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CD8 T cell Activation in Cancer is Comprised of Two Distinct Phases

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CD8 T cell Activation in Cancer is Comprised of Two Distinct Phases

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An abstract submitted to the Faculty of the

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Abstract

CD8 T cell Activation in Cancer is Comprised of Two Distinct Phases

By Nataliya Prokhnevska

The CD8 T cell response to tumors is extremely variable with heterogenous T cell subsets, a stem-like CD8 T cell (PD1+TCF1+) that sustains the CD8 response and gives rise to a terminally differentiated (TD) cytotoxic cell (TCF1-Tim3+). Although these subsets have been described, how tumor-specific CD8 T cells are activated and differentiate in tumors are not well defined. Using a prostate cancer model that expresses the LCMV-GP (TRAMPC1-GP), we studied tumor-specific CD8 T cell activation by transferring LCMV-GP specific P14 CD8 T cells into tumor-bearing mice. We found that P14s are activated in the tumor-draining LNs (TDLNs) and acquire a stem-like phenotype. These cells migrate into the tumor as stem-like CD8 T cells and only differentiate into a TD CD8 T cell in the tumor. We found that stem-like CD8 T cells need additional co-stimulation from antigen presenting cells within the tumor to fully differentiate, even though they have been previously activated in TLDNs. Similarly, stem-like CD8s from human kidney cancer require both TCR and co-stimulatory signals to divide and differentiation *ex-vivo* and can differentiate when co-cultured with autologous dendritic cells. The addition of IL12 with TCR alone was not sufficient to induce differentiation, but improved differentiation when co-stimulation was present. This demonstrates the necessity of additional TCR and co-stimulation once activated stem-like CD8 T cells migrate into the tumor. Overall, these data suggest two distinct phases of CD8 T cell differentiation, the first occurs in the TDLN where they are initially activated. The second occurs in the tumor, where they require additional co-stimulation to differentiate and acquire an effector phenotype.

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INTRODUCTION

This chapter is reproduced with edits from Prokhnevska, N., Emerson, D.A., Kissick, H.T., Redmond, W.L. (2019). Immunological Complexity of the Prostate Cancer Microenvironment Influences the Response to Immunotherapy. In: Dehm, S., Tindall, D. (eds) Prostate Cancer. Advances in Experimental Medicine and Biology, vol 1210. Springer, Cham.

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CD8 T cells are a crucial part of the immune response against tumors. Many studies have described that CD8 T cells found within tumors acquire an exhausted phenotype. These exhausted cells lose their ability to proliferate, have increased expression of inhibitory receptors, and have lower effector function [1]. Even though these cells have varying degrees of functionality, CD8 T cell infiltration into tumors predicts disease progression in melanoma, breast cancer, head and neck cancer, ovarian cancer, non-small cell lung cancer, esophageal cancer, small cell lung cancer, hepatocellular carcinoma, and renal cell carcinoma [2-4]. Recently, a group developed an "immunoscore" for tumors based on T cell infiltration by looking at CD8+ and CD45RO+ T cells in both the tumor core and invasive margin. Higher immunoscores indicated more CD8 T cell infiltration and patients with higher scores had better disease-free survival and overall survival compared to patients with low immunoscores [5, 6]. The number of CD8 T cells in the tumor can also predict response to PD-1 blockade, making it an important biomarker for both survival and response to current immunotherapies [7]. Understanding the mechanism behind the diversity of CD8 T cell infiltration amongst different cancers, as well as within the same cancer type, is crucial to improve current immunotherapies. Additionally, recent studies have changed our understanding of CD8+ T cell differentiation and exhaustion in the context of chronic infections and cancer. Why there is such a wide range of CD8 T cell infiltration in tumors is currently not known. To better

understand CD8 T cell response to tumors, where they are unable to clear their antigen, it is important to consider how CD8 T cells effectively respond to acute viral infections. Using the CD8 T cell response to acute viral infections, we can define the differences between a productive CD8 T cell response to a tumor-specific CD8 T cell response.

Immune response to viral infections

The majority of our knowledge of CD8 T cell activation and effector differentiation has been established in models of acute viral infections that are dependent on the CD8 T cell response. Since CD8 T cells are essential to the clearance of acute viral infections, understanding how the immune system promotes a functional CD8 T cell response in viral infections is important to compare to the response to tumors. Virus-specific CD8 T cell responses have been extensively characterized, with well-established models of CD8 T cell activation and memory formation.

CD8 T cell activation and effector function

CD8 T cells are critical for viral clearance in response to an acute viral infection, for this to occur they must undergo antigen-specific activation, clonal expansion and effective killing of virally infected cells. During viral infections the innate immune system is critical for the processing and presentation of viral antigens to T cells. CD8 T cell activation is dependent on antigen-presenting cells (APCs) or dendritic cells (DCs) that present a viral peptide on MHC I. Each peptide-MHCI complex on APCs is specific for cognate T cell receptor (TCR) on a CD8 T cells. Activated APCs that are presenting peptide-MHCI must find an antigen-specific CD8 T cell that has a T cell receptor (TCR) specific for the peptide. Once APCs and their cognate antigen-specific CD8 T cells find each other, usually within secondary lymphoid organs, CD8 T cells require several signals to fully activate and acquire an effector program. The first signal that is necessary for CD8 T cell

activation is through the TCR by cognate peptide-MHCI complex provided by APCs. The second main signal necessary for T cell activation is co-stimulation, predominantly through CD28, which binds its ligands CD80/86 expressed by activated APCs. Naïve CD8 T cells require both TCR and co-stimulation to proliferate and upregulate cytotoxic molecules and produce cytokines [8]. These signals are only necessary for a short period of time, with only 2 hours of stimulation being sufficient for the activation program to be initiated. Activated CD8 T cells undergo many rounds of proliferation, increase expression of activation markers such as CD25 and CD69, and gain cytotoxic capacity rapidly after receiving TCR and co-stimulatory signals. Transcriptional and epigenetic changes also occur rapidly after CD8 T cell activation. Transcriptional changes occur as rapidly as 1 hour after peptide stimulation, before division has even occurred [9]. Epigenetic changes have also been shown to occur rapidly, with the CD25 promoter and IL2 getting de-methylated within an hour of CD8 T cell activation [10]. Importantly, when this program is initiated, consistent TCR signaling is not necessary, recently activated CD8 T cells continue to proliferate when transferred into antigen-free conditions [11, 12]. This demonstrates that the CD8 T cell activation program is acquired hours after initial antigen encounter and these CD8 T cells are programmed to clonally expand and acquire an effector program. It has been well established that both TCR and co-stimulation is necessary for CD8 T cell activation, but there are other signals that promote the acquisition of an effector phenotype and cytotoxic. The third signal that promotes CD8 T cell activation are numerous cytokines produces by APCs as well as T cells themselves. The major cytokines that are considered to promote the acquisition of an effector phenotype are IL2, IL12 and Type I IFN. One of the first molecules upregulated during CD8 T cell activation is CD25, or the IL2 receptor alpha, IL2 is a critical signal that is necessary for the acquisition of differentiated effector phenotype. Signaling through IL2 signaling through STAT3/5 induces the

expression of Blimp1. Blimp1 has been shown to promote effector and terminal differentiation represses TCF1 and memory related markers, while promoting the effector program [13-16] . CD8 T cells that are primed in the absence of co-stimulation and cytokines, with physiological levels of TCR stimulation, undergo poor proliferation and express lower amounts of cytotoxic molecules [17-19] . Overall, for a productive CD8 T cell response to viral infections, CD8 T cells must be activated by APCs that are presenting cognate peptide-MHC, express necessary co-stimulatory molecules, and cytokines. Once this occurs the antigen-specific CD8 T cells are programmed to rapidly expand and acquire an effector phenotype.

Once activated CD8 T cells are activated in secondary lymphoid tissues they must migrate into the compromised tissue to find cognate target cells and kill them. Activated CD8 T cells upregulate chemokine receptors such as CXCR3 and S1PR1, and downregulate CD62L, which allows them to leave the secondary lymphoid organs and migrate into tissue [20]. These activated CD8 T cells express numerous effector molecules such as granzyme B, perforin, and death ligands such as Fas and TRAIL. CD8 T cells selectively kill cells that express the MHC-peptide complex they are specific for, leaving uninfected cells intact while targeting only infected cells. When activated CD8 T cells encounter an infected and antigen-specific cell, the engagement of its TCR leads to the release of cytotoxic granules. Perforin polymerizes to form a pore on the target cell, allowing different granzymes to enter the target cell. Granzyme B is a protease which can activate caspase 3 and cleave BID. This induces an intrinsic apoptotic pathway as the activation of caspase 3 leads to DNA degradation and eventually apoptosis. The cleaved truncated BID also interacts with Bax and Bad, leading to the release of cytochrome C from the mitochondria, which also leads to an intrinsic apoptosis. Overall, the selective killing of infected cells by cytotoxic CD8 T cells prevents unnecessary inflammation and damage as the apoptotic cells are phagocytosed and cleared.

Activated CD8 T cells main function is to induce apoptosis of antigen-specific infected cells. In a viral infection when the antigen is cleared, some activated CD8 T cells survive and become memory CD8 T cells. CD8 T cells activated in response to acute viral infections are functional throughout the infection and undergo a contraction phase where 90-95% antigen-specific CD8 T cells die after the antigen has been cleared. The remaining cells seed the memory CD8 T cell pool, that have undergone an effector phase and retain the ability to rapidly expand and produce effector cytokines [1, 13]. This is an important function of CD8 T cells, since having long-lived memory to a pathogen leads to fast recall responses upon reinfection. In the context of progressively growing tumors, CD8 T cells do not become memory cells and instead gain an exhausted phenotype, upregulate expression of numerous inhibitory receptors, and lose their ability to proliferate.

CD8 T cells in cancer and chronic infections

The CD8 T cell response to acute viruses has been extensively characterized, but how CD8 T cells activate and differentiate in response to tumor antigens has not been well described. The CD8 T cell activation paradigm was established in acute viral models, where CD8 T cells rapidly expand and acquire effector function and can clear virally infected cells. In tumors, CD8 T cells lose both proliferative and effector capacity within the tumor. Understanding how the CD8 T cell response to tumors is different compared to viral infections will give us important insights into CD8 T cell biology. The immune response to cancer differs in how the immune system is activated, due to the chronic nature of tumor development as well as differences in the types of antigens to which the immune system responds. Increased CD8 T cell infiltration in tumors predicts better overall survival, but there is an extremely wide range of infiltration, why there is such a wide range of CD8 T cells within tumors is not clear. How CD8 T cells are activated in response to tumor and

how they are supported within the tumor microenvironment (TME) is important to understand in the context of clinical outcomes and future immunotherapies.

Progressive model of T cell exhaustion

The LCMV model has been used to discover and understand many immunological phenomena, spanning from CD8 T cell memory to exhaustion. Two strains of LCMV allow for the study of an acute versus chronic viral infection. LCMV Armstrong is an acute viral infection that is cleared through CD8 T cells and elicits a strong memory CD8 T cell response. The LCMV clone 13 strain models a chronic infection, and by depleting CD4 T cells and then infecting the mice with clone 13 the infection becomes truly chronic and is not cleared by the immune system [14, 21]. The early model of exhaustion described a gradual increase in inhibitory receptor expression such as PD-1 or Tim-3 and loss of function. CD8 T cells first lose their ability to produce IL-2 and cytotoxic function, followed by the loss of proliferation and production of IFN-y and TNF-a [1, 22]. PD-L1 blockade was shown to rescue antigen-specific exhausted CD8 T cells in chronic LCMV clone 13 infected mice, thus restoring their ability to proliferate and produce IFN-γ [23]. This was the first example of PD-1 blockade restoring CD8 T cell functionality in a chronic antigen setting.

The CD8 T cells that are found within tumors have the canonical exhausted phenotype. They have upregulated numerous inhibitory receptors, such as PD-1, CTLA-4, Lag-3 and Tim-3. They also have a diminished ability to proliferate and produce effector cytokines [24, 25]. This shows that tumor infiltrating CD8 T cells have a comparable phenotype to the antigen-specific CD8 T cells found in the chronic viral model of LCMV clone 13 infected mice.

While this model of T cell exhaustion offers an understanding of why T cells in tumors have lost functionality, it does not explain why some tumors have very low T cell numbers, or more importantly why some patients do not respond to checkpoint therapy.

Stem-model of CD8 T cell exhaustion

Work in the field of CD8 T cell exhaustion has caused us to update our model of how T cell exhaustion occurs. This work described two main populations of CD8 T cells in a chronic viral infection that both express PD-1. One is a stem-like CD8 T cell which expresses CXCR5, TCF1 and has higher expression of CD28 and the other is a terminally differentiated (TD) effector-like Tim-3+ CD8 T cell. The stem-like CD8 T cells reside in a DC-rich white-pulp of the spleen (Figure 3B), while the Tim-3+ subset localizes to sites of infection and is not restricted to lymphoid tissues, where those cells act as effectors who express granzyme B and IFN-y. The CXCR5+ TCF1+ stem-like CD8 T cell subset can self-renew and give rise to the Tim-3+ effector TD cells (Figure 3C). Recent work in the field of T cell exhaustion has updated this model further, by defining two populations of Tim3+ effector-like CD8 T cells. The first is defined as a transitory CD8 T cell which is defined by the expression of CX3CR1 and are both migratory and proliferative. These transitory cells express the highest amount of Gzmb and have maintained the ability to produce cytokines such as IFNg and TNFa. These transitory CX3CR1+ CD8 T cells give rise to the terminally exhausted subset, defined by CD101 and Tim3 expression. These cells have the lowest capacity to produce cytokines and have lost the ability to proliferate [26]. Our understanding of CD8 T cell exhaustion is constantly evolving, but the distinct function of a stem-like CD8 T cells and Tim3+ effector-like CD8 T cells greatly increased our understanding of how CD8 T cells respond to chronic antigens.

During PD-1 blockade the CXCR5+TCF1+ stem-cells proliferate and differentiate into more effector cells, inducing a large increase in antigen specific Tim-3+ CD8 T cells capable of killing infected cells. PD-1 blockade also affects the Tim-3+ CD8 T cells by blocking negative signaling and increasing their effector functions at the site of infection [27]. Recent work showing that PD-1 blockade promotes differentiation into CX3CR1+ transitory CD8 T cells, that have highest cytotoxic capacity [26]. This new model of CD8 T cell exhaustion helps us understand how the immune system responds to chronic antigen as well as how PD-1 blockade works within this model. Since this model was described in a chronic viral setting there have been many groups who have also discovered similar CD8 T cell subsets in mouse tumor models and in human tumor samples.

Stem-like CD8 T cells in cancer

A stem-model of T cell exhaustion has also been described in numerous cancers and mouse tumor models. A TCF1+ CD8 T cell that has stem cell properties has been described in MC38 sarcoma, B16 melanoma, TRAMP-C1 prostate cancer, Lewis Lung Carcinoma, and CT26 colon tumors [28, 29]. Likewise, a terminally differentiated Tim-3+ CD8 T cell was detected in these tumor models, paralleling the stem-model of exhaustion that was described in the context of a chronic viral infection. The cells that were found in mouse tumor models closely resemble CD8 T cells from chronic viral infection where the model was first described. The TCF1+ CD8 T cells have higher expression of IL-7R, CCR7 and CD62L similar to CXCR5+ CD8 T cells from LCMV Cl13. The effector TCF1-Tim-3+ CD8 T cells were also found to express higher levels of effector molecules such as IFN-y and granzyme B, which parallels the effector cells that were originally discovered in chronic viral infections. An important part of the stem-model of CD8 T cell exhaustion is the ability of TCF1+ CD8 T cells to proliferate as well as give rise to the Tim-3+ effector cells. the

Tim-3+ CD8 T cells lack the ability to proliferate but express more effector molecules. These studies demonstrate that the stem-model of CD8 T cell exhaustion can be applied to numerous mouse models of both chronic viral infections as well as tumor models.

The stem-model of CD8 T cell exhaustion is also translatable to human cancer. Many recent studies have shown that in different human cancers there are similar stem-like and effector CD8 T cell populations. In lung cancer patients, a CXCR5+ CD8 T cell population, and a CXCR5-Tim-3+ population was found by using high dimensional CyTOF analysis. These two subsets closely resemble what has been described in mouse models, where CXCR5+ CD8 T cells retain proliferative capacity and give rise to CXCR5-Tim-3+ effector CD8 T cells [30]. In melanoma, using single-cell RNAseq of the tumor a transitional and dysfunctional CD8 T cell subset was found. The transitional CD8 T cells from the tumors retain the expression of TCF1, while the dysfunctional CD8s have higher expression of inhibitory receptors such as PD-1 and Lag-3 [31]. We have identified the presence of a CD28+TCF1+Tim-3- stem-like CD8 T cell in prostate, kidney, bladder cancer and head and neck squamous cell carcinoma (HNSCC) [32, 33]. When these cells are sorted from human tumors and stimulated *ex-vivo* with anti-CD3/28, they undergo many rounds of proliferation, as they divide, they also upregulate Tim3 and Gzmb signifying their differentiation into a TD effector-like CD8. When the Tim-3+ CD8 T cell population was *in-vitro* stimulated with anti-CD3/28 they cannot proliferate, but still express high levels of effector molecules as was described previously in mouse models. This work also showed that CD8 T cell infiltration could predict patient outcomes, with higher CD8 T cell % of total cells predicting better progression-free survival than patients with tumors with low CD8 T cell infiltration. The Tim-3+ CD8 T cell population correlated better with total CD8% with the highly infiltrated tumors containing a large Tim-3+ CD8 T cell population (Carey paper). Due to the numerous potential

tumor antigens, and many MHC subtypes in humans it is extremely difficult to study tumor-specific CD8 T cell responses in human cancer. Recent work done in HNSCC, which is often caused by HPV infection, it was possible to study HPV protein specific CD8 T cells that allowed for the analysis of tumor-specific CD8 T cells of patients with known MHCI in human tumors. This work showed a distinct stem-like TCF1+ CD8 T cell within HNSCC tumors, as well as a Tim3+ TD population. When stem-like CD8 T cells were stimulated by cognate peptide *in-vitro* they could proliferate and differentiate to Tim3+ TD CD8 T cells and gain the expression of GzmB [33]. This work has shown that tumor-specific CD8 T cells are composed of the same stem-like and TD CD8 T cell subsets, that have been previously described in bulk activated CD8 T cells from other cancer types. These data contradict the current CD8 T cell exhaustion paradigm of inhibitory receptors signifying poor immune response to tumors. However, in the stem-model of CD8 T cell exhaustion, the presence of stem-cells that are proliferating and giving rise to terminally differentiated Tim-3+ CD8 T cells signifies a robust CD8 T cell response. When there are no effector Tim-3+ CD8 T cells present, either the stem-like CD8 T cell compartment doesn't exist or doesn't have the necessary signals to produce effector CD8 T cells. This model of CD8 T cell exhaustion has helped explain the variability of CD8 T cell infiltration in human cancer, as well as the lack of response to immunotherapy in many patients, but many questions remain unanswered.

Unanswered questions in tumor immunology

Through these numerous studies, it is evident that stem-like CD8 T cells are a crucial part of the CD8 T cell response to chronic antigen in both viral infections and tumors. These cells must be present within the tumor microenvironment to produce Tim3+ TD CD8 T cells that can kill tumor cells. Even though the stem-like CD8 T cell model of CD8 T cell exhaustion has been well

established in both mouse chronic viral infections (LCMV), tumor models and more recently in human tumors, how CD8 T cells acquire this stem-like phenotype has not been thoroughly investigated. This is especially important in the context of tumor immunology since many studies have now shown that stem-like and total CD8 T cell infiltration in tumors predicts patient survival and response to immunotherapy. Our current model of CD8 T cell activation in cancer is based on the cancer-immunity cycle [34], which implies that CD8 T cells are activated in tumor-draining LNs and then migrate into the tumor. This model does not consider the newly described stem-like CD8 T cell model of exhaustion. The development of stem-like CD8 T cells in response to chronic antigen is critical to support the overall CD8 T cell response. The new stem-model of CD8 T cell exhaustion raises questions which have not currently been addressed in tumor immunology, such as how CD8 T cells activate in response to tumor antigen and how CD8 T cells acquire a stem-like phenotype within the tumor. In the following thesis we aim to better understand how CD8 T cells are activated in response to tumors, and how the stem-like CD8 T cell response within the tumor is established.

PART 1

CD8 T cells activate in tumor draining lymph nodes to acquire a stem-like phenotype

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CD8 T-cell activation in cancer is comprised of two distinct phases

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**In press at Immunity*

ABSTRACT

This work aimed to understand the source of TCF1+ stem-like CD8 T cells in tumors. By studying human tumor draining lymph-nodes (TDLNs), we found a population of activated (PD1+CD45RA-) CD8 T cells that have not acquired a typical effector phenotype. Instead, these CD8 T cells share functional, transcriptional, and epigenetic traits with TCF1+ stem-like cells in the tumor. This suggests that these activated cells are a precursor to the stem-like CD8 T cells in tumors. By using mouse models, we show that tumor-specific CD8 T cells are activated in TDLNs and not within the tumor directly using a prostate cancer and melanoma mouse model. We compared the activation seen in TDLNs in response to tumor to the response seen to an acute viral infection of LCMV Armstrong. Even though in both models, antigen-specific CD8 T cells proliferate, upregulate activation markers, tumor-specific CD8 T cells fail to acquire a canonical effector CD8 T cell phenotype, transcriptional and epigenetic program. Overall, we have demonstrated that CD8 T cells are activated in TDLNs to acquire a stem-like phenotype, and fail to attain a canonical effector program, in both human TDLNs as well as mouse models.

INTRODUCTION

CD8 T cell infiltration in human tumors has been shown to predict overall survival as well as response to immunotherapy, but there is a wide range of CD8 T cell infiltration within tumors. Recent work that has described the stem-like CD8 T cell model of exhaustion has helped us understand possible mechanisms that control the differences in CD8 T cell infiltration. The stem-like CD8 T cell population is crucial to support the total CD8 T cell response within the tumor, since without this subset the overall CD8 response is diminished [27, 28]. Even though this subset is clearly important for the response to tumors, how these stem-like CD8 T cells are activated and how they establish the anti-tumor response is currently not known.

The current paradigm of CD8 T cell activation is based on the response to acute viral infections. This paradigm has been applied to cancer and is central to ideas like the cancer immunity cycle where T cells are primed in tumor draining lymph-nodes (TDLNs) and then migrate to tumors where they can kill cancer cells [34-37]. However, canonical T cell activation does not explain the occurrence of stem-like CD8 T cells that are found within the tumor. Stem-like CD8 T cells have encountered antigen, undergone many rounds of proliferation, but do not express cytotoxic genes like perforin or granzyme B. These cells remain in a relatively quiescent state, despite showing signs of ongoing TCR signaling [27, 28, 38]. Because this cell does not fit with ideas predicted by the standard T cell activation model, this study aimed to investigate how this TCF1+ stem-like CD8 T cell arises and generally how T cell activation in cancer compares to what decades of work has described in viral infections.

RESULTS CHAPTER 1

1. Activated PD1+ CD8 T-cells in human TDLNs are the precursor of stem-like cells in tumors

To understand how CD8 T-cells are primed in cancer we analyzed matching tumor and non-metastatic tumor-draining lymph-nodes (TDLNs) from patients undergoing surgery for prostate, kidney, and bladder cancers. As we had previously found in over 100 patients, there was a wide range of CD8 T-cell infiltration in these tumors [1], and the activated CD8 population was always comprised of both a TCF1+ stem-like and a TIM3+ terminally differentiated (TD) CD8 T-cell (Fig 1A, Fig S1A,B). Stem-like CD8 T-cells expressed high levels of CD127, CD28 and low levels of TIM3, CD39, and Granzyme B (GZMB) (Fig 1C). In comparison, the TCF1-TIM3+ TD population expressed high levels of CD39, GZMB and lower levels of CD28 and CD127 (Fig 1A,C). Due to their co-expression of TIM3 and CD39, we have used both markers interchangeably to denote the TD CD8 TIL population. When we analyzed TDLNs from prostate and kidney

tumors, we could identify a population of activated PD1+CD45RA- cells (Fig S1C). These cells expressed markers like CD69 and CXCR3 that distinguished them from naïve CD8 T-cells (Fig S1D). Most importantly, all these activated cells in the TDLNs were phenotypically similar to the stem-like cells in the tumor, having high expression of TCF1, CD127, CD28 and no expression of GZMB, CD39, TIM3 (Fig 1B,C, Fig S1D).

Based on the phenotypic similarity of these activated T-cells in TDLNs to stem-like cells in tumors, we were interested if these populations were clonally related. To test this, we performed TCR sequencing on activated CD8 T-cells from TDLNs (PD1+CD45RA-CD28+CD39-), and stem-like (PD1+CD39-CD28+) and terminally differentiated (PD1+CD39+) cells from matching kidney or prostate tumors. In every patient we detected TCR overlap between the activated T-cells from TDLN with the stem-like subset in the tumor (Fig 1D). Additionally, several TCRs found in the LN population were detected in both stem-like and TD CD8 tumor-infiltrating lymphocytes (TILs) from matching tumors implying a clear lineage relationship (Fig 1E). Importantly, PD1- CD8 T-cells sorted from the same TDLNS showed significantly less overlap or similarity with tumor populations (Fig S1E). Together with the phenotype data above, these data suggest a lineage relationship between these 3 populations of cells, where the PD1+CD45RA-TCF1+ cells in TDLNs, from here on referred to as LN-stems, appear to be the precursor to stem-like cells in the tumor, which then give rise to the TD CD8 TILs.

We were next interested if the LN-stem CD8s shared functional characteristics with stem-like cells in tumors. To determine if the LN-stem CD8 T-cells also share these functional roles, we labelled LN-stems with Celltrace Violet (CTV) and stimulated them *in-vitro* with anti-CD3/28/2 beads for 7 days. These cells all diluted CTV and upregulated GZMB, CD39 and TIM3, as we had previously

seen for stem-like CD8 T-cells isolated from tumors, suggesting a shared ability to proliferate and differentiate (Fig 1F).

Given the proposed relationship between LN-stem CD8 T-cells, stem-like and TD cells in tumors, we were interested in the transcriptional and epigenetic changes that occurred through these stages. To do this we first performed RNA-seq on sorted naive (CD45RA+ CCR7+), LN-stem, tumor stem-like and TD CD8 T-cells from kidney tumors and TDLNs. Principal component analysis (PCA) shows that all three subsets of activated CD8 T-cells clustered away from naïve CD8 T-cells, and the LN-stem CD8 T-cells clustered more closely to the stem-like population from the tumor (Fig 1G). Unbiased K-means clustering was performed to identify common and differential gene expression patterns in the data. The first cluster identified genes that were highly expressed in naïve CD8 T-cells and were progressively downregulated from LN-stems to tumor subsets. These genes were significantly enriched with genes downregulated during normal CD8 effector differentiation and enriched with genes downregulated in effector CD8 T-cells from both Yellow Fever (YF) and LCMV Arm (Fig 1H,I, Fig S1F). The second cluster contained genes that were progressively upregulated in LN-stems, further upregulated in tumor subsets. This second cluster was mostly enriched in genes found upregulated in effector CD8 T-cells from YF and LCMV Arm (Fig 1H,I). The last cluster identified genes that are upregulated in LN-stems but progressively decrease as they differentiate, such as *CXCR4 and CD28* (Fig 1H,I, Fig S1F). We also performed gene set enrichment (GSEA) analysis comparing the LN-stem to tumor-stem population found higher levels of IFN-beta signaling and many proliferative pathways active in the tumor-stem populations (Fig S1G). Finally, comparing LN-stem to terminally differentiated cells in tumors found energy production and cell cycle were upregulated in the TD cells (Fig 1H).

Whole genome DNA methylation analysis of these cell populations found a similar trend. Most epigenetic changes occurred between naïve and LN-stems, with 51,602 demethylated and 1,777 methylated regions compared to naïve CD8s. Epigenetic changes continue in the tumor subsets with 17,540 new regions demethylated in tumor stem-like TILs, and 3,935 demethylated regions occurring specifically in tumor TD TILs (Fig 1J, Fig S1I,J). These methylation changes clustered into similar patterns as what was found in the transcriptional analysis. The first cluster showed genes that are progressively methylated as CD8 T-cells differentiate. (Fig 1H,K, Fig S1H). For example, in Fig1K, there are two regions in the *TCF7* loci that get progressively methylated as LN-stems differentiate into stem-like and then TD CD8 TILs. The second cluster included genes that were progressively demethylated and transcriptional upregulated, such as *CTLA4*, *EOMES*, and *PRDM1* (Fig 1H, Fig S1H). The last cluster contained regions that were demethylated in the LN-stems and tumor stems, but then re-methylated in tumor TDs (Fig S1H). Together, these data suggest that CD8 T-cells in tumor draining lymph-nodes are generally more similar to the stem-like population in the tumor.

2. *Tumor antigen specific cells in TDLNs of mouse models are in a stem-like state*

Although our data suggests a two-step differentiation model, several other models of CD8 T-cell activation are possible. One plausible explanation for the observed data in Fig 1 is that naïve cells are initially primed in tumors and then only stem-like cells migrate back to lymph-nodes [30] (Fig S2A). Another possibility is that effector CD8 T-cell activation occurs first, and those cells de-differentiate to a stem-like phenotype later. A third possibility is that these subsets both activate in the TDLNs and migrate to the tumor independently (Fig S2B).

To better identify which of these models explains the origin of CD8 T-cell phenotypes in cancer we looked to study these cells in mouse models. We first examined if mouse models of cancer had

similar populations of CD8 T-cells to what we had found in patients. We first examined TRAMPC1 tumors that expresses the LCMV glycoprotein (GP), (TRAMPC1-GP) and used both GP33 (LCMV GP epitope) as well as the endogenous SPAS1 antigen [31] to analyze the tumor specific CD8 T-cell response in TDLNs and tumors. Tumor-specific CD8 T-cells were present in both tumor and TDLNs and had an activated CD44+Pd1+ phenotype (Fig 2A, Fig S2D). In the tumor, both GP33+ and SPAS1+ CD8 T-cells had stem-like (Tcf1+ Tim3-) and TD (Tcf1-Tim3+) phenotypes (Fig 2B). Both tumor-specific and bulk activated TD CD8 TILs had higher expressions of Gzmb, Ki67, Blimp1 while stem-like CD8 TILs expressed higher levels of CD127 (Fig 2C). When we analyzed B16-GP, a melanoma tumor that grows in C57BL/6 mice, and RENCA-HA, a kidney tumor that grows in Balb/c mice, the tumor antigen specific (GP33+ or PR8+) CD8 TILs contained the same stem-like and TD populations. In the TDLN of each mouse model, all of the tumor-antigen specific CD8 T-cells expressed high levels of Tcf1 and did not express Tim3 (Fig 2B, Fig S2E). The LN-stems upregulated Pd1, CD44, Ki67, downregulated Cd127 and overall expressed no effector associated molecules Gzmb, Blimp1 (Fig 2C, Fig S2F). These data show that in three separate mouse models across different mouse strains, tumor-specific CD8 T-cells in TDLNs are activated, retain Tcf1 expression, and do not express effector molecules like Gzmb, which parallels the phenotype we observed in human TDLNs.

3. T-cell activation in tumor draining lymph nodes is distinct from the response to a viral infection

Since these mouse models replicated what we found in human cancers, we designed an experiment to test which of the proposed models (Fig S2A-C) best explains how T-cell activation occurs in cancer. In this experiment we transferred CTV labeled tumor-antigen specific P14 T-cells into mice bearing B16-GP or TRAMPC1-GP tumors to track the kinetics and location of CD8 T-cell activation by division (Fig 2D). As a positive control to represent canonical T-cell activation, we

also transferred CTV labeled P14 T-cells into mice and then infected them with acute LCMV Armstrong (Arm). P14 cells responding to LCMV Arm in the spleen and axillary LNs (AxLN) adopted a typical effector CD8 T-cell activation program, upregulating Pd1, CD25, Gzmb and Prf and downregulating CD62L and Tcf1 (Fig 2E, Fig S2G). This phenotypic change was rapid and observed prior to the P14 cells even undergoing division. In comparison, in both B16-GP and TRAMPC1-GP TDLNs, P14 cells underwent many rounds of division and upregulated genes associated with activation such as Pd1, CD44, but failed to downregulate CD62L (Fig 2E, Fig S2H). When we compared the expression of Tcf1 and Tim3 on P14s activated in LCMV Arm AxLN to TDLNs, we found that in LCMV Arm P14s rapidly upregulate Tim3 and lose the expression of Tcf1. Importantly, in TDLNS, P14s never downregulated Tcf1 and never upregulated Tim3 in any of the early divisions.

We next wanted to test if CD8 T-cells in the TDLNs might be dividing and upregulating the various markers due to local cytokines from the tumor instead of exposure to tumor antigen. P14 cells transferred into TRAMPC1 bearing mice with no GP antigen remained naïve in TDLNs (CD44-Pd1-CD62L+), showing that the activation, upregulation of CD44 and Pd1, was antigen dependent (Fig S2I). We next considered if low amounts of antigen in TDLNs might be the reason for failure of CD8 T-cells to acquire effector function upon initial activation, as several studies have suggests amount or affinity of antigen is related to differentiation state [32-35]. To investigate, we transferred P14s into B16-GP bearing mice and injected (I.V.) 100ug of cognate GP33 peptide at the time of transfer and every other day for 5 days. P14s in mice treated with peptide expressed far higher levels of Pd1 and Irf4, both of which are driven by TCR signaling [36] (Fig S2J). Activated P14s in TDLNs remained Tcf1+ and failed to upregulate Tim3 even after 5 days of GP33 peptide treatment

(Fig S2K). This implies that lack of antigen is not the reason CD8 T-cells fail to acquire effector function within the TDLN.

Together, these data rule out models A and B in Fig S2 and only model C, where cells become stem-like cells on initial activation then migrate to the tumor fits with the data presented.

4. CD8 T-cells initially activated in TDLN fail to undertake a canonical effector transcriptional or epigenetic program

We next sought to understand the early transcriptional and epigenetic events in these tumor-specific CD8 T-cells and how they compare to a canonical acute viral effector program. To do this, we sorted activated undivided P14s as they underwent division in response to LCMV Arm, in the spleen, or TRAMPC1-GP tumors in TDLNs (Fig 3A). PCA analysis found that P14s activated in LCMV Arm and in TDLNs cluster separately (Fig 3B), and cells were mostly similar between the undivided and later divisions. P14s responding to LCMV Arm immediately acquired an effector transcriptional program before even undergoing division, with high expression of effector molecules *Ifng, Tnf, Gzmb,* inhibitory receptors, *Pdcd1, Havcr2, Ctla4, a*nd transcription factors related to terminal differentiation such as *Prdm1* and *Tbx21.* In comparison, P14 cells responding to the tumor underwent a completely different program. These cells failed to upregulate any of the genes usually associated with a typical LCMV Arm CD8 effector response (Fig 3C). *Gzmb* transcription, for example, went from 41 normalized counts in naive cells to 375,000 in LCMV Arm, but only to 1,200 counts in TDLNs. Other effector molecules like *Tnf, Il2* and *Ifng* followed a similar trend. Many transcription factors associated with the stem-program like *Tcf7* (TCF1) and *Bach2* all remained at high levels[37-40]. Using gene set enrichment analysis, we found that P14s from TDLNs significantly enrich for the tumor-specific stem-like program compared to P14s activated in LCMV Arm (Fig 3D). When these data were clustered

using K-means, 4 main clusters were found that showed differences in gene expression patterns between P14s from LCMV Arm and TDLNs (Fig S3A). Comparing these clusters to canonical effector signatures from LCMV-Armstrong, LM-OVA, and VSV-OVA infections using GSEA found that genes rapidly upregulated during activation in LCMV Arm enrich with genes upregulated in effector CD8 T-cells from these acute infections (Fig S3A). Finally, we performed pathway analysis using reactome gene sets that showed pathways related to interleukin signaling were upregulated in P14s from LCMV Arm, while pathways related to TCR signaling were upregulated in P14s from TDLNs (Fig S3B,C).

When we analyzed the DNA methylation changes of these cells, we found a similar trend. Even before undergoing division, activated P14 cells responding to LCMV Arm acquired many DNA methylation changes, with 3,228 methylated and 16,554 de-methylated regions compared to naïve cells. In comparison, P14 T-cells activated in TDLNs underwent far less epigenetic changes (Fig 3E,F, Fig S3D). We analyzed methylation changes further by unbiased K-means clustering and found two dominant clusters, with two other minor clusters (Fig 3G,H, Fig S3E). The first represented regions that were progressively demethylated in P14 cells responding to LCMV Arm and included genes that are highly expressed in P14s from LCMV Arm, such as genes related to effector CD8 T-cells like *Prdm1*, *Il2ra*, *Havcr2* (Fig 3G), as well as the *Gzmb*, *Ifng* loci shown in Fig3I,J. In comparison, no changes are found in these genes in activated P14 cells isolated from TDLNs. The second cluster identified the relatively small number of genes that became methylated in P14 cells responding to LCMV Arm but were unchanged in cells isolated from TDLNs. Importantly, *Tcf7* was methylated in LCMV Arm but remains demethylated in TDLNs (Fig 3H). Overall, these data suggest that the early priming conditions in TDLNs imprint an alternative transcriptional and epigenetic program on the tumor-specific CD8 T-cells and strongly suggests

that T-cells do not go through an effector stage in tumor draining lymph-nodes, but instead retain something similar to the stem-like program identified in other chronic antigen models.

DISCUSSION

The goal of this work was to investigate how CD8 T cells are activated in response to tumor antigens. We found that activated CD8 T cells within non-metastatic TDLNs in human kidney and prostate cancer maintain a stem-like phenotype and fail to upregulate canonical effector molecules. In humans, these activated undifferentiated CD8 T cells could exist in TDLNs for years and constantly seed the CD8 T cells found within the tumor. Using mouse models of both prostate cancer and melanoma we have been able to analyze tumor-specific CD8 T cell activation within TDLNs. Endogenous tumor-specific CD8 T cells in TDLNs of TRAMPC1-GP bearing mice maintain a stem-like phenotype and do not gain effector-associated molecules, such as GzmB, the same phenotype we have seen in activated CD8 T cells in human TDLNs. When we studied the early activation of antigen-specific CD8 T cells we found that unlike canonical CD8 T cell activation seen against viral infections, where T cells rapidly acquire cytotoxic molecules, tumor-specific CD8 T cells that are activated in TDLNs proliferate but fail to upregulate effector molecules. These activated tumor-specific CD8 T cells instead upregulate chemokine receptors and maintain high expression of co-stimulatory receptors. Antigen-specific CD8 T cells activated in LCMV Arm infection compared to TDLN, also have distinct transcriptional and epigenetic programs. Strikingly, the transcriptional program is acquired very rapidly during CD8 T cell activation, in both LCMV Arm and in TDLNs, with most of the transcriptional program acquired before division has even occurred. This corresponded to epigenetic changes we saw, with virus-specific CD8 T cells rapidly de-methylating genes associated with effector differentiation such as Gzmb and IFNg before division had occurred. Meanwhile tumor-specific CD8 T cells maintained

DNA methylation of genes associated with the effector program, and kept genes associated with the stem and naive programs de-methylated. The undifferentiated but activated transcriptional and epigenetic program is specific to tumor-specific CD8 T cell activation. Even though these cells have acquired an activated phenotype and undergone numerous rounds of proliferation they have failed to differentiate to acquire the effector program seen in response to an acute viral infection. The paradigm of CD8 T cell activation has assumed that all CD8 T cells must acquire an effector phenotype during the early stages of activation, we have shown that tumor-specific CD8 T cells that are activated do not undergo canonical CD8 T cell activation. Instead, these cells fail to upregulate effector molecules and maintain expression of markers associated with a stem-like CD8 T cell program.

Why CD8 T cells that are activated in response to tumor antigen, but do not undergo the canonical effector CD8 T cell activation is not clear. Work in chronic LCMV CL13 has also shown the development of stem-like CD8 T cells during the early stages of chronic viral infection [39]. Even though these cells are activated in response to a viral infection, a stem-like CD8 T cell is found within 5 days of infection, demonstrating that the early acquisition of a stem-like program is not specific to tumors alone. Whether these virus-specific stem-like CD8 T cells have gone through an effector phase is currently not known, and their early activation has not yet been described. Since the stem-like CD8 T cell is crucial for maintaining the CD8 T cell pool in both chronic viral infections as well as tumors, the development of this subset early during the response could be a crucial adaptation of CD8 T cells to maintain a pool of antigen-specific CD8 T cells during chronic antigen exposure. This stem-like CD8 T cell subset does not occur in response to an acute viral infection, which is rapidly cleared with a small percentage of cells becoming memory. Stem-like CD8 T cells arise in both chronic viral infections and tumors, both of which are a chronic source

of antigen, but tumor-specific CD8 T cells are activated to acquire this stem-like phenotype during the early stages of activation.

Even though the stem-like CD8 T cell subset occurs in multiple chronic antigen models, there are many differences in the environment within each of these models, acute virus, chronic virus, and tumor that could cause the distinct CD8 T cell lineages that have been observed. The lack of sufficiently activated APCs within TDLNs could be a potential mechanism for the stem-like CD8 T cell activation program. Since APCs must process antigen and migrate from the tumor to TDLNs to activate tumor-specific CD8 T cells, in viral infections APCs can become infected and activated rapidly in response to virus and viral TLRs. Lack of sufficiently activated APCs, that express high levels of co-stimulation and pro-inflammatory cytokines could maintain CD8 T cells in a stem-like state within TDLNs. Previous work on CD8 T cell activation has described the necessity of co-stimulation and cytokines for effector CD8 T cell activation and differentiation [8, 11, 12]. In TDLNs, where APCs have limited DAMP and PAMP activation signals, expression of co-stimulatory molecules and pro-inflammatory cytokines could be limited. In this case tumor-specific CD8 T cells are activated through TCR, with little co-stimulations and limited cytokines, which is sufficient to activate tumor-specific CD8 T cell and induce proliferation but is not enough to induce effector differentiation. Recent work has shown that when tumor-bearing mice are treated with both IL2 and IL12, tumor-specific CD8 T cells within TDLNs can differentiate and acquire a more effector-like TD CD8 T cell phenotype with loss of TCF1 and expression of Gzmb [40]. Overall, it is not clear if the acquisition of the stem-like CD8 T cell phenotype during activation in TDLNs is an effective CD8 T cell response to tumors and whether lack of sufficient co-stimulation or pro-inflammatory signals within the TDLN drives this CD8 T cell phenotype. Further studies are necessary to understand if lack of effector CD8 T cell differentiation within

TDLNs is an effective response to tumors, as well as why there is a lack of effector differentiation during activation in TDLNs.

MATERIALS AND METHODS

Human Sample Collection, processing, and flow staining

Tumor samples were collected after patients underwent partial or radical nephrectomy, prostatectomy or undergoing transurethral resection of a bladder tumor (TURBT). Tumor draining lymph nodes were collected directly after resection. Samples were collected immediately after tumor resection in Hank's Balanced Salt Solution. They samples were then processed by getting cut into small pieces, digested with a Liberase enzyme cocktail, and then homogenized using a MACS Dissociator, digested tumor was washed through a 70 um filter to get a single cell suspension. Red blood cells were lysed using RBC ACK Lysis buffer, fat was removed using a 44% Percoll/RPMI gradient, and samples were frozen in freezing media (FBS+10% DMSO) at -80C.

Single cell suspensions from processed human tumor samples were stained with antibodies from Table 1. Live/dead staining was done using fixable near-IR or aqua dead cell staining kit (Invitrogen). Cells were permed using the FOXP3 Fixation/Permeabilization kit (eBioscience) for 45 minutes with fixation/permeabilization buffer at 4C and stained with intracellular antibodies in permeabilization buffer for 30 mins at 4C. Samples were acquired on Becton Dickinson LSRII and Symphony instruments and analyzed using Flowjo (v10).

Human CD8 T cell RNA-seq and TCR analysis

Single cell suspensions from human tumor and TDLN samples were stained with antibodies, using near-IR live/dead cell staining kit to discern live cells. Populations were sorted on the Becton

Dickinson FACS Aria II Cell Sorter using the gating scheme shown in supplemental figure 16. DNA and RNA were isolated using the Qiagen RNA/DNA micro kit. RNA was used for TCR analysis and bulk RNA-seq. TCR libraries were made using the SMARTer Human TCR a/b Profiling Kit (Takara Biosciences) following the manufacturer's instructions. TCR libraries were sequenced using a Miseq. Complete T cell receptor V(D)J clonotype sequences were obtained using MIXCR. Aligned CDR3 regions and frequency of clonotypes were exported for further evaluation. In post processing steps, TCR analysis was performed using the immunarch R package (v 0.6.5) and custom R scripts. TCR beta chains were used for population overlap and diversity analysis, given its uniqueness to each cell and higher combinatorial potential. CDR3 amino acid sequences from sorted human TILS (CD28+ and CD39+) and LN PD1+ CD8 T cells were cross referenced with known published iNKT and MAIT cell TCRs [41-44]. The CDR3 regions used for the overlap of LN activated CD8s with CD28+ stem-like TILS did not contain CDR3 regions that match iNKT or MAIT cell TCRs [41-44].

RNA libraries were prepared using the clontech SMART-Seq V4 and sequenced HiSeq1000. Data was normalized and differentially expressed genes were determined using DeSeq. RNA-seq analysis was done using custom R scripts. Briefly, gene expression patterns were determined using K means clustering based on the fold change from naïve generated by Deseq. Normalized gene counts, heatmaps and volcano plots were generated using ggplot2 R package and excel.

Whole genome bisulfite sequencing (WGBS)

DNA was isolated using the AllPrep DNA/RNA Mini kit (Qiagen) and sonicated to generate random 300 to 500-bp fragments. DNA was end-repaired and A-tailed using the Hyper Prep Kit (KAPA Biosystems) following the manufacturer's protocol. Sequencing adapters that contained fully methylated cytosine residues (Integrated DNA Technologies) were ligated using the Hyper

Prep Kit (KAPA Biosystems). Adapter-ligated DNA was bisulfite converted using the EpiTect Bisulfite Kit (Qiagen), with a denaturation time of 10 min. Final libraries were PCR amplified 8–11 times using Tru-seq (Illunima)-compatible custom index primers, as previously described in [45], and HiFi Uracil (KAPA Biosystems). The resulting WGBS libraries were quality checked by Bioanalyzer, pooled at equimolar ratios and sequenced on a NovaSeq S4.

WGBS analysis

Sequenced data was aligned to the human or mouse genome using Hisat2 and analyzed using custom R and Python scripts. Fisher's exact test was used to define regions of statistically (p value less that $1x10^{-4}$) differentially methylated CpG motifs. Continuous regions of differentially methylated CpGs were identified by finding regions where at least 6 out of 10 CpGs in a continuous stretch were differentially methylated. These regions were then collapsed and analyzed as single 'differentially methylated regions' (DMRs).

Mouse tissue processing and flow staining

Tumor, axillary and inguinal TDLNs, and spleens were harvested and digested in Collagenase D (2 mg/mL) for 30 minutes in a shaker at 37C. Digested tissue was washed through a 70um filter using wash buffer (RPMI + 2% FBS) to produce a single cell suspension. Tumor and spleen were RBC ACK lysed and resuspended in FACS wash (PBS + 2%FBS + EDTA). Tumor samples underwent an additional step of a 44% Percoll/RPMI gradient to get rid of excess fat before staining. Mouse tissues were stained with antibodies listed in the antibody table. Extracellular staining was done in FACS wash for 30 min at 4C using antibodies described in Table 1. For intracellular staining the FOXP3 fixation/permeabilization kit (eBiosciences) was used. Cells were fixed in fixation/permeabilization buffer for 1-2 hours at 4C, and then stained with intracellular

antibodies in permeabilization buffer for 30 min at 4c. Data was acquired on the Beckton Dickinson LSRII and analyzed in Flowjo (v10).

Adoptive transfers and LCMV Armstrong infection

Spleens from CD45.1+ LCMV D^bGP33-specific TCR transgenic P14 mice were isolated. Cells were washed through a 70 um filter with wash buffer, RBC ACK lysed and resuspended in FACS wash. CD8 T cells were isolated using EasySep CD8 negative selection kit (StemCell). 1 mil P14 CD8 T cells were transferred intravenously directly after isolation, into either naïve CD45.2+ recipients or tumor-bearing CD45.2+ mice. For LCMV infection, 2.5 mil PFU of LCMV Armstrong was injected intravenously after P14 CD8 T cell transfer. For proliferation studies P14 CD8 T cells were CTV labelled at 1 ul of CTV per 1 million cells, and 1 million CTV labelled P14s were transferred intravenously.

Mouse T cell sorting for RNA and DNA methylation

Single cell suspension from processed mouse tissues were stained with antibodies described in Table 1 and sorted as shown in Figure S16. Tumor and TDLN samples were sorted on the Becton Dickinson FACS Aria II Cell Sorter. For RNA-seq and DNA methylation analysis, RNA and DNA were extracted using the Qiagen RNA/DNA micro Kit. RNA libraries were prepared and sequenced as previously described. Data was normalized and differentially expressed genes were determined using DeSeq. Data was then analyzed using custom R scripts and visualized using ggplot2 R package, as previously described. For Gene Set Enrichment Analysis (GSEA) each subset was analyzed using the pre-ranked list mode with 1,000 permutations. The gene sets that were used for GSEA and gene set enrichment were from available Reactome gene sets as well as

[14, 25, 27, 46-48]. Enrichment scores were visualized using the corrplot and ggplot2 R packages. Pathway analysis was visualized using Cytoscape.

FIGURES

Figure 1. Activated CD8 T-cells in human TDLNs have a similar phenotype to tumor infiltrating stem-like CD8 T-cells.

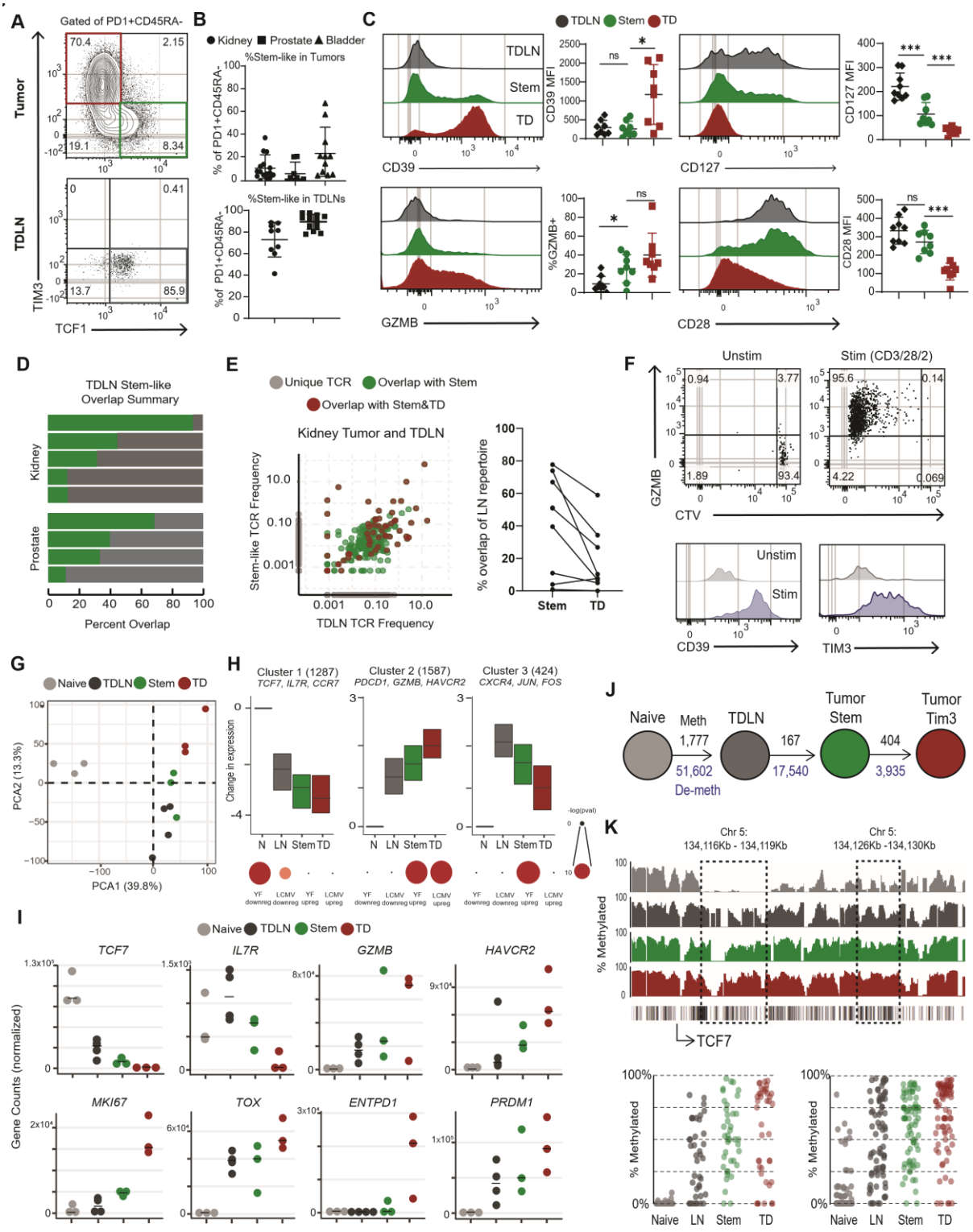

Figure 1. Activated CD8 T-cells in human TDLNs have a similar phenotype to tumor infiltrating stem-like CD8 T-cells. A) Representative plots showing activated CD8 T-cells in human tumor and tumor draining lymph nodes (TDLN). B) Summaries of the proportion of stem-like CD8 T-cells in human kidney (n=16), prostate (n=32), bladder (n=12) tumors and kidney (n=9), prostate (n=14) TDLNs. C) Phenotype of CD8 T-cell populations in TDLN and Tumor. D) Summary of TCR repertoire overlap between activated CD8 T-cells from TDLNs and tumor stem-like CD8 T-cells in human kidney and prostate cancer. E) TCR repertoire overlap in a representative patient between activated CD8 T-cells from TDLN, stem-like CD8 TILs, TD CD8 TILs. The proportion of the detected TCR clonotype in each patient that is unique or shared between the populations is shown. Summary showing the TCR overlap between stem-like and TD CD8 TILs as a proportion of the LN CD8 T-cell repertoire. F) Sorted CTV labelled activated CD8 T-cells from human TDLNs were cultured *in-vitro* with anti-CD3/28/2 beads for 7 days. G) PCA of RNA-seq of naïve CD8 T-cells, activated LN-stem CD8, stem-like CD8 and TD CD8 TILs H) Gene expression patterns from K-means clustering of all CD8 subsets. Gene set enrichment using genes from each cluster, compared to common effector CD8 T-cell signatures from Yellow Fever D14 and LCMV Arm I) Normalized gene counts showing expression of selected genes in the sorted CD8 T-cell populations. J) Schematic of the number of methylated regions that are at least 25% different from naïve in the TDLN and tumor CD8 T-cells populations. Black numbers show methylated regions, blue numbers show demethylated regions. K) Specific methylation changes in *TCF7*. Traces show total methylation in regions. Boxed regions show significantly differentially methylated regions. Dot plots showed methylation of each CpG in boxed region. Median and 95% confidence intervals (CI) are shown. *, $P<0.05$

Figure 2. Tumor-specific CD8 T-cells activate in TDLNs to acquire a stem-like phenotype.

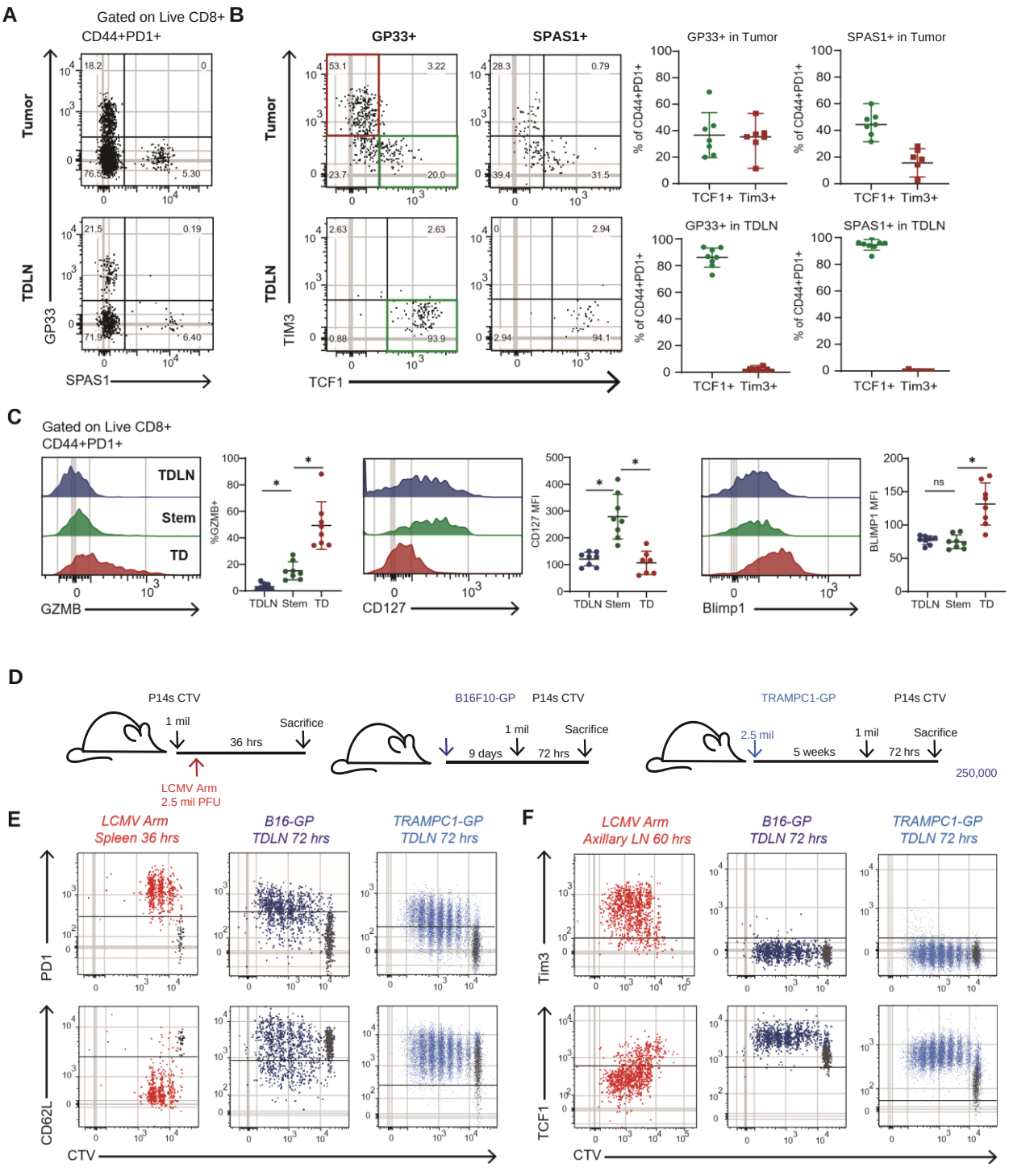

Figure 2. Tumor-specific CD8 T-cells activate in TDLNs to acquire a stem-like phenotype.

A) Representative plot of tumor-specific CD8s in 5 week TRAMPC1-GP tumors and TDLNs B)

Phenotype of CD44+Pd1+ tumor-specific CD8 T-cells in tumor and TDLN C) Phenotype of CD44+Pd1+ CD8 T-cell populations. D) Experimental set-up of early activation of P14 CD8 T-cells E) Flow cytometry of Pd1 and CD62L expression by CTV of P14s activated in each respective model F) Flow cytometry of TIM3 and Tcf1 expression by CTV of P14s activated in each respective model. Median and 95% confidence intervals (CI) are shown. *, $P<0.05$ determined by Mann-Whitney test.

Figure 3. Tumor-specific CD8 T-cells activated in TDLNs do not acquire an effector transcriptional or epigenetic program

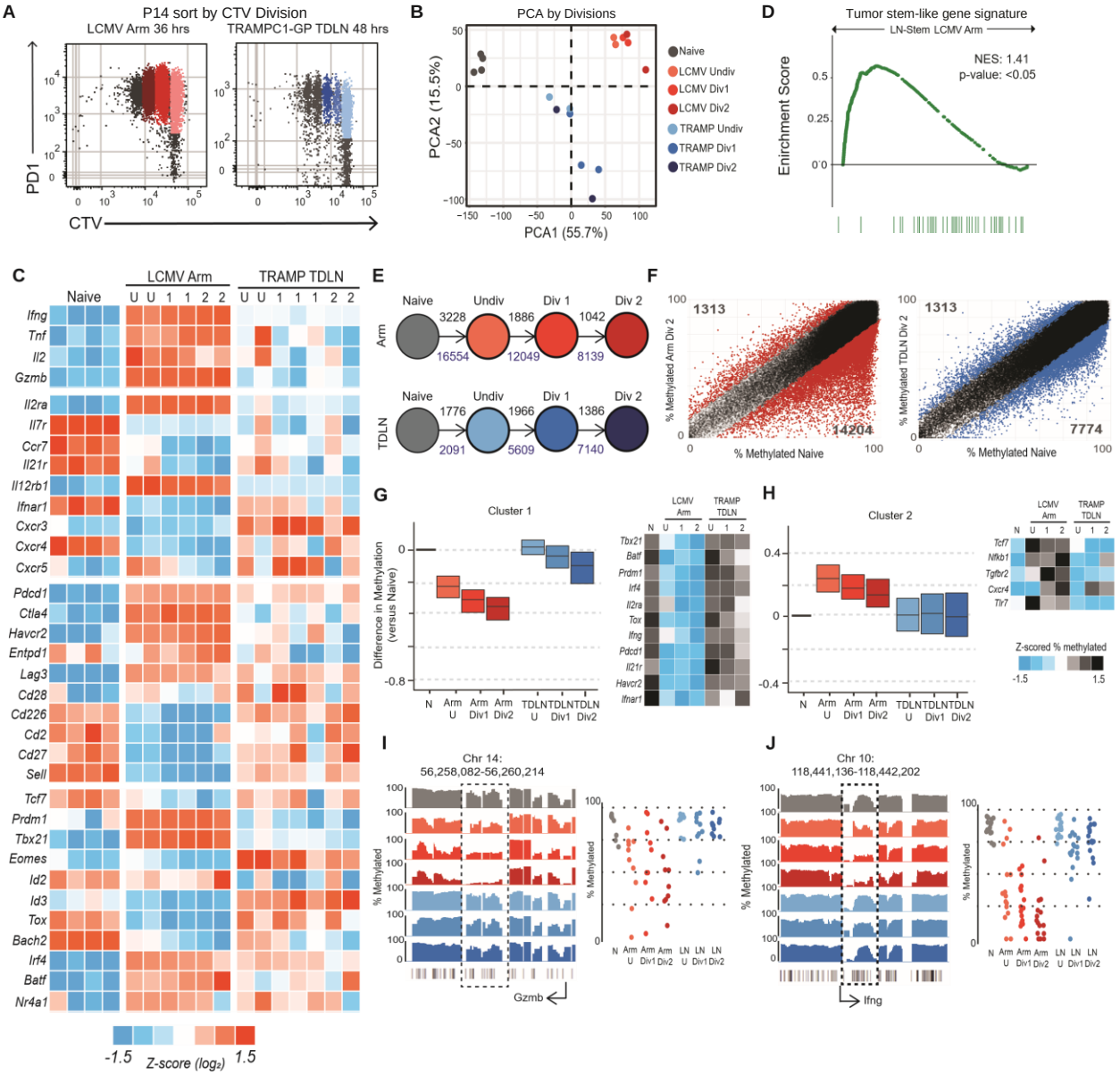

Figure 3. Tumor-specific CD8 T-cells activated in TDLNs do not acquire an effector transcriptional or epigenetic program A) Sorting scheme to isolate P14s by division in LCMV Armstrong spleen and TDLNs from TRAMPC1-GP bearing mice B) PCA of naïve P14s, P14s activated in LCMV Arm, and P14s activated in TRAMPC1-GP TDLNs by division C) Heatmap of z-score $\log_2$ expression of selected genes D) GSEA using the gene signature from mouse tumor-

specific stem-like CD8 T-cells, compared to P14s from LCMV Arm and TDLN. Enrichment score is plotted E) Schematic of number of regions with at least 15% difference in methylation from naïve P14s, as P14s divide, black numbers represent methylated regions, blue numbers represent de-methylated regions F) Regions of methylation in P14s Div2 from either LCMV Arm or TRAMPC1-GP TDLN plotted versus naïve P14s. Colored regions represent at least 15% difference in methylation compared to naïve P14s G) and H) Clustering using unbiased K-means of regions demethylated in P14s from LCMV Arm compared to naive, the same regions were plotted in P14s from TRAMPC1-GP TDLNs. Heatmap of z-scored %methylation of select genes is shown. I) and J) Traces show total methylation from 0 to 100% in regions near Gzmb and Ifng. Boxed regions show significantly differentially methylated regions. Dot plots showed methylation of each CpG in boxed region.

Figure S1. Human CD8 T cells are activated and acquire a stem-like phenotype in TDLN.

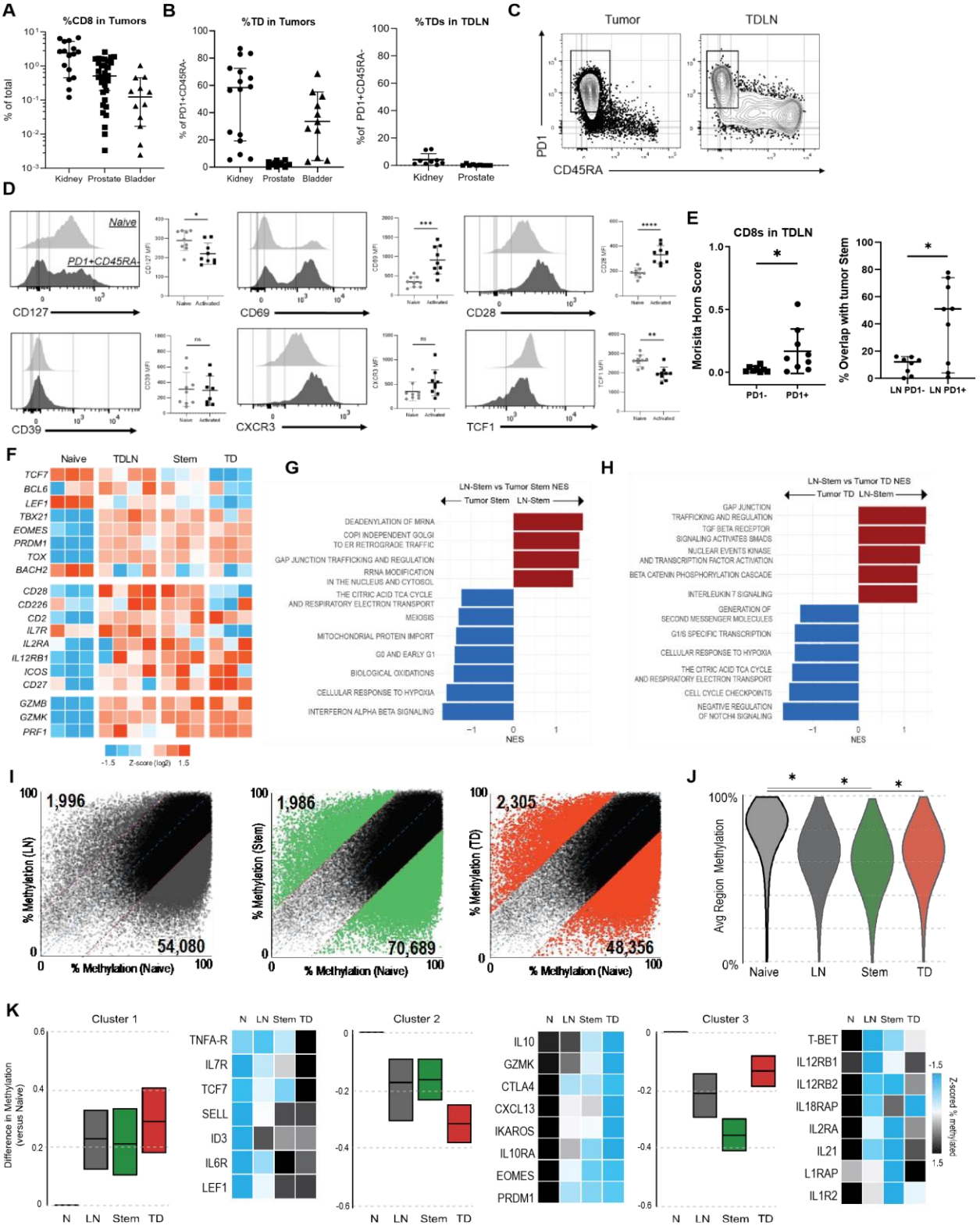

Figure S1. Human CD8 T cells are activated and acquire a stem-like phenotype in TDLN. A) Proportion of CD8 T cells in kidney, prostate and bladder tumors shown as percent of total cells (n=16, n=32, n=12). B) Proportion of activated (PD1+ CD45RA-) CD8 T cells that have a TD phenotype (CD28-TIM3+/CD39+) in tumors and TDLNs. C) Representative gating of activated CD8 T cells based on expression of PD1 and CD45RA in tumor and TDLN D) Expression of markers in naïve (PD1- CD45RA+) and activated (PD1+ CD45RA-) CD8 T cells in TDLNS. Summaries show geometric MFI. E) Morisita-Horn index between PD1+ and PD1- CD8 T cells from TDLNs to tumor infiltrating stem-like CD8 T cells within each individual patient. Summary of TCR repertoire overlap between activated CD8 T cells and non-activated PD1- CD8 T cells from TDLNs from kidney and prostate cancer. F) Heatmap of z-score $\log_2$ expression of selected genes in naïve, LN-stems and stem-like and TD CD8 TILs. G) and H) GSEA comparing LN-stem, tumor stem, tumor TD CD8 T cells subsets using the Reactome gene sets, normalized enrichment score (NES) is shown. I) Methylated regions in LN-stems, tumor stem-like and tumor TD CD8 T cells plotted versus methylation of naïve CD8s. Colored regions represent at least 25% difference in methylation. J) Global methylation changes of identified regions shown as violin plots K) Clustering using K-means clustering of differentially methylated regions in LN-stems, stem-like and TD CD8 TILs. Three main clusters are shown, with examples of genes in each cluster shown in heatmap of z-scored percent methylation of each gene.

Figure S2. CD8 T cells acquire a stem-like phenotype in the TDLN in an antigen dependent manner

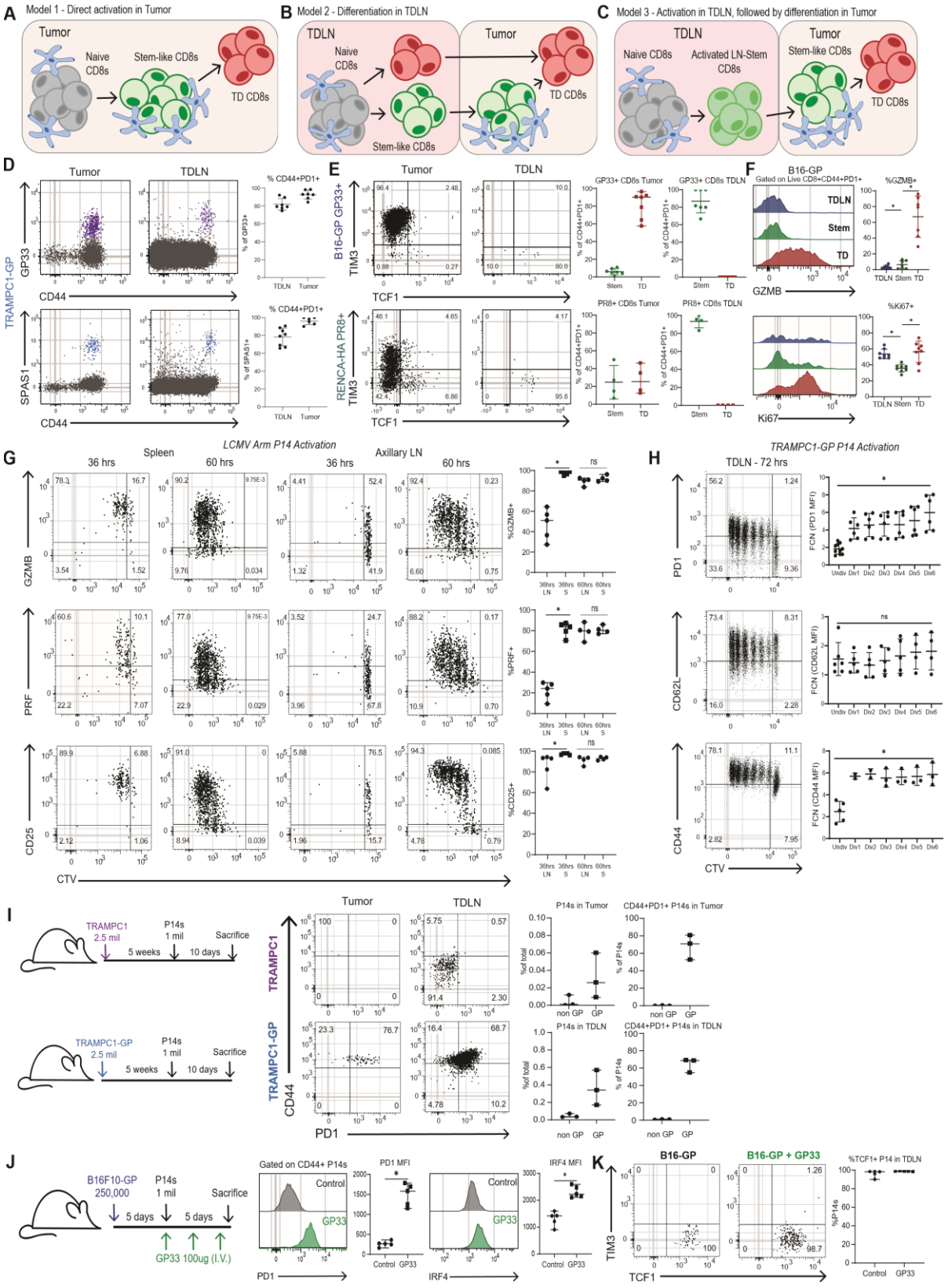

Figure S2. CD8 T cells acquire a stem-like phenotype in the TDLN in an antigen dependent manner A-C) Models of CD8 T cell activation in tumor D) Representative staining of tumor specific (GP33 and SPAS1) CD8 T cells in TDLN and tumors from 5-week subcutaneous TRAMPC1-GP tumor bearing mice. E) Representative TCF1 and TIM3 staining of CD44+PD1+ tumor specific (GP33+ top, and PR8+ bottom) CD8 T cells in the TDLN and tumors of B16-GP and RENCA-HA tumor bearing mice. F) Phenotype of CD44+ PD1+ CD8 T cell populations in B16-GP bearing mice. G) Kinetics of P14 activation in spleen and axillary LNs in LCMV Arm. H) P14 activation in TRAMPC1-GP TDLNs 72hrs post-transfer. I) Experimental layout of P14 transfer into TRAMCP1-GP and TRAMPC1 tumor bearing mice and phenotype of P14 cells 10 days post transfer in non-GP and GP bearing TRAMPC1. J) Experimental layout of GP33 treatment after P14 transfer into B16-GP tumor bearing mice. Pd1 and Irf4 expression of P14s in TDLNs K) Phenotype of P14s in TDLNs. Median with 95% confidence intervals (CI) are shown. *, $P<0.05$ determined by Mann-Whitney test.

Figure S3. Transcriptional and epigenetic program of activated CD8 T cells is acquired before division in both LCMV Arm and TRAMPC1-GP TDLNs

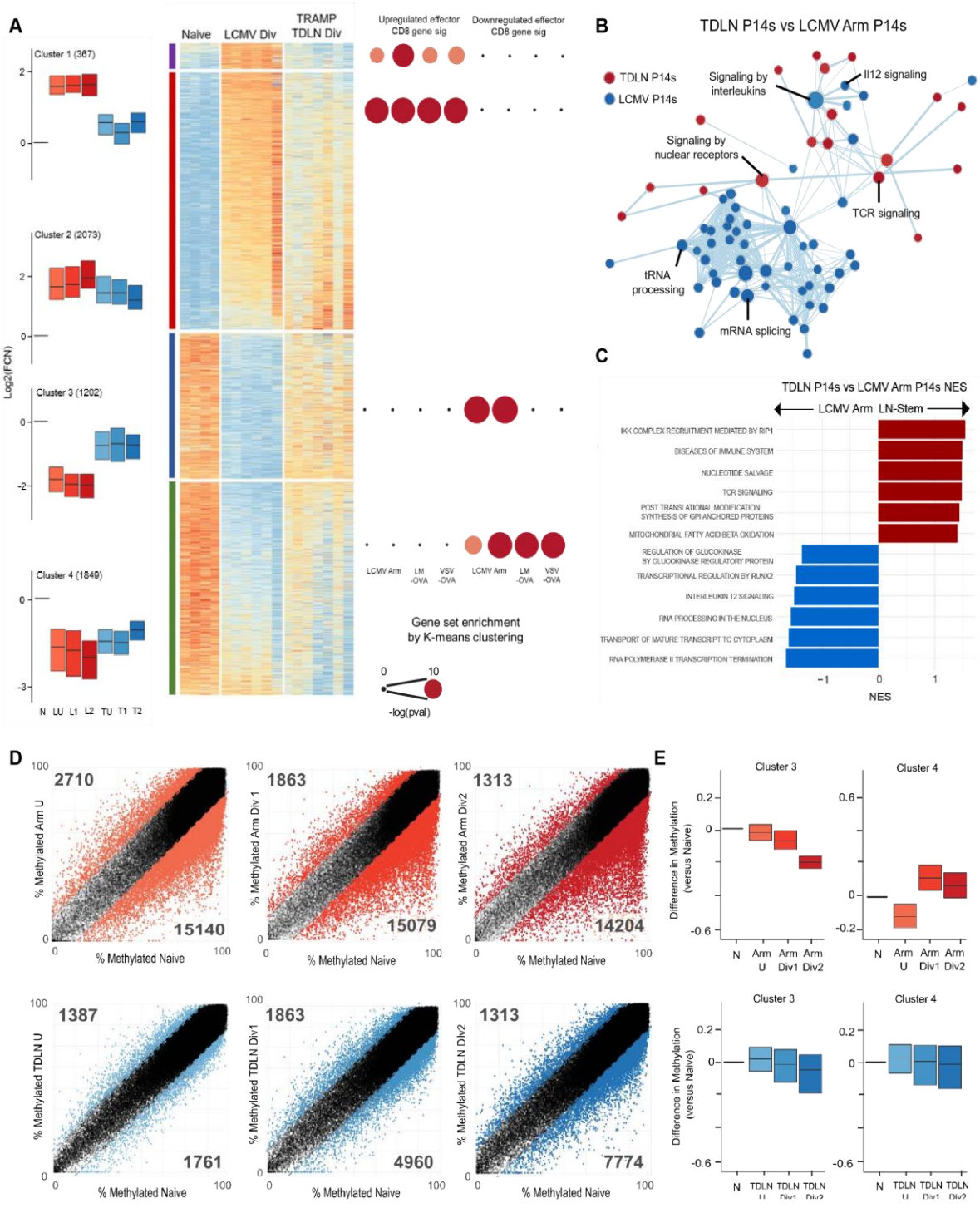

Figure S3. Transcriptional and epigenetic program of activated CD8 T cells is acquired before division in both LCMV Arm and TRAMPC1-GP TDLNs A) Boxplots and heatmap showing 4 main patterns of gene expression using unbiased K-means clustering of gene expression of P14s from LCMV Arm and TRAMPC1-GP TDLNs. Heatmap of log_2 expression of naïve P14s, LCMV Arm P14 divisions, TRAMPC1-GP TDLN P14 divisions. Gene set enrichment using genes from each cluster, compared to common effector CD8 T cell signatures from LCMV Arm, LM-OVA, VSV-OVA B) Pathway analysis of RNA-seq comparing P14s activated in LCMV Arm and TDLN. Circle size represents the number of genes in the gene set and length of connecting lines is proportional to how many genes overlap F) GSEA comparing P14s activated in LCMV Arm and TDLN using the Reactome gene sets, normalized enrichment score (NES) is shown. D) Regions of methylation in activated P14s from both models versus naïve P14s. Colored regions represent 15% methylation change compared to naïve P14s. E) Cluster 3 and 4 from unbiased K-means clustering analysis based on methylation of P14s in LCMV Arm, same genes plotted for P14s from TRAMPC1-GP TDLNs

PART 2

LN-Stem CD8 T cells give rise to tumor infiltrating CD8 T cells

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CD8 T-cell activation in cancer is comprised of two distinct phases

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**In press at Immunity*

ABSTRACT

This work aimed to understand the origin of stem-like CD8 T cells within the tumor. In previous studies we determined that tumor-specific CD8 T cells are activated in TDLNs, and not directly within the tumor. These activated CD8 T cells do not acquire an effector phenotype, but instead acquire the LN-stem program. We next wanted to determine if the tumor-specific CD8 T cells that are activated in TDLNs give rise to tumor stem-like and TD CD8 T cells. We found that these LN-stem CD8 T cells are functional, can produce cytokines and migrate into the tumor to establish the anti-tumor response as stem-like CD8 T cells. Only once they are in the tumor, did they differentiate into TD CD8 T cells and upregulate effector molecules such as GzmB and Prf. Overall, we have shown that the origin of stem-like CD8 T cells within the tumors is a pool of LN-stem CD8 T cells that are activated in TDLNs and only fully differentiate within the tumor.

INTRODUCTION

Recently described stem-like CD8 T cells in chronic infections and tumors have changed the way we view CD8 T cell exhaustion. Initially described in chronic LCMV Cl13 infection, these antigen-specific CD8 T cells have now been extensively studied in both mouse cancer models as well as human cancer. These stem-like CD8 T cells lack cytotoxic capacity but retain proliferative capacity and ability to differentiate to terminally differentiated effector CD8 T cells [27, 28]. Importantly, the stem-like CD8 T cell subset is crucial to sustain the CD8 T cell response to tumors and chronic viral infections, when the stem-like CD8 T cell population is lost the overall CD8 T cell response is also diminished [27, 28]. The importance of stem-like CD8 T cells within tumors is evident, but the origin of these cells within tumors has not been described.

There are numerous hypotheses on how the stem-like and TD CD8 T cells within the tumor originate. One plausible explanation is that the activated tumor-specific CD8 T cells migrate from TDLN to tumor to establish the response and differentiate within the tumor. Another plausible explanation is that tumor-specific CD8 T cells gain an effector phenotype gradually in TDLNs, after initial activation, and both subsets migrate into the tumor from TDLN. In the previous chapter we have shown that tumor-specific CD8 T cells are activated in TDLNs to acquire a stem-like phenotype. In human non-metastatic TDLNs, activated CD8 T cells also maintain a stem-like phenotype and fail to upregulate markers associated with terminal differentiation and effector function. We have found TCR overlap between these activated undifferentiated CD8 T cells in human TDLNs to both stem-like and TD subsets found within the tumor, suggesting that activated CD8 T cells in TDLNs could be precursors to the population found within the tumor. Using mouse models, we have also shown that tumor-specific CD8 T cells are activated in TDLNs and not within the tumor directly. These cells fail to acquire an effector program seen in canonical CD8 T cells differentiation in acute viral infections. These cells have undergone many rounds of division, upregulated activation markers but have maintained an undifferentiated transcriptional and epigenetic program. Where and when these recently activated but undifferentiated tumor-specific CD8 T cells acquire the effector program is not clear. Because tumor-specific CD8 T cells activated in TDLNs acquire a stem-like program, we sought to determine if activated tumor-specific CD8 T cells are the origin of CD8 T cell subsets within the tumor and where they undergo effector differentiation.

RESULTS CHAPTER 2

1. Tumor-specific CD8 T-cells migrate to the tumor in the stem-like state and only acquire effector function within tumors

Our data suggests CD8 T-cells responding to tumors in both humans and mice initiate an alternate program than what occurs in viral infections such as LCMV Arm. Based on these observations, we were interested in the kinetics of cells moving from TDLN to eventually establish the stem-like and TD cells in tumors. To investigate, we transferred P14s into either B16-GP or TRAMPC1-GP tumor bearing mice and analyzed TDLNs and tumors at varying timepoints post transfer (Fig 1A, Fig S1B,C). In both tumor models, P14 cells were found in TDLNs and maintained the LN-stem phenotype throughout the time course (Fig 1A,B), and were even detected 5 weeks after initial transfer (Fig S1A). Four days post transfer P14 T-cells could be detected in the tumors. At this early timepoint all the P14s in the tumor were Tcf1+Tim3-, suggesting that after activation in TDLNs they can establish the stem-like CD8 response within the tumor (Fig 1A). Between day 5 and 10 after transfer the P14s in the tumor begin to differentiate towards a TD phenotype (Tcf1-Tim3+) in both TRAMPC1-GP and B16-GP tumors, with a gradual increase in the relative frequency of terminally differentiated P14s in the tumor (Fig 1 A-B, S4B). P14s within the tumor maintain Ki67 and lose the expression of CD62L (Fig S1D). Together, these data show that CD8 T-cells move from TDLNs to tumor in the stem-like state, only once they are established in the tumor can they fully differentiate to acquire a TD effector phenotype.

One possible explanation for the alternate activation program in these tumor specific CD8 cells is that they were anergic or dysfunctional in some way. To test this, we first performed intracellular cytokine staining (ICCS) on these cells. Two weeks after transfer, P14s in both TDLNs and tumor

produced high levels of IFNγ, TNFα and IL2 after peptide stimulation (Fig 1C). We were next interested in determining if the tumor antigen specific cells in TDLNs had functional traits of stem-like cells; the ability to regenerate their own population and both the stem and effector populations in the tumor. To do this, we generated activated LN-stem CD8 T-cells in TDLNs. Two weeks post-transfer, CD44+Pd1+ P14s were sorted from TDLNs and transferred into a TRAMPC1-GP tumor bearing mice, who were treated with intra-tumoral CpG to promote migration of the transferred cells to the tumor (Fig 1E). The LN-stem cells repopulated the TDLN and were almost entirely found in the Tcf1+ state (Fig 1E-F). These cells did not express any Gzmb, Tim3 and retained expression of CD62L (Fig 1F-H). In comparison, the transferred cells that migrated to the tumor were found in both the Tcf1+ stem-like and Tcf1-Tim3+ TD states, further demonstrating effector differentiation only occurs within tumors (Fig 1F-H). Together, these experiments demonstrate that although CD8 T-cells primed in TDLNs do not undergo normal effector differentiation, they retain functionality and most importantly, retain the ability to re-generate their own population as well as the stem and effector populations within tumors.

2. The canonical effector transcriptional and epigenetic programs are initiated in tumors.

Given that tumor specific CD8 T-cell differentiation only occurs within the tumor, we were interested in how the transcriptional and epigenetic features of the LN-stem CD8 T-cells changed as they differentiated within the tumor. To do this we sorted LN-stem P14s from TDLNs, 7 days after transfer into established TRAMPC1-GP tumor bearing mice, and endogenous stem-like (CD44+Pd1+Tim3-CD127+) and TD (CD44+Pd1+Tim3+CD127-) CD8s from tumors. PCA analysis showed that P14s from TDLNs cluster away from both naïve, and tumor CD8 T-cells (Fig 1J), confirming that LN-stem P14s have a distinct transcriptional program compared to tumor CD8 T-cell subsets. For example, 912 genes were specifically upregulated in tumor stem-like CD8 T-

cells and 560 genes were upregulated in LN-stem P14s (Fig S1E). We next performed unbiased K-means clustering and found 5 distinct gene expression clusters. These cluster were used to perform GSEA on previously described effector CD8 T-cell gene sets. This analysis showed that clusters of genes that were highly upregulated in tumor subsets significantly enriched for effector CD8 T-cell signatures, demonstrating the acquisition of an effector program of CD8 T-cells within the tumor (Fig S1F). Pathway analysis indicated enrichment of co-stimulation, interferon signaling and chemokine signaling in the tumor stem population when compared to LN-stems, suggesting potential signals that might drive the differentiation in this location (Fig S1G).

We next performed whole genome DNA methylation analysis on LN-stem P14s from TDLNs at day 7 post-transfer and endogenous tumor stem and TD subsets. We used the previously defined cluster of genes that are demethylated in P14s activated in LCMV Arm (Fig 3H (Chapter 1)) to compare to both TDLN D7 P14s and tumor infiltrating subsets. P14 cells in TDLNs still had not epigenetically changed most of these loci. In comparison, once these cells reached the tumor, the stem and TD CD8 T-cells demethylated more genes associated with effector function and differentiation, (Fig 1K, Fig S1H,I), such as *Gzmb* and *Ifng* loci (Fig 1L). These transcriptional and epigenetic data demonstrate that LN-stem CD8s maintain an activated undifferentiated state and migrate into the tumor to establish the stem-like CD8 tumor subset. These cells then further differentiate into TD CD8 TILs to acquire the effector program.

DISCUSSION

In this work we have demonstrated that tumor-specific CD8 T cells activated in TDLNs are functional LN-stem CD8 T cells that migrate into the tumor. These cells migrate into the tumor 5 days after initial activation as stem-like CD8 T cells and establish the CD8 T cell response within the tumor. Once the stem-like CD8 T cell pool is established, they can differentiate to acquire an

effector-like TD phenotype, lose the expression of TCF1 while gaining effector associated markers. Importantly, activated tumor-specific CD8 T cells in TDLNs never differentiate into the TD subset and maintain a LN-stem phenotype throughout the timepoints in our analysis. Even though these cells are activated to acquire a stem-like program, they are functional and have the capacity to produce cytokines such as TNFa, IFNg and IL2 after *ex-vivo* peptide stimulation. They also retain the properties of a stem-like CD8 T cells. When LN-stem CD8 T cells were transferred into second tumor-bearing mice, they re-populate the LN-stem population as well as both tumor subsets in secondary recipient. Overall, we have shown that tumor-specific CD8 T cells that are activated in TDLNs have stem-like properties and give rise to both stem-like and TD CD8 T cells subsets within the tumor.

After activation in TDLNs, tumor-specific CD8 T cells do not acquire an effector phenotype until they have reached the tumor, which occurs several days later. This gradual acquisition of the effector program is not seen in acute viral infections, as antigen-specific CD8 T cells acquire an effector phenotype rapidly during activation. In contrast the CD8 T cell response to tumors occurs in two distinct phases. The first phase occurs in TDLNs, where tumor-specific CD8 T cells are activated to acquire a stem-like phenotype. Even though, these cells have undergone many rounds of division they have not acquired an effector phenotype, and maintain markers associated with the stem program such as TCF1. These cells then migrate into the tumor, to establish the response within the tumor as stem-like CD8 T cells. The second phase of activation occurs only within the tumor, where stem-like CD8 T cells differentiate to TD effector CD8 T cells. At this second phase the tumor-specific CD8 T cells acquire an effector phenotype, transcriptional and epigenetic program. At this stage of activation tumor-specific TD CD8 T cells lose the expression of TCF1 and gain the expression of effector markers such as CX3CR1 and GzmB. Why stem-like CD8 T

cells only differentiate within the tumor after initial activation in TDLNS remain unanswered. CD8 T cells acquiring an effector-like epigenetic and transcriptional program in the tumor implies something within the tumor microenvironment must drive this phenotypic change.

Work from other groups have shown that several signals contribute to stem-like CD8 T cell differentiation and acquisition of an effector phenotype in chronic viral infections and tumor models. One of the cytokines that has been shown to impact this differentiation is IL21. This cytokine is crucial to CD8 T cell differentiation specifically in environments with low levels of IL2, which occurs in both chronic viral infections as well as tumors. IL21 signaling can compensate for the lack of IL2 and induce transcription factors known to cause effector CD8 T cell differentiation such as Blimp1 [49]. Recent studies in tumor models have shown that IL21 produced by CD4 T cells aids the differentiation of CD8 T cells into the CX3CR1+ transitory effector state, that express the highest level of GzmB [50]. Since tumor-specific CD8 T cells in TDLN and tumors express very low levels of CD25 (IL2RA), IL21 is a potential mechanism that allows for stem-like CD8 T cells to overcome insufficient IL2 and acquire an effector program. This work clearly demonstrates that cytokines can play an important role for the differentiation of stem-like CD8 T cells within the tumor. Other cytokines that have been shown to promote CD8 T cell effector differentiation could also contribute to the differentiation of stem-like CD8 T cells within the tumor. Notable other cytokines include type I IFNs and IL12. These are usually expressed at high levels during acute viral infections and have been shown to promote effector CD8 T cell differentiation in these settings. Recent work in tumors has shown that IL12 from DCs is crucial for the response to anti-PD1 [51]. Other work has also been shown that the combination of IL2 and IL12 treatment can induce effector differentiation of tumor-specific CD8 T cells within TDLNs, and reduced tumor growth. Importantly IL12 has been shown to enhance effector

differentiation only in the presence of either IL2 or co-stimulation, demonstrating that there might be multiple signals within the tumor microenvironment that contribute to stem-like CD8 T cell differentiation. Cytokines have been shown to promote both stem-like CD8 T cell differentiation as well as effector differentiation in viral infections and must be considered as a possible signal within the tumor.

Lastly, other than cytokines, one of the major signals necessary for CD8 T cell activation and effector differentiation is co-stimulation [8, 17-19]. Tumor-specific CD8 T cells that have been activated to acquire a stem-like program, maintain high expression of numerous co-stimulatory molecules including CD28 in both mouse and human TDLNs. Lack of sufficient co-stimulation during T cell activation has been shown to induce anergy or tolerance, but in environments of low co-stimulation CD8 T cells can become activated. During chronic LCMV CL13 DCs express significantly lower levels of CD80 and CD86, compared to early stages of the infection. When naïve virus-specific CD8 T cells are transferred into this chronic LCMV CL13 environment, they activate but with a higher proportion of stem-like CD8 T cells. When mice were treated with CD3 and CD28 agonists, this phenotype was reversed and effector CD8 T cell differentiation was restored. The same phenotype was observed if chronically infected mice were treated with antigen pulsed activated APCs. This work demonstrates that lack of sufficient levels of co-stimulation can hinder effector CD8 T cell differentiation in chronic viral infections [52]. Additional work has also shown that response to PD1 blockade in both chronic viral infections and tumor models, is dependent on CD28 signaling. When CD28 signaling was blocked during PD1 blockade, antigen-specific CD8 T cells failed to expand and did not clear the viral infection or tumor [53]. This work shows the importance of CD28 signaling for both CD8 T cell effector differentiation as well as responsiveness to PD1 blockade. The signals that are directly affecting tumor-specific CD8 T cell

differentiation in tumors have not been well characterized. Most likely many signals contribute to the stem-like to TD CD8 T cell differentiation, and other cells such as CD4 T cells and APCs are necessary to provide these additional signals. Moreover, the distinct phases of CD8 T cell activation that we have described implies that signals necessary for this effector differentiation are present specifically within the tumors where CD8 T cells can gain their effector phenotype.

MATERIALS AND METHOD - ADDITIONAL METHODS IN CHAPTER 1

Mouse T cell sorting for RNA and DNA methylation

Single cell suspension from processed mouse tissues were stained with antibodies. Tumor and TDLN samples were sorted on the Becton Dickinson FACS Aria II Cell Sorter. For RNA-seq and DNA methylation analysis, RNA and DNA were extracted using the Qiagen RNA/DNA micro Kit. RNA libraries were prepared and sequenced as previously described. Data was normalized and differentially expressed genes were determined using DeSeq. Data was then analyzed using custom R scripts and visualized using ggplot2 R package, as previously described. For Gene Set Enrichment Analysis (GSEA) each subset was analyzed using the pre-ranked list mode with 1,000 permutations. The gene sets that were used for GSEA and gene set enrichment were from available Reactome gene sets as well as [14, 25, 27, 46-48]. Enrichment scores were visualized using the corrplot and ggplot2 R packages. Pathway analysis was visualized using Cytoscape.

Figure 1. Tumor-specific CD8 T-cells only acquire effector phenotype after migration into the tumor

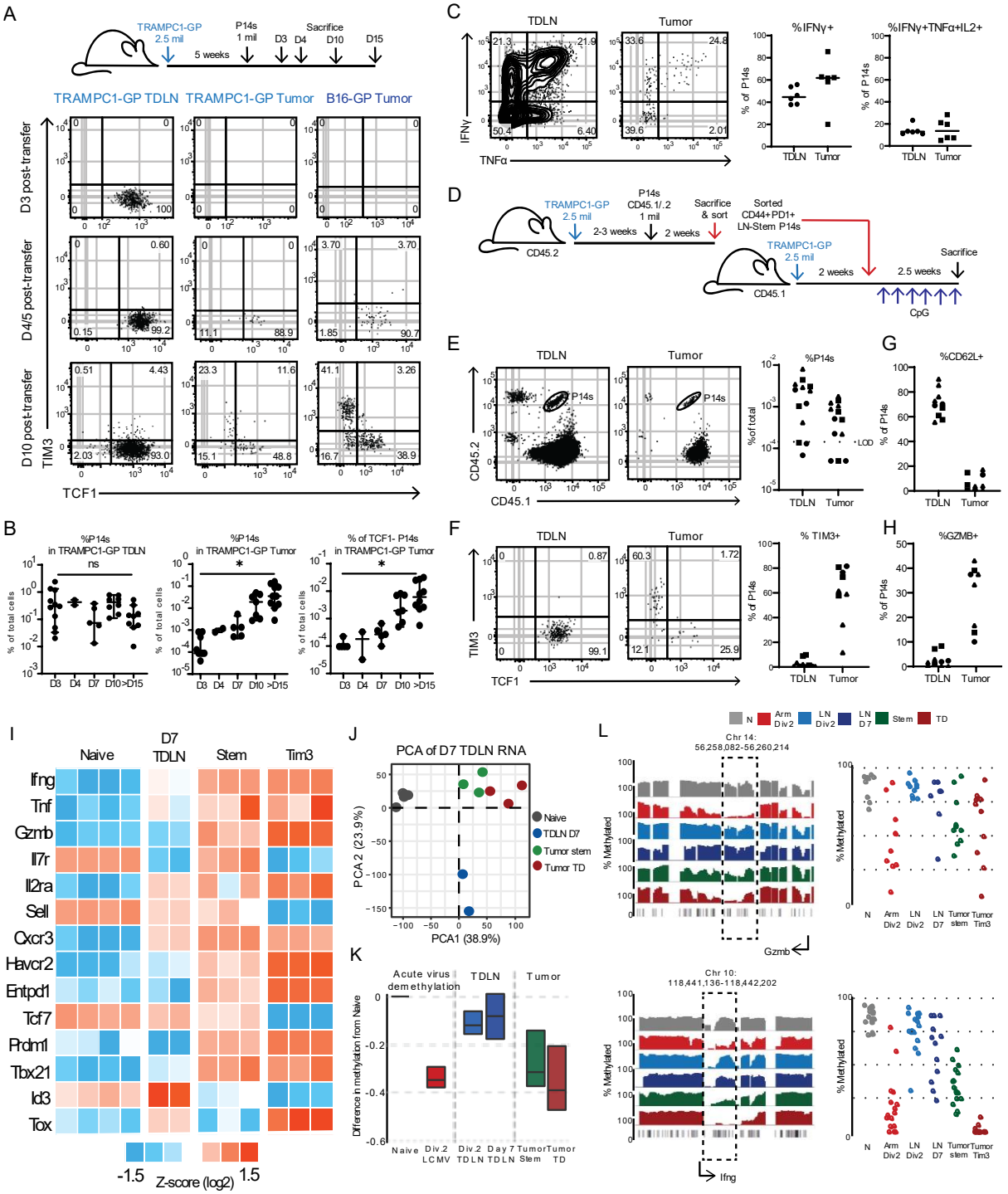

Figure 1. Tumor-specific CD8 T-cells only acquire effector phenotype after migration into the tumor A) Experimental setup for P14 transfers to study kinetics of tumor infiltration. Analysis of P14 phenotype in both TRAMPC1-GP and B16-GP tumors over the time course. P14s gated on

CD44+Pd1+. B) Summaries of total P14s in TRAMPC1-GP TDLNs and tumors over time course. C) Representative flow cytometry of intra-cellular cytokine staining (ICCS) of IFNγ and TNFα, gated on CD44+Pd1+ P14s D) Experimental setup for P14 TDLN re-transfer into tumor matched mice. E) Flow cytometry gating of re-transferred P14s in TDLN and tumors of congenically mismatched recipients F) Analysis of phenotype of re-transferred P14s. G) and H) Summary plot of CD62L and GzmB expression of re-transferred P14s. Different shapes (circle/square/triangle) represent from which of the 3 separate experiments each sample belongs to. I) Heatmap of z-scored $\log_2$ expression of genes in naïve P14s, sorted activated P14s D7 post-transfer from TRAMPC1-GP TDLNs, endogenous tumor stem-like CD8s, endogenous TD CD8s J) PCA of T-cell subsets K) Analysis of differentially methylated regions using previously defined cluster of genes demethylated in P14s from LCMV Arm (cluster 1, Fig 3G). Plot shows cluster 1 differentially methylated regions T-cells from various conditions. L) Specific methylation changes in *Gzmb* and *Ifng*. Traces show total methylation from 0 to 100% in regions near both genes. Boxed regions show significantly differentially methylated regions. Dot plots showed methylation of each CpG in boxed region. Median and 95% confidence intervals (CI) are shown. *, $P<0.05$ determined by Mann-Whitney test.

Figure S1. Stem-like phenotype is maintained in tumor draining LN, while differentiation occurs in the tumor

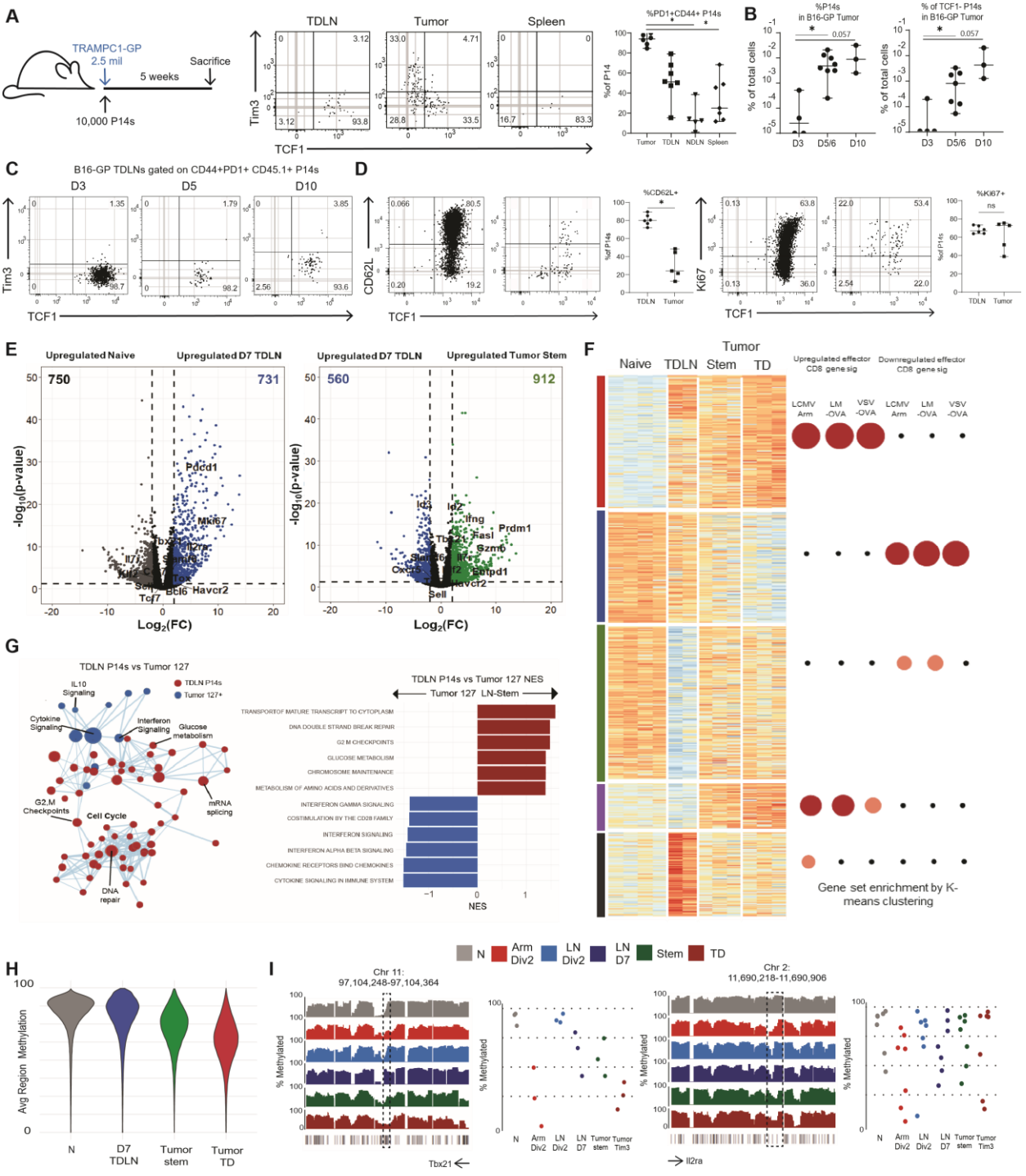

Figure S1. Stem-like phenotype is maintained in tumor draining LN, while differentiation occurs in the tumor A) Experimental setup of P14 transfer D0 of TRAMPC1-GP tumor

inoculation. P14s gated on CD44+PD1+ in TDLN, tumor and spleen B) Summary of total P14s and TCF1- P14s in B16-GP tumors over the time course C) Analysis of P14 phenotype in B16-GP TDLN over the time course G) Flow cytometry analysis of P14s two weeks after transfer in TRAMPC1-GP bearing mice. Median and 95% confidence intervals (CI) are shown. *, $P<0.05$ determined by Mann-Whitney test. E) Volcano plots of differentially expressed genes (2 log FC, $p<0.05$). F) Heatmap of $\log_2$ expression of P14s Day7 TRAMPC1-GP TDLN, endogenous tumor stem-like and TD CD8 subsets based unbiased K-means clustering. Gene set enrichment using genes from each cluster, compared to common effector CD8 T cell signatures from LCMV Arm, LM-OVA, VSV-OVA G) Pathway analysis of RNA-seq comparing P14s activated in LCMV Arm and TDLN. Circle size represents the number of genes in the gene set and length of connecting lines is proportional to how many genes overlap. GSEA comparing LN-stem P14s and tumor stem and TD subsets using the Reactome gene sets, normalized enrichment scores (NES) are shown. H) Global methylation changes of identified regions shown as violin plots of average methylation in naïve P14s, P14s D7 TRAMPC1-GP TDLNs, endogenous tumor stem and TD CD8s. I) Specific methylation changes in *Tbx21* and *Il2ra*. Traces show total methylation from 0 to 100% in regions near both genes. Boxed regions show significantly differentially methylated regions. Dot plots showed methylation of each CpG in boxed region.

PART 3

Antigen presenting cell phenotype predicts CD8 T cell differentiation

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CD8 T-cell activation in cancer is comprised of two distinct phases

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**In press at Immunity*

ABSTRACT

This work aimed to understand what signals within the tumor microenvironment promote stem-like CD8 T cell differentiation. We have previously shown that tumor-specific CD8 T cells are activated in the TDLN, to acquire a stem-like phenotype, but only fully differentiate to acquire an effector phenotype within the tumor. This points to a signal within the tumor microenvironment promoting the differentiation of stem-like CD8 T cells. Our work and previous work from others have shown the importance of APCs within the tumor to promote the CD8 T cell response, and the close localization of stem-like CD8 T cells to APCs. Here we have shown the co-stimulation is a critical signal for the differentiation of stem-like CD8 T cells within the tumor and that activation of APCs promotes CD8 T cell differentiation. Using ex-vivo cultures of human stem-like CD8 T cells we show that they can only proliferate and differentiate with co-stimulation from CD28, and CD3 stimulation alone does not induce differentiation. Overall, we demonstrate that once stem-like CD8 T cells migrate into the tumor they require both TCR and co-stimulation, likely from APCs that they reside next to.

INTRODUCTION

This chapter is reproduced with edits from Prokhnevska, N., Emerson, D.A., Kissick, H.T., Redmond, W.L. (2019). Immunological Complexity of the Prostate Cancer Microenvironment Influences the Response to Immunotherapy. In: Dehm, S., Tindall, D. (eds) Prostate Cancer. Advances in Experimental Medicine and Biology, vol 1210. Springer, Cham.

The maintenance and differentiation of stem-like CD8 T cells within the tumor is crucial for sustaining the CD8 T cell response. We have previously shown that stem-like CD8 T cells originate from TDLNs and differentiate into a TD effector-like CD8 T cell only within the tumor. This

implies that signals specifically within the tumor microenvironment induces this stem-like CD8 T cell differentiation. Which signals induce this differentiation are not clear, previous work in chronic viral infections and tumor models have suggested cytokines such as IL21 and Type I IFN, as well as co-stimulation [50, 53, 54]. Many of these signals can come from other immune cells within the tumor microenvironment, for this reason understanding how immune cells are organized within the tumor is critical.

Lymphoid organization in tumors:

Recent work has shown that in certain instances tertiary lymphoid structures (TLS), which contain CD8 T cells, DCs, follicular DCs and high endothelial venules, can form near the tumor [55]. Tumors containing a TLS were associated with higher T cell infiltration and improved disease-free survival in both breast and colorectal cancer [56, 57]. In viral infections, the organization of antigen presenting cells and CD8 T cells is crucial for activating and promoting effector differentiation of antigen-specific CD8 T cells. Interestingly, in non-small cell lung cancer, TLS-associated mature DCs correlate with CD8 T cell infiltration and improved survival [58, 59]. This demonstrates the power of having an organized structure that supports T cells and DCs near the tumor that is comparable to the organization of lymphoid tissues during a viral infection. In prostate cancer, TLS comprised of more pro-inflammatory Th1 and CD8 T cells are associated with improved tumor regression [60]. In kidney cancer, we have previously shown that CD8 T cells reside in immune niches [32]. These immune niches are not characterized as a TLS but support the CD8 T cell infiltration, since tumors without these immune niches also have less overall CD8 T cell infiltration. Importantly, stem-like CD8 T cells within these immune niches reside significantly closer to APCs compared to the terminally differentiated CD8 T cells. Since

DC infiltration as well as presence of immune niches predict overall higher CD8 T cell infiltration, there is a clear role for CD8 T cells interacting with APCs within human kidney tumors.

Antigen presenting cells in cancer:

Antigen presenting cells are the interface between foreign or tumor-associated antigen and T cell activation. Understanding how this population of cells operates in cancer is key to understanding the initial generation of the anti-tumor T cell response. APCs and DCs are also a crucial part of TLS's and immune niches within tumors, the presence of which promotes CD8 T cell infiltration within the tumor.

Several subsets of DCs have been classified based upon their phenotype and function in mice and humans. DCs can be broadly classified by the high expression of CD11c and MHC-II. One crucial subset of DCs for CD8 T cell activation is the cross-presenting DC, which refers to the processing and presentation of exogenous antigens on MHC class I molecules [61]. These cells are of interest in the context of tumor immunology since most tumor antigens are exogenous proteins and must be presented on MHC-I to activate tumor-specific CD8 T cells. The cross-presenting DC (cDC1) subset has been thoroughly characterized in mice. These cells are defined by CD8α and CD103 expression and show increased antigen uptake, processing, and presentation on MHC-I [62]. This DC subset has also been characterized in human tissue, distinguished by the expression of CD141 and CLEC9A, and has been seen in the lung, liver, skin, and blood compartments [63]. The cDC1 subset is indispensable in the activation of CD8 T cells in infection and tumor progression as cDC1-deficient mice do not control influenza infection or immunogenic tumors in a T cell-dependent manner [64, 65]. Overall, this is a key DC subset in the immune response that specializes in the activation of viral and tumor-specific CD8 T cells. Another major DC subset (cDC2) in mice is characterized by the expression of CD11b on CD11c+ MHC II+ DCs. CD11b+

DC (mice) and CD1c (humans) are less efficient at cross presentation of exogenous antigens and therefore thought to mainly activate CD4 T cells through MHC-II [66]. Another DC subset that has not been extensively described is a monocyte-derived DC (Mo-DC), which are thought to originate from monocytes that acquire DC-like properties in tissues. These cells are of particular interest in tumors since they possess the ability to cross-present exogenous antigens (murphy paper) and are one of the major DC populations in numerous human cancer types (max papers). The role of each DC subset separately in the response to tumors has not been defined yet. Since some DC subsets may also have roles in presenting antigen in TDLNs, while others could have a role directly in the tumor.

There have been numerous studies showing the prognostic power of DC infiltration in tumors. For example, one study used data from the cancer genome atlas (TCGA) and analyzed CD103/141-associated genes to determine cross-presenting DC infiltration (cDC1). The ratio of CD103/141+ signature genes to genes not associated with CD103/141 DCs acts as a prognostic marker that predicted overall survival in numerous cancer types including breast cancer, head-neck squamous cell carcinoma, and lung adenocarcinoma [66, 67]. This shows how the extent of DC infiltration alone can have prognostic power over a wide variety of cancers. Other studies using TCGA data have also determined that the CD141 gene signature correlated with CD8 transcript levels [64].This demonstrates the importance of DCs in the TME to promote and support CD8 T cell infiltration and how DC and CD8 T cell infiltration can be used to predict survival in patients with cancer. Studies have also shown in human melanoma tumors the crucial role of CD141+ DCs expressing CCR7, which allows for the migration into lymph nodes to present tumor antigens to tumor-specific CD8 T cells. The tumors which contain higher levels of CCR7 transcripts correlated with more CD3+ T cell infiltration and better survival [67]. DCs need to be able to bring tumor

antigens into the lymphoid organs to activate tumor-specific CD8s more efficiently due to the higher concentration of CD8 T cells and DCs in lymphoid tissues. Recent work has similarly described an activated migratory DC within both human and mouse tumors called mature DCs enriched with immunoregulatory molecules or "mreg" DCs, these DCs express CCR7 and have been also referred to as "DC3s" or "migratory DCs". Interestingly, these migratory mreg DCs can be of either cDC1 or cDC2 origin, have an activated phenotype with high expression of co-stimulatory molecules CD40 and CD80 and express higher levels of cytokines, IL12 and IL15 [68, 69]. The role of these migratory CCR7+ DCs ("mregs") in tumors is correlated to improved CD8 T cell infiltration in tumors, even though this DC population is the rarest within tumors.

These studies collectively show that the presence of DCs, correlates with more T cell infiltration and better prognosis and survival in many tumor types and have an essential role for CD8 T cell response to tumors. In our work we have determined that stem-like CD8 T cells are within dense APC niches, and specifically differentiate within the tumor and not TDLNs., raising numerous questions on how APCs contribute to stem-like CD8 T cell differentiation. In this study we aimed to investigate what signals, including co-stimulation and cytokines from APCs, contribute to stem-like CD8 T cell differentiation within the tumor.

RESULTS CHAPTER 3

1. Co-stimulatory molecules and cytokines in the tumor microenvironment contribute to stem to effector CD8 differentiation.

CD8 T-cells acquiring an effector-like epigenetic and transcriptional program in the tumor implies something within the tumor microenvironment must drive this phenotypic change. In our previous work, we had found that stem-like CD8 T-cells in tumors always reside near areas of high antigen presenting cells (APCs) density [1]. In addition, transcriptional analysis above suggested that

pathways related to costimulatory (signal 2) and cytokines (signal 3) were most different in tumor stem-like cells compared to those in the TDLNs. Based on these data, we hypothesized that APCs in the tumor might provide these important signals to cause stem-like CD8 T-cells to differentiate into TD CD8 TILs. To investigate this idea, we first characterized the APCs from naïve lymph-nodes, TDLNs and TRAMPC1-GP tumors using scRNAseq. Seven clusters were identified based on expression of lineage specific markers (Fig 1A,B, Fig S1A). This clustering designation was confirmed by comparison with known myeloid cell signatures [41] (Fig S1B).

The primary DC populations present in the tumor were cDC2s and mo-DCs, while all APC subsets were found in the TDLNs (Fig 1B). We further characterized these DC populations by flow cytometry, using CD11b for a combined cDC2 and mo-DC marker [42]. As in the scRNAseq analysis, in TDLNs both cDC1s (CD8a+) and CD11b+ DCs were found, while in the tumor only CD11b+ DCs were present (Fig 1C, Fig S1C). We also analyzed migratory DCs by using CD103 and CD40, and found a small percentage in the tumor, mostly within the CD11b+ subset (Fig 1C, Fig S1D). We further characterized CD11b+ DCs, and found significantly higher expression of CD80, CD86, in the tumor when compared to TDLN (Fig 1D). These data show that the primary DCs within the tumor are CD11b+ cDC2/mo-DCs that express high levels of co-stimulatory molecules.

Based on the observation that DCs in tumors expressed more co-stimulatory molecules, and predicted co-stimulatory pathways enriched in tumor stem-like CD8 T-cells, we hypothesized that these signals might be what causes differentiation from a stem-like CD8 T-cell to a TD CD8. To test this, we transferred P14s into TRAMPC1-GP tumor bearing mice, and 5 days post-transfer treated mice with 10ug of CpG intratumorally (I.T.) (Fig 1E). CpG was used to activate APCs and determine whether increased APC activation can alter CD8 T-cell differentiation within the tumor.

After CpG treatment, P14s in TDLNs maintained an LN-stem phenotype (Tcf1+), meanwhile in the tumor there were significantly more differentiated (Tcf1-) P14s and Gzmb+ P14s (Fig 1F,G). To test if co-stimulatory molecules were involved in this process of differentiation, we blocked CD80 and CD86 during CpG treatment (Fig 1E). Blockade of these critical molecules returned differentiation, and numbers of P14s, to control levels, and the percent of Gzmb+ P14s significantly decreased compared to CpG alone suggesting co-stimulation is one of the important signals for stem to effector differentiation in the tumor (Fig 1G, Fig S1I,J).

We were next interested to see how cytokines contributed to this process. In comparison to co-stim blockade, blocking IL12 or type I IFN had no significant impact on the proportion of Tcf1- P14s within the tumor or TDLNs, indicating that these cytokines are not the main drivers of CD8 T-cell differentiation with CpG treatment (Fig S1E-H). However, IFNAR blockade significantly decreased Gzmb expression by Tcf1- P14s suggesting this cytokine is important for effector function (Fig S1H). Together, these data indicate co-stimulation in the tumor microenvironment is one of the major contributors to the differentiation of Tcf1+ stem-like cells to the effector state in the tumor.

2. Co-stimulation in human tumors correlates with the presence of terminally differentiated CD8 cells

Based on what we had observed in mouse models, we wanted to know if co-stimulatory molecule expression on DCs in human tumors might serve a similar function of regulating the stem to TD transition. We first performed scRNAseq to broadly determine what APC populations infiltrate human kidney tumors. Four clusters of APCs were identified based on differences in gene expression using tSNE clustering: monocytes, DCs, NK cells, B cells (Fig 2A). Using published human APC cell signatures and previously defined markers, we confirmed our clustering of APCs

from human kidney tumors (Fig 2B,C, Fig S2A) [41,43-50]. To expand this characterization, we analyzed 152 kidney tumors and 34 prostate tumors by flow cytometry for the DC populations we identified by scRNAseq. We defined cDC2s as CD11c+CD1c+ cells, while cDC1s and mo-DCs were defined as CD11c+CD1c- and expressed CD141 and CD11b, respectively (Fig 2D). In both kidney and prostate tumors, cDC2s and Mo-DCs were the dominant DC populations (Fig 2D,E). All DC subsets had high expression of FLT3 as well as PDL1, further validating that they are activated DCs, as others have reported [43]. Mo-DCS were the only DC subset expressing CD14, corresponding to derivation from the monocyte lineage (Fig S2B). Importantly, the presence of all DC subsets correlated with higher CD8 T-cell infiltration (Fig S2C,D).

We were next interested in expression of co-stimulatory molecules by DCs in human kidney tumors and how it might correlate with generation of TD CD8 TILs. In our prior work, we found patients with higher total CD8 T-cell infiltration in tumors, generally also have more TD CD8 TILs within the tumor [1]. We therefore split patients into high and low CD8 infiltration, based on the median CD8 infiltration of the patients analyzed, to investigate how co-stimulation might correlate with CD8 differentiation in the tumor (Fig 2F). Mo-DCs from tumors with higher total CD8 T-cell infiltration expressed significantly higher levels CD86 and CD40 (Fig 2G) and cDC2s expressed more CD40 in tumors with higher CD8 T-cell infiltration (Fig 2G). In contrast, expression of co-stimulatory molecules on cDC1s was not significantly different between low and high CD8 T-cell infiltrated tumors (Fig S2E). We next used the TCGA database to determine how CD8 effector molecules might correlate with co-stimulation in the tumor across the top 8 most represented tumor types. We compared expression of various co-stimulatory molecules with cytotoxic genes that are typically only expressed by terminally differentiated CD8s in tumors, we found that CD80, CD86, CD40 as well as CD48, CD58, and LIGHT expression correlated with

expression of the cytotoxic genes (Fig 2H, Fig S2F). Overall, in both mouse and human tumors, cDC2s and mo-DCs make up the majority of DCs and expression of co-stimulatory molecules correlate with CD8 T-cell differentiation.

To determine if these major populations found in the tumor had the functional capacity to cause differentiation of stem-like CD8s, Mo-DCs and cDC2s were sorted from tumors as shown in Fig 2D, and co-cultured with patient matched CTV labelled tumor stem-like CD8 cells (Fig 2I). In all patients analyzed, both Mo-DCs and cDC2s were capable of inducing proliferation of the stem-like CD8s (Fig 2J, Fig S2G,H). Both DC subsets were also capable of inducing differentiation in stem-like CD8 T-cells, as shown by upregulation of GZMB, CD39 (Fig 2J, Fig S2H). These data demonstrate the capacity of Mo-DCs and, to an extent, cDC2s to induce stem-like CD8 T-cell differentiation.

3. Co-stimulation is required for human stem-like CD8 T-cells to differentiate to the effector state

These data suggest that co-stimulation is a major signal that drives T-cells to acquire cytotoxic function in tumors. To functionally test this, we sorted CTV labelled stem-like TILs (PD1+CD39-CD28+) from human kidney tumors and stimulated them *in-vitro* with either anti-CD3 alone, anti-CD3/CD28 or anti-CD3/CD28/CD2 beads (Fig 3A). After five days *in-vitro*, stem-like CD8 T-cells that received only TCR stimulation (anti-CD3) did not proliferate and did not acquire a TD CD8 phenotype (Fig 3B,C). In comparison, stem-like CD8s stimulated with anti-CD3/CD28 proliferated and upregulated proteins related to effector differentiation (Fig 3B,C). Most importantly, when we provided additional co-stimulation with both anti-CD28 and anti-CD2 beads, more stem-like cells divided and differentiated into a TD, effector-like state (Fig 3B,C). These data illustrate that co-stimulatory molecules are required to induce stem-like CD8 T-cell differentiation and that TCR signaling alone is not sufficient. Furthermore, and in line with the

correlative data we collected in human tumors (Fig 2F-H), more co-stimulation appears to accelerate this process.

Since signal 3 cytokines are an important mechanism regulating CD8 T-cell function, and several recent papers have highlighted the important role of these cytokines in the immune response to cancer, we tested how these cytokines contribute to stem- to effector differentiation [51,52]. We found that IL12 with TCR alone (anti-CD3) was not sufficient to cause any division, or increase in GZMB, TIM3, TBET expression of the stem-like CD8 T-cells (Fig 3D,E). Similarly, when IL12 was added to anti-CD3/28/2, there was no increase in proliferation or differentiation, with a minor increase in Tim3 expression (Fig 3D,E). When IL12 was added to a lower co-stimulatory condition, anti-CD3/28 stimulation, there were still no differences in proliferation or GZMB expression, but both Tim3 and TBET underwent a small but significant increase (Fig S3A,B). The addition of IL12 to tumor TD (TIM3+CD39+) stimulations did not induce proliferation or alter the expression of TIM3 (Fig S3C). We also analyzed the effects of IL12 on naïve CD8 T-cells stimulated with anti-CD3 or anti-CD3/28/2 (Fig S3D). Similar stem-like CD8 T-cells, naïve CD8 T-cells do not proliferate with CD3 stimulation alone or with the addition of IL12, but there was an increase in GZMB expression when naïve cells were stimulated with anti-CD3/28/2 with IL12 (Fig S3D,E). We next analyzed the amount of IFNγ in supernatants of *in-vitro* cultures of cells stimulated in these conditions and found that there was not a significant increase in IFNγ with the addition of IL12 (Fig S3F). We next tested the effect of Type I IFN on stem-like CD8 T-cell differentiation by adding IFNb to the anti-CD3/28/2 stimulations. Unexpectedly, we found that addition of IFNb significantly reduced the proliferation of stem-like CD8 T-cells and TBET expression, but not GZMB, CD39 or TIM3 expression (Fig S3G-I).

From these data we conclude that for stem-like CD8 T-cells to undergo differentiation to the effector state, both TCR stimulation and co-stimulation is required. In addition, signal 3 cytokines like type I IFNs and IL12 may contribute to regulation of some genes associated with effector function. Together, these data highlight co-stimulation as a critical signal in the tumor microenvironment that regulate anti-tumor CD8 T-cell differentiation and acquisition of an effector phenotype.

DISCUSSION

The goal of this work was to determine the signals that promotes stem-like CD8 T cell differentiation specifically within the tumor. From previous work we know that stem-like CD8 T cells reside in immune niches with dense APC infiltration, and that DC infiltration within tumors correlates with total CD8 T cell infiltration. We hypothesized that APCs can promote CD8 T cell differentiation through co-stimulation or cytokines, due to close localization within the tumor. To better understand the potential signals from APCs that contribute to T cell differentiation we analyzed the DCs that infiltrate both our mouse prostate cancer model, as well as human kidney and prostate cancer. Surprisingly, we found that cDC1s are rarest population of DCs within both our mouse model and human tumors, even though they have been shown to be crucial to CD8 T cell infiltration and patient survival [67-70]. Instead, we find that cDC2s and Mo-DCs are the major DC subsets that were found in human kidney and prostate cancer, and CD11b+ DCs within TRAMPC1-GP tumors. Interestingly, all three DC populations, cDC1s, cDC2s, Mo-DCs, correlated with CD8 T cell infiltration. From this work it is not clear which DC subset is crucial for CD8 T cell activation and differentiation, moreover different DC subsets could have different roles in TDLNs compared to tumor.

We next analyzed how DCs within TDLNs and tumors were different, to determine potential signals that promote CD8 T cell differentiation. We found significantly higher expression of both CD80 and CD86 on CD11b+ DCs within the tumor in our TRAMPC1-GP mouse model, compared to TDLNs. Additionally, in human kidney cancer, higher expression of CD86 on Mo-DCs predicted higher CD8 T cell infiltration in the tumor. These data suggested that co-stimulation may contribute to stem-like CD8 T cell differentiation within the tumor. We further showed this by blocking CD80 and CD86 *in-vivo*, which significantly reduced stem-like CD8 T cell differentiation during CpG treatment. Lastly, using sorted human stem-like CD8 T cells we have shown that with CD3 stimulation alone (TCR alone) these cells do not proliferate or differentiate, *ex-vivo*. When they receive co-stimulation from CD28, they proliferate and differentiate into TD CD8 T cells. Overall, these data suggested that co-stimulation is one of the critical signals for CD8 T cell differentiation within the tumor. Importantly, IL12 or Type I IFN could not compensate for the lack of co-stimulation and had little effect on stem-like CD8 T cell differentiation *in-vivo* or during *ex-vivo* stimulation. These cytokines may be able to augment the CD8 T cell response when low levels of co-stimulation are present but are not sufficient to replace co-stimulation entirely. Overall, we have shown that co-stimulation, most likely from DCs within the tumor, is crucial for the differentiation of stem-like CD8 T cells and overall CD8 T cell infiltration within tumors.

The difference in DC populations and phenotype within TDLNs and tumors, demonstrates that APCs could have two distinct roles in the immune response to tumors. The first role is in TDLNs, where they must find and activate tumor-specific CD8 T cells in order to initiate the adaptive anti-tumor response. The second role is within the tumor, where APCS are found in lymphoid-like areas of the tumor such as immune niches and TLSs [32, 56, 58]. Within the tumor their role seems to be related to stem-like CD8 T cell differentiation, through antigen-presentation and additional

co-stimulation. Numerous studies have found that cDC1s are crucial to sustain the CD8 T cell response within the tumor, through whole mouse knockouts using Batf3 KO or Zbtb46 KO mice, but it is not clear whether this is due to deficiency in CD8 T cell priming within TDLNs, or differentiation within the tumor [67, 70]. Antigen draining to TDLNs is dependent on CCR7 expression on migratory DCs and is critical for CD8 T cell activation in TDLNs [71]. These cells must migrate from within the tumor into TDLNs, which could explain why they are the rarest population within tumors. Surprisingly, newly designated "mreg" or migratory CCR7+ DCs within tumors have been found to predict patient survival. but their role in TDLNs versus tumor is not clear. The critical role of CCR7+ migratory/mreg DCs is to migrate to TDLNS with antigen from tumor, by this logic they could predict higher CD8 T cell infiltration due to improved CD8 T cell priming in TDLNS and have a smaller role within the tumor [67-69, 72]. Recent work has also shown the role of migratory CD103+ DCs in bringing antigen from the tumor to TDLNs and transferring antigen to resident TDLN DC populations. The trafficking of antigen from tumor to TDLN is a critical part of the immune response to tumors, and migratory DCs within the tumor are necessary for this to occur. Within the tumor they are usually the rarest DC population but have an activated phenotype with high expression of co-stimulatory molecules such as CD80/86 and CD40, as well as cytokines such as IL12 and IL15 [68-70]. Recent work suggested that migratory DCs within the tumor can promote survival and differentiation of CD8 T cells, but the role of migratory DCs compared to tumor-resident DCs within the tumor needs to be further investigated.

DCs within the tumor can predict CD8 T cell infiltration, demonstrating a necessary secondary role of DCs specifically within the tumor. The major DC populations within tumors are cDC2 or mo-DCs, with a small percentage of cDC1s and migratory DCs, as many groups and we have shown[68, 70, 73]. The role of cDC2s and mo-DCs within the tumor and how they can contribute

to the anti-tumor response has not been thoroughly characterized. We showed that the major DC subset in kidney tumors, Mo-DCs, were capable of inducing stem-like CD8 T cell differentiation *ex-vivo*. Other reports have also shown that Mo-DCs are capable of cross-presentation of exogenous antigen, possibly due to their monocyte lineage, and could be a potential mechanism to present tumor antigens to CD8 T cells within the tumor [74]. The other major DC subset resident within the tumor are resident cDC2s, these cells were also able to induce some CD8 T cell differentiation *ex-vivo*, but it is not clear if their major role within the tumor is related to CD8 T cells or CD4 T cells [73]. With higher expression of CD40 on cDC2s also predicting higher CD8 T cell infiltration, but it is not clear if it is through activation of beneficial CD4 subsets or directly on CD8 T cells. Overall, the major DC subsets that are resident within tumors are not cDC1s but still predict overall CD8 T cell infiltration within tumors and are functionally capable of inducing CD8 T cell differentiation.

These data support that DCs have two different roles to promote the CD8 T cell response, with one role in the TDLN and the second role within the tumor. This mirrors the two phases of CD8 T cell activation we have described in our work. The lack of sufficiently activated DCs within TDLNs, with lower expression of co-stimulatory molecules, could contribute to the distinct activation of tumor-specific CD8 T cells where they do not acquire an effector program within the TDLNs. Instead, these tumor-specific CD8 T cells require additional co-stimulation from APCs within the tumor to fully differentiate and acquire the effector program. This demonstrates the importance of tumor resident APCs, and organization of immune cells within the tumor for stem-like CD8 T cells to be near the necessary APCs. Since cDC1s seem to be a minor population within most tumors, additional work on the origins of tumor-resident Mo-DCs and cDC2s is necessary to understand how these cells contribute to the CD8 T cell response within the tumor specifically.

Mo-DCs are particularly interesting since they can be of monocyte origin and recruited into the tumor and differentiate to acquire properties of a DC [75-77]. In other infection models, monocytes could differentiate to acquire a DC-like phenotype and activate both CD8 and CD4 T cells [76]. Since monocytes are a major innate immune cell population and capable of acquiring different phenotypes within tissues, these cells could be an underappreciated APC that can promote the CD8 T cell response within tumors. Understanding the origin of DCs that are resident within the tumor is critical to better understand the second phase of CD8 T cell activation within the tumor, and eventually develop therapeutics that can target both APCs and CD8 T cells.

MATERIAL AND METHODS - ADDITIONAL METHODS IN CHAPTERS 1/2

In vivo treatments (CpG, anti-CD80/CD86, anti-IL12, anti-IFNAR)

For CpG treatments, 10ug was injected intra-tumorally (I.T.) at week 4 of tumor growth. CpG was injected every other day for 10 days. For combination CpG and CD80/86 blockade, anti-CD80 (B7.1) (200 ug) and anti-CD86 (B7.2) (200ug) antibodies were injected every 3 days IP, as previously described [53]. For cytokine blocking experiments anti-IL12p40 (250ug) or anti-IFNAR (250ug) antibodies were injected every other day IP, as previously described [54, 78].

In vitro stimulation and co-cultures

Single cell suspension from fresh and frozen human tumor samples were stained with CellTrace violet (CTV) (Thermo), using 1 ul of CTV per 1 million cells. CTV labelled cell suspension were sorted on the Becton Dickinson FACS Aria II Cell Sorter. Sorted CD8 T cells were cultured in U-bottom plates in T cell media (RPMI. 10% FBS, 1% Pen-Strep, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, 0.0005% 2-Mercaptoethanol) and 20-50 U/mL of IL2 (Peprotech). Stimulation assays were performed using anti-CD3, anti-CD3/28, anti-CD3/28/2

beads at a ratio of 1 bead for 2 CD8 T cells. For *in-vitro* stimulations with cytokines, either 10ng/ml of human Il12 or human IFNb (Peprotech) were added to cultures D0 of culture. Samples were analyzed by flow cytometry for proliferation and expression of various proteins are 5-7 days after stimulation. IFN-gamma production was measured by taking supernatants at day 5 post stimulation. Using the high sensitivity Human IFN-gamma ELISA kit, we measured IFN-gamma production above the limit of detection of 0.16pg/mL (Thermo Fisher).

For DC co-culture experiments, DCs were sorted as shown in figure 6F. DC populations were irradiated with 10 Gy before being put in culture. Co-cultures were plated at 2 DCs per 1 T cell, as much as possible based on cell number recovered, with CTV labelled stem-like CD8 T cells sorted from matching tumors. Samples were analyzed 7 days post co-culture on the Beckton Dickinson Symphony.

FIGURES

Figure 1. Co-stimulation from antigen presenting cells promotes tumor specific CD8 T-cell differentiation

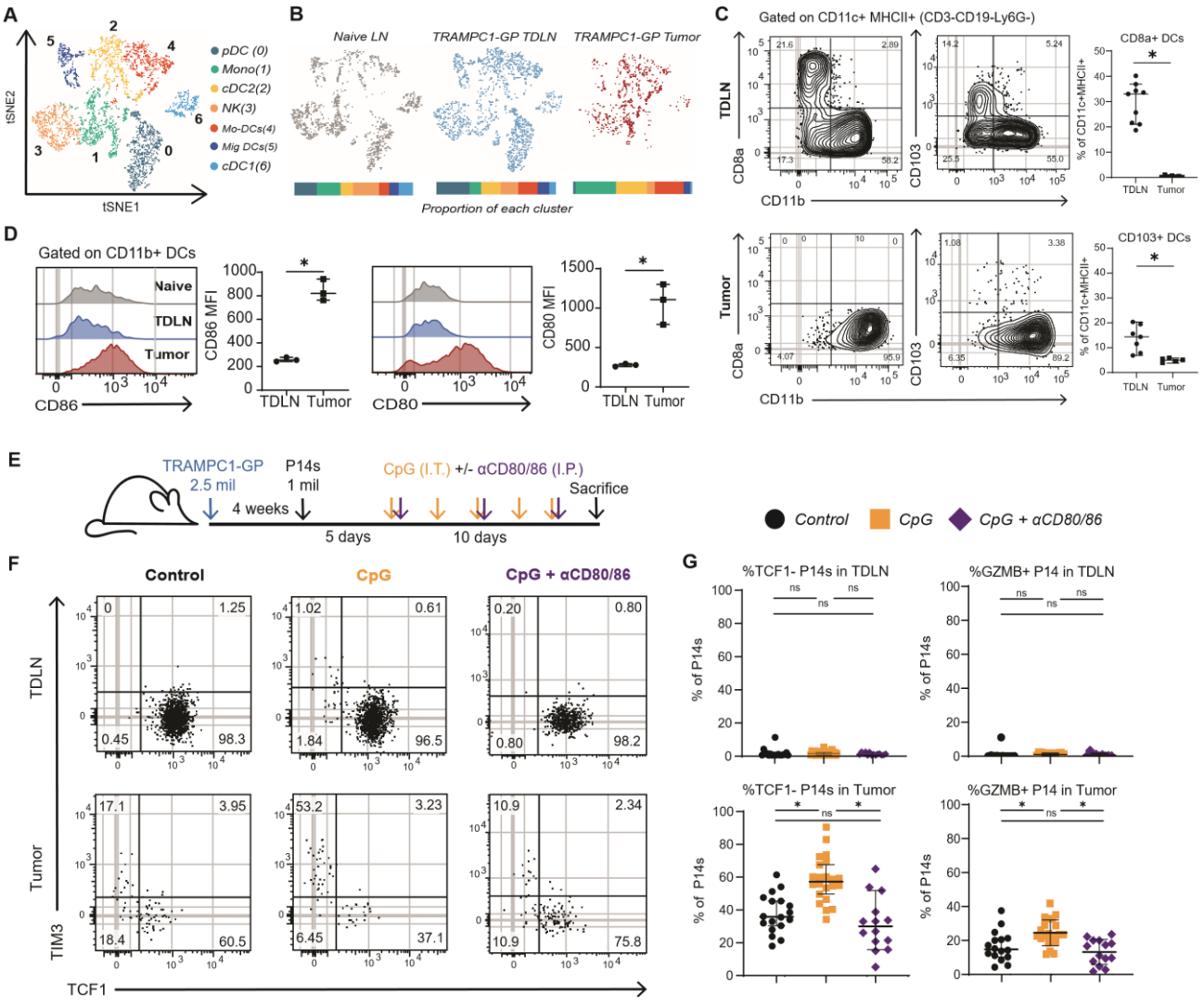

Figure 1. Co-stimulation from antigen presenting cells promotes tumor specific CD8 T-cell differentiation A) tSNE clustering of antigen presenting cells (APCs) sorted from naïve LN, TDLN, and tumors of 5-week TRAMPC1-GP bearing mice. B) tSNE clustering split by tissue origin, with proportions of each cluster from each tissue represented in a bar graph, each APC population is significantly different between TDLN and Tumor C) Flow cytometry analysis of DC

subsets from TDLN and tumor from TRAMPC1-GP bearing mice. D) Flow cytometry analysis of co-stimulatory molecules in CD11b+ DCs in naïve LNs, TDLNs and tumors from TRAMPC1-GP bearing mice. E) Experimental setup. P14s were transferred into 4-week TRAMPC1-GP tumor-bearing mice and 5 days post transfer treated with CpG and/or CD80/CD86 blocking antibodies for 10 days. F) and G) Phenotype of transferred Pd1+ CD44+ P14s 15 days post-transfer in the TDLN and tumors of all treatment groups. Median and 95% confidence intervals (CI) are shown. *, $P<0.05$ determined by Mann-Whitney test.

Figure 2. Co-stimulation from dominant CDC2s and mo-DCs in human tumors predicts CD8 T-cell infiltration.

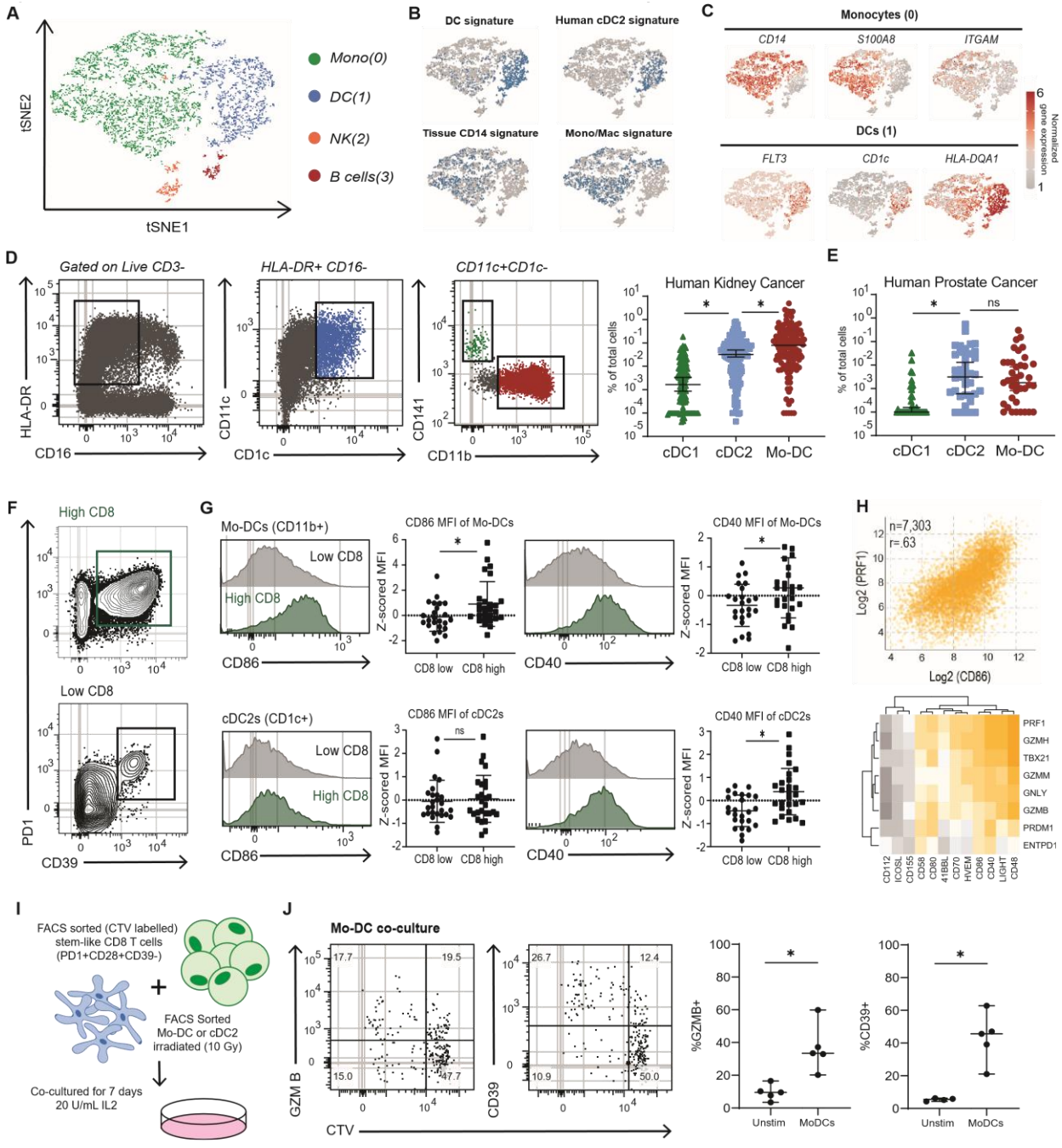

Figure 2. Co-stimulation from dominant CDC2s and mo-DCs in human tumors predicts CD8 T-cell infiltration. A) tSNE clustering of single cell RNA-seq of APCs from two patient kidney tumor samples B) VISION analysis of gene signatures associated with APC subsets from human

tissues. tSNE plots show the top quintile of cells enriched for the signature shown highlighted in blue C) Normalized gene expression of selected genes that define the Monocyte and DC cluster are shown D) Flow cytometry analysis of 152 kidney tumors, gating to distinguish three DC subsets E) Summary of three DC subsets in 34 prostate tumors F) Representative flow cytometry plots of high and low CD8 infiltrated kidney tumors. PD1 and CD39 expression shown to denote TD CD8 TILS G) Expression of costimulatory molecules on mo-DCs and cDC2s in kidney tumors from high and low CD8 T-cell tumors H) TCGA data from top 8 represented tumor types. Correlation of *CD86* expression versus Perforin (*PRF1*) expression shown. Heatmap of correlations between other co-stimulatory molecules versus effector and cytotoxic genes. I) Experimental layout to test the capacity of different DC subsets from tumors to induce differentiation of autologous tumor stem-like CD8 T-cells. J) Representative plots showing CTV dilution and phenotype after 7 days of stem-like CD8 T-cell co-culture with irradiated mo-DCs. Medians and 95% CIs are represented. *, $P<0.05$ determined by Mann-Whitney test

Figure 3. Co-stimulation is necessary for the differentiation of human tumor stem-like CD8 T-cells

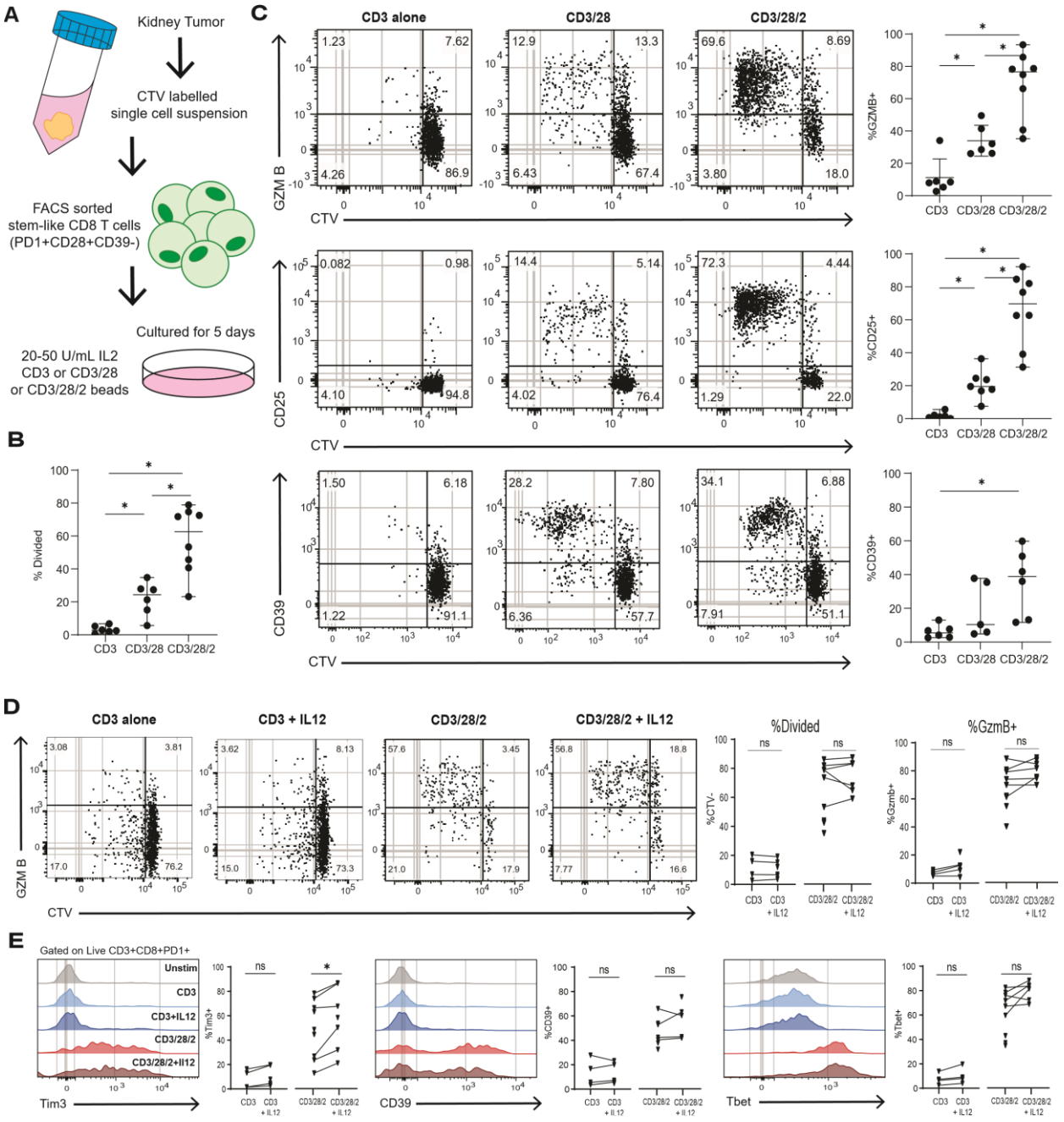

Figure 3. Co-stimulation is necessary for the differentiation of human tumor stem-like CD8 T-cells. A) Experimental layout to test the requirement of co-stimulation to differentiate sorted tumor stem-like CD8 T-cells. B) Summary plot shows the proportion of divided CD8 T-cells in each *in-vitro* condition, based on CTV dilution. C) Representative plots showing CTV dilution and

phenotype after 5 days of culture in each condition. D) Representative plots showing CTV dilution and expression of GZMB of control *in-vitro* stimulations or with added IL12. E) Histograms showing expression of TIM3, CD39, and Tbet for all conditions. Medians and 95% CIs are represented. *, $P<0.05$ determined by Mann-Whitney test. *, $P<0.05$ determined by Wilcoxon test when sufficient paired samples were analyzed, shown as connected by a line.

Figure S1. Effect of signal three cytokines, Il12 and Type I IFN, on effector differentiation in the tumor.

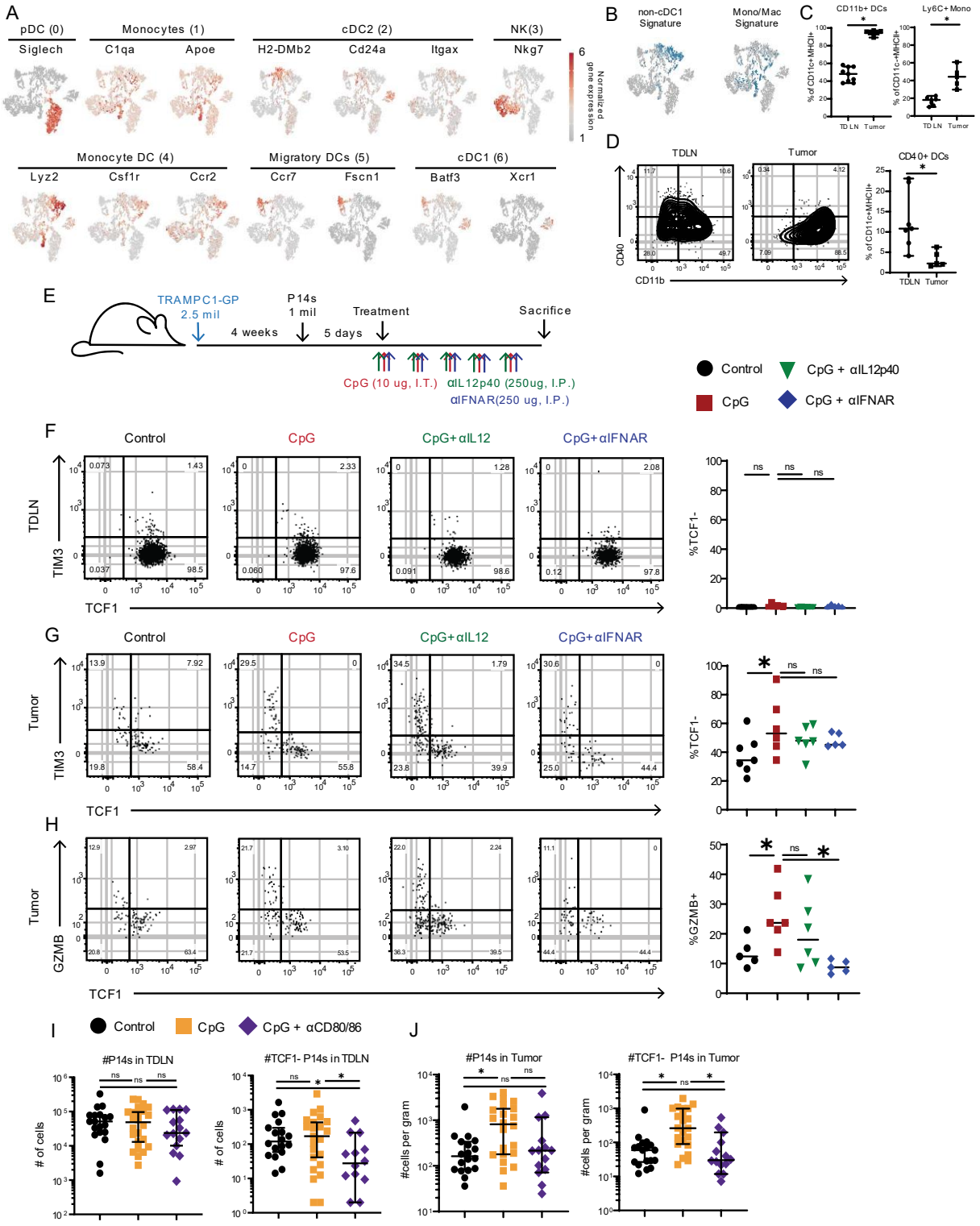

Figure S1. Effect of signal three cytokines, Il12 and Type I IFN, on effector differentiation in the tumor. A) Normalized gene expression of selected genes that define each individual cluster are shown, from APCs in TRAMPC1-GP mice (from Fig5A) B) VISION analysis of Immgen gene signatures associated with APC subsets. tSNE plots show the top quintile of cells enriched for the signature shown highlighted in blue C) Summary of flow cytometry data from Fig 1C of CD11b+ DCs and Ly6C+ Monocytes in TDLN and tumor D) Flow cytometry analysis of CD40 expression of DCs with summary. E) Experimental setup of P14 transfer and treatment. F-) Phenotype of transferred PD1+ CD44+ P14s 15 days post-transfer in each treatment group in TDLNs and Tumors Medians are shown. *, $P<0.05$ determined by ANOVA I) and J) Numbers of PD1+ CD44+ P14s 15 days post-transfer in the TDLN and tumors of all treatment groups. Median and 95% confidence intervals (CI) are shown. *, $P<0.05$ determined by Mann-Whitney test

Figure S2. All DC subsets correlate to total CD8 T cell infiltration

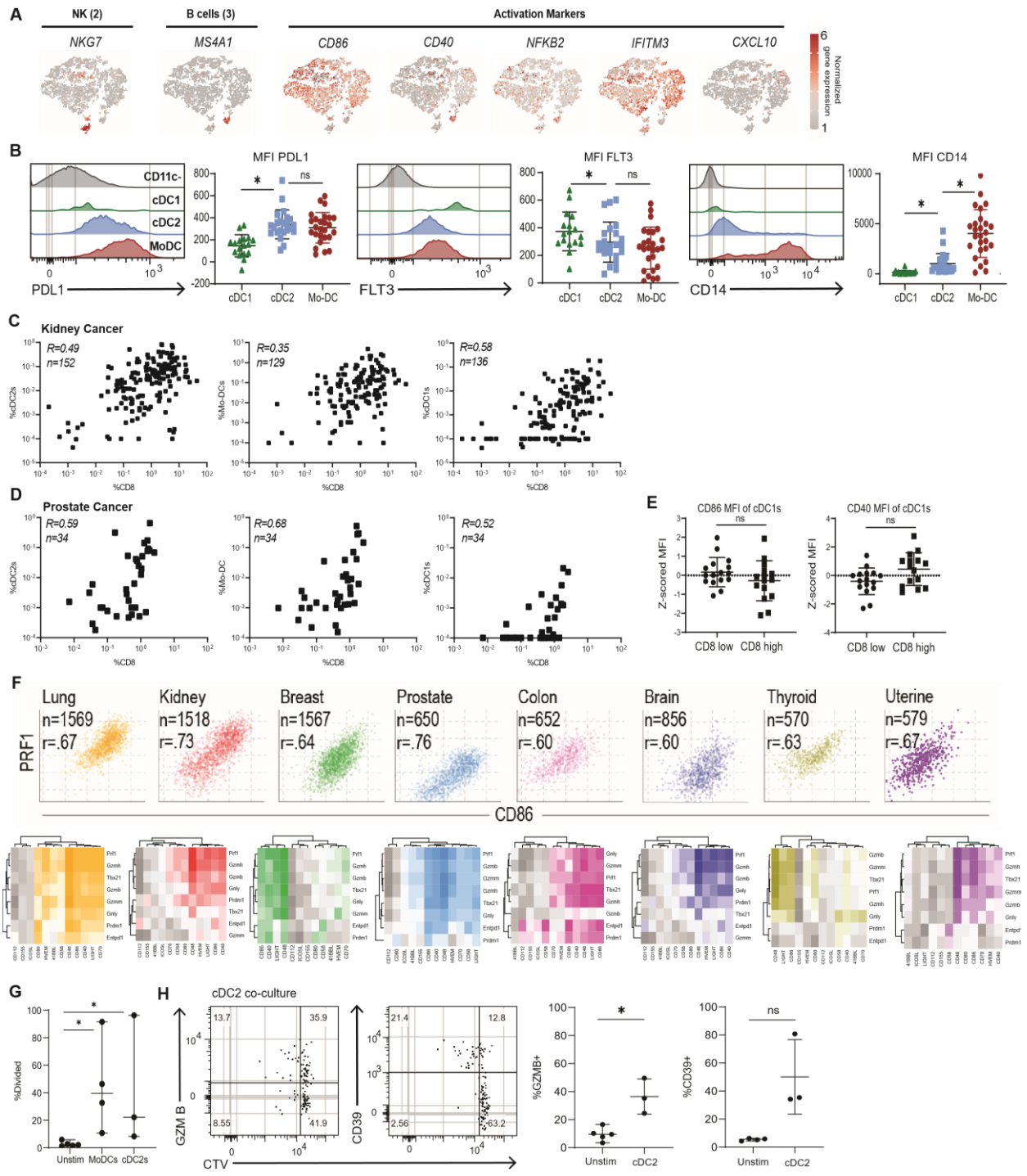

Figure S2. All DC subsets correlate to total CD8 T cell infiltration A) Normalized gene expression of selected genes from human kidney tumor single-cell RNA-seq analysis B)

Phenotype of CD11c- cells, cDC1s, cDC2s, mo-DCs in human kidney tumors shown. Summaries of geometric MFI C) and D) Correlation of each DC subset to CD8 T cell infiltration as percent of total cells in 152 kidney tumors and 34 prostate tumors E) Expression of CD86 and CD40 on cDC1s in kidney tumors, high and low CD8 T cell tumors stratified based on median of samples analyzed F) TCGA analysis of top 8 tumor types shown by tumor. Correlation of *CD86* expression by *PRF1* for each tumor shown, with heatmap correlations between other co-stimulatory molecules versus effector and cytotoxic genes below G) Summary plot shows the proportion of divided (based on CTV dilution) CD8 T cells alone or in the presence of each DC subset H) Representative plots showing CTV dilution and phenotype after 7 days of stem-like CD8 T cell co-culture with cDC2s. Summary plots show the percentage of cells that are positive for the indicated marker after co-culture. Medians and 95% CIs are represented. *, $P<0.05$ determined by Mann-Whitney test

Fig S3. Effect of signal three cytokines, Il12 and Type I IFN, on human stem-like CD8 differentiation.

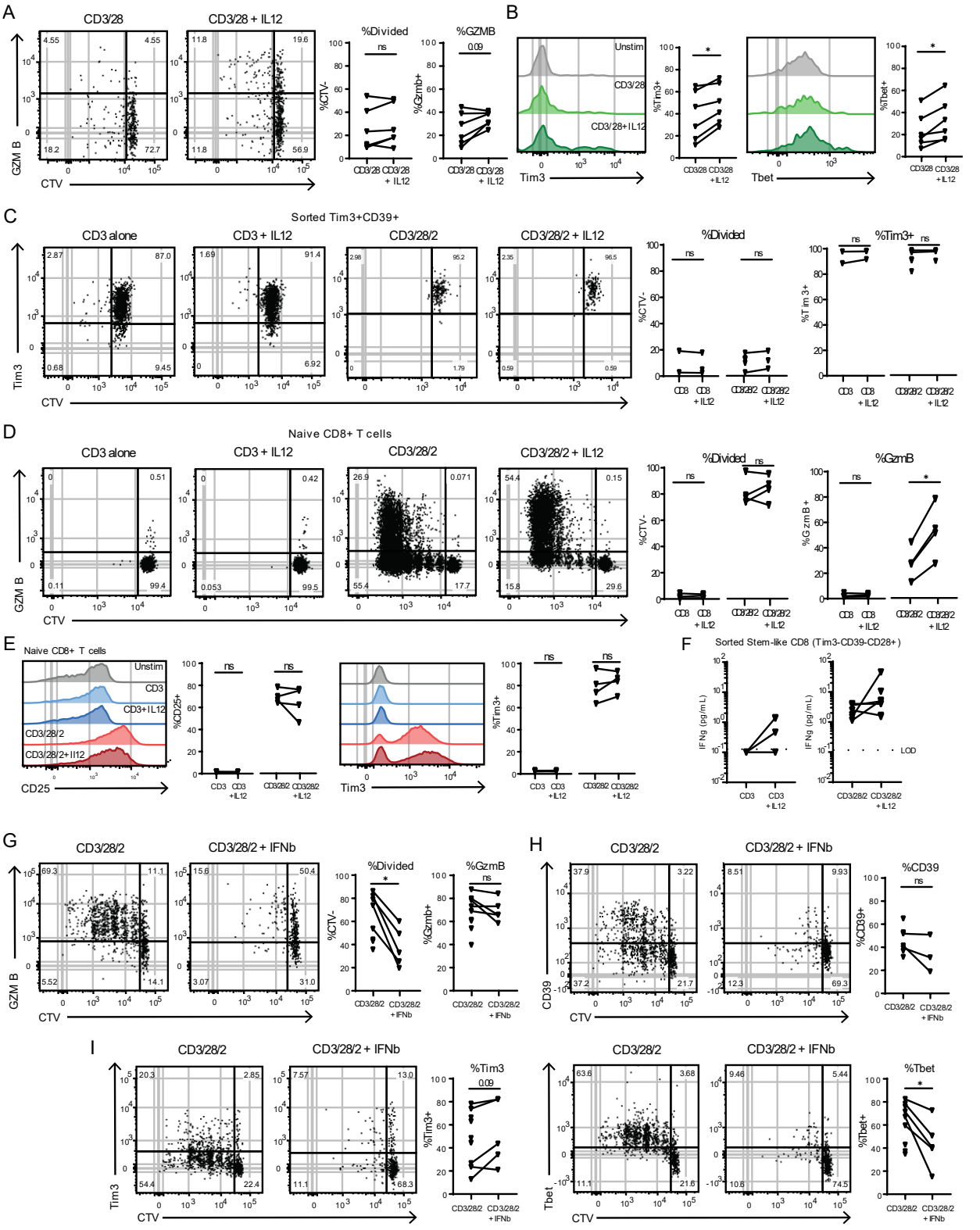

Figure S3. Effect of signal three cytokines, Il12 and Type I IFN, on human stem-like CD8 differentiation. A) Representative plots showing CTV dilution and expression of GZMB of *in-vitro* stimulations of stem-like CD8 T cells with CD3/28 and added IL12. B) Histograms showing expression of TIM3, and Tbet C) Representative plots showing CTV dilution and expression of TIM3 of *in-vitro* stimulations of sorted TIM3+CD39+ CD8 T cells are shown. D) Representative plots showing CTV dilution and expression of GZMB of naïve CD8 T cells stimulated with CD3 alone, or CD3/28/2 with added IL12. E) Histograms showing expression of CD25 and TIM3 for naïve CD8 T cells stimulations. F) Concentration of IFNγ (pg/mL), measured by ELISA, in supernatants of stem-like CD8 T cell *in-vitro* cultures in CD3 and CD3/28/2 stimulating conditions, with and without IL12. G) Analysis of *in-vitro* CD3/28/2 stimulation of stem-like CD8 T cells with added IFNβ. H) and I) Representative plots showing CTV dilution and phenotype of after 5 days of stimulation with or without added IFNβ. *, $P<0.05$ determined by Wilcoxon test when sufficient paired samples were analyzed, shown as connected by a line.

DISCUSSION

In this thesis we aimed to understand how CD8 T cells activate in response to tumor antigen, and how the newly described stem-like CD8 T cells arise in the tumor. The current paradigm of T cell activation, described as the cancer immunity cycle, assumes that CD8 T cells are activated in TDLNs and migrate into the tumor. We wanted to advance the current paradigm and take the stem-like CD8 T cell model into account by studying the early activation of tumor-specific CD8 T cells and their differentiation within the tumor. We found that unlike canonical CD8 T cell activation seen against viral infections where T cells rapidly acquire effector function, tumor-specific CD8 T cells proliferate in tumor draining lymph-nodes but fail to upregulate effector molecules. Instead, they acquire an activated but undifferentiated phenotype resembling the stem-like program. In human TDLNs, these cells could have been in this state for years. These undifferentiated cells instead upregulate co-stimulatory receptors and several chemokine receptors and slowly migrate to the tumor. Only once in the tumor do these cells acquire a transcriptional and epigenetic program that resembles normal effector cells. The differentiation in the tumor from the stem- to effector state requires both TCR signaling and co-stimulation, which is an unexpected requirement for T cells to need again several days after their primary activation. Cytokines like Type I IFNs and IL12 can also play an important role in regulating this differentiation but are not sufficient to differentiate stem-like CD8 T cells in the absence of co-stimulation. Based on these observations we propose the two-step activation model as the best explanation for how T cells respond to cancer. This model has several implications for how we understand the T cell response to cancer and develop future therapies.

The most important point that this work highlights is that the signals that drive differentiation of T cells to a cytotoxic state in cancer do not occur during the initial activation phase, as what we

and many others have found in viral infections [17-19, 79, 80]. Instead, the canonical 3 signals that people have described in viral infections are required, but only once these cells have previously been activated in TDLNs and migrated to the tumor [80-82]. This finding highlights a critical role of the tumor microenvironment to provide the correct signals to cause this stem- to effector differentiation and highlights an obvious defect that can occur during the immune response to cancer; the breakdown of this signaling resulting in a loss of cytotoxic CD8 response to the cancer.

Differences between viral infections and tumor initiation and innate immune response

Since CD8 T cells must be activated through APCs presenting cognate antigen on MHC-I, they have a critical role at both phases of CD8 T cell activation. We have shown the strikingly different phenotype of activated CD8 T cells from acute viral infection of LCMV Arm, and in response to tumors. It is possible that the differences that we have seen in CD8 T cell activation is mediated by differences in the activation of APCs. The innate immune system has a crucial role to sense DAMPS and PAMPs from viral infections or tumors to initiate the immune response, and then activate the adaptive immune response. To understand the differences in CD8 T cell activation in response to viral infections and tumors we must understand how the innate immune response also differs. One of the major differences in the response to viral infections and cancer is the initiation of the innate immune response during the early stages of infection compared to tumorigenesis.

Innate immune response to viral infections

The innate immune response is rapidly activated in response to viral infections, where infected cells rapidly release pro-inflammatory cytokines such as IL1, IL6 and Type I IFN that can recruit and rapidly activate antigen presenting cells. These APCs can aid in clearing infected cells through phagocytosis and take up antigen and migrate to secondary lymphoid organs to activate T cells.

This pro-inflammatory response of the innate immune system rapidly activates CD8 T cells to acquire an effector phenotype in acute viral infections. How the innate immune system activates in response to tumorigenesis is not as well defined compared to the response to viral infections. The striking differences of tumorigenesis compared to infections is the lack of a rapid pro-inflammatory response that activates the innate immune system. Since early phases of tumorigenesis occurs through the transformation of normal cells to cancerous cells that have escaped the immune system [83], there are no viral PAMPS to activate the innate immune system. This leads to an entirely different innate immune response, where APCs are not directly infected or rapidly activated by release of pro-inflammatory cytokines by virally infected cells. In tumors, the signals that activate the immune system rely on danger-associated molecular patterns (DAMPs) such as extracellular ATP, heat shock proteins, hydrophobic aggregates, reactive oxygen species and nucleic acids. These signal that there has been tissue damage and cell death, which can elicit an immune response [84]. Importantly, the DAMPs released by necrotic cells can activate DCs and induce T cell proliferation, whereas apoptotic cell death does not induce the same type of DC activation and T cell activation [85]. This has been shown in both mouse and human DCs that are stimulated and activated by necrotic syngeneic cells or necrotic tumor cells, from melanoma, kidney adenocarcinoma, and thymoma cell lines. DCs activated by necrotic tumor cells are capable of activating antigen-specific CD8 T cells [86]. Numerous tumor derived factors that are released after necrotic cell death can activate APCs and promote the production of pro-inflammatory cytokines. Studies have shown the STING pathway, which senses cytosolic DNA and leads to IRF3 activation and production of Type I IFN, activates APCs through tumor-derived DNA [87]. Other possible factors that activate PRRs and can activate innate immune cells include HMGB1, a chromatin binding protein, that may signal through TLR4 to activate APCs [88]. Overall, the

activation of the innate immune system is drastically different in viral infections compared to tumors. This shows one of the potential mechanisms that leads to either the acquisition of an effector program or a stem-like program during CD8 T cell activation due to the different activation of APCs in both of these immune responses. The innate immune system is indispensable to activate CD8 T cells, by understanding how the innate immune system responds to tumors we can learn how to augment the CD8 T cell response as well.

Innate immune response to tumors

The innate immune response to tumors is not fully understand, especially during early tumorigenesis when the initial activation of both APCs and CD8 T cells occurs. Several studies have now shown the critical role of Type I IFN early during tumorigenesis to control immunogenic tumors that are normally rejected in a T cell dependent manner[89]. When Type I IFNs were blocked early after tumor inoculation, tumor control was completely lost, but when they are blocked at later timepoints of tumor growth there was no significant effect on tumor control. The specific necessity of type I IFN early shows the necessity of pro-inflammatory response for the innate immune response. Follow up studies showed that type I IFN signaling was specifically needed on DCs and not directly on CD8 T cells, confirming that activation of DCs is critical for the CD8 response to tumors. Lack of Type I IFN signaling on cDC1s also led to diminished CD8 T cell activation in TDLNs [34, 90, 91]. Recent work in progressing versus regressing tumor models showed that CD11b+ DCs from regressing tumors had a distinct Type I IFN response gene signature (ISG). Importantly, these ISG+ DCs could present tumor antigens to CD8 T cells, expressed higher levels of co-stimulatory molecules, and could induce protective immunity [92]. The production of type I IFN from either tumor cells or innate cells through PRR sensing is a crucial mechanism of innate cell activation in response to cancer that has drastic effects on the

activation of CD8 T cells. It is not clear if other pathways in non-regressing tumors that activate DCs are also critical for CD8 T cell activation in both TDLNs and tumors. These studies show the clear importance of activation of APCs early during tumorigenesis, and the vastly different CD8 T cell response and tumor clearance. Compared to viral infections where the innate immune system is activated rapidly and there is a rapid increase in pro-inflammatory cytokines, in tumors the release of DAMPs could often occur slowly and asynchronously as more tumor cells undergo necrotic cell death. Compared to the large burst of both antigen and immune activation we have seen in viral infections, in tumors the initial innate response might not be sufficient to mount a rapid CD8 T cell response. Since the T cell response heavily relies on APCs becoming activated and presenting tumor antigens a lack of necrotic cell death or activation of APCs through PRRs and DAMPs, could lead to a failure to activate CD8 T cells in TDLNs and differentiate in the tumor. We have shown that tumor-specific CD8 T cells must be activated in TDLNs, before migrating into the tumor to acquire an effector program. If DCs are not activated in response to the tumor to migrate into TDLNs, and fewer CD8 T cells are activated in TDLNs this potentially leads to impaired CD8 T cell infiltration into tumors. The activation of both DCs and CD8 T cells early during tumorigenesis is clearly important since the lack of early activation of DCs leads to impaired tumor control. Further work is necessary to understand how the innate immune responds to very early tumor transformation and how that can affect the activation of CD8 T cells in TDLNs.

From our work on the two phases of CD8 T cell activation, the second phase that occurs within the tumor also requires APCs within the tumor to provide additional antigen presentation and importantly additional co-stimulation. There are clearly two roles of APCs in response to tumors, one within the TDLNs and one within the tumor. Many studies have now shown the necessity of migratory DCs to activate T cells within TDLNs, but our study raises additional questions about

the role of different DC subsets within the tumor. We have shown that in both human kidney and prostate cancer the major DC subset within the tumor are mo-DCs, followed by cDC2s and with limited infiltration of cDC1s. Others have also reported that cDC1s makeup a rare population of DCs within the tumor, even though they are thought to be critical for the CD8 T cell response due to their ability to cross-present exogenous antigens [69, 70, 72]. The role of the resident tumor DCs has not been well described, we have shown that expression of CD86 on Mo-DCs correlates with improved CD8 T cell infiltration, and importantly *ex-vivo* these APCs can differentiate autologous stem-like CD8 T cells. These data suggest a previously underappreciated role for Mo-DCs within tumors to induce CD8 T cell differentiation but does not definitively prove that these are the DCs that are crucial for the second phase of CD8 T cell activation *in-vivo*. The activation of APCs in our prostate cancer mouse model promoted CD8 T cell differentiation and the acquisition of the effector phenotype within the tumor, demonstrating a connection between CD8 T cells and APC activation within the tumor *in-vivo*. Even though the importance of DCs for the CD8 T cell response to tumors has been extensively studied, the two separate roles of DCs within TDLNs and tumors has not been defined. Important questions remain in how DCs are recruited into the tumor, how they are activated within the tumor, what signals maintain them within the tumor or induce migration.

Implication of two phases of T cell activation on immunotherapy

Two distinct phases of T cell activation in tumors are a crucial addition to our understanding of why there is such a wide range of CD8 T cell infiltration in human cancer. We now understand that there are many steps that must occur for CD8 T cells to infiltrate tumors, and therefore many possible breakdowns in the immune response that can prevent high CD8 T cell infiltration. Current immunotherapies have focused on increasing CD8 T cells within the tumor by blocking inhibitory

receptors, through checkpoint blockade, but few therapies have considered the activation of tumor-specific CD8 T cells in secondary lymphoid organs. Most studies have focused exclusively on CD8 T cells within the tumor, and therefore missed the first phase of CD8 T cell activation in TDLNs that we have defined in this work. These LN-stem CD8 T cells that are activated in TDLNS, share TCR overlap with both stem-like and TD CD8 T cells within the tumor, in kidney and prostate cancer, and are most likely the precursors to the CD8 T cell subsets within the tumor. Additionally, we have shown using mouse models that tumor-specific CD8 T cells are activated in TDLNs before migrating to the tumor to establish both stem-like and TD CD8 T cells. These data show that the critical first step of the CD8 T cell activation occurs in TDLNs, therefore the first breakdown of the CD8 T cell response to tumors can also occur in TDLNs. For a productive CD8 T cell response within the tumor, there must be tumor-specific CD8 T cells within the TDLNs that are activated, proliferate, upregulate chemokine receptors and can migrate into the tumor. Without this step, it is unlikely that tumor-specific CD8 T cells will migrate into the tumor to establish the anti-tumor response. In the cases where patients lack CD8 T cell infiltration within the tumor, the breakdown of the immune response could be lack of sufficient CD8 T cell activation within TDLNs. The lack of the CD8 T cell activation within the TDLNs is most likely related to APCs that are not presenting tumor-associated antigens in the secondary lymphoid organs. Recent studies have shown the necessity of migratory CD103+/CCR7+ DCs that can cross-present exogenous antigens, migrate to TDLNs and activate tumor-specific CD8 T cells. Lack of migratory cDC1s, or lack of CCR7 expression by DCs greatly diminished the activation of CD8 T cells and resulted and lack of tumor control [64, 70]. This demonstrates the irreplaceable role of APCs migrating from tumor to TDLN to activate CD8 T cells, and how the lack of activated APCs within the tumor can lead to lack of sufficient CD8 T cell activation. In patients with extremely low CD8

T cell infiltration in tumors, checkpoint blockade such as anti-PD1, which increases CD8 T cell stem- to effector- differentiation, most likely will not be effective since there could be a lack of CD8 T cell activation in TDLNs. This subset of patients could instead benefit from other therapeutics that can activate APCs within the tumor that can transport tumor-antigen to TDLNs to initiate the CD8 T cell response, such as vaccination or TLR agonists. Currently the only successful vaccine-based immunotherapy is used in prostate cancer, GVAX, which is typically a "cold" cancer with very limited CD8 T cell infiltration [93]. TLR agonists such Imiquimod, which activate APCs through TLR7, are also currently used to treat basal cell carcinoma [94]. Both therapeutics are directed to increase APC activation within the tumor, and most likely leads to increased antigen presentation and migration of APCs to TDLNs. In line with this, increased percentage of DCs in the blood of prostate cancer patients that receive the GVAX vaccine in combination with anti-CTLA4, predicted improved response [93]. Understanding how current available therapies modulate the response within the TDLNs as well as the tumor is crucial in improving the responses in patients with low overall CD8 T cell infiltration in tumors. Overall, the activation of APCs and improved presentation of tumor antigens to CD8 T cells in TDLNs is a possible therapeutic approach to improve the first phase of CD8 T cell activation. Current immunotherapies that are focused on the CD8 T cell response within the tumors alone are only effective when there are already CD8 T cells there. This has been shown in clinical responses to checkpoint blockade, since CD8 T cell and stem-like CD8 T cell infiltration predicts response [95]. Since only around 20-30% of patients respond to checkpoint blockade it is important to take activation of CD8 T cells within the TDLN into account in the subset of patients that are non-responders, and whether combination therapies with cancer vaccines or TLR agonists will be more effective. There have been limited studies on the effect of PD1 blockade specifically in TDLNs.

Recent work shows that targeting anti-PDL1 antibodies to TDLNs leads to improved tumor control and increased proliferation of tumor-specific CD8 T cells within the TDLNs [96]. Further work is necessary to determine if checkpoint blockade is effective in both TDLNs and tumor to improve CD8 T cell activation and differentiation. How to improve the first phase of CD8 T cell activation in cancer is an important question for the future of immunotherapy and could improve the overall response rates to current checkpoint blockade-based immunotherapies.

The CD8 T cell response to tumors can also breakdown in the second phase of CD8 T cell activation, the stem- to effector- differentiation within the tumor itself. Since patients with high overall CD8 T cell differentiation also have a larger proportion of TD effector-like CD8 T cells, a constant differentiation from stem-like to effector CD8 T cells is necessary for a successful CD8 T cell response [32]. In the second phase of CD8 T cell activation that we have described in this work, we show that the CD8 T response in tumors is established by stem-like CD8 T cells migrating from TDLNs. Even though these cells have been previously activated, they require additional TCR as well as co-stimulation to fully acquire the effector program and differentiate into TD CD8 T cells. When human stem-like CD8 T cells are stimulated *ex-vivo* without co-stimulation, they fail to divide or differentiate. This differentiation can also be augmented partially by cytokines such as IL12, that can improve the acquisition of the CD8 T cell effector program in situations with low co-stimulation. Current immunotherapy approaches focus on checkpoint blockade that can improve stem-like CD8 T cell differentiation, proliferation, and function by blocking the negative signaling. Anti-PD-1, for example, promotes stem-like CD8 T cell differentiation to the effector TD subset, and improves CD8 T cell cytokine expression and proliferation [23, 27]. Previous studies have also shown that the CD8 T cell response to anti-PD1 is dependent on co-stimulation through CD28. When the CD28 was blocked, antigen-specific CD8

T cells failed to proliferate in response to PD1 blockade, which correlated with decreased viral and tumor control [53]. Together these data demonstrate the critical role of co-stimulation through CD28, for both CD8 T cell differentiation and proliferation in tumors and response to checkpoint blockade. This shows the necessity of not only blocking inhibitory receptors and negative signals, but the necessity of additional positive signals through co-stimulation or pro-inflammatory cytokines for stem-like CD8 T cell differentiation. This is an important consideration for future immunotherapy, that focuses on the differentiation of CD8 T cells within tumor since the addition of co-stimulation could promote increased CD8 T cell proliferation and differentiation.

The second phase of CD8 T cell activation within the tumor is an obvious part of the CD8 T cell response that breaks down in the response to tumors. In our work we have shown that patients with high CD8 T cell infiltration had Mo-DCs (CD11b+) that express higher levels of both co-stimulatory molecules of CD86 and CD40. Since stem-like CD8 T cells require both TCR and co-stimulation, the most likely source of this within tumors are APCs that have been activated and present tumor-associated antigens to stem-like CD8 T cells. This is corroborated by previous work that shows that stem-like CD8 T cells reside within an immune niche in kidney cancer and are significantly closer to APCs compared to TD CD8 T cells. The organization of stem-like CD8 T cells near APCs in immune niches also correlated to overall CD8 T cell infiltration and response to anti-PD1[95]. These studies combined with previous work on anti-PD1, show the clear necessity of positive signals from APCs within the tumor for response to PD1. Other than co-stimulation from APCs, recent studies have also shown that IL12 from DCs in the tumor promotes CD8 T cell differentiation and response to PD1 [51]. Many of these studies have focused on activated DCs that express both cytokines such as IL12, but also have high expression of co-stimulatory molecules. This demonstrates that both co-stimulation and pro-inflammatory cytokines contribute

to CD8 T cell differentiation and can promote improved responses to checkpoint blockade [51]. Future combinations of immunotherapies should focus not only on blocking inhibitory receptors but also providing additional co-stimulation through activation of APCs that promotes effective responses to checkpoint blockade.

The final therapeutic implication for the two phases of CD8 T cell activation in cancer is related to adoptive cell therapy (ACT) or engineered chimeric-antigen-receptor (CAR) T cells. ACT and CAR-T therapy has historically not been as successful in solid tumors compared to blood cancers [97]. Recent work has shown that responders to ACT have an enrichment of stem-like TILS within their tumors. Importantly, these cells retained proliferative capacity and maintained tumor recognition, and most likely retain ability to differentiate to effector-like CD39+ CD8 TILs [98]. This shows how the stem-like model of CD8 T cell exhaustion needs to be considered when working on expanding TILS *ex-vivo* for reinfusion and ACT, since stem-like CD8 T cells seem to provide improved responses. These stem-like TILs from ACT resemble the endogenous stem-like CD8 T cells that our group and many others have described in human tumors. The clear advantage of having stem-like TILs in ACT is their ability to survive, proliferate and give rise to a constant stream of effector-like TILs. It is currently not clear if ex-vivo expanded ACT products or CAR-T cells require the same signals as endogenous tumor-specific CD8 T cells to respond to tumors after re-infusion. We have shown that when naïve tumor-specific CD8 T cells need to be activated in TDLNs before they can migrate into the tumor and fail to acquire an effector phenotype within the TDLNs. It is currently not clear whether CD8 T cells that are activated and expanded *ex-vivo* must first migrate to TDLNs to receive additional activation signals, or if they can directly migrate into the tumor. A possible reason that ACT and CAR-T cell therapy has not been as successful in solid tumors is the necessity of peripheral activation in TDLNs to increase expression of crucial

chemokine receptors such as CXCR3 to allow CD8 T cells to migrate into the tumor [64, 99]. Unpublished data from our lab shows the necessity of CXCR3 for activated CD8 T cells to migrate from TDLNs to tumors to initiate the response within the tumor. These data demonstrate the necessity of proper chemokine receptors for ACT or engineered CAR-T cells to migrate into the tumor. Additional setbacks for ACT could arise from a lack of a sustained ACT/CAR T cell populations within the tumor. It is not clear if other cells within the tumor microenvironment that support the endogenous CD8 T cell response are necessary for ACT. The necessity for APCs for continual differentiation of ACT/CAR-T cells is also not clear, both within the TDLNs as well as tumor. Stem-like ACT TILs might still require additional signals within the tumor microenvironment to fully differentiate to acquire and effector phenotype, like endogenous tumor-specific CD8 T cells. We have shown the necessity of co-stimulation within the tumor to induce the differentiation of endogenous tumor-specific CD8 T cells. It is not clear whether ex-vivo expanded TILS that are used for ACT have the same requirements, specifically stem-like ACT products that have been shown to predict better response to therapy. This is an important question for ACT therapy, since tumors with low T cell infiltration also have lower DC infiltration, thus when these patients receive ACT they may not have sufficient APCs within the tumor to support the large numbers of TILs that are re-infused. Even though ACT has improved over the last decade, lack of response in solid tumors remains a major issue for this therapy to be broadly effective. By considering the two phases of CD8 T cell activation, CAR-T cells could be engineered to bypass the endogenous CD8 T cell step of initial activation in TDLNs and directly migrate into the tumor. Additionally, the role of APCs must be considered for ACT and CAR-T cell therapy and other signals that are necessary to maintain and differentiate these cells within the TME.

Overall, there are many clinical implications for the two phases of CD8 T cell activation that we have shown in this work. This model updates the current stem-like CD8 T cell model of exhaustion in tumors by describing two distinct phases that must occur for CD8 T cells to acquire an effector-program in response to cancer. Tumor-specific CD8 T cells activate to acquire a stem-like program and fail to acquire an effector program, until they migrate into the tumor to receive additional TCR and co-stim. This new model of CD8 T cell activation must be considered when designing future immunotherapies, since the breakdown of the CD8 T cell response can occur either in TDLN or tumor. Since CD8 T cells need to see cognate antigen in both TDLNs and within the tumor, APCs have a critical role in controlling the CD8 T cell response. The presence and activation of APCs within the tumor must be considered, both to migrate into the TDLNs to present antigen and to support the CD8 T cell response within the tumor directly. The future of immunotherapy must combine the activation of APCs within the tumor that provide necessary signals to CD8 T cells in both locations to promote CD8 T cell activation at both phases. Why the innate immune response breaks down and fails to initiate a sufficient CD8 T cell response remain an important unanswered question

CONCLUSIONS

In this thesis we have described a two-step model of CD8 T cell activation in cancer. This has changed the current paradigm of CD8 T cell activation. In this work we have shown that naive tumor-specific CD8 T cells activation occurs in TDLNs, and not directly within the tumor. Moreover, these activated tumor-specific CD8 T cells fail to acquire a canonical CD8 T cell effector program that is rapidly acquired in response to acute viral infections, such as LCMV Arm. Instead, the acquire a LN-stem program, where they fail to express effector-associated molecules and retain a more stem-like phenotype with high expression of TCF1, chemokine receptors and co-stimulatory molecules. These cells are extremely functional, can produce numerous cytokines such as IFNg, TNFa and IL2. We have also determined that LN-stem CD8 T cells are the precursors to both tumor stem-like and TD CD8 T cells. Importantly, the same populations of CD8 T cells exist in both human kidney and prostate cancer, where we have shown TCR overlap between LN-stem, tumor stem-like and TD CD8 T cells in numerous patients. This work has clearly demonstrated the origin of tumor stem-like CD8 T cells are activated tumor-specific CD8 T cells that are activated within TDLNs and then migrate into the tumor as a stem-like CD8 T cells

The second part of this thesis focused on understanding the signals that drive CD8 T cell differentiation within the tumor. Since LN-stem CD8 T cells seed the anti-tumor response as stem-like CD8 T cells, we focused on understanding what signals specifically within the tumor could drive differentiation of stem-like CD8 T cells into TD. From previous work we have shown that stem-like CD8 T cells reside near dense areas of APCs, called immune niches. We analyzed the APC populations further, to find that tumor-resident APCs expressed significantly higher co-stimulatory molecules CD80 and CD86. We found that stem-like CD8 T cells need additional co-stimulation within the tumor to differentiate and acquire the more effector TD program. In

conclusion the novel two-step model of CD8 T cell activation in cancer changes the current paradigm of T cell activation and improves upon the widely accepted cancer immunity cycle [34]. This work shows the importance of CD8 T cell priming within TDLNs, and a secondary hit of TCR and co-stimulation within the tumor. It also shows the indispensable role of APCs, both antigen presentation of tumor antigens within TDLNs, and additional co-stimulation within the tumor. Overall, this work has improved our current understanding of how CD8 T cells response to cancer and has wide implications for future immunotherapies, from checkpoint blockade to adoptive T cell therapy.

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