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Studying the Antagonistic Relationship Between NKG2D and TGF-β Signaling in CD8+ T Cells

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STUDYING THE ANTAGONISTIC RELATIONSHIP BETWEEN NKG2D AND TGF-β SIGNALING IN CD8+ T CELLS

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Live as if you were to die tomorrow. Learn as if you were to live forever.

– Mahatma Gandhi

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CHAPTER I

INTRODUCTION

Cancer Immunotherapy and the Importance of CD8+ T Cells

Over the past few decades, the cancer immunotherapy has become a fourth pillar of cancer care and therapeutics after surgery, radiation, and chemotherapy [1][2]. As the name implies, this approach utilizes the power of the immune system in selectively eliminating the cancer cells in patient's body. Although the idea of an immune system controlling or fighting against cancer cells has existed since hundreds of years of human history [2–6], the scientific advancement in our basic understanding of the cancer immunology and transformation of this knowledge into clinics has only begun to happen after the late 19th century [1,2,7–12].

Our current knowledge has highlighted the involvement of many different immune cell types in shaping the anti-cancer immune response [13]. These cell types include the major components of innate immune system such as dendritic cells, natural killer cells, myeloid derived suppressor cells (MDSCs), and macrophages as well as key players of the adaptive immune system which include CD8+ and CD4+ T cells [13]. Among these cell types, T cells of the adaptive immune system are particularly important as they can not only recognize and eliminate the cancer cells but possess a capacity to form an immunological memory and provide a durable anti-tumor protection [14]. T cells identify their target cells using a T cell receptor (TCR) that can bind to a specific antigen presented on the target cells in the context of major histocompatibility complex (MHC). Thus, T cells carrying a TCR reactive against antigens presented on tumors, termed as tumor associated antigens (TAA), can recognize and mount an immune response against them [15]. CD4+ T cells, termed often as 'helper T cells', have been shown to possess majorly a helper function i.e. they secrete

inflammatory cytokines upon antigen-encounter to help and promote the function of CD8+ T cells and other immune cells [16]. However, it should be noted that there is evidence of CD4+ T cells mediated cytolysis [17]. CD8+ T cells, also termed as 'killer' or 'cytotoxic' T cells in the literature, are the final executers of the adaptive immune response which can kill the target cells upon their recognition [18]. Because of their direct, indispensable role in eliminating the cancer cells, the biology of CD8+ T cells has received an extensive attention in the field of cancer immunology research.

CD8+ T Cells Mediated Immunity in Cancer

The immune response of CD8+ T cells against cancer is a result of series of biological processes that take place in the body [19]. In tumor-bearing hosts, the dying cancer cells release the TAA in the systemic circulation which are taken up by the dendritic cells. Dendritic cells process and present these antigens to the T cells in the lymph node in the form of peptide-MHC I or II complex. CD8+ T cells in the lymph node identifies TAA in the context of MHC I via their TCR and undergo a process of 'activation' or 'priming'. In this priming process, CD8+ T cells undergo variety of biochemical, biophysical, and genetic changes including rapid proliferation, production of inflammatory cytokines, and acquisition of cytotoxic capacity by up-regulation of effector molecules such as granzyme B and perforin. These primed T cells then traffic to the tumor site and detect their target cancer cells by the interaction between TCR and peptide-MHC I complex. Then, CD8+ T cell form pores in the membrane of cancer cells using a glycoprotein named perforin, and subsequently release granzyme B inside the cancer cells through these pores. Granzyme B, a serine protease, then causes the apoptosis of cancer cells in a caspase-dependent manner. This cycle of CD8+ T cells' immunity against cancer is further propagated by the release of more TAA form the lysed cancer cells→ processing and presentation by DCs→ priming of T cells and so on...

Despite this inherent capacity to eliminate cancer cells, the killer T cells system often

fails to control cancer naturally. Although, the inability of spontaneous T cell responses to eliminate cancer cells remains an active area of investigation, few major reasons have been outlined. The T cells reactive against 'self' antigens are negatively selected in the thymus and hence the strength of the interaction between their TCRs and shared tumor antigens remains weak, preventing strong anti-tumor immune responses [20]. The other reasons attributed for it are inefficient antigen presentation to the T cells, peripheral mechanisms to promote self-tolerance and importantly, various mechanisms adopted by cancer cells to evade the immune system [21]. Nevertheless, the scientists have been able to develop powerful cancer immunotherapy approaches in the past few decades by harnessing the function of these very cytolytic CD8+ T cells [1,2]. Two major FDA approved cancer immunotherapies: chimeric antigen receptors (CAR) T cell therapy and check-point blockade (Anti-PD 1/CTLA-4) therapy have been at the forefront of a battle between the immune system and a cancer.

CAR T Cell Therapy

CAR therapy boosts anti-tumor CD8+ T cell responses by improving their abilities to identify and kill the cancer cells. CAR was originally designed as a chimera of extra-cellular ligand binding domain (variable region of the antibody that binds to the desired antigens) and the intra-cellular CD3ζ signaling domain of the TCR [22]. Hence, CAR expressing CD8+ T cells can recognize the surface antigens on cancer cells that are either poorly detected or not detected by the TCR. In the clinics, T cells from cancer patients' blood are extracted, engineered to express appropriate CAR and transfused back into the patients to allow efficient cancer cell recognition and killing [23]. Over the years, CAR has been modified with the goal of further enhancing T cell cytolytic function and as a result many different generations of CARs exist currently [22]. The latest CAR approaches involve the use of co-stimulatory signaling domains such as CD28, 4-1BB or OX40 in addition to the TCR signaling domain to increase the recognition of and the effector response against cancer cells [22].

Check-Point Blockade (Anti-PD 1/CTLA-4) Therapy

The check-point blockade therapeutic approach is based on the natural mechanisms used by the immune system to control the hyperactivity of CD8+ T cells. As a self-regulatory mechanism, T cells up-regulate the check-point receptors such as PD-1, CTLA-4, LAG3, TIM3 and TIGIT after chronic or persistent encounter to the target antigens (such as in the case of cancer) [24,25]. When the check-point receptors on T cells bind to their ligands on the target cells or other immune cells, their effector function is remarkably 'checked' or reduced. Such T cells are also often termed as 'exhausted' in the literature. The cancer cells use this mechanism to their favor. The ligands for CTLA-4 are present mainly on APCs [26], whereas the studies have shown that a vast majority of tumors abundantly express the ligands to check-point receptors PD-1 (PD-L1) and suppress the T cell activity [27,28]. In check-point blockade therapy, the interaction between PD1-PD-L1 and CTLA-4-CD80 is blocked by monoclonal antibodies, and the functions of CD8+ T cells are thus revived from the exhaustion [24,29].

Success and Challenges of the Cancer Immunotherapy

CAR T cell and check-point blockade immunotherapies have revolutionized the cancer therapy field with their outstanding clinical outcomes in advanced melanomas as well as hematological cancers such as leukemia and lymphomas [30,31]. Despite the success, these approaches face significant challenges in their paths of becoming versatile cancer treatments mainly due to their poor clinical outcomes against solid tumors [30,31]. Even in the case of melanoma where check-point blockade is highly effective in regressing established tumors in the responding patients, anti-CTLA-4 and anti-PD1 therapies have objective response rate (ORR) of less than 20% and about 40% respectively [32]. Combination of CTLA-4 and PD-1 has led to the ORR of about 55% in metastatic melanoma patients [32]. Unfortunately, in case of other solid cancers, the overall clinical benefits are even lower. Anti-PD-L1 mono-therapy in triple negative breast cancer (TNBC) show ORR

ranging from 4 to 19% [33]. In metastatic pancreatic cancer, combination of gemcitabine with anti-CTLA4 yielded the response rates of 7 to 18% [34]. Anti-PD1 treatment results in about 12-18% and 15-20% ORR in head and neck and non-small cell lung cancers (NSCLC) respectively [35,36]. In refractory colorectal cancers (CRC), the response rates of anti-PD-1 and CTLA-4 have been as low as 0 to 2% [37]. Even the CAR T cell therapy, despite achieving outstanding complete response rates of 70-90% in children and young patients of B cell acute lymphoblastic leukemia, has failed to demonstrate significant clinical benefits against solid tumors [30]. One of the major reasons implicated in the failure of immunotherapies against solid tumors is highly immune-suppressive tumor micro-environment (TME) in which the functions of CD8+ T cells are largely compromised [38].

Tumor Induced Immune-Suppression

As mentioned in the previous section, tumors have evolved to develop various mechanisms to evade the immune responses. These mechanisms could broadly be classified into two categories. One in which cancer cells ‘hide’ themselves from the immune system, and the other wherein the cancer cells ‘actively’ suppress the functions of immune cells [39]. The former strategy, which includes mechanisms such as down-regulation of the components of antigen presentation (MHC, TAA) and the ligands for co-stimulatory receptors such as B7 family, is extensively reviewed elsewhere [40,41] and will not be discussed here. For the scope of this dissertation, we define the latter set of mechanisms as tumor-induced immune suppression. This is mediated by variety of potent immune-inhibitory soluble factors such as TGF-β, IL-10, IDO which are secreted by tumors or certain immune cells [42–44]. Thus, rescuing the function of either endogenous or engineered CD8+ T cells from these immune-suppressive factors is a major current objective in the field to achieve full potential of existing cancer immunotherapies.

Clinical Evidence of Tumor Induced Immune-Suppression

Although the direct link between the type/stage of the tumor and the extent of

immune-suppression is missing, majority of the advanced solid tumors have been shown to exert potent suppressive effects on the immune system and T cells [42]. Delayed-Type Hyper-sensitivity (DTH) test, which measures the cell-mediated immunity (CMI) against common microbial antigens *in vivo* [45], revealed that CMI function was defective in cancer patients compared to normal subjects and was inversely related to the clinical stages of various hematological and solid tumors [44,46–49]. Anti-CD3 induced IFN-γ and IL-2 production in T cells from melanoma patients was reduced compared to the normal controls and was positively correlated with the prognosis [50]. The production of IFN-γ in whole blood cell cultures from patients having urinary bladder cancer, renal cell carcinoma, and colorectal cancer was decreased compared to benign tumor bearing or healthy controls [44,51–56]. Further, the cytotoxic capacity of T cells from melanomas and cervical cancer against influenza virus is lower compared to healthy controls [57,58]. Whiteside and colleagues have also shown that T cells and NK cells from about half of the ovarian and prostate cancer patients respond less to the antigens or mitogens *in vitro* [59–61]. The negative effects of cancer on the immune system, as evidenced by above mentioned clinical manifestations, could also be replicated in mouse experimental models. Various studies including ours have shown suppression of the T cells or immune system following transplantation of 4T1, C3, DA3-mammary cancer [62,63], Lewis and LP07-lung carcinoma [64,65], B16-melanoma [66,67] and RENCA-renal cell carcinoma [68] in mice. Taking together all the clinical and physiological evidences discussed above, it is clear that in tumor bearing hosts the functionality of immune system and T cells is compromised.

Mechanisms of Tumor Induced Immune-Suppression

Cancer cells have evolved numerous different mechanisms to ‘actively’ suppress the host immune system and escape the T cell mediated killing. These mechanisms include recruitment of the suppressive immune cells at tumor site as well as production and/or secretion of immune-suppressive cytokines and metabolic enzymes that directly or indirectly

suppress the activity of immune cells. Although discussion of all of these mechanisms is out of the scope of this dissertation, important immune-suppressive mechanisms are reviewed below.

Regulatory T Cells (Tregs).

In a variety of tumors, including but not limited to ovarian, lung, breast, and colorectal, regulatory T cells (Tregs) are increased in numbers and recruited at the tumor sites [69]. Tregs produce suppressive cytokines IL-10, TGF-β, and IL-35 that can directly inhibit the effector function of CD8+ T cells [69]. It has also been reported that Tregs can cause cytolysis of effector T cells using granzyme B, galenctin-1 or TRAIL pathway [69–72]. Tregs could also increase IDO expression in DCs and reduce their ability to present antigens to T cells [73].

Myeloid Derived Suppressor Cells (MDSCs).

As the name suggests, MDSCs are heterogenous, immature immune cells of myeloid origin. Increased presence of MDSCs at the tumor sites and in periphery has been reported in the cancer patients [43,74,75] and there is a positive correlation between the numbers of MDSCs and immune-suppression in patients [76]. MDSCs can suppress the T cell activity and DC maturation by production of IL-10, arginase and nitric oxide synthase [77].

IL-10.

IL-10 is produced majorly by MDSCs, tumor associated macrophages (TAM) and the tumor cells [43]. IL-10's prominent mechanism of immune-suppression includes inhibition of antigen presentation by down-regulation of MHC and co-stimulatory molecules on DCs and macrophages [78]. The defect in the antigen presentation to T cells ultimately leads to poor T cell activation and diminished anti-tumor effector function.

Vascular Endothelial Growth Factor (VEGF).

Vascular endothelial growth factor (VEGF) is produced by many solid tumors, and is known to prevent the maturation of myeloid cells and DCs [79]. It is also implicated in the

recruitment of MDSCs and macrophages to the tumor [79]. In 2003, Ohm *et al.* showed that pathophysiological concentrations of VEGF attenuate T cell development from the early hematopoietic progenitors [80].

Arginase I and Nitric Oxide Synthase.

T cells need the amino acid L-arginine for the sustained expression of TCR and effective anti-tumor function. Both arginase I and NOS enzymes digest L-arginine, reducing its availability, and could generate the analogues of L-arginine that suppress T cell function [43]. MDSCs are the major producers of arginase I and NOS at the tumor site [81].

Indoleamine 2,3-Dioxygenase (IDO).

IDO is another potent immune-suppressive enzyme that is highly expressed by tumor and the cells of stroma [43,82]. IDO catalyzes the conversion of tryptophan, an essential amino acid for immune cells, to N-formyl-kynurenine in the tumor micro-environment. The resultant depletion of tryptophan and formation of toxic by-products following this metabolic process suppress T cell activation, induce apoptosis in T cells, and favor CD4+ T cell differentiation towards Tregs [83].

Transforming Growth Factor-β (TGF-β).

TGF-β is produced in large quantities mainly by tumor cells and Tregs, and exerts multitude of inhibitory effects on immunity [43,84,85]. It can directly suppress T cell activation, proliferation as well as the cytotoxic function [84–87]. TGF-β also suppresses antigen presentation by DCs by preventing their maturation and reducing MHC II levels [88,89]. Further, TGF-β can recruit more Tregs and MDSCs to the tumor site, and promote the survival and function of Tregs to propagate the immune-suppression [90].

Clinical Relevance of TGF-β in Cancer

TGF-β is produced in very high quantities by most of the advanced, solid tumors including breast, prostate, colon, esophagus, lung, liver, stomach, osteosarcoma, pancreas as well as melanomas and hematological malignancies [91,92]. In addition, higher levels of

TGF-β have been found in the patients with metastatic lesions compared to the ones with only primary tumors [93,94]. Importantly, increased amounts of TGF-β in the tumor lesions were positively correlated with disease progression, metastasis and mortality in colon and breast cancer and with poor prognosis in lung cancer [95–97]. In pancreatic cancer, higher expression of TGF-β in circulating tumor cells (CTC) was linked to worse duration free and overall survival [98]. Thus, TGF-β is highly prevalent cytokine in the cancer patients and appears to play a crucial role in dictating their clinical outcome.

Introduction to TGF-β

The name of the superfamily Transforming Growth Factors (TGF) perhaps stems from the fact that these pleiotropic molecules induced a reversible, 'transformed' phenotype and caused anchorage independent growth in certain non-cancerous cells [99]. TGF superfamily consists of at least 35 members that include TGF-βs, bone morphogenic proteins (BMPs), activins, inhibins, and growth differentiation factors (GDFs). TGF-βs are multi-faceted cytokines implicated in a host of physiological processes including but not limited to development, cell differentiation, tumorigenesis, immune-surveillance and tolerance, angiogenesis, wound healing, chemotaxis [84,85,90,100]. There are three structurally related isoforms of TGF-β expressed in mammals (TGF-β1, TGF-β2, and TGF-β3). These isoforms of TGF-β share about 70-80% homology in their amino acid sequences and are transcribed from the separate genes [101]. The genes for TGF-β are located in the chromosome 19 (19q13.1, for TGF-β1), the chromosome 1 (1q41, for TGF-β2), and a the chromosome 14 (14q24, for TGF-β3) [101].

TGF-β1 (referred to as TGF-β from here on), the best characterized isoform, is predominantly expressed in the immune cells. It is originally synthesized as 'pre-pro-TGF-β' form which contains signaling peptide on amino terminus followed by latency associated peptide (LAP) and the mature TGF-β sequence on the carboxy terminus. The signaling peptide from pre-pro-TGF-β is removed and two pre-pro-TGF-β molecules are dimerized by

formation of di-sulfide bonds at their cysteine residues. This dimer, where each monomer consists of LAP at amino terminal and mature TGF-β at carboxy terminal, is called ‘pro-TGF-β’. The enzyme Furin cleaves this pro-TGF-β and separates the LAP dimer from the mature-TGF-β dimer. These dimers remain non-covalently attached to each other after Furin-induced cleavage. This form of TGF-β, comprising a homodimer of 12.5 kDa active TGF-β peptide non-covalently linked to 25 kDa of LAP, is known as ‘latent TGF-β’ or ‘small latent complex (SLC)’. In this form, LAP keeps TGF-β inactive by preventing it from binding to its receptors.

Since TGF-β is generally secreted in its inactive SLC form and hence, this latent TGF-β needs to be activated by unmasking the active TGF-β moiety from LAP and allowing its binding to the TGF-β receptors. Stimuli such as heat, acidic pH, stress or molecules such as reactive oxygen species, certain matrix metallo-proteases (MMP), thrombospondin-1 and integrins are shown to activate the latent TGF-β [85,102–105]. This process of TGF-β activation serves as an important regulatory mechanism to control its activity depending on the changes in temperature or biochemical and spatial environments.

There are also mechanisms by which the latent TGF-β could be linked to other molecules via cysteine 33 residue on the LAP before its production or secretion. In some cells, latent TGF-β binds to latent TGF-β binding protein (LTBP) in the endoplasmic reticulum and the resulting tri-molecules are termed as ‘large latent complex (LLC)’ [101,106,107]. After secretion of LLC, LTBP can associate with the extra-cellular matrix (ECM) components such as collagen and fibronectin and thus could serve as a means for the deposition of latent TGF-β in the ECM. LAP has also been shown to associate with transmembrane domain containing Glycoprotein A Repetition Predominant (GARP), which is expressed in many cell types such as Tregs, hepatic stellate cells, megakaryocytes and some cancer cells [108]. GARP has been found to link with both pro- and latent TGF-β, indicating that GARP binds to LAP at the early stages of TGF-β production [108]. In Tregs, GARP

anchors latent TGF-β on to the cell-membrane and presents it to the other cells in a contact-dependent manner [108]. It is believed that activation of TGF-β in the GARP-latent TGF-β complex occurs near the surface of Tregs [108]. Based on this mechanism, Cuende et al. have shown that the blockade of GARP using monoclonal antibodies abrogated human Tregs mediated immune-suppression in NSG mice *in vivo* [109].

TGF-β Signaling

TGF-β signaling is transduced via a heterodimeric TGF-β receptor complex anchored on the cell membrane. This complex consists of two serine/threonine kinase receptors: activin like kinase (ALK) or TGF-β receptor I (TGFβRI), and TGF-β receptor II (TGFβRII). With identification of five isoforms of TGFβRI and seven isoforms of TGFβRII, TGF-β receptors are expressed ubiquitously in the body and hence essentially every cell in the body can respond to TGF-β. In T cells, expression of TGFβRs increases upon activation. In its activated form, TGF-β binds to the constitutively active TGFβRII extra-cellularly and brings it into the proximity of TGFβRI, resulting in its phosphorylation. Phosphorylated TGFβRI then causes phosphorylation of the receptor-associated Smads 2 and 3. This is followed by the association of Smads 2/3 with the co-Smad 4, and the translocation of Smad 2/3/4 complex into the nucleus. In nucleus the complex can bind to the Smad Binding Element (SBE) in the DNA resulting in either expression or repression of the Smad responsive genes. Smad complex is also capable of partnering with other transcriptional co-activators and repressors, and modulate the associated gene transcription events [110–112]. In addition to the receptor-associated and co-Smads, the role of inhibitory Smad-7 is also important in regulation of TGF-β signaling. Smad-7 inhibits TGF-β pathway inside the cell by competing with Smads 2 and 3 in binding to TGFβRI as well as causing the degradation of TGFβRI by Smurfs ubiquitin ligase in proteasomal and lysosomal dependent manner [110–112].

Although canonical Smad pathway is pivotal in mediating a spectrum of TGF-β's crucial effects in the cells, TGF-β also activates other non-canonical signaling pathways.

PI3K-Akt, Ras-Erk, TAK-MKK4-JNK, TAK-MKK3/6-p38 and Rho-Rac-cdc42-MAPK signaling pathways are found to be activated by TGF-β [112]. Although these non-Smad pathways downstream of TGF-β are not well-defined, they are indicative of a wide and complex signaling network initiated by this pleiotropic cytokine in the cells.

TGF-β's Suppressive Effects on the Immune System

TGF-β's role as a prominent immune-suppressor has been supported by extensive investigations over many years. Perhaps the most compelling evidence of its inhibitory effects on essentially every component of the immune system came in early 1990s when Shull *et al.* and Kulkarni *et al.* generated TGF-β1-deficient mice models by disrupting ORF of mouse TGF-β1 gene [113,114]. In both of these seminal studies, the mice showed severe, multifocal inflammatory disease and died of organ failure within 3 to 4 weeks after birth [113,114]. In the latter study in particular, TGF-β1-null mice displayed extreme infiltration of lymphocytes and macrophages in numerous organs with many lesions resembling auto-immune diseases, graft vs host disease or viral diseases [113]. Importantly, subsequent studies evaluated the involvement of T cell mediated immune responses in the phenotype of TGF-β1 deficient mice by specific abrogation of TGF-β signaling in T cells. Richard Flavell's group generated mice expressing dominant negative TGFβRII (dnTGFβRII; which contains kinase dead intra-cellular domain) specifically in T cells, and reported the auto-immunity, excessive inflammation infiltration in multiple organs and death of these mice at early age [115]. Along the similar lines, Ronald Gress' group studied the phenotype of a mouse model with dnTGFβRII expressed under T-cell specific CD2 promoter [116]. These mice developed a lymphoproliferative disorder characterized by lymphoid organ expansion which was attributed to hyperproliferation and lack of apoptosis in CD8+ T cells in the periphery. Supporting these observations, T cell-specific over-expression of Smad 7, inhibitor of TGF-β signaling, resulted in heightened T helper cell type 1 (Th1) and Th2 cytokine production in response to antigen-induced airway inflammation [117]. In human patients with

Sezary syndrome, a type of T-cell lymphoma, the surface expression of TGFβRII is remarkably reduced, resulting in impaired TGF-β mediated growth inhibition of these cells and development of the disease [118]. Collectively, these findings establish that TGF-β has pronounced regulatory effects on the homeostasis and peripheral tolerance of CD4+ and CD8+ T cells, and suggested that the severe auto-immune phenotype observed in TGF-β1 deficient mice is largely mediated by lack of TGF-β's inhibition on T cells.

A study by Won *et al.* determined the relevance of TGF-β in tumor immunology by inhibiting TGF-β signaling in the immune-microenvironment of the thymoma EL-4, which produces large amounts of TGF-β [119]. They showed that transduction of an extra-cellular domain of TGFβRII (soluble TGFβRII), which sequesters TGF-β and thus reduces its signaling in the environment, into EL-4 cells reduced their tumorigenicity *in vivo*. More importantly, the lymphocytes isolated from mice bearing soluble TGFβRII-EL4 tumors had 3-5 folds higher cytotoxic capacity *ex vivo* compared to EL4 bearing mice [119]. It has also been shown that in mice expressing dn-TβRII specifically in T cells, engraftments of murine B16-F10 melanoma and EL-4 thymoma is delayed in a T cell-dependent manner [120]. TGF-β driven suppression of the function of antigen or tumor-specific T cells was also confirmed by Zhang *et al.* [121]. In this study, Pmel-1 CD8+ T cells (reactive against gp-100) and Trp-1 CD4+ T cells (reactive against tyrosinase related protein 1) over-expressing dn-TβRII showed dramatically high anti-tumor immunity against B16 melanoma [121]. In a nutshell, TGF-β's inhibitory effects on T cells and the recovery of their anti-tumor function upon blocking this inhibition have been shown in mouse experimental systems by numerous studies.

TGF-β's Inhibitory Effects on T cells

TGF-β affects multitude of processes in the T cells including development, activation/differentiation, proliferation, acquisition of effector/inflammatory molecules, and survival/apoptosis [84,85,90,102]. It was first shown to inhibit T cells proliferation by

blocking production of IL-2, which enhances proliferation of T cells [99]. The other studies have shown that TGF-β down-regulates of c-Myc, cyclin D and cyclin E in T cell lines, suggesting role of these mechanisms in TGF-β induced inhibition in proliferation [122–124]. It is interesting to note that in CD4+ T cells, but not in CD8+ T cells, TGF-β's effects on proliferation are dependent on Smad 3 [125]. TGF-β prevents the differentiation of naïve CD4+ T cells into Th1 and Th2 cells and promotes the central memory phenotype [126–128]. This phenomenon is linked to TGF-β's effects on the expression of Gata-3, IL-12 receptor β chain in CD4+ T cells [102,129–131]. However, consistent with its role as a suppressor, TGF-β promotes the differentiation of CD25- CD4+ T cells into CD25+ CD4+ Tregs by up-regulating the expression of the forkhead family transcription factor Foxp3 [132]. Ranges *et al.* examined the effect of TGF-β on CD8+ T cell differentiation where addition of TGF-β at the beginning of the mixed lymphocyte culture (MLC) *in vitro* led to a reduced generation of CTL from naïve CD8+ T cells [133]. Interestingly, the CTL differentiation was not as much affected when TGF-β was added in the MLC at later time points [133]. TGF-β can also directly inhibit TCR signaling by inactivating key TCR signaling molecule IL2-inducible T cell kinase (Itk) as well as preventing the phosphorylation of ERK [134]. In contrast to these effects, various studies have reported positive impact of TGF-β on T cell survival. In TGF-β1-null mice, thymic and peripheral T cells undergo spontaneous apoptosis, which is greatly enhanced upon anti-CD3 antibody injection [135]. TGF-β inhibits T cell apoptosis and increases expansion in the presence of CD28 stimulation [136,137]. It also promotes survival of effector and memory T cells [137–141]. Moreover, TGF-β synergizes with IL-2 to repress activation induced cell death (AICD) in Th1 and Th2 CD4+ T cells [138,141–143]. The underlying mechanism, although not clear, has been associated with the reduction in Fas ligand induced apoptosis in T cells [144,145].

In regard to the function of CD8+ T cells in the immune system, the most important effect of TGF-β is the attenuation of their cytolytic capacity observed also in many of the

above discussed studies. This impairment in cytotoxicity results from TGF-β's potent suppressive effects on the expression of crucial effector molecules. In earlier studies, TGF-β was reported to down-regulate the expression of pore forming protein (PFP), which is required for T cell killing, and IFN-γ production in human T cells [146,147]. The results from former study suggested that TGF-β's effect on PFP was independent of its effect on proliferation in T cells. In 2005, an important study by Thomas and Massague showed that TGF-β down-regulated the expression of key effector molecules perforin, granzyme A, granzyme B, IFN-γ at the mRNA level in purified mouse CD8+ T cells [148], the effects which were again found independent of growth-inhibition. Importantly, TGF-β induced suppression in the gene-expression of granzyme B and IFN-γ, but not others, was found to be dependent on Smad pathway [148]. All of these molecular changes were also confirmed in an *in vivo* setting and associated with diminished cytotoxicity of CTL [148]. Work from Ahmadzadeh and Rosenberg brought these discoveries into the context of tumor immunology where they assessed the effects of exogenous TGF-β on tumor-specific memory T cells isolated from the melanoma patients, who were previously vaccinated with modified melanoma antigen g209-2M [149]. Addition of TGF-β during the re-stimulation of memory T cells with g209-2M antigen led to profound reduction in the production of inflammatory cytokines IFN-γ, TNF-α and GM-CSF. The authors obtained identical results in the TIL isolated from the same patients [149]. In addition to the effects on individual cytokine production, we and others have also demonstrated that TGF-β highly suppresses poly-cytokine (more than one cytokine) production in CD8+ T cells [150]. This may be an important mechanism of TGF-β suppression as polyfunctionality has been positively associated with higher effector function in T cells [151–154].

Despite its potent negative effects on activation, differentiation or cytotoxic function, TGF-β does not appear to inhibit the phenotypic activation of CD8+ T cells. Studies have shown that TGF-β signaling in T cells either increase or does not alter the surface expression

of CD44, which is a marker of antigen-experienced T cells [126,148,155]. This counter-intuitive paradigm is not completely understood yet. In numerous studies addressing TGF-β responses in T cells, it is also consistent that naïve T cells are far more susceptible to TGF-β's effects compared to activated or differentiated ones [103,133,139]. In fact, Filippi *et al.* reported that TGF-β, while suppressing naïve T cell functions, enhanced the functions of activated T cells in their system [156]. These differential effects of TGF-β are attributed to the differences in the expression levels of TGF-β receptors in different state of T cell activation as well as the presence of other cytokine or co-stimulatory factors during TGF-β signaling.

TGF-β's Positive Effects on T cells

It is important to note that given its multi-faceted nature, TGF-β's effects on many cellular processes could also be opposite to those described in the previous section [103,157]. For instance, TGF-β signaling enhances the antigen processing and co-stimulatory function of Langerhans cells, a type of dendritic cells present in the epithelium [88,158,159]. In CD8+ T cells, TGF-β was reported to act as a co-stimulator in certain culture conditions and induce CD44 expression and proliferation [155]. Gray *et al.* demonstrated enhanced production of TNF-α upon TGF-β treatment in human lymphocytes [160]. Moreover, there is evidence that TGF-β augments apoptosis and cell death in the activated T cells [161,162]. What triggers these completely opposite functions of TGF-β is not clear yet. Despite these discrepancies, TGF-β mediated impairment in the cytotoxic potential of CD8+ T cells is consistent throughout the literature.

Therapeutic Strategies to Counter TGF-β

Based on our current knowledge and the findings discussed above, it is clear that inhibiting suppressive effects of TGF-β on CD8+ T cells is an effective approach to rescue and enhance their cytotoxic functions against cancer. Along these lines, numerous therapeutic strategies targeting TGF-β signaling have been investigated in pre-clinical animal models.

Some of the earlier strategies included anti-TGF-β monoclonal antibodies, soluble TGF-β receptors, small molecule TGFβR kinase inhibitors, oligo-nucleotides silencing the expression of TGF-β1/2, and Smad-interacting peptide aptamers [86,163–170]. Although many of the above studies reported desired results of augmented anti-tumor immunity, only two approaches advanced into the clinics. AP12009, oligonucleotide targeting TGF-β2 mRNA was tested intra-tumorally in high-grade glioma patients [171], and Belagenpumatucel-L, an allogeneic tumor vaccine expressing TGF-β2 vector was tested in patients with advanced NSCLC [172]. Although both the agents were well-tolerated in patients in phase I and II clinical trials, the treatments failed to show any efficacy benefit at their primary end-points [171,172]. Galunisertib (LY2157299), a small molecule inhibitor of TGFβRI kinase, has shown improved over-all survival in advanced hepatocellular carcinoma patients in phase II studies and is currently being investigated further [173]. Recently, novel bi-specific antibody-ligand traps (Y traps), comprising a fusion of CTLA-4 or PD-1 antibody and extra-cellular domain of TGFβRII, have been invented to simultaneously target check-point receptors and TGF-β in the tumor micro-environment. In pre-clinical studies, both the antibody-ligand traps (i.e. blocking PD-L1 or CTLA-4 in addition to TGF-β) demonstrated superior anti-tumor responses compared to anti-PD1 or CTLA-4 monotherapies with evidence of augmented adaptive immune responses [174–176]. MS827, a bifunctional PD-L1 and TGF-β blocking Y-trap, has already entered the phase I clinical trial and has shown encouraging results in patients with advanced solid tumors [177]. The conclusive clinical benefits of TGF-β blockade in patients with advanced solid tumors are yet to be seen.

Although some of the above-mentioned studies have shown desired results in the clinic, approach of TGF-β signaling blockade to improve immunotherapies faces crucial challenges. The neutralization of TGF-β in tumor microenvironment or periphery is not perceived as a viable therapeutic strategy since TGF-β is produced abundantly by tumor and non-tumor cells [86,178]. In such a scenario, sheer prevalence of TGF-β may outcompete any

intervention to neutralize this cytokine. On the other hand, systematic inhibition of TGF-β signaling by using the small kinase inhibitors or other means must be considered with caution from the safety standpoint. As stated before, the manifestation of lethal auto-immune, inflammatory phenotype in TGF-β KO mice raises serious concerns of auto-immune events in the patients receiving these treatments [86]. Similar to the observations in mouse models, these adverse immune effects may not be evident initially in humans, but may surface after prolonged or chronic exposure to the treatments inhibiting TGF-β pathway. This caution is even heightened in the case of bi-functional PD-L1/CTLA-4-TGF-β traps or the proposed strategies combining anti-TGF-β and anti-PD-1/CTLA-4 mAb therapies. One major clinical problem with the PD-1 and CTLA-4 check-point blockade therapies is that majority of the treated patients show occurrence of a distinct toxicity, known as immune-related adverse events (irAES), with a possibility of becoming lethal in its severe form [179]. Targeting a prominent immune-suppressor like TGF-β in conjunction with check-point blockade could worsen the incidences and intensities of irAES. Furthermore, down-modulating TGF-β signaling ubiquitously poses a risk of preventing its plethora of essential biological functions such as cell development, differentiation, angiogenesis, tissue repair, fibrosis, chemotaxis and some of the immune-stimulatory effects. Hence, in lieu of reducing the amount of TGF-β or signaling systematically, novel pharmacological approaches that make only CD8+ T cells resistant to TGF-β are warranted to improve the efficacy of cancer immunotherapies. In order to do that, we seek to identify novel regulators of TGF-β signaling in $CD8^+$ T cells in this dissertation research.

NKG2D Receptor in the Immune System

Natural killer group 2 member D (NKG2D) is a transmembrane, stimulatory receptor encoded by the killer cell lectin-like receptor subfamily K member 1 (Klrk1) gene [180–182]. NKG2D is expressed on all murine and human NK cells. It is also constitutively expressed on human CD8+ T cells, whereas its expression on mouse CD8+ T cells only occurs after the

activation [183]. In addition to NK and CD8+ T cells, NKG2D is also present on natural killer T (NKT) cells, γδ T cells, activated mouse macrophages and a subgroup of CD4+ T cells [184].

NKG2D receptor was originally discovered in NK cells, wherein NKG2D signaling was shown to be sufficient in mediating direct killing of the target cells [184]. In CD8+ T cells, however, NKG2D requires simultaneous activation of the T-cell receptor (TCR) to be functional [183–185]. Thus, it acts as a co-stimulatory receptor enhancing the TCR activation and function in CD8+ T cells.

NKG2D-DAP10 Signaling and its Co-Stimulatory Function in CD8+ T cells

NKG2D consists of a short intracellular domain that is devoid of any signaling motifs. In CD8+ T cells, NKG2D signals through the adaptor protein DNAX-activating protein 10 (DAP10), whereas in NK cells, it can pair with either DAP10 or its homolog DAP12 [182,183,186,187]. The NKG2D-DAP10 complex is expressed as a hexamer consisting of a homodimer of two NKG2D molecules, each bound to two DAP10 molecules [188]. DAP10 consists of the tyrosine-isoleucine-asparagine-methionine (YINM) signaling motif. Upon NKG2D activation, YINM is phosphorylated by Src kinase on its tyrosine motif, which leads to the recruitment of the adaptor protein-Grb2 and p85 subunit of PI3K to the asparagine and methionine residues, respectively [189,190]. This in turn results in the activation of a wide array of well-characterized Grb2 → Vav → SOS → Ras and p85 → PDK1 → Akt → Rac → MEK 1/2 signaling pathways (Figure 1) [191–193]. In addition to these canonical pathways, NKG2D-DAP10 also activates JNK2 pathway downstream of Grb2 [194], which was shown to mediate repression of transcription factor T-bet, and memory formation in CD8+ T cells [195]. Collectively, activation of these signaling cascades in CD8+ T cells result in AP-1, NFAT and NF-κB nuclear translocation and subsequent enhancement in cell survival, proliferation, expression of effector molecules and cytokines, as well as the release of cytolytic granules in CD8+ T cells (Figure 1) [196,197].

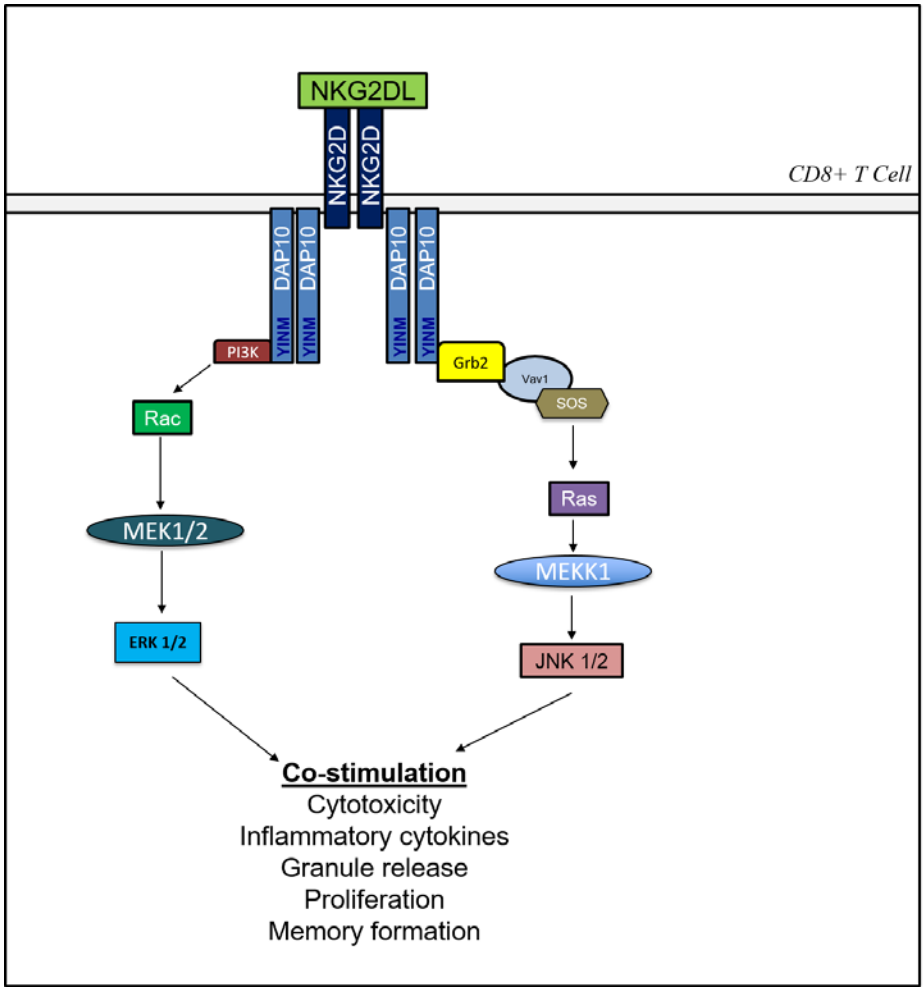


Figure 1. NKG2D Signaling and its Co-Stimulatory Effects in CD8+ T Cells.

Non-Canonical Functions of NKG2D in CD8+ T cells

In addition to its co-stimulatory function, many studies have reported NKG2D's non-canonical functions in CD8+ T cells [182]. For instance, it has been shown that memory CD8+ T cells can be activated only by cytokines under specific conditions, which transform them into NK-like killer cells. In such states, CD8+ T cells can kill the target cells in an NKG2D-dependent manner without the need of TCR engagement [198,199]. This TCR-independent and NKG2D-dependent killing mediated by CD8+ T cells has been linked to the genesis of several autoimmune disorders, highlighting the importance of NKG2D receptor in pathophysiological conditions. The priming of CD8+ T-cell in the absence of CD4+ T-cell help leads to the formation of so-called 'helpless' or 'unhelped' CD8+ T cells which are defective in their effector functions and memory formation [200–202]. Study from our

laboratory has shown that NKG2D signaling on CD8+ T cells can substitute CD4+ T cell 'help' in the memory formation [195]. We demonstrated that overexpressing NKG2D ligands during the immunization of mice depleted of CD4+ T cells completely restored the quality and quantity of the CD8+ T cell memory recall response. Using NKG2D-deficient CD8+ T cells and NK cell depletion in mice, we also showed that this rescue was dependent on NKG2D expression on CD8+ T cells and was NK cells-independent. Interestingly, despite rescuing the memory formation, NKG2D signaling was unable to restore in the initial CD8+ T cell effector response in this study.

Few studies have also investigated the contribution of NKG2D in CD8+ T cell memory formation in the intact system (i.e. without abrogating CD4+ T cell help). Wensveen *et al.* showed that NKG2D augmented IL-15-mediated PI3K activity in activated CD8+ T cells and enhances their memory commitment [203]. In this study, the authors reported that memory formation in mice adoptively transferred with NKG2D deficient OT-I cells was impaired following immunization against LCMV expressing OVA. In contrast, a study by Andre *et al.* indicated that NKG2D only improves the effector responses of memory T cells during recall, and does not play substantial role in the formation and expansion of memory CD8+ T cells [204]. Further investigation is necessary to completely define the role of NKG2D in CD8+ T cell memory formation.

Taken together, these findings point out that the biological role of NKG2D on CD8+ T cells extends beyond it's known canonical functions of co-stimulation and enhancement of target killing.

Ligands of NKG2D Receptor

The ligands for NKG2D receptor are major histocompatibility complex (MHC) class-I-related chain (MIC) A/B and UL16 binding proteins 1–6 in humans, and Raε1, MULT1, H60 a-c families in mouse [205–207]. As expected for a stress-receptor signaling, the surface expression of NKG2D ligands is highly regulated in the body. NKG2D ligands are either not

expressed or expressed at negligible levels on the healthy tissues. They are transcriptionally up-regulated as when the cells undergo any stress-related stimuli such as infection, transformation or exposure to DNA-damaging agents [206,207]. TLR signaling, cell proliferation or exposure to certain cytokines have also been shown to induce the expression of ligands for NKG2D [185,205,208]. Cancer cells often consistently undergo multiple of the above-mentioned stress-related events. Indeed, various tumors have been reported to have abundant surface expression of NKG2D ligands [209], which points towards the important role of NKG2D receptor in cancer immunology.

NKG2D Receptor in Cancer Immunology

Studies from Cerwenka *et al.* and Diefenbach *et al.* were perhaps the first ones to pinpoint the role of NKG2D in cancer immunity wherein the mice were able to reject the tumor cells over-expressing NKG2D ligands, and not the unmanipulated counterparts [185,207]. The authors showed that the tumor rejection was mediated by NK cells and CD8+ T cells in these studies. In 2005, it was reported that in vivo NKG2D neutralization in BALB/c and C57BL/6 mice led to higher incidence of methylcholanthrene-induced sarcoma [210]. Following that, experiments conducted on NKG2D-deficient mice crossed with TRAMP mice (SV40 large T-driven prostate cancer) by Guerra *et al.* demonstrated that the absence of NKG2D in TRAMP mice leads to higher incidence of prostate adenocarcinomas [211]. These observations demonstrated that the tumor onset and progression were accelerated in the absence of NKG2D or NKG2D ligands. A study by Liu *et al.* to address the role of NKG2D in cancer led to interesting outcomes. Here, the authors used the transgenic mice that over-express native human MICB or membrane-restricted mutant MICB (which will not shed and activate NKG2D) under the control of a prostate tissue-specific promoter [212]. These mice were crossed to TRAMP mice and the prostate tumor onset and growth was measured. Paradoxically, TRAMP/MICB mice showed significantly more incidence and metastasis of prostate tumors, whereas TRAMP/membrane-restricted-MICB mice remained

tumor-free for a long period [212]. The authors attributed this dichotomy to the elevated levels of soluble MICB and the resultant depletion of NK cells in TRAMP/MICB mice. Earlier mentioned study by Andre *et al.* also investigated the role of NKG2D in driving memory CD8+ T cells' anti-tumor function by using transgenic mice that constitutively express MICA and as a result show profound dysfunction in NKG2D signaling [204]. These mice were adoptively transferred with OT-I CD8+ T cells with simultaneous immunization of OVA peptide, and about 40 days later EG.7 tumors (EL-4 thymoma expressing OVA) were implanted in them. While in control mice EG.7 tumors were unable to grow, MICA transgenic mice failed to control the growth of EG.7 tumors [204]. Taken together, these findings outline the important role of NKG2D receptor in the tumor immunology and provide a rationale to further dissect the role of NKG2D in governing the biology of CD8+ T cells in cancer and tumor micro-environments.

Relationship Between TGF-β and NKG2D Receptor

Given the potent inhibitory and stimulatory effects on NK and CD8+ T cells exerted by TGF-β and NKG2D signaling respectively, it is intuitive to imagine a cross-talk between these two pathways for the regulation of the immune system. In fact, numerous studies have shown the negative effects of TGF-β on the expression of NKG2D receptor. Chul Lee *et al.* reported that in lung and colorectal cancer patients TGF-β specifically down-regulated expression of NKG2D receptor, but not other stimulatory receptors such as NKG2A, CD16, 2B4, on NK cells [213]. In a study by Crane *et al.*, sera from glioma patients led to the down-regulation of NKG2D on NK and CD8+ T cells which could be reversed by blocking TGF-β [214]. Notably, this change in the NKG2D expression was at the transcriptional level. Similar effects of TGF-β on NK cells from the patients with persistent hepatitis B virus (HBV) infections was discovered by Sun and colleagues [215]. Castriconi *et al.* demonstrated that *in vitro* TGF-β treatment of freshly isolated human NK cells results in reduced NKG2D surface expression [216]. In 2004, it was shown that TGF-β treatment of human PBLs decreases

NKG2D levels on the surface of CD8+ T cells [217]. Interesting study by Pheel Park *et al.* provided some mechanistic insight into this phenomenon where the authors showed that TGF-β inhibits NKG2D surface expression in NK cells by significantly repressing DAP10, but not NKG2D, expression at the transcriptional level [218]. In many of the above discussed studies, TGF-β mediated down-modulation of NKG2D was also accompanied by diminished NK cell functions, indicating TGF-β induced, NKG2D dependent suppressive mechanisms. Importantly, work from Friese *et al.* determined that NK cells isolated from mice bearing TGF-β-silenced LNT-229 glioma cells had significantly more lytic capacity than the control LNT-229 cells [217]. Overall, these studies strongly suggest that one of the mechanisms by which TGF-β suppresses the functions of NK cells or CD8+ T cells is through down-regulation of NKG2D-DAP10 receptor complex. Despite a widely reported literature on TGF-β mediated effects on NKG2D, the impact of NKG2D signaling on TGF-β signaling and its associated suppressive effects is not known yet.

Hypothesis I

Based on the opposing roles of NKG2D and TGF-β signaling reported in the literature, we propose the following central hypothesis (Figure 2) for this dissertation project. *NKG2D receptor signaling directly opposes the inhibitory effects of TGF-β on CD8+ T cells and makes them more resistant to the TGF-β mediated immune-suppression.*

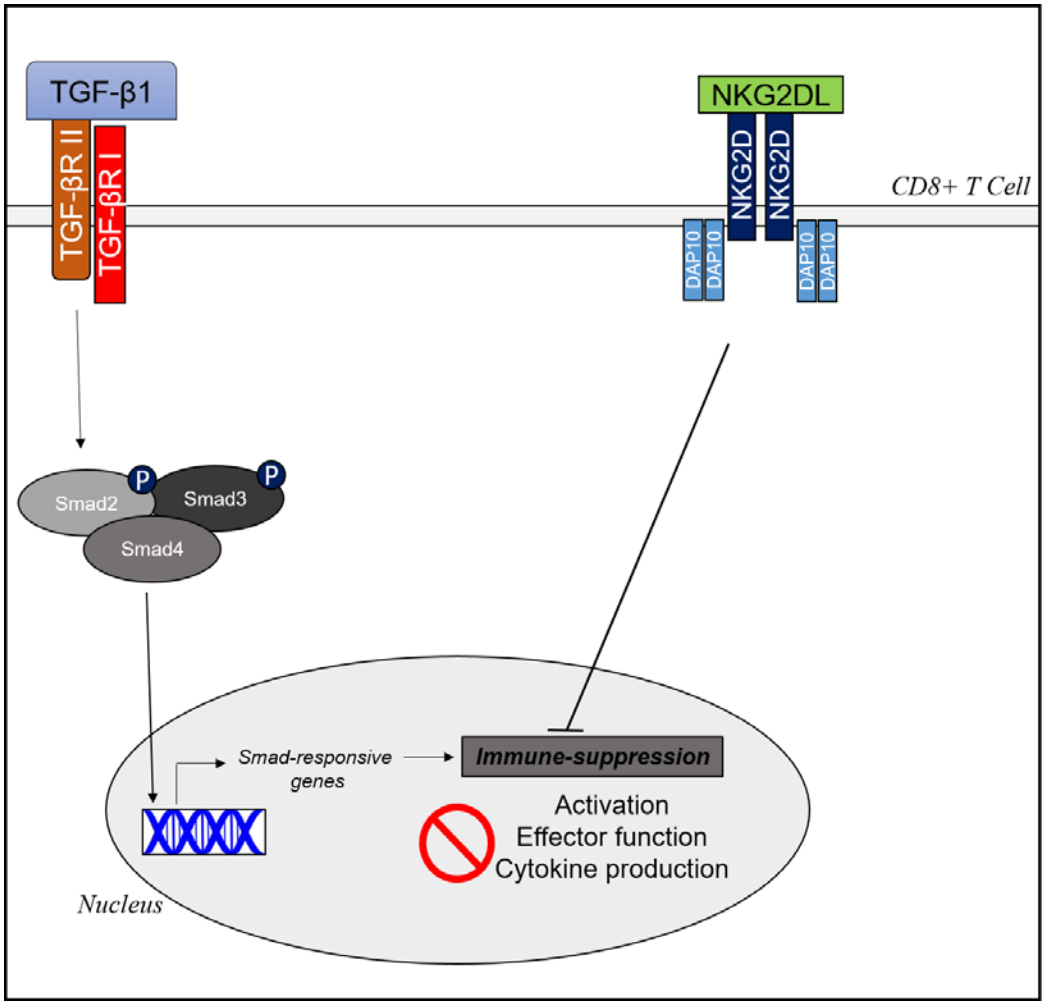


Figure 2. Hypothesis I.

Cytokines IL-2 and IL-15 in TGF-β Resistance

The common gamma chain (γc) cytokines IL-2 and IL-15 enhance the activation, proliferation and effector function of CD8+ T cells [219], the effects that are contrary to TGF-β induced suppressive effects. Reduction in the IL-2 production and its associated proliferation were among the first suppressive effects of TGF-β described on T cells [99]. Later, it was also reported that TGF-β down-regulates IL-2 mRNA expression in a Smad 3 dependent manner in T cells [220]. When naïve mouse CD4+ T cells were activated under Th1 culture conditions *in vitro*, TGF-β blocked the up-regulation of CD122, which is an important receptor for IL-2 and IL-15 signaling [221]. Interesting study by Benahmed *et al.* evaluated the interplay between IL-15 and TGF-β in the intestinal lymphocytes of active celiac disease and reported that IL-15 counteracts TGF-β-Smad 3 signaling possibly by up-

regulating c-jun-N-terminal kinase [222]. IL-15 and TGF-β were also shown to have opposite effects on the expansion of effector T cells following Listeria infection in mice [223]. These findings raise a possibility that IL-2 and/or IL-15 signaling could rescue CD8+ T cells from TGF-β's negative effects. Indeed, in previously stated study by Thomas and Massague, IL-2 could reverse TGF-β's effects on proliferation, expression of IFN-γ and granzyme B in T cells that were pre-treated with TGF-β [148]. Interestingly, IL-15 could only partly restore proliferation and had no effect on effector molecules expression in these experiments. Brownlie and colleagues studied the role of tyrosine phosphatase Ptpn22 in enhancing TGF-β sensitivity in CD8+ T cells [224]. In this study, IL-2 prevented TGF-β induced suppression of CD8+ T cells proliferation in a dose-dependent manner. Collectively, the γc cytokines IL-2 and IL-15 have the potential to override TGF-β mediated suppression in CD8+ T cells.

Interestingly, there is also a substantial literature showing a cross-talk between IL-15 and NKG2D-DAP10 pathways [198,199,225]. Wensveen *et al.* showed that NKG2D signaling increased IL-15-mediated PI3K activity in activated CD8+ T cells, and assisted in memory commitment [203]. In celiac disease, an autoimmune disease caused by gluten intolerance and characterized by the destruction of the small intestine, work by Bana Jabri and colleagues have shown that IL-15 upregulates NKG2D, ultimately allowing CD8+ T cells to kill in a TCR-independent manner through NKG2D mediated mechanisms [198,199]. The authors found that NK cells lacking NKG2D-DAP10 expression were not capable of responding to IL-15 stimulation. Further, the same study showed that IL-15-activated Jak3 is able to phosphorylate DAP10 and prime the NKG2D-DAP10 signaling pathway. Thus, IL-15 was able to transform effector T cells into NK-like 'lymphokine-activated killers' (LAK cells) both in vitro and in vivo in the celiac disease. Based on these findings, it has also been proposed that DAP10 couples with the IL-15 receptor in the cell membrane [226]. A recent study demonstrated that NKG2D activation leads to the re-organization of nanoclusters of IL-2 and IL-15 receptor subunits [227]. Nevertheless, the interplay between IL-2, IL-15 and

NKG2D receptors in the regulation of TGF-β resistance in CD8+ T cells has not been studied.

Hypothesis II

Based on the literature discussed above, it is possible that IL-2 and IL-15 could have redundant function shared with NKG2D in mediating TGF-β resistance in CD8+ T cells. We propose the following hypothesis:

Cytokines IL-2 and IL-15 can substitute NKG2D mediated TGF-β resistance in CD8+ T cells.

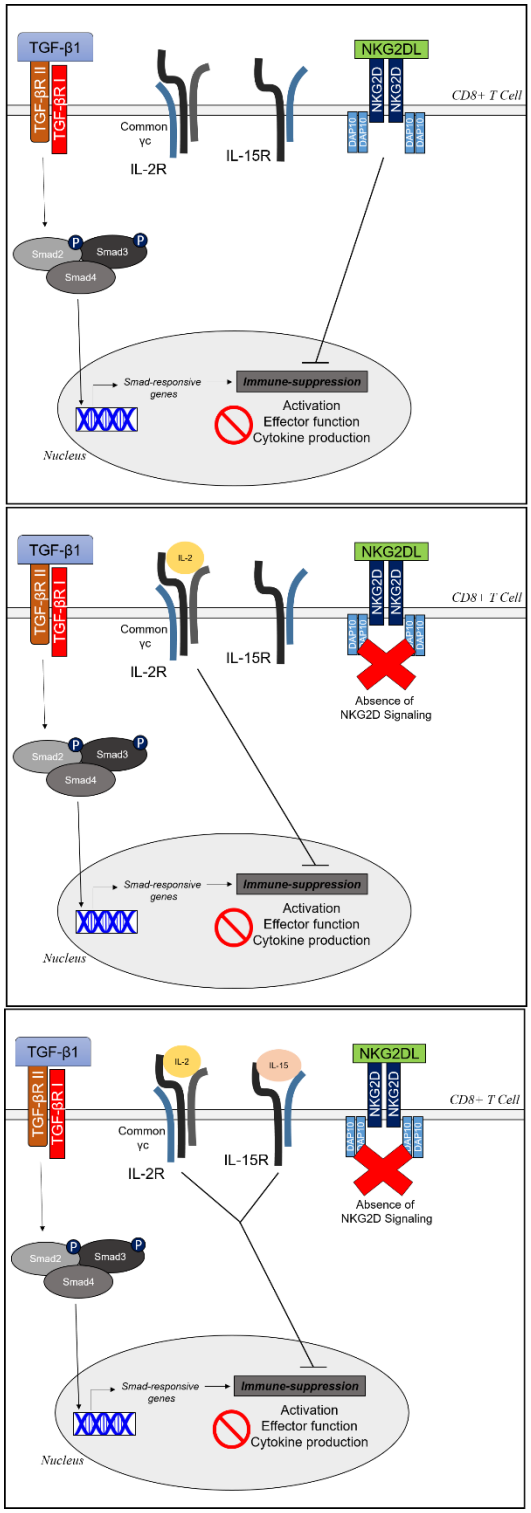


Figure 3. Hypothesis II.

Co-Stimulatory & Co-Inhibitory Receptor Signaling in TGF-β Resistance

Like NKG2D, there are number of other co-stimulatory and co-inhibitory receptors that modulate TCR signaling and play important role in regulating the biological processes in CD8+ T cells [25]. Majorly studied co-stimulatory receptors in CD8+ T cells include CD28

(prototype co-stimulatory receptor), ICOS, 4-1BB, OX40, and GITR which receive signals from their cognate ligands B7-1/2 (shared by CD28 and ICOS), 4-1BBL, OX40L, and GITRL present on APCs. As per their described co-stimulatory roles, these receptors enhance TCR signaling and positively regulates the activation, proliferation, effector function and survival in general with some receptor-specific differences [25]. Co-inhibitory receptors, perhaps more important in the current immunotherapy landscape, include PD-1, CTLA-4, TIM3, and TIGIT with their ligands being PD-L1, B7-H1/2 (CTLA-4), galectin 9 (TIM3), and CD155/CD112/CD113 (TIGIT) [25]. Upon binding to their ligands on APC or cancer cells, co-inhibitory receptors negatively impact the proliferation and effector function as well as causes apoptosis in CD8+ T cells [25]. The blockade of co-inhibitory (also known as 'check-point') signaling pathways PD-1 and CTLA-4 have led to a remarkable enhancement of CD8+ T cell effector function in cancer [228].

Inhibition of the co-inhibitory pathways PD-1 and CTLA-4 has been a highly successful strategy in boosting the cytotoxic capacity of CD8+ T cells against cancer cells in the tumor micro-environment. Therefore, it is plausible to imagine a relationship between these check-point pathways and immune-suppressive pathways such as TGF-β in CD8+ T cells. TGF-β increases the expression of PD-1 on antigen-specific T cells in Smad 3 dependent manner [229]. It was recently published that TGF-β is responsible for rendering cancer cells resistant to PD-1/PD-L1 blockade therapy by halting T cells' infiltration at the tumor site [230]. Work by Zheng *et al.* using CTLA-4 deficient mice showed that TGF-β requires CTLA-4 to drive the conversion of CD25- CD4+ T cells into Tregs by up-regulating Foxp3 [231]. CTLA-4, on the other hand, was shown to exert its inhibition on T cells independent of TGF-β [232]. Although these reports have increased our understanding of the interplay between check-point signaling and TGF-β, the effect of PD-1 and CTLA-4 blockades in antagonizing TGF-β's effects on CD8+ T cells is unknown.

Hypothesis III

We hypothesize that:

Blocking PD-1 or CTLA-4 check-point signaling on CD8+ T cells makes them more resistant to TGF-β induced immune-suppression.

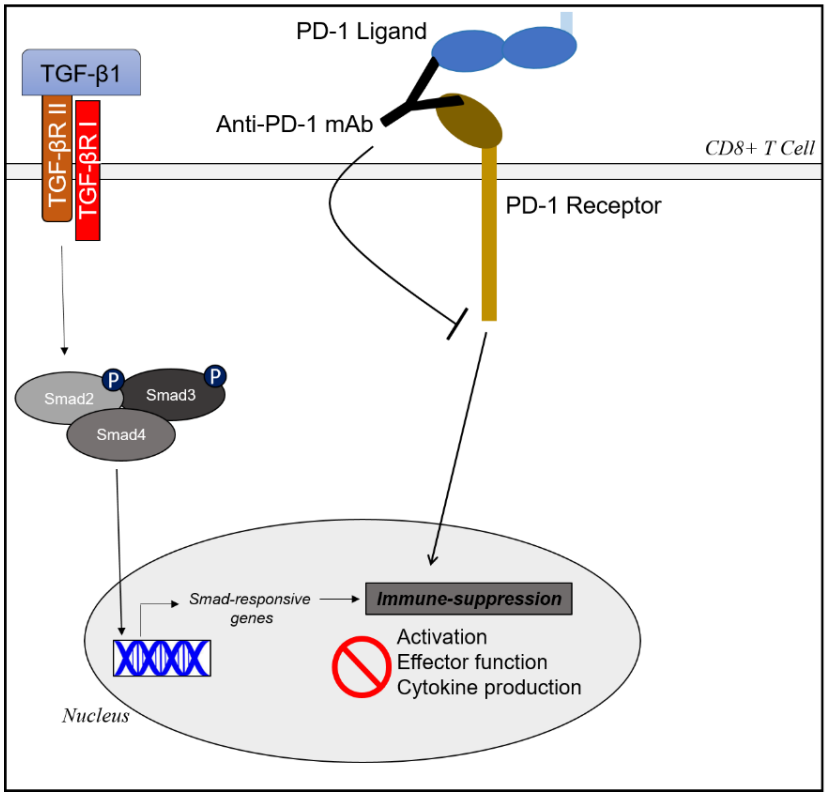


Figure 4. Hypothesis III-A.

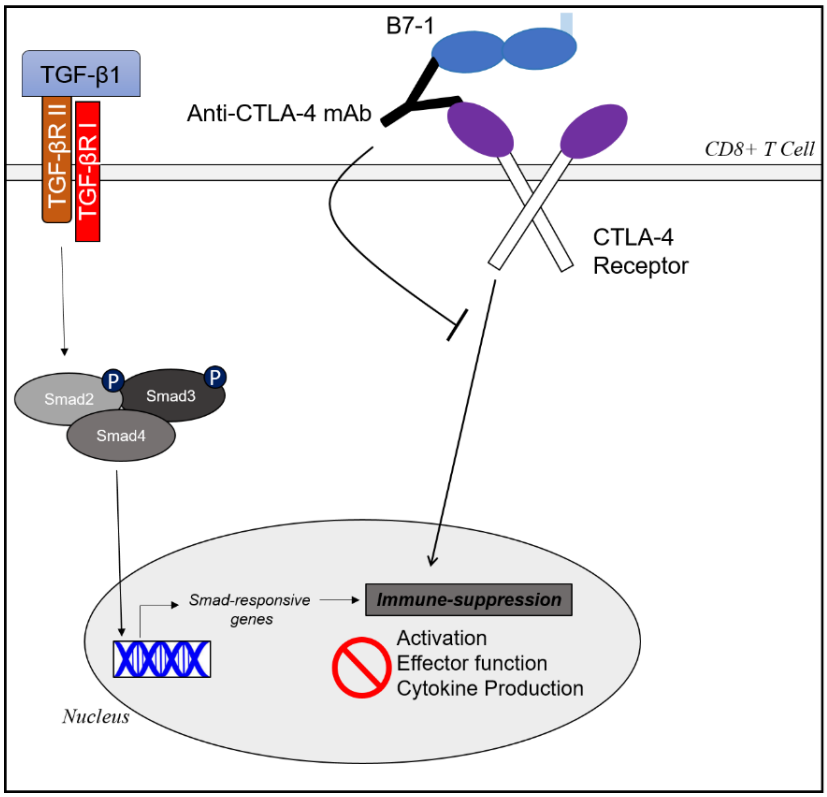


Figure 5. Hypothesis III-B.

In contrast to the co-inhibitory signaling, there is existing evidence that some of the co-stimulatory receptors can overcome the suppressive effects of TGF-β in T cells. June-Kim *et al.* demonstrated that 4-1BB co-stimulation in human cord blood CD8+ T cells reversed TGF-β induced Smad 2 phosphorylation and suppression in cytotoxic capacity and expression of granzyme B, IFN-γ [233]. In mouse system, it was shown that OX40 co-stimulation prevented TGF-β driven conversion of naïve CD25- CD4+ T cells into CD25+ Foxp3+ CD4+ Tregs by inhibiting Foxp3 mRNA expression [234]. In experiments performed on naïve human CD4+ and CD8+ T cells, Gunnlaugsdottir and colleagues showed that activating CD28 during anti-CD3 activation decreases TGF-β induced suppression on proliferation as well secretion of IL-2 and IFN-γ [136]. Loskog *et al.* tested the inhibitory effects of TGF-β in human T cells transduced with CD19 specific CAR carrying intra-cellular CD3ζ chain with or without CD28 signaling domain [235]. The authors reported that CD19 CAR T cells carrying CD28 signaling domain were far more resistant to TGF-β's negative

effects on cytotoxic capacity and proliferation compared to the ones lacking it. In human T cells expressing carcinoembryonic antigen (CEA) specific TCR, CD28 co-stimulation opposed TGF-β mediated inhibition of antigen-induced proliferation [236]. Studies showing increased in vitro and in vivo susceptibility to TGF-β in mouse T cells deficient in Cbl-b, which negatively regulates CD28 signaling, also supported CD28 receptor's function of antagonizing TGF-β's suppressive effects on T cells [237]. It should be noted however that in studies from Thomas *et al.* and June-Kim *et al.* CD28 signaling did not protect T cells from TGF-β mediated inhibition [148,233], suggesting a possibility that CD28 confreres resistance to TGF-β only in certain conditions.

Due to the common signaling domain YxNM shared by NKG2D-DAP10 and CD28, both the NKG2D and CD28 receptors are often subjected to the comparison in terms of their co-stimulatory and non-canonical functions. Despite sharing many similarities, both the NKG2D and CD28 receptors possess distinct expression patterns, structures, signaling pathways and functional outcomes. A review of some important literature comparing NKG2D and CD28 in the immune cells is presented below [182].

CD28 and NKG2D: Similarities and Differences

In humans, both CD28 and NKG2D are expressed in CD8+ T cells; however, the proportions of these cells expressing these receptors differ. NKG2D is expressed by all CD8+ T cells [183], whereas CD28 is expressed by ~ 50% of mature human CD8+ T cells, in addition to the vast majority (~95%) of human CD4+ T cells [238]. The NKG2D receptor has gained increasing research interest based on the widespread expression of its ligands compared to those of CD28 (CD80/CD86), which are expressed only in antigen-presenting cells (APCs). NKG2D ligands can be expressed in any cell type upon cell stress, including viral infection and DNA damage. This is also true in cancer cells, underscoring the relevance of this receptor under pathophysiological conditions. While NKG2D ligands do not have any known association with inhibitory receptors, the CD28 ligands B7.1 and B7.2 are shared by

the inhibitory receptor cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), which blocks CD28-dependent T-cell responses [239–241].

Both NKG2D and CD28 receptors use a similar YxNM motif to transduce signaling pathways. However, they differ remarkably in their structural properties and assembly, which determine the unique functional outcomes of their signals. CD28 is expressed as a homodimer on the cell surface with an extracellular ligand-binding domain, a transmembrane domain and an intracellular domain containing the YMNM signaling motif, among other motifs (Figure 6) [182,191,193]. NKG2D, lacking any signaling motif, relies on the YINM motif-containing adaptor protein DAP10 to initiate its signaling pathways [194,242]. The NKG2D-DAP10 complex is expressed as a hexamer consisting of a homodimer of two NKG2D molecules, each bound to two DAP10 molecules. Thus, each CD28 and NKG2D-DAP10 complex consists of two and four YxNM signaling motifs, respectively (Figure 6) [182,188]. In addition to these structural and stoichiometric differences, CD28 expresses additional non- YxNM signaling domains. These include the membrane proximal proline-rich PRRP domain, which can recruit inducible-2 (IL-2) T-cell kinase, and the distal PYAP domain, which is capable of binding to Lck, Filamin A and Grb2 [191].

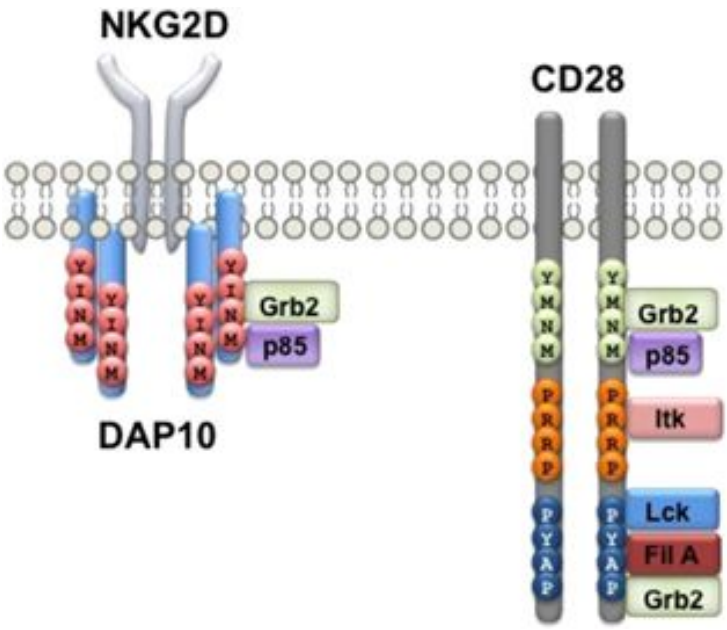


Figure 6. Differences in the Structure and Signaling Motifs of NKG2D and CD28 [182]**.**

For both CD28 and NKG2D-DAP10 receptors, activation leads to phosphorylation of tyrosine within the YxNM motif by Src family kinase, which is followed by the recruitment of Grb2 and p85 subunits to asparagine and methionine residues, respectively. This in turn results in the activation of a Grb2→Vav and p85→PDK1→Akt signaling pathways, ultimately resulting in AP-1, NFAT and NF-κB nuclear translocation and subsequent increases in cell survival, proliferation and expression of effector molecules and cytokines, as well as the release of cytolytic granules [191–193,196,197].

In NK cells, it has been shown that NKG2D-DAP10 specifically stimulates phospholipase C-γ2 (PLC-γ2) downstream of the Grb2-Vav1 pathway [243]. For CD28, no such preference for PLC-γ1 has been described. It is thought that PLC-γ1 and PLC-γ2 have redundant functions [244,245], and thus the functional relevance of this preferential signaling of NKG2D is not clear. It is important to note that although both of these isoforms are equally expressed in NK cells, PLC-γ1 is preferentially expressed over PLC-γ2 in T cells [246]. Based on previous studies that have established PLC-γ as a key signaling molecule for costimulatory signaling, it is plausible that NKG2D's costimulatory potential is reduced compared with CD28 in CD8+ T cells. The same study demonstrated that overexpression of

PLC-γ2, but not PLC-γ1, led to increased NKG2D-mediated cytotoxicity in the majority of human CTL clones [243]. However, the differences between NKG2D and CD28 signaling in the context of differential PLC-γ isoform expression were not addressed in this work.

In 2003, it was reported that NKG2D-DAP10 does not activate LAT, an important adaptor molecule that recruits key proteins in T-cell signaling, in NK cells [247]. Notably, CD28 has been shown to induce phosphorylation of LAT independent of Syk or ZAP70 kinases [248]. One study reported that although CD28 is necessary to phosphorylate LAT, its signaling motif containing cytoplasmic tail is not involved in the initiation or persistence of TCR-induced LAT phosphorylation [249]. NKG2D's effects on LAT in the context of TCR stimulation remain to be determined. As mentioned above, CD28 has two additional signaling domains that are absent in DAP10, namely, PRRP and PYAP. Itk binds to the PRRP domain, the importance of which is not yet clear in CD28 signaling. PYAP recruits Lck, Filamin A and Grb2, and activates signaling pathways that lead to cytoskeletal rearrangement and greater cytokine mRNA stability [191]. Although it apparently lacks this signaling potential, NKG2D-DAP10 has been linked to other non-YINM signaling pathways. In experiments performed with human NK cells, Sutherland et al [250] demonstrated that NKG2D stimulation leads to specific phosphorylation of JAK2 and STAT5. However, the molecular basis and functional relevance of this pathway is not yet completely understood.

Both CD28 and NKG2D stimulation have been shown to have canonical costimulatory effects on T cells, including increases in cell survival, proliferation, expression of effector molecules and cytokines, and release of cytolytic granules [191–193,196,197]. Many studies have reported that NKG2D is capable of co-stimulating human and mouse T cells to an extent similar to that of CD28 at the level of proliferation, cytolytic function and interferon-γ (IFN-γ) production [184,251–253]. It is important to note that factors such as cell activation status and cytokine treatment can alter the responsiveness of CD8+ T cells to NKG2D. For instance, Lanier and co-workers were unable to co-stimulate human and murine

CD8+ T cells by engaging the NKG2D receptor (unlike CD28), presumably due to different culture conditions and/or cytokine milieu [254]. Owing to the unique characteristics of the signaling pathways discussed above, it is not altogether surprising that NKG2D and CD28 would have different functional effects on T cells. In experiments performed on DUC18 transgenic CTLs, only CD28 could promote survival upon antigen encounter, whereas only NKG2D could form immunological synapses in the absence of antigen [251]. Interestingly, in another study, Barber and Sentman [255] demonstrated that in human CD8+ T cells, NKG2D engagement specifically activated β-catenin, increased the expression of β-catenin-induced genes and led to reduced production of the anti-inflammatory cytokines IL-9, IL-10, IL-13 and vascular endothelial growth factor-α in a peroxisome proliferator activated receptor-γ-dependent manner. CD28 stimulation either failed to exert these effects or caused effects opposite to that of NKG2D in this study. It was also found that both CD28 and NKG2D stimulation decreased the migration of human CD8+ T cells; however, only NKG2D activated Cdc42, a Rho GTPase involved in T-cell motility, and only NKG2D's effects on T-cell migration were dependent on N-WASP [256]. These data suggest that CD28 and NKG2D may also use different signaling pathways to produce similar functional outcomes in T cells. There is also evidence that both receptors can synergize with each other or modulate the other's function [252,255], suggesting possible crosstalk between the two receptors at certain signaling notches. Hu *et al.* studied the effects of CD28 signaling on the expression of NKG2D and found that CD28 costimulation resulted in sustained NKG2D expression in murine and human CD8+ T cells via Lck/JAK/ STAT3 signaling [257].

Collectively, both NKG2D and CD28, despite sharing a similar YxNM motif and associated upstream signaling events, lead to unique signaling cascades, most likely due to different structures, recruitment of adaptor proteins, downstream effectors and possible crosstalk with other immune receptors. This results in NKG2D- or CD28-mediated costimulation of CD8+ T cells with unique features/functional outcomes. In addition to the

above factors, differential expression levels of these receptors on T cells as well as those of the activating ligands in different tissues and cytokine environments may dictate the relevance of NKG2D and CD28 at the physiological level.

Despite this extensive work on the biology of NKG2D and CD28, NKG2D's involvement in CD28 mediated TGF-β resistance in CD8+ T cells remains unexplored.

Hypothesis IV

In this dissertation, we test the following hypothesis to unravel the interplay between NKG2D, CD28 and TGF-β.

CD28 co-stimulation makes CD8+ T cells more resistant to the TGF-β induced immune-suppression independently of NKG2D signaling.

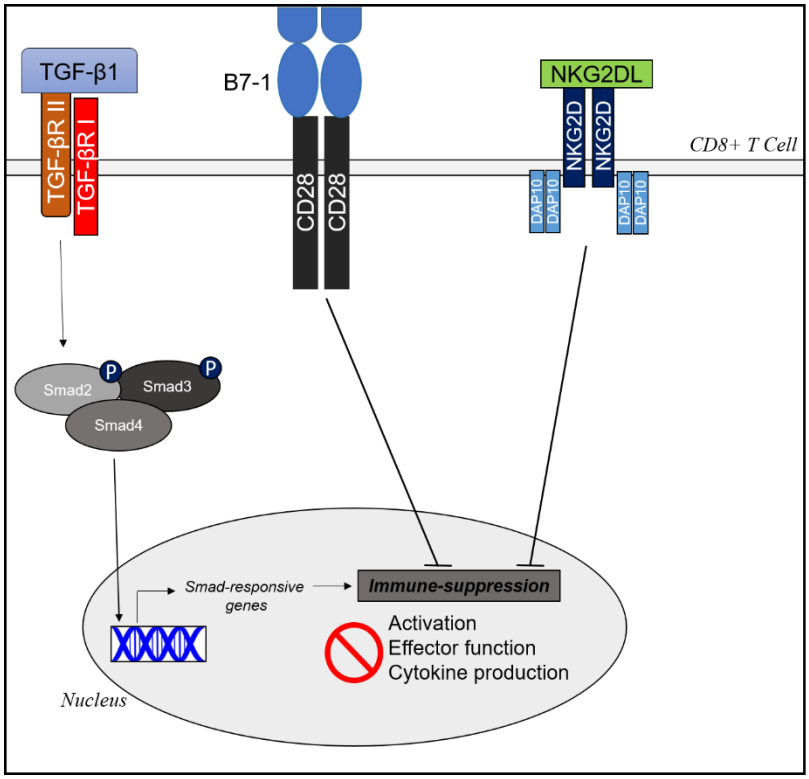


Figure 7. Hypothesis IV.

Relevance of the Studies

As discussed above, the objectives and hypotheses proposed in this dissertation are of biological and translational relevance. Dysfunctional state of CD8+ T cells in TGF-β prevalent immune-suppressive environments remains a major hurdle in the way of advancing

cancer immunotherapies in the clinics. NKG2D's influence on TGF-β suppressive pathway will not only add new knowledge to the body of scientific literature but present novel therapeutic opportunities to negate the adverse effects of TGF-β on CD8+ T cells, and ultimately improve the clinical responses of T cell based cancer immunotherapies. Whether the effects of NKG2D on TGF-β pathway are unique or redundant with other stimulatory factors such as IL-2 and IL-15 is also important in determining the relevance of this receptor in T cell biology. PD-1 and CTLA-4 signaling pathways have been extensively studied and utilized in the clinics to boost the T cell effector function, however, their relationships with other aspects of T cell biology (such as tumor-induced immune suppression) are not fully understood. Through this research, we will try to fill this gap by unravelling the role of check-point signaling in controlling TGF-β's suppressive effects in CD8+ T cells. Further, NKG2D's cross-talks with CD28 in the context of co-stimulatory functions has been of substantial interest in CD8+ T cell field. From this standpoint, our study will investigate a novel interplay between NKG2D and CD28 in regulating the TGF-β responsiveness in CD8+ T cells.

CHAPTER II

MATERIALS AND METHODS

Cell culture and Antibodies

Human and mouse cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, catalog # SH30228.01, GE Healthcare) supplemented with 10% FBS (Seradigm) and 1% penicillin and streptomycin (catalog # MT30001CI, Corning) (referred as complete IMDM). Platinum-E (Plat-E) cells used for transductions were cultured in Dulbecco's Modified Eagle Medium (DMEM, catalog # 10-013-CV, Corning) supplemented with 10% FBS and 1% penicillin and streptomycin (referred as complete DMEM). TGF-β1 was purchased from Biolegend (catalog # 580702). For human T cell cultures, anti-CD3 mAb (clone OKT3, Bio X Cell), and for mouse T cell cultures, anti-CD3 mAb (clone 2C11, Bio X Cell), anti-CD28 mAb (clone 37.51, BioLegend), anti-PD-1 mAb (RMP1-14), rat IgG2a mAb isotype control (clone 2A3, Bio X Cell), anti-CTLA-4 mAb (clone 9D9, Bio X Cell), mouse IgG2b mAb isotype control (clone MPC-11, Bio X Cell) were used. For flow cytometry stainings of human cells, fluorochrome-conjugated mAb against CD3 (clone SK7), CD8 (clone HIT8A), NKG2D (clone 1D11) were purchased from BioLegend, and unconjugated TGFBR2 mAb (clone MM0056-4F14) was purchased from Abcam. For mouse cells staining, fluorochrome-conjugated mAb against CD3 (2C11 and 17A2), CD8 (53-6.7), CD44 (IM7), CD28 (clone 37.51), IFN-γ (clone XM61.2), TNF-α (clone MP6-XT22), IL-2 (clone JES6-5H4), HLA-A2 (clone BB7.2) were purchased from BioLegend, and unconjugated polyclonal anti-TGFBR1 (catalog # 31013, Abcam) and biotinylated anti-TGFBR2 (catalog # baf532, R & D Systems) were used. To detect human TGFBR2, secondary anti-mouse IgG1 mAb (clone A85-1), and to detect mouse TGFBR1 and TGFBR2, secondary anti-rabbit IgG

(catalog 11-4839-81, eBiosciences) and streptavidin (catalog # 405214, BioLegend), were used respectively. Vβ12 antibody to stain h3T cells was purchased from Beckman Coulter (clone VER2.32.1). Granzyme B was stained using anti-granzyme B (clone GB11, BioLegend) for both human and mouse cells.

Flow Cytometry

Standard flow cytometry staining protocols were followed for measuring extra and intra-cellular markers analyzed in this study. Briefly, the cells were spun down in either flow cytometry tubes (BD Biosciences) or 96 well-plates and washed with PBS twice to remove any residual media. Then, extra-cellular markers were stained by re-suspending the cell-pellet in extra-cellular staining master mix, which consisted of viability dye and fluorochrome tagged antibodies diluted in FACS buffer (PBS with 1% BSA, catalog # 130-091-221, Miltenyi Biotec). Following incubation with extra-cellular master mix for 20 minutes at 4 C in dark, the cells were washed twice with PBS to remove any unbound antibodies. Thereafter, cells were incubated in fixation/permeabilization buffer (catalog # 51-2090KZ, BD Biosciences) at 4 C for 15 minutes. This was followed by two washes of the cells with permeabilization buffer (catalog # 421002, BD Biosciences) and incubation with intra-cellular staining master mix, which contained fluorochrome-tagged antibodies diluted in permeabilization buffer, for 45 minutes at 4 C in dark. Then, the cells were washed three times with permeabilization buffer.

For human TGFBR2 staining, the cells were incubated with primary, unconjugated TGFBR2 antibody for 1 hour at 4 C, followed by incubation with secondary, anti-mouse IgG antibody for 20 minutes at 4 C with three PBS washes after each step. This was followed by staining for CD3, CD8 or other surface markers using a standard protocol. For mouse TGFBR1 and TGFBR2 stainings, unconjugated TGFBR1 and biotinylated TGFBR2 primary antibodies were added in the antibody master mix of CD3, CD8, CD44, or other extra-

cellular surface markers. Then the cells were washed three times with PBS, and stained with anti-rabbit IgG and streptavidin secondary antibodies for 20 minutes at 4 C.

Stained cells were either analyzed immediately in flow cytometer or preserved in fixation buffer (2-4% paraformaldehyde, catalog # 420801, Biolegend) at 4 C to analyze later. The data were collected on either LSR Fortessa (BD Biosciences) or FACSCanto II (BD Biosciences). For all the flow cytometry data analysis, the dead cells were excluded from the analysis by using Zombie Aqua viability dye (catalog # 423102, BioLegend).

Human PBMC Culture and Activation

Blood samples from human donors were obtained from Key Biologics, LLC. Human PBMCs were isolated from the blood by using Ficoll-Paque method and stored in 10% DMSO in FBS in the liquid nitrogen. Some cryovials of frozen human PBMC samples were obtained from Dr. Michael Nishimura (Loyola University Chicago). Frozen vials of human PBMCs from healthy donors were thawed by rapidly incubating them in 37 C water bath and immediately adding 10x warm complete media on the thawed cells. Then, the cell suspension was spun down and the supernatant containing DMSO was discarded. The cells from each donor were then suspended in complete media and counted. Human PBMCs were cultured in complete IMDM supplemented with 300 IU/mL recombinant human (rh) IL-2 (NCI) and 50 ng/mL rh IL-15 (NCI) in round-bottom 96 well-plates at a density of 1 million cells per mL. They were activated with 2 ng/mL anti-CD3 mAb, and TGF-β was added at the same time in some wells at a final concentration of 1 ng/mL. Five days later, PBMCs were stained and analyzed by flow cytometry for the indicated markers following the flow cytometry staining procedure described above.

Dendritic Cell Maturation and Pulsing

Bone marrows were extracted from femur and tibia bones of a donor mouse. Red blood cells (RBCs) were lysed by incubation with ACK lysing buffer (catalog # A10492-01, Gibco) for 1 minute, followed by neutralization with 10x complete media. RBC free bone

marrow cells were then cultured with complete IMDM supplemented with 10% mouse granulocyte-macrophage colony stimulating factor (GM-CSF). The next day, i.e. on day 1, the bone-marrow cell suspension was pooled , supplemented with additional 10% GM-CSF, and re-plated in new 6 well-plates. On day 3 and 5, the cells were split either 1:2 or 1:3 (depending on the cell density) and re-suspended in a fresh 10% GM-CSF. On day 6, lipopolysaccharide (LPS, catalog # L2880, Sigma-Aldrich) was added in the cultures at the final concentration of 1 μg/mL to activate the DCs overnight. On day 7, mature DCs were trypsinized and pulsed with indicated concentrations of OVA peptide at the density of 3 million cells per mL for 2 hours at room temperature with intermittent shaking, followed by 3 washes with complete media. The pulsed DCs were then counted and used for co-culture with purified CD8+ T cells.

In vivo **Experiments**

Female C57BL/6 mice of 6-8 weeks of age (Jackson Laboratories) were tumor challenged by injecting 200,000 B16 cells per mouse intra-dermally (i.d.) on the flanks. Ten days later, OVA-pulsed DCs were injected subcutaneously (s.c.) into the mice bearing tumors of comparable sizes or tumor-free mice at 400,000 DCs per mouse. At the same time, 100 μg of blocking anti-TGF-β mAb was injected intraperitoneally (i.p) in some mice. Seven days after DC vaccination, splenocytes were isolated from all the mice. Following removal of RBCs using ACK lysing buffer, the splenocytes were counted and cultured overnight with or without 10^{-1} or 10^{-5} μg/mL OVA peptide in complete IMDM media in 24 well plates at 2 million cells per mL. Brefeldin A (catalog # 420601, Biolegend) was also added at the same time in the culture to block the protein transport. The next day, the cells were stained and analyzed by flow cytometry for the indicated markers.

For h3T experiment, similar procedures were followed except that 8 weeks old male B6/A2 mice were tumor challenged i.d. with 200,000 B16 cells. Ten days later CD8+ T cells

from splenocytes of 6-8 weeks old male h3T WT and NKG2D KO mice were purified and labelled with CFSE (using the methods described below). DCs matured from male B6/A2 mouse were pulsed with 10 µg/mL human tyrosinase peptide on the same day. CD8+ T cells and DCs were injected into the mice at 400,000 cells per mouse intravenously (via retro-orbital route) and s.c. respectively. Seven days post-adoptive transfer of T cells, splenocytes from mice were isolated, and stained ex vivo for the flow cytometric analysis after RBC lysis.

Activation of OT-1 CD8+ T Cells with DCs

Splenocytes from male or female OT-1 mice of 8-12 weeks were isolated, and RBCs were lysed by incubation with ACK lysing buffer for 1 minute, followed by neutralization with complete media. CD8+ T cells were isolated using mouse $CD8a^+$ T cell isolation kit (catalog # 130-104-075, Miltenyi Biotec) following manufacturer's instructions. Briefly, splenocytes were first incubated with biotin-conjugated monoclonal antibodies specific against non-CD8+ T cell markers (such as CD4, CD19, CD11) in FACS buffer at 4 C for 5 minutes. Then, anti-biotin microbeads were added in the cell suspension at 4 C for 10 minutes. Thereafter, the cell suspension was run through LS column placed in the magnetic field to retain non-CD8+ T cells in the column. The negative fraction flowing through the column, consisting of untouched CD8+ T cells, was collected in a 15 mL conical tube. Next, the purified CD8+ T cells were spun down, re-suspended in complete media, and counted.

Purified OT-1 CD8+ T cells were cultured with OVA-pulsed DCs at a 10:1 ratio (270,000 T cells to 30,000 DCs) in complete IMDM in 24 well plates. In some experiments, 20 IU/mL rh IL-2 and 50 ng/mL recombinant mouse (rm) IL-15 (Peprotech) were added into the culture. TGF-β was added in the cultures at 20 ng/mL final concentration simultaneously. Four days later, OT-1 cells were stained and analyzed by flow cytometry for indicated markers.

CFSE Labeling

Purified h3T CD8+ T cells were suspended in PBS + 1% BSA at 10 million/mL.

CFSE (catalog # 92557-80-7, Cayman Chemical) was added at 10 µM final concentration and the cell-suspension was incubated at 37 C for 10 minutes. Following incubation, the excess of CFSE was neutralized by addition of ice-cold serum-supplemented media and incubation on ice for 15 minutes. After labeling, the cells were washed three times with PBS to remove excess CFSE, counted and used immediately for injection into the mice.

Activation of OT-1 Splenocytes with Antibodies

Splenocytes from male or female OT-1 mice of 8-12 weeks were isolated and counted after lysing RBCs. For experiments involving PD-1 and CTLA-4 blockade, OT-1 splenocytes were activated using 0.1 µg/mL anti-CD3 mAb in complete IMDM with rh IL-2 and rm IL-15 at density of 2 million cells per mL in 96 well plates. TGF-β was added to the activation culture at 20 ng/mL. 0.1 µg/mL anti-CD28 mAb, 10 µg/mL of anti-CTLA-4 mAb, anti-PD-1 mAb or corresponding isotype controls (mouse IgG and rat IgG respectively) were added into some wells at the beginning of activation. Three days later, cells were stained and analyzed by flow cytometry for indicated markers. For CD28 co-stimulation experiments using WT and KO mice, OT-1 splenocytes were activated similarly in 24 well plates in the presence or absence of TGF-β (20 ng/mL). Three days later, the cells were stained and analyzed by flow cytometry for indicated markers.

Purification of CD44 Expressing OT-1 Cells and RNA Sequencing

CD8+ T cells from WT and NKG2D KO OT-1 splenocytes were purified and cultured with peptide-pulsed DCs in the presence of rh IL-2 and rm IL-15 as described above. Four days post-activation, CD44 expressing cells were purified from the culture using Magcellect Mouse Naïve CD8+ T cell isolation kit (catalog # MAGM207, R & D Systems) according to manufacturer's instructions. Briefly, the cultured cells were washed with PBS to remove media and incubated with cocktail containing biotinylated antibodies binding to the cells expressing CD44 at 4 C for 15 minutes. This was followed by incubation of cell-suspension with streptavidin ferrofluid, which consisted of nano-particles conjugated with streptavidin, at

4 C for additional 15 minutes. Then, the flow cytometry tube containing cell suspension was placed in a magnet to allow the separation of CD44 expressing cells bound to biotinylated antibodies and streptavidin nano-particles. The negative fraction, enriched in naïve CD8+ T cells, was removed gently from the magnet-placed tube using pasteur pipette. Thereafter, the tubes were removed from the magnet and the remaining positive fraction was recovered by rinsing the tube multiple times with FACS buffer. The collected cells enriched in CD8+ T cells expressing CD44 were used immediately for the RNA isolation.

Total mRNA was isolated by using miRvana miRNA isolation kit (catalog # AM1561, Thermo Fisher) according to manufacturer's instructions. RNA samples were subjected to single-end 75 base pairs sequencing on NextSeq (Illumina) at Northwestern University Sequencing Core Facility with an output of 25 million reads per sample.

Mouse Interferon-γ ELISA

Supernatants from the T cell cultures were collected and IFN-γ levels were measured by Mouse IFN-γ ELISA set (catalog # 551866, BD Biosciences) following the manufacturer’s protocol. Briefly, 96 well-ELISA plates (catalog # 9018, Corning) were coated with capture antibody diluted in carbonate coating buffer (catalog # 421701, Biolegend) overnight at 4 C. The next day, the plates were blocked using assay diluent (PBS with 10% FBS) for 1 hour, and subsequently the standards and samples diluted in assay diluent were incubated for 2 hours at room temperature. This was followed by incubating the plate with a mixture of biotinylated detection antibody and stepravidin-horseradish peroxidase (HRP) conjugate for 1 hour at room temperature. The HRP enzyme reaction was then initiated by adding tetramethylbenzidine (TMB) substrate and stopped by adding 1 M phosphoric acid (H_3PO_4). The absorbance (HRP enzyme activity) was read at 450 nm in iMark Microplate absorbance reader (Bio Rad). After each step of this process, the plate was thoroughly washed 4-5 times with PBS containing 0.05% Tween-20.

Site Directed Mutagenesis of DAP10

pCMV6 expression vector carrying full length mouse DAP10 ORF with Myc/FLAG tag at C-terminus (Fig 9) was purchased from Origene (catalog # MR226262) and the following point mutations were inserted into the YINM domain of WT DAP10 using QuickChange II Site-Directed Mutagenesis Kit (catalog # 200523, Agilent).

Table 1. Point Mutations Inserted in YINM Signaling Domain of DAP10. The sequence encoding YINM motif has been highlighted in yellow color and the introduced mutations are indicated in red color. Y = Tyrosine I = Isoleucine N = Asparagine M = Methionine Q = Glutamine F = Phenylalanine.

Number	Mutation/Description	Name/Code	Sequence
1	WT DAP10 gene	Wildtype (WT)	ATGGACCCCCCAGGCTACCTCCTGTTC CTGCTTCTGCTCCCAGTGGCTGCAAGT CAGACATCGGCAGGTTCCTGCTCCGG ATGTGGGACTCTGTCTCTGCCACTCCT GGCAGGCCTAGTGGCTGCAGATGCGGT CATGTCACTCCTAATTGTAGGGGTGGT GTTTGTATGTATGCGCCCACACGGCAG GCCTGCCCAAGAAGATGGTAGAGTC TACATCAACATGCCTGGCAGAGGC
2	M mutated with Q (Deficient in p85 recruitment)	MQ	ATGGACCCCCCAGGCTACCTCCTGTTC CTGCTTCTGCTCCCAGTGGCTGCAAGT CAGACATCGGCAGGTTCCTGCTCCGG ATGTGGGACTCTGTCTCTGCCACTCCT GGCAGGCCTAGTGGCTGCAGATGCGGT CATGTCACTCCTAATTGTAGGGGTGGT GTTTGTATGTATGCGCCCACACGGCAG GCCTGCCCAAGAAGATGGTAGAGTC

			TACATCAACCAGCCTGGCAGAGG C
3	N mutated with Q (Deficient in Grb2 recruitment)	NQ	ATGGACCCCCCAGGCTACCTCCT GTTC CTGCTTCTGCTCCCAGTGGCTGC AAGT CAGACATCGGCAGGTTCCTGCTC CGG ATGTGGGACTCTGTCTCTGCCAC TCCT GGCAGGCCTAGTGGCTGCAGATG CGGT CATGTCACTCCTAATTGTAGGGG TGGT GTTTGTATGTATGCGCCCACACG GCAG GCCTGCCCAAGAAGATGGTAGA GTC TACATCCAGATGCCTGGCAGAGG C
4	Y mutated with F (Deficient in p85 and Grb2 recruitment)	YF	ATGGACCCCCCAGGCTACCTCCT GTTC CTGCTTCTGCTCCCAGTGGCTGC AAGT CAGACATCGGCAGGTTCCTGCTC CGG ATGTGGGACTCTGTCTCTGCCAC TCCT GGCAGGCCTAGTGGCTGCAGATG CGGT CATGTCACTCCTAATTGTAGGGG TGGT GTTTGTATGTATGCGCCCACACG GCAG GCCTGCCCAAGAAGATGGTAGA GTC TTTATCAACATGCCTGGCAGAGG C

Table 2. Primers Designed to Introduce Mutations in DAP10.

Number	Mutation	Primer Set
1	MQ	GTAGAGTCTACATCAACCAGCCTGGCAGAGGCAC Complement: GTGCCTCTGCCAGGCTGGTTGATGTAGACTCTAC
2	NQ	GGTAGAGTCTACATCCAGATGCCTGGCAGAGGCACG Complement: CGTGCCTCTGCCAGGCATCTGGATGTAGACTCTACC
3	YF	GATGGTAGAGTCTTTATCAACATGCCTGGCAGAGGCAC Complement: GTGCCTCTGCCAGGCATGTTGATAAAGACTCTACCATC

The following PCR reactions were set-up for each experimental group listed in Table 4 at thermal cycling conditions described in Table 5.

Table 3. PCR Reactions for Site Directed Mutagenesis.

Component	Volume
Mouse DAP10-pCMV6 (5 ng/µL)	2 µL
Primer 1 (1 µg/µL)	1.25 µL
Primer 2 (1 µg/µL)	1.25 µL
DNTP mix	1 µL
Quick solution	3 µL
10x Reaction buffer	5 µL
Water	36.5 µL
Pfu Ultra HF DNA Polymerase*	1 µL
Total	**51 µL**

Table 4. Experimental Groups for Mutagenesis PCR. For no polymerase control, 1 µL water was added instead of DNA polymerase.

Number	Group
1	No polymerase
2	No polymerase + DpnI
3	YINM-FINM
4	YINM-FINM + DpnI
5	YINM-YIQM
6	YINM-YIQM + DpnI
7	YINM-YINQ
8	YINM-YINQ + DpnI

Table 5. Thermal Cycling Conditions for Mutagenesis PCR.

Cycles	Temperature (C)	Duration
1	95	1 minute
18	95 60 68	50 secs 50 secs 5 minutes
1	68	7 minutes
	4	Infinite

After completion of PCR, 1 µL of DpnI restriction enzyme was added into the reactions (water for no DpnI control) and they were incubated at 37 C for 1 hour. The PCR amplification of DAP10 expressing vectors was verified for each reaction (Figure 9). Rest of the reactions were incubated on ice for 30 minutes before transformation into NEB Turbo Competent E. Coli bacteria (Catalog # C2984I, New England Biolabs). 2 µL of each reaction was transformed into 50 µL bacteria using NEB protocol except following changes. After 1 hour incubation of transformation reaction with 950 µL SOC media, the bacterial culture was spun down and 800 µL of supernatant was discarded. Bacterial pellet was then resuspended in 200 µL remainder media. Then, 50 or 100 µL of bacterial suspension was plated on LB agar plates containing 50 µg/mL kanamycin. The next day, 10 colonies for each +DpnI experimental group were picked up for colony PCR.

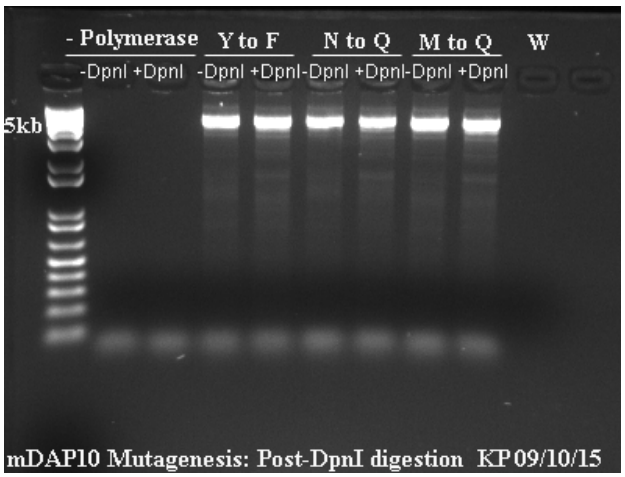


Figure 8. Verification of Mutagenesis-PCR. After completion of PCR to introduce point mutations in DAP10 gene, 10 µL of each reaction was ran on 1% agarose gel at 40 V to verify the amplification.

Colony PCR was performed with forward primer (BglII-DAP10) - TTTTAGATCTATGGACCCCCCAGGCTACC and reverse primer (EcoRI-DAP10) - TTTTTTGAATTCACGCGTGCCTCTGCCAGG at 500 nM final concentration and using DreamTaq Green PCR master mix (catalog # K1081, ThermoFisher Scientific). The following thermal cycling conditions were used (Table 6).

Table 6. Thermal Cycling Conditions for Colony PCR.

Cycle	Temperature (C)	Duration
1	95	3 minutes
30	95 57 72	30 secs 30 secs 1 minute
1	72	3 minutes
	4	Infinite

All the colonies were positive for mouse DAP10 (Figure 9). The presence of DAP10 gene and the desired mutation in each group was verified by sequencing. Importantly, there were no other mutations found in the DAP10 gene.

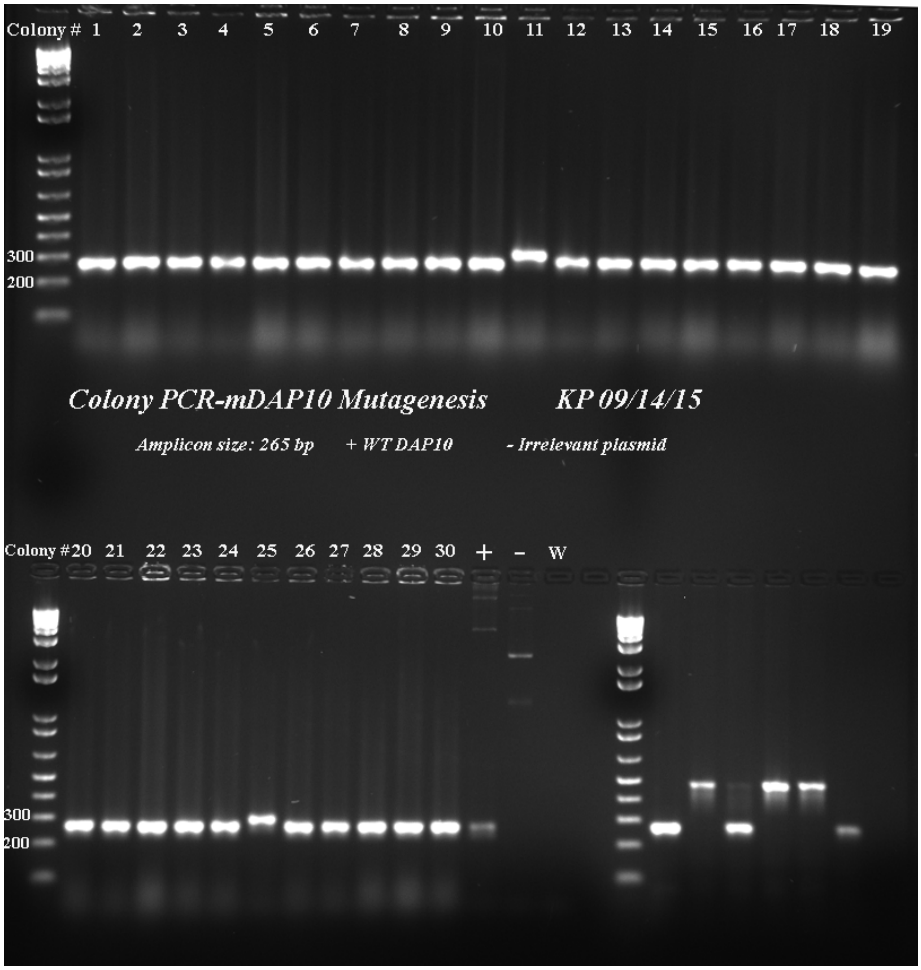


Figure 9. PCR to Identify the Bacterial Colonies Carrying DAP10 Genes. Colonies for MQ-DAP10 (#1-10), NQ-DAP10 (#11-20), and YF-DAP10 (#21-30) were picked up from the bacterial plates and diluted in 10 µL water, followed by heating at 95 C for 10 minutes to exude out DNA content. Then, substrate DNA from colonies was amplified as described for colony PCR and 10% of each PCR reaction was ran on 1% agarose gel at 80 V. W = water control, + = positive control (WT DAP10 amplified from pCMV6), - = negative control (a vector devoid of DAP10 gene). Amplicon size of DAP10 gene was 265 bp.

Sub-Cloning DAP10 Mutants into pMIG Retroviral Vector

WT and mutant DAP10 genes generated above were amplified by PCR to insert BglII and EcoRI restriction sites at their upstream and downstream ends respectively. The primers and PCR conditions were similar to the ones used for colony PCR above. DAP10 inserts were then sub-cloned into GFP expressing pMIG retroviral vector between its Bgl II and EcoRI sites using standard sticky-end sub-cloning procedures (Figure 11).

Retroviral Transduction of T Cells

Platinum E (Plat-E) cells, a retroviral packaging cell line [258], were plated in

complete DMEM in 6 well plates at 700,000 cells per well. The next day, 2.5 µg DNA of retroviral vector was transfected into the cells using Lipofectamine 2000 (catalog # 11668-019, ThermoFisher Scientific) following standard protocol. One day following transfection, the media on the cells was changed to complete IMDM supplemented with IL-2 and IL-15 (the media used to culture T cells) to collect viral titers. Then, the supernatants were collected from Plat-E cell cultures for two consecutive days (day 2 and 3 following transfection), passed through 0.45 µm filter, and supplemented with 10 µg/mL polybrene before adding on the T cell culture (described below).

Mouse splenocytes were isolated and counted after the removal of RBCs. Two million splenocytes per mL per well were plated in complete IMDM containing 1 µg/mL anti-mouse CD3, 0.1 µg/mL anti-mouse CD28 mAbs and IL-2, IL-15 in 24 well plates. On the next two consecutive days (day 1 and 2), most of the media on T cells was removed and replaced with supernatants containing viral titers (as in previous paragraph). This was followed by the centrifugation of the cell culture plates at 2000 rpm and 32 C for at least 2 hours. Post-centrifugation, the media on T cells was replaced with fresh complete IMDM supplemented with IL-2 and IL-15. The transduced cells were used either on day 4 or on split twice in complete IMDM with cytokines on day 4 and used on day 5 for experimentation.

Cytokine Assays on Transduced T Cells

Transduced T cells were spun down, re-suspended in complete IMDM and counted. They were plated in a 96 well plate pre-coated overnight with anti-mouse CD3 mAb (clone 2C11) at 200,000 cells per well in complete IMDM without cytokines. TGF-β was added in the cultures at the same time at final concentration of 20 ng/mL. Brefeldin A was added into the cultures 8 hours later, and the cells were collected and stained for flow cytometric analysis 24 hours after beginning the stimulation.

Statistics

All the statistical analysis were conducted using Microsoft Excel or Graphpad Prism.

For human PBMCs and *in vitro* OT-1 experiments, the resistance and susceptibility to TGF-β were calculated by the following equations:

% TGF-β resistance =

(% granzyme B+ in TGF-β treated / % granzyme B+ in untreated cells) x 100

% TGF-β susceptibility =

(1 - % granzyme B+ in TGF-β treated / % granzyme B+ in untreated cells) x 100

For human PBMC experiments, donors whose CD8+ T cells displayed geometric means equal to or more than the median values of distribution were considered 'Hi' for TGFBR2 and NKG2D. Similarly, donors having a % TGF-β resistance of CD8+ T cells equal to or more than the median values of distribution were considered high TGF-β resistance. For all the experiments, statistical significance was determined by unpaired, two-tailed Student's T-test using Graph Pad Prism, and differences with $p < 0.05$ were considered significant. For RNA sequencing data, only gene expression changes that showed $p < 0.05$ were considered significant and included in the analysis.

Vector Maps

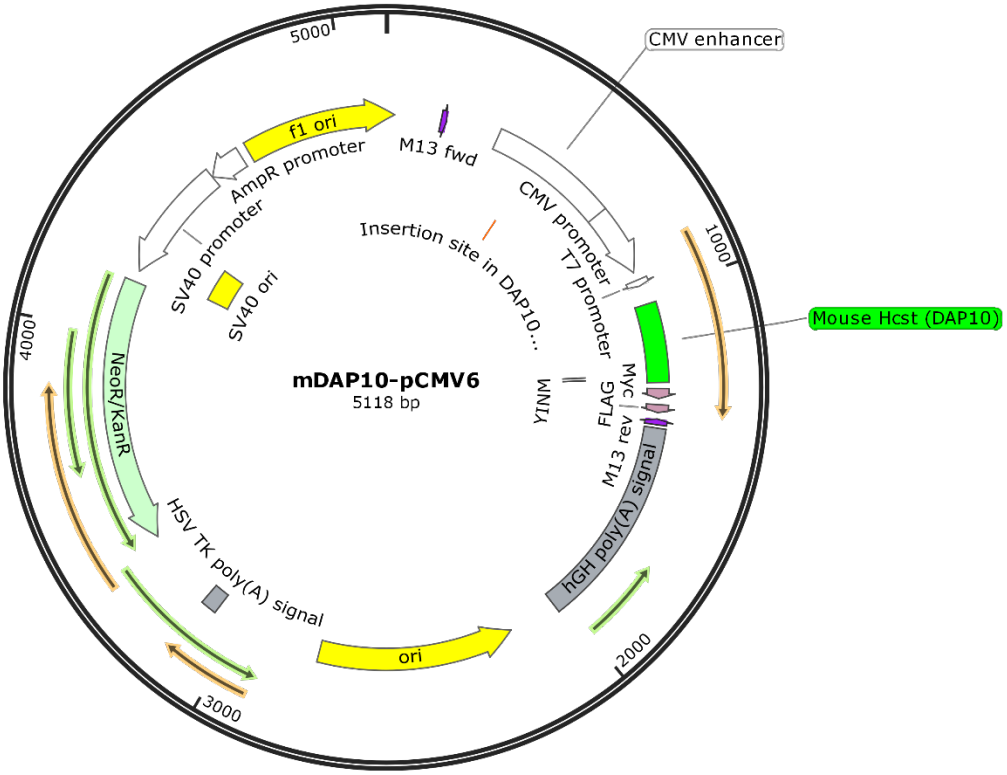


Figure 10. Vector Map of Mouse DAP10-pCMV6. Mouse DAP10 (237 bp) is shown in green color with Myc and FLAG tags at its C-terminus. NeoR/KanR is the gene conferring resistance to neomycin, kanamycin and G418.

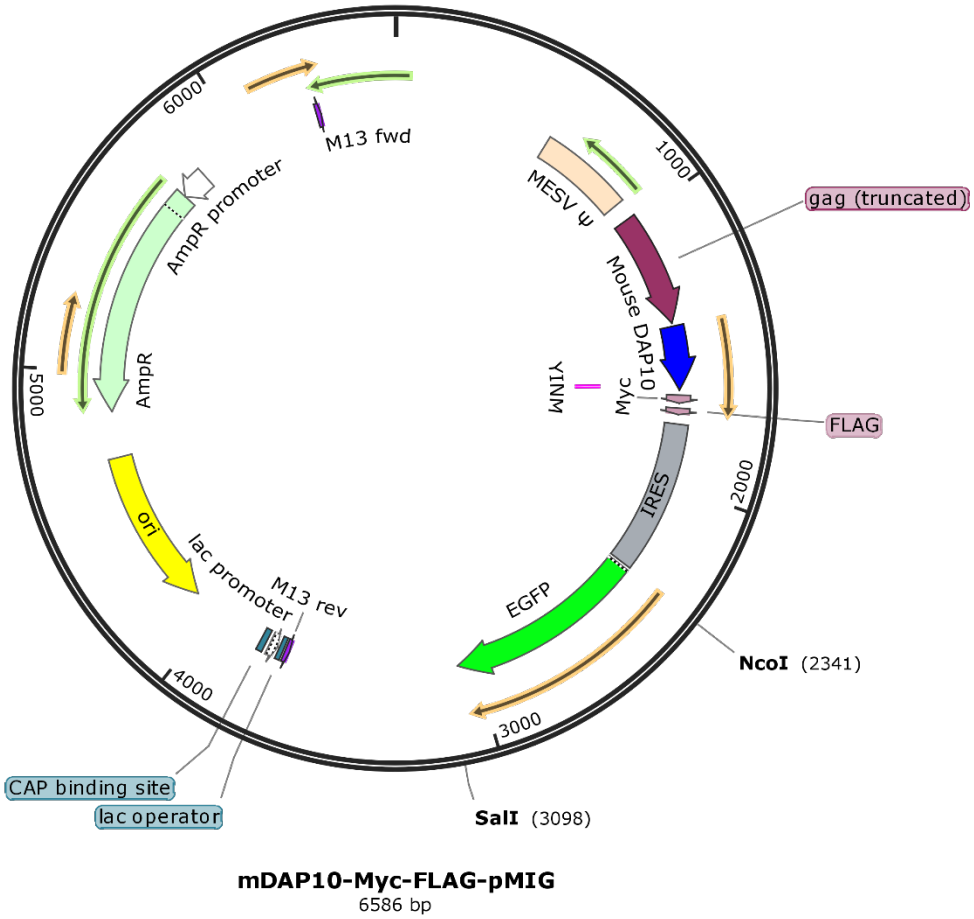


Figure 11. Map of Mouse WT DAP10-pMIG Retroviral Vector (representative of MQ, NQ and YF-DAP10-pMIG). Mouse DAP10 (237 bp) is shown in blue color with Myc and FLAG tags at its C-terminus. Enhanced GFP (EGFP) serves as a marker of vector-expression. AmpR is the gene conferring resistance to ampicillin.

CHAPTER III

RESULTS

The Role of NKG2D Receptor in Mediating Resistance to TGF-β in CD8+ T Cells

The Relationship Between TGF-β Resistance and the Expression Levels of TGFβR2 and NKG2D in Human CD8+ T Cells.

In order to establish a translational relevance of our study, first we sought to evaluate TGF-β suppression in human CD8+ T cells. Studies have shown that the expression levels of TGFBR2 are important in positively regulating TGF-β response [259], whereas stimulatory receptors such as NKG2D could negatively impact the effects of TGF-β on CD8+ T cells. Early investigations have shown that TGF-β inhibits the expression of the effector molecules such as granzyme B at the mRNA level in a Smad-dependent manner in T cells [148]. Thus, protein expression levels of granzyme B serve as an accurate readout to measure the sensitivity of cytolytic T cells to TGF-β suppression. We sought to determine the relevance of both TGFBR2 and NKG2D receptors in the context of TGF-β suppression in human CD8+ T cells. To this end, we activated human PBMCs from 18 different healthy donors using anti-CD3 Ab in the presence or absence of TGF-β and analyzed CD45 RA, CD45 RO (activation markers) and granzyme B expression after 5 days. On TGF-β untreated CD8+ T cells, we also measured the surface expression of NKG2D and TGFBR2.

After 5 days of activation, we confirmed the OKT3-dependent activation of CD8+ T cells by measuring CD45 RA to CD45 RO conversion and acquisition of granzyme B; both of which are indicative of TCR/CD3 complex activation in CD8+ T cells. As shown in Figure 12, majority of CD8+ T cells from all the donors converted from CD45 RA ^{Hi} CD45 RO ^{Lo} to CD45 RA ^{Lo} CD45 RO ^{Hi} phenotype and acquired granzyme B when stimulated with OKT3

mAb. $CD8^+$ T cells from various donors displayed variable levels of TGF-β resistance (Figure 13, A and B; Table 7) as well as TGFBR2 (Figure 13, C and D; Table 7) and NKG2D expression (Figure 13, C and D; Table 7). To study the relationship between TGF-β resistance and TGFBR2 or NKG2D, we determined medians for each of these parameters (45% for TGF-β resistance, 552 geometric mean for TGFBR2, and 2459 geometric mean for NKG2D; Table 7) and categorized all the donors into low TGF-β resistance (<45%, 9 donors) or high TGF-β resistance (>45%, 9 donors), $TGFBR2^{Lo}$ (<552, 9 donors) or $TGFBR2^{Hi}$ (>552, 9 donors), and $NKG2D^{Lo}$ (<2459, 9 donors) or $NKG2D^{Hi}$ (>2459, 9 donors). Contrary to our expectations, 77% (7 out of 9) of low TGF-β resistance donors were also $TGFBR2^{Lo}$ (Figure 13E) whereas only 22% (2 out of 9) of high TGF-β resistance donors showed $TGFBR2^{Lo}$ profiles (Figure 13E). On the other hand, 33% (3 out of 9) of low TGF-β resistance donors were $NKG2D^{Lo}$ (Figure 13F), and 66% (6 out of 9) of high TGF-β resistance donors fell into the $NKG2D^{Hi}$ category (Figure 13F). Taken together, we found that among different donors higher NKG2D levels, but not lower TGFBR2 levels, coincided with increased TGF-β resistance in CD8+ T cells.

(A)

(B)

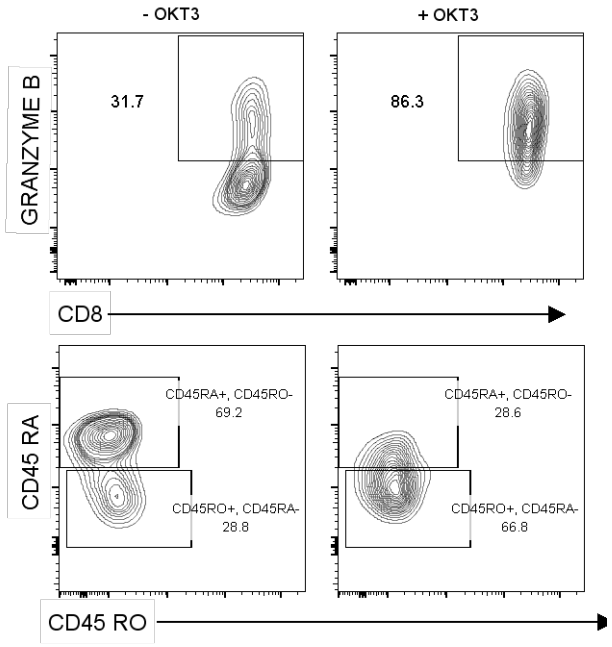


Figure 12. Confirmation of Activation of Human CD8+ T Cells by Anti-CD3 (OKT3) mAb. Human PBMCs isolated from 18 different, healthy human donors were activated with 2 ng/mL anti-CD3 mAb (OKT3) and IL-2, IL-15 in the presence or absence of 1 ng/mL TGF-β. 5 days post-activation, (A) CD45 RA, CD45 RO and (B) granzyme B levels were compared between OKT3 stimulated and unstimulated samples to confirm the activation. TGFBR2, and NKG2D levels were measured on CD3+, CD8+ T cells. The data shown are representative of CD8+ T cells from all the 18 human donors.

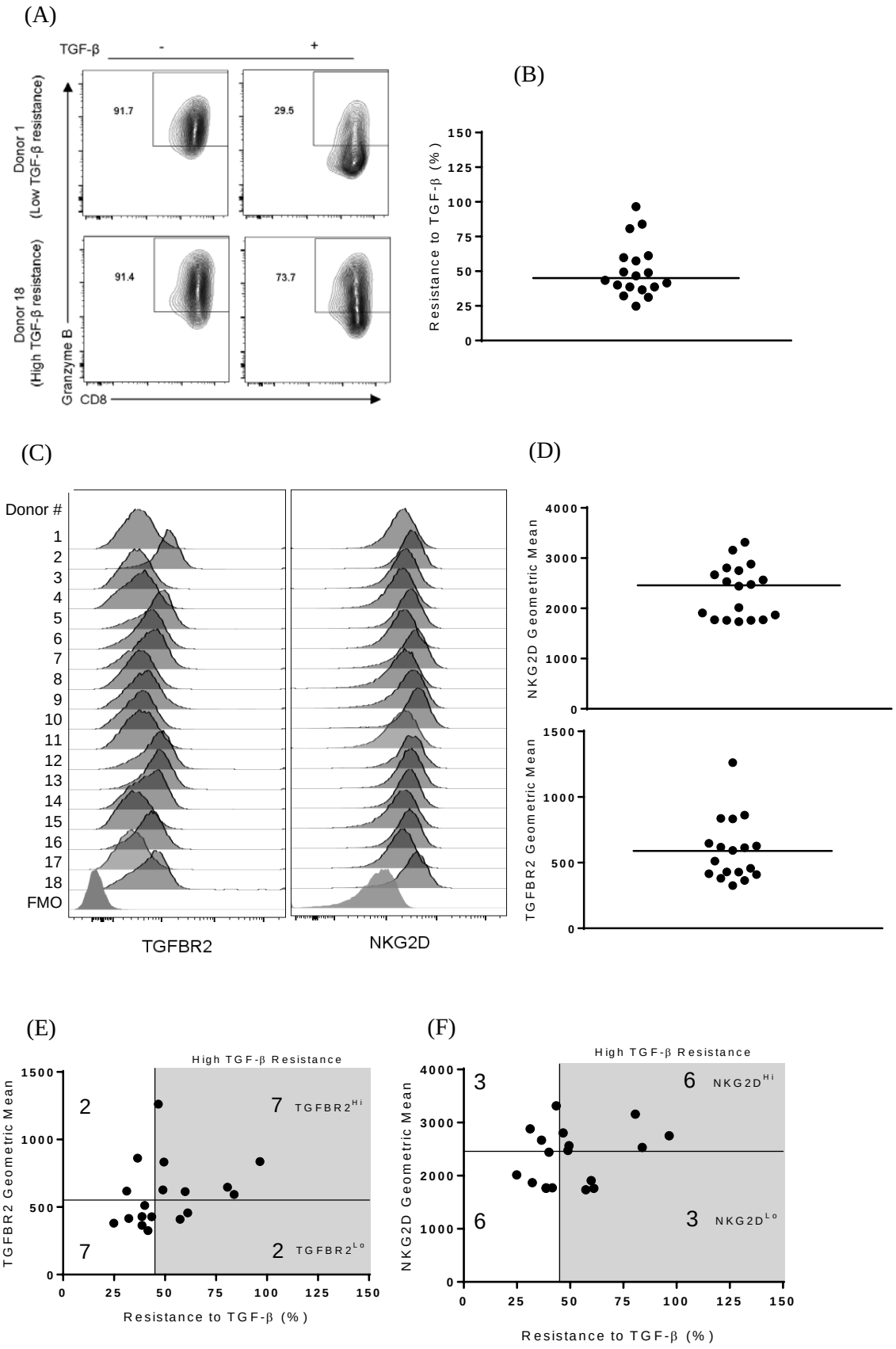


Figure 13. NKG2D Expression, but not TGFBR2, is Related to TGF-β Resistance in Human CD8+ T Cells. Human PBMCs isolated from 18 different, healthy human donors were activated with 2 ng/mL anti-CD3 mAb and IL-2, IL-15 in the presence or absence of 1 ng/mL TGF-β. Five days post-activation, granzyme B, TGFBR2, and NKG2D levels were

measured on CD3+, CD8+ T cells. (A) Two representative examples of donors 1 and 18 showing low (< 45%) and high (> 45%) TGF-β resistance respectively. (B) The distribution of variable TGF-β resistance among different donors. (C) Histograms show surface staining of TGFBR2 and NKG2D for all the donors. (D) The distribution of geometric means of TGFBR2 and NKG2D among different donors. For (B) and (D), the horizontal bar represents the median value of the distributions. (E-F) % TGF-β resistance was plotted against geometric means of (E) TGFBR2, and (F) NKG2D to study their inter-relationships. For (E) and (F), horizontal and vertical bars represent the median values determined in (B) and (D), and the numbers displayed denote the number of donors in the corresponding quartile. Quartiles containing high TGF-β resistance donors are shaded in grey. For, (B), (D), (E), and (F), each dot represents an individual donor. The data presented are representative of two independent experiments performed on human PBMCs from a total of 22 healthy donors with similar results.

Table 7. Levels of TGFBR2, NKG2D, and TGF-β resistance in Individual Donors.

Donor number	TGFBR2 Geometric Mean	NKG2D Geometric Mean	% TGF-β Resistance
1	416	1869	32.17
2	1262	2805	46.7
3	380	2016	24.8
4	409	1736	57.4
5	833	2565	49.5
6	614	1908	59.8
7	618	2883	31.2
8	429	1770	38.7
9	512	2443	40
10	428	3314	43.4
11	457	1760	61.1
12	837	2750	96.5
13	862	2667	36.5
14	593	2530	83.9
15	364	1762	38.6
16	627	2475	48.9
17	326	1770	41.6
18	647	3157	80.6
Median Values	**552**	**2459**	**45**

The Role of NKG2D in Resisting TGF-β's Suppressive Effects on CD8+ T Cells *In Vivo.*

Our findings in human CD8+ T cells suggested the inverse relationship between NKG2D signaling and TGF-β suppression, and provided a strong rationale to test the hypothesis that NKG2D signaling opposes TGF-β's negative effects in CD8+ T cells. We began to test the hypothesis by assessing the role of NKG2D in CD8+ T cells' susceptibility to tumor-mediated immune suppression *in vivo*. To this end, we used a highly immune-suppressive B16 melanoma model which is known to produce large quantities of TGF-β *in*

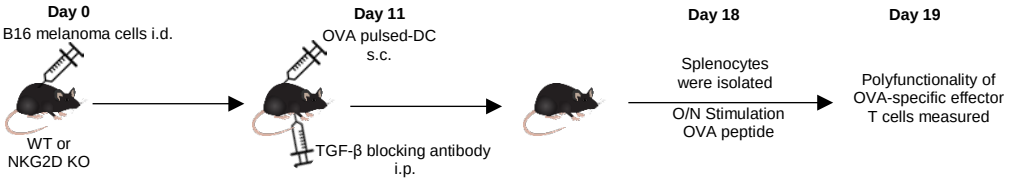
vivo [66]. B16 tumor cells were injected intra-dermally into WT and NKG2D KO B6 mice. Eleven days after tumor implantation when the tumors measured approximately 5 x 5 mm (length x width), mice were vaccinated with dendritic cells (DCs) pulsed with a foreign peptide derived from chicken ovalbumin (OVA_{257}) (Figure 14A). We chose OVA as antigen in order to study immune-suppression independently from the effects of T cells on the tumor, which would be a confounding element in our experiment. At the time of DC-injection, some mice also received blocking anti-TGF-β mAb to measure the contribution of TGF-β in immune-suppression (Figure 14A). Seven days after DC vaccination, splenocytes were isolated, and CD8+ T cells were tested for their ability to produce IFN-γ, TNF-α, and IL-2 upon OVA re-stimulation (Figure 14A).

When we first analyzed the percentages of the cells producing cytokines IFN-γ, TNF-α, or IL-2 individually in the CD44 hi CD62L lo CD8+ T cells (effector T cell population), we found that both the WT and NKG2D KO cells were remarkably suppressed in B16 tumor-bearing mice compared to tumor free controls. Figure 14B and show that the percentages of IFN-γ+ cells in tumor free WT and KO mice were 10% and 18.5% respectively, whereas in tumor bearing mice they were 2.52% and 2.14% for WT and KO mice respectively (similar results were obtained for TNF-α and IL-2). Although in the presented experiment percentages of NKG2D KO T cells producing cytokines were higher than WT, this difference was not consistent in the second experimental repeat (Figure 15). In WT tumor-bearing mice, TGF- β blockade resulted in the increase in the percentages of IFN-γ+ cells from 2.52% to 11.2%; similar results were obtained for TNF-α, IL-2 (Figure 14B; Figure 15). These data confirm that TGF-β was a major immune-suppressive factor in our B16-experimental model.

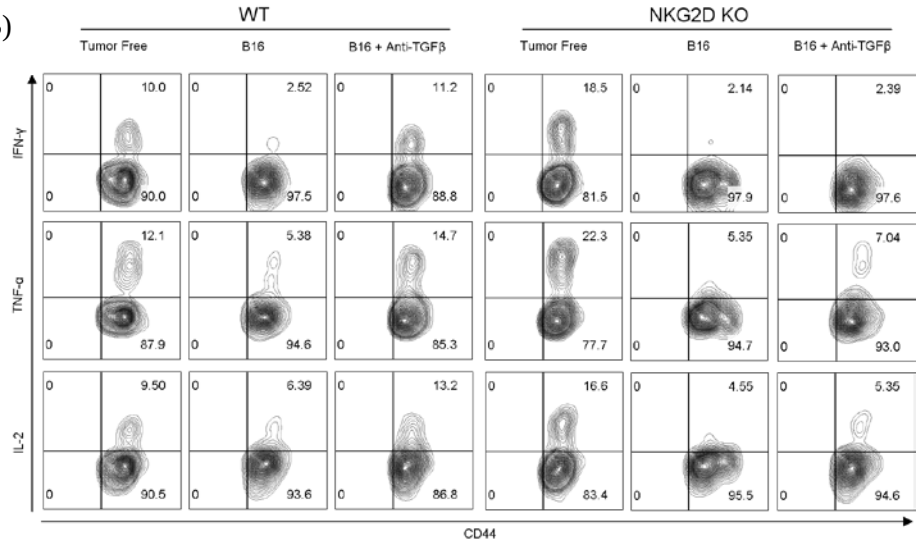
Interestingly, TGF-β blockade was not able to restore the production of individual cytokines by CD8+ T cells lacking NKG2D (% IFN-γ+ cells 2.14% for KO B16 vs. 2.39% for KO B16 + Anti-TGFβ with similar results for TNF-α, IL-2 in Figure 14B; Figure 15). These data indicate that NKG2D KO CD8+ T cells are susceptible to tumor-induced

suppressive mechanisms other than TGF-β. To conclude, contrary to our expectations, the ability of CD8+ T cells to produce individual cytokines was suppressed equally under tumor-bearing conditions in both the WT and NKG2D KO mice.

(A)



(B)



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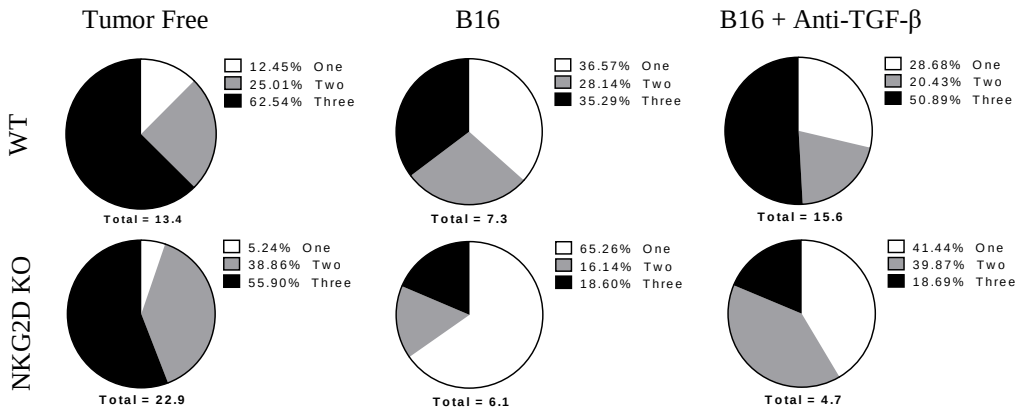


(D)

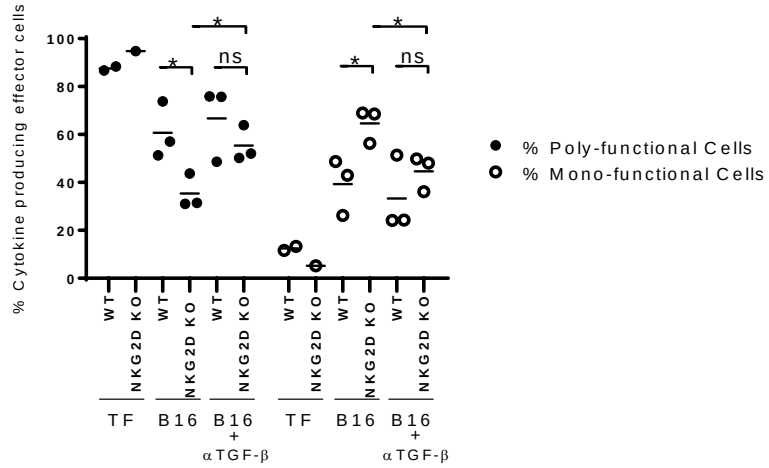


Figure 14. NKG2D KO CD8+ T cells are More Susceptible to Tumor-Induced Immune Suppression *in vivo*. (A) Schematic representation of the design and time-course of the experiment. (B) Representative flow cytometry example of IFN-γ, TNF-α, or IL-2 producing cells in effector (CD44 hi CD62L lo) CD8+ T cell population from B16 tumor-bearing WT and NKG2D KO mice with or without anti-TGF-β treatment, and the tumor-free WT and NKG2D KO mice. (C) Normalized proportions (averaged for all mice in each experimental group) of one, two or three cytokine-producing cells are presented as pie charts. Numbers shown below pie charts denote the percentages of total cytokine producing cells for the groups. (D) The results in (C) quantified as mono and polyfunctional cells and shown as a dot plot where bars represent means and each dot represents one mouse. n = 3 for all the tumor-bearing mice. For tumor-free control mice, n = 2 for WT and n = 1 for NKG2D KO. * $p < 0.05$ determined by two-tailed, unpaired Student's t test. All the data are representative of two independent experiments with similar results. TF = tumor free, ns = not significant.

In T cells, the effector function has been positively associated with polyfunctionality (the capacity to produce multiple cytokines) [152,153,260], which is shown to be suppressed by TGF-β [150]. Hence, after assessing the cytokine production individually, we further sought to determine the effect of immune-suppression on the capacity of T cells to produce multiple cytokines. To this end, we dissected the mono and poly-functionality profiles of cytokines producing populations of all experimental groups using Boolean-gating strategies (Figure 14B and Table 8).

Table 8. Representative Cytokine Analysis in CD44 hi CD62L lo CD8+ T Cells.

Population	% of CD44 hi CD62L lo	% of total Cytokine Producing	Functionality	% of total Cytokine Producing
IFN-Y+	0.61	4.6	One Cytokine Producing	13.2
TNF-α	0.91	7		
IL-2	0.21	1.6		
IFN-Y+ TNF-α+	2.0	15.38	Two Cytokines Producing	29.9
IFN-Y+ IL-2+	0.1	0.007		
TNF-α+ IL-2+	1.9	14.6		
IFN-Y+ TNF-α+ IL-2+	7.3	56	Three Cytokines Producing	56
Total Cytokine Secreting Cells	13.0	100		100

We found that in the tumor-free group, the majority of WT and NKG2D KO effector T cells were polyfunctional, i.e., most of them produced three cytokines and two cytokines (Figure 14C). In tumor-bearing WT and NKG2D KO mice, the effector T cells lost their polyfunctionality as seen by the reduction in three cytokines (35.2% in WT vs 18.6% in KO, Figure 14C) and two cytokines production (28.1% in WT vs 16.1% in KO, Figure 14C) and corresponding increase in one cytokine-production (36.5% in WT vs 65.2% in KO, Figure 14C). However, this loss of polyfunctionality was more profound in tumor-bearing NKG2D KO T cells as the percentages of polyfunctional cells were reduced to half (63.3% in WT vs. 34.7% in KO, Figure 14, C and D), and that of monofunctional cells were doubled (36.5% in WT vs. 65.2% in KO, Figure 14, C and D) in NKG2D KO tumor-bearing group compared to WT. Further, blockade of TGF-β in an immune-suppressed host reversed the polyfunctionality profile in WT mice only from 63.3% to 71.3% (Figure 14, C and D), however it significantly rescued the multi-cytokine production of T cells from 34.7% to 58.5% in NKG2D KO mice (Figure 14, C and D). Thus, while there were no significant changes at the level of individual cytokine production, TGF-β mediated stronger suppression in CD8+ T cells lacking NKG2D at the level of multiple cytokines production.

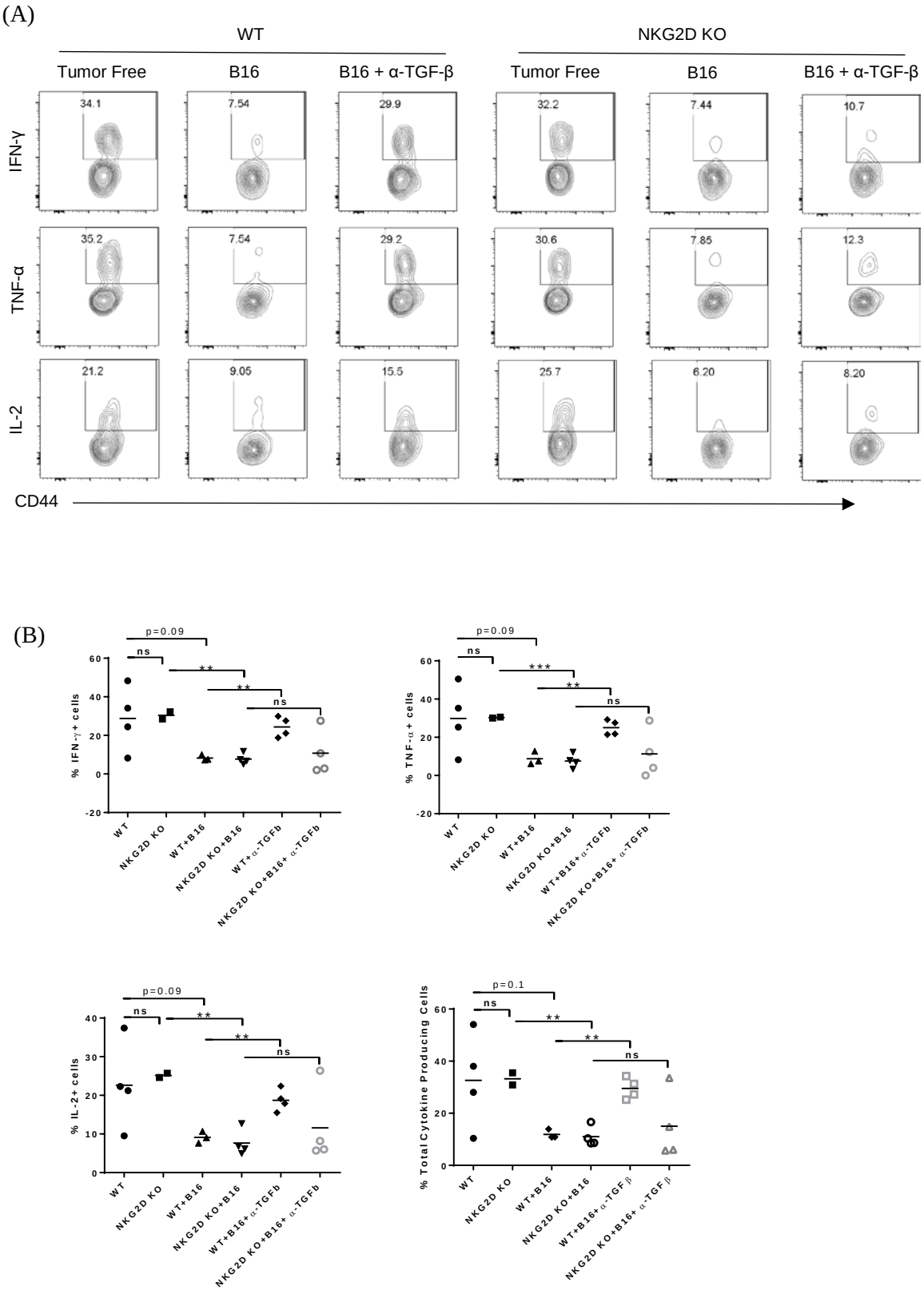


Figure 15. Effect of Tumor-Induced Immune Suppression on Individual Cytokine Production in WT and NKG2D KO CD8+ T Cells *in vivo.* Experiment was performed as depicted in Figure 3. (A) Representative flow cytometry example from each experimental group depicting percentages of IFN-γ, TNF-α, or IL-2 producing cells in effector (CD44

hiCD62L lo) CD8+ T cell population (B) Quantification of the data shown in (A) for all the mice. Bars represent mean and each dot represents one mouse. n = 4 for all the tumor-bearing mice and for tumor-free control mice, n = 4 for WT and n = 2 for NKG2D KO. ns = not significant. ** $p < 0.01$ *** $p < 0.001$ determined by unpaired, two-tailed Student's t-test. All the data are representative of two independent experiments with similar results.

NKG2D's Role in Resisting TGF-β's Suppressive Effects on CD8+ T Cells *In Vitro.*

Following our findings indicating that OVA-primed NKG2D KO CD8+ T cells are highly susceptible to TGF-β *in vivo* (Figure 14), we developed an *in vitro* system to further study NKG2D mediated TGF-β resistance and involvement of other stimulatory factors in this phenomenon. To mimic the *in vivo* physiological activation of CD8+ T cells, we activated purified CD8+ T cells from OT-1 WT and OT-1 NKG2D KO mice by co-culturing them with mature DCs pulsed with OVA peptide. TGF-β was added to the culture from the beginning of activation. Because many cytokines have been shown to play roles in overcoming TGF-β's effects on T cells, no cytokines were supplemented in the co-culture in order to study the relationship between NKG2D and TGF-β in a direct manner. Four days post-activation, the intra-cellular granzyme B content of CD8+ T cells in the activated (CD44 hi) CD8+ T cells were analyzed by flow cytometry.

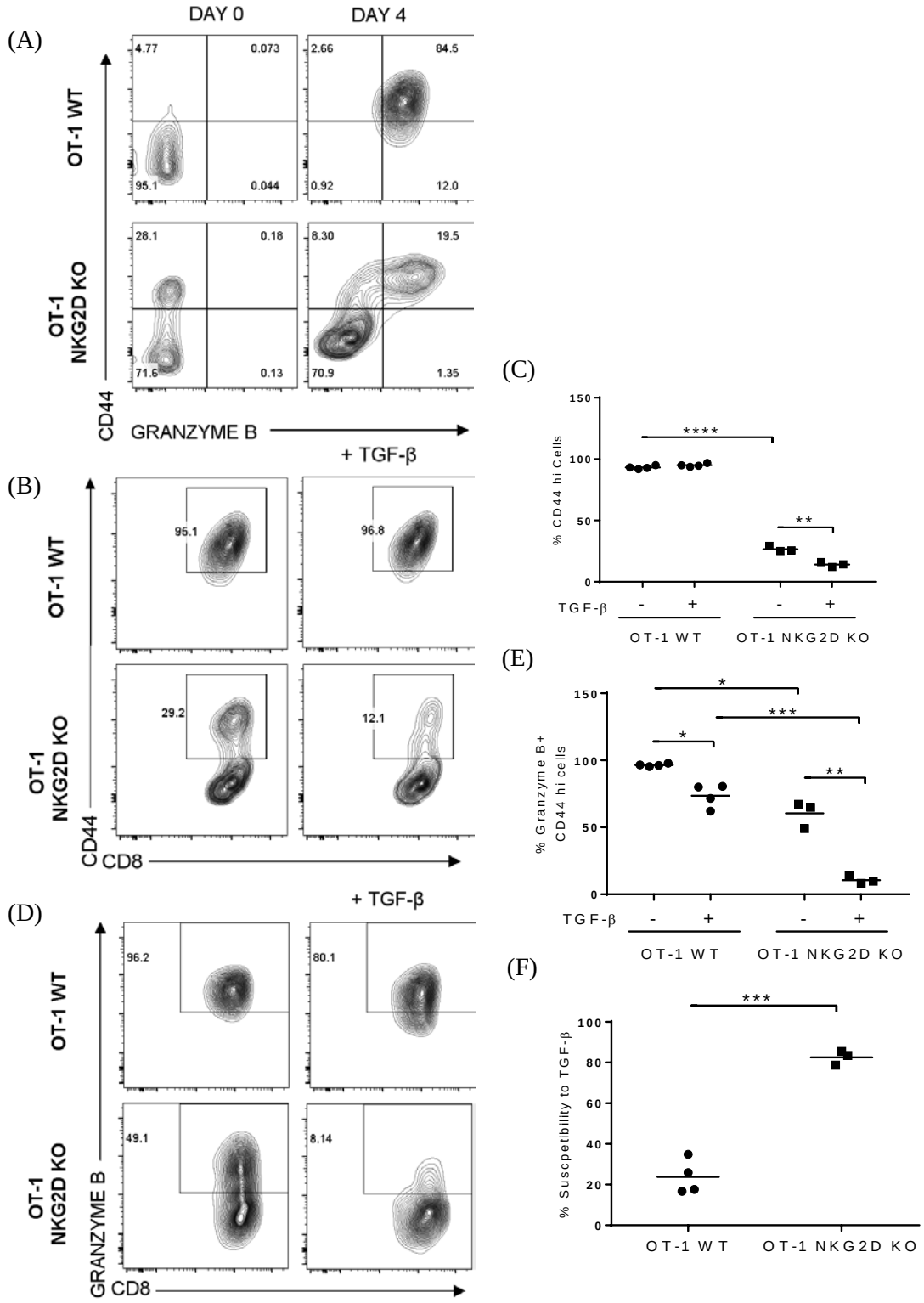


Figure 16. NKG2D Deficient OT-1 CD8+ T Cells are More Susceptible to TGF-β Mediated Suppression in the Absence of Exogenous Cytokines. CD8+ T cells from WT and NKG2D KO OT-1 splenocytes were isolated and activated by co-culturing with dendritic cells pulsed with 10 μg/mL OVA peptide in the absence of exogenous cytokines. 20 ng/mL of TGF-β was added into the culture at the beginning of activation. 4 days after activation, CD3+, CD8+ cells were analyzed by flow cytometry for CD44 and granzyme B expression. (A) Representative flow cytometry examples of CD44 and granzyme B expression on day 0

(before activation) and 4 (after activation) (B) Representative flow cytometry examples of CD44 expression on TGF-β untreated or treated CD8+ T cells on day 4 (C) Quantification of the data shown in (B) for all the mice. (D) Representative flow cytometry examples of granzyme B expression in TGF-β untreated or treated CD44 hi CD8+ T cells on day 4. (E) Quantification of the data shown in (D) for all the mice. (F) Quantification of susceptibility to TGF-β suppression (% reduction in granzyme B+ cells upon TGF-β treatment) for all the mice. In all the dot plots, bars represent means and each dot represents one mouse. n = 4 for WT and n = 3 for NKG2D KO. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ determined by two-tailed, unpaired Student's t test. All the data are representative of at least two independent experiments with similar results.

While the majority of the WT OT-1 CD8+ T cells were converted to CD44 hi upon co-culturing with OVA-pulsed DCs, OT-1 NKG2D KO CD8+ T cells did not up-regulate CD44 during the four day activation period (93% for WT vs. 30% for KO on day 4, Figure 16, A and C). Both the cell types acquired granzyme B upon activation (Figure 16, A, D, and E), although the percentages of granzyme B positive cells in CD44 hi population was significantly lower in OT-1 NKG2D KO compared to WT OT-1 cells (Figure 16, A, D, and E). This defective activation of OT-1 NKG2D KO T cells in the absence of exogenous cytokines again highlights the crucial co-stimulatory role of NKG2D receptor in CD8+ T cells activation and effector function. We observed no change in the percentages of OT-1 WT CD44 hi cells upon TGF-β treatment (Figure 16C). However, it is worth noting that TGF-β treatment of OT-1 NKG2D KO cells under same conditions led to significant reduction in the percentages of CD44 hi cells (Figure 16, B and C). TGF-β also caused a significant reduction in the percentages of granzyme B positive cells within CD44 hi population, however, this reduction was dramatically higher in NKG2D KO group compared to WT (Figure 16, D and E). Thus, NKG2D deficient OT-1 CD8+ T cells showed significantly higher (4 fold higher) susceptibility to TGF-β (% reduction in granzyme B levels upon TGF-β treatment, see Methods) than their WT counterparts (Figure 16F). These different sensitivities to TGF-β were not attributed to any inherent difference in the levels of TGF-β receptors, as CD8+ T cells from the spleens of both WT and NKG2D KO OT-1 mice showed similar surface expression of TGFBR1 and TGFBR2 (Figure 17).

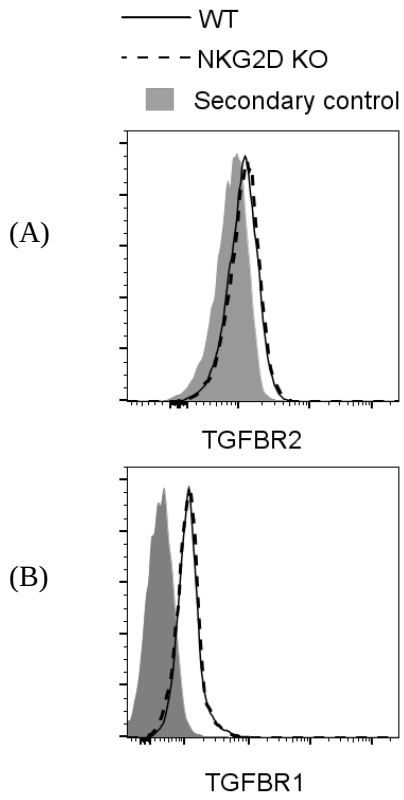
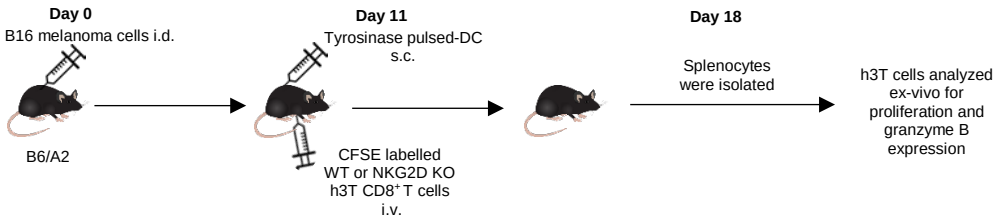


Figure 17. TGFBR2 and TGFBR1 are Similarly Expressed on WT and NKG2D KO OT-1 CD8+ T Cells. Surface expression of (A) TGFBR2 and (B) TGFBR1 on CD8+ T cells from freshly isolated WT and NKG2D KO splenocytes. The histograms are representatives of n = 6 WT and 7 KO mice from two independent experiments with similar results. For secondary control, no primary antibodies were added while staining.

After discovering higher susceptibility of NKG2D deficient CD8+ T cells to TGF-β mediated suppression in granzyme B production *in vitro*, we sought to confirm these findings in vivo using h3T mice, which express a human 1383I TCR reactive to melanoma antigen-tyrosinase when presented in context of HLA-A2 [261]. 1383I TCR is being used in the clinic to treat advanced stage melanoma patients using ACT and hence h3T mouse model is highly relevant from the clinical viewpoint of cancer immunotherapy. We injected B16, but not B16/A2, tumor cells intra-dermally into B6/A2 mice (Figure 18A) to prevent h3T T cells from reacting against implanted tumor and evaluate the immune-suppression separately from anti-tumor responses. Eleven days after tumor implantation when the tumors measured approximately 5 x 5 mm (length x width), CFSE labeled, purified CD8+ cells from either WT or NKG2D KO h3T mice were injected into tumor bearing or tumor free B6/A2 mice (Figure

18A). At the same time, the mice were vaccinated with DCs (derived from B6/A2 donor mouse) pulsed with a human tyrosinase peptide (Figure 18A). Seven days after adoptive T cell transfer and DC vaccination, splenocytes were isolated, and CFSE as well as granzyme B levels in CD44 hi h3T CD8+ T cells were measured *ex vivo* (Figure 18A).

(A)



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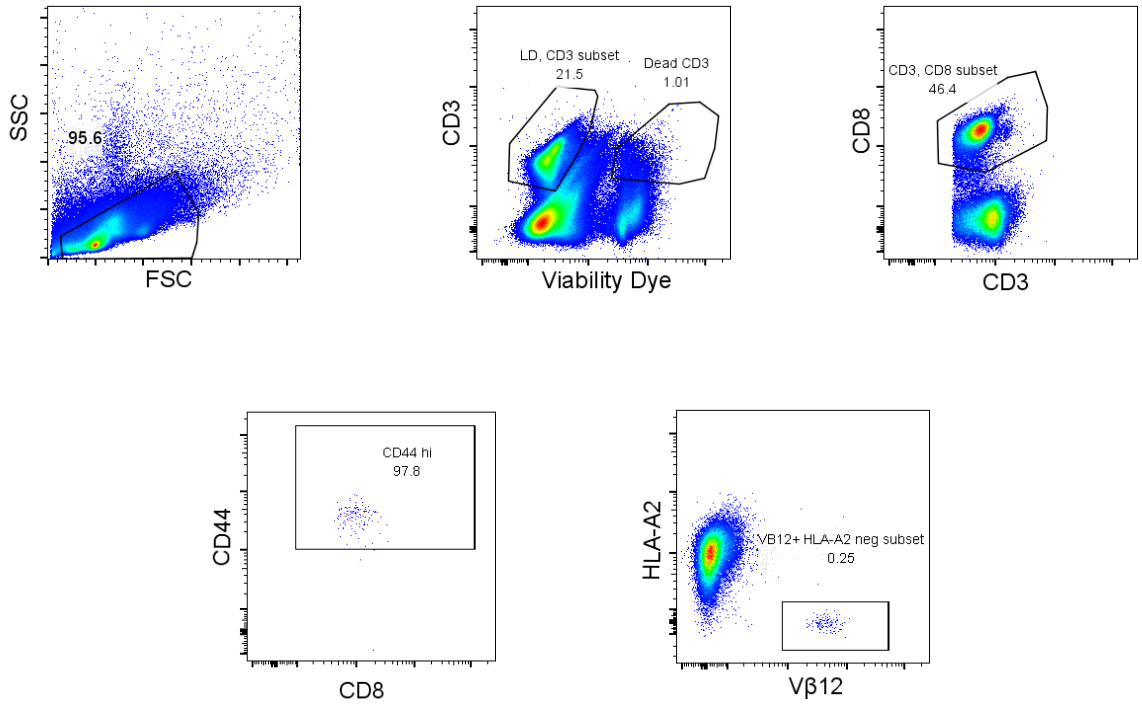


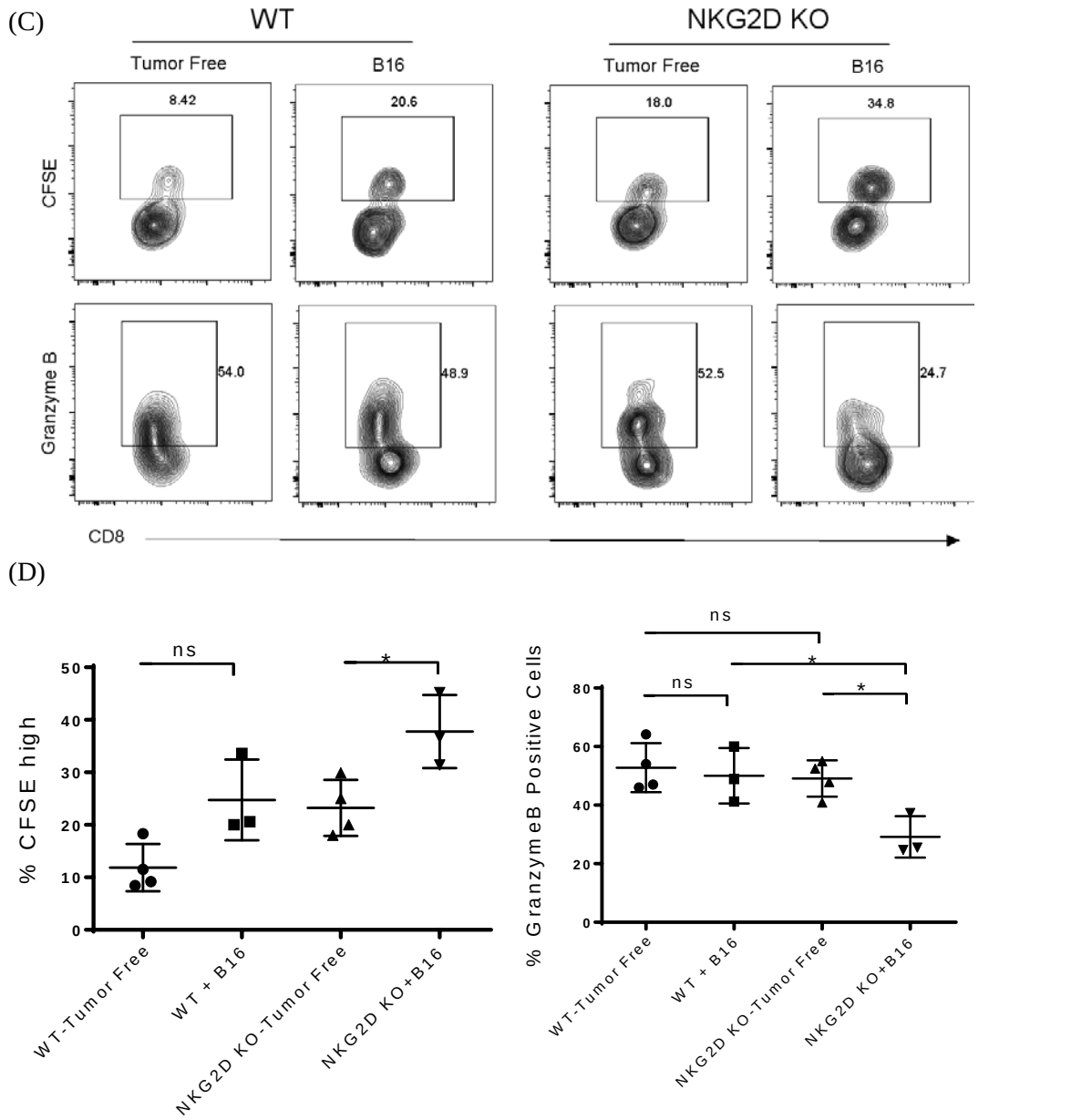


Figure 18. NKG2D Deficient h3T CD8+ T cells are More Susceptible to TGF-β Mediated Suppression in Granzyme B *in vivo.* (A) Schematic representation of the design and time-course of the experiment. (B) Gating strategy to study CD44 hi, CD8+ h3T cells. (C) The levels of CFSE and granzyme B in WT or NKG2D KO h3T cells from tumor bearing or tumor free B6/A2 mice (D) The quantification of the results shown in (C). n = 3 for all the tumor-bearing mice and n = 4 for tumor-free control mice. * $p < 0.05$ determined by two-tailed, unpaired Student's t test. ns = not significant.

A representative example of gating strategy is shown in Figure 18B where live CD8+ T cells were gated first by using viability dye. Then, CD44 hi h3T CD8+ T cells were gated for analysis by excluding HLA-A2 positive cells from host mice and staining for Vβ12 chain of 1383I TCR (Figure 18B). When we analyzed the effect of immune suppression on proliferation, NKG2D KO, but not WT, h3T cells showed significant increase in the

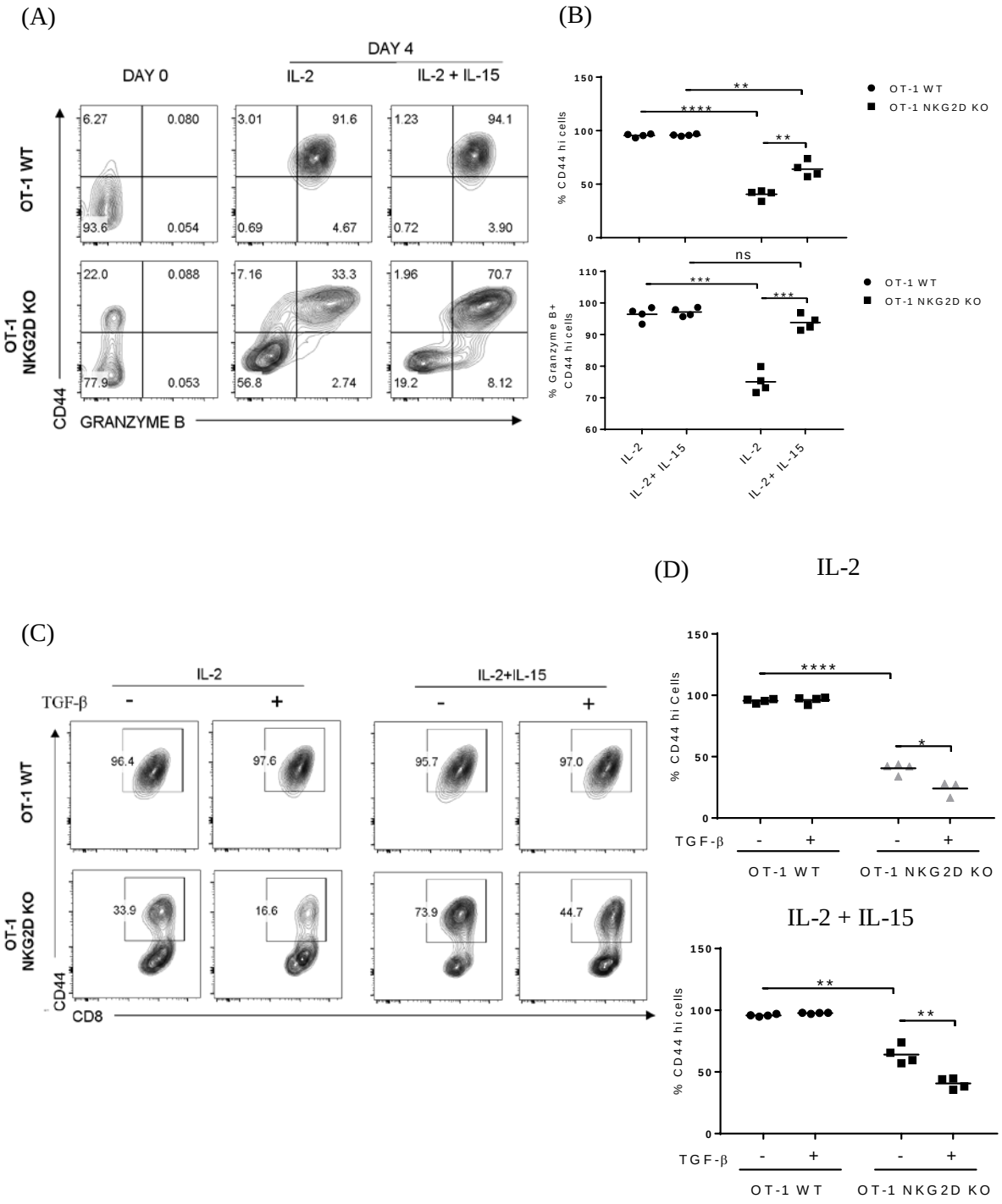
proportions of CFSE positive, non-proliferating cells in tumor-bearing mice compared to tumor-free controls (Figure 18, C and D). Both the WT and NKG2D KO h3T cells expressed comparable levels of granzyme B in tumor free mice (Figure 18, C and D). While proportions of granzyme B positive, WT h3T cells were not changed (53% in tumor free vs 50% in B16) , those of NKG2D KO h3T cells were significantly reduced under tumor bearing conditions (49% in tumor free vs 29% in B16; Figure 18, C and D). These results confirmed that the lack of NKG2D rendered CD8+ T cells highly susceptible to TGF-β's inhibition in granzyme B expression *in vivo*.

The Role of Cytokines IL-2 and IL-15 in Mediating TGF-β Resistance in NKG2D Deficient CD8+ T cells

Stimulatory cytokines IL-2 and IL-15 have been shown to oppose the inhibitory effects of TGF-β on CD8+ T cells [262,263]. Interestingly, many redundancies between NKG2D and IL-15 regarding signaling and stimulatory effects on T cells have also been reported [199,225,264]. These observations raise the possibility that IL-2 and IL-15 could rescue the functions as well as reduce the hyper-sensitivity to TGF-β in CD8+ T cells lacking NKG2D. Hence, we conducted experiments similar to those shown in Figure 16, but with the addition of IL-2 alone or IL-2 and IL-15 in the co-culture.

We first assessed the activation and granzyme B production in CD8+ T cells not treated with TGF-β. Compared to previous results obtained in the absence of exogenous cytokines, supplementing IL-2 increased the percentages of CD44 hi cells (Figure 19, A and B; Figure 16, B and C) and granzyme B positive cells in the OT-1 NKG2D KO group (Figure 19, A and B; Figure 16, D and E). Addition of IL-15 with IL-2 further increased the CD44 hi percentages significantly in the OT-1 NKG2D KO group; however, they still could not reach the WT levels (Figure 19, A and B). Moreover, the addition of IL-15 completely restored the percentages of granzyme B positive cells in OT-1 NKG2D KO group back to the WT levels (Figure 19, A and B). Taken together, supplementing exogenous IL-2 and IL-15 could

overcome the defect in the granzyme B production due to the lack of NKG2D in CD8+ T cells.



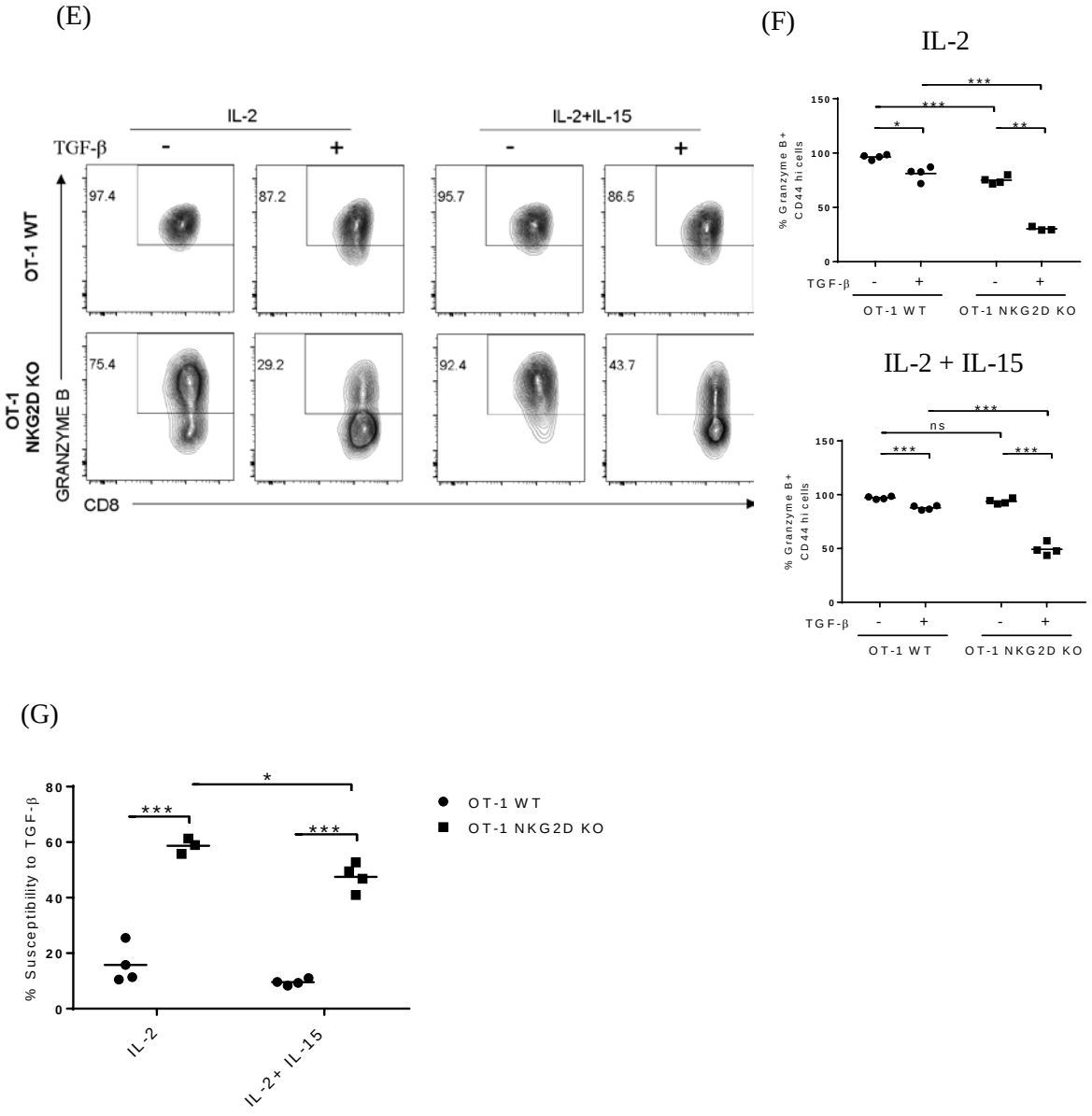


Figure 19. IL-2 and IL-15 Cannot Overcome the Defect in TGF-β Resistance in NKG2D Deficient OT-1 CD8+ T Cells. CD8+ T cells from WT and NKG2D KO OT-1 splenocytes were isolated and activated by co-culturing with dendritic cells pulsed with 0.01 μg/mL OVA peptide in presence of 20 IU/mL IL-2 or combination of 20 IU/mL IL-2 and 50 ng/mL IL-15. 20 ng/mL of TGF-β was added into the culture at the beginning of activation. 4 days after activation, CD3+, CD8+ T cells were analyzed by flow cytometry for CD44 and granzyme B expression. (A) Representative flow cytometry examples of CD44 hi and granzyme B expression day 0 (before activation) and 4 (after activation) (B) Quantification of CD44 hi % and granzyme B positive % CD8+ T cells on day 4 for data shown in (A). (C) Representative flow cytometry examples of CD44 expression in TGF-β untreated or treated CD8+ T cells. (D) Quantification of the data shown in (C) for all mice. (E) Representative flow cytometry examples of granzyme B expression in TGF-β untreated or treated CD44 hi CD8+ T cells (F) Quantification of the data shown in (E) for all mice. (G) Quantification of susceptibility to TGF-β suppression calculated from (F). In (B), (D), and (F), same data for CD44 and granzyme B in TGF-β untreated group have been presented to make different comparisons. n = 4 for WT and n = 4 for NKG2D KO for all the panels except n = 3 for NKG2D KO in IL-2

+ TGF-β treated panel. In all the dot plots, bars represent means and each dot represents one mouse. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ determined by two-tailed, unpaired Student's t test. All the data are representative of at least three independent experiments with similar results.

When we looked at the TGF-β responsiveness on CD44 expression, there were no striking changes in OT-1 NKG2D KO cells supplemented with or without cytokines (Figure 19, C and D; Figure 16, B and C). At the level of granzyme B, IL-2 indeed conferred some protection to TGF-β in OT-1 NKG2D KO cells as seen by reduced susceptibility (55%, Figure 19, D-F) compared to no cytokine added cultures (80%, Figure 19, E-G). Addition of IL-15 further increased resistance in OT-1 NKG2D KO, as seen by a lower reduction in granzyme B positive upon TGF-β treatment compared to IL-2 alone (susceptibility 58% in IL-2 vs 47% in IL-2 + IL-15; Figure 19, E-G). Nonetheless, OT-1 NKG2D KO CD8+ T cells were profoundly and significantly more susceptible (4 to 5 folds) to TGF-β than WT control even after supplementation with IL-2 alone or combination of IL-2 and IL-15 (Figure 19, E-G). Overall, despite enhancing the activation (CD44 expression) and granzyme B production, both IL-2 and IL-15 were not able to rescue the impaired TGF-β resistance in OT-1 NKG2D KO CD8+ T cells.

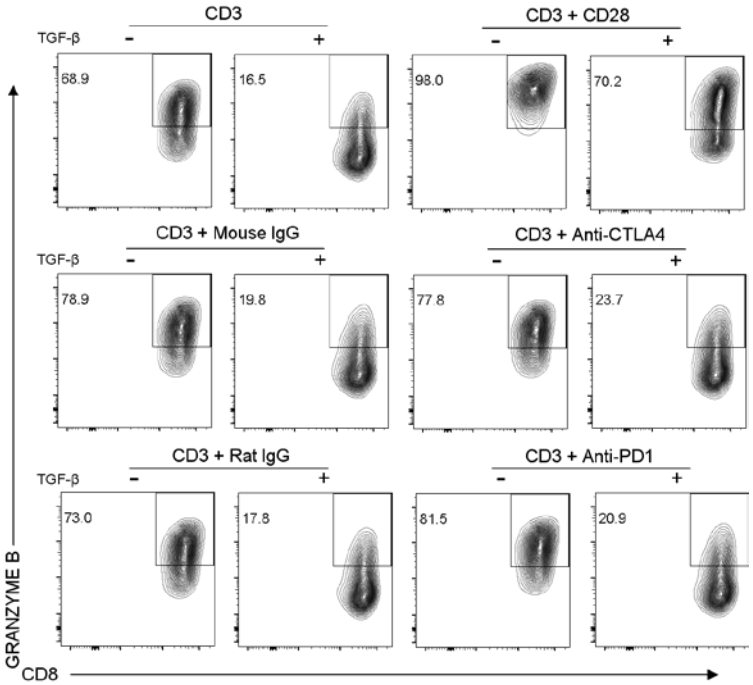
Evaluating the Inter-Relationship Between Co-Stimulatory Signals, TGF-β Resistance and NKG2D Signaling in CD8+ T Cells

The Impact of PD-1 and CTLA-4 Blockade on TGF-β Resistance in CD8+ T Cells.

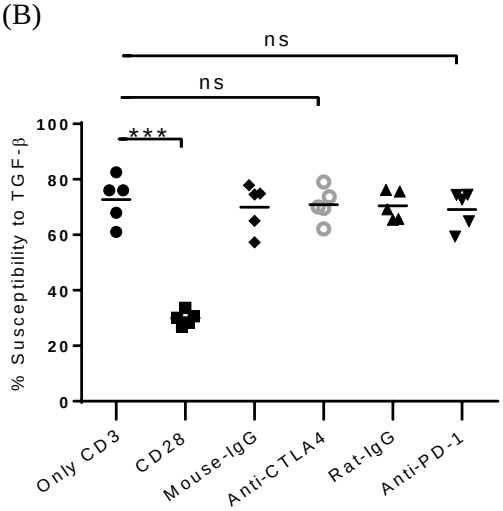
As described in the introduction, various co-stimulatory signals in addition to TCR/CD3 complex can govern TGF-β signaling in CD8+ T cells. We were interested in teasing out the contributions of TCR/CD3 complex and other widely studied co-stimulatory and co-inhibitory signals in driving TGF-β resistance in CD8+ T cells. To this end, we first activated WT OT-1 splenocytes with only activating anti-CD3 mAb in presence or absence of TGF-β and measured the levels of granzyme B production 3 days later. To our surprise, under these conditions when only the CD3 complex was engaged, WT CD8+ T cells were

dramatically susceptible to TGF-β (72% susceptibility for CD3 group, Figure 20, A and B) in contrast to their extraordinarily low susceptibility under DC activation (10-15% susceptibility for WT group, Figure 19G). These sharply different susceptibility to TGF-β under different methods of T cell activation pointed towards a major role of factors additional to TCR in regulating TGF-β responsiveness in CD8+ T cells. The higher TGF-β susceptibility of CD3-activated WT CD8+ T cells in this system allowed us to study the elevation of TGF-β resistance by biologically and therapeutically important molecules such as CD28, an archetype co-stimulatory receptor in $CD8^+$ T cells, and check-point receptors PD-1 and CTLA-4, targets of highly successful immunotherapy approaches [265]. When we activated CD28 or blocked PD-1 and CTLA-4 pathways in combination with CD3 activation, the cells that received CD28 co-stimulation were highly protected from TGF-β's inhibition of granzyme B compared to CD3 alone (Figure 20A). Hence, CD28 co-stimulation significantly reduced susceptibility to TGF-β (72% for CD3 vs 29% for CD3 + CD28, Figure 20B). Surprisingly, CD8+ T cells treated with either anti-PD-1 or anti-CTLA-4 mAb did not provide any resistance to TGF-β in CD8+ T cells, as seen by similar TGF-β induced suppression in granzyme B production compared to their corresponding IgG controls or CD3 alone group (Figure 20, A and B).

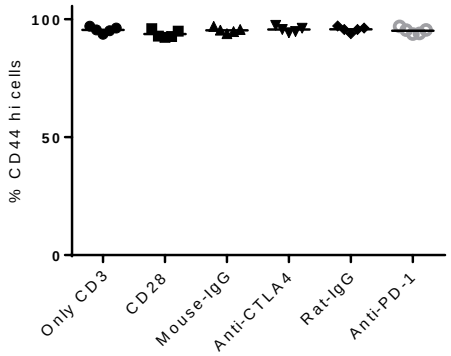
(A)


CD3
CD3 + CD28
TGF-β
-
+
68.9
16.5
98.0
70.2
CD3 + Mouse IgG
CD3 + Anti-CTLA4
78.9
19.8
77.8
23.7
CD3 + Rat IgG
CD3 + Anti-PD1
73.0
17.8
81.5
20.9
GRANZYME B
CD8

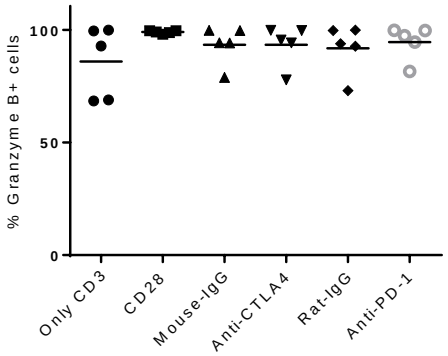
(B)


ns
ns
***
% Susceptibility to TGF-β
100
80
60
40
20
0
Only CD3
CD28
Mouse-IgG
Anti-CTLA4
Rat-IgG
Anti-PD-1

(C)


% CD44 hi cells
100
50
0
Only CD3
CD28
Mouse-IgG
Anti-CTLA4
Rat-IgG
Anti-PD-1

(D)


% Granzyme B+ cells
100
50
0
Only CD3
CD28
Mouse-IgG
Anti-CTLA4
Rat-IgG
Anti-PD-1

(E)

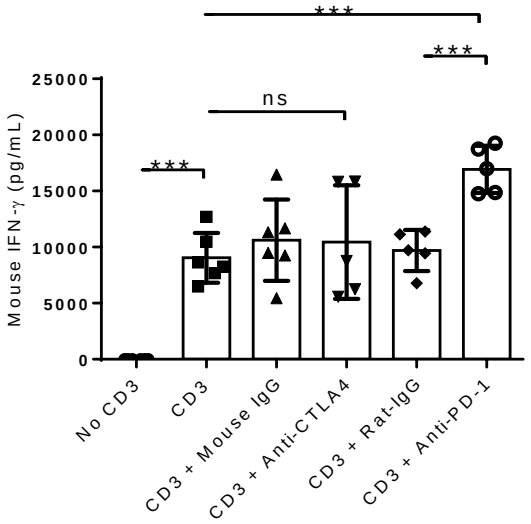


Figure 20. Blockade of PD-1 Receptor Fails to Confer TGF-β Resistance in OT-1 CD8+ T Cells. OT-1 mouse splenocytes were activated with 0.1 µg/mL anti-CD3 mAb and 20 IU/mL IL-2 and 50 ng/mL IL-15 in presence or absence of 20 ng/mL TGF-β. Some culture wells also received 0.1 µg/mL anti-CD28 agonistic mAb, 10 µg/mL-anti-CTLA-4 blocking mAb, control mouse-IgG mAb, anti-PD-1 blocking mAb, or control rat-IgG mAb at the beginning of activation. 3 days post-activation, CD3+, CD8+ T cells were analyzed by flow cytometry for CD44 and granzyme B expression. (A) Representative flow cytometry examples of granzyme B expression in TGF-β untreated or treated CD44 hi CD8+ T cells. (B) Quantification of susceptibility to TGF-β suppression. (C) CD44 expression on CD8+ T cells and (D) Granzyme B expression on CD44 hi CD8+ T cells 3 days post-activation. (E) Supernatants from different cell culture conditions were collected 3 days post-activation, and amounts of mouse IFN-γ were measured by ELISA. For (B-D) pooled data of n = 5 mice from 3 independent experiments are presented, where bars represent means and each dot represents one mouse. In (E) each bar represents data from n = 2 mice with 3 technical replicates per mouse and error bars denote standard deviations, presented data are representative of three independent experiments with similar results. ns = not significant *** $p < 0.001$ determined by two-tailed, unpaired Student's t-test.

In all the experiments, CD8+ T cells among all the different treatment groups had comparable frequencies of CD44 hi and granzyme B hi population (Figure 20, C and D). Therefore, these results could not be attributed to the unequal activation or granzyme B acquisition. Further, to determine if both the PD-1 and CTLA-4 blocking mAbs were indeed stimulating OT1 CD8+ T cells in our system, we collected the supernatants from T cells activated with these treatments and measured IFN-γ secretion by ELISA. Anti-PD-1, but not anti-CTLA-4 mAb, nearly doubled the secretion of IFN-γ by OT-1 cells compared to IgG control or CD3 alone (Figure 20E). IFN-γ was not detected in unstimulated T cell cultures,

confirming that IFN-γ in our cultures was a measure of T cell activity (Figure 20E). Although anti-CTLA-4 mAb did not augment IFN-γ secretion or provide TGF-β resistance, it indeed bound to CTLA-4 on CD8+ T cells as seen by the significant reduction in CTLA-4 surface staining upon treatment (Figure 21A). It was also possible that on the CD8+ T cells the ligands for CTLA-4 were either not expressed or expressed at lower levels, precluding the inhibitory CTLA-4 signaling and consequent positive effects of CTLA-4 blocking. To rule out this possibility, we confirmed that activated OT-1 CD8+ T cells strongly expressed CD80, one of the ligands for CTLA-4 (Figure 21B). Taken together, despite significantly augmenting the function, PD-1 blockade failed to confer TGF-β resistance in OT-1 CD8+ T cells. CD28 co-stimulation, which was our positive control for these experiments, made OT-1 CD8+ T cells highly resistant to TGF-β mediated suppression as previously reported [136,236,266].

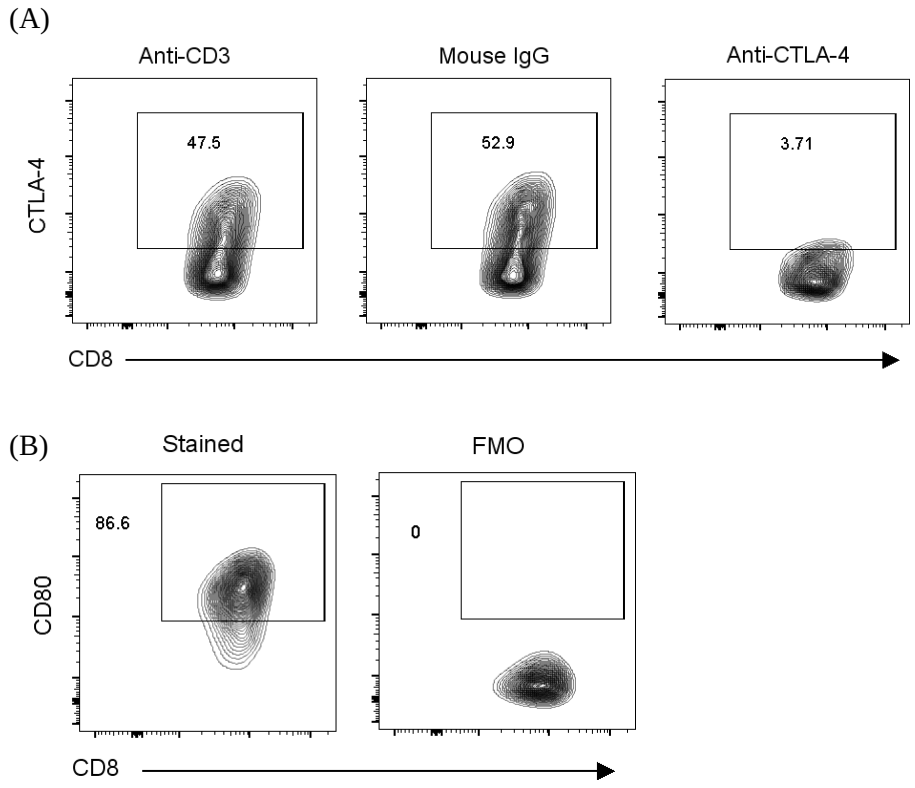


Figure 21. CTLA-4 and its Ligand CD80 are Expressed on OT-1 CD8+ T Cells. OT-1 mouse splenocytes were activated with 0.1 µg/mL anti-CD3 mAb and 20 IU/mL IL-2 and 50 ng/mL IL-15. Some culture wells also received 10 µg/mL-anti-CTLA-4 blocking mAb or

control mouse-IgG or at the beginning of activation. Three days post-activation, CD44 hi CD8+ T cells were analyzed by flow cytometry for CTLA-4 expression. (A) Representative example of CTLA-4 staining on mouse-IgG or anti-CTLA-4 mAb treated OT-1 CD8+ T cells. The data are representative of n = 4 mice from two independent experiments with similar results. (C) CD80 surface expression on CD44 hi OT-1 CD8+ T cells activated with 0.1 µg/mL anti-CD3 mAb and 20 IU/mL IL-2 and 50 ng/mL IL-15 for 4 days. The data is representative of n = 6 mice from two independent experiments with similar results.

Investigating the Cross-Talk Between CD28 and NKG2D in Mediating TGF-β Resistance in CD8+ T Cells

The co-stimulatory molecules NKG2D and CD28 have been shown to share many similarities regarding signaling and function in CD8+ T cells [182]. Accordingly, we sought to study the involvement of NKG2D in CD28 mediated TGF-β resistance. To do that, OT-1 WT and OT-1 NKG2D KO splenocytes were CD3-activated with or without CD28 co-stimulation in the presence or absence of TGF-β, and the amounts of CD44 hi, granzyme B positive cells were analyzed 3 days later. When activated with CD3 mAbs, NKG2D KO cells showed reduced frequencies of CD44 hi cells compared to WT (92% for WT vs. 80% for KO), regardless of the addition of CD28 stimulation (Figure 22, A and B). Although this difference was statistically significant, the amplitude was much smaller than what we observed in our DC activation experiments (Figure 16, A and C; Figure 19, A and B) indicating that non-specific forms of activation such as CD3/CD28 could overcome the defect in the activation of NKG2D KO OT-1 T cells to a large extent. Interestingly, opposite to our observations in DC-based activation experiments, we found that frequencies of CD44 hi NKG2D KO OT-1 CD8+ T cells were significantly up-regulated in response to TGF-β when activated with CD3 or CD3+CD28 mAbs (Figure 22, A and B). This was also true for WT cells, although not apparent at the level of frequency due to already high basal levels of CD44 hi, but certainly at the per cell basis in CD44 hi population (Figure 22, A and B; Figure 23).

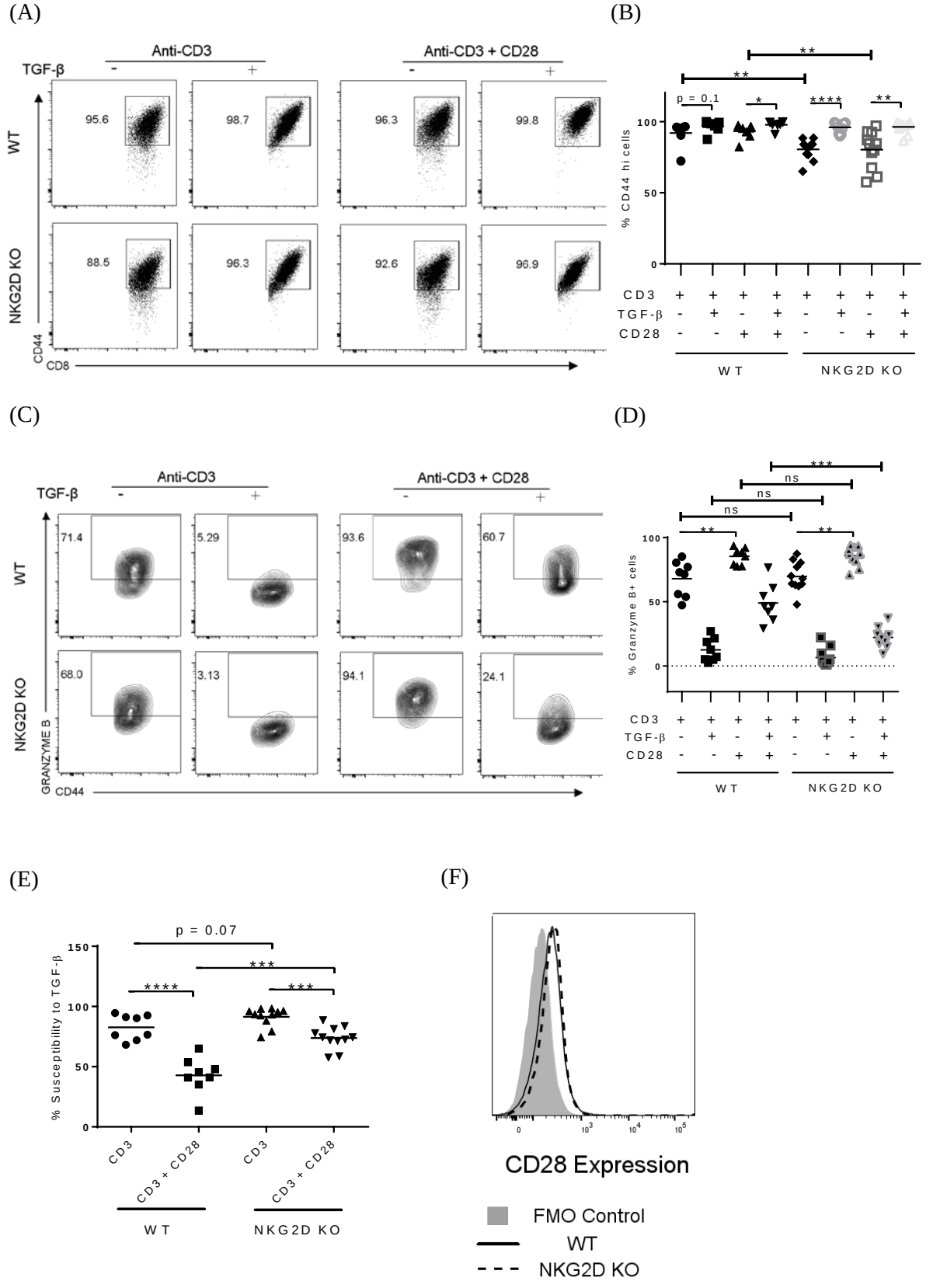


Figure 22. CD28 Mediated TGF-β Resistance in CD8+ T cells is Dependent on NKG2D. WT and NKG2D KO OT-1 splenocytes were activated with 0.1 µg/mL anti-CD3 mAb and 20 IU/mL IL-2 and 50 ng/mL IL-15 in presence or absence of 20 ng/mL TGF-β. Some culture wells also received 0.1 µg/mL anti-CD28 mAb at the beginning of activation. Three days post-activation, CD3+, CD8+ T cells were analyzed by flow cytometry for CD44 and

granzyme B expression. (A) Representative flow cytometry examples of CD44 expression in TGF-β untreated or treated CD8+ T cells. (B) Quantification of data shown in (A) for all the mice. (C) Representative flow cytometry examples of granzyme B expression in TGF-β untreated or treated CD44 hi CD8+ T cells (D) Quantification of data shown in (E) for all mice. (E) Quantification of susceptibility to TGF-β suppression calculated from (D). (F) CD28 expression on CD8+ T cells from freshly isolated WT and NKG2D KO OT-1 splenocytes. For (B), (D), and (E), pooled data of n = 8 WT and n = 11 NKG2D OT-1 mice from 4 independent experiments are presented, each dot represents one mouse. The histogram in (F) is representative of n = 7 WT and n = 9 KO mice from three independent experiments. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ determined by unpaired, two-tailed Student's t test. FMO = fluorescence minus one, ns = not significant.

CD3 activated CD44 hi WT and NKG2D KO cells also showed similar frequencies of granzyme B positive cells, and as expected, the addition of anti-CD28 mAb in the cultures significantly enhanced the levels of granzyme B expressing cells in both groups (Figure 22, C and D). These results confirmed that the canonical CD28 co-stimulatory pathway was intact in both WT and NKG2D KO OT-1 CD8+ T cells. When TGF-β responses at the level of granzyme B were assessed following CD3 activation, similar to our findings in Figure 16, WT cells were highly prone to TGF-β mediated suppression (Figure 22, C-E). While NKG2D deficient cells were slightly more sensitive to TGF-β, this difference did not reach statistical significance when multiple experiments were analyzed together (Figure 22, D and E). As expected, WT cells that received CD28 co-stimulation showed a significantly higher percent of granzyme B expressing cells under the influence of TGF-β (Figure 22, C and D), and therefore were highly resistant to its suppression when compared to CD3 alone (Figure 22E). However, contrary to our expectations, CD28 was unable to elevate the frequencies of granzyme B positive cells in TGF-β treated NKG2D KO cells as much as their WT counterparts (50% for WT vs. 22% for KO in TGF-β treated group, Figure 22, C and D). Accordingly, although CD28 significantly reversed the TGF-β mediated suppression in NKG2D KO group, the magnitude of this reversal was remarkably lower, rendering them still significantly more susceptible to TGF-β compared to WT group (Figure 22E). To rule out any inherent dissimilarities in the CD28 expression on CD8+ T cells of WT and NKG2D KO OT-1 mice, we confirmed that the basal levels of CD28 were comparable on CD8+ T cells

from the spleens of these two mice (Figure 22F). To conclude, despite being able to provide its canonical co-stimulatory function, CD28 failed to confer TGF-β resistance in NKG2D KO CD8+ T cells to the degree observed in WT cells.

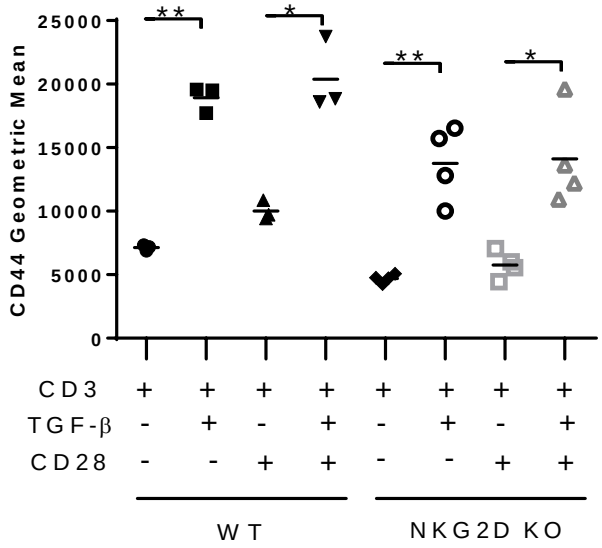


Figure 23. TGF-β Up-Regulates CD44 Expression on Per Cell Basis in CD3/CD28 Activated WT and NKG2D KO OT-1 CD8+ T Cells. WT and NKG2D KO OT-1 splenocytes were activated with 0.1 µg/mL anti-CD3 mAb and 20 IU/mL IL-2 and 50 ng/mL IL-15 in presence or absence of 20 ng/mL TGF-β. Some culture wells also received 0.1 µg/mL anti-CD28 mAb at the beginning of activation. Three days post-activation, cells were analyzed by flow cytometry for CD44 expression. Geometric means of CD44 on CD44 hi CD8+ T cells are shown. Error bars denote standard deviation. n = 3 for all groups. * $p < 0.05$ ** $p < 0.01$ determined by unpaired, two-tailed Student's t-test. The data are representative of at least three independent experiments with similar results.

NKG2D's Mechanisms of TGF-β Resistance

Genetic Disruption of DAP10 Signaling to Determine NKG2D's Proximal Mechanism Driving TGF-β Resistance.

NKG2D receptor does not contain of any signaling motif in its structure. However, its adaptor DAP10, which consists of YINM motif, could recruit PI3K and GrB2 upon NKG2D activation and trigger activation of PI3K→Akt and GrB2→Erk or JNK2 pathways. We extended our studies to investigate which of NKG2D's two signaling arms i.e. PI3K or GrB2 is responsible for resistance to TGF-β. In order to do that, we generated mutants of DAP10 that are deficient in the recruitment and sub-sequent phosphorylation of either PI3K or GrB2

or both. We obtained full-length DAP10 gene, and generated following point mutations using site directed mutagenesis approach.

1. M88V (YINM→YINQ) to abrogate PI3K signaling
2. N87Q (YINM→YIQM) to abrogate Grb2 signaling
3. M88V and N87Q (YINM→FINM) to abrogate PI3K and Grb2 signaling pathways

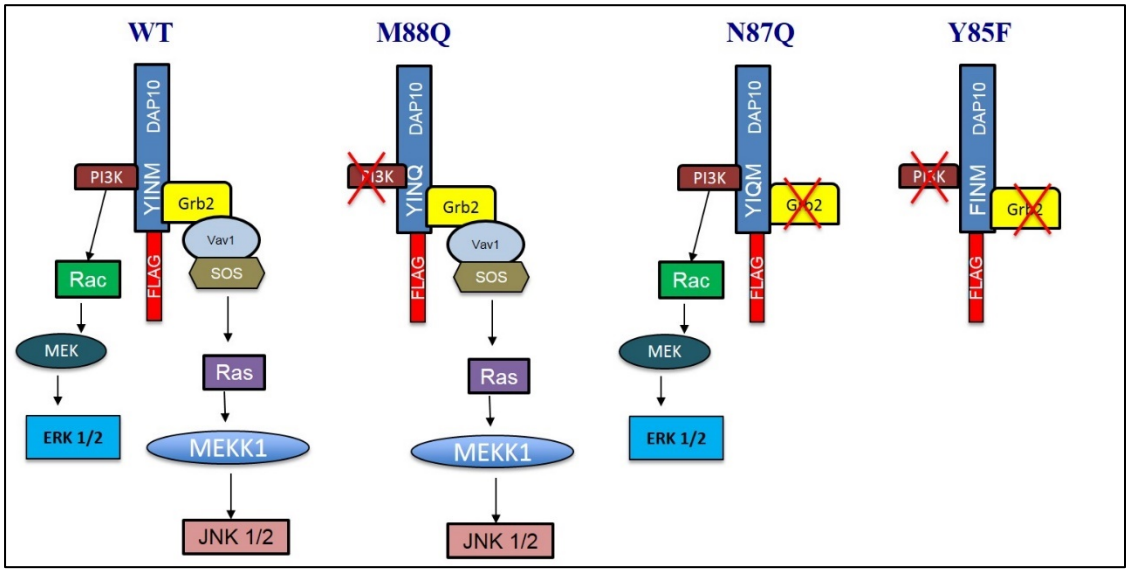


Figure 24. Generated DAP10 Mutants to Study NKG2D's Proximal Mechanism Responsible for TGF-β Resistance. The point mutations inserted in the YINM signaling motif of WT DAP10 and the resultant signaling changes are depicted in this schematic. All the WT and mutant DAP10 genes were sub-cloned into a vector so that they express FLAG tag at their C terminus (shown in red).

As mentioned earlier, NKG2D is expressed on the surface of CD8+ T cells in association with its signaling adaptor protein DAP10. Hence, CD8+ T cells from mice that lack DAP10 do not express NKG2D on their surface [187]. When we retro-virally transduced GFP-tagged vectors carrying WT and mutant DAP10 genes (shown in Figure 24) in the DAP10 KO T cells, NKG2D surface expression was restored in the GFP positive DAP10 KO cells (Figure 25, A and B). Further, in GFP positive cells expression of NKG2D was correlated with the expression of FLAG, indicating that the rescue of NKG2D was indeed due to the expressed DAP10 (Figure 25C). Thus, all the DAP10 constructs we generated were able to restore the surface expression of NKG2D in DAP10 KO CD8+ T cells.

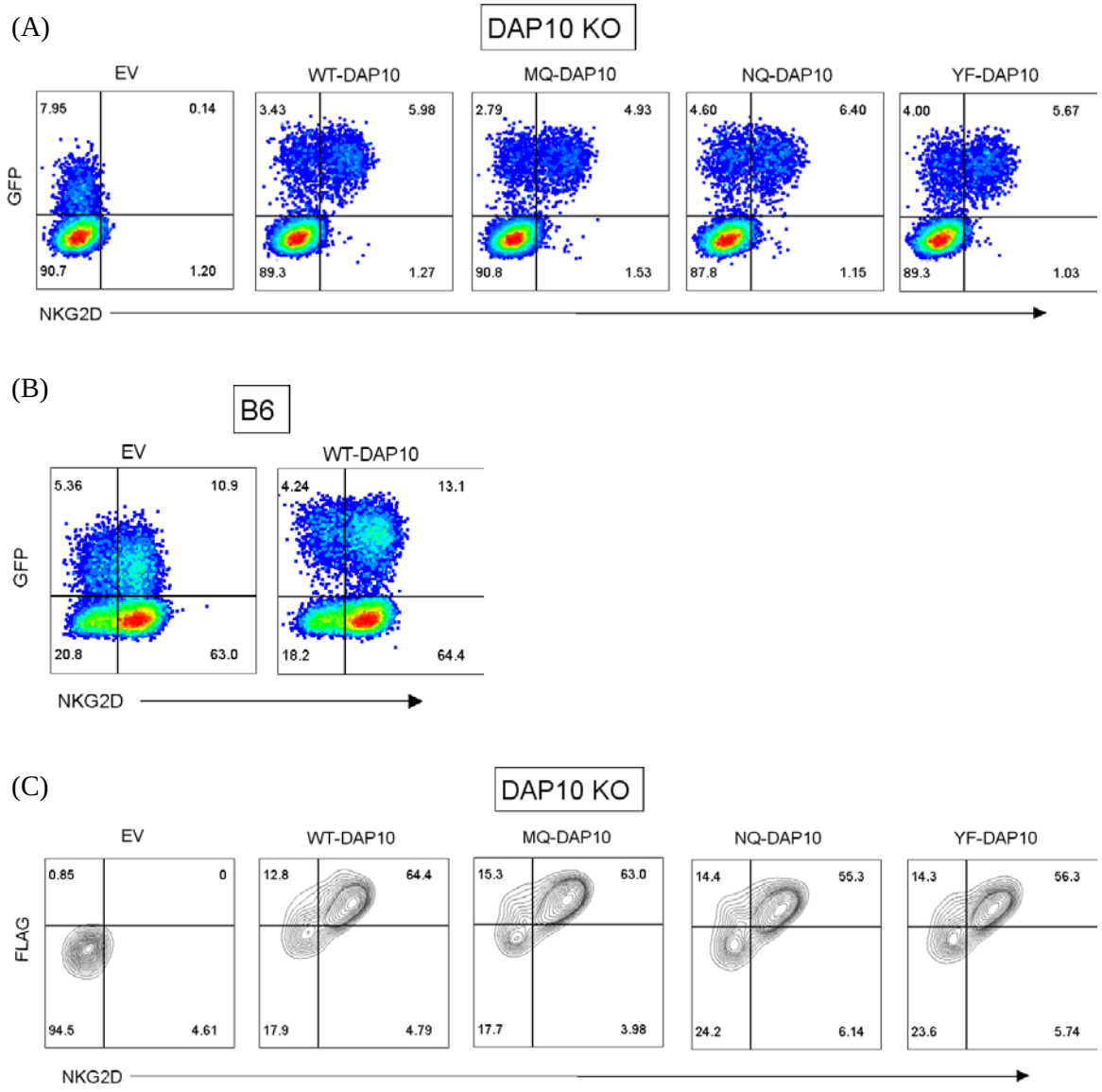


Figure 25. Forced DAP10 Expression Rescues Surface Expression of NKG2D in DAP10 KO CD8+ T Cells. The splenocytes from DAP10 KO mice were isolated and retro-virally transduced with the indicated vectors as described in materials and methods for four days. On day 5, CD8+ T cells were analyzed for (A, B) GFP and NKG2D expression. GFP positive CD8+ T cells were further analyzed for (C) FLAG and NKG2D expression. B6 cells shown in (B) served as positive control for NKG2D expression. The data are representative of at least three independent experiments with similar results.

Having confirmed the expression and adaptor function of DAP10 genes in DAP10 KO cells (Figure 25), we next sought to establish a TGF-β/granzyme B assay in transduced CD8+ T cells to study DAP10 signaling in TGF-β resistance. Accordingly, DAP10 KO splenocytes were activated and transduced with EV, WT or YF-DAP10. Following transduction (day 0), the cells were re-stimulated with increasing concentrations of coated

anti-CD3 mAb and granzyme B expression was measured 3 days post-stimulation (day 3). We found that upon TCR re-stimulation, transduced T cells did not appreciably up-regulate granzyme B expression (Figure 26) presumably due to already saturating levels of granzyme B. This prevented us from using intra-cellular granzyme B expression as a functional read-out for transduced T cells.

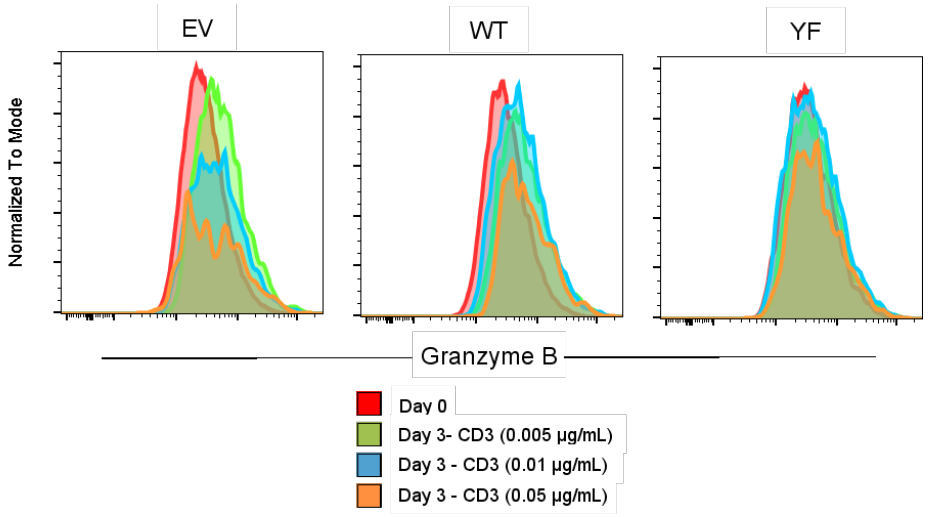
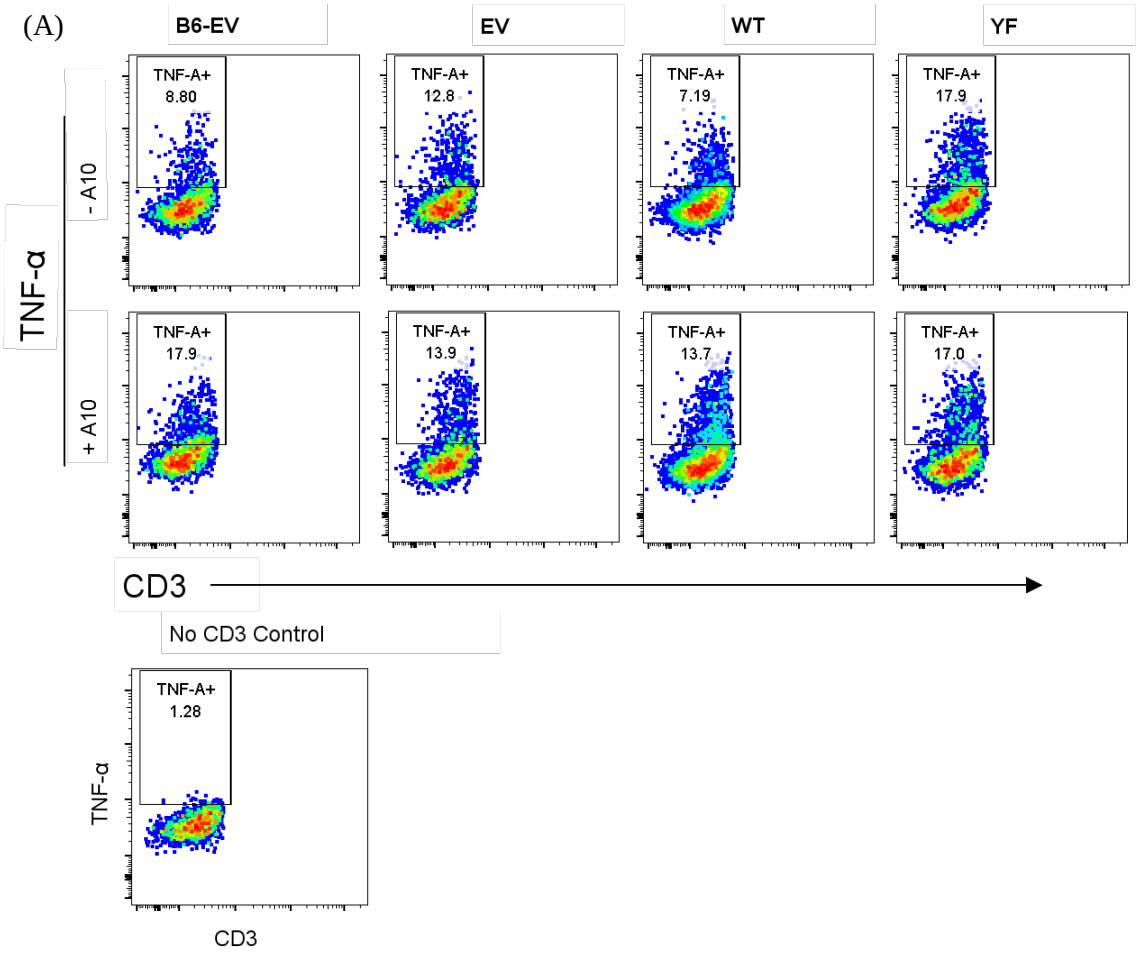


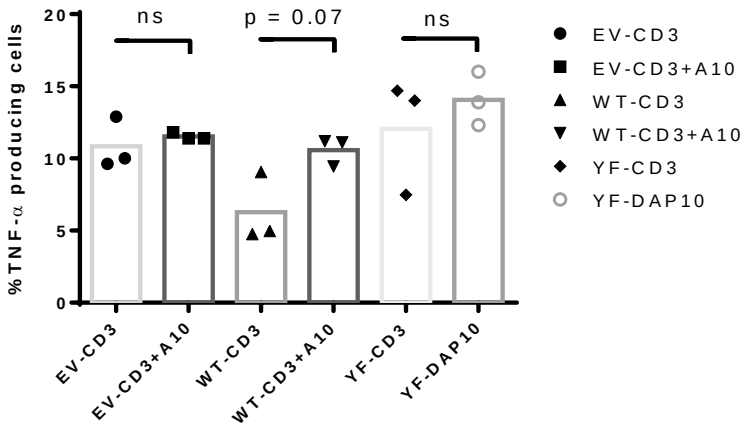
Figure 26. Granzyme B Levels in Activated CD8+ T Cells are Saturated. The splenocytes from DAP10 KO mice were isolated and retro-virally transduced with the indicated vectors as described in materials and methods for four or five days. Following transduction (day 0), the cells were supplemented with fresh IL-2 and IL-15 in complete IMDM and cultured in a plate coated with indicated concentrations of anti-CD3 mAb for 3 days. On day 0 (before re-stimulation) and day 3 (after re-stimulation), granzyme B expression in GFP positive CD8+ T cells was measured. The data are representative of two independent experiments with similar results.

As granzyme B expression was not a reliable functional read-out in the transduced T cells, we wanted to determine if production of cytokines IFN-γ, TNF-α and IL-2 could be used to assess the functionality of DAP10 KO T cells transduced with WT or mutant DAP10 constructs. To this end, DAP10 KO T cells transduced with EV, WT or YF- DAP10 constructs were re-stimulated with plate-bound 0.05 µg/mL mouse anti-CD3 mAb in complete IMDM for 4 hours and production of TNF-α, IFN-γ, and IL-2 was measured by flow cytometry. We found that only upon re-stimulation with anti-CD3 mAb, intra-cellular levels of TNF-α (Figure 27, A and B) and IFN-γ (data not shown) were increased in all the transduced cells. Therefore, intra-cellular TNF-α and IFN-γ expression were a reliable read-

out to measure the function of transduced CD8+ T cells. Next, using this read-out, we also tested the functionality of WT and YF-DAP10 constructs in DAP10 KO CD8+ T cells by crosslinking NKG2D receptor using activating mouse NKG2D mAb (A10) in conjunction with TCR. As expected, DAP10 KO CD8+ T cells transduced with WT DAP10, but not non-functional YF DAP10, showed increased production of TNF-α (Figure 27) upon NKG2D cross-linking. B6 CD8+ T cells transduced with EV served as positive control for this experiment. These results confirmed that our WT DAP10 construct indeed restored the NKG2D-DAP10 signaling in DAP10 KO T cells whereas mutating tyrosine (Y) to phenylalanine (F) in its YINM signaling motif completely abrogated this signaling pathway.



(B)



(C)

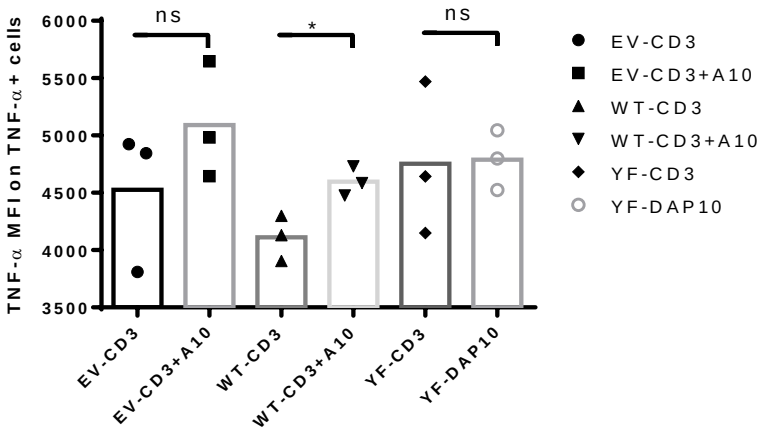
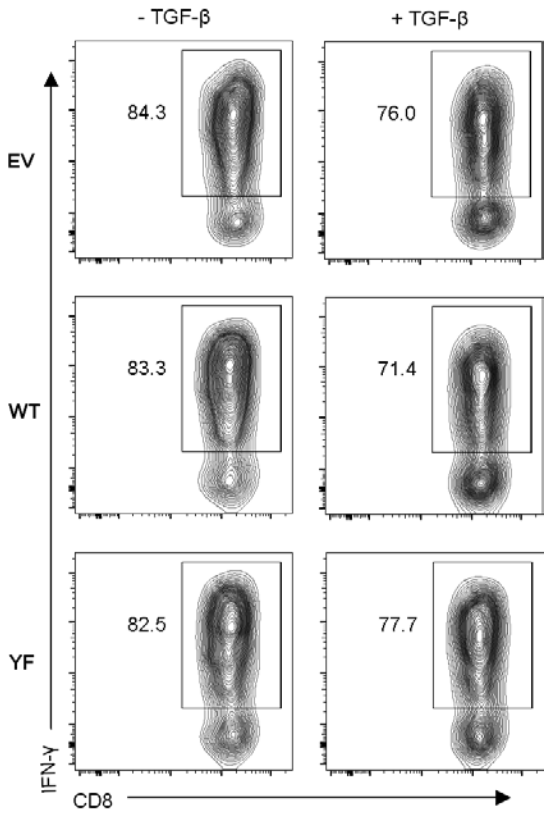


Figure 27. Cytokine Production as a Functional Read-Out and NKG2D Activation. The splenocytes from DAP10 KO mice were isolated and retro-virally transduced with the indicated vectors as described in materials and methods for four or five days. Transduced cells were re-stimulated with 0.05 µg/mL plate-bound mouse anti-CD3 mAb with or without 10 µg/mL activating mouse anti-NKG2D mAb in complete IMDM and the levels of intra-cellular cytokines were measured 4 hours later by flow cytometry. Brefeldin A was added into the cultures at the beginning of the stimulation. (A) Representative flow cytometry example and (B) quantification of TNF-α positive cells among GFP+ CD8+ T cells is shown. B6 cells transduced with EV shown in (A) served as positive control for this experiment. In (C), mean fluorescence intensities of TNF-α within TNF-α positive population were quantified. n = 3 technical replicates for all groups, each data point represents one experimental well. * $p < 0.05$ determined by unpaired, two-tailed Student's t-test. The data are representative of two independent experiments with similar outcome. MFI = mean fluorescence intensity, ns = not significant.

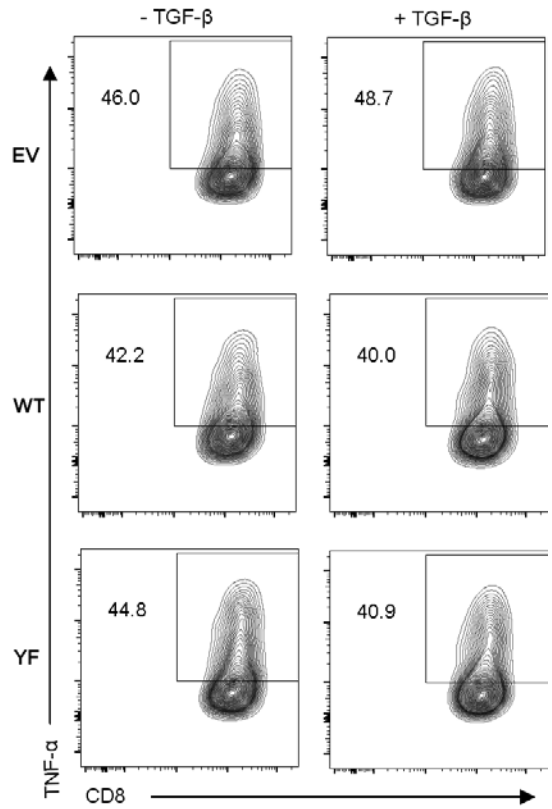
Our goal for the generation of DAP10 mutants and an accurate read-out to measure the function of transduced T cells was to determine DAP10's signaling pathway responsible for mediating resistance to TGF-β in CD8+ T cells. Hence, our next objective was to establish a robust TGF-β suppression assay on DAP10 KO T cells transduced with various DAP10

mutants. In order to do that, DAP10 KO splenocytes were transduced with EV, WT or YF-DAP10 constructs, and five days later the cells were stimulated with plate-bound mouse anti-CD3 mAb in the presence or absence of 20 ng/mL TGF-β for overnight. The next day, intra-cellular expression of TNF-α and IFN-γ in GFP positive CD8+ T cells was assessed by flow cytometry. We found that anti-CD3 stimulation resulted in a remarkable production of IFN-γ and TNF-α (about 82% and 40% respectively, Figure 28) in all the experimental groups. Addition of TGF-β in the cell culture, induced either a negligible or no suppression in the proportions of transduced CD8+ T cells producing IFN-γ or TNF-α (Figure 28). The % susceptibility to TGF-β observed in Figure 28 was only 3-10% for IFN-γ production and 0% for TNF-α (calculated based on mean values shown in Figure 28, C and D). To increase the robustness of this assay, we performed similar experiment with differing conditions such as increased concentration of TGF-β, reduced concentrations of IL-2/IL-15, pre-treatment of the cells with TGF-β, and varying time point/duration of brefeldin A incubation in the culture. However, in all these conditions the suppression to TGF-β continued to remain weak (data not shown). This low TGF-β susceptibility of transduced CD8+ T cells did not permit us to dissect the contribution of DAP10 signaling pathways in TGF-β resistance in a conclusive manner.

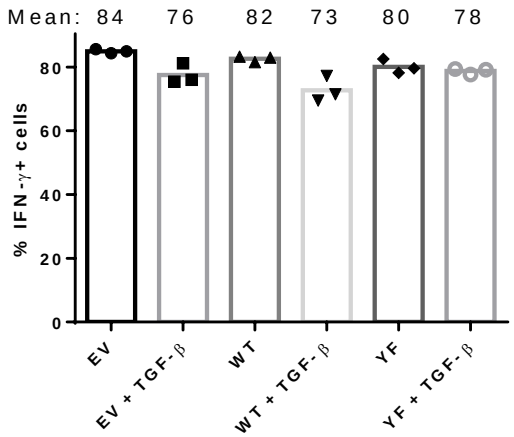
(A)


- TGF-β
+ TGF-β
EV
WT
YF
84.3
76.0
83.3
71.4
82.5
77.7
IFN-γ
CD8

(B)


- TGF-β
+ TGF-β
EV
WT
YF
46.0
48.7
42.2
40.0
44.8
40.9
TNF-α
CD8

(C)


Mean: 84 76 82 73 80 78
% IFN-γ+ cells
0
20
40
60
80
EV
EV + TGF-β
WT
WT + TGF-β
YF
YF + TGF-β

(D)

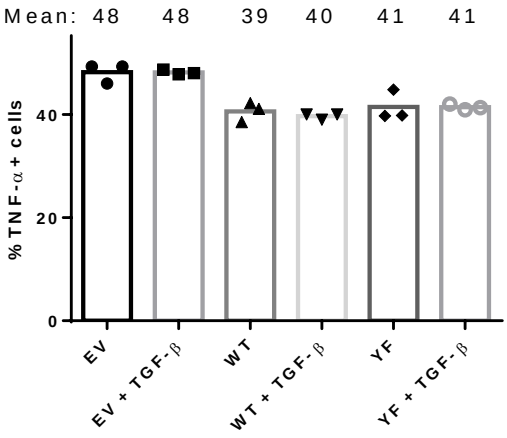


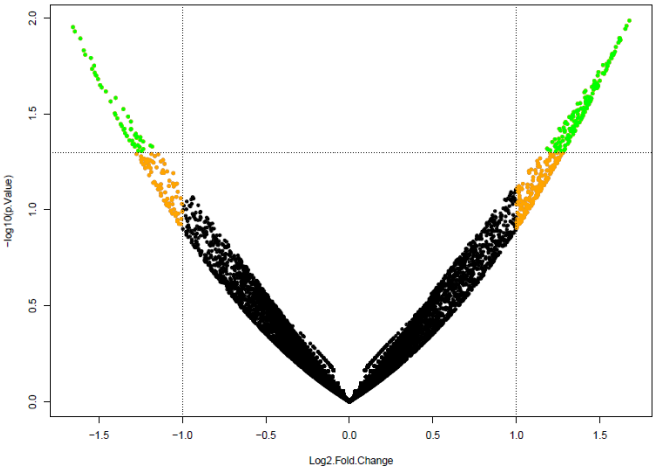
Figure 28. Transduced T Cells are Not Potently Suppressed by TGF-β. The splenocytes from DAP10 KO mice were isolated and retro-virally transduced with the indicated vectors as described in materials and methods for four or five days. Transduced cells were re-stimulated with 1 µg/mL plate-bound mouse anti-CD3 mAb with or without 20 ng/mL TGF-β in complete IMDM supplemented with cytokines IL-2 and IL-15, and the levels of intra-cellular cytokines were measured 24 hours later by flow cytometry. Brefeldin A was added into the cultures 8 hours after beginning of the stimulation. (A, B) Representative flow cytometry examples and (C, D) quantification of IFN-γ and TNF-α positive cells among GFP+ CD8+ T cells is shown. n = 3 technical replicates for all groups, each data point represents one experimental well. The data are representative of two independent experiments with similar outcome.

Identification of NKG2D Signaling Associated Genetic Changes Implicated in TGF-β Resistance in CD8+ T Cells.

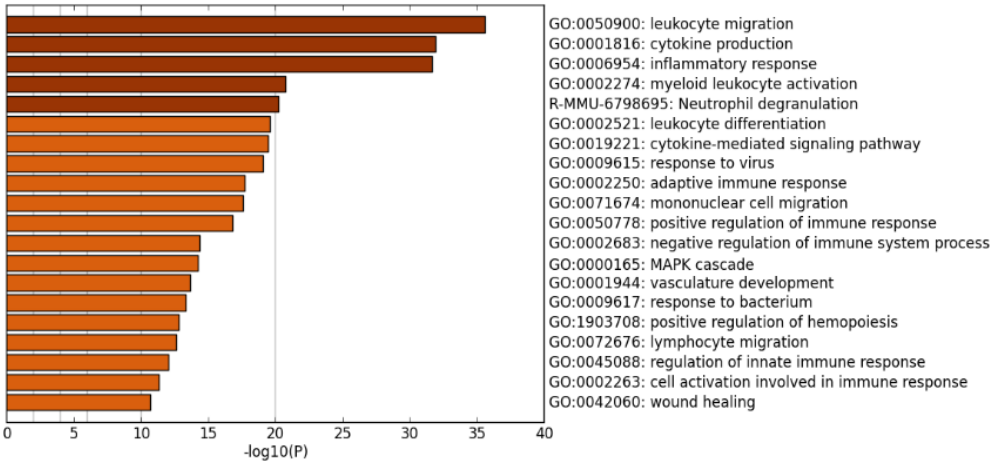
Due to the technical problems encountered in previous approach to study DAP10 signaling, we shifted our focus towards an alternative approach of identifying NKG2D's mechanisms involved in opposing TGF-β signaling. In this approach, we sought to determine changes in the genetic program associated NKG2D signaling induced TGF-β resistance in CD8+ T cells. To this end, we performed RNA sequencing analysis on mRNA samples isolated from CD44 expressing CD8+ OT-1 WT or NKG2D KO cells activated with DC in the presence of IL-2 and IL-15 as described in Fig 18. We found expression of a total of 324 genes significantly different in NKG2D KO CD8+ T cells compared to WT (Figure 29A). Gene enrichment analysis revealed significant changes in at least 20 biological clusters (Figure 29, B and C), wherein major ones were leukocyte migration, cytokine production,

inflammatory response, and cytokine-mediated signaling pathway (Figure 29, B and C). As expected, we also identified significant changes between WT and NKG2D KO cells in a group of 34 genes that have been shown to affect TGF-β's biological activity directly or indirectly (Figure 29D, Table 9 and 10). In this group, the majority (27) of the genes were positive regulators of TGF-β activity, and among them, 23 genes were up-regulated in NKG2D KO CD8+ T cells (Figure 29D, Table 9). On the other hand, among other 7 genes that negatively regulate TGF-β function, 5 were down-regulated in NKG2D KO (Figure 29D, Table 10). Few noteworthy genes that induce TGF-β activity were *IRF7, S100A4*, *Ahnak*, *NRP2*, *EGR1*, *LGALS3*, *MMPs*, and *CTSD* (Table 9) whereas the ones that inhibit TGF-β activity included *CD80*, *PTPN14*, *NDRG2*, and *TNFRSF4* (Table 10). Taken together, loss of NKG2D in CD8+ T cells led to significant changes in the transcriptional program that elevate the responsiveness to TGF-β.

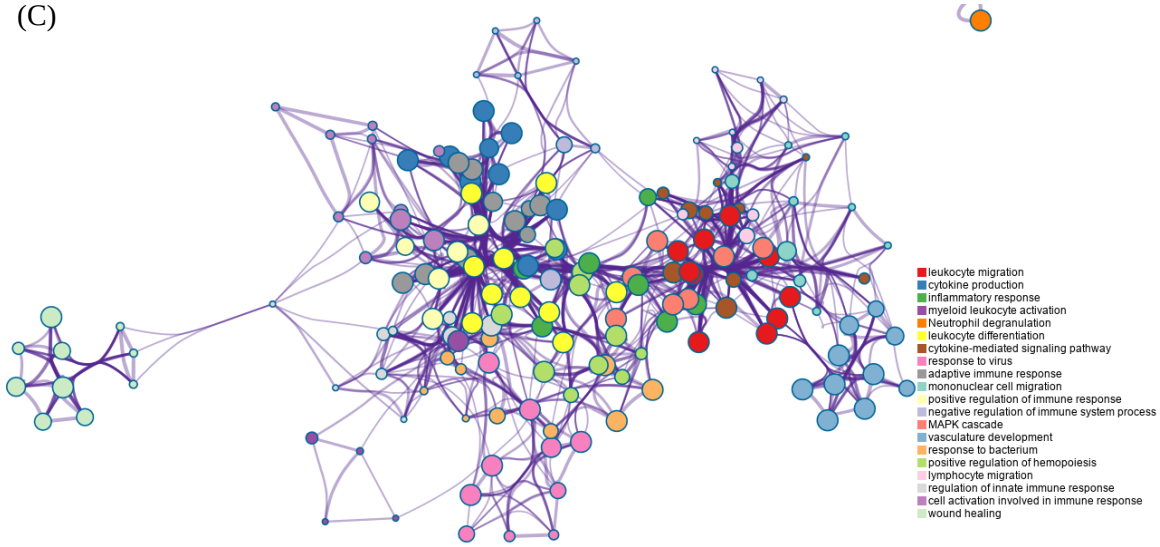
(A)


−log10(p.Value)
Log2.Fold.Change

(B)


GO:0050900: leukocyte migration
GO:0001816: cytokine production
GO:0006954: inflammatory response
GO:0002274: myeloid leukocyte activation
R-MMU-6798695: Neutrophil degranulation
GO:0002521: leukocyte differentiation
GO:0019221: cytokine-mediated signaling pathway
GO:0009615: response to virus
GO:0002250: adaptive immune response
GO:0071674: mononuclear cell migration
GO:0050778: positive regulation of immune response
GO:0002683: negative regulation of immune system process
GO:0000165: MAPK cascade
GO:0001944: vasculature development
GO:0009617: response to bacterium
GO:1903708: positive regulation of hemopoiesis
GO:0072676: lymphocyte migration
GO:0045088: regulation of innate immune response
GO:0002263: cell activation involved in immune response
GO:0042060: wound healing
-log10(P)

(C)


leukocyte migration
cytokine production
inflammatory response
myeloid leukocyte activation
Neutrophil degranulation
leukocyte differentiation
cytokine-mediated signaling pathway
response to virus
adaptive immune response
mononuclear cell migration
positive regulation of immune response
negative regulation of immune system process
MAPK cascade
vasculature development
response to bacterium
positive regulation of hemopoiesis
lymphocyte migration
regulation of innate immune response
cell activation involved in immune response
wound healing

(D)

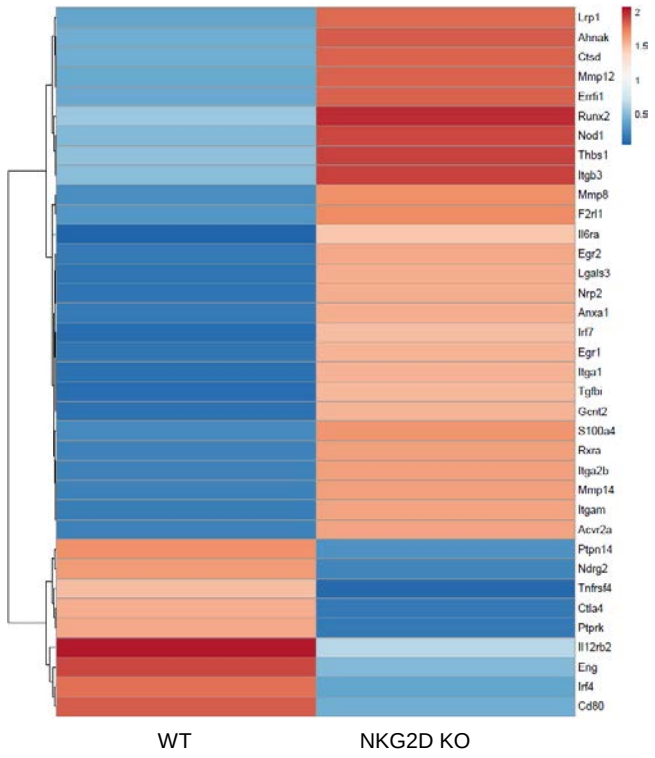


Figure 29. NKG2D Predominantly Represses the Expression of Genes Linked to Positive Regulation of TGF-β Pathway. CD8+ T cells from WT and NKG2D KO OT-1 splenocytes were isolated and activated by co-culturing with dendritic cells pulsed with 0.01 μg/mL OVA peptide in presence 20 IU/mL IL-2 and 50 ng/mL IL-15. Four days after activation, CD44 expressing cells were isolated by magnetic selection followed by total mRNA extraction. Efficiency of CD44 isolation and the phenotypic differences in the TGF-β susceptibility for WT and NKG2D OT-1 cells were confirmed for this experiment by flow cytometry (data not shown). Isolated mRNA samples were subjected to RNA sequencing and gene expression profiles were compared between WT and NKG2D KO (A) Volcano plot for differentially expressed genes (B) Top 20 significantly changed gene ontology (GO) clusters identified in gene set enrichment analysis with their significance levels (C) Network plot of differentially expressed clusters. (D) Heat map showing changes in the expression of genes associated with the regulation of TGF-β activity.

Table 9. Genes Associated with Positive Regulation of TGF-β or Smad Activity.

Number	Gene	Protein	Change in KO	Reference(s)
1	*IRF7*	Interferon regulatory factor 7	Upregulated	[267]
2	*S100A4*	S100 Calcium Binding Protein A4	Upregulated	[268]
3	*AHNAK*	AHNAK	Upregulated	[269]
4	*NRP2*	Neuropilin 2	Upregulated	[270]
5	*ANXA1*	Annexin A1	Upregulated	[271]
6	*ACVR2A*	Activin A Receptor Type 2A	Upregulated	[272]
7	*F2RL1*	Proteinase-Activated Receptor-2	Upregulated	[273]
8	*GCNT2*	Glucosaminyl (N-Acetyl) Transferase 2, I-Branching Enzyme	Upregulated	[274]
9	*LRP1*	Low density lipoprotein receptor-related protein 1	Upregulated	[275]
10	*RUNX2*	Runt Related Transcription Factor 2	Upregulated	[276]
11	*EGR1*	Early Growth Response 1	Upregulated	[277]
12	*EGR2*	Early Growth Response 2	Upregulated	[278]
13	*LGALS3*	Galectin 3	Upregulated	[279]
14	*NOD1*	Nucleotide Binding Oligomerization Domain Containing	Upregulated	[280]
15	*IL6RA*	IL-6 Receptor Subunit Alpha	Upregulated	[281]
16	*MMP14*	Matrix Metallopeptidase 14	Upregulated	[282]
17	*MMP12*	Matrix Metallopeptidase 12	Upregulated	[283]
18	*MMP8*	Matrix Metallopeptidase 8	Upregulated	[283]
19	*CTSD*	Cathepsin D	Upregulated	[284]
20	*THBS1*	Thrombospondin 1	Upregulated	[285]
21	*ITGB3*	Integrin subunit beta 3	Upregulated	[286]
22	*ITGA1*	Integrin subunit alpha 1	Upregulated	[286]
23	*TGFBI*	Transforming Growth Factor Beta Induced	Upregulated	-
24	*CTLA4*	Cytotoxic T-Lymphocyte Associated Protein 4	Downregulated	[231]
25	*IRF4*	Interferon regulatory factor 4	Downregulated	[287]
26	*PTPRK*	Protein Tyrosine Phosphatase, Receptor Type K	Downregulated	[288]
27	*ENG*	Endoglin	Downregulated	[289]

Table 10. Genes Associated with Negative Regulation of TGF-β or Smad Activity.

Number	Gene	Protein	Change in KO	Reference(s)
1	*CD80*	CD80	Downregulated	[136,235,236]
2	*PTPN14*	Protein Tyrosine Phosphatase, Non-Receptor Type 14	Downregulated	[290]
3	*NDRG2*	N-Myc Downstream-Regulated Gene 2 Protein	Downregulated	[291]
4	*Tnfrsf4*	TNF Receptor Superfamily Member 4 (OX40)	Downregulated	[234]
5	*Il12rb2*	Interleukin 12 Receptor Subunit Beta 2	Downregulated	[292]
6	*RXRA*	Retinoid X Receptor Alpha	Upregulated	[293]
7	*ERRFI1*	ERBB Receptor Feedback Inhibitor 1	Upregulated	[294]

CHAPTER IV

DISCUSSION

TGF-β's suppressive effects on CD8+ T cells is a well-recognized biological problem that is expected to limit clinical outcomes of cancer immunotherapies [42,86,295]. Although many strategies to neutralize TGF-β's effects have been designed [43,86,170], molecular blockade of TGF-β must be approached with caution as it has resulted in lethal, auto-immune pathologies in mice [113,114]. In this context, our objective in this study was to better understand the biological mechanisms utilized by CD8+ T cells to regulate the TGF-β responsiveness. NKG2D, a YxNM co-stimulatory receptor exerts potent stimulatory effects in CD8+ T cells that are opposite to that of TGF-β [182]. Perhaps, representative of its importance in this context is the observation that one of the mechanisms by which TGF-β suppresses function of CD8+ T cells is by down-regulating surface expression of NKG2D-DAP10 complex [213,214]. The studies addressing relationship between TGF-β and NKG2D have been mostly unidirectional, namely, focusing on the biology of TGF-β's negative effects on NKG2D. However the impact of NKG2D activation on TGF-β's activity in CD8+ T cells has not been studied. Here, we show for the first time that NKG2D signaling makes CD8+ T cells resistant to the suppressive effects of TGF-β.

Our data on human CD8+ T cells from 22 healthy donors was particularly important in hypothesizing NKG2D's role in controlling susceptibility to TGF-β. First, we found that despite TGFBR2's described role in augmenting TGF-β signaling [259], the majority (77%) of TGF-β resistant donors expressed higher levels of TGFBR2. These counter-intuitive findings suggested crucial roles of other mechanisms in driving TGF-β resistance in CD8+ T cells. Interestingly, the majority (66%) of donors that were resistant to TGF-β, also expressed

high levels of NKG2D. However, the observation showing that 33% of NKG2D Hi donors are susceptible to TGF-β must be attributed to NKG2D-unrelated mechanisms of TGF-β resistance. Collectively, these results indicated an important role of NKG2D in opposing TGF-β's effects in human CD8+ T cells.

To be able to study the function of NKG2D under immune suppressive conditions we used the highly immune suppressive B16 melanoma. This model mimics the clinical scenario of tumor-induced, TGF-β driven immune-suppression in WT and NKG2D KO mice. Importantly, to study the effects of immune-suppression independently of anti-tumor responses, we measured and characterized CD8+ T cells responses elicited by a foreign antigen OVA. We have shown that one prominent way TGF-β exerts its inhibitory effects on immune system is by suppressing polyfunctionality of CD8+ T cells (i.e. their ability to make more than one stimulatory cytokine) [150]. To this end, we found that NKG2D KO cells lost their polyfunctionality by tumor induced immune-suppression more than their WT counterparts. Moreover, blocking TGF-β restored the polyfunctionality in NKG2D KO cells back to WT levels, and induced no significant changes in WT group. These results clearly demonstrated the higher sensitivity of NKG2D KO CD8+ T cells to TGF-β in vivo. When we analyzed the effects of immune-suppression on the production of individual cytokines IFN-γ, TNF-α, or IL-2, we found that proportions of cells producing each of these cytokines were suppressed in all the tumor-bearing mice, however blockade of TGF-β could reverse this immune-suppression in WT but not in NKG2D KO mice. This suggests that absence of NKG2D could also render CD8+ T cells more susceptible to immune-suppressive mechanisms other than TGF-β such as IL-10 or IDO. Overall, we conclude that NKG2D receptor makes CD8+ T cells highly resistant to TGF-β mediated loss in cytokine polyfunctionality *in vivo*.

Our in vivo findings were corroborated in OVA-reactive OT-1 transgenic T cells by two different in vitro activation methods: physiological activation of purified CD8+ T cells

by OVA-pulsed DCs, and non-physiological CD3/CD28 mAb activation. We used activation (conversion to CD44 hi) as well as cytotoxic capacity (granzyme B positive cells) as read outs of TGF-β's activity in CD8+ T cells. Work by Man Lee and Rich has showed that TGF-β treatment increases CD44 expression and proliferation in CD8+ T cells, where authors described these effects indicative of TGF-β's co-stimulatory function on T cells during activation [155]. Our studies show similar positive effects of TGF-β on CD44 expression in WT OT-1 CD8+ T cells. Although in case of DC-activation, the differences in CD44 levels were difficult to detect due to saturating levels of CD44 hi in untreated group, CD44 expression on per cell basis was indeed increased upon TGF-β treatment (data not shown). Surprisingly, this effect of TGF-β on CD44 was completely inverted in NKG2D KO CD8+ T cells when activated with DCs, but not CD3/CD28. Although we cannot fully explain these intriguing results, they point out towards the involvement of NKG2D receptor in mediating TGF-β's co-stimulatory effects during CD8+ T cell activation. Despite many contrasting reports of TGF-β's various effects on T cells, it is important to note that its suppressive effects on granzyme B expression and cytotoxicity of CD8+ T cells is highly consistent across the literature including in earlier mentioned report by Man Lee and Rich [148,155,157]. In concurrence with these studies, we show that TGF-β reduced granzyme B expression in CD44hi CD8+ T cells in all our in vitro experiments. We demonstrate that CD8+ T cells that lacked NKG2D receptor were dramatically more susceptible to TGF-β induced suppression in granzyme B compared to their WT counterparts. Collectively, our results from in vitro studies demonstrate that NKG2D signaling makes CD8+ T cells highly refractory to TGF-β driven inhibition in granzyme B.

In some of our experiments, we purposely excluded the addition of cytokines in the T cell cultures. The rationale for this was cytokines such as IL-2 and IL-15 are shown to counter the inhibitory effects of TGF-β on T cells [222,262,263] and could have potentially overcome TGF-β suppression in NKG2D KO cells. Moreover, stimulatory effects of IL-2 and

IL-15 on T cells are very similar to NKG2D [219], suggesting that they could mask the defects of NKG2D KO cells in our assays. These possibilities were investigated in DC-activation experiments with addition of IL-2 or combination of IL-2, IL-15 in the culture. With no exogenous cytokine support, percentages of NKG2D KO cells producing granzyme B were significantly lower than WT. The combination of IL-2 and IL-15, but not IL-2 alone, reverted the lower granzyme B levels of NKG2D KO cells back to the normal, WT levels. Similar trends were observed at the level of CD44 hi percentages. Nevertheless, the combo could not overcome the defect in TGF-β resistance in NKG2D KO cells. These data clearly show that IL-2 and IL-15 can substitute for NKG2D's positive effects on activation and effector molecule expression, but they are unable to replace NKG2D mediated TGF-β resistance in CD8+ T cells.

One of the goals of our study was to understand the roles of well-known co-stimulatory (CD28) and inhibitory pathways (PD-1 and CTLA-4) in regulating TGF-β responses in CD8+ T cells. Indeed, OT-1 cells were strikingly more sensitive to TGF-β when activated with CD3 compared to DC which clearly points out the role of co-stimulatory factors additional to TCR in driving TGF-β resistance. Manipulation of inhibitory pathways such as PD-1 and CTLA4 have proven to be very successful in augmenting CD8+ T cells' function against cancer [24,26]. Nonetheless, TGF-β has been shown to negatively affect the outcomes of anti-PD-1 and CTLA4 therapy [174–176,296], and mediate exclusion of T cells from TME specifically in the case of PD-1 blockade [297]. To our knowledge, this is the first study that addressed if blocking PD-1 and CTLA4 pathways protect CD8+ T cells against TGF-β suppression. Opposite to our expectation, PD-1 blockade failed to confer TGF-β resistance despite enhancing the effector function of OT-1 T cells. Thus, we conclude that anti-PD-1 mediated enhancement of CD8+ T cells function does not underlie increased resistance to TGF-β. In other words, PD-1 blockade may augment CD8+ T cells effector function only by its 'brute-force'. This also explains the improved efficacy of anti-PD-

1/PDL-1 when it is combined with TGF-β blockade [174–176,296]. On the other hand, inhibition of CTLA4 did not stimulate the effector function in our system despite the presence of CTLA4 and CD80 the surface of OT-1 cells, and the evidence of blocking antibody binding to CTLA4. It is worth noting that CTLA-4 has been shown to primarily act on CD4+ T cells [298] and may explain our observations on CD8+ T cells. Nonetheless, further investigation will be necessary to conclude the role of CTLA4 blockade in regulating sensitivity to TGF-β in CD8+ T cells.

CD28, another YxNM domain containing co-stimulatory receptor, is considered the gold standard of co-stimulation for T cells and has been widely investigated in the context of TGF-β resistance in T cells [136,235,236,299,300]. Various studies have shown that CD28 engagement in conjunction with CD3 overcome suppressive effects of TGF-β on proliferation, cytokine production as well as cytotoxic capacity in T cells [136,235,236]. Our results are consistent with this already described role of CD28 in opposing TGF-β's influence on T cells. In CD8+ T cells expressing CEA-specific immunoreceptors, Koehler *et al.* showed that CD28 could overcome TGF-β's inhibitory effects in proliferation, but not in granzyme B production on per cell basis [236]. In contrast, our results demonstrate that CD28 engagement also reduces TGF-β's suppressive effect on percentages of granzyme B producing cells. It is worth noting however that study by Koehler and colleagues used fully activated, transduced cells unlike naïve CD8+ T cells in our case which could explain different experimental outcomes.

Due to their shared signaling domain and co-stimulatory roles, the cross-talk and inter-relationship between CD28 and NKG2D receptors have received considerable attention [182]. From this standpoint, we present an important discovery that CD28 driven TGF-β resistance in CD8+ T cells is highly dependent on the activity of NKG2D receptor. Interestingly, CD28's canonical co-stimulatory function in NKG2D-deficient cells was not compromised in our study evidenced by significant increase in the granzyme B production.

These results suggest that NKG2D and CD28 synergies with each other in a non-canonical pathway to make CD8+ T cells resistant to TGF-β suppression. The mechanism by which CD28 represses TGF-β's effects on T cells is not fully understood. Studies from Gunnlaugsdottir *et al.* and Koehler *et al.* suggested that CD28's TGF-β antagonistic activity depends on the avidity of TCR/CD3 in T cells [136,236]. They linked the higher TGF-β resistance in T cells activated by high avidity TCR/CD28 stimulation to lower expression of protein Tob, which associates with Smads 2/4 and enhances their DNA binding [301]. Hence, it could be speculated that NKG2D helps CD28 overcome TGF-β suppression by increasing the avidity of TCR and exerting associated molecular changes such as down-regulation of Tob.

We sought to extend this study to determine the mechanisms responsible for NKG2D mediated TGF-β resistance in CD8+ T cells. In order to identify NKG2D's proximal signaling pathway opposing TGF-β's effects in CD8+ T cells, we adopted the approach of genetic disruption of DAP10 signaling motif [194,302]. Despite being successful in generating the DAP10 mutants, earlier shown to be defective in the recruitment of p85 or Grb2 or both, technical and biological problems precluded us from determining NKG2D-DAP10's immediate signaling arm responsible for conferring TGF-β resistance. We show that all the DAP10 constructs i.e. WT and mutants (MQ, NQ and YF) were able to restore NKG2D surface expression in the CD8+ T cells, and at least two of them (WT and YF) were functional in transducing DAP10 signals. The major issue in identifying the TGF-β antagonistic DAP10 pathway was a very low TGF-β sensitivity of fully activated, transduced CD8+ T cells. This is not surprising considering the existing knowledge about CD8+ T cells' differential responsiveness to TGF-β at different activation states and under different culture conditions. At least two independent studies have reported refractoriness of activated, but not naïve, T cells to TGF-β's inhibitory effects on the CTL generation [99] and proliferation [139]. Interestingly, the latter study by Cottrez and Groux also showed that expression of

TGFβRII was lower on activated T cells compared to naïve cells and restoring its levels by IL-10 treatment also restored the sensitivity to TGF-β [139]. These results suggested that lower TGF-β response in differentiated T cells is due to lower availability of its receptor. While we did not compare TGF-β susceptibility in naïve vs activated T cells at the same time in our systems, our collective results from multiple experiments are in concurrence with these findings.

To obtain the clues about the molecular mechanisms by which NKG2D antagonizes TGF-β pathway, we conducted RNA sequencing analysis on WT and NKG2D KO OT-1 CD8+ T cells activated with DCs. We identified significant changes in expression of about 34 genes, which have been linked to TGF-β's biological effects in the literature, among two groups. Twenty-seven of these genes have been shown to enhance TGF-β pathway, and importantly, most them were up-regulated in NKG2D KO group. Notably, the genes encoding proteins that enhanced TGF-β signaling by interacting with Smads (*IRF7, S100A4, AHNAK*) were expressed at higher levels in NKG2D KO. It was shown that Interferon Regulatory Factor-7 (IRF-7) physically interacts with Smad 3 and enhances Smad induced IFN-β promoter activity in HepG2 cells [267]. S100A4, also known as metastatin-1, was shown to bind to N-terminal region of Smad 3 and enhance its transcriptional activity [268]. In the same study S100A4 also increased TGF-β induced expression of MMP-9, which activates latent TGF-β. Interesting work from Lee *et al.* evaluated involvement of Ahnak, a nuclear phosphoprotein, in TGF-β signaling and found that Ahnak binds to MH2 domain of Smad 2 and promotes its nuclear translocation and transcriptional activity [269]. The authors also showed that Ahnak binds to Smad 7 and prevents its interaction with TGFβRI, which would further augment TGF-β signaling.

A set of genes (*NRP2, ANXA1, ACVR2A, F2RL1, GCNT2, LRP1, RUNX2*) up-regulated in NKG2D KO CD8+ T cells was implicated in augmenting TGF-β/Smad pathway by other mechanisms. In 2011, Grandclement *et al.* identified neuropillin-2 (Nrp2), a

neuronal transmembrane receptor, as a co-receptor for TGF-β signaling [270]. Nrp2 promoted TGF-β induced phosphorylation of Smad 2/3, resulting in epithelial to mesenchymal transition (EMT) in colorectal cancer cells. A calcium and phosphoprotein binding protein Annexin A1 increases metastasis in breast cancer cells also by increasing TGF-β/Smad signaling [271], however the underlying mechanism is not known. As mentioned in the introduction, activins also belong to the TGF superfamily and are closely related to TGF-βs in terms of signaling (for example, they also initiate phosphorylation and nuclear translocation of Smad upon activation) and biological effects. Recently, activin signaling was described as an essential component of TGF-β signaling in metastatic colorectal cancer [272]. Hence, one could imagine that increase in the receptors of activins such as Activin receptor type-2A will potentiate TGF-β's effects in T cells. Proteinase activated receptor 2 (PAR2; encoded by gene *F2RL1*) is known to mediate TGF-β induced phosphorylation of Smad 2 and 3, and positively regulate the expression of TGF-β receptor type I. Its functional similarities and cross-talks with TGF-β signaling have been extensively reported in the normal and cancerous cells [273]. Work by Zhang and colleagues demonstrated that the expression of glucosaminyl transferase 2, I-branching enzyme (encoded by gene *GCNT2*) in metastatic breast cancer lines was necessary in the induction of EMT by TGF-β [274].

LDLR related protein-1 (LRP-1), another gene with elevated expression in absence of NKG2D, is a member of low-density lipoprotein receptor (LDLR) family. It is generally involved in the endocytosis and degradation of various ligands but can also modulate activities of many signaling pathways [303]. Tseng *et al.* showed positive contribution of LRP-1 in TGF-β pathway where LRP1 deficient CHO cells were less prone to TGF-β's growth-inhibitory effects [275]. In conjunction with its ligand decorin, LRP1 could enhance TGF-β/Smad signaling in myoblast [304]. It is also interesting that LRP1 is structurally very similar to TGF-β receptor type V [305], hinting at its even direct involvement in TGF-β

pathway. The transcription factor Runx2 was found to transcriptionally up-regulate TGFβRI expression and link TGF-β pathway with Wnt pathway in a work by McCarthy *et al.* [276].

Interestingly, we also found increased expression of group of genes involved in the conversion of latent TGF-β to its activated form in NKG2D KO CD8+ T cells. These genes included *CTSD, MMP 8/12/14, THBS1, ITGA, ITGB3,* which express cathepsin D, matrix metalloprotease 8/12/14, thrombospondin 1, integrin alpha and integrin beta 3. The function of these proteins in activating latent TGF-β is well established [110–112,283,285,286]. Furthermore, the genes found to be important in transducing TGF-β/Smad signaling in the fibrosis were up-regulated in the absence of NKG2D signaling in CD8+ T cells. These genes encoded transcription factors- early growth response 1 and 2 (EGR 1,2) [277,278], intra-cellular NOD1 [280] (nucleotide binding oligomerization domain containing 1) receptor, and galactoside binding protein galectin 3 [279].

On the opposite side, we found 7 genes capable of directly or indirectly antagonizing TGF-β's effects. As expected, 5 of them (*PTPN14, NDRG2, CD80, TNFRSF4, IL12RB2*) were significantly down-regulated in the CD8+ T cells that lacked NKG2D receptor. Non-receptor tyrosine phosphatase 14 (PTPN14) blocks the activity of Yes Associate Protein (YAP), which was recently found to positively regulate TGF-β/Smad signaling [290]. In rat models, N-Myc downstream regulated gene-2 (NDRG2) attenuates hepatic fibrosis by antagonizing TGF-β/Smad pathway [291]. *TNFRSF4* gene encodes co-stimulatory receptor OX-40 which counters TGF-β dependent conversion of CD4+ T cells into Foxp3+ CD25+ T cells [234]. Reduction in the CD80 expression in T cells may also lead to lower CD28 signaling and associated TGF-β resistance, ultimately increasing susceptibility to the TGF-β suppression. Finally, we observed NKG2D mediated changes in the genes that could alter the TGF-β responsiveness by altering cytokine signaling in CD8+ T cells. The alpha subunit of IL-6 receptor (*IL6RA*) and beta-2 subunit of IL-12 receptor (*IL12RB2*) in NKG2D KO T cells were up and down-regulated respectively. Work by Zhang and colleagues have shown that

IL-6 increases compartmentalization/turnover of TGF-β receptors and enhances down-stream signaling [281]. IL-12 on the other hand opposed TGF-β's T cell development programs and its differentiation of Th1 cells [292].

Based on these results, we propose that NKG2D confers TGF-β resistance by predominantly repressing the expression of set of genes that promote TGF-β activation and signaling in CD8+ T cells. We hope that these findings will provide a rationale for the future studies addressing NKG2D's mechanism responsible for mediating TGF-β resistance.

Working Model.

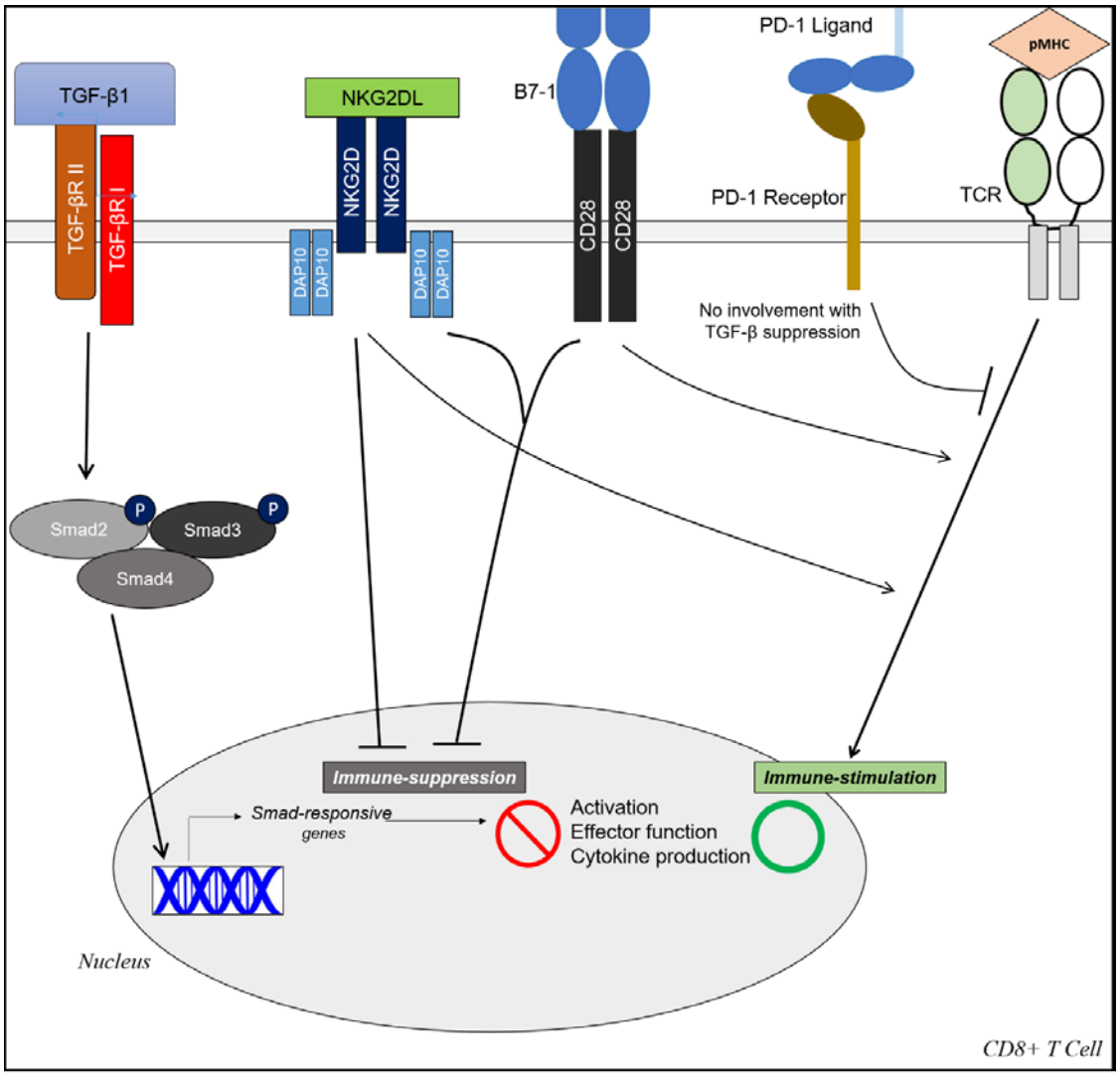


Figure 30. Working model of the findings of this dissertation. In CD8+ T cells, co-inhibitory pathway PD-1 inhibits the TCR mediated activation and enhancement of the effector function, and plays no role in mediating TGF-β's suppressive effects. Co-stimulatory receptors NKG2D and CD28 augment TCR activation and acquisition of effector molecules independently. NKG2D signaling potently inhibits TGF-β mediated tumor-induced immune-

suppression. Further, NKG2D is required for CD28 driven TGF-β resistance (but not co-stimulation) in CD8+ T cells.

Proposed Model of NKG2D's Mechanism of TGF-β Resistance.

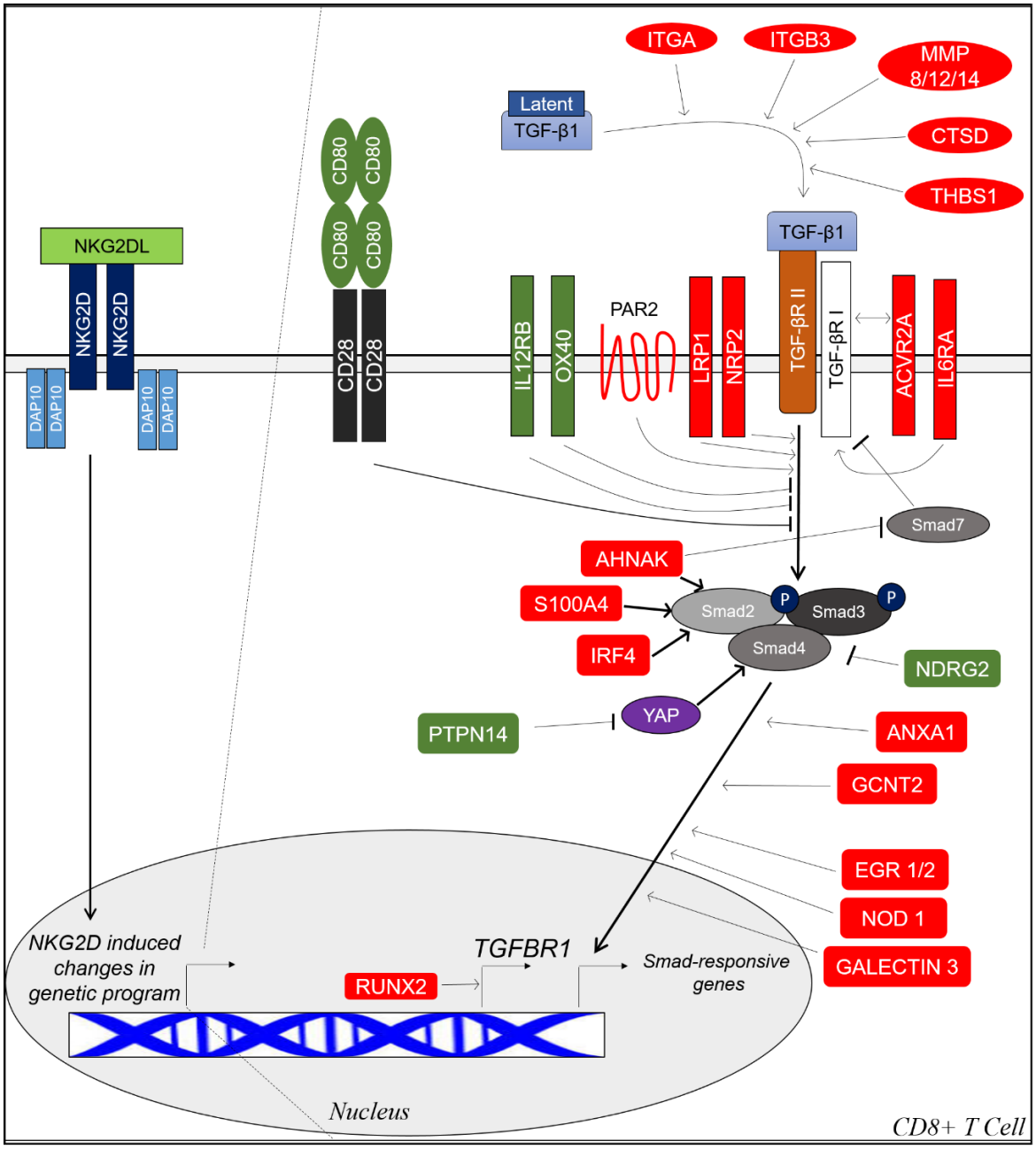


Figure 31. Proposed model of NKG2D mediated mechanisms conferring TGF-β resistance. NKG2D alters the expression of the indicated genes to diminish TGF-β/Smad signaling in CD8+ T cells. The genes shown in red are repressed and the genes in green are up-regulated by NKG2D signaling. The genes with the bold arrows interact directly with Smads.

Future Directions

The findings of this dissertation shall open the doors to new research endeavors and

therapeutic opportunities to improve the clinical outcomes of cancer immunotherapies. The focus of this study was on the interplay between NKG2D and TGF-β signaling and our data clearly show that these pathways counteract each other, which could be visualized as nature's evolutionary mechanism to control CD8+ T cells activity via two opposing forces. In this context, it is plausible to hypothesize that NKG2D receptor also inhibits other immune-suppressive mechanisms such as IL-10, VEGF, or IDO in CD8+ T cells. The combination of IL-2 and IL-15, although failed to restore TGF-β resistance, could enhance the activation in NKG2D deficient CD8+ T cells to some extent. It will be interesting to test other various γc cytokines (e.g. combinations of IL-7, IL-4) or non-γc cytokines (e.g. TNF-α, IFN-γ, GM-CSF) in the same setting to identify the individual or combination of cytokines that have either redundancy with NKG2D signaling or superior capacity to counter TGF-β's inhibitory effects in T cells.

One of the most intriguing discoveries of this study is CD28 receptor's dependency on NKG2D to resist TGF-β signaling, but not to provide canonical co-stimulation in CD8+ T cells. At what point do these pathways converge to co-operate for TGF-β resistance will be of significant interest in the field. RNA sequencing analysis on WT and NKG2D KO CD8+ T cells activated with and without CD28 co-stimulation in the presence of absence of TGF-β will provide clues to this question and the molecules of interest for further investigation. Moreover, it could be anticipated that NKG2D mediated TGF-β resistance is also dependent on CD28 signaling. This hypothesis could be tested by generating CD28/NKG2D double KO mice, and performing DC based TGF-β suppression assays (similar to Figure 16) on WT, NKG2D KO, CD28 KO, and CD28/NKG2D double KO CD8+ T cells. Further, these systems and methodologies could be applied to investigate the relationship of NKG2D with other co-stimulatory signaling such as 4-1BB, OX40 and ICOS in the context of TGF-β suppression. This will be relevant specially in the case of 4-1BB co-stimulation, which has been shown to attenuate TGF-β inhibition [233] as well as modulate NKG2D co-stimulation

[306].

This work has laid foundation to determine the immediate signaling pathway utilized by NKG2D-DAP10 receptor complex to abrogate TGF-β's suppressive effects in T cells. With all the DAP10 signaling mutants (MQ, NQ, and YF) available, the next step would be to determine the conditions wherein these constructs could be expressed in DAP10 KO T cells while preserving their sensitivity to TGF-β. This can be achieved by generating the DAP10 KO mice that express MQ, NQ, YF, and WT DAP10 genes. Alternatively, the retrogenics approach, where the mouse bone marrow progenitor stem cells are retro-virally transduced with gene of interest [307], could also be used to generate these mice. This way contribution of each DAP10 signaling arm in TGF-β resistance can be measured in naïve CD8+ T cells. In addition, the problem of low transduction efficiency would be resolved and the impact of expressed DAP10 constructs in DAP10 deficient cells would be maximized.

We have determined NKG2D induced alterations in the genetic program that likely down-regulates the intensity of TGF-β pathway in CD8+ T cells. Although the heightened TGF-β resistance by NKG2D is probably a collective outcome of all the described gene changes, it is possible that few genes are the key mediators. We propose that the signaling molecules having direct consequences on Smad phosphorylation and nuclear translocation, i.e. IRF7, S100A4, AHNAK, neuropilin 2, annexin A1, activin receptor type-2A, proteinase activated receptor 2, and RUNX2 will be interesting candidates to pursue this direction. The first step would be to confirm these gene changes at the protein level. Then, the individual roles could be determined by treating NKG2D KO and WT CD8+ T cells with small molecule inhibitors of these proteins during TGF-β suppression assay. For instance, GB83 [308], a selective PAR-2 antagonist, CADD522 [309], a recently characterized RUNX2 inhibitor could be used. The activity of S100A4 can be attenuated by FPS-ZM1 [310], which blocks its binding partner RAGE receptor [311]. Interruption at the crucial signaling notches should reduce the susceptibility to TGF-β in NKG2D KO, but not in WT, CD8+ T cells.

Furthermore, identification of NKG2D's prominent mechanism to counter TGF-β suppression may present a therapeutic opportunity. The inhibitors showing promising results in the above experiments could be tested in the mouse models to rescue dysfunctional CD8+ T cells from TGF-β driven immune-suppression as these cells display phenotype similar to NKG2D KO cells (NKG2D signaling/surface expression is also profoundly reduced in these cells).

Findings of this study will also have important therapeutic implications in the cancer immunotherapy field. Our results clearly point out that TGF-β induced down-modulation of NKG2D-DAP10 would not only repress the effector function of CD8+ T cells but will also simultaneously increase their susceptibility to TGF-β mediated suppression. Therefore, we propose therapeutic interventions that increase or maintain surface expression of NKG2D-DAP10 complex in a constitutive manner on CD8+ T cells to make them refractory to TGF-β mediated immune-suppression. In the case of adoptive T cell transfer (ACT) therapies, a strategy of cross-linking NKG2D on T cells *in vitro* before transferring them into patients can also be envisioned. Lastly, this dissertation also provides rationale for development of potent NKG2D agonistic antibody or small molecule to strengthen the shield of CD8+ T cells against inhibitory effects of TGF-β.

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VITA

The author, Kushal Prajapati, was born in Ahmedabad, India on September 23, 1989 to Hema and Pravin Prajapati. He attended the Sardar Patel University in Anand, India where he earned a Bachelor's of Pharmacy in April 2011. During his college days, Kushal got interested in studying the biology of drug action and moved to the US to obtain Master of Science in the Molecular Pharmacology Program at Loyola University Chicago in 2011. His thesis work involved the development of high throughput, cell-based PKC-δ reporter assay to screen compounds for squamous cell carcinoma treatment in the laboratory of Dr. Mitchell Denning. Upon completion of MS, he enrolled in the PhD program at Loyola in the Molecular Pharmacology department to extend his research training.

Intrigued by the cancer immunology field, Kushal joined the laboratory of Dr. Jose A. Guevara-Patino in the Cardinal Bernardin Cancer Center in 2014 to pursue a doctoral research. His dissertation work in Dr. Guevara's lab investigated the interplay between co-stimulatory receptor NKG2D and inhibitory TGF-β signaling in CD8+ T cells with the goal of improving the effectiveness of T cell based cancer immunotherapies. After completion of his PhD, Kushal would like to pursue a research career in the pharmaceutical or biotech industry within Immuno-Oncology field.