The Modulation Effect of Inflammatory Cytokines on T cell Proliferation in Hypertension

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Thesis Submitted to the University of Glasgow in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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May 2024

Abstract

Hypertension is a common medical condition with very serious target organ consequences, increasing the risk of heart disease, stroke, and severe health complications. Despite the identification of various mechanisms (vascular, renal, and central mechanism) contributing to the pathogenesis of hypertension, the majority of cases lack a clear aetiology. Emerging evidence has established a significant association between hypertension and immune responses, particularly involving adaptive immune cells and inflammatory cytokines. Immunosuppressive drugs and cytokine inhibitors have shown potential in mitigating hypertension, suggesting a crucial role of the immune system in this condition. Given the central role of T lymphocytes in the adaptive immune response, this study hypothesises that, in the context of hypertension, inflammatory cytokines can modulate T cell activation independently of antigen stimulation. To test this hypothesis, total T cells were isolated from the spleens and PBMCs of normotensive and hypertensive mice and exposed to a range of cytokines, including TNF-α, IL-6, IL-15, IFN-γ, IL-7, IL-1β, IL-17A, IL-2, and IL-12, using different stimulation protocols. Aiming to understand the effects of these cytokines on T cell proliferation, differentiation, and the expression of activation markers such as CD69.

Our findings highlight the varying abilities of cytokines to sustain T cell viability, with IL-7, IL-15, and IL-6 demonstrating a tendency for greater efficacy compared to other cytokines. In addition, IL-7 and IL-15 significantly impact T cell proliferation, notably affecting the CD8+ T cell population. However, despite these effects, no significant difference was detected between normotensive and hypertensive T cells in response to IL-7 and IL-15. This suggests that while these cytokines are potent in driving T cell proliferation, their influence is not specifically heightened in the context of hypertension. In GSEA and KEGG analyses, the Ca2+ signalling pathway was distinctively activated in response to IL-7 and IL-15 in Ang II induced hypertension.

Conclusion: These data imply that most studied cytokines linked to hypertension pathology do not substantially affect normotensive or hypertensive T cells in a murine model. However, T cell proliferation was elevated in both Sham and Ang II mice in response to IL-15 and IL-7. Together, the data presented in this thesis warrant further investigations into the role of cytokines in hypertension and may point to IL-15 or IL-7 as biological targets for antihypertension therapy.

Acknowledgement

In the name of Allah, the Most Merciful and Most Compassionate,

First and foremost, I express my heartfelt gratitude to Allah, who has given me the will, ability, power, belief, and patience throughout my journey. I feel His presence beside me in every circumstance, guiding me with his wisdom and grace. This gratitude also extends to my beloved mother, who has nurtured a profound passion for knowledge and continuous learning within me. Her constant prayers and support have been my steadfast companions throughout life's journey.

I am deeply indebted to my supervisors, Professors Marta Czesnikiewicz-Guzik and Tomasz Guzik, whose unwavering support and guidance enabled me to complete my PhD, turning my dream into a tangible reality.

To my esteemed postdoctoral mentor, Ryszard Nosalski, I offer my sincerest thanks and appreciation for your constant kindness, support, and invaluable assistance in and out of the laboratory. I am grateful to all members of Guzik lab for valuable interactions throughout this challenging but rewarding scientific Journey.

To my beloved husband, Khalid, you have been the backbone of this journey, and I am profoundly grateful for your love and enduring support, which has made every step of this endeavour more manageable.

To my sisters, Ola and Zeinab, your presence in my life has been a source of immense blessing and encouragement. You are the backbone of our family, and the way we take care of and comfort each other is a testament to the unbreakable bond we share.

To my cherished children, Abdullah, Omar, and Alya, everything I have achieved and will continue to strive for is for your sake. You are my greatest motivation and inspiration, and I am forever grateful for the love and joy you bring into my life. With immense appreciation, I extend my thanks to the University of Umm AlQura and The Royal Embassy of Saudi Arabia (Cultural Bureau) for their gracious support. Their assistance has not only eased the financial burden but also granted me the opportunity to further my education and research, a privilege that will undoubtedly shape my future endeavours

The Messenger of Allah (ﷺ) said,

''Whoever takes a path upon which to obtain knowledge, Allah makes the path to Paradis easy for him''.

Author's Declaration

I declare that this thesis is the result of my own work. AI tools, specifically OpenAI (© 2024) and Grammarly (© 2024 Grammarly Inc.), were used in this thesis to assist in correcting grammar and sentence structure. These tools were not used to generate any information or data in my thesis. All content and research findings presented are my own original work. No part of this thesis has been submitted for any other degree at The University of Glasgow or any other institution.

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Abbreviations

EH	Essential Hypertension
CVD	Cardiovascular Disease
HBP	High Blood Pressure
RAA	Renin Angiotensin Aldosterone
TOD	Target Organ Damage
TCR	T cell Receptor
MHC	Major Histocompatibility Complex
SHR	Spontaneously Hypertensive Rat
ROS	Reactive Oxygen Species
BP	Blood Pressure
Rag-/-	Recombination Activating Gene 1
Ig	Immunoglobulin
Ang II	Angiotensin II
DAMPs	Damage-Associated Molecular Patterns
PAMPs	Pathogen-Associated Molecular Patterns
PRRs	Pattern Recognition Receptors
DCs	Dendritic Cells
DOCA	Deoxycorticosterone Acetate
APC	Antigen-Presenting cells
TLRs	Toll-like Receptors

SLE	Systemic Lupus Erythematosus
VSMCs	Vascular Smooth Muscle Cells
VCAM	Vascular Cell Adhesion Molecule
HAV	Hepatitis A Viruses
NKG2D	Natural Killer Group 2D (Cell Receptors)
eNOS	Endothelial Nitric Oxide Synthase
PCNA	Proliferating Cell Nuclear Antigen
GFR	Glomerular Filtration Rate

Chapter 1 Introduction

1.1 Hypertension

Hypertension affects over one billion people worldwide and is a major contributor to cardiovascular disease (CVD) events such as coronary heart disease (CHD), heart failure, and stroke (Rodriguez-Iturbe et al., 2017). The prevalence of hypertension in adults was estimated at 31.1% (1.39 billion) in 2010, and this figure continues to rise, making the prevention and control of hypertension a critical public health priority (Mills et al., 2020). Hypertension is diagnosed when repeated measurements in a clinical setting show SBP of ≥140 mmHg and/or DBP of ≥90 mmHg, and it remains the leading cause of premature death due to CVDs (Unger et al., 2020). The emphasis on evidence related to CVD outcomes of blood pressure (BP)-lowering treatments not just reducing blood pressure is one of the important changes in the 2024 ESC Guidelines (McEvoy et al., 2024). Hypertension is categorized as essential (primary) hypertension, which accounts for over 90% of cases and lacks a clear cause, or secondary hypertension, which is linked to an identifiable cause. While the exact mechanisms behind essential hypertension are still unclear, current treatment focuses on long-term blood pressure management rather than curing the condition, and it often requires a combination of medications and lifestyle changes (Fang et al., 2020).

1.1.1 Pathophysiology of Hypertension

The development of hypertension involves a complex aetiology involving various contributing risk factors. Factors such as RAAS, and endothelial and nervous system disruptions are recognised as critical contributors to hypertension (Figure 1.1). However, despite these known causes, approximately 90% of cases lack clear origins, resulting in their classification as essential hypertension. This form often accompanies ageing, high salt intake, obesity, lipid metabolism disorders, and insulin resistance (Caillon et al., 2019). Conditions commonly associated with hypertension, like obesity, sleep disturbances, and chronic stress, are all associated with increased sympathetic outflow (Norlander et al., 2018). Moreover, there is broad recognition that low-grade inflammation plays a significant role in starting and sustaining high blood pressure and could contribute to its occurrence in association with chronic inflammatory diseases (Norlander et al., 2018; Rodriguez-Iturbe et al., 2017; Caillon and Schiffrin, 2016). The migration of activated immune cells to vital organs such as the kidneys, arteries, brain, and heart, where they release effector cytokines, was linked to increased blood pressure, vascular remodelling, cardiac hypertrophy, renal damage, dementia, and cognitive impairment (Guzik et al.,

2024). Moreover, evidence suggests that hypertension is more than a hemodynamic disorder. It is recognized as a complex condition involving multiple abnormalities, including inflammation, immune system activation, metabolic issues, and irregular fat distribution. This complexity underlies treatment failures in numerous patients (Formanowicz et al., 2020). A deeper understanding of the immune inter-communication between the upstream regulators and the mechanism of activating the immune system in hypertension will provide new insights into potential therapeutic targets (Figure 1.1).

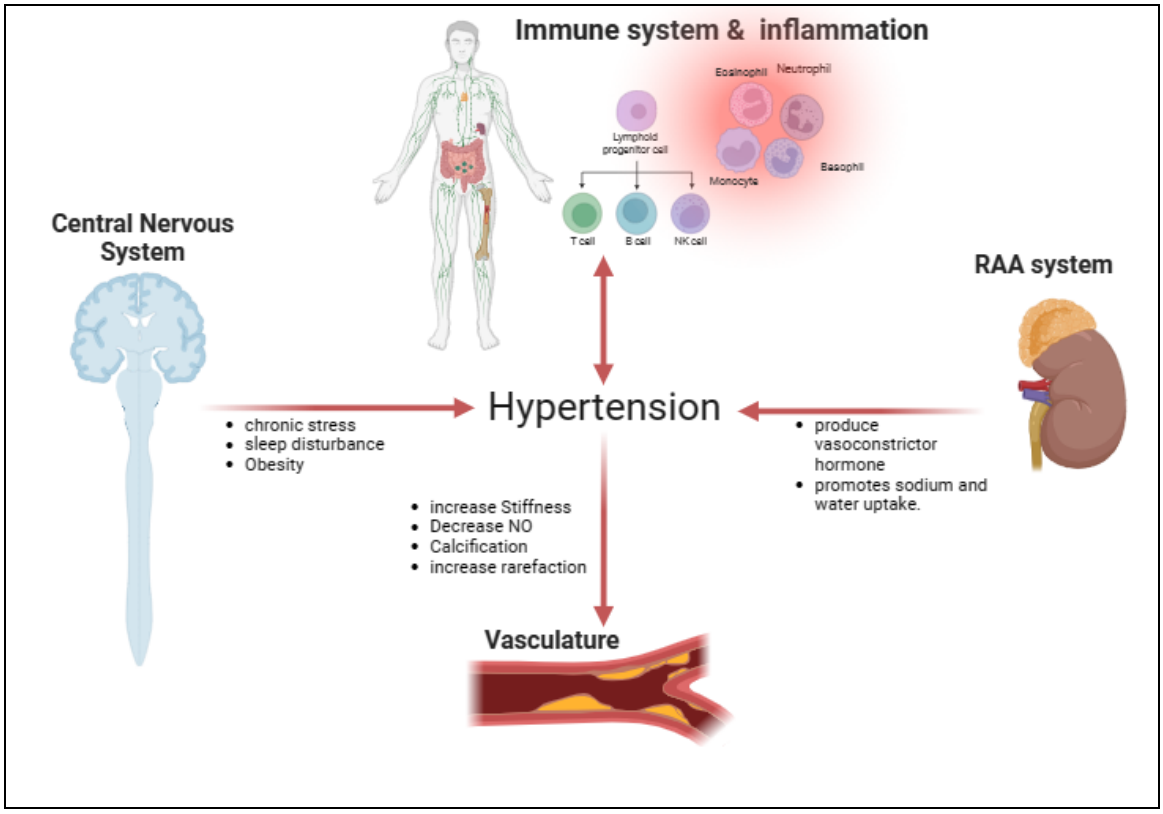

Figure 1.1. Aetiology of hypertension.

This figure illustrates the mechanisms contributing to hypertension, focusing on both immune and non-immune pathways. Chronic activation of the sympathetic nervous system (CNS) and the renin-angiotensin-aldosterone system (RAAS) leads to the excessive production of angiotensin II and aldosterone. These, in turn, stimulate adrenoceptors, angiotensin II receptor type 1 (AT1), and mineralocorticoid receptors. This stimulation results in increased vascular tone, renal sodium, and water reabsorption, which contribute to elevated blood pressure. Additionally, hypertension and immune system activation form a cyclical relationship. Hypertension induces immune activation through pressure effects, altered blood flow, and CNS signalling. Also, low-grade inflammation plays a role in both initiating and sustaining elevated blood pressure.
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Hypertension is a chronic condition driven by numerous interacting factors, making its treatment and control a persistent challenge. Blood vessels play a crucial role in regulating blood flow through vasoconstriction and vasodilation, thereby modulating peripheral resistance. The kidneys regulate blood volume by sodium and water excretion (Drummond et al., 2019). The first-line medications for treating hypertension include thiazide diuretics, calcium channel blockers, and either angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (Carey et al., 2022). Even in patients treated with more than four medications, including diuretics, around 30% are considered treatment-resistant (Cai and Calhoun, 2017). Additionally, abnormal metabolism and end-organ damage can persist even when blood pressure is controlled (Dai et al., 2018). As many of these drugs mainly target lowering blood pressure rather than addressing the main causes of the condition (Murray et al., 2021). Although progress in developing new treatments has been limited (Freeman et al., 2023; Harrison et al., 2011), novel approaches such as bispecific peptides are emerging in hypertension drug development. However, further studies are needed to fully investigate the therapeutic properties of these drugs (Ghatage et al., 2021). Overall, these clinical features of hypertension suggest that there might be additional mechanisms playing a role in hypertension.

1.1.2 Target organ damage in hypertension

Hypertension is often termed a "silent killer" as it can persist for many years without noticeable symptoms, potentially leading to target organ damage (TOD) before clinical detection. Most of the patients with hypertension are unaware of their condition, leading to a significant number of patients presenting with TOD at their first hospital visit (GuidelinesWHO, 1999). TOD is a consequence of sustained high blood pressure, which leads to damage to vital organs. This damage is characterized by both macrovascular and microvascular abnormalities, including stroke, structural heart disease, coronary artery disease, chronic kidney disease, retinopathy, proteinuria, and atherosclerosis (Hitha et al., 2008). These structural and functional changes within the affected organs often lead to serious complications and increased risks of cardiovascular and renal issues (Prakash, 2019). In addition, microalbuminuria was found to be significantly associated with the duration and severity of hypertension, as well as target organ failure, left ventricular hypertrophy, retinopathy, and cerebrovascular accidents (Kanjolia et al., 2022).

Recent research confirms a strong association between hypertension and damage to vital organs in newly diagnosed hypertensive patients. In a study involving a total of 150 participants, of whom 91 exhibited at least one TOD, the impact on critical organs such as the heart, kidneys, brain, and blood vessels was more pronounced than anticipated in hypertensive patients (Prakash, 2019). Another study also revealed the involvement of both the innate and adaptive immune systems in hypertension and subsequent long-term organ damage (Perrotta and Carnevale, 2023). This, in turn, emphasises the crucial role of tight blood pressure control in managing essential hypertension. Such control not only prevents immediate complications but also safeguards against long-term organ damage associated with high blood pressure.

In experimental settings, early reports using animal models of hypertension demonstrated a relationship between excessive fluctuations in BP and the development of TOD (Su and Miao, 2005). BP variability is recognised as a physiological indicator of autonomic nervous system regulation and may be involved in elevated cardiovascular risk in individuals with hypertension. In addition, the decrease in BP variability was associated with a reduction of TOD and a better cardiovascular outcome (Irigoyen et al., 2016). Several studies investigated whether different antihypertensive medications lowered blood pressure levels and minimised variability. Some of these studies found that lowering blood pressure variability with, e.g. calcium channel blockers was linked to effective reduction in blood pressure, less damage to vital organs, and improved cardiovascular outcomes. However, the association with medications targeting the renin-angiotensin system did not consistently show the same effects (Rothwell et al., 2010; Webb et al., 2010).

1.2 Central role of the immune system in the pathogenesis of hypertension

The concept of the relationship between the immune system and hypertension started decades ago. In the 960s, Okuda and Grollman proved that rats develop hypertension when they receive lymphocytes obtained from another hypertensive animal, i.e., a unilateral renal-infarction rat (Okuda and Grollman, 1967). Years later, Ba *et al.*, in the 1980s, found that blood pressure in the spontaneously hypertensive rat (SHR) had decreased after thymus transplantation from normotensive Wistar-Kyoto rat (Okuda and Grollman, 1967). Moreover, with the immunosuppressive drug cyclophosphamide and anti-thymocyte serum, there was a noticeable decrease in the level of blood pressure (Dzielak, 1991). These early studies suggested that the immune system has a key role in hypertension. In

2007, Guzik *et al*. demonstrated the involvement of lymphocytes in hypertension by utilising mice deficient in the recombination activating gene-1 (Rag-/-). This gene is crucial for the rearrangement and recombination of immunoglobulin (Ig) and T cell receptor (TCR) genes, leading to a deficiency in both T and B cells in these mice. The mice lacking Rag1 exhibited diminished Angiotensin II (Ang II)-induced vascular remodelling and decreased superoxide production (Guzik et al., 2007).

1.3 Key Immune regulators of hypertension

The primary role of the immune system is to protect the host from antigens, involving a complex interplay between two primary branches: innate immunity, which orchestrates initial reactions, and adaptive immunity, a more specialised and delayed response. Innate and adaptive immune cells emerge as critical players implicated in hypertensive pathology. Classically, immune activation initiates from antigens like damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) when they activate innate immune response via pattern recognition receptors (PRRs) such as Toll-Like R receptors (TLRs). Ongoing inflammation in essential hypertension is contributing to increased levels of DAMPs, which might be neoantigens produced by stressed or injured tissues due to high blood pressure. However, the origin of DAMPs remains unclear; molecules such as Ang II, HMGB-1, HSP60, HSP70, fibrinogen, uric acid, and mitochondrial DNA identified as key DAMPs persistently elevated in hypertension (McCarthy et al., 2014). Detecting DAMPs and PAMPs by PRRs can activate innate cells (macrophages, dendritic cells, NK cells) and trigger the initiation of the immune response (Mian et al., 2014).

Dendritic cells (DCs), macrophages and neutrophils are primary cell types in the innate immune response. DCs are identified as a potential source of DAMPs in hypertension. This is due to an increase in NADPH oxidase-dependent superoxide production in DCs, leading to the creation of highly reactive γ-ketoaldehydes, also known as isoketals or isolevuglandins (IsoLG) (Kirabo et al., 2014). This process promotes renal and vascular dysfunction, contributing to the development of hypertension. In experimental models and hypertensive patients, there is a noted infiltration of macrophages into the vascular wall, kidneys, and myocardium (Justin Rucker and Crowley, 2017; Hulsmans et al., 2018). Studies involving mice lacking genetic macrophage deficiency showed protection from vascular dysfunction induced by Ang II (De Ciuceis et al., 2005) and deoxycorticosterone acetate (DOCA)-salt hypertension (Ko et al., 2007). M1 and M2-type macrophage

responses activate and direct T cells/adaptive immunity. M1 macrophages are associated with activating T helper 1 (Th1) lymphocytes, while M2 macrophages are linked to inducing Th2 lymphocyte responses. Although limited data exist regarding the polarisation of M1 and M2 phenotypes in hypertension, the interaction between T lymphocytes and macrophage subsets indirectly indicates the potential role of macrophage polarisation in hypertension (Kossmann et al., 2013).

T and B lymphocytes are important components of adaptive immunity, exhibiting interactions with the innate immune system. B cells play a crucial role in adaptive immunity by detecting and processing antigens and generating antibodies. Studies reported elevated levels of IgG, IgM, and IgA antibodies in severely hypertensive individuals (Suryaprabha et al., 1984; Hilme et al., 1989; Chan et al., 2014). IgG can interact with Fcγ receptors found on macrophages, which affects the macrophages' polarisation and triggers cytokine release (Tanigaki et al., 2015). While the transfer of B cells did not restore hypertension in Ang II-infused RAG1-/- mice (Guzik et al., 2007), the activation of B cells appears to rely significantly on specific interactions with T cells (Parker, 1993; Guzik et al., 2007), which are absent in RAG1-/- animals. Ang II infusion induces antibody production from activated B cells. Genetic deficiency of the B-cell-activating factor receptor or depletion of B cells via pharmacological methods prevents blood pressure elevation and the end-organ consequence of Ang II, such as aortic stiffness and collagen deposition. These effects can be reversed by reintroducing B cells through adoptive transfer (Chan et al., 2015; Dingwell et al., 2019). T and B lymphocytes are essential elements of adaptive immunity and are intricately intertwined with the innate immune system, where they play a crucial role in the pathophysiology of hypertension.

1.3.1 T cell in hypertension

Typically, T cells exhibit the T cell receptor (TCR), which consists of two TCR chains, specifically αβ, surrounded by the CD3 complex. The CD3 complex, composed of CD3γ, δ, ε, and ζ-chains, which are responsible for transmitting the TCR signal. T cells were shown to be implicated in hypertension, with their role becoming increasingly clear through various studies. Their maturation in the thymus precedes their dormant lymphoid organs until activation by APC triggers their response. Early investigations using athymic mice highlighted that lymphocyte depletion prevented or corrected hypertension in multiple models (Svendsen, 1977; Horan and Lovenberg, 1986). Athymic DOCA-treated mice resisted salt-induced hypertension, but thymus transplantation restored their salt

sensitivity (Svendsen, 1976). Similarly, lymphocyte or splenocyte transfers from hypertensive rats to normal ones increased blood pressure in recipients, emphasising the necessity of an intact T cell immune system for hypertension development (Olsen, 1980; Okuda and Grollman, 1967). The absence of B and T cells in RAG-1-/- mice blunted hypertension responses to various stimuli (Mattson et al., 2013; Guzik et al., 2007), whereas reintroducing CD8+ T cells restored the hypertensive response (Guzik et al., 2007; Trott et al., 2014). Moreover, mice with severe combined immune deficiency (SCID) and Dahl salt-sensitive rats with selective T cell signalling gene removal were protected from hypertension despite hypertensive stimuli (Crowley et al., 2010; Rudemiller et al., 2014).

T cells express all the necessary parts of the renin-angiotensin system, such as Ang II, ACE, renin, AT1, and AT2 receptors (Hoch et al., 2009). When activated, this system produces Ang II, which acts within the T cells themselves, boosting the expression of these components and triggering the production of TNF (Coppo et al., 2011; Silva-Filho et al., 2011). Interestingly, this process happens specifically in T cells that were previously activated with a TCR ligand (Hoch et al., 2009), which could indicate that Ang II plays a role in influencing T cell behaviour. In Dahl salt-sensitive rats, increasing salt consumption enlarged renal infiltration of T lymphocytes and increased arterial blood pressure and albumin excretion, resulting in renal glomerular and tubular damage. Infiltrating T cells produced Ang II, which caused renal disease. Suppression of T cells decreased intrarenal Ang II and prevented Dahl SS hypertension (De Miguel et al., 2010).

T cell subsets play a crucial role in the secretion of cytokines like TNFα during hypertension. T cells produce cytokines like TNFα, inducing ROS production and activating sodium retention, both implicated in hypertension (Guzik et al., 2007; Huang et al., 2016a). Furthermore, the deletion of IFN-γ and IL-17, secreted by T cells, significantly reduced elevated blood pressure in some hypertension models (Kamat et al., 2015; Madhur et al., 2010), however, the role of immune cells can vary across different models (Krebs et al., 2014).

Mechanistically, T cell subsets (CD4+ and CD8+) infiltrate kidneys and vasculature during hypertension, triggering endothelial dysfunction and sodium retention, mediated through pathways like ROS development and pro-inflammatory cytokine release (Crowley et al., 2010; Guzik et al., 2007; Miguel et al., 2011). Interestingly, Itani *et al.* used a humanised mouse model in which the human immune cells replaced the murine immune system. They

observed increased infiltration of human T cells, especially CD4+ subsets in thoracic lymph nodes, thoracic aorta, and kidneys in response to Ang II infusion. Also, CD8+ infiltration was higher in both lymph nodes and thoracic aorta in hypertensive animals compared with normotensive. The aortas and lymph nodes also noted an increase in memory T cells CD3+CD45RO+ (Itani et al., 2016a). This highlights the multifaceted role of T cells in hypertension, involving both their infiltration and the secretion of cytokines.

On the other hand, studies highlight T-regulatory (Treg) cells as potential hypertension regulators. Adoptive transfer of Treg cells mitigates hypertension, vascular damage, and inflammation induced by Ang II or aldosterone (Barhoumi et al., 2011) (Guzik et al., 2007). Conversely, the transfer of T cells lacking Treg cells exacerbates endothelial dysfunction and oxidative stress (Mian et al., 2016). This oxidative stress is associated with hypertensive conditions through the activation of adaptive immunity (Kirabo et al., 2014; Mikolajczyk et al., 2019; Guzik et al., 2007; Loperena and Harrison, 2017). Enhanced Treg function in rodent models was found to be associated with reduced hypertension, emphasising their pivotal role in hypertension regulation (Viel et al., 2010).

1.3.1.1 CD4+ T cell

T cells are distinguished by their specific cell surface markers that determine their role. T cells undergo maturation in the thymus, where CD3+ T lymphocytes transition into single-positive cells (CD4+ or CD8+). Certain cells remain double-negative (CD4−CD8−), while others differentiate into double-positive cells (CD4+CD8+). Two signals are required to activate T cells: the first signal through TCR activation by an antigenic peptide presented by APCs via their major histocompatibility complex (MHC) molecules and the co-stimulatory signal, often through interactions between B7 family ligands on APCs and the T cell surface co-stimulatory molecules. CD4 and CD8 act as co-receptors for the TCR, attaching to MHC class I and II molecules. While it was established that both types of T cells play a role in the onset and advancement of hypertension, findings from *in vivo* models indicate that CD4+ T cells are the primary contributor to promoting hypertension (Itani et al., 2016a). Upon stimulation by antigen, co-stimulators, and specific cytokines, naive CD4+ T helper cells differentiate into Th1, Th2, and Th17 effector cells or Treg, each producing its unique set of cytokines and performing distinct functions (Bomfim et al., 2019). In hypertensive patients, the presence of circulating Th1 and Th17 cells was higher in uncontrolled hypertensive patients compared to normotensive individuals (Ji et al., 2017). However, a different study showed no significant difference in circulating

senescent CD4+ T cell frequencies in newly diagnosed hypertensive patients compared to age- and sex-matched normotensive controls. However, these patients exhibited higher frequencies of senescent (CD28−CD57+) CD8+ T cells (Youn et al., 2013). Ang II infusion led to higher systolic and diastolic blood pressure in WK and CD4 KO mice, unlike mice lacking CD8 T cells, which showed a reduction in blood pressure elevation with Ang II infusion. Similarly, transferring CD4+/CD25− cells from Ang II-treated mice to RAG-1 KO mice did not impact BP, while transferring CD8+ cells increased BP (Trott et al., 2014).

The roles of Th1, Th2, and Th17 cells in hypertension centre on their specific cytokine profiles, which influence blood pressure regulation and the development of target organ damage. In hypertension, Th17 cells and their associated cytokine IL-17 influence kidney sodium balance and contribute to endothelial dysfunction, ultimately leading to increased blood pressure (Madhur et al., 2010). However, the role of Th2 cells in hypertension remains unclear and necessitates further investigation (Wenzel et al., 2021). Studies using T-bet knockout mice suggest that Th1 cells might not notably affect blood pressure, but they could induce damage to target organs, particularly the kidneys. Th1 cells express key cytokines such as IFN-γ, TNF-α, and IL-2 via the transcription factor T-bet. Although the impact of IFN-γ on blood pressure remains varied, mice lacking TNF-α showed reduced hypertensive responses and were shielded from organ damage, highlighting a pivotal role for Th1 cell-derived TNF-α, specifically from renal parenchymal cells, in driving hypertension and its associated complications (Zhang et al., 2014; Guzik et al., 2007).

1.3.1.2 CD8+ T cell

CD8+ T cells have an essential specific cytotoxic function. A study demonstrated that Ang II infusion in wild-type (WT) and CD4 KO mice increased systolic and diastolic BP. However, BP elevation was blunted in CD8 KO mice under Ang II infusion. Additionally, the adoptive transfer of $CD4^{+}/CD25^{-}$ cells from Ang II-treated mice to RAG-1 KO mice did not affect BP. However, the adoptive transfer of $CD8^{+}$ cells led to an increase in blood pressure (Trott et al., 2014). Elevation in the number of IFN-γ+ CD8+ T cells was observed in the spleen and kidneys of Lnk-/- mice compared to their WT mice upon Ang II administration (Saleh et al., 2015). Interestingly, the T cell population analysis from hypertensive individuals revealed an increased percentage of immunosenescence, pro-inflammatory, and cytotoxic CD8+ cells. These cells were characterised by the expression of CD57 antigens and lack of expression of CD28 molecules ($CD8+CD57+CD28^{null}$).

Furthermore, CD8+ cells derived from patients with hypertension produced more perforin and granzyme B than normotensive individuals. This effect was not observed in CD4+ T cells; however, in these cells, a higher percentage of perforin-positive cells was noticed in hypertensive subjects compared to normotensive individuals. Moreover, renal tissue biopsies confirmed higher infiltration of T cells and their subset CD8+ T cell in patients with hypertensive nephrosclerosis (Youn et al., 2013).

CD8+ T cells, known for their IFN-γ production, are mass in the kidneys during hypertension. Studies on mice lacking CD8+ T cells revealed their protection against hypertension, while mice lacking CD4+ T cells exhibited heightened blood pressure responses. This heightened response in CD4+ cell-deficient mice, which also lack regulatory T cells (Tregs), could contribute to worsened hypertension in these animals (Trott et al., 2014). Notably, vascular rarefaction in the kidney was observed in WT and CD4-/- mice but not in CD8-/- mice. These changes potentially induce sodium and volume retention (Trott et al., 2014). Liu *et al.* discovered that CD8+ T cells interact with the distal convoluted tubule in DOCA-salt mice, leading to increased expression and activation of the NCC (sodium-chloride cotransporter). This process resulted in salt-sensitive hypertension by promoting sodium retention through chloride influx triggered by CD8+ T cell contact with tubular cells (Liu et al., 2017).

1.3.1.3 Double Negative (DN) T cell

In Ang II-dependent hypertension, the percentage of CD4+TNF+ and CD8+TNF+ T cells in perivascular adipose tissue was higher compared to control animals. This was also extended to TNF+CD3+CD4−CD8− (Double negative) T cells. However, implementing anti-inflammatory interventions led to a decrease in the percentage of TNF+CD8+ T cells without affecting other subsets (Mikolajczyk et al., 2019). Guzik *et al.* demonstrated that Ang II boosted the presence of double negative (DN) (CD3+CD4−CD8−) T cells in specific tissue regions like the perivascular adipose tissue and adventitia. These T cells exhibited a significant increase, nearly sevenfold, in response to Ang II stimulation, indicating their enhanced accumulation in the aorta under hypertensive influence (Guzik et al., 2007), similar to another finding in 2010 (Vinh et al., 2010).

DN T cells exhibit a complex role in inflammation across various diseases. They possess a diverse cytokine profile, contributing to proinflammatory and regulatory responses in autoimmune diseases and infections. Notably, they play a crucial role in disease

pathogenesis by instigating systemic inflammation, organ damage, and the generation of autoantibodies, evident in conditions such as systemic lupus erythematosus (SLE) and autoimmune lymphoproliferative syndrome (Crispin et al., 2008; Li et al., 2020; Shivakumar et al., 1989; Bleesing et al., 2002). Moreover, their presence within organs is closely associated with the severity and progression of diseases like SLE-related nephritis (Crispin et al., 2008; Alexander et al., 2020; Qiao et al., 2015). The involvement of DN T cells in ailments like Sjögren's syndrome and psoriasis further underscores their contribution to tissue damage and skin pathology (Hayashi et al., 1994; Ueyama et al., 2017; Brandt et al., 2017; Alunno et al., 2014). However, while DN T cells play a pivotal role in driving inflammation and immune-related disorders, the specific roles and underlying mechanisms are unclear.

1.3.1.4 Memory, Effector T Cell

Memory T cells (T_M) that encountered an antigen previously possess the ability to mount a faster and stronger immune response upon re-exposure to the same antigen. The formation of these T_M cells requires interactions between naive T cells ($CD44^{low}$ $CD62L^{high}$ $CCR7^{high}$) and activated APCs expressing specific co-stimulatory molecules like CD70, CD80, CD83, and CD86. In hypertension, APCs from affected individuals exhibit heightened levels of these co-stimulatory molecules (Itani et al., 2016a; Kirabo et al., 2014; Itani et al., 2016b). This interaction between APCs and T cells leads to the generation of specialised memory and effector T cell subsets, such as central memory T cells (T_{CM}) ($CD44^{high}$ $CD62L^{high}$ CCR7+), effector memory T cells (T_{EM}) ($CD44^{high}$ CD62Llow CCR7−), and resident memory T cells ($CD44^{high}$CD62L−CD103+CD69+) (Itani et al., 2016b). This memory enables a quicker and more efficient response when encountering antigens (Barski et al., 2017).

Immunological memory appears to be implicated in hypertension, as highlighted by several studies. In one instance, increased BP was observed in control rats following the transfer of splenocytes from hypertensive rats. Also, mice experiencing initial hypertensive challenges developed T_M cells that, upon subsequent mild challenges, significantly heightened the susceptibility to severe hypertension. These T_M cells, generated by the first hypertensive encounter induced by Ang II or L-NAME, substantially amplified the response to later mild hypertensive triggers like high salt or low doses of Ang II. Mice lacking T_M cell formation did not exhibit this heightened response, emphasising the crucial role of these cells in hypertensive memory. Interestingly, mice deficient in a co-stimulatory molecule

CD70 did not accumulate T_M cells or show a sensitised response to repeated hypertensive challenge (Itani et al., 2016b). In addition, the accumulation of memory cells was observed in hypertensive animal models (Itani et al., 2016b) and humans (Itani et al., 2016a). Itani *et al.* stated that elevation in blood pressure and kidney injury would not occur without T_M cells (Itani et al., 2016b). Additionally, Xiao *et al.* and Carnevale *et al.* found increased T_M cells in the aorta and kidney during pre-hypertensive stages and after hypertension development in mice due to Ang II infusion (Xiao et al., 2015; Carnevale et al., 2014). The sympathetic nervous system also plays a role in accumulating hypertension-specific T_{EM} cells in the bone marrow, contributing to their homing and survival. Inhibition of sympathetic nerve activity and β2 adrenergic receptor blockade reduced these cells, preventing the elevation of BP and renal inflammation upon re-exposure to hypertension stimuli (Xiao et al., 2020).

Hypertension is associated with an increase in the number of CD3+CD45RO+ T_M cells, as demonstrated by Itani *et al.* Single-cell transcriptome profiling further substantiated this finding, revealing distinct T_M cell subsets, including CD4+ and CD8+ T_{EM} cells (CD45RA−CCR7−), along with T_{CM} (CD45RA−CCR7+) (Itani et al., 2016a). Among these groups, patients with hypertension displayed notably higher levels of CD8+ T_{EM} cells compared to those with normal blood pressure. These hypertensive patients' CD8+ cells exhibited increased activation of pathways linked to mitochondrial oxidative metabolism, vascular function, and inflammation (Wang et al., 2023). It was also proposed that CD4+ T_{EM} lymphocytes, particularly those expressing choline acetyltransferase (ChAT), play a distinct role in cholinergic anti-inflammatory pathways and BP regulation. Activation of these cells in the spleen, triggered by sensory afferent vagal nerve signals, results in acetylcholine release. This acetylcholine inhibits macrophages' TNF-α production through α7-nicotinic acetylcholine receptors, suppressing inflammation (Okusa et al., 2017). Olofsson *et al.* suggest that CD4+ ChAT+ T_{EM} cells may contribute to BP reduction by promoting acetylcholine-induced vasodilation, as evidenced by increased endothelial nitric oxide synthase phosphorylation and nitric oxide production during co-culture with endothelial cells (Olofsson et al., 2016). The elevated percentage of T_M (CD45RO) in both CD4+ and CD8+ T cell subsets in hypertensive individuals indicates increased circulating T_M cells, suggesting immune system activation and potential pathophysiological relevance in sustaining hypertension in humans.

1.3.2 Role of cytokines in the immune system and hypertension

Cytokines, glycoproteins, are produced by different cell types. They regulate immunity, inflammation, and haematopoiesis. Cytokines regulate several physiologic and pathologic functions, including innate immunity, acquired immunity, and many inflammatory responses (Khan and Khan, 2016). They are generated from several sources during the effector stages of innate and adaptive immune responses. Additionally, they are released during non-immune events and have a function unrelated to the immune response in many tissues. Typically, its secretion is short-lived, and several cell types generate these molecules, influencing various cell types and tissues (Khan and Khan, 2016). Cytokines frequently have pleiotropic effects on the same target cell, capable of stimulating and suppressing the production and actions of other cytokines. They exert their specific effects by attaching to specific receptors on the cell surface of target cells. Multiple signals regulate the expression of cytokine receptors. The target cells exhibit a specific biological response by producing new mRNA and proteins responding to cytokines (Khan and Khan, 2016).

Cytokines participate in various cellular processes, encompassing inflammation, tissue damage, and regeneration. They stimulate the recruitment and activation of immune cells and contribute significantly to the development of atherosclerosis (Spinas et al., 2014; Ma et al., 2014). Proinflammatory signals prompt mast cells to produce cytokines, initiating the expression of endothelial adhesion molecules such as vascular cell adhesion protein 1 (VCAM-1), P-selectin, and plated activating factor (Mulvany, 2012). The recruitment and adhesion of leukocytes foster vascular and extracellular matrix remodelling, leading to increased fibrosis and hypertrophy and subsequently reducing the diameter of the vascular lumen (Singh et al., 2014; Ishibashi et al., 2004). In addition, interleukins, serving as crucial mediators of inflammation, appear to play a role in target organ damage among hypertensive individuals (Singh et al., 2014; Ishibashi et al., 2004). During the progression of hypertension, immune cells accumulate in target organs, such as in kidneys and vasculature (Itani et al., 2016a; Mikolajczyk et al., 2016). These immune cells secrete potent cytokines that significantly impact vascular and renal function, thereby playing an essential role in the development of hypertension.

1.3.3 Cytokines involved in inflammatory responses

Cytokines such as IL-15, IL-17, TNF-α, and IFN-γ are key contributors to hypertension through their roles in promoting inflammation. These cytokines are involved in inflammatory pathways that influence vascular health and blood pressure regulation. The table (Table 1.1) provides an overview of the regulatory and pro-inflammatory cytokines, detailing their known associations with hypertension and pathological effects.

Table 1.1. Cytokines involved in hypertension and their functional and pathological roles.

Cytokines	Source	Cytokines/ cytokines receptors inhibitor effect	Hypertension Link	Present in serum	Physiological and Pathological Effects	References
IL-1β	T cells, monocytes and neutrophils	- Lowering blood pressure. - Decrease release of IL-6.	Yes	Yes	- Induce expression of inflammatory genes (E-selectin, VCAM-1, ICAM-1, IL-6, IL-8, and COX-2 genes). - Tissue injury. -Inflammation-related events such as myocardial infarction and hypertension. -Vascular remodelling.	(Krishnan et al., 2019), (Sims and Smith, 2010; Xu et al., 2019; Dinarello et al., 2010), n (Krishnan et al., 2014), (Urwyler et al., 2020), (Veiras et al., 2022; Kułdo et al., 2005).

TNF-α	Monocytes and macrophages	- Lower PB in both patients and animal models.	Yes	-	-Regulating inflammatory processes, such as cytokines secretion, cellular differentiation, and programmed cell death. - Promotes cell apoptosis, NADPH oxidase activation, and activation of nuclear factor kappa B (NFκB). - Increase inflammation and blood vessel damage by increasing the expression of chemokines and adhesion molecules in blood vessels. - Lead microvascular remodelling and sodium retention. - Destabilizes eNOS mRNA, leading to eNOS degradation and reduced NO synthesis in kidney and endothelial. - Increased expression of TNF-α with hypertension	(Segiet et al., 2019). (Aggarwal, 2003). (Guzik et al., 2007; Yoshida et al., 2014). (Kleinbongard et al., 2010), (Landry et al., 1997), (Neumann et al., 2004; Alonso et al., 1997). (Ramseyer et al., 2012; Garvin et al., 2011).
INF-γ	Th1 cells, CD4+ and CD8+ T cells		Yes	-	- Enhancing the expression of angiotensinogen in rat renal proximal tubule cells in a JAK2/STAT3-dependent manner, leading to increased blood volume. - Conversely, Reduces BP and alleviates proteinuria and glomerular injury in Dahl salt-sensitive rats.	(McMaster et al., 2015), (Itani et al., 2016a), (Satou et al., 2012), (Ishimitsu et al., 1992)
IL-6	Monocytes, macrophages, dendritic cells	- Significant therapeutic effects on rheumatoid and juvenile arthritis. - Reduce BP	Yes	Yes	- Activate genes involved in cell proliferation, differentiation, and apoptosis. - Activation of JAK and Ras-mediated signal pathway. - Enhances the expression and activity of sodium channels in mouse cortical collecting duct cells *In vivo*.	(Heinrich et al., 1998). (Ogata and Tanaka, 2012), (Barbaro and Harrison, 2019), (Vazquez-Oliva et al., 2005), (Lee et al., 2006; Brands et al., 2010), (Li et al., 2010)

IL-12	Dendritic cells, macrophages, and B cells	-	Yes	Yes	- Elevation in IL-12 expression is linked to cardiovascular conditions like atherosclerosis, coronary artery disease, atrial fibrillation, aortic dissection, cardiomyopathy, and viral myocarditis. - IL-12 may also have anti-inflammatory effects in specific inflammatory microenvironments. - Prompts T cells to release IFN-γ and TNF-α.	(Tripp et al., 1993; Hsieh et al., 1993), (Cheng et al., 2005), (Ye et al., 2020), (Sharma et al., 2019; Ye et al., 2019).
IL-17	CD8+ cells, neutrophils, natural killer T cells, Th17 cells	- IL-17 neutralisation (via gene deletion or antibodies) limits the progression of Ang II-induced hypertension; and reduces arterial immune cell infiltration, superoxide production, and aortic T cell infiltration.	Yes	Yes	- Increase proinflammatory cytokine involved in rheumatoid arthritis, inflammatory bowel disease, psoriasis, and airway inflammation. - Contributes to hypertension by increasing arterial immune cell infiltration, superoxide production, and reducing endothelium-dependent vasodilation. - IL-17 facilitates chemotaxis of other inflammatory cells, leading to inflammation and fibrosis.	(Witowski et al., 2004), (Kondo et al., 2009), (Madhur et al., 2010; Kamat et al., 2015), (Madhur et al., 2010; Saleh et al., 2016), (Amador et al., 2014), (Kleinewietfeld et al., 2013; Wu et al., 2014), (Kao et al., 2005; Hartupee et al., 2007), (Li et al., 2014).

IL-15	Monocytes, macrophages, dendritic cells, and vascular endothelial cells	-	Yes	Yes	- Associated with end-organ damage, including left ventricular hypertrophy, retinal artery narrowing, proteinuria, and carotid plaque. - Induces JAK/STAT-related pathways; promotes differentiation, proliferation, and survival of immune cells, particularly memory CD8+ T cells. - Enhances effector functions of memory CD8+ T cells. - High IL-15 levels are associated with complications like coronary artery disease and lacuna infarction in essential hypertension.	(Xie et al., 2020), (Gokkusu et al., 2010), (Wuttge et al., 2001), (Hu et al., 2007; El-Baradie et al., 2009), (Bullenkamp et al., 2021; Kalantar et al., 2013; Kaibe et al., 2005; Perera, 2000).

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1.3.4 Cytokines involved in immune regulation

Table 1.2. Cytokines involved in immune regulation and their roles in hypertension

Cytokines	Source	Cytokines/ cytokines receptor inhibitor effect	Hypertension Link	Present in serum	Physiological and Pathological Effects	References
IL-2	Activated T cells	- Blocking Type1 Ang II receptor inhibits IL-2.	Mixed effects studies.	-	- Stimulate the proliferation of effector T cells. - Generating and sustaining Treg cells	(Tawinwung et al., 2018), (Ogbole and Moroyei, 2023), (Given et al., 1992; Pascual et al., 1990), (de la Rosa et al., 2004), (Ishimitsu et al., 1994; Tuttle and Boppana, 1990).
IL-4	Produced by Th2 cells.	Suppress hyperten-sion.	- IL-4 infusion improved mean arterial pressure and decreased inflammato ry markers	-	IL-4 is proposed as a possible contributing mechanism to the increased risk of chronic kidney disease associated with persistent asthma. - Elevated IL-4 levels can exacerbate susceptibility to hypertension and dilated cardiomyopathy. - Linked to asthma and chronic kidney disease. - Mixed results on its role in chronic hypertension and preeclampsia.	(Peng et al., 2015), (van Heuven-Nolsen et al., 1999), (Liu et al., 2013), (Taylor et al., 2016), (Liu et al., 2013).

IL-7	Stromal cells in lymphoid organs, tissue-specific tertiary lymphoid organs.	- Impair joint inflamma-tion, osteoclast formation, and neovasculari zation.	No evidence.	No	- IL-7 is essential for T cell proliferation, survival, and co-stimulation. - Upregulation of the α-apoptotic molecule Bcl-2. - Increase in serum with auto-immune diseases like rheumatoid arthritis.	(Kim et al., 2008; Meyer et al., 2022), (Pachynski et al., 2021; Kim et al., 2008), (McInnes et al., 1997; Laurent et al., 2021).

1.4 Cytokine receptors expression in hypertension.

Cytokines, as signalling molecules, exert their effects through specific receptors, and research illuminated their pivotal role in the pathophysiology of hypertension. Cytokine receptors, such as CD25, and interleukin receptors, like IL-6R and TNFRs, contribute to diverse immune responses that impact BP regulation and cardiovascular outcomes. Moreover, IL-7R was implicated in various immune responses. IL-7R blockade showed effectiveness in impairing joint inflammation, osteoclast formation, and neovascularisation, particularly relevant in patients with rheumatoid arthritis, where increased levels of IL-7 identified (Meyer et al., 2022). IL-1R1 is as well a subject of investigation in the context of hypertension. An IL-1R1 inhibitor, Anakinra, demonstrated a significant reduction in systolic blood pressure and peripheral vascular resistance in patients with obesity (Urwyler et al., 2020).

CD25, also known as the IL-2R alpha chain, is a crucial component involved in the activation and function of T cells, which play a key role in immune regulation and maintaining tolerance. Recent findings in mouse studies shed light on the intricate role of α-CD25 treatment in the context of hypertension, specifically in the development of Ang II-induced hypertension. The protective effects of knocking down complement receptors 3a and 5a in the development of Ang II-induced hypertension were eliminated by α-CD25 therapy (Chen et al., 2018). Studies in male C57BL/6 mice demonstrate that the adoptive transfer of CD4+ CD25+ T cells can attenuate hypertension induced by Ang II or aldosterone (Barhoumi et al., 2011; Kasal et al., 2012). In addition, the adoptive transfer of these T cells to Ang II-infused hypertensive mice showed to improve microvascular function (Radwan et al., 2019) and cardiac damage independently of high BP (Kvakan et al., 2009). In contrast, Compared to normotensive Wistar Kyoto rats, male SHR expresses less than half as many CD4+ CD25+ T cells in the spleen, which they interpreted as indicating that the absence of this population plays a role in the development of hypertension in the SHR (Zhang et al., 2018).

Chronic hypertension was shown to be associated with an upregulation of IL-6, and constitutive IL-6/sIL-6Rα stimulation was also shown to induce cardiac hypertrophy (Matsushita et al., 2005). This effect is mediated through the interaction of IL-6/IL-6R with gp130, leading to downstream signalling that impacts various cellular events, contributing to hypertensive characteristics in the heart (Melendez et al., 2010). Furthermore, TNFR plays a crucial role in blood pressure regulation. Deletion of TNFR1 was linked to higher

systolic pressure and altered renal function in response to angiotensin II infusion. TNFR1 activation is implicated in mediating acute renal vasoconstriction and natriuretic actions, while TNFR2 influences positive inotropic effects in cardiac myocytes and may contribute to increased susceptibility to albuminuria (Chen et al., 2010; Defer et al., 2007). TNF-α receptor activation contributes to cell apoptosis, NADPH oxidase activation, and nuclear factor kappa B (NFκB) activation (Kleinbongard et al., 2010). Evidence suggests that NFκB and NADPH oxidase activation, driven by TNF-α, promotes hypertension by upregulating the expression of chemokines and adhesion molecules in blood vessels. This, in turn, facilitates microvascular remodelling and sodium retention (Landry et al., 1997). NADPH oxidase-produced superoxide can react with endothelial NO to generate the potent oxidant peroxynitrite, severely impacting vasodilation and causing a significant elevation in BP due to reduced NO levels.

Furthermore, a previous study indicated that IFN-γR deficiency significantly attenuates ventricular hypertrophy and ventricular electrical remodelling (Marko et al., 2012). Despite injecting high doses of angiotensin, BP levels did not significantly increase in mice lacking the IFN-γR1. At the same time, renal fibrosis decreased, and the glomerular filtration rate (GFR) was maintained compared to WT mice (Marko et al., 2012).

Lentiviral targeting of IL-17R showed promise in improving cardiac function and reducing fibrosis in hypertensive rat models. The knockdown of IL-17R was associated with decreased Collagen 1A expression, concentration, and cross-linking, suggesting a potential therapeutic approach for hypertensive cardiac complications (Liu et al., 2011). In contrast to other cytokines, a study by Testa *et al.* revealed that IL-2 and sIL-2R were not elevated in patients with heart failure secondary to coronary artery disease (CAD) or hypertension (Bakhshi et al., 2020), indicating a nuanced cytokine expression profile in different cardiovascular conditions. These findings collectively contribute to our understanding of the role of cytokine receptor expression in hypertension, offering valuable insights for potential therapeutic interventions in hypertensive disorders.

1.5 Targeting inflammation as a potential treatment for hypertension

As discussed previously, hypertension, characterised by elevated blood pressure, is intricately linked with the immune system, making inflammation a crucial player in its pathogenesis. Beyond conventional BP control, addressing inflammation is a promising avenue for potential therapeutic interventions. A holistic understanding of the immune mechanisms involved in hypertension opens the door to novel strategies for treating hypertension and mitigating end-organ damage associated with this condition.

In this context, various targets were identified, including specific immune cell types, cytokines, TLRs, and components of inflammasomes. Notably, cytokines, as key mediators of the inflammatory response, play a central role in the complex interplay between immune function and hypertension (De Miguel et al., 2015). IL-15, a pleiotropic pro-inflammatory cytokine, garnered attention for its regulatory role in glucose metabolism and its potential as a therapeutic target in CVD (Guo et al., 2020). Other cytokines like IL-6, IL-2, and IFN-γ were implicated in hypertension, with studies showing elevated levels in patients with associated conditions like pulmonary arterial hypertension and cardiac heart disease (Tomaszewski et al., 2023). The precise role and potential of these cytokines as diagnostic markers or therapeutic targets in hypertension warrant further investigation (Tomaszewski et al., 2023). Researchers explored the use of recombinant human IL-4 receptor in asthma and BP control, showcasing its potential to improve respiratory function (Kassem et al., 2020). This highlights the diverse applications of cytokine modulation and underscores the need for continued experimental studies and clinical trials to unravel the full scope of their beneficial effects.

While targeting inflammation, mainly through immunosuppressive therapies, holds promise for hypertensive patients, it is imperative to weigh the potential benefits against the risks (Tanase et al., 2019). Monoclonal antibodies targeting specific interleukins present a new frontier in therapeutic options, with future prospective trials required to assess their impact on PB values, end-organ damage, and systemic inflammation (Tanase et al., 2019). Targeting cytokine receptors represents a promising avenue for treating hypertension, where the intricate interplay between immune responses and vascular health plays a pivotal role. Cytokines, including interleukins like IL-15, exert their effects by binding to specific receptors and activating signalling pathways. IL-15, for instance, shares signalling receptor subunits (IL-2/15R β and γC) with IL-2, and its membrane-proximal

signalling pathways are similar to those induced by IL-2 (Kim et al., 2008). Understanding and modulating these receptor-mediated signalling cascades makes it possible to regulate immune responses and potentially mitigate the inflammatory processes contributing to hypertension and associated end-organ damage.

Another potential avenue for managing hypertension involves targeting cytokine receptors with antibodies against TNF-α, such as infliximab, and soluble recombinant TNF receptor 2 fusion proteins like etanercept. These agents demonstrated preventive effects on hypertension in experimental models (Filho et al., 2013). Similarly, inhibiting IL-6 with a neutralising antibody (nAb) demonstrated efficacy in attenuating hypertension and reducing glomerular inflammation (Hashmat et al., 2016). However, it is crucial to note that while these experimental studies showcase the potential benefits, clinical studies reveal potential drawbacks. For instance, infliximab infusions in clinical settings may lead to cytokine release syndrome, anaphylactic reactions, and fever with degranulation of mast cells and basophils (Lichtenstein et al., 2015). On the other hand, the monoclonal antibody against the IL-6 receptor, tocilizumab, did not alter cardiovascular risk or events in rheumatoid patients (Kim et al., 2017). Balancing the risks and benefits of these immunosuppressant drugs is crucial for their potential application in hypertension treatment. Overall, the intricate relationship between inflammation and hypertension prompts a shift in therapeutic approaches. Understanding cytokines signalling pathways provides a foundation for the development of innovative treatments (De Miguel et al., 2015). However, the clinical translation of these findings necessitates comprehensive research and careful consideration of the diverse roles played by immune components in hypertension and associated diseases.

1.6 Effects of antihypertensive medications on immune and inflammatory responses

Antihypertensive drugs play a crucial role in managing high blood pressure and reducing the risk of cardiovascular diseases. Among these**,** angiotensin-converting enzyme inhibitors (ACEi) work by blocking the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor. This results in blood vessels relaxation, and blood pressure reduction (Byrd et al., 2019). Ang II receptor blockers (ARBs) similarly target the RAS but act by blocking Ang II receptors, preventing vasoconstriction and promoting vasodilation. Moreover, calcium channel blockers (CCBs) reduce blood pressure by inhibiting calcium entry into vascular smooth muscle cells, thereby causing blood vessels to relax and dilate. Beta-blockers (BB) are responsible for decreasing sympathetic activity within the central nervous system (CNS). It exerts its antihypertensive and cardioprotective effects primarily by blocking beta receptors. Lastly, diuretics reduce blood pressure by promoting the excretion of sodium and water through urine, effectively decreasing blood volume and vascular load (Bomfim et al., 2019).

Hypertension is associated with inflammation, and immune dysregulation plays a crucial role in its pathogenesis. The current antihypertensive treatments not only target blood pressure regulation but also exhibit immunomodulatory effects, offering additional benefits by influencing the immune responses involved in hypertension. In the following, each antihypertensive drug will be discussed in detail, highlighting its specific role in immune modulation.

1.6.1 Angiotensin-converting enzyme inhibitors (ACEi)

ACEi are commonly used to treat hypertension, but their role goes beyond just lowering blood pressure. They have significant effects on the immune system, especially on adaptive immunity, which could explain their ability to protect against organ damage. One major way ACEi affects immunity is by influencing T cells. For example, enalapril enhanced the production of Tregs in mice, specifically $CD4^+CD103^+CD25^-$ Tregs. These Tregs help control excessive immune reactions and reduce inflammation (Albuquerque et al., 2010). In lupus nephritis, linked to hypertension, ACEi treatment reduces Th2 polarization and cytokine production, leading to lower blood pressure with less kidney damage (De Albuquerque et al., 2004). ACEi also interferes with antigen presentation processes by altering both MHC I and MHC II pathways, which could increase innate and adaptive immune responses (Bernstein et al., 2018). The effect of ACEi on T cell functions is also supported by studies that showed how blocking the RAS system can prevent inflammation. In an experimental model of autoimmune encephalomyelitis, blocking Ang II with ACEi reduced autoreactive Th1 and Th17 cells, while increasing antigen-specific Tregs (Platten et al., 2009).

Captopril showed the ability to modulate immune responses in various ways. It was demonstrated to decrease inflammatory cytokine expression by inhibiting NF-κB, which plays a crucial role in immune and inflammatory responses (Boskabadi et al., 2019). Captopril also reduces the level of IFN-γ, a key cytokine secreted by Th1 cells and macrophages, as well as IL-12, both *in vivo* and *in vitro* models of LPS stimulation. Additionally, captopril has a preventive effect on LPS-induced lung inflammation by regulating T lymphocytes in the bronchoalveolar fluid (Boskabadi et al., 2019). In autoimmune myocarditis, captopril was found to reduce myosin-specific immunity and cardiac inflammation in myosin-immunized mice, though it did not influence Ag-specific T cell proliferation (Godsel et al., 2003). Moreover, it has been observed to decrease chronic renal lesions in lupus nephritis without affecting anticardiolipin autoantibody or IgG anti-dsDNA production, indicating its role in modulating immune-related renal damage (De Albuquerque et al., 2004).

Although ACE is widely expressed peripherally in human T cells, it is not present in B cells (CD19+) (Petrov et al., 2000). However, in a rat study, enalapril was found to decrease IgG production following myocardial infarction (Waltman et al., 1995). Additionally, in rats with nephropathy, there was renal interstitial inflammation with B cell

infiltration, but this infiltration was reduced after enalapril treatment (Ding et al., 2014). In contrast, Cucak *et al.* showed that mice with type 2 diabetes had fewer B cells in their kidneys compared to the control group, though enalapril did not affect the total B cell count in non-diabetic mice (Cucak et al., 2015).

1.6.2 Angiotensin II receptor blockers (ARBs)

ARBs work by inhibiting the RAS by blocking the AT1 receptor (AT1R) (Ashcheulova et al., 2018). In inflammatory conditions, Ang II enhances AT1R expression via positive feedback, while AT2R levels remain unchanged, contributing to inflammation (Silva-Filho et al., 2013). Ang II receptors are not limited to endothelial cells but are also found on T lymphocytes (Huang et al., 2016b; Zhang et al., 2016; Cheng et al., 2004), lung tissue (Kim and Im, 2019), endometrial stromal cells (Nenicu et al., 2014), and brain cells. Activation of AT1R promotes ROS production and triggers proinflammatory signalling via transcription factors like NF-κB, AP-1 (Cheng et al., 2004), and NF-AT (Zhang et al., 2016; Huang et al., 2016b). It also appears to increase and activate TLR4 expression (Biancardi et al., 2016), further enhancing the inflammatory response.

The action of ARBs significantly reduces proinflammatory responses and inflammation. ARBs also suppress leukocyte maturation, mobilization, and activity. However, their effect on different leukocyte subpopulations may vary due to differences in Ang II receptor functions e.g. AT1R seems to activate NF-κB in monocytes but not in lymphocytes (Kranzhofer et al., 1999). ARBs also limit inflammation by inhibiting the formation of inflammasomes, partly through NF-κB inhibition. This inhibition reduces the expression of cytokines and other proteins essential for leukocyte activity (Lian et al., 2018). ARBs can also modulate T lymphocytes, particularly by inhibiting their activation and proliferation. Candesartan, for instance, reduces CD25 and IL-2 expression, limiting T cell activation (Tawinwung et al., 2018). Moreover, Telmisartan was shown to inhibit the expression of potassium channel proteins Kv1.3 and KCa3.1, which in turn blocks the increase in calcium ion influx. This leads to the inhibition of T cell activation and proliferation by interfering with calcineurin and NF-AT-dependent pathways (Zhang et al., 2016; Huang et al., 2016b). ARBs tend to reduce the proinflammatory Th1 and Th17 populations while having a more complex effect on Th2 and Treg cells. ARBs can lower both pro- and anti-inflammatory cytokines, including IL-4, IL-5, and IL-13, in asthma models, which may alleviate Th2-driven inflammation (Kim and Im, 2019). ARBs also showed to increase

Treg cell populations, as seen in studies on fimasartan (Kim et al., 2019), although opposite results have been observed in malaria models (Silva-Filho et al., 2013).

In term of cytokines, ARBs inhibit the production of several proinflammatory cytokines, such as TNF-α (Cheng et al., 2004) (Robles-Vera et al., 2020), IL-1 (Nenicu et al., 2014; Pang et al., 2012), IL-6 (Huang et al., 2016b), IFN-γ (Cheng et al., 2004; Silva-Filho et al., 2013), and IL-17 (Silva-Filho et al., 2013) (Zhang et al., 2016; Robles-Vera et al., 2020). However, some cytokines, like IL-4, appear less sensitive to ARBs, with mixed results showing either a reduction (Kim and Im, 2019) or an increase (Meng et al., 2015). ARBs have also been observed to reduce other inflammatory mediators like IL-5, IL-13 (Kim and Im, 2019), IL-8 (An et al., 2010), and TGF-β (Zhang et al., 2016), while promoting anti-inflammatory cytokines such as IL-10 (Robles-Vera et al., 2020) and TGF-β (Meng et al., 2015).

Adhesion molecules and chemotactic proteins were reduced by ARBs, which inhibits the migration of leukocytes to inflamed tissues (Robles-Vera et al., 2020). For instance, losartan was found to reduce the expression of CCR2, CCR5, CD11a, VCAM-1, and ICAM-1 in models of malaria (Silva-Filho et al., 2013), and Telmisartan also inhibited ICAM-1 (Pang et al., 2012). This reduction in adhesion molecules limits the migration of monocytes and macrophages, as well as other immune cells such as eosinophils in asthma (Kim and Im, 2019). In humans, losartan was found to reduce T cell activity and lower levels of the inflammatory cytokine IL-6 with atorvastatin and captopril (Sonmez et al., 2001; Sepehri et al., 2016).

1.6.3 Beta-Blockers (BBs)

BBs or adrenergic beta receptor antagonists are another class of drugs used to treat hypertension. Adrenergic receptors, particularly ADRβ1 and ADRβ2, are found on the surface of nearly all immune cells and play a key role in regulating their activity (Scanzano and Cosentino, 2015). ADRBs are found on CD8+ T cells, similar to other white blood cells. After sympathetic stimulation, the levels of CD8+ cells and monocytes in circulation increase, suggesting that stress may alter some cellular immune functions (Bachen et al., 1995). In response to short-term biological stress, there is a rapid mobilization of lymphocytes and monocytes into the bloodstream. This movement is part of the fight-or-flight response, allowing immune cells to reach sites of potential injury quickly (Dhabhar, 2018).

ADRBs are not just present in immune cells but also in organs like the spleen, making the immune system partially dependent on the adrenergic system (Graff et al., 2018). Studies demonstrated that administering propranolol before stressful tasks can inhibit the stress-induced mobilization of lymphocytes and monocytes, preventing their rise in circulation, whether due to mental stress or catecholamine infusion (Benschop et al., 1996). One hypothesis is that stress-induced lymphocytosis results from decreased adhesion molecule expression, causing more lymphocytes to remain in circulation. However, propranolol does not significantly affect adhesion molecule density (such as CD11a, CD54, and CD62 L) in humans after exercise, although it does alter the distribution of activated lymphocytes (Kuhlwein et al., 2001).

A study by Murray *et al.* explored how BBs affect immune function during exercise. In this research, healthy volunteers took propranolol or metoprolol before performing exhaustive treadmill exercises. Propranolol was found to reduce the exercise-induced rise in lymphocyte levels, while metoprolol did not have a significant impact. This shows that different BBs can have varying effects on the immune system (Murray et al., 1992). Labetalol, a drug targeting both beta and alpha adrenergic receptors, restores the CD4++ ratio to normal after stress-induced changes (Bachen et al., 1995), while nadolol, a nonselective BB, reduces the rise in highly differentiated CD8+ T cells triggered by physical activity (Graff et al., 2018). In contrast, bisoprolol, a selective beta-1 antagonist, has little effect on stress-induced lymphocytosis (Graff et al., 2018).

The effect of ADRB2 activation differs depending on various factors. In the presence of IL-12, ADRB2 stimulation of activated naive T cells leads to an increased production of IFN-γ. Similarly, ADRB2 stimulation in the presence of IL-4 enhances cytokine production in Th2 cells (Sanders, 2012). However, the timing of ADRB stimulation (whether before, during, or after T-cell activation) can affect cytokine levels differently. In Th2 cells, ADRB2 activation does not change IL-4 levels compared to controls (Sanders, 2012). Propranolol also showed to block norepinephrine-induced IFN-γ production, highlighting its role in immune modulation (Swanson et al., 2001). The stimulation of ADRB2 on naive T cells also promotes Th1 differentiation, a process that can be inhibited by BBs (Tsai et al., 2020). Propranolol was shown to restore immune function in an animal model of cirrhosis-associated immune dysfunction by normalizing T cell subset activity in the spleen (Tsai et al., 2020). However, more research is required to fully understand how BBs affect T cell populations.

In B cells, ADRB2 agonists increase CD86 expression, especially when combined with an antigen (Sanders, 2012). Additionally, adrenergic signalling increases IgG1 and IgE antibody production, as demonstrated by studies using terbutaline, an adrenergic agonist, and nadolol, a BB that blocks this effect (Kasprowicz et al., 2000). Propranolol also acts on B cells in a non-receptor-mediated manner, stabilizing the cell membrane at high concentrations and inhibiting cap formation, a process essential for B cell function (Anderton et al., 1981). Interestingly, only BBs with local anaesthetic properties, such as propranolol, alprenolol, and oxprenolol, inhibit cap formation, while others like metoprolol, sotalol, and practolol do not have this effect (Anderton et al., 1981)

1.6.4 Calcium Channel Blockers (CCBs)

CCBs mainly used to manage blood pressure by reducing calcium influx into the vascular system, were also shown to modulate the immune system in various studies. Early research indicated that CCBs have immunomodulatory effects in both *in vitro* and *in vivo* models (Rödler et al., 1995; Katoh et al., 1997). Nifedipine was found to reduce CCL2 mRNA through NF-κB inhibition in rat vascular smooth muscle cells exposed to Ang II (Wu et al., 2006). Similarly, Amlodipine prevented the expression of NADPH oxidase, VCAM-1, and CCL2 in hypertensive rats without affecting blood pressure (Toma et al., 2011). Another study demonstrated that Amlodipine could reverse the effects of TNF superfamily activation, linked to left ventricular hypertrophy in hypertensive rats (Lu et al., 2016).

Calcium, as a secondary messenger, is crucial for lymphocyte activation, particularly in cytokine production and proliferation. CCBs suppress these processes by inhibiting Ca^{2+} influx into lymphocytes (Rödler et al., 1995). Verapamil, for instance, blocks the L-type calcium channels and modulates IL-4 production in Th2 cells, while reducing the secretion of IL-5 and IL-6 in sinus polyp explants, suggesting potential use in treating chronic rhinosinusitis with nasal polyps (Bleier et al., 2015). Th2 cells express the L-type calcium channels, unlike Th1 cells, indicating a selective effect on Th2-mediated immune responses. Nicardipine inhibits Th2-driven autoimmune diseases, including glomerulonephritis and chronic graft versus host reaction in rats (Savignac et al., 2004). This highlights the potential therapeutic use of CCBs in Th2-mediated conditions like allergic rhinitis and asthma.

Programmed death-ligand 1 (PD-L1), essential for immune self-tolerance, can be targeted by CCBs. In addition, Amlodipine was found to inhibit PD-L1 expression in cancer cells,

enhancing CD8+ T cell infiltration in tumors (Grzywnowicz and Giannopoulos, 2012). This pathway is also involved in autoimmune disorders, indicating CCBs' potential role in therapies for these conditions. There is limited research on how CCBs affect B cells. B cells, which possess voltage-dependent calcium channels, showed impaired proliferation and antibody production in patients with elevated Ca2+ levels, such as those on hemodialysis. Nifedipine was shown to reverse impaired B cell proliferation in uremic patients, indicating its beneficial effects in calcium-related B-cell dysfunction (Alexiewicz et al., 1997)

IL-1β is a key regulator of immune responses, particularly in increasing endothelial permeability and adhesion molecule expression. CCBs, including Amlodipine, Losartan, and Captopril, were shown to reduce IL-1β levels in hypertensive and non-hypertensive patients (Nemati et al., 2011). Nifedipine suppresses IL-1β and NO production in a murine model, as well as IL-1β mRNA expression in macrophages activated by LPS (Choe et al., 2021). Additionally, Amlodipine downregulates TNF-α, IL-6, and VCAM-1 expression by inhibiting p38 MAPK and NF-κB in hypertensive mice (Benjamin et al., 2015). Verapamil also inhibits LPS-induced TNF-α and IL-6 production in the liver while increasing IL-10 expression (Eickelberg et al., 1999).

1.6.5 Diuretics

Thiazide and loop diuretics are often prescribed for BP control, alone or combined with other antihypertensive drugs. While the effects of diuretics on immune modulation in hypertension remain controversial, there is some evidence connecting diuretics with immune system changes in hypertensive patients. Thiazide diuretics were found to upregulate the immune system in newly diagnosed hypertensive patients by increasing IgM anti-ApoB-D autoantibodies (Fonseca et al., 2015). Another study observed that diuretic treatment reduced levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, in elderly hypertensive patients (Toledo et al., 2015). In animal models, such as Dahl salt-sensitive rats, hydrochlorothiazide (HTZ) and chlorthalidone were effective in controlling BP but did not reduce oxidative stress or inflammation associated with hypertension (Zhou et al., 2008). Combining diuretics with other antihypertensive therapies did show better immune modulation and improved hypertension outcomes in both experimental (Jin et al., 2014) and clinical studies (Agabiti-Rosei et al., 2014). However, long-term inflammation caused by diuretics may be considered a threatening condition, and additional therapies aimed at reducing immune responses might be beneficial alongside diuretic treatment.

Hydrochlorothiazide (HTZ) works by inhibiting sodium-chloride cotransporters in the nephron's distal tubule, leading to increased sodium and water excretion, along with the loss of potassium and magnesium (Peri and Mangipudy, 2014). However, studies showed that HTZ can have genotoxic effects on human lymphocytes, particularly in older women, who were more prone to chromosomal damage during HTZ treatment (Andrianopoulos et al., 2006). T cells are critical in the development of hypertension, and experimental models showed that hypertension can lead to T cell accumulation in organs like the kidney and aorta. Treatment with HTZ and hydralazine prevented this accumulation in both CD4+ and CD8+ cells in these organs (Itani et al., 2016a). HTZ treatment increased the number of T_M cells in the lymph nodes, indicating a complex effect on immune cell trafficking. In another study, HTZ did not significantly affect the proportion of T cell subtypes in the spleen of SHR but increased the percentage of naive T cells in both normotensive and hypertensive rats. There was no significant effect on Tregs or activated T cells across different groups (Aloud et al., 2020). HTZ influenced macrophage secretion profiles. HTZ reduced IL-6 levels in mice, whether or not they were stimulated with LPS (Bryniarski et al., 2021). However, other studies reported differing results regarding HTZ's impact on IL-1β and IL-6 (Fukuzawa et al., 2000). In addition, HTZ decreased IL-17A, which plays a role in the remodelling of small arteries (Bryniarski et al., 2021). Despite these findings, HTZ had no significant impact on the production of TNF-α or TGF-β1 (Bryniarski et al., 2021), aligning with other research that showed no effect on TNF-α activity or immunoreactivity (Fukuzawa et al., 2000). Bendroflumethiazide, a related diuretic, also did not affect these cytokine levels or contribute to renal fibrosis (Das et al., 2010). It was reported that after 4 weeks of indapamide therapy, the levels of monocyte chemotactic protein (MCP-1), MIP-1α, and P-selectin showed no significant difference from their initial concentrations compared to other antihypertensive drugs like valsartan (Xie et al., 2006).

Overall, Antihypertensive drugs, such as ACEi, ARBs, BBs, CCBs, and diuretics, are traditionally used to manage hypertension but also play a significant role in modulating the immune system. Despite these findings, there are still gaps in fully understanding how these drugs modulate the immune system across various conditions. In addition, numerous studies explored the relationship between antihypertensive therapy and immune system function, revealing various interactions that suggest the potential for modulating immune responses. Clinical trials have begun to apply these insights, such as using antihypertensive drugs as modulators of autoimmune responses or as adjuvants for recovery after myocardial infarction. However, significant gaps remain in our understanding of the

precise immunomodulatory effects and underlying mechanisms of these drugs. Most of the available data are based on animal models, limiting the ability to generalize these findings to humans.

Antihypertensive drugs such as thiazides, beta-blockers, ARBs, and ACEi have shown varying effects on immune function, influencing T cell populations, cytokine production, and immune responses. Despite their promise, the observed effects are often inconsistent and dependent on the specific drug, experimental model, and condition being studied. The potential of these drugs to modulate immune responses in patients with conditions such as diabetes, cancer, or immunotolerance dysfunction underscores the importance of further clinical research. Additionally, treatments targeting immune function could provide promising therapeutic strategies for hypertensive patients.

1.7 Thesis aim and hypothesis.

Systolic blood pressure (SBP) exhibits a positive correlation with pro-inflammatory cytokines, as indicated by findings from both experimental and clinical studies (Chae et al., 2001; González-Amor et al., 2022; Madhur et al., 2010). Activating the RAA system and sympathetic nervous system stimulates the synthesis of proinflammatory cytokines (Zhang et al., 2003; Sanz-Rosa et al., 2005; Ruiz-Ortega et al., 2002). This may support the potential involvement of cytokines in PB regulation. An imbalance between pro-inflammatory cytokines (Kany et al., 2019), responsible for initiating inflammation in response to tissue injury, and anti-inflammatory cytokines like IL-10 (Opal and DePalo, 2000), which inhibit excessive inflammatory reactions, may contribute to the development of hypertension and other severe cardiovascular complications, including coronary artery disease and heart failure. Moreover, emerging evidence suggests that chronic increases in plasma levels of cytokines, comparable to concentrations observed in hypertension, can lead to significant elevations in blood pressure.

While anti-inflammatory cytokines like IL-10 play an essential role in modulating immune responses, this research specifically targets pro-inflammatory cytokines due to their direct involvement in the initiation and propagation of immune responses in hypertensive conditions. Targeting pro-inflammatory pathways may provide a more immediate approach to addressing the exaggerated immune activation observed in hypertension, as these cytokines are more relevant to the early stages of the disease. The role of anti-inflammatory cytokines, while important in resolving inflammation, was not the primary focus of this investigation.

Considering the important role of T cells and various cytokines in hypertension development, we hypothesise that inflammatory cytokines can modulate T cell activation independently of antigen stimulation, and this process could be enhanced in hypertension. Therefore, this study aims to comprehend the proliferation and differentiation effects of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, IL-2, IL-12, IL-15, IL-7, IFN-γ, IL-4) on T cells and further elucidate the potential mechanisms through which cytokine stimulation sustains immune cell activation in hypertension, employing RNA-seq analysis.

Chapter 2 Material and Method

2.1 Animal models

2.1.1 C57BL/6J mice

8–12-week-old male wildtype (WT) mice were purchased from Charles River Laboratories (CRL), Margate, UK (Reg. No. 8457759).

2.1.2 Angiotensin II-induced hypertension in mice

Mice were subjected to Ang II-induced hypertension by administering Ang II at a dose of 490 ng/min/kg/day for either 5 or 14 days. The Ang II was delivered via subcutaneous osmotic minipumps (Model 2002, AlZET® Corporation) using Ang II buffer (a solution of NaCl and acetic acid in distilled water) (Guzik et al., 2007). Sham surgery involved minipumps containing the vehicle for Ang II (0.5M NaCl, 0.01M CH3COOH in sterile saline) to serve as a control for the experiment.

Mice were anaesthetized with 2.5% isoflurane and oxygen at a flow rate of 2 L/min. Then, the implementation site was shaved and sanitised with povidone-iodine (Betadine), followed by a 1-2 inches incision made at the upper back area, just below the neck. The forceps were used to make a pocket to insert the osmotic minipumps. The incision was then closed with three sutures. All surgery was performed in a sterile field and with an aseptic technique. Mice were given 1mg/ml ibuprofen injection subcutaneously (1000ul) as analgesia before the surgery was performed. Soft food was introduced, and weight monitoring was done by scaling the mice daily after the surgery.

Following a period of training, BP was measured before and after minipumps were implanted using non-invasive tail cuff plethysmography with the BP 2000 Blood Pressure analysis system (Visitech Systems Inc.). BP was recorded daily at the same time with the platform heated to 36°C. For each session, ten baseline measures and fifteen true measurements were taken to ensure that the mice were affected by hypertension (Figure 2.1). To avoid channel-specific variances, animals were rotated among the channels daily. The BP-200 Software automatically detected and eliminated the outliers based on Chauvenet's criterion, which is defined as measurements more than two standard deviations from the mean. On days 5 or 14, mice were sacrificed by CO2 asphyxiation, followed by cervical dislocation to confirm death and spleens and PBMCs were collected.

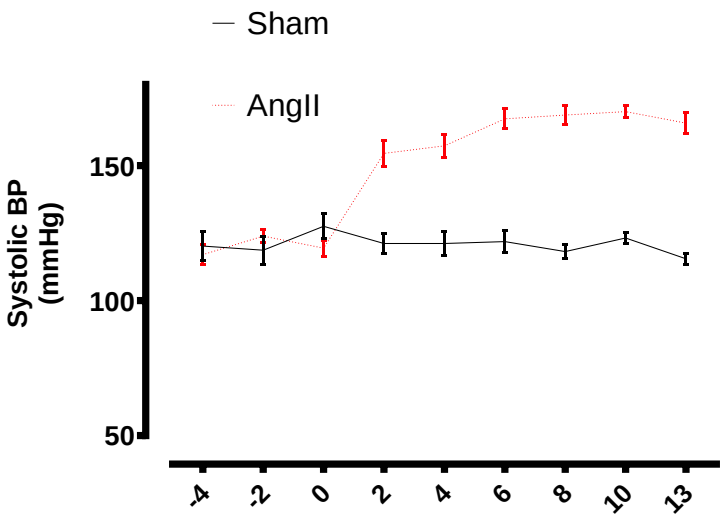


Figure 2.1. Measurements of blood pressure after the implementation of the Ang-II mini pump.
Line plot showing tail cuff blood pressure changes over 14 days in Sham and Ang II-infused mice following minipump implantation. The graph illustrates SBP elevation in Ang II-treated mice compared to the sham group, which maintained normal BP levels. Blood pressure was measured daily using non-invasive tail cuff plethysmography at 36°C, with ten baseline and fifteen true measurements recorded per session. The data are presented as mean ± SD to reflect the variability of blood pressure measurements. Figure produced by the author to ensure hypertension was introduced to mice in response to Ang II.

2.2 *In vitro* cell culture

Spleen and blood were taken from mice, processed under laminar flow hoods, and cultured in 5% CO2 at 37C° atmosphere. Cells were cultured in complete medium in 96- or 12-well plates.

2.2.1 T Cells isolation

Single-cell suspension is prepared from spleens that were collected from WT or Sham/hypertensive C57BL/6J mice. C57BL/6J sub-strain mice were selected for the experiment due to its genetic consistency and well-documented immune and cardiovascular phenotypes, including neutrophil recruitment, systolic blood pressure regulation, and ventricular remodelling, making them an ideal model for studying hypertension (Mekada and Yoshiki, 2021). Additionally, they are easier to house and handle than larger animals like rats, which is an important consideration for experimental design and resource management. Mouse models are also valuable for studying inflammation, and immune response due to their similar immune systems to humans (Miles et al., 2020). In addition, the Ang II infusion model is noted for its ability to define

the impact of the local RAS on cardiovascular disease more effectively than genetically complex models (Falcon et al., 2004). Before isolation of the T cell, the spleen was minced in copious amounts of fetal bovine serum (FBS) and passed through a 70 μm micro strainer, which was placed on a 50ml falcon tube. Suspended spleen solution was centrifuged for 7 min at 400 x g and 4 °C. The supernatant was discarded, and cells were resuspended in 10 ml FB to be ready for restrain at 40 μm and counting.

The subsequence process is the isolation of T cells, and a magnetic negative selection technique was used for T cell isolation. Cells were resuspended in 100 μl/10^7 cells of sorting buffer. 10 μl/10^7 cells of Bitotin-Antibody cocktail were mixed and incubated on ice for 15 min, followed by 10 μl/10^7 of streptavidin nanobeads for 15 min on ice. 2.5 ml of sorting buffer was added to the cells before placing the tube in the magnet for 5-10 min. T cells were poured out in a different tube. Purity was >90% as determined by FACS analysis of $CD3^+$ cell surface expression.

2.2.2 T Cells activation

1- For α-CD3 Ab concentration titration: 2 - 4 x 10^5 cells were incubated with/without α-CD28 antibody (2 mg/mL) in flat 96-well plates that had been coated with various concentrations of α-CD3 antibody (1 - 2.5 - 5 ug/ml) in the absence of cytokines for 6- days at 37°C in complete RPMI 1640 medium supplemented with 10% FBS and antibiotic pen/step. 5 mM of 2-β mercaptoethanol (2-βME) was added on a daily basis. For re-stimulation, cells were harvested, counted, and seeded again at new α-CD3 antibody-coated wells with new culture medium and soluble α-CD28 antibody (Figure 2.1/P1).

2- Isolated splenic T cell was cultured for 6 days in α-CD3 coated plate (2.5 ug/ml) in the presence of α-CD28 (2 ug/ml) with cytokines IL-6 (50 ng/ml), IL-12 (10 ug/ml), IL-15 (50 ng/ml), TNF-α (100 ng/ml), IFN-γ (10 ng/ml). Cells were restimulated on day 3 (Figure 2.1/P2).

3- T cells (2-4 x 10^5) were cultured with α-CD3 and α-CD28 for 48 hours. Recombinant murine IL-2 (50 U/ml) was added on day 2. Then, IL-6 (50 ng/ml), TNF-α (100 ng/ml), and IFN-γ (10 ng/ml) were introduced to the cells three days later (day 5), and analyses were conducted on day 7 and day 8 (Figure 2.1/P3).

4- T cells (2-4 x10^5) were cultured with low concentration α-CD3 (1ug/ml) coated plate and kept for 3 days in media supplemented with cytokines IL-6 (50 ng/ml), IL-12 (10 ug/ml), IL-15 (50 ng/ml) without CD28 (Figure 2.1/P4).

5- Priming: 3-4 x 10^5 T cells were primed with individual cytokines IL-2 (50 U/ml), IL-1β (20 ug/ml), IL-4 (25 ug/ml), IL-6 (50 ng/ml), IL-7 (25 ug/ml), IL-12 (10 ug/ml), IL-15 (50 ng/ml), TNF-α (100 ng/ml), IFN-γ (10 ng/ml) for 24 hours. Then, the cells were washed and re-cultured in α-CD3 coated plate for 48 hours. 2-βME was added daily to the cells with all protocols (Figure 2.2).

The cytokine concentrations used in this experiment were determined based on both published research (Table 2.1) and previous experiments conducted in our lab. To ensure optimal stimulation of T cells, cytokine concentrations were collected from studies that applied similar stimulation protocols using recombinant cytokines.

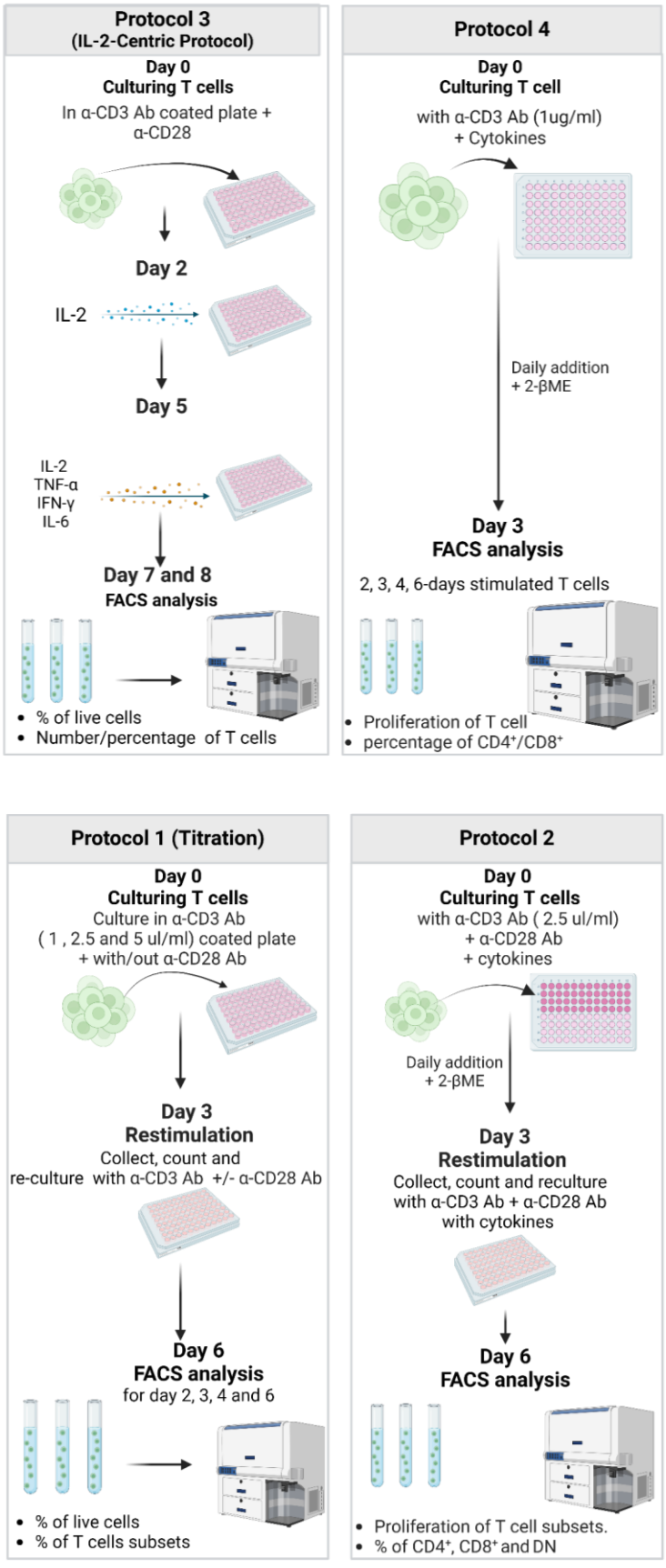


Figure 2.1. Used protocols to stimulate T cells *In vitro*.

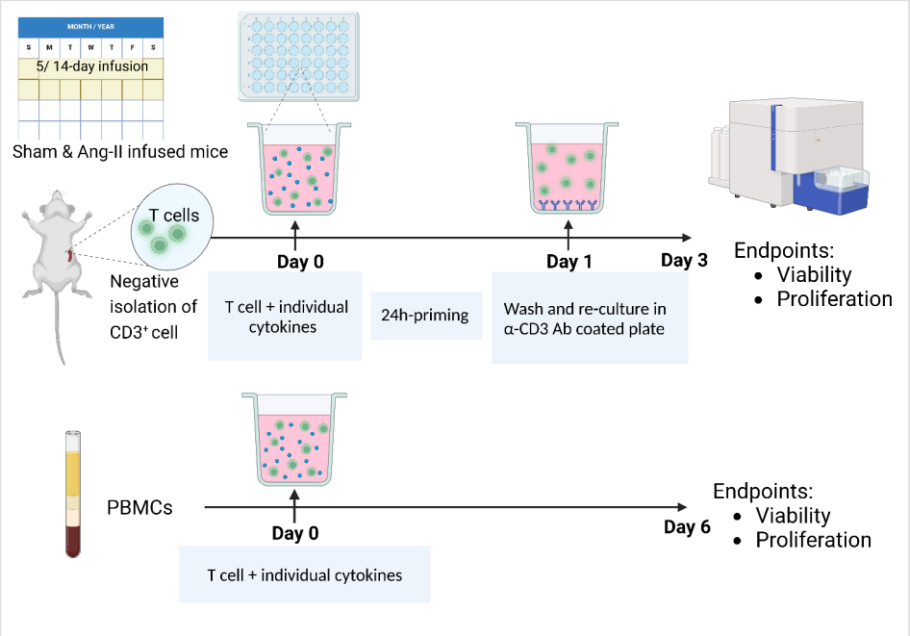


Figure 2.2. Applied protocol for priming T Cells with cytokines.

2.2.3 PBMCs isolation

Heparinised blood was collected via cardiac puncture from Sham and Ang II-treated mice; PBMCs were diluted 5 times with PBS and isolated by density gradient centrifugation. Diluted PBMCs were layered onto 5 ml HistoPaque® (Sigma) and then centrifuged at 400 X g for 30 minutes without break. Buffy coats were collected after the centrifugation in an FBS-filled tube with a plastic Pasteur pipette and centrifuged again for 7 min at 400 x g at 4 °C. After discarding the supernatant, PBMCs were then resuspended in 5 ml of RBC lysis buffer (BioLegend) for 7 min at RT. Up to 10 ml of FBS was added to the cells and centrifuged again for 7 min at 4 °C and 400 x g. The supernatant was discarded, and cells were resuspended in 1 ml medium for counting and culturing.

2.2.4 PBMCs activation

Isolated PBMCs were cultured for 6 days with various individual cytokines in 96 well-plates without further activation.

2.2.5 Cell counting

TURKA solution was used for counting by diluting the cells suspension 10 X, or 20 X. Trypan Blue was also used to count the number of life cells by preparing 1:1 dilution. Cells were counted using a Fuchs-Rosenthal counting chamber under the microscope.

Calculating formula

$$number\ of\ cells = \frac{numberof\ cells\ in\ counted\ square}{mubnumber\ of\ squares} \times\ dilution$$

2.3 Flow cytometry

2.3.1 PBMC receptors expression

Isolated PBMCs were collected and stained with CD3, CD4, CD8 antibodies, and different cytokine receptors antibodies for 20 minutes at dark. Samples were measured using a FACS machine (Figure 2.3).

2.3.2 Assessment of T Cell proliferation: Carboxyfluorescein Succinimidyl Ester (CFSE)

Cells were labelled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) in PBS only for 20 min at 37 °C in the dark with gentle agitation (1ul / 6 × 106 cells), then washed with a plentiful amount of medium (RPMI 1640 medium + 10 % FBS + 2-βME) to quench any remaining dye. They were then resuspended in the medium for culturing.

2.3.3 Assessment of T Cells viability and cell surface staining

Zombie violet™ fixable viability dye (Biolegend) was prepared by diluting 1/1000 in PBS. Cell suspensions were added to FACS tubes and washed with FB by centrifuge for 10 min at 4 C and 400 x g. After discarding the supernatant, 100 ul of Zombie viability dye dilution was added and incubated in the dark for 15 min at room temperature. Cells were rewashed FB and prepared to be stained with mixed monoclonal antibodies in dark for 20 min on ice. Cells were washed for the last time and resuspended in 200ul FB to be ready for analysis or fixed for 30-1 hour in FluoroFix™ buffer at room temperature, then washed and kept at 4C for next-day analysis.

2.3.4 Flow cytometry analysis.

Flow cytometric analysis was performed on BD FACS Canto (BD bioscience). Data results were analysed by FlowJo v10 software. All samples had the same strategy in gating T cell population, single and live T cells (Figure 2.3). Cells were identified based on FSC-A vs SSC-A. Single cells were selected based on FSC-A vs FSC-H, and live cells were identified using the viability dye.

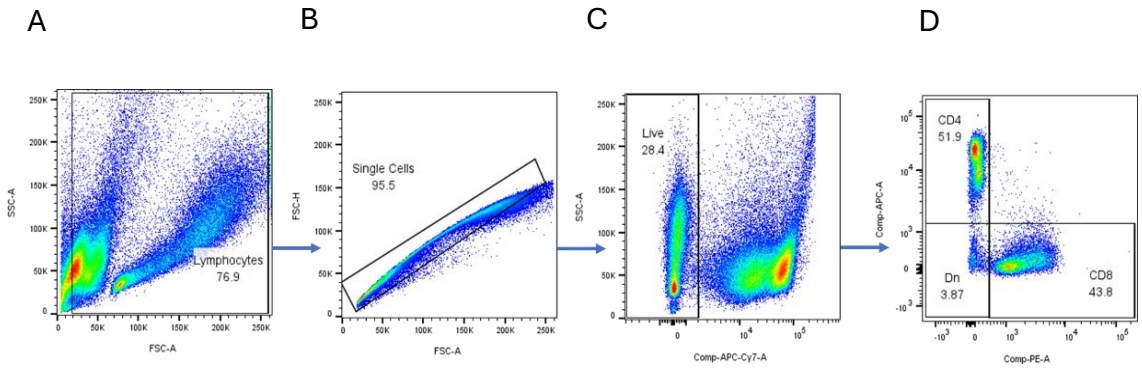


Figure 2.3. Flow cytometer gating strategies for T Cell analysis.
Flow cytometry scatterplots illustrating the gating strategy for identifying CD4+ and CD8+ T cell populations. Lymphocytes were initially gated based on size and granularity using FSC/SSC plot **(A)**. Then gating on single cells was performed to exclude debris **(B).** CD4+ and CD8+ T cell subpopulations **(D)** were gated from the live cell population **(C)** based on viability staining.

2.4 Study design including sample size and power analysis

For this study, which focused on measuring proliferation response, receptor expression, and marker expression in sham-treated and Ang II-infused mice, the sample size was determined using the sample size calculator for unpaired t-tests from the Experimental Design Assistant (EDA) (RRID: SCR_017019, https://eda.nc3rs.org.uk). Accordingly, based on preliminary results in Prof. Guzik's lab and the expected variability in immune cell responses between the groups, it indicates that 3 mice per group are needed to assess the percentage of proliferation changes (measured by flow cytometry) with 90% power, a significance level of 0.05, and a standard deviation of 0.75. In addition, to assess receptor expression differences, it was calculated that 5 mice per group would be required to achieve 90% statistical power with a significance level of 0.05 and detect the difference between sham (0.6 %) and Ang II-infused mice (2.6%).

To further confirm the results, additional spleen samples were obtained from mice used in related experiments by colleagues, where the spleens were otherwise not required for their analyses. This collaborative approach ensured efficient use of resources and helped avoid unnecessary animal use.

For Bulk RNA-seq, we plan to use total RNA samples from 4 Sham and 6 Ang II WT animals, as well as 4 Sham-treated and 6 Ang II-treated WT mice. The discrepancy in group size arises from the higher variation in Ang II-infused animals. Based on previous experience in our lab, this experimental design and group sizes are expected to provide sufficient power to detect significantly different RNA transcript expressions between hypertensive and control animals after applying FDR correction (Nosalski et al., 2020; Józefczuk et al., 2020).

2.5 Ethical approval and declaration

All experimental protocols involving mice in this thesis were performed in accordance with the United Kingdom Animals Scientific Procedures Act 1986, under the project license (PPL: PP3412366) held by Professor Tomasz Guzik, and in accordance with the ARRIVE Guidelines. Additionally, all experiments conform to Directive 2010/63/EU of the European Parliament, which outlines the protection of animals used in scientific research.

In this study, all experiments, including surgeries and the collection of samples (blood and spleen) for the Ang II model, were performed by the thesis author. I also conducted all experimental work related to T cell culture following appropriate training. Moreover, cell sorting for RNA-seq was carried out by professional laboratory technicians at the Flow Core Facility, School of Infection & Immunity. RNA-seq analysis was performed by myself with the guidance and supervision of Dr. Ryszard Nosalski (postdoctoral researcher).

2.6 Statistical analysis

GraphPad Prism® software version 9 (GraphPad Software, *California* USA) was used to analyse data. Using unpaired two-tailed Student's T-test if the mean of 2 samples was normally distributed, two-way ANOVA to compare more than 2 means and one-way ANOVA for normally distributed variables. In cases where the data did not meet the assumption of normality, the U Mann-Whitney and Kruskal-Wallis tests were applied. Post-hoc multiple comparison tests such as Bonferroni, Tukey, or Dunn's were then conducted to compare means across multiple treatment groups. Error bars in the figures indicate the Standard Error of Mean (SEM), and statistical significance was determined at a $P\ value < 0.05$.

2.7 Reagents and antibodies

Appendix I provides details on the sources of reagents used in the experiments, and Appendix II outlines the antibodies used for flow cytometry staining in this thesis.

Table 2.1. Summary of cytokine concentrations reported in various studies.

Cytokine	Concentration	Cell type	References
TNF-α	40 ng/ml	Human T cells	(Sebbag et al., 1997)
	100 ng/ml	Murin Spleen T cells	(Lahn et al., 1998)
	100 ng/ml	Human T cells	(McInnes et al., 1997) (Santis et al., 1992)
IL-6	50 ng/ml	Human T cells	(Jiang et al., 2014)
	100 ng/ml	Murine PBMCs - T cells	(Greenfield et al., 1995)
	50 ng/ml	Human T cells	Kuhweide et al., 1990)
	25 ng/ml	Murine Spleen - T cells	(Hirahara et al., 2012)
	1 ng /ml	Murine Spleen - T cells	(Teague et al., 1997)
	10 ng/ml	Murine Spleen - T cells	(Dienz et al., 2009)
	5 ng/ml	Murine Lymph node - T Cells	(Vink et al., 1990)
IFN-γ	50 U/ml	Murine Spleen - T cells	(Resta-Lenert and Barrett, 2006)
	10 ng/ml	Murine Spleen - T cells	(Dienz et al., 2009), and (Dalton et al., 2000)
	100 U/ml	Murine Spleen - T cells	(Tsutsui et al., 1996), and (Kingston et al., 1989)
	10 ng/ml	Human PBMCs - T cells	(Chomarat et al., 1993)
IL-2	10 ng/ml IL-2	Murine Spleen - T cells	(Berg et al., 2002)
	25 ng/ml	Human T cells	(Mujib et al., 2012)
	100 U/ml	Human T cells	(Wang et al., 2000)

	50 U/ml	Human T cells	(Kwoczek et al., 2018)
IL-7	1 ng/ml	Human T cells	(Catalfamo et al., 2011)
	0.5 – 62.5 ng/ml	Human T cells	(Lu et al., 2009)
	20 ng/ml	Human T cells	(Wang et al., 2006)
	10 -100 ng/ml	Human T cells	(Crawley et al., 2014)
	Low dose 20 ng/ml High dose 100 ng/ml	Murine Spleen - T cells	(Pulle et al., 2006)
	5 ng/ml	Murine Spleen - T cells	(Wherry et al., 2004)
IL-12	10 ng/ml	Human T cells	(Schilbach et al., 2020) (Kwoczek et al., 2018)
	50 ng/ml	Murine Spleen - T cells	(Tchaptchet et al., 2010)
	10 ng/m	Murine Spleen - T cells	(Suarez-Ramirez et al., 2014)
	5 ng/ml	Murine Lung - T cells	(Vesosky et al., 2006)
	10 ng/ml	Murine Spleen - T cells	(Freeman et al., 2012)
	20 ng/ml	Murine Spleen - T cells	(Ingram et al., 2011)
	10 ng/ml	Murine Spleen - T cells	(Lee et al., 2017)

IL-17	10 ng/ml	Human T cells	(Hsu et al., 2013)
	100ng/ml	Murine Spleen - T cells	(Feng et al., 2011)
	10 ng/ml	Murine Spleen -T cell line	(Wang et al., 2009)
	20 and 100 ng/ml	Murine T cells	(Hou et al., 2014)
IL-1β	10 ng/ml	Murine Spleen - T cells	(Bremser et al., 2015)
	1,000U/ml	Murine Spleen - T cells	(LeClair et al., 1989)
	50 ng/ml	Human T cells	(Jiang et al., 2014)
IL-4	1,000 U/ml	T cells line	(Geginat et al., 2003)
	25 ng/ml	Human T cells	(Mujib et al., 2012)
	10 ng/ml	Human T cells	(Unutmaz et al., 1995; Ingram et al., 2011)
	20 ng/ml	Murine Spleen - T cells	(Morris et al., 2009)
	20 ng/ml	Murine Spleen - T cells	(Bremser et al., 2015)
	40 ng/ml	Murine Spleen & lymph node - T cells	(Ilangumaran et al., 2003)
	5 ng/ml	Murine Spleen - T cells	(Wherry et al., 2004)

IL-15	Low dose 20ng/ml. High doses 100ng/ml	Murine Spleen - T cells	(Pulle et al., 2006)
	10 ng/ml	Human T cells	(Wang et al., 2006)
	25 ng/ml	Human T cells	(Abdelsamed et al., 2017)
	Low does 10 ng/ml – 100 ng / mL	Murine Spleen -T cells	(Freeman et al., 2012)

This table summarizes the cytokine concentrations reported in various published studies, which were reviewed to help determine the optimal cytokine concentrations for T cell stimulation in the experiments.

Chapter 3 Role of Cytokines in TCR-independent Activation of T cells: A Systematic Review

3.1 Introduction

The immune system comprises complex organ systems like the spleen, lymph nodes, bone marrow, and thymus, providing organised and successive defencing responses known as innate and adaptive immune responses. These two branches, innate and adaptive, form the fundamental pillars of the immune system. As a first-line defence, innate immune cells' role is to recognise PAMPs and to express invariant antigen receptors such as PRRs to other adaptive cells. Induction of adaptive immune response, antigen-specific response, relies not only on direct antigen recognition but also on signals received from the innate immune cells to the adaptive immune cells like T and B lymphocytes (Lee et al., 2020). The adaptive immune response is a key factor in cardiovascular disorders such as heart failure, atherosclerosis, hypertension, and cardiac remodelling. The interaction between T and B cells in driven immune-mediated pathology is well-known (Ren and Crowley, 2019). In many cardiovascular diseases, adaptive immune cells are reported to be activated and accumulated in the affected tissue, e.g. atherosclerotic plaque, ischemic heart, and kidney in hypertension leading to TOD (Raddatz et al., 2019; Bansal, 2017).

T cell activation is pivotal in initiating the adaptive immune response, which requires two signals: antigen presentation by MHC molecules to the TCR or BCR on lymphocytes, and a co-stimulatory signal provided by interactions between APCs (B7 family ligand) and T cell co-stimulatory molecules (CD28, CTLA-4, OX40) (Caillon et al., 2019). In contrast to the above-mentioned classical dogma, bystander activation, an alternative activation pathway, occurs independently of TCR stimulation and can contribute to autoimmune diseases and cardiovascular pathology. This activation, which can occur in CD4+/CD8+ T cells, is mediated by indirect signals such as cytokines, chemokines, or PAMPs, and leads to functional responses such as proliferation, differentiation, and cytokine production (Bangs et al., 2006; Lee et al., 2020).

Bystander activation of lymphocytes is mediated by indirect signals favouring an inflammatory milieu, such as chemokines, cytokines, ligands of co-signalling receptors, extracellular vesicles with microbial particles, or PAMPs signals through TLRs (Pulle et al., 2006). Recently, it was reported that the neoantigen-like IsoLG can elicit TCR activation and induce robust proliferation in CD4+ T cells (Ngwenyama, 2021). Furthermore, various cytokines showed their ability to activate T cells independently from presenting antigens (Bastidas et al., 2014). Interestingly, bystander activation may occur in plaque-derived T cells in atherosclerosis or during viral infections (Houtkamp et al., 2001;

Kim and Shin, 2019). Thus, the mechanism of bystander activation, unlike classical activation, is characterised by stimulating the functional role of the lymphocytes, such as proliferation, differentiation, cytokine production, or apoptosis, independently of BCR/TCR specificity (Pacheco et al., 2019).

The pathological role of bystander activation was demonstrated in many diseases accompanied by exacerbation of symptoms during infections. Activated T cells are implicated in the pathology of autoimmune conditions, hypertension, and microbial infection. For example, in acute hepatitis A infection (HAV), CD8+ T cells activated by IL-15 cytokine without antigen recognition exhibit innate-like cytotoxicity through the ligation of natural killer cell receptors (NKG2D) with their ligands, which leads to hepatocyte damage in the liver (Kim and Shin, 2019). Within the adaptive immune cells, activated T cells show their role in the pathogenesis of hypertension, enhancing infiltration and accumulation of various T cell subtypes in kidneys, heart, and vasculature, resulting in end-organ damage (Ren and Crowley, 2019).

The immune system exhibits bystander proliferation effects in activated cells, leading to the acquisition of effector functions, including cytokine secretion and cytotoxicity. In response to antigenic stimuli, various cell types, such as NK cells, T cells, neutrophils, and macrophages, secrete IFN-γ. Similarly, IFN-γ secretion is reported in response to the activation of different cell types by IL-15, IL-2, IL-12, and IL-18 (Younes et al., 2016; Tietze et al., 2012; Domae et al., 2017). Additionally, cytotoxic molecules like Granzyme B and perforin, secreted with other cytokines, perpetuate inflammation and mediate cytolysis (Kim et al., 2018). Perforin molecules create small pores within targeted cells, allowing granzyme B entry and leading to increased circulating apoptotic bodies, inducing inflammation (Zhang and Bevan, 2011). NKG2D receptors expressed on T cells play a dual role, inducing immune senescence or directly killing cells expressing NKG2D ligands. These effects are triggered by T cell subsets activated in an antigen-independent or dependent manner (Abbas and Akbar, 2021). Notably, IL-15 exemplifies this, mediating cytolytic activity by inducing NKG2D expression on CD8+ T cells upon activation in an antigen-independent manner (Bastidas et al., 2014).

The bystander effect, where cells are activated through mechanisms other than direct TCR/BCR signals, plays a role in various diseases. For example, cytokines can trigger autoreactive T cells, worsening conditions like multiple sclerosis, autoimmune encephalomyelitis, and RA. In cardiovascular diseases such as hypertension, the

mechanisms of T cell activation, potentially involving neoantigens or molecules like IsoLG, remain under investigation. In addition, studies have shown that inhibiting TNF-α can attenuate blood pressure in hypertension models. Therefore, based on these reported studies, this systematic review aims to identify the most frequently reported and implicated cytokines in activating T cells in an Ag-independent manner. Specifically, it focuses on cytokines' ability to stimulate adaptive immune cells in a bystander manner in various autoimmune and cardiovascular diseases and in the context of the absence of disease. Not to mention, the gathered comprehensive data from existing literature also serves as a resource for guiding and informing our upcoming laboratory work in the hypertensive model.

3.2 Systematic review aim

Our objective in conducting the systematic review is to confine the most reported cytokines used to stimulate adaptive immune cells in a way where cells are activated by only being exposed to the cytokines in a TCR-independent manner.

3.3 Methods

3.3.1 Protocol

The study design and the research questions were based on and followed the PICO outline:

Population: Adaptive immune cells including (B cells, T cells including γδ-T cells, Dendritic cells, and NK T cells)

Intervention: Cytokines, including (Interleukins, Interferons, and necrotic tumor factors) and α-CD3/CD28 Antibodies.

Comparison: Control (unstimulated cells or α-CD3/CD28 antibodies stimulated cells) or another individual/combined cytokine.

Outcome:

1. ***Primary outcome***: Proliferation response.
2. ***Secondary outcome***: Apoptosis or cytotoxic activity, receptors, markers, gene expression, cytokines secretion, mechanisms/pathways, and the protocol used for activation.

Research questions:

- Which inflammatory cytokines are commonly used to stimulate adaptive immune cells?
- Which adaptive immune cells are dominantly targeted in bystander stimulation?
- The mechanisms and possible pathways involved in T cell activation and marker expression.
- The applied Protocol.

3.3.2 Criteria for the inclusions of studies

This systematic review contained only the research papers with hands-on lab experiments to address the research question regarding the cytokines involved in activating adaptive immune cells in a bystander (Ag-independent) manner. Therefore, all reviews, editorials, conference abstracts, and book chapters were excluded. In addition, to reduce data variability between papers, only papers that study the direct effect of cytokine or α-CD3/CD28 on the targeted cell were included, whether human or animal cells. Studies that utilise other approaches to stimulating immune cells were eliminated (Table 3.1).

Table 3.1. Study inclusion and exclusion criteria.

Inclusion criteria:
• Direct cells-cytokines stimulation • Cells that were stimulated with α-CD3/α-CD28 antibody (TCR-dependent manner) (Control)
Exclusion criteria:
• Studies target non-adaptive immune cells (e.g., innate-immune cells, Neuron, and epithelial cells). • Studies using anti-cytokines. • Activation using antibodies like (α-CD40, α-CD80, α-CD6, α-CD69, α-CD2, α-CD5, α-CD46, α-CD137). • Studies used cytokine/Cytokines receptor KO mice. • Ag-dependent stimulation (bacteria, virus). • Activation cells with Supernatant. • Bystander suppression.

- Inappropriate control.

3.3.3 Search strategies and review process.

The search query was performed systemically on three databases from Jun 2020 to August 2021 (updated in November 2022) to identify all studies with no year restriction:

1. MEDLINE (PubMed)
2. EMBASE (OVID): Embase 1947-Present, updated daily.
3. Web of Science

The advanced search included no restrictions and non-English language manuscripts in databases were excluded later. Controlled vocabulary (MeSH, Map, and full-text terms) was used for a detailed database search. In addition, to broaden our search, truncation was applied by adding an asterisk symbol (*) at the end of the keyword along with boolean operators to combine or exclude the keywords (Table 3.2).
The MeSH terms/Map terms and subheadings are cytokine, T lymphocytes, B lymphocytes, dendrites, bystander activation, and bystander effect.
Keywords: NK cell, Dendritic cell, antigen independent.

In this systematic review, the key terms for the search strategy were determined in collaboration with my supervisor. The search was conducted across all relevant databases, followed by the removal of duplicates and the screening of titles and abstracts, which were undertaken by the thesis author. The full-text article assessments were conducted by the author and a second reviewer, Dr. Ryszard Nosaloski (postdoctoral researcher), to ensure a thorough evaluation. A third reviewer was involved to resolve any discrepancies. The inclusion and exclusion criteria were established before the review process and strictly adhered to throughout. All procedures were conducted following ethical standards, ensuring the transparency and integrity of the systematic review.

Table 3.2. Search strategy for three databases.

	MEDLINE (PubMed)	EMBASE (OVID)	Web of Science
1-	"cytokine*"[MeSH Terms]	bystander activation.mp	(TS=(T lymphocyte*) AND LA=(English)) AND DT=(Article)
2-	"t lymphocytes"[MeSH Terms]	*bystander effect/	(TS=(B lymphocyte*) AND LA=(English)) AND DT=(Article)
3-	"b lymphocytes"[MeSH Terms]	*bystander effect/ or bystander activation. Mp	(TS=(NK cell*) AND LA=(English)) AND DT=(Article)
4-	"NK"[All Fields] AND "cell*"[All Fields]	*cytokine/ or interferon/ or interleukin 1/ or interleukin 10/ or interleukin 11/ or interleukin 12/ or interleukin 12p35/ or interleukin 12p40/ or interleukin 12p70/ or interleukin 13/ or interleukin 14/ or interleukin 15/ or interleukin 16/ or interleukin 17/ or interleukin 17c/ or interleukin 17f/ or interleukin 18/ or interleukin 19/ or interleukin 1alpha/ or interleukin 1beta/ or "interleukin 1beta[163-171]"/ or interleukin 2/ or interleukin 2 derivative/ or interleukin 20/ or interleukin 21/ or interleukin 22/ or interleukin 23/ or interleukin 23p19/ or interleukin 24/ or interleukin 25/ or interleukin 26/ or interleukin 27/ or interleukin 28/ or interleukin 28b/ or interleukin 3/ or interleukin 31/ or interleukin 32/ or interleukin 33/ or interleukin 34/ or interleukin 35/ or interleukin 37/ or interleukin 4/ or interleukin 5/ or interleukin 7/ or interleukin 9/ or tumor necrosis factor/	(TS=(Dendritic Cell*) AND LA=(English)) AND DT=(Article)

5-	(("dendrites"[MeSH Terms] OR "dendrites"[All Fields] OR "dendrite"[All Fields] OR "dendritic"[All Fields] OR "dendritically"[All Fields]) AND "cell*"[All Fields]))	t lymphocyte/ or cd25+ t lymphocyte/ or cd3+ t lymphocyte/ or cd4+ cd25+ t lymphocyte/ or cd4+ t lymphocyte/ or cd8+ t lymphocyte/ or cytotoxic lymphocyte/ or cytotoxic t lymphocyte/ or effector cell/ or gamma delta t lymphocyte/ or helper cell/ or intraepithelial lymphocyte/ or memory t lymphocyte/ or natural killer t cell/ or pre t lymphocyte/ or regulatory t lymphocyte/ or splenic t cell/ or t lymphocyte subpopulation/ or t lymphocyte activation/	#1 OR #2 OR #3 OR #4
6-	"bystander effect"[MeSH Terms]	*B lymphocyte/ or b lymphocyte*.mp.	(TS=(cytokine*)) AND LA=(English) AND DT=(Article)
7-	"Antigen-Independent"[All Fields]	natural killer cell/ or nK cell*.mp.	((TS=(interleukin*)) AND LA=(English)) AND DT=(Article)
8-	#2 OR #3 OR #4 OR #5	*dendritic cell/ or Dendritic cell*.mp.	#6 OR #7
9-	#6 OR #7	antigen-independentdent.mp.	(TS=(bystander activation) AND LA=(English)) AND DT=(Article)
10-	#1 AND #8 AND #9 "cytokine*"[MeSH Terms] AND ("T lymphocytes"[MeSH Terms] OR "b lymphocytes"[MeSH Terms] OR ("NK"[All Fields] AND "cell*"[All Fields]) OR (("dendrites"[MeSH Terms] OR "dendrites"[All Fields] OR "dendrite"[All Fields] OR "dendritic"[All Fields] OR "dendritically"[All Fields]) AND "cell*"[All Fields])) AND ("bystander effect"[MeSH Terms] OR "Antigen-Independent"[All Fields])	5 or 6 or 7 or 8	(TS=(antigen-independent) AND LA=(English)) AND DT=(Article)
11-		1 or 2 or 3 or 9	#9 OR #10
12-		4 and10 and 11	#5 AND #8 AND #11

3.3.4 Comparators

The comparisons will include the following:

1. Cells activated via individuals or combined cytokines vs. nonactivated T cells group (control).

2. Cells activated via individuals or combined cytokines vs. α-CD3/CD28 antibody-stimulated (activated) T-cells.

3. Cells activated via individual cytokines vs. combined cytokines.

3.3.5 Data items

The data items extracted are the papers' title, including PMID and DOI, the type of the targeted immune cells, and the studied cytokine, along with its effect on proliferation, activation, cell markers, receptors, and gene expression. In addition, details of the protocol and steps of activation, such as concentration, time points, and type of stimulation, were extracted. Extraction data also included the possible pathways and mechanisms of activation mentioned in the study.

3.3.6 Risk of bias assessment

There is no standard assessment tool to evaluate the risk of bias and heterogeneity. Possible sources of such heterogeneity are between-study differences in the type of protocol used to stimulate cells and different time points. To overcome this, the condition of stimulation was stabilised, such as

- Source of cytokines (recombinant cytokines).
- Include only *in Vitro*-study.

3.4 Results

3.4.1 Study selection.

Three scientific databases (Medline, Embase, and Web of Science) were searched using a study selection process. First, removing duplicate articles (n=390) was followed by a title and abstract screening process, resulting in 168 pieces for eligibility assessment. Eventually, 56 papers were included in this systematic review after assessing the full-text studies.

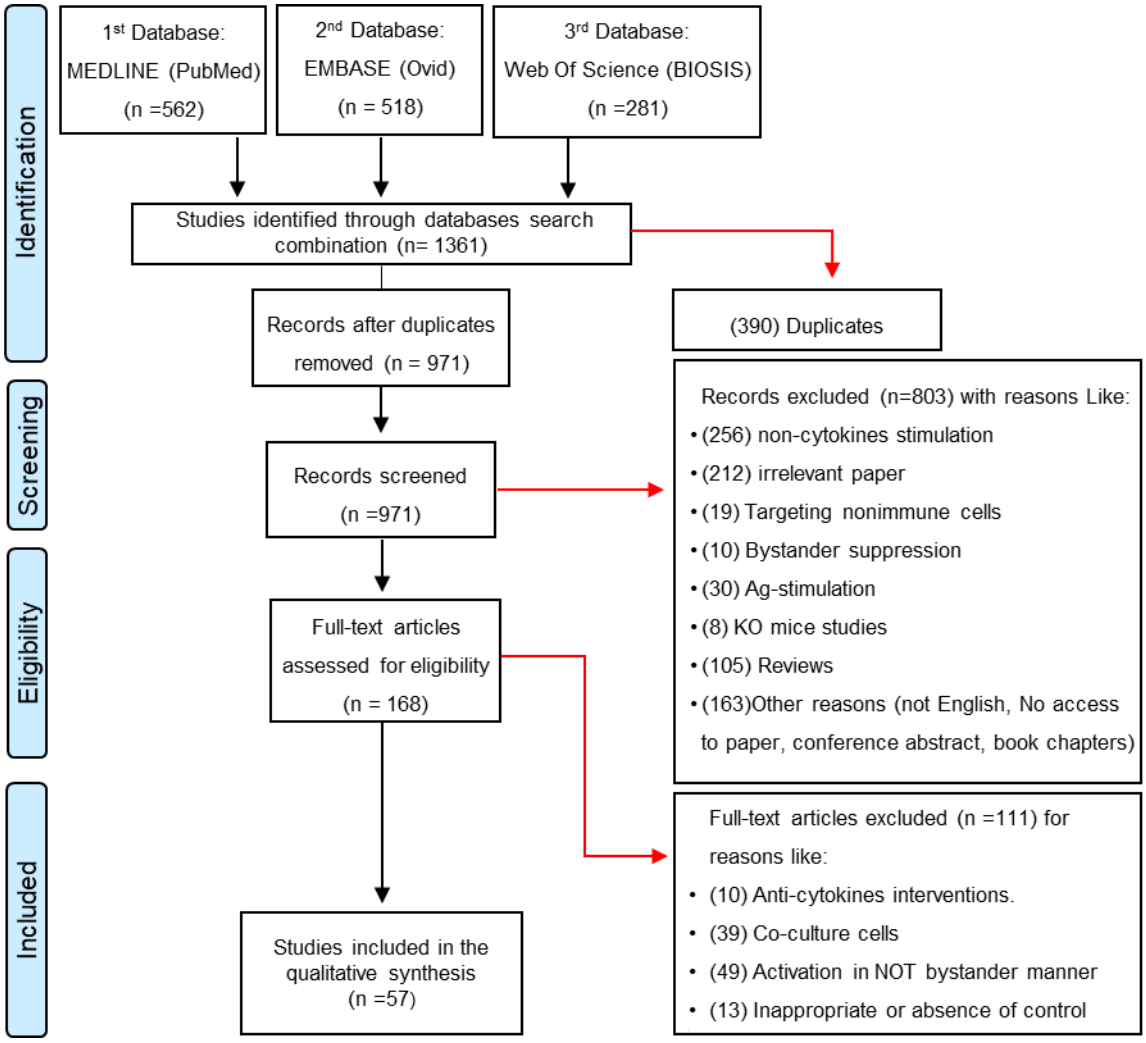


Figure 3.1. PRISMA flow diagram of the study selection process.

3.4.2 Study characteristics.

Upon identifying eligible studies and analysing the data, all quantitative extracted data from the studies were categorised and summarised in an Excel file. The model type of the study was defined; there were thirty-four human studies (Skold et al., 2018; Martino et al., 1998; Alves et al., 2003; Yurova et al., 2016; Unutmaz et al., 1994; Crawley et al., 2014; Kohlmeier et al., 2010; Nolz and Harty, 2014; Clark and Kupper, 2007; Lu et al., 2002; Vakkila et al., 2001; Houtkamp et al., 2001; Fukui et al., 1997; Sebbag et al., 1997; Unutmaz et al., 1995; Jiang et al., 2014; Weber et al., 2006; Younes et al., 2016; Holmkvist et al., 2015; Gruenbacher et al., 2014; Brod et al., 1990; Schilbach et al., 2020; Mujib et al., 2012; Tietze et al., 2012; Kwoczek et al., 2018; Liu et al., 2002; Seo et al., 2019; Wang et al., 2000; Munk et al., 2011; Bastidas et al., 2014; Bou Ghanem et al., 2011; Domae et al., 2017; Geginat et al., 2003; Abdelsamed et al., 2017) and twenty-two animal models studies (Pulle et al., 2006; Tchaptchet et al., 2010; Ilangumaran et al., 2003; Lee et al., 2017; Hirahara et al., 2012; Ghanem et al., 2011; Ingram et al., 2011; Wong and Pamer, 2004; Freeman et al., 2012; LeClair et al., 1989; Guo et al., 2009; Suarez-Ramirez et al., 2014; Lertmemongkolchai et al., 2001; Bremser et al., 2015; Anfossi et al., 2004; Vesosky et al., 2006; Zhao et al., 1999; Andrada et al., 2017; Berg et al., 2002; Morris et al., 2009; Louahed et al., 1995; Wherry et al., 2004), all of them are mice. Research experiments purify T cells from different body organs/tissues. Among 56 articles, twenty-eight studies extracted cells from PBMCs (Skold et al., 2018; Martino et al., 1998; Alves et al., 2003; Yurova et al., 2016; Unutmaz et al., 1994; Crawley et al., 2014; Munk et al., 2011; Lu et al., 2002; Vakkila et al., 2001; Sebbag et al., 1997; Unutmaz et al., 1995; Jiang et al., 2014; Weber et al., 2006; Younes et al., 2016; Holmkvist et al., 2015; Gruenbacher et al., 2014; Brod et al., 1990; Schilbach et al., 2020; Mujib et al., 2012; Kwoczek et al., 2018; Abdelsamed et al., 2017; Liu et al., 2002; Seo et al., 2021; Wang et al., 2000; Bastidas et al., 2014; Bou Ghanem et al., 2011; Domae et al., 2017; Geginat et al., 2003), four studies were from cord blood (Alves et al., 2003; Lu et al., 2002; Fukui et al., 1997; Kwoczek et al., 2018), and twenty-four purified cells from different organs, such as the spleen (Pulle et al., 2006; Kohlmeier et al., 2010; Nolz and Harty, 2014; Wherry et al., 2004; Ilangumaran et al., 2003; Hirahara et al., 2012; Lee et al., 2017; Ingram et al., 2011; Wong and Pamer, 2004; Freeman et al., 2012; Tietze et al., 2012; Guo et al., 2009; Suarez-Ramirez et al., 2014; Lertmemongkolchai et al., 2001; Bremser et al., 2015; Zhao et al., 1999; Anfossi et al., 2004; Tchaptchet et al., 2010; Ghanem et al., 2011; Andrada et al., 2017), lung (Vesosky et al., 2006; Kohlmeier et al., 2010) , lymph node (Ilangumaran et al., 2003). In

three studies, cell lines were also used)Berg et al., 2002; Louahed et al., 1995; LeClair et al., 1989(

Various recombinant cytokines were studied in the extracted articles; IL-15 was used as a cell stimulator in twenty-five research papers (Pulle et al., 2006; Alves et al., 2003; Yurova et al., 2016; Nolz and Harty, 2014; Crawley et al., 2014; Geginat et al., 2003; Bastidas et al., 2014; Andrada et al., 2017; Wherry et al., 2004; Ilangumaran et al., 2003; Houtkamp et al., 2001; Sebbag et al., 1997; Anfossi et al., 2004; Seo et al., 2021; Liu et al., 2002; Abdelsamed et al., 2017; Kwoczek et al., 2018; Mujib et al., 2012; Gruenbacher et al., 2014; Holmkvist et al., 2015; Younes et al., 2016; Weber et al., 2006; Jiang et al., 2014; Lu et al., 2002; Freeman et al., 2012) , IL-12 in twenty papers (Munk et al., 2011; Vakkila et al., 2001; Ghanem et al., 2011; Lee et al., 2017; Holmkvist et al., 2015; Gruenbacher et al., 2014; Ingram et al., 2011; Schilbach et al., 2020; Freeman et al., 2012; Guo et al., 2009; Kwoczek et al., 2018; Suarez-Ramirez et al., 2014; Lertmemongkolchai et al., 2001; Bremser et al., 2015; Vesosky et al., 2006; Wang et al., 2000; Tchaptchet et al., 2010; Bastidas et al., 2014; Berg et al., 2002; Bou Ghanem et al., 2011; Domae et al., 2017), IL-7 in nineteen papers (Alves et al., 2003; Domae et al., 2017; Bastidas et al., 2014; Munk et al., 2011; Yurova et al., 2016; Crawley et al., 2014; Vakkila et al., 2001; Fukui et al., 1997; Jiang et al., 2014; Weber et al., 2006; Younes et al., 2016; Mujib et al., 2012; Kwoczek et al., 2018; Abdelsamed et al., 2017; Liu et al., 2002; Geginat et al., 2003; Pulle et al., 2006; Wherry et al., 2004; Ilangumaran et al., 2003), IL-18 was in fifteen papers (Munk et al., 2011; Ghanem et al., 2011; Lee et al., 2017; Holmkvist et al., 2015; Gruenbacher et al., 2014; Ingram et al., 2011; Schilbach et al., 2020; Freeman et al., 2012; Guo et al., 2009; Suarez-Ramirez et al., 2014; Lertmemongkolchai et al., 2001; Vesosky et al., 2006; Tchaptchet et al., 2010; Berg et al., 2002; Bou Ghanem et al., 2011; Domae et al., 2017), and IL-2 in thirty articles (Martino et al., 1998; Unutmaz et al., 1994; Domae et al., 2017; Bastidas et al., 2014; Yurova et al., 2016; Clark and Kupper, 2007; Lu et al., 2002; Unutmaz et al., 1995; Jiang et al., 2014; Schilbach et al., 2020; Mujib et al., 2012; Tietze et al., 2012; Wang et al., 2000; Andrada et al., 2017; Ilangumaran et al., 2003; Wong and Pamer, 2004; Lertmemongkolchai et al., 2001; Zhao et al., 1999). Seven papers studied IL-4 (Mujib et al., 2012; Morris et al., 2009; Bremser et al., 2015; Guo et al., 2009; Geginat et al., 2003) and IFNs (Martino et al., 1998; Skold et al., 2018). TNF-α was in nine research papers (Martino et al., 1998; Domae et al., 2017; Geginat et al., 2003; Sebbag et al., 1997; Unutmaz et al., 1994; Unutmaz et al., 1995), IL-21 in five (Ingram et al., 2011; Kwoczek et al., 2018; Bastidas et al., 2014; Mujib et al., 2012; Guo et al., 2009) , IL-1 in four papers (Guo et al., 2009; LeClair et al., 1989), IL-33 in three papers (Freeman et al., 2012;

Bremser et al., 2015), and only one paper in IL-23 and IL-27 (Munk et al., 2011), (Hirahara et al., 2012) respectively.

Some of these cytokines were studied in relation to different diseases like Viral infection (Wherry et al., 2004; Suarez-Ramirez et al., 2014; Kwoczek et al., 2018) including HIV (Jiang et al., 2014; Younes et al., 2016; Bastidas et al., 2014) and bacterial infection(Ingram et al., 2011; Lertmemongkolchai et al., 2001; Vesosky et al., 2006; Tchaptchet et al., 2010), Multiple Sclerosis (Martino et al., 1998) , Rheumatoid Arthritis (Sebbag et al., 1997) , and Atherosclerosis (Houtkamp et al., 2001). Two study on Cancer cells (Schilbach et al., 2020; Tietze et al., 2012).

Various cytokines target different immune cells to examine their effect. Total Naïve T cells were targeted in eight research papers(Skold et al., 2018; Martino et al., 1998; Yurova et al., 2016; Sebbag et al., 1997; Mujib et al., 2012; Zhao et al., 1999; Tchaptchet et al., 2010; Houtkamp et al., 2001), CD8+ T cell was the most common T cell subtype which was included in twenty-four papers(Pulle et al., 2006; Alves et al., 2003; Crawley et al., 2014; Kwoczek et al., 2018; Nolz and Harty, 2014; Morris et al., 2009; Wherry et al., 2004; Ilangumaran et al., 2003; Weber et al., 2006; Younes et al., 2016; Ingram et al., 2011; Wong and Pamer, 2004; Freeman et al., 2012; Tietze et al., 2012; Abdelsamed et al., 2017; Suarez-Ramirez et al., 2014; Lertmemongkolchai et al., 2001; Liu et al., 2002; Vesosky et al., 2006; Seo et al., 2021; Anfossi et al., 2004; Andrada et al., 2017; Bastidas et al., 2014; Berg et al., 2002; Bou Ghanem et al., 2011) , and CD4+ T cell was in fourteen papers (Unutmaz et al., 1994; Munk et al., 2011; Vakkila et al., 2001; Fukui et al., 1997; Unutmaz et al., 1995; Louahed et al., 1995; Hirahara et al., 2012; Jiang et al., 2014; Lee et al., 2017; Holmkvist et al., 2015; Guo et al., 2009; Lertmemongkolchai et al., 2001; Bremser et al., 2015; Geginat et al., 2003). γδ T cell (Gruenbacher et al., 2014; Schilbach et al., 2020; Geginat et al., 2003). NK cells were in four studies(Wang et al., 2000; Andrada et al., 2017; Berg et al., 2002; Geginat et al., 2003) and one paper study T reg cells (Clark and Kupper, 2007).

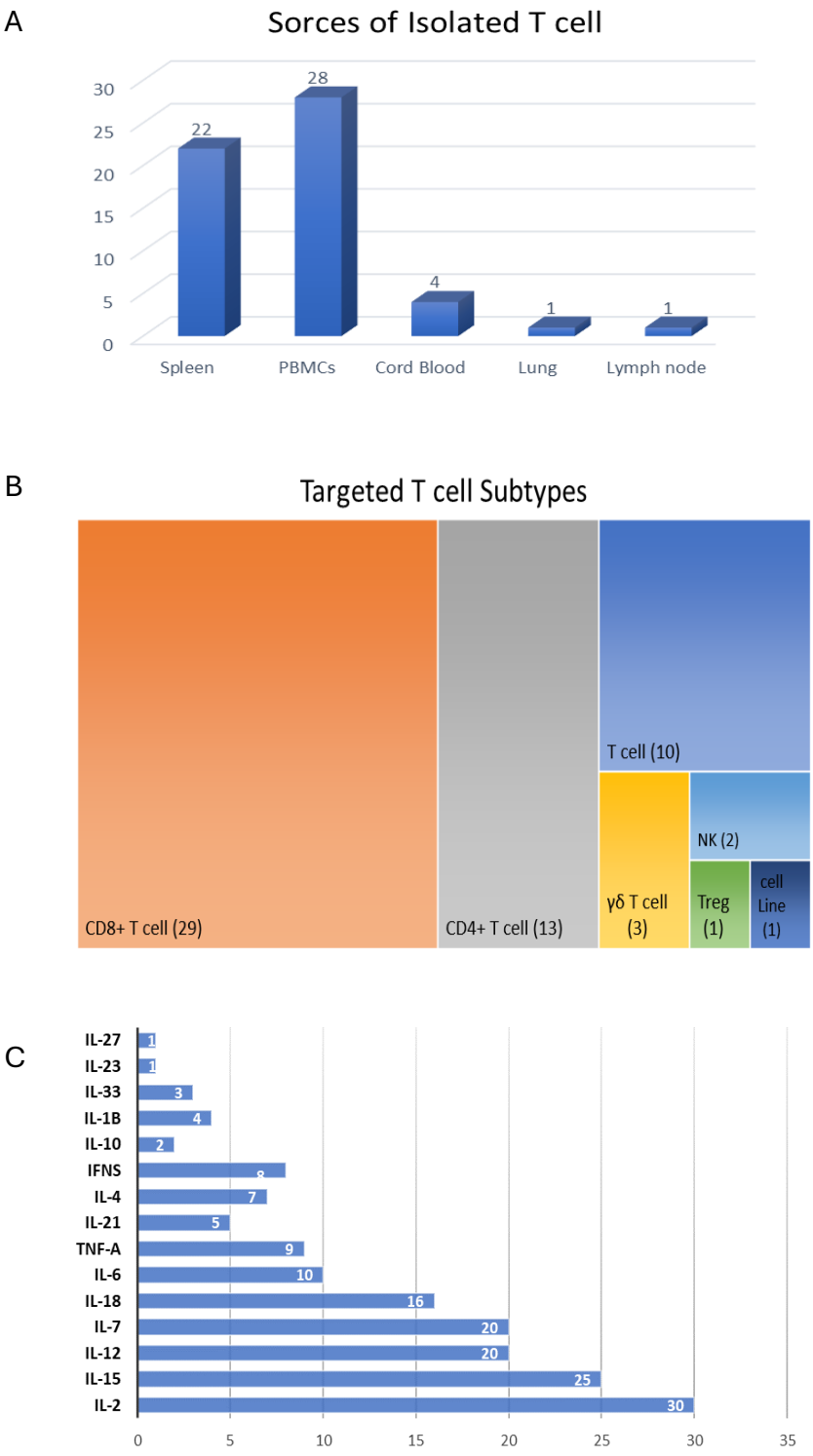


Figure 3.2. Characteristics of systematic review data.

The figures summarize key characteristics from the systematic review data**. (A)** presents the sources of isolated T cells used across studies, whether they were derived from the spleen, PBMCs, lung, cord blood, or lymph nodes. **(B)** shows the number of studies targeting different T cell subtypes (e.g., CD4+, CD8+, etc.), providing an overview of the most studied T cell populations**. (C)** represents the cytokines used to stimulate the T cells, and the frequency of cytokines involved in TCR-independent activation.

Table 3.3. Studies on Human Isolated T Cells.

References			Population	Intervention	Variables				Protocol
No.	DOI	Author, title, journal, year published	Target immune Cell /subpopulation	Cytokine	proliferation	Receptors expression//antigens	Cytokines production	Mechanism and other effect/s of gene	Protocol
1	https://doi.org/10.1007/s00262-018-2204-2.	Sköld, Annette E., Till SM Mathan, Jasper JP van Beek, Georgina Flórez-Grau, Michelle D. van den Beukel, Simone P. Sittig, Florian Wimmers, Ghaith Bakdash, Gerty Schreibelt, and I. Jolanda M. de Vries. "Naturally produced type I IFNs enhance human myeloid dendritic cell maturation and IL-12p70 production and mediate elevated effector functions in innate and adaptive immune cells." *Cancer Immunology, Immunotherapy* 67, no. 9 (2018): 1425-1436.	Stimulated PBL T cells NKT cell NK cell	IFN-α	↓	- CD69 on T cells (20%) - CD69 on NKT cells (60%)	Dose dependants increase in IFN-γ (0.1-2%)		T cells from 9 Peripheral Blood donors cultured for five days with IFN-α (1; 10; and 100 ng/ml) **Compared to α-CD3 + α-CD28 and medium.**

2	https://doi.org/10.1002/ana.410430312	Martino, Gianvito, Fabio Grohovaz, Elena Brambilla, Franca Codazzi, Antonella Consiglio, Emilio Clementi, Massimo Filippi, Giancarlo Comi, and Luigi ME Grimaldi. "Proinflammatory cytokines regulate antigen-independent T-cell activation by two separate calcium-signaling pathways in multiple sclerosis patients." *Annals of neurology* 43, no. 3 (1998): 340-349.	Stimulated PBL T cell, CD4+ T cell	TNF-α + IL-6 + IL-2 ± IFN-γ Higher proliferation of T cells from MS patients.	↑			Ca^{+} Elevation in MS patient samples via 2 pathways: - IFN-γ-activated influx-positive cells in MS patients. - IP_3-mediated pathway	PBL was incubated for 48 hours in the presence of the cytokines TNF-α 25 ng/ml, IFN-γ 50 U/ml, IL-6 500 U/ml, and IL-2 300 U/ml, individually or in combination. **Compared to other cytokines and unstimulated cells**
2			PBL T cell	TNF-α	No Effect				
2			PBL T cell	IL-6	No Effect				
2			PBL T cell	IFN-γ	No Effect	Induce additive effect with TNF-α, IL-6 and IL-2.			
2			PBL T cell	IL-2	No Effect				

3	DOI: 10.1182/blood-2003-01-0183	Alves, Nuno L., Berend Hooibrink, Fernando A. Arosa, and René AW van Lier. "IL-15 induces antigen-independent expansion and differentiation of human naive CD8+ T cells in vitro." *Blood* 102, no. 7 (2003): 2541-2546.	PBMCs UCBMC $CD8^+$ T cell	IL-15	↑	**Naive CD8 + T cell:** - Express IL-2/15Rβ - Down-modulation of CD45RA - Down-regulated CD28, CCR7, and CD62L in cells that underwent more than 3 to 4 rounds of divisions - maintain CD27 expression. - Upregulation IL-2/15Rγ (CD132) on Naïve, (CD27-CD45RAbright) cytotoxic, and (CD27+CD45RAdull) noncytotoxic CD8+ T cells. - IL-15Rα undetectable on all CD8+ T cell subsets.	IFN-γ, TNF-α, perforin, and granzyme B.		$CD8^+$ T cell purified from PBMCs or UCBMCs cultured with/ without IL-15 10ng/ml for seven days. **Compare to Isotype control and medium.**
4	DOI: 10.1134/S1990750815020146	Yurova, K. A., N. A. Sokhonevich, O. G. Khaziakhmatova, and L. S. Litvinova. "Cytokine-mediated regulation of expression of Gfi1 and U2afll4 genes activated by T-cells with different differentiation status i*n vitro* " *Biomeditsinskaya khimiya* 62, no. 2 (2016): 180-186.	PBMCs Naive $CD45RO^+$	IL-15 / IL-7 / IL-2				- Dose-dependent decrease of the U2af1l4 gene expression and an increase of Gfi1 gene expression	CD45RA+ and CD45RO+ cells were cultivated for 48 hours in various concentrations of recombinant IL-2, IL-7, IL-15, and (control).
4			$CD45RA^+$	IL-15 / IL-7 / IL-2				- Decrease in expression of both genes with increased concentration of IL-7 - Increase in expression of both genes with increased concentration of IL-15.	

5	DOI: 10.1084/jem.180.3.1159	Unutmaz, Derya, Piero Pileri, and Sergio Abrignani. "Antigen-independent activation of naive and memory resting T cells by a cytokine combination." *The Journal of experimental medicine* 180, no. 3 (1994): 1159-1164.	PBMCs Naive CD45RA$^+$ and Memory CD45RO$^+$ resting CD4$^+$ T cell	TNF-α + IL-6 + IL-2 TNF+ IL-2 IL-6 + IL-2	↑	<u>CD45RA$^+$:</u> express CD69 with TNF-α + IL-6 + IL-2 <u>CD45RO$^+$:</u> - Express CD69 with TNF-α + IL-2 ± IL-6 - No CD69 expression with IL-2 alone. - High proliferation with TNF-α +IL-6+ IL-2 - No proliferation with TNF-α + IL-2 or IL-6 + IL-2		- IFN-γR and IL-4 mRNA with only combination of IL-2 + TNF-α + IL-6 but not IL-2 alone.	Resting T cells were cultured for 8 days with various combinations of rIL-2 (200-300 U/ml), lL-6 (500 U/ml), and TNF-α (25 ng/ml). **Compared to other cytokines.**
6	DOI: 10.18632/oncotarget.17498	Domae, Eisuke, Yuya Hirai, Takashi Ikeo, Seiji Goda, and Yoji Shimizu. "Cytokine-mediated activation of human ex vivo-expanded Vγ9Vδ2 T cells." *Oncotarget* 8, no. 28 (2017): 45928.	PBMCs γδ T Cells	IL-12 + IL-18	↑	- Proliferate *ex vivo* expanded γδ T Cells, not freshly isolated. - <u>Synergistically:</u> increase ICAM-1, CD25, IL-12Rβ2, and IL-18R exp and decrease BTLA exp. - <u>IL-12:</u> increase IL-18R expression	IFN-γ, Granzyme B + ve Annivax	- Receptors express phosphorylation of STAT4 and NF-κB p65. - IFN-γ through IκBζ expression.	γδ T cells were cultured with medium alone or with media with IL-12 (10 ng/mL), IL-18 (10 ng/mL), or IL-12 and IL-18 (10 ng/mL each). **Compared to medium and isotype**
6				IL-12 / IL-18	No E.		No IFN-γ Production		
6			PBMCs γδ T Cells	IL-12 + IL-18 ± IL-15 or IL-7	IL-15: ↑ IL-7: No E.				Vγ9Vδ2 T cells were stimulated with IL-12/IL-18 for 16 h, washed extensively, and cultured for three days with medium alone, IL-2 (10 U/mL), IL-7 (1 ng/mL) or IL-15 (1 ng/mL). **Compared to medium and isotype**

7	https://doi.org/10.4049/jimmunol.1302027	Bastidas, Sonia, Frederik Graw, Miranda Z. Smith, Herbert Kuster, Huldrych F. Günthard, and Annette Oxenius. "CD8+ T cells are activated in an antigen-independent manner in HIV-infected individuals." *The Journal of Immunology* 192, no. 4 (2014): 1732-1744.	PBMCs CD8+ T cell CD4⁺ T cell	IL-15	↑	- No proliferation effect on CD4+ T cell - HLA-DR+CD38+: high on CD8⁺ (50%). No expression on CD4⁺ T cell (4-5%). - Expression CD25, CD69, and CD38 - Expression of IL-15Rβ on CD8 $T_{EM.}$ - Selective proliferation effect on memory CD8+ T cell.		- Memory CD8⁺ T cells are more efficiently activated by IL-15 stimulation. - IL-15Rβ mRNA expressed on T_{CM} T_{EM} CD8+ T cell not CD4+ T cell.	T cells were stimulated with IL-15 (50 ng/ml, IL-7 (50 ng/ml), IL-12 (3 ng/ml), IL-21 (25 ng/ml), and IL-2 (300 U/ml) and cultured for 3–6 day. **Compared to medium and other cytokines.**
7				L-12 + IL-15	↑↑	HLA-DR+CD38+: On CD8⁺ (50-54%). No expression on CD4⁺ T cell (5-6%).			
7				L-7	↑	HLA-DR+CD38+: On CD8⁺ (15-20%) No expression CD4⁺ T cell (5-7%)			
7				IL-21	No E.	HLA-DR+CD38+: On CD8⁺ (2.9-3.5%) (Not sig.) Expression on CD4⁺ T cell (1-1.3%)			
7				IL-12	No E.	HLA-DR+CD38+: (5.1% ± 0.6%) on CD8+ T cell. No expression (1-3 %) on CD4+ T cell.			
7				IL-2	No E.	NO HLA-DR+CD38+ expression (4.9% ± 2.8%) on CD4+ T cell, and (15.3% ± 5.9%) CD8+ T cell.			

8	**https://doi.org/10.1189/jlb.0313122**	Crawley, Angela M., Agatha Vranjkovic, Elliott Faller, Michaeline McGuinty, Aurelia Busca, Stephanie C. Burke, Sophie Cousineau, Ashok Kumar, Paul A. MacPherson, and Jonathan B. Angel. "Jak/STAT and PI3K signaling pathways have both common and distinct roles in IL-7-mediated activities in human CD8+ T cells." *Journal of leukocyte biology* 95, no. 1 (2014): 117-127.	PBMCs $CD8^+$ T cell	IL-7	↑	Low concentration: Induce Bcl-2 production and glucose uptake. Higher concentration: Induce cell proliferation and intracellular accumulation of perforin and glucose uptake.	Perforin	- At low concentration: Jak-STAT5 signalling pathways. - Induce p-Akt via PI3K activation. - Cellular metabolic activity by increasing glucose uptake.	Isolated cells were cultured with medium only (Ctl) or IL-7 (10 –50,000 pg/ml) for 5 days. **Compared to medium**
9	DOI: 10.1371/journal.pone.0018553	Munk, Rachel B., Katsuki Sugiyama, Paritosh Ghosh, Carl Y. Sasaki, Louis Rezanka, Kasturi Banerjee, Hidenori Takahashi, Ranjan Sen, and Dan L. Longo. "Antigen-independent IFN-γ production by human naïve CD4+ T cells activated by IL-12 plus IL-18." PLoS One 6, no. 5 (2011): e18553.	PBMCs Naïve and memory $CD4^+$ T cell	IL-12 + IL-18	No E.		IFN-γ when only combined	- Expression of IFN-γ mRNA. - STAT4 phosphorylation - IFN-γ produced in cRel/NFkB dependent mechanism. - No cell death. -	CD4+ T cells were purified from PBMC and stimulated with IL-12 (1 ng/ml) and/or rh IL-18 (40 ng/ml) for various periods of time. **Compared to other cytokines**
10	https://doi.org/10.1016/j.cellimm.2011.09.003	Ghanem, Elsa N. Bou, and Sarah EF D'Orazio. "Human CD8+ T cells display a differential ability to undergo cytokine-driven bystander activation." Cellular immunology 272, no. 1 (2011): 79-86.	PBMCs $CD8^+$ T cell	IL-12 + IL-18		- Less response with individual cytokines (IL-12 / IL-18)	IFN-γ only when combined	- Induce $IFN\gamma^+$ $CD8^+$ T cells in murine cells. - Induce IFNγ secretion by $CD8^+$ T cells	Human PBMC, murine splenocytes, or T cells were cultured and stimulated either with 50 ng/ml recombinant IL-12 + IL-18 or media alone. **Compared to other cytokines**

11	10.1016/j.immuni.2010.06.016	Kohlmeier, Jacob E., Tres Cookenham, Alan D. Roberts, Shannon C. Miller, and David L. Woodland. "Type I interferons regulate cytolytic activity of memory CD8+ T cells in the lung airways during respiratory virus challenge." *Immunity* 33, no. 1 (2010): 96-105.	Lung, spleen CD8⁺ memory T cell	IFN-α/ IFN-β		- Enhance Lytic activity	Granzyme B in murine and human	- STAT1 is required for the expression of gzmB protein in memory CD8+ T cells.	Human PBMCs were cultured with or without IFN-α or IFN-β t IFN-α or IFN-β (2×104 units/ml, PBL Interferon Source). for 36 hours. **Compared to unstimulated cell & isotype**
12	DOI: 10.1172/JCI72039	Nolz, Jeffrey C., and John T. Harty. "IL-15 regulates memory CD8+ T cell O-glycan synthesis and affects trafficking." *The Journal of clinical investigation* 124, no. 3 (2014): 1013-1026	Spleen CD8+ Memory T cell	IL-15		- Regulate core 2 O-glycan expression.		- Upregulate binding with E-selectin and P-selectin on memory Not Naïve CD8+ T cell. - Regulate Gcnt1 gene expression.	Human CD8+ T cells were purified and cultured for three days with or without 250 ng/ml IL-15. **Compared to unstimulated cell**
13	DOI: 10.1182/blood-2006-02-002873	Clark, Rachael A., and Thomas S. Kupper. "IL-15 and dermal fibroblasts induce proliferation of natural regulatory T cells isolated from human skin." *Blood* 109, no. 1 (2007): 194-202.	Skin T cell Treg	IL-15 + IL-2 / α-CD3+α-CD28		- skin-resident T cells did not proliferate when treated with IL-2 and IL-15 alone (IL2/15) but proliferated at low levels when stimulated α-CD3+α-CD28 antibodies - upregulate CD69 and CD25 expression			Skin T Cells were harvested at 3 weeks or at the interval specified, stimulated with IL-15 (20 ng/mL) and/or IL-2 (100 IU/mL). **Compared to unstimulated T cell OR α-CD3/CD28 antibody**
14	PMID: 12473603	Lu, Jun, Robert L. Giuntoli, Ryusuke Omiya, Hiroya Kobayashi, Richard Kennedy, and Esteban Celis. "Interleukin 15 promotes antigen-independent in vitro expansion and long-term survival of antitumor cytotoxic T lymphocytes." *Clinical cancer research* 8, no. 12 (2002): 3877-3884.	CBMCs PBMCs CD8+ T cell CD8+ CTL clone CD4+ T cell	IL-15 / IL-2	/ No E. ↑	- High concentrations of IL-2 stimulate the antigen-independent expansion of CD8+ but not CD4+ T cells (1000 IU/ml IL-2) - Human CD4+ T lymphocytes do not proliferate but are able to survive in the presence of IL-15 or IL-2.	perforin	- IL-15 increases the proliferation and inhibits apoptosis of CD8+ T lymphocytes more than IL-2. IL-2 had higher DNA debris (apoptotic cell death indication) compared IL-15.	CD8+ T cell and CTL clones were cultured in either 50 IU/ml IL-2 or 10 ng/ml IL-15, and the numbers of viable cells were determined at different time points.

15	10.1046/j.1365-3083.2001.01001.x	Vakkila, Jukka, S. Äystö, Ulla M. Saarinen-Pihkala, and Hannu Sariola. "Naive CD4+ T cells can be sensitized with IL-7." *Scandinavian Journal of Immunology* 54, no. 5 (2001): 501-505.	PBMCs, CB Naïve CD4+ T cell	IL-1 + IL-2 + IL-7	↑				Naive T cells were cultures with (IL-1β, 10ng/ml; IL-2, 5ng/ml; IL-6, 10ng/ml, IL-7, 10ng/ml; IL-10, 10ng/ml; IL-12, 1ng/ml; IFN-γ, 10 ng/ml; TNF-α, 10ng/ml). Cells were then collected and washed twice with PBS. Viable cells were counted and used for proliferation assays (primed cells). **Compared to unstimulated cells**
15				IL-2 + IL-6 +TNF-α	↑				
15				IL-7	↑				
15				IL-7 + IL-10	↓	IL-10 Abrogated the effect of IL-7			
15				IFN-γ -IL-12+IL-1	No E.				

16	DOI: 10.1161/hq0701.092162	Houtkamp, Mischa A., Allard C. van der Wal, Onno J. de Boer, Chris M. van der Loos, Piet AJ de Boer, Antoon FM Moorman, and Anton E. Becker. "Interleukin-15 expression in atherosclerotic plaques: an alternative pathway for T-cell activation in atherosclerosis?." *Arteriosclerosis, thrombosis, and vascular biology* 21, no. 7 (2001): 1208-1213.	T cell form atherosclerosis T cell plug	IL-15	↑				From endarterectomy tissue, T-cell lines were cultured with or without rIL-15 (1, 5, and 10 ng/mL) for 3 days. **Compared to unstimulated cell**
17	DOI: 10.1016/s0165-2478(97)00093-x	Fukui, Tetsuya, Kenji Katamura, Nobutaka Abe, Takahiro Kiyomasu, Jun Iio, Hideki Ueno, Mitsufumi Mayumi, and Kenshi Furusho. "IL-7 induces proliferation, variable cytokine-producing ability and IL-2 responsiveness in naive CD4+ T-cells from human cord blood." *Immunology letters* 59, no. 1 (1997): 21-28.	CBCs Naïve CD4+ T cell	IL-7	↑	- CD45RA (112 MFI), CD45RO (3.7 MFI) - CD25 and CD40 - CD62L stable			Freshly isolated cord blood CD4 + T cells were cultured with the indicated concentrations of IL-7 for 7 days. **Compared to unstimulated cells**
18	DOI: 10.1002/eji.1830270308	Sebbag, Mireille, Sarah L. Parry, Fionula M. Brennan, and Marc Feldmann. "Cytokine stimulation of T lymphocytes regulates their capacity to induce monocyte production of tumor necrosis factor-α, but not interleukin-10: possible relevance to pathophysiology of rheumatoid arthritis." European journal of immunology 27, no. 3 (1997): 624-632.	PBMCs Naïve T cell	TNF-α + IL-6 + IL-15		- Increase expression of CD45RO (165 MFI), activation markers CD25, CD69 and adhesion molecules CD11a (LFA-l), CD11b (MAC-l), ICAM-1, and CD44. - Decrease in expression of CD45RA. - No changes in the expression of CD40L or HLA-DR.		- Cytokine-induced T cells had a higher mean survival rate of 70% compared to 30% of unstimulated T cells.	T cells isolated from peripheral blood (0 days, uncultured, unstimulated T cells) or culture with 25 ng/ml TNF-a, 100 ng/ml IL-6, or 100 ng/ml IL-15 for 8 days. **Compared to unstimulated cells.**

19	Doi: 10.1093 /intimm/ 7.9.141 7	Unutmaz, Derya, Fabiana Baldoni, and Sergio Abrignani. "Human naive T cells activated by cytokines differentiate into a split phenotype with functional features intermediate between naive and memory T cells." *International immunology* 7, no. 9 (1995): 1417-1424.	PBMCs Naïve CD4+ T cell	TNF-α + IL-6 + IL-2	↑	- IL-4 maintains viability with no proliferation induction. - IL-4 can replace IL-2 T cells can be activated to proliferate by combination of TNF-a and IL-6 with either IL-2 or IL4.		- Upregulate adhesion molecules (ICAM-1, LFA-3, CD2, LFA-1 and CD29), CD38 - L-selectin - The majority is CD45RA+ cell, (3-10%) double-positive (CD45RA+RO+ -IL-2R expressed	Naive T cells were cultured in the presence of different combinations: IL-2 (250 U/ml), IL-4 (10 ng/ml), TNF-a (40 ng/ml) and IL-6 (500 U/ml). **Compared to fresh unstimulated cells.**
20	DOI: 10.1128 /JVI.000 17-14	Jiang, Wei, Souheil-Antoine Younes, Nicholas T. Funderburg, Joseph C. Mudd, Enrique Espinosa, Miles P. Davenport, Denise C. Babineau, Scott F. Sieg, and Michael M. Lederman. "Cycling memory CD4+ T cells in HIV disease have a diverse T cell receptor repertoire and a phenotype consistent with bystander activation." *Journal of virology* 88, no. 10 (2014): 5369-5380.	PBMCs Memory CD4+ T cell	IL-15 / IL-2/ IL-7		- OX40 sig. reduced (5-10%) with IL-15, IL-7 and IL-2 vs α-CD3 (40-50%).		- Upregulate CD38 by IL-7 and α-CD3	PBMCs from healthy control were cultured with or without anti-CD3 (50 ng/ml, recombinant human IL-2, IL-7, or IL-15 (50 ng/ml), IL-6 (50 ng/ml), IL-1β (50 ng/ml), or IFN-α (500 units/ml). **Compared to unstimulated cells.**
20				IL-1β, IL-6, IFN-α.		- No OX40 expression (1-3%).			

21	doi.org/10.1002/eji.200535616	Weber, Walter P., Chantal Feder-Mengus, Alberto Chiarugi, Rachel Rosenthal, Anca Reschner, Reto Schumacher, Paul Zajac et al. "Differential effects of the tryptophan metabolite3-hydroxyanthranilic acid on the proliferation of human CD8+ T cells induced by TCR triggering or homeostatic cytokines." *European journal of immunology* 36, no. 2 (2006): 296-304.	PBMCs CD8+ T cell	IL-15 / IL-2 / IL-7		IL-2 and IL-15: - Upregulate HLA-DR, CD69 and CD38. IL-7: Upregulate HLA-DR and CD69.			Cells were cultured in the presence of cytokines 50 ng/mL, anti-CD3, or the absence of any stimuli. On day 2 for anti-CD3 stimulation and on day 5 for cytokine stimulation, cultures were pulsed with [3H] thymidine (1 μCi/well) for 18h and then harvested. **Compared to unstimulated cells and α-CD3 stimulation.**
22	10.1172/JCI85996	Younes, Souheil-Antoine, Michael L. Freeman, Joseph C. Mudd, Carey L. Shive, Arnold Reynaldi, Soumya Panigrahi, Jacob D. Estes et al. "IL-15 promotes activation and expansion of CD8+ T cells in HIV-1 infection." *The Journal of clinical investigation* 126, no. 7 (2016): 2745-2756.	PBMCs CD8+ T cell	IL-15 + IL-7		- Upregulate ki-67 expression. - Activate CD8+ T cell.	granzyme B		PBMCs from healthy subjects were incubated with IL-2 (50 ng/ml), IL-7 (10 ng/ml), IL-15 (20 ng/ml), IL-6 (10 ng/ml), or IL-1β (10 ng/ml) for 48 hours. **Compared to medium**
22				IL-1β , IL-6 , IL-2		- No ki-67 expression	No granzyme B		

23	10.1038/mi.2014.87	Holmkvist, Petra, K. Roepstorff, Heli Uronen-Hansson, Caroline Sandén, Sigurdur Gudjonsson, O. Patschan, Olof Grip et al. "A major population of mucosal memory CD4+ T cells, coexpressing IL-18Rα and DR3 display innate lymphocyte functionality." *Mucosal immunology* 8, no. 3 (2015): 545-558.	PBMCs CD45RO+ CD4+ T cells	L-12 +IL-18 +IL-15+ TL1a IL-12+ IL-18 + TL1a	↑	- IL-18Rα with IL-12 + IL-18 + IL-15.	IFN-γ and IL-6, TNF-α, GM-CSF, IL-5, IL-13, IL-22	- TL1a receptor and death receptor 3 (CR3) - Cytokines secreted in MAPKs and PI3Ks dependent way.	Peripheral blood CD45RO+CD4+ T cells were cultured in medium alone (control) or with IL-12 (2 ng ml−1), IL-18 (50 ng ml−1), IL-15 (25 ng ml−1), and TL1a (100 ng ml−1). **Compared to unstimulated cells.**
24	10.4161/21624011.2014.953410	Gruenbacher, Georg, Oliver Nussbaumer, Hubert Gander, Bernhard Steiner, Nicolai Leonhartsberger, and Martin Thurnher. "Stress-related and homeostatic cytokines regulate Vγ9Vδ2 T-cell surveillance of mevalonate metabolism." *Oncoimmunology* 3, no. 8 (2014): e953410.	PBMCs γδ T cell	IL-18 / I L-12	↑	- Induced the differentiation of effector cells in an antigen-independent manner. - Expression of Il-18Ra (CD218a). - CD25		- Activate isopentenyl pyrophosphate (IP P)	PBMCs or enriched γδ T cells were stimulated in triplicates with (10–50 μM) in the presence or absence of 100 U/mL IL-2, 100 ng/mL IL-18 or 25 ng/mL IL-15. **Compared to unstimulated cells**
24				IL-1 5	↑	- Upregulation of CD56 - IL-15Rα, perforin, cytotoxic cell markers CD161, and CD96.			

25		Schilbach, Karin, Christian Welker, Naomi Krickeberg, Carlotta Kaißer, Sabine Schleicher, and Hisayoshi Hashimoto. "In the Absence of a TCR Signal IL-2/IL-12/18-Stimulated γδ T Cells Demonstrate Potent Anti-Tumoral Function Through Direct Killing and Senescence Induction in Cancer Cells." *Cancers* 12, no. 1 (2020): 130.	PBMCs γδ T cell	IL-12 + IL-18 + IL-2		- Increase expression of FasL - Increased NKG2D - Expression of NKG2D decreases by TCR stimulation.	Granzyme B/perforin IFN-γ / TNF-α	- Combined IL-2/IL-12/IL-18 Induces Senescence.	γδ T cells were isolated, and IL-12 or IL-18 (10 ng/mL, respectively), IL-2 (50 U/mL) or combinations of them were added for four days. **Compared to unstimulated cells, or TCR stimulation.**
26		Mujib, Shariq, R. Brad Jones, Calvin Lo, Nasra Aidarus, Kiera Clayton, Ali Sakhdari, Erika Benko, Colin Kovacs, and Mario A. Ostrowski. "Antigen-independent induction of Tim-3 expression on human T cells by the common γ-chain cytokines IL-2, IL-7, IL-15, and IL-21 is associated with proliferation and is dependent on the phosphoinositide 3-kinase pathway." *The Journal of Immunology* 188, no. 8 (2012): 3745-3756.	PBMCs CD8+ T cell CD4+ T cell	IL-7, IL-21, IL-2, IL-15	 IL-15	- Induce expression of T cell Ig mucin domain-containing molecule 3 (Tim-3) on naïve, memory, effector T cell. - Tim-3 expression is marked on proliferating cells.		- Tim-3 induced via PI3K pathway.	T cells selected negatively were stimulated with complete medium alone or with IL-2 (0.04–125 ng/ml), IL-4 (25 ng/ml), IL-7 (25 ng/ml), IL-10(25 ng/ml), IL-15 (0.04–125 ng/ml), IL-2 (25 ng/ml), anti-CD3/CD28 at 1 µg/ml each. **Compared to medium alone**
27		Tietze, Julia K., Danice EC Wilkins, Gail D. Sckisel, Myriam N. Bouchlaka, Kory L. Alderson, Jonathan M. Weiss, Erik Ames et al. "Delineation of antigen-specific and antigen-nonspecific CD8+ memory T-cell responses after cytokine-based cancer immunotherapy." *Blood, The Journal of the American Society of Hematology* 119, no. 13 (2012): 3073-3083.	PBMCs Spleen CD8+ T cell	IL-2 / α-CD3 +CD28		- CD25 and PD-1 expression on human and mouse T cells with α-CD3/CD28, whereas IL-2 stimulation alone did not. - NKG2D upregulation also occurred after cells were cultured in IL-2.			Mouse splenocytes were cultured with α-CD3/α-CD28 or rhIL-2 for three days. Human Lymphocytes were isolated and cultured with rhIL-2 and analysed after three or 14 days. **Compared to medium alone, α-CD3 +CD28**

28		Kwoczek, Julian, Sebastian B. Riese, Sabine Tischer, Szilvia Bak, Julia Lahrberg, Mathias Oelke, Holger Maul, Rainer Blasczyk, Martin Sauer, and Britta Eiz-Vesper. "Cord blood–derived T cells allow the generation of a more naïve tumor-reactive cytotoxic T-cell phenotype." *Transfusion* 58, no. 1 (2018): 88-99.	CB and PB Spleen CD8+ T cell	IL-7 + IL-15 ± IL-21 or IL-7 + IL-15 ± IL-12 or IL-7 + IL-15 + IL-12 + IL-21	↑	- IL-21 enhances the expansion of MART1-reactive CD8+ T cells from CB and PB.	TNF-α higher in IL-7 + IL-15 + IL-12 combination	- CB T cells showed more IFN- γ, and TNF-α, CD107a expression. - Higher proliferative capacity of CD8+ T cell CB compared to PB.	CD8 T cells were stimulated with CD3/CD28 and 50 U/mL IL-2, supplemented with IL-7 and IL-15 alone and in combination with IL-12 and/or IL-21 (all 10 ng/mL) for 7 days. **Compared to Cord blood and unstimulated cells.**
29	10.1084 /jem.20 161760. Epub 2017 May 10.	Abdelsamed, Hossam A., Ardiana Moustaki, Yiping Fan, Pranay Dogra, Hazem E. Ghoneim, Caitlin C. Zebley, Brandon M. Triplett, Rafick-Pierre Sekaly, and Ben Youngblood. "Human memory CD8 T cell effector potential is epigenetically preserved during in vivo homeostasis." *Journal of Experimental Medicine* 214, no. 6 (2017): 1593-1606.	PBMCs CD8+ T cell	IL-7, IL-15	↑	- Increased methylation of the CCR7 and Tcf7 loci.	IFN-γ	- Increase methylation of CCR7 and TCF1 - Down-regulation of CCR7 expression in both T_{CM} and T_{SCM} cells and conversion to T_{EM}-like cells.	CD8 T cells were maintained in culture in RPMI containing IL-7/IL-15 (25 ng/ml each) for seven days. **Compared to medium alone, and α-CD3 + CD28**
30	doi: 10.1 073/pna s.09267 5799	Liu, Kebin, Marta Catalfamo, Yu Li, Pierre A. Henkart, and Nan-ping Weng. "IL-15 mimics T cell receptor crosslinking in the induction of cellular proliferation, gene expression, and cytotoxicity in CD8+ memory T cells." *Proceedings of the National Academy of Sciences* 99, no. 9 (2002): 6192-6197.	PBMCs Memory CD8+ T cell	IL-15/ IL-7 / TCR	↑	- CD53 - Cytotoxicity with IL-15	IFN-γ – TNF-β – perforin – Granzym B	- Upregulation in 6 genes: two IFN-induced proteins (15- and 56-kDa proteins), two chemokine receptors (CCR1 and CCR2), CD26, and CD53.	Cells were resuspended in medium, mixed with anti-CD3 beads, immobilised on plates, or IL-15, and incubated for different time points. **Compared to α-CD3.**

31	DOI:https://doi.org/10.1016/j.celrep.2021.109438	Seo, In-Ho, Hyuk Soo Eun, Ja Kyung Kim, Hoyoung Lee, Seongju Jeong, Seong Jin Choi, Jeewon Lee et al. "IL-15 enhances CCR5-mediated migration of memory CD8+ T cells by upregulating CCR5 expression in the absence of TCR stimulation." *Cell Reports* 36, no. 4 (2021): 109438.	PBMCs CD8+ T Cell	IL-15	↑		Granzyme B / perforin	- CCR5 /ki67 /granulysin - IL-15-treated memory CD8+ T cells migrate in a CCR5-dependent manner. - CCR5 decrease in response to α-CD3.	PBMCs of healthy donors were cultured with or without IL-15 (10 ng/mL) for 48 hours. **Compared to medium/α-CD3**
32	https://doi.org/10.1182/blood.V95.10.3183	Wang, Kathy S., David A. Frank, and Jerome Ritz. "Interleukin-2 enhances the response of natural killer cells to interleukin-12 through up-regulation of the interleukin-12 receptor and STAT4." *Blood, The Journal of the American Society of Hematology* 95, no. 10 (2000): 3183-3190.	PBMCs NK cell	IL-2 / IL-12		- IL-12 induced cytotoxicity in cytokine-primed NK cells. - Increase expression of IL-12Rβ2 and reduction in IL-12Rb1.	IFN-γ	- IL-12–induced STAT4-DNA–binding activity in NK cells primed with cytokines. - STAT4/ STAT5	The NK cells were primed for 3 days with or without cytokines, and supernatants were collected on the third day. Cells were then washed and cultured with or without IL-12 for 3 days. **Compared to unstimulated cells.**
33	10.1073/pnas.09267580 1	Geginat, Jens, Federica Sallusto, and Antonio Lanzavecchia. "Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4+ T cells." *The Journal of experimental medicine* 194, no. 12 (2003): 1711-1720.	PBMCs Naive and memory CD4⁺ T cells	IL-15 ± IL-7	↑	- Higher proliferation response with memory T cell.			Cells were stimulated with a cytokine mixture (IL-7, IL-15, TNF-α, IL-6, and IL-10) for three - six days. All cytokines were used in culture at 25 ng/ml. **Compared to unstimulated cells/α-CD3**
33			Naïve CD4+ T cell	IL-15 + IL-6 + IL-10 + TNF-α	↑				

33			CD4+ naïve, T_{CM}, and T_{EM}.	TNF-α + IL-6 + IL-10	No E.			- No IL-4Rα expression on Naïve and T_{CM}. - IL-2/15Rβ on T_{CM}, T_{EM} cell.	
33			CD4+ Naive, T_{CM}, and T_{EM}	TNF-α + IL-6 + IL-10 + IL-15 + IL-7	↑	- **Naïve:** CD45RA and CCR7 - **Memory:** Downregulate CD45RA, CCR7. Upregulate CD40L and CCR5.		- Naïve: IFN-γ, IL-4 secretion. - T_{CM}: IFN-γ - ERK, and P-P38 MAP phosphorylation	
33			CD4+ Naive, T_{CM}, and T_{EM}	TNF-α + IL-6 + IL-10+IL-4	↑	- Higher response in naïve T cell		- IL-4Rα-chain on naive, but not on memory T cells both at the RNA and protein level	
34		Carey L. Shive, Joseph C. Mudd, Nicholas T. Funderburg, Scott F. Sieg, Benjamin Kyi, Doug A. Bazdar, Davide Mangioni, Andrea Gori, Jeffrey M. Jacobson, Ari D. Brooks, Jeffrey Hardacre, John Ammori, Jacob D. Estes, Timothy W. Schacker, Benigno Rodriguez, Michael M. Lederman, Inflammatory Cytokines Drive CD4+ T-Cell Cycling and Impaired Responsiveness to Interleukin 7: Implications for Immune Failure in HIV Disease, *The Journal of Infectious Diseases*, Volume 210, Issue 4, 15 August 2014, Pages 619–62	CD4+ T cells CD8+ T cells	IL-1β / IL-7	↑ ↑	Downregulate CD127 expression on CD4+ T cell. No change in the expression of CD127 on CD8 T cell		- IL-7Rα gene expression downregulated in response to IL-6 and IL-1β. - IL-1β or IL-6 Impairs T-Cell Responses to IL-7	PBMCs were purified and cultured in a complete medium with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, and 1% sodium pyruvate at 37°C and 5% CO2. PBMCs were stimulated by rhIL-6, IL-1β or IL-7. **Compared to unstimulated cells.**

			CD4+ T cells CD8+ T cells	IL-6	No E			-	

- No E = No Effect

Table 3.4. Studies on animal Isolated T Cells

No.	DOI	Author, title, journal, year published	Target immune Cell /subpopulation	Cytokine	Proliferation	Receptors expression//antigens	Cytokines	Mechanism and other effect/s gene	Protocol
35	https://doi.org/10.4049/jimmunol.176.5.2739	Pulle, Gayle, Mariana Vidric, and Tania H. Watts. "IL-15-dependent induction of 4-1BB promotes antigen-independent CD8 memory T cell survival." *The Journal of Immunology* 176, no. 5 (2006): 2739-2748.	Spleen CD8+ T cell	IL-15	↑	- Induces the expression of 4-1BB (CD137) on CD8+CD44high T cell - Induce CD69 expression on CD8+ T cell. - No expression of OX40 on CD8+ and CD4+ T cell. - OX40 induced in response to anti-CD3/CD28 stimulation.		- Activation of MAPK p38 and ERK pathways.	T cells were cultured with various doses (10–250 ng/ml) of recombinant human IL-15 or IL-7 for 2–3 days. **Compared to unstimulated cells.**
				IL-7	No E.	- Induce T cell survival. - No 4-1BB and CD69 induction.			
36	doi: 10.1016/j.ebiom.2017.04.024	Andrada, Elena, Rosa Liébana, and Isabel Merida. "Diacylglycerol kinase ζ limits cytokine-dependent expansion of CD8+ T cells with broad antitumor capacity." *EBioMedicine* 19 (2017): 39-48.	Spleen T Cell NK cell CD8+ T Cell	IL-15	↑	- Upregulation CD122		- DGKζ Limits Antigen-independent IL-15-mediated Peripheral Expansion of CD8+ T Cells. - DGKζ limits IL-15-induced STAT5 and S6 phosphorylation in CD44hi CD8+ T cells.	Splenocytes from BALB/c WT or DGKζ−/− mice were cultured in RPMI complete medium containing IL-2 (200 U/ml) or IL-15 (100 ng/ml) for 6–7 days. **Compare to α-CD3 stimulated cell.**
				IL-2	Less than IL-15				

37	https://doi.org/10.1002/eji.200939860	Tchaptchet, Sandrine, Jörg Kirberg, Nikolaus Freudenberg, Wolfgang WA Schamel, Chris Galanos, and Marina A. Freudenberg. "Innate, antigen-independent role for T cells in the activation of the immune system by Propionibacterium acnes." *European journal of immunology* 40, no. 9 (2010): 2506-2516.	Spleen Naïve T cell	IL-18 + IL-12			IFN-γ	- IFN-γ mRNA - IL-12RB2 mRNA	Murine (C57BL/6) or human CD8⁺ enriched T cells and unfractionated murine splenocytes or human PBMC were incubated with 50 ng/ml rIL-12 + rIL-18 or media alone. **Compared to unstimulated cells .**
38	10.4049/jimmunol.182.3.1429	Morris, Suzanne C., Stephanie M. Heidorn, De'Broski R. Herbert, Charles Perkins, David A. Hildeman, Marat V. Khodoun, and Fred D. Finkelman. "Endogenously produced IL-4 nonredundantly stimulates CD8+ T cell proliferation." *The Journal of Immunology* 182, no. 3 (2009): 1429-1438.	Spleen CD8⁺T cell (*In VIVO – In Vitro)*	IL-4		- Stimulates T cell proliferation *in vivo* but not in vitro.			Cells were cultured with or without IL-4 (20 ng/ml). **Compared to unstimulated cells.**
39	DOI:10.1073/pnas.0407192101	Wherry, E. John, Daniel L. Barber, Susan M. Kaech, Joseph N. Blattman, and Rafi Ahmed. "Antigen-independent memory CD8 T cells do not develop during chronic viral infection." *Proceedings of the National Academy of Sciences* 101, no. 45 (2004): 16004-16009.	Spleen CD8+ T cell	IL-7 ± IL-15	↑	- Chronic CD8+ T cell response less efficiently than acute memory CD8+ T cell. <u>Reduction in chronic compared to acute:</u> CD127, CD122, IL-15Rα, and Bcl-2 expression (142,28 compared to acute. <u>No change</u>: CD132		- Reduction pSTAT5 in chronic (50MFI) compared to acute (80MFI)	Purified CD8 T cells were mixed with naïve spleen as antigen-presenting cells. Cytokines (IL-7 or IL-15; each 5 ng/ml) or peptide (0.2 µg/ml) were added for 60 hours. **Compared to chronic CD8+ T cell**
40	https://doi.org/10.1002/1521-4141(200210)32:10<2807::AIDIMMU2807>3.0.CO;2-0	Berg, Rance E., Christoph J. Cordes, and James Forman. "Contribution of CD8+ T cells to innate immunity: IFN-γ secretion induced by IL-12 and IL-18." *European journal of immunology* 32, no. 10 (2002): 2807-2816.	Spleen CD8⁺T cell NK T cell	IL-12 + IL-18	↑		IFN-γ		Splenocytes where cultured with rm IL-12, IL-15, and IL-18 were overnight with (10 ng/ml IL-2, 5 ng/ml IL-12, and 10 ng/ml IL-18). **Compared to other cytokines**

41	10.4049/jimmunol.171.5.2437	Ilangumaran, Subburaj, Sheela Ramanathan, Jose La Rose, Philippe Poussier, and Robert Rottapel. "Suppressor of cytokine signaling 1 regulates IL-15 receptor signaling in CD8+ CD44high memory T lymphocytes." *The Journal of Immunology* 171, no. 5 (2003): 2435-2445.	Spleen Lymph Node CD8+ T cells	IL-15 / IL-2 / IL-7	↑	- Up-regulation of antiapoptotic protein Bcl-2. - SOCS1 to control cell proliferation.		- Increase IL-2Rβ - SOCS1 is a critical regulator of cytokine-induced TNF-α production in CD8+ T cells	Purified T cell subsets were stimulated with cytokines for 48 h stimulated with IL-15 (40 ng/ml), IL-2, or IL-7 (both at 1/20 dilution of the CS) for 72 hours. **Compare to unstimulated cells.**
42	https://doi.org/10.1016/j.immuni.2012.03.024	Hirahara, Kiyoshi, Kamran Ghoreschi, Xiang-Ping Yang, Hayato Takahashi, Arian Laurence, Golnaz Vahedi, Giuseppe Sciumè et al. "Interleukin-27 priming of T cells controls IL-17 production in trans via induction of the ligand PD-L1." *Immunity* 36, no. 6 (2012): 1017-1030.	Spleen Naïve CD4+ T cell	IL-27 / IL-6 / IFN-γ		- Upregulated expression of programmed death ligand 1 (PD-L1) by IL-27		- Activation of STAT1 and STAT3 by IL-6 and IL-27.	CD4+ T cells from spleens and lymph nodes were purified and stimulated with IL-6 (20 ng ml-1), IL-27 (20 ng ml-1), IFN-α (10000U ml−1), IFN-β (10000U ml−1) or IFN-γ (20ng ml-1) for 3 hours. **Compare to unstimulated cells.**
43	https://doi.org/10.4049/jimmunol.1001960	Bou Ghanem, Elsa N., Christina C. Nelson, and Sarah EF D'Orazio. "T cell-intrinsic factors contribute to the differential ability of CD8+ T cells to rapidly secrete IFN-γ in the absence of antigen." *The Journal of Immunology* 186, no. 3 (2011): 1703-1712.	Spleen CD8⁺ T cell	IL-12 + IL-18		- Upregulation of IL-12Rb2 and IL-18Ra.	IFN-γ	- IL-12 induce STAT4 activation in ~6% of splenocytes - Upregulation of IL-12Rβ2 and IL-18Ra mRNA protein expression.	CD8⁺ T cells Splenic CD8⁺ T cells or whole splenocytes harvested from either a naive BALB/c or B6 mouse were incubated with (10 and 50 ng/ml) rIL-12 plus rIL-18 or in media alone. **Compare to unstimulated cells**
44	10.1371/journal.pone.0186352	Lee, Yun Kyung, Ashley E. Landuyt, Stefani Lobionda, Panida Sittipo, Qing Zhao, and Craig L. Maynard. "TCR-independent functions of Th17 cells mediated by the synergistic actions of cytokines of the IL-12 and IL-1 families." *PLoS One* 12, no. 10 (2017): e0186351.	Spleen Lumph node CD4+ T cell (Th1 / Th17)	IL-12 + IL-18 /α-CD3			IFN-γ	- STAT4 activation	In vitro-polarised Th17 or Th1 cells or ex vivo CD4 T cells were incubated with recombinant cytokines: IL-1β (10 ng/ml), IL-6 (20 ng/ ml), IL-18 (50 ng/ml), IL-12 (10 ng/ml). Control cultures were left without additional cytokines or plated in wells coated with anti-CD3 and supplemented with soluble anti-CD28 (5 μg/ml). **Compared to unstimulated T cell or α-CD3 α-CD28 stimulated T cell.**

45	10.1371/journal.ppat.1002273	Ingram, Jennifer T., John S. Yi, and Allan J. Zajac. "Exhausted CD8 T cells downregulate the IL-18 receptor and become unresponsive to inflammatory cytokines and bacterial co-infections." *PLoS pathogens* 7, no. 9 (2011): e1002273.	Spleen memory, effector CD8+ T cell Vs exhausted CD8+ T cell	IL-12 + IL18 + IL-21		- Upregulate CD25	IFN-γ when combined.		Splenocytes were cultured in the presence or absence of recombinant murine IL-12, IL-18, and IL-21, all in concentration (20 ng/ml) or various combinations of the three cytokines. For 5.5 hours. **Compared to exhausted CD8+ T cell**
46	https://doi.org/10.4049/jimmunol.172.4.2171.	Wong, Phillip, and Eric G. Pamer. "Disparate in vitro and in vivo requirements for IL-2 during antigen-independent CD8 T cell expansion." *The Journal of Immunology* 172, no. 4 (2004): 2171-2176.	Spleen CD8+ T cell	TCR + IL-2		- IL-2 is required for CD8+ T cell proliferation in vitro but not in vivo - up-regulation of CD25.		- The addition of anti-IL-2 to the cultures significantly inhibited Ag-independent division as well as CD25 up-regulation on CD8 T cells. - Ag-independent CD8+ T cell proliferation in vivo occurs in the absence of IL-2.	T cells were coated at 30 µg/ml with anti-CD3/CD28 mAbs. rm IL-2 was added (20 ng/ml), and then T cells were transferred into new wells (not coated). T cell proliferation was analysed after 3–6 days of culture. **Compared to anti-CD3/CD28 stimulated cells.**
47	10.1073/pnas.1203543109	Freeman, Bailey E., Erika Hammarlund, Hans-Peter Raué, and Mark K. Slifka. "Regulation of innate CD8+ T-cell activation mediated by cytokines." *Proceedings of the National Academy of Sciences* 109, no. 25 (2012): 9971-9976.	Spleen CD8+ T cell memory	IL-12 + IL-18 / IL-12 + IL-15		- Increased CD69	IFN-γ		Cells were cultured with cytokines (individual or combined) with final concentration (100 ng/mL or 10 ng/mL) for six days. **Compared to medium and other cytokines**
			Spleen CD8+ T cell effector/ memory	IL-15 + IFN-a/b or IL-33 +IFN-a/b		- Increased CD69	Low INF-γ production		

48	DOI: 10.1128/mBio.01978-14	Suarez-Ramirez, Jenny E., Margarite L. Tarrio, Kwangsin Kim, Delia A. Demers, and Christine A. Biron. "CD8 T cells in innate immune responses: using STAT4-dependent but antigen-independent pathways to gamma interferon during viral infection." *MBio* 5, no. 5 (2014): e01978-14.	Spleen CD8+ T cell	IL-18+IFN-α / IL-18 /IL-12/ IFN-α and α-CD3		- CD25 expressed high with CD3 than IL-18 + IFN-α. - Low with individual cytokines IL-12, IL-18 or IFN-α.	IFN-γ	- IFN-γ high with anti-CD3 stimulated CD8+ T cell, then IL-18 + IFN-α. - Low IFN-γ secretion with IL-12, IL-18, or IFN-α	Cells were incubated under control or stimulated with cytokines (IL-12, 10 ng/ml), IL-18, 10 ng/ml and rmIFN-α (1,000 U/ml). Anti-CD3ε coated plate was used at 10 μg/ml for 24 hours. **Compared to unstimulated cells and α-CD3 stimulated cells.**
49		Lertmemongkolchai, Ganjana, Guifang Cai, Christopher A. Hunter, and Gregory J. Bancroft. "Bystander activation of CD8+ T cells contributes to the rapid production of IFN-γ in response to bacterial pathogens." *The Journal of Immunology* 166, no. 2 (2001): 1097-1105.	Spleen CD8+ T cell CD4+ T cell +	IL-12 / IL-12+IL-18+IL-2 / IL-18+IL-18+TNF/ IL-12+TNF/IL-12+IL-2/ IL-12+IL-			IFN-γ	- IFN-γ+ cell in response to IL-12 + IL-18 + IL-2 and IL-12 + IL-18 +TNF from CD8+ T cell -T cell.	Cells were stimulated with 1 ng/ml IL-12 plus 5 ng/ml IL-18 and assayed for intracellular IFN-γ-positive cells by flow cytometry for 48 hours. **Compared to unstimulated cells**
50	https://doi.org/10.4049/jimmunol.180.12.7958	Gagnon, J., Ramanathan, S., Leblanc, C., Cloutier, A., McDonald, P.P. and Ilangumaran, S., 2008. IL-6, in synergy with IL-7 or IL-15, stimulates TCR-independent proliferation and functional differentiation of CD8+ T lymphocytes. *The Journal of Immunology*, *180*(12), pp.7958-7968.	Spleen CD8+ T cell	IL-6 / IL-7 / IL-15	↑	- IL-6 stimulates proliferation in synergy with IL-7 or IL-15. - IL-6 and IL-21 individually did not induce proliferation.			Purified T cells were stimulated with cytokines IL-7 (5 ng/ml), IL-6 (1 ng/ml), or IL-15 (10 ng/ml) in medium in 96-well culture plates for 72 h, or cells were stimulated with anti-mouse CD3/CD28 Dynabeads. **Compared to medium and α-cd3 stimulated cells**

51	https://doi.org/10.1074/jbc.274.20.13744	Zhao, Jinmin, Keiko Furukawa, Satoshi Fukumoto, Masahiko Okada, Reiko Furugen, Hiroshi Miyazaki, Kogo Takamiya et al. "Attenuation of interleukin 2 signal in the spleen cells of complex ganglioside-lacking mice." *Journal of Biological Chemistry* 274, no. 20 (1999): 13744-13747.	Spleen T cell	IL-2	No E.	- No expression on IL-2Rα/β/γ		- C-myc and c-fos Gene expression - JAK/STAT5 activation	Spleen cells from mice were plated with IL-2 (1 unit/ml) and cultured for five days. **Compared to medium and α-cd3 stimulated cells**
52	doi.org/10.4049/jimmunol.173.6.3773	Anfossi, Nicolas, Scott H. Robbins, Sophie Ugolini, Philippe Georgel, Kasper Hoebe, Cécile Bouneaud, Catherine Ronet et al. "Expansion and function of CD8+ T cells expressing Ly49 inhibitory receptors specific for MHC class I molecules." *The Journal of Immunology* 173, no. 6 (2004): 3773-3782.	Spleen CD8+ T cell	α-CD3 / IL-15	↑	- IL-15 preferentially promotes the expansion of the Ly49+CD8+ T cell subset		- produce IFN-γ and TNF-α upon anti-CD3 mAb stimulation in the CD44+ CD8+ T cell	Naive cells were stimulated for 6 days in the presence of different combinations of anti-CD3ε mAb, anti-CD28 mAb at 1 μg/ml, IL-2 at 1000 U/ml, and IL-15 at 100 ng/ml. **Compared to medium and α-cd3 stimulated cells**

53	10.1073/pnas.0906988106	Guo, Liying, Gang Wei, Jinfang Zhu, Wei Liao, Warren J. Leonard, Keji Zhao, and William Paul. "IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells." *Proceedings of the National Academy of Sciences* 106, no. 32 (2009): 13463-13468.	Spleen CD4+ T cell (Th)	IL-12 ± IL-18 / IL-1 + IL-23 / IL-1+IL-6 / IL-1+IL-21		- IL-18Rα on Th1 with IL-12 + IL-18. - IL-17A secretion with IL-1 plus IL-23, IL-21, or IL-6.	IL-17A	- Elevation in IL1r1 and Rorc mRNA levels are found in Th17 cells cultured with IL-23 or IL23 plus IL-1. - Elevation in Tbet and Il18r1 mRNA with IL-12 ± IL-18	Rested Th17 and Th1 cells were cultured in cRPMI with cytokines for 24 h. **Compared to unstimulated cells.**
54	https://doi.org/10.4049/jimmunol.154.10.5061	Louahed, Jamila, Abdenaim Kermouni, Jacques Van Snick, and Jean-Christophe Renauld. "IL-9 induces expression of granzymes and high-affinity IgE receptor in murine T helper clones." *The Journal of Immunology* 154, no. 10 (1995): 5061-5070.	Clones T helper cell clone $CD4^+$ T cell	IL-9		- Induce IgE receptor	granzyme A, B	- Expression of FcεRIα mRNA on TS3 cell line in the presence of IL-9 but not IL-2. - FceRI γ mRNA expressed with IL-2	TS2 cells were cultured with IL-2 (100 U/ml) or IL-9 (200 U/ml) for 4 days.
55	10.1002/eji.1830190713	Leclair, Kenneth P., Margot M. Bridgett, Francis J. Dumont, Roger GE Palfree, Ulrich Hämmerling, and Alfred LM Bothwell. "Kinetic analysis of Ly-6 gene induction in a T lymphoma by interferons and interleukin 1, and demonstration of Ly-6 inducibility in diverse cell types." *European journal of immunology* 19, no. 7 (1989): 1233-1239.	T cell Lines	IL-1 / IFN α/β				- Increase cell surface Ly-6 antigen expression.	Cells were treated with 1000 U/ml purified murine IFN-α/β; mr IFN-γ (100 U/ml); rIL-1 (800 pg/ml). **Compared to exhausted CD8+ T cell**

56	10.1371/journal.pone.0137393	Bremser, Anna, Maria Brack, and Ana Izcue. "Higher sensitivity of Foxp3+ Treg compared to Foxp3- conventional T cells to TCR-independent signals for CD69 induction." *PLoS One* 10, no. 9 (2015): e0137393.	Spleen $CD4^+CD25^+$ T cells (Treg)	IL-33, IL-4, IL-12, TNF, IL-6, IFN-γ, α-CD3		- No effect on the level of CD69 with cytokines on T reg. - TCR stimulation increase CD69 expression on both Foxp3- and Foxp3+ cells - IL-33 induce CD69 expression on T reg Foxp3+. - No changing effect on Nur77 expression after adding IFN-α, TNF-α or IL-2, but Nur77 upregulated after TCR activation.	IFN-γ	- Cytokine activation affected neither the production of IL-10 nor the expression of CD25 on Treg.	$CD4^+CD25^+$ T cells or $CD4^+Foxp3^{RFP+}$ were sorted from $CD4^+$-enriched splenocytes and the culture medium was supplemented with 5,000U/ml recombinant murine IL-1β 1,000U/ml recombinant murine TNF-α, 1,000U/ml recombinant murine IFN-α, **Compared to unstimulated cells.**
57	Mehrotra, P.T., Grant, A.J. and Siegel, J.P., 1995. Synergistic effects of IL-7 and IL-12 on human T cell activation. *Journal of immunology (Baltimore, Md.: 1950)*, *154*(10), pp.5093-5102.	Mehrotra, P.T., Grant, A.J. and Siegel, J.P., 1995. Synergistic effects of IL-7 and IL-12 on human T cell activation. *Journal of immunology (Baltimore, Md.: 1950)*, *154*(10), pp.5093-5102.	T cell CD8 T cell. CD4 T cell	IL-7 / IL-12		- IL-7 and IL-12 together enhance T-cell proliferation. - CD8+ T cells were more responsive to IL-7 and IL-12 at lower concentrations than were CD4+ T cells	Synergistically induce IFN-γ	- Augmented the cytotoxic activity of CD8+ T cells. - Induce expression of IL-12R	Isolated T lymphocytes, or CD4+ or CD8+ T cells, were cultured overnight in a medium with a-CD3 mAb at 1 pg/ml. Cells were harvested after overnight incubation with a-CD3, washed, and recultured with different cytokines. **Compared to unstimulated cell.**

- No E = No Effect

3.5 Discussion

3.5.1 TCR-dependent activation of T cells

Proliferation and activation of T cells in a TCR-dependent (α-CD3/CD28)-manner was demonstrated in many studies (Zhao et al., 1999; Skold et al., 2018). JAK3 and PI3 kinase pathways are important in T cell proliferation, and blocking these pathways led to suppression of TCR-induced and cytokines-induced CD4+ T cell proliferation. In addition, blocking γc cytokine-dependent pathway abrogates cell proliferation in response to both TCR and cytokines stimulation. Nevertheless, Lck and MEK1/2 were key pathways in cell proliferation in a TCR-dependent way. Reciprocally, TCR and cytokine stimulation activated MAPK, but blocking this pathway inhibited cytokines-induced proliferation, not TCR-induced proliferation (Geginat et al., 2003).

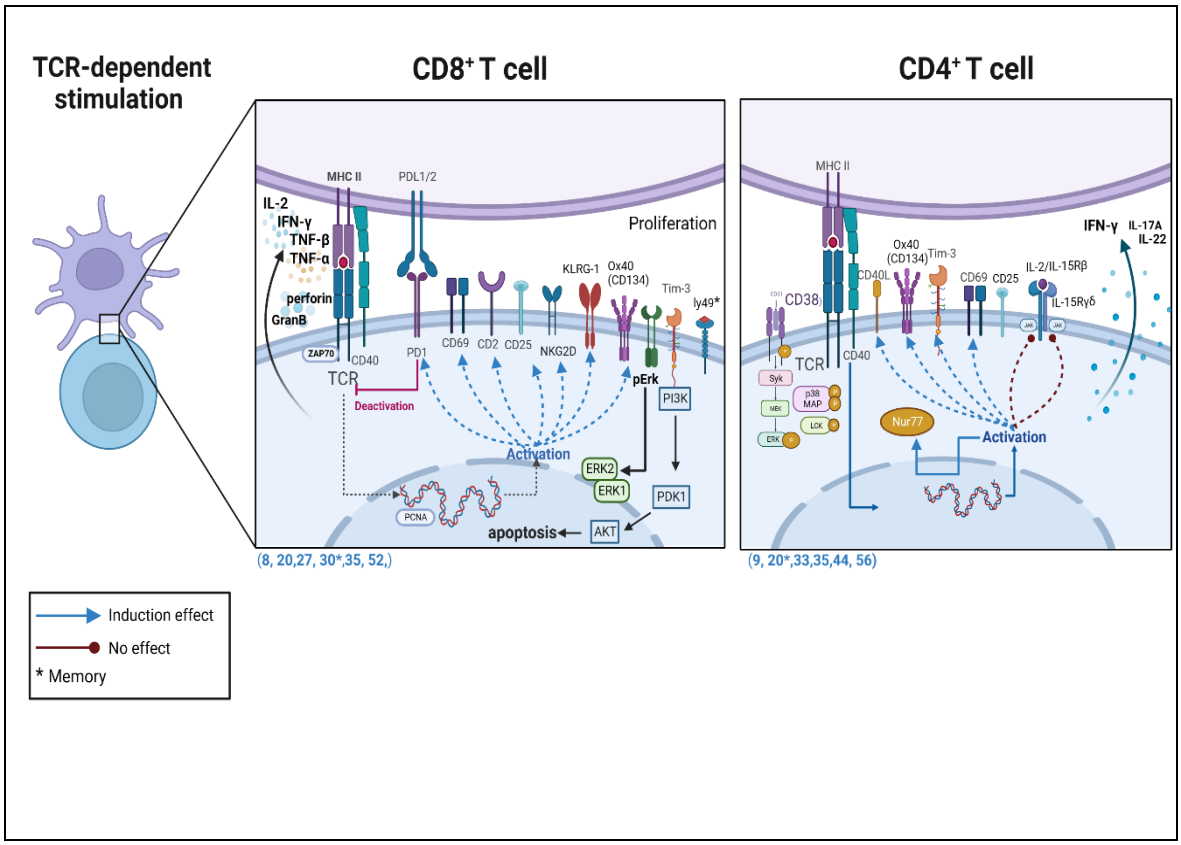


Figure 3.3. TCR-dependent activation profiles of CD4+ and CD8+ T cells. This figure illustrates the differential expression of surface markers on CD4+ and CD8+ T cells in response to TCR-dependent activation. The cytokine profiles show that CD4+ T cells predominantly release IL-17, IL-22, and IFN-γ, while CD8+ T cells release TNF-α, TNF-β, IFN-γ, and IL-2. Additionally, the figure highlights the intracellular signalling pathways activated in both T cell subsets following stimulation. **N.B.** The figure was created by the author using BioRender, based on data provided in this thesis, and is published under a license.

The immune activation marker CD40L (CD154) exhibited high expression on naïve CD4+ T cells in response to α-CD3/CD28 as a costimulatory molecule. This was accompanied by a decrease in the expression of the homing receptor CCR7, with no expression observed for CCR5 and CD45RA. As well as CD40L, the co-stimulatory molecule OX40 was expressed markedly on CD45RO CD4+ T cell along with CD38 (Pulle et al., 2006). Adding to this, a regulating transmembrane receptor called T-cell immunoglobulin and mucin domain 3 (Tim-3) was expressed highly on both CD4+ and CD8+ T cells with TCR stimulation, and under cytokines stimulation (IL-15, IL-7 and IL-2) were thereafter (Mujib et al., 2012).

In response to TCR stimulation, a programmed cell death protein (PD-1) and CD25 are expressed on CD8+ T cells, along with the secretion of IFN-γ cytokine from both CD4+ and CD8+ T cells (Tietze et al., 2012). Other cytokines like TNF-α/β and apoptotic molecules like Granzyme B and perforin were reported to be secreted from CD8+ T cells but none from CD4+ T cells (Liu et al., 2002). In addition, TCR-stimulation can lead to IL-17F, IL-17A and IL-22 secretion from CD4+ T cells (Lee et al., 2017), along with activation markers CD69 and nur77 (Bremser et al., 2015). Although CD69 was shown to be expressed on T cells following the exposure to TCR agonist molecules or cytokines, Nur77 was only present on TCR-stimulated cells (Figure 3.3).

3.5.2 TCR-independent activation of T cells

3.5.2.1 Proliferation and proliferation-dependent pathways

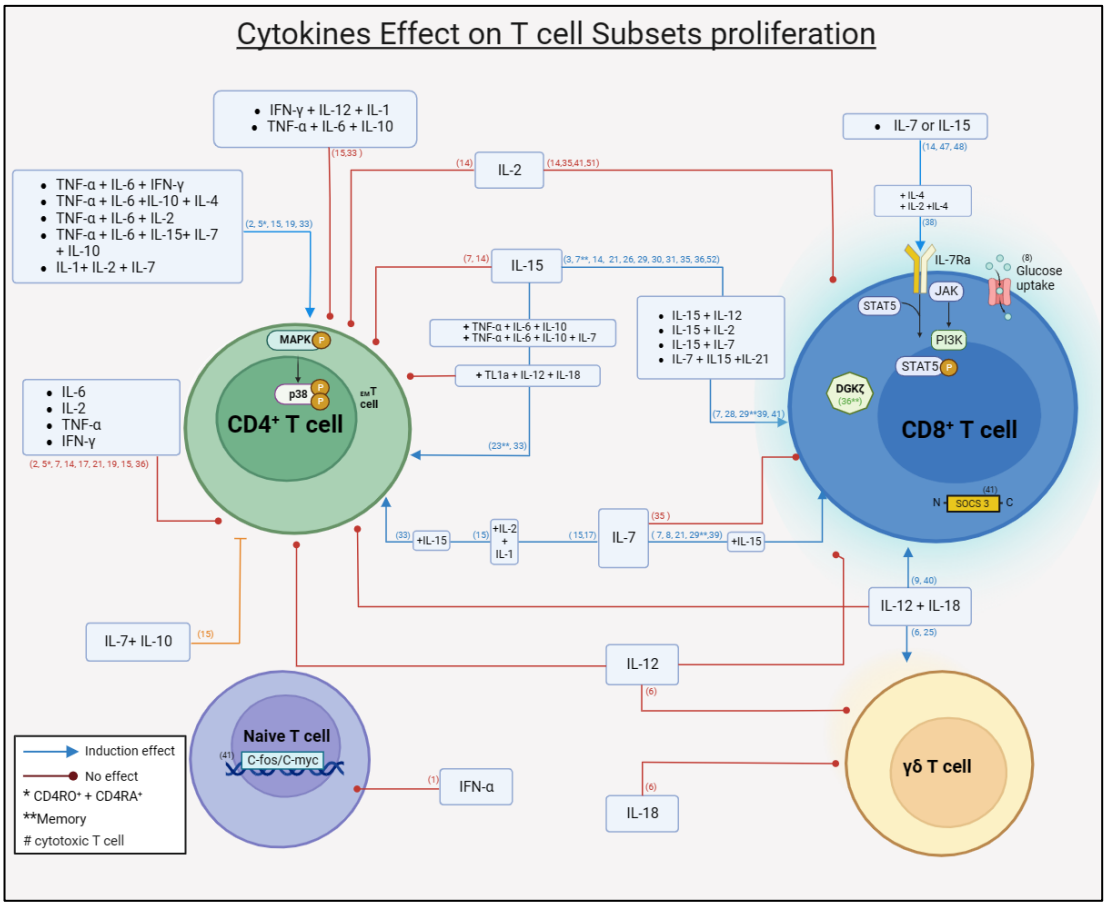


Figure 3.4. Effect of various pro-inflammatory cytokines on the proliferation of T cell subsets (naïve, CD4+, CD8+, and γδ T Cells).
This figure illustrates the proliferation response of different T cell subsets—Naïve, CD4+, CD8+, and γδ T cells—when stimulated with various pro-inflammatory cytokines. The figure also highlights the intracellular signalling pathways involved in mediating these proliferation responses across the different T cell subsets. **N.B.** The figure was created by the author using BioRender, based on data collected from the systematic review presented in this chapter, and is published under a license.

3.5.2.1.1 IL-15

IL-15 is one of the γ chain cytokines essential for cell survival and proliferation. Most studies demonstrated the effect of IL-15 alone on the CD8+ T cell population to induce a distinct proliferation response. IL-15 shows a proliferative response of human and murine CD8+ T cells and the enhancement contribution in augmentation T cell viability (Alves et al., 2003; Pulle et al., 2006; Weber et al., 2006; Lu et al., 2002) (Figure 3.4). In human studies, peripheral and umbilical cord naïve CD8+ T cells and their subtypes, like cytotoxic (CD27-CD45RA^{bright}) and noncytotoxic (CD27+ CD45RA$^{dull/neg}$) CD8+ T cells,

showed similar proliferation responses to IL-15 alone compared to Ag-independent cell culture (Alves et al., 2003; Lu et al., 2002). However, comparing the response of peripheral blood (PB) to umbilical cord blood (UCB), the latter demonstrated a higher proliferation capacity in the presence of different cytokines combinations (IL-15 + IL-7 with/Without IL-21+IL-12) among $CD8^+$ T cell population (Kwoczek et al., 2018). However, not all cytokines show a synergetic effect in combination with IL-15. In the presence of IL-15, co-stimulation of T cells with IL-2 or IL-7 did not exhibit an additional proliferative effect, compared to IL-12, which enhanced the proliferation of CD8+ T cells by 25%; and this effect was not observed among the $CD4^+$ cells (Lu et al., 2002; Bastidas et al., 2014). Among the CD4+ T cell population, no proliferation was even detected with IL-15. Nevertheless, CD4+ T cell subsets show the differential requirement for cytokines-induced proliferation. Combined cytokines: IL-15 + IL-7 or IL-15 + TNF-a + IL-6 could change $CD4^+$ cell subtype proliferation response (Figure 3.4). IL-15, together with IL-7, induce the proliferation of both effector memory T_{EM} and central memory T_{CM} CD4+ subset only, whereas TNF + IL-6 + IL-7 + IL-10 with IL-15 induce naive and memory CD4+ T cells proliferation response (Lu et al., 2002; Bastidas et al., 2014; Geginat et al., 2003). In addition, IL-15 and Tumor Necrosis Fctor-like cytokine 1A (TL1a) induce proliferation of $IL\text{-}18^+Ra^+$ $CD4^+$ T cell subtype (activated previously with IL-12/IL-18), while no significant cell division was observed with TL1a cytokine in the absence of IL-15 (Holmkvist et al., 2015). On skin resident T cells, IL-15 stimulation induces a maximal production of $CD25^{hi}$ $CD69^{lo}$ on T cells at day 21 of the cell culture (Clark and Kupper, 2007). *Domae et al* reported that 1 ng/mL of IL-15 was enough to show an enhancement effect on IL-12/IL-18-activated Vγ9Vδ2 T cells by increasing the proliferation 30% more compared to IL-12/IL-18 stimulated T cells (Domae et al., 2017).

Bystander activation was also studied on different T cell life stages, where isolated T cells from chronic and acute infected mice responded differently to cytokine stimulation. T cells from acutely infected mice exhibited efficient cell proliferation, unlike those from chronically infected mice, where they failed to go through more than one division (Wherry et al., 2004). In addition, the Ly49+ CD8+ T cell subset was reported to increase with mice age and proliferate to IL-15 stimuli compared to naïve and Ly49- CD8+ T cells (Anfossi et al., 2004). In some studies, CD8+ T cell proliferation was assessed by stimulating PBMCs with IL-15, which also showed that proliferation was high among T cell-expressed Tim-3 co-inhibitory receptors (Mujib et al., 2012). Moreover, atherosclerotic plaque-derived T cells significantly respond to IL-15, and this response was demolished upon anti-IL-15 inhibitor supplementation (Houtkamp et al., 2001). The proliferation was selectively

apparent among memory T cells in both CD4+ and CD8+ subsets, in particular, T_{EM} cells in the human model, and this corresponded with the expression of proliferating cell nuclear antigen (PCNA) (Liu et al., 2002; Abdelsamed et al., 2017; Geginat et al., 2003). In addition, the Ki-67 nuclear antigen, a marker of active cell proliferation, was high among the IL-15-treated cell population (Younes et al., 2016).

Regulation of cell proliferation occurs through two different signals: Suppressor of cytokine signalling 1 (SOCS1) and Diacylglycerol Kinase ζ (DGKζ), which showed a restrictive effect on T cell proliferation. In $SOCS1^{-/-}$ and $DGK\zeta^{-/-}$ mice, $SOCS1^{-/-}$ and $DGK\zeta^{-/-}$ CD8+ T cells exhibit a hyperproliferative response to IL-15 stimuli whilst there was less proliferation among $SOCS1^{+/+}$ and $DGK\zeta^{+/+}$ T cell (Ilangumaran et al., 2003; Andrada et al., 2017).

3.5.2.1.2 IL-12 and IL-18

IL-12 and IL-18 are frequent cytokines that are mostly combined to stimulate T cells in a bystander manner. Studies show that they can stimulate the proliferation of in *ex vivo* expanded γδ T cells synergistically. Another study showed (Schilbach et al., 2020) that isolated human γδ T cells could respond and proliferate to IL-12 and IL-18 with and without previous TCR activation. On the contrary, freshly isolated human CD4+ and γδ T cells demonstrate no proliferation response upon cytokines stimulation (Munk et al., 2011; Bastidas et al., 2014; Domae et al., 2017). Neither IL-12 nor IL-18 could stimulate the proliferation of γδ and CD4+ T cells individually (Bastidas et al., 2014; Domae et al., 2017) (Figure 3.4). However, $IL\text{-}18R\alpha^{+}CD4^{+}$ T cells subset proliferated only when IL-12 and IL-18 were combined with IL-15 or IL-15 and TL1a (Holmkvist et al., 2015). Alongside that, CD8+ T cells and NK cells showed a similar response to IL-12 and IL-18, where both exhibit a selective proliferation to IFN-γ^{+} T cells (Berg et al., 2002).

3.5.2.1.3 IL-7

IL-7 has a controversial proliferation effect on T cells. Splenic CD8+ T cells in mice failed to enter the proliferative state even when exposed to a relatively high concentration of 250 ng/ml of IL-7. Conversely, at a lower concentration of (10ng/ml), IL-7 demonstrated a distinct enhancement in cell viability (Pulle et al., 2006). The same results were observed among human CD8+ and CD4+ T cell subsets with 18.9% ± 1.1% and 4.4% ± 1.1% of the proliferated cell, respectively. In contrast, Weber et al. (2006) and Crawley *et al.* studied the effect of a wider range of IL-7 concertation (10 – 50,000 pg/ml) on human CD8+ T

cells, determining that a minimum of ≥5000 pg/ml was required to stimulate cell division (Weber et al., 2006; Crawley et al., 2014). Additionally, memory CD8+ T cells from acutely infected mice's spleen responded to (5 ng/ml) of IL-7 and proliferated with around 53%, which was not observed in chronic CD8+ T cells (Wherry et al., 2004; Fukui et al., 1997).

Metabolic activity, known to precede cell proliferation measured by Glucose uptakes, increases significantly in T cells after using a high concentration of IL-7 treatment. Induction of glucose uptakes on CD8+ T cells depends on JAK/STAT5 and PI3K signalling, and inhibiting these pathways was associated with a reduction in T cell proliferation (Crawley et al., 2014). IL-7 elicited proliferation responses from both CB and PB CD4+ T cells. Nevertheless, CB cells demonstrated a notably higher response compared to adult PB cells (Kwoczek et al., 2018; Geginat et al., 2003), and no changes in cell response were noticed with the addition of anti-IL-2 to cell culture. In a distinct context, IL-7-induced Th2 cell proliferation was contingent on its combination with IL-33 but not IL-33 alone. This combination led to the upregulation of T1ST2 and activation of STAT5 (Guo et al., 2009).

3.5.2.1.4 IL-6, TNF, and IL-2 / IL-4

Stimulating T cells in macrophage-depleted PBLs with IL-6 or TNF alone showed no proliferation response. However, they proliferate significantly when IL-2 is combined with IL-6 or TNF (Martino et al., 1998). In line with this, naive CD45RA+ T cells respond to TNF-α only in the presence of IL-6 + IL-2, unlike CD45RO+ T cells, where the cells respond to TNF in the presence of either IL-2 or IL-2 + IL-6 (Unutmaz et al., 1994). In addition, priming naïve T cells with IL-6 + TNF-α demonstrated higher proliferation after α-CD3 stimulation than un-primed T cells (Vakkila et al., 2001).

On CD4+ T cells, the presence of IL-2 with TNF-α and IL-6 together induces higher cell division than IL-4 with TNF-α and IL-6. However, IL-4 was an excellent survival cytokine for the cells (Unutmaz et al., 1995). IL-6 studied on human CD4+ T cell in combination with (IL-7+ IL-15 + TNF-α + IL-10) and with (TNF + IL-10). Their response was different as a result of the accompanying cytokines. CD4+ T cells were not capable to proliferate with TNF and IL-6 in the presence of IL-10, whereas adding IL-7 and IL-15 to (TNF + IL-6 + IL-10) induced the proliferation significantly and demolished IL-10 proliferation inhibitory influence (Geginat et al., 2003). The P38 MAPK pathway is one of the pathways that are necessary for cytokines-dependent proliferation along with the ERK MAPK

pathway, which is activated at a weaker level than P38 MAPK. A combination of IL-7, IL-15, TNF-α, IL-6, and IL-10 cytokines induces an activation effect of the P38 MAPK pathway on CD4+ T cell (Geginat et al., 2003) (Figure 3.4). In Multiple Sclerosis patients, a distinct pathway was activated in response to IL-6 and TNF-α combination, inducing intracellular Ca2+ elevation via 2-independent pathways (Martino et al., 1998).

3.5.2.1.5 IL-2 and IL-4

IL-2 cytokines exhibited a slight proliferation effect on T cells in PBLs from healthy donors (Martino et al., 1998). Conversely, studies on resting CD45RA+ CD45RO+ T cells showed no proliferation in both subsets with IL-2, even when combined with IL-6 or TNF-α (Unutmaz et al., 1994; Ilangumaran et al., 2003; Andrada et al., 2017; Bastidas et al., 2014). This response was consistent among CD8+ CTLs and CD4+ T-helper clones (Lu et al., 2002). Furthermore, no additive effect from IL-2 was observed on CD4+ and CD8+ T cells when combined with IL-15 (Bastidas et al., 2014). Studies comparing the effects of IL-2 or IL-4 combinations with TNF-α and IL-6 in CD4+ T cells revealed that IL-2 + IL-6 + TNF-α induced higher proliferation than IL-4 + IL-6 + TNF-α, with no proliferation detected with IL-2, IL-4, IL-6, or TNF-α alone. However, IL-4 acted as a survival factor in cell culture (Unutmaz et al., 1995). Noteworthy, IL-2 showed no induction of proliferation in γδ T cells (Schilbach et al., 2020). Although IL-2 was found necessary for Ag-independent proliferation in vitro, this effect was not observed in vivo (Wong and Pamer, 2004).

3.5.2.1.6 IFNs

IFN-α has a reduction effect on T cell proliferation after stimulating PBL with (1, 10 and 100 ng/ml), even in the presence of α-CD3/CD28 (Skold et al., 2018). In addition, IFN-γ-stimulated PBL had demonstrated a similar effect on CD4+ T cells. IFN-γ showed an additive effect with TNF, IL-6 and IL-2 on T cell proliferation (Martino et al., 1998).

3.5.2.2 Cell markers expression and pathways involved.

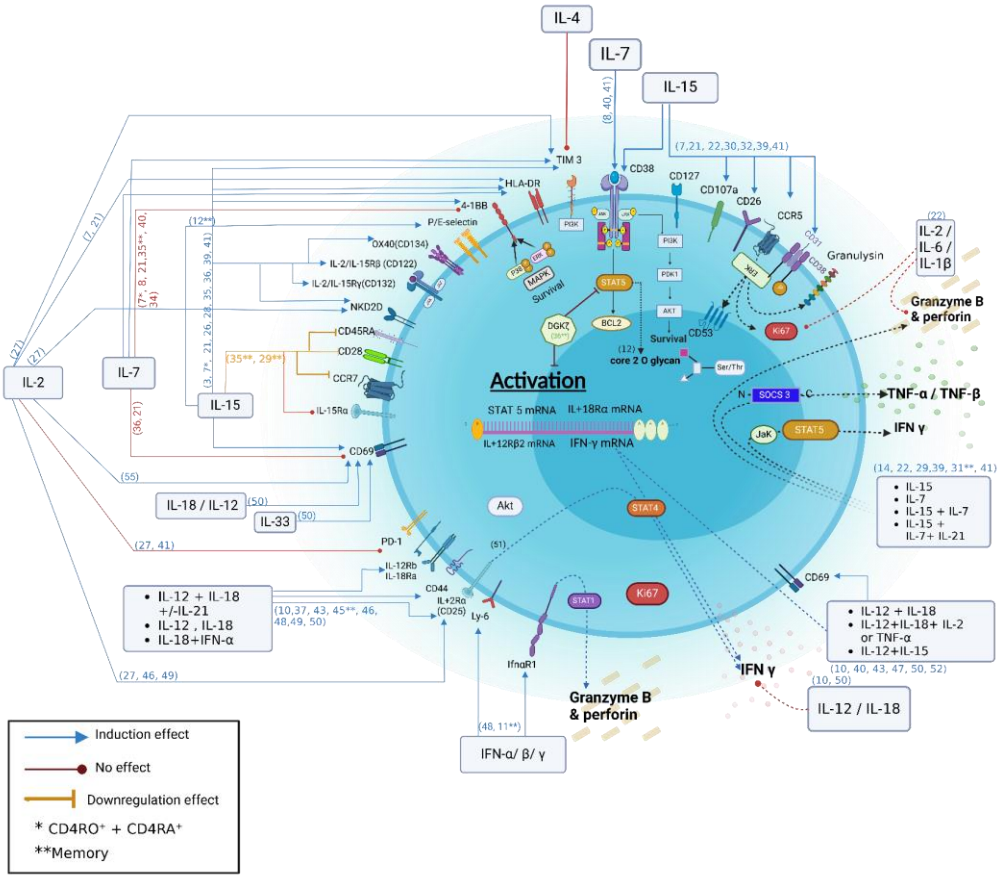


Figure 3.5. Surface markers expressed on CD8+ T cells in response to cytokines.

This figure demonstrates the expression of surface markers on CD8+ T cells following stimulation with various cytokines. It also details the cytokines released by the CD8+ T cells and the intracellular signalling pathways activated as a result of the cytokine stimulation. **N.B.** The figure was created by the author using BioRender, based on the data collected from this systematic review, and is published under a license.

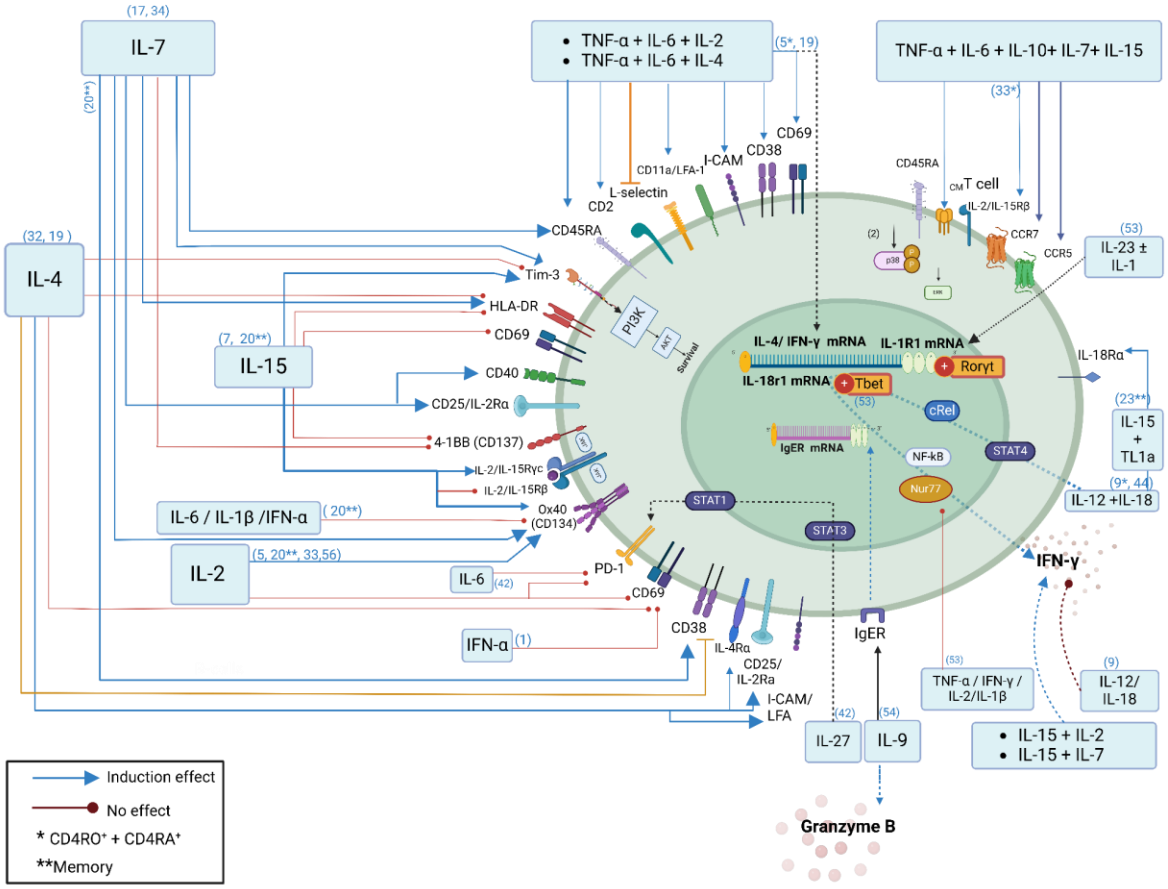


Figure 3.6. Surface marker expression on CD4+ T cells in response to different cytokine stimulation.

This figure illustrates the expression of surface markers on CD4+ T cells in response to different cytokines. Additionally, it shows the cytokines released by the CD4+ T cells and the signalling pathways that are induced upon cytokine stimulation. N.B. The figure was created by the author using BioRender, based on the data collected from this systematic review, and is published under a license.

3.5.2.2.1 IL-15

4-1BB, OX40 and HLA-DR surface receptors markers and MHC class II molecules were found on IL-15-activated T cells, playing crucial roles in T cell activation, co-stimulation, and the regulation of immune responses. Evidence supported the role of 4-1BB, a member of the TNF-α receptor family, in the maintenance of T cell response after activation and its involvement in survival signalling (Hurtado et al., 1997; Cannons et al., 2001; Pulle et al., 2006). Through the MAPK pathway, the expression of 4-1BB on T cells is induced in response to IL-15 (Pulle et al., 2006). After treating splenic T cell subsets (CD8+ and CD4+) with low (20 ng/ml) and high (100 ng/ml) doses of IL-15 for 2-3 days, expression of 4-1BB was detected on CD8+ T cell surface, especially among memory phenotype T cell. In contrast, no expression was detected on the CD4+ T cell surface (Pulle et al.,

2006). It was demonstrated that OX40, a costimulatory molecule that conveys activating signals to T cells, was absent on both CD4+ and CD8+ murine T cell surfaces after the IL-15 activation (Pulle et al., 2006). Another study showed the contrary, where OX40 was present in the human stimulated-CD4+ T cells (Jiang et al., 2014). LA-DR+ and CD38+ were expressed on CD8+ T cells but not CD4+ subsets after IL-15 stimulation (Figure 3.5) (Bastidas et al., 2014).

In addition, mRNA and surface expression of IL-15Rβ was expressed at higher levels on naïve, effector and central memory, cytotoxic (CD27-CD45RA^{bright}) and noncytotoxic CD8+ T cells (CD27+CD45RAdull/neg) than CD4+ T cell isolated from UCB or PBMCs. IL-15Rα, was not detectable on all previously mentioned subsets (Alves et al., 2003; Geginat et al., 2003; Bastidas et al., 2014).

Markers like CD69, CD11b, ICAM-1, LFA-1, and CD40L are elevated after treating human T cells with IL-15 + IL-6 + TNF-α (Sebbag et al., 1997; Weber et al., 2006). Many studies report the induction of cytotoxic function, secretion of granzyme B and perforin and IFN-γ production by CD8+ T cells upon IL-15 stimulation (Alves et al., 2003; Liu et al., 2002; Younes et al., 2016; Seo et al., 2021; Freeman et al., 2012). IFN-γ production robustly increases with the addition of more than one cytokine (Kwoczek et al., 2018) (Figure 3.5). The level of production IFN-γ and TNF-α from CD8+ T cells upon α-CD3 stimulation was demonstrated to be increased when α-CD3 combined with cytokines such as IL-15 (Anfossi et al., 2004; Munk et al., 2011).

On CD8+ memory T cells, IL-15 could regulate genes like *Gcnt1, which encodes* the enzyme initiating core 2 O-glycan synthesis. A core 2 O-glycan is a molecule that is usually attached to P- E- selectin and has a role in modulating trafficking to inflamed tissues. In addition, other genes like U2 small nuclear RNA auxiliary factor 1like 4 (U2af1l4) was downregulated, and growth factor independent 1 (Gfi1) gene expression was upregulated in response to IL-15 (Nolz and Harty, 2014; Yurova et al., 2016). The Gfi1 gene is involved in T cell development and T cell receptor signalling and potentially regulates effector functions and exhaustion. U2af1l4 gene, as a splicing factor, may modulate alternative splicing events in T cells, potentially impacting T cell activation, differentiation, and signalling pathways (Yurova et al., 2016).

During the chronic CD8+ T cell expansion, the expression of IL-15β receptor, the antiapoptotic molecule Bcl-2, and STAT5 phosphorylation were reduced upon IL-15

treatment when compared to acute CD8+ T cell (Pulle et al., 2006). Furthermore, diacylglycerol kinases (DGK) constitute a family of enzymes responsible for metabolising diacylglycerol generated upon T lymphocyte antigen recognition. Specifically, diacylglycerol kinase ζ was identified as capable of restraining IL-15-triggered T cell proliferation, STAT5 phosphorylation, and the expression of CD122 while not affecting CD25 and IL-15Rα levels (Andrada et al., 2017). Additionally, IL-15 upregulates CCR5 on memory CD8+ T cells without TCR stimulation via ERK signalling pathways (Seo et al., 2021).

3.5.2.2.2 IL-12 + IL-18

In response to IL-12 and IL-18 together (not individually). CD25 and ICAM-1 were reported to increase on γδ T cells significantly. On the contrary, B- and T-lymphocyte attenuator (BTLA), a protein belonging to the CD28 immunoglobulin superfamily, downregulated with IL-18 or IL-12+IL-18 combined but no change in the expression with IL-12 alone. Other activating markers for tumer cell recognition, like DNAX Accessory Molecule-1 (DNAM-1) and NKG2D, also upregulate on γδ T in response to IL-12 or IL-12 and IL-18 combined (Figure 3.7). In addition, IFN-γ production was not detectable when γδ T cells were treated with IL-12 or IL-18 separately and were markedly high when combined (Domae et al., 2017; Schilbach et al., 2020). Combining IL-18 with IL-10 suppresses IFN-γ secretion (Domae et al., 2017; Freeman et al., 2012). The expressions of IL-18Rα and IL-12Rβ2 on γδ T cells are significantly heightened upon stimulation with IL-12 and IL-18, working synergistically. Interestingly, IL-18Rα showed a slight increase with IL-12 alone, whereas IL-12Rβ2 is upregulated with IL-18 alone (Domae et al., 2017) (Figure 3.7).

On CD8+ T cells, IL-12R and IL-18R expression upregulated with Cytokine-driven bystander activation, occurring in both the CD122lo and CD122hi subsets of memory phenotype CD8+ T cells (Bou Ghanem et al., 2011; Ghanem et al., 2011), and IFN-γ also produced from CD8+ with IL-12 and IL18, in present and absent of IL-21 (Ingram et al., 2011; Ghanem et al., 2011) (Figure 3.5). On the IFN-γ+-secreted CD8+ subset, CD62L, CD44 and Ly6C expression were significantly high between (62 – 92%) (Berg et al., 2002). However, no IFN-γ was detected on exhausted CD8+ T cells (Ingram et al., 2011). Similar to CD8+ T cell, IFN-γ induced gradually on CD4+ T cell with increasing concentrations of IL-12 and IL-18, which aligns with elevation in the mRNA expression for IFN-γ (Munk et al., 2011). On the Th cell, IL-18 acts synergistically with IL-23 and IL-

12 on the TH17 cell to induce the production of IFN-γ and IL-17A cytokines in a STAT3-dependent manner (Bremser et al., 2015).

Eomes and T-bet transcription factors are critical for IFN-γ production on γδ T cells. They were expressed on ex vivo expanded γδ T cells after IL-12 and IL-18 stimulation (Domae et al., 2017), but not induced on freshly isolated γδ T cells (Schilbach et al., 2020). In addition, the IκBζ pathway plays an important role in IFN-γ secretion. Silencing IκBζ suppresses the production of IFN-γ on γδ T cells with IL-12 and IL-18 (Domae et al., 2017). Furthermore, activation of NF-κB p65 in γδ T cells in response to IL-12 and IL-18 was independent of IκBζ expression.

Although IL-12 alone did not elevate the expression of HLA-DR and CD38 on CD4+ and CD8+ T cells, it exhibited an additive effect with IL-15 on the expression of these activation markers specifically on CD8+ T cells (Bastidas et al., 2014), showing selective activation among CD8+ T cell (Bastidas et al., 2014). Among Foxp3-CD4+ T cells subsets, no CD69 expression on IL-12-treated T cells. CD25 expression and IFN-γ induction on both CD4+ and CD8+ T cells in response to IL-12 and IL-18 was induced in a STAT4-dependent pathway (Munk et al., 2011; Bou Ghanem et al., 2011; Suarez-Ramirez et al., 2014). As well as STAT4, cRel transcription factor and NFκB/Rel family member, were activated in IL-12/IL-18-induced IFN-γ transcription (Munk et al., 2011).

Along with T cell, NK cell treated with IL-12 with/wo IL-2 induce secretion of IFN-γ, expression of IL-12 receptors (IL-12RB1- IL-12RB2) and activate STAT4 pathway (Wang et al., 2000; Berg et al., 2002). Among different immune cells subpopulations treated with IL-12 and IL-18, the production of IFN-γ was highest on NK cell then CD8+ T cell, CD4+ T cell and γδ T cell after that (Berg et al., 2002).

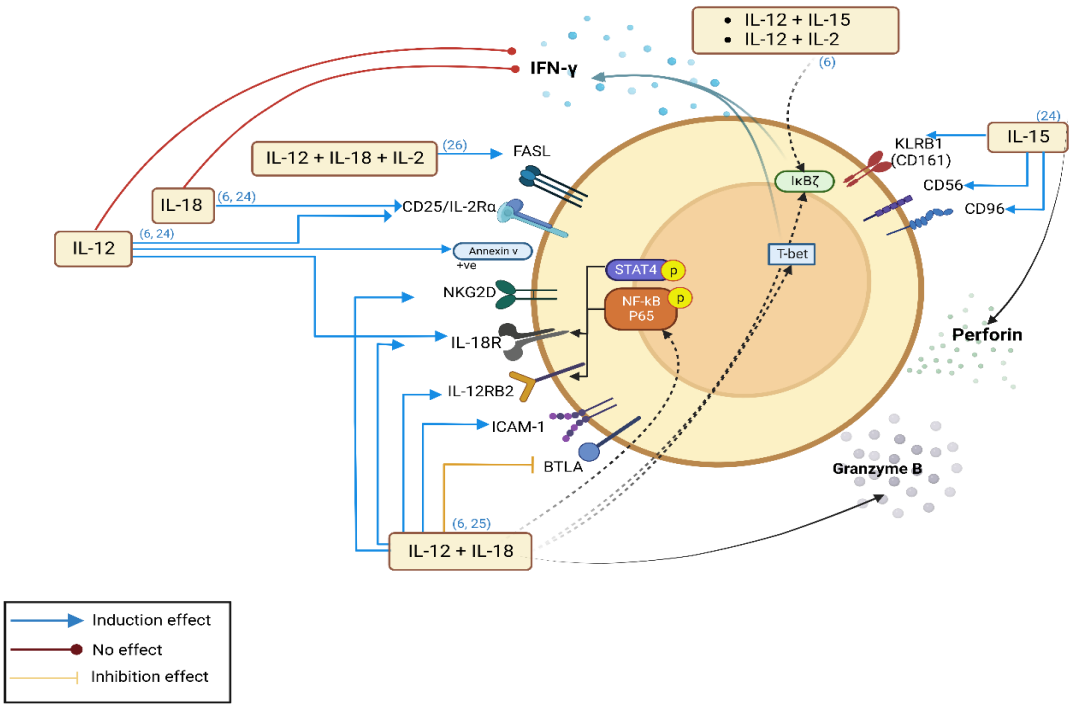


Figure 3.7. Surface marker expression on γδ T cells in response to various cytokines stimulation.

This figure illustrates the expression of surface markers on γδ T cells following stimulation with various cytokines. It also details the cytokines released by the γδ T cells and the intracellular signalling pathways activated as a result of the cytokine stimulation**. N.B.** The figure was created by the author using BioRender, based on data collected from this systematic review, and is published under a license.

3.5.2.2.3 IL-7

Unlike IL-15, IL-7 with 20 or 100ng/ml did not induce 4-1BB expression on CD8+/CD4+ T cells. Interestingly, even with the increase in the cell size and granularity of cells in the side scatter/ forward scatter/profile, CD69 expression was not detected on CD8+ T cells in mice. However, in healthy human PBMCs, CD69 and HLA-DR activation markers were expressed on IL-7-stimulated CD8+ T cells (Weber et al., 2006). IL-7 induces the expression of markers like OX40 and Tim-3 on CD4+ T cells (Jiang et al., 2014). IL-7 shows capability to induce cell cycling, Ki-67, CD25, granzyme B and perforin production but less than IL-15 (Younes et al., 2016; Fukui et al., 1997). Bcl-2 and perforin production were highly expressed in CD8+ T cells, depending on the activation of the STAT5 pathway (Crawley et al., 2014). INF-γ was reported to be also produced from IL-7-treated CD4+ T cells (Fukui et al., 1997). IL-7 with IL-33, but not IL-33 alone, exhibit T1ST2 upregulation and STAT5 activation on CD4+ T cells (Guo et al., 2009).

3.5.2.2.4 IL-6 and TNF-α

Naïve T cell remains CD45RA+ expression on the cell surface after activation with TNF-α + IL-6 + IL-2 or TNF-α + IL-6 + IL-4 cytokines combinations (Unutmaz et al., 1995; Geginat et al., 2003). On CD4+ T cells, CD69 expression was observed upon activation with the combination of TNF-α, IL-6 and IL-2, while it was not detected with the combination of TNF-α, IL-6, and IL-4. Various markers such as IL-2R, VLA-2, ICAM-1, CD2, CD29, LFA-1, LFA-3, and CD38 were upregulated on the cell surface in response to combinations of TNF-α with IL-6 and either IL-2, IL-4, or IL-15. However, neither HLA-DR nor CD39 were expressed (Sebbag et al., 1997; Unutmaz et al., 1995). L-selectin dramatically downregulated upon TNF-α + IL-6 + IL-2 stimulation and was only a slight decrease in response to TNF-α + IL-6 + IL-4. Receptors like IL-2/IL-15Rβ and γc-chain were expressed highly on T_{EM}, intermediate level on T_{CM} and low on naïve with no expression for IL-4Rα on all T cell subsets cultured with TNF-α, IL-6, and IL-10.

After exposure to IL-7, IL-15, TNF-α, IL-6, and IL-10, naïve CD4+ T cells maintained CCR7 expression, while central memory CD4+ T cells showed decreased CCR7 and increased CCR5 expression. Both naïve CD4+ T cells and central memory cells secreted IFN-γ and IL-4 in response to this cytokine combination (Geginat et al., 2003). The combination of TNF-α with IL-12 and IL-18 led to a higher population of IFN-γ+ CD8+ T cells compared to IL-12 alone, with CD8+ T cells expressing more intracellular IFN-γ than CD4+ T cells (Lertmemongkolchai et al., 2001). Incubation of naïve T cells with IL-6 + TNF-α + IL-2 for 100 hours resulted in an increase in IL-4 and IFN-γ mRNA expression (Unutmaz et al., 1994).

IL-6 did not affect PD-L1or OX40 on CD4+ T cells (Hirahara et al., 2012; Jiang et al., 2014) and Granzyme B or Ki-67 were not expressed on CD8+ T cells in response to IL-6 alone (Younes et al., 2016). Nur77, a gene involved in T cells cycle mediation, did not show modulation in response to TNF and IL-1 despite high CD69 expression (Bremser et al., 2015).

3.5.2.2.5 IL-2 and IL-4

Upon exposure to IL-4, the IL-4 cytokine receptor (IL-4Rα) was upregulated on naïve cells but not memory cells, while the γ chain receptor was downregulated on both subsets. (Geginat et al., 2003). In response to IL-4, markers like: ICAM-1, LFA-3, CD2, LFA-1, CD29, were all upregulated while no expression changes were detected on CD69, IL-2R,

VLA-2, HLA-DR, and CD39, In contrast, CD38, and L-selectin downregulated on naïve CD4+ T cell (Unutmaz et al., 1995). IL-4 does not affect Tim-3, regulating T cell tolerance and expression on CD4 and CD8+ T cells (Mujib et al., 2012).

3.5.2.2.6 IL-1 and IFNs

Various cytokine combinations have distinct effects on T cell responses, as observed in studies exploring their impact on T cell markers and functions. Surface expression of lymphocyte antigen 6 (Ly-6) activation protein was induced in response to IL-1 and IFN-α/β (LeClair et al., 1989). Conversely, IL-1β or IFN-α did not elicit Ox40 expression on CD4+ T cells (Jiang et al., 2014). Furthermore, combining IL-1β with IL-2 failed to induce granzyme B or Ki-67 expression on CD8+ T cells (Younes et al., 2016). Notably, there was a significant elevation in IL1R1 and Rorc mRNA expression in Th17 cells when cultured with IL-1 plus IL-23. In contrast, in Th2 CD4+ cells, the combination of IL-1 with IL-23 + IL-21, and/or IL-6 induced the secretion of IL-17A (Guo et al., 2009). Interestingly, Th1 cells secreted IL-17A only when exposed to IL-6 combined with IL-1, whereas IL-6 alone did not induce the same response (Guo et al., 2009).

3.6 Conclusion

In conclusion, our systematic review underscores a distinct disparity in phenotypic responses between T cell stimulation through the TCR and cytokines despite both inducing activation and proliferation effects. Cytokine stimulation induces quantitatively and qualitatively distinct phenotypic changes on T cells compared with those induced by TCR signalling (Gagnon et al., 2008). Notably, the observed effects are highly dependent on the specific cytokines or combinations thereof and the T cell subtypes that were under investigation. While most studies focused on T cell responses, variations in effects were evident based on T cell subtypes, particularly in memory or effector cells.

The complexity of cytokine combinations became apparent, with different effects observed on specific T cell subtypes. Some cytokines demonstrated minimal impact individually; however, they exhibited additive effects when synergising with others. Furthermore, the distinction between in vivo and ex vivo responses highlighted the dynamic nature of cytokine effects. Certain cytokines failed to express their effects ex vivo but were impactful in an in vivo setting, and vice versa. This emphasises the need to consider the microenvironment in different experimental contexts and the dynamic conditions in vivo, including various cellular interactions, signalling molecules, and tissue-specific factors, which can significantly impact cytokine stimulation outcomes.

Furthermore, the diverse effects of cytokines on T cell activation, proliferation, and marker expression were evident, influencing activation markers, adhesion molecules, and cell surface receptors. Notably, the source of T cell subtypes, whether from PBMCs, cord blood CB or spleen, played a significant role in determining the response to cytokine stimulation. These findings collectively underscore cytokines' intricate and various role in modulating T cell behaviour, providing valuable insights into the nuanced regulation of immune responses. Together, stimulation through TCR or cytokines had shown an apparent disparity in pathway and phenotypic response, although both have an activation and proliferation effect.

Chapter 4 The Effect of Cytokines on T Cell and Receptor Expressions on Canonical T Cell Activation in the Context of Ang II-induced Hypertension

4.1 Introduction

T cell activation is crucial in the adaptive immune response and is pivotal in defending the host against various foreign bodies. T cells proliferate and differentiate into effector subsets that orchestrate the immune response by recognising specific antigens presented by MHC on the APC and the co-stimulatory signal (Alegre et al., 2001). Dysregulated T cell activation is involved in the pathogenesis of various inflammatory and autoimmune diseases, as well as activated T cells were found to accumulate in multiple cardiovascular and autoimmune diseases.

The investigation started in 2007 in Guzik *et al.'s* study demonstrated that T lymphocytes are essential determinants of hypertension and the necessity of T cells for developing Ang II-induced hypertension in mice (Guzik et al., 2007). Since then, extensive efforts were made to unravel T cell-mediated mechanisms involved in blood pressure regulation. Researchers focused on identifying pathogenic T cell subtypes in relevant tissues, particularly the kidneys and vasculature, aiming to understand the underlying disease pathways. Renal biopsies from patients with hypertensive nephrosclerosis and experimental hypertension models further confirm the accumulation of CD4+ and CD8+ T cells in the tubulointerstitium. Although the infiltration of these T cells in hypertensive models is well-documented, recent research, exemplified by Trott et al.'s study, emphasised their specific roles and significance in hypertension (Trott et al., 2014). Notably, the study highlighted a potentially harmful role for CD8+ T cells in hypertension. These cells in hypertensive patients express heightened levels of proinflammatory molecules, indicating an elevated proinflammatory state (Youn et al., 2013). Besides, CD4+ T cells were also found to produce elevated levels of IL-17A and IFN-γ, associated with inflammation. This increased activity of CD4+ T cells, particularly the subset producing IL-17A, seems to play a role in sustaining hypertension by contributing to inflammatory responses that could affect vascular function and blood pressure regulation. Particularly, higher levels of memory CD4+ T cells suggest a potential pathophysiological significance in maintaining the hypertensive state through repeated immune responses (Itani et al., 2016a). This collective research underscores the evolving understanding of T cell subsets involvement in hypertensive pathology (Abais-Battad et al., 2015).

The activation of T cells initiates a complex process involving proliferation and differentiation into various subsets, including effector, memory, and naive T cells. Upon encountering an antigen or stimuli like cytokines, T cells become activated, undergoing

rapid proliferation to increase their numbers. During this proliferation phase, these cells differentiate into distinct subsets based on their functions and surface marker expression (Li et al., 2006; Kieper et al., 2001). The presence of cytokines during this activation modulates receptor expression, influencing the differentiation trajectory and function within the evolving T cell subsets.

Stimulation of T cells play a critical role in understanding their activation mechanisms and functional effects and harnessing their potential in various immunological applications. T cell stimulation can be achieved in experimental settings using specific antibodies targeting key cell surface molecules involved in T cell activation. CD3 and CD28 antibodies emerged as valuable tools for studying and manipulating T cell responses (Riddell and Greenberg, 1990). CD3 antibodies specifically bind to the CD3 receptor complex on T cells, mimicking the interaction between T cell receptors and APCs. This activation step is essential for studying T cell responses and understanding their signalling pathways. CD28, another important co-stimulatory molecule expressed in T cells, interacts with its ligands, such as CD28 antibody, CD80 (B7-1) and CD86 (B7-2) on APCs. Utilising CD28 antibodies in experimental systems allows for the investigation of their impact on T cell responses (Riddell and Greenberg, 1990). The ligation of TCR-CD3 activates both stimulatory and inhibitory signal transduction pathways. These include the MAP kinases and NFAT-dependent pathways, which promote T cell activation, and the PKA-dependent pathway, which exerts inhibitory effects on T cell activation. Therefore, the engagement of TCR-CD3 leads to the activation of multiple signalling pathways that can have both stimulatory and inhibitory effects on T cell function (Li et al., 1999). PKA exerts an inhibitory effect on cell proliferation in multiple cell types, including fibroblasts, smooth muscle cells, adipocytes, and T cells. Hence, for T cells to achieve full activation, they necessitate supplementary signals independent of TCR stimulation to overcome the inhibitory effects of PKA (Li et al., 1999). Activation of T cells with TCR-CD3 signal alone without further costimulatory signal causes cells to go to an anergy or apoptosis state, which is critical in the case of self-reactive T cells (Schwartz, 1990; Janeway and Bottomly, 1994). The co-stimulatory signal is required for optimal T cell activation and effector functions. Many molecules can control T cell activation and differentiation, and the effector functions as a second signal. These molecules are members of the CD28 family and include molecules that promote T cell activation and cytokine production, such as CD28 and inducible co-stimulator (ICOS), and molecules that inhibit T-cell activation, such as CTLA-4 and PD-1, which result in T-cell anergy or death (Clay and Sperling, 2007). CD28, as the primary co-stimulatory receptor, is necessary for survival and

upregulation of anti-apoptotic molecules (Boise et al., 1993). In the presence of suboptimal TCR, CD28 co-stimulation signal results in increased transcription of multiple cytokines, up-regulation of IL-2R α-, β-, and γ, T cell proliferation, and effector function (Li et al., 1999; Appleman et al., 2000).

Along with antigens and the costimulatory signal, cytokines play important roles in regulating immune responses. Any external signals from the surrounding environment can also impact the differentiation pathway of T cells, leading to the generation of diverse phenotypic and functional outcomes. Additionally, T cell activation ensures that the cytokine signalling occurs within the appropriate cellular context and helps coordinate a controlled immune response. Clinical data suggests that inflammatory cytokines produced by T cells act as indicators and cause treatment-resistant hypertension (Barbaro and Harrison, 2019; Chen et al., 2019). For instance, the level of IL-6 was elevated in the plasma of hypertensive patients compared to controls, and severe PPH and gestational hypertension demonstrated significantly increased TNF-α and IL-6 serum levels (Liu et al., 2022; Humbert et al., 1995; Naya et al., 2007), suggesting a potential modulation role for cytokines in hypertension pathophysiology. Therefore, to mimic this process in vitro, activation of T cells was applied by using α-CD3/CD28 antibody molecules to achieve optimal subthreshold stimulation for T cells; it was essential to investigate the concentration of α-CD3 antibody required for activation. The optimal concentration of α-CD3 antibody for T cell activation can help to examine the potential additive effect of the cytokines on CD4+ and CD8+ T cells when added subsequently.

The complex process involving T cell activation, proliferation, marker expression, and differentiation is critical for initiating an effective immune response (Li et al., 2006) in hypertension. Therefore, in this chapter, we aim to assess the impact of cytokine supplementation on α-CD3 or α-CD3/CD28 antibody-activated T cells, analysing aspects such as viability, proliferation, receptors expression (post and prior-stimulation) and the differentiation of CD4+ /CD8+ T cell subsets. T cells were obtained from the spleen and PBMCs of WT, Sham, and Ang II mice.

4.2 Chapter Aims

- Investigate cytokines receptors' expression on T cells derived from spleen and PBMCs *in vivo*.

- Investigate the suboptimal concentration of α-CD3 antibody and the addition of α-CD28 to activate T cells.

- Assess alterations in receptor expression level following T cell stimulation with TNF-α, IL-6, IL-2, and IFN-γ cytokines.

- Investigate the impact of cytokines (TNF-α, IL-6, IL-15, IL-12 and IFN-γ) stimulation on T cell proliferation, differentiation into effector and memory subsets, and the expression of activation markers.

4.3 Results

4.3.1 Evaluation of cytokine receptor expressions on T cells.

4.3.1.1 Expression of cytokines receptors on T cell subtypes from PBMCs in Sham and Ang II-infused mice (*In VIVO)* (14-day infusion).

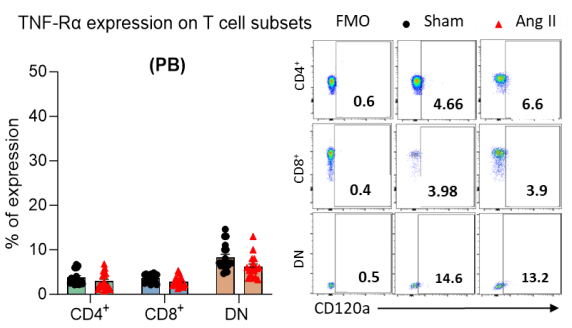
As hypertension has characteristics with dysregulated immune responses, as in autoimmune disorders or chronic infections, it may lead to altered receptor expression patterns in T cell subsets (Dunham et al., 2008). To study the receptor expressions, the presence of TNF-αRs (CD120a & CD120b), IL-6R (CD126), IL-7R (CD127), IL-1R1 (CD121a), IL-4R (CD124), IL-2Rs (CD122 & CD25), IL-12R, IL-17Rs (IL-17Rα and IL-17Rβ), and IFN-γRs (IFN-γR1 & IFN-γR2) on CD4+, CD8+ and DN T cell subsets from Sham and Ang II-treated PBMCs was assessed using flow cytometry. The results depicted in (Figure 4.1) show no difference between Sham vs Ang II was observed in the expression of TNF-αRs: CD120a (18% ± 0.3% vs 17% ± 0.4%), and CD120b (16% ± 1.7% vs 12% ± 0.7%), IL-1R1 (8.4% ± 0.3% vs 6.7% ± 0.7%), IL-12Rβ1 (24.3% ±3 % vs 20.3% ± 2%), IFN- γR2 (14.2% ± 1.3% vs 17.6% ±1.4 %) and IL-17Rβ (8.2% ±1.4% on Sham vs 7.8% ±1.7% on Ang II) on CD4+ T cell. On CD8+ T cells, there was also no difference in the expression of TNF-αRs CD120a (14% ± 0.3% vs 14% ± 0.4%), and CD120b (14% ± 1.9% vs 14% ± 1.4%); IL-1R1 (6.6% ± 1.4% vs 6.1% ± 1.2%); IL-12Rβ1 (6.1% ± 0.9% vs 8.6% ±2%); IFN- γR2 (19.8% ±1.3% vs 22% ± 1.9%) and IL-17Rβ (7.7% ± 1.6% vs 7.6% ± 1.2%) on Sham and Ang II group, respectively (Figure 4.1 A, D, G, H and I), indicating lower expression levels.

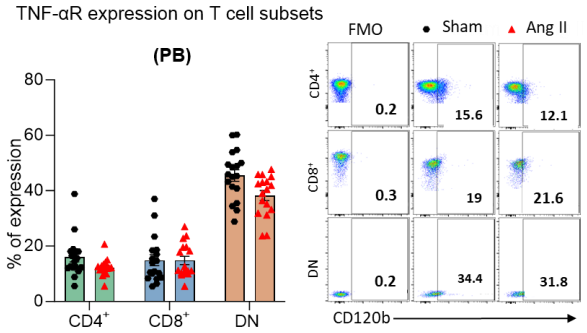
In addition, in both CD4+ and CD8+ cells, the expression levels of IL-7R (84% ± 2.3% on Sham vs 91% ± 0.6% on Ang II, and 86% ± 1.8% vs 91% ± 3.8%, respectively), CD126 (74% ± 1.8% on Sham vs 80 % ± 1.2% on Ang II and 48% ± 4.4% on Sham vs 55% ± 3.1% on Ang II, respectively), and IFN-γR1 CD119 (98% ± 0.5% vs 95% ± 1.7% and 84% ± 2.1% vs 80% ± 3.3%, respectively) showed no significant difference between Sham and Ang II groups (Figure 4.1B, C and I).

Higher expression levels of IL-4R (CD124) and IL-17Rα (CD217) were observed on CD4+ T cells, 39.2% ± 17.3% and 45.2% ± 3%, in comparison to CD8+ T cells, 12.4% ± 4.0% and 14.8 ±2.8%, respectively (Figure 4.1 E and H). On the contrary, IL-2Rβ showed a higher expression on CD8+ T cells (67.5± 8.9% on Sham vs 73.8 ± 7.5% on Ang II) than CD4+ T cells (Figure 4.1G). However, there were no statistically significant differences in

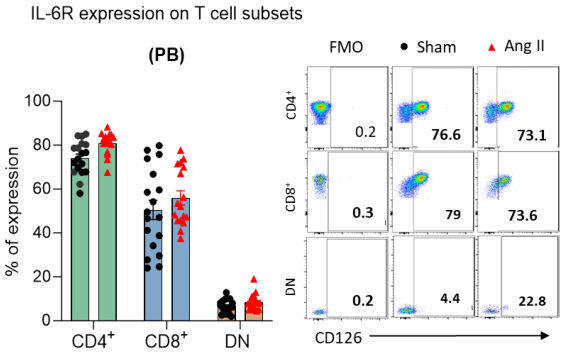
the expression level of any receptor between Sham and Ang II T cells. These results suggest that receptor expression varies across T cell subsets and is unaffected by Ang II treatment.

A

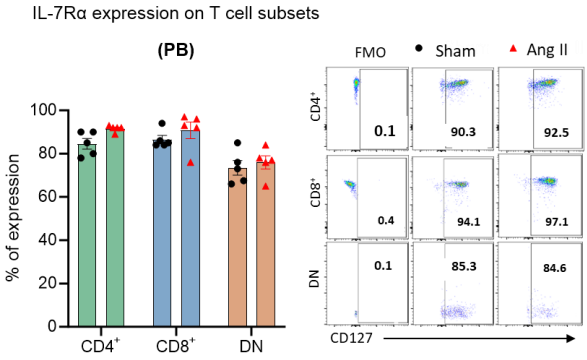

TNF-Rα expression on T cell subsets
(PB)
% of expression
CD4+
CD8+
DN
FMO
Sham
Ang II
0.6
4.66
6.6
0.4
3.98
3.9
0.5
14.6
13.2
CD120a


TNF-αR expression on T cell subsets
(PB)
% of expression
CD4+
CD8+
DN
FMO
Sham
Ang II
0.2
15.6
12.1
0.3
19
21.6
0.2
34.4
31.8
CD120b

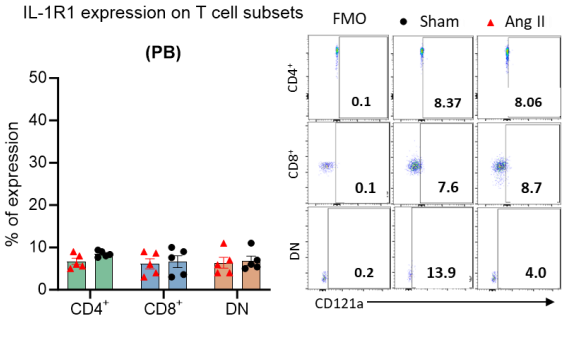
B


IL-6R expression on T cell subsets
(PB)
% of expression
CD4+
CD8+
DN
FMO
Sham
Ang II
0.2
76.6
73.1
0.3
79
73.6
0.2
4.4
22.8
CD126

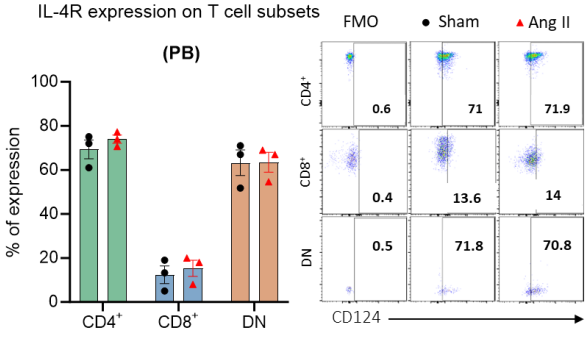
C


IL-7Rα expression on T cell subsets
(PB)
% of expression
CD4+
CD8+
DN
FMO
Sham
Ang II
0.1
90.3
92.5
0.4
94.1
97.1
0.1
85.3
84.6
CD127

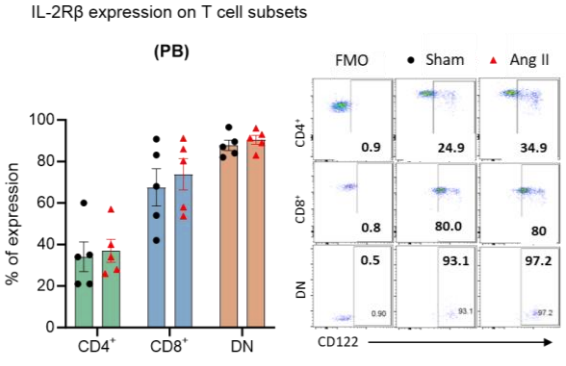
D


IL-1R1 expression on T cell subsets
(PB)
% of expression
CD4+
CD8+
DN
FMO
Sham
Ang II
0.1
8.37
8.06
0.1
7.6
8.7
0.2
13.9
4.0
CD121a

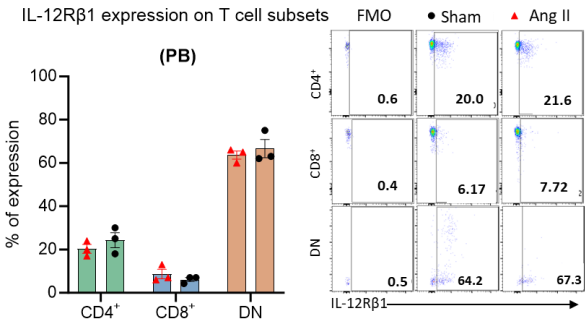
E


IL-4R expression on T cell subsets
(PB)
% of expression
CD4+
CD8+
DN
FMO
Sham
Ang II
0.6
71
71.9
0.4
13.6
14
0.5
71.8
70.8
CD124

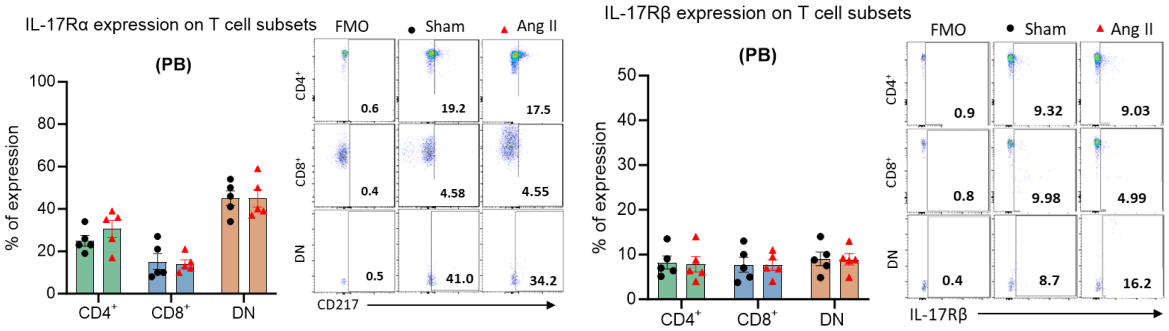
F


IL-2Rβ expression on T cell subsets
(PB)
% of expression
CD4+
CD8+
DN
FMO
Sham
Ang II
0.9
24.9
34.9
0.8
80.0
80
0.5
93.1
97.2
CD122

G


IL-12Rβ1 expression on T cell subsets
(PB)
% of expression
CD4+
CD8+
DN
FMO
Sham
Ang II
0.6
20.0
21.6
0.4
6.17
7.72
0.5
64.2
67.3
IL-12Rβ1

H



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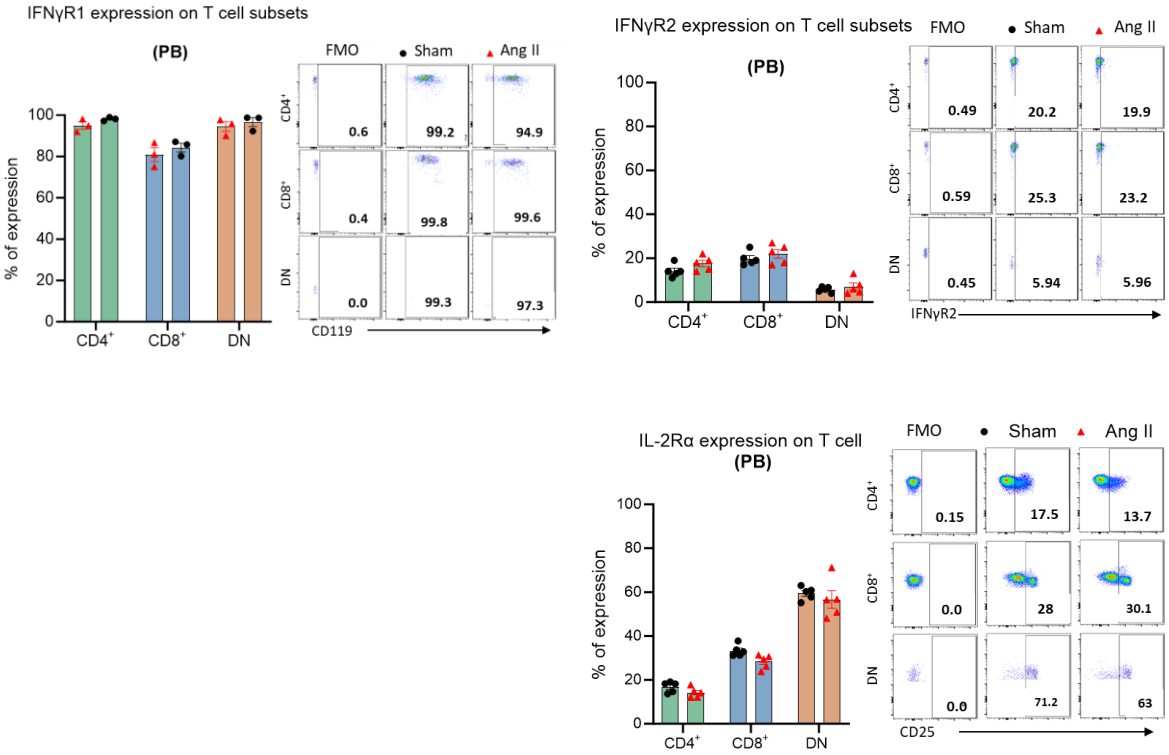


Figure 4.1. Expression of cytokine receptors on T cell subsets derived from PBMCs (*in vivo*).

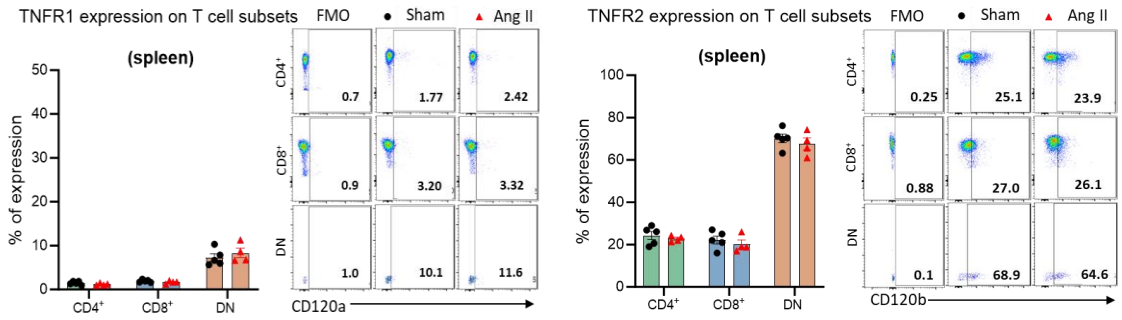
Dot Plots and bar graphs illustrating the expression levels of various receptors, including CD120a, CD120b (TNF-αRs) **(A),** CD126 (IL-6R) **(B),** CD127 (IL7R) **(C),** CD121a (IL-1R1) **(D),** CD124 (IL-4R) **(E),** CD122 (IL-2R) **(F),** IL-12Rβ (IL-12R) **(G),** IL-17Rα and β, IL-17Rs **(H),** and IFN-γR1 and 2 Receptors **(I)** in unstimulated PBMCs isolated from Sham and 14 days Ang II-infused mice. The data are represented as Mean ± SEM (n=3) per group for IFNγR1, IL-12Rβ, and IL-4R, (n=18) for IL-6R and TNF-αRs, (n = 5) for IL-7Rα, IL-6R, IL-1R1, IL-2Rbβ, IL-17Rα&β IFN-R2 B. FMO - fluorescence minus one. The statistical test (**** P<0.0001, *** P<0.001, ** P<0.01) was performed using a 2-way ANOVA test followed by a Bonferroni multiple comparisons test.

4.3.1.2 Expression of cytokines receptors on the splenic T cell in Sham and Ang II infused animal (*in vivo*) (14-d infusion)

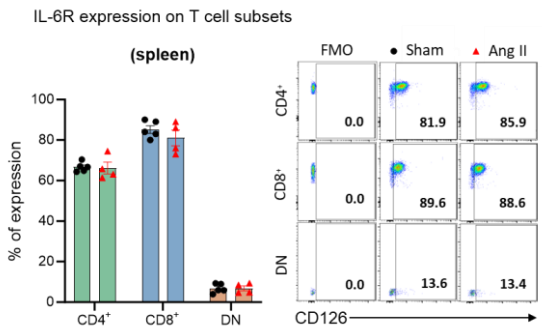
The cytokine receptor expression in T cells derived from spleens of Sham and Ang II-infused groups was compared to discern differences between the two groups. The expression of receptors displayed consistent patterns between Sham and Ang II-treated animals. In CD4+ T cells from both Sham and Ang II-infused groups (Figure 4.2A, D, F, H, I), the expression levels of TNF receptors: CD120a (1.4% ± 0.1% vs 1.1% ± 0.1%), and CD120b (24.4% ± 1.9% vs 22% ± 0.9%); IL-1R1 (5% ± 0.8% vs 5% ± 0.5%); CD122 (12% ± 1.8% vs 12% ± 1.2%); IL-12Rβ1 (8% ± 1.8% vs 7% ± 1%); IL-17Rα (5% ± 0.6% vs 6% ± 0.7%); IL-17Rβ (3% ± 0.2% vs 3.9% ± 0.4%), and IFN-γR1 CD119 (21% ± 1.8% vs 22% ± 2%) showed no significant differences observed between both groups. Similarly, in CD8+ T cells from Sham and Ang II-infused animals, there were no differential expressions of TNF-αRs CD120a (1.9% ± 0.1% vs 1.6% ± 0.2%), and CD120b (22% ± 1.8% vs 20% ± 1.9%); IL-1R1 (4.1% ± 0.3% vs 4.6% ± 0.3%); CD122 (20% ± 2.4% vs 23% ± 1.5%); IL-12Rβ1 (5% ± 0.8% vs 6% ± 1%); IL-17Rα (4% ± 0.8% vs 4.4% ± 0.3%); IL-17Rβ (3% ± 0.2% vs 3% ± 0.4%) and IFN-γR1 CD119 (17% ± 2% vs 17% ± 1.6%).

IL-6R, IL-4Rα, and IL-7Rα (Figure 4.2 B, C, E) showed expression on CD4+, with levels between 66%, 85%, and 75% on Sham vs 66%, 62%, and 75% on Ang II, respectively, with mean ± 1.6-3.3%. On CD8+ T cells, the level of these receptors was 85%, 72%, and 88% on Sham vs 81%, 72%, and 88% on Ang II ±1 - 4%, respectively. Importantly, no discernible differences in the expression of these receptors were observed between T cells derived from Sham and Ang II-treated groups.

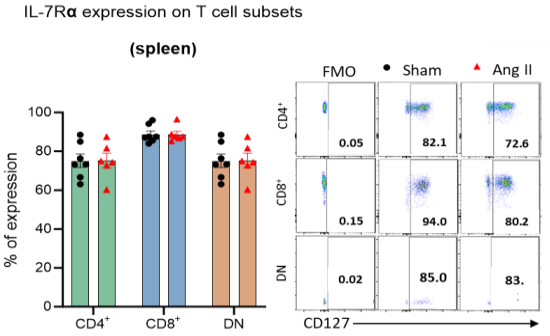
A


TNFR1 expression on T cell subsets
FMO
Sham
Ang II
(spleen)
% of expression
CD4+
CD8+
DN
0.7
1.77
2.42
0.9
3.20
3.32
1.0
10.1
11.6
CD120a
TNFR2 expression on T cell subsets
(spleen)
0.25
25.1
23.9
0.88
27.0
26.1
0.1
68.9
64.6
CD120b

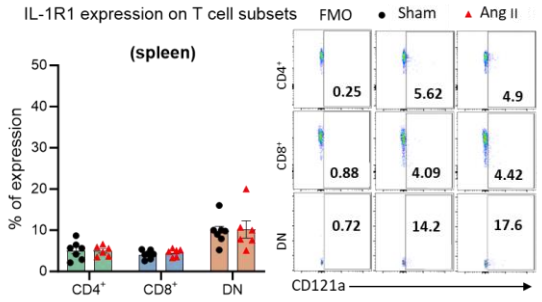
B


IL-6R expression on T cell subsets
(spleen)
FMO
Sham
Ang II
% of expression
CD4+
CD8+
DN
0.0
81.9
85.9
0.0
89.6
88.6
0.0
13.6
13.4
CD126

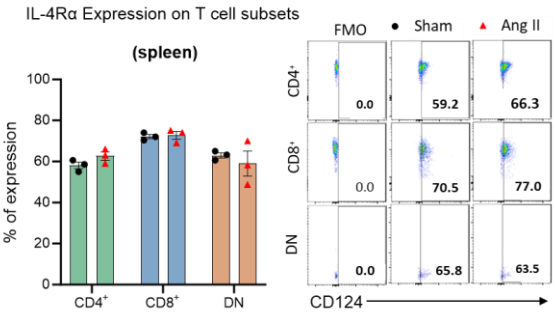
C


IL-7Rα expression on T cell subsets
(spleen)
FMO
Sham
Ang II
% of expression
CD4+
CD8+
DN
0.05
82.1
72.6
0.15
94.0
80.2
0.02
85.0
83.
CD127

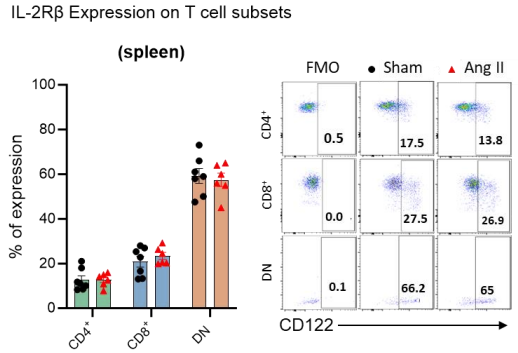
D


IL-1R1 expression on T cell subsets
(spleen)
FMO
Sham
Ang II
% of expression
CD4+
CD8+
DN
0.25
5.62
4.9
0.88
4.09
4.42
0.72
14.2
17.6
CD121a

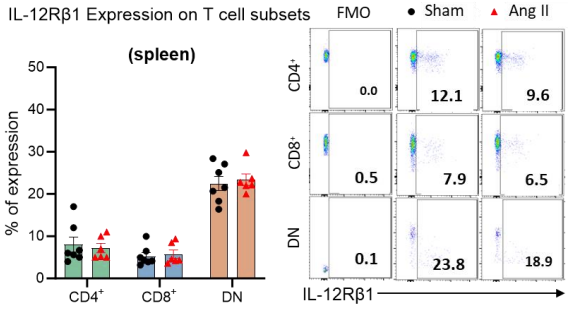
E


IL-4Rα Expression on T cell subsets
(spleen)
FMO
Sham
Ang II
% of expression
CD4+
CD8+
DN
0.0
59.2
66.3
0.0
70.5
77.0
0.0
65.8
63.5
CD124

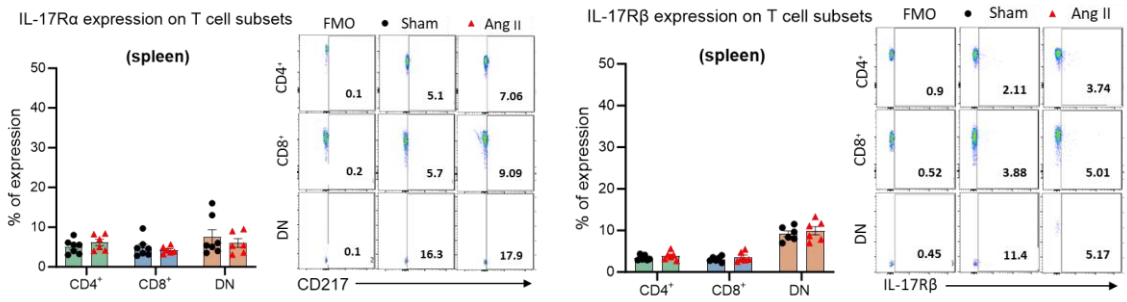
F


IL-2Rβ Expression on T cell subsets
(spleen)
FMO
Sham
Ang II
% of expression
CD4+
CD8+
DN
0.5
17.5
13.8
0.0
27.5
26.9
0.1
66.2
65
CD122

G


IL-12Rβ1 Expression on T cell subsets
(spleen)
FMO
Sham
Ang II
% of expression
CD4+
CD8+
DN
0.0
12.1
9.6
0.5
7.9
6.5
0.1
23.8
18.9
IL-12Rβ1

H



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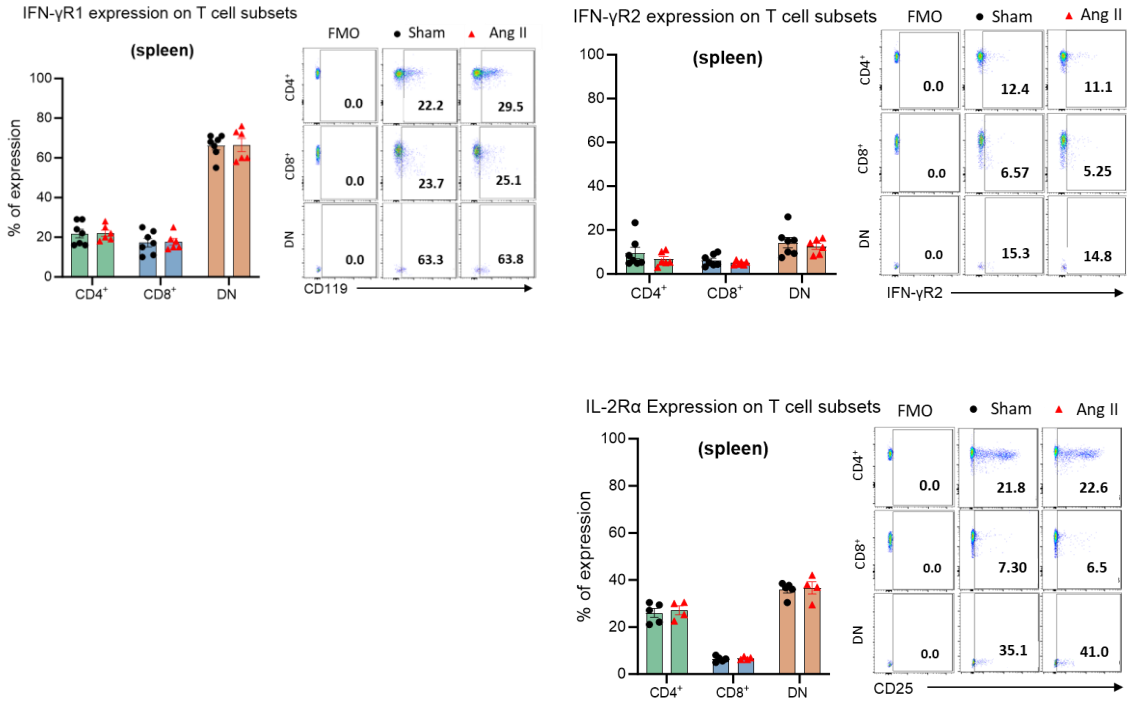


Figure 4.2. Expression of cytokines receptors on splenic T cell subsets. Representative dot plots and graphs of CD120a, CD120b (TNF-αR) **(A),** CD126 (IL-6R) **(B),** CD127 (IL7R) **(C),** CD121a (IL-1R1) **(D),** CD124 (IL-4R) **(E),** CD122 (IL-2) **(F),** IL-12Rβ (IL-12R) **(G),** IL-17Rα and β (IL-17Rs) **(H),** and IFN-γ receptors (CD119-IFN- γR2) **(I),** expressed on splenic T cell isolated from sham and 14-days Ang II-infused mice. Mean ± SEM (n = 3, 5 and 7) Sham group / (3, 4 and 6) Ang II group. FMO -fluorescence minus one. Statistical test (**** P<0.0001, *** P<0.001, ** P<0.01) was performed using a 2-way ANOVA test followed by a Bonferroni multiple comparisons test.

4.3.1.3 Expression of TNF and IL-6 receptors on the splenic T cell *(In vivo)* (5-day Ang II-infusion).

To evaluate receptor expression on T cells at various stages of hypertension, T cells from mice infused for 5 days were examined. The expression of TNF-αRs (CD120a and CD120b) showed no significant difference between the Sham and Ang II groups, indicating similar levels of expression. Likewise, the expression of IL-6R (CD126) on CD4+, CD8+, and DN T cells subsets did not vary significantly between the two groups. These findings suggest comparable receptor expression profiles in both hypertensive and control conditions (Figure 4.3).

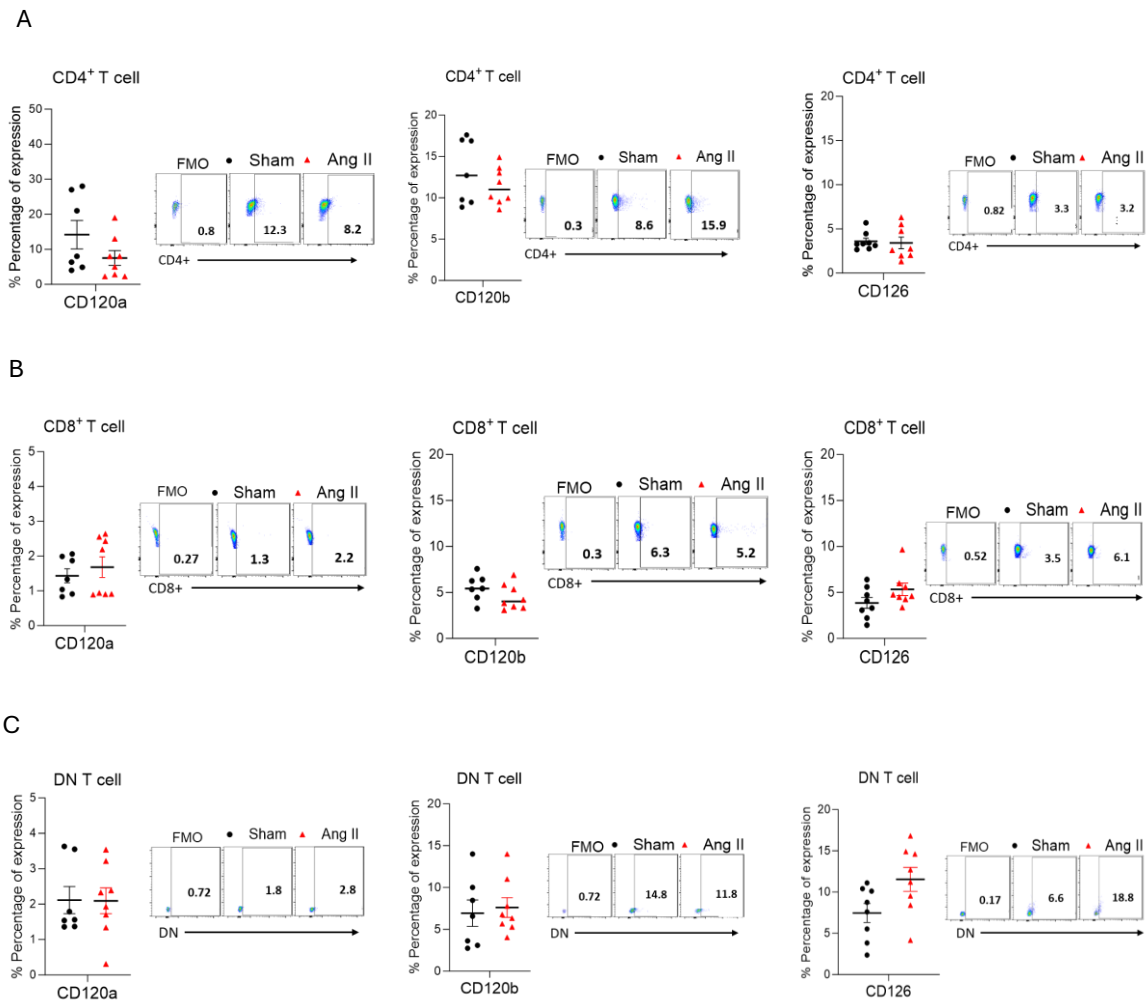


Figure 4.3. Cytokines receptors expression on splenic T cell subsets (5-day infusion).
Representative dot plots and graphs of CD120a, CD120b (TNF-αR) and CD126 (IL-6R) expression on CD4+ **(A),** CD8+ **(B)** and DN **(C)** splenic T cells isolated from Sham and 5-day Ang II-infused mice. Data were presented as mean ± SEM from (n =7-8) Sham group / (n=8) Ang II group. FMO -fluorescence minus one. Statistical test was (**** P<0.0001, *** P<0.001, ** P<0.01) performed using a T-test followed by the Mann-Whitney test.

4.3.2 T cell activation at different conditions to identify optimal stimulation for T cell (*In Vitro*).

4.3.2.1 Response of T cells to various concentrations of α-CD3 antibody

To study the effect of the cytokines on T cells, the α-CD3 antibody was used to activate T cells, as in various studies (Pulle et al., 2006; Crawley et al., 2014; Abais-Battad et al., 2015; Domae et al., 2017) and used as an initial activation for T cells prior to adding cytokines (Tormo et al., 2012). However, the concentrations utilised varied across different studies. A titration of α-CD3 Ab concentration was performed using **protocol 1** to identify the required optimal subthreshold concentration for studying the cytokines' effect (Figure 2.1/ P1). Spleen-isolated T cells from control mice were cultured with different concentrations of α-CD3 (1 - 2.5 - 5 ug/ml) to investigate the response in terms of viability (Figure 4.4A), differentiation (CD8+ : CD4+ Ratio) (Figure 4.4B) and the proliferation of CD4+ and CD8+ subsets (Figure 4.4 C and D). The results showed that T cell viability experienced a gradual decline over a span of six days of cell culture, irrespective of the various concentrations used. However, on the 4^{th} day, 2.5 ug/ml of α-CD3 maintained a higher level of cell viability, unlike 5 ug/ml, where the percentage of live cells significantly dropped on day 4 (Figure 4.4A). Stimulation T cells with α-CD3 differentiate the T cell toward CD8+, which was notable from day 3 onward (Figure 4.4B). On day 2, the expansion and proliferation response of CD4+ and CD8+ T cells was significantly induced.

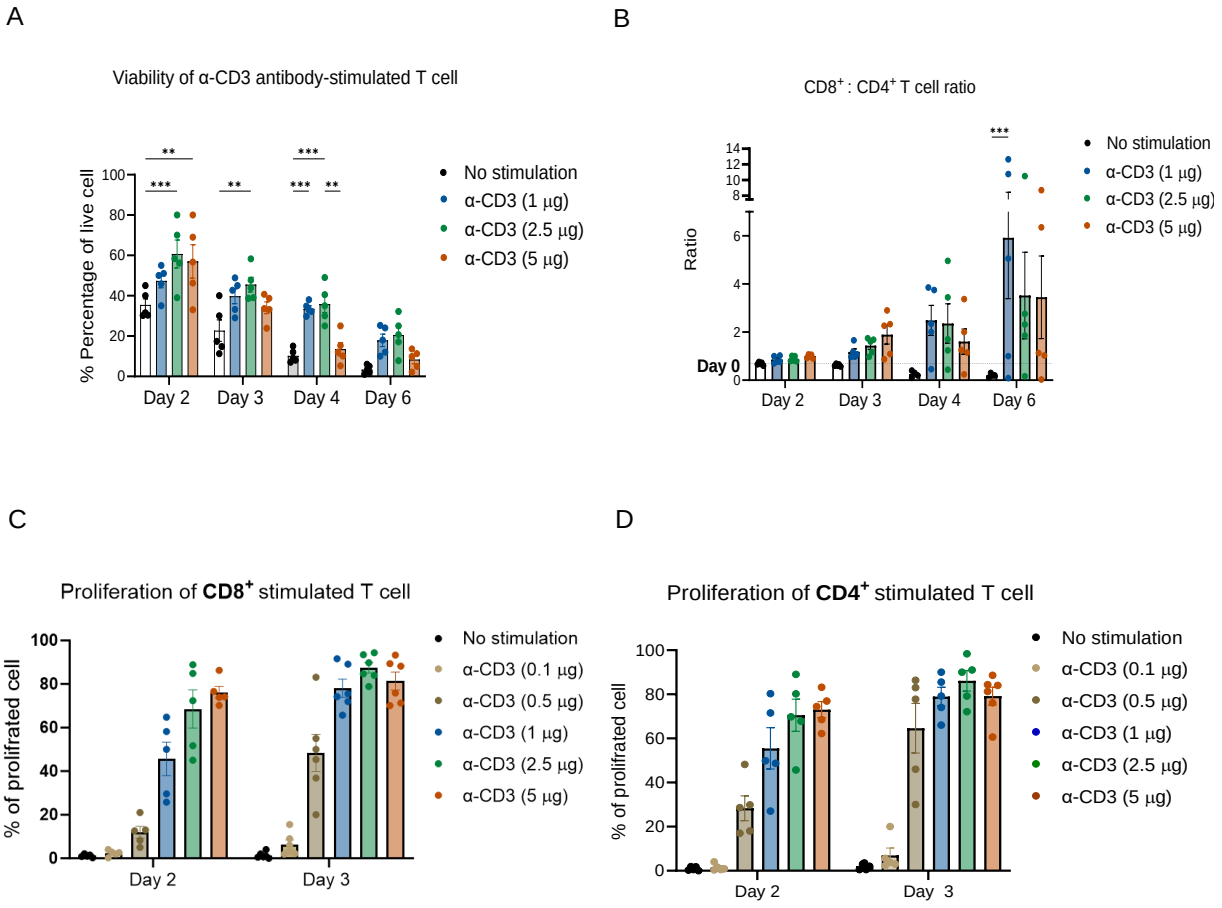


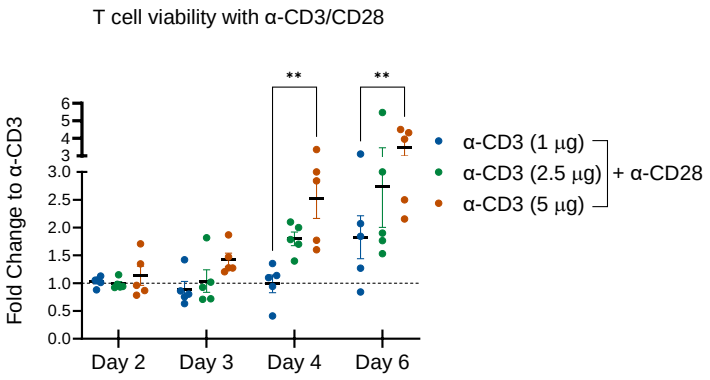
Figure 4.4. Effect of α-CD3 stimulation on T cell viability and proliferation after 2 and 3 days *In Vitro*.

T cells were stimulated with plate-bound α-CD3 Ab for 6 days, and the percentage of live T cells on the 2nd, 3rd, 4th, and 6th day were analysed **(A).** Characterisation of $CD8^+$: $CD4^+$ ratio **(B)** and the proliferation of $CD8^+$ **(C)** and $CD4^+$ **(D)** T cells upon α-CD3 stimulation were assessed. Data was analysed using flow cytometry and presented as mean ± SEM from n=5 independent experiments. Statistical significance (**** P<0.0001, *** P<0.001, ** P<0.01) was determined by a 2-way ANOVA test followed by a Tukey test.

4.3.2.2 Characterisation of T cell activation upon α-CD3 and α-CD28 antibody stimulation.

To study the additive effect of the co-stimulatory signal on the T cells, the CFSE dilution method was used to track cell division (Kashef et al., 2022). Spleen-isolated T cells were cultured in α-CD3 Ab-coated plates in the presence or absence of soluble α-CD28 antibody (2 ug/ml) (Figure 2.1/P1). α-CD28 showed an impact on prolonging T cell survival, especially with higher concentrations of α-CD3 antibody (2.5 and 5 ug/ml). However, no significant difference was detected in cell viability compared to α-CD3 alone on days 2 and 3 (Figure 4.5A). α-CD28 has no additive effect regarding CD4+ and CD8+ T cell proliferation over the 6 days with 1 and 2.5 ug/ml of α-CD3. Only a slight acceleration in the proliferation of CD4+ T cells was noticed with the addition of α-CD28 co-stimulation on day 3 (Figure 4.5B and C). Moreover, as shown in Figure 4.6 and Figure 4.7, both α-CD3/CD28 and α-CD3 stimulated T cells induced the proliferation of CD4+ and CD8+ T cells, and the peak point was at day 4. Although the addition of α-CD28 did not alter the proliferation response when added to 1 and 2.5 ug/ml conditions, adding α-CD28 to T cells treated with 5 µg/ml of α-CD3 boosted CD4+ T cell growth on day 4 compared to only α-CD3 (Figure 4.6A and B).

A



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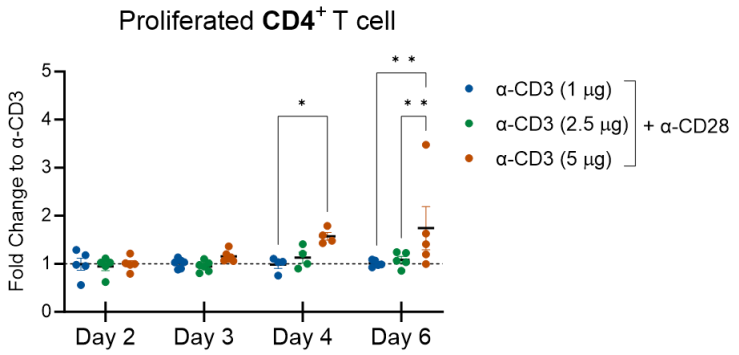


C

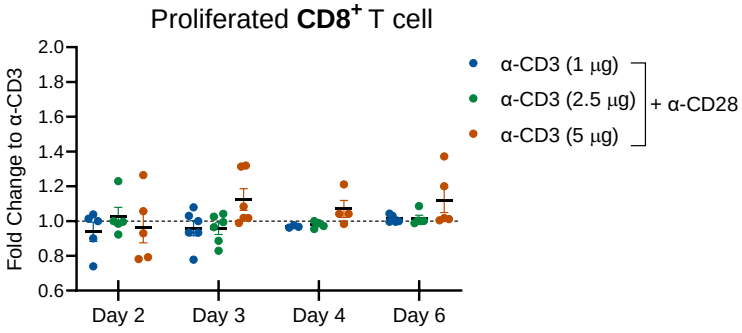


Figure 4.5. Fold change effect of plate-bound α-CD3 and soluble α-CD28 antibodies on T cell viability and proliferation.

Spleen-isolated T cell was cultured in α-CD3 (1, 2.5, 5 ug/ml) Ab-coated plated supplemented with or without α-CD28 (2 ug/ml) for 6 days. Data were collected at 4 different time points. The fold change of the percentage in viability **(A),** CD4+ **(B)** and CD8+ **(C)** proliferation of α-CD3/CD28 stimulated T cell, compared to α-CD3 stimulated T cell. Data were presented as mean ± SEM from 5 independent experiments. Statistical significance (**** P<0.0005, *** P<0.005, ** P<0.01, * P≤0.05) was determined by a 2-way ANOVA test followed by a Tukey post hoc test.

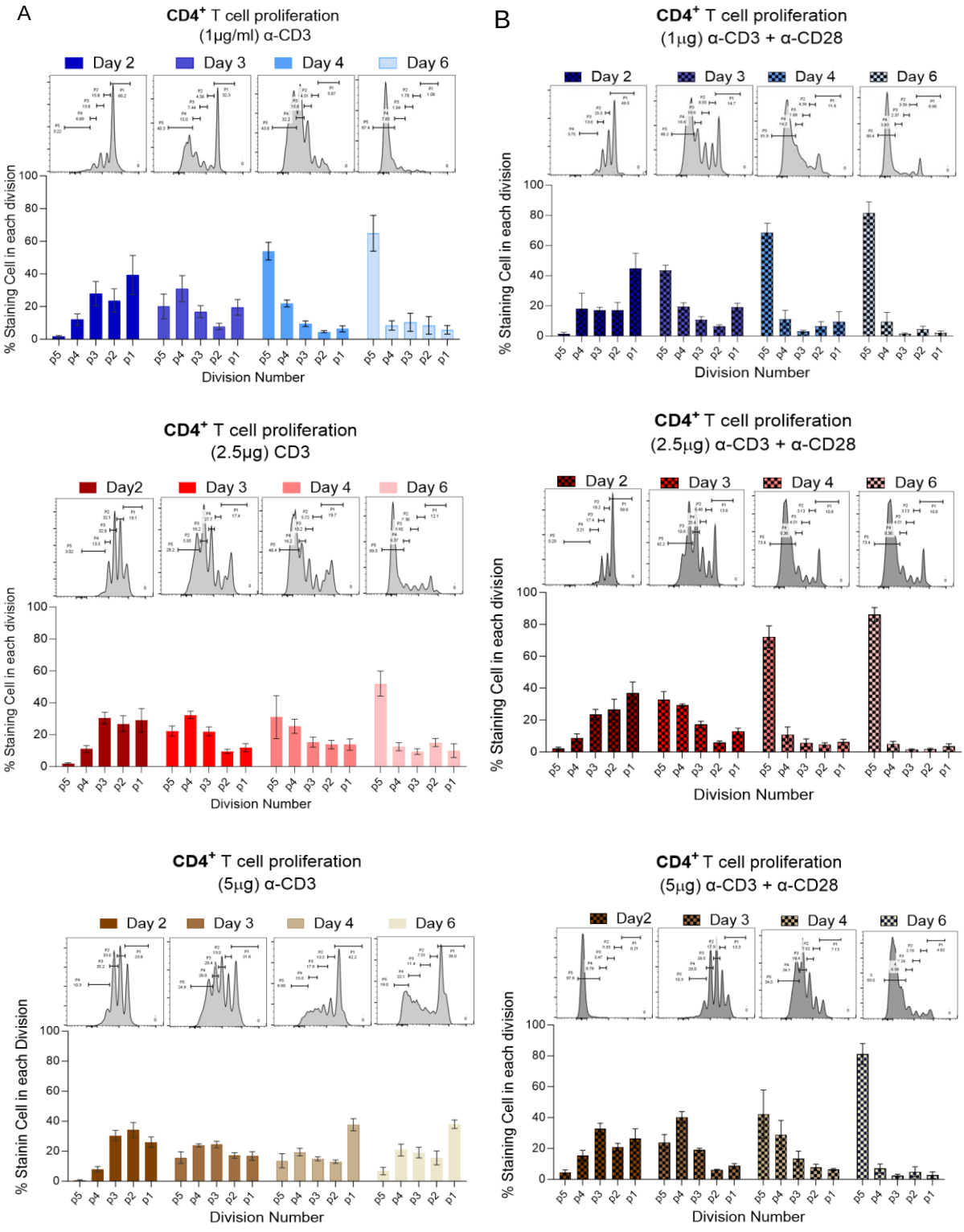


Figure 4.6. Proliferation of CD4+ T cell over 6 days stimulation *In Vitro*.
T cells labelled with CFSE were cultured with α-CD3 antibodies (1, 2.5, 5 ug/ml) in the presence **(A)** or absence **(B)** of α-CD28 (2 ug/ml). CFSE Intracellular fluorescent dye was used to track cell proliferation upon stimulation. Peaks were counted and represented on graphs from n=5 independent experiments. A representative histogram example for cell proliferation was shown.

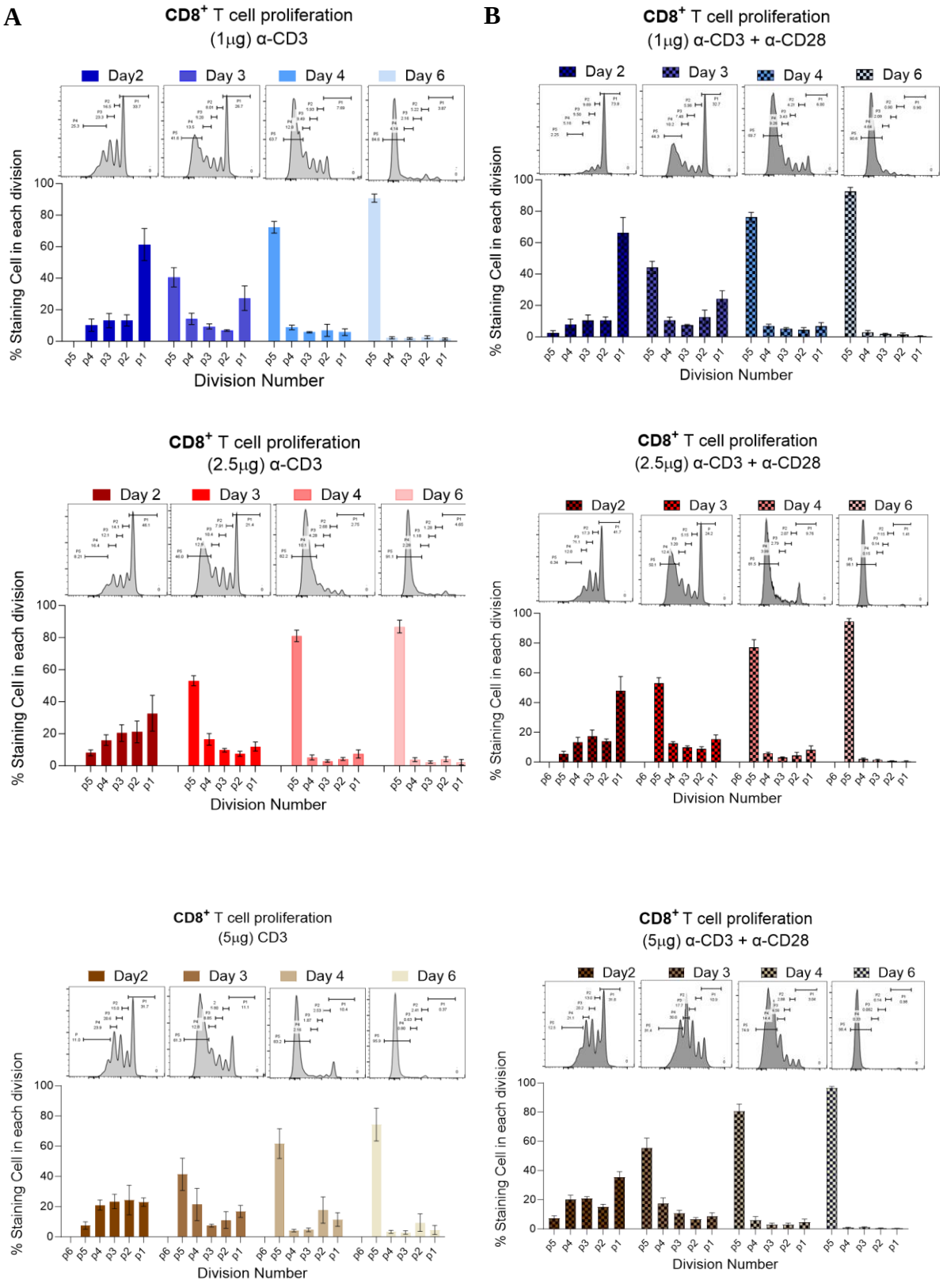


Figure 4.7. Proliferation of CD8+ T cell over 6 days stimulation *In Vitro*.
T cells labelled with CFSE were cultured with α-CD3 (1, 1.5, 5 ug/ml) in the present **(A)** or absence **(B)** of α-CD28. CSFE intracellular staining was used to track cell proliferation following stimulation. Peaks were counted and represented on graphs from n=5 independent experiments. A representative histogram example for cell proliferation was shown.

4.3.3 Expression of TNF-α and IL-6 receptors on splenic T cell surface upon cytokine stimulation *ex vivo*

After evaluating the expression of cytokine receptors from PBMCs (Figure 4.1) and spleen (Figure 4.2*) In vivo*, we examined the expression of cytokine receptors on T cells post-stimulation with TNF-α, IL-6, IL-2, and IFN-γ using protocol 3 (Figure 2.1/P3). In splenic T cells stimulated with IL-2, the expression of TNF-αRs (CD120a and CD120b) and IL-6R (CD126) on day 5 revealed significantly high expression levels of CD120b (TNF-αR1) on CD4+, CD8+, and DN T cell subsets (Figure 4.8B) averaging 73% ± 4.7%, 81.54% ± 4.6%, and 92.75% ± 1.7%, respectively. Meanwhile, CD120a (TNF-αR2) (Figure 4.8A) showed significant expression on CD4+ (14.5% ± 3.2%), CD8+ (23.5% ±2.4%), and DN (40.1% ± 4%) subsets on day 5 when compared to day 0. CD126 (IL-6R) (Figure 4.8C) was significantly downregulated on CD4+, CD8+, and DN T cell subsets, with 2.5% ±0.5%, 1.7% ±0.3, and 10.8% ±2.2% respectively. These expression patterns were observed on day 5 of post-IL-2 treatment.

Then, the expression of the TNF-αRs (CD120a &CD120b) and IL-6R (CD126) was further investigated on T cell subsets on day 7 and day 8 after exposure of the cells to IL-2 and to individual cytokines (TNF-α, IL-6, IFN-γ, and IL-2). No significant expression was detected among T cell subsets in response to cytokines compared to T cells stimulated with α-CD3/CD28 antibody (control). Overall, the CD120a, CD120b, and CD126 expression were either maintained or decreased in response to the cytokines compared to the mean expression level on day 5, before the addition of cytokines. However, IL-2, IL-6, and IFN-γ could maintain the expression on CD4+ T cells, unlike TNF-α. (Figure 4.9). In addition, this effect was further observed on day 8, where the expression was also either maintained or further reduced compared to day 7.

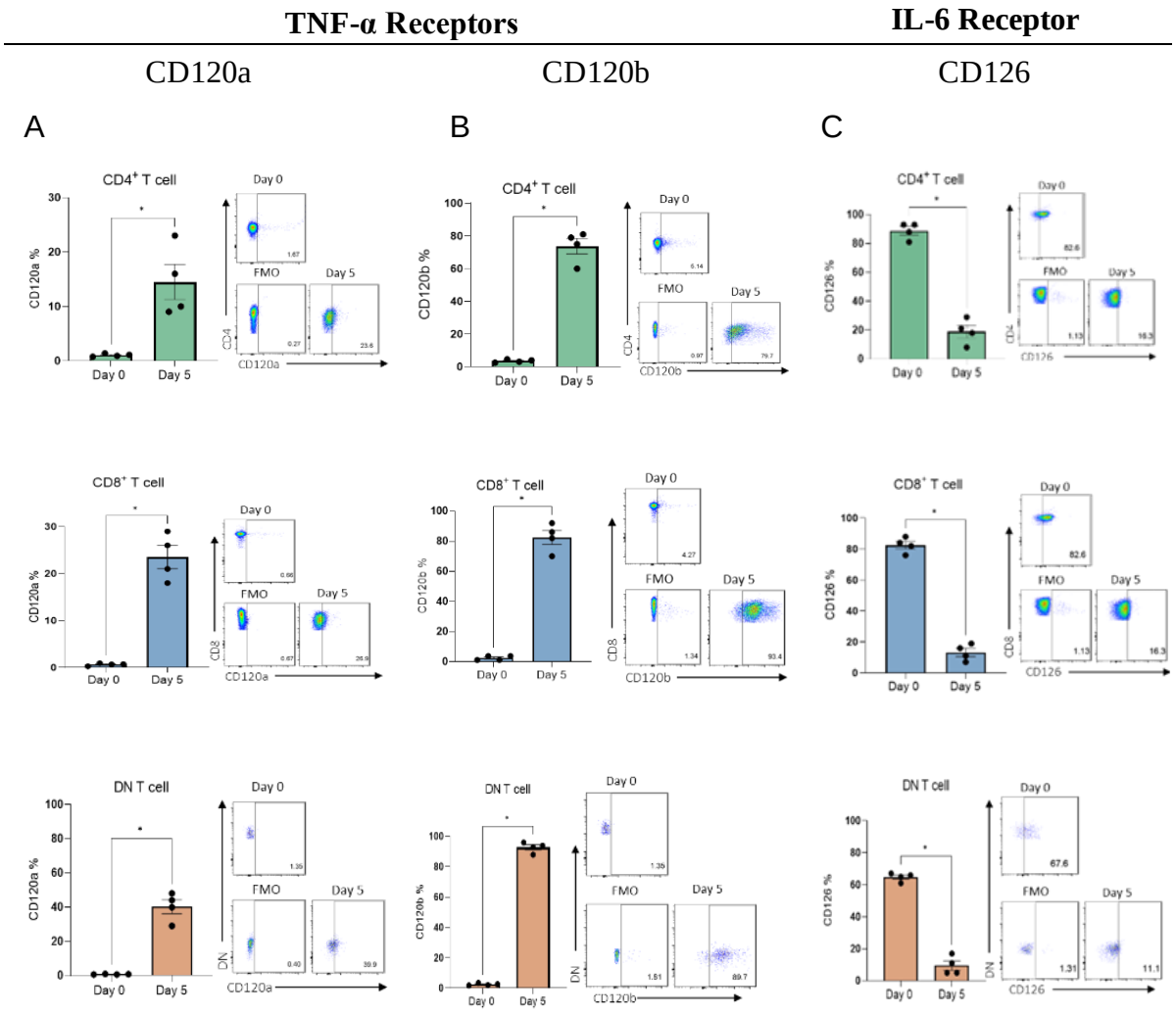
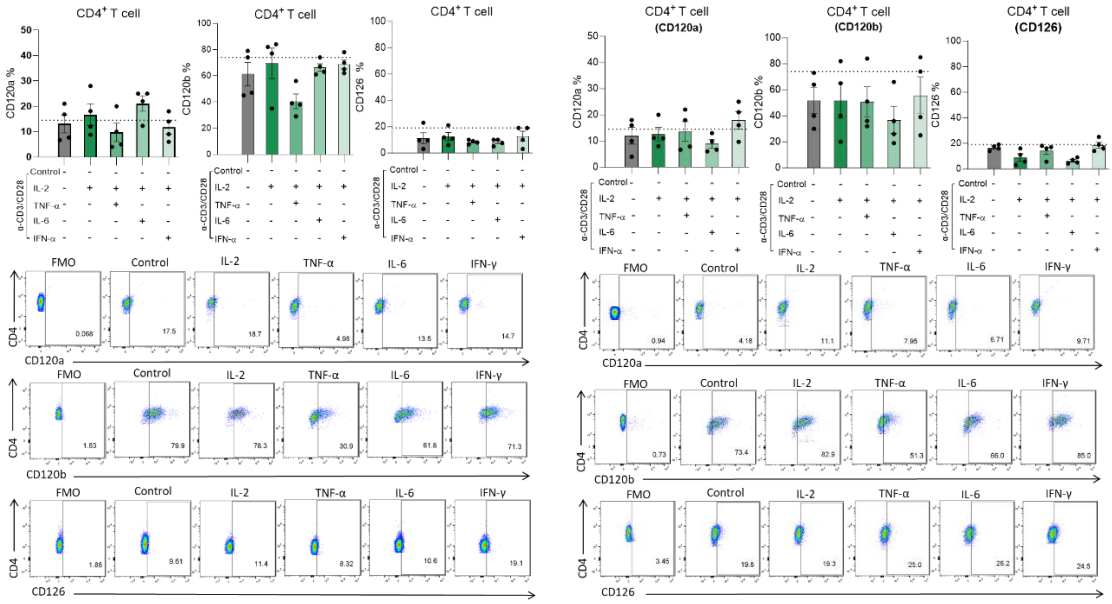


Figure 4.8. Expression of CD120a, CD120b, and CD126 receptors on T cell subsets after stimulation *(in vitro).*

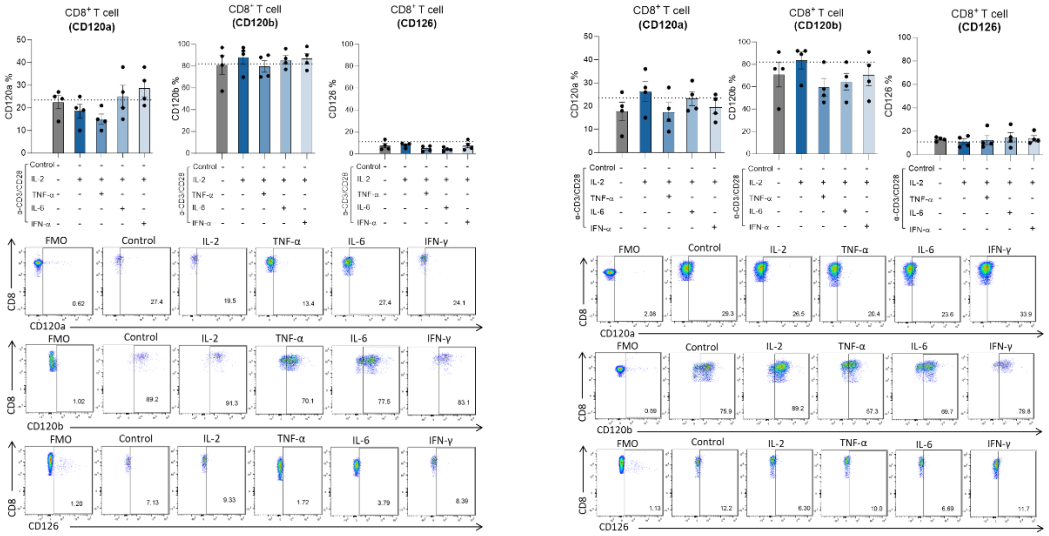
CD4+, CD8+ and DN T cells were analysed on day 0 (after isolation) and day 5 after α-CD3/α-CD28 + IL-2 stimulation, prior to the addition of cytokines. FACS was used to measure the expression of type 1 TNF-αR (CD120a) **(A),** type 2 TNF-αR (CD120b) **(B),** and IL-6R (CD126) on CD4+, CD8+ and DN cell **(C).** A T-test was performed, followed by the Mann-Whitney test (* P≤0.05). Data are expressed as mean ± SEM (n=4).

Day 7 **Day 8**

A


CD4+ T cell
CD4+ T cell (CD120a)
CD4+ T cell (CD120b)
CD4+ T cell (CD126)
CD120a %
CD120b %
CD126 %
α-CD3/CD28
Control
IL-2
TNF-α
IL-6
IFN-γ
FMO
CD4
CD120a
CD120b
CD126

B


CD8+ T cell
CD8+ T cell (CD120a)
CD8+ T cell (CD120b)
CD8+ T cell (CD126)
CD120a %
CD120b %
CD126 %
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CD8
CD120a
CD120b
CD126

C

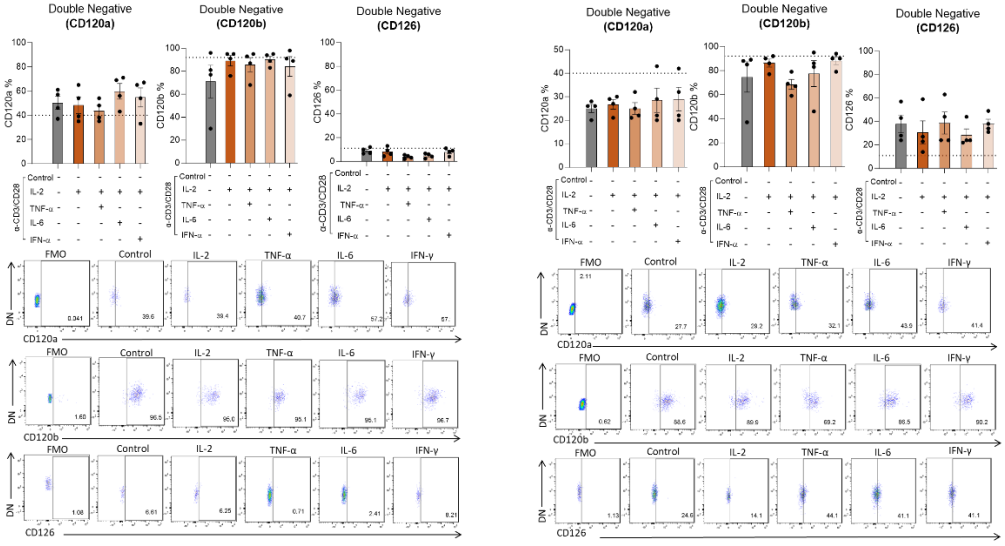


Figure 4.9. The Expression of CD120a, CD120b and CD126 receptors on stimulated T cells.

Receptors expression on CD4+**(A),** CD8+ **(B)** and double negative **(C)** T cells on days 7 and 8 after adding cytokines on day 5. The dash-dot line represents the mean of the receptor's expression on day 5. A one-way ANOVA (Kruskal-Wallis) test was performed, followed by Dunn's multiple comparisons test (**** P<0.0001, *** P<0.001, ** P<0.01). Data are expressed as mean ± SEM (n=4).

4.3.4 Cytokines effect on splenic T Cell differentiation.

With protocol 2 (Figure 2.1/p2), analysis was conducted to examine the influence of individual cytokines (TNF-α, IL-6, IFN-γ, IL-12 and IL-15) on T cell subsets, CD4+ and CD8+. The findings show the differential impact wherein IL-15 and IFN-γ notably direct the proliferation tendencies towards the CD8+ subset. In contrast, the IL-6 cytokine exhibited a tendency to drive cellular proliferation toward the CD4+ subset. Moreover, the cytokine milieu demonstrated an intriguing capacity to drive the differentiation of cells into memory T cell phenotypes within both CD4+ and CD8+ subsets (Figure 4.10B, C).

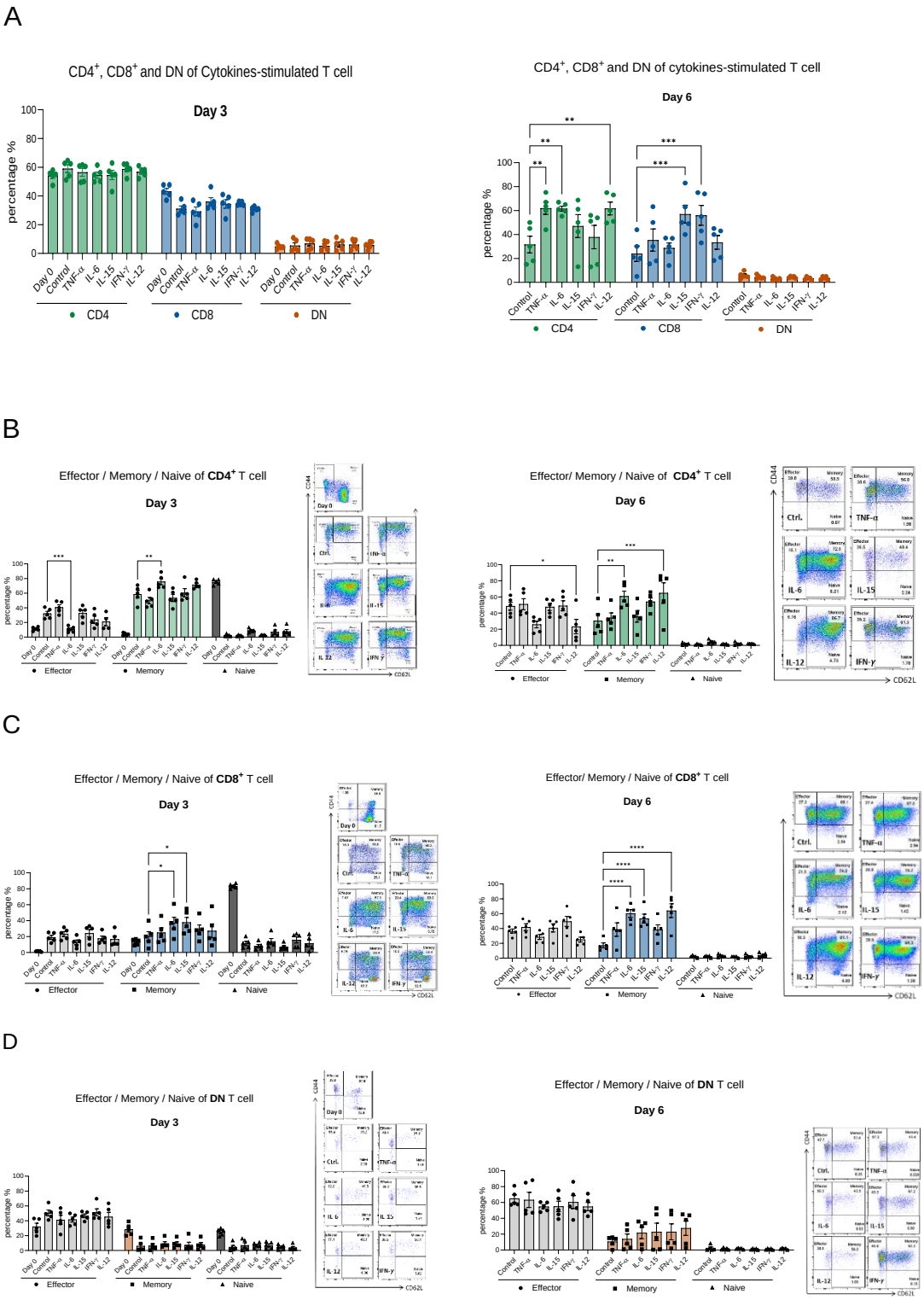


Figure 4.10. The Percentage of Effector, Memory, and Naïve CD4+, CD8+, and DN T cells upon various cytokines stimulation.

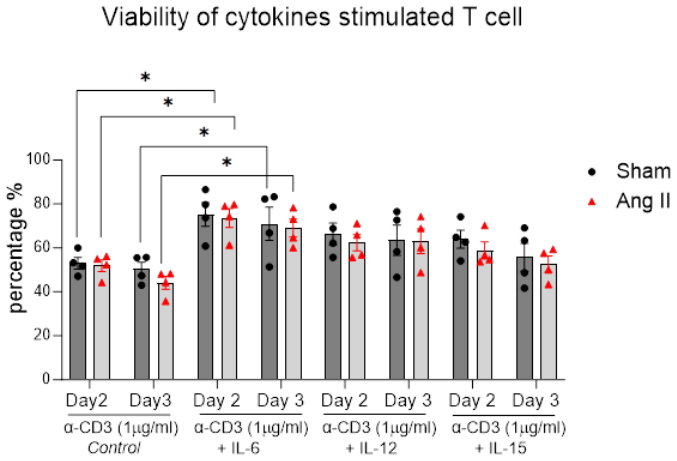
T cells were stimulated with α-CD3 (2.5ug/ml) and α-CD28 (2ug/ml) Ab in addition to individual cytokines **(A)** percentage of CD4+, CD8+ and DN T cells on day 3 and day 6 and CD4+/CD8+ ratio **(B)** percentage of effector, memory naïve of CD4+ T cells on day 3 and day 6. **(C)** percentage of effector, memory naïve of CD8+ T cell on day 3 and 6 **(D)** percentage of effector, memory naïve of DN T cell on day 3 and 6 upon stimulation. A one-way ANOVA test was performed, followed by Dunn's test for multiple comparison adjustment. (**** P<0.0001, ** P<0.001, ** P<0.01, * P≤0.05). Mean ± SED (n = 5) per group FMO -fluorescence minus one.

4.3.4.1 Effect of selected cytokines on T Cells differentiation in Sham and Ang-II infused mice

Using protocol P4 (Figure 2.1) involving a lower concentration of α-CD3 antibody (1ul/ml) and a shorter incubation time (3 days) was aimed to elucidate cytokine effects on T cells isolated from both Sham and Ang II-animals. Within the CD4+ subset, the response to all cytokines exhibited a consistent trend, promoting differentiation toward effector T_E and memory T_M cells, with a higher proportion directed toward T_M cells. In the CD8+ population, IL-15 demonstrated a capacity to promote the differentiation of T cells into memory phenotype compared to other cytokines. However, by day 3, most cells across all other cytokines had differentiated into T_M cells. Conversely, IL-6 exhibited a less pronounced effect on CD8+ T cells, where roughly 34% of the cells remained naive on day 3, and the rest transitioned to T_M cells. Nevertheless, the differentiation response to cytokine additions remained similar between T cells isolated from both Sham and Ang II-infused mice (Figure 4.12).

CD69+ activation marker expression was assessed across all T cell subsets following α-CD3 stimulation (control) and α-CD3 combined with cytokine exposure. Interestingly, the expression remained consistent in both the control and cytokine-stimulated conditions across CD4+ and CD8+ T cell subsets (Figure 4.13). Surprisingly, no significant distinctions were observed in CD69 expression among T cells from sham and Ang II-infused groups in response to cytokine stimuli (Figure 4.14).

A



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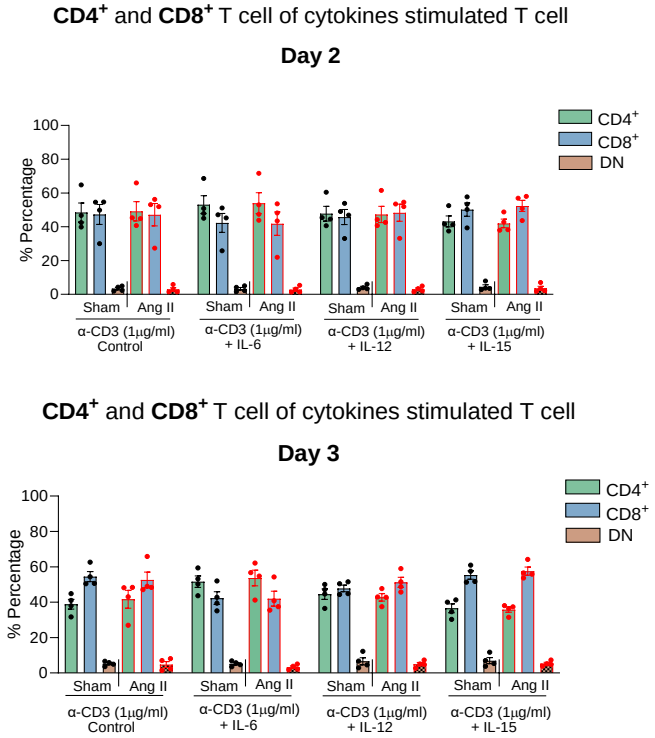
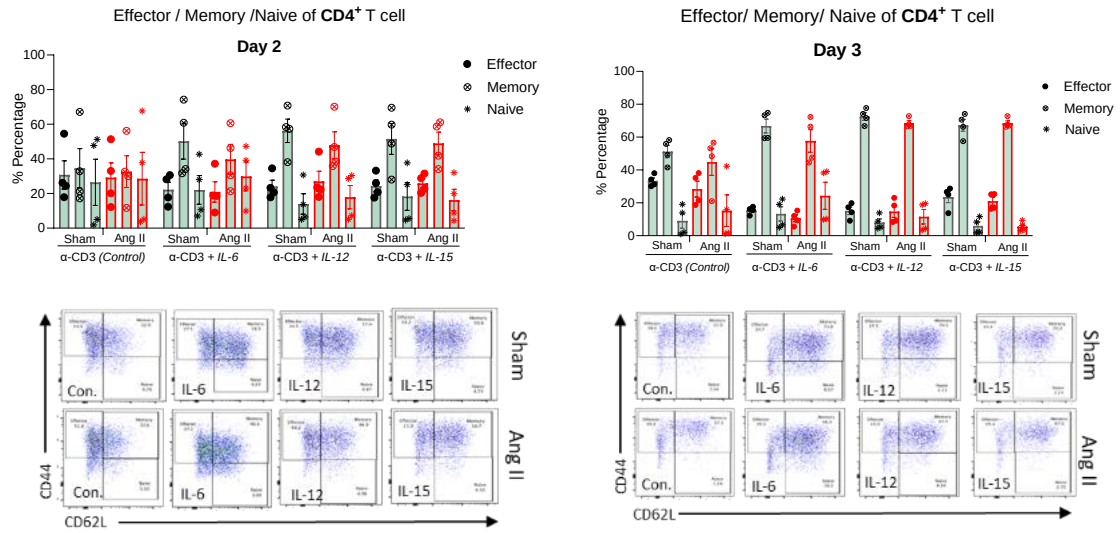


Figure 4.11. The impact of cytokine-stimulation on T cell viability and percentage of CD4+ and CD8+ subsets.

T cells were isolated from the spleen of Sham and Ang II-infused mice (14 days). T cells were stimulated with α-CD3(2.5ug/ml) and α-CD28 (2ug/ml) in the presence of individual cytokines. The percentage of live T cells **(A)** and the percentage of CD4+ and CD8+ **(B)** of cytokines-treated T cells on days 2 and 3 post-stimulation were analysed. A 2-way ANOVA test was performed, followed by the Tukey test for multiple comparison adjustment (* P≤0.05). Mean ± SED (n=4) per group.

A



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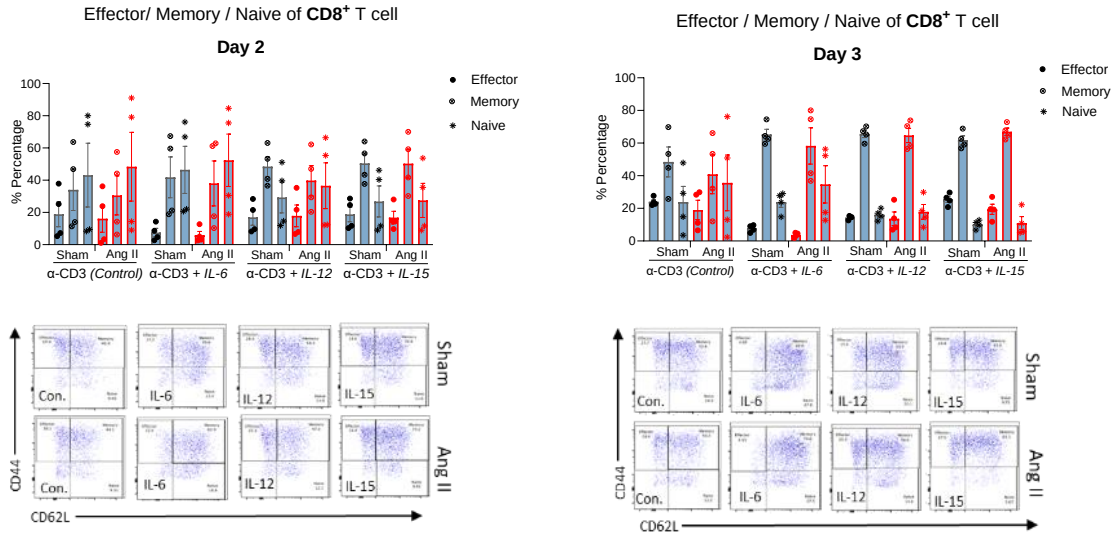


Figure 4.12. Cytokines effect on T cells differentiation toward effector and memory phenotype.

The percentage of CD4+ **(A)** and CD8+ **(B)** effector, memory, and naïve T cell subsets was analysed on days 2 and 3. Mean ± SED (n=4) per group. A 2-way ANOVA test was performed, followed by the Tukey test for multiple comparison adjustment. (P≤0.05) Mean ± SED (n=4) per group.

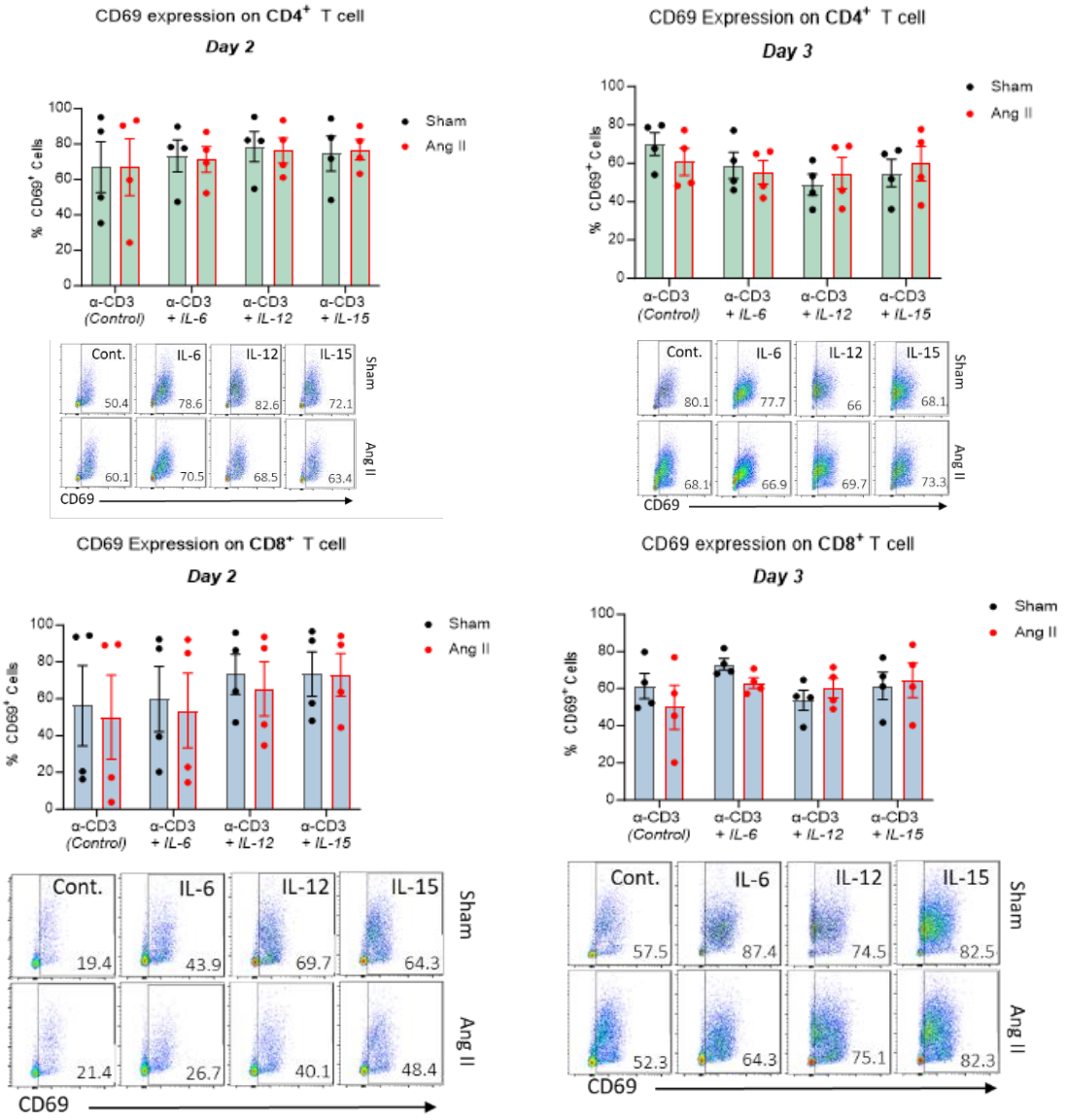


Figure 4.13. Expression of CD69 activation marker on CD4+ (A) and CD8+ (B) effector, memory, and naive T cells.

T cells were stimulated with α-CD3(2.5ug/ml) and α-CD28 (2ug/ml) Ab in addition to individual cytokines**.** Mean ± SED (n=4) per group. Statistical significance was determined by a 2-way ANOVA test followed by a Tukey multiple comparisons test ($p<0.05$).

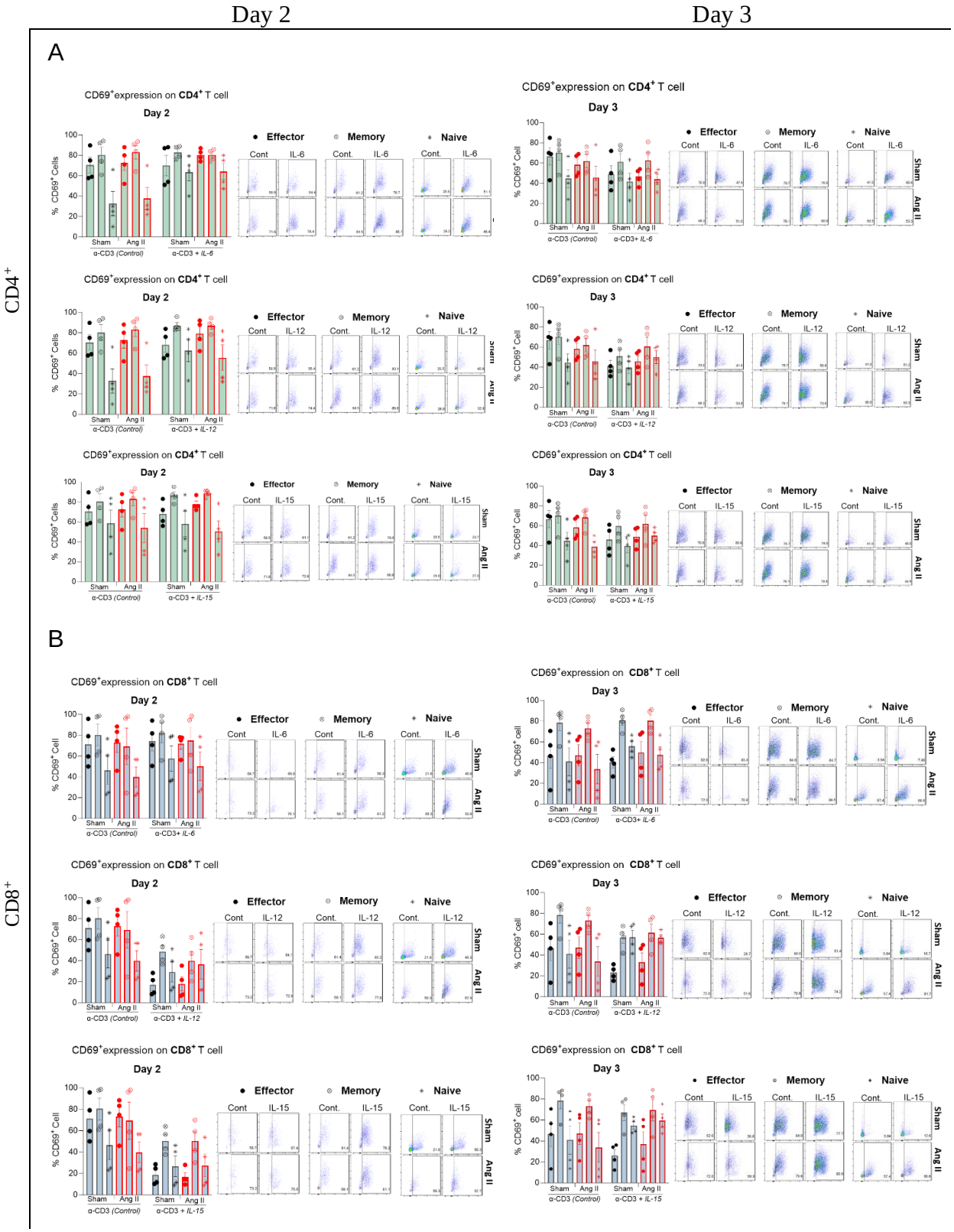


Figure 4.14. Expression of CD69 activation marker on CD4+(A) and CD8+ (B) proliferated T cells.

T cells were stimulated with α-CD3 (1ug/ml) in the presence of indicated cytokines for 3 days. The mean ± SED (n=4) per. Statistical significance was determined by a 2-way ANOVA test followed by a Tukey multiple comparisons test.

4.3.5 Cytokines effect on splenic T Cell proliferation.

The proliferation response of activated T cells to cytokine stimulation was assessed (Figure 2.1/P2), aiming to gain comprehensive insights into the nuanced interplay between cytokine signalling and T cell proliferation. Analysis was conducted over a 6-day stimulation period. The results demonstrated that adding TNF-α, IL-6, IL-12, IL-15, and IFN-γ to the T cells did not elicit any discernible proliferation response compared to the control condition, where cytokines were absent. By the third day, all cells had entered the cell cycle and underwent division in response to α-CD3/CD28 stimulation, regardless of the presence or absence of cytokines.

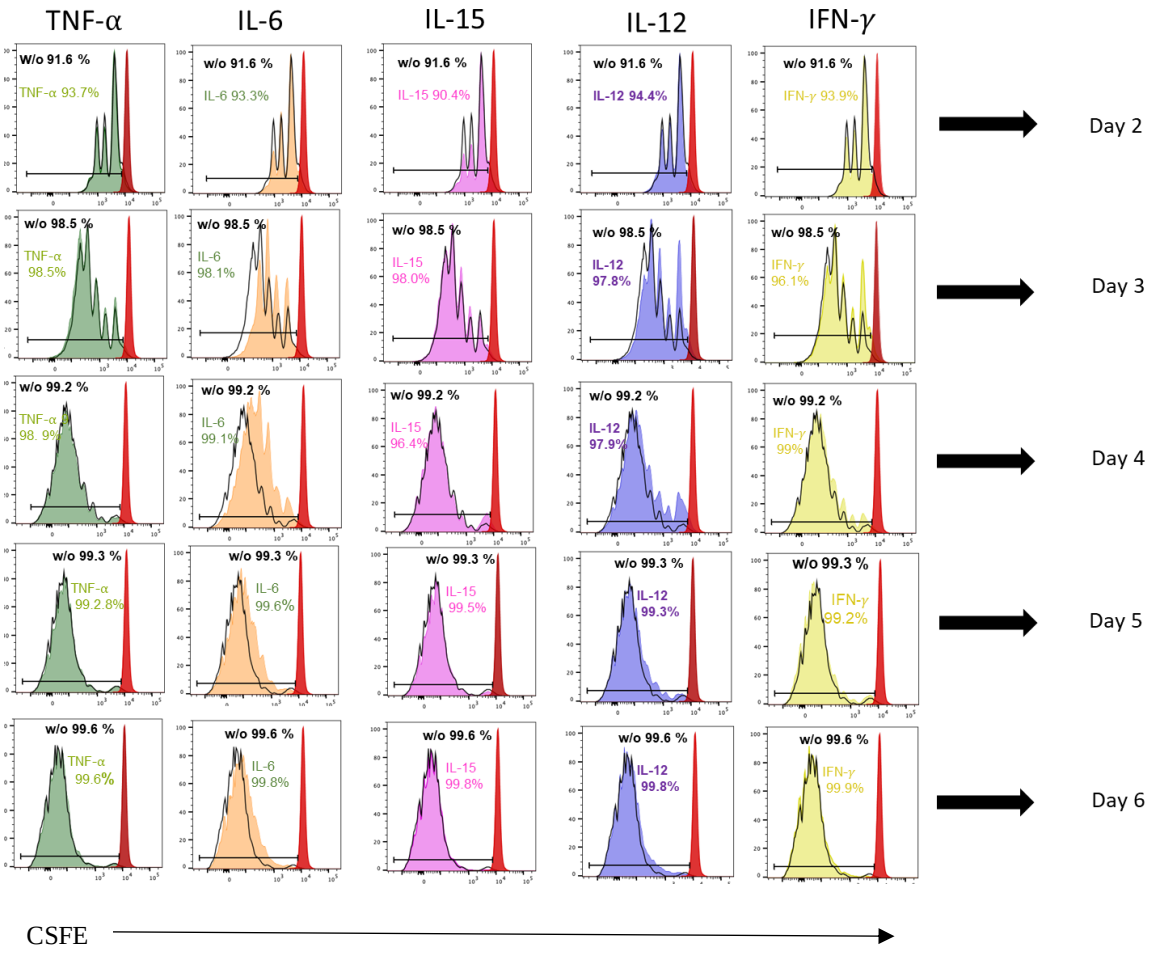


Figure 4.15. Proliferation of T Cells Induced by Diverse Cytokine Stimulation

Representative histogram of proliferated $CD3^+$ T cell in response to α-CD3/CD28 Ab in present and absence of different individual cytokines. The proliferation was measured by flow cytometer on days 2, 3, 4, 5 and 6 following cytokines stimulations. (n=3). Day 0 =Red histogram, α-CD3 (2.5ug/ml) + α-CD28 (2ug/ml) (control) = black line histogram.

4.3.5.1 Effect of cytokines on T Cells proliferation in Sham and Ang II-infused mice

Protocol 4 was used to stimulate T cells with a reduced concentration of α-CD3 antibody (1 µl/ml) and a shortened incubation period to examine the proliferation effects of cytokines on T cells from both sham and Ang II-infused animals. The results showed a consistent proliferation response of T cells to α-CD3, comparable to the response observed with α-CD3 stimulation combined with cytokines, indicated by similar patterns of cell division peaks in the CSFE histogram. There were no noticeable distinctions in the observed effects between the T cells from the sham and Ang II-infused groups.

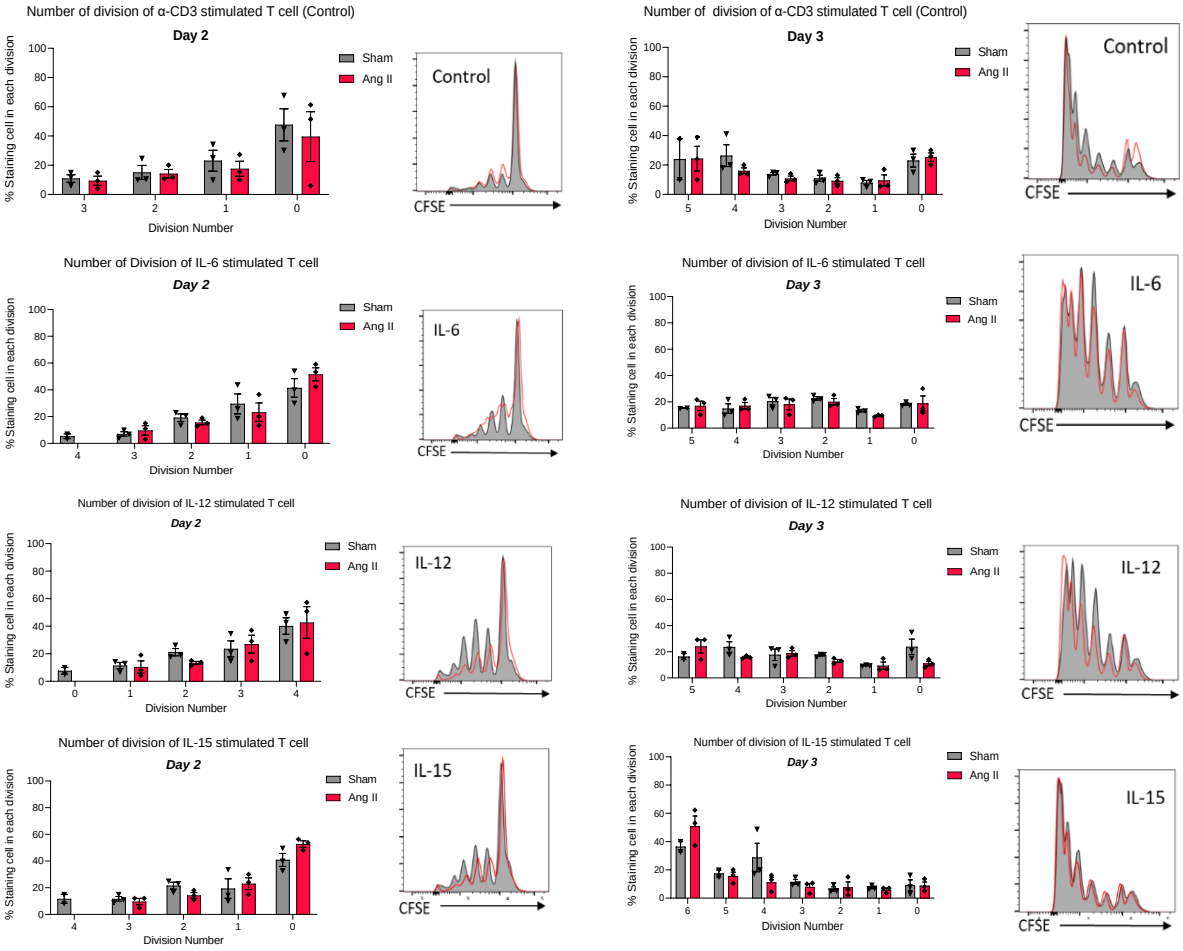


Figure 4.16. T cells proliferation in response to stimulation with various cytokines in Sham and Ang II-infused mice.
Representative histogram of the percentage of proliferating $CD3^+$ T cell in response to IL-6, IL-12, and IL-15 addition. The proliferation was measured by flow cytometer on day 2 **(A)** and day 3 **(B)** following stimulations. Mean ± SED, n=3 per group.

4.1 Discussion

In this chapter, we aimed to identify optimal subthreshold conditions for T cell-stimulating studies while retaining the ability to observe the effects of cytokine addition. Three concentrations of α-CD3 (1, 2.5, and 5 µg/ml) were tested. The findings demonstrate that the concentration of 2.5 µg/ml of α-CD3 antibody delivers an adequate stimulation level to T cells, as it maintained the viability level ≥ 60%. In contrast, with a 5 µg/ml concentration of α-CD3 Ab, the viability significantly dropped on days 4 and 6. In addition, the combination of α-CD3 and α-CD28 as a costimulatory signal resulted in a moderate degree of proliferation when 2.5 µg/ml of α-CD3 antibody was used. This level of proliferation was between the effects reported with 1 µg/ml and 5 µg/ml, providing a balanced outcome in terms of both proliferation and viability.

Then, cytokine receptor expression IL-6R (CD126) and TNF-αRs receptors (CD120a & CD120b) were evaluated in response to IL-6, TNF-α, IL-2, IFN-γ stimulation. There were no significant differences in receptor expression on T cells in response to these cytokines' *ex vivo*. Similarly, *in vivo* experiments using freshly isolated PBMCs, and T cells isolated from the spleen of mice infused with Ang II for 14 days showed no variation in the expression of cytokines receptors (TNF-αRs, IL-6R, IL-4R, IL-12Rs, IL-2/IL-15Rs, IFN-γRs) compared to mice in the Sham group. The same lack of alterations was observed in mice infused with Ang II for 5 days (TNF-αRs, IL-6R).

While certain cytokines, such as IL-6 and IL-12, promote the differentiation of T cells toward CD4+ subsets and IL-15, IFN-γ toward CD8+ differentiation, there were no notable distinctions between Sham and Ang II-infused mice regarding cell differentiation into effector or memory phenotypes in response to these cytokines. Moreover, no significant differences were observed in the expression of the CD69 activation marker or in T cell proliferation among various stimulation protocols, indicating that hypertension did not affect these parameters.

Achieving optimal activation of T cells necessitates successful binding of both the antigen-specific T cell receptor and an additional co-receptor, such as CD28 (Clay and Sperling, 2007). In literature, various stimuli such as concanavalin A (ConA), phorbol myristate acetate (PMA) with a calcium ionophore (Ca-I), or a combination of antibodies targeting CD3 and CD28, are commonly used to activate T cells (Lin et al., 2000; Farrar and Ruscetti, 1986; Samelson et al., 1987). Each stimulus operates at distinct sites and employs different mechanisms to activate the T cells. α-CD3 and α-CD28 were widely used to provide the essential activation signals to activate T cells (Pulle et al., 2006; Crawley et al., 2014; Domae et al., 2017). However, the concentration of α-CD3 used in various studies varied depending on factors such as the responsiveness of the T cells and the experimental design (Mujib et al., 2012; Koenen et al., 2013; Deenick et al., 2003). This chapter examined the T cell response to 3 different concentrations of α-CD3 to study the viability, proliferation, and expansion of CD8+ and CD4+ T cells. A reduction in the percentage of live T cells occurred, starting with around 60% and reaching 20% by day 6 (Figure 4.4A and 4.5A). This decline is particularly pronounced on days 4 and 6, coinciding with the higher concentration (5 µg/ml). The result was in line with many *in vitro* studies showing that naïve T cells had a half-life of 1-2 days, where the cells undergo a rapid apoptotic phase during cell culture experiments (Deenick et al., 2003; Vella et al., 1997). When T cells are exposed to mitogenic stimulation using agonistic antibodies to the TCR/CD3 complex, no impact was on the cells' viability within the first 24 hours, and a portion of the cells undergo their first cell division (Koenen et al., 2013). Hildeman *et al* stated that naïve T cells might undergo cell death through a pathway similar to activated T cells, which involves mitochondria and proteins like Bcl-2 and Bim. Bcl-2, a key regulatory and anti-apoptotic protein, inhibits cell death by preserving mitochondrial integrity. In contrast, Bim, a pro-apoptotic protein, promotes apoptosis by regulating mitochondrial outer membrane permeabilisation. These proteins collectively contribute to the intricate balance governing T cells' fate. Naïve T cells can be protected from death by specific scavengers such as reactive oxygen species (ROS) and exhibit longer lifespans when lacking Bim (Hildeman et al., 1999). The survival of T cells seems to depend on a balance between different factors controlling cell life and death. For instance, Bim-deficient T cells have lower levels of antiapoptotic proteins like Bcl-2 and Bcl-xl, suggesting that other death-signalling proteins may regulate their survival. In normal conditions, naïve T cells likely die when the balance between Bcl-2 and Bim decreases beyond a certain threshold (Hildeman et al., 1999). *In vitro*, naïve T cells without Bim have a significantly extended

lifespan compared to normal T cells (Hildeman et al., 2002). Koenen P *et al* were shown a sharp reduction as well in the number of purified CD4+ and CD8+ T cells after 20h of stimulation with α-CD3 (Koenen et al., 2013). Overall, the short half-life of T cells *in vitro* is attributed to the absence of essential growth factors, limited physiological cues, and the lack of immune regulation in the natural environment of T cells within the body.

Deenick, Gett *et al*. investigated the impact of various concentrations of α-CD3 on T cell proliferation and found that reduction in α-CD3 concentration increased the average time cells required for their initial division (Deenick et al., 2003). This implies that weaker stimulation through the CD3 receptor delays the initiation of cell division. Moreover, α-CD3 may influence the proportion of cells entering the first division. Notably, their study encompassed a broad concentration range (5 - 40 μg/ml) (Deenick et al., 2003). This potentially explains the difficulty in detecting and observing proliferation differences within the narrower range of α-CD3 (1, 2.5, 5 μg/ml) in (Figure 4.7 & Figure 4.7). Lucas PJ *et al*. demonstrated that naive T cells exhibited a lack of proliferative response when cultured on plates coated with α-CD3 concentrations below 2.5 ug/ml; however, higher levels of α-CD3 led to observable proliferation. This is contrary to the current finding in (Figure 4.6-Figure 4.7) where T cells respond markedly to 1 ug/ml of α-CD3.

The inclusion of α-CD28 in T cell cultures exhibited an impact on cell viability, as evidenced by a substantial increase in the fold change of live cell percentages, particularly when higher concentrations of α-CD3 were maintained (Figure 4.5A). CD28 signalling enhances the survival of T cells by stimulating upregulated expression of the anti-apoptotic protein Bcl-xl, which belongs to the Bcl-2 family (Boise et al., 1993). CD28, through phosphorylation on specific tyrosine residues (Y170 in mice, Y173 in humans), recruits SH2-containing signalling molecules to support T cell survival. Disrupting these interactions with a mutated CD28 impairs Bcl-xl expression, rendering T cells more susceptible to radiation-induced cell death while still preserving anergy prevention, T cell proliferation, interleukin 2 secretion, and B cell assistance (Okkenhaug et al., 2001). This underscores CD28's role in regulating more prolonged cell survival. While CD28 co-stimulation is a crucial element in the two-step signalling process for T cell proliferation, IL-2 production, and the prevention of anergy (Janeway and Bottomly, 1994), some evidence suggests that the requirement for CD28 co-stimulation may not be absolute. This was demonstrated by the results, which showed that the addition of α-CD28 did not exhibit an additive effect on the proliferation of CD4+ or CD8+ T cells (Figure 4.6 and Figure 4.7). However, α-CD28 promoted an accelerated proliferation of CD4+ cells on day

2. This corresponds to other studies illustrating that naive T cells display a moderate reliance on B7 co-stimulation for their proliferation and IL-2 production. In the absence of B7 co-stimulation, naive T cells fail to proliferate at low peptide concentrations. However, when peptide concentrations are optimal to supra-optimal, naive T cells still undergo proliferation with B7 double knockout APCs (McAdam et al., 1998; Lucas et al., 1995; Harding et al., 1992). D28-deficient T cells exhibit an ability to initiate proliferation without maintaining the proliferative response. Some attempt was made to stimulate T cells with α-CD28 alone, and no proliferation was detected (Lucas et al., 1995; Ledbetter et al., 1985).

The optimal stimulation dose of α-CD3 and α-CD28 antibodies was observed to vary depending on the cellular state, whether naïve or previously activated T cells were used (Renate Siefken, 1998; Kalamasz et al., 2004). Additionally, the proliferative effect of CD28 depends on the type of T cell, with varying responses observed between CD4+ and CD8+ T cells despite the presence of CD28 expression in both subpopulations (Linsley et al., 1991; Damle et al., 1992; Singh et al., 2008). Notably, the results (Figure 4.6) of the stimulation among the CD4+ T cell population with/without α-CD28 demonstrated the capability of α-CD28 to induce cell cycle progression and accelerate the proliferation on day 3, with the majority of cells undergoing cell division by day 6. Maximal proliferation, reaching three- to fivefold higher levels compared to the highest concentrations achieved with α-CD3 alone, can be attained by co-stimulating cells with both α-CD28 and α-CD3 (Harding et al., 1992). Results in this chapter show that α-CD3 alone had the ability to activate and promote the proliferation of CD8+ and CD4+ T cells. In June CH *et al.* study, T cell proliferation capacity in response to α-CD3 is higher at lower concentrations compared to higher concentrations of α-CD28. Nevertheless, both α-CD3 and α-CD28 induce a dose-dependent response (June et al., 1990). *In vivo*, CD28 is not required for all T cell responses, suggesting that alternative costimulatory pathways may exist (Shahinian et al., 1993). Overall, the impact of CD28 co-stimulation on T-cell activation and proliferation involves various mechanisms. These include enhancing signal amplification upon antigen recognition, facilitating the activation and translocation of transcription factors necessary for cell-cycle progression, and IL-2 production (Diehn et al., 2002; Beyersdorf et al., 2015).

Cytokines' receptors expression in response to cytokines stimulation:

The study on IL-6, TNF-α, INF-γ, IL-2, IL-15, and IL-12, along with their receptors, should help to delineate the immunological abnormalities in hypertension. TNF-α cytokine plays a crucial role in activating or inhibiting various biological processes in immune cells. This cytokine primarily exerts its effects by interacting with two receptors, TNFR type1 (CD120a) and TNFR type 2 (CD120b). Membrane-bound TNF initiates signals through TNFR1 or TNFR2, but soluble TNF activates TNFR1 signalling (Wortzman et al., 2013). TNF plays an important role in the pathophysiology of several human inflammatory diseases (McDevitt et al., 2002). Freshly isolated T cells from both spleen and PBMCs exhibit low CD120a and CD120b expression levels on both CD4+ and CD8+ T cell subsets (Figure 4.1A and Figure 4.2A). Similarly, a study on healthy patients observed the low CD120a and CD120b expression on freshly isolated circulating T cells (DOBOSZ; Santis et al., 1992; Nagar et al., 2010). Consistent with these findings, low expression of CD120b was also detected on unstimulated T cells (Santis et al., 1992). However, after T cell stimulation with α-CD3 antibody and IL-2, the expression of CD120a and CD120b increased (Figure 4.8A and Figure 4.9A, B). The addition of cytokines did not induce any alteration in the expression levels of CD120a. Ware C *et al.* observed that CD120a expression depended on the T cell activation state induced by TCR activation for antigen or IL-2 stimulation (Ware et al., 1991). Rapid downregulation (trans-modulation) of CD120a occurs in differentiated effector T cells upon TCR or protein kinase C activation (Nagar et al., 2010). This can explain the further decrease observed in CD120a expression on CD4+ and CD8+ T cells after TNF-α addition (Figure 4.9).

TNF-α helps T cells grow and differentiate by boosting IL-2 receptor expression (Owen-Schaub et al., 1989). This creates a loop where IL-2, in turn, induces CD120a expression, making the immune response stronger. However, mature T cells quickly shed their TNFR when activated, preventing an autocrine loop with TNF. This shedding might be a short-term defence against TNF's cell-killing effects, limiting its reach and maintaining a localised inflammatory response (Ware et al., 1991). Despite the link between elevated circulating CD120a and CD120b levels and higher risks of cardiovascular events and mortality in patients with stable coronary heart disease (Carlsson et al., 2018), results showed no significant differences in the expression of these receptors on T cells isolated from the spleen or PBMCs of Sham and Ang II-infused mice. The role of TNFR1 & TNFR2 was demonstrated when the deletion of TNFR 1 and TNFR 2 genes in animal models revealed their impact on kidney diseases. Removing TNFR 1 increased glomerular

filtration rate (GFR) in a hypertension model induced by Angiotensin II. Moreover, in hypertensive mice lacking TNFR1, heightened TNFR2 mRNA expression in the kidneys corresponded to higher urinary albumin levels, suggesting TNFR2 might contribute to albuminuria (Chen et al., 2010). In an Anglo-Celtic sibling-pairs study, the TNFR2 locus was linked to hypertension and elevated TNFR2 levels (Glenn et al., 2000).

The association between Ang II-induced hypertension and T cells is closely linked to the cytokine IL-6, which exerts its biological activities through two molecules: IL-6Rα (CD126/gp80) and IL-6Rβ (CD130) (Tanaka et al., 2014; Chamarthi et al., 2011). CD126 from freshly isolated T cells showed a high CD126 expression (Figure 4.8C). Thymocytes known to express IL-6Rβ throughout their development. IL-6Rα starts to be expressed at the stage where they become either CD4+ or CD8+ T cells (Betz and Muller, 1998). However, upon stimulation of T cells with α-CD3 + IL-2, a downregulation in CD126 was observed, and the addition of the cytokine (TNF-α, IL-2, IL-6, and IFN-γ) did not show an effect on the receptor expression. Similarly, Betz U. A *et al.* showed that peripheral T cells mainly express both IL-6Rβ and IL-6Rα, but their expression decreases when TCR is engaged, both in laboratory settings and live organisms. T cells with low or absent levels of both IL-6Rβ and IL-6Rα in the periphery exhibit surface markers typical of memory T cells (Betz and Muller, 1998). The expression of gp130 and IL-6Rα in T cells is regulated based on their developmental and functional stages, which impacts their maturation and response (Betz and Muller, 1998). The presence or absence of IL-6R in T cells is closely connected to other markers like CCR7 and CD62L. Effector T cells in instances of peritonitis and those activated in vitro tend to lose IL-6R expression. IL-6 signalling through the membrane-bound IL-6R is mainly limited to naive or central memory T cells. Activated T cells that lose IL-6R may still respond to IL-6 through trans-signalling, where the cytokine interacts with a soluble form of its receptor (sIL-6R). This mechanism maintains the potential for a cellular response to IL-6, even in the absence of membrane-bound IL-6R (Jones et al., 2010). In inflamed areas, CD4+ T cells produce sIL-6R, which allows cells without IL-6R but with gp130 to still respond to IL-6 (Dienz and Rincon, 2009), and this could interpret the response observed among the CD4+ population when stimulated with IL-6 in the results (Figure 4.10). Based on the results in this chapter, the stimulated T cells from both the Sham and Ang II groups exhibited no discernible differences in their response. However, considering a relevant study revealing distinct roles of IL-6Rα and IL-6Rβ in Ang-II induced thrombosis, with IL-6Rα suggested to impact initial platelet recruitment predominantly and IL-6Rβ implicated in both thrombogenesis and thrombosis, it raises a question about the precise involvement of IL-6, IL-6R, and T

cell-dependent IL-6 signalling in Ang-II induced thrombo-inflammation within the context of hypertension (Senchenkova et al., 2019).

High IL-4R (CD124) expression was observed on both CD4+ and CD8+ T cells isolated spleen and on only CD4+ T cells from the PBMCs (Figure 4.1 & Figure 4.2). Nevertheless, the increase in IL-4R expression was IL-4-dependent, specifically when CD4+ T cells from B6 mice were cultured with Con A and IL-2; 38% of these cells up-regulated IL-4R. Conversely, cultures of CD4+ T cells from IL-4-deficient mice with Con A and IL-2 did not demonstrate an up-regulation of IL-4R. Interestingly, the addition of IL-4 from an external source induced a strong expression of IL-4R on CD4+ T cells, regardless of whether they were from B6 or IL-4-deficient mice. However, IL-4R was expressed on peripheral blood lymphocytes from healthy donors, and the expression increased in response to the activation of T cells (Dienz and Rincon, 2009; Dokter et al., 1992).

IL-7 receptor is highly expressed on CD4+ and CD8+ T cells from PBMCs and spleen (Figure 4.1 & Figure 4.2). It was demonstrated that IL-7R is essential for T cell survival, growth and proliferation following stimulation. Deletion of IL-7R in T cells resulted in reduced responses to IL-7, leading to impaired signalling, decreased survival both in vitro and *in vivo*, delayed growth, proliferation, and reduced glycolytic activity (Carrio et al., 2007; Maraskovsky et al., 1996; Jacobs et al., 2010). Both CD4+ and CD8+ T cells exhibited high expression of IL-7R, as evident from (Figure 4.1and Figure 4.2) in both PBMCs and spleen samples. This highlights the crucial role of IL-7R in sustaining T cell metabolism, impacting their development, homeostatic proliferation, and survival. Many studies underscore the significance of CD127 (IL-7R) expression in distinguishing memory and effector T cell subsets. CD127 is a marker for long-lasting memory T cells, aiding in the differentiation between central and peripheral effector memory T cells (Huster et al., 2004). It is pivotal in regulating the balance between effector and central memory T cell subsets, influenced by factors like antigen exposure and time (Bachmann et al., 2005). In HIV-infected individuals, IL-7-mediated down-regulation of CD127 on CD8+ T cells, particularly affecting naive cells, suggests implications for T-cell dysfunction (Vranjkovic et al., 2007). These findings collectively highlight CD127 as a critical factor in T cell responses, and immune memory lower CD127 expression appears to contribute to the regulatory function and potential longevity of T cell by limiting their activation and proliferation (Hartigan-O'Connor et al., 2007). No differences in the expression of IL-7 were detected in our results among T cell subsets from sham and Ang II-infused mice.

In humans, IL-12Rβ1 was shown to be barely detectable in CD4+ and CD8+ T cells (Zaki et al., 2001), which is compatible with the results (Figure 4.1 & Figure 4.2). The expression of IL-12Rβ1 on T cells can be markedly enhanced after activation or under disease conditions (Zaki et al., 2001). According to De Beaucoudrey L's study, mutations in IL12Rβ1 could impact the development of IL-17–producing T cells, highlighting the significance of IL-12Rβ1–dependent signals (de Beaucoudrey et al., 2008). Furthermore, Miller HE and Robinson RT demonstrate that individuals lacking IL12Rβ1 exhibit increased susceptibility to recurrent mycobacterial infections, emphasising the role of IL12Rβ1 in combating infections like tuberculosis through T cells and IFN-γ production (Miller and Robinson, 2012). These findings collectively emphasise IL12Rβ1's importance in shaping immune responses, particularly in combating infectious diseases. Similarly, Low IL-1R1 (CD121a) expression was detected in T cells. Research indicated that mice lacking IL-1 signalling, either deficient in both IL-1α and IL-1β or lacking IL-1R1, develop normally in stable conditions (Labow et al., 1997; Horai et al., 1998; Glaccum et al., 1997). Moreover, although IL-1R1 expression is not abundant on the cell surface, only a few ligand-occupied receptors per cell are already sufficient to induce a strong response, as the high-affinity membrane-bound receptor binds (Subramaniam et al., 2004). However, no expression difference was detected on T cells from Sham and Ang II-infused mice.

Similar to IL-12R and IL-1R, IL-17R showed low expression on both T cell subsets. A study with immunoprecipitation and immunoblot analysis showed that total cellular IL-17R protein quantities were undetectable in naive T cells. However, IL-17R was upregulated at late stages during the Th1 differentiation program, with moderate amounts of cell surface IL-17R detectable by day 4 (Subramaniam et al., 2004). The data shows no difference in the expression among Ang II and Sham groups. This was similar to other study data, which showed that the increase in IL-17 protein level in the aorta is not due to increased IL-17 receptor expression, as this was similar between Sham and Ang II-treated aortas (Madhur et al., 2010). It was reported that inhibiting IL-17 receptor unit A by a neutralising antibody lowered blood pressure in Ang II-infused mice (Saleh et al., 2016).

The data reveals expression of Ifn-γR1 (CD119) levels is low in spleen T cells but high in PBMCs, whereas Ifn-γR2 was low in both spleen and PBMC T cells. The observed variation in Ifn-γR1 and Ifn-γR2 expression between spleen and PBMC T cells aligns with a study outlining the complex regulation of these receptors upon ligand engagement and internalisation (Claudinon et al., 2007). Such differential expression might suggest distinct

regulatory mechanisms governing receptor internalisation, recycling, and responsiveness to external cues in these T cell populations (de Weerd and Nguyen, 2012).

The expression of IL-2Rs on T cells varies in response to stimulation, reflecting their activation status and involvement in modulating immune responses, which can be crucial in both physiological immune reactions and pathological conditions. Under normal "unstimulated" conditions, T cells, including both CD4+ and CD8+ subsets, exhibit varying expression levels of CD25 and CD122. Typically, a small fraction of resting T cells expresses CD25 at low levels, while CD122 is constitutively expressed on most T cells, which is in line with the current results (Figure 4.1). However, both receptors are low expressed in the spleen (Figure 4.2). During stimulation or pathological conditions, T cell activation can significantly affect the expression of CD25 (Kmieciak et al., 2009) and CD122 (Kmieciak et al., 2009; Suarez-Ramirez et al., 2014). Activation often leads to increased CD25 expression, which serves as a marker of T cell activation and proliferation (Suarez-Ramirez et al., 2014). Both CD4+ and CD8+ T cells can upregulate CD25 upon encountering antigens and cytokines or during inflammatory responses (Clénet et al., 2017). CD122 expression remains relatively stable but may also increase under activated or disease states, particularly in response to IL-2 or other cytokine signals (Bastidas et al., 2014), promoting T cell survival and effector functions (Kmieciak et al., 2009; Bastidas et al., 2014). However, under hypertensive conditions, the differential expression of CD25 and CD122 on T cells was not observed. In addition, despite observed differences in the expression of CD122 in SLE patients indicating lymphocyte activation (Chan et al., 1996). The lack of correlation between cytokine receptor expression, such as IL-2Rs, IL-4Rs, IL-6R, IL-12R, IL-1R, TNFRs, IL-17Rs, IL-7, and IFN-γ, and hypertension suggests that these specific cytokine receptor systems might not directly contribute to disease activation in hypertension.

Differentiation in response to cytokines stimulation:

IL-6 plays a pivotal role in the expansion and persistence of memory and effector CD4+ T cells. It also extends the lifespan of T cells in vitro by preserving Bcl-2 levels and reducing the expression of a pro-apoptotic molecule, Fas ligand. The observed significant increase in cell viability in response to IL-6 in the results in (Figure 4.11) can be attributed to IL-6's pivotal role in promoting the expansion and persistence of memory and effector CD4+ T cells. Additionally, IL-6 extends the lifespan of T cells in vitro by preserving Bcl-2 levels and reducing the expression of the pro-apoptotic molecule, Fas ligand (Rochman et al., 2005; Teague et al., 1997). The percentage of memory CD4+ T cells was significantly high, while the percentage of the effector cells was low (Figure 4.10). IL-6 found to facilitate the activation and proliferation of T cells. Exposure of T cells to IL-6 resulted in a long-term increase in the frequency of memory CD4+ T cells. When IL-6 is present during the initial phase of immunisation, there is a more robust expansion of antigen-specific CD4+ T cells, resulting in increased frequency and numbers of these cells in various organs. Furthermore, it was found that blocking IL-6 or using IL-6 knockout mice reduces the frequency of antigen-specific CD4+ T cells, indicating that IL-6 is involved in their expansion and persistence over extended periods (Rochman et al., 2005). IL-6 plays a role in enhancing the magnitude of specific immune responses by increasing the yield of long-lived effector/memory CD4+ T cells. Likewise, the memory response against a second, heterologous influenza infection is impaired in IL-6 gene-deficient mice, which coincides with reduced T cell numbers in the lung (Longhi et al., 2008; Nish et al., 2014). On CD8+ T cell, on the other hand, IL-6 might not be a critical factor in this specific context for generating CD8+ T-cell memory, whereas, in APC-based vaccination models, IL-6 provided by APCs seems to be dispensable for the proper generation of CD8+ T-cell memory (Daudelin et al., 2013).

Various studies showed the role of IL-15 on CD8+ T cells and its ability to induce T cell proliferation and differentiation in an antigen-independent manner. IL-15 demonstrated the ability to keep memory CD8+ T cells up to day 6 (Figure 4.10C), and this is similar to Weng NP *et al.*, who found that IL-15 did not only help these CD8 memory T cells proliferate but also acts as a trigger for their activity independently of encountering the specific antigen secondary challenge (Weng et al., 2002; Prlic et al., 2002). IL-15 plays a crucial role in the characteristics of the secondary memory CD8+ T cell pool after the immune system encounters the same pathogen for a second time. The absence of IL-15 affected the generation and characteristics of these secondary memory CD8 T cells, impacting their ability to proliferate, survive, and display specific surface markers such as

CD27 and CCR7 compared to normal mice with an intact IL-15 signalling pathway (Sandau et al., 2010). In animals lacking IL-15, the immune response exhibited significant changes in CD8+ memory cell types. These animals could not generate effector memory cells, particularly those with low levels of IL-7Rα. Another subset of memory cells, characterised by high CD27 levels but minimal granzyme B essential for eliminating infected cells, was notably affected by the absence of IL-15. Furthermore, the deficiency of IL-15 led to reduced cell growth and impaired survival among these memory CD8 cells, attributed to alterations in the expression of Bcl-2, a critical molecule for cell survival (Sandau et al., 2010). L-15Rα−/− animals exhibit a decrease in the quantity of peripheral CD8 T cells, particularly in memory-phenotype CD8 T cells, compared to naive CD8 T cells (Lodolce et al., 1998). The reduced abundance of naive CD8 T cells in the absence of IL-15Rα is mainly caused by a reduction in the survival of these cells (Berard et al., 2003). In the context of an *in vivo* study utilising tetanus toxoid (TT) and influenza (Flu) (antigen-dependent) vaccines in primate models, the investigation aimed to discern the impact of IL-15 and IL-2 on memory T cell dynamics post-primary and secondary immunisations. IL-2 exhibited efficacy in augmenting the immediate response of specific cell populations to both Flu and TT antigens. However, its influence on the establishment of enduring memory cells was limited. In contrast, IL-15's effect on immediate response augmentation was relatively modest; nevertheless, its role in substantially amplifying the development of persistent, long-term memory cell pools following the administration of Flu and TT vaccines was markedly evident (Villinger et al., 2004). Despite the various roles elucidated for IL-15 in memory CD8+ T cell populations and its impact on immune responses, there appeared to be no discernible differentiation effect on T cells from hypertensive mice compared to sham mice. This lack of differentiation effect might align with the absence of differences observed in cytokine receptor expression between the two groups.

In transgenic mice with heightened IL-7 levels, there's a notable increase in memory phenotype CD8+ cells. Elevated IL-7 assist TCR recognition of self-MHC ligands, prompting naive CD8+ cells to become memory cells as well. Additionally, in mice lacking IL-15 but with increased IL-7, there's a significant rise in CD8+ memory cells, indicating that IL-7 can compensate for the usual dependency on IL-15 (Kieper et al., 2002). It was proven that IL-15 specifically drives the proliferation of the human CD4+ T_{EM} subset in vitro (Geginat et al., 2003). However, while rodent studies emphasise IL-15's pivotal role in regulating CD8+ memory T cell proliferation, the exact impact hasn't been consistently observed in the CD4+ memory compartment (Tan et al., 2002; Surh and Sprent, 2005; Villinger et al., 2004). Notably, in hosts lacking both IL-7 and IL-15, the

homeostatic proliferation of CD44hi CD8 cells is nearly abolished, contrasting with the unimpaired proliferation of CD44hi CD4 cells (Tan et al., 2002).

IL-12 promotes the activation and differentiation of naive CD4+ and CD8+ T cells into effector T cells upon encountering antigens. It stimulates T cells to produce cytokines like IFN-γ, vital for combating infections (Kieper et al., 2001). Pearce and Shen showed that IL-12 contributes to the generation and maintenance of memory T cells, ensuring a robust and prolonged immune response upon subsequent encounters with a pathogen. It aids in differentiating effector cells into long-lived memory cells, which is crucial for sustained immunity (Pearce and Shen, 2007). Additionally, IL-12 supports the proliferation and survival of CD8+ memory T cells, helping to maintain a pool of memory cells that can rapidly respond upon re-exposure to antigens (Kieper et al., 2001). IL-12 plays a crucial role in directing memory CD4 cells towards becoming effector subsets. This is evident in the results, showing a notable increase in memory T cells within both the CD4+ and CD8+ populations (Figure 4.10). Specifically, it prompts the development of effector cells that produce high levels of IFN-γ while producing minimal IL-2, contributing to the polarisation of these subsets towards a Th1-like phenotype (Bradley et al., 1995). IL-12 serves as a pivotal factor in the generation, functionality, and maintenance of effector and memory CD4+ and CD8+ T cell populations, contributing significantly to the immune system's ability to fight infections and maintain long-term immunity. Pearce EL, and Shen H found that IL-12-deficient mice displayed a weaker initial CD8 T cell response but developed significantly more memory CD8 T cells, leading to enhanced protective immunity upon reinfection with Listeria monocytogenes (Pearce and Shen, 2007). This suggests that IL-12 plays a dual role: promoting immediate effector responses while impeding the formation of memory T cell precursors. In the context of T cell responses, IL-12 is crucial in promoting robust effector differentiation in CD8 T cells, fostering expansion, and enhancing sensitivity to homeostatic cytokines like IL-7 and IL-15, ultimately bolstering immunity and controlling infections (Li et al., 2006). In our results, IL-12 did not induce a differential response in T cell differentiation in hypertensive animals.

Proliferation in response to cytokines stimulation:

Although many cytokines were proven to induce proliferation (Stonier et al., 2008; Kieper et al., 2002; Villinger et al., 2004). The investigation into individual cytokine-induced proliferation of T cells, such as TNF-α, IL-6, IL-15, and IL-12, revealed no distinct impact on T cell proliferation (Figure 4.15 & Figure 4.16). The response observed in T cells stimulated with α-CD3+ α-CD28 or α-CD3 alone was comparable to those stimulated with α-CD3+ α-CD28 or α-CD3 alongside cytokines, suggesting that TCR stimulation might overshadow or obscure the effects of these cytokines on proliferation. It is also possible that the T cells reached a saturation point in responsiveness, where optimal activation through α-CD3 resulted in a plateauing of signalling pathways, potentially hindering further proliferation with the addition of α-CD28 or cytokines (Roosnek et al., 1987). Additionally, it was found that once a threshold dose of antigenic stimulation was reached, antigen-independent T cells expansion did not significantly increase (Tripathi et al., 2007). Moreover, the proliferation responses remained similar in T cells obtained from Sham and Ang II-infused mice, indicating a consistent lack of discernible modulation effect in this context. Modifications in the stimulation protocol could be considered to enhance the understanding of cytokine-induced proliferation. Overall, these findings suggest a potential dominance of α-CD3-induced TCR activation and possible saturation or threshold sensitivity, constraining additional effects of individual cytokines or CD28 co-stimulation on T cell proliferation within this experimental context

Chapter 5 Cytokine-Priming of T cells in response to Ang II Induction in Mice Spleen and PBMCs

5.1 Introduction

Cytokines are central in regulating the immune system, and their effects on T cells are fundamental. Stimulating T cells with cytokines is essential to achieve a stable cellular response (Unutmaz et al., 1999). Cytokines can activate and modulate T cell functions, influencing their proliferation, differentiation, and effector capabilities. This is crucial for mounting effective immune responses against various pathogens and maintaining immune homeostasis (Khan and Khan, 2016; Unutmaz et al., 1999). In the context of hypertension, loads of evidence suggests a significant involvement of T cells in the development of hypertension. Hypertension is increasingly recognised as an immune-related condition, and alteration of the pro-inflammatory cytokines profile can contribute to the complex network of immune dysregulation associated with high blood pressure (Peeters et al., 2001). Understanding how cytokines stimulate T cells in hypertensive conditions is pivotal for deciphering the immune mechanisms underlying hypertension and exploring potential avenues for therapeutic intervention. Examining the interplay between cytokine-driven T-cell activation and hypertension presents promise as a robust initial step in investigating the intricate involvement of T cells in this complex cardiovascular disease.

Cytokine priming is an alternative approach for investigating T cell activation and response mechanisms. The role of priming is evident (Banerjee et al., 2005) when cytokines-primed T cells exhibit heightened responsiveness to TCR signals, which quickly act on the IκBα pathway. This response leads to rapid mobilisation of c-Rel to the nucleus and the subsequent c-Rel-dependent gene expression. The absence of priming via inflammatory cytokines in c-Rel-deficient T cells, both *in vivo* and *ex vivo*, underscores the importance of c-Rel in this process (Banerjee et al., 2005).

Mescher *et al.* proposed that, aside from signals from antigen and co-stimulatory receptors, naïve CD8+ T cells require a 'third' signal from cytokines such as IL-12 or IFN-I for optimal proliferation and effector functions (Mescher et al., 2006). While the molecular events controlling this third signal remain unclear, recent evidence suggests that IL-12 is essential for upregulating Bcl-3, whereas antigen- and co-stimulatory-derived signals alone upregulate Bcl-2 and Bcl-xl. When IL-12 was primed in vitro with CD8+ T cells, it provided better *in vivo* protection against viral infection (Chang et al., 2004). However, primed CD8+ cells under conditions lacking IL-12 did not exhibit defects in primary or memory T cell responses *in vivo*. Moreover, following short-term stimulation of T cells from the lungs of M. tuberculosis-infected mice with α-CD3/α-CD28, a distinctive pattern

emerges in regulating cytokine (IFN-γ) production between CD4+ and CD8+ T cells. This suggests that the observed differential regulation between CD4+ and CD8+ subsets may arise due to either antigen presentation of mycobacterial antigens or the influence of immunoregulatory cytokines within the pulmonary microenvironment (Serbina and Flynn, 1999). In essence, the presence of these cytokines contributes to the existing differential regulation in the production of IFN-γ between CD4 and CD8 T cells. Consequently, priming T cells with cytokines can provide valuable insights into the underlying regulatory processes and give distinctive T cell responses in vitro.

In this chapter, an alternative method was applied to study CD4+ and CD8+ T cell proliferation, where T cells were primed with various cytokines before a short-term stimulation with α-CD3 antibody. This process might become compelling when examined within the context of Ang II-induced hypertension, a condition closely linked to immune system dysregulation. This dysregulation is evident by local inflammation occurring in the target organs affected by hypertension, where the expression of pro-inflammatory cytokines is increased.

5.2 Chapter Aim

- To assess the effect of various cytokine pre-priming on T cell function in the context of Ang II-induced hypertension

5.3 Results

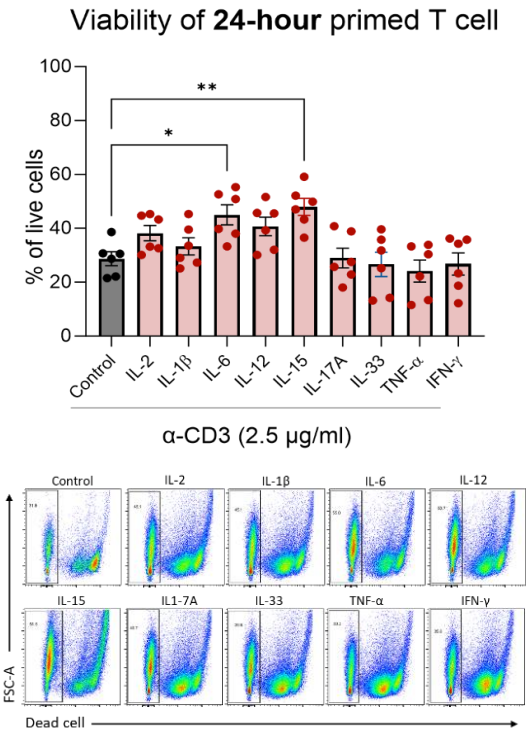
5.3.1 24- Vs 48-hour priming T cell with cytokines

To investigate the capability and capacity of individual cytokines to induce T cell differentiation and proliferation independent of TCR-mediated signals, isolated splenic T cells were primed with IL-2, IL-1β, IL-6, IL-12, IL-15, IL-17A, IL-33, TNF-α and IFN-γ. This priming process was conducted over two intervals, 24 hours (Figure 5.1) and 48 hours (Figure 5.2), to determine the optimal priming period. Notably, the 24-hour priming period maintained higher cell viability than the 48-hour duration, while both intervals yielded similar effects on proliferation and differentiation. IL-6 and IL-15 showed the ability to induce the survival of T cells significantly. The percentage of live cells with IL-6 and IL-15 was 44 ± 5 % - 47 ± 5 % for 24 hours compared to 21± 4 – 24 ± 4 % for 48 hours, respectively (Figure 5.1A).

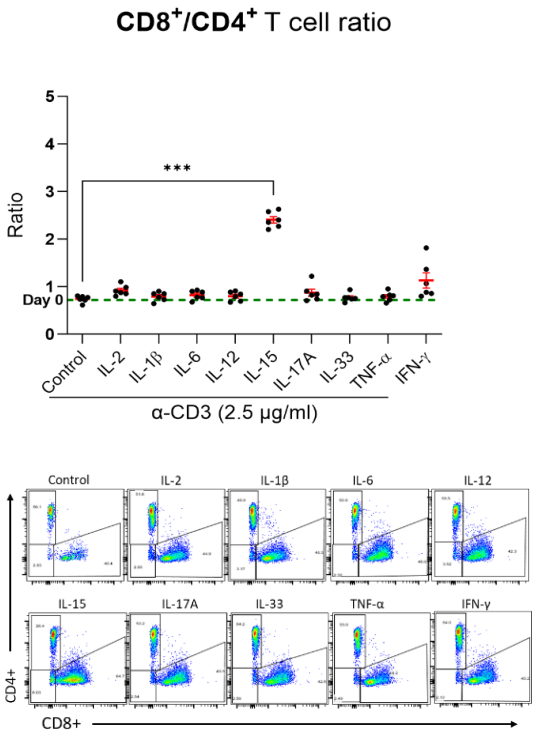
Despite the relatively high percentage of proliferated CD4+ T cells, similar to CD8+ T cells, in response to IL-15, it is worth noting that this level of proliferation **(**Figure 5.1**C)** only resulted in pushing the cell cycle to two generations, as evidenced by the presence of two peaks in the histogram. In contrast, among CD8+ T cells, IL-15 pushed the cell's cycle up to 5 ± 1 peaks. With 48-hour priming, the reduction effect on the viability manifested that among IL-6 life cells, the majority were CD4+ T cells, unlike IL-15, where CD8+ T cell was the majority (Figure 5.2C).

With both durations, IL-15 markedly directed cell expansion toward CD8+ T cells, as reflected by significantly higher proliferation among CD8+ T cells (Figure 5.2C). IL-2 exhibited pronounced proliferative effects within the CD8+ T cell subset with a 48-hour priming period, while IL-6 demonstrated a proliferation effect, impacting CD8+ and CD4+ T cell populations.

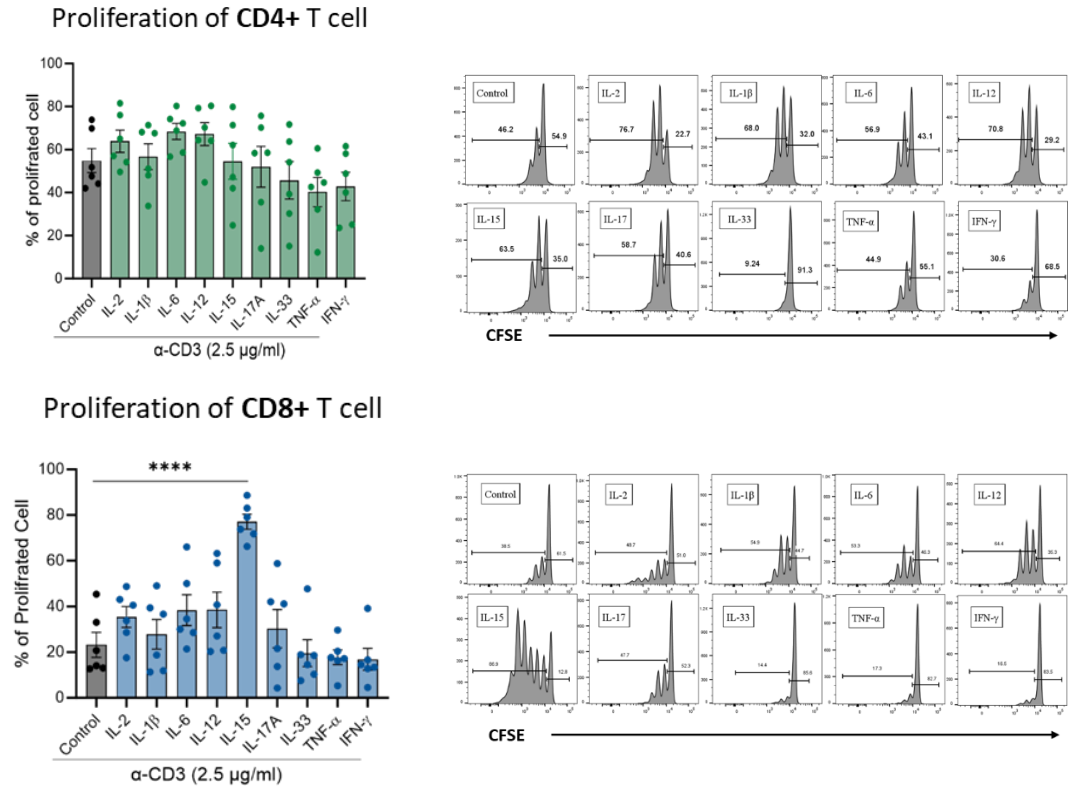
A


Viability of **24-hour** primed T cell
% of live cells
100
80
60
40
20
0
**
*
Control
IL-2
IL-1β
IL-6
IL-12
IL-15
IL-17A
IL-33
TNF-α
IFN-γ
α-CD3 (2.5 μg/ml)
Control
IL-2
IL-1β
IL-6
IL-12
IL-15
IL1-7A
IL-33
TNF-α
IFN-γ
FSC-A
Dead cell

B


CD8⁺/CD4⁺ T cell ratio
Ratio
5
4
3
2
1
Day 0
0
***
Control
IL-2
IL-1β
IL-6
IL-12
IL-15
IL-17A
IL-33
TNF-α
IFN-γ
α-CD3 (2.5 μg/ml)
Control
IL-2
IL-1β
IL-6
IL-12
IL-15
IL-17A
IL-33
TNF-α
IFN-γ
CD4+
CD8+

C


Proliferation of **CD4+** T cell
% of prolifrated cell
100
80
60
40
20
0
Control
IL-2
IL-1β
IL-6
IL-12
IL-15
IL-17A
IL-33
TNF-α
IFN-γ
α-CD3 (2.5 μg/ml)
Control
46.2
54.9
IL-2
76.7
22.7
IL-1β
68.0
32.0
IL-6
56.9
43.1
IL-12
70.8
29.2
IL-15
63.5
35.0
IL-17
58.7
40.6
IL-33
9.24
91.3
TNF-α
44.9
55.1
IFN-γ
30.6
68.5
CFSE
Proliferation of **CD8+** T cell
% of Prolifrated Cell
100
80
60
40
20
0
****
Control
IL-2
IL-1β
IL-6
IL-12
IL-15
IL-17A
IL-33
TNF-α
IFN-γ
α-CD3 (2.5 μg/ml)
Control
IL-2
IL-1β
IL-6
IL-12
IL-15
IL-17
IL-33
TNF-α
IFN-γ
CFSE

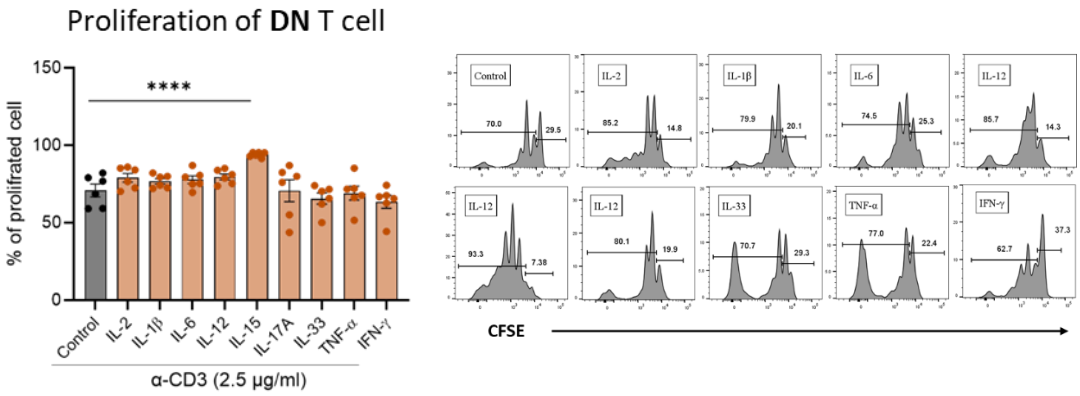
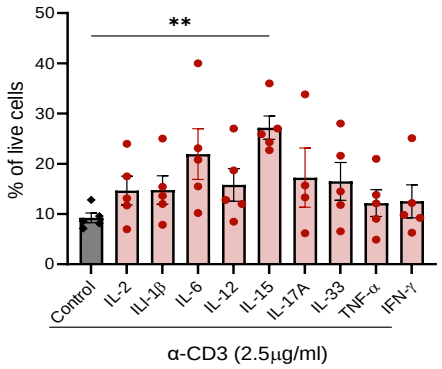


Figure 5.1. Effect of 24-hour cytokine priming on T cells Viability and proliferation.

Spleen isolated-T cells were primed with cytokines (IL-2, IL-1β, IL6, IL-12, IL-15, IL-17A, IL-33, TNF-α, IFN-γ) individually for 24 hours prior to stimulating with α-CD3 (2.5mg/μl) coated plate. **(A)** T cell Viability, **(B)** CD8+: CD4+ subsets ratio and **(C)** proliferation response of CD4+, CD8+, DN T cells were analysed using FACS on day 2 after stimulation**.** Data are expressed as mean ± SEM from n=6 experiments. Statistical significance (**** P<0.0001) was determined by a one-way ANOVA (Kruskal-Wallis) test, followed by Dunn's multiple comparison adjustment.

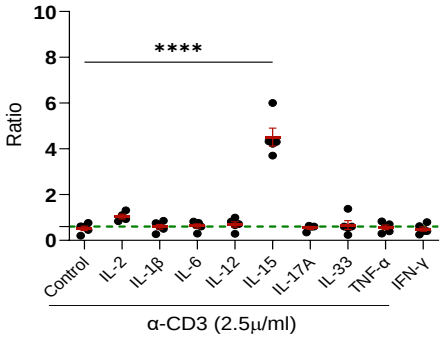
A

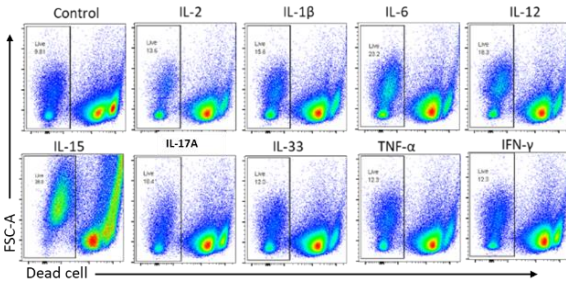
Viability of **48-hour** Cytokine-primed T cell

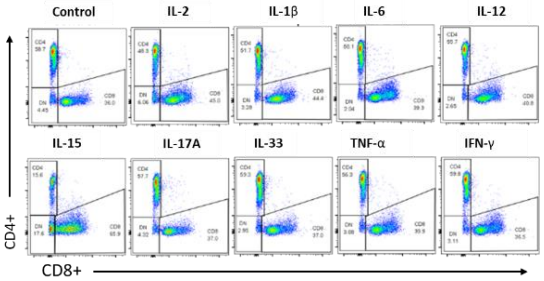


B

CD8⁺ : CD4⁺ T cell ratio

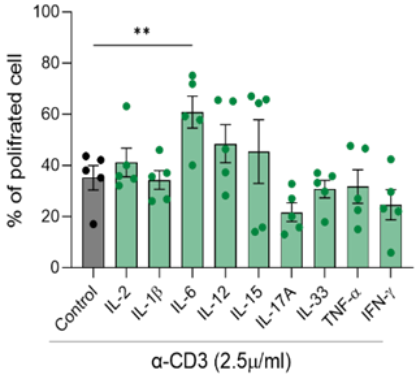


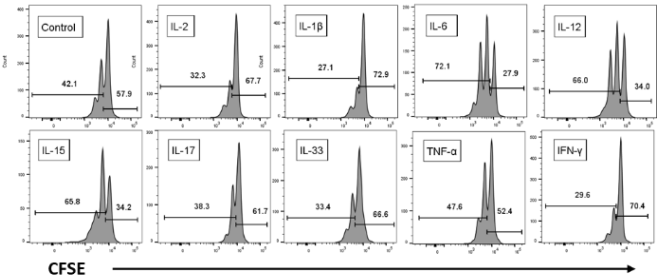




C

Proliferation of **CD4+** T cell





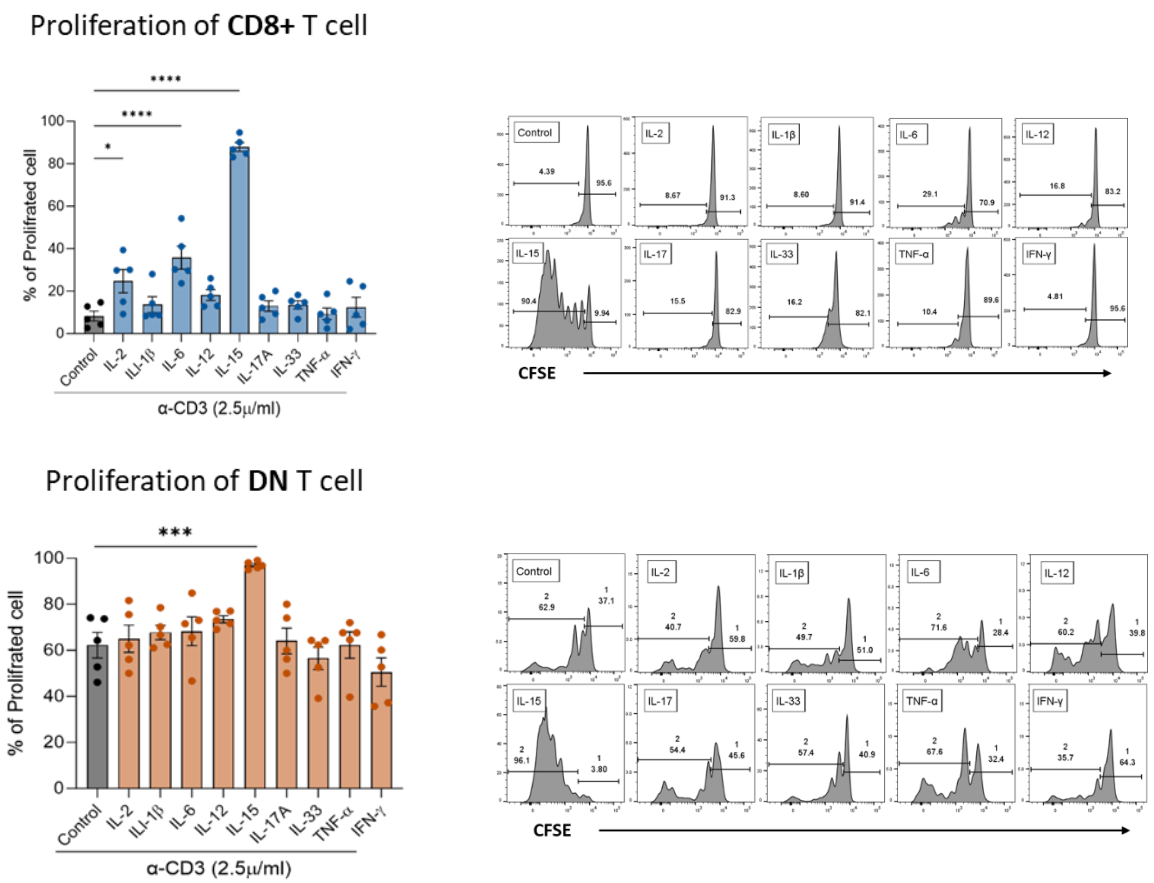


Figure 5.2. Effect of 48-hour cytokine priming on T cells Viability and proliferation.

Spleen isolated-T cell was primed with individual cytokines (IL-2, IL-1β, IL-6, IL-12, IL-15, IL-17A, IL-33, TNF-α, IFN-γ) for 48 hours prior stimulating with α-CD3 antibody (2.5mg/μl) coated plate. **(A)** T cell viability, **(B)** $CD8^{+}$: $CD4^{+}$ subsets ratio and **(C)** proliferation response were analysed using FACS on day 2 after stimulation**.** Data are expressed as mean ± SEM from n=5 experiments for IL-1β,IL-6,IL-12, IL-15, IL-33,TNF-α and IFN-γ , n=4 experiments for IL-2 and IL-17A. Statistical significance (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001) was determined by a one-way ANOVA (Kruskal-Wallis) test, followed by Dunn's test for multiple comparison adjustment.

5.3.2 24-hour cytokine priming of T cells isolated form spleens of normotensive and hypertensive animals

Based on the previous results, which show a distinctive effect of IL-15 on T cells, IL-7 and IL-4 were included to be studied in a hypertensive context. The stimulation protocol was used with a 24-hour priming period to measure the effect of the cytokines on T cells from the spleen of sham vs hypertensive mice. IL-7 and IL-15 showed a distinctive impact on T cells where the percentage of the CD8+ population was higher than CD4+ (

Figure 5.5 A & B). In addition, the proliferation of T cells was induced significantly among CD8+ and DN T cells in response to IL-15 and IL-7. However, a similar response was detected in T cells collected from Sham and Ang II animals. In addition, IL-6 had also increased the CD8+ proliferation in both studied groups, although to a lesser extent than IL-15 and IL-7.

Other cytokines, including IL-2, IL-1β, IL-17, IL-33, IL-4, and IFN-γ, exhibit no significant proliferation effect compared to the control (α-CD3). The expansion of T cells with these cytokines was directed towards CD4+ T cells (Figure 5.3), leading to the proliferation of the CD4+ T cell population (Figure 5.4A). Conversely, the proliferation among CD8+ T cells was minimal (Figure 5.4B). In addition, the DN population showed a high proliferation response to all these cytokines (Figure 5.4C); however, no discernible difference was detected compared to the sham Ang II animal.

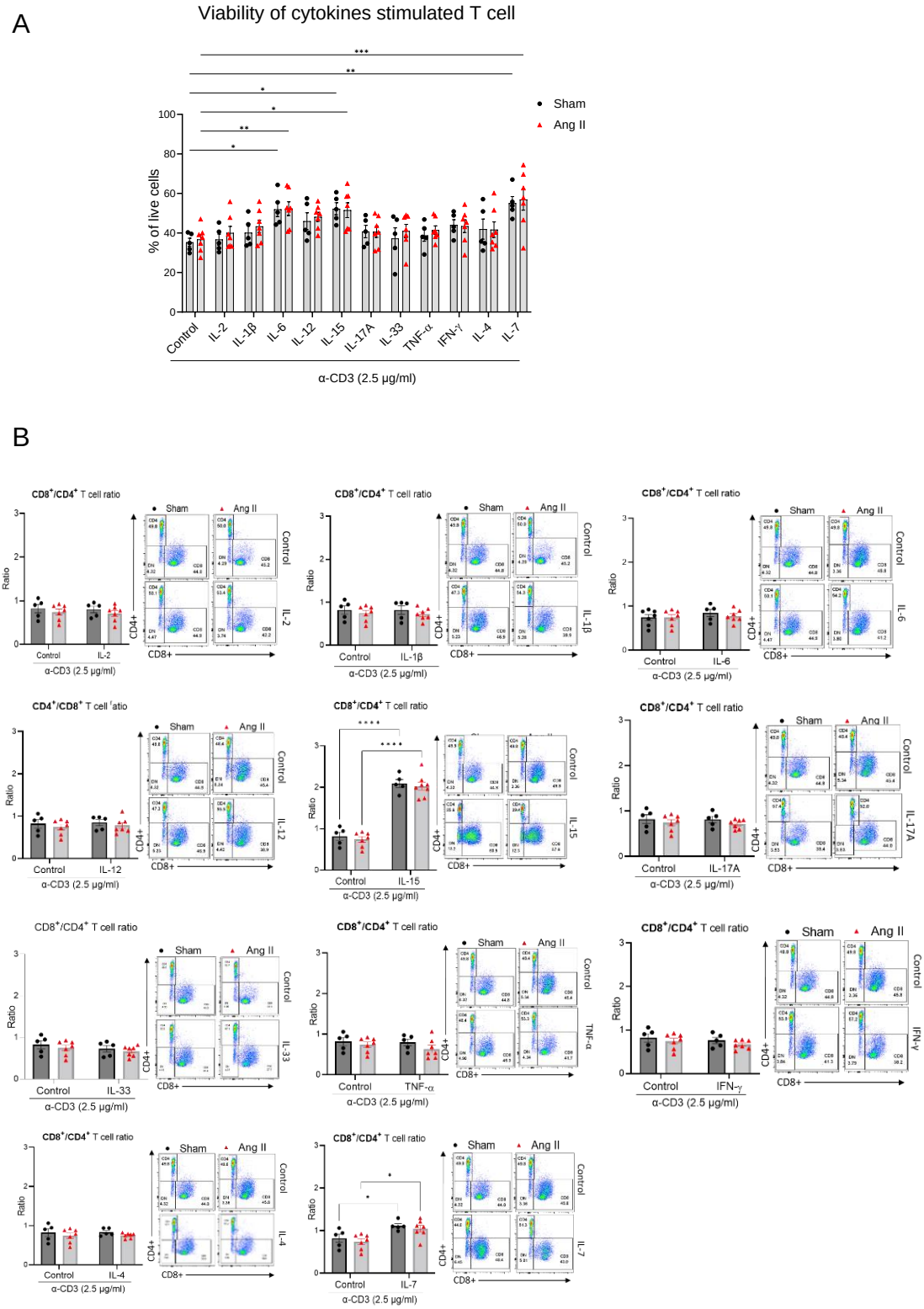
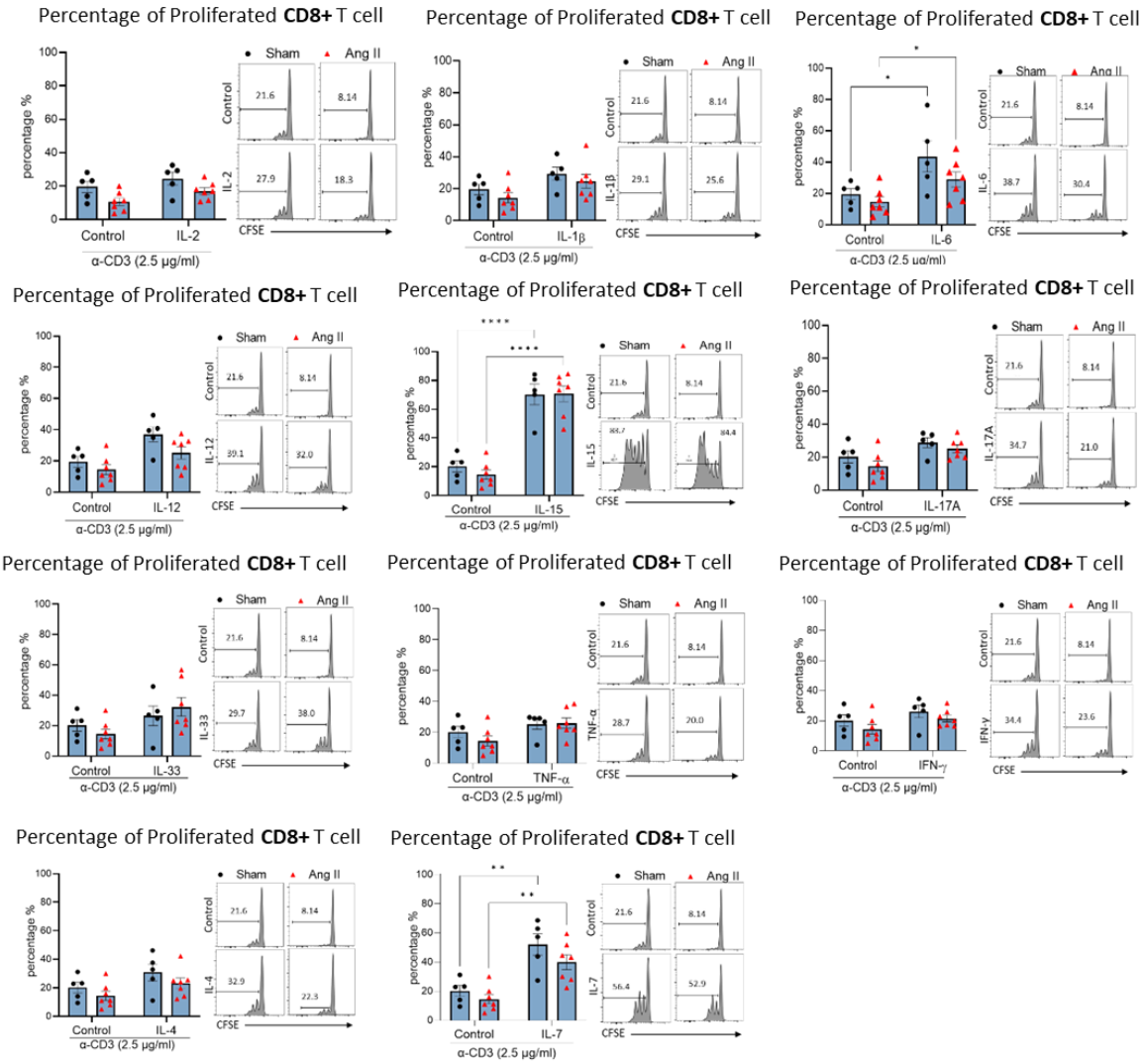


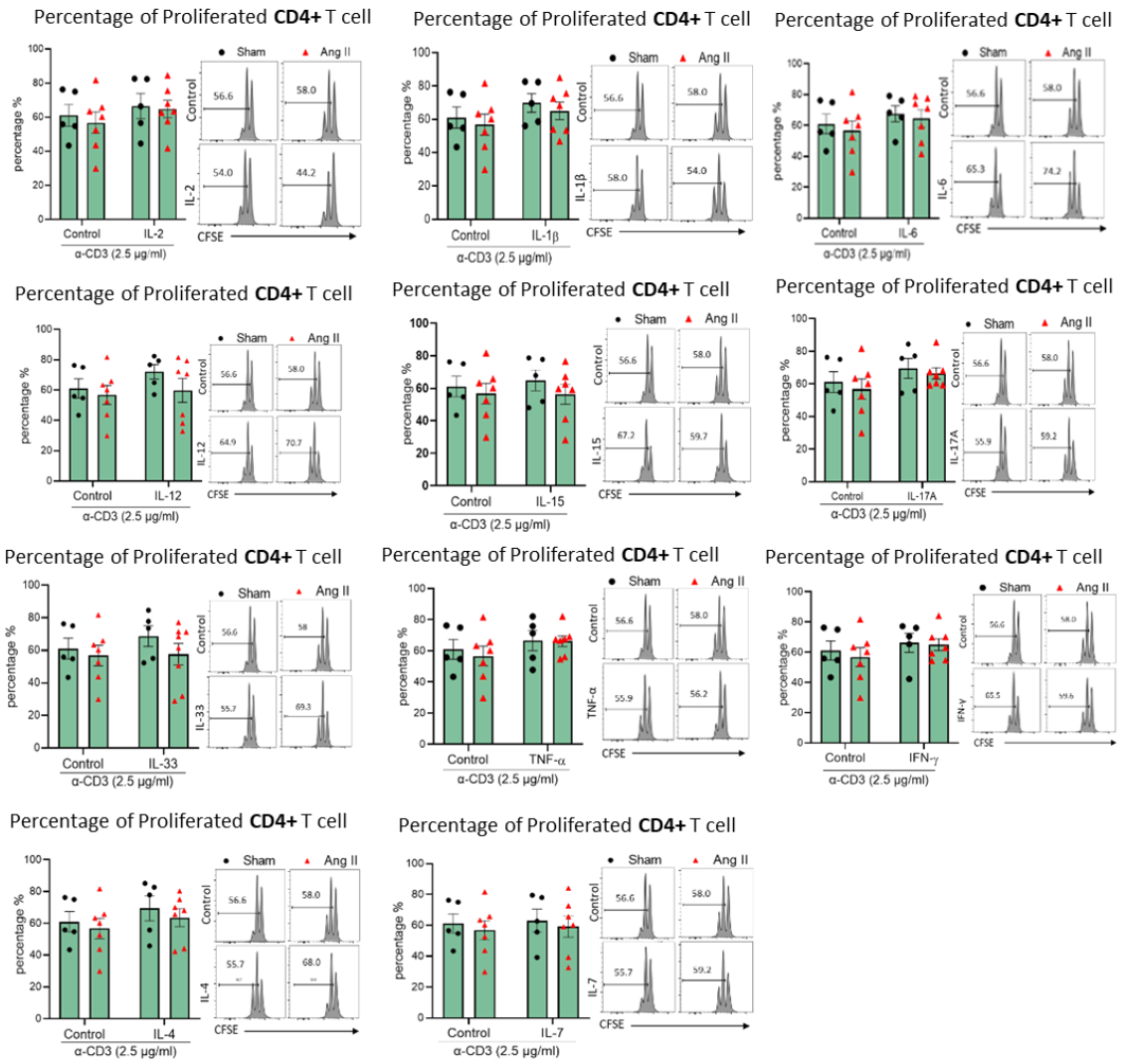
Figure 5.3. Effect of cytokine priming (24 hours) on viability and expansion of T cells from Sham and Ang II infused mice.

Spleen isolated T cells were primed with individual cytokines (IL-2, IL-1β, IL-6, IL-12, IL-15, IL-17A, IL-33, TNF-α, IFN-γ, IL-7 and IL-4) for 24 hours prior stimulating with α-CD3 coated plate (2.5mg/μl). T cell viability **(A),** $CD8^+$:$CD4^+$ subsets ratio **(B)** was analysed using FACS on day 2 after stimulation**.** Data are expressed as mean ± Standard error of the mean (SEM) from sham=5 /Ang II=7 experiments. Statistical significance (* P<0.05, ** P < 0.01, *** P<0.001, **** P<0.0001) was determined by a 2-way ANOVA test followed by Tukey test multiple comparison adjustment

A


Percentage of Proliferated CD8+ T cell
percentage %
Sham
Ang II
Control
IL-2
IL-1β
IL-6
IL-12
IL-15
IL-17A
IL-33
TNF-α
IFN-γ
IL-4
IL-7
CFSE
α-CD3 (2.5 μg/ml)

B


Percentage of Proliferated CD4+ T cell
Sham
Ang II
percentage %
Control
IL-2
α-CD3 (2.5 μg/ml)
CFSE
56.6
58.0
54.0
44.2
Percentage of Proliferated CD4+ T cell
IL-1β
65.3
74.2
Percentage of Proliferated CD4+ T cell
IL-6
Percentage of Proliferated CD4+ T cell
IL-12
64.9
70.7
Percentage of Proliferated CD4+ T cell
IL-15
67.2
59.7
Percentage of Proliferated CD4+ T cell
IL-17A
55.9
59.2
Percentage of Proliferated CD4+ T cell
IL-33
55.7
69.3
Percentage of Proliferated CD4+ T cell
TNF-α
55.9
56.2
Percentage of Proliferated CD4+ T cell
IFN-γ
65.5
59.6
Percentage of Proliferated CD4+ T cell
IL-4
55.7
68.0
Percentage of Proliferated CD4+ T cell
IL-7
55.7
59.2

C

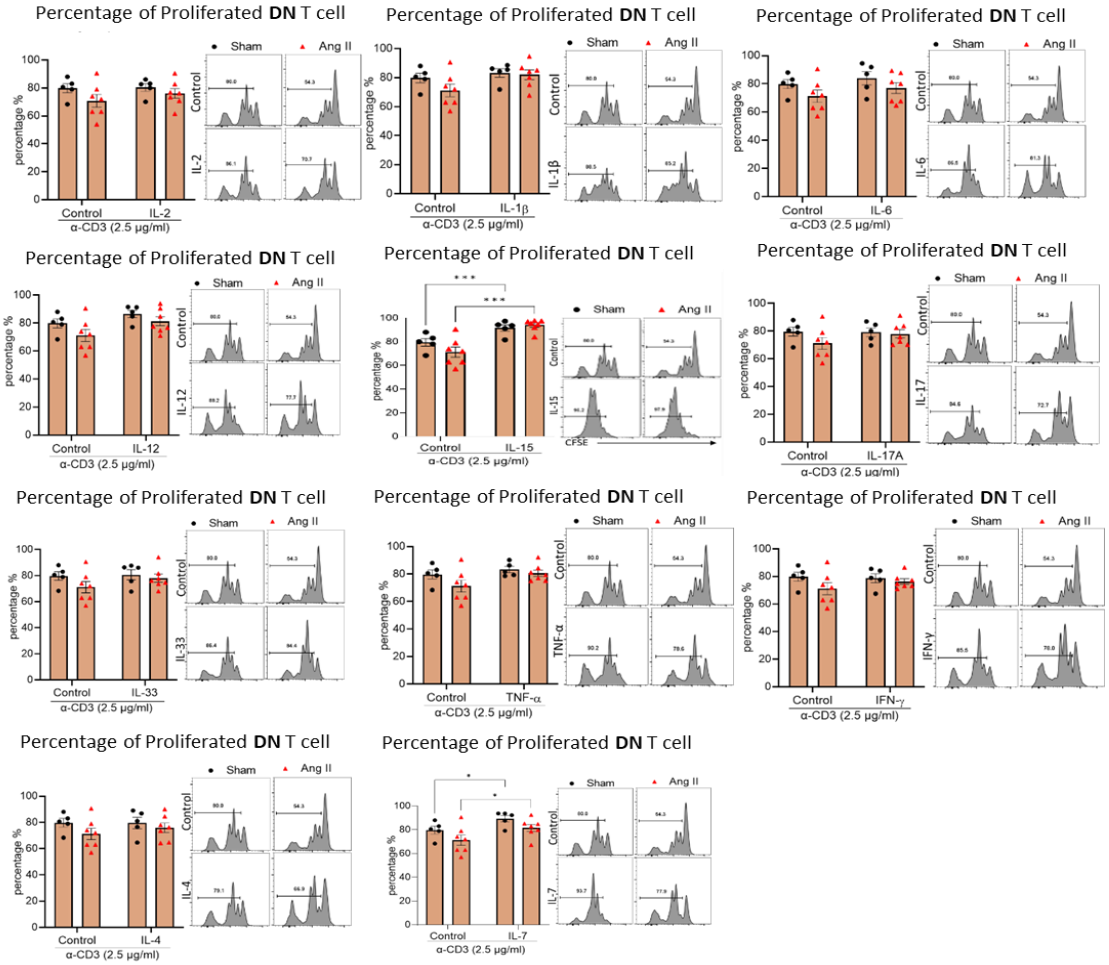
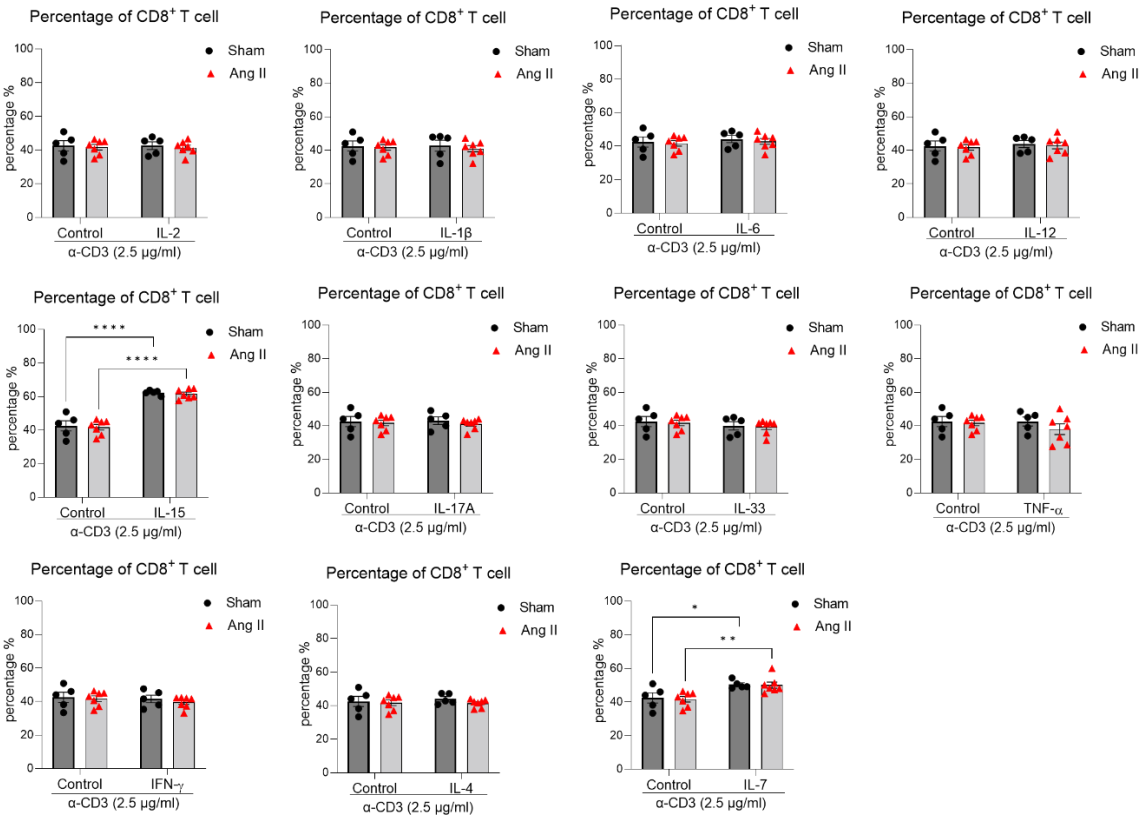


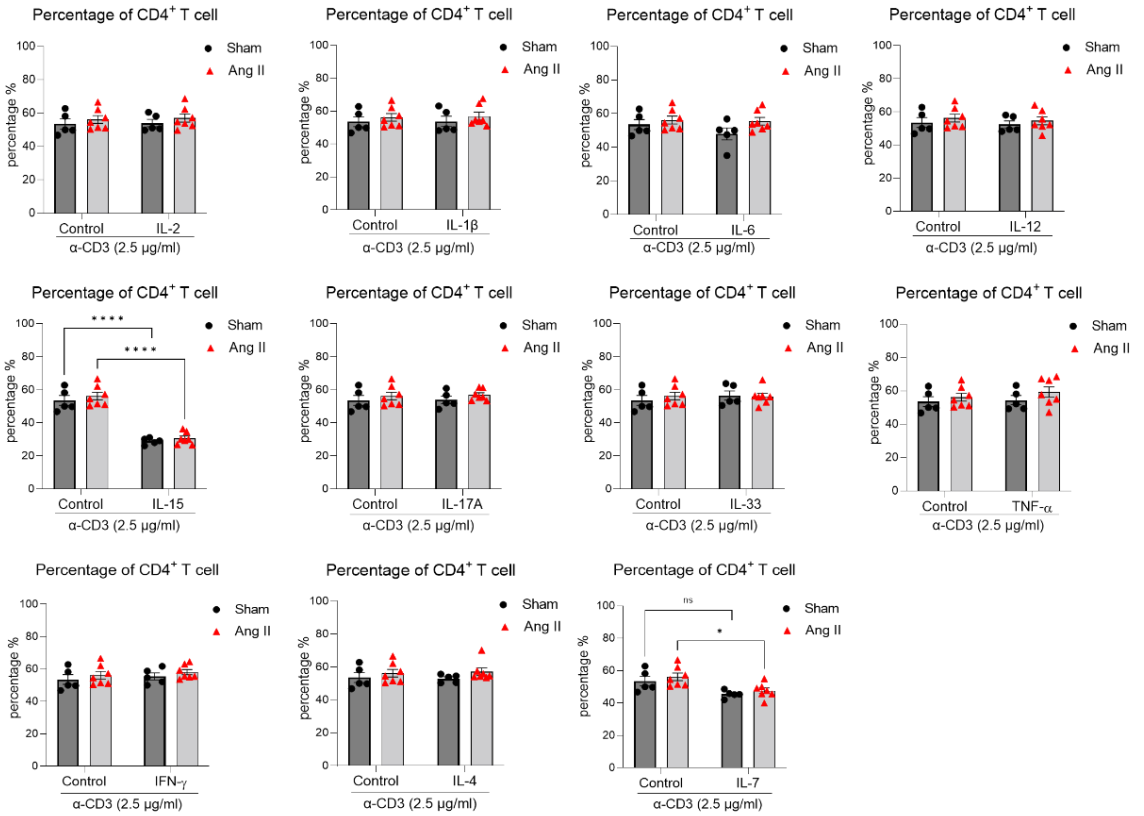
Figure 5.4. Proliferation response of cytokines primed-T Cell on CD4+, CD8+ & DN subsets from Sham vs Ang II mice.

Spleen isolated-T cell was primed with individual cytokines for 24 hours prior to stimulating with α-CD3 coated plate (2.5mg/ μl). CD4+ **(A),** $CD8^{+}$ T cell **(B)** and DN proliferation **(C)** response was analysed on day 2 after stimulation with a Flow cytometer**.** Data are expressed as mean ± SEM from Sham=5 experiments and Ang II = 7 Ang II experiments. Statistical significance (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001) was determined by the 2-way ANOVA test followed by the Tukey test for multiple comparison adjustment.

A


Percentage of CD8+ T cell
Sham
Ang II
percentage %
Control
IL-2
IL-1β
IL-6
IL-12
IL-15
IL-17A
IL-33
TNF-α
IFN-γ
IL-4
IL-7
α-CD3 (2.5 μg/ml)
****
*
**
ns

B


Percentage of CD4+ T cell
Sham
Ang II
percentage %
Control
IL-2
IL-1β
IL-6
IL-12
IL-15
IL-17A
IL-33
TNF-α
IFN-γ
IL-4
IL-7
α-CD3 (2.5 μg/ml)
****
*
ns

C

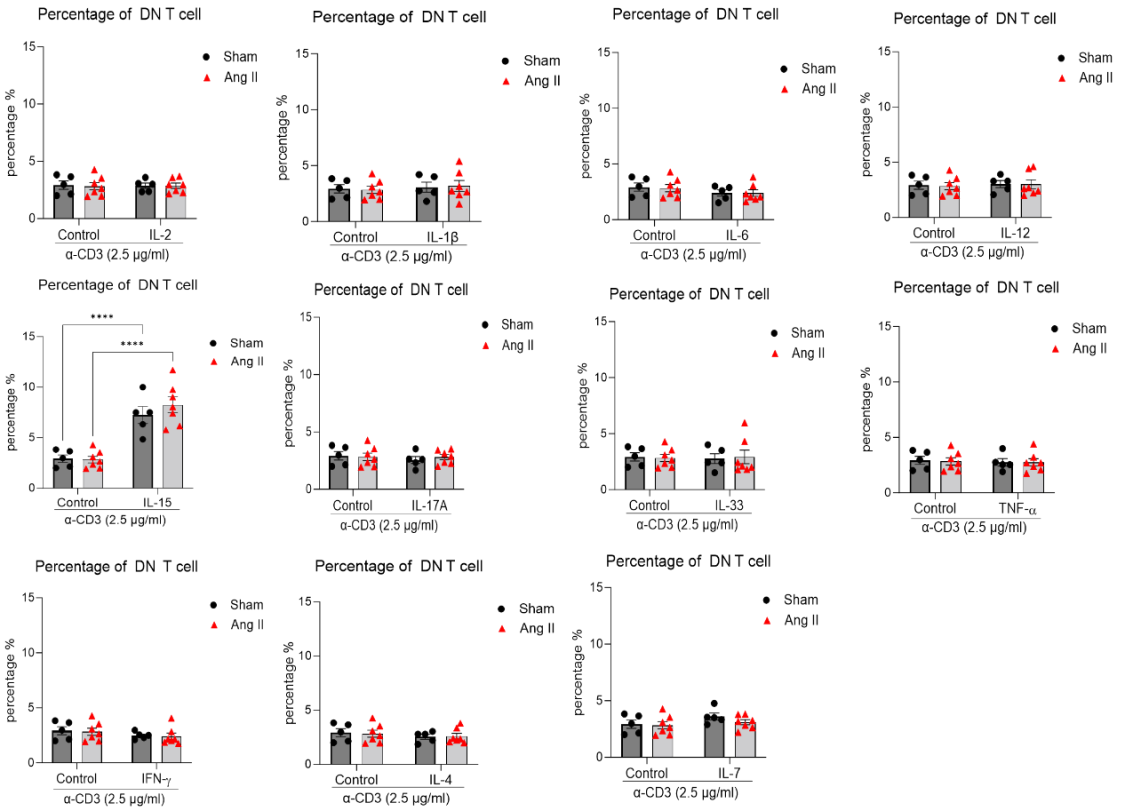


Figure 5.5. The Percentage of CD4+, CD8+, and DN T cells following cytokines priming in T cells isolated from sham vs. Ang II mice.
Spleen isolated T cells were primed with individual cytokines for 24 hours prior to stimulation with α-CD3 coated plate (2.5mg/μl). The percentage of CD4+ **(A),** CD8+ **(B)** and DN T cells was analysed on day 2 after stimulation with FACS. Data are expressed as mean ± SEM from Sham=5/Ang II=7 experiments. Statistical significance (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001) was determined by a 2-way ANOVA test followed by the Tukey test for multiple comparison adjustment.

5.3.3 Effect of 24-hour cytokine priming on peripheral T cells isolated from Sham and Ang II-treated mice.

The experiment was conducted using circulating T cells isolated from PBMCs of both Sham and hypertensive mice. This was made considering the potential for distinct responses in circulating T cells originating from different microenvironments. Following cytokine priming, T cell subsets from PBMCs exhibited a response similar to that observed in splenic T cells. Specifically, IL-15 and IL-7 induced the proliferation of T cells within the CD8+ population, while no proliferation was observed among the CD4+ subset (Figure 5.7A & B).

A

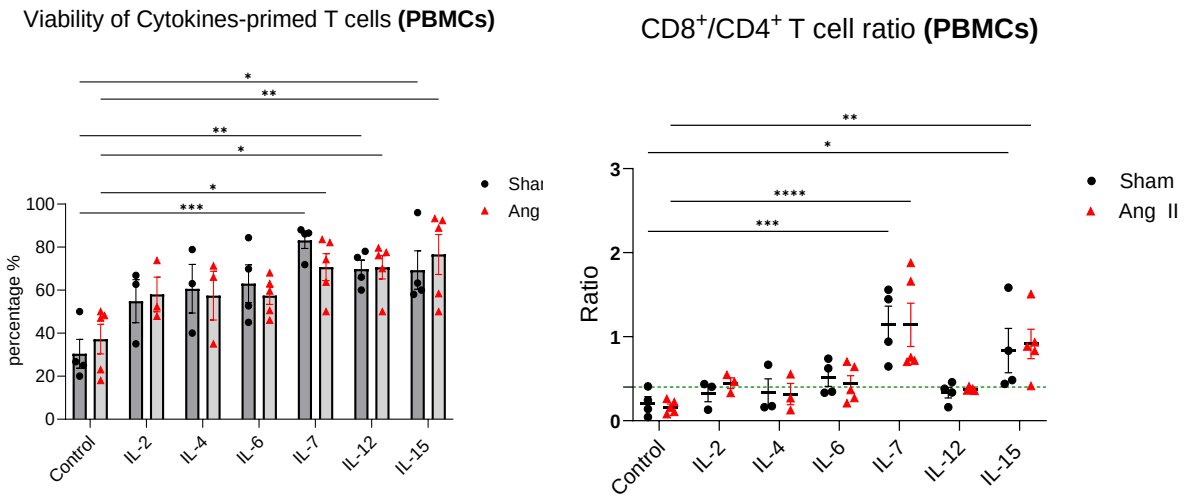
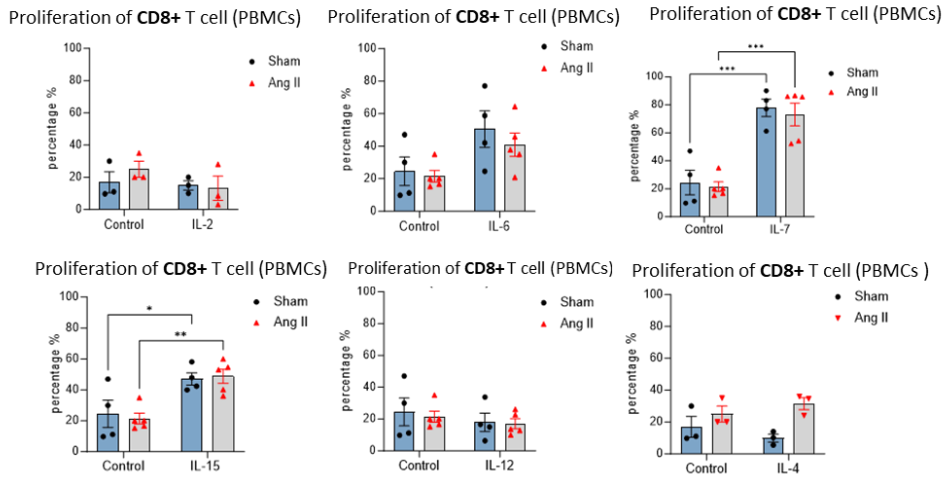


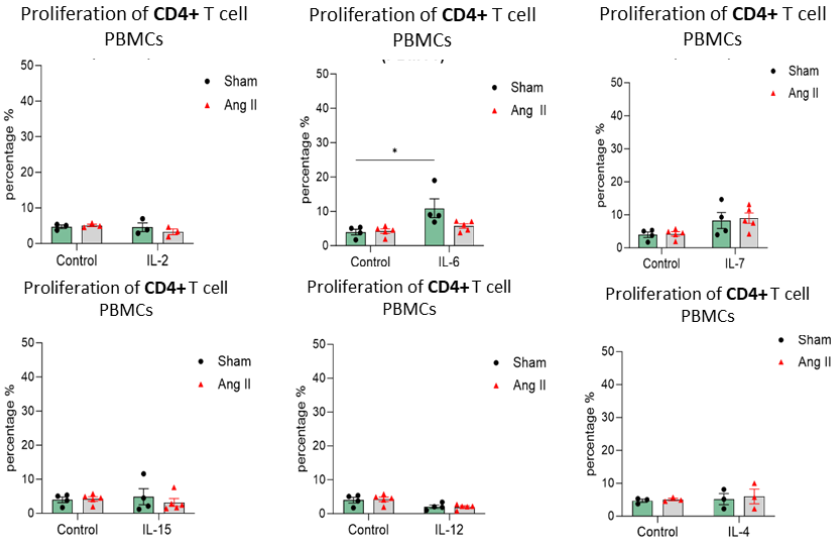
Figure 5.6. Effects of cytokines on T cells isolated from PBMCs of both Sham and 14-day Ang II-infused mice.

PBMCs were primed with individual cytokines for 6 days without further stimulation. Cells were stained with α-CD3, α-CD4 and α-CD8 mAbs and analysed by FACS. Percentage of live T cells **(A)** and CD4+:CD8+ T cell ratio **(B)** was analysed on Day 6. Data are expressed as mean ± SEM from sham=4/Ang II=5 experiments and sham=3/Ang=3 for IL-2 and IL-4. Statistical significance (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001) was determined by the 2-way ANOVA test followed by the Tukey test for multiple comparison adjustment.

A


Proliferation of CD8+ T cell (PBMCs)
Proliferation of CD8+ T cell (PBMCs)
Proliferation of CD8+ T cell (PBMCs)
Proliferation of CD8+ T cell (PBMCs)
Proliferation of CD8+T cell (PBMCs)
Proliferation of CD8+ T cell (PBMCs)
Sham
Ang II
percentage %
Control
IL-2
IL-6
IL-7
IL-15
IL-12
IL-4

B


Proliferation of CD4+ T cell PBMCs
Proliferation of CD4+ T cell PBMCs
Proliferation of CD4+ T cell PBMCs
Proliferation of CD4+T cell PBMCs
Proliferation of CD4+T cell PBMCs
Proliferation of CD4+T cell PBMCs
Sham
Ang II
Ang I
percentage %
Control
IL-2
IL-6
IL-7
IL-15
IL-12
IL-4

C

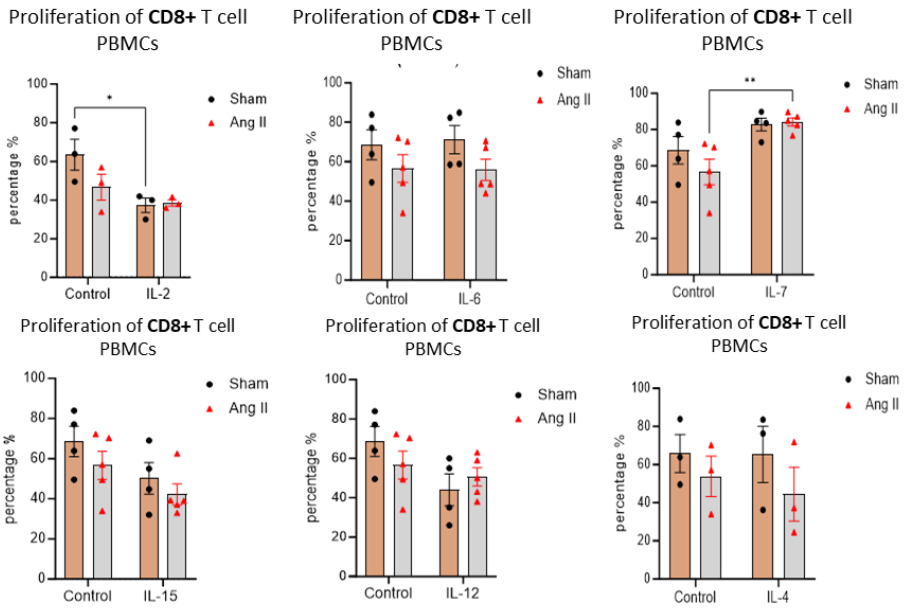


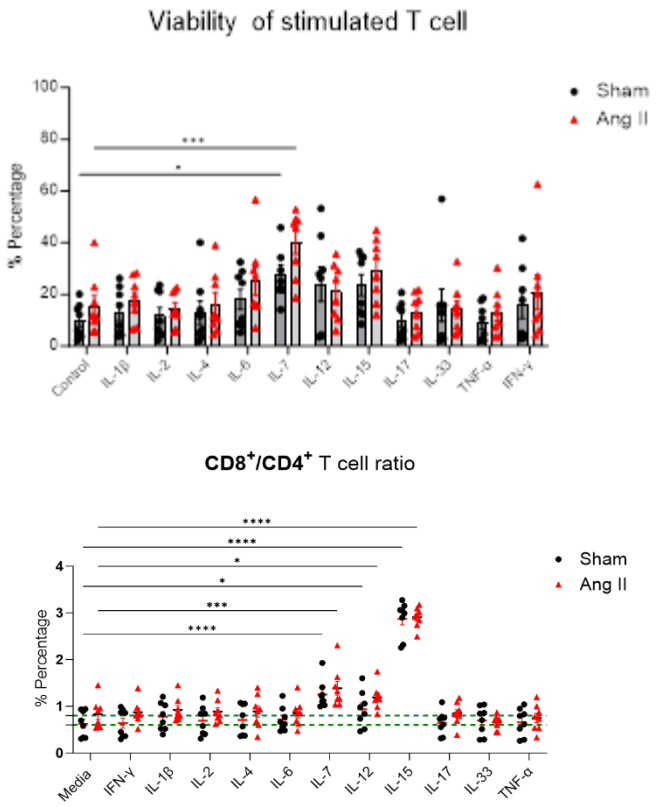
Figure 5.7. Effect of cytokine Priming on the proliferation of T Cell Subsets (CD4+, CD8+ and DN) isolated from PBMCs in Sham/Ang II mice.

PBMCs were primed with individual cytokines for 6 days without further stimulation. The percentage of CD4+ **(A),** CD8+ **(B)** and DN **(C)** T cell was analysed on day 6 with FACS from total live CD3+ T cell. Data are expressed as Mean ± SEM from sham=4/Ang II=5, and Sham= 3/Ang= 3 for IL-2 and IL-4. Statistical significance (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001) was determined by the 2-way ANOVA test followed by Tukey test for multiple comparison adjustment.

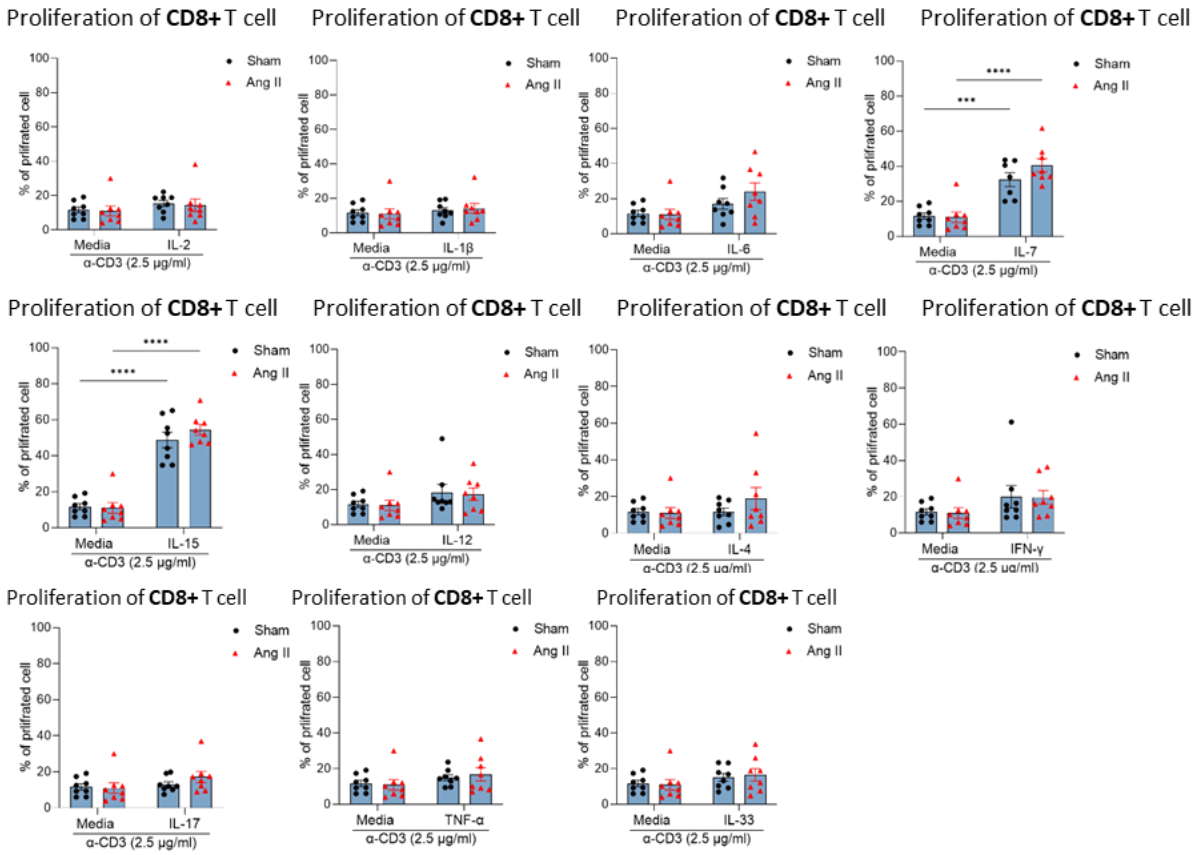
5.3.4 Effect of 24-hour cytokine priming on peripheral and Splenic T cells isolated from Sham and Ang II-treated mice (5-day infusion).

Further T cells from sham and 5-day Ang II infused mice were collected to investigate the cytokine modulation effect. 5 days of Ang II infusion reflects different stages of hypertension, whereas shorter infusions aim to capture initial responses and early changes induced by Ang II. In comparison, longer infusions allow more prolonged or chronic effects. IL-7 and IL-15 had shown proliferative effects on T cells from the spleen (Figure 5.8), and PBMCs (Figure 5.9) from sham and 5-day Ang II infusion mice. The proliferation was observed towards CD8+ T cells. However, no differential effect was observed between sham and Ang II conditions.

A


Viability of stimulated T cell
% Percentage
Sham
Ang II
Control
IL-1β
IL-2
IL-4
IL-6
IL-7
IL-12
IL-15
IL-17
IL-33
TNF-α
IFN-γ
CD8+/CD4+ T cell ratio
Media
IFN-γ

B


Proliferation of CD8+ T cell
% of prlifrated cell
Sham
Ang II
Media
IL-2
IL-1β
IL-6
IL-7
IL-15
IL-12
IL-4
IFN-γ
IL-17
TNF-α
IL-33
α-CD3 (2.5 μg/ml)

C

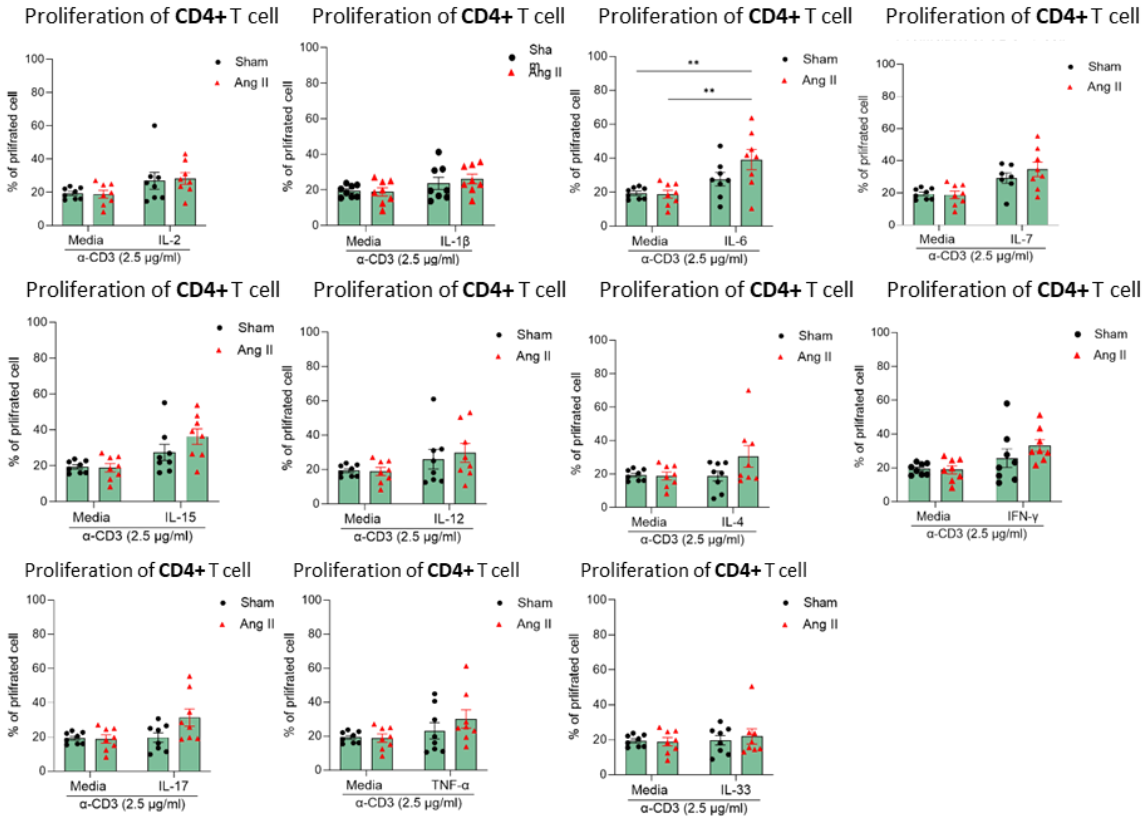


Figure 5.8. Proliferation effect of cytokines priming on T Cell from Sham vs 5-day Ang II infused mice.

Spleen isolated T cells were primed with individual cytokines (IL-2, IL-1β, IL-6, IL-12, IL-15, IL-17A, IL-33, TNF-α, IFN-γ, IL-7 and IL-4) for 24 hours prior stimulating with α-CD3 coated plate (2.5mg/μl). T cell viability, $CD8^{+}$:$CD4^{+}$ subsets ratio **(A),** and percentage of CD8+ **(B)** CD4**+ (C)** T cell proliferation was analysed using FACS on day 2 after stimulation**.** Data are expressed as mean ± SEM from sham=8/Ang II=8 experiments. Statistical significance (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001) was determined by a 2-way ANOVA test followed by Tukey test multiple comparison adjustment.

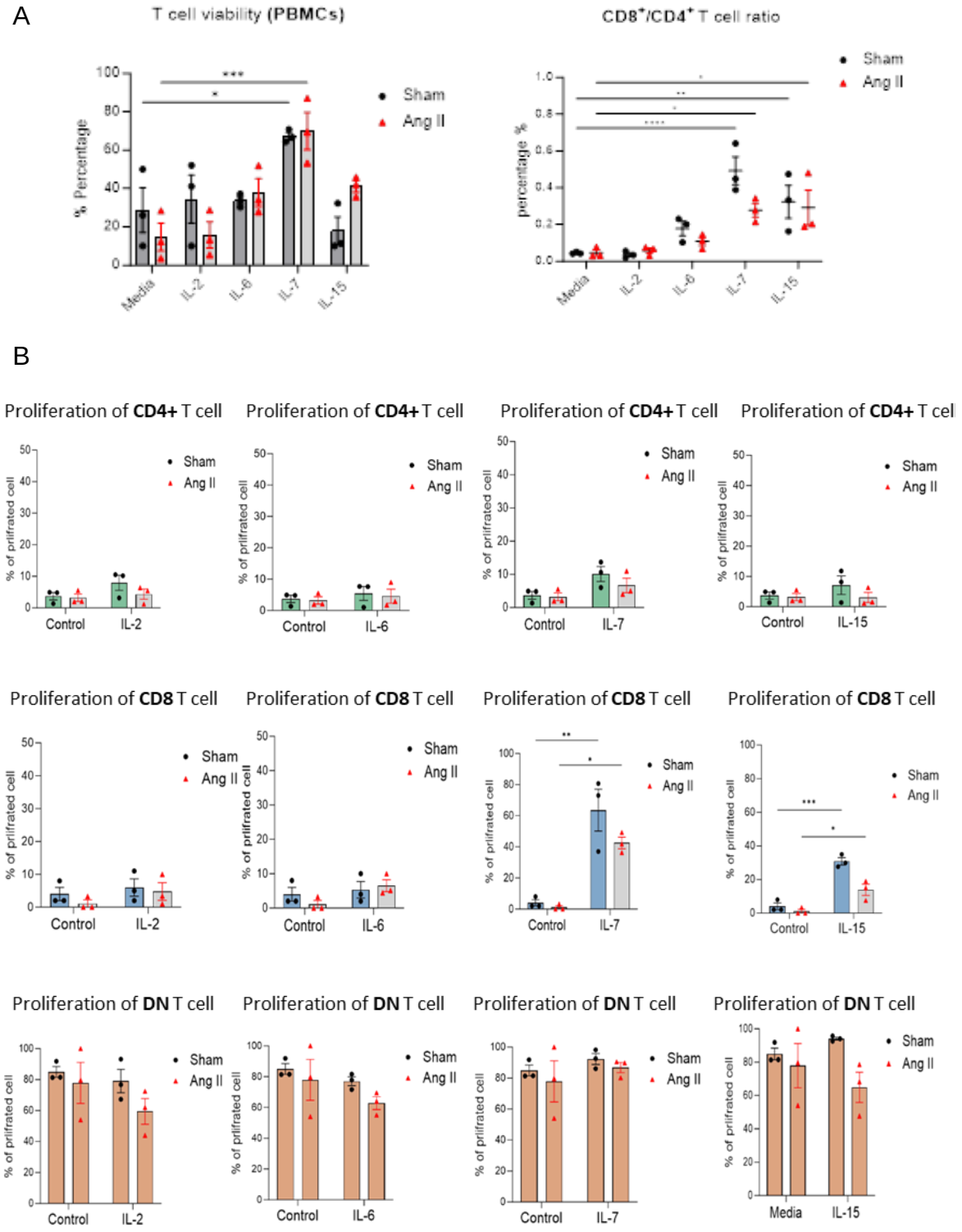


Figure 5.9. Proliferation effects of cytokines on PBMCs isolated-T cell from Sham and 5-day Ang II infused Mice.

PBMCs were primed with individual cytokines for 6 days without further stimulation. Cells were stained with α-CD3, α-CD4 and α-CD8 mAbs, and analysed by FACS. Percentage of live T cells, CD4+:CD8+ T cell ratio **(A)** and percentage of CD4+, CD8+, and DN **(B)** T cell proliferation was analysed on Day 6. Data are expressed as mean ± SEM from Sham=3/Ang II=3 experiments. Statistical significance (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001) was determined by a 2-way ANOVA test compared to control followed by the Tukey test for multiple comparison adjustment.

5.4 Discussion

The findings presented in this chapter indicate that among a comprehensive array of pro-inflammatory cytokines analysed, which included IL-2, IL-1β, IL-6, IL-12, IL-15, IL-17A, IL-33, TNF-α, IFN-γ, IL-7, and IL-4, only IL-7 and IL-15 exhibited a substantial increase in the percentage of proliferative T cells. This was notably characterised by an augmentation in the CD8+ T cell population, while the CD4+ T cell population remained unaffected. The remaining cytokines, on the other hand, failed to induce a proliferative response. Additionally, there were no distinguishable variations in the responses between the T cells isolated from the spleen or PBMCs. Likewise, no significant differences were observed between the Sham (normotensive) and the 14-day or 5-day Ang II-infused mice (hypertensive).

IL-7 significantly increased when oxidation-reduction potential was utilised to assess redox status in patients with pulmonary arterial hypertension (PAH). Both male and female patients exhibited elevated levels of IL-7, alongside several other cytokines, compared to healthy controls. These findings suggest a potential role for IL-7 in the inflammatory response associated with PAH (Rafikov et al., 2022). However, in the results, no differential response of T cells to IL-7 was detected in Sham compared to hypertensive T cells. Experimental evidence suggests that such molecules may individually or collectively contribute to the role of T cells in hypertension; however, the presence of more than 3000 unique T cell receptor sequences in Ang II-induced hypertensive mice indicates that clonal expansion may not be occurring due to antigen presentation and recognition. Under certain pathological conditions such as lymphopenia (Jameson, 2002) or exposure to cytokines such as IL-6 or IL-21 with IL-17 or IL-15, CD8+ T cells can be activated without direct antigen presentation (Benson et al., 2022; Gagnon et al., 2008; Zeng et al., 2005; Ramanathan et al., 2008). IL-7 was also implicated in activating autoimmune CD8+ T cells (Benson et al., 2022). Winer *et al* stated that antigen-independent activation of T cells can be induced in vitro by incubating the cells with various cytokines. Cytokines like the γc family have distinct effects on the regulation of survival and proliferation of T cells. Particularly, IL-7 and IL-15 are considered survival factors for naive and memory αβ T cells, as well as γδ T cells (Winer et al., 2022).

It's challenging to determine whether or not IL-7's impact on the proliferation of naive T cells is due to improved cell survival during culture. However, the results revealed that IL-

12 and IL-6, like IL-7 and IL-15, boosted the viability of primed T cells compared to unprimed ones (Figure 5.1). Nevertheless, it is important to highlight that while IL-6 and IL-12 positively affected cell survival, they did not significantly increase T cell proliferation. Another explanation may be that priming naive T cells with IL-7 might lower the threshold for TCR-mediated activation (Litvinova et al., 2013). Thus, impacting the proliferation of naïve T cells. Moreover, cell sensitivity to activation signals (Boichuk and Dunaev, 2008) and proliferation could be enhanced by IL-7.

IL-7 exerts its effect through a high-affinity receptor complex composed of the IL-7Rα chain and the IL-2Rγ common chain, shared by the receptors for IL-2, IL-4 and IL-15 (Noguchi et al., 1993). In the absence of IL-7R signalling, IL-7R -/- mice exhibit severe impairments in lymphocyte development and function. Peripheral lymphocyte number was also significantly decreased. A study of IL-7R -/- mice revealed several important findings (Maraskovsky et al., 1996). Firstly, IL-7R -/- T cells reduced the frequency of clonogenic cells compared to wild-type T cells. This suggests that IL-7R signalling is necessary for optimal T cell proliferation. Secondly, IL-7R -/- T cells are less responsive to receptor-dependent stimuli. They exhibit impaired proliferation and reduced ability to respond to allo-antigens. Additionally, IL-7R -/- T cells show impaired survival and increased apoptosis upon stimulation. A significant proportion of IL-7R -/- T cells undergo programmed cell death within the first 72 hours of *in vitro* CD3 Abs-stimulation (Maraskovsky et al., 1996). This suggests that IL-7R signalling is essential for T cells survival. Nevertheless, the proliferation of hypertensive T cell did not show any difference in kinetic proliferation response to IL-7.

CD8+ and CD4+ T cells undergo homeostatic proliferation when transferred into a RAG-/- host but not in an IL-7-/- RAG-/- host (Schluns et al., 2000). IL-7R was not required for antigen-induced expansion but is necessary for CD8+ memory T cell production. IL-7R expression at the memory cell stage promotes survival and is required for memory cell homeostasis (Schluns et al., 2000). In addition, CD45RO+ CD4+ memory cells exhibited less sensitivity to IL-7-induced proliferation; however, CD4+ memory cells showed more sensitivity to antigenic stimuli and less dependence on cytokines and membrane co-stimulation for their functional activity (Litvinova et al., 2013). This observation could explain the lack of proliferation observed among the CD4+ population in response to IL-7 priming, as depicted in (Figure 5.4B) and (Figure 5.7B).

The protocol of stimulation T cell, designed to assess the proliferation effects, demonstrates that the CD4+ population displays resistance to proliferation induced by various cytokines, including IL-7 and IL-15, in contrast with findings from a previous study (Vakkila et al., 2001), where IL-7-primed naive CD4+ T cells proliferated more vigorously than unprimed cells. In response to IL-7, Litvinova detected several markers' expression on CD4+ and CD8+ memory T cells from PBMCs. These markers included CD25, which serves as the IL-2 receptor α chain and indicates T cell activation and proliferation, as well as CD71, the transferrin receptor, a surrogate cell proliferation marker and CD69, an early activation marker associated with antigen-independent stimulation, which were all upregulated (Litvinova et al., 2013; Shmarov et al., 2016). However, despite the activation marker expression, our results showed that IL-7 had a dissimilar effect on CD8+ and CD4+ memory T cells. CD4+ memory cells exhibited relative resistance to proliferation by IL-7, as in other studies (Litvinova et al., 2013; Geiselhart et al., 2001). Boichuk, S.V. and Dunaev, P.D demonstrate that following α-CD3 stimulation, naive CD4+ T cells lose their capacity to IL-7 response. However, when cells are pre-exposed to IL-7 before antigenic stimulation, their proliferative responses are amplified (Webb et al., 1999; Boichuk and Dunaev, 2008).

The importance of IL-7 lies in its role as a critical cytokine for regulating the survival and homeostatic proliferation of naive T cells, acting in coordination with TCR signalling (Tan et al., 2001). The results demonstrate IL-7's capacity to augment the proliferation of CD8+ T cells with IL-7 and 2.5 ug/ml of α-CD3, which aligns with a published study (Geiselhart et al., 2001). Geiselhart research showed that IL-7 treatment enhanced T cell proliferation, particularly within the CD8+ T cell subset, leading to increased numbers of CD4+ and CD8+ T cells. In addition, IL-7 was observed to induce an increase in basal proliferation of T cells, consequently expanding the T cell number. This increase in T cell numbers is mainly due to the ability of IL-7 to induce additional T cells to enter the cell cycle, a pattern consistent with the findings in (Figure 5.4). Geiselhart study indicated that IL-7's promotion of T cell proliferation occurred independently of cytokine production and alterations in activation and memory markers in mice (*in vivo*). Moreover, the administration of IL-7 resulted in a disproportionate increase in CD8 T cells, causing an alteration in the CD4+: CD8+ T cell ratio (Geiselhart et al., 2001), as depicted in our results (

Figure 5.5). Overall, IL-7 enhances the functionality of T cells, particularly CD8 T cells, through a non-activating mechanism without triggering the typical activation signals (Geiselhart et al., 2001).

While IL-7 plays a pivotal role in regulating the survival and homeostatic growth of naive T cells, in contrast, IL-15 predominantly contributes to the generation and maintenance of memory CD8+ T cells. The results in (Figure 5.4A, Figure 5.7A, and Figure 5.8C) showed that IL-15 could induce potent proliferation of CD8+ T cells after priming. A study showed that mice lacking IL-15Rα had a deficiency in CD8+ T cells in the thymus and experience marked lymphopenia among CD8+ T cells (Liu et al., 2002). Furthermore, IL-15 has the ability to induce effector T cell function, which was supported by functional and DNA microarray data from Liu *et al* (Liu et al., 2002), and demonstrated that IL-15 could mimic TCR activation in cellular proliferation, gene expression profiles, and the induction of cytotoxic activity in memory CD8+ T cells. In various inflammatory conditions, the induction of IL-15 primes memory CD8+ T cells for enhanced effector functions, including priming, proliferation and trafficking into non-lymphoid tissues (Nolz and Richer, 2020). IL-15 was found to activate and induce the proliferation of CD8+ memory T cells in a manner that closely resembles the signals received from the TCR. This suggests that IL-15 serves as an antigen-independent activator for CD8+ memory T cells, contributing to their survival and effector functions independently of TCR engagement (Weng et al., 2002). In hypertensive mice, IL-15 did not demonstrate any differential proliferation effect among T cells from spleen and PBMCs compared to normotensive mice.

Homeostatic mechanisms controlling memory CD4+ T cells are differ and distinct from those of memory CD8+ T cells, which is likely due to differences in cytokine receptor expression, lipid rafts, and cellular metabolism (Carrette and Surh, 2012). IL-7 and IL-15 are critical in maintaining homeostasis among memory CD4+ T cell (Boyman et al., 2009; Surh and Sprent, 2008), although the results from this chapter did not reveal a distinct proliferation effects. Thus, homeostasis of memory CD4+ T cells is regulated by IL-7 and IL-15, with IL-15 signals being less important for these cells, likely because memory CD4+ T cells express CD122 levels that are comparable to naive CD8+ T cells and about 4-10-fold lower than that of memory CD8+ T cells (Purton et al., 2007; Boyman et al., 2006; Zhang et al., 1998). In loss-of-function experiments, the homeostatic proliferation of antigen-specific memory CD4+ T cells was reduced in IL-15-/- mice, which was even more evident in IL-7-/- and IL-7-/- IL-15-/- animals (Raeber et al., 2018). Tripathi P. observed that IL-15 was not necessary for sustaining effector CD4+ T cell responses. Although another study demonstrated that IL-15 plays a role in supporting the survival of memory CD4+ T cells (Tripathi et al., 2010; Purton et al., 2007). In the mouse collagen-induced arthritis model, both wild-type and IL-15 -/- mice exhibited similar disease

susceptibility. However, the transgenic overexpression of IL-15 exacerbated the disease by increasing CD4+ T cell proliferation and enhancing their production of IFN-γ and IL-17A. However, it remains unclear whether or not IL-15 directly influences IL-17A expression in CD4+ cells (Yoshihara et al., 2007). Conversely, another findings showed that IL-15 act as a negative regulator in modulating IL-17A production and Th17-mediated inflammation, reducing IL-17A through STAT5 activation (Pandiyan et al., 2012).

Both IL-7 and IL-15 can activate similar signalling pathways within T cells, including PI3K/protein kinase B (PKB) and STAT5. In T cells, both the PI3K/PKB and Jak3/STAT5 pathways are critical for T cell homeostasis (Yao et al., 2006; Sasaki et al., 2000). Furthermore, pathways were implicated, but not directly examined, in both the proliferative and cell survival effects of IL-7 and IL-15 *in vivo* (Seki et al., 2007; Swainson et al., 2007). However, TCR and cytokine stimulation activate PI3K/PKB signalling (Okkenhaug et al., 2002; Mazzucchelli and Durum, 2007), which complicates interpretations of the specific role of the PI3K/PKB pathway on particular stages of T cell homeostasis. Deletion of STAT5 during the infection resulted in the loss of CD8+ T cells while leaving effector CD4+ T cell populations unaffected. STAT5 played a critical role in maintaining Bcl-2 expression in effector CD8+ T cells, with no such impact on CD4+ T cells. Both IL-7 and IL-15 depended on STAT5 to induce Bcl-2 expression and sustain effector CD8+ T cell responses. These observations highlight how IL-7 and IL-15 signalling converge through STAT5 to support effector CD8+ T cell responses (Tripathi et al., 2010). In a study that investigated the impact of IL-7 and IL-15 on the growth of activated CD4+ T cells using RNA sequencing for genetic analysis. The researchers provided insights into how each cytokine affected gene expression in CD4+ T cells. IL-7 was associated with regulation genes related to chloride transport, cell chemotaxis, chloride transmembrane activity, and cytokine receptor activity. IL-15 influenced genes associated with cell growth, zinc ion responses, cellular response to metal ions, and the regulation of calcium ion transport into the cell (Coppola et al., 2020).

Chapter 6 RNA Sequencing of Differential Gene Expression in Cytokine-treated CD8+ T cell from Sham and Ang-II-induced Hypertensive mic

6.1 Introduction

Hypertension, a chronic condition characterised by persistently elevated blood pressure, has traditionally been attributed to factors, e.g., lifestyle, genetics, and vascular mechanics. However, evidence illuminated the critical role of the immune system, particularly T cells, in the development, progression, and complications of hypertension (Svendsen, 1976; Chan et al., 2015; Guzik et al., 2007). The mechanism by which T cells contribute to hypertension is unclear. T cells respond to signals like that released from stressed vascular tissues (ROS, cytokines), inflamed microenvironments, and neoantigens Iso LGs, leading to their activation (Kirabo et al., 2014; Curtsinger and Mescher, 2010; Wu et al., 2013; Ren and Crowley, 2019). Cytokines, chemokines, and key inflammatory signalling pathways, such as the NF-κB and MAPK, were found to be amplified in hypertension (Youn et al., 2013; Luft, 2001).

Cytokines are pivotal in regulating the immune system by orchestrating various cellular responses. Among these, IL-15 and IL-7 are structurally related cytokines that belong to the common γc family, sharing similarities in receptor subunit usage. Both cytokines are critical for the development, homeostasis, and functional competence of T cells (Bradley et al., 2005). IL-15 and IL-7 became crucial players in stimulating CD8+ T cells, critical adaptive immune response components. IL-15 primarily supports memory CD8+ T cell proliferation and survival, thereby sustaining long-term immune responses (Deshpande et al., 2013). While IL-7 is essential for developing and maintaining CD8+ T cells, ensuring a diverse T cell repertoire capable of responding to various antigens (Bradley et al., 2005). Although there is currently no research on the role of IL-7 in hypertension, individuals with essential hypertension exhibiting signs of "severe organ damage" are reported to had elevated levels of IL-15 in comparison to patients with mild or no organ damage (Kaibe et al., 2005).

T cells, specifically CD8+, play a critical role in the immune-mediated processes that underlie hypertension, contributing to inflammation, vascular dysfunction, hypertensive pathologies, and immune memory in this condition. IL-15 and IL-7, being potent stimulators of CD8+ T cells, likely play a role in enhancing this immune response. In hypertension, CD8+ T cells infiltrate vascular tissues and contribute to tissue damage, aggravating the underlying pathology (Trott et al., 2014). A study showed that mice with a deficiency in CD8+ lymphocytes displayed a significantly reduced hypertensive reaction in response to Ang II, while $CD4^{-/-}$ mice had not (Thabet et al., 2010). In hypertensive

patients, CD57+ CD8+ T cells with cytotoxic ability significantly increased compared to control (Youn et al., 2013).

The pathways within T cells differ significantly based on the activation mode, whether it's the classical antigen-dependent route or the activation triggered by cytokines. T cell activation is initiated by recognising specific APCs presenting specific antigens through their MHCs. This interaction triggers a series of signalling events, primarily mediated by the TCR complex and its associated co-receptors. The TCR complex undergoes phosphorylation, leading to the recruitment of kinase enzymes such as Lck, which phosphorylates key motifs in immunoreceptor tyrosine-based activation motifs (ITAMs) (Mujib et al., 2012). This cascade of events activates downstream pathways, including the activation of protein kinase cascades (MAPK and PI3K-Akt), culminating in the production of transcription factors like NFAT and AP-1. These factors drive T cell proliferation, differentiation into effector or memory cells, and the secretion of cytokines that shape immune responses (Mujib et al., 2012). Cytokines, as stimulators, also orchestrate the intricate pathways of T cell activation in diverse immune responses. For example, receptors for IL-2, IL-21, and IL-15 contain distinct ligand-binding α subunits and the common γc (Kovanen and Leonard, 2004). IL-2/IL-15R is a pivotal cytokine receptor that drives T cell proliferation and differentiation via JAK-STAT pathways (Gagnon et al., 2008). The γc receptor employs JAK3 and STAT5 for signalling, while IL-7Rα, IL-21Rα, and IL-2/15Rβ utilise JAK1 to trigger STAT3 activation (Tagaya et al., 1996; Kovanen and Leonard, 2004; Hofmeister et al., 1999). In addition, another cytokine-like IFN-γ from Th1 cells activates cytotoxic CD8+ T cells through JAK-STAT, enhancing immune surveillance and antigen presentation. These pathways collectively govern T cell responses, ensuring tailored and effective immune reactions in health and disease.

6.2 Chapter Aims

In the previous chapter, IL-7 and IL15 were shown to have an apparent proliferation effect on T cell proliferation. However, no distinctive difference in the proliferation of T cells from Sham and hypertensive mice was detected.

This chapter aims to determine.

- The differential gene expression between IL-15-treated CD8+ T cells and IL-7-treated CD8+ T cells from Sham and Ang II-induced mice.

- Determine the common pathway(s) activated in both conditions and elucidate their points of divergence.

- To determine distinctive pathway(s) that regulate T cell activation in Ang II animals.

6.3 Method

6.3.1 Preparation of T cell for stimulation, sorting, and RNA extraction.

6.3.1.1 T cell stimulation and sorting

T cells were isolated using negative isolation KIT as mentioned in (3.2.3), then cultured with IL-7 and IL-15 cytokines for 24 hours before washing and re-culture in α-CD3 antibody coated-plate for 48 hours. Cells were then collected and prepared for sorting. Stimulated T cells were sorted by flow cytometry after staining with DRAQ7 viability dye (DRAQ7; ThermoFisher), CD4+ APC CD8+ PE, and APC-Cy7 for the exclusion of nonviable cells (**Figure 6.1**). Sorted cells were collected in RNA*later* stabilisation solution.

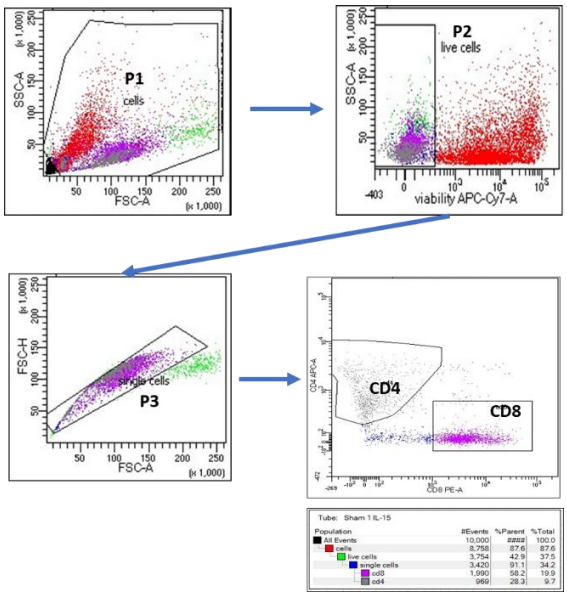


Figure 6.1. Gating strategy for separation of CD3+CD8+ T cells.
A representative gating strategy plot for sorting CD3+ CD8+ T cells from the spleen. Gate p1 selects T lymphocytes. Gate P2 selects DRAQ7 negatively stained live T cells. Gate P3 selects single-stained T cells.

6.3.1.2 RNA extraction

CD8+ T cells from IL-15 and IL-7 stimulated T cells were sorted in RNAlater (Thermofisher). Following centrifugation for 30 min at 25000 g X at RT, the samples were resuspended in 700 ul QIAzol® lysis reagent, and the pellet was vortexed robustly. RNA was extracted with Direct-zol™ RNA MiniPrep Kit (Zymo Research) following the manufacturer's protocol and treated with DNase I. Once extracted, RNA was stored at –80°C (Figure 6.2).

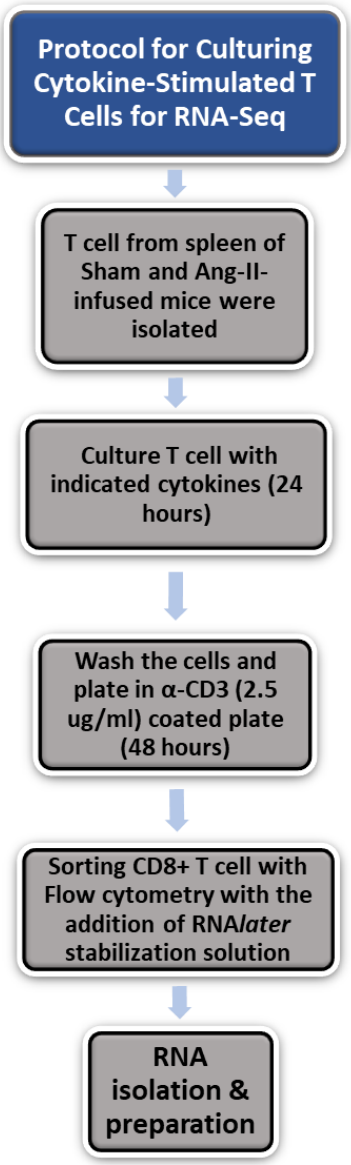


Figure 6.2. Flow diagram depicting the protocol utilised for RNA sequencing of cytokine stimulated CD8+ T cells.

6.3.2 RNA-seq analysis

6.3.2.1 RNA-sequencing and Quality Control.

The RNA quality control was assessed through the utilisation of Agilent 2100 Bioanalyzer System. Samples with RIN score between 8.0-10 were used for further sequencing. Sequencing was performed with HiSeq 4000 (Illumina) with 150 bp paired-end reads (PE150). To eliminate possible adapter sequences and nucleotides with low quality (Quality score < 20), Trimmomatic v.0.36 (Bolger et al., 2014) was used. Samples underwent paired-end read sequencing, and a Poly A selection method was applied before sequencing.

6.3.2.2 Genome Mapping

Clean reads were mapped to the Mus musculus GRCm38 reference genome available on ENSEMBL using the STAR aligner v.2.5.2b. FeatureCounts (v. 1.11) from the Subread package v.1.5.2 was used to count the number of reads per annotated gene. The hit counts were summarised, and only unique reads within exon regions were counted. The gene counts were used for downstream differential expression analysis (DESeq2).

6.3.3 Statistical analysis

Differential gene expression analysis from the RNA-Seq experiment was performed using raw gene counts and DESeq2 package in R (ver. 3.5.1) (Love et al., 2014). Primary analysis employed the Wald test and investigated the effect of IL-7 / IL-15 on transcriptome profile in Sham (n=4) and Ang II (n=4 for IL-7, n=6 for IL-15 Ang II group) T cells separately. Wald test was employed in order to identify genes with significant interaction terms between Sham and Ang II treatment (IL-7/IL-15). The number of samples represents biological replicates, and independent mice were used for experiments. The differential expression analysis was conducted using DESeq2, based on a negative binomial generalised linear model and Benjamini-Hochberg correction to correct for multiple comparisons with a false discovery cut-off <0.05 (p.adj<0.05) was used.

Gene set enrichment analysis (GSEA) for functional annotation was performed utilising the Goseq tool (v. 1.44.0) (Young et al., 2010). This analysis aimed to identify and characterise the biological functions and molecular pathways that exhibited notable enrichment within the pool of Differentially Expressed Genes (DEGs).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was analysed using clusterProfiler package that supports all organisms, including Mus musculus, and has KEGG annotation data available in the KEGG database (Wu et al., 2021). The browseKEGG function was applied to view the KEGG pathways, highlighting enriched genes.

6.4 Results

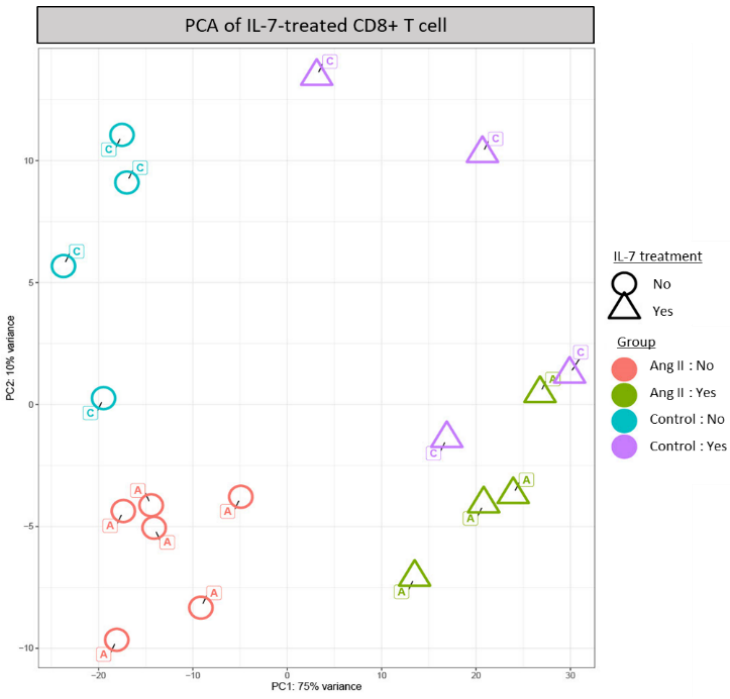
6.4.1 Gene expression effect of IL-7 on CD8+ T cell.

6.4.1.1 Differential Expression Analysis

Principal component analysis (PCA) was applied to analyse the data's structure and variability. PCA showed a separation between IL-7-treated CD8+ T cells (Sham and Ang II) compared to controls (Sham and Ang II without IL-7 treatment) based on all expressed genes and transcriptomic data. An overlap was observed between Sham + IL-7 and Ang II + IL-7 conditions. The separation was primarily represented by the first principal component 1, PC1, vs principal component 2, PC2, accounting for 75% and 10%, respectively, of the observed variance (Figure 6.3A).

Differential gene expression was analysed to gain deeper insights into the distinctions between the Sham + IL-7 and Ang II + IL-7 samples. This analysis combined the results from n=4 for the sham group treated with IL-7 and Ang II treated with IL-7 to improve statistical power. The gene expression level between Sham vs. Sham + IL-7 and Ang II vs. Ang II + IL-7-treated CD8+ T cells was assessed and visually presented through volcano plots (Figure 6.3B).

A



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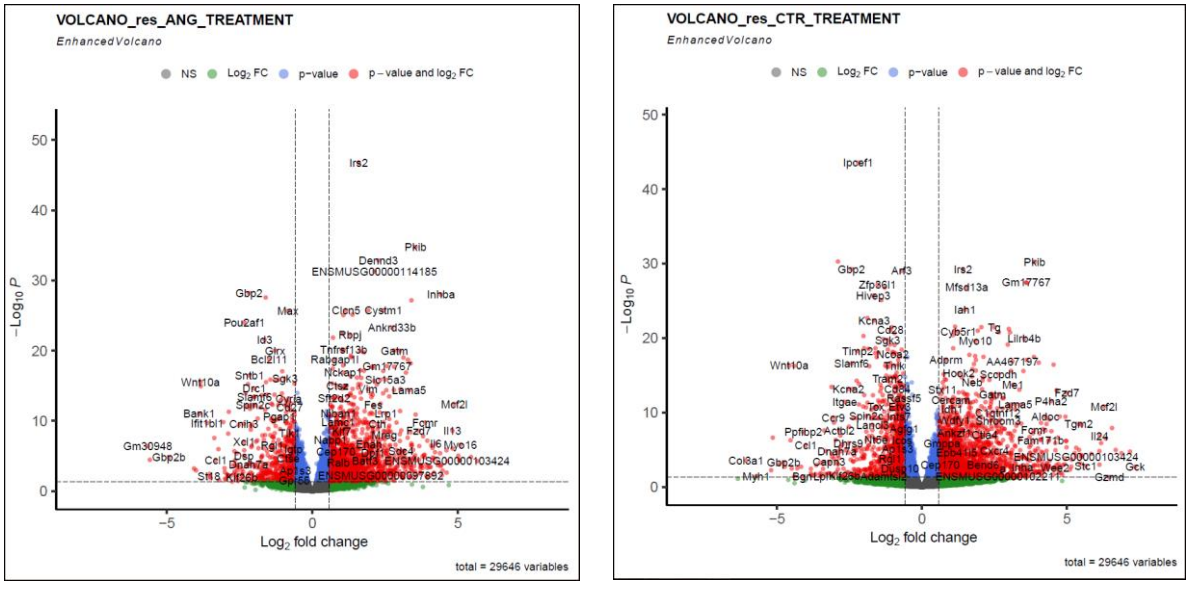


Figure 6.3. PCA and Volcano plot for Sham IL-7-treated vs Ang II IL-7-treated CD8+ T cell.

PCA plots representing CD8+ T cell + IL-7 from sham and Ang II-infused mice, samples analysed by RNA-Seq **(A).** Volcano plots demonstrate an overview of the differential expression of all genes in Sham + IL-7 and AngII + IL-7 conditions **(B).** The threshold for the volcano plot was p.adj >0.05. Green=not sig. & FC =1.5, bule =sig. & FC<1.5, and Red= Sig. & FC>1.5.

A Venn diagram for all significantly changed genes in two samples was obtained from DESeq2 analysis. P.adj is used to screen and filter gene expression. There are 6075 DEGs in the Sham + IL-7 conditions compared to 4759 DEGs in the Ang II + IL-7 condition. These genes reflect the specific effect of adding IL-7 to the Sham and Ang II condition compared to the baseline, which is Sham and Ang II without treatment. Notably, only one gene, CHCHd2, was identified as an interaction gene. The CHCHd2 gene is uniquely influenced by the interaction between Ang II and IL-7, and this differential expression is attributed to the presence of Ang II or IL-7 factors (P.adj<0.05) (Figure 6.4A).

The Venn plot revealed distinct expression patterns in analysing upregulated genes (Figure 6.4B). Specifically, 1230 genes were upregulated in the Sham + IL-7 condition, while 743 genes exhibited upregulation in the Ang II + IL-7 condition. Additionally, a set of 1833 genes showed common upregulation in both conditions. Interestingly, the interaction group demonstrated upregulation in 127 genes, highlighting unique expression patterns influenced by the combined presence of Ang II and IL-7.

Among downregulated genes, 1587 genes exhibited downregulation in the Sham + IL-7 condition, while 726 genes displayed downregulation in the Ang II + IL-7 condition. A set of 1424 genes showed common downregulation in both conditions. Additionally, the interaction group demonstrated downregulation in 23 genes, highlighting unique expression patterns influenced by Ang II and IL-7 combined. Notably, 19 genes were expressed in common between the interaction genes group and the Ang II + IL-7 group, which exhibit a similar pattern of downregulation in response to the combined presence of Ang II and IL-7. These genes likely play a role in the interaction effect observed in this experimental condition, where the impact on gene expression is different from what would be expected based on the individual effects of Ang II and IL-7 (Figure 6.4).

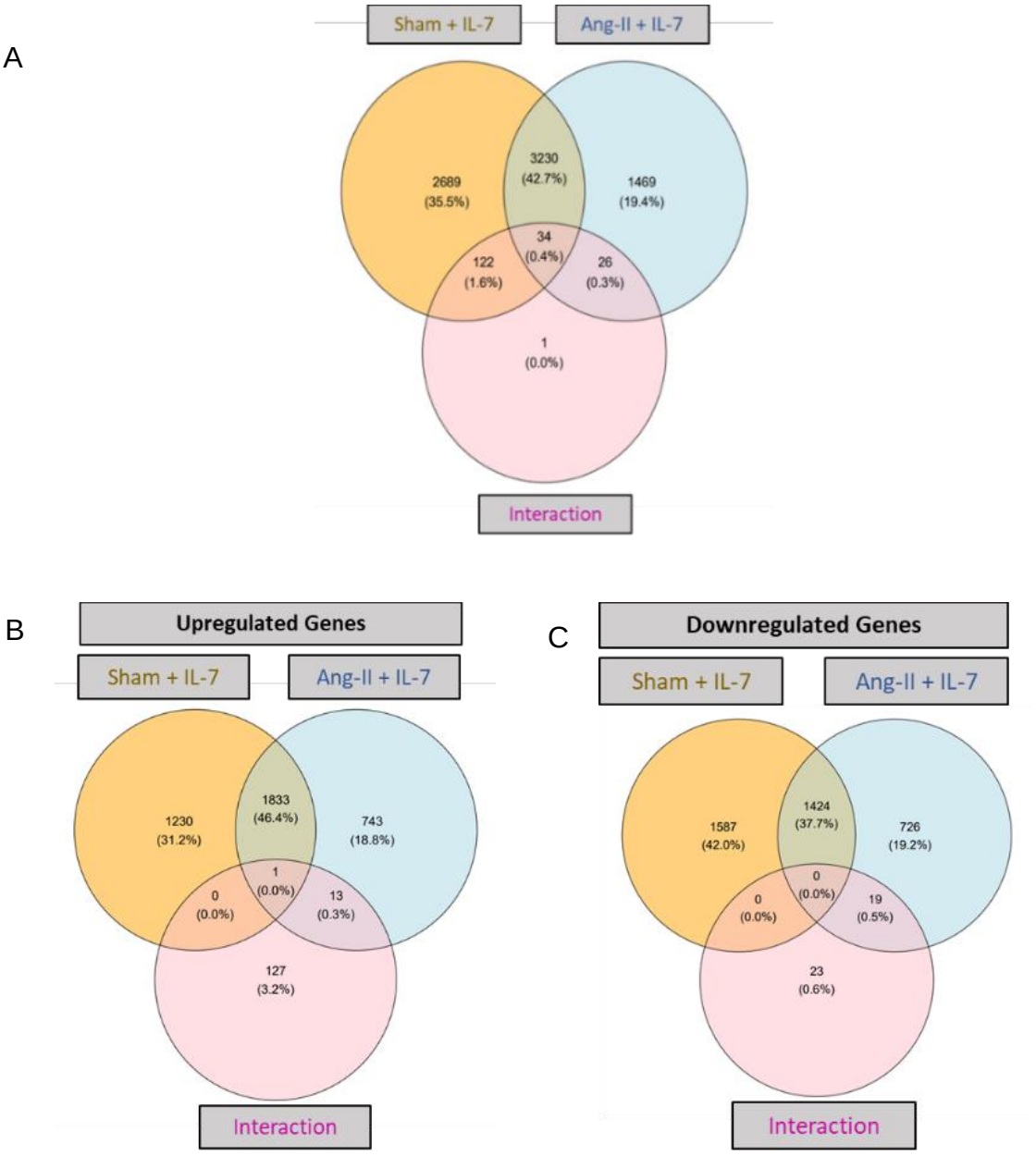


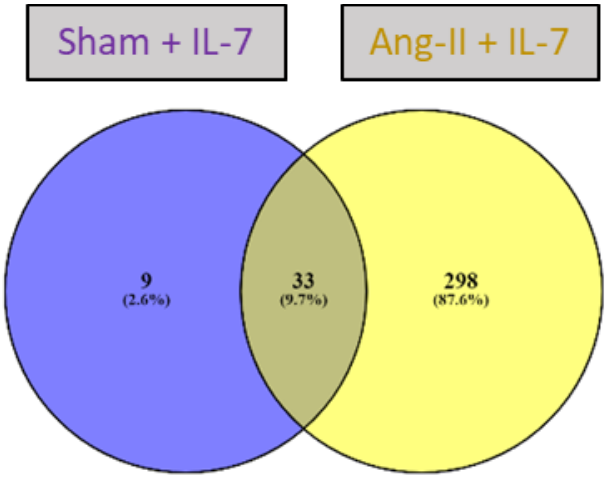
Figure 6.4. Differential gene expression of Sham + IL-7 and Ang II + IL-7 treated CD8+ T cell.

Venn plots illustrate 3 groups comparing Sham + IL-7 to Ang II-IL7 total genes expression profile, including interaction group **(A),** upregulated **(B)** and downregulated **(C)** DEGs.

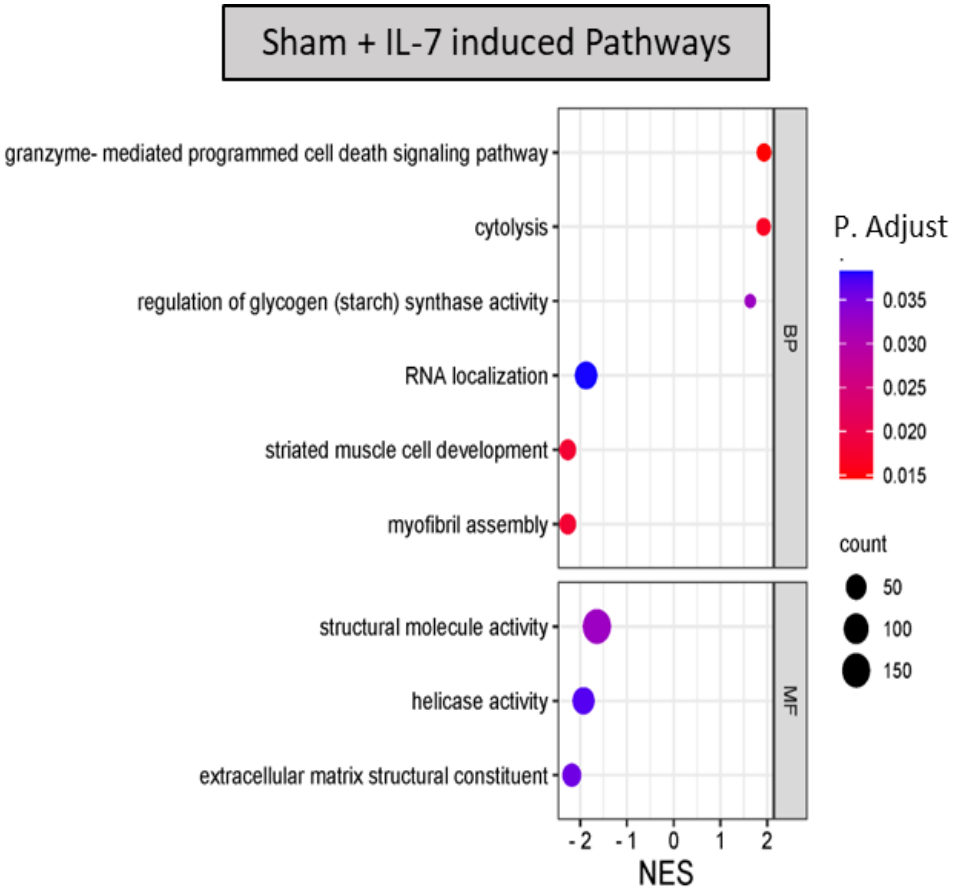
6.4.1.2 Gene Set Enrichment Analysis (GSEA)

GSEA identified gene sets in each condition. GSEA revealed the induction of 42 pathways in the Sham +IL-7 conditions, whereas the Ang-II + IL-7 condition exhibited 331 pathways (Figure 6.5A). Notably, 33 pathways were induced in common in both Sham + IL-7 and Ang II + IL-7 conditions, indicating a degree of overlap in the enriched pathways across these two experimental settings. Common top 10 upregulated and downregulated pathways were presented in (Figure 6.5D) All nine Sham + IL-7 pathways are presented on a dot plot (Figure 6.5B)On the other hand, more than 290 pathways were induced explicitly in the Ang II +IL-7 condition, and the top 20 upregulate pathways were presented on the dot-plot. However, no down-regulated pathways were detected (Figure 6.5C) All detected pathways are with p.adj ≥ 0.05.

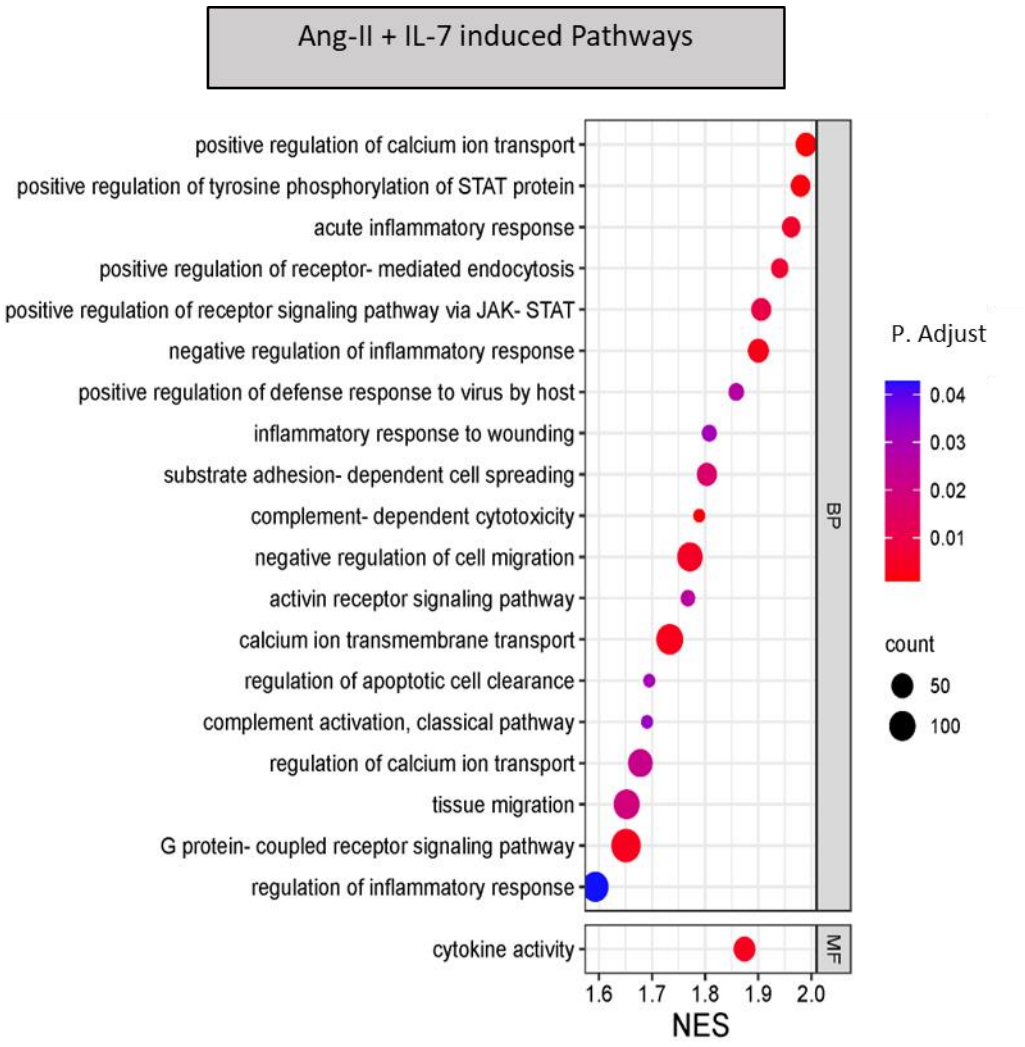
A


Sham + IL-7
Ang-II + IL-7
9
(2.6%)
33
(9.7%)
298
(87.6%)

B


Sham + IL-7 induced Pathways
granzyme- mediated programmed cell death signaling pathway
cytolysis
regulation of glycogen (starch) synthase activity
RNA localization
striated muscle cell development
myofibril assembly
structural molecule activity
helicase activity
extracellular matrix structural constituent
BP
MF
-2
-1
0
1
2
NES
P. Adjust
0.035
0.030
0.025
0.020
0.015
count
50
100
150

C


Ang-II + IL-7 induced Pathways
positive regulation of calcium ion transport
positive regulation of tyrosine phosphorylation of STAT protein
acute inflammatory response
positive regulation of receptor- mediated endocytosis
positive regulation of receptor signaling pathway via JAK- STAT
negative regulation of inflammatory response
positive regulation of defense response to virus by host
inflammatory response to wounding
substrate adhesion- dependent cell spreading
complement- dependent cytotoxicity
negative regulation of cell migration
activin receptor signaling pathway
calcium ion transmembrane transport
regulation of apoptotic cell clearance
complement activation, classical pathway
regulation of calcium ion transport
tissue migration
G protein- coupled receptor signaling pathway
regulation of inflammatory response
cytokine activity
BP
MF
1.6
1.7
1.8
1.9
2.0
NES
P. Adjust
0.04
0.03
0.02
0.01
count
50
100

D

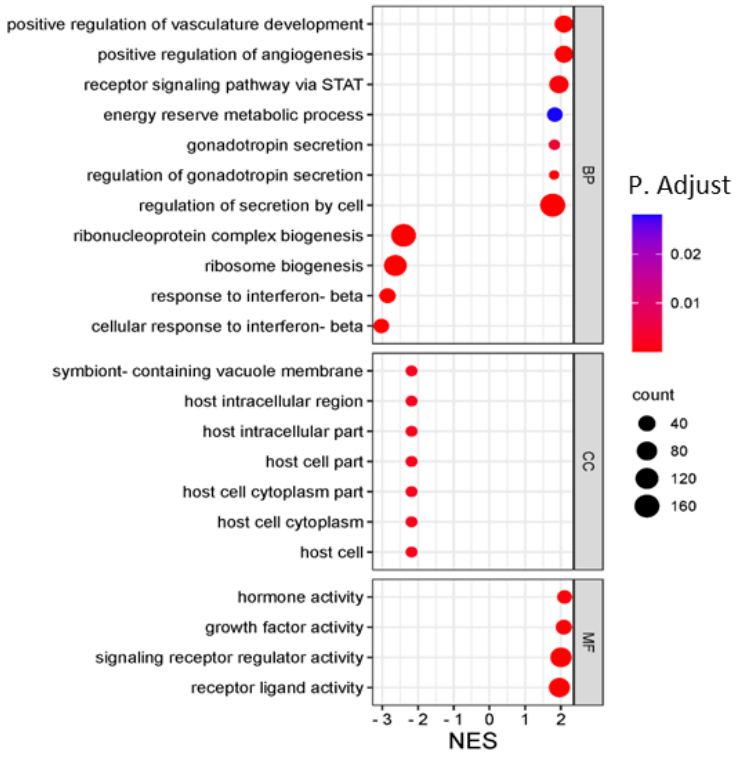


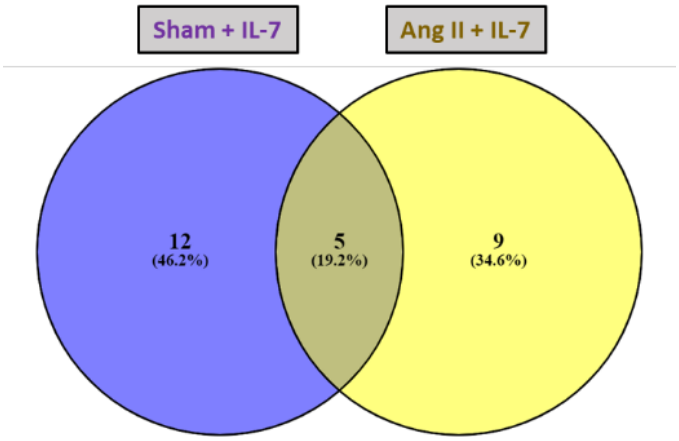
Figure 6.5. GSEA depicting pathways induced by Sham + IL-7, Ang II + IL-7, and pathways commonly induced by both conditions.
Venn plot showing pathways induced in Sham + IL-7 and Ang II + IL-7 treatment conditions **(A)**. The dot plot of GSEA analysis illustrates up-regulated and down-regulated pathways in Sham + IL-7 condition **(B),** the top 20 up-regulated pathways in Ang II + IL-7 condition **(C),** and the top 20 common pathways induced in the 2 conditions **(D)** (p.adj<0.05).

6.4.1.3 The Kyoto Encyclopedia of Genes and Genomes Analysis (KEGG)

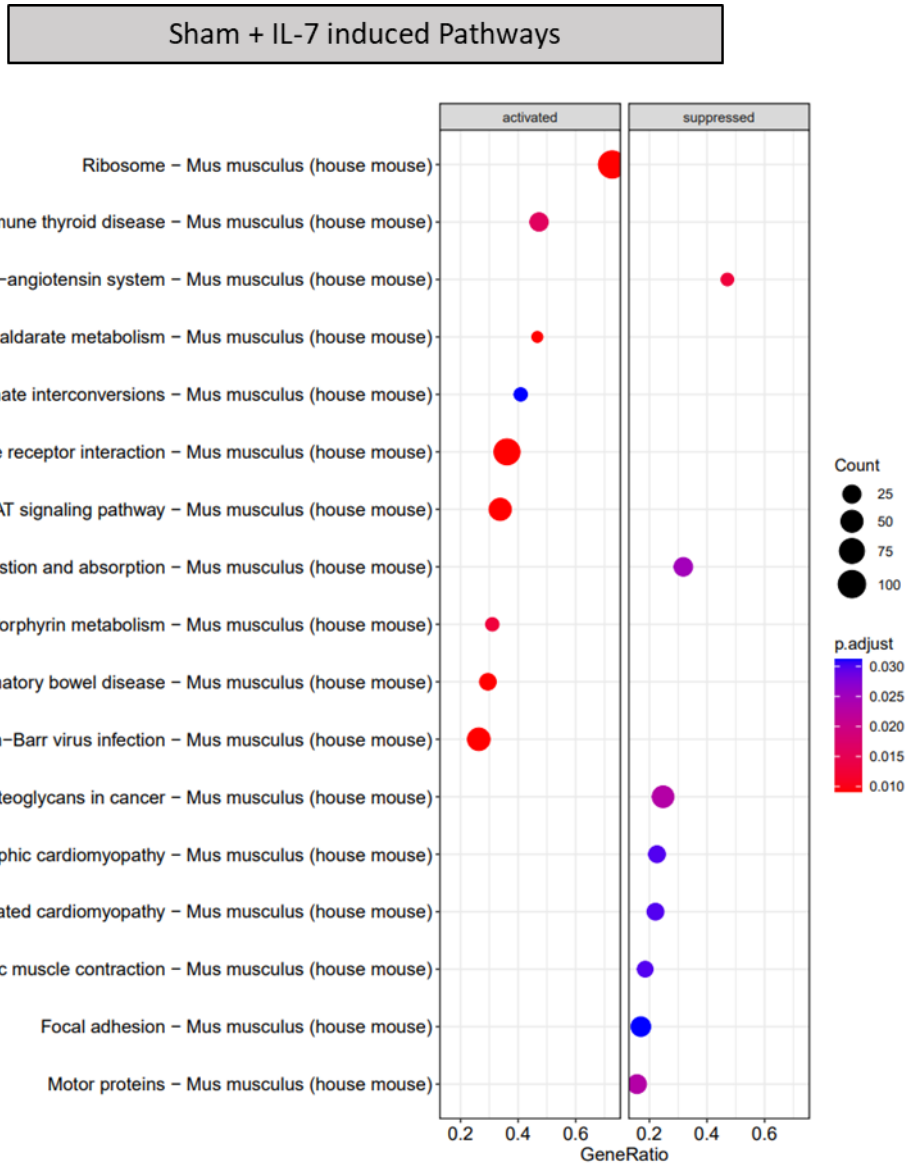
A KEGG enrichment analysis was conducted to investigate the possible distinct response of T cells from Sham and Ang II-infused mice to IL-7. In addition, the KEGG database was used to identify the potential signalling pathways implicated in regulating Sham and Ang II CD8+ T cells. Examination of the 6075 DEGs within the Sham + IL-7 condition revealed the high-ranked pathways of KEGG analysis. The ribosome was the pathway that covered the highest proportion of genes. Cytokine-cytokine receptor interaction and the JAK-STAT signalling pathway, which has a crucial role in T cell biology, regulating various aspects of T cell development, differentiation, activation, and immune response (Robinette et al., 2018), were also among the most significantly enriched pathways. Inflammatory and autoimmune diseases such as autoimmune thyroid disease and inflammatory bowel disease were also present in the results. On the other hand, the renin-angiotensin system and protein digestion and absorption were significantly suppressed in Sham + IL-7 compared to Sham T cells. This encompassed a total of 17 pathways, where P.adj $\geq$ 0.05 (Figure 6.6C). Five pathways were commonly induced in both conditions (Figure 6.6D).

In contrast, the KEGG analyses of the 4759 DEGs within the Ang II + IL-7 condition revealed an exclusive upregulation of pathways linked to insulin and calcium signalling, which has crucial roles in signal transduction during effector function and dysregulation in calcium regulation within lymphocytes is implicated in several conditions, including autoimmune, inflammatory, and immunodeficiency syndromes (Trebak and Kinet, 2019). Other pathways like inflammatory and autoimmune diseases, JAK-STAT signalling, and cytokine-cytokine receptor interaction are accompanied. These trends were observed among a total of 14 pathways (with P.adj $\geq$0.05) (Figure 6.6C). Five pathways, as depicted in (Figure 6.6D) were consistently activated in both conditions. These include the activated pathways: JAK-STAT signalling, cytokine-cytokine receptor interaction, and inflammatory bowel disease, as well as the suppressed pathways, dilated cardiomyopathy, and hypertrophic cardiomyopathy.

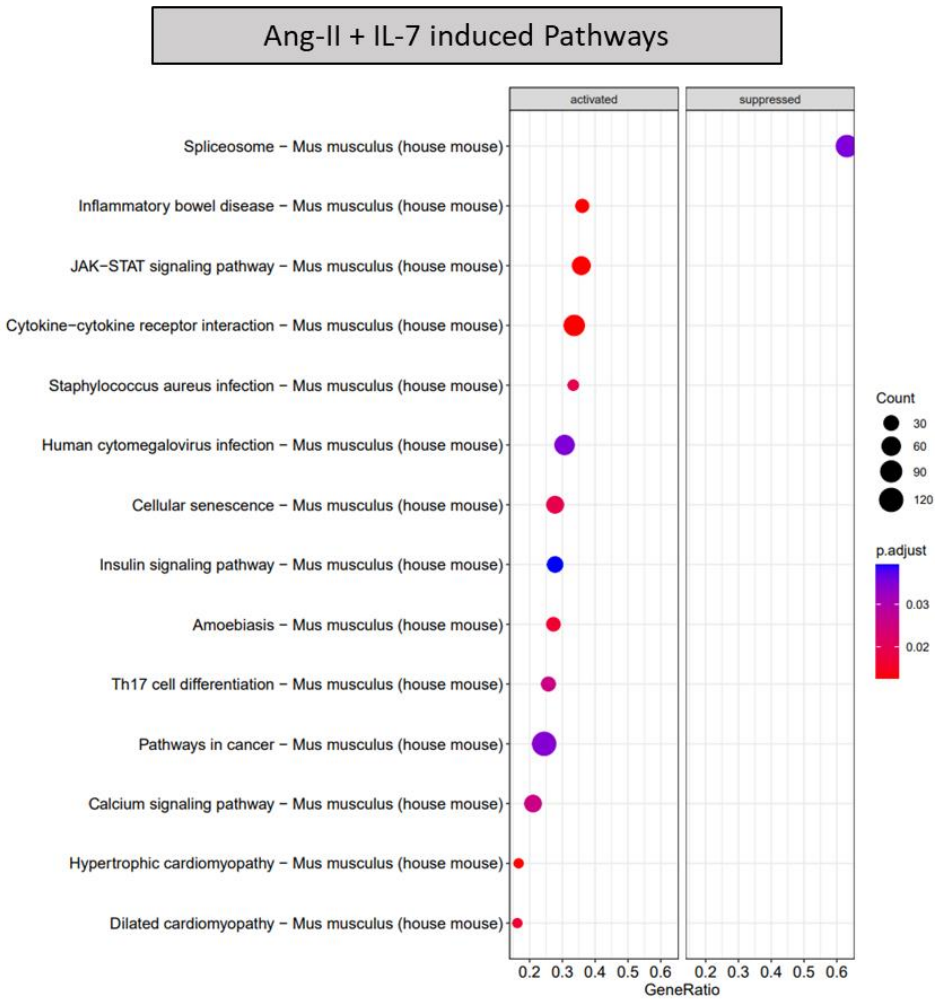
A


Sham + IL-7
Ang II + IL-7
12
(46.2%)
5
(19.2%)
9
(34.6%)

B


Sham + IL-7 induced Pathways
activated
suppressed
Ribosome − Mus musculus (house mouse)
Autoimmune thyroid disease − Mus musculus (house mouse)
Renin−angiotensin system − Mus musculus (house mouse)
Ascorbate and aldarate metabolism − Mus musculus (house mouse)
Pentose and glucuronate interconversions − Mus musculus (house mouse)
Cytokine−cytokine receptor interaction − Mus musculus (house mouse)
JAK−STAT signaling pathway − Mus musculus (house mouse)
Protein digestion and absorption − Mus musculus (house mouse)
Porphyrin metabolism − Mus musculus (house mouse)
Inflammatory bowel disease − Mus musculus (house mouse)
Epstein−Barr virus infection − Mus musculus (house mouse)
Proteoglycans in cancer − Mus musculus (house mouse)
Hypertrophic cardiomyopathy − Mus musculus (house mouse)
Dilated cardiomyopathy − Mus musculus (house mouse)
Cardiac muscle contraction − Mus musculus (house mouse)
Focal adhesion − Mus musculus (house mouse)
Motor proteins − Mus musculus (house mouse)
0.2
0.4
0.6
0.2
0.4
0.6
GeneRatio
Count
25
50
75
100
p.adjust
0.030
0.025
0.020
0.015
0.010

C



D

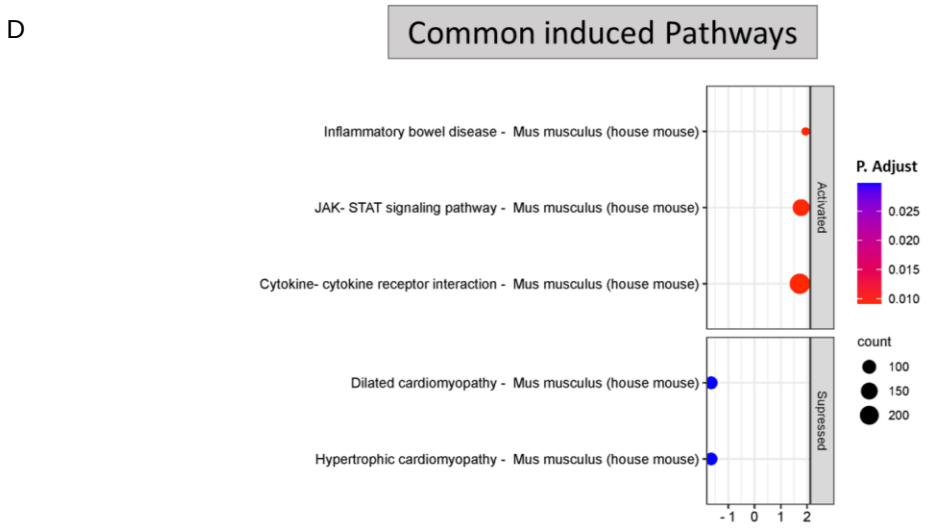


Figure 6.6. KEGG pathway analysis of differentially expressed genes associated with Sham + IL-7 and Ang II + IL-7 conditions.

Venn plot for induced KEGG pathways in the 2 groups **(A).** The dot plot illustrates the KEGG-enriched pathways in Sham + IL-7 **(B),** Ang II + IL-7 **(C)** and the common pathways in 2 conditions **(D).**

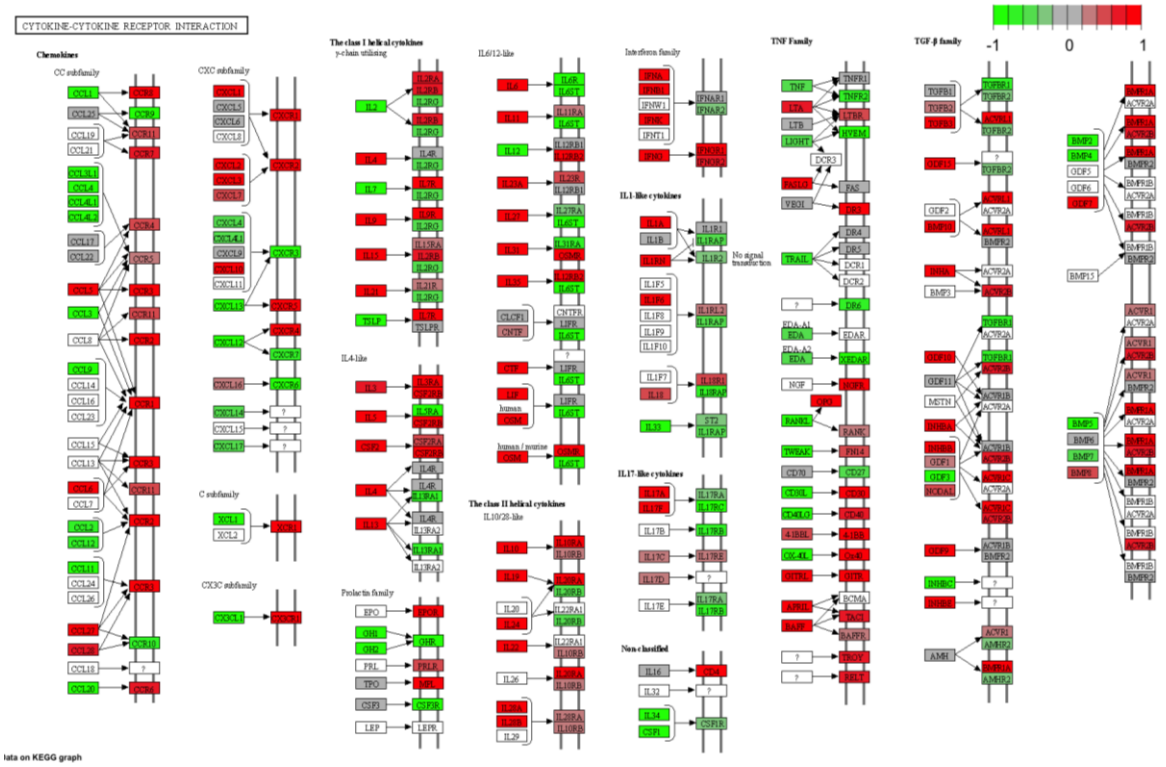
Based on KEGG analysis, the differential gene expression in CD8+ T cells between Sham + IL-7 and Ang II + IL-7 was indicated in the heatmaps (Figure 6.8). In the JAK-STAT pathway, 6 genes were significantly expressed in Ang II + IL-7, including Ifna4, Ifna2, Il9r, Aox2, Ifnk, Ifnl2, and Pias3. on the contrary, 3 genes significantly expressed in Sham + Il-7 (IL23r, Prlr, and Ifngr1) and 30 genes were significantly up-regulated in IL-7 treated CD8+ T cell whether sham or Ang II compared to untreated sham/Ang II CD8+ T cell (Figure 6.8A).

Ca2+ pathway was shown 3 significantly changed genes (Tnnc1, Mylk, Cacna1d) expressed in Ang II + IL-7 CD8+ T cell, compared to Ntrk1, Pdela, Ltb4r2 genes in Sham + IL-7 CD8 T cell. 25 genes were significantly expressed in both conditions compared to untreated CD8+ T cells (Figure 6.8B).

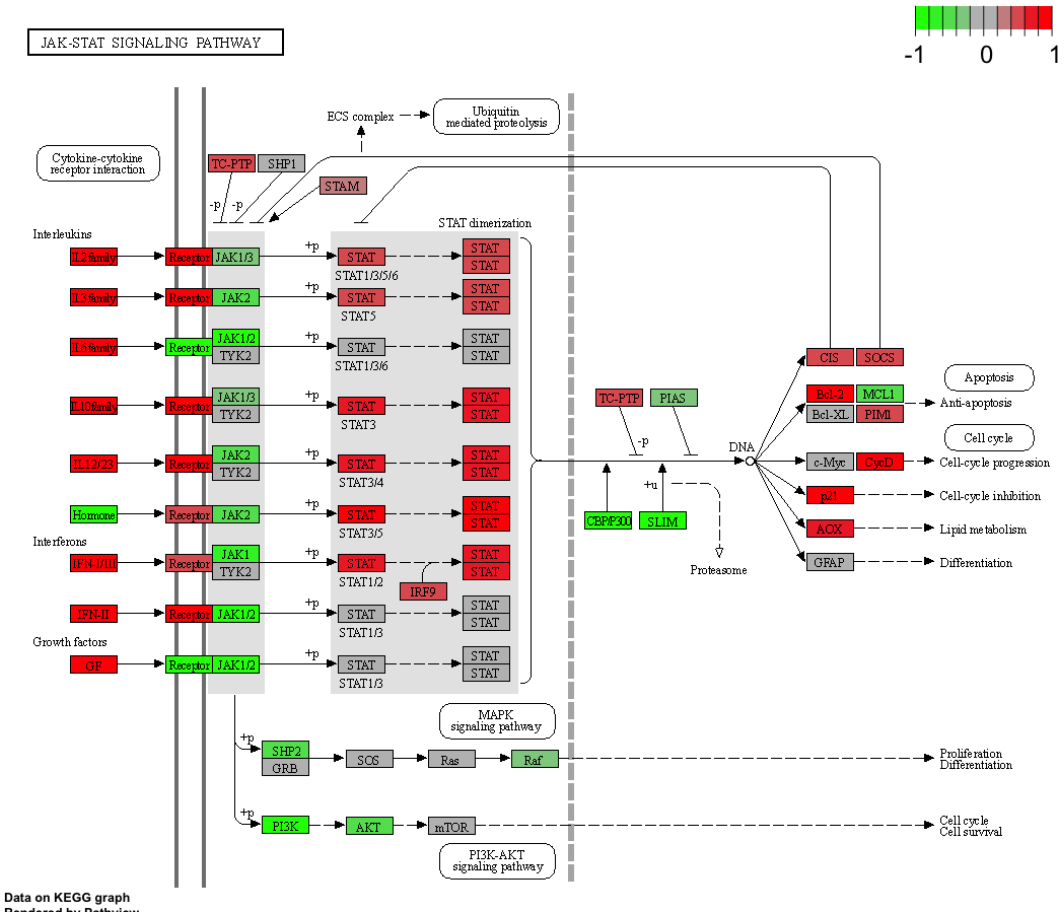
Expression patterns across different samples in cytokine-cytokines receptor interaction were shown 6 genes (Il23r, Bmp8b, Tnfrsf11a, Ifnl2, Tnfsf15, Prlr) significantly changed in Ang II + IL-7 CD8+ T cell, while Ifna4, Ifnlr1, IL-33 genes in Sham + IL-7 CD8 T cell. 47 genes were significantly changed in both conditions compared to untreated CD8+ T cells (Figure 6.8C).

Sham + IL-7 induced Pathways.

A

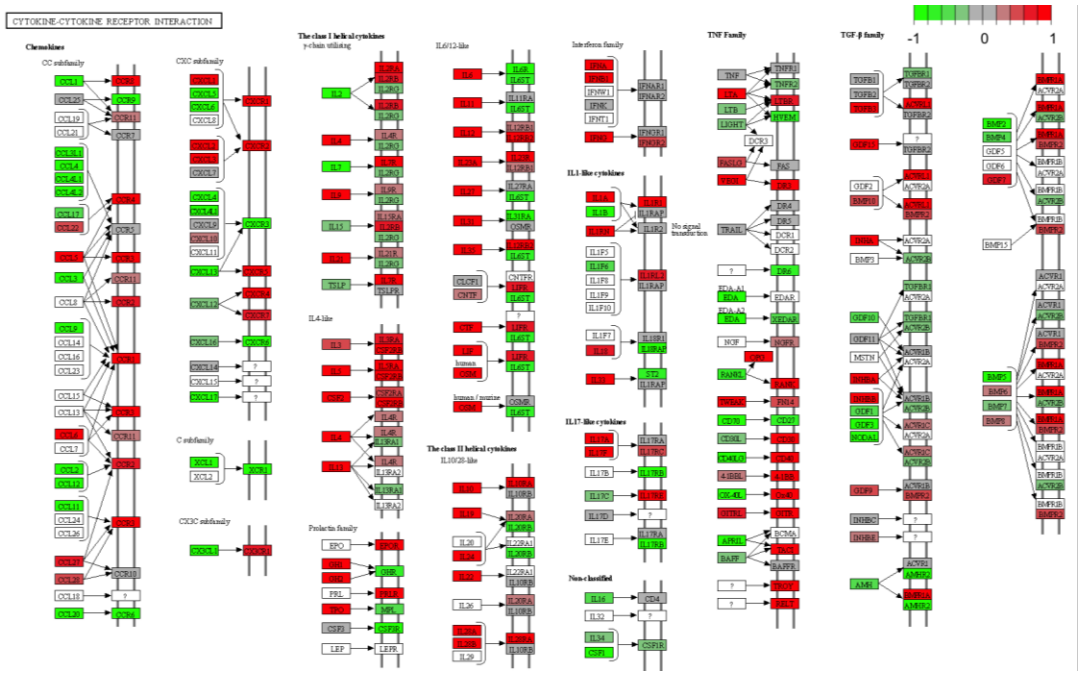


B

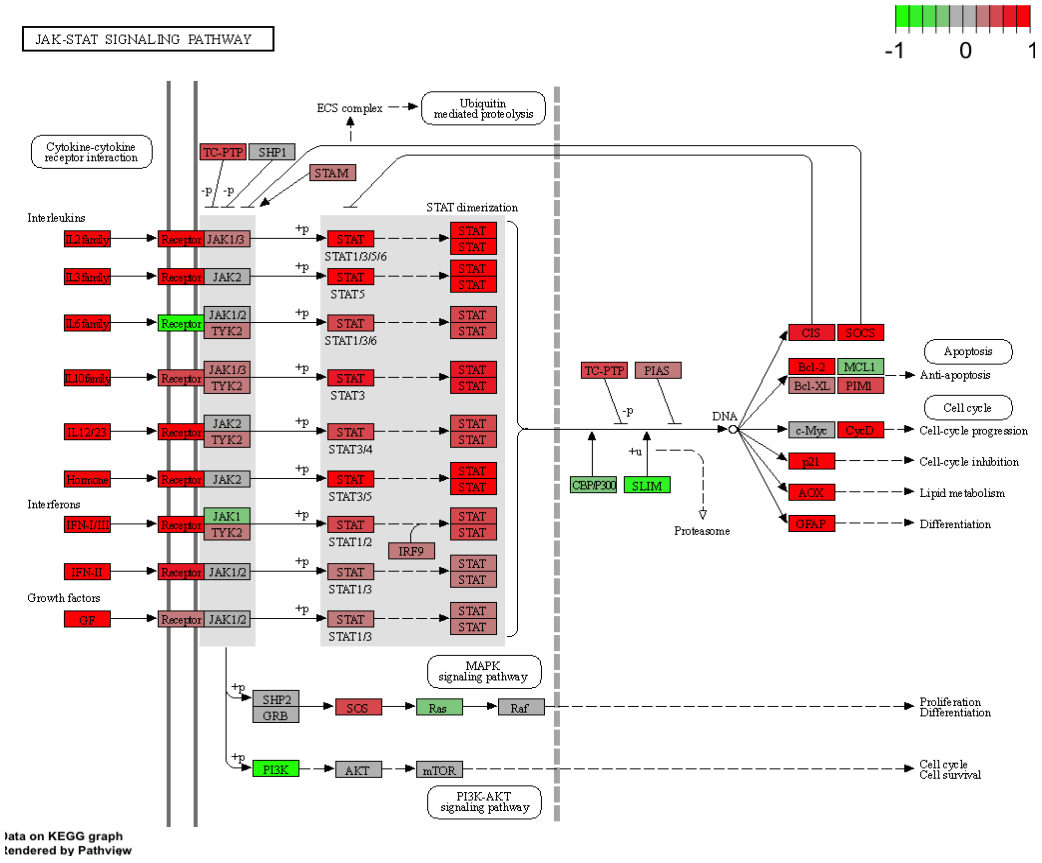

JAK-STAT SIGNALING PATHWAY
-1
0
1
ECS complex
Ubiquitin mediated proteolysis
Cytokine-cytokine receptor interaction
TC-PTP
SHP1
STAM
STAT dimerization
Interleukins
IL2 family
IL3 family
IL6 family
IL10 family
IL12/23
Hormone
Interferons
IFN-I/III
IFN-II
Growth factors
GF
Receptor
JAK1/3
JAK2
JAK1/2
TYK2
STAT
STAT1/3/5/6
STAT5
STAT1/3/6
STAT3
STAT3/4
STAT3/5
STAT1/2
STAT1/3
IRF9
CBP/P300
SLIM
PIAS
DNA
Proteasome
CIS
SOCS
Bcl-2
MCL1
Bcl-XL
PIM1
c-Myc
CycD
p21
AOX
GFAP
Apoptosis
Anti-apoptosis
Cell cycle
Cell-cycle progression
Cell-cycle inhibition
Lipid metabolism
Differentiation
MAPK signaling pathway
SHP2
GRB
SOS
Ras
Raf
Proliferation
Differentiation
PI3K
AKT
mTOR
Cell cycle
Cell survival
PI3K-AKT signaling pathway
Data on KEGG graph

Ang II + IL-7 induced Pathways.

C



D



E

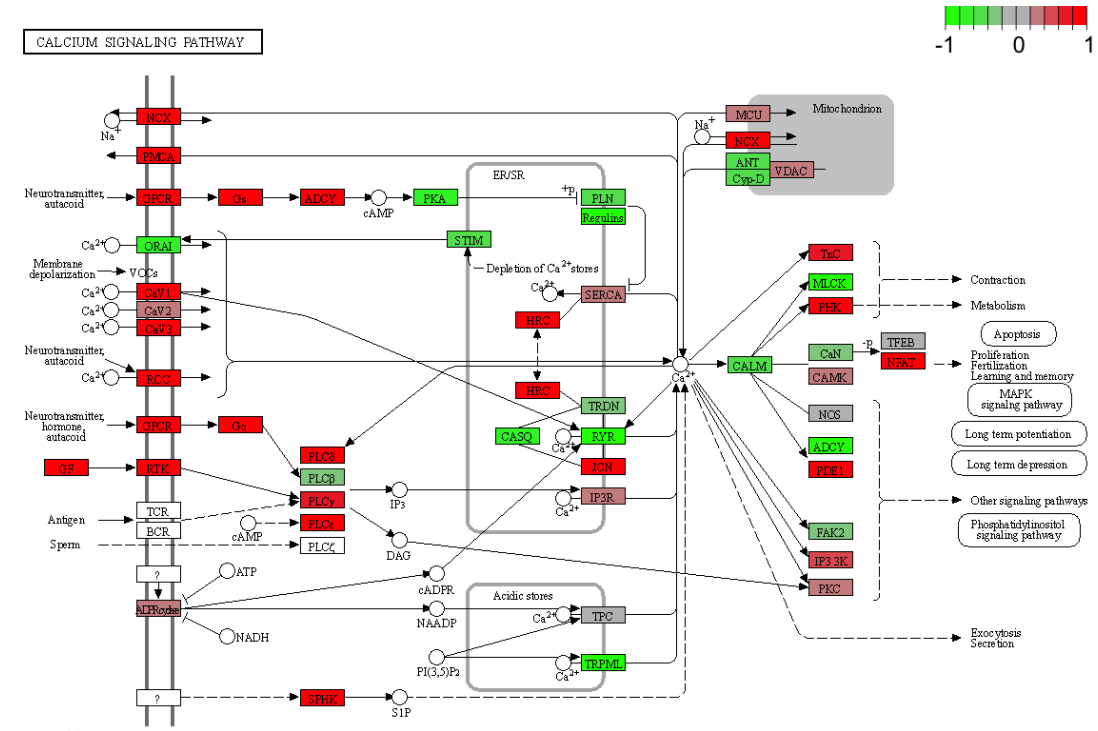
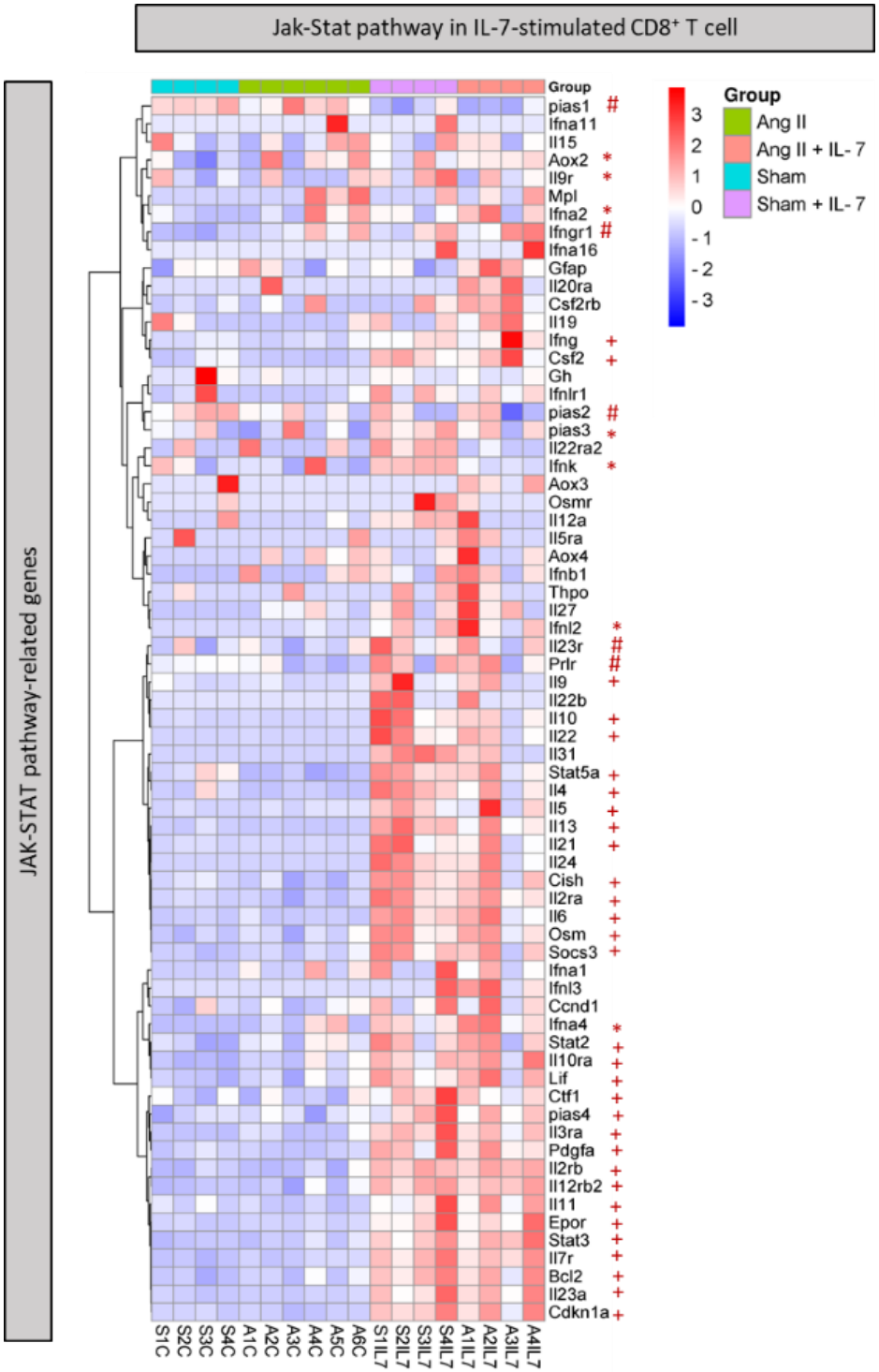


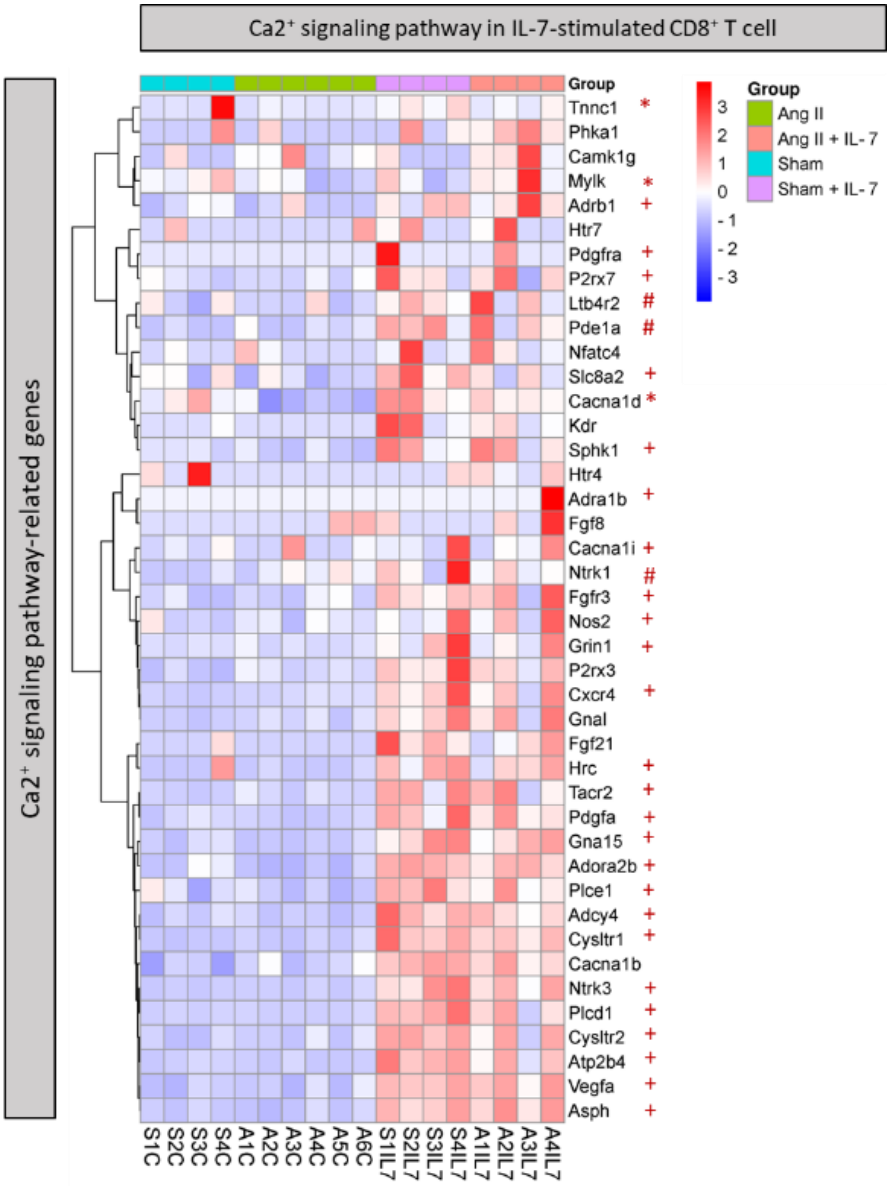
Figure 6.7. The Identified KEGG pathways diagram from Sham + IL-7 and Ang II + IL-7 conditions.

Visualisation of the cytokines-cytokines receptor interaction pathway **(A and C)** and the JAK-STAT pathway **(B and D)** in IL-7-treated CD8+ T cells in Sham and Ang II conditions. Calcium signalling pathway in Ang II + IL-7 condition **(E)**.

A


Jak-Stat pathway in IL-7-stimulated CD8⁺ T cell
JAK-STAT pathway-related genes
Group
Ang II
Ang II + IL- 7
Sham
Sham + IL- 7
3
2
1
0
- 1
- 2
- 3
pias1 #
Ifna11
Il15
Aox2 *
Il9r *
Mpl
Ifna2 *
Ifngr1 #
Ifna16
Gfap
Il20ra
Csf2rb
Il19
Ifng +
Csf2 +
Gh
Ifnlr1
pias2 #
pias3 *
Il22ra2
Ifnk *
Aox3
Osmr
Il12a
Il5ra
Aox4
Ifnb1
Thpo
Il27
Ifnl2 *
Il23r #
Prlr #
Il9 +
Il22b
Il10 +
Il22 +
Il31
Stat5a +
Il4 +
Il5 +
Il13 +
Il21 +
Il24
Cish +
Il2ra +
Il6 +
Osm +
Socs3 +
Ifna1
Ifnl3
Ccnd1
Ifna4 *
Stat2 +
Il10ra +
Lif +
Ctf1 +
pias4 +
Il3ra +
Pdgfa +
Il2rb +
Il12rb2 +
Il11 +
Epor +
Stat3 +
Il7r +
Bcl2 +
Il23a +
Cdkn1a +
S1C
S2C
S3C
S4C
A1C
A2C
A3C
A4C
A5C
A6C
S1IL7
S2IL7
S3IL7
S4IL7
A1IL7
A2IL7
A3IL7
A4IL7

B


Ca2⁺ signaling pathway in IL-7-stimulated CD8⁺ T cell
Ca2⁺ signaling pathway-related genes
Group
Ang II
Ang II + IL-7
Sham
Sham + IL-7
3
2
1
0
-1
-2
-3
Tnnc1 *
Phka1
Camk1g
Mylk *
Adrb1 +
Htr7
Pdgfra +
P2rx7 +
Ltb4r2 #
Pde1a #
Nfatc4
Slc8a2 +
Cacna1d *
Kdr
Sphk1 +
Htr4
Adra1b +
Fgf8
Cacna1i +
Ntrk1 #
Fgfr3 +
Nos2 +
Grin1 +
P2rx3
Cxcr4 +
Gnal
Fgf21
Hrc +
Tacr2 +
Pdgfa +
Gna15 +
Adora2b +
Plce1 +
Adcy4 +
Cysltr1 +
Cacna1b
Ntrk3 +
Plcd1 +
Cysltr2 +
Atp2b4 +
Vegfa +
Asph +
S1C
S2C
S3C
S4C
A1C
A2C
A3C
A4C
A5C
A6C
S1IL7
S2IL7
S3IL7
S4IL7
A1IL7
A2IL7
A3IL7
A4IL7

C

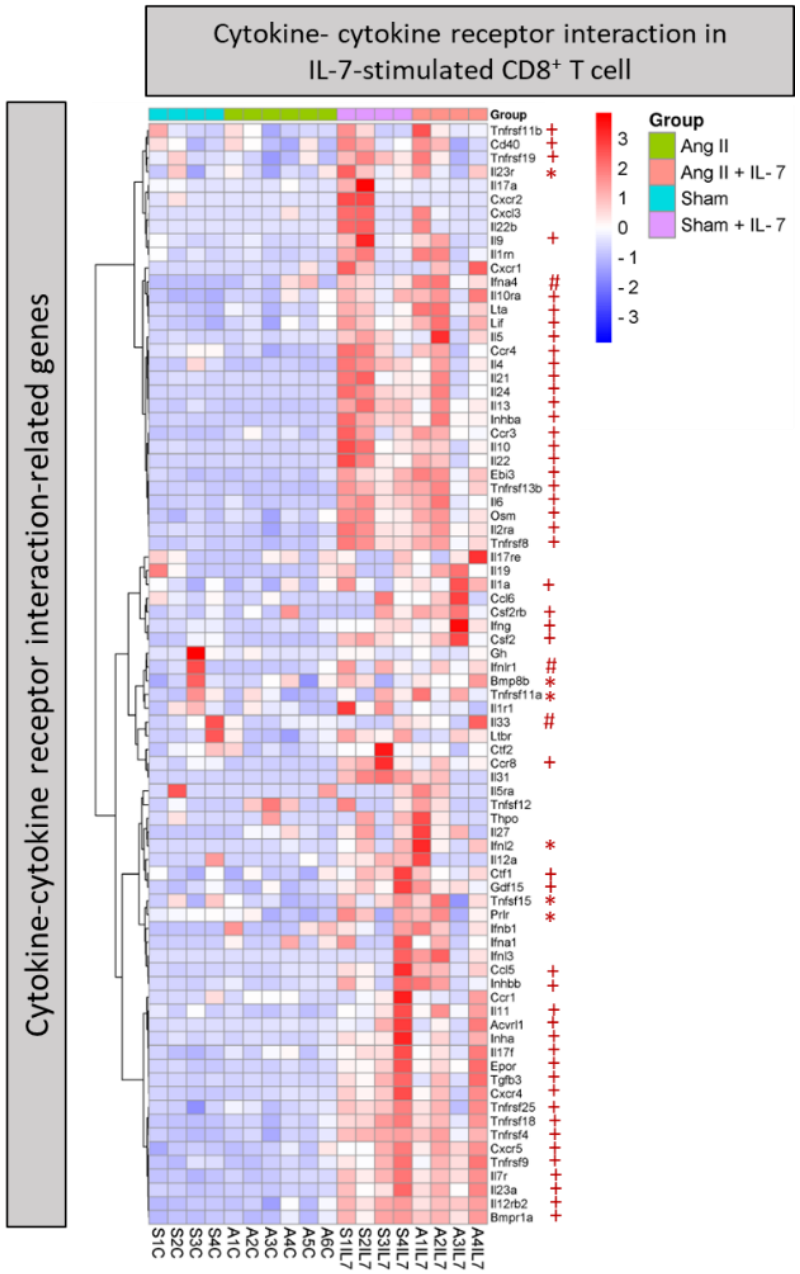


Figure 6.8. Heatmap illustrating genes involved in induced pathways in IL-7 treated CD8+ T cells.

Representative genes in the JAK-STAT pathway **(A),** cytokines-cytokines interaction **(B),** and Ca2+ signalling pathway **(C)** induced in Sham and Ang II IL-7-treated CD8+ T cells. **(#)** refer to the genes significantly expressed in Sham + IL-7 compared to Sham, **(*)** refers to the genes significantly expressed in Ang II+ IL-7 compared to Ang II CD8+ T cell, and **(+)** refers to the genes significantly expressed in both.

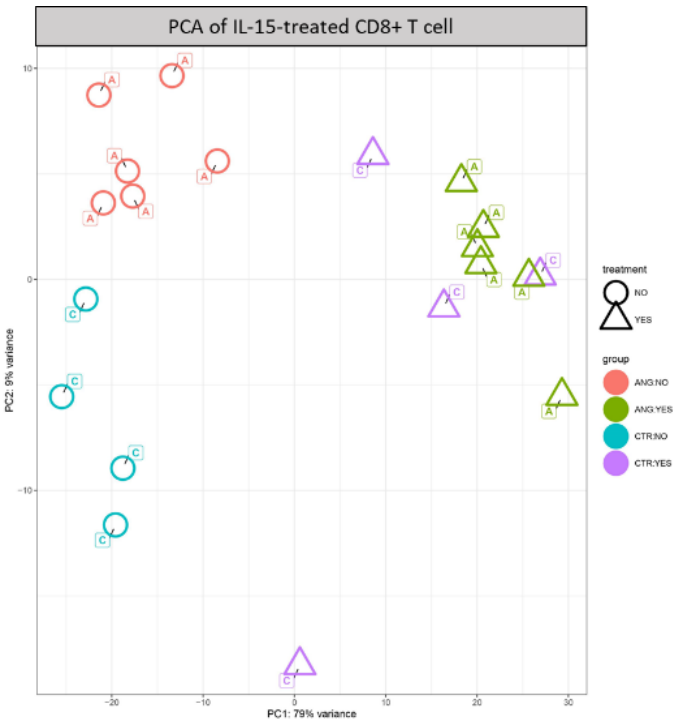
6.4.2 Gene expression effect of IL-15 on CD8+ T cell

6.4.2.1 Differential Expression Analysis

The dataset was analysed by PCA (Figure 6.9). The separation was primarily represented by the PC1 vs PC2, accounting for 79% and 9%, respectively, of the observed variance (Figure 6.9A). Separation was observed between IL-15-treated CD8+ T cells (Sham and Ang II) when compared to untreated CD8+ T cells (Sham and Ang II). However, there was no separation between Sham + IL-15 and Ang II + IL-15 conditions.

To gain deeper insights into the distinctions between the Sham + IL-15 and Ang II + IL-15 samples, we analyzed the differential gene expression. This analysis involved pooling the outcomes from the four (n=4 for sham / n=6 for Ang II) repetitions to enhance statistical power. The gene expression level between Sham vs. Sham + IL-15 and Ang II vs. Ang II + IL-15-treated $CD8^+$ T cells was assessed and visually presented through volcano plots (Figure 6.9B).

A



B

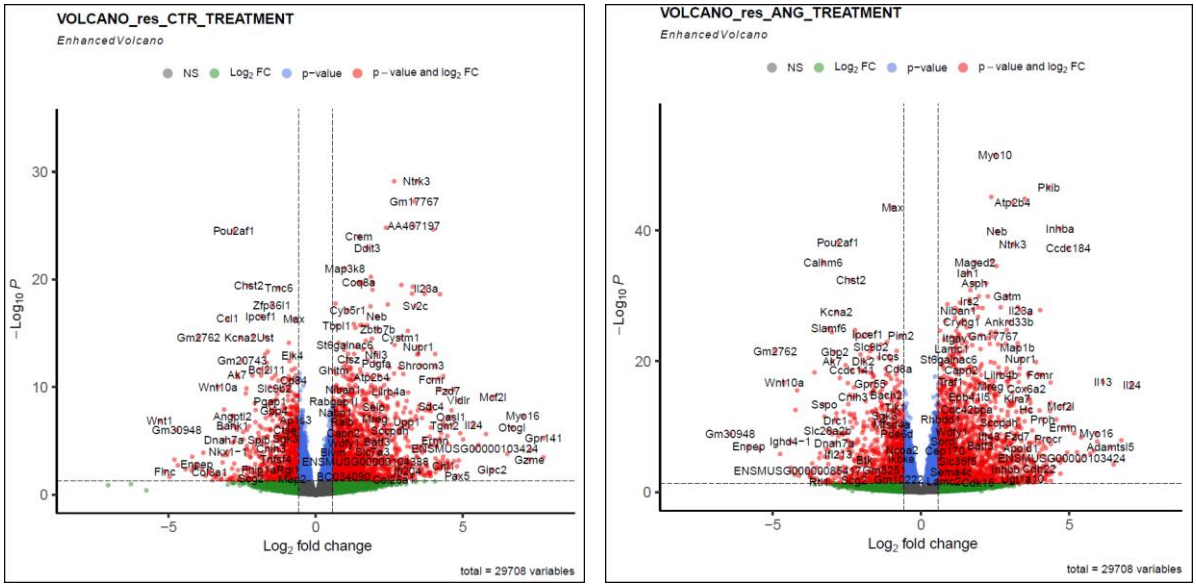


Figure 6.9. PCA and Volcano plot for Sham Vs Ang II IL-15-treated CD8+ T cell.

PCA plots representing CD8+ T cell + IL-15 from sham and Ang II-infused mice, samples analysed by RNA-Seq **(A).** Volcano plots demonstrate an overview of the differential expression of all genes in Sham + IL-15 and Ang II + IL-15 conditions **(B).** The threshold for the volcano plot was p.adj >0.05. Green=not sig. & FC =1.5, bule =sig. & FC<1.5, and Red= Sig. & FC>1.5.

A Venn diagram is in (Figure 6.9) illustrating gene expression comparisons between two sample groups was generated post-DESeq2 analysis. Using the P.adj parameters for screening and filtering gene expression, 5226 DEGs were identified in the Sham + IL-15 treatment condition, compared to 6438 DEGs in Ang-II-T cells + IL-15 treatment (P.adj<0.05, Log2FC>0.1). These genes specifically represent the impact of adding IL-15 to both the Sham and Ang II conditions compared to their respective untreated baselines. Within the 5226 DEGs in Sham + IL-15, 578 genes were upregulated, and 689 were downregulated. In Ang II-T cells + IL-15, among the 6438 DEGs, 2475 were upregulated, and 1153 were downregulated. Unexpectedly, no interaction genes were detected among upregulated and downregulated genes; only 3 interaction genes (IL1l1, Pkp4, Pdgfb) were detected, which are likely to play a role in the interaction effect observed in this experimental condition.

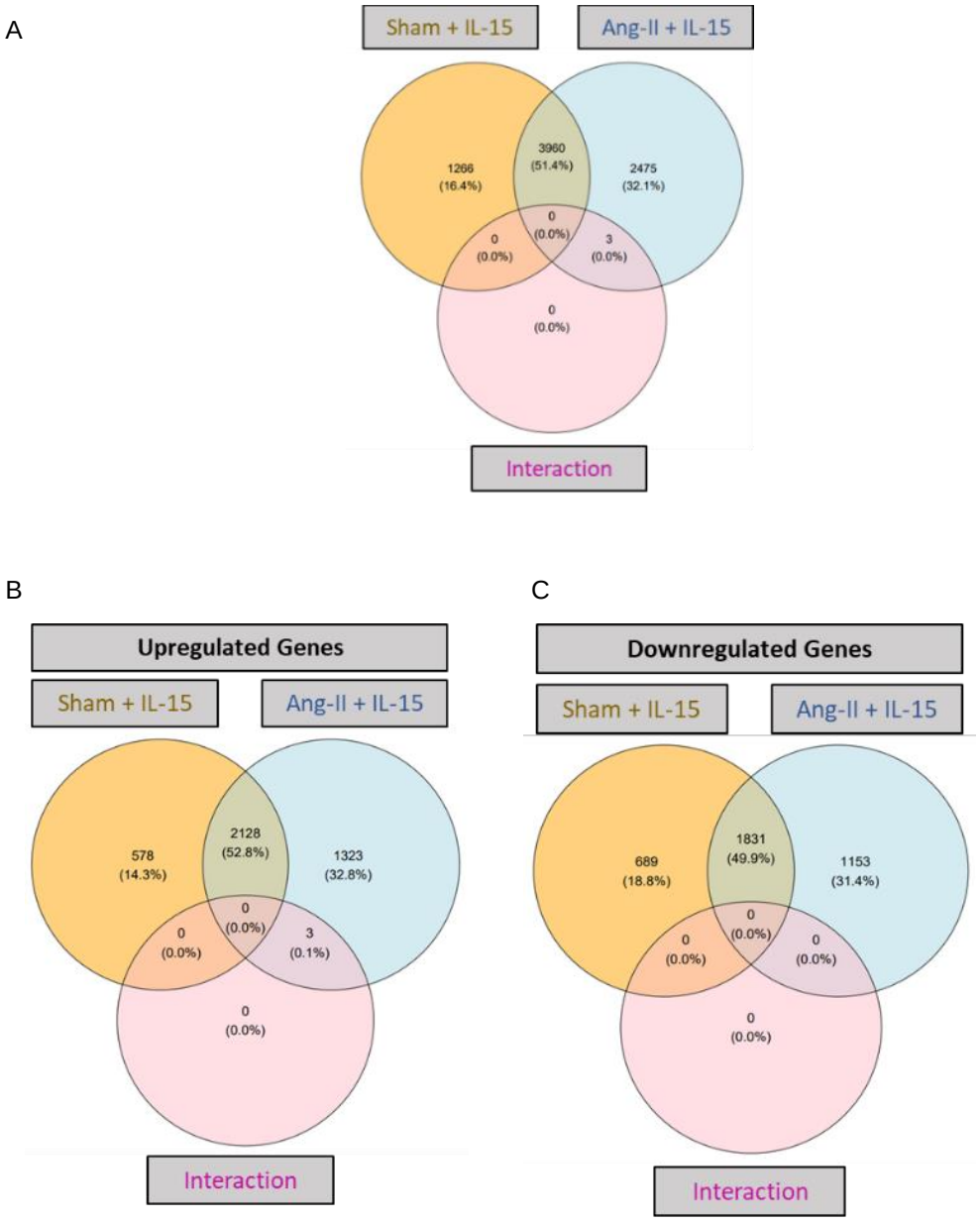


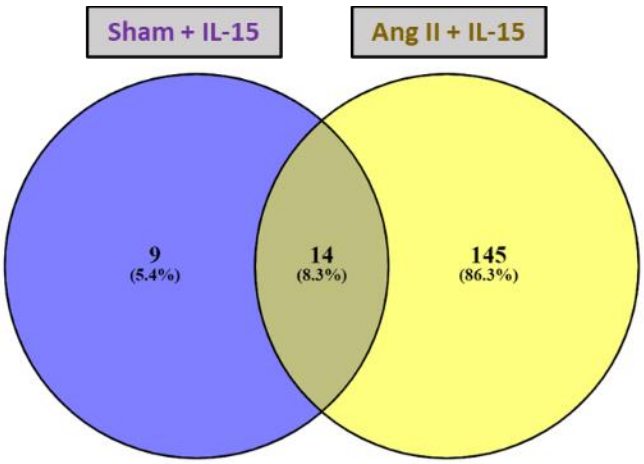
Figure 6.10. Differential gene expression of Sham and Ang-II IL-15-treated CD8+ T cell.

Venn plots illustrate 2 pairs of groups comparing Sham + IL-15 to Ang II + IL-15 gene expression profiles **(A).** Venn plots illustrate 3 groups, including interaction group **(B).** upregulated **(C)** and downregulated **(D)** DEGs.

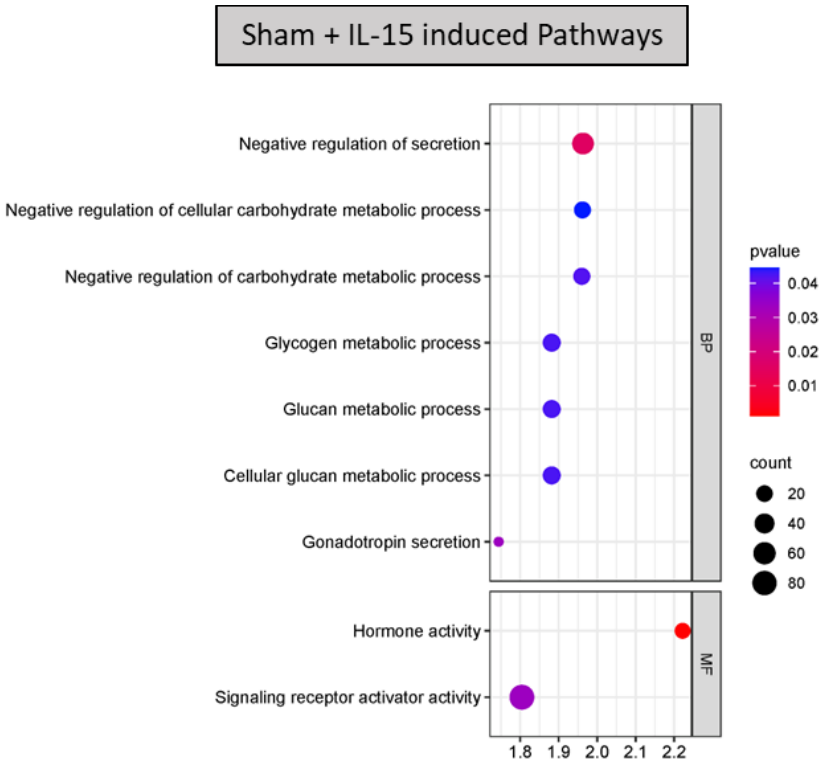
6.4.2.2 GSEA analysis.

The GSEA threshold setting here includes P.adj≤0.05. GSEA revealed the induction of 23 pathways in the Sham + IL-15 condition, whereas the Ang II + IL-15 condition exhibited the induction of 159 pathways. Notably, 14 pathways were found to be shared between the sham + IL-15 and Ang II + IL-15 conditions, presented all on dot-plot (Figure 6.11B-D). The nine induced pathways in the Sham + IL-15 condition are presented on a dot plot (Figure 6.11B) where all are up-regulated. On the other hand, more than 145 pathways were specifically induced in the Ang II + IL-15 condition, and the top 20 up-regulated and down-regulated pathways were presented (Figure 6.11C). All induced pathways are NES ≥1.

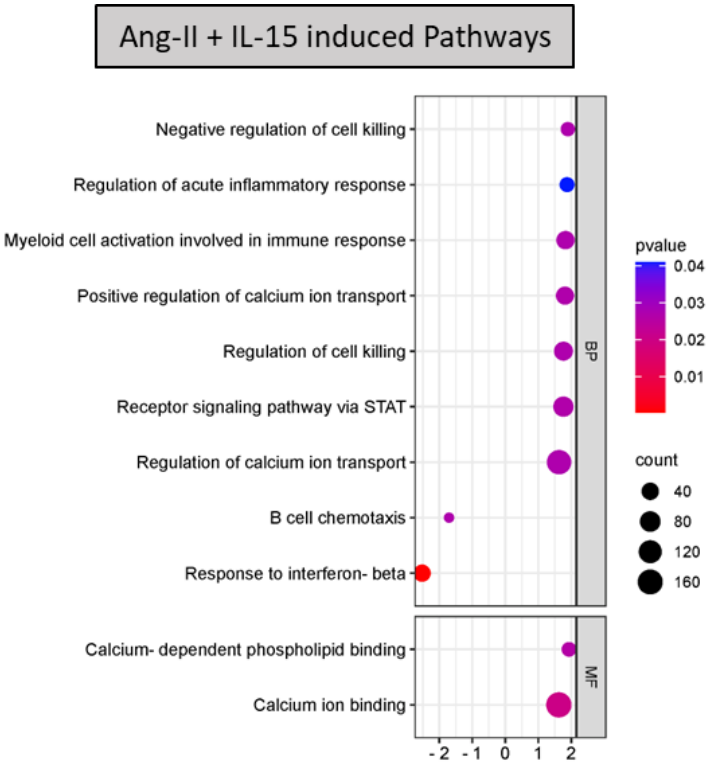
A


Sham + IL-15
Ang II + IL-15
9
(5.4%)
14
(8.3%)
145
(86.3%)

B


Sham + IL-15 induced Pathways
Negative regulation of secretion
Negative regulation of cellular carbohydrate metabolic process
Negative regulation of carbohydrate metabolic process
Glycogen metabolic process
Glucan metabolic process
Cellular glucan metabolic process
Gonadotropin secretion
Hormone activity
Signaling receptor activator activity
BP
MF
1.8
1.9
2.0
2.1
2.2
pvalue
0.04
0.03
0.02
0.01
count
20
40
60
80

C



D

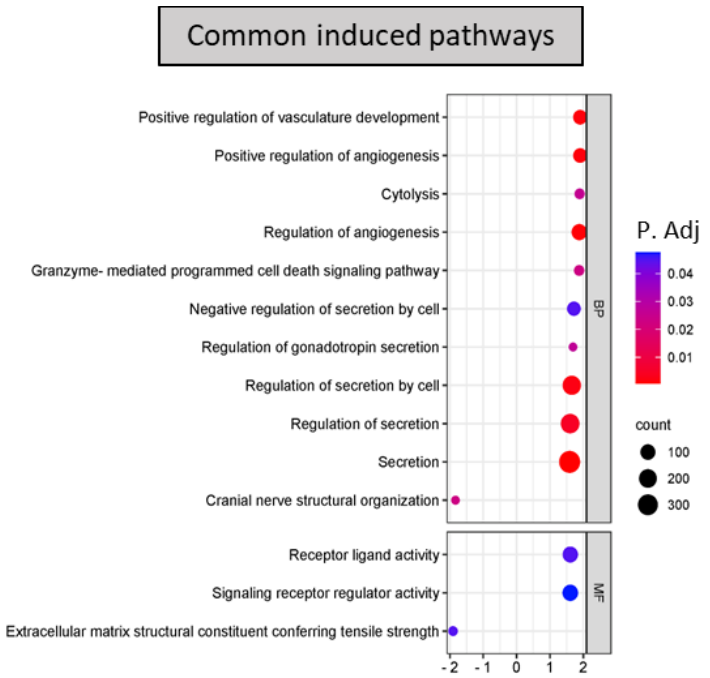
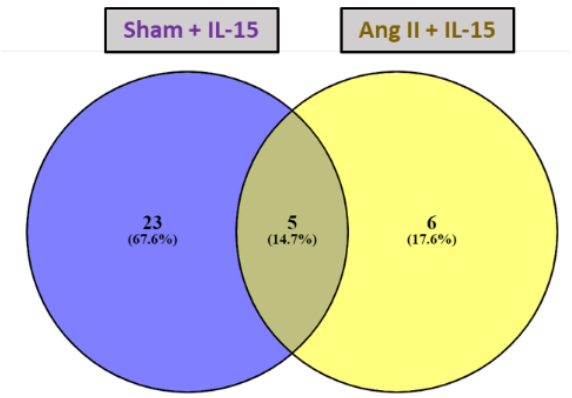


Figure 6.11. GSEA of Sham, Ang II, and common induced pathway.
Venn plot of the pathways induced in Sham and Ang II + IL-15 conditions **(A).** Dot plot of GSEA analysis illustrate the induced pathway in Sham + IL-15 **(B),** the top 20 up-regulated and down-regulated Ang II + IL-15 induced pathway **(C)** and top 10 common upregulated and down-regulated pathways**(D).** P.adj<0.05.

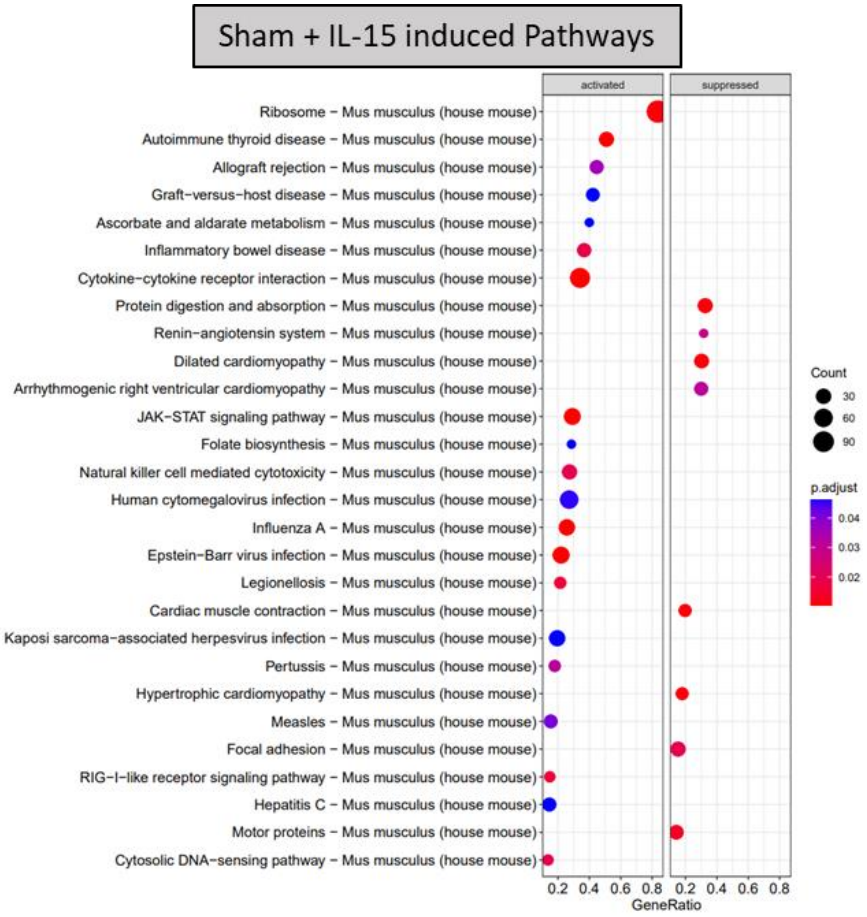
6.4.2.3 KEGG analysis.

KEGG analysis was performed to investigate the differential effect of IL-15 on T cells from sham versus Ang II-infusion mice. Examination of the 5226 DEGs within the control+IL-15 condition revealed that pathways of KEGG analysis included JAK-STAT signalling pathway, cytokine-cytokine receptor interaction, as well as inflammatory and autoimmune disease, which are absent in the Sham vs Ang II condition. This encompassed a total of 27 pathways, where P.adj ≥0.05 (Figure 6.13A). On the other hand, KEGG analyses of the 6435 DEGs identified within the Ang II + IL-7 condition revealed the upregulation of pathways such as calcium signalling, MAPK and PI3K-Akt signalling. These were accompanied by the presence of inflammatory and autoimmune diseases, along with the activation of pathways like JAK-STAT signalling and cytokine-cytokine receptor interaction. Among a total of 11 pathways (with P.adj ≥ 0.05), these trends were observed (Figure 6.13B).

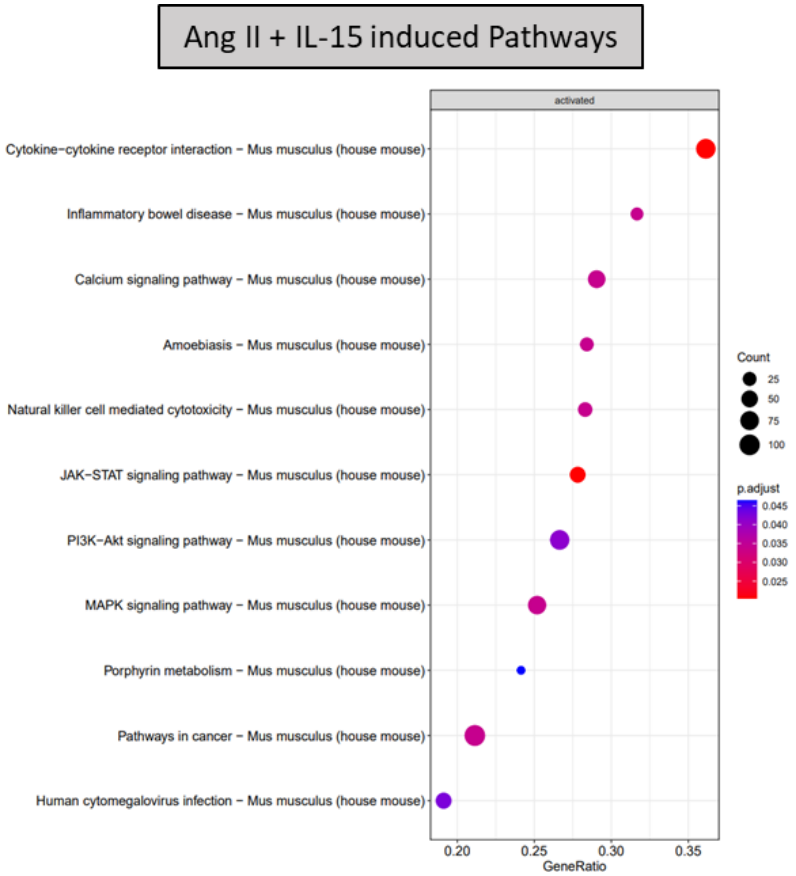
A



B


Sham + IL-15 induced Pathways
activated
suppressed
Ribosome − Mus musculus (house mouse)
Autoimmune thyroid disease − Mus musculus (house mouse)
Allograft rejection − Mus musculus (house mouse)
Graft−versus−host disease − Mus musculus (house mouse)
Ascorbate and aldarate metabolism − Mus musculus (house mouse)
Inflammatory bowel disease − Mus musculus (house mouse)
Cytokine−cytokine receptor interaction − Mus musculus (house mouse)
Protein digestion and absorption − Mus musculus (house mouse)
Renin−angiotensin system − Mus musculus (house mouse)
Dilated cardiomyopathy − Mus musculus (house mouse)
Arrhythmogenic right ventricular cardiomyopathy − Mus musculus (house mouse)
JAK−STAT signaling pathway − Mus musculus (house mouse)
Folate biosynthesis − Mus musculus (house mouse)
Natural killer cell mediated cytotoxicity − Mus musculus (house mouse)
Human cytomegalovirus infection − Mus musculus (house mouse)
Influenza A − Mus musculus (house mouse)
Epstein−Barr virus infection − Mus musculus (house mouse)
Legionellosis − Mus musculus (house mouse)
Cardiac muscle contraction − Mus musculus (house mouse)
Kaposi sarcoma−associated herpesvirus infection − Mus musculus (house mouse)
Pertussis − Mus musculus (house mouse)
Hypertrophic cardiomyopathy − Mus musculus (house mouse)
Measles − Mus musculus (house mouse)
Focal adhesion − Mus musculus (house mouse)
RIG−I−like receptor signaling pathway − Mus musculus (house mouse)
Hepatitis C − Mus musculus (house mouse)
Motor proteins − Mus musculus (house mouse)
Cytosolic DNA−sensing pathway − Mus musculus (house mouse)
0.2 0.4 0.6 0.8
0.2 0.4 0.6 0.8
GeneRatio
Count
30
60
90
p.adjust
0.04
0.03
0.02

C


Ang II + IL-15 induced Pathways
activated
Cytokine−cytokine receptor interaction − Mus musculus (house mouse)
Inflammatory bowel disease − Mus musculus (house mouse)
Calcium signaling pathway − Mus musculus (house mouse)
Amoebiasis − Mus musculus (house mouse)
Natural killer cell mediated cytotoxicity − Mus musculus (house mouse)
JAK−STAT signaling pathway − Mus musculus (house mouse)
PI3K−Akt signaling pathway − Mus musculus (house mouse)
MAPK signaling pathway − Mus musculus (house mouse)
Porphyrin metabolism − Mus musculus (house mouse)
Pathways in cancer − Mus musculus (house mouse)
Human cytomegalovirus infection − Mus musculus (house mouse)
0.20
0.25
0.30
0.35
GeneRatio
Count
25
50
75
100
p.adjust
0.045
0.040
0.035
0.030
0.025

D

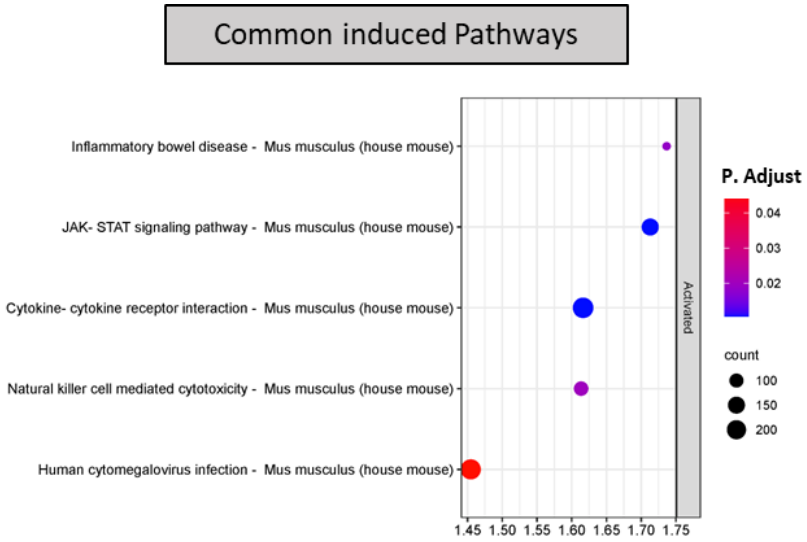


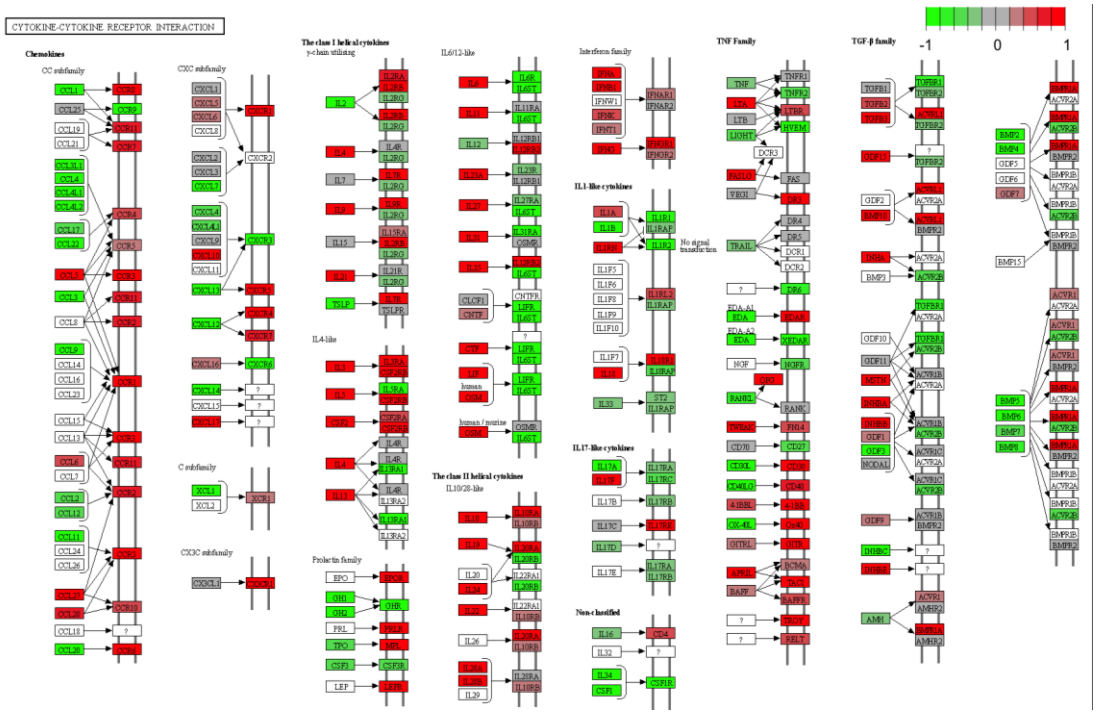
Figure 6.12. KEGG pathway analysis of differentially expressed genes associated with Sham + IL-15 and Ang II + IL-15 conditions.
Representative Venn plot of the pathways induced in IL-15 treated-CD8+ T cell from Sham and Ang II mice **(A).** Dot plot of the pathways induced in Sham + IL-15 **(B),** Ang II + IL-15 **(C),** and in both conditions **(D).**

The differential gene expression was evaluated, as in IL-7, in 4 different conditions comparing Sham, Sham + IL-15, Ang II and Ang II + IL-15. 2 pathways were found to be induced commonly in Sham + IL-15 and Ang II + IL-15 conditions, which are JAK-STAT and cytokine-cytokine receptor interaction. MAPK and Ca2+ signalling pathways were found exclusively in the Ang II + IL-15 condition. In JAK-STAT, 4 genes were found to be significantly expressed in Ang II + IL-15 treatment, which are Ifnlr1, Ifnb1, Ctf2 and Ifnl2. 26 genes were markedly induced in response to IL-15, whether in sham or Ang II (Figure 6.14A **(+)**).

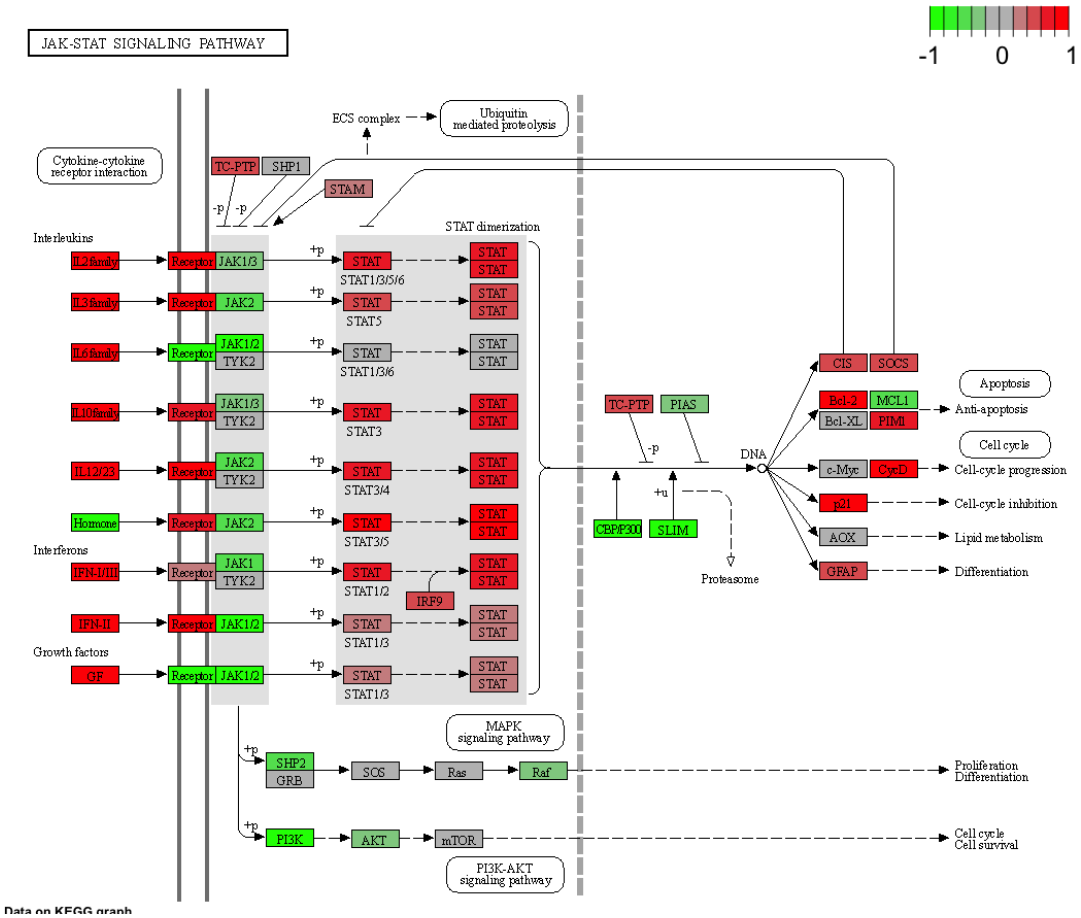
In cytokine-cytokine receptor interaction, Ifnlr1, Ctf2, Ltbr, Ifnb1, Inhbe, Inhba, Ifnl2, Ifnl3, CCR5, CCR10 and Bmpr2 significantly expressed in Ang II +IL-15condition out of 54 genes significantly expressed in both conditions (Figure 6.14B **(+)**). In Ca2+ signalling pathway, 6 genes (Cacna1d, Nfatc4, Pln, Cacna1i, Ntrk1, Slc8a2) were significantly induced in Ang II + IL-15 condition (Figure 6.14C **(*)**). Cacna1d, Cacna1g, Cacna1i, Cacnb4, Fgf21, Pgf, Mapk13, HsPb1, Artn, Prkcg, Rasgrp3, Igf1, Ntrk1 were also significantly expressed in Ang II + IL-15 in MAPK signalling pathway (Figure 6.14D **(*)**).

Sham + IL-15 induced Pathways.

A

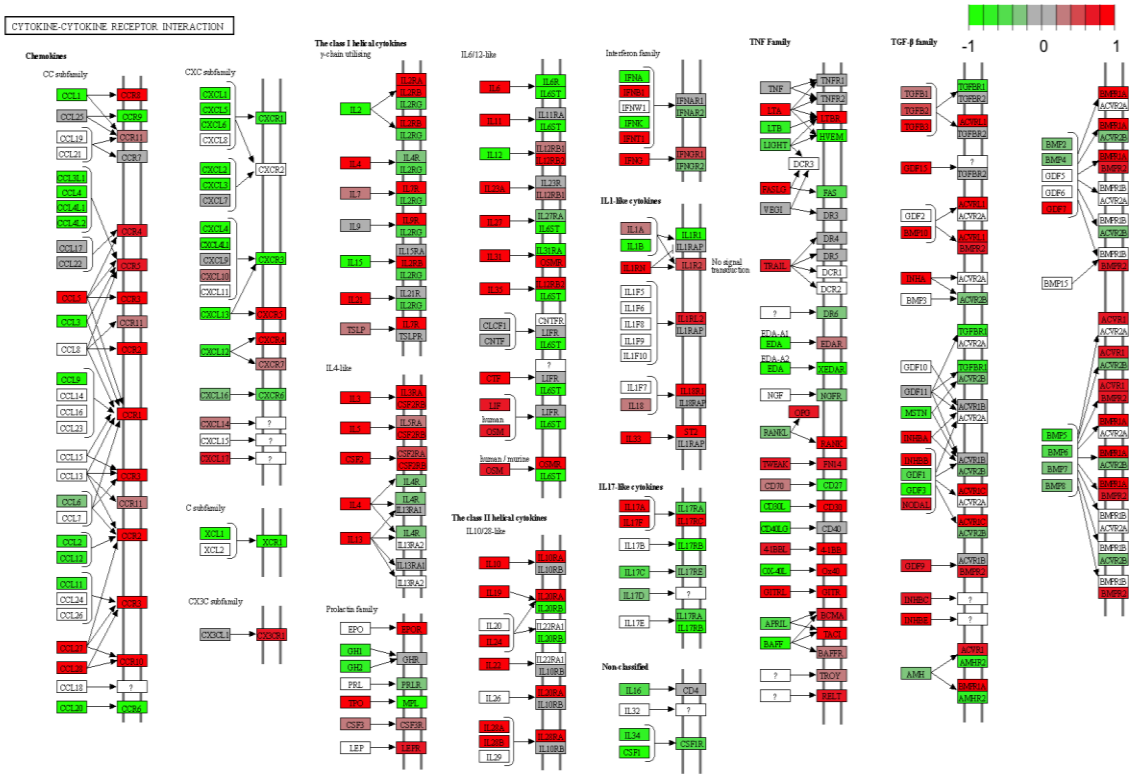


B

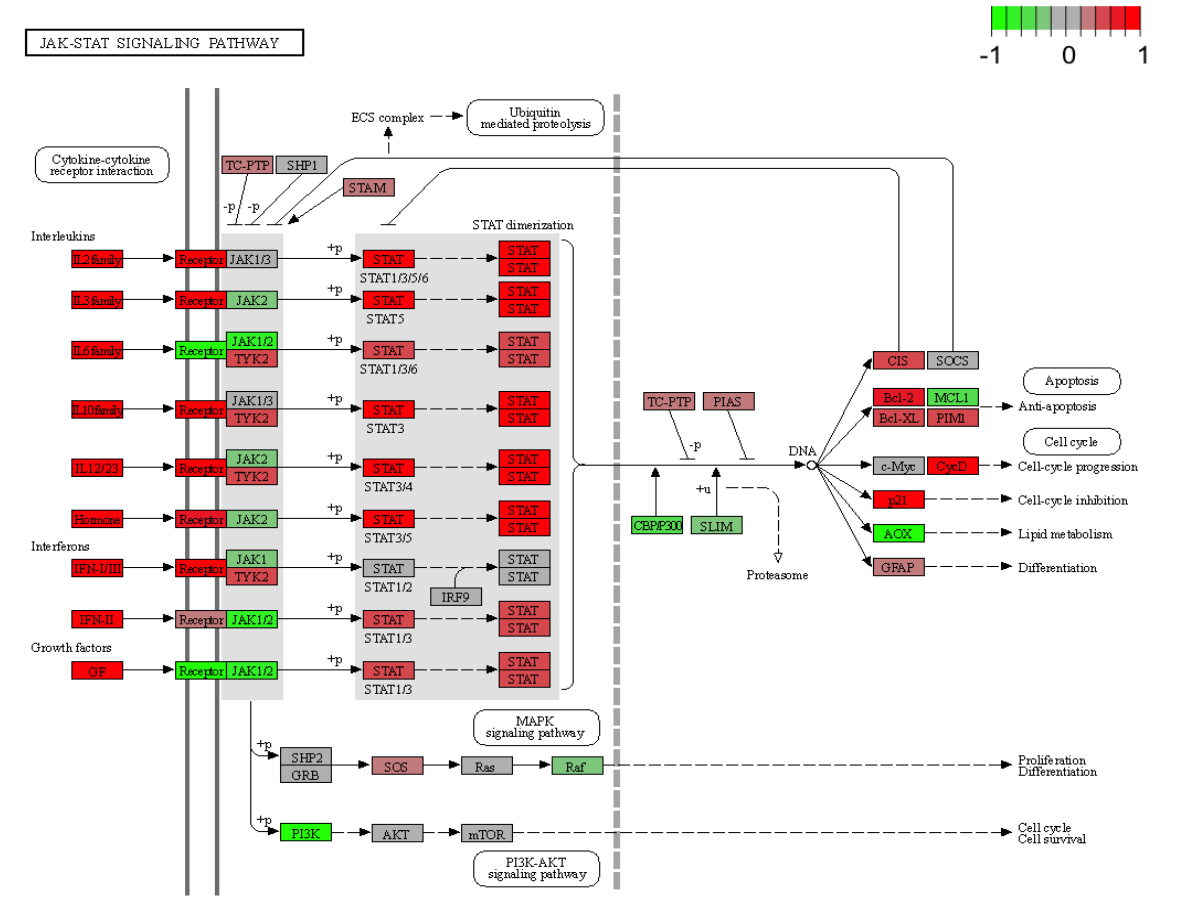

JAK-STAT SIGNALING PATHWAY
-1
0
1
ECS complex
Ubiquitin mediated proteolysis
Cytokine-cytokine receptor interaction
TC-PTP
SHP1
STAM
STAT dimerization
Interleukins
IL2 family
IL3 family
IL6 family
IL10 family
IL12/23
Hormone
Interferons
IFN-I/III
IFN-II
Growth factors
GF
Receptor
JAK1/3
JAK2
JAK1/2
TYK2
JAK1
STAT
STAT1/3/5/6
STAT5
STAT1/3/6
STAT3
STAT3/4
STAT3/5
STAT1/2
STAT1/3
IRF9
+p
-p
TC-PTP
PIAS
CBP/P300
SLIM
+u
DNA
Proteasome
CIS
SOCS
Bcl-2
MCL1
Bcl-XL
PIM1
c-Myc
CycD
p21
AOX
GFAP
Apoptosis
Anti-apoptosis
Cell cycle
Cell-cycle progression
Cell-cycle inhibition
Lipid metabolism
Differentiation
MAPK signaling pathway
SHP2
GRB
SOS
Ras
Raf
Proliferation
Differentiation
PI3K
AKT
mTOR
Cell cycle
Cell survival
PI3K-AKT signaling pathway
Data on KEGG graph

Ang II + IL-15 induced Pathways.

C

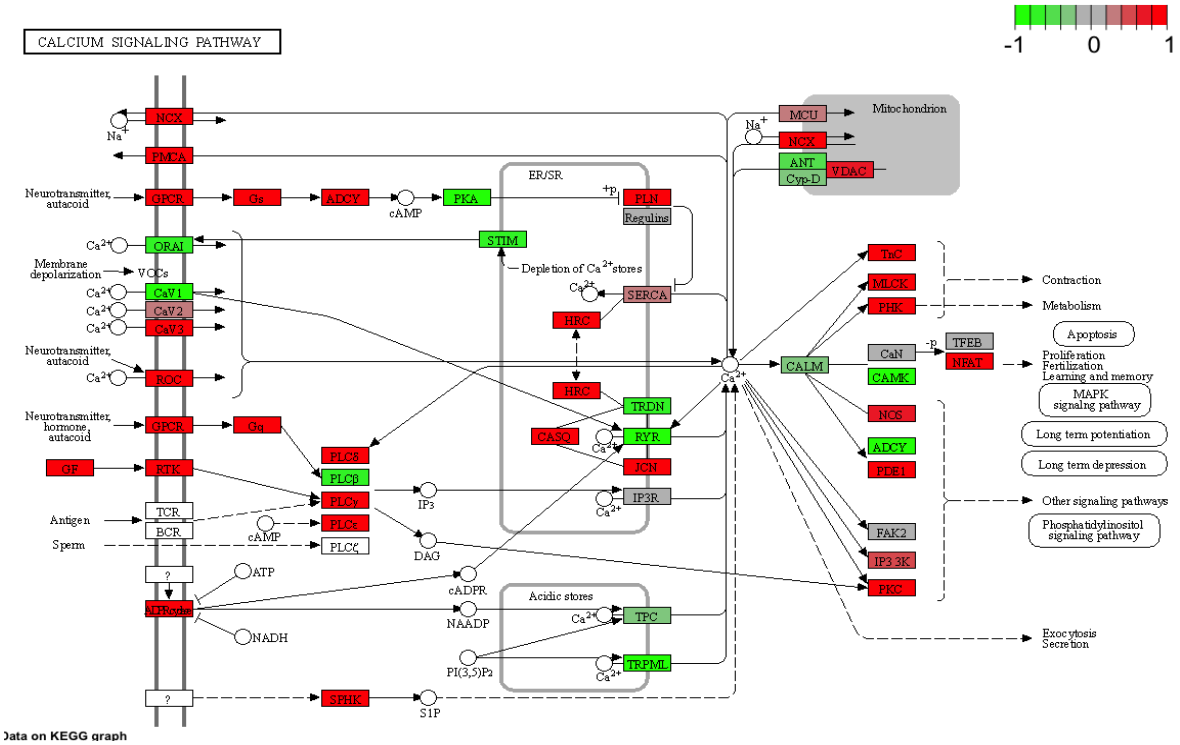


D



Data on KEGG graph

E



Data on KEGG graph

F

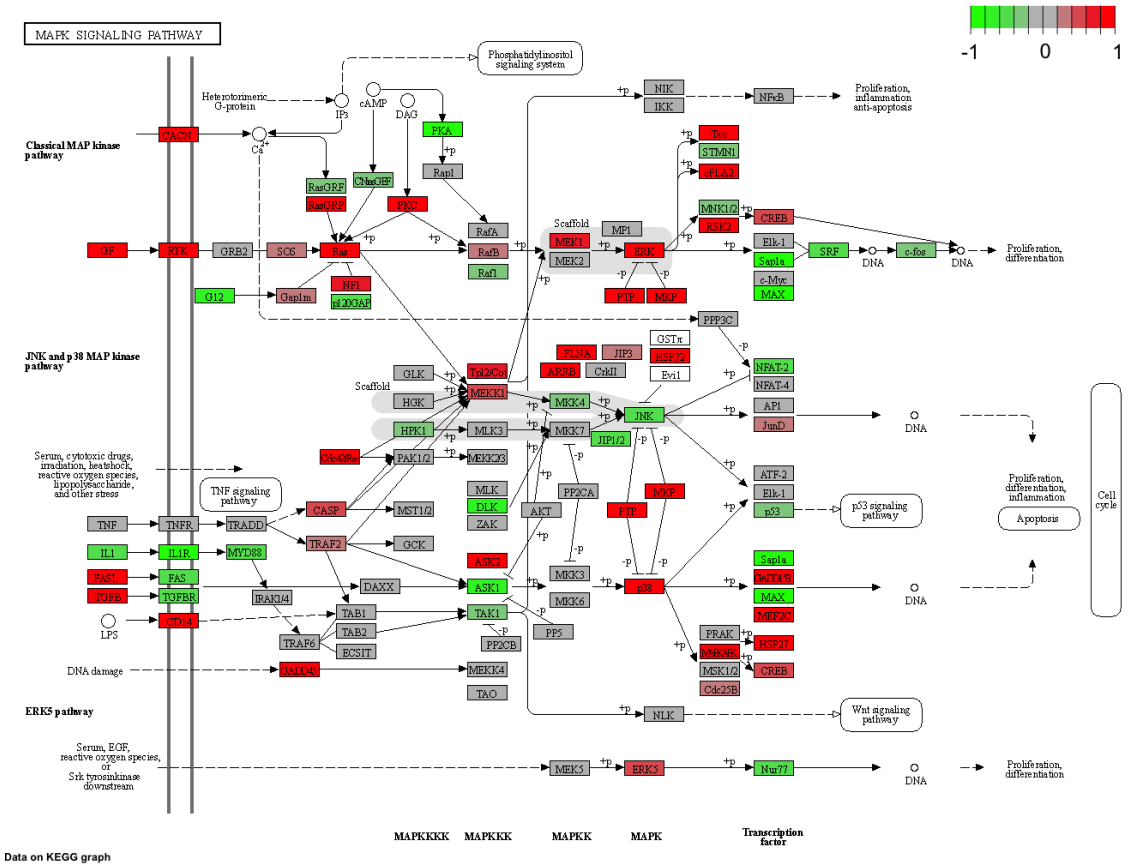
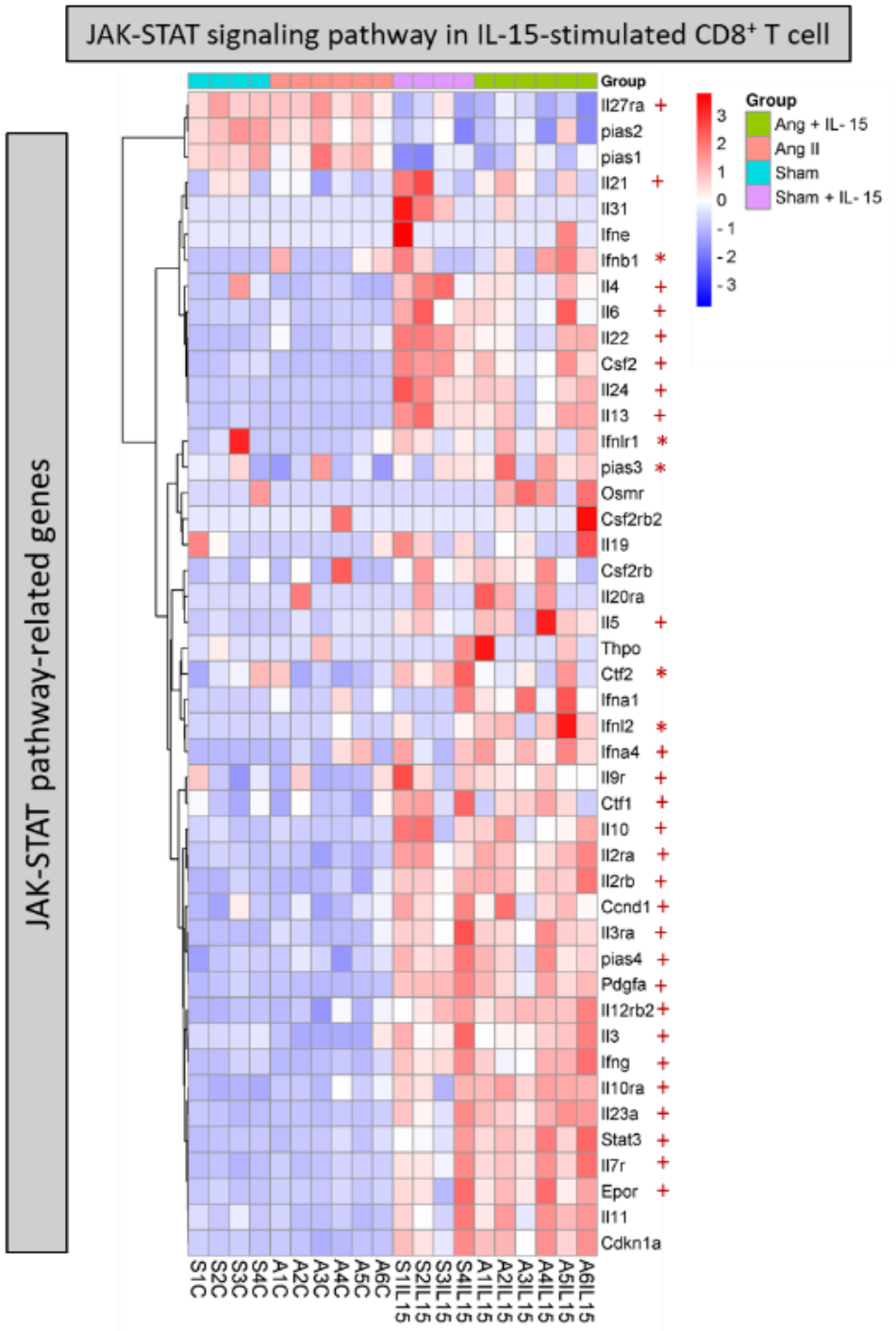


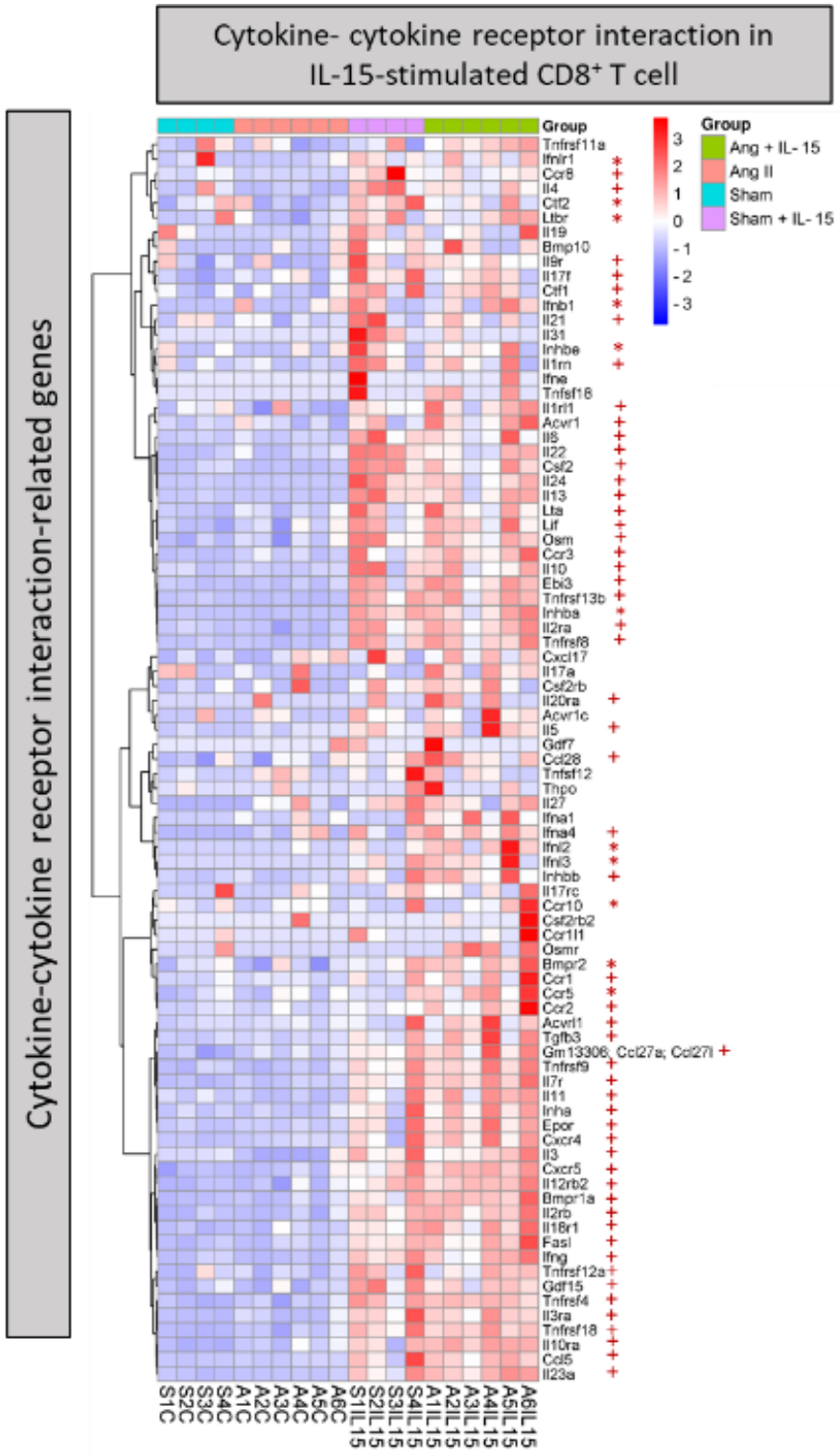
Figure 6.13. The Identified KEGG pathways in both sham + IL-15 and Ang II + IL-15 conditions.

Visualisation of gene expression relating to cytokines-cytokines receptors interaction **(A and C)** and JAK-STAT signalling pathway **(B and D)** in sham and Ang II IL-15-treated CD8 T cells. Ca2+ **(E)** and MAPK **(F)** pathways induced only in Ang II + IL-15-treated CD8+ T cells.

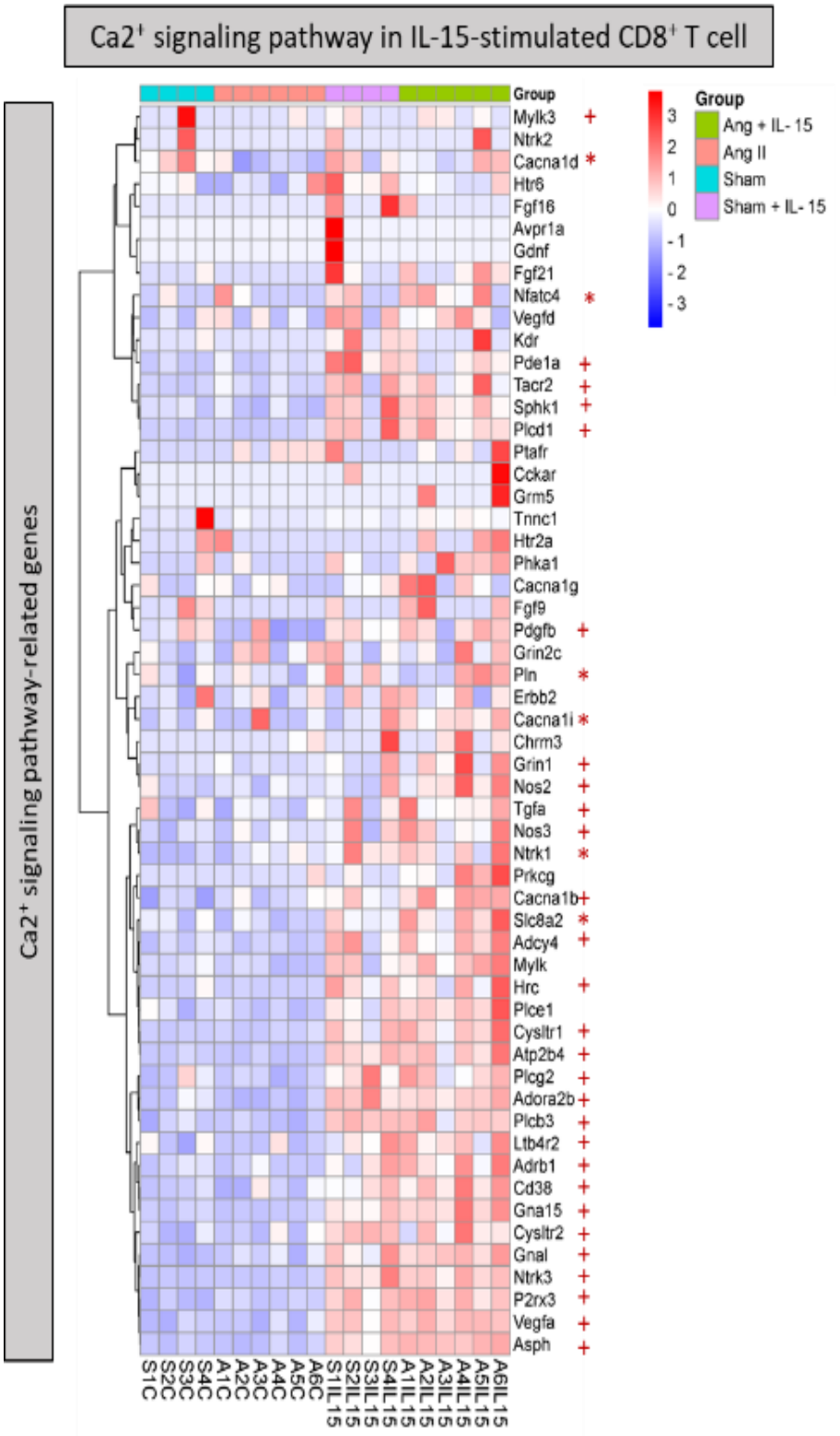
A


JAK-STAT signaling pathway in IL-15-stimulated CD8+ T cell
JAK-STAT pathway-related genes
Group
Ang + IL- 15
Ang II
Sham
Sham + IL- 15
3
2
1
0
- 1
- 2
- 3
Il27ra
pias2
pias1
Il21
Il31
Ifne
Ifnb1
Il4
Il6
Il22
Csf2
Il24
Il13
Ifnlr1
pias3
Osmr
Csf2rb2
Il19
Csf2rb
Il20ra
Il5
Thpo
Ctf2
Ifna1
Ifnl2
Ifna4
Il9r
Ctf1
Il10
Il2ra
Il2rb
Ccnd1
Il3ra
pias4
Pdgfa
Il12rb2
Il3
Ifng
Il10ra
Il23a
Stat3
Il7r
Epor
Il11
Cdkn1a
S1C
S2C
S3C
S4C
A1C
A2C
A3C
A4C
A5C
A6C
S1IL15
S2IL15
S3IL15
S4IL15
A1IL15
A2IL15
A3IL15
A4IL15
A5IL15
A6IL15

B


Cytokine- cytokine receptor interaction in IL-15-stimulated CD8⁺ T cell
Cytokine-cytokine receptor interaction-related genes
Group
Ang + IL-15
Ang II
Sham
Sham + IL-15
3
2
1
0
-1
-2
-3
Tnfrsf11a
Ifnlr1
Ccr8
Il4
Clf2
Ltbr
Il19
Bmp10
Il9r
Il17f
Clf1
Ifnb1
Il21
Il31
Inhbe
Il1rn
Ifna
Tnfsf18
Il1rl1
Acvr1
Il6
Il22
Csf2
Il24
Il13
Lta
Lif
Osm
Ccr3
Il10
Ebi3
Tnfrsf13b
Inhba
Il2ra
Tnfrsf8
Cxcl17
Il17a
Csf2rb
Il20ra
Acvr1c
Il5
Gdf7
Ccl28
Tnfsf12
Tnpo
Il27
Ifna1
Ifna4
Ifnl2
Ifnl3
Inhbb
Il17rc
Ccr10
Csf2rb2
Ccr1l1
Osmr
Bmpr2
Ccr1
Ccr5
Ccr2
Acvrl1
Tgfb3
Gm13306; Ccl27a; Ccl27l
Tnfrsf9
Il7r
Il11
Inha
Epor
Cxcr4
Il3
Cxcr5
Il12rb2
Bmpr1a
Il2rb
Il18r1
Fasl
Ifng
Tnfrsf12a
Gdf15
Tnfrsf4
Il3ra
Tnfrsf18
Il10ra
Ccl5
Il23a
S1C
S2C
S3C
S4C
A1C
A2C
A3C
A4C
A5C
A6C
S1IL15
S2IL15
S3IL15
S4IL15
A1IL15
A2IL15
A3IL15
A4IL15
A5IL15
A6IL15

C


Ca2⁺ signaling pathway in IL-15-stimulated CD8⁺ T cell
Ca2⁺ signaling pathway-related genes
Group
Ang + IL-15
Ang II
Sham
Sham + IL-15
3
2
1
0
-1
-2
-3
Mylk3 +
Ntrk2
Cacna1d *
Htr6
Fgf16
Avpr1a
Gdnf
Fgf21
Nfatc4 *
Vegfd
Kdr
Pde1a +
Tacr2 +
Sphk1 +
Plcd1 +
Ptafr
Cckar
Grm5
Tnnc1
Htr2a
Phka1
Cacna1g
Fgf9
Pdgfb +
Grin2c
Pln *
Erbb2
Cacna1i *
Chrm3
Grin1 +
Nos2 +
Tgfa +
Nos3 +
Ntrk1 *
Prkcg
Cacna1b +
Slc8a2 *
Adcy4 +
Mylk
Hrc +
Plce1
Cysltr1 +
Atp2b4 +
Plcg2 +
Adora2b +
Plcb3 +
Ltb4r2 +
Adrb1 +
Cd38 +
Gna15 +
Cysltr2 +
Gnal +
Ntrk3 +
P2rx3 +
Vegfa +
Asph +
S1C
S2C
S3C
S4C
A1C
A2C
A3C
A4C
A5C
A6C
S1IL15
S2IL15
S3IL15
S4IL15
A1IL15
A2IL15
A3IL15
A4IL15
A5IL15
A6IL15

D

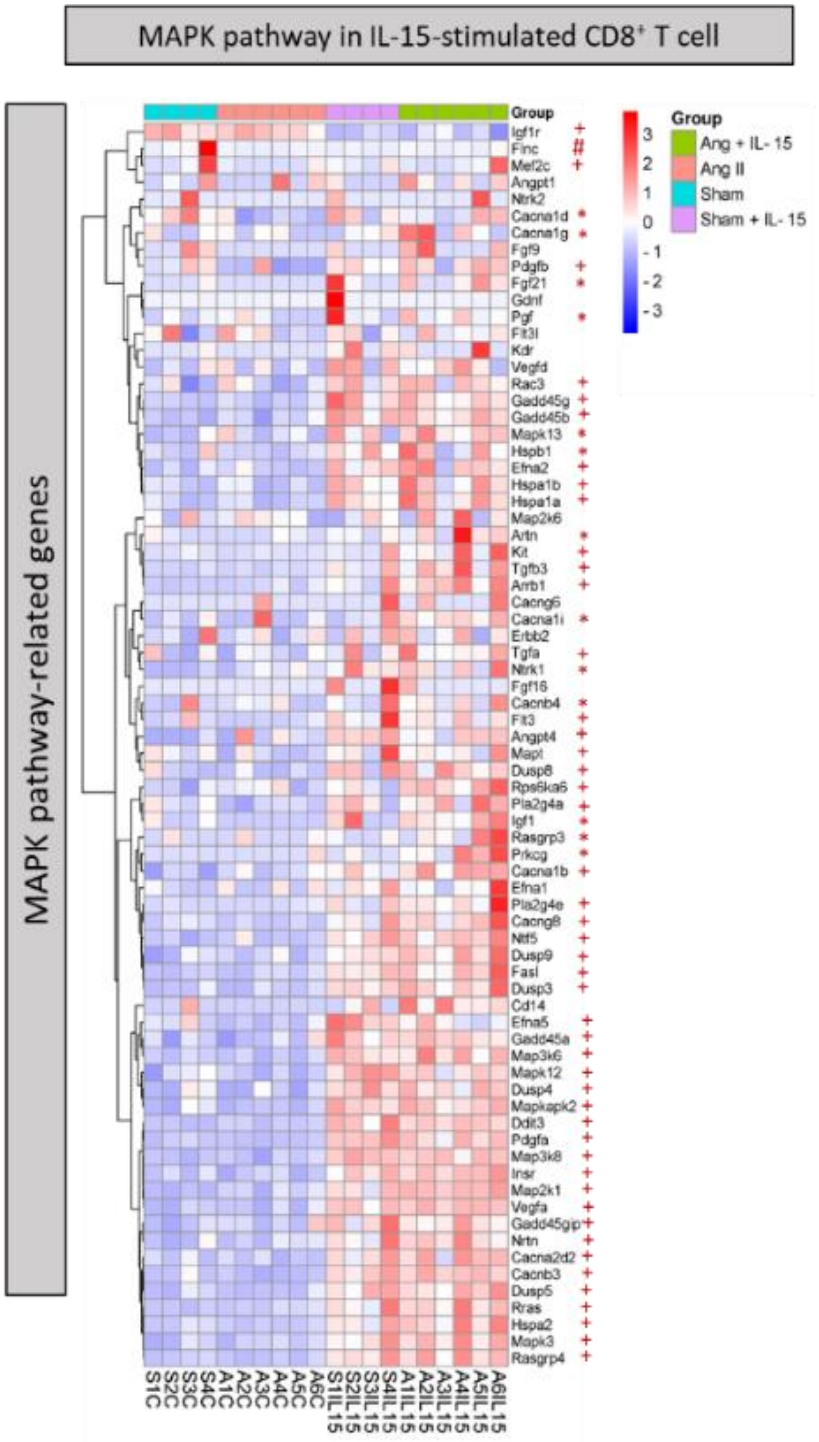


Figure 6.14. Heatmap for the expression profiles of genes associated with pathways induced in CD8+ T cells between Sham and Ang II in the presence and absence of IL-15.

Representative genes in the JAK-STAT pathway **(A),** cytokines-cytokines interaction **(B),** Ca2+ signalling pathway **(C),** and MAPK pathway **(D)** were induced in Sham and Ang II IL-15-treated CD8+ T cells. **(#)** refer to the genes significantly expressed in Sham + IL-15 compared to Sham, **(*)** refers to the genes significantly expressed in Ang II+ IL-15 compared to Ang II CD8+ T cell, and **(+)** refers to the genes significantly expressed in both.

6.5 Discussion

Despite the challenge of reliably detecting distinct phenotypic changes in Ang II-treated T cells, the differential in cellular response to various cytokines in an experimental lab setting still needed to be fully evident. For this reason, bulk RNA-seq was performed to investigate the effect of IL-7 and IL-15 cytokines on transcriptome changes in CD8+ T cells. Furthermore, it generates deeper insights into pathways involved in activating CD8+ T cells in response to IL-7 and IL-15. In the previous chapter, IL-7 and IL-15 demonstrated an apparent proliferation effect on CD8+ T cells, distinct from the other cytokines, which did not exhibit this effect. Therefore, the aim was to uncover the underlying transcriptomic changes and pathways associated with activating CD8+ T cells induced by IL-7 and IL-15.

In this chapter, the RNA-seq analysis findings show that there is an overlap in the PCA between the Ang II + IL-7 and Sham + IL-7 conditions, as well as between the Ang II + IL-15 and Sham + IL-15 conditions. Overall gene expression was downregulated in response to IL-7. CHCHD2 interaction gene significantly downregulated in the presence of both IL-7 and Ang II factors. In addition, both GSEA and KEGG analyses indicated that the Ca2+ signalling pathway was distinctively activated in response to IL-7 in the Ang II condition. while cytokine-cytokine interactions and the JAK-STAT pathways were commonly induced in both Sham + IL-7 and Ang II + IL-7 conditions. In response to IL-15, gene expression was upregulated. IL-1R1 and PDGFb interaction genes were significantly upregulated in the presence of both IL-15 and Ang II factors. KEGG pathway analysis revealed that the Ca2+ signalling pathway and MAPK were specifically activated in the Ang II + IL-15 condition. On the other hand, the JAK-STAT pathway and cytokine-cytokine receptor interactions were commonly stimulated in both the Sham + IL-15 and Ang II_ + IL-15 situations.

The number of genes deemed statistically significant in Ang II-treated CD8+ T cells in response to IL-7 (4759 genes) was lower than that in Sham+IL-7-treated CD8+ T cells (6075 genes). Notably, among the 4759 significant genes in the Ang II-treated group, approximately an equal proportion was distributed between upregulated and down-regulated genes. The decrease in gene expression in Ang II cytokine treated-CD8 T cells might result from Ang II's role in regulating various cellular processes, such as vascular tone and blood pressure and promoting inflammation, which could influence gene expression. Or cytokine treatments often involve activating proinflammatory pathways and downregulating other pathways. Thus, combining Ang II and cytokine stimulation may

lead to a complex interplay of signalling pathways that modulate gene expression. Moreover, in hypertensive animals, the cells might already be activated, leading to a reduced population of naive cells. This pre-existing activation could contribute to fewer significantly changed genes observed in response to Ang II.

It is known that Ang II regulates T cell function and the expression of associated marker molecules. Ang II, a key component of the RAS system, exerts multifaceted influences on immune responses, including T cell responses. Ang II may modulate intracellular signalling through its receptors on T cells, such as AT1R, and subsequently impact T cell activation, proliferation, differentiation, and marker expression (Hoch et al., 2009). However, unlike IL-7, it is noteworthy that IL-15 demonstrated a substantially greater number of significantly expressed genes in Ang II-treated CD8 T cells compared to Sham IL-15-treated CD8 T cells. This discrepancy suggests that the observed effect may not stem from the initial cellular activation by Ang II, but rather, IL-5 might have the ability to enhance this activation. Meanwhile, changes in the Sham group were likely a result of cytokine addition alone (Figure 6.10/Figure 6.4).

To identify the genes that respond differently to the cytokine's treatment in Sham and Ang-II CD8+ T cells, a multifactor analysis was performed using DESeq2. Gene-cytokine interactions may manifest as differences in how genes are expressed in response to IL-7/IL-15 within each experimental condition (Sham and Ang II). Specifically, it evaluates how Ang II influences the response to cytokines, controlling for baseline effects. This approach allows for the assessment of the impact of IL-15 and IL-7 treatments on gene expression and the exploration of potential interactions between Ang II treatment and cytokine responses (Figure 6.4 and Figure 6.10), Coiled-coil-helix-coiled-coil-helix domain 2 (CHCHD2) is the only gene influenced by the interaction between both IL-7 and Ang II (Figure 6.4A). CHCHD2 is identified as a binding partner of the mitochondrial protein p32 and is involved in apoptotic effects; CHCHD2, a small mitochondrial protein, inhibits Bax activation and oligomerisation by binding to Bcl-xl. CHCHD2 is a novel regulator of Bax oligomerisation and apoptosis. It is also found to regulate cellular metabolism and migration and plays a vital role in cell survival under genotoxic stress (Liu et al., 2015). In addition, CHCHD2 down-regulation could also lead to inhibition of cell proliferation, decreased migration ability, and aggravation of mitochondrial-mediated apoptosis (Ren et al., 2022). However, despite being identified as one of the genes uniquely influenced by the interaction between Ang II and IL-7, the CHCHd2 gene was

found to be downregulated in response to IL-7, contrary to the anticipated outcome based on the experimental results.

In response to IL-15, only three genes (IL1rl1, Pkp4, and Pdgfb) were upregulated with significant interaction in CD8+T cells, as depicted in (Figure 6.10B). Il1rl1, interleukin-1 receptor-like, encodes a protein receptor involved in the IL-1 family signalling pathway. This receptor, the IL-33 receptor, plays a role in immune responses and inflammatory processes. A study involving 170 men grouped by essential hypertension (EH) presence and severity discovered that the IL1RL1 gene variation (rs950880) wasn't directly correlated to EH or its severity. However, it did show a potential association with specific cardiovascular phenotypes, particularly LV hypertrophy, in those with EH (Katayose et al., 1993). Platelet-derived growth factor (PDGF) appears to play critical in hypertensive conditions, being linked to increased gene expression in vascular smooth muscle cells (VSMCs) and potentially contributing to vascular hypertrophy, myocardial inflammation, and altered gene regulation across different tissues, showcasing its multifaceted involvement in hypertension. High blood pressure was found to be linked to increased expression of the PDGF gene in VSMCs of spontaneously hypertensive rats (SHRs). Treatment with different antihypertensive drugs led to a reduction in PDGF mRNA levels, indicating a potential association between PDGF gene activation, elevated blood pressure, and vascular hypertrophy in hypertensive conditions (Negoro et al., 1995). Sarzani R *et al.* highlight the differential regulation of the PDGF ligand and receptor genes in various tissues under hypertensive conditions, indicating a complex relationship between PDGF and hypertension that may vary across tissue types (Sarzani et al., 1991). Another study suggests that in hypertensive conditions, there are alterations in the expression levels of certain components (PDGF) system. Specifically, it indicates an increase in the mRNA levels of the PDGF beta-receptor in the aorta of hypertensive rats compared to normotensive controls. This suggests a potential role for PDGF in the growth of VSMCs associated with hypertension. In rats with hypertensive conditions, excessive activation of the PDGF signalling pathway was observed, particularly in myocardial inflammation. The findings suggest a possible link between PDGF overactivity and the pathophysiology of hypertension-related myocardial issues (Katayose et al., 1993).

Genes KEGG and GSEA showed that the JAK-SAT pathway activated in both Sham and Ang II cytokines (IL-7 / IL15) stimulated-CD8+ T cell. Regardless of the Ang II effect, analysis reveals that IL-7 and IL-15 play a key role in activating the JAK-STAT pathway, as evidenced by the significant up-regulation of most genes in both conditions compared to untreated conditions. Both cytokines bind to receptors that utilise the common γ-chain to signal through JAK3, JAK1, and STAT proteins, triggering gene expression and promoting the maintenance of T lymphocyte populations, survival and proliferation. The role of IL-7 in T cell development and homeostasis is well-established (Melchionda et al., 2005; Tan et al., 2001). It is known to activate the JAK-STAT and PI3K signalling pathways, which play crucial roles in various IL-7-associated activities in CD8+ T cells. Previous studies showed that IL-7-induced activation of JAK-STAT5 signalling is necessary for producing Bcl-2, an anti-apoptotic protein, and perforin, which is involved in cytotoxic activity (Crawley et al., 2014). Inhibition of JAK activation was found to reduce IL-7-induced Bcl-2 and perforin production. In addition, JAK-STAT5 signalling and the PI3K pathway were also implicated in IL-7-mediated activities. It was demonstrated that inhibition of both JAK/STAT5 and PI3K pathways leads to a decrease in glucose uptake and proliferation in response to IL-7 (Crawley et al., 2014). Similarly, the pathways involved in IL-15-mediated Bim induction include the JAK/STAT pathway and the PI3K/AKT pathway (Shenoy et al., 2014). Both pathways are essential for Bim induction by IL-15. The JAK/STAT pathway is activated by the IL-15 receptor, which signals through the common γ chains. This pathway involves the activation of JAK3 and STAT5. The PI3K/AKT pathway is activated by signals through the common γ chains as well. Inhibition of either of these pathways blocks the induction of Bim protein by IL-15 (Shenoy et al., 2014).

KEGG and GSEA highlighted the key pathways involved in CD8+ T cells treated with IL-7/IL-15 cytokines, where the JAK-STAT signalling pathway was one of the pathways found to be induced in both Sham and Ang II treated cells. In response to IL-7 (Figure 6.8A), Ifnl2, Aox2, IL9r, Ifna4, and Ifna2 (JAK-STAT involved genes) were found to be significantly upregulated in Ang II CD8+ T cell, while Ifngr1, Il23r and Prlr were t up-regulated in IL-7 Sham-CD8+ T cell. IFN-IIIs (Ifnl2) and IFN-Is (Ifna2, Ifna4) are well-known activators of the JAK-STAT pathway. In T cells, exposure to interferon α can lead to the phosphorylation of STAT proteins, particularly STAT1 and STAT3. This activation can regulate various aspects of T cell function, including antiviral responses and immunomodulation (Wang et al., 2019). Deletion of IFN-γR (Ifngr1), one of the genes that expressed in sham + IL-7 condition, was found to reduce cardiac hypertrophy, macrophage and T cell infiltration and fibrosis without affecting BP elevation caused by Ang II in

129SV mice (Marko et al., 2012). Moreover, the upregulation in the expression of the IL9R gene in Ang II CD8+ T cells was also observed. Strong crosstalk between IL-9 signalling and the STAT3 pathway in CD8+ T cells was revealed by IPA analysis (Xiao et al., 2022). Suggests that IL-9 could be a potential novel target for treating and preventing clinical hypertension. The study showed that IL-9 knockout can alleviate inflammatory response, prevent the phenotypic transformation of smooth muscle, reduce vascular dysfunction, and lower blood pressure in Ang II-infused mice. These effects are mediated through the STAT3 pathway, as IL-9 KO decreases p-STAT3 levels (Yang et al., 2020).

In response to IL-15 (Figure 6.14A) other IFN-IIIs member's expression was significantly up-regulated: Ifnl2, Ifnb1, Ifnlr1, and Ctf2. Interferon activates the JAK-STAT pathway, as indicated by the increased phosphorylation of Jak1 and STAT1. The knockdown of Ifnl2, or Ifnb1, also reduces the phosphorylation of Jak1 and STAT1 (Zhang et al., 2022). This further supports the involvement of these interferons in activating the pathway. Ifnlr1 encodes the receptor for type III interferons (IFN-λ or lambda interferons), such as IFN-λ2 (Ifnl2). While IFN-λs are primarily associated with antiviral, antitumor responses and immune regulation, Ifnlr1 engagement can activate the JAK-STAT pathway (Qin et al., 2021). In the context of IL-15 stimulation, it is important to note that Ifnl2 may not have a direct role as IL-15 belongs to different classes (molecular structure, function, or biological properties) from type III interferons. However, the JAK-STAT pathway activated by Ifnl2 shares similarities with the pathway activated by IL-15, highlighting potential crosstalk or synergy in immune responses.

Furthermore, the PIAS gene was one of the genes implicated in the JAK-STAT pathway and was observed to exhibit differential expression in response to Ang II stimulation with both IL-7 and IL-15 stimulation (Figure 6.13B & Figure 6.7B). PIAS family consists of PIAS1, PIAS2, PIAS3, and PIASy; these genes are important in innate immunity (Shuai, 2000; Liu et al., 1998). JAK-STAT pathway regulates the PIAS3 gene in Ang II + IL-7/IL-15 compared to Sham + IL-7/IL-15. It is a protein known to be an inhibitor of activated STAT proteins. PIAS proteins regulate the activity of certain transcription factors such as STATs, nuclear factor-κB and SMADs (SMA (small body size)- and MAD (mothers against decapentaplegic)-related proteins) in cytokine-mediated signalling, using distinct mechanisms (Liu et al., 1998). The gene PIAS1, which exhibits significant downregulation in both Ang II and sham-treated cells, exerts distinct effects on cytokine-mediated signalling by selectively regulating a subset of interferon- or TNF-responsive genes as well as inhibits STAT1 signalling (Liu et al., 1998). A deficiency in PIAS1 increases immunity,

providing increased protection against viral and bacterial infections (Shuai and Liu, 2005). PIAS4 was also found to inhibit STAT1-mediated gene activation. However, it did not affect STAT1 DNA-binding activity. PIAS4 rather appears to act as a transcriptional co-repressor of STAT1 (Liu et al., 2001). However, the PIAS3 gene was the only member that distinctly upregulated in our result in Ang II IL-7 and IL-15-treated T cells and was found to be specific for inhibiting STAT3-mediated gene expression after IL-6 stimulation (Chung et al., 1997).

In resting lymphocytes, calcium influx from external sources occurs upon antigen receptor engagement and calcium acts as a vital secondary messenger. The mechanism of Ca2+ entering lymphocytes is by store-operated calcium (SOC) channels, which comprise CRACM1 (channel pore) and STIM1 (calcium sensor) (Trebak and Kinet, 2019; Vig and Kinet, 2009). Studies in mice deficient in CRACM1 or lacking both STIM1 and STIM2 reveal significant impairments in critical T cell functions, including proliferation, cytokine secretion, and naive T cell differentiation. These findings highlight the important role of SOC channels, CRACM1, and STIM1/STIM2 in lymphocytes, emphasising their significance in immune responses (Birx et al., 1984). While calcium signalling in T cells primarily relates to their immune functions, such as activation and cytokine production, it can indirectly influence hypertension through immune responses and inflammation. Thus, inflammation is a contributing factor to hypertension. Activated T cells release pro-inflammatory cytokines. Calcium signalling can modulate the production and release of cytokines, contributing to inflammation and endothelial dysfunction in blood vessels, which are key components of hypertension development.

The Ca2+ pathway was found to be exclusively induced in Ang II + IL-7 and Ang II + IL-15 conditions. Only *Tnnc1, Mylk, and Cacna1d* genes were statistically expressed in Ang II+ IL-7 (Figure 6.8C) induced-Ca2+ pathway, while *Ntrk1, Pln, Nfatc4, Cacna1d, Cacna1i* , and *Slc8a2* genes in Ang II+ IL-15 condition (Figure 6.14C). Notably, Cacna1d was upregulated in response to IL-7 and IL-15 in Ang II CD8+ T cell. In patients with hypertension, genetic variations (SNPs) in *Cacna1d* or *Cacna1c* genes make them more responsive to the blood pressure-lowering effects of L-type calcium channel blockers (Kamide et al., 2009). The *Cacna1d* gene was found to be a key player in the regulation of blood pressure (Morihara et al., 2017). Moreover, the *Cacna1i* gene encodes the alpha subunit responsible for forming CaV3.3 channels, a voltage-gated calcium channel. In response to IL-15, there was a notable increase in the expression of this gene. CaV3.3 channels are expressed in a subset of central nervous system neurons (Ghoshal et al.,

2020), particularly in GABAergic neurons and T cells (Erdogmus et al., 2022). The Phospholamban (*Pln*) gene was also observed to exhibit a substantial up-regulation in Ang II in response to IL-15 treatment. The finding showed that the inhibition of *Pln* through a solitary administration of Pln-targeting Locked Nucleic Acid Antisense Oligonucleotide (LNA-ASO) yielded enhancements in contractile function in the context of cardiac dysfunction induced by pressure overload, which shows a the potential viability of Pln-targeting therapeutic strategies as a promising avenue for the management of hypertensive heart failure (Morihara et al., 2017).

Although no studies directly demonstrated the induction of IL-15 or IL-7 on the activation of the Ca2+ channel pathway, available data indicate the possibility of indirect activation. For example, electrophysiological investigations and patch-clamp experiments showed that IL-1β and TNF-α effectively augment calcium ion (Ca2+) currents by influencing voltage-gated channels in isolated vascular smooth muscle cells (VSMCs) from the rat tail artery (Wilkinson et al., 1996). Exposure to cytokines like TNF-a can increase the expression of transient receptor potential channel (TRPC) homologues in endothelial cells, leading to an increase in Ca2+ influx and vascular permeability (Lawson et al., 2015). In Atherosclerosis-prone apolipoprotein E-deficient (ApoE)-deficient mice on a high-cholesterol diet, it revealed that amlodipine, an L-type calcium channel blocker, effectively suppresses the formation of atherosclerotic lesions and even reverses atherosclerosis.(Yoshii et al., 2006). Moreover, IL-7 is critical for the induction of the PI3K/Akt pathway, STAT5 phosphorylation, and Ca2+ mobilisation, and ensuing optimal T cell activation. On naive T cells from IL-7- deficient mice markedly reduced upregulation of CD25 and CD69 as well as failed to mobilise Ca2+ in response to TCR triggering(Sprague and Khalil, 2009).The evidence illustrates that immune-mediated mechanisms, including T cell activation and inflammation, can contribute to hypertension development. Dysregulated calcium signalling in T cells could potentially contribute to this process.

IL-15 signals through both the PI3K-AKT and RAS-MAPK pathways, as demonstrated in T cell populations and natural killer cells (NK) (Lawson et al., 2015). The MAPK is activated by both TCR- and cytokine stimulation (Waldmann, 2006; Lawson et al., 2015). However, while PP2 blocked TCR-dependent p38 MAPK activation and was partially blocked by CsA, cytokine-dependent p38 MAPK activation was not affected by these drugs but was blocked by a JAK3 inhibitor. This indicates that the TCR and cytokine receptors activate p38 MAPK by different mechanisms and that p38 activation is required for cytokine-dependent proliferation but not TCR-dependent proliferation (Geginat et al., 2003).

Signalling initiated by IL-15 in immune cells showed to trigger the activation of the MAPK pathway and the phosphatidylinositol 3-kinase (PI3K)/Protein Kinase B (AKT)/mammalian target of the Rapamycin (mTOR) pathway. These signalling pathways work together to induce a transcriptional program that regulates lymphocyte populations' growth, activation and survival, including memory CD8+ T cells (Fehniger and Caligiuri, 2001; Nolz and Richer, 2020). Ang II plays a significant role in the activation of MAPK. Ang II significantly activates the MAPK pathway in both the heart and aorta, and inhibition of pMAPK may offer a therapeutic approach to cardio-vascular disease (Meijles et al., 2020).

The MAPK and JNK cascades serve as critical regulators in maintaining the delicate balance between cell survival and apoptosis. They are activated in response to various pathophysiological stresses, including redox stress and the presence of proinflammatory cytokines. Importantly, hypertension is closely associated with heightened levels of oxidative stress and chronic inflammation, suggesting that the modulation of p38-MAPK and JNK activities could hold promise in the management of hypertensive heart failure (Bao et al., 2007). It is worth noting that a comprehensive blockade of the entire p38 MAPK or JNK signalling pathways may not be the most desirable course of action, as these cascades also play pivotal roles in mediating inflammatory responses. Therefore, a more judicious and targeted therapeutic approach involves pinpointing specific inputs within these cascades for intervention. Upstream kinases such as MAP3K5 (ASK1) and MAP3K7 (TAK1) emerge as key regulators responsible for orchestrating the activities of both p38-MAPKs and JNKs.

Chapter 7 General Discussion

7.1 Discussion

The immune system plays a crucial role in the pathogenesis of hypertension, and studies demonstrated the protective impact of depleting T cells, B cells, monocytes, and macrophages in various hypertensive models (Chan et al., 2015; Thang et al., 2015; Guzik et al., 2007). Additionally, hypertension leads to inflammation in the vasculature and kidneys, contributing to end-organ damage, as observed in many studies (Moore et al., 2015; Ozawa et al., 2007). This underscores the intricate relationship between immune responses and the development of hypertension, where immune cell depletion is associated with protective effects against organ damage.

The systematic review provided a comprehensive overview of the roles of TCR activation and cytokine modulation in regulating T cell proliferation and marker expressions. The elucidated information conveys the intricate mechanisms underlying T cell responses, distinguishing between TCR-dependent and cytokine-dependent stimulation. In addition, TCR-dependent signalling, initiated through the TCR complex, is pivotal in triggering pathways like Lck and MEK1/2, essential for cell proliferation, and culminates in the secretion of cytokines crucial for T cell responses. Conversely, cytokine stimulation, exemplified by IL-15, IL-12, and IL-18, modulates T cell proliferation and activation through distinct mechanisms (JAK/STAT, PI3K/Akt, NF-κB and MAPK pathways). While IL-15 enhances CD8+ T cell viability and proliferation, IL-12 and IL-18 act as a bystander, promoting proliferation synergistically with other cytokines. However, cytokines like IL-7 exhibit a more nuanced effect, enhancing cell viability while potentially inhibiting specific signalling pathways associated with reduced proliferation. Overall, the interplay between TCR-dependent and cytokine-dependent pathways intricately regulates T cell responses, highlighting the complexity of T cell activation mechanisms.

Moreover, the systematic review underscores the diverse effects of cytokines on T cell marker expression, further shaping immune responses and T cell functions. For instance, IL-15, IL-12, and IL-18 induce the upregulation of markers such as CD25, ICAM-1, DNAM-1, and NKG2D on T cells, influencing their activation and function. In contrast, IL-7 and IL-4 modulate the expression of markers like CD69, HLA-DR, OX40, and Tim-3 on T cells, reflecting their distinct roles in immune regulation. Additionally, IL-1 and IFNs impact the expression of various activation markers, such as Ly-6 and OX40, further illustrating the intricate interplay between cytokines and T cell markers. Understanding these diverse effects assists in identifying candidate cytokines for experimental modules,

facilitating a more targeted approach to studying T cell activation and immune regulation. Overall, Stimulation through TCR or cytokines revealed apparent disparities in pathway and phenotypic response, despite both pathways having an activation and proliferation effect. These disparities were even observed among individual cytokines.

As regulators in T cell differentiation and inflammation, cytokines and their receptors were implicated in hypertension. For example, TNF, IL-6, and IL-17 are proven to be elevated in hypertension conditions (Loperena et al., 2018). Other cytokines demonstrate their capability to modulate T cell activation in experimental models. In addition, T cells are activated and accumulate in different organs in hypertension. however, despite these facts, the modulatory effect of cytokines on T cells were not previously studied in hypertension. T cells obtained from both Sham and Ang II-infused mice were utilised to investigate the modulating effects of cytokines in a hypertension context. T cells were stimulated with various cytokines associated with hypertension or other cardiovascular diseases, as identified from the systematic review. The experiments in this study outlined the influence of cytokines in the context of hypertension. Firstly, through characterising the expression of cytokine receptors on T cells in hypertension induced by Ang II. Secondly, by exploring T cells' proliferation and differentiation responses when cultured with these cytokines. T cells were analysed from the spleen and PBMCs after 14 days and 5 days of Ang II-induced hypertension using flow cytometry to characterise cytokine expression. Interestingly, there were no discernible changes in the expression of cytokine receptors, including TNF-αR, IL-6R, IL-1R, IL-7R, IL-17R, IL-12R, IFN-γR, and IL-2/IL-15R *in vivo* on T cells from both the spleen and PBMCs. However, it is noteworthy that deletion of some cytokine receptors, such as TNFR1 and IL-6R, are implicated in cardiovascular conditions and hypertension (Chen et al., 2010). The absence of alterations or changes in these receptors suggests potential tissue-specific regulation. Cytokine receptor expression may be regulated in a tissue-specific manner, implying that changes in receptor expression might occur in specific tissues or cell types not captured in the analysed T cells from the spleen and PBMCs. Another consideration is that cytokine receptor expression might undergo dynamic changes over time (Chen et al., 2010). The analysis conducted at a specific time point (14 days /5 days of Ang II-induced hypertension) may not capture potential fluctuations that could occur during earlier or later stages of hypertension development.

Despite the unique effects that cytokines like IL-15, IL-7, and IL-6 may exert on cell proliferation, differentiation, and viability, it was observed that these effects were

consistently similar on T cells, irrespective of the stimulation protocols used, in both sham-operated and Ang II-treated mice. This suggests that Ang II may not directly impact the cytokine-mediated functions of T cells under the conditions tested. Nevertheless, IL-7 and IL-15 are key players in autoimmune inflammation and CVDs, with elevated levels observed in conditions like unstable angina and hypertension. They drive lymphocyte expansion and activation, with IL-15 linked to end-organ damage in hypertension. Additionally, IL-6 signalling in Ang II-induced thromboinflammation presents therapeutic avenues for hypertension management (Senchenkova et al., 2019; Kaibe et al., 2004; Guzik et al., 2024). Therefore, further investigation was performed using RNA-seq specifically to determine the responsiveness of the T cell subset, CD8+ T cells, under the influence of implicated cytokines (IL-15 and IL-7). This comprehensive approach aims to unravel the intricate interplay between cytokines and T cells in the context of hypertension.

The RNA-seq analysis from CD8+ T cell findings showed that there is an overlap in the PCA between the Ang II + IL-7 and Sham + IL-7 conditions, as well as between the Ang II + IL-15 and Sham + IL-15 conditions. Additionally, gene expression was downregulated in response to IL-7, and the CHCHD2 interaction gene was significantly downregulated in the presence of both IL-7 and Ang II factors. In addition, both GSEA and KEGG analyses indicated that the Ca2+ signalling pathway was distinctively activated in response to IL-7 in the Ang II condition. while cytokine-cytokine interactions and the JAK-STAT pathways were commonly induced in both Sham + IL-7 and Ang II + IL-7 conditions. On the other hand, in response to IL-15, gene expression was upregulated in Ang-II CD8 T cells. IL-1R1 and PDGFb interaction genes were significantly upregulated in the presence of both IL-15 and Ang II factors. Like IL-7, KEGG pathway analysis revealed that the Ca2+ signalling pathway and MAPK were specifically activated in the Ang II + IL-15 condition. while the JAK-STAT pathway and cytokine-cytokine receptor interactions were commonly stimulated in both the Sham + IL-15 and Ang II + IL-15 conditions.

TLRs are pivotal PRRs essential for the immune system's defence against pathogens by detecting PAMPs. Upon recognition of PAMPs, TLRs initiate immune responses that include the production of interferons and proinflammatory cytokines, which recruit immune cells to infection sites (Takeuchi and Akira, 2010). While TLRs were traditionally associated with APCs, accumulating evidence demonstrates their expression on T cells, where they significantly modulate T cell functions. For example, TLR2 is upregulated on naïve human T cells upon stimulation with anti-TCR antibodies and IFN-alpha, enhancing IFN-γ production in response to bacterial lipopeptides (Komai-Koma et al., 2004). This

TLR2-mediated pathway contributes to both CD4+ T cell development and memory maintenance.

In the context of Ang II and cytokine treatment, TLR activation, particularly of TLR2 and TLR4, plays a pivotal role in shaping T cell responses. Ang II has been shown to upregulate TLR4 expression, leading to increased production of inflammatory mediators and ROS, which are closely linked to vascular dysfunction and hypertension (Ding and Liu, 2019). The activation of TLR2 and TLR4 can synergize with TCR signalling, lowering the activation threshold and enhancing T cell proliferation, survival, and effector functions, including cytokine production (Salerno et al., 2019). Moreover, TLR signalling contributes to the formation and maintenance of T_M cells, as evidenced by the reduced frequency of memory CD8+ T cells in TLR2 knockout mice (Cottalorda et al., 2009).

Furthermore, TLRs are involved in renal inflammation and fibrosis through the activation of NF-κB, a common pathway in TLR signalling cascades, implicating TLRs in the inflammatory responses observed in hypertension and related cardiovascular diseases (Akira et al., 2006). The interplay between Ang II, TLRs, and cytokines represents a critical pathway through which TLR activation exacerbates the inflammatory environment, contributing to both vascular and renal damage and modulating T cell responses. Therefore, understanding TLR-mediated pathways is essential for elucidating the effects of combined Ang II and cytokine treatments on T cells and their role in hypertension-related immune regulation. Overall, it is important to consider TLR-mediated pathways when investigating the effects of such treatments on T cells, particularly in the context of hypertension and its associated immune dysregulation.

Therapeutic targeting of TLR-4 has shown promising effects in reducing damage related to hypertension. TLR-4 antagonist was found to lower BP, reduce cardiac hypertrophy, and minimize renal damage in rats infused with aldosterone and salt for 28 days (De Batista et al., 2014). Additionally, neutralizing anti-TLR4 antibodies used in different models, such as WT mice treated with Ang II and SHR rats, showed a reduction in BP, vascular inflammation, and vascular remodelling (Hernanz et al., 2015; Bomfim et al., 2015). In another study, using anti-TLR4 antibodies in mice treated with Ang II improved vascular dysfunction, although BP remained unaffected (Nunes et al., 2017). These findings suggest that TLR-4 inhibition reduces vascular and cardiac damage from hypertension, even without lowering blood pressure, and anti-TLR4 antibodies can diminish cardiac hypertrophy and inflammation.

Current antihypertensive medications, including ACEi, ARBs, BBs, CCBs, and diuretics, demonstrate a role in modulating immune cytokines. For instance, ACEi like captopril and lisinopril reduced levels of inflammatory cytokines such as IFN-γ and IL-12, as well as decreased TLR4 expression, thus lowering proinflammatory cytokines like IL-1β, IL-6, and TNF-α (Yang et al., 2013). ARBs similarly inhibit the expression of multiple proinflammatory cytokines (Wang et al., 2020), and diuretics were found to downregulate circulating levels of IL-6 and TNF-α in an elderly hypertensive patient (Toledo et al., 2015). CCBs can have a dual effect, like amlodipine, reversing hypertensive left ventricular hypertrophy by targeting cytokine pathways despite potentially increasing TNF-α and IL-1β expression (Lu et al., 2016). Therefore, targeting the cytokines identified, such as IL-7R and IL-15, could offer significant therapeutic benefits, like in rheumatoid arthritis and MS conditions, where IL-7 and IL-15 levels are elevated and its blockade showed effectiveness in reducing inflammation and neovascularization (Meyer et al., 2022; McInnes et al., 1997; Laurent et al., 2021). However, despite these findings, there are still gaps in fully understanding how these drugs modulate the immune system, and the interactions highlight the complex ways existing antihypertensive drugs modulate the immune system, suggesting the need for further research to understand their precise immunomodulatory effects and mechanisms. As hypertension is closely linked to immune dysregulation, exploring new therapeutic targets focused on immune modulation could significantly enhance treatment strategies for hypertensive patients.

7.2 Conclusion

Overall, the work described here has significantly advanced our understanding of how cytokines influence T cell responses in hypertension. Characterising receptor expression in Ang II-induced hypertension revealed that Ang II does not impact cytokine receptor expression. Additionally, most studied cytokines demonstrate no discernible difference in their differentiation effect on Ang II-treated T cells. However, both IL-15 and IL-7 exhibit an increased proliferation response when primed with T cells from both Sham and Ang II-infused mice. In relation to these, cytokines were chosen for studies of differential global gene expression changes comparing T cells from sham and Ang II-infused mice. RNA-seq results provided crucial insights, demonstrating the involvement of pathways such as JAK-STAT and Ca2+ in Ang II CD8+ T cell responses to cytokines. In line with functional studies, IL-7 was the most pronounced inducer of T cell activation in hypertensive conditions. This comprehensive analysis underscores the intricate interplay between cytokines and T cell activation pathways, shedding light on the mechanisms underlying hypertension-associated immune dysregulation.

7.3 Future work

The unique proliferative effects of IL-15 and IL-7 on T cells after cytokine priming highlight the need for further investigation. To explore the broader physiological effects, future studies should investigate how these cytokines influence blood pressure regulation and vascular function. This could be achieved through *ex vivo* analysis of vascular tissues, such as the aorta or mesenteric arteries, to assess changes in vascular tone, endothelial function, and smooth muscle cell behaviour in response to cytokines stimulation. Additionally, isolating cells from other tissues, including the kidney, heart, and brain, could reveal tissue-specific responses to IL-15 and IL-7, offering insights into how these cytokines contribute to organ-specific pathophysiological processes in hypertension. By evaluating these effects in hypertensive versus normotensive models, researchers could determine whether cytokine-induced changes are exacerbated under hypertensive conditions. This approach is essential for understanding the broader implications of cytokine activity in hypertension.

To further elucidate the role of cytokines in T cell behaviour and their contribution to hypertension, studies using KO mice lacking key cytokines and their receptors should be proposed. These studies will allow examination of the effects of specific cytokines on blood pressure regulation and target organ damage as endpoints. Comparing the responses of KO mice to WT controls will give insights into the mechanisms through which these cytokines influence T cell function and their impact on hypertensive pathology.

Extending observations beyond the initial 14 days of Ang II treatment, or examining earlier time points, could help identify potential temporal variations in receptor expression and T cell responses that evolve over time. Investigating the underlying mechanisms, including their associations with specific T cell subsets or signalling pathways, could provide valuable insights into the role these cytokines play in hypertension. By addressing these aspects, researchers can assess whether cytokines initiate hypertension and expand upon current findings, contributing to a more comprehensive understanding of the intricate interplay between cytokines and T cell responses in the context of Ang II-induced hypertension.

Moreover, refining experimental conditions to enhance the responsiveness of T cells from the spleen and PBMCs is critical, considering their sensitivity and rapid cell death in vitro. *In vivo* studies could further validate these findings and address the issue of low T cell

viability, offering insights into how cytokines influence T cell behaviour in a physiological context. This comprehensive approach will contribute to a deeper understanding of the pathophysiology of hypertension and the potential development of new therapeutic strategies.

Appendix I: List of reagents used in the experiments

Supplier Cat. No.	Description	Cat. No.
RPMI + GlutaMAX™-I (1X)	Gibco	61870-036
Dulbecco's phosphate buffer saline DPBS (1X)	Gibco	14190-094
HyPure™ WFI Quality Water	HyClone	SH30221.17
Turka	Merck	109277
Trypan Blue Stain	Gibco	15250061
MojoSort™ buffer (5X)	BioLegend	480017
2 β-Mercabtoethanol	Merck	60-24-2
MojoSort™ Mouse CD3 T Cell Isolation Kit	BioLegend	480031
HistoPaque®-1077	Sigma	10771
RBC lysis buffer (10X)	BioLegend	420301
FluoroFix™ buffer	BioLegend	422101
QIAzol™ Lysis Reagent	QIAGEN Science	79306
Recombinant murine IL-15	PeproTech	210-15
Recombinant murine IL-12	PeproTech	210-12
Recombinant murine IL-6	PeproTech	216-16
Recombinant murine IL-7	PeproTech	217-17
Recombinant murine IL-17A	PeproTech	210-17
Recombinant murine IL-2	PeproTech	212-12
Recombinant murine TNF-α	PeproTech	315-01A
Recombinant murine INF-γ	PeproTech	250-33
Recombinant murine IL-1β	PeproTech	211-11B
Recombinant murine IL-33	PeproTech	210-33

Appendix II: Antibodies and staining reagents used in flow Cytometry

Antibodies	Vendor	Cat. No. #	Concentration	Fluorochrome
CD3	BioLegend	100306	1:100	FITC
CD3	BioLegend	100227	1:100	BV421
CD4	BioLegend	100414	1:200	APC/Cy7
CD4	BioLegend	100551	1:100	BV786
CD8	BioLegend	100722	1:200	PE/Cy7
CD8	BioLegend	344765	1:100	BB700
CD120a	BioLegend	113006	1:50	APC
CD120b	BioLegend	113406	1:100	PE
CD126 (IL-6Ra)	BioLegend	115806	1:100	PE
IL-12Rβ1	R & D systems	FAB1998P-025	1:100	PE
CD25 (IL2Rα)	ThermoFisher	12-0259-80	1:50	PE
CD122 (IL-2Rβ)	ThermoFisher	11-1228-41	1:50	FITC
CD119 (IFN-γR1)	ThermoFisher	12-1199-42	1:50	PE
IFN-γR2	R & D systems	AF1185-SP	1:50	PE
CD127 (IL-7Rα)	BioLegend	135013	1:50	PE-cy7
CD217 (IL-17Ra)	ThermoFisher	17-7182-80	1:50	APC
IL-17Rβ	BioLegend	146305	1:50	PE
CD121a (IL-1R1)	BioLegend	113509	1:50	APC
7-AAD	BioLegend	420404	1.25	PerCP/cy7
IgG	BD Biosciences	12-479-81	1:100	PE
CD44	BioLegend	103022	1:50	FITC

CD62L	BD Biosciences	553151	1:50	PE
Zombie	BioLegend	423105	1:1000	Apc/cy7
DRAQ7	ThermoFisher	D15105	3 µM	Apc/cy7

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