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Unleashing Immunity:
Expanding our Immune Arsenal with Treg Depletion

By
Chenyu Zhang

A thesis submitted in partial satisfaction of the
requirements for the degree of
Doctor of Philosophy
in
Molecular and Cell Biology
in the
Graduate Division
of the
University of California, Berkeley

Committee in charge:

Professor David H. Raulet, Co-Chair
Professor Michel J. DuPage, Co-Chair
Professor Russell E. Vance
Professor Gregory M. Barton
Professor Ellen A. Robey

Fall 2025

Unleashing Immunity:
Expanding our Immune Arsenal with Treg Depletion

Abstract

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Chenyu Zhang

Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor David H. Raulet, Co-Chair
Professor Michel J. DuPage, Co-Chair

Our understanding of immune cell crosstalk in the tumor microenvironment (TME) has evolved significantly in recent years, revealing a complex network of interactions beyond the classical CD4⁺ T cell help to other adaptive immune cells such as CD8⁺ cytotoxic T cells, B cells and macrophages. The TME is a highly dynamic and immunologically rich landscape, where both innate and adaptive immune cells, such as natural killer (NK) cells and CD4⁺ T cells, engage in complex, reciprocal signaling that shapes therapeutic outcomes. NK cells, as key components of the innate immune system, can detect and eliminate tumor cells that evade cytotoxic T lymphocytes by downregulating MHC class I molecules. Meanwhile, CD4⁺ T cells serve as potent cytokine producers and have emerged as critical amplifiers of antitumor immunity, not only by supporting CD8⁺ T cell function but also through direct effector activity and coordination of broader immune responses.

Recent advances in immunotherapies such as checkpoint immunotherapies that are predicated on amplifying CD8⁺ T cell responses have led to vastly improved patient outcomes. While CD8⁺ T cells have long been the cornerstone of cancer immunotherapy, some tumors lack neoantigens and others acquire defects in antigen presentation or MHC I expression either before therapy or as a result of it, acquiring resistance to CD8⁺ T cell-dependent therapies. There is therefore a need for alternative regimens that can overcome tumor immune resistance. NK cells

and CD4⁺ T cells represent two emerging immune cell populations that are favorable targets for novel and improved immunotherapy, as NK cells and CD4⁺ T cells have the potential to control MHC I-deficient tumors that can resist CD8⁺ T cell immune responses.

Regulatory T cells (Tregs) play a pivotal role in maintaining immune homeostasis by suppressing autoimmune responses mediated by conventional T cells. Systemic depletion of Tregs is known to unleash lethal autoimmune responses. On the other hand, Tregs also suppress beneficial antitumor responses mediated by CD8⁺ T cells. Continuing Moreno *et al.*'s work on investigating CXCR3⁺ Tregs in suppressing cDC1 cross-presentation and CD8⁺ T cell control of cancer, I proposed to explore the hypothesis that Tregs also suppress antitumor immune responses mediated by NK cells and CD4⁺ T cells. If true, this raises the possibility that selective Treg depletion may provide an approach to generate responses against MHC I-deficient tumors, which are resistant to CD8⁺ T cell recognition and killing.

The work I describe in this dissertation in Chapter 3 demonstrates that both systemic and intra-tumoral depletion of Tregs leads to effective control of *B2m*^{-/-}, and thus MHC I-deficient, colon carcinoma, melanoma and lymphoma tumors implanted into mice. The findings reveal that Tregs suppress anti-tumor responses independent of CD8 T cell responses. Specifically, depletion of NK cells or CD4⁺ T cells restored tumor growth in Treg-depleted settings, while CD8⁺ T cell depletion had no effect. This indicates that NK and CD4⁺ T cells are the primary effectors responsible for tumor control under these conditions. Furthermore, NK cell and CD4⁺ T cells were found to act synergistically to mediate the clearance of MHC I-deficient tumors. Importantly, intra-tumoral—rather than systemic—Treg depletion elicited potent antitumor effects mediated by NK cells and CD4⁺ T cells without inducing significant autoimmunity. In Chapter 3, I detail how intra-tumoral depletion of Tregs unleashes anti-tumor function of tumor-infiltrating NK cells. I characterize this enhanced activity by documenting changes in NK cell surface marker expression, cytokine production, degranulation, and cytotoxic capacity. I further demonstrate that CD4⁺ T cell help is essential for optimal NK cell-mediated tumor control, and investigate the underlying mechanisms driving this interaction. My results identify a critical role for conventional dendritic cells (cDCs) in orchestrating the crosstalk between Tregs, CD4⁺ T cells, and NK cells within the tumor microenvironment via a key cytokine—IL-2. To dissect and validate these mechanisms of

cellular crosstalk, I employ physiologically relevant tumor models and integrate high-resolution techniques, including immune cell sorting, transcriptomic profiling, and imaging. My approach combines *in vivo* tumor modeling, *ex vivo* functional assays, and spatial analysis to map the timing, location, and nature of NK–CD4–Treg interactions in the context of MHC I-deficient tumors. Collectively, this work offers new insights into alternative immune effector pathways in tumor control and provides a framework for enhancing cancer immunotherapy, particularly in tumors that escape CD8⁺ T cell recognition.

From the experiments targeted at understanding NK cell-mediated tumor control following intra-tumoral Treg depletion, I identified that beyond providing essential help for robust NK cell tumor control, CD4⁺ T cells in parallel were also able to elicit tumor control independent of NK cells. In Chapter 4, I systematically characterize the CD4⁺ T effector cell response after intra-tumoral Treg depletion, and study the mechanism by which the CD4⁺ T cells contribute to anti-tumor immunity. We used a combination of bulk RNA sequencing data supplemented with spectral flow analyses, direct *ex vivo* killing assays, and the expression of different activation markers and cytokines of these CD4⁺ cytotoxic T cells to carefully phenotype these cells. I also noted that these CD4⁺ T cells upregulated expression of canonical NK cell receptors. I examined whether the cytotoxic CD4⁺ T cell population is induced locally within the tumor microenvironment, or if a more systemic response is also observed following Treg depletion. I also generated evidence to suggest that tumor cell killing occurs independently of MHC II-recognition or death receptor-engagement on tumor cells, but is highly dependent on granzyme and perforin activity. Taken together, these data uncover a potent population of cytotoxic CD4⁺ T cells which have high anti-tumor potential, further expanding on the immune arsenal for cancer immunotherapy.

While it is widely accepted that Treg depletion enhances conventional T cell effector functions by removing suppressive constraints, emerging evidence suggests a more complex picture in inflamed tissues (e.g. autoimmune sites or cancer). Specifically, Tregs that have lost Foxp3 expression, termed “ex-Tregs”, may themselves contribute to the effector immune response under these conditions. My work in Chapter 5 tests the hypothesis that ex-Tregs play a central role in shaping the immune response following Treg depletion both in autoimmunity and tumor contexts. Demonstrating that the inflammatory effector responses originate primarily from ex-

Tregs will significantly shift our understanding of the immunological consequence of Treg depletion. Such insights could inform the development of novel therapeutic strategies to selectively target ex-Treg function to modulate immune responses in diseases. To investigate this, we are currently optimizing two new genetic mouse models to study ex-Tregs that will allow, for the first time, ex-Treg ablation *in vivo* and ex-Treg tracing *in situ* by fluorescence microscopy.

To explore immune modulation in clinically relevant settings, I also employed and further developed multiple spontaneous models of cancer using genetically engineered mouse models (GEMMs) harboring *K-ras* and *Tp53* mutations (KP), including sarcomas, lung adenocarcinomas and glioblastomas. These models more accurately reflect the complexity and immune resistance of human cancers. In combination with CRISPR-Cas9-mediated gene editing—such as *B2m*^{-/-} to model MHC class I deficiency—these systems provide a robust platform to investigate mechanisms of immune evasion and response to therapy. In Chapter 6, I will detail a series of experiments setting up and optimizing these KP-Cas9 models.

Finally, in Chapter 7, I will present preliminary findings on a novel pathway of tumor control uncovered through the use of tetracycline derivatives 9-TB (9-tert-butyl doxycycline) and AD (7-ethylphenyl 4'-methoxysancycline). These compounds have been chemically modified to eliminate antimicrobial activity while preserving their ability to inhibit mitochondrial protein translation and remain well tolerated *in vivo*. Treatment with 9-TB and AD activates a mitochondrial unfolded protein response (UPR^{mt}), a stress response pathway that remains poorly understood in comparison to the more extensively characterized endoplasmic reticulum UPR (UPR^{er}). In collaboration with Dr. Andrew Dillin, a leading expert on mitochondrial stress responses, as well as postdoctoral fellows in the Raulet lab (Hannah Ghasemi and Harrison Sudholz), this project seeks to elucidate the molecular mechanisms driving this UPR^{mt}-mediated anti-tumor effect and to identify key regulators within this pathway that may be exploited for therapeutic benefit.

Collectively, the studies in this thesis uncover previously unrecognized mechanisms of immune cooperation within the tumor microenvironment and expand our understanding of how tumor control can occur independently of classical CD8⁺ T cell responses. Through targeted

depletion of regulatory T cells (Tregs), I demonstrate that both NK cells and CD4⁺ T cells can act as potent effectors capable of mediating robust antitumor immunity. I show that intra-tumoral Treg depletion restores NK cell cytotoxicity through IL-2–dependent crosstalk with CD4⁺ T cells and dendritic cells. A population of cytotoxic CD4⁺ T cells capable of directly eliminating MHC I–deficient tumors via granzyme–perforin activity also arises after IT Treg ablation, revealing an alternative effector pathway with therapeutic promise. Further, ex-Tregs have the potential to influence both immune activation and tissue inflammation. With tools such as genetically engineered mouse models of cancer combined with CRISPR editing platforms, we can now test interventions such as localized Treg depletion, cytokine delivery, cyclic dinucleotides, and other therapies with more physiologically relevant tumor models. Finally, I describe a novel line of investigation demonstrating that non-antimicrobial tetracycline derivatives activate the mitochondrial unfolded protein response (UPR^{mt}), revealing a new link between mitochondrial stress and antitumor immunity. Together, these findings delineate a broader and more flexible immune network that can be harnessed to target tumors resistant to CD8⁺ T cell recognition. By integrating mechanistic studies with translationally relevant models, this work establishes a foundation for therapies that mobilize NK and CD4⁺ T cell immunity, modulate Treg and ex-Treg dynamics, and exploit stress-response pathways to achieve durable tumor control.

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DEDICATION

This thesis is dedicated to my brother, Wenyu Zhang, for paving the way and showing me the bravery to step out of our comfort zones and to pursue the things we are most passionate about. From moving across the world from Singapore to the United States for our studies, to ending up in California together for the past six years, you are the home away from home. Thank you, for being the best elder brother a sister can ever ask for.

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They say it takes a village to raise a child. As it turns out, it takes one to finish a PhD too. Behind the completion of this thesis and my degree stands a village of mentors, colleagues, family and friends whose support made this endeavor possible.

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To my parents Zhengu Zhang and Xiaoman Wang—your countless sacrifices and steadfast devotion have carried me farther than words can express. Thank you for teaching me the quiet strength of hard work, the honesty that grounds every endeavor, and the resilience to begin again after every fall. Though my work may sometimes feel distant from your world, you have met it always with patience, pride, and unconditional love. Everything I have accomplished rests upon the foundation you built, and I hope, in some small way, I have made you proud. To my grandparents watching over me from beyond, I wish that you all could be here to celebrate this milestone together, but know that I always keep you in my heart.

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Last, but never least, my dearest cat Banana: you are the purest joy and constant love of my life, and you more than deserve an honorary PhD simply for being there since day one in Berkeley.

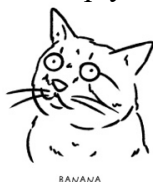


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Chapter 1: Introduction

The Dichotomous Roles of our Immune System

When we pause to reflect on the capabilities of our immune system, some of the first qualities that come to mind seem almost paradoxical: we need our immune system to rapidly respond to external threats like infectious agents, yet we require a delicate regulatory balance to ensure that we do not activate autoimmune responses against normal self-cells, and at the same time we need to launch desired responses against foreign pathogens and abnormal cells, such as tumor cells. How our immune system manages these different outcomes remains an important topic that is not fully understood. The simplistic categorization of innate versus adaptive immunity can help us begin to appreciate the intricate coordination that underlies our immune system.

Innate Immune System

/iˈnāɪt/

Adjective: inborn; natural.

Our innate immune system acts as our body's first line of defense, responding rapidly and ready for any attack. Physical barriers like our skin and mucosal surfaces act as a first impediment to trap any unwanted pathogens or foreign objects from entering our tissues (González 2013).

The innate immune system comprises a range of specialized cells that provide rapid, non-specific defense against invading pathogens. Neutrophils arrive rapidly to the site of injury and aid in the recruitment of other immune cell subsets. Macrophages are primary phagocytes that ingest and destroy microbes, identifying the foreign invaders or dead host cells through generalized features of infection (Unanue 1984). Broadly, these features are called pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs consist of components of bacterial or viral parts, such as the cell wall or nucleic acids, that distinguish the intruders from the host. DAMPs are generated from cell death as a consequence of infections, which cause the mislocalization of nuclear components when leak out of dying cells. The PAMPs and DAMPs are recognized with pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) (Lemaitre, Nicolas et al. 1996, Medzhitov, Preston-Hurlburt et al. 1997, Barton and Medzhitov 2002, Barton and Medzhitov 2003), NOD-like receptors (NLRs) (Chen, Shaw et al. 2009), C-type lectin receptors (CLRs) (Geijtenbeek and Gringhuis 2009), and scavenger receptors. Macrophages also express CD14, which, in association with

TLR4, detects bacterial lipopolysaccharide and triggers pro-inflammatory cytokine release (Meng, Gong et al. 2011). Dendritic cells sense pathogens via TLRs and CLRs, then migrate to lymphoid tissues to present antigens and activate adaptive immunity (Harding, McArthur et al. 1992, Larsen, Ritchie et al. 1992, Inaba, Witmer-Pack et al. 1994). NK cells, distinguished by the expression of natural cytotoxicity receptors (such as NKp30, NKp46, and killer immunoglobulin-like receptors (KIRs)), survey for cells lacking normal self MHC-I and eliminate infected or transformed cells (Karre, Ljunggren et al. 1986, Raulet and Held 1995, Diefenbach and Raulet 2001). Mast cells, eosinophils, and basophils respond to helminths and allergens primarily through Fc receptors and release of histamine or granule contents (Stone, Prussin et al. 2010).

Collectively, innate immune cells rely on germline-encoded receptors to recognize conserved molecular patterns, rapidly activating antimicrobial functions, producing cytokines and chemokines, and shaping the subsequent adaptive immune response (Carroll, Pasare et al. 2024).

Adaptive Immune System

/əˈdaptiv/

Adjective: To change something so that it functions better or is better suited for a purpose

To achieve the specificity and magnitude of effector cells necessary to control infections, adaptive immunity requires about a week to fully develop after initial antigen exposure (Cooper and Alder 2006, Bonilla and Oettgen 2010). A T or B cell specific for a particular pathogen is extremely rare—at baseline, there are not enough cells to drive an effective immune response. The week-long process required for adaptive immunity involves a rapid, Darwinian-like selection process in which T and B cells that recognize a particular pathogen undergo multiple rounds of cell division, called clonal expansion, to generate a clonal army of cells, each with a pathogen-specific receptor tailored to recognize the invading pathogen. Upon recognition of their cognate antigen, these lymphocytes activate effector functions and clonally expand several thousand-fold, enabling effective pathogen clearance. Importantly, a subset of these cells differentiates into long-lived memory cells that persist after infection resolution and mediate faster, more robust responses upon re-exposure (Kaech and Ahmed 2001, Kaech, Wherry et al. 2002, Kaech, Hemby

et al. 2002). This capacity for immunological memory is a defining hallmark of adaptive immunity, enabling lasting protection and forming the basis of vaccination strategies.

Cancer vs. Autoimmunity

Cancer presents a unique challenge to our immune system as cancerous cells are derived from the host, and there are limited antigens (neoantigens) that effectively distinguish tumors from the host (DuPage, Cheung et al. 2011, McGranahan, Rosenthal et al. 2017, Xie, Shen et al. 2023). Cancer is often viewed as a “maximalist” disease in which cells escape normal regulatory mechanisms, proliferate without restraint, and establish malignant tumors that hijack resources from surrounding tissues. The seminal review of Hanahan and Weinberg identified several hallmark features underlying this transformation, including self-sufficiency in growth signaling, resistance to growth-inhibitory cues, evasion of apoptosis, limitless replicative capacity, sustained angiogenesis, and the ability to invade tissues and metastasize (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011). The concept that the immune system can surveil and eliminate malignant cells was first articulated in the “cancer immunosurveillance” hypothesis, posited by Lewis Thomas and Sir Frank Macfarlane Burnet in the late 1950s and early 1960s (Burnet , Thomas and Lawrence 1959), that immune cells detect and target tumor-specific features that mark these cells as “non-self,” such as reduced expression of MHC class I molecules.

In parallel, the immune system possesses robust mechanisms to maintain self-tolerance, primarily by eliminating or suppressing self-reactive lymphocytes during their development. Central tolerance mechanisms, such as clonal deletion of autoreactive T cells in the thymus, are complemented by peripheral tolerance processes. In particular, CD4⁺ regulatory T (Treg) cells play a critical role in restraining potentially autoreactive T cells that escape central tolerance/thymic deletion, thereby safeguarding against unwanted immune activation (Klein, Robey et al. 2019). Impaired Treg cell frequency or function is associated with a range of autoimmune diseases, including autoimmune thyroiditis, gastritis, type 1 diabetes, and inflammatory bowel disease. In cancer, Treg cells prevent anti-cancer immune responses simply by doing their job “too well”.

Thus, immune homeostasis is achieved through a delicate balance between eliminating dangerous self-cells, such as infected cells or cancer cells, and restraining the activity of self-

reactive immune cells to prevent autoimmunity. The mechanisms and key immune populations that enforce this balance will be discussed in detail in the following sections.

Natural Killer Cells

Natural killer (NK) cells are cytolytic lymphocytes that target and eliminate transformed or virus-infected cells by employing germline-encoded receptors to recognize specific stimulatory ligands expressed on stressed or unhealthy cells—a process known as ‘induced-self recognition’. Additionally, NK cells detect the absence or reduction of major histocompatibility complex class I (MHC I) molecules on target cells through inhibitory receptors, enabling ‘missing-self recognition’ to identify abnormal cells that evade T cell immunity (Shifrin, Raulet et al. 2014). NK cells in homeostasis are kept in check by the balance of activating and inhibitory signals via the respective receptors, which allows normal healthy cells to not be targeted for NK cell killing (Figure 1). The name “natural killer” was derived from studies that highlighted the ability of NK cells, but not T or B cells, to spontaneously kill cultured tumor cells (Herberman, Nunn et al. 1975).

NK cell Receptors

Among the principal activating receptors on NK cells are the natural cytotoxicity receptors (NCRs)—NKp30, NKp44, and NKp46—as well as the natural killer group 2D receptor (NKG2D) and Ly49 family receptors (Kruse, Matta et al. 2014, Barrow, Martin et al. 2019). NKp30 and NKp46 are constitutively expressed on resting NK cells, whereas NKp44 expression is restricted to mature NK cells. These receptors detect ligands associated with viral infection or cellular stress. For example, NKp46 recognizes viral hemagglutinin, vimentin, complement factor P, and ecto-calreticulin on endoplasmic reticulum (ER)-stressed cells (Sen Santara, Lee et al. 2023). NKp44 binds ligands such as proliferating cell nuclear antigen (PCNA), linking DNA repair and cancer cell proliferation. Glycosaminoglycans like heparan sulfate (HS), abundant in proteoglycans that contribute to tumor growth, serve as ligands for NKp30, NKp44, and NKp46 receptors (Bloushtain, Qimron et al. 2004, Hecht, Rosental et al. 2009).

NK cells also express Fc gamma receptors (FcγRs), notably CD16 (FcγRIIIA), which mediate antibody-dependent cellular cytotoxicity (ADCC) by binding to the Fc portion of IgG

antibodies produced by B cells. Human NK cells express five low-affinity FcγRs: one inhibitory receptor (CD32B/FcγRIIB/FCGR2B) and four stimulatory receptors (CD32A, CD32C, CD16A, CD16B). Murine NK cells express two activating FcγRs (CD16/FcγRIII/Fcgr3 and CD16.2/FcγRIV/Fcgr4) and one inhibitory receptor (CD32b). CD16 is a well-established marker of mature NK cells, enabling effective killing of antibody-coated target cells. This ADCC mechanism underlies the efficacy of therapeutic monoclonal antibodies such as rituximab, trastuzumab, and cetuximab (Bhatnagar, Ahmad et al. 2014).

The NKG2D receptor was first identified in a human NK cell screen, along with NKG2A/C/E, as a type 2 transmembrane receptor with sequence similarities to C-type lectins. Although the NKG2D gene is located with the other NKG genes in the NK gene complex (NKC), NKG2D differs in sequence and recognizes numerous MHC I-like ligands, including MICA, MICB, and ULBP1-6 in humans, and RAE-1 α -RAE-1 ϵ , MULT1, and three H60 isoforms in mice (Zingoni, Ardolino et al. 2012, Raulet, Gasser et al. 2013, Zingoni, Molfetta et al. 2018). NKG2D ligands are usually upregulated in response to stressors such as heat-shock, DNA damage, cellular hyperproliferation and activated p53 signaling (Nice, Coscoy et al. 2009). While NKG2D ligands in humans and mice have diversified independently, the NKG2D receptor itself is highly conserved, with up to 70% similarity between the human and mouse receptors. The diversification of ligands but not the receptor strongly indicate that NKG2D ligands are likely functionally redundant, ensuring robust responses across species.

Inhibitory NK cell receptors primarily recognize MHC I molecules on target cells, providing a mechanism to prevent NK cell-mediated killing of healthy cells (Liao, Bix et al. 1991). Human inhibitory receptors include the killer immunoglobulin-like receptor (KIR) family, which binds various HLA class I molecules. Mice lack KIRs and instead possess a family of lectin-like Ly49 receptors, with about seven inhibitory members that distinguish various H-2 class I molecules. Both humans and mice express the heterodimeric CD94-NKG2A receptor, which recognizes non-classical class I molecules (HLA-E in humans and Qa-1 in mice). These inhibitory signals regulate NK cell activation thresholds and prevent autoimmunity (Yokoyama and Seaman 1993, Raulet, Vance et al. 2001). Importantly, the various inhibitory receptors can differentiate between MHC I allelic products and are distributed across different subsets of NK cells in an

overlapping pattern. This distribution allows for a nuanced response to cells with varying MHC I expression profiles. For instance, NK cells can be activated if they engage tumor cells that lose even one of its MHC I molecules. Alternatively, even in cases where tumor cells express normal levels of MHC I, if they express sufficiently high levels of activating ligands, this can allow NK cells to overcome inhibitory signaling and become activated (Cerwenka, Baron et al. 2001, Diefenbach, Jensen et al. 2001).

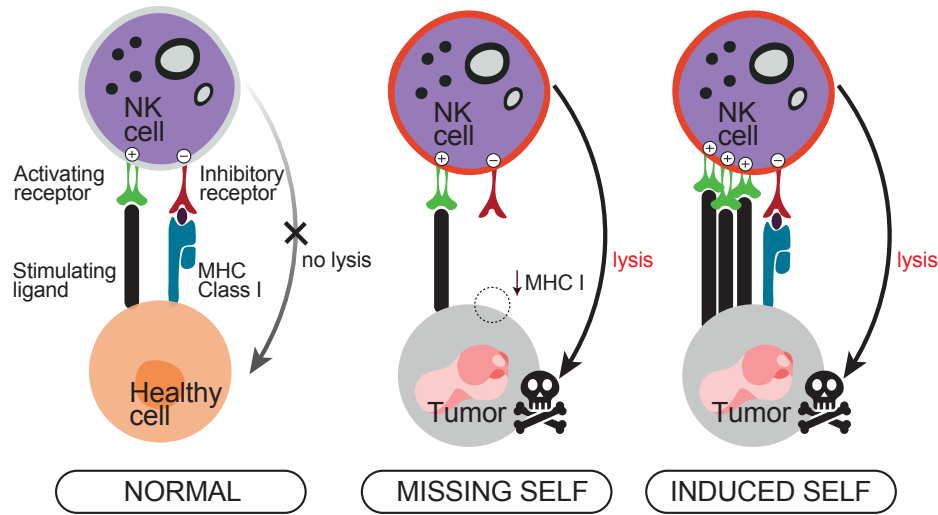


Figure 1.1: Mechanisms for NK cell lysis of target cells. Normal healthy cells escape NK cell target recognition by the balance of activating signals from the engagement of stimulating ligands with activating receptors on NK cells, and the inhibitory signals via binding of MHC I to the inhibitory receptors on NK cells. Two conditions can unleash NK cell target recognition: (1) **MISSING SELF**—The loss of MHC I expression on target cells via infection or transformation, leading to the loss of inhibitory signals to NK cells and a net activating “kill” signal; (2) **INDUCED SELF**—In the presence of MHC I inhibition, the inhibitory signals can be overcome with the overexpression or upregulation of activating ligands on target cells, resulting in a net activating signal instructing NK cells to lyse target cells.

NK cell Effector Functions

NK cell cytotoxicity mainly involves the production of cytotoxic granules that contain pore-forming protein perforin along with procaspase-cleaving granzyme B, thereby triggering apoptosis in target cells. In addition to granzyme B, cytotoxic granules in NK cells (and T cells) also contain granzyme A, which induces pyroptotic death in cells expressing gasdermin B (Davis, Smyth et al. 2001, Fehniger, Cai et al. 2007). NK cells also employ death receptor-mediated apoptosis via Fas ligand (FASL) and TNF-related apoptosis-inducing ligand (TRAIL), which engage receptors containing death domains to activate caspases (Arase, Arase et al. 1995, Eischen, Schilling et al. 1996, Lee, Spielman et al. 1996, Zamaï, Ahmad et al. 1998, Taylor, Ward et al. 2002, Prager, Liesche et al. 2019). Fas, a member of the TNF receptor family, contains a conserved intracytoplasmic "death domain" that indirectly triggers the caspase enzymatic cascade, leading to apoptosis in various cell types. Two other members of this family, DR4 and DR5, initiate apoptotic signals when bound by soluble TNF-related apoptosis-inducing ligand (TRAIL)(Zamaï, Ahmad et al. 1998).

Beyond direct killing, NK cells secrete cytokines and chemokines such as interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α), CCL5, and XCL1 to recruit and activate other immune cells, including effector T cells and dendritic cells. Effector functions of NK cells also highly depend on the activation state of NK cells, which is regulated by cytokines produced by dendritic cells and monocytes, including IL-2/IL-15 to support proliferation, IL-12/IL-18 to stimulate IFN- γ production and type I IFNs to induce granzyme B production (Scharton and Scott 1993, Wang, Jaw et al. 2011, Ferlazzo and Morandi 2014, Barry, Hsu et al. 2018, Nicolai, Wolf et al. 2020).

NK cell response to Cancer

Activated NK cells are known to control cancer, and it is well established that NK cells play a role in immunosurveillance and eliminating various tumor cells lines injected at low doses in syngeneic mice. In spontaneous models of cancer such as the carcinogen-induced (methylcholanthrene-induced) model of sarcoma (MCA), NK cells have been shown to prevent development of tumors (Smyth, Crowe et al. 2001, Street 2004). NK cell infiltration is also positively associated with better survival outcomes in The Cancer Genome Atlas databases, including in melanoma, breast cancer, gliomas and lung adenocarcinomas. The critical role of NK

cells in tumor surveillance is demonstrated in the TRAMP transgenic model of prostate adenocarcinoma, where tumors that progressed aggressively in wild-type mice exhibited reduced or absent expression of NKG2D ligands on their surface. In contrast, tumors arising in NKG2D-deficient mice maintained or upregulated these ligands, indicating that NKG2D-mediated recognition exerts immunoselective pressure, driving the loss of activating ligands to enable immune evasion (Guerra, Tan et al. 2008). Other evidence of cancer immunoediting to escape NK cell immunosurveillance include the loss of NKp46 ligands expressed on tumors arising in wildtype versus NKp46-deficient littermates in MCA-induced fibrosarcomas (Elboim, Gazit et al. 2010). The process of ligand-shedding, which reduces the density of ligands on tumor cell surface that engages with NK cell activating receptors, can also severely impair NK cell activation, limiting effectiveness of NK cell-dependent tumor control (Deng, Gowen et al. 2015).

NK cells as a therapeutic—CAR NKs and NK engager molecules

Chimeric antigen receptor (CAR)–engineered natural killer (NK) cells represent a rapidly advancing frontier in cancer immunotherapy, combining the innate cytolytic power of NK cells with synthetic CAR-mediated specificity (Burger, Zhang et al. 2019, Liu, Marin et al. 2020, Pfefferle and Huntington 2020, Christodoulou, Ho et al. 2021, Biederstadt, Basar et al. 2025). Unlike CAR-T cells, which rely solely on CAR engagement, CAR-NK cells can kill tumor cells through both CAR-directed recognition and native activating receptors, including NKG2D, NKp30, NKp44, and DNAM-1, providing dual and more versatile mechanisms of tumor lysis that mitigate antigen escape and heterogeneity (Marin, Li et al. 2024, Balkhi, Zuccolotto et al. 2025). Clinical data highlight key advantages of CAR-NK therapy: a reduced risk of cytokine release syndrome and graft-versus-host disease, an improved safety profile allowing “off-the-shelf” use from allogeneic or cord-blood sources, and minimal HLA restriction (Burger, Zhang et al. 2019, Marin, Li et al. 2024). Preclinical and early-phase trials show that CAR-NK cells effectively target CD19⁺ malignancies and exhibit durable control in acute myeloid leukemia and multiple myeloma while showing emerging promise in solid tumors through the modulation of the immunosuppressive tumor microenvironment (Liu, Marin et al. 2020).

Another important therapeutic application of NK cells is the use of bispecific NK cell engagers, which are engineered molecules that simultaneously bind to activating receptors on NK cells (such as CD16) and antigens on tumor cells. These bispecific engagers, including BiKEs and TriKEs (Gleason, Verneris et al. 2012, Gleason, Ross et al. 2014, Vallera, Felices et al. 2016), efficiently redirect and activate NK cells to target and kill cancer cells, often with improved specificity and reduced adverse effects compared to traditional approaches. By facilitating direct contact between NK cells and tumor cells and enhancing NK cell cytotoxicity, bispecific NK cell engagers are emerging as a promising strategy in cancer immunotherapy, expanding the clinical utility of NK cells beyond natural surveillance and adoptive cell therapies.

Regulatory T cells

Regulatory T (Treg) cells maintain immune homeostasis by controlling immune responses to self-antigens, transplantation antigens, dietary antigens, and gut microbiota. Initially characterized by the expression of CD25, the alpha chain of the IL-2 receptor, these CD25⁺CD4⁺ T cells displayed potent suppressive activity, preventing autoimmunity and transplant rejection when adoptively transferred into thymectomized mice. In the landmark 1995 study, Sakaguchi and co-workers showed that CD4⁺ T lymphocytes expressing the surface marker CD25 possessed immune regulatory functions (Sakaguchi, Sakaguchi et al. 1995).

While CD25 was initially used to identify Tregs, the upregulation of CD25 on activated T cells made it difficult to discriminate between Tregs and activated T cells. The insights into the biology and function of Tregs was made possible with the later identification of the transcription factor FOXP3 as the definitive marker and master regulator of Treg lineage commitment, which was shown to be stably expressed in mouse CD25⁺CD4⁺ Treg cells, but not in naïve CD25⁻CD4⁺ or activated CD4⁺ T cells (Sakaguchi 2000, Hori, Nomura et al. 2003). Genetic defects in *Foxp3* lead to fatal autoimmune syndromes such as scurfy in mice and IPEX in humans (Bennett, Christie et al. 2001, Brunkow, Jeffery et al. 2001, Wildin, Ramsdell et al. 2001). Later, it was reported that *Foxp3*-deficient mice displayed the same phenotype as the scurfy strain (Fontenot, Gavin et al. 2003). Rudensky's group subsequently showed that deleting *Foxp3* in established Tregs was sufficient to induce an early onset lymphoproliferative syndrome and autoimmunity, similar to that found in *Foxp3*-deficient mice (Fontenot, Dooley et al. 2005).

Treg Development

Thymic development of regulatory T cells (thymic, tTregs) is driven by intermediate T cell receptor (TCR) signal strength, which allows cells to escape negative selection and induce FOXP3 expression. High-strength TCR signals generally lead to apoptosis of most CD4 single-positive thymocytes, but some thymocytes with high TCR affinity are enriched as precursors for FOXP3⁺ Tregs (Moran, Holzapfel et al. 2011, Hsieh, Lee et al. 2012, Malhotra, Linehan et al. 2016). Co-stimulation through CD28 and its ligands CD80/CD86 is crucial for Treg development, with deficiencies markedly reducing thymic Treg pools (Salomon, Lenschow et al. 2000, Tang, Henriksen et al. 2003, Tai, Cowan et al. 2005). Downstream transcription factors activated by the TCR/CD28 signaling cascade—including NFAT, NF-κB, and AP-1—play critical roles in *Foxp3*

gene regulation and Treg differentiation (Wu, Borde et al. 2006, Passerini, Allan et al. 2008). Cytokine signaling, notably IL-2, is vital for tTreg differentiation and survival; mice deficient in IL-2 or the IL-2 receptor α subunit (CD25) display profound Treg depletion (Burchill, Yang et al. 2008, Malek 2008). Moreover, combined loss of multiple common γ -chain cytokines like IL-2, IL-7, and IL-15 results in almost complete ablation of thymic and peripheral Tregs (von Freeden-Jeffry, Vieira et al. 1995, Lodolce, Boone et al. 1998).

Transcriptomic and epigenomic profiling revealed that the *Foxp3* locus, which defines Treg lineage commitment and suppressive function, undergoes multilayered regulation via specific histone modifications that distinguishes Tregs from conventional CD4⁺ T cells (Zheng, Josefowicz et al. 2010, Feng, Arvey et al. 2014, Li, Liang et al. 2014). In Tregs, the *Foxp3* promoter and its conserved non-coding sequences (CNS1, CNS2, CNS3) are decorated with active histone marks such as H3K4me3 and H3K27ac, features consistent with open chromatin and productive transcription (Zheng, Josefowicz et al. 2010, Josefowicz, Lu et al. 2012, Josefowicz, Niec et al. 2012, Lu, Barbi et al. 2017). Notably, CNS2, a Treg-specific demethylated region (TSDR), is enriched for H3K4me3 and hypomethylated CpG DNA exclusively in lineage-committed, stable Tregs, correlating with durable *Foxp3* expression and suppressive activity (Li, Liang et al. 2014). Beyond *Foxp3* itself, neighboring *Foxp3* family genes, including *Foxp1* and *Foxp4*, display distinct histone modification and enhancer-binding profiles consistent with their unique yet cooperative roles in maintain Treg identity and immune homeostasis (Konopacki, Pritykin et al. 2019, Dong, Sindhava et al. 2025).

In contrast, conventional CD4⁺ T cells or newly induced Tregs (iTregs) that fail to maintain FOXP3 expression, exhibit repressive chromatin signatures such as H3K27me3 at the promoter and extensive DNA methylation at CNS2, resulting in unstable or transient *Foxp3* transcription. Together, these findings highlight how lineage-specific histones and DNA methylation landscapes across the *Foxp3* locus, and adjacent *Foxp* family members orchestrate Treg differentiation, stability and effector programming potential.

Treg suppression mechanisms

It is known that due to their high expression of IL-2R α (CD25), Tregs serve as an IL-2 sink, reducing the availability of IL-2 to act on other T cells, while also suppressing the production of IL-2 by other T cells (Gasteiger, Hemmers et al. 2013, Gasteiger, Hemmers et al. 2013, Gerner, Torabi-Parizi et al. 2015, Liu, Gerner et al. 2015). Inhibition of NK cell proliferation and function by Tregs has been described in homeostatic or autoimmune conditions in studies where Tregs were depleted systemically (Trzonkowski, Szmit et al. 2004, Feuerer, Shen et al. 2009, Gasteiger, Hemmers et al. 2013, Sitrin, Ring et al. 2013). Previous studies have highlighted Treg suppressive mechanisms including the secretion of inhibitory cytokines (TGF- β , IL-10, IL-35, IL-37), their expression of CTLA-4 and PD-L1, and their capacity to block key interactions of NK cells with DCs and macrophages (Terme, Chaput et al. 2008, Tekguc, Wing et al. 2021, Pereira, Lanzar et al. 2023). Furthermore, contact-dependent inhibition of NK cells by Tregs can occur by cell surface-bound TGF- β on Tregs (Trzonkowski, Szmit et al. 2004).

Growing research in the field suggests that Treg cells deploy a broad repertoire of suppressive mechanisms, rather than relying on a single, or universal mode of action (Figure 2). The context-dependent choice, employing a combination of these mechanisms or even the switch between suppressive strategies by Tregs, remain incompletely understood. Variables such as the tissue microenvironment, type of target cell and the Treg cell's own activation status can influence the mode of suppression employed. Moreover, accumulating evidence points to the differentiation of distinct Treg cell subsets, specialized for suppression in different immune contexts (Chaudhry, Rudra et al. 2009, Koch, Tucker-Heard et al. 2009, Zheng, Chaudhry et al. 2009, Duhon, Duhon et al. 2012, Moreno Ayala, Campbell et al. 2023) (Table 1).

Targeting Tregs in the Clinic In Autoimmune and Inflammatory Disease

Tregs are indispensable for maintaining immune tolerance and preventing aberrant immune activation against self-antigens. In autoimmune diseases, defects in Treg number, stability, or suppressive function often underlie pathogenic immune responses. For example, reduced Treg frequencies or impaired function have been documented in diseases such as type 1 diabetes, multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis.

Mechanistically, impaired Treg stability is associated with a loss of FOXP3 protein. Therapeutic strategies in autoimmunity aim to expand, stabilize, or enhance Treg function and numbers to suppress pathogenic immune responses.

One of the most promising approaches involves low-dose interleukin-2 (IL-2) therapy, which preferentially expands CD25-high Tregs due to their high-affinity IL-2 receptor expression (Rosenberg, Spiess et al. 1986, Rosenberg, Lotze et al. 1993, Rosenberg 1994, Rosenberg 2014). Multiple clinical trials have demonstrated that low-dose IL-2 selectively increases Treg populations in diseases such as graft-versus-host disease (GVHD) (Matsuoka, Koreth et al. 2013), type 1 diabetes (Hartemann, Bensimon et al. 2013), systemic lupus erythematosus (SLE), and autoimmune vasculitis (He, Zhang et al. 2020, Graßhoff, Comdühr et al. 2021), leading to improved clinical outcomes and reduced inflammation. Beyond IL-2, novel modalities explore adoptive Treg cell therapy, where *ex vivo* expanded autologous Tregs are reinfused to patients to promote durable immune tolerance (Mathew, H.-Voss et al. 2018, Alhosseini, Ebadi et al. 2024). Early-phase trials in transplantation and autoimmunity have shown safety and some efficacy. Additional strategies focus on targeting metabolic and epigenetic pathways to promote Treg stability, as both are necessary for maintenance of suppressive function. Small molecule drugs that inhibit histone deacetylases (HDACs) (Huang, Wang et al. 2017), or modulate mTOR signaling (Chapman and Chi 2014, Gedaly, Orozco et al. 2023), are under investigation to enhance Treg phenotypes. Collectively, these therapies aim to restore the balance between effector and regulatory immune cells without generalized immunosuppression.

Tregs In Cancer Immunotherapy

Cancers frequently harbor abundant Tregs that suppress anti-tumor immune responses and inhibit effective immunotherapy. Tumor-infiltrating Tregs exploit multiple immunosuppressive mechanisms, including secretion of inhibitory cytokines like IL-10 and TGF- β , expression of immune checkpoint molecules (e.g., CTLA-4, PD-1), metabolic disruption of effector T cells through adenosine production, and modulation of dendritic cell function (Wing and Sakaguchi 2012, Tay, Tanaka et al. 2023).

Clinical strategies to target Tregs in tumors include depletion, functional inhibition, or reprogramming. However, systemic depletion of Tregs may cause autoimmune toxicities, limiting therapeutic windows and generating caution surrounding Treg-targeted therapeutic approaches. To develop more selective approaches to eliminate Tregs only in tumors, substantial efforts were made in identifying unique surface molecules expressed distinctly on tumor Tregs to target tumor-specific deletion rather than broad systemic Treg depletion. This repertoire includes various activation cell surface markers including cytotoxic T-lymphocyte antigen-4 (CTLA)-4, programmed cell death (PD)-1, inducible T-cell costimulator (ICOS), glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), OX40, CD137, CCR4, and CCR8 (Ishida and Ueda 2006, Piconese, Valzasina et al. 2008, Wing, Onishi et al. 2008, Sugiyama, Nishikawa et al. 2013, Miyara, Chader et al. 2015).

Antibodies against CD25 (IL-2R α), such as daclizumab and basiliximab, target the high-CD25-expressing intra-tumoral Tregs, although they can also impact activated effector T cells (Arce Vargas, Furness et al. 2017, Solomon, Amann et al. 2020). More recently, immune checkpoint inhibitors (e.g., anti-CTLA-4 and anti-PD-1 antibodies) have been shown to modulate Treg activity as part of their anti-cancer efficacy. Several studies revealed that depletion of tumor-infiltrating Tregs correlates with improved responses to checkpoint blockade. Early Treg-based cancer therapy studies using anti-CD25 and anti-CTLA-4 antibodies to deplete Tregs demonstrated rejection of transplanted tumors (Sutmoller, van Duivenvoorde et al. 2001, Golgher, Jones et al. 2002, Jones, Dahm-Vicker et al. 2002, Read, Greenwald et al. 2006, Quezada, Peggs et al. 2008, Setiady, Coccia et al. 2010, Simpson, Li et al. 2013). In addition, while anti-CTLA4 treatments have been shown to deplete Tregs in mice, Treg depletion in tumors in patients treated with Ipilimumab is highly controversial and is rarely observed *in situ* in human patients (Sharma, Subudhi et al. 2019).

Mogamulizumab, an anti-CCR4 antibody, has been approved for T-cell lymphoma and is being tested in combination with checkpoint inhibitors (Ishida and Ueda 2006, Sugiyama, Nishikawa et al. 2013). However, as CCR4 is also expressed on multiple cell populations other than Tregs, including on CD8⁺ T cells, it is not specific for Tregs and has been ineffective in clearing only tumor Tregs (Ni, Jorgensen et al. 2015, Maeda, Wada et al. 2021). Additionally,

strategies that target Treg metabolism, such as adenosine production inhibitors and PI3K δ inhibitors (Abu-Eid, Samara et al. 2014, Eschweiler, Ramírez-Suástegui et al. 2022), are emerging to reduce Treg suppressive function more selectively in the tumor microenvironment.

Anti-CCR8 therapy is the most recent and promising strategy for selectively targeting tumor-infiltrating Tregs, offering potentially improved specificity compared to other approaches. CCR8 (C-C- chemokine receptor type 8) is highly expressed on Tregs within the tumor microenvironment but is minimally expressed on peripheral Tregs or other immune cells, making it more a tumor-restricted target (Plitas, Konopacki et al. 2016, Simone, Arrigoni et al. 2016). Preclinical studies have demonstrated that antibodies or antagonists against CCR8 can effectively deplete or inhibit suppressive intra-tumoral Tregs, leading to enhanced anti-tumor immune responses and tumor regression in various murine cancer models (Weaver, Stack et al. 2022, Villarreal, L'Huillier et al. 2023). Unlike CCR4, which is also expressed on other T cell subsets and can cause broader immune depletion, CCR8 targeting appears to only preferentially affect tumor Tregs, reducing systemic toxicity and autoimmune risk. Moreover, anti-CCR8 therapies are being developed as both monotherapies and in combination with checkpoint inhibitors (Damme, Dombrecht et al. 2021), with early-phase clinical trials underway to assess safety and efficacy in human cancers. This targeted approach aims to dismantle the immunosuppressive niche more precisely, thereby improving the effectiveness of cancer immunotherapies while preserving systemic immune homeostasis.

Combination therapies leveraging Treg modulation alongside vaccines, checkpoint inhibitors, or adoptive T cell therapies represent promising future directions, aiming to remodel the immunosuppressive tumor niche to unleash potent anti-cancer immunity while minimizing systemic autoimmunity. In Chapter 3, my main thesis project explores the effect of utilizing a targeted tumor-specific Treg depletion model and how it unleashes an immune response against MHC I-deficient tumors.

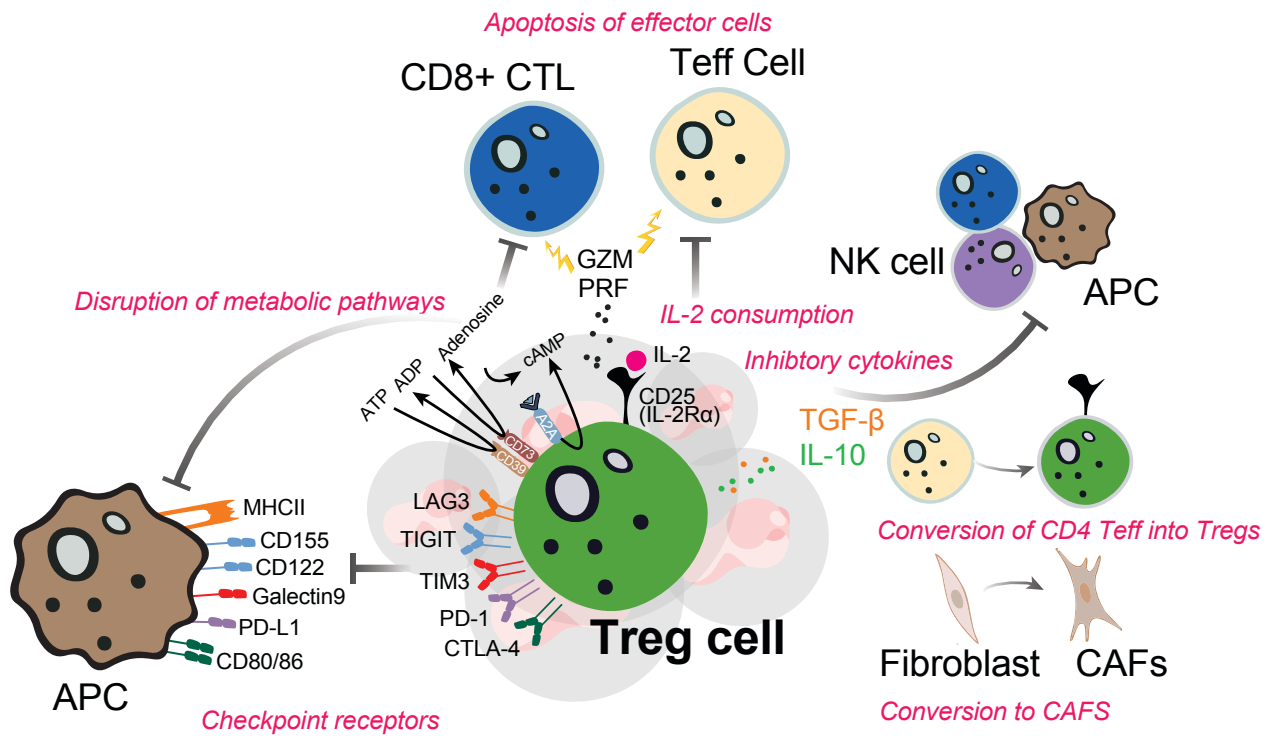


Figure 1.2: Mechanisms of Treg Suppression. Summary of the multiple mechanisms of immune suppression. Schematic overview of the diverse suppressive strategies employed by regulatory T cells (Tregs) in the tumor microenvironment. Tregs directly inhibit effector CD8+ cytotoxic T lymphocytes (CTLs), CD4+ Teff cells, and natural killer (NK) cells through secretion of inhibitory cytokines (TGF-β, IL-10), withdrawal of key growth factors (IL-2 consumption), checkpoint receptor engagement (CTLA-4, PD-1, LAG3, TIGIT, TIM-3), and release of cytolytic molecules (GZM, PRF) that result in the apoptosis of effector cells. They disrupt local metabolic pathways, mediate the conversion of Teffs to induced Tregs, suppress antigen presentation by dendritic cells (APCs), and promote transformation of fibroblasts into cancer-associated fibroblasts (CAFs). Tregs further suppress anti-tumor activity by disrupting local metabolic pathways, such as by the depletion of extracellular ATP and generation of immunosuppressive adenosine via CD39/CD73 ectoenzymes. These mechanisms combine to enforce immune tolerance and facilitate tumor immune escape.

CD4+ Tconv cells

CD4+ T cells, often termed helper T (Th) cells, are pivotal orchestrators of adaptive immunity, differentiating into specialized subsets that execute distinct effector functions. Each Th subset is characterized by unique transcription factors, surface markers, and cytokine secretion profiles that tailor immune responses to specific pathogens or immunological contexts (Table 1).

For example, Th1 cells, identified by the transcription factor T-bet and surface markers such as CXCR3, produce IFN- γ to mediate cellular immunity against intracellular pathogens (Szabo, Kim et al. 2000, Lighvani, Frucht et al. 2001, Szabo, Sullivan et al. 2002, Szabo, Sullivan et al. 2003). Th2 cells, regulated by GATA3 and expressing CCR4, secrete IL-4, IL-5, and IL-13 to facilitate humoral responses and combat extracellular parasites (Zheng and Flavell 1997, Imai, Nagira et al. 1999, Zheng, Chaudhry et al. 2009). Th17 cells, marked by ROR γ t and CCR6, produce IL-17 and IL-22 and are key mediators of mucosal immunity and inflammation (Ivanov, McKenzie et al. 2006, Annunziato, Cosmi et al. 2007). Other subsets include T follicular helper (Tfh) cells, which express Bcl6 and CXCR5 and provide essential help for B cell maturation within germinal centers (Crotty 2014, Crotty 2019, Liang, Tang et al. 2019, Ruggiu, Guerin et al. 2024), and Th9 cells producing IL-9, implicated in allergy and parasite immunity (Veldhoen, Uyttenhove et al. 2008, Schmitt, Klein et al. 2014). The differentiation and functional regulation of CD4+ CTLs add further complexity to the CD4+ T cell compartment, underscoring the diversity and plasticity of helper/effector CD4+ T cell responses. In Chapter 4, I will outline preliminary efforts in characterizing cytotoxic CD4+ T cells that arise as a result of Treg depletion in the tumor microenvironment.

Critically, an additional layer of immune regulation arises from specialized Treg subsets that correspond to each Th effector lineage (Wing and Sakaguchi 2012). These Tregs express the same lineage-specifying transcription factors—such as T-bet in Th1-like Tregs (Koch, Tucker-Heard et al. 2009), GATA3 in Th2-like Tregs, and ROR γ t in Th17-like Tregs, enabling them to migrate to similar inflammatory milieus and specifically suppress their corresponding Th effectors. This precision prevents excessive or inappropriate immune activation, maintaining immune homeostasis while calibrating pathogen-specific responses (Vignali, Collison et al. 2008, Campbell

and Koch 2011, Vignali 2012). The complexity and specificity of this interplay highlight the sophisticated regulatory networks that preserve host protection while limiting immunopathology.

Th Subset	Signature Transcription Factor(s)	Surface Markers	Key Cytokines	Immune Response Role	Corresponding Treg Subset	Treg Key Transcription Factor(s)	Treg Role
Th1	T-bet	CXCR3, CCR5	IFN- γ , TNF- α	Cellular immunity against intracellular pathogens (e.g., viruses, some bacteria)	Th1-like Treg	T-bet	Suppresses Th1-mediated inflammation
Th2	GATA3	CCR4, CCR6	IL-4, IL-5, IL-13	Humoral immunity; defense against helminths and allergens	Th2-like Treg	GATA3	Controls Th2-driven allergic and parasitic responses
Th17	ROR γ t	CCR6	IL-17A, IL-17F, IL-22	Mucosal immunity; defense against extracellular bacteria and fungi; inflammation	Th17-like Treg	ROR γ t	Limits Th17-mediated inflammation and autoimmunity
Tfh (T follicular helper)	Bcl6	CXCR5, PD-1, ICOS	IL-21, IL-4	B cell help, antibody production, germinal center responses	Tfr (follicular regulatory T cells)	Bcl6 + FoxP3	Suppress Tfh functions and control germinal center reactions
Th9	PU.1, IRF4	CCR6	IL-9	Immune responses to parasites, allergy	Th9-like Treg	IRF4	Regulates Th9-related inflammatory responses

Table 1: Summary of T helper subsets and their corresponding Treg subsets in immune responses.

Immune Cross-talk

Immune Cell Cross-Talk in the Tumor Microenvironment

Effective antitumor immunity depends on coordinated communication between innate and adaptive immune compartments. Dendritic cells (DCs), T cells, and natural killer (NK) cells engage in continuous bidirectional signaling that governs antigen recognition, cytotoxic activation, and immune tolerance within the tumor microenvironment (Barry, Hsu et al. 2018, Böttcher, Bonavita et al. 2018, Binnewies, Mujal et al. 2019). These communication networks, collectively termed immune crosstalk, ultimately determine whether cancer immunity proceeds toward elimination or evasion.

DC–T Cell Interactions

Dendritic cells (DCs) serve as the principal link between innate sensing and adaptive immune responses in tumors. Specialized subsets of DCs play distinct roles in antigen presentation: conventional type 1 dendritic cells (cDC1s) are critical for cross-presenting tumor-associated antigens to CD8⁺ T cells, whereas cDC2s are more involved in priming CD4⁺ helper T cells (Hildner et al., 2008; Dudziak et al., 2007; Schlitzer et al., 2013). Upon activation, DCs secrete key cytokines such as IL-12, IL-15, and IFN- β , alongside chemokines CXCL9 and CXCL10, which facilitate the recruitment of effector and memory T cells to regions of inflammation within the tumor microenvironment (Roberts et al., 2016; Broz et al., 2014). In a critical reciprocal interaction, CD4⁺ T cells license DCs through CD40–CD40L signaling, amplifying antigen cross-presentation and sustaining CD8⁺ T cell priming necessary for effective cytotoxic responses (Castellino et al., 2006; Bevan, 2004).

However, the tumor microenvironment can reprogram DCs towards a tolerogenic phenotype through chronic exposure to factors such as prostaglandin E₂, lactic acid, or IL-10. These dysfunctional DCs exhibit diminished IL-12 secretion and increased expression of inhibitory molecules including PD-L1 and arginase-1, which impairs their capacity to activate T cells efficiently and contributes to the recruitment and function of regulatory T cells (Tregs), leading to immune evasion (Maier et al., 2020; Binnewies et al., 2019).

Tregs further modulate the immune response both by directly suppressing effector T cell activation and indirectly by inhibiting DC function (Trzonkowski et al., 2004; Misra et al., 2004;

Wing et al., 2008; Cederbom, Hall, and Ivars, 2000). Systemic depletion of Tregs leads to expansion and maturation of conventional DCs, with cDC1s (CD8 α ⁺/CD103⁺) playing a key role in activating CD8⁺ T cells, and cDC2s (CD11b⁺/Sirp α ⁺) primarily stimulating CD4⁺ T cell responses (Broz et al., 2014; Hildner et al., 2008; Roberts et al., 2016; Dudziak et al., 2007; Schlitzer et al., 2013; Binnewies et al., 2019).

The CXCR3⁺ Treg–DC–T Cell Axis

Our recent work identified a specialized subset of CXCR3⁺ Tregs that organize within inflammatory niches of both murine and human tumors. These cells cluster around BATF3⁺CXCL9⁺ DC1s, positioning themselves to outcompete effector T cells for antigen-loaded DC contact. Mechanistically, CXCR3 expression enables Tregs to exploit CXCL9/10 gradients—normally guiding effector migration—to access high-affinity antigen-presenting zones rich in IFN- γ , where they suppress CD8⁺ T cell priming (Moreno Ayala, Campbell et al. 2023). CXCR3 expression on Treg cells enhances their co-localization with CD11c cells in the tumor microenvironment. The co-localization of CXCR3⁺ Treg cells with DC1s limits DC1 cross-presentation of tumor antigens, impeding antitumor CD8⁺ T cell control of cancer progression. In mouse genetic models where CXCR3 was selectively deleted in Tregs, DC–CD8 T cell synapse formation increased, while tumor burden decreased. Part of my rotation work and research in the DuPage lab has contributed to the *Immunity* paper (Moreno Ayala, Campbell et al. 2023) that emphasizes how intra-tumoral Tregs use chemokine receptors as spatial positioning cues to integrate within classical DC–T cell circuits.

NK–DC Cross-Talk

NK cells mediate anti-tumor effects not only through direct cytotoxicity but also by recruiting conventional type I dendritic cells (cDC1s) via chemokine secretion (CCL5 and XCL1), enhancing the priming and activation of tumor-specific T cells. This crosstalk underscores the potential for harnessing NK cells therapeutically to improve cancer immunotherapy outcomes (Barry, Hsu et al. 2018, Böttcher, Bonavita et al. 2018).

Moreover, there is reciprocal activation between DCs and NK cells, demonstrated both in vitro and during viral infections. NK cells, through secretion of IFN- γ , TNF- α , and GM-CSF, can promote DC maturation toward a type 1-polarized IL-12-producing phenotype (Fernandez, Lozier

et al. 1999, Ferlazzo, Tsang et al. 2002, Cooper, Fehniger et al. 2004). Once matured, DCs reciprocate by producing IL-12, IL-15, IL-18, and type I interferons that enhance NK cytotoxic capacity and survival (Diefenbach, Jamieson et al. 2000, Jamieson, Diefenbach et al. 2002). This positive feedback loop—the NK–DC axis—helps recruit secondary waves of cDC1s through XCL1 and CCL5 secretion and supports cytotoxic T cell priming (Barry, Hsu et al. 2018, Böttcher, Bonavita et al. 2018). Disruption of NK–DC communication, as observed in suppressive TMEs such as pancreatic or lung carcinomas, leads to diminished DC recruitment and reduced IL-12 signaling, and further NK cell “disarming” by Tregs or tumor-derived TGF- β limits crosstalk efficiency (Ghiringhelli, Menard et al. 2005, Ghiringhelli, Puig et al. 2005, Terme, Chaput et al. 2008).

Therapeutic Perspectives

The interplay between DCs, T cells, NK cells, and Tregs exemplifies a multi-layered communication network essential for immune regulation. Targeting these axes holds substantial therapeutic interest. Collectively, these studies redefine the TME not as a passive immunosuppressive milieu, but as a highly structured ecological system shaped by cooperative and competitive interactions among immune lineages (Figure 3). In the chapters to follow, I will outline my efforts in understanding the mechanisms driving such interactions, and how perturbations can shift immune responses significantly.

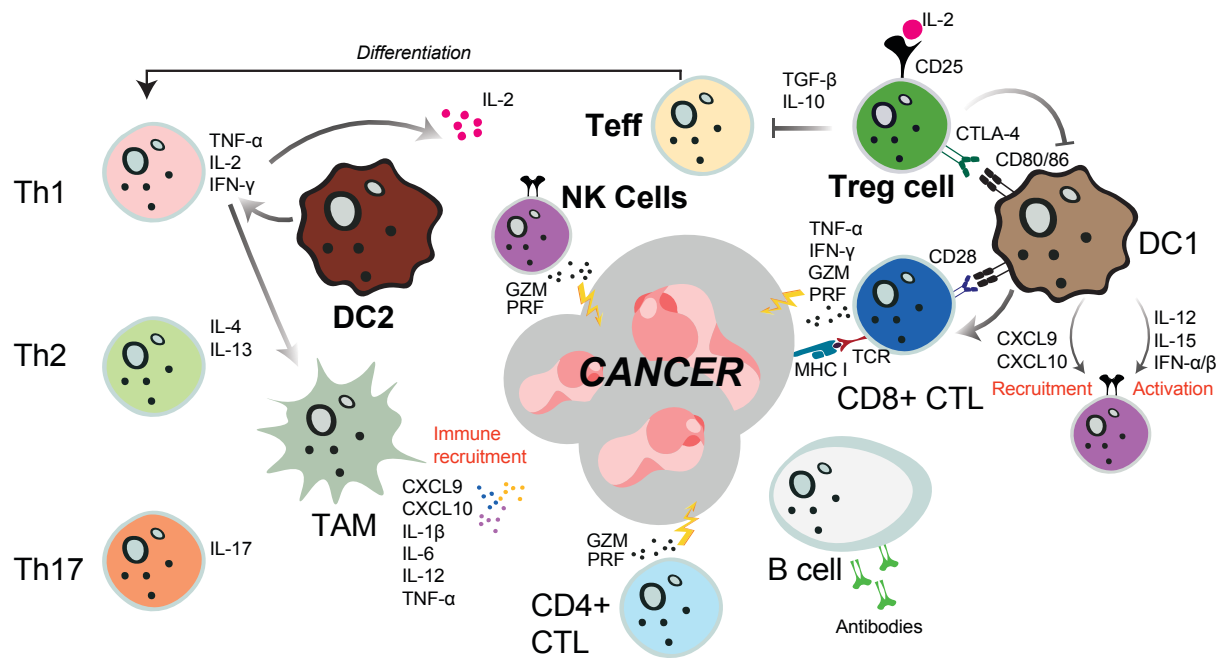


Figure 1.3: Immune Cross-talk within the Tumor Microenvironment. Antigen-presenting cells (APCs), especially dendritic cell subsets DC1 and DC2, process tumor antigens and activate T helper (Th) cells, cytotoxic CD8⁺ T lymphocytes (CTLs), and natural killer (NK) cells via cytokines (IL-12, IL-15, IFN- α/β) and costimulatory signals (CD80/CD86, CD28, MHC-TCR engagement). Th1 cells secrete IFN- γ and TNF- α , promoting DC1 activation and type 1 immune responses, whereas Th2 cells produce IL-4, IL-13, supporting tumor-associated macrophage (TAM) differentiation and a more suppressive microenvironment. Th17 cells generate IL-17 to drive inflammation and regulate neutrophil and TAM recruitment. T follicular helper (Tfh) cells and B cells support antibody production. Treg cells reinforce immunosuppression by producing inhibitory cytokines (TGF- β , IL-10), consuming local IL-2, and suppressing activation of effector T cells and NK cells via CTLA-4 and other surface checkpoints. CD8⁺ CTLs and cytotoxic CD4⁺ T cells mediate direct tumor cell killing through perforin and granzyme granule exocytosis. NK cells provide additional tumor cytotoxicity and shape DC maturation via IFN- γ . Chemokines (CXCL9, CXCL10, CCL5) facilitate immune cell recruitment and positioning within the tumor. The central TME network is tuned by the status, activation, and spatial interactions of these multiple immune populations, collectively determining the balance between tumor immunity and immune evasion.

Chapter 2: Methodology

Cell lines

RMA-*B2m*^{-/-}, B16F10-*B2m*^{-/-} and MC38-*B2m*^{-/-} tumor lines were previously described (Nicolai, Wolf et al. 2020, Wolf, Blaj et al. 2022). RMA is a lymphoma, MC38 is a colorectal cancer line, B16F10 is a melanoma cell line, EO771 is a breast cancer cell line, and KPDL3 is a sarcoma cell line, all from B6 mice. RMA-*B2m*^{-/-} cells were cultured in RPMI 1640 (ThermoFisher) medium, whereas B16-F10-*B2m*^{-/-}, MC38-*B2m*^{-/-}, EO771-*B2m*^{-/-} and KPDL3-*B2m*^{-/-} cells were cultured in DMEM (ThermoFisher), in all cases in 10% FBS (Omega Scientific), 0.2 mg/ml glutamine (Sigma-Aldrich), 100 U/ml penicillin (Thermo Fisher Scientific), 100 µg/ml streptomycin (Thermo Fisher Scientific), 10 µg/ml gentamycin sulfate (Lonza), 50 µM β-mercaptoethanol (EMD Biosciences), and 20 mM HEPES (Thermo Fisher Scientific, in 5% CO₂). All cell lines tested negative for mycoplasma contamination.

In vivo animal studies

All mice used were between 8-12 weeks of age and were compared to littermates or age-matched control mice. *Foxp3*^{DTR-GFP} mice, kindly provided by Dr A. Rudensky (Memorial Sloan Kettering Institute, JAX:016958)(Teng, Ngiew et al. 2010), express human diphtheria toxin receptor and EGFP genes at the *Foxp3* locus without disrupting endogenous expression of the *Foxp3* gene. *Zbtb46*^{DTR} mice (Meredith, Liu et al. 2012), *Batf3*^{-/-} mice (Hildner, Edelson et al. 2008), *Zeb3*^A (Liu, Kim et al. 2022) and *Prfl*^{-/-} (Kägi, Ledermann et al. 1994) were obtained from Jackson laboratories (JAX:019506; JAX:013755, JAX:037704, JAX:002407 respectively) and backcrossed with *Foxp3*^{DTR-GFP} mice in our laboratory. In *Foxp3*^{DTR-GFP} mice, Tregs were depleted intra-tumorally by injecting 250 ng DT i.t. in 50 µl every other day, or depleted systemically by injecting 1 µg DT in x 200 µl every other day. Diphtheria toxin was prepared in sterile HBSS solution at 1000 ng/ml and aliquoted and stored in -80 deg until use.

In vivo tumor transplant experiments

Syngeneic C57BL/6J mice were inoculated with 2x10⁶ MC38-*B2m*^{-/-} cells, 1x10⁶ B16-F10-*B2m*^{-/-} cells or 1x10⁶ RMA-*B2m*^{-/-} cells, in 100 uL of RPMI medium without serum. Tumor measurements were performed blindly across the entire experiment by a single operator with electronic calipers three times a week. Tumor volume was estimated using the ellipsoid formula: $V = (4/3)\pi abc$ where a, b and c correspond to height, width and length of the tumors. Five days

after tumor cell inoculation, when tumors reached volumes of 50 mm³, they were injected intratumorally (IT) with PBS or DT. Tumor experiments were terminated once the average of the length and width of tumors exceeded 20 mm. All experiments were conducted according to the Institutional Animal Care and Use Committee guidelines of the University of California, Berkeley.

Orthotopic tumor transplantations

For the breast cancer model, mice were anesthetized, and the area around the nipples were cleaned with ethanol wipes. 2.5×10^5 tumor cells were injected into the orthotopic second or fourth mammary fat pads (left or right side) of mice, according to described non-surgical methods (Kocatürk and Versteeg 2015). In detail, on the day before tumor injection, syngeneic C57BL/6J mice were placed in an anesthesia induction chamber with 2-4% isoflurane and 0.2 L/min oxygen flow until the mice breathe calmly. Once ready, the mouse is removed from the chamber and placed on the surgical table with a nose cone connected with tubing which allows inhalational anesthesia. The area near the nipples were shaven using an electronic hair trimmer prior to a short 20 second Nair application using a cotton bud. Hair was then removed with a wet paper towel and animals were observed for recovery. On the day of tumor injections, mice were similarly anesthetized, and the area around the nipples were cleaned with ethanol wipes. 2.5×10^5 tumor cells were injected into the orthotopic second or fourth mammary fat pads (left or right side) of mice.

For the sarcoma cancer model, syngeneic C57BL/6J mice were injected with 2.5×10^5 tumor cells intramuscularly into the right hind leg (calf muscle) according to previously described procedures (Thompson, Jackson et al. 2018).

All *in vivo* experiments were performed in accordance with the Institutional Laboratory Animal Care and Use Committee (IACUC) and the Office of Laboratory Animal Care (OLAC) at the University of California, Berkeley.

Spontaneous GEMM induction

Foxp3^{DTR};Kras^{G12D};p53^{fl/fl} mice were kindly provided by Dr. Jacks (Massachusetts Institute of Technology) and crossed to R26_Cas9^{fl/fl} to generate KP-Cas9 mice in our facilities. To induce spontaneous tumors, 5×10^4 lentiviral particles expressing Cre recombinase (Lenti-Cre) were

injected in mice based on the type of tumors desired. Tumors were palpable 3-4 months after Lenti-Cre injection.

Lung adenocarcinomas were induced in by intratracheal injection of the lungs with Lenti-sgB2m Cre or control non-targeting Lenti sgNT Cre viruses (plasmid construction described later). Sarcomas were induced by injection of virus intramuscularly into the right hind leg (calf muscle). Brain tumors were induced by intracranial injection as previously described²⁷. Briefly, mice were anesthetized with 2% isoflurane, given pre-emptive analgesics (Buprenorphine 0.1 mg/kg and Meloxicam 10 mg/kg), and arranged on Angle-two stereotactic frame (Leica, Nussloch, Germany). The incision area was swabbed with three alternating wipes of 70% ethanol and betadine scrub with sterile applicators prior to performing minimally damaging craniotomies. The stereotaxic surgery coordinates used for targeting each region listed below, are:

Striatum: 2mm lateral right, 0.5mm anterior and 3mm ventral

Hippocampus: 0.75mm lateral right, -1.43 mm anterior and 2.25mm ventral

SVZ: 1.5mm lateral right, 0.5mm anterior and 2.5mm ventral

Infusion of lentivirus was performed with a syringe to deliver 0.5 μ L per minute for a total volume of 5 μ L. Postinfusion, the syringes were left in position for 2 min before slow removal from the injection site, which was then cleaned, sutured, and surgically glued. Throughout the procedure, mice were kept at 37 °C for warmth and Puralube Vet Ointment (Dechra, NDC #17033-211-38, Northwich, England) was applied to the outside of the eyes. All *in vivo* experiments were performed in accordance with the Institutional Laboratory Animal Care and Use Committee (IACUC) and the Office of Laboratory Animal Care (OLAC) at the University of California, Berkeley.

MRI Imaging

MRI imaging is performed using the Bruker Pharmascan 70/16 7T MRI scanning system, which is a state of the art small animal MRI unit that consists of a 7 Tesla magnet, the Bruker Avance III spectrometer that provides direct digitalization of radio frequency signals, imaging gradients that provide up to 370 mTesla/meter gradient strength, and a collection of imaging coils. The largest coil has a 7.2 cm inner diameter into which the subject/specimen must fit. The system

is equipped with full physiologic support for small animals during scans with thermal support and real time display of rectal temperature, respiration and ECG waveforms. Prior to imaging, animals are anesthetized with steady isoflurane flow, and weighed. Localizer and RARE T1/T2 images were collected, and where stated, 0.15mmol/25g (triple dose) of Magnevist contrast agent was given to detect and visualize areas with disrupted blood brain barrier and/or abnormal vascularity of the central nervous system. As a comparison diffusion weighted MRI was performed to confirm if abnormalities observed were a result of cytotoxic cerebral edema.

***In vivo* cellular depletions and cytokine blockade**

To deplete NK cells, CD8 cells and/or CD4 cells, mice were injected IP with 200 µg of depleting antibodies specific for NK1.1 (clone PK136, Leinco), CD8b.2 (clone 53-5.8, Leinco) or CD4 (clone GK1.5, Leinco), respectively, 1 day before and on the day of initiating Treg depletions, and again every six days thereafter until mice were euthanized or no palpable tumors were detected for one week. Anti-CTLA4 (clone 9D9) and anti-CCR8 (Gilead Sciences) were dosed at 200 µg IP every 3 days. To neutralize cytokines, mice were injected IP with 200 µg doses of neutralizing antibodies specific for IL-2 (clone JES6-1) TNF- α (clone TN3-19.12) or IFN-g (clone XMG1.2), or control whole rat IgG (Jackson ImmunoResearch) starting on the day of Treg depletion and repeated every 3 days. Depletions were confirmed by flow cytometry.

Generation of bone marrow chimeras

Host CD45.1 C57/BL6 mice were irradiated using an Xrad320 (Precision X-Ray Irradiation) with two doses of 5 Gy, 16h apart. Four hours after the second dose of irradiation, mice received 8-10 million bone marrow cells via tail vein injection. Bone marrow cells were prepared by surgically resecting femurs and tibias from euthanized mice, which were then cleaned to remove excess muscle tissue, and bone marrows were flushed into a 15 mL conical tube using 27-gauge syringes containing ice-cold complete RPMI. Red blood cells were removed using ACK lysis buffer and the white cells were washed three times with PBS before being resuspended in serum-free RPMI and injected into mice via the tail vein. Following the bone marrow injections, mice received water supplemented with sulfamethoxazole-trimethoprim oral suspension (Ani Pharmaceuticals) for 30 days with daily monitoring.

Flow cytometry

Tumors were resected from animals and diced into small fragments with a razor blade, followed by enzymatic digestion in RPMI medium containing 125 U/mL Collagenase D (Roche Diagnostics) and 20 mg/mL DNase I (ThermoFisher Scientific) using an orbital shaker for 45 min at 37°C. Cells were stained directly for determining NK cell and T cell numbers and phenotypes, or incubated for 4 hours in medium containing Brefeldin A (Biolegend) and Monensin (Biolegend) for intracellular staining of granzyme B and perforin. LIVE/DEAD Fixable blue or aqua stain (Molecular Probes) was used to exclude dead cells. Before staining with antibodies, FcγRII/III receptors were blocked by resuspending the cells in 50 μL undiluted culture supernatant of the 2.4G2 hybridoma cell line (prepared in the lab) and incubating for 10 minutes at 4°C. Cells were washed in PBS containing 2.5% FCS and stained with antibodies directly conjugated to fluorochromes for 1 h at 4°C in the same buffer.

For **intracellular staining** of granzyme B and perforin, cells were fixed and permeabilized using the FoxP3/ Transcription Factor Staining Buffer Kit (Tonbo) and stained with antibodies directly conjugated to fluorochromes for 1 hr at RT in 1X Perm/Wash buffer (BD Biosciences).

For *ex vivo* pSTAT5 staining, tumor lysates and single cell suspensions from spleen and lymph nodes were immediately fixed by adding an equal volume of pre-warmed FOXP3 fixation buffer to the wells and kept in incubator for a further 10 mins. Cells were washed in FACS buffer and resuspended in 100 μL BD phosflow perm buffer III (cat #558050) and kept on ice for 30 mins before another wash. Cells were then incubated with Fc Block for 10 mins on ice, washed and then stained with an antibody cocktail for both intracellular and extracellular markers together (diluted in FACS buffer). Phospho antibodies were diluted at 1:10.

For staining of **intracellular IL-2**, 1:1000 Brefeldin A (Biolegend) and Monensin (Biolegend) was added to all medium, including tumor digest solutions and PBS solutions. Omission of Brefeldin A and Monensin in the preparation media resulted in weaker or no detection of *ex vivo* IL-2 levels in cells.

For **tetramer staining**, cells were incubated with PE-conjugated CD1d tetramers loaded with α-galactosylceramide or unloaded controls (NIH Tetramer Core Facility contract number

75N93020D00005) for 30 min at 4°C, washed and resuspended before subsequent co-staining. NK cells were gated as viable, CD45⁺, CD3⁻, CD19⁻, F4/80⁻, Ter119⁻, NK1.1⁺, NKp46⁺ cells.

Flow cytometry was performed using an LSRFortessa, LSRFortessa X-20 (BD Biosciences) or Cytex Aurora Spectral Cytometer. Data were analyzed using FlowJo software (Tree Star).

RNA Sequencing

Tumors from mice treated with PBS, IT-DT or ITDT + anti-CD4 were resected, and single cell suspensions were prepared as described above. Tumor immune cells were enriched by layering on a lympholyte-M gradient (Cedarlane Laboratories Ltd., Canada) then stained with DAPI, CD19-PECy5, F4/80-PECy5, Ter119-PECy5, CD8a-PECy5, CD3e-APC, CD45-FITC, CD4-BV605, and NK1.1-APC-Cy7 for 30 min on ice. NK cells and CD4⁺ T cells (*not excluding Tregs*) were sorted from tumors pooled from 2-4 mice per replicate, with a yield ranging from 20,000-200,000 cells, with three replicates total. To prepare RNA, cells were lysed in RLT buffer (Qiagen) containing 1% 2-mercaptoethanol (Thermo Fisher Scientific) before being purified with RNeasy Miniprep kits (Qiagen) and validated with Qubit, bioanalyzer and fragment analyzer (QB3 Genomics, UC Berkeley, Berkeley, CA, RRID:SCR_022170). Sequencing libraries were prepared using KAPA Hyper Prep Kit (KAPA Biosystems) and sent for bulk RNA sequencing with NovaSeq 6000 150PE (QB3 Genomics, UC Berkeley, Berkeley, CA, RRID:SCR_022170). Samples were processed and normalized to account for sequencing depth differences using Kallisto.

Sequencing Analyses

Differential Gene Expression Analysis: Genes with zero counts across all samples were removed prior to conducting differential expression analysis. Differential gene expression analysis was conducted using the *limma-voom* package (Version 3.58.1) in R([54](#)) using the formula $Y_i \sim 0 + T + R$, where Y_i denotes the log-CPM value for gene i , T denotes the treatment (i.e. PBS, IT-DT, or IT-DT+ α CD4) and R denotes the experimental replicate. Replicates were encoded using **sum-to-zero contrasts** (contr.sum), which centers the replicate effects and prevents over-

parameterization when using a no-intercept (~ 0) model formulation. This ensures that condition coefficients remain interpretable as group-level effects independent of replicate-specific biases. We then performed two pairwise comparisons (IT-DT vs PBS, and IT-DT+ α CD4 vs IT-DT) between the three treatment groups. For each pairwise comparison, $\log_2$ fold-changes ($\log_2FC$) and BH-adjusted p-values were computed using empirical Bayes moderation. Genes were classified as significantly up- or down-regulated based on an adjusted p-value < 0.05 and $|\log_2FC| > 1.5$. Volcano plots were generated in **R** (v4.3.0) using the **ggplot2** (Wickham 2016) and **ggrepel** (Slowikowski 2025) packages. Points were color-coded according to significance: red (significant up-regulated), blue (significant down-regulated), and grey (non-significant). Threshold lines marking $\log_2FC = \pm 1.5$ and BH-adjusted p-value = 0.05 were added for reference. Selected genes of interest (e.g., *Gzma*, *Gzmb*) were annotated directly on the plots to highlight biologically relevant changes. This superset of significantly changing genes was used in downstream analyses.

GSEA: Gene Set Enrichment Analysis was conducted using the *fGSEA* package (version 1.26.0). Gene lists were pre-ranked according to the BH-adjusted p-value calculated from the IT-DT vs PBS and ITDT + α CD4 vs IT-DT comparisons. Only MSigDB Hallmark and KEGG pathways obtained from *msigdb* (Version 7.5.1) were considered for pathway enrichment analysis.

***In vivo* BrdU Labeling**

To label proliferating cells, mice were injected IP with 1 mg of BrdU in 100 μ L sterile 1X DPBS and sacrificed 18-24h later. Cells were stained with surface markers as described earlier. Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm kit according to the manufacturer's instructions. Intracellular BrdU staining was performed using anti-BrdU antibody (clone Bu20a, BioLegend) for 30 minutes at room temperature.

***Ex vivo* cytotoxicity assay**

In all cases cytotoxicity was assessed with a standard 4-hour ^{51}Cr -release assay, using NK sensitive MC38-*B2m*^{-/-} tumor cells as target cells, as previously described (Nicolai, Wolf et al. 2020). NK cells from tumors were sorted by flow cytometry from single cell suspensions of tumor cells as *DAPI*⁻*CD45*⁺*CD3*⁻*CD8 α* ⁻*CD19*⁻*F480*⁻*NK1.1*⁺ cells. For testing cytotoxicity by splenocytes, NK cells were in some cases enriched from splenocytes using MojoSort™ Mouse

NK cell isolation kit, or depleted from splenocytes with biotin-anti-NK1.1 antibodies using the EasySep™ Mouse Streptavidin RapidSpheres™ Isolation Kit.

For CD4 cytotoxicity assays, CD4⁺ T cells are first enriched with EasySep™ CD4 T cell enrichment kit and then sorted for Foxp3-negative T conventional cells, before being co-cultured with target cells for a standard 4-hour ⁵¹Cr-release assay as described above. As an alternative, column-based CD25 removal (after CD4 enrichment) using biotinylated anti-CD25 antibodies followed by streptavidin beads from the standard EasySep kit was performed to remove Tregs. We note that the column-based method might also inadvertently remove CD25⁺ activated Tconv populations.

To enrich for NK receptor (NKR)-expressing CD4 T cell populations, we attempted a cocktail of biotinylated NKR receptor antibodies including NKp46, NK1.1, NKG2ACE, NKG2D, Ly49I, Ly49C, were incubated with CD4 T cell enriched cells for 1h at 4 deg, washed three times with PBS + 2% FBS and incubated with Streptavidin for 15 min at 4 deg, before sorting on Streptavidin⁺ cells.

Ex vivo stimulation assay

For assessing NK degranulation (Betts, Brenchley et al. 2003, Alter, Malenfant et al. 2004) and cytokine production, single cell suspensions of splenocytes were incubated for 4 hours in high-binding 96-well plates pre-coated overnight at 4 deg with antibodies for the NK1.1 activating receptor (5 ug/ml), or isotype control antibodies (5 ug/ml), in DMEM medium containing Alexa Fluor-conjugated CD107a antibodies, 2.4G2 hybridoma supernatant to block FcγRII/III receptors, brefeldin A (Biolegend), and monensin (Biolegend) before performing surface and intracellular staining followed by flow cytometric analysis, gating on CD3-NK1.1⁺ cells.

Transfer Colitis Experiments

Rag2^{-/-} mice were used as recipients, and mice were transferred with sorted Tnaive, Teffector, Tregs and ex-Tregs.

Weight loss scoring:

0 -- No weight loss,

1 -- 1-5% loss

2 -- 6-10% loss

3 -- 11-15% loss

4 -- >15% loss

Tumor tissue cryo sectioning

Tumors were removed and fixed in 4% paraformaldehyde in ice in the dark for four hours, washed twice with PBS, then dehydrated in 10% sucrose, 20% sucrose, and 30% sucrose for at least 6 hours each at 4°C. Tumors were imbedded in OCT (Fisher) cut side-down and frozen in a slurry of dry ice and ethanol, then stored at -80°C until sectioning. Tumors were cut into 12-micron sections on a cryostat microtome (Leica Biosystems) onto SuperFrost Plus slides (Fisher Scientific) and stored at -80°C until used for immunofluorescence.

Two-photon imaging

Two-photon imaging of lymph node slices has been adapted from imaging of thymic slices as previously described (Melichar et al., 2013; Dzhagalov et al., 2012; Ross et al., 2014; Dzhagalov et al., 2013; Au-Yeung et al., 2014). Lymph node slices were glued onto glass cover slips and fixed to the bottom of a petri dish being perfused at a rate of 1 ml/ min with 37°C oxygenated, phenol red-free DMEM during imaging using an MPII Mini-Peristaltic Pump (Harvard Apparatus) and a 35 mm Quick Exchange Platform (QE-1) in conjunction with a TC324B temperature controller (Warner Instruments). Images were collected using a Zeiss LSM 7 MP upright, two-photon microscope with a 20/1.0 immersive Zeiss objective and a Coherent Chameleon laser. Image areas of up to 212 x 212 mm to a depth of up to 55 mm were acquired every 10 s for 15 or 20 min with 3 mm z steps starting from beneath the cut surface, using Zen 2010 software from Zeiss.

Lenti-sg-Cre Construct Generation

To optimize and select oligos for B2m knockout, we first tested efficiency of knockouts in 3T3 cells using PX458 construct. Once the desired oligo sequences are cloned into PX458, the plasmids are transfected into 3T3 cells using Lipfectamine 2000, and GFP⁺ cells were sorted one week after transfection. DNA were extracted from sorted GFP⁺ cells, PCR amplified and sent for Sanger sequencing using the primer sequences as listed below.

Primer Sequences:

primer set 1: **383bp** product

Fwd: CAGCTAGGAGACTGGTGACGA

Rev: GCTGACCGTCAGCACACTCG

→ Covers Oligos 1,2,3 and 5

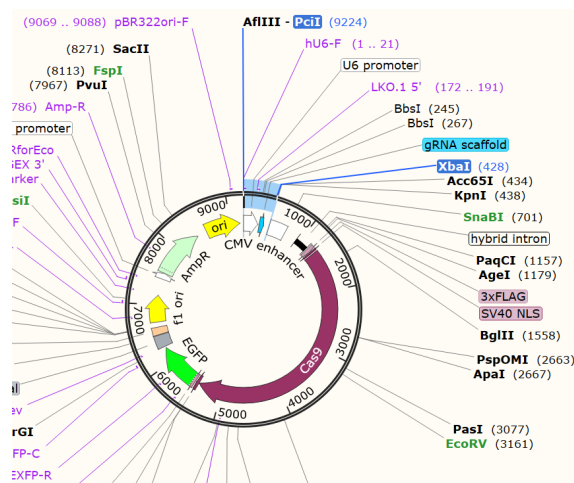
primer set 2: **326bp** product

Fwd2: CAA AAT GTG GGT AGA CTT TGG GGG

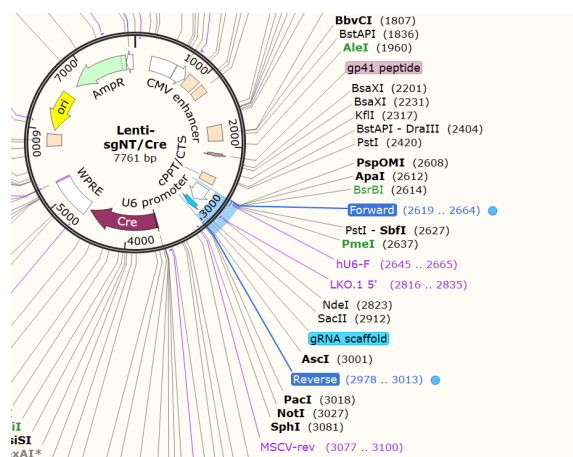
Rev2: AGG ATA TAG AAA GAC CAG TCC TTG CTG

→ Covers Oligos 4,6,7,8 and 9

To first replace the enzyme cut sites in the Lenti sgT/Cre plasmid to the ones in PX458 near the guide sequence, site-directed mutagenesis (SDM) was performed.



PX458 plasmid map



Lenti sgNT/Cre plasmid map

Cut Lenti sgNT/Cre with **SbfI** and **AscI** and design primers based on this vector sequence with homology regions with the U6 promoter and the end of the cuTregion to use for amplifying and introducing the SbfI and AcsI cut sites for the PX458 swap region of interest. The region between SbfI and AscI is 374bp; rest is **7387bp**. Amplify the PX458 region with the F and R primers and cut the PCR product with **SbfI** and **AscI**.

Primer design for SDM:

Forward: gcgaggtattcggtccgcgGTTTATAGAGCTAGAAATAGCAAG

2-2: TCCTTAGTTCTCCTTCCCGA

3-1: AGTCGTCAGCATGGCTCGCT

Reverse: GGTGTTTGGTCCTTTCCAC

Tetracyclines Preparation

All tetracycline compounds are kept protected from light and stored in -80 deg until use. The lyophilized powder should be reconstituted with sterile water for injection, but not with other solutions containing calcium because a precipitate may form especially in neutral and alkaline solutions. When diluted in compatible solutions, the pH usually ranges from 4.5 to 6.0. Compounds have been tested to be injected via IP, IT routes at a range from 0.02 ug to 50 ug per animal.

Chapter 3: Intra-tumoral Treg ablation Activates a CD4-NK Cell Axis to Control CD8 T cell Resistant Tumors

Adapted from submission to *Science Immunology* (2025):

Intra-Tumoral Treg ablation Unleashes NK Cell-Mediated Control of CD8 T Cell-

Resistant Tumors

Chenyu Zhang[†], Charles Chien^{†,‡,§}, Eglė Jurgaitytė[†], Koharu Sakiyama[†], Alissa Bockman[†], Yeara Jo[†], Seungwon Lee[†], Stephanie Silveria[†], Elizabeth Andrews[†], Abigail Mende[†], Lily Zhang[†], K. Christopher Garcia[¶], Allon Wagner^{‡,§}, Michel DuPage^{†*}, David Raulet^{†*}

[†]Division of Immunology and Molecular Medicine, Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720, USA

[‡]Department of Electrical Engineering and Computer Sciences, University of California, Berkeley, CA 94720, USA

[§]Center for Computational Biology, University of California, Berkeley, CA 94720, USA

[¶]Department of Molecular & Cellular Physiology, Stanford University School of Medicine, Palo Alto CA 94305

* Co-corresponding authors.

Email: dupage@berkeley.edu; raulet@berkeley.edu

Abstract

Cancer cells frequently lose MHC I to evade CD8⁺ T cell recognition. While Natural Killer (NK) cells are poised to target MHC I-deficient cancer cells, MHC I loss alone is often insufficient to unleash fully effective NK cell responses. Here we show that selective intra-tumoral (IT) ablation of regulatory T (Treg) cells elicited potent antitumor NK cell responses that controlled MHC I-deficient and even MHC I⁺ cancers that expressed NKG2D ligands. Treg_s controlled the activation, maturation, and anti-tumor cytotoxic activity of NK cells within the tumor microenvironment. Mechanistically, depletion of intra-tumoral Treg_s relieved the inhibition of cDC2-dependent induction of IL-2 production by CD4⁺ T_{conv} cells that was necessary for NK cell activation. Systemically administered antibodies that selectively depleted IT Treg_s similarly empowered NK-dependent tumor control. These findings expand the breadth of Treg-mediated cancer immunosuppression to encompass antitumor NK cells and suggest that therapeutic targeting of Treg_s in tumors can control CD8⁺ T cell-resistant cancers.

INTRODUCTION

Downregulation or loss of MHC I is a frequent mechanism of tumor immune evasion in human tumors (Garrido, Aptsiauri et al. 2016, Dhatchinamoorthy, Colbert et al. 2021). MHC I loss occurs in multiple tumor types and is associated with mutations occurring in the genes encoding MHC proteins themselves, as well as numerous genes that regulate MHC or are associated with antigen processing and presentation (Hicklin, Dellaratta et al. 1997, Rooney, Shukla et al. 2015, Mcgranahan, Rosenthal et al. 2017, Rodems, Heninger et al. 2022). By reducing or eliminating MHC I expression, tumor cells become resistant to being killed by CD8⁺ T cells, which recognize tumor antigens presented by MHC I molecules. Thus, tumor cells undergo strong selective pressure to lose MHC I antigen presentation to evade CD8-mediated immune control, which is especially apparent in the setting of checkpoint blockade immunotherapies that strengthen CD8⁺ T cell responses (Zaretsky, Garcia-Diaz et al. 2016, Sade-Feldman, Jiao et al. 2017, Schoenfeld and Hellmann 2020).

Natural Killer (NK) cells are cytolytic lymphocytes that recognize transformed and virus-infected cells using germline-encoded receptors to recognize stimulatory ligands upregulated on unhealthy cells. Activated NK cells control cancer by directly killing tumor cells via perforin/granzyme secretion or death receptor engagement, or kill indirectly by producing cytokines (e.g. IFN- γ , TNF- α) and chemokines that enhance immune infiltration and activation at the site of the tumor (Vivier, Raulet et al. 2011, Böttcher, Bonavita et al. 2018). Loss of MHC I enhances tumor cell killing by NK cells by preventing engagement of MHC I-specific inhibitory receptors on NK cells (“missing self” recognition). Nevertheless, loss of MHC I alone is frequently insufficient to elicit tumor-controlling NK cell responses, since MHC I-deficient tumors typically progress in the absence of therapeutic interventions. However, recent studies demonstrate that agents that mobilize and activate NK cells can effectively treat multiple tumor types, including established solid tumors (Ardolino, Azimi et al. 2014, Zhang, Bi et al. 2018, Nicolai, Wolf et al. 2020, Wolf, Blaj et al. 2022). We hypothesize that without intervention, NK cell anti-tumor responses are held in check by immunosuppressive mechanisms and/or because NK cells receive insufficient activation signals.

In normal tissues, Tregs maintain tissue homeostasis by preventing inappropriate or excessive immune responses while also directly promoting tissue repair (Barton and Rudensky 1999, Kim, Rasmussen et al. 2007, Feuerer, Shen et al. 2009, Josefowicz, Lu et al. 2012). How Tregs are recruited to tumors and how they protect tumor cells from elimination by the immune system are active areas of investigation. Early Treg-based cancer therapy studies using anti-CD25 and anti-CTLA-4 antibodies to deplete Tregs demonstrated rejection of transplanted tumors (Sutmoller, van Duivenvoorde et al. 2001, Golgher, Jones et al. 2002, Jones, Dahm-Vicker et al. 2002, Read, Greenwald et al. 2006, Quezada, Peggs et al. 2008, Setiady, Coccia et al. 2010, Simpson, Li et al. 2013). However, understanding the impact of each antibody is difficult due to their effects on other immune cells, especially tumor-controlling effector T cells, as well as their impact systemically. In normal tumor-free mice, the depletion of Tregs using the *Foxp3^{DTR-GFP}* system results in massive expansion and activation of many immune cell types including conventional CD4⁺ T cells (CD4⁺ Tconv cells) and CD8⁺ T cells, B cells, granulocytes, dendritic cells (DCs), and NK cells (Kim, Rasmussen et al. 2007, Klages, Mayer et al. 2010, Teng, Ngiew et al. 2010, Bos, Plitas et al. 2013, Joshi, Akama-Garren et al. 2015). Tregs execute their suppressive activity by several different mechanisms (Trzonkowski, Szmit et al. 2004, Kim, Rasmussen et al. 2007, Lahl, Loddenkemper et al. 2007, Klages, Mayer et al. 2010, Liu, Gerner et al. 2015, Moreno Ayala, Li et al. 2019). It is known that due to their high expression of IL-2R α (CD25), Tregs serve as an IL-2 sink, reducing the availability of IL-2 to act on other T cells, while also suppressing the production of IL-2 by other T cells (Gasteiger, Hemmers et al. 2013, Gasteiger, Hemmers et al. 2013, Gerner, Torabi-Parizi et al. 2015, Liu, Gerner et al. 2015). Inhibition of NK cell proliferation and function by Tregs has been described in homeostatic or autoimmune conditions in studies where Tregs were depleted systemically (Trzonkowski, Szmit et al. 2004, Feuerer, Shen et al. 2009, Gasteiger, Hemmers et al. 2013, Sitrin, Ring et al. 2013). Previous studies have highlighted Treg suppressive mechanisms including the secretion of inhibitory cytokines (TGF- β , IL-10, IL-35, IL-37), their expression of CTLA-4 and PD-L1, and their capacity to block key interactions of NK cells with DCs and macrophages (Terme, Chaput et al. 2008, Tekguc, Wing et al. 2021, Pereira, Lanzar et al. 2023). Furthermore, contact-dependent inhibition of NK cells by Tregs can occur by cell surface-bound TGF- β on Tregs (Trzonkowski, Szmit et al. 2004). However, the impact of Treg depletion on antitumor NK cell responses,

especially in the setting of local Treg depletion within the tumor environment, has not been investigated.

Here, we show that selective intra-tumoral (IT) depletion of Tregs unleashes a powerful NK cell response locally within tumors that controls the growth of MHC I-deficient tumors without inciting autoimmunity. Local NK cell activation, proliferation, and cytolytic function required CD4⁺ Tconv cells that produced IL-2. IL-2 was both necessary for tumor control, and sufficient to restore NK cell antitumor activity when CD4⁺ Tconv cells were depleted in combination with Tregs. In addition, conventional type 2 dendritic cells (cDC2s) were critical for NK-mediated tumor control, at least in part by supporting CD4⁺ Tconv cell production of IL-2 in tumors after IT Treg ablation. After treatment, STAT5 phosphorylation was elevated specifically in intra-tumoral NK cells, but not tumor-draining LN or splenic NK cells, suggesting that IL-2 produced locally in tumors acted on NK cells. Finally, therapeutically translatable approaches to deplete IT Tregs with antibodies led to the control of CD8⁺ T cell-resistant tumors in a manner that recapitulated the genetic *Foxp3^{DTR-GFP}* experimental model. Taken together, our findings show that IT Treg ablation can activate NK cells within tumors via a cDC2-CD4-IL2 axis that can control tumors that resist CD8⁺ T cell responses.

RESULTS

Intra-tumoral Treg ablation promotes control of MHC I-deficient tumors without autoimmunity.

We employed three different C57BL/6 (B6) MHC I-deficient tumor models that were engineered by CRISPR-Cas9-mediated disruption of the beta-2-microglobulin (*B2m*) gene: the MC38-*B2m*^{-/-} (colon carcinoma), B16F10-*B2m*^{-/-} (melanoma) and RMA-*B2m*^{-/-} (lymphoma) cell lines (Nicolai, Wolf et al. 2020, Wolf, Blaj et al. 2022) (fig. 3.S1A). We depleted Treg_s using *Foxp3*^{DTR-GFP} mice, wherein diphtheria toxin (DT) injection leads to Treg apoptosis (Kim, Rasmussen et al. 2007). Mice bearing subcutaneous tumors of each type at a size of ~50 mm³ (five days after inoculation) began receiving DT systemically by intraperitoneal (IP) injection every other day (Fig. 3.1A). IP DT treatments resulted in the significant control of all three *B2m*^{-/-} tumor types, and in Treg depletion in all organs assessed (Fig. 3.1B-C, fig. 3.S1C-D). Thus, Treg ablation can lead to tumor control even when tumors lack MHC I molecules. However, systemic Treg depletion led to autoimmune toxicity, as evidenced by weight loss (Fig. 3.1D, fig. 3.S1B), enlarged lymph nodes and spleens (fig. 3.S1E), increased spleen weight (Fig. 3.1E), and morbidity by day 15 that required euthanasia.

To test whether localized Treg ablation within tumors could also mediate tumor control, but without autoimmune toxicity, we tested a low dose of DT injected IT in an attempt to generate localized intra-tumoral-specific Treg depletion (Fig. 3.1A). Compared to IP DT, IT DT treatments resulted in comparable depletion of Treg_s within tumors, but little or no depletion of Treg_s in the spleen and lymph nodes, indicative of significant tumor-specific Treg depletion (Fig. 3.1C, fig. 3.S1E, F). In both tumor models tested (MC38-*B2m*^{-/-} and B16F10-*B2m*^{-/-}), low-dose IT DT injections resulted in control of tumor growth that was equivalent to systemic Treg depletions by IP DT (Fig. 3.1F, G, fig. 3.S1C, D). However, mice treated with IT DT did not lose weight (Fig. 3.1D, fig. 3.S1B), or exhibit enlargement of non-tumor-draining lymph nodes (ndLN) or spleens (Fig. 3.1E, fig. 3.S1E) and survived long-term. The percentages of CD4⁺ T_{conv} cells and CD8⁺ T cells expressing the activation marker CD25 increased in the spleens and ndLN of IP DT-injected mice. In contrast, IT DT-injected mice showed no significant increase in T cell activation in spleens and ndLN, despite a small increase in CD25⁺ CD4⁺ T_{conv} cells in the tumor-draining lymph nodes (tdLN), which was, however, smaller than seen with systemic Treg depletion (fig. 3.S1F).

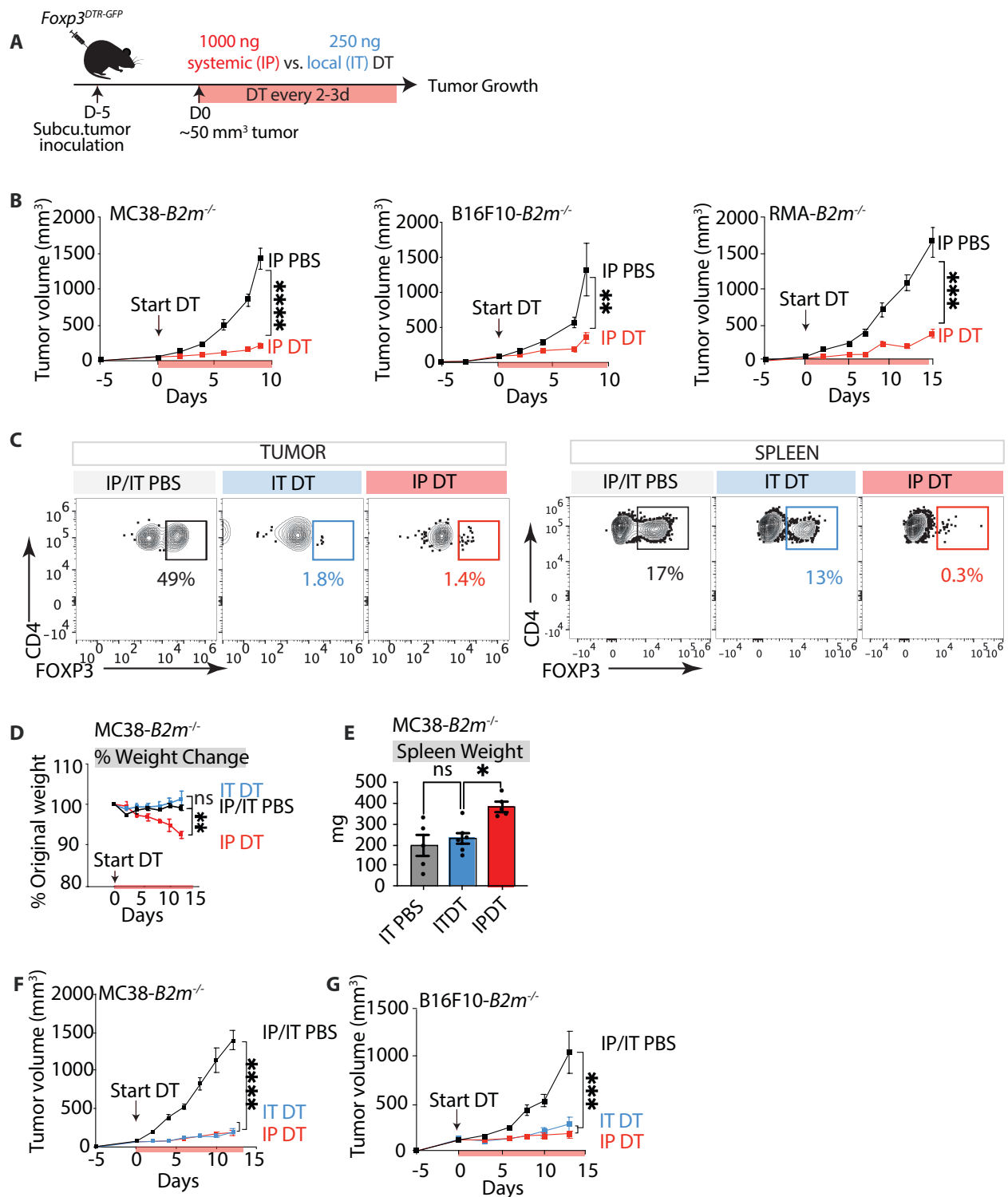


Figure 3.1. Intra-tumoral Treg depletion leads to potent tumor control without toxicity

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- (A) Schematic of tumor inoculation and treatment regimen in *Foxp3^{DTR-GFP}* mice depleted of Tregs either systemically by IP injections of PBS or 1000 ng DT or locally (intra-tumorally, IT) with 250 ng of DT. DT inoculations began when tumors reached ~50 mm³, typically 5 days after tumor inoculations.
- (B) Tumor growth curves in *Foxp3^{DTR-GFP}* mice systemically depleted of Tregs (IP DT) or control (IP PBS). Data are representative of 3-10 experiments performed for each tumor type.
- (C) Representative flow cytometry plots showing Treg frequencies in tumors and spleens from *Foxp3^{DTR-GFP}* mice with MC38-*B2m*^{-/-} tumors 12 days after PBS, IP DT, or IT DT treatment. Gated CD4⁺CD3⁺ cells shown. Tregs were identified by anti-FOXP3 staining.
- (D) Body weight changes in *Foxp3^{DTR-GFP}* mice bearing MC38-*B2m*^{-/-} tumors that were depleted of Tregs systemically (IP DT) or intra-tumorally (IT DT) compared to no depletion (IP/IT PBS).
- (E) Spleen weight of *Foxp3^{DTR-GFP}* mice bearing MC38-*B2m*^{-/-} tumors at D12 post DT initiation of groups described in (D).
- (F) Tumor growth curves in *Foxp3^{DTR-GFP}* mice that were depleted of Tregs intra-tumorally with IT DT compared to IP DT injections. MC38-*B2m*^{-/-} and B16F10-*B2m*^{-/-} tumors were tested. Data are representative of 2 experiments performed for each tumor type.

Data represent means ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

B, F, G: RM two-way ANOVA followed by Geiser-Greenhouse correction. (n=5-8 mice/group). All comparisons are performed pairwise against the DT group with time as a factor. Statistic shown reflect total column (treatment) effect.

D, E: Ordinary one-way ANOVA with Dunnett's multiple comparison test.

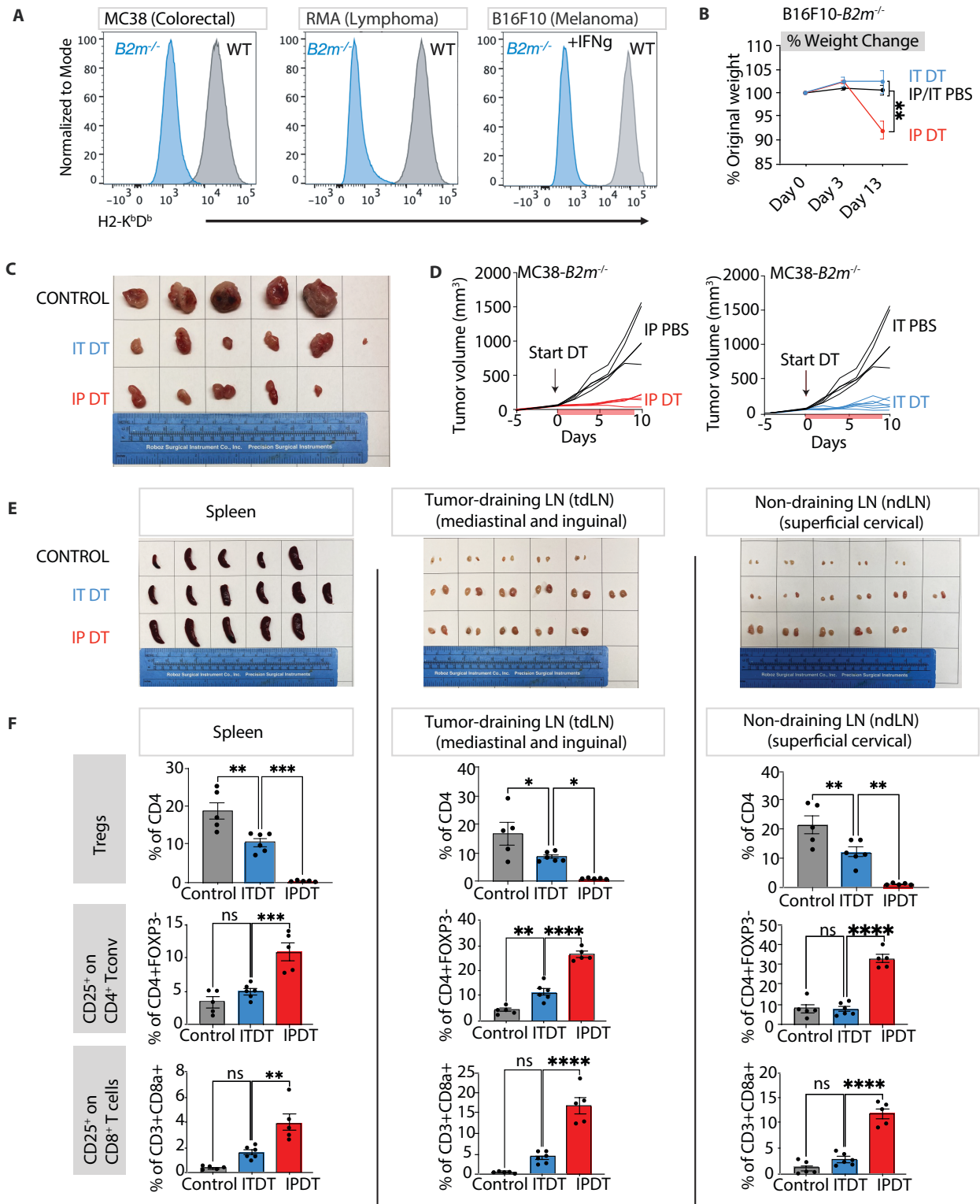


Figure 3.S1. MHC I-deficient tumor lines and comparison of systemic vs. intra-tumoral Treg depletion models

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- (A) Histograms showing staining of H2-K^b/D^b antigens on WT (MHC I-expressing, grey) vs *B2m*^{-/-} cell lines (blue). The B16F10 cells were stimulated overnight in vitro with 20 ng/mL IFN- γ to induce MHC I expression.
- (B) Body weight changes in *Foxp3*^{DTR-GFP} mice with B16F10-*B2m*^{-/-} tumors that were not depleted or depleted of Tregs with IT or IP DT injections. Representative of 2 independent experiments.
- (C) Representative experiment showing relative MC38-*B2m*^{-/-} tumor sizes at 12 days post Treg ablation (D17 post-tumor inoculation).
- (D) Spider plots for *Foxp3*^{DTR-GFP} mice with MC38-*B2m*^{-/-} tumors that were depleted of Tregs with IT vs IP DT injections.
- (E) Images of spleens, tdLN and ndLN from mice that were not depleted or depleted of Tregs with IT or IP DT injections.
- Percentages of Tregs and CD25⁺ cells among CD4 Tconv cells (CD4⁺CD3⁺FOXP3⁺) or CD8⁺CD3⁺ T cells from the spleens, tumor draining LN (tdLN), and nondraining LN (ndLN) 12 days after DT initiation. Representative of 2 independent experiments.

Data represent means $\pm$ SEMs; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001

B,F: Ordinary one-way ANOVA with Tukey's multiple comparisons test

NK cells and CD4⁺ T_{conv} cells control MHC I-deficient tumors upon intra-tumoral Treg ablation.

To identify the effector cell types responsible for tumor rejection after Treg ablation, different effector cell types were depleted *in vivo* with antibody treatments starting one day before Treg depletion in mice with 50 mm³ established tumors (Fig. 3.2A). With systemic (IP DT) (Fig. 3.2B-D) or intra-tumoral (IT DT) (Fig. 3.2E-G, fig. 3.S2A-C) Treg ablation, tumor control was significantly diminished by CD4⁺ T_{conv} cell or NK cell depletion in all three tumor models tested. Notably, the numbers of NK cells per mm³ of tumors were not significantly increased after Treg depletion, potentially implicating an enhancement in antitumor NK activity within tumors as opposed to increased proliferation or recruitment of cells upon IT Treg ablation (fig. 3.S2D-E). We did observe an increase in CD4⁺ T_{conv} cells in MC38-*B2m*^{-/-} tumors but not in B16F10-*B2m*^{-/-} tumors after Treg ablation (fig. 3.S2D-E). While NK and CD4⁺ T cells were clearly required for tumor control with IT Treg ablation, depletion of CD8⁺ T cells never reduced tumor control in any of the MHC I-deficient tumor models (Fig. 3.2B-D, fig. 3.S2F-G). Thus, local IT ablation of Tregs was sufficient to mediate significant tumor control of MHC I-deficient tumors independently of CD8⁺ T cells.

IT Treg ablation elicits NK-dependent control of MHC I⁺ tumors that ectopically express NKG2D ligands.

Other than killing tumors cells via the loss of MHC I, i.e. “missing-self” recognition, NK cell recognition and killing of target cells also occurs against MHC I⁺ cells when NK-activating receptors, such as NKG2D or NKp46, are activated by binding their ligands expressed on cancer cells ("induced-self recognition") (Jamieson, Isnard et al. 2004, Gasser, Orsulic et al. 2005, Guerra, Tan et al. 2008, Unni, Bondar et al. 2008, Raulet and Guerra 2009, Schuster, Berger et al. 2011, Raulet, Gasser et al. 2013). We examined whether Treg depletion induced rejection of MHC I⁺ tumors by NK cells only when they expressed sufficient NKG2D ligands to overcome MHC I-mediated inhibitory signaling. We used MHC I⁺ RMA tumor lines that do not naturally express NKG2D ligands and are poorly killed by NK cells (Diefenbach, Jamieson et al. 2000, Diefenbach, Jensen et al. 2001, Jamieson, Isnard et al. 2004). However, RMA cells become sensitive to NK cell killing when transduced with the NKG2D ligand H60a (Diefenbach, Jensen et al. 2001, Gasser, Orsulic et al. 2005) (Fig. 3.2H, fig. 3.S2H). RMA-*H60a* tumor cells were implanted in B6 mice and CD8⁺ T cells were depleted to prevent antitumor responses mediated by CD8⁺ T cells

against H60a (Malarkannan, Shih et al. 1998, Diefenbach, Jensen et al. 2001). IT depletion of Treg_s elicited significant control of MHC I⁺ RMA-*H60a* tumors that was significantly inhibited by NKG2D blocking antibodies (Fig. 3.2H) or NK cell depleting antibodies (Fig. 3.2I). In contrast, IT Treg ablation did not elicit control of the RMA-WT tumors that are known to be resistant to NK cells (Fig. 3.2J) (Jamieson, Isnard et al. 2004). Overall, we hypothesize that Treg ablation can induce NK cell control of MHC I-deficient tumors with little or no activating ligand expression, but can also elicit NK cell control of MHC I⁺ tumors if tumor cells express sufficient NK activating ligands to overcome inhibition via inhibitory receptors specific for MHC I. Thus, Treg ablation enhances NK cell control of tumors mediated by both missing-self recognition and "induced-self" recognition, including MHC I⁺ tumors that express the H60a NKG2D ligand.

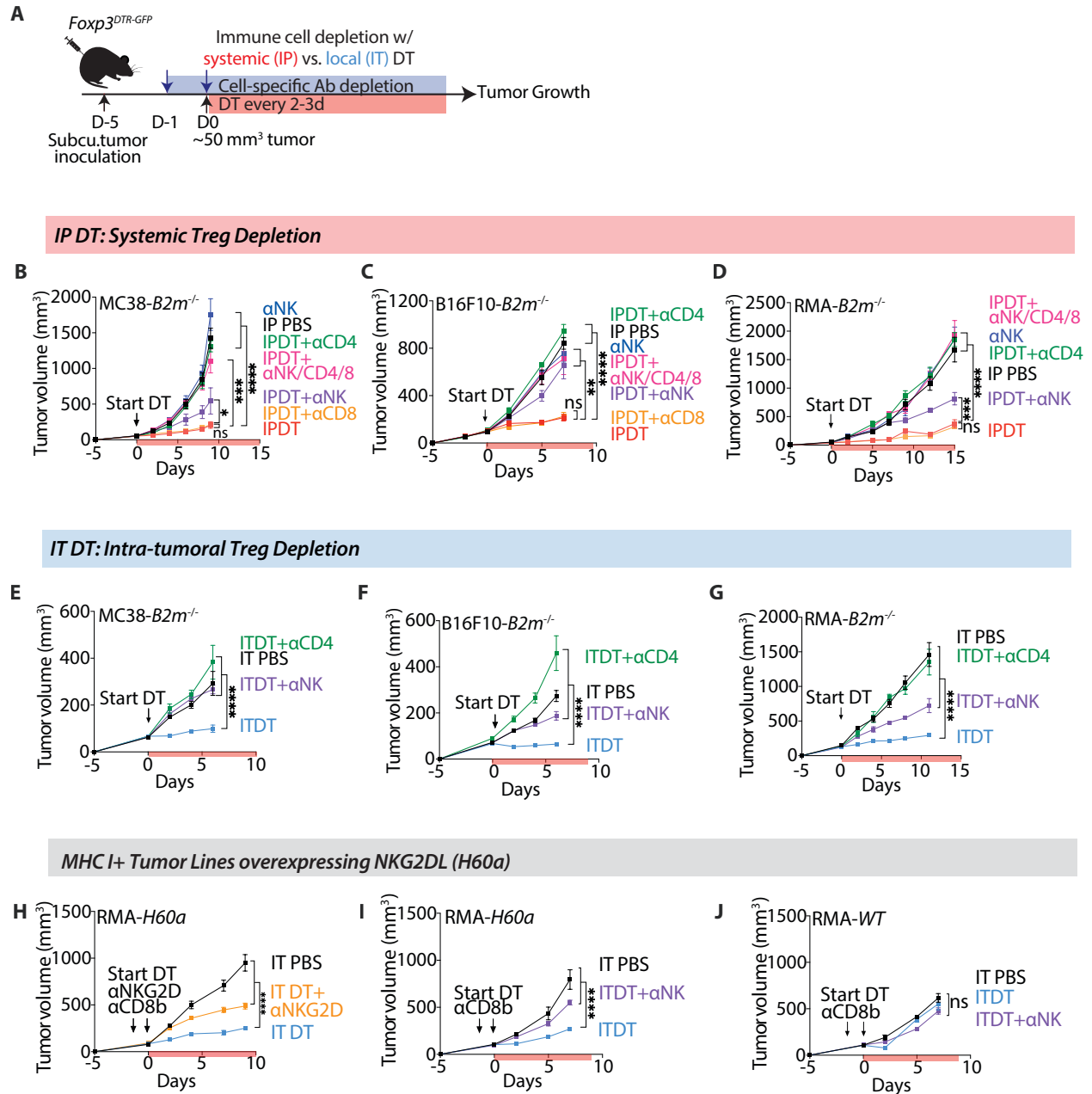


Figure 3.2. NK cells and CD4⁺ T cells, but not CD8⁺ T cells, are required for the control of tumors after Treg depletion.

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(A) Schematic of tumor inoculation and treatment regimen in *Foxp3^{DTR-GFP}* mice depleted of Tregs systemically (IP) or locally (IT). DT was administered every 48-72 hours, while cell depleting antibodies were administered on D1, D0 and every six days thereafter. Control mice were treated with either IP PBS or IT PBS as controls to account for local inflammation at injection sites.

(B-D) Growth curves of indicated *B2m^{-/-}* tumors in mice that were systemically depleted of Tregs alone (red) or in combination with depletion of all CD4⁺ T cells (green), NK cells (blue), CD8⁺ T cells (orange) or all three cell types (NK/CD4/CD8 cells) (pink). NK cell depletion alone without Treg depletion (purple) was included as an additional control. Data are representative of 2-3 experiments for each tumor type.

(E-G) Growth curves of indicated *B2m^{-/-}* tumors in mice that were intra-tumorally (IT) depleted of Tregs alone (blue) or in combination with depletion of all CD4⁺ T cells (green) or NK cells (purple). Data are representative of 8 (MC38-*B2m^{-/-}*), 3 (B16F10-*B2m^{-/-}*) or 1 (RMA-*B2m^{-/-}*) experiments.

(H) Growth curves of RMA-*H60a* tumors in mice that were depleted of CD8b⁺ T cells before IT-Treg depletion without or with injections of blocking NKG2D antibody (clone MI-6). Data are representative of 3 experiments.

(I) Growth curves of RMA-*H60a* tumors in mice that were depleted of CD8b⁺ T cells before IT-Treg depletion without or with anti-NK1.1 antibody (clone PK136). One experiment performed.

(J) Growth curves of RMA-WT tumors in mice that were depleted of CD8b⁺ T cells before IT-Treg depletion without or with anti-NK1.1 antibody (clone PK136). One experiment performed.

Data represent means $\pm$ SEM; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

B, C, D, E, F, G, H, I, J: RM two-way ANOVA followed by Geiser-Greenhouse correction. (n=5-8 mice/group). All comparisons are performed pairwise against the DT group with into account time as a factor. Statistic shown reflect total column (treatment) effect.

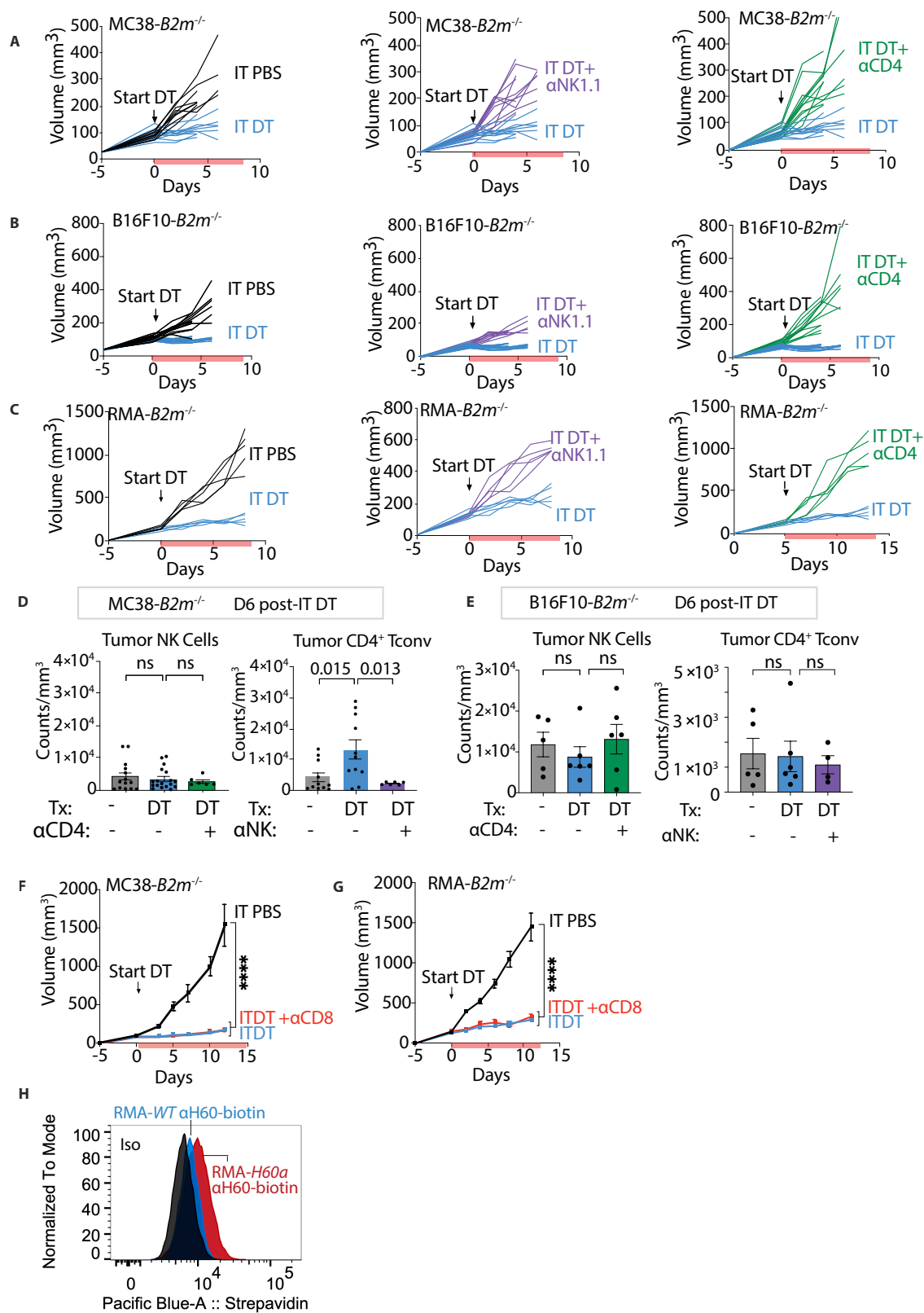


Figure 3.S2 (associated with Fig 2). Cellular requirements for tumor rejection

Figure 3.S2 (associated with Fig 2). Cellular requirements for tumor rejection

(A, B, C) Spider plots from Figure 2E, 2F and 2G

(D-E) Bar graphs showing counts per mm³ of tumor NK cells and CD4⁺ T cells six days after intra-tumoral Treg depletion (blue) or either Treg plus Tconv depletion (anti-CD4, green) or Treg plus NK depletion (anti-NK1.1, purple) in mice with MC38-*B2m*^{-/-} tumors. Data are representative of 2 experiments.

(F) Growth curves of MC38- and (G) RMA-*B2m*^{-/-} tumors in mice that were intra-tumorally depleted of Tregs alone (blue) or in combination with depletion of all CD8⁺ T cells (red). Control mice are treated with IT PBS (black). Data are representative of 2-3 experiments for each tumor type.

(H) Staining validation for ectopic expression of NKG2D ligand H60a on RMA lymphoma tumor cell lines (RMA-*WT* vs RMA-*H60a*) using biotinylated polyclonal H60 antibodies.

Data represent means $\pm$ SEM; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

E: Ordinary one-way ANOVA with Dunnett's multiple comparisons test, representative of at least 1 independent experiment per tumor cell line).

D, G: RM two-way ANOVA followed by Geiser-Greenhouse correction. All comparisons are performed pairwise against the DT group with into account time as a factor. Statistic shown reflect total column (treatment) effect.

IT Treg ablation elicits transcriptional changes in NK cells that depend on CD4⁺ Tconv cells.

To investigate changes in NK cells using an unbiased approach, we performed bulk RNA sequencing analysis of NK cells (*DAPI*⁺*CD45*⁺*CD3*⁺*CD8α*⁺*CD19*⁺*F480*⁺*NK1.1*⁺) FACS purified from MC38-*B2m*^{-/-} tumors in *Foxp3*^{DTR-GFP} mice six days after IT injections of PBS or DT, or IT DT with systemic depletion of all CD4⁺ T cells (Fig. 3.3A, fig. 3.S3A). Principal component analysis (PCA) was first performed on z-score-transformed transcripts per million (TPM) values to assess sample clustering and detect potential batch effects. PCA analysis showed a shift in the NK cell transcriptome associated with IT Treg depletion compared to the PBS control (fig. 3.S3B). Differential expression analysis was then carried out using the *limma* linear modeling framework (Ritchie, Phipson et al. 2015), incorporating batch as a covariate and identifying significantly regulated genes in the PBS vs. ITDT and ITDT vs. ITDT + anti-CD4 comparisons. We represented these differentially expressed genes (DEGs) with a volcano plot and saw increased expression of many genes associated with NK cell activation and cytotoxic status, including *Gzmb* (encoding granzyme B), *Gzma* (encoding granzyme A) and *Prfl* (encoding perforin) (Fig. 3.3B, Supplementary Table 1). Interestingly, orphan granzyme family members (*GzmC-N*), which are poorly studied in both humans and mice, were more upregulated than *Gzmb* and *Gzma* (Fig. 3.3B-C) (Grossman, Revell et al. 2003). Notably, the shifts in the NK cell transcriptome with IT Treg ablation were largely prevented by depleting all CD4⁺ T cells at the same time DT treatments were initiated (Fig. 3.3C-D, fig. 3.S3B,C (Wagner, Wang et al. 2021), Supplementary Table 2), which suggests that CD4⁺ T_{conv} cells are necessary for the altered transcriptome of NK cells upon IT Treg ablation. While CD4⁺ T_{conv} are recognized for their roles in priming, activating, and helping CD8⁺ T effector cell responses (Janssen, Lemmens et al. 2003, Shedlock and Shen 2003, Bevan 2004, Castellino, Huang et al. 2006), less is understood about their role in helping NK cell antitumor responses *in vivo*.

Genes representing distinct biological pathways were selected for visualization using z-score-scaled TPM values and ranked by lowest to highest adjusted p-values. IT Treg ablation led to significant or trending increases in the expression of genes encoding common NK cell activation markers, i.e. *Tigit*, *Ly6a* (SCA-1), *Klrg1*, *Icos*, *Fas*, *CD69* and *CD226* (DNAM-1) (Fig. 3.3D), and immune checkpoint (ICP) genes *Havcr2* (TIM3), *Tigit*, *Lag3*, *Pdcd1* (PD-1) and *Ctla4* (Fig. 3.3F). Key metabolic pathway genes were also upregulated in NK cells, including *Nos2* (nitric oxide

synthesis pathway), *Gys1* (glycogen synthesis pathway), *Pgk1* (glucose metabolism and ATP production), and *Sod2* (mitochondrial reactive oxygen species metabolism), consistent with an oxidative and/or hypoxic stress response, metabolic reprogramming, and a general inflammatory state (Fig. 3.3G).

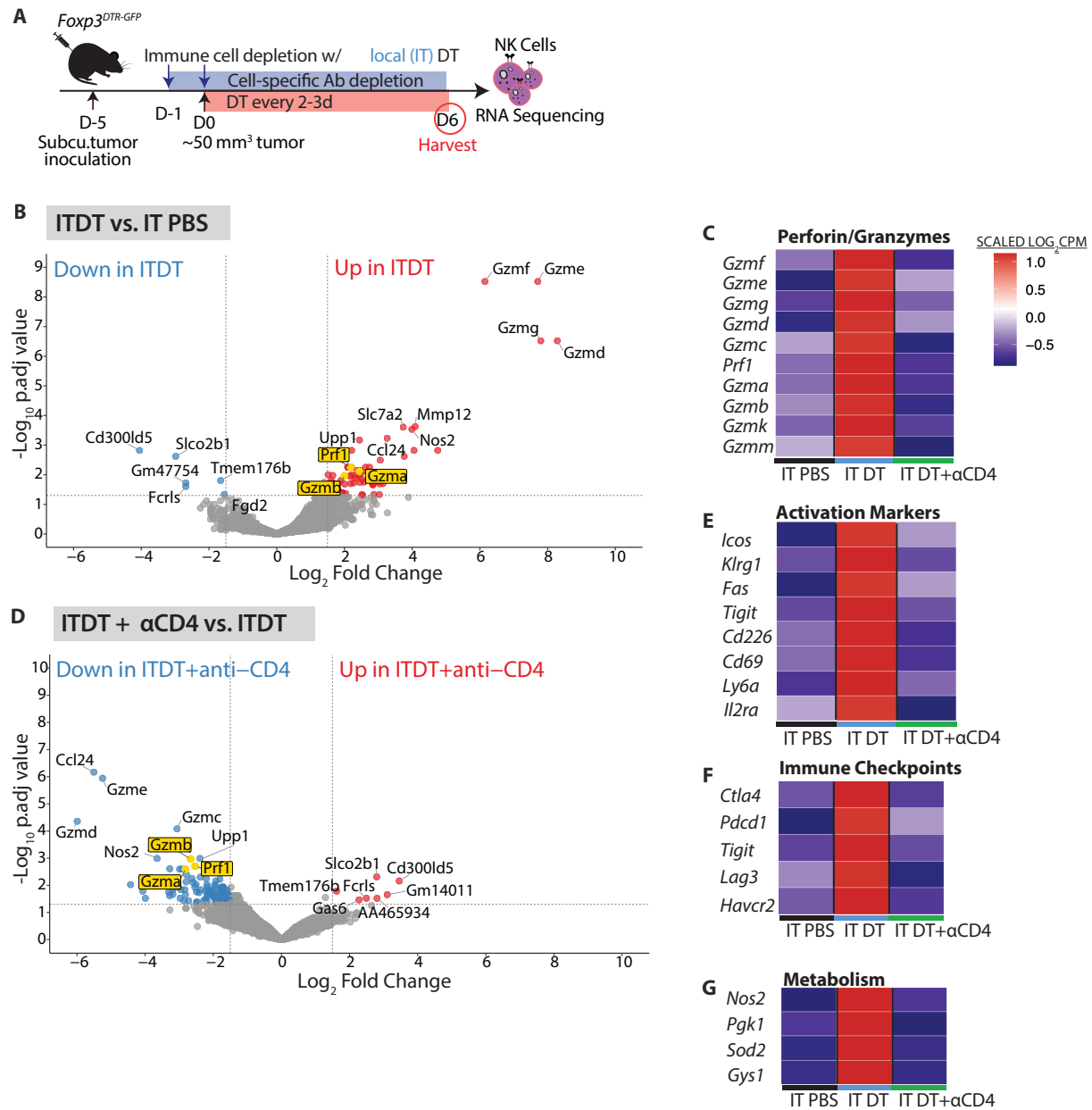


Figure 3.3: Global transcriptomic changes in NK cells following IT-Treg ablation depends on CD4⁺ T cells.

Figure 3.3: Global transcriptomic changes in NK cells following IT-Treg ablation depends on CD4+ T cells.

(A) Schematic of MC38-*B2m*^{-/-} tumor inoculation and treatment regimen in *Foxp3*^{DTR-GFP} mice depleted of IT Tregs, followed by processing and sorting of NK cells from tumors of mice treated as indicated after 6 days. RNA sequencing was performed on three individual replicates, each comprised of sorted cells from a pool of 3-4 tumors.

(B, D) Differential expression analysis was conducted with limma-voom (Methods). Volcano plots show upregulated and downregulated genes in NK cells in two pairwise comparisons (+/- IT DT and IT DT +/- anti-CD4 depletion). Genes were labeled as significantly upregulated (red) or downregulated (blue) if adjusted p-value < 0.05 and absolute log 2 fold change > 1.5. Selected genes (*Gzma*, *Gzmb*, *Prfl*) are annotated to show relative position to orphan granzyme members.

(C, E-G) Heatmaps of z-normalized replicate-controlled expression for selected genes encoding activation markers, granzyme family members, immune checkpoint receptors (ICP), and metabolic markers. Genes are ranked from lowest to highest adjusted p-value.

IT Treg ablation increases NK cell activation, maturation, and cytotoxicity in a CD4⁺ T cell-dependent manner.

To assess the impact of these transcriptomic findings on protein levels and functional activities of NK cells, we collected MC38-*B2m*^{-/-} tumors at the same timepoint as the RNAseq data (day 6 after IT DT), as well as an earlier timepoint (day 4), and performed spectral flow cytometry analyses (Fig. 3.4A). At day 4, NK cells in the tumors from IT Treg-depleted mice were more proliferative, as demonstrated by *in vivo* labeling with BrdU (Fig. 4B), and expressed higher levels of the early activation marker CD69 (Fig. 3.4C). By day 6, additional activation markers for NK cells, SCA-1 and KLRG1, were also significantly upregulated, and NK cells exhibited a more mature phenotype (CD11b⁺ CD27⁻) (Fig. 3.4D-F). These phenotypic changes were abolished when CD4⁺ T_{conv} cells were co-depleted with IT Tregs, confirming that the activation and maturation of NK cells was dependent on CD4⁺ T_{conv} cells. IT Treg depletion also resulted in significant upregulation of activation markers (KLRG1, SCA-1) on NK cells in MHC I⁺ RMA-*H60a* tumors (fig. 3.S3C). These data suggest that upon Treg depletion, an absence of MHC I-inhibition or the engagement of NK activating receptors can both contribute to NK cell activation.

As observed by RNAseq transcriptome analysis (Fig 3.3E, H), IT Treg depletion also increased the intracellular levels of granzyme B and perforin (Fig. 3.5A-B). Importantly, *ex vivo* cytotoxicity of MHC I-deficient tumor cells by NK cells sorted from tumors was elevated with IT Treg-depletion (Fig. 3.5C). In addition, spleen cells from mice with Tregs depleted systemically also exhibited elevated cytotoxicity *ex vivo*, which depended on the presence of CD4⁺ T_{conv} cells (fig. 3.S3D). The splenic effector cells were confirmed to be NK cells by both depleting (fig. 3.S3E, G-H) or enriching (fig. 3.S3F, G-H) them from spleen cell preparations (fig. 3.S3D-H). Notably, tumor rejection after IT Treg ablation was perforin-dependent, because *Prf*^{-/-}; *Foxp3*^{DTR-GFP} mice lacking perforin were unable to control MC38-*B2m*^{-/-} tumors compared to control *Prf1*^{+/+}; *Foxp3*^{DTR-GFP} mice (Fig. 3.5D) (Kägi, Ledermann et al. 1994). Thus, after IT Treg depletion, NK cells in tumors exhibited increased *ex vivo* cytotoxicity and capacity to control tumors *in vivo* in a perforin-dependent fashion.

Tumor NK cells from IT Treg-depleted mice also showed significantly increased IFN- γ production compared to non-depleted mice when stimulated *ex vivo* with plate-bound NK1.1

antibodies (Fig. 3.5E,G), but also when the cells were not restimulated *ex vivo* (fig. 3.S3I) presumably reflecting stimulation that occurred *in vivo*. The increased IFN- γ production was abolished when the mice were also depleted of CD4⁺ T_{conv} cells (Fig. 3.5E,G). In contrast, we observed no increase in *ex vivo* degranulation (CD107a) upon NK1.1 stimulation, despite the increased killing noted earlier (Fig. 3.5E-F, fig. 3.S3I). We hypothesize that although the level of degranulation in NK cells was not noticeably increased, the increased granzymes and perforin in the granules of these NK cells with Treg depletion likely increased their cytotoxic potential. Thus, NK cells in tumors that were Treg-depleted exhibited increased proliferation, maturation, cytokine production, tumor cell cytotoxicity and capacity to reject tumors *in vivo*. For each of these phenotypes observed in NK cells with Treg ablation, CD4⁺ T_{conv} cells were required.

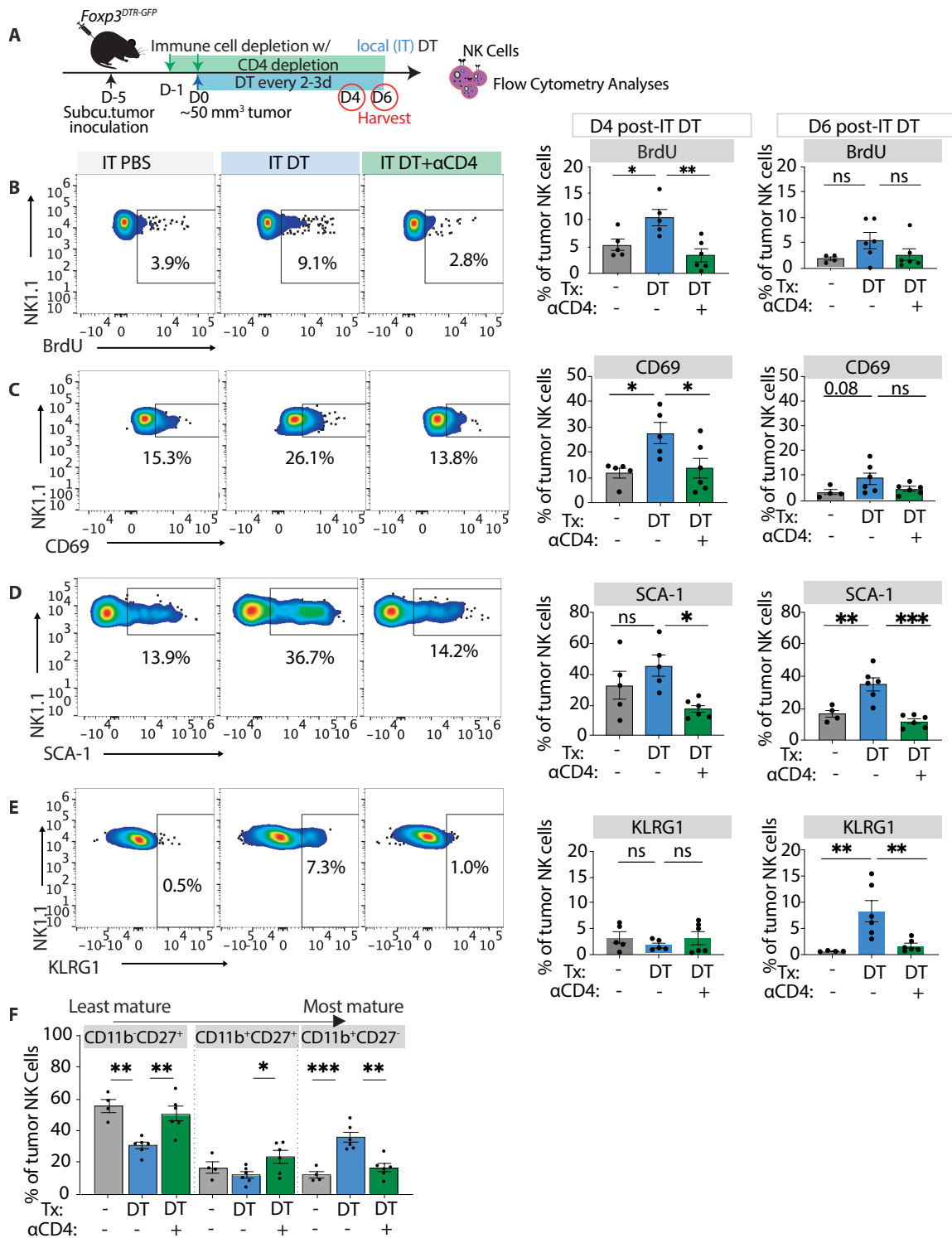


Figure 3.4. The NK cell activation phenotype with IT-Treg ablation is dependent on CD4+ T cells.

Figure 3.4. The NK cell activation phenotype with IT-Treg ablation is dependent on CD4⁺ T cells.

(A) Schematic of MC38-*B2m*^{-/-} tumor inoculation in *Foxp3*^{DTR-GFP} mice, IT depletion of Tregs, immune cell depletions, and tumor resection 4 or 6 days after treatment initiation, for phenotypic analysis of intra-tumoral NK cells. After processing, NK cells were sorted from tumors from three treatment conditions: IT PBS, IT DT and IT DT+ anti-CD4.

(B-E) Representative flow plots (from day 4) and summary data (from days 4 and 6) of markers of proliferation (BrdU incorporation, B) and activation (CD69, C; SCA-1, D; and KLRG1, E) on gated NK cells in MC38-*B2m*^{-/-} tumors from various treatment groups (IT PBS control in black, IT DT in blue, and IT DT + anti-CD4 in green). n=5-8 mice/group, representative of at least 3 independent experiments.

(F) Percentages of NK cells with the indicated maturation phenotypes defined by CD11b and CD27 expression at D6 after starting IT treatments in MC38-*B2m*^{-/-} tumors.

The BrdU experiment was done twice, and the other analyses were done 4 times.

Data represent means ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001

B-F: Unpaired t test with Welch's correction

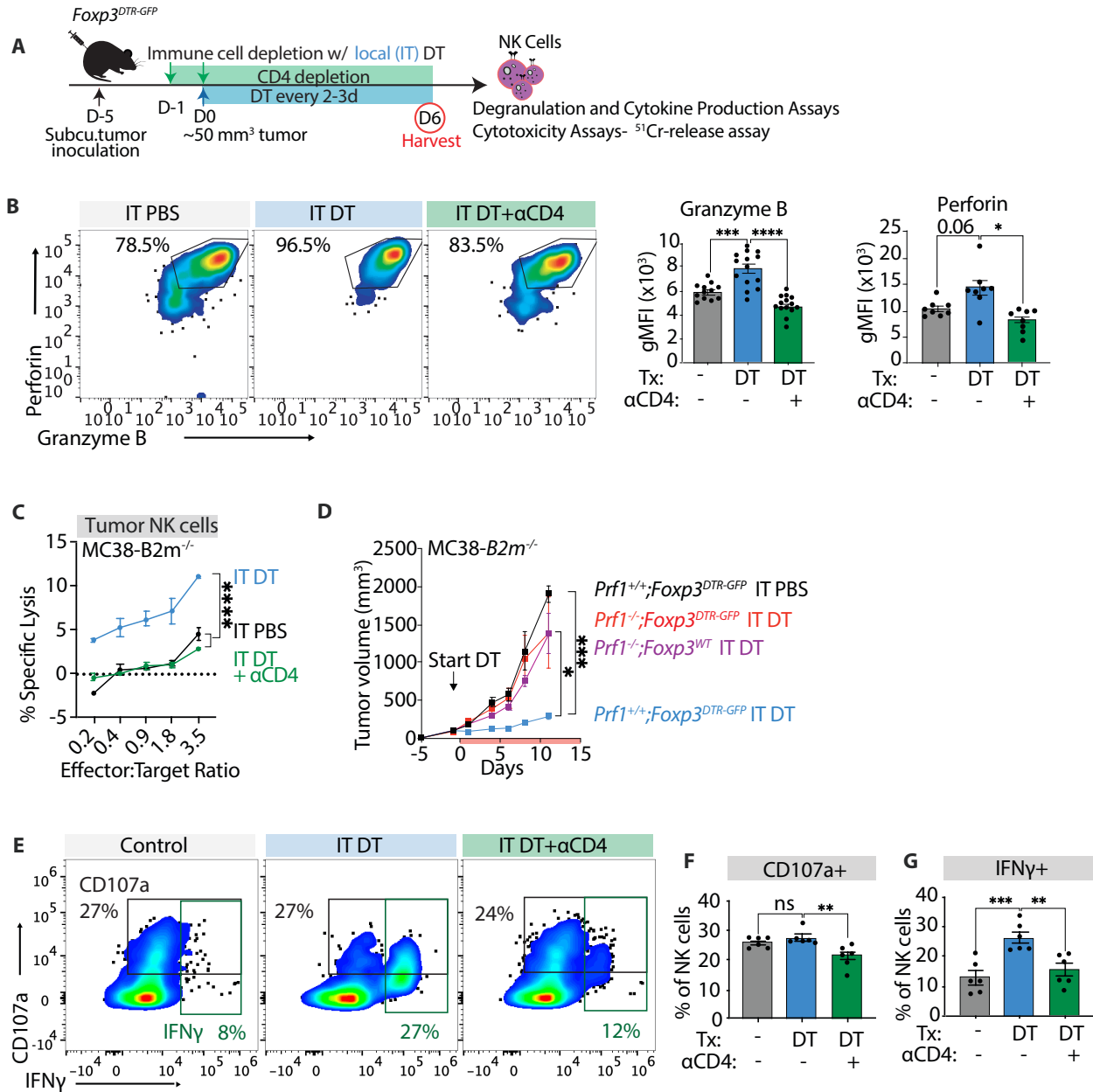


Figure 3.5. Induction of NK cell cytotoxicity with IT-Treg ablation is CD4 T cell-dependent.

Figure 3.5. Induction of NK cell cytotoxicity with IT-Treg ablation is CD4 T cell-dependent.

(A) Schematic of MC38-*B2m*^{-/-} tumor inoculation and treatment regimen in *Foxp3*^{DTR-GFP} mice to prepare NK cells from tumors for RNA and phenotypic analysis, from three treatment conditions: IT PBS, IT DT and IT DT+ anti-CD4.

(B) Representative flow plots and quantification of MFI of intracellular granzyme B and perforin in NK cells from MC38-*B2m*^{-/-} tumors of various treatment groups 6 days after initiation of DT treatment. Bar graphs are of data pooled from 2 independent experiments.

(C) Cytotoxicity of MC38-*B2m*^{-/-} target cells measured with 4-hour chromium release assays of sorted NK cells from MC38-*B2m*^{-/-} tumors in mice treated with IT PBS, IT DT or IT DT+anti-CD4 (n=5-6 from each group). The target cell spontaneous release was 10.8% in this assay.

(D) Growth curves of MC38-*B2m*^{-/-} tumors in *Foxp3*^{DTR-GFP} mice crossed to *Prfl*^{-/-}, including Perforin-expressing mice that were depleted of Tregs intra-tumorally (blue) or treated with IT PBS (black), and Perforin-deficient mice that were depleted of Tregs intra-tumorally (red) or Perforin-deficient mice with WT *Foxp3* alleles treated with IT DT (green). Data are representative of 3 independent experiments.

(E) Representative flow plots and quantification of NK cell functional markers for (F) degranulation (CD107a) and (G) IFN- γ production across various treatment groups after stimulation with plate-bound NK1.1 antibodies *ex vivo*. n=5-8 mice/group, representative of 3 independent experiments.

Data represent means $\pm$ SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001

B, C, G: Ordinary one-way ANOVA with Dunnett's multiple comparisons tests

E: RM two-way ANOVA followed by Geisser-Greenhouse correction. All comparisons are performed pairwise against the DT group with into account time as a factor. Statistic shown reflect total column (treatment) effect.

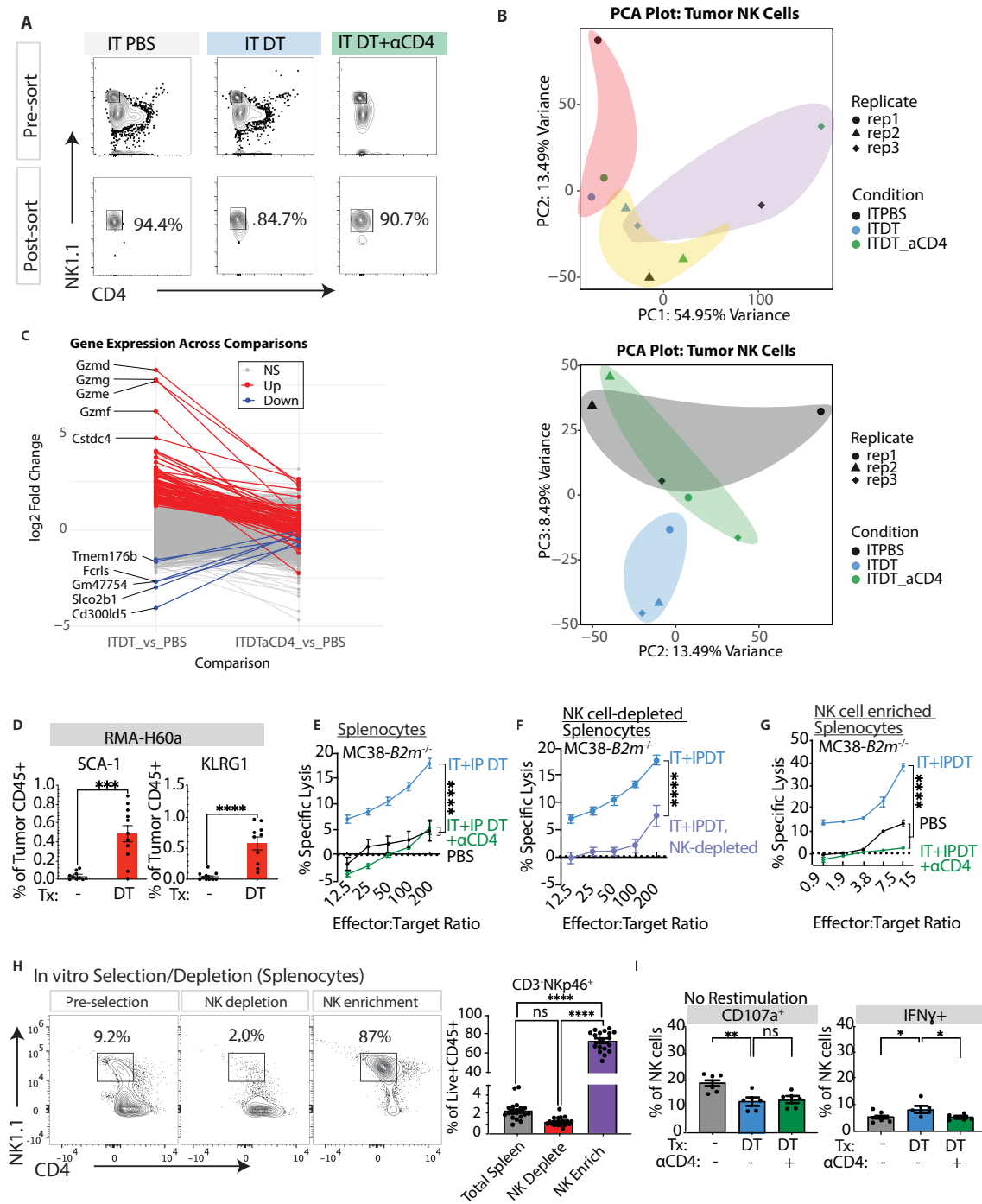


Figure 3.S3 (associated with Fig 3,4,5). Analyses of NK cell populations and splenic cytolytic killing assays and stimulation assays

Figure 3.S3 (associated with Fig 3,4,5). Analyses of NK cell populations and splenic cytolytic killing assays and stimulation assays

(A) Representative flow plots of NK1.1 versus CD4 pre- and post-sort for RNA Sequencing.

(B) Principal component analysis (PCA) of log-transformed NK cell transcriptomes with PC1, PC2 and PC3 scores visualized. Colors indicate treatment conditions while shapes indicate replicate information. PCA plots showing PC1 vs PC2 with groups shaded by replicates, and PC2 vs PC3 with groups shaded by condition.

(C) Line plot of gene log2 fold changes of ITDT v.s. PBS and ITDT_aCD4 v.s. PBS with lines connecting the same gene across comparisons. Genes significantly upregulated or downregulated in the ITDT v.s. PBS comparisons are colored. Significance threshold is based on adjusted p-value < 0.05 and $\text{abs}(\log\text{FC}) > 1$. Top up and down genes based on Log FC are labeled on plot.

(D) Expression of SCA-1 and KLRG1 on tumor NK cells from RMA-*H60a* tumor-bearing mice treated with IT HBSS or IT DT. Data pooled from 2 independent experiments, with n=5-6 per group. Welch's t-tests (n=5-8 mice/group, representative of 2 independent experiments).

(D-F) Cytotoxicity of MC38-*B2m*^{-/-} target cells measured with 4-hour chromium release assays.

(D) Cells were prepared from the spleens of MC38-*B2m*^{-/-} tumor bearing mice treated as shown with DT (both IT and IP) 6 days previously, with or without IP depletion of CD4 T cells or with PBS alone. (E) Splenocytes from these mice were tested after depleting NK cells *in vitro*, or not, before the assay. (F) NK cells were enriched from the spleens of all three conditions. The target cell spontaneous release averaged 8.7% in these assays.

(G) Representative analysis of NK-depleted splenocytes and enriched NK cell populations. Cells were pre-gated for live, CD45⁺CD19⁻F4/80⁻Ter119⁻ cells.

(H) Percentages of NK cells (CD3-NKp46⁺) among splenocytes, splenocytes depleted of NK cells, or enriched NK cells.

(I) Baseline percentages of degranulation and IFN- γ production by NK cells without *ex vivo* stimulation with plate-bound NK1.1 antibodies.

Data represent means $\pm$ SEM; *p < 0.05 , **p < 0.01 , ***p < 0.001 , and ****p < 0.0001

D, F, G: Ordinary one-way ANOVA with Dunnett's multiple comparisons tests. Data is representative of 2 independent experiments.

Conventional dendritic cells are required for NK cell-dependent tumor control.

Treg_s regulate the activation and priming of effector T cells directly and also indirectly by suppressing dendritic cells (Cederbom, Hall et al. 2000, Misra, Bayry et al. 2004, Trzonkowski, Szmit et al. 2004, Wing, Onishi et al. 2008). Specifically, conventional dendritic cells expand and mature after systemic depletion of Treg_s, and cDC1s (CD8 α ⁺/CD103⁺) have been shown to be important for activating CD8⁺ T cell responses, while cDC2s (CD11b⁺/Sirp α ⁺) play a dominant role in stimulating CD4⁺ T cell responses (Dudziak, Kamphorst et al. 2007, Hildner, Edelson et al. 2008, Schlitzer, McGovern et al. 2013, Broz, Binnewies et al. 2014, Roberts 2016, Binnewies, Mujal et al. 2019). In addition, NK cells have been implicated in recruiting cDC1s to boost CD8⁺ T cell responses (Barry, Hsu et al. 2018, Böttcher, Bonavita et al. 2018), and various lines of evidence suggest that DCs can stimulate NK cells *in vitro* or during viral infections (Fernandez, Lozier et al. 1999, Ferlazzo, Tsang et al. 2002, Cooper, Fehniger et al. 2004). However, the roles of DCs in stimulating antitumor NK cell responses *in vivo* after Treg depletion, and the types of DCs required, have not been established.

To probe the contribution of cDCs in the antitumor response after Treg depletion, we utilized three different genetic models for DC deficiencies, namely *Zbtb46*^{DTR} mice (Meredith, Liu et al. 2012) (which lack all cDCs upon DT treatment), *Batf3*^{-/-} mice (Hildner, Edelson et al. 2008) (which lack cDC1s but retain cDC2s) and *Zeb 1+2+3* (abbreviated *Zeb*^{3A}) triple enhancer knockout mice (Liu, Kim et al. 2022) (which lack cDC2s), all crossed to *Foxp3*^{DTR-GFP} to allow for testing their importance upon depletion of Treg_s. We generated bone marrow chimeras (BMCs) in which donor bone marrow cells from *Foxp3*^{DTR-GFP}; *Zbtb46*^{DTR} mice and control *Foxp3*^{DTR-GFP} mice were used to repopulate lethally irradiated B6 mice for 6 weeks to allow immune cell reconstitution (Fig. 3.6A-B). BMCs were necessary for these studies because DT treatment of intact *Zbtb46*^{DTR} mice is lethal (Meredith, Liu et al. 2012). The *Batf3*^{-/-}; *Foxp3*^{DTR-GFP} and *Zeb*^{3A}; *Foxp3*^{DTR-GFP} mice were tested in separate experiments, without the use of BMCs (Fig 6C-F). Tumors were established in the mice and once tumors were palpable, IT DT treatments were initiated to deplete IT Treg_s alone or IT Treg_s and various DC populations. In the *Foxp3*^{DTR-GFP}; *Zbtb46*^{DTR} BMCs, the combined depletion of Treg_s and all CD11c⁺MHCII⁺ cDCs prevented tumor control with IT Treg ablation (Fig. 6A-B, fig. S4A-C). However, *Batf3*^{-/-}; *Foxp3*^{DTR-GFP} mice, which lack cDC1s but retain cDC2s, had no defect in controlling tumors after IT Treg depletion (Fig 3.6C-D). Finally,

Foxp3^{DTR-GFP};Zeb^{3Δ} mice, which lack CD11b⁺ cDC2s but retain cDC1s, had a significantly diminished capacity to control tumors (Fig 3.6E-F, fig 3.S4D-E). These results demonstrate that cDC2s, but not cDC1s, are necessary for tumor control following IT Treg ablation.

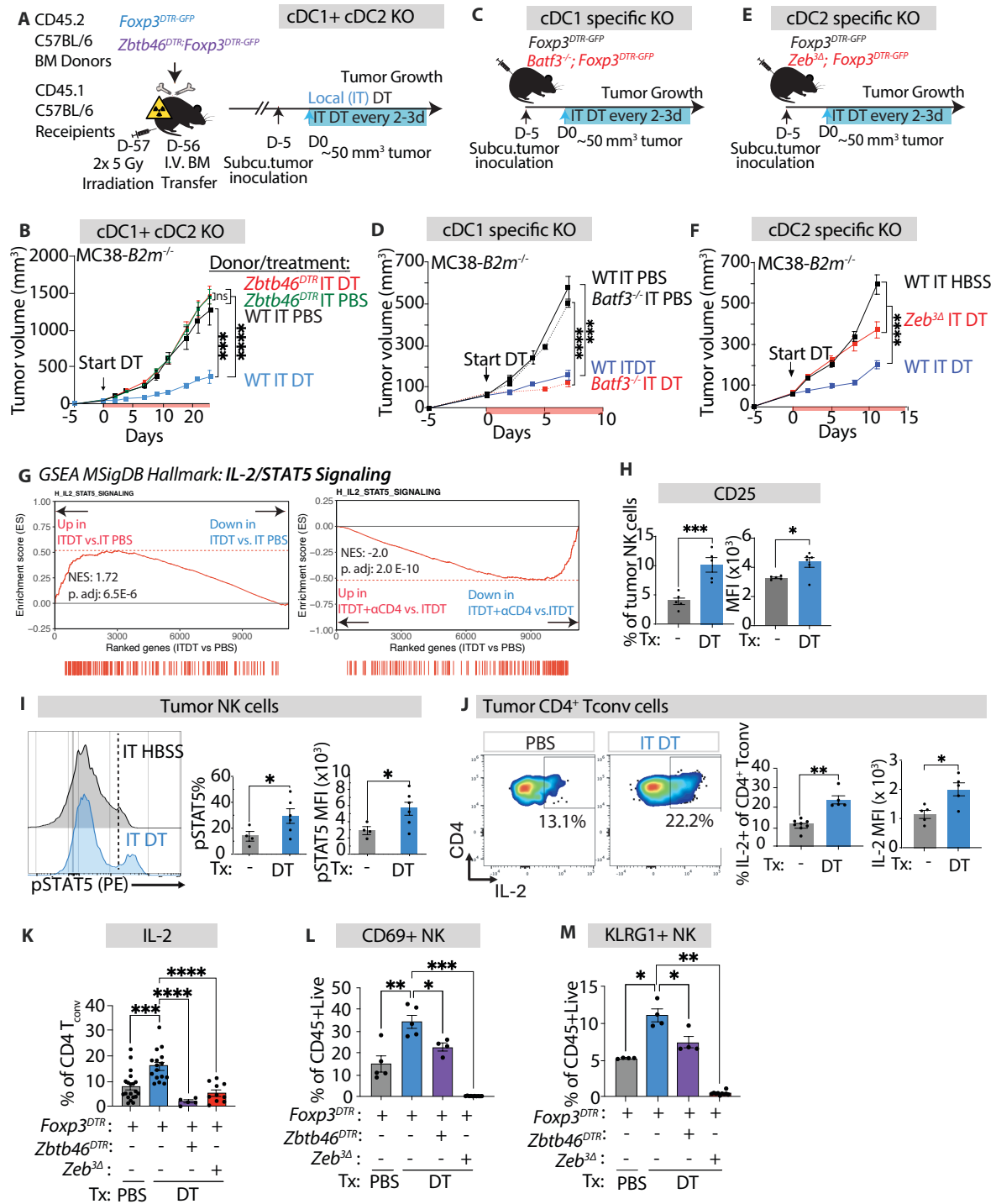


Figure 3.6. NK cell activation and anti-tumor activity is dependent on cDC2s and IL-2

Figure 3.6. NK cell activation and anti-tumor activity is dependent on cDC2s and IL-2

- (A) Schematic for the generation of bone marrow chimeras from *Foxp3^{DTR-GFP}* or *Zbtb46^{DTR};Foxp3^{DTR-GFP}* hematopoietic cells. After 8 weeks of reconstitution, MC38-*B2m*^{-/-} tumor cells were inoculated and 5 days later the treatment regimen was initiated, to deplete Treg_s or Treg_s and *Zbtb46* expressing cDCs intra-tumorally. Tumors were resected at day 6 after Treg depletion for NK cell analysis in panels L-N.
- (B) Growth curves of MC38-*B2m*^{-/-} tumors in mice that were IT depleted of Treg_s (blue) or IT depleted of both Treg_s and cDC1/2s (red). Control groups in mice were treated with IT PBS are shown for each mouse phenotype (black and green). The results shown are representative data from one experiment of three performed.
- (C) Schematic for testing cDC1 specific knock-out using *Foxp3^{DTR-GFP}* and *Batf3*^{-/-}; *Foxp3^{DTR-GFP}* mice inoculated with MC38-*B2m*^{-/-} tumors
- (D) Growth curves of MC38-*B2m*^{-/-} tumors in mice that were IT depleted of Treg_s (blue) or IT depleted of both Treg_s and lack cDC1s (red). Control groups in mice were treated with IT PBS are shown for each mouse phenotype (black solid and dotted lines). The results shown are representative data from one experiment of three performed.
- (E) Schematic for testing cDC2 specific knock-out using *Foxp3^{DTR-GFP}* and *Zeb3^Δ;Foxp3^{DTR-GFP}* mice inoculated with MC38-*B2m*^{-/-} tumors.
- (F) Growth curves of MC38-*B2m*^{-/-} tumors in mice that were IT depleted of Treg_s (blue) or IT depleted of both Treg_s and lack cDC2s (red). Control groups in mice were treated with IT PBS are shown for each mouse phenotype (black solid and dotted lines). The results shown are pooled data from two experiments performed. Tumors were resected at day 6 after Treg depletion for NK cell analysis in panels L-N.
- (G) GSEA plot for Hallmark IL-2/STAT5 signaling pathway on list of genes upregulated in ITDT versus PBS and upregulated in ITDT_aCD4 v.s. ITDT respectively. Normalized enrichment score (NES) and BH-adjusted p-value are labeled in plot.
- (H) CD25 expression and MFI on tumor NK cells on day 6 following either IT PBS or IT-Treg ablation.
- (I) Phosphorylation of STAT5 on tumor NK cells, comparing between mice treated with either IT HBSS (control; black) or IT DT (Treg deplete; blue), taken 6 days after treatment.

Quantification of percent psTAT5 positive NK cells and pSTAT5 gMFI are shown. Representative of two independent experiments.

- (J) Intracellular staining of IL-2 in tumoral CD4 T_{conv} cells (CD3⁺CD4⁺FOXP3⁻) 6 days after treating MC38-*B2m*^{-/-} tumor-bearing mice with IT-DT (blue) or PBS (grey). Representative flow plots showing IL-2 staining are presented on top with quantification below. Representative of 2 independent experiments.
- (K) Intracellular staining of IL-2 in CD4 T_{conv} cells (CD3⁺CD4⁺FOXP3⁻) on day 6 following either PBS, IT-Treg ablation, or IT-Treg IT-cDC ablation or IT-Treg ablation in cDC2-deficient mice in MC38-*B2m*^{-/-} tumors. Two experiments performed for WT HBSS and IT DT and *Zeb3*^Δ conditions; one experiment for *Zbtb46*^{DTR}; plots show combined quantified percent of IL-2 produced by T_{conv} cells in experiments described.
- (L) CD69 expression on tumor NK cells on day 6 following either IT PBS, IT-Treg ablation, or IT-Treg and IT-cDC1/2 ablation in MC38-*B2m*^{-/-} tumors. Representative of 3 experiments performed.
- (M) KLRG1 expression on tumor NK cells on day 6 following either IT PBS, IT-Treg ablation, or IT-Treg and IT-cDC1/2 ablation in MC38-*B2m*^{-/-} tumors. Representative of 3 experiments performed.

Data represent means ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

B, D, F: RM two-way ANOVA followed by Geiser-Greenhouse correction. (n=5-8 mice/group, representative of at least 3 independent experiments). All comparisons are performed pairwise against the DT group with into account time as a factor. Statistic shown reflect total column (treatment) effect.

H, I, J, K, L, M, N: Ordinary one-way ANOVA with Dunnett's multiple comparisons tests.

Increased IL-2 signaling in intra-tumoral NK cells promotes their activation

To interrogate how cDC2s and CD4⁺ T cells may contribute to NK cell activation, we determined whether Molecular Signatures Database (MSigDB) Hallmark and KEGG datasets were enriched in NK cells following IT Treg depletion from our RNA-seq dataset. Among the enriched pathways were multiple cytokine signaling pathways induced by cytokines that can be produced by T cells (fig. 3.S5A, Supplementary Table 3). Using Gene Set Enrichment Analyses (GSEA) (Mootha, Lindgren et al. 2003, Subramanian, Tamayo et al. 2005), we found that NK cells from Treg-depleted tumors were significantly enriched in genes associated with pathways commonly associated with effector functions and tumor immunity such as "IL-2-STAT5 signaling", "TNF- α signaling via NF- κ B" and "Interferon Gamma Response" from the Hallmark dataset, in the top 20 pathways ranked by absolute Normalized Enrichment Score (NES) (Fig. 3.6G, fig. 3.S5A-B, Supplementary Table 3). The enrichment in NK cells of genes associated with these datasets was largely dependent on the action of CD4⁺ T_{conv} cells, as shown by the comparison of gene sets from ITDT vs ITDT + anti-CD4 (Fig. 3.6G, fig. 3.S5B).

Tregs suppress CD4⁺ T_{conv} cell activation by limiting their production of IL-2 and by acting as an IL-2 sink, among other mechanisms (Shimizu, Yamazaki et al. 1999, Feinerman, Veiga et al. 2008, Gasteiger and Kastenmuller 2012, Vignali 2012, Harris, Berdugo et al. 2023). We found that cell surface IL-2R α (CD25) was increased on NK cells after IT Treg depletion (Fig. 6H). We performed pSTAT5 staining in NK cells from tumors and observed higher percentages of pSTAT5⁺ cells and a higher pSTAT5 staining intensity after IT Treg depletion, strongly indicating direct IL-2 signaling within NK cells in tumors (Fig. 3.6I). In contrast, we noted no significant pSTAT5 increases in NK cells from the draining LN or the spleen, providing evidence that these effector cells are activated by IL-2 locally within the tumor following IT Treg ablation (fig. 3.S5D). The percentages of pSTAT5⁺ NK cells were also significantly increased after IT Treg ablation in MHC I⁺ RMA-H60a tumors (fig. 3.S5E).

Following the observation of a tumor-specific activation of NK cells, we carried out experiments in which tumor bearing mice were treated with Fingolimod/FTY720 just before IT Treg ablation to try to block the egress of immune cells from lymphoid tissues (fig. 3.S5F). Importantly, FTY720 did not impair tumor control after IT Treg ablation (fig. 3.S5G), in marked

contrast to another report examining anti-tumor CD4⁺ T cell responses against MHC I⁺ tumors after systemic Treg depletion (Binnewies, Mujal et al. 2019). As expected, FTY720 treatment substantially decreased the numbers of CD4⁺ and CD8⁺ T cells in the blood (fig. 3.S5H). These data argue that the local IT Treg ablation did not impact tumor control by recruiting T cells from lymph nodes or spleen to tumors. However, FTY720 did not decrease NK cell numbers in the blood, consistent with published reports that the egress of NK cells from lymphoid tissues is largely independent of SIP receptor 1 (S1P₁) (Jenne, Enders et al. 2009), the target of FTY720 (fig. 3.S5H). Furthermore, NK cell numbers were not decreased in the tumor, but NK cells did exhibit increased activation in tumors after IT Treg ablation, as they did in mice not treated with FTY720 (fig. 3.S5I, J). These results, in combination with our data demonstrating that pSTAT5 levels were elevated in tumor NK cells (Fig 3.6I), but not in spleen and dLN NK cells (fig. 3.S5D), support the conclusion that activation of immune cells *in situ* within tumors promotes tumor control by NK cells after IT Treg ablation.

To determine the source of IL-2 production following IT Treg ablation, we performed intracellular cytokine staining of immune cells from tumors by preparing single cell suspensions from tumors directly in Golgi-blockers, and without additional *ex vivo* stimulation, to capture IL-2 production occurring *in vivo*. This revealed that the production of IL-2 from CD4⁺ T_{conv} cells increased with IT Treg ablation (Fig. 3.6J). CD8⁺ T cells also produced IL-2, but the percentage of IL-2⁺ CD8⁺ T cells did not increase with IT Treg depletion, and the MFI levels were significantly lower than in CD4⁺ T cells (fig. 3.S6A,B).

Importantly, analysis of the aforementioned mice lacking all cDCs (*Zbtb46^{DTR}*) or cDC2s (*Zeb^{3Δ}*), demonstrated that the increased IL-2 production from CD4⁺ T_{conv} cells (Fig. 3.6K), as well as increased CD69 and KLRG1 expression on tumor NK cells after IT Treg ablation (Fig. 3.6L-M), depended on cDC2s. Taken together, these data suggest that cDC2s are necessary to stimulate CD4⁺ T_{conv} cell production of IL-2, as well as for elevating activation marker expression on NK cells upon IT Treg ablation.

IL-2 availability induced by Treg depletion is required for antitumor NK responses

Analysis of genes differentially expressed in NK cells after IT DT treatment identified the cytokine pathway “IL-2/STAT5 Signaling,” which we confirmed to be enhanced by pSTAT5 staining (Fig. 3.6I, fig. 3.S5A). To test the contribution of IL-2 to tumor control, mice with either MC38-*B2m*^{-/-} or B16-F10-*B2m*^{-/-} tumors were treated with an IL-2 neutralizing antibody. IL-2 neutralization completely or largely prevented IT Treg ablation NK cell-mediated tumor control (Fig. 3.7A,B, fig. 3.S6C,D). In addition, IL-2 neutralization prevented the induction of CD25, SCA-1, granzyme B, and the acquisition of a mature CD11b⁺CD27⁻ phenotype by NK cells (Fig. 3.7C-F). In addition to IL-2, the cytokine pathway “TNF- α signaling via NF- κ B” was also significantly enriched (fig. 3.S5A, B). While multiple pathways in addition to TNF- α , e.g. IL-18 and NK activating receptors, can signal through NF- κ B to regulate NK cell activation, cytotoxicity, and effector function, only TNF- α can be produced by CD4⁺ T_{conv} cells, and therefore fits with the defined requirement for CD4⁺ T_{conv} cells (Wiedemann, Santosa et al. 2021, Pedde, Kim et al. 2024). To test the importance of TNF- α in promoting NK cell tumor control, we used TNF- α neutralizing antibodies and found that this did not prevent tumor control with IT Treg ablation (Fig. 3.7G). Furthermore, as with anti-TNF- α blockade, anti-IFN- γ blockade did not prevent tumor control with IT Treg ablation (Fig 3.7H). Overall, these data suggested that IL-2 signaling, but not TNF- α or IFN- γ signaling, is required for NK cell-dependent tumor control after IT Treg ablation.

If IT Treg ablation resulted in the increased availability of IL-2, which activated the antitumor NK cell response, we reasoned that excess IL-2 alone may drive tumor control even in the presence of Treg_s. Administration of half-life extended IL-2 (IL-2 fused to mouse serum albumin (IL-2-MSA) (Momin, Mehta et al. 2019, Wolf, Blaj et al. 2022) promoted the control of MC38-*B2m*^{-/-} tumors without IT Treg ablation (Fig. 3.7I, fig. 3.S6E). However, the depletion of NK cells prevented the tumor control elicited by IL-2-MSA, confirming that increased IL-2 mobilized NK cell-mediated antitumor activity (Fig. 3.7I, fig. 3.6E).

To test whether IL-2 could replace the requirement for CD4⁺ T_{conv} cells in the control of tumors with IT Treg ablation, we supplemented mice with IL-2-MSA when CD4⁺ T cells were depleted with IT Treg depletion. Indeed, supplementation of IL-2-MSA restored NK cell-mediated tumor control in the absence of CD8⁺ or CD4⁺ T cells (Fig. 3.7J, fig. 3.S6F). Together, these data,

along with the evidence of an activated IL-2 signaling pathway in NK cells, argue that after IT-Treg ablation, cDC2s stimulate CD4⁺ T_{conv} cells to produce IL-2 that acts directly on NK cells to drive NK-mediated tumor control, highlighting an important axis of cell interactions unleashed by local IT Treg ablation.

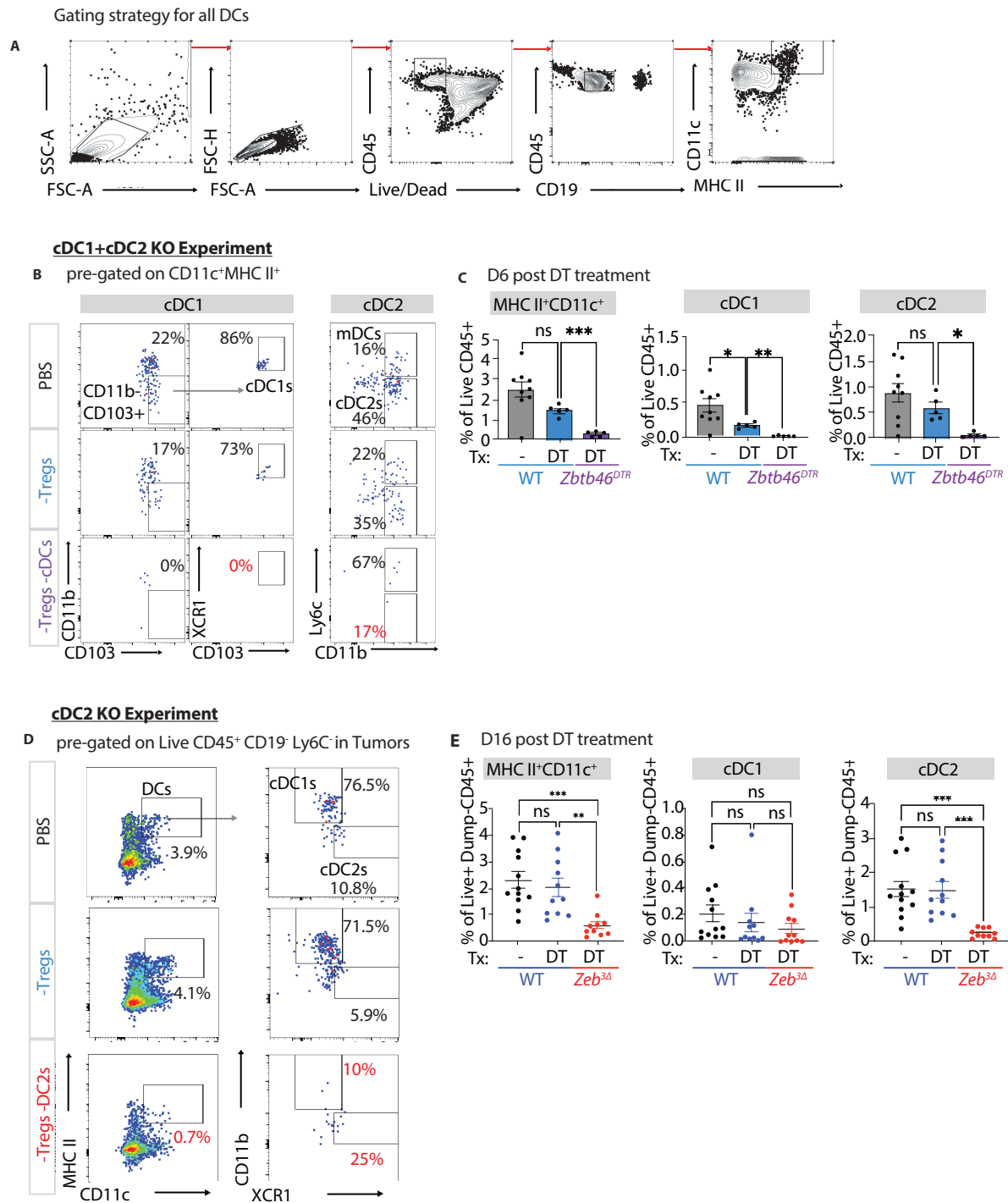


Figure 3.S4 (associated with Fig 6): Bone marrow chimera validation and DC phenotyping

Figure 3.S4 (associated with Fig 6): Bone marrow chimera validation and DC phenotyping

- (A) Representative flow gating strategy to characterize dendritic cells.
- (B) Representative flow gating strategy for *Foxp3^{DTR-GFP}* and *Zbtb46^{DTR};Foxp3^{DTR-GFP}* to characterize dendritic cells, showing the reduction in bulk DCs in mutant mice shown in panel C.
- (C) The bar graphs show CD11c⁺MHCII⁺ DCs in *Foxp3^{DTR-GFP}* and *Zbtb46^{DTR};Foxp3^{DTR-GFP}* as percentages of CD45⁺Live cells, cDC1s (XCR1⁺CD103⁺CD11b⁻) and DC2s (CD11b⁺Ly6C⁺) in each treatment group with corresponding bar graphs pre-gated on Live⁺CD45⁺CD19⁻MHCII⁺CD11c⁺ as shown in S6A. One experiment is shown of two performed.
- (D) Representative flow gating strategy for *Foxp3^{DTR-GFP}* and *Zeb3^Δ;Foxp3^{DTR-GFP}* to characterize dendritic cells, and showing the reduction in bulk DCs and cDC2s in mutant mice as shown in panel E.
- (E) The bar graphs show CD11c⁺MHCII⁺ DCs in *Foxp3^{DTR-GFP}* and *Zeb3^Δ;Foxp3^{DTR-GFP}* as percentages of CD45⁺Live, DC1s (XCR1⁺CD11b⁻) and DC2s (XCR1⁻CD11b⁺) in each treatment group with corresponding bar graphs pre-gated on Live⁺CD45⁺CD19⁻Ly6C⁻MHCII⁺CD11c⁺ as shown in ext. data Fig 4e. Representative data for 2 independent experiments are shown.

Data represent means $\pm$ SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 from C, E: Ordinary one-way ANOVA with Dunnett's multiple comparisons tests

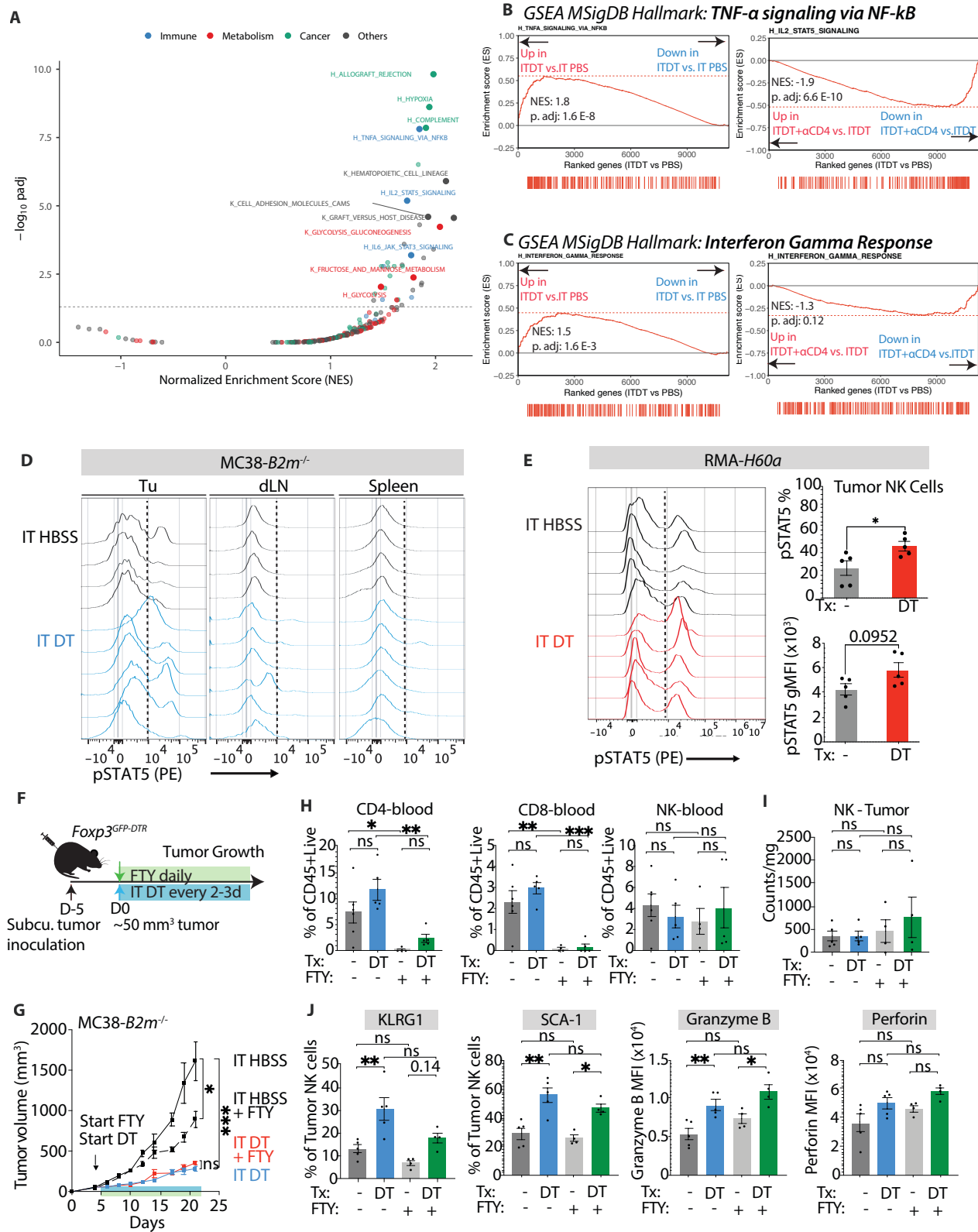


Figure 3.S5: Local priming and activation mechanism

Figure 3.S5: Local priming and activation mechanism

(A) Scatter plot depicting top 20 MSigDB Hallmark and KEGG pathways as determined by normalized enrichment scores (NES) calculated by GSEA on list of genes upregulated in ITDT vs. PBS NK cells. Bars are colored according to BH-adjusted p-value (purple < 0.05; red 0.05–0.1; black 0.1–0.25; gray 0.25). Pathways are annotated by functional category.

(B-C) GSEA plots for Hallmark TNF-alpha signaling and IFN-gamma signaling pathways on list of genes upregulated in ITDT versus PBS and upregulated in ITDT_aCD4 vs. ITDT respectively. Normalized enrichment score (NES) and BH-adjusted p-value are labeled in plot.

(D) Phosphorylation of STAT5 on tumor, dLN and spleen NK cells, comparing mice treated with either IT HBSS (control; black) or IT DT (Treg deplete; blue), taken 6 days after treatment. Representative staining of two experiments shown.

(E) Phosphorylation of STAT5 on tumor NK cells, comparing mice with RMA-*H60a* tumors, treated with either IT HBSS (control; black) or IT DT (Treg deplete; red/blue), taken 6 days after treatment. Quantification of percent pSTAT5 positive NK cells and pSTAT5 gMFI are shown.

(F) Schematic outlining FTY treatment that starts at the same time of IT DT, 5 days after mice were inoculated with MC38-*B2m*^{-/-} tumors.

(G) Growth curves of MC38-*B2m*^{-/-} tumors in mice that were IT depleted of Treg_s (blue) or IT depleted of both Treg_s and treated with FTY (red). Control groups in mice were treated with IT HBSS (black solid) or IT HBSS with FTY (black dotted) are shown. The results shown are from one experiment performed.

(H) Frequencies of CD4, CD8, and NK cells in circulation one week after FTY treatment.

(I) NK cell counts per mg of tumor at takedown (D16 post IT DT and FTY).

(J) Activation marker (KLRG1, SCA-1, Granzyme B and Perforin) expression on NK cells for each group as described in (b) are shown. Statistical test with Ordinary one-way ANOVA with Dunnett's multiple comparisons tests shown.

Data represent means ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001

G: Statistical test with two-way ANOVA with Greenhouse Geiser correction shown. All comparisons are performed pairwise against the DT group with into account time as a factor. Statistic shown reflect total column (treatment) effect.

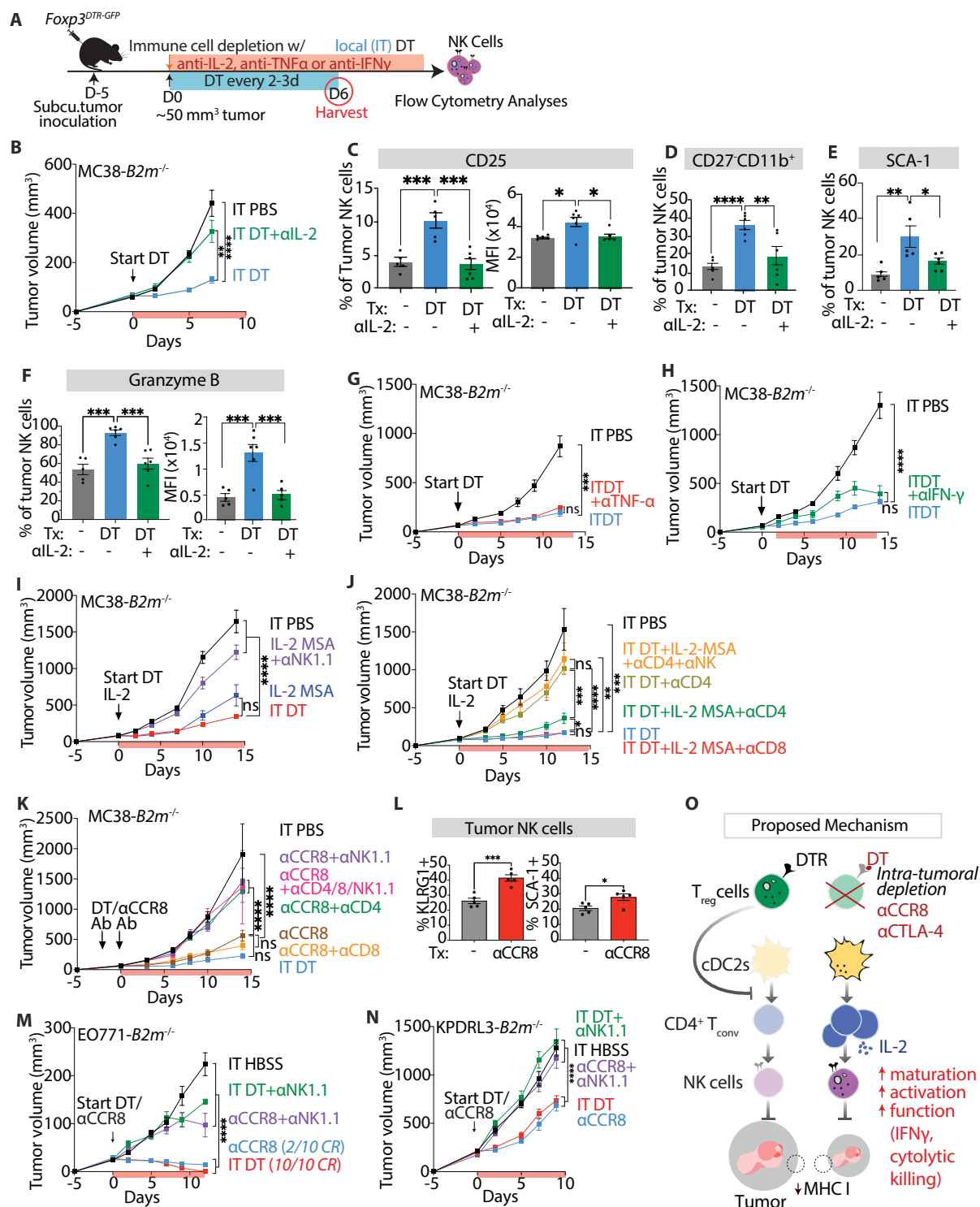


Figure 3.7: IL-2 signaling is essential for tumor control after IT-Treg ablation

Figure 3.7: IL-2 signaling is essential for tumor control after IT-Treg ablation

(A) Schematic of MC38-*B2m*^{-/-} tumor inoculation and treatment regimen in *Foxp3*^{DTR-GFP} mice.

Tumors were resected at day 6 after IT-DT for processing to obtain NK cells from various conditions for phenotypic analysis.

(B) Growth curves of MC38-*B2m*^{-/-} tumors in mice that were intra-tumorally depleted of Tregs (blue) in combination with anti-IL-2 (JES6-1) (green), or IT PBS (black). Representative of two independent experiments. B16F10-*B2m*^{-/-} tumors show similar trend (ext data Fig 6c).

(C) Growth curves of MC38-*B2m*^{-/-} tumors in mice that were intra-tumorally depleted of Tregs (blue) in combination with anti-TNFα (TN3-19.12) (red), or IT PBS (black). Representative of two independent experiments.

(D) Growth curves of MC38-*B2m*^{-/-} tumors in mice that were intra-tumorally depleted of Tregs (blue) in combination with anti-IFNγ (XMG1.2) (green), or IT PBS (black). Representative of two independent experiments.

(E) Percentages and MFI of CD25⁺ cells among tumoral NK cells in mice bearing MC38-*B2m*^{-/-} tumors that were treated with DT IT 6 days previously, without (blue) or in combination with anti-IL-2 (JES6-1) treatments (green). Control (grey) mice were not Treg depleted (IT PBS). Representative of 3 independent experiments.

(F-H) CD27⁺CD11b⁺ (mature phenotype), SCA-1 and Granzyme B staining of tumoral NK cells in the treatment groups described in (C). Percentages or mean fluorescence intensities are shown. Representative of four independent experiments.

(I) Growth curves of MC38-*B2m*^{-/-} tumors in mice with intact Tregs that were injected with IL-2-MSA or depleted of NK cells before treatment with IL-2-MSA, in comparison to IT PBS (control). IT-Treg deleted mice are shown for comparison (red). Representative of 3 independent experiments.

(J) Growth curves of MC38-*B2m*^{-/-} tumors in IT-Treg depleted mice (blue), that were also depleted of CD4 cells (gold), or CD4-depleted and supplemented with IL-2-MSA (green), or the latter treatments combined with NK depletion (orange). Control mice in which Tregs were not depleted and treated with IT PBS are shown for comparison (black). Representative of 3 independent experiments.

(K) Growth curves of MC38-*B2m*^{-/-} tumors in mice with treated with CCR8 antibody, combined with various cellular depletions. One representative experiment of three is shown.

(L-M) Growth curves of orthotopic tumor models EO771-*B2m*^{-/-} and KPDR3-*B2m*^{-/-} tumors in mice with treated with IT DT or CCR8 antibody, combined with NK cellular depletions with PK136. One representative experiment of two for each tumor model is shown.

(N) KLRG1 and SCA-1 staining of tumoral NK cells in PBS or anti-CCR8 treated mice.

(N) Model of NK cell activation and tumor control with IT Treg ablation.

Data represent means $\pm$ SEM; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

B, C, D, I, J, K, M, N: RM two-way ANOVA followed by Geiser-Greenhouse correction. (n=5-8 mice/group). All comparisons are performed pairwise against the DT group with into account time as a factor. Statistic shown reflect total column (treatment) effect.

E, F, G, H, L: Ordinary one-way ANOVA with Dunnett's multiple comparisons tests.

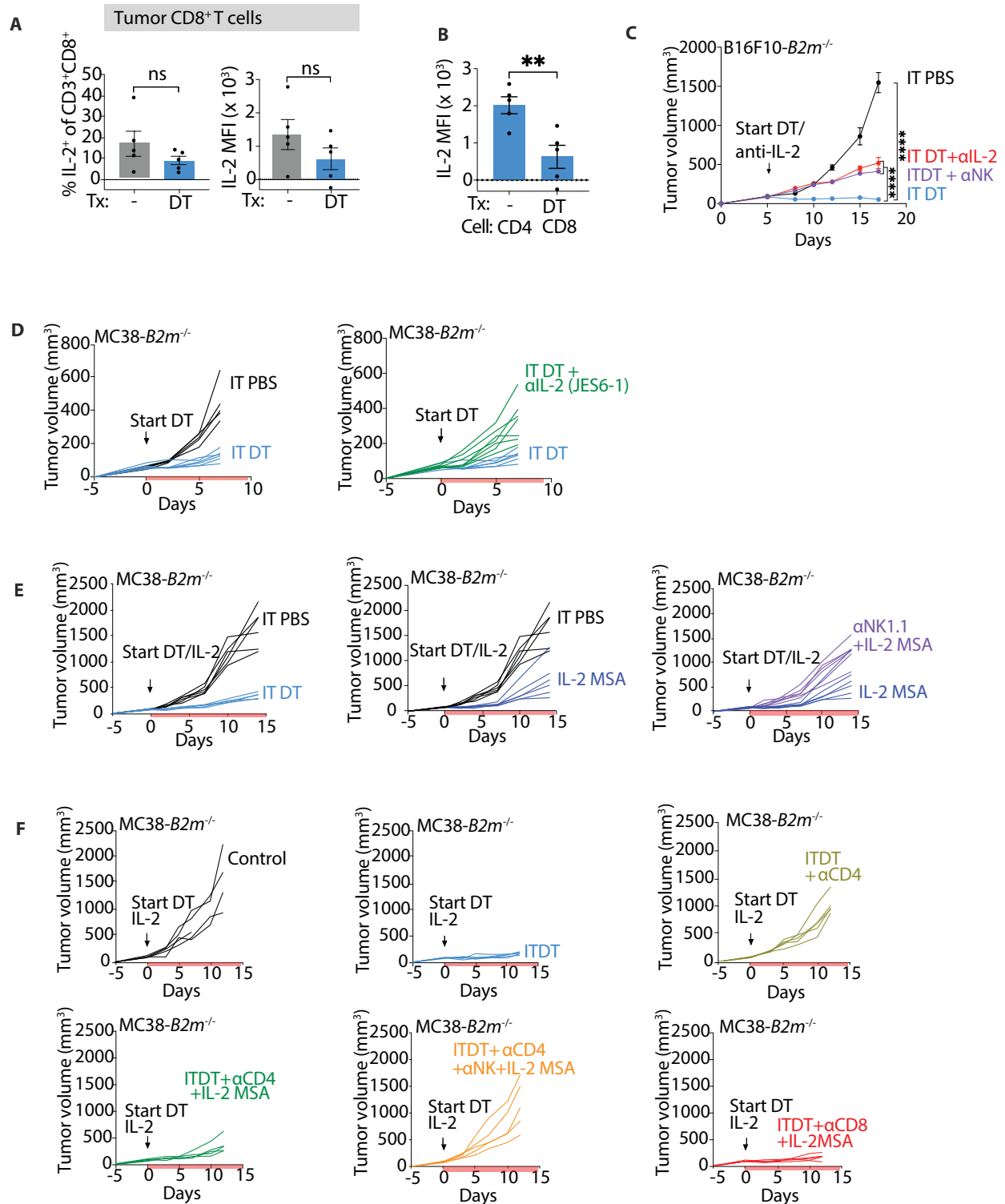


Figure 3.S6 (associated with Fig 7): Tumor growth dependence on cytokines

Figure 3.S6 (associated with Fig 7): Tumor growth dependence on cytokines

(A) Intracellular staining of IL-2 in tumoral CD8⁺ T cells (CD3⁺CD8⁺) 6 days after treating MC38-*B2m*^{-/-} tumor-bearing mice with IT-DT (blue) or IT PBS (grey). Percent of IL-2⁺ CD8⁺ T cells and gMFI of IL-2 are shown. Representative of 2 independent experiments.

(B) Comparison of IL-2 MFI between CD4 and CD8⁺ T cells following IT Treg ablation.

(C) Tumor growth curves of *Foxp3*^{DTR-GFP} mice that were depleted of Tregs intra-tumorally with IT DT injections (blue), or IT DT in combination with IP administration of anti-IL-2 mAb JES6 (red), or with anti-IL-2+anti-NK1.1 (purple). One experiment was performed with B16F10-*B2m*^{-/-} tumors.

(D-F) Spider plots of tumor growth shown in Figure 7.

Data represent means $\pm$ SEM; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001.

B: RM two-way ANOVA followed by Geiser-Greenhouse correction. All comparisons are performed pairwise against the DT group with into account time as a factor. Statistic shown reflect total column (treatment) effect.

Clinically applicable intra-tumoral Treg depleting antibodies promote tumor control dependent on NK cells and CD4⁺ T_{conv} cells.

Studies have demonstrated that certain systemic antibody treatments in mice, including anti-CTLA4 and anti-CCR8, can selectively deplete IT Treg_s without depleting Treg_s systemically throughout the body (Selby, Engelhardt et al. 2013, Simpson, Li et al. 2013, Villarreal, L'Huillier et al. 2023). To extend these findings to MHC I-deficient tumors, we assessed CCR8 expression on IT Treg_s and confirmed earlier findings that it is highly elevated on Treg_s infiltrating MHC I-deficient tumors compared to those in the spleen (Plitas, Konopacki et al. 2016, Lax, Palmeri et al. 2023) (fig. 3.S7A). Anti-CCR8 treatment significantly decreased IT Treg percentages without decreasing splenic Treg percentages (fig. 3.S7B). Most importantly, systemic administration of anti-CTLA4 or anti-CCR8 resulted in control of established MHC I-deficient tumors, comparable to the impact of IT DT treatments in *Foxp3^{DTR-GFP}* mice (Fig. 3.7K, fig. 3.S7C-E). Similar to our findings with depletion of IT Treg_s in *Foxp3^{DTR-GFP}* mice, tumor control elicited with anti-CCR8 antibodies depended on NK cells and CD4⁺ T_{conv} cells, but not CD8⁺ T cells (Fig. 3.7K). In addition, similar to MC38-*B2m*^{-/-} tumors treated with IT DT, Treg depletion with anti-CCR8 resulted in increased NK cell activation as manifested by elevated expression of KLRG1 and SCA-1 (Fig. 3.7L).

To bolster the physiological relevance of our findings, we further tested the impact of IT Treg ablation in two orthotopic cancer models with DT treatment in *Foxp3^{DTR-GFP}* mice (administered IT) or CCR8 antibody depletion in normal mice (administered IP). To do so, we generated MHC I-deficient variants of the B6 EO771 mammary tumor cell line (Naour, Koffi et al. 2020) and the B6 sarcoma cell line KPDRL3 (Thompson, Jackson et al. 2018), which we derived from the *Kras-p53* genetically engineered mouse model of cancer (fig. 3.S7F). To establish orthotopic models, EO771-*B2m*^{-/-} tumor cells were inoculated in the mammary fat pad, whereas KPDRL3-*B2m*^{-/-} tumor cells were inoculated in the calf muscle. In tumor-bearing *Foxp3^{DTR-GFP}* mice treated IT with DT, substantial tumor control occurred in both tumor models (Fig. 3.7M-N). Similarly, substantial tumor control occurred in both models when tumor-bearing B6 mice were depleted of IT Treg_s by injecting anti-CCR8 systemically in wildtype mice (Fig. 3.7M-N). Tumor control after Treg depletion resulting from both IT DT and anti-CCR8 treatments was NK cell-dependent, as the administration of anti-NK1.1 reversed tumor control (Fig. 3.7M-N). Therefore,

clinically applicable strategies using systemically infused antibodies to selectively deplete intra-tumoral Treg_s can lead to NK cell-dependent control of MHC I-deficient orthotopic tumors.

Since both CD4⁺ T_{conv} cells and NK cells played significant roles in the tumor control following IT Treg ablation, we considered the possible role in the response of invariant NK T cells, which recognize a lipid antigen presented by CD1d (Benlagha, Weiss et al. 2000, Gapin, Matsuda et al. 2001, Borg, Wun et al. 2007). Notably, however, CD1d is *B2m*-dependent and therefore absent from the surface of *B2m*^{-/-} tumor cells. Nevertheless, using CD1d tetramer staining to detect NK T cells, IT Treg depletion did not change the frequencies of CD4⁺CD1d-specific T cells in either the tumors or spleens of mice after intra-tumoral Treg depletion (fig. 3.S8A). We also examined a possible role of ILC1 cells in the response, since they also express NK1.1. Analysis of tumoral NK1.1⁺CD3⁻ cells showed that nearly all of these cells were EOMES⁺, consistent with an NK cell identity (fig. 3.S8B-C). Examination of the EOMES^{low} NK1.1⁺ cells revealed few were CD49a⁺CD49b⁻, which is consistent with an ILC1 identity (fig. 3.S8B-C). These data suggests that the vast majority of NK1.1⁺CD3⁻ cells in the tumors were NK cells, suggesting that NK cells play the major role in tumor rejection.

Based on our findings, Tregs can regulate effective antitumor NK cell responses against MHC I-deficient tumors that are resistant to most immunotherapies that are CD8⁺ T cell-based. In addition, IT Treg depletion can mobilize NK cells even against MHC I⁺ tumors that express high levels of NKG2D ligands. Our data argue that intra-tumoral Treg depletion enables CD4⁺ T_{conv} cells to produce IL-2 in a manner that is dependent on cDC2s, which then acts on NK cells locally within tumors to promote their cytotoxic function against tumor cells, thus highlighting an important cDC2-CD4-IL2 axis that activates NK cells within the tumor microenvironment (Fig. 3.7O).

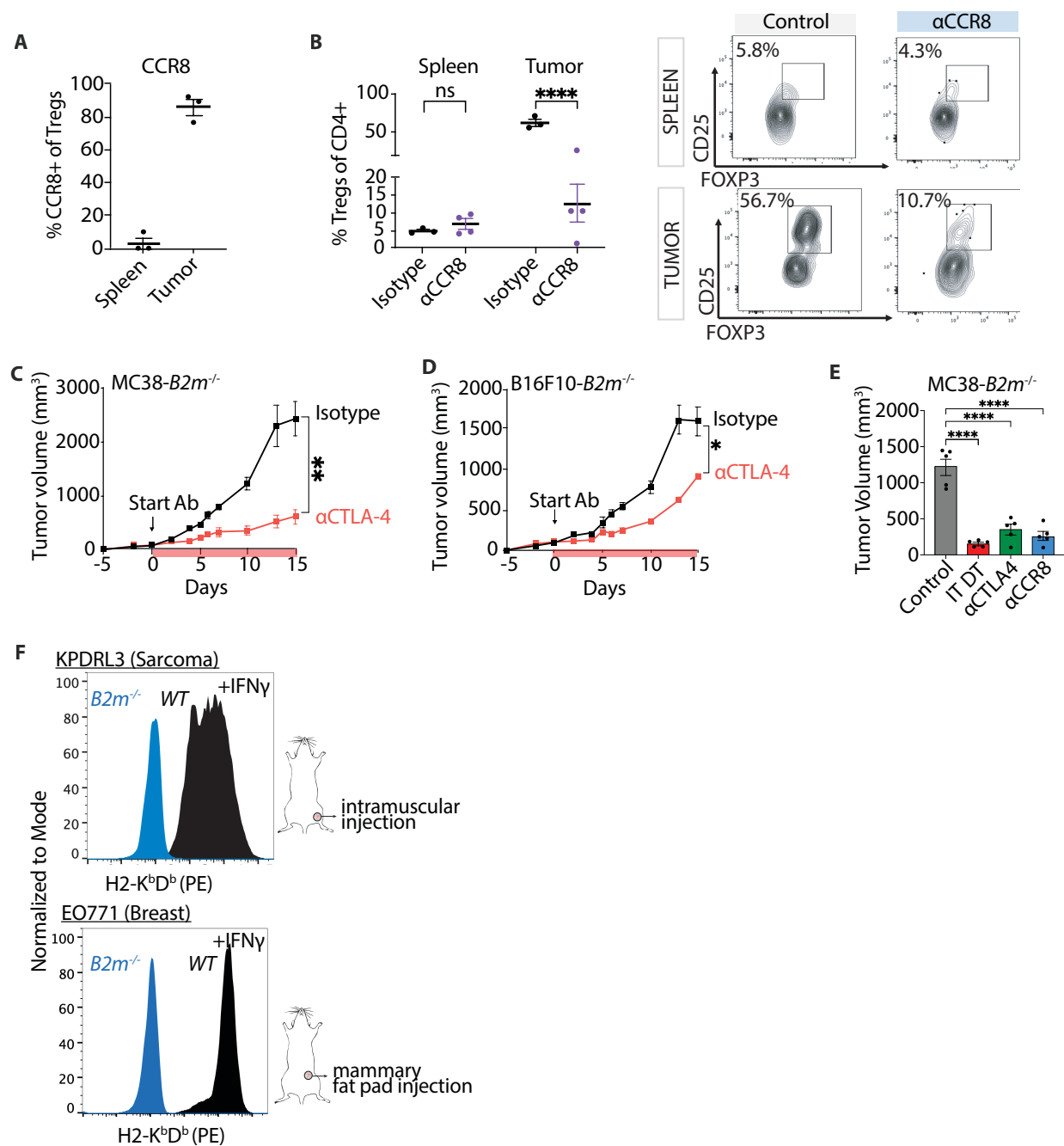


Figure 3.S7: Alternatives to intra-tumoral Treg ablation and orthotopic tumor models

Figure 3.S7: Alternatives to intra-tumoral Treg ablation and orthotopic tumor models

(A) Expression of CCR8 on Tregs in the spleen and tumor of MC38-*B2m*^{-/-} tumor-bearing mice.

One experiment performed.

(B) Representative flow plots and quantified frequencies of Tregs in spleen and tumor of mice treated with isotype control and anti-CCR8 (same experiment as in (a))

(C-D) Growth curves of MC38-*B2m*^{-/-} and B16F10-*B2m*^{-/-} tumors in mice treated with isotype control IgG or CTLA-4 antibody. Representative data from 4 (MC38-*B2m*^{-/-}) or 2 (B16F10-*B2m*^{-/-}) experiments performed.

(E) Bar plots comparing tumor volume at day 15 after different methods of IT Treg ablation in MC38-*B2m*^{-/-} tumors in mice.

(F) Histograms showing MHC I (K^b/D^b) staining on tumor cell lines KPDRL3 and EO771, comparing WT to *B2m*^{-/-} counterparts after overnight 10ng/ml IFN- γ stimulation.

Data represent means $\pm$ SEM; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001

B: Welch's t Tests

C-D: RM two-way ANOVA followed by Geiser-Greenhouse correction. (n=5-10 mice/group)

E: Ordinary one-way ANOVA with Dunnett's multiple comparisons tests.

DISCUSSION

Based on our findings, Treg_s can regulate effective antitumor NK cell responses against MHC I-deficient tumors that are resistant to most immunotherapies that are CD8⁺ T cell-based. In the present study, we demonstrated that depleting Treg_s locally within tumors can control multiple tumor types that were deficient for MHC I. IT Treg ablation also elicited tumor control without autoimmune pathologies associated with systemic Treg depletion. Control of MHC I-deficient tumors required NK cells and CD4⁺ T_{conv} cells. Importantly, local Treg depletion also sensitized MHC I⁺ tumors to NK cell killing if tumors highly expressed NK activating ligands. *In vivo* cellular depletions combined with bulk RNA sequencing and phenotypic and functional analyses of tumor infiltrating NK cells *ex vivo* suggested that a major contribution of CD4⁺ T_{conv} cells to the NK cell response was their production of IL-2, which activated NK cell functions directly within tumors. The co-depletion of CD4⁺ T_{conv} cells with Treg_s prevented NK cells from upregulating markers of activation and maturation, increasing expression of granzymes and perforin, and acquiring cytotoxic activity. Whereas conventional dendritic cells (cDCs) were essential for the NK cell-mediated antitumor response, cDC1s were dispensable while cDC2s were necessary for tumor control. These effects were shown to be independent of additional recruitment of lymphocytes from lymph nodes or spleen, and highly localized within the tumor. Thus, we propose that IT Treg depletion unleashes cDC2-mediated CD4⁺ T_{conv} cell IL-2 production while preventing IL-2 sequestration by IT Treg_s; the increased available IL-2 acts on NK cells in tumors to promote their maturation into cytotoxic killers of tumor cells, thus highlighting an important cDC2-CD4-IL2 axis that activates NK cells within the tumor microenvironment (Fig. 3.70).

Previously, the impact of regulatory T cell (Treg) depletion has been studied extensively in various contexts—in homeostasis (Shimizu, Yamazaki et al. 1999, Kim, Rasmussen et al. 2007, Gasteiger, Hemmers et al. 2013), autoimmune disease (Lund, Hsing et al. 2008), during viral infections (Klages, Mayer et al. 2010), and in the setting of cancer (Ghiringhelli, Menard et al. 2006, Klages, Mayer et al. 2010, Selby, Engelhardt et al. 2013, Joshi, Akama-Garren et al. 2015, Arce Vargas, Furness et al. 2017, Wang, Quiros et al. 2018, Caudana, Nunez et al. 2019, Moreno Ayala, Li et al. 2019, Nunez, Tosello Boari et al. 2020, Dees, Ganesan et al. 2021, McRitchie and Akkaya 2022, Moreno Ayala, Campbell et al. 2023, Bockman, Gittins et al. 2025). However, the focus has primarily been on Treg suppression of CD8⁺ T cell responses, or how suppression of

CD4⁺ T_{conv} cells or APCs diminishes CD8⁺ T cell responses (Chen, Pittet et al. 2005, Oderup, Cederbom et al. 2006, Kastenmuller, Gasteiger et al. 2011). There are in fact reports in the literature where CD8⁺ T cells were critical for mediating therapy-induced rejection of MHC I-deficient tumors (Lerner, Woroniecka et al. 2023, Walsh, Stump et al. 2023), but here we ruled out the involvement of CD8⁺ T cells, as we also did in the context of STING agonist therapy (Wolf, Blaj et al. 2022). Studies that have investigated the impact of Treg depletion on NK cell responses have done so primarily in homeostatic conditions (Gasteiger, Hemmers et al. 2013, Gasteiger, Hemmers et al. 2013). In those studies, activated NK cells killed MHC-deficient normal cells *in vitro* whereas our findings show NK-mediated rejection of established tumors *in vivo*, including MHC-deficient tumors and MHC I⁺ tumors that express NKG2D ligands. These earlier studies also did not explore the critical role of DCs in the NK response, which we show is dependent on cDC2s. Finally, none of these previous studies addressed the impact of local, IT Treg depletion on modulating the NK cell anti-tumor response *in vivo*. Because MHC I-deficient tumors that are resistant to CD8⁺ T cell recognition arise frequently, especially in the context of checkpoint blockade cancer immunotherapy, our findings suggest that tumor-localized Treg depletion may provide therapeutic benefit beyond checkpoint blockade by promoting NK cell responses that eliminate MHC I-deficient cancer cells. We acknowledge that while MHC I-deficient tumor models are valuable for dissecting mechanisms of Treg-mediated suppression of CD4⁺ T_{conv}/NK cell activation, human tumors often express a more nuanced and heterogenous pattern of MHC I loss. Treg depletion in human samples is also exceptionally rare, and immunotherapies such as anti-CTLA-4 (ipilimumab) only deplete intra-tumoral Treg_s to a small extent or not at all (Sharma, Subudhi et al. 2019), making spatially resolved samples from IT Treg ablated tumors unavailable for immune interaction analyses. To investigate the potential of direct cell–cell interactions within the heterogeneous tumor microenvironment, and in the context of human tumors that typically display a mixed MHC I phenotype, we analyzed human melanoma samples from a recently published high-plex cyclic immunofluorescence (CyCIF) method for three-dimensional tissue imaging (Yapp, Nirmal et al. 2025). Tumor image sections were stratified based on MHC I expression, and key immune and stromal cell markers were identified from the available antibody panel (fig. 3.S9A). For each section, we quantified cell-type abundance and measured spatial interactions between selected immune subsets. Notably, regions with low MHC I expression contained fewer CD8⁺ T cells but a higher frequency of NK cells, together with more frequent NK–

DC and NK–CD4 T cell contacts (fig. 3.S9B). These findings provide a preliminary view of coordinated immune topology in human melanoma, but much additional analysis will be necessary to corroborate our findings in human cancer.

Our approach of using local Treg depletion within tumors is promising as an immunotherapy given that it avoids lethal pathology and autoimmunity that have been shown to accompany systemic Treg ablation (Kim, Rasmussen et al. 2007). Systemically administered antibodies, such as anti-CTLA4 and anti-CCR8, selectively deplete IT Treg_s in mice, and our results show that both treatments were indeed effective against MHC I-deficient tumors. Dissecting the impact of anti-CTLA-4 treatments is complicated by the capacity of these treatments to block inhibitory signaling in effector T cells, as well as poor evidence that Ipilimumab depletes IT Treg_s in human cancer patients (Leach, Krummel et al. 1996, Romano, Kusio-Kobialka et al. 2015, Sharma, Subudhi et al. 2019, Dees, Ganesan et al. 2021). CCR8 antibody administration, in contrast, appears to function more exclusively by depleting Treg_s within tumors (Weaver, Stack et al. 2022, Villarreal, L'Huillier et al. 2023). Importantly, local Treg ablation in tumors with IT DT or anti-CCR8 was not associated with detectable adverse pathologies. The efficacy of IT Treg depletion is suggestive of local immune cell modulation. Indeed, we found that increased IL-2 signaling appears to be restricted to NK cells in tumors because they exhibited significantly increased STAT5 phosphorylation compared to NK cells in the draining LN and the spleen.

Our data suggest that a major function of CD4⁺ T_{conv} cells in the antitumor response to MHC I-deficient tumors is to provide IL-2 for NK cells. Indeed, we found that IL-2-MSA injections could replace the requirement for CD4⁺ T_{conv} cells in mediating tumor control with IT Treg depletion. Thus, our data support the hypothesis that IT Treg_s suppress antitumor NK cells locally by acting as an IL-2 sink (Gasteiger, Hemmers et al. 2013), inhibiting IL-2 production by CD4⁺ T_{conv} cells, or both. IL-2 is known to promote NK cell activation, cytotoxic function, and proliferation (Robertson and Ritz 1990, Cooper, Fehniger et al. 2001, Waldmann 2006). We previously reported that an IL-2 superkine elicits NK-dependent therapeutic efficacy against MHC I-deficient tumor cells at a stage when NK cell desensitization was established *in vivo* (Ardolino, Azimi et al. 2014, Wolf, Blaj et al. 2022). Thus, the excess IL-2 associated with Treg depletion in

our studies may promote tumor rejection in part by reversing or delaying NK cell desensitization. In addition, we noted that neutralizing IL-2 also reduced the expression of CD25 on intra-tumoral NK cells, but it remains possible that the role of IL-2 in inducing or sustaining CD25 expression on NK cells is indirect or requires additional signals *in vivo*, such as IL-18, IL-12 and/or NK receptor activation (fig 3.S10A). Indeed, a high dose of IL-2 alone failed to induce CD25 on purified splenic NK cells, but adding IL-2 to IL-12 or IL-18 or in conjunction with anti-NK1.1 stimulation did result in increased CD25, suggesting that IL-2 may enhance or sustain CD25 expression induced by other cytokines or NK receptor stimuli (fig. 3.S10A). We further observe a strong correlation of genes upregulated in the IL-2/STAT5 GSEA pathway between the NK receptor stimulated- and IL-18-stimulated NK cell datasets. While the source of NK cells differs significantly between tumor NK cells versus *in vitro* stimulated purified NK cells, genes upregulated in NK cells from Treg-deplete tumors appeared to be more similar to IL-18-stimulated NK cells (fig. S10B). As such, corroborating with *in vitro* stimulation assays in fig 3.S10A, other cytokines such as IL-18 can also drive strong IL-2 signaling in NK cells.

Although exogenous IL-2-MSA could replace CD4⁺ T_{conv} cells in promoting tumor control with IT Treg ablation, additional IL-2-independent mechanisms whereby CD4⁺ T cells promote NK cell responses may exist. Regardless, these results bolster the evidence that IL-2 or IL-2 superkines can exert potent antitumor effects alone or in combination with other immunotherapies against both MHC I-deficient and MHC I⁺ tumors (Ardolino, Azimi et al. 2014, Pouxvielh, Marotel et al. 2024). Interestingly, we found that IL-2-MSA injections stimulated NK cell-dependent antitumor responses even in the presence of Treg_s. While these data are consistent with the possibility that Treg_s suppress NK cell responses solely by regulating IL-2 availability, it remains possible that Treg_s utilize additional mechanisms to suppress NK cell responses (Trzonkowski, Szmit et al. 2004, Dasgupta, Bhattacharya-Chatterjee et al. 2005, Ghiringhelli, Menard et al. 2005, Ghiringhelli, Menard et al. 2006, Sarhan, Hippen et al. 2018). Thus, further investigation of whether Treg_s directly suppress NK cell activation independently of CD4⁺ T_{conv} cells is warranted.

We observed an essential role of cDCs in the antitumor response following IT Treg ablation. The results go further in demonstrating that cDC2s and not cDC1s are required, which aligns with previous studies showing that cDC2s activate CD4⁺ T_{conv} cells across various types of

immune responses (Binnewies, Mujal et al. 2019). Specifically, it was previously shown that subsets of cDC2s can traffic from tumor to tdLN, present tumor-derived antigens to CD4⁺ T_{conv} cells and that, following Treg depletion, the frequencies of these cDC2s and their capacity to elicit strong CD4⁺ T_{conv} responses against tumors were increased (Binnewies, Mujal et al. 2019, Chudnovskiy, Castro et al. 2024). Here we show that cDC2s can also influence antitumor NK cell responses directly within tumors. While no major changes were observed in the bulk population of cDC2s with IT Treg ablation in this study, results reported in a separate study show changes in cDCs when looking specifically at cDCs that had endocytosed tumor antigens (Bockman, Gittins et al. 2025). Whereas Treg_s may suppress cDC2 functions per se, they may also act to inhibit CD4⁺ T_{conv} cells directly or disrupt productive interactions of CD4⁺ T_{conv} cells with cDC2s, either by directly interacting with these cells or via suppressive cytokines.

In conclusion, our findings open new avenues for cancer immunotherapy by revealing the potential to mobilize NK cells and CD4⁺ T_{conv} cells against CD8⁺ T cell-resistant cancers following localized, intra-tumoral depletion of Treg_s. Because Treg depletion can also drive immune responses against MHC I⁺ tumors, this strategy may effectively complement current checkpoint blockade immunotherapies that drive tumor resistance to CD8⁺ T cell responses. The fact that IT Treg depletion was sufficient to drive tumor control without inciting systemic autoimmunity provides a strong foundation for developing more effective and broadly applicable IT Treg-depleting strategies for treating cancer.

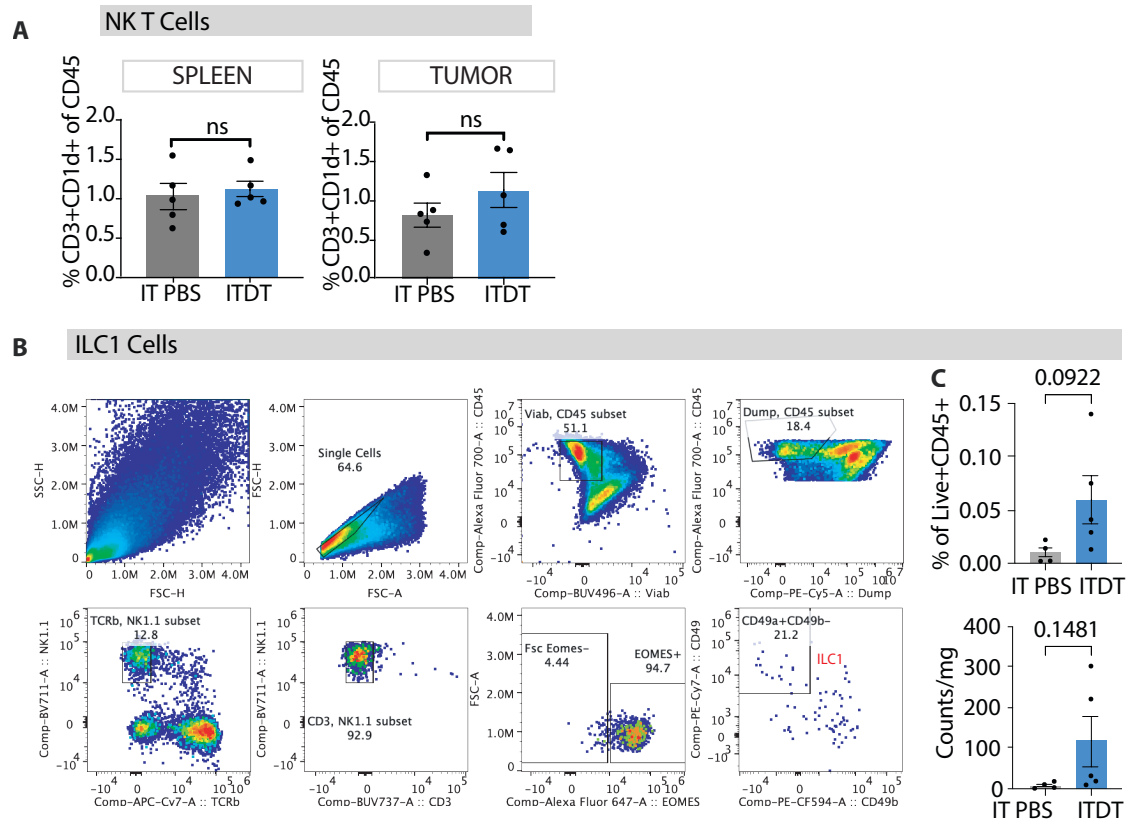


Figure 3.S8: ILC1 cells and invariant NK1.1⁺ T cells in tumors and spleens

(A) Percentages of invariant NKT cells detected by staining with α -galactosylceramide loaded CD1d tetramers (gated as CD3⁺Tetramer⁺) in spleens and tumors of IT DT treated mice compared to PBS-treated controls. Invariant NKT cells were rare in the tumors.

Note: recognition of CD1d on these tumor cells does not occur because functional CD1d expression is B2-microglobulin-dependent.

(B-C) Gating strategy, percentages and counts of ILC1 cells (gated as viable CD45⁺ NK1.1⁺Tcrb⁻ CD3^eEOMES⁻CD49a⁺CD49b⁻) in tumors of mice treated with PBS or IT DT.

Data represent means $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$

A, C: Welch's t-tests. (n=5-8 mice/group, one experiment)

A Yapp *et. al* Nat. Methods PMID: 41023436

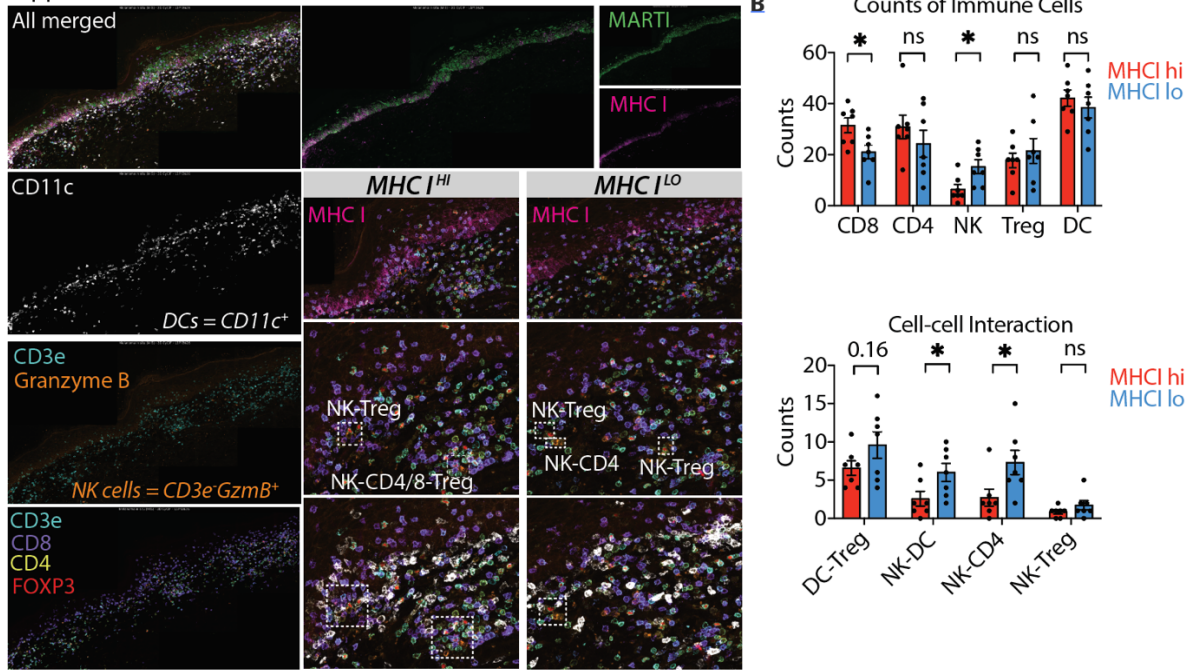


Figure 3.S9: Human Melanoma Tumor Sections with varying MHC I Expression

(A) Selected confocal images from PMID:41023436 human Stage IIC, anatomic level IV malignant melanoma, showing cell types of interest and tumor marker (MART I, green) as well as MHC I (pink). Color code of IHC markers: Magenta=CD4, Yellow=CD3e, Green= GZMB, Blue=MHC I, White=CD11c, Cyan = CD11b, Red=FOXP3; NK cells are marked as CD3⁺GZMB⁺

(B) Absolute counts of each cell type and interactions between cell types as defined in (A) from 7 representative areas in two melanoma sections. Welch's t Test performed.

Data represent means $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$

Chapter 4: Treg Depletion Promotes Expansion of Cytotoxic CD4⁺ T cells

Abstract

Regulatory T cells (Tregs) are essential for maintaining immune homeostasis but simultaneously act as a key suppressive barrier against effective antitumor activity. Their depletion in tumor-bearing hosts profoundly remodels the immune landscape, leading to unrestrained expansion of effector populations and the emergence of cytotoxic CD4⁺ T cells. These cells express transcription factors such as T-bet, Eomesodermin (EOMES), and Runx3 and acquire potent cytolytic activity characterized by granzyme B, perforin and IFN- γ production. In this section, we explore how Treg depletion reconfigures the CD4⁺ Tconv cell compartment, driving an expansion of the cytotoxic CD4⁺ T cell subset. These mechanisms parallel findings observed with deletion of *Bcl11b*^{-/-} or *Dnmt1*^{-/-} or inhibition of *Ezh2*, where CD4⁺ T cells reprogrammed into NK cell-like cytotoxic cells. Collectively, these studies reveal that beneath their helper identity, CD4⁺ T cells possess an intrinsic cytotoxic potential that can be unmasked when suppressive or transcriptional constraints are lifted, providing new insights into adaptive plasticity and avenues to optimize immunotherapy.

Introduction

To date, cancer immunotherapy approaches have been heavily reliant on targeting CD8⁺ T cell responses, such as via the blockade of immune checkpoints like anti-PD-1. However, cancer cells employ multiple immune evasion strategies to evade recognition and killing, and one specific mechanism involves the downregulation or mutations in MHC I molecules, which are essential for antigen recognition by CD8⁺ T cells. As such, there is a need to mobilize different immune cell subsets and overcome various challenges in current immunotherapy approaches to develop more effective anti-tumor strategies. In Chapter 3, we outlined the strong NK cell-mediated tumor control of these CD8⁺ T cell-evaded, MHC I-deficient tumor models. CD4⁺ T cell effector functions have been heavily focused on their role in cytokine production and in boosting other effector cell, such as CD8⁺ T cells, macrophages or B cell functions. Until recently, accumulating evidence demonstrated the critical role CD4⁺ T cells can play in antitumor immunity (Sutmoller, van Duivenvoorde et al. 2001, Quezada, Peggs et al. 2008, Quezada, Simpson et al. 2010, Simpson, Li et al. 2013, Sledzinska, Vila de Mucha et al. 2020). Here, we investigate the role of CD4⁺ T cells beyond their conventional role of providing essential help to other immune subsets and explore the potential of their independent cytotoxic capabilities in controlling tumor progression.

Cytotoxic CD4⁺ T cells are identified by their expression of cytotoxic molecules such as granzyme B, perforin, and Fas ligand, traditionally associated with CD8⁺ T cells, although additional surface markers and transcription factors have also been associated with these cells that do not have a direct link to the cytolytic activity. Cytotoxic CD4⁺ T cells often display distinct transcriptional profiles characterized by upregulation of T-bet, Eomesodermin (EOMES), and BLIMP-1 (Quezada, Simpson et al. 2010, Oh, Kwek et al. 2020, Sledzinska, Vila de Mucha et al. 2020, Oh and Fong 2021), which supports their cytolytic programming. This population was most commonly described in chronic viral infections in mice and humans, such as in mice infected with lymphocytic choriomeningitis virus and gamma-herpes virus, and patients with human immunodeficiency virus (Sun, Ghanekar et al. 2001, Soghoian, Jessen et al. 2012), cytomegalovirus (Leeuwen, Remmerswaal et al. 2004, Saez-Borderias, Guma et al. 2006), Epstein-Barr Virus (EBV) (Khanna 1997, Long, Haigh et al. 2005), as well as with acute viral infections, such as influenza (Brown, Lee et al. 2012).

Importantly, the presence of cytotoxic CD4⁺ T cells in tumors correlates with improved clinical outcomes in some cancer types, highlighting their potential as targets for immunotherapy. Under steady-state conditions, FOXP3⁺ Tregs maintain immune tolerance by limiting effector T cell activation and restraining inflammatory cytokines. In the tumor microenvironment (TME), however, this suppression hampers effective antitumor immunity. Genetic or antibody-mediated Treg depletion, for example in *Foxp3*^{DTR} or anti-CD25/anti-CTLA-4 settings, leads to rapid expansion of antigen-specific CD4⁺ T cells, many of which adopt atypical effector programs. It was further shown by multiple groups that the anti-tumor function of cytotoxic CD4⁺ T cells was enhanced with the depletion of Tregs with anti-CTLA-4 (Sledzinska, Vila de Mucha et al. 2020), or shown with increased killing of target cells with co-cultures comparing CD4⁺ TILs from the same patient that were depleted or not of Tregs (Oh, Kwek et al. 2020). In human bladder tumors, Oh *et al.* further described heterogeneous states of cytotoxic CD4⁺ T cells differing in their expression of GZMB, GZMK, PRF1 as well as GNLY and NKG7. In tumors expressing MHC II, these CD4⁺ CTLs can recognize and lyse antigen-bearing malignant cells directly, acting in synergy with CD8⁺ and NK cells to curb tumor growth (Quezada et al., 2008; Sitrin et al., 2013).

CD4⁺ T cells contribute to anti-tumor immunity not only through perforin-granzyme-mediated killing but also via their production of effector cytokines, most notably, interferon-gamma (IFN- γ) and tumor necrosis factor-alpha (TNF- α). Upon activation in the tumor microenvironment, CD4⁺ T cells secrete IFN- γ , a cytokine that exerts potent antitumor activity by inducing apoptosis, senescence, and growth arrest in susceptible tumor cells, while also promoting antigen presentation and modulating the local immune milieu. Recent studies, including the work of Boulch *et al.*, have underscored that the effectiveness of CD4⁺ CAR T cell therapy depends critically on the intrinsic sensitivity of tumors to the pro-apoptotic effects of IFN- γ (Boulch, Cazaux et al. 2021, Boulch, Cazaux et al. 2023, Boulch, Cazaux et al. 2023). In both murine and humanized models, CD4⁺ CAR T cells were shown to eradicate tumors not merely through direct cytolytic activity, but importantly by releasing IFN- γ , which acts in a paracrine manner to kill both antigen-positive and antigen-loss variants within the tumor mass. Complementing the action of IFN- γ , TNF- α can also synergize to promote tumor cell death via activation of death receptors and enhancement of apoptotic signaling pathways (Josephs, Ichim et al. 2018). This dual action of

CD4⁺ T cell-derived IFN- γ and TNF- α represents a powerful, non-canonical mechanism of tumor control, highlighting how the tumor's own susceptibility to cytokine-induced apoptosis can become a major determinant of immunotherapeutic efficacy. It is, however, important to also acknowledge that the sensitivity of tumor cells to IFN- γ and TNF- α is highly variable and depends on the tumor type, its genetic alterations, and the tumor cell's intrinsic sensitivity to cytostatic or pro-apoptotic effects of these cytokines. Tumor cells have been shown to develop resistance to IFN- γ or TNF- α via mutations to JAK/STAT signaling pathway, defects in the cytokine receptors, as well as in upregulating anti-apoptotic molecules which activate NF- κ B survival signaling (Kearney, Vervoort et al. 2018). As such, evaluating IFN- γ and TNF- α pathway responsiveness in tumors is emerging as an important consideration for predicting and optimizing responses to T cell-based immunotherapies.

Further, work by Peng Li *et. al* revealed that deletion of *Bcl11b*, a transcription factor indispensable for T lineage fidelity, can reprogram conventional T cells into NK-like cytotoxic cells capable of tumor killing (Li, Burke et al. 2010). The expression of the zinc finger transcription factor BCL11B is recognized as one of the earliest checkpoints in T cell development in mice (Ikawa, Hirose et al. 2010, Li, Leid et al. 2010). Genetic deletion of *Bcl11b* in developing T cells inhibited commitment to the T cell lineage. Under conditions that should have stimulated T lineage differentiation, *Bcl11b*-deficient T cell progenitors failed to up-regulate genes associated with lineage-committed T cells and maintained stem cell- and progenitor cell-associated gene expression. In both developing and committed T cells, loss of *Bcl11b* resulted in the generation of cells that resembled natural killer (NK) cells in both phenotype and function. These NK-like cells could be expanded easily *in vitro* and possessed antitumor cytotoxicity, but they did not exhibit cytotoxicity against normal cells and were not tumorigenic. Follow-up studies by Rui Liao *et. al.* revealed how BCL11B cooperates with the Nucleosome Remodeling and Deacetylase (NuRD) complex to maintain T-cell identity by repressing a mitochondrial-metabolic program that predisposes T cells to reprogramming (Liao, Wu et al. 2023). Specifically, BCL11B and the NuRD components CHD4, MTA2, and MBD2 together silence OPA1, a key regulator of mitochondrial fusion. Loss of either BCL11B or NuRD components derepresses OPA1, driving excessive mitochondrial fusion and elevated oxidative phosphorylation (OXPHOS). This metabolic shift enhances acetyl-CoA levels, which in turn promotes histone H3K27 acetylation at NK

lineage-associated genes including *Id2*, *Zbtb16* (PLZF), and *Znf683* (Hobit). The result is an epigenetic–metabolic feedback loop that reprograms T cells toward an NK-like state with elevated expression of cytotoxic effectors and NK receptors.

In this light, the conversion of CD4⁺ T cells into cytotoxic effectors following Treg depletion mirrors the mechanistic principles uncovered in these reprogramming systems, where cytokine-driven environmental cues and intrinsic transcriptional control may jointly determine the emergence of cytotoxic competence from within the canonical T cell pool. Understanding these processes not only informs the design of adjunct Treg-modulating strategies but also redefines the hierarchy of antitumor effector subsets, placing CD4⁺ cytotoxic T cells as active contributors to tumor surveillance and potential therapeutic targets for the treatment of cancer.

RESULTS

Intra-tumoral depletion of Tregs leads to NK cell-independent control of MHC I-deficient tumors

We employed three different C57BL/6 (B6) MHC I-deficient tumor models that were engineered by CRISPR-Cas9-mediated disruption of the beta-2-microglobulin (*B2m*) gene: the MC38-*B2m*^{-/-} (colon carcinoma), B16F10-*B2m*^{-/-} (melanoma) and RMA-*B2m*^{-/-} (lymphoma) cell lines. We depleted Tregs using *Foxp3*^{DTR-GFP} mice, wherein diphtheria toxin (DT) injection leads to Treg apoptosis. Mice bearing subcutaneous tumors of each type at a size of ~50 mm³ (five days after inoculation) began receiving DT every other day by IT injection. IT DT treatments resulted in tumor control even when animals were depleted of NK cells using anti-NK1.1 (PK136). Especially in B16F10-*B2m*^{-/-} and RMA-*B2m*^{-/-}, the NK cell-dependent component only contributed to an intermediate level of tumor control, while the depletion of CD4⁺ T cells completely eradicated tumor control (Figure 4.1). While we showed in Chapter 3 that intra-tumoral Treg depletion can unleash NK cell responses to MHC I-deficient tumors, the role of NK cell-independent tumor control was not investigated in these models. Thus, IT Treg ablation can not only drive an NK cell-mediated tumor control, but in some tumor models, IT Treg ablation also causes potent NK cell-independent, CD4⁺ T cell-mediated tumor control.

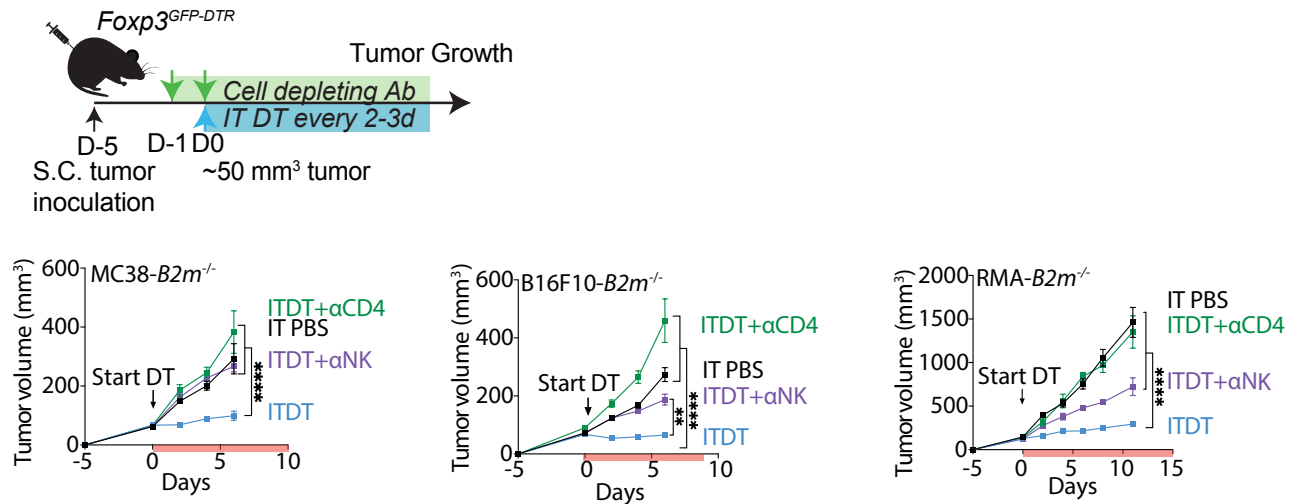


Figure 4.1: NK cell-independent tumor control across MHC I-deficient tumors.

Schematic of tumor inoculation and treatment regimen in *Foxp3^{DTR-GFP}* mice depleted of Tregs locally (IT). DT was administered every 48-72 hours, while cell-depleting antibodies were administered on D1, D0 and every six days thereafter. Control mice were treated with IT PBS to account for local inflammation at injection sites. Growth curves of various *B2m^{-/-}* tumors in mice that were systemically depleted of Tregs alone (red) or in combination with depletion of all CD4⁺ T cells (green) or NK cells (purple). Data are representative of 2-3 experiments for each tumor type. Data represent means ± SEM; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001 from RM two-way ANOVA followed by Geisser-Greenhouse correction.

Intra-tumoral depletion of Tregs leads to global transcriptomic changes in CD4⁺ Tconv cells

To investigate changes in CD4⁺ T cells using an unbiased approach, we performed bulk RNA sequencing analysis of CD4⁺ T cells (DAPI-CD45⁺ CD19-F480-CD3⁺CD4⁺) FACS sorted from MC38-*B2m*^{-/-} tumors in *Foxp3*^{DTR-GFP} mice six days after IT injections of PBS or DT (Figure 4.2). We want to emphasize that in this sequencing run, we did not sort to purify Tregs from the CD4⁺ T cell population, and hence the gene expression data results from a mix of both CD4⁺ Tconv and Treg cells in the group not treated with DT. Differential expression analysis was then carried out using the *limma* linear modeling framework (Ritchie, Phipson et al. 2015), incorporating the batch as a covariate and identifying significantly regulated genes in the PBS vs. ITDT. Volcano plot analysis showed increased expression of several genes associated with activation and cytotoxic status, as well as the de-enrichment of *Foxp3* given the sorting conditions. IT Treg ablation led to a prominent increase in the expression of genes encoding common cytotoxic markers, i.e. numerous granzyme genes (Figure 4.2).

Similar to the NK cell sequencing dataset, principal component analysis (PCA) was performed to assess sample clustering and detect potential batch effects. We overlaid the NK cell sequencing dataset from Chapter 3, and noted a shared transcriptomic landscape in the CD4⁺ T cells and NK cells following IT Treg depletion, as PCA analysis showed a similar shift in the CD4 T cell and NK cell transcriptome associated with IT Treg depletion compared to the PBS control in PC2 and PC3 (Figure 4.2). Performing GSEA enrichment against MSigDB Hallmark and KEGG pathways also revealed an enrichment of genes related to NK cell-mediated cytotoxicity, and the T cell receptor signaling pathway. Interestingly, while expression of the IFN- γ gene itself was strongly upregulated in our dataset, the Hallmark IFN- γ response pathway was among the most de-enriched gene sets, as were the IFN- α and IL-2/STAT5 pathways. In contrast, genes associated with TNF- α responses were positively enriched. This can indicate either cell-intrinsic resistance, negative regulatory feedback or a selective signaling blockade downstream of these cytokines.

To look further at what contributed to the shared NK-CD4 gene signature, we generated GSEA enrichment analyses to compare the genes in our CD4⁺ T cell dataset to the GOBP Natural Killer cell Mediated Immunity dataset (Liberzon, Birger et al. 2015, Castanza, Recla et al. 2023),

and saw an enrichment of genes from IT Treg deplete tumor-derived CD4⁺ T cells to the genes upregulated in activated NK cells (Figure 4.3A). We also further ran our dataset against Li et al.'s *Bcl11b* deletion-induced TNK cells, and saw that DT treatment induced similar upregulated genes in the *Bcl11b* deletion, and that control CD4⁺ T cells registered a concomitant downregulation of genes from *Bcl11b* deleted T cells (Figure 4.3B).

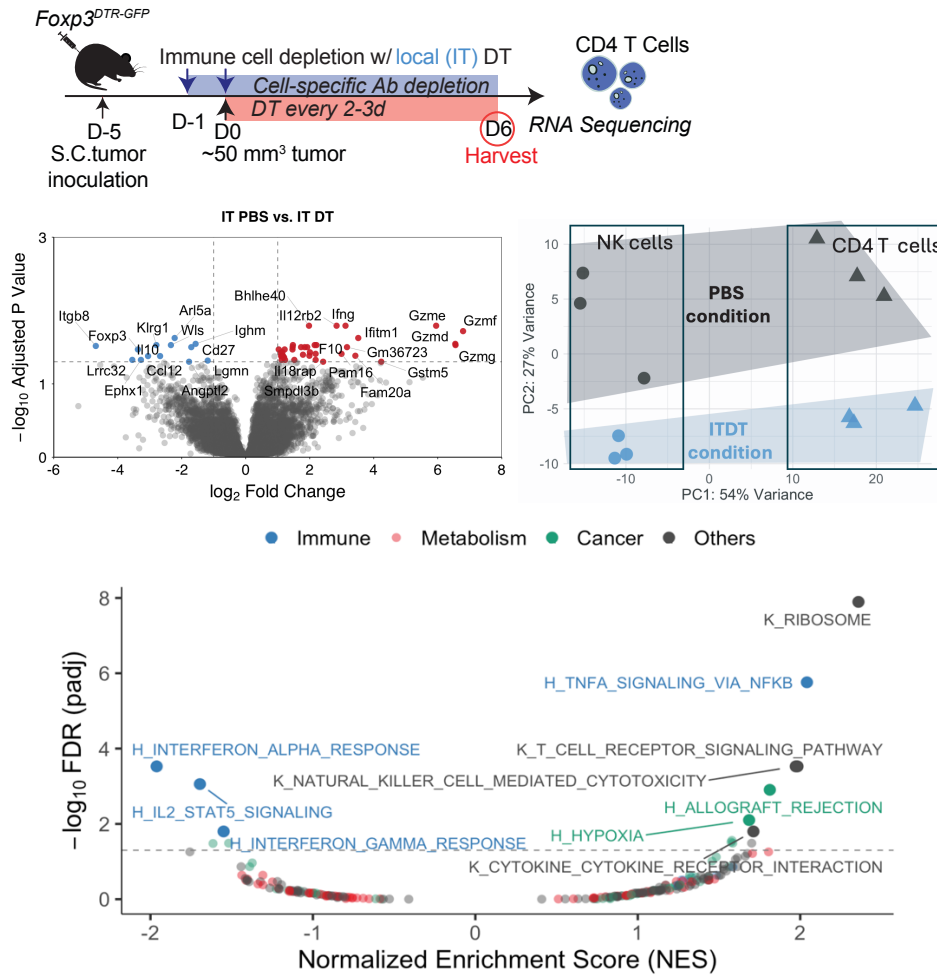


Figure 4.2: Global transcriptomic changes in CD4⁺ T cells following IT-Treg ablation. Schematic of MC38-*B2m*^{-/-} tumor inoculation and treatment regimen in *Foxp3*^{DTR-GFP} mice depleted of Tregs intra-tumorally, before tumor resection 6 days later for downstream processing and sorting to obtain CD4⁺ T cells from IT PBS and IT DT treated tumors. RNA sequencing was performed on three individual replicates, each comprised of sorted cells from a pool of 3-4 tumors. Differential expression analysis was conducted with limma (Methods). Volcano plots show upregulated and downregulated genes in CD4⁺ T cells. Genes were selected as differentially upregulated (red) or downregulated (blue) and highlighted if adjusted p-value < 0.05 and log₂ fold change > 1.5 in absolute value. Principal component analysis (PCA) plot of PC1 vs PC2 for combined NK cell (circles) and CD4⁺ T cell (triangles) RNA-seq datasets, showing shared PC2 between NK and CD4⁺ T cells. Scatter plot depicting top 10 MSigDB Hallmark and KEGG pathways as determined by absolute normalized enrichment scores (NES) calculated by GSEA on list of genes upregulated in ITDT v.s. PBS CD4⁺ T cells. Pathways are annotated by functional category as shown.

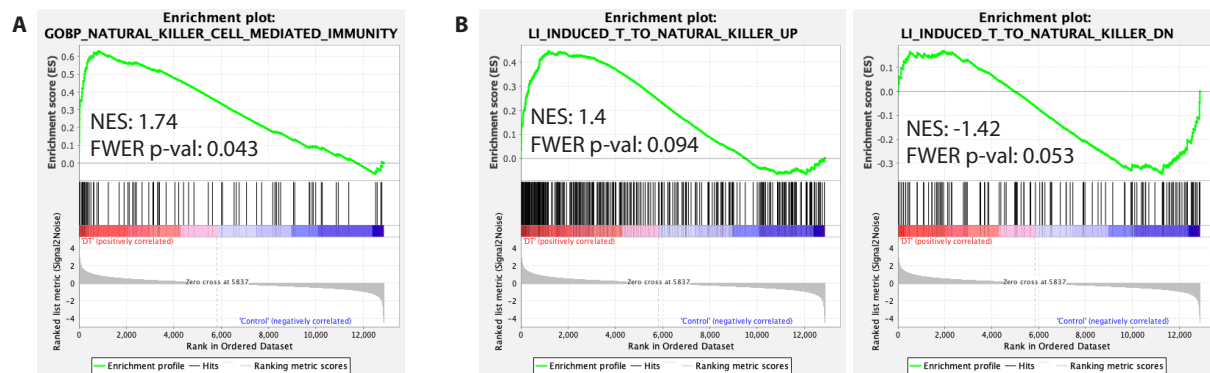


Figure 4.3: GSEA plots for CD4+ T cell datasets. (A) GSEA for GO Biological Processes Natural Killer Cell Mediated Immunity using list of genes upregulated in IT DT versus IT PBS CD4+ T cells. FWER p-value and normalized enrichment score (NES) are labeled in plot. (B) GSEA using list of genes upregulated in IT DT versus IT PBS CD4+ T cells against the published dataset from upregulated and downregulated genes in Bcl11b deletion-induced TNK cells (Li, Burke et al. 2010). FWER p-value and normalized enrichment score (NES) are labeled in plot.

Mechanism of tumor killing by CD4⁺ Tconv cells

CD4⁺ T cells are also known to use other death pathways to result in target cell lysis. CD4⁺ T cells, especially activated or cytotoxic CD4 subsets, have been shown to upregulate FAS ligand (FASL/CD95L) on their surface upon activation. When these T cells encounter target cells expressing FAS, (CD95), FASL binds to FAS, inducing trimerization of FAS. This triggers recruitment of the adaptor protein FADD (Fas-associated death domain), which in turn recruits and activates CASPASE-8, initiating a caspase cascade that results in apoptosis of the target cell. We investigated if there were differences in expression of FAS on CD4⁺ T cells following Treg ablation and observed an increase in FASL transcripts per million (Figure 4.4).

TRAIL (TNF-related apoptosis-inducing ligand) is another death ligand expressed by some activated CD4⁺ T cells. When TRAIL binds to its cognate death receptors, primarily DR5 (also called TRAILR2) on the target cell, it similarly induces receptor trimerization and formation of the death-inducing signaling complex (DISC). This again leads to FADD recruitment and caspase-8 activation, resulting in apoptotic cell death. TRAIL–DR5 killing is particularly relevant in tumor immune surveillance, as many tumor cells upregulate DR5 and become susceptible to TRAIL-mediated lysis. However, there was no change in TRAIL expression on CD4⁺ T cells upon Treg depletion (Figure 4.4).

To test for the roles of death receptor pathways in mediating CD4⁺ T cell tumor control *in vivo*, Elizabeth Andrews and Emily Shaw generated RMA-B2m^{-/-}Fadd^{-/-} tumors in the lab to test if Fas-FasL, TRAIL and/or TNF-driven death receptor pathways contribute to tumor control with IT Treg ablation. Tumors were established in the mice and once tumors were palpable, IT DT treatments were initiated to deplete IT Tregs alone or IT Tregs and NK cell or CD4⁺ T cells as well. In mice with RMA-B2m^{-/-}Fadd^{-/-} tumors, IT Treg depletion still resulted in delayed but strong tumor control compared to RMA-B2m^{-/-}. However, while NK cell-depletion did not affect tumor control, CD4⁺ T cell depletion nearly abolished control back to control levels (Figure 4.5). These results demonstrate that the CD4⁺ T cell-dependent tumor control was likely independent of death receptor-mediated killing by FAS or TNFR engagement, whereas NK cells likely use death receptors, at least to some extent, for controlling tumors following IT Treg depletion.

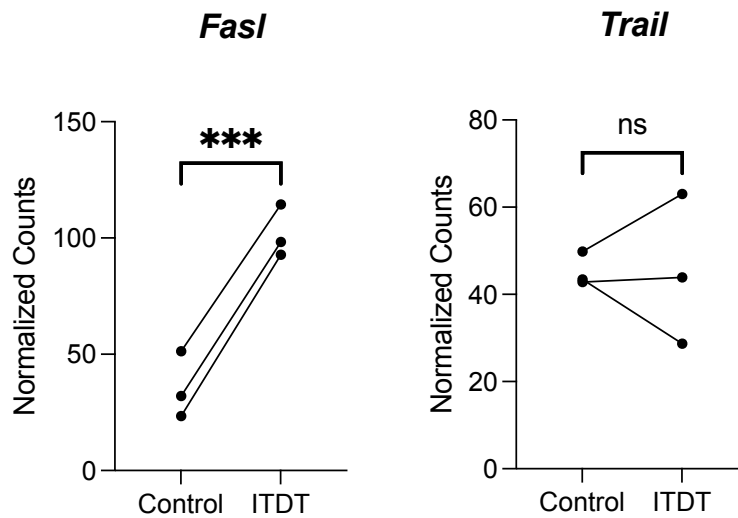


Figure 4.4: Expression of death pathway markers on CD4 Tconv cells following IT Treg ablation in tumors. Normalized transcripts per million of *FasI* and *Trail* in CD4+ Tcells isolated from MC38-*B2m*^{-/-} tumors of various treatment groups 6 days after initiation of DT treatment. Bar graphs are of data pooled from 3 independent experiments sent for bulk RNASeq. Data represent means $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ from Unpaired t test with Welch's correction.

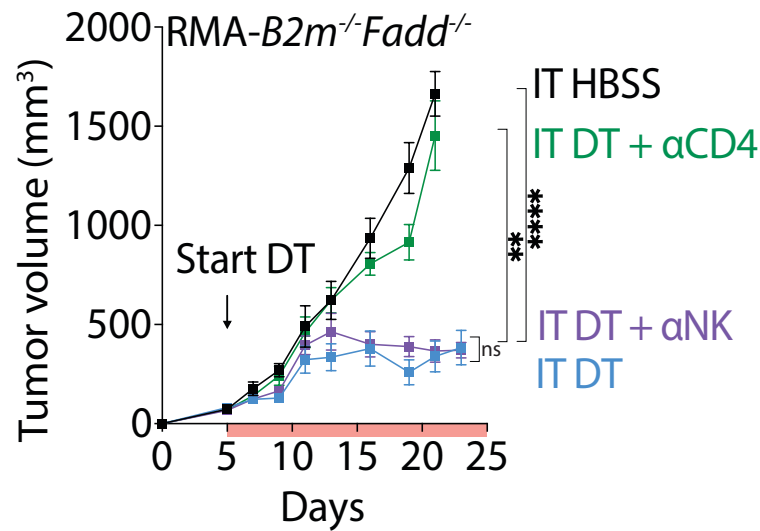


Figure 4.5: CD4⁺ T cell control of tumor is FADD-independent. Growth curves of RMA-*B2m*^{-/-}-*Fadd*^{-/-} tumors in mice that were IT depleted of Tregs (blue) or IT depleted in addition to NK cell-depletion (purple) or with CD4⁺ T cell depletion (green). Control groups in mice were treated with IT PBS (black). Data represent means $\pm$ SEM; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001. RM two-way ANOVA followed by Geiser-Greenhouse correction. (n=5-8 mice/group, representative of 2 independent experiments). All comparisons are performed pairwise against the DT group.

CD4⁺ Tconv cells upregulate cytotoxic and NK cell receptors following IT Treg ablation

To assess the impact of these transcriptomic changes on protein levels and functional activities, we collected MC38-*B2m*^{-/-} tumors at the same timepoint as the RNAseq data (day 6 after IT DT), and performed spectral flow cytometry. Confirming RNAseq data, CD4⁺ Tconv cells from Treg-depleted tumors exhibited significantly higher levels of intracellular granzyme B, perforin, and EOMES (Figure 4.6). The expression of granzyme B, perforin, as well as increased degranulation by CD107A staining, suggests that the CD4⁺ Tconv cells take on an “NK cell-like” cytotoxic phenotype, and might have the capacity to directly kill cancer cells, a function traditionally associated with NK cells and CD8⁺ T cells.

To delve deeper into the shared transcriptomic landscape between the NK cells and CD4⁺ Tconv cells, we examined the RNAseq data with respect to common NK receptor genes, comparing CD4⁺ Tconv from IT PBS vs. IT DT treated tumors (Figure 4.7). The data suggested that with Treg depletion, CD4⁺ T cells had increased expression of genes encoding various NK activating receptors, including *Klrblc* (encoding NK1.1), *Klrkl* (encoding NKG2D), *Klrcl* (encoding CD94), *Klrc2* (encoding NKG2C), *Klrc3* (encoding NKG2E) and *Cd226* (encoding DNAM1). Flow cytometric analysis confirmed increases in the percentages of CD4⁺ Tconv cells with membrane expression of NKG2D, one or more of the NKG2A, C or E molecules and the canonical Nkp46 activating receptor in Treg-depleted tumors. We attempted to co-stain NKG2A (16A11) and NKG2ACE (20D5), but it proved challenging to tease apart NKG2CE levels with this method, potentially attributed to the low levels of expression. When comparing normalized counts per TPM, while NKG2A, NKG2C and NKG2E were all upregulated, NKG2A transcript levels were about 10-fold higher than NKG2C and almost 50-fold higher than NKG2E. NKG2A is an inhibitory receptor with an ITIM motif, while NKG2C and E are activating receptors and associated with DAP12 and contain ITAM motifs. However, unlike NKG2C, NKG2E is largely retained inside the cell, specifically in the endoplasmic reticulum, rather than being expressed on the cell surface, likely due to hydrophobic residues in its extracellular domain. These differences are interesting, and it remains to be investigated if the CD4⁺ Tconv use these receptors to engage and bind to targets, since the tumors are MHC I-deficient but NKG2A-CD94 is known to interact with non-classical MHC I molecules (HLA-E in humans and Qa-1 in mice) (Braud, Allan et al. 1998, Vance, Kraft et al. 1998).

Notably, the degranulation marker CD107a was also significantly increased (Figure 4.7). All staining results of NK cell receptors were also compared to NK cells to confirm positive staining (Figure 4.8) We noted that there was a tumor-specific induction of NK cell receptor expression on the CD4⁺ Tconv cells after IT Treg ablation, since there was no similar induction on CD4⁺ Tconv cells isolated from the spleens of the same animals (Figure 4.8).

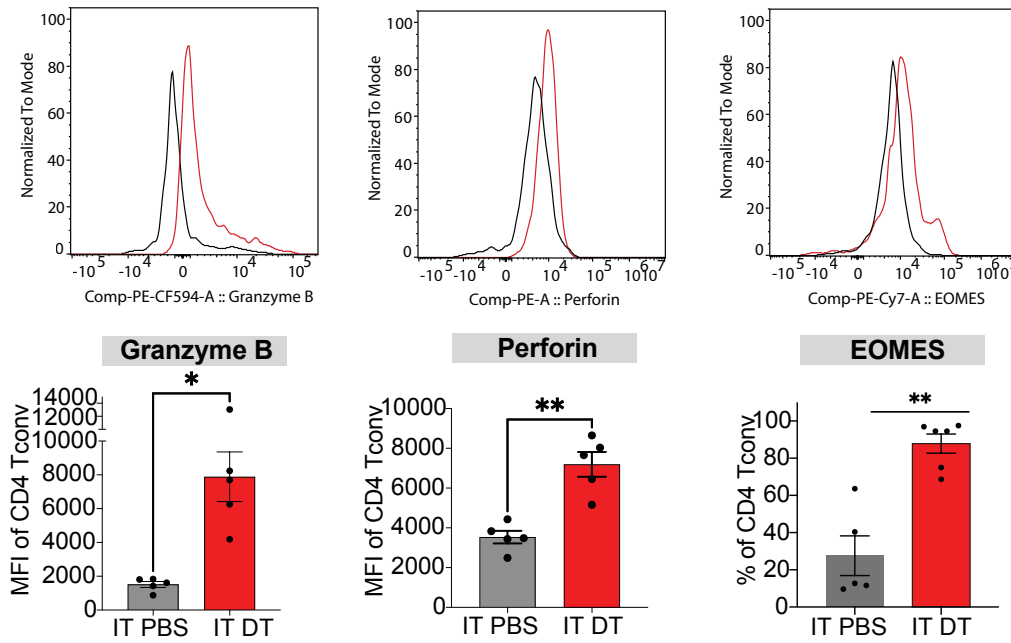


Figure 4.6: Expression of cytotoxic markers on CD4 Tconv cells following IT Treg ablation in tumors. Representative histograms and quantification of MFI of intracellular granzyme B and perforin, and frequencies of EOMES⁺ CD4 Tconv cells in MC38-*B2m*^{-/-} tumors of various treatment groups 6 days after initiation of DT treatments. Bar graphs are of data pooled from 2 independent experiments. Data represent means $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ from Unpaired t test with Welch's correction.

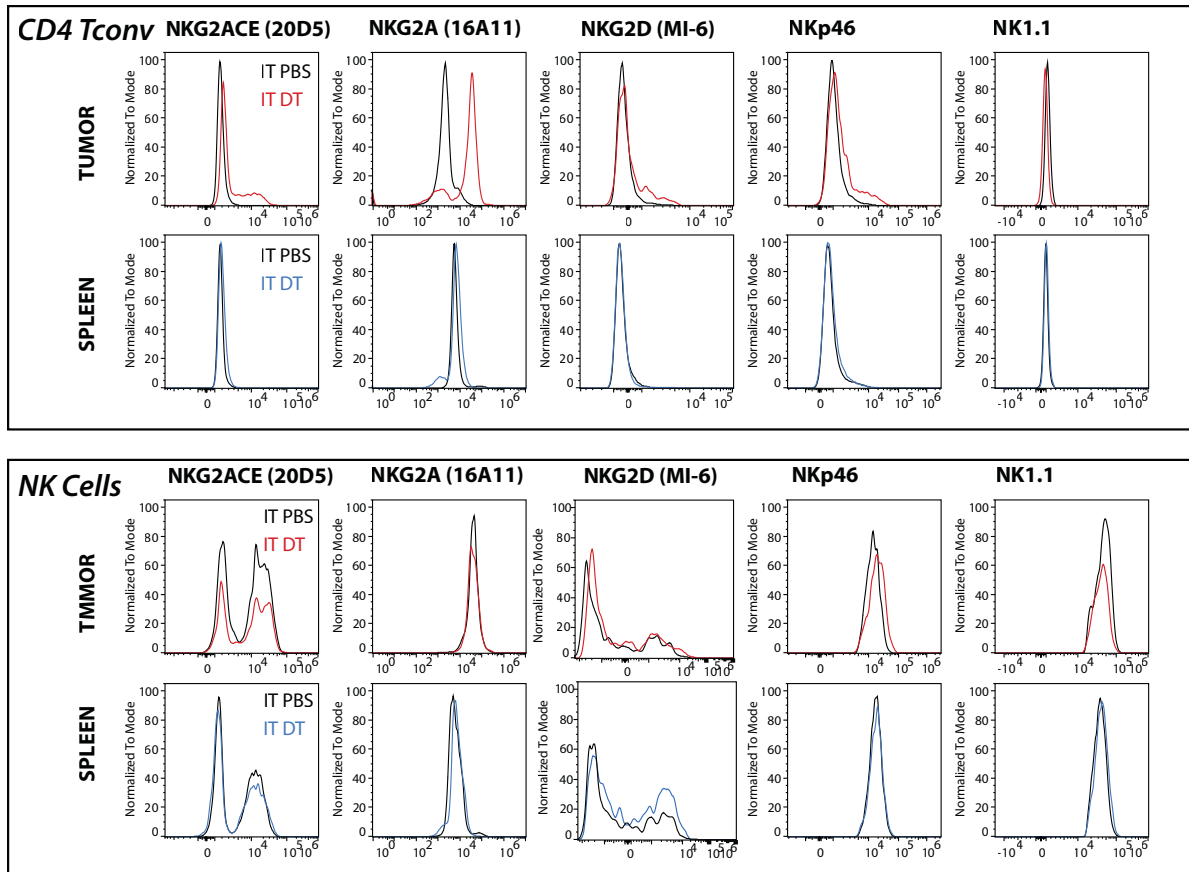


Figure 4.8: Expression of NK cell receptor markers on CD4 Tconv cells following IT Treg ablation in tumors. Representative histograms of NKG2ACE⁺, NKG2A⁺, NKD2D⁺ NKp46⁺ or NK1.1⁺ CD4 Tconv cells in MC38-*B2m*^{-/-} tumors of IT PBS and IT DT 6 days after initiation of DT treatments. Corresponding staining of markers on tumor NK cells are shown below.

CD4⁺ Tconv cells do not use cytotoxic cytokines in anti-tumor responses

Along with cytotoxicity and NK receptor genes, the RNAseq dataset also provided insight into different cytokine pathways, including upregulation in the expression of genes encoding IFN- γ , and IL-21 and receptors for IL-12 and IL-18, in addition to chemokines CCL1, CCL8, CCL24 and CXCL3 among the top upregulated genes. (Figure 4.9). As noted in Chapter 3, experiments using anti-TNF- α and anti-IFN- γ blockade *in vivo* suggested that there was no critical involvement of these cytokines in driving tumor control following Treg ablation (Figure 3.7G,H). Tumors lacking IFN- γ R expression were still controlled with IT Treg ablation, but mice that are *Ifngr1*^{-/-} showed a slight reversal of tumor control, which might indicate that IFN- γ -sensing by the host is necessary for the response, while direct IFN- γ recognition and signaling in tumor cells is not (Figure 4.10).

We plan to repeat these experiments in the context of NK cell-depletions, to confirm whether CD4⁺ T cell-mediated tumor control, as opposed to NK cell-mediated control, is independent of TNF- α and IFN- γ . In addition, intracellular staining and ELISA assays will be performed to test if the CD4⁺ Tconv cells increase their production of any cytokines other than IL-2 following IT Treg ablation, as shown in Chapter 3. It would also be of interest to test co-staining of the cytokine-producing populations to see if there are overlaps with the granzyme- or perforin-expressing CD4⁺ Tconv cell populations. This will inform us if the cytotoxic CD4⁺ T cell compartment is distinct in their killing capacities versus helper phenotypes.

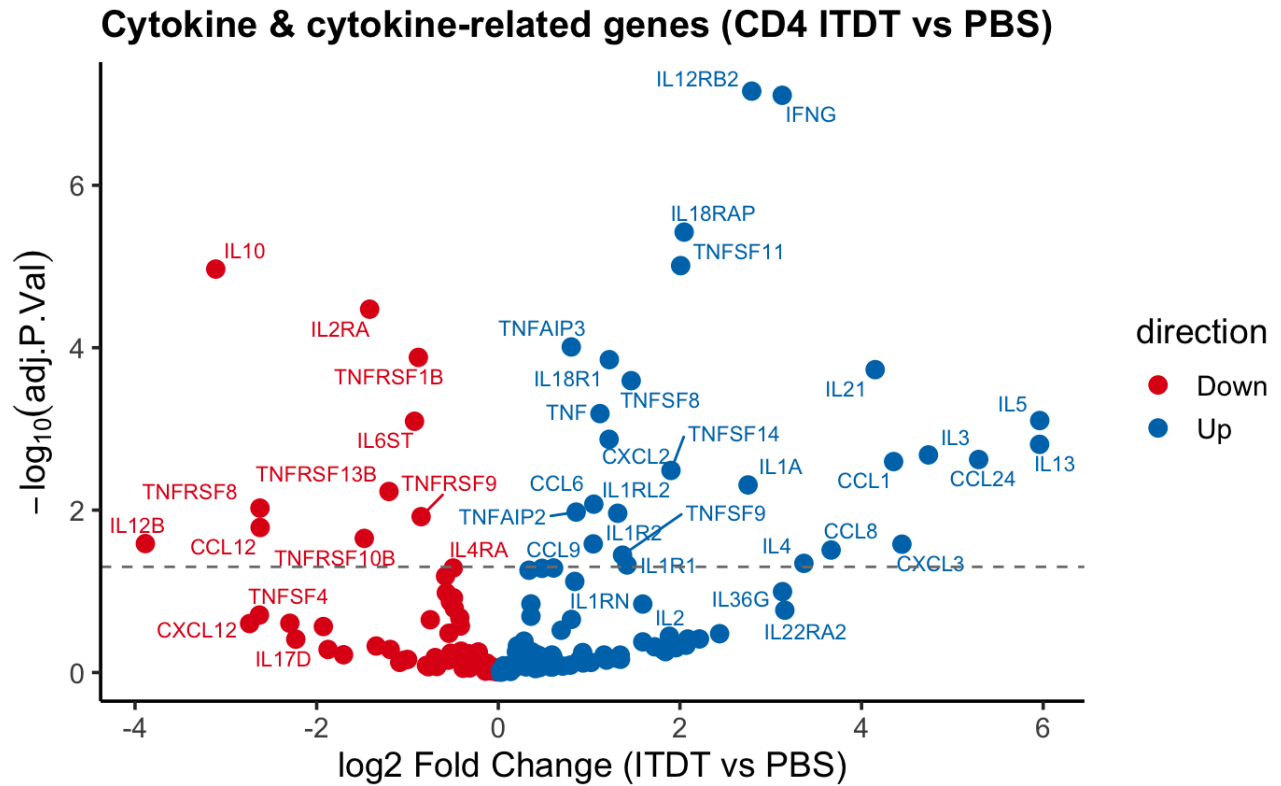


Figure 4.9: Extended transcript level expression of cytokines and cytokine receptor genes expressed by CD4 Tconv cells following IT Treg ablation in tumors. Scatter plot shows Limma processed differential analyses for cytokine and chemokine genes, showing log₂ fold change on the x-axis and -log₁₀ adjusted p-value on the y axis (red = log FC < 0; blue = logFC

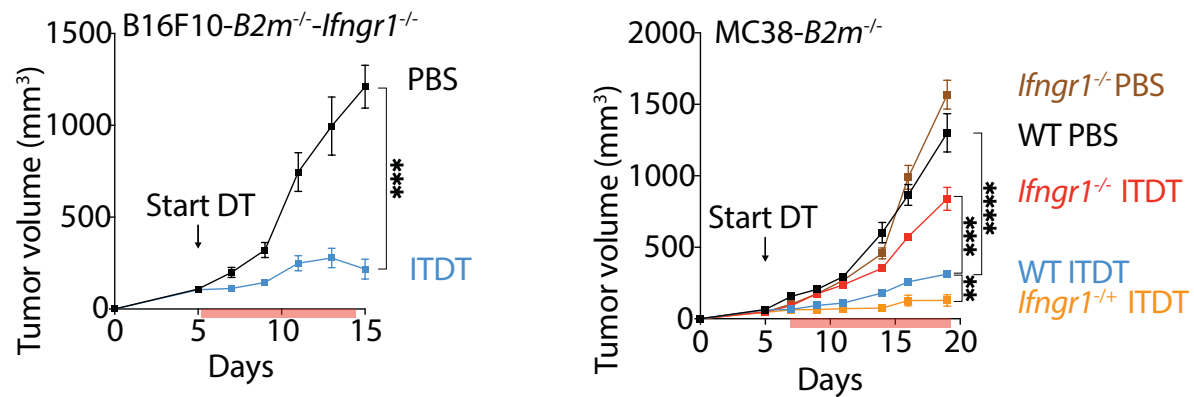


Figure 4.10: Tumor control following IT Treg ablation is not dependent on IFN- γ R expression on tumor or on host. Growth curves of B16F10-B2m^{-/-}Ifngr1^{-/-} tumors in mice that were intra-tumorally depleted of Tregs (blue), or MC38-B2m^{-/-} tumors implanted in IFN γ R^{-/-} mice treated with IT PBS (brown), or IT DT (red), or in Ifngr1^{+/-} mice with IT DT (orange). One experiment performed for each. Data represent means $\pm$ SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. RM two-way ANOVA followed by Geiser-Greenhouse correction. (n=5-8 mice/group).

Direct cytotoxicity exhibited by CD4⁺ Tconv following IT-Treg ablation

To test if the CD4⁺ Tconv cells that arise with IT Treg ablation have direct cytotoxic activity, we performed *ex vivo* killing assays of MC38-*B2m*^{-/-} target cells using as effector cells CD4⁺ Tconv cells sorted from tumor, spleen and lymph nodes of tumor-bearing mice. Only tumor-derived CD4⁺ Tconv cells showed elevated cytotoxicity *ex vivo* in the IT Treg-depleted condition, while no difference was observed in spleen or draining lymph-node CD4⁺ Tconv cells (Figure 4.12). These results suggest a localized expansion of cytotoxic CD4⁺ Tconv cells in the tumor. Further, we also observed that CD4⁺ Tconv cells isolated from MC38-*B2m*^{-/-} and B16F10-*B2m*^{-/-} tumors were able to kill *ex vivo* target cells of the same tumor origin (Figure 4.12). Preliminary experiments were conducted to test whether killing could occur when target cells were mismatched from the original tumors from which the CD4⁺ Tconv cells were isolated (data not shown), but more work needs to be done to confirm the conclusions of these preliminary assays.

Notably, tumor rejection after IT Treg ablation was perforin-dependent, because *Prfl*^{-/-}; *Foxp3*^{DTR-GFP} mice lacking perforin function were unable to control MC38-*B2m*^{-/-} tumors in contrast to control *Prfl*^{+/+}; *Foxp3*^{DTR-GFP} mice tested in parallel (Figure 4.13). CD4⁺ Tconv cells isolated from tumors also exhibited increased *ex vivo* cytotoxicity and capacity to control tumors *in vivo* in a perforin-dependent fashion. This result, taken together with FADD-independence (Figure 4.5), strongly suggest that the granzyme-perforin pathway is critical for tumor clearance by CD4⁺ T conv cells. Since NK cells also make use of the perforin pathway in direct cytotoxicity of tumor cells, we plan to repeat these experiments in the context of NK cell- and CD4⁺ T cell depletions, to confirm if CD4⁺ T cell-mediated tumor control is perforin dependent. To address the same question in a different way, we will test if perforin in NK cells or CD4⁺ T cells is sufficient to drive tumor control by employing mixed 50:50 bone marrow chimeras. In these mice, a cellular compartment of interest (e.g. NK cells or CD4⁺ T cells) is solely *Prfl*^{-/-} whereas other blood cell types are composed of a ~50:50 mix of WT and *Prfl*^{-/-} cells (Table 2). Control group 1 should reject the tumors after intra-tumoral Treg depletion, whereas control group 2 should be defective in tumor control if perforin is required in hematopoietic cells. Reduced tumor rejection in Experimental groups 1 or 2 will implicate perforin/granzyme killing in tumor control by NK cells and/or T cells.

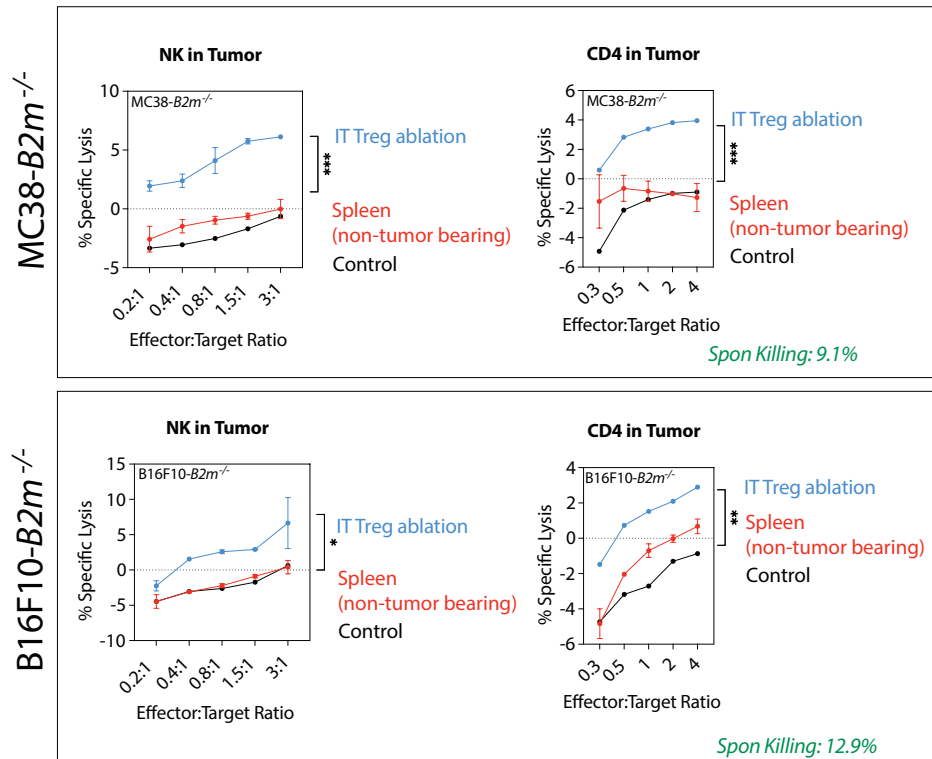
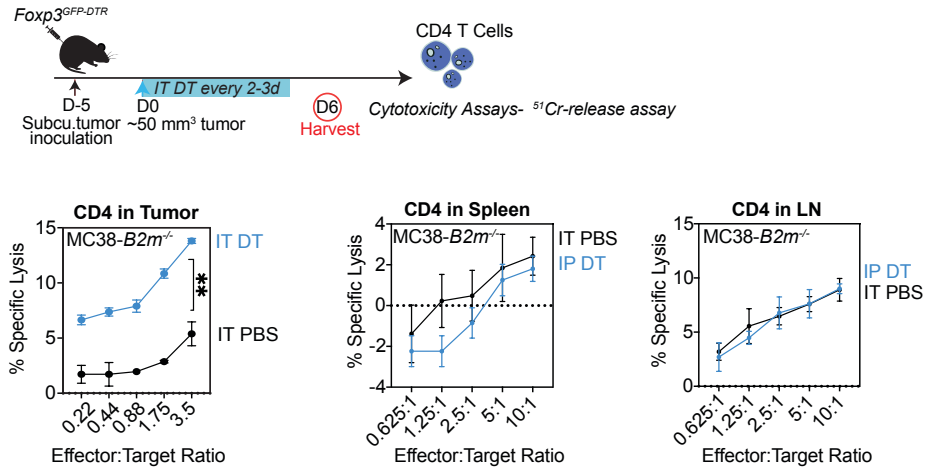


Figure 4.11: Ex vivo cytotoxicity of CD4⁺ Tconv cells following IT Treg ablation in tumors. Schematic of MC38-B2m^{-/-} tumor inoculation and treatment regimen in *Foxp3^{DTR-GFP}* mice depleted of IT Tregs, before tumor resection 6 days later to obtain CD4⁺ T cells from IT PBS and IT DT treated tumors for 4-h ⁵¹-chromium release assay. Percent specific lysis of MC38-B2m^{-/-} tumor targets incubated at various E:T ratios, with CD4⁺ Tconv cells sorted from tumor, spleen or lymph nodes. CD4⁺ T cells isolated from MC38-B2m^{-/-} and B16F10-B2m^{-/-} tumors both show cytolytic killing capabilities *ex vivo*. Data represent means $\pm$ SEM; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001 from Unpaired t test with Welch's correction.

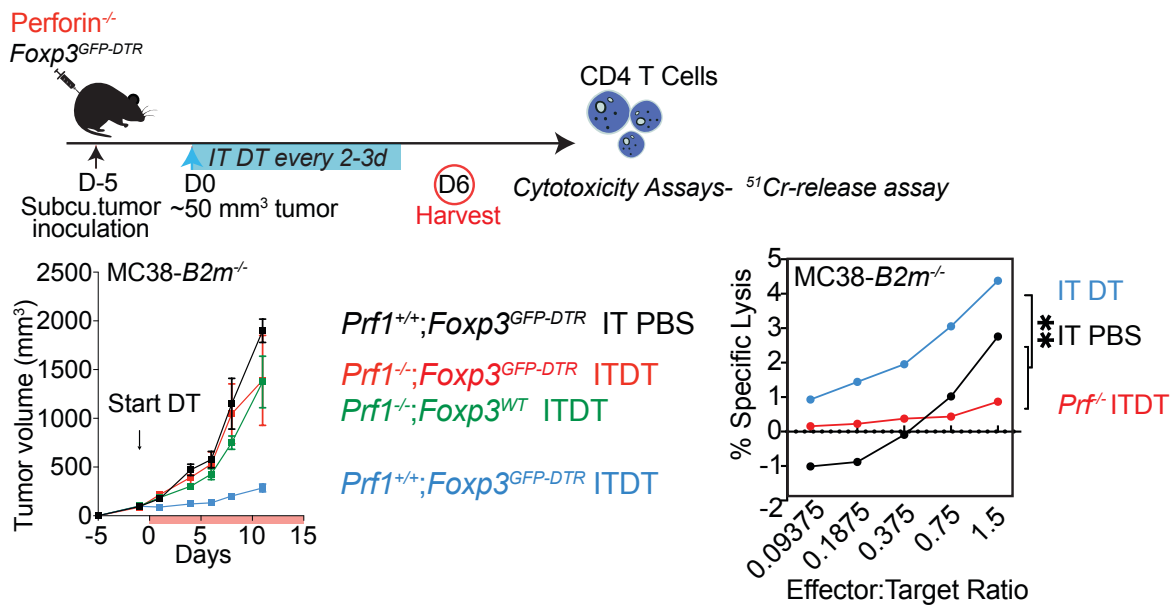


Figure 4.12: Perforin dependence of tumor rejection and CD4⁺ Tconv cell-mediated cytotoxicity. Schematic of MC38-B2m^{-/-} tumor inoculation and treatment regimen in *Foxp3*^{DTR-GFP} mice depleted of Tregs intra-tumorally, before tumor resection 6 days later for cytotoxicity assays. Growth curves of MC38-B2m^{-/-} tumors in mice that were intratumorally depleted of Tregs alone (blue) or in perforin knock-out animals (red). Control mice of *Prf1*^{-/-}; *Foxp3*^{WT} or *Prf1*^{+/+}; *Foxp3*^{DTR-GFP} were treated with IT DT or IT PBS respectively. Data are representative of 2-3 experiments for each tumor type. Data represent means ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 from RM two-way ANOVA followed by Geiser-Greenhouse correction

Group	Donor 1	Donor 2	Composition of mature immune system		
			NK compartment	T (and B) cell compartment	Other blood cells
Control 1	<i>Prfl</i> ^{-/-}	WT	50:50 <i>Prfl</i> ^{-/-} and <i>Prfl</i> ^{+/+}	50:50 <i>Prfl</i> ^{-/-} and <i>Prfl</i> ^{+/+}	50:50 <i>Prfl</i> ^{-/-} and <i>Prfl</i> ^{+/+}
Control 2	<i>Prfl</i> ^{-/-}	<i>Prfl</i> ^{-/-}	<i>Prfl</i> ^{-/-} only	<i>Prfl</i> ^{-/-} only	<i>Prfl</i> ^{-/-} only
Expt 1	<i>Prfl</i> ^{-/-}	NKDTA	<i>Prfl</i> ^{-/-} only	50:50 <i>Prfl</i> ^{-/-} and <i>Prfl</i> ^{+/+}	50:50 <i>Prfl</i> ^{-/-} and <i>Prfl</i> ^{+/+}
Expt 2	<i>Prfl</i> ^{-/-}	<i>Rag</i> ^{-/-}	50:50 <i>Prfl</i> ^{-/-} and <i>Prfl</i> ^{+/+}	<i>Prfl</i> ^{-/-} only	50:50 <i>Prfl</i> ^{-/-} and <i>Prfl</i> ^{+/+}

Table 2. 50:50 bone marrow chimeras to test role of perforin/granzyme killing by NK cells and CD4 cells. All the mice are crossed to *Foxp3*^{DTR-GFP}. NK-DTA mice are NK-deficient mice.

IL-2 further enhances CD4⁺ T cell-dependent control

As previously investigated in Chapter 3, our data suggested that IL-2 signaling, but not TNF- α or IFN- γ signaling, is required for NK cell-dependent tumor control after IT Treg ablation, and that the supplementation of IL-2-MSA restored NK cell-mediated tumor control in the absence of CD8⁺ or CD4⁺ T cells. In the same experiment, we also saw that Treg ablation with the addition of IL-2-MSA can also restore tumor control when NK cells are depleted, uncovering a NK cell-independent mechanism of tumor control (Figure 4.13). This tumor control is likely dependent on CD4⁺ T cells. Therefore, IL-2 may be important in either the differentiation or the maintenance of a cytotoxic CD4⁺ T cell phenotype. While very preliminary, we also tested if H9-MSA synergizes with IT Treg depletion in boosting the cytotoxic CD4⁺ T cell phenotype. The superkine H9, also known as super-2, is a variant of IL-2 that was selected to bind with high affinity to the IL-2R β / γ complexes that NK cells express (Levin, Bates et al. 2012). H9 activates NK cells at much lower concentrations than IL-2, and was shown to stimulate antitumor responses in mouse tumor models with lower toxicity than IL-2 (Levin, Bates et al. 2012). We observed that there was a mixed effect on the expression of NK cell receptors, where a further increase in expression of NKG2D and NKp46 was noted, with a concomitant decrease in expression of NKG2ACE (20D5) and NK1.1. Notably, granzymes A and B expression was significantly higher in CD4⁺ T cells isolated from IT Treg-deplete and H9-MSA co-treated tumors compared to just IT Treg deplete tumors.

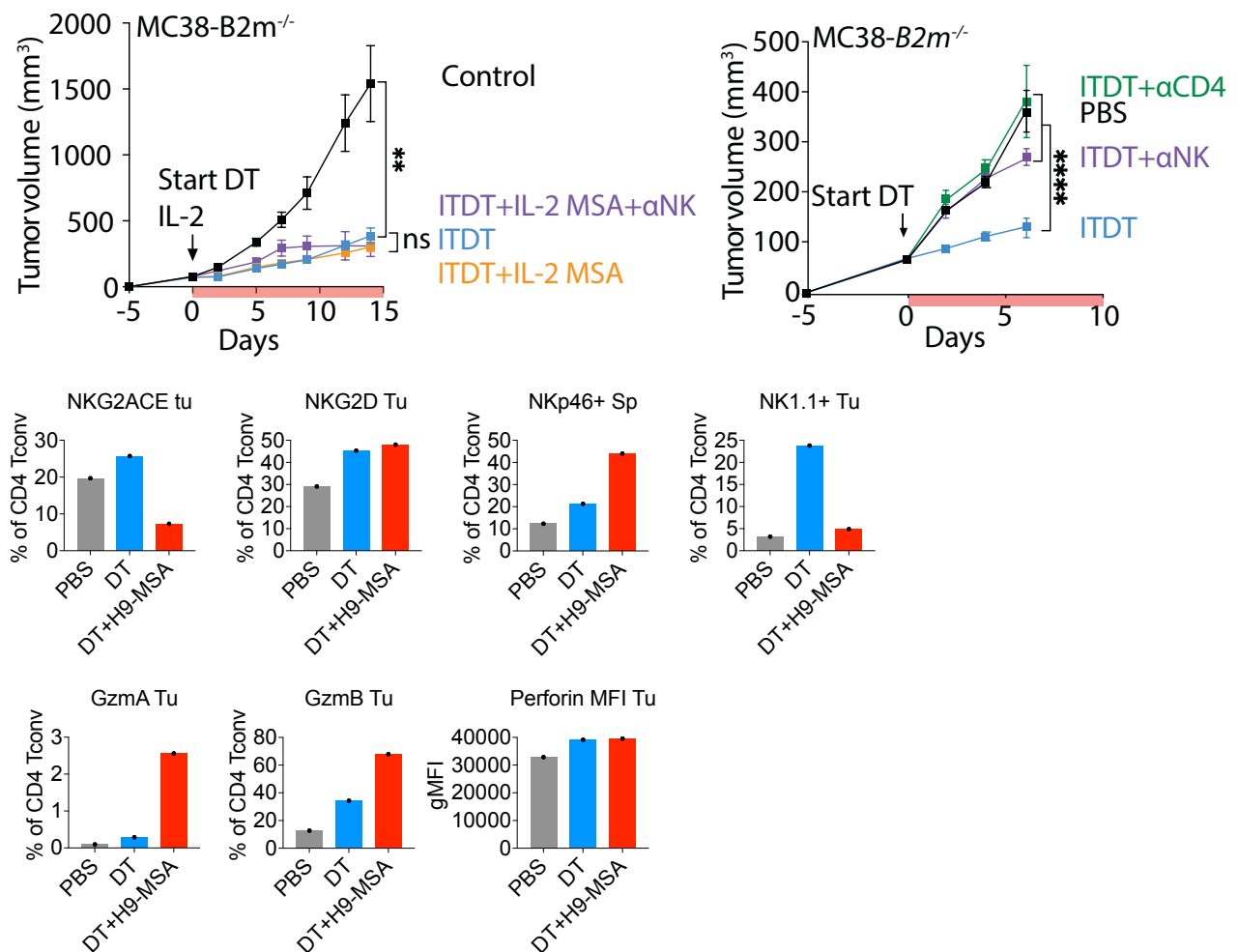


Figure 4.13: IL-2 enhances NK cell-independent, CD4+ T cell-dependent tumor control.

Growth curves of MC38-B2m^{-/-} tumors in mice that were intra-tumorally depleted of Tregs (blue) in combination with IL-2-MSA (orange), or in addition to NK cell depletion (purple). This is in comparison to the graph on the right from Figure 3.2E, without addition of IL-2-MSA. Representative of two independent experiments. Data represent means ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. RM two-way ANOVA followed by Geiser-Greenhouse correction. (n=5-8 mice/group).

Bar graphs of separate preliminary experiment using CD4⁺ Tconv cells isolated from MC38-B2m^{-/-} tumors six days after initial IT DT, or IT DT + H9-MSA or control (IT PBS), stained for NK cell receptors and cytotoxic marker expression. One experiment with n=1 tumor performed.

MHC II-Independence in CD4 T cell- dependent tumor control

To investigate the potential mechanism for tumor recognition by the CD4⁺ Tconv cells, we used tumor cell lines engineered to be MHC II-deficient in addition to being MHC I-deficient (B16F10-*B2m*^{-/-} *Abi*^{-/-}) and observed no change in the control of these tumors following IT Treg ablation (Figure 4.14). The depletion of NK cells alone still resulted in significant tumor control, but tumor control was also significantly less than that in the just IT Treg deplete group (purple vs. blue). However, the co-depletion of CD4⁺ T cells and NK cells (green) reversed tumor control back to near control levels, suggesting that CD4⁺ T cell mediated mechanisms of tumor control dominates in the MHC II-deficient tumor lines. This is all in consideration that the B16F10-*B2m*^{-/-} *Abi*^{-/-} tumor line was generated with first the transfection of Cas9 plasmid containing the guide sequence targeting *Abi*, followed by transient induction of MHC II-expression by a CIITA-plasmid, before MHC II-negative cell populations were enriched by FACS enrichment of MHC II-negative cells. There was no confirmation of *Abi* knock-out by sequencing, and hence, these cells may not represent a true MHC II-deficient tumor cell line.

Currently, both MC38-*B2m*^{-/-} *Abi*^{-/-} and RMA-*B2m*^{-/-} *Abi*^{-/-} double knockout cell lines are being generated by Joanna Zhang with CRISPR-Cas9 engineering using multiple pooled guides, followed by stringent cell clonal selection and a pool of 5-10 confirmed knock-out clones via ICE analyses. These new cell lines will enable us to further test the importance of MHC II-expression on tumor cells for the CD4⁺ T cell response following IT Treg ablation. We tested the RMA-*B2m*^{-/-} *Abi*^{-/-} tumors in one preliminary experiment, and saw a late but significant control of these tumors following IT Treg ablation (Figure 4.14)

Experiments using MHC II-knockout tumor cells, together with the mismatched *ex vivo* target-cell killing assays described earlier, can help determine whether cytotoxic CD4⁺ T cells can recognize and kill targets through antigen-independent mechanisms.

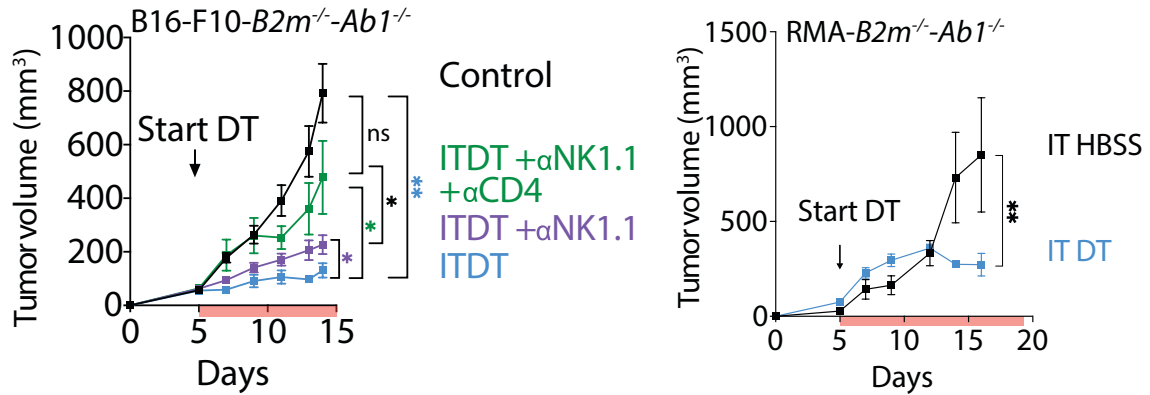


Figure 4.14: MHC II on tumor cells is not required for tumor recognition and control by CD4⁺ Tconv cells.

Growth curves of B16F10-B2m^{-/-}-Ab1^{-/-} or RMA-B2m^{-/-}-Ab1^{-/-} tumors in mice that were intra-tumorally depleted of Tregs (blue) in combination with NK cell depletion (purple) or CD4 T cell depletion (green). One experiment performed each. Data represent means ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. RM two-way ANOVA followed by Geiser-Greenhouse correction (n=5-8 mice/group). Colored asterisks represent comparisons to IT DT group.

CD4⁺ T cells in MHC I⁺ vs MHC I⁻ tumors

Work by another graduate student in the lab, Alissa Bockman, also showed that MHC I⁺ tumors are efficiently controlled by IT Treg depletion, and that control was dependent on CD4⁺ T cells (Bockman, Gittins et al. 2025). To test if the CD4⁺ T cells also gain a similar NK-like phenotype in MHC I⁺ tumors, we took a similar approach and surveyed for cytotoxic and NK cell receptor marker expression on CD4⁺ T cells isolated from tumors that were IT Treg depleted and compared them to CD4⁺ T cells from control tumors treated with IT PBS. Surprisingly, the CD4⁺ T cells from MHC I⁺ tumors, while displaying some level of increased activation (CD44, Ki67 ICOS, PD-1 and KLRG1 (Figure 4.15), also showed decreased expression of CD25, CD69, and SCA-1, indicating a mixed phenotype. Importantly, CD4⁺ Tconv cells from MC38-WT tumors did not show any significant increase in expression of Granzyme B, Perforin or EOMES (Figure 4.16), and in some experiments even showed a dramatic decrease in the MFI of granzyme B in the IT Treg depleted tumors compared to control, but a surprising increase of granzyme B⁺ in CD4⁺ Tconv cells in the spleen (Figure 4.16). Similarly, we did not observe any increased expression of NK cell receptors on these CD4⁺ Tconv cells as we had observed in MHC I-deficient tumors, and even a decrease in the MFI of NKG2A (Figure 4.17, Figure 4.8). These data suggest that, while CD4⁺ T conv cells might be important for the control of MHC I⁺ tumors, the mechanism of control between MHC I⁺ and MHC I-deficient tumors are likely distinct.

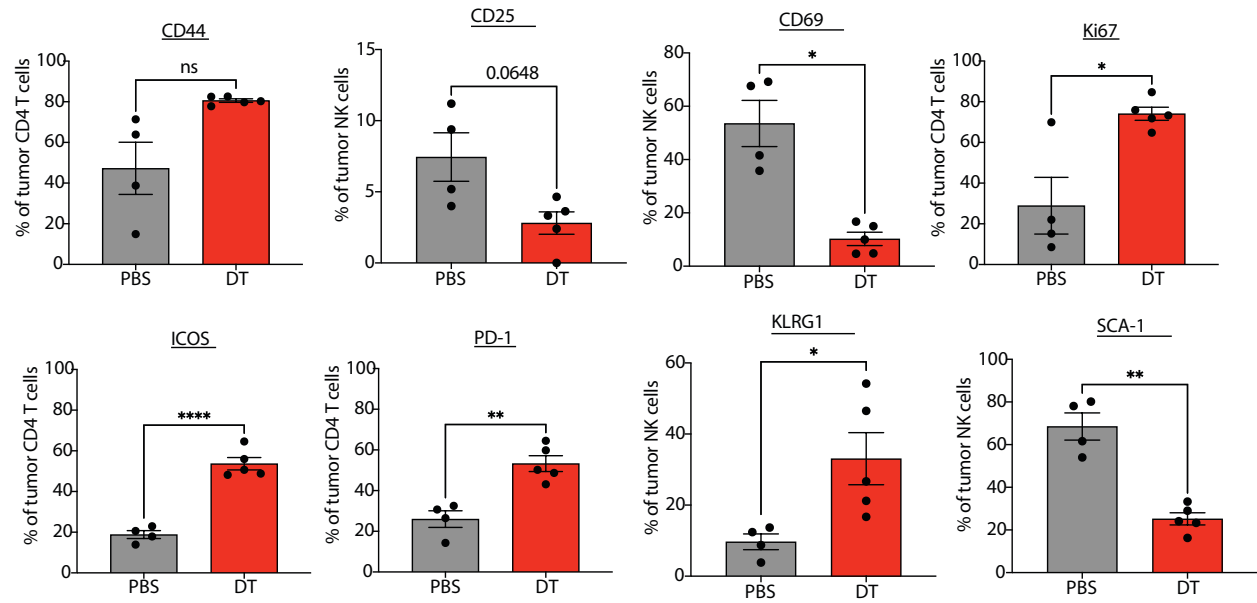


Figure 4.15: Expression of activation markers on CD4⁺ T cells from MHC I-expressing tumors.

Expression of CD44, CD25, CD69, Ki67, ICOS, PD-1, KLRG1 and SCA-1 on CD4⁺ Tconv cells isolated from MC38-*WT* tumors 6 days after IT Treg depletion. Data represent means $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Ordinary one-way ANOVA with Dunnett's multiple comparisons tests.

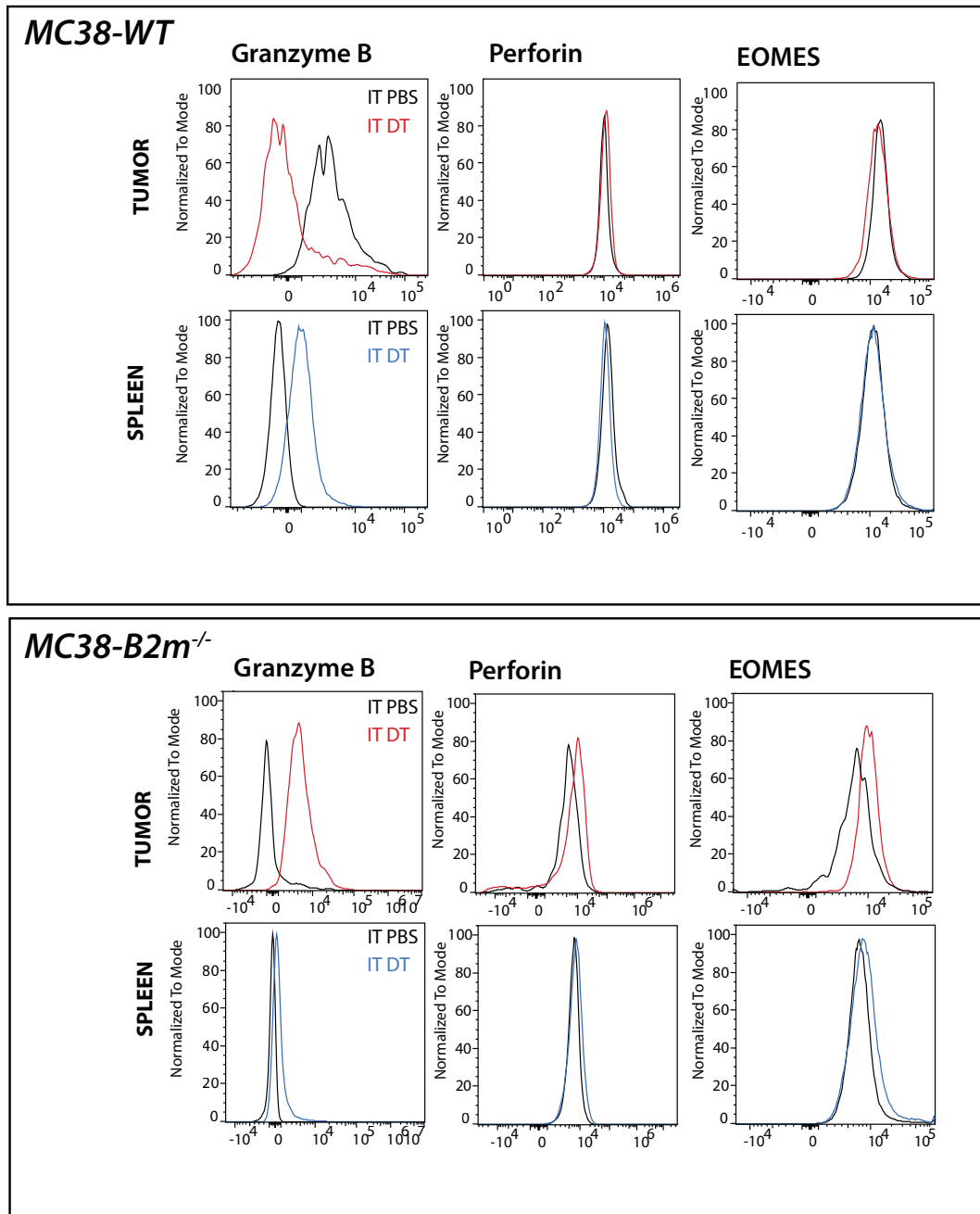


Figure 4.16: Expression of cytotoxic markers on CD4⁺ T cells on MHC I-expressing tumors versus MHC I-deficient tumors

Expression of granzyme B, perforin and EOMES on CD4⁺ Tconv cells isolated from MC38-*WT* tumors 6 days after IT Treg depletion. Data represent means $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Ordinary one-way ANOVA with Dunnett's multiple comparisons tests.

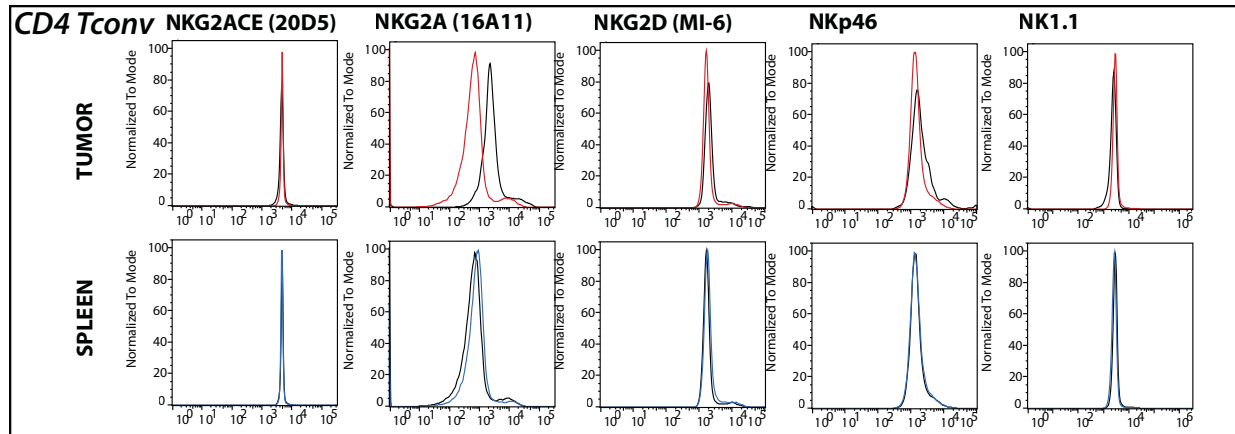


Figure 4.17: Expression of NK cell receptor markers on CD4⁺ T cells from MHC I-expressing tumors.

Expression of NKG2ACE, NKG2A, NKG2D, NKp46 and NK1.1 on CD4⁺ Tconv cells isolated from MC38-*WT* tumors 6 days after IT Treg depletion. Data represent means $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Ordinary one-way ANOVA with Dunnett's multiple comparisons tests.

DISCUSSION

The discovery that intra-tumoral (IT) Treg ablation—specifically in MHC I-deficient tumors—drives the robust expansion of cytotoxic, NK cell receptor-expressing CD4⁺ conventional T (Tconv) cells highlights a previously underappreciated adaptation of the adaptive immune response within highly immune evasive tumor settings. While CD4⁺ T cells have long been characterized in terms of their “helper” and regulatory roles, it is now clear that under specific conditions of immune pressure, and especially in the absence of suppressive Treg cues, CD4⁺ T cells can acquire potent cytolytic capacity, including expression of perforin and granzymes, as well as NK cell-associated receptors. Our data show that these CD4⁺ T cells are maintained in the tumor microenvironment (TME) with a possible dependence on IL-2 availability, and this is consistent with emerging evidence that cytokine milieu and inflammatory cues tightly regulate the differentiation, expansion, and functional stability of cytotoxic CD4⁺ T cells.

Notably, we show preliminarily that some modes of cytolysis proposed to be important for CD4⁺ T cell-mediated tumor control, such as those mediated by TNF- α , IFN- γ , or death receptor-ligand interactions (e.g., Fas/FasL, TRAIL), may be dispensable for CD4⁺ T cell-mediated tumor killing upon IT Treg ablation. Instead, perforin-dependent mechanisms are essential. However, FADD, together with caspase-8, forms a critical inhibitory checkpoint that prevents spontaneous activation of RIPK1 and RIPK3, key drivers of necroptosis. When FADD is ablated or inhibited, this checkpoint is lost, leading to activation of RIPK1/RIPK3 and necroptosis as described in recent studies (Rodriguez, Quarato et al. 2022, Rodriguez, Tummers et al. 2024). Therefore, the use of *Fadd*^{-/-} tumor lines to rule out the role of death receptor recognition and killing by CD4⁺ T cells may unintentionally activate necroptotic pathways within the tumors, confounding interpretation. This points to the need for deeper exploration of the mechanisms through which CD4⁺ T cells recognize and eliminate tumor targets, particularly in the context of comparing the difference in their responses against MHC I-expressing versus MHC I-deficient tumors. Whether the upregulation of NK cell receptors enables these CD4⁺ T cells to bypass classical antigen recognition requirements and directly sense stress ligand upregulation on tumor cells remains an open question of significant mechanistic and therapeutic interest. One potential way to test if the

cytotoxic CD4⁺ T cells are using an NK-like approach to recognize and kill tumor cells could be to conduct cytotoxicity assays in conjunction with various NK cell receptor-blockade.

Descriptions of a small population of CD4⁺ T cells capable of eradicating tumors that have lost both MHC-I and MHC-II expression—tumors typically resistant to classical CD8⁺ T cell—have been previously described (Kruse, Buzzai et al. 2023). Activated CD4⁺ T cells can cluster at tumor margins, engage MHC-II⁺ antigen-presenting cells, and reprogram tumor-associated myeloid cells toward an IFN- γ -driven, iNOS-expressing, tumoricidal phenotype (Kruse, Buzzai et al. 2023, Bawden, Wagner et al. 2024). Rather than relying on direct cytolysis of cancer cells, these CD4⁺ T cells and the reprogrammed myeloid cells cooperate to induce a potent, remote inflammatory cell death that eliminates immune-evasive and interferon-unresponsive tumors. Other studies have also showed that the primary mechanism by which CD4⁺ CAR T cells eliminate tumors is through the production of interferon gamma (IFN- γ), rather than direct cell contact or perforin-mediated cytotoxicity (Boulch, Cazaux et al. 2023). These findings emphasize that CD4⁺ T cells can orchestrate tumor eradication indirectly, through the activation of myeloid cell effector programs or by secreting cytolytic cytokines. However, in our studies, we suggest a possible alternative direct cytotoxic mechanism exerted by the CD4⁺ T cells that may more closely resemble NK cell-mediated killing that is independent of TCR-MHC II recognition, however, more mechanistic understanding is required to confirm the recognition and killing pathway unleashed from IT Treg ablation. The combination of both indirect and direct mechanisms of killing by CD4⁺ T cells suggest a powerful alternative therapeutically pathway to target tumors that escape other arms of adaptive immunity.

Looking forward, further dissection of the molecular signals and spatial cues that direct CD4⁺ T cell differentiation into cytotoxic or "helper" phenotypes—and whether these are mutually exclusive or plastic—will be essential. Deciphering the contributions of tumor-derived factors, Treg and cytokine gradients, and the exact role of NK cell receptor signaling in target engagement will clarify whether cytotoxic CD4⁺ T cells can be therapeutically harnessed, particularly for tumors that evade classic adaptive immunity by the loss of antigen presentation machinery or the creation of a profoundly suppressive tumor microenvironment. Collectively, these insights position cytotoxic CD4⁺ T cells as a flexible, multifaceted effector population whose clinical

utility may extend well beyond conventional dogma, especially as new strategies for Treg modulation, cytokine manipulation, and adoptive cell transfer are developed.

Chapter 5: Role of Ex-Tregs in Autoimmunity and Cancer

Abstract

Regulatory T cells (Tregs), defined by the expression of the transcription factor FOXP3, are essential for maintaining immune homeostasis by restraining effector T cell activity and preventing autoimmunity. However, Tregs display remarkable plasticity, and under inflammatory or metabolic stress can lose *Foxp3* expression and their suppressive capacity, giving rise to “ex-Tregs.” These ex-Tregs retain TCRs with high affinity for self-antigens but convert into inflammatory effector cells that can produce IFN- γ and IL-17, thus amplifying immune activation. In cancer, emerging evidence indicates that this fate conversion may shift the immune balance toward tumor rejection, particularly when Treg stability is disrupted by epigenetic or cytokine cues. Using genetic mouse models that enable us to visualize and to deplete ex-Tregs, we can now ask questions on how these ex-Tregs contribute to antitumor immunity or the onset of autoimmunity. Our preliminary findings suggest that, beyond serving as byproducts of instability, ex-Tregs act as direct pro-inflammatory mediators that drive effective T-cell priming. Understanding how ex-Tregs arise, localize, and function will provide insight into both autoimmune pathology and the optimization of Treg-targeted cancer immunotherapies.

Introduction

Regulatory T cells are indispensable for immune tolerance, yet paradoxically pose a major obstacle to durable antitumor responses (Sakaguchi 2000, Simon, Jones et al. 2007, Feuerer, Shen et al. 2009, Selby, Engelhardt et al. 2013). Tregs enforce suppression via IL-2 sequestration, cytotoxic mediator release, and checkpoint engagement (CTLA-4, LAG-3, TIGIT) with dendritic cells (DCs) in the tumor microenvironment (TME) (Sitrin, Ring et al. 2013). However, Tregs exhibit intrinsic lineage plasticity governed by *Foxp3* stability, epigenetic state, and metabolic adaptation, all of which determine whether they maintain suppressive identity or revert to effector-like phenotypes (Zheng, Josefowicz et al. 2010, DuPage, Chopra et al. 2015). Loss of *Foxp3*, either genetically, by inflammatory cytokines such as IL-6 or IL-1 β , or through metabolic stress, precipitates reprogramming into ex-Tregs, characterized by renewed TCR signaling, proliferation, and secretion of IFN- γ , IL-17, or TNF- α (Floess, Freyer et al. 2007, Zhou, Bailey-Bucktrout et al. 2009, Duarte, Agua-Doce et al. 2010, Joller and Kuchroo 2014, Overacre-Delgoffe, Chikina et al. 2017).

However, the inflammation-induced reprogramming and the origin of ex-Tregs remain controversial in the field. Rubtsov et al. used *Foxp3*^{GFP-Cre-ERT2}; *Rosa26*^{YFP} mice to label all *Foxp3*⁺ Tregs, and showed that under physiological and inflammatory conditions Tregs remain largely stable, and thus suggested that mature Tregs are able to self-renew and maintain steady state cellularity within adult animals (Rubtsov, Nieuwenhuis et al. 2010). In contrast, Zhou et al. crossed *Foxp3* bacterial artificial chromosome (BAC) transgenic mice expressing a green fluorescent protein (GFP)-Cre recombinase fusion protein with ROSA26-yellow fluorescent protein (ROSA26YFP) Cre-reporter mice, and observed in their system that a substantial percentage (up to 20%) of cells that initially express *Foxp3* have transient or unstable expression of *Foxp3*. TCR repertoire studies indicated that many ex-*Foxp3* cells shared TCRs with tTreg cells (Zhou, Bailey-Bucktrout et al. 2009). Later, it was shown that activation induced *Foxp3* expression does not confer a regulatory cell phenotype (i.e. low CD25 and Helios expression compared to FOXP3⁺ cells) and is unstable, with partially and heavily methylated Treg-specific demethylated region (TSDR) and *Foxp3* promoter regions (Zhou, Bailey-Bucktrout et al. 2009). With adoptive transfer experiments, it was also confirmed that the majority (~90%) of *Foxp3*⁺ donor T cells maintained

Foxp3 expression in *Rag1*^{-/-} recipients when co-transferred with *Foxp3*⁻ T cells, showing that *Foxp3*⁻ T cells can impede the accumulation of ex-*Foxp3* cells by inhibiting their expansion, likely by their production of IL-2, which is essential for the stabilization of *Foxp3* expression in Tregs (Miyao, Floess et al. 2012). Most importantly, Miyao et al. argue that expansion of non-Treg cell-derived ex-*Foxp3* T cells (and/or restrained expansion of Treg cells), rather than Treg cell reprogramming, accounts for the observed plasticity of *Foxp3*⁺ Treg cells.

These transitions have clear functional implications. Since Tregs express TCRs with a heightened affinity for self-peptide MHC complexes, these ex-Tregs have the potential to become pathogenic. In autoimmunity models, ex-Tregs act as potent effector T cells driving tissue inflammation (Miyao, Floess et al. 2012). In contrast, within tumors, partial Treg conversion or destabilization has been associated with improved immunogenicity and increased infiltration of CD8⁺ T cells and dendritic cells (Zhou, Bailey-Bucktrout et al. 2009). It was also shown that *Ezh2*-deficient Tregs show elevated *Foxp3* instability and that accumulation of ex-Tregs correlates closely with stronger tumor control following transient Treg depletion, highlighting ex-Tregs as active participants in antitumor immunity rather than mere byproducts of disturbed homeostasis (Fig 1A,B) (DuPage, Chopra et al. 2015, Wang, Quiros et al. 2018). Mechanistically, the loss of EZH2, which is a catalytic component of the Polycomb Repressive Complex 2 (PRC2), resulted in reduced H3K27me3 marks on effector genes, facilitating transcriptional access to cytokine loci and T-bet binding sites (DuPage, Chopra et al. 2015). This epigenetically “unlocked” chromatin landscape predisposes former Tregs to acquire Th1- and Th17-like signatures, producing IFN- γ and IL-17 while losing expression of CD25 and CTLA-4.

Despite these insights, major questions remain: (1) Is the presence of ex-Tregs required to initiate autoimmune disease or augment antitumor immunity following Treg ablation? (2) Do ex-Tregs adopt distinct tissue localization or cell–cell interaction signatures that define their mechanism of action? Addressing these questions will clarify whether controlled Treg destabilization can be harnessed therapeutically to enhance immunotherapy without eliciting autoimmunity.

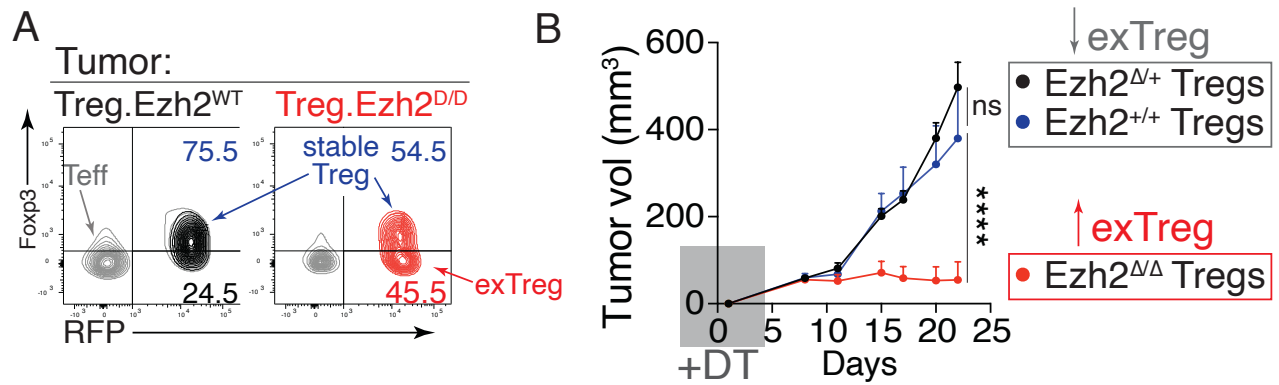


Figure 5.1: Generation of Ex-Tregs in mice with Treg-specific deficiency in Ezh2. (A) Representative flow plots comparing *Ezh2*^{WT} and *Ezh2*^{Δ/Δ} in Treg lineage trace mice. Tregs are Foxp3-GFP and RFP double positive, while Ex-Tregs are RFP single positive. (B) Tumor growth curves comparing the effects of *Ezh2*-deficiency in Tregs and the level of tumor control. Experiment results adapted with permission from (Wang, Quiros et al. 2018).

To dissect the role of ex-Tregs in immunological processes following Treg ablation, we undertook a three-pronged experimental strategy. First, we sought to determine whether ex-Tregs are required for the induction of autoimmunity after Treg depletion. This was accomplished by comparing the onset and severity of autoimmune symptoms in mice with selective ablation of Tregs versus those with combined ablation of both Tregs and ex-Tregs. By analyzing these cohorts, we clarified whether the presence of ex-Treg cells mitigates or accelerates autoimmune pathology following loss of regulatory T cell populations.

Second, we investigated the importance of ex-Tregs in tumor immunity specifically in the context of Treg ablation. Here, tumor growth and immune control was compared between mice lacking only Tregs and those lacking both Tregs and ex-Tregs. This experiment enabled us to assess whether ex-Tregs contribute to or are dispensable for tumor control mechanisms following the removal of canonical Tregs within the tumor microenvironment.

Finally, we addressed the spatial dynamics of Treg and ex-Treg populations by testing our hypothesis that these two cell types occupy distinct niches within tumors and lymph nodes. Utilizing our established Treg lineage trace mouse model, we visualized and monitored ex-Treg localization by confocal microscopy in both tumor-bearing and tumor-free animals. This approach allowed us to characterize the tissue distribution and potential functional compartmentalization of Tregs and ex-Tregs during steady-state and tumor-driven immune responses.

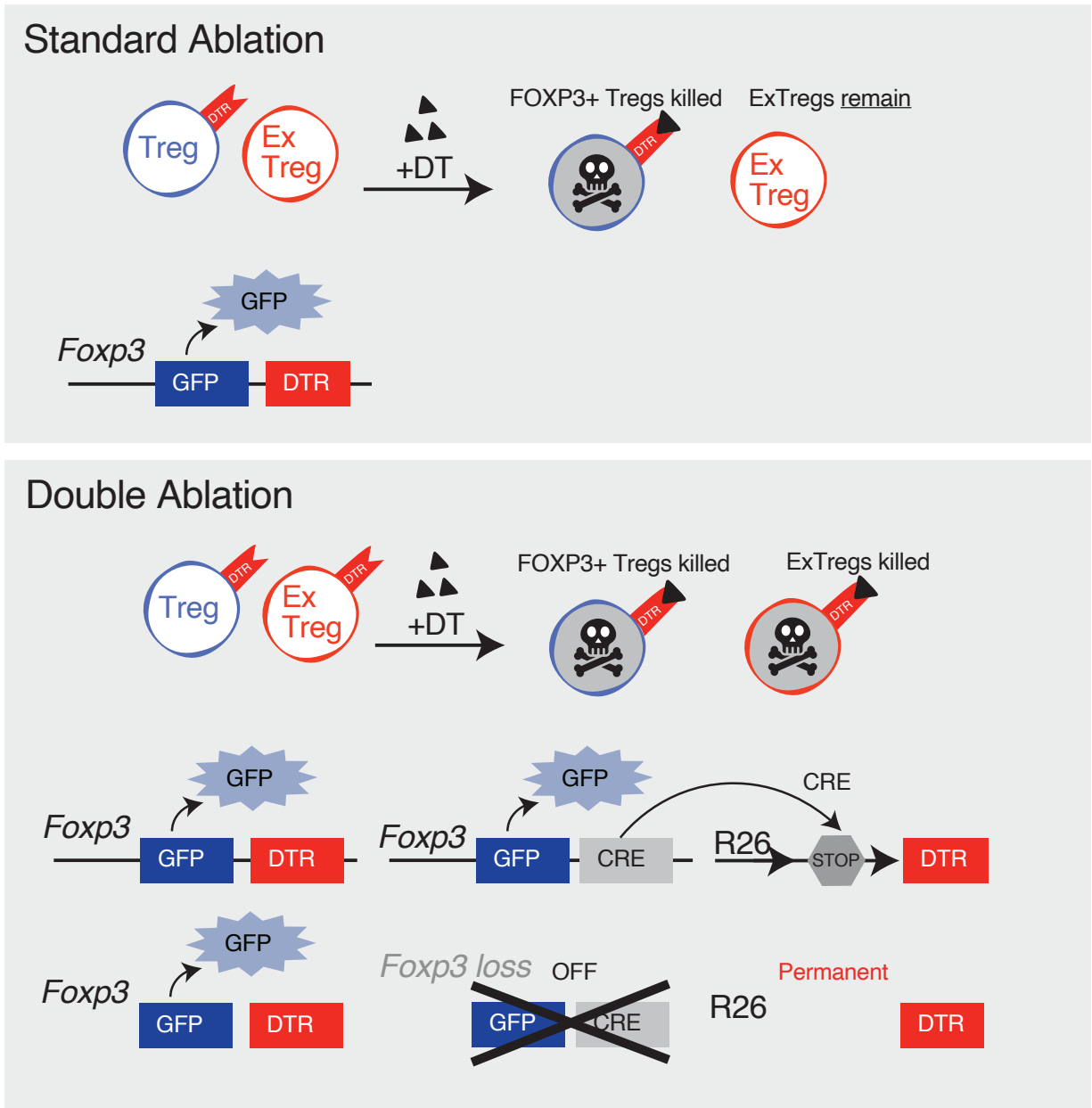


Figure 5.2: Mouse genetics of double ablation model. Overview of approach to test importance of Ex-Tregs in autoimmunity and anti-cancer responses with the double ablation mouse model.

RESULTS

Generating a mouse model to deplete ex-Tregs and Tregs

To test the requirement of ex-Tregs in autoimmunity, we first developed the dual depletion model in which the application of DT will enable us to deplete not just FOXP3-expressing Tregs, but also ex-Tregs that have lost current expression of FOXP3. These "dual deplete mice" are an extension of the original lineage trace mice, in which the R26 locus drives the expression of RFP, and the mice also harbor a Bac transgene containing a *Foxp3*^{Cre-GFP} segment that marks any cells that are currently FOXP3+ (Tregs) as GFP+RFP+, and any cell that once expressed Foxp3 but does no longer (ex-Tregs) as GFP-RFP+. We generated mice that had DTR expression driven by lox-stop-lox R26, and further crossed these mice to generate *Foxp3*^{DTR/DTR(Y); R26^{ls}-DTR/RFP; *Foxp3*^{Cre1a(high)} mice to simultaneously deplete Tregs and ex-Tregs. As controls, we have mice of the genotype *Foxp3*^{DTR/DTR(Y); R26⁺-RFP/RFP (or RFP/+); *Foxp3*^{Cre1a(high)} mice to deplete only Tregs and visualize the remaining ex-Tregs following DT treatment.}}

Compared to standard Treg ablation which leaves ex-Tregs in mice, we observed a significant decrease in frequencies of ex-Tregs remaining in the animals treated with DT, indicating a successful method for us to deplete Tregs and ex-Tregs with our initial model of the "dual deplete mice" that was not back-crossed to endogenous *Foxp3*^{DTR/DTR(Y)} (Fig. 5.3). Experiments to show depletion of Tregs and ex-Tregs with the newly generated mouse models are underway, to better quantify and demonstrate the effective sustained depletion of Tregs by use of the *Foxp3*^{DTR} allele.

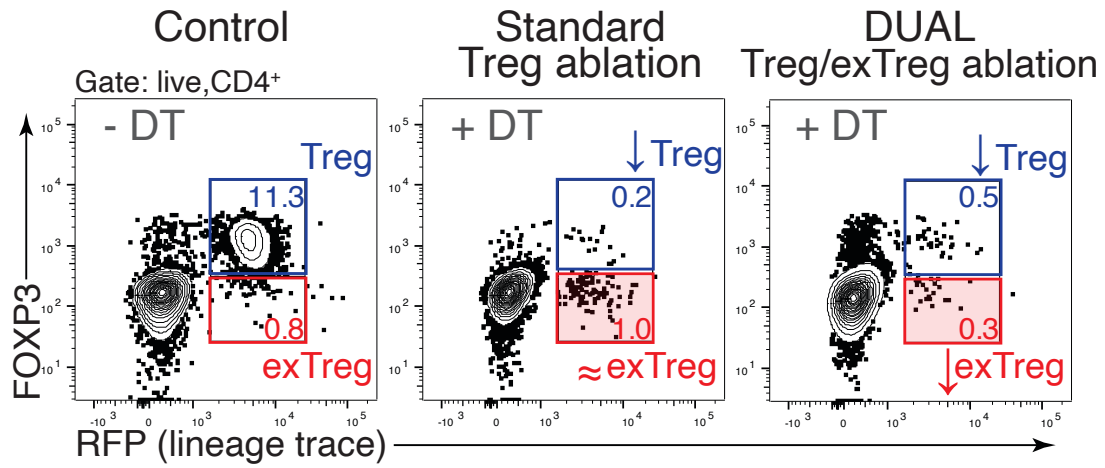


Figure 5.3: Comparison of standard and dual ablation methods. Representative flow plots comparing control mice to standard Treg and dual Treg/ex-Treg ablation.

Role of ex-Tregs in the onset of autoimmune pathologies in mice

To test the requirement of ex-Tregs in driving autoimmunity, we used our dual deplete mice and carried out long term systemic Treg depletion with the treatment of DT over a period of two weeks. Compared to standard Treg depletion, dual depletion mice showed a recovery of weight loss and a significant rescue in splenomegaly and lymphadenopathy (Figure 5.4A-E). Further, we observed a less severe onset of dermatitis in mice, as marked by the scabbing and flaking of skin around the eye regions (Figure 5.4F). Along with visual scoring and secondary lymphoid organ analyses, we also collected various other organs, including kidney, liver, lungs and colon, to investigate potential autoimmune pathologies (Figure 5.5). We observed higher immune infiltration in the organs of Treg-depleted mice compared to control and to dual-deplete mice, especially in lung sections, along with loss of tubular structures in the kidney and thickening of colon structures, suggesting a greater degree of autoimmune onset. Together, these data suggest that ex-Tregs contribute critically to the autoimmune pathologies observed in mice that are depleted of Tregs, as the co-depletion of ex-Tregs and Tregs reversed the severity of these pathologies.

Role of ex-Tregs in anti-tumor immunity

Tumor cells are strongly controlled after Treg depletion, as shown by treating *Foxp3^{DTR-GFP}* mice with IP DT and IT DT, or WT mice treated IP with anti-CCR8 antibodies (chapters 3 and 4). To test if ex-Tregs contribute to driving anti-tumor immunity in the context of standard Treg ablation models, we employed C57BL/6 (B6) tumor model of MC-38. We depleted Tregs using *Foxp3^{DTR-GFP}* mice and our dual deplete mice, wherein diphtheria toxin (DT) injection leads to Treg or Treg and ex-Treg apoptosis³⁴. Mice bearing subcutaneous tumors of each type at a size of ~50 mm³ (five days after inoculation) began receiving DT systemically every other day by intraperitoneal (IP) injection. IP DT treatments resulted in the significant control of both Treg and dual deplete mice. However, we consistently observed a delay in tumor control in the dual deplete mice at early time-points, in the first week post-DT initiation (Figure 5.6). These data point to a potential for ex-Tregs to be poised and crucial for immediate and rapid tumor control in the context of Treg depletion.

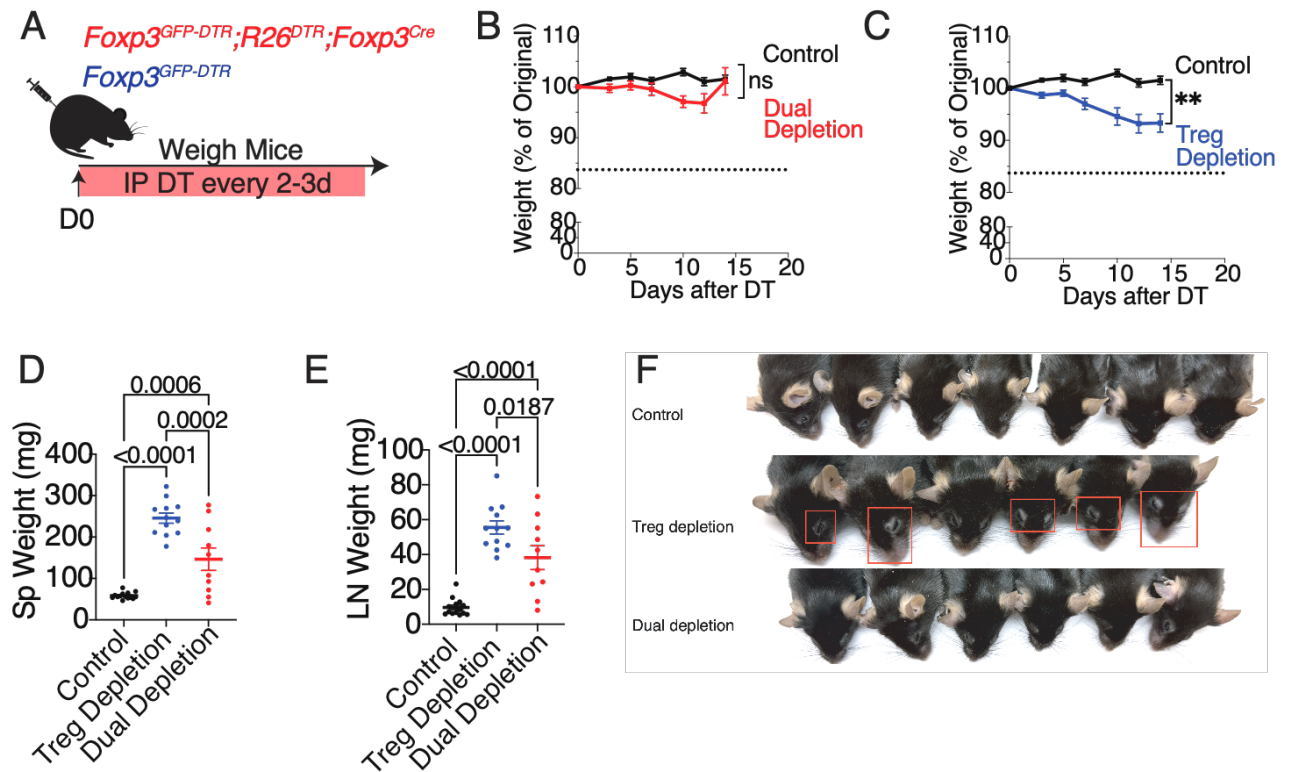


Figure 5.4: Ex-Tregs contribute to onset of autoimmunity. (A) Schematic of treatment regimen in $Foxp3^{DTR-GFP}$ or $Foxp3^{DTR-GFP}; R26^{DT}; Foxp3^{Cre}$ mice depleted of Tregs systemically. (B-C) Mice treated with systemic DT and weighed over time show differences in extent of weight loss and spleen weights and lymph node weights 16 days after initiation of DT. (D-E) Spleen and lymph node weights at D16 post DT initiation at takedown, comparing across the three different groups. (F) Representative photos of mice in each group, highlight dermatitis symptoms around eye regions of mice.

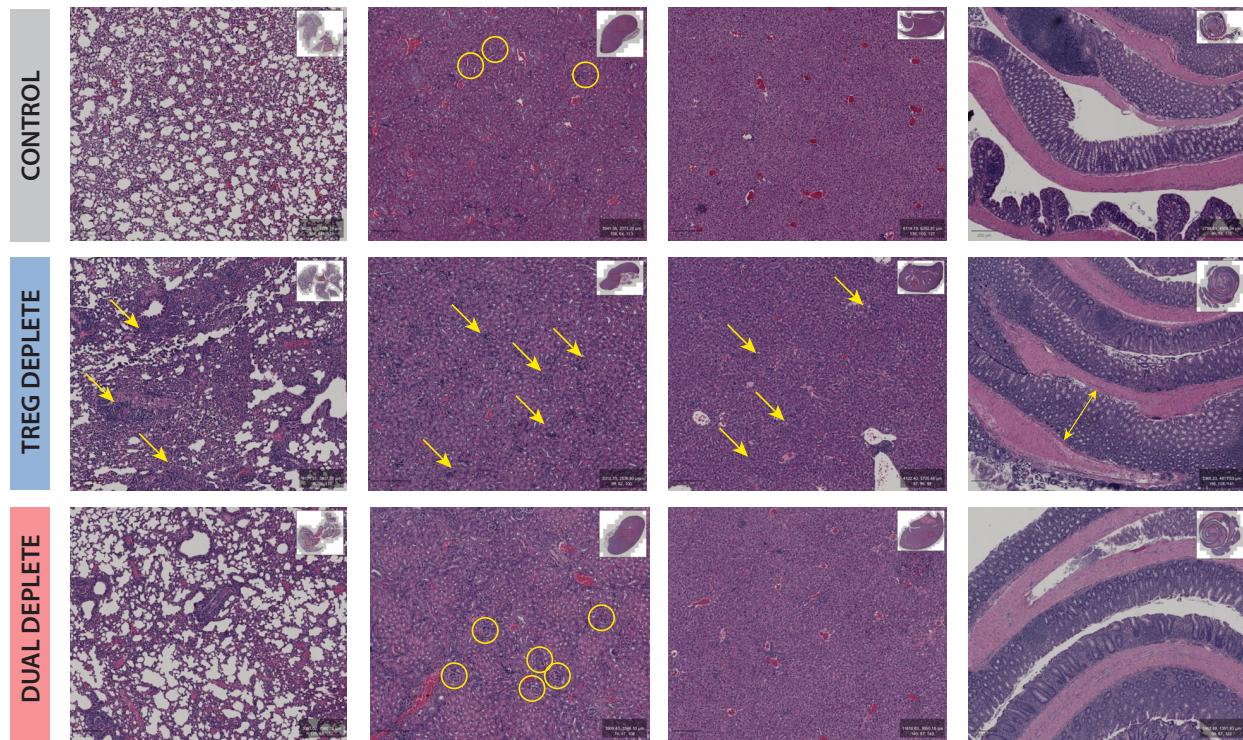


Figure 5.5: H&E staining of organs. Representative images from mice from control (no depletion), Treg only depletion or ex-Treg+Treg (Dual) depletion collected at D15 post-initiation of DT. Organs were paraffin embedded, and H&E staining was performed. Image sections were viewed on QuPath. Arrows point to regions of immune infiltration; circles show tubular kidney structures; double-headed arrows indicate thickening of colonic structures.

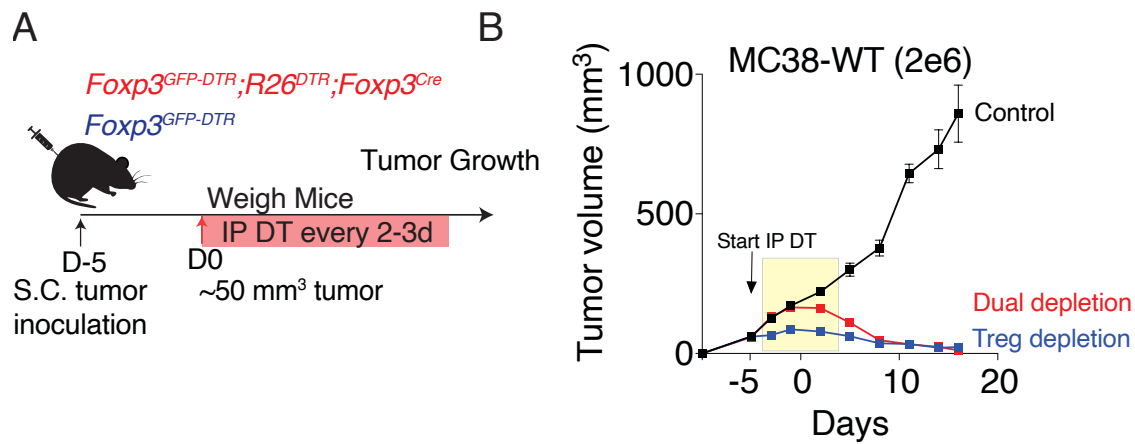


Figure 5.6: Ex-Tregs contribute to early anti-tumor immunity. (A) Schematic of MC38-*B2m*^{-/-} tumor inoculation and treatment regimen in *Foxp3^{DTR-GFP}* or *Foxp3^{DTR-GFP}; R26^{DT};Foxp3^{Cre}* mice depleted of Tregs systemically. (B) Tumor growth curves of groups described, with yellow box showing early time-points where dual depletion mice demonstrated a delay in tumor control as compared to Treg depleted mice. Representative of 2 experiments performed shown.

Localization of Tregs and Ex-Tregs

To investigate if Tregs and ex-Tregs occupied different niches in secondary lymphoid organs, we utilized our Treg lineage trace mice to perform confocal imaging and two-photon live imaging. Work from previous undergraduate in the lab (Michael Jiang) demonstrated a preferential accumulation of ex-Treg cells vs Treg cells in the B cell zone of lymphoid organs, as shown by the significantly increased ex-Treg: Treg ratios (Figure 5.7A, B). In the two-photon live video of lymph nodes from naïve mice, we also observed an increased motility of ex-Treg cells compared to Treg cells, by following a specific ex-Treg cell across time. While preliminary, these data taken together suggest that ex-Treg cells may have increased capacities to migrate and interact with other lymphoid cells, contributing to an inflammatory environment by the secretion of their cytokines and enhancing priming of neighboring lymphocytes.

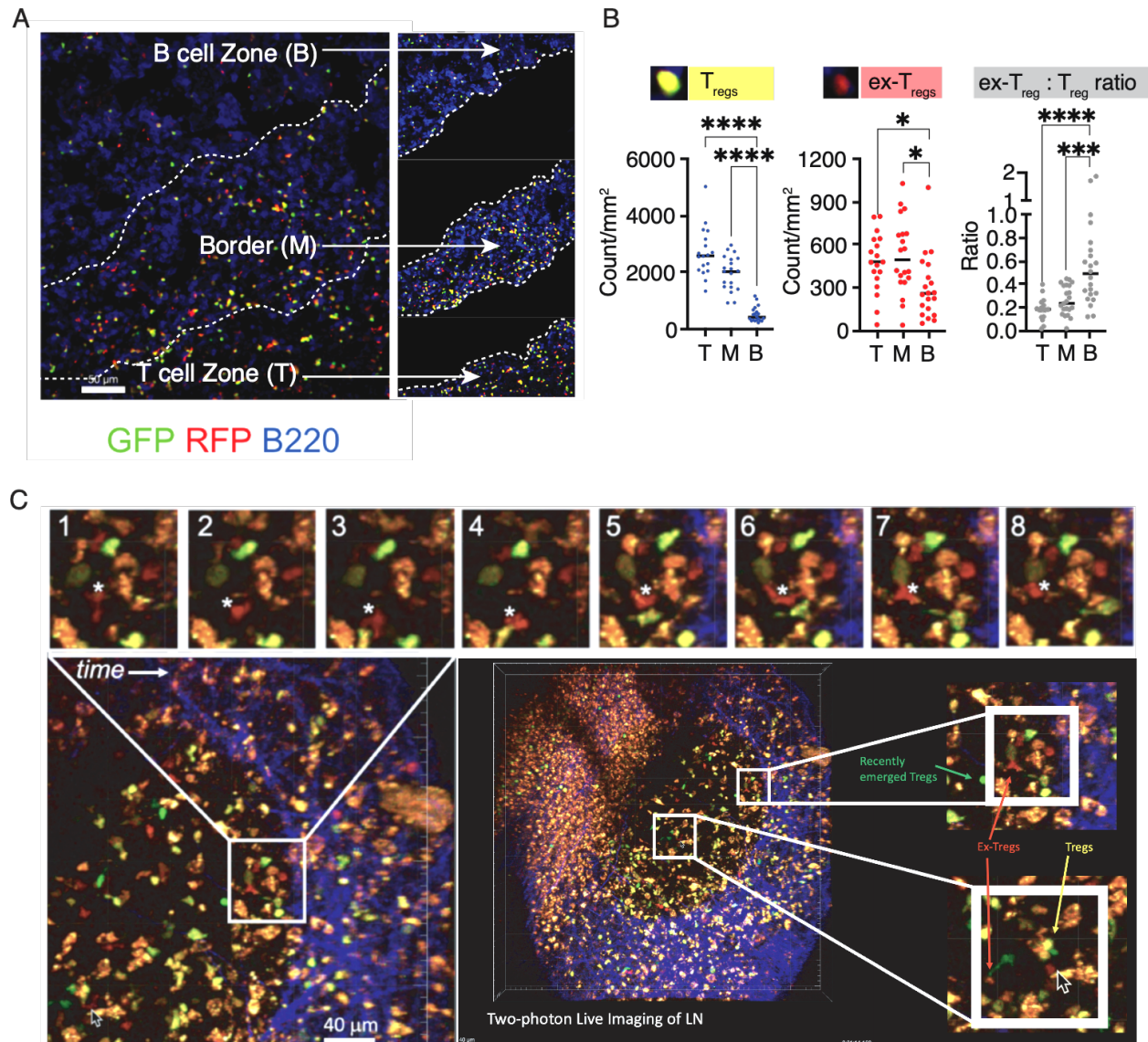


Figure 5.7: Treg and Ex-Treg Localization in Tissues. We used our Treg lineage trace mice to distinguish Tregs from ex-Tregs in lymph nodes in situ. (A,B) Using CellProfiler, we saw Tregs and ex-Tregs accumulate most prevalently in the T cell zone and its border with the B cell zone (anti-B220+) in lymph nodes, ex-Tregs were found at a significantly greater proportion to Tregs in the B cell zone. (C) Still images from two-photon microscopy of lymph node in Treg lineage trace mice acquired over five minutes. Top, sequential images left to right over time. A single ex-Treg (*) is highlighted to move and interact with other Tregs. Below, large picture of lymph node shows majority of cells are positive for red and green fluorescence (yellow) indicative of normal Tregs. RFP only (red) cells are ex-Tregs of which there are few. Some recent thymic emigrant Tregs (GFP only, green) can also be identified. Blue = second harmonic generation.

DISCUSSION

The conversion of regulatory T cells (Tregs) into effector-like “ex-Tregs” represents a critical interface between immune tolerance and immunity. Our findings showing that ex-Tregs can directly contribute to inflammatory amplification and cytotoxic T cell recruitment within the tumor microenvironment. Such ex-Tregs could thus represent an unappreciated immunogenic module arising destructively in autoimmunity but beneficially in cancer immunotherapy scenarios.

An important conceptual advance from these results is the recognition that ex-Tregs might not merely be “dead” or “dysfunctional” remnants of Tregs but can actively participate as cytokine-producing, metabolically primed effector cells. This raises the possibility that *Ezh2*-dependent chromatin remodeling is one of several molecular thresholds establishing Treg lineage rigidity. Comparing epigenetic and transcriptional profiles of *Ezh2*-deficient ex-Tregs to other instability models, such as cytokine induced destabilization, may help identify shared instability nodes. Similarly, spatial mapping of ex-Treg niches relative to dendritic cells compared to other effector cells using high-plex imaging could clarify whether ex-Tregs act locally through IFN- γ -dependent conditioning of antigen-presenting cells or more distantly via cytokine gradients or other mechanisms.

An interesting parallel that could be drawn between Chapters 4 and 5 is the link between cytotoxic CD4⁺ T cells and ex-Treg cells. IFN- γ has been shown to play a central role in converting stable FOXP3⁺ Tregs within the tumor microenvironment into a “fragile” state (Overacre-Delgoffe, Chikina et al. 2017). Using neuropilin-1 (Nrp1)-deficient mice, the authors show that loss of *Nrp1*-deficiency destabilizes intra-tumoral Tregs, driving their fragility and augmenting anti-tumor immunity. IFN- γ signals were both necessary and sufficient to induce this fragile (or ex-Treg-like) program, and elevated IFN- γ within tumors drove Tregs to upregulate Th1 genes and lose suppressive function. Notably, the presence of fragile Tregs was essential for the therapeutic effect of anti-PD1 checkpoint blockade, underscoring that Treg destabilization by IFN- γ is a prerequisite for effective immune-mediated tumor clearance (Overacre-Delgoffe, Chikina et al. 2017). We noted a similar delay in tumor control in ex-Treg deplete mice and in *Ifngr1*^{-/-} mice. Given that ex-Tregs require intact IFN- γ signaling for their induction and functional activity, this

delay may reflect the absence of ex-Treg-mediated early tumor control following IT Treg depletion. These findings suggest that IFN- γ -driven conversion or activation of ex-Tregs may be crucial for the rapid engagement of immune effector pathways, linking CD4⁺ T cell cytotoxicity with Treg plasticity in orchestrating timely anti-tumor responses. In Chapter 3, we did also note increased IFN- γ production by NK cells after IT Treg ablation, but we did not investigate if other cells such as CD8⁺ T cells, CD4⁺ T cells, or even these ex-Treg cells may be the primary contributors to the elevated IFN- γ signature within the tumor microenvironment. In Chapter 4, bulk RNA sequencing data showed an enriched IFN- γ signature on the CD4⁺ T cells when comparing IT DT to IT PBS samples, strongly suggesting that CD4⁺ T cells themselves can secrete IFN- γ . We plan to also use our existing lineage trace and depletion mouse models to characterize the ex-Treg population, to see if they similarly express cytotoxic markers and/or NK cell receptors. Further, it will be interesting to see if these self-reactive ex-Treg populations within the tumor act as “catalysts” in driving the differentiation of cytotoxic CD4⁺ T cells, such that the depletion of ex-Tregs in addition to Tregs result in a lower induction of NK cell-like, cytotoxic CD4⁺ T cells.

Further, we have yet to dissect the origin of these ex-Treg cells as coming from initial FOXP3⁺ Tregs, or from Tconv cells that have transiently upregulated *Foxp3* and then lost it in subsequent inflammatory conditions. In a preliminary transfer colitis experiment, we expected the highly self-reactive ex-Treg cells co-transferred with naive T cells to contribute to an earlier onset of disease compared to T effector cells co-transferred with naive T cells. Instead, we did not observe any difference in disease score or onset (Figure 5.8). While cell numbers in this pilot experiment were small compared to our standards (note no disease with Tnaive only, or Teff + Tnaive, and minimal weight loss with Teff only) and might address the disease phenotype, it would be possible that like Miyao et al had observed, the co-transfer of FOXP3⁻ Tconv cells may actually prevent the accumulation of ex-Treg cells. It would be interesting to track the populations using genetic fate mapping (e.g. Ly5.1 cells) and also to TCR map the FOXP3⁻ cells to determine the origin of these cells. In addition, we also think that how these ex-FOXP3 cells were derived can make a difference in terms of their inflammatory activity or expansion capacity in lymphocyte-depleted recipients. As an example, the FOXP3⁻ cells sorted for transfer were derived from naïve mice and represented only 5-10% of the FOXP3-lineage trace population, but if we were to first

induce autoimmunity or other conditions to destabilize FOXP3 expression in the host mice before isolating these ex-FOXP3 cells, the effects might potentially be different.

These data have important therapeutic implications. Clinical efforts to deplete Tregs are hampered by severe immune-related toxicities and the risk of unchecked autoimmunity, highlighting an urgent need for safer strategies. Rather than pursuing broad systemic ablation, selectively inducing intrinsic Treg instability, either pharmacologically or through transient epigenetic modulation, could stimulate in situ ex-Treg conversion and weaken intra-tumoral immune suppression without compromising systemic tolerance. It would also be interesting to test if CCR8 is expressed on ex-Tregs, and whether anti-CCR8 depletion might (or might not) deplete ex-Tregs. Furthermore, the finding that immunotherapy responsiveness may depend on the degree of Treg fragility reinforces the concept that precise modulation of Treg stability in the tumor microenvironment could enhance anti-tumor immune activation while minimizing collateral autoimmune risks. This shifts the focus toward understanding and harnessing Treg plasticity as a therapeutic target, possibly opening novel avenues to potentiate immunotherapy efficacy and safety in cancer.

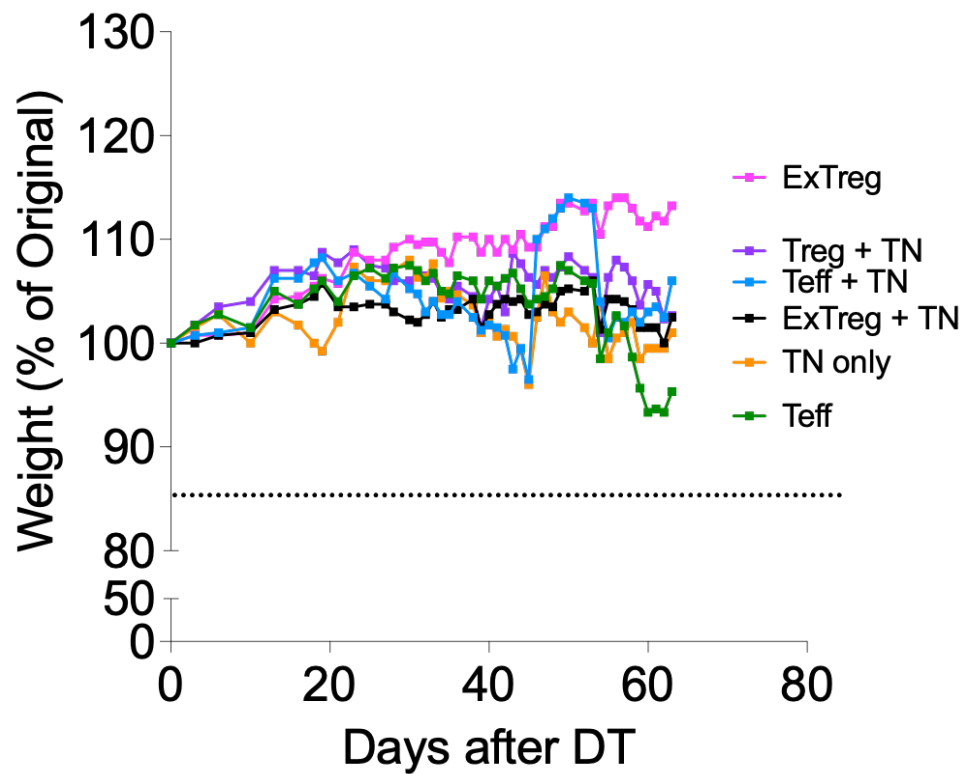


Figure 5.8: Transfer Colitis experiment. Tracking changes in weight over time for *Rag2*^{-/-} recipients transferred with cells derived from lineage trace hosts. 100K cells in total were transferred to each recipient mice; in cases with mixed transfers, cells are mixed at a 1:1 ratio.

Chapter 6: Genetically Engineered Mouse Models of Cancer

Abstract

Genetically engineered mouse models (GEMMs) have transformed the study of tumor biology by enabling precise, tissue-specific control of gene expression to model human cancers in their native microenvironment. Traditional approaches for modeling cancer in mice have enabled many key discoveries in cancer biology and treatment strategies. These include transplantable tumor models by the injection of established murine tumor cell lines (syngeneic models), or the implantation of human tumor fragments or cell suspensions into immunodeficient mice (xenograft models). However, limitations still hinder the translation of these tumor models back into effective therapies for patients in the clinic. The most significant limitation in these models is the assumption that cancer is homogenous, arising from a clone of cultured cells from the dish and thus respond synchronously to host immune responses. GEMMS permit tumor initiation from autochthonous cells, preserving interactions between stromal, immune, and epithelial compartments. The development of conditional recombination systems, notably the Cre/loxP platform, allowed the spatial and temporal activation of oncogenes, such as *K-ras*, and inactivation of tumor suppression genes, such as *Tp53*, to mirror the same mutational trajectories observed in patients. More recently, the integration of CRISPR-Cas9-based gene editing has enabled high-throughput *in vivo* modeling of compound genotypes and immune-escape mechanisms. Here, we describe the generation of various tumor models that are deficient or mixed in MHC I-expression, leveraging *K-ras^{LSL-G12D};Trp53^{fl/fl}* crossed to Cas9 backgrounds in combination with lentiviral delivery of Cre recombinase and *B2m*-targeting guide RNAs. This design faithfully recapitulates physiologic tumor initiation while enabling conditional loss of MHC I, allowing exploration of how immune pressure shapes tumor emergence and influences therapeutic responses.

Introduction

From the establishment of immortalized cell lines *in vitro* to the development of xenograft and syngeneic *in vivo* models, cancer research has progressively advanced toward greater mimicry of cancer initiation and progression in humans. The advent of genetically engineered mouse models (GEMMs) represented a true paradigm shift, allowing precise modeling of tumor initiation and progression within immune-competent hosts (Jacks, Remington et al. 1994, Jonkers and Berns 2002, Sharpless and Depinho 2006, Frese and Tuveson 2007, Nobel Prize Outreach 2007). In GEMMs, mouse oncogenes or tumor suppressor genes can be conditionally activated or deleted in defined cell types through Cre/loxP recombination. This approach has generated models such as the well-characterized *Kras*^{LSL-G12D};*Trp53*^{fl/fl} (“KP”) system, developed by Tyler Jacks and collaborators, which mirrors the histopathological and molecular features of human non-small cell lung cancer. Advances by Michel DuPage and colleagues further refined this framework, establishing next-generation lentiviral vectors capable of introducing *Cre* and engineered antigens into epithelial tissues, thereby modeling tumor–immune cell interactions and uncovering mechanisms of immunoediting and antigen loss (DuPage, Dooley et al. 2009, Feldser, Kostova et al. 2010, DuPage, Cheung et al. 2011, DuPage, Mazumdar et al. 2012). Such autochthonous models preserve the full immune architecture, making them invaluable for studying immune surveillance, T cell exclusion, and checkpoint blockade responses in comparison to cell-line transplants. Furthermore, Monte Winslow and others have pioneered the use of *in vivo* CRISPR/Cas genome engineering—combined with barcoding technologies like Tuba-seq—to enable multiplexed interrogation of tumor suppressor gene loss and epistatic interactions during lung adenocarcinoma evolution (Sánchez-Rivera, Papagiannakopoulos et al. 2014, Chiou, Winters et al. 2015, Rogers, Mcfarland et al. 2018).

As with previous chapters, I was highly interested in studying tumors that have escaped CD8⁺ T cell recognition due to loss of MHC I expression on tumors. In these studies, I applied this integrated GEMM-CRISPR system to explore immune escape through loss of MHC class I. By delivering *lenti-Cre-sgB2m* constructs into *KP-Cas9* mice, I generated *de novo* tumors that exhibit both oncogenic *Kras*^{LSL-G12D};*Trp53*^{fl/fl} mutations in addition to *B2m* loss, thus modeling MHC I-deficient tumor evolution within an immune-intact environment. This approach permits

real-time investigation of how tumor–immune interactions drive selective pressure, how cytotoxic CD8⁺ T cell recognition shapes clonal outgrowth, and how MHC I loss influences therapy resistance. Beyond its immediate scope, this model establishes a flexible platform for studying multigenic cooperation and adaptive immune editing under physiologic conditions—bridging the gap between traditional GEMMs and translational immuno-oncology.

RESULTS

Guides 3-1 (KO score: 17) and 2-2 (KO score: 50) were determined to be the most efficient editors and chosen to be cloned into the Lenti sgNT/Cre plasmid for in vivo editing.

After the generation and validation of constructs (see Methods section for construct engineering), virus was made by transfecting Lenti-X cells to test *B2m* knockout by transduction in Cas9 expressing cell lines. As seen in Figure 6.1, a significant portion of the Cas9-expressing cells lost MHC I expression as compared to the non-transduced control, confirming that the viral construct was effective. With these viruses, I proceeded to use them in a series of proof-of-concept experiments to generate multiple different GEMM cancers with MHC I-deficiency.

KP Sarcoma with Lenti sgB2mCre

Once confirmed with *in vitro* cultured cells, viruses were titrated and used *in vivo* in KP-Cas9 mice (*Kras*^{LSL-G12D/+}; *p53*^{fl/fl} *R26_LSL_Cas9/Cas9*) to induce spontaneous tumors. I inject the virus into the calf muscle of mice with approximately 10^4 – 10^5 infectious particles of Lenti-sgNT-Cre or Lenti-sgB2m-Cre. Tumors were palpable in mice after around three to four months post-virus injection. Our preliminary results with the KP-Cas9 sarcoma generation suggests that MHC I loss resulted in faster tumor formation, consistent with the conclusion that MHC I recognition by T cells delays tumor formation in this system, and contrary to the expectation that innate cells such as NK cells could recognize and eliminate MHC I-deficient tumors early after initiation based on missing-self recognition (Figure 6.2). While NK cells theoretically can recognize and kill MHC I-deficient tumor cells, this surveillance is often inefficient, particularly in cold tumor contexts, as well as poorly infiltrated tumors such as sarcomas. KP-driven tumor models also exhibit strong recruitment of suppressive subsets such as MDSCs and Tregs which can inhibit NK cell activity, or drive NK cell desensitization over long term non-engagement by inhibitory signaling in NK cells. While these inhibitory influences can also limit the antitumor activity of T cells, one or more of them may be more limiting for T cell function. In addition, NK cell engagement with target cells also depends on expression of activating ligands, which have been shown to be variably expressed in KP tumors. The loss of recognition by CD8⁺ T cells, along with the aforementioned

limitations of intra-tumoral NK cell function in the presence of Tregs, may explain the faster tumor progression that was observed in Figure 6.2.

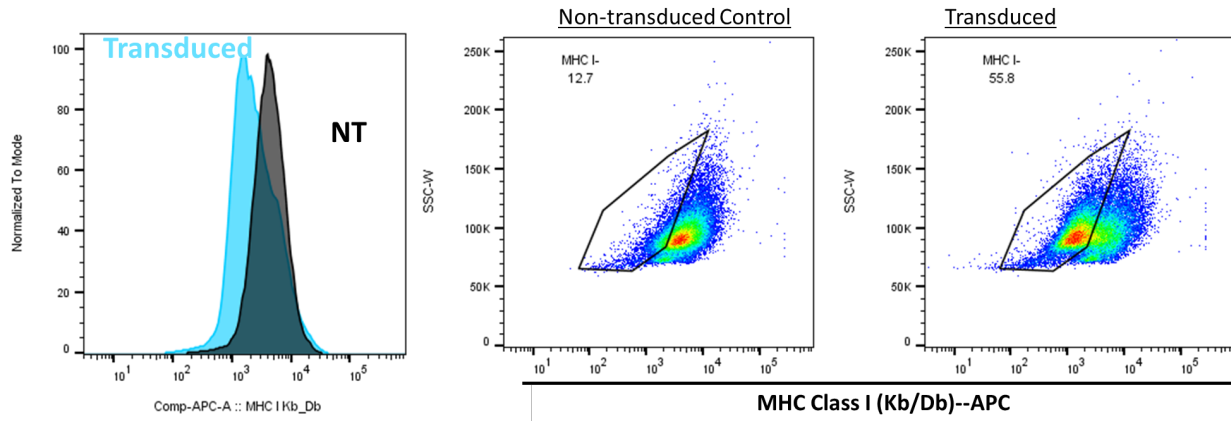


Fig 6.1: In vitro validation of MHC I knock-down with Lenti-sgB2m Cre. Representative histogram and flow plots showing downregulation of MHC I expression in mouse inguinal WAT pre-adipocyte cell line expressing dcas9 with and without transduction with virus containing the Lenti-sgB2m Cre plasmid. Cells were collected for analyses 24h after transduction. Validation was also repeated in neuronal Cas9-expressing cell line and immortalized cas9+ mouse macrophage progenitors.

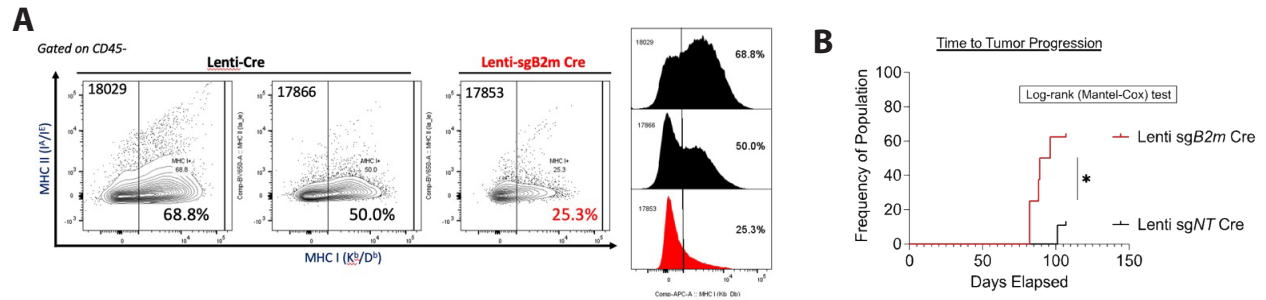


Fig 6.2: In vivo KP GEMM sarcoma induction with Lenti-sgB2m Cre constructs formed spontaneous tumors with varying levels of MHC I loss. (A) Representative flow plots and histograms showing downregulation of MHC I expression in sarcoma tumors collected from mice injected with virus containing either the Lenti-sgB2m Cre plasmid (red) or Lenti-sgNT Cre (black). (B) Tumor progression curve recording the days elapsed post virus injection for the emergence of tumors in mice injected with the Lenti-sgB2m Cre plasmid or Lenti-sgNT Cre.

KP Lung Adenocarcinoma with Lenti-sg*B2m*Cre

To test our Lenti-viral constructs in a different KP tumor model, I inoculated mice intra-tracheally as previously described (DuPage, Dooley et al. 2009), to induce lung adenocarcinomas. Similar to the KP sarcoma model, I observed that the lung model also revealed significant differences based on MHC I loss. There were visibly more and larger tumor nodules in the lung lobes from mice inoculated with Lenti-sg*B2m*-Cre compared to Lenti-sg*NT*-Cre viruses (Figure 6.3A), and I observed downregulation of MHC I expression in the individual tumor nodules with flow cytometry staining of H2-K^b/D^b (Figure 6.3B). We note that even in the mice inoculated with Lenti-sg*NT*-Cre viruses, there is also lower expression of MHC I than that from whole lung lysates (Figure 6.3B). Lower MHC I expression in tumor nodules compared to whole lung is commonly driven by a combination of tumor-intrinsic and selection-based mechanisms. Tumor cells frequently downregulate MHC I through genetic or epigenetic alterations as a means of immune evasion and escape from CD8⁺ T cell-mediated killing. Over time, immunoediting can selectively eliminate MHC I-high tumor cells, resulting in outgrowth of MHC I-low variants within the tumor mass, which is possibly the case in mice inoculated with Lenti-sg*B2m*-Cre. Additionally, whole lung contains a mixture of non-tumor stromal, endothelial, and immune cells that retain high MHC I expression, whereas isolated tumor nodules are enriched for transformed cells more likely to have lost surface MHC I. Thus, both cellular composition differences and tumor cell-autonomous loss of MHC I contribute to the lower overall MHC I levels observed in tumor nodules versus lung tissue.

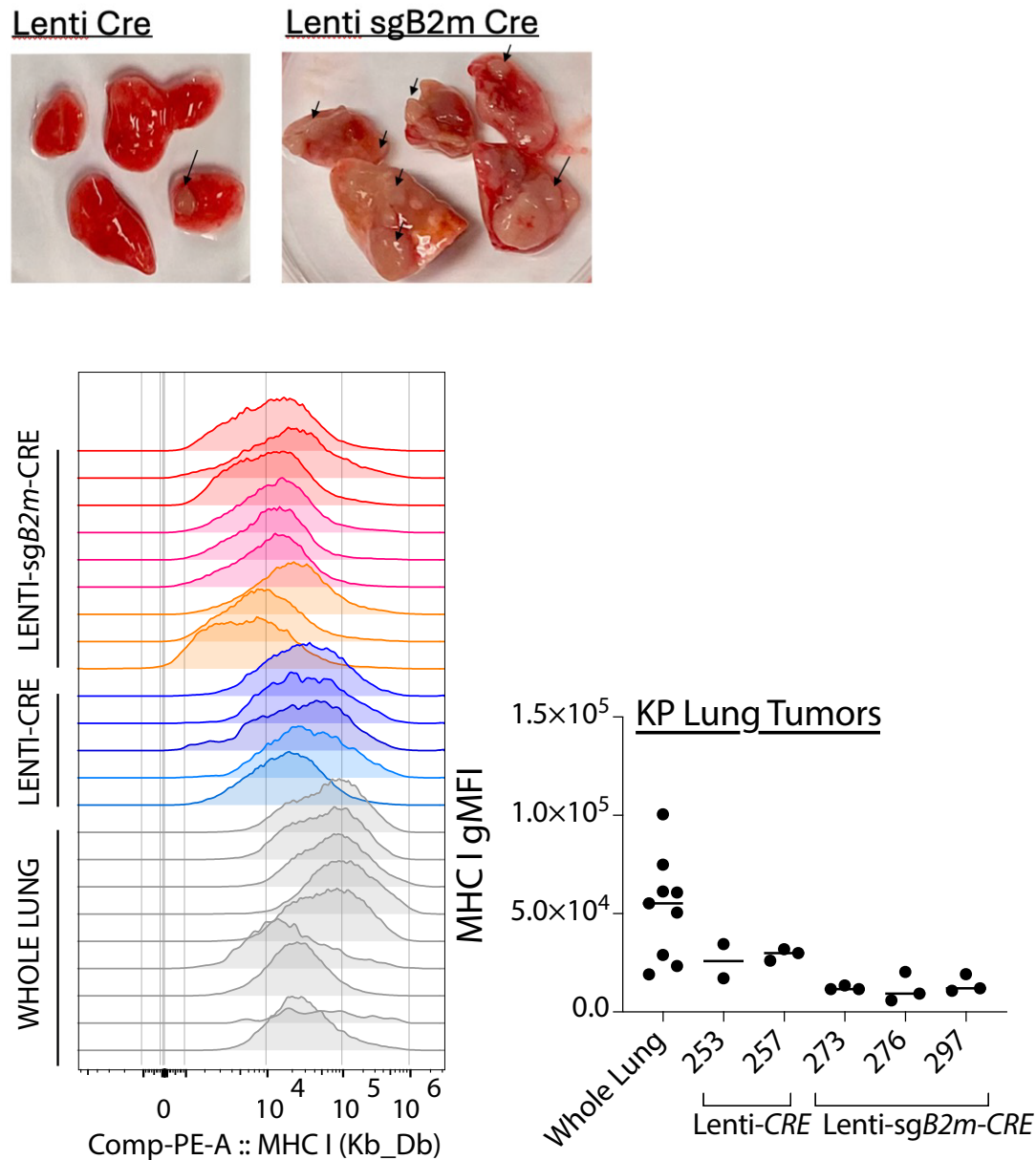


Fig 6.3: In vivo KP GEMM lung adenocarcinoma induction with Lenti-sgB2m Cre constructs formed spontaneous tumors with varying levels of MHC I loss. Representative images of lung lobes with arrows pointing to clonal tumor nodules from mice injected with virus containing either the Lenti-sgB2m Cre plasmid or Lenti-sgNT Cre. Quantification of MHC I staining in tumor nodules from mice injected with the Lenti-sgB2m Cre plasmid or Lenti-sgNT Cre. Mice 253 and 273 were inoculated with Lenti-Cre plasmid while mice 273, 276 and 297 were inoculated with Lenti-sgB2m-Cre plasmid.

KP Glioblastoma Model

Glioblastoma remains one of the most lethal neurological malignancies, as it is notoriously heterogeneous, with an abundance of signaling pathways even within the same tumor mass, thereby limiting the options for targeted therapies (Qazi, Vora et al. 2017). Moreover, the glioblastoma tumor microenvironments are also often considered immunologically “cold” and are devoid of important immune subsets such as T cells which can recognize and eliminate the tumors (Jackson, Choi et al. 2019, Frederico, Hancock et al. 2021, Simonds, Lu et al. 2021, Chen, Varanasi et al. 2023). The present failures of effect immune-checkpoint blockade therapy in glioblastomas highlight the therapeutic options for glioblastoma, and a need for using relevant glioblastoma tumor models in mice to parse out the mechanisms for resistance to immune control.

To facilitate tumor formation in the brain microenvironment, we injected 10^4 infectious units of Lenti-Cre virus directly into the brain parenchyma of KP mice via intracranial stereotactic injection. To mimic different subtypes of glioblastomas, we also varied the location of injections, to target different stem cell populations within the brain (see Methods section for coordinates of injections).

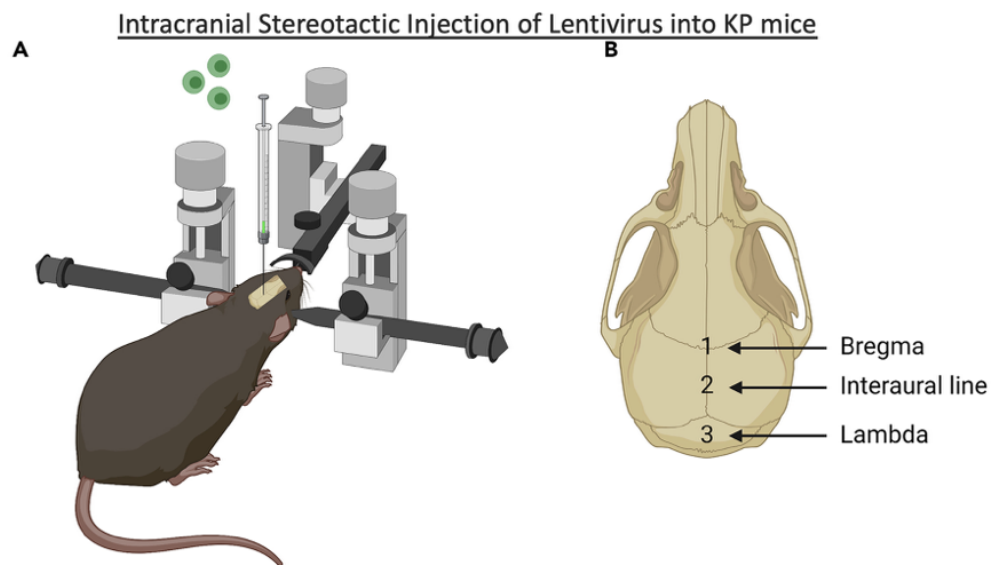


Fig 6.4: Stereotactic intracranial injection with Lenti-Cre constructs to generate spontaneous glioblastomas. (A) Representative images stereotactic set-up for intracranial injections/ Briefly, mice were anesthetized, and a region of the head was shaven to expose the scalp for a short incision to be made. Based on the locations of the bregma and lambda as shown in (B), the needle is localized to the coordinates of interest, and a burr hole is created before the needle is lowered into the region of interest in the brain.

MRI IMAGING

In order to capture the progression of glioblastoma, I used Magnetic resonance imaging (MRI) of the mouse brain, which is a noninvasive, high-resolution anatomical and pathological visualization that also allowed for repeated monitoring of mice throughout the experiment (Koutcher, Hux et al. 2002, Mcconville, Hambardzumyan et al. 2007). This work was in collaboration with Michael Wendland from UC Berkeley's Pre-clinical Imaging Core Facility.

MRI is based on the magnetization properties of atomic nuclei. In a typical imaging session, one begins with a localizer scan, often a fast 2D gradient-echo or spin-echo acquisition, low resolution in three orthogonal planes, to orient and prescribe subsequent axial, coronal, and sagittal slices. After positioning via the localizer, T_1 -weighted images (longitudinal relaxation time; rate at which spinning protons realign with the external magnetic field) are acquired to delineate anatomical contrast: gray matter, white matter, ventricles, and lesion boundaries. Next, T_2 -weighted imaging (transverse relaxation time, rate at which spinning protons lose phase coherence among the nuclei spinning perpendicular to the main field) highlights edema, necrosis, and heterogeneous tumor components.

To identify and delineate tumor masses, two different protocols were attempted:

First, we used diffusion weighted imaging (DWI), which was designed to detect the random movements of water protons. As water movement becomes restricted intracellularly, this results in an extremely bright signal on DWI. The phases of stationary spins are unaffected by the DG pair since any phase accumulation from the first gradient lobe is reversed by the second. Diffusing spins, however, move into different locations between the first and second lobes, falling out of phase and losing signal. Therefore, we should expect to see that areas that are white in T_2 -w scans become black in the diffusion-w scans if they are fluid (use ventricles as control for comparison); if it is a lesion, in contrasts, it will remain white.

The second method is to use gadolinium (Gd)-based contrast agents. Gadolinium is not directly seen in an MRI image, but these agents are paramagnetic chelates, and manifest their presence indirectly by facilitating the relaxation of nearby hydrogen protons (shorten the T_1),

thereby increasing signal in tissues where the contrast agent accumulates and rendering them bright (Ibrahim, Hazhirkarzar et al. 2025). A standard workflow is: acquire a pre-contrast T_1 image, then inject contrast (often ~ 0.1 mmol Gd/kg in rodents), allow a delay (e.g. ~ 4 – 10 minutes for uptake), and then acquire post-contrast T_1 images using the same geometry and parameters. Subtraction or comparison between pre- and post-contrast images highlights enhancing tumor regions.

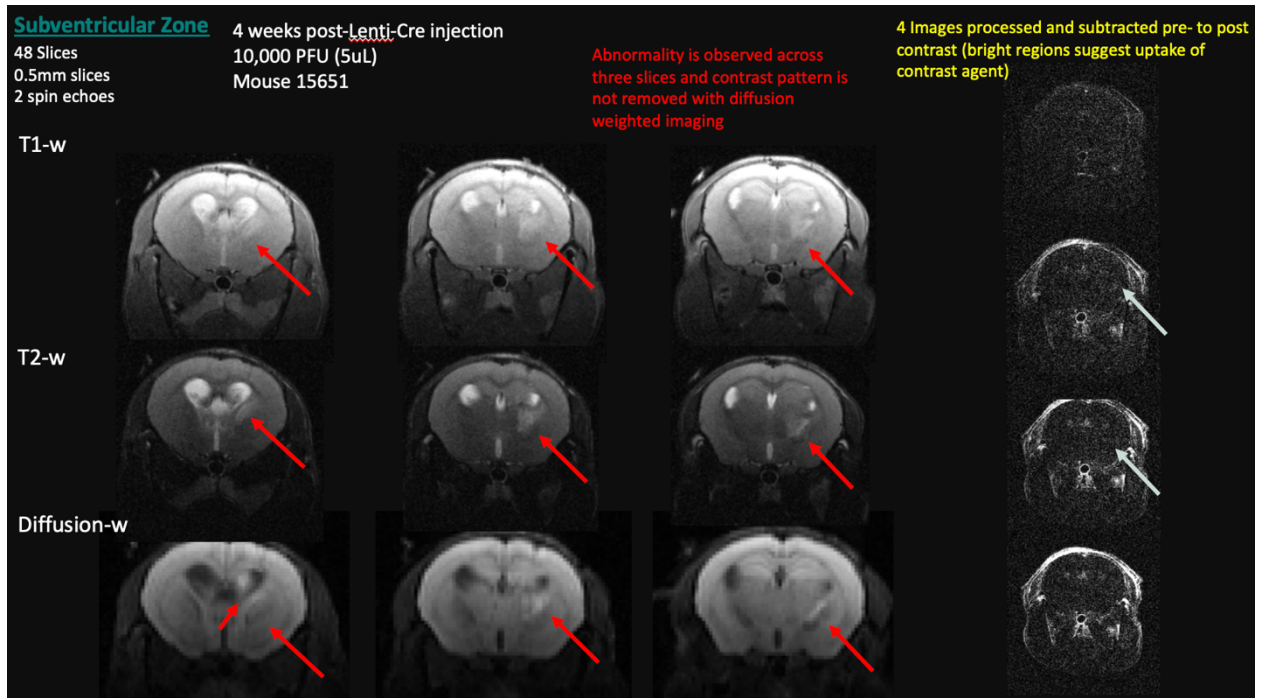


Fig 6.5: Early-stage MRI brain imaging with diffusion weighted imaging (DWI) and contrast agent. T1, T2, DWI and processed post to pre-contrast images of selected slices of brain from mice four weeks after injection with Lenti-Cre. Red arrows point to potential areas of tumor masses.

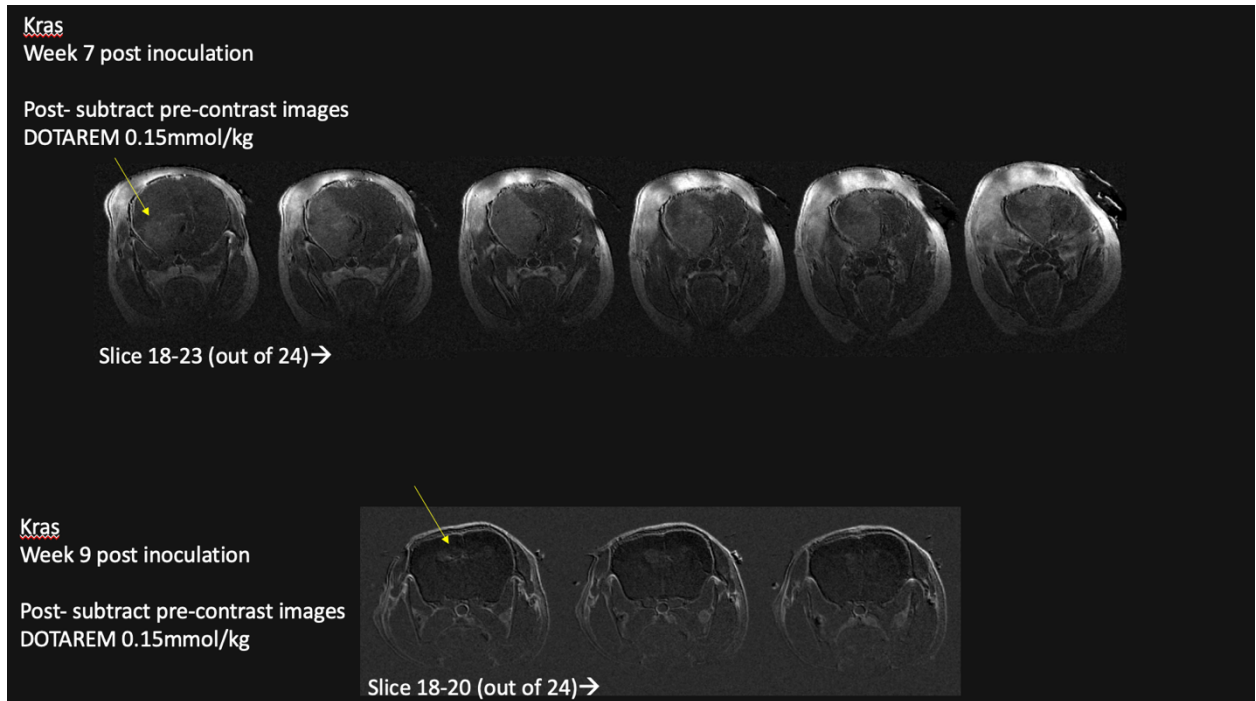


Fig 6.6: Late-stage MRI brain imaging with contrast agent. Processed post to pre-contrast images of selected slices of brain from mice 7- and 9 weeks post injection with Lenti-Cre. Yellow arrows point to potential areas of tumor masses.

FUTURE DIRECTIONS

The genetically engineered mouse models (GEMMs) discussed in this chapter provide a versatile and powerful platform to explore immune cell dynamics within complex, physiologically relevant tumor microenvironments that closely mirror human cancer heterogeneity. Traditional transplantable tumor models often lack the genetic diversity, native stromal architecture, and evolving immune landscapes characteristic of spontaneous cancers. However, GEMMs allow tumors to arise *de novo* in immunocompetent hosts, preserving the spatial and functional interactions between tumor cells, stromal components, and immune populations. This fidelity is critical for dissecting how genetic alterations, immune evasion mechanisms, and microenvironmental cues unfold during tumor development and progression.

Implementing GEMMs that model variable MHC class I expression adds an additional layer of clinical relevance by recapitulating tumor immune escape via partial or complete loss of antigen presentation, a common feature in human cancers. These models facilitate investigations into how immunotherapies impose selective pressures that shape tumor evolution, the emergence of resistant clones, and the remodeling of tumor-immune cell crosstalk under conditions of heterogeneous antigen expression. The intra-tumoral heterogeneity achieved in GEMMs also enables study of niche-specific immune regulation and the spatial organization of immune infiltrates.

The integration of CRISPR-based genome editing enhances GEMM utility by allowing precise, combinatorial targeting of cancer-relevant oncogenes and tumor suppressors (for e.g., PTEN, TP53, EGFR, PIK3CA, NF1, RB1, and PDGFRA) in a tissue- and time-specific manner. This approach facilitates rapid generation of genetically complex tumors that more faithfully represent the mutational landscapes and clonal heterogeneity seen in patients. Furthermore, CRISPR-GEMMs enable high-throughput functional screening of mutations and their impacts on tumor immunogenicity, immune editing processes, and responsiveness to checkpoint blockade or other immunomodulatory therapies. This capability provides a powerful preclinical platform for identifying vulnerability nodes and rational combination therapies tailored to specific genetic contexts.

In summary, these GEMM and CRISPR-GEMM models serve as indispensable tools to bridge the gap between simplified tumor systems and the clinical complexity of cancer. They empower incisive exploration of mechanisms underlying tumor-immune interactions, immune escape, and therapeutic resistance in settings that incorporate native immune regulation, genetic diversity, and microenvironmental heterogeneity. These models thus represent critical advances for designing and testing next-generation immunotherapies with improved precision and translational relevance. My preliminary findings that introduced MHC I-deficiency accelerates primary sarcoma formation and results in increased tumor incidence in the lung KP model, thus providing an important tool for future investigations of the consequences of MHC I loss in primary tumors.

Chapter 7: Novel Activation of Immune Responses with Tetracycline Compounds

Abstract

Tetracyclines, originally developed as broad-spectrum antibiotics, are potent inhibitors of both bacterial and mitochondrial translation. Inhibition of translation in the mitochondria of *C. elegans* or mammalian mitochondria has been shown to activate a conserved stress response known as the mitochondrial unfolded protein response (UPR_{mt}). It has been reported that certain tetracycline analogs, presumably by virtue of inhibiting mitochondrial translation and thus stimulating the UPR_{mt}, may activate the immune response. Because tetracyclines alter the microbiota, which may indirectly impact the outcomes of infections, the most informative studies have employed variants of tetracyclines with reduced or absent anti-microbial activity, which nevertheless retain the capacity to activate the UPR_{mt}.

One such drug is 9-tert-butylodoxycycline (9-TB). 9-TB administration was shown to protect mice from lethal influenza virus infections. An in vitro study suggested that 9-TB may induce production of type I interferons by immune cells. I investigated whether 9-TB and a second novel tetracycline variant anhydro-doxycycline (AD-11 or AD hereafter), which may also lack antimicrobial activity, exhibit antitumor activity when administered to mice with established MC38 colorectal tumors. Both compounds were effective when administered intra-tumorally. The antitumor effects were independent of the canonical cGAS–STING cytosolic DNA sensing pathway but relied heavily on T cell activation and interferon signaling. Animals that cleared tumors after treatment exhibited long-term immunological memory, as the mice rejected subsequent tumor rechallenges. Collectively, these findings uncover a previously unappreciated mitochondrial–immune coupling mechanism: mild mitoribosomal stress via tetracycline analogs serves as a danger signal that mobilizes adaptive immunity and can elicit durable tumor control.

INTRODUCTION

Mitochondria are central organelles that sustain cellular fitness and energy homeostasis through ATP production, biosynthesis, and metabolic signaling (Harrington, Ryter et al. 2023). As such, cells have evolved sophisticated surveillance mechanisms to monitor mitochondrial health and mount adaptive responses to stress. Under adverse conditions, a variety of mitochondrial stress responses (MSRs) can be activated to restore organellar and cellular equilibrium (Houtkooper, Mouchiroud et al. 2013). The nature of these responses depends on the type and severity of the insult: for example, mitophagy targets and degrades dysfunctional mitochondria, while accumulation of unfolded or misfolded proteins in the mitochondrial matrix triggers the mitochondrial unfolded protein response (UPR^{mt}) (Houtkooper, Mouchiroud et al. 2013, Moullan, Mouchiroud et al. 2015).

The Mitochondrial Unfolded Protein Response (UPR^{mt})

The UPR^{mt} was initially described in mammalian cell culture models but subsequently dissected in genetic screens in *C. elegans* (Yoneda, Benedetti et al. 2004, Nargund, Pellegrino et al. 2012, Haynes and Hekimi 2022), and later in yeast (Schleit, Johnson et al. 2013) and *D. melanogaster* (Pareek and Pallanck 2018). The UPR^{mt} is a specific stress response in the mitochondria triggered by the accumulation of unfolded or misfolded proteins within the mitochondrial matrix (Zhao, Wang et al. 2002). A key player in this pathway is the transcription factor ATFS-1 (or ATF5 in mammals), which coordinates the induction of nuclear genes encoding mitochondrial chaperones, proteases, antioxidant enzymes, and components of the mitochondrial import machinery (Fiorese, Schulz et al. 2016, Shpilka and Haynes 2018). ATFS-1/ATF5 possesses both a mitochondrial targeting sequence (MTS) and a nuclear localization sequence (NLS). Under physiological conditions, it is imported into mitochondria and rapidly degraded. However, during mitochondrial dysfunction (e.g., loss of membrane potential or increased proteostatic burden), mitochondrial import efficiency drops, allowing ATFS-1/ATF5 to be imported instead into the nucleus, where it drives expression of genes that help alleviate mitochondrial stress (Nargund, Pellegrino et al. 2012, Fiorese, Schulz et al. 2016).

In mammals, the UPR^{mt} is closely linked to the integrated stress response (ISR). Mitochondrial stress activates one of several upstream kinases—including GCN2, which senses amino acid depletion and ROS and can be triggered by mitoribosomal stalling—to phosphorylate the translation initiation factor eIF2 α . This results in a global decrease in protein synthesis, but paradoxically increases translation of select transcription factors, including ATF4, CHOP, and ATF5, due to their 5' UTR upstream open reading frames (uORFs) (Torres, Fleischhart et al. 2024). These transcription factors, in turn, upregulate genes necessary to recover proteostasis and redox balance, thus reinforcing and amplifying the mitochondrial stress response. It has been reported that the UPR^{mt} is activated in different diseases where mitochondrial dysfunction is critical in driving the disease, such as in cardiac diseases (Smyrniak, Gray et al. 2019), kidney diseases (Liu, Zhang et al. 2023), mitochondrial diseases (Suárez-Rivero, Pastor-Maldonado et al. 2022), neurodegenerative diseases (Beck, Mufson et al. 2016, Cooper, Machiela et al. 2017) and cancer (Kumar, Lu et al. 2007, Sun, Jefferson et al. 2020, Inigo, Kumar et al. 2021). In cancer, it has been shown that the activation of UPR^{mt} is related to cancer progression, and research on the therapeutic approaches have focused on the inhibition of the UPR^{mt}. Still, the regulation of UPR^{mt} is less studied, and more information on how to safely modulate the UPR^{mt} while avoiding the detrimental effects from its long-term activation is crucial for designing therapeutics.

The link between UPR^{mt} activation and host resilience was recently exploited through the discovery of novel tetracycline derivatives, such as 9-TB and AD. 9-TB was generated through position-specific modifications of the tetracycline scaffold, were screened in *C. elegans* and mammalian cells for their ability to activate mitochondrial stress responses without antimicrobial activity. Notably, 9-TB was also found to enhance survival in lethally infected influenza models while preserving gut microbiota integrity, highlighting the therapeutic potential of selective UPR^{mt} inducers (Mottis, Li et al. 2022). AD, on the other hand, is a completely novel compound and has not been published as of yet.

The relationship between mitochondrial stress responses and immune activation has typically been framed in the context of the cGAS–STING pathway, which detects cytosolic mitochondrial DNA (mtDNA) as a danger signal and activates type I interferon signaling

(Rongvaux, Jackson et al. 2014). Our lab has shown that injections of STING agonists, such as cyclic dinucleotides (CDNs), can super-activate STING in non-tumor cells in the tumor microenvironment, thereby stimulating T- and NK cell-mediated tumor control (Nicolai, Wolf et al. 2020, Wolf, Blaj et al. 2022). However, our findings presented here demonstrates that UPR^{mt} induction by tetracycline derivatives such as 9-TB and AD leads to potent antitumor immunity even in the absence of functional cGAS–STING signaling. This indicates that mitochondrial stress can engage immunosurveillance programs and promote antitumor responses via alternative, STING-independent routes. Consistent with this model, the therapeutic benefits of UPR^{mt} activation are abolished in T cell–deficient hosts, highlighting a critical role for adaptive immunity—specifically, the expansion and activation of cytotoxic T cells—in mediating tumor control. This positions mitochondrial proteotoxic stress as a key upstream modulator of antigen-presenting cell function, inflammatory cytokine production, and T cell–dependent immunological memory, establishing a new axis through which metabolic cues can influence tumor immunity independently of canonical innate DNA sensing.

RESULTS

Low doses of tetracycline derivatives delivered intra-tumorally elicit tumor control in mice.

We have previously demonstrated the strong anti-tumor effects of a single intra-tumoral dose of STING agonist cyclic dinucleotide (CDN) in mice bearing MC-38 tumors. While anti-viral responses have been demonstrated with 9-TB, the potential anti-tumor effects of the tetracycline derivatives have not been studied. We first performed a dose titration of 9-TB along with either intra-peritoneal (IP) or intra-tumoral (IT) delivery in mice bearing MC-38 tumors, either a single dose or three total doses (once every three days) ranging from 5 ug to 500 ug and monitored tumor growth over a period of three weeks (Figure 7.1B). Strikingly, we saw that three 5 ug doses gave rise to significant tumor control, while three higher doses (50ug) failed to do so (Figure 7.1C). Single doses were generally ineffective. With AD we saw a similar dose titration where significant tumor control was achieved with low (0.1-5 ug, 3X) doses of AD, and was lost with a high (50 ug, 3X) IT dose (Figure 7.1D). In experiments to date, we have not seen an effect with IP dosing.

To test if the TLR signaling pathway is responsible for the tumor control by tetracycline derivatives, we performed similar tumor experiments, comparing wild-type C57BL/6 mice with *Unc/TLR2/4^{-/-}* mice, which lack all TLR function. The TLR-deficient mice were co-housed with the WT controls post-weaning at 21 days of age for a period of 3-4 weeks. We saw that the tumor control was independent of TLR signaling, as *Unc/TLR2/4^{-/-}* mice displayed similar levels of tumor control as WT mice when dosed with 9-TB (1 ug, 3X) (Figure 7.1E). The tumor control was also independent of the cGAS-STING pathway, as demonstrated with the use of *Sting^{gt/gt}* (STING-deficient) and *Cgas^{-/-}* mice (Figure 7.1F-G). However, type I IFN signaling was shown to play a significant role in 9-TB-dependent tumor control, as tumor control in 9-TB treated tumors was lost in *Ifnar1^{-/-}* mice, which lack the receptor for type I interferon, or in WT mice in which the receptor was blocked by administering IFNAR antibodies (Figure 7.1H).

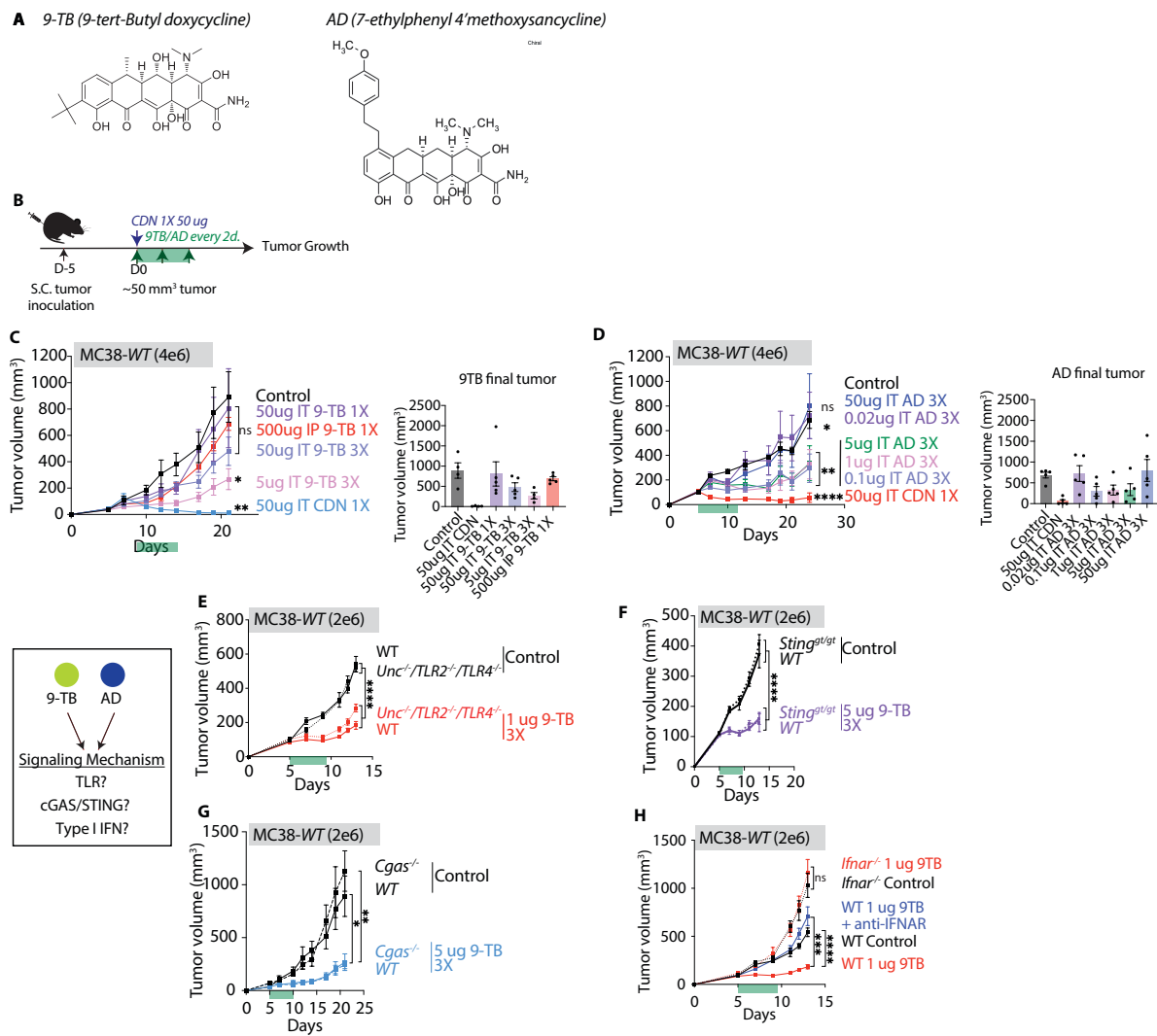


Figure 7.1: Immune activation by tetracycline derivatives induces anti-tumor responses.

Figure 7.1: Immune activation by tetracycline derivatives induce anti-tumor responses.

(A) Chemical structures of 9-TB and AD. (B) Schematic of tumor inoculation and treatment regimen. (C) Growth curves of indicated *MC38* tumors in mice that were treated with varying doses (5, 50, or 500 ug) of 9-TB via either systemic intra-peritoneal (IP) or intra-tumoral (IT) injections, and either for 1X dose or 3X dose once every 2 days. CDN treated mice were included as an additional control with known tumor control effect. Bar graph shows comparison of final tumor volume at D21 post-tumor inoculations. (D) Growth curves of indicated *MC38* tumors in mice that were treated with varying doses (0.02, 0.1, 1, 5, or 50 ug) of AD via IT injections, for 3X dose once every 2 days. CDN treated mice were included as an additional control with known tumor control effect. Bar graph shows comparison of final tumor volume at D24 post-tumor inoculations. (E) Growth curves of indicated *MC38* tumors in WT or *Unc^{-/-}/Tlr2^{-/-}/Tlr4^{-/-}* mice that were treated with 1 ug of 9-TB via IT injections for 3X dose once every 2 days. (F) Growth curves of indicated *MC38* tumors in WT or *Sting^{gt/gt}* mice that were treated with 5 ug of 9-TB via IT injections for 3X dose once every 2 days. (G) Growth curves of indicated *MC38* tumors in WT or *Ifnar1^{-/-}* mice, or WT mice given anti-IFNAR antibodies, that were treated with 1 ug of 9-TB via IT injections for 3X dose once every 2 days. (H) Growth curves of indicated *MC38* tumors in WT or *Cgas^{-/-}* mice that were treated with 5 ug of 9-TB via IT injections for 3X dose once every 2 days.

RM two-way ANOVA followed by Geiser-Greenhouse correction. (n=5-8 mice/group). All comparisons are performed pairwise against the PBS group with into account time as a factor. Statistic shown reflect total column (treatment) effect.

T cells are required for tetracycline-driven tumor control

To confirm if immune cells are responsible for the control of tumors, we performed a series of *in vivo* cellular depletions immediately before administering 9-TB therapy (3 doses of 1 ug). The results showed that tumor control was dependent on CD4⁺ and/or CD8⁺ T cells, but not NK cells (Figure 7.2A). Furthermore, *Rag2*^{-/-} *Il2rgc*^{-/-} mice, which lack T, B and NK cells, were unable to reject the tumors after 9-TB administration (Figure 7.2B). One important conclusion from these studies is that the antitumor effect is not mediated by direct inhibition of tumor cell growth, or initiation of tumor cell death, but instead requires participation of the immune system. The results suggest that 9-TB is acting to elicit a T-cell-dependent anti-tumor response, though the data do not address whether the drug is acting directly on T cells or indirectly on other cells. To test if a memory response was driven by exposure to the tetracycline derivatives, we re-challenged mice that had been previously cured 9 months earlier as a result of AD administration (from Fig. 7.1D). The results showed that these mice, similar to mice cured as a result of CDN administration, rejected subsequently administered doses of MC-38 tumor cells, indicating that a strong memory recall response had been elicited (Figure 7.2C).

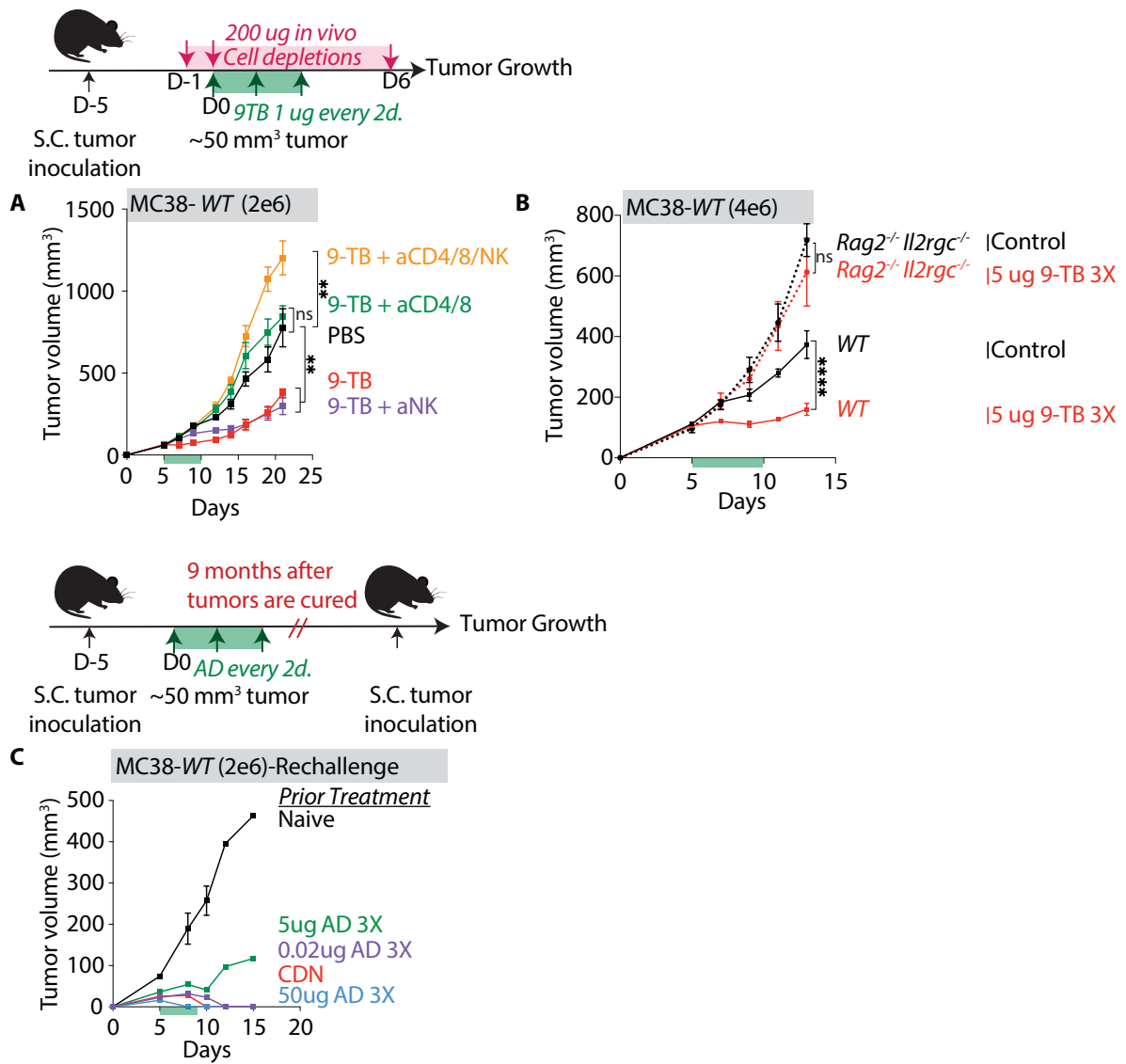


Figure 7.2: Tetracycline derivatives drive T-cell dependent anti-tumor responses.

Figure 7.2: Tetracycline derivatives drive T-cell dependent anti-tumor responses.

(A) Growth curves of indicated *MC38* tumors in mice that were treated with 5 ug of 9-TB via intratumoral (IT) injections, for 3X dose once every 2 days. Mice were treated with 200 ug of various antibodies for *in vivo* cellular depletions, started one day before 9-TB and dosed every six days. (B) Growth curves of indicated *MC38* tumors in WT or *Rag2Il2rgc^{-/-}* mice that were treated with 5 ug of 9-TB via IT injections for 3X dose once every 2 days. (C) Rechallenge of mice that had been cured of MC-38 tumors 9 month earlier as a result of receiving various doses of AD (see Fig. 7.1D) with the same tumor cells (MC-38). Naïve mice and mice cured as a result of CDN therapy were used as controls.

RM two-way ANOVA followed by Geiser-Greenhouse correction. (n=5-8 mice/group). All comparisons are performed pairwise against the PBS group with into account time as a factor. Statistic shown reflect total column (treatment) effect.

DISCUSSION

The control of tumors following the injection of 9-TB indicates the engagement of a novel innate immune pathway with striking capacity to elicit robust antitumor immune responses. Going forward, many exciting questions remain. With the removal of antibacterial effects of tetracyclines on host microbiome, and the superior ability to induce UPR_{mt} as compared to doxycycline, 9-TB and AD show therapeutic potential in decreasing the severity of infection (Mottis, Li et al. 2022), as well as driving a T cell-mediated tumor control as shown herein.

To continue to probe at the mechanism of immune activation by tetracycline derivatives, efforts are being made in understanding the change in immune landscape following 9-TB and AD treatment in mice. The lab will focus efforts at identifying a marker which can be used to sort on activated cells and perform an unbiased screen. This would be a combination of immune activation markers and mitochondria activation markers that can be assayed via spectral flow cytometry. To identify molecular mechanisms that may be contributing to the immune-mediated control of 9-TB/AD treated tumors, bulk and single-cell RNA sequencing (scRNA seq) of immune cells (macrophages, dendritic, NK, CD4 and CD8 T cells) from the tumor, spleen and draining lymph nodes of tumor-bearing mice at various times after treatment with 9-TB will be conducted. We will also profile various cytokine and mitochondrial pathways that may be altered in either tumor or immune cells following exposure to 9-TB/AD. Further, we are also interested in testing different tumor models beyond MC-38, such as B16-F10 and RMA tumor cell lines, as well as whether MHC class I expression is required for response by using tumor cell lines that have been engineered to be MHC I deficient (*B2m*^{-/-}). Together, these analyses should identify innate immune pathways that are key regulators of UPR_{mt} and elucidate the mechanisms that contribute to the antitumor responses seen following intra-tumoral treatments with the tetracycline derivatives. A key aim will be to develop a molecular understanding of how the UPR_{mt} engages the immune response.

One interesting link of UPR^{mt} and tetracyclines is through the widespread use of antibiotics such as Doxycycline in the control of gene expression. Since many genes are essential either for cellular function and/or animal development, total knock-outs are lethal and thus spatial and

temporal control of gene expressions are desirable. The two main systems developed over the years are the use of chimeric Cre recombinase proteins (Uto, Nikitin et al. 1999), and the Tet-On/Tet-Off systems (Gossen and Bujard 1992, Gossen, Freundlieb et al. 1995). The Tet-On/Tet-Off system employs a tetracycline, doxycycline, to activate or inactivate the tetracycline-responsive promoter. In Tet-On systems, doxycycline binds the tetracycline transactivator protein and thereby allows binding to a tetracycline response element and transcriptional activation to occur. In Tet-Off systems, doxycycline binding to a slightly modified tetracycline transactivator protein impairs its ability to activate the responsive promoter, thus preventing transcriptional activation. Although the Tet-On/ Tet-Off system provides exquisite flexibility to study gene function, few researchers consider the potential detrimental effects of the use of tetracyclines themselves, although prolonged antibiotic use is known to cause adverse effects in the clinic (Brummett and Fox 1989, Mingeot-Leclercq and Tulkens 1999, Selimoglu 2007).

Collectively, these analyses aim to dissect the genetic mechanisms that drive innate immune stimulation and antitumor control following the induction of a mitochondrial stress response. Understanding the mechanisms may reveal new intermediates that have potential as therapeutics, and/or will aid in optimizing the use of the existing drugs therapeutically. Furthermore, the mechanisms are likely to also contribute to spontaneous antitumor immune responses and have general interest.

Chapter 8: Concluding Remarks

Concluding Remarks

At the outset of this thesis, my work was driven by the question that initiated my PhD journey:
Do Tregs suppress NK cell-mediated anti-tumor responses, and by what mechanisms?

This one question led to a union of the Raulet and DuPage labs' expertise in the study of each cell type, and a series of elegant experiments which uncovered a mechanism which was described earlier in the homeostatic control of NK cells by Tregs (Gasteiger, Hemmers et al. 2013, Gasteiger, Hemmers et al. 2013), extending these insights into tumor immunity and revealing novel mechanisms by which Tregs modulate NK cell activity in the tumor microenvironment.

Both IT DT administration and anti-CCR8 antibody treatment elicit robust NK cell-driven anti-tumor responses through selective ablation of CCR8-expressing Tregs within the tumor microenvironment. Although both methods efficiently relieve immune suppression and promote NK cell activation, we are interested in investigating if there may be fundamental differences. For instance, the influence on the spectrum of non-Treg populations affected, as well as the Treg populations that remain after each strategy. Importantly, both strategies are likely to remodel local cell-cell interactions. We aim to characterize these shifts in immune cell dynamics and quantify changes in the propensity and frequency of immune cell interactions in tumor sections via immunofluorescence analyses. Elucidating these similarities and differences will clarify how distinct Treg ablation methods reshape the tumor microenvironment and may identify mechanisms by which combinatorial immunotherapies can most effectively exploit NK cell-mediated tumor eradication.

Through the combined application of genetic mouse models and classical immunological techniques, our studies have demonstrated that CD4⁺ conventional T cell-derived IL-2 is both necessary and sufficient to drive robust NK cell-mediated anti-tumor immunity, especially under conditions of intra-tumoral Treg depletion. This finding isolates a key cytokine axis within the tumor microenvironment, highlighting IL-2's central role in enabling NK cell activation against tumors. However, data from subsequent in vitro experiments and emerging literature suggest that optimal NK cell activation often requires more than IL-2 alone. The complex milieu of cytokines

and accessory signals, or even direct interactions with other cell types residing in the tumor microenvironment, may all be critical in ensuring a robust NK cell activation. The regulation, expression, and maintenance of high-affinity IL-2 receptor CD25 on NK cells post-Treg depletion remain unresolved and warrant further investigation. In in vitro cultures of pure NK cells isolated from splenocytes, the addition of IL-2 did not lead to a significant upregulation of CD25. This was in contrary to whole splenocyte cultures, where CD25 expression was increased even after 24 hours (Figure 3S10A, plus whole splenocyte data not included). With the addition of cytokines like IL-12 and IL-18 in pure NK cell cultures, CD25 expression was induced strongly, and this could suggest that additional signals within the TME from the production of IL-12/18 could be necessary to catalyze the CD25 expression, which later is essential for NK cell activation via the increased IL-2 levels within the Treg-deplete TME. Dissecting the IL-2 pathway is further complicated by the dual impact of Treg ablation: there is not only reduction in IL-2 consumption by Tregs but concurrently increased IL-2 availability and production from CD4⁺ Tconv cells.

The use of MHC I-expressing tumor cell lines engineered to overexpress NKG2D ligands in my studies established that Treg depletion can also potentiate “induced self” recognition by NK cells, extending the relevance of this immune surveillance axis beyond MHC I-deficient tumors. Given the endogenous expression of NKG2D ligands on MC38 cells, it becomes essential to dissect the relative contributions of “missing-self” (recognition of MHC I loss) versus “induced-self” (recognition of upregulated stress ligands) mechanisms in driving NK cell-mediated cytotoxicity in tumors where both modes of activation may coexist. Preliminarily, we did not observe substantial changes in the overall repertoire of NK cell activating receptors following IT Treg depletion (Figure 4.8), suggesting that Treg ablation may principally enhance NK cell function through extrinsic modulation of target ligand presentation or local cytokine environment, rather than intrinsic reprogramming of NK cell activation thresholds. However, much more detailed characterization is necessary to ascertain whether Treg depletion also induces intrinsic changes in NK cell responsiveness or modifies their activation receptor landscape more subtly.

Most investigations for my projects have focused on early post-depletion time points; future studies employing longitudinal and image-based analyses are needed to assess whether Treg depletion can also counteract NK cell desensitization and dysfunction in MHC I-deficient tumors

over time. Ultimately, incorporating time-course and spatially resolved approaches will yield a more nuanced understanding of how immune cell interactions and the cytokine landscape shape anti-tumor responses within the complex TME landscape.

The study of cytotoxic CD4⁺ T cells has revealed alternative immune pathways that compensate when classical CD8⁺ T cell responses are impaired, particularly in MHC I-deficient tumors. We observed an expansion of perforin-dependent, NK receptor-expressing CD4⁺ conventional T cells with potent tumor-killing abilities. Notably, while neutralizing TNF- α , IFN- γ , or canonical death receptor pathways reversed tumor control, these experiments were conducted in the presence of NK cells, which mediate tumor control with significant help from CD4⁺ T cells as shown in Chapter 3. Thus, although CD4⁺ T cell-mediated support of NK cells may be independent of TNF- α , IFN- γ , or FADD, CD4⁺ T cells' ability to directly kill tumors in the absence of NK cells may still depend on these mechanisms. Ongoing experiments targeting these cytolytic pathways under NK cell depletion aim to clarify the unique contributions of CD4⁺ T cells alone. However, key questions remain about which ligands or stress signals on tumor cells are recognized by these NK receptor-expressing cytotoxic CD4⁺ T cells, and how these recognition events induce tumor cell killing. More broadly, the mechanisms governing expansion and maintenance of these cytotoxic CD4⁺ T cell populations are yet to be elucidated.

Questions remain about the precise role of CD8⁺ T cells in tumor microenvironments following Treg depletion. While extensive studies by Alissa Bockman and my own research demonstrated that CD8⁺ T cells were not essential for direct tumor control in either MHC I-expressing or MHC I-deficient tumors post-Treg ablation, these cells may nonetheless contribute to the shaping of the tumor immune microenvironment and support "tumor landscaping". In data not shown in this thesis, I observed increased frequencies of CD8⁺ T cells in MHC I-deficient tumors after IT Treg depletion, along with also enhanced expression of NK cell receptors on these CD8⁺ T cells. This is consistent with literature describing "bystander" CD8⁺ T cells that, although not directly cytotoxic, can influence other immune responses, support cytokine production, or mediate tissue reorganization within the tumor (Tough, Borrow et al. 1996, Schietinger, Philip et al. 2010, Kim and Shin 2019). Overall, further scrutiny is warranted to tease apart the non-canonical roles of

CD8⁺ T cells and to assess how Treg ablation may foster functional heterogeneity or indirect contributions to immune activation in diverse tumor contexts.

Comparing MHC I-expressing with MHC I-deficient tumors may reveal distinct mechanisms by which CD4⁺ T cells mediate tumor control, as well as underscore the versatility and adaptability of this lymphocyte subset in varying immune contexts. While CD4⁺ T cells do not directly engage MHC I for antigen recognition, their effector functions (cytokine production, support of myeloid cell reprogramming, to direct cytolytic activity) can be heavily influenced by the broader tumor microenvironment, including MHC I status and the relative abundance of other immune populations. Outstanding questions remain regarding whether, and how, CD4⁺ T cells “sense” or respond to changes in MHC I expression, and whether MHC I-associated molecules or the dynamics of antigen presentation shape CD4⁺ T cell activation, localization, or function within tumors. Additionally, it is unclear if MHC I itself could interact with accessory receptors on CD4⁺ T cells, analogous to its inhibitory effects on NK cells. The mixed tumor model system being developed and refined by Abigail Mende in the Raulet lab offers a powerful experimental platform to disentangle these complexities, where we can perform spatial and functional comparisons of CD4⁺ T cell responses against MHC I-high versus MHC I-low tumor populations within the same host. Such a system will be instrumental in determining whether MHC I expression is sufficient to drive divergent CD4⁺ T cell effector programs, and whether one response mode predominates in settings of tumor heterogeneity.

Recent work on ex-Tregs has deepened our understanding of their critical role in immune tolerance and revealed both therapeutic opportunities and challenges in modulating suppressive networks *in vivo*. The identification of ex-Treg populations raises important questions regarding their functional heterogeneity, molecular regulation, and specific contributions to tumor immunity. For example, it remains unclear whether ex-Tregs retain any residual suppressive capacity or instead acquire cytotoxic or helper functions. The signals that drive FOXP3 instability and Treg plasticity within the tumor microenvironment need to be thoroughly investigated, as they may dictate whether Tregs convert into proinflammatory or cytotoxic effectors. Understanding these pathways could reveal novel opportunities to therapeutically reprogram or stabilize Tregs *in situ* to enhance immunotherapeutic efficacy. A key next step is to fully elucidate the transcriptional

and epigenetic programs governing ex-Treg formation and their diverse functional states. It is also crucial to determine whether ex-Tregs contribute directly to anti-tumor immunity.

Importantly, the possibility remains that the CD4⁺ T cells mediating NK cell-independent tumor control in our models could actually be derived from ex-Tregs that have lost Tregulatory function and acquired effector properties, a hypothesis that warrants direct investigation. Increasing evidence indicates that IFN- γ is a key pro-inflammatory cytokine capable of destabilizing tumor-infiltrating regulatory T cells (Tregs), driving their loss of suppressive function and potentially facilitating their conversion into ex-Tregs with effector properties (Overacre-Delgoffe, Chikina et al. 2017, Munn, Sharma et al. 2018). Importantly, this Treg destabilization process appears to be necessary for the full efficacy of certain immunotherapies, such as checkpoint blockade, as Tregs rendered unresponsive to IFN- γ or unable to mount an IFN- γ -driven response are less likely to lose suppressive function and more resistant to therapeutic intervention (Overacre-Delgoffe, Chikina et al. 2017, Liu, Zhang et al. 2024). In our own models, the possibility remains that the CD4⁺ T cells mediating NK cell-independent tumor control could originate, at least in part, from ex-Tregs that have lost *Foxp3* expression and acquired new effector functions, including cytotoxic or helper phenotypes—a hypothesis directly linked to IFN- γ 's destabilizing influence in highly inflamed tumors. Further, the presence of ex-Tregs in the tumor microenvironment may fundamentally reshape immune cell recruitment, activation dynamics, and spatial organization, potentially acting as catalysts for the differentiation of cytotoxic CD4⁺ T cells or amplifying pro-inflammatory feedback loops.

Exploring tetracycline derivatives revealed their unexpected capacity to modulate mitochondrial stress responses in immune cells, influencing metabolic pathways foundational to immune activation, exhaustion, and survival within the tumor microenvironment (TME). These findings suggest new strategies for targeting mitochondrial function and stress signaling to enhance immune effector fitness and overcome the metabolic constraints imposed by tumors. Notably, mitochondrial stress can engage the mitochondrial unfolded protein response (UPR^{mt}), which is increasingly appreciated as a key regulator of innate immune activation. UPR^{mt} signaling upregulates stress-adaptive transcriptional programs, including antioxidant defense, metabolic rewiring, and cytokine production, that can promote dendritic cell maturation, support the antigen

presentation machinery, and stimulate the secretion of proinflammatory mediators such as type I interferons and IL-6. These immune-modulatory pathways not only facilitate T cell and NK cell effector functions, but can also shape the recruitment and activation of myeloid and other innate cells within the TME, collectively enhancing antitumor immunity. Nevertheless, a critical challenge remains in delineating the threshold at which mitochondrial modulation transitions from beneficial immune potentiation to detrimental toxicity, potentially compromising immune homeostasis or cell viability. Therefore, unraveling the molecular circuitry that couples UPR^{mt} activation to anti-tumor responses, whether through direct innate immune signaling, metabolic crosstalk, or coordination of immunogenic cell death, will be central to optimizing therapeutic strategies that leverage mitochondrial stress for cancer immunotherapy.

Recent studies have highlighted mitochondrial transfer between cells as a novel mechanism influencing immune cell function and metabolic fitness within the tumor microenvironment. Notably, Ikeda *et al.* demonstrated that cancer cells transfer dysfunctional mitochondria bearing mutated mitochondrial DNA to tumor-infiltrating lymphocytes (TILs) (Ikeda, Kawase et al. 2025). These transferred mitochondria evade normal mitophagy, accumulate within TILs, and induce metabolic defects, senescence, and impaired effector functions, which collectively suppress antitumor immunity both *in vitro* and *in vivo*. This mitochondrial exchange represents a novel tumor-driven immune evasion strategy that could synergize with mitochondrial stress modulation and activation of the UPR^{mt} by replenishing or altering mitochondrial content critical for cell signaling and energy production. Understanding this process and its molecular underpinnings may reveal new therapeutic opportunities to enhance immunotherapy efficacy by targeting mitochondrial dynamics and metabolic crosstalk in the tumor microenvironment.

The advanced genetically engineered mouse models (GEMMs) and CRISPR-based tools developed in this thesis provide a versatile and physiologically relevant platform to interrogate immune-tumor cell interactions under conditions that recapitulate clinical complexity, including location-specific tumor induction and diverse mutational landscapes. These models enable in-depth studies of immune editing, therapy-induced selection, and emergence of resistant tumor clones, all of which are pivotal for understanding translational responses to immunotherapy. While our models were designed to capture specific mutations such as the knockout of the *B2m* gene,

careful interpretation is needed: in autochthonous or clonal lung tumor models, MHC I expression may be uniformly lost across the tumor cell population, and MHC I-positive cells detected may represent non-tumor stromal or infiltrating leukocytes rather than malignant cells. Future studies should make use of lineage or reporter tagging to definitively track tumor cell-intrinsic MHC expression and distinguish these populations from their microenvironment. Additionally, these platforms are uniquely positioned to dissect how immune cell spatial organization and cell-cell interactions evolve following interventions that deplete Tregs or reprogram the TME, using multiplexed imaging and single-cell spatial analyses. Collectively, the continued refinement and application of these systems will facilitate the understanding of the mechanisms of immune evasion, aid in the development of strategies to re-sensitize tumors with MHC I loss, and clarify the contributions of specific immune subsets in shaping tumor fate.

Together, these complementary lines of research converge to deepen our mechanistic understanding of immune regulation in cancer, uncovering novel cellular players, pathways, and models. This integrated knowledge lays a foundation for the development of more effective, precision immunotherapies capable of overcoming tumor immune evasion and improving clinical outcomes.

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