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LYMPHATIC ENDOTHELIAL CELLS AS POTENT MODULATORS OF CD8 T CELL PHENOTYPE DURING INFLAMMATION

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To my Bebetos,

for fostering and nurturing my sense of wonder

and curiosity from an early age, I miss you dearly.

To my mom Elisa Salles and

to my brothers Emi and Marcel:

gracias por siempre estar conmingo,

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I am eternally thankful.

Smile not because everything is good,

but because you can see the good in everything.

— Unknown

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ABSTRACT

The expanding range of CD8 T cell phenotypes deepens the need to address how specific signals and contexts lead to this subset diversity. As lymphocytes enter the lymph node, they encounter and interact with the stromal cells that form the lymph node itself, so it's also crucial to understand the role that lymphatic endothelial cells (LECs) of the lymph node play in adaptive immunity.

Our lab has shown that at homeostasis, LEC education of naïve CD8 T cells results in a population that skews to central memory. Here, we address some of the dynamics that govern CD8 T cell memory differentiation when primed or maintained by LECs and uncover how LECs cooperate with dendritic cells (DCs) to establish a memory niche not only at homeostasis, but also at different cytokine microenvironments crucial for active immune responses. *In vitro* models reveal that priming of T cells by LECs stimulated under Th1 and Th2 related cytokine conditions induced a T cell population that is 80 percent central memory in phenotype that grows generationally. Interestingly, when LECs were treated with immunosuppressive cytokines associated with the tumor microenvironment the T cells developed into an effector memory-like phenotype with reduced proliferation.

At all conditions, LEC-primed CD8 T cells result in a phenotype that has stem-like qualities (Tscm) defined by high expression of TCF1 and PD1, and low expression of Tim3 when compared to those primed by DCs. LEC priming for 24 hours is sufficient to confer this phenotype, even if T cells are maintained by DCs after LEC priming. In contrast, if T cells are primed by DCs and maintained in a LEC rich environment, the resulting T cells skew towards central memory more than those primed by DCs alone.

Together this shows that LECs are potent modulators of T cell phenotype in vitro which has incredible translational potential to modulate phenotypes for different immunotherapies; in particular, adoptive T cell therapies and CAR T therapies would benefit from this, as the phenotype we observe LECs developing is the desired phenotype for these therapies.

CHAPTER 1

INTRODUCTION

1.1 The Adaptive Immune Response

Upon encountering pathogens, the immune system orchestrates a highly efficient response wherein the initial responders, referred to as the innate immune system, act rapidly and non-specifically.[1, 2] Here, we will delve into another branch of the immune system: the adaptive immune system. The adaptive immune system, while slower, mediates an exceptionally specific response, customized to the particular pathogens invading the body.[1, 2]

The adaptive immune system is comprised of B lymphocytes (B cells) and T lymphocytes (T cells), which orchestrate the adaptive immune response against pathogens. B and T cells identify specific pathogen components, referred to as antigens throughout this thesis. B cells are responsible for recognizing structural epitopes on pathogens to produce antibodies that neutralize and mark them for destruction by other components of the immune system.[1, 2]

In this thesis, we place focus on the T cell response. T cells utilize their T cell Receptor (TCR) to identify linear peptides derived from pathogens, which are presented by affected cells on Major Histocompatibility Complex (MHC) molecules.[1, 2] T cells are significantly potent, and excessive activity can result in heightened inflammation and autoimmunity, requiring strict regulation. Naïve T cells need activation by professional antigen presenting cells (APCs), which deliver two distinct signals: initially, they present the antigen on MHC molecules for T cell recognition through their TCR, and additionally, they offer a costimulatory signal via various costimulatory molecules, which transition the T cell into an activated state prepared for response.[1, 2] The T cell response can be further deconstructed into two distinct components.

The CD4 T cell response is orchestrated by CD4 T cells that identify peptides presented via MHCII molecules.[1, 2] These CD4 T cells are responsible for generating a cytokine milieu

that is optimal for defending against the particular pathogen.[2] Various phenotypes of CD4 T cells exist, leading to diverse inflammatory responses. Notably, Th1, Th2, and Tregs have been extensively characterized and will be expounded upon in a subsequent section addressing inflammatory cytokines. In this thesis, the primary emphasis will be placed on the response of CD8 T cells. CD8 T cells, often referred to in informal contexts as "killer" T cells, possess the capacity to identify and eliminate cells that have been compromised by pathogens or are malignant, particularly in oncological settings.

A fundamental aspect of the adaptive immune system is the establishment of immunological memory. Throughout the immune response, memory B and T cells emerge, characterized by their longevity and persistence following the attenuation of the immune response.[2–6] Upon subsequent infections, these cells can mount a swifter and more effective response to pathogens due to their prior activation from a naïve state, rendering them "ready to attack" upon antigen detection. These memory cells are the intended outcome of vaccinations, designed to safeguard individuals against future infections. In concert, B cells, CD4 T cells, and CD8 T cells orchestrate a potent and specific adaptive immune response.

1.2 The T Cell Response

1.2.1 CD4 T Cells: Subsets and Cytokine Production

Inflammation is quite diverse, influenced by a multitude of factors including various cytokines that modulate the inflammatory response to pathogens requiring elimination for host defense.[2, 7] As noted previously, CD4 T cells, also referred to as "helper" T cells, play a crucial role in generating a wide array of cytokines that elicit intricate inflammatory signals, thereby facilitating the customization of the response to the specific pathogen that has breached the host. [2, 8, 9]

CD4 T cells are categorically defined as Th1, Th2, Th17, and Tregs, contingent upon the

specific transcription factor they express, which determines their functional properties and the spectrum of cytokines they produce.[2, 8, 9]

Th1 CD4 T cells are crucial for mounting an effective immune response against intracellular pathogens like viruses and small bacteria. Th1 CD4 T cells are defined by their expression of the transcription factor Tbet, and their production of cytokines like IFNγ , TNFα, and IL-2. [2, 8–11]

Th2 CD4 T cells are involved in the response against parasites, worms, as well as allergy. They are defined by their expression of the transcription factor Gata3, and the production of cytokines like IL-4, IL-5, and IL-13. [2, 8–12]

Th17 CD4 T cells defend against extracellular bacteria and they are quite important in the gut. They are characterized by their expression of the transcription factor RORγT and their production of IL-17. We will not discuss much about Th17 any further, but wanted to mention them for completeness. [2, 8, 9, 13, 14]

Regulatory CD4 T cells constitute an essential phenotype of CD4 T cells responsible for maintaining immune tolerance and averting both autoimmunity and excessive immune responses. These cells are distinguished by the expression of the transcription factor FoxP3, the synthesis of immunosuppressive cytokines TGFβ and IL-10, and the presence of CD25. This CD25 acts as an IL-2 receptor, functioning as a sink to inhibit other cells from receiving IL-2 stimulation, which is pivotal for cell proliferation and survival signals.[2, 8, 9, 15–17]

Collectively, CD4 cells function as "helpers," offering the essential framework within which the immune response develops. They provide critical signals and guide the immune response to ensure it is appropriately adapted to the specific invading pathogen. While additional phenotypes and subphenotypes have been identified, their examination lies beyond

the scope of this thesis.

1.2.2 The CD8 T cell response

As previously indicated, the CD8 T cell response plays a vital role in the elimination of malignant and infected cells. These cells are particularly significant in the context of viral infections, intracellular pathogens in general, and cancer.[2, 18, 19] They effectively eradicate virus-infected cells that might otherwise propagate the virus to adjacent cells, as well as cancerous cells.[2, 18, 20] CD8 T cells are subject to stringent regulation by the immune system, incorporating several mechanisms to avert excessive activity, as excessive cell destruction and inflammation could be detrimental, potentially resulting in autoimmunity.[2, 21, 22]

Naïve CD8 T cells originate in the thymus, where they undergo a selection process to ensure their ability to recognize the host's MHC I molecules, as their antigen presentation is dependent on these molecules. Concurrently, they are also selected to avoid responding to self-antigens, which are constitutively expressed by the host, thereby mitigating the risk of autoimmunity.[2, 23, 24] Cells that fail to recognize MHC I molecules or that recognize self-peptides are subject to deletion.[2, 23, 24] Upon leaving the thymus, naïve T cells enter the circulatory system and localize to secondary lymphoid organs, such as lymph nodes and the spleen, where they monitor for their respective antigen.[2, 25]

Naïve CD8 T cells require activation by an APC, with dendritic cells (DCs) serving as the primary APC. These cells deliver TCR signaling by presenting the antigenic peptide to CD8 T cells via MHC I and provide a co-stimulatory signal that authorizes T cell expansion, proliferation, and the activation of effector functions, including the production of the membrane-perforating enzyme perforin and the apoptosis-inducing enzyme granzyme B.[2, 26–28] Upon activation, CD8 T cells migrate from the lymph node to the site of infection to eradicate infected cells. During the progression of the CD8 T cell response, they increasingly express regulatory molecules such as PD1 and CTLA4, which function as brakes

and checks to prevent an excessive immune response.[2, 29, 30] Furthermore, they differentiate into various phenotypes of CD8 T cells, which will be elaborated upon in the next section.[31]

1.2.3 CD8 T Cell Heterogeneity and Memory

As the field investigates the multifaceted roles of CD8 T cells, it has been recognized that these cells exist along a continuum of diverse phenotypes. Several theoretical models have been proposed to explain the emergence of these phenotypes.[5, 32, 33] Here we will go over the most salient phenotypes that will be covered throughout the thesis.

Naïve CD8 T cells (Tn): The initial phenotype, which has been previously discussed, pertains to naïve CD8 T cells. These T cells emerge from the thymus following their development and enter systemic circulation, where they monitor lymph nodes for activation by APCs upon infection. Within murine models, such cells are characterized by the expression of L-Selectin (CD62L), a surface molecule used in the homing of CD8 T cells to lymph nodes. Additionally, they express CCR7, a chemokine receptor for CCL21, permitting their extravasation from circulation into the lymph node via its gradient. Conversely, these cells are devoid of CD44, a surface glycoprotein that is up-regulated upon activation and experience with antigens.[2, 32, 34–37] For the rest of this thesis, we will define naïve CD8 T cells as CD62L+CD44-. Once naïve CD8 T cells are activated, there is a plethora of phenotypes that arise.

Central memory CD8 T cells (Tcm): Central memory cells, having encountered antigens during initial infection or vaccination and subsequently survived, are primed to initiate an immune response upon antigen recognition. These cells circulate systemically, entering lymph nodes to actively search for their specific antigens, and mount a rapid and efficient immune response upon reactivation.[2, 38–41] Central memory T cells exhibit the capacity

for rapid expansion and proliferation, differentiating into effector CD8 T cells that possess potent cytotoxic capabilities.[32, 39, 40] These cells constitute an essential component of durable immunological memory, as they respond faster compared to naïve T cells, which undergo extensive activation processes. In mouse models, these cells are identified by the co-expression of CD62L and CD44, indicating their localization in secondary lymphoid organs and previous antigen exposure.[32, 39, 42, 43] Central memory cells or Tcm will be defined as CD62L+CD44+ double positives.

Effector/Effector memory-like CD8 T cells (Tem): Effector cells are short-lived, and they are effective at killing cells that present their cognate antigen.[2, 40, 41, 44, 45] Effector memory cells are long-lived, effector-like cells that actively monitor tissues for their specific antigens. Upon antigen recognition, they are capable of initiating effector functions, such as cytotoxic activity. These cells are characterized by the absence of CD62L expression and enhanced expression of CD44.[32, 39, 43, 45] Since these phenotypes are quite similar and differ when it comes to short versus long-lived, we will define them as CD62L-CD44+ and use the terms "effector", "effector memory" and "effector memory-like", interchangeably throughout this thesis unless otherwise noted.

Exhausted CD8 T cells (Tex): Those that have been responding against their antigen for an extended time and loose effector functions over time.[46, 47] These cells are particularly relevant in chronic viral infections and cancer, as they are dysfunctional, and their presence can be a sign of poor immunological effectiveness against their antigen.[46, 48, 49] There are several features of exhausted cells, specifically, high PD1 and Tim3 expression is an important characteristic. These molecules are involved in stopping T cells from being effective by providing a "brake" signal and are therefore defined as checkpoint or inhibitory molecules.[29, 30] Many cancer therapies for example are designed to reinvigorate these cells by blocking checkpoint molecules to "accelerate" rather than "brake" the response against the cancer.[49–

51] We define exhausted cells here as PD1+Tim3+ double positives.

Stem Cell Memory T cells (Tscm): A relatively new phenotype of CD8 T cell that has been recognized as important to the immune response. These cells have less defined characteristics, and it is a phenotype of T cell that lies in between a naïve cell and a central memory cell.[52] Tscm is an interesting phenotype of CD8 T cells as they have the ability to self-renew, they can differentiate into several other phenotypes of CD8 T cells and they reside in the lymph nodes.[53–56] These cells are characterized by their expression of the T cell transcription factor TCF1 (transcription factor involved in T cell stemness, self-renewal and differentiation potential), the expression of PD1 and the lack of Tim3.[54, 55, 57] These are crucial cells that have been found to be the population of T cells that proliferates and differentiates into effector cells upon the aforementioned checkpoint therapies, in particular anti-PD1 therapies, thus making them crucial for the CD8 response against cancer.[58–61]

1.3 Lymphatics: Key Players in Adaptive Immune Responses

1.3.1 An Overview of the Lymphatic System

Lymphatics are a network of vessels that connect all tissue to regional lymph nodes where the immune system can survey and sample for possible infection in an effective manner. [62–65] These vessels form complex networks even inside the lymph nodes themselves. This system plays a crucial role in regulating fluid homeostasis, assisting in immune surveillance, and transporting dietary lipids.[62–65] The lymphatic vasculature is essential for efficient uptake of fluid and macromolecules, while also expressing chemotactic and adhesion molecules that allow for trafficking of specific immune cell subsets. Additionally, the lymphatic system serves as a conduit for immune cells and antigens in lymph fluid, enabling the immune system to effectively monitor and respond to potential threats.

1.3.2 Lymphatics in Adaptive Immunity

Historically, lymphatics have been ignored and assumed to be passive conduits of fluid transport, however studies from the past decade have shown that the cellular unit of lymphatics, lymphatic endothelial cells (LECs), play several roles in the immune response.[66–73] Lymphatics are key players in the immune response induced by inflammation as lymphatics are the routes through which antigen and immune cells drain from the inflamed site to the lymph node where the immune response can be mounted.[65]

LECs are components of the lymph node stromal cell compartment, lining the sinuses of the lymph node; and the architecture of the lymph nodes and their sinuses is generally conserved among rats, mice, humans and other mammals.[62, 63, 74, 75] Not only do they contribute to structural integrity of the lymph node, but we and others have recently uncovered roles that they have within the adaptive immune response at modulating cell interactions and actively partaking in antigen directed responses.[76] Inflammation induces lymphangiogenesis, the expansion of lymphatics, through vascular endothelial growth factor C (VEGF-C) stimulation. Inflammatory signals up-regulate the LEC-specific transcription factor Prox1, and the receptor VEGFR3 in LECs, which makes them more susceptible to VEGF-C stimulation.[77] IFNγ is a very potent stimulator of LEC functions, which promotes up-regulation of the co-inhibitory receptor PD-L1 as well as adhesion molecules like ICAM-1.[65, 67, 68] LECs have also been thought to regulate T cell activation by modulating antigen-presenting functions of dendritic cells during inflammation as inflamed LECs have been shown to decrease expression of CD86 on DCs, which dampens their ability to co-stimulate T cells.[78] Another interesting feature of LECs has been shown to be the potential to archive antigen and transfer it to DCs for maintenance of memory T cells and enhance protective immunity.[79] In addition to this, we have recently found that lymphatic vessel clotting can also be involved in the progression or worsening of conditions like ARDs in humans.[80]

The role of lymphatics and T cell survival is not fully understood; however, it is known that in the lymph node, LECs are a major source of IL-7.[81, 82] IL-7 interacts with cells that express IL-7R and is a key survival cytokine for naïve and central memory T cells as it provides anti-apoptotic and proliferative signals to naïve and memory T cells.[83, 84] Upon inflammatory stimuli, LECs up-regulate IL-15, a cytokine that is involved in naïve and memory T cell homeostasis. IL-7 and IL-15 have also been found to be necessary for the development of memory T cells including Tscm.[85] CCL21 is an important chemokine used by T cells to extravasate from high endothelial venules (HEVs), and LECs are a source of CCL21 in the lymph node.[86] LECs express MHC class I and MHC class II for antigen presentation and are capable of inducing T cell tolerance.[67, 68]

1.3.3 Lymphatic Endothelial Cell Interactions with T cells: A paradox

The interactions between LECs and T cells are quite interesting. In the CD4 compartment, there has been little to no evidence that LECs can activate naïve CD4 T cells; however, we have recently shown that in humans, LECs can reactivate memory CD4 T cells in an allogenic context.[87] LECs have been shown to be involved in peripheral tolerance of CD8 T cells. They can present antigen to CD8 T cells and through their expression of PDL1 and lack of co-stimulation, they induce anergy of CD8s.[68] However, some studies suggest that PDL1 signaling is crucial for developing effective memory during the primary response.[88, 89] We have recently shown that LECs not only sample whatever drains into the lymph node, but they can also scavenge and cross present antigen to CD8 T cells at steady-state (homeostasis).[90] We then showed that LECs at steady-state can actually activate and prime naïve CD8 T cells into a central memory phenotype.[91] These cells are long lived after the primary response stops, they home and survey for antigen in secondary lymphoid organs, and they can respond accordingly to a secondary challenge, all key features of true central memory cells. These findings provide an interesting paradox that LECs fall under; they can

both partake in peripheral tolerance, but they can also be one of the sources of CD8 central memory cells that arise early in the immune response.

1.3.4 Bridging the Knowledge Gap

Lymphatic endothelial cells (LECs) are strategically localized within regions of the lymph node where immune cells either directly drain into the lymph node or extravasate from the circulatory system. This positioning enables LECs to engage with immune cells actively participating in immune response initiation or antigen surveillance. Furthermore, LECs secrete chemokines that facilitate the homing and extravasation of naïve T cells into the lymph nodes, thereby enhancing the likelihood of interactions between naïve T cells and LECs. As previously demonstrated by our laboratory, under steady-state conditions, LECs possess the capacity to scavenge antigens and cross-present these to CD8 T cells. Additionally, we have elucidated that steady-state LECs can prime naïve CD8 T cells into a predominantly central memory phenotype that is long-lived *in vivo*, exhibiting effective responses to secondary challenges, as evidenced by the successful suppression of Listeria infection. This finding prompted further inquiry into the capacity of LECs to prime naïve CD8 T cells within inflammatory environments, which are critical for the development of effective immune responses. For this purpose, we utilized models *in vitro* to thoroughly investigate the interactions between LECs and T cells. Moreover, we examined the potential interactions between LECs, professional antigen-presenting cells such as dendritic cells (DCs), and T cells, given that LECs are never isolated in the lymph node with naïve T cells. In summary, our investigation focused on delineating the role of LECs in the priming of CD8 T cells within inflammatory settings, both in the presence and absence of DC interactions.

CHAPTER 2

PHENOTYPES AND KINETICS: LEC PRIMING OF NAÏVE CD8 T CELLS AT DIFFERENT CYTOKINE MICROENVIRONMENTS

2.1 Chapter Summary

In this chapter, we focus on lymphatic endothelial cell (LEC) activation of naive CD8 T cells in the context of different inflammatory microenvironments. Different cytokine microenvironments are needed to guide and shape the response to specifically kill the invading pathogen. Here, we look at LEC stimulation by cytokines characteristic of Th1, Th2 and suppressive responses that would be produced by CD4 T cells in the lymph node. We model these responses with *in vitro* stimulation of LECs with cytokines.

We interrogate the CD8 T cell priming capabilities of LECs at these different microenvironments, and look at the kinetics of the CD8 T cell response in a 3-day period upon activation, comparing them to activation of CD8 T cells by mature Dendritic Cells (mDCs). In essence, we observe that LECs induce a central memory phenotype in Th1 and Th2 environments, similar to how they behave at steady-state, and in contrast, CD8 T cells activated by LECs in a suppressive environment yield an effector population that proliferates to a lesser extent.

We not only phenotype the T cell populations that arise from these activating conditions, but also seek to phenotype and understand how these cytokine microenvironments affect the LECs themselves, since they are susceptible to changes as the immune response takes place and they experience stimulation from these cytokines.

2.2 Introduction

2.2.1 Cytokine Microenvironments

Inflammatory signals are vast and diverse, and as previously examined in Chapter 1, in order to develop an effective immune response, CD4 T cells create a microenvironment of cytokines that appropriately directs the response to the category of pathogen that has invaded the host organism. This chapter will focus on three distinct inflammatory microenvironments: Th1, Th2 and suppressive microenvironments. These environments were modeled by stimulating lymphatic endothelial cells with cytokines associated with the specific inflammatory response, selecting those cytokines for which lymphatic endothelial cells possess specific receptors.

Th1 Cytokine Microenvironment

IFNγ + TNFα: To model a Th1 environment, LECs were stimulated with interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα). IFNγ and TNFα are two key cytokines produced by Th1 CD4 cells,[11, 92] and in conjunction with other cytokines like IL-2 and IL-12 they facilitate a response against intracellular pathogens like virus and small bacteria.[2, 93] Since we only wanted to stimulate LECs with cytokines they are susceptible to, we only used IFNγ and TNFα. These cytokines are potent modulators of LECs, inducing expression of important molecules like PD-L1, ICAM-1 and MHC-II upon coming in contact with lymphatic endothelial cells. [67, 71]

Th2 Cytokine Microenvironment

IL-4 + IL-13: In order to create a microenvironment conducive to killing parasites, or induce an allergic response, CD4 Th2 cells produce IL-4, IL-5 and IL-13. IL-4 is essential in Th2 responses as it is responsible at both differentiating and maintaining CD4 Th2 cells,[11, 94] it promotes IgE class switch on B cells and helps polarize macrophages into an M2

phenotype.[95, 96] IL-13 shares some functions with IL-4 like promoting class switch into IgE, but it also induces tissue remodeling as well as airway hyperactivity.[97–99] We focus on IL-4 and IL-13 since LECs do not have the IL-5 receptor.

Suppressive Cytokine Microenvironment

TGFβ + IL-10: The primary cytokines produced by regulatory CD4 T cells are transforming growth factor beta (TGFβ) and IL-10, thus we used these cytokines to model a suppressive environment by stimulating LECs *in vitro* with them.[17] TGFβ is a crucial cytokine that helps promote Treg development and function which are critical for immune tolerance, and it also inhibits T cell proliferation.[100–103] IL-10 suppresses the production of inflammatory cytokines like TNFα and IFNγ, and can also suppress antigen presentation after T cell activation.[104–108] Both TGFβ and IL-10 contribute to tumor immune evasion by suppressing anti-tumor responses. These cytokines have been found in the tumor microenvironment (TME) as well as the gut and gut associated lymphoid organs, which also contributes to our curiosity about this combination of cytokines.[101, 106]

In this chapter, we examine the phenotypic changes of LECs following stimulation in distinct cytokine microenvironments. While LECs have been previously analyzed post IFNγ and TNFα exposure, comprehensive studies under IL-4 + IL-13 stimulation and TGFβ + IL-10 stimulation conditions remain limited. We also explore how these varied cytokine microenvironments modulate LECs' capacity to activate and prime naive CD8 T cells and determine the phenotypic outcomes of CD8 T cells subsequent to LEC activation in these environments. Throughout this thesis, the terms "Th1 LECs", "Th2 LECs", and "Sup LECs" or "Tsup LECs" will be utilized. It is important to clarify that this nomenclature does not imply that the LECs themselves synthesize the cytokines typically associated with these CD4 cell phenotypes; rather, it denotes that the LECs were subjected to stimulation

by those specific cytokines.

2.2.2 T Cell Phenotypes: Main Markers of Interest

Throughout this chapter, we seek to identify different phenotypes of CD8 T cells. Here, we will cover the main markers of interest that we use to define the many phenotypes that CD8 T cells can develop into.

TCF1 - T cell factor 1 (TCF1) is a transcription factor that plays a crucial role in CD8 T cell differentiation and function, especially in the context of CD8 responses against cancer and chronic viral infection.[109–111] TCF1 helps maintain the stem-like properties of CD8 T cells, as CD8 T cells that express high levels of TCF1 show a progenitor and stem-like phenotype which is important for maintaining long term immunity.[57, 112] TCF1+ CD8 T cells have the capability to self-renew, which allows for the persistence of an antigen-specific T cell response during prolonged immune challenges. Finally, CD8 T cells that express TCF1 have the ability to differentiate into several phenotypes, including cental memory as well as many effector subsets. [113]

In cancer, the role of TCF1 has been found to be quite important as the presence of TCF1+ CD8 T cells generally correlates with better responses to immune checkpoint blockade (ICB) therapy, and TCF1+ progenitors have been found to often respond to subdominant antigens.[111, 114–117]

TCF1 regulation is closely related to T cell fate. TCF1 can be asymmetrically partitioned during T cell activation leading to an unequal distribution of the transcription factor in the two daughter cells after mitosis contributing to the generation of diverse T cell subsets.[118] In contrast the loss of TCF1 is associated with commitment to an effector T cell fate and exhaustion, and inflammatory cytokines can suppress TCF1 expression which in conjunction promote the differentiation of effector CD8 T cells.[113, 118–120]

CD62L - Also known as L-Selectin, CD62L is an important molecule involved in cell trafficking, differentiation and function. As T cells are activated, CD62L is shed from the cell surface by the metalloprotease ADAM17 through proteolytic cleavage.[121–123] CD62L is crucial for T cell homing into the lymph nodes as it is involved in the initial rolling of T cells on high endothelial venules (HEVs) facilitating entry into the lymph nodes which is essential for naive T cell surveillance to generate an effective immune response. [124, 125]

CD62L+ CD8 T cells, especially central memory-like T cells have been shown to have an enhanced anti-tumor efficacy in adoptive cell therapy models, especially if these cells express other markers like CXCR3.[126, 127] CD62L is also linked with the proliferative potential of CD8 T cells, as cells that express high levels of CD62L correlate with higher proliferative potential than those with low or no expression.[125] Finally, CD62L+ T cells demonstrate better tumor control in both solid and non-solid tumors, and synergize better with immune checkpoint blockade therapies.[127]

CD44 - A cell surface glycoprotein, CD44 is an adhesion molecules that aids T cells migrate into sites of inflammation and infections. CD44 is an important marker that is up-regulated during CD8 T cell activation, and its expression is closely correlated with the activation status and antigen experience of CD8 T cells, with higher expression showing a more activated state. This glycoprotein is commonly used to distinguish several T cell subsets, particularly differentiate between naive, central memory and effector memory subsets in conjunction with CD62L. [128–132]

CD27 - CD27 is a member of the tumor necrosis factor receptor (TNFR) superfamily, and it functions as a co-stimulatory molecule to CD8 T cells. This marker is particularly expressed by naive and memory T cells and plays a role in T cell activation, differentiation and survival. CD27 is important for the generation and maintenance of memory CD8 T cells, and supports their long-term survival and function. CD27 has also been associated

with TCF1+ stem cell memory CD8 T cells, playing a role in maintaining the pool of these cells in chronic infection and cancer. [133–138]

CXCR3 - CXCR3 is a G protein-coupled receptor whose expression is up-regulated primarily on activated T cells, and can be used as a marker of effector and memory T cells. CXCR3 is a chemokine receptor that responds to CXCL9, CXCL10, and CXCL11. This receptor is involved in guiding CD8 T cells to sites of inflammation, and infected tissue. CXCR3 is important in CD8 T cell infiltration of tumors and is critical for an effective anti-tumor immune response. Signaling through CXCR3 enhances the cytotoxic capabilities of CD8 T cells by promoting the expression of effector-associated molecules like perforin and granzyme B. [139–141]

PD1 - Programmed Cell Death 1 (PD-1) is an inhibitory receptor expressed on the surface of CD8 T cells that binds to the ligand PD-L1 and PD-L2. It is up-regulated quite rapidly after TCR engagement and activation of naive CD8 T cells, and even though it is an inhibitory receptor, it is also associated with the development of an effective memory CD8 T cell pool.[54, 55, 88, 142] PD1 plays a significant role in the early stages of CD8 T cell activation as it helps regulate and tightly control the effector functions like granzyme B expression during an acute T cell response.[51, 89, 143, 144] PD1 regulation of T cell function is crucial for maintaining a balance between an effective immune response and potential tissue damage and autoimmunity. [145]

In chronic viral infections and cancer, PD1 is highly expressed by exhausted CD8 T cells.[146, 147] The presence of PD1 is associated with dysfunctional CD8 T cells characterized by reduced effector functions and proliferative ability. PD1 inhibits TCR signaling and it also suppresses the expression of the co-stimulatory molecule CD28, all contributing to the dampening of the T cell response. PD1 expression is associated with decreased production of effector cytokines as well as reduced proliferative capacity by the T cells.[148–150] In the

tumor, PD1-expressing CD8 T cells exhibit impaired anti-tumor activity, which contributes to the wide interest for anti-PD1 therapies in cancer.[151, 152]

Tim3 - T cell immunoglobulin and mucin domain-containing protein 3 (Tim3) is an inhibitory receptor expressed by CD8 T cells especially when there is chronic antigen stimulation. It is often co-expressed with other inhibitory and immune checkpoint receptors like PD1, and the co-expression is associated with a more severe state of exhaustion of the CD8 T cells.[147, 148] Tim3 signaling can lead to decreased production of effector cytokines which reduces overall cytotoxic activity in CD8 T cells. It also plays a role in the transition from effector phenotype to an exhausted T cell state, and its expression is associated with a more terminally differentiated and exhausted phenotype. Tim3 serves as a marker of terminal exhaustion in CD8 T cells, and the co-expression of Tim3 and PD1 has been linked to immune exhaustion, reduced T cell function, and poor outcomes in various diseases including cancer.[153–156]

Many more markers were looked at, however the ones mentioned here are the most salient markers that will be observed throughout the project. I will introduce any other markers, molecules and receptors as we dissect the results and when they are necessary, but they do not need an entire introduction for themselves as they are not as important to these studies.

2.2.3 The Transgenic OT System: A Mouse Model

Throughout this thesis, the OT mouse model is highly used, in particular the OT1 mouse model. In immunology, transgenic models are a great tool that has been used for years, and when we are interested in antigen specific responses, it is beneficial to have a model that reacts to a known antigen. This is the role of the OT1 transgenic mouse models, in which all of the T cells of the mouse react to the ovalbumin (OVA) chicken protein, which is a component of chicken egg whites. We leverage OVA as a model antigen in immunology as

it has many favorable qualities, including its solubility, the well defined CD4, CD8 and B cell responses, its lack of enzymatic activity and the fact that laboratory mice lack prior exposure to it. The mice contain the transgenic inserts for mouse Tcrα-V2 and Tcrβ-V5 chains of the T cell receptor which provide the T cell specificity to OVA. The OT II model (B6.Cg-Tg(TcrαTcrβ)425Cbn/J) corresponds to CD4 T cells so it was not used for these studies, but the CD4 T cells respond to the chicken OVA 323-339 peptide in the context of the MHC II haplotype I-A^b. The OT1 (C57BL/6-Tg(TcrαTcrβ)1100Mjb/J) model on the other hand corresponds to mice whose CD8 T cells respond to the chicken OVA 257-264 SIINFEKL peptide in the context of the MHC I haplotype $H2K^b$.[157–159] SIINFEKL was used as the model antigen for most of this project.

2.3 Methods

Lymphatic endothelial cell isolation: We first isolated primary lymphatic endothelial cells utilizing a protocol adapted from the Turly lab.[160] We first digested mouse skin draining lymph nodes with Liberase DH. We expanded the lymph node stromal cell population for a week, and sorted them using magnetic bead negative selection by sorting out any remaining CD45+ cell and the FRC population by CD140a. The population that remained was 85-95% LEC **2.1** determined by flow cytometry looking at the negative expression of CD45, and co-expression of endothelial cell marker CD31 and podoplanin/gp38.

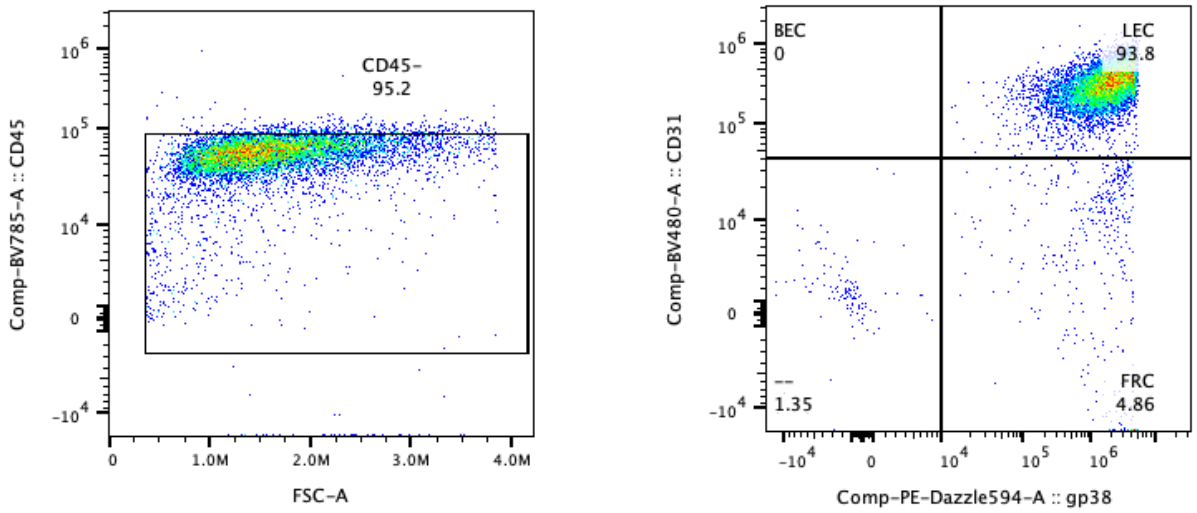

Figure 2.1: **LEC Purity.** Left panel shows the live population where we gate for all cells that do not express CD45 as LECs do not express this marker and there might be some leftover CD45 cells from the lymph node. Right panel shows LECs by identifying the CD31+ and gp38+ double positive population which demark LEC identity.

BMDC Isolation: We used the bone marrow of young (6-8 week old) C57Bl/6 mice following the published Lutz BMDC protocol [161]

T cell isolation: We first obtained CD45.1 OT1 mice from the UChicago CIIC and dissected the spleens. We then mechanically digested them with syringe needles and then passed

them through a 70 uM cell strainer using a syringe stopper before utilizing either the Stem Cell mouse CD8 T cell isolation kit or MojoSort Mouse Cd8 T cell isolation kit and followed the manufacturer's protocol.

CFSE Labeling: We stained the isolated CD8 T cells with Carboxyfluorescein succinimidyl ester (CFSE) in order to be able to track the generations as T cells expanded and divided. CFSE is a dye that binds to free amine groups in the cytosol of the cell, and it dilutes as the cells divide and dilute the original cytosol of the mother cell. The entire staining protocol is done in a tube covered in aluminum foil to avoid photo-bleaching of the CFSE with light from the room. In order to stain for this I make a solution that is 10mM CFSE in PBS, and add it to one milliliter of cell suspension (10-20 million cells per mL, or the T cells from one spleen) for a total of 2 milliliters of solution at 5uM CFSE. I incubate this solution for 7 minutes at room temperature, and after that I quench the CFSE solution with 8 milliliters of complete media and incubate on ice for five minutes. After the second incubation, I spin the cells down at 1500 rpm for 5 minutes and take the supernatant, they are now ready to use as CFSE labeled CD8 T cells.

LEC and BMDC pulsing and stimulation: After both LECs and BMDCs were obtained, we pulsed them with antigen and stimulated with cytokines overnight, before washing them and adding OT1s for co-culture. We pulsed both cell populations with 1ng/mL of the immunodominant MHC-I restricted OVA peptide SIINFEKL. For DC maturation we pulsed BMDCs with 1ng/mL of LPS in conjunction to the OVA peptide. For our LEC stimulating conditions, we used peptide only for Steady State, peptide plus recombinant mouse IFNγ and TNFα for our Th1 condition, peptide plus recombinant mouse IL-4 and IL-13 for our Th2 condition, and peptide plus recombinant TGF-β and IL-10 for our suppressive condition. All cytokines were used at a concentration of 20ng/mL as per past lab experience and experimentation. LECs and mDCs were rinsed and washed with PBS before addition of the

CD8 OT1 cells.

Full Spectrum Flow Cytometry, Staining, and Analysis I first collected the cells from the experiments and plated them in a 96 well plate. I stained for dead cells utilizing Live Dead Blue fixable dye and stained for 25 minutes on ice in the dark. After the 25 minutes I washed the cells with FACs buffer (PBS + 2% FBS + 10mM EDTA) and spun the down at 2000 rpm for 5 minutes. After tossing the supernatant, I stained for surface molecules with antibody solutions made in Brilliant Stain Buffer for 30 minutes. I repeated the wash step and if chemokine receptors had to be stained I stained those with antibody solutions also made in Brilliant Stain Buffer for one hour at 37 °C . After washing again, I stain fix the cells and do intracellular staining using the manufacturer's protocol for the eBioscience Foxp3 / Transcription Factor Staining Buffer Set. Finally I re-suspend the cells in 100 uL of my FACs buffer, put them on bullet tubes and run them through our 5 laser Cytek Aurora.

LEC-T cell and BMDC-T cell cocultures: Once we had isolated both LECs and T cells, we pulsed them with the model antigen SIINFEKL and stimulating proteins (LPS for BMDCs and the aforementioned cytokines for LECs). We pulsed and stimulated the LECs and BMDCs overnight, then rinsed them with PBS. After T cell isolation, we CFSE labeled the OT1 CD8 T cells. Once the T cells were labeled and the APCs washed, we added the OT1s to the APCs and cocultured them for 72 hours. After 72 hour co-culture, we stained the cells for flow cytometry and analyzed the using the Cytek Aurora 5L Flow Cytometer. The general method is shown in 2.2. I did not made any changes to this protocol of co-culture throughout the thesis unless otherwise stated.

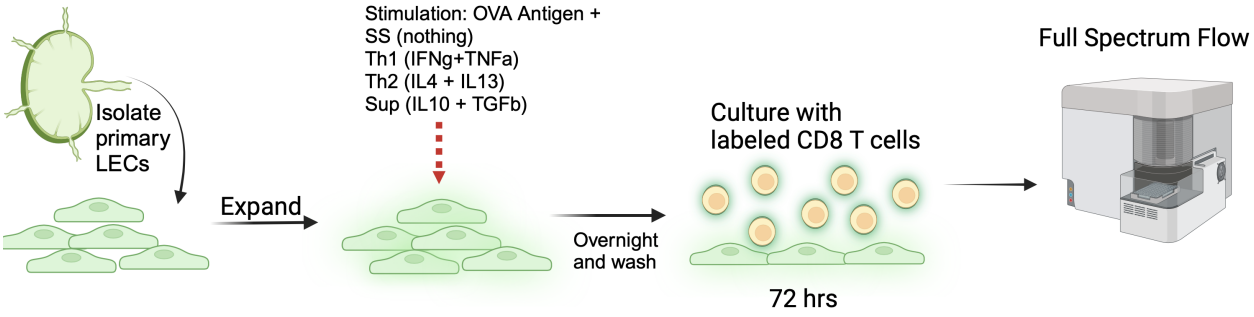

Figure 2.2: **Coculture General Method**

Proliferation index: I calculated the proliferation index by using the following equation. Where i is the generation number, and Ni is the number of T cells in that generation. We used CFSE to gate the generations and used those as i, where we have generations 0-5.

$$ProliferationIndex = \frac{\sum_0^i iN_i/2^i}{\sum_1^i Ni/2^i} \tag{2.1}$$

Mice: We obtained our lymphatic endothelial cells from WT C57BL/6 mice from Charles River labs, our BMDCs from C57BL/6 mice from UChicago's CIIC (Chicago Immunoengineering Innovation Center), and the OT1s from UChicago's CIIC bred CD45.1 OT1 transgenic mice. All mice used were 6-12 weeks old.

Statistics: For all experiments, otherwise stated each dot on the plot represents the LECS of one mouse interacting with OT1s, or mDCs, so each dot represents LECs from individual mice. We utilized Brown-Forsythe and Welch ANOVA tests, Dunnett's T3 multiple comparisons test, with individual variances computed for each comparison. We only show the important statistically significant comparisons. For those that are grouped together with a line, they were all statistically significantly different to the compared group, and they were not statistically significant against each other.

2.4 Results

2.4.1 Lymphatics surround high endothelial venules in the lymph node

Initially, our investigation focused on the feasibility and likelihood of encounters between lymphatic endothelial cells (LECs) and naive T cells. Lymphatic vessels are responsible for draining interstitial fluids, along with antigens and immune cells, but naive CD8 T cells do not follow this route. Instead, they enter lymph nodes via high endothelial venules (HEVs). Therefore, our objective was to determine the proximity of lymphatics to HEVs within lymph nodes since this inquiry is novel and conventional diagrams typically depict HEVs located within T cell zones.

Interestingly, our findings revealed that HEVs tend to cluster in regions closely surrounded by lymphatics, encompassing LECs, with several within the T cell zone itself. This was evident in steady-state lymph nodes, where the majority of HEVs (illustrated as red vessel-like structures) were encircled by lymphatics (depicted as green networks), with only a limited number deep within the T cell zones (denoted as the yellow zone) **Fig. 2.3A**.

Subsequently, we assessed whether this spatial arrangement persisted in post-vaccine and tumor-draining lymph nodes. We were gratified to observe that in lymph nodes examined three days post-vaccination, similar patterns emerged: most HEVs were either completely surrounded by or located in lymphatic-rich regions **Fig. 2.3B**, with few residing deep within T cell zones. This spatial distribution persisted in tumor-draining lymph nodes **Fig. 2.3C**. These observations highlight the high probability of naive CD8 T cell interactions with LECs prior to or during activation in T cell zones. Such findings underscore the physiological significance of investigating LEC-CD8 T cell interactions, as these entities are integral components of immune responses and are positioned in close spatial proximity.

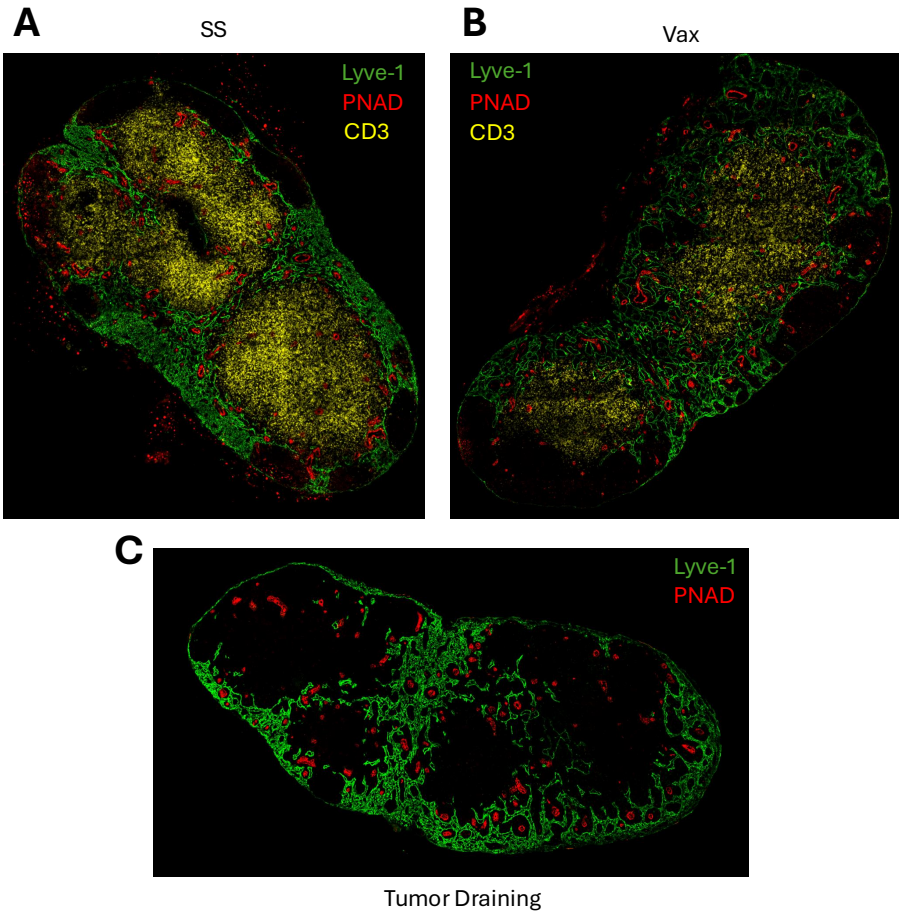

Figure 2.3: **Lymphatics surround HEVs in lymph nodes at steady-state, after vaccination, and in tumor draining lymph nodes** Lymph nodes stained with Lyve-1 a (LEC marker in lymph nodes) in green, PNAD (an HEV marker) in red, and CD3 in yellow. **A** Representative image of steady-state lymph nodes. **B** Representative image of vaccinated lymph nodes (OVA + CpG) **C** Representative of tumor draining lymph nodes (CD3 did not work in this panel)

2.4.2 Lymphatic endothelial cells in inflammatory environments induce a central memory phenotype and effector-like phenotype in suppressive environments

As previously indicated in Chapter One, our laboratory has demonstrated that, at a steady state, lymphatic endothelial cells (LECs) are capable of not only cross-presenting antigens to CD8 T cells but also activating and priming naive CD8 T cells into a central memory phenotype. Consequently, we sought to investigate whether LECs exhibit similar behavior in inflammatory microenvironments, which are critical for mounting effective immune responses against various pathogens, along with the cytokines present in the lymph node during these immune responses. To this end, we treated LECs with cytokines associated with Th1, Th2, and suppressive inflammatory environments as outlined in the introduction of this chapter.

We compared the activation of LECs in these contexts with that of mDC activation, as typically observed in an immune response, as well as with steady-state LECs (antigen only), which serves as our laboratory's control. Our observations revealed that upon activation by LECs treated in both Th1 and Th2 microenvironments, similar responses were elicited as when T cells are activated by LECs at steady state **Fig.2.4A**, while LECs stimulated in a suppressive environment exhibited contrary responses. The CFSE dilution is relatively consistent when comparing mDC, steady-state LEC, Th1 LEC, and Th2 LEC activation of T cells; however, diminished dilution is apparent when T cells are activated by LECs in suppressive environments **Fig.2.4A**. Notably, the response in terms of total T cell numbers indicates that LECs at steady state and mDC induce comparable responses, as similar numbers of T cells are present towards the conclusion of the 72-hour co-culture **Fig.2.4B**.

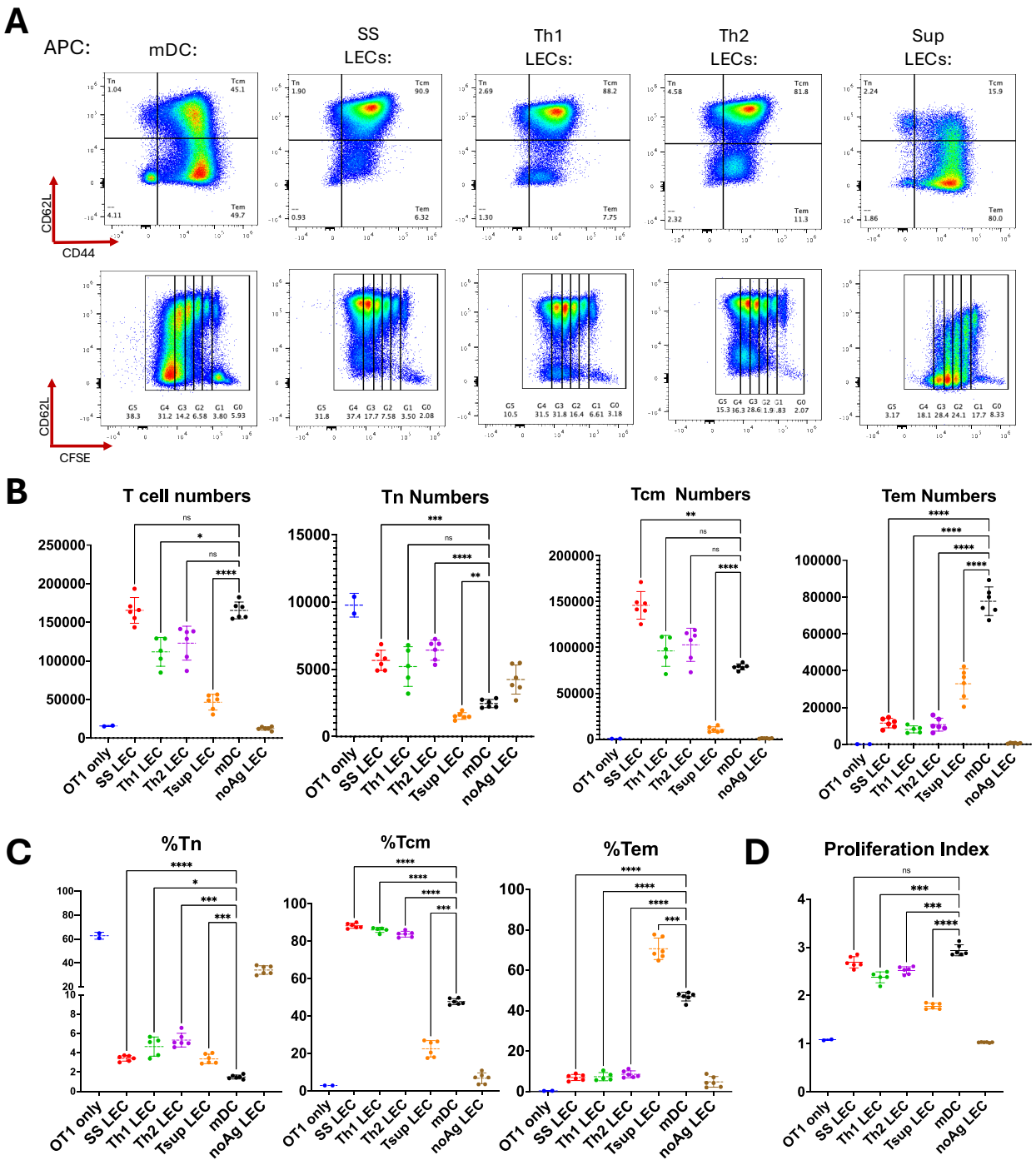

Figure 2.4: **LEC education of Naive CD8 T cells programs central memory in Th1 and Th2 cytokine environments and effector in Suppressive environments** **A** Representative flow plots of both the phenotype of the T cell pool as well as the CFSE dilution observed. **B** Quantified numbers of total T cells, naive T cells (Tn), central memory T cells (Tcm) and effector memory T cells (Tem) from A. **C** Percentages of naive, central memory and effector memory from the total T cell pool. **D** Proliferation index of the T cell pool after activation by the stimulated LECs or mDCs.

It is evident that LECs stimulated in Th1 and Th2 microenvironments result in a slightly reduced total number of T cells, whereas suppresive LECs induce T cell activation to a lesser degree, particularly in terms of proliferation and CFSE dilution. This response is comparatively weaker than that observed from T cell activation by SS, Th1 and Th2 LECs, and mDCs **Fig.2.4B,D**. Although statistical significance is attained only at steady state, we note a similar pattern where there is generally a higher number of central memory cells when naive CD8 T cells are activated by Th1 and Th2 LECs, and a greater presence of effector-like cells upon activation by mDCs **Fig.2.4B**. This analysis also includes OT-1 only and LEC without antigen conditions as negative controls to demonstrate that: first, LEC induce T cell activation in an MHC-TCR dependent manner and second, that OT-1 cells alone do not undergo proliferation or differentiation. When T cells are activated by suppressive LECs, the overall T cell count is lower compared to all other activation conditions. However, there is a notable development of effector memory cells (effector-like), albeit less than those resulting from mDC activation. It is also interesting to note that LEC activation across all environments appears to support the survival of a naive T cell population **Fig.2.4B**.

From the total T cell pool that expanded, LEC activation of naive CD8 T cells yielded exceptionally high percentages of central memory T cells when LECs were stimulated with Th1 and Th2 cytokines. If they were stimulated with suppressive cytokines they primed the T cells into a predominantly effector like pool, even at greater extents than mDCs, since mDC activation induces around a 50% central memory and 50% effector pool **Fig.2.4C**.

We were also interested in looking at the proliferation index of the CD8 T cells after the different activating conditions **Eq.2.1**. As expected, mDC activation induced the most proliferation however, LECs at steady state induced similar non statistically significant expansion of the CD8 T cells, albeit it trended lower. Th1 and Th2 LECs, those that were stimulated with inflammatory cytokines induced lower expansion than mDC and SS LEC activation, however it was significantly higher than T cells that were activated by LECs that had been

treated with suppressive cytokines, with those T cells having the lowest proliferation index of all the groups **Fig.2.4D**.

2.4.3 LEC activation of naive CD8 T cells results in a population that is highly stem like in nature in all cytokine microenvironments

Our interest extended beyond the general phenotypic characteristics of T cells (i.e., naive, central memory, effector) to a more profound understanding of the changes involved when naive CD8 T cells are activated by LECs. Considering prior evidence indicating that LECs in a steady state bestow a stem-like phenotype upon the activation of naive CD8 T cells, we aimed to assess whether this phenomenon persists in inflammatory and suppressive cytokine environments, as well as to examine the expression levels of several markers and receptors pertinent to memory and stem cell memory. An extensive panel was utilized for full spectrum flow cytometry, which encompassed various molecules including transcription factors, chemokine receptors, interleukin receptors, co-stimulatory molecules, inhibitory molecules, and proliferation markers **Fig.2.5A**. The primary markers of interest, which are discussed in detail throughout this thesis, were introduced earlier in the chapter: TCF1, PD1, Tim3, and CD27. Nonetheless, a comprehensive overview of all investigated markers is provided for completeness. We focused on three transcription factors: TCF1, EOMEs, and Tbet. TCF1 is predominantly present in naive T cells and recognized as a crucial transcription factor in the stem cell memory phenotype of CD8 T cells, endowing them with capabilities for self-renewal and differentiation into other CD8 T cell phenotypes. EOMEs and Tbet represent two transcription factors that operate synergistically to facilitate memory and effector functions, as well as differentiation signals [32, 45, 162].

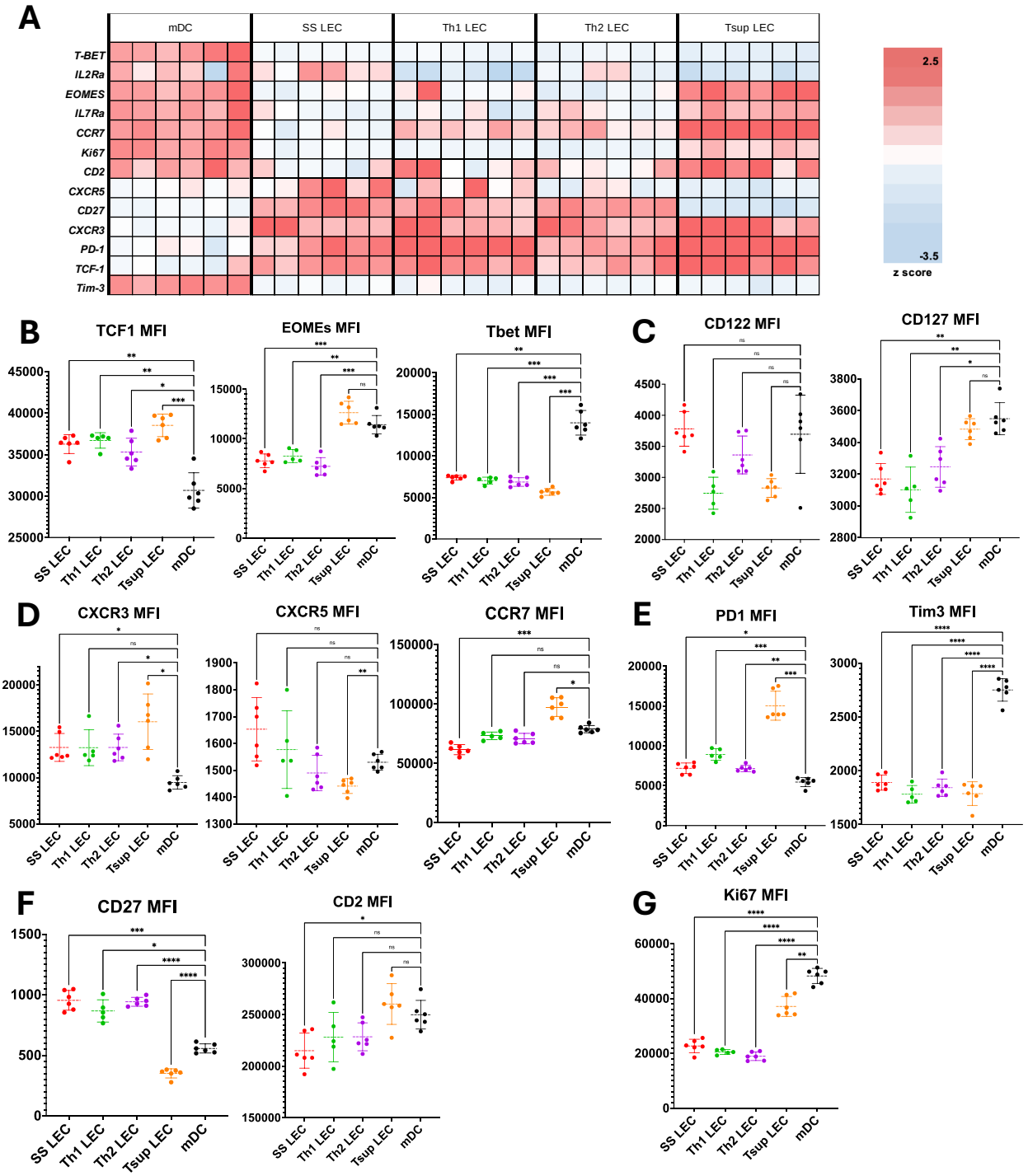

Figure 2.5: **LEC education programs receptors and transcription factors associated with stem like memory A** Heatmap of markers of interest and their relative expression across groups **B** MFI quantification of transcription factors. **C** MFI quantification of interleukin receptors. **D** MFI quantification of chemokine and cytokine receptors. **E** MFI quantification of inhibitory receptors. **F** MFI quantification of costimulatory markers. **G** MFI quantification of the proliferation marker Ki67.

A higher expression of EOMEs generally indicates a state of reduced differentiation, yet it also promotes effector function and is correlated with cell exhaustion at elevated levels, particularly when expressed by effector cells. Conversely, Tbet is linked to effector functions as well as functional responses, but is associated with more terminal differentiation without leading to exhaustion. LEC activation is observed to result in increased levels of TCF1, which is notable because this transcription factor is predominantly associated with stem cell stemness and the stem cell memory phenotype **Fig.2.5B**. LEC activation in Th1 and Th2 environments results in reduced expression of both EOMEs and Tbet, suggesting a generally less differentiated state that aligns with higher TCF1 expression levels. However, when LECs are initially stimulated with suppressive cytokines, they exhibit EOMEs expression levels similar to mDC activation but with diminished Tbet activation compared to other LEC conditions **Fig.2.5B**.

We also looked at several cytokine and chemokine markers as these are important in T cell function and some could point to certain phenotypes. LEC activation and mDC activation at all conditions promote similar levels of CD122 which is part of the IL2 and IL15 receptors which are important for T cell homeostasis and expansion, and LEC activation in inflammatory conditions lowers the expression of the IL7 receptor CD127 which is interesting as this is also important for T cell homeostasis and LECs are known to be sources of IL7 **Fig.2.5C**. Notably, IL7 receptor is downregulated upon IL7 contact. CXCR3 is up-regulated by T cells that were activated by LECs at all conditions, this is a chemokine receptor that is associated with better effector responses as well as the stem cell memory phenotype of CD8 T cells **Fig.2.5D**. CXCR5 does not change statistically in inflammatory conditions but trends towards increasing in expression, and it decreases when LECs are treated with suppressive cytokines **Fig.2.5D**. Interestingly, CCR7 is up-regulated also when LECs are treated with suppressive cytokines and decreases when LECs are treated with Th1 and Th2 cytokines compared to mDC activation **Fig.2.5D**.

We would not be thorough if we didn't investigate the expression levels of inhibitory receptors, so we wanted to look at PD1 and Tim3, both of which are inhibitory. PD1 is associated with activation and is one of the main checkpoints of the CD8 T cell response. Tim3 is another inhibitory marker that signals exhaustion particularly when co-expressed with PD1. Interestingly we can see that LEC activation at all conditions promotes the expression of PD1, a key marker of stem cell memory T cells as well, but it actually inhibits the expression of Tim3 **Fig.2.5E**. It seems like another feature of Tsup LEC activation is the over-expression of PD1, compared to all other activating conditions **Fig.2.5E**.

The expression of the co-stimulatory molecules CD2 and CD27 was of interest as they are both involved in memory CD8 functions. There is no statistical significance in the expression of CD2 but it trends down at all LEC conditions. CD27, a co-stimulatory molecule highly associated with memory formation and reactivation is up-regulated by T cells activated by Th1 and Th2 LECs, and down-regulated when they're activated by Tsup LECs **Fig.2.5F** when compared to mDC activation. This was interesting, as we observe more central memory when naive CD8 T cells are primed by Th1 and Th2 LECs, and more effector when Tsup LECs prime them. Finally, an intriguing finding was the expression of Ki67, a molecule found in highly proliferative cells or with a high proliferation potential. It was perplexing to find that of all LEC activating conditions, Tsup LEC activation induced the higher expression of Ki67 **Fig.2.5G** even though they possess the lowest proliferation index **Fig.2.4D**.

We were quite intrigued by the observation that TCF1 and PD1 increased and Tim3 dicreased when T cells were activated by LECs at all conditions compared to mDC activation, as clearly seen in the bottom three rows of **Fig.2.5A** especially since these are key characteristics of the relatively new phenotype of CD8 T cells known as stem cell memory T cells (Tscm) also known as precursor exhausted T cells (Tpex). This phenotype of T cell was introduced in chapter one, but as a refresher, they have been demonstrated to be important in the T cell response against cancer and chronic viral infections. These cells are

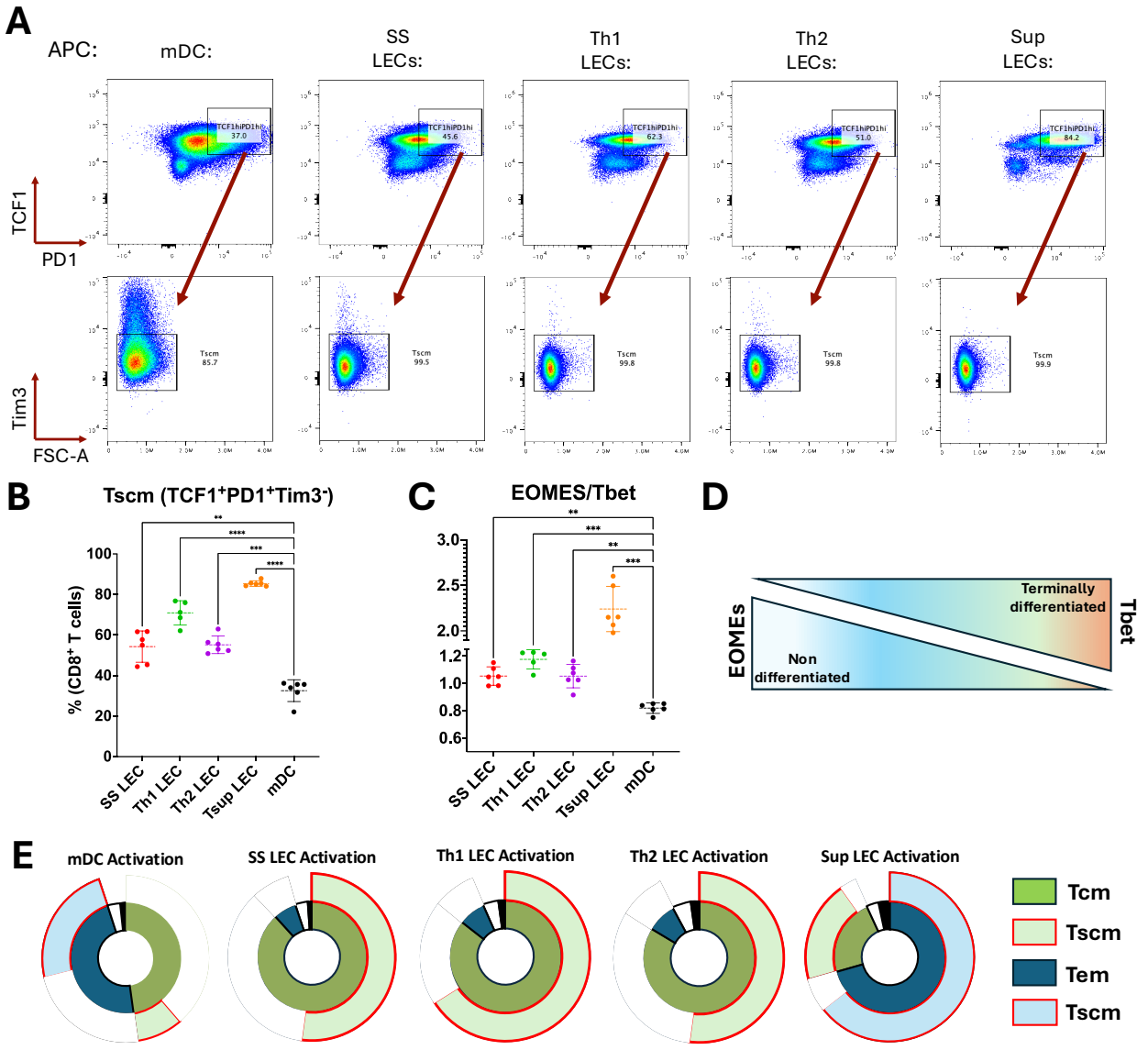

Figure 2.6: **LEC education programs a stem cell memory phenotype and less terminally differentiated T cells at all conditions A** Flow plots of stem cell memory T cells Tscm at all activating conditions. **B** Quantified percentage of Tscm in the overall T cell pool. **C** Ratio of EOMEs to Tbet MFIs denoting level of terminal differentiation. **D** Schematic of EOMEs and Tbet interplay in differentiation. **E** Sunburst charts showcasing the percentage of Tscm within Tcm and Tem.

responsible for proliferating and responding to some immunotherapies like PD1 checkpoint blockade, and it is clear that LEC activation of naive CD8 T cells at all conditions programs for this stem cell memory phenotype **Fig.2.6A,B,E**. In order to obtain this population of

T cells, we first gated the CD8 T cells on TCF1+ PD1+ double positive T cells as this shows that they are activated, and express the transcription factor associated with a less differentiated state and self renewal potential **A.1**. After that, we looked at their expression of Tim3 where the negative population represents the Tscm population **Fig.2.6A**. It was clear just by looking at the flow plots that LEC activation of T cells induced a high percentage of Tscm, particularly by Tsup LECs. After quantifying the percentage of the total T cell pool that is Tscm, we can conclude that LECs program for this phenotype, as no less than 50% of the population is this phenotype in all LEC environments, with an average of around 70% when Th1 LECs activate T cells and around 80% when Tsup LECs activate T cells **Fig.2.6B**. This discovery is noteworthy as it elucidates a potential role that LECs may assume in the modulation of the immune response in the lymph node.

Since stem cell memory T cells are considered less differentiated, we looked at the ratio of EOMEs and Tbet, since a higher expression of EOMEs relative to Tbet is observed in less differentiated T cells, whereas an increase in Tbet expression coupled with a decrease in EOMEs expression is indicative of more terminal differentiation in T cells, as demonstrated by **Fig.2.6D**. It is evident that the activation of T cells by LECs across various environments leads to a higher ratio of EOMEs to Tbet compared to mDC activation **Fig.2.6C**. We also show that Tsup LECs induce a higher ratio of EOMEs to Tbet than any other condition, which is consistent with the fact that they also induce higher EOMEs expression overall **Fig.2.5B**.

Finally, we examined the distribution of stem cell memory T cells within central memory and effector-like T cells, particularly because although LECs elicit entirely opposite phenotypes **Fig.2.4A,C**, they uniformly give rise to high percentages of Tscm **Fig.2.6B**. As expected, in T cells activated by Th1 and Th2 LECs, the Tscm population originates from the central memory compartment, and in T cells activated by Tsup LECs the majority come from the effector memory pool, while a small fraction comes from the central memory pool.

It is apparent that LECs program for this phenotype in more than 50% of the naive T cells that interact with them in an antigen-dependent priming manner, while mDC priming results in approximately 30% Tscm **Fig.2.6E**.

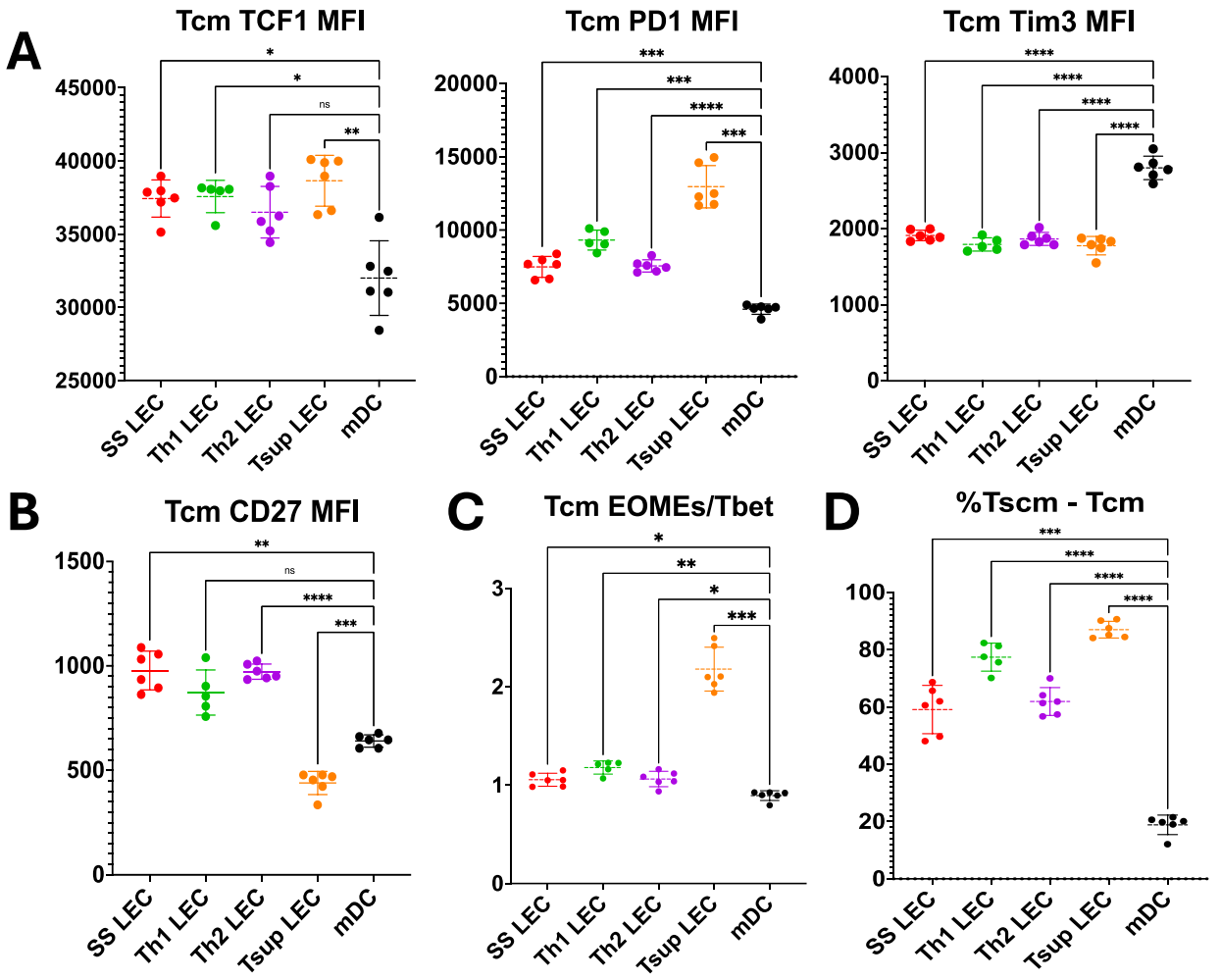

Figure 2.7: **LEC induced central memory T cells exhibit stem cell memory qualities A** Quantified stem cell memory associated markers in the Tcm compartment. **B** Quantified CD27 MFI, on of the only markers that was different across LEC activating conditions. **C** Ratio of EOMEs to Tbet MFIs denoting level of terminal differentiation. **D** Percentage of central memory cells that have stem cell memory qualities.

We sought to investigate both the central memory and effector memory compartments directly. As observed in **Fig.2.6E**, the proportion of stem cell memory within these compartments is notably substantial when LECs prime T cells, with more than 50% of the total

T cell compartment acquiring stem cell memory characteristics. When we looked at the central memory compartment, we noticed that LEC priming induced high TCF1 expression in all stimulating conditions, as well as higher levels of PD1 than mDC activation, and lower expression of Tim3, all of which point to the stem cell memory phenotype as well **Fig.2.7A**. As previously observed, a notable distinction in expression within LEC-activated T cells across all conditions (with the exception of CD62L which differentiates Tcm from Tem) was the expression of CD27. This pattern was equally evident within the central memory compartment, where Th1 and Th2 LEC activation of T cells prompted the expression of CD27. Conversely, Tsup LEC activation resulted in the down-regulation of CD27, all in comparison to mDC activation **Fig.2.7B**. In general, the ratio of EOMEs to Tbet is elevated in LEC-activated central memory T cells, indicating a lower level of differentiation compared to mDC activation, in which the ratio approaches 1 **Fig.2.7C**. Finally, central memory T cells induced by LEC activation exhibit a markedly elevated stem cell memory phenotype compared to mDC activation **Fig.2.7D**.

Similarly, we probed the effector compartment induced by LEC activation and compared it to mDC activation. We do not observe much Tem formation by Th1 and Th2 LEC priming, however, from the Tem that arise, we observe that those have significantly lower TCF1 expression than Tem that arise from both Tsup activation, and mDC activation, with Tsup activation inducing the highest expression of TCF1 across all groups **Fig.2.8A**. The PD1 expression mirrors TCF1 expression, where inflamed LECs induce low expression of PD1 compared to Tsup LECs that induce the highest expression of the inhibitory receptor in the effector memory compartment **Fig.2.8A**. Tim3 mirrors what we see not only in the central memory compartment, but also in the overall T cell compartment, where LECs induce low expression of the inhibitory molecule at all conditions **Fig.2.8A**. Interestingly, even though we do not observe much effector differentiation after Th1 and Th2 LEC activation, we still observe an increase in the expression of CD27 compared to mDC activation, and Tsup LECs

induce the lowest expression out of all of the activating conditions **Fig.2.8B**. Even though Th1 and Th2 LECs induce the most central memory, the few effector cells seem to be more terminally differentiated by the EOMEs to Tbet ratio. They have the lowest ratio, meaning they express more Tbet compared to EOMEs even compared to mDC activation, and Tsup LECs once again induce the highest ratio, showing that the effector cells they produce are less terminally differentiated **Fig.2.8C**.

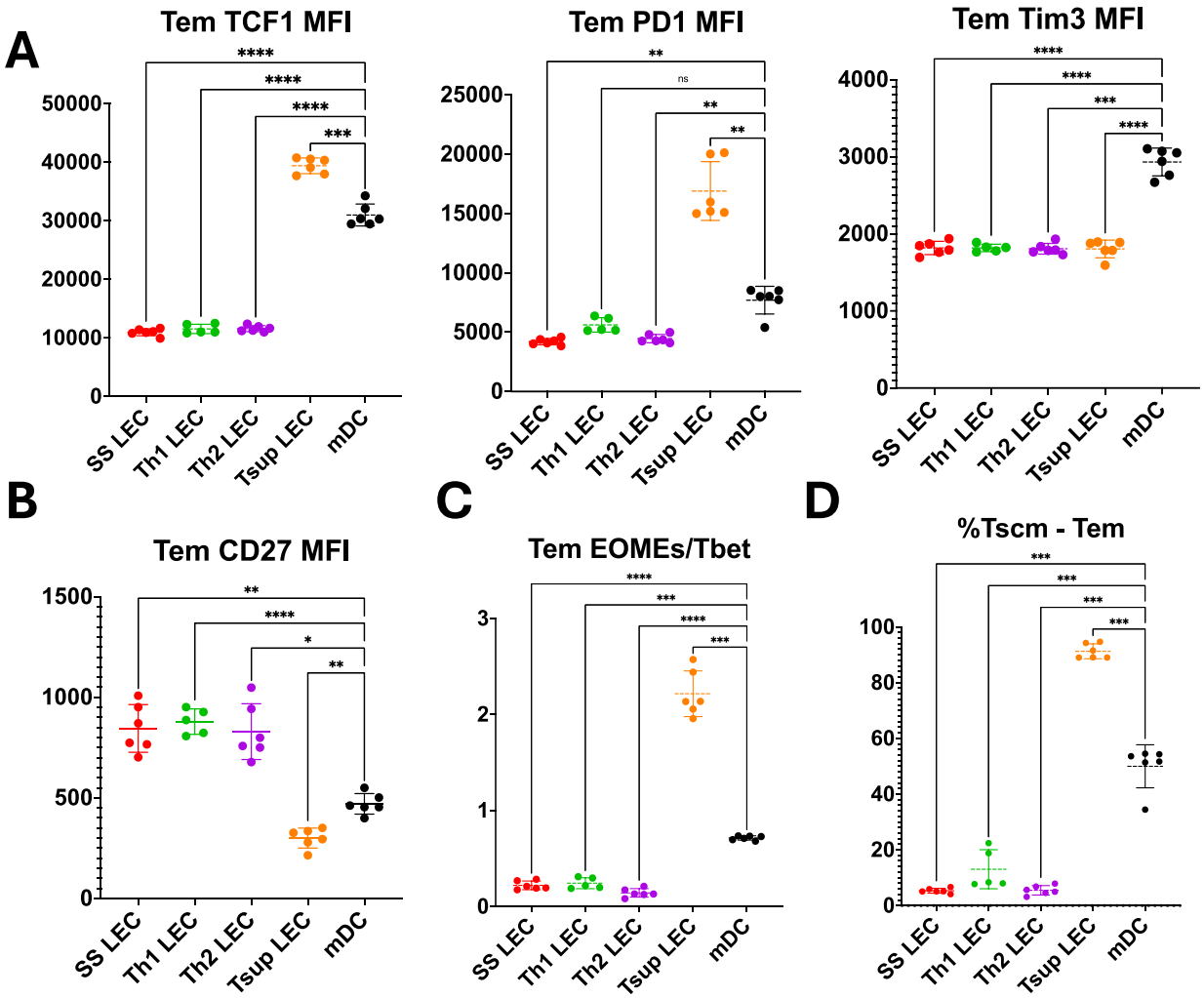

Figure 2.8: **Suppressive LEC induced effector-memory T cells exhibit stem cell memory qualities** **A** Quantified stem cell memory associated markers in the Tem compartment. **B** Quantified CD27 MFI, on of the only markers that was different across LEC activating conditions. **C** Ratio of EOMEs to Tbet MFIs denoting level of terminal differentiation. **D** Percentage of effector memory cells that have stem cell memory qualities.

Finally, Tsup LECs induce the highest percentage of stem cell memory like cells, where almost 90% of the effector population can also be defined as stem cell memory by our standards, while Th1 and Th2 LECs induce a low percentage of Tscm from the already low number of effector cells they prime **Fig.2.8D**.

2.4.4 Dynamics of the CD8 T cell response upon LEC activation

Our research aimed to examine the dynamics of T cell responses and the expression dynamics of markers on T cells. The utilization of CFSE staining proved to be an invaluable technique, as it enabled us to gate individual generations. This allowed us to examine the marker expression and phenotype distribution across each generation.

The difference in the responses between mDC activation and LEC activation of naive CD8 T cells is conserved across LEC activating groups, at least in the dynamics of the general response. When LECs activate T cells, the expansion of T cells is relatively linear, peaking at generation three (Th1 and Tsup) or four (SS and Th2), while the expansion of T cells is more exponential when mDCs activate the naive T cell pool **Fig.2.9A**. The difference in the dynamics is interesting, as the expansion not only slows down but also decreases as the response unfolds when LECs activate T cells, but on the other hand, when mDCs activate T cells the response and expansion of T cells increase after each generation. The central memory compartment behaves similarly to the total T cell numbers when it comes to the groups that experienced LEC activation, observing the same trends **Fig.2.9B**. When the T cells were activated by mDCs, the central memory compartment mirrors LEC activation, peaking in numbers at generation 4 and then decreasing, but when we look at the effector compartment we clearly see that effector cells arise on generation 3 and increase linearly when mDCs activate naive CD8 T cells **Fig.2.9B**. We can distinctly see that SS, Th1 and Th2 LECs induce almost no effector cells, however Tsup LECs induce effector memory cells that peak at generation 3 in terms of numbers, and decrease after; however, not to the extent

that mDC activation induces effector cells **Fig.2.9B**. We included

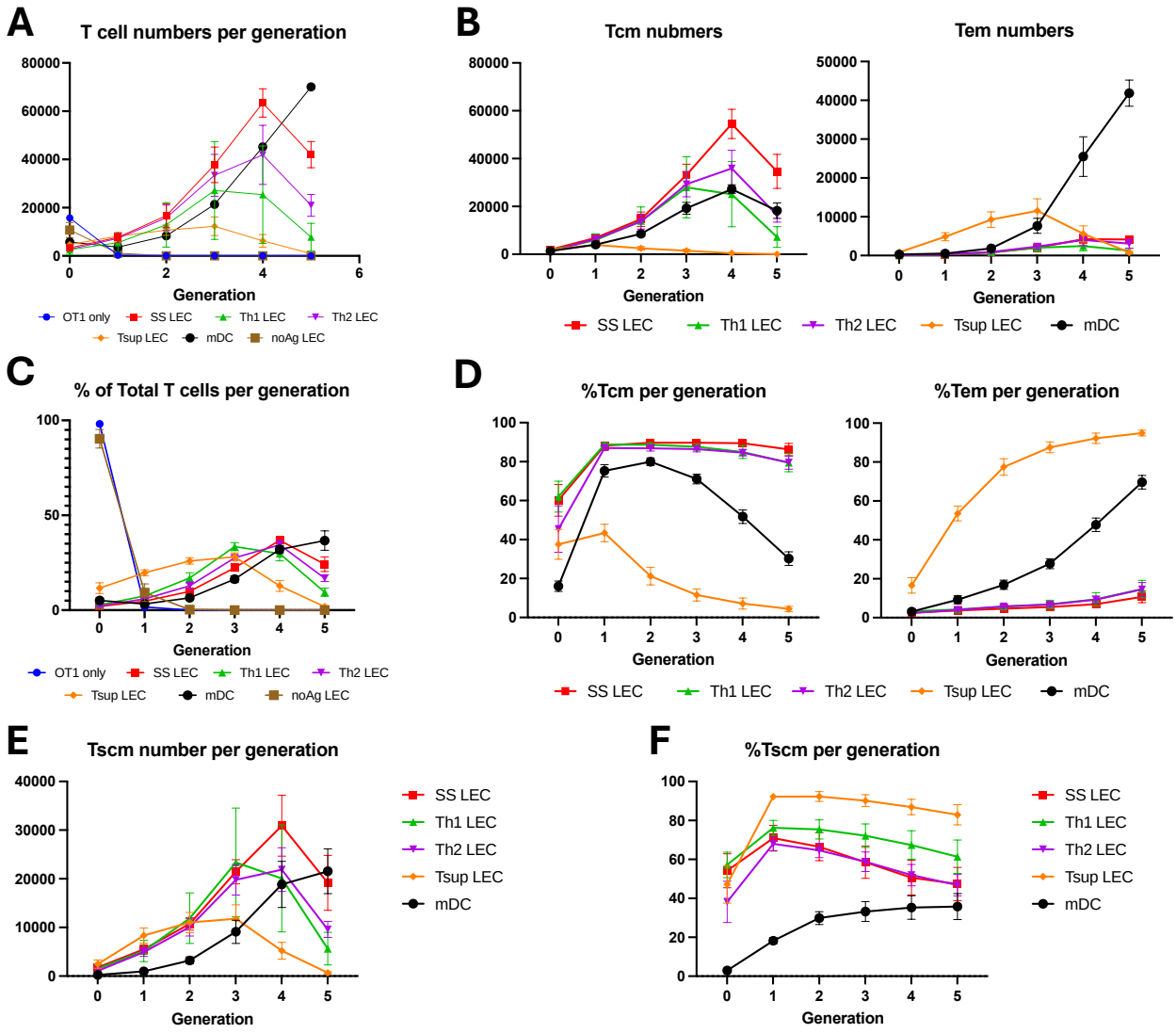

Figure 2.9: **Generational breakdown of T cell phenotypes A** Numbers of T cells in each generation. **B** Numbers of both central memory and effector memory cells in each generation. **C** Percent of the total T cell pool per generation. **D** Percent Tcm and Tem per generation. **E** Numbers of Tscm in each generation. **F** Percent Tscm per generation.

the OT1 only as well as the LEC with no antigen controls to show that they do not induce a response, all or the majority of the T cells lie within the first generation and they do not expand **Fig.2.9A,C**. We see that the percent of total T cells mirrors the T cell numbers response as expected **Fig.2.9C**.

An examination of the percentage distribution of central memory and effector memory across generations reveals that SS, Th1, and Th2 LECs elicit a response transitioning to central memory immediately following the first generation, sustaining this state throughout the response **Fig.2.9D**. In contrast, mDC activation leads to a central memory population that reaches its peak in the second generation followed by a percentage decline in the third generation **Fig.2.9D**.The LECs that were stimulated with suppressive cytokines induced a growing effector population throughout the response, and a central memory population that peaked at 40% in generation 2 and decreased from there **Fig.2.9D**.

Finally, we wanted to check the stem cell memory phenotype that we've described. We see that the stem cell memory numbers completely mirror the dynamics of the overall T cell numbers **Fig.2.9E**, which is consistent with the fact that the majority of the T cells that develop with LEC priming are stem cell memory in nature. It is clear when we look at the percentage of T cells that express stem cell memory characteristics defined by TCF1+PD1+Tim3-, that education and priming of naive CD8 T cells results in a T cell population that is programmed to be stem cell memory in nature. The percentage of stem cell memory peaks at generation one in all LEC activating conditions, with Tsup inducing the highest percentage of Tscm throughout the response, while SS and Th2 behave almost identically **Fig.2.9F**. Th1 LECs induce a response that lies in between Tsup and the other two (Th2 and SS), however the numbers of Tscm still peak at generation one and steadily decrease as the response unfolds, while mDCs induce a Tscm population that steadily increases however it never reaches the levels that all LEC activating conditions do **Fig.2.9F**.

Altogether, this shows that inflammatory (Th1 and Th2) LECs directly induce and maintain a central memory population while Tsup LECs induce a growing effector memory population. And all LEC conditions induce and maintain a Tscm population.

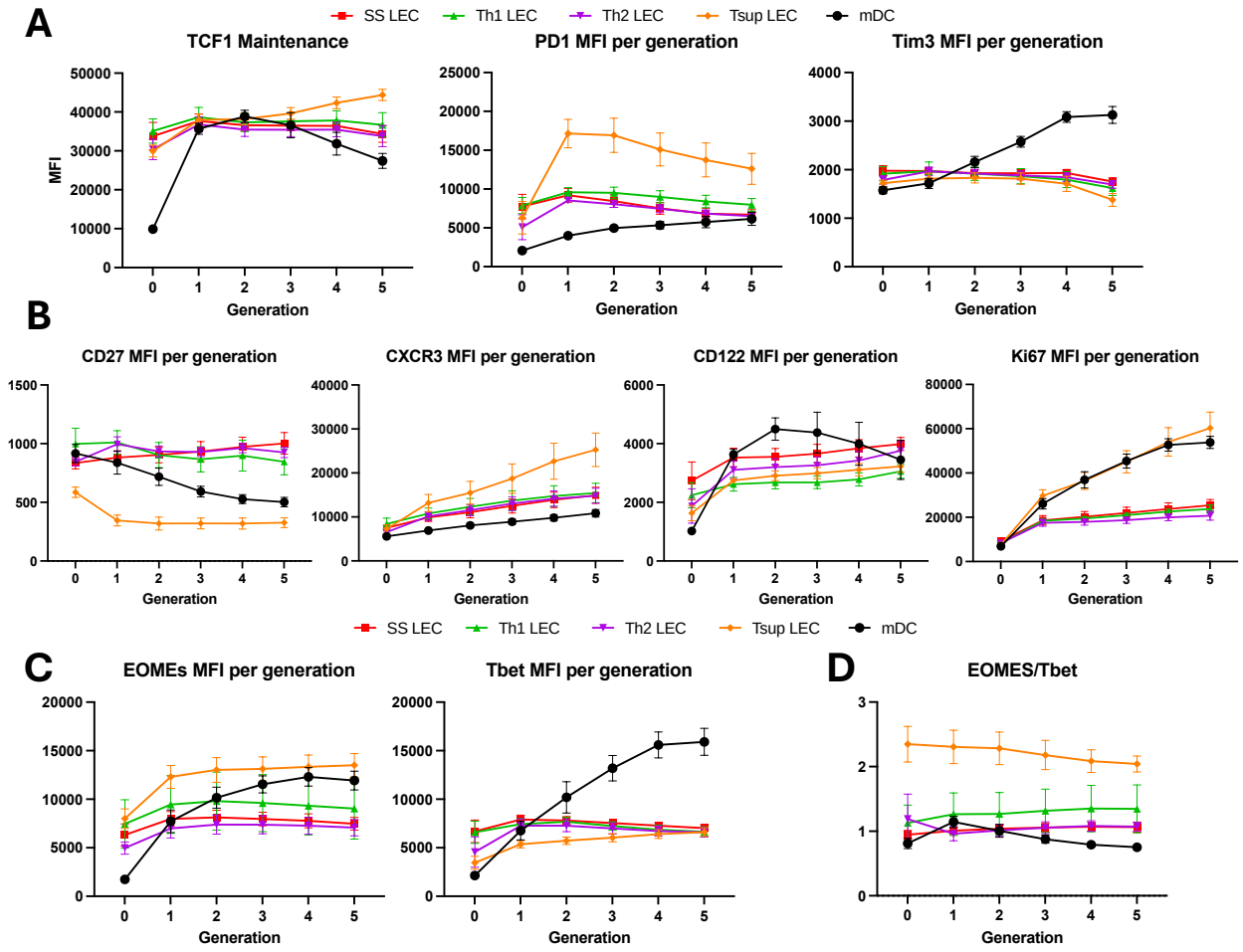

Figure 2.10: **Generational breakdown of T cell marker expression A** Generational expression of Tscm associated markers. **B** Generational expression of markers of interest. **C** Generational expression of differentiation transcription factors. **D** Generational ratio of EOMEs to Tbet.

Aside from the general breakdown per generation, we also wanted to look at the generational marker expression of the T cells as they were activated for 72 hours. As demonstrated in **2.9C,F**, LEC education resulted in a substantial proportion of Tscm within each generation. The observation that LECs also generate a high expression of TCF1 in each generation was not unexpected; however, it should be noted that LECs not only cause an elevated expression of this transcription factor upon activation, but also maintain it throughout the response. In contrast, mDC activation leads to TCF1 expression that reaches its peak in the

second generation, subsequently declining as the response progresses **2.10A**. As expected by the general expression of PD1, we also observe that LEC activation at all conditions induces a high expression of PD1 immediately upon activation, peaking in generation one and steadily decreasing as the response unfolds, with Tsup LECs inducing the highest expression of all conditions, and Th1 and Th2 LECs inducing the same as SS LECs **2.10A**. mDC activation seems to induce a steady increase of PD1 that does not reach the maximum expression of LEC activation towards the end of the response, however it induces a Tim3 expression that increases significantly after the second generation while LEC activation maintains a low Tim3 expression throughout the response **2.10A**.

In addition to the stem cell memory-associated markers that we were highly interested in, we also looked at the expression of other markers that we found interesting in **2.5**. Since CD27 was one of the only markers that differed between LEC groups we were interested in looking at the dynamics of it. SS, Th1, and Th2 LECs induce high expression of CD27 and maintain it throughout the response, and on the other hand, Tsup LECs induce a low expression that decreases further as the response unfolds. The same is true for mDC activation but at relatively higher levels than Tsup activation **2.10B**. The CXCR3 chemokine receptor grows generationally in all LEC activating conditions, with Tsup inducing higher expression than all towards the end of the response and mDC activation follows the same trend but at lower levels in all generations **2.10B**. The expression of the IL2Rβ receptor CD122 which is the receptor for IL2 and IL15 was quite interesting. Even though all conditions induce non statistically significant levels of CD122 **2.5C**, the dynamics of the marker are completely different between LEC activation and mDC activation. When LECs activate the T cells, we see the same dynamics, where CD122 increases generationally at all conditions while mDC activation peaks in CD122 expression at generation 2 and then decreases as the response winds down **2.10B**. Lastly, since we saw Ki67 expression differ between inflammatory and suppressive LEC activating conditions, we wanted to look at the kinetics and dynamics of the

marker expression as well. It was surprising to observe that despite Tsup LECs promoting the lowest proliferation index, they simultaneously elicited the highest expression of Ki67 throughout the response across all LEC groups, paralleling the pattern observed in mDC activation **2.10B**.

The examination of the expression of the transcription factors EOMEs and T-bet, as well as their ratios proved to be intriguing. We see that inflammatory LECs induce a relatively maintained expression of EOMEs, while both Tsup LECs and mDCs induce a growing expression of EOMEs. In particular, in terms of Tbet, mDC activation elicits a markedly different expression pattern, which increases significantly as the response unfolds, while all LEC activating conditions induce a low expression of Tbet that all converged to the same level towards the end **2.10C**. Analyzing the ratio of these transcription factors to assess T cell differentiation reveals that mDC activation induces a population whose less differentiated cells peak in the first generation after initial activation, while inflammatory LECs induce a relatively maintained population that expresses more EOMEs over Tbet. Tsup LEC activation results in a T cell population which expresses significantly more EOMEs than Tbet, resulting in a highly undifferentiated effector pool which is of interest as this would be counterintuitive **2.10D**.

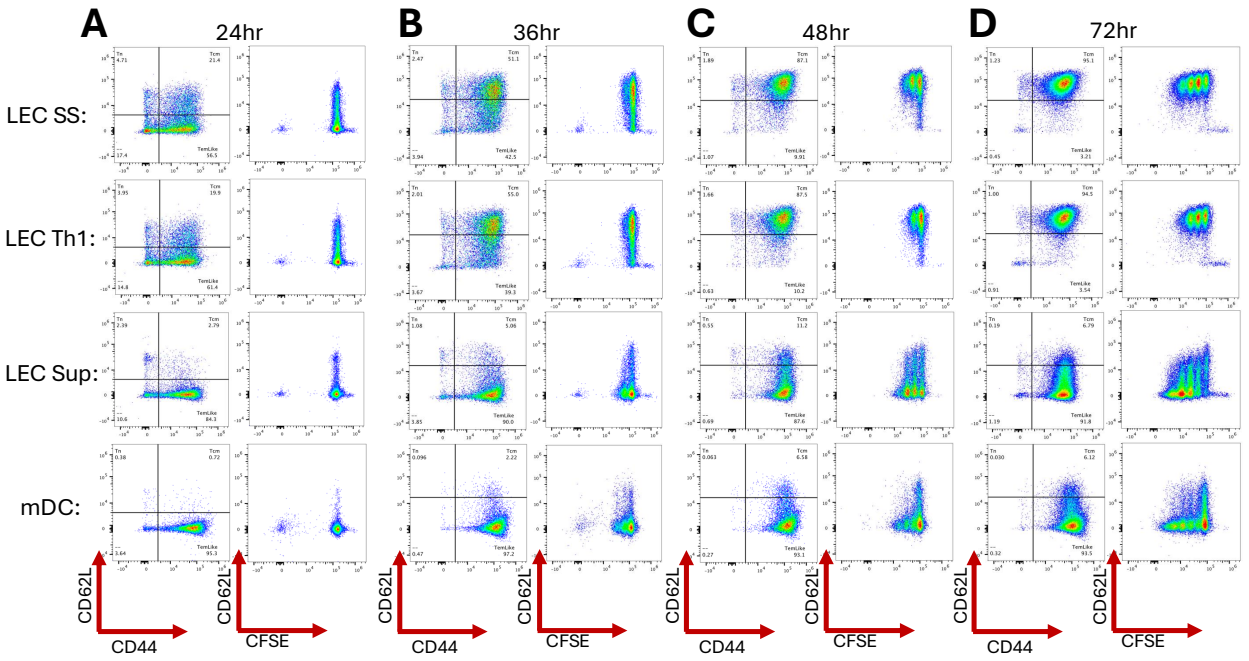

Figure 2.11: **Timeline evolution of CD8 T cell response in 72 hours** General phenotype breakdown of T cells and CFSE dilution showing division after activation for **A** 24 hours, **B** 36 hours, **C** 48 hours, and **D** 72 hours.

Although we can monitor the response using CFSE, there remains a possibility of overlooking certain phenotypic differences, particularly due to the reliance on endpoint analyses in recent figures. This constraint might have led to the omission of populations that die at earlier time points. To address this potential issue, we conducted a straightforward timeline experiment, where T cells were collected at 24 hours, 36 hours, 48 hours, and 72 hours following the initial co-culture. Given the limited availability of LECs, and the observed similarity in behavior between Th1 and Th2 LECs, the study was restricted to the Th1 condition while maintaining the SS condition as the LEC control, alongside Tsup LECs and mDCs.

It is noteworthy that after 24 hours, CD62L shedding is observed prior to the first cell division upon activation, a phenomenon previously documented to occur when CD8 T cells are effectively activated. This observation is consistent in all groups **2.11A**.[121] Although T cells had not yet undergone division, it is evident that within the initial 24-hour period, they had already received signals from LECs and mDCs, as CD62L shedding serves as an

indicator of activation signals. Upon the first division at 36 hours, we observed that SS and Th1 LECs have already started programming the central memory phenotype seen at the end of the response, whereas Tsup LECs and mDCs initially program for an effector-like phenotype **2.11B**. After 48 hours the T cells have divided two more times and we observe the same trends, SS LECs and Th1 LECs induce an already central memory population by day 2, while Tsup LECs and mDCs induce and effector like population with a growing central memory population **2.11C**. Finally, at 72 hours after the full response and T cell expansion, we observe what we traditionally see, SS and Th1 LECs induce a predominantly central memory T cell population while Tsup LECs induce a predominantly effector memory T cell pool.

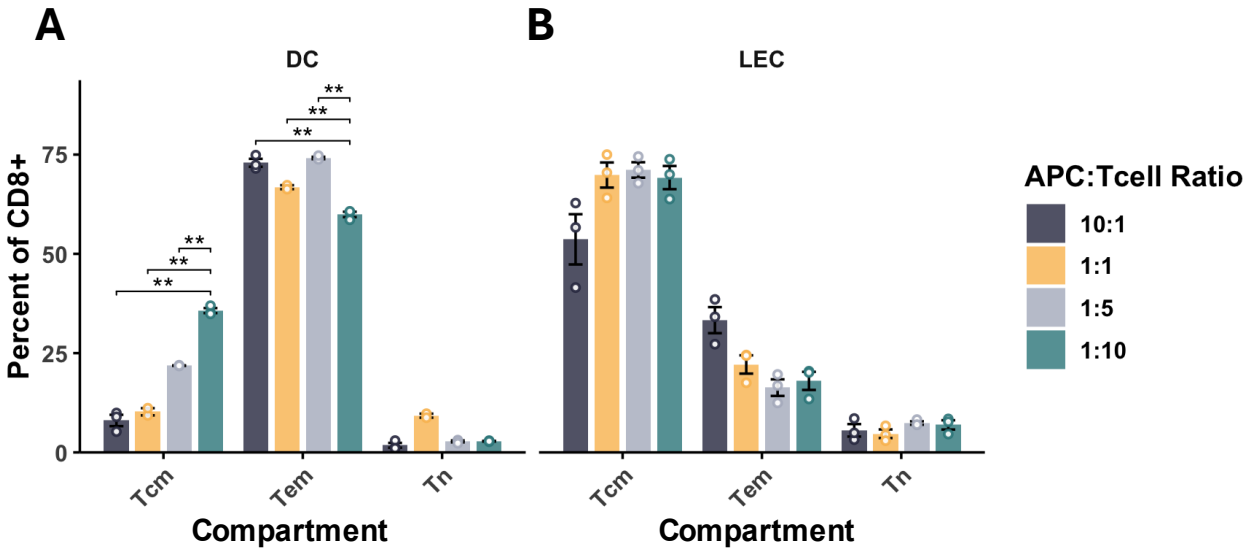

Figure 2.12: **Phenotypes of T cells activated by different ratios of APCs A** Phenotype of T cells activated by different ratios of mDCs to T cells **B** Phenotype of T cells activated by different ratios of SS LECs to T cells.

In investigating the dynamics of the overall T cell response, we sought to determine whether varying the ratios of APCs to T cells elicited different outcomes. Initial experiments were conducted exclusively with SS LECs to evaluate the potential significance of this research direction. Interestingly, regardless of the ratios of LEC to T cells employed (10:

1, 1:1, 1: 5, 1:10), SS LECs consistently induced a central memory phenotype **2.12B**. In contrast, the ratio was significant in the context of mDC activation, where a higher central memory response was observed with fewer mDCs interacting with T cells, while a higher presence of mDCs favored the development of effector cells **2.12A**.

2.4.5 LECs confer their associated phenotype regardless of activation of naive T cells or maintenance of 24hr activated CD8 T cells

Upon observing that a 24-hour LEC activation induced proficient shedding of CD62L **Fig.2.11A** before the first round of division, we sought to determine if 24 hour contact was enough for LECs to program the CD8 T cells into the phenotypes associated with their stimulation condition (Tcm for SS, Th1, and Th2 LECs, and Tem for Tsup LECs). In order to do this, we designed an experiment in which we activated the naive CD8 T cells with LECs for only 24 hours, then carefully removed them from the LEC containing wells, and moved them to empty wells where they could finish the response for the remaining 48 hours of the co-culture, as depicted in **Fig.2.13**.

It was unexpected to observe that LEC activation for 24 hours was sufficient to establish central memory. The 24 hour flow plots looked quite similar than our 72 hour co-culture plots, however since the population was lower in numbers, we had to use the contour feature in order to fully visualize it **Fig.2.14A**.

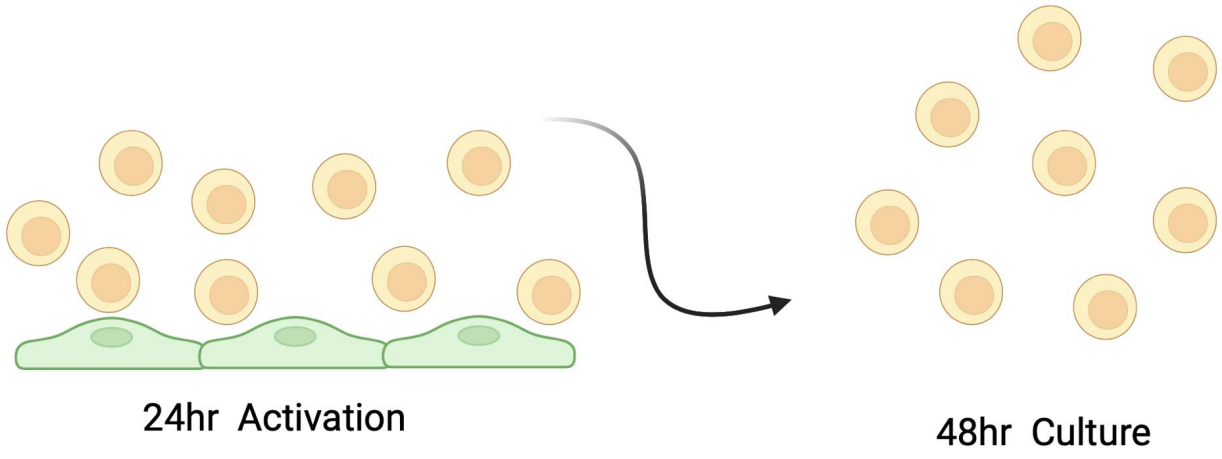

Figure 2.13: **Schematic of 24 hour LEC activation experiment**

SS and Th1 LECs induce non statistically significant differences in central memory formation **Fig.2.14C**, with the majority of the population developing into the phenotype and the differences being due to a higher number of naive cells left over (assuming that not all of them were able to interact with the LECs) and the effector memory population was maintained the same **Fig.2.14B,D**. Interestingly, suppressive LECs that interacted with T cells for only 24 hours induced a higher percentage of Tcm than their 72 hour co-culture counterparts **Fig.2.14C**. We also observe a decrease in the Tem population that Tsup LECs induce which is consistent with said increase **Fig.2.14D**. Even though there was an increase in the central memory compartment, it was not to the extent of SS and Th1 LECs.

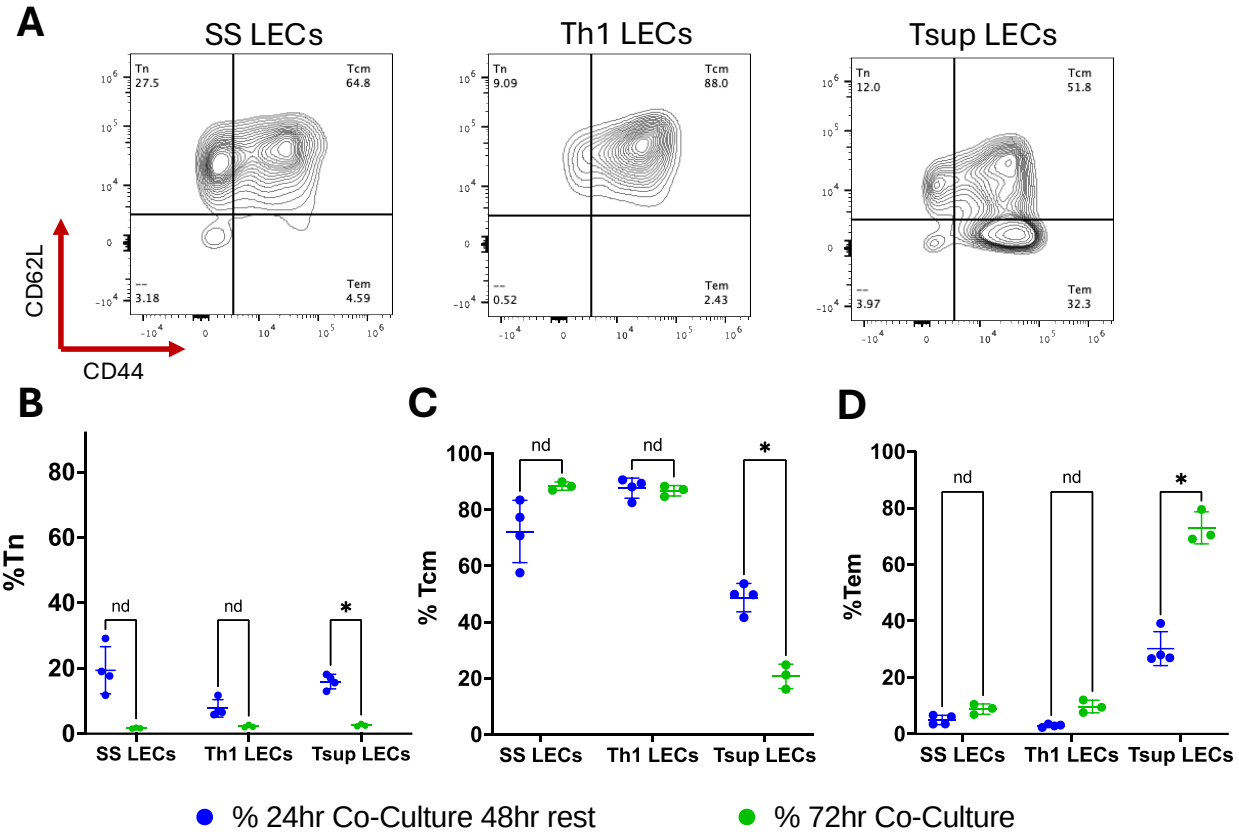

Figure 2.14: **LEC activation for 24 hours is enough to program LEC associated phenotypes A** Flow plots depicting the phenotypes based on CD62L and CD44 after LEC education for only 24 hours only. **B** Quantified percentage of naive CD8 T cells left over. **C** Quantified percentage of the central memory compartment. **D** Quantified percentage of the effector memory phenotype. All were compared to 72 hour co-culture as the control.

After observing that LEC activation for 24 hours was sufficient to confer a central memory phenotype, we wanted to test the stability and intensity of this programming. So the first question we asked was if mDC maintenance of LEC activated T cells ablated the phenotype conferred by LECs. To this end, we designed an experiment in which we activated naive CD8 T cells with LECs, and then carefully moved them from the LECs and moved them to wells containing matured Dendritic cells that had been pulsed with the SIINFEKL peptide **Fig.2.15**.

It was unexpected to observe that even though mDCs are considered to be a quite strong antigen presenting cell, LEC activation for 24 hours still conferred their LEC associated

phenotype. Even though not to the same extent as when T cells are primed by LECs for the full 72 hours, which results in about 85-95% central memory, T cells that were first activated by SS, Th1 and Th2 LECs and then maintained by mDCs developed into around 60-75% central memory **Fig.2.16A**. In this case, Tsup LECs activation and mDC maintenance also conveyed an effector memory phenotype, however it was to the same extent as mDC only activation **Fig.2.16B**. We see that even though the T cells interacted with mDCs, an APC that normally activates more than 90 percent of the T cell pool, initial LEC activation also maintained a naive T cell population alive at all LEC conditions compared to mDC only activation **Fig.2.16C**.

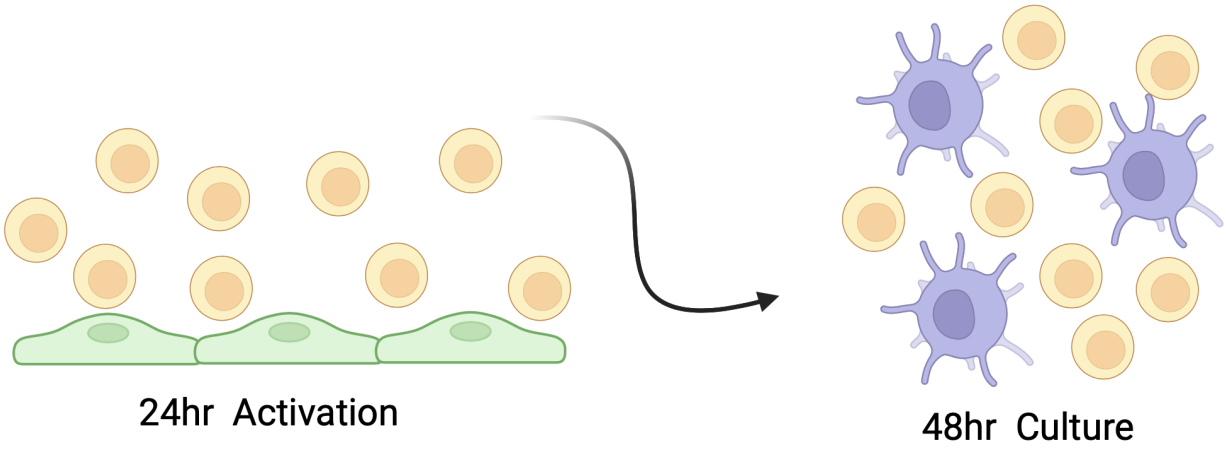

Figure 2.15: **Schematic: mDC maintenance of LEC activated T cells**

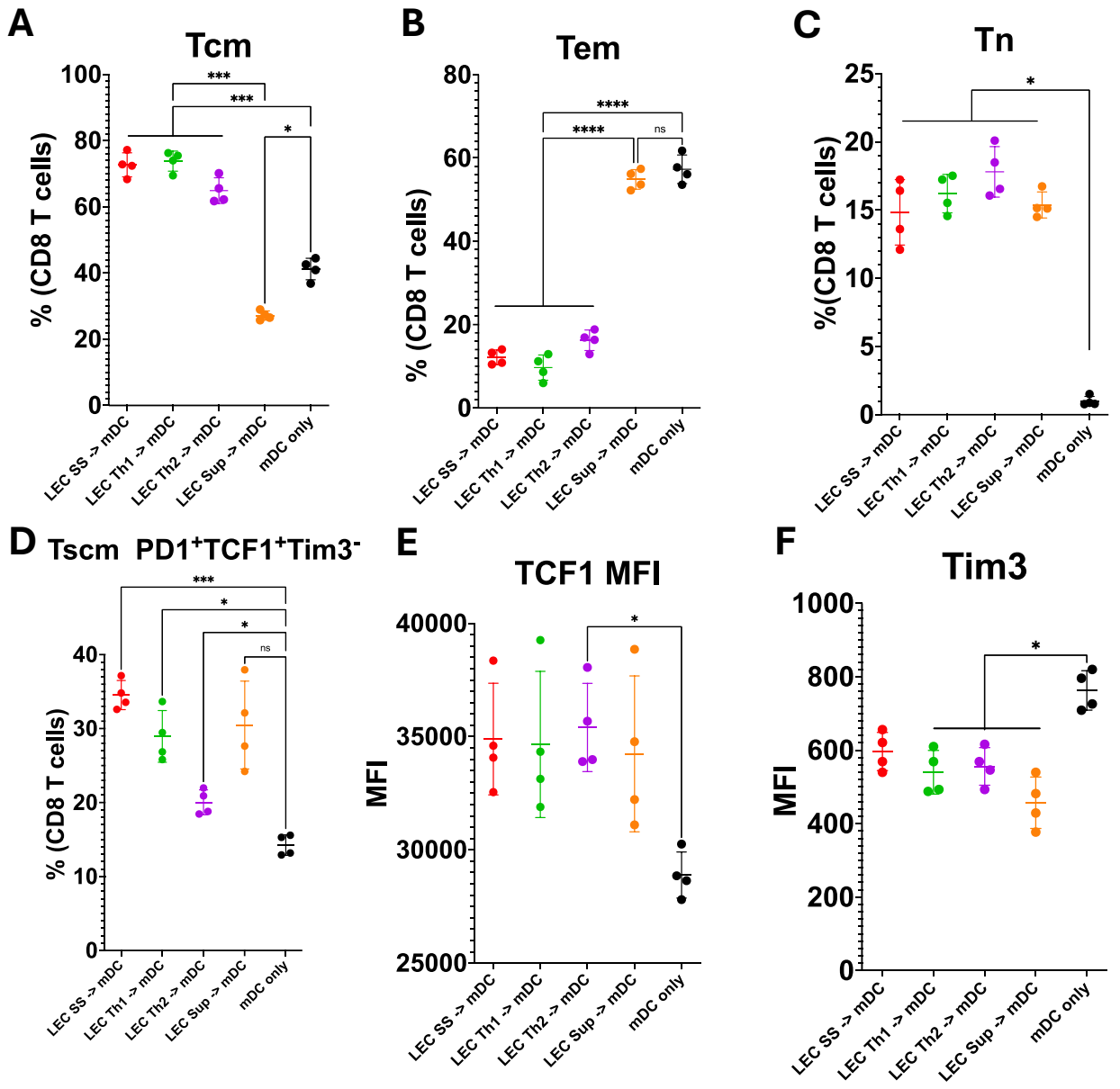

Figure 2.16: **LEC activation for 24 hours programs associated phenotypes regardless of mDC maintenance A** Quantified percentage of the central memory compartment. **B** Quantified percentage of the effector memory compartment. **C** Quantified percentage of the naive compartment. **D** Quantified percentage of the stem cell memory cells from the overall T cell pool. **E** Quantified MFI of the TCF1 expression by all T cells. **F** Quantified MFI of the Tim3 expression by all T cells.

Although mDC maintenance appeared to ablate the statistically significant increase in TCF1 in most LEC activation conditions (excluding Th2 LECs), the trend holds, with initial LEC

activation inducing higher expression of TCF1 compared to mDC activation only. We observe a statistical decrease in the exhaustion molecule Tim3 under all LEC activation conditions, except SS LECs. With this, the stem cell memory like cells that developed from initial LEC activation and mDC maintenance is statistically higher than mDC activation alone at all conditions except Tsup LEC activation, although it still trends higher **Fig.2.16D**; however, the percentage is still lower than when LECs activate T cells for the full 72 hours. All together, these data show that initial LEC activation is sufficient to not only confer central memory at SS, Th1 and Th2 conditions and Tem in a suppressive environment, but also enough to program a stem cell memory like pool and decrease exhaustion markers.

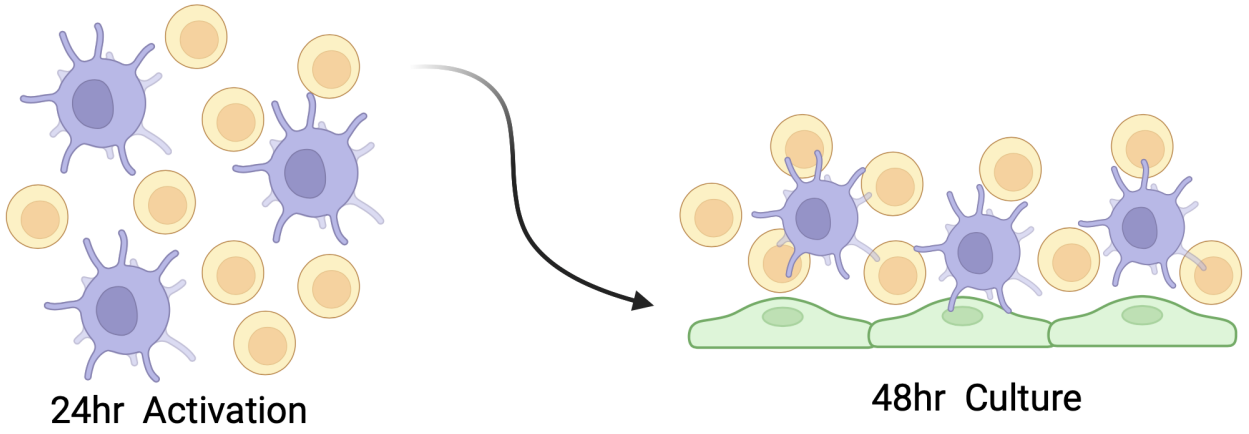

Figure 2.17: **Schematic: LEC maintenance of mDC activated T cells**

The findings were notable as they demonstrate that initial interactions between LECs and naive T cells result in robust programming. Consequently, our study explored whether the maintenance of mDC-activated T cells by LECs could either ablate the programming imparted by mDCs or induce LEC-specific programming. To investigate this, we conducted a similar experiment where T cells were primed with mDCs for 24 hours, after which the DC-T cell mixture was transferred to a well containing LECs that had been previously stimulated and pulsed with antigen **Fig.2.17**. Removing DCs from T cells posed significant challenges due to their non-adherent nature. Thus, the solution containing both DCs and T cells was

transferred to the LECs, creating an intriguing scenario where the T cells received signals from both LECs and mDCs, which more accurately mirrors in vivo conditions within the lymph node, with initial stimulation solely by mDCs.

To our delight, we observed the hypothesized effect. When LECs maintain T cells and DCs that had previously been in co-culture together for 24 hours, LECs still confer the programming associated with their stimulated conditions (SS, Th1 and Th2 LECs pushing for Tcm, while Tsup LECs pushing for Tem). We see that if inflamed and steady state LECs maintain mDC-activated T cells, even though the mDCs are still providing stimuli in conjunction to the LECs, the T cells develop into a predominantly central memory T cell pool **Fig.2.18A**. On the other hand, suppressive LECs induce more central memory than in previous experiments when they maintain mDC-activated T cells, but still induce higher levels of effector cells compared to the other LEC maintenance conditions **Fig.2.18A,B**. We also observe that when LECs maintain these activated T cells, they maintain a population of naive T cells alive, while Tsup LECs maintain the highest percentage of naive T cells alive **Fig.2.18C**. We notice the opposite effect as to when mDCs maintain LEC-activated cells, where if LECs maintain mDC-activated cells they are still able to induce a statistically significant increase in the TCF1 expression, while they lower Tim3 expression in a non statistically significant way except for suppressive LECs **Fig.2.18D,E**. With that we also see that the overall stem cell memory-like percentage of the total T cell pool increases significantly when LECs maintain mDC-activated T cells when compared to mDC activation alone **Fig.2.18C**, and to a greater extent than LEC activation and mDC maintenance.

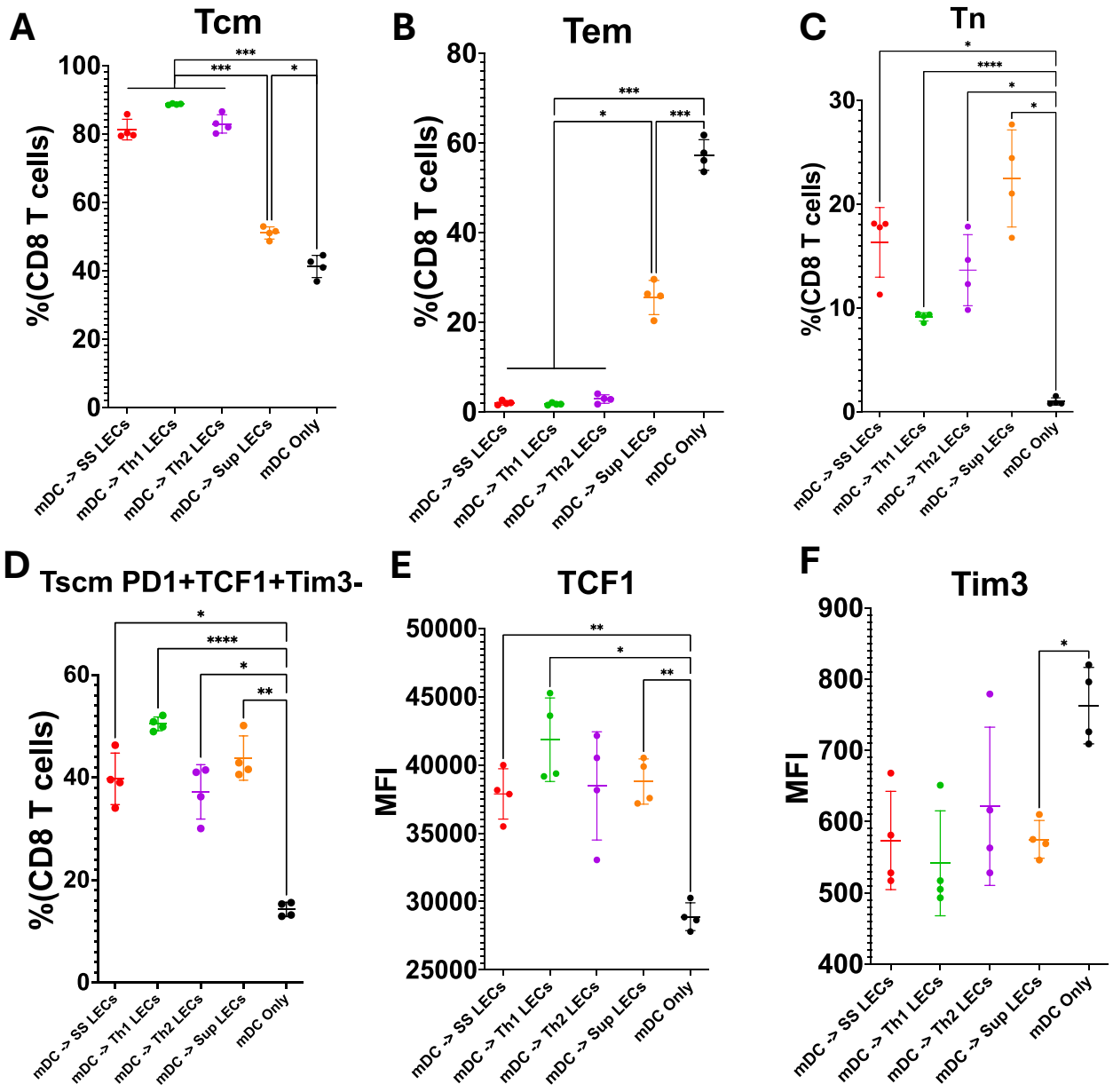

Figure 2.18: **LEC maintenance of mDC activated T cells is sufficient to program associated phenotypes A** Quantified percentage of the central memory compartment. **B** Quantified percentage of the effector memory compartment. **C** Quantified percentage of the naive compartment. **D** Quantified percentage of the stem cell memory cells from the overall T cell pool. **E** Quantified MFI of the TCF1 expression by all T cells. **F** Quantified MFI of the Tim3 expression by all T cells.

2.4.6 Confirmation of LEC programming in another transgenic T cell model

We aimed to validate our observations regarding the phenotypes induced by LECs in an alternative transgenic T cell mouse model, acknowledging the variability in behavior these models may exhibit. This variability can be attributed to differences in antigen affinity by TCRs and inherent biological variations in the response of T cells, which require verification of our findings with a different transgenic model. Accordingly, we co-cultured LECs with Pmel T cells, a transgenic model that recognizes the gp100 peptide of melanoma. We are pleased to report that the trends observed in this model parallel those identified in OT1s. Specifically, SS,, Th1 and Th2 LECs predominantly induce a central memory T cell population upon activation of naive Pmel T cells **Fig.2.19A**. Additionally, we confirm that Tsup LECs induce the highest percentage of effector memory T cells, compared to other activation conditions **Fig.2.19B**. Furthermore, LEC activation of Pmel T cells results in a higher percentage of Tscm compared to mDC activation, along with elevated TCF1 expression **Fig.2.19C,D**. Collectively, these results indicate that our data maintain consistency across transgenic models.

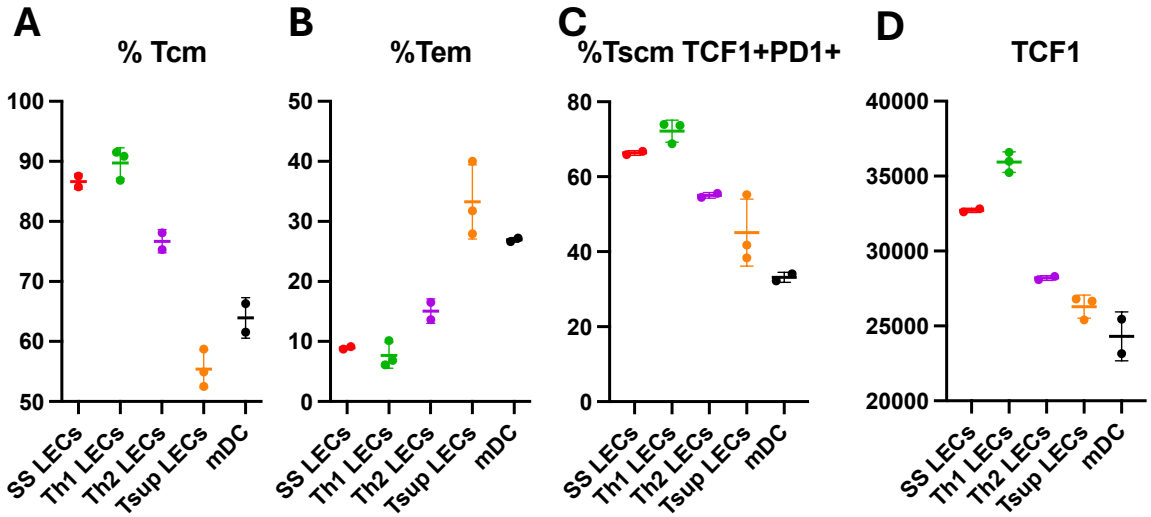

Figure 2.19: **LECs induce the same phenotypes in the Pmel T cell transgenic model** **A** Quantified percentage of the central memory compartment. **B** Quantified percentage of the effector memory compartment. **C** Quantified percentage of the naive compartment.

2.5 Conclusions

2.5.1 LECs as antigen-presenting cells in inflammatory and suppressive microenvironments

Our initial investigation focused on the interaction between lymphatic endothelial cells (LECs) and CD8 T cells in both inflammatory and suppressive contexts. This inquiry stemmed from our laboratory's prior findings that, under steady-state conditions, lymphatic endothelial cells of the lymph node exhibit the capability to scavenge antigens and cross-present them via MHC class I to CD8 T cells. This cross-presentation facilitates the activation of naive CD8 T cells, predominantly driving their differentiation into a central memory phenotype. Our observations led to an investigation of whether LECs elicit similar responses within inflammatory environments, which are essential for effective immune responses. Concurrently, an examination of suppressive contexts was warranted, given LEC presence in secondary lymphoid organs subject to suppressive influences, such as Peyer's Patches in the gastrointestinal tract and tumor-draining lymph nodes.

We tested LECs stimulated with Th1-associated cytokines and Th2-associated cytokines, particularly IFNγ and TNFα, and IL-4 and IL-13 respectively. These cytokine microenvironments model the inflammation present in antiviral responses (Th1), and allergy and anti-parasitic (Th2) responses. Our objective was to contrast these effects with those of mature dendritic cell activation as this is the convention. As hypothesized, Th1 and Th2 LEC activation of naive CD8 T cells resulted in a predominantly central memory population, similar to that at SS. Interestingly, the opposite was observed in suppressive microenvironments, as TGFβ and IL-10 stimulated (Tsup) LECs induce a predominantly effector-like population upon activation of naive CD8 T cells. We see that LEC-activated T cells proliferate less than mDC-activated T cells in particular those activated by Tsup LECs.

Looking at the generational data, we observe that LEC activation at all conditions re-

sults in a response that peaks and decreases, while mDC activation results in a continuously increasing response that resembles exponential growth. SS, Th1 and Th2 LECs program for central memory immediately after the first round of division and maintain it throughout, while inducing a virtually non-existent effector memory pool that slightly increases towards the end of the response. Tsup LECs program an effector-like population that grows generationally in a logarithmic manner and a central memory population that decays after the first round of division.

2.5.2 LEC activation induces a highly stem cell memory and less differentiated population of T cells

When we demonstrated that LECs at steady-state induce a central memory phenotype, we also showed that they induce a generally more stem cell memory-like population. So we wanted to validate that the same was occurring at these cytokine microenvironments. As we had hypothesized, we also observed an remarcably high percentage of stem cell memory cells, marked by their co-expression of TCF1 – the main transcription factor associated with T cell stemness –, and PD1 – a receptor associated with activation and crucial for checkpoint inhibition –, and their lack of Tim3 expression – a receptor associated with T cell exhaustion –. Interestingly, we noticed that LECs at all conditions induce this phenotype, so we show that stem cell memory can be a phenotype possessed by both central memory and effector memory cells. In fact, LECs at all conditions induce a T cell population that is predominantly Tscm upon the first round of T cell division and is highly maintained throughout the T cell response; while mDCs induce a growing Tscm population that never reaches the levels of the LEC-activated T cell compartment.

We demonstrate that LECs generally induce a higher expression of TCF1, PD1 and CXCR3 all associated with stem cell memory and effective T cell responses. Notably, CD27 is exclusively upregulated in T cells activated by inflamed LECs, while it is downregulated

following suppressive LEC activation. This observation is of particular interest given CD27's role as a co-stimulatory molecule associated with memory and its ligand's capacity to mitigate tolerance under steady-state conditions. The suppressive microenvironment programs for an effector-like population that we refer to as effector memory because they express high levels of CD127, EOMEs, CCR7, and the Tscm markers. However, it is interesting that they are quite less proliferative than the rest of the LEC activating conditions yet they express the highest level of Ki67 out of all of them so they maintain proliferative abilities. It would seem that these cells are possibly exhausted however they do not express Tim3 at all. This warrants further investigation due to its intriguing nature.

In general, the degree of differentiation exhibited by LEC-activated T cells is comparatively lower, as evidenced by their elevated expression of TCF1 and a ratio of EOMEs to Tbet exceeding one, indicating a greater presence of EOMEs and thus a lesser state of differentiation. mDC activation results in an EOMEs to Tbet ratio of less than one, demonstrating that these T cells are in a more advanced stage of differentiation. The temporal expression of Tbet is quite interesting. While mDC activation results in a Tbet expression that grows generationally, LEC activation at all conditions induces the same Tbet expression as mDCs on the first generation, but then maintains it low throughout the response, to the point that they converge. Showcasing that Tbet expression is not induced at high levels by LECs. EOMEs on the other hand responds similarly at all conditions albeit at different levels. TCF1 is maintained highly expressed by LEC-activated T cells, while DCs induce a quick up-regulation of the transcription factor that decreases as the T cell response unfolds.

In essence, all of this shows that LEC activation induces an undifferentiated stem cell memory T cell pool, at both central memory (by SS, Th1 and Th2 LECS) and effector memory (by Tsup LECs) phenotypes.

2.5.3 LEC activation or maintenance of CD8 T cells is sufficient to program their associated phenotypes

LECs are strong inducers of their associated phenotypes, since at steady-state we observe that LECs induce central memory irrespective to the LEC to T cell ratio. In our investigation of the timeline and kinetics of the response, we observed that within 24 hours following T cell activation, the cells initiate CD62L shedding, despite not having undergone division. This also signals that even before the first division and within the first 24 hours, the T cells have already interacted with the LECs and received signals from them. Subsequent to receiving these signals, upon their initial division, the T cells are already differentiated into a central memory phenotype when stimulated by Th1 and SS LECs, and into an effector memory phenotype when stimulated by Tsup LECs.

Since LECs demonstrate robust programming capabilities, we wanted to test if within the first 24 hours of activation, they effectively provide signals to induce central memory (SS, Th1 and Th2 LECs) or effector memory (suppressive LECs). They indeed induce central memory, even suppressive LECs induce higher levels of central memory than when they activate T cells for 72 hours. Following this observation, we sought to determine whether the programming induced by LECs could be nullified by the maintenance of LEC-activated T cells by mDCs. Unexpectedly, the programming by LECs is sufficiently robust that mDCs are unable to ablate it.

We observe that the T cells still develop into a significantly higher percentage of central memory compared to mDC activation alone albeit not to the level that LEC activation for 72 hours does. We see a definite decrease in Tim3 and a non statistically significant increase in TCF1 with initial LEC activation. On the flip side, since initial LEC activation is a quite strong phenotype programmer, we wanted to investigate if maintenance by LECs could also contribute to phenotype induction. When we moved 24 hour mDC-activated T cells to LECs, we observed that LEC maintenance was also a strong contributor to the

T cell phenotype. SS, Th1 and Th2 LEC maintenance of mDC activated T cells resulted in the expected high percentage of central memory, interestingly, Tsup LECs induced a significantly higher central memory population than mDC alone, however it was still lower than the other LEC maintenance groups. We noticed that in the LEC maintenance case, Tim3 was not significantly down-regulated by all groups, but TCF1 was up-regulated. This showcases that initial interaction and activation by LECs is needed for significant decrease of Tim3, but prolonged LEC exposure induces higher levels of TCF1. All conditions that had LEC contact with T cells however induced a higher percentage of stem cell memory cells than mDC activation alone.

2.5.4 Conclusion schematic

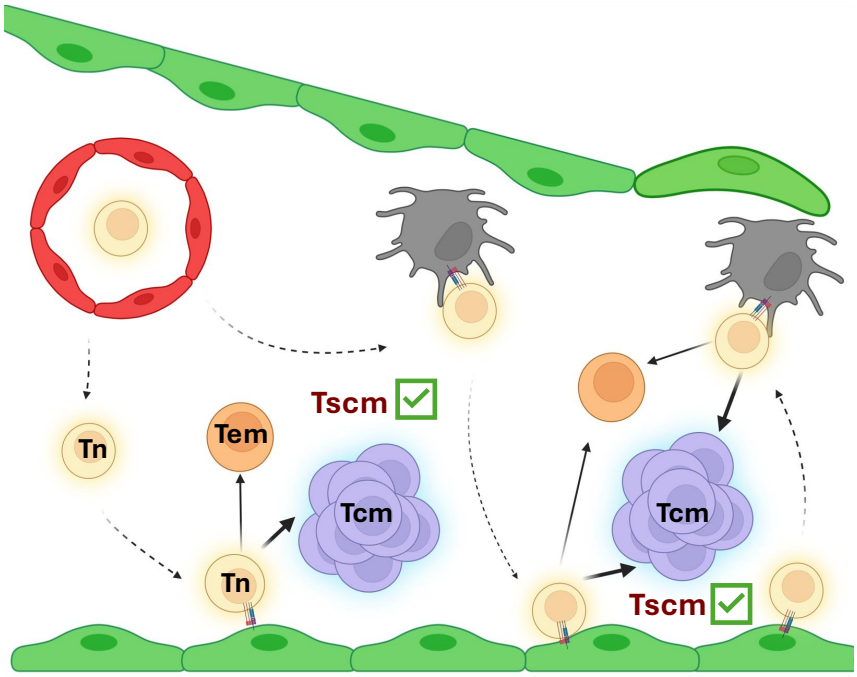

Figure 2.20: **Chapter 2 Conclusions**

CHAPTER 3

LEC COOPERATION WITH MDCS TO INDUCE CENTRAL MEMORY CD8 T CELLS

3.1 Chapter Summary

In this chapter, we look at different receptors and ligands of interest that LECs express. Since we observe this development of memory upon naive CD8 T cell activation by LECs, we wanted to investigate what specific markers LECs express that could induce memory. We look at the expression of several types of proteins in all of the cytokine microenvironments we explored on Chapter 2.

We also wanted to investigate what the T cell population would look like if we activate naive CD8 T cells in the presence of both antigen pulsed LECs and DCs. We were curious about this since in the previous chapter we observed that LEC activation or maintenance of DC-activate T cells is sufficient to induce LEC-associated phenotypes. After performing the triculture experiments, we were curious to investigate if direct contact was always necessary for LECs to induce their associated phenotypes. We performed activation experiments where mDCs activated naive CD8 T cells in the presence of LEC-T cell co-culture conditioned media in order to see if there were any secreted factors at play. To confirm the findings of the conditioned media experiment, we also performed transwell experiments where we cultured the stimulated LECs in the transwell, and mDCs with T cells in the well.

In this chapter we show that LECs express memory-associated co-stimulatory markers while expressing low levels of the classic co-stimulatory molecules. LECs also express inhibitory molecules, adhesion molecules and suppressive cytokine docking proteins. We show that in a triculture setting where both LECs and DCs present antigen, the T cells still develop into the LEC-associated phenotype, and we see that indirect LEC contact by possible secreted factors induces the memory phenotype but not the stem cell memory phenotype.

3.2 Introduction

3.2.1 LEC-DC interactions

Lymphatic endothelial cells play some crucial roles in modulating dendritic cell functions, which in turn impacts CD8 T cell activation, differentiation and function. The interplay and interactions between these two cells types plays an essential role for regulating the immune response and maintenance of immunological memory. [66, 163–165]

Antigen Archiving and Presentation

LECs have a unique ability to archive antigens. This process involved the acquisition, retention, and exchange of antigens between LECs and dendritic cells. This archiving mechanism is particularly important for maintaining long-term immune memory, as LECs can present the archived antigens to dendritic cells, which can then stimulate antigen-specific CD8 T cells. In addition, as discussed in the previous chapter, LECs can directly present the antigen to CD8 T cells in an inflammatory environment. [79, 90, 91, 166]

Regulation of DC Migration

LECs also play a role in the regulation of DC trafficking from peripheral tissues to the draining lymph nodes where the immune response unfolds, and where DCs are needed to initiate the response. LECs express molecules like Stabilin-1, ICAM1 and Sema3A which are involved in the process of trafficking DCs and DC migration into the lymph node. [66, 163–165]

Immunomodulatory Effects

LECs can also exert immunosuppressive effects on migrating dendritic cells. This modulation can affect the magnitude of the immune response created by dendritic cells that enter the

lymph node. Interestingly, in mice with reduced DC trafficking due to stabilin-1 knockout on LECs, there is a stronger proliferative response of antigen-specific T cells in the draining lymph node. [164, 165, 167, 168]

The antigen archiving function of LECs is crucial for maintaining long-term memory. During subsequent pathogenic challenges, LEC archived antigens can be released and presented, leading to an increase in archived antigen-specific memory CD8 T cells, boosting the response, resulting in increased protection against pathogens expressing archived antigens. [79, 169]

3.2.2 LEC Phenotyping: Main Markers of Interest

In this section we delve into some key receptors and ligands that antigen-presenting cells use to activate CD8 T cells, many of which interact with the molecules mentioned in the previous section. We are interested in the expression levels of these molecules by LECs and mature dendritic cells in order to compare how they activate T cells. We investigate a wide variety of molecules, from co-stimulatory molecules that provide stimulation and activation signals to CD8 T cells, inhibitory molecules that provide dampening signals, adhesion molecules that provide structural support and adhesion of T cells to the APCs, as well as molecules associated with cytokines, as activators or docking proteins for them.

Co-Stimulatory Ligands

CD80 and CD86 - These two are crucial co-stimulatory molecules members of the B7 family that play a significant role in CD8 T cell activation and function. CD80 and CD86 are key players in the co-stimulatory pathway that complements T cell receptor signaling. These two molecules are primarily expressed by antigen-presenting cells and both interact with the ligand CD28 which is expressed by T cells. Binding of these molecules to CD28 provides the second signal necessary for full T cell activation, and this interaction is part

of the classical two-signal model of T cell activation, where signal one comes from the TCR and antigen-MHC complex recognition and signal two comes from co-stimulatory molecules like CD80 and CD86. CD28 signaling through CD80 and CD86 is crucial for the ability of CD8 T cells to produce IL-2, an important cytokine for T cell proliferation and survival. Signaling provided by CD80 and CD86 through CD28 also promotes the development of cytotoxic activity in CD8 T cells. [2, 170–174]

CD70 - A co-stimulatory molecule that belongs to the tumor necrosis factor (TNF) superfamily that works as the ligand for the CD27 receptor on CD8 T cells. CD70-CD27 is an important co-stimulatory axis that is vital for the activation, survival and differentiation of CD8 T cells. CD70 engagement leads to enhanced effector functions of CD8 T cells including increased ability to secrete IFNγ. CD70 is crucial for the generation of a robust primary CD8 T cell response, where it has been reported that CD70-deficient mice have a lower magnitude of CD8 antigen-specific response against the LCMV virus, which results in impaired viral clearance. It supports the maintenance of tumor-specific CD8 T cells in the tumor microenvironment. CD70 co-stimulation enhances T cell expansion, survival, and the formation of long-term memory. Expression of CD70 can also prevent tolerance induced by antigen presentation in the steady-state. [133, 135, 137, 138, 175–179]

ICOSL - Inducible T-cell co-stimulator ligand is a member of the B7 family of co-stimulatory molecules, like CD80 and CD86. It is the ligand to the ICOS (inducible T-cell co-stimulator) receptor that is expressed by T cells. The ICOS-ICOSL interaction plays several roles, such as T cell activation, memory formation, and even thymic selection. ICOSL-ICOS signaling can influence the differentiation of CD8 T cells into memory T cells, and there have been some studies that have explored targeting this pathway to enhance anti-tumor immune responses.[133, 180–182]

OX40L - A member of the tumor necrosis factor (TNF) superfamily which binds to OX40, serves as a co-stimulatory molecule to CD8 T cells. OX40L is expressed in various cell types including antigen-presenting cells. Signaling through this pathway promotes the division, expansion and survival of CD8 T cells, contributing to the expansion of effector and memory populations during the immune response. It also modulates the production of cytokines which influences their functional capabilities, and by supporting the survival of activated T cells, signaling through the OX40L-OX40 axis likely plays a role in the generation of memory CD8 T cells.[133, 138, 183–187]

Co-Inhibitory Ligands

PD-L1 - Also know as programmed death-ligand 1, PDL1 is a critical molecule in the immune system that plays a significant role in regulating T cell responses, in particular those mounted by the CD8 T cell pool. PDL1 is a ligand that binds the PD1 receptor on CD8 T cells. This interaction is important, and it is part of the immune checkpoint pathway that modulates T cell responses, acting as a dampener of the T cell response. PDL1 binds to PD1 and it typically delivers inhibitory signals that can suppress CD8 T cell activity. PDL1 signaling plays a crucial role in maintaining immune homeostasis by preventing over-activation of the immune system and the CD8 T cell response which is critical to prevent autoimmune diseases and reactions. PDL1 signaling is also involved in chronic infections and cancer, as persistent PDL1 signaling by the cancer cells can lead to T cell exhaustion and therefore less effective CD8 T cell responses. [68, 88, 143, 149, 188–193]

PVR - The Poliovirus Receptor (PVR) is a member of the nectin-like molecule family that plays a significant role in cancer biology and CD8 T cell immune responses. PVR interacts with various T cell receptors, influencing CD8 T cell behavior and function. It has been shown that PVR treatment decreases the CD8 T cell pool while increasing the CD4

T cell pool, and that PVR exhibits an immunosuppressive role by decreasing cytotoxic T cell responses and inhibiting the secretion of key cytokines like IFNγ, TNFα, as well as IL2, IL6 and IL10. PVR plays a role in CD8 T cell exhaustion as well, as PVR is observed to be over-expressed in many human cancers, and has been reported to increase in expression after radiation therapy in some breast cancers. [194–198]

Adhesion Molecules

ICAM-1 - Intercellular Adhesion Molecules 1, is a member of the immunoglobulin gene superfamily and is an important protein involved in immune cell interactions and adhesion. ICAM1 is expressed by many cell types including both hematopoietic and non-hematopoietic cells, and highly expressed by endothelial cells. ICAM1 is the ligand to Lymphocyte Function-Associated Antigen 1 (LFA-1) and Membrane Attack Complex 1 (MAC-1). It plays a role in the activation of T cells as an accessory molecule, forming part of the immune synapse where adhesion molecules on the APC form an outer adhesion barrier to where the MHC-TCR interactions and the co-stimulation occur. ICAM1 has also been reported to be necessary for the asymmetric division of CD8 T cells upon activation.[199, 200] ICAM1 is also crucial for the extravasation of T cells (particularly naive CD8 T cells) into the lymph nodes, which is the movement of the T cells from the high endothelial venules (HEVs) through the junctions in between the endothelial cells into the lymph node.[167, 201–205]

TGFβ-Associated Molecules

GARP - Glycoprotein A Repetitions Predominant (GARP), is a cell surface protein that is involved in immune regulation, in particular in the context of TGFβ. GARP is a trans-membrane protein that serves as a docking receptor for the latent form of TGFβ. GARP is expressed on the surface of activated Tregs, some cancer cells, platelets and some endothelial cells. The primary function of GARP is to promote the activation of TGFβ

which as previously stated, is a cytokine with potent immunosuppressive capabilities. GARP does not work directly on T cells but since it is crucial for latent TGFβ activation, it is a molecule of interest as it is immunosuppressive. It has been reported that GARP-mediated TGFβ activation contributes to creating a suppressive tumor microenvironment, impairing CD8 T cell function and infiltration. Blocking of GARP has shown some potential as a cancer therapy as it enhances CD8 T cell-mediated anti-tumor responses by reducing the immunosuppressive environment of the tumor, particularly in combination with PD1 blockade.[206–209]

LAP- Latency-Associated Peptide, LAP is a component of the regulation of immune responses. It is an adapter and regulator of TGFβ, as it forms a complex with TGFβ keeping it inactive and in its latent state until it is activated and released by factors such as integrins or matrix metalloproteinases. LAP works together with GARP in regulating TGFβ, as GARP binds and presents the latent TGFβ (which is a LAP-TGFβ complex) on the cell surface and GARP facilitates the release of mature, active TGFβ from LAP. There have been some anti-LAP therapies that target LAP and inhibit the release of the active form of TGFβ.[210–214]

This is not an exhaustive list of molecules that co-stimulate, inhibit and generally affect CD8 T cells as they are activated. We cannot cover all of them in just one thesis, so these are the most salient ones to our studies and the ones that we found the most interesting to investigate due to their complex involvement in general CD8 T cell activation, memory formation, or exhaustion.

3.3 Methods

LEC and mDC stimulation and Phenotyping: We first isolated LECs and mDCs as described in Chapter 2. We stimulated the LECs with 1 ng/mL of SIINFEKL peptide and 20 ng/mL of each cytokine (IFNγ + TNFα for Th1, IL-4 + IL-13 for Th2, and TGFβ + IL-10 for Tsup) and the DCs with 1 ng/mL of SIINFKEL and LPS each. After overnight stimulation, we detached the LECs with accutase to avoid cleavage of some surface molecules that are susceptible to trypsin like CD31, and collected the mDCs that were in suspension. We looked at the surface expression of the co-stimulatory, co-inhibitory, adhesion and cytokine docking proteins introduced in part 3.2.2 of this chapter utilizing full spectrum flow cytometry.

Triculture Experiments: For the triculture experiments, we pulsed LECs and DCs with the SIINFEKL antigen, and we stimulated LECs in our SS, Th1 (IFNγ + TNFα), Tsup (TGFβ + IL-10) and mDCs with LPS. After overnight pulse and stimulation, we washed both LECs and mDCs, and added them together in a 1:1 ration. We then added isolated CD8 T cells, and tri-cultured them for 72 hours as we usually do. We then analyzed the resulting T cell population with full spectrum flow cytometry.

Conditioned Media Co-culture: For the conditioned media experiments, we pulsed the dendritic cells same as above. After overnight stimulation, we washed the mDCs and plated them with 100 μL of T cells at a ratio of 1 DC to 10 T cells as we usually do in fresh IMDM media. We also added 100 uL of conditioned media that came from the co-culture of LECs with T cells at all LEC activating conditions. After this, we cocultured the mDCs and T cells for 72 hours and analyzed using full spectrum flow cytometry with the same panels as in chapter 1.

Transwell Experiments: For these experiments we used 0.4 μm pore size 24 well transwell plates in order for the LECs to not be able to move across the pores. . We first plated the

LECs on the transwells, and stimulated them with the cytokines and antigens as explained above. We also separately stimulated and pulsed the dendritic cells as explained above. After overnight stimulation, I washed the LECs with PBS, and moved the transwell to a separate well in order to have a fresh well for the experiment. I then plated the mDCs with T cells at a ratio of 1:10 mDCs to T cells in the wells of the 24 well plate which was around 60,000 mDCs with 600,000 T cells. We then cocultured the mDCs and T cells with the LECs in the transwell for 72 hours.

Mice: We obtained our lymphatic endothelial cells from WT C57BL/6 mice from Charles River labs, our BMDCs from C57BL/6 mice from UChicago's CIIC (Chicago Immunoengineering Innovation Center), and the OT1s from UChicago's CIIC bred CD45.1 OT1 transgenic mice. All mice used were 6-12 weeks old.

Statistics: For all experiments, otherwise stated each dot on the plot represents the LECS of one mouse interacting with OT1s, or mDCs, so each dot represents LECs from individual mice. We utilized Brown-Forsythe and Welch ANOVA tests, Dunnett's T3 multiple comparisons test, with individual variances computed for each comparison. We only show the important statistically significant comparisons. For those that are grouped together with a line, they were all statistically significantly different to the compared group, and they were not statistically significant against each other.

3.4 Results

3.4.1 Lymphatic endothelial cells express several molecules involved in CD8 T cell activation and differentiation

In the preceding chapter, we established that lymphatic endothelial cells possess the capacity to activate naive CD8 T cells across diverse cytokine microenvironments. Additionally, they are capable of inducing memory phenotypes, such as central memory within SS, Th1, an d Th2 environments, as well as stem cell memory across all conditions. This observation prompted our investigation into the receptors and ligands potentially expressed by LECs that facilitate these phenotypic inductions. Accordingly, we examined the expression profiles of various co-stimulatory and co-inhibitory receptors that have been implicated not only in the activation of CD8 T cells but also in the development and differentiation of memory phenotypes.

Initially, we examined the expression levels of traditional B7 co-stimulatory molecules CD80/CD86 in LECs compared to mature dendritic cells. Our findings indicate that LECs exhibit minimal expression of CD80, merely at baseline levels; however, they do express CD86, a crucial co-stimulatory molecule that interacts with CD28 on CD8 T cells during T cell activation **Fig.3.1A**. We were further interested in analyzing the expression of co-inhibitory receptors such as PDL1, given its association not only with the inhibition of T cell activity but also its reported critical role in effective memory T cell responses and differentiation via interactions with PD1. Additionally, we explored the expression of PVR, a relatively novel co-inhibitory molecule not traditionally linked to memory, to ascertain if LECs express it. Interestingly, LECs demonstrated high levels of PVR expression under all conditions, while mDCs did not exhibit high levels of this inhibitory molecule **Fig.3.1B**. Furthermore, we observed that LECs express significantly higher levels of PDL1 compared to mDCs across all conditions **Fig.3.1B**, particularly after stimulation with IFNγ and TNFα.

This finding aligns with existing literature suggesting that the expression of PDL1 by LECs could be associated with peripheral tolerance; however, we propose that it may also be linked to memory formation.

Finally, we were pleased to find that LECs express a variety of co-stimulatory molecules, which are implicated not only in the reactivation of memory but also in the formation and differentiation of memory. Our findings indicate that LECs express two co-stimulatory molecules that are members of the TNF superfamily. They express significantly higher levels of CD70 compared to mature dendritic cells, as well as similar expressions of OX40L that trend on the higher end at all conditions **Fig.3.1C**. CD70 is the ligand for CD27, a co-stimulatory receptor that we see over-expressed by LEC-activated T cells at all conditions except Tsup LEC activation.

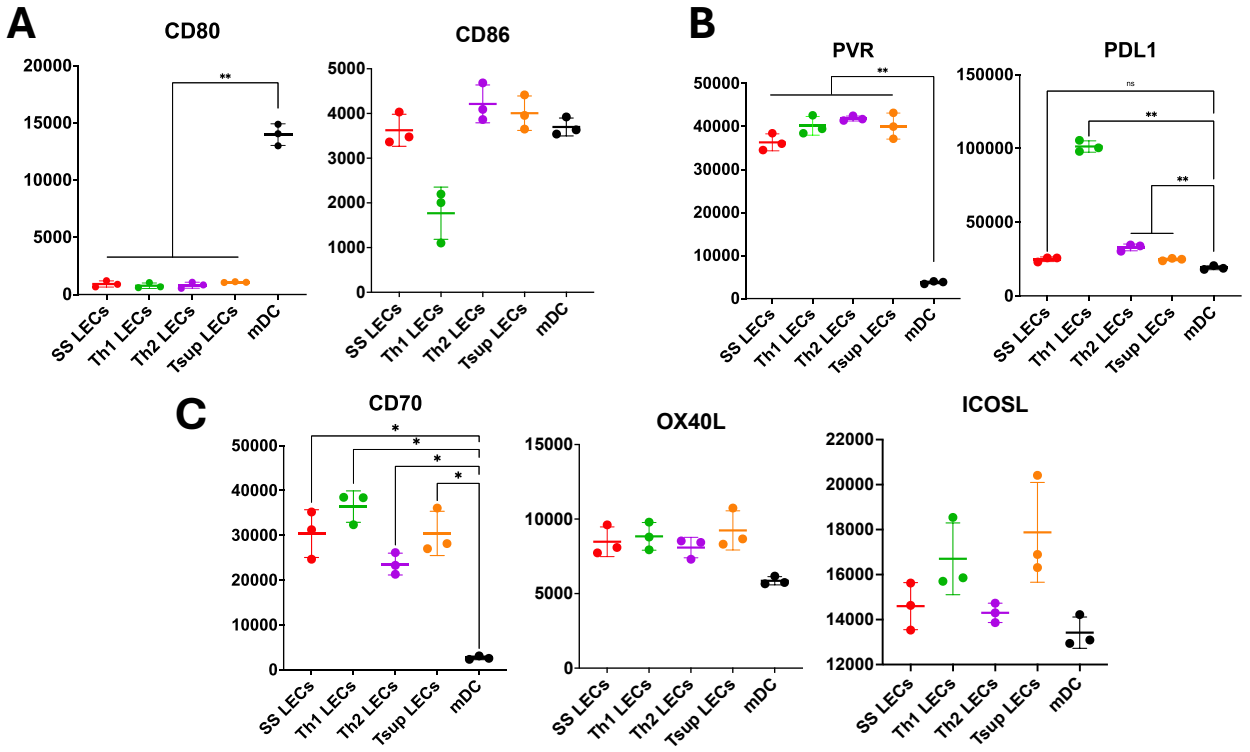

Figure 3.1: **LECs express traditional costimualtory molecules, inhibitory molecules, and memory costimulatory molecules A** Expression levels of costimualtory molecules CD80/CD86. **B** Expression levels of inhibitory molecules. **C** Expression levels of memory associated costimulatory molecules.

This is an important molecule that is also associated with tolerance escape at the steady-state. OX40L binds to OX40 on T cells and is involved in memory differentiation and activation. We also see the expression of the B7 superfamily co-stimulatory molecule ICOSL which is not significantly up-regulated by LECs compared to mDCs but it trends higher. ICOSL binds to ICOS on T cells and is also associated with memory induction.

Collectively, the data indicate that LECs (Lymphatic Endothelial Cells) not only express classical co-stimulatory molecules but also exhibit a range of memory-associated co-stimulatory ligands, in addition to certain inhibitory molecules. These inhibitory molecules may play a role in potentially inhibiting effector differentiation and guiding T cells toward establishing a memory phenotype.

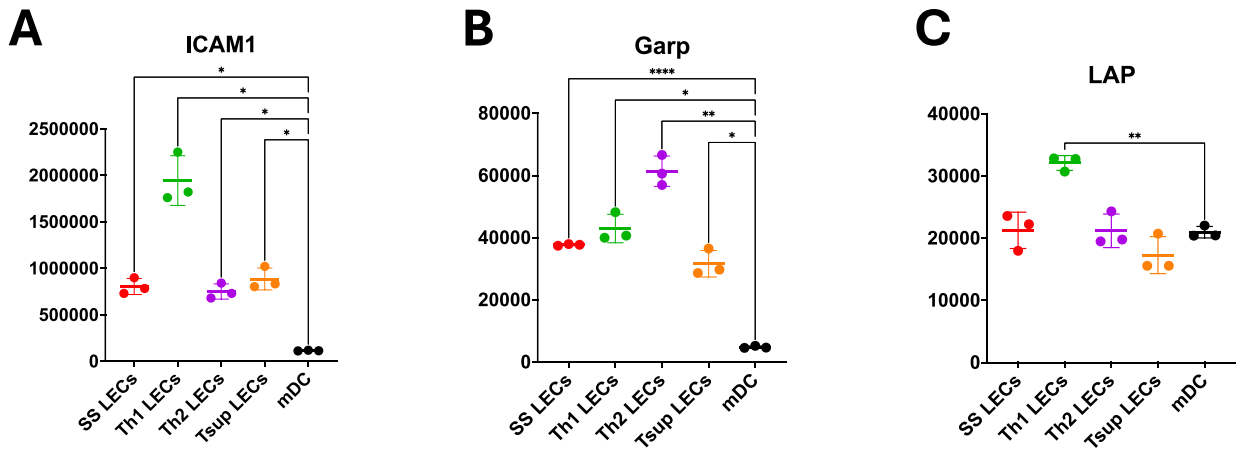

Figure 3.2: **LECs express adhesion molecules, and cytokine docking proteins at all conditions A** Expression of the adhesion molecule ICAM1 **B** Expression of the latent TGFβ docking molecule Garp. **C** Expression of the latent TGFβ associated protein LAP.

We also investigated the expression levels of other important molecules that can affect the phenotype of CD8 T cells. For example, ICAM1 is an adhesion protein that is necessary for the induction of asymmetric division of CD8 T cells which has been associated with memory differentiation and maintenance. We saw that LECs express ICAM1 significantly higher than mDCs at all conditions with Th1 LEC stimulation inducing the highest expression of them all **Fig.3.2A**.

We were interested in examining various proteins related to TGFβ, as our lab's findings have demonstrated the significance of this cytokine in the reactivation of memory CD4 T cells by LECs. We decided to investigate the LEC expression of the TGFβ docking protein Garp, which serves as a holder for the latent version of TGFβ and is released when LAP (the latent associated protein) of TGFβ is cleaved from the cytokine by Garp. We see that LECs express significantly higher levels of Garp than mDCs **Fig.3.2B**. Interestingly, the highest expression occured when LECs were stimulated with IL-4 and IL-13, instead of Tsup LECs as we had expected. LAP was similarly expressed by LECs and mDCs at all conditions except when LECs were stimulated with IFNγ and TNFα which induced a significantly higher expression than mDCs **Fig.3.2C**. We thought there would be a pattern between LAP and Garp expression, but we do not observe one.

Subsequently, we aimed to investigate the dynamics of these markers following the interaction of LECs and mDCs with T cells. To this end, we examined their expression levels 24 hours post-contact with T cells and computed the fold change in expression between the post-overnight stimulation state and the state following 24-hour T cell interaction. We observed that LECs exhibit complete down-regulation of CD86, whereas mDCs demonstrate up-regulation of the same marker following T cell contact, with both cell types up-regulating CD80, which possesses a higher affinity for CTLA4 than CD28 **Fig.3.3A**. The modulation of PDL1 presented intriguing results, as both up-regulation and down-regulation were observed in LECs. Specifically, PDL1 was up-regulated in SS and Th2 LECs, while down-regulated in Th1 and Tsup LECs **Fig.3.3B**. However, the magnitude of change in PDL1 expression was considerably less pronounced in LECs compared to mDCs, which nearly doubled their PDL1 expression following T cell contact **Fig.3.3B**. Despite observing changes in PDL1 expression in LECs, there was negligible alteration in their PVR expression, whereas mDCs exhibited a substantial increase in PVR expression, increasing it nearly sixfold compared to their expression level following LPS stimulation **Fig.3.3B**.

In terms of the fold change of the memory-associated molecules we observe that LECs down-regulated all of them **Fig.3.3C**. They significantly down-regulated CD70 to almost half, in comparison to mDCs which up-regulated it to three times as much **Fig.3.3C**. CD70 down-regulation is associated with engagement of CD70 with CD27 on T cells. ICOSL was down-regulated by all LEC conditions and mDCs, however Th1 LECs down-regulated it the most upon T cell contact **Fig.3.3C**. OX40L was also down-regulated by all LEC conditions and mDCs, although at different levels **Fig.3.3C**. The expression of the adhesion molecule ICAM1 remained relatively the same by LECs with some down-regulation by Th1 LECs, and mDCs up-regulated it almost three times as much **Fig.3.3D**.

Garp remained unchanged by SS, Th1 LECs and mDCs, while Th2 and Tsup LECs down-regulated their expression of it **Fig.3.3E**. Finally Lap was equally down-regulated by all LEC conditions and mDCs **Fig.3.3F**, which could point at the release of this cytokine upon T cell interactions.

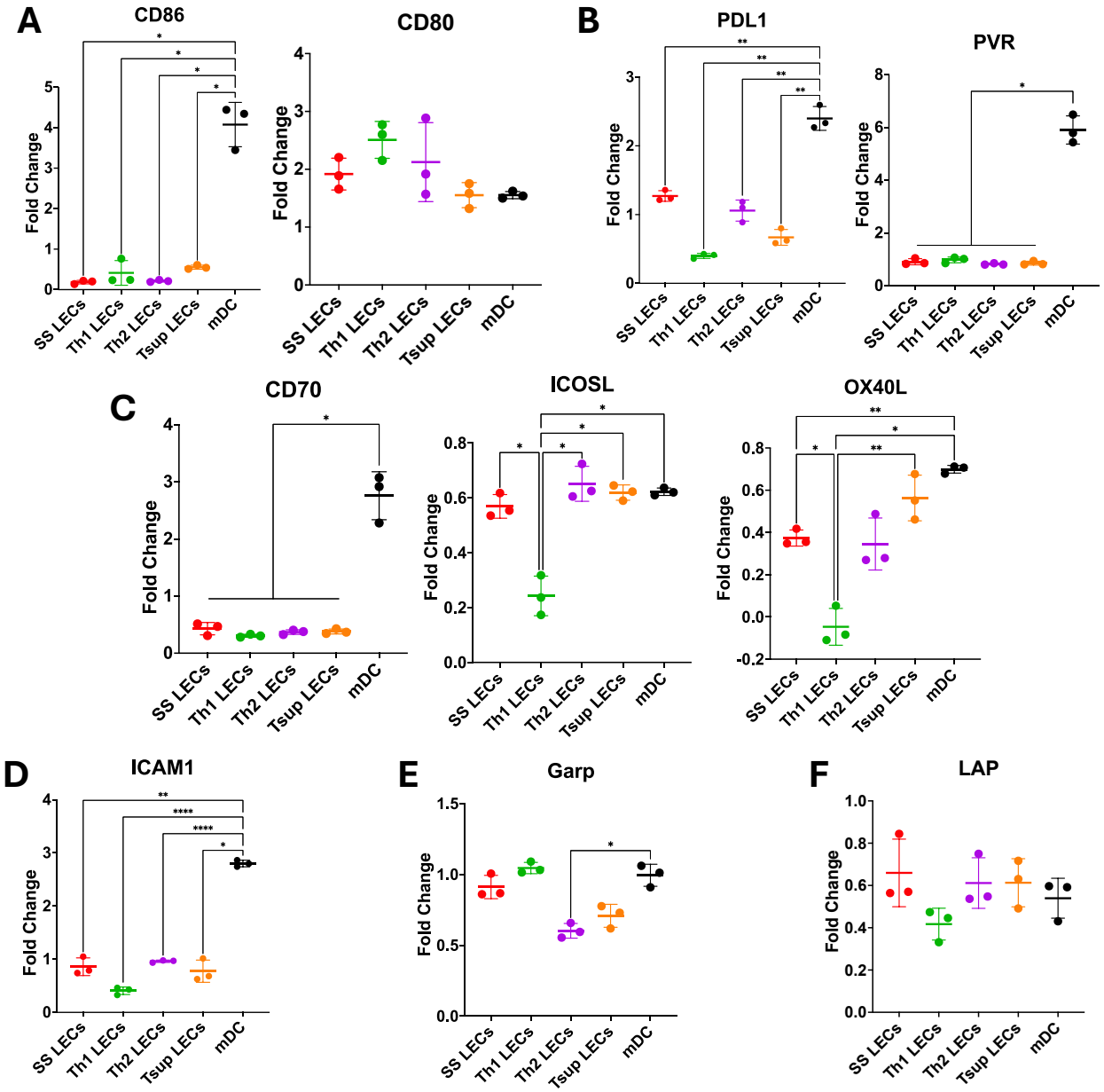

Figure 3.3: **LEC and mDC marker response to interactions with CD8 T cells A** Fold change in costimualtory markers CD80/CD86. **B** Fold change in inhibitory markers. **C** Fold change in memory associated costimualtory molecules. **D** Fold change in adhesion molecule ICAM1. **E** Fold change in Garp. **F** Fold change in LAP.

3.4.2 Lymphatic endothelial cells induce their associated phenotypes in mDC-T cell-LEC tricultures

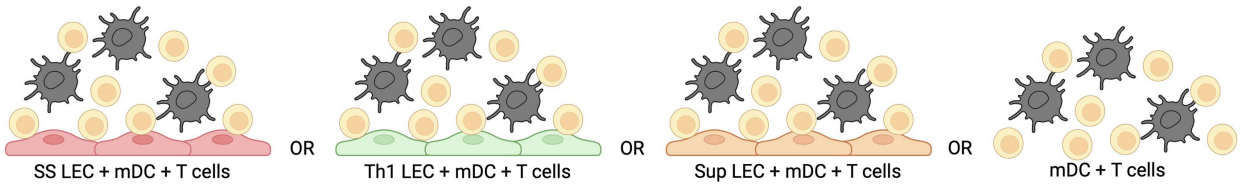

Figure 3.4: **LEC-mDC-T cell triculture schematic**

Given that LEC maintenance of mDC-activated T cells resulted in LEC's programming, we sought to investigate what would happen in a full triculture setting in which both LECs and mDCs are pulsed and presenting antigen. To this end we performed a triculture experiment in which we compared the T cell pools that developed after SS LEC + mDC, Th1 LEC + mDC, and Tsup + mDC activation of CD8 T cells with mDC only activation. The setup can be seen in the figure above **Schematic.3.4**. Given the comparable behavior observed between Th1 and Th2 LECs, we opted to include only one condition representative of these in this experiment.

Initially, we sought to determine whether these activating conditions resulted in comparable numbers of viable cells, and our observations confirmed that they all yielded approximately similar populations of live cells **Fig.3.5A**. It was particularly gratifying to observe that, as hypothesized, SS LEC and Th1 LECs in conjunction with mDCs promote a T cell population predominantly characterized by a central memory phenotype **Fig.3.5B**, while Tsup LECs in tandem with mDCs lead to a population with a pronounced effector memory phenotype **Fig.3.5C**. This outcome is noteworthy as it aligns with our findings on LEC activation alone, suggesting a robust programming effect mediated by LEC contact with T cells, even in the constant presence of mDCs. Lastly, our findings indicate that when mDCs are present, T cells tend to express more Tbet than EOMEs, signifying a higher de-

gree of differentiation **Fig.3.5D**. LECs continue to program a notably higher proportion of stem cell memory that mirrors LEC activation alone, even when in conjunction with mDCs. Conversely, mDCs induce a T cell pool consisting of approximately 40% Tscm **Fig.3.5E**.

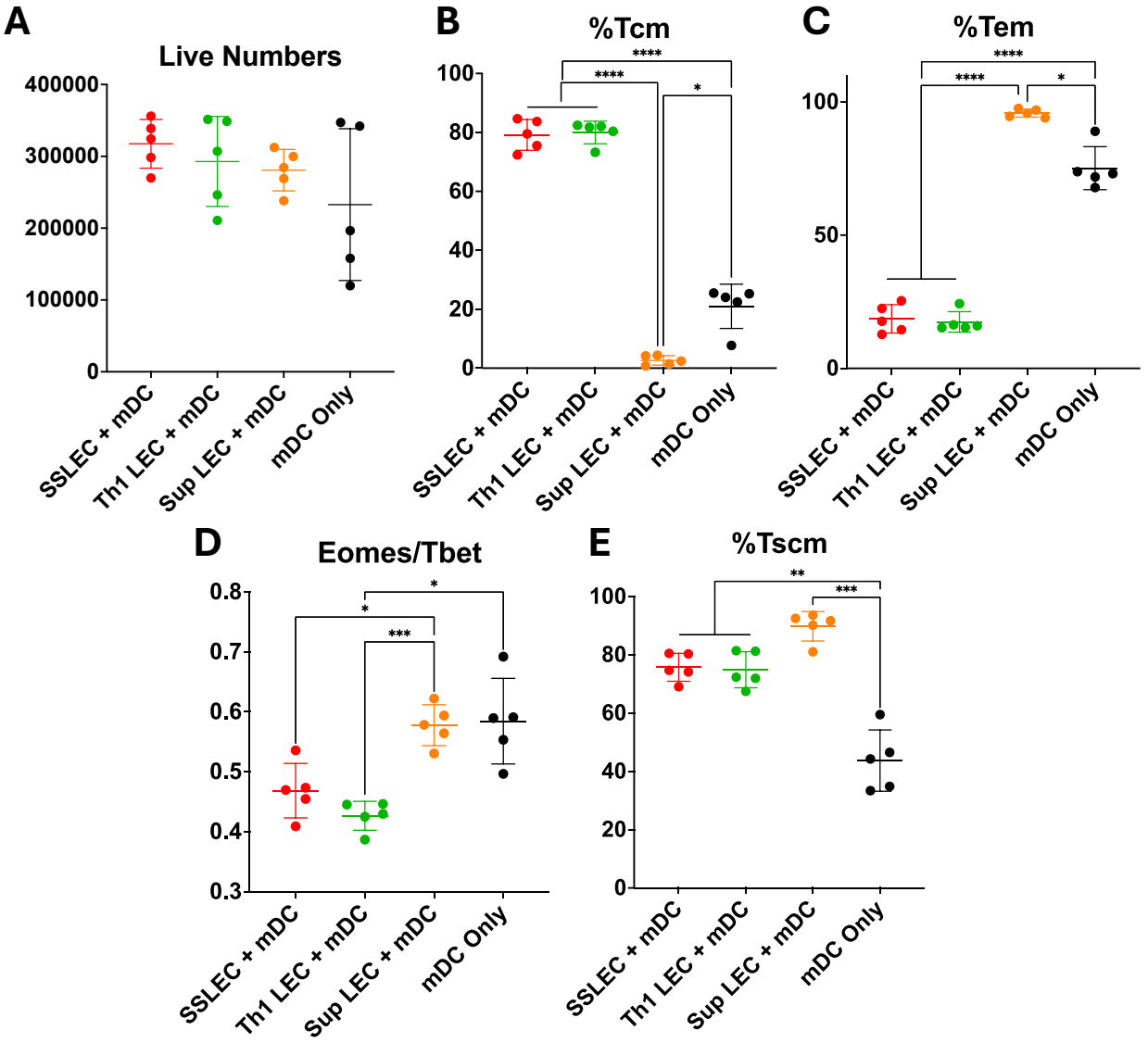

Figure 3.5: **LECs induce their associated phenotypes when interacting with mDCs and T cells in a triculture setting A** Quantified live cell numbers. **B** Percentage of central memory in the T cell compartment. **C** Percentage of effector memory in the T cell compartment. **D** Ratio of EOMEs to Tbet. **E** Percentage of Tscm in the T cell pool.

Finally, we aimed to quantify the markers associated with stem cell memory, as well as other receptors that exhibited differences in our initial co-culture experiments. We initially

quantified the expression levels of the stem cell memory-associated markers TCF1, Tim3, and PD1. Notably, TCF1 was similarly expressed by the T cells across all experimental conditions **Fig.3.6A**. Consistent with previous observations where LECs activate T cells independently, we noted a significant down-regulation of Tim3 in the presence of LECs, indicative of reduced exhaustion **Fig.3.6A**. Intriguingly, with respect to PD1, we observed the converse trend compared to when LECs activate T cells alone. Specifically, when LECs and mDCs collaboratively activate T cells, there is a marked reduction in PD1 expression, suggesting diminished inhibition of T cells **Fig.3.6A**. While both Tim3 and PD1 expression were down-regulated, there was an increase in the proportion of T cells co-expressing PD1 and TCF1 alongside low levels of Tim3 (Tscms) in cultures with LECs **Fig.3.5E**. Additionally, the presence and interaction of LECs with T cells significantly suppressed the expression of Tbet and EOMEs relative to mDC-only activation **Fig.3.6B**. No significant alterations were detected in the expression of CD122 **Fig.3.6C**. Furthermore, no substantial differences were observed in CXCR3 expression within a triculture setting, except for Tsup LECs, and no significant differences were recorded for CXCR5 expression; however, an upward trend was noted with SS and Th1 LECs **Fig.3.6D**.

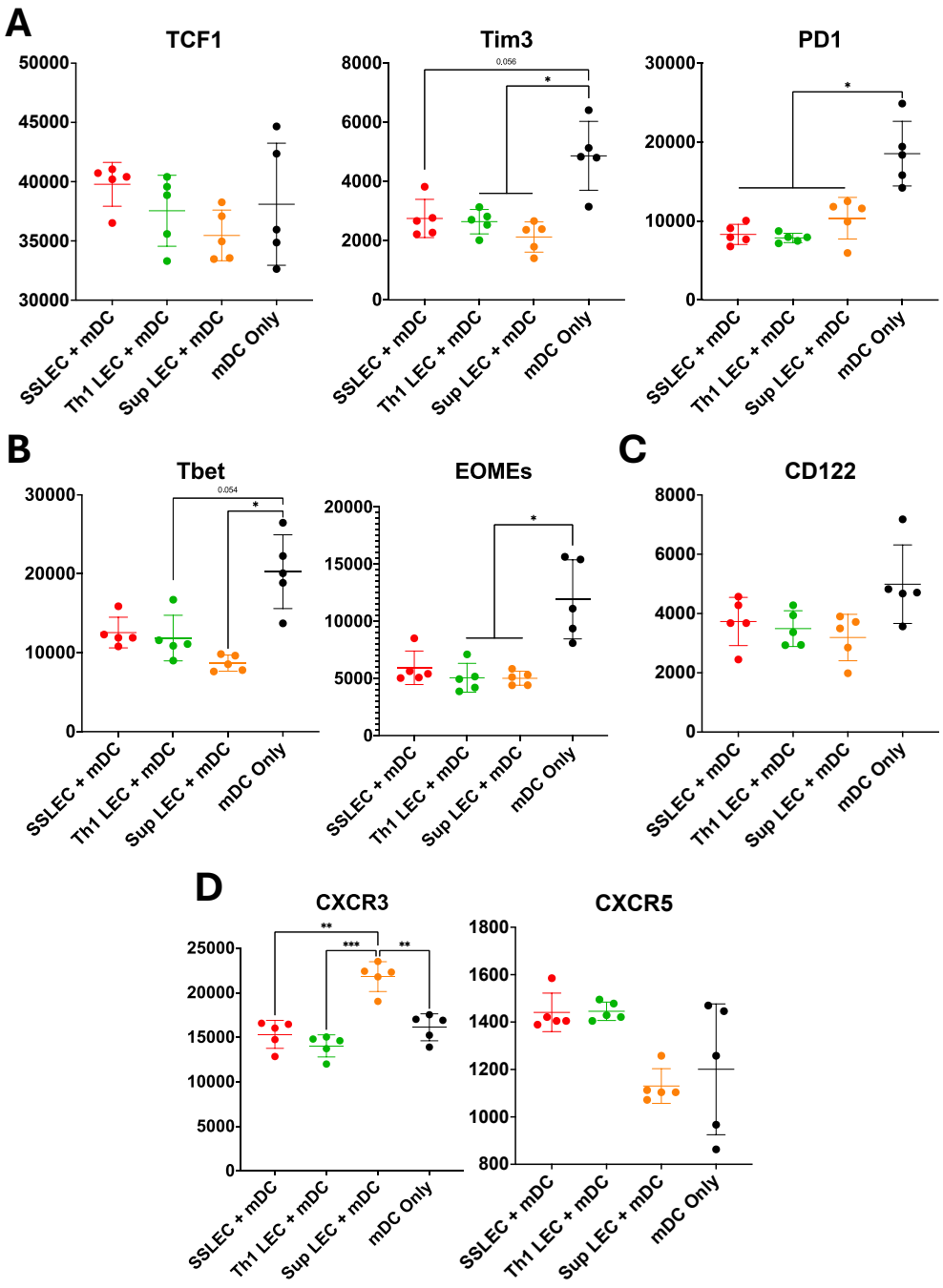

Figure 3.6: **LECs program a phenotype that is less exhausted and inhibited when in a triculutre setting A** Stem cell memory associated markers quantified by MFI. **B** Quantification of EOMEs and Tbet. **C** Quantification of the IL-2 and IL-15 high affinity receptor CD122 . **D** Quantified expression of chemokine receptors.

3.4.3 Lymphatic endothelial cell indirect contact programs central memory phenotype

We sought to determine whether lymphatic endothelial cells (LECs) could induce their associated phenotypes through indirect mechanisms for two main reasons. Firstly, LECs are recognized as critical contributors within the lymph node, producing numerous factors essential for T cell homeostasis, maintenance, and survival. Secondly, our findings from the 24 hour LEC activation experiment demonstrated that LEC activation for 24 hours is sufficient to impart these phenotypes. We hypothesized that one possible explanation for this phenomenon is that secreted factors transferred along with T cells indirectly influenced the phenotype of the responding cells.

To explore this hypothesis, we initially conducted a co-culture experiment where naive CD8 T cells were activated with mature dendritic cells, both in the presence and absence of LEC-T cell co-culture conditioned media. We utilized conditioned media derived from co-cultures involving SS LECs and T cells (SS CM - steady state conditioned medium), Th1 LECs and T cells (Th1 CM - Th1 conditioned medium), and Tsup LECs and T cells (Sup CM - Tsup conditioned medium). These media were collected at the end of the 72-hour co-culture period. Additionally, we added a condition where mDC-T cell co-cultures were supplemented with IL-7 and IL-15, both cytokines produced by LECs in the lymph node and known foe their crucial role in memory T cell development and maintenance.

Conditioned media from SS (SS CM) and Th1 (Th1 CM) cocultures elicited elevated levels of central memory and naive cells in comparison to mDCs alone. Conditioned media from Tsup LECs and T cells (Sup CM) appeared to induce a modest increase in central memory **Fig.3.7A**. Upon quantification, it was confirmed that SS CM and Th1 CM resulted in the highest levels of central memory. Notably, Sup CM also enhanced central memory levels; however, this was not significantly greater than that observed with mDC activation alone, paralleling the effect of IL-7 and IL-15 supplementation **Fig.3.7B**.

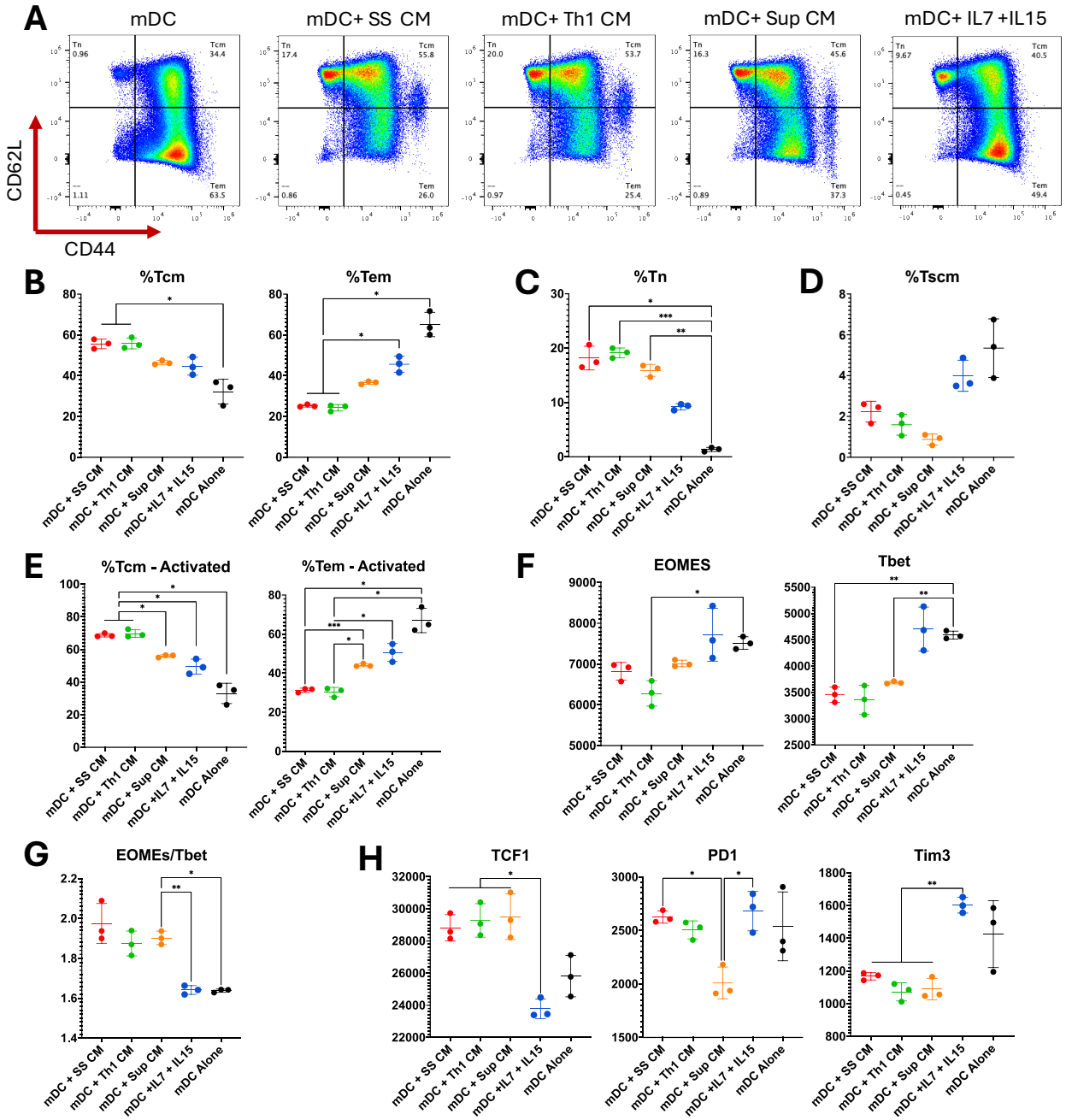

Figure 3.7: **LEC-T cell conditioned media programs for LEC associated phenotypes but not stem cell memory when mDC-T cell cocultures are exposed to it A** Flow plots of T cell phenotypes after coculture with conditioned medias. **B** Quantification of central memory and effector memory phenotypes. **C** Quantification of naive T cell percentage. **D** Quantified percentage of stem cell memory. **E** Quantified percentage of central memory and effector memory within the activated T cell pool. **F** Quantification of EOMEs and Tbet. **G** Ratio of EOMEs to Tbet. **H** Quantification of Tscm associated markers.

We observed that mDCs elicited the highest proportion of effector-like cells; unexpectedly, Sup CM did not result in a high proportion of effector memory as originally hypothesized **Fig.3.7B**. Notably, the conditioned media derived from LEC-T cell cocultures appeared to sustain a population of naive T cells in a viable and inactive state, despite mDCs serving as the primary APCs in this context **Fig.3.7C**. Although SS and Th1 CM were associated with an increased proportion of central memory cells, no increase in stem cell memory cell percentages was noted; instead, a decrease was observed in all LEC CM conditions **Fig.3.7D**. Given the persistence of a maintained naive T cell pool, a re-evaluation of whether the activated pool exhibited similar phenotypic trends was conducted. The findings reaffirmed that SS and Th1 CMs promote higher proportions of central memory within the activated compartment, while mDCs elicit the highest percentage of effector-like cells **Fig.3.7E**.

Regarding the expression of EOMEs and Tbet, there is no substantial reduction in EOMEs except in the case of Th1 CM. However, these levels generally tend to be lower than those observed during mDC activation, as well as mDC activation with IL-7 and IL-15 supplementation **Fig.3.7F**. Furthermore, it was observed that LEC CMs universally result in a reduction in Tbet expression in T cells, and conditioned media with SS and Sup significantly decrease this transcription factor **Fig.3.7F**. These findings corroborate our previous observations that LECs suppress the expression of this transcription factor. Upon examining the ratio of these transcription factors, it remains evident that LEC conditioned media in all conditions consistently promote a higher ratio of EOMEs to Tbet. This suggests that, despite these cells not exhibiting traits typical of stem cell memory-like cells, they remain relatively less differentiated.

We noticed that even though the conditioned media did not induce the Tscm phenotype itself, we still observe higher levels of TCF1 than both mDC conditions (alone and supplementation with IL-7 and IL-15) **Fig.3.7H**. The LEC-T cell conditioned media did not induce a higher expression of PD1 as in LEC only activation cocultures, but we observe a

decrease in Tim3 expression by T cells that were in the presence of all LEC conditioned media **Fig.3.7H**.

The principal limitation of this experiment is that the conditioned media were depleted of nutrients, which may influence the T cell phenotypes, given that metabolism plays a significant role in T cell differentiation. To validate the outcomes observed with the conditioned media, we devised a transwell experiment designed to mitigate this limitation substantially. This experiment aimed to determine whether indirect interaction of LECs with T cells, or during T cell activation, is sufficient to induce the T cell phenotypes associated with our LEC conditions (central memory by SS, Th1 and Th2 LECs, and effector memory by Tsup LECs). The experimental setup is illustrated in **Fig.3.8**.

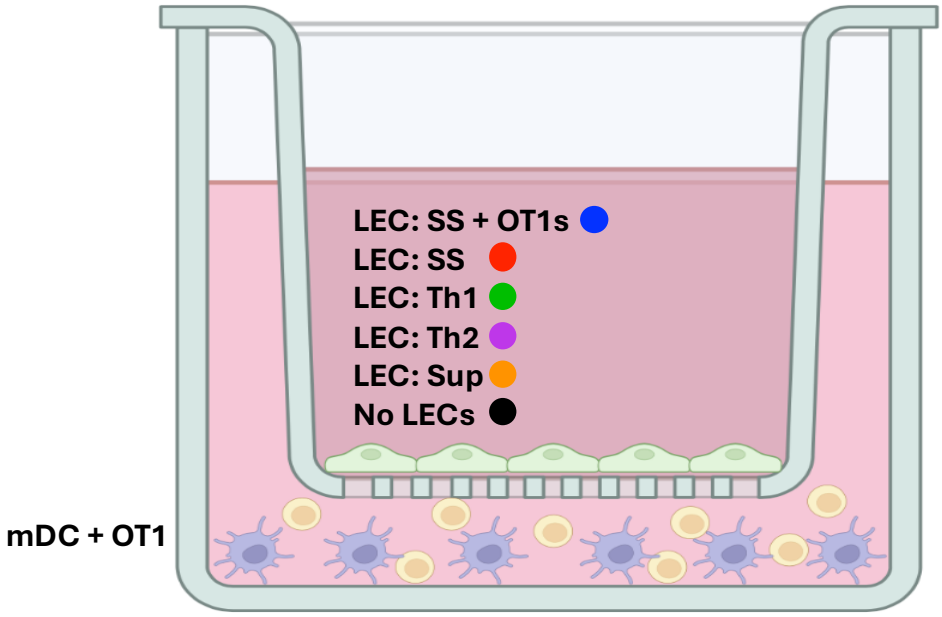

Figure 3.8: **Schematic of Transwell experiment set up**

We initially seeded the LECs within a transwell to prevent any interaction with the mDC-T cell cocultures occurring in the well. Subsequently, we stimulated the LECs overnight using our established cytokine cocktails, followed by washing before transferring them to the wells where the mDC and T cell co-cultures were initiated. We used every LEC stimulating

condition (SS, Th1, Th2, and Tsup), alongside a control condition without LECs (mDCs and T cells in co-culture). We hypothesized that interactions between T cells and LECs might be essential for the induction of these secreted factors. Therefore we included a condition where SS LECs and T cells where in co-culture in the transwell, while analyses were restricted to T cells present in the well with the mDCs.

Initially, we observed that despite the lack of direct contact between LECs and both T cells and mDCs, LECs appeared to sustain a naive T cell population that persisted until the end of the co-culture. This resulted in a generally lower number of activated cells within wells containing LECs in the transwell, with the exception of SS LECs **Fig.3.9A,B**. The Th2 and Tsup LEC conditions induced the least activation, a pattern corroborated by the percentage of cells retaining a naive phenotype **Fig.3.9A,B**. Notably, the presence of SS LECs still allowed for a residual population of naive cells; however, there was no notable reduction in the quantity of activated T cells **Fig.3.9A,B**.

We were pleased to confirm our hypothesis, as the findings were consistent with the conditioned media experiment. The presence of SS and Th1 LECs in the transwell was sufficient to program a population exhibiting a significantly greater central memory phenotype compared to mDC activation alone **Fig.3.9C**. Notably, Th2 LECs did not sufficiently induce central memory, as the T cell pool displayed behavior akin to mDCs activation. Conversely, the behavior of Tsup LECs was as anticipated. The presence of Tsup LECs effectively ablated central memory formation and predominantly programmed an effector memory pool **Fig.3.9C,D**. Additionally, it was evident that all LEC conditions, except for Tsup LECs, resulted in a significantly lower proportion of effector memory cells, whereas Tsup LECs led to a notably higher percentage of effector memory T cells compared to mature dendritic cell activation.

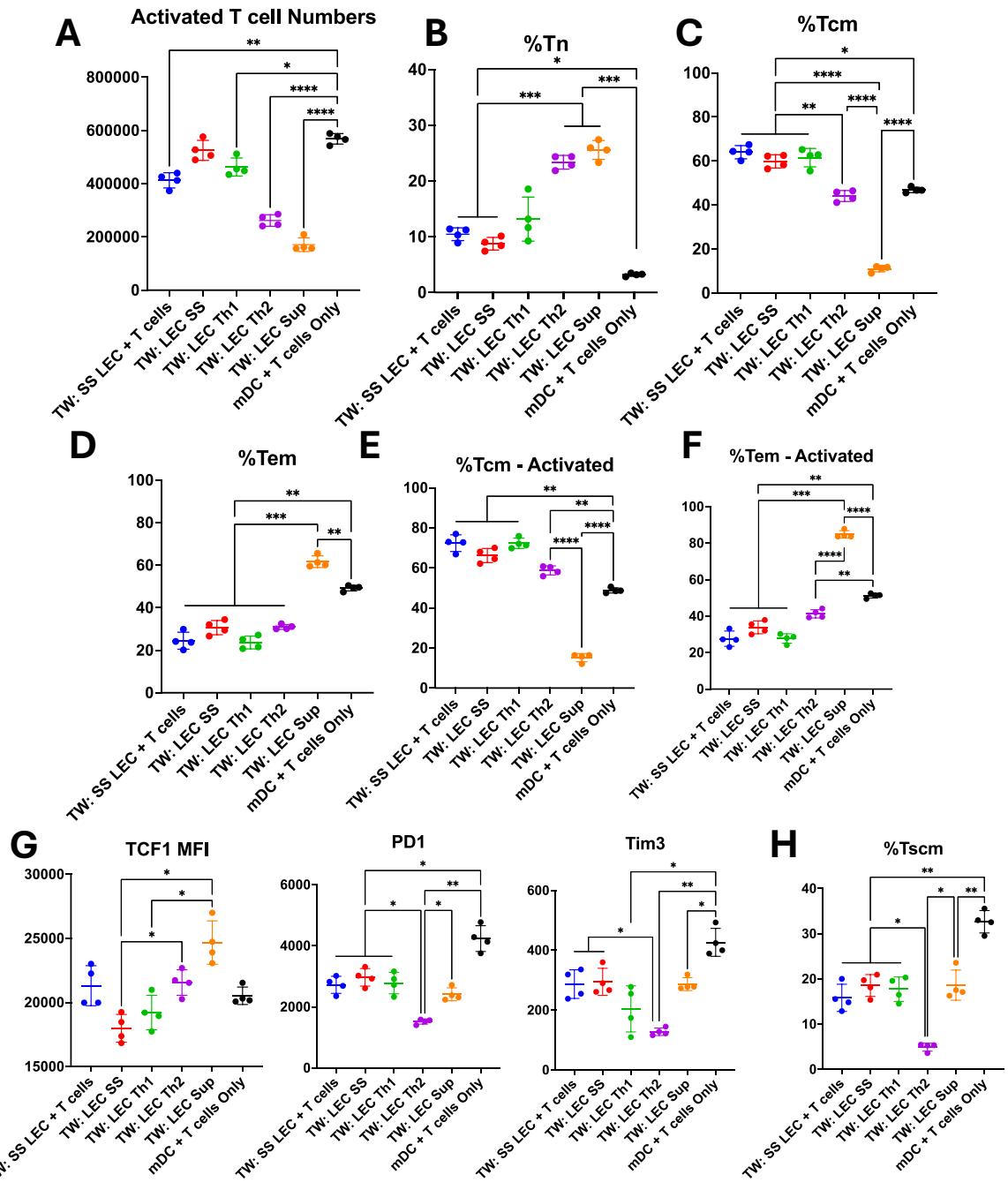

Figure 3.9: **LECs can induce their associated phenotypes with indirect contact, while Tscm does not effectively develop with indirect contact A** Number of activated cells. **B** quantified percentage of central memory from the total T cell pool. **C** Quantified percentage of Naive T cells. **D** Quantified percentage of effector from the total T cell pool. **E** Quantified percentage of central memory in the activated T cell compartment. **F** Quantified percentage of effector memory in the activated T cell compartment **G** Tscm associated marker quantification. **H** Quantified percentage of stem cell memory in the total T cell compartment.

Given our observations that all LEC conditions preserved a viable population of naive cells, with Th2 and Tsup LECs maintaining naive cells comprising approximately 25% of the total T cell population **Fig.3.9B**, we aimed to examine more closely the activated T cell pool to ascertain the proportions of central memory and effector memory within the activated compartment. When we gated on activated T cells, we saw that all LEC inflammatory conditions induced a population of T cells that was significantly more central memory than mDC-activated T cells only, raising the percentage of central memory about 20 percent **Fig.3.9E**. Tsup LECs induce the highest percentage of effector memory T cells at a staggering 80% effector memory, which is significantly higher than any other group, while all other LEC transwell conditions were significantly lower than mature dendritic cell-activated T cells **Fig.3.9F**. We also noticed that this was all due to indirect interactions between LECs and mDC-T cell cocultures, as the LEC SS + T cell in transwell control behaved almost identically as the LEC SS only group **Fig.3.9A-F**.

To further our investigation into the stem cell memory phenotype, we examined the expression levels of three primary markers associated with this phenotype. Notably, the presence of LECs alone does not significantly increase the expression of TCF1 in activated T cells. The sole condition indicating an upward trend in TCF1 expression occurs with the presence of Tsup LECs in the transwell; nonetheless, this increase is not statistically significant compared to mDC activation **Fig.3.9G**. Although differences were observed among the LEC transwell groups, none exhibited significant differences when compared to mDC activation. The patterns aligned with observations from the triculture and conditioned media experiments, particularly concerning PD1 and Tim3 expression. The general presence of LECs in the transwell was associated with a reduction in PD1 expression by T cells, with Th2 LECs in the transwell producing the most pronounced decrease **Fig.3.9G**.Furthermore, nearly all LEC transwell conditions resulted in a reduction in the expression levels of the inhibitory and exhaustion-associated receptor Tim3. All LECs, with the exception of Th1

LECs, demonstrated a significant reduction in Tim3 expression; however, Th1 LECs did exhibit a downward trend when compared to the activation of T cells by mature dendritic cells **Fig.3.9G**. Once more, we observe that the SS LECs in the transwell and the SS LECs + T cells in the transwell groups exhibited nearly identical behaviors. The only notable difference was a trend towards higher TCF1 expression in the SS LEC + T cell in the transwell group. This result indicates that direct contact with LECs is not necessary to induce central memory (in the case of SS, Th1, and Th2 LECs) or effector memory (as seen with Tsup LECs), nor to decrease exhaustion and inhibitory markers **Fig.3.9G**. In the context of these markers, we also investigated the presence of the stem cell memory population. Mature dendritic cells activation resulted in a higher induction of Tscm compared to any of the LECs in transwell conditions, with Th2 LECs eliciting the lowest induction. This finding aligns with our results from the conditioned media experiments.

Collectively, these data suggest that lymphatic endothelial cells are capable of programming the phenotype of T cells through indirect contact. Both inflamed and steady-state LECs have the capacity to program T cells towards a central memory phenotype, whereas suppressive LECs facilitate the induction of an effector memory population. Despite their pronounced ability to induce these phenotypic states, they are unable to impart a stem cell memory state to CD8 T cells via indirect contact.

3.5 Conclusions

In this chapter, we examined the expression of molecules that interact with T cells by LECs, highlighting and substantiating their comprehensive potential as antigen-presenting cells capable of activating and priming naive CD8 T cells. Furthermore, we demonstrate that lymphatic endothelial cells can induce their associated phenotypes when simultaneously activating naive CD8 T cells with mature dendritic cells. Lastly, we illustrate that although LECs can induce central memory and effector memory through indirect contact with T cells, direct contact is required to program for stem cell memory.

3.5.1 Lymphatic endothelial cell expression of molecules that stimulate T cells

The initial focus of our investigation in this chapter was LEC's expression of molecules that can interact with T cells. It has been reported that LECs can induce peripheral tolerance by their expression of PDL1, but we have also shown at steady state, Th1, Th2 and even suppressive conditions that they can activate naive CD8 T cells into central memory (SS, Th1 and Th2 LECs) and effector memory (in the suppressive environment). Consequently, we aimed to elucidate the dual role of LECs in both situations by identifying key molecules that may facilitate interactions with CD8 T cells.

To this end, we used full spectrum flow cytometry to investigate the expression levels of several molecules that are involved in T cell activation and differentiation. We focused on LECs that had been stimulated overnight with our standard cytokine cocktails. Initially, we examined a range of co-stimulatory molecules and determined that LECs do not exhibit the conventional co-stimulatory molecule CD80. However, they do express CD86 in conjunction with other co-stimulatory molecules intimately linked to memory induction and reactivation. In particular, we observed the expression of the TNF receptor superfamily co-stimulatory

molecules CD70 and OX40L, as well as ICOSL, a member of the B7 superfamily, all of which play a role in the development of memory T cells. Furthermore, our findings identified two inhibitory molecules: PDL1, whose expression on LECs is well-documented in existing literature, and PVR, an emerging inhibitory receptor related to checkpoint inhibition. These results indicate that LECs are capable of delivering both positive signals via co-stimulatory ligands and negative signals via checkpoint molecules.

In our investigation of molecules implicated in LEC-T cell interactions, we examined the expression levels of ICAM1, Garp, and LAP, an adhesion molecule, a TGFβ docking protein, and a TGFβ latency protein, respectively. Our findings confirmed that LECs exhibited higher ICAM1 expression compared to mDCs. Moreover, Garp expression was generally elevated in LECs, with Th2 LECs displaying the highest levels. Conversely, LAP did not exhibit the anticipated expression pattern; rather, Th1 LECs were observed to express the greatest levels of LAP, while the remaining LECs demonstrated equivalent expression levels to mDCs.

Notably, LECs across all stimulating conditions exhibit consistent trends in the expression of these molecules. Although there are variations in the levels of expression, the same molecules are consistently up-regulated and down-regulated when compared to mature dendritic cells.

The dynamics of marker expression upon T cell interactions were further examined by comparing the expression levels of these molecules following overnight stimulation and subsequent 24-hour contact with T cells. To achieve this, fold changes in expression were calculated by comparing the levels before and 24 hours after the addition of T cells. LECs consistently down-regulated all analyzed co-stimulatory molecules with the exception of CD80, which was up-regulated under all conditions. In terms of inhibitory molecules, their expression was relatively stable, although PDL1 was down-regulated specifically in Th1 LECs, while the expression of ICAM1 and Garp remained unchanged. Both LECs and mDCs demonstrated an

equivalent reduction in LAP expression. Notably, LECs exhibited consistent trends across all conditions.

3.5.2 LECs can induce and maintain their associated phenotype in tandem with mDCs activation and stimulation of CD8 T cells

When LECs and mDCs worked together to activate naive CD8 T cells in a triculture setting, LECs strongly promote their associated phenotypes. They promote a predominantly central memory population at SS and Th1 conditions, and they induce a large effector memory population in suppressive conditions. We also noticed that LEC contact programs a stem cell memory state quite strongly, with all LEC conditions inducing more than 70% of the T cell population into a stem cell memory state.

Finally we saw that LEC activation of CD8 T cells, when collaborating with mDCs induces a lower expression of the inhibitory and exhaustion molecules PD1 and Tim3 respectively compared to mature dendritic cell activation alone. Interestingly LECs also induce lower expression of the transcription factors Tbet and EOMEs.

All together, this shows that LEC activation works in tandem to mDC activation to induce central memory in the case of SS, Th1 and Th2 LECs, and effector memory in the case of Tsup LECs. LEC contact and activation at all conditions programs for a population that is highly stem cell memory in nature, and is also less exhausted and inhibited.

3.5.3 Indirect contact by LECs can strongly induce central or effector memory but not stem cell memory

We aimed to determine whether LECs could engage in indirect interactions with T cells, especially given that LECs are known to secrete numerous factors and cytokines essential for the differentiation, survival, and maintenance of memory T cells.

We first investigated this by conducting cocultures of mDCs and T cells in the presence of LEC-T cell conditioned media (CM) that were collected at the end of LEC and T cell cocultures. Upon supplementation of mDCs with LEC CM, it was observed that a naive T cell population remained inactivated by the conclusion of the experiment. Furthermore, examination of the activated T cell compartment revealed that the SS and Th1 LEC CM predominantly induced a central memory T cell population. Notably, Sup CM also resulted in higher levels of central memory compared to mDC activation alone. Interestingly, contrary to our hypothesis the proportion of Tscm was reduced in mDC and T cell cocultures supplemented with LEC CM.

We hypothesized that the nutrient depletion within the LEC conditioned media might have influenced T cell phenotype. To test this hypothesis, we conducted a transwell experiment. In this setup, LECs were placed within a transwell with small pore size to maintain complete separation from the mDC-T cell cocultures, preventing initial media nutrient depletion. Consistent with our observations in the conditioned media experiment, the presence of LECs under various stimulating conditions (SS, Th1, Th2, and Tsup) led to an increased population of naive CD8 T cells that were viable at the conclusion of the response. It was gratifying to observe that the inclusion of SS and inflamed (Th1 and Th2) LECs in the transwell promoted the differentiation of the activated T cell pool predominantly into central memory cells. Tsup LEC presence in the transwell notably resulted in a pronounced effector memory T cell population, constituting the highest percentage of such cells. These findings suggest robust LEC-driven programming, achieved indirectly through secreted factors or metabolites. Furthermore, the lack of stem cell memory T cell development observed parallels our earlier conditioned media experiment results, indicating that LEC presence alone does not suffice to induce a stem cell memory T cell pool. Thus, LECs facilitate strong programming both directly and indirectly: indirectly inducing central or effector memory and necessitating direct contact for stem cell memory phenotype induction. This elucidates

additional functional roles of LECs within the lymph node. As T cells become activated, proximity to LECs and their secreted factors can foster their development into the central memory pool, whereas those in direct contact with LECs can transform into the Tscm pool.

3.5.4 Conclusion schematic

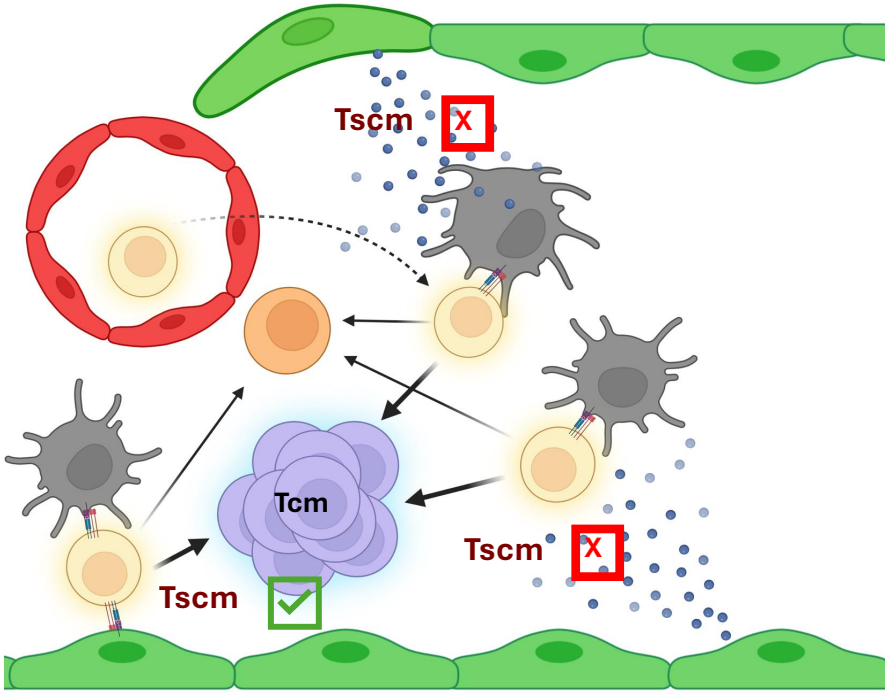

Figure 3.10: **Chapter 3 Conclusions**

CHAPTER 4

TRANSLATIONAL POTENTIAL: ADOPTIVE CELL THERAPIES AND MOLECULAR MECHANISM

4.1 Chapter Summary

In this chapter, we focus the mechanisms by which LEC activation influences the phenotypic characteristics of the T cells they encounter. As an immunoengineering laboratory, we further aim to investigate potential translational aspects of our findings, as this underpins the rationale for conducting our research.

In light of our previous observations that LECs can facilitate the induction of central end effector memory through the secretion of specific factors or metabolites, while needing direct contact with T cells for comprehensive programming (i.e., stem cell memory), we aimed to examine whether the presence of LECs during either mDC or dynabead-mediated activation of naive CD8 T cells, under LEC antigen-independent conditions, would similarly reproduce the effects documented in prior chapters. Our interest in this is driven by recognizing that the combined phenotype of central memory and stem cell memory in CD8 T cells is highly advantageous for adoptive T cell therapies such as tumor-infiltrating lymphocyte (TIL) and chimeric antigen receptor (CAR) T cell therapies. This is attributable not only to the long-term memory capacity of such cells but also to their established role as the reservoir from which proliferative responses and effector cells emerge following checkpoint blockade.

We investigated the effectiveness of LEC+bead activation of T cells in the anti-tumor response, and we tested the memory response of this activation method in a vaccine model.

Having identified the interaction and intercellular mechanisms involved, our aim was to elucidate the molecular mechanism by which LECs induce these phenotypes. However, this proved to be more challenging than anticipated. Additionally, this study highlights the molecules that are not implicated, or only partially implicated, in the molecular mechanism.

4.2 Introduction

4.2.1 Rationale and Unmet Clinical Need

Adoptive T cell therapies and CAR-T therapies are a line of immunotherapy in which we harness the CD8 T cells from cancer patients and utilize them as a therapeutic. This is done by identifying those that respond against the cancer, or forcing them to express a chimeric antigen receptor (CAR) that recognizes the immunodominant antigen of the cancer. The CD8 T cells are subsequently expanded *in vitro* and reintroduced into the patient to serve as a therapeutic modality against the cancer. These therapies have shown remarkable success in treating certain cancers, particularly those that are hematological in nature. However, there are still several unmet clinical needs and challenges, especially regarding the phenotypes of transferred CD8 T cells. These challenges are more evident in the treatment of solid tumors and in achieving long-term remission.[17, 215–222]

Unmet Clinical Need

T cell Exhaustion: A major challenge in adoptive T cell therapies is the rapid loss of effector function in transferred T cells, often due to T cell exhaustion. This exhaustion is characterized by a decrease in cytotoxic activity and proliferative capacity, limiting the long-term efficacy of the therapy.[215, 218, 219]

Limited Persistence: The inability of transferred T cells to persist long-term in patients is another significant challenge. This limited persistence can result in incomplete tumor elimination and potential relapse.[216, 220, 221]

Durable Remission: Achieving durable remission remains a challenge in many cases, particularly in patients with advanced or refractory disease.[215, 217, 218]

Desired CD8 T cell phenotypes

Memory-like T Cells: T cells with memory-like characteristics are highly desirable for adoptive cell therapies. These cells have enhanced persistence and can provide long-term anti-tumor immunity.[215, 220, 223, 224]

Resistance to Exhaustion: T cells that are resistant to exhaustion are also highly desirable. These cells maintain their effector functions even under chronic antigen stimulation, which is common in the tumor microenvironment.[215, 218, 219, 225]

While adoptive T cell therapies have shown a lot of promise, there are still significant challenges to overcome. Focusing on generating T cells with optimal phenotypes, including memory-like characteristics, and resistance to exhaustion is crucial for improving the efficacy and durability of these therapies, and in this chapter we explore if utilizing lymphatic endothelial cells during the expansion process could provide these desired characteristics.

4.3 Methods

mDC-LEC-Tcell antigen dependence tricultures: We isolated primary dendritic cells and lymphatic endothelial cells as described in chapter 1. After isolation and expansion of both the bone marrow derived dendritic cells and LECs, we pulsed and stimulated the DCs with the SIINFEKL peptide and LPS. We only treated the LECs with the cytokines cocktails that we've used throughout this project with no antigen. We then cultured the antigen pulsed mDCs, with the LECs and isolated naive T cells. We cultured them for 72 hours and analyzed them through full spectrum flow cytometry.

Bead-LEC and Bead-mDC T cell cocultures: After isolation and expansion of the BMDCs and LECs, we treated them with the normal stimulating conditions, with or without antigen, and added CD3/CD28 Dynabeads to the cultures according to manufacturer's protocol. We then added naive CD8 T cells at the usual ratio of 10:1 T cells to APCs ratio. We cultured these conditions for 72 hours and analyzed them using full spectrum flow cytometry.

***in vivo* experiments:** For the tumor experiments, we used C57BL/6 mice, and injected them with 250k B16 cells. We activated the naive CD8 T cells with beads and beads + LECs, for 6 days with IL2 supplementation. We adoptively transferred 1 million activated OT1s to each mice to treat the tumors. We measured tumors and administered aPD1 therapy (50 ug per mouse) every other day. On day 22 we resected the tumors and analysed them. For the vaccination experiments, we used C57BL/6 mice, and adoptively transferred one million activated OT1s (with the previous two methods). We waited 6 weeks for a memory time point, and then vaccinated with 40ug of OVA + 15 ug of CpG per mouse. We collected lymph nodes and spleens for analysis.

Blocking Experiments: After isolation and expansion of the LECs, we stimulated them as previously described. Once we washed them, we added media that had been supplemented with 10 ug/mL of the blocking antibodies and let them incubate for a minimum of 10 minutes before adding the isolated CD8 T cells. We then left them in co-culture for 72 hours as we do in the rest of our experiments.

Statistics: For all experiments, otherwise stated each dot on the plot represents the LECS of one mouse interacting with OT1s, or mDCs, so each dot represents LECs from individual mice. We utilized Brown-Forsythe and Welch ANOVA tests, Dunnett's T3 multiple comparisons test, with individual variances computed for each comparison. We only show the important statistically significant comparisons.

4.4 Results

4.4.1 Lymphatic endothelial cells can induce their associated phenotypes in an antigen independent manner

Upon recognizing that LECs are capable of inducing central memory (SS, Th1, and Th2) and effector memory (Tsup) through indirect mechanisms, yet require direct contact to elicit Tscm, we questioned whether this process is antigen-dependent. To investigate this matter, we conducted tricultures in which dendritic cells were pulsed with antigen. Furthermore, we varied the experimental conditions by either pulsing or not pulsing the LECs with the SIINFEKL peptide **Schematic.4.1**. This experimental design enabled us to compare the phenotypic outcomes following interactions between antigen-presenting LECs, mDCs, and T cells, alongside LECs lacking antigen exposure under identical conditions. We hypothesized that LECs would facilitate the generation of phenotypes such as central memory and effector memory in an antigen-independent manner, consistent with previous findings, whereas the induction of stem cell memory would be contingent upon the presence of antigen.

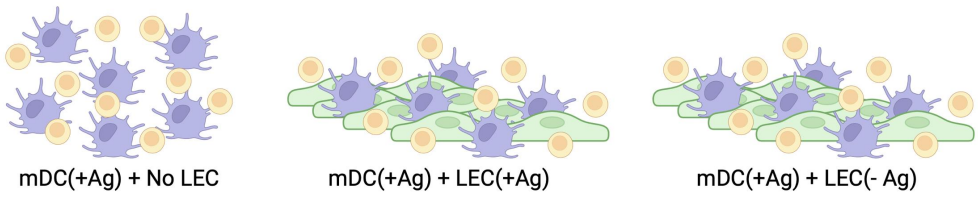

Figure 4.1: **Schematic of triculture set up**

Contrary to our initial expectations, LECs have the capacity to confer their complete associated phenotype in an antigen-independent manner, yet there are benefits associated with LEC antigen presentation as well. Our initial observation was that when LECs present antigen in conjunction with mDCs, there is an increase in live cell numbers during the

response, particularly from SS and inflamed LECs, whereas antigen-less LECs result in non-statistically significant differences in live cell numbers when compared to mDC activation alone **Fig.4.2A**. Furthermore, we corroborate our previous findings that the presence of LECs sustains a naive T cell population; however, this population is significantly larger when LECs do not present antigen, compared to when they do **Fig.4.2B**. Unsprisingly, while the naive T cell population is greater in scenarios where LECs do not engage in antigen presentation, the central memory T cell population is notably enhanced compared to mDC activation alone, influenced by the presence of SS, Th1, and Th2 LECs, irrespective of antigen presentation **Fig.4.2C**. The effector memory T cell population is larger in the presence of Tsup LECs within the triculture system, also independent of antigen presentation **Fig.4.2D**. Intriguingly, the percentage of stem cell memory T cells remains consistently high across all LEC conditions, regardless of their engagement in antigen presentation **Fig.4.2E**.

Similar to previous studies, we observed a higher proportion of naive T cells in the absence of antigen-pulsed LECs, prompting us to verify whether these patterns persisted within the activated T cell population. As anticipated, the trends were consistent. Upon examining the activated T cell pool, we noted that in the presence of SS, Th1, and Th2 LECs, irrespective of antigen presentation, the majority of the activated T cell compartment, exceeding 90%, differentiated into a central memory phenotype **Fig.4.2F**. A similar trend is observed in the presence of Tsup LECs, where, irrespective of antigen presentation, the majority of the activated T cell population differentiates predominantly into an effector memory phenotype. Approximately 60% of this population is effector memory, exceeding the proportion induced by mDC activation alone**Fig.4.2G**. The majority of the activated T cell population exhibited a stem cell memory phenotype in the presence of LECs independent of antigen presentation by them. This observation was both unexpected and intriguing, as it indicates that lymphatic endothelial cells possess a significant capacity to program their phenotypes. Nonetheless, direct contact with T cells is essential for the induction of the stem cell memory phenotype.

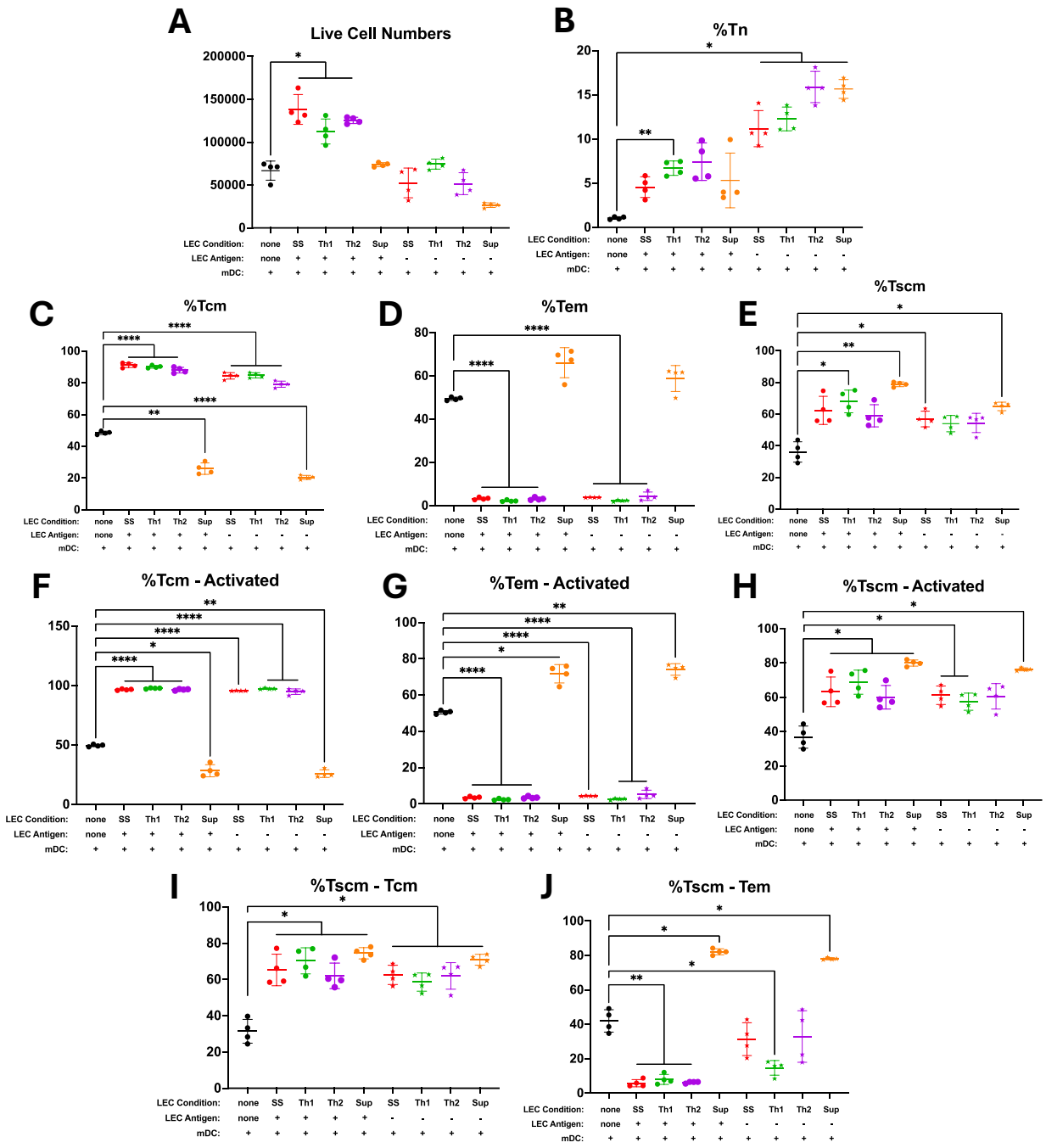

Figure 4.2: **LECs can program their associated phenotypes by contact in an antigen independent manner A** Quantified live cell numbers. **B** Quantified percentage of naive T cells left over. **C** Quantified percentage of central memory T cells. **D** Quantified percentage of effector memory T cells. **E** Quantified percentage of stem cell memory T cells. **F** Percentage of Tcm within the activated compartment. **G** Percentage of Tem within the activated compartment. **H** Percentage of Tscm within the activated compartment. **I** Percent Tscm of the Tcm pool. **J** Percent Tscm of the Tem pool.

Finally, we sought to independently assess the central memory and effector memory pools for the acquisition of stem cell memory characteristics. Similar to our initial co-culture experiment, we observed that a substantial proportion of the central memory pool, induced by either LEC antigen presentation or mere contact with LECs, adopted a stem cell memory fate across all LEC stimulation conditions **Fig.4.2I**. Regarding the effector memory pool, it was exclusively the Tsup LECs that elicited an effector population capable of acquiring stem cell memory characteristics **Fig.4.2J**, aligning with the outcomes of our initial co-culture experiment.

All together these data show that LEC contact is necessary to induce stem cell memory fate, but it does not depend on LEC antigen presentation, which showcases LECs as potent modulators of T cell phenotype in the lymph node.

In this experiment, a marginal up-regulation of TCF1 was observed when LECs did not engage in antigen presentation, whereas no up-regulation of TCF1 occurred under conditions where LECs presented the antigen **Fig.4.3A**. Furthermore, the presence of LECs, irrespective of antigen presentation, led to an increased expression of PD1 at levels that did not reach statistical significance, with the exception of the Tsup LEC condition, which exhibited a significant increase in PD1 expression regardless of antigen presentation **Fig.4.3A**. Previously we have observed that LECs contribute to the down-regulation of Tim3 expression on T cells, even during indirect interactions. In the current experiment, this observation is reaffirmed as LECs significantly attenuate Tim3 expression in an antigen-dependent manner. Specifically, the reduction in Tim3 was noted to be insignificant when LECs did not present the antigen. However, Tim3 expression was significantly down-regulated in all LEC conditions when antigen presentation occurred in conjunction with mDCs **Fig.4.3A**.

We quantified the expression of granzyme B in these T cells to provide an estimate of their effector functions. LEC antigen presentation at SS, Th1, and Th2, in conjunction with mDCs, does not result in a significant alteration in the T cell expression of granzyme B

Fig.4.3B. Interestingly, when LECs are in contact with T cells without presenting antigen, there is an up-regulation of granzyme B expression, though this increase is not statistically significant **Fig.4.3B**. Furthermore, our findings reveal that the presence of Tsup LECs, regardless of antigen presentation, significantly reduces the expression of granzyme B in T cells. Additionally, we investigated the transcription factor TOX, known to be associated with cellular exhaustion. Our analyses indicate that LEC antigen presentation leads to a down-regulation of TOX, notably by Th1 LECs to a substantial degree **Fig.4.3C**. When LECs did not present antigens, we observed that both Th1 and Th2 LECs reduced TOX expression, with the reduction by Th1 LECs being statistically significant **Fig.4.3C**. Collectively, the data indicate that interaction between LECs and T cells results in lower levels of exhaustion compared to activation by mDCs alone, suggesting a potential role for lymph node LECs in influencing the immune response as it unfolds in the lymph node.

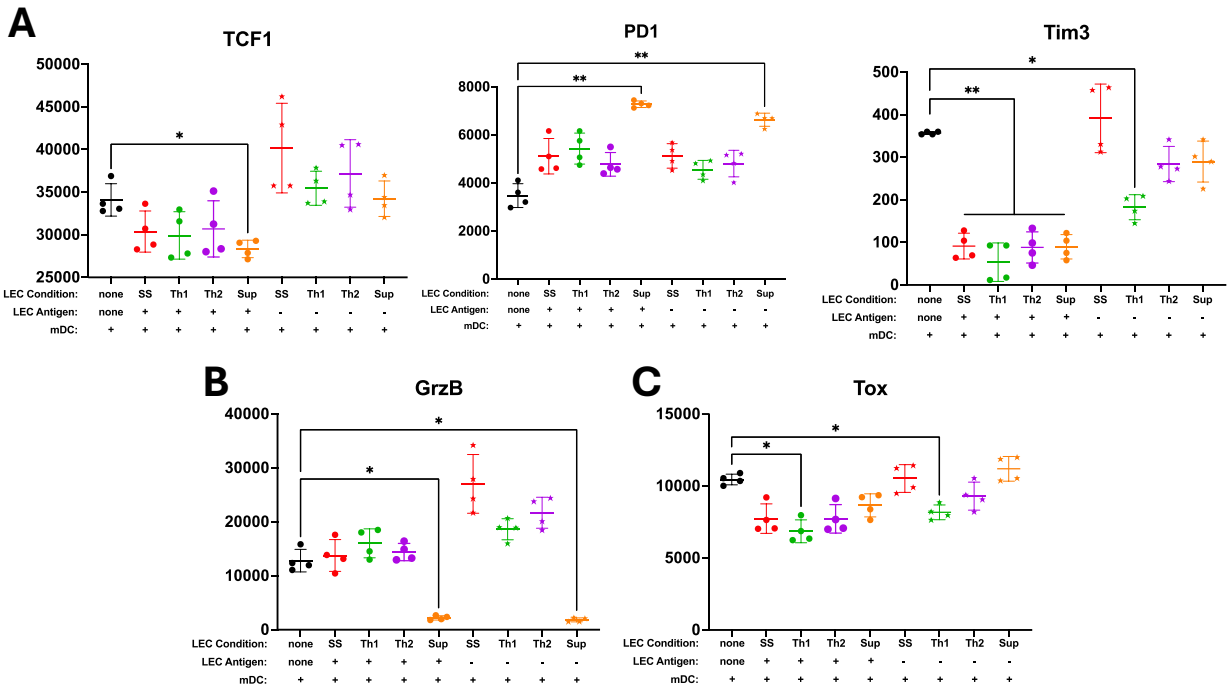

Figure 4.3: **Lymphatic endothelial cells program a less exhausted T cell pool in an antigen independent manner A** Quantified MFIs of stem cell memory associated markers. **B** Quantified MFI of granzyme B. **C** Quantified MFI of exhaustion transcription factor Tox.

Upon observing that LECs are capable of inducing both central memory and stem cell memory in an antigen-independent manner, our initial thought was to explore the potential of activating T cells using dynabeads, a method more relevant in adoptive T cell therapy research, with LECs concurrently present in the same culture well. To this end, we designed a co-culture experiment where T cells were activated using dynabeads in conjunction with LECs present in the culture wells. The experimental conditions mirrored those utilized in the prior experiment, with groups stratified based on whether LECs presented antigen or not **Schematic.4.4**. Additionally, we incorporated a group containing mDCs, either with or without antigen presence, to examine if the inclusion of APCs generally enhanced the efficacy of bead-induced activation **Schematic.4.4**. Furthermore, IL-2 was supplemented during T cell activation, as it is a conventional practice when activating CD8 T cells with beads or antibodies, specifically when expanding them for adoptive cell therapies.

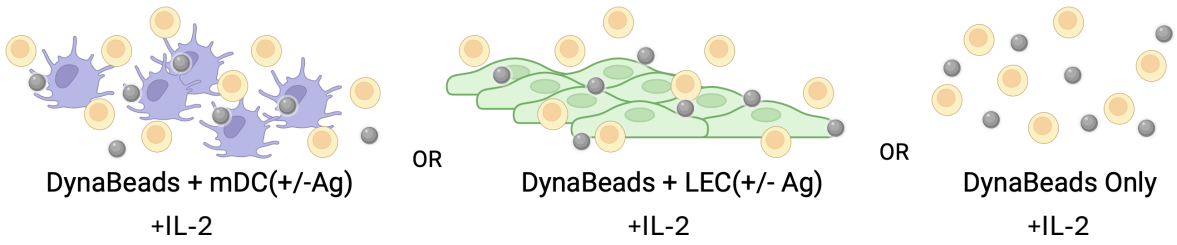

Figure 4.4: **Schematic of bead cocultures set up**

As anticipated, the contact and presence of LECs during bead activation led to the differentiation of the CD8 T cell population predominantly into central memory cells when SS, Th1, and Th2 LECs were present **Fig.4.5A**, and into effector memory cells when Tsup LECs were present **Fig.4.5B**. No substantial difference was observed in the presence or absence of antigen presentation by LECs, consistent with prior experimental results, apart from a non-significant increase in the naive T cell population when LECs did not present antigens **Fig.4.5D**, except for antigenless SS and Th2 LECs.

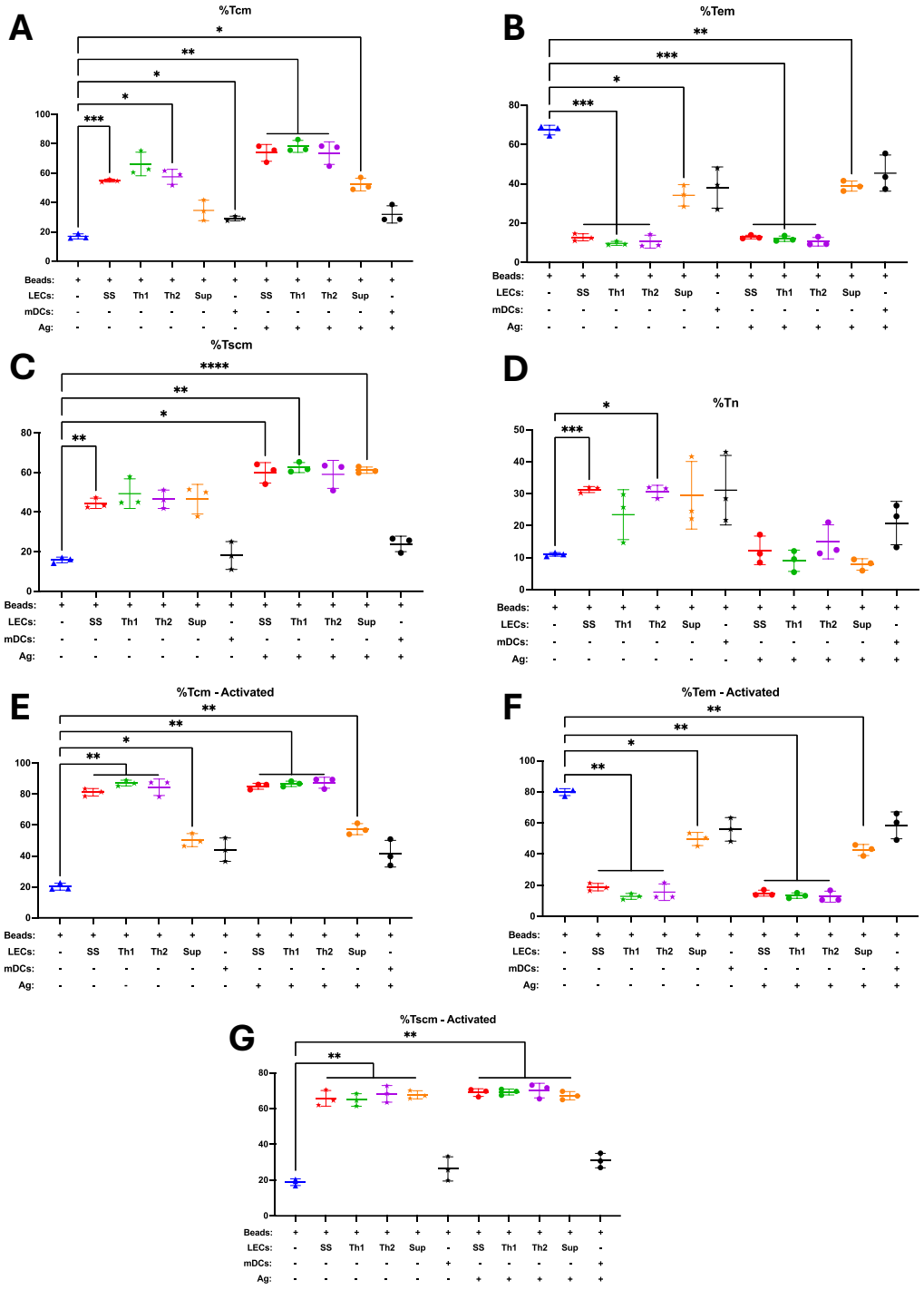

Figure 4.5: **Lymphatic endothelial cells can adjuvant bead activation to induce their associated phenotypes A** Percentage of Tcm in the T cell compartment. **B** Percentage of Tem in the T cell compartment. **C** Percentage of Tscm in the T cell compartment. **D** Percentage of naive T cell left over. **E** Percentage of Tcm in the activated T cell pool. **F** Percentage of Tem in the activated T cell pool. **G** Percentage of Tscm in the activated T cell pool.

A compelling outcome of this experiment was the observed increase in the proportion of stem cell memory within the total T cell compartment, attributable to the presence of non-antigen pulsed LECs during bead activation across all conditions **Fig.4.5C**. Although this increase, which exceeded 20%, did not reach statistical significance due to multiple comparisons, it was notable in contrast to bead activation alone and mDC supplementation to bead activation. Furthermore, a higher percentage of Tscm was detected when LECs presented antigen in conjunction with bead activation **Fig.4.5C**.

Given our previous observation that non-antigen presenting LECs sustain a substantial proportion of naive CD8 T cells, we examined the activated compartment to verify whether these trends were sustained in that context. As anticipated, it was observed that non-antigen presenting SS, Th1, and Th2 LECs induce an approximately 90% central memory population, which significantly surpasses the approximately 20% central memory induced by bead activation alone — thereby elevating the percentage of central memory within the activated compartment by 70% **Fig.4.5E**. Consistent with the findings of our previous experiments, it is evident that Tsup LECs elicit the highest percentage of effector memory among the various LEC conditions, akin to the effect of mDC supplementation during bead activation **Fig.4.5F**.

Finally, the percent of Tscm within the activated compartment increases 70%, from 20% when T cells are activated by beads only to 90% when LECs supplement the bead activation **Fig.4.5G**. Moreover, it is noteworthy that LECs facilitate this effect in an antigen-independent manner, as there is no significant difference in the percentage of stem cell memory from the activated T cell pool between LECs + Antigen groups and LECs - Antigen groups **Fig.4.5G**. Additionally, all of the LEC conditions induced a higher percentage of stem cell memory compared to mDC supplementation.

Consistent with the methodology used throughout this project, we aimed to examine the individual expression of several markers, namely TCF1, PD1, Tim3, and Granzyme B. Our

initial focus was on assessing the expression levels of TCF1, as it has consistently served as our most reliable and significant readout.

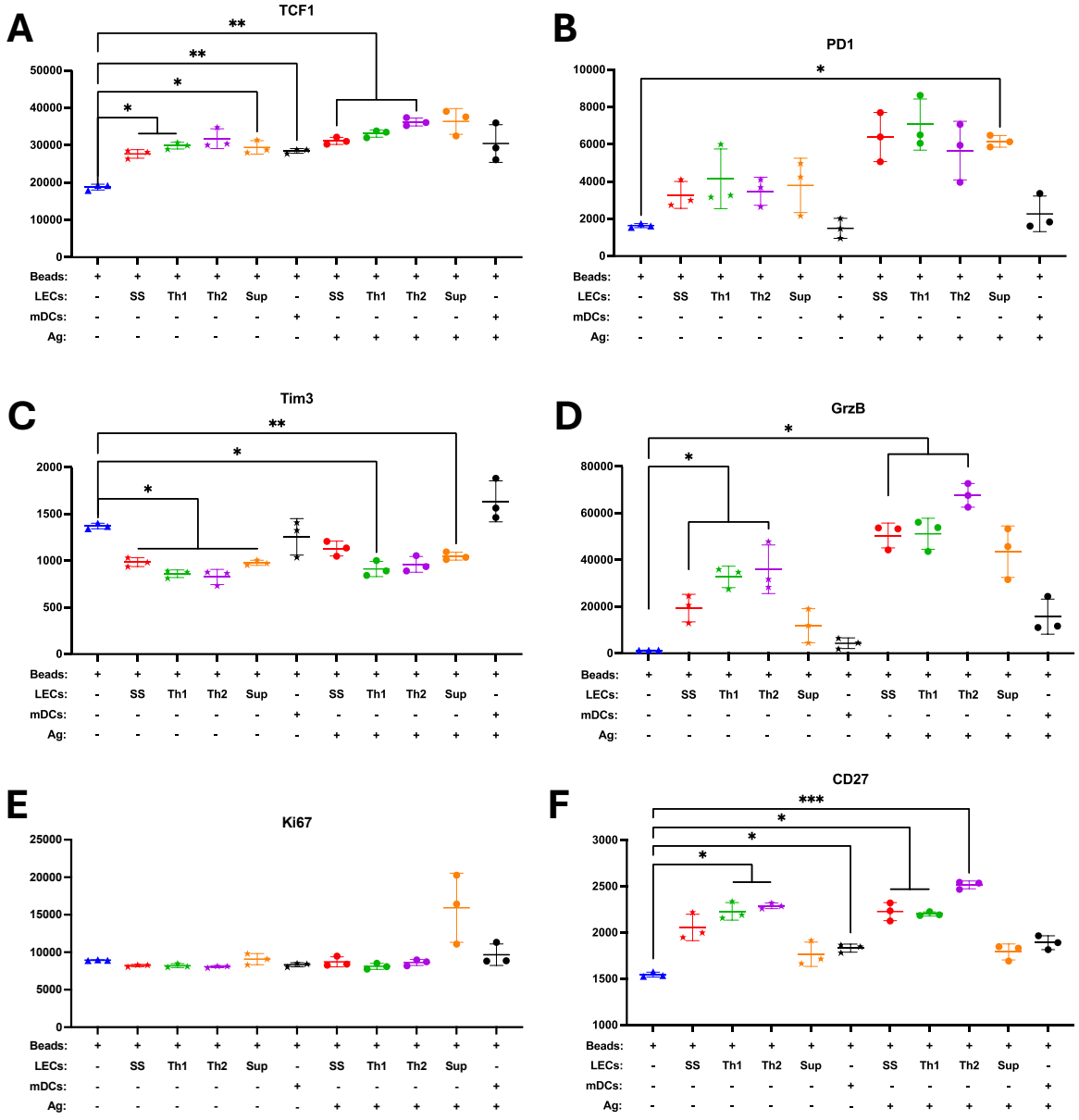

Figure 4.6: **LEC presence during bead activation induces a less exhausted T cell pool A** Quantified TCF1 expression. **B** Quantified PD1 expression. **C** Quantified Tim3 expression. **D** Quantified granzyme B expression. **E** Quantified Ki67 expression. **F** Quantified CD27 expression.

During bead activation of T cells, the presence of additional APCs was crucial in either

promoting higher levels of TCF1 expression or preventing the inhibition of this transcription factor's expression **Fig.4.6A**. This may be attributed to the strong activation signals provided by beads, which have the potential to suppress the expression of TCF1. When LECs are utilized to supplement bead activation, a general elevation in PD1 expression occurs compared to bead activation alone. However, this increase is not statistically significant, with the exception of antigen-pulsed Tsup LECs, which induce a marked and significant increase in PD1 expression **Fig.4.6B**. A decrease in Tim3 expression is consistently observed when compared to strong activation, in this case by beads **Fig.4.6C**. Notably, this reduction in Tim3 expression occurs in an antigen-independent manner, suggesting potential benefits for utilizing LECs as supplementary cells in T cell expansion for adoptive therapies. To assess effector functions, we evaluated the expression of granzyme B, showing that supplementation of bead activation with SS, Th1, and Th2 LECs enhances granzyme B expression in both antigen-pulsed and antigenless LECs, with antigen-presenting LECs exhibiting a slightly greater increase in granzyme B expression **Fig.4.6D**. We sought to determine whether LEC supplementation influenced the expression of the proliferation marker Ki67; however, no differences were observed across all groups except for the antigen-presenting Tsup LECs, which notably increased Ki67 expression **Fig.4.6E.**. Nonetheless, all T cells exhibited comparable proliferation levels. Furthermore, we investigated whether the T cells upregulated any additional LEC-associated markers and observed a similar trend to the initial cocultures: SS, Th1, and Th2 LECs elevated CD27 expression on CD8 T cells in an antigen-independent manner. This finding demonstrates the robust capacity of LECs to program T cell phenotypes through LEC-T cell interactions, even in the absence of antigen presentation **Fig.4.6F**.

4.4.2 In vivo confirmation of LEC adjuvanted bead activation of CD8 T cells

A key translational application of utilizing LECs during antigen-independent bead activation of T cells is adoptive T cell therapies. To elucidate the behavior of LEC-adjuvanted CD8 T

cells *in vivo*, we devised two experimental setups aimed at evaluating their memory potential and effector functions.

Initially, we sought to investigate whether the presence of LECs within the culture well during T cell activation affects or enhances their effector functions. Accordingly, we conducted an experiment where mice were inoculated with either B16-OVA or B16-VEGFC-OVA (melanoma models available to us) and treated with CD8 T cells activated either solely with beads or with beads in conjunction with LEC presence in the culture well. Additionally, the mice received aPD1 therapy, due to the synergistic response of the Tscm population with this treatment. The experimental timeline is depicted in **Fig.4.7A.**.

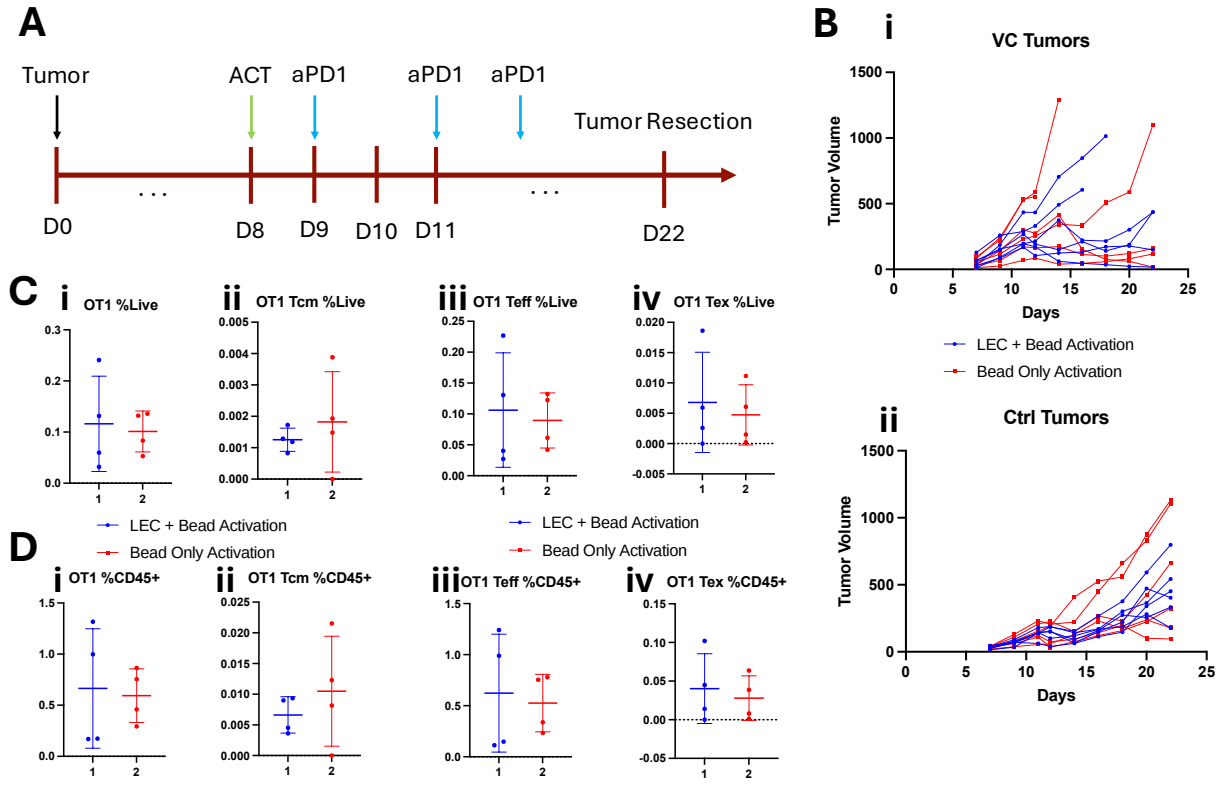

Figure 4.7: **LEC supplementation during bead activation results in a T cell population equally as capable of responding against B16 Melanoma A** Timeline of experiment. **B** Tumor growth curves of (i) B16-OVA-VEGFC and (ii) B16-OVA. **C** Percentage of tumor live cells that are (i) OT1 (ii) OT1 Tcm (iii) OT1 Teff and (iv) OT1 Tex. **D** Percentage of tumor CD45+ cells that are (i) OT1 (ii) OT1 Tcm (iii) OT1 Teff and (iv) OT1 Tex.

Remarkably, both activation conditions elicited comparable anti-tumor responses, as evidenced by the tumor growth curves and tumor burden **Fig.4.7B.** in both tumor models. After tumor extraction, our objective was to examine the OT1 CD8 T cell populations that had infiltrated the tumor microenvironment. Upon analysis of the T cell populations, no discernible difference in OT1 infiltration was detected between the activation conditions **Fig.4.7Ci,Di**. Analysis was conducted based on both the percentage of live T cells and the percentage of CD45+ cells, with both approaches yielding consistent outcomes. The addition of LECs in the culture wells during CD8 T cell activation neither augmented the percentage of OT1s infiltrating the tumor nor altered the phenotypic composition within the tumor **Fig.4.7Cii-iv, Dii-iv**. Both tumor models showed consitent results; however, to prevent redundancy, we present only the results pertaining to the B16-OVA-VEGFC model.

Subsequently, we aimed to demonstrate that the supplementation of bead activation with LECs would result in a population capable of eliciting an effective memory response or potentially enhancing it. To this end, we performed adoptive transfer of either bead-activated T cells or those activated with the combination of LECs and beads. Following a period of six weeks to allow for the establishment of a memory population, we proceeded with vaccination using OVA and CpG, as demonstrated in **Fig.4.8A**. Post-vaccination, we harvested the lymph nodes and spleens from the immunized mice for subsequent analysis of T cell populations. While both the percentage of live cells and CD45+ cells were calculated, owing to similar observed trends, only the percentage of live cells is presented to avoid redundancy. It is clear that both activating conditions induce OT1 populations that perform quite similarly to secondary challenge. Both activation conditions led to an equivalent percentage of OT1 cells within both the lymph node and spleen following vaccination **Fig.4.8Bi, Ci**. Upon analyzing the phenotypic distribution of OT1 cells within the lymph nodes and the spleen, we observed that the percentages were consistent across different activation conditions **Fig.4.8Bii-v, Cii-v**.

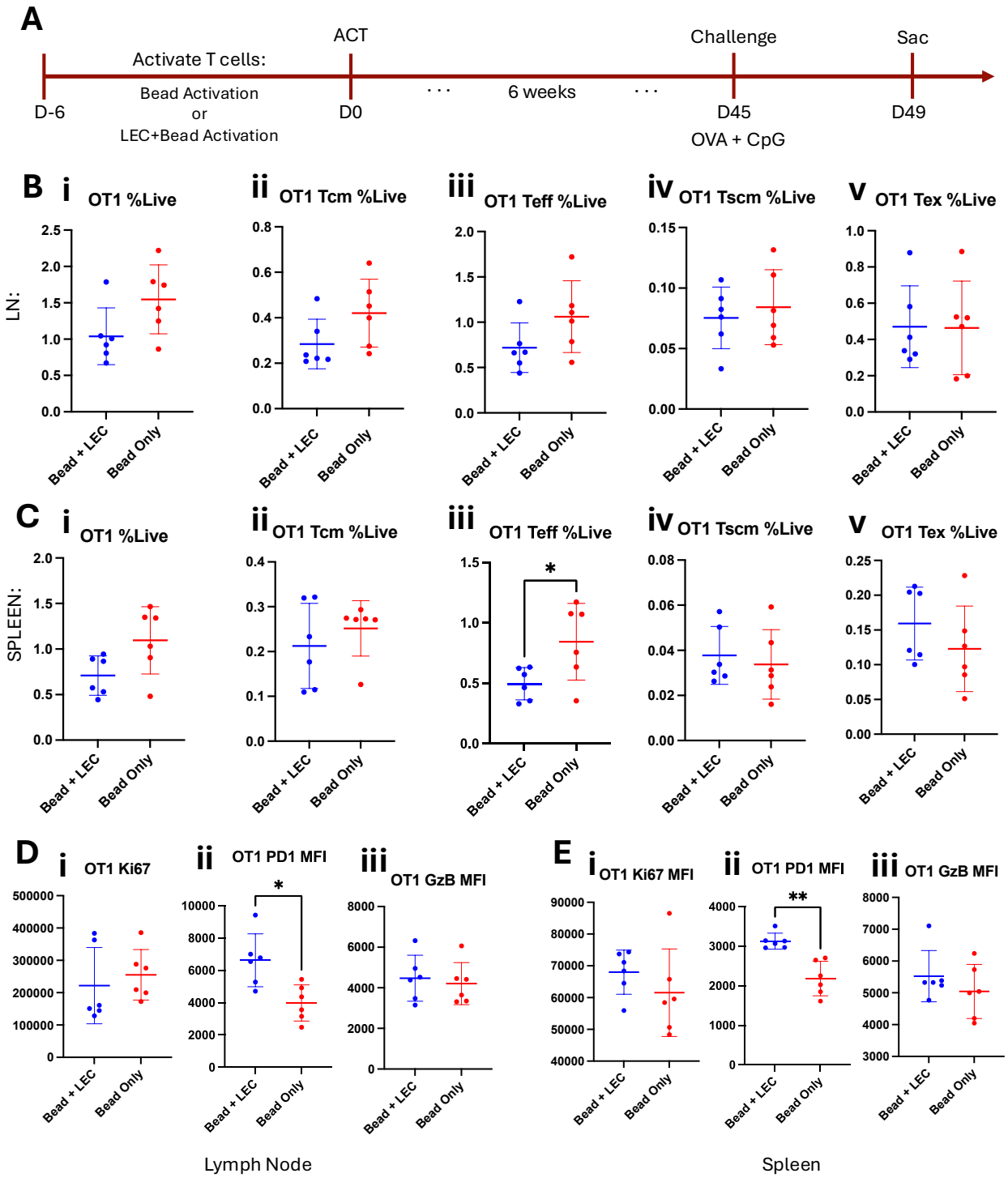

Figure 4.8: **LEC supplementation during bead activation results in a T cell population equally as capable of mounting a memory response upon vaccination A** Timeline. **B** Percentage of live LN cells that are (i)OT1 or OT1 (ii)Tcm (iii)Teff (iv)Tscm and (v)Tex. **C** Percentage of live spleen cells that are (i)OT1 or OT1 (ii)Tcm (iii)Teff (iv)Tscm and (v)Tex. **D** (i)Ki67, (ii)PD1 and (iii)Granzyme B MFIs of LN OT1s. **E** (i)Ki67, (ii)PD1 and (iii)Granzyme B MFIs of spleen OT1s.

The sole statistically significant difference identified was an increased percentage of effector OT1 cells in the spleen when activated exclusively by beads **Fig.4.8Ciii**. Finally, we hypothesized that LEC+bead activation would cultivate a population exhibiting heightened proliferation and efficacy. Consequently, we evaluated the expression levels of Ki67 and granzyme B. Contrary to our initial hypothesis, the expression levels of Ki67 were found to be consistent across both activation conditions in OT1 cells within the lymph node and spleen **Fig.4.8Di, Ei**. Notably, an increased expression of PD1 was observed when LECs were employed to supplement activation **Fig.4.8Dii,Eii**, whereas no differential expression of granzyme B was detected between the activation conditions **Fig.4.8Diii,Eiii**.

4.4.3 A story of negative data: our stab at molecular mechanism

A key objective was to elucidate the molecular mechanisms by which LECs facilitate the induction of central memory and effector memory phenotypes. Additionally, we sought to understand the mechanism of induction of stem cell memory fate attributed to T cell-LEC interactions. Unfortunately, our efforts to decipher this mechanism were unsuccessful. Nevertheless, it is crucial to document negative data, as it can significantly aid future researchers by narrowing down possibilities in subsequent investigations, as we believe this is a valuable direction for future research.

To elucidate the underlying molecular mechanisms, we initially sought to inhibit several molecules that we identified in chapter 3 as differentially expressed by LECs under varying conditions, compared to mDCs. Our initial focus was on the LEC expression of the inhibitory molecule PDL1 and the adhesion molecule ICAM1, given their reported involvement in memory formation and differentiation. PDL1 is essential for the development of an effective memory response, while ICAM1 is required for the asymmetric division of T cells, a process associated with the preservation of memory. Notably, the inhibition of these molecules revealed no discernible impact on the development of central and stem cell memory fates

Fig.4.9A.

Our subsequent investigation focused on the co-stimulatory molecules identified in Chapter 3, specifically ICOSL, OX40L, and CD70, due to their association with memory formation and reactivation. Upon inhibition of these molecules, no discernible effects on central memory development or stem cell memory fate were observed **Fig.4.9B**. Considering that OX40L and CD70 belong to the TNF family of co-stimulatory molecules, we extended our approach to include the blockade of 41BBL, another TNF family co-stimulatory molecule implicated in memory formation. Nonetheless, the simultaneous and individual inhibition of these molecules did not result in any changes in either central memory development or stem cell memory fate **Fig.4.9C**. Additionally, recognizing that LECs may indirectly promote central memory, we sought to inhibit two cytokines produced by LECs, IL7 and IL15, known to be involved in memory maintenance. However, the blockade of these cytokines yielded no alterations in phenotypes **Fig.4.9D**.

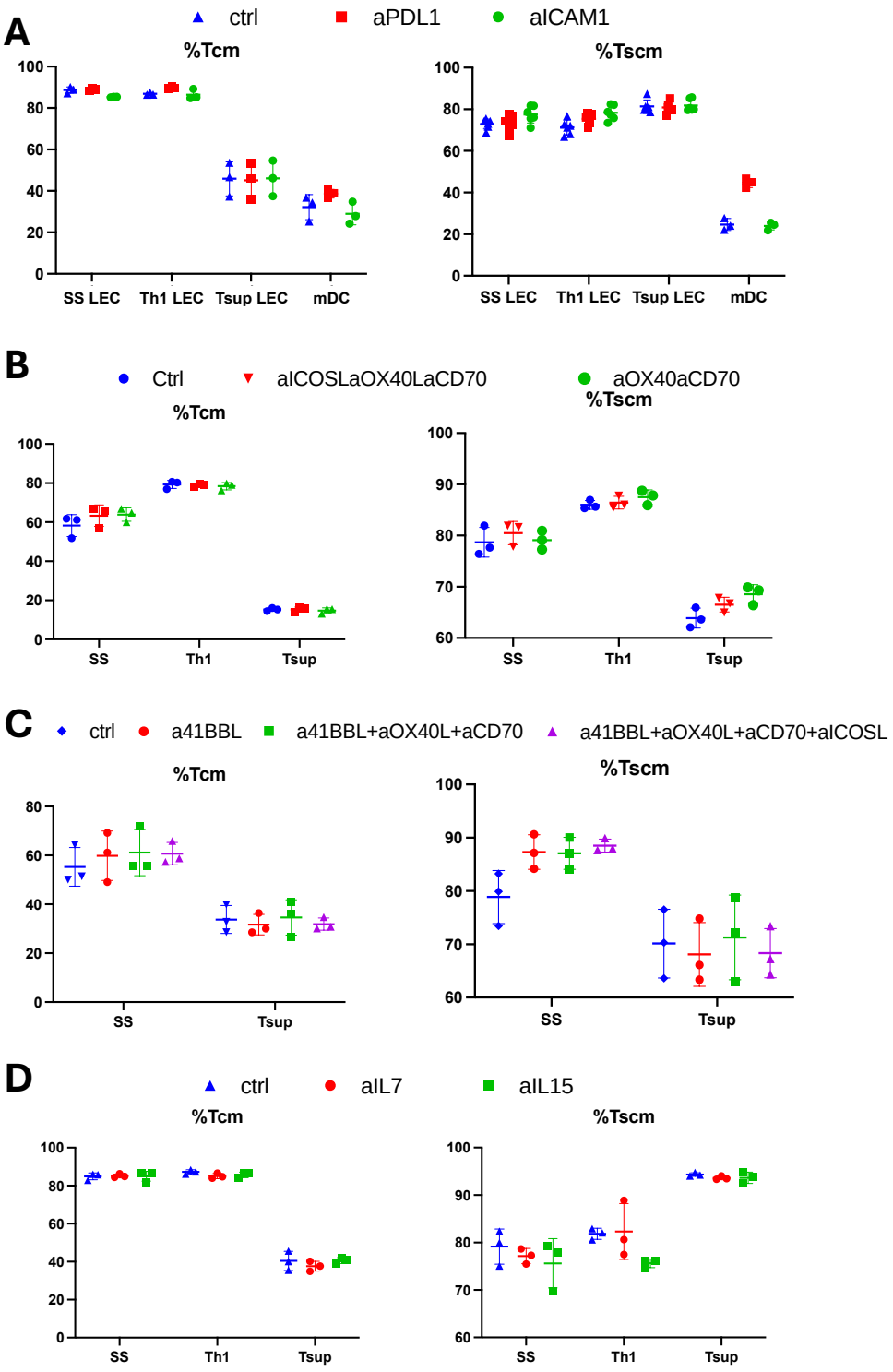

Figure 4.9: **LECs do not program their associated phenotypes through PDL1, ICAM1, CD70, OX40L, ICOSL, 41BBL, IL7 or IL15 A** Blocking of PDl1 and ICAM1 on LECs. **B** Blocking of ICOSL, OX40L and CD70 on LECs. **C** Blocking of TNF family costimualtory molecules. **D** Blocking of IL7 and IL15 produced by LECs.

4.5 Conclusions

4.5.1 Lymphatic endothelial cells are strong modulators of CD8 T cell phenotype regardless of antigen presentation

In this chapter, we sought investigate why LECs failed to induce the stem cell fate we had previously documented in an indirect way, unlike their ability to indirectly induce central memory. We had established that direct contact between T cells and LECs was essential for programming this T cell fate. However, it was unclear if the induction was dependent on the presence of antigen. This was the primary question we aimed to address in this chapter. Consequently, we conducted triculture experiments similar to those in the previous chapter, with the major difference being the inclusion of groups where the LECs were not pulsed with any antigen.

We observed notable variations depending on whether LECs presented antigens or not, predominantly showing an increase in viable cells with LEC and mDC antigen presentation and a decrease in residual naive cells at the end of the culture. However, upon examining the T cell phenotypes arising from both conditions, LECs consistently induced their specific phenotypes regardless of antigen presentation. Supplementation of mDC-T cell cultures with SS, Th1, and Th2 LECs, irrespective of antigen presence, led to a central memory population, while Tsup LECs encouraged an effector memory population. Notably, in all conditions, LEC supplementation significantly increased the percentage of stem cell memory compared to mDC activation alone, showing that LECs are potent modulators of CD8 T cell phenotypes even without antigen presentation.

As an immunoengineering laboratory, we were thrilled when we observed this effect and considered the potential of using LECs as an adjuvant cell in adoptive T cell therapies, such as TIL and CAR-T therapies. The LEC-associated phenotypes we observed are precisely what we seek for the CD8 T cells involved in these therapies. Noting that LECs could induce

desired phenotypes in mDCs without reliance on antigens, we decided to conduct similar experiments with Dynabeads to activate and expand T cells, as is standard in adoptive cell therapy studies. We were thrilled to discover that SS, Th1, and Th2 LECs significantly increased the percentage of central memory cells compared to bead activation alone—by almost 40%. Although antigen presentation by LECs results in a somewhat higher increase in central memory due to fewer remnants of naive T cells, optimizing this could yield more activated T cells. Importantly, LECs consistently programmed a stem cell memory fate, independent of antigen presence, underscoring their potential as a supplement to bead or antibody T cell activation in these therapies due to the highly sought-after phenotype they induce.

LECs contribute to the maintenance of a less exhausted T cell population, which is highly desirable for adoptive cell therapies. LEC supplementation results in decreased expression of Tim3 independent of antigen presentation and enhances granzyme B expression in T cells, indicating robust effector functions. This represents a promising area for further investigation as it may facilitate the development of more efficient CAR-T and TIL therapies. This is particularly relevant given that LECs have the capability to impart these phenotypes to T cells previously activated by other APCs.

4.5.2 Lymphatic endothelial cells as adjuvants for T cell expansion in vivo

A significant translational application of utilizing LECs in the antigen-independent bead activation of T cells is adoptive T cell therapies. Consequently, we aimed to investigate whether T cells activated with LEC-supplemented beads exhibited enhanced responsiveness against tumors and to evaluate their behavior at memory timepoints within a vaccination model. Observing that both activation methods elicit comparable anti-tumor responses was intriguing. This result validated that LEC-supplemented activation triggers a potent effector response, which remains unaffected by the presence of LECs in the culture. We did not see

any enhanced outcomes in the tumor-bearing mice, and the phenotypic composition of T cells infiltrating the tumors was consistent under both activation conditions. Contrary to our hypothesis, activation with LEC-supplemented beads did not stimulate an enhanced memory response post-vaccination; both spleen and lymph node OT1s expanded into identical phenotypes with comparable expression of granzyme B and Ki67.

4.5.3 Molecular mechanism: An intriguing future direction

A component of this project was to elucidate the underlying molecular mechanisms; however, this proved to be a challenge due to the involvement of not merely a surface molecule or a secreted element, but rather a synergistic interaction of both that directs the programming of central memory and stem cell memory fates. We conducted a series of blocking experiments targeting pivotal receptors, ligands, and cytokines expressed by LECs, which are implicated in the formation, differentiation, activation, and maintenance of memory. Our investigation concentrated on PDL1, ICAM1, the B7 family co-stimulatory molecule ICOSL, and the TNF superfamily co-stimulatory molecules CD70, OX40L, and 41BBL, as well as the cytokines IL7 and IL15. Regrettably, none of these blocking experiments resulted in changes in either central memory or stem cell memory.

One notable limitation of these experiments is the difficulty in identifying a suitable positive control for the antibody blockade of these molecules. Although we trust the results and the manufacturing certifications that confirm these antibodies as effective blockers, there remains uncertainty about whether the blockade failed *in vitro*. However, this presents an intriguing avenue for future research, as understanding the underlying molecular mechanisms could facilitate therapeutic advances to induce the observed LEC-associated phenotypes, rather than employing LECs as auxiliary cells for T cell therapy expansion. The most straightforward method I propose is to conduct RNA sequencing on the stimulated LECs and compare expression levels of surface molecules, such as receptors, ligands, integrins, and

secreted elements like cytokines and metabolites. Identifying variances in the expression of these molecules between SS, Th1 LECs, and Tsup LECs, for instance, could significantly narrow the possibilities. While the molecular mechanism was not pinpointed, we did elucidate and describe the mechanisms of cell-to-cell interactions involved.

CHAPTER 5

GENERAL CONCLUSIONS, DISCUSSION, AND FUTURE DIRECTIONS

5.0.1 Lymphatic endothelial cells as APCs in cytokine microenvironments crucial for immune responses

Central and Effector Memory Induction:

The origin of this project can be traced back to our lab's discovery that lymph node lymphatic endothelial cells (LECs) have the capability to capture antigens during steady-state conditions. This revelation was significant, as it led to the identification of additional immune roles for LECs, categorizing them as active participants in the immune system rather than merely structural components of the lymph nodes and lymphatic vessels. Following this, we were able to demonstrate that these LECs, under steady-state conditions, not only capture antigens but also cross-present them on MHC I molecules to CD8 T cells. This process can prime naive CD8 T cells into a central memory phenotype, analogous to stem cell memory T cells. Given the novelty and implications of LECs not just inducing tolerance, we sought to further investigate their interactions with T cells within cytokine-rich environments crucial for effective immune responses, beyond just steady-state. Thus, we embarked on an experiment where our lymph node LECs were treated with cytokines mirroring those of active immune responses. These included Th1 and Th2 cytokines associated with anti-viral and anti-parasitic (or allergic) responses, along with suppressive cytokines typically found in the gut and tumor microenvironments.

We were delighted to discover that in inflammatory settings, when LECs are stimulated by Th1 and Th2 cytokines, they also promote the formation of a central memory population upon activation of naive CD8 T cells. This finding is particularly exciting as it suggests

that during inflammation, LECs serve as one of the several antigen-presenting cells (APCs) that interact with naive CD8 T cells, contributing to the memory cell pool that remains post-pathogen clearance. Notably, we found that high endothelial venules (HEVs), which are pathways for naive T cells to enter lymph nodes, are mainly located near lymphatics or in lymphatic-rich areas. Interestingly, when LECs were exposed to suppressive cytokines, they still managed to activate naive T cells; however, these cells matured into an effector memory population characterized by reduced proliferation and increased PD1 expression. Although these Tsup LEC activated cells exhibit limited proliferation, they surprisingly express high levels of Ki67, presenting an intriguing paradox as this marker indicates a high potential for proliferation, contrary to their actual behavior.

Our goal was to compare T cell response dynamics in LEC activation versus mDC activation. We observed that T cell responses differ: LEC activation triggers a response that peaks and diminishes by the third or fourth generation across all LEC stimulating conditions within 72 hours, whereas mDCs provoke an exponentially growing response within the same timeframe. When Th1 and Th2 LECs activate, T cells rapidly develop into central memory with Tcm percentages reaching approximately 80% after the initial division and maintaining this level. In contrast, mDC-activated T cells see Tcm percentages peaking in the second generation, which then steadily declines. Tsup LEC activation leads to a population moving towards an effector memory phenotype as the response progresses. Both LEC and mDC activations produce similar T cell numbers, except Tsup, which results in the lowest count, aligning with its lowest proliferation index.

LEC activation programs stem cell memory T cell fate

Earlier, we observed that LEC activation of naive CD8 T cells leads to the programming of central memory in a steady state. Additionally, these cells expressed markers and characteristics associated with stem cell memory. To confirm if this pattern held true in various

cytokine microenvironments of interest, we initially speculated that this phenomenon would be restricted to Th1 and Th2 LEC activation. However, we were pleased to discover that LECs stimulate stem cell memory across all conditions, including SS, Th1, Th2, and Tsup environments.

The field has not yet agreed upon a precise definition of stem cell memory, but three markers are generally recognized as essential molecules for identifying this T cell phenotype: the transcription factor TCF1, the inhibitory receptor PD1, and the absence of the exhaustion and inhibitory receptor Tim3. In this thesis, we used these markers to characterize our stem cell memory population. It is important to note, we are not distinguishing them from central or effector memory populations; instead, we view stem cell memory as a potential attribute of these two phenotypes. Our findings indicate that LEC activation under all conditions results in a much higher expression of TCF1, compared to mDC activation. This transcription factor is a hallmark of stem cell memory T cells. When we calculated the proportion of T cells co-expressing TCF1 and PD1 while lacking Tim3, we discovered that SS, Th1, and Th2 LEC activation stimulates about 60% of the total T cell population to become Tscm. Unexpectedly, Tsup LECs trigger over 80% of the Tscm. These findings highlight the critical role of LEC activation and interactions with naive CD8 T cells in Tscm development. This is logical, as these cells are primarily found in secondary lymphoid organs, where LECs are also located. Moreover, both central and effector memory can adopt a stem cell memory state, evidenced by Tsup LEC-induced effector memory cells also manifesting as stem cell memory, thus qualifying as true effector memory cells since they also express CD127. We examined Tscm generation and compared the response following both LEC and mDC activation. Similar to Tcm, LEC activation consistently prompts immediate Tscm development after the first T cell division, with this population slowly diminishing over time but consistently remaining above 40% across all generations. In contrast, mDC activation causes a gradual increase in the Tscm percentage, yet it never surpasses 40%. LEC activation

ensures sustained high TCF1 expression throughout all stimulation conditions.

Future Directions

Conducting RNAseq on T cell populations resulting from LEC activation seems promising, particularly to analyze the distinction between SS, Th1, Th2, and Tsup LEC activations. While SS, Th1, and Th2 activations appear to generate similar populations, RNAseq might reveal more subtle differences among them. Our results showed that Tsup LEC activation led to increased Ki67 and PD1 expressions and reduced CD27 expression, though the population was effector memory rather than central memory. Investigating whether Tsup-activated cells epigenetically express an exhausted state could be valuable; however, this seems improbable, given their low Tim3 and high TCF1 expressions. Demonstrating this *in vivo* would be beneficial but poses significant challenges, as LEC activation is merely one among various T cell activations during immune responses. Existing models (mouse chimeras and LEC expressed antigens) are not ideal and present their own limitations, yet this remains a fascinating project worth pursuing.

5.0.2 Lymphatic endothelial cells are potent modulators of CD8 T cell phenotype and can induce central and effector memory by indirect contact

LEC initial activation, maintenance, or even collaboration with mDCs is sufficient to program their associated phenotypes

Having demonstrated that LECs under all conditions are capable of programming T cells into stem cell memory, we sought to evaluate the robustness of this programming. We conducted a timeline experiment by examining the T cells generated after 24, 36, 48, and 72 hours of LEC and mDC activation. Our findings revealed that both LECs and T cells triggered

CD62L shedding after 24 hours, signifying effective T cell activation. This indicated that by 24 hours, LECs and T cells had successfully interacted. We then questioned whether the 24-hour interaction and activation between LECs and T cells were sufficient for this phenotype development, and to our surprise, it was indeed sufficient. Observations showed that just 24 hours of LEC activation led to predominantly central memory populations 48 hours following T cell activation.

This observation illustrates that LEC activation induces robust programming. Consequently, we investigated the subsequent fate of T cells initially activated by LECs for 24 hours and maintained by mDCs, which are recognized as professional antigen-presenting cells. The findings revealed that the robust programming induced by LEC activation was preserved during mDC maintenance. Our analysis identified that these T cells predominantly exhibited central memory characteristics (by SS, Th1, and Th2 LECs), as well as effector memory ones (by Tsup LECs), alongside an enhanced stem cell memory state compared to exclusive mDC activation. Additionally, LEC activation generally resulted in elevated expression levels of TCF1 and reduced expression levels of Tim3, independent of mDC maintenance. This is noteworthy as it further substantiates the significant influence of LEC activation on determining T cell fate. It seemed reasonable to explore the reverse scenario by questioning the outcome when LECs sustain mDC activated T cells. We initially assumed that mDC activation would significantly influence T cell programming, but unexpectedly, we discovered that when LECs maintained mDC activated T cells, they induced their own characteristic phenotypes. T cells maintained by SS, Th1, or Th2 LECs predominantly differentiated into central memory cells, while those supported by Tsup LECs intriguingly also pushed for central memory, albeit not to the extent seen with other LEC activations. Tsup LECs did induce higher percentages of effector memory cells however. Finally, LEC maintenance resulted in high percentages of Tscm, even higher than when LECs do the initial activation and mDCs maintain them. This was also apparent by the significantly higher expression of

TCF1. However, even though Tim3 trended to be down-regulated by LEC maintenance, it was not significant. This showed that prolonged LEC contact and interactions was important for induction of their associated phenotypes, as the cells were more central memory upon LEC maintenance than mDC maintenance, and were also more Tscm when LECs maintained them than when mDCs did.

Given that LECs are powerfully inducing these phenotypes, we wanted to investigate which receptors and ligands they express that interact with T cells during activation and maintenance. Our observations indicated that LECs express co-stimulatory molecules linked with memory induction and reactivation, notably CD70 and OX40L—members of the TNF superfamily—and ICOSL, a member of the B7 family. We also found that LECs express certain inhibitory receptors, such as PDL1 and PVR, along with TGFβ-associated molecules like Garp and LAP. These elements are all recognized for their roles in some type of memory induction, including ICAM1, an adhesion molecule expressed by LECs.

In an effort to mimic lymph node dynamics, we opted to study LECs, T cells, and mDCs in a triculture setup. This approach mirrors the interactions in the T cell zone, where T cells engage with varying cell types, notably LECs at their entry and mDCs within the zone. Within the triculture, we noted analogous patterns: SS and Th1 LEC activation alongside mDC activation cultivated a central memory T cell pool, while Tsup LECs promoted an effector memory population. All LEC-activating conditions coupled with mDC activation significantly enhanced stem cell memory more than mDC activation did alone, underscoring that the synergistic interaction between mDCs and LECs is crucial for Tscm development in the lymph node, driving the induction of their specific phenotypes.

LECs can induce their associated phenotypes through indirect contact

Due to the strong induction of LEC-associated markers, and knowing that LECs secrete various factors and cytokines crucial for T cell development and maintenance, we explored

whether these secreted factors by LECs could trigger these phenotypes. Initially, we cocultured mDCs and T cells with conditioned media from LEC - T cell cocultures. We discovered that all LEC - T cell cocultured conditioned media increased the percentage of central memory, with SS and Th1 specifically causing a significant increase in central memory T cells. Interestingly, LEC conditioned media kept a high percentage of naive cells alive but did not promote stem cell memory, even though TCF1 expression was promoted and Tim3 expression was down-regulated. To verify these results, we utilized a transwell setup to allow LEC presence and secretion of factors or metabolites during the response while preventing direct interaction with mDCs and T cells. Remarkably, merely having LECs present was enough to induce central memory with SS, Th1, and Th2 LECs, and effector memory with Tsup LECs. However, their mere presence could not elevate TCF1 levels or promote stem cell memory, and notably, in the absence of direct contact with T cells, they remarkably decreased the percentage of Tscm cells.

All together, these findings indicate that LECs act as highly effective programmers and modulators of CD8 T cell phenotypes, reinforcing their significance in the lymph node's immune response. Given the strategic positioning of LECs in regions where cells not only enter the lymph node but also where T cells, especially naive and central memory cells, migrate into it, we propose that LECs frequently interact with naive or newly activated T cells, serving as modulators and initiators of central memory (both directly and indirectly) and stem cell memory (particularly for those directly in contact with LECs).

Future Directions

We described the interaction mechanism by which LECs manifest their related phenotypes. This lays the groundwork for identifying the underlying molecular mechanisms, which, as discussed in Chapter 4, have been challenging to determine due to the synergy between cell-to-cell contact and secreted factors, which may include cytokines and metabolites. Con-

ducting RNAseq on the LECs could yield significant insights, particularly when paired with the RNAseq mentioned in the previous section regarding T cells. This paired analysis would enable a detailed examination of how T cells and LECs both upregulate receptors in response to ligands and secreted factors. Exploring this pathway could be crucial for inducing these phenotypes *in vitro*.

5.0.3 LECs can induce their associate phenotypes by contact in an antigen independent manner

Given our focus as an immunoengineering lab, we were interested in exploring how these effects might be leveraged for translational applications. Understanding that LECs must interact with T cells to trigger the combined phenotypes of central memory and stem cell memory, both advantageous for adoptive T cell therapies, we aimed to determine if LECs could induce these phenotypes in an antigen-independent manner. This approach could simplify the use of LECs during T cell expansion for such therapies.

Initially, we conducted experiments within a mDC triculture framework, where LECs were not exposed to an antigen. This approach led to the expression of the LEC-associated phenotypes in a manner independent of LEC antigen presentation. While successful in inducing both central memory and stem cell memory, a considerable proportion of naive cells persisted. This outcome was attributed to the utilization of a fixed ratio of LECs and DCs to T cells, as consistently applied throughout this project. This warrants further investigation into whether an optimization of these ratios could enhance T cell activation. Our observations indicated a more pronounced reduction of Tim3 and Tox in an antigen-dependent context. Therefore, in instances where the cancer's immunodominant antigen is identified, such as gp100 in the case of B16 melanoma, it is advisable to pulse the LECs with the antigen to mitigate levels of cellular exhaustion.

Instead of mDCs, which aren't typically utilized in CAR T or adoptive cell therapy

research, we opted to replicate the experiment with Dynabeads. This choice is due to their prevalent use in *in vitro* T cell activation and expansion for these therapies. We were pleased to find consistent trends, with LECs effectively programming both central memory and stem cell memory—excluding Tsup LECs, as expected—though there was still a notable proportion of naive cells remaining. With bead-based activation, LECs successfully reduced Tim3 expression in a manner independent of antigen presence, and also enhanced granzyme B expression, a key indicator of robust effector functions.

Together, the data indicate that LECs must interact directly with T cells to promote the development of a stem cell memory T cell fate. Interestingly, this process occurs independently of antigen presentation, highlighting the influential role of LEC-T cell interactions in shaping the phenotype and fate of CD8 T cells. We propose that within the lymph node, LECs secrete cytokines and chemokines, such as CCL21, which attract T cells to them. This attraction can lead to the induction of central memory in those T cells activated by other antigen-presenting cells nearby, as well as the promotion of central and stem cell memory in T cells directly contacting LECs, whether following LEC antigen processing or simply through direct interaction.

Our research demonstrated that using LECs as an adjunct to bead activation did not improve the antitumor response in two B16 melanoma models. This outcome might be due to B16's resistance to immunotherapies, prompting us to recommend further investigation with alternative tumor models. We also investigated the secondary response of LEC-supplemented bead-activated T cells by vaccinating mice that had been adoptively transferred with these T cells six weeks prior. We observed that both activation modalities yielded comparable secondary responses, with OT1s from both the lymph node and spleen demonstrating similar behavior. Given our *in vitro* results that LEC supplementation leads to T cells showing reduced signs of exhaustion, it would be interesting to investigate their response *in vivo* with prolonged antigen exposure to test whether these cells can withstand it more and prevent

exhaustion.

Future Directions

These data provide several compelling opportunities for future research. It would be intriguing to conduct an in-depth investigation into the timescale required for LEC-T cell interactions to elicit the observed phenotypes. While we established that these phenotypes are conferred after 24 hours, it remains unclear whether this process is antigen-independent. Could the LECs engage with T cells for a shorter duration yet effectively program them into central memory or stem cell memory? Furthermore, is there a discernible variation in the induction periods for central memory and stem cell memory programming? Conversely, could LECs be utilized in culture to sustain live T cells for prolonged durations? These questions are of significant interest, and answering them would be advantageous for the utilization of LECs in the expansion of T cells for adoptive cell therapies. Moreover, the ratio of LECs to beads and T cells warrants refinement to maximize T cell activation while preserving the phenotypes associated with LECs. The most promising avenue to pursue would be conducting this experiment again using dynabeads, human LECs, and human T cells to verify whether the trends observed thus far hold true for human cells, and if this hypothesis is confirmed, it would be insightful to repeat with expanding CAR-T cells. Moreover, it could be advantageous to explore the application of these cells in longitudinal survival studies involving immune-sensitive tumor models, such as MC38, given that the primary drawback of our B16 research is its limited responsiveness to immunotherapy. We also consider it intriguing to examine the memory responses of LEC-supplemented, bead-activated cells in a chronic infection model like LCMS Cl 13, as we have demonstrated that these cells tend to exhibit reduced exhaustion traits.

5.0.4 Key Takeaways

I trust that this thesis has effectively demonstrated the significant role of lymphatic endothelial cells (LECs) in influencing CD8 T cell phenotypes. LECs achieve this both directly and indirectly through the release of soluble factors and through direct cellular interactions, which promote the formation of central and stem cell memory. While these effects occur under all inflammatory conditions, there is a shift in LEC behavior in suppressive environments that diminishes their ability to foster central memory, although they still proficiently program stem cell memory differentiation. This process can involve either the initial activation of T cells or the maintenance of previously activated T cells. While the secreted factors from LECs are sufficient to promote central memory, direct LEC-T cell contact is necessary for programming stem cell memory. I hope that I have demonstrated these findings comprehensively and rigorously, illustrating the potential for further research projects that our lab can embark on. This marks the beginning of an exciting journey, exploring not only the role of LECs in adaptive immunity but also their potential in developing novel immunotherapies!

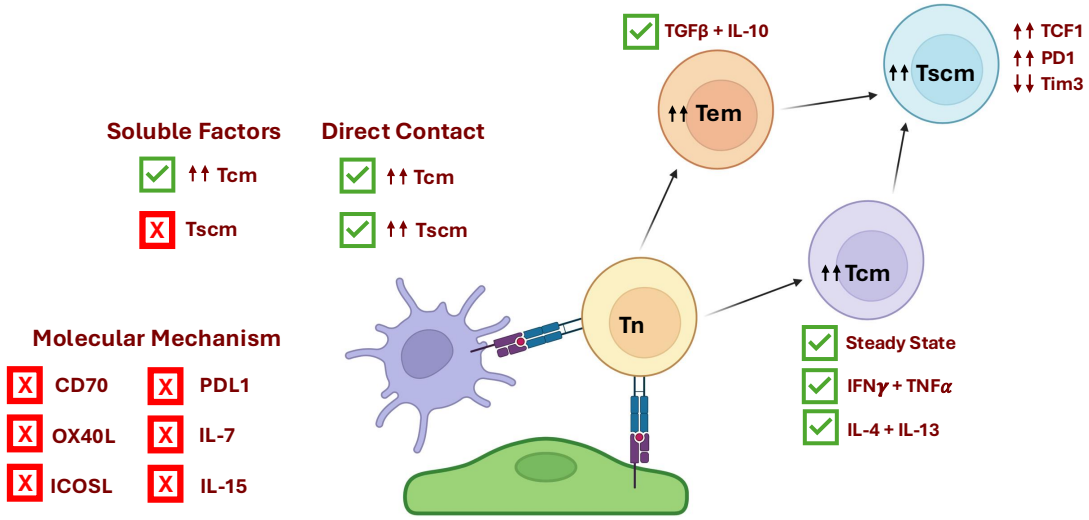

Figure 5.1: **Key Takaways**

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APPENDIX A

GATING STRATEGIES FOR FLOW CYTOMETRY

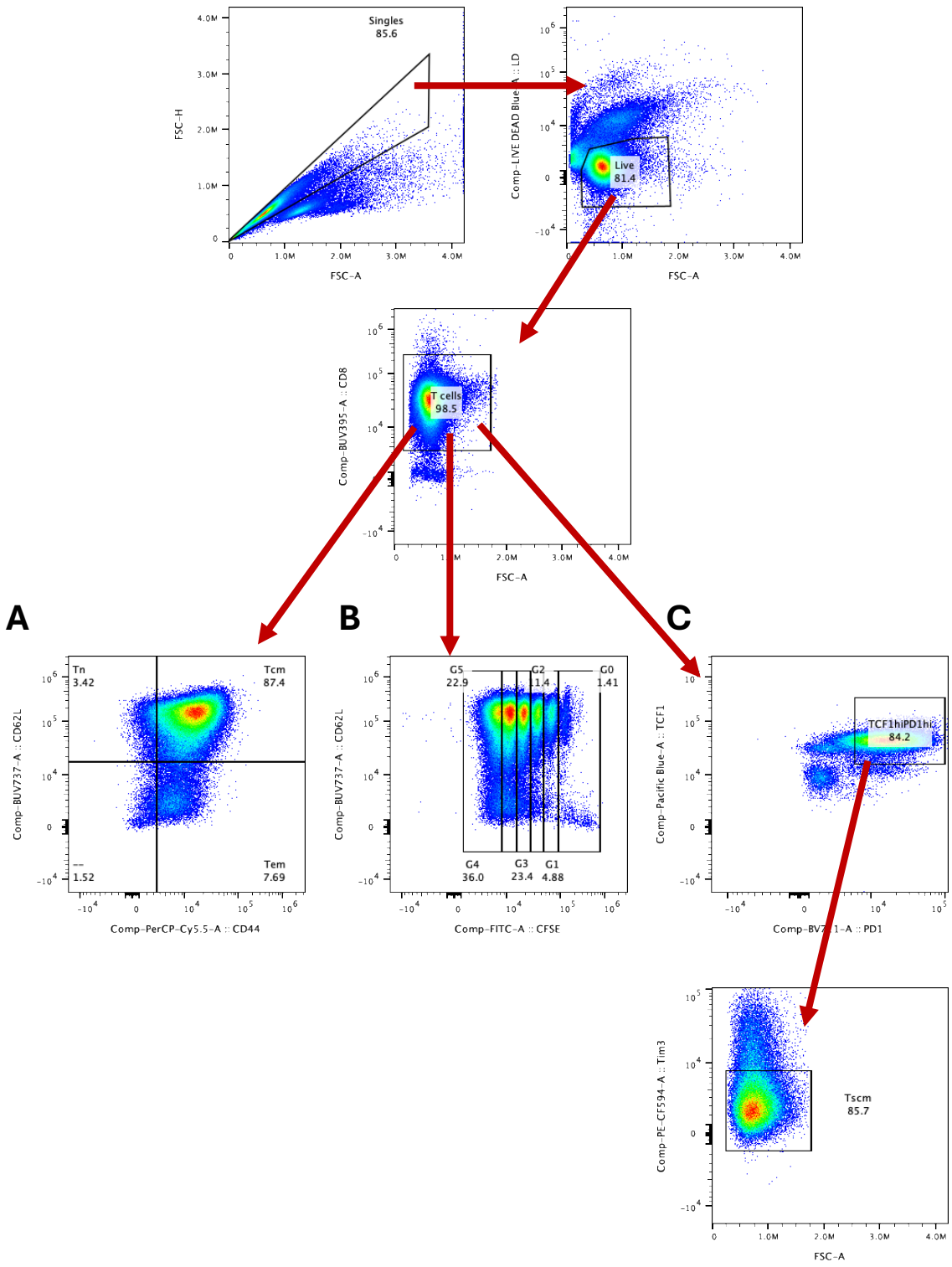

Figure A.1: **Gating strategies used throughout the thesis A** CD44 vs CD62L gate used to identify Tcm, Tem, and Tn. **B** CFSE v CD62L gate used to identify generations. **C** Gates used to identify Tscm, first used TCF1 vs PD1, then FSCA vs Tim3.