Immunomodulatory Effects of Inhibitor of Apoptosis (IAP) Antagonists on Dendritic Cells

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A thesis submitted in partial fulfillment of the requirements for the
Master's degree in Microbiology and Immunology

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ABSTRACT

The Inhibitor of Apoptosis (IAP) proteins are a highly conserved group of anti-apoptotic proteins that regulate various pathways, particularly those that affect proliferation and cell death. Smac mimetics compounds (SMCs) are IAP antagonists that induce the degradation of two IAPs, cellular IAP 1 (cIAP1) and cellular IAP 2 (cIAP2). cIAP1 and cIAP2 are negative regulators of the alternative NF-κB pathway, which is critical to the regulation, activation, proliferation, and survival of immune cells. Consequently, SMCs can affect immunological responses by providing co-stimulatory signals for antigen-presenting cells or promoting proliferation and activation of T cells. Due to their potent immunomodulatory properties, SMCs are an ideal candidate for new vaccine adjuvants. I sought to demonstrate the potential of SMCs as a vaccine adjuvant by evaluating SMCs effects on dendritic cells (DCs). I demonstrated that SMC treatment of bone marrow derived dendritic cells (BMDCs) induces degradation of both cIAP1 and cIAP2 and leads to activation of the alternative NF-κB signalling pathway. Furthermore, SMC treatment led to upregulation of proteins associated with DC maturation, as well as secretion of pro-inflammatory cytokines. Despite the activating effects elicited by SMCs *in vitro*, the use of SMCs as an adjuvant for peptide vaccination failed to prevent tumour growth. Further work to determine the best use of SMCs as adjuvants *in vivo* needs to be done to explore the potential of this class of drugs. Thus, these findings will guide the use of SMCs in adjuvant vaccine therapies for robust protective immunity.

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APC	Antigen Presenting Cell
BAFF	B-Cell Activating Factor
BCR	B-Cell Receptor
BIR	Baculovirus IAP repeat
BMDC	Bone Marrow Derived Dendritic Cell
CAR	Chimeric Antigen Receptor
CARD	Caspase Recruitment Domain
CD	Cluster Differentiation
cIAP	Cellular Inhibitor of Apoptosis
CM	Central Memory
DAMP	Danger Associated Molecular Pattern
DC	Dendritic Cell
DR	Death Receptor
EM	Effector Memory
FADD	Fas Associated Death Domain
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HPV	Human Papilloma Virus
IAP	Inhibitor of Apoptosis
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IκB	Inhibitors of κB
IκK	Inhibitor of IκB kinase
IL	Interleukin
I.P.	Intraperitoneal
LPS	Lipopolysaccharide
NEMO	NF-κB Essential Modulator
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
NIK	NF-κB Inducing Kinase
NK	Natural Killer
MHC	Major Histocompatibility Complex

MLKL	Mixed Lineage Kinase Domain-Like Protein
Mo-DC	Monocyte Derived Dendritic Cell
OVA	Ovalbumin
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
pDC	Plasmacytoid DC
PRR	Pattern Recognition Receptor
RING	Really Interesting New Gene
RIPK	Receptor Interacting Kinase
SD	Standard Deviation
SMAC	Second Mitochondrial-Derived Activator of Caspases
SMCs	Smac Mimetic Compounds
TAC	T-Cell Antigen Couplers
TAK1	Transforming Growth Factor-β-Activated Kinase 1
TCR	T-Cell Receptor
TNF-α	Tumour Necrosis Factor-α
TNFRSF	Tumour Necrosis Factor Receptor Superfamily
TRADD	TNF-Receptor-1-Associated Death Domain
TRAF	TNF Receptor-Associated Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
UBA	Ubiquitin-Associated Domain

ACKNOWLEDGMENTS

First and foremost, my deepest thanks go to my supervisors Dr. Robert Korneluk and Dr. Shawn Beug for accepting me into your lab and giving me the opportunity to flourish into a better scientist. I am immensely grateful for your support and guidance which has allowed me to complete this Master's program.

Extended thanks go to my thesis advisory committee members, Dr. Subash Sad and Dr. Tommy Alain for all your help and advise that allowed me to stay on track and submit this thesis.

A special thank you to Nathalie Earl for all your assistance and guidance with my animal work and training. Additional thanks go to Martine St. Jean and Allan Humphrey for your help in everyday lab work and answering my endless questions. I would also like to thank all my fellow lab members in the Kornleuk/Beug lab for your guidance and support and making these past two years enjoyable.

I would like to thank my parents, Pamela Jones and Martin Labelle for supporting me throughout this endeavour and allowing me to focus on my science. I'd also like to thank my grandfather, Allen Jones for encouraging me to pursue higher education and continue to be critical and curious. Lastly, I'd like to thank my friends and support system, Julia Loiselle, Anthea Mavridis, and Tara Sabloff for being there when I needed you.

I could not have done this without you all.

CHAPTER ONE: INTRODUCTION

1.1 Vaccines and Adjuvants

The goal of vaccination is to induce an appropriate immune response against a desired target and provide long-lasting protective immunity. There are many different vaccination formulations that will elicit varying immune responses[1–3]. For some viruses or pathogens, stimulation of the innate immune response is adequate to clear infection and generate necessary immunity against recurrence. However, for some viruses, pathogens, or even cancer cells, these responses are not enough[1–3]. One of the only vaccines to have been shown to induce a high magnitude of antigen-specific $CD8^+$ T cell responses in humans are those pertaining to live viral "attenuated" vaccines, such as fore yellow fever or smallpox[1–3]. These vaccines are created by attenuating the virulence of the pathogen via serial passage which allow the virus to mutate such that it is no longer harmful to the recipient of the vaccine[1–3]. However, these vaccines have some drawbacks such as the rare possibility that the attenuated virus can revert to a pathogenic form and cause disease. As well these vaccines typically have long manufacturing and production times[1–3]. To overcome these problems, there has been a shift in vaccine formulation towards nucleic acid vaccine such as DNA, RNA and peptide vaccines[3]. These vaccines are much quicker to produce and relatively safer, however they often don't induce the required immune response for the target diseases[3].

New adjuvants that will help stimulate the appropriate immune response to generate long-term protection are therefore being sought. Adjuvants were first described as a substance that helped the uptake of specific antigens provided by the vaccine to induce a more robust immune response[4]. Aluminum salts (alum) represent one of the oldest adjuvants used in vaccines, however, there has been a recent push to remove aluminum-based vaccines from the market due to a theoretical potential for neurotoxicity[5,6]. Furthermore, alum and many other adjuvants, primarily induce an innate and antibody immune response, and despite extensive studies the specific mechanism of

action remains undefined or poorly understood[7]. This has led to an increase in demand to develop modern adjuvants that will induce adaptive immune responses and better stimulate antigen-presenting cell activity[4]. This approach may improve antigen recognition and increase the effectiveness of vaccines long term.

1.2 Vaccine Induced Immune Response

Immune responses are generated by a two-prong approach, the first branch being the innate response, which induces a non-specific immune response and only requires the recognition of non-self pathogens/particles[1,2]. The adaptive response, as the second branch, occurs later in the immune response and is specific to the invading pathogen or foreign particle. The adaptive response follows clonal expansion of a T cell targeting the invading pathogen or foreign particle [1–3]. The adaptive immune response is also responsible for inducing long-lasting immunity[1–3].

After vaccination, antigen presenting cells (for example dendritic cells, see **Figure 1**) will capture the foreign vaccine antigens which allows for maturation of dendritic cells (DCs) and their trafficking from the site of injection to peripheral lymphoid organs, where the antigen will be presented through major histocompatibility complex (MHC) molecules to T cells[1–3]. There are 3 signals that are required for T cell activation to occur, the first is presentation of the antigen by MHC to the T cell receptor (TCR), the second consists of co-stimulatory signals, and the third and last signal consists of the secretion of specific cytokines to allow for the differentiation of the now activated T cells[1–3] (**Figure 2**). T cells themselves can further be delineated into effector $CD8^+$ cytotoxic T cells that induce programmed cell death in specific target cells and $CD4^+$ helper T cell which enhance/activate other immune cells by secreting specific cytokines[1,2]. Furthermore, the

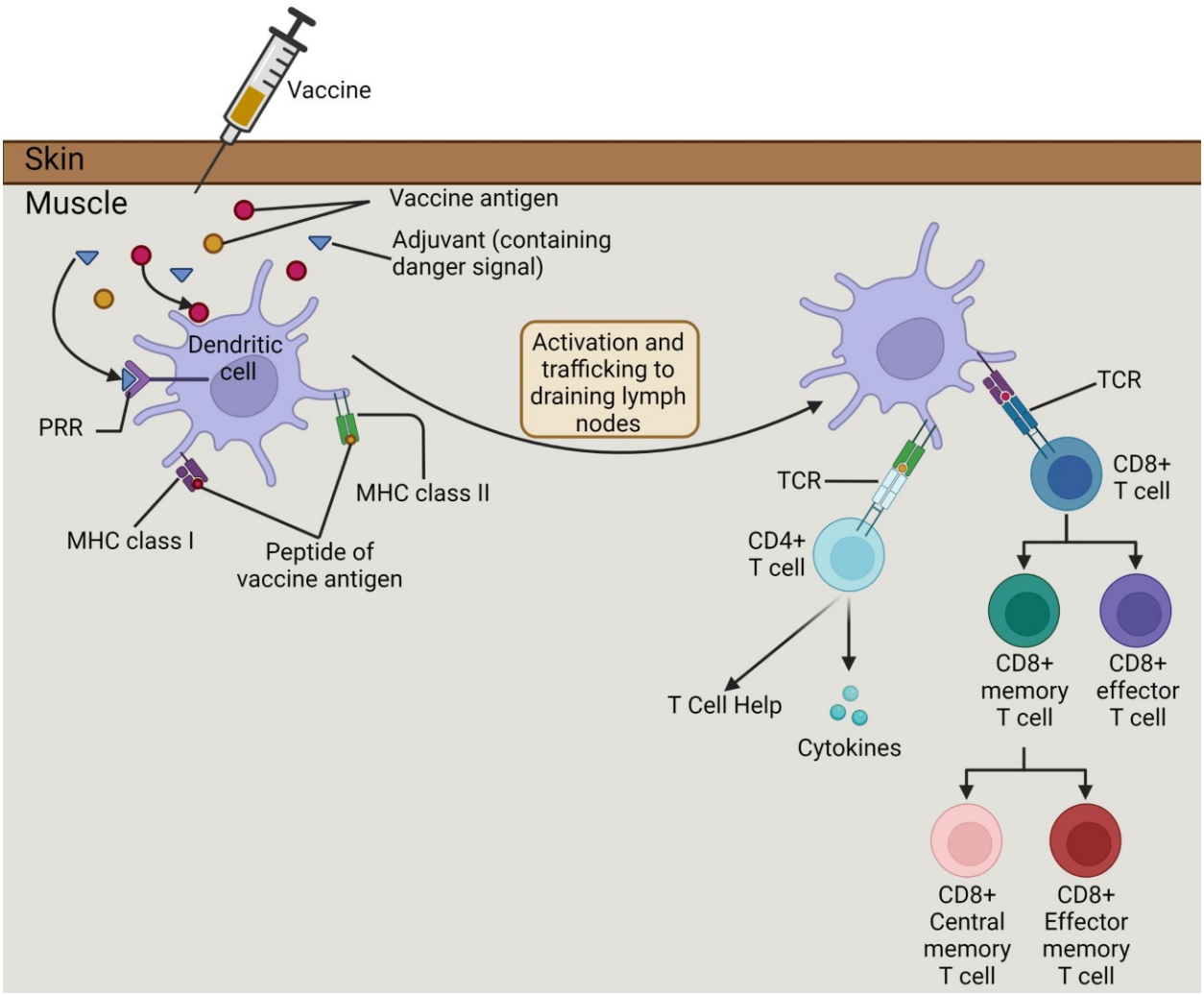


Figure 1. Generation of vaccine induced immune response. Vaccine antigens injected into the muscles are taken up by local dendritic cells, which are activated through pattern recognition receptors (PRRs) by danger signals in the adjuvant, and then trafficked to lymphoid organs. Here, the presentation of peptides of the vaccine antigen by MHC molecules on the dendritic cell activates T cells through their T cell receptor (TCR)[1,3].

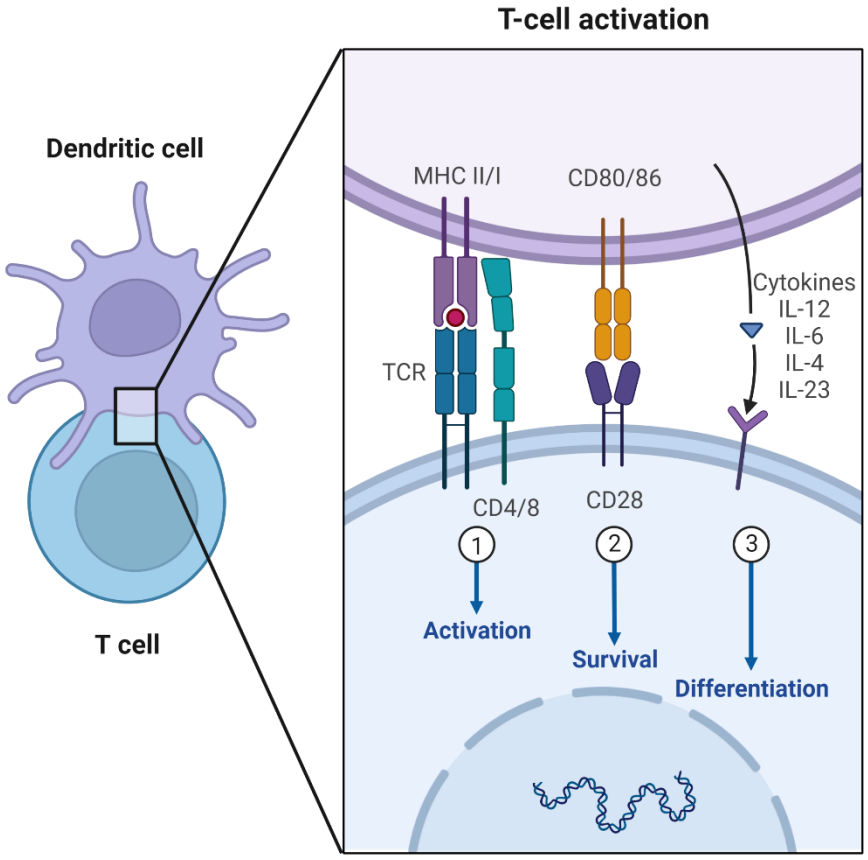


Figure 2. The three signals required for activation of naïve T cells by antigen presenting cells. Binding of peptide:MHC molecule by the T cell receptor (TCR) consists of the first activating signal (1). The second signal (2), also known as the co-stimulatory signal, promotes the survival and expansion of the T cell, in this example, the T cell expresses CD28 and recognizes a B7 receptor (CD80/86) on the dendritic cell. The last signal (3) includes secretion of cytokines from the dendritic cell which will allow for the differentiation of the T cell into a subset that is required for an appropriate immune response [1,2].

adaptive immune response also involves B cells, which can become activated when B cell receptors (BCRs) on the surface of mature naïve B cells encounter and bind a specific antigen[1,2].

This activation sometimes requires the help of helper T cells. Once B cells are activated, they travel to lymph nodes where they will undergo somatic hypermutation and isotope class switching of the immunoglobulins (Ig) to achieve an Ig that is specific to the antigen[1,2]. B cells with the highest affinity Ig receptors are selected to survive, proliferate, and differentiate into memory B cells and antibody-producing plasma cells[1,2]. The plasma cells will either secrete the antibodies or have membrane bound antibodies that can subsequently neutralize, opsonize or mediate killing of target antigens by natural killer (NK) cells[1,2].

1.3 Dendritic Cells (DCs)

1.3.1 Maturation of DCs

Dendritic cells are professional antigen-presenting cells that bridge the innate and adaptive immune responses. DCs mature upon encountering and phagocytosing foreign pathogens, cytokines, damage-associated molecular pattern (DAMPs) molecules, or pathogen-associated molecular pattern (PAMPs) molecules[1,8,9] (**Figure 3**). This encounter and phagocytosis lead to the generation of peptide antigens for presentation to adaptive immune cells[1]. Mature DCs are trafficked to peripheral lymphoid organs via CCR7 upregulation, which facilitates interactions between DCs and adaptive immune cells, such as T cells[1] (**Figure 2, Figure 3**). Mature DCs upregulate expression of MHC molecules and co-stimulatory molecules CD86 and CD80, which allows for activation and proliferation of T cells (**Figure 2**)[1,8,9]. Additionally, DCs secrete cytokines such as IL-12, IL-6, IL-23, IL-4, and IL-10 to induce differentiation of T cells (**Figure**

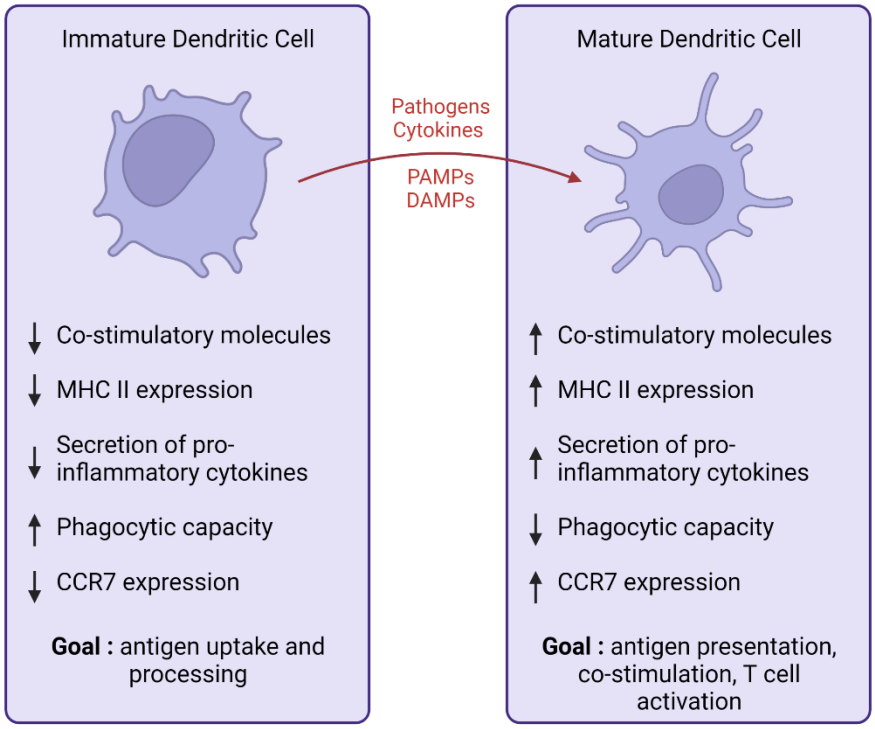


Figure 3. Dendritic cell maturation. There are many phenotypical and functional changes that occur when dendritic cells are matured. The most prominent morphological difference is that the dendritic cells take on a more dendrite like appearance, which is linked with the upregulation of multiple receptors and ligands. These changes allow for the dendritic cell to migrate from the site of antigen uptake to peripheral lymphoid organs where they can present antigens to T cells [1,8,9].

2)[1,8]. The cytokines produced depend on the stimuli that induced the maturation of the DC[1,8–11]. Moreover, the subset of DC also plays a role in the cell's maturation response[8–11].

1.3.2 Mouse Dendritic Cell Subsets

One of the key features of DCs is their ability to present foreign antigens to other immune cells. This key role as well as the CD11c marker helps distinguish DCs from other immune cells. However, as with many immune cells, DCs are a heterogeneous cell population with variety in their location, phenotype, and immunological function[12–14]. DCs plasticity allows for varied immune responses when presented with different pathogens. DC subsets have been elucidated through mouse studies in which lymphoid and non-lymphoid DC subsets have been characterized[12–14]. Lymphoid tissue-based DC subsets are characterized as such due to their primary location in lymphoid organs such as the spleen and lymph nodes. There are two subsets of lymphoid DCs, $CD8\alpha^{+}CD11b^{-}$ and $CD8\alpha^{+}CD11b^{+}$ (**Figure 4, Table 1**)[12–14]. The non-lymphoid tissue resident DCs, also known as migratory DCs, are found in various organs across the body from lungs, stomach, and intestines. There are three subsets of non-lymphoid DCs, $CD103^{-}CD11b^{+}$, $CD103^{+}CD11b^{-}$, and $CD103^{+}CD11b^{+}$ (**Figure 4, Table 1**)[12–14]. Once these DCs interact with a foreign pathogen and are matured they will migrate to lymphoid organs to interact with T cells and B cells. Lastly, there are two more subsets of DCs known as plasmacytoid DCs (pDCs) and monocyte-derived DCs (**Figure 4, Table 1**)[12–14]. Both are found circulating in the blood, however, when pDCs interact with a pathogen they will migrate to lymphoid organs to present antigens, while monocyte-derived DCs will exit the blood stream and mature into inflammatory monocyte-derived DCs[12–14]. These inflammatory monocyte-derived DCs will attract other immune cells to the inflamed tissue by secreting cytokines and chemokines[12–14].

Table 1. Mouse DC markers. List of markers used to distinguish mouse dendritic cell subsets generated from bone marrow cultured with GM-CSF[12–14].

Markers	**Migratory DCs**			**Lymphoid DCs**		**Plasmacytoid DCs**	**Monocytic DCs**
	CD103+ CD11b-	CD103- CD11b+	CD103+ CD11b+	CD8α+	CD8α-		
CD11c	+	+	+	+	+	+	+
CD8α	-	-	-	+	-	-	-
CD103	+	-	+	-	-	-	-
Siglec-H	-	-	-	-	-	+	-
Ly6c	-	-	-	-	-	+	+
CD11b	-	+	+	-	+	-	+
CD64	-	+	-	-	-	-	-

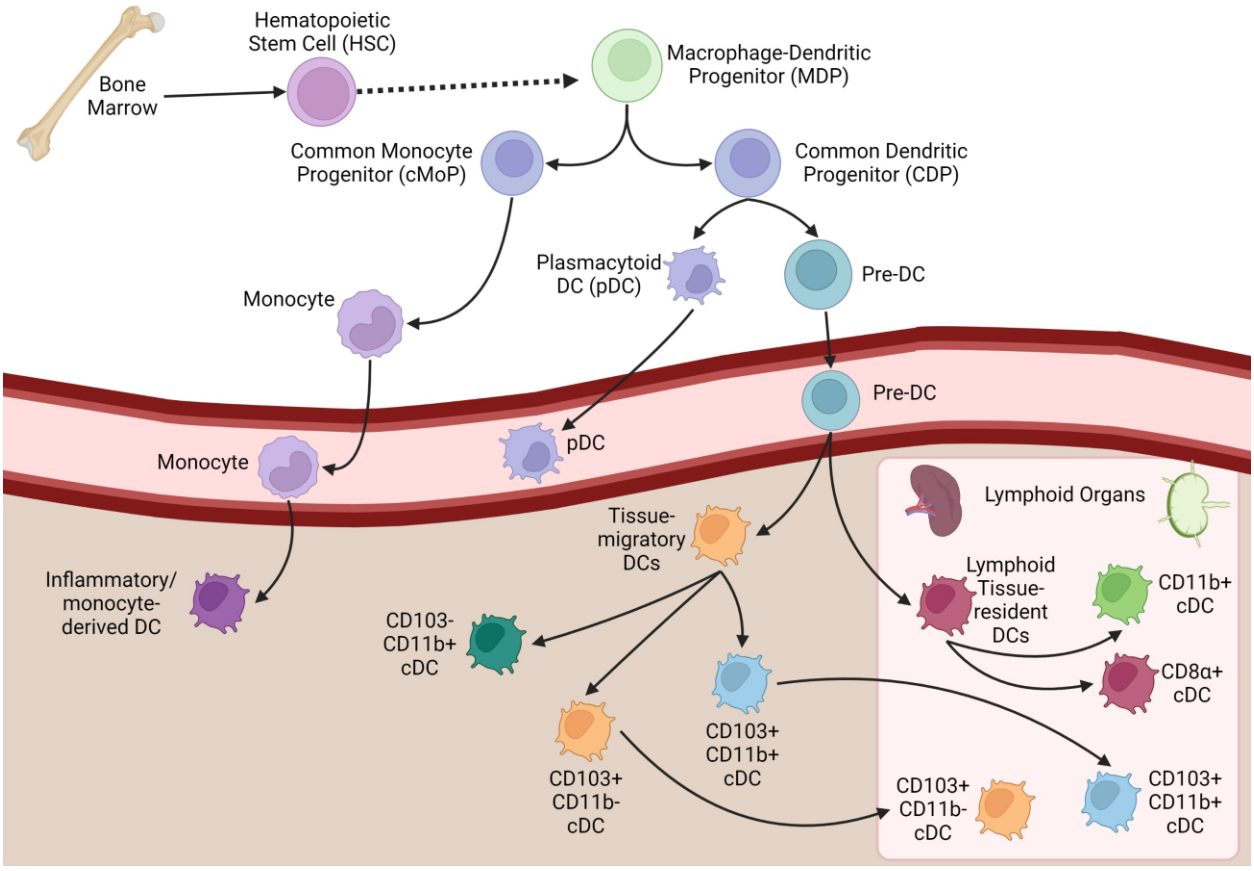


Figure 4. Mouse DC subsets and their precursors. This illustration demonstrates the development pathways of subsets of DCs that can arise from mouse bone marrow cells[12–14]. Dashed line indicates precursor cells between HSC and MDP that are not indicated.

1.4 Adaptive Immune Response – T cells

Adaptive immunity is the response of antigen-specific lymphocytes (B cells and T cells) to antigens. An adaptive immune response is generated by 'clonal selection' of antigen-specific lymphocytes and can generate immunological memory[1–3]. T cells differentiate into two subtypes, cytotoxic $CD8^+$ T cells and helper $CD4^+$ T cells. Effector $CD8^+$ cytotoxic T cells induce target specific cell death, while $CD4^+$ helper T cells enhance/activate other immune cells by secreting specific cytokines[1–3].

As previously stated, T cell activation relies on three signals (**Figure 2**). $CD8^+$ T cells recognize antigens presented via MHC class I, while $CD4^+$ T cells are activated by recognition of antigens presented by MHC class II [1–3]. Following activation, T cells proliferate and differentiate through IL-2 autocrine production [1,2,15] (**Figure 2**). These activated and proliferating T cells will also secrete various other cytokines, IFN-γ, TNF-α, IL-4, IL-7, etc., to mediate the required immune response[1,2,15–17].

T cells are pivotal in protective immune responses [3], thus contributing to diverse memory T cell pools. Two of these pools are formed from memory $CD8^+$ T cells (**Figure 1**). Central memory (CM) $CD8^+$ T cells reside in peripheral lymphoid organs and are re-activated once presented with the target antigen by APCs that encountered the antigen in circulation[18,19]. Once re-activated, CM $CD8^+$ T cells will initially proliferate and secrete IL-2, until eventually differentiating into effector $CD8^+$ T cells and exiting peripheral lymphoid organs[18,19]. Effector memory (EM) $CD8^+$ T cells circulate in the blood and become re-activated once they encounter the target antigen in circulation[18,19]. Once reactivated, EM $CD8^+$ T cells will migrate to the inflamed tissue and display immediate effector function[18,19].

1.5 NF-κB Pathways

Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) are a family of transcription factors that act as master regulators of various pathways of anti-apoptotic, immune, and inflammatory responses[20–23]. This family consist of 5 members, p50/p105 (NF-κB1), p52/p100 (NF-κB2), RelA (p65), RelB, and c-Rel, which mediates activation and transcription of certain target genes by binding to the DNA in various dimers. These proteins are normally located in the cytoplasm and are sequestered by the inhibitors of κB (IκB) proteins[20–23]. Activation of the NF-κB pathway can occur due to a variety of stimuli including ligands of various cytokine receptors, pattern-recognition receptors, ligands of TNF receptor superfamily (TNFRSF), as well as T cell-receptors and B-cell receptors[20–23]. Downstream signalling pathways may be initiated based on various stimuli presented, however, many of these will congregate to the inhibitor of κB kinase (IKK) complex, composed of its two catalytic subunits IKKα and IKKβ and a regulatory subunit named NF-κB essential modulator (NEMO) or IKKγ[20–23]. Two of these signalling pathways are known as the classical NF-κB pathway and the alternative NF-κB pathway.

Upon activation of the classical NF-κB pathway, the IKK complex consisting of IKKα/β and NEMO phosphorylate IkBα leading to it's proteasomal degradation[20–22]. This allows for the previously sequestered p50/p65 dimer complex to be translocated to the nucleus, wherein p65 is phosphorylated and the dimer binds the DNA and induces transcription of genes under the control of the classical NF-κB pathway[20–22,24]. In contrast, activation of the alternative NF-κB signalling pathway leads to accumulation of NF-κB inducing kinase (NIK)[20,22,23,25]. NIK complexes with IKKα/α which phosphorylates p100 to induce proteasomal degradation and processing of p100 into p52[20,22,23,25]. The p52 molecule then complexes with RelB and is translocated to the nucleus

to bind to the DNA as a dimer and induce the transcription of genes specific to the alternative NF-κB pathway[20,22,23,25].

1.5.1 Inhibitor of Apoptosis (IAP) Proteins

The inhibitor of apoptosis proteins are a highly conserved group of anti-apoptotic proteins that function as regulators in various pathways of proliferation, differentiation, and cell death[26–31]. IAPs have been well established in their ability to regulate the NF-κB pathway, cytokine signalling, effector functions in immune cells, as well as caspase signalling[26–29]. There are eight known human IAPs, with cellular IAP 1 (cIAP1) and cellular IAP 2 (cIAP2) being well studied, particularly with respect to their involvement in immunity and apoptosis control[29,32]. This is due to them being highly expressed in cancer and immune cells[33]. IAP family members are defined by a Baculovirus IAP Repeat (BIR) domain located at the N-terminal of the proteins[27,29,34,35] (**Figure 5**). Five of the IAPs, including cIAP1 and cIAP2 are known to have a really interesting new gene (RING) domain located at the C-terminal, which can function as an E3 ubiquitin ligase controlling binding protein stability or signalling[29,34–37] (**Figure 5**). Through the ubiquitination of certain target proteins, cIAP1 and cIAP2 positively and negatively regulate the classical and alternative NF- κB pathway, respectively[26,28,29]. In the classical NF-kB pathway, cIAPs are recruited to the TNFR1 via their involvement in the TNF-receptor-1-associated death domain (TRADD) complex following receptor stimulation with TNF-α. cIAPs ubiquitinate receptor-interacting protein kinase (RIPK) 1 and begin the signaling cascade to induce the expression of genes under the control of the classical NF-κB pathway (**Figure 6**), which encode pro-survival signals[26,28,29,38]. cIAPs also ubiquitinate NIK in the cytosol, which leads to degradation of NIK and the negative regulation of the alternative NF-κB pathway[26,29] (**Figure 6**).

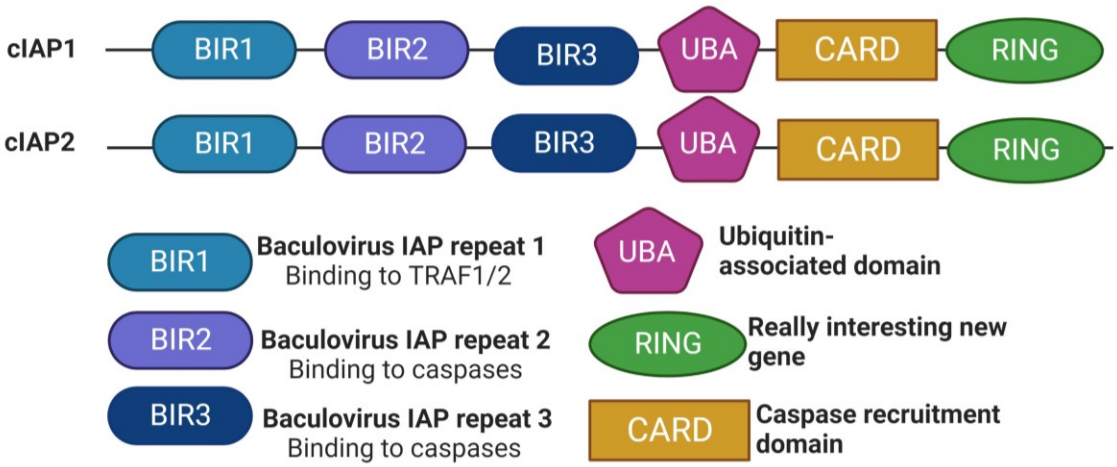


Figure 5. Structural domains of cIAP proteins. The IAP gene family are characterised by the presence of at least one BIR domain, which participates in many protein-protein interactions, such as TRAFs, caspases, and second mitochondria-derived activator of caspases (Smac). Certain IAPs contain a RING domain which contains an E3 ubiquitin ligase domain which allows it to ubiquitinate various proteins. Some IAPs contain a UBA domain which allows the IAPs to bind ubiquitin, and/or they will contain a CARD domain which allows for the suppression of the E3 ligase activity under steady-state conditions[29]. Abbreviations: cellular IAP 1 (cIAP1), cellular IAP 2 (cIAP2).

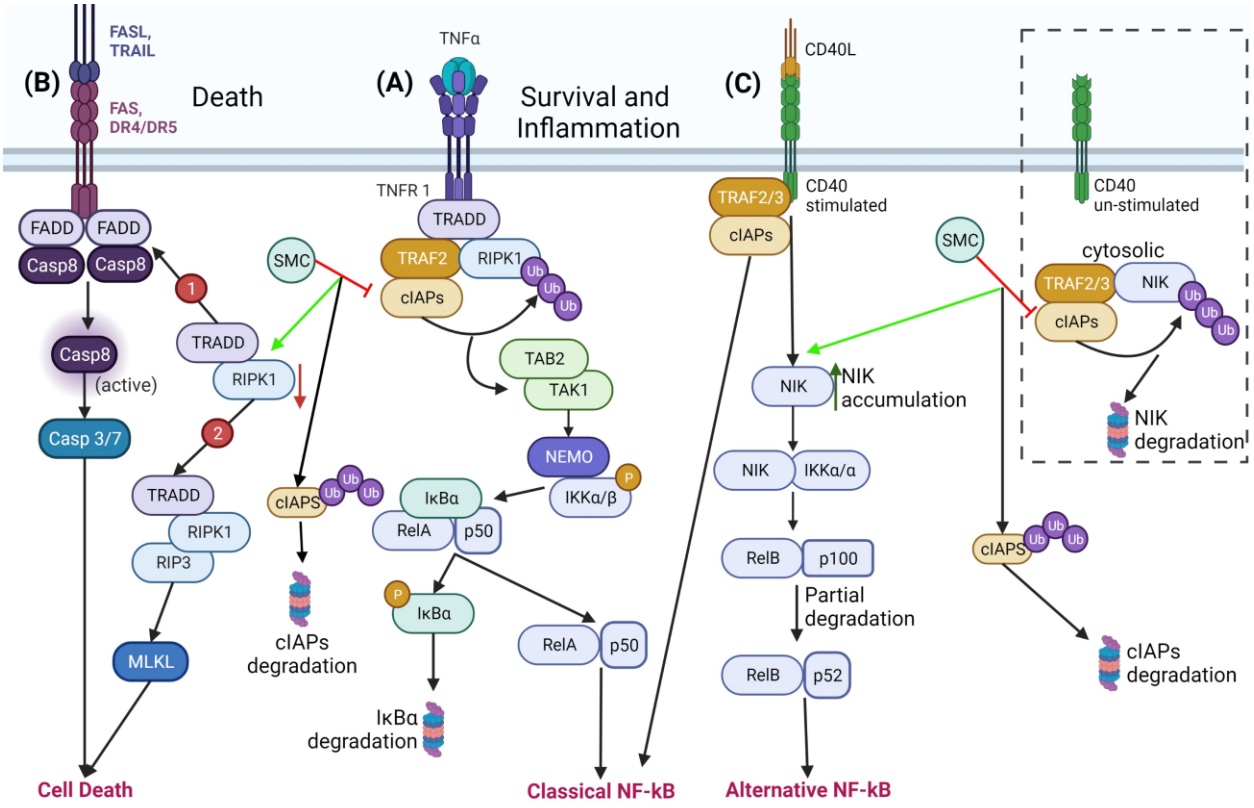


Figure 6. The IAP proteins regulate NF-κB signaling and cell survival.[29] **(A)** Activation of classical NF-κB pathway by TNF-α. cIAPs are recruited to the TNF receptor 1 (TNFR1) via their involvement in the TNF-receptor-1-associated death domain protein (TRADD) and TNF-receptor-1-associated factor (TRAF)2 complex following receptor stimulation with TNF-α. The cIAPs ubiquitinate receptor-interacting protein kinase (RPIK)1, which leads to the recruitment and activation of TGF-β activated kinase 1/MAP3K7 binding protein 2 (TAB2) and transforming growth factor-β-activated kinase 1 (TAK1). TAK1 phosphorylates and activates the IKK complex consisting of NF-κB essential modulator (NEMO), IKKα, and IKKβ, which in turn phosphorylates IkBα leading to its proteasomal degradation. This degradation allows the NF-κB dimer p50/p65 to be translocated to the nucleus to activate transcription of target genes. **(B)** Antagonism of cIAPs by SMC leads to RIPK1 not being ubiquitinated and the formation of cytosolic complexes with Fas-associated death domain protein (FADD) and Caspase 8 or RIPK3, which will both lead to cell death. **(C)** Activation of alternative NF-κB pathway by CD40 ligand (CD40L) or SMC. Under resting conditions, the alternative NF-κB pathway is suppressed due to the constitutive ubiquitination and degradation of NIK by the TRAF2/3 and cIAP1/2 complex in the cytosol. Upon receptor stimulation by CD40L, the TRAF2/3 and cIAP1/2 complex is recruited to the receptor and initial activation of the classical NF-κB pathway occurs until NIK accumulates enough and is stabilized. SMC induces degradation of cIAPs, which leads to eventual NIK stabilizing and accumulation. NIK complexes with IKKα/α which phosphorylates p100 to induce proteasomal degradation and processing of p100 into p52. The NF-κB dimer, p52/RelB, will be translocated to the nucleus to activate transcription of target genes. Abbreviations: TNF-related apoptosis-inducing ligand (TRAIL), death receptor (DR)4/5, caspase (Casp), receptor-interacting protein (RIP)3, mixed lineage kinase domain-like protein (MLKL), ubiquitin (Ub).

1.5.2 Smac Mimetic Compounds (SMCs)

SMCs are a class of drugs that are derived from the endogenous pro-apoptotic protein, the second mitochondria-derived activator of caspase (SMAC)[39]. SMCs inhibit the activity of IAP proteins which in turn sensitizes cancer cells to apoptosis[29]. Given this, many SMCs, including LCL161 and Birinapant, have progressed to Phase II/III clinical trials[40–42]. SMCs bind to the BIR domain of IAPs to induce their auto-ubiquitination and subsequent degradation[29,39,40]. In TNFRSFs that possess a death domain (i.e.. TNFR1, FAS, DR4/DR5), the degradation of the cIAPs leads to the deubiquitination of RIPK1, which in turn results in the formation of cytosolic complexes with Fas-associated death domain (FADD) and Caspase 8 or RIPK3, leading to cell death[28,29,34,36] (**Figure 6**). Through this mechanism, SMCs help sensitize infected or cancerous cells to apoptosis by TNF-α mediated killing[28,29] (**Figure 6**). In TNFRSFs that do not posses a death domain (i.e.. TNFR2, CD40, 4-1BB, OX40, BAFF), the degradation of the cIAPs allows for the accumulation of NIK and activation of the alternative NF-κB pathway[22,29,32,43] (**Figure 6**). Many of the genes that the NF-κB signalling pathway will express lead to cell proliferation, maturation/activation, survival, and growth[22,28,40,44]. However, the alternative pathway induces the expression of genes that are unique and specific for immune/lymphoid cells, based on their upstream receptor stimulation, and express genes that will propagate this signal[22].

1.5.3 cIAP Proteins and SMCs in Immune Cells

SMCs have often been referred to as an immunomodulatory drug as it acts on IAP proteins that are critical in various pathways of anti-apoptotic, immune, and inflammatory responses regulated by the NF-kB signalling pathway[29,33,35,42,45]. This has led to SMCs being used in various therapeutic approaches that capitalize on their immunomodulating effect. SMCs have been shown

to prolong B cell survival *in vitro* through the activation of the alternative NF-κB pathway downstream of B cell-activating factor (BAFF), a TNFRSF[43]. SMCs have been demonstrated to increase the number of natural killer (NK) cells that infiltrate the tumour[46] and to increase NK cell cytolytic activity in response to tumour cell death[47]. One study showed that SMCs could induce a change in macrophage phenotype towards an M1 pro-inflammatory phenotype within a tumour microenvironment, which aided in tumour clearance, as well as helped form an immunological memory that protected against recurrence[48]. SMCs have been demonstrated to induce activation and proliferation, and to increase cytokine production by T cells, when combined with a TCR stimulant[49,50]. A dimeric form of SMC, AZD5582, has been shown to increase expression of genes involved in cross-presentation of antigens in dendritic cells[51]. SMCs have also been demonstrated to have widespread immune modulating responses when in combination with other immunotherapies, such as checkpoint blockade inhibitors[52–54], oncolytic viruses[55–57], chimeric antigen receptor (CAR) T cells[58], and T cell antigen couplers (TAC)[59]. In a mouse study, SMCs has been shown to delay tumour growth and prolong survival when used as an adjuvant in a prophylactic vaccine using an inactivated "killed" vaccine base[49]. As such, SMCs hold much clinical promise as they are a chemically defined synthetic molecules having a mechanism of action that stimulates both the innate and adaptive responses in various immune cells (**Figure 7**).

1.6 Rational and Hypothesis

Since SMCs have been shown to have profound effects on various aspects of immune responses, I hypothesized that SMCs possess immunomodulatory properties that would **enhance vaccine induced immune responses through activation of dendritic cells**. The ***specific aims*** of

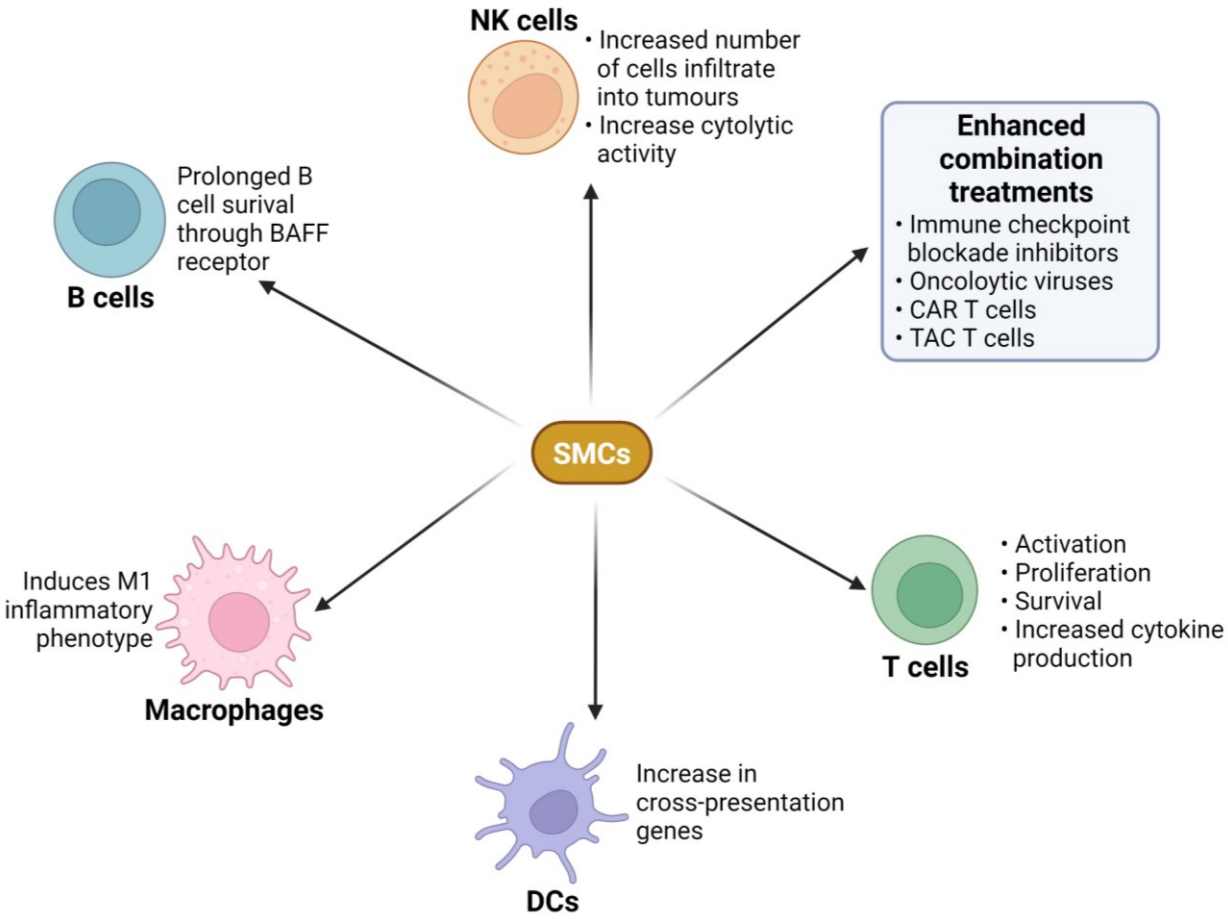


Figure 7. Known immunomodulatory effects of SMCs. Illustration of the known effects of IAP antagonists on various immune cells and immune responses in the context of various therapeutic approaches.

this study were **(1)** to evaluate the effect of SMCs on the maturation of dendritic cells, **(2)** to determine the antigen-presentation response of dendritic cells to SMC treatment and **(3)** to investigate whether vaccination with an SMC as an adjuvant confers protective immunity against tumours.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Reagents

The monomeric Smac mimetic compound, LCL161, was provided by Novartis. The H-2b–restricted E7 epitope (RAHYNIVTF) was synthesised by the Peptide Synthesis Core at the University of Ottawa, ON, Canada with a purity greater than 95%. The ovalbumin (OVA) peptide (SIINFEKL – aa257-264) were synthesized by IvivoGen (vac-sin). The ODNs, CpG-containing ODN 1826 (5′-TCC ATG ACG TTC CTG ACG TT-3′) and non-CpG–containing ODN 1982 (5′-TCC AGG ACT TCT CTC AGG TT-3′), were synthesized by Invitrogen Corp, Carlsbad, Calif (tlrl-1826 and tlrl-1826c-1 respectively). Lipopolysaccharides (LPS) was purchased from Sigma Aldrich (L4391-1MG).

2.2 Cell Line

The TC-1 cell line was obtained from Dr. T.C. Wu. This cell line was derived from C57BL/6 mice lung epithelial cells and immortalized by transforming the cells with HPV16 E6 and E7 oncoproteins, as well as *ras* oncogene[60]. As such, this cell line mimics the natural tumour progression of E6 and E7 expressing cancers. This cell line was grown in culture with RPMI 1640, 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 ug/mL), L-glutamine (2 mM), and sodium pyruvate (1 mM).

2.3 Primary Cell Isolation and Culture

2.3.1 Bone Marrow Derived Dendritic Cell (BMDC) Isolation

Primary bone marrow derived dendritic cells were isolated from C57BL/6 mice as outlined by Inaba et al. (2009)[61] with some modifications. The Femurs and tibias were removed from the

mice, cut on an angle, and placed in a small Eppendorf tube with a hole at the bottom punctured with the help of an 18G needle and placed in a larger 1.5 mL Eppendorf tube. These tubes were centrifuged at 4000 RPM for 2 minutes and the inner tube with bones discarded. The bone marrow suspension was washed with RPMI 1640 medium and centrifuged for 10 minutes at 300 x *g*. Viable cells were resuspended in complete R10 medium (RPMI 1640, 10% FBS, penicillin (100 U/mL), and streptomycin (100 ug/mL)) at $1x10^6$ cells/mL. The cells were plated in 10 cm tissue culture plates and supplemented with 20 ng/mL of recombinant murine granulocyte macrophage colony-stimulating factor (rmGM-CSF) (Peprotech, 250-05). On days 2 and 4, the cells were washed and fed by removing the old medium with non-adherent cells (neutrophils, granulocytes, eosinophils, etc.) and replaced with fresh R10 medium supplemented with 20 ng/mL of rmGM-CSF. On day 6, non-adherent and semi-adherent cells were considered immature dendritic cells (DCs) and were ready to be collected for experiments.

2.3.2 T Lymphocyte Isolation

Lymphocytes were isolated from C57BL/6 mice spleens. Spleens were collected and dissociated in 1X phosphate-buffered saline (PBS) in gentleMACs C tubes (Miltenyi, 130-093-237) using the gentleMACs Octo Dissociator with Heaters (Miltenyi, 130-096-427) following the mouse spleen protocol. Single cell suspension was washed through a 70-µm filter and centrifuged at 500 x *g* for 5 minutes. Cells were resuspended in ACK lysis buffer (150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA in H_2O) and incubated on ice for 5 minutes, before washing with cold 1X PBS and centrifuging at 500 x *g* for 5 minutes. The T cells were isolated from splenocytes following the manufacturing instructions from a Negative Pan T Cell Isolation Kit (Miltenyi, 130-095-130).

2.3.3 BMDC Treatment and Co-Culture Experiments

After culturing BMDCs for 6 days, the non-adherent cells are collected and re-plated. For DC analysis, BMDCs were treated with LPS (100 ng/mL), LCL161 (0.1 μM to 5 μM), or vehicle and collected after 24 hours for analysis of DC subsets and maturation, while the supernatant was collected for later analysis of cytokines.

For the co-cultures, BMDCs were treated with LCL161 (1 μM) or vehicle for 24 hours and stimulated with the $OVA_{257-264}$ (SIINFEKL) peptide at varying doses (0.001 μg/mL to 1 μg/mL) 2 hours before the addition of OT-I T cells. Isolated OT-I cells were plated at a 1:1 ratio with the BMDCs and the addition of recombinant human IL-2 (200 IU/mL, StemCell Technologies, 78036.3) and the supernatant was then collected at 24, 48, and 72 hours for cytokine analysis.

2.4 Western Blotting

After culturing BMDCs for 6 days, the non-adherent cells were collected and re-plated. BMDCs were treated with LPS (100 ng/mL), LCL161 (1 μM), or vehicle and collected at three different time points (1, 6, and 24 hours). Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (10mM Tris pH 7.4, 150mM NaCl, 10mM KCl, 1mM EDTA 0.5% deoxycholic acid, 0.5% Tween-20, 0.5% NP-40) with the addition of proteinase and phosphatase inhibitors (Sigma, PPC1010). The lysates were incubated on ice for 25 minutes, then spun down at 13,800 RPM for 15 minutes at 4ºC. The supernatants were transferred to new tubes and protein quantification was performed using the BioRad Bradford Assay (5000002). Samples (25 ug) were mixed with 5X loading buffer and loaded onto eight or ten percent 1.5 mm polyacrylamide gels depending on the size of the protein of interest. The gels were run at 80 volts for 15 minutes, then 120 volts until completion. Proteins were transferred to a 0.2 nm nitrocellulose membrane via

Trans-Blot Turbo Transfer according to the manufacturer's protocol (BioRad, 1704150). The membranes were blocked with 5% skim milk in 1X Tris-buffered saline with 0.1% Tween-20 (TBST) for 30 minutes at room temperature. The membranes were incubated with the primary antibody overnight at 4ºC. The following day, the primary antibody was removed, and the membranes washed thrice for 5 minutes with 1X TBST. The membranes were incubated with the secondary antibody in the dark for 1 hour at room temperature. Following this incubation, the membranes were kept in the dark and washed thrice for 5 minutes with 1X TBST. The membranes were scanned on the LICOR Odyssey. The following primary antibodies were used: anti-phospho-p65 (Cell signalling, 3033), anti-p65 (Cell Signalling, 8242), anti-p100/p52 (Cell Signalling, 4882), anti-cIAP1/2 (Cyclex, CY-P1041), and anti-GAPDH (Sigma, PLA0302). For biotin detection, Streptavidin IRDye 680 and 800 (P/N 926-68079 and P/N 926-32230) from LICOR were used.

2.5 Immunofluorescence

Immunofluorescence analysis was carried out on TC-1 cells following protocol from Grasso et al. (2013)[62] with modifications. Briefly, coverslips were placed in a 6-well plate and the cells seeded at 80% confluency and allowed to adhere for 24 hours. The following day, the cells were fixed with 3% paraformaldehyde for 30 min at room temperature. The cells were washed twice with 1X PBS, permeabilized for 10 min with 0.1% Triton X-100 (in 1X PBS), and saturated with 3% bovine serum albumin (BSA)-PBS. The cells were incubated overnight with anti-E7 monoclonal antibody (1:50; ThermoFisher Scientific, MA5-14132) in 1% BSA-PBST (1% BSA and 0.1% Tween-20 in 1X PBS) at 4ºC. The following day the cells were washed twice with 1X PBST and incubated for 1 hour with an anti-mouse AF488-conjugated IgG (1:500; ThermoFisher,

A-10680) in 1% BSA at room temperature. The cells were washed twice with 1X PBST and counterstained with DAPI (1:40 dilution) for 2 minutes at room temperature. Finally, cells were washed twice with 1X PBS and coverslips removed from 6-well plate and mounted on slides with a drop of fluorescent mounting medium (DAKO, S3023). Images were attained at room temperature using Zeiss Axio Imager M2 and Zeiss Zen imaging software.

2.6 Flow Cytometry Analysis

Flow cytometric workflow was performed in accordance with the Yale Flow cytometry (FACS) staining protocols for cell surface markers[63]. After experiments, cells were collected and washed with fluorescence-activated cell sorting (FACS) buffer (1% BSA and 0.1% NaN_3 in 1X PBS). Cells were stain for viability (Zombie Violet; Biolegend, 423114) and Fc-blocked using CD16/CD32 antibody (Biolegend, 101302) at a 1:300 dilution. Subsequent staining with primary fluorochrome conjugated antibodies was done at a 1:200 dilution (**Table 2**). Single stain UltraComp compensation beads (ThermoFisher Scientific, 01-3333-41) were used to ensure minimization of crossover between fluorophores in multi-fluorophore panels. In each experiment, unstained and heat-killed controls were utilized to account for auto- or non-specific fluorescence. Samples were run on LSRFortessa X20 Flow Cytometer (BD Biosciences). Flow cytometric data was analysed with FlowJo Version 10.8.1 Software, including compensation analyses.

2.7 ELISA

Supernatant were collected following BMDC maturation experiments, as well as co-culture experiments. Supernatant for analysis of IL-6 cytokine was concentrated using a centrifugal

Table 2. List of Antibodies Used for Various Flow Cytometry Analyses.

Antibody / Probe	Fluorochrome	Vendor / Cat. No. / Clone	Analysed for
Anti-mouse CD11c	APC	Biolegend / 117310 / N418	DC subsets
	FITC	Biolegend / 117306 / N418	DC maturation
Anti-mouse CD11b	APC	Biolegend / 101212 / M1/70	DC subsets
	AF700	Biolegend / 101222 / M1/70	
Anti-mouse CD103	PE	Biolegend / 121405 / 2E7	DC subsets
Anti-mouse Ly6c	AF488	Biolegend / 128022 / HK1.4	DC subsets
Anti-mouse Siglec-H	PE	Biolegend / 129606 / 551	DC subsets
Anti-mouse CD64	PE/dazzle	Biolegend / 139319 / X54-5/7.1	DC subsets
Anti-mouse CD8α	BV785	Biolegend / 100150 / 53-6.7	DC subsets
	FITC	Invitrogen / MA5-16759 / KT15	*In vivo* T cells
Anti-mouse MHCII	AF700	Biolegend / 107622 / M5/114.152	DC maturation
Anti-mouse MHCI	PE	Biolegend / 114708 / 34-1-2S	TC-1 cell line
Anti-mouse CD86	BV785	Biolegend / 105043 / GL-1	DC maturation
Anti-mouse CD80	AF488	Biolegend / 104716 / 16-10A1	DC maturation
Anti-mouse CD40	PE	Biolegend / 124609 / 3/23	DC maturation
Anti-mouse CD3	BV785	Biolegend / 100225 / 17A2	*In vivo* T cells
Anti-mouse CD4	AF700	Biolegend / 100536 / RM4-5	*In vivo* T cells
Anti-mouse E7 MHC tetramer	PE	MBL / TB-5008-1	*In vivo* T cells
Anti-mouse CD44	APC	Biolegend / 103012 / IM7	*In vivo* T cells
Anti-mouse CD62L	BV785	Biolegend / 104440 / MEL-14	*In vivo* T cells
Anti-mouse CD90.2	APC	Biolegend / 105311 / 30-H12	*In vivo* T cells
Anti-mouse SIINFEKL MHC tetramer	PE	MBL / TB-5001-1	OT-I mice confirmation

concentrator (10 kDa) (Millipore Sigma, UFC801096). Presence of IL-12 (DY2398-05), IL-6 (DY406-05), TNF-α (DY410), IFN-γ (DY485), and IL-2 (DY402-05) cytokines was determined via ELISA in duplicates according to the manufacturer's instruction (R&D Systems). Plates were read on the BioTek Synergy Multi-Mode Plate Reader and preliminary analysis to obtain standard curve equations and sample concentrations was performed using Gen 5 2.06 software. Remaining analysis was performed with MyCurveFit Microsoft Excel Add In and GraphPad Prism Version 9 software.

2.8 Mice and Animal Care

Mice were handled in accordance with the guidelines established by the University of Ottawa Animal Care Veterinary Service and the Canadian Council on Animal Care. C57BL/6 female mice and OT-I TCR transgenic female mice (age 5 to 6 weeks) were obtained from Charles River Laboratories. Mice were housed in a controlled facility at the University of Ottawa (22°C on a 12-h light/dark cycle) and had ad libitum access to food and water.

2.9 Prophylactic Vaccination Experiment

Female C57BL/6 mice 6- to 8-week-old were divided into 6 groups of 5 mice and received treatments as follows[64]:

Treatment 1: E7 peptide and LCL161.

Treatment 2: E7 peptide alone.

Treatment 3: Control peptide (OVA – SIINFEKL) and LCL161.

Treatment 4: E7 peptide and ODN 1826 (a CpG-containing ODN).

Treatment 5: E7 peptide and ODN 1982 (a non-CpG-containing ODN).

Treatment 6: Unvaccinated naïve mice.

LCL161 was solubilized in 30% 0.1N HCl and diluted to a final concentration of 10 mg/kg with 100 mM NaAcetate. LCL161 was administered via oral gavage at a dose of 75 mg/kg. All other treatments were administered intraperitoneally (i.p.). The E7 peptide were solubilized in a minimal amount of dimethyl sulfoxide (1% of final vaccine volume) and brought to a final concentration of 100 µg per dose in 200 µL of 1X PBS. The OVA peptide was solubilized in 1X PBS to a final concentration of 100 µg per dose. For treatments 4 and 5, the E7 peptide was mixed with ODNs and brought to a final concentration of 100 µg per dose of peptide and 20 µg per dose of ODN in 200 µL of PBS and kept on ice until injection. Vaccines were administered twice using two dosing regimens. For the first dosing regimen mice were vaccinated on days -14 and -7. For the second dosing regimen, mice were vaccinated on days -21 and -7 (**Figure 18B**). On day 0, the mice were either challenged with $5x10^4$ TC-1 cells injected subcutaneously into the left flank, or spleens were collected for analysis of immune response. Tumors were measured every 3 days with electronic calipers. Tumour growth was calculated using the equation: V (tumor volume) = $(\pi \times (\text{Width}^2) \times \text{Length})/4$.

2.10 Statistical Analysis

Data are expressed as the mean ± standard deviation for each group, with individual data points shown as scattered points. Statistical assessments were performed using one-way ANOVA with Dunnett's multiple comparison analysis comparing vehicle control to the varying treatments using GraphPad Prism Software. Statistical differences were considered significant when the probability value (p) was <0.05. Statistical differences are shown as *p<0.05, **p<0.01, ***p<0.001, and ****p < 0.0001.

CHAPTER THREE: RESULTS

3.1 Aim 1: Evaluate the Effect of SMCs on the Maturation of BMDCs

3.1.1 Generation and characterization of BMDCs

To evaluate the effects of SMCs on DC maturation, bone marrow derived DCs (BMDCs) were treated with LCL161. To generate BMDCs, primary mouse bone marrow cells were cultured over a period of 6 days with recombinant murine GM-CSF[61] (**Figure 8**). I characterized the proportion of DC subsets after treatment with SMCs (**Figure 9 and 10**). As expected, I detected four DC subsets: monocyte-derived DCs (Mo-DCs), $CD8\alpha^{+}CD11b^{-}$ lymphoid DCs, $CD11b^{+}CD103^{-}$ migratory DCs, and $CD11b^{-}CD103^{+}$ migratory DCs[65] (**Figure 10A, B**). The DC subsets that were detected at low levels to the point of being negligible consists of: plasmacytoid DCs (pDCs), $CD11b^{+}CD103^{+}$ migratory DCs, and $CD8\alpha^{-}CD11b^{+}$ lymphoid DCs. SMC treated BMDCs lead to a decrease in number of Mo-DCs and $CD11b^{+}CD103^{-}$ migratory DCs detected, while no change was observed for $CD8\alpha^{+}CD11b^{-}$ lymphoid DCs and $CD11b^{-}CD103^{-}$ migratory DCs (**Figure 10B**). CD11b expression was further analysed as it is a shared marker of Mo-DCs and $CD11^{+}CD103^{-}$ migratory DCs. A decrease in CD11b expression was observed in SMC-treated BMDCs (**Figure 10C**). When viability was assessed downstream of DC subset gating, no change in viability was observed in SMC-treated and CD11b downregulated subsets compared to vehicle-treated. This indicates that SMC-treated BMDCs induces a downregulation of CD11b, thus affecting DC subsets.

3.1.2 Confirmation of NF-κB activation in SMC treated BMDCs

SMCs have been shown to activate the alternative NF-κB signalling pathway[22,29,32,43], a pathway that is essential for DC maturation[9,66]. I then analyzed if SMCs activate NF-κB signalling in BMDCs. LPS treated BMDCs was used as an alternative immune stimulating agent. A decrease

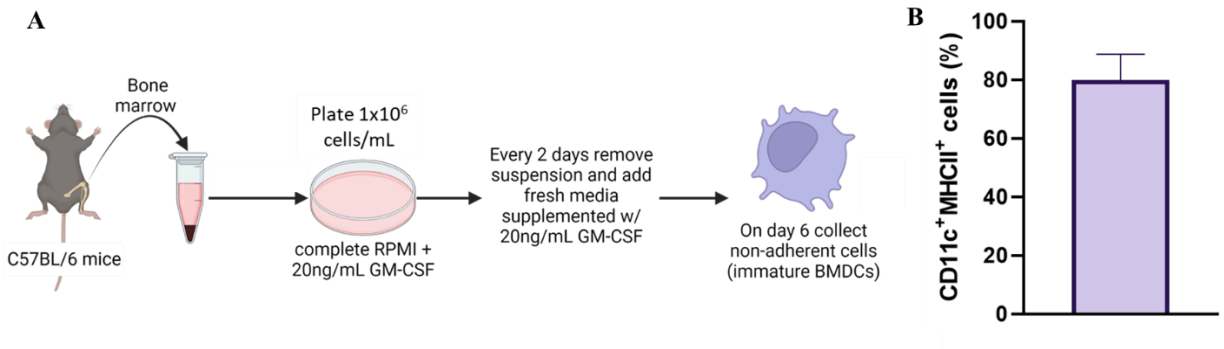


Figure 8. Generation of bone marrow derived dendritic cells. (A) BMDCs were generated according to the protocol by Inaba et al. (2009)[61]. After 6 days of culturing, BMDCs were collected from the non-adherent population and used for downstream experiments. **(B)** BMDC purity was determined based on $CD11c^+$ and $MHCII^+$ expression by flow cytometry, which are common DC markers[61,67]. This protocol results in ~80% purity (mean, SD).

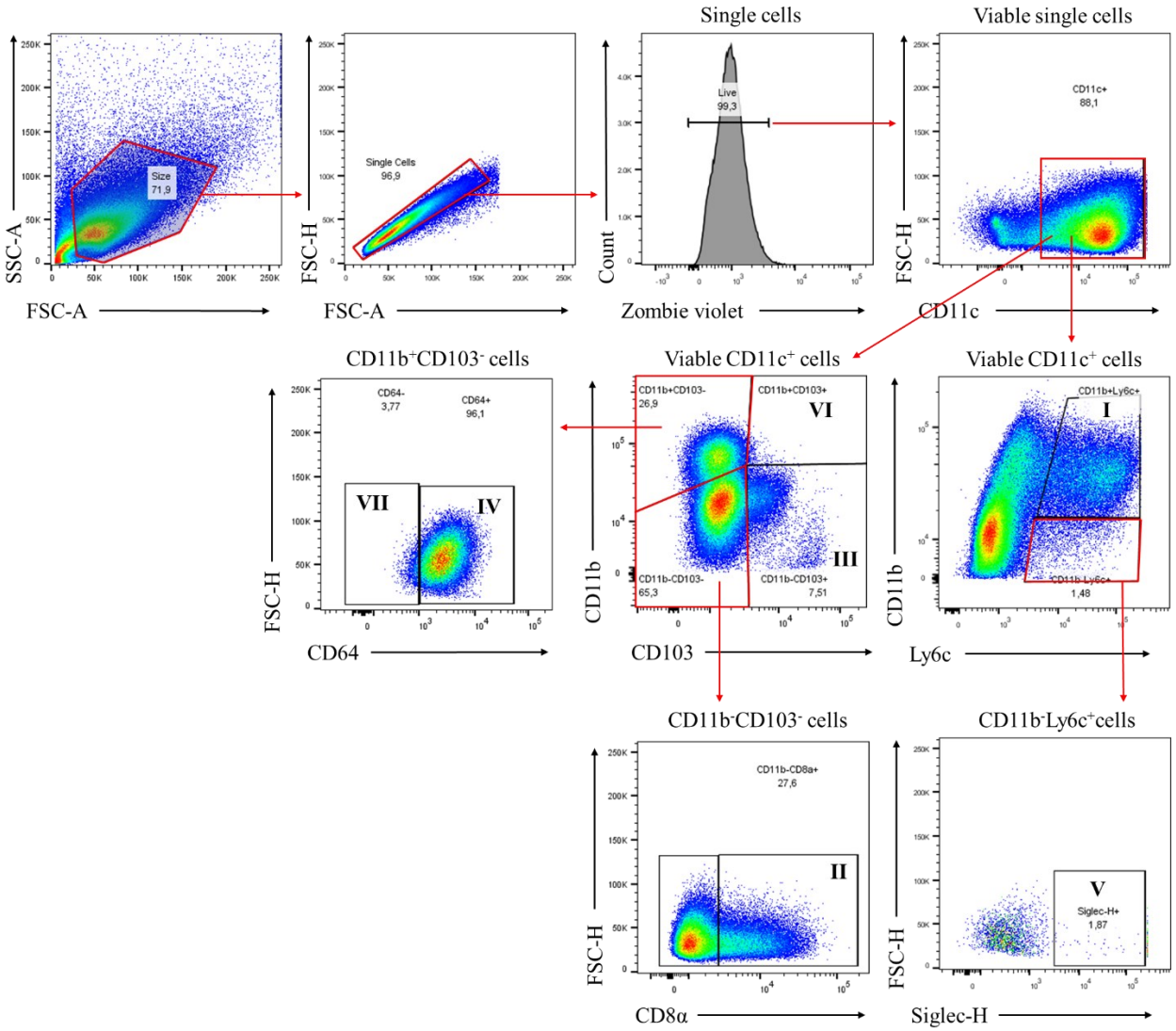


Figure 9. Sequential gating strategy to identify BMDC subsets. BMDCs were stained with the following antibodies for analysis via flow cytometry: panel #1 – Zombie violet (BV421), CD11c (APC), CD11b (AF700), Ly6c (AF488), and Siglec-H (PE); panel #2 – Zombie violet (BV421), CD11c (FITC), CD11b (APC), CD8α (BV785), CD103 (PE), and CD64 (PE/dazzle™ 594). Mouse DC subsets were defined based on markers in **Table 1**. **(I)** Monocyte derived DCs (Mo-DCs), **(II)** $CD8a^{+}CD11b^{-}$ lymphoid DCs, **(III)** $CD103^{+}CD11b^{-}$ migratory DCs, **(IV)** $CD103^{-}CD11b^{+}$ migratory DCs, **(V)** plasmacytoid DCs (pDCs), **(VI)** $CD103^{+}CD11b^{+}$ migratory DCs, and **(VII)** $CD8a^{-}CD11b^{+}$ lymphoid DCs.

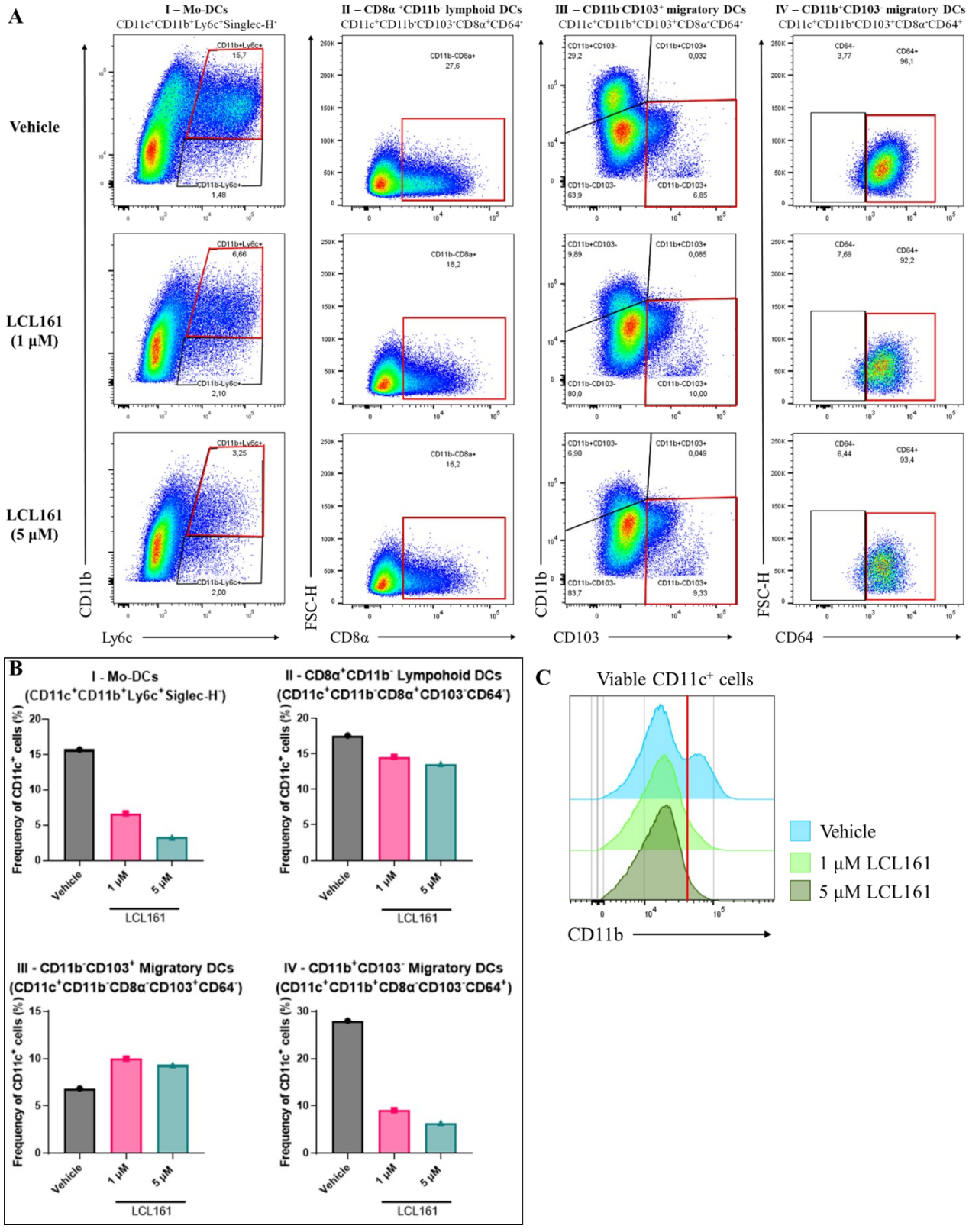

A
I – Mo-DCs
CD11c+CD11b+Ly6c+Singlec-H-
II – CD8α +CD11b- lymphoid DCs
CD11c+CD11b-CD103-CD8α+CD64-
III – CD11b-CD103+ migratory DCs
CD11c+CD11b+CD103+CD8α-CD64-
IV – CD11b+CD103- migratory DCs
CD11c+CD11b-CD103+CD8α-CD64+
Vehicle
LCL161 (1 µM)
LCL161 (5 µM)
CD11b
Ly6c
FSC-H
CD8α
CD103
CD64
B
I - Mo-DCs (CD11c+CD11b+Ly6c+Siglec-H-)
II - CD8α+CD11b- Lympohoid DCs (CD11c+CD11b-CD8α+CD103-CD64-)
III - CD11b-CD103+ Migratory DCs (CD11c+CD11b-CD8α-CD103+CD64-)
IV - CD11b+CD103- Migratory DCs (CD11c+CD11b+CD8α-CD103-CD64+)
Frequency of CD11c+ cells (%)
Vehicle
1 µM
5 µM
LCL161
C
Viable CD11c+ cells
CD11b
Vehicle
1 µM LCL161
5 µM LCL161

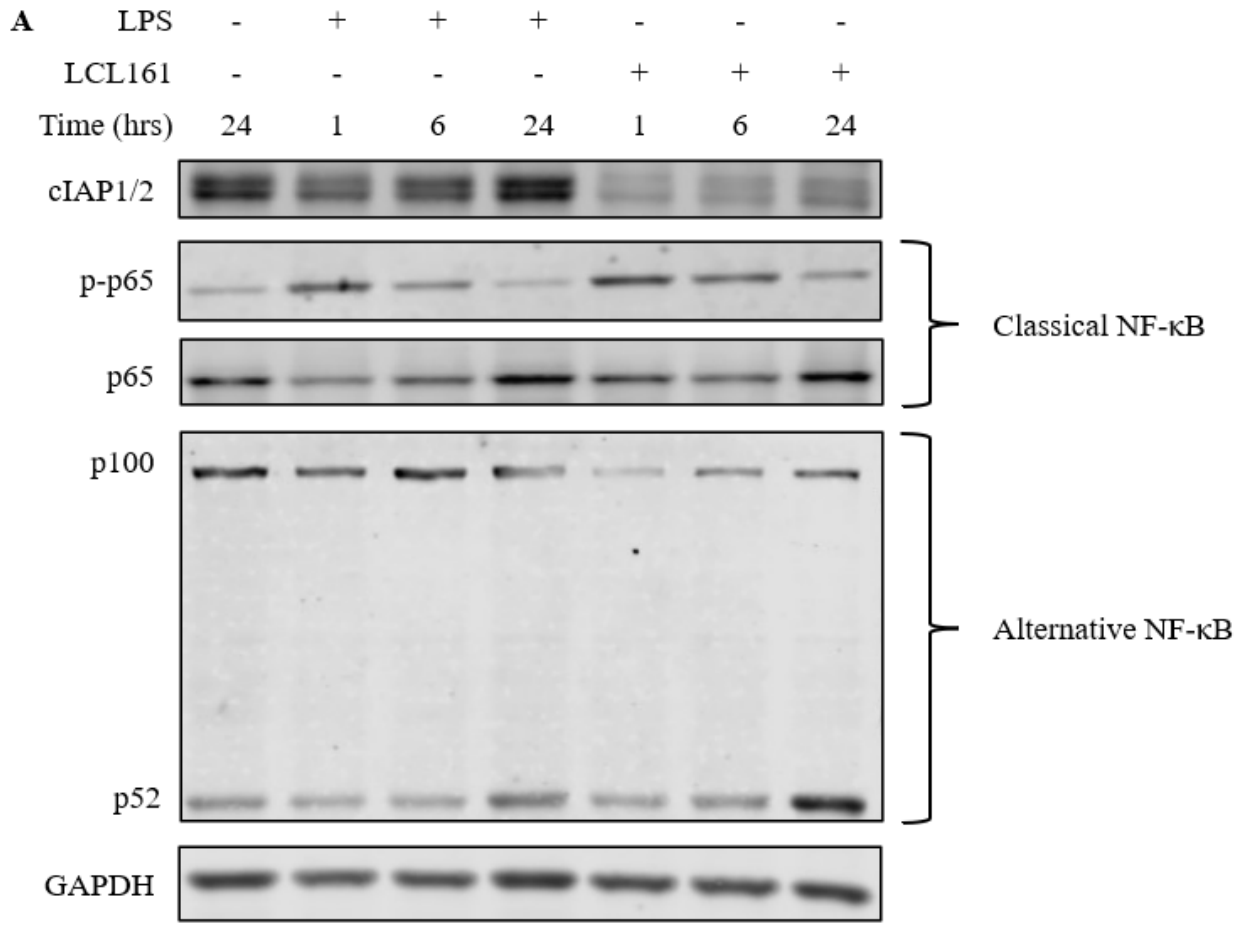
Figure 10. SMCs induced a decrease in CD11b$^+$ expression in DC subsets. BMDCs were treated with varying doses of LCL161 (1 μM and 5 μM) or vehicle for 24 hours. **(A)** Flow cytometry data with percentages of each of the 4 DC subsets generated: monocyte-derived DCs (Mo-DCs), CD8α$^+$CD11b$^-$ lymphoid DCs, CD11b$^-$CD103$^+$ migratory DCs, and CD11b$^+$CD103$^-$ migratory DCs. **(B)** The flow cytometry data represented as frequency of each DC subsets based on CD11c$^+$ gated cells. Roman numerals match to **Figure 9** for individual subset gating strategy. **(C)** Histogram representation of CD11b expression on CD11c$^+$ gated cells for all three treatments.

in cIAP1/2 expression was observed in LCL161-treated BMDCs across all three time points analysed, while a slight decrease was observed at the 1-hour time point in LPS treated BMDCs with return to normal levels by the 24-hour time point (**Figure 11A**). Phosphorylation of p65 was observed in LCL161-treated BMDCs at the 1-hour and 6-hour time point, indicating activation of the classical NF-κB pathway (**Figure 11A, B**). Phosphorylated p65 was observed in LPS treated BMDCs (**Figure 11A, B**). Furthermore, processing of p100 to p52 was observed starting at the 1-hour time point and increased at the 24-hour time point in LCL161-treated BMDCs indicating activation of the alternative NF-κB pathway (**Figure 11A, B**). Lower levels of processing of p100 to p52 was also observed in LPS-treated BMDCs at the 24-hour time point compared to LCL161-treated BMDCs (**Figure 11A, B**). These results indicate that SMCs decrease the expression of cIAP1/2 in BMDCs allowing for phosphorylation of p65 and the processing of p100 to p52 and thus activation of the classical and alternative NF-κB pathway.

3.1.3 Evaluation of SMC immunomodulatory effect on BMDCs

Adjuvants are classically used to induce maturation and activation of immune cells, particularly APCs to help the uptake, processing, and presentation of antigens. As such, if SMCs are to be used as an adjuvant, SMCs would need to stimulate maturation of DCs. To assess the ability of LCL161 to mature DCs, I evaluated the expression of surface maturation markers and secretion of cytokines.

BMDCs were treated with various doses of LCL161 (0.1 μM to 5 μM) and analysed via flow cytometry for expression of surface maturation and co-stimulatory markers: MHCII, CD80, CD86, and CD40 (**Figure 12**). LPS-treated BMDCs was used as a positive control for DC



B

LPS	-	+	+	+	-	-	-
LCL161	-	-	-	-	+	+	+
Time (hrs)	24	1	6	24	1	6	24
p-p65/p65	0.02	0.38	0.08	0.01	0.17	0.16	0.02
p52/p100	0.57	0.57	0.37	1.55	2.86	1.68	3.04

Figure 11. SMC treatment activates the NF-κB pathway in BMDCs. BMDCs were treated with LCL161 (1 μM) or LPS (100 ng/mL) and collected at three different post-treatment time points. **(A)** Expression of the indicated proteins was analyzed via Western blotting. **(B)** Ratios of p100 processing to p52 (p52/p100) and phosphorylation of p65 (p-p65/p65) by semi-quantitative analysis of band intensity.

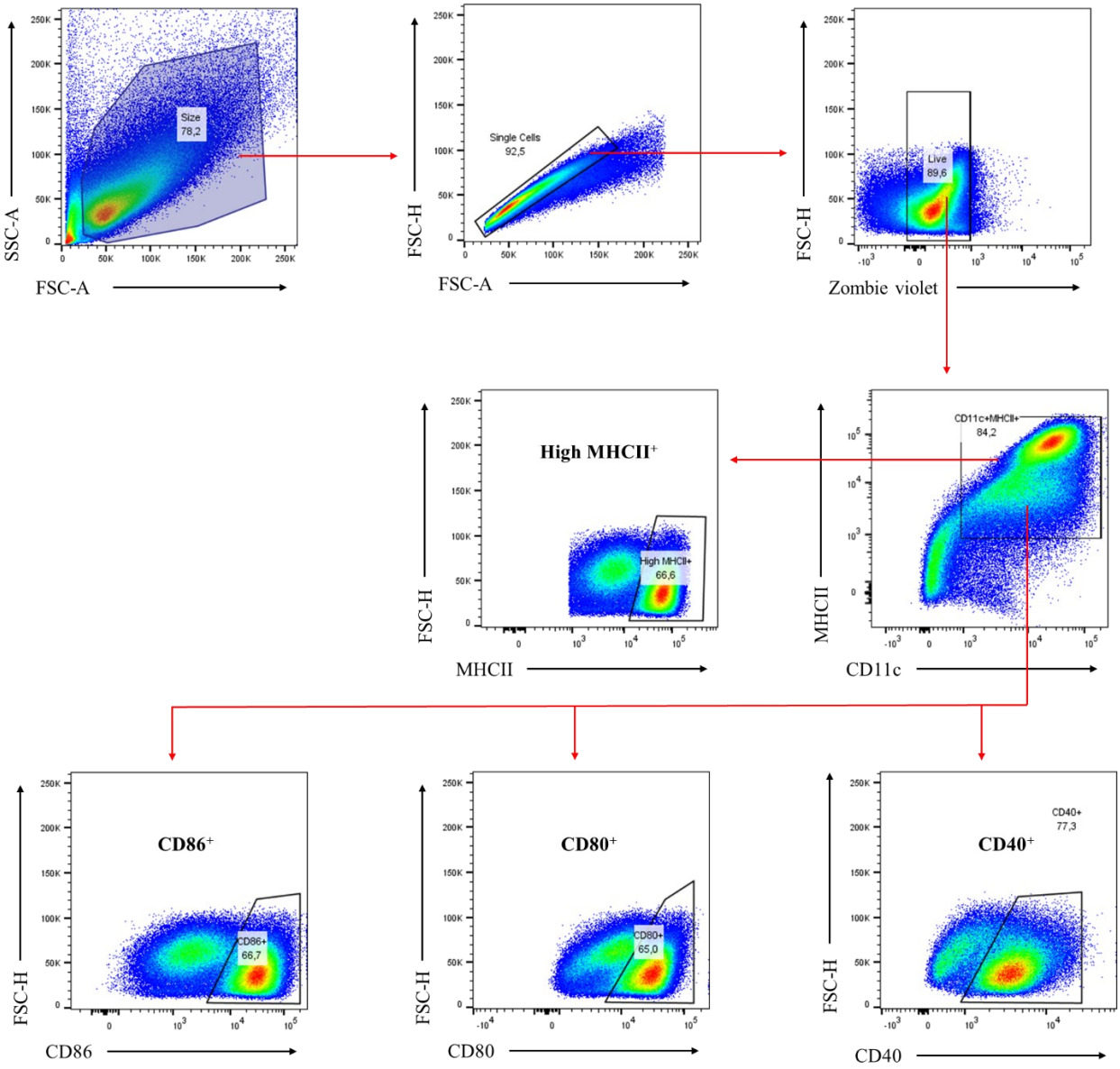


Figure 12. Sequential gating strategy to identify mature BMDCs. BMDCs were stained with the following antibodies for analysis via flow cytometry: Zombie violet (BV421), CD11c (APC), MHCII (AF700), CD86 (BV785), CD80 (AF488), and CD40 (PE). From the $CD11c^+$ and $MHCII^+$ gate, cells were gated for high MHCII expression, CD86 expression, CD80 expression and CD40 expression.

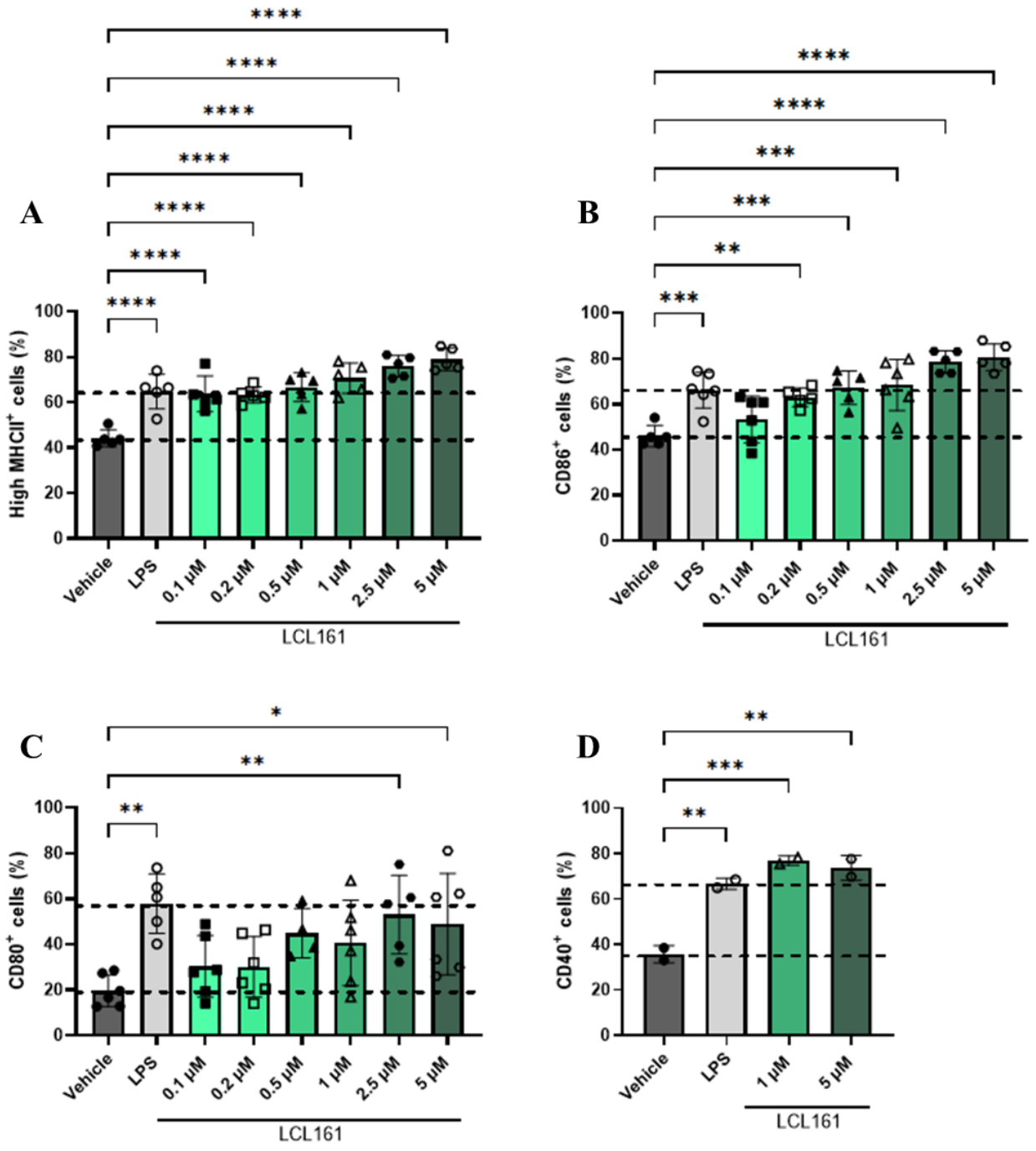


Figure 13. SMCs induces upregulation of maturation markers in BMDCs. BMDCs were treated with varying doses of LCL161 (0.1 µM to 5 µM) for 24 hours. BMDCs treated with LPS (100ng/mL) was used as a positive control. Data is represented as percent expression of various DC maturation markers from $CD11c^{+}MHCII^{+}$ for **(A)** High MHCII, **(B)** CD86, **(C)** CD80, and **(D)** CD40 (n=2-6, mean, SD). Statistical significance was calculated using a one-way ANOVA with a Dunnett multiple comparisons test, comparing vehicle control to the experimental groups. *, P<0.05, **, P <0.01, ***, P <0.001, and ****, P < 0.0001.

maturation. LCL161 induced upregulation of MHCII across all dosages whereby upregulation of CD86 was observed at doses of 0.2 μM and higher of LCL161 and upregulation of CD80 was only observed with doses of 2.5 and 5 μM (**Figure 13A to C**). I also observed increased expression of CD40 in BMDCs following LCL161 treatment (**Figure 13D**). When compared to LPS, doses of 1 μM and higher of LCL161 induced similar and higher levels of upregulation of the maturation markers, except for CD80. I also analyzed for the secretion of IL-12, IL-6, and TNF-α from SMC-treated BMDCs (**Figure 14A-C**). When looking at fold changes in expression of the cytokines, LCL161 significantly increased the secretion of IL-12 at doses of 0.5 μM, 1 μM, and 5 μM (**Figure 14D**), while all doses of LCL161 significantly increased the secretion of IL-6 (**Figure 14E**). However, only the highest dosage, 5 μM of LCL161 induced a significant increase in secretion of TNF-α (**Figure 14F**). When compared to LPS-treated BMDCs, no LCL161 treatment induced similar levels of cytokine secretion, except for IL-12. Altogether, these results indicate that LCL161 is able to induce maturation and a pro-inflammatory state in BMDCs.

3.1.4 Minimal cell death observed in BMDCs after treatment with high doses of SMC

SMCs were initially developed as cancer therapeutics with the goal to kill cancerous and infected cells[28,29,36,37]. I then evaluated whether SMCs can induce cell death in BMDCs. When normalized to the vehicle treated BMDCs, only the highest doses of LCL161 (5 μM) induced statistically significant cell death (**Figure 15**). This indicates that SMCs induces death of BMDCs at high concentrations.

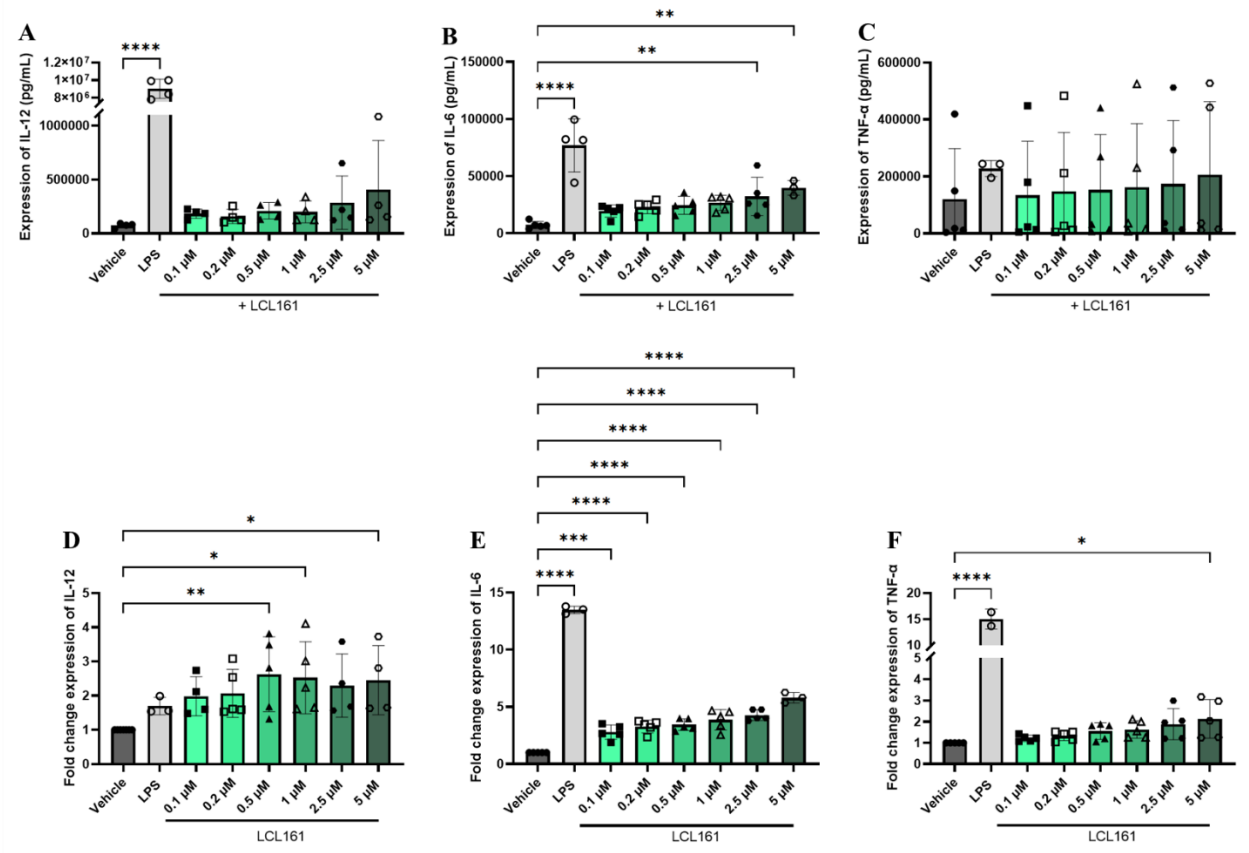


Figure 14. SMC induces the secretion of inflammatory cytokines in BMDCs. BMDCs were treated with varying concentration of LCL161 (0.1 µM to 5 µM) for 24 hours. BMDCs treated with LPS (100ng/mL) was used as a positive control. Supernatant from cultures were collected and the secretion of cytokines **(A)** IL-12, **(B)** IL-6, and **(C)** TNF-α, were measured compared to vehicle treated BMDCs via ELISA in pg/mL (n= 2-5, mean, SD). Fold change in expression of cytokines **(D)** IL-12, **(E)** IL-6, and **(F)** TNF-α was calculated as: treatment (pg/mL)/vehicle treatment (pg/mL). Statistical significance was calculated using a one-way ANOVA with a Dunnett multiple comparisons test, comparing vehicle control to the varying treatments. *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$, and ****, $P<0.0001$.

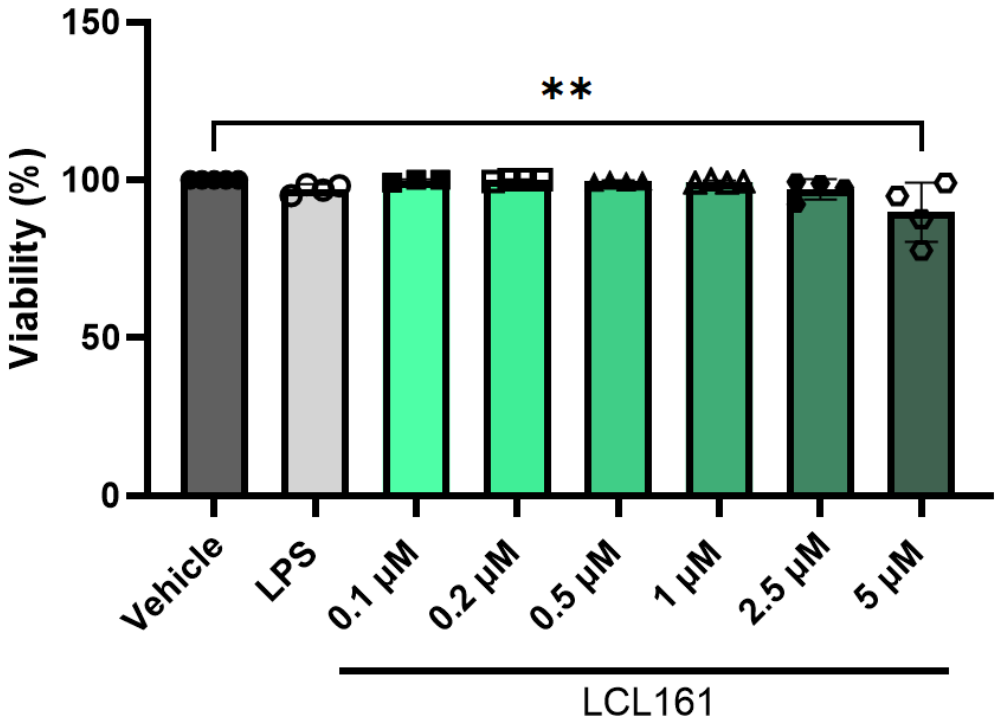


Figure 15. SMC induced minimal death of BMDCs at high concentrations. BMDCs were treated with varying doses of LCL161 (0.1 µM to 5 µM) for 24 hours. Viability of cells was analysed via live/dead staining of cells with zombie violet and normalized to vehicle treated BMDCs (n = 4, mean, SD). Statistical significance was calculated using a one-way ANOVA with a Dunnett multiple comparisons test, comparing vehicle control to the varying treatments. *, P<0.05, **, P <0.01, ***, P <0.001, and ****, P < 0.0001.

3.2 Aim 2: Evaluate the Effects of SMCs on BMDC Antigen Presentation

3.2.1 Experimental model

SMCs have been demonstrated to induce activation, proliferation, and cytokine production of T cells when combined with a TCR stimulant[49,50]. However, if SMCs are to be used in vaccine adjuvants, they will not interact with T cells directly, but through DCs. Therefore, I sought to evaluate SMCs effect on BMDCs antigen presentation. To accomplished this, I used a model commonly used to evaluate immune responses, the OT-I/ovalbumin (OVA) model. OT-I mice have a transgenic TCR that recognizes a singular peptide sequence of chicken ovalbumin (aa257-264)[68]. Since the OT-I TCR is MHC Class I restricted, this mouse model is ideal to study $CD8^+$ T cell responses[68].

3.2.2 Verification of OT-I mice

Verification of OT-I mice was accomplished via flow cytometry analysis using a fluorophore conjugated MHC tetramer that recognises $OVA_{257-264}$ (SIINFEKL) specific T cells. Whole blood and splenocytes were collected from OT-I mice and the cells were stained with the following flow antibodies: zombie violet (BV421), CD3 (BV785), CD8α (FITC), and SIINFEKL MHC tetramer (PE). On $CD3^+$ gated cells, 95% of both whole blood and splenocytes were observed to be SIINFEKL specific $CD8^+$ T cells (**Figure 16A**).

3.2.3 SMCs likely induce the activation of antigen specific $CD8^+$ T cells

To partially evaluate the effects of SMCs treatment on antigen presentation, BMDCs were treated with LCL161 24 hour prior and $OVA_{257-264}$ peptide 2 hours prior to the addition of OT-I T

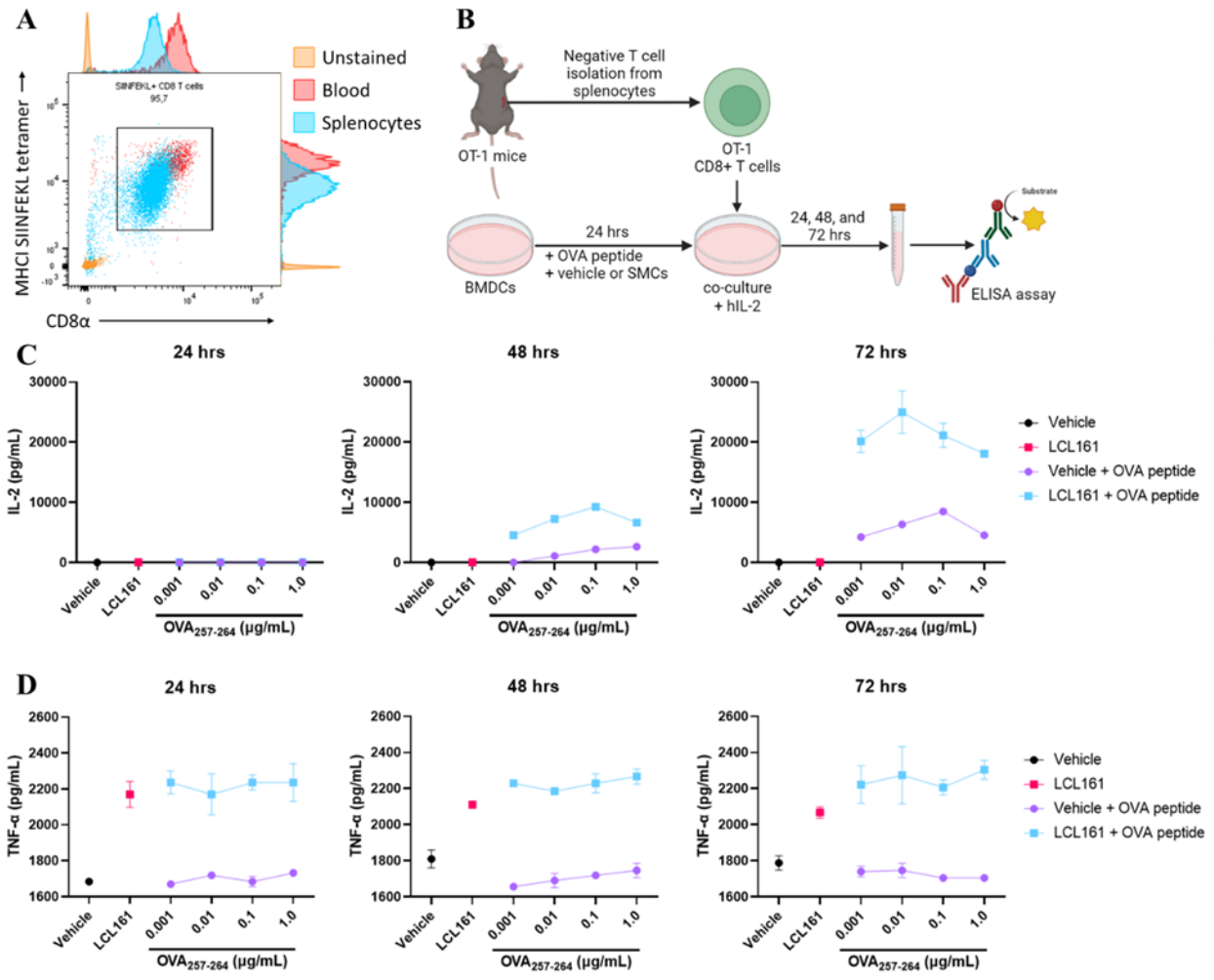


Figure 16. Antigen-specific T cells co-cultured with SMC-treated BMDCs leads to increased secretion of IL-2. (A) Verification of $OVA_{257-264}$ specific TCR from blood cells and splenocytes derived from OT-I mice. **(B)** Schematic of the co-culture experiment. BMDCs were treated with LCL161 (1 μM) for 24 hours and stimulated with various doses of $OVA_{257-264}$ peptide (0.001 to 1 μg/mL) 2 hours prior to the addition of OT-I cells. The supernatant from the co-cultures were collected at three different time point to evaluate for secretion of **(C)** IL-2 and **(D)** TNF-α (technical replicate n = 2, mean, SD).

cells (**Figure 16B**). Antigen presentation in these co-cultures was indirectly analysed via the secretion of IL-2, TNF-α, and IFN-γ. Regardless of treatment I observed no secretion of IFN-γ (data not shown). On the other hand, I observed an increase of IL-2 secretion in co-cultures with LCL161- and peptide-treated BMDCs at the 48- and 72-hour time points (**Figure 16C**).

Co-cultures in which BMDCs were treated with vehicle or LCL161 but were not pulsed with $OVA_{257-264}$ peptide induced no secretion of IL-2 (**Figure 16C**). Co-cultures of T cells with LCL161-treated BMDCs regardless of being pulsed with $OVA_{257-264}$ peptide induced an increase in secretion of TNF-α (**Figure 16D**). These results indicate that SMCs have the potential to induce antigen specific activation of T cells with the caveat that the evaluated cytokine expression of T cells from the co-culture is limited and this is only a partial analysis of T cell activation and antigen presentation.

3.3 Aim 3: Investigate the Ability of SMCs to Function as an Adjuvant

I next evaluated the ability of SMCs to function as an adjuvant for cancer vaccines, particularly for Human papilloma virus (HPV) induced cervical cancers. The expression of the HPV E7 oncoprotein in HPV-infected people leads to development of cancer[69]. The TC-1 cancer cell line is a mouse cell line that expresses the E6 and E7 oncoproteins[70–72]. The E6 oncoprotein inhibits p53, while E7 targets retinoblastoma protein (pRb) for ubiquitination and degradation[69,70]. The activation of these oncogenes in human HPV infections leads to uncontrolled cell proliferation due to the deregulation of growth suppressors[73]. Since cancers have variable and patient specific expression of tumor antigens, the TC-1 cancer model utilizes a predefined shared antigen[74]. Since there is a known target and using an immunodominant E7 peptide sequence, this model can be used to test vaccine responses.

3.3.1 SMCs as an adjuvant does not curtail TC-1 tumour growth.

I first confirmed that the TC-1 cell line expressed the E7 oncoprotein and MHC I (**Figure 17**). With this confirmation, I moved onto prophylactic tumour protection experiments with the TC-1 cell line. Before evaluating SMCs for its ability to serve as functional cancer vaccine adjuvant, I optimized the number of cells to be implanted into mice to obtain consistent tumour growth. There was little difference in survival across the three TC-1 tumour cell quantities evaluated (**Figure 18A**). However, of the three quantities, implantation of $5x10^4$ TC-1 cells resulted in consistent tumour growth across three biological replicates, consistent with a similar study by Gendron et al. (2006)[64] (**Figure 18A**).

I used two different treatment regimens to evaluate the ability of SMCs to function as a vaccine adjuvant against E7-expressing tumor cells (**Figure 18B**). The first regimen was modelled as in Gendron et al (2006)[64] whereby a booster dose was administered 7 days post primary dose. In the second regimen, the booster dose was administered 14 days post primary dose. Mice were vaccinated with the E7 peptide and TC-1 cells were injected 7 days post booster. The CpG ODN 1826 was used as a positive control[64].

In the first dosing regimen, the E7 peptide and CpG vaccinated mice reached endpoint due to tumour burden; however, their tumour growth rate was relatively slower than all other treatments (**Figure 18C**). For the second dosing regimen, I observed no protection against establishment of tumors and no difference in overall tumor growth regardless of treatment (**Figure 18D**). However, depending on the dosing regimen, certain treatments had slower tumour growth (**Figure 18E**). For example, mice treated with only E7 peptide had a slower tumour growth rate with the two-week dosing regimen, while mice treated with E7 peptide and CpG had a slower

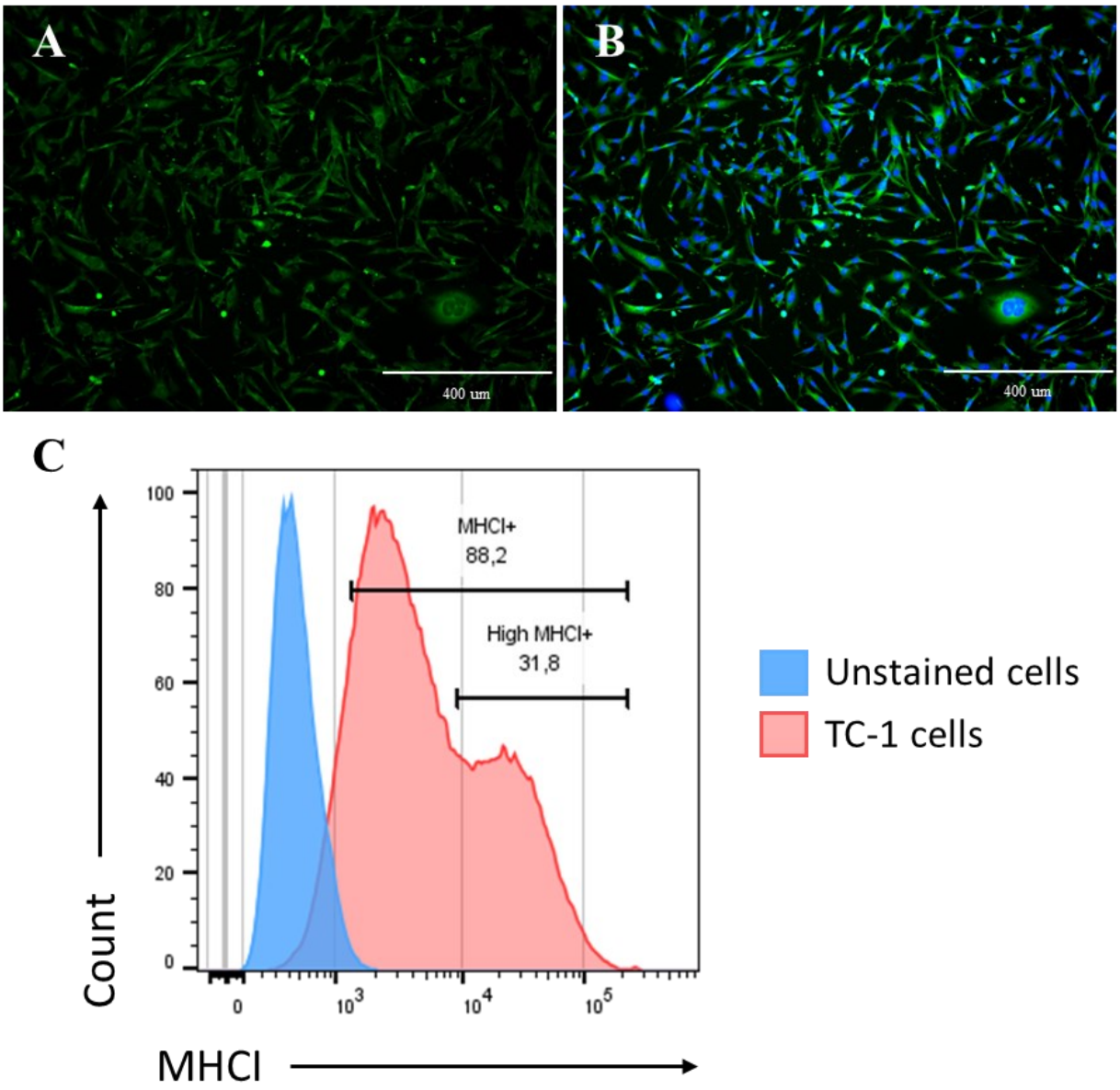


Figure 17. The TC-1 cell line expresses E7 and MHCI. Fluorescent microscopy analysis on TC-1 tumour cells. E7 expression was confirmed on TC-1 cells using anti-E7 monoclonal antibody. The micrograph shows the **(A)** E7 protein coloured in green by AF488 anti-mouse IgG staining and the **(B)** nuclei coloured in blue by DAPI staining. **(C)** Confirmation of MHCI expression on TC-1 cells. TC-1 cells were stained with the following antibodies for analysis via flow cytometry: zombie violet (BV421) and MHCI (PE).

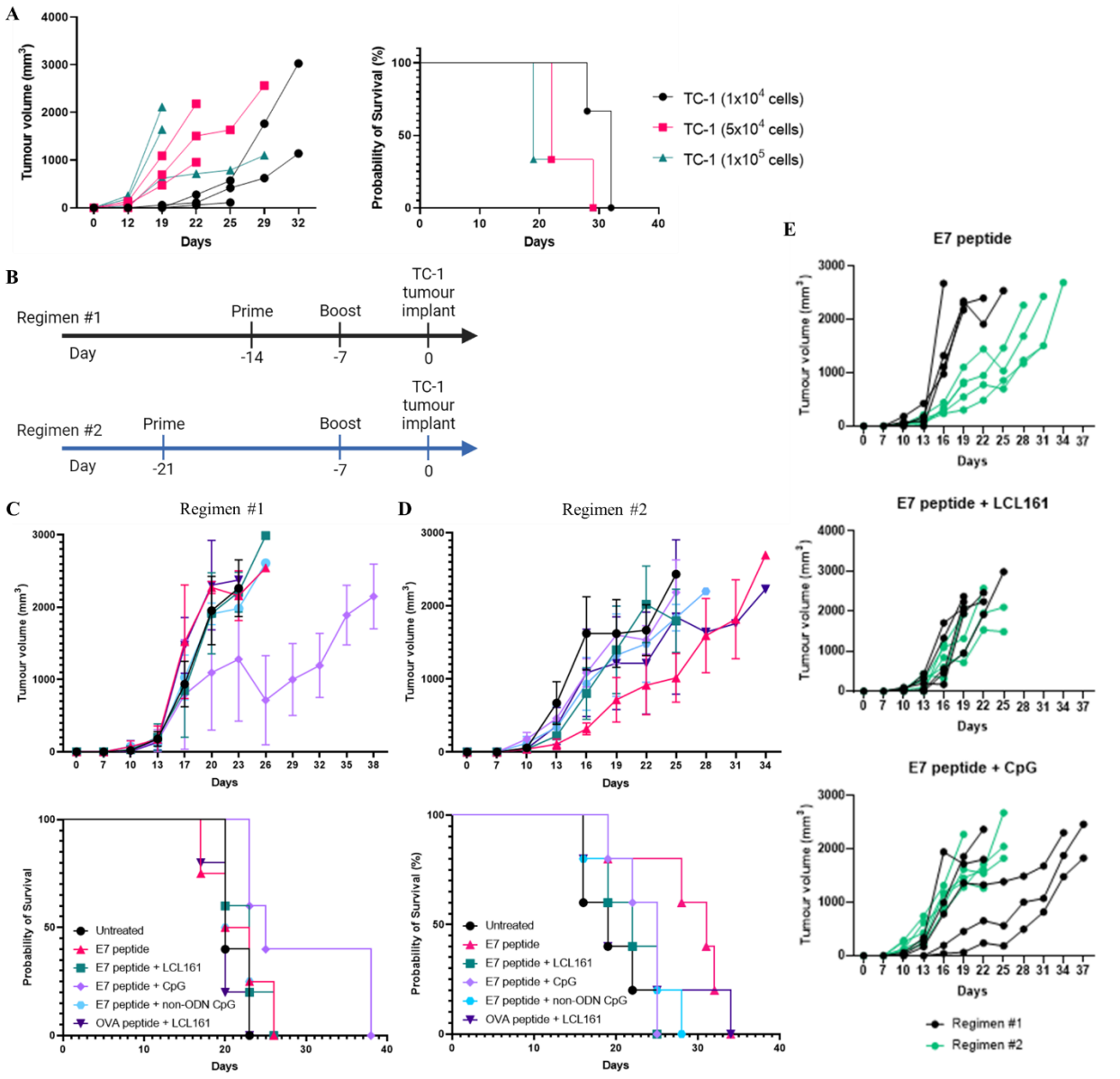


Figure 18. Mice vaccinated with SMC as an adjuvant does not curtail TC-1 tumour growth. TC-1 cells were implanted subcutaneously into the left flank of C57BL/6 female mice at three different cell numbers: $1x10^4$, $5x10^4$, or $1x10^5$. Tumour growth was measured every 3 days via calipers. Kaplan-Meier curve depicting mouse survival and tumour growth curve for each TC-1 cell quantity (n = 3). **(B)** Schematic of two vaccine regimens tested. Mice were vaccinated twice with combinations of E7 or $OVA_{257-264}$ peptide (100 µg) via i.p. injection, LCL161 (75 mg/kg) via oral gavage or vehicle. CpG ODN 1826 and non-ODN CpG (20 µg, i.p.) served as positive and negative controls. Prime refers to the first vaccine dose and boost refers to the second vaccine. Seven days after the booster dose $5x10^4$ TC-1 tumour cells were implanted subcutaneously. **(C, D)** Tumour growth curves and Kaplan-Meier curves depicting mouse survival for both treatment regimens (n = 5, mean, SD). **(E)** Comparison of three vaccine treatments as measured by tumour growth.

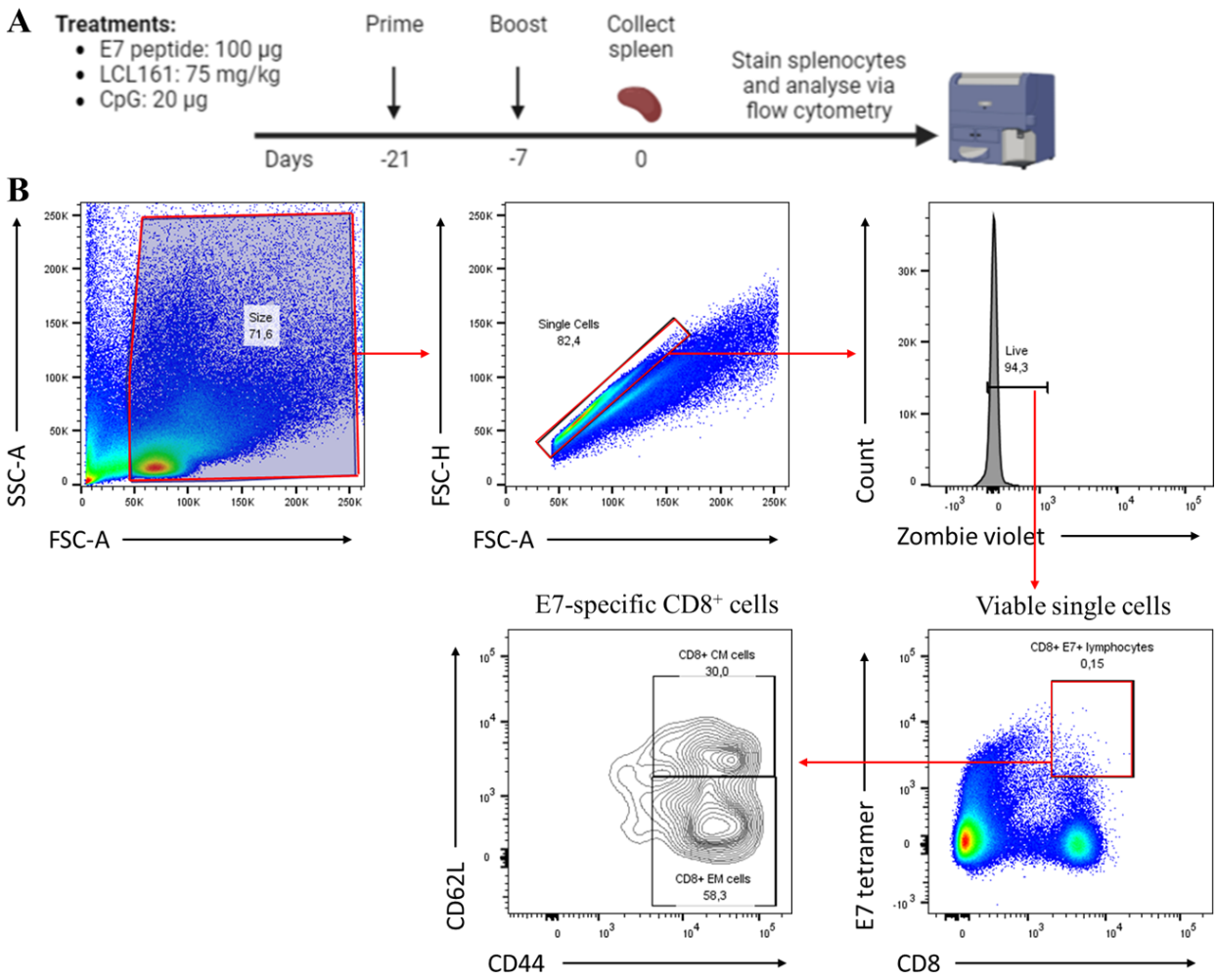
tumour growth rate with the one-week dosing regimen (**Figure 18E**). These results indicate that the SMCs do not induce a protective response against E7-expressing tumor cells.

3.3.2 SMC as an adjuvant does not induce an antigen specific $CD8^+$ T cell response.

Since SMCs as an adjuvant in a peptide vaccine failed to curtail tumour growth, I sought to evaluate the presence of an antigen specific $CD8^+$ T cell response. This experiment was modeled after a similar experiment run by Gendron et al (2006)[64] that demonstrated a significant increase in expression of antigen specific effector $CD8^+$ T cells of mice vaccinated with E7 peptide and ODN CpG, which I used as a positive control. Female C57BL/6 mice were vaccinated in the same fashion of Treatment Regimen #2 (**Figure 19A**). At 7 days post boost, the spleens of mice were collected and stained for analysis via flow cytometry (**Figure 19B**). I analyzed for E7 specific expression in bulk $CD8^+$ T cells. Mice vaccinated with E7 peptide and CpG as well as mice vaccinated with E7 peptide and LCL161 demonstrated a decrease in E7 specific $CD8^+$ T cells compared to naïve mice (**Figure 19C**). On the other hand, mice vaccinated with only the E7 peptide showed no difference in E7 specific $CD8^+$ T cells expression compared to naïve mice (**Figure 19C**). As such, vaccination with LCL161 as an adjuvant does not induce an antigen specific $CD8^+$ T cell response.

3.3.3 SMC as an adjuvant does not change the memory phenotype of antigen specific $CD8^+$ T cells.

One of the important steps in vaccination is to induce a memory response to protect against the target antigen[1,3,4]. Therefore, I also analyzed $CD8^+$ T cell memory responses. Memory


A
Treatments:
E7 peptide: 100 µg
LCL161: 75 mg/kg
CpG: 20 µg
Prime
Boost
Collect spleen
Stain splenocytes and analyse via flow cytometry
Days
-21
-7
0
B
SSC-A
FSC-A
Size
71,6
FSC-H
FSC-A
Single Cells
82,4
Count
Zombie violet
Live
94,3
E7-specific CD8⁺ cells
Viable single cells
CD62L
CD44
CD8+ CM cells
30,0
CD8+ EM cells
58,3
E7 tetramer
CD8
CD8+ E7+ lymphocytes
0,15

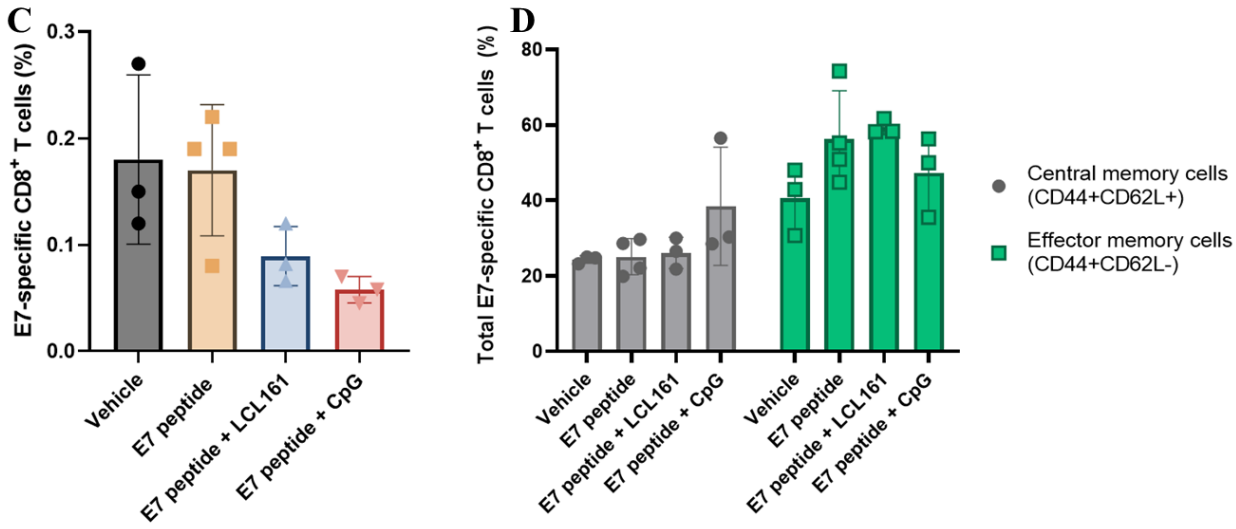

C
E7-specific CD8⁺ T cells (%)
Vehicle
E7 peptide
E7 peptide + LCL161
E7 peptide + CpG
D
Total E7-specific CD8⁺ T cells (%)
Central memory cells (CD44+CD62L+)
Effector memory cells (CD44+CD62L-)

Figure 19. Vaccination with SMC as an adjuvant does not induce enhanced $CD8^+$ T cell response. (A) Schematic of vaccine regimen for $CD8^+$ T cell analysis. On days −21 and −7, mice were vaccinated with the E7 peptide (100 μg) via i.p. injection and LCL161 (75 mg/kg) via oral gavage (p.o.) or control (E7 peptide alone or E7 peptide and ODN CpG (20 μg)). On day 0, the spleens of mice were collected and stained for analysis via flow cytometry. **(B)** Sequential gating strategy to identify in vivo T cell response to SMC as an adjuvant. The following flow cytometry antibodies were used for analysis: zombie violet (BV421), CD8 (FITC), CD62L (BV785), and $E7/D^b$ tetramer (PE). Antigen-specific T cells were defined as $CD8^+$ $E7/D^b$ $tetramer^+$, central memory (CM) T cells were defined as $CD8^+$ $E7/D^b$ $tetramer^+CD44^+CD62L^+$, and effector memory (EM) T cells were defined as $CD8^+$ $E7/D^b$ $tetramer^+CD44^+CD62L^-$. **(C)** Flow cytometry analysis for E7-specific $CD8^+$ T cells (n =3-4, mean, SD). **(D)** Flow cytometry analysis of central memory $CD8^+$ T cells and effector memory $CD8^+$ T cells (n =3-4, mean, SD). Statistical significance was calculated using a one-way ANOVA with a Dunnett multiple comparisons test, comparing vehicle control to the varying treatments. *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$, and ****, $P < 0.0001$.

responses were analysed via flow cytometry based on E7-specific $CD8^+$ gated T cells whereby $CD44^+CD62L^+CD8^+$ T cells correspond to central memory (CM) T cells and $CD44^+CD62L^-CD8^+$ T cells correspond to effector memory (EM) T cells (**Figure 19B**). All mice regardless of treatment demonstrated a predominantly effector memory response (**Figure 19D**). As such, vaccination with LCL161 as an adjuvant does not alter the memory phenotype of antigen specific $CD8^+$ T cells.

CHAPTER FOUR: DISCUSSION

Smac mimetic compounds (SMCs) are a relatively well studied class of drugs that have been clinically tested in the context of cancer therapeutics[28,29,36,37,39–42]. Although many studies highlight their immunomodulatory properties[29,33,35,42,43], more insight is required in order to exploit the potential of this class of drugs as immunotherapeutic compounds. In my thesis, I have investigated the immunomodulatory properties of SMCs in dendritic cells (DCs) and examined their potential as adjuvant vaccine therapeutics.

4.1 SMC Effect on BMDCs *In Vitro*.

4.1.1 SMC induced a downregulation of $CD11b^+$ expression in BMDCs.

Dendritic cell (DC) subsets play an important role in the inflammatory immune response[10,75]. DCs have some variety in phenotype and location, and consequently also express different activation patterns, resulting in varied interactions with other immune cells and in differences in the type of cytokines/chemokines secreted[10,75].

Throughout my thesis, I used bone marrow derived dendritic cells (BMDCs) that were cultured and differentiated using a particular 6-day protocol with GM-CSF[61] (**Figure 8**). I then sought to determine which DC subsets were generated and how these cells responded when treated with SMCs. Previous studies have shown that $CD11b^+$ DC subsets were predominantly obtained when bone marrow cells were cultured with GM-CSF[65]. I was able to validate this result and confirm the presence of four DC subsets: monocyte-derived DCs (Mo-DCs), $CD8\alpha^+CD11b^-$ lymphoid DCs, $CD11b^+CD103^-$ migratory DCs, and $CD11b^-CD103^+$ migratory DCs (**Figure 10A**). When the BMDCs were treated with SMCs a decrease in two of the four DC subsets was observed (**Figure 10A**). Remarkably, the only observed difference between the DC subsets that decreased with SMC treatment compared to those that did not change was $CD11b^+$ expression.

Thus, when evaluating CD11b expression on $CD11c^+$ gated cells, a decrease in $CD11b^+$ expression was observed in only BMDCs treated with LCL161 (**Figure 11C**). However, when looking at the overall DC population using the common DC markers ($CD11c^+MHCII^+$) LCL161-treated BMDCs demonstrated no change in population size when compared to vehicle-treated BMDCs. This raises the question as to how LCL161 is affecting these cells such that a decrease in CD11b expression is occurring, but the overall cell type numbers are still observed at comparable levels. Since CD11b plays a role in trafficking of DCs to peripheral lymphoid organs by binding to ICAM and rolling across endothelial cells[76], this downregulation in CD11b expression could affect DCs ability to mature and complete its function. SMC treatment could thus prevent matured DCs from reaching lymph nodes and presenting antigens to T cells. However, there are other subsets of DCs that do not express CD11b and are still able to traffic to peripheral lymphoid organs[12,65]. These results further highlight the need to determine the precise mechanism(s) by which SMCs act on DCs and other immune cells.

4.1.2 Activation of alternative NF-κB signalling pathway.

Previous studies have shown that, in some immune cells, SMCs induce the degradation of IAPs resulting in NIK accumulation and subsequent activation of the alternative NF-κB pathway [1,11,15,16] (**Figure 6**). I confirmed that over a period of 24 hours, 1 μM of LCL161 induced the degradation of cIAP1 and 2, and the eventual activation of the alternative NF-κB pathway via the processing of p100 to p52 (**Figure 6, Figure 11**). The 1 μM dose of LCL161 is low compared to that typically used for cancer therapeutic purposes[77]. However, as my purpose was to observe maturation of DCs, I believe the lower dose was optimal. This lower dose induced a partial degradation of cIAP1 and 2 and not a complete loss of these molecules (**Figure 11**). Interestingly,

the classical NF-κB pathway was activated at the early time points, as indicated by the phosphorylation of p65 (**Figure 11**). This result is consistent with previous findings in immune cells demonstrating that the classical pathway can be activated early in response to SMC treatment and that the activation of the alternative pathway activation is a later event[25]. Indeed, by the 24-hour time point there was strong and clear activation of the alternative NF-κB pathway by LCL161 in BMDCs as indicated by the processing of p100 to p52 (**Figure 11**).

4.1.3 Maturation of BMDCs via upregulation of surface maturation markers.

One of the signs of maturation in DCs is the upregulation of MHC and particular co-stimulatory molecules, which is necessary for downstream activation of T cells (signal 1 and 2)[1,9,10,14,75,78] (**Figure 2**). Therefore, I sought to determine if treatment of BMDCs with LCL161 would cause an upregulation of MHCII, CD80, CD86, and CD40, thus indicating maturation. Treatment of BMDCs with LCL161 induced upregulation of both MHCII and CD86 across all dosages (**Figure 13A, B**). Upregulation of CD80 was observed in BMDCs treated with LCL161 doses of 2.5 μM and higher (**Figure 13C**). Treatment of BMDCs with LCL161 also induced upregulation of CD40 across the two doses tested (**Figure 13D**). The upregulation of MHCII and CD86 indicates that LCL161 induces proper maturation of BMDCs at low concentrations. SMCs upregulate expression of MHCII on DCs, which is necessary to form complexes with the TCR on T cells (signal 1). In addition, SMCs upregulate expression of co-stimulatory molecules, a prerequisite for T-cell activation (signal 2). This indicates that SMC treatment can be potentially used as an adjuvant in vaccines as it accomplishes two of the three signals required for T cell activation (**Figure 2**).

Initial studies regarding the role of CD40 revolved primarily around its expression on B cells and subsequent increase in B cell proliferation and survival[75,78]. However, CD40 has now been found to be expressed on other immune cells as well as non-immune cells[75,78]. Ligation of CD40 on DCs has been shown to increase secretion of inflammatory cytokines and chemokines, as well as upregulating MHC and CD80/86[1,10,14,75,78]. It has been suggested that activated DCs are able to interact with each other to regulate their functions through the CD40L-CD40 signalling pathway[75,79]. CD40 is one member of the TNF receptor superfamily (TNFRSF). Maturation of DCs by SMC treatment may be due to the activation of the CD40L-CD40 signalling pathway, which could be sustained through activated DCs interacting with each other. To confirm this, CD40L surface expression on DCs and T cells should be examined further.

T cell regulation is controlled by two receptors, CD28 and CD152; in which CD28 stimulates T cells and CD152 inhibits T cells. These two receptors share two ligands, CD80 and CD86, which perform similar functions and are expressed on the same cell types. However, for all the similarities between CD80 and CD86, there has not been any study that determines whether the ligands promote or inhibit T cell regulation. Therefore, in most studies these ligands are used interchangeably[80,81]. Despite their similarities, CD80 forms a bivalent dimer with two binding sites, while CD86 is a monomer with a single binding site[82]. Scientists have thus stipulated that a delay in CD80 upregulation, in contrast to CD86, arises since CD86 binding affinity leans towards CD28, which is a monovalent dimer [80,82–84]. It has been proposed that CD80 plays a role in immune tolerance and would thus be downregulated upon immune stimuli, while CD86 would be upregulated[80]. However, in my thesis, CD80 upregulation is observed at higher concentrations of immune stimulus, with LCL161 (**Figure 13C**). Could a specific immune stimulation threshold dictate CD80 upregulation? I suggest that the concentration and nature of immune stimuli might

influence CD80 upregulation. However, my thesis examined these ligands at a single time point, leaving open the possibility of a time delay in CD80 upregulation due to lower binding affinity to CD28 compared to CD86.

4.1.4 Activation of pro-inflammatory state in BMDCs.

IL-12 is considered a pro-inflammatory cytokine as it primarily functions to induce IFN-γ production from natural killer (NK) cells and cytotoxic T cells. As well, IL-12 will promote the differentiation of naïve T cells into Th1 effector cells[85]. IL-12 is produced by activated monocytes, macrophages, DCs, and B cells[85]. Accordingly, I evaluated the dosage effect of LCL161 on BMDCs ability to produce IL-12 and found that doses of 0.5 μM, 1 μM, and 5 μM caused significant increase in secretion of IL-12 from BMDC's compared to the vehicle treated control BMDCs (**Figure 14D**). Considerable variety was observed in each treatment group, as indicated by the large error bars; however, there seemed to be a correlation demonstrating that a dosage increase of LCL161 induced an increase in IL-12 production from the BMDCs. This indicates that SMC treatment is able to induce a pro-infammatory state of activation in DCs, which could therefore further activate T cells.

Unlike IL-12, IL-6 is considered a pleiotropic cytokine, meaning that it possesses both pro-inflammatory and anti-inflammatory properties[10]. However, when IL-6 is produced by DCs, it tends to mediate crosstalk between DC-T cells and DC-B cells, and also regulate the differentiation of Th2 and Th17 cells[10]. Furthermore, IL-6 can act in an autocrine fashion on DCs to maintain an immature and semi-mature cell state[10]. As mentioned earlier, three distinct signals are required for T cell activation (**Figure 2**). Briefly, the first signal required for T cell activation is presentation of an antigen by an MHC molecule to the T cell receptor (TCR) and the second signal consists of co-

stimulatory signals[1–3] (**Figure 2**). The third signal involves the production of cytokines by DCs whose expression levels depend largely on the type of stimulation received by the DCs[1,10]. Therefore, I sought to determine the dosage effect of LCL161 on DC cytokine production. All doses of the SMC tested on BMDCs (0.1 – 5 μM) induced a significant increase in secretion of IL-6 compared to vehicle-treated BMDCs (**Figure 14E**). Similar to that found with IL-12 expression, an increase in LCL161 dose induced a corresponding increase in IL-6 production from BMDCs. In addition to the results pertaining to IL-12 secretion (**Figure 14D**), these results indicate that the LCL161-treated BMDCs secrete IL-6 to induce a pro-infammatory immune response. Therefore, SMCs can act to stimulate the immune response of DCs.

Although TNF-α is considered to be a pleiotropic cytokine, it is more commonly known for its pro-inflammatory properties, which promote survival and proliferation[86]. TNF-α is secreted downstream of NF-κB activation, and as I demonstrated earlier, SMC-treated BMDCs induce latent activation of alternative NF-κB signalling (**Figure 11**). Theoretically, this indicates that SMC treatment of BMDCs should induce secretion of TNF-α. Only the highest dose of LCL161 (5 μM) induced a significant increase in secretion of TNF-α when compared to vehicle-treated BMDCs (**Figure 14F**). When assessing activation of the alternative NF-κB pathway by LCL161 treatment, only a 1 μM dose was used (**Figure 11**). However, no increase in TNF-α secretion was observed for that dosage (**Figure 14F**). It is possible that since lower dosages of LCL161 only induce a partial degradation of the cIAPs, this could result in little to no secretion of TNF-α, compared to that found with higher doses of LCL161. Alternatively, the maturation and immune response induced by SMCs on DCs may not induce the secretion of TNF-α to levels comparable to that found with other pro-inflammatory cytokines and chemokines.

Based on my analyses, I propose that SMCs stimulate DCs to secrete cytokines resulting in a significant inflammatory immune response. However, the cytokines that I evaluated were limited, and as such, further studies should be expanded upon to validate this conclusion. In addition, a more comprehensive analysis of cytokine expression could possibly indicate whether SMC treatment would lead to a Th1 or a Th2 $CD4^+$ T cell response.

4.1.5 Dosage of SMCs decides fate of BMDCs.

When viability of BMDCs was assessed, the highest dose of LCL161 (5 μM) induced minimal cell death that was of low statistical significance (**Figure 15**). Interestingly, the 5 μM dose of LCL161 was the only one to induce a statistically significant increase in secretion of TNF-α (**Figure 14F**). SMC treatment is thus a double edged sword. On the one hand, when acting downstream of TNFRSFs that possess death domains, such as TNFR1, SMCs induce TNF-α mediated cell death (**Figure 6**). On the other hand, when very little TNF-α is present, SMCs act on TNFRSFs without death domains to activate the alternative NF-κB pathway and stimulate immune cells. Notably, even though statistically significant cell death is observed at 5 μM of LCL161, this dose should be considered to be a result of non-specific toxicity.

4.2 SMCs Effect on Antigen Presentation of BMDCs.

4.2.1 SMC treated BMDCs likely lead to antigen specific activation of $CD8^+$ T cells.

The direct effect of SMC exposure on T cells has been well studied[49,50,58,59]. However, SMCs are likely to alter the function of antigen presenting cells (APCs), an avenue of research that needs to be further studied. As such, I sought to evaluate the effect of SMCs on antigen presentation

by BMDCs. I treated BMDCs with LCL161 and various doses of $OVA_{257-264}$ peptide before co-culturing these BMDCs with OT-I T cells and then analysed for secretion of T cell cytokines.

I have shown that SMCs induce the maturation and generation of a pro-inflammatory state in BMDCs (**Figure 13, Figure 14**). There was an increase of IL-2 secretion in co-cultures of OT-I cells with BMDCs that were treated with LCL161 and $OVA_{257-264}$ peptide (**Figure 16C**). Furthermore, IL-2 secretion was not detected in co-cultures in which BMDCs were treated with only LCL161, while a basal level of IL-2 secretion was observed in co-cultures in whereby BMDCs were only pulsed with $OVA_{257-264}$ peptide (**Figure 16C**). These results indicate that IL-2 expression is most likely coming from antigen-specific OT-I T cells, and not BMDCs themselves. Activated T cells will secrete cytokines, such as IL-2, TNF-α, and IFN-γ, that are essential for their survival, growth, and proliferation[15]. Thus, BMDCs treated with LCL161 and $OVA_{257-264}$ peptide are likely activating antigen specific T cells. These results highlight the potential for SMC stimulated DCs to activate T cells and mount an adaptive immune response.

I have shown that SMC-treated BMDCs secrete low levels of TNF-α (**Figure 14F**). In a co-culture setting with T cells and BMDCs, TNF-α was secreted regardless of treatment (**Figure 16D**). However, there was a slight increase of TNF-α secretion in all SMC-treated BMDC co-cultures at all three evaluated time points (**Figure 16D**). Future experiments would need to be conducted to determine whether T cells or BMDCs are the source of TNF-α secretion.

Another cytokine commonly associated with T cell effector function that was evaluated is IFN-γ[87]. IFN-γ is produced by activated T cells and is necessary for the generation of effector and memory T cell populations[87–89]. When analysed, none of the treatments induced secretion of IFN-γ. Lack of INF-γ secretion indicates the possibility of non-functional activated $CD8^+$ T cells.

These results indicate that the stimulation of BMDCs by SMC treatment likely led to the antigen specific activation of T cells. However, it is important to note that the assessed cytokine expression in the co-culture is limited, providing only partial insight into antigen presentation and T cell activation. Nonetheless, these results further highlight the potential of SMCs to act as immunomodulatory adjuvants in vaccine therapies. Future work should involve the further evaluation of the T cell response by BMDCs that are stimulated by SMC treatment (i.e., T cell proliferation and expression of T cell activation and functional markers). Lastly, my results are limited to only that found for $CD8^+$ T cells and should thus also be evaluated in $CD4^+$ T cells to see if a similar response will be observed.

4.3 Effect of SMC as an Adjuvant in a Tumour Model.

4.3.1 SMC treatment fails to curtail tumour growth.

To assess the potential of SMCs as adjuvants, I used an HPV tumor model with a known antigenic target, E7. When mice were vaccinated with one week between the primary and booster doses, only mice treated with peptide and CpG demonstrated slower tumour growth rate and prolonged survival (**Figure 17C**). On the other hand, when using a schedule that involved two weeks between the primary and booster doses, none of the mice from all treatment cohorts showed no difference in tumour growth or survival (**Figure 18D**). However, the different treatment regimens seemed to influence differential tumour growth rates (**Figure 18E**). Mice treated with only the peptide demonstrated slower tumour growth for the regimen #2, while mice treated with peptide and CpG demonstrated slower tumour growth for the regimen #1 (**Figure 18B, E**). Either regimen did not have an effect on mice treated with peptide and LCL161 (**Figure 18E**). This is

surprising as LCL161 stimulated BMDCs pulsed with a peptide is likely inducing activation and proliferation of T cells (**Figure 16C**).

There are two potential reasons why I did not see a protective effect. LCL161 is administered orally to mice, however, the E7 peptide in this study was administered via i.p. injections. Since these two components were administered simultaneously, this highlights the first limitation/pitfall of this experiment. Since LCL161 is administered orally it must first pass through the stomach before being able to diffuse across the circulatory system. Therefore, in these evaluated treatment regimens, it is probable that LCL161 was not systematically present alongside the E7 peptide, thus limiting the induction of an E7-specific immune response. A second reason could be the use of peptides for vaccination. Dougan et al. (2010) conducted a similar experiment in which an SMC was used as an adjuvant in a vaccine and demonstrated significant reduction in tumour growth rate and prolonged survival[49]. Therefore, the main difference could be related to the nature of the vaccine component. In my experiments a peptide vaccine model was utilized, while the study by Dougan et al. (2010) utilized an inactivated "killed" vaccine in which mice were administer irradiated tumor cells[49].

There are several ways to circumvent these pitfalls. The timing of LCL161 administration could be adjusted by administering LCL161 orally 6-12 hours before delivery with the peptide. This would allow for LCL161 to be circulating, increasing the chances of the two components interacting *in vivo* to induce an immune response. Another solution would be to utilize a DC-based vaccine approach, which include loading of DCs *ex vivo* with a source of target antigens (e.g., peptides, mRNA, viral vectors, or allogenic tumour cells)[72,90–92]. These loaded-DCs are then administered to patients which then can present a target antigen to T cells and B cells and mount an antigen specific immune response [72,90–92]. I was able to demonstrate the immune stimulating

effect of LCL161 on BMDCs *in vitro*, and thus DCs could be activated and loaded with the necessary target antigen *ex vivo* before being administered intravenously.

4.3.2 SMC as an adjuvant had no effect on $CD8^+$ T cell and memory immune responses.

In addition to evaluating the ability of SMCs to prevent tumour growth, I analysed whether SMCs could expand antigen specific $CD8^+$ T cells and its memory cell subsets. Mice vaccinated with E7 peptide and CpG as well as mice vaccinated with E7 peptide and LCL161 demonstrated a decrease in E7 specific $CD8^+$ T cells compared to naïve mice (**Figure 19C**). On the other hand, mice vaccinated with only E7 peptide showed no difference compared to naïve mice (**Figure 19C**). Since no antigen specific $CD8^+$ T cell immune response was observed when mice were vaccinated with the E7 peptide and LCL161 compared to naïve mice, this correlates with the inability of this combination to curtail tumour growth (**Figure 18C**). During this same experiment, $CD8^+$ T cell memory responses were evaluated (**Figure 19B**). All mice regardless of treatment demonstrated a predominantly effector memory cell ($CD44^+CD62L^-$) phenotype (**Figure 19D**). Since spleens were collected only 7 days after the second vaccination, a predominantly effector memory response is consistent with what is expected at that time point. Furthermore, the profiling of T cell memory subsets was conducted on T cells isolated from spleens; it is likely that the results may vary if the T cells were isolated from draining lymph nodes.

4.4 Future Work

My thesis has provided a solid platform for future research into how SMCs can be utilized in a vaccine therapeutic setting. SMCs have been demonstrated by myself and others to affect the function of immune cells; however the precise mechanisms and associated pathways that are

impacted by SMC treatment need further delineation. For example, determining the transcriptional responses of SMC-treated immune cells could provide further insight into how SMCs function in the regulation of the classical and alternative NF-kB pathway.

One of the key steps for activation of T cells by DCs is the migration of mature DCs to peripheral lymphoid organs. If SMC-treated DCs are unable to migrate, then these DCs will be unable to present antigens to T cells. The analysis of DC migration in SMC treated mice can be determined by measuring upregulation of CCR7 and other migratory receptors.

The most challenging aspect of SMCs for its utility in vaccine therapeutics is to co-exist with the vaccination products in the proper signalling and cellular context. The delay in activation of the alternative NF-κB pathway, attributed to the gradual buildup of NIK in SMC treated DCs, should be considered when evaluating the efficacy of the vaccine. Additionally, this delay in alternative NF-κB activation impacts decisions about dosage, route of administration, and treatment regimen, potentially impacting the ability of mature DCs to present an MHC complex containing a peptide antigen. Changing the way SMCs are formulated and/or administered may provide the solution to how to properly adjuvant SMCs. Additionally, testing compatibility with different types of vaccines, such as mRNA and whole cell, may reveal which combinations would work well with SMCs. Moreover, as my study focuses on the effects of SMCs on DCs, this opens the door to exploring the use of SMCs for DC-based vaccines.

4.5 Significance

New vaccines being developed use safer nucleic bases that are sometimes unable to induce the required immune response for a target[3]. The NF-κB pathway is a target of certain

adjuvants/immune stimulants, leading to immune cell activation and in this regard, SMCs are a relatively well studied class of drugs that regulate both the classical and alternative NF-κB pathway[26–29]. My thesis highlights the impact of SMCs on immunomodulatory responses in dendritic cells, a key immune cell subset. In this regard, SMCs in combination with target antigens could be used to specifically activate DCs and promote antigen-specific adaptive immune responses and potentiate long-term immune cell memory. Additionally, my work demonstrates the gap in knowledge that needs to be filled to understand how to utilize this class of drug in vaccine therapies. SMCs have the potential to be used in a wide range of immunotherapeutic capacities that would pave the way for a new generation of adjuvants.

4.6 Conclusion

In this thesis I have demonstrated the immunomodulatory properties of SMCs on DCs. More specifically, I have demonstrated that SMCs induce maturation of BMDCs and a pro-inflammatory immune response. Furthermore, I found that SMCs stimulated BMDCs likely led to the antigen specific activation of T cells. Further work needs to be done to elucidate the mechanisms by which SMCs activate DCs, as well as determine the best use of SMCs as adjuvants *in vivo* to explore the potential of this class of drugs. Thus, these findings will guide the use of SMCs as adjuvants in vaccine therapies tin induce robust protective immunity.

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