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The Role of Diacylglycerol Kinase Isoforms in T cell Differentiation

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by

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The thesis of Bianca Parisi is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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EPIGRAPH

We cannot solve problems with the kind of thinking we employed when we came up with them.

Albert Einstein

Every failure brings with it the seed of an equivalent success

Napoleon Hill

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ABSTRACT OF THE THESIS

The role of diacylglycerol kinase isoforms in T cell differentiation

By

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Master of Science in Biology

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Professor Susan Kaech, Chair

Professor Elina Zuniga, Co-Chair

$CD8^+$ T cells are an important arm of adaptive immune responses critical for protection against intracellular pathogens and cancer. $CD8^+$ T cells that recognizing tumor cells often exhibit a state of dysfunction and despite promising clinical progress in immunotherapies

targeting immunoregulatory pathways, the fundamental mechanisms underlying T cell dysfunction remain poorly understood. Diacylglycerol kinases (DGK), a class of enzymes involved in the catalysis of diacylglycerol (DAG) into phosphatidic acid (PA), is critical for TIF induced T cell suppression. Deletion of DGKH enhanced the differentiation of effector T cell responses especially though the expression of IFNγ in $CD8^+$ T cells. Increased effector cytokine production by DGKH knockdown is associated with increased expression of IL-2 receptor-alpha (CD25) and PD-1. Finally, adoptive transfer of tumor specific T cells lacking DGKH into liver tumor bearing mice resulted in increased expression of exhaustion marker TIM-3. In conclusion, we demonstrate the previously unexplored role of DGKH in $CD8^+$ T cell effector responses and how DGKH could serve as a potential therapeutic target to potentiate anti-tumor T cell response.

INTRODUCTION

Diacylglycerol kinases (DGKs) are a class of enzymes conventionally involved in the catalysis of diacylglycerol (DAG) to phosphatidic acid (PA). Both DAG and PA are lipids that play important roles as second messengers. DAG mediates signaling downstream of the TCR by recruiting RasGRP1 and PKCθ to the plasma membrane though its C1 domain and activating both the RasFRP1/Ras/ERK and the PKCθ/IKK/NF-kB pathways (Krishna & Zhong, 2013). PA on the other hand, can bind and induce activation of mTOR, PIP_5, SHP_1 and KSR_1 (Chen, Hu, & Zhong, 2016).

DGKs are shown to play heterogeneous roles across different cell type, and have functions in regulating cell signaling, metabolism, and lipid synthesis (Eichmann & Lass, 2015). There are ten different DGK isoforms that are conventionally divided into five subtypes based on which catalytic domains they contain, however a kinase domain and two C1 domains are conserved across all isoforms (Kume, Kawase, Komenoi, Usuki, Takeshita, Sakai, & Sakane, 2016). Besides structural differences, these isoforms express tissue specific expression profiles and localize to different subcellular compartments after activation (Massart & Zierath, 2019). Two DGK isoforms, alpha and zeta, have been studied in T cells as they have higher expression in immune cells, including mast cells and macrophages, compared to any other isoform (Krishna & Zhong, 2013). In mature T cells, DGKA and DGKZ are known to regulate self-tolerance by promoting T cell anergy; a state of functional inactivation following insufficient co-stimulatory signals during antigen encounter (figure 1A, figure 1B) (Krishna & Zhong, 2013; Schwartz, 2003). Additionally, several studies have shown that DGK expression is higher in exhausted T cells, suggesting they might play a direct role in contributing to loss of cytotoxicity (figure 1C) (Li, van der Leun, Yofe, Lubling, Gelbard-Solodkin, van Akkooi, van den Braber, Rozeman,

Haanen, Blank, Horlings, David, Baran, Bercovich, Lifshitz, Schumacher, Tanay, & Amit, 2020; Zheng, Zheng, Yoo, Guo, Zhang, Guo, Kang, Hu, Huang, Zhang, Liu, Dong, Hu, Ouyang, Peng, & Zhang, 2017). Given their primary role in leading to a dysfunctional T cell state as well as their location in signaling pathways downstream of the TCR, it was originally hypothesized, and later proven, that their ablation could increase T cell effector function, by indirectly causing increased production of effector cytokines IFNγ and TNFα (figure 1D) (Chen, Hu, & Zhong, 2016; Jing, Gershan, Holzhauer, Weber, Palen, McOlash, Pulakanti, Wesley, Rao, Johnson, & Riese, 2017; Shin, O'Brien, Grayson, & Zhong, 2012). These two isoforms have additionally been studied in the context of tumor immunity as potential targets in-conjunction to anti-PD1 therapies. DGKA has in fact been shown to contribute to the PD1/PDL1 suppression axis and to enhance exhaustion and ablation of DGKA and DGKZ have shown to increase response to anti-PD-1 treatment in mice with B16-OVA tumors (Fu, Li, Xiao, Yu, Li, Yuan, Shen, Dong, Fang, Zhang, Chen, Li, You, Xia, Kang, Tan, Chen, Yang, Gao, & Zhou, 2021; Jing, Gershan, Holzhauer, Weber, Palen, McOlash, Pulakanti, Wesley, Rao, Johnson, & Riese, 2017). Immune checkpoint inhibitors have shown incredible potential, however with many patients still not showing favorable responses, the search for mechanisms that could better therapy outcomes are still ongoing.

Although DGKA and DGKZ knockouts have shown to be associated with enhanced primary response against tumors and viruses, there are a few downfalls with targeting them therapeutically (Krishna & Zhong, 2013). First, these isoforms have medium to high expression levels across a wide range of lymphocytes, as shown by single-cell sequencing data on infiltrating T cells hepatocellular carcinoma, suggesting that their inhibition would affect a variety of immune cells (Zheng, Zheng, Yoo, Guo, Zhang, Guo, Kang, Hu, Huang, Zhang, Liu,

Dong, Hu, Ouyang, Peng, & Zhang, 2017) (table 1). In addition, DGKA and DGKZ also show extremely high expression in Foxp3$^+$ T cells suggesting that gene knockouts could be increasing Foxp3$^+$ T cells effector function within the tumor microenvironment (TME) (table 1). Foxp3$^+$ T cells, or regulatory T cells (Tregs), play pro-tumorigenic roles in that they suppress effector T cell function in the TME, thus increasing their cytotoxic activity wouldn't be beneficial in hindering tumor growth (Dowling, Kan, Heinzel, Marchingo, Hodgkin, & Hawkins, 2018).

DGK studies have been mostly linked to CD8+ cytotoxic T lymphocytes (CTLs), as these cells make up an important line of defense in anti-viral and anti-tumor immunity. However, one of the main reasons why T cells alone are not successful at halting tumor growth and proliferation is a phenomenon known as T cell exhaustion; a state of disfunction reached following prolonged antigen presentation in the TME. The exact mechanisms that regulate and lead to this state of dysfunction are still unknown, however both cell extrinsic and intrinsic (PD-1) regulatory pathways are known to play a role (Wherry, 2011). Common hallmarks of exhausted T cells are the loss of production of effector cytokines such as interferon gamma (IFNγ), which is a type II interferon, and tumor necrosis factor (TNFα) as well as increased expression of surface markers such as programmed cell death protein 1 (PD-1), lymphocyte activation gene (LAG-3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and SLAM family member 6 (SLAMF-6) (Avery, Filderman, Szymczak-Workman, & Kane, 2018; Wherry, 2011; Yigit, Wang, Ten Hacken, Chen, Bhan, Suarez-Fueyo, Katsuyama, Tsokos, Chiorazzi, Wu, Burger, Herzog, Engel, & Terhorst, 2019). Additionally recent advances in the field have led to identification of transcription and epigenetic markers that are uniquely expressed at different time-points within chronic antigen settings. Based on these findings T cells have been classified into stem-like PD1$^+$

TCF-1$^+$ T cells, transitory PD1$^+$ CXCR1$^+$ Tim3$^+$ TCF-1$^-$ T cells and terminally exhausted PD1$^+$ CD101$^+$ Tim3$^+$ TCF-1$^-$ T cells (Hudson, Gensheimer, Hashimoto, Wieland, Valanparambil, Li, Lin, Konieczny, Im, Freeman, Leonard, Kissick, & Ahmed, 2019). Transitory exhausted T cells produce cytotoxic cytokines and granzymes and are thus important for tumor clearance.

Here we try to find novel DGK isoforms that are selectively involved in promoting T cell exhaustion in hopes of developing more effective immunotherapies. The type II isoform DGKH has conventionally been studied with respect to bipolar disorders as it is highly expressed in the brain (Baum, Akula, Cabanero, Cardona, Corona, Klemens, Schulze, Cichon, Rietschel, Nothen, Georgi, Schumacher, Schwarz, Abou Jamra, Hofels, Propping, Satagopan, Detera-Wadleigh, Hardy, & McMahon, 2008). However, single-cell sequencing data on infiltrating T cells in hepatocellular carcinoma (HCC) which was made available by Dr. Zheng's groups, shows high expression of DGKA in all cell clusters, but DGKH expression to be limited to CD8-LAYN T cells and CD4-CTLA4 T cells (table 1) (figure 2). It also reveals that DGKD has expressions patters almost opposite to those of DGKH, with noticeable lower expression in CD8-LAYN+ exhausted T cells and CD4-LAYN+ FoxP3+ T cells. CD8-LAYN cells are unique in that they were found to be mostly derived from tumor tissue and expressed high levels of exhaustion markers *CTLA4*, *PDCD1*, and *HAVCR* and a high percentage of these cells associate to reduced disease-free survival rate (Zheng, Zheng, Yoo, Guo, Zhang, Guo, Kang, Hu, Huang, Zhang, Liu, Dong, Hu, Ouyang, Peng, & Zhang, 2017). In addition, high amounts of *LAYN* are found in tumor infiltrating FoxP3+ regulatory T cells, which also had higher expression levels of CTLA4 and were thus classified into the CD4-CTLA4 cluster. Given that both exhausted and regulatory T cells are pro-tumorigenic, the fact that DGKH is uniquely expressed in these lymphocytes withing the TME suggests that a DGKH knockout in T cells could have potential in enhancing

anti-tumor immunity. In addition to DGKH knockouts possibly affecting a more selective pool of lymphocytes, we also found evidence that this isoform contributes to T cell exhaustion across different tumor types in humans. Although not many studies have looked at DGKA and DGKZ expressions in human cancer patients, DGKH has been shown to be upregulated in T cells expressing high levels of LAG3 (a dysfunctional cell molecule) in human melanoma studies suggesting DGKH plays an important role in human tumor infiltrating lymphocytes (Li, van der Leun, Yofe, Lubling, Gelbard-Solodkin, van Akkooi, van den Braber, Rozeman, Haanen, Blank, Horlings, David, Baran, Bercovich, Lifshitz, Schumacher, Tanay, & Amit, 2020). 40%-65% of melanoma patients still show resistance to anti-PD1 therefore finding a target that is known to be overexpressed in this specific tumor-type is also beneficial (Imbert, Montfort, Fraisse, Marcheteau, Gilhodes, Martin, Bertrand, Marcellin, Burlet-Schiltz, Peredo, Garcia, Carpentier, Tartare-Deckert, Brousset, Rochaix, Puisset, Filleron, Meyer, Lamant, Levade, Segui, Andrieu-Abadie, & Colacios, 2020). Single-cell sequencing results from lymphocytes collected from mice with either LCMV Clone 13 or liver cancer, which are both disease models with chronic antigen exposure, have also shown that DGKH expression is highest in terminally exhausted $CD101^+$ T cell subsets, whereases DGKA is higher in naïve T cells and DGKZ in stem-like exhausted T cells proving that this differential expression of various DGK isoforms applies to *in vivo* murine models as well (Hudson, Gensheimer, Hashimoto, Wieland, Valanparambil, Li, Lin, Konieczny, Im, Freeman, Leonard, Kissick, & Ahmed, 2019; Scott, Dundar, Zumbo, Chandran, Klebanoff, Shakiba, Trivedi, Menocal, Appleby, Camara, Zamarin, Walther, Snyder, Femia, Comen, Wen, Hellmann, Anandasabapathy, Liu, Altorki, Lauer, Levy, Glickman, Kaye, Betel, Philip, & Schietinger, 2019). Together, these findings lead us to hypothesize DGKHs role in diminished T cell effector function and in promoting exhaustion.

CHAPTER 1

Chapter 1.1 - Metabolic screening experiment revealed DGKH knockouts to correlated to increased IFNγ+ populations

DGKs have already been shown to be great targets for promoting an effector like phenotype in T cells. However, only two (DGKA and DGKZ) out of ten isoforms have been studies in immune cells. Although protein levels for DGKA and DGKZ are highest in spleen and lymph nodes, the presence of other isoforms in the lymphatic system is still significant. Specifically, type II DGK, DGKH, has peak expression in the cerebral cortex and cerebellum but is also expressed in secondary lymphoid organs.

To try and establish if DGKH plays a role in T cells we first ran a few preliminary experiments. We started by performing a CRISPR/Cas9 screening to try and identify a range of genes involved in regulating $CD8^+$ T cell effector function. To do this we cultured $CD8^+$ T cells, added a metabolic gene library which genetically modulated the pool of cells by inserting a variety of different gene knockouts, and stimulated the cells for 4 hours with PMA/ionomycin (figure 3a). From this pool of cells, we sorter out IFNγ+ cells and IFNγ- cells respectively and performed sequencing and bioinformatics analysis which revealed IFNγ+ populations to have gRNA for DGKH. Furthermore, T cells with the DGKH knockout that were cultured with tumor interstitial fluid (TIF), didn't lose their ability to produce IFNγ (figure 3b). Given that we had previously shown that TIF from hepatocellular carcinoma bearing mice has immunosuppressive functions and that cells cultured with different TIF concentrations *in vitro* produce significantly less effector cytokines (figure 4), we were surprised to see that DGKH knockout cells cultured in TIF did not lose their ability to produce IFNγ. Given these results, the single-cell RNA sequencing data and the potential relevance of DGKH in exhausted T cells in HCC and human melanoma, we concluded that we had enough evidence to hypothesize that DGKH could play

important roles in regulating $CD8^+$ T cell effector function within the TME (Li, van der Leun, Yofe, Lubling, Gelbard-Solodkin, van Akkooi, van den Braber, Rozeman, Haanen, Blank, Horlings, David, Baran, Bercovich, Lifshitz, Schumacher, Tanay, & Amit, 2020).

Chapter 1.2 - DGKH knockouts in $CD8^+$ T cells contribute to increased effector phenotype

Naïve CD8+ cells are primed in secondary lymphoid organs by dendritic cells expressing specific tumor antigens. Once activated, naïve T cells differentiate into effector T cells which can migrate to different sites where they can interact with tumor cells through their T cell receptor (TCR) (Menares, Galvez-Cancino, Caceres-Morgado, Ghorani, Lopez, Diaz, Saavedra-Almarza, Figueroa, Roa, Quezada, & Lladser, 2019). Tumor antigens interactions with the TCR lead to a variety of downstream effects which end with the production of effector cytokines such as IFNγ and TNFα. Although the transcriptional and epigenetic changes that lead to T cell dysfunction are still being studies, T cell effector function within the TME is generally determined by expression of certain surface markers (CD25, CD69), low levels of inhibitory signals (PD-1, CTLA-4, TIM-3, LAG-3) and activation of transcription factors that lead to subsequent effector cytokine production (table 2). Exhausted T cells in the TME, which have undergone prolonged antigen stimulation, will instead lose the ability to produce effector cytokines.

Once we determined, through the metabolic screening, that there was a correlation between DGKH and T cells ability to produce effector cytokines, we decided to run further assays to assess to what extent a DGKH knockout would enhance $CD8^+$ T cell effector function. We performed several *in vitro* experiments on T cells isolated from Tag Cas9+ thy1.1/1.2 mice, and activated using *tag* peptide, as we showed this to be the most effective activation method. These T cells were transduced with virus containing plasmids with two different DGKH gRNAs. We ran an experiment to compare MFI levels for CD25, CD69 and PD-1 in DGKH knockouts

compared to controls and found significant increase in all three parameters in cells that received the knockout (figure 5). After confirming that in fact DGKH activity could play central roles in the regulation of $CD8^+$ effector function, we decided to compare the effects of DGKH knockouts to DGKA knockouts. Both knockouts were validated with western blots (figure 6). Consistently to what other groups had shown, we observed significant increases in expression of effector like surface molecules in DGKA knockouts. Interestingly however we found no substantial difference in percent of cells that produced CD25, PD1 and CD69 between T cells with DGKH and DGKA knockouts, although both showed significant increases in all three markers compared to control cells (figure 7). After establishing that both DGKA and DGKH knockouts increase the expression of effector like molecules, we wanted to determine if they had similar effects on T cell functionality as well. We stimulated transduced cells with PMA/ionomycin for 4 hours after which we stained for intracellular IFNγ and TNFα levels. Both knockouts showed significant increases in cytokines compared to controls, with DGKA however showing slightly greater MFIs and percentage for both IFNγ and TNFα (figure 8). The MFIs of effector cytokines increased in both knockouts following cell culture with IL-2 as well as IL-7 and IL-15, however when comparing percent of IFNγ and TNFα generated following culture with IL-7 and IL-15, DGKA knockouts but not DGKH knockouts lead to increases (figure 9). Together our data seems to strongly and consistently suggest that an increased effector phenotype is not unique to DGKA knockouts and that other DGK isoforms might play a role in regulating various stages of T cell differentiation.

Chapter 1.3 - Proliferation assay reveals no change in proliferative capabilities in cells with DGK knockouts

To make sure DGK knockouts did not have unwanted secondary effects such as that of hindering cell proliferation rates and viability we performed a cell proliferation assay. To do this we added Invitrogen's Cell Trace Violet (CTV) fluorescent dye to cells at day 0 and ran them using flow cytometry at day 1, day 2 and day 3 (figure 10A). Proliferation is assessed by dye dilutions, as at each round of proliferation the dye will become more and more diluted. CTV assays were performed on cells electroporated with DGKA and DGKH gRNAs respectively. Analyzed cells were classified into groups P0 to P8, where P0 cells had proliferated the least and P8 cells had proliferate the most. At day 2 most cells were in the P3-P5 subsets whereases at day 3 they all appeared to the left of the chart past P5 (figure 10B). This trend was to be expected as cells proliferate more with time and after culture with IL-2. When comparing proliferation rates of individual knockout conditions there was no strickling difference, indicating that DGK knockouts do hinder $CD8^+$ T cells proliferation rates.

Chapter 1.4 - Increase in effector cytokine production is not observed across all DGK isoform knockouts

Given that we hypothesized the role of DGKH in T cell effector function and provided evidence that suggests it plays a role in effector cytokine production, we started to ask if other type II DGKs could potentially have similar effects. As previously stated DGKs are commonly classified into five different subtype which are characterized by the presence of certain kinase domains (table 3). Type II DGKs (DGKH, DGKD and DGKK) have two C1a/b domains, a conserved catalytic domain, and an accessory domain in common with other subtypes but are unique in that they have a pleckstrin homology domain (PH) domain and a SAM domain. The PH domain in DGKH is found to preferentially bind phosphatidylinositol (PI) 4,5-bisphosphate ($PI(4,5)P_2$) but all PH domains have some degree of affinity for phosphatidylinositol in the

plasma membrane (Kume, Kawase, Komenoi, Usuki, Takeshita, Sakai, & Sakane, 2016). This protein is therefore thought to be important for establishing the cellular localization of the enzyme, however most of the functional consequences of type 2 DGKs translocating to the plasma membrane remain unknown. Given the similarities in the structural domains that make up type II DGKs we expected them to play similar roles, however our results reveal that this might not be the case.

Decreased DGKD, another type II DGK, is found to correlate to decrease in lipid oxidation, increased lipid storage and reduced glucose uptake in skeletal muscles. DGK-D accumulation has been linked to insulin resistance and metabolic inflexibility however its role in T cell regulation remain to be studies (Manneras-Holm, Kirchner, Bjornholm, Chibalin, & Zierath, 2015). By looking at data sets that reveal expression levels of these enzymes in different cells we noticed that expression levels of DGKH and DGKD in lymphocyte populations are almost opposite. As previously mentioned DGKH is found to have higher expression levels in exhausted T cells, however DGKD is highest in naïve and effector CD8s as well as regulatory T cells (FoxP3+) and helper T cells. This led us to hypothesize that DGKD possibly plays opposite roles to DGKH in regulating T cell exhaustion and effector state. If this were the case a DGKD knockout would not be an optimal target in the context of anti-tumor immunity.

Although further experiments are needed to reach this conclusion, we did show that the increases in surface markers observed in DGKA and DGKH knockouts were not observed in DGKD knockouts. For these experiments we used RNP delivery of Cas9 instead of plasmid transduction, as this method yielded higher levels of live, $CD8^+$ cells. We observed that CD25 MFI was on average slightly lower in DGKD knockouts compared to the controls suggesting that, consistently to what we had hypothesized, this isoform could hinder T cell function (figure

11). Given that the decrease in CD25 was however unsignificant and that CD69 levels did not show any differences between DGKD knockouts and controls it could be that DGKD plays no role whatsoever in regulating $CD8^+$ differentiation.

The fact that DGKD does not cause changes in CD25 or CD69 expression however doesn't exclude that it could play roles in regulating T cell metabolism. T cells ability to adapt to the changing metabolic demands within the TME is necessary for their survival, therefore if DGKD did play roles in T cell metabolic adaptation it could be a great therapeutic target.

Chapter 1.5 - DGKH and DGKA knockouts affect signaling pathways downstream of the TCR

Production of effector cytokines is a process that happens downstream of many molecules and intermediate reactions. Effector T cells get activated through a co-stimulatory process in which the T cell receptor (TCR or CD3 receptor) and CD28, which both lay on the cell membrane, are stimulated. Following TCR stimulation by antigens on major histocompatibility (MHC) receptors, PIP_2 gets converted to IP_3 and DAG. DAG is then a precursor to three different intermediates: PKCθ, RasGRP1 and PA. PKCθ activates the IKK/IkB/NF-kB pathway, RasGRP1 activates the Ras/Raf1/MEK/ERK/AP-1 pathway and PA indirectly activates Raf1 and mTORc1, which is found downstream of CD28 stimulation (Hwang, Byeon, Kim, & Park, 2020). Activation levels of these intermediates modulate T cell function by regulating the differentiation stage of the lymphocyte, the role it will play in autoimmunity and inflammation and how it will respond to a tumorigenic environment (Hwang, Byeon, Kim, & Park, 2020). Increased phosphorylation of intermediates downstream of TCR signaling can indirectly lead to increased transcription of effector cytokines such as IFNγ and TNFα, which in turn enhances T cells cytotoxic properties. Our initial hypothesis stemmed by

the fact that we expected a decrease in DGK to lead to an increase in DAG, which would then lead to activation of both the IKK/IkB/NF-kB and the Ras/Raf1/MEK/ERK/AP-1 pathways, and finally an increased production of IFNγ and TNFα. As shown in our results, DGK knock outs do in fact lead to increase in IFNγ and TNFα levels, however the exact mechanisms behind this and how pathway intermediates are affected by DGKA and DGKH knockouts respectively is still largely unknown. It also remains to be established if all DGK isoforms modulate phosphorylation of pathway intermediates equally within T cells. Difference in subcellular localization and functional motifs between isoforms suggests that they might act differently on intermediates, however the mechanisms behind this have not been explored. To try and better understand the intracellular mechanisms regulated by DGKs in T cells we looked at changed in phosphorylation of AKT, ERK and mammalian target of rapamycin (mTOR) following DGKA and DGKH knockouts.

PA and the 3-kinase (P13K)-AKT pathway, downstream of the CD28 receptor, are known to lead to mTOR activation, therefore we expected DGK knockouts to decrease PA and in turn mTOR phosphorylation. We assessed changes in mTOR by looking at pS6, a reliable marker for mTORc1 activation (Yang, Rudge, Koos, Vaidialingam, Yang, & Pavletich, 2013). Baseline pS6 levels in unstimulated cells were equal in controls and DGKA knockouts whereases DGKH knockouts showed slightly lower levels, although the difference wasn't significant. After stimulation with anti-CD3 antibody we saw pS6 levels induced by DGKH knockouts raise past those in both control and DGKA knockout cells (figure 12A). Although the increase wasn't significant the difference in levels following DGKA and DGKH knockouts suggest the two isoforms might regulate mTOR differently. In addition to regulating effector function, mTOR is a major regulator of memory CD8+ T cell differentiation. At early stages

mTOR promotes primary T cells response by promoting clonal expansion and effector T cell differentiation, however at later stages of chronic infection mTOR inhibitors have been correlated to enhanced memory T cell formation (Krishna & Zhong, 2013). DGKA and DGKZ knockouts in LCMV-specific memory T cells have already been shown to correlate to increase in mTOR levels (Shin, O'Brien, Grayson, & Zhong, 2012). Therefore, DGKs role in regulating mTOR phosphorylation could also contribute to modulating memory formation, however the extent of this and whether it applies to DGKH knockouts remains to be studied.

In addition to mTOR, we wanted to assess differences in ERK phosphorylation following DGKA and DGKH knockouts since DAG leads to activation of the Ras/Raf1/MEK/ERK/AP-1. As expected, studies have shown DGKA inhibition to increase pERK over prolonged time intervals in humans, suggesting this is one of the main pathways DGKs act on to enhance effector functionality (Prinz, Mendler, Masouris, Durner, Oberneder, & Noessner, 2012). We did not observe significant increases in pERK, probably due to variability in control replicates, but nonetheless we show an increase in pERK MFIs in both DGKA and DGKH knockouts following anti-CD3 stimulation (figure 12B). It is also of note that our unstimulated samples showed significant levels of pERK, suggesting there was a baseline levels of ERK activation caused by T cell activation that interfered with the accuracy of our results. Further experiments are needed to quantify differences in how DGKA and DGKH regulate pERK, although this is still thought to be the primary pathway though which DGKs regulate T cell effector function.

Finally, we decided to look at changes in AKT phosphorylation, as this intermediate is not directly tied to pathways downstream of DAG or PA. Noticeable changes in AKT following DGK knockouts could therefore help determine if these kinases have secondary effects on other pathways downstream of TCR stimulation. Interestingly, we noticed DGKA knockouts lead to

increases in pAKT MFIs in both stimulated and unstimulated cells (figure 12C). DGKH knockouts showed no difference compared to controls in unstimulated cells, but significant increases in pERK MFIs in stimulated cells. Similarly, to what we observed in cells stained for pERK, we notice high baseline levels of pAKT that interfere with experimental accuracy. Nonetheless these results suggested that DGKA and DGKH could be regulating this pathway differently. Since AKT is directly upstream of mTOR this could help explain why DGK knockouts lead to increase mTOR phosphorylation, regardless of inevitable decreases in PA. More studies are however needed to conclude how the two isoforms differentially regulate pathway intermediates and how they act on AKT levels.

Chapter 1.6 - FoxP3 expression is reduced in DGKH, but not DGKA knockouts

Given our initial hypothesis of that DGKH knockouts would be a better target for halting regulatory T cells, which play pro-tumorigenic roles within the tumor microenvironment, we ran an *in vitro* experiment to establish if in fact a DGKH knock out would result in decreases production of FoxP3+ Tregs. In addition, we also wanted to confirm that DGKD does not regulate neither $CD8^+$ nor $CD4^+$ T function, by looking at the effect its knockouts would have on Treg and Th1 production.

For this experiment we harvested lymphocytes from B6 IL10+ mice, isolated CD4's and cultured them for five days with optimal concentrations of Treg inducing cytokine TGFβ (figure 13A). Interestingly, when looking at percentage of FoxP3+ T cells there was no difference between the amounts generated in control cells and in DGKA knockouts, however when looking at DGKH knockouts FoxP3 expression was significantly reduced (figure 13B). Consistently with the previously made observation that DGKD is more highly expressed in tumor infiltrating regulatory T cell subsets we found that expression of FoxP3 was reduced to a similar degree in

DGKD knockouts. Furthermore, DGKA knockouts produced 10% more IL-10 compared to control cells, suggesting this knockout might enhance Treg functionality (figure 13C). DGKD knockouts, however, did not seem to have significant effects on neither the percent of Tregs produced nor their functionality.

We also wanted to make sure that although DGKH knockouts seemed to hinder Treg production, they don't have similar impacts on other CD4+ subsets, which are anti-tumorigenic. To assess this, we looked at the effect of these same knockouts of Th1 production and functionality. High amounts of Th1s are associated with better prognosis in patients with HCC and their ability to produce cytotoxic cytokines generally classifies them as anti-tumorigenic cells (Lee et al., 2019). For Th1 differentiation we culture $CD4^+$ for 5 days with optimal concentrations of IL-12 (figure 14A). When comparing CD4+ Tbet+ FoxP3- cells with different knockouts we found that DGKA and DGKH knockouts showed no significant differences in expression of Tbet compared to controls (figure 14B). DGKD knockouts, however, lead to a significant decrease in Tbet expression, suggesting that they might not be as good of a target as DGKH. When assessing for functionality we noted no significant fluctuations in the amount of IFNγ and TNFα produced in neither DGKA, DGKH or DGKD knockouts (figure 14C). Together this data suggests that DGKH could be a better therapeutic target in the context of tumor immunity as it has a more aimed effect on effector $CD8^+$ T cells. Although its effects on Tregs and Th1's were only assessed *in vitro*, single-cell sequencing data on DGKHs expression levels in tumor infiltrating lymphocytes suggest their role could be maintained *in vivo,* but this possibility remains to be explored. Furthermore, we confirmed that DGKD knockouts do not seem to affect $CD4^+$ or $CD8^+$ effector phenotypes supporting the idea that different type II DGKs play separate roles in lymphocytes.

Chapter 1.7 - DGKH knockouts in vivo increase expression of exhaustion markers

Given that *in vitro* results showed that DGKH knockouts yield higher effector cytokine production we decided to test the effects of DGKH and DGKA knockouts on anti-tumor $CD8^+$ T cell function *in vivo*. Additionally, because of the reported increase in DGKH expression in pro-tumorigenic tumor infiltrating T cells in human HCC, we decided to look at the effects of DGKH and DGKA knockouts in murine liver tumor models specifically. CRISPR-Cas9 DGKA knockouts in mice have already been found to yield $CD8^+$ T cell mediated anti-tumor activity through increases in ERK signaling and IFNγ production, however no studies have looked at how DGKA knockouts mediate lymphocyte function in HCC (Jung, Kim, Yu, Lee, Kim, & Lee, 2018).

We ran our experiments by first inducing liver tumors in mice by inoculating AST transgenic mice with AAV-Cre, which induces TAG expression causing tumor onset (figure 15A) (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5119632/). Fourteen days after tumor induction we transferred equal amounts of $Thy1.1^+Thy1.2^-$ WT $CD8^+$ T cells and $Thy1.1^+$ $Thy1.2^+$ $CD8^+$ DGKA or DGKH knockout cells respectively (figure 15B). Additionally, we also tested the effects of a DGKA/DGKH double knockout *in vivo* by transferring $Thy1.1^+$ $Thy1.2^+$ $CD8^+$ T cells electroporated with both DGKA and DGKH gRNAs. Two weeks after transfer we processed the livers and isolated tumor infiltrating $CD8^+$ T cells for analysis by flow cytometry. We found that compared to DGKA knockouts and DGKA/DGKH double knockouts, DGKH knockouts survived better and generated higher percentages Tag+ $CD8^+$ T cells (figure 15C). To determine if $CD8^+$ T cells with DGK knockouts performed functionally better we stained Tag expressing lymphocytes for exhaustion markers TIM-3, CD38, Slamf-6, LAG-3 and PD1

(Wherry, 2011) . Interestingly, we noticed that the percent of $CD8^+$ T cell expressing exhaustion markers TIM-3 and CD38, increased in all knockouts compared to controls, and that PD-1 was highly expressed in all groups (Figure 16A, figure 16B). PD-1^{hi} Tim-3^{hi} T cells are commonly classified as terminally exhausted, thus indicating that DGK knockouts drove dysfunction in tumor infiltrating $CD8^+$ T cells. Expression of exhaustion markers Slamf-6 and Lag-3 however, did not change significantly between control cells and DGK knockouts (figure 16B, figure 16C). We also noted that Granzyme B levels increased in DGKH knockouts compared to both controls and DGKA knockouts.

Additionally, we observed no changes in neither IFNγ nor TNFα production but increases in $CXCR6^+$ $CD8^+$ T cells (figure 16D). CXCR6 positions T cells to receive critical survival signals in the TME thus, although increases in TIM-3 and CD38 production suggest knockouts lead to a more exhausted T cell phenotype, high expression of CXCR6 suggest otherwise (Di Pilato, Kfuri-Rubens, Pruessmann, Ozga, Messemaker, Cadilha, Sivakumar, Cianciaruso, Warner, Marangoni, Carrizosa, Lesch, Billingsley, Perez-Ramos, Zavala, Rheinbay, Luster, Gerner, Kobold, Pittet, & Mempel, 2021). Because Granzyme B and CXCR6 are upregulated in DGKH knockouts and production of IFNγ and TNFα is not hindered, we have reason to believe Tim-3 and PD-1 increases at this stage are due to enhance T cell activation and could lead to formation of short-lived effector T cells (Avery, Filderman, Szymczak-Workman, & Kane, 2018). Together these results highlighted the ambiguous and previously unreported effects of DGKA and DGKH knockouts in tumor infiltrating T cells in murine HCC (Noessner, 2017).

METHODS

Western blots

To run Western blots cells were grown 3 days post plasmid transduction or RNP in RPMI with 10% FBS and IL2 then harvested and lysed in a RIPA lysis buffer containing protease inhibitor (1:30). 20ul of protein (form approximately 1 million cells) were added to 5ul of loading buffer and ran on a Bio-Rad 10% to 12% acrylamide gel in 1x running buffer. The gel was ran for 1 hour at 120V and then transferred to nitrocellulose membrane that was activated in methanol prior to being run for 90 minutes at 100V in 1X transfer buffer (made from 100ml 10X transfer buffer, 200ml of methanol and 700ml of DI water). After transfer the membrane was blocked in 5% milk in 1X TBST and blotted with rabbit, anti-DGKH and anti-DGKA primary

monoclonal antibodies form Cell Signaling technology which were diluted 1:1000 in 1X TBST with 1X BSA (1:10) and sodium azide (1:100). The membrane was incubated in primary antibody mix overnight at 4 ºC, washed in 1X TBST and then incubated at room temperature for one hour with anti-rabbit secondary antibody in 5% milk. After one hour the membrane was washed 3 times in 1X TBST and visualized after exposure to detection reagents from Thermo Fisher Scientific.

Cloning

To make a plasmid containing gRNA for the gene of interest we had to take a few steps. First, we set up an oligo duplex reaction with 1ul of foreword oligo for the desired gene, 1ul of reverse oligo, 10ul of 10X T4 buffer, 6.5ul of water and 0.5ul of T4 PNK. We ordered oligos based on the following sequence of the gene available on Brie Library: DGKA_1 (CACACCAATATCCGGAACTG), DGKA_2 (CAGTACCCGAAAAGCCTCAG), DGKA_3 (GTCTTTCCGAGACTTGGCAT), DGKH_1 (CAACTCTGATGAGCATGCGG), DGKH_2 (GTGCTATTTAGTGCAATCGG), DGKH_3 (CTTTCGCATAATAAAGTGTG). These sequences were chosen because they have the highest reported Rule Set 2 score, which is a measure of on-target specific activity of the gRNA. This mixture was then put in the thermocycler to anneal. Simultaneously, 2.5ul of mg guide were added to 4ul of restriction enzyme BbSI, 4ul of recombinant shrimp alkaline phosphatase (rSAP), 10ul of CutSmart and 80ul of water and left to incubate for 2 hours at 37ºC. Following the oligo duplex reaction and vector digestion and purification 1ul of BbSI digested plasmid was added to 1ul of 20-fold diluted oligo duplex, 1ul of T4 ligase buffer, 1ul of T4 ligase and 6ul of water. This mixture was incubated at room temperature for 10 minutes following which 5ul were collected and added to

50ul of competent cells, which were left on ice for 10 minutes before getting heat shocked for 1 minute at 42ºC. The ligated plasmid and primer were then incubated in the shaker for 1 hours at 37ºC at 200 rpm with 500ul LB media without ampicillin. After 1 hour the pellet was resuspended in 100ul of media, plated onto agar plates with ampicillin and incubated at 37ºC overnight. The next day colonies were picked and sent to the Salk Institute Mini Core in 5ml LB media with ampicillin or DNA was extracted using miniprep plasmid DNA extraction kit from QIAprep. Extracted DNA was then sent to Eton for sequencing. Sequencing results were viewed and compared to desired sequences using SnapGene Viewer.

Transfection and Transduction

293 HEK cells were cultured in f12 DMEM and 100ul Opti-MEM, 6ul X-tremeGENE9 DNA transfection reagent, 500 ng/well ECO helper and 1ug/well of plasmid were added per well (each well contained 450K HEK cells). 24 hours later the media was replaced with 10% FCS DMEM. 42 hours later the supernatant was collected and stored in -80C. In vitro activation of Tag Cas9 splenocytes was accomplished by overnight culture with tag peptide (1:2000) and IL-2 (1:2000) in 10% RPMI medium. Splenocytes were also collected in 10% RPMI medium and ACK lysis was performed. 1ml of virus and 1ul of polybrene transfection reagent were added to 1 million activated T cells and spined down at 1500g/ 32ºC for 90 minutes. After transduction, the media was replaced with fresh RPMI 1640 and IL-2 (1:2000). Cells were then cultured for 3 days after which flow cytometry was performed. Data analysis was performed using FlowJo and gates were made around lymphocytes, single cells, live cells and finally GFP+ cells (figure 17).

Ribonucleoprotein (RNP) Cas9 and gRNA delivery

CD8+ isolated T cells were spined down again before beginning electroporation and resuspended in PBS to remove potential RNase contamination from FBS in the MACS buffer. A Cas9/RNA complex was then made by adding 0.6uL of Cas9 protein to 1ul of DGKA and DGKH gRNA respectively. To reach a final volume of 5ul we also added 3.4ul of RNase free water. For each knock out we used two gRNAs to increase knock out efficiency. gRNAs for the following genes were ordered from Synthego: DGKA_1 (CACACCAATATCCGGAACTG), DGKA_2 (CAGTACCCGAAAAGCCTCAG), DGKH_2 (GTGCTATTTAGTGCAATCGG), DGKH_3 (CTTTCGCATAATAAAGTGTG), DGKD_1 (AGAGGGCTTGTACAAGACGG), DGKD_2 (AGAGCTGTGTGATTGCCAAG). We flicked and briefly spun down the tube containing the gRNA and Cas9 and incubated it for 10 minutes at room temperature for complex formation. In the meantime, we made P3 buffer by mixing 3.6ul of reagent 1 to 16.4ul of P3. Immediately after complex formation we spun down the isolated CD8+ cells, took out the supernatant and added 20ul of P3 buffer and 5ul of the gRNA/Cas9 complex. All 25ul were then transferred to the bottom hole of a well of the Lonza nucleofector strip. We electroporated the cells by putting the strip in the Lonza nucleofector machine and selected program DN100. After electroporation 130ul of pre-warmed 10% RPMI were added to the bottom of the strip and cells were collected and incubated in the incubator at 37C for 10 minutes. After incubation we counted the cells using Trypan Blue and plated 1 million cells per well with 10% RPMI. Cells were then cultured for 3 days after which flow cytometry was performed. Data analysis was performed using FlowJo and gates were made around lymphocytes, single cells, live cells and CD8+ cells (figure 18).

$CD8^+$ and $CD4^+$ T Cell Isolation and Differentiation

Splenocytes were harvested from Thy1.1/Thy1.2 Cas9 for $CD8^+$ isolation mice or IL-10/IL-$12^{+/+}$ B6 mice for CD4 isolation. The spleen was isolated and mashed through a 70um filter in 10% RPMI. Cells were spined at 400g for 4 min and resuspended in 500ul of MACS buffer (2% PSB). The following biotinylated antibodies were then added to the cells for CD8 extraction: α-B220 (2.5ug/mL), α-CD4 (2.5ug/mL), α-CD11c (2.5ug/mL), α-CD49b (2.5ug/mL), α-CD11b (5ug/mL), α-Ter119 (5ug/mL). For $CD4^+$ extraction α-B220 (2.5ug/mL), α-CD8 (2.5ug/mL), α-CD11c (2.5ug/mL), α-CD49b (2.5ug/mL), α-CD11b (5ug/mL), α-Ter119 (5ug/mL) and α-CD25 (2.5ug/mL) were added instead. Antibodies and lymphocytes were incubated at room temperature for 15 minutes. Following incubation 60ul of STEMCELL streptavidin beads were added and left to incubate for another 5 minutes at room temperature. After 5 minutes 2ml of MACS were added and the solution was placed on a magnet for 15 minutes. Finally, cells were spined at 400g for 3min and resuspended in PBS without FBS. CD8s were then either cultured on plates coated with anti-CD3 (1:500) and anti-CD28 (1:500) or in the presence of tag peptide (1:1000, 1:2000). We performed a T cell proliferation assay (using CellTrace Violet) on $CD8^+$ T cells cultured with either tag or anti-CD3 and anti-CD28, to establish the preferred method of T cell activation *in vitro* and found that cells cultured with tag proliferated more (figure 19A). We also tested weather different activation methods affected expression of surface markers CD25, CD69 and PD-1 but found no significant changes (figure 19B). We thus used tag to activate T cells *in vitro* in out following experiments. For each condition we also added IL-2 either in 1:2000 or 1:5000 dilutions. As a negative control we also cultured CD8s with no antigen or antibody but still applied the same IL-2 conditions. CD4 T cells were also cultured in several different conditions to determine which one gave the

best results. CD4s were differentiated into Tregs following 5-day culture with TGFβ in a 1:200 dilution and into Th1s following 5-day culture with IL-12 in a 1:1000 dilution. Different concentrations of both TGFβ and IL-12 were tested but 1:200 and 1:1000 dilutions were found to produce higher amounts of FoxP3+ and Tbet+ respectively (figure 20A, figure 20B).

Flow Cytometry Staining

To assess for functionality cells were first stimulated with PMA and ionomycin (1:1000) for 4 hours or tag peptide for 6 hours in RPMI with 10% FBS. In both cases a protein transfer inhibitor was also added in 1:100 dilations (BD GolgiPlug™). After stimulation cells were stained for live dead (Invitrogen by Thermo Fisher Scientific) for 15 min in PBS (1:10000). Following live/dead staining cells were fixed in 100ul of fixation buffer (eBioscience™) for 30 minutes and then stained for IFNγ and TNFα. Cells stained for transcription factors (Tbet, Eomes and Gzmb) were first incubated overnight in 4°C in 10X permeabilization buffer (eBioscience™) and then stained with respective antibodies in FoxP3 buffer. Non-stimulated samples were stained in FACS with CD8, PD-1, CD25, CD69, CXCR6, CD62L, CD90. Cells from *in vivo* experiments were harvested and stained in FACS with surface markers CD8, thy1.1, thy1.2, PD-1, CD69, CXCR6, Lag3, Slamf-6, CD38 and Tim3 and for transcription factor Granzyme B (Thermo Fisher ™). Cells that were stimulated *ex vivo* with PMA/Ionomycin were stained for IFNγ and TNFα. To assess cell proliferation, we used CellTrace™ Violet Cell Proliferation Kit.

Phospho-Flow Staining

$CD8^+$ T cells were isolated and collected after 3-day incubation with IL-2 (1:2000). To determine the better stimulant, we first ran trials using PMA, CD3 alone, CD28 alone or CD3

and CD28 together. Although PMA led to more significant changes in phosphorylation, we discarded this method as we deemed it to not lead to biologically accurate results since PMA acts by activating PKC, which is downstream of DGKs. We found that CD28 alone was not a significant stimulant (figure 21). Therefore, we ran experiments by seeding cells in a 96 well plate (approximately 1 million cells per well) and stimulating them for 30 minutes with anti-CD3. After incubation cells were fixed with 100 ul of room temperature fixation buffer (eBioscience™). Fixed cells were incubated at room temperature, in the dark for 10 minutes following which 50ul of 90% methanol were added. Following this step cells were incubated for 10 minutes at -20°C. Next, cells were washed with FACS twice and 10ul of pre-heated (35°C) normal goat serum were added per well. After 10-minute incubation at room temperature with the NGS, primary antibody was added for either phospho-ERK1/ERK2 (Thr185, Tyr187) rabbit polyclonal antibody, phosphor-AKT1 (Ser473) rabbit monoclonal antibody or phosphp-S6 (Ser235, Ser236) rabbit monoclonal antibody (eBioscience™). Cells were incubated with primary antibody for 30 minutes at 4°C after which mouse secondary antibody was added for 30 minutes. After secondary antibody incubation cells were washed in FACS buffer and run using Flow Cytometry.

Liver digestion

For *in vivo* experiments mice were sacrificed 14 days after T cell transfer and livers were processed as described here. First, livers were mashed in RPMI into 50ml tubes and spun at 60g for 2 minutes. After centrifugation the supernatant was collected and spun again at 400g for 4 minutes. After this step 40% percoll was added to the pellet and the vortexed solution was sup at 850g for 25 minutes. Following this step supernatant was removed and ACK lysis reagent was

added to the pellet. Cells were then resuspended in RPMI, counted, and stained for respective surface markers.

DISCUSSION

As cancer rates reach all-time highs the search for more effective immunotherapies continues. Over the past few years checkpoint inhibitors such as anti-PD1 and anti-CLTA4 have shown tremendous therapeutic potentials, however many patients remain unresponsive to such therapies. These checkpoint inhibitors act by blocking the PD-1 protein on the surface of T cells from binding PD-L1 on tumor cells, as PD-1/PD-L1 engagement is one of the mechanisms by which tumor cells send immunosuppressive signals to T cells (Han, Liu, & Li, 2020). Age, tumor mutational burden, expression levels of selected $CD8^+$ surface markers and even gut microbiota all affect how a patient might respond (Kugel, Douglass, Webster, Kaur, Liu, Yin, Weiss, Darvishian, Al-Rohil, Ndoye, Behera, Alicea, Ecker, Fane, Allegrezza, Svoronos, Kumar, Wang, Somasundaram, Hu-Lieskovan, Ozgun, Herlyn, Conejo-Garcia, Gabrilovich, Stone, Nowicki, Sosman, Rai, Carlino, Long, Marais, Ribas, Eroglu, Davies, Schilling, Schadendorf, Xu, Amaravadi, Menzies, McQuade, Johnson, Osman, & Weeraratna, 2018; Vetizou & Trinchieri, 2018; Yarchoan, Hopkins, & Jaffee, 2017). With so many possibilities, finding the underlaying mechanisms for why some patients respond to these therapies better than others has been central to many studies, and so has seeking molecular targets, that could provide better outcomes when given in-conjunction to checkpoint inhibitors.

Here we explored the potential therapeutic effects of DGK knockouts, as their proven ability to increase effector like function, suggest they could be a good target in conjunction to immune-checkpoint inhibitors. Previous studies have already highlighted the potential of DGKA and DGKZ knockouts in T cells as well as show that DGKA knockouts enhance the efficacy of

anti-PD1 therapies (Fu, Li, Xiao, Yu, Li, Yuan, Shen, Dong, Fang, Zhang, Chen, Li, You, Xia, Kang, Tan, Chen, Yang, Gao, & Zhou, 2021). In addition, DGK overexpression has previously been shown to decrease TCR signaling via decreased activation of the RasGRP1/Ras/ERK-pathway (Noessner, 2017).

The beta and zeta isotypes have been chosen as targets in T cell studies thus far because they have the highest expression profiles in these lymphocytes compared to other cell types (Riese, Moon, Johnson, & Albelda, 2016). However, one big downfall of targeting these two isoforms is that they are highly expressed across all lymphocytes, and it would therefore be challenging to knock them out in only one cell type, without having negative effects on others.

In the context of tumor immunity and the tumor microenvironment effector, memory, regulatory and exhausted T cells all play a role (Li, van der Leun, Yofe, Lubling, Gelbard-Solodkin, van Akkooi, van den Braber, Rozeman, Haanen, Blank, Horlings, David, Baran, Bercovich, Lifshitz, Schumacher, Tanay, & Amit, 2020). Besides tumor cells, which continuously send suppressive signals to $CD8^+$ T cells, regulatory T cells also suppress $CD8^+$ function within the tumor microenvironment. Usually, regulatory T cells play important roles in defending our body against autoimmunity. Within the TME however their functionality is altered as they perceive anti-tumorigenic cytotoxic $CD8^+$ T cells as attacking self-antigens and send inhibitory signals to suppress their function. Therefore, within the TME it is essential to maintain high numbers of $CD8^+$ effector T cells and a lower number of regulatory T cells.

Here, we hypothesized and gave convincing evidence that DGKH could be an important target for preferentially enhancing effector T cells functionality. Although its role in conjunction to anti-PD1 therapies remains to be studies, our results suggest that it could be an effective target against HCC. By analyzing existing single cell RNA sequencing data sets from liver tumors, we

found evidence that DGKH is expressed in $LAYN^+$ cells which can be either exhausted T cells or regulatory T cells. The preferential expression of DGKH in these two pro-tumorigenic cell subsets makes it a great therapeutic target. In addition, we were able to provide convincing evidence that similarly to DGKA, DGKH also increases $CD8^+$ T cells effector phenotype by increasing expression of effector like markers such as CD25, CD69 and PD-1 *in vitro*. Furthermore, we explore the possibility that DGKH knockouts increased $CD8^+$ functionality by showing that these knockouts yield increased expression of effector cytokines IFNγ and TNFα. Consistently to published data we also observed increases in functionality in cells with DGKA knockouts, which however didn't greatly exceed that of DGKH knockouts.

To prove that DGKH knock outs could potentially hinder Treg functionality and survival within the tumor microenvironment to a higher extent than DGKA knockouts, we looked at the amount of FoxP3+ cells generated after each knockout. We confirmed in one experiment that following DGKH knockouts the amount of FoxP3+ cells decreased compared to in DGKA knockouts. We also aimed to look at Treg functionality by staining for IL-10 production, however we were only able to collect this data for DGKA and DGKD knockouts. Nonetheless we showed Tregs with DGKA knockouts resulted in slightly higher IL-10 production compared to controls, suggesting this knockout might be enhancing Treg functionality within the TME. Scientists are constantly seeking for methods to inhibit Treg function while not hindering $CD8^+$ effector function within the TME, making DGKH a potentially great target. The risk with hindering Treg functionality and production is always that of generating autoimmune reactions, however this still needs to be quantified *in vivo*. Furthermore, we confirmed that a DGKH knock out would not have negative effects on other $CD4^+$ T cell subsets that have anti-tumorigenic roles. T helper cells, which develop from naïve CD4s following antigen-dependent activation,

have the ability of recognizing antigens on MHC-II complexes on tumor cells and hinder tumor growth (Kennedy & Celis, 2008). We looked at Type I effector Th cells ($Tbet^+$) production and function following DGKH, DGKA and DGKD knockouts and showed no significant change in IFNγ and TNFα production. The change in the amount of $Tbet^+$ cells generated wasn't huge, but we did show a slight increase in the amount of $Tbet^+$ cells following DGKH knockouts compared to both DGKA or DGKD knockouts.

At this point in our studies, we sought to understand by which mechanism DGK knockouts regulate changes in effector functions. Understanding the molecular pathways and cellular intermediates that DGKs bind could help understand much more about their functions. Previous studies have shown that different DGK isotypes can affect intracellular signaling intermediates in distinct, at times even opposite, ways (Topham & Epand, 2009). With the intracellular location of DGKs, as well as the domains they bind to varying, there is much that needs to be understood about how they regulate signal transduction in lymphocytes. We tried to assess which intermediates downstream of TCR stimulation increase in phosphorylation following DGKA and DGKH knockouts respectively and found noticeable increases in pERK and pS6. The increase in pS6 in DGKH seemed to be higher, although further experiments are needed to determine the diverging mechanisms by which DGKA and DGKH knockouts regulate T cell effector function. Understanding more about their roles in signal transduction could potentially reveal if DGKA and DGKH regulate metabolic changes within the TME. Other DGK isotypes have already been shown to regulate lipid and glucose metabolism in distinct cell types (Massart & Zierath, 2019). DGKD for example, which has the same structural elements as DGKH, accumulation has been linked to insulin resistance and metabolic inflexibility (Massart & Zierath, 2019). We knocked out DGKD in T cells and did not observe neither an increase in

effector cytokine production nor in expression of effector like surface molecules. This proved that in fact DGKD and DGKH do not have the same effects in T cells, however weather their differences extend to metabolic regulation remains to be studied. In the context of tumor immunity, T cells ability to adapt and respond to the changing metabolic demands within the TME is crucial for their survival. Therefore, future studies aimed at understanding the metabolic implications of a DGKH knockout could shed more light on the extents of its therapeutic potential.

Finally, we assessed the roles of DGKH knockouts in tumor infiltrating $CD8^+$ T cells in *in vivo* liver tumor models. Our results show that DGKH and DGKA knockouts increased expression of exhaustion marker TIM-3, suggesting that DGK knockouts could potentially impede lymphocyte driven tumor clearance in murine models of HCC. Interestingly, previous studies have already shown that tumor infiltrating DGK-A and DGK-Z double knockout 139 CAR-T cells expressed high levels of inhibitory immune checkpoints such as PD-1, TIM-3 and LAG-3 (Jung, Kim, Yu, Lee, Kim, & Lee, 2018). The same study also found that nonetheless knockouts proliferated better and showed increased cytotoxicity (Noessner, 2017). Although we did not observe significant increases in IFNγ and TNFα production, tumor infiltrating T cells with DGK knockouts retained the ability to produce effector cytokines and yielded increases in Granzyme B and CXCR6, which are both traditionally associated with enhanced T cell survival in the TME (Di Pilato, Kfuri-Rubens, Pruessmann, Ozga, Messemaker, Cadilha, Sivakumar, Cianciaruso, Warner, Marangoni, Carrizosa, Lesch, Billingsley, Perez-Ramos, Zavala, Rheinbay, Luster, Gerner, Kobold, Pittet, & Mempel, 2021) (Wherry, 2011). This led us to hypothesize that increases in TIM-3 and high PD-1 expression could be due to increased T cell activation, and that these cells could retain cytotoxic abilities in the long run. It must also be noted that, human

T cells loose CD28 expression during development and CD28 inhibits DGK-A activity, thus DGK knockouts could have different roles in modulating anti-tumor T cell function in human and mouse models (Jung, Kim, Yu, Lee, Kim, & Lee, 2018).

In conclusion we were able to explore the previously unreported role of DGKH in T cell exhaustion and showed that DGKH knockouts in $CD8^+$ T cells *in vitro* are functionally enhanced compared to control cells. Additionally, we provide preliminary data which suggests DGKH knockouts enhance effector function though AKT mediates signaling. Although DGKH knockouts in tumor-specific T cells in HCC upregulate exhaustion marker Tim-3, single cell RNA sequencing results from patients suggest that DGKH knockouts could affect anti-tumorigenic CD8 T cell function in humans specifically.

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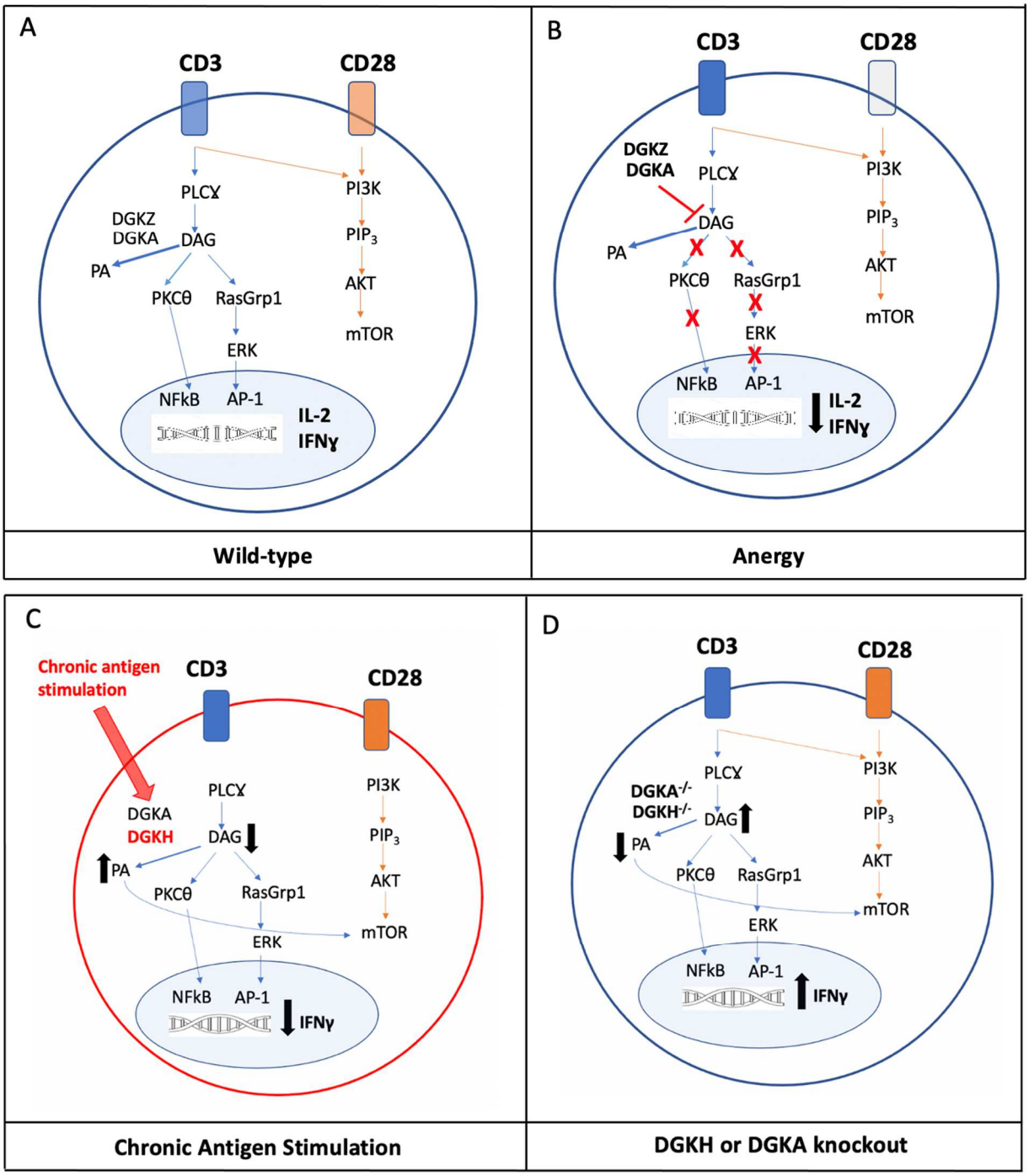
Figure 1 - DAG Pathway Downstream of TCR Stimulation

T cells get activated by co-stimulation of CD3 and CD28 receptors on the cell surface. DGKs and DAGs are found downstream of the CD3 receptor. Production of DAG is essential to ensure further activation of the NF-kB pathway as well as the RasGrp1/ ERK/ AP-1 pathways which lead to activation of transcription factors essential for production of effector cytokines (A). However, in situations when full immune activation is not desirable, such as in the presence of self-antigens or in the absence of co-stimulatory signals, T cells become anergic (hyporesponsive). Amongst different signals which are thought to lead to the induction of T cell anergy is the transcriptional upregulation of DGKα, which in turn acts by decreasing DAG and downstream production of IL-2 and IFNɣ (B). Another state of T cell hyperresponsiveness, is T cell exhaustion which occurs following chronic antigen stimulation. Although mechanistically it is still not fully understood what would lead to increased DGK expression in dysfunctional T cells, many exhausted T cell subsets have been found to upregulate DGKA and DGKH (C). Finally, we propose that by using CRISRP/Cas9 to knockout DGKA or DGKH in T cells we can induce increased effector cytokine production according to the signaling pathway depicted and we suggest that this strategy could be employed to maintain cytotoxic T cell functionality following chronic antigen stimulation (D).


A
CD3
CD28
PLCɣ
PI3K
DGKZ
DGKA
DAG
PIP3
PA
AKT
PKCθ
RasGrp1
mTOR
ERK
NFkB
AP-1
IL-2
IFNɣ
Wild-type
B
CD3
CD28
DGKZ
DGKA
PLCɣ
PI3K
DAG
PIP3
PA
AKT
PKCθ
RasGrp1
mTOR
ERK
NFkB
AP-1
IL-2
IFNɣ
Anergy
C
Chronic antigen stimulation
CD3
CD28
PLCɣ
PI3K
DGKA
DGKH
DAG
PIP3
PA
AKT
PKCθ
RasGrp1
mTOR
ERK
NFkB
AP-1
IFNɣ
Chronic Antigen Stimulation
D
CD3
CD28
PLCɣ
PI3K
DGKA-/-
DGKH-/-
DAG
PIP3
PA
AKT
PKCθ
RasGrp1
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NFkB
AP-1
IFNɣ
DGKH or DGKA knockout

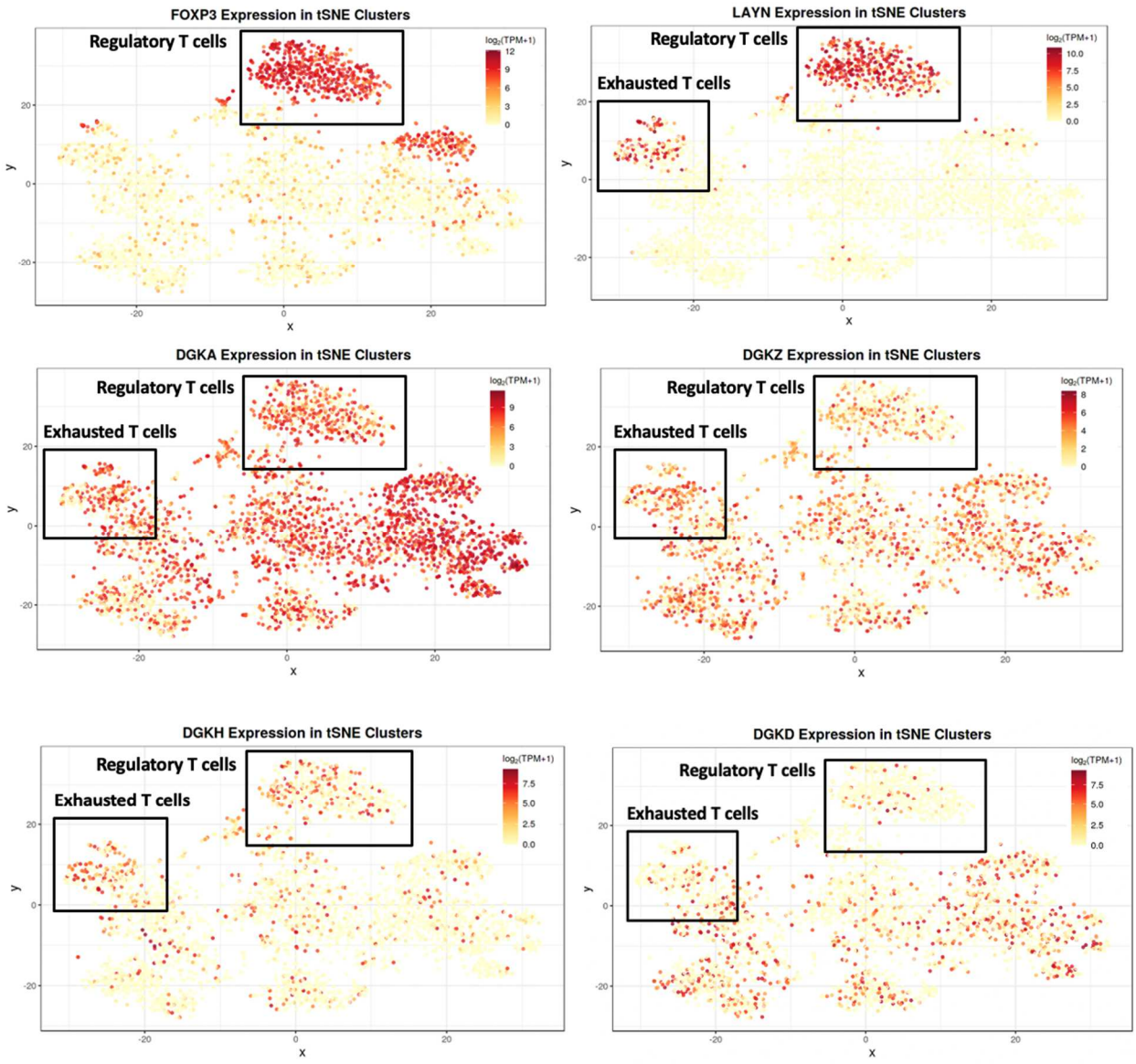
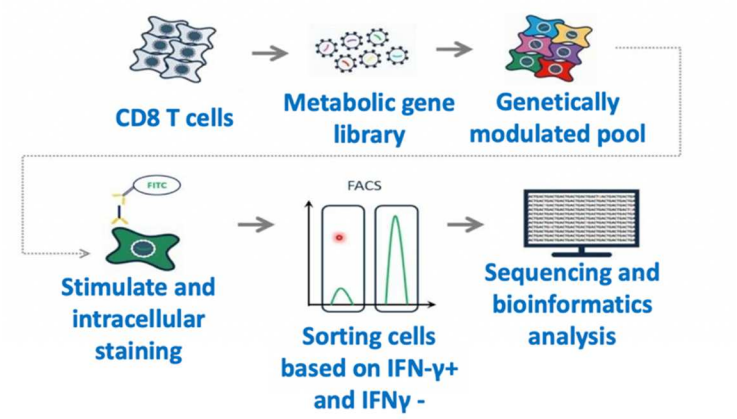


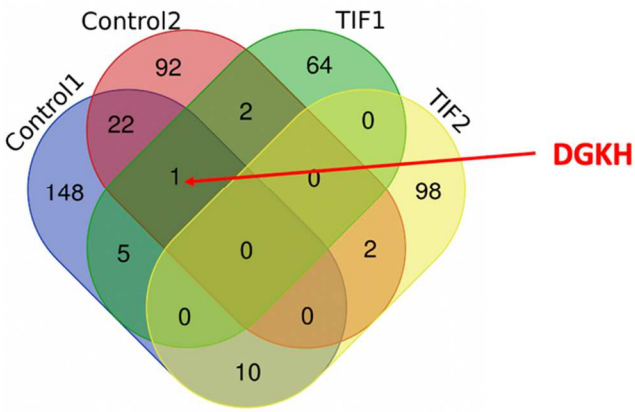
Figure 2 - Single-Cell Sequencing on Tumor Infiltrating T cells in HCC

This data was collected from an online data viewer on the Landscape of Infiltrating T cells in Liver Cancer Revealed by Single-Cell Sequencing made public by Chunhong Zheng. EGA: EGAS00001002072 and GEO: GSE98638. This information can be found at the following link: http://hcc.cancer-pku.cn. We compared single-cell sequencing data on expression of FoxP3, LAYN, DGKA, DGKD and DGKH in tumor infiltrating T cells in hepatocellular carcinoma and revealed that DGKH is upregulated in $FoxP3^{+}$ and LYN^{+} clusters specifically. Additionally, we gated on the regions which represent of regulatory T cells and exhausted T cells.

A



B



C

Control1 Control2 TIF1	1	Dgkh
Control1 Control2	22	Ugt2b34 Slc2a6 Pon1 Sat2 Sv2b Uqcrh Gad1 Cox10 Stt3b Slc1a4 Cps1 Acox2 Abcb5 Mgat5 Mat1a Slc5a7 Cyp1a2 Umps Nat1 Suclg1 Polr3f Galnt12
Control1 TIF1	5	Slc22a8 Rptor Gatm Prodh Slc4a10
Control1 TIF2	10	Slc5a8 Nagk Mthfs Msmo1 Slc3a1 Tph1 Slc24a2 Slc35b1 B4galnt1 Khk
Control2 TIF1	2	Polr1c Man1b1
Control2 TIF2	2	Ppt2 Me1

Figure 3 - Screening experiment to assess genes overexpressed in IFNγ+ populations

Initial screening experiment to identify DGKH as a potential target were performed following the above experimental layout. $CD8^+$ T cells were harvested from murine spleens and cultured with virus containing plasmids with metabolic gene inserts. The genetically modulated pool of cells was than stimulated with PMA/ionomycin for 4 hours and these cells were stained for IFNγ and sorted. Through sequencing and bioinformatics analysis we were then able to identify key gRNAs overexpressed in IFNγ+ populations (A). In addition, we looked for which genes were upregulated while yielding IFNγ+ production after culture with tumor interstitial fluid (TIF) (B). Amongst the gRNAs expressed in cell populations that produced IFNγ+ both in control setting and after culture with tumor interstitial fluid was DGKH (C).

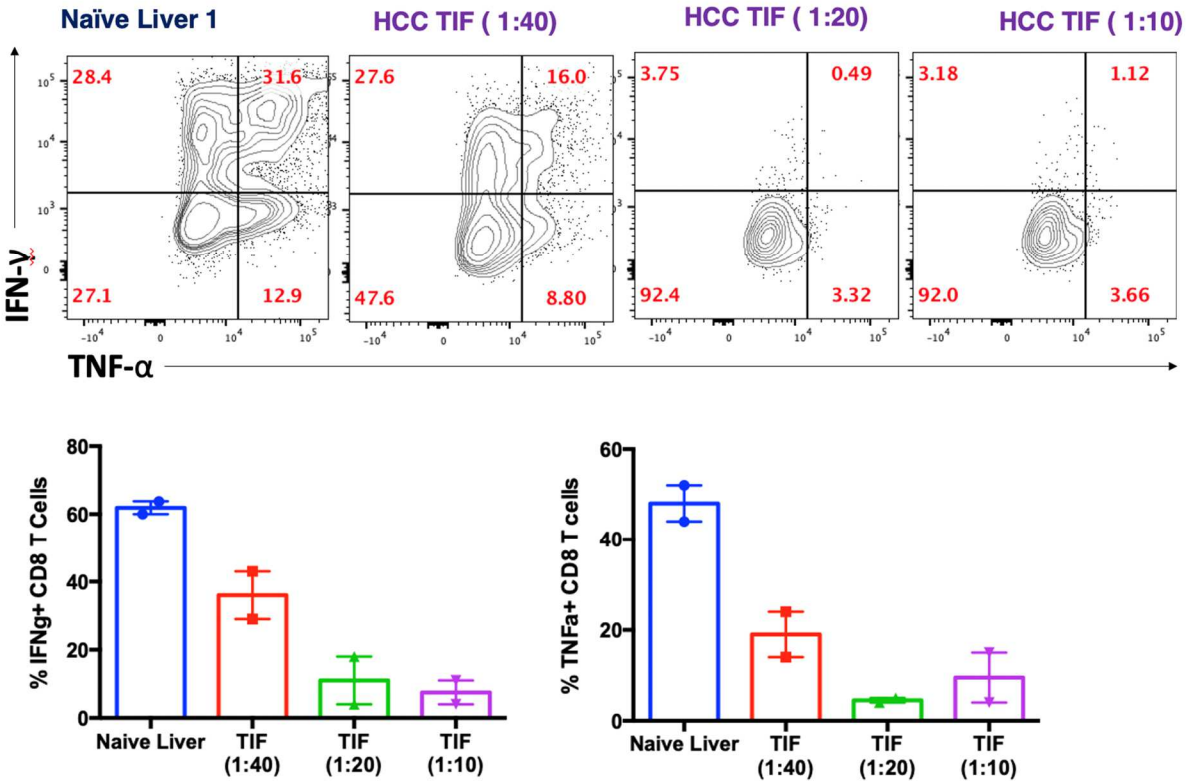


Figure 4 - TIF from HCC is immunosuppressive

$CD8^+$ lymphocytes were isolated and cultured with different concentrations of tumor interstitial fluid (1:40, 1:20 and 1:10) from hepatocellular carcinoma bearing mice. CD8s from a naïve liver were used as a control. After X days of culture with TIF $CD8^+$ T cells were stimulated with PMA/ionomycin for 4 hours and then stained for IFNγ and TNFα. Stained cells were ran using Flow Cytometry and analyzed using FlowJo and Prism. This experiment was executed with two biological replicates per condition.

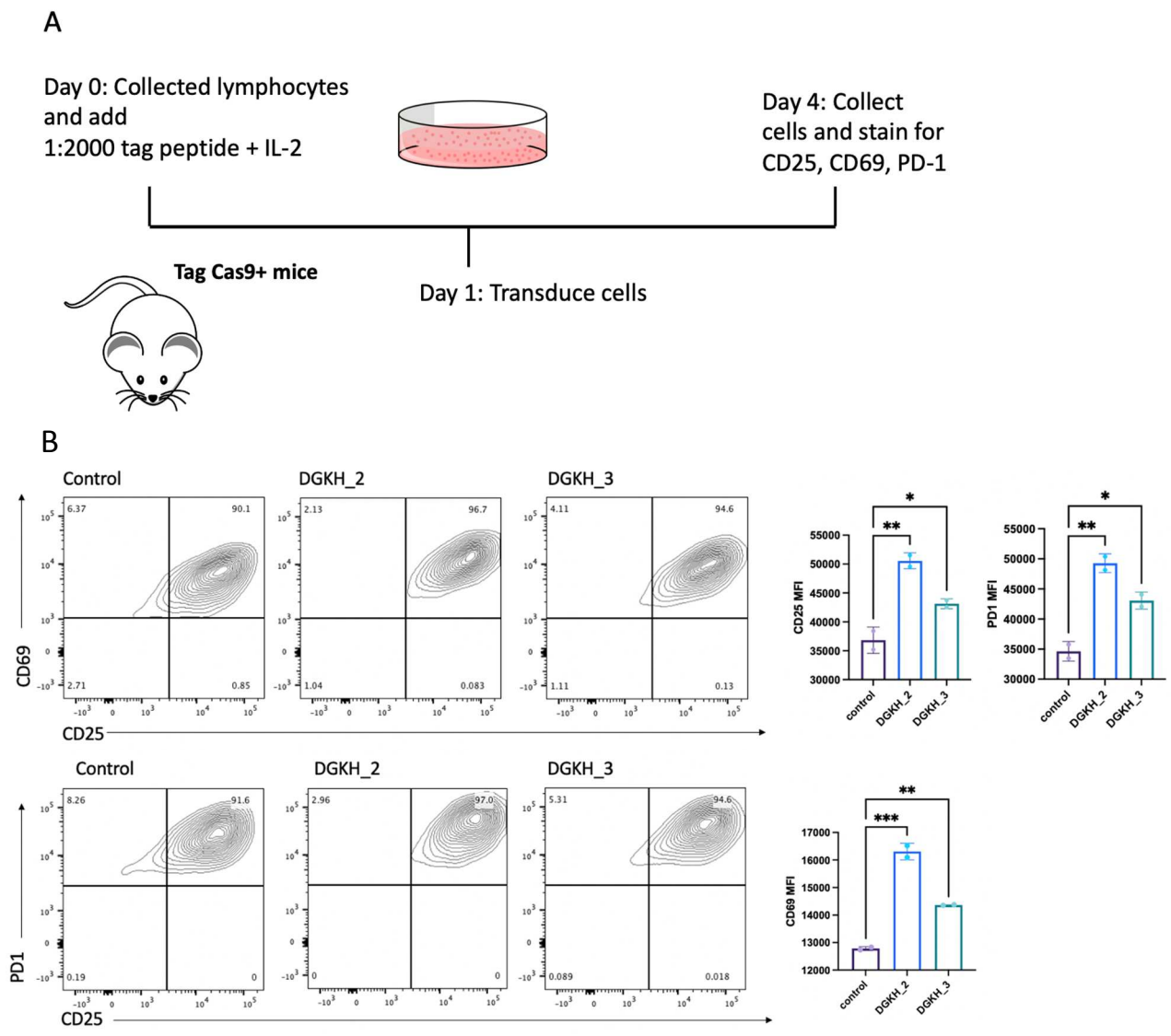


Figure 5 - DGKH knockouts showed increases in effector cytokine production

Tag activated $CD8^+$ T cells were transduced with virus containing two separate DGKH gRNAs and cultured with IL-2 in RPMI at 37ºC. Three days later cells were collected and stained for CD25, CD69 and PD-1 (A). Each condition had two biological replicates. Stained cells were ran using Flow Cytometry and analyzed using FlowJo and Prism. The above figures show statistically significant increases in CD25, PD1 and CD69 MFIs with both DGKH guides compared to controls (B). Analysis was performed on gated live, GFP+ cells. This experiment was repeated three times, under the same conditions and similar results were obtained. Statistical significance was calculated based on p-values (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

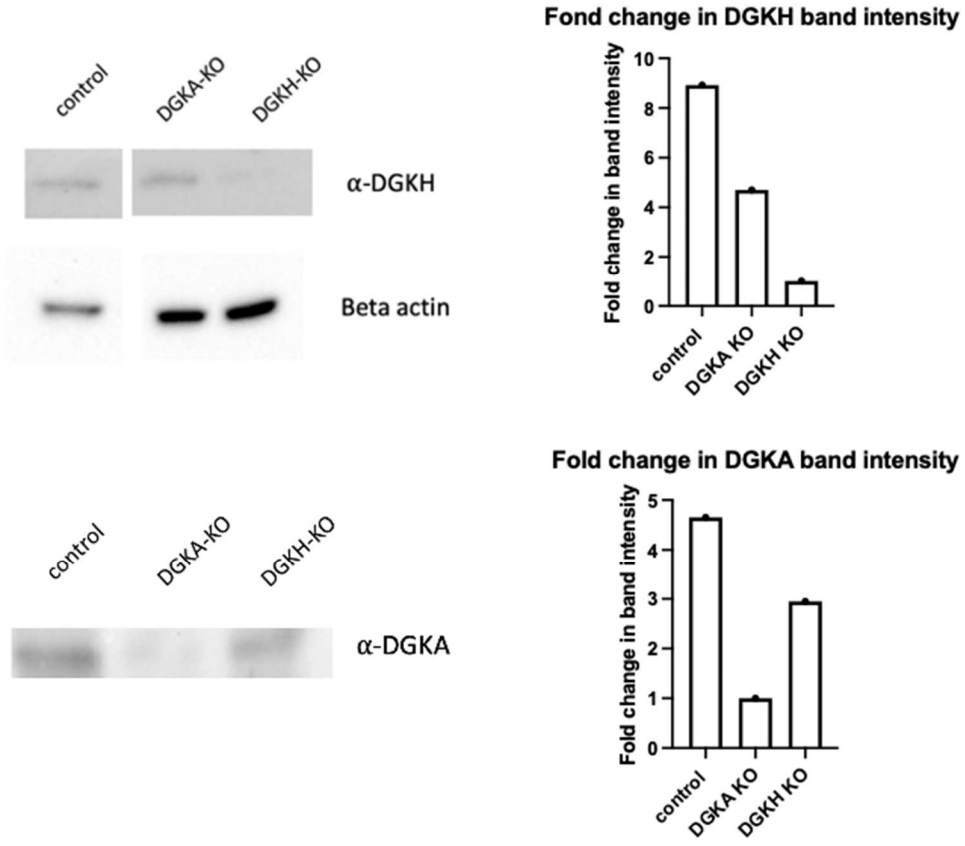


Figure 6 - Western Blots confirm the absence of DGKH and DGKA

To validate that transduced cells contained the desired knockout, we performed Western Blots. Cells were cultured for 3 days with in RMPI and IL-2 (1:5000). At day 3 one million cells where harvested and protein was extracted. We used rabbit-DGKH and rabbit-DGKA primary antibodies and anti-rabbit secondary antibody. We then blotted with beta actin as out loading control. We used Image-J to calculate band intensity and confirm lower intensity of knockout bands.

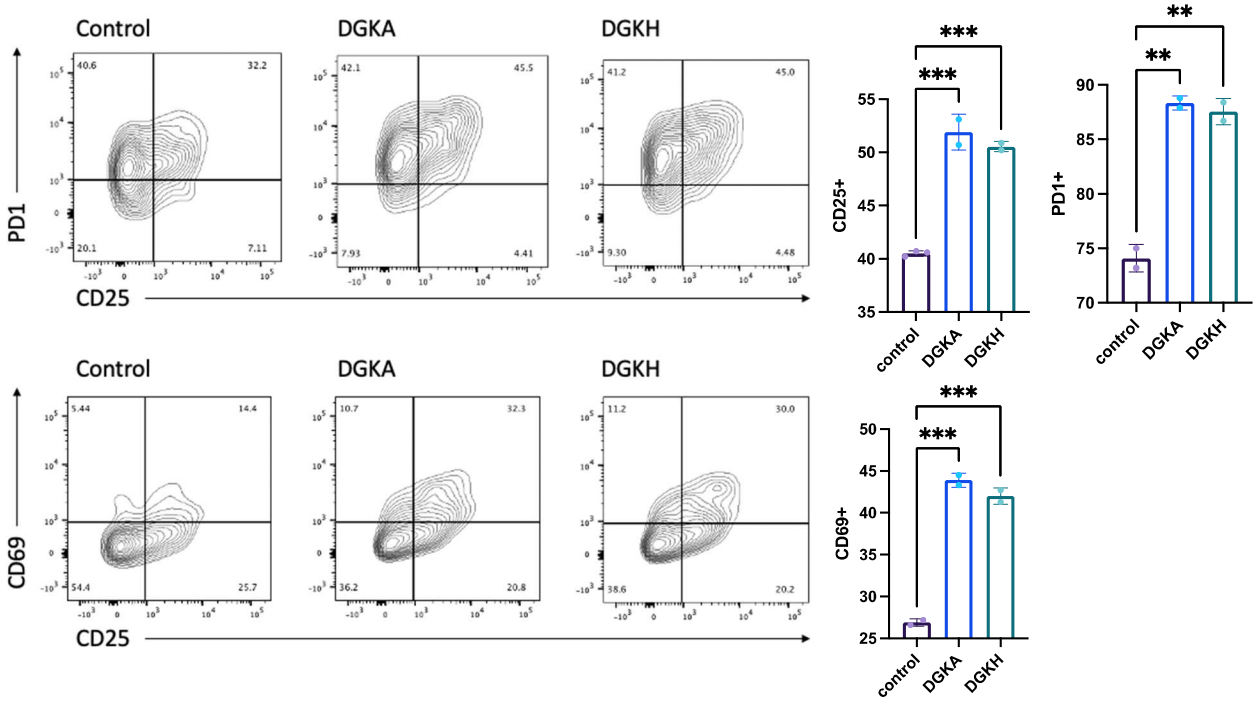


Figure 7 - CD8+ T cells with DGKH and DGKA knockouts show increased expression of effector-like surface markers

Tag activated $CD8^+$ T cells were transduced with virus containing DGKH gRNA or DGKA gRNA and cultured with IL-2 in RPMI at 37ºC. Transduced cells were then cultured for 3 days with IL-2 and then stained for CD25, PD1 and CD69. Each condition had two biological replicates. Stained cells were ran using Flow Cytometry and analyzed using FlowJo and Prism. Analysis was performed on gated live, GFP+ cells. The above figures show that DGKA and DGKH knockouts yielded statistically significant increases in CD25, PD1 and CD69 compared to control cells. This experiment was repeated fix times, under the same conditions and similar results were obtained. Statistical significance was calculated based on p-values (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

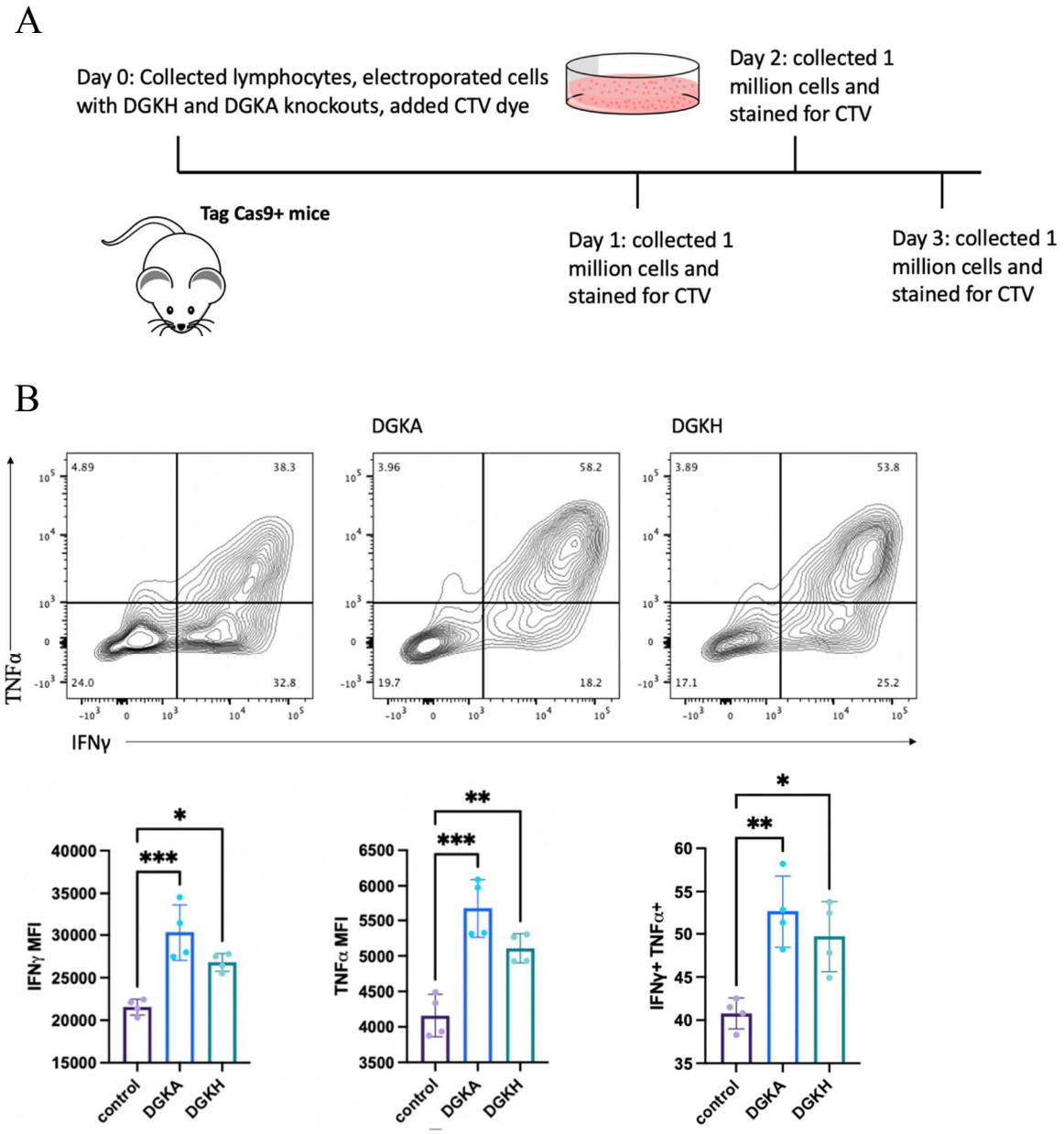
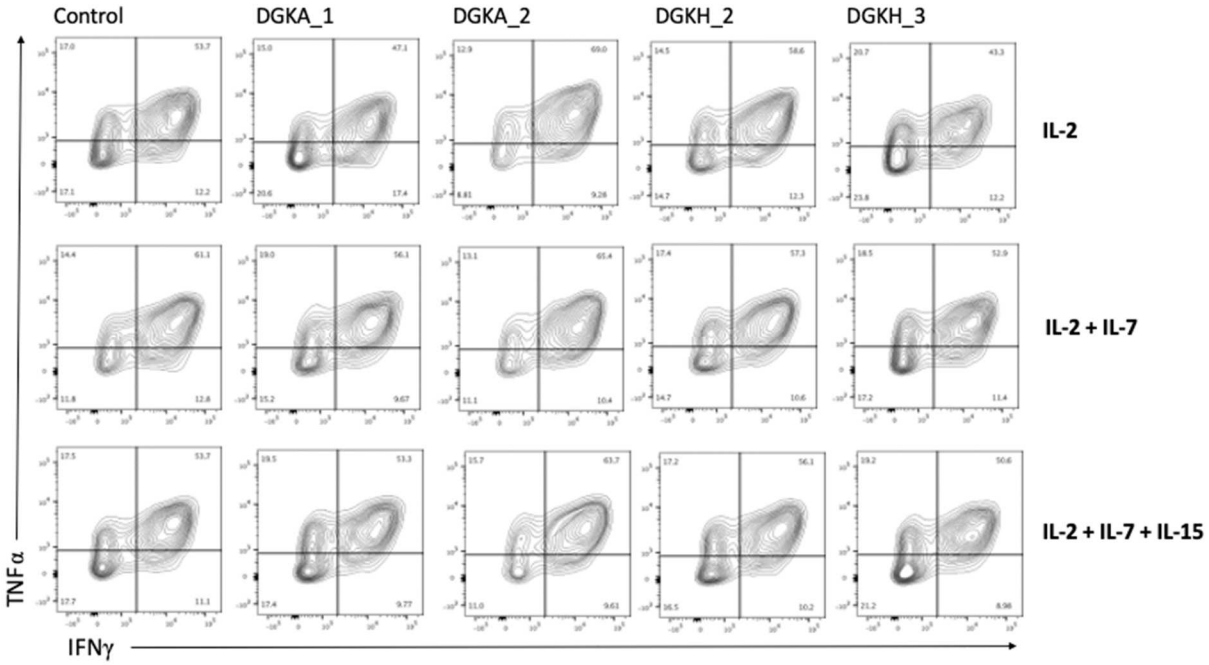


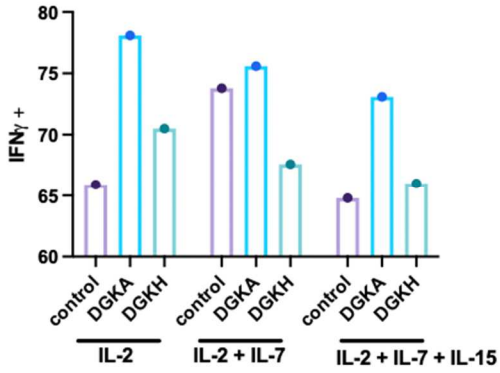
Figure 8 - CD8+ T cells with DGKH and DGKA knockouts show increased effector cytokine production

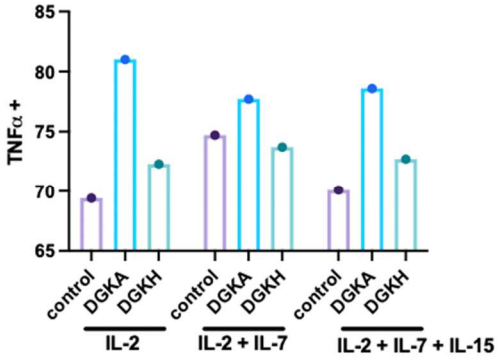
Tag activated $CD8^+$ T cells were transduced with virus containing DGKH gRNA or DGKA gRNA and cultured with IL-2 in RPMI at 37°C. Transduced cells were then cultured for 3 days with IL-2 and then stimulated with PMA/ ionomycin and stained for IFNγ and TNFα. Each condition had three biological replicates. Stained cells were ran using Flow Cytometry and analyzed using FlowJo and Prism. Analysis was performed on gated live, GFP+ cells. The above figures show statistically significant increases in IFNγ and TNFα percentage and MFIs with both DGKH and DGKA knockouts compared to controls. This experiment was repeated fix times, under the same conditions and similar results were obtained. Statistical significance was calculated based on p-values (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

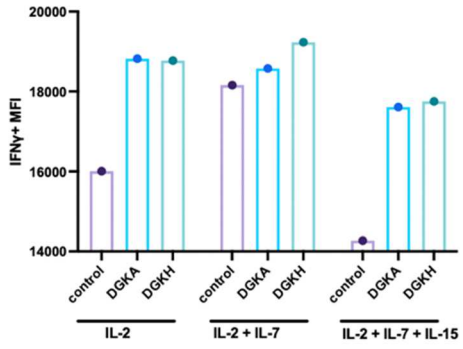
Figure 9 - CD8+ T cells with DGKH and DGKA knockouts show increased effector cytokine production

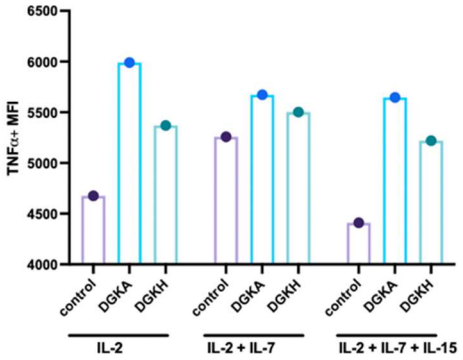
Tag activated $CD8^+$ T cells were transduced with virus containing DGKH gRNA or DGKA gRNA and cultured with IL-2 in RPMI at 37ºC. Transduced cells were then cultured for 3 days with IL-2 and then stimulated with PMA/ ionomycin and stained for IFNγ and TNFα. Each condition had one biological replicates. Stained cells were ran using Flow Cytometry and analyzed using FlowJo and Prism. Analysis was performed on gated live, GFP+ cells. The following figures show increases percentage of IFNγ and TNFα in DGKA and DGKH knockouts compared to controls after culture with IL-2 alone or IL-2, IL-7 and IL-15. The percent of IFNγ and TNFα was slightly lower then controls in DGKH, but not DGKA knockouts. Additionally, MFIs increased for both IFNγ and TNFα in DGKA and DGKH knockouts compared to controls after culture with IL-2, IL-2 plus IL-7 or IL-2, IL-7 and IL-15 Because IL-7 and IL-15 are cytokines that promote memory formation, we aimed to test if DGK knockouts would still yield increases in effector cytokine production under these conditions.


Control
DGKA_1
DGKA_2
DGKH_2
DGKH_3
IL-2
IL-2 + IL-7
IL-2 + IL-7 + IL-15
TNFα
IFNγ


IFNγ +
control
DGKA
DGKH
IL-2
IL-2 + IL-7
IL-2 + IL-7 + IL-15


TNFα +
control
DGKA
DGKH
IL-2
IL-2 + IL-7
IL-2 + IL-7 + IL-15


IFNγ+ MFI
control
DGKA
DGKH
IL-2
IL-2 + IL-7
IL-2 + IL-7 + IL-15


TNFα+ MFI
control
DGKA
DGKH
IL-2
IL-2 + IL-7
IL-2 + IL-7 + IL-15

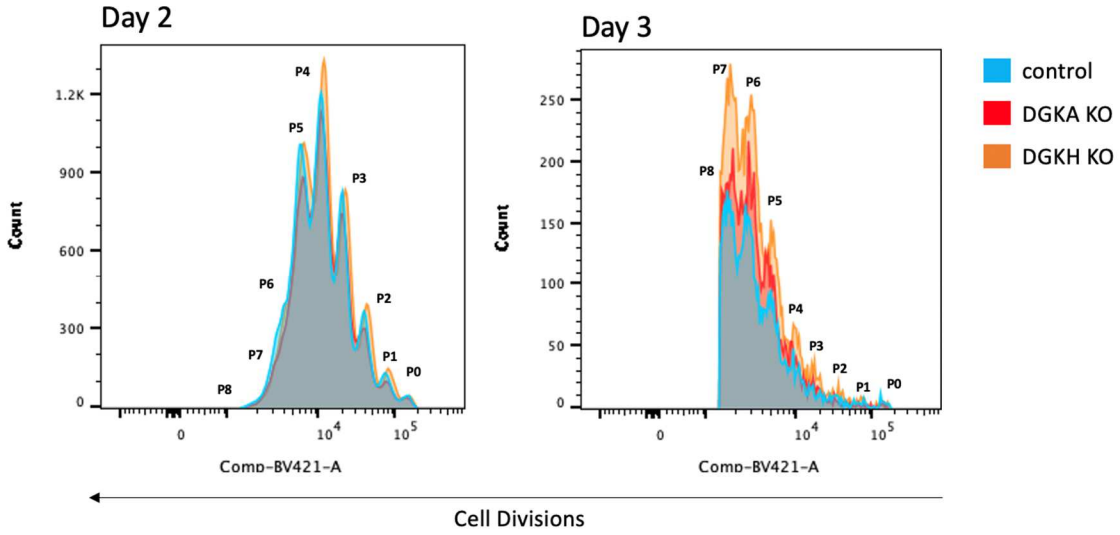


Figure 10 - CD8+ T cells with DGKA and DGKH knockouts do not affect proliferation rates

After electroporating with Cas9 and gRNAs for DGKA and DGKH, isolated $CD8^+$ T cells were cultured for 3 days with IL-2 in RPMI, and CellTrace Violet Cell Proliferation Kit (ThermoFisher) was added (A). Cells were collected for each condition at day 1, day 2 and day 3 and ran using Flow Cytometry. Using FlowJo cells were gates in P0 to P8 subpopulations according to dye dilution. Cells in the P0 gate are the ones that proliferated less and cells in the P0 gate are the ones that proliferated most. CTV assays revealed that cell had proliferated significantly more at day 3 compared to day 2 but that DGK knockouts did not affect proliferation rates (B).

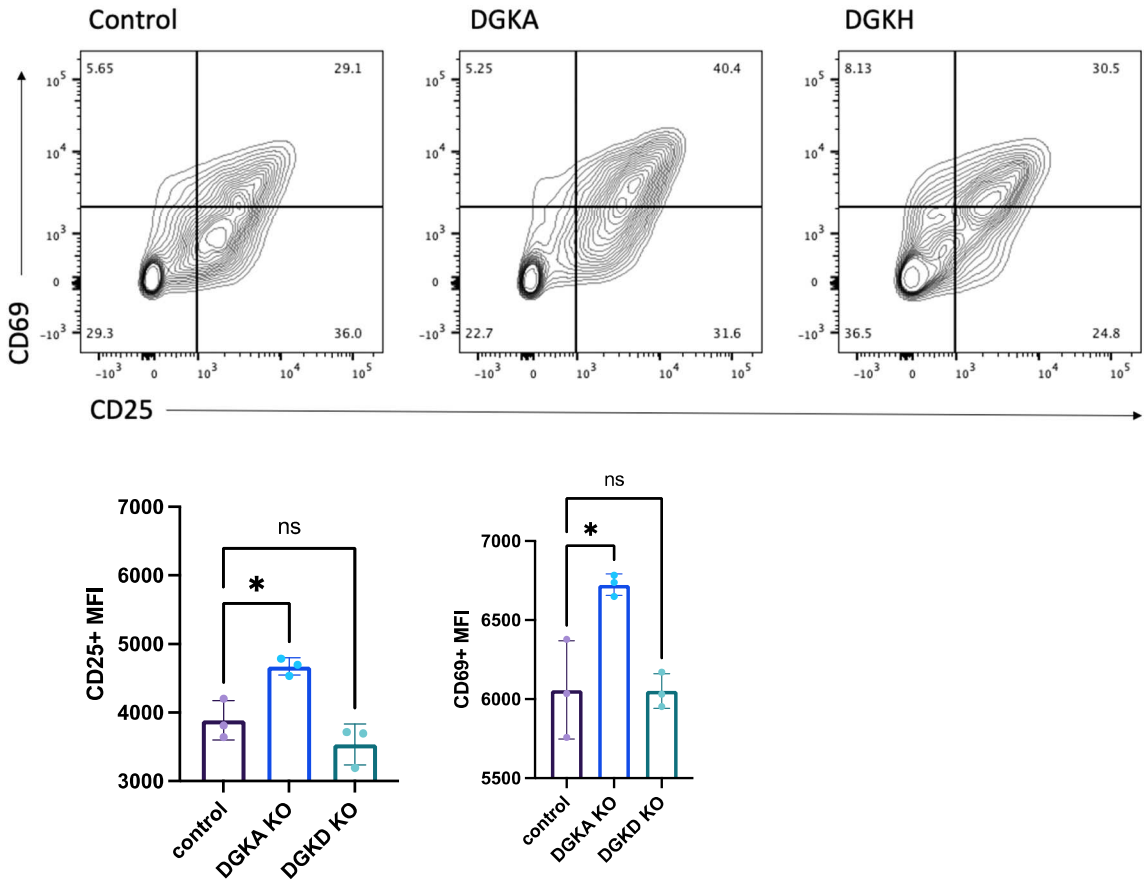
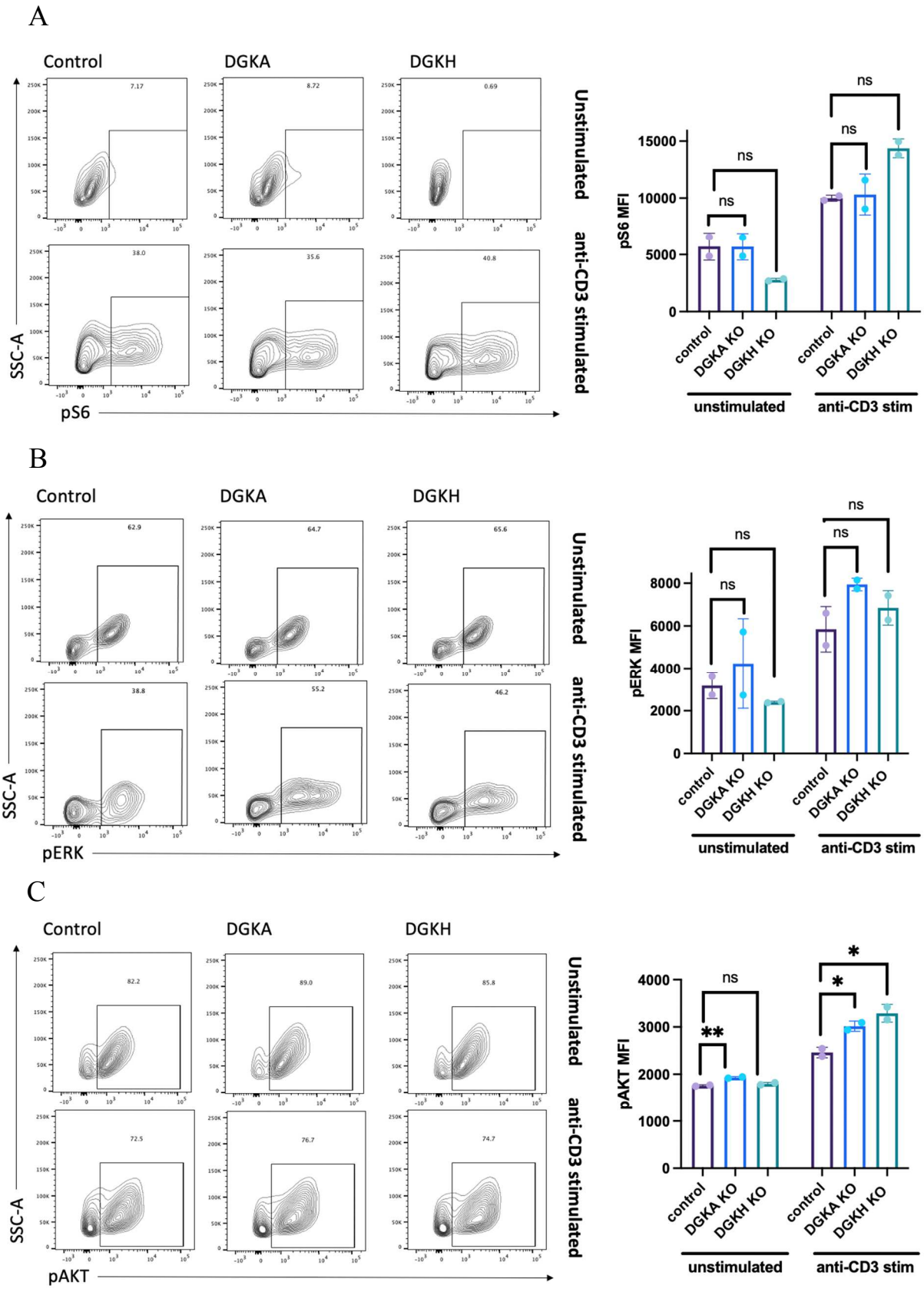


Figure 11 - DGKD knockout did not affect production of effector-like surface markers

After electroporating with Cas9 and gRNAs for DGKA and DGKD, isolated $CD8^+$ T cells were cultured for 3 days with IL-2 in RPMI. After 3 days cells were collected and stained for surface markers CD25 and CD69. Each condition had three biological replicates. Stained cells were ran using Flow Cytometry and analyzed using FlowJo and Prism. Results showed no significant difference in neither CD25 nor CD69 MFIs in DGKD knockouts. Statistical significance was calculated based on p-values (ns $p > 0.05$, * $p \leq 0.05$).

Figure 12 - Effects of DGK KO on TCR signaling

After electroporating with Cas9 and gRNAs for DGKA and DGKH, isolated $CD8^+$ T cells were cultured for 3 days with IL-2 in RPMI. After 3 days cells were seeded into a 96-well plate and stimulated for 30 minutes with anti-CD3 antibodies. Following stimulation cells were fixed and permeabilized with methanol. Rabbit-primary antibodies were added for p44/42 MAPK (ERK1/2 Thr202/Tyr204), pAKT (Ser473) and pS6 (Ser235/236) and addition of anti-rabbit goat secondary antibodies conjugated to APC followed. Samples were then run using a BD Accuri ™ Flow Cytometer. Flow plots and MFI calculations revealed increases in pS6 in DGKH knockouts although these were not significant (A). MFIs for pERK increased in both DGKH and DGKA knockouts compared to controls, but once again these increases were not significant (B). Finally, we observed that both DGKA and DGKH knockouts yielded significantly higher MFIs for AKT phosphorylation indicating this is the primary pathway they target (C).


A
Control
DGKA
DGKH
7.17
8.72
0.69
38.0
35.6
40.8
Unstimulated
anti-CD3 stimulated
SSC-A
pS6
pS6 MFI
15000
10000
5000
0
ns
ns
ns
ns
ns
control
DGKA KO
DGKH KO
unstimulated
anti-CD3 stim
B
Control
DGKA
DGKH
62.9
64.7
65.6
38.8
55.2
46.2
Unstimulated
anti-CD3 stimulated
SSC-A
pERK
pERK MFI
8000
6000
4000
2000
0
ns
ns
ns
ns
ns
control
DGKA KO
DGKH KO
unstimulated
anti-CD3 stim
C
Control
DGKA
DGKH
82.2
89.0
85.8
72.5
76.7
74.7
Unstimulated
anti-CD3 stimulated
SSC-A
pAKT
pAKT MFI
4000
3000
2000
1000
0
ns
**
*
*
control
DGKA KO
DGKH KO
unstimulated
anti-CD3 stim

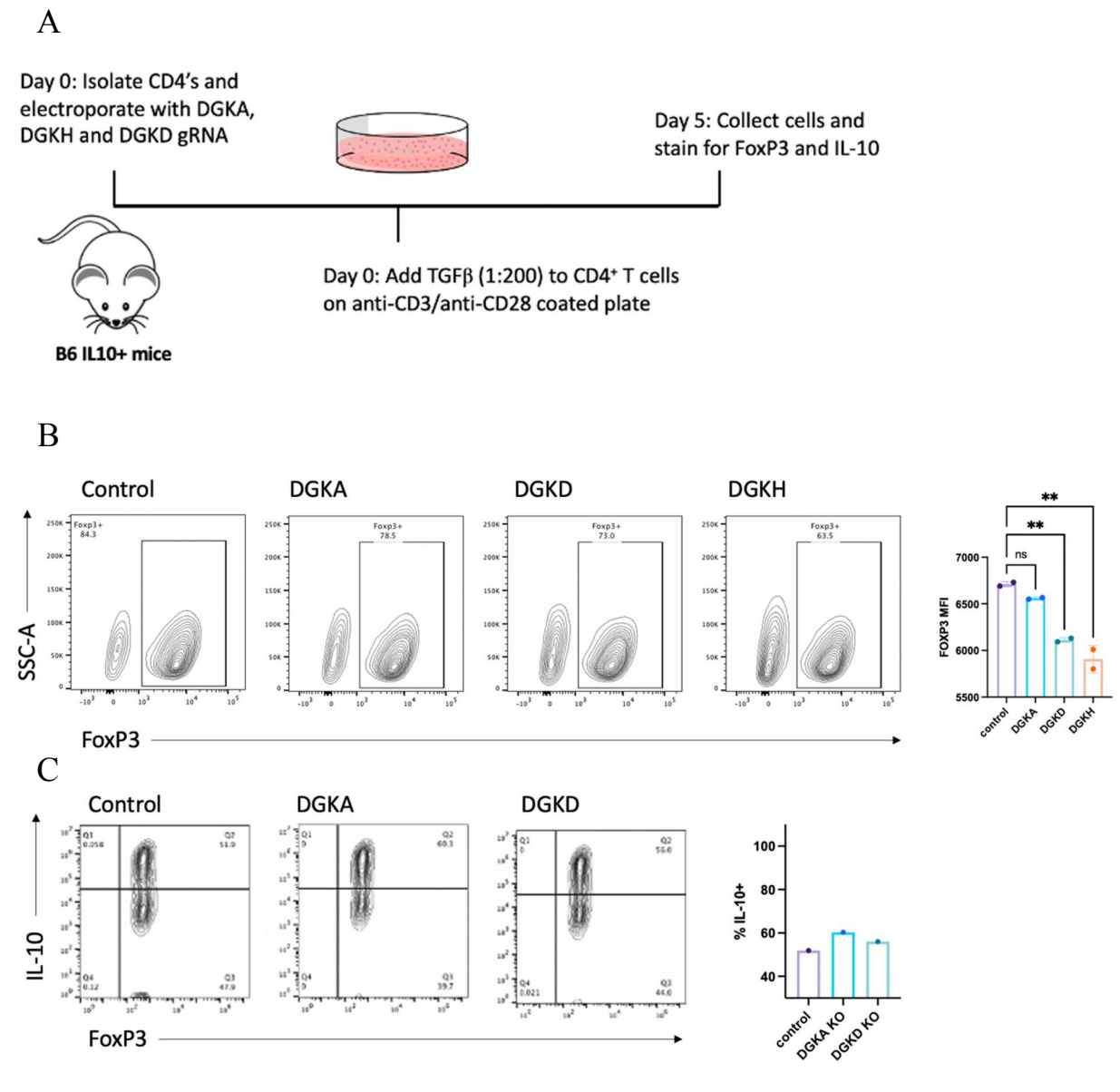


Figure 13 - FoxP3 expression is reduced in DGKH KO, but not DGKA KO

To determine if DGKH knockouts hinder Treg differentiation more than DGKA knockouts, we looked at percent FoxP3+ cells after 5-day culture of isolated $CD4^+$ T cells with TGFβ (1:200) (A). Cells were isolated and harvested from B6 IL10/IL12 + transgenic mice. We observed no difference in expression of FoxP3 between control cells and DGKA knockouts, however DGKH knockouts significantly decreased FoxP3 MFIs (B). Similarly, to DGKH, we observed that DGKD knockouts also lead to significant decreases in FoxP3 expression. To assess for Treg functionality we looked at percent IL-10 generated (C). This experiment would need to be repeated as it was executed only once due to challenges in obtaining well differentiated $CD4^+$ Tregs. Furthermore, due to there being enough cells only for one biological replicate we were unable to calculate statistical significance.

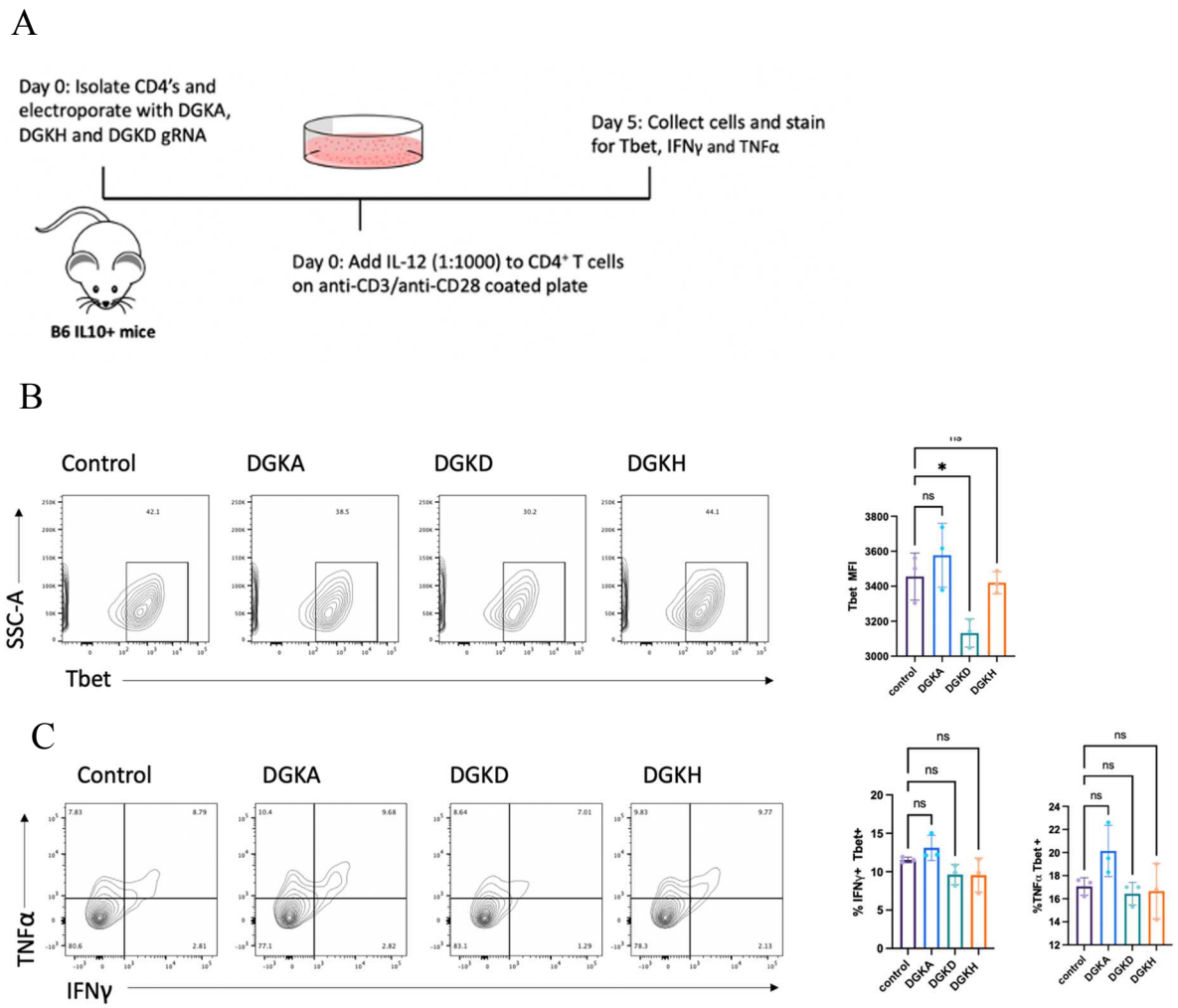


Figure 14 - Th1 production is unaffected in both DGKH and DGKA knockouts

To determine if DGKH knockouts hinder Th1 differentiation, we looked at percent Tbet+ cells after 5-day culture of isolated CD4$^+$ T cells with IL-12 (1:1000) (A). Cells were isolated and harvested from B6 IL10/IL12 + transgenic mice. We observed no striking difference in the expression of Tbet between control cells, DGKA knockouts and DGKH knockouts(B). DGKD knockouts lead to a significant decrease in expression of Tbet. To assess for Th1 functionality we looked at percent IFNγ and TNFα generated and observed no significant changes across all conditions (C). This experiment would need to be repeated as it was executed only once due to challenges in obtaining well differentiated CD4$^+$ Th1s. We ran the cytokine assay with three technical replicates.

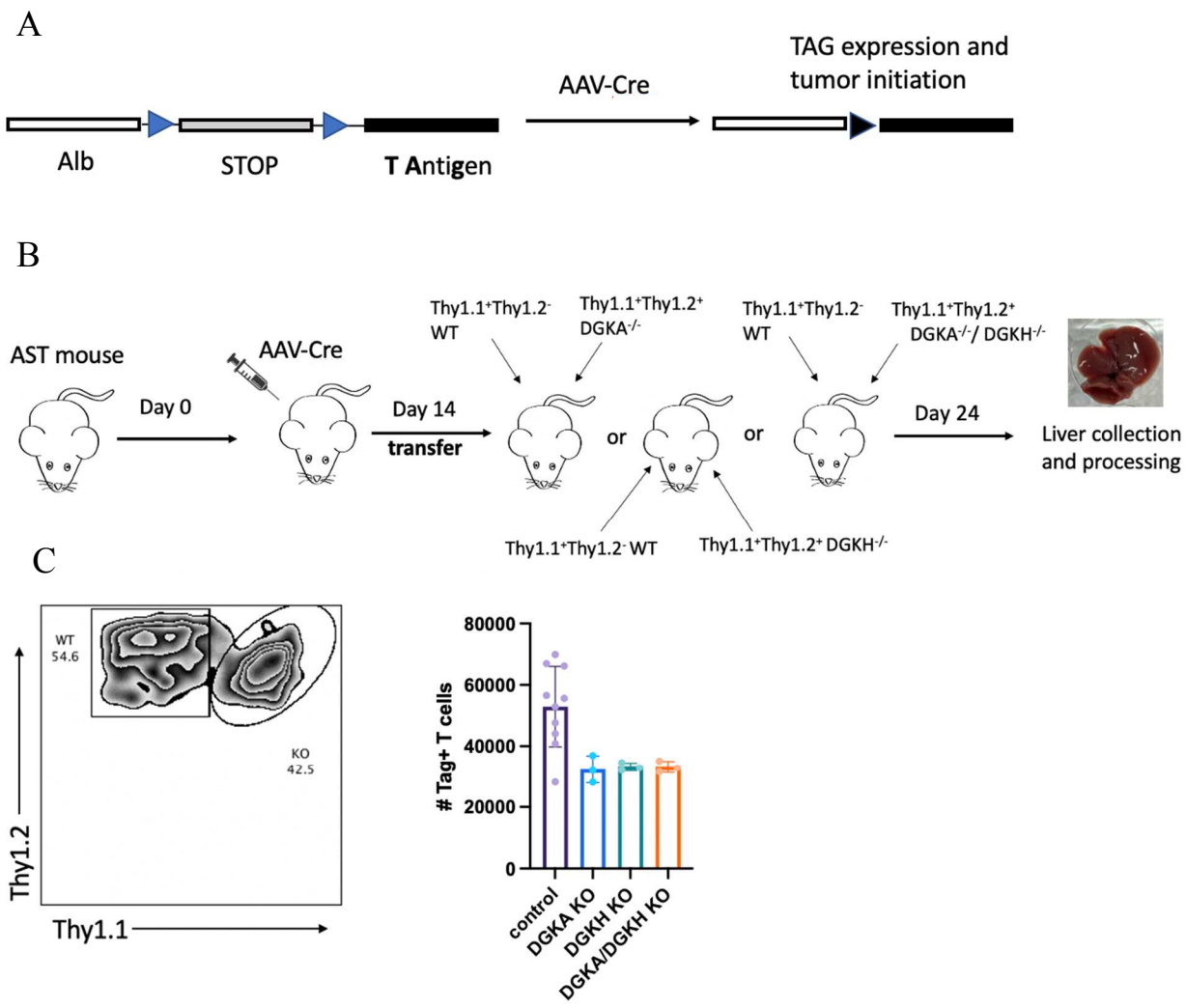
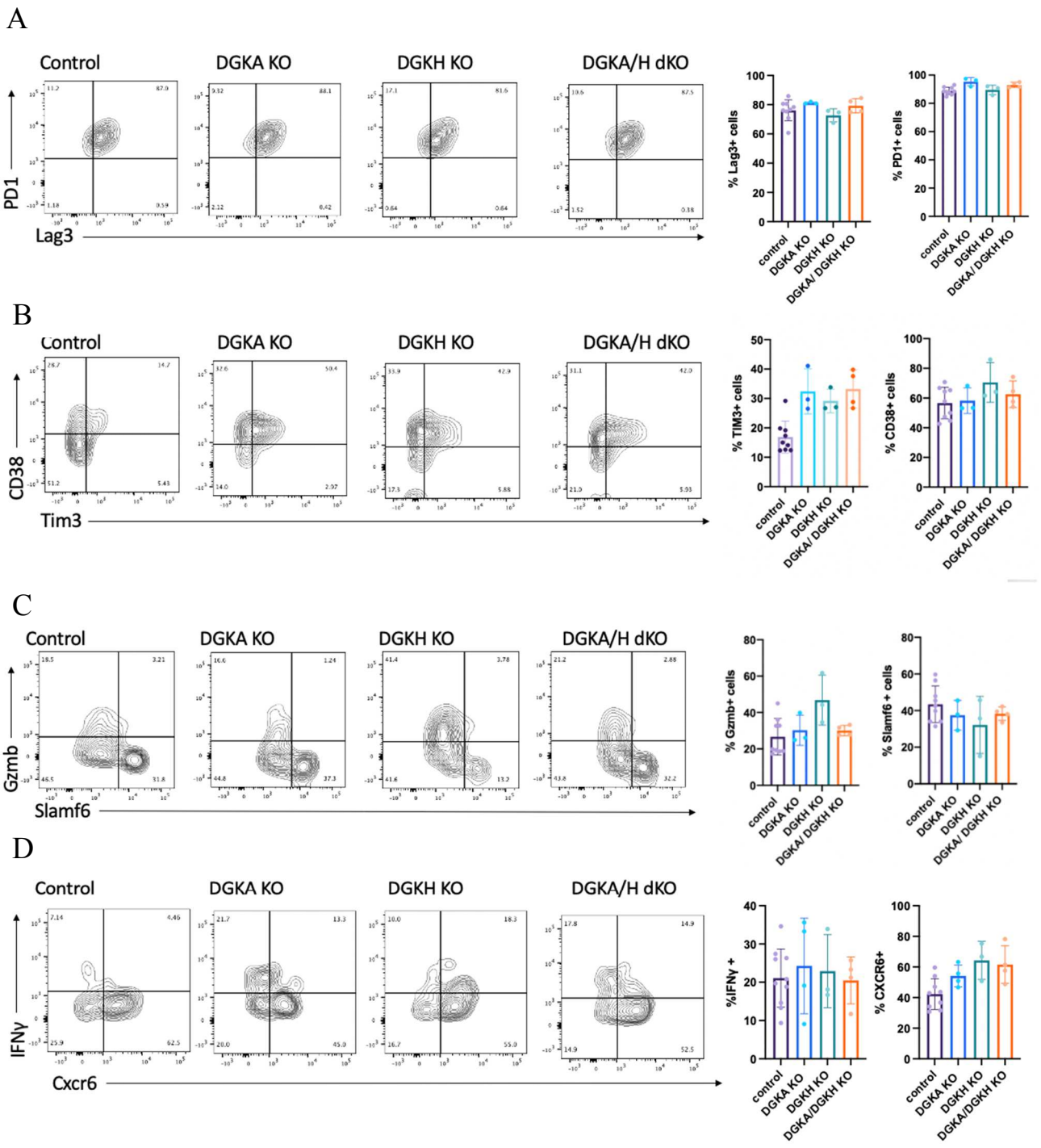


Figure 15 – Methods for liver cancer induction in vivo

To study the effects of DGK knockouts *in vivo* we used an AAV-Cre method for liver induction in AST transgenic mice, which allows tracking of tumor specific cells. AST mice are characterized by albumin-stop-TAG cassettes. Cre- mediated excision of flox-stop cassette leads to SV40 large T antigen expression in albumin expressing cells. Tag expression is tumor specific and drives tumor initiation (A). Using this model for tumor induction we took AST transgenic mice and induced TAG expression by AAV-Cre injections. 14 days after TAG induction we then transferred Thy1.1^{+}Thy1.2^{+} CD8^{+} T cells with electroporated DGKH, DGKA or DGKH and DGKA knockouts in different mice. We also transferred Thy1.1^{+}Thy1.2^{-} CD8^{+} T cells with no knockout as control cells into all mice (B). For each condition we had at least three mice as biological replicates. 14 days after T cell transfer, we then sacrificed mice and collected livers for Percol processing. We transferred equal amounts of Thy1.1^{+}Thy1.2^{+} CD8^{+} T cells and Thy1.1^{+}Thy1.2^{-} CD8^{+} T cells into each mouse and analyzed different subsets using FlowJo and Prism. Overall, we observed that control cells survived slightly better than DGKA, DGKH or DGKA/H double knockouts *in vivo* (C).

Figure 16 - DGKH knockouts increase Tim3 expression in vivo

After collecting livers from mice with HCC we processed them and isolated tumor-infiltrating lymphocytes. We then stained cells *ex vivo* with antibodies against several different exhaustion markers (PD-1, Lag-3, CD38, Tim-3 and Slamf-6) and for serine protease Granzyme B. Although we reported no significant changes in expression of Lag3, PD-1 (A), CD38 (B) and Slamf-6 (C), we show increases in Tim-3 in all knockouts compared to controls (B) and increase in Gzmb in DGKH knockouts specifically (C). We also additionally stimulated cells *ex vivo* with PMA/ionomycin for 4 hours following which we stained cells for chemokine receptor CXCR6 and effector cytokines IFNγ and TNFα. Following stimulation, we observed increases in CXCR6 in all knockouts (D), but no significant changes in IFNγ (E).


A
Control
DGKA KO
DGKH KO
DGKA/H dKO
PD1
Lag3
% Lag3+ cells
% PD1+ cells
control
DGKA KO
DGKH KO
DGKA/ DGKH KO
B
Control
DGKA KO
DGKH KO
DGKA/H dKO
CD38
Tim3
% TIM3+ cells
% CD38+ cells
control
DGKA KO
DGKH KO
DGKA/ DGKH KO
C
Control
DGKA KO
DGKH KO
DGKA/H dKO
Gzmb
Slamf6
% Gzmb+ cells
% Slamf6+ cells
control
DGKA KO
DGKH KO
DGKA/ DGKH KO
D
Control
DGKA KO
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DGKA/H dKO
IFNγ
Cxcr6
%IFNγ +
% CXCR6+
control
DGKA KO
DGKH KO
DGKA/DGKH KO

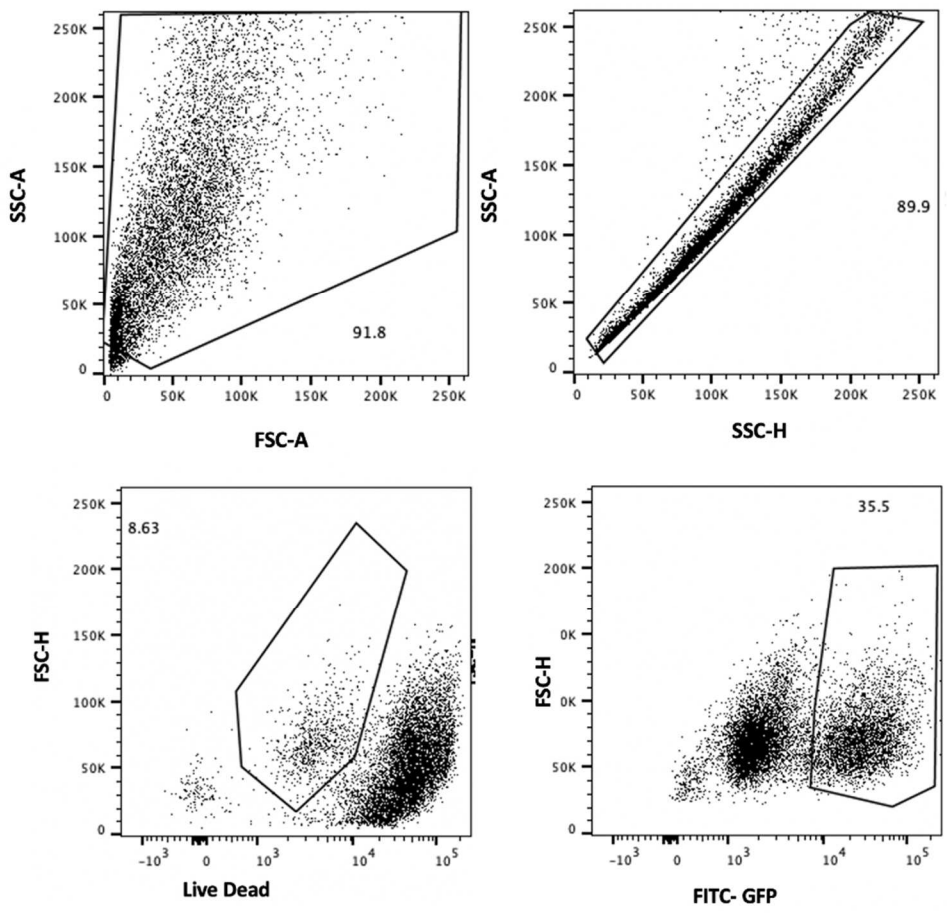

Figure 17 - Gating for transduced cells

Cells that were cultured for 3 days under different conditions after transduction were then stained for different surface and intracellular markers. After staining cells were ran using Flow Cytometry and analyzed in Flow Jo. This analysis if for cells conventionally transduced with virus containing plasmids with gRNA for the desired gene knockout. First, I gated on all live lymphocytes, then on single cells, then on live cells and finally on GFP+ lymphocytes, which contain the desired knock out. Plots show that with this transduction method only about 8% of cells survive, 35% of which contain the desired knockout.

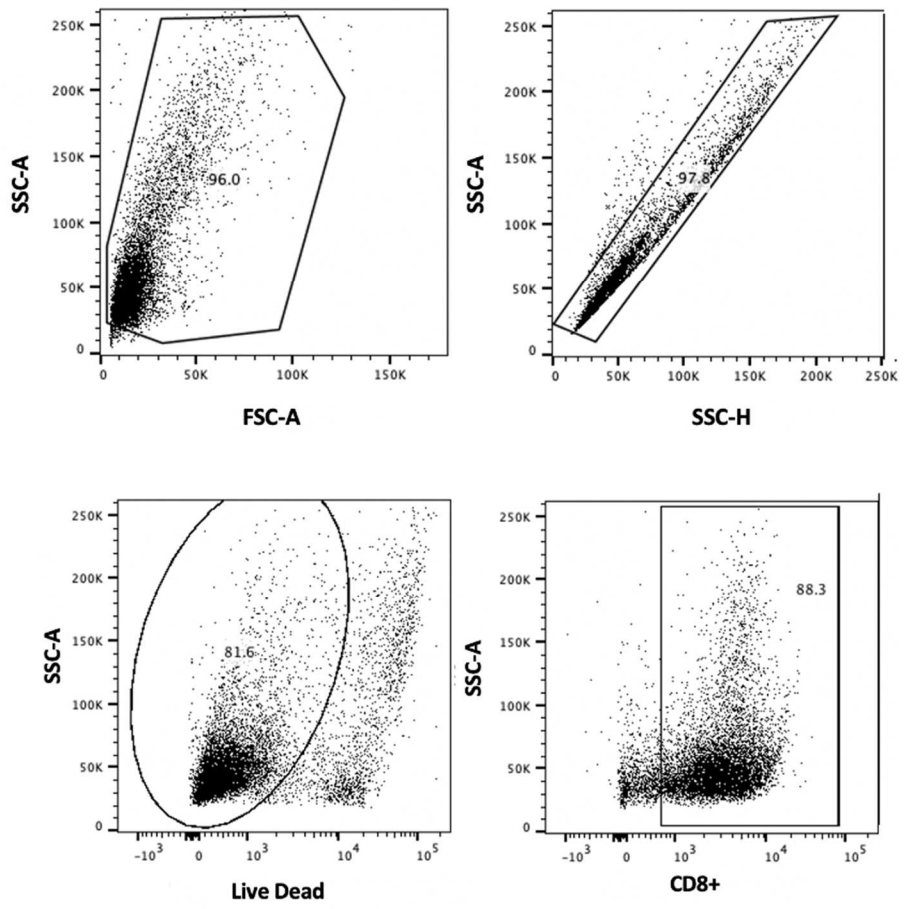

Figure 18 - Gating on cells with knockout performed with RNP

Cells that were cultured for 3 days under different conditions after electroporating isolated $CD8^+$ T cells with gRNAs for desired knockouts using RNP delivery of Cas9. Cells were then stained for different surface and intracellular markers. After staining cells were ran using Flow Cytometry and analyzed in Flow Jo. First, I gated on all live lymphocytes, then on single cells, then on live cells and finally on $CD8^+$ T cells. This method yielded 81% of live cells, 88% of which were $CD8^+$.

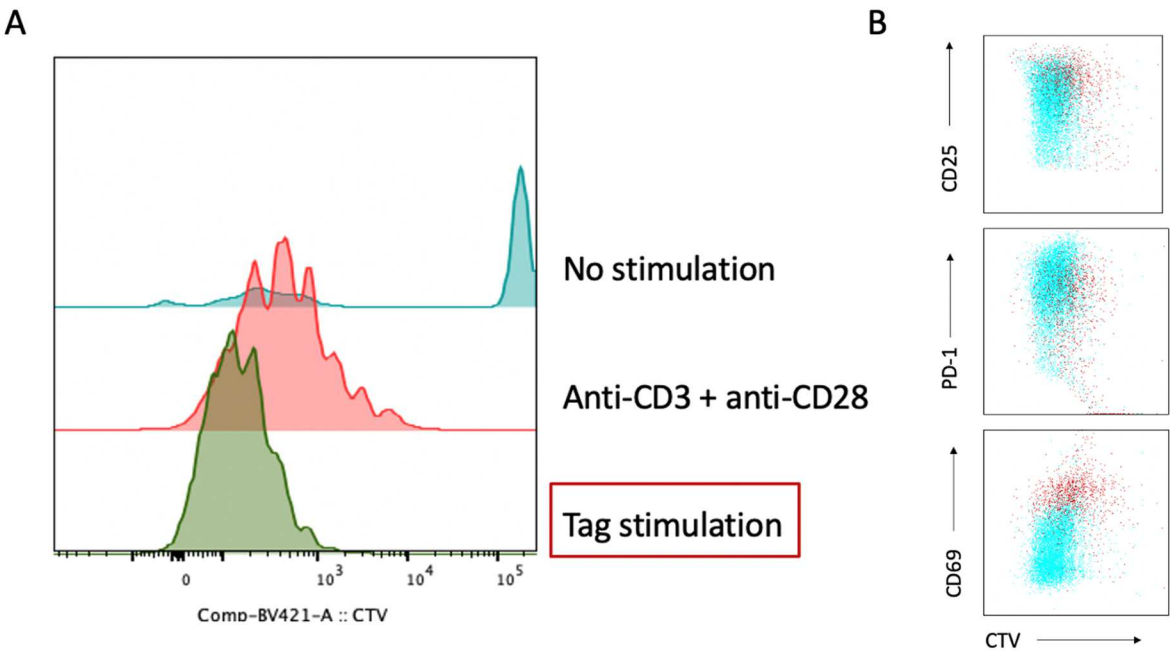

Figure 19 - T cell proliferation assay using dye dilutions by flow cytometry

T cells were activated with either anti-CD3 and anti-CD28 or tag peptide (1:2000) in 10% RPMI. After 3 days of culture cells were collected and stained using a dilution dye, the intensity of which was measured by Flow Cytometry. Detection of a lower fluorescent intensity indicated that cells had proliferated more and were therefore better activated. Our results showed that the tag peptide stimulation better activated CD8 T cells when compared to anti-CD3/anti-CD28 stimulation, although those cells also appeared sufficiently activated (A). We also stained for common surface markers such as CD25, PD-1 and CD69 to test whether different activation strategies affected their expression. We noticed no major different in expression profiles of these cell surface markers (B).

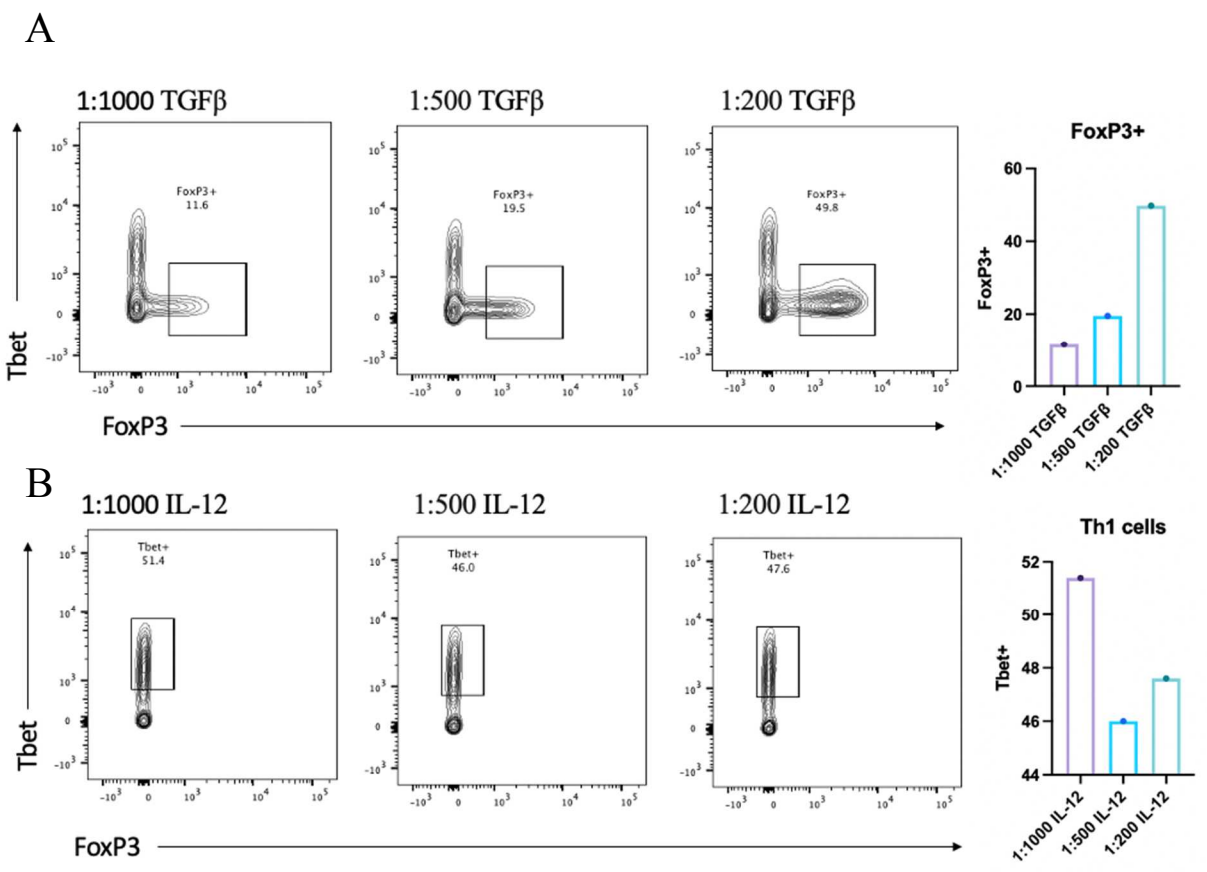

Figure 20 - Preferential methods for cytokine induced Treg and Th1 differentiation

To determine what cytokine concentrations yielded better CD4 differentiation into regulatory T cells and Helper T cell subsets we ran trial experiments by culturing isolated CD4 cells with different amounts of either TGFβ or IL-12 (1:1000, 1:500 or 1:200). Stained cells were ran using Flow Cytometry and analyzed using FlowJo and Prism. We observed that a 1:200 dilution of TGFβ stocks yielded the highest amount of $FoxP3^+$ regulatory T cells (A), whereases IL-12 in 1:1000 dilutions yielded the highest amount of $Tbet^+$ Th1 cells (B). We thus adopted these differentiation techniques for future experiments.

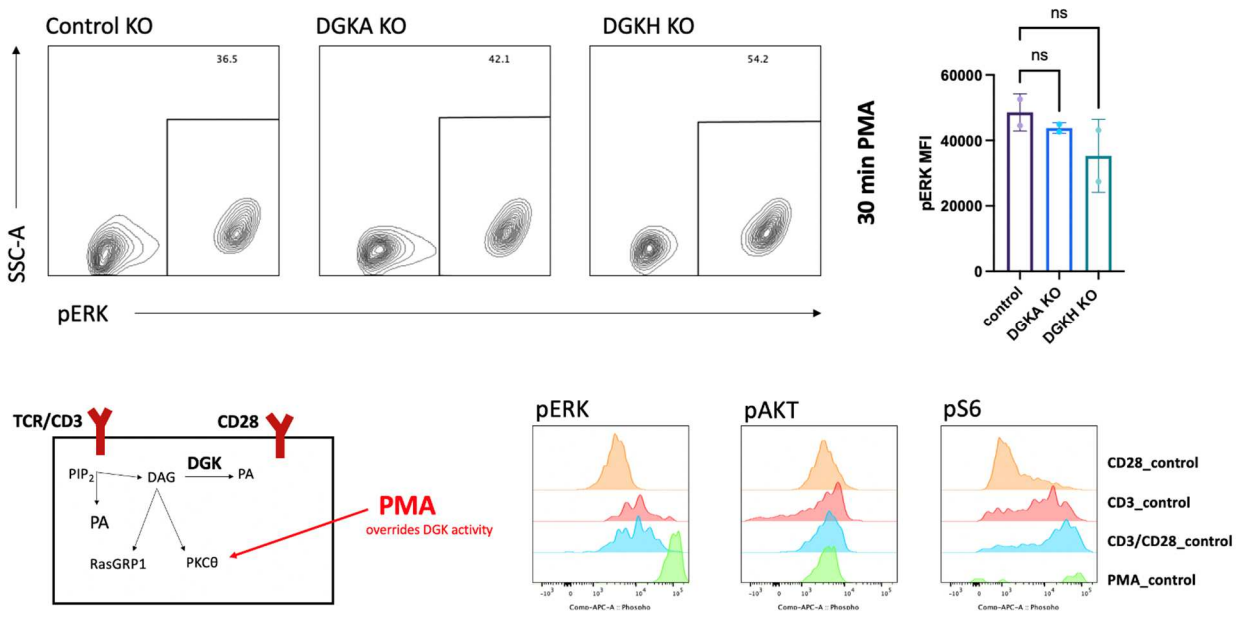

Figure 21 - anti-CD3 as a stimulant for measuring effects of DGK knockouts on phosphorylation of signaling intermediates by flow cytometry

To assess what the best method was to determine phosphorylation levels of pathway intermediates ERK, AKT and mTOR we stimulated cells under four different conditions for 30 minutes before adding fixation buffer. Phospho-flow protocols usually suggest stimulation with PMA however since it acts as a PKC activator, which bypasses DGK activity we established this process wouldn't give the most biologically accurate results when looking at the effects of DGK knockouts. We therefore decided to use PMA as a positive control and compared it to anti-CD28 stimulation, anti-CD3 stimulation and anti-CD3 combined with anti-CD28 stimulation (B). The best results were given by stimulation with anti-CD3 alone, so we chose to use this as a stimulant for phospho-flow experiments.

Table 1 - Expression levels of DGK isoforms in liver cancer T cell subsets

Single cell sequencing data revealed different expression levels of DGK isoforms in each one of the 11 subsets outlined by Chunhong Zheng et al., in *Landscape of Infiltrating T cells in Liver Cancer Revealed by Single-Cell Sequencing.*

T cell subset	cell type name	DGKA level	DGKH level	DGKD level
C1_CD8-LEF1	naïve CD8+	high	low	medium
C2_CD8-CX3CR1	effector CD8+	medium	low	medium
C3_CD8-SLC4A10	mucosal associated invariant T cell	medium	low	low
C4_CD8-LAYN	exhausted CD8+ in tumor tissue	medium	medium	low
C5_CD8-GZMK	exhausted CD8+	medium	low	low
C6_CD4-CCR7	naïve CD4+	high	low	medium
C7_CD4-FOXP3	regulatory T cell in blood	high	low	medium
C8_CD4-CTLA4	regulatory T cell in tumor	medium	low	low
C9_CD4-GZMA	T helper cells	medium	low	low
C10_CD4-CXCL13	Exhausted T helper cells	medium	medium	low
C11_CD4-GNLY	Cytotoxic T helper cells	medium	low	medium

Table 2 - Surface marker expression at various stages of T cell differentiation

Here we show the expected expression of certain surface markers at 3 main stages of T cell differentiation: naïve, effector and memory. The negative sign indicates little to no expression, the positive moderate expression, and the double positive high expression. Ideally in out knock outs we would want to see an effector T cell phenotype, which is therefore mainly characterized by high levels of CD25, IL18RA, CD44, CD69 and PD1 and low expressions of CD62L and IL7R.

	naïve	effector	memory
CD25	-	++	+
IL7R (CD127)	+	-	++
IL18RA	-	++	+
CD62L	+	-	+
CD44	-	+	+
CD69	-	++	+
PD1	-	+	

Table 3 - DGK isoforms

DGKs are classified into five different subtypes based on the catalytic domains they have. Here we highlight the five main subtypes, which DGKs are associated to each subtype and what kinase domains they have. A C1a/b domain, a conserved catalytic domain and an accessory domain are common to all five subtypes, however each class has other domains that correlate to unique functions and specify subcellular localization.

Type	Isoforms	Domains
I	DGK-A, DGK-B, DGK-G	Recoverin, EK-hands, C1a/b domain, conserved catalytic domain, accessory domain
II	DGK-D, DGK-H, DGK-K	PH domain, 2 C1a/b domain, conserved catalytic domain, accessory domain, SAM domain
III	DGK-E	C1a/b domain, conserved catalytic domain, accessory domain
IV	DGK-Z, DGK-I	C1a/b domain, MARCKS homology domain, conserved catalytic domain, accessory domain, ankyrin reporters
V	DGK-Θ	Proline rich, C1a/b domain, PH+RA domain, conserved catalytic domain, accessory domain