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"ROLE OF A CONDITIONED MELANOMA CELL LYSATE IN THE PROMOTION OF A CD8^{+} T CELL MEDIATED ANTI-TUMOR RESPONSE"

Camila Alejandra Fuentes Villegas

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Director de Tesis: Prof. Mercedes López Nitsche MD/PhD

Codirector de Tesis: Prof. Flavio Salazar-Onfray PhD

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CAMILA ALEJANDRA FUENTES VILLEGAS

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Prof. Mercedes López Nitsche MD/PhD
Director de Tesis
Directora Programa de Inmunología, ICBM
Facultad de Medicina, Universidad de Chile

COMISION INFORMANTE DE TESIS

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A la Camila
del pasado que siguió
luchando hasta el final

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I. TABLE OF CONTENTS

II. TABLE OF FIGURES

III. ABREVIATIONS

ACT: adoptive cell therapy

APC: Antigen presenting cell

B16-F10: Murine melanoma cell line

B16 lysate: Heat conditioned B16-F10 tumor cell lysate

CAR-T cells: chimeric antigen receptor T cells

CCH: *Concholepas concholepas* hemocyanin (adjuvant)

cDC: Conventional dendritic cells

cDC1: Conventional dendritic cells type 1

cDC2: Conventional dendritic cells type 2

CTLA-4: Cytotoxic T lymphocyte antigen-4

DAMPs: Danger associated molecular patterns

DC: Dendritic cells

DC3: Dendritic cell state/type 3

DCs-FL: FLT3-L bone marrow derived dendritic cells

EOMES: Eomesodermin

FLT3-L: ms-like tyrosine kinase 3 ligand

FMO: Fluorescence minus one control

GM-CSF: granulocyte-macrophage-colony -stimulating factor

ICB: Immune checkpoint blockade

ICOS: inducible costimulatory molecule

IFN-γ: Interferon gamma

IL-2: Interleukin 2

IL-10: Interleukin 10

IL-12: Interleukin 12

IL-15: Interleukin 15

IL-15R: Interleukin 15 receptor

IL-21: Interleukin 21

Iono: Ionomycin

LAG-3: lymphocyte activation gene-3

LN: Lymphnodes

LPS: lipopolysaccharide

MAGE: melanoma associated antigen

MDSC: Myeloid derived suppressor cells

Mel1, 2 and 3: human melanoma cell line 1,2 and 3

MFI: Mean fluorescence intensity

MoDCs: Monocyte derived dendritic cells

mReg: mature DCs enriched in immunoregulatory molecules

NK: natural killer cell

TAMs: Tumor associated macrophages

TANs: Tumor associated neutrophils

TAPcells: Tumor antigen presenting cells

T-bet: T-box transcription factor TBX21

TCR: T cell receptor

TCF-1: T cell factor 1

TdLN: Tumor draining lymphnodes

TNF-α: Tumor necrosis factor Alpha

TIGIT: T cell immunoglobulin and ITIM domain

TILS: tumor infiltrating lymphocytes

TIM-3: T cell immunoglobulin and mucin domain 3

TME: Tumor microenvironment

TOX: Thymocyte selection-associated high mobility group box transcription factor

Tregs: $CD4^+$ Regulatory T cells

TRIMEL: Heat conditioned human melanoma cell lysate

TRIMELvax: Heat conditioned human and murine melanoma cell lysate-based vaccine

T_{CM}: Central memory T cell

T_{EM}: Effector memory T cell

T_{NL}: Naïve like T cell

T_{RM}: Resident memory T cell

T_{scm}: T memory stem cells

PD-1: programed cell death protein 1 (PD-1)

PD-L1: Programmed death ligand-1

pDC: Plasmacytoid dendritic cells

PMA: Phorbol 12-myristate 13-acetate

ROS: Reactive oxygen species

SFB: Serum fetal bovine

UMAP: Uniform Manifold Approximation and Projection

VEGF-A: Vascular endothelial growth factor A

IV.ABSTRACT

In the tumor microenvironment, high and sustained antigen stimulation can lead to altered $CD8^+$ T cell differentiation inducing a dysfunctional state so-called exhaustion. This phenomenon impedes the elimination of tumor cells, therefore impeding an effective antitumor response. Hence, hindering this process is key to obtain an optimal clinical response.

In Chile, our laboratory developed an immunotherapy based on heat conditioned melanoma cell lysate loaded dendritic cells, which has had successful clinical results. Recently, we have optimized this therapy using an innovative approach through the direct inoculation of a heat-conditioned melanoma cell lysate, in conjunction with the hemocyanin derived from the Chilean mollusk *concholepas concholepas* as an adjuvant, called TRIMELvax vaccine. Our preclinical data showed that mice immunized with TRIMELvax show a marked decrease in the tumor growth rate using the B16-F10 murine melanoma model which was $CD8^+$ T cell dependent.

In this work, we characterized the $CD8^+$ T cell population within the tumor site, expecting to understand possible mechanisms behind TRIMELvax antitumor effect. Further analysis of this $CD8^+$ T cell population using flow cytometry showed that immunotherapy based on tumor cell lysates induced an important decrease in the expression and frequency of tumor infiltrating $CD8^+$ T cells expressing exhaustion associated receptors such as PD-1, Lag-3 and ICOS in addition to preliminary data indicating lower levels of expression of exhaustion-associated TOX transcription factor. Unfortunately, no differences between treatments were determined when analyzing effector cytokine production (IL-2, IFN-γ and TNF-α). Remarkably, TRIMELvax vaccination not only reduced the exhaustion-associated phenotype of $CD8^+$ tumor infiltrating lymphocytes but also increased the frequency of central memory T cells rather than effector memory T cells as observed on control mice. Interestingly, TRIMELvax promoted an increase on a naive-like phenotype on tumor infiltrating $CD8^+$ T cells, which might have progenitor-like features which is currently being further studied.

Additionally, we analyzed TRIMELvax effect modulating the tumor myeloid compartment since it's complex interaction with tumor infiltrating $CD8^+$ T cell population. As expected, TRIMELvax vaccination strongly increased tumor infiltrating conventional dendritic cells and *in vitro* promoted maturation markers expression and IL-12 production, inducing an antitumoral DC associated phenotype (DC3 state).

Overall, this new knowledge will help elucidating possible mechanisms by which TRIMELvax boosts the antitumor response improving the development of a TRIMELvax clinical trial phase I that is currently taking place.

V. INTRODUCTION

Carcinogenesis is a process described as a multi-stage event, in which the effect of genetic alterations leads to the progressive transformation of normal cells into highly malignant tumor cells [1].

Currently, it is well described that the immune system is continuously detecting and eliminating cancer cells, while managing diverse immune evading mechanisms developed by tumor cells [2]. To achieve this, antigen presenting cells (APCs), such as dendritic cells (DC) within the tumor, capture tumor derived antigens and activate antigen specific T cells in tumor draining lymph nodes. Through this activation, T cells acquire the capacity to migrate back and eliminate tumor cells using diverse cytotoxic mechanisms [3]. Unfortunately, the immunosuppression of the tumor microenvironment and the persistence of tumor antigens promotes the induction of a dysfunctional state on T cells called “exhaustion” impeding an efficient, long lasting antitumoral response.

T cell exhaustion

The presence of tumor infiltrating T cells has been described as a positive prognosis marker in several types of cancer, such as melanoma, ovarian, colorectal, bladder, breast, and pancreatic cancers [4–9]. ***However, their mere presence does not ensure an effective antitumor response.*** Tumor cells and tumor associated cells have developed several mechanisms to survive through diminishing T cell effector function. These immunosuppressive mechanisms include recruitment or development of immunosuppressive cells, like myeloid derived suppressor cells (MDSCs) and regulatory T cells (Tregs), inhibition of T cell function by the production of suppressive cytokines, compounds, enzymes and metabolites by tumor and tumor associated cells [10–19] and physiological changes in the tumor microenvironment, such as hypoxia, low pH, and deprivation of nutrients among others [20,21]. All these factors cooperate to allow the immune evasion performed by the tumoral cells [22,23].

In addition, T cell activation is not only impaired in the tumor site by extrinsic factors. The constant and persistent T cell receptor (TCR) stimulation by tumor asso-

ciated antigens drives a specific epigenetic program on T cells, which diminishes their function hence impeding a proper antitumor response, called T cell exhaustion [24]. Exhausted T cells were primarily identified in chronic viral infections and are characterized by an altered transcriptional program which translates into diminished effector function and the co-expression of multiple inhibitory receptors, such as programed cell death protein 1 (PD-1), T cell immunoglobulin and mucin domain 3 (Tim-3), Lymphocyte and activation gene 3 (Lag-3), Cytotoxic T lymphocyte antigen-4 (CTLA-4) and T cell immunoglobulin and ITIM domain (TIGIT) among others [25–27].

Another important feature of exhausted T cells, is the progressive loss of effector functions, including cytokine production, such as interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ)[28]. Thymocyte selection-associated high mobility group box transcription factor (TOX) has been described by several groups to promote the acquisition of an exhausted phenotype in $CD8^+$ T cells, following chronic antigen stimulation [29–31], therefore proposing **TOX** as a central regulator of the exhausted T cell state. TOX is a critical factor for the normal progression of T cell dysfunction and the maintenance of exhausted T cells during chronic infection. In cancer patients, T-cell exhaustion associated receptors is concurrent with the elevated expression of TOX in T cells considering it a potential target for reversing T cell exhaustion and improving T cell function [32–35]

T cell exhaustion compartment

Initially the T cell exhausted population was described as a one homogeneous T cell subset. However, the fact that throughout the years different authors have described this T cell population using different receptors and different lack of functions, raised the question about the real homogeneity of this subpopulation. **This heterogeneity besides being disease related, has also been described within the T cell exhausted compartment as a progressive process.** These stages form a proliferative hierarchy during steady state chronic infections and cancer, that is critical to maintain the population of exhausted T cells. At first, it was described that exhausted T cells with intermediate PD-1 and high T-bet expression ($T\text{-}bet^{hi}$ subset) were less differentiated being defined as progenitor cells, which continually gave rise to a termi-

nal differentiated exhausted T cells with high PD-1 and Eomes expression (Eomes^{hi} subset). While the T-bet^{hi} subset was slightly better in terms of cytokine production, only the Eomes^{hi} subset maintained modest cytotoxic capacity, indicating that this terminally exhausted subset had an important role in containing (but not fully eliminating) persisting infections and tumor cells. Importantly, PD1 pathway blockade seemed to only reinvigorate the T-bet^{hi} subset, while having little to no impact on the Eomes^{hi} cells, showing an important aspect of population dynamics in ICB mediated reversal of T cell exhaustion [26,36].

This transitional model has been recently updated and amplified by the definition of four progressive stages that develop during chronic viral infections and cancer. These subsets are mainly described by the expression of CD69 and Ly108 (slamf6), and the interplay between T cell factor-1 (TCF1), TOX and T-bet transcription factors, proposing the existence of two progenitor exhausted T cells stages that progress into an intermediate exhausted stage that finally declines into terminal exhausted T cells. Specifically, in cancer using samples from murine B16-F10 tumor infiltrating lymphocytes (TILs) and from human melanoma TILs, it was suggested that the predominant tumor infiltrating subset within the tumor site are terminally differentiated exhausted T cells [37].

Therefore, a better characterization of the different stages of exhausted CD8^{+} T cells on cancer patients, focusing on the different factors that might contribute in their progression towards terminally exhausted T cells and their response to different therapeutic strategies is highly relevant.

Tumor derived factors that cooperate in T cell exhaustion

During the past few years, several factors have been described to have an important role in cooperating with the generation of this exhausted population. Although diverse mechanisms have been described for the generation of exhausted T cells addressing chronic viral infections [38], the complexity of the tumor microenvironment suggests possible new mechanisms may be involved in this setting.

As already mentioned, Tregs are one of the main tumors infiltrating immunosuppressive populations, where through a variety of mechanisms avoid T cell effector

function hence impeding tumor cell elimination. Recent data described that through the secretion of immunosuppressive cytokines such as interleukin-10 (IL-10) and interleukin-35 (IL-35), Tregs can cooperatively promote intratumoral T cell exhaustion on $CD8^+$ TILs by modulating B lymphocyte-induced maturation protein-1 (BLIMP1) transcription factor expression which induces several inhibitory receptors expression and an exhaustion associated transcriptomic signature [39,40].

In addition to the secretion of immunosuppressive cytokines, it is well described that Tregs can produce adenosine, a strong immunosuppressive metabolite [41]. Adenosine is produced from the degradation of ATP via ectonucleotidases CD39 and CD73, which are highly expressed on Tregs and play a key role in their inhibitory role over effector T cells [42]. Specifically, *in vitro* studies have shown the inhibitory role of adenosine over cytokine production on anti-melanoma specific $CD8^+$ and $CD4^+$ T cells, suggesting that intratumor-produced adenosine could impair the function of TILs [43]. Recent data showed that the density of $FoxP3^+$ Tregs and adenosine receptor ($A2aR^+$) versus $CD8^+$ T cells density was higher in gastric cancer (GC) tissue compared to peritumoral normal tissue and significantly correlated with the TNM stage, lymph node metastasis, and distant metastasis of GC. Also, authors suggested that Tregs within the TME might reduce $CD8^+$ T cell function through diminishing IFN-γ, TNF-α and perforin production [44]. Indeed, it has been shown that adenosine blockers in combination with other therapeutic strategies, exhibited a significantly increased reduction in tumor progression compared to control groups in preclinical models. However, it is still unclear the action mechanisms involved in this therapeutic effect [45].

Another relevant immunosuppressive population that resides on tumors are myeloid derived suppressor cells (MDSCs). Specifically, chemokine receptor CXCR2 expressing MDSCs, predominantly accumulates and expands during breast cancer progression and upregulates the expression of immunosuppressive molecules such as PD1, PD-L1, LAG3, CTLA-4 and TIM3 on murine $CD4^+$ and $CD8^+$ T cells [46]. In myelodysplastic syndromes (MDS), it has been recently suggested that MDSCs promote $CD8^+$ T cell exhaustion through the galectin 9/TIM-3 pathway indicating that the

use of TIM3/Gal-9 pathway inhibitors might be promising candidates for target therapy of MDS in the future [47].

However, not only MDSCs are the only myeloid population who seems to participate in promoting the T cell exhaustion program on $CD8^+$ T cells. Tumor-associated macrophages or TAMs are a macrophage subpopulation with well described pro-tumoral functions [48]. Within the TME it was recently determined that monocyte-macrophage lineage cells produced glucocorticoids which might activate glucocorticoid receptor signaling on $CD8^+$ T cells promoting the expression of multiple checkpoint receptors and inducing the expression of dysfunction-associated genes upon T cell activation. Hence, the genetic ablation of steroidogenesis in tumor associated macrophages (TAMs) as well as localized pharmacologic inhibition of glucocorticoid biosynthesis was able to improve tumor growth control on preclinical models. This suggests glucocorticoid signaling pathway may be a relevant therapeutic target in some kinds of cancer [49].

Besides tumor antigen presentation, tumor cells acquire several mechanisms to promote tumor evasion through inducing T cell exhaustion. Vascular endothelial growth factor-A (VEGF-A) is a proangiogenic molecule produced by the tumors, which plays a key role in the development of an immunosuppressive microenvironment. In particular, it has been shown that VEGF-A enhances co-expression of inhibitory receptors associated to $CD8^+$ T cell exhaustion such as PD-1, LAG3 and CTLA-4 in *in vitro* experiments. Additionally, VEGF-A neutralization decreases expression of exhaustion-associated receptors on $CD8^+$ T cells in murine tumor models [50]. Recent data obtained on microsatellite stable (MSS) colorectal cancer (CRC) showed that VEGF-A induces the expression of transcription factor TOX in T cells to drive exhaustion-specific transcription program on T cells. Through a combinatorial immunotherapeutic approach using combined blockade of PD-1 and anti-VEGF-A, the antitumor function of T cells was restored, resulting in better control of MSS CRC tumors [51].

The intratumoral microenvironment indeed, is a very hostile environment for T cells. Aside from the high production of immunosuppressive factors, the highly hypoxic conditions within the tumor site, alters the TILs metabolic state affecting other sig-

naling pathways. Continuous antigen stimulation in hypoxic conditions promoted Blimp-1-mediated repression of Peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) dependent mitochondrial reprogramming. This consequently leaves T cells poorly responsive to hypoxic environments. Loss of mitochondrial function generated high levels of reactive oxygen species (ROS), which promoted T cell terminal exhaustion. Decreasing ROS or hypoxia exposure hinders T cell differentiation to exhaustion and improves response to immunotherapy [52]. In fact, it was shown that mitochondrial fitness directly affects the process of exhaustion since enforced accumulation of depolarized mitochondria using pharmacological inhibitors induced epigenetic reprogramming toward terminal exhaustion, indicating that mitochondrial deregulation cooperates in T cell exhaustion development. Furthermore, supplementation with nicotinamide riboside, which enhanced T cell mitochondrial fitness, improved responsiveness to anti-PD1 immunotherapy [53].

Finally, it was suggested that the lack of specific signals provided to $CD8^+$ T cells during the activation process might also be relevant in the acquisition of this dysfunctional state. It is well described the relevance of $CD4^+$ T cell help during the activation and the generation of $CD8^+$ T cell memory response during viral infections [54–57]. Remarkably, this cooperative function has also been described in cancer, where $CD4^+$ T cell help promotes a specific cytotoxic effector program on $CD8^+$ TILs, downregulating exhaustion associated receptors and improving migratory potential [58–60]. It has been suggested that the mechanism by which $CD4^+$ T cell help sustains $CD8^+$ T cells effector functions might be by the production of interleukin 21 (IL-21) which is required for the formation of a specific subset of cytotoxic $CD8^+$ T cells that has effector functions against chronic infection and cancer. In fact, it was recently shown that at least on chronic infections, the main producer of this cytokine supporting $CD8^+$ T cell effector responses are $CD4^+$ T follicular helper cells [61–63].

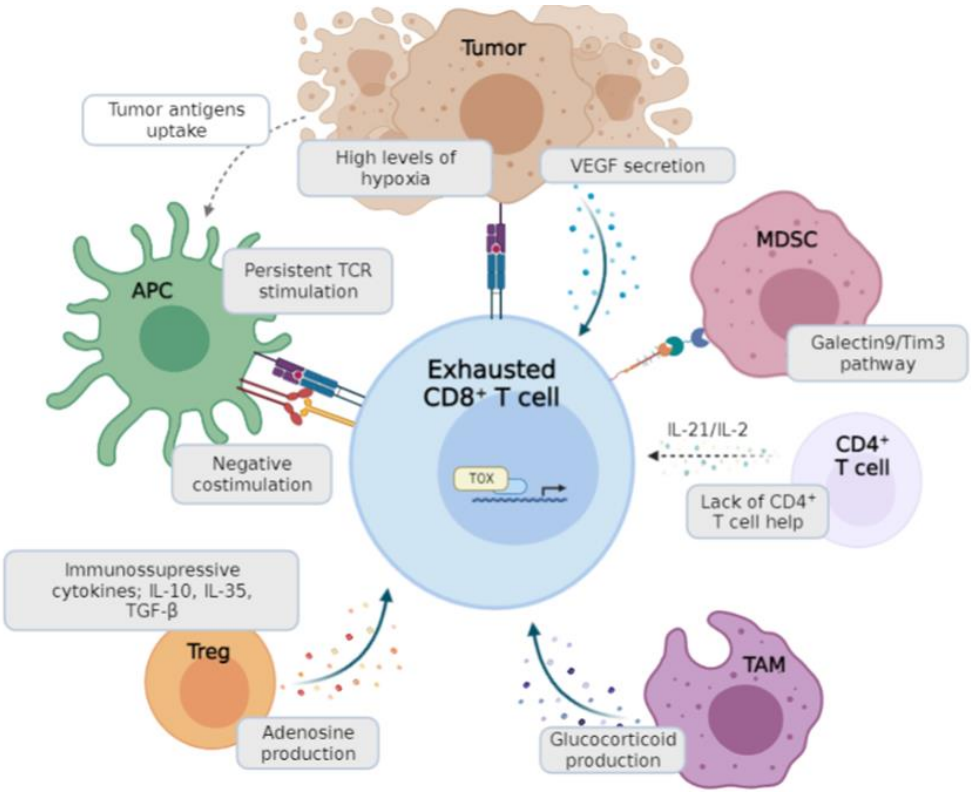

Figure 1. ***Mechanisms of T cell exhaustion on the tumor microenvironment***

In summary, several mechanisms that cooperates with persistent TCR stimulation in the promotion of an exhaustion state have been described (Figure 1). However, undoubtedly there might be more due to the high diversity of populations present within tumors. Altogether, they describe how the complexity of the tumor microenvironment affects the effector state of $CD8^+$ TILs pointing out that although T cell exhaustion is intrinsic to T cells, diverse variables might be contributing to the development of this state, hence impeding a proper antitumor response. Till now, it is unclear how T cells progress from a progenitor exhausted T cell towards a terminal exhausted state. It is possible that other immune and tumor associated populations might collaborate in this antigen-driven process. Additionally, even though most of the knowledge about T cell exhaustion comes from studying chronic viral infections, the fact is that tumor development has clear differences to viral infection, thus suggesting they might not share every step and every variable affecting the process.

Although there is some knowledge on populations in the tumor site that may cooperate in the induction of the exhaustion state of the $CD8^+$ tumor-infiltrating T cell population, less it is known about possible populations that might contribute maintaining healthy efficient activated antitumor $CD8^+$ T cells less prone to dysfunctionality in the tumor site. However, one classical population has always been linked to $CD8^+$ T cell function and activation: Dendritic Cells.

Conventional Dendritic Cells: cDC1 critical role on Cancer

As already mentioned, a critical phase of the antitumor response is the effective activation of tumor specific $CD8^+$ T cells by dendritic cells, mainly conventional dendritic cells (cDCs), which allows $CD8^+$ T cells to gain the ability to eliminate cancer cells. The activation of such cells is critically dependent on type 1 conventional Dendritic Cells (cDC1s), which excel in cross-presentation of tumor-associated antigens to cytotoxic $CD8^+$ T lymphocytes [64,65], and secrete soluble factors (including cytokines such as IL-12 and type III interferons (IFNs), and the chemokines CXCL9 and CXCL10), which recruit and boost $CD8^+$ T cell function at the tumor site [66–71]. cDC1 infiltration correlates with improved prognosis and better response to immune checkpoint blockade (ICB) therapy in several cancer types [72–76]. Furthermore, therapeutic strategies focused on expanding and activating the cDC1 compartment have shown promising results in animal models and clinical trials [77]. **Interestingly, a novel DC activation state termed 'DC3' or 'mReg DCs' (mature DCs enriched in immunoregulatory molecules) originating from cDCs has been proposed to contribute to antitumor $CD8^+$ T cell responses.** It has been suggested that this new state arises from conventional dendritic cells after phagocytizing tumor cells in the tumor site, and it may be characterized by the expression of PD-L1, CD40, CCR7, and the production of IL-12 [70,78,79].

As already mentioned, IL-12 promotes $CD8^+$ T effector cells [80,81]. Interestingly, although little is known about possible mechanisms that might reduce dysfunctionality on $CD8^+$ T cells, a few data describe IL-12 as a possible cytokine that in specific settings might reduce the expression of TOX transcription factor [82,83].

Some authors have also suggested that in the tumor site, DCs might also express IL-15 receptor, which may allow them to trans present IL-15 to $CD8^+$ T cells in specific niches in the tumor as a survival signal for this population, augmenting the antitumor response [79]. This may be a possible mechanism describing how within the tumor site, $CD8^+$ T cells can maintain the antitumor response functioning for long periods of time without necessarily becoming exhausted and how if this process is completely understood, it could become useful for future generations immunotherapies.

Indeed, taking advantage on this natural capacity of the immune system to destroy tumoral cells, several therapeutic strategies have been used. Hence, cancer Immunotherapies aim to boost the natural defense to eliminate malignant cells through diverse mechanisms.

Cancer Immunotherapies

One of the first attempts came from scientist William Bradley Coley who proposed a direct association between bacterial infection and cancer remission. Therefore, in 1981 he began injecting a bacterial mixture (Coley's toxin) into patients' tumors. Hence, Coley's work supported the fact that the immune system of the cancer patients could still respond and be enhanced to eliminate tumor cells [84].

Since then, a diversity of different approaches has been used in the field of cancer immunotherapies with different levels of success.

Cytokines are the primary messengers of the immune system; thus, they have been used for many years as antitumor response enhancers. Cytokines such as interleukin-2 (IL-2) and several interferons have been used as promoters of immune responses against several kinds of cancer. However, studies showed that even though they produce good clinical responses, poor tolerability and high toxicity frustrated their further use as monotherapies in clinical settings [85–89]. Instead, their use in much lower doses in combination with other immunotherapies is still being investigated.

Based on the principle that T cells are key in the antitumor response, adoptive cell therapy (ACT) has been used. In ACT, autologous immune cells, most commonly T cells, are isolated, expanded ex-vivo are reinfused into the patients to eliminate ma-

lignant cancer cells. In fact, due to the advances in genetic engineering, either chimeric antigen receptor (CAR) T cells or T cell receptor engineered T cells have been used in ACT with successful clinical results mainly on hematologic malignancies. However, this approach has not been as successful on solid tumors, and commonly patients discontinue treatment due to live-threatened levels of toxicity [90–93].

In addition, cancer vaccines were developed to promote a specific-antitumor immunity by targeting tumor or tumor associated antigens. First attempts used vaccination with tumor- specific antigens such as MAGE (melanoma associated antigen) family and human melanoma antigen Gp100 [94–97]. With the advances in genomics, neoantigens have risen as interesting targets for the development of personalized cancer vaccines [98]. Although the use of tumor associated peptides have shown to be tumor-specific and safe for use, the translation to the clinic has not been as successful due to tumor heterogeneity, self-tolerance, and immune suppression [99]. Other attempts have used whole genetically modified tumor cells to trigger antitumor responses such as GVAX. This treatment is composed of genetically modified autologous tumor cells, thus becoming able to secrete granulocyte-macrophage-colony - stimulating factor (GM-CSF). This strategy alone or in combination with other immunotherapies had some promising results in multiple cancer types [100–102].

Aside from direct vaccination with tumor antigens or whole tumor cells, vaccination using autologous dendritic cells has also shown significant clinical outcomes [103–105]. One of the most successful attempts was the use of a dendritic cell-based vaccine on prostate cancer patients, denominated Sipleucel-T that showed evidence of efficacy [106,107]. However, the use of Dendritic cell vaccines has shown discrete clinical benefits in a large patient population. Current technologies have widened the knowledge on this key immune population, which generates positive expectations in this field of study [108,109].

Currently, Immune Checkpoint Blockade (ICB) has been the breakthrough in the immunotherapy field for the past few years. Immune checkpoints are molecules within the co-inhibitory signaling pathways, with a critical role in maintaining immune tolerance. In the tumor microenvironment, tumor cells either express or promote the expression of these molecules on immune cells, mainly T cells, hence inhibiting the

promotion of an effective antitumor response [110,111]. The use of antibodies designed against immune checkpoints (ICB) aims to disrupt this inhibitory signaling pathway therefore promoting the antitumor response [112]. The most common examples of ICB targets are cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), programmed cell death receptor-1 (PD-1), and programmed cell death ligand-1 (PD-L1). Although the use of ICB has achieved impressive clinical results on different cancer types this response is cancer type dependent. Additionally, a significant percentage of patients are refractory to primary or secondary ICB-based therapies, primarily due to resistance development to the treatment and lack or weak immune tumor infiltration.

Thus, although diverse strategies have been used in pre-clinical models the fact is that their translation into the clinic hasn't been fully successful since most patients only develop partial, short-term, or no clinical response. As already mentioned, ICB treatment induces a partial reinvigoration of the T cells exhausted compartment suggesting that some subsets of exhausted T cells can be rescued or reinvigorated using this therapeutic tool [26]. In fact, a strong correlation between clinical response to anti-PD-1 ICB therapy and the ratio between the responding circulating exhausted T cells and tumor burden on melanoma patients has been shown [113]. Additionally, pre-clinical data showed that the use of a half-life-extended interleukin-10–Fc fusion protein enhanced expansion and effector function of terminally exhausted $CD8^+$ TILs which was independent of the progenitor exhausted T cells subset [114]. Indeed, a combination of a pegylated IL-10 with an PD-1 ICB was relatively safe and activated the anti-tumor immunity in patients with advanced solid tumors during clinical trials [115].

Unfortunately, recent data showed that exhausted $CD8^+$ T cells, although can partially present some phenotypic and transcriptional features of memory T cells after antigen clearance, have durable epigenetic changes that might affect the strength of future immune responses [116]. This epigenetic "scar" might be critical during long-term processes such as cancer, where the development of an efficient T cell memory response becomes critical.

Additionally, even though the use of ICB therapies alone or as combinatorial strategies have had an important rescue effect over exhausted T cells, ICB therapies

only induced a clinical response in a minority of patients. Besides, patients can develop resistance to this kind of therapies highlighting the need of new therapeutic approaches.

Conditioned melanoma cell lysate as a promising promoter of the antitumor response

In this regard, our group, using a treatment based on conditioned tumor cell lysates (TRIMEL) loaded autologous monocyte derived DCs called TAPcells (for tumor antigen presenting cells), has conducted phase I clinical studies on advanced melanoma patients. These studies demonstrated that 60% of the treated patients developed antitumoral immune responses, with a positive correlation between the delayed hypersensitivity reaction (DTH) against tumor antigens and their survival [117].

Although evidence shows that an important percentage of patients respond to the use of DC vaccines, such as TAPcells, this is a time-consuming process with several logistic challenges. The requirement of special equipment, technically trained specialists, and GMP approved facilities hinders the global implementation of this kind of approaches. Furthermore, *ex-vivo* mature monocyte derived DCs, which are commonly used in this kind of vaccines, do not strictly correlate with DCs type 1 [118]. These cells are the principal DC subtype with the ability to cross-present antigens to $CD8^+$ T cells [119,120], which is a crucial process in the generation of an antitumor response [121,122]. However, the isolation of the cDC1 population remains elusive due to the very low frequency of this population in human peripheral blood, hindering their use for immunotherapy. Therefore, the development of new immunotherapies, where we avoid the use of monocyte derived DCs, is a desirable therapeutic tool.

Therefore, we have developed an innovative approach by directly using a **conditioned tumor cell lysate derived vaccine as a potent antitumor treatment in a preclinical B16-F10 melanoma murine model**. This treatment consists of the administration of a mix of human and murine heat conditioned melanoma cell lysates plus hemocyanin from *concholepas concholepas* (CCH) as adjuvant, called TRIMELvax. Heat-conditioned tumor cell lysates provides a complex source of tumor associated antigens and contains several DAMPs, such as HMGB1 and ATP release

hence inducing DCs maturation, and promoting cross-presentation of tumor derived antigens required for establishing an antitumor response in several types of cancers, such as melanoma, gallbladder, and ovarian cancer [117,123–125]. **Our previous results using B16-F10 murine melanoma model showed that TRIMELvax immunotherapy reduces the tumor growth rate in a $CD8^+$ and $CD4^+$ T cell dependent manner**. Immunohistochemistry analysis showed a higher infiltration of $CD8^+$ T cells inside the tumor of TRIMELvax vaccined mice compared to control injected mice [125]. Interestingly, the use of only murine B16-F10 lysate plus CCH, in the absence of human lysate, lacks antitumoral effect, although a high presence of $CD8^+$ T cells within the tumor site was described (Unpublished results). This indicates that even though $CD8^+$ T cells tumor infiltration is necessary for an effective antitumor response but not sufficient. Hence, other variables such as their phenotype and/or functionality may be critical.

Therefore, we propose TRIMELvax efficacy may rely on maintaining $CD8^+$ T cell activity within the tumor site which may be associated with the presence and activity of other tumor infiltrating immune populations such as cDCs and the production of effector cytokines like IL-12. (Figure 2)

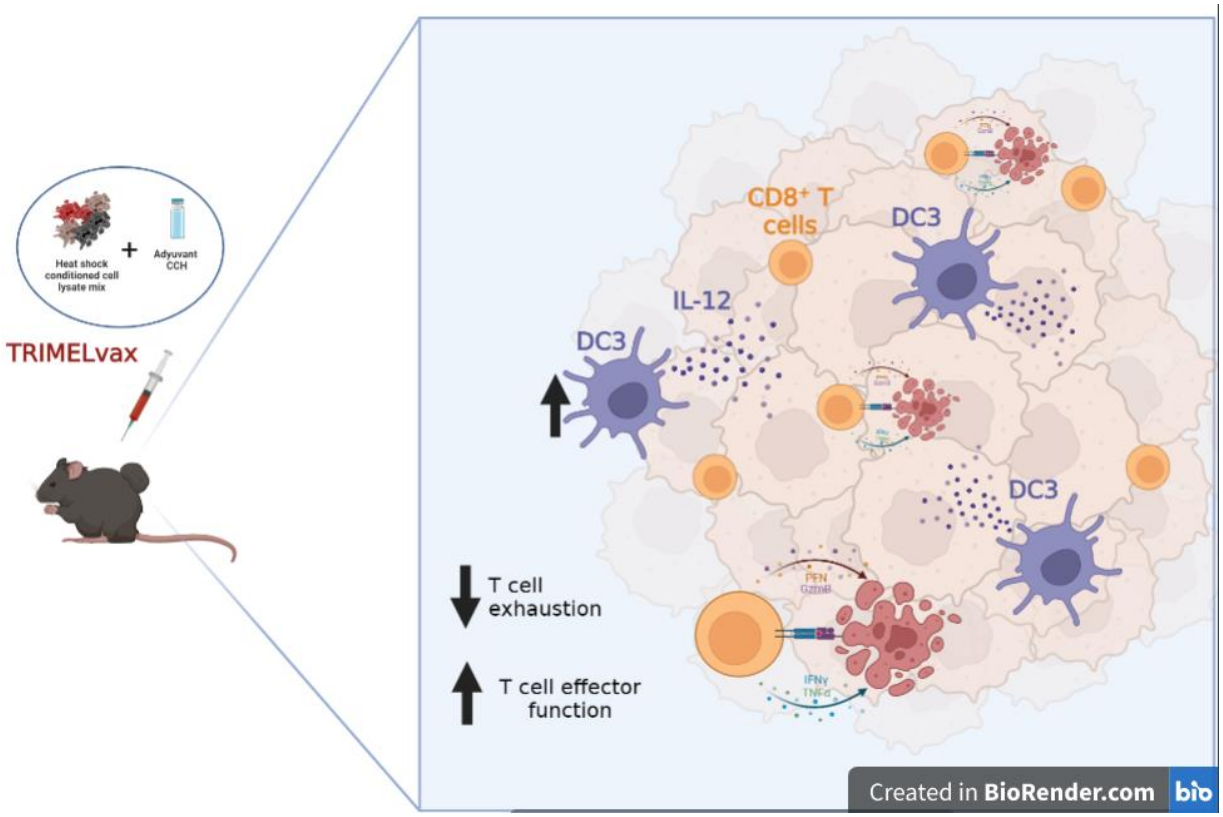

Figure 2. ***Heat-conditioned melanoma cell lysate derived vaccine promotes the anti-tumor capacity of CD8⁺ T cells by diminishing the acquisition of an exhausted profile and the induction of DC3 state on tumor infiltrating DCs.***

VI. HYPOTHESIS

Heat-conditioned melanoma cell lysate derived vaccine (TRIMELvax) promotes the anti-tumor capacity of CD8$^+$ T cells by diminishing the acquisition of an exhausted profile and the induction of DC3 state on tumor infiltrating DCs.

VII. MAIN AIM

Study the effect of heat-conditioned melanoma cell lysates derived vaccine over the exhaustion state of tumor infiltrating CD8$^+$ T cells and frequency and activation of cDCs in the tumor site in the B16-F10 melanoma murine model.

SPECIFIC AIMS

1. Characterization of circulating and tumor infiltrating CD8$^+$ T cells in immunized mice versus control mice in the B16-F10 melanoma murine model.

2. Analyze frequency and activation state of tumor infiltrating cDCs in immunized mice versus control mice in the B16-F10 melanoma murine model.

VIII. MATERIALS AND METHODS

- **Preparation of tumor cell lysate derived vaccine TRIMELvax** (Figure 3)

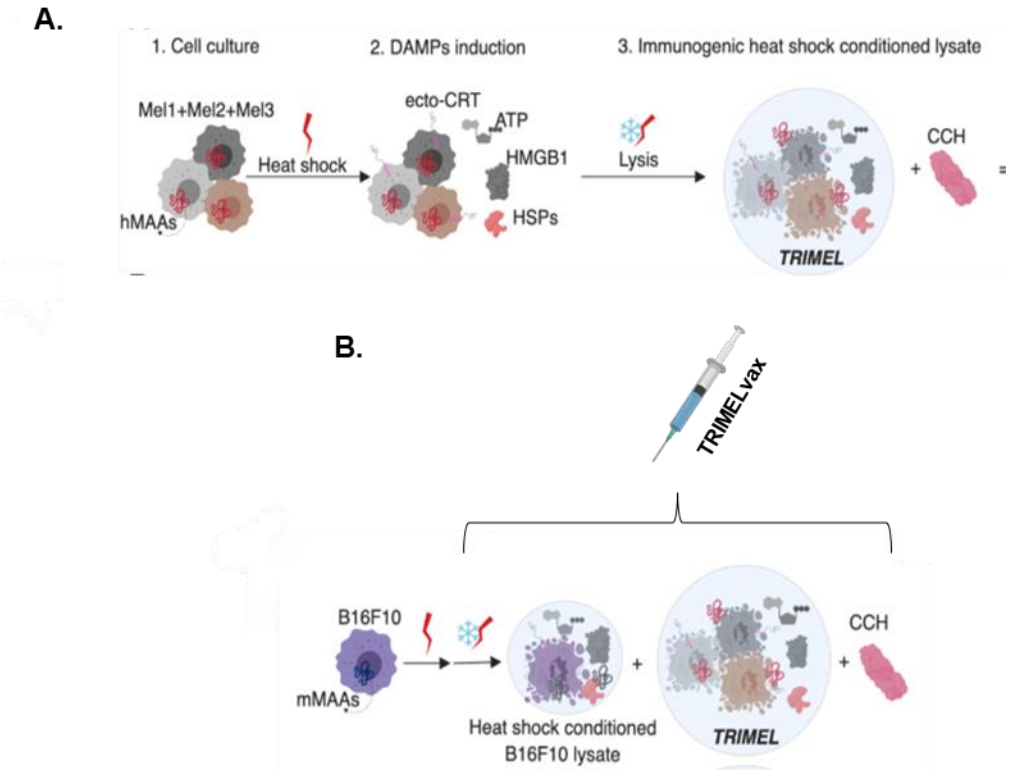

Figure 3. ***Schematic representation of TRIMELvax vaccine production***

A. Mel1, Mel2 and Mel3 human melanoma lines are cultivated, harvested, and subjected to heat shock treatment (HS), inducing the production of several molecular signals associated with danger (DAMPs), such as the translocation of calreticulin (CRT), ATP release, HMGB1 translocation and heat shock proteins induction (HSP). Then, cells are lysed by freeze/thaw cycles to produce the TRIMEL immunogenic lysate. **B.** Scheme showing the composition of the experimental vaccine TRIMELvax used to induce antitumor activity in B16-F10 melanoma murine model.

For the generation of tumor cell lysates either using B16.F10 cells or a mixture of human melanoma cell lines (MEL1, MEL2, MEL3 for TRIMEL), heat-conditioning and cell lysis was performed as previously described [117]. For vaccine generation, additionally, c*oncholepas concholepas* hemocyanin (CCH) was used as an adjuvant.

➢ **TRIMELvax therapeutic model** (Figure 4)

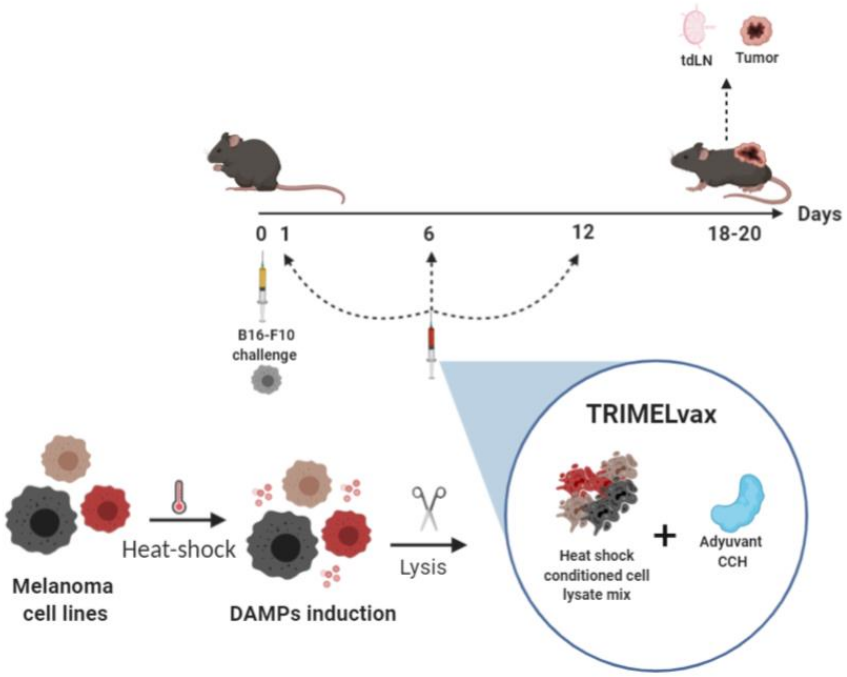

Figure 4. ***TRIMELvax vaccination therapeutic strategy.***
Therapeutic scheme used to evaluate antitumor activity in B16F10 melanoma murine model. 18 days post tumor inoculation, tumor, lymphnodes were dissected and FACS analysis was performed.

B16-F10 cells were thawed from our cell nitrogen deposit at least two weeks before starting any vaccination procedure, allowing the tumor cells to be in optimal metabolic and viability conditions prior to being injected. Also, during this period, it was concluded that prior tumor cell injection, cell culture must be over 80% viability measured using LUNA-FL™ Dual Fluorescence Cell Counter (Logos Biosystems). Since a very low tumor cell number is injected on each mouse, cells must acquire optimal metabolic and viability conditions to grow *in vivo*. Hence, after checking B16-F10 cell viability, C57BL/6 female mice were subcutaneously injected with $2.5x10^4$ B16-F10 murine melanoma cells on day 0. On days 1, 6, and 12 after tumor cell inoculation, either PBS, B16-F10 lysate + CCH or TRIMELvax (TRIMEL+ B16 lysate + CCH) was administered on the opposite flank of the spine to the injection of tumor cells (Figure 4). For each experiment, 4-6 mice per group were used and tumor

growth was evaluated from day 8-10, when the tumor mass becomes visible and measurable, every two days following bioethical statements. For tumor measurements the following formula was used:

$$Tumor\ volume = \frac{lenght \times width^2}{2}$$

Animals were kept during the therapeutic procedures either at the Central Bioterium at the Faculty of Medicine, Universidad de Chile, or at Fundación Ciencia & Vida Bioterium, under Bio-ethical protocol CBA N°1069 FMUCH until the end of each experiment.

- **Obtaining samples from lymph node and tumor of treated mice**

On day 18-20 mice were sacrificed, and organs were dissected. At the beginning, spleen, lymphnodes and tumor samples were evaluated by FACS, however since no difference was detected in the parameters evaluated between lymphnodes and spleen, only lymphnodes and tumor samples were evaluated for posterior experiments. Tumor and lymphnodes (Inguinal, braquial and axiliary) were collected on PBS, and manually dissociated using scissors. Cell suspension was incubated in 1X PBS medium + 5% SFB + Collagenase D (10 mg / ml, Roche) + DNAse I (10 mg / ml, Roche) for 30 minutes at 37 ° C.

For lymphnode samples, after digestion, cells were passed through a 70uM cell strainer and washed with RPMI medium + 10% SFB. Finally, cells were counted using LUNA-FL™ Dual Fluorescence Cell Counter (Logos Biosystems). For tumor samples, after digestion, cells were passed through a 70 µm cell strainer, received in a 50 ml tube, and washed with RPMI medium + 10% SFB. Then cells were centrifuged at 500g for 5 minutes and Percoll (GE Healthcare) gradient was performed. After density gradient generation leukocytes were recovered from the intermediate phase, transferred to a new tube, and washed with RPMI + 10% SFB medium for posterior utilization.

- **Cell activation for cytokine measurement**

To analyze cytokine production by $CD8^+$ T cells, lymphnode and tumor samples were activated separately using PMA + Ionomycin + Brefeldin-A. For this, cells were resuspended at $2x10^6$ cells/ml in RPMI+10%FCS and PMA (50 ng/ml; Sigma-Aldrich), Ionomycin (1 ug/ml; Sigma-Aldrich) and Brefeldin A (1X; Biolegend) was added. After a 4-hour incubation at 37°C and 5% CO_2 were recovered for posterior antibody staining and FACS analysis.

- **Staining of samples and flow cytometry**

For extracellular staining, cells were incubated for 10 minutes at 4°C in 1X PBS + 2% FCS plus mouse FcBlock® (Biolegend). After the cells were washed, the corresponding antibodies were added and a 30-minute incubation at 4°C protected from light was performed. For intracellular staining of either TOX transcriptional factor or cytokine analysis, samples were permeabilized and fixed using FoxP3 staining buffer (eBiosciences) following manufacture instructions. Then, acquisition was performed using BD LSR Fortessa or BD FACSVerse cytometer and posterior analysis using Flowjo 10.4 software.

- **Data analysis**

The FCS files obtained were analyzed using Flowjo 10.8.1 software and Plug-ins from Flowjo exchange webpage. Statistical analysis was conducted using GraphPad Prism software (v9.1.2). All results are expressed as the mean ± SD. Multiple groups were compared using non-parametric Kruskal-Wallis analysis with Dunn's post-test. A p-value < 0.05 was considered statistically significant.

IX.RESULTS

SPECIFIC AIM 1. *Characterization of circulating and tumor infiltrating CD8^{+} T cells in immunized mice versus control mice in the B16.F10 melanoma murine model.*

To begin with, since different authors have identified tumor infiltrating exhausted CD8+ T cells analyzing the expression of different exhausted associated receptors, a working definition of an exhausted CD8+ T cell. For the purpose of this work a tumor infiltrating CD8^{+} T cell is characterized by a high expression of multiple exhaustion associated receptors such as PD-1, LAG3 and TIM3, expression of TOX transcription factor and low production of effector cytokines; IL-2, IFN-γ and TNF-α as seen on Figure 5.

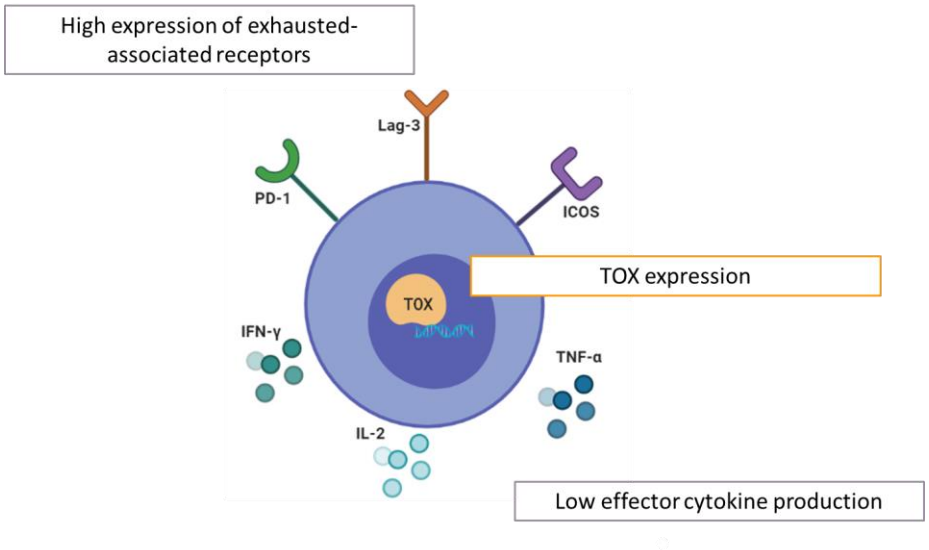

Figure 5. ***Definition of an Exhausted CD8^{+} T cell.***

Exhausted CD8^{+} T cells will be described by a high expression of multiple exhaustion associated receptors such as PD-1, LAG3 and ICOS, expression of TOX transcription factor and low production of effector cytokines; IL-2, IFN-γ and TNF-α

We began analyzing the expression of exhausted-associated receptors on the CD8+ T cell population from mice vaccinated with either TRIMELvax, B16 lysate + CCH or PBS (Control) following the therapeutic design as shown on Figure 6.

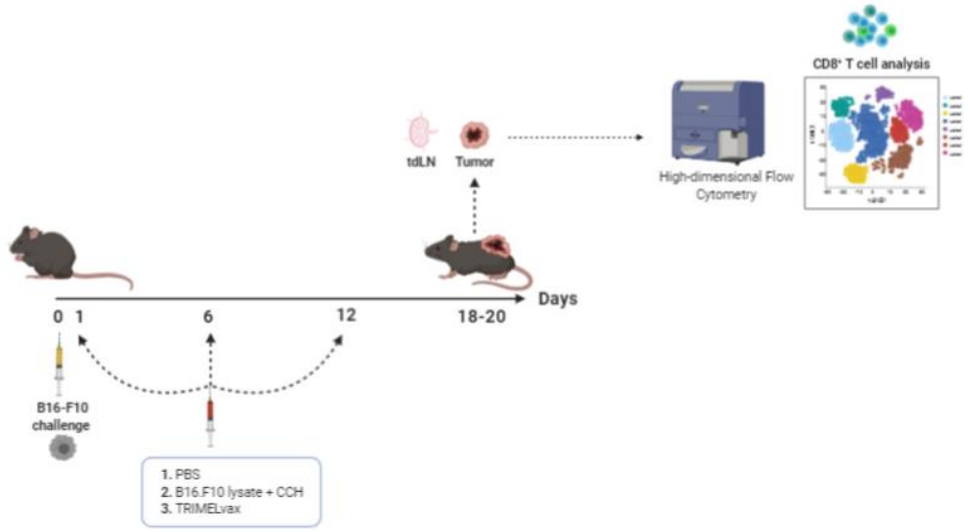

Figure 6. ***Scheme of the Therapeutic design used to evaluate CD8⁺ T cell population in B16F10 melanoma murine model.***

18 days post tumor inoculation, tumor, lymphnodes were dissected and FACS analysis was performed focusing on T cell exhaustion phenotype analysis.

Since only tumor infiltrating $CD8^+$ T cells showed significant expression of exhausted associated receptors such as PD-1, Lag-3 (Figure 7) and ICOS (CD278) (data not shown) indicating that the generation of exhausted T cells was mainly tumor dependent on this model, we focused on the characterization of tumor infiltrating $CD8^+$ T cells and $CD8^+$ T cells from lymphnodes.

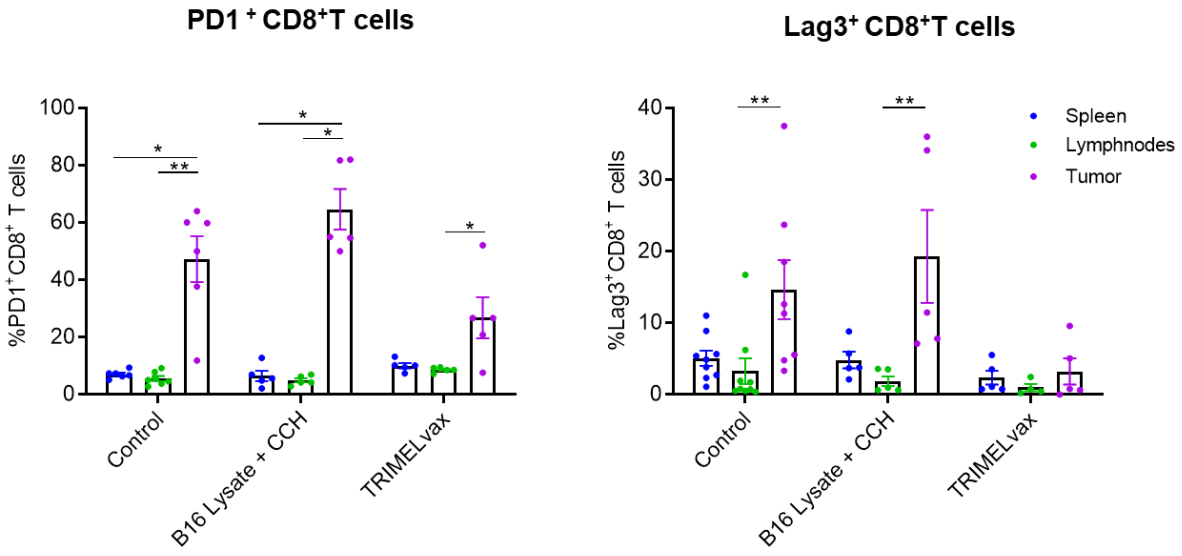

Figure 7. ***PD1⁺ and Lag3⁺CD8⁺ T cells are predominantly present on the tumor site.***
Analysis of the spleen, lymphnode and tumor infiltrating $CD8^+$ T cell population in mice receiving TRIMELVAX, B16 lysate + CCH or no treatment (Control) Bar graphs show mean and SD. Kruskal-wallis test with Dunn's posttest is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ n=5-10 mice per group

To perform a deep characterization of tumor infiltrating $CD8^+$ T cells, FACS data acquired using BD LSR Fortessa was further analyzed using Flowjo v10. For this analysis, first, a concatenate from three different tumor samples from each treatment was done. Then, after gating on the live $CD8^+$ T cell population (lymphocyte$^+$viability-stain$^-$CD45$^+$CD3$^+$CD8$^+$) (Figure 8), a down sample was done limiting to 1800 events each concatenate. Finally, a final concatenate was done using the previous down sample concatenates for each treatment.

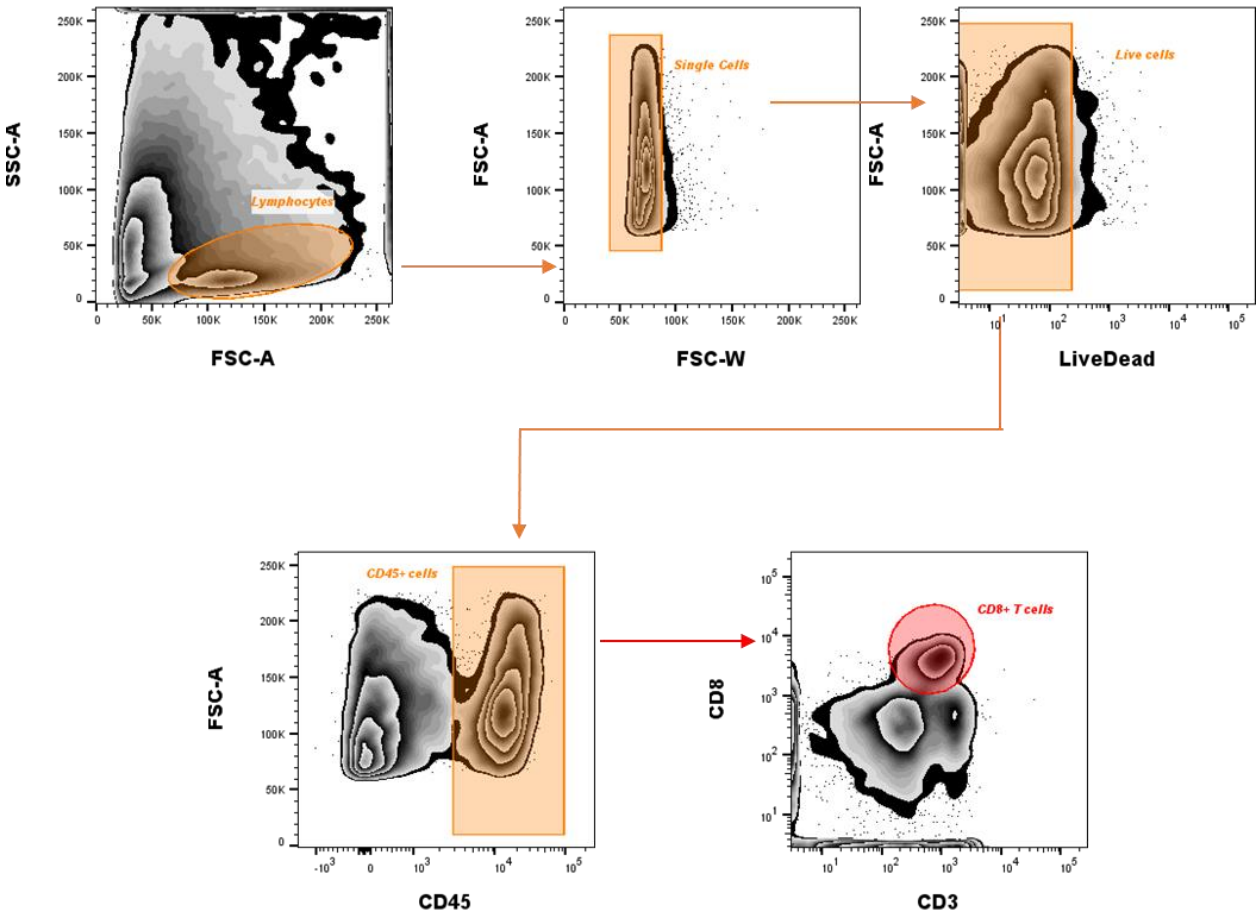

Figure 8. ***Gating strategy for CD8⁺ TILs analysis of flow cytometry data acquired from TRIMELvax, B16 lysate + CCH or Control mice***

Then using the phenograph Flowjo plug-in (with preset values using compensated values for ICOS, LAG3, TIM3, CD62L, PD-1,.CD103, OX40, CD69 and CD44), 8 clusters were determined. This information was used to perform an UMAP analysis on this final concatenate using the preset flowjo plug-in parameters; minimum distance= 0,5, number of components= 2 and 8 nearest neighbors as determined by the previous phonograph analysis. Interestingly, UMAP visualization analysis determined a striking difference on cluster distribution when comparing tumor infiltrating CD8⁺ T cells from control, B16 lysate + CCH and TRIMELvax mice (Figure 9).

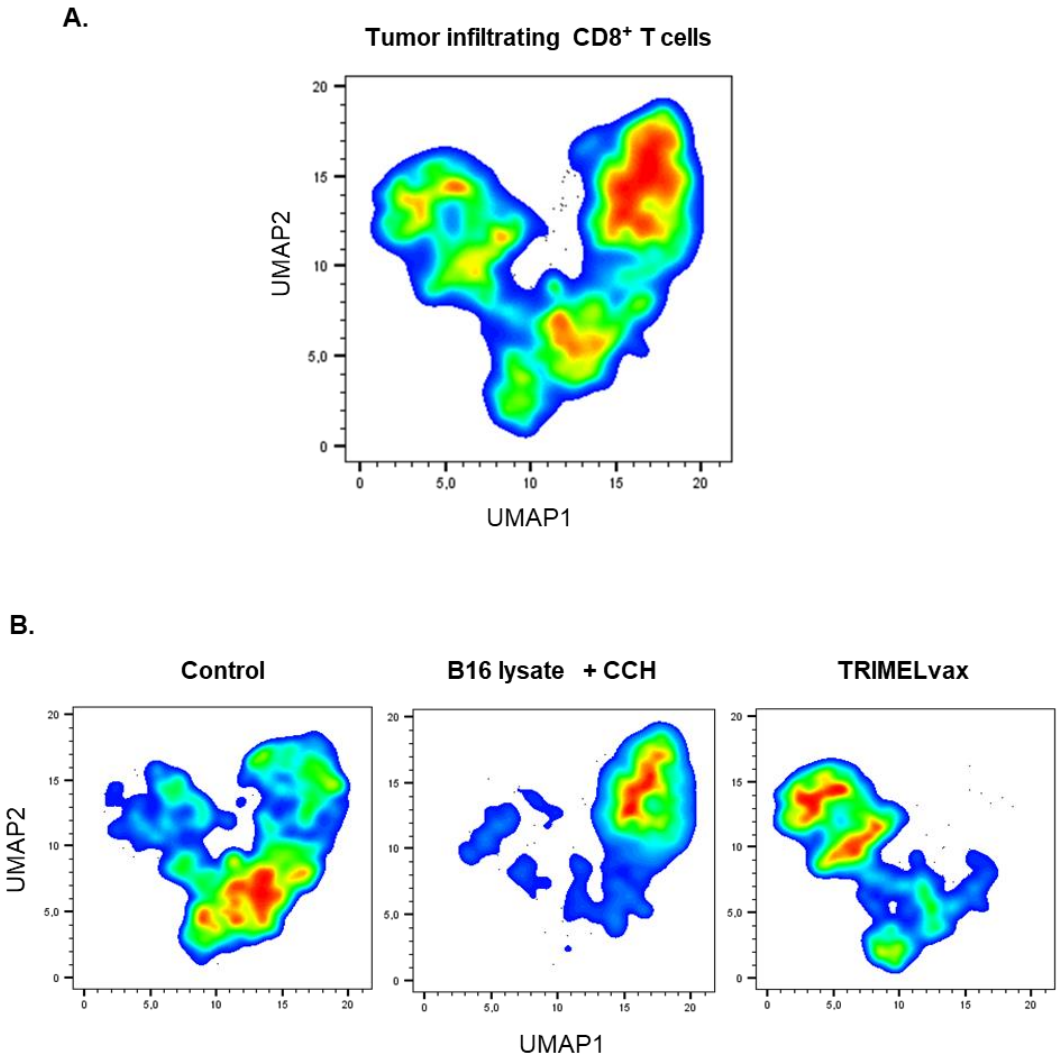

Figure 9. ***Different treatments generate different cluster distribution of tumor infiltrating CD8⁺ T cells.***

UMAP analysis based on tumor infiltrating CD8⁺ T cells expression of 9 parameters: PD-1, LAG3, TIM3, ICOS, CD44, CD62L, OX-40, CD69 and CD103. A. UMAP visualization using data from Control, B16 lysate + CCH and TRIMELvax vaccinated tumors. B. UMAP visualization using data from each treatment. n=3 mice per group

Since, the co-expression of multiple exhausted-associated receptors is strongly associated with increased level of dysfunctionality, UMAP visualization colored by each exhausted associated receptor (ICOS, LAG3, TIM-3, PD-1) signal intensity was performed. As shown on Figure 10, the highest expression of these receptors is focused on the top right zone, which is mainly present on $CD8^+$ TILs from B16 lysate + CCH and control mice. PD-1 is the most recognized exhausted-associated receptor, even being used as the main target for immune blockade immunotherapy, highlighting its relevance in T cell function in the tumor. The difference on PD-1 expression was the most evident where B16 lysate + CCH vaccinated, and control mice showed a higher expression than TRIMELvax treated mice. Indeed, TRIMELvax treated mice even lacks the clusters where the expression of PD-1, Lag3, ICOS and TIM-3 was the highest (top right area).

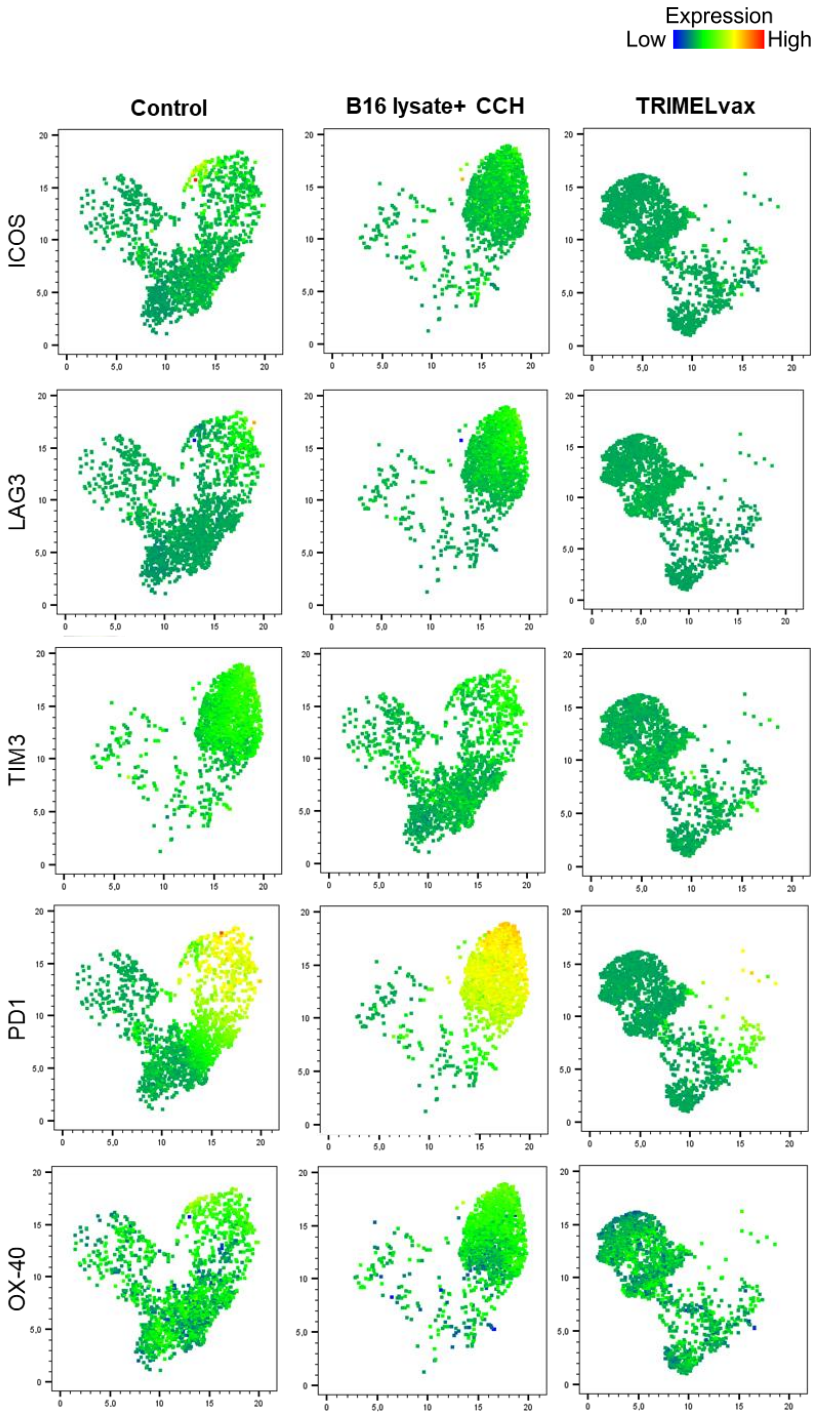

Figure 10. ***Exhausted-associated receptors expression across the different cell clusters.***

UMAP visualization colored by each receptor signal intensity from tumor infiltrating $CD8^+$ T cells from control (left), B16 lysate + CCH (middle) and TRIMELvax (right). n=3 mice per group

Interestingly, when analyzing each treatment separately, a striking difference between $CD8^+$ TILs from control, B16 lysate + CCH and TRIMELvax vaccinated mice was shown. Analysis of each distribution, using the cluster explorer plug in from Flowjo, showed that the 8 previously determined cluster present different frequencies on each treatment data set (Figure 11).

$CD8^+$ TILs from control mice predominantly presents higher frequencies of clusters 2 and 6, $CD8^+$ TILs from B16 lysate + CCH vaccination on clusters 1 and 3, where those from TRIMELvax treated mice predominantly did on clusters 4 and 5 (Figure 11.B). These striking differences on cluster distribution supports the fact that TRIMELvax vaccination promotes a completely different phenotype of $CD8^+$ TILs in comparison to control and B16 lysate + CCH treated mice. Indeed, although control and B16 lysate + CCH both have similar tumor growth rates, the phenotype of $CD8^+$ TILs from both treatments differ completely. When analyzing the expression of each parameter measured, using heatmap visualization (Figure 11.C) it was observed that $CD8^+$ TILs from TRIMELvax mice have a higher expression of CD62L on the clusters that were predominantly increased in comparison with control and B16 lysate + CCH where PD-1 had a higher expression on the clusters that were predominantly observed. Using these tools of analysis, it was possible to suggest that the three different treatments have differences in $CD8^+$ TILs phenotype when measuring this set of receptors, where TRIMELvax vaccinated mice clearly distinguishes between control and even more with B16 lysate + CCH vaccinated mice. Interestingly, B16 lysate + CCH had strong differences in comparison with control mice which was unexpected.

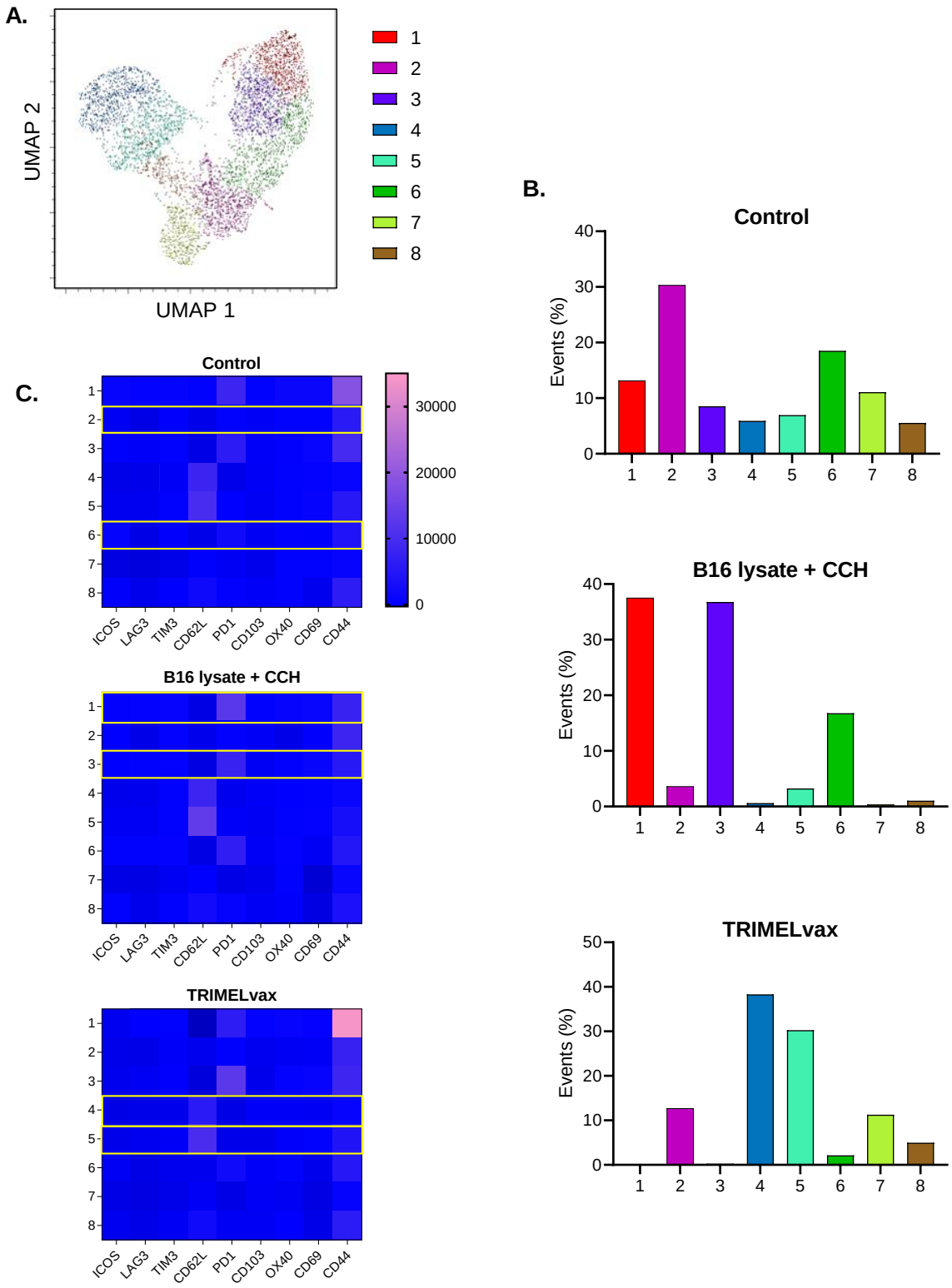

Figure 11. ***Tumor infiltrating CD8⁺ T cells distribute in 8 different clusters.***

A. UMAP visualization of the 8 clusters determined within the tumor infiltrating CD8⁺ T cells

from all treatments. **B.** Bar graphs showing frequency of cells on each cluster shown on A. C. Heatmaps showing the level of expression of 9 parameters used to describe the population; ICOS, LAG3, TIM3, CD62L, PD-1,.CD103, OX40, CD69 and CD44 for each cluster for all treatments. The most frequent clusters for each treatment are highlighted in yellow: Control clusters 2 and 6, B16 lysate + CCH cluster 1 and 3, and TRIMELvax clusters 4 and 5. n=3 mice per group

Then, using manual gating, exhausted associated receptors level of expression was analyzed separately on tumor infiltrating $CD8^+$ T cells for each treatment, showing that TRIMELvax decreases the level of expression of PD-1 when compared to $CD8^+$ TILs from B16 lysate + CCH treated mice. Overall, there was subtle differences observed between treatments, but no statistical difference was determined when comparing the three treatments (Figure 12).

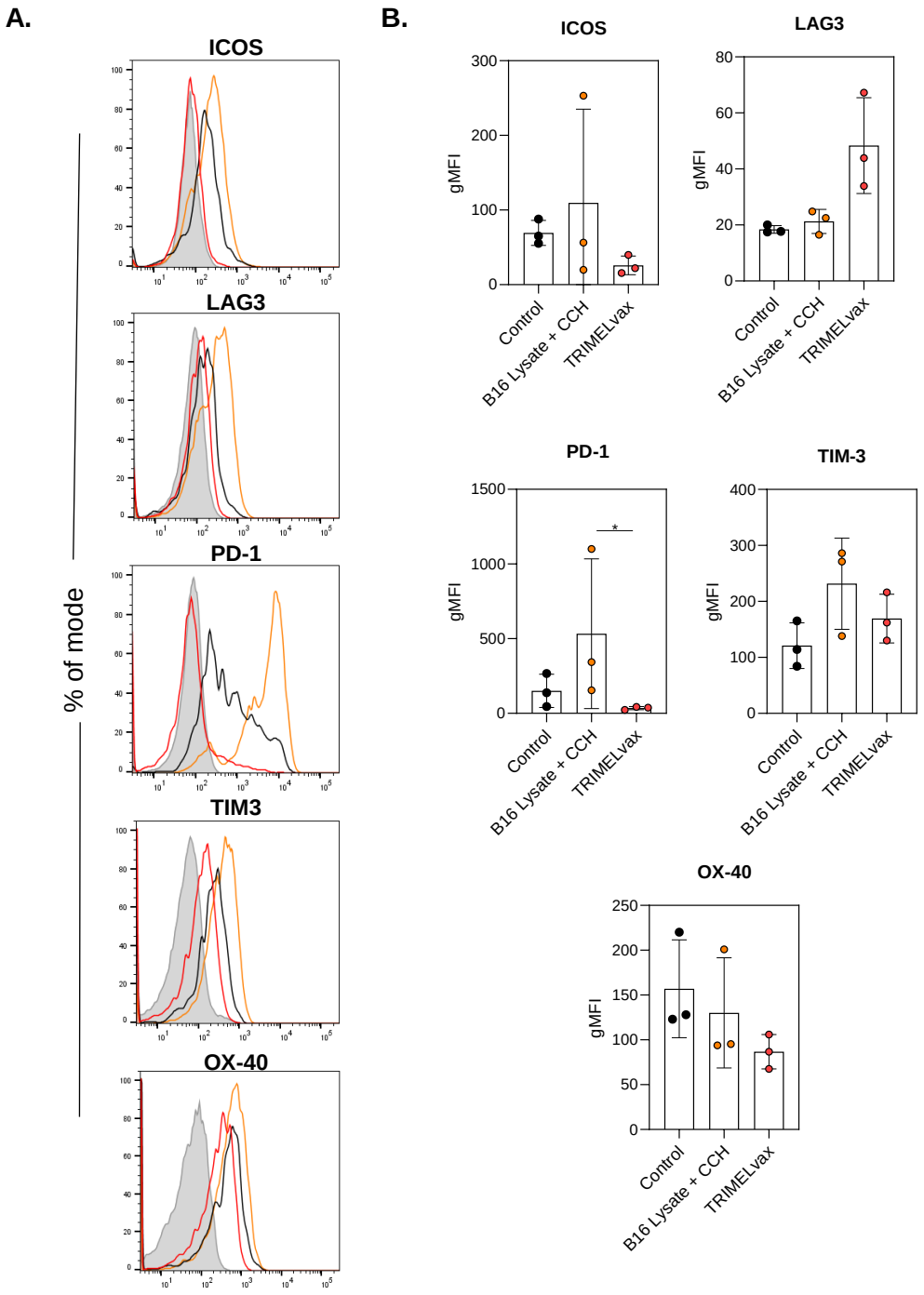

Figure 12. ***Analysis of exhausted associated receptors expression level on CD8⁺ TILs from Control, B16 lysate + CCH and TRIMELvax vaccinated mice.***

A. Representative histograms of ICOS, LAG-3, PD-1, TIM-3 and OX-40 expression on $CD8^+$ TILs from control (black), B16 lysate + CCH (orange) and TRIMELvax (red) treatments. **B.** Quantification of A., Bar graphs show mean and SD. Kruskal-wallis test with Dunn's post test is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ n=3 mice per group

Additionally, frequency of tumor infiltrating CD8$^+$ T cells with exhausted associated receptors was analyzed for each treatment. TRIMELvax decreases the expression of PD-1, Lag-3 and ICOS when compared to CD8$^+$ TILs from B16 lysate + CCH treated mice. Although no statistical difference was determined when comparing TRIMELvax treatment versus control, an important decrease on the frequency of cells expressing these receptors was observed (Figure 13). No difference was observed when analyzing TIM3 and OX-40. The latter was used as a specificity inside control since it is not associated with the exhausted profile.

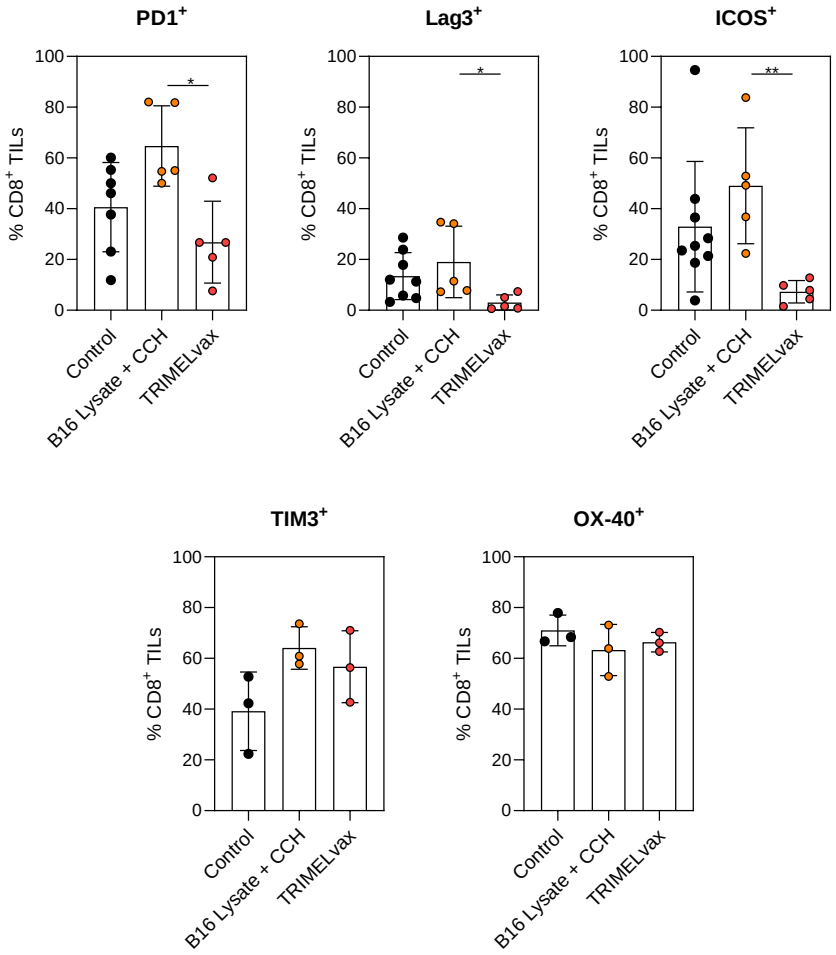

Figure 13. ***TRIMELvax vaccinated mice have lower frequency of tumor infiltrating CD8$^+$ T cell that express exhausted associated receptors*****.**
Graph bars showing the frequency of CD8$^+$ TILs expressing each receptor, comparing different treatments. Bar graphs show mean and SD. Kruskal-wallis test with Dunn's post test is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ n=8-3 mice per group.

Exhausted state on T cells is proposed as a progressive phenomenon. In this regard, data suggested PD-1 expression level could distinguish PD-1 low expressing CD8$^+$ T cells (PD-1^{int}) that could be reinvigorated with anti-PD-1 therapies and PD-1 high expressing CD8$^+$ T cells (PD-1^{hi}) which are terminally exhausted and refractory to anti-PD-1 therapies. Hence, PD-1^{neg}, PD-1^{lo} and PD-1^{hi} population were evaluated on CD8$^+$ TILs from control, B16 lysate + CCH and TRIMELvax treated mice (Figure 14).

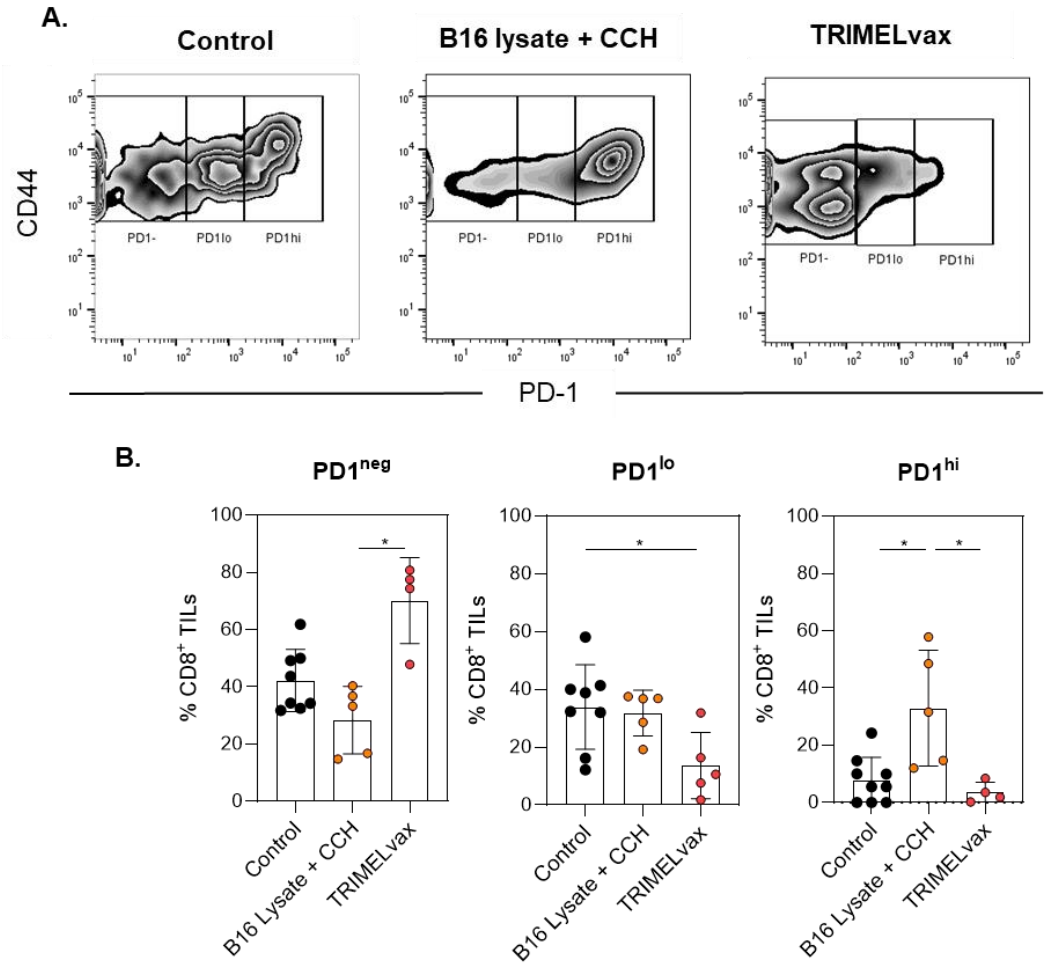

Figure 14.*TRIMELvax vaccinated mice have a lower frequency of PD-1^{hi} and PD-1^{lo} CD8$^+$ TILs.*

A. Representative zebra-plots showing PD-1 negative (PD-1^{neg}), PD-1 low (PD-1^{lo}) and PD-1 high (PD-1^{hi}) CD8$^+$ TILs frequency comparing; control, B16 lysate + CCH and TRIMELvax treatments. **B.** Graph bars showing frequency of PD-1^{neg} PD-1^{lo} or PD-1^{hi} (left to right) CD8$^+$ TILs comparing different treatments. Bar graphs show mean and SD. Kruskal-wallis test with Dunn's post test is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ n=8-5 mice per group

TRIMELvax treatment increases the frequency of $CD8^+$ TILs lacking PD-1 expression ($PD\text{-}1^-$) and decreases the frequency of PD-1 intermediate expressing $CD8^+$ TILs ($PD\text{-}1^{int}$) compared to control mice (Figure 14.B). Interestingly, PD-1 high expressing $CD8^+$ TILs ($PD\text{-}1^{hi}$) were predominantly present in tumors from B16 lysate + CCH treated mice. This phenomenon might partly explain why, although there is an increase in $CD8^+$ tumor infiltrating cells on B16 lysate + CCH treated mice no effect on tumor growth rate is seen as shown on previous results [125].

When correlating the frequency of $PD1^{neg}$, $PD\text{-}1^{lo}$ and $PD\text{-}1^{hi}$ $CD8^+$ TILs with tumor volume, as expected, frequency of $PD\text{-}1^{hi}$ and $PD\text{-}1^{lo}$ expressing $CD8^+$ TILs positively correlated to B16-F10 tumor volume measured at day 19 and frequency of $PD\text{-}1^{neg}$ expressing $CD8^+$ TILs negatively correlated to B16-F10 tumor volume measured at day 19 (Figure 15). These results agree with literature showing that high levels of exhaustion in $CD8^+$ TILs are strongly associated with a poor clinical outcome in cancer patients [126–130].

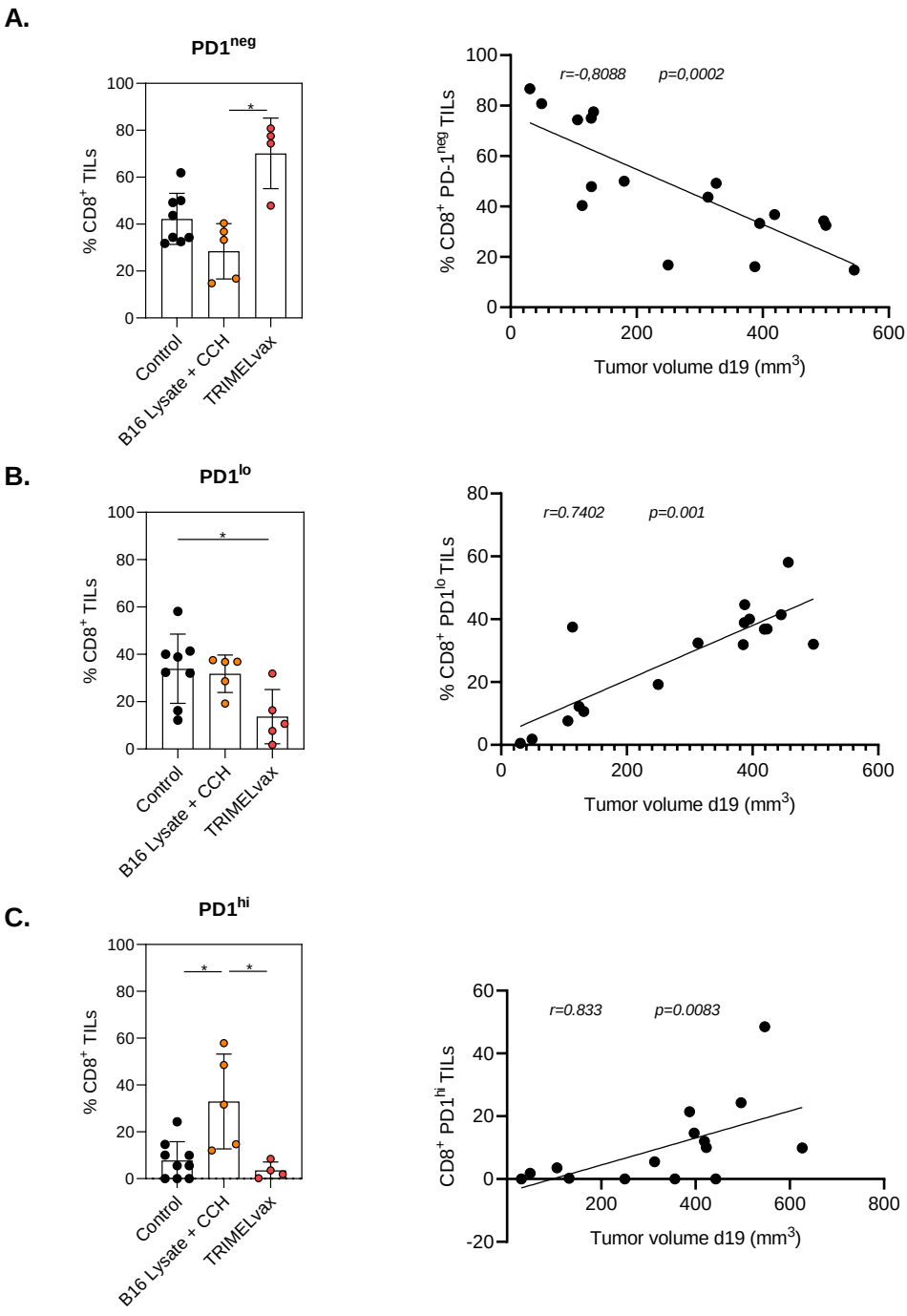

Figure 15. ***PD-1 expression on CD8⁺ TILs correlate with B16-F10 tumor volume.***

A. Bar graph (left) shows frequency of PD-1^{neg} expressing CD8^{+} TILs for each treatment, Dot-plot (right) shows negative correlation between frequency of PD-1^{neg} CD8^{+} TILs and tumor volume measured on day 19. **B.** Bar graph (left) shows frequency of PD-1^{lo} expressing CD8^{+} TILs for each treatment, Dot-plot (right) shows positive correlation between frequency of PD-1^{lo} CD8^{+} TILs and tumor volume measured on day 19. **C.** Bar graph (left) shows frequency of PD-1^{hi} expressing CD8^{+} TILs for each treatment, Dot-plot (right) shows positive

correlation between frequency of PD-1^{lo} CD8+ TILs and tumor volume measured on day 19. Bar graphs (left) show mean and SD. Kruskal-wallis test with Dunn's post test is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ n=8-5 mice per group. Non-parametric Spearman correlation was calculated for each dot-plot (right), r and p values are shown n=17-18.

Exhausted T cells are characterized by high expression of multiple inhibitory receptors and diminished functionality described as low effector cytokine production. Hence, production of IL-2, TNF-α and IFN-γ separately and in combination was analyzed through PMA/Iono/BFA induced activation and flow cytometry of lymphnode and tumor samples. Unexpectedly, no differences were determined either in $CD8^+$ T cell population from lymphnode nor tumor samples from each treatment (Figure 16 and data not shown).

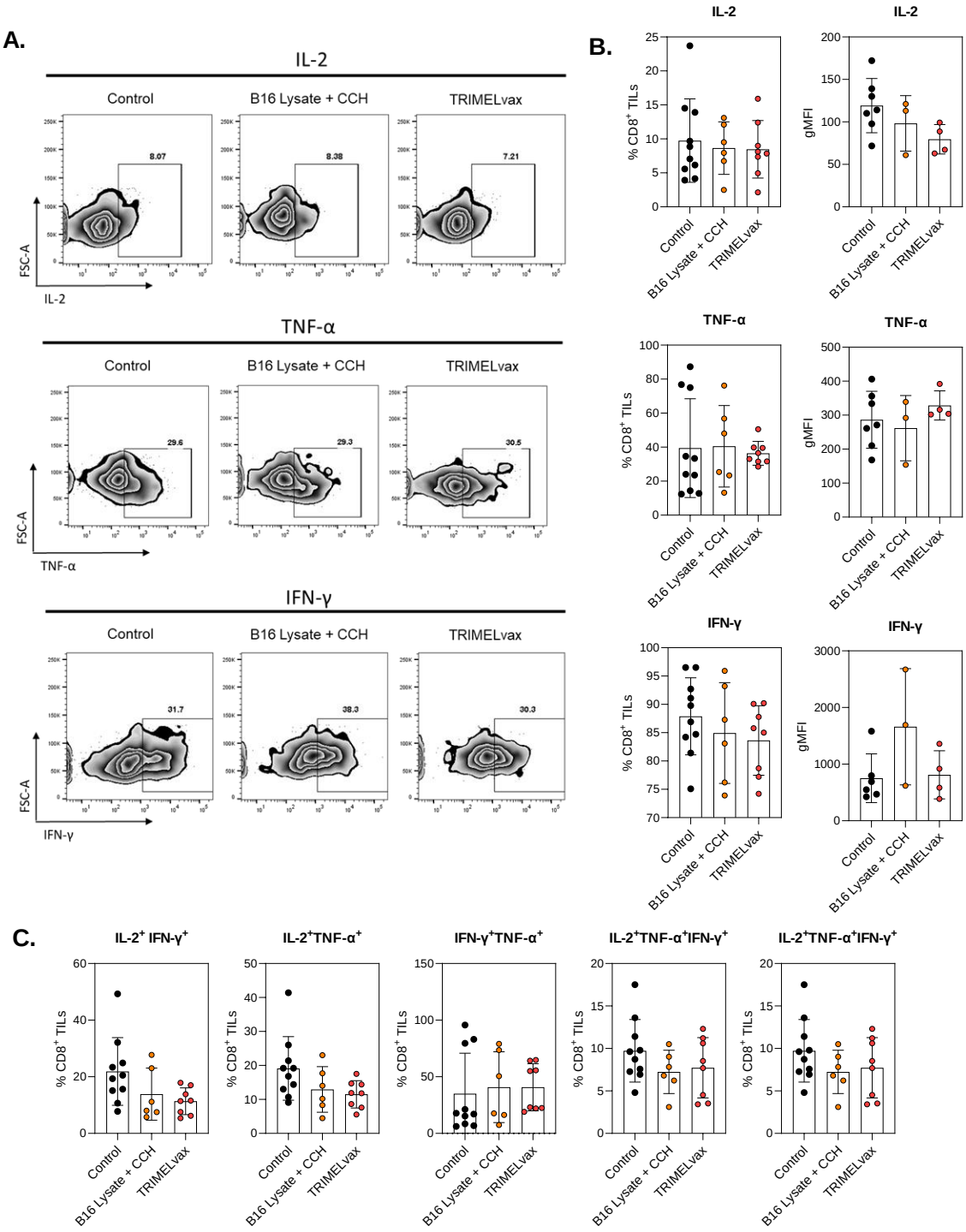

Figure 16. ***TRIMELvax vaccination does not alter CD8⁺ TILs effector-cytokine production***

A. Representative zebra-plots showing IL-2 (upper), TNF-α (middle) and IFN-γ (lower) production of CD8⁺ TILs for control, B16 lysate + CCH and TRIMELvax treatments. **B.** Quantification of A. showing frequency (left column) and geometric mean fluorescence intensity

(right column) per each effector cytokine measured. **C.** Frequency of $CD8^+$ TILs producing multiple cytokines. Bar graphs show mean and SD. Kruskal-wallis test with Dunn's post test is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ n=10-6 mice per group

Transcriptional factor TOX (Thymocyte selection-associated high mobility group box protein) has shown to be a critical regulator of exhausted $CD8^+$ T cells. Therefore, TOX expression was analyzed on $CD8^+$ T cells from lymphnode and tumor samples for each treatment. Results obtained evaluating $CD8^+$ TILs from the different treatments suggest that TOX expression is decreased on $CD8^+$ TILs from TRIMELvax treated mice when compared to control and B16 lysate + CCH treated mice (Figure 17.A.). Also, when analyzing the co-expression of PD-1 and TOX on $CD8^+$ TILs, results obtained suggest that TRIMELvax treatment decreased the frequency of $PD\text{-}1^+TOX^+$ $CD8^+$ TILs compared to $CD8^+$ TILs from control and B16 lysate + CCH treated mice (Figure 17.B.).

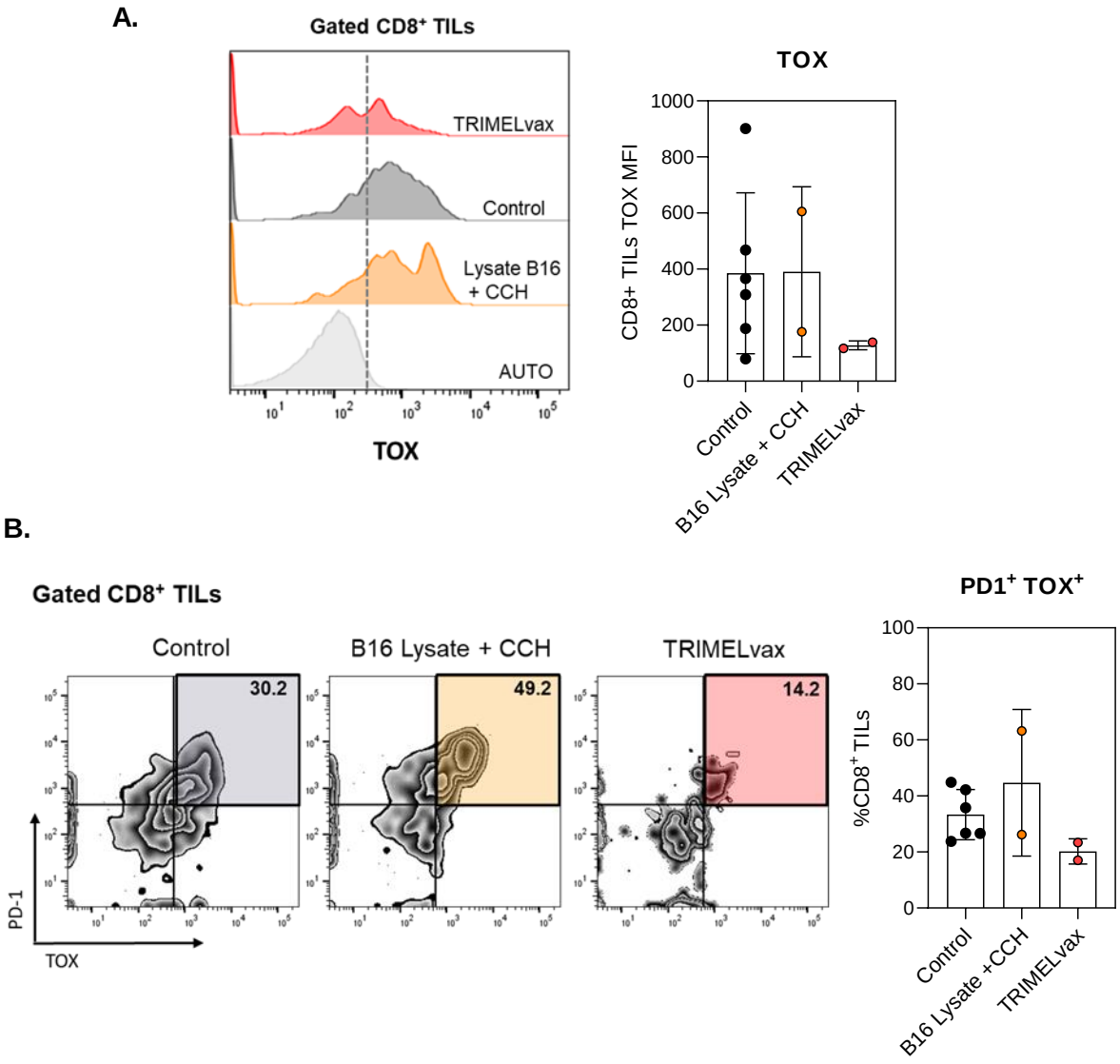

Figure 17. ***TRIMELvax vaccination likely diminishes TOX expression on CD8⁺ TILs and decreases frequency of PD1⁺TOX⁺CD8⁺ TILs***

A. Representative histogram of TOX expression (left) and quantification (right) for CD8⁺ TILs from control, B16 lysate + CCH and TRIMELvax treatments **B.** Representative zebra plot showing PD-1 and TOX expression of CD8⁺ TILs (left) and frequency quantification (right). Bar graphs show mean and SD. n=4-2 mice per group

Promoting immune memory is one of the most important challenges when developing vaccines. Furthermore, it is critical in cancer treatment which can be a long-term process. Using cancer immunotherapies, it has been shown that central memory T cells and stem cell memory T cells confer improved antitumor responses when adoptively transferred in tumor murine models and clinical trials, making these populations an interesting differentiation target. Hence, in addition to studying the exhausted associated phenotype, the evaluation of the T cell differentiation within the $CD8^+$ T cell population was performed. As shown on Figure 18, TRIMELvax treatment increased the frequency of $CD8^+$TILs with central-memory ($CD62L^+CD44^+$) and naïve-like ($CD62L^+CD44^-$) phenotypes while decreasing the frequency of effector-memory T cells ($CD62L^-CD44^+$) in comparison to B16 lysate + CCH vaccinated mice and control mice which present higher frequencies of effector-memory T cells ($CD62L^-CD44^+$). This increase in the frequency of a naïve-like ($CD62L^+CD44^-$) population was unexpected, however since it is very rare to observe tumor infiltrating naïve $CD8^+$ T cells, it is suggested that this population might be T memory stem cells (T_{scm}).

T memory stem cells (T_{scm}) are a newly identified population, which have similar expression of CD62L and CD44 with naïve T cells. However, T_{scm} show robust self-renewal and the multipotent capacity to generate central memory and effector T cells as progenitor cells, hence their designation as T memory stem cells. With the data obtained, only CD62L and CD44 were analyzed on $CD8^+$ tumor infiltrating cells. Since, their location, within the tumor site, and their CD62L high expression and lack of CD44 expression, it is strongly suggested that these cells that are increased in tumors from TRIMELvax treated mice correspond to progenitor like T cells. However, further data is needed to have a better characterization of this population.

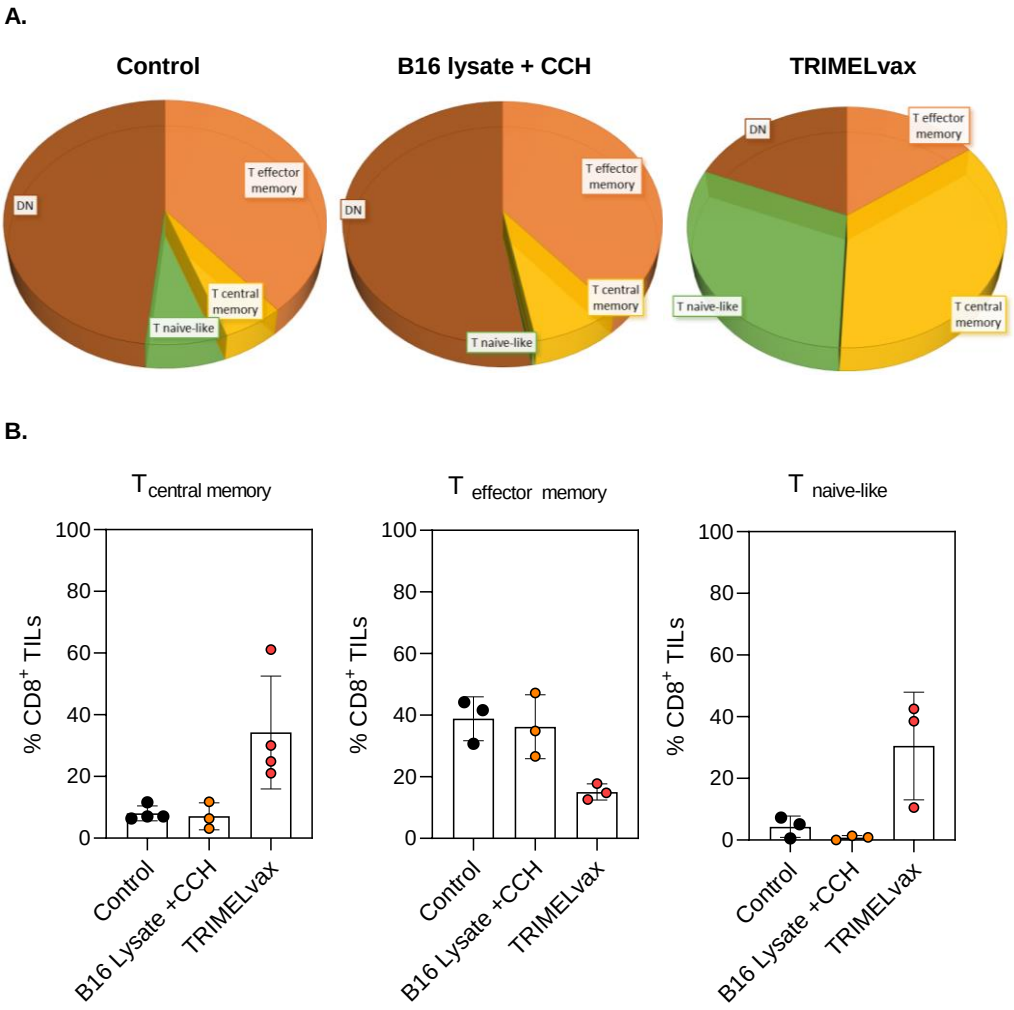

Figure 18. ***TRIMELvax treatment increases CD8⁺ TILs with central memory and naive-like phenotype***

A. Pie chart showing the population distribution observed among $CD8^+$ TILs for each treatment. Effector-memory T cells (T_{em}) were described as $CD62L^-CD44^+$, central-memory T cells (T_{cm}) as $CD62L^+CD44^+$ and naïve-like T cells (T_{nl}) as $CD62L^+CD44^-$ among the $CD45^+CD3^+CD8^+$ population. DN= double negative **B.** Quantification of A. showing frequency of each population. Bar graphs show mean and SD. Kruskal-wallis test with Dunn's post test is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ n=3 mice per group

Overall, these results showed that TRIMELvax treated mice predominantly present tumor infiltrating $CD8^+$ T cells with a central memory or naïve-like phenotype which might be represented by clusters 4 and 5 observed on the UMAP visualization and cluster analysis. Additionally, these populations showed a low expression of ex-

hausted associated receptors which were highly expressed on tumor infiltrated $CD8^+$ T cells from control and particularly from B16 lysate + CCH vaccinated mice that were mainly represented by clusters 1,2 and 3.

Additionally, a recent population of memory T cells present within the tumor has been characterized named resident memory T cells. This population has been suggested to have an important role in antitumor immunity residing within tumors and metastatic lesions being even associated to better clinical outcomes in cancer patients [131–135]. Therefore, the expression of CD69 and CD103, as characteristic markers for this T cell subset, was performed (Figure 19).

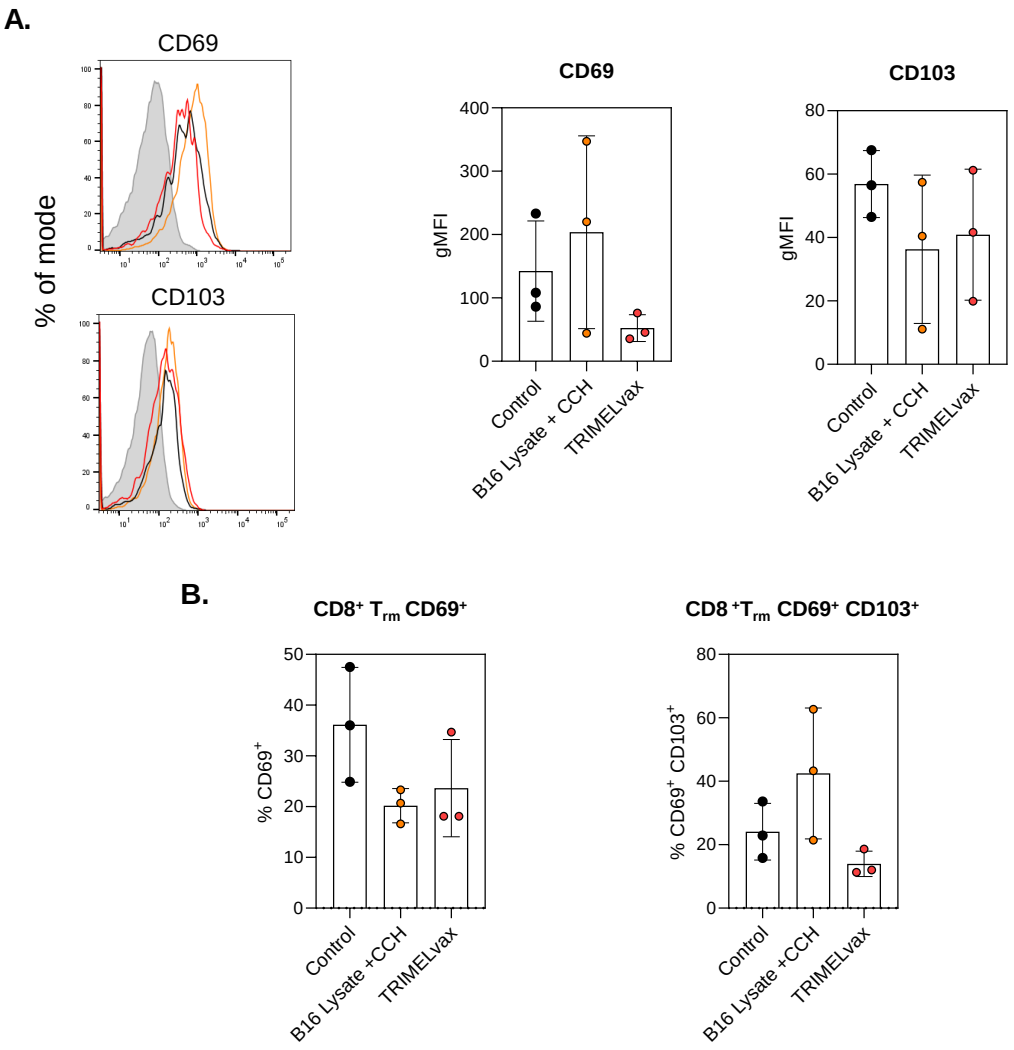

Figure 19. ***TRIMELVAX, B16 lysate + CCH nor Control tumors have significant differences on CD8⁺ Trm CD69⁺ nor CD8⁺ Trm CD69⁺ CD103⁺ cells.***

A. Representative histograms of CD69 and CD103 expression on $CD8^+$ TILs from control, B16 lysate + CCH and TRIMELvax treatments (left) and bar graphs showing the quantification of $CD8^+$ TILs $CD69^+$ and $CD69^+$ $CD103^+$ gMFI. **B.** Frequency of tumor infiltrating $CD8^+$ T_{rm} $CD69^+$ or $CD8^+T_{rm}$ $CD69^+$ $CD103^+$ cells from control, B16 Lysate + CCH and TRIMELvax vaccinated mice. Bar graphs show mean and SD. Kruskal-wallis test with Dunn's post test is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ n=3 mice per group.

No statistical difference on frequency or level of expression was observed comparing the three treatments. However, a slight tendency of lower CD69 levels of expression and $CD8^+T_{rm}$ $CD69^+$ $CD103^+$ cells frequency on the tumors from TRIMELvax vaccinated mice was observed (Figure 19).

SPECIFIC AIM 2. *Analyze frequency and activation state of tumor infiltrating cDCs in immunized mice versus control mice in the B16.F10 melanoma murine model.*

Previous results showed that TRIMELvax vaccine was able to diminish the acquisition of the exhausted profile on tumor infiltrating $CD8^+$ T cells. As already mentioned, T cell exhaustion is mainly driven by persistent antigen stimulation. However recent has shown that several immune populations may promote this profile cooperating with the tumor derived antigen stimulation (Figure 1). Published results showed that TRIMELvax vaccination increases the frequency of cDCs within the tumor [125], however no data of other myeloid populations had been obtained. Hence, tumor myeloid compartment analysis on mice receiving different treatments was performed as explained for specific aim 1 for the $CD8^+$ T cell population (Figure 20).

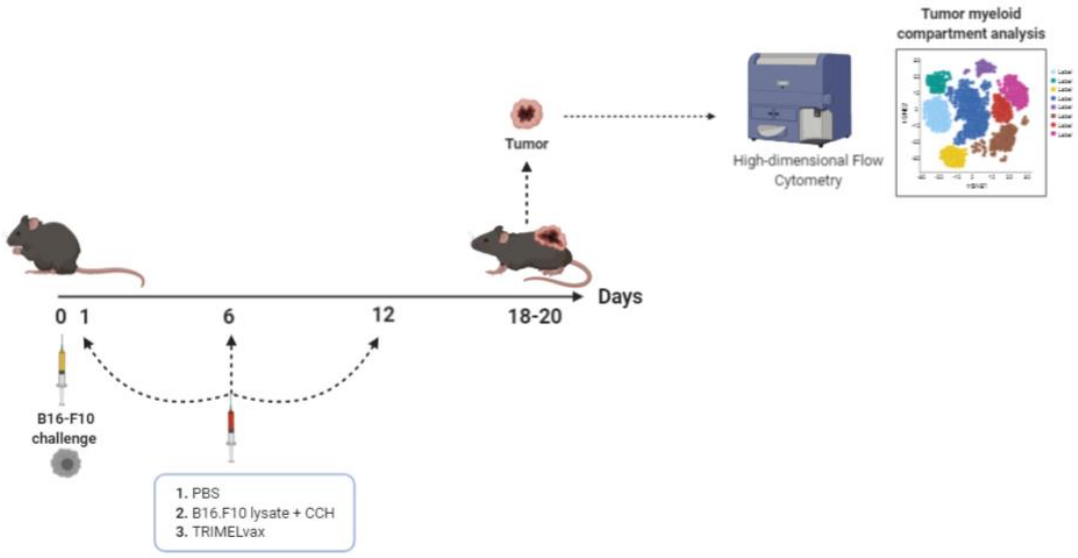

Figure 20. *Therapeutic design used to evaluate tumor myeloid population in B16-F10 melanoma murine model.*

18 days post tumor inoculation, tumors were dissected and FACS analysis was performed focusing on non-T cell population analysis.

To perform a first characterization of tumor myeloid compartment, FACS data acquired using BD LSR Fortessa was further analyzed using Flowjo v10 as for the tumor infiltrating $CD8^+$ T cells. For this analysis, first, a concatenate from three different tumor samples from each treatment was done. Then, after gating on the live immune non-T cell population (viability-$stain^-$ $CD45^+$ $CD3^-$ cells), a down sample was done limiting to 5000 events each concatenate. Finally, a final concatenate was done using the previous down sample concatenates for each treatment.

Then using the phenograph Flowjo plug-in (with preset values using compensated values for IA/IE, XCR1, CD24, CD86, Ly6C, Ly6G, F4/80, CD11c, Sirpa and CD11b), 12 clusters were determined. This information was used to perform an UMAP analysis on this final concatenate using the preset flowjo plug-in parameters; minimum distance= 0,5, number of components= 2 and 12 nearest neighbors as determined by the previous phenograph analysis. Interestingly, using UMAP visualization analysis determined a different cluster distribution when comparing the tumor myeloid compartment from control, B16 lysate + CCH or TRIMELvax mice (Figure 21). Hence, a similar result compared to what was observed in the $CD8^+$ T cell population

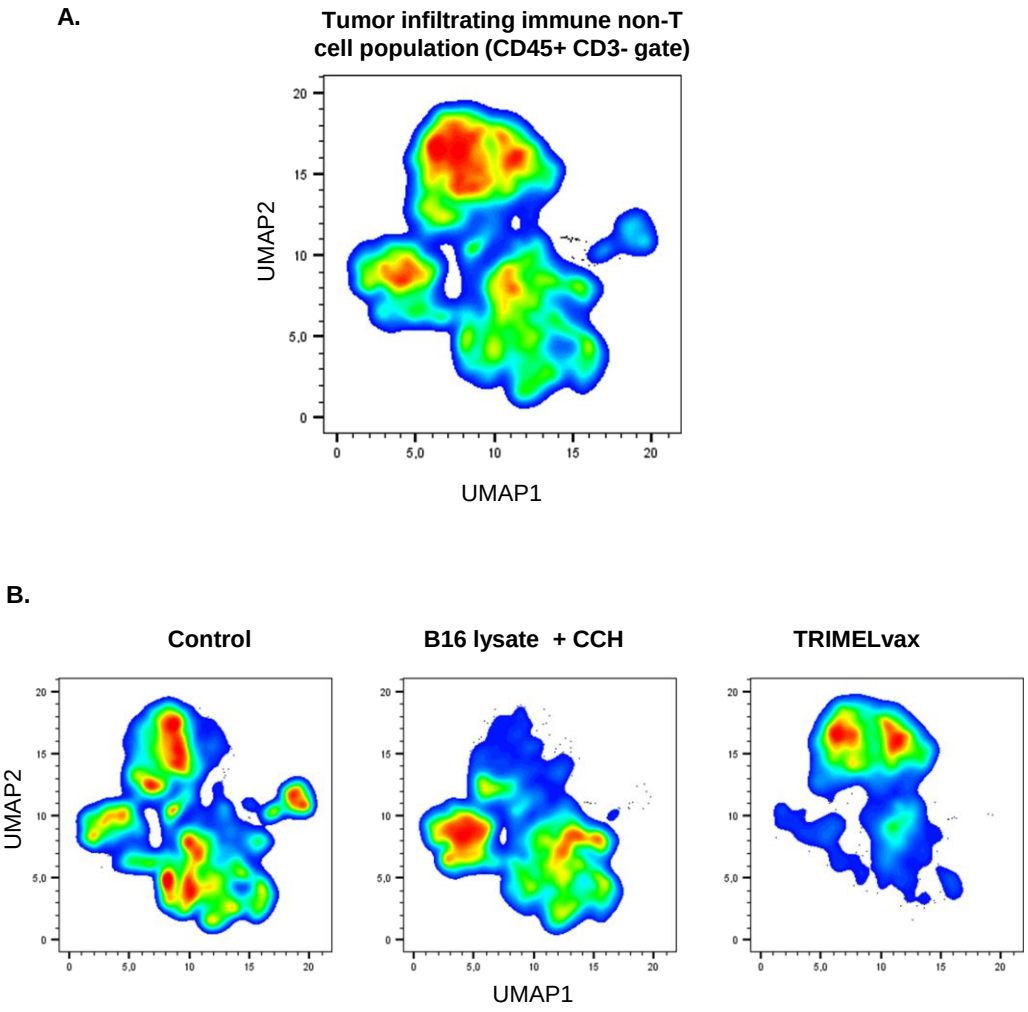

Figure 21. ***Different treatments generate different cluster distribution of tumor infiltrating Myeloid population***

UMAP analysis based on tumor infiltrating immune non-T cell population ($CD45^{+}CD3^{-}$) expression of 9 parameters; CD86, Ly6C, Ly6G, F4/80, IA/IE, XCR1, CD24, CD11c, Sirpa and CD11b.**A.** UMAP visualization using data from Control, B16 lysate + CCH and TRIMELvax vaccinated tumors. **B.** UMAP visualization using data from each treatment. n=3 mice per group

Later, analysis of each cluster determined by phenograph, using the cluster explorer plug in from Flowjo, showed that the 12 previously determined cluster present different frequencies on each treatment data set (Figure 22).

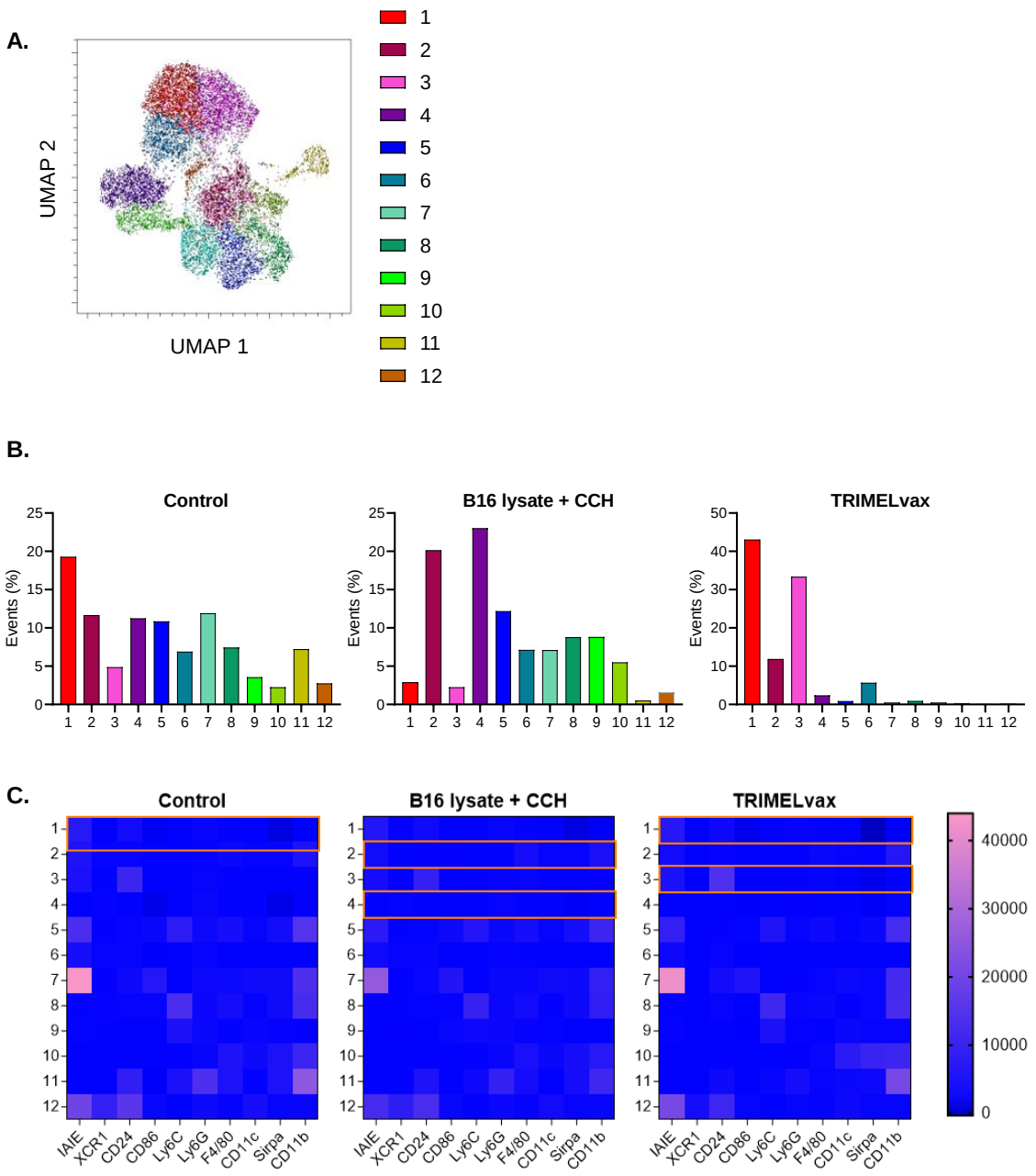

Figure 22. ***Tumor myeloid compartment distribute in 12 different clusters.***

A. UMAP visualization of the 12 clusters determined within the tumor myeloid compartment from all treatments. **B.** Bar graphs showing frequency of cells on each cluster shown on A. **C.** Heatmaps showing the level of expression of 9 parameters used to describe the population: IAIE, XCR1, CD24, CD86, Ly6C, Ly6G, F4/80, CD11c, Sirpa, CD11b for each cluster for each treatment. The most frequent clusters for each treatment are highlighted in orange: Control cluster 1, B16 lysate + CCH cluster 1 and 3, and TRIMELvax cluster 2. n=3 mice per

group

Analysis of the tumor myeloid compartment from control mice predominantly presents higher frequencies of cluster 1, B16 lysate + CCH vaccination on clusters 1 and 3, where those from TRIMELvax treated mice predominantly did on cluster 3 (Figure 22.B). These differences on cluster distribution supports the fact that TRIMELvax vaccination promotes a completely different tumor microenvironment in comparison to control and B16 lysate + CCH treated mice. However, the distribution between tumor myeloid compartment from B16 lysate + CCH vaccinated mice present a similar distribution to the one observed on the analysis of control mice when compared to TRIMELvax vaccinated tumors. This observation differs to what was observed on the tumor infiltrating $CD8^+$ T cells, where the three different treatments have different cluster distribution. No differences on levels of expression were seen in the comparison of the three heatmaps obtained (22.C).

Next, through manual gating the frequency of each myeloid population was obtained. Several tumor myeloid populations were analyzed: Tumor-associated Neutrophils (TANs), Monocytes, Monocyte-derived Dendritic Cells (moDCs), Tumor-associated Macrophages (TAMs) and Conventional Dendritic cells (cDCs). (Figure 23).

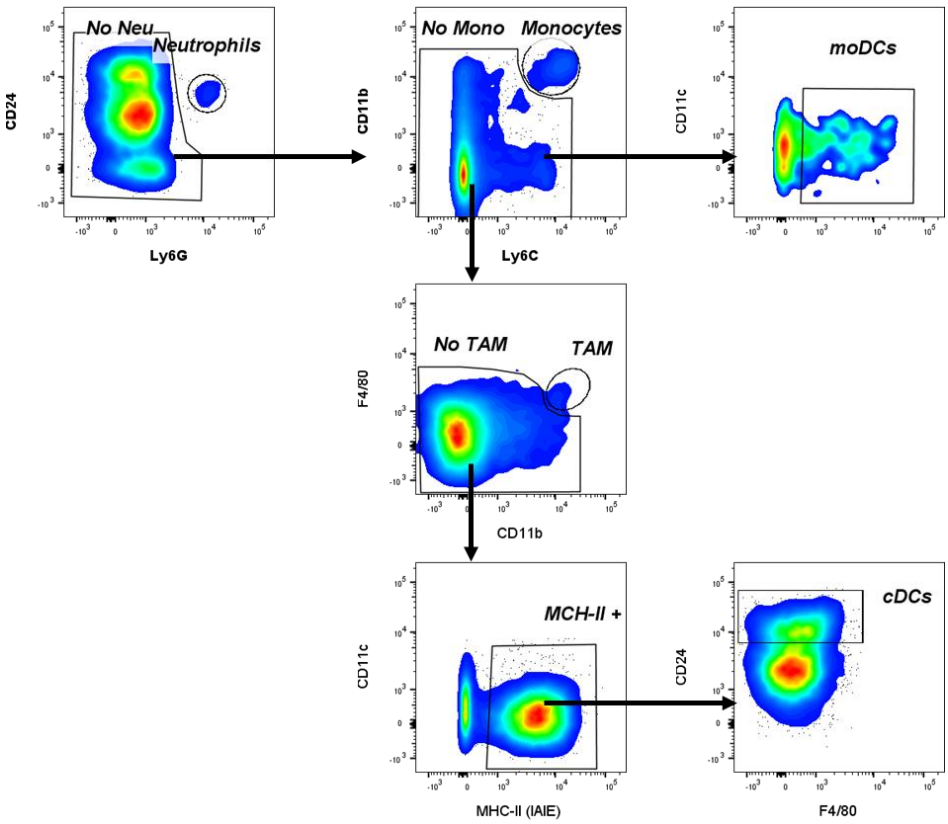

Figure 23. ***Representative gating analysis used on FACS data to characterize tumor myeloid compartment for each treatment.***

FACS data analysis shows tumor infiltrating immune non-T cell population (gate $CD45^{+}CD3^{-}$)

Results suggest TRIMELvax treatment induces a decrease in frequency of TANs, Monocytes, moDCs, and TAMs and an increase in frequency of cDCs compared to control treated mice (Figure 24). When comparing TRIMELvax versus B16 lysate + CCH vaccinated mice the results suggested similar frequencies on TANs and TAMs, and a decrease of frequency on TRIMELvax vaccinated mice tumors when analyzing monocytes and moDCs. Interestingly, only cDCs population present an increase on TRIMELvax vaccinated mice tumors, but a different result is obtained between control and B16 lysate + CCH vaccinated mice tumors. Indeed, tumors from B16 lysate + CCH vaccinated mice have very low frequencies of this population (<20%) in comparison to control (~50%) and TRIMELvax vaccinated mice (~80%). Looking back on the results obtained using the UMAP visualization and cluster analysis, is it possible that

cluster 3, which is highly represented on the tumor myeloid compartment from TRIMELvax vaccinated mice but almost absent on the other two conditions might correspond to this cDC population. Cluster 3 as shown on figure 22, has an slight increase on CD24 expression which is usually expressed by tumor infiltrating cDCs.

Unfortunately, due to the low experimental number it wasn't possible to determine statistical differences on Monocyte and MonoDCs analysis which showed very high dispersion among the individuals measured. Indeed, increasing the experimental number will allow to clarify how TRIMELvax affects other tumor myeloid populations.

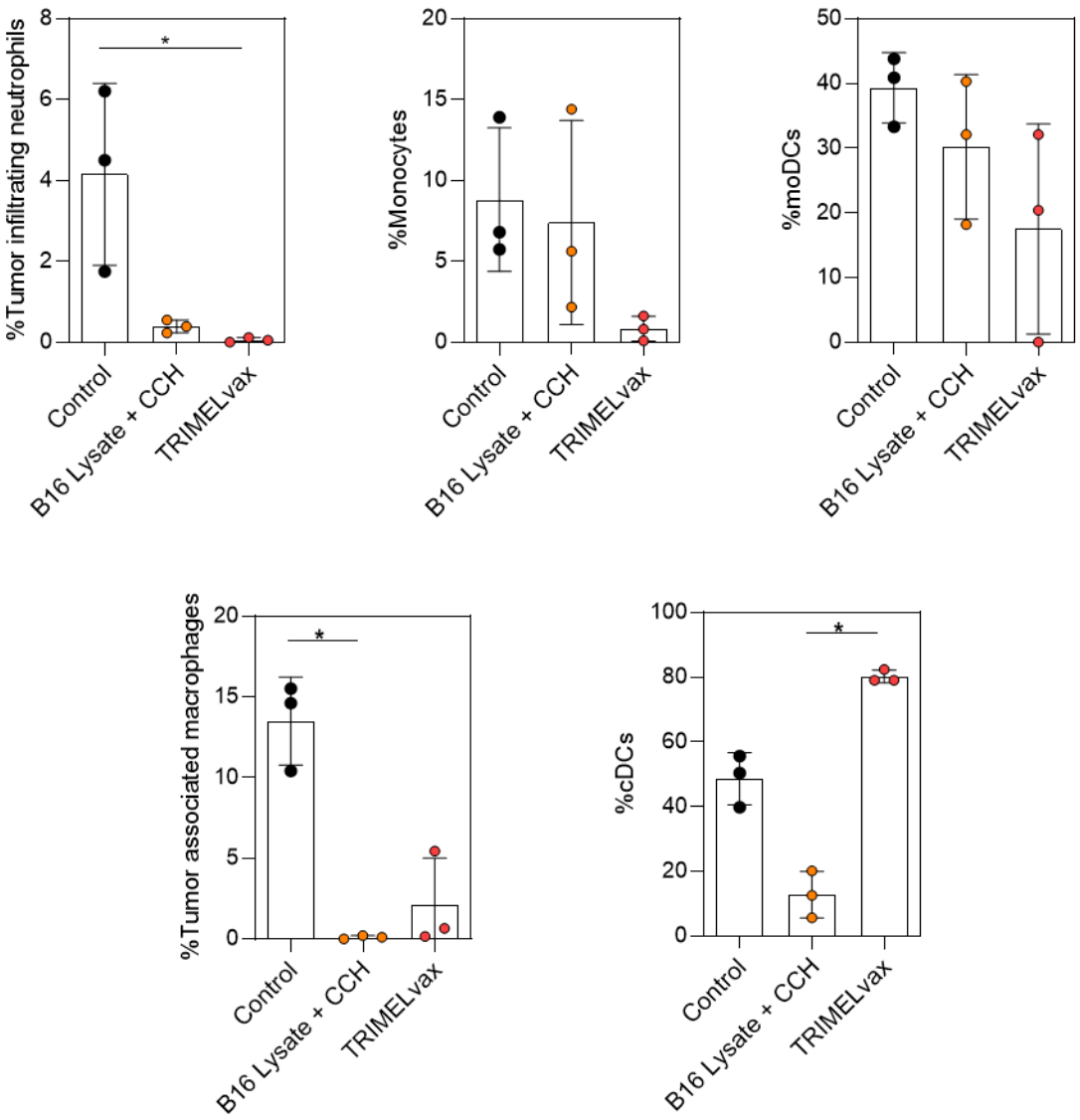

Figure 24. ***TRIMELvax modulates the tumor myeloid compartment.***
Bar graphs show frequency of each myeloid population for different treatments. Bar graphs show mean and SD. Kruskal-wallis test with Dunn's post test is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ n=3 mice per group

Next, due to pandemic associated problems an *in vitro* approach was used to analyze the TRIMELvax activation profile on cDCs. Bone marrow derived dendritic cells were generated using the cytokine ms-like tyrosine kinase 3 ligand (FLT3-L) (DCs-FL) which were used to determine the DC3 activation profile induced by the different treatments on cDC, cDC1 and cDC2 (Figure 25.A). Novel DC3 activation state is

characterized by high expression of PD-L1, CD40, CCR7 and IL-15R plus high production of IL-12 [78,79]. These experiments were performed in collaboration with Dr Fabiola Osorio's Laboratory.

DCs were stimulated for 18 hours using either:

- TRIMELvax (TRIMEL + B16 Lysate + CCH)
- B16 lysate+ CCH
- Lysates (TRIMEL + B16 lysate)
- CCH
- Lipopolysaccharide (LPS) (positive control for DCs activation)
- Unstimulated (basal status control)

For IL-12 analysis, Brefeldin A (BFA) was added to the culture during the last 2 hours of stimulation. BFA is a protein transport inhibitor commonly used to enhance intracellular cytokine staining signals by blocking transport processes during cell activation.

After the stimulation time was completed, cells were recovered, and flow cytometry was used to determine the DCs activation profile. For this through manual gating, cDCs, cDCs1 and cDCs2 were analyzed (Figure 25.B).

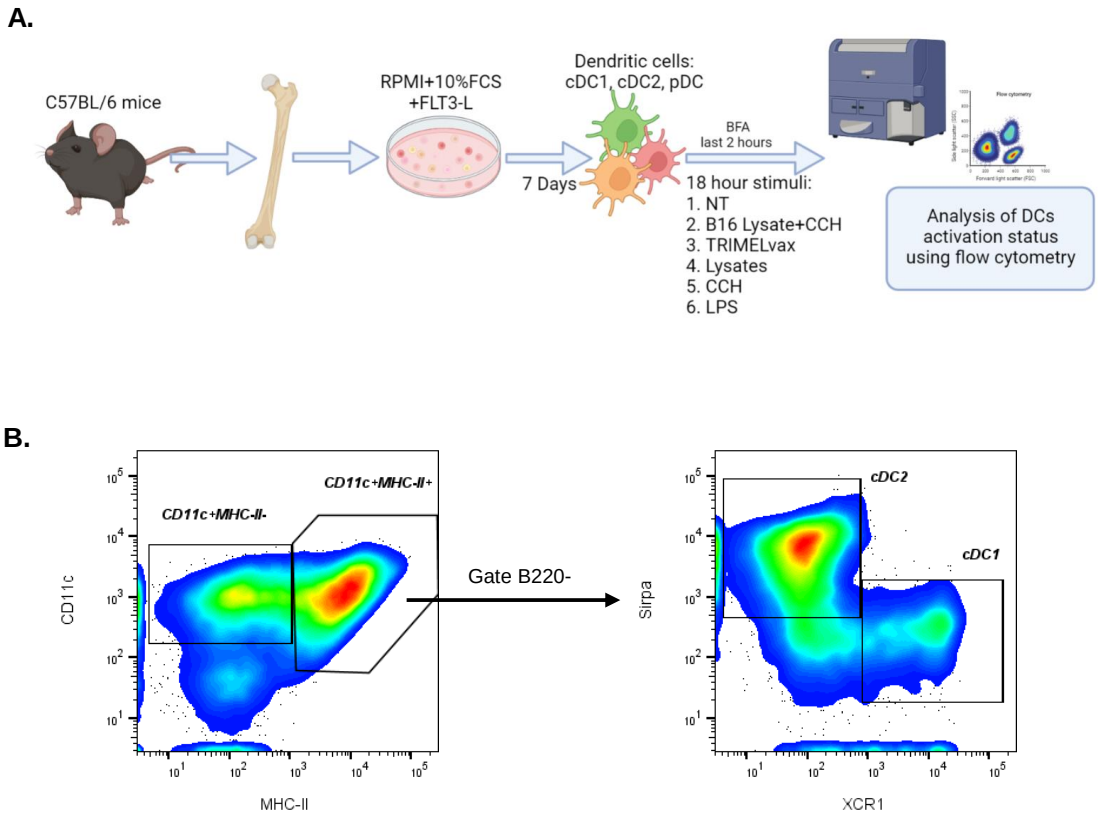

Figure 25. ***Evaluation of FL-DCs activation profile.***

A. Scheme of the experimental design used to evaluate DCs activation status. Bone marrow derived DCs-FL were stimulated using either; B16 lysate + CCH, TRIMELvax, Lysates (B16 lysate + TRIMEL), CCH, LPS (as positive control for activation) or left untreated (NT). After 18 hours, activation profile was measured using flow cytometry. For IL-12 analysis, cells were left with BFA for 2 hours before intracellular cytokine measurement. **B.** Representative gating analysis to determine cDC1s and cDC2s after the 7 days stimulation.

First, the expression level of several molecules was measured on the total cDC population using flow cytometry since it has been determined that the DC3 activation state can be acquired by cDC1 and cDC2 subpopulations. It was observed that non-treated cDCs express lower levels of expression of CD86, PD-L1, CD40, CCR7 and IL-15 when compared with LPS stimulated cells which was used as an activation control. These results were expected because it is well known that LPS activated DCs mainly through toll like receptor 4, promoting its activation [136].

Interestingly, TRIMELvax, B16 lysate + CCH and Lysates stimulation promoted similar activation levels on all the measured parameters, which were higher that NT cells but usually lower than LPS stimulated cells. Additionally, CCH stimulated cells present low expression levels of all the measured parameters, similar to NT cells suggesting that CCH does not have an activating effect over cDCs when stimulated using TRIMELvax, at least on the measured parameters. This is supported by the results obtained when stimulating cDCs with Lysates, which contains the tumor cell lysates that compose TRIMELvax but without adding CCH (Figure 26).

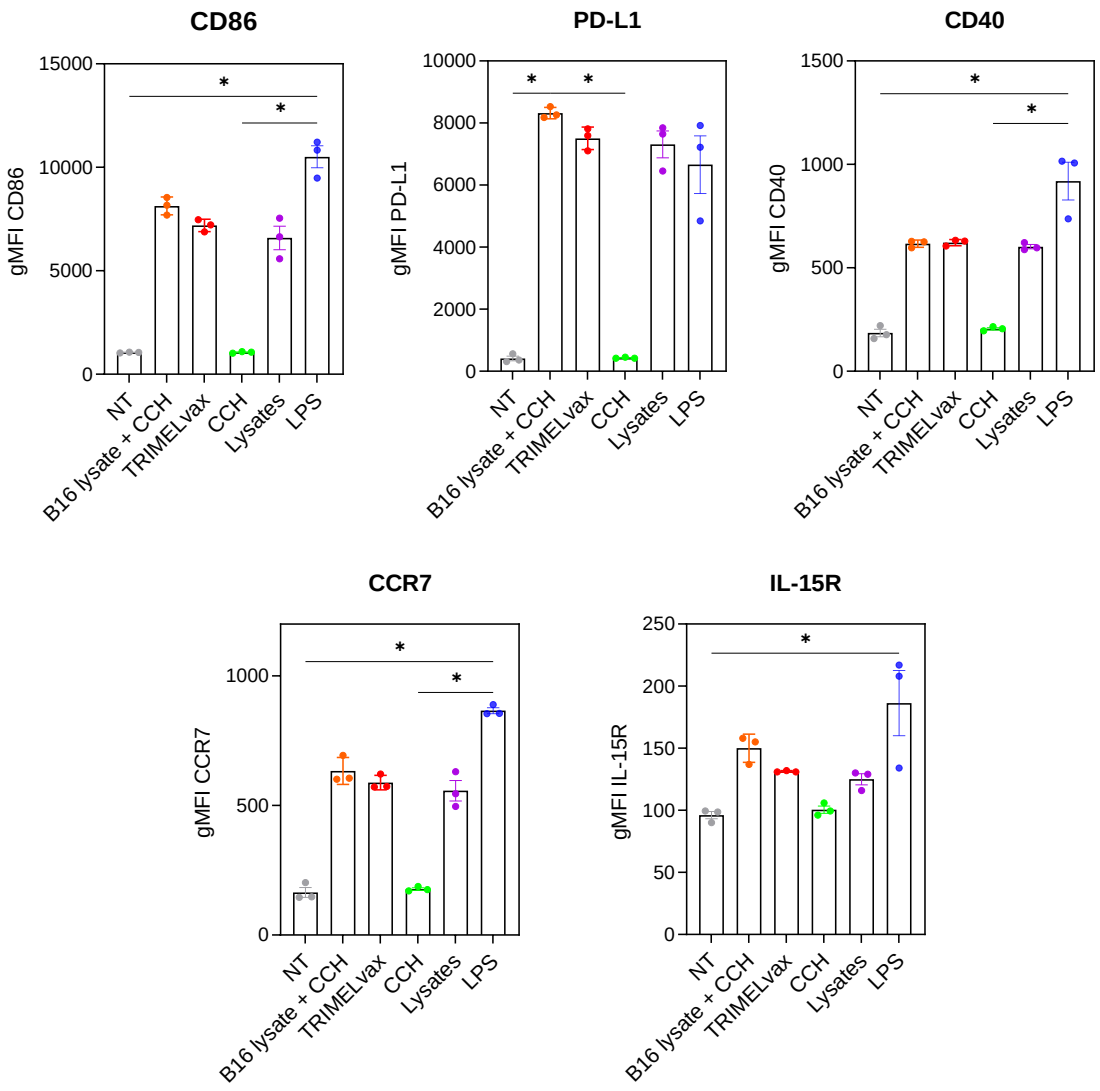

Figure 26. ***Tumor cell lysate induces a cDCs activation profile on FL-DCs.***

Bar graphs show gMFI of different markers on FL-DCs stimulated for 18 hours with either; B16 lysate + CCH, TRIMELvax, CCH, Lysates, LPS (positive control) or left untreated (NT). Bar graphs show mean and SD. Kruskal-wallis test with Dunn's post test is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ n=3 per stimuli.

cDC1s are the main drivers of the antitumor response. Therefore, the cDC1 population was analyzed separately. The results obtained were equivalent to those obtained when analyzing the whole cDC population. TRIMELvax, B16 lysate + CCH

and Lysates stimulation promoted similar activation levels on all the measured parameters, which were higher that NT cells but usually lower than LPS stimulated cells (Figure 27).

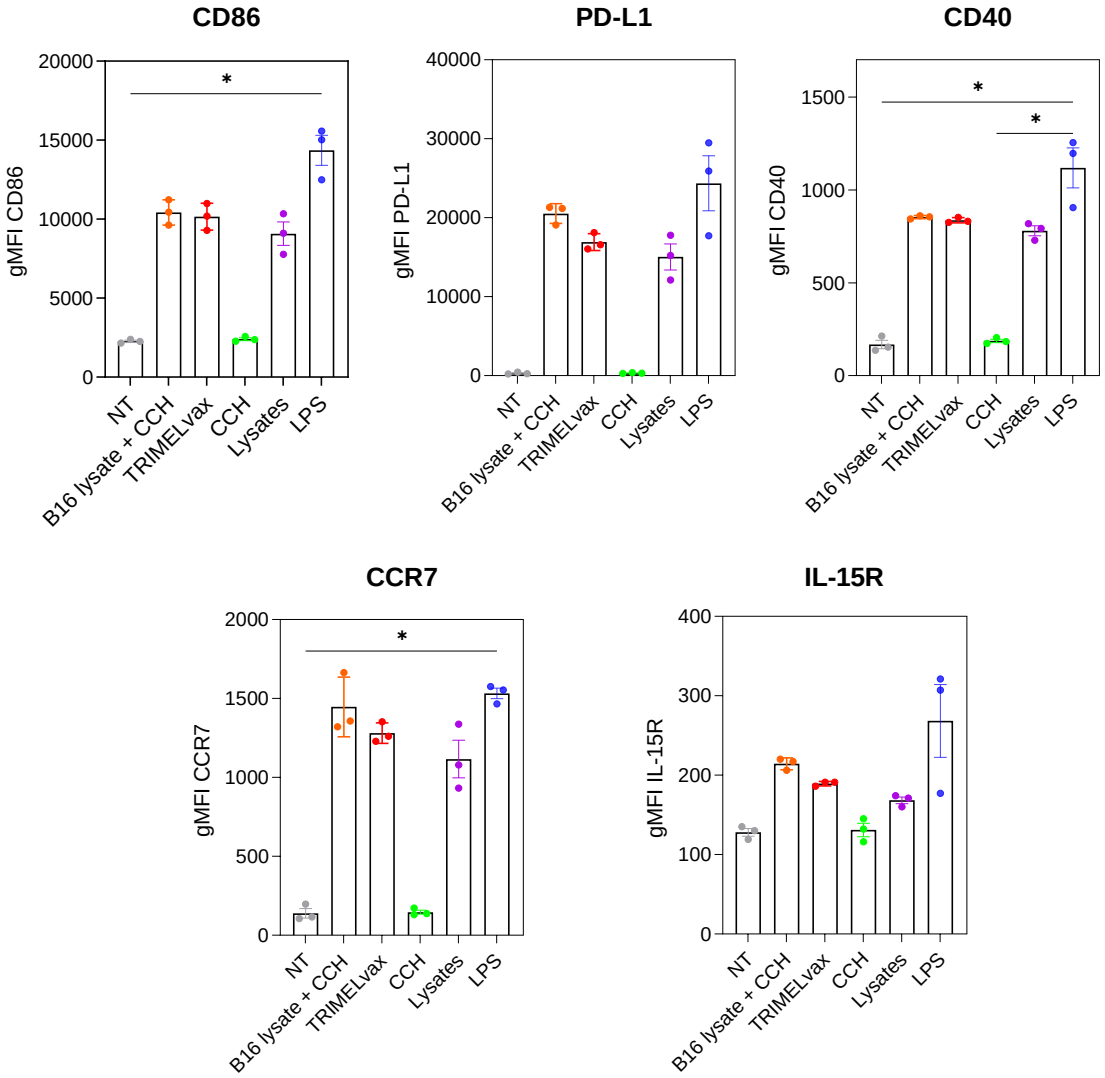

Figure 27. ***Tumor cell lysate induces cDCs activation on FL-DCs cDC1s sub population.***

Bar graphs show gMFI of different markers on DCs-FL cDC1s subpopulation (as described on Figure 24.B) stimulated for 18 hours with either; B16 lysate + CCH, TRIMELvax, CCH, Lysates, LPS (positive control) or left untreated (NT) Bar graphs show mean and SD. Kruskal-wallis test with Dunn's post test is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ n=3 per stimuli.

CD4+ T cells have emerged as an important actor of the antitumor response. CD4+ T helper cells can license DCs to enhance immunity and stimulate pro-inflammatory myeloid cell programs [137]. Additionally, cDC2s can present tumor-derived antigens to CD4+ T cells, promoting the antitumor response [138]. Therefore, cDC2s subpopulation was analyzed separately. The results observed are like those obtained when analyzing cDCs and cDC1s (Figure 28).

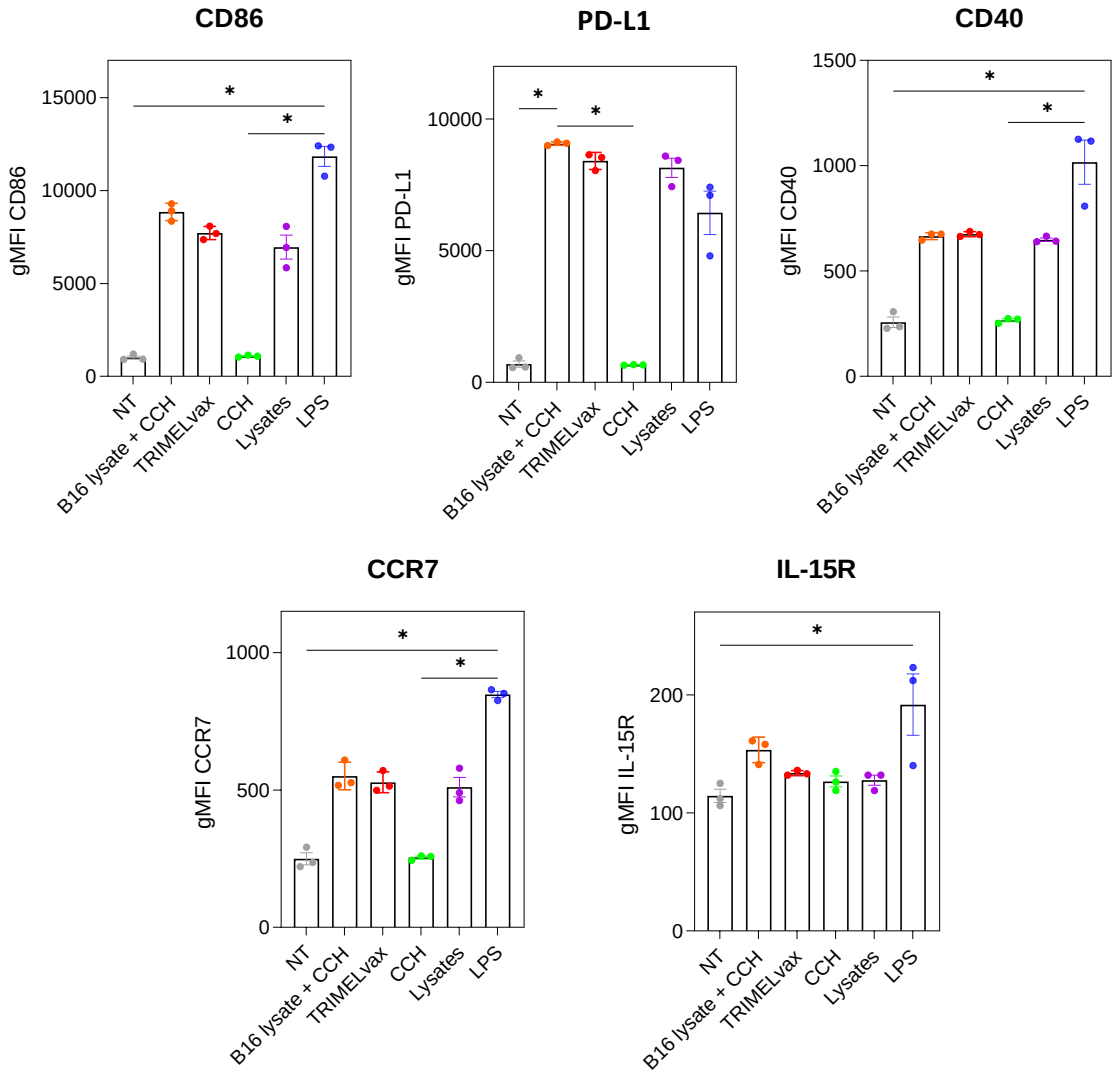

Figure 28. ***Tumor cell lysate induces activation of FL-DCs cDC2s sub population.***
Bar graphs show gMFI of different markers on DCs-FL cDC2s subpopulation (as described on Figure 24.B) stimulated for 18 hours with either; B16 lysate + CCH, TRIMELvax, CCH,

Lysates, LPS (positive control) or left untreated (NT) Bar graphs show mean and SD. Kruskal-wallis test with Dunn's post test is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ n=3 per stimuli.

As a novel described state, DC3s have been characterized using different parameters according to different authors. However, it has been suggested that DCs acquire this specific program of activation when up taking autoantigens in particular tumor associated antigens. DCs that up take these antigens acquire a high expression of CD40 and PD-L1, therefore the analysis of co-expression between those two molecules was performed (Figure 29) [78].

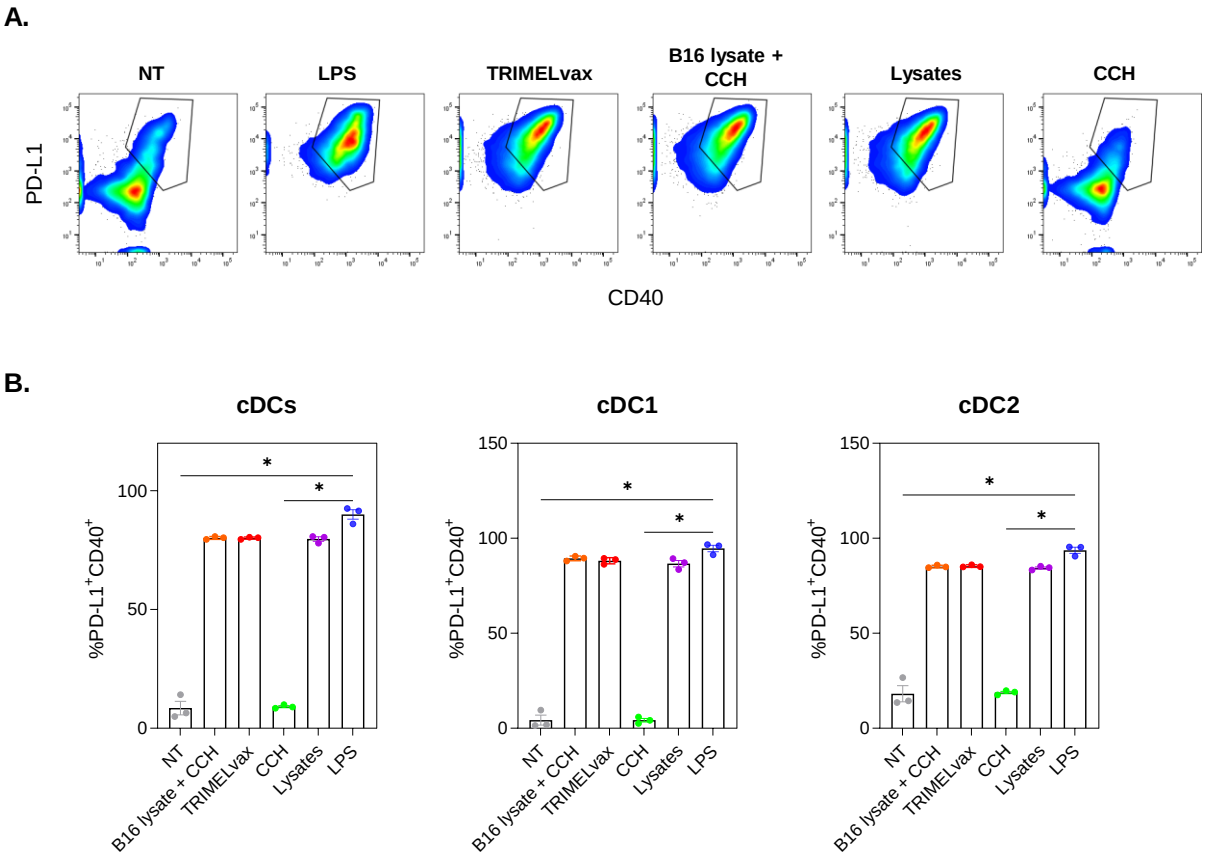

Figure 29.*Tumor cell lysate induces a DC3-like activation profile on cDCs, cDC1s and cDC2s subpopulation.*

A. Representative pseudo color plots showing $CD40^{+}PD\text{-}L1^{+}$ population analysis on FL-DCs after stimulated for 18 hours using either; B16 lysate + CCH, TRIMELvax, CCH, Lysates, LPS

(positive control) or left untreated (NT) n=3 for each stimulus. **B.** Bar graphs showing frequency of PD-L1+CD40+ cells on cDCs (left), cDC1s (middle) or cDC2s (right). Bar graphs show mean and SD. Kruskal-wallis test with Dunn's post test is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ n=3 per stimuli.N=3 * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Results obtained show that tumor cell lysates (TRIMELvax, B16 lysates + CCH and lysates) promoted the co-expression of CD40 and PD-L1 on cDCs, cDC1s and cDC2s. Interestingly, the frequency of double positive expressing cells was as high as those obtained when activating using LPS, supporting the fact already published that tumor cell lysates have a strong activation capacity on DCs [125] but also suggesting this activation state might be similar to the DC3 activation program (Figure 29). However, it has been suggested that the physiological uptake of tumor antigens drives an immunoregulatory program that might promote a protumor microenvironment. However, the modulation of this program through IL-4 signaling blockade enhances IL-12 production promoting an antitumoral phenotype which expands the pool of tumor infiltrating effector T cells and reduces tumor growth. Therefore, IL-12 production was measured after 18-hour stimulation with the different treatments (Figure 30)

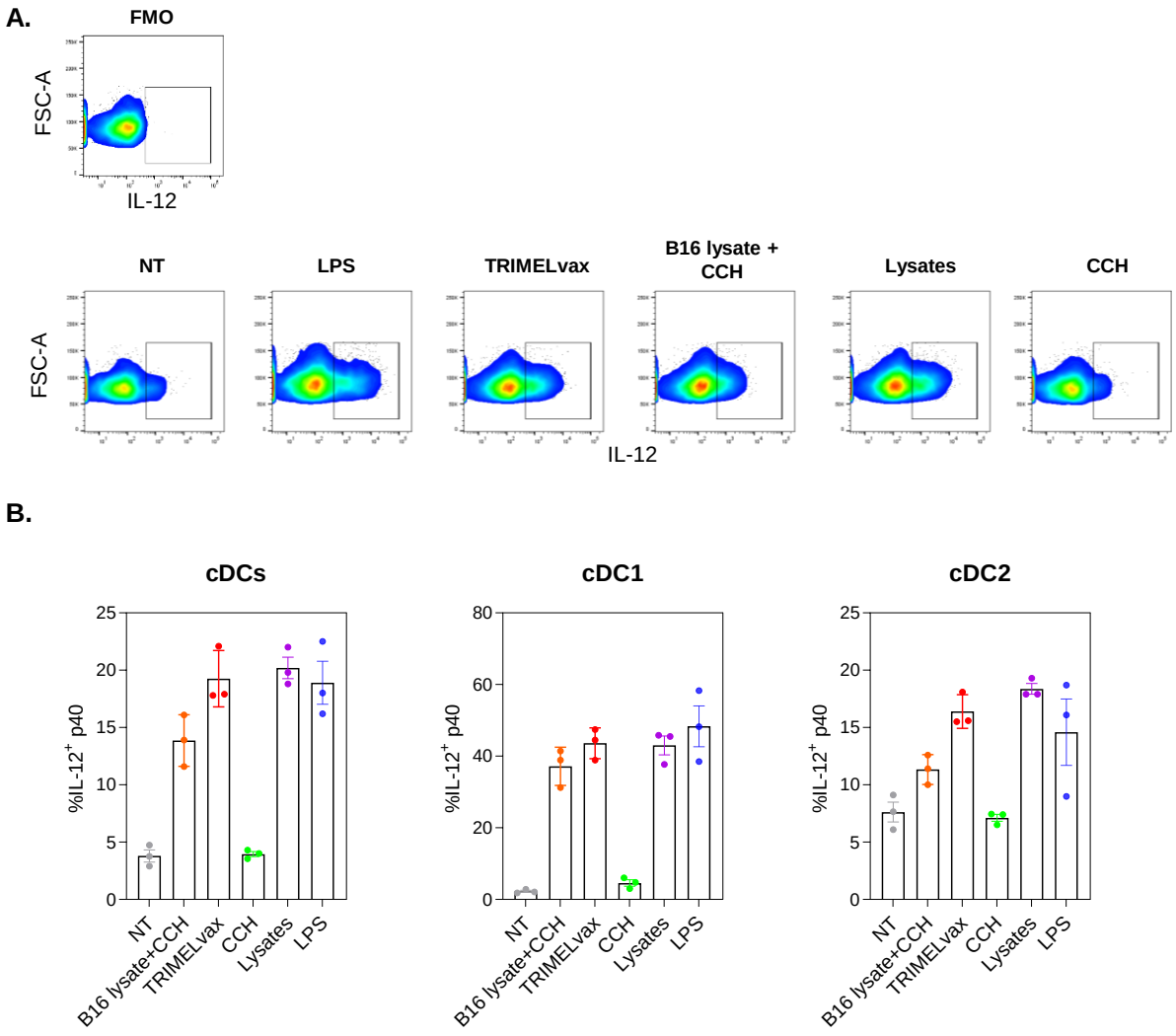

Figure 30. ***Tumor cell lysate induces a DC3-like activation profile IL-12 producing FL-DCs.***

A. Representative pseudocolor plots showing IL-12^{+} population analysis on FL-cDCs after stimulated for 18 hours with either; B16 lysate + CCH, TRIMELvax, CCH, Lysates, LPS (positive control) or left untreated (NT). BFA was added in the last two hours of the 18 hours stimulation. n=3 for each stimulus. **B.** Bar graphs showing frequency of IL-12^{+} cells on cDCs (left), cDC1s (middle) or cDC2s (right) population. Bar graphs show mean and SD. Kruskal-wallis test with Dunn's post test is shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ n=3 per stimuli.

Interestingly, tumor cell lysates induce IL-12 production on cDCs, cDC1 and cDC2s from stimulated FL-DCs culture. As expected cDC1s had higher frequency of

IL-12^{+} cells when compared to cDCs2, highlighting their critical role in antitumor immunity.

Overall, these results suggest that TRIMELvax and B16 lysate + CCH vaccines can promote an DC3-like program on FL-DCs *in vitro*. Therefore, indicating TRIMELvax not only induces DCs tumor infiltration but suggesting it might promote a DC3 activation program which may partly explain how TRIMELvax vaccination produces a T cell dependent response. Undoubtedly, further studies are required to explore this hypothetic action mechanism.

X. DISCUSSION

Several strategies are currently used in cancer immunotherapies being ICBs the one with better clinical results. However, the economic costs of their development and application, makes it almost inaccessible for most patients. Unfortunately, treated patients can also be refractory to primary or secondary ICB-based therapies, primarily due to resistance development to the treatment and low immune tumor infiltration. Therefore, the need for cheaper and better immunotherapies becomes critical.

Regarding this, our group through the development of a DC-based cancer vaccine (TAPcells) or by a tumor lysate-based vaccine (TRIMELvax) has shown how conditioned tumor cell lysate can be an interesting source of tumor associated antigens also including several DAMPs promoting DC maturation and activation of tumor specific T cells [117,125]. However, the mechanisms behind their antitumoral effect are still unknown with several studies on this topic being currently developed.

In this work, tumor infiltrating $CD8^+$ T cells where the focus since previous results had shown their critical role in TRIMELvax antitumoral effect. The lack of $CD8^+$ or $CD4^+$ T cells makes TRIMELvax vaccination completely useless, indicating its effect is T cell dependent. In addition, current data have shown that tumor infiltrating $CD8^+$ T cells undergo a persistent antigen stimulation driven process called exhaustion which limits antitumor immunity. Results showed TRIMELvax vaccination diminished the acquisition of an exhausted phenotype on tumor infiltrating $CD8^+$ T cells in comparison to control PBS vaccinated mice. As previously mentioned, although TRIMELvax and B16 lysate + CCH vaccination promoted $CD8^+$ T cells tumor infiltration, only TRIMELvax showed an antitumoral effect. Hence, suggesting different tumor infiltrating $CD8^+$ T cell phenotype and functionality among the two vaccines. According to this result, we also expected some level of immunomodulation by the B16 lysate + CCH vaccination since using this vaccine promoted $CD8^+$ T cell tumor infiltration contrary to what was observed for control mice which lacks it. However, it was striking that B16 lysate + CCH vaccination strongly aggravated the normal exhaustion described for this model (as seen for control mice), partly explaining why although it promoted $CD8^+$ T cell tumor infiltration no antitumoral effect was observed. Indeed,

when performing phenograph and UMAP visualization it was clear to observe that the three conditions generated completely different distributions (Figure 9). The high frequency of exhausted tumor infiltrating $CD8^+$ T cells seen on B16 lysate + CCH vaccinated mice cannot be explained yet, however it is suggested that increasing the amount of B16-F10 derived antigens might promote this antigen driven process. In addition, exhaustion might be promoted by the high level of B16-F10 antigens plus the lack of an undetermined signal that TRIMEL (human melanoma cell lysate) provides. From previous results, DAMPs emerge as possible candidates since heat shocked treatment has been proven to induce translocation of calreticulin, ATP release, High mobility group box 1 (HMGB1) translocation and heat shock proteins induction [117,123,124] (Figure 3). Additional published results have determined that even though both human and murine tumor cell lysates have similar DC activation capacity, some differences in phagocytic levels have been determined. Indeed, the generation of a xenogeneic immune response induced by the inoculation of human tumor cell lysate on murine models cannot be discarded, since previous results showed that TRIMELvax vaccinated mice possess serum IgGs that bind B16-F10 and human melanoma cells. However, TRIMELvax vaccinated mice sera also reacts against human PBL indicating some level of xenogeneic immune response [125].

When analyzing different exhausted-associated markers, PD-1 emerged as the more distinctive due to the low expression observed on tumor infiltrating $CD8^+$ T cell population on TRIMELvax vaccinated mice in contrast to control and B16 lysate + CCH (Figure 10, 12,13, 14). Indeed, it was possible to observe higher frequencies of tumor infiltrating $CD8^+$ T cells expressing high levels of PD-1 after B16 lysate + CCH vaccination. High levels of PD-1 expression have been linked to terminally exhausted $CD8^+$ T cells which can't be reinvigorated after ICB therapy, indicating how B16 lysate + CCH vaccination aggravates the induction of an exhausted phenotype.

PD-1 receptor has been widely studied since being used in immune checkpoint blockade therapy on cancer patients. Nivolumab (an anti-PD-1 monoclonal antibody) in combination with Ipilimumab (an anti-CTLA-4 monoclonal antibody) has shown successful clinical results when treating malignant melanoma patients highlighting the important role PD-1 expression has on antitumor immunity [139,140]. Indeed, previ-

ous published results showed no improvement of the combinatorial use of anti-PD-1 plus TRIMELvax vaccination in the B16-F10 melanoma murine model [125]. The lack of PD-1 expression seen on tumor infiltrating $CD8^+$ T cells from TRIMELvax vaccinated mice could partly explain the non-improvement observed when using the combinatorial therapy.

Decrease functionality is another characteristic of exhausted T cells. Unfortunately, using this setting, no differences in cytokine production was observed (Figure 16). Hence, functionality of tumor infiltrating $CD8^+$ T cells from control and B16 lysate + CCH vaccinated mice might not be affected in terms of cytokine production. This was a completely unexpected result since the striking differences observed when analyzing exhausted associated receptors. Although other authors have used PMA + Ionomycin + BFA stimulation to analyze tumor infiltrating $CD8^+$ T cells cytokine intracellular production, this method showed no difference between different treatments. The use of other techniques to induce cytokine production such as antigen specific stimulation using tumor lysate pulsed bone marrow derived dendritic cells or anti-CD3 + anti-CD28 antibodies, could be tested since the activation stimuli chosen could not be optimal. However, during the last period of this work, the TRIMELvax model showed several issues not allowing its reproducibility. The COVID pandemic besides hindering the progress seen on the phenotype analysis, intensified this issue which nowadays hadn't been completely solved. This problematic might have affected the cytokine analysis where TRIMELvax still showed antitumor efficacy, but tumor growth became erratic. Anyhow, tumor infiltrating $CD8^+$ T cells from control and B16 lysate + CCH vaccinated high and multiple expression of exhausted associated receptors, suggests these cells wouldn't be allowed to function properly within the tumor site affecting at least partially their cytokine production. However, it is expected to repeat these experiments in the future.

B16-F10 tumor growth rate is highly aggressive even with the low tumor cell number inoculated for this work. Indeed, due to ethical issues, the model could not be maintained past the 18–19-day post-inoculation. However, it is impressive how fast tumor $CD8^+$ T cells acquire an exhausted phenotype on control and B16 lysate + CCH vaccinated mice. Regarding this, the experimental design used caused a big

difference on tumor size by the end of each experiment and the evaluation performed only at day 18-19 didn't allow the description of this progressive process. It is possible that the differences observed between treatments might be caused by the big differences on tumor size at the experimental end point, where bigger tumors might have little or no immune infiltration in comparison to smaller tumors. In addition, as exhaustion is an antigen driven process it is feasible that smaller tumors translate into less tumor derived antigens hence fewer exhaustion, so to resolve these questions the analysis at earlier times and at different time points is desirable. Nevertheless, as already mentioned, the fast growth rate observed on the B16-F10 model hinders its use when analyzing at earlier timepoints. Since TRIMELvax vaccination delays tumor cell growth rate early, at approximately day 11-12, analyzing these tumors implies technical difficulties due to low cell counts. Hence, the use of different, less aggressive tumor cell lines inoculations as for example MC38 murine colon carcinoma which has been previously used in tumor cell lysate-based vaccination, should be evaluated for future experiments [125]. Certainly, the use of less aggressive murine cancer models will allow a bigger therapeutic frame, favoring the use of different therapeutic schemes such as delaying the beginning of treatment. The B16-F10 model aggressiveness growth induced the use of a therapeutic scheme where mice are vaccinated just one day after tumor cell inoculation. When translating this therapy into the clinic this scenery is far from real. Most treated patients have already developed long termed malignant melanoma, hence the use of a longer animal model with a longer therapeutic frame is highly desirable and will also allow the study of long-term processes like memory responses and possibly metastasis.

Anti-PD-1 ICB therapies have shown the capacity to reinvigorate exhausted tumor infiltrating $CD8^+$ T cells. Indeed, as already mentioned, early exhausted $CD8^+$ T cells can be rescued and even present some phenotypic and epigenetic resemble to memory T cells after antigen clearance. This is a desirable effect as exhausted tumor infiltrating $CD8^+$ T cells are indeed tumor antigen specific T cells.

However, recent data has shown that acquiring the exhausted epigenetic program leaves traces that might alter future immune responses. Cancer vaccines like TRIMELvax aim to boost the immune response towards a specific antitumor effect.

However, the search for better ways to induce memory responses becomes crucial when dealing with chronic diseases such as cancer. Besides being long-lasting with some periods of latency, the antitumoral response must maintain a local response within the tumor site and at the same time search for new sites where metastasis might take place. Hence, the need for memory responses becomes highly relevant and processes like exhaustion, that might hinder this kind of response, must be avoided. TRIMELvax vaccination decreased the frequency of tumor infiltrating $CD8^+$ T cells with high expression of PD-1, and overall diminished the exhausted associated receptors expression on this population. This finding is remarkable considering that early hindering this process should lead to better and stronger memory T cell anti-tumor responses which is highly desirable.

The most striking result of this work was the finding that TRIMELvax vaccination also promoted the differentiation towards central memory cells rather than effector memory cells as seen on control and B16 lysate + CCH vaccinated mice (Figure 18). ACT experiments have shown that less differentiated T cells, such as central memory T cells, rather than more differentiated effector memory T cells, have better antitumor effects. In fact, a subset of mature T cells with similar phenotype to naive T cells ($CD44^{low}$ and $CD62L^{high}$) but high expression of stem cell antigen-1 (Sca-1), IL-2Rβ (CD122), B-cell lymphoma 2 (Bcl-2) and chemokine receptor CXCR3 (CD183), exhibit the stem cell-like attributes of self-renewal, multipotency and the ability to undergo asymmetric division, therefore called T memory stem cells (T_{scm}). This subset possesses high antitumor capacity and can be induced by Wnt-B-catenin signaling, promoting the induction of T cell factor 1 (TCF-1) activity [141–143]. Additionally, a tumor infiltrating naïve-like $CD8^+$ T cell population was observed with high frequency on TRIMELvax vaccinated mice (Figure 18.B). This was completely unexpected since it wasn't this work main aim, however it is coherent with the observation that TRIMELvax hindered T cell exhaustion at the tumor site. As already mentioned, exhausted T cells develop through a progressive process. Accordingly, a gene signature of redistribution from progenitor-like to terminal exhausted T cell states, described by the loss of TCF-1 expression has been described. This loss of expression was associated with poor survival in lung and other cancer cohorts supporting the importance

of the progenitor exhausted T cell pool maintenance [144]. Tumor infiltrating T cells present dynamic epigenetic, transcriptional, and metabolic states. It has been proposed that meanwhile tumor progression takes place, T cells loose progenitor-like properties to differentiate into effector T cells that later develop an exhausted state through strong and persistent antigen stimulation in addition to the hostile tumor microenvironment [145,146]. Interestingly, the ectopic expression of TCF-1 on $CD8^+$ T cells on murine models of lymphocytic choriomeningitis virus (LCMV) chronic infection and modified melanoma B16-F10 cells, ameliorated T cell exhaustion and promoted T stem-cell features, highlighting the relevance that TCF-1 expression has on maintaining T cell function and boosting antiviral and antitumor immunity [147]. Hence, further characterization of this naïve-like population and TCF-1 expression analysis is currently being done.

The existence of specific niches within the tumor site, where progenitor-like $CD8^+$ T cells locate in proximity to antigen presenting cells (APC) resembling tertiary lymphoid structures has been described. These data established a strong correlation between the presence of DCs and the number of progenitor-like T cells in human tumor samples from several types of cancer. Even so, the authors proposed that the decline of the T cell antitumor response is due to the failure of the establishment of these specific APC/progenitor-like $CD8^+$ T cells niches. Impeding T cell proper stimulation hindered the generation of terminally differentiated $CD8^+$ T cells in the tumor. Therefore, failure of niche establishment might be a new mechanism of immune evasion that requires further study [148].

Tim-3 has emerged as an important immune-checkpoint molecule, being studied in multiple types of cancer. Hence, its expression has been usually associated with T cells. However, it has been recently described that Tim-3 is also expressed on DCs, showing that the loss of expression promoted a strong antitumor response preventing the acquisition of a DCs immunoregulatory program and expanding the progenitor-like and effector T cell pool within the tumor site [149]. In addition, recent data showed that Tim-3 blockade enhances the ability of intratumoral conventional cDC1s to promote $CD8^+$ T cell effector function by increasing the proximity between these two immune populations, once again highlighting the necessity of cDC-$CD8^+$ T cell

niches in the tumor site [150]. Besides, it was described that tumor infiltrating progenitor-like T cells (expressing TCF-1) differentiated into effector T cells (that lacked TCF-1 expression) in a chemokine receptor CXCR6 (CD186) dependent manner. This colocalization allows activated cDCs to sustain the effector response by trans-presenting interleukin-15 (IL-15) to effector $CD8^+$ T cells prolonging their survival in the TME. Hence delaying or impeding the fast development of an exhaustion state [79]. Furthermore, it has been shown that during the tumor progression, $TCF1^+$ progenitor-like T cells not only maintain the T cell effector pool within the tumor site, but they are also present in tumor draining lymph nodes (TdLN). Indeed, is suggested that TdLN T cells are the developmental precursors of, and be clonally related to, their more differentiated intratumoral counterparts with a continuous flux of these cells towards the tumor site feeding this tumor infiltrating progenitor-like pool [151]. These TdLN progenitor-like T cells would be supported by the action of TdLN cDC1s. It was observed that as the tumor progresses, TdLN cDC1s decreased in number as the progenitor-like T pool decreased. Indeed, the use of FMS-like tyrosine kinase 3 ligand (Flt3L) + anti-CD40 treatment, which supports and promotes cDC development, drove an improved tumor-specific $CD8^+$ T cell response. Therefore, suggesting that during antitumor responses TdLN cDC1s keep a pool of TdLN $TCF\text{-}1^+$ progenitor-like $CD8^+$ T population that supplies the tumor with new progenitor-like $CD8^+$ T cells. Thereby, the decrease of this cDC1s population, as the tumor progresses, contributes to a failed antitumor response [152].

Overall, these results highlight the importance of TdLN during the antitumor response, specifically addressing $TCF1^+$ $CD8^+$ T cells interaction with cDC1s. Therefore, performing multiple analysis of the tumor and TdLN during TRIMELvax treatment will allow for a better understanding of the antitumor response promoted by this vaccination. In this analysis, not only the $CD8^+$ T cell should be observed, but also cDCs should be included.

Unfortunately, all studies about progenitor CD8 T cells and cDC1s interaction is quite recent, hence it was not addressed in this work, however it is currently being studied. Indeed, cDC1s have a key role to establish a successful antitumor immune response. Previous published results showed that TRIMELvax vaccination induced

high frequency of tumor infiltrating cDC1s, but no information about this population and other tumor myeloid populations on B16 lysate + CCH vaccinated mice had ever been collected. Therefore, a broad analysis of these populations was performed to expand the knowledge of the tumor myeloid compartment immunomodulation due to vaccination (Figure 20).

According with the previous results showing that both vaccines immunomodulated the tumor infiltrating $CD8^+$ T cells to opposing phenotypes far from the results obtained when analyzing PBS vaccinated mice (control), it was expected to observe differences in the tumor myeloid compartment due to the described relevance these populations have on the tumor microenvironment and on tumor infiltrating $CD8^+$ T cells. Indeed, striking differences were observed in the tumor myeloid compartment clustering from vaccinated versus control mice as shown in the UMAP visualization (Figure 21). Data clustering using phenograph flow jo plug-in showed the existence of 12 different clusters which had different frequencies among different treatments. Both control and B16 lysate + CCH tumors from vaccinated mice showed events distributed among all the 12 different clusters, showing a wide diversification of this compartment. Although they have different frequencies, both treatments showed a similar cluster distribution having representation on all 12 clusters, distinct to what was observed on TRIMELvax treated mice where most of the events were distributed mainly on clusters 1 and 3 (Figure 22). However, due to the complexity of the tumor myeloid compartment, it was not possible to suggest specific immune populations from the expression of the parameters measured using this analysis. Hence, using manual gating analysis, as shown in figure 23, it was possible to determine tumor associated neutrophils (TANs), monocytes, monocyte derived DCs (moDCs), tumor associated macrophages (TAMs) and conventional DCs frequencies (Figure 24). Unfortunately, because of technical issues, it was not possible to determine cDC1s and cDC2s, but it is expected to repeat this analysis in the future. Additionally, due to low experimental number, only some of the populations analyzed showed statistical differences, however clear tendencies were observed.

It has been shown that an increase in circulating neutrophil numbers is an indicative of a worsened prognosis in several cancer types such as gliomas, lung, and

esophageal cancer [153–155]. Indeed, in a large cohort of advanced melanoma patients who received ipilimumab, baseline neutrophilia was significantly associated with an increased risk of death and disease progression [156]. Additionally, tumor cells can secrete chemokines, like IL-8, as well as GRO chemokines (CXCL1/2/3) and TGF-β to induce neutrophil migration to the primary tumor at early stages and then allowing their infiltration at advanced stages [157,158]. Neutrophils can promote tumor development through several mechanisms such as reactive oxygen species production, secretion of Matrix Metalloproteinase 9 (MMP9), Interleukin 17 (IL-17), Vascular Endothelial Growth Factor (VEGF) and angiogenesis induction among other effects [92,159–163]. Therefore, it was expected to observe a decrease in this population frequency on TRIMELvax vaccinated tumors in comparison to control mice. However, B16 lysate + CCH vaccinated mice, although not statistically significant, also showed a decrease in this population frequency when compared to control mice (Figure 24). This was unexpected, but it may be possible that in this setting, TANs may not have a critical role or that at this timepoint no differences can be observed. Beside tumor neutrophil recruitment, neutrophil polarization towards either a pro or anti tumoral phenotype should also be addressed. Therefore, analyzing TANs at an earlier stage and/or circulating neutrophils in TRIMELvax pre-clinical model is desirable.

In this regard, neutrophils can promote macrophage polarization and recruitment to inflammation sites by Interleukin 8 (IL-8), TNF-α and myeloperoxidase secretion [164,165]. Although direct interaction between TANs and TAMs in the TME has not been well established, data obtained from hepatocellular carcinoma suggests TANs can recruit TAMs to the TME promoting tumor progression highlighting the importance of this interaction [166]. Interestingly, a similar result to what was observed with TANs frequency was observed when analyzing TAMs frequency in the TME. Both TRIMELvax and B16 lysate + CCH vaccinated mice showed lower frequencies of TAMs when compared to tumors from control mice (only statistically significant when comparing B16 lysate + CCH vaccinated mice with control mice), which supports the hypothesis of a possible TANs-TAMs interaction in the TME (Figure 24). Additionally, the study of TAMs origin has shown that both tissue-resident macro-

phages and circulating inflammatory monocytes could be sources of TAMs development [46,167,168]. In this regard, monocytes are circulating cells able to migrate inside tissues in response to damage signals and can be recruited to the TME, where they locally mainly differentiate into: TAMs, tumor infiltrating DCs and myeloid derived suppressor cells [169]. Although no statistical differences were determined in tumor infiltrating monocyte frequencies between different treatments, it was suggested that TRIMELvax might diminish this population in comparison to B16 lysate + CCH vaccinated mice and control mice. This result is in accordance with what was observed when evaluating monocyte derived DCs, where TRIMELvax seems to decrease the frequency of this population. Although these results can only be suggested, they would agree with the hypothesis that TRIMELvax vaccination promotes an immunostimulatory tumor microenvironment rather than an immunosuppressive one as observed on B16 lysate + CCH vaccinated mice and control mice. Therefore, these experiments should be reproduced to determine if these differences are statistically significant.

Finally, tumor infiltrating cDCs frequency was evaluated (Figure 24). As already published, TRIMELvax vaccinated mice showed high frequencies of this population when compared to control mice (no statistical difference), but this increase was even higher when compared to B16 lysate + CCH vaccinated mice. Although it might have been expected to observe a lower frequency of cDCs on B16 lysate + CCH vaccinated mice due to the lack of antitumor response observed with this treatment, the fact that cDCs frequency was even lower than control mice was unexpected. Not only B16 lysate + CCH vaccination promoted PD-1 expression on tumor infiltrating $CD8^+$ T cells (Figure 14) but also it seems to diminish cDCs tumor infiltration.

Even though several differences were observed in the tumor myeloid compartment, the frequency of cDCs was the only parameter where a clear difference between TRIMELvax and B16 lysate + CCH was observed (Figure 24). Although it is not possible to determine possible differences between tumor infiltrating cDC1s and cDC2s in this analysis, previous published results showed that TRIMELvax promoted only an increase in cDC1s frequency. cDC1s are specialized on taking up tumor associated antigens and transport them to tumor-draining lymph nodes where they can

cross-prime anti-tumor $CD8^+$ T cells [65,74]. Additionally, cDC1 play a key role within tumors themselves by attracting T cells [67], re-stimulating and expanding tumor specific $CD8^+$ T cells [66] and supporting T cell effector function by IL-12 secretion [10]. Indeed, therapeutic strategies aimed to increase tumor infiltrating cDC1 abundance or activation may promote anti-tumor immunity potentially increasing cancer patients' responsiveness to immunotherapy [66,67,74]. Unfortunately, the mechanisms underlying cDC1 recruitment at the tumor site are still not totally clear. However, it has been described that natural killer (NK) cells that produce the cDC1 chemo attractants CCL5 and XCL1 promote cDC1 tumor infiltration. Remarkably, tumor cells can produce prostaglandin E2 (PGE2) which leads to evasion of the NK cell-cDC1 axis, partly by impairing NK cell viability and chemokine production, along with downregulating cDC1s chemokine receptor expression [170]. Unfortunately, previous published results showed no differences in tumor infiltrating NK cells in tumors from TRIMELvax vaccinated mice compared to control mice suggesting other mechanisms might be involved [125].

Since the aim of this analysis was to find possible mechanisms by which TRIMELvax could diminish tumor infiltrating $CD8^+$ T cell exhaustion, which was exacerbated on B16 lysate + CCH vaccinated mice, cDC population raised as an interesting target for further analysis. As previously mentioned, a recently described DC activation state termed "DC3"/" mREG" has been proposed to be a conservated state among different types of human cancers that when modulated might contribute to antitumor responses [70,78,79].

DC3s IL-12 production has been suggested one of their main characteristics when IL-4 signaling has been blocked. This was quite interesting since IL-12 signaling has been shown to diminish TOX expression [83]. However, during the experiments of tumor myeloid compartment determination, it was not possible to address any data about activation status. Hence, the next step was to evaluate the activation status of tumor infiltrating cDCs after vaccination, expecting that tumor infiltrating cDCs from TRIMELvax vaccinated mice should have a DC3-like phenotype.

Unfortunately, due to pandemic issues, it was not possible to evaluate this phenotype in the B16-F10 *in vivo* murine model, hence an *in vitro* approach was

used. Several strategies have been used to generate DCs *in vitro*, being the most used either GM-CSF generated BMDCs or FLT3-L generated BMDCs (FL-DCs). In collaboration with Dr. Fabiola Osorio, FLT3-L model was used to obtain cDCs with a closer resemblance to physiologically cDCs since GM-DCs had been reported to resemble monocyte derived DCs and macrophages rather than cDCs. FL-DCs cultures allow the segregation of 3 populations resembling: cDC1s, cDC2s and to a lesser extend plasmacytoid DCs (pDCs) [171,172]. Therefore, using FL-DCs approach, characterization of cDCs after vaccines stimulation was determined.

As seen on figure 25.A, FL-DCs were stimulated for 18 hours with; TRIMELvax, B16 lysate + CCH, LPS (positive control of activation) or NT (not treated). Additionally, to further characterize the role of each component of TRIMELvax vaccine, FL-DCs were stimulated with either lysates (TRIMELvax without CCH) or CCH. *In vitro* experiments had an important delay since technical issues were detected in the culture rooms and incubators. However, after a few attempts it was possible to perform this experiment. On figure 25.B, it is shown a representative zebra plot of the FL-DCs obtained after the 7-day bone marrow DC generation culture and before any stimuli is applied. It is possible to determine the generation of cDCs-like cells, which can be segregated into cDC1s (XCR-1^{+} expressing cells) and cDC2s (Sir1a^{+} expressing cells). To further characterize the activated DC3-like phenotype, induction of several key receptors has been described. Hence, CD86, PD-L1, CD40, CCR7 and IL-15R were evaluated after 18 hours of stimulation. The determination of the levels of expression of each receptor, using mean fluorescence intensity, was determined in the 3 populations evaluated: total cDCs (Figure 26), cDC1s (Figure 27) and cDC2s (Figure 28). For every parameter measured, LPS treatment showed the highest induction level when compared to unstimulated DCs (NT). In general, both vaccines and lysates treatment induced the expression of the different receptors analyzed when compared to NT condition but usually it was lower than those obtained after LPS treatment. This was expected since previous results had shown that CD86 is upregulated after stimulation using conditioned tumor cell lysates or TRIMELvax. Additionally, CCH treatment didn't promote the induction of any of the receptors evaluated being similar to what was observed in the NT condition. This was also expected

since the lack of CD86 induction after CCH stimulation on FL-DCs had previously been published [125]. In addition, cDC1s showed higher induction levels of PD-L1 and CCR7 when compared to cDC2s after stimulation using conditioned tumor cell lysates containing conditions (TRIMELvax, B16 lysate + CCH and lysates) and LPS. It is suggested that there is also a similar situation with CD40 induction levels, however, is not as clear as CCR7 and PD-L1 induction. Interestingly, as already mentioned, cDC1s are the main immune population to uptake tumor dead cells and perform cross presentation of tumor associated antigens to T cells. Therefore, it seems coherent that this cDC subpopulation had higher levels of activation, since previous published results had suggested cDC1s have higher phagocytic abilities when up taking conditioned tumor cell lysates *in vitro* [125]. Interestingly, although there was not a statistical significance when evaluating IL-15R expression, it is suggested that conditioned tumor cell lysate stimulation promoted IL-15R expression in cDC1s rather than cDC2s. Nonetheless with such a small experimental number (n=3) it is not possible to determine if there is a real effect mainly on cDC1s, hence increasing the experimental number would be interesting to address this phenomenon.

Additionally, the analysis of CD40/PD-L1 co-expression was performed as a hallmark for DC3 activation. Remarkably, results showed that conditioned tumor cell lysate have similar induction levels as LPS. Indeed, when evaluating IL-12 production which is a key cytokine for antitumoral responses, it is suggested conditioned tumor cell lysate also promoted IL-12 production being TRIMELvax and lysates condition the highest ones (Figure 29).

Therefore, these results confirmed previously published results from our group, indicating that conditioned tumor cell lysates have a strong stimulating capacity over murine and human DCs [117,125]. Also, it has been described that conditioned tumor cells lysates comprise several DAMPs such as ATP, HMGB1 and calreticulin which account for lysates DC maturation capacity [117,123,124]. Indeed, patients with polymorphisms in TLR4 showed lower levels of DC maturation after conditioned tumor cell lysate stimuli, indicating the importance of tumor cell lysates as DC maturation signals (DAMPs) providers as their role as sources of tumor associated antigens [173].

As seen on figures 26, 27, 28, 29 and 30, B16 lysate + CCH showed similar capacity on promoting DC maturation and possibly a DC3-like state as TRIMELvax *in vitro*. These results are not coherent with the fact that B16 lysate + CCH vaccination lacks antitumoral effect on the B16-F10 murine model. Hence, it may possible that *in vivo* results might show differences in their activation levels when compared to TRIMELvax. Additionally, B16 lysate + CCH vaccination does not promote tumor DC recruitment as seen on tumors from TRIMELvax vaccinated mice, hence suggesting that although cDCs may be activated with B16 lysate + CCH vaccination, other tumor DC recruitment signals are lacking or their migration and/or infiltration capacity is lower than cDCs from TRIMELvax vaccinated mice. As the TME from TRIMELvax compared to B16 lysate + CCH is clearly different as seen on most of the results showed using the *in vivo* approach, it is likely that the presence of other tumor immune populations might be relevant as already mentioned with NK cells. Therefore, this might be an interesting phenomenon to address in the future.

Overall, these results suggest TRIMELvax promotes a specific tumor microenvironment with low presence of immunomodulatory populations such as TANs and TAMs, and highly enriched on tumor infiltrating cDCs which might partly account for the striking differences observed on tumor infiltrating $CD8^+$ T cells phenotype. It is likely, that these differences on the TME promoted by TRIMELvax vaccination, might affect T cell differentiation promoting central memory and naïve-like T cells with possible progenitor features. In this regard, as new literature has shown the relevance of tumor draining lymphnodes in the maintenance of an effective antitumor response, by feeding the tumoral niche with progenitor $CD8^+$ T cells, it might be possible that TRIMELvax also immunomodulates the initial response occurring on this site. As the experimental design used for this work only analyzed tumor and LNs at day 18-20, it is highly desirable to, in future experiments, study the role of TdLN during TRIMELvax treatment, suggesting early analysis of TdLN might show interesting effects of vaccination that might account for the antitumoral effect of this vaccine.

Finally, these results unraveled possible ways by which TRIMELvax promotes an effective antitumor response. Additionally, using the B16 lysate + CCH vaccination, it was possible to determine how conditioned tumor cell lysates might promote

antitumoral activity, such as DC tumor infiltration and tumor infiltrating $CD8^+$ T cell phenotype. Additionally, this knowledge will be increased as a TRIMELvax clinical trial on advanced melanoma patients is now taking place. All the data obtained from this work helped in the selection of immune parameters evaluated on enrolled patients, hence contributing with the current work from our group.

As combinatorial immunotherapies have arisen as an interesting therapeutic approach for cancer patients due to this highly multifactorial disease. TRIMELvax might be an interesting candidate for this kind of combinatorial therapies. Although, when combined with anti-PD-1 treatment no improvement was observed [125], it is possible that other therapeutic strategies or other therapeutic schemes or even different cancer types might enable an enhancement of TRIMELvax antitumoral capacity making it an interesting field to work on.

XI. FINAL REMARKS

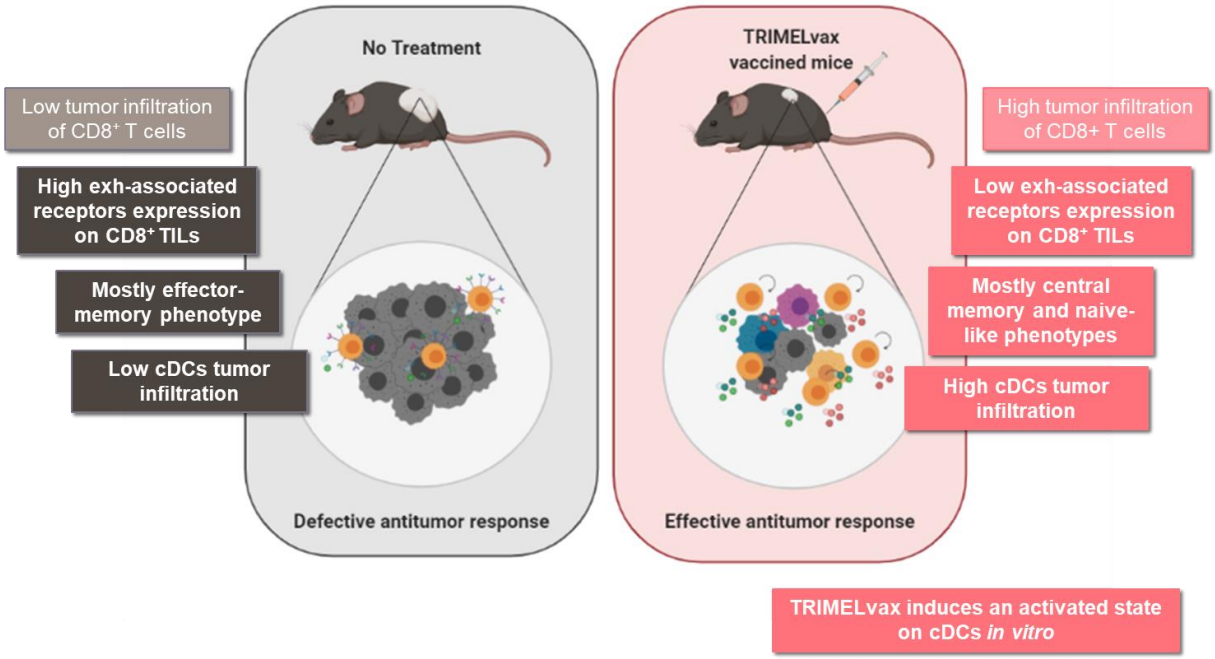

Figure 31. ***Results summary***

As shown on Figure 31, several conclusions were obtained from the present work and are described below.

1. TRIMELvax vaccination diminished the acquisition of an exhausted profile on tumor infiltrating $CD8^+$ T cells when compared to control PBS vaccinated mice.

2. The use of B16 lysate + CCH vaccination, as a “control lysate”, showed that tumor infiltration of $CD8^+$ T cells is not sufficient to promote an effective antitumor response. This vaccination in addition to inducing $CD8^+$ T cell infiltration, promoted a $CD8^+$ T cell exhausted phenotype in concordance with its lack of antitumor capacity.

3. TRIMELvax vaccination promoted tumor infiltration of $CD8^+$ T cells with a central memory or naïve-like phenotypes rather than effector memory phenotype as seen on control and B16 lysate + CCH vaccinated mice. This naïve-like population might have progenitor potential, hence further studies are taking place.

4. TRIMELvax vaccination modulated the tumor myeloid compartment by decreasing TANs, TAMs and possibly monocytes/mono DCs when compared to control mice. This is coherent since, TANs, TAMs and tumor infiltrating monocytes/mono DCs have been described to have pro tumoral effects.

5. TRIMELvax vaccination promoted cDCs tumor infiltration, as already published [125]. However, it was possible to determine that B16 lysate + CCH vaccination lacked this capacity in accordance with its absence of antitumoral capacity. This result might be associated with the $CD8^+$ T cell exhausted phenotype seen on these mice, since the relevance of cDCs on promoting $CD8^+$ T cell antitumor responses. However more studies are necessary addressing this interaction.

6. Conditioned tumor cell lysates promoted FL-cDC maturation *in vitro*, possibly inducing a DC3-like activation state.

Finally, TRIMELvax vaccination not only promoted DC and $CD8^+$ T cell tumor infiltration but also immunomodulated tumor infiltrating CD8+ T cells diminishing T cell exhausted phenotype and inducing a possible $CD8^+$ T cell progenitor-like population. Additionally, conditioned tumor cell lysates showed an interesting capacity to induce not only DC maturation but possibly a DC3-like activation state with high antitumor capacity. This activation might be relevant in the promotion of tumor infiltrating $CD8^+$ T cell progenitor like phenotype seeing on TRIMELvax vaccinated mice. Therefore, TRIMELvax might exert it antitumoral effect by modulating different immune populations which are relevant for an effective antitumor response (Figure 32).

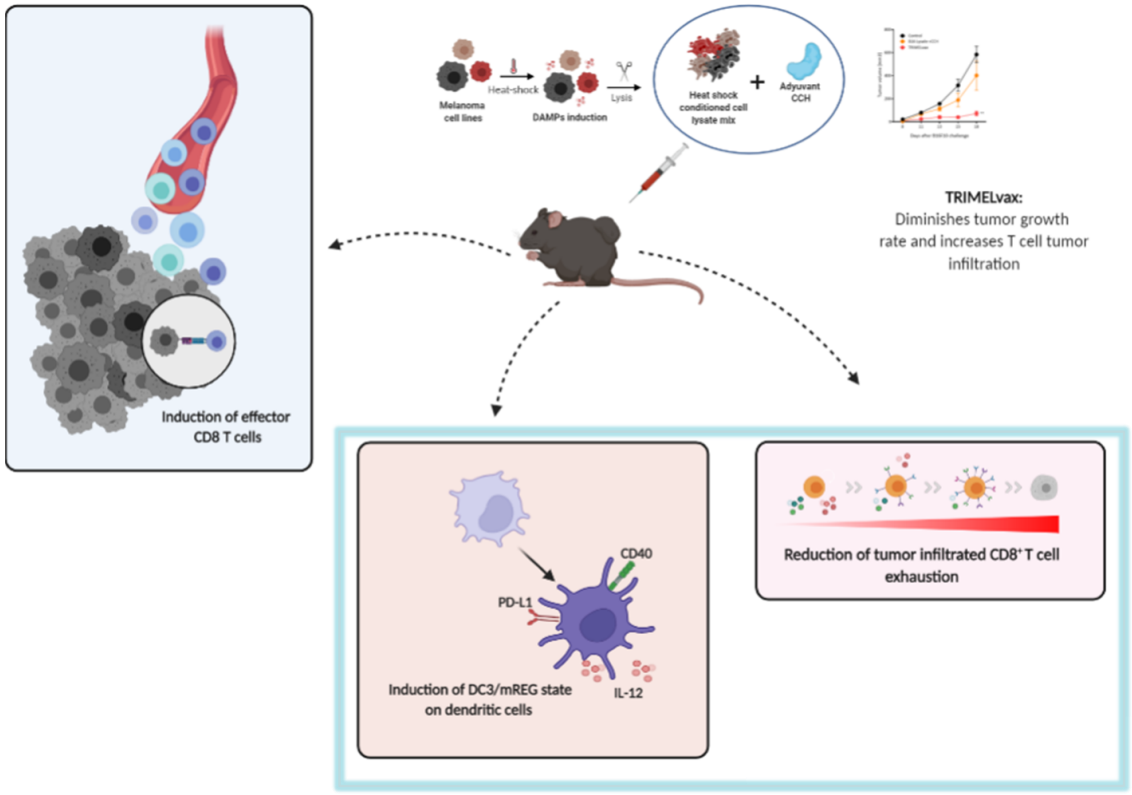

Figure 32. ***TRIMELvax exerts its antitumor effect by modulating different immune populations.***

In the present work TRIMELvax role in diminishing T cell exhaustion, and promotion of infiltration/ activation of cDCs was addressed. Additionally, it is suggested the $CD8^+$ T cell effector response might be also improved.

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XIII. JOURNAL PUBLICATIONS

- F.Flores-Santibañez , S.Rennen , D.Fernandez , C.De Nolf , S.Gaete , C.Fuentes , C.Moreno , D.Figueroa , A.Lladser , T.Iwawaki , MR Bono , S.Janssens, F. Osorio. “Activation of IRE1 RNase in dendritic cells curtails antitumor adaptive immunity in melanoma” In review.
- P. García-González, F. Tempio, C. Fuentes, C. Merino, L. Vargas, V. Simon, M. Ramirez-Pereira, V. Rojas, E. Tobar, G. Landskron, JP Araya, M. Navarrete, C. Bastias, R. Tordecilla, M. Varas, P. Maturana, A. Marcoleta, ML. Allende, R. Naves, M. Hermoso, F. Salazar-Onfray, M. Lopez, MR. Bono, F. Osorio. "Dysregulated Immune Responses in COVID-19 Patients Correlating with Disease Severity and Invasive Oxygen Requirements" Frontiers in Immunology, vol 12, 2021
- MA. Gleisner, C. Pereda, A. Tittarelli, M. Navarrete, C. Fuentes, I. Ávalos, F. Tempio, J. Araya, MI. Becker, F. González, M. López, F. Salazar-Onfray. “A heat-shocked melanoma cell lysate vaccine enhances tumor infiltration by prototypic effector T cells inhibiting tumor growth” Journal for Immunotherapy of cancer, 2020
- Flores, D. Hevia, A. Tittarelli, D. Soto, D. Rojas-Sepúlveda, C. Pereda, F. Tempio, C. Fuentes, C. Falcón-Beas, J. Gatica, F. Falcón-Beas, M. Galindo, F. Salazar-Onfray, F. González, M. López “Dendritic Cells Loaded with Heat Shock-Conditioned Ovarian Epithelial Carcinoma Cell Lysates Elicit T Cell-Dependent Antitumor Immune Responses In Vitro” Journal of Immunology Research, 2019

XIV. AWARDS

- **AAI/IUIS Travel grant:** Advanced Immunology course July 2020 (COVID-19 cancelled), Boston USA. Award defered to 2021 virtual course.
- **ANID Internship grant for doctoral studies 2021:** resign due to COVID-19 pandemic

XV. MEETING PRESENTATIONS

- ***“A Melanoma Vaccine based on Heat Shock Conditioned Tumor Cell Lysates Inhibits Tumor Growth and increases mice survival, even in the absence of anti-PD1 Treatment”*** M. Navarrete, M. Gleisner, C. Pereda, I. Ávalos, F. Tempio, C. Fuentes, D.Rojas-Sepúlveda, M.Becker, F. González, A. Tittarelli, M. López, F. Salazar-Onfray. CICON 2019, Paris, France. Poster presentation.

- **"*Unravelling the CD8$^+$ T cell response during cell lysate vaccine immunotherapy*"** *C.* Fuentes, M.Navarrete, C.Merino, F.Tempio, M.Gleisner, F.Salazar-Onfray, M.López. ASOCHIN Meeting, November 2019 (cancelled). Poster presentation.

- ***"Unravelling the CD8$^+$ T cell response during cell lysate vaccine immunotherapy"*** C. Fuentes, M.Navarrete, C.Merino, F.Tempio, M.Gleisner, F.Salazar-Onfray, M.López. Advances in immunotherapy, Keystone Symposia March 2020, Whistler, Canada (COVID-19 cancelled). Poster presentation.

- ***"Cell-Lysate vaccine immunotherapy induces a CD8+ T cell mediated response hindering the progressive transition to a dysfunctional state"*** C. Fuentes, M.Navarrete, C. Merino, F. Salazar-Onfray, M.N. López. 4th Annual meeting of the Chilean Society of Immunology, ASOCHIN, Virtual Conference, Santiago, Chile. Poster presentation.

A Melanoma Vaccine based on Heat Shock Conditioned Tumor Cell Lysate Inhibits Tumor Growth and increases mice survival, even in the absence of anti-PD1 Treatment

AUTHORS: M. Navarrete[1,2], MA. Gleisner[1,2], C.Pereda[1,2], I.Ávalos[1,2], F.Tempio[1,2], C.Fuentes[1,2], MI.Becker, F.González[1], A.Tittarelli[1,2], M.López[1,2], F.Salazar-Onfray[1,2]

INSTITUTIONS: [1]Millennium Institute on Immunology and Immunotherapy, Faculty of Medicine, University of Chile, 8380453 Santiago, Chile; [2]Disciplinary Program of Immunology, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, 8380453 Santiago, Chile.

Clinical immunotherapeutic strategies using immune-checkpoint blockers (ICB), such as anti-CTLA4, anti-PD1 or anti-PD-L1 antibodies, have demonstrated durable survival benefits in patients with melanoma and other solid tumors. Nevertheless, an important percentage of these patients remain refractory, suggesting that its combination with active immunization may improve this response rate. In this context, cancer vaccines become a complementary and attractive alternative for cancer treatment. Optimal delivery of antigens (Ags) and adequate use of adjuvants are crucial factors for vaccine success. Here, a prototype of a generic therapeutic vaccine for malignant melanoma treatment was tested in an experimental C57BL/6 murine melanoma preclinical model. This vaccine, named TRIMELVax is based on a heat shock-conditioned melanoma cell lysate (TRIMEL) combined with the *Concholepas Concholepas* hemocyanin (CCH) as a specific adjuvant. Vaccination with TRIMELVax has demonstrated to activate immune responses against B16F10 tumors in vivo, in both prophylactic and therapeutic approaches, inhibiting tumor growth and prolonging animals' survival. These immune responses were associated with increased $CD8^+$ T lymphocytes and dendritic cell tumor infiltration, in contrast to reduced neutrophils intratumor presence, measured by immunohistochemistry and multiparametric flow cytometry. Moreover, increased antibody production against tumors was detected by ELISA. Also, TRIMELVax delays tumor growth at comparable levels with anti-PD1 therapy. Remarkably, while anti-PD1 treatment doesn't increase survival of tumor bearing mice, TRIMELVax prolongs mice survival even in the absence of combinatorial therapy with anti-PD1 antidody. As TRIMELVax seems to be a potent vaccine against melanoma, this encourages the testing of this therapy in future clinical trials.

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Unravelling the CD8$^+$ T cell response during cell lysate vaccine immunotherapy

AUTHORS: C. Fuentes[1,2], M. Navarrete[1,2], C. Merino[1,2], F. Tempio[1,2], M. Gleisner[1,2], F. Salazar-Onfray[1,2], M. López[1,2].

INSTITUTIONS: [1]Faculty of Medicine, ICBM, Immunology Program, Universidad de Chile, Chile; [2]Millenium Institute of Immunology and Immunotherapy, Santiago, Chile.

In the tumor microenvironment, high and sustained antigen and inflammatory stimulation can lead to altered CD8$^+$ T cell differentiation inducing a dysfunctional state so-called exhaustion. This phenomenon affects the antitumor capacity of specific CD8$^+$ T cells against the tumor, inhibiting their action in the elimination of tumor cells. The rescue of this dysfunctional population has been described of great relevance in the success of several immunotherapies, such as checkpoint immune blockade therapies. In Chile, our laboratory developed an immunotherapy based on dendritic cells stimulated with lysates obtained from tumor cell lines, which has had successful results in patients. Recently, we have optimized this therapy using an innovative approach through the direct inoculation of a heat-conditioned melanoma cell lysate, in conjunction with the hemocyanin derived from the Chilean mollusk *concholepas concholepas* used as an adjuvant, called TRIMELvax vaccine. Our preclinical data shows that mice immunized with TRIMELvax show a marked decrease in the tumor growth rate in the murine melanoma model B16-F10. Although the use of this immunotherapy based on tumor lysates has shown to have successful results, the mechanisms of action behind this effect are not fully elucidated. Recent results using immunohistochemistry have shown that there is an important increase in the infiltration of CD3$^+$ and CD8$^+$ cells within the tumor. Further characterization of this CD8$^+$ T cell population using flow cytometry showed that immunotherapy based on tumor cell lysates induced an important decrease in the PD-1^{hi}Lag3$^+$ CD8$^+$ T cell population within the tumor, which has been associated to the phenotype of dysfunctional T cells. These results might suggest our therapy affects CD8$^+$ T cell population by diminishing the dysfunctionality of these cells, which might explain the anti-tumoral effect seen on immunized mice. Elucidating the effect of tumor lysate-based immunotherapy over the CD8$^+$T cell response will contribute to understanding the action mechanisms of this kind of therapies, helping to improve current treatments and predict future therapy outcomes.

Keywords: Immunotherapy, lymphocytes, melanoma

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XVI. ANIMAL ETHICS COMITTEE CERTIFICATE

VID INVESTIGACIÓN INNOVACIÓN CREACIÓN ARTÍSTICA
Vicerrectoría de Investigación y Desarrollo
UNIVERSIDAD DE CHILE

CICUA
COMITÉ INSTITUCIONAL DE CUIDADO Y USO DE ANIMALES

Santiago, 15 de Enero de 2020

Certificado Nº: **20344-MED-UCH**

CERTIFICADO

El Comité Institucional de Cuidado y Uso de Animales (CICUA) de la Universidad de Chile, certifica que en el Protocolo **CBA 1069,** del Proyecto de Investigación titulado: *"***Rol de una vacuna derivada de lisado condicionado de células de melanoma (TRIMELvax) en la promoción de una respuesta antitumoral mediada por linfocitos T CD8+",** de la Investigadora **Camila Fuentes**, Candidata a PhD en Ciencias Biomédicas y la Académica Responsable la **Dra. Mercedes López**, Profesora Asociada, ambas del Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, no se plantean acciones en sus procedimientos que contravengan las normas de Bioética de manejo y cuidado de animales, así mismo la metodología experimental planteada satisface lo estipulado en el Programa Institucional de Cuidado y Uso de Animales de la Universidad de Chile.

Las Investigadoras se han comprometido a la ejecución de este proyecto dentro de las especificaciones señaladas en el protocolo revisado y autorizado por el CICUA, a mantener los procedimientos experimentales planteados y a no realizar ninguna modificación sin previa aprobación por parte de este Comité.

Se otorga la presente certificación para el uso de un total de **357** ratones *Mus musculus*: **294** de la cepa C57BL/6 y **63** de la cepa Pmel-1, provenientes del Laboratorio Jackson (Bar Harbor, ME, USA), desde Marzo de 2020 hasta Marzo de 2022, tiempo estimado de ejecución del estudio, el cual será financiado por **Proyecto P09/16F Instituto Milenio de Inmunología e Inmunoterapia (IMII), Financiado con fondos ICM Minecom.**

*El CICUA de la Universidad de Chile, forma parte de la Vicerrectoría de Investigación y Desarrollo, y está constituido por 53 miembros: 5 médicos veterinarios, 39 académicos (12 de ellos médicos veterinarios), y 9 miembros no asociados a la academia o investigación, y que cuentan con experiencia en bioética relacionada a mantención y uso de animales. El certificado que emite el Comité procede de la aprobación del "**Protocolo de Manejo y Cuidado de Animales**" después de un estudio acucioso y de la acogida de los investigadores de las observaciones exigidas por el Comité.*

Comité Institucional de Cuidado y Uso de los Animales ★ CICUA VID ★ Universidad de Chile

Ronald Vargas Casanova
Director
CICUA – VID
Universidad de Chile

Dr. Emilio Herrera Videla
Presidente
CICUA - VID
Universidad de Chile