Naïve T Cell Functional Heterogeneity

by

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DEDICATION

I would like to dedicate this to my family and friends without whom this work would not have been possible.

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God for guiding me here.

I would like to thank my mentors Weiping Zou and Ilona Kryczek for all their support throughout these past years. Their knowledge and expertise have guided me as I become a better scientist. I hope to one day emulate your way of thinking and approaching a scientific question. My co-workers for always offering input and ideas into the work I was carrying out, the laughs we have shared, and the family we have become. Especially Tomasz, Nisha, Weimin, Weider, Linda, and Mirna for their friendship. My committee members, collaborators and members of the immunology program for offering their knowledge, time, and resources throughout the years.

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ABSTRACT

Naive T cells are recently shown to be a heterogeneous compartment of cells with distinct phenotypical, regulatory, and functional characteristics. Currently, little is known in regards to their functional characteristics prior to antigen stimulation and their involvement in anti-tumor immunity. This dissertation aims to understand the contributions of naïve T cells to tumor immunity.

The first project explores CXCL8$^+$ naïve T cells in humans and how they can promote tumorigenesis. We found naïve T cells express CXCL8 without prior stimulation, and are primed for CXCL8 expression upon activation as compared to memory T cells and other effector cytokines. We suggest CXCL8$^+$ naïve T cells may be important for tumorigenesis as co-injection of naïve T cells with cancer cells in a mouse model promotes tumor cell growth in a CXCL8-depenedint manner.

The second project studies distribution of CD28H, a novel B7-family member, and the functional state at which naïve and memory T cells find themselves in adult peripheral blood, ovarian cancer patient blood and multiple tumors. CD28H was detected on T cells, primarily on naïve T cells, innate lymphoid cells (ILCs), natural killer (NK) cells, and plasmacytoid dendritic cells (PDCs). We found that CD28H marks undifferentiated T cells with lower effector function and suggest a potential role of CD28H shaping innate and adaptive immunity.

The third project studies the consequences of loss of Dot1L in T cell phenotype, function and gene regulatory networks. Loss of Dot1L led to loss of naïve T cells in the periphery, increased apoptosis due to decreased STAT5 signaling and

decreased cell cycle progression. Furthermore, Dot1L-deficeint T cells showed decreased anti-tumor and effector functions *in vivo* and *in vitro*.

Very little is known regarding naïve T cells in the tumor microenvironment due in part to their discreet intra-tumoral numbers. Here we explore how naïve T cells promote tumor growth and their differentiation state in various tumors. Lastly, we show Dot1L is important for T cell homeostasis and function.

Chapter 1

Introduction

This dissertation describes three projects that aim to understand naïve T cell functional heterogeneity and their involvement in tumor biology. The following introduction describes what is known in regards to naïve T cell subsets, underlying regulatory mechanisms, and functional capacity. We will argue that naïve T cells, similar to effector and memory T cells, are heterogeneous and perform active functions prior to experiencing specific antigen activation. We also study epigenetic mechanisms which directly affect T cell homeostasis and anti-tumor function in various *in vivo* tumor models. This chapter will provide a specific introduction and objectives for each project that was undertaken.

1.1 Naïve T cell biology

Our immune system is equipped with various tools aimed for immediate elimination of antigenic insult and development of long-term defenses that depend on innate vs. adaptive immunity. As such, we are born with all the tools needed for our survival but in a naïve state that asks for education against such antigens. Naïve T cells, as per the current dogma, exist in a quiescent state and acquire effector characteristics upon specific antigen stimulation in the context of co-stimulatory molecules. Nevertheless, recent studies reveal the inherent differences of naïve T cells based on phenotype, cytokine expression, and genetic regulatory mechanisms.

1.1a Naïve T cell homeostasis and maturation

Human naïve T cells are commonly described by their surface marks $CD45^{+}CD3^{+}(CD4^{+}$ or $CD8^{+})CD45RA^{+}CD45RO^{-}CD62L^{+}CCR7^{+}$, their restricted cytokine expression patterns, high T cell receptor excision circles (TRECs) and increased T cell receptor repertoires (De Rosa, Herzenberg, Herzenberg, & Roederer, 2001; Hebel et al., 2014; Naylor et al., 2005; Zaghouani, Hoeman, & Adkins, 2009). Naïve T cells are developed in the thymus where they are selected through their T cell receptors capable of reacting to antigens presented in the context of major histocompatibility complex (MHC). Various studies have shown that naïve T cell heterogeneity can be traced to those that have recently egressed from the thymus, $CD31^{+Thymic}$ naïve T cells, and $CD31^{-Central}$ naïve T cells which derive from $CD31^{+Thymic}$ naïve T cells that have homeostatically proliferated in the periphery (Fig 1.1).

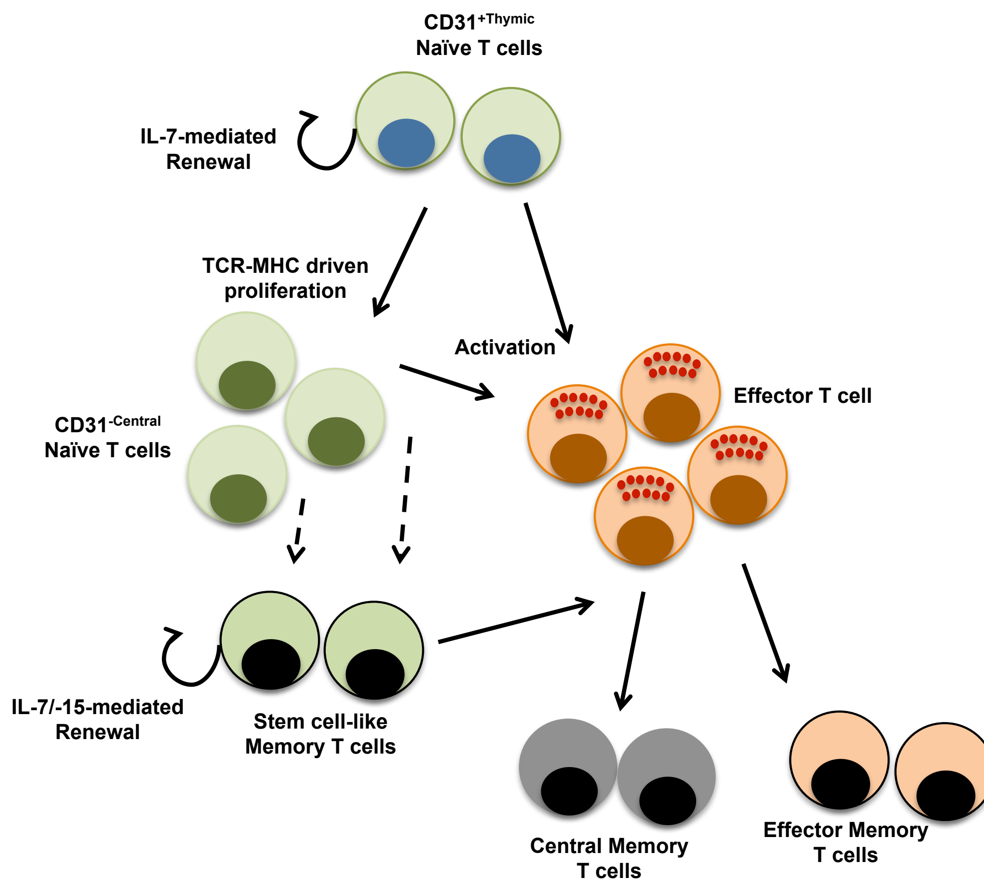


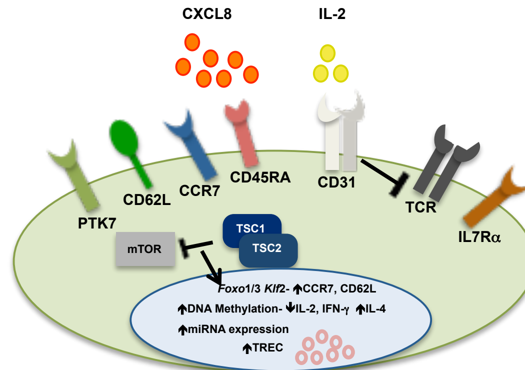
Figure 1.1. Naïve T cell subsets and maturation. CD31$^{+Thymic}$ naïve T cells that have been selected for in the thymus depend on IL-7 for homeostatic proliferation and self-renewal. Upon low-affinity TCR-MHC interactions, CD31$^{+Thymic}$ naïve T cells will proliferate and lose CD31 thereby becoming CD31$^{-Central}$ naïve T cells. Both CD31$^{+Thymic}$ and CD31$^{-Central}$ naïve T cells can be activated in the context of TCR and co-stimulation and give rise to effector T cells which may either die off or become central or effector memory T cells. Stem cell-like memory T cells are thought to arise by incomplete differentiation following antigen stimulation and represent a rare subset of long-lived, multipotent T cells.

CD31$^{+Thymic}$ naïve T cells constitute the major naïve T cell population in umbilical cord blood and newborns (Kilpatrick et al., 2008). In the periphery, they can be identified using PTK7 and/or CD31 on their surface (Haines et al., 2009; Junge et al., 2007; Kimmig et al., 2002). CD31$^{+Thymic}$ naïve T cells, as well as CD31$^{-Central}$ naïve T cells, navigate through the blood homing towards secondary lymphoid organs via CCR7 ligands CCL19/CCL21 and CD62L in search of specific antigen stimulation by antigen presenting cells (APC) (Butcher, Williams, Youngman, Rott, &

Briskin, 1999; Mitra et al., 1999; Picker et al., 1993). Throughout this process, $CD31^{+Thymic}$ naïve T cells will undergo various rounds of proliferation losing PTK7 and CD31 and becoming $CD31^{-Central}$ naïve T cells. This process is actively regulated by common-γ chain cytokine IL-7 and low-affinity interactions between the T cell receptor (TCR). IL-7 signaling depends on the binding between IL-7 and CD132/CD127 receptor which promotes anti-apoptosis Bcl-family member expression while maintaining CD31 expression in $CD31^{+Thymic}$ naïve T cells (Hassan & Reen, 2001; Schluns, Kieper, Jameson, & Lefrancois, 2000; Tan et al., 2001). In contrast, low-affinity TCR-MHC interactions induce maturation and proliferation of $CD31^{+Thymic}$ naïve T cells as they enter the cell cycle and lose CD31 surface expression (Brocker, 1997; Hassan & Reen, 2001; Moses, Thorstenson, Jameson, & Khoruts, 2003; Viret, Wong, & Janeway, 1999; Witherden et al., 2000). As such, IL-7 signaling is crucial for maintaining naïve T cell TCR heterogeneity whereas TCR-MHC interactions induce proliferation of naïve T cells with certain TCR specificity (Fig. 1.2) (Hassan & Reen, 2001; Kohler et al., 2005; Tanchot, Lemonnier, Perarnau, Freitas, & Rocha, 1997; van der Geest et al., 2015).

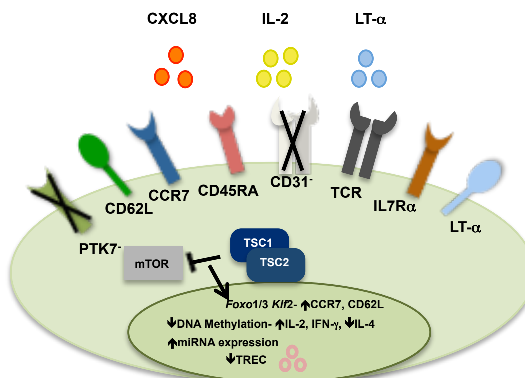
CD31$^{+Thymic}$ Naïve T cells

-Recently exited the thymus
-Slow proliferation rate, IL-7 mediated
-Express CXCL8
-High number of TRECs
-High TCR diversity
-Skewed towards
T_H2 response



CD31$^{-Central}$ Naïve T cells

-Major population in adults
-Maintained homeostatically
-Maintain/sustain lymph nodes
through Lymphotoxin-α



Stem cell-like memory T cells

-Long-lived cells
-IL-7/-15 self-renewal
-Give rise to various subsets (Multipotent)
-Rapid effector responses
-Important against chronic infections

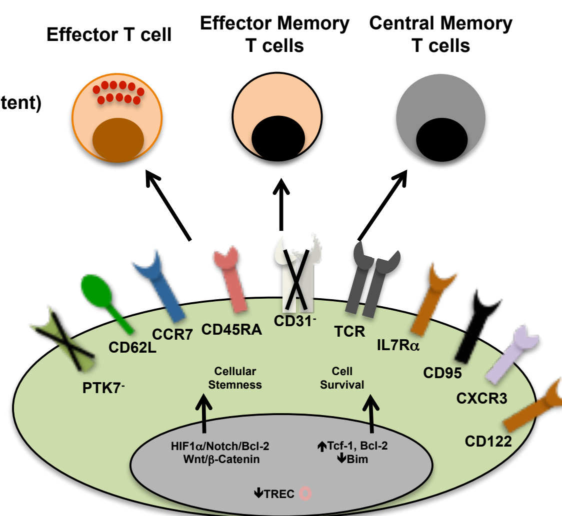


Figure 1.2. General characteristics of CD31$^{+Thymic}$, CD31$^{-Central}$ naïve T cells and stem cell-like memory T cells. Recent studies into the inherent characteristics of these cell stages reveal similarities/differences which drive and maintain functional heterogeneity. (i) CD31$^{+Thymic}$ naïve T cells can be identified based on CD31 surface expression, show high number of TRECs as they have recently been selected for in the thymus, depend on IL-7 for homeostatic proliferation, express CXCL8 and are skewed to T_H2 responses upon activation. (ii) CD31$^{-Central}$ naïve T cells arise from CD31$^{+Thymic}$ naïve T cells that have had TCR-MHC interactions and lose CD31 surface expression and TREC numbers. In contrast to CD31$^{+Thymic}$ naïve T cells, CD31$^{-Central}$ cells have lower CXCL8 expression and are not skewed towards T_H2 responses as they can express IFN-γ and IL-2. (iii) Stem cell-like memory T cells are T cells that have not completely differentiated following activation and therefore

represent a transient subset between the naïve and memory compartments. Stem cell-like memory T cells have high capacity for self-renewal, are long-lived, and are multipotent cells.

Various studies looking into the long-term homeostasis of naïve T cells have shown $CD31^{+Thymic}$ naïve T cells are progressively lost throughout age mainly due to decreased thymic output as thymus involution occurs while $CD31^{-Central}$ naïve T cells are maintained. Newborns and infants are shown to have an increased population of $PTK7^{+}$ and/or $CD31^{+}$ naïve T cells as opposed to adults and people of advanced age (Junge et al., 2007; Kilpatrick et al., 2008). These $CD31^{-Central}$ naïve T cell population is maintained throughout age as observed by longitudinal aging studies where their absolute cell numbers are maintained (Kilpatrick et al., 2008; Sauce et al., 2009). Infant patients which have had their thymus removed resulting in contraction of the naïve T cell pool, specifically the $CD31^{+Thymic}$ naïve T cell population (Sauce et al., 2009). Naïve T cells are also stable in the context of chronic viral infections such as HIV and CMV where these viruses preferentially target proliferating memory T cells (Brenchley et al., 2004; Douek et al., 1998; Kuijpers et al., 2003; Sauce et al., 2009; Snyder et al., 2008). Importantly, TCR diversity in naïve T cells is not lost in elderly humans, despite overall loss of TCR heterogeneity in $CD3^{+}$ T cells (Qi et al., 2014). It is therefore understood that the major source of naïve T cells in adults are maintained through homeostatic proliferation. The progression from $CD31^{+Thymic}$ naïve T cell to $CD31^{-Central}$ naïve T cell is not only marked by surface phenotype, but also by loss of TRECs, epigenetic changes that result in distinct effector responses. Nevertheless, quiescence must be ensured and enforced in the periphery while naïve T cells recirculate through the blood and lymph nodes.

A third population (≈2-3%) of T cells has been described as displaying a naïve phenotype with expression of memory T cell-associated markers CXCR3, CD122,

CD95 and CD11a (Fig. 1.2) (Gattinoni, Speiser, Lichterfeld, & Bonini, 2017). Unlike $CD31^{+Thymic}$ and $CD31^{-Central}$ naïve T cells, stem cell-like T cells (T_{SCM}) rapidly proliferates upon interaction with antigen (similar to memory T cells), express high levels of effector cytokines, have decreased TRECs. Similar to naïve T cells, T_{SCM} cells rely on IL-7 and IL-15 for cellular turnover and display similar distribution in the host's body to naïve T cells. T_{SCM} cells are thought to bridge naïve and memory T cell maturation given they display intermediate phenotype and functions. Their defining characteristic, and where they receive their name from, is their stem-like capacity to self-renew and ability to derive multiple T cell subsets (Cieri et al., 2015; Oliveira et al., 2015; Roberto et al., 2015). Specifically, T_{SCM} cells were shown to reconstitute effector memory, central memory and T_{SCM} cell subsets *in vivo*. Secondly, T_{SCM} cells were able to persist *in vivo* (>10yrs) in humans following transfer of chimeric antigen receptor (CAR) T cells and hematopoietic stem cell transplantation (Biasco et al., 2015; Oliveira et al., 2015; Xu et al., 2014). CAR T cells are T cells engineered with a specific TCR to recognize a specific antigen (Gattinoni et al., 2017). T_{SCM} cells are thought to normally develop throughout the course of disease as naïve T cells become activated (Fig. 1.1). Some evidence suggests T_{SCM} arise due to incomplete differentiation of the naïve T cell to the effector cell. For example, blocking of naïve T cell differentiation to effector/memory, Wnt/β-catenin pathway promoted T_{SCM} formation *in vitro* (Gattinoni et al., 2009). T_{SCM} cells arise in the context of chronic inflammation as observed in bacterial, parasitic and viral infections (Gattinoni et al., 2017). T_H17 cells were also shown to have stem-like characteristics of long-term survival, self-renewal and multipotency (Kryczek et al., 2011; Muranski et al., 2011). As such, they may not be considered strictly speaking as naïve T cells since they have been selected for this function following

activation.

1.1b Molecular regulators of naïve T cell homeostasis

Naïve T cells are classically thought to exist in a quiescent state in their host's body. This quiescent state is characterized by a specific phenotype that allows for recirculation through the blood to secondary lymphoid organs, restricted proliferative and effector responses, and metabolic program. Below we discuss some surface molecules, transcription factors and miRNA which play a regulatory role in naïve T cell homeostasis (Fig. 1.2).

CD31

CD31 (PECAM-1) is an adhesion molecule and immunoglobulin-like receptor involved in leukocyte transmigration, angiogenesis, integrin activation and immune function. CD31 contains an immune-receptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail which upon phosphorylation recruits SH2 domain-containing tyrosine phosphatases (e.g., SHP-1, SHP-2) that directly increase TCR activation threshold by dephosphorylating TCR-engaged tyrosine kinases important for T cell activation, provided CD31 is co-engaged upon antigen stimulation by the antigen presenting cell (Hua, Gamble, Vadas, & Jackson, 1998). This has direct implications on the homeostatic proliferation in secondary lymphoid organs as well as the activation threshold of $CD31^{+Thymic}$ vs. $CD31^{-Central}$ naïve T cells. By hampering TCR signaling, $CD31^{+Thymic}$ naïve T cells require higher affinity binding for homeostatic proliferation to occur, over $CD31^{-Central}$ naïve T cells, and may serve as a peripheral tolerance checkpoint (Clement et al., 2014; Newton-Nash & Newman, 1999; M. X. Wong, Hayball, & Jackson, 2008). Furthermore, low TCR engagement promotes

homeostatic proliferation of naïve T cells but leads to the loss of CD31 expression on $CD31^{+Thymic}$ naïve T cells as opposed to IL-7 signaling which allows CD31 expression be retained (Fornasa et al., 2010; Hassan & Reen, 2001; Moses et al., 2003; Tanchot et al., 1997). Similarly, $CD31^{+Thymic}$ naïve T cell activation requires higher antigenic load to be presented, over $CD31^{-Central}$ naïve T cells, in order to achieve full activation (Newton-Nash & Newman, 1999). In line with this, we know $CD31^{+Thymic}$ naïve T cells proliferate slower and express lower cytokines (IFN-γ and TNF-α) than $CD31^{-Central}$ naïve T cells (Boursalian, Golob, Soper, Cooper, & Fink, 2004). In this, CD31 serves to define a naïve T cell subset and is also a regulatory molecule in itself.

Foxo family and Krupel-like factor 2

Low-affinity TCR-MHC interactions and IL-7 stimulation can lead to the inappropriate activation of naïve T cells in circulation. Transcription factors of the Foxo family (FOXO1 and FOXO3) and Krupel-like factor 2 (KLF2) were originally thought to enforce quiescence and survival in naïve T cells (Modiano, Johnson, & Bellgrau, 2008). These first studies showed that LKLF-deficient T cells showed spontaneous marks of activation on their surface and increased cell death *in vivo* and following activation (Kuo, Veselits, & Leiden, 1997). Similarly, Foxo3-deficient T cells showed spontaneous activation, increased effector cytokine expression, and proliferation compared to wild-type counterparts (Lin, Hron, & Peng, 2004). Later, studies identified these mainly contribute to the naïve phenotype by promoting homing towards lymphoid organs through CD62L and CCR7 expression (Carlson et al., 2006; Kerdiles et al., 2009; Takada et al., 2011).

Tuberous Sclerosis-1

Tuberous Sclerosis-1 (TSC1) has been characterized as a key component regulating naïve T cell quiescence and survival. TSC1 is a tumor suppressor protein that complexes with TSC2 to negatively regulate mammalian target of rapamycyn (mTOR) activity in the cell. Loss of TSC1 in T cells showed decreased naïve T cells in the periphery, larger cell size, increased cell cycling and predisposed cells to hyperactivation and apoptotic cell death, despite stimulation by pro-survival cytokine IL-7 (Q. Wu et al., 2011; Yang, Neale, Green, He, & Chi, 2011; Zhang et al., 2012). In line with this, unregulated mTOR activity in T cells led to decreased Foxo1 and Foxo3 phosphorylation and function as T cells lost their naïve phenotype.

Micro-RNA networks

Micro-RNA (miRNA) are small non-coding RNA molecules which function to modulate gene translation and are shown to have vast regulatory effects in the immune system (Lu & Liston, 2009; Mehta & Baltimore, 2016). As such, specific miRNA programs are observed for T cell subsets that underlie their cellular differentiation and function (Baumjohann & Ansel, 2013; Rossi et al., 2011; Smigielska-Czepiel et al., 2014; H. Wu et al., 2007). miR-125b was shown to enforce naïve T cell quiescence and its loss led to differentiation and gaining of effector responses (Rossi et al., 2011). Further, miR-193b and miR-188-5p were singularly expressed by human naïve T cells showing that miRNA networks have specific signatures and function in distinct cell subsets. In line with this, activation of naïve T cells was shown to dramatically change miRNA expression profiles in naïve T cells whereby effector cytokines and proliferation may progress (Bronevetsky et al., 2013; Muljo et al., 2005). Further, it has been suggested that these miRNA networks are

dynamically regulated in naïve T cells throughout age (G. Li et al., 2012; Li et al., 2007; Palin, Ramachandran, Acharya, & Lewis, 2013).

Taken together, naïve T cell quiescence and homeostasis is dynamically regulated to ensure proper activation occurs in peripheral tissues against specific antigens. By modulating the strength of TCR signaling and enforcing quiescence through transcription factors, naive T cells ensure their naïve status is overcome upon high-affinity TCR-MHC binding in the context of co-stimulation. With this being said, it is becoming apparent that naïve T cells may perform important functions prior to specific antigen stimulation *in vivo*.

1.1c Naive T cell functional heterogeneity

Naïve T cell subsets display a restricted cytokine expression repertoire as part of their quiescent state. Different studies showed naïve T cell subsets have distinct epigenetic signatures skewing them towards a specific response upon activation and underlie their homeostatic characteristics. Together, these underlie heterogeneity in the naïve T cell pool as identified by autocrine survival signals, lymph node homeostasis, and rapid recruitment of innate immune cells by naïve T cell secreted factors.

Naïve T cells are functionally important in lymph node homeostasis. Lymphotoxin (LT) is known to shape immune responses at secondary lymphoid organs. Deficiency of this pathway is known to disrupt compartmentalization of T and B cells, marginal zone and follicular dendritic cell networks, and failure to develop germinal centers in the spleen following immunization (Alimzhanov et al., 1997; Banks et al., 1995; De Togni et al., 1994; Koni et al., 1997). Naïve T cells are a known source of LT and their depletion in non-human primates showed

consequential loss of fibroblastic reticular cell networks (Ohshima et al., 1999; Zeng et al., 2012). LT is also important for tertiary lymphoid organ (TLO) development in autoimmune and malignant diseases (Hiraoka, Ino, & Yamazaki-Itoh, 2016). Consequently, naïve T cells are known to accumulate at TLO's and is very possible they help maintain these structures by similar means used in secondary lymphoid organs. At this moment, no studies have identified whether $CD31^{+Thymic}$ or $CD31^{-Central}$ naïve T cells preferentially express LT and their specific contributions to lymphoid organ development and homeostasis.

Inherent differences are observed between $CD31^{+Thymic}$ vs. $CD31^{-Central}$ naïve T cells. As previously mentioned, CD31 may modulate TCR signaling and $CD31^{+Thymic}$ naïve T cells acquisition of effector function. $CD31^{+Thymic}$ naïve T cells display skewing towards T_H2 responses characterized by high IL-4 and low IFN-γ expression vs. $CD31^{-Central}$ naïve T cells (Berkley & Fink, 2014; Hendricks & Fink, 2011). This led to the discovery that $CD31^{+Thymic}$ naïve T cell loci for IL-2 and IL-4 are hypermethylated compared to $CD31^{-Central}$ naïve T cells and that inhibition of this methylation allowed for better IL-4 secretion (Berkley, Hendricks, Simmons, & Fink, 2013). These differences in methylation were traced to higher expression of DNA methyltransferases DMNT1, DMNT3, and TET1. Histone acetylation has also been shown to be involved in the maturation of $CD31^{+Thymic}$ to $CD31^{-Central}$ naïve T cell as losing histone deacetylase-3 limits progression from naïve to memory phenotype and great loss of cellularity in the periphery (Hsu et al., 2015).

T_{SCM} cells represent a unique subset in the naïve-memory compartment. T_{SCM} cells have distinct gene expression signatures, highly express effector cytokines as opposed to $CD31^{+Thymic}$ and $CD31^{-Central}$ naïve T cells, and give rise to multiple cell lineages as described above (Cieri et al., 2013; Gattinoni et al., 2011; Gattinoni et

al., 2017). They are especially important in chronic inflammation where effector cells are chronically activated and result in functional exhaustion and/or senescence as T_{SCM} cells serve as a source of new effector cells against specific antigens.

Most recently, naïve T cells from umbilical cord and adult peripheral blood were shown to express pro-inflammatory human chemokine CXCL8 (Das et al., 2017; Gibbons et al., 2014; van den Broek et al., 2016). Collectively, these results show naïve T cells from the umbilical cord blood are primed for rapid CXCL8 expression upon activation. These naïve T cells were also present in adult blood but at much lower percentages. Concordant with these cells being naïve, they were enriched in the $CD31^{+Thymic}$ naïve T cell subset and were progressively lost in infants following thymectomy. $CXCL8^{+}$ naïve T cells are lost as a function of age therefore it is possible these cells specifically derive from the fetal liver and linger for the first decades of life whereupon memory responses are developed and there is less need for these cells (Mold et al., 2010). CXCL8 is a pro-inflammatory chemokine capable recruiting and activating innate immune cells such as neutrophils and macrophages, promoting wound healing, angiogenesis and cellular growth. As such, $CXCL8^{+}$ naïve T cells may bridge the early adaptive to innate immune response by attractive and activating innate immune cells in a host that is developing memory/adaptive responses. Given these cells are activated in lymphoid organs, they may also help in the remodeling and supporting lymphoid structures by promoting vascularization. Co-expression of CXCL8 and LT-α has not been explored.

1.2 Naïve T cell (dys)function and the tumor microenvironment

Effective T cell-mediated anti-tumor responses depend on the recognition of tumor-antigens in the context of co-stimulatory molecules. The immediate problem

naïve T cells face in the tumor microenvironment is the immune-suppressive molecules leading to dysfunctional T cell responses. Classically, naïve T cells home towards lymph nodes where they are primed by APCs then home towards the site of the pathogenic insult. However, recent studies have shown naïve T cells are present at tumor sites. This section will discuss naïve T cells exist in tumors and dysfunctional programs they fall into in the tumor microenvironment.

Naïve T cells mainly home towards secondary lymphoid organs as discussed above and are found at discreet amounts outside lymphoid tissues (Thome et al., 2014; M. T. Wong et al., 2016). Recent evidence points to naïve T cells being present in tumors which develop tertiary lymphoid structures (TLS) (de Chaisemartin et al., 2011; Thompson, Enriquez, Fu, & Engelhard, 2010; Weninger et al., 2003). TLS are organized similar to SLO with specialized T cell and B cell areas, high endothelial venules, and secretion of chemokines specialized in the recruitment of lymphocytes (Sautes-Fridman et al., 2016). Recruitment of naïve T cells to these sites is facilitated by TLS expression of CCR7- and CD62L- ligands similar to recruitment by canonical SLO. Increased densities of TLS are shown to correlate with better disease outcome in a variety of cancer patients (Dieu-Nosjean et al., 2008; Remark et al., 2013; Sautes-Fridman et al., 2016). These TLS are rich in tumor antigens and pro-inflammatory molecules which stimulate effector responses (Sautes-Fridman et al., 2016; Thompson et al., 2010). Naïve T cells can therefore be actively recruited to- and primed against tumors to limit tumor progression. Nevertheless, TLS are privileged sites that localize pro-inflammatory immune responses distinct from other parts of the tumors where immune-suppressive networks are the norm.

Tumors usually exist in an immune-suppressive environment characterized by

low co-stimulatory but high co-inhibitory molecules, dysfunctional effector T cells produced in response to chronic inflammation, and increased populations of regulatory cell subsets such as myeloid-derived suppressor cells and regulatory T cells (Crespo, Sun, Welling, Tian, & Zou, 2013; Zou, 2005, 2006). As such recruited T cells may be improperly activated. T cell anergy is functionally described as the state of hyporesponsiveness with low IL-2 production and incomplete activation naïve T cells fall into by low co-stimulatory or high co-inhibitory stimulation (Schwartz, 2003). This hyporesponsive state is thought to serve as a tolerance mechanism to protect the host from developing autoimmunity. In line with this theory, induction of anergy was recently proposed to generate regulatory T cell (T_{REG}) precursors *in vivo* (Kalekar et al., 2016). There is currently a lack of markers which would allow the selective enrichment of anergic T cells for further study. However we know human tumors and tumor associated antigen presenting cells express high levels of co-inhibitory molecules B7-H1 (CD274 or PD-L1), B7-H2, (CD275 or ICOS-L), B7-H3 (CD276), B7-H4 (B7S1 or B7x), and B7-DC (CD273 or PD-L2) and lack or express little co-stimulatory B7-1 (CD80) and B7-2 (CD86) (Pardoll, 2012; Zou, 2005; Zou & Chen, 2008). This high co-inhibitory, low co-stimulatory environment can promote anergy in T cells. In line with this, introducing B7-1 or blocking co-inhibitory B7 family members results promotes anti-tumor responses *in vivo* (Blank et al., 2006; Curiel et al., 2003; Gajewski, 1996; Zou, Wolchok, & Chen, 2016). These T cells upon being transferred into a lymphopenic host will homeostatically proliferate and promote anti-tumor responses suggesting active suppression of their activation (Brown, Blank, Kline, Kacha, & Gajewski, 2006). In line with this, tumor-antigen specific T cells are dysfunctional in the tumor microenvironment (Broderick et al., 2006; Nazareth et al., 2007). Taken together, naïve T cells may not be completely

activated in the tumor microenvironment because higher co-inhibitory network leading to T cell anergy which can be overcome by shifting to balance to that of co-stimulation.

On the other hand, chronically activated T cells become functionally exhausted at sites of chronic inflammation such as cancer, autoimmunity and chronic inflammation (Wherry, 2011). Functionally exhausted T cells display decreased effector cytokine expression, reduced cell cycle progression and are resistant to activation. T cell exhaustion occurs progressively as T cells are repeatedly activated and gain expression of various co-inhibitory surface molecules including PD-1, T cell immunoglobulin and mucin-domain-containing molecule-3 (Tim-3), lymphocyte-activation gene (LAG)-3, B and T-cell lymphocyte attenuator (BTLA, CD272), 2B4 (CD244), CTLA-4, and CD160 (Blackburn et al., 2009; Fourcade et al., 2012; H. Li et al., 2012; Wherry et al., 2007; Woo et al., 2012). Exhausted T cells do not necessarily co-express all of these molecules. Exhausted T cells represent a fraction of antigen-specific T cells making them an attractive therapeutic target because they already respond against tumor antigens. It is generally accepted that B7-H1/PD-1 signaling mediates CD8^{+} T cell exhaustion and PD-1 is therefore used as a marker for T cell exhaustion. In line with this PD-1 or B7-H1 blockade promotes better anti-tumor responses (Curiel et al., 2003). Human tumors and tumor-associated APCs express B7-H1 and PD-1^{+} T cells have also been found in various kinds of cancers leading to various clinical trials targeting this pathway and shown better prognosis (Zou et al., 2016). Current research is focusing on combination therapies where PD blockade is used in the context of other immunotherapies or chemotherapies to enhance clinical prognosis.

Taken together, naïve T cells can be recruited to cancer sites where they can

mount efficient responses at TLS sites. Naïve T cells may contribute to the formation of TLS through expression of LT-α, though this has yet to be shown. However, tumors do not always develop TLS and immune-suppressive networks develop impeding proper activation leading to dysfunctional T cell programs such as T cell anergy and exhaustion.

1.3 Scope of dissertation

Naïve T cells form a heterogeneous population with functional capacity. The functional impacts of naïve T cells are incompletely understood in patients with cancer and tumor-bearing murine experiments though it is known that tumor masses support the infiltration of naïve T cells and that these are present at tertiary lymphoid organs. This dissertation research is divided into three projects, which together aimed at understanding naïve T cell functional heterogeneity, their role in cancer biology, and the epigenetic mechanisms in place of their homeostasis and function.

Context for Chapter 2 – Human naïve T cells express functional CXCL8 and promote tumorigenesis

It was a long held notion that naive T cells require activation to acquire effector functions. Upon undertaking this project, the immunological field did not know $CXCL8^+$ naïve T cells are existed and were present in umbilical cord blood. We aimed to characterize these cells and their impacts in tumors. As previously stated, tumor masses sustain naïve T cell infiltration but their functional impacts are incompletely understood (Thompson et al., 2010; Xia et al., 2017). Further, CXCL8 is able to promote tumorigenesis through enhanced tumor growth, induction of stemness, and angiogenesis.

In this work, we show CXCL8^{+} T cells are enriched in naïve markers while lacking memory phenotypical markers and activated/effector T cell cytokines. Naïve T cell-derived CXCL8 was able to promote neutrophil migration *in vitro* and tumor growth *in vivo*. Through this work we challenge previously held notions that naïve T cells are functionally silent during homeostasis. We show that naïve T cell-derived CXCL8 is biologically active and may be involved in shaping anti-tumor immune responses.

Context for Chapter 3 – Phenotype and tissue distribution of CD28H^{+} immune cell subsets

Memory T cells exist in different flavors during homeostasis and fall into specific programs following activation with inherent characteristics that define them. For example, memory T cells that are chronically activated against a specific antigen may fall into an exhausted state or become terminally differentiated with decreased effector functions (Crespo et al., 2013; Schwartz, 2003; Wherry, 2011). Similarly, naïve T cells are heterogeneous during homeostasis as discussed above and may come in different flavors. This study focuses on the tissue and cellular distribution of CD28H^{+} immune cells and characterizing the effector state in which T cells are in human blood, secondary lymphoid organs and multiple types of cancer tissues.

CD28H is a novel co-receptor of the human B7 family which interacts with its ligand B7-H5 and regulates T cell responses. CD28H was detected in T cells, innate lymphoid cells, natural killer cells, and plasmacytoid dendritic cells. In T cells, CD28H is primarily expressed by naïve over memory T cells. We build from our work in Chapter 2 and study the functional state naïve T cells exist in and characterize them based on CD28H. We find CD28H^{+} T cells display less differentiated phenotype and

lower effector functionality when compared to CD28H$^-$ T cells across tissues. This study further supports naïve T cells come in distinct functional programs which can be targeted for better immunotherapeutic treatments. Further, our findings suggest an important role of CD28H in the regulation of T cell responses in the context of inflammation.

Context for Chapter 4 – Dot1L regulates STAT5-signaling and T cell anti-tumor function.

Naïve T cell subsets have molecular and genetic networks in place to regulate homeostasis and their acquisition of effector functions. In chapter 4, we question the underlying epigenetic network as it pertains to naïve T cell homeostasis and function. Disruptor of telomeric silencing 1-like (Dot1L) is the sole epigenetic enzyme currently identified that catalyzes the methylation of H3K79, wherein target gene expression is induced. Upon starting this project, no studies have determined the importance of Dot1L in naïve T cell biology and function. We found that conditional knockout of Dot1L in murine T cells abrogated their homeostasis, survival and effector functions as characterized by increased apoptosis of naïve T cells and abrogated anti-tumor responses. Further, Dot1L-knockout abrogates proper cell cycle gene expression and led to reduced STAT5 expression preventing cell cycle progression and cell survival which may underlie naïve T cell dysfunction. These findings may have important impacts in cancer where T cells are dysfunctional.

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Chapter 2

Human naïve T cells express functional CXCL8 and promote tumorigenesis

2.1 Abstract

Naïve T cells are thought to be functionally quiescent. Here, we studied and compared the phenotype, cytokine profile, and potential function of human naïve $CD4^+$ T cells in umbilical cord and peripheral blood. We found that naïve $CD4^+$ T cells, but not memory T cells, spontaneously expressed high levels of chemokine CXCL8. $CXCL8^+$ naïve T cells were preferentially enriched $CD31^+$ T cells and did not express T cell activation markers and typical T helper effector cytokines including IFN-γ, IL-4, IL-17, and IL-22. In addition, upon activation, naïve T cells retained high levels of CXCL8 expression. Furthermore, we showed that naïve T cell-derived CXCL8 mediated neutrophil migration in the *in vitro* migration assay and promoted tumor growth in an *in vivo* human xenograft model. We also identified differences in micro-RNA's expressed between $CD4^+$ naïve and memory T cell subsets from cord and peripheral blood. Thus, human naïve T cells are phenotypically and functionally heterogeneous and can carry out active functions in immune responses.

2.2 Introduction

Naïve T cells are thought to be functionally quiescent. These cells are largely in the lymph nodes and may maintain lymph node integrity through lymphotoxin expression and be epigenetically poised in newborns to Th2 type responses

(Berkley, Hendricks, Simmons, & Fink, 2013; Gramaglia, Mauri, Miner, Ware, & Croft, 1999; Ohshima et al., 1999; Opiela, Koru-Sengul, & Adkins, 2009; Yoshimoto, Yoder, Guevara, & Adkins, 2013; Zeng et al., 2012). Micro-RNA's have been shown to regulate various aspects of T cell naivity, effector function and stability. (Bronevetsky et al., 2013; Rossi et al., 2011; Wu et al., 2007; Zhao et al., 2016; Zhou et al., 2008) Interestingly, recent reports have shown that human naive T cells can express chemokine interleukin-8 (CXCL8) (Gibbons et al., 2014; van den Broek et al., 2016). However, the phenotype, cytokine profile, and functional potential of $CXCL8^+$ naïve T cells in tumor are poorly defined in human literature.

CXCL8 is a pro-inflammatory chemokine with a variety of functions in the immune response. In cancer, CXCL8 promotes neutrophil migration, endothelial cell proliferation, wound healing responses, and metastatic potential (Li, Dubey, Varney, Dave, & Singh, 2003; Luster, 1998; Waugh & Wilson, 2008). CXCL8 may promote cancer cell growth through increased angiogenesis and tumor cell proliferation (Huang et al., 2002; Kassim et al., 2004; Merritt et al., 2008; Xu & Fidler, 2000). Such effects are abrogated in the presence of the drugs targeting the CXCL8 protein (Huang et al., 2002). CXCL8 is highly expressed in the advanced tumor cancer microenvironment and predicts poor prognosis (Kassim et al., 2004). For example, we have shown that in patients with advanced ovarian cancer a subset of regulatory T cells (Kryczek et al., 2016) and plasmacytoid dendritic cells (Curiel, Cheng, et al., 2004) can express CXCL8 and contribute to tumor immunopathology. In line with this observation, we observed that naive T cells selectively and spontaneously expressed high levels of CXCL8. Given the role of CXCL8 in tumor and the location of naïve T cells in the lymph nodes, we suggest that naïve T cell-derived CXCL8 may be functionally important in tumorigenesis and particularly in tumor lymphoid

metastasis. Lastly, we offer insights into the differences in miRNA expressed between $CD4^+$ naïve and memory T cells from cord and peripheral blood which may underlie functional differences.

2.3 Materials and Methods

Human samples, cell isolation and flow cytometric analysis (FACS)

Human tonsil was obtained tissue procurement lab in the University of Michigan. Study was approved by the local Institutional Review Board. Human peripheral blood samples were obtained from healthy volunteers by venipuncture or cytopheresis. Human umbilical cord blood was collected by C.S. Mott Children's Hospital (University of Michigan) in blood collection tubes (Becton Dickinson Biosciences). Mononuclear cells were collected by Ficoll-Hypaque or Lymphoprep density gradient centrifugation. Peripheral blood naïve T cells were isolated by RossetteSep (Stemcell Technologies) followed by $CD45RA^+$ or $CD45RO^+$ microbeads (Myltenyi) column selection. Naïve T cells from umbilical cord blood and human tonsil were isolated by first depleting $CD14^+$ monocytes followed by positive selection of $CD4^+$ T cells using $CD14^+$ monocyte selection EasySep and $CD4^+$ T cell selection EasySep (Stemcell Technologies). $CD4^+CD45RA^+CD31^{+/-}$ naïve T cells were sorted from single-cell suspensions by high-speed cell sorter (FACSAria, Becton Dickinson Immunocytometry Systems). Neutrophils were isolated as described from adult blood (Nuzzi, Lokuta, & Huttenlocher, 2007). Cellular purity was measured by flow cytometry (LSRII, BD). Surface and cytokine profiles were measured through surface and intracellular staining, respectively, and analyzed by LSRII, DIVA and FloJo software (Curiel, Coukos, et al., 2004; Kryczek et al., 2011; Kryczek et al., 2006).

Reagents

Recombinant-human cytokines and chemokines were from R&D systems. Antibodies for flow cytometry were CCR7, CD3, CD4, CD8, CD25, CD31, CD45RA, CD45RO, CD62L, CD69, CD154, CXCR2, CXCR3, CXCL8, IFN-γ, IL-4, IL-10, IL-17, IL-22 (BD), CD57, CD122, Foxp3, TNF (eBiosciences/ThermoFischer), KLRG-1 (BioLegend). Antibodies for T cell stimulation were CD3, CD28 (BD or Affymetrix). Small-molecule CXCR1/2 chemical inhibitor Reparixin (RPX) was purchased from MedChem Express.

***In vitro* T cell and OC8 tumor cell line cultures**

T cell subsets (1 million cells/mL) were stimulated with αCD3/αCD28 beads (BD Biosciences) for up to two weeks. Cells were supplemented with fresh medium and recombinant-human IL-2 (5 ng/mL) every three days and re-stimulation on days six and twelve. Cytokine expression was measured at three-day time points through either FACS. CXCL8 was quantified following 3day culture *in vitro* with αCD3/αCD28 beads (BD Biosciences) ELISA (R&D). In other cases, T cell subsets (10 million cells/mL) were cultured for up to three days in medium supplemented with recombinant-human IL-7 and IL-15 (10 ng/mL) or vehicle. Supernatants were saved and measured for specific cytokines. Human tonsil naïve T cells were cultured *in vitro* in the presence of IL-7 (10ng/mL) and IL-15 (10ng/mL) for up to twelve days. Human primary OC8 ovarian cancer cells (Peng et al., 2015; Wang et al., 2016) were cultured *in vitro* in RPMI supplemented with 10% FBS and 1% PennStrep (ThermoFischer).

Flow cytometry and cytokine detection

Freshly isolated mononuclear cells or enriched *in vitro* stimulated T cell subsets were stimulated *in vitro* with PMA/Ionomycin, GolgiStop and GolgiPlug (BD Biosciences) for 4 hours, then stained against extracellular and intracellular antigens. Single-cell suspensions from tissues were stained for extracellular surface antigens with specific antibodies, followed by fixation and permeabilization using Perm/Fix solution (ThermoFischer), then stained against intracellular antigens. Viable single cells were analyzed.

Tumor models

NOD.Scid.IL-2R$\gamma c^{-/-}$ (NSG) male mice aged 14-18 weeks (Jackson Laboratories) were used (Peng et al., 2015; Wang et al., 2016). Primary human ovarian cancer cells (OC8) were cultured in RPMI supplemented with 10% FBS (Cui et al., 2013; Kryczek et al., 2012). NSG mice were subcutaneously co-injected with $1x10^6$ OC8 and $1x10^6$ human T cells for two weeks (Cui et al., 2013). Mice received peritoneal injection with (200uL) PBS-solubilized Reparixin daily (5mg/kg) or PBS (Kim et al., 2011). In a separate experiment, OC8 cells were cultured in the presence of recombinant human CXCL8 (10 ng/mL) for 24 hours and $1x10^6$ single-cells were subcutaneously injected into mice.

Sphere formation assay

OC8 cells were cultured *in vitro* in X-VIVO medium (Lonza) and ultra-low attachment plates (Corning) for 5d supplemented with 0, 1, or 10(μg/mL) recombinant human CXCL8. Separately, supernatants from umbilical cord blood naïve $CD4^+$ T cells cultured *in vitro* from above was mixed 1:1 with X-VIVO medium and cultured as

described above at a density of 1,000-10,000 viable cells/well. Spheres (>100μm) were counted after 5 days.

Neutrophil migration assay

T cells were cultured *in vitro* for three days at 1 million/mL in the presence of αCD3/αCD28 beads (BD Biosciences). Supernatants were collected and placed on the bottom of 3-μm-Pore, 12-mm-diameter Transwells while neutrophils were placed on the upper chamber. Reparixin was added as indicated. Neutrophil migration was performed for two hours (Nuzzi et al., 2007).

Enzyme-linked Immunoassay

ELISA to detect CXCL8 was carried out as per the manufacturer's protocol (R&D Systems).

Micro-RNA MicroArray

Naïve T cells from umbilical cord blood and from adult peripheral blood were isolated with high purity. mRNA was extracted via Trizol reagent. Isolated mRNA was applied for miRNA profiling using Affymetrix miRNA 4.1 Array Strip (Affymetrix, Cat: 902404) at University of Michigan MicroArray Core.

Quantitative real-time PCR for micro-RNAs

microRNAs were detected by TaqMan MicroRNA Assay kit (Applied Biosystems).

T cell nucleofection

Freshly isolated CD4^{+} T cell subsets were nucleofected as per the manufacturer's instructions (Lonza). miR-29b, -106b, and -125 mimics or inhibitors were used to manipulate miRNA function (InVivoGen).(Zhao et al., 2016) Following 72hrs of nucleofection, T cells were measured for cytokine expression through FACS.

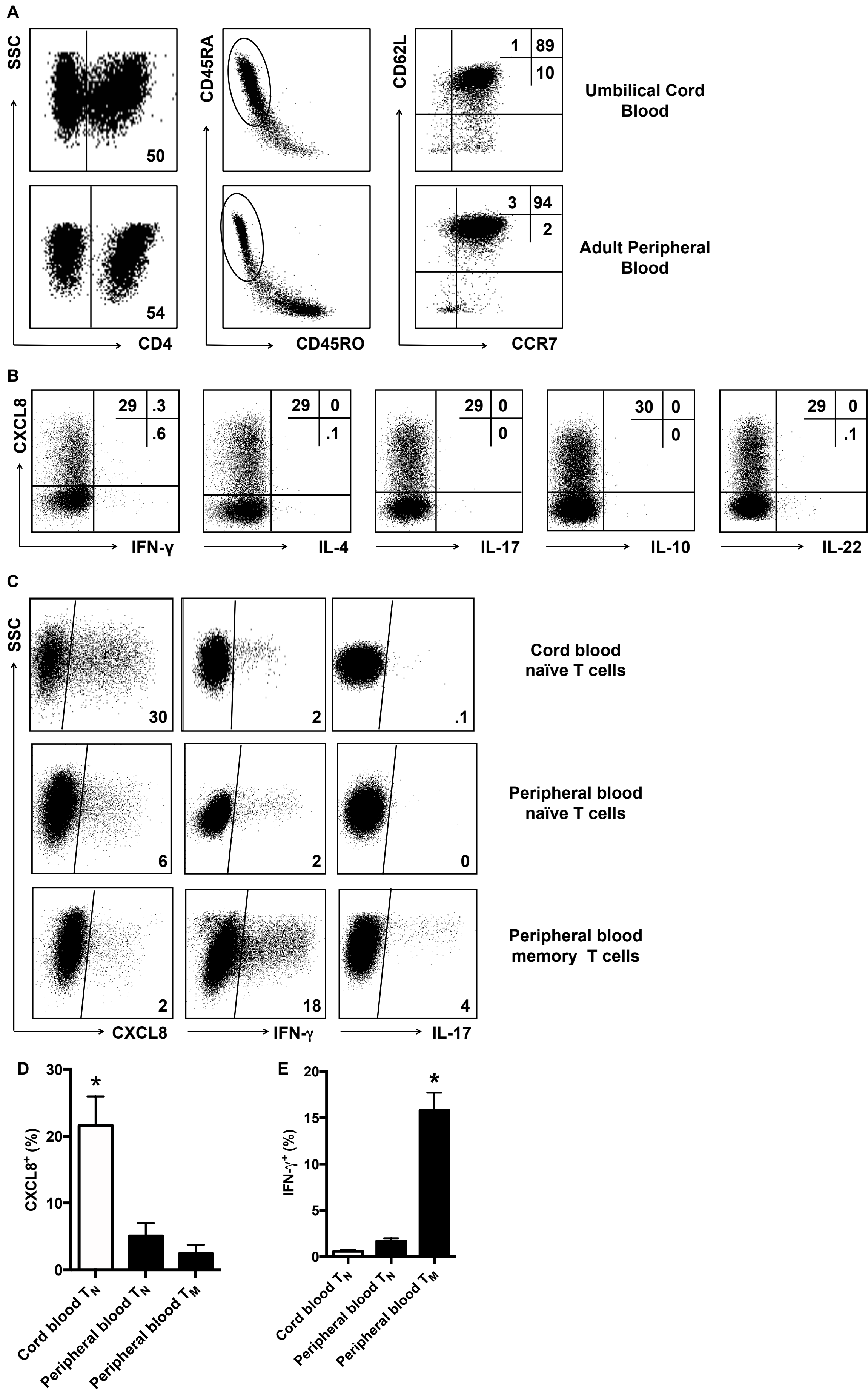
Statistical analysis

Student's T-Test was carried out to determine significant difference between expression levels, tumor weight, and neutrophil migration numbers.

2.4 Results

2.4a Naïve T cells spontaneously express CXCL8

To determine whether naïve T cells are functionally quiescent, we studied and compared the cytokine profiles of naïve and memory CD4^{+} T cells in fresh umbilical cord blood and adult peripheral blood. CD45RA^{+}CD45RO^{-}CD62L^{+}CCR7^{+} naïve T cells and CD45RA^{-}CD45RO^{+} memory T cells were defined by flow cytometric analysis (FACS) (Fig. 2.1A). We found that umbilical cord blood naïve CD4^{+} T cells spontaneously expressed 22% CXCL8, and less than 1% IFN-γ, IL-4, IL-17, IL-10 and IL-22 (Fig. 2.1B). The expression level of CXCL8 was the highest among detectable cytokines in umbilical cord blood naïve CD4^{+} T cells (Fig. 2.1B). Next we compared the cytokine expression levels in cord blood and adult peripheral blood (Fig. 2.1C, D). Furthermore, naïve T cells expressed higher levels of CXCL8 than memory T cells (Fig. 2.1C, D). In contrast, the expression levels of IFN-γ and IL-17 were higher in memory T cells than naive T cells (Fig. 2.1C, E). Thus, human naïve T cells spontaneously and selectively express CXCL8.


A
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CD45RA
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Cord blood naïve T cells
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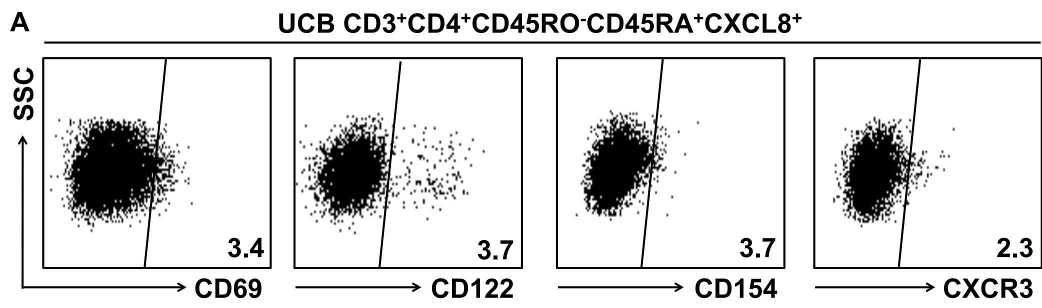
Peripheral blood memory T cells
CXCL8
IFN-γ
IL-17
D
CXCL8+ (%)
Cord blood TN
Peripheral blood TN
Peripheral blood TM
E
IFN-γ+ (%)
Cord blood TN
Peripheral blood TN
Peripheral blood TM

Figure 2.1. Human naïve T cells spontaneously express CXCL8. (A) Gating strategy used to identify T cell subsets. Naïve T cells: $CD3^{+}CD4^{+}CD45RA^{+}CD45RO^{-}CD62L^{+}CCR7^{+}$. Memory T cells: $CD3^{+}CD4^{+}CD45RA^{-}CD45RO^{+}CD62L^{-}CCR7^{-}$. (B-E) Cytokine profile of cord blood and peripheral T cells. Intracellular cytokines were analyzed by FACS. Representative FACS plots and percentages are shown (B, C). (D-E) Graphical summary of CXCL8 (D) and IFN-γ (E) FACS values in human T cell subsets. n = 4-8, *, $P < 0.05$.

2.4b $CXCL8^{+}$ naïve T cells are enriched in $CD31^{+}$ T cells

We defined naïve T cells based on $CD45RA^{+}CD45RO^{-}CD62L^{+}CCR7^{+}$ surface phenotype. We could not exclude whether $CXCL8^{+}$ naïve T cells were activated and/or differentiated *in vivo*. To address this possibility, we analyzed the activation and differentiation markers in $CXCL8^{+}$ naïve T cells. $CXCL8^{+}$ naïve T cells expressed neither of several activation markers including CD69, CD122, CD154, and CXCR3 (Fig. 2.2A) nor differentiation markers including CD57 and KLRG1 (Fig. 2.2B). We previously reported that a subset of regulatory T cells expressed in the tumor microenvironment (Kryczek et al., 2016). We found that $CXCL8^{+}$ naïve T cells did not express Foxp3 and CD25 (Fig. 2.2C). Thus, $CXCL8^{+}$ naïve T cells are not regulatory T cells. Interestingly, $CXCL8^{+}$ naïve T cells expressed minimal levels of CXCR2, the receptor for CXCL8 (Fig. 2.2D), suggesting that $CXCL8^{+}$ naïve T cells may not consume CXCL8 either from their own or from the microenvironment. CD31 is a marker for recent thymic emigrant cells (Kimmig et al., 2002). Adult peripheral blood naïve T cells can be divided into $CD31^{+}$ cells and $CD31^{-}$ mature cells (Kimmig et al., 2002). We sorted $CD31^{+}$ and $CD31^{-}$ naïve cells. We found that the levels of CXCL8 were higher in $CD31^{+}$ T cells than $CD31^{-}$ T cells (Fig. 2.2E). Thus, peripheral blood $CXCL8^{+}$ T cells are enriched in $CD31^{+}$ naïve T cells.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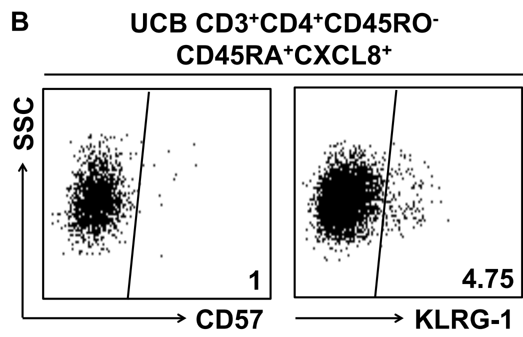
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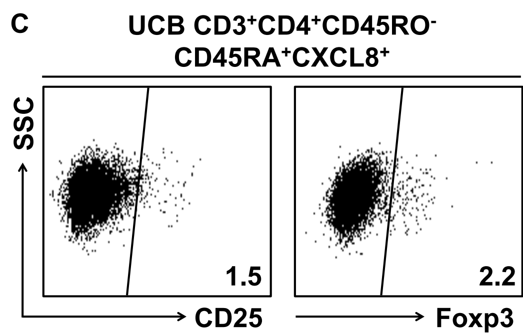
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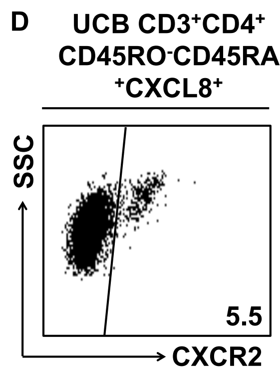
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CD45RO-CD45RA
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SSC
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CXCR2

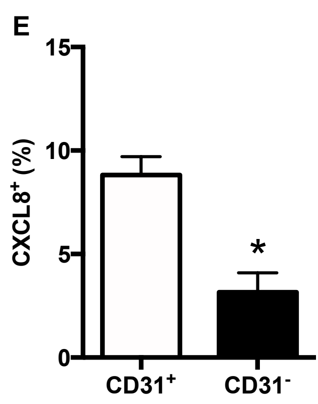
E
CXCL8+ (%)
15
10
5
0
*
CD31+
CD31-

Figure 2.2. CXCL8$^+$ naïve T cells are enriched in CD31$^+$ T cells. (A-D) Fresh CXCL8$^+$ umbilical cord blood naïve T cells were analyzed for expression of surface and intracellular marks of activation and differentiation. Representative FACS plots and percentages are shown. Gated on CXCL8$^+$ naïve T cells. (E) Graphical summary of CXCL8 expression in CD31$^+$ and CD31$^-$ T cells (E). n = 4-8, *, $P < 0.05$.

2.4c Activated naive T cells remain to express CXCL8

CXCL8$^+$ naïve T cells are enriched in CD31$^+$ T cells. It suggests a potential dynamic expression of CXCL8 in naïve T cells. To test this, we activated naïve T cells from umbilical cord and adult peripheral blood with αCD3/αCD28 stimulation and kinetically analyzed CXCL8 expression. We found that TCR engagement initially maintained and subsequently stimulated CXCL8 expression in naïve T cells from umbilical cord blood (Fig. 2.3A) and adult peripheral blood (Fig. 2.3B) naïve CD4$^+$ T cells. As expected, the levels of IFN-γ expression were low and gradually increased following T cell activation in CD4$^+$ naïve T cells from umbilical cord blood (Fig. 2.3C) and adult peripheral blood (Fig. 2.3D). In line with this, activated naïve T cells from cord blood gained IFN-γ expression following *in vitro* activation (Fig. 2.3E). Naïve T cells mainly home to secondary lymphoid organs. We therefore cultured CD4$^+$ naïve T cells enriched from human tonsil with homeostatic cytokines IL-7 and IL-15 and kinetically analyzed CXCL8 expression. We showed that homeostatic cytokines initially maintained and subsequently stimulated CXCL8 expression in these cells (Fig. 2.3F). As expected, homeostatic cytokines did not stimulate IFN-γ expression in human tonsil CD4$^+$ naïve T cells (Fig. 2.3G). The data suggest that CXCL8 expression may be a functional feature for certain naïve T cells.

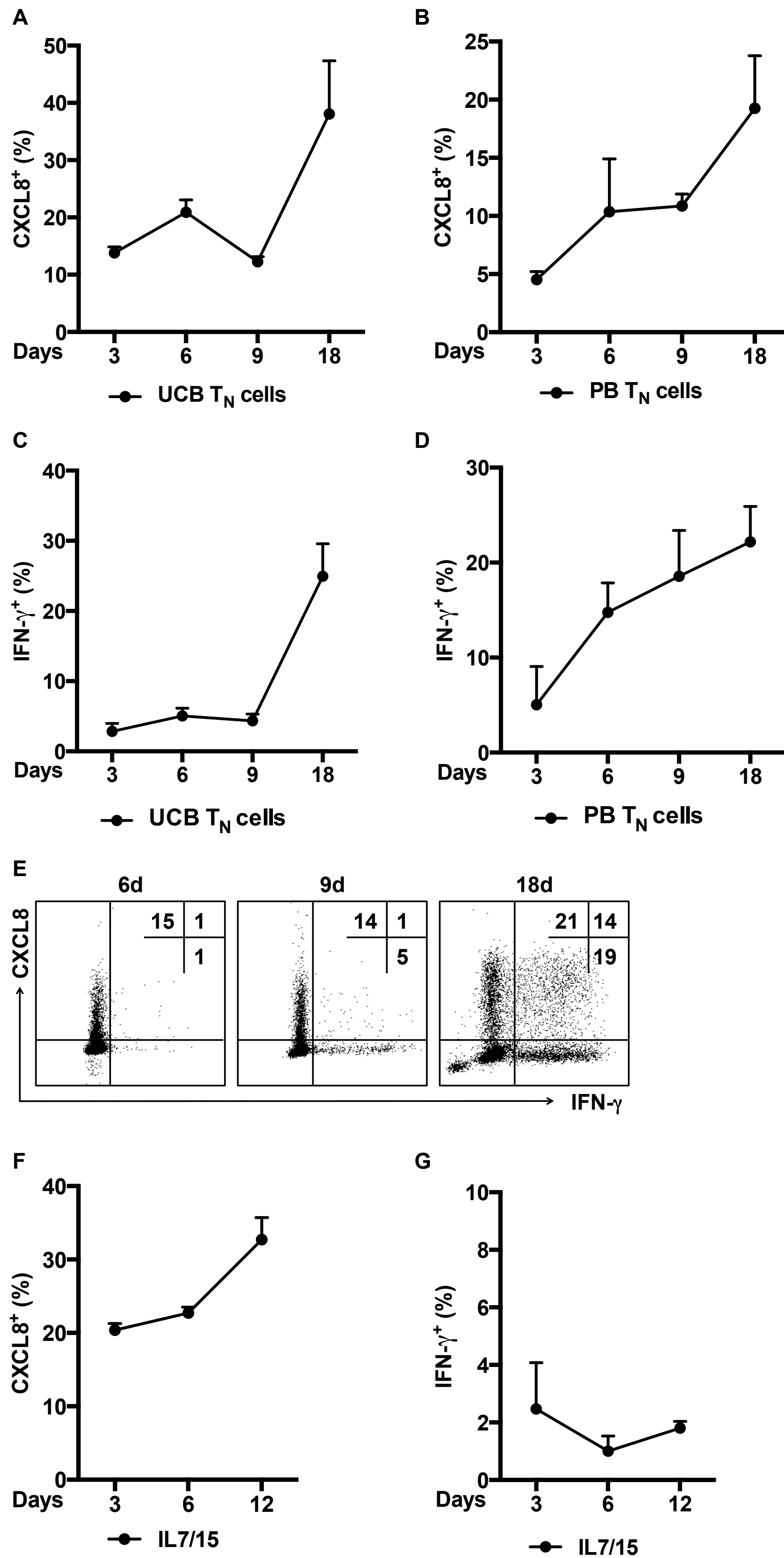

Figure 2.3. Human naïve T cells retain CXCL8 expression *in vitro* under homeostatic and activation conditions. (A-D) Results are shown as the kinetic

CXCL8 (A, B) and IFN-γ (C, D) expression in naïve T cells in cord blood (A, C) and peripheral blood (B, D) upon activation with αCD3/αCD28 beads. (E) Expression plots for CXCL8 and IFN-γ following *in vitro* activation. (F-G) Results are shown as the kinetic CXCL8 (F) and IFN-γ (G) expression in naïve T cells in the presence of IL-7 and IL-15 from human tonsil. Average of 3-4 experiments is shown.

2.4d Naïve T cell-derived CXCL8 is biological active

To study the potential role of naïve $CD4^+$ T cell-derived CXCL8, we carried out an *in vivo* model of tumor growth in the NSG mice. We inoculated primary human ovarian cancer cells into the NSG mice with or without umbilical cord blood naive $CD4^+$ T cells at a 1:1 ratio. We treated the mice with Reparixin, a small-molecule chemical inhibitor for CXCL8 receptors, CXCR1 and CXCR2 (Gorio et al., 2007; Moriconi et al., 2007). We observed that mice that received umbilical cord blood naïve $CD4^+$ T cells showed enhanced tumor volume and weight (Fig. 2.4A, 4B) as compared to mice which did not receive naïve $CD4^+$ T cells. This effect was abrogated by Reparixin administration (Fig. 2.4A, 4B). In support of this observation, we incubated primary human ovarian cancer cells with recombinant human CXCL8 for 24 hours and subsequently injected into the NSG mice. CXCL8 treatment resulted in increased tumor weight (Fig. 2.4C). Thus, naïve T cell-derived CXCL8 is biologically active. We suggest CXCL8 works by promoting stemness qualities on ovarian cancer cells. We tested this by incubating OC8 cells in cord blood $CD4^+$ naive T cell-conditioned medium with or without Reparixin and found Reparixin inhibited OC8 cell sphere formation (Fig. 2.4D). Similarly, OC8 cells cultured with increasing concentrations of recombinant human CXCL8 showed increased sphere formation as well (Fig. 2.4E). Furthermore, CXCL8 can mediate neutrophil migration. We tested the potential effect of naive T cell-derived CXCL8 on neutrophil migration in an *in vitro* transwell assay. We observed that neutrophils migrated toward naive T cell supernatants. This effect was blocked by Reparixin (Fig. 2.4F). As confirmation,

we detected high levels of CXCL8 in umbilical cord blood naive $CD4^+$ T cells (Fig. 2.4G). Thus, naive T cell-derived CXCL8 may be functionally important *in vivo*.

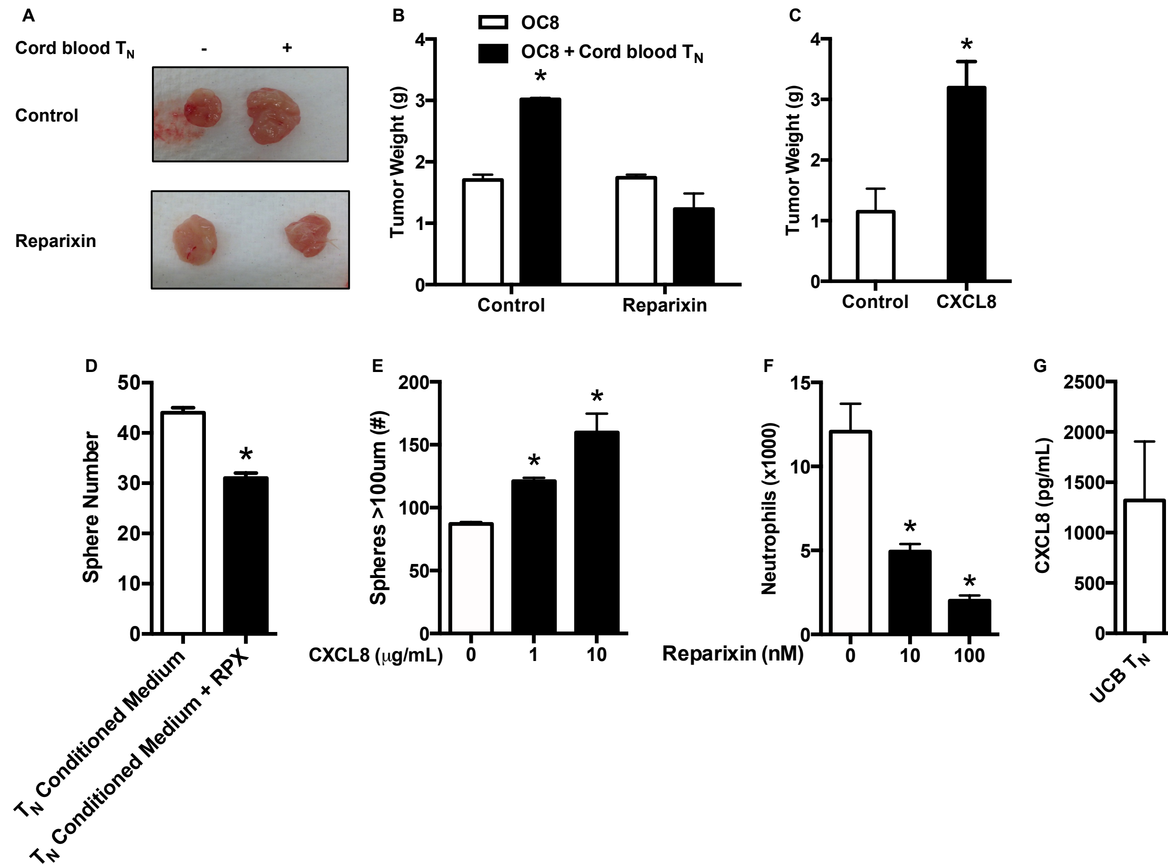

Figure 2.4. Naive T cell-derived CXCL8 promotes tumor growth and neutrophil migration. (A-B) Human primary cells (OC8) were mixed with cord blood naïve T cells and subcutaneously injected into NSG mice. Mice received daily intraperitoneal PBS or Reparixin (5mg/kg) injection. Following two-weeks, tumors were recovered and weighted. Representative tumors are presented in (A) and graphical summary of tumor size (B). n = 5/group. *, P <0.05. (C) OC8 tumor cells were pre-incubated with recombinant human CXCL8 *in vitro* for 24 hours and subcutaneously injected into NSG mice and tumor growth at endpoint was measured. n = 10/group. *, P <0.05. (D) Effects of recombinant human CXCL8 on cancer sphere formation. OC8 cells were cultured with increasing concentrations of CXCL8 in sphere condition. Sphere formation assay was performed. (E) Effects of cord blood $CD4^+$ naïve T cell conditioned medium on cancer sphere formation. OC8 cells were cultured with $CD4^+$ naïve T cell conditioned medium with or without Reparaxin in sphere condition. Sphere formation assay was performed. (F) Neutrophil migration assay was carried out on cord blood naïve T cell-derived supernatants with increasing concentrations of Reparixin. Migrated neutrophil numbers were counted. n = 5, *, P <0.05. (G) CXCL8 secretion by naïve T cells. Cord blood naïve $CD4^+$ T cells were cultured with anti-CD3 and anti-CD28 for 3 days. CXCL8 was detected by ELISA in the supernatants. n = 3.

2.4e Umbilical cord blood and adult peripheral naïve T cells show distinct miRNA expression profiles

miRNA's have been shown to regulate T cell functionality and cytokine expression (Jeker & Bluestone, 2013). miRNA's mainly function to silence target genes from being translated into protein from mRNA. It has also been shown that naïve T cells have high expression of miRNA as compared to memory T cells regulating T cell quiescence and naivity (Fig 2.5A) (Hsu et al., 2011; Landgraf et al., 2007; Rossi et al., 2011). Furthermore, naïve T cells show robust remodeling of miRNA's expressed upon activation resulting in their acquisition of effector functions (Bronevetsky et al., 2013). Based on this, we hypothesized CXCL8 was regulated on T cells through miRNA. We compared miRNAs expressed on naïve T cells from cord and peripheral blood through a micro-array for miRNAs. Naïve T cells from peripheral blood expressed more miRNAs than cord blood nauce T cells (Fig. 2.5B). We focused on miRNA's that have been hypothesized to target CXCL8 and were downregulated on cord vs. peripheral blood naïve T cells and confirmed these differences on $CD4^+$ naïve T cells and memory T cells from cord and peripheral blood by qRT-PCR (Fig. 2.5C). We identified miR-29b and -106b as possible miRNA's that could influence CXCL8 expression in T cells. $CD4^+$ naive T cells were nucleofected with miRNA mimics and activated them for 72hrs but no significant changes were detected through FACS for CXCL8, IFN-γ, and TNF expression (Fig. 2.5 D). In contrast, $CD4^+$ memory T cells were nucleofected with miRNA inhibitors and activated them for 72hrs but no changes were detected for CXCL8 either (Fig. 2.5 E). Nevertheless, miRNA micro-array shows differences in miRNA expression between naïve T cells from umbilical cord blood vs. adult peripheral blood. We

hypothesize these may regulate specific differences inherent to naïve T cells from cord vs. peripheral blood that have remain untested.

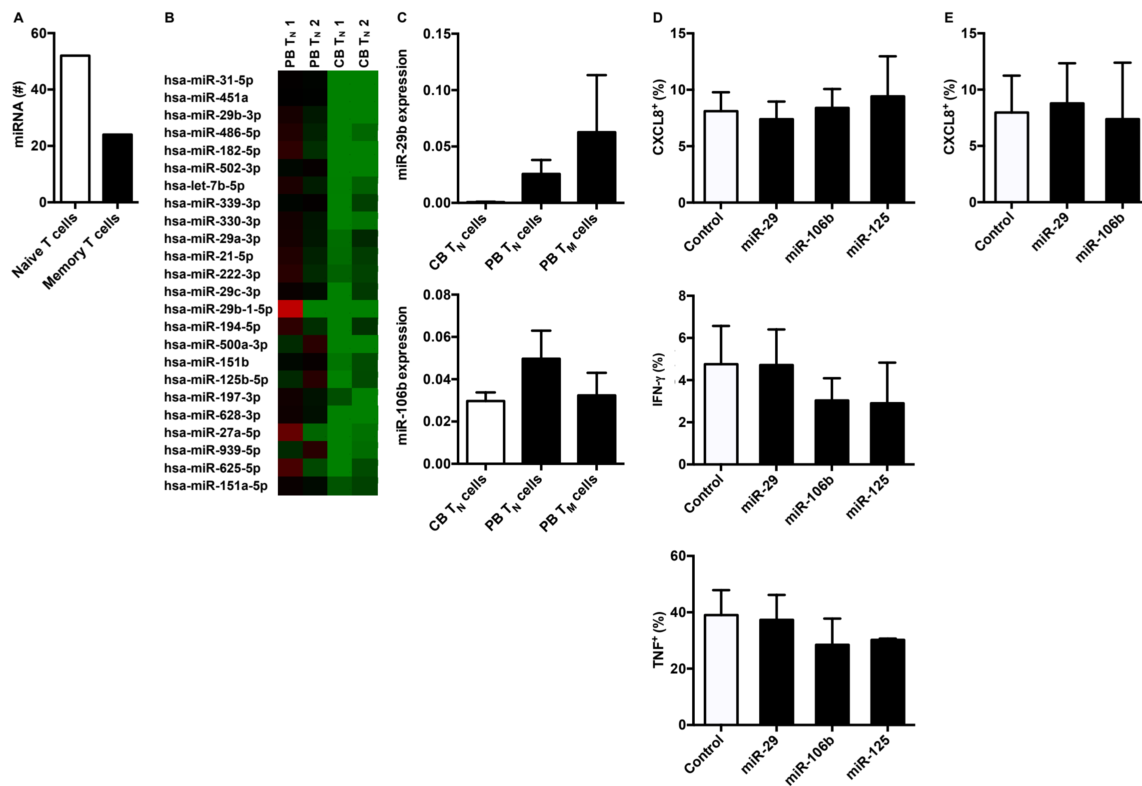

Figure 2.5. miRNA profiles of UCB and PB T_N cells. (**A**) Number of miRNA genes expressed by naïve vs. memory $CD4^+$ T cells in miRbase database. (**B**) Heatmap showing miRNA downregulated by *ex vivo* cord blood $CD4^+$ naïve T cells compared to *ex vivo* peripheral blood $CD4^+$ naïve T cells. (C) Expression of specific miRNA in *ex vivo* $CD4^+$ naïve T cells from cord and peripheral blood and memory T cells from peripheral blood. (D) CXCL8, IFN-γ and TNF expression by flow cytometry of $CD4^+$ naïve T cells nuclofected with specific miRNA mimics and activated for 3 days. (E) CXCL8 expression by flow cytometry of $CD4^+$ memory T cells nucleofected with specific miRNA inhibitors and activated for 3 days.

2.5 Discussion

Naïve T cells are thought to require activation to acquire effector functions. In line with this report, naïve T cells undergo remodeling of their miRNA expression profiles which allow their acquisition of effector functions.(Bronevetsky et al., 2013) Recent reports suggest that naive T cells carry out active functions prior to their activation such as maintaining lymph node integrity (Ohshima et al., 1999). Previous

studies have shown that human umbilical cord blood $CD4^+$ naïve T cells express CXCL8 and naive T cell CXCL8 expression is lost in adulthood (Gibbons et al., 2014; van den Broek et al., 2016). Here we show that human $CD4^+$ naive T cells spontaneously express high levels of CXCL8 and are poised to express CXCL8 upon activation. Furthermore, adult human tonsil naïve T cells express CXCL8 spontaneously and this expression may be a functional feature of these cells. Typical T helper effector cytokines including IFN-γ, IL-4, IL-17 and IL-22 are not co-expressed with CXCL8 in naïve $CD4^+$ T cells. Phenotypic experiments reveal that these cells are enriched in $CD4^+CD45RA^+RO^-CD62L^+CCR7^+CD31^+$ population. These CXCL8 producers show no signs of prior activation or differentiation, indicating that they are naïve T cells.

Interestingly, although naïve T cells in both fresh umbilical cord blood and adult peripheral blood express high levels of CXCL8, the percentage of $CXCL8^+$ T cells are substantially higher in umbilical cord blood than that in adult peripheral blood. Naïve T cells from human tonsil also expressed CXCL8 under homeostatic conditions. In addition, *ex vivo* memory T cells express high levels of other effector cytokines including IFN-γ, rather than CXCL8. The data suggest that spontaneous CXCL8 expression may be a functional feature for naïve T cells. In support of this, we have found that naïve T cell-derived CXCL8 mediates neutrophil migration *in vitro* and promotes primary ovarian cancer growth in the NSG model. Spontaneous CXCL8 expression in naïve T cells may allow humans be prepared against pathogens encountered in early in life. Thus, $CXCL8^+$ naive T cells may regulate innate cell trafficking and shape early immune responses. Given the well-defined role of CXCL8 in angiogenesis, $CXCL8^+$ naïve T cells may support vascularization in newborn tissues. Of course, these cells may represent a remnant of the fetal

immune system and their potential role in fetus development has yet to be determined (Mold et al., 2010). Moreover, the pro-tumor role of CXCL8 has been well-defined in many types of tumor (Nagarsheth et al., 2016; Zou, 2005). As the CXCL8, CXCR1 and CXCR2 signaling is involved in the regulation of cancer stem cells (Ginestier et al., 2010) and tumor often metastasizes into lymph nodes, it is speculated that $CXCL8^{+}$ naïve T cells may support tumor lymphoid metastasis via CXCL8 production. Altogether, naïve T cell-derived CXCL8 may play an important role in physiologic homeostasis and specific pathological conditions.

Lastly, we observe differences in miRNA expression between naïve T cells obtained from cord vs. adult peripheral blood. Upon testing these miRNA, we were unable to show direct correlation of miRNA in regulation of CXCL8 expression. Future studies should look at miRNA that are differentially expressed between these two subsets to determine how they might relate to homeostatic and/or effector function. For example, it is known that cord blood naïve T cells differ in their proliferation and cytokine responses. It might be interesting to determine if there is a link between the miRNA differentially expressed between these subsets.

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Chapter 3

Phenotype and tissue distribution of CD28H$^+$ immune cell subsets

Excerpts from this chapter were published:

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3.1 Abstract

CD28H is a newly discovered co-receptor of the human B7 family. CD28H interacts with its ligand B7-H5 and regulates T cell response. Here we showed that CD28H was not expressed on granulocytes, monocytes, myeloid dendritic cells (MDCs), and B cells, but constitutively expressed with moderate levels on memory T cells and with high levels on naïve T cells, innate lymphoid cells (ILCs), natural killer (NK) cells, and plasmacytoid dendritic cells (PDCs) in human peripheral blood. Similar CD28H$^+$ cell profile existed in secondary lymphoid organs and pathological tissues including multiple types of cancers. Further analysis demonstrated that CD28H$^+$ naïve and CD28H$^+$ memory T cells were characterized with increased naïve feature and less effector functional phenotype, respectively. High levels of constitutive CD28H expression on naïve T cells and innate immune cells suggest a potential role of CD28H in innate and adaptive immunity.

3.2 Introduction

The B7-CD28 co-signaling pathway is crucial in acquisition of effector

functions by the immune system. Recent studies have demonstrated that inhibitory B7 family members and their receptor signaling pathways mediate dysfunctional T cell immunity in the tumor microenvironments (Curiel et al., 2003; W. Zou, 2005; W. Zou & Chen, 2008) and blockade of PD-L1 (B7-H1) and PD-1 pathway induces important objective clinical responses in many types of human cancer (Pardoll, 2012; Weiping Zou, Wolchok, & Chen, 2016). It is well known that CD28 ligation in the context of TCR engagement is regarded as the most important co-signaling pathway to activate T cells and to prevent T cell anergy. In addition, CD28 may promote NK cell cytolytic activity and cytokine production (Galea-Lauri et al., 1999; Nandi, Gross, & Allison, 1994). A recent report shows that ILCs can express CD28 at different levels in inflammatory conditions,(Roan et al., 2016) however, the potential immunological role of CD28 in ILCs remains unknown.

In an effort to identify novel B7-CD28 co-signaling member and particularly explore potential new B7 target for cancer immunotherapy, CD28H (TMIGD2) is a newly discovered co-receptor of the human B7 family that interacts with its ligand B7-H5 (B7-H7, HHLA2) and regulates T cell responses (Zhu et al., 2013). CD28H is constitutively expressed on naive T cells. Repetitive antigenic exposure induces a complete loss of CD28H on T cells (Zhu et al., 2013). It has been reported that CD28H signaling pathways promotes T cell proliferation and effector responses (Zhu et al., 2013). However, additional studies demonstrate an inhibitory role of B7-H5 signaling in T cell activation (Zhao et al., 2013) and show a negative association between tumor tissue B7-H5 expression and patient outcomes (Cheng et al., 2017; Janakiram et al., 2015). In this study we systemically analyzed CD28H expression profile, the phenotype and the distribution of CD28H$^+$ cells in different immune cell subsets in peripheral blood, secondary lymphoid organs, and multiple types of

human pathogenic tissues including cancer tissues. Our study suggests a potential role of CD28H in innate and adaptive immunity.

3.3 Materials and Methods

Human subjects and human samples

Peripheral blood was obtained from healthy volunteers and patients with ovarian cancer. Mononuclear cells were enriched by Ficoll-Hypaque (GE Healthcare and Life Sciences) or Lymphoprep (STEMCELL Technologies) density gradient centrifugation. Naïve CD4$^+$ T cells were enriched from healthy donors using CD4$^+$ RossetteSep enrichment cocktail (STEMCELL Technologies) followed by anti-CD45RA microbeads (Miltenyi Biotec) for *in vitro* T cell cultures. Pathological tissues from breast cancer, colon carcinoma, lung cancer, ovarian cancer, and ulcerative colitis were used. Human tissues were from patients presenting for diagnostic biopsy, prophylactic colectomy, or tumor debulking. Mononuclear cells from healthy or patient donors were used fresh unless described otherwise. All donors provided written, informed consent. In addition to these pathological tissues, human spleen and tonsil tissues were obtained from the tissue procurement core at the University of Michigan Hospital. Human pathological tissue cells were obtained as described (Curiel et al., 2004; Curiel et al., 2003; Wang et al., 2016). The Institutional Review Boards of the University of Michigan School of Medicine approved the study.

Reagents

Antibodies for flow cytometry analysis were CD3 (cat. 557943, 562280, 340662, clone UCHT1), CD4 (cat. 470049, clone RPA-T4), CD8 (cat. 557760, clone RPA-T8), CD11c (cat. 559877, clone B-Ly6), CD14 (cat. 555397, clone M5E2), CD15 (cat.

642917, clone MMA), CD28 (cat. 555729, clone CD28.2), CD31 (cat. 561654, clone WM59), CD33 (cat. 341640, clone P67.6), CD45RA (cat. 562298, 560675, clone HI100), CD45RO (cat. 340438, 555492, 560608, clone UCHL1), CD127 (cat. 562436, clone HIL-7R-M21), CXCL8 (cat. 554720, clone G265-8), IFN-γ (cat. 557844, 557995, 562392, clone 4S.B3), TNF-α (cat. 557996, 554514, clone MAb11), NKp46 (cat. 562101, clone 9E2), T-bet (cat. 561264, clone 4B10), GATA-3 (cat. 563510, clone L50-823), Rorγ-t (cat. 563081, clone Q21-559) (BD Biosciences), CD28H (Amplimmune), CD7 (cat. 47-0079, clone 124-1D1), CD16 (cat. MHCD1630, clone 3G8), CD19 (cat. 25-0199-42, clone HIB19), CD45 (cat. MHCD4530, clone HI30), CD57 (cat. 48-0577, clone NK-1), Foxp3 (cat. 48-4776, clone PCH101), HLA-DR (cat. 47-9956-42, clone LN3), IL-2 (cat. 46-7029, MQ1-17H12) (ThermoFischer), Armenian Hamster isotype control (cat. 400916, clone HTK888), and anti-Armenian Hamster (cat. 405503, clone Poly4055) (Biolegend). Antibodies for T cell activation were anti-CD3 (cat. 555329/14-0038-82, clone UCHT1) and anti-CD28 (cat. 555728/16-0289-81, clone CD28.2) (BD Biosciences and ThermoFischer).

Flow cytometry

Single-cell suspensions from tissue were stained for extracellular surface antigens with specific antibodies, followed by fixation and permeabilization using Perm/Fix solution (ThermoFischer), then stained against intracellular antigens as described (Peng et al., 2015; Wang et al., 2016). Viable single cells were analyzed.

T cell culture

Naïve $CD4^+$ T cell subsets were stimulated with 2.5 μg/mL anti-CD3 and 1.25 μg/mL anti-CD28 mAb and 3 ng/mL rhIL-2 for up to 20 days. Medium was refreshed every 3

days with 2.5 ug/mL anti-CD3, 1.25 ug/mL anti-CD28 and rhIL-2 3 ng/mL. T cells were then were measured against CD28H at indicated time-points.

T cell stimulation

Freshly isolated mononuclear cells were stimulated *in vitro* with PMA/Ionomycin, GolgiStop and GolgiPlug (BD Biosciences) for 4 hrs then stained against extracellular and intracellular antigens as described above.

3.4 Results

3.4a CD28H expression on immune cell subsets from healthy human peripheral blood

We systemically examined the CD28H expression profile across immune cell subsets in healthy human peripheral blood following mononuclear cell enrichment. We found that CD28H was negligible on granulocytes, monocytes, myeloid dendritic cells (MDCs), and B cells (Fig. 3.1A-C). As expected, NK and T cells expressed CD28H (Zhu et al., 2013), and naïve T cells expressed higher levels of CD28H than memory T cells (Fig. 3.1C-E). Interestingly, we detected high levels of CD28H expression on plasmacytoid dendritic cells (PDCs) and innate lymphoid cells (ILCs), which had not been previously reported (Fig. 3.1B-D). We compare the phenotypic and potential functional characteristics of CD28H expression on different immune cell subsets in peripheral blood, secondary lymphoid organs, and pathological tissues including human cancer tissues in subsequent sections of this study (Fig. 3.2-3.6).

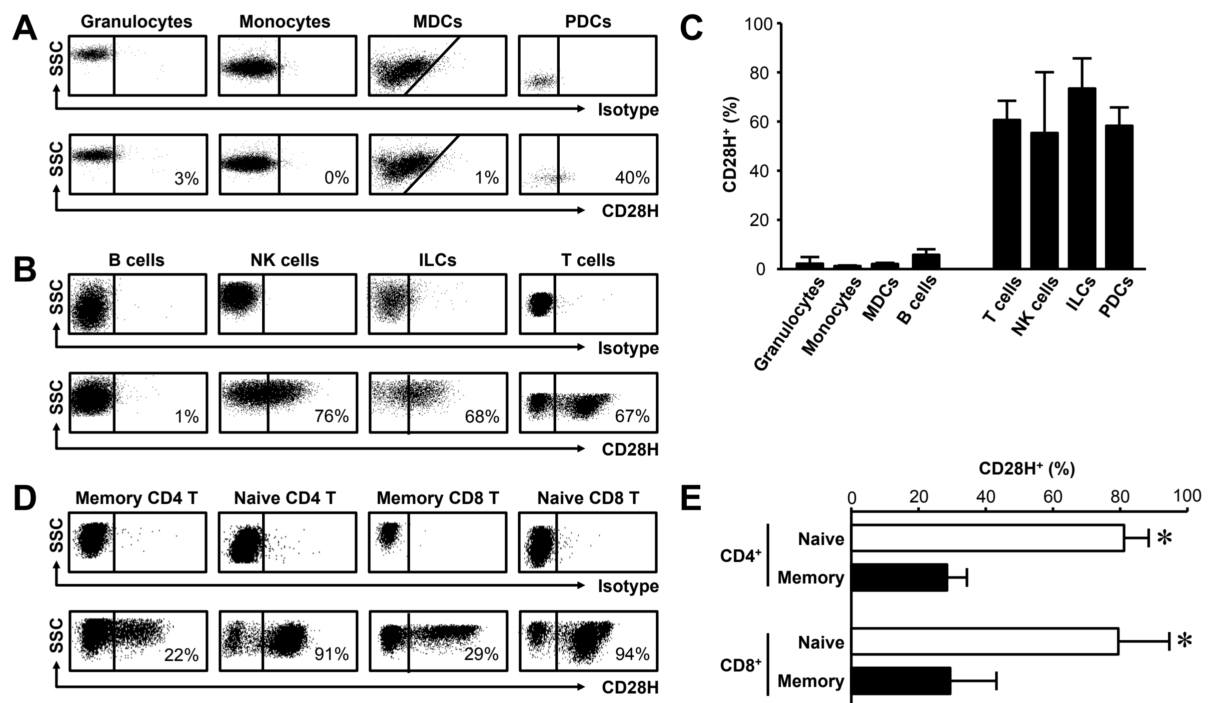

Figure 3.1 CD28H is expressed on immune cell subsets in healthy human peripheral blood. (A) Representative flow cytometric plots showing staining against CD28H or isotype control on FSC/SSC^{SINGLETS}CD45^{+}CD3^{-}CD19^{-} granulocytes (CD33^{+}CD14^{-}CD15^{+}), monocytes (CD33^{+}CD14^{-}CD15^{+}), myeloid dendritic cells (MDCs) (CD33^{+}CD14^{-}CD15^{-}CD11c^{+}HLA-DR^{+}) and plasmacytoid cells (PDCs) (CD33^{-}HLA-DR^{+}CD123^{+}). (B) Representative flow cytometric plots showing staining against CD28H or isotype control on FSC/SSC^{LOW}CD45^{+}CD3^{-} B cells (CD19^{+}), NK cells (CD7^{+}CD19^{-}CD16^{+}NKp46^{+}), innate lymphoid cells (ILCs) (lin^{-}CD7^{+}CD19^{-}CD16^{-} CD127^{+}), and FSC/SSC^{LOW}CD45^{+}CD19^{-} T cells (CD3^{+}). (C) Bar graphs summarize CD28H expression from (A) and (B). (D) Representative flow cytometric plots showing staining against CD28H or isotype control on FSC/SSC^{LOW}CD45^{+}CD33^{-} CD19^{-}CD3^{+} naïve (CD45RA^{+}CD45RO^{-}CCR7^{+}CD62L^{+}) and memory (CD45RA^{-} CD45RO^{+} CCR7^{-}CD62L^{-}) CD4^{+} and CD8^{+} T cells. (E) Bar graphs summarize CD28H expression from (D). *, $P < 0.05$. 6-10 donors.

3.4b CD28H^{+} naïve T cells possess enriched naïve characteristics

6-15% naïve T cells were CD28H^{-} (Fig. 3.1D, E). We analyzed and compared the potential phenotypic differences between CD28H^{+} and CD28H^{-} naïve T cells. We found that the majority of CD28H^{+}, but not CD28H^{-} naïve T cells expressed CD31 (Fig. 3.2A), a recent thymic emigrant marker (Kimmig et al., 2002). In line with this, CD28H^{-} naïve cells expressed higher levels of T_H1-regulating transcription factor T-bet (Fig. 3.2B), as well as effector cytokines IFN-γ (Fig. 3.2C) and TNF-α (Fig. 3.2D). Similar levels of IL-2 and CXCL8 were detected in CD4^{+}CD28H^{+} and CD4^{+}CD28H^{-}

naïve T cells (Fig. 3.2G-H). Interestingly, CD28 frequency was lower on CD28H⁻ than CD28H⁺ naive cells (Fig. 3.2E). Furthermore, enriched naïve CD4⁺ T cells cultured *in vitro* progressively lost CD28H surface expression (Fig. 3.2F). Taken together, compared to CD28H⁻ naïve T cells, CD28H⁺ naïve T cells show enriched naïve characteristics.

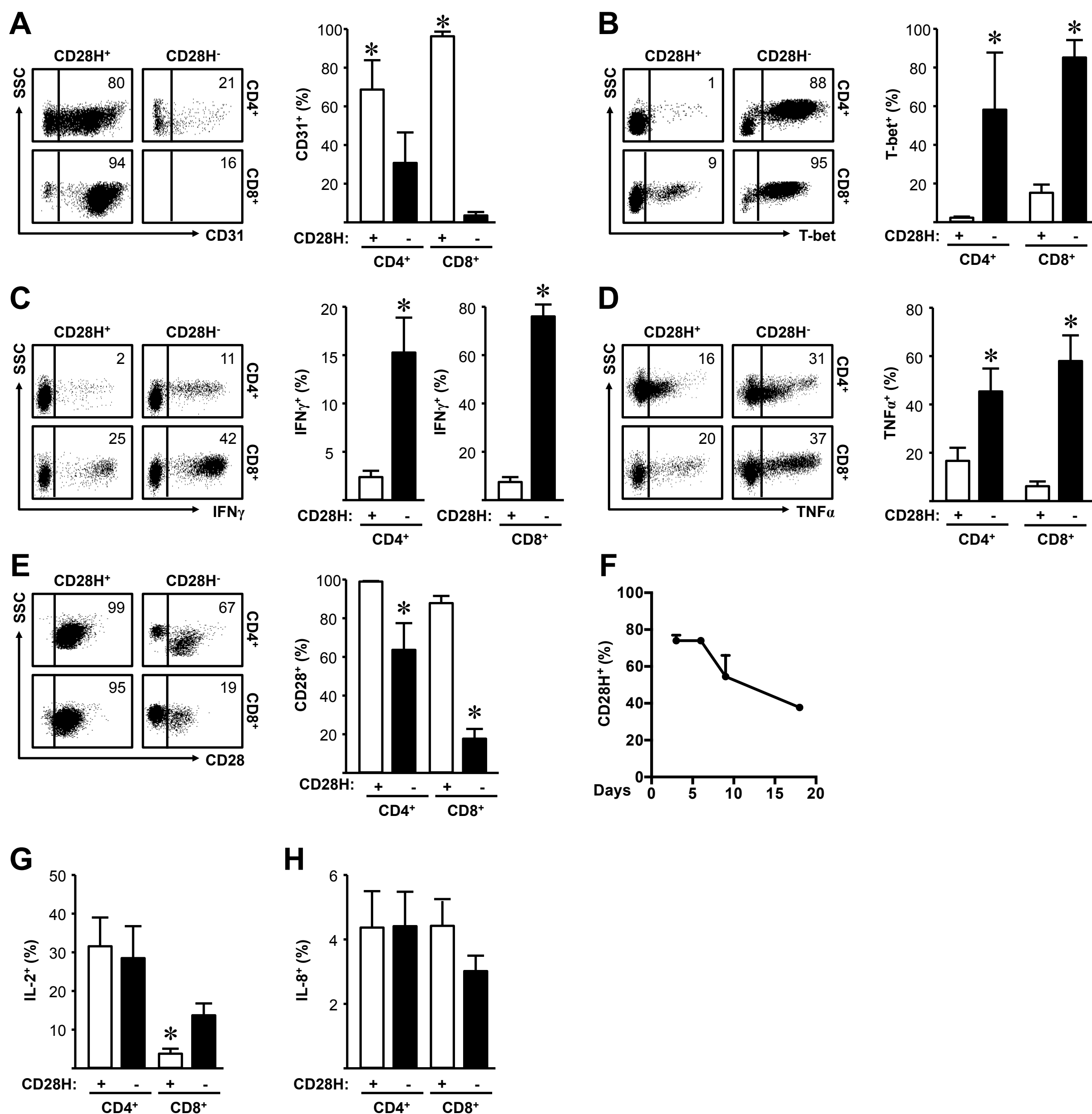

Figure 3.2 CD28H⁺ naïve T cells show increased naïve characteristics. CD28H⁺ naïve T cells show increased naïve characteristics. (A) Expression of CD31 on naïve T cells. Representative plots and the mean percentages + SEM showing CD31 expression on CD28H⁺ and CD28H⁻ naïve T cells. (B) Expression of T-bet on naïve

T cells. Representative plots and the mean percentages + SEM showing T-bet expression on $CD28H^+$ and $CD28H^-$ naïve T cells. (C) Expression of IFN-γ on naïve T cells. Representative plots and the mean percentages + SEM showing IFN-γ expression on $CD28H^+$ and $CD28H^-$ naïve T cells. (D) Expression of TNF-α on naïve T cells. Representative plots and the mean percentages + SEM showing TNF-α expression on $CD28H^+$ and $CD28H^-$ naïve T cells. (E) Expression of CD28 on naïve T cells. Representative plots and the mean percentages + SEM showing CD28 expression on $CD28H^+$ and $CD28H^-$ naïve T cells. 6-8 donors, *, $P < 0.05$. (F) Kinetic CD28H expression in *in vitro* cultured enriched naïve T cells. Enriched peripheral blood $CD4^+$ naïve T cells were stimulated with anti-CD3 and anti-CD28 every 3 days for up to 20 days as described. CD28H surface expression was measured by FACS. 3 donors with repeats. (G-H) Graphical summary shows the mean percentages + SEM of IL-2 (G) and CXCL8 (H) expression by naïve T cells from healthy donor blood.

3.4c $CD28H^+$ memory T cells show less effector function and differentiation

Approximately 20% of memory T cells expressed CD28H (Fig. 3.1D, E). We compared the phenotype of $CD28H^+$ and $CD28H^-$ memory T cells as we did with $CD28H^+$ and $CD28H^-$ naïve T cells (Fig. 3.3). $CD28H^-$ memory T cells showed increased IFN-γ to $CD28H^+$ memory T cells, as had been observed for $CD28H^-$ vs. $CD28H^+$ naïve T cells (Fig. 3.3A-B). $CD28H^-$ T cells might be replicative senescent following cellular division (Zhu et al., 2013). CD57 may serve as a marker of T cell terminal differentiation (Crespo, Sun, Welling, Tian, & Zou, 2013). In line with this, we detected an increased frequency of CD57 on $CD28H^-$ over $CD28H^+$ memory T cells (Fig. 3.4C, D). No changes were detected in IL-2 or CXCL8 expression between $CD28H^-$ vs. $CD28H^+$ memory T cells (Fig. 3.3E-F). Interestingly, we found higher levels of CD28H expression on RORγ-t^+CD4^+ and $Foxp3^+CD4^+$ T cells as compared to T-bet^+CD4^+ and GATA-3^+CD4^+ T cells (Fig. 3.4). Thus, $CD28H^+$ memory T cells show less effector function and differentiation, as independently measured by IFN-γ and CD57 expression. No correlation was drawn between $CD57^+$ and IFN-γ^+ T cells.

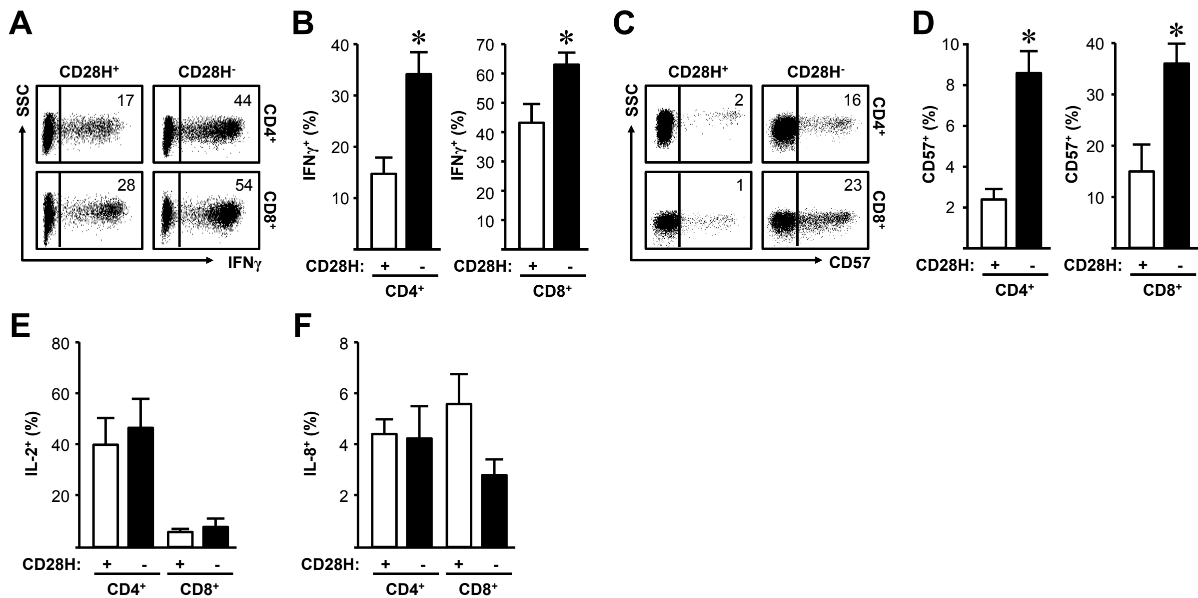

Figure 3.3 CD28H$^+$ memory T cells show less effector function marks. (A, B) Expression of IFN-γ on memory T cells. Representative plots and the mean percentages + SEM showing IFN-γ expression on CD28H$^+$ and CD28H$^-$ memory T cells. (C, D) Expression of CD57 on memory T cells. Representative plots and the mean percentages + SEM showing CD57 expression on CD28H$^+$ and CD28H$^-$ memory T cells. 6-8 donors, *, P < 0.05. (E-F) Graphical summary mean percentages + SEM of IL-2 (E) and CXCL8 (F) expression by memory T cells from healthy donor blood. N=5-6, P<0.05

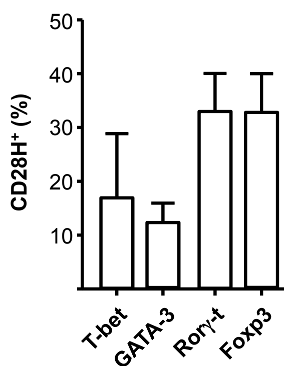

Figure 3.4 CD28H expression by T-Helper cell subsets. Graphical summary shows the mean percentages + SEM of CD28H by T-bet$^+$, GATA-3$^+$, Rorγ-t$^+$, or Foxp3$^+$ CD4$^+$ T cells from healthy donor peripheral blood. N=2-6.

3.4d CD28H$^+$ T cells exist in lymphoid organs and pathological tissues

CD28H is expressed in the majority of naïve T cells. Naïve T cells are largely located in secondary lymphoid organs. CD28H ligation by B7-H5 may have a

stimulatory (Zhu et al., 2013) or inhibitory (Zhao et al., 2013) effect on T cells. We analyzed CD28H$^+$ T cells in the human secondary lymphoid organs including tonsil and spleen. CD28H expression was not different between blood, tonsil, and spleen (Fig. 3.5A-B). Next we examined CD28H expression on T cells from ovarian cancer patient blood, ovarian cancer tissues, colon cancer tissues, and colon colitic tissues. We found CD28H$^+$ T cells in different pathological tissues (Fig. 3.5C-D). The levels of CD28H$^+$ T cells were slightly or moderately reduced, but not statistically different, in different pathological tissues as compared to healthy blood and ovarian cancer patient blood (Fig. 3.5C-D). We further evaluated the effector state of CD28H-expressing tissue-infiltrating T cells. We observed that the expression levels of CD57 (Fig. 3.6E) and IFN-γ (Fig. 3.5F) were significantly (Fig. 3.5E) and moderately (Fig. 3.5F) lower in CD28H$^+$ T cells than CD28H$^-$ CD8$^+$ T cells in different tissues. Thus, CD28H$^+$ T cells can be recruited into tumor and inflammatory tissue sites and tissue CD28H$^+$ T cells show less activation and differentiation.

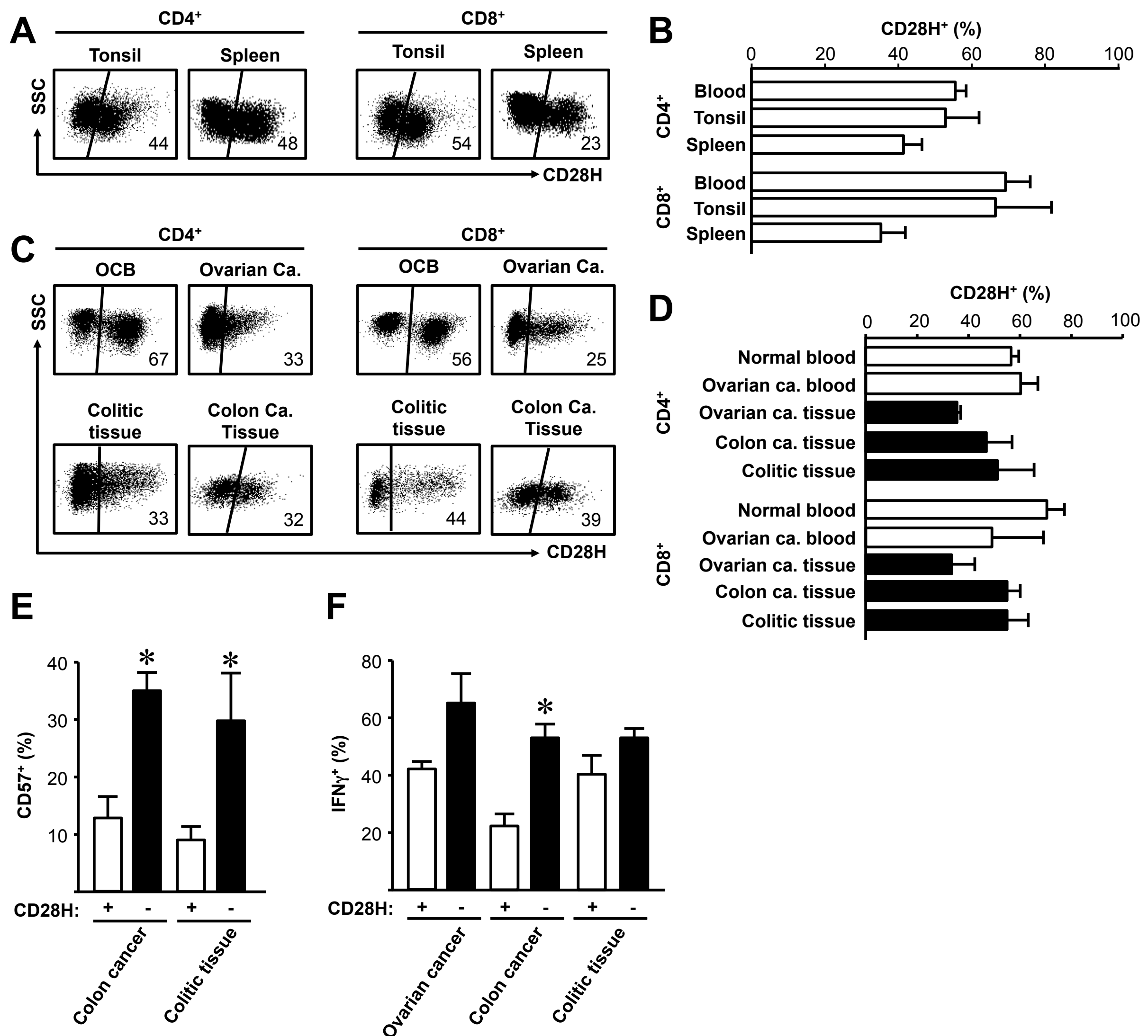

Figure 3.5 CD28H expression on tissue T cell subset. (A, B) Representative flow cytometric plots for isotype and anti-CD28H staining (A) and the percentages + SEM of CD28H expression (D) on secondary lymphoid tissue T cells. 4-6 donors. (C, D) Representative flow cytometric plots for isotype and anti-CD28H staining (C) and the percentages + SEM of CD28H expression (D) on pathological tissue T cells. 4-6 donors. (E, F) The mean percentages + SEM of CD57 (E) and IFN-γ (F) expressing $CD8^+$ T cells in $CD28H^+$ and $CD28H^-$ subsets from pathological tissue T cells. 4-6 donors, *, $P < 0.05$. OCB, blood from ovarian cancer patient; Ca, Cancer.

3.4e $CD28H^+$ ILCs and NK cells exist in lymphoid organs and pathological tissues

Expression of CD28H by tissue-infiltrating NK and innate lymphoid cells has not been studied. In human tissues, CD56 and NKP46 can be expressed by ILCs (Cella et al., 2009; Cupedo et al., 2009; Rankin et al., 2013; Spits & Di Santo, 2011).

Thus, we used CD127 along with other markers to define and distinguish NK cells from ILCs in human tumor tissues. We analyzed CD28H expression on $CD45^+CD3^-CD7^+CD16^+CD19^-CD33^-CD34^-CD127^-$ NK cells in secondary lymphoid organs including tonsil and spleen and different types of human cancer tissues. We detected high levels of CD28H expression on NK cells in tonsil and spleen (Fig. 3.6A, B) and several types of cancer tissues (Fig. 3.6C). It appeared that the percentage of $CD28H^+$ NK cells was lower in tissues than in peripheral blood (Fig. 3.6C). We also analyzed CD28H expression on $CD45^+CD3^-CD7^+CD16^-CD19^-CD33^-CD34^-CD127^+$ ILCs in secondary lymphoid organs including tonsil and spleen and different types of human cancer tissues (Fig. 3.6D). ILCs expressed high levels of CD28H in secondary lymphoid organs (Fig. 3.6E) and different types of human cancer tissues (Fig. 3.6F). Notably, the percentages of $CD28H^+$ ILCs were comparable in different pathological tissues (Fig. 3.6F). Further studies should measure the functional consequences of CD28H ligation on pDCs, ILCs and NK cells.

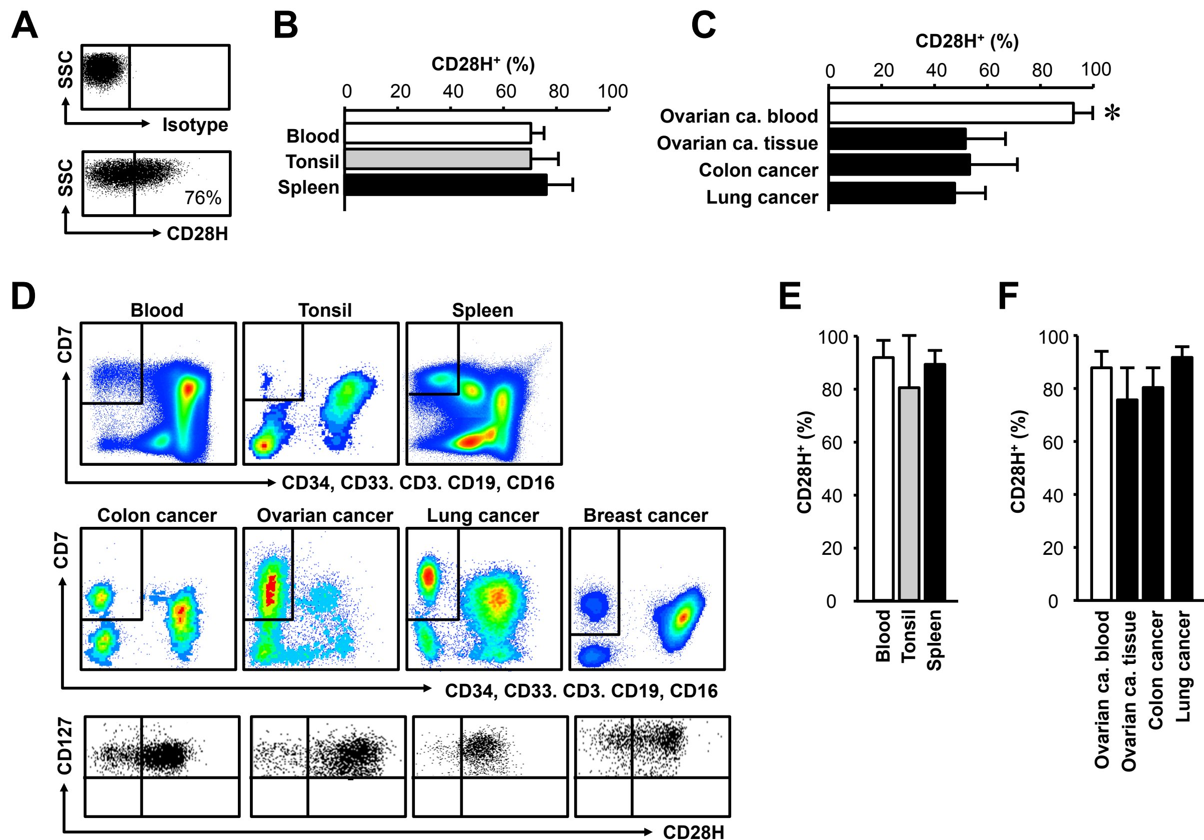

Figure 3.6 CD28H expression on NK cells and ILCs. (A-C) NK cells were gated on $CD45^+CD3^-CD7^+CD16^+CD19^-CD33^-CD34^-CD127^-$. (A) Representative flow cytometric plots for isotype and anti-CD28H staining in NK cells. (B) The mean percentage + SEM of CD28H positive cells in NK cells in peripheral blood of healthy donors (n = 8), tonsil (n = 5) and spleen (n = 4). (C) The mean percentage + SEM of CD28H positive cells in NK cells in ovarian cancer patient blood (n = 5) and cancer tissues from patients with ovarian cancer (n = 5), colon cancer (n = 5), and lung cancer (n = 4). * , $P < 0.05$. (D-F) ILCs were gated on $CD45^+CD3^-CD7^+CD16^-CD19^-CD33^-CD34^-CD127^+$. (D) Representative flow cytometric plots of ILCs in secondary lymphoid organs and cancer tissues for isotype and anti-CD28H staining. (E) The mean percentage + SEM of CD28H positive cells in ILCs in peripheral blood of healthy donors (n = 8), tonsil (n = 5) and spleen (n = 4). (F) The mean percentage + SEM of CD28H positive cells in ILCs in ovarian cancer patient blood (n = 5) and cancer tissues from patients with ovarian cancer (n = 5), colon cancer (n = 5), lung cancer (n = 4), and breast cancer (n = 3).

3.5 Discussion

In this work we have systemically examined CD28H expression in different immune cell subsets. In line with the previous report (Zhu et al., 2013), we have shown that CD28H is constitutively expressed on the majority of naive T cells and a small fraction of memory T cells in human peripheral blood, secondary lymphoid

organs, and different pathological tissues including breast cancer, colon cancer, lung cancer, ovarian cancer, and colitic colon tissues. Furthermore, we have not detected CD28H expression on B cells. Based on the phenotype and effector cytokine profile, we have found that CD28H$^+$ memory T cells show less effector function and minimal differentiation features. It has been described that engagement of CD28H (Zhu et al., 2013) and its ligand B7-H5 (Zhao et al., 2013) result in T cell activation and inhibition, respectively. This discrepancy is poorly understood in literature. Non-existence of murine CD28H and B7-H5 and lack of reliable, consistent, and reproducible reagents including specific neutralizing antibodies against human CD28H and B7-H5 significantly dampen our efforts toward a comprehensive understanding of this B7 family pathway. Nonetheless, CD28H expression profile and tissue distribution suggest a potential role of CD28H in adaptive T cell immunity.

In addition to T cells and B cells, we have examined CD28H expression on antigen presenting cell subsets and innate immune cell subsets. Granulocytes, monocytes, and MDCs do not express CD28H. However, 50% PDCs express CD28H. As peripheral blood (Siegal et al., 1999) and tumor associated (W. Zou et al., 2001) PDCs are major type-I IFN producers, PDCs are considered innate immune cells. CD28H expression is highly expressed on NK cells and ILCs. CD28 can support NK cell function (Galea-Lauri et al., 1999; Nandi et al., 1994). ILCs may express different levels of CD28 but the effect of its ligation has not yet been elucidated (Roan et al., 2016). Given the homology of CD28 and CD28H, CD28H may carry out a potential stimulatory and/or survival signal to ILCs and NK cells. Although high levels of CD28H expression on PDCs, ILCs, and NK cells in blood, secondary lymphoid organs, and pathological tissues suggest a potential role of CD28H in innate immunity, functional studies are in urgent need to define biological

and pathological relevance of CD28H and B7-H5 signaling pathway. Similar to the CD28 and B7 signaling pathway, we speculate that the CD28H and B7-H5 signaling pathway may add a novel layer of immune regulation in innate and/or adaptive immunity. Nonetheless, functional studies are essential to determine the basic immunological activity of this interaction and to assess whether targeting this pathway is an effective therapeutic approach for cancer immunotherapy.

3.6 References

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Chapter 4

Dot1L regulates STAT5-signaling and T cell anti-tumor function.

4.1 Abstract

Histone modifiers have been shown to regulate various aspects of cellular fitness. In this paper, we report Dot1L regulates T cell homeostasis, cell cycle progression and effector responses. Dot1L-deficient T cells displayed increased apoptosis *in vivo* that was further apparent upon *in vitro* activation. Decreased cellular fitness was characterized by gene expression patterns that affect cell cycle progression and survival. We found STAT5 was singularly affected in T cells lacking Dot1L which resulted in increased apoptosis levels *in vitro*. Lastly, anti-tumor responses were abrogated upon Dot1L deficiency in various models due to decreased TNF-α expression and higher apoptosis levels. Overall, we are the first to show Dot1L plays an important role in T cell homeostasis and effector responses.

4.2 Introduction

Epigenetic modifiers are known to regulate immune responses against cancer (Kryczek et al., 2014; Nagarsheth et al., 2016; Peng et al., 2015; Zhao et al., 2016). For example, H3K27-trimethylation was shown to mediate silencing of CXCL9 and CXCL10 expression by cancer cells thereby impeding tumor T cell infiltration in ovarian and colon cancer. On the other hand, EZH2 was observed to promote $CD8^+$ T cell polyfunctionality which results in better anti-tumor responses. Epigenetics are

therefore a rich area for research in cancer immunity (Nagarsheth, Wicha, & Zou, 2017).

Disruptor of telomeric silencing 1-like (Dot1L) is recognized as the sole epigenetic enzyme that catalyzes the methylation of H3K79 (Feng et al., 2002; Min, Feng, Li, Zhang, & Xu, 2003). H3K79 methylation is involved in driving gene expression in yeast cells and mammalian cells (Ng, Ciccone, Morshead, Oettinger, & Struhl, 2003; Schubeler et al., 2004; Steger et al., 2008). One study in humans suggests H3K79-trimethylation may be involved in gene repression (Barski et al., 2007). Dot1L and H3K79 methylations are known to have regulatory functions in cell cycle progression, DNA damage response, development and cellular stemness (Kim, Choi, & Kim, 2014; Kim, Kim, Park, Park, & Kim, 2012; Kryczek et al., 2014; Nguyen & Zhang, 2011). Our own studies showed Dot1L promoted colon cancer stemness and tumorigenicty but no studies have yet determined its role on T cell function (Kryczek et al., 2014).

In this study, Dot1L is shown to regulate homeostasis and its loss primes T cells for cellular death. Dot1L-deficiency leads to decreased T cell numbers, especially naïve T cells. Dot1L$^{-/-}$ T cells showed increased cell death *in vivo* and *in vitro*. Mechanistically, Dot1L was found to regulate cell death by establishing problems with cell cycle progression as well as decreased STAT5 expression. Pro-survival STAT5 was found to be singularly decreased and common-γ chain cytokines did not recover T cell apoptosis under activation or homeostatic conditions. Increased cell death hampered the acquisition of effector functions by T cells *in vitro* and in various *in vivo* tumor models. Our results show Dot1L is important in the switch from immune quiescence to activation.

4.3 Materials and Methods

Animal studies

Dot1L^{Flox} mice were generously donated by Dr. Jay Hess. *CD4*-Cre, CD45.1^{+}, and mice deficient in recombination-activating gene 1 (Rag1^{KO}) were obtained from Jackson Laboratory. Mice used were 6-10 weeks old and were in the C57BL/6 background. Bone marrow chimeras were generated by transfer of T cell-depleted bone marrow (1 x 10^7) into sublethally irradiated (5 Gy) Rag1^{KO} mice and allowing for reconstitution for 10 weeks. MC38 and LLC cell lines were s. c. injected (10^6 cells) into mice for up to two weeks before euthanizing at endpoint and carrying out studies. LLC cell line was injected i. v. into mice for up to two weeks before recovering lungs for cellular studies. Lung tumor burdens were separately recorded. All mice were kept in specific pathogen-free conditions in the BSRB mouse facilities under approval of the University of Michigan Committee on Use and Care of Animals.

Cell isolation

Mouse T cell subsets were enriched using CD3^{+} T cell negative selection followed by CD4^{+} negative selection (EasySep StemCell Technologies) from peripheral lymph nodes and/or spleens.

Cell culture

Homeostatic conditions: Mouse T cells were cultured (10^6 per mL) in 10% FCS, RPMI 1640 with recombinant mouse IL-7 and IL-15 (R&D Systems) at 10ng/mL for up to 24hrs.

T cell activation: Mouse T cell subsets (10^6 per mL) were activated with plate-bound anti-CD3 (clone HIT3a) and soluble anti-CD28 (clone CD28.2) (BD Biosciences or eBiosciences) for 24hrs in medium in presence or absence of recombinant mouse IL-2, IL-7 and/or IL-15 at final concentration of 10ng/mL (Kryczek et al., 2011).

Tumor cell lines: MC38 and EL4 tumor cells were cultured RPMI 1640 with 1% pennstrep and 10% FBS. LLC tumor cells were cultured DMEM with 1% pennstrep and 10% FBS.

T cell stimulation with PMA and Ionomycin, golgi stop and golgi plug was carried out as described previously (Kryczek et al., 2011).

Flow cytometric analysis

Cells were first stained against surface antigens then fixed and permeabilized using Fixation/Permeabilization solution (ThermoFischer Scientific) and stained for intracellular antigens. 7-AAD (559925), Annexin-V (556420, 550475), Brdu (560209), CD3 (557984), CD4 (552775), CD8 (557654, 552877), CD11b (553310), CD11c (553801), CD19 (cat. 553786), CD25 (551071), CD44 (560569, 553135), CD62L (553152, 553151, 553150), CD122 (553362), CD132 (554457), CD45.2 (560693), Foxp3 (560408), IFN-γ (560660), IL-2 (554427) and TNF-α (557644), (BD Biosciences), CD4 (47-0042), CD45.1 (12-0453), CD45RB (11-0455), CD69 (12-0691), CD90.2 (25-0902), CD127 (57-1271), and F4/80 (15-4801) (Thermofischer), and CD45 (cat. MCD4530) (Invitrogen). Samples were acquired using LSRII flow cytometric analyzer (BD Biosciences) and data was analyzed using FlowJo software (TreeStar).

Cellular proliferation assay

In vivo: Mice were injected intraperitoneally with Brdu solution as per the manufacturer's direction (Invitrogen) and cells were recovered from lymph nodes or spleens following 16 hours.(Yang, Neale, Green, He, & Chi, 2011)

In vitro: T cells were cultured as described above or otherwise stated in the text. Cells were added Brdu solution (Invitrogen) 1:100 dilution for the last hour of incubation following manufacturer's instructions.

Recovered cells were stained extracellular and fixed and permeabilized as described above. Cells were then incubated for 1 hour at 37*C in 300mg/mL DNAse (Sigma) in DPBS. Cells were stained against Brdu, 7-AAD, and other antigens as described above.

Cell apoptosis detection.

Cell apoptosis was measured with an Annexin-V/7-AAD apoptosis detection kit (BD Biosciences).

Gene-expression profile by microarray analysis.

RNA samples obtained from enriched Dot1L$^{+/+}$ or Dot1L$^{-/-}$, CD4^{+} or CD8^{+} T cells with Mouse Gene ST2.1 strips using the Affy plus kit. 3 biological repeats were analyzed. Samples were run by the University of Michigan DNA Sequencing Core. Principal component analysis was carried out to visualize profile of each sample and grouping to one another.

Quantitative real-time PCR.

Total RNA was isolated using TRIzol reagent (Invitrogen) obtained from cells at specified timepoints. Quantitative real-time PCR was carried out to quantify β-actin, p16, p19, p21, CDCA5, CDK4, TTK, and STAT5B).

Immunoblot analysis.

Western blotting was performed with the following antibodies against:

Milk was used for β-actin, H3K79-me2 and me3, Dot1L, Caspase-3 and -8, STAT1, 3, and 5, phosphorylated-STAT5, and Bcl-2. Signals were detected by ECL reagents (GE Healthcare)

Statistical analysis.

Statistical significance was determined by calculating P values using Student's T-test. P values less than 0.05 were considered significant.

4.4 Results

4.4a Dot1L deletion in T cells leads to loss of T naïve T cells.

H3K79-methylations are known to have diverse functions in the cell such as proliferation, apoptosis, cell cycle, stemness, etc. yet no studies have specifically elucidated its role on T cell survival and function.(Kim et al., 2014; Kim et al., 2012; Kryczek et al., 2014; Nguyen & Zhang, 2011) We crossed Dot1L$^{Flox/Flox}$ mice with transgenic expression of Cre recombinase under the *CD4* promoter to delete Dot1L in T cells specifically (referenced 'Dot1L$^{-/-}$' here). We found activated T cells upregulate Dot1L protein levels following activation through αCD3/αCD28 as well as H3K79-di- and tri-methylation (Fig. 4.1A-C). Dot1L$^{-/-}$ showed both loss of Dot1L

protein and H3K79-methylations. No changes in thymocyte frequency $CD4^-CD8^-$ double negative, $CD4^+CD8^+$ double positive, $CD4^+$ single-positive and $CD8^+$ single positive were observed between $Dot1L^{+/+}$ vs. $Dot1L^{-/-}$ (Fig. 4.1D) suggesting Dot1L loss does not affect thymocyte development. However, $Dot1L^{-/-}$ mice showed a considerable loss of T cells in peripheral lymph nodes (Fig. 4.1E) with an altered ratio of $CD4^+$ to $CD8^+$ T cells in $Dot1L^{-/-}$ mice (Fig. 4.1F-G). We analyzed the distribution of T cell subsets in $Dot1L^{-/-}$ against $Dot1L^{+/+}$ mice. $CD4^+$ and $CD8^+$ T cells both showed a decrease in frequency and absolute cell numbers of the naïve population $CD62L^+CD44^-$ (Fig. 4.1H-J). Naïve marker CD45RB on both $Dot1L^{-/-}$ $CD4^+$ and $CD8^+$ T cells was significantly decreased (Fig. 4.1K-L). We reconstituted recombination-activating gene 1 mice with bone marrow obtained from $CD45.1^+$ $Dot1L^{+/+}$ and $CD45.2^+$ $Dot1L^{+/+}$ mixed at 1:1 ratio after T cell depletion to determine whether the T cell loss and altered phenotypes are inherent to the Dot1L loss (Fig. 4.1M). $CD45.2^+$ $Dot1L^{-/-}$ derived T cells were unable to properly engraft in peripheral organs as evidenced by $Dot1L^{+/+}$ vs. $Dot1L^{-/-}$ T cell ratios. Further, CD4:CD8 and $CD62L^+$ T cell ratios were not recovered. Dot1L therefore directly affects peripheral T cell population homeostasis.

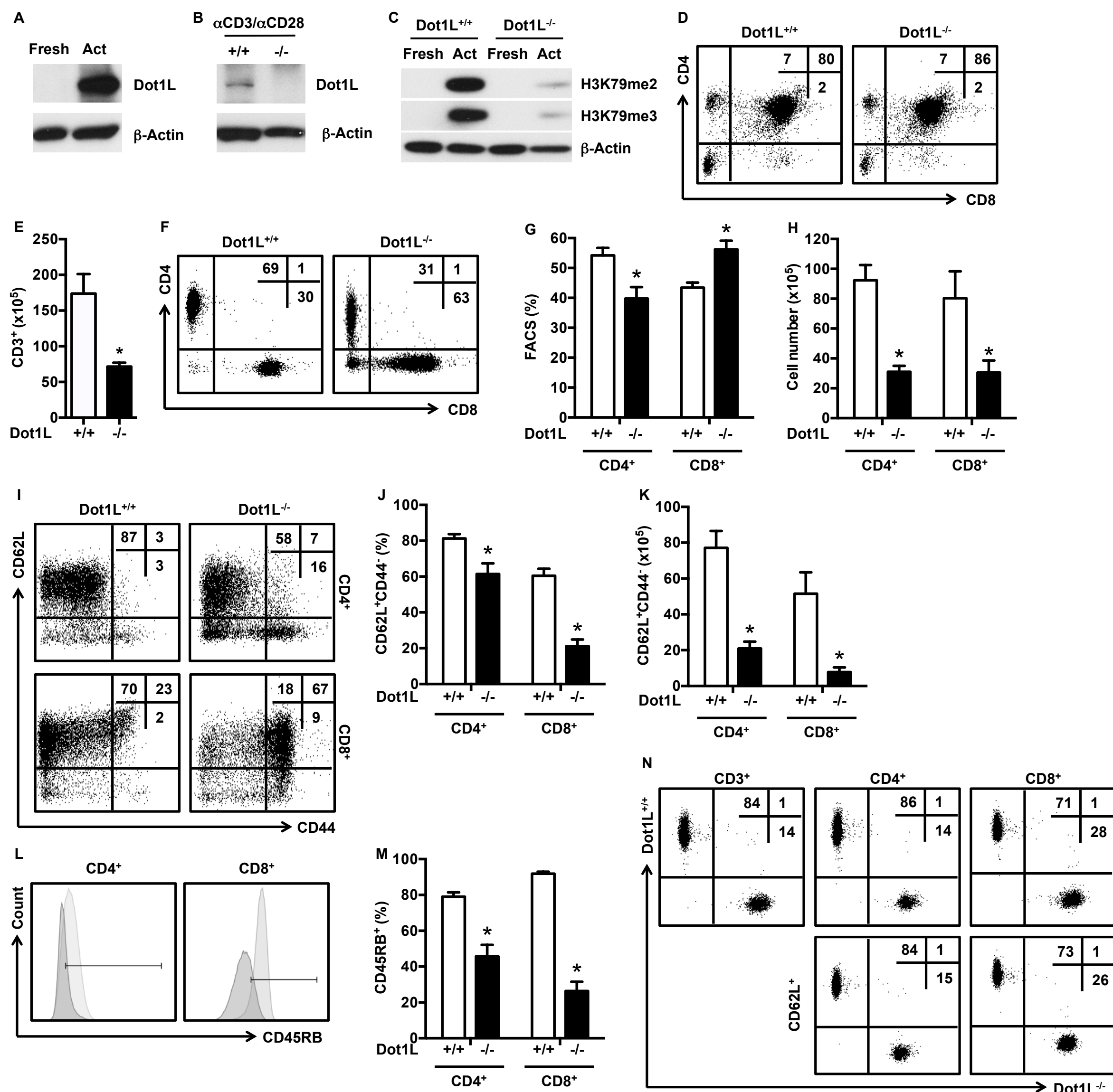


Figure 4.1. Dot1L regulates naïve T cell homeostasis. (A) Immunoblot against Dot1L on freshly enriched and 24hrs *in vitro* activated with αCD3/αCD28 Dot1L$^{+/+}$ T cells. (B) Immunoblot against Dot1L on 24hrs *in vitro* activated with αCD3/αCD28 Dot1L$^{+/+}$ vs. Dot1L$^{-/-}$ T cells. N=1-2 (C) Immunoblot against H3K79-me2/me3 on fresh and following 24hrs αCD3/αCD28 activated Dot1L$^{+/+}$ and Dot1L$^{-/-}$ T cells. (D) Representative flow cytometric plots of thymocytes from Dot1L$^{+/+}$ vs. Dot1L$^{-/-}$ mice. (E) Absolute CD4^{+} and CD8^{+} T cell numbers obtained from lymph nodes from Dot1L$^{+/+}$ vs. Dot1L$^{-/-}$ mice. (F) Representative flow cytometric plots of CD4^{+} and CD8^{+} T cells gated on CD45^{+}CD3^{+} cells from Dot1L$^{+/+}$ vs. Dot1L$^{-/-}$ mice. (G) Graphical summary of CD4^{+} and CD8^{+} frequency obtained by flow cytometry from Dot1L$^{+/+}$ vs. Dot1L$^{-/-}$ mice. (H) Representative flow cytometric plots of CD62L and CD44 expression by CD4^{+} (top) and CD8^{+} (bottom) T cells in Dot1L$^{+/+}$ vs. Dot1L$^{-/-}$ mice. (I-J) Graphical summary of CD62L^{+}CD44^{-} frequency (I) and cell numbers (J) in CD4^{+} and CD8^{+} T cells from Dot1L$^{+/+}$ vs. Dot1L$^{-/-}$ mice. (K) Representative flow cytometric plots against CD45RB on CD4^{+} (Left) and CD8^{+} (Right) obtained from Dot1L$^{+/+}$ vs. Dot1L$^{-/-}$ mice. N=3-6, $p<0.05$ (L) Graphical summary of CD45RB expression frequency on CD4^{+} and CD8^{+} T cells from Dot1L$^{+/+}$ vs. Dot1L$^{-/-}$. (M)

Representative flow cytometric plots from bone-marrow chimeric lymph nodes generated by mixing (1:1) T cell depleted bone marrow from CD45.1$^+$ Dot1L$^{+/+}$ vs. CD45.2$^+$ Dot1L$^{-/-}$ cells. Plots show relative percentages of T cells in reconstituted Rag$^{-/-}$ mice. N=4

4.4b Dot1L$^{-/-}$ T cells suffer decreased survival upon activation.

Given the decreased T cell numbers observed in the periphery of Dot1L$^{-/-}$ mice, we measured T cell apoptosis levels. We found significantly higher apoptosis levels, as measured by Annexin-V staining, on both CD4$^+$ and CD8$^+$ T cells freshly obtained from Dot1L$^{-/-}$ as compared to Dot1L$^{+/+}$ lymph nodes (Fig. 4.2A-B). Dot1L$^{-/-}$ T cells cultured *in vitro* under αCD3/αCD28 activation conditions were also found to show increased cell death vs. Dot1L$^{+/+}$ T cells (Fig. 4.2C-D). In line with this, activated T cells showed increased cleaved caspase-3 and -8 protein levels (Fig. 4.2E-F).

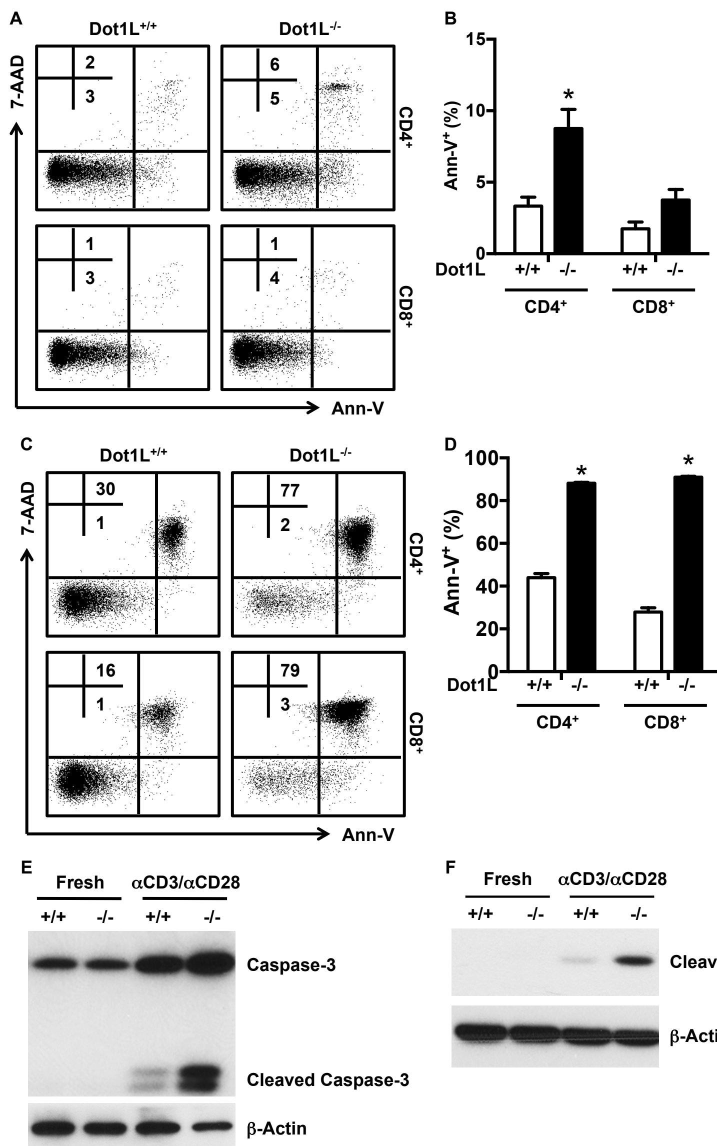
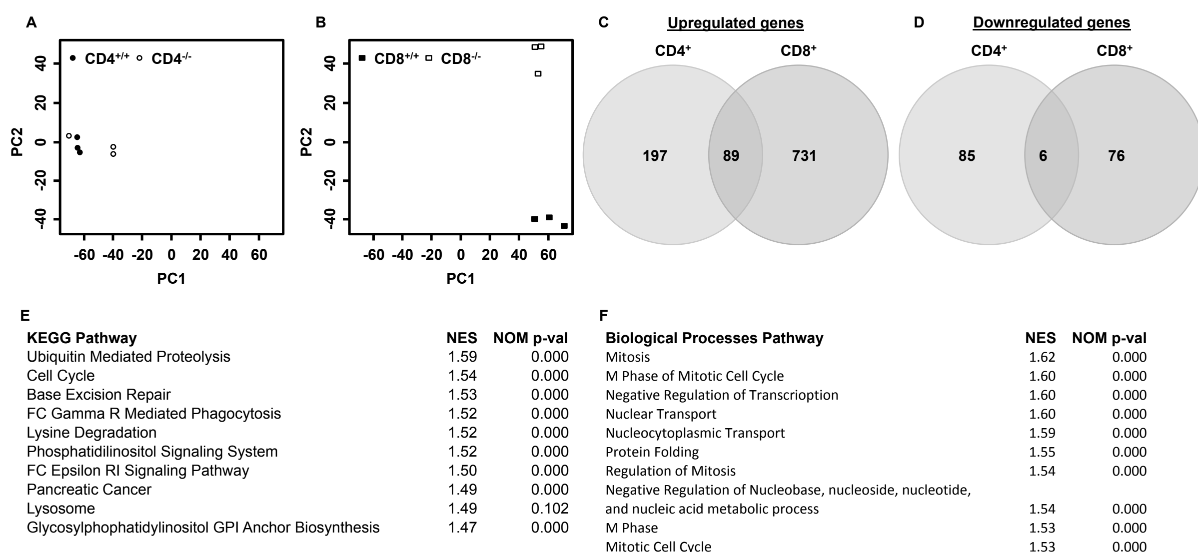


Figure 4.2. Dot1L-deficient T cells display exacerbated cell death upon activation. (A) Representative flow cytometric plots showing Annexin-V and 7-AAD on $CD4^+$ (Top) and $CD8^+$ (Bottom) T cells freshly isolated from $Dot1L^{+/+}$ vs. $Dot1L^{-/-}$ mice. (B) Graphical summary of Annexin-V staining from (A). (C) Representative flow cytometric plots showing Annexin-V and 7-AAD on enriched $CD4^+$ (Top) and $CD8^+$ (Bottom) T cells *in vitro* activated with aCD3/aCD28 for 24hrs obtained from $Dot1L^{+/+}$ vs. $Dot1L^{-/-}$ mice. (**D**) Graphical summary of Annexin-V staining from (C). (E) Immunoblot showing total and cleaved caspase-3 (E) or cleaved caspase-8 (F) protein level on freshly isolated or *in vitro* activated T cells.

4.4c Dot1L is important for cell cycle progression.

We performed a gene expression microarray and found significant differences in gene expression patterns as a consequence of Dot1L deletion in T cells (Fig. 4.3 and 4.4). Principal component analysis showed $Dot1L^{+/+}$ vs. $Dot1L^{-/-}$ $CD8^+$ T cells

have the higher difference in gene expression than do Dot1L$^{+/+}$ vs. Dot1L$^{-/-}$ CD4^{+} T cells (Fig. 4.3A-B). H3K79-methylation is described to promote gene expression. Interestingly, Dot1L deletion on T cells led to more genes being upregulated than downregulated in both CD4^{+} and CD8^{+} T cells (Fig. 4.3C-D). Further, gene set enrichment analysis of KEGG and biological processes pathways found several pathways related to the cell cycle were affected (Fig. 4.3E-F and Fig. 4.4A-B). Therefore, we measured cell cycle progression on T cells deficient vs. wild-type in Dot1L. We activated T cells with αCD3/αCD28 for 24hrs and measured cell cycle progression by 7-AAD staining and Brdu incorporation through flow cytometry. Dot1L$^{-/-}$ were arrested in $G_{0/1}$ phase of the cell cycle whereas Dot1L$^{+/+}$ were able to progress through S and G_2 phases (Fig. 4.4C-D). We confirmed aberrant gene expression of cell cycle regulators on CD4^{+} and CD8^{+} T cells by qRT-PCR and found p16, p19, p21, Cdca5, CDK4, and TTK genes were negatively regulated in Dot1L$^{-/-}$ T cells (Fig. 4.4E-F). qRT-PCR was normalized to Gapdh in order to control for cellular death on Dot1L$^{-/-}$ T cells.



KEGG Pathway	NES	NOM p-val
Ubiquitin Mediated Proteolysis	1.59	0.000
Cell Cycle	1.54	0.000
Base Excision Repair	1.53	0.000
FC Gamma R Mediated Phagocytosis	1.52	0.000
Lysine Degradation	1.52	0.000
Phosphatidilinositol Signaling System	1.52	0.000
FC Epsilon RI Signaling Pathway	1.50	0.000
Pancreatic Cancer	1.49	0.000
Lysosome	1.49	0.102
Glycosylphophatidylinositol GPI Anchor Biosynthesis	1.47	0.000

Biological Processes Pathway	NES	NOM p-val
Mitosis	1.62	0.000
M Phase of Mitotic Cell Cycle	1.60	0.000
Negative Regulation of Transcrioption	1.60	0.000
Nuclear Transport	1.60	0.000
Nucleocytoplasmic Transport	1.59	0.000
Protein Folding	1.55	0.000
Regulation of Mitosis	1.54	0.000
Negative Regulation of Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	1.54	0.000
M Phase	1.53	0.000
Mitotic Cell Cycle	1.53	0.000

Figure 4.3. Dot1L deletion leads to aberrant gene expression in T cells. (A-B) Principal component analysis of Affymetrix 2.1 gene expression array. CD4^{+} (A) and CD8^{+} (B) T cells from Dot1L$^{+/+}$ vs. Dot1L$^{-/-}$ were enriched at >97%purity and submitted for analysis. N=3 per group. (C) Venn diagram visualizing up-regulated

genes in Dot1L$^{-/-}$ over Dot1L$^{+/+}$ CD4^{+} and CD8^{+} T cells and their corresponding overlap. (D) Venn diagram visualizing down-regulated genes in Dot1L$^{-/-}$ over Dot1L$^{+/+}$ CD4^{+} and CD8^{+} T cells and their corresponding overlap. (E-F) Gene set enrichment analysis (GSEA) of Dot1L affected genes. KEGG (E) and Biological processes (F) pathway analysis are shown in Normalized Enrichment Score order with nominal-p value.

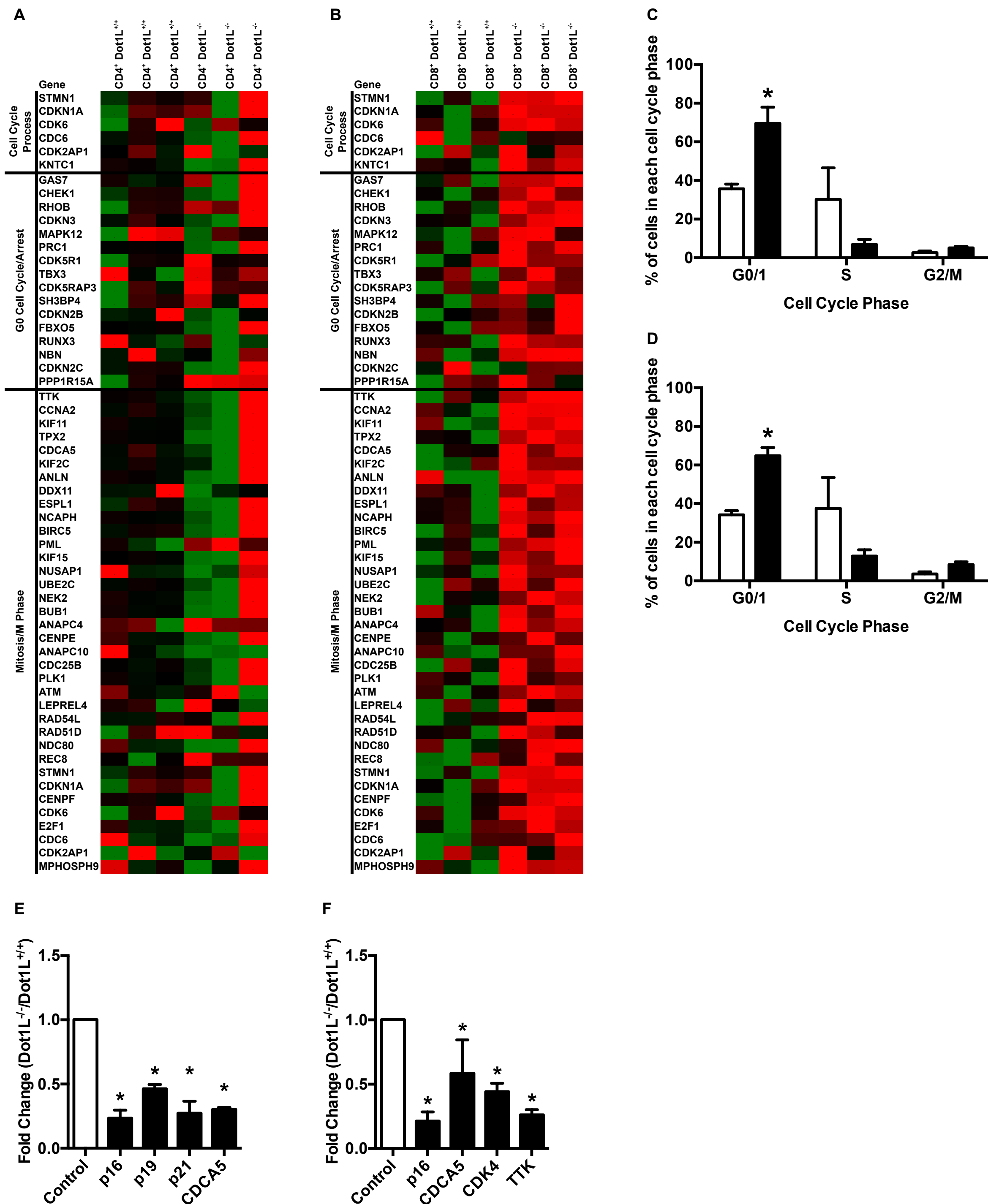


Figure 4.4. Dot1L deletion enforces cell cycle inhibtion. (A-B) CD4^{+} (A) and CD8^{+} (B) T cells were activated *in vitro* with aCD3/aCD28 for 24hrs and cell cycle

progress was measured through FACS. N=4, $p<0.05$. (C-D) Heatmaps of cell cycle-associated genes significantly changed in freshly enriched $CD4^+$ (C) or $CD8^+$ (D) T cells from $Dot1L^{+/+}$ vs. $Dot1L^{-/-}$ mice. (E-F) Fold change of cell cycle proteins affected in $CD4^+$ (E) and $CD8^+$ (F) $Dot1L^{-/-}$ vs. $Dot1L^{+/+}$ T cells activated with αCD3/αCD28 for 24hrs *in vitro*.

4.4d Dot1L regulates survival by regulating STAT5 regulation.

Dot1L-deficient T cells numbers are decreased *in vivo* in peripheral organs and show increased cell death upon culture *in vitro*. We tested T cell survival *in vitro* following activation and under homeostatic conditions with the addition of common-γ chain cytokines IL-2, -7, and -15 (Fig. 4.5A-D). Specifically, although treatment with common-γ chain cytokines did lower Annexin-V staining on cultured T cells, cell death remained significantly higher on $Dot1L^{-/-}$ T cells. This led us to hypothesize Dot1L-deficient T cells have abrogated STAT5 signaling. STAT5 is known to promote survival in T cells (Kimura et al., 2013; Tripathi et al., 2010). We measured STAT5 total protein and gene expression levels by freshly obtained T cells through immunoblot and qRT-PCR and found STAT5 was consistently decreased on $Dot1L^{-/-}$ vs. $Dot1L^{+/+}$ T cells (Fig. 4.5E-F). In contrast, STAT1 and STAT3 were not affected (Fig. 4.5D). We further cultured enriched $CD4^+$ or $CD8^+$ T cells and found $Dot1L^{-/-}$ cells also showed decreased phosphorylated-STAT5 following 15 mins incubation with IL-7 *in vitro* (Fig. 4.5G). Previously, STAT5 gene was shown to have H3K79 regulatory sites.

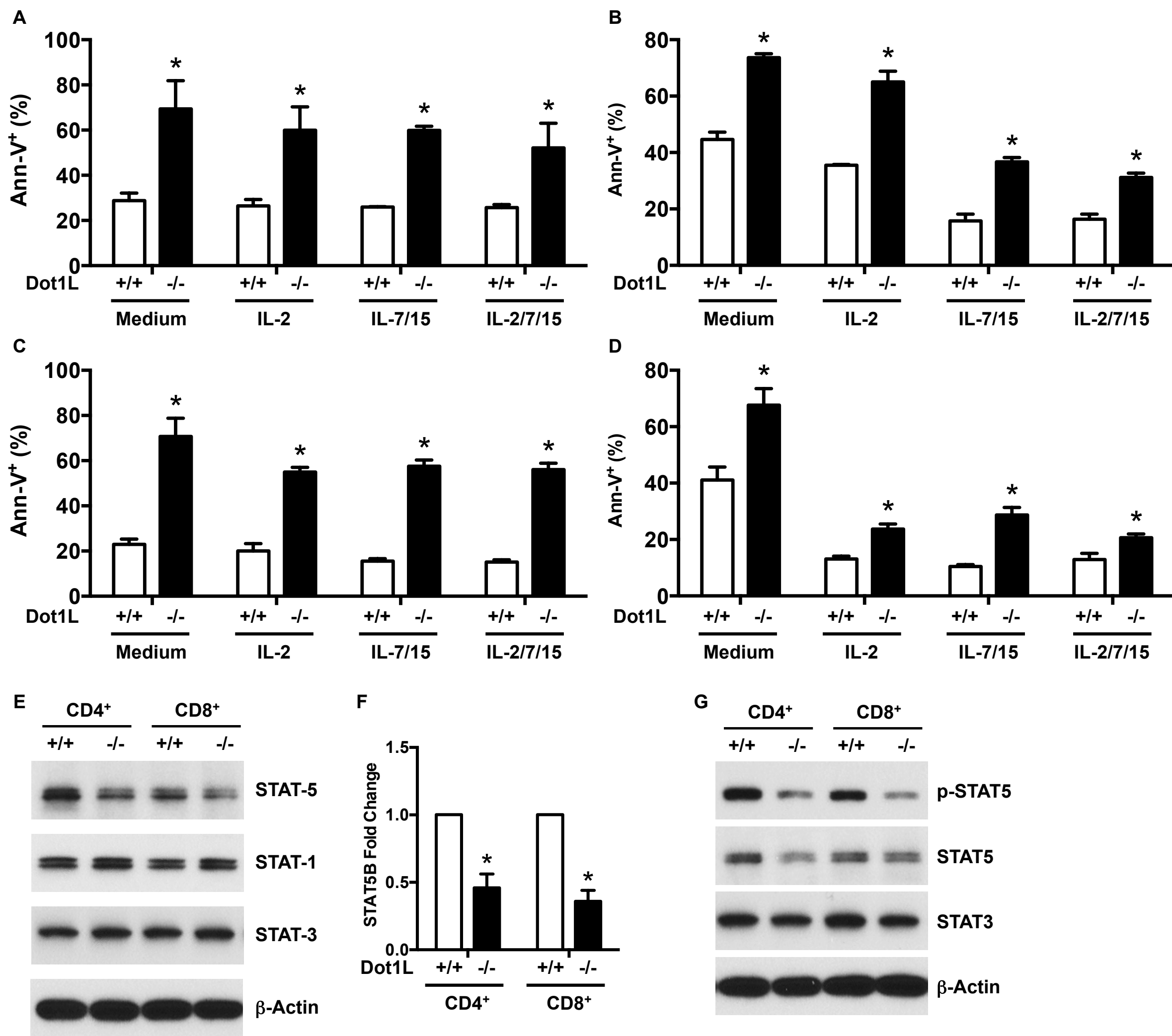


Figure 4.5. STAT5 expression is deregulated on Dot1L$^{-/-}$ T cells leading to cell death. (A-D) Enriched CD4^{+} (A-B) or CD8^{+} (C-D) T cells were cultured *in vitro* in the presence of αCD3/αCD28 (A, C) or medium (B, D) and mouse recombinant IL-2, -7, and/or -15. (E-F) Freshly enriched CD4^{+} or CD8^{+} T cells were measured for total protein levels by immunoblot (E) and gene expression by qRT-PCR (F) of STATs-1, -3, and -5. (G) Freshly enriched CD4^{+} or CD8^{+} T cells were treated with recombinant mouse IL-7 and phosphorylated STAT-5, total STAT-3 and -5 were measured by immunoblot.

4.4e Dot1L$^{-/-}$ T cells show abrogated anti-tumor responses

We examined the role of Dot1L in T cell immune responses *in vivo* by challenging Dot1L$^{-/-}$ mice with various tumor cell lines. Dot1L$^{-/-}$ mice exhibited increased tumor burden 12 days after sub cutaneous injection of MC38 tumor cells lines (Fig. 4.6A-B). Similar results were observed upon sub cutaneous and intravenous injection of LLC tumor cell lines where s. c. tumors showed bigger

tumors and i. v. showed increased tumor burden in the lungs of Dot1L$^{-/-}$ vs. Dot1L$^{+/+}$ mice (Fig. 4.6C-D). Dot1L$^{-/-}$ T cells activated *in vitro* with αCD3/αCD28 showed decreased effector and survival responses (Fig. 4.5A-D and 4.7A-D). This could underlie defective *in vivo* anti-tumor responses. We measured the T cell anti-tumor response and found TNF-α expression is downregulated in tumor-draining lymph nodes and tumor-infiltrating T cells from Dot1L$^{-/-}$ vs. Dot1L$^{+/+}$ mice (Fig. 4.6E-F). We also found increased apoptosis levels in these T cells (Fig. 4.6G). Taken together, Dot1L affects anti-tumor responses by decreasing T cell effector responses and survival in tumors.

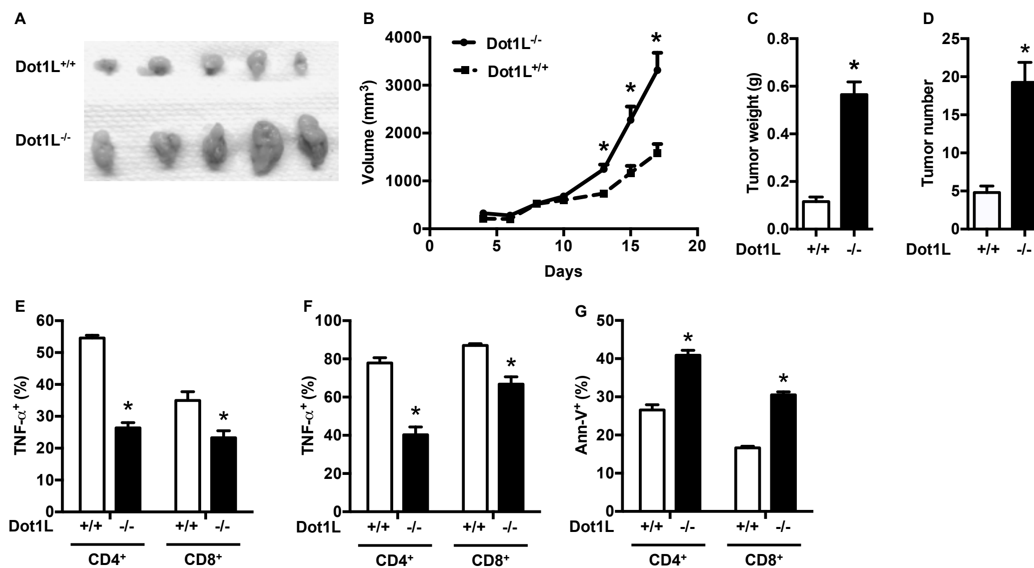


Figure 4.6. Dot1L-deficient T cells show impaired anti-tumor responses. 10^6 MC38 (subcutaneously) LLC (subcutaneously or intravenously through tail vein) tumor cell lines were injected into Dot1L$^{+/+}$ vs. Dot1L$^{-/-}$ mice for 18 days. Tumor growth, T cell death and effector responses were then measured. (A-B) MC38 tumor size at endpoint (A) and growth curve (B). (C) LLC tumor size at endpoint. (D) Number of tumors in tumor-bearing lungs 18 days post-injection. (E) Graphical summaries of TNF-α expression by CD4$^+$ and CD8$^+$ T cells (FACS) obtained from tumor-draining lymph nodes of LLC s.c. injected mice. (F) Graphical summaries of TNF-α expression by CD4$^+$ and CD8$^+$ T cells (FACS) obtained from tumor-bearing lungs of LLC i.v. injected mice. (G) T cell apoptosis as measured by annexin-V staining. CD4$^+$ and CD8$^+$ T cell apoptosis levels in tumor-draining lymph nodes of LLC s. c. injected mice.

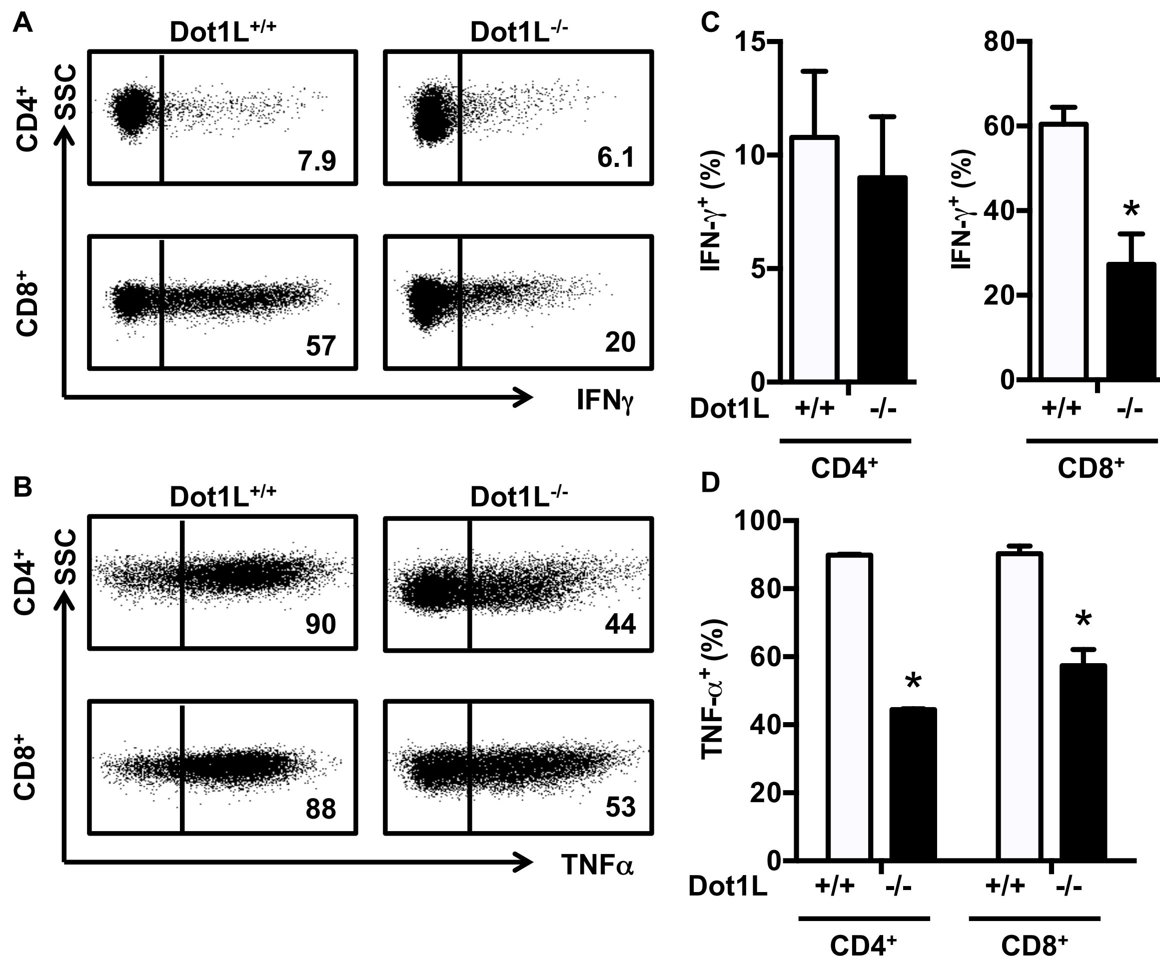


Figure 4.7. Loss of Dot1L leads to decreased T cell effector function. (A-D) T cells were enriched and activated *in vitro* αCD3/αCD28 for 36hrs and cytokine expression was measured. (A-B) Representative flow cytometric plots against IFN-γ (A) and TNF-α (B) in $CD4^+$ or $CD8^+$ T cells from $Dot1L^{+/+}$ vs. $Dot1L^{-/-}$ mice. (C-D) Graphical summaries of IFN-γ (C) and TNF-α (D) in $CD4^+$ and $CD8^+$ $Dot1L^{+/+}$ vs. $Dot1L^{-/-}$ mice. N=3 $p<0.05$.

4.5 Discussion

Various studies have looked at epigenetic modifiers, such as EZH2/H3K27-methylation, and their relationship to immune responses. These studies show the importance of the epigenetic regulatory axis in recruitment of T cells into the tumor microenvironment and their effector functions (Nagarsheth et al., 2016; Nagarsheth et al., 2017; Peng et al., 2015; Zhao et al., 2016). In this work, we show Dot1L is

important for T cell biology as it regulates T cell homeostasis, survival, and effector function.

Dot1L is the sole identified driver of H3K79-methylations in mammalian cells. Dot1L's roles are diverse which include cell cycle progression, the DNA damage response, cellular development and stemness (Kim et al., 2014; Kim et al., 2012; Kryczek et al., 2014; Nguyen & Zhang, 2011). However, Dot1L's involvement in T cell biology has not been studied. We have found that Dot1L is induced upon activation and important for naïve T cell homeostasis and survival. Dot1L-deficient T cells showed decreased survival *in vivo* and *in vitro,* as well as impaired anti-tumor responses. In support of this, naïve T cell numbers were significantly decreased in Dot1L-defcient T cell-bearing mice and were predisposed to apoptosis *in vivo*. Dot1L-deficient T cells were inherently impaired as Dot1L$^{-/-}$ T cells were unable to repopulate properly against Dot1L$^{+/+}$ T cells in RAG$^{-/-}$ hosts bone marrow chimera experiments.

We believe a Dot1L loss in T cells leads to increased naïve T cell death through impaired cell cycle progression and decreased STAT5 expression and signaling. As an epigenetic enzyme, Dot1L promotes the expression of various genes. Gene microarray and gene set enrichment analysis showed Dot1L-deletion leads to impaired cell cycle gene expression patterns. This aberrant gene expression profile led to decreased cell cycle progression. Various genes including *p16*, *p19* and *p21* were decreased on Dot1L-deficient T cells. Future experiments will determine the causal link between Dot1L and cell cycle gene expression. For example, H3K79-methylations are increased as the cell progresses through the cell cycle and we observe that Dot1L gene expression is increased following activation of T cells suggesting epigenetic enzyme is engaged upon proliferation being induced (Nguyen

& Zhang, 2011). Gene array results comparing Dot1L$^{+/+}$ vs. Dot1L$^{-/-}$ T cells did not show differences in Klf2, Foxo1, or Tsc1 known drivers of naïve T cell quiescence and homeostasis (Carlson et al., 2006; Kerdiles et al., 2009; Ouyang, Beckett, Flavell, & Li, 2009; Takada et al., 2011; Yang et al., 2011). STAT5 expression was also diminished in unmanipulated fresh T cells and incubation with common-γ chain cytokines IL-2, IL-7, and/or IL-15 was unable to completely rescue cell death. We hypothesize that Dot1L promotes STAT5 expression in T cells through H3K79-methylation. Future experiments will directly measure H3K79-methylation occurring at the STAT5 promoter as well as whether overexpression of STAT5 on Dot1L-deficient T cells rescues naïve T cell homeostasis and effector function.

We found Dot1L-deficient T cells showed abrogated anti-tumor responses. We believe increased tumor burden was due to increased T cell death and decreased cytokine expression. Sub-cutaneous and intra-venous tumor models both showed increased tumor burden, increased apoptosis and decreased cytokine expression. Given memory T cells derive from naïve T cells and Dot1L-deficient T cells are themselves impaired under homeostatic conditions, effector/memory T cells do not properly develop throughout the anti-tumor immune response. In support of this, *in vitro* activated T cells were shown to become apoptotic and displayed decreased cytokine expression. Future experiments will test whether overexpressing STAT5 in Dot1L-deficient mice recovers or partially recovers anti-tumor responses *in vivo*.

In summary, Dot1L is shown to have various impacts in T cell homeostasis and function. Naïve T cells were largely impaired and unable to properly develop immune responses *in vivo* and *in vitro*. We believe Dot1L is directly involved in the progression of T cells through the cell cycle and survival through STAT5 expression.

Given Dot1L-deficient T cells show decreased survival, these cells do not develop proper anti-tumor responses *in vivo*. Future experiments will aim at determining whether Dot1L expression is affected at the tumor microenvironment. Dot1L inhibitors are currently being studied in the clinic for MLL and AML with some efficacy. It will be important to determine the effects of these small-molecule inhibitors in the anti-tumor immune response by T cells and other immune cells.

4.6 References

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Chapter 5

Conclusions and future directions

5.1 Summary

This dissertation investigated distinct naïve T cell subsets and their functional state in blood, secondary lymphoid organs and pathological tissues, as well as the epigenetic machinery in place that regulates naïve T cell quiescence and survival. We first characterized a naïve T cell population in newborns with enhanced CXCL8 expression profile and pro-tumoral capability which was previously unappreciated in the field. We further show that CD28H$^-$ naïve T cells display a more differentiated phenotype over CD28H$^+$ naïve T cells which is maintained across secondary lymphoid organs, cancer and colon colitic pathological tissues. Lastly, we explore the epigenetic networks that regulate naïve T cell phenotype, quiescence and survival and how its loss leads to improper activation and anti-tumor responses *in vivo*. The following section will discuss the specific contributions of each chapter, potential future directions, and major implications for these studies.

5.2 Objectives, major findings and implications for chapter 2

In chapter 2 our objective was to characterize the cytokine expression profile and functional impacts of naïve T cells from cord blood. Currently, naïve T cells are thought as being homogenous and functionally quiescent cells that acquire effector cytokine expression following activation. We provide evidence that naïve T cells from cord blood selectively and spontaneously express CXCL8 and are poised for its

expression following activation. We show CXCL8$^+$ cord blood naïve T cells expressed several naïve-associated markers while lacking activation or differentiation marks that are gained following activation. Their pro-inflammatory state was further enhanced following activation as CXCL8 increased *in vitro* along with gaining IFNγ (Fig. 5.1). In contrast, memory T cells, which rapidly express various effector cytokines, did not readily express CXCL8. CXCL8 is unique in that naïve T cells spontaneously express this chemokine while memory T cells do not. Lastly, we observe naïve T cell-derived CXCL8 is capable of promoting neutrophil migration and tumor progression. Therefore, CXCL8$^+$ cord blood naïve T cells are a unique subset of T cells with naïve phenotype and pro-inflammatory state that challenge previous notions of naïve T cell functionality and can have a direct role on shaping early immune responses in humans.

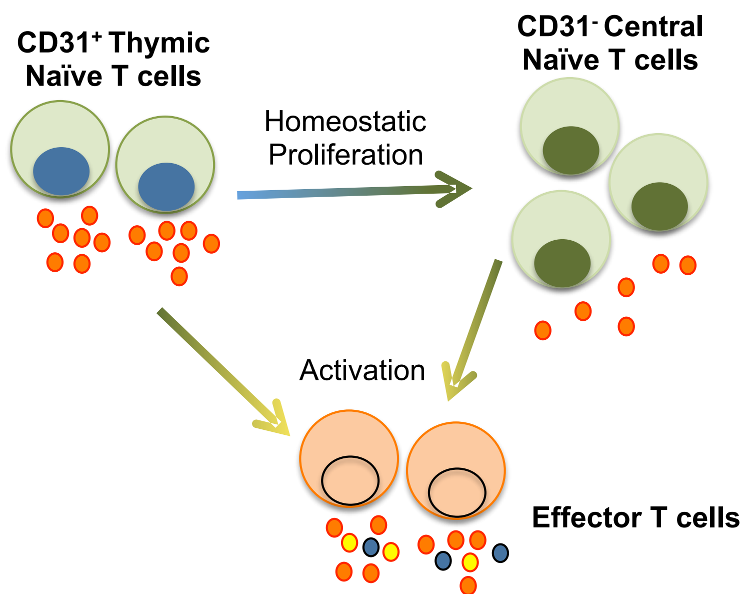

Figure 5.1. Human naïve T cells spontaneously express CXCL8. Naïve T cells from umbilical cord blood spontaneously express CXCL8 which is lost in adulthood as cells differentiate due to homeostatic proliferation. Further, naïve T cells are poised for CXCL8 expression following activation.

Naïve T cell functionality

Naïve T cells are generally defined as uniform in surface phenotype, having slow proliferative rate, and being functionally quiescent cells that gain effector functions following activation and differentiation into specific lineages. We challenge this notion by demonstrating the human naïve T cell compartment is heterogeneous and may be functionally active as measured by CXCL8 expression, age, and tissue distribution. $CXCL8^+$ naïve T cells do not show any activation or differentiation marks that would suggest differentiation as they express CD31 and lack other activation marks such as CD69 and CD25. In fact, these cells are enriched in cord blood and newborns which have had limited exposure to pathogenic antigens (Das et al., 2017; Gibbons et al., 2014). $CXCL8^+$ naïve T cells are also found in $CD31^+$ naïve T cells from peripheral blood which are undifferentiated cells and are understood not to have undergone much proliferation as CD31 is rapidly lost following low-affinity TCR-MHC interaction.

We show spontaneous CXCL8 expression is found in naïve T cells from cord blood followed by $CD31^{+\ Thymic}$, and $CD31^{-\ Central}$ naïve T cells, in that order and this phenomena is not found on memory T cells (Fig. 5.1). Previous work has also looked at the plasticity in the naïve T cell compartment and possible functions of naïve T cells prior to activation through CXCL8 and Lymphotoxin-α (LT-α) expression (Das et al., 2017; Gibbons et al., 2014; Ohshima et al., 1999; van den Broek et al., 2016; Zeng et al., 2012). Newborns lack developed adaptive immune responses from B and T lymphocytes and as such primarily rely on antibodies that are passed through breast milk and innate immune responses. $CXCL8^+$ naïve T cells would serve to bridge the lack of memory responses by promoting innate immune cell recruitment and activation at sites of inflammation. $CXCL8^+$ naïve T cells were also found on

human secondary lymphoid organs where they may promote lymph node vascularization under homeostasis or following antigenic insult. Naïve T cells have been described as being a source of LT-α and to promote lymphoid organ homeostasis and integrity. Taking our studies together, naïve T cells offer support to the developing immune system in newborns and maintain lymphoid organs *in vivo*. These results therefore expand the field by showing naïve T cells may carry out active functions prior to activation.

$CXCL8^+$ naïve T cells in cancer

Naïve T cells are observed to infiltrate tumor tissues but their contributions to tumor immunity has not been directly explored. Current understanding is that naïve T cells are recruited to tertiary lymphoid structures in solid tumors through similar homing mechanisms as homing to secondary lymphoid organs (de Chaisemartin et al., 2011; Thompson, Enriquez, Fu, & Engelhard, 2010; Weninger et al., 2003). These tertiary lymphoid structures are thought to be unique pro-inflammatory sites rich in tumor-associated antigens and neoantigens that develop as tumors progress. Therefore naïve T cells may be functionally important at these sites as they will interact with cancer neoantigens to which they may respond and develop anti-tumor responses.

Unfortunately, our results suggest naïve T cells promote tumor growth in a CXCL8-dependent manner. However, these experiments were carried out in immune-impaired hosts. CXCL8 is known to recruit other immune cell subsets and activate them. It is therefore possible that $CXCL8^+$ naïve T cells may promote recruitment and activation of immune cell subsets to tumor sites though our experiments do not address these questions.

CXCL8$^+$ naïve T cell are mainly found in newborns and their frequency decreases in humans as they age and only regain CXCL8 following activation. In adults, CXCL8$^+$ naïve T cell frequency in blood is low indicating these may not play an important role at the tumor site. Naïve T cells obtained from adult peripheral blood gain CXCL8 following activation but it is currently unknown whether CXCL8 is retained following activation *in vivo* and following T_H-lineage specialization. We have also observed regulatory T cells enriched in naïve markers that express CXCL8 in colon cancer and colitic tissue which promote neutrophil migration (Kryczek et al., 2016). Another study from our lab shows naïve T cells at sites of cancer are prone to apoptosis which may limit CXCL8 expression capacity (Xia et al., 2017). Nonetheless, naïve T cells enriched from human tonsil express CXCL8 under homeostatic condition. It is possible that cancer metastasis to lymph nodes is facilitated by CXCL8$^+$ naïve T cells by promoting survival and/or stem-like capacity to metastatic cancer cells homing to lymph nodes.

Given these cells were identified in 2014, study into the specific phenotype, mechanism by which these cells express CXCL8, stability in humans, and how they play an active role in fetal development, juvenile diseases, immunizations, and early immune responses is currently unknown. Future studies should aim at further characterizing how CXCL8$^+$ naïve T cells achieve a pro-inflammatory state at baseline and why these cells exist. For example, these cells exist at a time in human life where memory responses have not yet developed. It is possible CXCL8$^+$ naïve T cells are rapid responders that recruit and activate innate immune responses. CXCL8 is also conducive towards angiogenesis and as such may promote neo-vascularization in developing secondary lymphoid organs. The development of these cells is also largely unknown. It is known that the fetal liver is the source of the T cell

lineage until it moves to the bone marrow shortly before being birthed. Thus CXCL8$^+$ naïve T cells may be developed specifically from this source and not the mature bone marrow.

Our studies focused on CXCL8 expression by CD4$^+$ naïve T cells from cord blood. It is of interest to determine whether CD8$^+$ T cells, B cells, natural killer cells and innate lymphoid cells from cord blood may share CXCL8 expression dynamics and their own contributions in shaping early immune responses.

5.3 Objectives, major findings and implications for chapter 3

In chapter 3 our objective was to measure the cellular distribution and functional status of CD28H-expressing cells in human healthy and pathological tissues. We found CD28H expression occurs across plasmacytoid dendritic cells, innate lymphoid cells, natural killer cells and T cells. We confirmed that naïve T cells have a higher frequency of CD28H than memory T cells. In both naïve and memory T cell compartments, CD28H$^+$ cells exhibit lower cytokine and less differentiation as compared to more mature CD28H$^-$ cells. CD28H was similarly expressed by immune cell subsets across blood, spleen, tonsil, ovarian cancer blood, ovarian cancer, colon cancer and colitic tissue, and lung cancer. Reduced effector and differentiation state was observed in CD28H$^+$ T cells, compared to CD28H$^-$ T cells, infiltrating pathological tissues as well (Fig. 5.2).

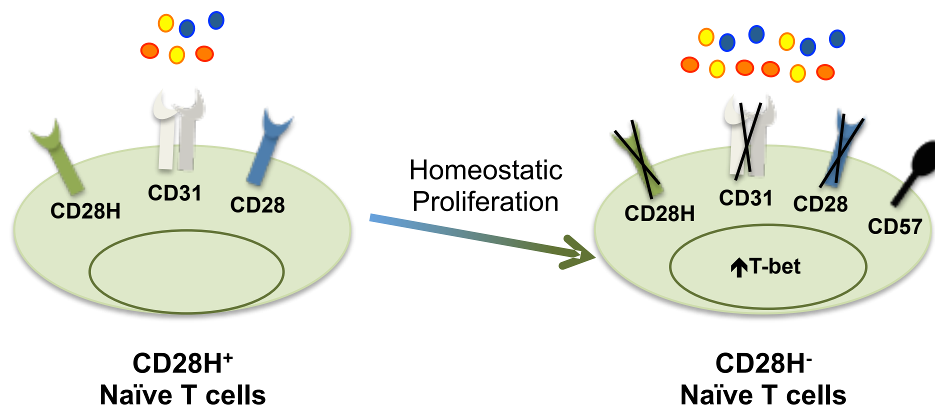

Figure 5.2. CD28H$^+$ naïve T cells feature more naïve characteristics over CD28H$^-$ naïve T cells. CD28H$^+$ naïve T cells display an undifferentiated phenotype characterized by higher CD31 and CD28 and lower T-bet, IFN-γ and TNF expression when compared to their CD28H$^-$ naïve T cell counterparts. CD28H is progressively lost on naïve cells following activation and possibly homeostatic proliferation.

CD28H/B7H5 are a newly identified pair in the B7-family whose signaling and effect on the immune system is not well described (Zhu et al., 2013). Co-stimulatory/inhibitory molecules have been shown to have vast effects on T cells such as promoting proliferation, survival, specific cytokine expression, T helper-lineage polarization and/or differentiation towards memory (Chen & Flies, 2013). CD28H engagement on T cells promoted cellular proliferation and enhanced cytokine expression (Zhu et al., 2013). Although we were unable to directly study the effects of CD28H engagement in T cells, we measured the effector state in blood and pathological tissues and suggest CD28H$^+$ T cells are less differentiated based on phenotype and cytokine expression patterns. As discussed above, CD31 may be used as marker for less differentiated naïve T cells. Lack of CD28 is associated with replicative senescence in T cells. CD28H$^+$ naïve T cells displayed increased CD31$^+$ frequency whereas CD28H$^-$ cells surprisingly showed decreased frequency of CD28$^+$ cells, suggesting CD28H$^+$ cells were antigen inexperienced while CD28H$^-$ had replicated *in vivo*. Following activation, naïve T cells rapidly lose CD28H suggesting CD28H may play a more important role shaping the naïve over the memory T cell

response. These results further suggest naïve T cells are a heterogeneous pool of cells with distinct functional states under homeostasis.

CD28H was detected in memory T, plasmacytoid dendritic, natural killer and innate lymphoid cells. $CD28H^+$ vs. $CD28H^-$ memory T cells showed a similar functional and less differentiated phenotype as $CD28H^+$ vs. $CD28H^-$ naïve T cells. Our studies also observed CD28H was stably expressed throughout healthy and pathological tissues in the aforementioned $CD28H^+$ cell subsets that would allow for co-stimulation through this ligand-receptor pair to occur in pathological tissues. It is currently not known how engagement of CD28H affects plasmacytoid dendritic, natural killer and innate lymphoid cell responses.

CD28H-ligand B7-H5 is expressed on monocyte-derived dendritic cells and macrophages and was further upregulated upon interaction with bacterial components (Zhu et al., 2013). B7-H5 was not detected on freshly isolated monocytes, NK, T or B cells though it is unknown whether they may gain B7-H5 expression following inflammation, as is B7-H1 (Yamazaki et al., 2002). It is therefore possible that B7-$H5^+$ T lymphocyte may provide $CD28H^+$ plasmacytoid dendritic cell signaling. Further, B7-H5 cellular distribution in healthy and pathological tissues has yet to be determined.

Ongoing studies on CD28H will focus on the functional consequence of CD28H/B7-H5 binding on naïve T cells, whether it leads to co-stimulation or co-inhibition and how it affects acquisition of effector cytokines, and specialization. One question from Chapter 2 is how CXCL8 expression is modulated and whether it can be specifically increased. Though $CD28H^+$ naïve T cells did not show increased CXCL8 expression, vs. $CD28H^-$ naïve T cells, it would be interesting to study whether CD28H engagement can enhance or decrease CXCL8 expression on T

cells. For example, CD28H may promote T cell polyfunctionality by stimulating simultaneous expression of multiple cytokines. Further, co-stimulatory molecules come in various flavors where they may promote specific T_H-lineages, memory formation and tissue homing. Studies should aim to determine whether CD28H is able to work in a similar manner. PD-1 marks differentiated or exhausted T cells in cancer tissues and it is being targeted to promote anti-tumor responses (Zou, Wolchok, & Chen, 2016). It will be interesting to test whether targeting CD28H could lead to better anti-tumor responses down the line. For example, if PD-1$^+$ CD28H$^+$ T cells exist and whether stimulation through CD28H recovers exhausted T cell effector functions.

5.4 Objectives, major findings and implications for chapter 4

In chapter 4 our objective was to evaluate Dot1L and its role in T cell quiescence and survival. We found Dot1L deletion on T cells leads to loss of T cells, markedly naïve T cell frequency and absolute number. We found T cells were prone to cell death *in vivo* and following *in vitro* incubation under homeostatic and activation conditions. Gene microarray showed dysfunctional gene networks in place and we confirmed cell cycle arrest following activation *in vitro*. Loss of Dot1L led to decreased STAT5 signaling which we hypothesize is one mechanism that inhibits survival. Further, Dot1L-deficient T cells mounted abrogated anti-tumor responses as compared to wild-type mice.

Naïve T cell quiescence is dynamically regulated through the mTOR signaling pathway. Loss of metabolic integrity regulated by this pathway leads to loss of quiescence, naïve phenotype, hyperactivation and decreased T cell survival (Zhang et al., 2012). Here we show Dot1L epigenetically enforces T cell quiescence and its

loss consequentially leads to decreased naïve T cells. Interestingly, gene microarray results did not show aberrant *Tsc1*, *Foxo1*, *Klf2*, *Sell*, *Ccr7* or *Cd44* gene expression on Dot1L$^{-/-}$ vs. WT T cells suggesting T cell quiescence was being regulated differently. Instead, T cells display increased cell death *in vivo* and following tumor challenge. T cells depend upon common-γ chain cytokines IL-7 and IL-15 for homeostatic survival and IL-2 during their effector state yet incubation of Dot1L$^{-/-}$ did not recover T cell survival *in vitro* to WT levels. ChIP-Seq data from a previous publication shows H3K79-methylation occurs at the STAT5 promoter in T cells (Zou et al., 2016). In line with this, STAT5 gene and protein expression and activation was decreased on Dot1L$^{-/-}$ vs. WT T cells. Chemical inhibition of Dot1L on EL4 cells also induced decreased STAT5 expression (data was not shown). Future experiments must question whether STAT5 overexpression on Dot1L$^{-/-}$ T cells recovers T cell death and STAT5-target gene expression i.e. Bcl-family members. Further, effector and anti-tumor capacity on STAT5-overexpressing Dot1L$^{-/-}$ T cells should also be studied.

Two striking observations from the gene micro-array are that Dot1L-deletion in T cells led to more genes being expressed (vs. WT) and that CD8^{+} Dot1L$^{-/-}$ vs. WT T cells show more profound gene expression differences than CD4^{+} Dot1L$^{-/-}$ vs. WT T cells. In humans, it has been shown that H3K79 may perform a negative regulation of gene expression in some genes therefore it is possible that this may be an operating mechanism in mouse T cells as well. Alternatively, H3K79 may modulate expression of other epigenetic modifiers such as Ezh2 and loss of a gene repressor results in increased gene expression on Dot1L$^{-/-}$ vs. WT T cells. Secondly, although quiescence and survival is lost on both T cell subsets, we do not why CD8^{+} T cells show more profound differences in gene expression. There are inherent differences

in $CD4^+$ vs. $CD8^+$ T cells such as TCR sensitivity, Runx3 vs. ThPOK in $CD8^+$ vs. $CD4^+$ T cells, and metabolic needs. It is possible that these may somehow play a role on gene expression following Dot1L-deletion on T cells. One limitation in this study was the use of whole $CD8^+$ T cells that could also explain these differences given the increase memory phenotype. This can be corrected by comparing cells with similar phenotypes based on CD62L and CD44 expression and depletion of regulatory T cells. This will allow a more clean comparison between cell subsets and understand Dot1L's role in gene expression by T cells.

In chapter 2 we studied $CXCL8^+$ naïve T cells but one question that remains is what the intrinsic mechanism for spontaneous CXCL8 expression by this cell subset is and its consequential increase following activation. We initially thought micro-RNA's may modulate CXCL8 expression in these cells but did not succeed in this. The epigenetic machinery is one hypothesis that may be tested. Dot1L is shown to regulate naïve T cell quiescence and be increased following activation. Similarly, Dot1L may be important for the stability of CXCL8 in T cells. CXCL8 is not expressed on mouse T cells and it has already been shown that CXCL8-homologues in mice (e. g. CXCL1, CXCL2, CXCL5) do not show the same patterns of expression as human cells therefore these studies will primarily be carried out in human samples.

Similarly, Dot1L may play a role in CD28H expression by T cell subsets and/or be downstream of CD28H signaling. H3K79 methylations promote gene expression. Given Dot1L's role in fresh T cells discussed in chapter 4, it is possible that CD28H expression in T cells may be partially regulated by H3K79-methylations. An alternative is that CD28H signaling may lead to stable epigenetic changes in T cells that regulate T cell responses as has been observed for CD28 signaling in $CD4^+$ T cells.

This is the first study that directly studies how the Dot1L/H3K79 epigenetic mechanism affects T cell quiescence, activation and effector responses and therefore many questions remain. Future studies should determine how Dot1L affects T cell metabolism, effector response maturation and is expressed by different T cell subsets and lineages. Finally, Dot1L/H3K79-mediated gene regulation is very probably involved in other immune cell subsets and their function. Further exploration on the impacts of Dot1L in the immune system should be investigated.

5.5 Final thoughts

The results in this dissertation challenge the dogma of homogeneity and functional quiescence of the naïve T cell compartment. We build from previous studies and show naïve T cells are heterogeneous in phenotype and effector state (Fig. 5.3). Further we show the importance of epigenetic networks and their influence on naïve T cell biology.

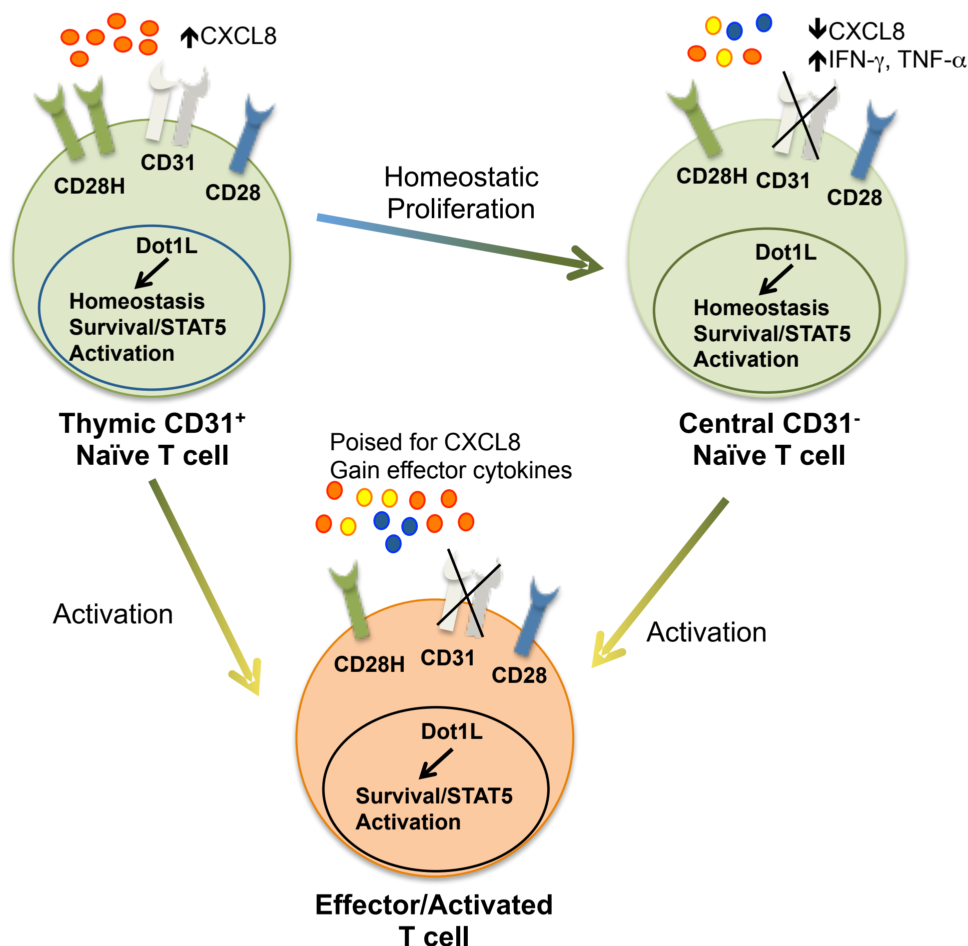

Figure 5.3. Model summarizing naïve T cell functional heterogeneity. CD31$^+$ naïve T cells from umbilical cord blood selectively and spontaneously express CXCL8 which is further increased following activation. Homeostatic proliferation of CD31$^+$ naïve T cells leads to loss of CD31 and decreased CXCL8 expression. In this same manner, CD28H$^+$ T cells are enriched in naïve features vs. CD28H$^-$ naïve T cells that are consequentially lost following homeostatic proliferation. Lastly, Dot1L was shown to regulate naïve T cell features by regulating cell cycle progression and survival. Dot1L-deficient T cells also showed increased apoptosis following activation.

These studies open the opportunity for other scientists to explore the naïve immune system from umbilical cord blood to pathological tissues and aged adults. Our experiments specifically focused on identifying naïve T cell functional heterogeneity and their possible impacts in cancer. Future studies should measure how different naïve T cells respond to immunizations, infections and cancer, can lead to autoimmune disorders or may be modified for anti-cancer treatments. As

humans age, so does the immune system and naïve T cells will become more homogenous in nature which is possibly why its heterogeneity not been fully appreciated before.

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