Investigating Functional Synergy Between PD-1 and TIGIT in Anti-Tumor Immunity and Autoimmunity

INVESTIGATING FUNCTIONAL SYNERGY BETWEEN PD-1 AND TIGIT IN ANTI-TUMOR IMMUNITY AND AUTOIMMUNITY

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ABSTRACT

Anti-PD-1 therapy has emerged as a promising therapeutic for cancer. PD-1 blockade can increase tumor clearance in pre-clinical mouse models and this has translated to therapy with FDA approval of PD-1 pathway inhibitors for cancer immunotherapy. However, this therapy only works in a subset of patients and for only a subset of cancers. Thus, targeting multiple co-inhibitory pathways seems to be a promising strategy to increase response to immunotherapy. TIGIT is another co-inhibitory receptor expressed on a variety of cells including different T-cell subsets. Both PD-1 and TIGIT are expressed in cancer and chronic infections leading to T cell dysfunction. There have been some studies showing synergy between PD-1 and TIGIT in the context of anti-tumor immunity in mice treated with antibody blockade. However, the mechanism of synergy is unknown. The goal of this thesis is to understand the mechanism of this synergy and identify different cell types involved in tumor clearance using conditional PD-1/TIGIT double knockout mouse models. To achieve this goal, we identified a tumor model that could be used to study the synergy in conditional knockout mice. Since immunotherapy is also associated with a high frequency of immune-related side effects in patients and PD-1 and TIGIT also regulate T cell tolerance, we also investigated the autoimmune phenotype in these mice using the experimental autoimmune encephalomyelitis (EAE) model of autoimmunity. Our studies show that inhibiting PD-1 and TIGIT does not cause more severe disease as compared to inhibiting PD-1 alone. We further studied the phenotype of Tregs from different conditional

knockout mice by in vitro methods. Our findings provide insights relevant for development of strategies to improve anti-tumor response while minimizing autoimmune side effects.

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Acknowledgements

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This work was done in close collaboration with Juhi Kuchroo. The in vivo experiments were labor intensive and Juhi Kuchroo's help was taken for the same. Data analysis for flow cytometry shown in figures 3.2, 3.8, 3.9B, 3.13B, and 6.1 was done independently.

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Chapter 1 Background

Cancer Immunotherapy

The immune system is capable of recognizing and thus, eliminating tumors through immune surveillance yet maintaining tolerance[1,2]. However, tumors find multiple ways to evade the immune system such as by establishment of an immunosuppressive tumor microenvironment[1,2,3]. Immune responses are regulated by multiple pathways in order to keep the immune system in check to maintain tolerance and prevent immune mediated tissue damage[1,2,3,4]. Cancer immunotherapy is based on the idea of harnessing the body's immune system to kill the tumor.

Co-inhibitory receptors

Co-inhibitory pathways function to regulate T cell activation and tolerance. Signals through co-inhibitory receptor on T cells are important to ensure proper contraction of effector T cell responses to maintain homeostasis[5]. These inhibitory receptors are also known as checkpoints as they keep the immune system in check in conditions of chronic immune response. These immune checkpoints that normally maintain self-tolerance are utilized by cancer to evade immune mediated destruction. Blocking these inhibitory checkpoints can thus lead to anti-tumor responses[1,3,6]. Checkpoint blockade has revolutionized the field of cancer immunotherapy due to its remarkable efficacy to generate durable anti-tumor immune responses[3]. Blocking checkpoints such as CTLA-4 leads to improved anti-tumor response as seen in pre-clinical studies in mice treated with anti-CTLA-4 antibody leading to its translation to therapy and approval by FDA for the treatment of melanoma [6,7,8]. Since then a landscape of co-inhibitory pathways has been discovered and studied for their potential to be targets in cancer immunotherapy. Multiple co-inhibitory receptors such as PD-1, Lag-3, Tim-3, VISTA and TIGIT have been studied in cancer as well as tolerance[5,9]. Co-inhibitory pathways differ in their potency, kinetics and signalling

pathways to dampen T cell activation[3,5,9]. Here we will discuss two co-inhibitory receptors- PD-1 and TIGIT.

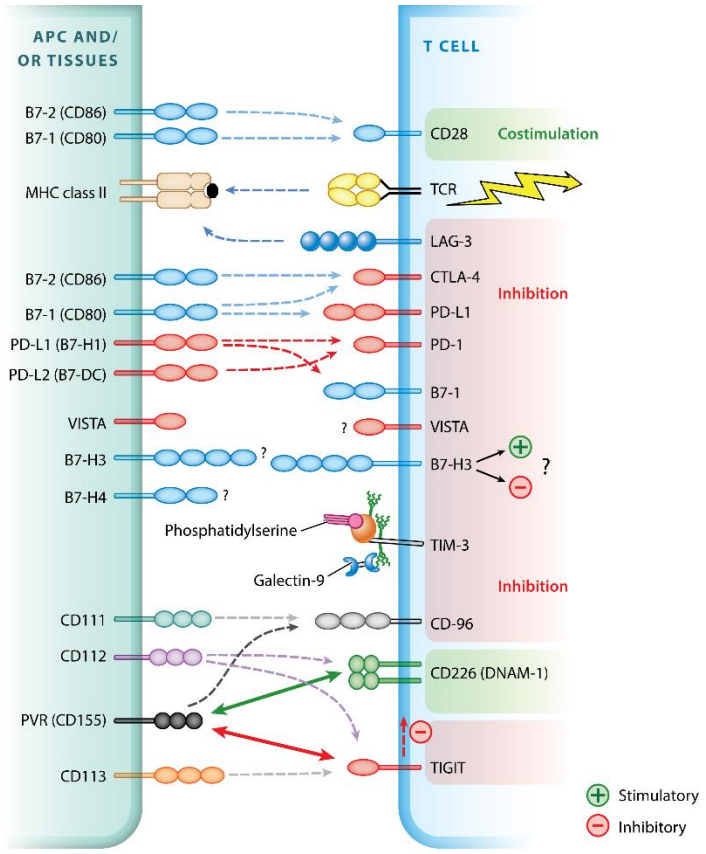

Figure 1.1 Overview of co-inhibitory pathways After T cell activation, various inhibitory receptors are expressed by cells that inhibit T cell activation. TIGIT, LAG-3 and PD-1 are also expressed on other cells such as NK cells (Figure taken from Baumeister SH, et al. 2016)

PD-1 (Programmed Death-1)

The PD-1 pathway is one such pathway that plays a central role in cancer immunotherapy and maintenance of tolerance. PD-1 is a member of the Ig superfamily that has two tyrosine motifs in its cytoplasmic domain, an ITIM and ITSM motif[1,10,11]**.** It is a transmembrane co-inhibitory receptor that is expressed on activated T cells and plays a significant role in T cell activation[10,12]. It also is expressed by T cells in conditions of chronic antigen exposure such as in chronic viral infections and cancer[1,12]. PD-1

expression on T cells remains high when stimulated by antigen repetitively. During chronic viral infection and cancer, T cells enter a state of exhaustion which protects the tissue from damage due to excessive immune response[1,13]. T cell exhaustion is progressive loss of T cell function[9]. During exhaustion, T cells can express multiple inhibitory receptors which can be targeted in cancer therapy[14]. PD-1 is expressed on a variety of T cells including CD4+ and CD8+ helper T cells, follicular helper T cells and regulatory T cells. PD-1 also is expressed by NK cells and B cells inhibiting their effector functions[15,16].

PD-1 pathway: Mechanism of Action

PD-1 has two ligands, PD-L1 and PD-L2, which are both expressed on haematopoietic and non-haematopoietic cells. PD-L1 is more broadly expressed whereas PD-L2 expression is more restricted[17,18]. PD-1 engagement of the ligands leads to phosphorylation of tyrosine motifs in its cytoplasmic domain and recruitment of phosphatases such as SHP2 that leads to reduction in the phosphorylation of kinases downstream of the TCR and CD28. This leads to inhibition of signals for TCR stimulation downstream, decreased T cell activation and cytokine production[19]. PD-1 signalling inhibits T-cell cytotoxicity by decreasing the production of cytotoxic molecules from T cells[1]. PD-1 also asserts its function through modulating Tregs[1]. Thus, PD-1 inhibits T cell responses in multiple ways.

Anti-PD-1 therapy

PD-1 pathway inhibition by antibodies that block PD-1 engagement by its ligands leads to tumor killing by rescuing TILs from exhaustion and enhancing tumor killing[1,20]. Enhanced anti-tumor immunity due to blockade of the PD-1 pathway in pre-clinical models has been translated to therapy[1,21]. Anti-PD-1 therapy is now approved by the FDA for the treatment of variety of cancers including advanced melanoma, non-small cell lung cancer, Hodgkin's lymphoma and kidney cancer[1].

Combination therapy

Anti-CTLA-4 therapy was the first checkpoint blockade therapy to be approved by the FDA for treatment of melanoma[1,22]. Anti-PD-1 therapy has shown impressive anti-tumor effects in various tumors[3,4]. However, many patients do not respond to anti-CTLA-4 or anti-PD-1 monotherapy[1,3]. For instance, anti-PD-1 therapy does not work in a variety of cancers such as prostate cancer, pancreatic cancer and ovarian cancer[1]. To increase the benefit of PD-1 pathway blockade, a variety of combination therapy strategies are under investigation. One promising approach involves targeting multiple inhibitory pathways to improve response to immunotherapy. Pre-clinical studies in mice showed that co-blockade of CTLA-4 and PD-1 leads to better tumor clearance than blockade of each alone as T cells co-express these co-inhibitory receptors. This has been translated to therapy[23, 24].

The remarkable success of anti PD-1 and anti CTLA-4 therapy has motivated the study of other inhibitory pathways that can be targeted for cancer therapy including Lag3, Tim-3 and TIGIT[3,5]. A variety of combinations are being investigated to improve tumor clearance and clinical trials underway.

TIGIT (T-cell immunoreceptor with Ig and ITIM domain)

TIGIT is a co-inhibitory receptor and a member of the immunoglobulin superfamily. It is also known as Vstm3 or WUCAM and is expressed on NK cells and various T cell subsets including activated CD4+ and CD8+ helper T cells, follicular helper T cells and regulatory T cells[5,25]. It has an extracellular IgV domain and a type I transmembrane region. Its cytoplasmic tail is composed of an ITIM and an immunoglobulin tail tyrosine (ITT)-like motif[26]. TIGIT was discovered in a genomic search for genes expressed in T cells that had structures representative of potential inhibitory receptors[27].

TIGIT pathway

TIGIT has multiple ligands which are nectin and nectin-like proteins. TIGIT binds to CD155 (polio virus receptor PVR, NECL5) with a higher affinity and CD112 (PVRL2, nectin-2) with a lower affinity[5,27]. The significance of CD112/TIGIT interaction is not known[5]. These ligands are expressed on hematopoietic cells including APCs such as DCs, T cells, B cells as well as non-hematopoietic cells and tumor cells[5,27]. CD155 expression on tumor cells causes loss of contact inhibition and thus promotion of tumor growth[1].

The TIGIT pathway also includes a co-stimulatory receptor CD226 (DNAM-1) which has been linked to many autoimmune diseases such as type 1 diabetes, multiple sclerosis, and rheumatoid arthritis in GWAS studies[5]. CD226 binds to the same ligands as TIGIT but with a lower affinity. The TIGIT pathway is analogous to the CTLA-4/CD28 pathway as both pathways have a positive and negative receptor expressed on T cells and NK cells that share ligands expressed on APCs. In the CTLA-4/CD28 pathway, the B7 family members CD80 and CD86 engage CD28, leading to T cell activation. CD80 and CD86 engagement of their higher affinity receptor CTLA-4 results in T cell inhibition. Similarly, TIGIT competes with CD226 to bind to CD155 and causes T-cell inhibition[5]. In addition, TIGIT can also directly bind to CD226 and disrupt its homodimerization thus inhibiting its co-stimulatory function[5]. Like CTLA-4, TIGIT is also constitutively expressed by Tregs but its expression increases after activation. TIGIT is a direct target gene of Foxp3, the master transcription factor in Tregs[1,5]. CD112R is another co-inhibitory receptor expressed by T cells that causes T cell inhibition on engagement with CD112[28].

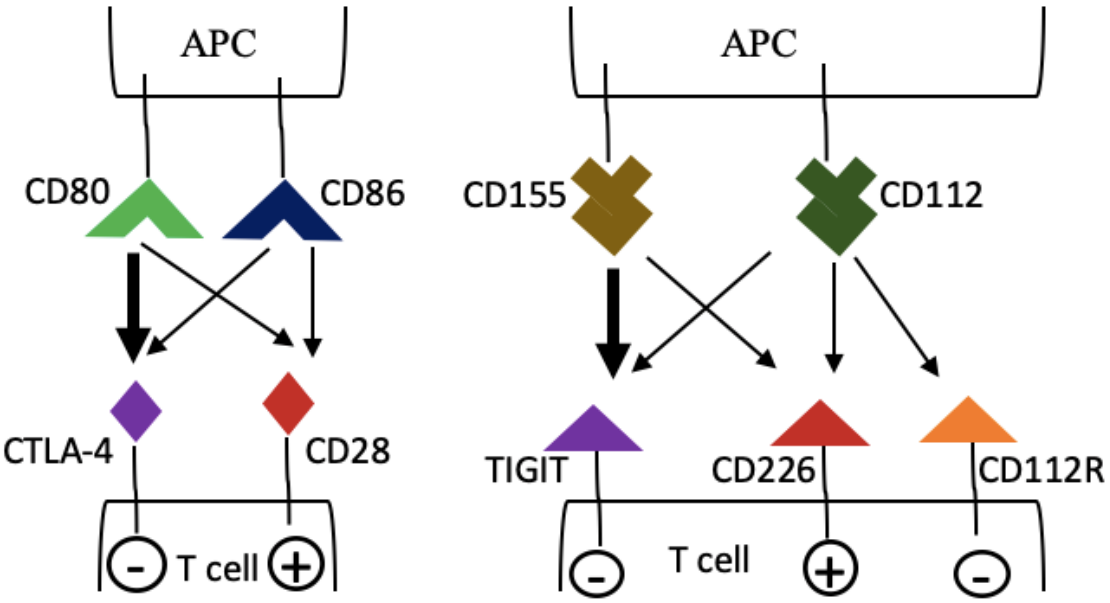

Figure 1.2 Analogy between CTLA-4 and TIGIT pathway

CTLA-4 and TIGIT are co-inhibitory receptors and share their ligands with the CD28 and CD226 co-stimulatory receptors, respectively. The co-inhibitory receptors in both the pathways bind their ligands with a higher affinity than the respective co-stimulatory receptors and inhibit T cell activation. CD112R is another co-inhibitory receptor expressed on T cells that binds to CD112. (Adapted from Anderson AC et al. 2016 and Zhu et al. 2016)

TIGIT pathway- Mechanism of action

In NK cells, TIGIT/CD155 interaction induces phosphorylation through Fyn and Lck and recruitment of SHIP1 through the cytosolic adaptor protein Grb2. SHIP1 recruitment to the TIGIT tail inhibits signalling through PI3K and MAPK pathways which results in NK cell inhibition. Phosphorylation also leads to the binding of ITT like motif to b-arrestin and recruits SHIP1 which then limits NFkB signalling[5].

In T cells, the TIGIT pathway does not cause activation and expansion of cells but neither does it cause the cells to undergo anergy and deletion from the repertoire. TIGIT plays a role in the maintenance and survival of T cells[5]. TIGIT expression is upregulated on human and mouse T cells after activation. It causes T cell inhibition through both cell extrinsic and intrinsic mechanism[5,21]. TIGIT when binds to CD155 expressed on DCs makes them tolerogenic[5,25,27,29]. CD155 has an ITIM motif in its cytoplasmic domain. Work of Grogan et al suggested that TIGIT binding to CD155 on DC could lead to signalling into DC, resulting in IL-10 production. TIGIT ligation of CD155 on DCs causes increase in the release of IL-10 and inhibits the production of IL-12[5,27]. TIGIT also causes direct T cell inhibition in a cell intrinsic manner by inhibiting T cell proliferation and effector functions by downregulating components of the TCR complex as well as molecules of the TCR signalling cascade such as PLCg[25]. TIGIT directly acts on components of the TCR complex such as TCRa chain. Thus, unlike PD-1 which causes T cell inhibition by reducing phosphorylation of kinases downstream of the TCR signalling pathway, TIGIT acts upstream as it directly acts on the TCR complex [1,11, 27]. It has recently been shown that TIGIT exerts its inhibitory effects on T cell function mainly by modulating Tregs. TIGIT induces IL-10 and Flg2 production in Tregs which suppress effector T cell functions[5,30].

PD-1 and TIGIT Synergy

PD-1 and TIGIT are promising therapeutic targets. Some studies have shown that there is functional synergy between PD-1 and TIGIT in anti-tumor immunity in pre-clinical models[27,31]. However, little is known about how these pathways synergize to limit tumor growth. TIGIT is co-expressed with PD-1 by CD8+ TILs in tumors in both mice and humans. When colon carcinoma CT26 cells were injected into WT mice that were treated with combination of anti-PDL1 and anti-TIGIT blocking antibody, there was improved tumor clearance and better survival rate as compared to mice treated with single agent blockade[27]. Blockade of both the pathways significantly restored the potency of CD8+ T cells

in the tumor infiltrating lymphocytes (TILs) and enhanced cytokine production as compared to single agent blockade[27]. However, the mechanism of this synergy between the two molecules to promote anti-tumor immunity is unknown.

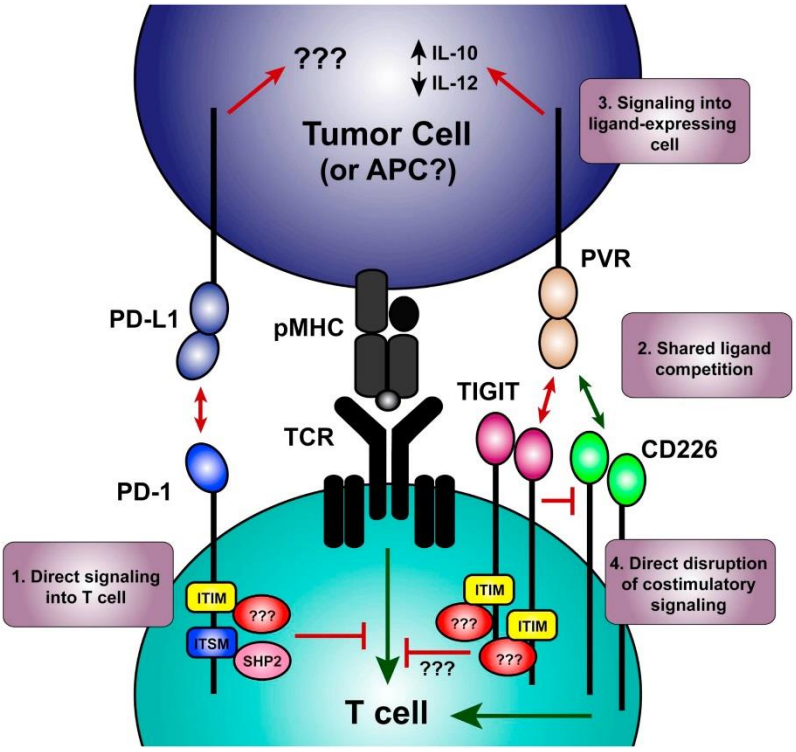

Figure 1.3 Possible mechanisms of synergy between PD-1 and TIGIT during co-blockade.

1. T cell inhibition by direct signalling into T cell by recruitment of intracellular proteins such as phosphatases in PD-1 signalling. 2. T cell inhibition by competition with between ligands shared by co-inhibitory and co-stimulatory receptors (e.g. TIGIT competes with CD226 to bind to PVR to cause T cell inhibition). 3. Making the APCs tolerogenic (e.g. TIGIT/PVR signalling into DCs induces IL-10). 4. Co-inhibitory receptors can disrupt homodimerization of co-stimulatory receptors (e.g. TIGIT disrupts CD226 homodimerization). Red lines indicate inhibitory signals and green lines indicate stimulatory signals. (Figure adapted from Pauken KE et al. 2014)

Tolerance and Autoimmunity

Co-inhibitory pathways prevent the initial activation of self-reactive T cells and thus play roles in induction and maintenance of tolerance. The significance of co-inhibitory receptors in tolerance was first appreciated by the development of lethal spontaneous autoimmunity in CTLA-4 -/- mice [1,32]. PD-1 also maintains tissue tolerance by regulating potentially pathogenic CD4 and CD8 T cells in target organs, as PD-1 ligands are also expressed on non-hematopoietic cells in tissues such as islet cells in pancreas[1]. Both antibody blockade and PD-1 deficiency in mice exacerbates autoimmune disease phenotype[1]. PD-1 also inhibits T follicular regulatory (Tfr) cell function, which is a subset of Tregs that inhibits the germinal center reaction and antibody production[1]. Similarly, TIGIT regulates T cell tolerance by controlling self-reactive T cell activation and Tregs mediated suppression[30]. Although TIGIT-/- mice do not develop spontaneous autoimmunity, deficiency or blockade of TIGIT exacerbated autoimmune disease as seen in mouse models of EAE[5,31]. Furthermore, agonizing TIGIT antibody can suppress pro-inflammatory response through Treg mediated suppression of effector T cells[31]. The roles of PD-1 and TIGIT in regulating T cell tolerance may explain why blockade of multiple co-inhibitory pathways in cancer carries the risk of autoimmune adverse effects in cancer patients.

1.2 Questions addressed in this thesis

In this project, we aim to understand the mechanism of synergy between PD-1 and TIGIT and explore the different cell types involved in regulating anti-tumor immunity and autoimmunity. It is not clear if the synergy is due to blockade of these receptors on the same cell or different cell types. We will investigate the relative roles and interaction between the PD-1 and TIGIT pathways in controlling CD4+ Tregs and CD4+ Foxp3- T cells as well as define the genetic circuits underlying these interactions. We will explore how TIGIT and PD-1 interact to control self-reactive CD4+ FoxP3- T cells during initiation and progression of autoimmunity. Further, we will examine the effects of TIGIT and PD-1 on individual T cell subsets in tumors and autoimmunity. Our studies will provide insights into how TIGIT and PD-1 exert their functions in regulation of tolerance and autoimmunity. Understanding this synergy will help in development of better strategies to improve anti-tumor response to co-inhibitory receptor blockade while minimizing autoimmune side effects, and how to modulate these receptors to treat chronic infection and autoimmunity.

Chapter 2 Materials and Methods

MICE

8-10 week old mice were used for all tumor and EAE experiments. For aging experiments, ~12 months old mice were used. WT C57BL/6 mice were purchased from the Jackson Laboratory.

UBC ERT Cre and CD4 Cre mice on C57BL/6 background were generated in the Sharpe laboratory by Juhi Kuchroo by crossing Tamox-Cre $Pdcd1^{fl/fl}$ X CD4 Cre $Vstm3^{fl/fl}$ mice. These were then validated by flow cytometry performed by Juhi Kuchroo. Tamox-Cre $Pdcd1^{fl/fl}$ mice were generated in house and CD4 Cre $Vstm3^{fl/fl}$ mice were obtained from Vijay Kuchroo lab at Harvard Medical School.

UBR ERT Cre and CD4 Cre PD-1 flox, TIGIT flox, PD-1/TIGIT double flox mice and Cre-controls or WT mice were compared in the tumor and EAE studies. All animals were kept in conventional, pathogen free facility at New Research Building, Harvard Medical School, Boston, MA and all experiments were carried out in accordance with guidelines prescribed by Institutional Animal Care and Use Committee at Harvard Medical School.

ANTIBODIES

Blocking TIGIT antibody (clone 1B4) and agonizing TIGIT (clone 1G9) were provided by Dr. Vijay Kuchroo's lab at Harvard Medical School.

TAMOXIFEN ADMINISTRATION

1 mg tamoxifen was injected i.p each day for 10 days for full deletion of PD-1 and TIGIT expression in inducible Cre PD-1 flox, TIGIT flox and PD-1/TIGIT double flox mice. Mice were then

rested for about a week before the start of any experiment. Tamoxifen dose administration was reduced to 5 days to reduce deletion of PD-1 and TIGIT expression in the inducible Cre PD-1 flox, TIGIT flox and PD-1/TIGIT double flox mice.

TUMOR CELL LINES

MC38 colon carcinoma, B16 melanoma and B16OVA tumor cells were cultured in vitro in DMEM supplemented with 10%FBS, 1% penicillin/streptomycin, and 20 µg/ml gentamicin. B16OVA cells were cultured in DMEM+10%FBS, 1% penicillin/streptomycin along with puromycin (1:5000) for a week to select for B16OVA cells. They were then switched to DMEM+10%FBS, 1% penicillin/streptomycin and 20 µg/ml gentamicin.

TUMOR MODELS

For MC38 colon carcinoma, $1x10^6$ cells MC38 cells were implanted s.c in the left flank of CD4 Cre mice and 5×10^5 cells were implanted in the left flank of inducible Cre mice. For B16 melanoma tumor, 2.5×10^5 cells and for B16OVA tumor, 3×10^5 cells were implanted s.c into the left flank of mice. All tumors were measured 2-3 times per week for 4-5 weeks by a caliper. Tumor volume was determined by the formula: $\frac{1}{2} \times D \times d^2$ where D is the major axis and d is the minor axis.

For antibody blockade experiments, mice were randomized when tumor size reached ~$25mm^3$ and treated as follows (adopted from Dixon KO et al. 2018): Anti-PD-1 antibody (Clone 29F)- 100µg per dose on days 1, 3, 5 after randomizing mice; Anti-TIGIT antibody (Clone 1B4)- 100µg per dose on days

0, 2, 4, 10, 17 after randomizing mice. Isotype control antibodies used were 1gG2 (100 µg/dose) and IgG1 (50 µg/dose).

EAE

EAE was induced by sub-cutaneous immunization of mice with 100µg myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide emulsified in CFA followed by 2 ng/dose of pertussis toxin given i.p on day 0 and day 3. Clinical signs of EAE were scored everyday on a scale of 0-5. Score- 0- no disease, 1- limp tail, 1.5- abnormal gait, 2- hind limb weakness, 2.5- extreme weakness of hind limbs or one hind limb completely paralysed, 3- paralysis of both hind limbs, 4- hind and forelimb paralysis, 5- moribund (adapted from Latchman YE et al. 2004)

For in vivo antibody administration, the following protocol was followed (adapted from Dixon KO et al. 2018)- Agonizing TIGIT antibody (1G9)- 100µg per dose on days 1, 3, 5, 10; Blocking TIGIT antibody (1B4)- 100µg per dose on days 0, 2, 4, 10, 17; Anti-PD-1 antibody (29F)- 100µg per dose on days 1, 3, 5; Anti PD-1 antibody (RPM14)- 100 µg per dose on days 1, 3, 5. Isotype control antibody administered for agonizing TIGIT (1G9) and blocking TIGIT antibody (1B4) was IgG1 from BioLegend (50µg per dose) and that for anti-PD-1 (29F and RMP14) was IgG2a from BioXCell (100µg per dose).

IN VITRO TREG SUPPRESSION ASSAY

CD4 Cre PD-1 flox, TIGIT flox, PD-1 TIGIT double flox and WT mice with Foxp3.GFP reporter were sacrificed and spleens were harvested. Single cell spleen suspensions were made in R10 media using nylon cell strainers (70µm). R10 media is RPMI-1640 (Gibco) supplemented with 10% FBS, 100U/ml penicillin/streptomycin (Gibco), 10 mM Hepes (Gibco) and 100 µM beta-mercaptoethenol (Gibco). CD4+ T cells from spleens of CD4 Cre PD-1 flox, TIGIT flox, PD-1/TIGIT double flox and WT mice

were isolated using a CD4+ T-cell isolation kit through MACS beads and columns (Miltenyi Biotec). Purified CD4+ T cells were then stained with fluorescent labelled CD4 BioLegend (1:100 dilution) and CD62L BioLegend (1:100 dilution) antibodies to sort for naïve T cells. CD4+ CD62Lhi GFP- cells were sorted on FACS Aria II cytometer as naïve conventional CD4+ T cells from WT mice. In parallel, purified CD4+ GFP+ cells were sorted as Tregs (CD4+ Foxp3+) from the conditional knockout mice WT controls. Sorted naïve conventional T cells from WT mice were then labelled with Cell Trace Violet (CTV) dye (Thermo Fisher Scientific). CTV is used to measure proliferation as it gets diluted with each replication cycle. CTV stained cells were incubated with Tregs from conditional knockout and control mouse strains in varying ratios in 96-well round bottom plates coated with anti-CD3 and irradiated APCs. After 72 hours of incubation, cells were harvested and analysed for CTV dilution to assess proliferation of WT conventional T cells on flow cytometer. Supernatant were harvested to measure cytokine levels using the method described below.

CYTOKINE MEASUREMENT

Supernatants from the in vitro Tregs suppression assay described above and blood serum from aged mice were analysed by cytokine bead array (CBA) kit from BD Biosciences.

FLOW CYTOMETRY

All cells from lymphoid organs were resuspended in staining buffer (PBS with 1% FBS and 2mM EDTA) and stained with directly conjugated antibodies. Antibodies used include: CD4 Biolegend (1:200 dilution), CD8b BioLegend (1:200 dilution), Foxp3 BioLegend (1:100 dilution), TIGIT BioLegend (1:100 dilution), Tim-3 BioLegend (1:200 dilution), Lag-3 BioLegend (1:200 dilution), CD62L BioLegend (1:200 dilution), CD25 BioLegend (1:200 dilution), CD44 BioLegend (1:200 dilution). For

intracellular staining, single cell suspensions from the indicated organs were fixed using the fixation/permeabilization solution kit from BD Biosciences. Flow cytometry data were acquired on LSR II or Symphony and analysed using FlowJo software.

HISTOPATHOLOGY AND NECROPSY

Mouse samples for histopathology and full necropsy were sent to Rodent Histopathology Core facility at Harvard Medical School.

STATISTICAL ANALYSIS

Analysis was performed using GraphPad Prism, statistical significance calculated using Student's t-test, and data plotted as mean±SEM. P values less than 0.05 were considered statistically significant.

Chapter 3 Results

To explore the mechanism of synergy between PD-1 and TIGIT in regulating tolerance and tumor immunity, we performed experiments using PD-1/TIGIT conditional double knockout mice as well as antibody blockade experiments. To assess the tolerance and autoimmunity, we used the EAE model of autoimmunity in PD-1/TIGIT conditional double knockouts and used different combinations of agonists and antagonists of PD-1 and TIGIT to alter the pathogenicity of myelin reactive CD4+ T cells. We further assessed how the function of Tregs is affected by targeting both PD-1 and TIGIT.

Inducible Cre PD-1/TIGIT double flox and CD4 Cre PD-1/TIGIT double flox strains generated by Juhi Kuchroo were validated by flow cytometry. T cells from spleens of CD4 Cre PD-1/TIGIT double flox and UBC ERT Cre PD-1/TIGIT double flox (after tamoxifen administration) were sorted to examine PD-1 and TIGIT expression on CD4+ Foxp3- T cells (Tcons) (Figure 3.1) and CD4+ Foxp3+ Tregs (data not shown), and efficacy of deletion.

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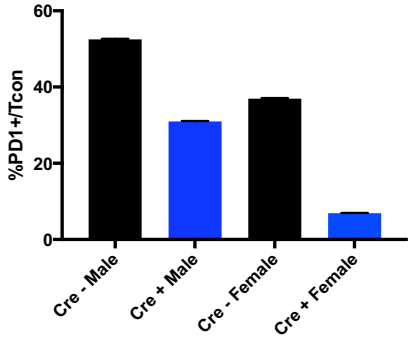

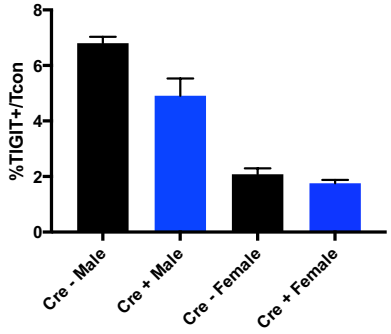

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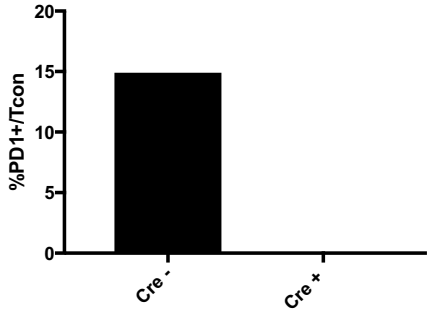

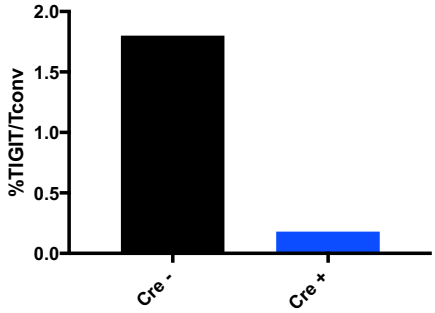

Figure 3.1 PD-1 and TIGIT expression on T cell subsets in UBC Cre PD-1/TIGIT flox and CD4 Cre PD-1/TIGIT flox mice generated in lab. Flow cytometry analysis of the effect of PD-1 and TIGIT deletion on expression of PD-1 and TIGIT in conventional T cells harvested from spleens of UBC Cre PD-1/TIGIT flox mice (Fig 2.1A) and CD4 Cre PD-1/TIGIT flox mice (Fig 2.1B) (In all graphs- frequency of cells ±SEM) (Unpublished data produced by Juhi Kuchroo)

Next, we evaluated the phenotype of CD4Cre PD-1 flox, TIGIT flox and PD-1/TIGIT conditional double flox mice (that were generated by Juhi Kuchroo) at homeostasis. We analyzed the phenotype of different T cell subsets in spleen and thymus of these mice to determine if there is any impact of PD-1/TIGIT deletion on thymic development as it is known that PD-1 deletion affects development of T cells[32]. We did not find any significant difference in the thymus of Cre+ and Cre- controls. On analyzing the T cells in spleen, we found a higher number of Tregs expressing Lag-3 in CD4 Cre PD-1 flox and PD-1/TIGIT double flox as compared to CD4 Cre TIGIT flox mice (Figure 3.2A). On analysing the

expression of another inhibitory receptor Tim-3, we found that there were higher Tim-3 expressing CD4+ conventional T cells (Figure 3.2B) and CD8+ T (Figure 3.2C) cells in CD4 Cre PD-1/TIGIT double flox mice as compared to the Cre- controls.

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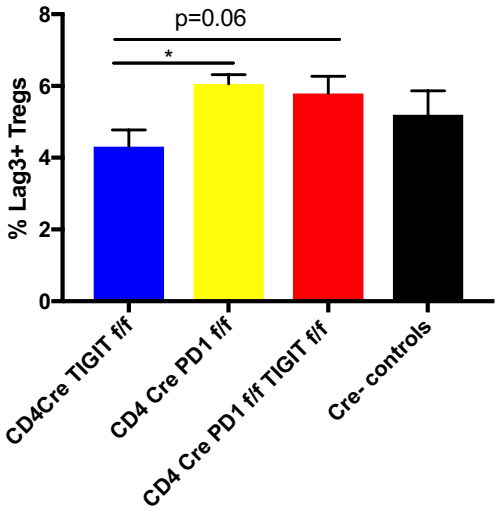

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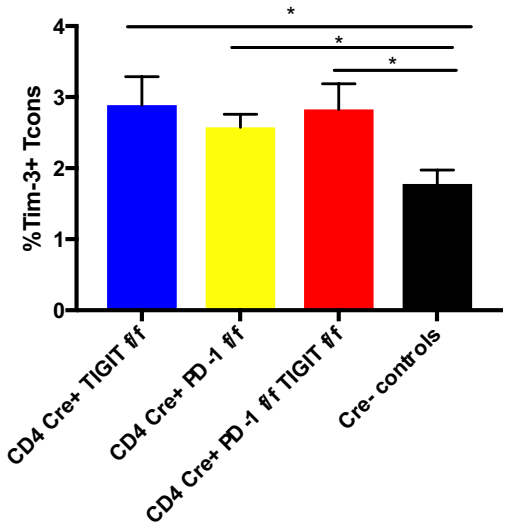

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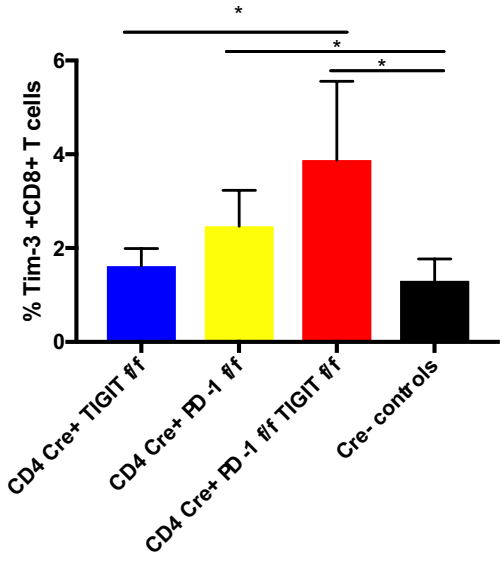

Figure 3.2 Assessment of Lag-3 and Tim-3 expression on T cells in CD4 Cre conditional knockout mice at homeostasis T cells were harvested from spleens of ~8 week old females and expression of different inhibitory receptors was assessed by flow cytometry. (A) shows Lag-3 expression on Tregs from the different conditional knockout mice. (B) and (C) show Tim-3 expression on CD4+ Tcons and CD8+ T cells. Data are shown as frequency±SEM

To explore the potential synergy of PD-1 and TIGIT in generating anti-tumor immunity, we first needed to identify the best tumor model to study the synergy in the PD-1/TIGIT CD4 Cre conditional knockout mice. Previous studies done in the lab with CD4 Cre conditional knockout mouse strains with the MC38 colon carcinoma model showed that it was difficult to separate MC38 tumor growth phenotype in CD4 Cre PD-1 flox and PD-1/TIGIT double flox mice, as this tumor is exquisitely PD-1 sensitive (Figure 3.3).

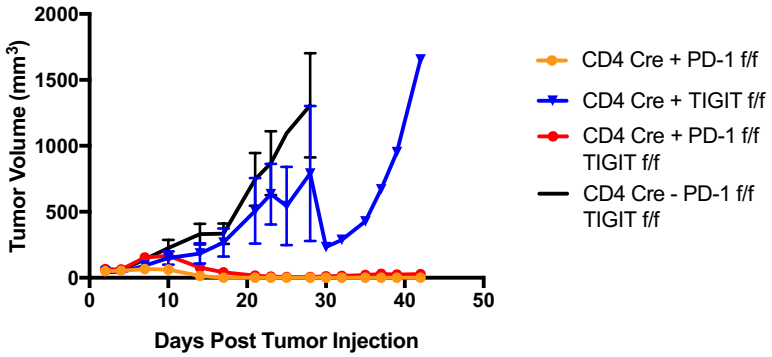

Figure 3.3 MC38 tumor growth in CD4 Cre conditional knockout mice Tumor growth was monitored 2-3 times per week for 4-5 weeks. Data are shown as mean tumor volume ±SEM of each group from 3-5 mice per group (unpublished data produced by Juhi Kuchroo)

This result led us to test the B16 melanoma model. This tumor model is less immunogenic. When implanted in the CD4 Cre conditional knockout mice, it was found that the tumors grew out (Figure 3.4). Thus, it was difficult to study the synergy between PD-1 and TIGIT using the B16 model.

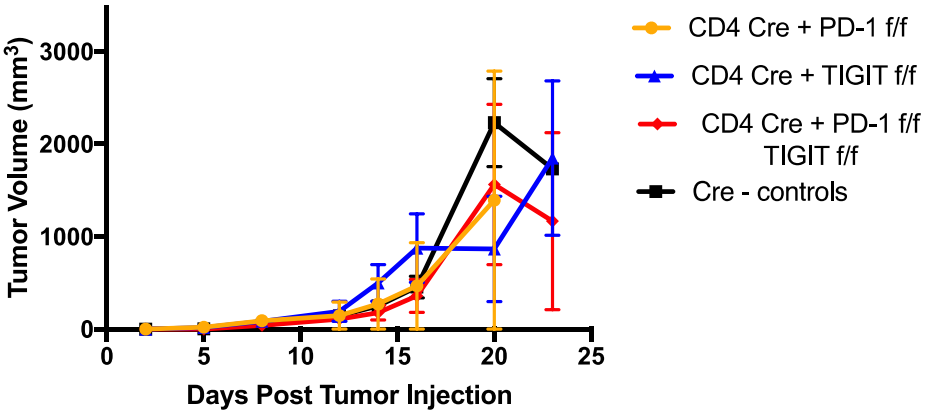

Figure 3.4 B16 melanoma tumor growth in CD4 Cre conditional knockout mice Tumor growth was monitored 2-3 times per week for 4-5 weeks. Data are shown as mean tumor volume ±SEM of each group from 3-5 mice per group (unpublished data by Juhi Kuchroo)

Hence, the best tumor model should neither be too PD-1 sensitive or too PD-1 insensitive. We next examined the B16OVA tumor model in which OVA antigen provides greater immunogenicity compared to the B16 melanoma but less than the MC38 tumor model. B16OVA tumors have slower growth in PD-1/TIGIT double floxed and there is an improved survival rate in PD-1/TIGIT double floxed mice as compared to the PD-1 or TIGIT single floxed mice (Figure 3.5).

A

B

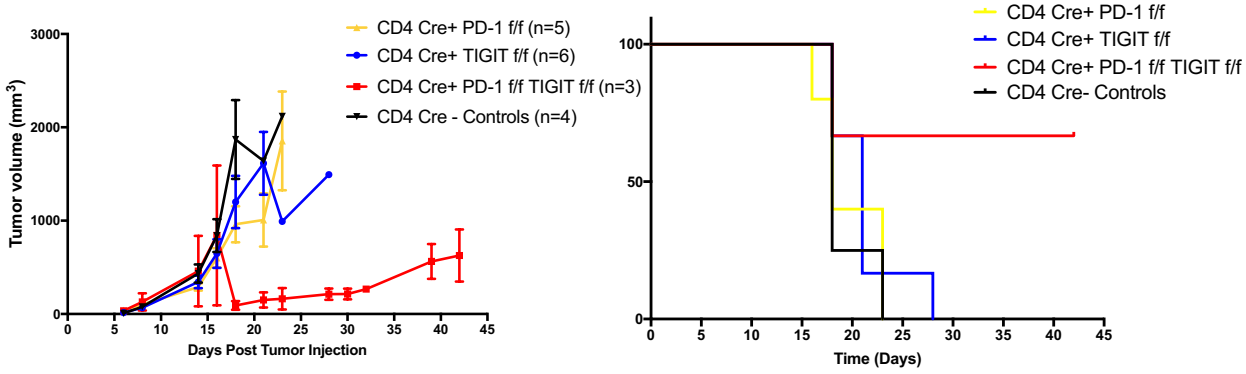

Figure 3.5 B16OVA tumor growth and survival curve in CD4 Cre conditional knockout mice Tumor growth (A) and survival (B) were monitored 2-3 times per week for 4-5 weeks. Data are shown as mean tumor volume ±SEM

We then investigated whether we could use the B16OVA model to replicate the phenotype observed in PD-1/TIGIT double conditional knockout mice using antibody blockade in WT mice. WT mice were implanted with B16OVA tumors and treated with blocking anti-PD-1 or anti TIGIT antibody or a combination of both. Mice treated with combination therapy had an improved anti-tumor response and better survival as compared to mice treated with single agent blockade (Figure 3.6).

A B

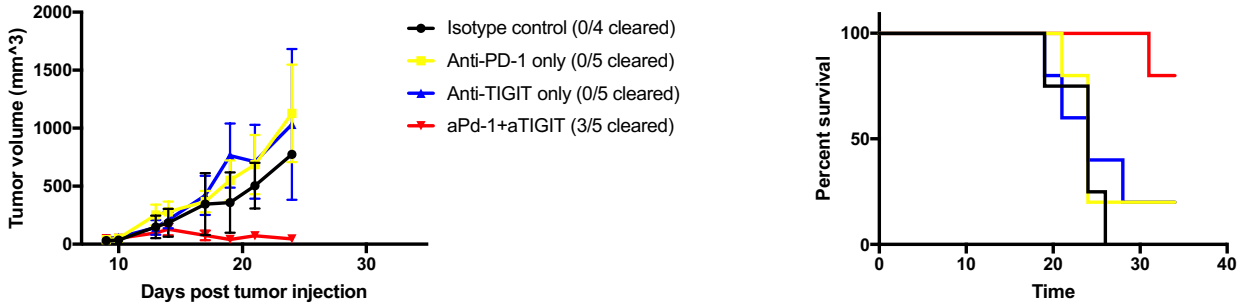

Figure 3.6 B16OVA tumor growth and survival curve in WT mice treated with blocking antibodies
Tumor growth (A) and survival (B) were monitored 2-3 times per week for 4-5 weeks. Blocking anti-TIGIT antibody clone 1B4 (100µg/dose) was administered on Day 0, 2, 4, 10 17 and anti-PD-1 clone 29F (100µg/dose) was administered on Day 1, 3, 5 along with IgG1 and IgG2 isotype controls respectively post randomization. Data are shown as mean tumor volume ±SEM

To assess the tumor phenotype in inducible cre mice, we used the MC38 colon carcinoma model. However, because MC38 tumors are so immunogenic, we tried to reduce the dose of tamoxifen by half to see if we could reduce the knockout of PD-1 expression on cells. We first implanted MC38 tumors in inducible cre mice and then administered tamoxifen from day 7 to 11 post tumor injection. We found that the tumor grew and was cleared very rapidly after tamoxifen administration in UBC Cre PD-1/TIGIT double flox mice (Figure 3.7).

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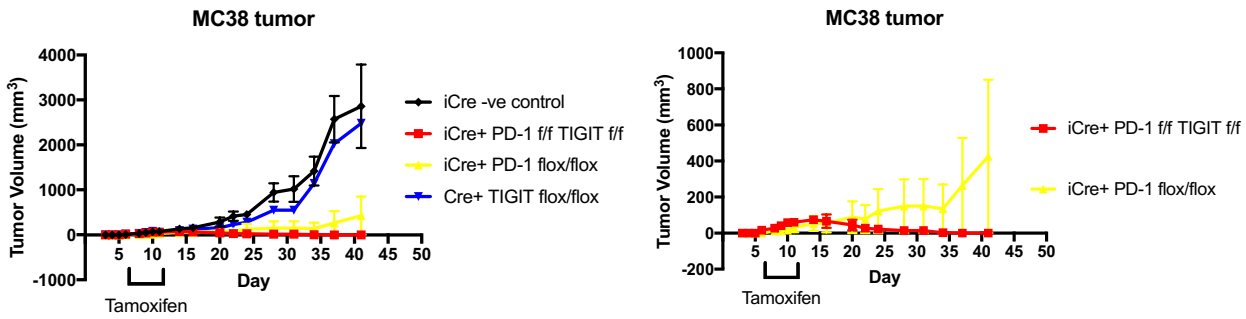
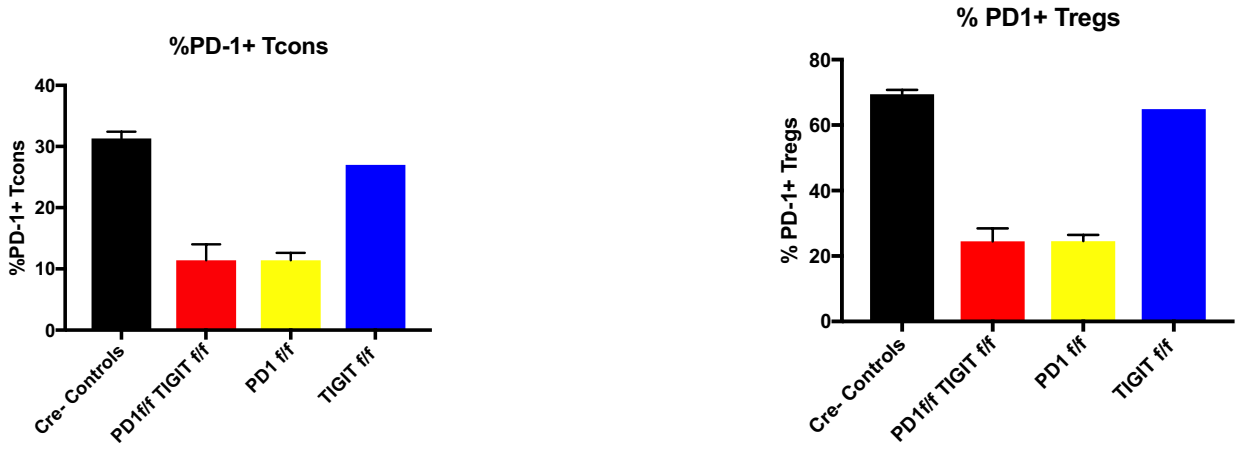


Figure 3.7 MC38 tumor growth and survival curve in ERT Cre mice Tumor growth curve in all mice (A) was monitored 2-3 times per week for 4-5 weeks. 5 doses of tamoxifen 1mg each were administered from days 7-11 post tumor injections. Tumor growth curves were separated to compare tumor growth in PD-1/TIGIT double flox and PD-1 alone flox mice (B). Data are shown as mean tumor volume ±SEM of each group from 3-5 mice per group.

We assessed the deletion of PD-1 and TIGIT expression on T cells in these mice at the end of experiment using flow cytometry. In the inducible mouse models used in the lab, 10 consecutive doses of 1mg each of tamoxifen are administred for a complete deletion of PD-1. Here we reduced the dose to half to see if we could reduce the deletion of PD-1 while still deleting TIGIT. We found that both PD-1 and TIGIT levels on conventional T cells and Tregs were reduced by half in the UBC Cre PD-1 alone flox and PD-1/TIGIT double flox mice was reduced by almost half as compared to the Cre- control mice (Figure 3.8).

A



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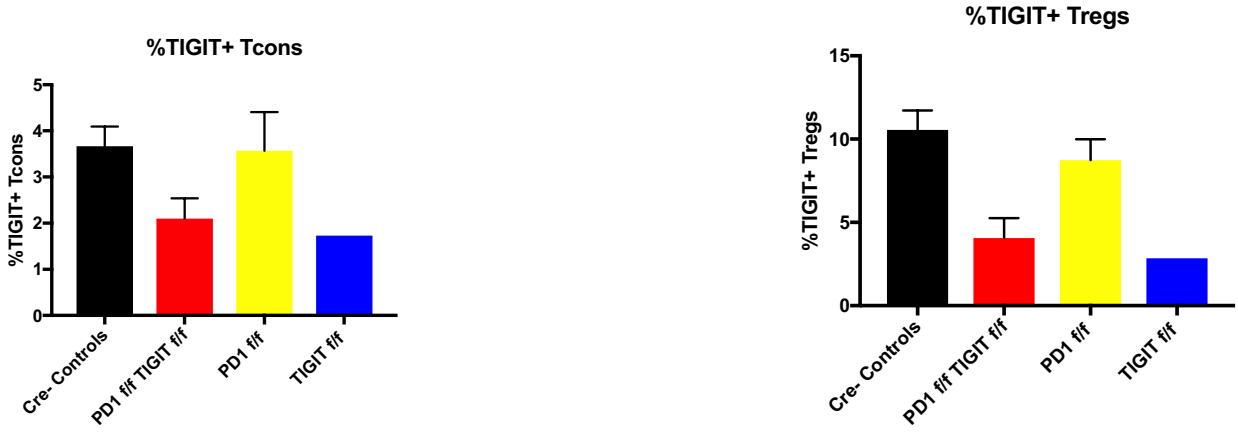

Figure 3.8 Assessment of PD-1 and TIGIT deletion on T cells by tamoxifen administration. At the end of MC38 tumor experiment (day 42), ERT Cre mice were sacrificed and T cells were harvested from the spleens of these mice to evaluate %PD-1 deletion (A) and %TIGIT deletion (B) on conventional T cells (Tcons) and Tregs by analysing PD-1 and TIGIT expression by flow cytometry. Data are shown as frequency±SEM.

Co-inhibitory receptors play a major role in tolerance and autoimmunity. CTLA-4 deficiency is lethal as CTLA-4 -/- mice die due to early development of spontaneous autoimmunity and CTLA-4 blockade causes various autoimmune side effects in patients with cancer [22, 33, 34]. Combination therapy for treatment of cancer causes even severe immune related side effects patients. Lag-3/PD-1 double deficient

mice die due to development of systemic autoimmune disease[35]. Both PD-1 and TIGIT play an important role in regulating tolerance[1,25]. CD4 Cre PD-1/TIGIT flox mice developed in the lab reproduced normally and did not die spontaneously. Thus, these mice were aged and we wanted to see if aged CD4 Cre PD-1/TIGIT double floxed mice develop any spontaneous autoimmunity.

Mice aged ~12 months were studied to assess spontaneous autoimmunity. Grossly, we found no significant differences between Cre+ and Cre- control mice. The spleens were weighed to look for splenomegaly and this was not seen (Figure 3.9A). We also assessed the level of different cytokines in the serum of these mice. We did not find any significant differences in the cytokine levels in Cre+ vs Cre- mice in both males and females (Figure 3.9B). The histopathology and full necropsy results showed that there was no significant difference in the level of inflammation between the Cre+ and Cre- mice in both males and females (data not shown). These results suggest that there is no spontaneous autoimmune inflammation in these mice even when aged for a year.

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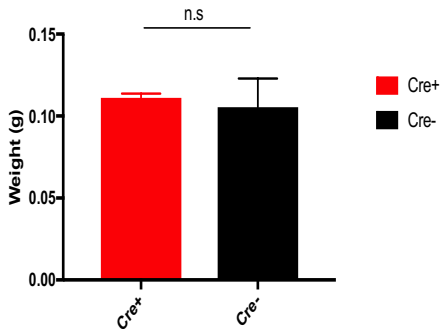

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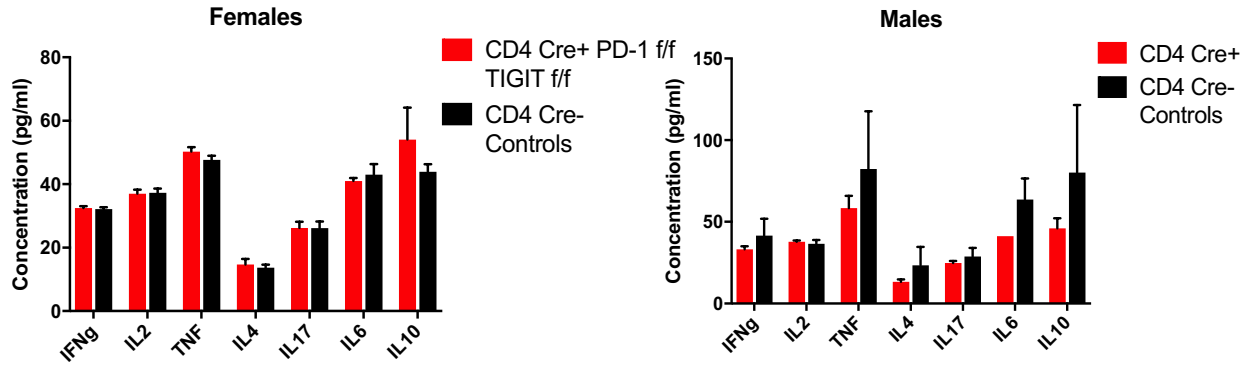

Figure 3.9 Assessment of spontaneous autoimmunity in aged CD4Cre PD-1/TIGIT double flox mice. Cre+ and Cre- mice aged ~12 months were sacrificed and their spleens were weighed (A). Cytokine levels in blood serum of aged mice were measured by Cytokine Bead Array (B). Data are shown as mean±SEM

Deficiency or blockade of PD-1 leads to exacerbated autoimmune disease [36]. Similarly, it has been found that TIGIT-/- mice have increased susceptibility to autoimmune disease[31]. To assess the autoimmune phenotype in PD-1/TIGIT conditional double knockout mice, we used the EAE model of autoimmunity. Previous studies in the lab showed that CD4Cre PD-1/TIGIT double knockout mice when immunized with MOG do not develop a higher disease score as compared to the PD-1 alone floxed mice, following immunization with MOG (Figure 3.10). However, the CD4 Cre TIGIT flox mice have a more severe disease phenotype as well as a delayed resolution as compared to the other strains.

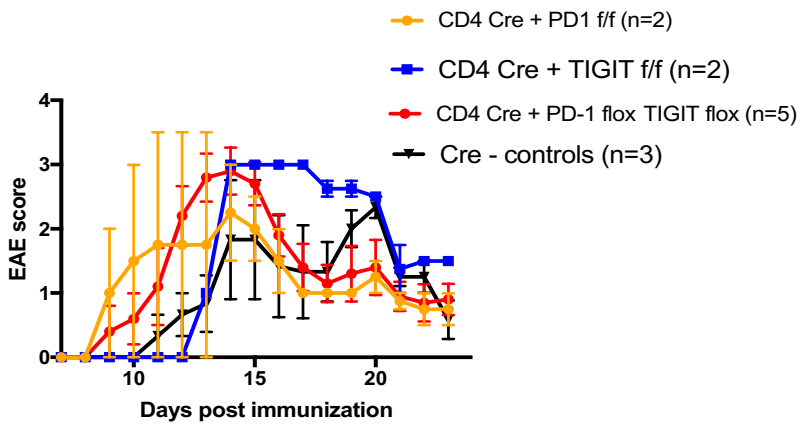

Figure 3.10 EAE disease score curve in CD4Cre conditional knockout mice. Female mice aged ~8 weeks were immunized with MOG peptide. Data are shown as mean±SEM (Unpublished data produced by Juhi Kuchroo)

Following this experiment, we tested if we could replicate this phenotype using antibody blockade. We immunized WT mice with MOG peptide and treated them with blocking TIGIT and high affinity blocking anti-PD-1 antibody. It was difficult to separate the effect in the different groups using high affinity anti-PD-1 antibody in combination with anti-TIGIT antibody (Figure 3.11).

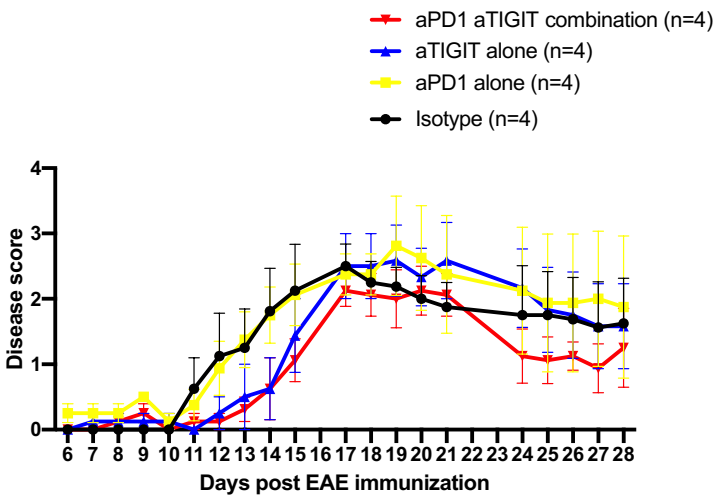

Figure 3.11 EAE disease score curve in WT mice treated with antibody blockade (blocking anti-TIGIT + high affinity blocking anti-PD-1 antibody). C57BL/6 female mice aged ~8 weeks were immunized with MOG peptide. Blocking anti-TIGIT antibody clone 1B4 (100ug/dose) was administered on Days 0, 2, 5, 10 and 17 and high affinity blocking anti-PD-1 antibody clone 29F (100ug/dose) was given on days 1, 3, 6 post immunization with IgG1 and IgG2 antibodies as isotype control. Data are shown as mean±SEM

We then did a similar experiment using a low affinity anti-PD-1 blocking antibody instead. Interestingly, we saw that the mice treated with combination of these blocking PD-1 and TIGIT antibodies were less sick as compared to the mice treated with anti-PD-1 antibody alone (Figure 3.12).

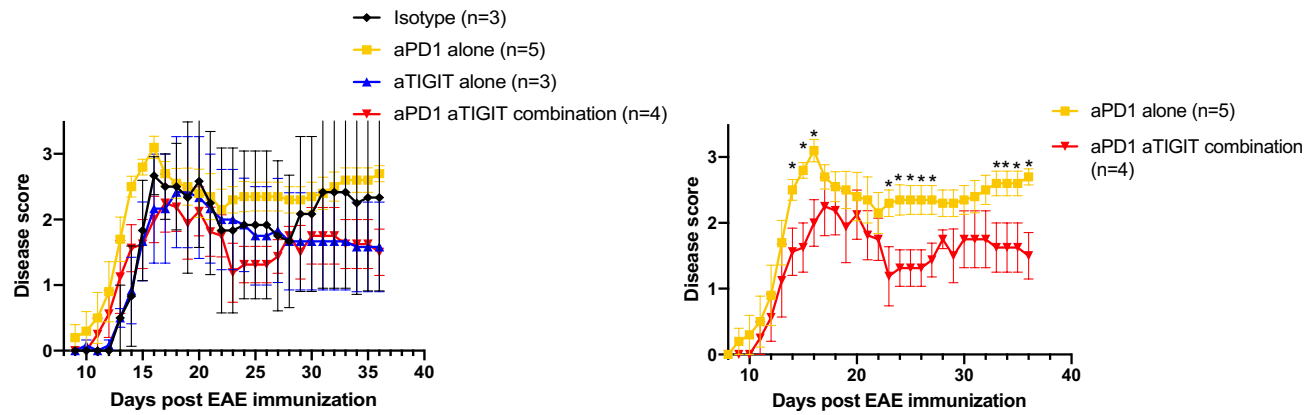

Figure 3.12 EAE disease score curve in WT mice treated with antibody blockade. (blocking anti-TIGIT + low affinity anti-PD-1 blocking antibody). C57BL/6 female mice aged ~8 weeks were immunized with MOG peptide. Blocking anti-TIGIT antibody clone 1B4 (100µg/dose) was administered on Days 0, 2, 5, 10 and 17 and low affinity anti-PD-1 blocking antibody clone RMP14 (100µg/dose) was given on days 1, 3, 6 post immunization with IgG1 (50µg/dose) and IgG2 (100µg/dose) antibodies as isotype control. EAE disease score was monitored every day from day 8 in the different groups (A). (B) shows disease curve to compare mice treated with anti-PD-1 antibody alone to those treated with co-blockade. Data are shown as mean±SEM

Recent studies have shown that TIGIT mainly asserts its suppressive function through Tregs. TIGIT deficient Tregs are less functional as compared to WT Tregs[30]. In our studies, the EAE disease phenotype in CD4 Cre conditional knockout mice showed delayed resolution of disease in TIGIT alone floxed mice. It is known that Tregs play a major role in resolution of disease in the EAE model of autoimmunity[37, 38]. Thus, we wanted to compare the suppressive functions of Tregs from the different conditional knockout mice. We cultured WT CD4+ conventional T cells with the different knockout Tregs and assessed the proliferation of CD4+ conventional T cells to measure Tregs suppressive function. We found that PD-1/TIGIT double deficient Tregs were more functional as compared to the WT Tregs. They are similar in their suppressive capacity to PD-1 deficient Tregs (Figure 3.13A). This is consistent with studies in the lab showing that Tregs from PD-1-/- mice are more suppressive than Tregs from WT mice (unpublished data by Cat Tan). This observation suggests that PD-1/TIGIT double floxed Tregs are

similar in their suppressive capacity to PD-1 alone floxed Tregs. We did not have enough cells from CD4 Cre TIGIT alone floxed mice, thus this assay needs to be repeated to compare their function with other types of Tregs.

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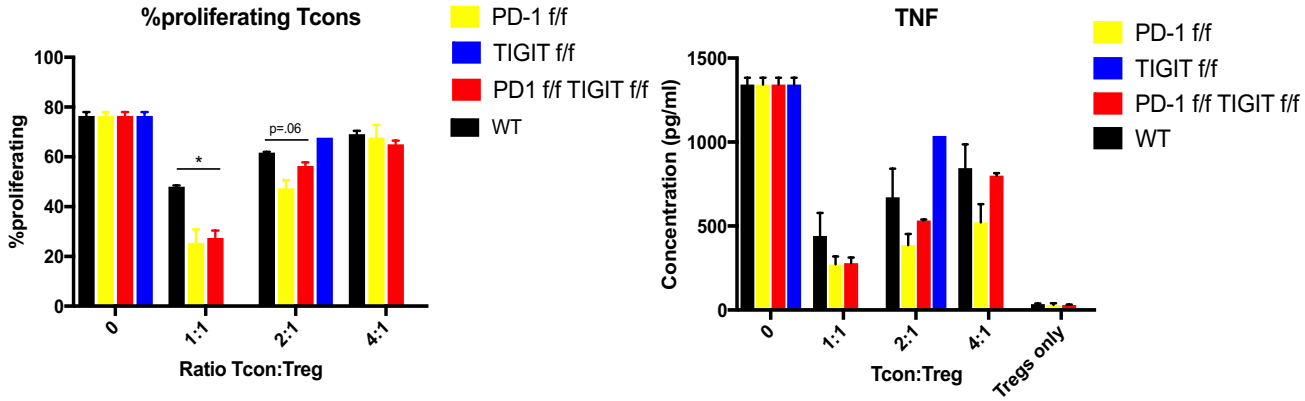

Figure 3.13 Assessing the function of Treg from CD4Cre conditional knockout mice using in vitro Treg suppression assay. WT Tcons were stained with CTV and cultured with Tregs from the indicated conditional knockout mouse strains. CTV dye dilution was assessed to measure proliferation of Tcons to assess Treg frunction (A). Supernatant from the different conditions was harvested and TNF levels measured by CBA (B). Data are shown as mean±SEM

Cytokine analysis of the supernatant from the culture plate showed a similar pattern. Lower TNF cytokine levels were observed when WT conventional T cells were cultured with PD-1 deficient Tregs as compared to when cultured with WT Tregs. Similar TNF levels were produced by WT conventional T cells were cultured with PD-1/TIGIT double deficient Tregs (Figure 3.13B). Previous studies have shown that TIGIT deficient Tregs are less suppressive as compared to WT Tregs. Again, because we did not have enough TIGIT deficient Tregs this assay needs to be repeated to compare cytokine levels produced when WT Tcons are cultured with TIGIT deficient Tregs.

We found that there is a significantly higher number of Tregs expressing TIGIT in CD4 Cre PD-1 flox mice as compared to Cre- controls (Figure 6.1 appendix) (also seen in unpublished data by Juhi Kuhroo). The increased expression of TIGIT in PD-1 deficient mice maybe due to compensatory

mechanisms. It is known that TIGIT is required for the suppressive function of Tregs [25, 30]. Thus, considering the Treg mediated function of TIGIT and higher expression of TIGIT on deletion of PD-1, we wanted to test whether an agonizing TIGIT antibody would have an effect on EAE disease phenotype in PD-1 deficient mice. WT mice when immunized with MOG peptide and treated with agonizing TIGIT antibody are protected from disease when compared to those treated with isotype control antibody[31]. Thus, we wanted to test if administration of agonizing TIGIT antibody in PD-1 deficient mice that have high TIGIT expression could protect them from disease. PD-1-/- mice develop more severe disease as compared to WT mice when immunized with MOG peptide[36]. We wanted to see if agonizing TIGIT rescues the PD-1 -/- mice from disease by modulating the function of Tregs. We found that PD-1 -/- mice treated with agonizing TIGIT antibody developed less severe disease as compared to those treated with the isotype control antibody (Figure 3.14). However, we did not have enough mice in each group and thus this experiment needs to be repeated.

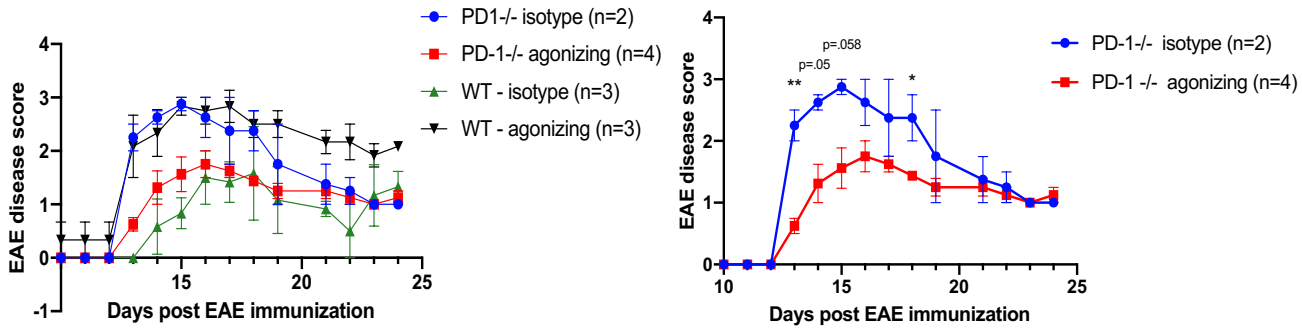

Figure 3.14 EAE disease score curve in PD-1-/- and WT mice treated with agonizing TIGIT antibody. PD-1-/- and C57BL/6 WT female mice aged ~8 weeks were co-housed and immunized with MOG peptide. Agonizing TIGIT antibody clone 1G9 (100µg/dose) was administered on Days 1, 3, 5, 10 with IgG1 (50µg/dose) as isotype control. EAE disease score was monitored every day from day 8 in the different groups (A). (B) shows disease curve to compare PD-1-/- mice treated with agonizing TIGIT vs isotype antibody. Data are shown as mean±SEM

Chapter 4

4.1 Discussion and future perspectives

Despite the tremendous benefits of checkpoint blockade in the treatment of cancer, a number of patients do not respond to these agents[1]. Using a combination of immunotherapies that work through different mechanisms maybe a promising approach[1,2]. Blockade of two inhibitory receptors such as CTLA-4 and PD-1 has shown improved response rates[1,2]. The remarkable success of checkpoint blockade has stimulated the study of a variety of co-inhibitory receptors and various combinations are being tested to improve response to immunotherapy in a wide range of tumors.

In this thesis, we investigated the role of two co-inhibitory receptors PD-1 and TIGIT in tumor immunity and autoimmunity. Recent studies have shown that there is synergy that exists between these pathways in generating anti-tumor immunity in antibody blockade experiments[27,30]. While clinical trials are underway for the treatment of certain tumors with this combination, the mechanism of synergy remains unclear. Furthermore, the enhanced anti-tumor benefits of combination therapy is associated with an increased risk of developing immune related side effects and the possibility of enhanced autoimmunity with this combination therapy will needs to be assessed.

To study the mechanism of synergy, conditional knockout mouse models- UBC Cre PD1/TIGIT double flox and CD4Cre PD-1/TIGIT double flox were developed in the lab by Juhi Kuchroo. On evaluating phenotype of T cells in CD4 Cre conditional knockout mice at homeostasis, we found that knocking out PD-1 causes increase in the number of Lag-3 expressing Tregs whereas knocking out TIGIT alone does not have any impact (Figure 3.2A). Similarly, genetic ablation of TIGIT alone does not affect Tim-3 expression on CD8+ T cells but knocking out PD-1 increases Tim-3+ CD8+ T cells which further increases in PD-1/TIGIT double knockout mice (Figure 3.2C). We also found that knocking out TIGIT alone or in combination with PD-1 leads to increase in the number of Tim-3 expressing CD4+ Tcons

(Figure 3.2B). This increase in the expression of other inhibitory receptors could be because of compensatory mechanisms.

To study the tumor phenotype in the CD4 Cre conditional knockout mice, we identified B16OVA as the best tumor model. We found slower tumor growth as well as improved survival in CD4Cre conditional double knockout mice as compared to the single knockouts (Figure 3.5). We also investigated whether we could replicate this phenotype using antibody blockade as genetic ablation in condition knockout mouse may have development effects. Antibody blockade experiments in WT mice also are more therapeutically relevant. We implanted B16OVA tumors in WT mice and treated them with anti-TIGIT, anti-PD-1 single agent blockade as well as co-blockade (Figure 3.6). Similar to previous studies, we found that mice treated with combination therapy had slower tumor growth and improved survival. Future studies are required to harvest the tumor infiltrating lymphocytes (TILs) and analyze their phenotype (cellular composition and activation status). To understand the genetic circuit underlying this synergy, it would be useful to perform RNA sequencing on these TILs.

To investigate whether deletion of PD-1 and TIGIT is able to rescue the T cells from exhaustion, we used inducible PD-1/TIGIT knockout mouse strains. Because MC38 tumor model is highly PD-1 sensitive, we tried to reduce the deletion of PD-1 and TIGIT expression by reducing the dose of tamoxifen post tumor injection in inducible PD-1/TIGIT knockout mice. We found no significant difference on the tumor growth as the tumor is rapidly cleared (Figure 3.7). Further work is needed to investigate the effect of further reduction of tamoxifen administration on tumor growth. Using UBC Cre mice to assess the tumor phenotype will help us understand if the timing of deletion of PD-1 and TIGIT is important to elicit a better anti-tumor response. It will be useful to study if the timing of tamoxifen administration- before vs. after tumor injection is important in tumor clearance.

Co-inhibitory receptors play a significant role in regulating tolerance. Patients on combination therapy can develop immune related adverse events. Both PD-1 and TIGIT have been shown to play a

major role in tolerance and autoimmunity[31, 39]. Both blockade and deficiency of PD-1 leads to increased autoimmune disease severity[39]. Similarly, TIGIT-/- mice are also more susceptible to developing autoimmune disease[31]. We assessed whether targeting both of these receptors would worsen autoimmune disease severity. We first examined whether CD4 Cre PD-1/TIGIT mice developed any spontaneous autoimmunity as PD-1/LAG3 double deficient mice develop lethal spontaneous autoimmunity[35]. The CD4 Cre PD-1/TIGIT double floxed mice generated in the lab did not die spontaneously and reproduced normally. We found that aged CD4 Cre PD-1/TIGIT double floxed mice did not develop any significant inflammation at steady state by histology (data not shown) and cellular analysis (Figure 3.9). Further studies are required to assess whether autoantibodies are present in these aged mice, and serum has been banked from these aged mice for that analysis.

On monitoring the EAE disease phenotype in CD4 Cre conditional knockout mice, we found that CD4 Cre PD-1 flox mice developed more severe disease compared to the Cre- controls (Figure 3.10). CD4Cre TIGIT alone floxed mice had a delayed onset of disease as compared to all the other conditional knockout mice as well as a delayed resolution of disease, whereas the PD-1/TIGIT conditional double floxed mice were similar in EAE severity to PD-1 alone floxed mice but not worse. Further studies are needed to harvest lymphocytes from the CNS of these mouse strains at different stages of disease-priming, peak and resolution to analyse cellular composition and activation status. It also would be useful to perform RNA sequencing on these cells to infer the transcriptional networks downstream of PD-1 and TIGIT in different T-cell subsets and determine how loss of a receptor impacts the transcriptome.

Based on the EAE data from conditional knockout mice, we determined whether a similar phenotype could be observed with antibody blockade. On treating WT mice with antibody blockade, we found that high affinity anti PD-1 antibody combined with anti-TIGIT antibody resulted in similar disease severity in the different groups of mice (Figure 3.11). It was hard to separate the EAE disease score curve in the different groups of mice with this combination of blocking antibodies as all groups of mice had a similar disease phenotype. To study the autoimmune phenotype using the EAE model, the optimum dose

of high affinity anti-PD-1 blocking antibody to be administered in combination with anti-TIGIT blocking antibody is still not clear. Thus, we then repeated the experiment with a low affinity anti-PD-1 blocking antibody. Surprisingly, we found that WT mice immunized with MOG peptide and treated with this combination of anti-PD-1 and anti-TIGIT antibody had less severe disease as compared to mice treated with anti-PD-1 antibody alone (Figure 3.12). Since the enhanced anti-tumor benefits of combination therapy is associated with an increased risk of developing immune related side effects, the above result suggests that blocking anti-TIGIT antibody has the potential of being used in combination with anti-PD-1 therapy in cancer, however, further studies are required to understand the mechanism.

TIGIT mainly asserts its immunosuppressive function through Tregs. Since we observed that CD4 Cre TIGIT alone floxed mice had a delayed resolution of disease when immunized with MOG peptide (Figure 3.10), we hypothesized that the TIGIT deficient Tregs were responsible for this delay in resolution. Thus, we compared the function of Tregs from these different conditional knockout mice. Interestingly, we found that PD-1/TIGIT double deficient Tregs were more functional as compared to WT Tregs and similar to PD-1 alone deficient Tregs (Figure 3.13). Because we did not have enough TIGIT deficient Tregs, we plan to repeat this experiment to assess their function. However, these results suggest that PD-1 and TIGIT work together to modulate autoimmunity, and that removing TIGIT from PD-1 deficient Tregs does not negatively impact the functional capacity of these Tregs. This assay was mainly performed to assess the function of Tregs from these conditional knockout mice but it will also be interesting to do a similar assay to assess the function of conventional T cells from these different conditional knockouts as they may also be playing a role in mediating the in vivo disease phenotypes observed above.

TIGIT acts as a negative regulator in a variety of autoimmune diseases[25]. On treatment with agonizing TIGIT antibody, WT mice immunized with MOG peptide are protected from disease as compared to the control group[25]. PD-1 -/- mice are more susceptible to autoimmunity[36] and previous studies in lab (unpublished data by Juhi Kuchroo) and our results suggest that PD-1 deficient mice have

high a higher number of CD4+ Foxp3+ Tregs expressing TIGIT (see appendix Figure 6.1). Thus, we wanted to see if PD-1-/- mice are protected from EAE when treated with agonizing TIGIT antibody. We found that agonizing TIGIT treatment in PD-1-/- mice protects them from disease (Figure 3.14). This suggests that agonizing TIGIT antibody might be enhancing the suppressive function of Tregs and dampening autoimmune T cell response. But this experiment needs to be repeated, as we did not have enough number of mice for each experimental group.

PD-1 asserts its immune inhibitory functions through various mechanisms. Blocking PD-1 leads to a significant anti-tumor response mainly because of activation of CD8+ cytotoxic lymphocytes[1,12]. On the other hand, TIGIT mainly causes immune suppression through Tregs[30]. Thus, it would be interesting to identify the different cell types involved in the synergy between these pathways by performing adoptive transfer experiments to study the autoimmune phenotype. It would be interesting to study the effect of different combinations of the various T cell subsets- Tregs, conventional T cells and CD8+ T cells that lack PD-1 alone, TIGIT alone and PD-1/TIGIT by adoptive transfer experiments to identify and isolate the role of each receptor on different T-cell subsets in autoimmune disease phenotype.

To identify the cell types involved in mediating anti-tumor responses and modulating autoimmunity, we are also developing novel mouse models in the lab including inducible CD8 T cell specific TIGIT knockout and PD-1/TIGIT double knockout mice and inducible Treg specific TIGIT knockout and PD-1/TIGIT double knockout mice. Further, to study the autoimmune phenotype we are developing mouse models on the 2D2 TCR transgenic background that lack TIGIT alone, PD-1 alone or both on CD4+ and CD8+ T cells.

Through pre-clinical studies, TIGIT has been identified as a promising target for cancer immunotherapy and its potential to work synergistically with PD-1 in cancer has been demonstrated as well. Through this project, we have tried to elucidate the mechanism of synergy between PD-1 and TIGIT in tumor immunity as well as whether the efficacy of this therapy is limited by immune-related adverse

events. Our results will help in the development of better strategies to improve anti-tumor response while minimizing immune related side effects.

4.2 Limitations of this study

One of the major limitations of this study was the number of conditional knockout mice available to perform the experiments. These knockout mouse models were being developed in the lab and it was difficult to get a large sample size with the appropriate genotypes, age and gender to perform any experiment. Further, we only used EAE as our autoimmune model. It would be useful to study the autoimmune phenotype using other disease models such as colitis.

5. References

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6. Appendix

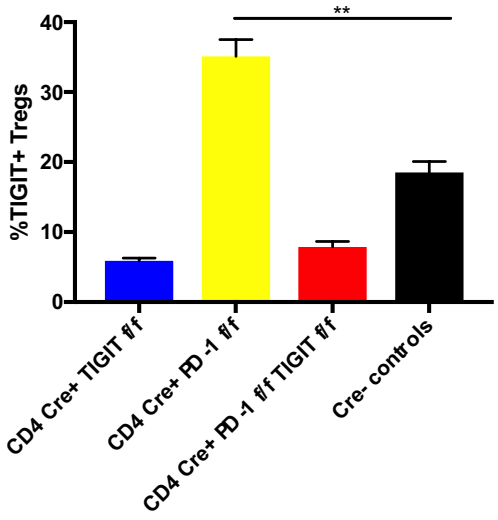

Figure 6.1 Assessing TIGIT expression on CD4+ Tregs and Tcons in CD4 Cre conditional knockout mice at homeostasis. CD4+ T cells were harvested from spleens of ~8 week old females and expression of TIGIT was assessed by flow cytometry on Foxp3+ Tregs. Data are shown as frequency±SEM (***$p<0.001$)