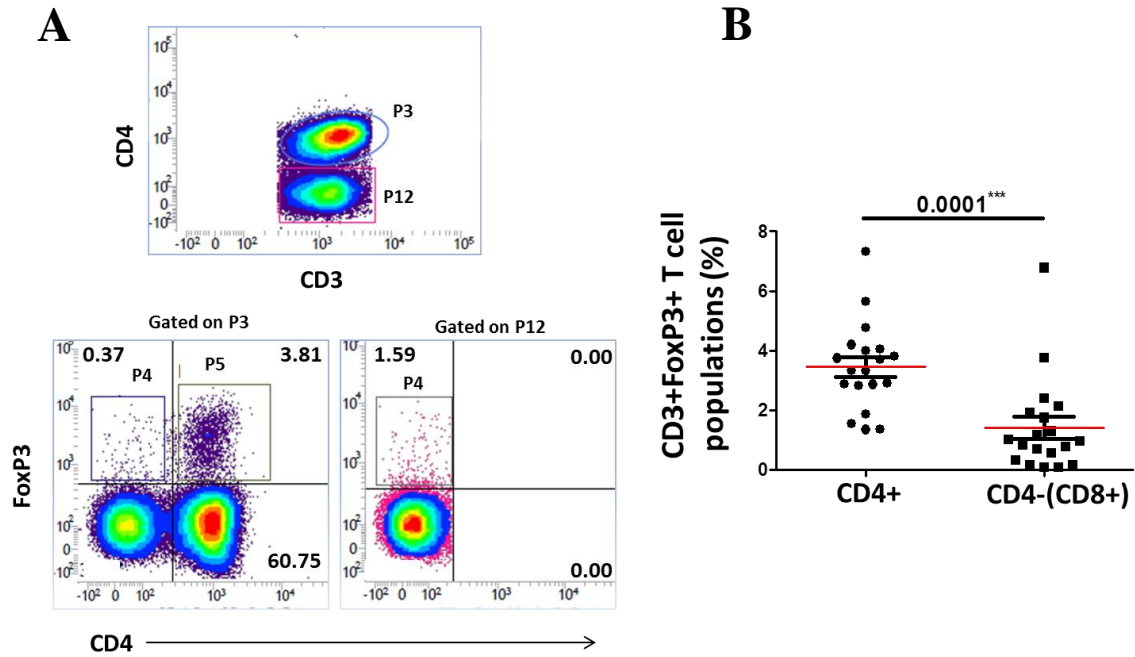


Figure 18: Expression level of CD4 on CD3⁺FoxP3⁺ activated T cells.

Representative flow cytometry plots showing the expression of CD4⁺FoxP3⁺ T cells (P5) gated on P3 and CD4⁺FoxP3⁺ T cells (P4) gated on P3 and P12 (A). Scatter plots show the mean percentages \pm SEM expression of CD4⁺CD3⁺FoxP3⁺ T cells. The expression of CD4 on PBMC was analysed from 19 healthy donors. The percentages of CD3⁺FoxP3⁺ Tregs that express CD4 were significantly higher than CD3⁺FoxP3⁺ Tregs that did not express CD4 and expressed CD8 on its surface (B).

3.1.2 Expression of GARP/LAP is significantly higher on CD4⁺FoxP3⁺ T cells compared to CD4⁻FoxP3⁺ T cells

The subpopulations of CD8⁺ Tregs are described based on the expression of different markers, such as; FoxP3, CD25, CD45, CD56, CXCR3, CD103, CD122, LAG-3 and HLA-G (Tang et al., 2005; Dinesh et al., 2010; Pomie et al., 2008; Tsai et al., 2010). However, only a small amount of CD8⁺ T cells express FoxP3, which represent 0.1 to 0.4% of PBMC in healthy human (Churlaud et al., 2015), and this indicates that the best characterizations of regulatory cell populations are CD4⁺ T cells. Therefore, to further investigate and understand the co-expression of different key Tregs markers for both CD4⁺ and CD4⁻ T cells, FoxP3 was analysed against GARP and LAP, gated on CD3⁺CD4^{+/-} T cells. The results elucidated that GARP/LAP are expressed higher on CD4⁺ than on CD8⁺ T cells for both rested (Figure 19) and activated (Figure 20A) PBMC. Moreover, the representative flow cytometric plots show that LAP is expressed at a greater level than GARP both in CD3⁺CD4^{+/-}FoxP3^{+/-} rested and activated T cells (Figure 19 & 20A and B). As can be seen in figure 20B, LAP was expressed in a highly significant level on CD4⁺FoxP3⁺ T cells ($1.06 \pm 0.17\%$) than on CD4⁻FoxP3⁺ T cells ($0.16 \pm 0.06\%$) ($P= 0.0001$).

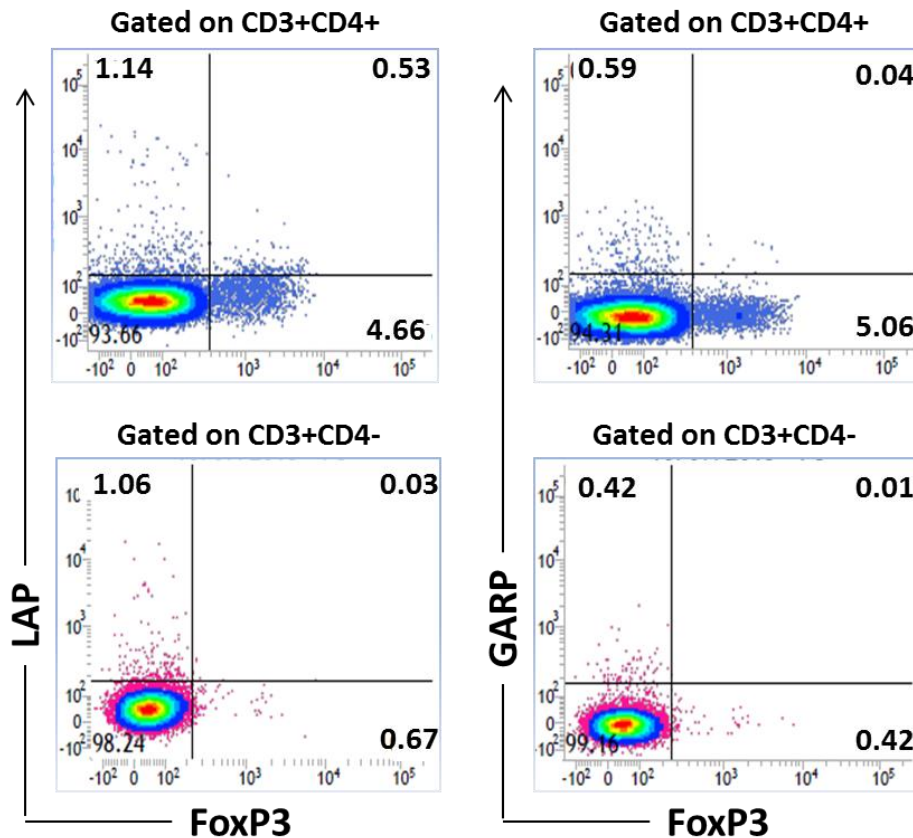
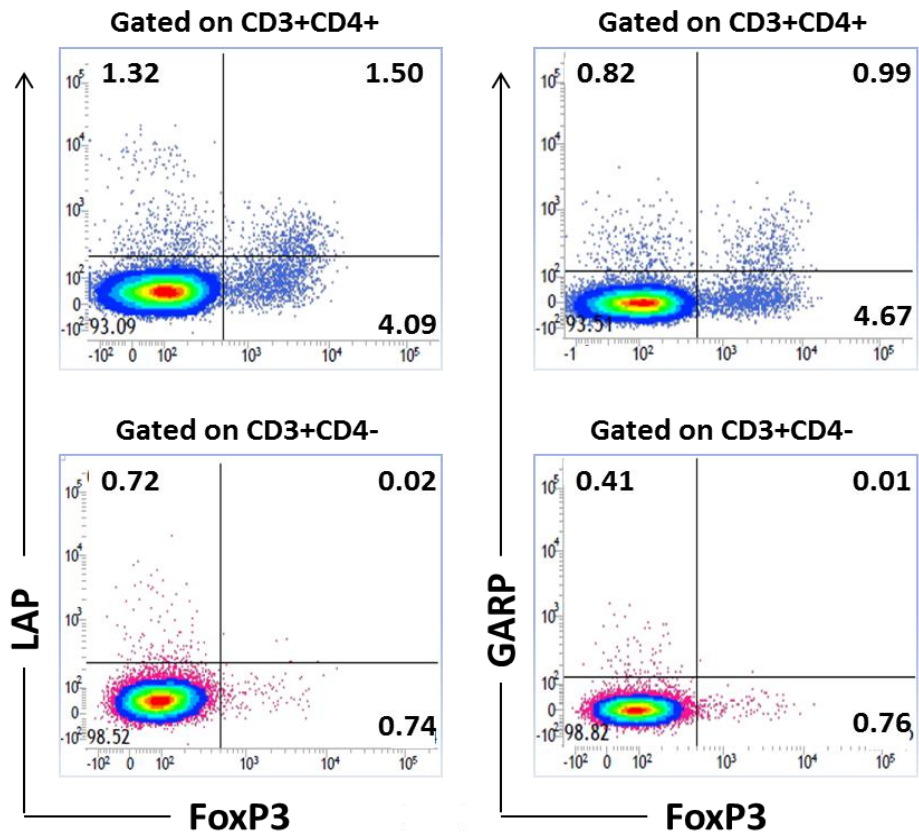


Figure 19: Expression of GARP and LAP on CD3⁺CD4^{+/-}FoxP3^{+/-} non-activated T cells.

Representative flow cytometric plots isolated for HD, showing the expression of GARP/LAP on non-activated CD3⁺CD4^{+/-}FoxP3^{+/-} T cells. The percentages of CD3⁺CD4⁺LAP^{+/-}FoxP3^{+/-} sub-population are higher than CD3⁺CD4⁻LAP^{+/-}FoxP3^{+/-} subpopulation. Furthermore, the percentage of CD3⁺CD4⁺GARP^{+/-}FoxP3^{+/-} sub-population is also higher than CD3⁺CD4⁻GARP^{+/-}FoxP3^{+/-} subpopulation.

A



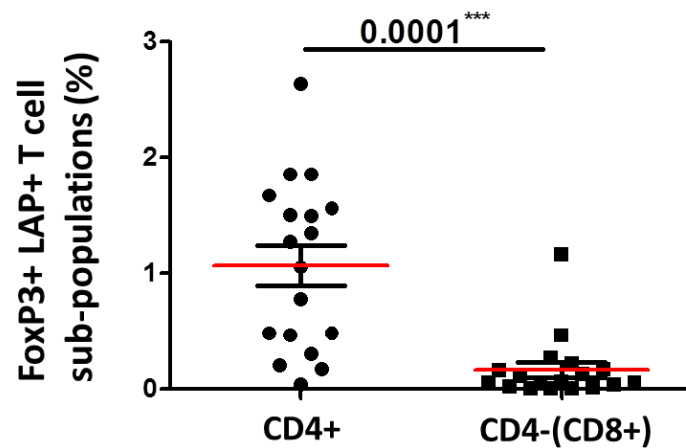
B

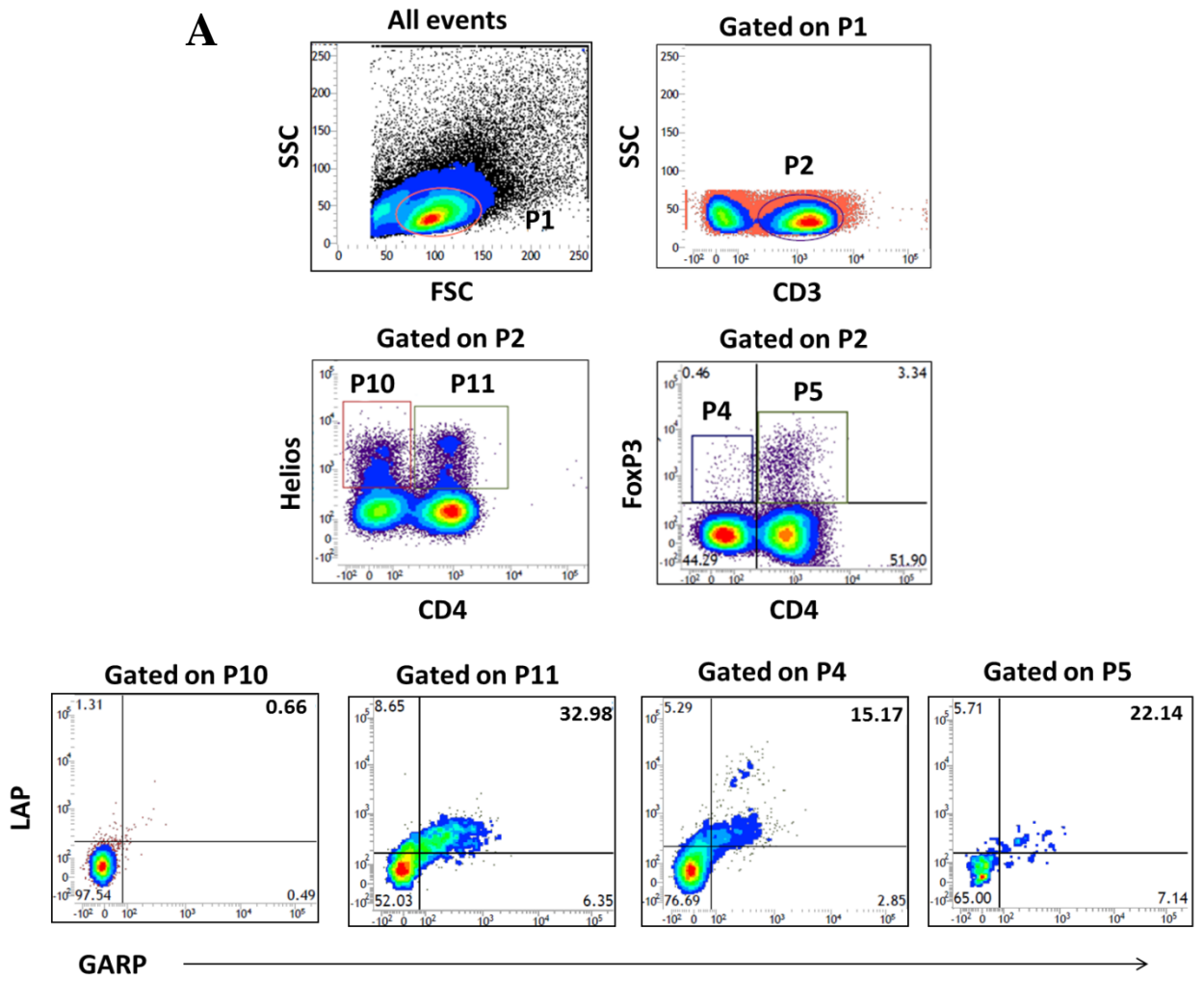
Figure 20: Expression of GARP and LAP on CD4⁺FoxP3⁺ activated T cells.

Activated PBMCs isolated from 19 healthy donors. Representative flow cytometric plots for HD, showing the expression of GARP/LAP on activated CD3⁺CD4⁺FoxP3⁺ T cells. The percentages of CD3⁺CD4⁺LAP⁺FoxP3⁺ subpopulation are higher than CD3⁺CD4⁻LAP⁺FoxP3⁺ subpopulation. The percentage of CD3⁺CD4⁺GARP⁺FoxP3⁺ subpopulation is also greater than CD3⁺CD4⁻GARP⁺FoxP3⁺ subpopulations (A). Scatter plots show the mean percentages \pm SEM expression of LAP on CD4⁺FoxP3⁺ and CD4⁻FoxP3⁺ T cells. CD4⁺ T cells expressed significantly higher LAP compared to CD4⁻ (CD8⁺) T cells (B).

3.1.3 CD4⁺GARP⁺LAP⁺ T cells express significantly higher level of Helios and FoxP3 compared to CD4⁻GARP⁺LAP⁺ T cells

To further understand Tregs mechanisms and to identify markers of the different functional subsets of Tregs, a comparison between CD4⁺ and CD4⁻ T cells were made within the co-expression of GARP and LAP, as markers of activated Tregs, and Helios and FoxP3, as Tregs transcription factors. To investigate the different subpopulations and investigate GARP/LAP co-expression, activated CD3⁺CD4^{+/-} Tregs were analysed by combining Helios and FoxP3 staining and analysing the co-expression of GARP/LAP on these different subsets. The representative flow cytometric plots in figure 21A illustrate that Helios⁺CD4⁺ T cells (P11) expressed approximately 33% of GARP/LAP on its surface, while FoxP3⁺CD4⁺ T cells (P5) expressed approximately 22% of GARP/LAP on its surface.

Interestingly, after comparing the co-expression level of GARP/LAP on the surface of CD4⁺Helios⁺ and CD4⁺FoxP3⁺ T cells with CD4⁻Helios⁺ and CD4⁻FoxP3⁺ T cells, the results demonstrate that CD3⁺CD4⁻Helios⁺ Tregs are expressing GARP/LAP ($2.04 \pm 0.79\%$) in very small amount, in contrast to CD3⁺CD4⁻FoxP3⁺ Tregs, which did express GARP/LAP but to some extent ($12.78 \pm 2.61\%$). However, the expression of GARP and LAP were significantly higher in CD3⁺CD4⁺Helios⁺ Tregs ($24.87 \pm 3.85\%$) compare to CD3⁺CD4⁺FoxP3⁺ Tregs ($17.68 \pm 2.13\%$). Furthermore, the expression level of GARP/LAP on CD3⁺CD4⁺Helios⁺ Tregs were significantly higher than CD3⁺CD4⁻Helios⁺ Tregs ($P= 0.0001$), and CD3⁺CD4⁺FoxP3⁺ Tregs ($17.68 \pm 2.13\%$) were significantly higher than CD3⁺CD4⁻FoxP3⁺ Tregs (Figure 21B).

A

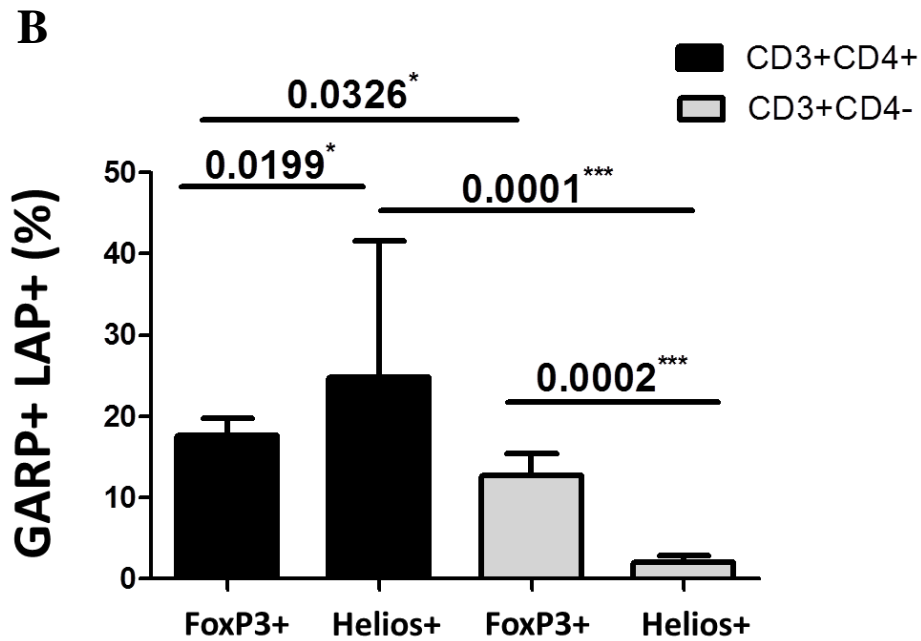


Figure 21: Percentages of CD4^{+/-} FoxP3⁺ and CD4^{+/-} Helios⁺ activated T cells expressing GARP and LAP.

Representative flow cytometric plots for HD, showing the expression level of GARP/LAP gated on activated CD3⁺CD4⁻Helios⁺ T cells (P10), GARP/LAP gated on CD3⁺CD4⁺Helios⁺ T cells (P11), GARP/LAP gated on CD3⁺CD4⁻FoxP3⁺ T cells (P4) and GARP/LAP gated on CD3⁺CD4⁺FoxP3⁺ T cells (P5) (A). The bar chart shows the mean percentage \pm SEM expression of GARP/LAP gated on CD4^{+/-}FoxP3⁺ and CD4^{+/-}Helios⁺ T cells. The expression of GARP/LAP on PBMC were analysed from 19 healthy donors. The expression level of GARP/LAP was high on CD3⁺CD4⁺FoxP3⁺, CD3⁺CD4⁺Helios⁺ Tregs and CD3⁺CD4⁻FoxP3⁺ T cells. However, the percentages were significantly higher in CD4⁺ Tregs than in CD4⁻ T cells. Additionally, CD4⁺GARP⁺LAP⁺ T cells are expressing both Helios and FoxP3, while CD4⁻GARP⁺LAP⁺ T cells are only expressing FoxP3 and no Helios (B).

3.2 Analysing the expression level of FoxP3, Helios, GARP and LAP in CD4⁺ T cells isolated from healthy donors

3.2.1 LAP expressed significantly higher than GARP on activated CD4⁺ T cells in healthy donors

The ability to discriminate CD4⁺ and CD4⁻ subsets was quite remarkable. Firstly, to classify which of CD4⁺FoxP3^{+/-}Helios^{+/-} and/or CD4⁻FoxP3^{+/-}Helios^{+/-} Tregs express GARP/LAP on its surface, and to further elucidate the expression level of GARP and/or LAP on CD4⁺FoxP3^{+/-}Helios^{+/-} subsets. Therefore, a comparison was made between activated CD4⁺FoxP3⁺LAP⁺ and CD4⁺FoxP3⁺GARP⁺ T cells. Apparently, the result shows that the population level of CD4⁺FoxP3⁺LAP⁺ Tregs (1.06 ± 0.17%) is significantly higher than CD4⁺FoxP3⁺GARP⁺ Tregs (0.87 ± 0.17%) as can be seen in figure 22. Likewise, activated CD4⁺Helios⁺LAP⁺ was compared with CD4⁺Helios⁺GARP⁺ T cells, and the results illustrate that the population level of CD4⁺Helios⁺LAP⁺ Tregs (1.42 ± 0.24%) is significantly higher than CD4⁺Helios⁺GARP⁺ Tregs (1.10 ± 0.20%) (Figure 23A & B).

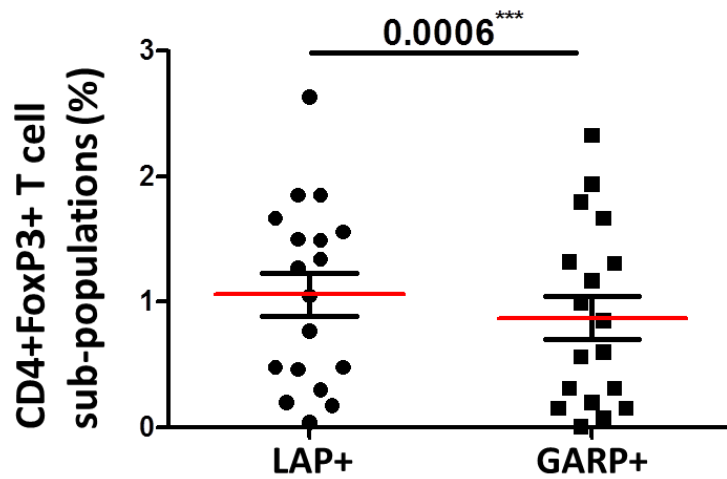


Figure 22: Percentages of the expression level of GARP and LAP on activated CD4⁺FoxP3⁺ T cells.

Scatter plots show the mean percentages \pm SEM of GARP and LAP within CD4⁺FoxP3⁺ T cells in activated PBMCs isolated from 18 healthy donors. The CD4⁺FoxP3⁺ Tregs express significantly higher LAP than GARP (P=0.0006).

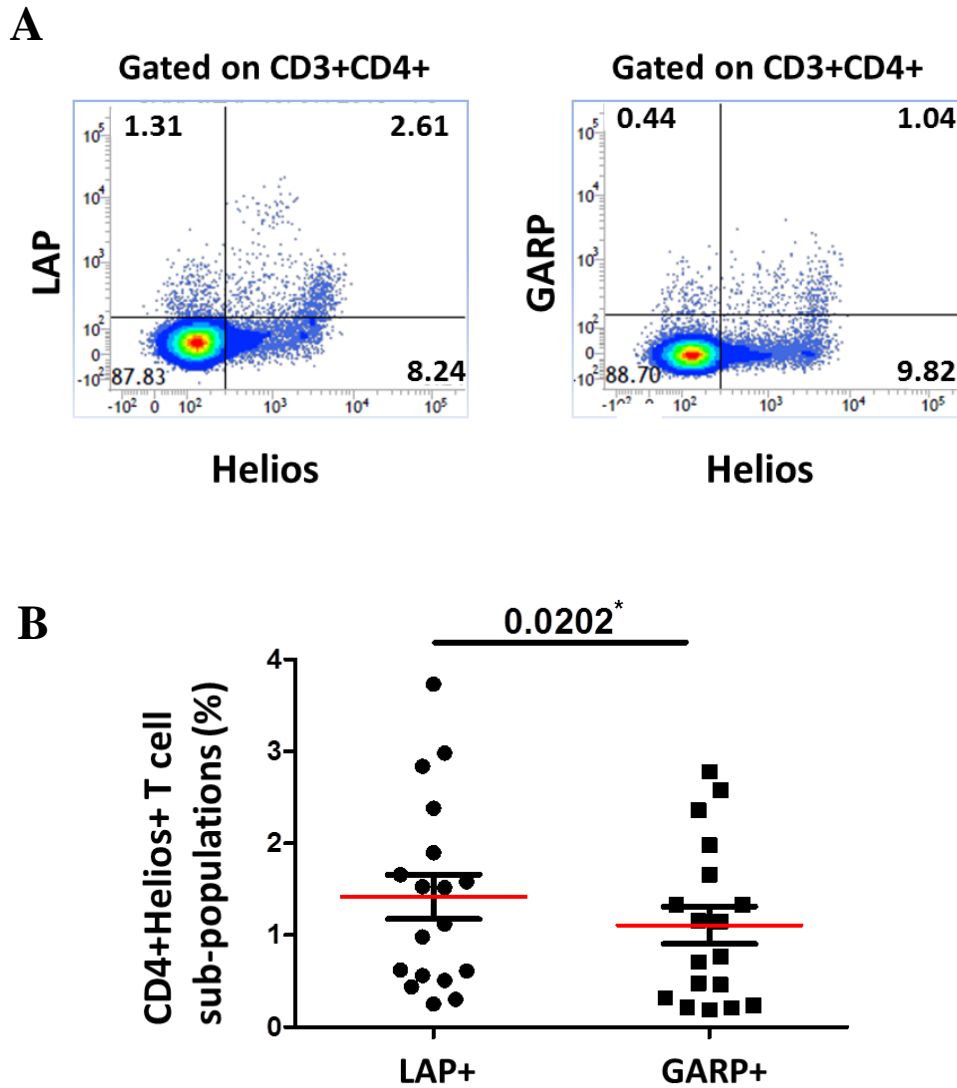


Figure 23: Percentages of the expression level of GARP and LAP on activated CD4⁺Helios⁺ T cells.

Representative flow cytometric plots showing Helios expression against LAP or GARP, as gated on CD3⁺CD4⁺ T cells (A). Scatter plots present the mean percentages \pm SEM of LAP and GARP within CD4⁺Helios⁺ T cells in activated PBMCs isolated from 18 healthy donors. The CD4⁺Helios⁺ Tregs express significantly higher LAP than GARP (P= 0.0202) (B).

3.2.2 GARP/LAP expressed mainly on CD4⁺Helios⁺ T cells compared to CD4⁺FoxP3⁺ T cells

The continual investigation compared the expression level of GARP/LAP between CD4⁺FoxP3⁺ T cells and CD4⁺Helios⁺ T cells in steady-state non-activated PBMCs. Of note, GARP/LAP was mainly expressed on CD4⁺Helios⁺ T cells ($5.03 \pm 1.16\%$) and to a significantly lower level of CD4⁺FoxP3⁺ T cells ($1.13 \pm 0.57\%$) (Figure 24).

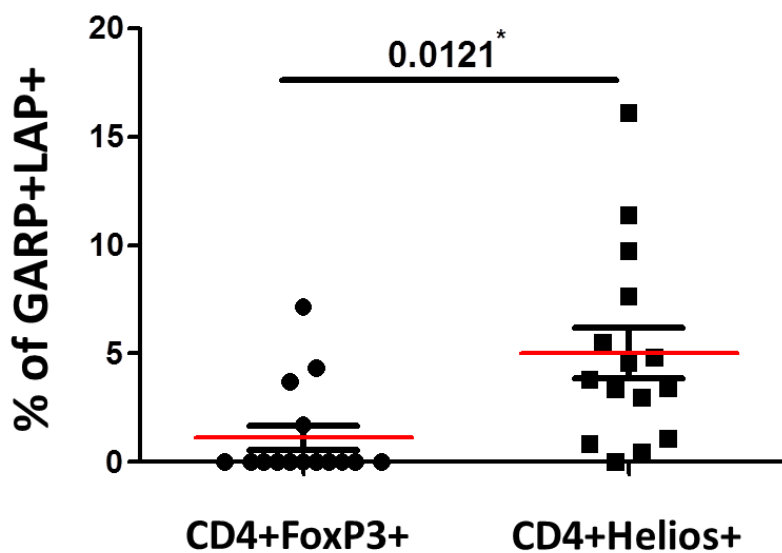


Figure 24: Percentages of the expression level of GARP and LAP on non-activated CD4⁺FoxP3⁺ and CD4⁺Helios⁺ T cells.

Scatter plots display the mean percentage \pm SEM of GARP⁺LAP⁺ within CD4⁺FoxP3⁺ and CD4⁺Helios⁺ T cell subsets in non-activated PBMCs isolated from 15 healthy donors. The expression level of GARP/LAP was significantly higher on CD4⁺Helios⁺ T cells compare to CD4⁺FoxP3⁺ T cells.

3.2.3 The percentages of FoxP3⁻Helios⁺ and FoxP3⁺Helios⁻ CD3⁺CD4⁺ T cells increases after activation

FoxP3 and Helios staining were combined, in order to explore the different CD4⁺ T cell subsets. In non-activated T cells from healthy donors, the percentages of CD3⁺CD4⁺ subpopulations were almost equally divided into FoxP3⁻Helios⁺ (2.95 ± 0.37%) and FoxP3⁺Helios⁺ T cells (2.96 ± 0.30%). Of note, a significant lower percentage of CD3⁺CD4⁺ T cells were FoxP3⁺Helios⁻ T cells (0.68 ± 0.06%) (Figure 25A & B). However, there were some differences in activated CD3⁺CD4⁺ T cells. The highest percentage of CD3⁺CD4⁺ T cells were FoxP3⁻Helios⁺ (4.06 ± 0.35%) T cells, which were significantly higher than FoxP3⁺Helios⁺ (2.96 ± 0.37%) and FoxP3⁻Helios⁺ (1.99 ± 0.37%) T cells (Figure 26A & B).

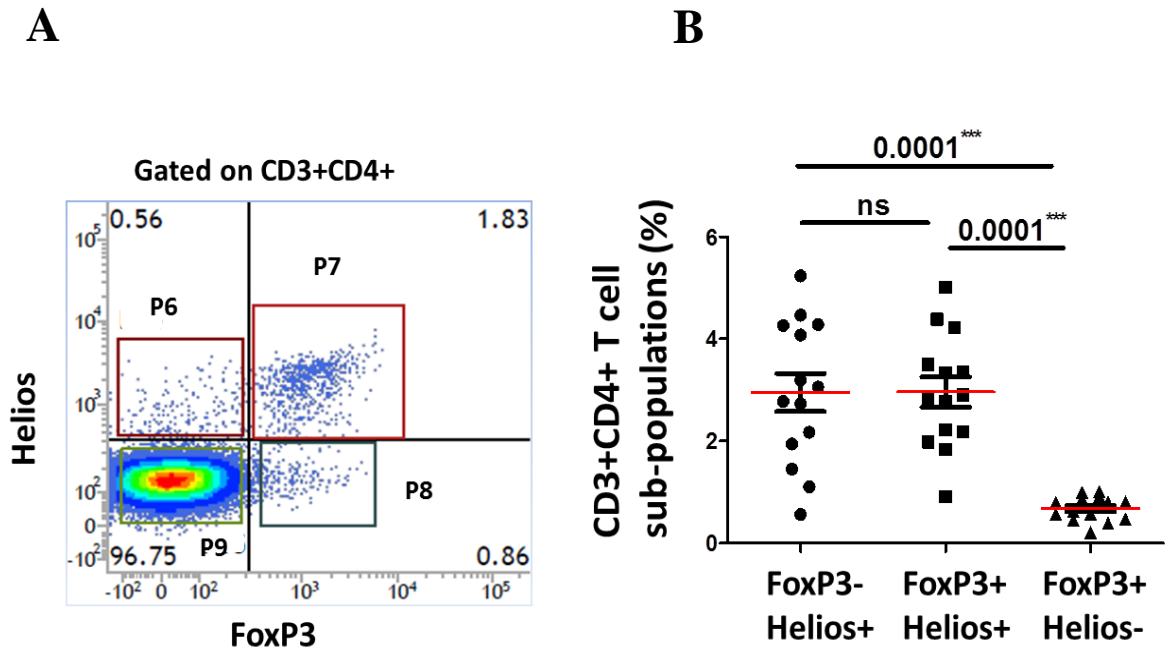


Figure 25: Expression of CD3 and CD4 on different FoxP3^{+/-} Helios^{+/-} non-activated T cell subsets.

Representative flow cytometric plots showing FoxP3 against Helios, as gated on CD3⁺CD4⁺ T cells (**A**). Scatter plots show the mean percentage \pm SEM of CD3⁺CD4⁺ within FoxP3⁻Helios⁺, FoxP3⁺Helios⁺, FoxP3⁺Helios⁻ T cell subsets in non-activated PBMC isolated from 14 healthy donors (**B**).

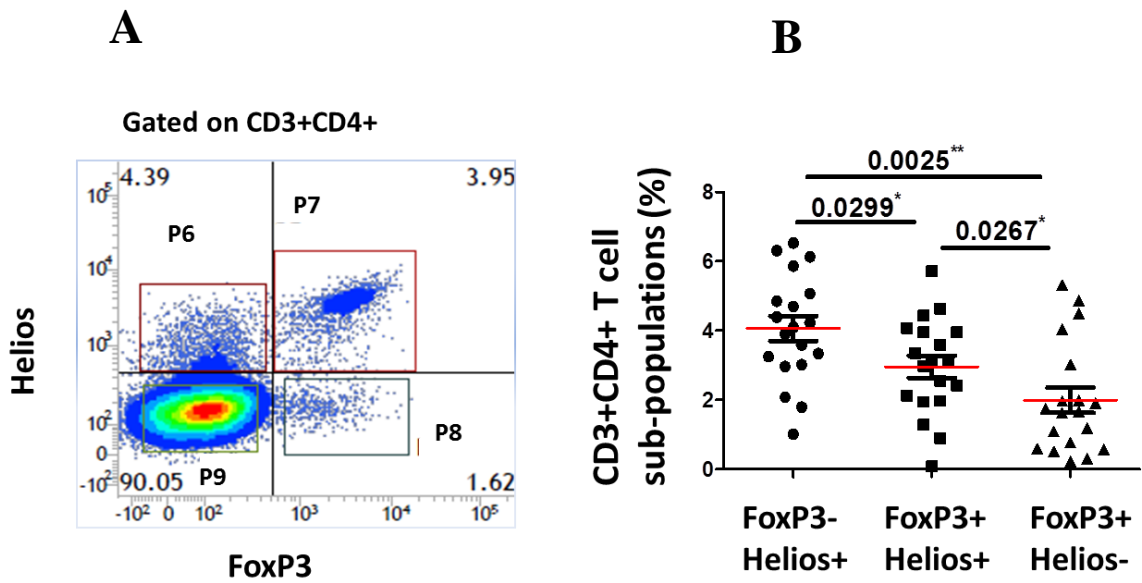


Figure 26: Expression of CD3 and CD4 on different FoxP3^{+/-}Helios^{+/-} activated T cell subsets.

Representative flow cytometric plots showing FoxP3 against Helios, as gated on CD3⁺CD4⁺ T cells (A). Scatter plots present the mean percentage \pm SEM of CD3⁺CD4⁺ within FoxP3⁻Helios⁺, FoxP3⁺Helios⁺, FoxP3⁺Helios⁻ T cell subsets in activated PBMCs isolated from 19 healthy donors (B).

3.2.4 GARP/LAP expressed on a subset of CD4⁺FoxP3⁻Helios⁺ in non-activated setting

The co-expression of GARP/LAP were then analysed on non-activated CD3⁺CD4⁺FoxP3^{+/-}Helios^{+/-} T cell subsets. Different FoxP3^{+/-}Helios^{+/-} CD4⁺ T cell subsets were gated (Figure 27A). GARP/LAP was expressed mainly on Helios⁺FoxP3^{+/-} T cells and it was expressed at negligible levels on non-activated CD4⁺FoxP3⁺ Tregs. Interestingly, the only subpopulation that expressed significantly higher levels of GARP/LAP was CD4⁺FoxP3⁻Helios⁺ (4.66 ± 0.86%) Tregs compared with other subpopulations (Figure 27B).

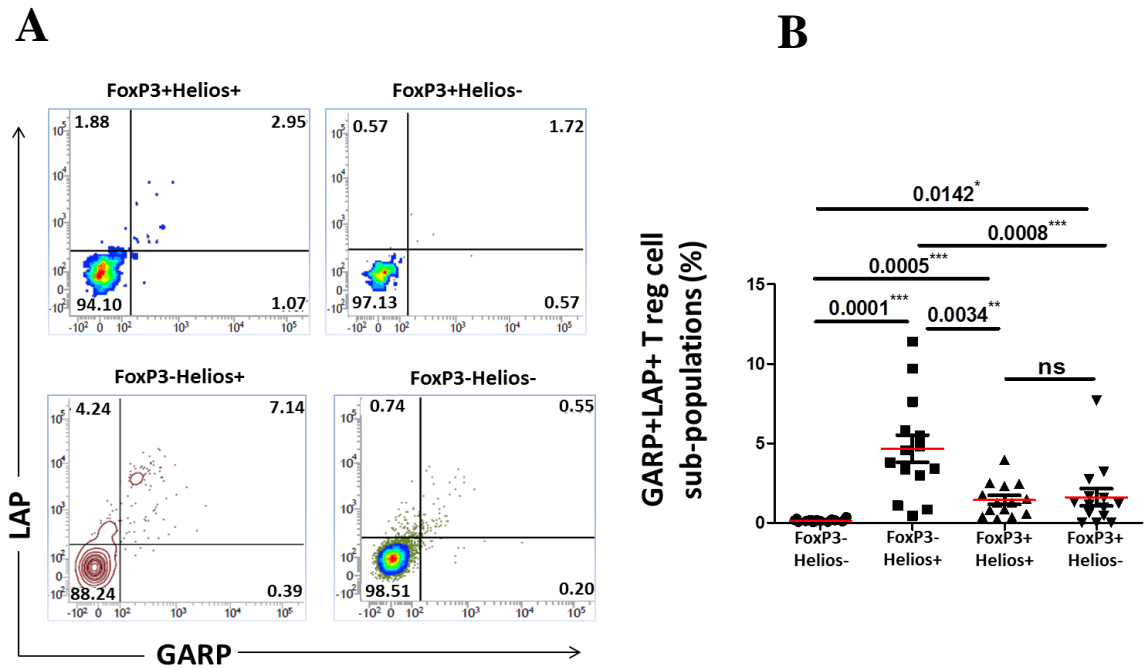


Figure 27: Percentages of non-activated CD3⁺CD4⁺FoxP3^{+/-}Helios^{+/-} Tregs expressing GARP and LAP.

Representative flow cytometry plots showing LAP against GARP, gated on FoxP3⁺Helios⁺, FoxP3⁺Helios⁻, FoxP3⁻Helios⁺, and FoxP3⁻Helios⁻ CD3⁺CD4⁺ T cells (A). Scatter plots show the mean percentage \pm SEM expression of GARP and LAP gated from FoxP3^{+/-}Helios^{+/-} T cells. The expression of GARP and LAP on PBMCs were analysed from 14 healthy donors. GARP and LAP were expressed at significantly higher levels on FoxP3⁻Helios⁺ T cells compared to FoxP3⁻Helios⁻, FoxP3⁺Helios⁺, and FoxP3⁺Helios⁻ T cells (B).

3.2.5 GARP/LAP expressed on Helios⁺, regardless of FoxP3 expression, in activated CD4⁺ T cells

GARP and LAP expression on FoxP3^{+/-}Helios^{+/-} CD4⁺ T cell subsets following TCR stimulation were further analysed. Different FoxP3^{+/-}Helios^{+/-} CD4⁺ T cell subsets were gated, as shown in Figure 28A. As expected, GARP and LAP were expressed at much higher levels on activated CD4⁺ T cells, compared with non-activated CD4⁺ T cells. Figure (28A & B) shows percentages of CD4⁺ T cells expressing GARP and LAP within the different subsets. Interestingly, GARP and LAP were mainly expressed on FoxP3⁺Helios⁺ (26.07 ± 3.55%) and FoxP3⁻Helios⁺ (13.68 ± 3.16%) T cells and to a significantly lower level on FoxP3⁺Helios⁻ T cells (6.51 ± 0.92%), but not on FoxP3⁻Helios⁻ (0.46 ± 0.06%) CD4⁺ T cells.

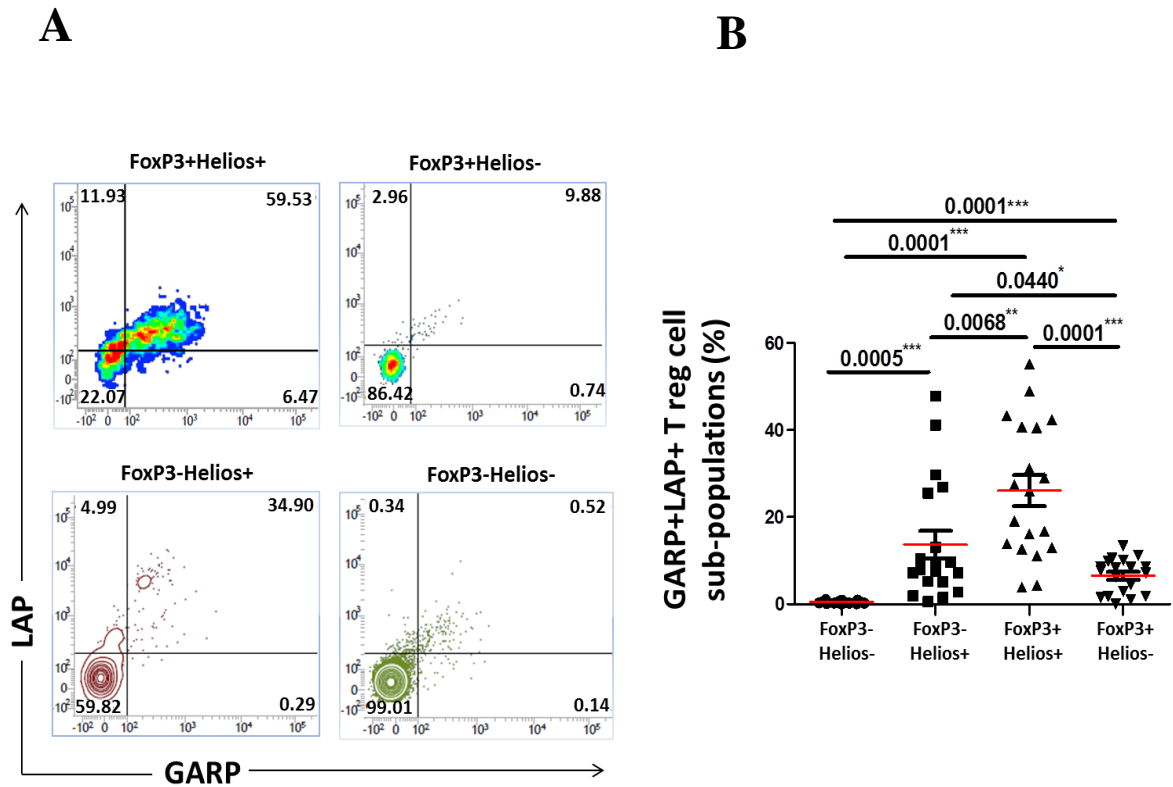


Figure 28: Percentages of activated $CD3^+CD4^+FoxP3^{+/-}Helios^{+/-}$ Tregs expressing GARP and LAP.

Representative flow cytometric plots showing $FoxP3^-Helios^-$, $FoxP3^-Helios^+$, $FoxP3^+Helios^+$ and $FoxP3^+Helios^-$ T cell subsets and the expression of GARP/LAP within these subsets in activated cells (A). Scatter plots show the mean percentage \pm SEM expression of GARP and LAP gated on $FoxP3^{+/-}Helios^{+/-}$ T cells. The expression of GARP and LAP on PBMCs were analysed from 19 healthy donors. In the activated T cells, GARP and LAP are mostly expressed on the surface of $FoxP3^+Helios^+$ followed by $FoxP3^-Helios^+$ Tregs, and to a significantly lower level on $FoxP3^+Helios^-$ and $FoxP3^-Helios^-$ $CD4^+$ T cells (B).

3.3 Investigating the expression level of FoxP3, Helios, GARP and LAP in CD4⁺ T cells isolated from healthy donors, chronic pancreatitis, pancreatic cancer, and liver metastatic from colorectal cancer patients

3.3.1 LAP is expressed significantly higher than GARP on activated CD4⁺ T cells in healthy donors and pancreatic cancer patients

The expression of GARP and LAP on T cells in the setting of cancer has been studied, but the nature of their expression remains uncertain. CD4⁺LAP⁺ Tregs subset accumulates at tumour sites, and it associates with cancer progression, regardless of FoxP3 expression. Therefore, the expression of GARP and LAP were compared on CD4⁺ T cells isolated from the peripheral blood of healthy donors (Figure 29A) and pancreatic cancer patients (Figure 29C). GARP and LAP were expressed at low levels on resting CD4⁺ T cells, but after activation with anti-CD3/28 within 18-20 hours, both GARP and LAP were significantly upregulated on activated CD4⁺ T cells, although expression of LAP was higher than GARP on CD4⁺ T cells. This difference was significant in both healthy donors (LAP: $3.15 \pm 0.35\%$ vs. GARP $2.46 \pm 0.39\%$; $P= 0.0256$; Figures 29B) and pancreatic cancer patients (LAP: $5.41 \pm 0.51\%$ vs. GARP: $4.73 \pm 0.52\%$; $P= 0.0341$; Figures 29D). This method was also investigated on CP and LI/CRC samples as can be seen in Table 5. The table shows the average of the expression level of GARP or LAP on CD3⁺CD4⁺ T cells isolated from healthy donors, chronic pancreatitis, pancreatic cancer, and liver metastases from colorectal cancer patients. The results demonstrated that in all samples, the population of T cells that expressed LAP were more than the T cells that expressed GARP on its surface (Table 5).

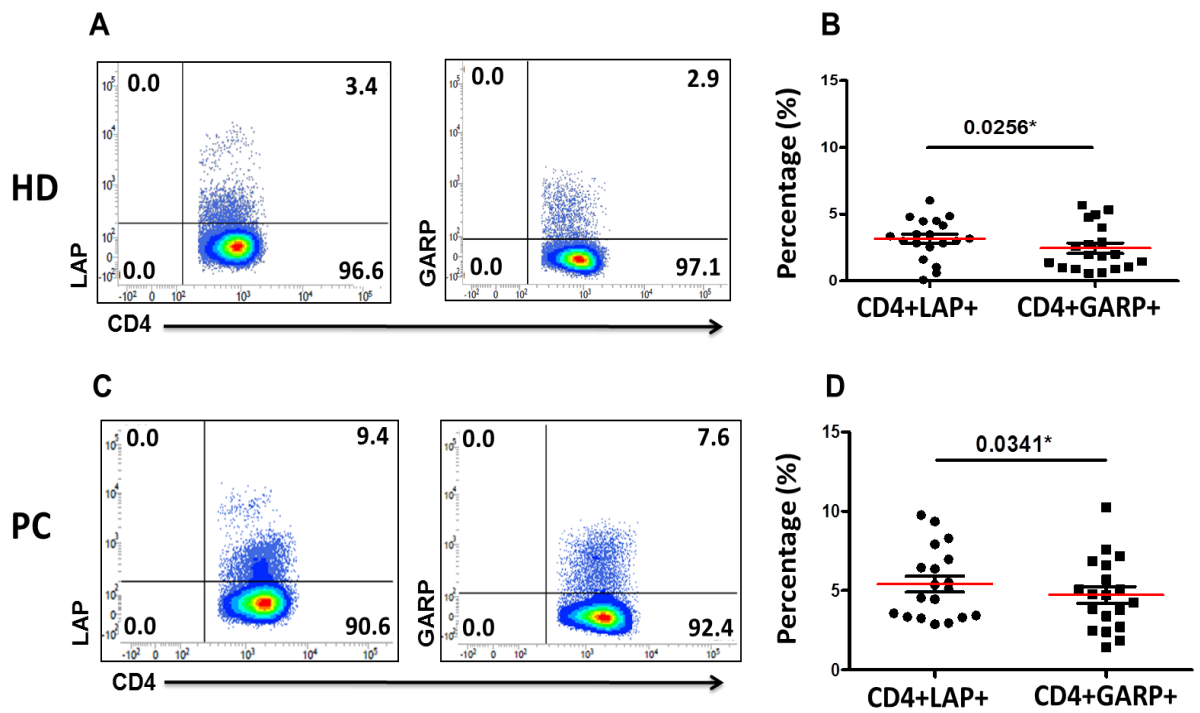


Figure 29: Expression of GARP or LAP on activated CD4⁺ T cells.

PBMCs from 19 healthy donors and 19 pancreatic cancer patients were activated and stained with GARP and LAP. Representative flow cytometric plots showing GARP or LAP expression on CD3⁺CD4⁺ T cells isolated from healthy donors (A) and PC patients (C). Scatter plots present the mean percentages \pm SEM of CD4⁺LAP⁺ T cells compared with CD4⁺GARP⁺ T cells in activated PBMCs isolated from healthy donors (B) and PC patients (D).

Table 5: Expression of GARP or LAP on activated PBMC isolated from HD and Patients

The table shows the average and the standard deviation of both GARP and LAP that are expressed on activated CD3⁺CD4⁺ T cells isolated from healthy donors, chronic pancreatitis, pancreatic cancer, and liver metastatic from colorectal cancer patients.

ACTIVATED CD3⁺CD4⁺ T cells	Healthy Donors (HD)	Chronic Pancreatitis (CP)	Pancreatic Cancer (PC)	Liver Metastatic from Clorectal Cancer (LI/CRC)
GARP (Average)	2.457	3.596	4.726	5.405
GARP (Standard deviation)	1.700	1.039	2.251	2.053
LAP (Average)	3.153	4.253	5.413	5.642
LAP (Standard deviation)	1.534	1.744	2.216	2.572

3.3.2 Levels of FoxP3⁺LAP⁻, FoxP3⁺LAP⁺, and FoxP3⁻LAP⁺ Treg subsets in cancer patients and controls

Different Tregs-related markers are also important to determine Tregs level. Therefore, several significant populations of Tregs have been found with regards to cancer environment, including natural CD4⁺FoxP3⁺ Tregs. In this study, the co-expression of FoxP3 and LAP on non-activated CD4⁺ T cells were investigated in Figure 30A. The results demonstrated that LAP was co-expressed with FoxP3 at deficient levels on non-activated CD4⁺ Tregs isolated from HD, CP, PC and LI/CRC. There were no any significant increases in FoxP3⁺LAP⁻ Tregs subset in PBMCs from PC ($3.46 \pm 0.28\%$), compared to HD ($3.04 \pm 0.36\%$) and CP ($3.46 \pm 0.41\%$). However, the PBMCs from LI/CRC patients ($6.90 \pm 1.11\%$) were significantly increased compared with PC patients ($P=0.0004$) and HDs ($P=0.0005$) (Figure 30A & B). In all samples, FoxP3⁺LAP⁺ T cell subsets were in a tiny population, but it was higher in LI/CRC patients ($0.19 \pm 0.03\%$) compared with healthy donors ($0.10 \pm 0.02\%$) (Figure 30C). Tregs as FoxP3⁻LAP⁺ subset showed an interesting result, there was significant increases both in LI/CRC ($1.98 \pm 0.37\%$) and PC ($1.39 \pm 0.21\%$) samples, compared with HDs ($0.70 \pm 0.11\%$) and CP controls ($0.60 \pm 0.25\%$) (Figure 30D).

The level of LAP^{+/-} and FoxP3^{+/-} CD4⁺ T cell subsets in healthy donors and patients were further analysed following *In vitro* activation with anti-CD3/28 (Figure 31A). The FoxP3⁺LAP⁻ Treg subset was significantly expanded only in LI/CRC ($6.25 \pm 0.85\%$) compared with HD ($3.82 \pm 0.42\%$) (Figure 31B), and they were higher than PC ($4.60 \pm 0.40\%$) although this did not reach significance. Noticeably, FoxP3⁺LAP⁺ Treg subset was greater inactivated samples (Range in all groups: 1.06 – 1.64) (Figure 31C), compared with the non-activated samples (Figures 30C). Furthermore, FoxP3⁻LAP⁺ Treg subset increased in activated cells

(Range in all groups: 1.77 – 3.63), compared with non-activated cells (Range in all groups: 0.6 – 1.98). Similar to non-activated cells, the FoxP3⁻LAP⁺ subset was expanded in activated cells both in LI/CRC ($3.63 \pm 0.50\%$) and PC ($3.27 \pm 0.37\%$), compared with HDs ($1.79 \pm 0.27\%$) and CP controls ($1.77 \pm 0.30\%$) (Figure 31D).

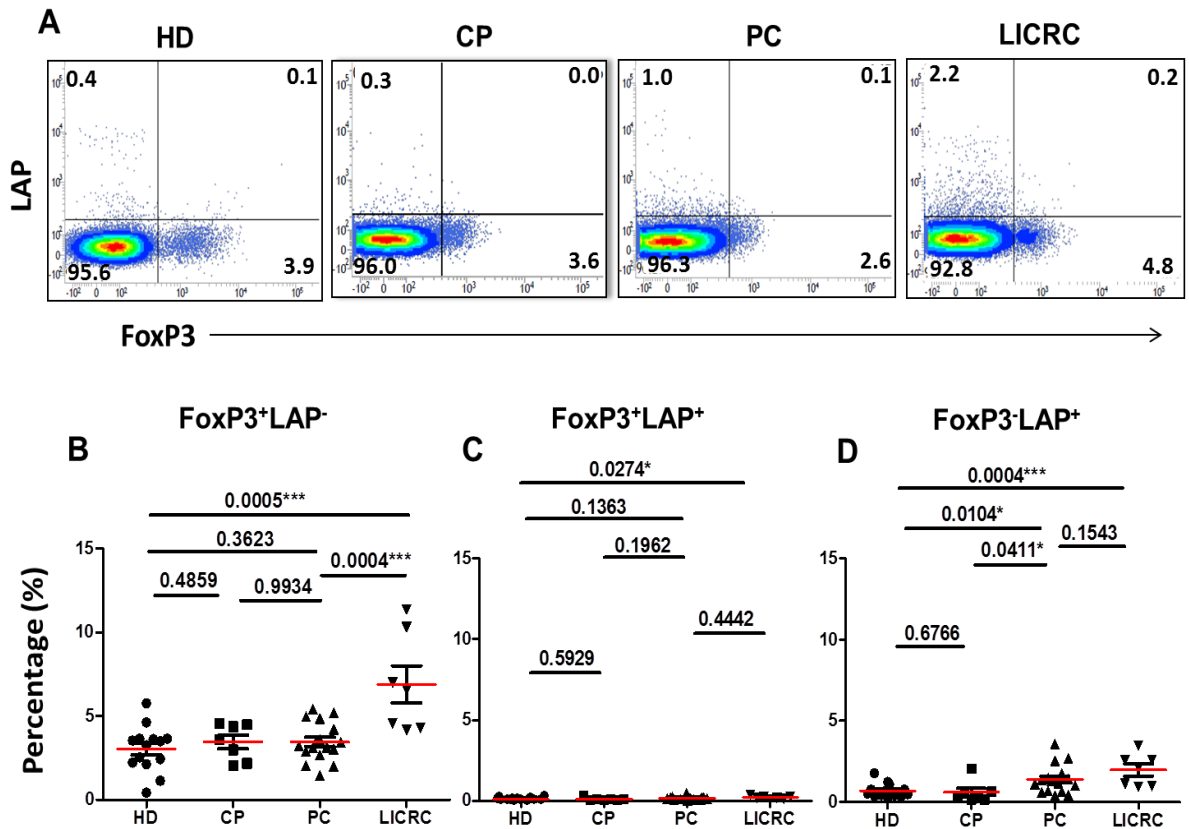


Figure 30: Comparisons between healthy donors and patients for the expression of LAP on non-activated FoxP3^{+/-} T cell subsets.

Thawed PBMCs isolated from 14 HD, 7 CP, 17 PC, and 7 LI/CRC patients were stained for surface and intracellular markers. Representative flow cytometric plots are demonstrating FoxP3 expression against LAP gated on CD3⁺CD4⁺ T cells from healthy donors and patients (A). Scatter plots display the mean percentages \pm SEM of FoxP3⁺LAP⁻ (B), FoxP3⁺LAP⁺ (C) and FoxP3⁻LAP⁺ T cells (D).

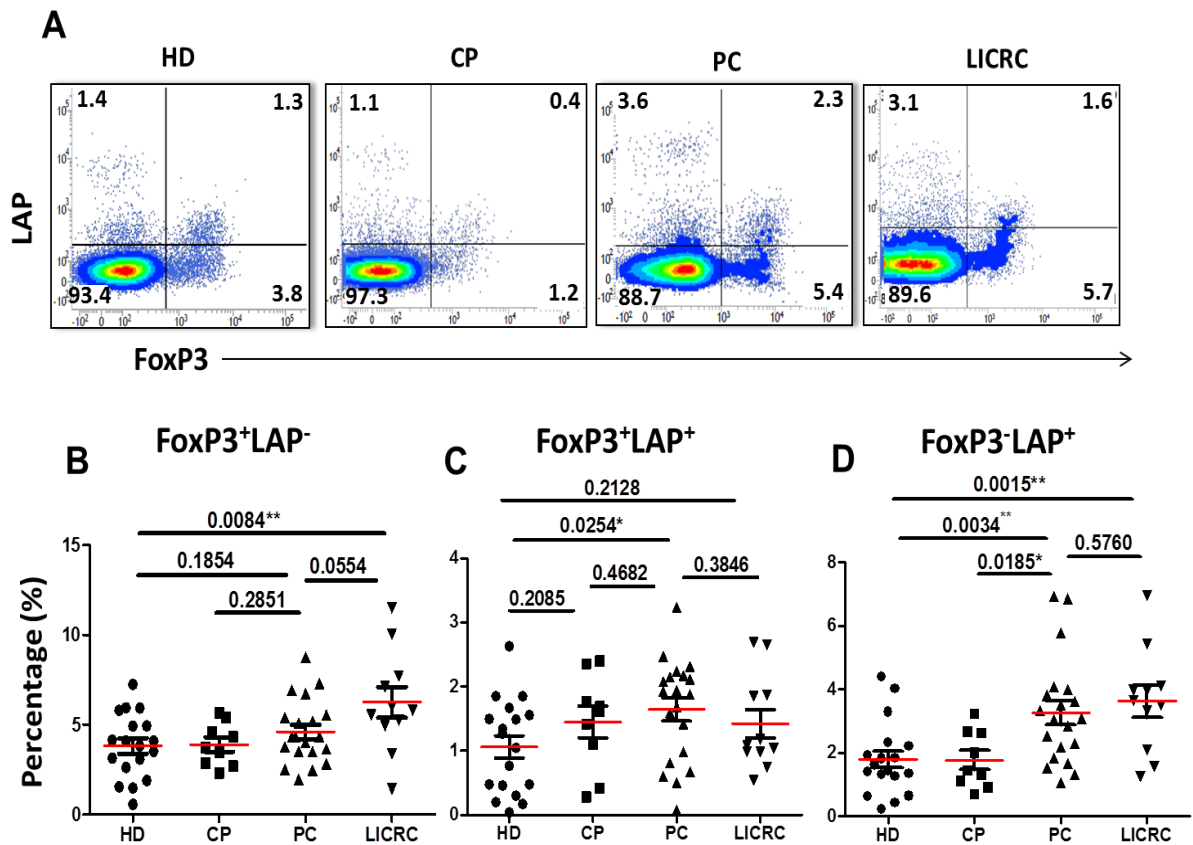


Figure 31: Comparisons between healthy donors and patients for the expression of LAP on activated FoxP3^{+/-} T cell subsets.

Thawed PBMCs isolated from 18 HD, 9 CP, 20 PC, and 11 LI/CRC patients were activated with anti-CD3/28 and then stained for intracellular and surface markers. Representative flow cytometric plots showing FoxP3 expression against LAP as gated on CD3⁺CD4⁺T cells from both healthy donors and patients (A). Scatter plots present the mean percentages \pm SEM of FoxP3⁺LAP⁻ (B), FoxP3⁺LAP⁺ (C) and FoxP3⁻LAP⁺ T cells (D).

3.3.3 Levels of Helios⁺LAP⁻, Helios⁺LAP⁺, Helios⁻LAP⁺ Treg subsets in cancer patients and controls

Multiple studies suggest that Helios expression has been associated with Tregs and for this reason, this research went on to examine CD4⁺ T cells by analysing the co-expression level of LAP and Helios on non-activated CD4⁺ T cell subsets (Figure 32A). The result shows that the expression level of Helios⁺LAP⁻ on non-activated CD4⁺ Tregs were significantly higher in LI/CRC ($9.27 \pm 1.27\%$) compared with HD ($4.97 \pm 0.46\%$) and PC ($5.05 \pm 0.35\%$) (Figure 32B). However, in the double positive Helios⁺LAP⁺ Tregs, the co-expression level of Helios and LAP were much lower. Despite the very small population; LI/CRC ($0.55 \pm 0.15\%$) was significantly higher than HD ($0.22 \pm 0.04\%$) (Figure 32C). On the other hand, in Helios⁻LAP⁺ subset, there were significant increases both in LI/CRC ($1.60 \pm 0.29\%$) and PC ($1.04 \pm 0.14\%$) samples, compared with HD ($0.44 \pm 0.08\%$) and CP ($0.31 \pm 0.09\%$) controls (Figure 32D).

To further determine CD4⁺ Treg subset, the expression level of LAP on Helios[±] was investigated on activated CD4⁺ Tregs (Figure 33A). The expression level of Helios⁺LAP⁻ CD4⁺Tregs in LI/CRC ($6.37 \pm 0.71\%$) patients were significantly higher compared with HDs ($4.52 \pm 0.55\%$) and PC ($4.03 \pm 0.28\%$) patients (Figure 33B). Remarkably, the expression level of the double positive Helios⁺LAP⁺ Treg subpopulations was higher than the non-activated Tregs. Moreover, there were significant increases both in LI/CRC ($2.42 \pm 0.41\%$) and PC ($2.73 \pm 0.33\%$) patients, compared with HDs ($1.42 \pm 0.24\%$) (Figure 33C). Of note, the CD4⁺ Treg subpopulations expressing LAP and not Helios were relatively significant in both LI/CRC ($2.16 \pm 0.39\%$) and PC ($2.31 \pm 0.18\%$) samples, compared with HD ($1.04 \pm 0.13\%$) and CP ($1.15 \pm 0.24\%$) controls (Figure 33D).

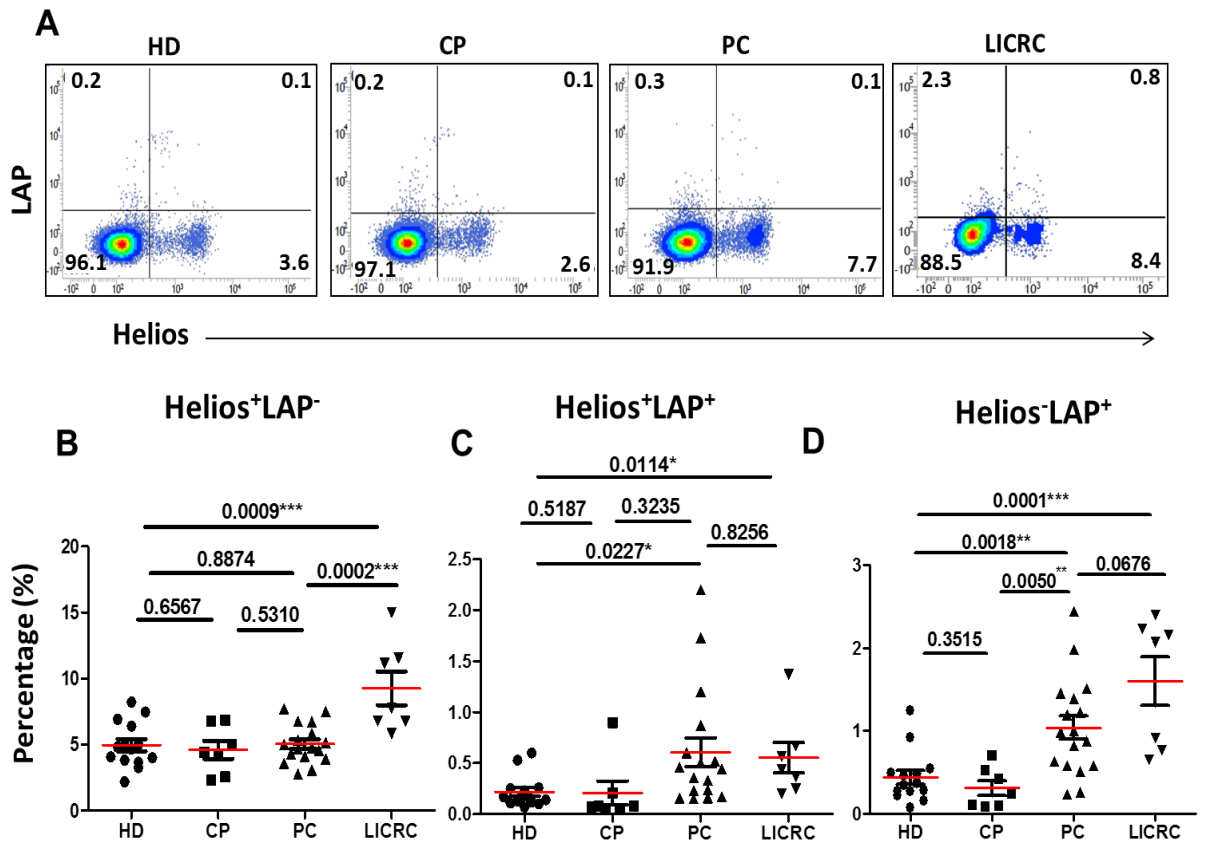


Figure 32: Comparisons between healthy donors and patients for the expression of LAP on non-activated Helios^{+/-} T cell subsets.

Thawed PBMCs isolated from 14 HD, 7 CP, 17 PC, and 7 LI/CRC patients were activated with anti-CD3/28 and then stained with surface and intracellular markers. Representative flow cytometric plots showing Helios expression against LAP, as gated on CD3⁺CD4⁺ T cells from both healthy donors and patients (A). Scatter plots present the mean percentages \pm SEM of Helios⁺LAP⁻ (B), Helios⁺LAP⁺ (C) and Helios⁻LAP⁺ T cells (D).

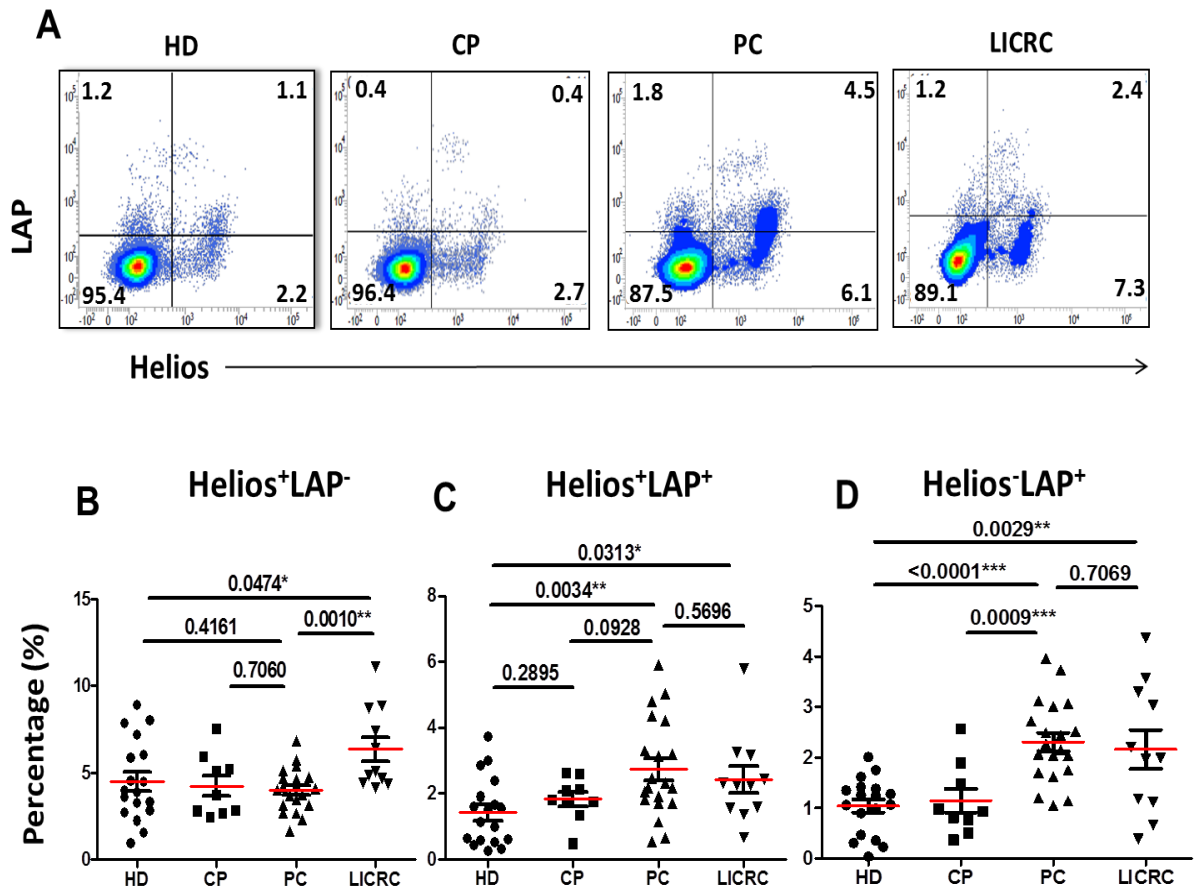


Figure 33: Comparisons between healthy donors and patients for the expression of Helios^{+/-} LAP^{+/-} T cell subsets in the activated setting.

Thawed PBMCs isolated from 18 HD, 9 CP, 20 PC, and 11 LI/CRC patients were activated with anti-CD3/28 and then stained with surface and intracellular markers. Representative flow cytometric plots showing Helios expression against LAP, as gated on CD3⁺CD4⁺T cells, from both healthy donors and patients (**A**). Scatter display the mean percentages \pm SEM of Helios⁺LAP⁻ (**B**), Helios⁺LAP⁺ (**C**), and Helios⁻LAP⁺ T cells (**D**).

3.3.4 FoxP3^{+/-}Helios⁺ T cells are expanded in liver metastatic from colorectal cancer patients in rested and activated setting

FoxP3 and Helios staining were combined, and FoxP3^{+/-}Helios^{+/-} T cell subpopulations isolated from HD, CP, PC and LI/CRC in non-activated (Figure 34A) and activated (Figure 35A) settings were analysed. In the non-activated PBMCs, the subpopulations of FoxP3⁻Helios⁺ and FoxP3⁺Helios⁺ were significantly higher compared to FoxP3⁺Helios⁻ T cells in all subgroups (Figure 34B). Interestingly, FoxP3⁺Helios⁺ and FoxP3⁻Helios⁺ T cell subsets were expanded only in peripheral blood of LI/CRC ($6.21 \pm 1.10\%$ & $5.50 \pm 0.70\%$) samples, compared with PC ($3.10 \pm 0.28\%$ & $3.21 \pm 0.33\%$) and HDs ($2.96 \pm 0.30\%$ & $2.95 \pm 0.37\%$). However, there was no significant difference in the level of FoxP3⁺Helios⁻ T cells, between patients and controls (Figure 34B).

The results in the activated PBMC showed similar pattern compared to the non-activated PBMC. Following TCR stimulation, the subpopulations level of FoxP3⁻Helios⁺ and Foxp3⁺Helios⁺ T cells expressed significantly higher on LI/CRC ($5.97 \pm 0.72\%$ & $5.11 \pm 0.67\%$) patients compare with PC ($3.6 \pm 0.28\%$ & $4.35 \pm 0.411\%$) and HD ($2.96 \pm 0.32\%$ & $4.06 \pm 0.37\%$). However, comparing to the non-activated cells, FoxP3⁺Helios⁺ and FoxP3⁻Helios⁺ T cell subsets in all four groups did not significantly expand following activation. On the other hand, FoxP3⁺Helios⁻ subset developed following activation, but there were no significant differences between the samples (Range in all groups: activated: $1.77 - 2.45\%$) (Figure 35B).

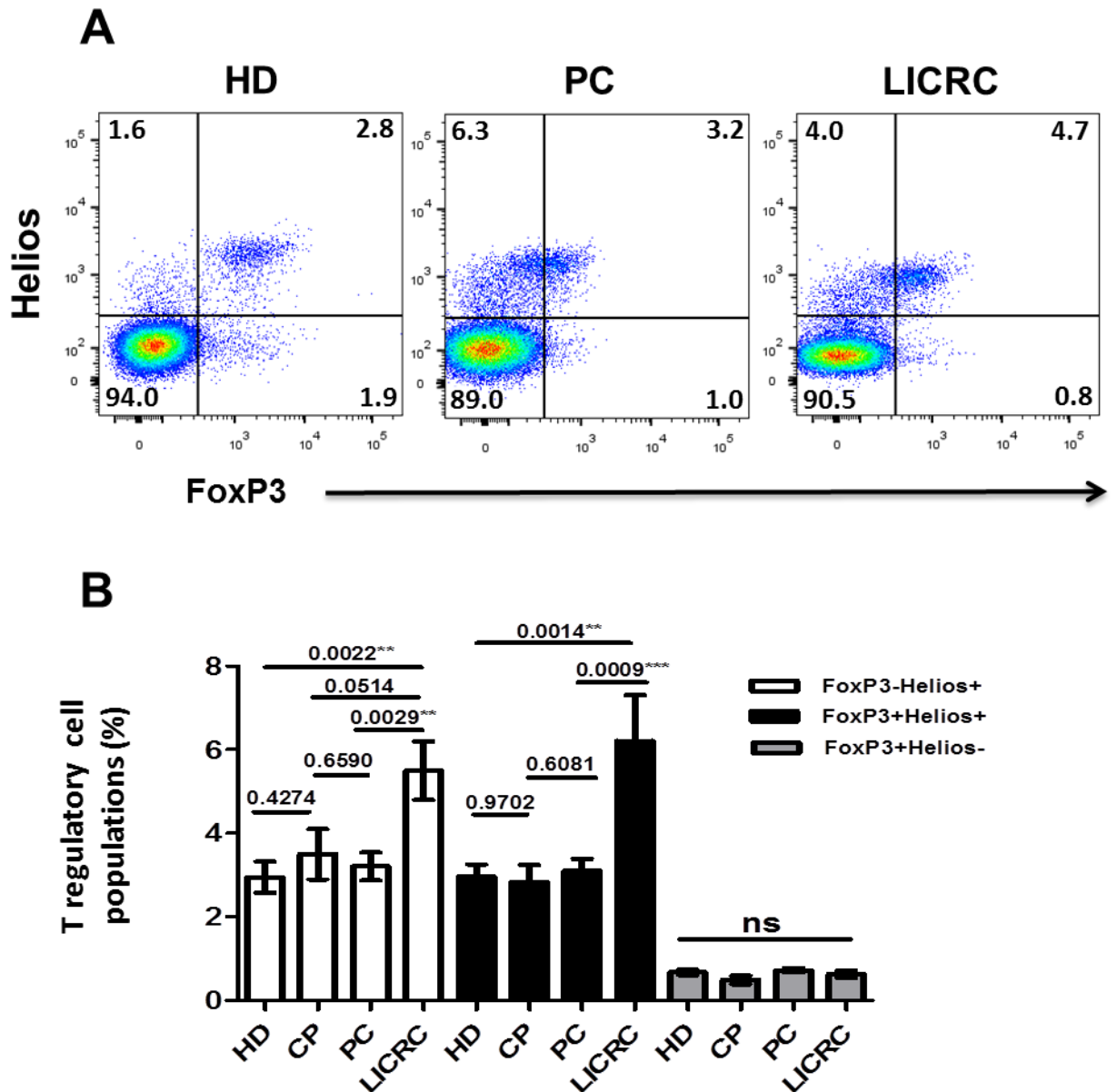


Figure 34: Expression of FoxP3 and Helios on CD3⁺CD4⁺ non-activated T cells.

Representative flow cytometric plots showing the expression level of FoxP3 against Helios in healthy donors and cancer patients (A). The bar chart shows the mean percentages \pm SEM of FoxP3⁻Helios⁺, FoxP3⁺Helios⁺ and FoxP3⁺Helios⁻ T cell subsets in non-activated PBMCs isolated from 14 HD, 7 CP, 17 PC, and 7 LI/CRC patients (B).

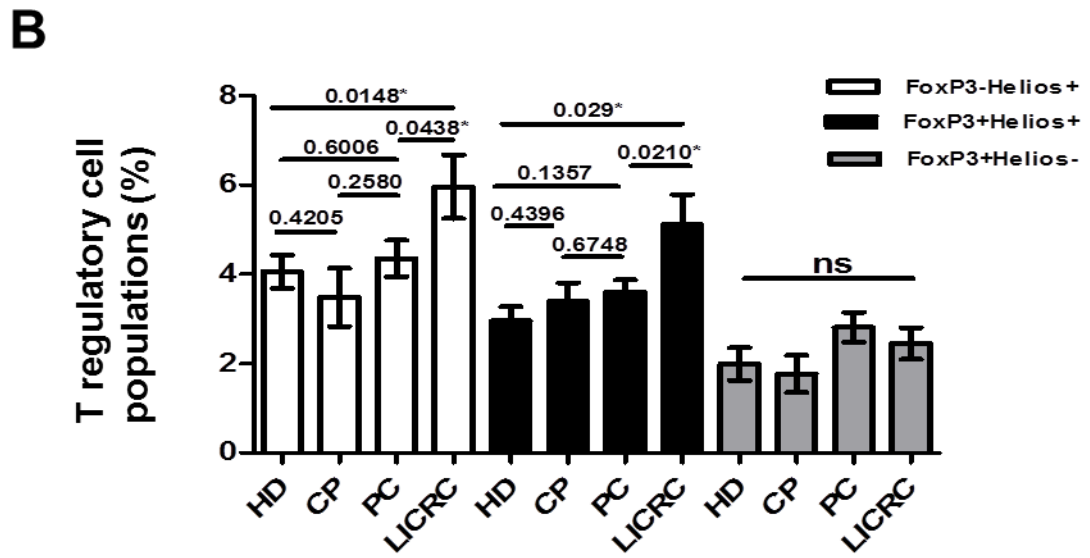
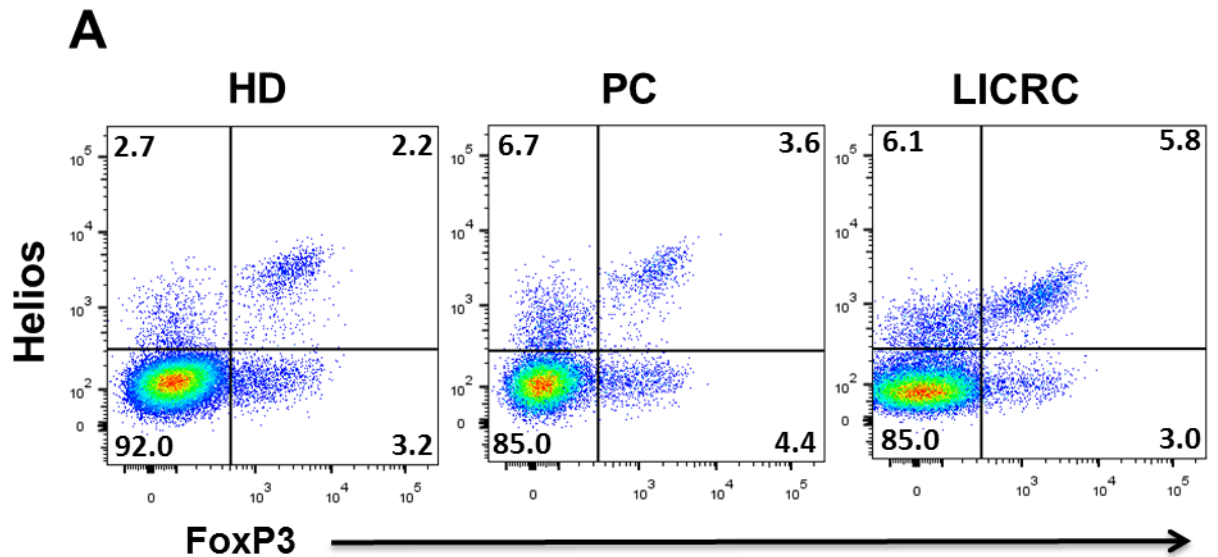


Figure 35: Expression of FoxP3 and Helios on activated CD3⁺CD4⁺ T cells.

Representative flow cytometric plots showing the expression level of FoxP3 against Helios in healthy donors and cancer patients (A). The bar chart shows the mean percentages \pm SEM of FoxP3⁻Helios⁺, FoxP3⁺Helios⁺ and FoxP3⁺Helios⁻ T cell subsets in stimulated PBMCs isolated from 18 HD, 9 CP, 20 PC and 11 LI/CRC patients (B).

3.4 Expression of GARP/LAP on FoxP3^{+/-}Helios^{+/-} T cell subsets in cancer patients, compared to controls

Since Tregs level increases in cancer patients and they stimulate suppressive cytokines against the immune responses, PBMCs were isolated from PC and LI/CRC patients. The co-expression of GARP and LAP on FoxP3^{+/-}Helios^{+/-} T cell subsets in cancer patients and controls investigated in the non-activated (Figure 36A) and the activated settings (Figure 37A & 38A).

This study provides clear evidence that in all samples; HD, CP, PC, and LI/CRC, the expression of GARP and LAP were significantly higher in non-activated FoxP3⁻Helios⁺ Tregs compare to the rest of the subpopulations (Figure 36B & C). The level of CD4⁺FoxP3⁻Helios⁺GARP⁺LAP⁺ Tregs was significantly expanded in LI/CRC samples ($10.41 \pm 3.09\%$) compared with HDs ($4.66 \pm 0.86\%$, $P=0.0302$) (Figure 36B). Furthermore, there was no significant difference between PC and HD, despite the elevated Treg subsets in PC ($9.60 \pm 2.36\%$) compared with HD (Figure 36B). Of note, the expression level of GARP/LAP are significantly higher in FoxP3⁺Helios⁺ T cells compared with FoxP3⁻Helios⁻ T cell subset in PC ($P=0.0156$), LI/CRC ($P=0.0190$), and in HDs ($P=0.0005$) (Figure 36C).

Following TCR stimulation, GARP and LAP were significantly higher in activated FoxP3⁺Helios⁺ compared with FoxP3⁺Helios⁻ Tregs (Figure 37A & B). Likewise, the data further supported the notion that the level of CD3⁺CD4⁺GARP⁺LAP⁺ FoxP3⁺Helios⁺ Tregs is significantly higher in cancer patients; PC ($40.65 \pm 3.96\%$, $P=0.0096$) and LI/CRC ($41.02 \pm 3.23\%$, $P=0.0087$), compared with controls; HD ($26.07 \pm 3.55\%$). Although, the level of CD3⁺CD4⁺GARP⁺LAP⁺FoxP3⁺Helios⁺ Tregs was lower in CP patients ($33.17 \pm$

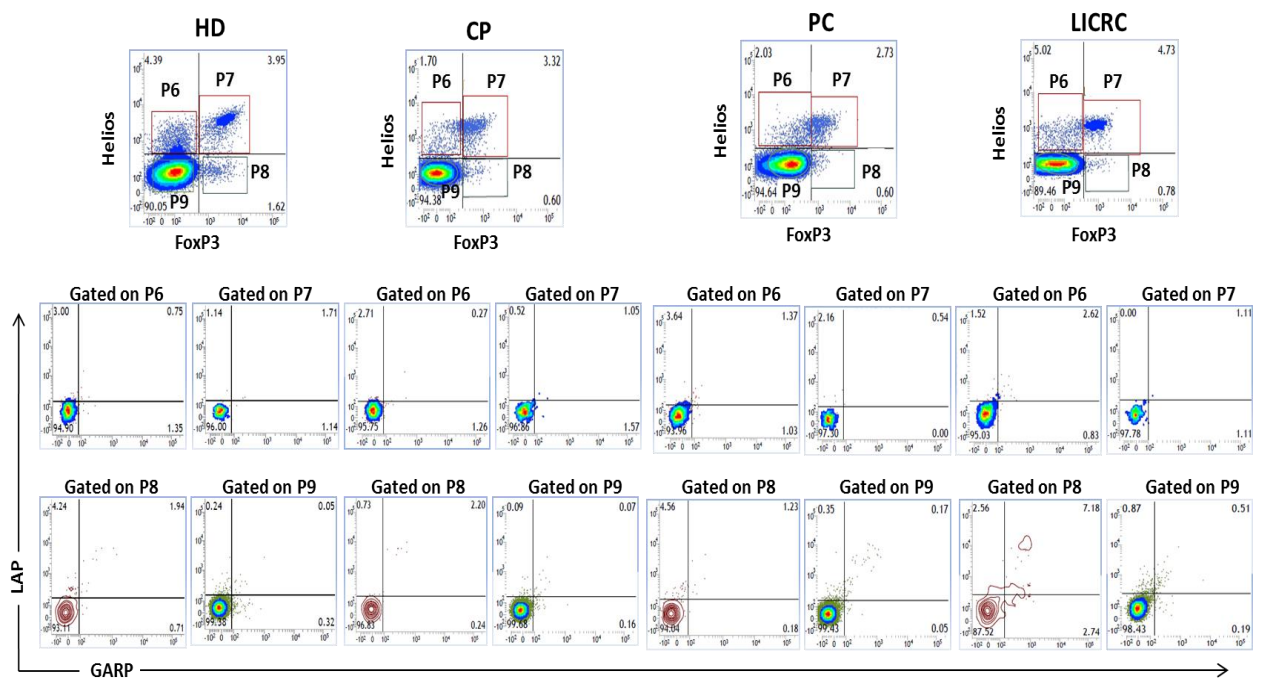
5.56%) compare with PC, but it did not reach significance (Figure 37B). Also, there were no differences between cancer patients and controls within CD3⁺CD4⁺GARP⁺LAP⁺FoxP3⁺Helios⁻ Tregs (Figure 37B).

Moreover, to further elucidate the nature of GARP and LAP expression on Tregs. A representative flow cytometry shows the expression level of GARP and LAP on FoxP3⁺Helios⁺ and FoxP3⁻Helios⁻ CD4⁺ T cells in cancer patients and controls (Figure 38A). The four groups were then compared with each other by analysing the expression of GARP and LAP on FoxP3⁻Helios⁺ and FoxP3⁻Helios⁻ CD4⁺ T cell subpopulations. In all four groups, the expression levels of GARP and LAP were significantly higher on FoxP3⁻Helios⁺ T cells compared to FoxP3⁻Helios⁻ T cells. Furthermore, GARP and LAP were mainly expressed in cancer patients compared with controls. The CD4⁺FoxP3⁻Helios⁺GARP⁺LAP⁺ subset was significantly expanded only in LI/CRC samples ($32.56 \pm 3.57\%$, $P= 0.0018$), compared with HDs ($13.68 \pm 3.16\%$). Despite the lack of significance, the level of CD4⁺FoxP3⁻Helios⁺GARP⁺LAP⁺ Tregs was higher in PC ($23.07 \pm 3.64\%$) compared to HD ($P= 0.0747$) (Figure 37B).

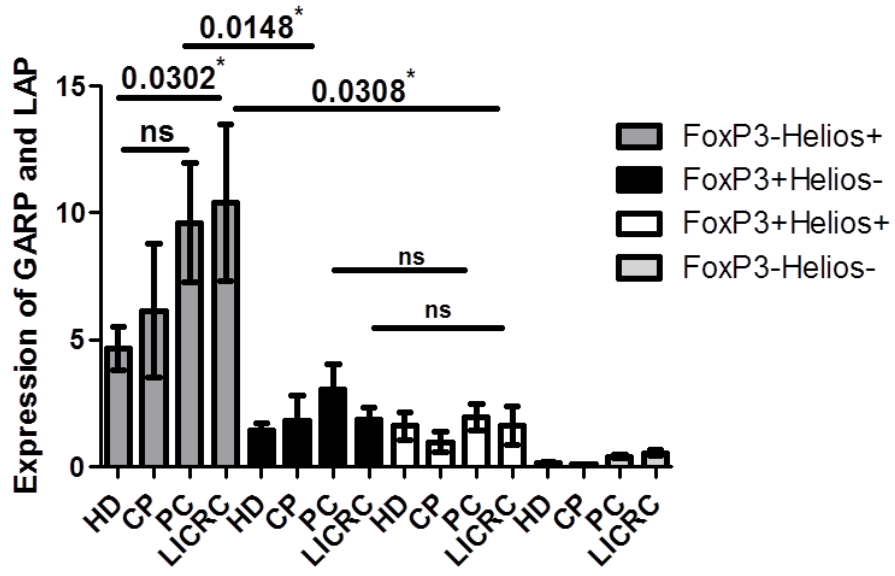
With different analysis, same result established GARP/LAP expressions on FoxP3⁺Helios⁻ and FoxP3⁻Helios⁻ T cells were significantly lower than their expressions on FoxP3⁺Helios⁺ and FoxP3⁻Helios⁺ T cells in HD and cancer patients (Figure 39A, B & C). There were no significant changes in GARP/LAP expression on FoxP3⁺Helios⁻ T cells between HD ($6.51 \pm 0.92\%$), CP ($7.57 \pm 1.99\%$), PC ($8.36 \pm 1.04\%$) and LI/CRC patients ($8.60 \pm 1.18\%$) (Figure 39D). Similarly, there were no significant differences of GARP and LAP between HD and patients in the double negative FoxP3⁻Helios⁻ T cells (Figure 39E).

Figures (36B), (37B), (38B) and (39B, C, D & E) compare the expression level of GARP/LAP between the different samples, while Figures (36C) and (39C) compare the expression level of GARP/LAP between the subpopulations.

A



B



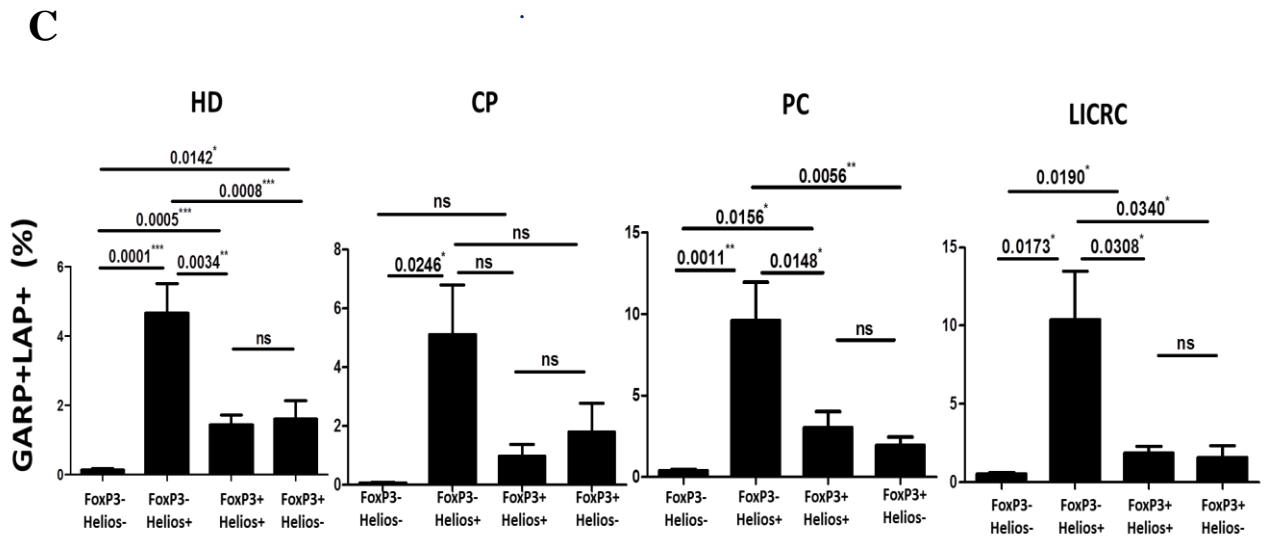
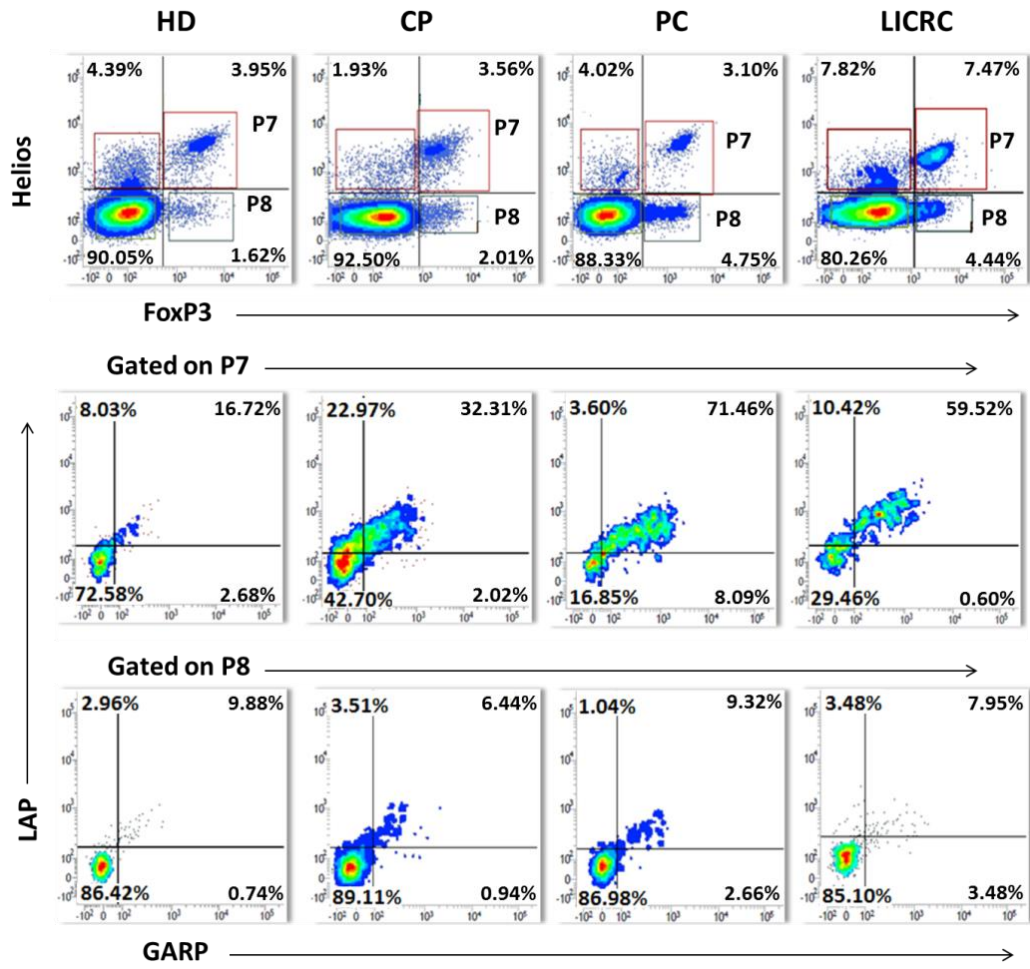


Figure 36: Expression of GARP and LAP on non-activated FoxP3^{+/-} Helios^{+/-} T cell subsets.

Representative flow cytometric plots showing GARP/LAP expression on non-activated FoxP3⁻Helios⁺ (gated on P6), FoxP3⁺Helios⁺ (gated on P7), FoxP3⁺Helios⁻ (gated on P8), and FoxP3⁻Helios⁻ (gated on P9) CD3⁺CD4⁺ T cell subsets isolated from HD, CP, PC, and LI/CRC patients (A). Bar charts comparing the mean percentages \pm SEM of GARP⁺LAP⁺ T cells within non-activated FoxP3⁻Helios⁺, FoxP3⁺Helios⁺, FoxP3⁺Helios⁻, and FoxP3⁻Helios⁻ T cell subsets isolated from 14 HD, 7 CP, 17 PC and 7 LI/CRC patients (B). Bar charts show the mean percentages \pm SEM of FoxP3⁻Helios⁺, FoxP3⁺Helios⁺, FoxP3⁺Helios⁻, and FoxP3⁻Helios⁻ T cell subsets in non-activated PBMC cells isolated from these various subgroups (C).

A



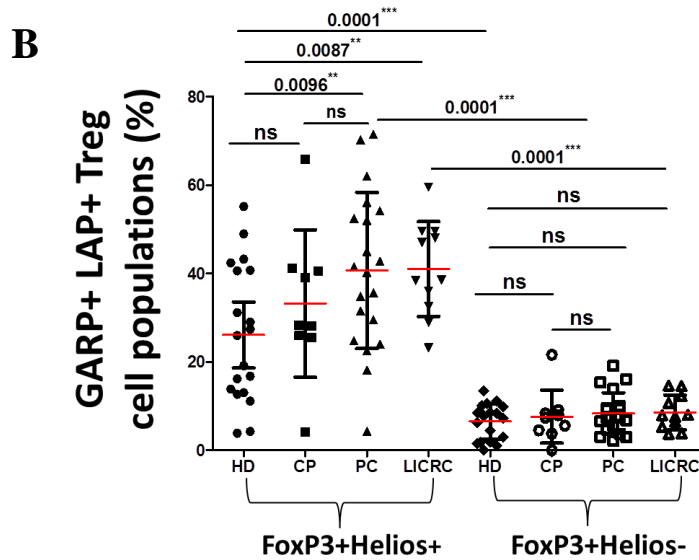
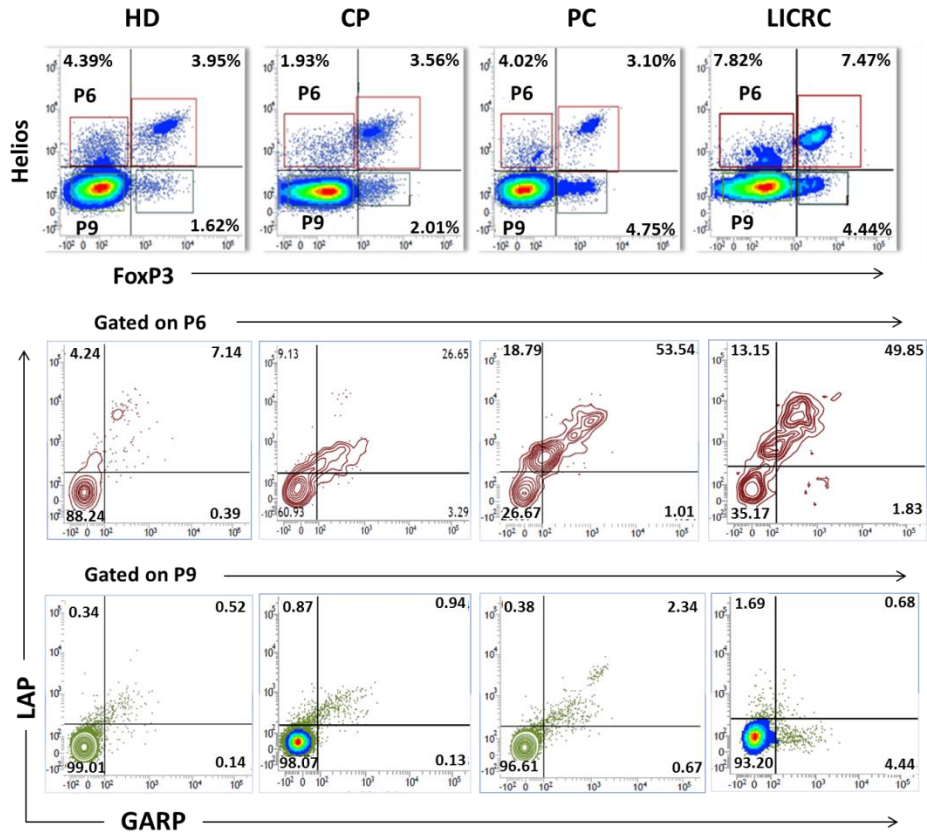


Figure 37: GARP and LAP expression on activated $CD3^+CD4^+Helios^{+/-}$ FoxP3⁺ Tregs.

Representative flow cytometric plots gated on $CD3^+CD4^+$ T cells shows the expression level of GARP and LAP on activated FoxP3⁺Helios⁺ (gated on P7) and FoxP3⁺Helios⁻ (gated on P8) Tregs isolated from HD, CP, PC, and LI/CRC cancer patients (A). Scatter plot diagram comparing the individual samples with each other, and show the mean percentages \pm SEM of Helios^{+/-} expressed on $CD4^+FoxP3^+GARP^+LAP^+$ Tregs (B).

A



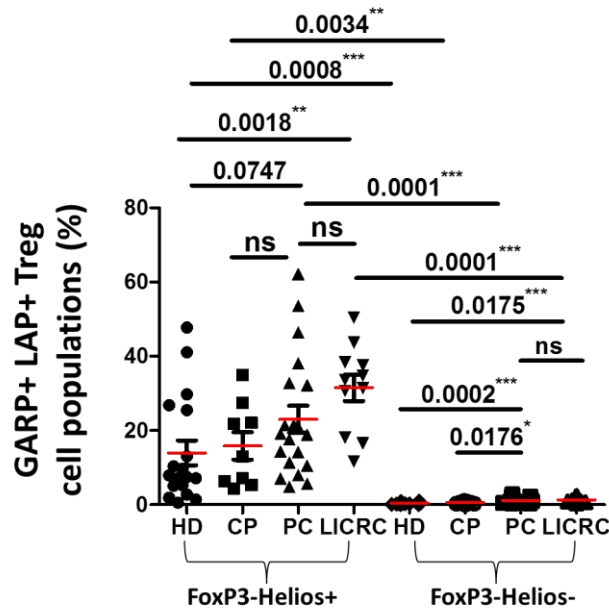
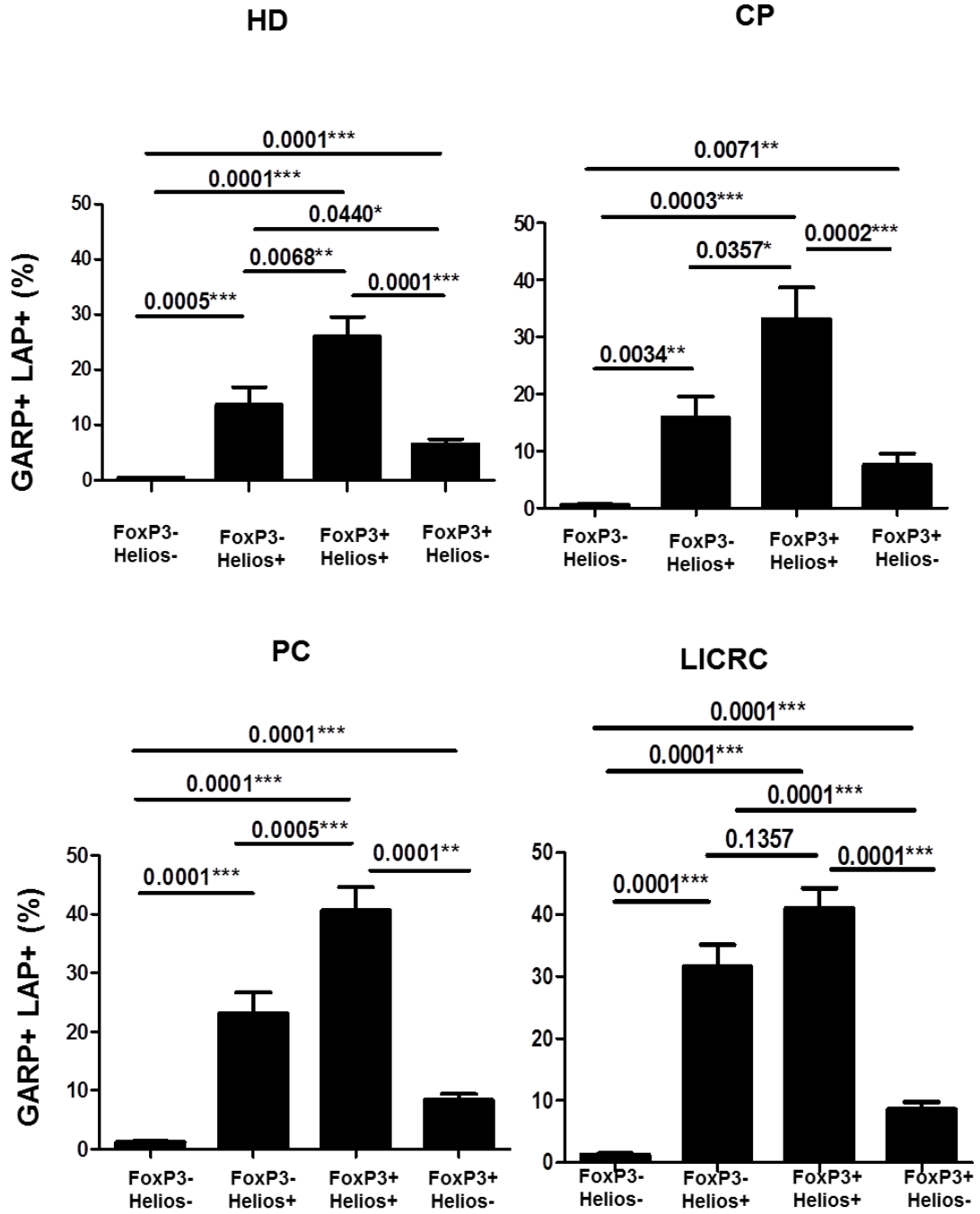
B

Figure 38: GARP and LAP expression on activated $CD3^+CD4^+Helios^{+/-}$ FoxP3⁻ Tregs.

Representative flow cytometric plots gated on $CD3^+CD4^+$ T cells shows the expression level of GARP and LAP on T regulatory cells of HD, CP, PC, and LI/CRC cancer patients. The activated FoxP3⁻Helios⁺GARP⁺LAP⁺ Tregs (gated on P6) and FoxP3⁻Helios⁻GARP⁺LAP⁺ Tregs (gated on P9) are shown (A). Scatter plot diagram comparing individual samples with each other's, and show the mean percentages \pm SEM of Helios expression (Helios⁺ and Helios⁻) in $CD4^+FoxP3^+GARP^+LAP^+$ Tregs (B).

A



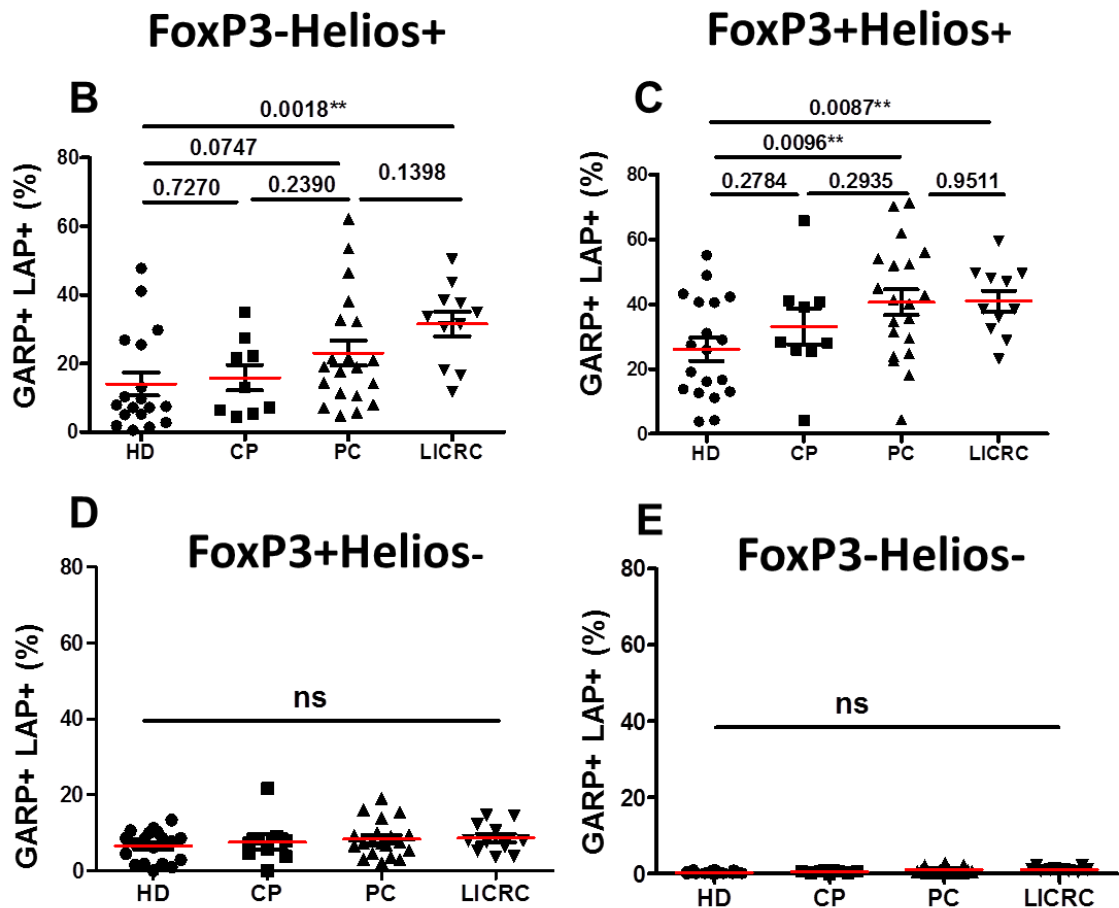


Figure 39: Expression of GARP and LAP on different FoxP3^{+/-}Helios^{+/-} T cell subsets in the activated setting.

The bar chart shows the mean percentages \pm SEM of GARP⁺LAP⁺ T cells within FoxP3^{+/-}Helios^{+/-} T cell subsets in activated PBMCs isolated from 18 HD, 9 CP, 20 PC and 11 LI/CRC patients (A). Scatter plots correlating the mean percentages \pm SEM of GARP⁺LAP⁺ T cells within activated FoxP3⁻Helios⁺ (B), FoxP3⁺Helios⁺ (C), FoxP3⁺Helios⁻ (D), and FoxP3⁻Helios⁻ (E) T cell subset in HD, CP, PC and LI/CRC patients.

Chapter 4

Results

4.1 The expression level of GARP or LAP on FoxP3^{+/-}Helios^{+/-} T cells

In vitro, the expression levels of GARP or LAP on FoxP3^{+/-}Helios^{+/-} T cells isolated from these four different groups (HD, CP, PC, and LI/CRC) were also assessed with non-activated and activated suppression assay.

4.1.1 LAP expression on FoxP3^{+/-}Helios^{+/-} non-activated T cells

In non-activated steady-state, figure 40 shows a comparison between the subsets for each group. In healthy donors, the result shows that LAP and not GARP (GARP⁻LAP⁺) was expressed significantly higher on FoxP3⁻Helios⁺ ($5.12 \pm 1.14\%$) T cells compared to FoxP3⁺Helios⁻ ($1.79 \pm 0.45\%$) and FoxP3⁻Helios⁻ ($0.37 \pm 0.10\%$) T cells. Furthermore, the expression level of LAP (GARP⁻LAP⁺) was significantly higher on the double positive (FoxP3⁺Helios⁺) ($2.98 \pm 0.47\%$) T cells compared to FoxP3⁺Helios⁻ ($P= 0.0135$) and FoxP3⁻Helios⁻ ($P= 0.0001$) T cells. Remarkably, FoxP3⁺Helios⁻ T cells also expressed a significantly higher level of LAP on its surface compared to FoxP3⁻Helios⁻ ($P= 0.0053$) T cells (Figure 40A). In chronic pancreatitis, on the other hand, LAP (GARP⁻LAP⁺) expression was significantly higher only on FoxP3⁺Helios⁺ ($5.52 \pm 1.69\%$) T cells compared to FoxP3⁺Helios⁻ ($0.88 \pm 0.35\%$, $P= 0.0283$) and FoxP3⁻Helios⁻ ($0.20 \pm 0.04\%$, $P= 0.0202$) T cells (Figure 40B). Similar results were shown in pancreatic cancer patients compare to healthy donors. In pancreatic cancer patients, the expression level of LAP was significantly higher on FoxP3⁻Helios⁺ ($6.52 \pm 1.15\%$) compare to FoxP3⁺Helios⁻ ($2.07 \pm 0.46\%$, $P= 0.0038$) and FoxP3⁻Helios⁻ ($0.81 \pm 0.31\%$, $P= 0.0001$) T cells. Moreover, LAP was expressed significantly higher on FoxP3⁺Helios⁺ ($4.12 \pm 0.61\%$) compared to FoxP3⁻Helios⁺ ($P= 0.0014$) and FoxP3⁻Helios⁻ ($P= 0.0002$) T cells (Figure 40C). Interestingly, patients with

liver metastases from colorectal cancer produced a significantly higher level of LAP on FoxP3⁻Helios⁺ ($2.81 \pm 0.41\%$) compared to FoxP3⁻Helios⁻ ($0.72 \pm 0.13\%$, $P= 0.0013$) T cells. Additionally, the expression level of LAP was significantly higher on FoxP3⁺Helios⁺ ($2.26 \pm 0.35\%$) compared to FoxP3⁻Helios⁻ ($P= 0.0047$) T cells (Figure 40D).

The expression level of LAP (GARP⁻LAP⁺) on non-activated FoxP3^{+/-}Helios^{+/-} T cells compared between cancer patients and controls (Figure 41). The result demonstrated that there were no significant differences between all four groups in association with the expression of LAP on rested FoxP3⁺Helios⁺, FoxP3⁺Helios⁻, and FoxP3⁻Helios⁺ T cells (Figure 41A, B, & C). However, LAP (GARP⁻LAP⁺) expression on FoxP3⁻Helios⁻ T cells were produced significantly higher in LI/CRC ($0.72 \pm 0.13\%$) and PC ($0.81 \pm 0.31\%$) patients compared to CP ($0.20 \pm 0.04\%$) patients and HDs ($0.37 \pm 0.10\%$) (Figure 41D).

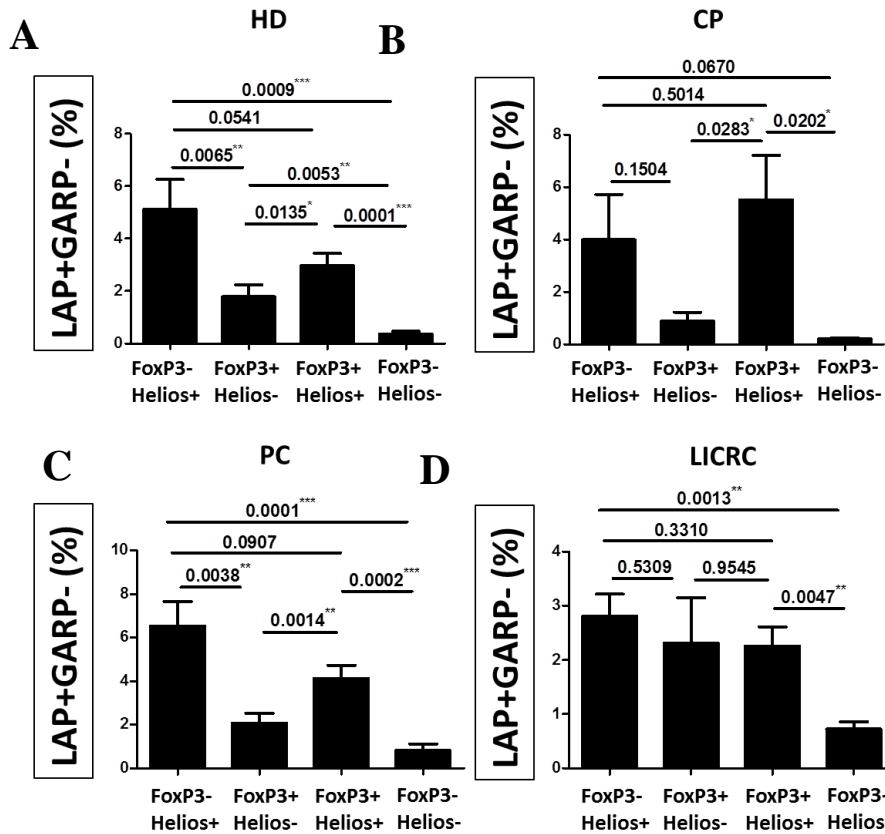


Figure 4014: Comparing the expression level of LAP on non-activated $CD3^+CD4^+GARP^+FoxP3^{+/-}Helios^{+/-}$ T cell subsets for each group.

The bar chart shows the mean percentages \pm SEM of $GARP^+LAP^+$ T cells within $FoxP3^{+/-}Helios^{+/-}$ T cell subsets in non-activated PBMCs isolated from 14 HDs, 7 CP, 17 PC and 7 LI/CRC patients. In HDs, LAP and not GARP was expressed a significantly higher on $FoxP3^-Helios^+$ T cells compared to $FoxP3^+Helios^-$ and $FoxP3^-Helios^-$ T cells (A). In CP patients, the double positive $FoxP3^+Helios^+$ T cells expressed a significantly higher level of LAP compared to $FoxP3^+Helios^-$ and $FoxP3^-Helios^-$ T cells (B). In PC patients, $FoxP3^-Helios^+$ following $FoxP3^+Helios^+$ T cells expressed a significantly higher level of LAP compared to $FoxP3^+Helios^-$ and $FoxP3^-Helios^-$ T cells (C). In LI/CRC patients, $FoxP3^-Helios^+$ following $FoxP3^+Helios^+$ T cells express a significantly higher level of LAP on their surface compare to $FoxP3^-Helios^-$ T cells (D).

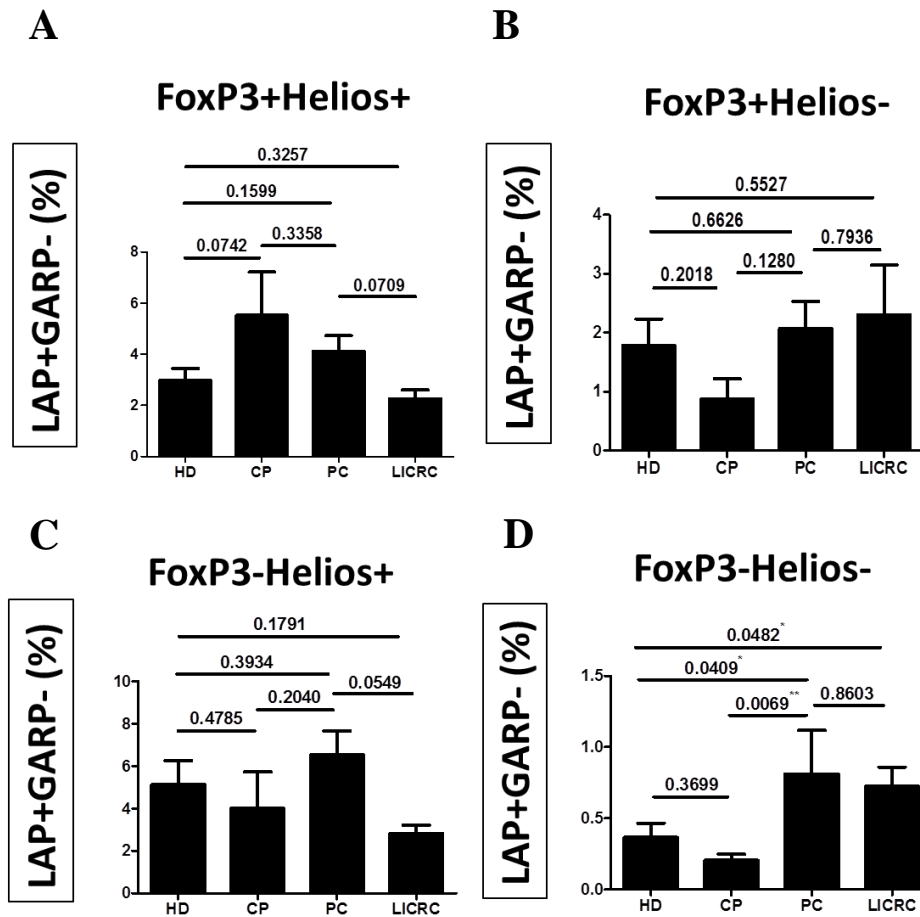


Figure 41: Comparing the expression level of LAP on non-activated $CD3^+CD4^+GARP^-FoxP3^{+/-}Helios^{+/-}$ T cell subsets between the patients.

The bar chart shows the mean percentages \pm SEM of $GARP^-LAP^+$ T cells within $FoxP3^{+/-}Helios^{+/-}$ T cell subsets in non-activated PBMCs isolated from 14 HDs, 7 CP, 17 PC, and 7 LI/CRC patients. The result shows no significant differences in the expression of $GARP^-LAP^+FoxP3^+Helios^+$ T cells between cancer patients and controls (A). The bar chart shows the mean percentages \pm SEM of the surface expression LAP on $FoxP3^+Helios^-$ T cells, the result performs no significant differences between cancer patients and controls (B). Results are shown no major differences in the expression of $GARP^-LAP^+FoxP3^-Helios^+$ T cell when comparing cancer patients with controls (C). The result shows significant differences in the expression level of $GARP^-LAP^+FoxP3^-Helios^-$ T cell between cancer patients and controls (D).

4.1.2 LAP expression on FoxP3^{+/-}Helios^{+/-} activated T cells

Further work was performed to delineate LAP expression on activated FoxP3^{+/-} Helios^{+/-} T cells. The results in figure 42 demonstrate the elevated expression of LAP on FoxP3⁻Helios⁺ and FoxP3⁺Helios⁺ T cells. In HDs, the expression level of LAP was significantly higher on FoxP3⁻Helios⁺ ($10.44 \pm 2.08\%$) T cells followed by FoxP3⁺Helios⁺ ($7.20 \pm 0.53\%$) T cells compare to FoxP3⁺Helios⁻ ($2.19 \pm 0.41\%$) and FoxP3⁻Helios⁻ ($0.54 \pm 0.09\%$) T cells (Figure 42A). However, in CP patients, LAP expression was significantly higher in FoxP3⁺Helios⁺ ($13.99 \pm 1.86\%$) T cells compare to FoxP3⁻Helios⁺ ($7.38 \pm 1.00\%$), FoxP3⁺Helios⁻ ($3.00 \pm 0.77\%$) and FoxP3⁻Helios⁻ ($0.48 \pm 0.13\%$) T cells (Figure 42B). In addition, FoxP3⁻Helios⁺ T cells were also significantly higher than FoxP3⁺Helios⁻ (P= 0.0035) and FoxP3⁻Helios⁻ (P= 0.0001) T cells (Figure 42B). Similar results established in PC patients compared to HDs. In PC patients, FoxP3⁻Helios⁻ ($11.62 \pm 1.61\%$) followed by FoxP3⁺Helios⁺ ($7.51 \pm 0.62\%$) expressed significantly higher level of LAP compared to FoxP3⁺Helios⁻ ($2.76 \pm 0.43\%$) and FoxP3⁻ Helios⁻ ($1.22 \pm 0.39\%$) T cells (Figure 42C). Moreover, LI/CRC patients produced also FoxP3⁻Helios⁺ ($10.03 \pm 1.21\%$) T cells that expressed a significantly higher level of LAP compare to FoxP3⁺Helios⁻ ($4.42 \pm 0.88\%$), FoxP3⁺Helios⁺ ($6.42 \pm 0.65\%$), FoxP3⁻Helios⁻ ($2.39 \pm 0.44\%$) T cells (Figure 42D). Likewise, the expression level of LAP was significantly higher on FoxP3⁺Helios⁺ T cells compared to FoxP3⁻Helios⁻ T cells (P= 0.0021) (Figure 42D).

The bar charts in figure 43 show a comparison of the expression level of LAP on activated FoxP3^{+/-}Helios^{+/-} T cells between the subgroups. Interestingly, CP (13.99 ± 1.86%) patients were the only group that produced a significantly higher level of LAP on FoxP3⁺Helios⁺ T cells compare to PC (7.51 ± 0.62%), LI/CRC (6.42 ± 0.65%) patients, and HDs (7.20 ± 0.53%) (Figure 43A). On the other hand, the expression level of LAP on FoxP3⁺Helios⁻ T cells were significantly higher in LI/CRC (4.42 ± 0.90%) patients compared to HDs (2.19 ± 0.41%) (Figure 43B). Remarkably, there were no significant differences in LAP expression on FoxP3⁻Helios⁺ T cells between the groups studied (Figure 43C). However, a significant increase in the expression level of LAP on FoxP3⁻Helios⁻ T cells was in LI/CRC (2.39 ± 0.44%) and PC (1.22 ± 0.39%) patients compared to CP (0.48 ± 0.13) patients and HDs (0.55 ± 0.09%) (Figure 43D).

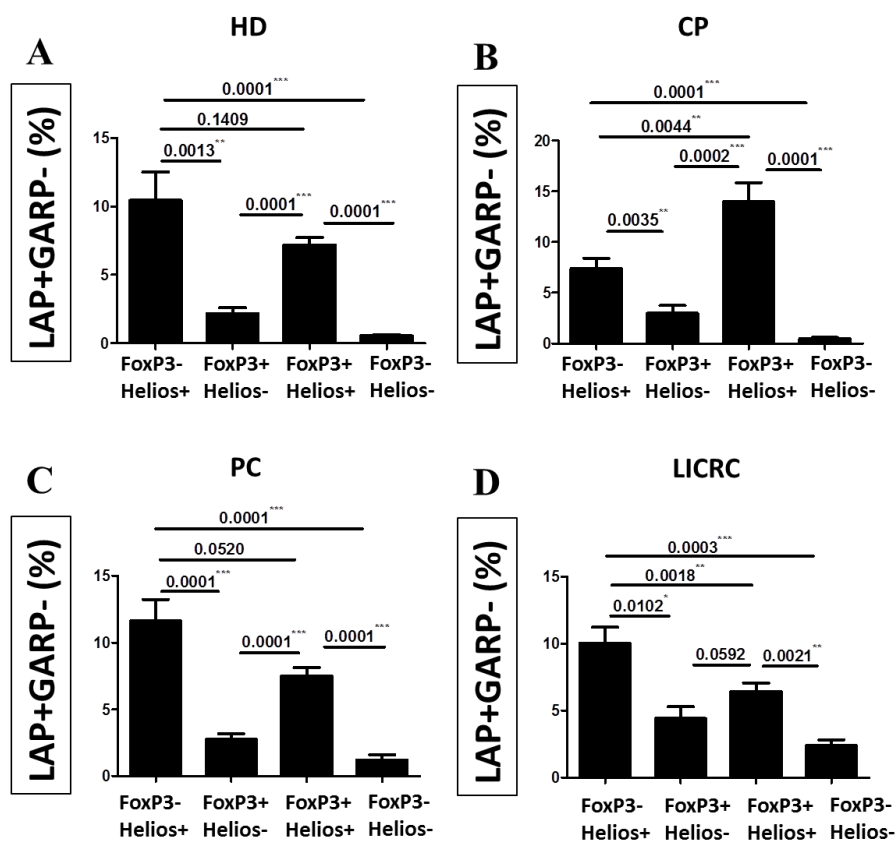


Figure 42: Comparing the expression level of LAP on activated $CD3^+CD4^+GARP^+FoxP3^{+/-}Helios^{+/-}$ T cell subsets for each group.

The bar chart shows the mean percentages \pm SEM of $GARP^+LAP^+$ T cells within $FoxP3^{+/-}Helios^{+/-}$ T cell subsets in stimulated PBMCs isolated from 18 HDs, 9 CP, 20 PC and 11 LI/CRC patients. Human Tregs activated with anti-CD3 and anti-CD28 antibodies. In HDs, LAP was expressed significantly higher on $FoxP3^-Helios^+$ T cells compared to other subpopulations (A). In CP patients, $FoxP3^+Helios^+$ T cells showed a significantly higher level of LAP compared to the other subpopulations (B). In PC patients, $FoxP3^-Helios^+$ following $FoxP3^+Helios^+$ T cells expressed a significantly higher level of LAP compared to the rest of subpopulations (C). In LI/CRC patients, $FoxP3^-Helios^+$ following $FoxP3^+Helios^+$ T cells represent a significantly greater level of LAP compare to the other subpopulations (D).

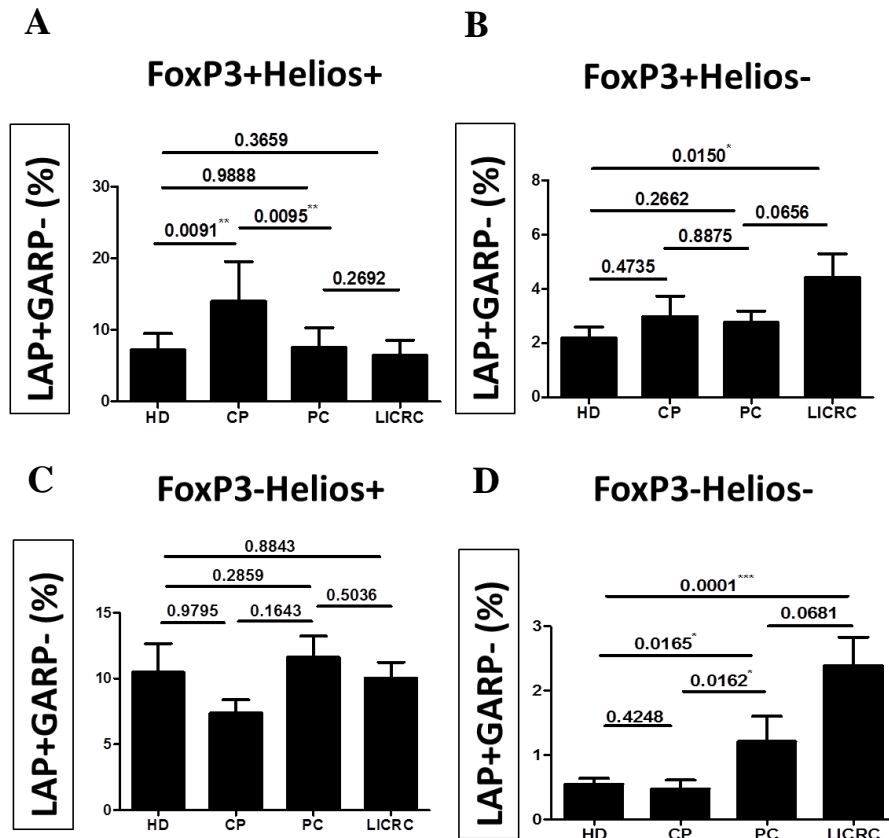


Figure 43: Comparing the expression level of LAP on activated $CD3^+CD4^+GARP^+FoxP3^{+/-}Helios^{+/-}$ T cell subsets between the patients.

The bar chart shows the mean percentages \pm SEM of $GARP^+LAP^+$ T cells within $FoxP3^{+/-}Helios^{+/-}$ T cell subsets in activated PBMCs isolated from 18 HDs, 9 CP, 20 PC and 11 LI/CRC patients. Human Tregs activated with anti-CD3 and anti-CD28 antibodies. LAP expression on $FoxP3^+Helios^+$ T cells was significantly higher in CP patients compare to the pancreatic cancer patients ($P= 0.0095$) and healthy donors ($P= 0.0091$) (A). LI/CRC patients produced a significantly higher level of LAP on $FoxP3^+Helios^-$ T cells compared to HDs ($P= 0.0150$) (B). There are no significant differences in LAP expression on $FoxP3^-Helios^+$ T cells between cancer patients and controls (C). Both LI/CRC and PC patients expressed a significantly higher level of LAP on the double negative $FoxP3^-Helios^-$ subsets compared to controls (D).

4.1.3 GARP expression on FoxP3^{+/-}Helios^{+/-} non-activated T cells

Further analysis was performed to examine the expression level of GARP, without LAP, on FoxP3^{+/-}Helios^{+/-} non-activated T cells, isolated from healthy donors, chronic pancreatitis, pancreatic cancer, and liver metastases from colorectal cancer patients (Figure 44). Figure 44A shows a comparison between the subpopulations in healthy donors. The level of GARP was expressed relatively low on all the subpopulations, there were no significant differences between FoxP3⁻Helios⁺, FoxP3⁺Helios⁻, FoxP3⁺Helios⁺, and FoxP3⁻Helios⁻ T cells (Figure 44A). In chronic pancreatitis, even though there were no significant differences between the subpopulations, FoxP3⁺Helios⁻ T cells expressed a barely detectable level of GARP compared to the other subpopulations (Figure 44B). Interestingly, the non-activated double positive FoxP3⁺Helios⁺ (2.07 ± 0.36%) T cells in pancreatic cancer patients expressed a significantly higher level of GARP compared to FoxP3⁻Helios⁺ (0.67 ± 0.15%, P= 0.0017), FoxP3⁺Helios⁻ (1.34 ± 0.31%, P= 0.0470), and FoxP3⁻Helios⁻ (0.43 ± 0.18%, P= 0.0024) non-activated T cells (Figure 44C). The data of the liver metastases from colorectal cancer patients illustrate that concerning to the surface expression of GARP, all the non-activated FoxP3^{+/-}Helios^{+/-} subset shows no significant differences to each other (Figure 44D).

In Figure 45, the results addressed the fact that GARP expression level on non-activated FoxP3⁺Helios⁺ T cells (Figure 45A), or on FoxP3⁺Helios⁻ (Figure 45B) T cells were not significant in cancer patients compared to chronic pancreatitis and healthy donors (Figure 45A & B). On the contrary, a significant level of GARP⁺FoxP3⁻Helios⁺ T cells was in LI/CRC (2.97 ± 0.62%) patients compare to HDs (1.28 ± 0.34%, P= 0.0174) and PC (0.67 ± 0.147%, P= 0.0001) patients (Figure 45C). Despite the reduced representation, the mean percentages ± SEM of

GARP⁺FoxP3⁻Helios⁻ T cells were significantly high in LI/CRC ($1.24 \pm 0.32\%$) patients compared to PC ($0.43 \pm 0.18\%$) patients (Figure 45D).

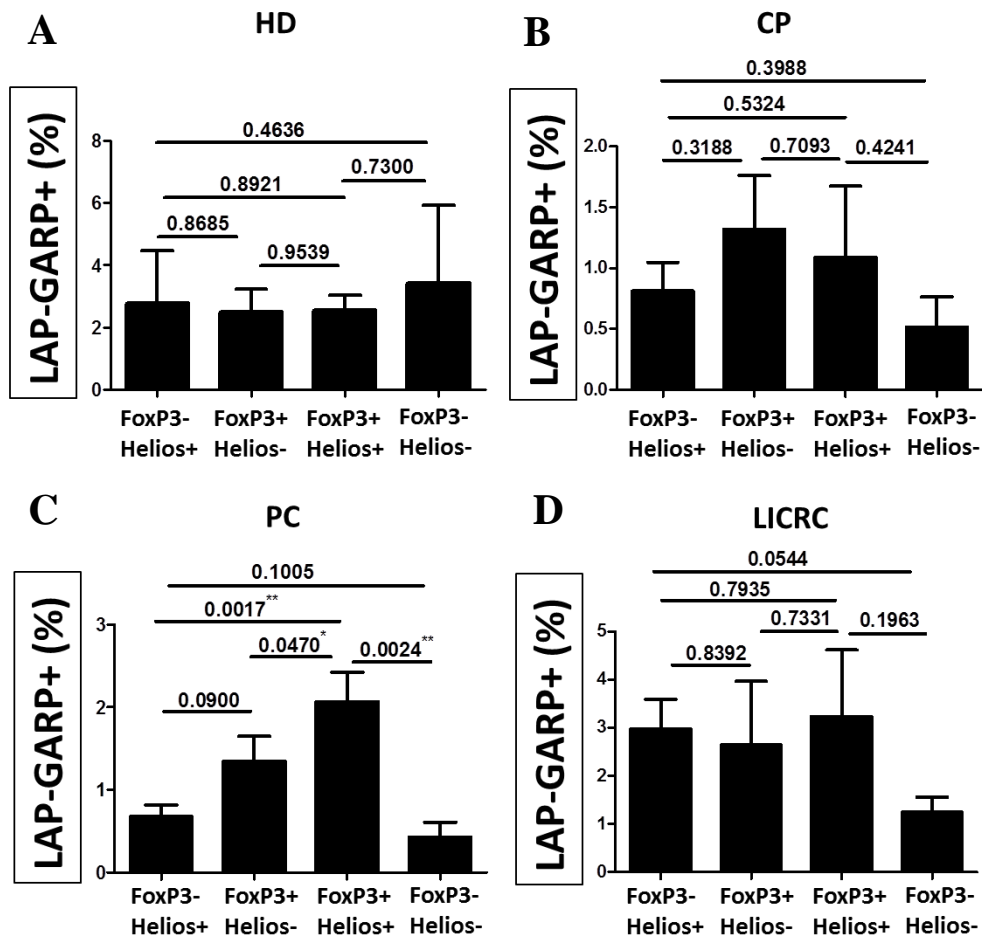


Figure 44: Comparing the expression level of GARP on non-activated CD3⁺CD4⁺LAP⁻FoxP3^{+/+}Helios^{+/+} T cell subsets for each group.

The bar chart shows the mean percentages \pm SEM of GARP⁺LAP⁻ T cells within FoxP3^{+/+}Helios^{+/+} T cell subsets in non-activated PBMCs isolated from 14 HDs, 7 CP, 17 PC, and 7 LI/CRC patients. There were no significant differences among the subpopulations that express GARP in HDs, CP patients, and LI/CRC patients (A, B, & D). However, in PC patients, FoxP3⁺Helios⁺ T cells expressed a significantly higher level of GARP compare to FoxP3⁻Helios⁻, FoxP3⁺Helios⁻, FoxP3⁻Helios⁺ T cells (C).

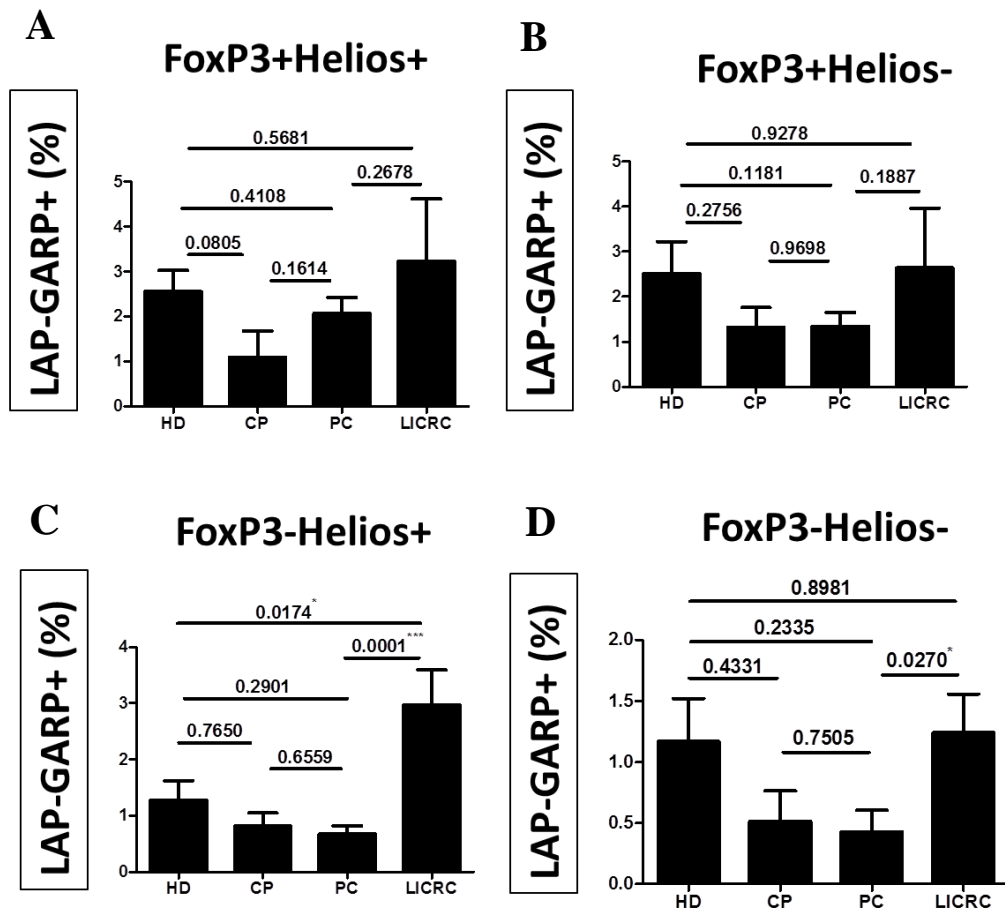


Figure 45: Comparing the expression level of GARP on non-activated $CD3^+CD4^+LAP^-FoxP3^{+/-}Helios^{+/-}$ T cell subsets between the patients.

The bar chart shows the mean percentages \pm SEM of $GARP^+LAP^-$ T cells within $FoxP3^{+/-}Helios^{+/-}$ T cell subsets in non-activated PBMCs isolated from 14 HDs, 7 CP, 17 PC, and 7 LI/CRC patients. The result shows no significant differences in the expression level of $GARP^+LAP^-FoxP3^+Helios^+$ T cells between cancer patients and controls (A). Furthermore, no significant differences observed between cancer patients and controls for GARP expression on $FoxP3^+Helios^-$ T cells (B). Conversely, GARP expressed significantly higher on $FoxP3^-Helios^+$ T cell in LI/CRC patients compared to HDs and PC patients (C). Moreover, the expression level of GARP was significantly greater on the double negative $FoxP3^-Helios^-$ T cells in LI/CRC patients compared to PC patients (D).

4.1.4 GARP expression on FoxP3^{+/-}Helios^{+/-} activated T cells

The co-expression of Helios and GARP in mice and human lymphocytes studied by Akimiva et al., (2011) showed that with or without the addition of IL-2, GARP expression did not increase in association of Helios and FoxP3 in activated T cells. Building upon this observation, figure 46 shows an opposite view of GARP expression on the double positive FoxP3⁺Helios⁺ T cells. In this study, in HDs, GARP expression was significantly higher in FoxP3⁺Helios⁺ ($6.13 \pm 0.95\%$) T cells compared to FoxP3⁻Helios⁺ ($1.97 \pm 0.71\%$) and FoxP3⁺Helios⁻ ($2.07 \pm 0.33\%$) T cells (Figure 46A). Furthermore, GARP expression, in CP patients, was significantly higher on FoxP3⁺Helios⁺ ($3.52 \pm 0.89\%$) T cells compared to FoxP3⁻Helios⁻ ($0.23 \pm 0.03\%$) T cells (Figure 46B). After investigating GARP expression level on FoxP3^{+/-}Helios^{+/-} subsets in PC patients, noticeably, the results show that GARP expression was significant high on FoxP3⁺Helios⁺ ($7.42 \pm 0.54\%$) T cells compared to FoxP3⁻Helios⁺ ($1.09 \pm 0.15\%$), FoxP3⁺Helios⁻ ($2.0 \pm 0.50\%$), and FoxP3⁻Helios⁻ ($0.47 \pm 0.13\%$) T cells (Figure 46C). Further investigation was on LI/CRC patients, showing GARP expression level on FoxP3^{+/-}Helios^{+/-} T cells. The results show that GARP expression level was significantly higher in FoxP3⁺Helios⁺ ($9.08 \pm 1.56\%$) T cells compared to FoxP3⁻Helios⁺ ($2.27 \pm 0.34\%$), FoxP3⁺Helios⁻ ($2.84 \pm 0.62\%$), and FoxP3⁻Helios⁻ ($1.62 \pm 0.39\%$) T cells (Figure 46D).

Building upon this observation, GARP expression level on activated FoxP3^{+/-}Helios^{+/-} T cells compared between the subgroups (Figure 47). The result shows that cancer patients (LI/CRC ($9.08 \pm 1.56\%$) and PC ($7.42 \pm 0.54\%$)) were significantly higher than CP ($3.52 \pm 0.89\%$), but they were not significantly high compared to HDs ($6.13 \pm 0.95\%$) (Figure 47A). Furthermore, there were not any significant differences between the subgroups, in regards to GARP expression on

FoxP3⁺Helios⁻ T cells (Figure 47B). However, GARP expression on FoxP3⁻ Helios⁺ T cells was significantly higher on LI/CRC ($2.30 \pm 0.34\%$) patients compared to HDs ($1.30 \pm 0.25\%$) and PC ($1.10 \pm 0.15\%$) patients (Figure 47C). In addition, GARP expression on the double negative FoxP3⁻Helios⁻ T cells was also significantly high on LI/CRC ($1.62 \pm 0.39\%$) patients compared to HDs ($0.56 \pm 0.13\%$) and PC ($0.47 \pm 0.13\%$) patients (Figure 47D).

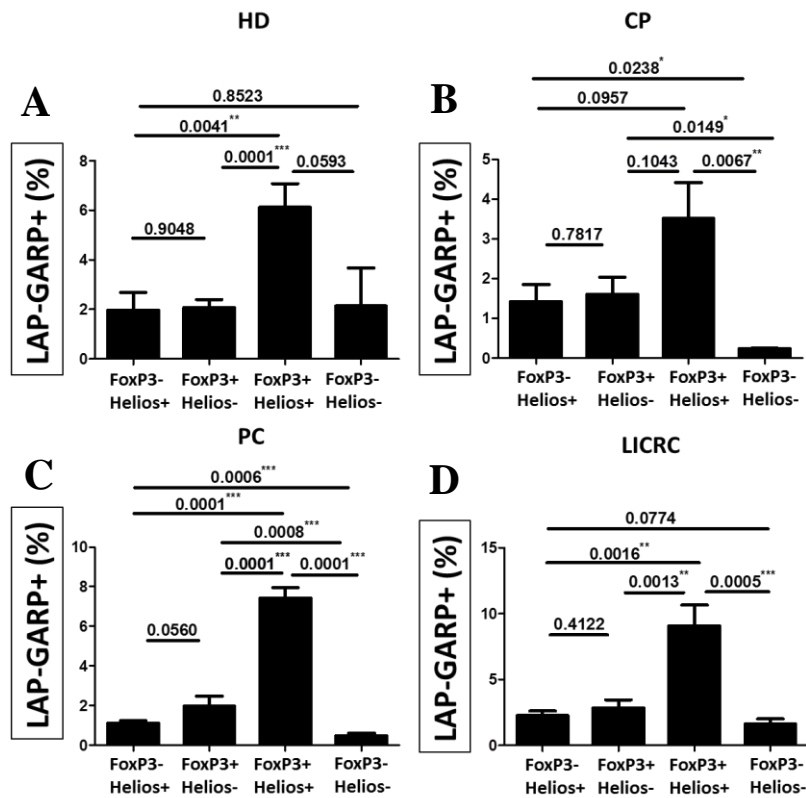


Figure 46: Comparing the expression level of GARP on activated $CD3^+CD4^+LAP^-FoxP3^{+/-}Helios^{+/-}$ T cell subsets for each group.

The bar chart shows the mean percentages \pm SEM of GARP⁺LAP⁻ T cells within FoxP3^{+/-}Helios^{+/-} T cell subsets in activated PBMCs isolated from 18 HDs, 9 CP, 20 PC and 11 LI/CRC patients. Human Tregs activated with anti-CD3 and anti-CD28 antibodies. In HDs, GARP was expressed significantly higher on FoxP3⁺Helios⁺ T cells compared to other subpopulations (A). In CP patients, FoxP3⁺Helios⁺ following by FoxP3⁻Helios⁺ and FoxP3⁺Helios⁻ T cells expressed a significantly higher level of GARP compared to FoxP3⁻Helios⁻ T cells (B). In PC patients, FoxP3⁺Helios⁺ T cells showed significantly higher GARP compare to all the other subsets. While, FoxP3⁺Helios⁻ following FoxP3⁻Helios⁺ T cells expressed a significantly higher level of GARP compared to FoxP3⁻Helios⁻ T cells (C). In LI/CRC patients, FoxP3⁺Helios⁺ T cells showed a significantly higher level of GARP compare to the other subpopulations (D).

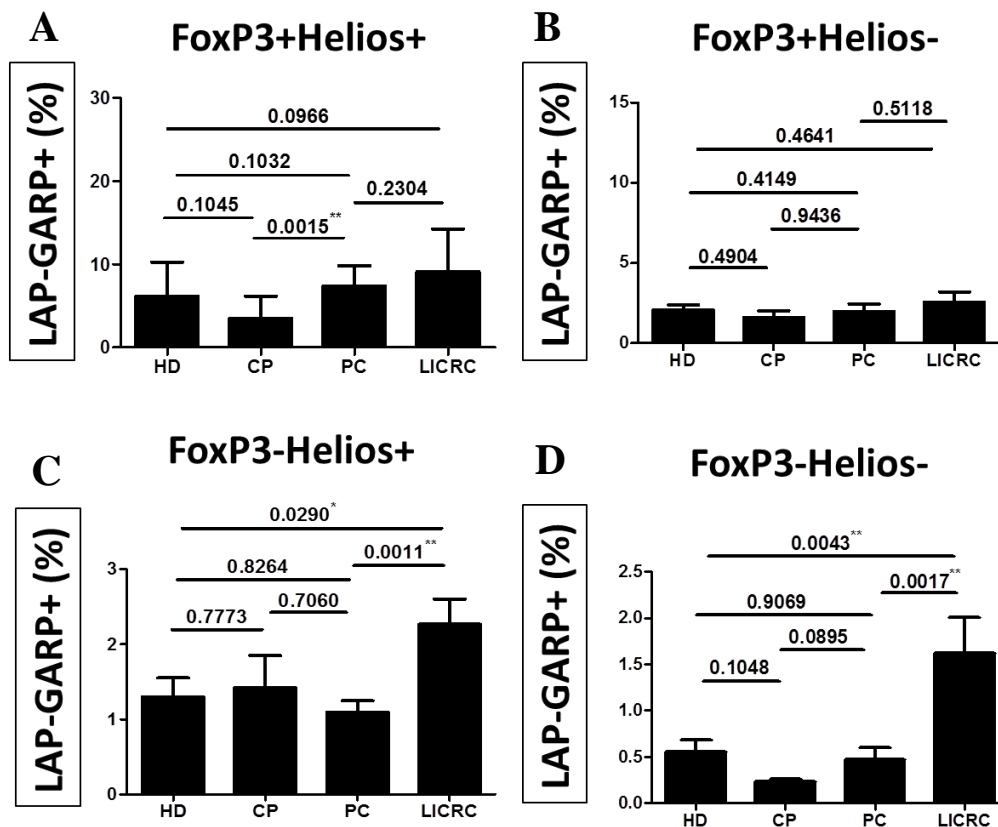


Figure 47: Comparing the expression level of GARP on activated $CD3^+CD4^+LAP^-FoxP3^{+/-}Helios^{+/-}$ T cell subsets between the patients.

The bar chart shows the mean percentages \pm SEM of GARP⁺LAP⁻ T cells within FoxP3^{+/-}Helios^{+/-} T cell subsets in activated PBMCs isolated from 18 HDs, 9 CP, 20 PC and 11 LI/CRC patients. Human Tregs activated with anti-CD3 and anti-CD28 antibodies. GARP expression on FoxP3⁺Helios⁺ T cells was significantly higher in PC patients compare to CP patients (P= 0.0015) (A). There are no significant differences of GARP expression on FoxP3⁺Helios⁻ T cells between cancer patients and controls (B). LI/CRC patients expressed a significantly higher level of GARP on FoxP3⁻Helios⁺ T cell subsets compared to HDs and PC patients (C). LI/CRC patients produced a significantly greater level of GARP on FoxP3⁻Helios⁻ T cells compared to HDs and PC patients (D).

Chapter 5

Results

5.1 Secretion of IL-10 and IFN- γ from GARP⁺LAP⁺ T cells and FoxP3⁺Helios⁺ T cells

5.1.1 CD4⁺ T cells expressing GARP/LAP secrete IL-10 but not IFN- γ

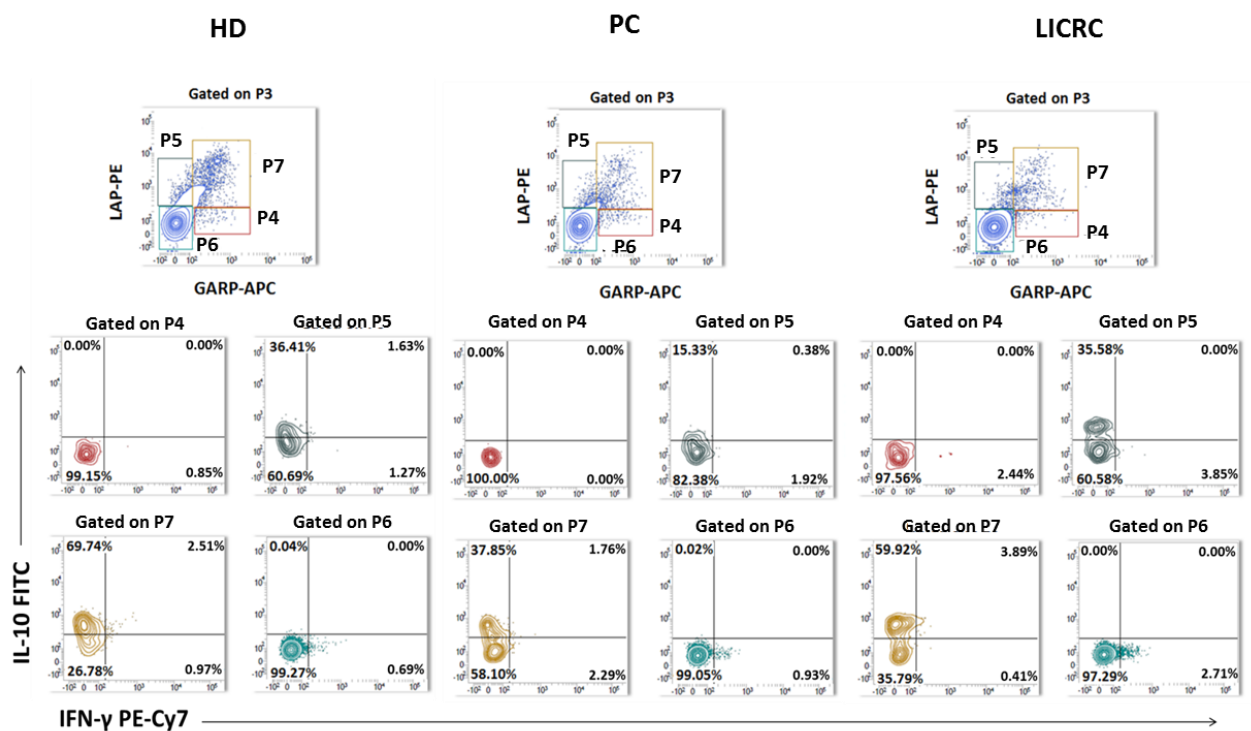
To further identify the characterisation of CD3⁺CD4⁺GARP⁺LAP⁺ Tregs, it was interesting to explore which cytokines this subset is secreting. Therefore, the secretion levels of IL-10 and IFN- γ that produced from these Tregs were analysed. PBMCs were activated with anti-CD3/28 and incubated for 18-20 hours to stimulate cytokine secretion. To increase the accumulation of cytokines in the cells and inhibit the cytokine from transporting outside the cells, Golgi Plug added to the cells. PBMCs from PC, LI/CRC patients and HDs were stained with the surface antibodies and intracellular anti-IL-10 and anti-IFN γ antibodies. There was no need to stain the cells with anti-TGF- β because LAP remains non-covalently associated with TGF- β to form the latent TGF- β complex (Edwards et al., 2013). In other words, the secretion level of TGF- β is associated with LAP expression.

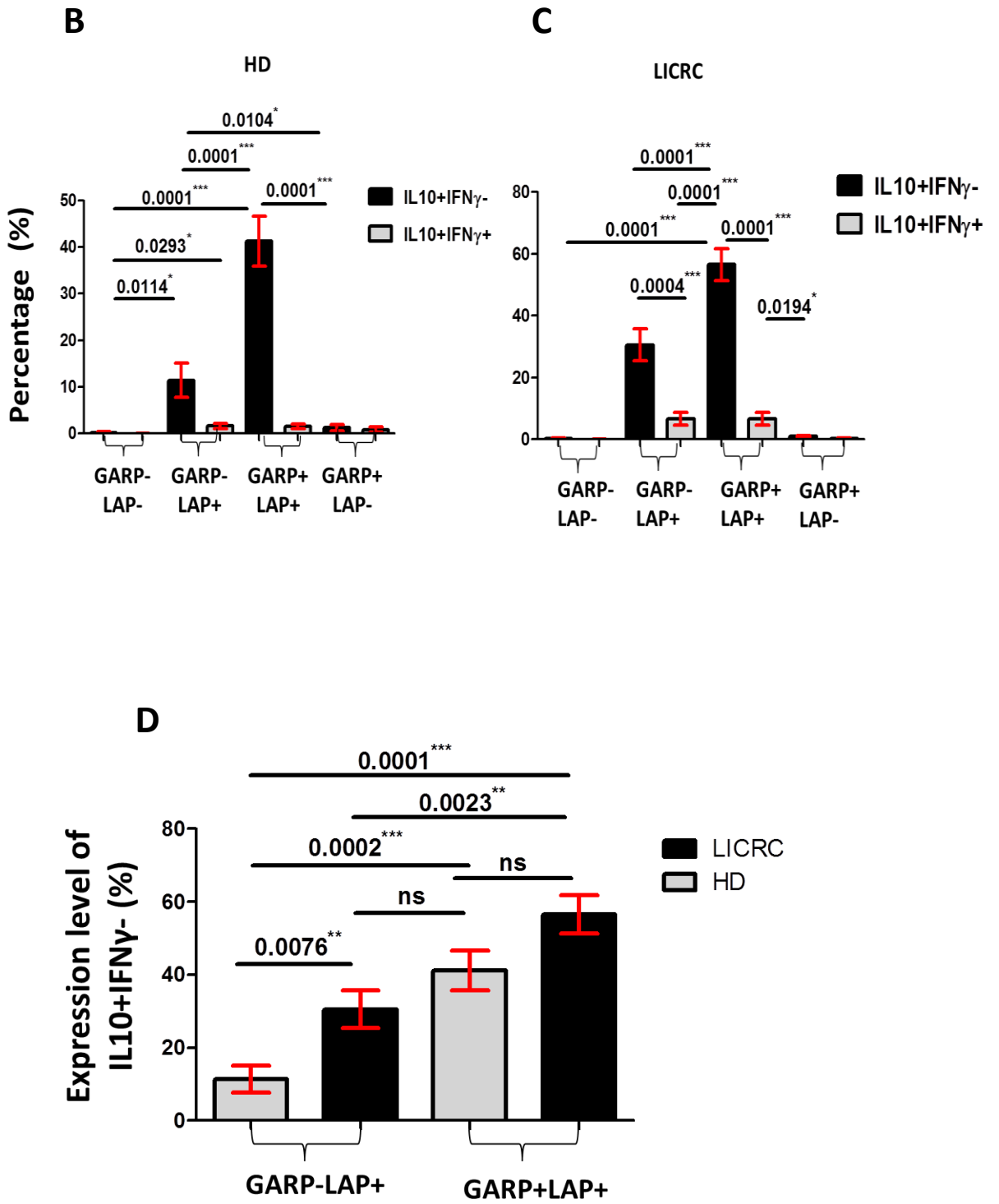
All the investigated samples showed a similar pattern; IL-10 and not IFN- γ secreted by GARP⁺LAP⁺ and GARP⁺LAP⁺ Tregs, (mainly from GARP⁺LAP⁺ Tregs) (Figure 48A). The secretion levels of these cytokines were examined with bar graphs showing the mean percentages \pm SEM expression of IL-10 and IFN- γ on GARP^{+/}LAP^{+/} PBMCs from activated HDs and LI/CRC patients statistically (Figure 48B & C). The result shows that in HDs and LI/CRC patients, the secretion level of IL-10 and not IFN- γ was mainly from GARP⁺LAP⁺ (HD: 41.26 \pm 5.40%; LI/CRC: 56.55 \pm 5.19%) Tregs followed by GARP⁺LAP⁺ (HD: 11.45 \pm 3.70%; LI/CRC: 30.59 \pm 5.18%) Tregs. The GARP⁺LAP⁻ (HD: 1.26 \pm 0.66%; LI/CRC: 0.99 \pm 0.25%) and GARP⁻LAP⁻ (HD: 0.23 \pm 0.17%; LI/CRC: 0.29 \pm

0.11%) T cell subsets produced negligible amounts of IL-10 in both HDs and LI/CRC patients (Figure 48 B & C).

As can be seen, GARP⁺LAP⁺ T cells that produced IFN- γ were significantly lower than GARP⁺LAP⁺ T cells that secreted IL-10. The secretion level of IL-10 from GARP⁺LAP⁺ Tregs was significantly higher in LI/CRC patients compared with HD (P= 0.0076). However, there were no significant differences between LI/CRC and HD regarding the level of IL-10 that secreted from GARP⁺LAP⁺ Tregs (Figure 48D). According to figure 48E, GARP⁺LAP⁺ Tregs produce significantly more IL-10 than IFN- γ (P= 0.0001).

A





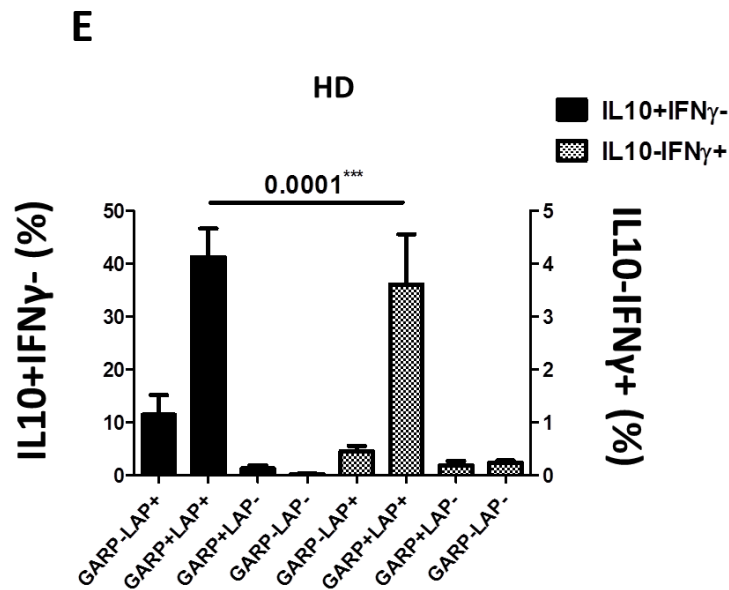


Figure 48: Intracellular cytokine secretion from different GARP^{+/-}LAP^{+/-} CD4⁺ T cell subsets.

Representative flow cytometric plots showing CD3⁺CD4⁺ GARP^{+/-}LAP^{+/-} T cell subsets and cytokine releases (IFN- γ and IL-10) from these subsets following PBMCs activation isolated from HD, PC, and LI/CRC. GARP⁺LAP⁻ T cells gated on P4, GARP⁻LAP⁺ T cells gated on P5, GARP⁻LAP⁻ T cells gated on P6, and GARP⁺LAP⁺ T cells gated on P7 (A). Bar charts show the mean percentage \pm SEM of IL-10⁺IFN- γ ⁻ and IL-10⁻IFN- γ ⁻ cells within CD4⁺GARP^{+/-}LAP^{+/-} T cell subsets in PBMCs isolated from 10 HDs (B) and 10 LI/CRC patients (C). A bar chart is correlating the mean percentage \pm SEM of IL-10⁺IFN- γ ⁻ cells within GARP⁻LAP⁺ and GARP⁺LAP⁺ CD4⁺ T cell subsets between HD and LI/CRC patients (D). Bar charts comparing the mean percentage \pm SEM of IL10⁺INF γ ⁻ and IL10⁻IFN γ ⁺ that released from GARP^{+/-}LAP^{+/-} T cells. The expression of GARP and LAP on PBMC were analysed from 10 HDs. More than 40% of IL-10 is mainly secreted from GARP⁺LAP⁺ Tregs, and this subset is significantly higher than the other subpopulations (E).

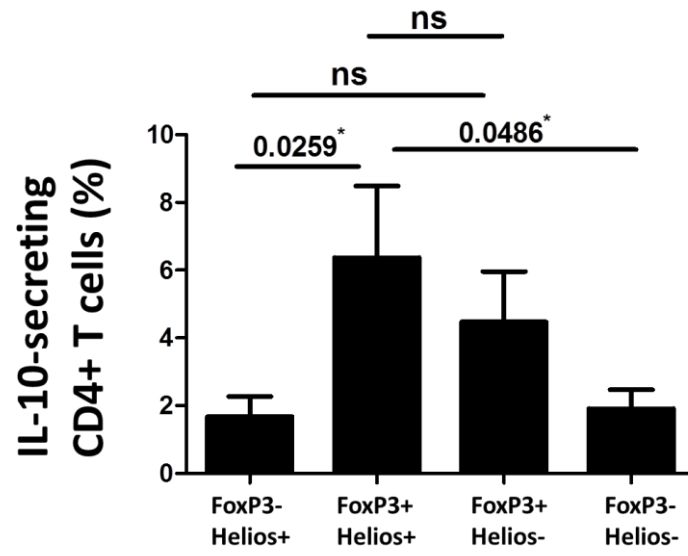
5.1.2 FoxP3⁺Helios⁺ Tregs secrete high level of the suppressive cytokine IL-10

To further elucidate the nature of Helios⁺FoxP3⁺ Tregs, this study investigated IL-10, IFN- γ , and IL-2 secretion by Helios^{+/-}FoxP3^{+/-} T cell subsets isolated from 8 HDs. IL-10 was mainly secreted by FoxP3⁺Helios⁺ (6.37 \pm 2.11%) followed by FoxP3⁺Helios⁻ (4.47 \pm 1.49%) Tregs. All the FoxP3⁻ T cell subsets produced significantly lower levels of IL-10 compared with FoxP3⁺ Tregs (Figure 49A).

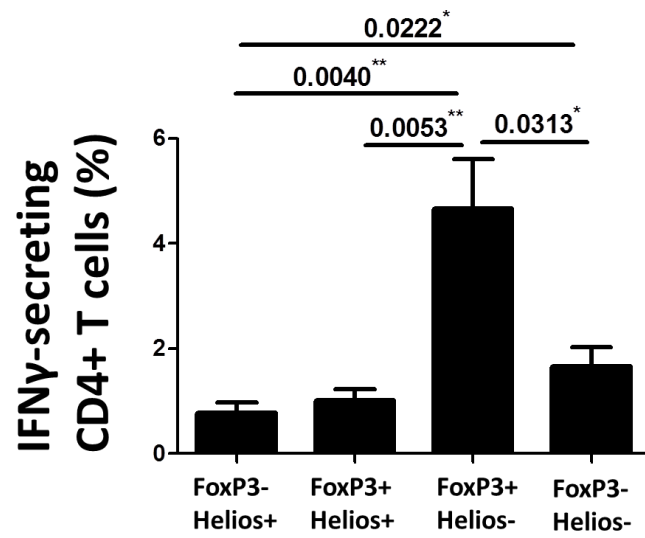
The level of IFN- γ that secreted from Helios^{+/-} and FoxP3^{+/-} T cell subsets were examined. The results showed that FoxP3⁺Helios⁻ (4.66 \pm 0.94%) T cells secreted higher level of IFN- γ than the other subsets. Besides that, as can be seen in (Figure 49B), IFN- γ are significantly higher in FoxP3⁻Helios⁻ T cells (1.66 \pm 0.37%) compared to FoxP3⁻Helios⁺ (0.77 \pm 0.19%) and FoxP3⁺Helios⁺ (1.02 \pm 0.21%) T cells.

The level of CD25 expression after TCR stimulation was measured to determine the level of IL-2 secreted by FoxP3^{+/-}Helios^{+/-} T cells. There were no significant differences between the different subpopulations in IL-2 secreting T cells although the double positive FoxP3⁺Helios⁺ Tregs secreted IL-2 much lower than the other subsets (Figure 49C).

A



B



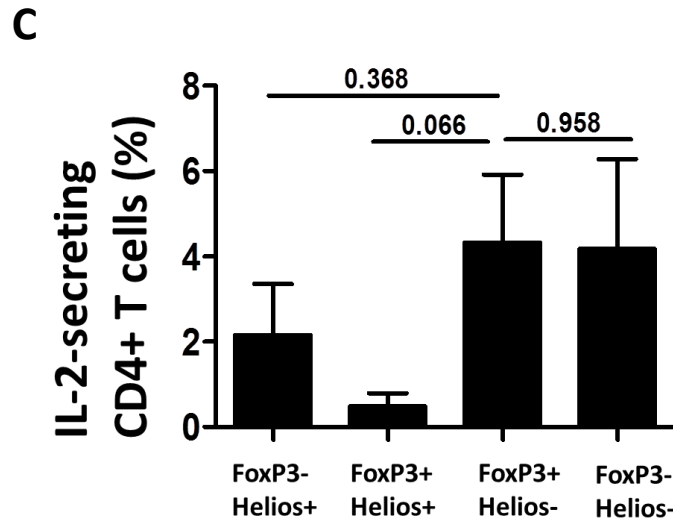


Figure 49: Intracellular cytokine secretion from different FoxP3^{+/-}Helios^{+/-} T cell subsets.

Bar charts show the mean percentage \pm SEM of FoxP3^{+/-}Helios^{+/-} T cell subsets and intracellular cytokine secretion of IL-10 (A), IFN- γ (B) and IL-2 (C) from these different subsets following PBMCs activation isolated from 8 HDs. IL-10 is mainly produced by FoxP3⁺Helios⁺ followed by FoxP3⁺Helios⁻ Tregs, and this subset is significantly higher than FoxP3⁻Helios⁺ and FoxP3⁻Helios⁻ T cells (A). FoxP3⁺Helios⁻ mainly produced IFN- γ followed by FoxP3⁻Helios⁻ Tregs, and this subset is significantly greater than FoxP3⁻Helios⁺ and FoxP3⁺Helios⁺ T cells (B). There are no significant differences between the subpopulations that produce IL-2 (C).

Chapter 6

Discussion

Despite our growing understanding of Tregs in mice, yet the dominant role of FoxP3 in the human system and its novel markers of human Tregs have been questioned. Therefore, more than FoxP3 is necessary for fully explaining the regulatory phenotype in humans. The reason for that is first; TCR stimulation of CD4⁺CD25⁻FoxP3⁻ T cells drives an induction of FoxP3 without hindering the expression of IL-2 and IFN- γ (Gavin et al., 2006; Francois et al., 2009). Secondly, the expression of FoxP3 by conventional CD4⁺CD25⁻ Th cells and even by Th lines and clones do not necessarily function as suppressor cells (Tran et al., 2007; Francois et al., 2009). Thirdly, the ectopic expression of FoxP3 in human helper T cells does not lead to the establishment of a stable regulatory phenotype (Allan et al., 2005). Finally, although the enhancement of FoxP3 expression by TGF- β in activated human CD4⁺CD25⁻ Th cells generates induced Tregs, this phenotype is rapidly lost (Tran et al., 2007). Altogether, these observations suggest that it is necessary to completely understand the regulatory phenotype. Therefore, to provide the missing link toward a better molecular definition of the regulatory phenotype, it is important to focus on other markers.

Recently, many studies have demonstrated that the surface receptor GARP might be Tregs-specific control mechanisms that keep providing high levels of FoxP3 and can control the regulatory function (Wang et al., 2008; Probst-Kepper et al., 2009). Depend on the study of gene expression profiling of Tregs and Th cells following TCR stimulation, GARP was identified (Wang et al., 2008; Probst-Kepper et al., 2009; Tran et al., 2009). GARP depends on FoxP3 because the expression of GARP is up-regulated in FoxP3-transduced Th cells (Probst-Kepper et al., 2009). For that reason, GARP is a special marker of activated Tregs, and through GARP, the quantitative and qualitative differences in FoxP3 expression and function in Th cells and Tregs can be measured and explained.

Further to that, when GARP receptor attaches to LAP, the TGF- β release and its signal regulate the autoimmunity. However, the selective induction of LAP has been reported only for human Tregs and clones (Stockis et al., 2009a; Nakamura et al., 2004). LAP is an important modulator of FoxP3 expression because the specific up-regulation of LAP on activated human Tregs has been shown to allow improvements in the purity of Tregs isolation procedures by separating activated LAP⁺FoxP3⁺ T cells from LAP⁻FoxP3^{-/low} Th cells (Tran et al., 2009).

Since Helios is also co-expressed on FoxP3⁺ Tregs, several studies demonstrated the expression of Helios on Tregs (Akimova et al., 2011) and also with other markers such as Nrp-1 on CD25⁺FoxP3⁺Tregs (Chaudhary & Elkord, 2014). Additionally, more than one study demonstrated the vital expression of GARP and LAP on FoxP3⁺Tregs. However, until now, there is no any study concerning the expression of GARP and LAP on FoxP3⁺Helios⁺ Tregs. Therefore, the novel expression of GARP and LAP on FoxP3^{+/+}Helios^{+/+} lymphocytes isolated from healthy donors, chronic pancreatitis, malignant pancreatic cancer and liver metastases from colorectal cancer patients has been reported in this study.

This study has shown that in both rested and activated PBMCs, FoxP3 is directly involved in the generation of CD4⁺ T cells and significantly less (P=0.0001) in CD8⁺ (CD4⁻) T cells (Figure 17A & B, 18A & B). Related studies have been performed by several scientists, indicating that CD4⁺FoxP3⁺ T cells are the well-characterised naturally occurring suppressive T cells (Nishioka et al., 2006; Adeegbe & Nishikawa, 2013; Azuma et al., 2003; Baecher et al., 2006). Consistent with the notion that CD4⁺FoxP3⁺ T cells play a significant role in the maintenance of tolerance. However, a population of CD8⁺FoxP3⁺ T cells has been identified as early inducing during graft-versus-host disease (GVHD). CD8⁺

Tregs is found to be capable of suppressing T cell responses in autoimmunity and allergen exposure (Hahn et al., 2005; Tsai et al., 2010; Wong et al., 2010), and this type of cells is known to be sufficient to prevent increased GVHD after allogeneic stem cell transplantation (Beres et al., 2012). Furthermore, several studies reported the presence of CD8⁺ Tregs in the tumour microenvironment of patients with colon and prostate cancer (Kiniwa et al., 2007; Chaput et al., 2009).

Accordingly, CD8⁺ Tregs express many of the same cell surface molecules as CD4⁺ Tregs, and *in vitro*, CD8⁺ Tregs are capable of suppressing effector T cell proliferation equally to CD4⁺ Tregs (Churlaud et al., 2015). In support of this idea, GARP and LAP was analysed against FoxP3 to further investigate the co-expression of different key Tregs markers for both CD4⁺ and CD8⁺ T cells. The results elucidated that the expression of LAP and/or GARP are higher on CD4⁺ than on CD8⁺ T cells in both rested (Figure 19) and activated (Figure 20A) PBMC. Furthermore, LAP is expressed significantly higher on FoxP3⁺CD4⁺ T cells (P= 0.0001) compared with FoxP3⁺CD4⁻ (CD8⁺) T cells (Figure 20B).

The co-expression of GARP/LAP on different activated subpopulations (CD3⁺CD4^{+/+}FoxP3^{+/+}Helios^{+/+}) was investigated. GARP/LAP has been shown to increase in conventional CD3⁺CD4⁺Helios⁺ and CD3⁺CD4⁺FoxP3⁺ Tregs after T cell activation. However, CD3⁺CD4⁻FoxP3⁺ Tregs do also express GARP/LAP but to some extent, in contrast to CD3⁺CD4⁻Helios⁺ Tregs that express GARP/LAP significantly less compared with the other subpopulations (Figure 21A & B). After comparing CD4⁺Helios⁺ or CD4⁺FoxP3⁺ T cells with CD8⁺Helios⁺ or CD8⁺FoxP3⁺ T cells, the results illustrate that GARP/LAP expression on human Tregs, are mostly from CD4⁺Helios⁺ or CD4⁺FoxP3⁺ T cells. The expression of GARP/LAP is mainly detected on activated functional

Helios⁺ Tregs (Figure 21B). A study by Tran et al. found out that GARP is a critical molecule for the surface expression of LAP by binding to the complex and functioning as its cell surface receptor. Conversely, the study did not find any significant expression of GARP or LAP in CD8⁺ T cells and many other different cell types including CD14⁺ monocytes, NK T cells, natural killer cells, immature or mature monocyte-derived DCs, and CD19⁺/CD20⁺ B cells. However, under certain conditions or during activation, these cells might express GARP (Tran et al., 2009). Few studies have demonstrated that there are CD8⁺Foxp3⁺ T cells, but with a low frequency of FoxP3 and it is tightly controlled population sharing certain developmental and phenotypic properties with CD4⁺FoxP3⁺ Tregs, but lacking potent suppressive activity (Mayer et al., 2011).

Assuming that both rested and activated CD3⁺CD4⁺FoxP3^{+/-} T cells express higher GARP/LAP than CD3⁺CD4⁻FoxP3^{+/-} T cells do, the question arises as do these CD3⁺CD4⁺FoxP3⁺ T cells express higher GARP or LAP on its surface. A comparison between GARP and LAP expression made with Prism software and the results indicated that there was a significant increase of LAP expression compared to GARP expression (P= 0.0006) on activated CD4⁺FoxP3⁺ T cells (Figure 22). Referring to Chen et al., 2008, CD4⁺CD25⁺LAP⁺ T cells express elevated levels of FoxP3. This experimental research is consistent with the observation that *in vitro* the suppressive function of CD4⁺CD25⁺LAP⁺ Tregs is both soluble factor and cell contact-dependent, while CD4⁺CD25⁺LAP⁻ T cells is only cell contact-dependent (Chen et al., 2008). CD4⁺CD25⁺LAP⁺ T cells are TGF-β dependent, and its function with more suppressive activity than CD4⁺CD25⁺LAP⁻ T cells do (Chen et al., 2008). Varies of kinetics GARP expression depends on the cell subset analysing and the type of cell activation. Of note, T helper cell clones do not express GARP protein upon activation, but they

express low basal levels of GARP mRNA, pointing out that there is post-transcriptional regulation of GARP expression (Tran et al., 2009).

To further define the expression level of GARP or LAP on different Tregs subpopulations, Helios was analysed against GARP and LAP (Figure 23A). Interestingly, the result shows that the expression level of LAP is greater than GARP on CD3⁺CD4⁺Helios⁺ activated T cells (P= 0.0202) (Figure 23B). Noticeable, these results demonstrate that LAP expression on CD4⁺FoxP3⁺ and CD4⁺Helios⁺ Tregs is significantly more than GARP expression. Accordingly, LAP does not have biological activity, and the release of TGF- β from LAP is an essential step to the suppressive functions (Edward et al., 2013). One recent murine study found out that GARP is expressed at low levels on non-activated Tregs and its expression increases via TCR stimulation (Tran et al., 2009). Furthermore, GARP is independent of TGF-B1, and it is cleaved intracellularly by furin. However, GARP is required for surface expression of LAP on human Tregs, as well as platelets (Tran et al., 2009).

Thus far, the data observed that GARP/LAP is mainly expressed on activated CD4⁺ T cells in contrast to activated CD8⁺ T cells. Also, the expression level of LAP is significantly higher than the expression level of GARP on both FoxP3⁺ T cells and Helios⁺ T cells. However, this data is not enough to fulfil the curiosity. Therefore, the co-expression of GARP/LAP was measured on CD4⁺FoxP3⁺ and CD4⁺Helios⁺ T cells. The results explore in depth that at steady state, GARP/LAP expression induced significantly in CD4⁺Helios⁺ T cells compared with CD4⁺FoxP3⁺ T cells (P= 0.0121) (Figure 24). Likewise, following *in vitro* induction of GARP/LAP expression by TCR stimulation, GARP/LAP was significantly higher on CD4⁺Helios⁺ compared with CD4⁺FoxP3⁺ T cells (P=

0.0199) (Figure 21B). These data support the idea that the existence of Helios in Tregs gives more suppressive functions than FoxP3⁺ Tregs does. A study by Akimova et al. reported that Helios⁻ T cells are enriched for naïve T cell phenotypes, whereas Helios expression increases markedly in Treg suppression assay (Akimova et al., 2011). Given that, this report further determined whether the expression of different CD3⁺CD4⁺Helios^{+/-}FoxP3^{+/-} subpopulation was stable under-rested and activated condition, or its proportion decreased during these conditions. Surprisingly, in resting cells, the CD3⁺CD4⁺ subpopulations were almost equally divided into FoxP3⁻Helios⁺ and FoxP3⁺Helios⁺ T cells, while a significantly lower percentage of CD3⁺CD4⁺ T cells were FoxP3⁺Helios⁻ T cells (Figure 25A & B). However, the expression level of CD3⁺CD4⁺ T cells expressing Helios and not FoxP3 following activation increased. The CD3⁺CD4⁺ T cells expressing Helios and not FoxP3 were significantly higher compared with the double positive (FoxP3⁺Helios⁺) and the FoxP3⁺Helios⁻ CD3⁺CD4⁺ T cells (Figure 26A & B). Similar results were demonstrated by Angela Thornton which has proposed that Helios is a marker of thymus-derived Tregs and FoxP3⁺Helios⁺ population expressed a more activated phenotype and had a higher suppressive capability in vitro than FoxP3⁺Helios⁻ populations.

Recent works have established the importance of GARP/LAP expression on CD4⁺FoxP3⁺ Tregs, but so far no study has reported the expression level of GARP/LAP on CD4⁺FoxP3^{+/-}Helios^{+/-} Tregs. Therefore, in this report, the expression of GARP/LAP was investigated on various subsets, such as; FoxP3⁻Helios⁻, FoxP3⁻Helios⁺, FoxP3⁺Helios⁺ and FoxP3⁺Helios⁻ for both activated and non-activated T cells. In resting cells, the expression levels of GARP/LAP were significantly higher on FoxP3⁻Helios⁺ T cells compared to all other subpopulations (Figure 27A & B). On the other hand, upon activation,

GARP/LAP expression was significantly in FoxP3⁺Helios⁺ following FoxP3⁻ Helios⁺ T cells. However, the expression level was significantly lower on FoxP3⁺Helios⁻ T cells, and there was no expression on FoxP3⁻Helios⁻ CD4⁺ T cells (Figure 28A & B). Along to the previous results, this data supports the idea that GARP and LAP are markers for Helios and not FoxP3 in activated PBMC isolated from healthy donors (Elkord et al., 2015).

Moreover, GARP and LAP expression on Tregs were also investigated in cancer patients. Using the flow cytometry setup, GARP and LAP were gated against CD4 for both healthy donor (Figure 29A) and pancreatic cancer patients (Figure 29C). The results show that the activated population of CD4⁺LAP⁺ Tregs were significantly higher than CD4⁺GARP⁺ Tregs in cancer patients and controls (Figure 29B & D) (Table 5).

To investigate the role of LAP in human CD4⁺FoxP3^{+/-} Tregs for cancer patients and controls, LAP analysed against FoxP3 (Figure 30A & 31A). At steady-state, the co-expression of LAP⁺FoxP3⁺ T cells was very low for all samples, but notable FoxP3⁺LAP⁺ T cells were expressed significantly higher in LI/CRC patients compared with HDs (P= 0.0274) (Figure 30C). However, upon TCR stimulation, the co-expression of LAP⁺FoxP3⁺ T cells was upregulated in all samples, and the results show that the expression of LAP⁺FoxP3⁺ T cells was significantly higher in PC patients compared with HDs (P= 0.0254) (Figure 31C). A recent study by Mahalingam et al., 2014 reported that the population of CD4⁺FoxP3⁺LAP⁺ Tregs increases significantly in peripheral blood and tissue of cancer patients compared with HDs. Additionally, LAP⁺ Tregs expresses more effector molecules such as tumour necrosis factor receptor II (TNFR-II), granzyme B, Ki67, CCR5, and perforin than LAP⁻ Tregs does (Mahalingam et al.,

2014). Therefore, *in vitro* LAP⁺ Tregs exert via the TGF- β mediated mechanisms, which is more efficient than that of LAP⁻ Tregs. Moreover, their study suggested that LAP should be a representative marker of tumour specific Tregs in colorectal cancer patients (Mahalingam et al., 2014).

Likewise, in this study, the co-expression of LAP and Helios on non-activated and activated CD4⁺ T cells went on to be examined (Figure 32A & 33A). The result shows that in resting cells, the co-expression of Helios/LAP were much lower in all samples (Figure 32C) compare to the other subpopulations (Figure 32B & D). However, despite the very small population, the co-expression of Helios/LAP in Tregs isolated from LI/CRC and PC patients were significantly higher compared with HDs (Figure 31C). However, there was an improvement of the co-expression level of Helios/LAP in activated Tregs compare to the non-activated Tregs. Moreover, the activated T cells expressing Helios and LAP were significantly higher in cancer patients compared with HDs (Figure 33C).

Because Helios might be another marker for thymic-derived Tregs and it can also express on induced Tregs in some conditions, a combination of FoxP3 and Helios expressed on Tregs was investigated on healthy donors as well as on chronic pancreatitis, pancreatic cancer and liver metastasis from colorectal cancer patients. A subpopulation of FoxP3^{+/-}Helios^{+/-} T cells in the non-activated (Figure 34A) and activated (Figure 35A) settings were analysed. In the non-activated PBMCs, all the samples represented FoxP3⁻Helios⁺ and FoxP3⁺Helios⁺ T cells as the highest subpopulation comparing to FoxP3⁺Helios⁻ T cells that had a lower incidence. Also, LI/CRC patients had the highest level of FoxP3⁻Helios⁺ and FoxP3⁺Helios⁺ T cells compared with the other subgroups (Figure 34B).

However, regarding the FoxP3⁺Helios⁻ T cell subset there was no any significant differences between patients and controls (Figure 34B).

Following TCR stimulation the expression level of FoxP3^{+/-}Helios^{+/-} T cell subpopulation expanded gradually compared with the non-activated PBMCs (Figure 35B). The subpopulations level of FoxP3⁻Helios⁺ and Foxp3⁺Helios⁺ T cells expressed significantly higher on LI/CRC patients compare with PC and HDs. However, following activation FoxP3⁺Helios⁻ subset also expanded to some extent, but it still did not reach any significance between the groups (Figure 35B).

It has been difficulties to study Tregs in a more precise way due to lack of finding markers. With the right vital markers, it creates an ability to expound Tregs characterisation and their mechanisms and relationships with other cells. Also, it will give us an understanding regarding the factors that control their expansion and contraction. Therefore, an investigation of the co-expression of GARP/LAP on FoxP3^{+/-}Helios^{+/-} T cell subsets in cancer patients and controls were done for both rested (Figure 36A) and activated T cells (Figure 37A & 38A). At steady-state, the data for all the four different subgroups showed that the co-expression of GARP/LAP was significantly higher on FoxP3⁻Helios⁺ T cells compared with FoxP3⁺Helios⁻, FoxP3⁺Helios⁺, and FoxP3⁻Helios⁻ T cells (Figure 36B & C). Moreover, the co-expression of GARP/LAP on FoxP3⁻Helios⁺ T cells was significantly higher in LI/CRC patients compared with HDs (Figure 36B). Figure 36C shows the percentages of GARP/LAP expression on the different subpopulations for all the four different samples. The result shows that the percentages of GARP⁺LAP⁺FoxP3⁻Helios⁺ Tregs in PC and LI/CRC patients were 3 to 5% higher than controls. The existence and function of surface LAP and GARP on Tregs and the rate of these cells in cancer patients have been a

matter of debate. As a part of these investigations, this interesting finding in figure 36B demonstrates that even at steady state the subpopulation of GARP⁺LAP⁺FoxP3⁻Helios⁺ Tregs shows higher impact in cancer patients compared to healthy donors.

To further analyse these different subpopulations of Tregs, activated PBMCs provided from HD, CP, PC, and LI/CRC were examined. Figure 37A shows representative plots of GARP and LAP gated on FoxP3⁺Helios⁺ (P7) and FoxP3⁺Helios⁻ (P8) T cells. The categorisation of GARP/LAP co-expression was based mainly on FoxP3⁺Helios⁺ Tregs compared with FoxP3⁺Helios⁻ T cells in all four groups (Figure 37B). Also, the data further supported the notion that the co-expression of GARP/LAP on FoxP3⁺Helios⁺ Tregs was significantly higher in cancer patients compared with controls, in contrast to FoxP3⁺Helios⁻ Tregs that did not show any differences between cancer patients and controls (Figure 37B). Representative plots of GARP and LAP gated on FoxP3⁻Helios⁺ (P6) and FoxP3⁻Helios⁻ (P9) CD4⁺ T cells for HD, CP, PC, and LI/CRC is shown in figure 38A. Cancer patients were then compared with controls to analyse the co-expression of GARP and LAP on FoxP3⁻Helios⁺ and FoxP3⁻Helios⁻ CD4⁺ T cells. In all four groups, the co-expression level of GARP and LAP was significantly higher on FoxP3⁻Helios⁺ T cells compared to FoxP3⁻Helios⁻ T cells. Furthermore, GARP and LAP were mainly expressed in cancer patients compared with controls (Figure 38B). Comparing the subpopulations with each other within the same group the same result has been established. GARP/LAP co-expression on FoxP3⁺Helios⁺ followed by FoxP3⁻Helios⁺ CD4⁺ T cells was significantly higher than their expression on FoxP3⁺Helios⁻ and FoxP3⁻Helios⁻ CD4⁺ T cells in healthy donors and CP, PC, and LI/CRC patients (Figure 39A). However, in a different perspective, figure 39B, C, D, and E shows a comparison of the

subgroups within the same subsets. Same results have been found, the co-expression of GARP/LAP on FoxP3⁻Helios⁺ T cells was significantly higher in LI/CRC patients compared with HD (P= 0.0018) (Figure 39B). Further to that, the co-expression of GARP/LAP on FoxP3⁺Helios⁺ T cells was significantly higher on PC (P= 0.0096) and LI/CRC (P=0.0087) patients compared with HDs. On the other hand, the co-expression of GARP/LAP on FoxP3⁺Helios⁻ and FoxP3⁻Helios⁻ T cells did not reach any significance between the subgroups. All these results indicate that the co-expression of GARP/LAP is mainly expressed on Helios⁺ Tregs compared to the other subpopulations. Moreover, this data confirms that GARP and LAP are predominantly on activated CD4⁺FoxP3⁺Helios⁺ Tregs, and the co-expression of GARP/LAP are certainly not on activated FoxP3⁻Helios⁻ T cells and it should be noted that GARP and LAP are suppressive mechanisms. Moreover, when comparing and analysing the different subgroups with each other, it is shown that the co-expression of GARP/LAP on CD4⁺FoxP3⁺Helios⁺ Tregs upon activation mainly expressed in patients who have cancer.

So far, this study confirming that GARP/LAP complex is mainly co-expressed on Helios⁺ Tregs. Therefore, to understand which of these markers are more suppressive, it is vital to investigate the expression level of LAP on Helios^{+/-} FoxP3^{+/-} T cells without the co-expression of GARP under rested and activated settings. The result indicates that in HDs, the non-activated GARP⁻LAP⁺ T cells mainly characterised as FoxP3⁻Helios⁺ followed by FoxP3⁺Helios⁺ T cells (Figure 40A). The result shows that the level of FoxP3⁻Helios⁺ T cells that expressed LAP and not GARP were significantly higher than FoxP3⁺Helios⁻ (P= 0.0065) and FoxP3⁻Helios⁻ (P= 0.0009) T cells. Moreover, the double positive FoxP3⁺/Helios⁺ T cells expressed a significantly higher level of LAP and not

GARP than FoxP3⁺Helios⁻ (P= 0.0135) and FoxP3⁻Helios⁻ (P= 0.0001) T cells. Predictably, even FoxP3⁺Helios⁻ T cells expressed a significantly higher level of LAP compared with the double negative FoxP3⁻Helios⁻ T cells (Figure 40A). This extensive comparison of LAP expression on FoxP3^{+/-}Helios^{+/-} subpopulation in non-activated PBMCs from healthy donor clearly indicate that not only the co-expression of GARP/LAP complex is highly significant on Helios⁺FoxP3⁻ T cells, as can be seen in figure 36B and C, but also, the expression of LAP alone is highly significant on Helios⁺FoxP3⁻ T cells compared to the other subpopulations (Figure 40A). In CP, however, LAP was mainly expressed on the non-activated FoxP3⁺Helios⁺ T cells compared to the other non-activated subpopulations, and it was significantly higher compared with FoxP3⁺Helios⁻ (P= 0.0283) and FoxP3⁻Helios⁻ (P= 0.0202) T cells (Figure 40B). On the other end of the spectrum, in CP patient, there was no significance in FoxP3⁻Helios⁺ T cells compared to the other subpopulations (Figure 40B). The various results of these two controls are making it interesting to continue to investigate the expression level of LAP on non-activated FoxP3^{+/-}Helios^{+/-} T cells provided from PC and LI/CRC patients. In PC patients, the result shows that the population of FoxP3⁻Helios⁺ followed by FoxP3⁺Helios⁺ T cells expressed significantly larger amount of LAP compared with FoxP3⁺Helios⁻ and FoxP3⁻Helios⁻ T cells (Figure 40C). Additionally, in LI/CRC patients, the most substantial proportion of LAP expression was also from FoxP3⁻Helios⁺ T cells (Figure 40D). Considering the elevated level of LAP expression on FoxP3⁻Helios⁺ Tregs, this finding exhibit that FoxP3⁻Helios⁺ Tregs shows more immunosuppressive characteristics compared to FoxP3⁺Helios⁻ Tregs. These results further corroborated by fine-grain analysis comparing the expression level of LAP on FoxP3^{+/-}Helios^{+/-} subpopulation between the different subgroups. The results show that LAP expression on the non-activated double

positive FoxP3/Helios subsets expanded mostly in CP followed by PC patients (Figure 41A). Furthermore, the expression level of LAP on FoxP3⁺Helios⁻ Tregs was higher on PC and LI/CRC patients compared to controls (Figure 41B). Also, there were no significances of LAP expression on FoxP3⁻Helios⁺ Tregs between the subgroups (Figure 41C). Remarkably, however, the double negative FoxP3⁻Helios⁻ T cells that are expressing LAP are significantly higher in PC and LI/CRC patients compared to controls (Figure 41D).

To assess if LAP expression on FoxP3^{+/+}Helios^{+/+} T cells *in vitro* was further enhanced after activation, human PBMCs were stimulated with anti-CD3/CD28 mAbs. The results of this study prove that the expression level of LAP on FoxP3⁻Helios⁺ and FoxP3⁺Helios⁺ Tregs has rapidly increased during activation in all four groups (HD, CP, PC, and LI/CRC). Moreover, FoxP3⁻Helios⁺ and FoxP3⁺Helios⁺ Tregs expressed a significantly higher level of LAP on their surface compared with FoxP3⁺Helios⁻ and FoxP3⁻Helios⁻ T cells, suggesting that Helios expression might be an activation marker for the suppressive Tregs (Figure 42A, B, C, & D). This study has been proposed to form a positive feedback loop confirming that LAP is capable of identifying Tregs after *in vitro* activation (Sun et al., 2012).

In addition, since all the four subgroups had a corresponding result, the data in figure 43 point out the expression level of LAP on FoxP3^{+/+}Helios^{+/+} T cells between these different subgroups. The result shows that FoxP3⁺Helios⁺ Tregs in CP patients expressed a significantly higher level of LAP on its surface compared with HD and PC patient (Figure 43A). Given the observation that Helios was associated with cellular activation and division, *in vitro*, LAP expression on FoxP3⁻Helios⁺ Tregs was tested and compared between the subgroups. The data demonstrated that compared with controls; there were no significances with PC

and LI/CRC patients (Figure 43C). However, observing all the four charts in figure 43, the percentages of LAP expression on the surface of FoxP3⁺Helios⁺ (Figure 43A) and FoxP3⁻Helios⁺ (Figure 43C) Tregs are still greater than on the surface of FoxP3⁺Helios⁻ (Figure 43B) and FoxP3⁻Helios⁻ (Figure 43D) T cells. Several researchers have referred that cancer cells employ Tregs to express this immune evasion mechanism to progress cancer and suppress tumour-specific immunity (Hollenbeak et al., 2005; Zhang et al., 2003; Martin et al., 2010).

Accordingly, the surface marker GARP discriminate the “true” suppressive Tregs from activated CD25⁺CD127^{low}FoxP3⁺CTLA-4⁺ expressing T effector cells (Wang et al., 2009). Wang study showed that silencing GARP in Tregs decreasing Tregs suppressive activity and CD25⁺GARP⁺ T cells displayed more suppressive activity than CD25⁺GARP⁻ T cells (Wang et al., 2009).

However, there have been limited investigations into GARP expression on FoxP3^{+/-}Helios^{+/-} T cells. Therefore, in this research, it was vital to characterise GARP expression without LAP on activated and non-activated FoxP3^{+/-}Helios^{+/-} T cells. At steady state, the results displaying that in PC patient, GARP expression was significantly higher on FoxP3⁺Helios⁺ Tregs compared with FoxP3⁻Helios⁺, FoxP3⁺Helios⁻, and FoxP3⁻Helios⁻ T cells (Figure 44C). Furthermore, comparing the expression level of GARP on FoxP3^{+/-}Helios^{+/-} Tregs between the subgroups, the results demonstrated that LI/CRC patient produced a significantly higher level of GARP⁺FoxP3⁻Helios⁺ T cells compared with PC patients (P= 0.0001) and HDs (P= 0.0174) (Figure 45C). Activated PBMC, on the other hand, showed more impressive results than the non-activated PBMC. GARP expression was significantly higher in FoxP3⁺Helios⁺ T cells compared to the other subpopulations within all the subgroups (Figure 46 A, B, C, & D). A

previous study has demonstrated that expression of GARP on activated FoxP3⁺ Tregs does not only correlate with their suppressive capacity but it also selectively identifies the activated human FoxP3⁺ Treg cells (Wang et al., 2009).

GARP is responsible for surface localisation of LAP and has been defined as a marker of activated human Tregs (Edwards et al., 2013). As mentioned above, the suppressive regulatory cells are responsible for producing IL-10 and TGF- β cytokines to maintain balance in the immune system, in contrast to IL-2 and IFN- γ , which are the mainly cytokines for T cell activation and proliferation (Zheng et al., 2004). CD4⁺ T cell secreting IL-10 and IFN- γ were first found in the early 1990s. One of the main reasons for Tregs cell-specific-inactivation is the IL-10 secretion, which is an important cytokine for Tregs to intensify immune-mediated lung hyper-reactivity and to increase skin sensitivity (Rubtsov et al., 2008). Therefore, upon further examination, the secretion level of IL-10 and IFN- γ produced from GARP^{+/-}LAP^{+/-} by human T cells were examined. The results demonstrated that *In vitro*, IL-10 was secreted mainly from GARP⁺LAP⁺ (P7) following GARP⁻LAP⁺ (P5) Tregs but these cells lacked the ability to produce IFN- γ (Figure 48A). Additionally, these cytokines were examined with bar graphs showing the secreted level of IL-10 and IFN- γ by GARP^{+/-}LAP^{+/-} T cells from activated HDs (Figure 48B) and LI/CRC patients (Figure 48C). The result shows that for both HDs and LI/CRC patients, the IL-10 secretion was mainly from GARP⁺LAP⁺ Tregs followed by GARP⁻LAP⁺ Tregs and these cells did not secrete IFN- γ . The GARP⁺LAP⁻ and GARP⁻LAP⁻ T cell subsets produced negligible amounts of IL-10 in both HDs and LI/CRC patients. Furthermore, The GARP⁺LAP⁺ T cells that secreted only IL-10 and not IFN- γ were significantly higher than GARP⁺LAP⁺ T cells that secreted both IL-10 and IFN- γ (Figure 48B

& C). Suggesting that, GARP and LAP are surely Tregs suppressive surface receptors.

Figure 48D shows a comparison between HDs and LI/CRC patients regarding the secretion level of IL-10 that produced from GARP⁻LAP⁺ and GARP⁺LAP⁺ Tregs. The bar graph demonstrates that IL-10 secreted by GARP⁻LAP⁺ Tregs was significantly higher in LI/CRC patients compared with HD. However, there were no significant differences between LI/CRC and HD regarding the level of IL-10 that secreted from GARP⁺LAP⁺ Tregs (Figure 48D). Moreover, the secretion level of IL-10 by GARP⁺LAP⁺ Tregs is significantly higher than the secretion level of IFN- γ by GARP⁺LAP⁺ Tregs (Figure 48E).

In 1989, IL-10 was first discovered in a Th2 T cell clone, and now it established that this cytokine could be secreted by many cell types including macrophages, B cells, dendritic cells, and CD4⁺ T cells (Fiorentino et al., 1989). The suppression function of IL-10 is linked to the expression of FoxP3 and Helios in CD4⁺ T cells. Tregs ability to secrete IL-10 prevents the autoimmune disorder, and that has been implicated in numerous of diseases. In this study, IL-10 secreted by Helios^{+/-} FoxP3^{+/-} CD4⁺ T cells was investigated. The data in figure 49A show that IL-10 was mainly secreted by FoxP3⁺Helios⁺ followed by FoxP3⁺Helios⁻ Tregs. Moreover, FoxP3⁻Helios⁻ and FoxP3⁻Helios⁺ T cell subsets produced a significantly lower level of IL-10 compared with FoxP3⁺Helios⁺ and FoxP3⁺Helios⁻ Tregs. According to many studies, tumour cells stimulate immature myeloid DCs to secrete TGF- β and/ or IL-10, inducing naïve T cells to convert to FoxP3⁺CD25⁺CD4⁺ Tregs (Ghiringhelli et al., 2005b). Additionally, FoxP3 negative T cells with suppressive activity are those secreting IL-10 (Tr1 cells) or TGF- β (Th3 cells) (Chen et al., 1994).

In the 1960s, IFN- γ was first discovered as the cytokines that have been mainly produced by NK cells, Th1 cells, CD8⁺ T cells and CD4⁺ T cells. The secretion level of IFN- γ produced by Helios^{+/-}FoxP3^{+/-} T cell was analysed. The data shows that FoxP3⁺Helios⁻ T cells secreted significantly higher level of IFN- γ compared with FoxP3⁻Helios⁺ (P= 0.0040), FoxP3⁺Helios⁺ (P= 0.0053), and FoxP3⁻Helios⁻ (P= 0.0313) T cells (Figure 49B). More than one study demonstrated that human CD4⁺CD25⁺FoxP3⁺ Tregs that have been derived from CD25⁻ T cells secrete the inflammatory cytokines IL-2 and IFN- γ . For the conversion of CD4⁺CD25⁻ T cells to CD4⁺ Tregs the IFN- γ cytokine is critically required (Stroopinsky et al., 2009). According to another study, the knockout of IFN- γ in mice reduced CD4⁺CD25⁺FoxP3⁺ Tregs frequency and function when compared with those of wild-type mice (Wang et al., 2006).

Figure 49C show the secretion level of IL-2 that is produced from FoxP3^{+/-} Helios^{+/-} T cells. The results indicated that there were no significant differences between the different subpopulations in IL-2 secreting T cells. Referring to current studies, Tregs generation and maintenance has been implicated by the IL-2 cytokine. Tregs do not produce IL-2, but these cells compete for IL-2 secreted by responder T cells. Activated Tregs in the presence of IL-2 induce IL-10 secretion which indicates that IL-2 uptake is also required to induce additional suppressive factors (Nelson, 2004).

Chapter 7

Conclusion

T regulatory cells have a significant position in the regulation of immune responses to tumours and infectious diseases. FoxP3 is specifically expressed by T regulatory cells, and therefore it is an appropriate marker for these cells. A few years ago, Helios was assumed to be a useful marker for tTregs (Thornton et al., 2010). However, more recent studies have shown that Helios is not a suitable marker for defining tTregs because it can also be expressed on pTregs (Lin et al., 2013). So far, many scientists focused on this latter aspect of Treg cell immunobiology to find a vital marker that can be used to distinguish tTregs from pTregs and iTregs. Identify new markers that can discriminate these subpopulations from each other would be an incredible opportunity and challenge. However, the researchers have found that achieving this can be difficult due to many reasons, including the requirement of; specific facilities for cell sorting, sufficient numbers of Tregs to get effective immunosuppression since Tregs are anergic and it is difficult to expand it, stability and homogeneity of Tregs therapeutics product and referring to Dhamne et al., 2013, LAP⁺ Tregs represent more homogeneous and stable population than the heterogeneous population. Therefore, this study focused on finding vital markers (GARP/LAP) for distinguishing and characterising FoxP3^{+/+}Helios^{+/+} Tregs subpopulations in healthy donors, chronic pancreatitis, and in cancer patients.

An argument has made that Helios induces epigenetic silencing of IL-2 gene expression in Tregs, and loss of Helios in Tregs cause a decrease in suppressive capacity, suggesting that Helios is capable of controlling T cell differentiation and/or function (Baine et al., 2013). Several studies reported a significant increase in Treg levels in most types of cancer. Currently, these studies are trying to establish additional surface markers to discriminate Tregs different subsets and to provide an effective solution to treat cancer patients. Therefore, to decrease Tregs

activity in cancer, it is necessary to find vital markers to target Helios⁺FoxP3⁺ Tregs. Many studies describe CD8⁺ (CD4⁻) Tregs as a phenotypic similar with CD4⁺ Tregs, but this study demonstrated that the expression of FoxP3, likewise the expression of GARP and LAP are gained mainly on CD4⁺ T cells and not on CD8⁺ T cells. Indeed, in activated PBMCs isolated from healthy donors, GARP/LAP is expressed significantly higher on CD4⁺Helios⁺ and CD4⁺FoxP3⁺ Tregs compared with CD4⁻Helios⁺ and CD4⁻FoxP3⁺ T cells. Furthermore, after comparing LAP expression against GARP expression on activated CD4⁺FoxP3⁺ T cells and CD4⁺Helios⁺ T cells, it shows that the expression level of LAP is greater than GARP on CD4⁺FoxP3⁺ and CD4⁺Helios⁺ T cells. A study by Edward et al. demonstrated that in generally GARP is responsible for the secretion of TGF- β from latent TGF- β (LAP), but the expression of GARP is independent of LAP. In this content, hypothetically, LAP and not GARP may frequently be expressed on Tregs surface even at steady-state but to be capable of secreting the suppressive-TGF- β cytokine, it needs to bind to GARP. Another alternative is that a culture of Tregs upregulates the expression of GARP in the presence of specific cytokines. Edward's group explored that knockdown of human GARP observe a reduction of the suppressor capacity, which indicates that GARP is ultimately required for the expression of latent TGF- β on the surface of T cells (Edward et al., 2013).

Additionally, the co-expression of GARP/LAP were significantly higher on CD4⁺Helios⁺ T cells compared with CD4⁺FoxP3⁺ T cells for both rested and activated cells, and that support the inkling that the existence of Helios in Tregs gives more suppressive functions than FoxP3⁺ Tregs does.

The results further demonstrated that rested suppressive CD3⁺CD4⁺ T cells are equally divided into FoxP3⁻Helios⁺ and FoxP3⁺Helios⁺ T cells, in contrast to the stimulated suppressive CD3⁺CD4⁺ T cells that mostly express Helios and not FoxP3. In this case, it is permissible to speculate that the rested FoxP3⁻Helios⁺ T cell may characterise as pTregs. Refereeing to Abbas et al., 2013, the peripherally derived Tregs are the conventional FoxP3⁻ T cells that located in the peripheral sites. Moreover, as described in this review, in healthy donors the co-expression of GARP/LAP was significantly higher on FoxP3⁻Helios⁺ non-activated T cells and on FoxP3⁺Helios⁺ activated T cells compared with the other subpopulations, which is another evidence demonstrating that the co-expression of GARP/LAP is expressed mainly on Helios⁺ Tregs, and they are markers for Helios and not FoxP3.

Comparing these healthy donors' results with cancer patients, a greater understanding of Tregs induction has established. These data suggest that in activated CD4⁺ Tregs, LAP expression was significantly higher than GARP expression in cancer patients and controls. Additionally, at steady-state, the co-expression of LAP/FoxP3 on Tregs was significantly greater in LI/CRC patients compared with HDs, but upon activation, the expression of LAP⁺FoxP3⁺ Tregs was significantly higher in PC patients compared with HDs. Analysing with the same method, in both rested and activated cells the expression of Helios⁺LAP⁺ Tregs in cancer patients were significantly higher compared with HDs. Remarkably, in both rested and activated settings, LI/CRC patients had the highest level of FoxP3⁻Helios⁺ and FoxP3⁺Helios⁺ Tregs compared with the other subgroups.

Further examination of GARP/LAP expression following activation revealed that the co-expression of GARP/LAP was significantly higher on FoxP3⁺Helios⁺ Tregs compared with the other subsets. Likewise, FoxP3⁺Helios⁺GARP⁺LAP⁺ Tregs was significantly higher in cancer patients compared with controls, in contrast to FoxP3⁺Helios⁻GARP⁺LAP⁺ Tregs that did not show any differences between cancer patients and controls. Notably, these results show that LAP and GARP are mainly expressed on Helios⁺ Tregs, and its level increases significantly in cancer patients.

This study has shown that the main subsets that produce IL-10 and not IFN- γ cytokines are GARP⁺LAP⁺ followed by GARP⁻LAP⁺ Tregs, and that was investigated in HDs and LI/CRC patients. Moreover, IL-10 secreted by GARP⁻LAP⁺ Tregs was significantly higher in LICRC patients compared with HD. Suggesting that, GARP and mostly LAP are surely Tregs immune-suppressive surface receptors.

A number of studies reported IL-10 secretion level from FoxP3⁺ Tregs, but very few studies reported the secretion level of IL-10 on FoxP3^{+/-}Helios^{+/-} Tregs. Consistent with this notion, this report shows that in healthy donors, IL-10 mainly secreted by FoxP3⁺Helios⁺ followed by FoxP3⁺Helios⁻ Tregs. In contrast to the secretion level of the anti-tumour IFN- γ cytokine, which was secreted significantly higher from FoxP3⁺Helios⁻ followed by FoxP3⁻Helios⁻ compared with FoxP3⁻Helios⁺ and FoxP3⁺Helios⁺ T cells. Notably, the results of this experiment showed that FoxP3⁺Helios⁺GARP⁺LAP⁺ Tregs that produced high level of IL-10 cytokines are the main cells that have to be targeted in cancer patients or in tumour microenvironments.

On the other hand, the main potential of this study is not only to find the vital markers to target Tregs in cancer patient with the intention of emerging the immune system to fight cancer cells and destroy it, but also to hopefully be a part of a more advance future techniques that can promote enhancements in diagnosis, estimating prognosis, cancer prevention, and potential treatments for cancer and autoimmune diseases.

The diagnostic and prognostic features of specific types of cancer are usually based on a combination of different types of tests e.g. blood test, different types of scan, MRI, X-Ray, ultrasound, and biopsy. However, the laboratory biomarkers are microinvasive or non-invasive indices and they provide faster results and cost less compared to other techniques (Fengming and Jianbing, 2014).

Therefore, a combination of (LAP, GARP, Helios, FoxP3) biomarkers, can be applied on different types of diagnosis. Finding high level of LAP⁺, GARP⁺, Helios⁺, FoxP3⁺ Tregs in the body can be a sign of cancer or, in contrary, finding low level of LAP⁺, GARP⁺, Helios⁺, FoxP3⁺ Tregs in the body can likely to be a sign of autoimmune disease and it also may confirm exactly which autoimmune disease the patient has.

In conclusion, the innovative data obtained during these three years provides an understanding of the mechanism and the vital markers of Tregs, and it also promotes the future studies to examine the origin, function and the translational potential of these cells. Furthermore, the main benefits of this study are the clinical parts that can be applied not only on cancer patients but also on patients with autoimmune disorder. Targeting the level of GARP⁺LAP⁺Helios⁺IL10⁺ Tregs in the body can be used as a vital way to diagnose cancer or any other

autoimmune diseases. This study has also explored in depth the role of GARP/LAP in vitro assay, and it has been highlighting the importance of GARP and LAP as a marker for Helios⁺ suppressive T cells. It also recommends the future studies to focus more on Helios⁺ Tregs with the aims to target these cells in cancer.

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Appendix 1

Appendix 2