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N-Acetyl Cysteine Protects Melanoma Specific Cytotoxic T Cells from T Cell Receptor Restimulation Induced Activation of the DNA Damage Response Pathway and Enhances Tumor Control in vitro and in vivo

Matthew James Scheffel
Medical University of South Carolina

N-Acetyl Cysteine Protects Melanoma Specific Cytotoxic T Cells from T Cell Receptor Restimulation Induced Activation of the DNA Damage Response Pathway and Enhances Tumor Control *In Vitro* and *In Vivo* by

Matthew James Scheffel

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Approved by:

Chairman, Advisory Committee

Christina Voelkel-Johnson

Craig Beeson

Shikhar Mehrotra

Michael Nishimura

Chrystal Paulos

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TABLE OF CONTENTS

List of Figures ..... **iv**

Abstract ..... **vi**

Chapter 1: Review of Literature **1**
The Development of Adoptive Cell Transfer Therapy 1
Melanoma 14
Immunotherapy Models for Melanoma 28
Activation-Induced Cell Death 31
T cell Receptor Generated ROS and Redox Regulation of Cellular Processes 35
N-acetyl cysteine 43
p53 50
ATM 53
T cell Exhaustion and PD-1 54
Foxo1 56
Concluding Remarks 58

Chapter 2: TCR restimulation Results in activation of the DNA Damage response pathway in the Onset of Activation-Induced Cell Death **60**

Chapter 3: Culturing T cells in N-acetyl cysteine Improves Their Anti-Tumor Functionality **83**

Chapter 4: Culture of TIL1383I TCR Transduced Human T Cells in NAC Attenuates Expression of Exhaustion Associated Receptors and Transcription Factors **108**

Chapter 5: Discussion and Future Directions **120**

Chapter 6: Materials and Methods **136**

List of References **146**

List of Figures

1 Chemical structure of N-acetyl cysteine 44

2 p53 is activated by phosphorylation and nuclear accumulation 52

3 p53 status does not alter expression of CD25 and CD69 activation markers 62

4 Lack of p53 increases m-IFNγ cytokine secretion 63

5 Presence of p53 exacerbates cell death upon TCR restimulation 65

6 TIL1383I TCR transduced $p53^{KO}$ mouse splenocytes are protected from AICD 67

7 p53 is phosphorylated on its Serine 15 residue upon TCR restimulation in human PBMCs 69

8 p53 translocates to the nucleus upon TCR restimulation 71

9 ATM is activated and required for cell death following TCR restimulation in human PBMCs 73

10 TCR restimulation results in the rapid upregulation of DNA damage markers in human PBMCs 74

11 DNA damage and ATM activation occurs in CD8+ TIL1383I TCR transduced human T cells restimulated with MEL624 melanoma cells 76

12 DNA damage and ATM activation occurs in CD4+ TIL1383I TCR transduced human T cells restimulated with MEL624 melanoma cells 77

13 Pretreatment with NAC prevents TCR restimulation induced ROS accumulation and JNK activation in human PBMCs 79

14 Pretreatment with NAC prevents the induction of γH2AX and activation of the DDR pathway upon TCR restimulation in human PBMCs 81

15 Signaling schematic of DDR pathway activation in AICD 82

16 Pmel-1 T cells cultured in NAC exhibit enhanced antioxidant capacity 85

17 Pmel-1 T cells cultured in NAC are more resistant to DNA damage and AICD upon TCR restimulation 86

18 Pmel-1 T cells cultured in NAC exhibit enhanced persistence once transferred *in vivo* 88

19 Pmel-1 T cells cultured in NAC maintain resistance to AICD and have enhanced expression of the cytotoxic effector molecule granzyme B after adoptive transfer 91

20 Pmel-1 T cells cultured in NAC demonstrate improved control of tumor burden and survivability of recipient mice 92

21 Production of NAC cultured TIL1383I TCR transduced human T cells 94
22 TIL1383I TCR transduced human T cells REPed in NAC have improved antioxidant capacity and resistance to MEL624 induced DNA damage 95
23 TIL1383I TCR transduced human T cells rapidly expanded in NAC exhibit enhanced anti-melanoma *in vitro* cytotoxicity 97
24 Effect of NAC supplementation during REP on the functional capacity of TIL1383I TCR transduced human T cells 99
25 Effect of NAC supplementation during REP on the functional capacity of TIL1383I TCR transduced human T cells 100
26 Lack of persistence of TIL1383I TCR transduced human T cells in NSG xenograft model 101
27 *In vitro* characterization of TRP-1 TCR transduced murine T cells cultured in NAC 103
28 TRP-1 TCR transduced murine T cells cultured in NAC demonstrate enhanced functional capacity after adoptive transfer 105
29 TRP-1 TCR transduced murine T cells cultured in NAC are more resistant to DNA damage and cell death while exhibiting enhancement in tumor burden control and survivability of recipient mice 106
30 REP in NAC does not alter expression of memory markers on TIL1383I TCR transduced human T cells 110
31 Effect of NAC REP supplementation on expression of co-stimulatory markers on TIL1383I TCR transduced human T cells 112
32 TIL1383I TCR transduced human T cells rapidly expanded in NAC display reduced expression of markers of exhaustion and senescence 113
33 TIL1383I TCR transduced human T cells rapidly expanded in NAC have reduced expression of the transcription factor EOMES 115
34 TIL1383I TCR transduced human T cells cultured in NAC have reduced expression of the Foxo1 transcription factor 116
35 Acute treatment of T cells with NAC rapidly phosphorylates and attenuates expression of Foxo1 118
36 T cells cultured in NAC exhibit activation of mTOR and AKT pathways 119
37 Addition of NAC to culture improves the phenotype of rapidly expanded TIL1383I TCR transduced human T cells via three distinct mechanisms 135
38 Genotype screening of p53 knockout mice 139

ABSTRACT

MATTHEW JAMES SCHEFFEL. N-Acetyl Cysteine Protects Melanoma Specific Cytotoxic T Cells from T Cell Receptor Restimulation Induced Activation of the DNA Damage Response Pathway and Enhances Tumor Control In Vitro and In Vivo. (Under the direction of CHRISTINA VOELKEL-JOHNSON).

The adoptive transfer of autologous melanoma antigen-specific T cells has demonstrated a remarkable improvement in clinical outcomes for patients with late-stage metastatic melanoma. However, the majority of patients do not achieve a durable response. To achieve a sufficient quantity of cells for transfer, T cells undergo a rapid expansion protocol which makes them more susceptible to activation-induced cell death (AICD). As the persistence of transferred T cells is necessary for optimal patient response, limitation of persistence via AICD is likely a constraint on clinical efficacy. The accumulation of oxidative stress caused by TCR restimulation has previously been demonstrated to be necessary for the onset of AICD. The data contained within in this thesis reveal that accumulation of ROS escalates into the incursion of the appearance of γH2AX foci, which are indicative of DNA damage, and activation of the DNA damage response pathway characterized by autophosphorylation of ATM on Ser1981 and ATM mediated phosphorylation of the tumor suppressor p53 on Ser15. Treatment with the glutathione pro-drug N-acetyl cysteine (NAC) significantly reduced the upregulation of γH2AX and subsequent ATM activation and cell death. Additionally, both murine Pmel-1 T cells and TIL1383I TCR transduced therapeutic human T cells exhibited less susceptibility to the upregulation of γH2AX and onset of AICD when NAC was added to the medium

during extended culture. Both Pmel-1 T cells and TRP-1 TCR transduced murine splenocytes cultured in NAC prior to adoptive transfer into B16F10 challenged mice exhibited enhanced control of tumor burden and survival of recipient mice. TIL1383I TCR transduced T cells cultured in NAC demonstrated reduced expression of the exhaustion and senescence markers PD-1 and CD57 as well as the exhaustion associated transcription factors EOMES and Foxo1. Taken together, the results contained in this thesis demonstrate the addition of NAC to the rapid expansion of therapeutic T cells bolsters the overall fitness and anti-melanoma functionality of the cells and could potentially improve the quality and therapeutic efficacy of adoptive T cell therapeutics infused into patients.

CHAPTER 1 – REVIEW OF LITERATURE

The Development of Adoptive Cell Transfer Therapy

In the late 1800s, William Coley, a bone surgeon from New York, observed several cases of cancer where the patient exhibited signs of remission upon the onset of fever. Based on these observations, Coley postulated that he could recapitulate such outcomes by purposefully injecting sarcoma and carcinoma patients with cultures of *Streptococcus pyogenes.* In 1893, he published his findings claiming such inoculations resulted in some degree of remission up to a curative potential (1). This seminal experiment in manipulating the immune system towards the eradication of cancer has been widely regarded as the origin of the cancer immunology field. However, the concept that the immune system could invoke remediation of cancer was resisted with much skepticism for several decades following Coley's work (2).

During the late 1950s and early 60s, the Laboratories of Robert Baldwin & Karl Hellström contemporaneously published several key experiments which reinvigorated the notion that the immune system was involved in cancer defense. Baldwin induced primary sarcomas in rats with methylcholanthrene, which were then removed via ischemia mediated atrophy. Subsequently, Baldwin demonstrated that these rats were resistant to a secondary tumor transplantation with syngeneic derived tumors, implying that the primary tumor had generated an inoculation in the rat against further tumor challenge (3). The Hellström Laboratory further established that previously tumor challenged mice were also resistant to a secondary transplantation of autologously

derived tumors, confirming that the anti-cancer immunity observed in these experiments was not a result of any unappreciated immunogenicity from donor derived tumors (4). Later on in the 1960s, reports demonstrated that tumors in rats and mice could be suppressed by the transfer of peripheral lymphocytes derived from tumor challenged donor mice (5,6), thus establishing that anti-cancer immunity could be "adoptively transferred" from one host to another.

Moving the field beyond controlling cancer through the use of peripheral lymphocytes, in 1986, Steven Rosenberg and colleagues successfully isolated lymphocytes embedded within the microenvironments of established murine sarcoma and adenocarcinoma tumors through mechanical dissociation of the tumor alongside elevated levels (1,000U/mL) of interleukin-2 (IL-2). Adoptive transfer of these isolated "tumor infiltrating lymphocytes" (TILs) was able to control the tumor burden of tumor challenged mice at a potency of "50 to 100 times" higher than peripherally-derived "lymphokine-activated killer cells" (7). The following year, the Rosenberg Laboratory successfully isolated human TILs from the tumor resections of metastatic melanoma patients. Furthermore, these TILs were found to be capable of *in vitro* cytotoxicity against autologous melanoma cells (8). Subsequently, pilot clinical trials were initiated to adoptively transfer these patient autologous TILs into patients with metastatic melanoma, as well as into patients with breast, colon, and renal cell carcinomas, which demonstrated an overall manageable safety profile and resulted in some cases of partial response (9,10). Most of the initial clinical trials of TIL therapy were purposed to treat metastatic melanoma where it was collectively observed that approximately one-third of

patients achieved an objective response when TILs were administered alongside high-dose IL-2 therapy (11), demonstrating the adoptive transfer of TILs to be a feasible and potentially efficacious treatment option.

Since these pilot trials, one of the key advancements in the efficacy of TIL based adoptive cell transfer (ACT) therapy has been the ability to preemptively condition the patient with a non-myeloablative lymphodepleting regimen prior to cell infusion, typically with the chemotherapeutic agents cyclophosphamide and fludarabine (12). There are multiple mechanisms by which lymphodepletion is thought to enhance ACT. As early as 1980, it was postulated that lymphodepletion thwarted host endogenous suppressor cells from restricting the efficacy of transferred effector T cells (13). Indeed, such suppressor cells are an impediment for ACT. In mice, Mac-1^+/Gr-1^+ myeloid derived suppressor cells have been shown to directly cause cell-death in effector CD8+ T cells (14), and in humans, peripheral derived myeloid cells can suppress T cell proliferation (15). Additionally, the level of host endogenous $CD4^+FoxP3^+$ T regulatory cells (Tregs) inversely correlates with the therapeutic outcome of ACT (16). Lymphodepletion prior to adoptive transfer has also been shown to make available homeostatic cytokines such as IL-7 and IL-15 both in mice and in humans (17,18). Lastly, lymphodepletion is thought to aid in activating the innate and antigen presenting compartments of the immune system. Lymphodepletion regimens likely cause some tumor cytotoxicity releasing tumor antigens. Moreover, in experimental mouse models, lymphodepletion via total body irradiation (TBI) has been shown to modulate gut microbiota to release LPS, subsequently enhancing dendritic cell activation (19). Attempts have also been made to intensify host lymphodepletion with

the addition of TBI to the cyclophosphamide and fludarabine regimen. An initial small-scale, non-randomized clinical trial demonstrated a complete response rate of 40% for those receiving the highest dosage of TBI (12 Gy) (20) with all but one of those patients maintaining the complete response beyond 5 years (21). However, a subsequent randomized clinical trial did not find any significant survival benefit with the addition of TBI to cyclophosphamide and fludarabine (22).

Beyond melanoma, TILs have been detected, and are good prognostic indicator, in renal cell carcinoma (23), head and neck squamous cell carcinoma (24,25), hepatocellular carcinoma (26), as well as in breast (27,28), gallbladder (29), bladder (30,31), ovarian (32–34), esophageal (35,36), colorectal (37–42), prostate (43,44), non-small cell lung (45,46), pancreatic (47), and cervical (48,49) cancers. In the clinic, TILs have produced positive clinical outcomes in viral associated cancers such as EBV positive nasopharyngeal carcinoma (50–52) and have demonstrated partial and complete responses in HPV positive cervical cancer (53). Additionally, TIL based ACT has been shown to mediate partial responses in glioma (54), and significantly enhance the 3 year survival in clinical trials for non-small cell lung cancer (55) and ovarian cancer (56).

Adoptive Transfer with TCR Transduced T cells

The use of TILs for ACT is limited by the ability of a patient surgical resection to yield TILs suitable for infusion with only approximately 45% of metastatic melanoma patients being appropriate candidates for the therapy (20). Moreover, the repertoire of antigen specificities, and affinities thereof, is limited to the inventory of TIL clonotypes

present within the harvested tumor. As an alternative approach, the T cell receptor (TCR) genes from tumor reactive TILs have been cloned and then transduced onto autologous peripheral T cells (57). Most TCRs are composed of an α-chain/B-chain heterodimer (with a minority of TCRs expressing γδ chains) (58). Each unique TCR recognizes a specific peptide antigen fragment presented on a major histocompatibility complex (MHC) molecule. There are two main classes of MHC molecules: MHC-I on the surface of virtually all nucleated cells, and MHC-II which is generally restrained to antigen presenting cells. MHC-I presents antigen to CD8+ T cells while MHC-II presents to CD4+ T cells. As MHC molecules are polymorphic, differing in the specific structural conformation of the groove binding the peptide, TCR recognition of a peptide is "MHC restricted" to the particular genetic variant of MHC presenting the peptide (59,60). In the context of melanoma, several TCRs specific for melanocyte differentiation antigens such as Melan-A (MART) (57,61–63), glycoprotein 100 (gp100) (64–66), and tyrosinase (67–70) have been cloned for transduction onto autologous T cells.

These melanocyte differentiation antigens are overexpressed on melanoma tumors, yet are still expressed in healthy melanocytes, which can in some instances potentiate the occurrence of autoimmunity (62). Alternatively, TCRs have been engineered on T cells to recognize another class of antigen: the cancer-testis (CT) antigen. CT antigens are a class of antigen of which expression is generally exclusive to male germline cells, normally absent from healthy adult somatic tissues, but can reappear during oncogenesis in various cancers (71). T cells transduced with a TCR directed against the CT antigen NY-ESO-1 and MAGE-3 were able to mount *in vitro* cytolytic lysis against

relevant antigen expressing melanoma and nonmelanoma cancers including neuroblastoma and non-small cell lung cancer, respectively (72,73). In the clinic, the NY-ESO-1 TCR has mediated objective responses against synovial cell sarcoma and melanoma (74) and has also shown clinical activity against multiple myeloma (75). Currently, the use of the NY-ESO-1 TCR is being expanded in clinical trials for breast, lung, esophageal, ovarian, and bladder cancers as well as neuroblastoma (76). Additionally, the MAGE-3 TCR elicited cancer regression in the clinic against melanoma and synovial sarcoma, though further use was discontinued due to the occurrence of fatal on-target off-tumor toxicity (77).

Achieving the appropriate TCR affinity for a target antigen remains an open avenue of optimization. The affinities of TCRs cloned from TILs targeting tumor associated antigens, which are co-expressed on non-malignant cells, are likely constrained by central tolerance (78,79). Moreover, limitation in antigen expression caused by tumor immune evasion maneuvers, such as the downregulation of MHC molecules (80), likely necessitates an appropriately high enough TCR affinity for optimal TCR-pMHC binding essential for T cell activation (81). In the original pilot trial of TCR engineered cells for ACT in 2006, 15 melanoma patients were treated with autologous peripheral lymphocytes transduced with a TCR specific for MART-1. Compared to TIL based therapy, the response was relatively low with only two (13%) of the patients exhibiting an objective response to the treatment (82). A subsequent trial was reported in Johnson, *et al*. where transferred T cells were transduced with a TCR of a higher affinity for MART-1 which resulted in an elevation in the objective response rate to approximately 30% (62).

This increase in efficacy highlights the importance of generating TCRs with an appropriately high affinity for the target antigen. However, several preclinical studies have demonstrated that there is likely an upper-limit to the increase in anti-tumor efficacy with enhanced TCR affinity. Dougan, *et al.* generated two different strains of transnuclear mice with T cells expressing either a high or low affinity TCR for the melanoma antigen tyrosinase-related protein 1 (TRP-1) using somatic cell nuclear transfer technology. The difference in affinity for TRP-1 between the two TCRs was nearly 100-fold, yet when these T cells were activated *ex vivo* and transferred into B16 challenged mice, both strains conferred a survival benefit and control of tumor burden that was indistinguishable in magnitude between the low and high affinity TCR (83). Other investigators have used a panel of increasing TCR affinities for the same antigen to more precisely determine the relationship between affinity and *in vivo* anti-tumor efficacy. Zhong, *et al*. used a panel of TCRs with increasing affinity towards gp100 and found that increasing the affinity towards gp100 did enhance anti-tumor efficacy, but this enhancement was plateaued at a TCR affinity of 10µM (84). A similar plateau was seen with a panel of increasing TCR affinities for NY-ESO-1 (85). Another study generated TCR affinities for NY-ESO-1 beyond the physiological range, which resulted in diminished T cell functionality (86). One potential explanation for this phenomenon is the increased expression of SHP-1, an inhibitor of TCR signaling (87), alongside increasing TCR affinity (88), implicating an intrinsic negative feedback mechanism against such supraphysiologic TCR stimulation. Moreover, it has been reported that T cells with higher affinity TCRs are more susceptible to tolerization within the tumor microenvironment (89). Enhancements in affinity also

increase the likelihood of autoimmune complications with therapy, particularly with TCRs that target antigens co-expressed on non-malignant tissues. As such, the T cells transduced with the higher affinity anti-MART1 TCR also targeted MART-1 expressing melanocytes in the skin, eyes, and ears resulting in the development of vitiligo and transient onsets of uveitis and hearing loss (62). In the context of tumor associated antigens shared between healthy and tumor cells, lowering the antigen affinity allows for preferential targeting of tumor cells as they significantly overexpress the antigen compared to normal healthy tissues (90,91). Additionally, more severe on-target off-tumor side effects have been observed with the use of TCR transduced T cells. A pilot clinical trial utilizing a TCR recognizing the CT carcinoembryonic antigen to treat gastrointestinal cancers had to be halted because all of the treated patients developed severe inflammatory colitis (92). More drastically, in the clinical trial utilizing the TCR directed against the CT antigen MAGE-A3, two patients died as a result of treatment induced neurotoxicity, as it was later determined that MAGE-A12, also recognized by the TCR, was previously unappreciated to be expressed in the brain (77). Another clinical trial with MAGE-A3 TCR transduced cells resulted in fatal cardiac toxicity potentially caused by a cross-reactive epitope on the striated muscle protein Titin (93). Altogether, the technology to engineer autologous T cells with a tumor-antigen specific TCR represents a feasible treatment option for cancer. However, there remains a disparity in the treatment efficacy compared to TIL therapy, and further enhancements are needed to fine-tune appropriate antigen specificities and affinities that are both efficacious and safe.

One approach to the generation of appropriate TCRs for transduction is to use whole exome sequencing to identify nonsynonymous mutations between healthy and malignant tissues which are subsequently screened for TIL recognition (94,95). The rationale for this approach builds off of the success of anti-PD1 therapy, which reports have suggested is mediated by T cells recognizing mutated antigens (96). Additionally, some reports have associated mutation specific TILs with positive outcomes in ACT (97,98), and T cells transduced with a KRAS-mutant specific TCR have shown efficacy in a preclinical pancreatic cancer model (99). Furthermore, this approach could potentially expedite the application of ACT immunotherapy towards cancers with lower mutation rates than melanoma (100), such as the clinically observed regression in a bile duct cancer patient treated with an ERBB2IP-mutation specific TIL (101).

Chimeric Antigen Receptor Engineered T Cells

An alternative approach to the transduction of T cells with a conventional αβ TCR is to transduce cells with a Chimeric Antigen Receptor (or CAR). CAR T cells, originally described by Gross and colleagues in 1989, are a fusion protein combining the antigen binding domain of an antibody with the intracellular signaling domain of a T cell (102). The use of an antibody antigen binding domain allows the CAR T cell to be MHC independent as well as able to bind to non-protein epitopes. Original CARs simply contained the CD3ζ domain of the TCR signaling complex and were lackluster in their clinical efficacy due to poor persistence (103,104). Subsequently, co-stimulatory

domains, such as CD28 or 4-1BB have been added, which have markedly improved both the persistence and therapeutic efficacy of CAR T cells (105,106).

CAR T cells have been primarily beneficial in the treatment of hematological cancers targeting the B-cell antigen CD19 where they have demonstrated an over 50% response rate in chronic B-cell leukemia (107) and complete response rates upwards of 90% in patients with acute lymphoblastic leukemia (108). Though currently the efficient use of CAR T cells has been restrained to hematological cancers, many investigators are looking at ways to target solid tumors with CAR engineered T cells. To that end, many CARs have been developed targeting relevant antigens, and have shown preclinical efficacy, in breast cancer (109–111), gastrointestinal cancers (112–114), glioblastoma (115–119), lung cancer (120,121), neuroblastoma (122,123), pancreatic cancer (124–126), osteosarcoma (127,128), ovarian cancer (103,129,130), cervical cancer (131), prostate cancer (132–135), renal cell carcinoma (136,137), and hepatocellular carcinoma (138,139). Though success in the clinic has remained more elusive, several small-scale clinical trials have demonstrated partial responses in patients with non-small cell lung cancer using anti-EGFR CAR T cells (120) and in prostate cancer with anti-PSMA CAR T cells (133). Additionally, there have been reports of complete responses in glioblastoma with an anti-IL13Rα2 CAR T cell (140) and in neuroblastoma with an anti-GD2 CAR T cell (122). However, other pilot clinical trials utilizing T cells transduced with CARs directed against solid tumors have demonstrated severe (92,137,141) and even fatal (142) toxic events. Unfortunately this has slowed down the clinical development of CAR engineered T cells

against solid tumors towards stringent dose-escalation trials, which have made it difficult to discern clinical efficacy (143).

To limit toxicities, investigators are currently working to engineer CARs with additional safety modifications. These approaches include incorporation of suicide genes such as Herpes simplex virus thymidine kinase or inducible caspase 9 which can be induced by either treatment with ganciclovir or the dimerization agent AP1903, respectively, to purposely eliminate transferred cells in order to modulate any escalation of on-target off-tumor toxicities (144,145). Alternatively, the CAR can be engineered to co-express a cell surface epitope that can be recognized by an already approved monoclonal antibody therapy such as rituximab or cetuximab, resulting in CAR T cell deletion (146,147). Conversely, the CARs may be inducible themselves, being only activated when given an exogenous peptide or small-molecule drug (148–150). Furthermore, the specificity of CARs can be enhanced by creating bi-specific "tandem" CARs whereby two antigens must be present on a cell to trigger CAR effector function (151,152).

Correlatives of Success with Adoptive Transfer Therapy

Unfortunately, reports of clinical success with adoptive cell transfer therapy must be balanced by the fact that the majority of patients do not generate a durable complete response. Understanding key correlatives of success and failure between responding and non-responding patients is therefore important for bridging this gap in therapeutic efficacy. Certain host factors can influence therapeutic success such as the individual

tumor's ability to evade immune detection via downregulation of MHC molecules (80). Similarly, development of resistance to CART19 therapy can be driven by either the mutation or loss of expression of CD19 (153,154). However, overall, there has not been a correlation between bulk of disease and/or the nature of metastasis with the therapeutic success of ACT (155).

Rather, the quality of the T cell used for infusion seems to be of paramount importance to therapeutic success. Both the age and differentiation status of the cell have been shown to correlate with clinical outcomes. In general, younger, less-differentiated cells consistently demonstrate superiority in therapeutic efficacy. A mouse study, which adoptively transferred cells stratified at different effector stages (naïve, early effector, immediate effector, effector) demonstrated an inverse correlation with differentiation status and ability to control tumor (156). This observation has also been extended to murine TCR transduced naïve cells being more anti-tumor efficacious than more differentiated memory cells (157). In fact, it has recently been shown that even the presence of memory cells during culture expansion can impair the anti-tumor efficacy of naïve cells (158). As such, there have been several attempts in the clinic to use shorter cultured "young" TILs, of which pilot studies have shown promising improvement in efficacy (159,160).

Consistently, telomeres, which degrade during cell-replication, and are therefore indicative of a cell's replication history, have also been shown to correlate with therapeutic outcomes. Patients receiving TILs with longer telomeres (i.e. have been through less replications) are significantly more likely to respond to therapy than patients

receiving TILs with shorter telomeres (18,161,162). Moreover, expression of costimulatory molecules also correlates with improved outcomes. Higher expression levels of CD27 (163), CD28 (162), and 4-1BB (164) associate with enhanced anti-tumor efficacy in preclinical and clinical studies. Additionally, as older, more differentiated cells are susceptible to senescence, and also lack the durability and proliferative capacity of younger, less differentiated cells (165), lack of cell persistence after transfer has also been shown to correlate with poor patient outcomes (166).

The quantity of cells infused is also important as studies have shown that responding patients receive a significantly higher number of cells than comparable non-responding patients. (159,167). In order to achieve enough cells for treatment, both TILs and TCR transduced cells undergo a Rapid Expansion Protocol (REP) whereby cells are typically stimulated polyclonally by anti-CD3 cross-linked on irradiated feeder cells with continued supplementation of IL-2 (168). However, counterproductively, cells that have undergone a REP have decreased expression of CD28 and CD27 (156,169). Additionally, post-REP cells have decreased telomere lengths compared to pre-REP cells (170). They are also more susceptible to deletion by activation-induced cell death (171). Thus, counter-acting the terminal differentiation promoting influence of the REP is likely a critical therapeutic intervention point to improve the quality and therapeutic efficacy of expanded anti-melanoma T cells.

Melanoma

The cancer of melanoma has been in the forefront of progress in cancer immunotherapy as it is notably one of the most immunogenic of cancers, likely due to its high mutation rate producing neoantigens (100). Several key observations have supported melanoma as a vanguard for the immunotherapy field. There are extremely rare cases where metastatic melanoma has spontaneously regressed without an attributable therapeutic cause (172–174). Incidences of melanoma are higher, and the prognosis is poorer, amongst immunocompromised individuals such as HIV patients or organ transplant recipients (175–177). Conversely, onset of the autoimmune condition vitiligo correlates with better outcomes for metastatic disease (178,179). The success gained by IL-2 therapy demonstrates that actively stimulating the immune system can modulate cancer regression. Moreover, the discovery of lymphocytes which have penetrated the tumor microenvironment evidences the direct targeting of melanoma cancer cells by cytotoxic T cells (8,180,181).

While the incidence rates of most cancers are on the decline in the United States, melanoma is one of a few cancers increasing in both occurrence and mortality in the US, and is one of the fastest growing cancers worldwide (182). Over the past two decades, the rate of people diagnosed with melanoma has increased 3.1% per year with an estimated 17-fold and 9-fold increase in incidence rates for men and women, respectively, within the US since the 1960s (183,184). Concurrently, since the 1960s, the mortality rate due to metastatic melanoma has been progressively increasing by 2% each year (183). In 2014, there were an estimated 76,100 new cases of melanoma in the US

with 1,350 of those cases occurring in South Carolina. Moreover, in 2014, it is estimated that there were 9,710 deaths attributed to melanoma (185). At this current incidence rate, it is now projected in the US, that men have a 1-in-33 chance and women have a 1-in-52 chance of developing melanoma within their lifetime (186).

The overall prognosis for melanoma is fairly encouraging with 5 and 10 year survival rates of 92% and 89%, respectively (187). However, these data are heavily weighted towards approximately 84% of melanomas being diagnosed at an early stage of development (187). Once the stage of initial detection is considered, the prognosis of melanoma is highly dichotomous based on when it is initially diagnosed. Melanomas detected while they are ≤1 mm in thickness, and have not metastasized, have a very high cure rate via surgical resection with 5 year and 10 year survival rates of 97% and 93%, respectively (188). However, once the primary melanoma tumor has grown to a depth of 4 mm, even before the detection of metastatic nodes, the 10 year survival rate of Stage IIC cancer quickly diminishes to 39% (188). For nearly one-in-five melanoma patients (20.5%), the cancer will metastasize into Stage IV disease (188) where the prognosis is exceedingly grim with a 5 year survival rate of only 15.2% (189).

Oncogenesis of Melanoma

Melanoma is a cancer originating from the melanocyte cell. The overwhelming majority of melanoma cases are cutaneous (~91.2%) with the additional rarer forms occurring as ocular and mucous membrane melanomas (190). Melanocytes, the pigment producing cells of skin and hair follicles, continually regenerate throughout an individual's

lifetime potentially making them more malleable to carcinogenic transformation (191). Melanomas typically arise from a cluster of melanocytes known as a nevus (or commonly referred to as a "mole"). The overwhelming majority of nevi are benign and do not progress to melanoma. However, genetic damage causes a rare minority of nevi to become dysplastic and progress into radial growth in the epidermis (192). The majority of melanomas present with mutations in either the N-RAS or BRAF cell proliferation pathways (192,193). The most prevalent mutation is in *BRAF*, estimated to occur in upwards of 66% of malignant melanoma cases (194) commonly presenting as a Valine-Glutamic Acid substitution at residue 600 ($BRAF^{V600E}$). This substitution causes an approximately 700-fold increase in B-raf kinase activity, resulting in constitutive activation of the Ras-Raf-MEK-ERK pathway promoting inappropriate cell proliferation (195,196).

In melanoma, as in most cancers, enhanced proliferation is generally coupled with loss of cell cycle control mechanisms. Typically, melanomas have been reported to thwart proper cell cycle control by either inactivating the *CDKN2A* or *PTEN* genes. Loss of function for the *CDKN2A* gene leads to a deficiency in the cell cycle regulator $p16^{INK4a}$ loosening restrictions on cell cycle progression (197). Similarly, mutations inactivating PTEN allow levels of PIP_3 to increase permitting an unregulated increase of activated Akt, promoting cell survival and proliferation (198).

After sufficient radial progression, melanomas begin to grow vertically and burrow through dermal layers. Loss of functional PTEN leads to a switch from E-cadherin to N-cadherin expression on the surface of melanoma cells (199). Loss of E-cadherin expression, which restrains melanoma proliferation via cell-to-cell contact with adjacent

keratinocytes, allows more migratory freedom for the expanding tumor mass (200–202). Additionally, as melanomas become more aggressive, they begin to express the αvβ3 integrin which secrets the active form of metalloproteinase MMP-2. MMP-2 is an enzyme that actively degrades collagen in the basement membrane eventually creating a path to metastasis for the evolving tumor (203).

Melanoma Treatment Options

For Stage I melanomas, the cancer can generally be cured via surgery, with a 5 year survival rate of approximately 98% (187). Unfortunately, once melanoma has metastasized into a Stage III–IV cancer, treatment options are more limited in their efficacy.

Chemotherapy

In the 1970s, the chemotherapeutic agent Dacarbazine emerged as one of the first approved therapeutic options for late stage melanoma and has represented the mainline standard-of-care since then. Preclinical studies demonstrated that Dacarbazine exhibited an *in vivo* antitumor effect against the leukemic cell line L1210 (204) as well as murine sarcoma, adenocarcinoma, and melanoma tumors (205,206).

Initial clinical trials administered Dacarbazine as an oral agent, which demonstrated incomplete and inconsistent absorption (207). Dacarbazine itself does not have anti-neoplastic activity. It must be first metabolized to the reactive byproduct 5-[3-methyl-triazen-1-yl]-imidazole-4-carboxamide (MTIC) in the liver via the cytochrome

P450 (208). The drug was henceforth administered intravenously. Phase III trials demonstrated a partial response rate of 14.8% and a complete response rate of 4.1% (209). However, these responses were not durable as 64.2% of complete responders relapsed within 37 weeks of treatment, and overall, only 1.4% of the original patient cohort was disease free at 74 months (209).

In the early 1990s, Temozolomide emerged in phase I trials as oral alternative to Dacarbazine (210,211). The drug demonstrates a near 100% bioavailability when administered orally and is capable of spontaneous conversion to the active agent MTIC without need of hepatic metabolism (210,212). While Temozolomide is more convenient to administer, it has unfortunately not demonstrated any significant improvement in therapeutic efficacy compared to Dacarbazine (213) with the exception that it can potentially cross the blood-brain barrier in the treatment of central nervous system metastases (212,214,215).

Dacarbazine and Temozolomide are generally regarded to have a manageable side effect profile (216). However, these agents have only produced a median overall survival of 5.6-7.8 months (217) and are likely, at best, only a palliative care option. Since the development of Dacarbazine, there have been many attempts and trials to combine various chemotherapeutic agents, including the often investigated “Dartmouth Regimen” of Dacarbazine alongside cisplatin and vinblastine. However, none of these have offered a serious advantage to life extension compared to Dacarbazine alone (218). Altogether, chemotherapeutics have not demonstrated any convincing potential as a curative agent for metastatic melanoma (219).

High Dose Interleukin-2

Since Dacarbazine, no drug or agent demonstrated any significant improvement in outcomes for metastatic melanoma patients until the FDA approval of high-dose Interleukin-2 (IL-2) in 1998 (220,221). IL-2 was originally discovered as a "T cell Growth Factor" in 1976 capable of stimulating *in vitro* lymphocyte proliferation (222). Thus, treatment with IL-2 exhibited the potential of manipulating the immune system towards tumor eradication (223). Treatment with high-dose IL-2 yields an objective response rate of approximately 16% with a subset of complete responders of around 6% (224). Moreover, nearly 70% of these complete responders never relapse (219), which made IL-2 a breakthrough in the durable management of metastatic melanoma. However, high-dose IL-2 treatment results in a complex side effect profile often requiring hospitalization for treatment. Consequently, current analysis reveals, at best, only 10% of eligible patients participate in this potentially curative treatment (219).

Interferon-α

The *in vivo* administration of interferon-α (IFN-α) has been characterized to bolster the immune system towards an anti-tumor response through several mechanisms. IFN-α promotes the maturation, and enhances the antigen presenting and co-stimulatory functionality, of dendritic cells (225,226). Additionally, IFN-α increases the Th1 immune response by increasing IFN-γ secretion by T cells (227), which is critical for the anti-tumor priming of T cells as well as promoting the upregulation of MHC expression (228) and oncogene induced senescence in melanoma cells (229). Ultimately, *in vivo*

tumor regression by IFN-α involves increased infiltration of dendritic cells and T cells into the tumor microenvironment (230).

Administration of IFN-α has not been very effectual for the eradication of unresected melanoma. Clinical trials which have attempted to combine IFN-α with various chemotherapeutic regimens were not able to show any increase in overall survival benefit from the addition of IFN-α (231–234). However, the use of high-dose IFN-α (specifically IFN-α2b) as an adjuvant therapy, to prevent relapse following surgical resection, has been shown to improve both the progression free survival and overall survival in patients with high-risk melanoma (235–238). Therefore, use of IFN-α2b in the adjuvant setting was approved by the FDA in 1995. The pegylated form of IFN-α2b, which results in a nearly 10-fold increase in the half-life compared to unmodified IFN-α2b (239), has also been developed and has been approved by the FDA for adjuvant treatment of melanoma. However, it is currently unclear if the pegylation has improved the therapeutic benefit of IFN-α2b as two phase III trials have showed no improvement in overall survival when compared to either observation (240) or unmodified IFN-α (241).

Additionally, it should be cautioned that a phase III trial of patients with "intermediate-risk" melanoma showed that there was no benefit from IFN-α2b adjuvant therapy, and patients had a reduced quality of life due to nearly 58% of the treatment group experiencing grade 3 or higher toxic side effects (242). Altogether, IFN-α is likely beneficial as an adjuvant therapy for post-resected, high-risk melanoma patients. Furthermore, while it has not demonstrated robust clinical efficacy in unresected disease, there is a renewed interest to determine if it can enhance the efficacy of immune

checkpoint blockade inhibitors. Clinical trials are currently underway to determine if there is any additive or synergistic effect in combining IFN-α with the anti-CTLA-4 agents Tremelimumab and Ipilimumab as well as with Pembrolizumab (anti-PD1) (243,244).

Vaccines

The immunogenic nature of melanoma has inspired many investigators to engineer vaccines that could potentially bolster a patient's immune system towards tumor eradication. Unfortunately, the overwhelming majority of clinical attempts have failed to exhibit a substantive patient response (245). The earliest vaccine attempts were whole cell vaccines utilizing irradiated autologous tumor cells obtained from surgical resections. Clinical trials using whole cell vaccines to treat melanoma have only been able to report an overall response rate in the range of approximately 3.85%-12.5% (246–249). Additionally, whole cells vaccines derived from allogenic melanoma cell lines have been developed (Melacine®, VMCL, VMO) and tested in the clinic for post-surgical adjuvant treatment. None of these have demonstrated an improvement in overall survival (250–252). In fact, a phase III trial of the allogenic whole cell vaccine Canvaxin™ had to be canceled when it was demonstrated that patients treated with Canvaxin™ had survival rates significantly lower than placebo treated controls (253).

Dendritic cell vaccines have also been used in an attempt to enhance the presentation of tumor antigens to the immune system by pulsing dendritic cells with melanoma peptides prior to patient infusion. While this approach has demonstrated encouraging results in increasing the overall survival rates in a post-surgical adjuvant

setting (254), in the treatment of unresected disease, phase I/II trials have only been able to demonstrate a handful of regression events (255–257). Moreover, a randomized phase III trial of dendritic cell vaccine treatment did not demonstrate an increase in efficacy when compared to Dacarbazine chemotherapy (258).

Other vaccines approaches include ganglioside vaccines that attempt to exploit the high levels of immunogenic gangliosides on the surface of melanoma cells (259). Currently, these have only been investigated within the context of post-surgical adjuvant therapy and have not shown any increase in clinical benefit in comparison to either observation (260) or INF-α2b therapy (236). DNA vaccines directly inject the genetic code for melanoma antigens either through the use of plasmid DNA or recombinant viral vectors. Clinical trials using DNA vaccines have either reported minimal (3.33%) to no clinical response (261,262) or were unable to distinguish a definitive benefit in contrast to IL-2 co-treatment (263,264). However, another viral vaccination approach, the oncolytic virus T-VEC (Talimogene Laherparepvec, formerly OncoVex^{GM-CSF}) has shown clinical benefit. T-VEC is a herpes simplex virus type 1 (HSV-1) which has been modified for enhanced targeting of tumors for replication and lysis (265) as well as to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) (266). In a phase III clinical trial, T-VEC had a higher overall response rate compared to GM-CSF control (26.4% vs 5.7%) as well as an improvement in median overall survival (23.3 vs 18.9 months, p=0.51) (267). As such, in 2015, T-VEC (trademarked as IMLYGIC) became the first oncolytic viral therapy to be approved by the FDA (268).

By far, peptide vaccines, which directly inject the peptides of melanoma antigens, have been the most extensively tested in trying to generate an immune response against unresected melanoma. Historically, trials experimenting with various antigen peptides (MART-1, TRP-1, TRP-2, gp100, Tyrosinase, MAGE-12, NY-ESO-1) have demonstrated a limited overall response rate of approximately 2.9% (245). One outlier to this trend is a phase III trial reporting a higher overall clinical response rate with gp100 peptide vaccine and high-dose IL-2 in comparison to IL-2 therapy alone (16% vs 6%) (269). However, it should be noted that the IL-2 alone treatment group grossly underperformed the historically observed response rate for high dose IL-2 therapy (~16%) (219,224). Other trials have demonstrated that adding gp100 peptide vaccine did not enhance the benefit of high dose IL-2 (270). Furthermore, peptide vaccine treatments have not been improved by the addition of INF-α2b or GM-CSF (271), nor have peptide vaccines demonstrated a benefit in a post-surgical adjuvant setting (272). Altogether, despite much effort to use vaccines towards the eradication of unrested melanoma, clinical success towards that goal has been minimal and sporadic (273). Besides T-VEC, the only other cancer vaccine to be approved by the FDA is Sipuleucel-T for castration-resistant prostate cancer, which has only been able to improve median survival by approximately 4.1 months (274,275).

B-raf and MEK Targeted Inhibitors

In 2011, the FDA approved Vemurafenib, which specifically targets and inhibits the *BRAF*^{V600E} mutation (276), as it demonstrated a significantly higher overall response rate

compared to Dacarbazine (48% vs 5%) in a phase III clinical trial, with interim analysis compelling a recommended crossover from Dacarbazine to Vemurafenib (217). Unfortunately, these responses have not demonstrated durability as, in most cases, the residual tumor burden develops resistance to the treatment (277) resulting in a progression free survival of only approximately 6.2 months (278). Additionally, by definition, this treatment option excludes nearly half of all melanoma patients that do not have *BRAF*^{V600E} mutant tumors (192), and of those that do, the heterogeneity of a patient's melanoma burden may likely contain *BRAF*-WildType (*BRAF*^{WT}) cells intermixed with mutant cells, unaffected by the treatment (279). Moreover, Vemurafenib has been shown to counterproductively activate MAPK/ERK pathways in *BRAF*^{WT} cells (280) making the development of secondary malignancies, such as the noticeable onset of squamous-cell carcinoma, a continuing point of concern (217,281).

Alternatively, inhibitors targeting downstream MEK, such as Trametinib, have been developed (282). Trametinib has shown a modest benefit in overall survival when compared to chemotherapy. However, MEK inhibition did not demonstrate a significant improvement in efficacy when compared to previous trials with Vemurafenib, excluding the caveat that treatment does not exclude patients with *BRAF*^{WT} tumors (283).

Based on the rationale that the principle resistance mechanism of melanoma to BRAF targeted therapy is reactivation of the MEK/MAPK pathway (284,285), investigators have recently been investigating the combination of BRAF and MEK inhibition. Initial clinical trials demonstrate a promising increase in efficacy compared to single agent treatment with the FDA approval of combining Vemurafenib with the MEK inhibitor

Cobimetinib (286). This was based on a phase III clinical trial which showed an overall response rate of 68% (versus 45% in the Vemurafenib alone group) with a median progression-free survival of 9.9 months (versus 6.2 months for Vemurafenib) (278).

CTLA-4 and PD-1 Immune Checkpoint Inhibitors

In 2011, Ipilimumab, an antibody targeting the T cell inhibitory receptor CTLA-4, was approved for the treatment of late-stage melanoma. CTLA-4 negatively regulates T cell activation and proliferation (287), and impediment of CTLA-4 via a blocking antibody has demonstrated pre-clinical *in vivo* improvement in tumor control as early as 1996 (288). Ipilimumab was developed by Keler and colleagues in 2003 as a human monoclonal antibody (MDX-010) against CTLA-4 (289). MDX-010 was later acquired by Bristol Myers Squibb and Madarex and subsequently termed Ipilimumab (with the trade name "Yervoy"). A phase III trial comparing Ipilimumab to patients receiving a gp100 peptide vaccine demonstrated a significant improvement in overall survival (10.1 months versus 6.4) with 23.5% of Ipilimumab treated patients being alive at 24 months post-treatment compared to 13.7% in the vaccine treated group (290). However, the side effects of Ipilimumab treatment were fairly severe as 60% of treated patients developed an immune-related adverse event, with 10-15% being a grade 3 or 4 event, and 2.1% resulting in a drug related fatality (290). Retrospective analysis of initial phase II/III trials report a 22% three-year survival rate for Ipilimumab treated patients, which was improved slightly (26%) for treatment-naïve patients (291). Beyond melanoma,

treatment with Ipilimumab has also shown positive clinical benefit in renal cell carcinoma (292), prostate cancer (293), and ovarian cancer (294).

More recently, the targeting of another T cell inhibitor receptor, PD-1, has demonstrated even more encouraging results than Ipilimumab. In 2015, the anti-PD-1 antibody, Pembrolizumab, demonstrated a significant enhancement in progression free survival compared to Ipilimumab (47.3% versus 26.5%) (295). In 2016, another clinical trial demonstrated an overall objective response rate of 33% (296). These results led Pembrolizumab to be approved the same year as a first-line treatment option for *BRAF*^{WT} melanomas. Additionally, another anti-PD-1 antibody, Nivolumab, has also demonstrated objective responses in the clinic for melanoma with a 30.8% increase in overall one-year survival and a 26.1% increase in the objective response in melanoma patients (*BRAF*^{WT}) compared to Dacarbazine (297), and has also shown clinical benefit for non-small-cell lung cancer, prostate cancer, and renal cell cancer (297–304). Nivolumab has also been shown to be a successful secondary treatment when Ipilimumab has failed (298). Moreover, in addition to being more effective than Ipilimumab therapy, both Pembrolizumab and Nivolumab were generally well tolerated without demonstrating the safety concerns associated with Ipilimumab (305).

Being that the inhibitory receptors CTLA-4 and PD-1 are relevant at different stages in the cytotoxic T cell response, with CTLA-4 interfering in initial activation and PD-1 being engaged by PD-L1 in the tumor microenvironment, it has been postulated that combining anti-CTLA-4 and anti-PD1 therapies may result in an even greater therapeutic optimization. Indeed, the combination of Nivolumab with Ipilimumab resulted in a

significantly higher objective response rate than Ipilimumab alone (61% vs 11%) (306). Moreover, while none of the Ipilimumab-alone cohort exhibited a complete response, 22% of those receiving the combination therapy had a complete response (306). A 2016 follow-up study of that trial and another phase III trial found that patients treated with the combination therapy had a 10.2% higher 2-year survival rate that patients treated with Ipilimumab alone (63.8% vs 53.6%) (307). Additionally, in a separate study, the combination of Nivolumab plus Ipilimumab resulted in an enhancement of progression free survival compared to either single agent therapy (11.5 months for combination therapy, 6.9 months for Nivolumab, 2.9 months for Ipilimumab) (308).

Emerging Combination Therapies

Several studies have shown that BRAF inhibitors can produce an immunomodulatory effect in the context of melanoma. The *BRAF*^{V600E} mutation has been reported to interfere with MHC-1 antigen processing (309), and conversely, *BRAF*^{V600E} inhibitors have been shown to enhance antigen presentation by melanoma cells (310). Moreover, BRAF inhibition does not appear to interfere with lymphocyte function (311) and some studies have suggested BRAF inhibition even enhances T cell activation (312). Consequently, BRAF inhibition also demonstrates enhanced tumor infiltration by T cells (313). In addition to promoting T cell activation, BRAF inhibition, conversely, decreases production of immunosuppressive factors such as IL-10, VEGF, and IL-6 by melanoma cells (314).

Based on these observations, the rationale has been put forth to combine *BRAF*^{V600E} inhibition with an immune checkpoint inhibitor. A pre-clinical study has shown that the combination of *BRAF* inhibition and PD-1 blockade produces a more durable anti-tumor response than either single agent alone (315). Currently, several trials are investigating the combination of targeted inhibitors with immune checkpoint blockade. An initial trial was limited by toxicity (316), and other trials are ongoing though have not been yet able to show data (317).

Immunotherapy Models for Melanoma

This thesis project has employed several models to determine how the administration of N-acetyl cysteine can modulate the phenotype and anti-tumor functionality of melanoma-specific T cells. As demonstration of the enhancement of *in vivo* efficacy was the overarching intent of these studies, two models were used to treat C57BL/6 wild-type mice challenged with B16-F10 murine melanoma tumors. One model, utilized Pmel-1 transgenic T cells to represent therapeutic T cells with native TCRs, while the other model transduced peripheral T cells with a TRP-1 specific TCR to demonstrate experimental modulation of engineered T cells. Additionally, to determine the effect of N-acetyl cysteine on human therapeutic cells, patient autologous T cells transduced with the TIL1383I TCR were obtained from patients undergoing a melanoma clinical trial alongside samples obtained from healthy donors.

The Pmel-1 Model

The repertoire of antigens available from any tumor is highly composed of non-mutated, self-antigens, which present a significantly high hurdle of poor immunogenicity that the immune system must overcome for an effective anti-tumor response. Therefore, effective *in vivo* models must adequately represent this handicap for optimal translational relevance, which is lacking from models which utilize foreign antigens as targets.

The murine B16 melanoma cell line has been regarded as an exceptionally "poorly immunogenic" tumor cell as it expresses no surface MHC-II and very little MHC-I (318,319). In 1997, one of the murine self-antigens expressed by B16 melanoma cells, gp100 (or PMEL17), was cloned (320). Gp100 is indeed a self-antigen as it is involved in the maturation of melanosomes, the pigment producing organelle in healthy melanocytes (321). The following year, Willem Overwijk and colleagues immunized mice with human gp100 (as murine gp100 did not elicit a proper immune response) and generated T cell "clone 9." This clonotype faithfully recognized and killed B16 melanomas cells implanted into mice *in vivo* (322). In 2003, the same research group, using the RNA isolated from "clone 9" generated transgenic mice expressed this MHC-1 restricted gp100 specific TCR named the Pmel-1 mouse (323). Greater than 95% of the CD8+ cells from generated transgenic mice expressed Vβ13, the specific TCR-β chain inserted into the transgene. However, despite the overwhelming presence of anti-gp100 specific T cells, these mice were just as vulnerable to a B16 melanoma challenge as wild-type C57BL/6 mice. However, isolated splenocytes from these Pmel-1 mice adoptively transferred alongside gp100 peptide stimulation and IL-2 effectively controlled the tumors in B16 challenged

C57BL/6 wild type mice. Thus, the Pmel-1 transgenic mouse does provide T cells with TCRs which recognize murine gp100 on B16 melanoma cells and mounts a cytolytic response *in vivo* which can serve as a platform to model the adoptive transfer of T cells with native bearing antigen specific TCRs. However, these cells must be activated via stimulation with the altered peptide ligand of the human homologue of gp100 for optimum anti-tumor efficacy (322), which does not completely parallel adoptive transfer protocols in the clinic.

Transduced TRP-1 TCR Model

To model the use of TCR engineered cells in the clinic, this thesis project utilized an institutionally available cellular transduction core to transduce wild type C57BL/6 mouse splenocytes with a TCR specific for the melanoma associated antigen tyrosinase-related protein-1 (TRP-1) (324). TRP-1 is another self/tumor antigen which functions in healthy melanocytes to stabilize tyrosinase activity and likely protects against oxidative stress in the process of melanogenesis (325). TRP-1 specific T cells, derived either from a native-TCR expressing transgenic mouse or from the transduction of a TRP-1 specific TCR, do mediate control of B16 tumors *in vivo*, and in contrast to the Pmel-1 model, do not require peptide stimulation prior to transfer to elicit anti-tumor functionality (324,326).

Human TIL1383I TCR Transduced Cells

For the most expeditious determination of the clinical relevance for the findings in this thesis project, T cells transduced with the therapeutic TIL1383I TCR currently being

generated as part of a phase I clinical trial (NCT01586403) were obtained for experimental use. The generation of these cells began when TILs from "patient 1383" (327) were cultured and tested for reactivity, demonstrating that culture "TIL 1383 I" was specifically reactive against HLA-A2 expressing melanoma cells yet was not reactive against non-HLA-A2 and/or non-melanoma tumor cells (328). Subsequently, the cognate antigen targeted by this TIL culture was determined to be the 368-376 peptide epitope of tyrosinase ($hTyr_{368-376}$) of either the 370D genetically encoded or the 370N post-translationally modified variation (328,329).

Originally discovered in CD4+ cells (328), the TIL1383I TCR has a high enough affinity for $hTyr_{368-376}$ that it can elicit recognition of the HLA-A2 presented peptide without the presence of the CD8 co-receptor. Subsequently, this TCR was successfully retrovirally transduced onto human donor peripheral blood lymphocytes (70). Both transduced CD4 and CD8 cells demonstrated antigen recognition, and CD8 cells additionally demonstrated HLA-A2 and Tyrosinase specific cytolytic functionality (70).

Activation-Induced Cell Death

Therapeutic T cells which have undergone a REP in order to achieve the necessary quantity of cells for infusion are susceptible to activation-induced cell death (AICD) upon TCR restimulation *in vitro* (171), which likely limits their persistence upon encountering tumor antigen when transferred *in vivo*. While AICD is likely counterproductive for adoptive transfer therapy, it is a necessary mechanism of immune regulation in the prevention of autoimmunity. During the context of a normal immune response, T cells

are activated by three separate signals consisting of cognate MHC-presented peptide to the TCR (signal 1), costimulatory receptor engagement such as CD28 ligation (signal 2), and inflammatory cytokines that instruct the T cell towards the development of effector functionality (signal 3). Once properly activated, T cells undergo clonal expansion and move out into the periphery whereby they mount effector responses against infected cells displaying their cognate antigen (330,331).

After activation and expansion of effector T cells, it is then necessary to prevent damage to the host caused by either the continual production of inflammatory cytokines or the potential onset of autoimmune or lymphoproliferative pathologies. As such, the immune system negatively regulates the expansion of activated T cells by limiting their persistence once the invading pathogen has been cleared (332). There are several mechanisms that restrain the persistence of T cells once they have been activated to undergo an immune response. As T cells undergo active replication in the process of clonal expansion, their telomeres continually erode eventually imposing the "Hayflick limit" of replicative senescence (333). T cells are also constrained by lack of cytokine support by the mechanism of "cytokine withdrawal-induced death" (334). This type of cell death has also been termed "activated cell autonomous death" as it occurs independent of any TCR or death receptor signaling (335,336). Rather, as T cells no longer receive cytokine support, they begin to undergo programmed cell death via the intrinsic apoptotic pathway mediated by a threshold of pro-apoptotic Bcl-2 family of proteins overcoming the resistance of anti-apoptotic factors (336).

In contrast, another form of programmed cell death that regulates the persistence of activated T cells is initiated by ligation of the TCR and is termed activation-induced cell death (AICD). Ligation of the TCR is absolutely required for the onset of this type of cell death and can occur via both αβ and γδ TCR subsets (337). The requirement of TCR signaling has been confirmed by many studies which have demonstrated that mutations in the ITAM signaling motifs of the CD3 co-receptor (338–340) and deletion of $p56^{lck}$ (341) thwart the occurrence of cell death upon TCR restimulation.

As a mechanism of peripheral tolerance, AICD is exclusively constrained to cells that have been previously activated via a primary TCR stimulus, as naïve T cells are resistant to cell-death induced by TCR ligation (342). Moreover, signaling from the activating cytokine IL-2 is also required to prime cells for AICD (343,344). Consequently, T cells from IL-2 and IL-2Rα deficient mice are resistant to AICD (345).

Modulation of any of the three signals involved in T cell activation can determine the susceptibly of an individual T cell towards AICD. A stronger TCR stimulus (signal 1), either through a higher affinity TCR or concentration of antigen, makes a T cell more susceptible to AICD (346,347). In parallel to TCR ligation, engagement of the co-stimulatory receptor CD28 (signal 2) can promote the proliferation of T cells through the activation of NF-κB (348), enhancement of glycolytic metabolism (349), upregulation of telomerase (350), as well as protection from AICD (351,352). Additionally, as T cells undergo a REP, they lose CD28 expression, but gain expression of another co-stimulatory receptor, 4-1BB, whose engagement also protects cells from AICD (171). However, such co-stimulatory engagement is rarely found within the tumor microenvironment (353)

leaving T cells that have trafficked to the tumor devoid of this protection. Consistent with the role of AICD in immune-constraint, inflammatory "signal 3" cytokines such as IL-12 and IFNγ aid in priming T cells for AICD (354,355). However, other cytokines, such as IL-4 and IL-15, have been shown bolster the resistance of T cells to AICD (356,357).

Many reports in the literature assert that Fas death receptor signaling is an integral part in the onset of AICD. Indeed, Fas deficient mice have a massive lymphoproliferative disorder indicating a Fas-critical deficiency in immune-regulation (358,359). Moreover, Fas expression is upregulated during TCR restimulation (360). However, multiple reports have shown that AICD still proceeds in the absence of Fas signaling either by the lack of Fas receptor expression (361–364) or utilization of a FasL blocking antibody (365,366) which suggests that Fas is not required for cell death to occur following TCR restimulation (367). Moreover, the downstream signaling components of AICD are not consistent with the requirement for FAS death receptor signaling. Fas operates independently of ITAM signaling (368). TCR restimulation results in the accumulation of reactive oxygen species (369) which is required for the onset of AICD as the ROS scavenger MnTBAP can protect T cells from death following TCR restimulation (370). Conversely, FasL induced cell death operates independently of ROS signaling (370,371). While Fas induced apoptosis is inherently dependent on caspase execution, inhibition of caspases cannot block AICD, nor are caspases activated during AICD (366,367,372,373). Moreover, the cell death morphology of AICD does not contain apoptotic blebs but appears more necrotic in nature (367). Finally, small scale DNA fragmentation consistent with an apoptotic phenotype is also absent from AICD

(373,374). Instead, AICD presents with large scale DNA damage mediated by Apoptosis Inducing Factor (AIF) (373). Thus, the cell death phenotype of TCR restimulated AICD is emerging to be more consistent with programmed necrosis (or "necroptosis") than classical apoptosis (375). Consistently, RIPK1 and RIPK3, involved in the onset of necroptosis (376), are induced during AICD, while conversely pharmacological inhibition of necroptosis protects T cells from cell death following TCR restimulation (377).

T cell Receptor Generated ROS and Redox Regulation of Cellular Processes

Generation of ROS Upon TCR Stimulation

Ligation of the TCR results in the rapid generation of reactive oxygen species (ROS) with both superoxide (O_2^-) and hydrogen peroxide (H_2O_2) species being distinctly generated within 15 minutes of TCR stimulation (369). In the cell, ROS are primarily generated in the mitochondria as a byproduct of metabolism. Within the mitochondria, Complex I and Complex III of the electron transport chain are major hubs of ROS generation (378). Complex I emits superoxide into the mitochondrial matrix whereby it is rapidly converted to hydrogen peroxide by the antioxidant enzyme manganese superoxide dismutase (MnSOD/SOD2) (378). Complex III releases superoxide into both the matrix and the intermembrane space (IMS) of the mitochondria (379,380). This superoxide in the IMS then has the potential to be transported into cytosol of the cell, without prior dismutation by MnSOD2, via voltage-dependent anion channels (381).

In the T cell, ROS has also been demonstrated to be a product of NADPH (nicotinamide adenine dinucleotide phosphate) Oxidase, which has classically been

characterized as responsible for the "respiratory burst" of pathogen killing free-radicals in the innate immune system (382). However, the Laboratory of Mark Williams has demonstrated NADPH Oxidase to also be relevant in the generation of ROS in the T cell. Devadas, *et al*, blocked the generation of superoxide by either preincubation of T cells with the NADPH Oxidase inhibitor diphenylene iodonium (383,384) or the overexpression a dominant negative form of Rac1 (N17 Rac1), which has been implicated in the ROS generating functionality of NADPH Oxidase (369,385). These data suggest a role for NADPH Oxidase in the production of superoxide in T cells upon TCR ligation. Subsequently, the lab demonstrated that T cells expressed mRNA for the NADPH Oxidase subunits $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$ and also had detectable protein expression for the $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$ subunits (386). Moreover, T cells from mice deficient in either $p47^{phox}$ or $gp91^{phox}$ NADPH Oxidase subunits have defective generation of ROS when undergoing TCR stimulation, supporting the role of NADPH Oxidase in TCR stimulation induced ROS generation (386).

Redox Regulation of Cellular Processes

Given the destructive nature of free radical species, the generation of ROS by T cells has classically, and appropriately, been viewed in terms of regulating T cell survival or death (387). Indeed, the scavenging of superoxide with the SOD mimic MnTBAP protects cells from AICD (370). Moreover, blocking ROS production through the inhibition of mitochondrial Complex I also protects T cells from AICD (388). However, more than just being a blunt force of cell destruction, the levels of ROS can exert influence in the

regulation of a wide array of cellular functions outside of the paradigm of cell death (389). For instance, oxidative stress has long been known to be a determinant of T cell proliferation. In general, the quenching of ROS through use of antioxidants has been shown to imped the activation, proliferation, and IL-2 secretion of mitogen stimulated T cells (390–393). Incubation of T cells with the Complex I inhibitor rotenone reduced ROS as well as T cell proliferation, cytokine production, and reduction in the degranulation marker CD107a, implying a decrease in cytolytic functionality (394). Disruption of Complex III through a T cell specific knockout of the Complex III subunit RISP resulted in T cells that were unable to undergo antigen-specific proliferation coinciding with defective NFAT activation, IL-2 secretion, and expression of CD25 and CD69 activation markers (395). Although, on the contrary, some reports have demonstrated that the exogenous addition of pro-oxidants can also block T cell activation (396–398). And moreover, decreasing a T cell's antioxidant capacity through depletion of glutathione (GSH), through the use of buthionine sulphoximine (BSO), can also thwart T cell proliferation (399–401).

The disagreement in these reports likely highlights the specific nature of the redox regulation of cellular processes, as indeed ROS levels not only dictate whether a T cell will die or proliferate but can have highly nuanced functional consequences for the T cell. Mice with a T cell specific knockout of SOD2 present with elevated levels of mitochondrial superoxide and a disruption in T cell thymic development rendering the mice more susceptible to influenza challenge (402). In manipulating the redox environment after thymic development, antioxidants can push a T cell towards an increase in the Th1 response (i.e. more IFNγ and TNFα) and a decrease in Th2 responses (i.e. less IL-4, IL-5)

(403–405), and pro-oxidants can have the opposite effect favoring a Th2 phenotype at the expense of Th1 (406,407). Consistently, the T cell specific deletion of the NADPH Oxidase subunit $p47^{phox}$ by Jackson, *et al* resulted in an increase of IFNγ and IL-2 with a concurrent decrease in IL-4 and IL-5 (386). This finding was further corroborated in a subsequent report demonstrating reduced expression of the Th2 promoting transcription factor Gata-3 in these $p47^{phox}$ deficient T cells (408,409).

The Cysteine Thiol Switch

Altogether, these studies affirm the importance of redox signaling in regulating a diverse array of cellular outcomes. Of free radical species, hydrogen peroxide has emerged as the chief signaling molecule (410). Hydrogen peroxide has several properties, particularly in contrast to superoxide, that make it an ideal molecule for signaling. Hydrogen peroxide has a significantly longer half-life (1ms vs 1μs) than superoxide (410). Moreover, the instability of superoxide is further compounded by its rapid dismutation into hydrogen peroxide catalyzed by superoxide dismutase (SOD) enzymes found in the cytoplasm (CuZnSOD/SOD1), the mitochondria (MnSOD/SOD2), and extracellularly (SOD3) (411). Additionally, hydrogen peroxide has a relatively tempered rate of oxidation which allows for a certain discernment in prioritizing which protein residues it oxidizes, in contrast to a more reactive species, such as the hydroxyl radical (OH•), that is more unsystematic in its oxidative activity, haphazardly attacking all biomolecules (410,412).

The preeminent target for hydrogen peroxide is the sulfur containing thiol moiety (R-SH) located on cysteine residues, of which there seems to a be selective targeting of certain cysteine thiols within the proteome (413). The reaction of hydrogen peroxide with the sulfur thiol (R-SH) produces the oxidized sulfenic acid (R-SOH) through the process of sulfenylation (414). Hydrogen peroxide oxidizes detoxifying enzymes, such as glutathione peroxidase (GPx) or peroxiredoxin (Prx), with an elevated priority due to the presence of catalytically advantageous thiols located within the active site of these enzymes that aid in a more favorable reaction (415,416). However, amongst less reactive cysteine residues, it is still unclear how hydrogen peroxide discerns which residues to oxidize. Though several, non-exclusive, guiding principles have emerged towards understanding the selectivity of certain cysteine residues by hydrogen peroxide. Certainly, a detoxifying enzyme may oxidize another cysteine residue as a result of its reaction with hydrogen peroxide, such as the oxidation of the transcription factor Yap1 by GPx3 in yeast (417). The localization of certain proteins relative to the production of hydrogen peroxide may also influence the likelihood for oxidation (414,418). For example, the localization of Nox4 (NADPH Oxidase 4) to the endoplasmic reticulum is key to the regulation via oxidation of the ER resident protein PTP1B (419). Additionally, steric hindrance and the polarity of adjacent residues can influence the pK_a value of an individual cysteine towards its reactivity with hydrogen peroxide (420).

The elucidation of proteins that are regulated by sulfenylation is ever evolving and relevant for many different cellular functions (414). For instance, sulfenylation has been implicated in the regulation of the PIP3/Akt proliferation pathway. PTEN, which represses

phosphatidylinositol 3,4,5-trisphosphate (PIP3), and subsequent activation of the AKT pathway, is inactivated via transient oxidation of its cysteine residues (421). Conversely, maintaining the Cys^{296} and Cys^{310} residues of Akt1 in their reduced form has been shown to be necessary for optimal Akt1 activation (422). Apoptosis can be regulated through the inactivation of the anti-apoptotic protein Bcl-2 by oxidation of its Cys^{158} and Cys^{229} residues (423). The status of protein ubiquitination can also be regulated through the cysteine oxidization induced inactivation of deubiquitinases such as USP1 or A20 (424,425). Cysteine oxidation can also provide critical feedback to launch antioxidant protective measures within the cell during periods of increasing oxidative stress. The transcription factor Nrf2 coordinates the upregulation of many antioxidant genes termed the "antioxidant response element" and is negatively repressed via nuclear exclusion and ubiquitination mediated proteasomal degradation by Keap1 (426). Consequently, Keap1 can be inactivated through oxidation of its Cys^{273} and Cys^{288} residues, allowing for the liberation of Nrf2 to traffic to the nucleus and transcribe pro-antioxidant genes (427).

For the T cell, sulfenylation based signaling is emerging as a key regulator of activation. Oxidation of the Cys^{195} residue of the calcium channel protein ORAI1 can inactivate it, impairing calcium influx during TCR activation (428). Moreover, the transcription factor NF-κB, necessary for T cell activation, requires that the Cys^{62} residue of its p50 subunit be in its reduced state for its DNA binding activity (429). Upon TCR stimulation, Michalek, *et al.* demonstrated that there is a global increase in the level of cysteine sulfenic acid (430). Moreover, treating T cells with dimedone, which covalently binds to cysteine sulfenic acid and does not allow further modification (431), prevented

T cell proliferation, implying that both the formation of sulfenic acid and its reversal are critical for the induction of T cell proliferation (430). The same study demonstrated that TCR stimulation results in cysteine sulfenic acid modification of the protein tyrosine phosphatases (PTP) SHP-1 and SHP-2 (430). This is consistent with previous studies that have shown sulfenylation to be a critical regulator of global PTP activity (432) as PTPs have a redox sensitive cysteine in their catalytic core (433). Moreover, in the case of SH2-domain containing PTPs, such as SHP-1 and SHP-2, unique regulatory "backdoor" cysteines have been identified which participate in disulfide bond formation critical for reversible oxidation (434).

Beyond sulfenylation, cysteines can achieve higher oxidative states such as sulfinic acid ($R\text{-}SO_2H$) and sulfonic acid ($R\text{-}SO_3H$) which are less amenable towards being reduced back towards a neutral thiol. Though, sulfiredoxin can catalyze the reduction of sulfinic acid with emerging signaling consequences (435). Sulfonic acid is generally regarded as an irreversibly oxidized state. Additionally, more aggressive oxidants, such as peroxynitrite or the hydroxyl radical, can oxidize thiols into thiyl radicals (436). In order to protect proteins from further oxidation, residues can undergo an S-thiolation modification whereby mixed disulfides are formed between the thiol residue and another low molecular weight thiol (437). When that particular low molecule weight thiol is glutathione (GSH), the modification is termed S-glutathionylation (438). The overall process of S-glutathionylation can be reversed by deglutathionylation facilitated by thioredoxin, glutaredoxin, (439), or sulfiredoxin (440). The reversible nature of glutathionylation allows it to serve in a signaling capacity beyond antioxidant protection.

Glutathionylation has been shown to regulate a diverse array of cellular functions. Glutathionylation promotes the inactivation of NF-κB both directly on the Cys^{62} residue of its p50 subunit (441) as well as upstream inactivation of IKKβ by glutathionylation of its Cys^{179} residue (442). Additionally the stress activated protein kinase MEKK1, also implicated in NF-κB signaling (443), is inhibited by glutathionylation on Cys^{1238} (444). Conversely, glutathionylation promotes Akt signaling by both activating upstream Ras on Cys^{118} (445) and inactivating PTEN (446). The tumor suppressor p53 is inactivated by glutathionylation of cysteines within its DNA binding domain, which prevent DNA binding and the necessary tetramerization of p53. (447). The cytoskeleton can also be regulated by glutathionylation as the rate of actin polymerization is reduced upon glutathionylation of its Cys^{374} residue (448). Glutathionylation in the α-rings aids to open the 20S proteasome facilitating the proteasomal degradation of proteins (449). Apoptosis can be promoted by glutathionylation of the Fas death receptor on its Cys^{294} residue which facilities its transport to lipid rafts, encouraging the formation of the death-inducing signaling complex (450). However, on the contrary, the cell death executioner Caspase 3 can be inactivated by glutathionylation (451). Glutathionylation of eNOS on Cys^{689} and Cys^{908} switches it from producing nitric oxide (NO) to superoxide (452). Additionally, glutathionylation can also regulate ion channels as it inhibits both the Kir4.1-Kir5.1 inward rectifier K^+ channel (by glutathionylation of the Cys^{158} residue of the Kir5.1 subunit) (453) and the Na,K-ATPase Na^+K^+ antiporter by an increase in the glutathionylation of multiple residues of its α-subunit (454). Within the mitochondria, glutathionylation of Complex I leads to a decrease of electron transfer activity with an inverse increase in ROS production

(455), as well as inhibition of several metabolic enzymes: α-ketoglutarate dehydrogenase (456), GAPDH (457), aldose reductase (458), succinyl-CoA transferase (459). As such, there are a diverse array of cellular process which are regulated by either sulfenylation or glutathionylation of cysteines which could like be altered modulating the redox environment or glutathione concentration in the cell by treatment with the glutathione pro-drug N-acetyl cysteine.

N-acetyl cysteine

N-acetyl cysteine (NAC) is a derivative of the sulfhydryl group containing amino acid cysteine whereby an acetyl group has been attached to the nitrogen atom of cysteine (Fig. 1). This modification enhances the stability of cysteine, which is rapidly oxidized in solution to form the biologically inactive Cys-Cys disulfide dimer Cystine. The acetyl moiety of NAC makes it less reactive than cysteine and thereby less susceptible to this oxidation/dimerization (460).

Biochemically, NAC has been reported to have four primary mechanisms of action. First, NAC's primary benefit to the cell is to serve as a pro-drug for glutathione (GSH) synthesis. NAC contributes the cysteine, which is then conjoined to glutamate via the enzyme glutamate-cysteine ligase. Subsequently, glycine is then added via the enzyme GSH synthase to complete the GSH tri-peptide. GSH synthesis is self-regulated as GSH itself inhibits glutamate-cysteine ligase (461,462). Therefore, NAC is only beneficial in this regard in situations where there is both depleted levels of GSH and sufficient levels of

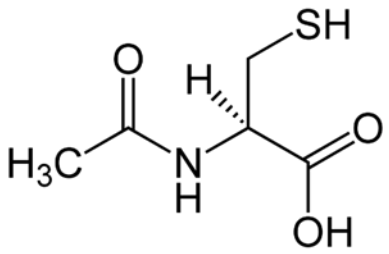

Figure 1. Chemical structure of N-acetyl cysteine

enzymes involved in GSH synthesis are maintained (460). In such situations, where there are competent levels of GSH, NAC administration can alternatively lead to an increase in cysteine levels in cells and in the plasma (463,464). Second, NAC is commonly regarded as an "antioxidant" and has often been shown to modulate oxidative stress in the literature (465). However, the interpretation of NAC as a direct reducing agent is complex as NAC is approximately 10-fold less efficient as a reducing agent than GSH (466) and both NAC and GSH are orders-of-magnitude less efficient at resolving oxidative stress than antioxidant enzymes such as superoxide dismutase (467). However, thirdly, NAC has been well documented to be important for the direct reduction of disulfide bonds. This has been specifically clinically relevant in bronchial disorders whereby the disruption of disulfide bonds in the glycoprotein matrix of mucus by NAC aids in reducing mucosal viscosity (468). Moreover, such modification of cysteine structure can have consequences in terms of alteration of ligand binding and structure-based functionality of proteins (465). Lastly, NAC can directly bind and chelate metals. Particularly, NAC has been documented to chelate the food and environmental contaminate methylmercury (469). Administration of NAC increases the urinary excretion of methylmercury in rats in a dose dependent manner (470). NAC also protects against mercury (471,472), cadmium (473), potassium dichromate, lead tetraacetate (474), and copper poising (475) in rat models as well.

NAC can be administered orally, intravenously, or by aerosol for use as an anti-mucosal agent. NAC, administered orally, is quickly metabolized in the liver rendering just 6-10% bioavailability in the plasma with a T_{max} of only 1-2 hrs (476). Intravenous

administration of NAC produces a higher initial concentration in the plasma (300µmol/L i.v. versus 19.9µmol/L oral) (477). However, retention of NAC in the plasma is not much improved with a half-life of only 2.27 hrs (477) likely due to the rapid oxidation of NAC in the plasma into NAC-cysteine and NAC-NAC formations (478).

Radiolabel studies suggest that NAC does not readily cross the cell membrane (479) likely due to the influence of negatively charged -COOH and -SH groups. However, protonation studies suggest that an acidic environment such as the stomach may put NAC at a more neutral isoform (480), though this has not been verified experimentally. NAC has primarily been reported to enter the cell via the System X_C^- cysteine-glutamate antiporter (481,482), though other transporters such as the System ASC (alanine-serine-cysteine) transporter system may also facilitate the transport of NAC (483).

The clinical utility of NAC was first demonstrated in the 1960s as an effective mucolytic agent in cystic fibrosis (CF) when used as an inhalant via breaking up disulfide bridges in the glycoproteins of the mucus (468,484). Beyond providing aid in the clearance of mucus, NAC can also benefit CF patients through the elevation of GSH levels which are notably depressed in the epithelial lining fluid (485) and the bronchoalveolar lavage fluid of CF patients (486). Additionally, NAC has also been shown to be beneficial in other respiratory conditions. NAC has been shown to reduce bronchial hypersecretion, exacerbations, and re-hospitalization episodes in chronic bronchitis and COPD (487–490). However, NAC does not prevent the overall decline of lung function associated with COPD (491). NAC also protects against smoking induced injury of endothelial cells (492) and the lungs of rats exposed to cigarette smoke (493).

After its initial use as an anti-mucolytic agent, the other most notable and long-standing use of NAC in the clinic has been to ameliorate the toxicity caused by acetaminophen poisoning (494). The metabolism of acetaminophen produces the by-product N-aceytl-p-benzoquinone imine (NAPQI) which is toxic to the cell (495,496). GSH neutralizes NAPQI back to acetaminophen; however, overwhelming this system via overdose can cause a rapid depletion of GSH allowing for NAPQI to accumulate in toxicity (497). As such, replenishment of GSH via NAC supplementation is thought to counteract this toxicity (498,499). Resolution of toxicity is most effective when NAC can be administered within 8 hours of overdose (500) with no notable difference in efficacy between oral and intravenous administration (501).

NAC enjoys a fairly benign safety profile even at very high doses (such as used in the case of acetaminophen poisoning). Most side-effects come about through intravenous administration where mild nausea and gastrointestinal symptoms can occur (489). More severe cases of anaphylactic reactions have been reported such as bronchospasm, angioedema, and hypotension, although attributed to an overestimation of i.v. dosage or with asthmatic patients (502). And though extraordinarily rare, a handful of fatal events have been reported with improper dosing of NAC (503).

NAC can be beneficial in certain cardiovascular applications. The cardiovascular complications of hyperhomocysteinaemia can be prevented by the conversion of homocysteine to methionine by NAC (504). Administration of NAC attenuates damage caused by myocardial infarction (505,506) in the clinic and damage caused by stroke in a rat model (507) most likely through the alleviation of ischemia/reperfusion injury (508).

Additionally, NAC has been shown to improve overall vascular health by preventing oxidative stress caused atherosclerotic plaques (509), inhibition of adhesion molecules such as ICAM and VCAM (510,511), thwarts platelet aggregation (512) and aids in vasodilation (513) particularly through the attenuation of angiotensin (514) and increased bioavailability of the vasodilator NO (515) through enhanced eNOS expression (516).

NAC has even been shown to be beneficial in certain neuropsychiatric disorders both by GSH replenishment where GSH depletion associates with a neuropsychiatric disorder (517,518) and also by increasing the extracellular levels of glutatmate, stimulating the release of vesicular dopamine via group II metabotropic glutamate receptors (519). In various trials, NAC has been able to demonstrate a beneficial effect of the symptoms associated of schizophrenia (520,521), obsessive-compulsive disorder (522), autism (523), and bi-polar depression (524). Additionally, administration of NAC has reduced addictive behavior associated with nicotine (525), cannabis (526), cocaine (527), methamphetamine (528), and even gambling (529).

Within the immune system, administration of NAC has exhibited a diversity of outcomes. For instance, NAC has been shown to mediate certain autoimmune parameters such suppression of anti-DNA antibodies and enhancing survival in a lupus mouse model (530) and quelling T cell hyporesponsiveness in rheumatoid arthritis patients (531) as well as reduction in TNFα-induced RA related cytokines through the inhibition of NF-κB (532–534). Additionally, administration of NAC has also been shown to attenuate neutrophil chemotaxis and ROS production (535,536). However, in other studies, NAC has been shown to enhance T cell functionality. Administration of NAC *in*

vitro can increase T cell secretion of IL-2 (537) as well as T cell proliferation (538). Moreover, NAC has also been shown to functionally restore the immune system in advanced cancer patients (539).

As the progression of HIV exacerbates the depletion in GSH levels, and as GSH levels inversely correlate with patient prognosis, it was proposed in the 1990s that NAC supplementation may be of benefit to HIV patients (540,541). Several studies have demonstrated a modest restoration in glutathione levels and immune function with NAC administration (542–544). However, no studies have demonstrated a prolonged benefit, and other studies have contradicted the utility of NAC administration in the ability to increase GSH levels in HIV patients (545). Altogether, benefit of NAC administration is likely transient at best as part of the pathology of HIV is interruption of the GSH synthesis machinery likely through a HIV-Tat mediated mechanism (545–548).

Administration of NAC may also affect the antigen-presenting compartment of the immune system as well. Cysteine supplementation to T cells by APCs has been shown to be critical for optimal T cell activation and proliferation (549,550) and endows them with protection against oxidative stress (551,552). Moreover, elevated intracellular GSH content has been shown to correlate with enhanced pro-inflammatory IFNγ secretion by dendritic cells and macrophages (553). As such, disruption of GSH synthesis in dendritic cells and T cells is one mechanism Tregs use to suppress T cell activation (554). However, some studies have suggested NAC may play a detrimental role as NAC has been shown to suppress dendritic cell activation by its repression of NF-κB as well as reduce expression of CD86 and CD40 co-stimulatory molecules (555). Altogether, studies with NAC have

demonstrated it can constitute a wide range of effects beyond simply being an antioxidant. However, most of these reported observations with NAC, some of which demonstrate contradictory outcomes, must be appreciated at the specific dosage and cell type or disease context reported.

p53

The tumor suppressor p53 has been implicated in the onset of AICD. In 2013, Chhabra & Mukherji demonstrated that the p53-inhibitor, pifithrin-u, protects T cells from cell death following TCR restimulation (556). The implication that p53 is involved in AICD is consistent with its role as a master regulator of cell death. First discovered in 1979 (557), p53 was recognized in the late 1980s to be a tumor suppressor (558–560). Since then, p53 has emerged to be one of the chief tumor suppressors earning the title as the "Guardian of the Genome" (561). The critical role of p53 in tumor suppression is evidenced by the fact that it has either been deleted or functionally inactivated in nearly half of all cancers (562,563). Consistently, p53-knockout mice quickly develop and succumb to lymphomas at approximately 6 months of age (564,565). In melanoma, p53 seems to be a hurdle in the initial dysplasia of benign nevi (566). In more advanced melanoma, p53 has a low level of mutations, but seems to be overexpressed (567).

The cell death functions of p53 have been characterized both within and outside of the nucleus. Within the nucleus, p53 functions as a transcription factor by coordinating the cellular response to a stress insult. Generally responding to DNA damage, p53 preferentially upregulates DNA repair genes while halting cell cycle progression (568),

giving the cell an opportunity to resolve the stress and repair any damage. However, if resolution of the stress insult cannot be accomplished, p53 then begins to target cell death genes such as Bax, Bak, Puma, etc (568). Outside of the nucleus, p53 functions in a transcriptionally-independent manner. It can promote the oligomerization of Bax and Bak at the outer membrane of the mitochondria towards the formation of the mitochondrial outer membrane permeabilization, and it can also sequester Bcl-2 and Bcl-xL which antagonize Bax/Bak oligomerization (569,570).

In unstressed cells, p53 levels are repressed through proteasomal degradation via the E3-ubiquitin ligase HDM2 whereby it binds and ubiquitinates the Transactivation Domain 1 of p53 targeting it for proteasomal degradation (571). In response to stress, p53 is phosphorylated, and the resulting conformational change results in dissociation from HDM2 (572,573). One of the primary residues to undergo phosphorylation in response to cellular stress (particularly DNA damage) is Serine-15 (574). In addition to facilitating the dissociation of p53 from HDM2, phosphorylation on Ser15 also promotes accumulation of p53 in the nucleus, another hallmark of p53 activation (Fig. 2) (575,576). Ser15 is located within a nuclear localization signal (577) as well as a nuclear export signal motif, which is inactivated via Ser15 phosphorylation retaining p53 in the nucleus (578). Furthermore, the phosphorylation of Ser15 is necessary for p53 functionality as an alanine substitution mutation protects cells from death following genotoxic stress (579). Moreover, cytoplasmic sequestration is one common mechanism whereby tumor cells

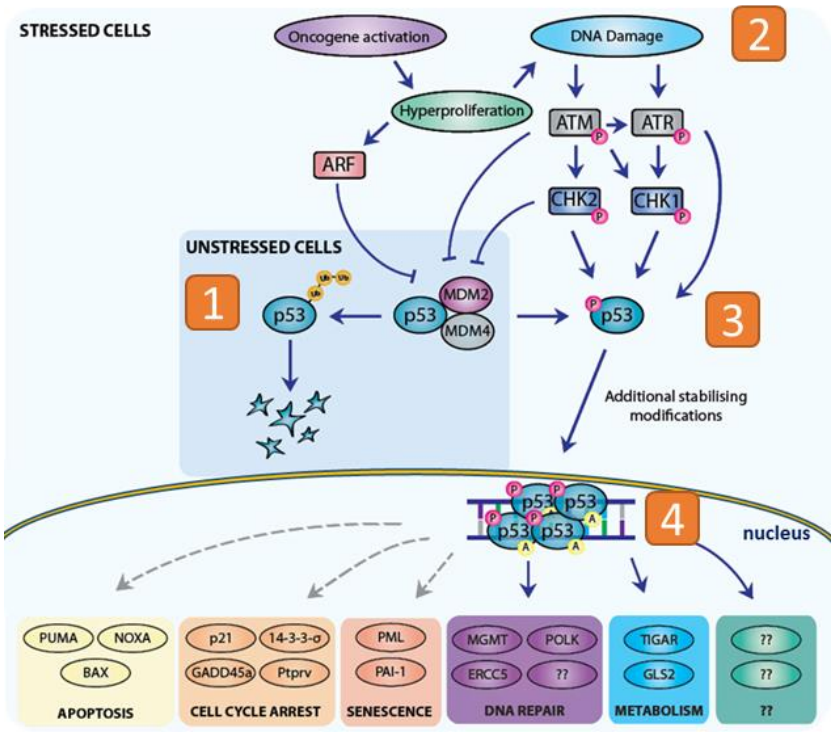

Figure 2. p53 is activated by phosphorylation and nuclear accumulation. (1) In unstressed cells, the E3 ubiquitin ligase MDM2 (HDM2 in the human) ubiquitnates p53 resulting in proteasomal degradation. (2) DNA damage results in the activation via autophosphorylation of ATM on Ser1981 (3) Activation of ATM results in the downstream phosphorylation of p53 on one or more of its N-terminal serines which promotes a conformational change allowing it to dissociate from MDM2. (4) p53 accumulates in the nucleus whereby it begins to promote the transcription of cell cycle arrest and repair genes, and then, if the stress cannot be resolved, p53 ultimately targets pro cell death genes (i.e. *BAX, NOXA, PUMA*) for transcription [Adapted from (580)].

deactivate p53 functionality in the nucleus, further supporting a cell death role of nuclear p53 (581).

p53 seems to play more complex role in the immune system than simply maintaining genomic integrity. Watanabe *et al*. demonstrated that p53 is critical to blocking non-specific IL-2 driven T cell proliferation, enforcing the requirement of antigen-specific TCR ligation for proliferation (582). p53 has also been shown to regulate autoimmunity. p53 promotes the differentiation of Tregs by conversely dampening the differentiation of Th17 cells through repression of STAT3 (583). Consistently, autoimmunity is exaggerated where there is a loss of p53 expression or function such as in arthritis (584–588), multiple sclerosis, (589,590), and Crohn's disease (591,592).

ATM

The chief kinase responsible for phosphorylation of p53 is Ataxia Telangiectasia Mutated (ATM). ATM derives its name from being functionally inactivated in the neurodegenerative disease Ataxia Telangiectasia (593). One of the many consequences of Ataxia Telangiectasia is a high rate of lymphoid malignancies, which is consistent with the failure of T cells from Ataxia patients to undergo cell death in response to DNA damaging insults (594). DNA damage, particularly the presence of double-strand DNA breaks (DSB), activates ATM. In response to DSB, the DNA damage sensing MRN complex targets ATM to the lesion, resulting in the autophosphorylation of ATM on Serine 1981. In its inactive form, ATM is in a dimer conformation, and autophosphorylation on Ser1981 causes it to disassociate into its active, monomeric form (p-ATM) which engages in

downstream kinase activity, such as phosphorylation of p53 on Ser15 (595). Consequently, T cells from Ataxia patients have reduced levels of p-p53^{Ser15} upon genotoxic stress (596).

However, while ATM has primarily been regarded to be activated by DNA damage, several reports have demonstrated that ATM can be activated by oxidative stress (597) or by hypoxia (598) in the absence of DNA damage. As such, activation of ATM alone is not sufficient to suggest the onset of DNA damage within a cell. Additional confirmation, such as the presence of γH2AX foci, should be examined as well to confirm that ATM is indeed being activated by *bona fide* DNA damage (599).

T cell Exhaustion and PD-1

An acute immune response effectively removes the foreign pathogen and its associated antigens allowing for contraction with a subset of cells remaining as memory cells. However, chronic infections or cancers, such as melanoma (600), which have evaded an initial immune response, are not resolved resulting in the continuation of antigen engaging the immune system (601). Such chronic stimulation leads to T cells exhibiting an exhausted phenotype denoted by lack of adequate effector functionality (602,603). Additionally, exhausted T cells express key inhibitory receptors such as PD-1, TIM-3, and/or LAG-3 (604).

T cells which have trafficked to the tumor microenvironment are reported to be exhausted even in the early stages of cancer (605), and exhaustion involves epigenetic modification to the T cell (606,607). Simply removing antigen does not revert an

exhausted phenotype (608). As such, PD-1 levels remain high on T cells from HIV patients even when viral load is significantly suppressed through retroviral therapy (606). However, modulation of inhibitory receptor signaling, such as PD-1/PD-L1 blockade can restore cells from an exhausted phenotype (609) given that PD-1 expression is not too high (610).

Programmed Death-1 (PD-1), aptly named first being discovered in T cells undergoing cell death, (611) is the most characterized marker of T cell exhaustion. PD-1 is initially induced upon TCR stimulation in T cells (612). During an acute infection, PD-1 levels peak at around Day 6 after stimulus and then contract back to basal levels. However, during chronic infection, where there is continued antigenic stimulation, PD-1 levels remain elevated (609). As such, PD-1 is expressed on viral-specific cells of patients with HIV (613), hepatitis B (614), and hepatitis C (615). Additionally, as the melanoma tumor microenvironment provides a source for continuous antigen exposure, PD-1 reliably marks melanoma specific TILs within the tumor microenvironment (616).

Expression of PD-1 is promoted by the transcription factor EOMES and repressed by T-bet expression (617,618). Ligands for PD-1 are PD-L1 (619) and PD-L2 (620). PD-L1 is expressed on melanoma cells (621). Ligation of PD-1 during activation can thwart proliferation, cytokine production, cytolytic function and impair T cell survival. PD-1 signaling attenuates phosphorylation of TCR signaling elements CD3ζ and ZAP70, reduces production of IL-2 (622), and depresses the PI3K-Akt cell proliferation pathway (623).

Consistent with its involvement in negative immune regulation, PD-1 knockout mice demonstrate an autoimmune phenotype via autoimmune dilated cardiomyopathy

(624), lupus-like disease (625) and are more susceptible to experimental autoimmune encephalomyelitis (626). Accordingly, mutational defects in PD-1 have been associated with human disease conditions such as multiple sclerosis (627), lupus (628), and Type I diabetes (629). Conversely, PD-1 deficient T cells demonstrate enhanced B16 tumor control in a murine adoptive transfer model (630). Together, these observations suggest that active PD-1 pathways quell the functionality of T cells.

Foxo1

Expression of PD-1 is regulated upstream, in part, by the activity of the transcription factor Foxo1 (631). Foxo1 (Forkhead Box O1) was discovered in 1993 (632) and has primarily been regarded as transcription factor acting as a tumor suppressor (633). For instance, Foxo1 can be activated via DNA damage in the cell and regulates cell death as siRNA knockdown of Foxo1 protects cells from DNA damage induced cell death (634).

As a transcription factor, Foxo1 is primarily regulated by its subcellular location in relation to the nucleus. Foxo1 is chiefly inactivated via phosphorylation by Akt at its Thr23, Ser256, and Ser319 residues. Phosphorylation of Foxo1 causes it to bind with 14-3-3 proteins sequestering it in the cytoplasm (635–637) in part through blockade of Foxo1's nuclear localization signals (638). Additionally, Akt mediated phosphorylation of Foxo1 can be enhanced by other post-translational modifications such as acetylation, which also interferes with its ability to bind to DNA (639). Moreover, beyond restricting nuclear access, sustained Akt signaling can also promote the ubiquitination and

subsequent proteasomal degradation of Foxo1 (640,641) via Skp2 of the Skp1/culin 1/F-box E3 ligase complex (642).

Activation of PI3K-Akt pathways during the initial activation of T cells results in the nuclear exclusion and inactivation of Foxo1 (643). However, after this initial repression of Foxo1 during T cell activation, studies over the past decade have demonstrated that the subsequent regulation of Foxo1 expression has major implications for T cell differential fate. For instance, differentiation of T follicular helper cells in response to ICOS signaling is dependent on the inactivation of Foxo1 (644). Memory markers and/or trafficking molecules such as CD62L, IL-7Rα, and CCR7 are amongst Foxo1 target genes (645,646). As such, Foxo1 has been shown to promote the differentiation of memory T cells (647) in part via the repression of T-bet and promotion of EOMES transcription factors (648,649). Consequently, repression of Foxo1 promotes T-bet expression and differentiation into effector cells including the expression of the cytotoxic effector molecule granzyme B (648). Concurrently, Foxo1 promotes the generation of Tregs with the T cell specific knockout of Foxo1 resulting in defective development of both thymic derived and inducible Tregs. As a consequence, these mice present with an exaggerated autoimmune phenotype characterized by enhanced anti-nuclear antibody production, organ infiltration by lymphocytes, and the onset of exocrine pancreatitis and hind limb paralysis (650,651). Conversely, Foxo1 represses Th17 differentiation through inhibition of RORγt (652). Additionally, Foxo1 expression has been shown to associate with exhausted T cells. Chronically stimulated exhausted T cells have more nuclear active

Foxo1 than acute stimulated cells, and moreover, Foxo1 promotes the expression of the exhaustion-associated transcription factor EOMES and inhibitory receptor PD-1 (631).

Concluding Remarks

The adoptive transfer of melanoma specific T cells has demonstrated a substantial improvement in outcomes, and even curative potential, for patients with late stage metastatic melanoma (219). However, the success of therapy has largely been dependent on the quality of the infused cells, particularly the ability of the cells to persist and continue to control tumor once they are transferred. The rapid expansion of T cells pushes the activation status of these cells more towards a terminally differentiated effector phenotype (170) which enhances their susceptibility to AICD when the cells are restimulated with tumor antigen (171). The ROS that are generated upon TCR restimulation (369) are a critical component in the onset of this type of programmed cell death (370). However, ROS generation has also been characterized as a necessary secondary messenger in lymphocyte activation (410). Therefore, it is necessary to further elucidate what pathways are responsible in escalating ROS accumulation from benign messenger to cell executioner in the context of AICD.

The glutathione pro-drug NAC has been shown to protect T cells from AICD and increase their persistence when transferred into a non-tumor bearing host (653). However, it has not been demonstrated whether this protection from AICD afforded by NAC is transient or if NAC provides a durable enhancement in phenotype that would be of benefit upon encounter with tumor antigen in a melanoma challenged host. As NAC

has previously demonstrated a wide range of phenotypic improvements for cultured T cells such as enhanced antioxidant capacity, proliferation potential, cytokine production, and improvement in the functional capacity of cells derived from advanced staged cancer patients (537,539,653,654), we reasonably hypothesize that culturing melanoma specific T cells in NAC prior to adoptive would improve their overall anti-melanoma performance, in part, through a reduced susceptibility to AICD.

CHAPTER 2 - TCR RESTIMULATION RESULTS IN ACTIVATION OF THE DNA DAMAGE RESPONSE PATHWAY IN THE ONSET OF ACTIVATION-INDUCED CELL DEATH

Introduction

Therapeutic T cells which have been subjected to a rapid expansion protocol (REP) are susceptible to activation-induced cell death (AICD) when restimulated *in vitro* with tumor antigen (171). As such, AICD likely restricts the durability of transferred T cells to persist and mount anti-tumor cytolytic effector functions *in vivo*, curtailing their overall abundance and therapeutic efficacy. AICD, which is triggered by restimulation of the T cell receptor (TCR) on a previously activated T cell, is independent of death receptor signaling and the downstream activation of caspases, but is dependent on the accumulation of reactive oxygen species (ROS) and activation of c-Jun NH2-terminal kinase (JNK) (366,370). Moreover, pharmacological inhibition of p53, a chief cell death regulator downstream of JNK and ROS (655), by pifithrin-α (Mehrotra Lab, unpublished data) and pifithrin-μ (556), has been shown to protect T cells from AICD.

However, off-target effects cannot be excluded from observations generated with pharmacological inhibitors. Pifithrin-α confers protection to both p53 null and competent cells from topoisomerase induced apoptosis (656), and pifithrin-μ has been additionally characterized as an HSP70 inhibitor advancing protein aggregation induced cell death (657). As such, this aim embarked to directly confirm the role of p53 in AICD by use of the p53 knockout mouse (658). The role of p53 during AICD in human cells was additionally explored by investigating two hallmarks of p53 activation: the phosphorylation status of p53 and its accumulation in the nucleus (572,659).

Furthermore, these observations acted as “barcode” (568) leading to the novel discovery that the DNA damage response pathway is activated in T cells upon TCR restimulation induced cell death.

T cells void of p53 are less susceptible to AICD

To directly determine the role of p53 in AICD, the p53-knockout ($p53^{KO}$) mouse was utilized (658). In order to make findings more relevant in a melanoma antigen-specific model, $p53^{KO}$ mice were crossbred with the h3T mouse to generate a $h3T^{+}p53^{KO}$ strain of mice. T cells from h3T mice constitutively express the human TIL1383I TCR conferring the ability of T cells to recognize and mount a cytolytic response against $hTyr_{368\text{-}376}$ expressing melanoma cells in a HLA-A2 restricted manner (660). To ensure that deletion of p53 did not interfere with the functionality of T cells, splenocytes from $h3T^{+}/p53^{KO}$ mice and $h3T^{+}/p53^{WT}$ littermate controls were assessed for their ability to recognize antigen as determined by the expression of activation markers and secretion of IFNγ. When stimulated overnight with $hTyr_{368\text{-}376}$ cognate peptide, there was no significant difference in the expression of CD25 or CD69 activation markers between splenocytes harvested from $h3T^{+}/p53^{KO}$ mice and those derived from $h3T^{+}/p53^{WT}$ controls (Fig. 3). However, splenocytes from $h3T^{+}/p53^{KO}$ mice did have a significant increase (~62.3%) in the amount of IFNγ secreted when restimulated with $hTyr_{368\text{-}376}$ pulsed T2 cells in comparison to splenocytes from $h3T^{+}/p53^{WT}$ littermates (Fig. 4). Together, these

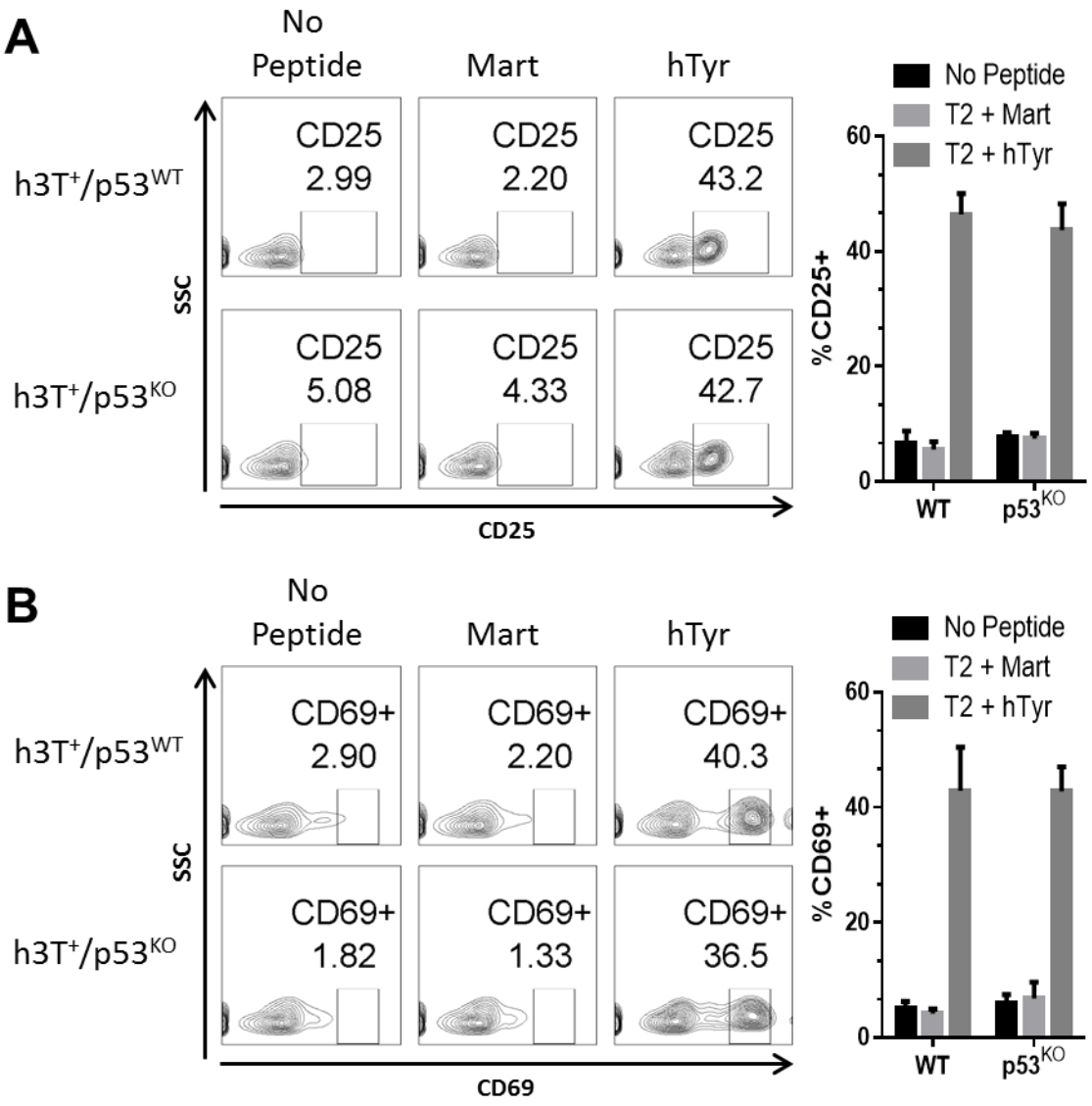

Figure 3. p53 status does not alter expression of CD25 and CD69 activation markers. Freshly isolated splenocytes from h3T^{+}/p53^{WT} and h3T^{+}/p53^{KO} mice were co-cultured at a 1:1 ratio overnight with T2 cells alone (no peptide) or T2 cells pulsed with either Mart (1.0µg/mL) or hTyr$_{368\text{-}376}$ (1.0µg/mL) peptide. After overnight co-culture, cells were then stained with fluorochrome conjugated antibodies for (A) CD25 or (B) CD69. Left panels display representative contour plot of Vβ12+CD8+ gated cells for each indicated marker. Right panels are mean ± SEM of percent positive cells for each indicated marker in Vβ12+CD8+ gated cells of n=5.

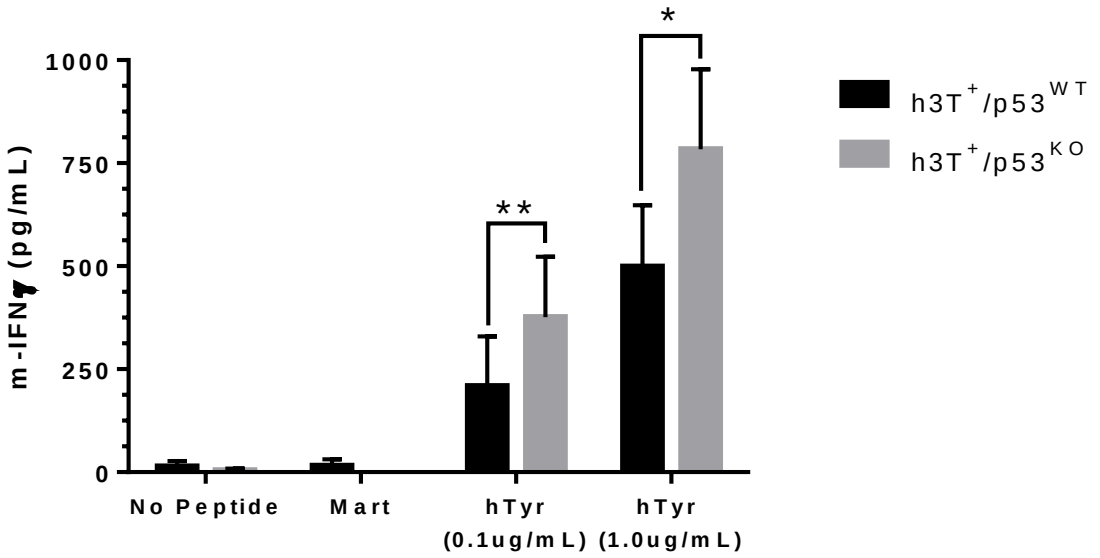

Figure 4. Lack of p53 increases m-IFNγ cytokine secretion. Freshly isolated splenocytes from $h3T^{+}/p53^{WT}$ and $h3T^{+}/p53^{KO}$ mice were co-cultured overnight at a 1:1 ratio with T2 cells alone (no peptide) or T2 cells pulsed with either Mart (1.0µg/mL) or $hTyr_{368\text{-}376}$ (0.1µg/mL or 1.0µg/mL) peptide. Supernatants were collected and analyzed for m-IFNγ concentration (pg/mL) via ELISA. Displayed is mean ± SEM of m-IFNγ (n=5, *p<0.05, **p<0.01).

data affirm that T cells derived from h3T^{+}/p53^{KO} are not defective in their ability to recognize antigen.

To determine if p53 status altered T cell susceptibility to AICD, freshly isolated splenocytes from h3T^{+}/p53^{KO} and h3T^{+}/p53^{WT} mice were activated for 48 hours with plate-bound anti-CD3 and anti-CD28, and then subsequently washed, rested overnight, and restimulated with hTyr$_{368\text{-}376}$ pulsed T2 cells. After 4 hours of peptide restimulation, T cells were stained with Annexin V, which binds to phosphatidylserine on the outer plasma membrane of the cell. Phosphatidylserine is normally restrained to the inside of the cell but is exposed on the surface when a cell losses plasma membrane asymmetry in the early phase of cell death (661). Restimulation with hTyr$_{368\text{-}376}$ for 4 hours caused an increase in Annexin V binding. T cells co-stained with 7-aminoactinomycin D (7-AAD), a membrane impermeant dye that stains cells with compromised plasma membrane integrity occurring in late-stage cell death (662), did not result in an increase in 7-AAD staining (Fig. 5a), indicating that 4 hours of peptide stimulation within our model system captured cells in the early stages of cell death kinetics. Splenocytes from h3T^{+}/p53^{WT} mice, when restimulated with hTyr$_{368\text{-}376}$ peptide pulsed T2 cells, exhibited a ~2.7-fold higher level of Annexin V than those restimulated by Mart control peptide. However, splenocytes from h3T^{+}/p53^{KO} mice demonstrated resistance to AICD as they consistently exhibited an approximately 17% decrease in Annexin V staining than their h3T^{+}/p53^{WT} littermate counterparts (Fig. 5).

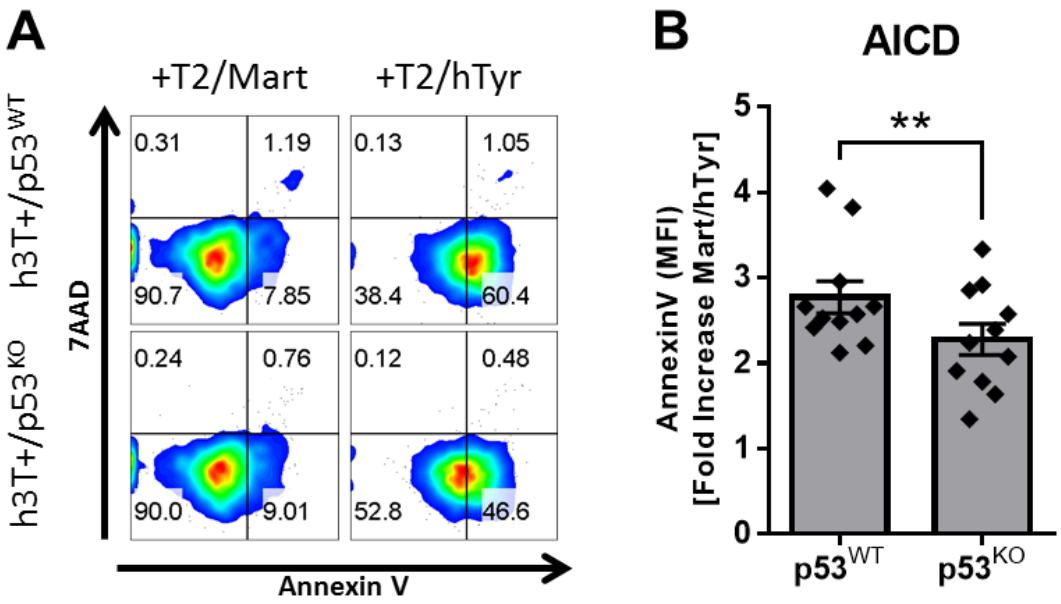

Figure 5. Presence of p53 exacerbates cell death upon TCR restimulation. Splenocytes from h3T^{+}/p53^{WT} and h3T^{+}/p53^{KO} mice were activated for 48 hrs with plate-bound anti-CD3 (1µg/mL) and anti-CD28 (2µg/mL), rested overnight, and then restimulated by co-culture at a 1:1 ratio with T2 cells pulsed with either Mart (1.0µg/mL) or hTyr$_{368\text{-}376}$ (1.0µg/mL) peptide for 4 hrs. (A) Representative flow plot of Annexin V and 7AAD expression following restimulation in Vβ12+CD8+ gated cells in representative experiment. (B) Quantification of mean ± SEM of fold change (hTyr/Mart) in Annexin V MFI expression in Vβ12+CD8+ gated cells (n=11, **p<0.01).

To further determine if protection afforded by p53 knockdown was relevant in the context of T cells that are transduced with an antigen-specific TCR, splenocytes from p53-knockout mice (and wild type controls) were activated and transduced with the TIL1383I TCR. Consistent with results observed in the h3T native TCR model, restimulation of transduced T cells with $hTyr_{368\text{-}376}$ peptide demonstrated a significant protection ($p=0.025$) for $p53^{KO}$ cells compared to $p53^{WT}$ cells. Additionally, TIL1383I TCR transduced murine T cells were restimulated with the HLA-A2 expressing B16(A2) murine melanoma cell line to undergo AICD relative to HLA-$A2^{-}$ B16 cells as a control. $p53^{KO}$ transduced T cells exhibited a 25.7% decrease in Annexin V expression when restimulated with HLA-A2 matched B16(A2) cells compared to $p53^{WT}$ cells (Fig. 6). Together, these data indicate that ablation of p53 results in a partial, but significant, reduction in AICD susceptibility suggesting that p53 is likely involved in the onset of AICD and is relevant for both native expressing TCRs and transduced engineered cells.

p53 is activated via phosphorylation and accumulation in the nucleus

Protection of T cells from AICD via p53 deletion prompted the investigation into the activation status of p53 upon TCR restimulation. An essential hallmark of initial p53 activation is the phosphorylation of one or more of its N-terminal serines, which facilitates the dissociation from its chief negative regulator HDM2 (572,573). To determine the phosphorylation status of p53 during AICD in human cells, PBMCs which were previously activated with plate-bound anti-CD3 and anti-CD28, were invoked to undergo AICD

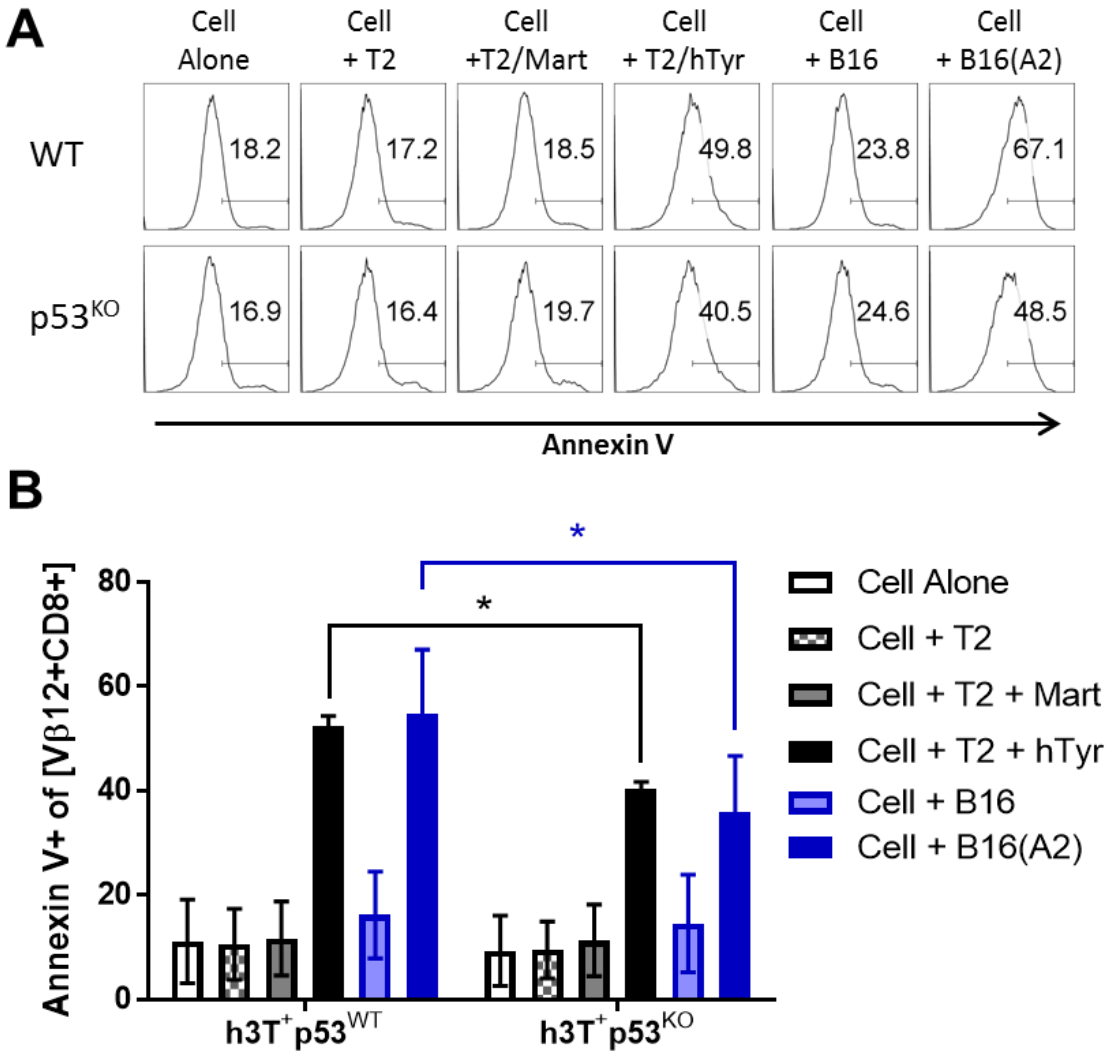

Figure 6. TIL1383I TCR transduced p53^{KO} mouse splenocytes are protected from AICD. WT and p53^{KO} mice were activated (with anti-CD3 & anti-CD28) and transduced with the TIL1383I TCR. Cells were cultured 3 more days after transduction and then restimulated by co-culture at a 1:1 ratio with T2 cells pulsed with either Mart (1.0μg/mL) or hTyr$_{368-376}$ (1.0μg/mL), or cells were co-cultured at a 1:1 ratio with B16 (HLA-A2^{-}) or B16-A2 (HLA-A2^{+}) murine melanoma cells to undergo AICD for 4 hrs. (A) Representative histograms of Annexin V expression in Vβ12+CD8+ gated cells. (B) Quantification of mean ± SEM of Annexin V+ amongst Vβ12+CD8+ gated cells (n=2, *p<0.05).

by restimulation with plate-bound anti-CD3. This polyclonal stimulation resulted in a time-dependent increase in Annexin V staining (Fig. 7a), indicative of these T cells undergoing AICD. In parallel, cells were stained intracellularly with a phospho-specific fluorochrome conjugated antibody for p-p53^{Ser15} as well as total p53. The amount of phosphorylated p-p53^{Ser15} increased in a similar time-dependent manner as observed with Annexin V staining with a 3.72-fold increase at 2 hours of anti-CD3 restimulation as well as an overall stabilization of total p53 expression (~2.94-fold increase) (Fig. 7b).

With its primary role as a transcription factor, nuclear accumulation is another indication of p53 activation (576,659). To investigate the subcellular distribution of p53 upon TCR restimulation, previously activated human PBMCs were induced to undergo AICD with polyclonal anti-CD3 restimulation and were examined using the Amnis ImageStream imaging flow cytometer which combines flow cytometry with cellular imaging. In addition to being stained with p53 and p-p53^{Ser15}, PBMCs were labeled with the nuclear marker Hoechst. Within two hours of TCR restimulation, there was nearly a 9-fold increase in the amount of p53 that co-localized with the Hoechst stained nucleus (Fig. 8a-c). Additionally, nearly all (>97%) of the Ser15-phosphorylated p53 was detected in the nucleus (Fig 8d), which is consistent with reports that have demonstrated Ser15 aids in nuclear accumulation and retention of p53 (577,578).

As p53 can have a transcriptionally independent role in cell death at the mitochondria, cells were additionally stained with the mitochondrial marker

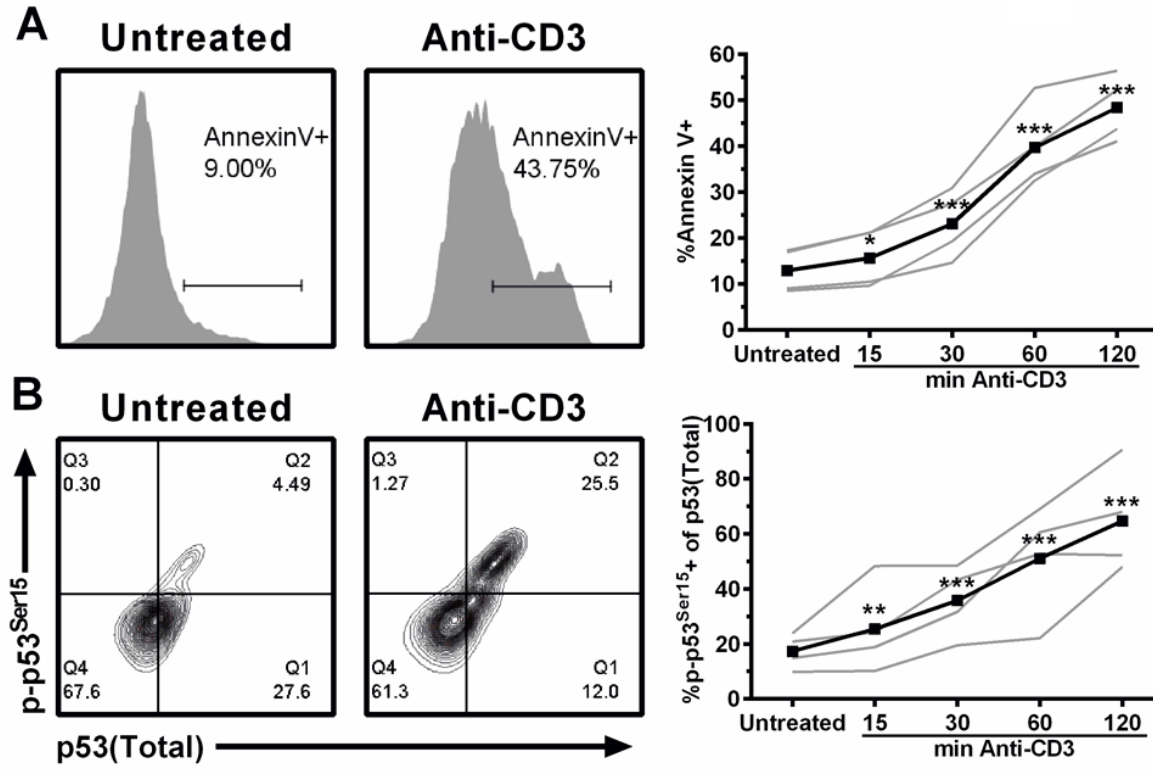

Figure 7. p53 is phosphorylated on its Serine 15 residue upon TCR restimulation in human PBMCs. Previously activated human PMBCs were restimulated with plate-bound anti-CD3 (5µg/mL) for up to 120 min. Left panels are representative histograms and contour plots of (A) Annexin V and (B) p53 and p-p53^{Ser15} expression after 120 min of TCR restimulation in CD8+ gated cells. Right panels denote quantification of each indicated maker in CD8+ cells of time course experiments with each of the four donors (grey lines) and the mean of all donors (black line). *p<0.05, **p<0.01, and ***p<0.0001.

MitoTracker- DeepRed. Visual and quantitative analysis revealed that virtually none of the p53 fluorochrome conjugated antibody co-localized with MitoTracker (Fig. 8e, <0.3%). Together these results demonstrate that p53 is activated via phosphorylation and accumulation in the nucleus following TCR restimulation, and that p53 is likely acting in its role as a transcription factor during the onset of AICD.

ATM is activated and required in the onset of AICD

Ataxia telangiectasia mutated (ATM), the chief kinase responsible for the phosphorylation of p53 on Ser15, is activated via autophosphorylation on its Ser1981 residue. As such, the activation status of ATM was investigated via probing for the expression levels of p-ATM^{Ser1981} in previously activated human PBMCs undergoing anti-CD3 restimulation. Indeed, ATM was found to be phosphorylated in a time-dependent manner similar to the upregulation of p-p53^{Ser15} upon TCR restimulation with an approximate 4.6-fold increase in the amount of p-ATM^{Ser1981} after 2 hours of TCR restimulation (Fig. 9a). As other kinases could potentially be responsible for the phosphorylation of p53, cells were additionally preincubated with the ATM inhibitors Caffeine or KU-55933 (663,664). Both inhibitors faithfully prevented the phosphorylation of ATM and additionally blocked the downstream phosphorylation of p53 (Fig. 9a), suggesting that ATM is necessary for the phosphorylation of p53 on Ser15 following TCR restimulation. Furthermore, in cells that were stained in parallel with Annexin V, inhibition of ATM nearly completely (>99%) prevented cell death resulting from TCR

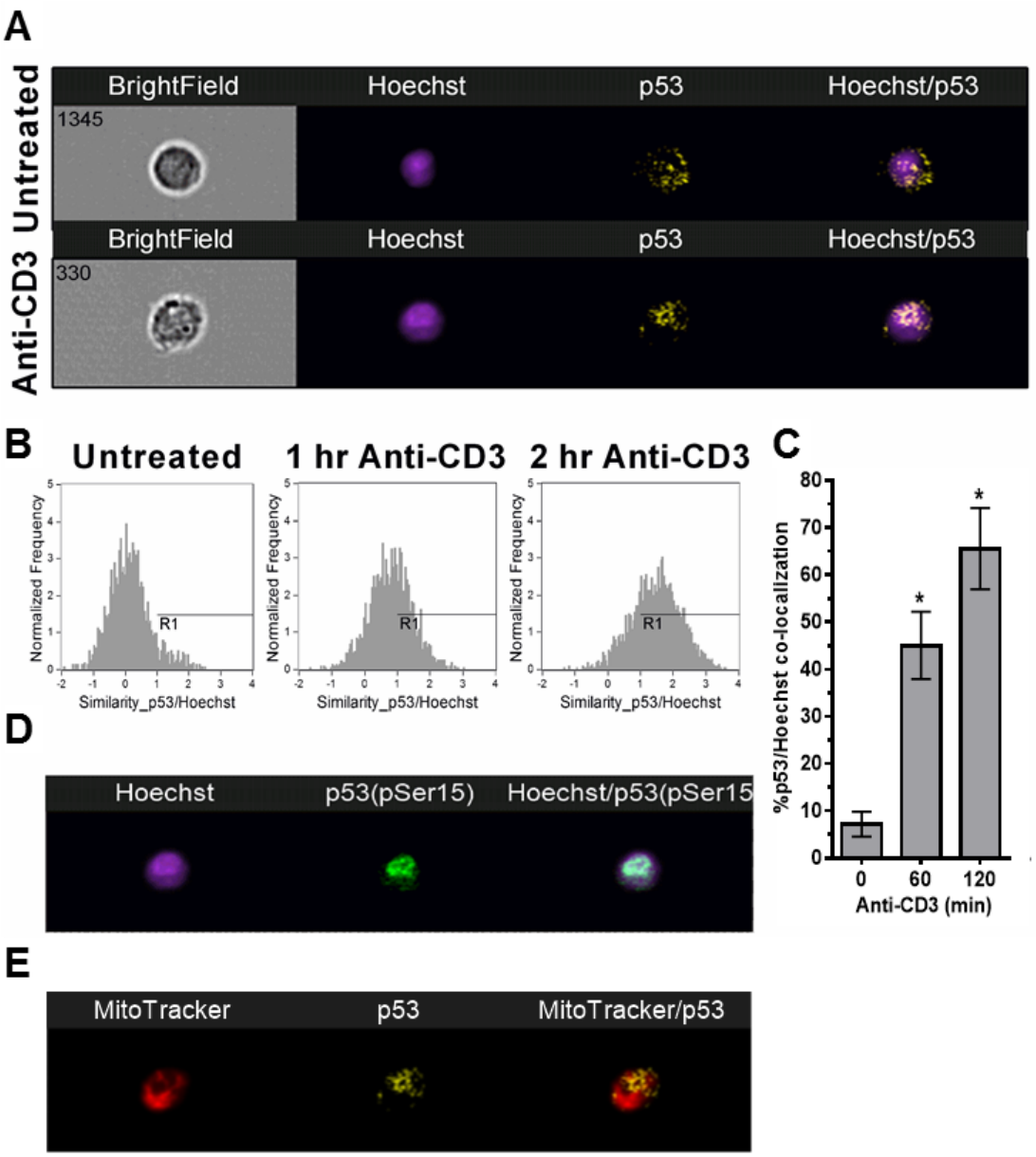

Figure 8. p53 translocates to the nucleus upon TCR restimulation. Previously activated human PMBCs were restimulated for 60 or 120 min with anti-CD3 and stained with Hoechst, MitoTracker-DeepRed, p53, and p-p53^{Ser15}. Cells were acquired on the Amnis ImageStream with at least 10,000 events collected. Cells were gated on CD8+ cells prior to further analysis. Representative images show localization (A) p53 and (D) p-p53^{Ser15} relative to Hoechst stained nucleus. (B) Representative similarity histogram of cells co-stained with p53 and Hoechst after TCR restimulation (C) Quantification (mean ± SEM) of percent positive for p53/Hoechst co-localization defined as a similarity score ≥1 (E) Same cell in 'D' exhibiting p53 relative to MitoTracker-DeepRed (n=2, *p<0.05).

restimulation (Fig. 9b). Together these data suggest that ATM activity is responsible for the phosphorylation of p53 upon TCR restimulation and is a novel upstream requisite factor in the onset of AICD.

TCR restimulation results in the rapid upregulation of DNA damage markers

Activation of the p-ATM^{Ser1981}/p-p53^{Ser15} pathway is classically defined as a DNA damage response pathway (665). However, this pathway can be activated independent of DNA damage via oxidative stress or hypoxia (595). As oxidative stress has already been implicated in the onset of AICD (370), the expression of two well defined markers of DNA double-strand breaks, γH2AX and p-SMC-1 (599), were examined to determine if activation of the p-ATM^{Ser1981}/p-p53^{Ser15} occurred in the presence of DNA damage. Indeed, polyclonal anti-CD3 restimulation of previously activated human PBMCs resulted in an increase of these DNA damage markers with a significant, 3-fold increase in expression within 15 min of restimulation and remained elevated throughout the 2 hour timecourse (Fig. 10). These data indicate that evidence of DNA damage parallels the onset of ATM activation and suggest that TCR restimulation results in DNA damage and subsequent activation of the DNA damage response pathway leading to AICD.

Detection of DNA damage response pathway activation in TIL1383I TCR transduced therapeutic T cells

As the DNA damage response pathway further elucidates signaling pathways involved in AICD, blocking components of that pathway, or engineering cells with

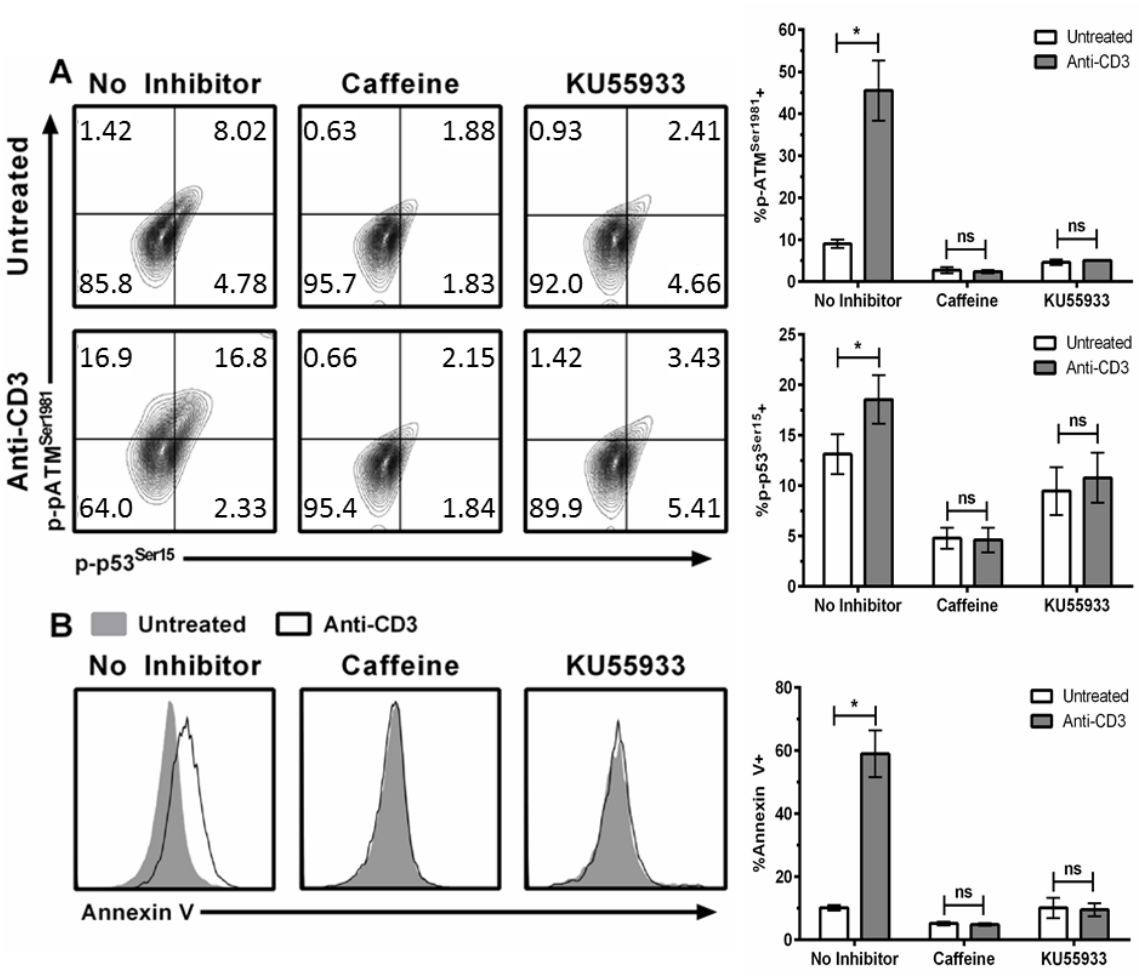

Figure 9. ATM is activated and required for cell death following TCR restimulation in human PBMCs. Previously activated human PBMCs were restimulated with anti-CD3 (5µg/mL) for 120 min with or without preincubation with Caffeine (10mM) or KU-55933 (100µM) 1hr prior to restimulation. After restimulation, T cells were stained in parallel for (A) expression of p-ATM^{Ser1981} and p-p53^{Ser15} or (B) Annexin V expression. Left panels show representative contour plot or histogram of CD8+ gated cells. Right panel displays quantification of mean ± SEM for each indicated marker in CD8+ gated cells (n=3, *p<0.05, ns=not significant).

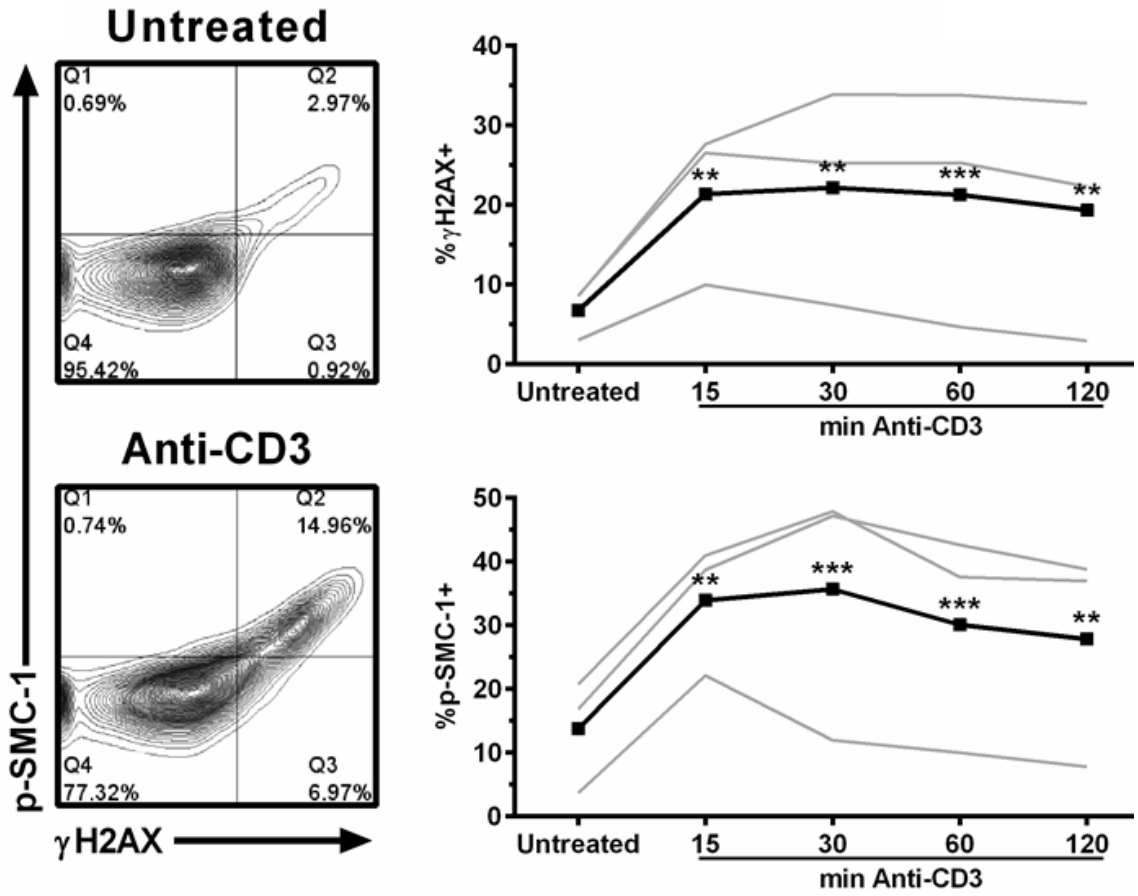

Figure 10. TCR restimulation results in the rapid upregulation of DNA damage markers in human PBMCs. Previously activated human PBMCs were restimulated with plate-bound anti-CD3 (5µg/mL) for up to 120 min. Left panels are representative contour plots of γH2AX and p-SMC-1 expression in CD8+ gated cells after 120 min of TCR restimulation. Right panels denote quantification of time course experiments for each indicated marker in CD8+ gated cells with each of the three donors (grey lines) and the mean of all donors (black line). n=3, **p<0.01, and ***p<0.0001.

enhanced DNA repair capacity, may improve therapeutic outcomes. Therefore, it was investigated whether these observations from restimulated polyclonal PBMCs could be applied to therapeutic T cells used in the clinic. To determine the clinical relevance of these findings, we obtained TIL1383I transduced T cells that had been prepared for adoptive cell transfer as part of an ongoing clinical trial (NCT01586403) from both melanoma patients enrolled in the trial and healthy donor controls. These TIL1383I transduced cells were co-cultured overnight with either HLA-$A2^{+}$ matched MEL624 cells or HLA-$A2^{-}$ MEL624-28 cells as a negative control. Previously activated by a REP, restimulation of the TCR of TIL1383I TCR transduced human T cells by co-culture with MEL624 cells caused the T cells to undergo cell death as evidenced by a 2.4-fold increase in Annexin V staining compared to cells co-cultured with MEL624-28 cells (Fig. 11). Concurrently, MEL624 co-cultured cells exhibited significant increases in p-$ATM^{Ser1981}$ (2.5-fold) and γH2AX (1.7-fold) expression levels (Fig. 11). Activation of the DNA damage response pathway was also observed in the CD4+ T cell population (Fig. 12). No significant difference was observed in Annexin V, γH2AX expression, or activation of ATM between melanoma patients and healthy controls. Altogether, these results suggest that antigen-specific restimulation of the TCR by tumor cells likely induces DNA damage in therapeutic T cells in the onset of AICD.

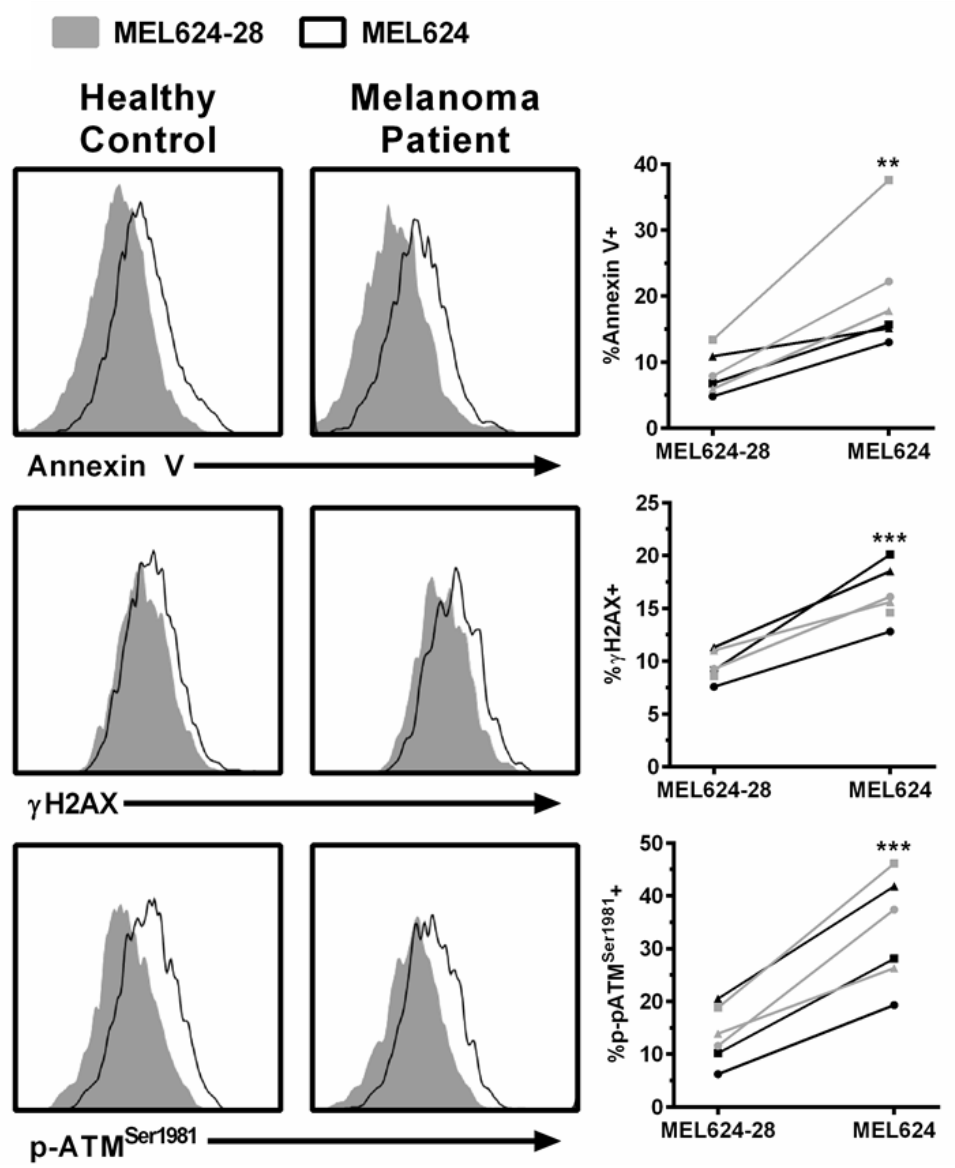

Figure 11. DNA damage and ATM activation occurs in CD8+ TIL1383I TCR transduced human T cells restimulated with MEL624 melanoma cells. TIL1383I TCR transduced human T cells were co-cultured overnight at a 1:1 ratio with either MEL624-28 (HLA-A2^{-}) or MEL624 (HLA-A2^{+}) cells. Left panels display Annexin V, γH2AX, and p-ATM^{Ser1981} expression amongst CD34^{+}CD8^{+} gated cells. Right panels denote quantification of percent positive for each labeled marker in 3 healthy donors (grey lines) and 3 melanoma patients (black lines) in CD34^{+}CD8^{+} gated cells. (n=3, **p<0.01, ***p<0.001).

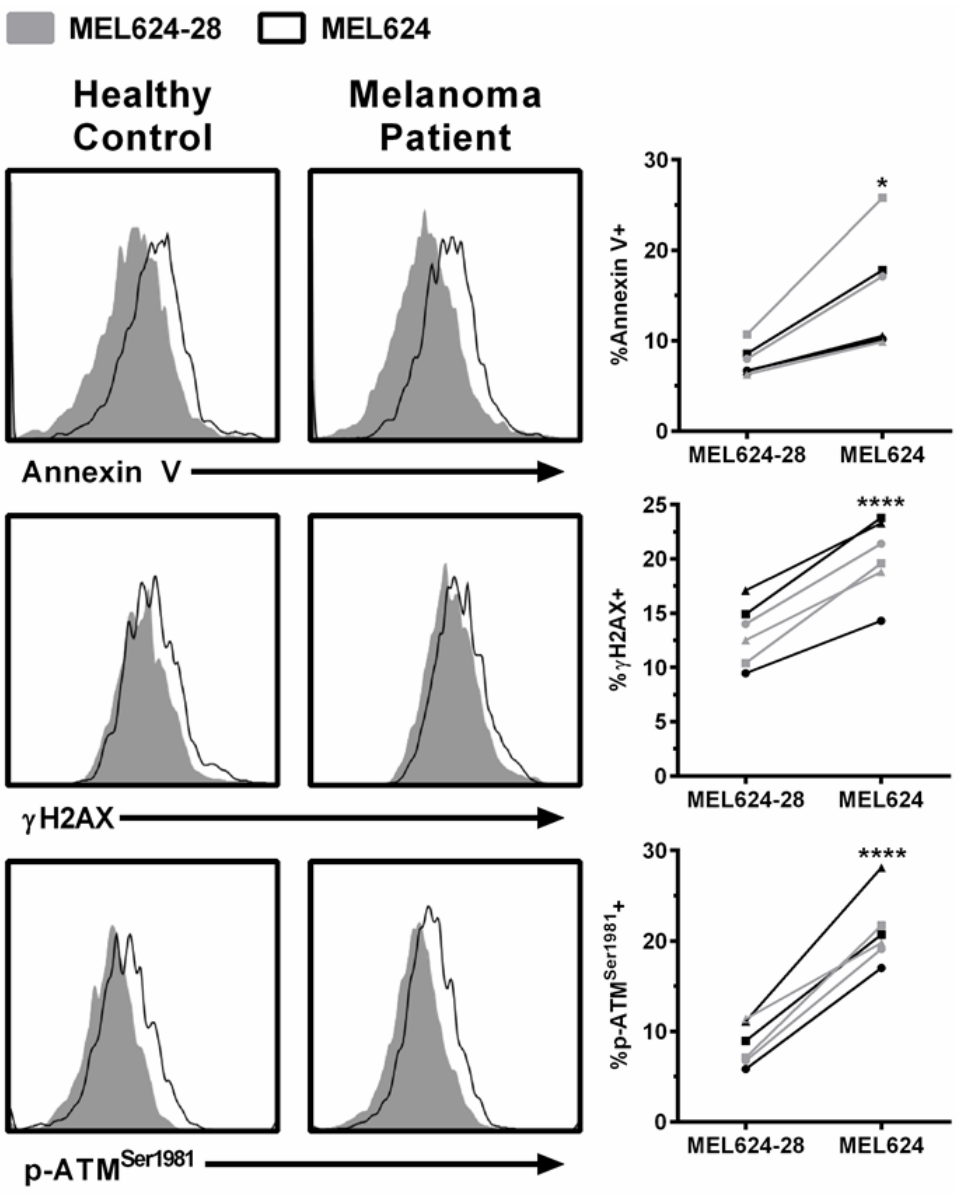

Figure 12. DNA damage and ATM activation occurs in CD4+ TIL1383I TCR transduced human T cells restimulated with MEL624 melanoma cells. TIL1383I TCR transduced human T cells were co-cultured overnight at a 1:1 ratio with either MEL624-28 (HLA-A2^{-}) or MEL624 (HLA-A2^{+}) cells. Left panels display Annexin V, γH2AX, and p-ATM^{Ser1981} expression amongst CD34^{+}CD4^{+} gated cells. Right panels denote quantification of percent positive for each labeled marker in 3 healthy donors (grey lines) and 3 melanoma patients (black lines) in CD34^{+}CD8^{+} gated cells. (n=3, *p<0.05, ****p<0.0001).

Accumulation of ROS is responsible for activation of the DNA damage response pathway upon TCR restimulation

Among endogenous sources of DNA damage, oxidative stress is a predominant assailant of DNA damage within the cell (666). ROS generated by TCR restimulation has previously been characterized to occur within a similar timeframe as our observation of DNA damage (≤15 min) (369). Additionally, as the accumulation of ROS has already been implicated in the onset of AICD (370), we sought to delineate whether oxidative stress was responsible for this observed incursion of DNA damage following TCR restimulation or, if rather, this was the result of a separately occurring phenomenon. To address this question, previously activated human PBMCs were preincubated with 50mM of the antioxidant N-acetyl cysteine (NAC) 60 min prior to restimulation with anti-CD3.

Anti-CD3 restimulated human PBMCs were stained with the ROS-reactive dye 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). Untreated cells exhibited an ~66.6% increase in the DCFDA MFI when restimulated with anti-CD3 which was reduced by ~38.8% when the cells were preincubated with NAC (Fig. 13a). Moreover, pretreatment with NAC prevented the downstream activation via phosphorylation (667) of p-$JNK^{Thr183/Tyr185}$, which has also been shown to be required for AICD (366) (Fig. 13b).

As previously reported, NAC treatment protects T cells from AICD following TCR restimulation (653) as demonstrated by the failure to increase the amount of Annexin V staining upon anti-CD3 restimulation (Fig. 14a). In parallel, pretreatment of cells with NAC also reduced the onset of DNA damage marker expression and subsequent activation

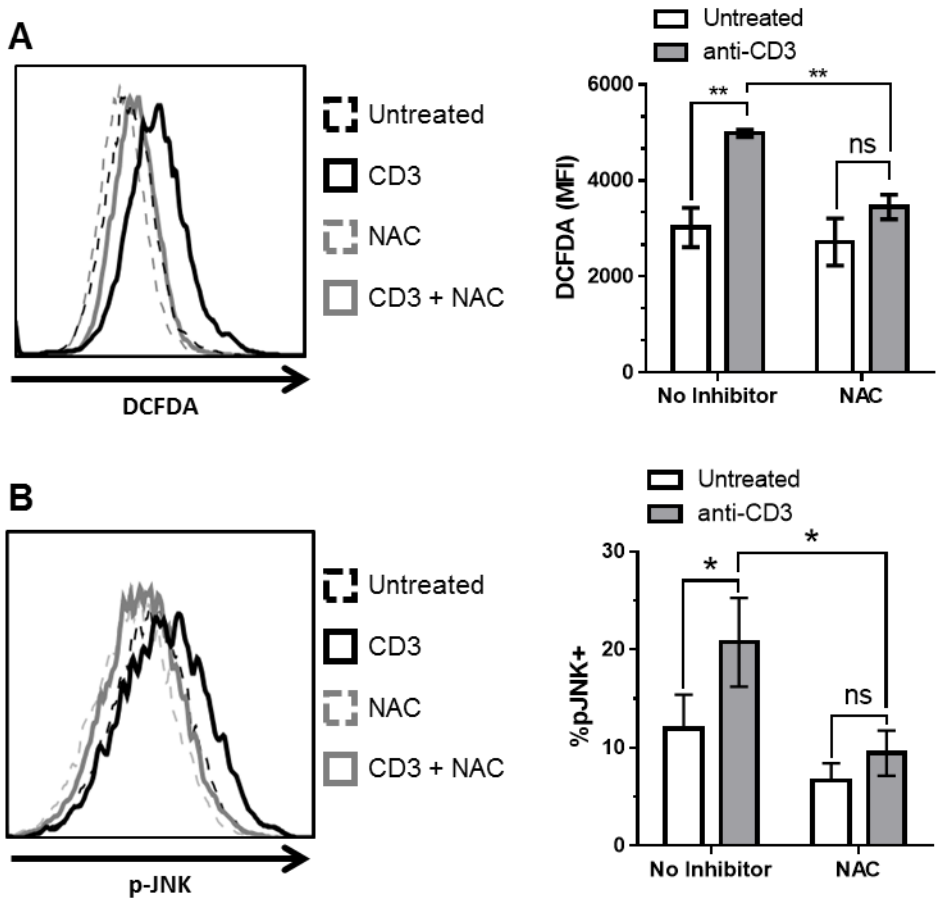

Figure 13. Pretreatment with NAC prevents TCR restimulation induced ROS accumulation and JNK activation in human PBMCs. Previously activated human PBMCs were pretreated with NAC (50mM) 60 min prior to restimulation with anti-CD3 (5µg/mL) for 4 hrs. Left panels are representative histograms of (A) DCFDA and (B) p-JNK expression in CD8+ gated cells. Right panels display quantification (mean ± SEM) of each indicated marker in CD8+ gated cells (n=3, *p<0.05, **p<0.01, ns=not significant).

of the DDR pathway as exhibited by a 94%, 69%, and 81% reduction in the expression of γH2AX, p-ATM^{Ser1981}, and p-p53^{Ser15} respectively (Fig. 14b-d).

Taken together, these results suggest that the accumulation of ROS, previously defined to be a requisite factor for AICD (370), is the cause for the onset of the DNA damage response pathway caused by TCR restimulation. Furthermore, this apparent onset of DNA damage activates p53 via phosphorylation of its Ser15 by ATM, confirming the likely role of p53 in the involvement of AICD demonstrated by previous inhibition and knockout studies (Fig. 15) (556,668).

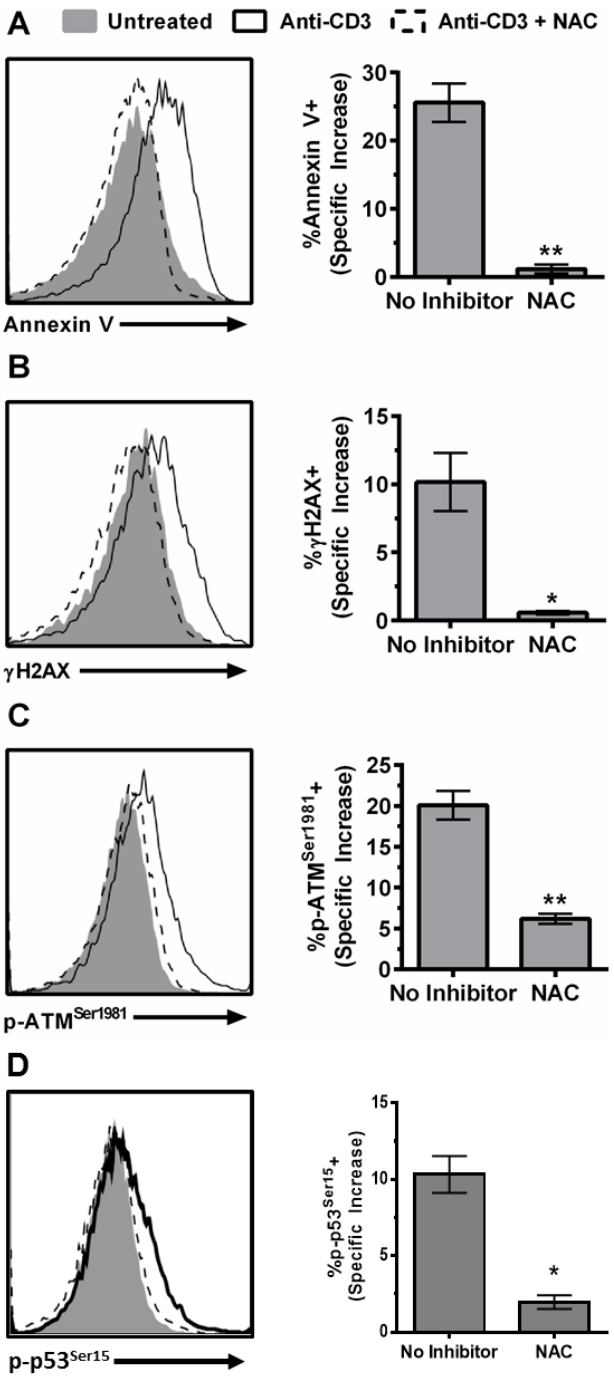

Figure 14. Pretreatment with NAC prevents the induction of γH2AX and activation of the DDR pathway upon TCR restimulation in human PBMCs. Previously activated human PBMCs were pretreated with 50mM NAC 60 min prior to restimulation with anti-CD3 (5µg/mL) for 4 hrs. Left panels are representative histograms of (A) Annexin V, (B) γH2AX, (C) p-ATM^{Ser1981}, and (D) p-p53^{Ser15} expression in CD8^{+} gated cells. Right panels display quantification of mean ± SEM (n=3, *p<0.05, **p<0.01).

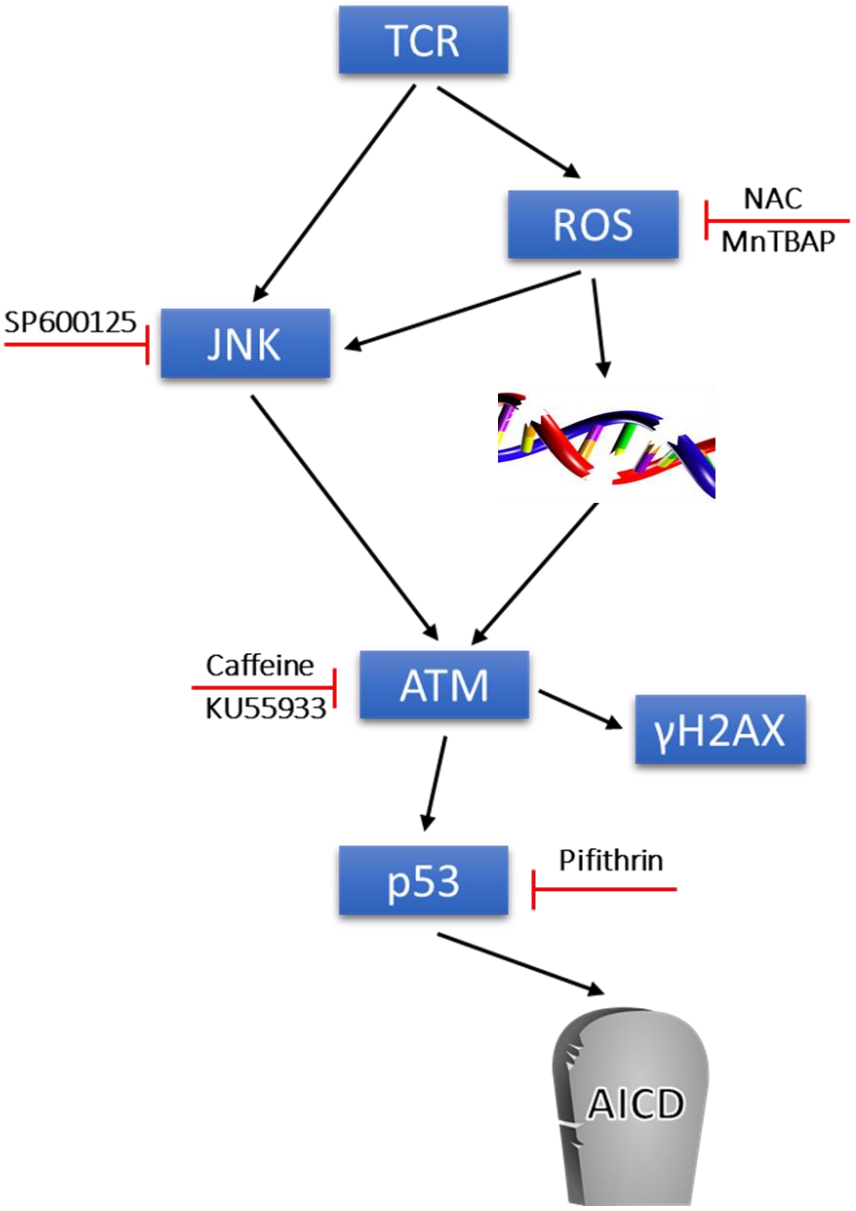

Figure 15. Signaling schematic of DDR pathway activation in AICD. TCR restimulation results in the activation of JNK and accumulation of ROS [Fig. 13 and (366,369,370)]. The accumulation of ROS results in the activation/phosphorylation of ATM (Fig. 9) and the downstream phosphorylation of γH2AX (Fig. 10), which is indicative of DNA damage within the cell. Downstream of ATM, p53 is phosphorylated on Ser15 (Figs 7,9) which can be blocked by the ATM inhibitors Caffeine or KU55933, which also inhibits AICD (Fig. 9). Upstream, neutralization of ROS accumulation by pre-treatment with the antioxidant NAC (Fig. 13) blocks ATM activation and subsequent phosphorylation of γH2AX and p53 protecting the T cells from death following TCR restimulation (Fig. 14).

CHAPTER 3 – CULTURING T CELLS IN N-ACETYL CYSTEINE IMPROVES THEIR ANTI-TUMOR FUNCTIONALITY

Introduction

As acute treatment of T cells with NAC protected them from the apparent onset of DNA damage and the onset of AICD, we postulated that enhancing the antioxidant capacity of therapeutic T cells used in the clinic with NAC would likely enhance the durability of T cells to persist and to control tumor once transferred *in vivo*. NAC has been commonly used in the clinic for a multitude of diseases and conditions for many decades and, even at very high doses, has demonstrated a consistent and well-regarded safety profile (669). However, direct administration of NAC to patients undergoing ACT may not effectively enhance therapy as NAC has a considerably short half-life in the plasma (477). Moreover, protection afforded to T cells by NAC may also confer protection to tumor cells in parallel, as NAC administration has been shown to increase tumor burden and metastasis in murine models (670,671). However, NAC supplementation does enhance the antioxidant capacity of T cells (653), which led us to hypothesize that culturing T cells in NAC prior to adoptive transfer would make them more resistant to the downstream occurrence of DNA damage and also impede the onset of AICD. Moreover, to what extent making T cells resistant to AICD through NAC supplementation would improve their therapeutic efficacy once transferred *in vivo* was examined in murine melanoma/self antigen-specific T cells, both from the native bearing transgenic Pmel-1 model and from T cells engineered with a TRP-1 specific TCR, both cultured in NAC prior to adoptive transfer into B16-F10 melanoma challenged mice. Additionally, human TIL1383I TCR

transduced cells, though not able to persist in a xenograft model, demonstrated enhanced *in vitro* cytolytic ability after rapid expansion in NAC.

Culturing Pmel-1 murine T cells in NAC enhances antioxidant capacity and diminishes their susceptibility to γH2AX induction and AICD

We used the Pmel-1 transgenic mouse as our first model, which has been a well-established model to demonstrate improvements in the efficacy of ACT therapy, notably in that T cells from the Pmel-1 transgenic mouse recognize the "self" melanoma associated antigen $gp100_{25-33}$ in the poorly immunogenic B16 melanoma tumor cell (323). Harvested splenocytes from Pmel-1 mice were activated with $gp100_{25-33}$ peptide and IL-2 and then were cultured with or without the presence of NAC (10mM) added to the culture medium for 6 days. Prior to use in adoptive transfer, the effect of NAC supplementation was characterized on these *in vitro* cultured cells. Indeed, Pmel-1 T cells cultured in NAC demonstrated an enhancement in antioxidant capacity as indicated by an increase in surface thiols as exhibited by the thiol-reactive maleimide dye (Fig. 16). Conversely, there was a decrease in the expression of the oxidative stress dye DCFDA (Fig. 16). Moreover, NAC cultured T cells exhibited a decrease in DNA damage as denoted by reduced levels of γH2AX when T cells were restimulated with $gp100_{25-33}$ pulsed irradiated splenocytes, which was consistent with these cells also being more resistant to AICD (Fig. 17). Together, these results affirm that culturing cells in NAC confers an increase in antioxidant capacity which subsequently makes cells more resistant to TCR restimulation induced DNA damage and AICD.

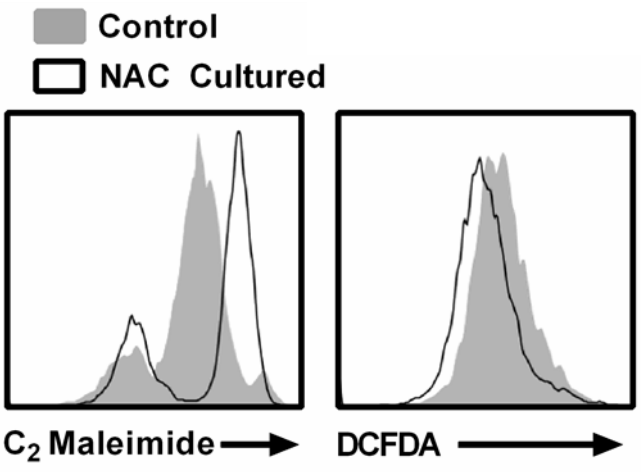

Figure 16. Pmel-1 T cells cultured in NAC exhibit enhanced antioxidant capacity. Representative histograms (n=2) of $V\beta13^{+}CD8^{+}$ gated Pmel-1 splenocytes which were activated with $gp100_{25\text{-}33}$ peptide and cultured for 6 days ± 10mM NAC. Cells were stained with the surface thiol reactive C_2Maleimide dye (left panel) or the oxidative stress indicating DCFDA dye (right panel).

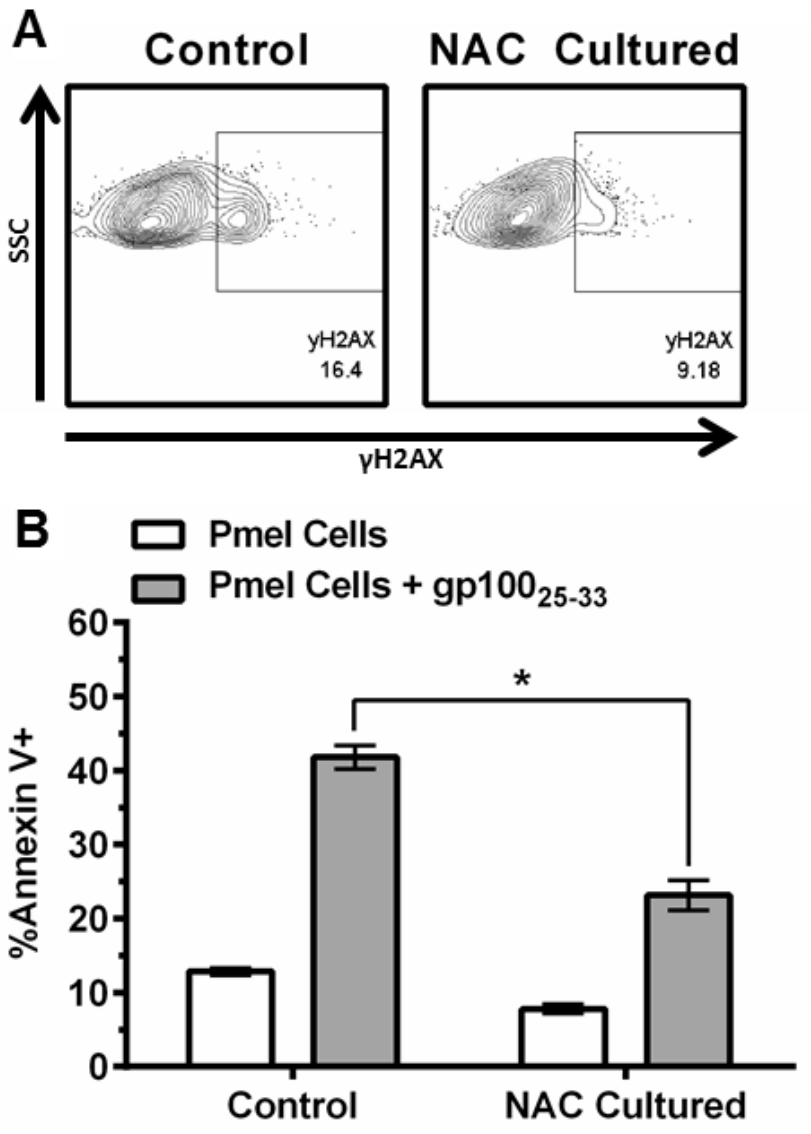

Figure 17. Pmel-1 T cells cultured in NAC are more resistant to DNA damage and AICD upon TCR restimulation. Pmel-1 splenocytes which were activated with $gp100_{25\text{-}33}$ peptide and cultured ± 10mM NAC for 6 days were then co-cultured overnight at a 1:1 ration with irradiated splenocytes pulsed with $gp100_{25\text{-}33}$ peptide (1µg/mL). (A) Representative contour plots denoting γH2AX expression of $V\beta13^{+}CD8^{+}$ gated cells restimulated with $gp100_{25\text{-}33}$ peptide (B) Quantification (mean ± SEM) of percent Annexin V positive cells amongst $V\beta13^{+}CD8^{+}$ gated cells (n=2, *$p<0.05$).

Pmel-1 murine T cells cultured in NAC demonstrate enhanced *in vivo* persistence after adoptive transfer

To determine if increased resistance to DNA damage and AICD conferred an improvement in *in vivo* tumor control, these Pmel-1 T cells, which had been cultured with or without NAC (10mM) for 6 days, were adoptively transferred via retro-orbital infusion into mice challenged with a subcutaneous injection of B16-F10 ($3x10^5$) murine melanoma cells 6 days prior to transfer. Mice additionally received a 5 Gy dose of whole-body irradiation the day before adoptive transfer in order to parallel patient lymphodepletion practiced in the clinic. To monitor the persistence of transferred cells, blood draws were taken from the tail veins of recipient mice 5 and 12 days after transfer which demonstrated that there was a significant enhancement in the percentage of $V\beta13^+CD8^+$ Pmel-1 T cells in the blood of mice which had received NAC cultured Pmel-1 T cells compared to control cultured cells (Fig. 18a). Similarly, there was a 2.2-fold increase in the percentage of $V\beta13^+CD8^+$ cells in the spleens of recipient mice receiving NAC cultured T cells (Fig. 18b). Strikingly, in mice receiving Pmel-1 T cells cultured in NAC, close to 40% of T cells within the tumor were $V\beta13^+CD8^+$ positive, whereas effector T cells in the tumors of mice receiving Pmel-1 T cells cultured in the absence of NAC were scarcely detectable (~1.2%, Fig. 18c). Together, these results demonstrate that Pmel-1 T cells cultured in NAC, which are resistant to AICD *in vitro*, exhibit enhanced persistence once they are transferred *in vivo* into a tumor challenged host.

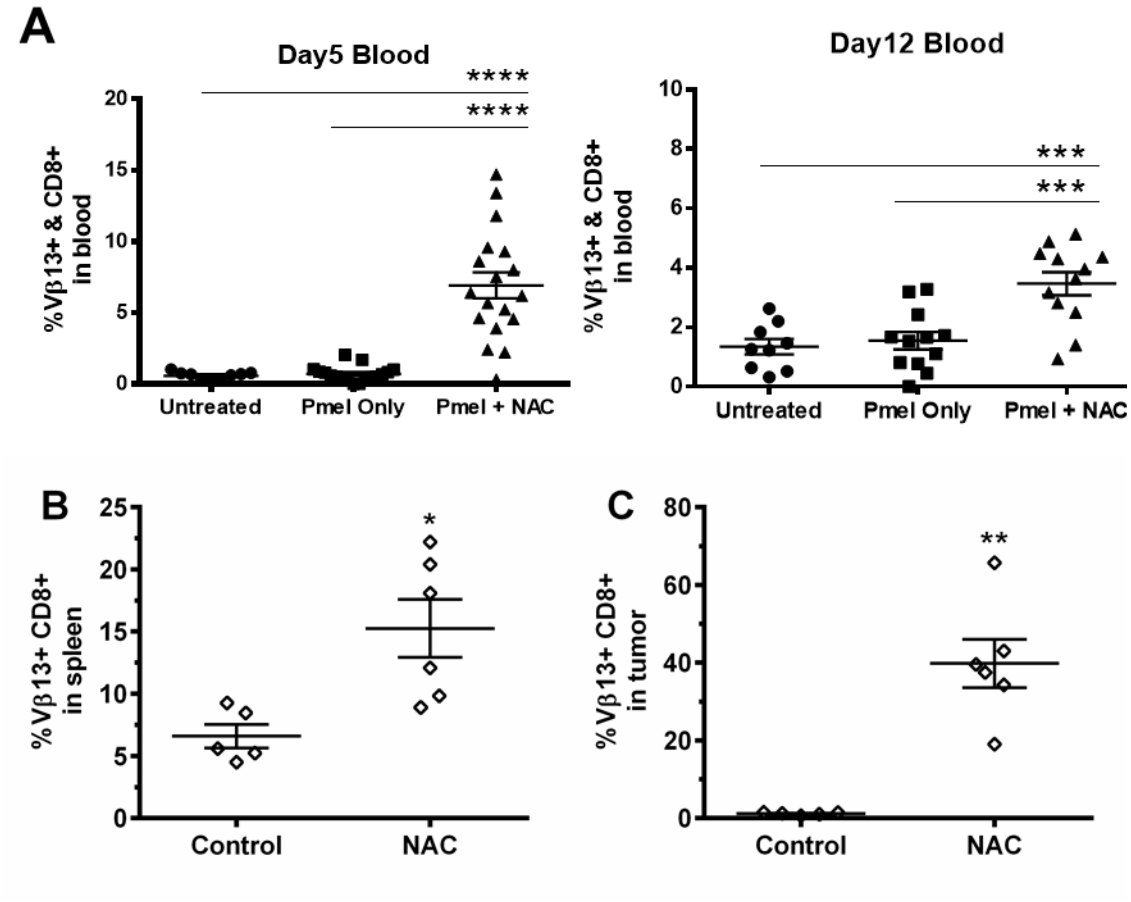

Figure 18. Pmel-1 T cells cultured in NAC exhibit enhanced persistence once transferred *in vivo*. C57BL/6 wild type mice were subcutaneously injected with $3x10^6$ B16-F10 murine melanoma cells. After 6 days of tumor establishment, mice were irradiated (5Gy) and treated with $2x10^6$ Pmel-1 T cells which had been cultured ± NAC (10mM) for 6 days and reactivated with $gp100_{25\text{-}33}$ peptide (1µg/mL) overnight prior to transfer. (A) Blood was collected from the tail vein of recipient mice on Days 5 and 12 post-transfer. Displayed is the percent of $V\beta13^+CD8^+$ cells of $CD3^+$ gated cells for each individual mouse. Bars in graph represent the quantification of mean ± SEM (B, C) Spleens and tumors were harvested from a subset of mice 6 days after adoptive transfer and processed into single-cell suspensions. Displayed is the percent of $V\beta13^+CD8^+$ cells of $CD3^+$ gated cells harvested from (B) the spleen or (C) the tumor for each individual mouse. Bars in graph represent the quantification of mean ± SEM (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Pmel-1 murine T cells cultured in NAC maintain resistance to DNA damage and AICD upon *ex vivo* restimulation

Recovered splenocytes and TILs were stimulated *ex vivo* with $gp100_{25\text{-}33}$ peptide to determine susceptibility to DNA damage and cell death. Because insufficient $V\beta13^+CD8^+$ cells were recovered from tumors of mice that received control cells, we compared Pmel-1 T cells isolated from tumors of mice receiving NAC treated cells to their corresponding splenocytes. Cells which were cultured in NAC retained their resistance to DNA damage even after adoptive transfer as evidenced by the amount of γH2AX+ cells recovered from the spleen being approximately half that of controlled cultured cells (Fig. 19a). Moreover, levels of γH2AX in NAC cultured cells which had trafficked to the tumor microenvironment, were comparable to adoptively transferred cells isolated from spleens (Fig. 19a). Consistent with a decrease in DNA damage susceptibility, cells cultured in NAC prior to adoptive transfer were less susceptible to AICD upon $gp100_{25\text{-}33}$ peptide stimulation. Restimulation of T cells isolated from either the spleen or tumor with $gp100_{25\text{-}33}$ peptide shows that a majority of Pmel-1 effector T cells become Annexin V positive and that *ex vivo* culture in NAC prior to adoptive transfer resulted in a significant decrease in Annexin V expression among cells that had trafficked to the spleen or the tumor (Fig. 19b). In addition, we found that granzyme B expression inversely correlated with Annexin V staining and that the strongest expression was observed on $V\beta13^+CD8^+$ NAC expanded cells that had trafficked to the tumor (Fig. 19c). Together, these results demonstrate that the protection against DNA damage and AICD conferred to Pmel-1 T

cells by *in vitro* culture is retained once cells are removed from NAC and transferred *in vivo*.

Pmel-1 murine T cells cultured in NAC demonstrate enhanced tumor control and improved survival status to recipient mice

To determine whether resistance to DNA damage and AICD translated into a reduction in the tumor burden of recipient mice, twice weekly tumor measurements as well as the overall survival were monitored. As expected, Pmel-1 T cells are capable of significantly delaying tumor growth compared to mice receiving no cells (Fig. 20a, $p=0.0025$), although under the conditions we used, this did not translate into a significant survival benefit (Fig. 20b). In contrast, transfer of Pmel-1 T cells that had been cultured in NAC resulted in highly significant delays in tumor growth compared to mice receiving no cells ($p<0.0001$) and to mice receiving Pmel-1 T cells cultured in the absence of NAC ($p<0.0001$). Expansion of cells in NAC also significantly increased median survival time compared to mice receiving no cells ($p=0.0004$) or those receiving Pmel-1 T cells (Fig. 20b, $p=0.0002$). Together these results suggest that expanding therapeutic T cells in the presence of NAC prior to adoptive transfer, can result in long-lasting benefits following transfer that enhance persistence, tumor control, and survival.

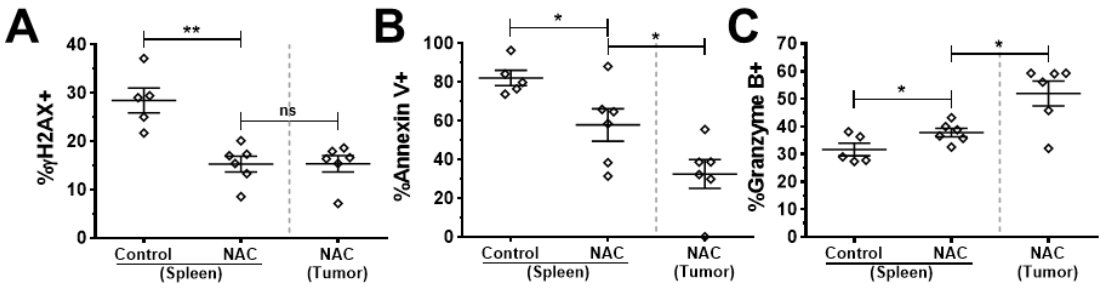

Figure 19. Pmel-1 T cells cultured in NAC maintain resistance to AICD and have enhanced expression of the cytotoxic effector molecule granzyme B after adoptive transfer. C57BL/6 wild type mice were subcutaneously injected with $3x10^6$ B16-F10 murine melanoma cells. After 6 days of tumor establishment, mice were irradiated (5Gy) and treated with $2x10^6$ Pmel-1 T cells which had been cultured ± NAC (10mM) for 6 days and reactivated with $gp100_{25-33}$ peptide (1µg/mL) overnight prior to transfer. Splenocytes and TILs harvested from mice 6 days after transfer were stimulated overnight by co-culture at a 1:1 ratio with irradiated splenocytes pulsed with $gp100_{25-33}$ peptide and then assayed for their expression of (A) γH2AX, (B) Annexin V, or (C) granzyme B. Displayed is percent positive of each indicated marker amongst $V\beta13^+CD8^+$ gated cells for each individual mouse. Bars in graph represent the quantification of mean ± SEM (*p<0.05, **p<0.01, ns=not significant).

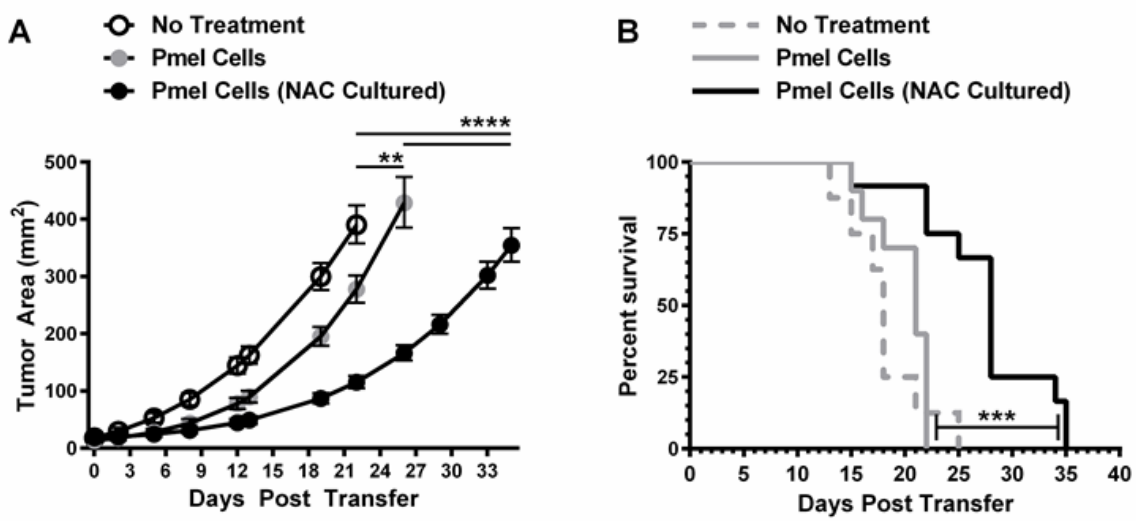

Figure 20. Pmel-1 T cells cultured in NAC demonstrate improved control of tumor burden and survivability of recipient mice. C57BL/6 wild type mice were subcutaneously injected with $3x10^6$ B16-F10 murine melanoma cells. After 6 days of tumor establishment, mice were irradiated (5Gy) and treated with $2x10^6$ Pmel-1 T cells which had been cultured ± NAC (10mM) for 6 days and reactivated with $gp100_{25-33}$ peptide (1µg/mL) overnight prior to transfer. (A) Tumor growth and (B) survival was determined for up to 35 days after adoptive transfer in untreated mice (n=8), Pmel-1 T cell treated mice (n=10), and mice receiving NAC treated Pmel-1 T cells (n=12) (**p<0.01, ***p<0.001, ****p<0.0001).

TIL1383I TCR transduced human T cells expanded in NAC demonstrate an enhanced antioxidant capacity, resistance to AICD, and superior anti-melanoma *in vitro* cytotoxicity

The improvement in cell persistence, tumor control, and survival of recipient mice conferred to Pmel-1 T cells by adding NAC to the expansion culture prior to adoptive transfer prompted us to investigate whether NAC would be a benefit to therapeutic TIL1383I TCR transduced human T cells. Autologous PBMCs derived from melanoma patients and healthy donor controls were activated and transduced with the TIL1383I TCR. Following transduction, NAC (2mM) was added to the culture medium and maintained through the rapid expansion of the cells (Fig. 21a). Though some reports have suggested NAC can enhance the proliferation of T cells (654), there was not any significant difference in the yield between cells that were expanded with or without the presence of NAC suggesting that NAC did not enhance the proliferation of T cells at this concentration (Fig. 21b). However, NAC did enhance the antioxidant capacity of TIL1383I TCR transduced human T cells. NAC REPed T cells exhibited an increased expression of surface thiols denoted by the C_2Maleimide dye (Fig. 22a). Conversely, TIL1383I transduced cells REPed in the presence of NAC demonstrate less evidence of oxidative stress via a decrease DCFDA staining expression (Fig. 22b). Moreover, consistent with oxidative stress being upstream of DNA damage, NAC REPed cells were less susceptible to upregulation of γH2AX than their control cultured counterparts when stimulated with MEL624 melanoma cells (Fig. 22c).

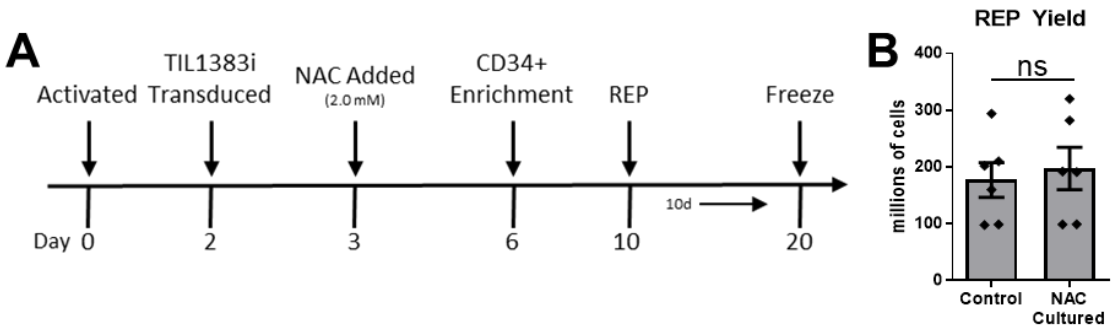

Figure 21. Production of NAC cultured TIL1383I TCR transduced human T cells. (A) PBMCs were activated for 2 days with soluble anti-CD3 and then transduced with the TIL1383I TCR. The day after transduction, NAC (2mM) was added to the culture medium after which cells were then enriched based on CD34+ expression and subject to a rapid expansion protocol whereby $1x10^6$ CD34+ cells are co-cultured with $2x10^8$ irradiated feeder cells supplemented with 30ng/mL anti-CD3 for 10 days with media replenishment at Day 5. (B) Cell counts following rapid expansion of each individual donor. Bars in graph represent the quantification of mean ± SEM (n=6, ns=not significant).

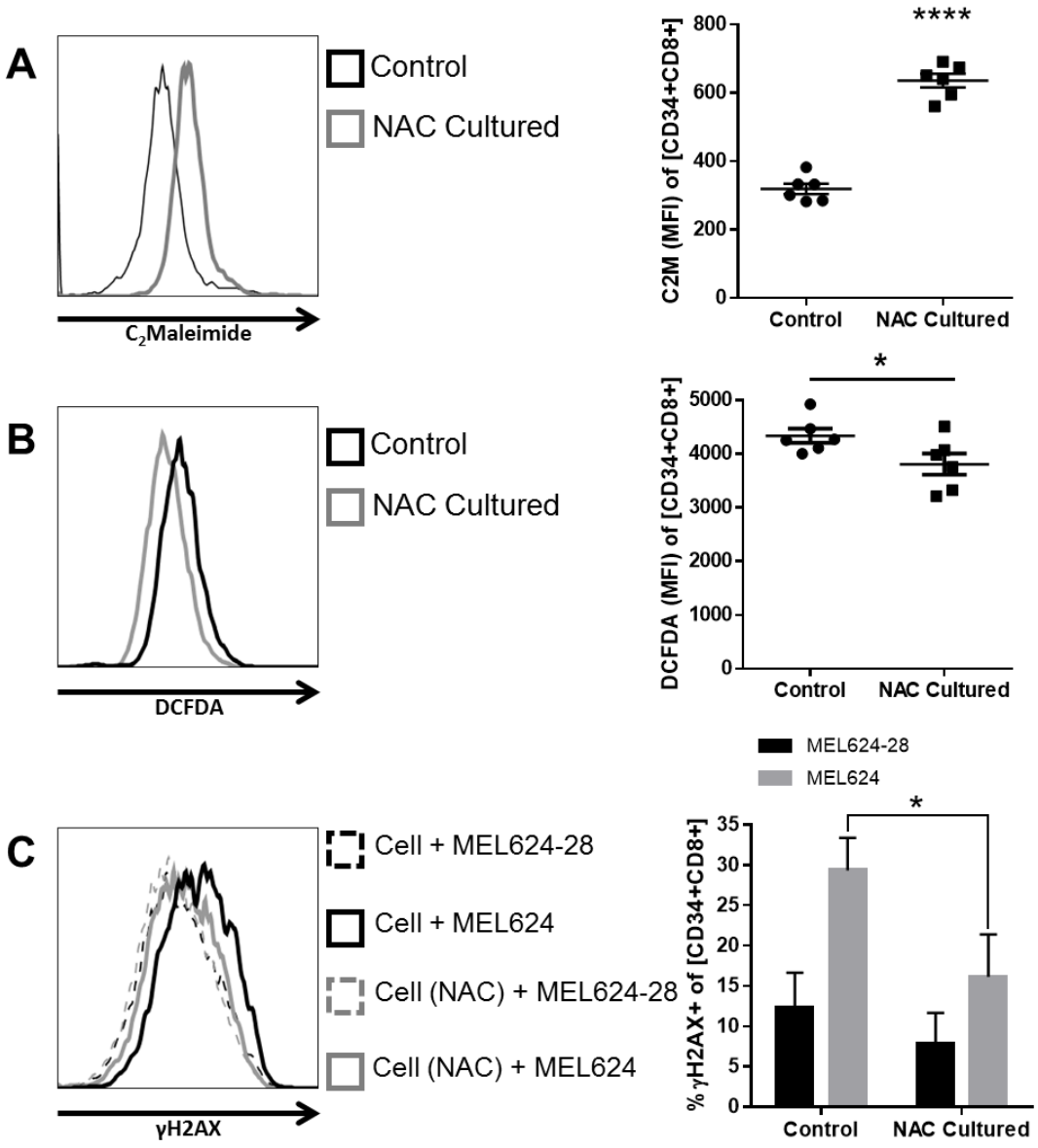

Figure 22. TIL1383I TCR transduced human T cells REPed in NAC have improved antioxidant capacity and resistance to MEL624 induced DNA damage. TIL1383I transduced human T cells rapidly expanded for 10 days (± 2mM NAC) were stained with (A) C_2Maleimide for detection of surface thiols and (B) DCFDA for detection of oxidative stress. (C) TIL1383I transduced cells were also co-cultured overnight with either MEL624 (HLA-A2$^+$) or MEL624-28 (HLA-A2$^-$) cells at a 1:1 ratio and then stained intracellularly for detection of γH2AX. Left panels display representative histogram overlays in CD34$^+$CD8$^+$ gated cells. Right panels display quantification (mean ± SEM) of each indicated marker in CD34$^+$CD8$^+$ cells (n=6, *p<0.05, ****p<0.0001).

As such, we investigated whether this improvement in antioxidant capacity and resistance to DNA damage would improve the functional ability of NAC REPed cells to kill melanoma tumor cells. TIL1383I TCR transduced human T cells which were rapidly expanded with or without the addition of NAC (2mM) were subsequently washed from the presence of NAC and co-cultured overnight with HLA-A2 matching MEL624 cells and HLA-A2-negative MEL624-28 cells. MEL624 and MEL624-28 cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) at different concentrations which allows for simultaneous gating of both melanoma cell types using MEL624-28 as an internal assay control (Fig. 23a). After overnight culture, cells were stained with Annexin V and 7AAD and analyzed via flow cytometry. Utilizing the differential CFSE staining, MEL624 and MEL624-28 were gated on their respective CFSE concentrations. MEL624 gated cells which were co-cultured with TIL1383I TCR transduced cells REPed in NAC demonstrated a consistent increase in the percentage of Annexin V and 7AAD double positive cells compared to MEL624 cells co-cultured with control REPed cells (Fig. 23a-b). Conversely, the TIL1383I TCR transduced human T cells themselves which were cultured in NAC exhibited increased resistance to cell death induced by tumor cells compared to control REPed counterparts (Fig. 23c). Together these data demonstrate that rapidly expanding TIL1383I TCR transduced human T cells in NAC enhances their ability to kill MEL624 melanoma cells while in parallel being more resistant to cell death themselves. We further investigated whether addition of NAC to the rapid expansion of TIL1383I TCR

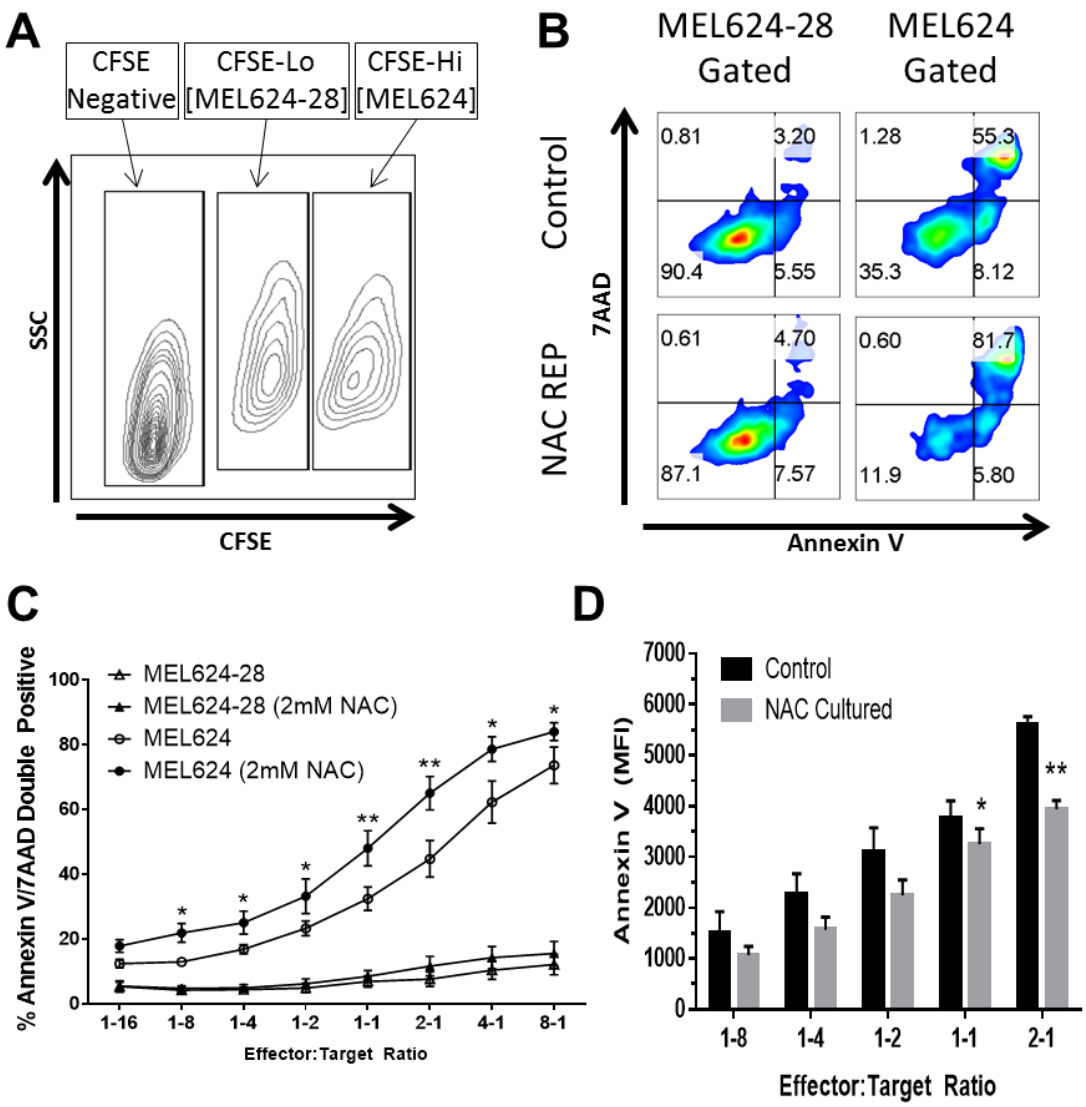

Figure 23. TIL1383I TCR transduced human T cells rapidly expanded in NAC exhibit enhanced anti-melanoma *in vitro* cytotoxicity. $2x10^4$ each MEL624 (HLA-$A2^+$) and MEL624-28 (HLA-$A2^-$) cells were co-cultured overnight with TIL1383I transduced human T cells (REPed with or without 2mM NAC) plated at increasing Effector:Target ratios. (A) Melanoma cells were gated based on being labeled with CFSE at differentiating concentrations. MEL624-28 cells were labeled with CFSE at 0.01 µM and gated as the "CFSE-Lo" population. MEL624 cells were labeled with CFSE at 0.1 µM and gated as the "CFSE-Hi" population. (B) Representative flow plot of Annexin V vs 7AAD expression at a 2:1 Effector:Target ratio. (C) Quantification (mean ± SEM) of Annexin V/7AAD double-positive cells at expanded Effector:Target ratios. (D) Quantification (mean ± SEM) of Annexin V MFI in TIL1383I transduced cells gated as $CFSE^{neg}CD34^+CD8^+$ (n=5, *p<0.05, **p<0.01).

transduced human T cells modulated their functional capacity. In our model system, NAC supplementation did not demonstrate any obvious enhancement in the functional capacity of cells to secrete IFNγ as there was no significant difference between NAC REPed and control cells (Fig. 24a). Moreover, the surface expression of CD107a, a marker indicative of the degranulation of cytotoxic effector molecules (672), was also unaltered between T cells REPed in NAC and control cultured cells (Fig. 24b). Additionally, there was no significant difference ($p=0.62$) between NAC and control REPed cells as to the expression of the cytotoxic molecule perforin (Fig. 25a). However, in contrast, there was a significant increase of cells positive for the cytotoxic molecule granyzme B amongst NAC cultured cells (Fig. 25b).

As these *in vitro* results suggested that NAC conferred an enhanced anti-melanoma cytolytic ability to human cells similar to what was observed *in vivo* with the murine Pmel-1 model, we sought out to investigate if culturing TIL1383I TCR transduced cells in NAC would improve their anti-melanoma functionality in a preclinical model. To do so, TIL1383I TCR transduced human T cells cultured with or without NAC were adoptively transferred into MEL624 challenged NSG mice. However, transferred TIL1383I TCR human transduced T cells did not persist once transferred as there was no detectable observance of $CD3^{+}CD34^{+}$ T cells in the spleens of any mice receiving cells above the "no cell" controls (Fig. 26). As such, there was no differentiation in tumor burden control amongst the treatment groups (Fig. 26). Unfortunately, the failure of transferred cells to

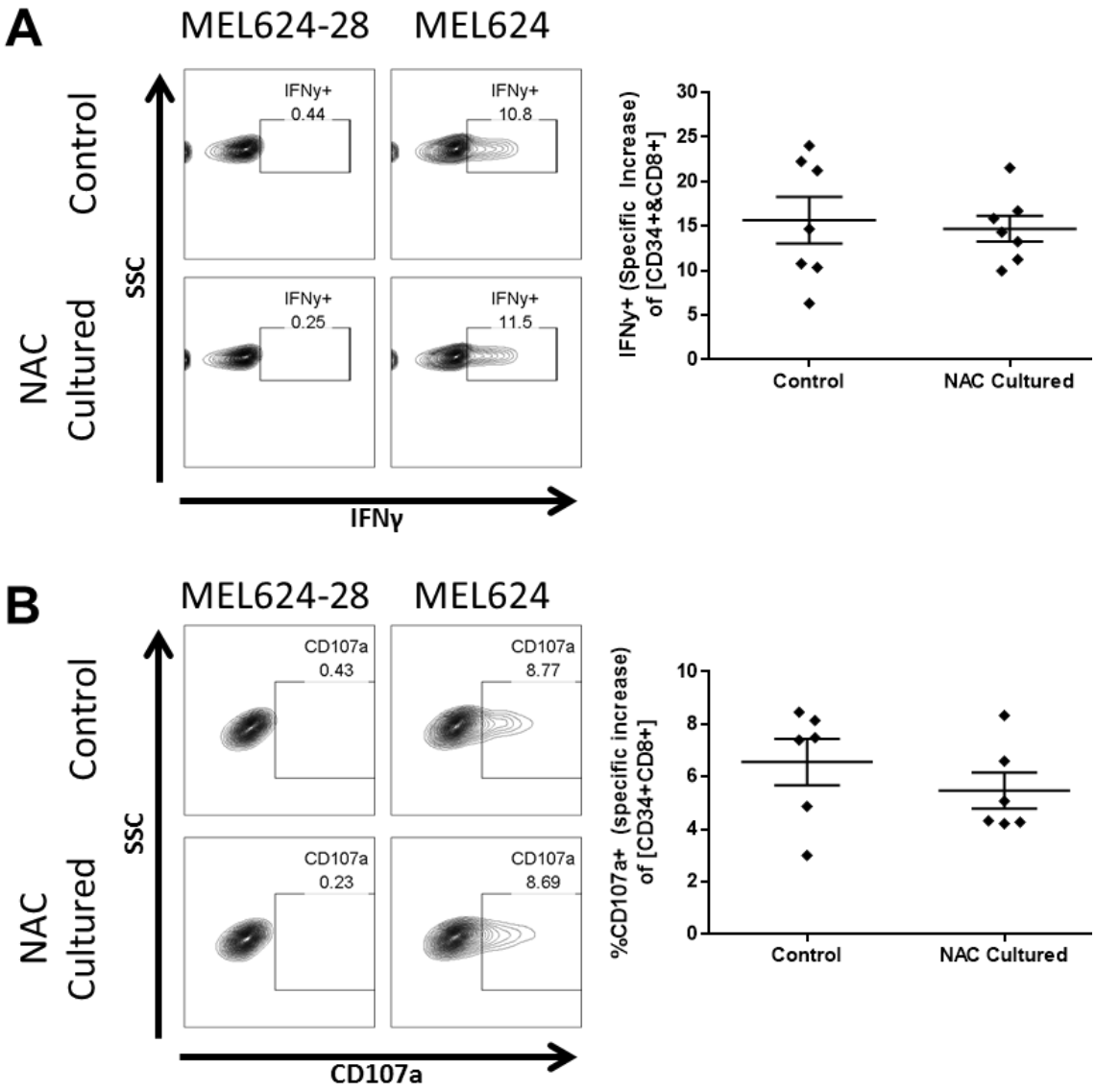

Figure 24. Effect of NAC supplementation during REP on the functional capacity of TIL1383I TCR transduced human T cells. TIL1383I TCR transduced human T cells which were rapidly expanded for 10 days (± 2mM NAC) were co-cultured overnight with MEL624 (HLA-A2^{+}) or MEL624-28 (HLA-A2^{-}) cells and then (A) stained intracellularly for IFNγ expression (B) or preloaded with a CD107a fluorochrome conjugated antibody in order to assess stimulation induced surface expression. Left panels are representative contour plots of indicated marker in CD34^{+}CD8^{+} gated cells. Right panels are quantification of percent positive for each indicated marker amongst CD34^{+}CD8^{+} gated cells. Bars denote mean ± SEM (n=6).

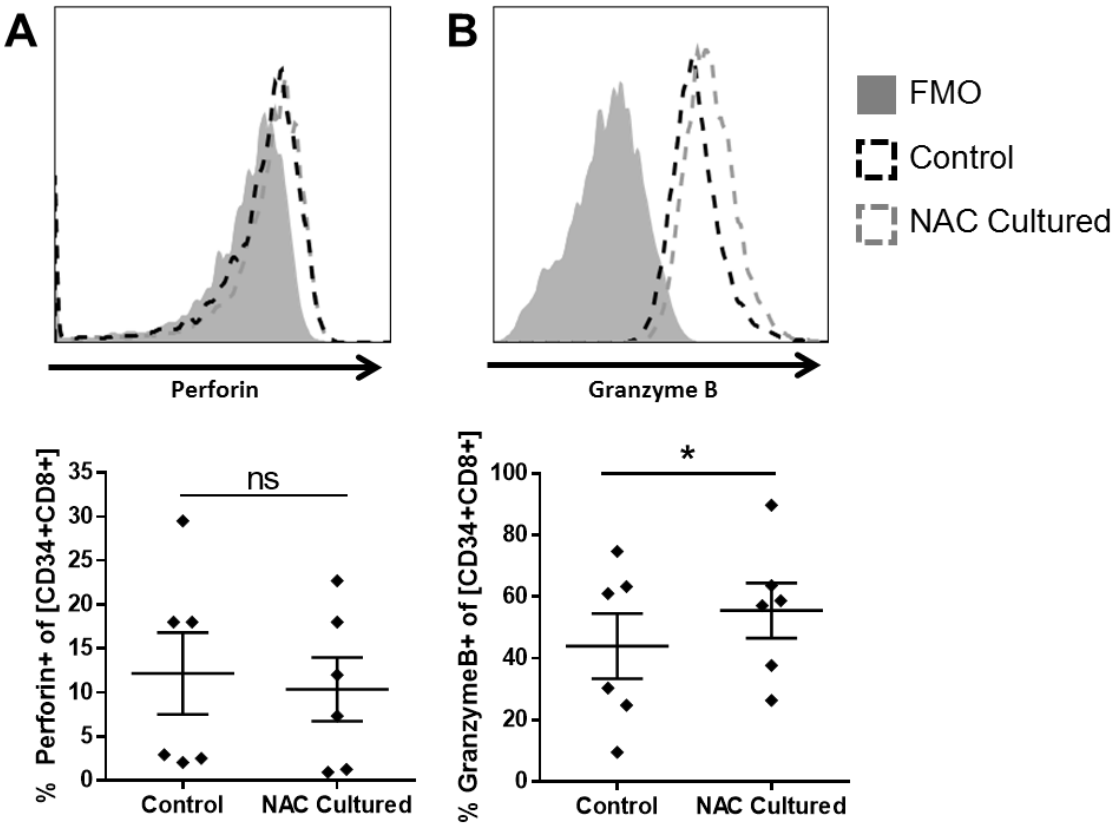

Figure 25. Effect of NAC supplementation during REP on the functional capacity of TIL1383I TCR transduced human T cells. TIL1383I TCR transduced human T cells which were rapidly expanded for 10 days (± 2mM NAC) were stained for intracellular expression of (A) perforin or (B) granzyme B. Top panels are representative histograms comparing NAC and control cultured cells to FMO controls in $CD34^{+}CD8^{+}$ gated cells. Bottom panels are quantification of percent positive for each indicated marker amongst $CD34^{+}CD8^{+}$ gated cells. Bars denote mean ± SEM (n=6, *$p<0.05$).

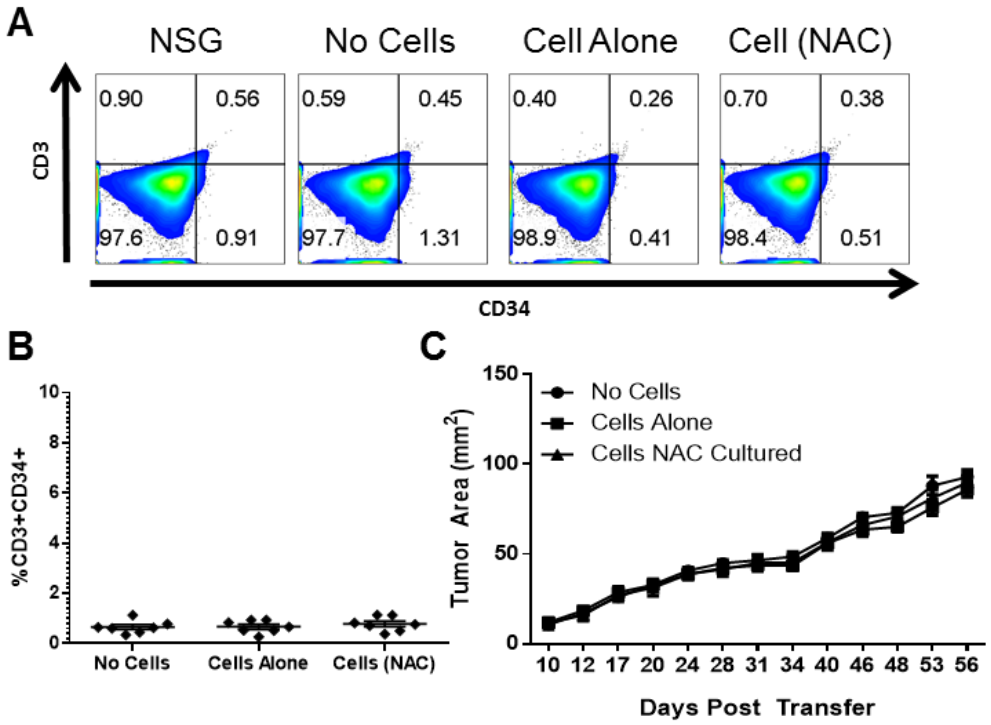

Figure 26. Lack of persistence of TIL1383I TCR transduced human T cells in NSG xenograft model. $10x10^6$ CD34+ enriched TIL1383I TCR transduced cells were adoptively transferred into NSG mice challenged with MEL624 tumors ($5x10^6$ cells). At 56 days post transfer, the experiment was terminated and mice were sacrificed. (A) Representative contour plots denoting CD3+ and CD34+ expression of spleens harvested from mice. (B) Quantification of percent $CD3^+CD34^+$ double-positive cells from spleen represented in 'A' for each individual mouse. (C) Tumor growth of mice receiving either no treatment, TIL1383I TCR transduced cells, or TIL1383I TCR transduced cells expanded in 2mM NAC (n=7 per treatment group).

persist in this model did not allow for any discernment as to whether TIL1383I TCR transduced human T cells would demonstrate enhanced *in vivo* functionality from being expanded in the presence of NAC, and thus, a humanized model for TIL1383I TCR transduced cells merits further optimization.

Effect of NAC supplementation on TCR transduced T cells in a murine model

As we were unable to investigate the effect of NAC on *in vivo* tumor control using TIL1383I TCR transduced human T cells in a NSG xenograft model, we alternatively used a completely murine model whereby splenocytes from C57BL/6 wild type mice were transduced with the TRP-1 TCR, which recognizes the melanoma associated antigen TRP-1 (324,326). After activation and transduction, these cells were expanded with or without NAC supplementation and adoptively transferred, into lymphodepleted, B16-F10 challenged C57BL/6 wild type mice. As observed in the Pmel-1 model, the addition of NAC (10mM) to the culture of TRP-1 splenocytes enhanced the expression of surface thiols while concurrently diminishing the susceptibly of cells to TRP-1 peptide induced DNA damage and AICD as indicated by a reduction in expression of γH2AX and Annexin V respectively (Fig. 27). However, in contrast to the Pmel-1 model, there was no significant increase in the percentage of adoptively transferred NAC cultured T cells detected in the spleens or tumors of mice sacrificed for biodistribution analysis (Fig. 28a,b). However, TRP-1 TCR transduced cells cultured in NAC did demonstrate an enhancement in their functional capacity when stimulated *ex vivo* with peptide when compared to control

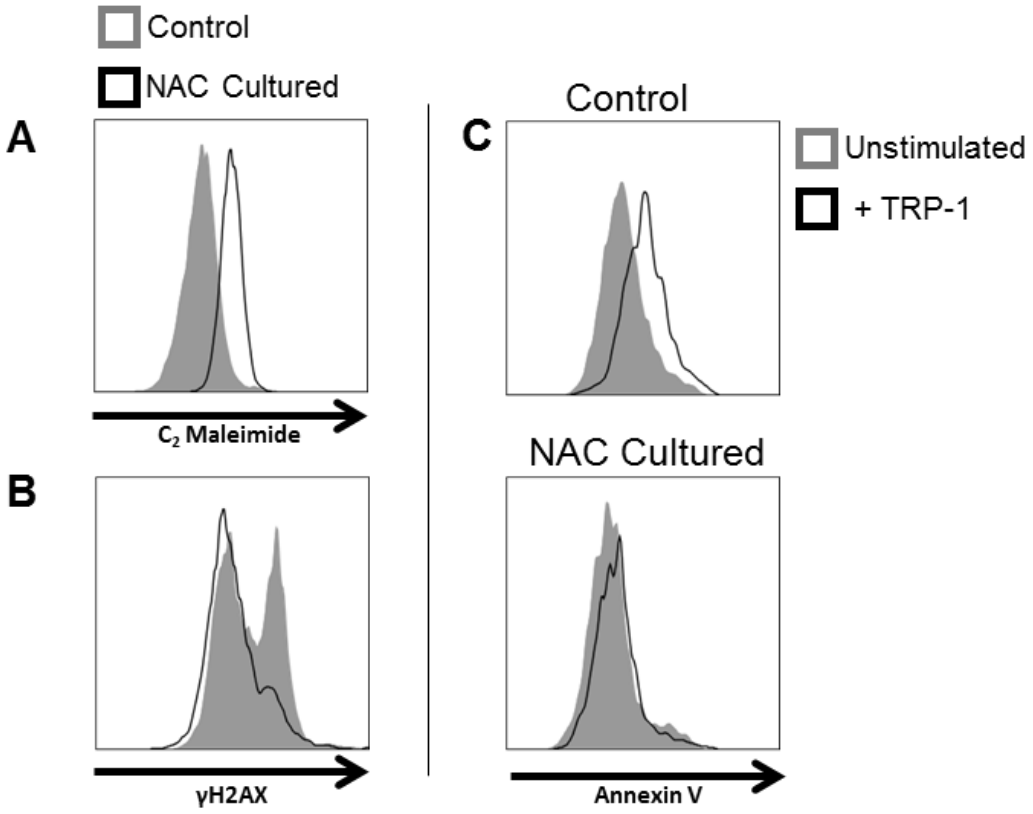

Figure 27. *In vitro* characterization of TRP-1 TCR transduced murine T cells cultured in NAC. (A) Representative histogram overlay of C_2maleimide dye expression in Vβ14$^+$CD8$^+$ gated TRP-1 TCR transduced murine T cells cultured for 6 days ± NAC (10mM). (B) Representative histogram overlay of γH2AX expression of Vβ14$^+$CD8$^+$ gated cells cultured ± NAC (10mM) after overnight restimulation with co-culture at a 1:1 ratio with irradiated splenocytes pulsed with TRP-1 peptide (4μg/mL). (C) TRP-1 cells cultured ± NAC (10mM) were restimulated or not by co-culture at a 1:1 ratio with irradiated splenocytes pulsed with TRP-1 peptide (4μg/mL). Displayed is representative histogram overlays demonstrating change in Annexin V expression in Vβ14$^+$CD8$^+$ upon TRP-1 restimulation for cells cultured with or without NAC (all panels are representative of n=2).

cultured cells. Similar to the Pmel-1 model, there was an enhancement in the expression of granzyme B (Fig. 28c), and moreover, TRP-1 TCR transduced cells cultured in NAC demonstrated an increase in the amount of cells positive for IFNγ cytokine expression as well as an increase in the proliferation marker Ki-67 (Fig. 28d,e). Additionally, TRP-1 TCR transduced cells cultured in NAC prior to adoptive transfer were less susceptible to peptide induced DNA damage and cell death in both the spleens and tumors of recipient mice (Fig. 29a,b). The amount of Annexin V+ T cells was reduced by nearly half in Vβ14+ cells harvested from the spleens of mice receiving NAC cultured cells, and in the tumors of mice receiving control cultured cells, nearly 60% of Vβ14+ cells were Annexin V+ which was reduced 5.1-fold amongst the cells cultured in NAC prior to adoptive transfer (Fig. 29a). Similar to observations in the Pmel model, mice receiving TRP-1 transduced cells that were cultured in NAC prior to adoptive transfer exhibited a significant delay in tumor growth compared to control cells ($p=0.025$), which themselves demonstrated a significant enhancement in outcome compared to untreated mice (Fig. 29c, $p<0.0001$). Moreover, the transfer of TRP-1 TCR transduced cells significantly extended the median survival time compared to untreated mice ($p=0.0289$), with TRP-1 cells previously cultured in NAC exhibiting an even further significant extension in survival compared to control cultured cells (Fig. 29d, $p=0.0243$). Together these results demonstrate that, similar to observations in native TCR bearing T cells, expanding TCR transduced cells in the presence of NAC can enhance their antioxidant capacity leading to a durable resistance to TCR

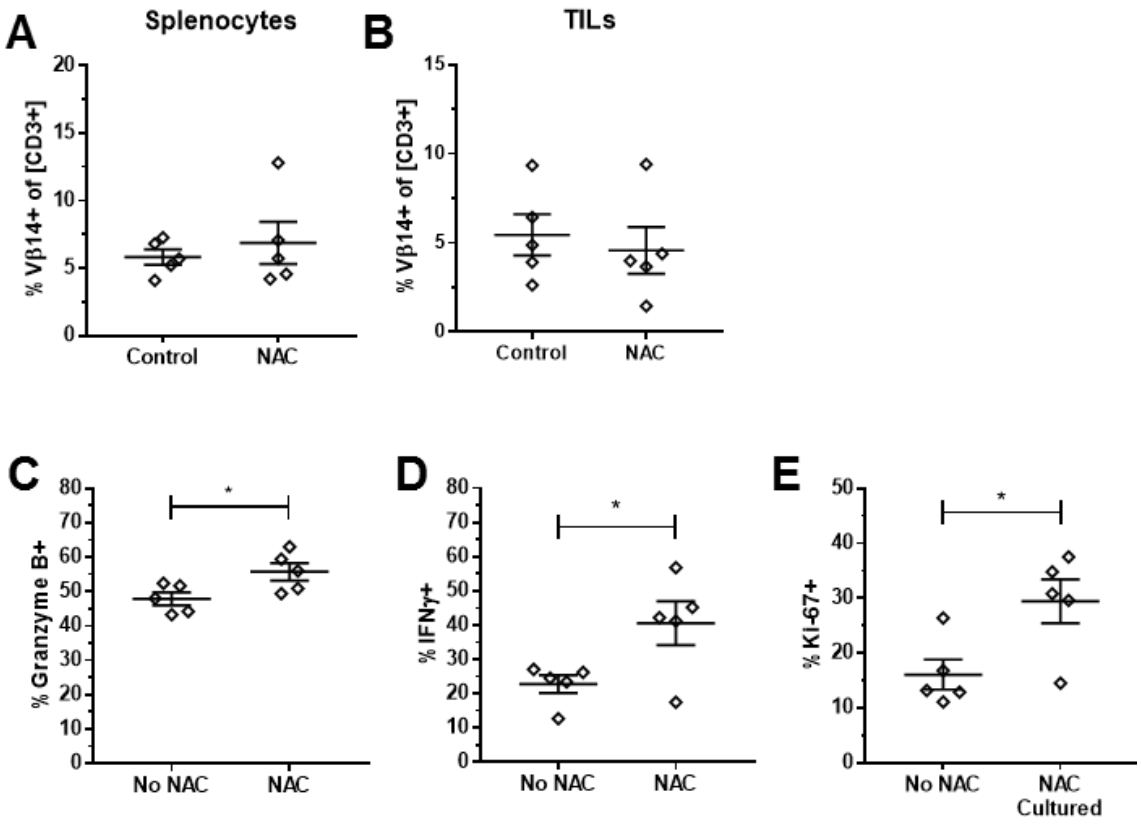

Figure 28. TRP-1 TCR transduced murine T cells cultured in NAC demonstrate enhanced functional capacity after adoptive transfer. C57BL/6 wild type mice were subcutaneously injected with $3x10^6$ B16-F10 murine melanoma cells. After 6 days of tumor establishment, mice were irradiated (5Gy) and treated with $2x10^6$ TRP-1 TCR transduced T cells which had been cultured ± NAC (10mM) for 6 days prior to transfer. (A) Spleens and (B) tumors were harvested from recipient mice 9 days post transfer and processed into single-cell suspensions. Displayed is the percent of $V\beta14^+$ cells of $CD3^+$ gated cells from each individual mouse. (C-E) Harvested splenocytes were additionally stimulated overnight with a co-culture at a 1:1 ratio with irradiated splenocytes pulsed with TRP-1 peptide (4μg/mL) and then stained intracellularly for the expression of (C) granzyme B, (D) IFNγ, or (E) Ki-67. Displayed is the percent positive for each indicated marker for each mouse in $V\beta14^+CD8^+$ gated cells (*$p<0.05$).

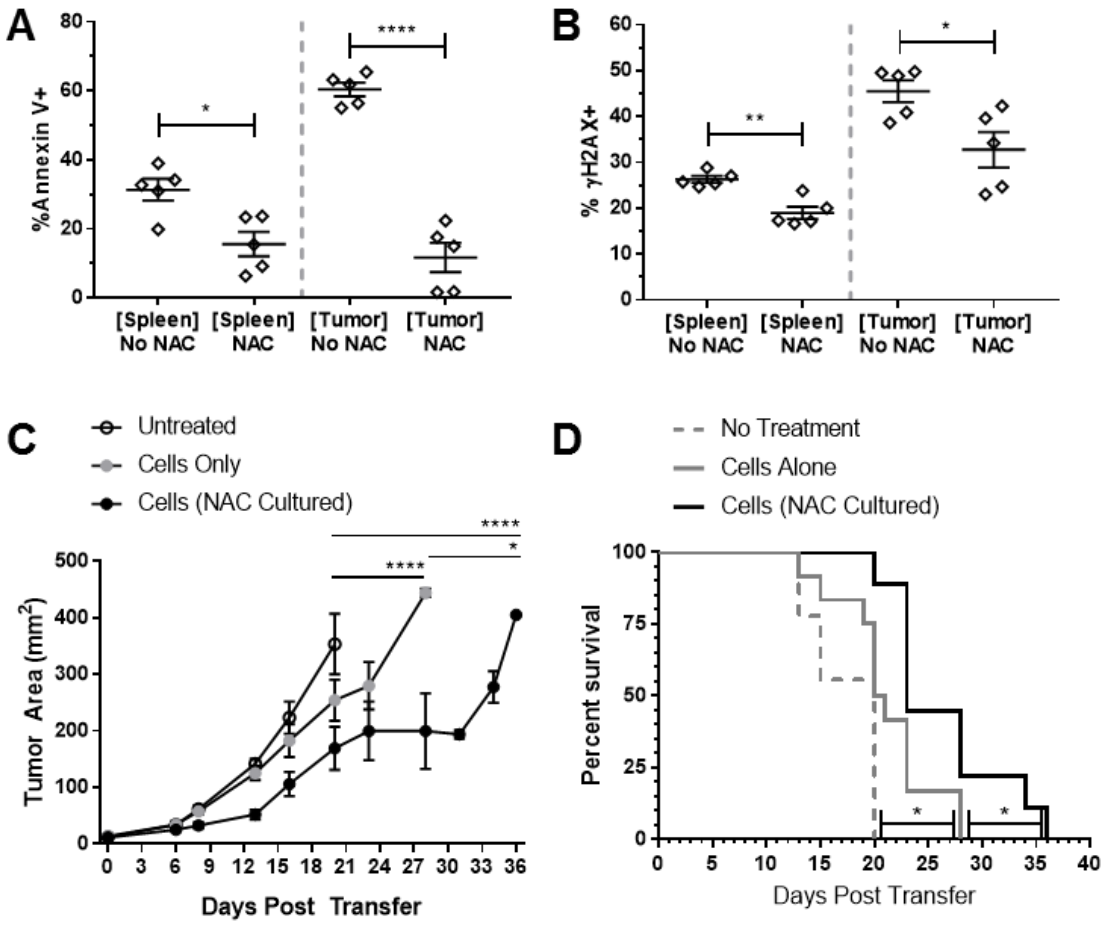

Figure 29. TRP-1 TCR transduced murine T cells cultured in NAC are more resistant to DNA damage and cell death while exhibiting enhancement in tumor burden control and survivability of recipient mice. C57BL/6 wild type mice were subcutaneously injected with $3x10^6$ B16-F10 murine melanoma cells. After 6 days of tumor establishment, mice were irradiated (5Gy) and treated with $2x10^6$ TRP-1 TCR transduced T cells which had been cultured ± NAC (10mM) for 6 days prior to transfer. (A-B) Splenocytes and TILS harvested from mice 9 days post transfer were stimulated overnight with co-culture at a 1:1 ration of irradiated splenocytes pulsed with TRP-1 peptide (4μg/mL) and then assayed for their expression of (A) Annexin V or (B) γH2AX. Displayed is percent positive of each indicated marker for each individual mouse in $V\beta14^+CD8^+$ gated cells. (C) Tumor growth and (D) survival was determined for up to 36 days after adoptive transfer in untreated mice, mice receiving TRP-1 TCR transduced cells, and mice receiving TRP-1 TCR transduced cells cultured in NAC. (*p<0.05, **p<0.01, ****p<0.0001).

restimulation induced DNA damage and cell death, while additionally conferring enhanced functional capacity exhibited by increased granzyme B expression and delay in tumor growth and morbidity. Together these results demonstrate that culturing cells in NAC prior to co-culture with tumor cells *in vitro* and adoptive transfer *in vivo* improves the anti-tumor cytolytic functionality of the T cells. Moreover, as the improvement in *in vivo* tumor control is maintained after T cells are removed from NAC and adoptively transferred, these data suggest that NAC supplementation to culture media imparts a durable alteration and improvement in the phenotype of T cells.

CHAPTER 4 – CULTURE OF TIL1383I TCR TRANSDUCED HUMAN T CELLS IN N-ACETYL CYSTEINE ATTENUATES EXPRESSION OF EXHAUSTION ASSOCIATED RECEPTORS AND TRANSCRIPTION FACTORS

Introduction

In Chapter Three, we observed that melanoma antigen-specific T cells expanded in NAC maintained a DNA damage and AICD resistant phenotype multiple days after removal from NAC and subsequent *in vivo* transfer both in the Pmel-1 native TCR model as well as in murine T cells which were transduced with the TRP-1 TCR. These observations suggest that NAC confers a durable alteration to the phenotype of T cells beyond transient antioxidant protection. Consistent with that premise, there have been many reports in the literature which have demonstrated phenotypic improvements in T cells which have been cultured in NAC. In addition to corroborating our observations that NAC increases the antioxidant capacity of T cells, NAC has also been shown to enhance the proliferation and cytokine production of T cells, as well as restore the functional capacity of T cells from advanced stage cancer patients (537,539,653,654). However, the rapid expansion of therapeutic T cells to achieve a sufficient quantity for infusion counterproductively results in T cells that are functionally less fit for durable tumor control. This decline in fitness is characterized by a degradation of telomeres (170), loss of the CD28 co-stimulatory receptor (170), an increase in effector-memory T cells with a parallel decrease in central-memory T cells (169), and an increase in the expression of exhaustion markers such as PD-1 (170). The enhanced ability of TIL1383I TCR transduced human T cells that were rapidly expanded in NAC to kill HLA-A2 matched MEL624 cells *in vitro* (Fig. 24) suggested that NAC may protect cells from the decline in fitness caused by

rapid expansion. As such, we sought to further interrogate how NAC may alter the phenotype of T cells when added to the culture medium during rapid expansion.

Enhancement of cytotoxicity amongst NAC cultured TIL1383I transduced human T cells is independent of alteration in memory phenotype or costimulatory receptors

As central memory cells (T_{CM}) have been reported to be more efficacious in tumor control than effector memory cells (T_{EM}) (673), as an initial approach, we investigated whether NAC altered the output of memory phenotype subsets of T cells at the conclusion of a REP. TIL1383I transduced human T cells which had been subjected to a REP (±2mM NAC) were stained with fluorochrome-conjugated antibodies against CD45RO, CD62L, and CCR7 with T_{EM} cells defined as $CD45RO^{+}CD62L^{LO}CCR7^{LO}$ and T_{CM} cells as $CD45RO^{+}CD62L^{HI}CCR7^{HI}$. Analysis of these memory markers did not reveal any major difference in T_{EM}/ T_{CM} subsets between T cells expanded with or without NAC as there was no significant difference in the expression of the central memory markers CD62L or CCR7 relative to CD45RO (Fig. 30).

Moreover, since the expression of CD28 positively correlates with the therapeutic efficacy of transferred cells (162), and the engagement of co-stimulatory receptors such as CD28 and 4-1BB can protect T cells against AICD (171,351,352), we examined whether NAC modulated the levels of these co-stimulatory receptors. As with memory markers, there was no enhancement in the expression of the co-stimulatory receptors CD28 or 4-1BB (Fig. 31a-b). However, we did detect an increase in the intensity for the expression

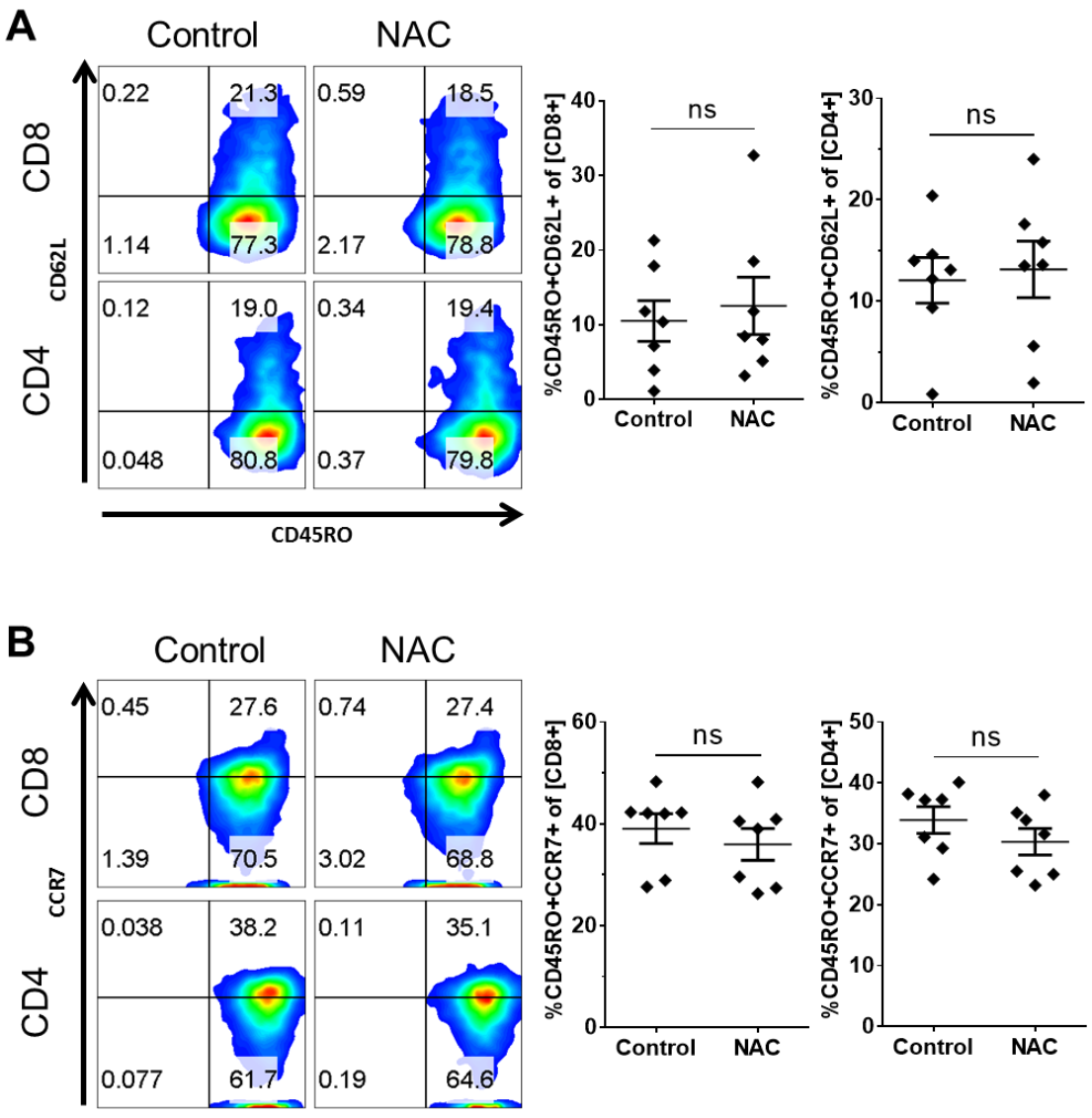

Figure 30. REP in NAC does not alter expression of memory markers on TIL1383I TCR transduced human T cells. TIL1383I TCR transduced human T cells which were rapidly expanded for 10 days ± NAC (2mM) were stained for the expression of (A) CD45RO and CD62L or (B) CD45RO and CCR7. Left panels are representative flow plots showing the expression of CD45RO vs CD62L or CCR7 in CD8+ or CD4+ gated cells as indicated. Right panels show quantification of percent positive for indicated marker for each individual donor. Bars denote mean ± SEM (n=7, ns=not significant).

of the ICOS co-stimulatory receptor in cells that were expanded in NAC (Fig 31c). Together, these results demonstrate that improvement of the cytotoxic function of NAC REPed cells seems to be independent of a major alteration of memory T cells subsets or enhancement of co-stimulatory receptors.

TIL1383I transduced human T cells rapidly expanded in the presence of NAC demonstrated an attenuation of exhaustion markers

The rapid expansion of therapeutic T cells has been shown to enhance the expression of the exhaustion marker PD-1 and the senescence marker CD57 on T cells (170), both of which associate with impaired functionality (674,675). Consistent with these previous reports, the rapid expansion of TIL1383I TCR transduced human T cells resulted in an increase in the expression of both PD-1 and CD57 (Fig. 32). However, supplementation with NAC impeded the upregulation of these markers culminating in a significant decrease in the expression of PD-1 and CD57 when NAC REPed T cells were compared to control cultured cells. (Fig. 32).

As exhaustion in T cells has been attributed to the relative expression levels of the transcription factors T-bet and EOMES, with exhausted cells exhibiting a T-bet^{Lo}Eomes^{Hi} phenotype (618), TIL1383I TCR transduced human T cells were further interrogated to determine if NAC modulated the expression of these transcription factors. There was not a significant difference in the expression of T-bet between cells which were REPed in NAC and control cells (Fig. 33a). However, in contrast, there was a significant decrease in the

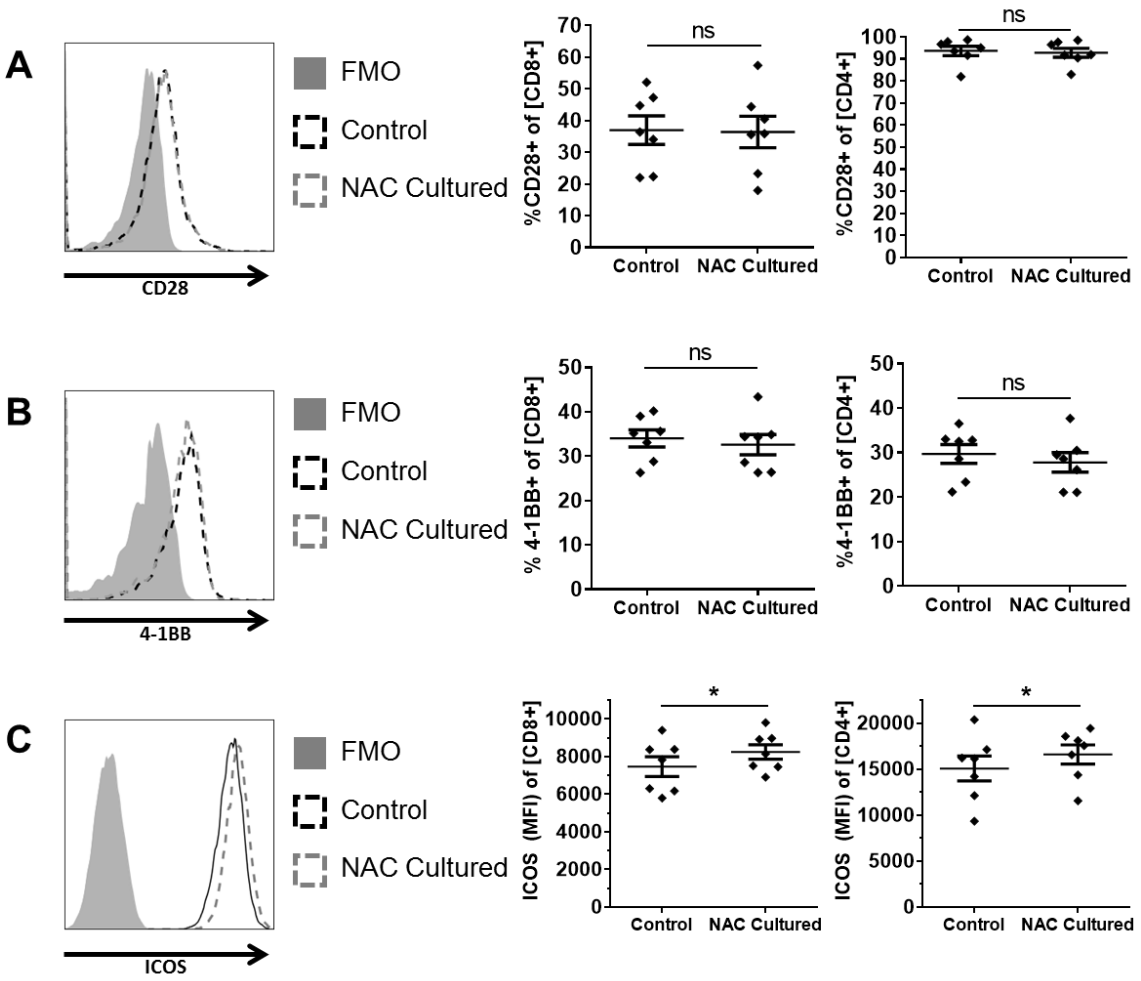

Figure 31. Effect of NAC REP supplementation on expression of co-stimulatory markers on TIL1383I TCR transduced human T cells. TIL1383I TCR transduced human T cells were rapidly expanded for 10 days (±2mM NAC) and were surface stained for the expression of (A) CD28, (B) 4-1BB, or (C) ICOS. Left panels display representative histogram overlay of NAC and controlled REPed cells compared to FMO control in CD8+ gated cells. Right panel display percent positive or MFI for each individual donor in either CD8+ or CD4+ gated cell as indicated. Bars denote mean ± SEM (n=7, *p<0.05, ns=not significant).

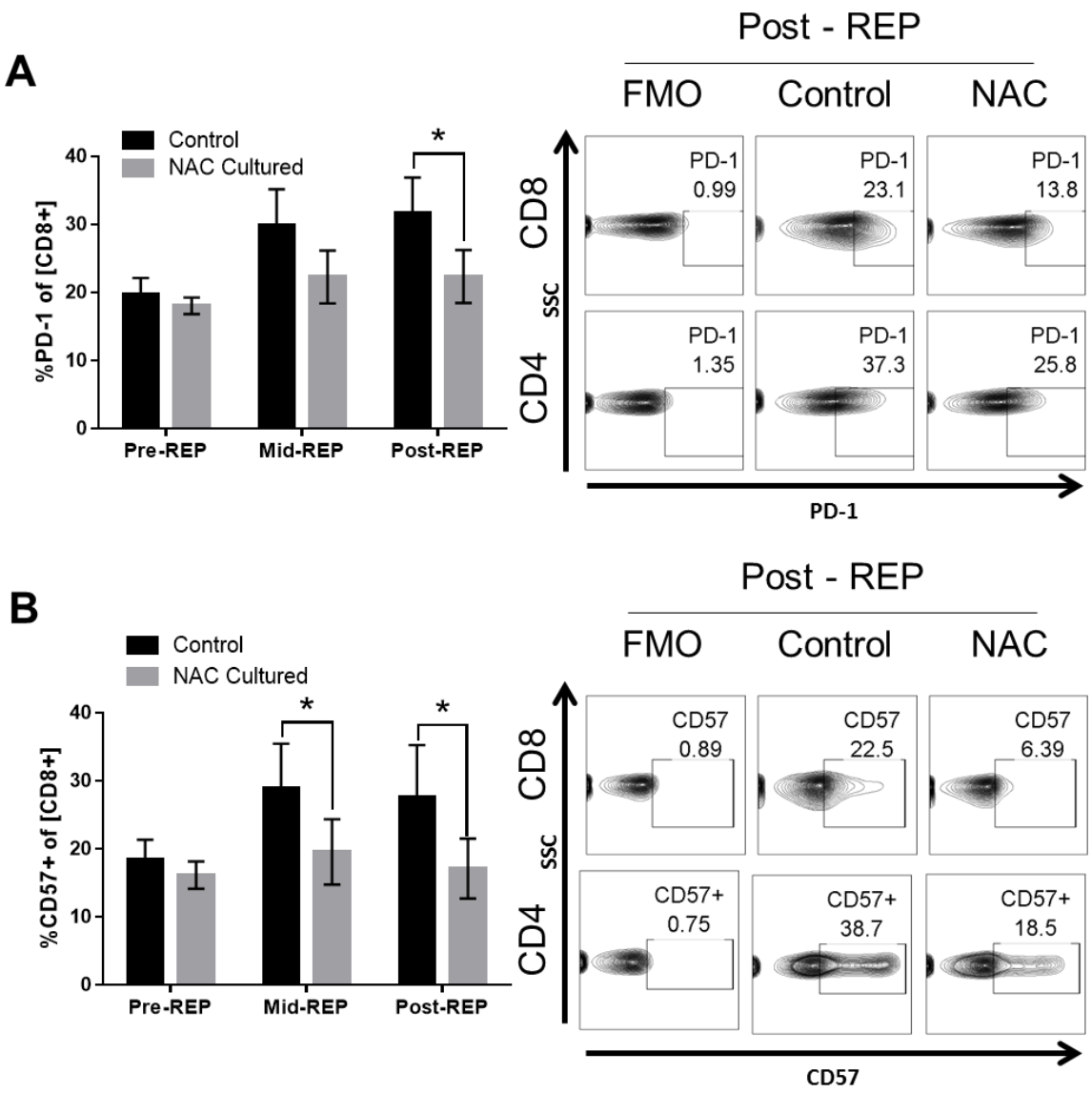

Figure 32. TIL1383I TCR transduced human T cells rapidly expanded in NAC display reduced expression of markers of exhaustion and senescence. TIL1383I TCR transduced human T cells which were rapidly expanded for 10 days (± 2mM NAC) and were surface stained for the expression of (A) PD-1 or (B) CD57. Left panels display quantification of mean ± SEM of the percent positive in CD8+ gated cells for each indicated marker on analysis performed on samples cryopreserved at indicated time-points throughout the REP. Right panels display representative contour plots of each indicated marker (vs side-scatter) in Post-REP samples in CD8+ or CD4+ gated cells as indicated. (n=7, *p<0.05).

percentage of cells which were EOMES+ amongst NAC REPed cells compared to control cells (Fig. 33b). Together these results demonstrate that addition of NAC to the culture medium of rapidly expanded T cells results in the cells being less exhausted which is likely regulated, in part, by a reduction in the activity of the exhaustion associated transcription factor EOMES.

TIL1383I TCR transduced human T cells rapidly expanded in NAC have reduced expression of the transcription factor Foxo1

Upstream of EOMES, the transcription factor Foxo1 has been implicated in modulation of the exhausted T cell phenotype, and specifically involved in PD-1 expression, in part through the promotion of EOMES (631). Additionally, Foxo1 represses granzyme B expression (648) which was observed to increase in NAC REPed cells (Fig. 25b). Therefore, we predicted that NAC likely restrains the expression of Foxo1 and interrogated TIL1383I TCR transduced human T cells cultured in NAC for Foxo1 expression. Indeed, cells cultured in NAC had a nearly 2.7-fold decrease in the expression of the Foxo1 transcription factor in comparison to control cultured cells (Fig. 34).

Foxo1 is inactivated via phosphorylation which both promotes its sequestration outside of the nucleus which restricts its activity as a transcription factor and ultimately aids in facilitating the ubiquitination and subsequent proteasomal degradation of Foxo1 (640,676). Therefore, to determine if NAC influenced the phosphorylation status of Foxo1, T cells were acutely treated with NAC for 60 min and then stained intracellularly

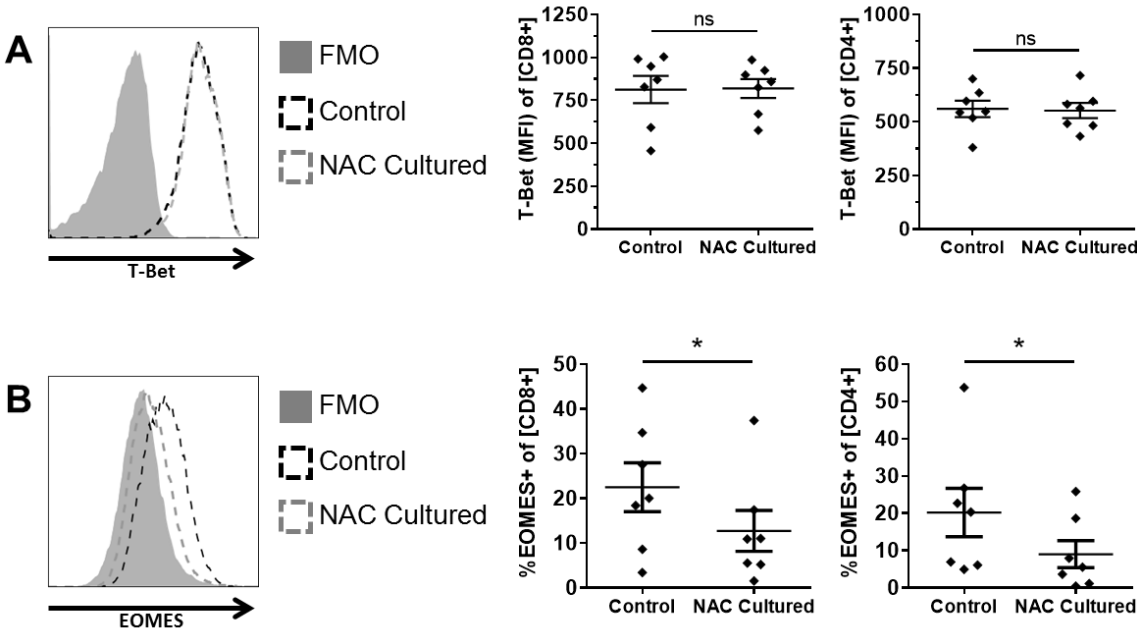

Figure 33. TIL1383I TCR transduced human T cells rapidly expanded in NAC display reduced expression of the transcription factor EOMES. TIL1383I TCR transduced human T cells were rapidly expanded for 10 days (± 2mM NAC) and were stained intracellularly for the expression of (A) T-bet or (B) EOMES. Left panels display representative histogram overlays of each indicated marker comparing NAC REPed and control cells to FMO control in CD8+ gated cells. Right panels display percent positive or MFI for each individual donor in CD8+ and CD4+ gated cells as indicated. Bars denote mean ± SEM (n=7, *p<0.05, ns=not significant).

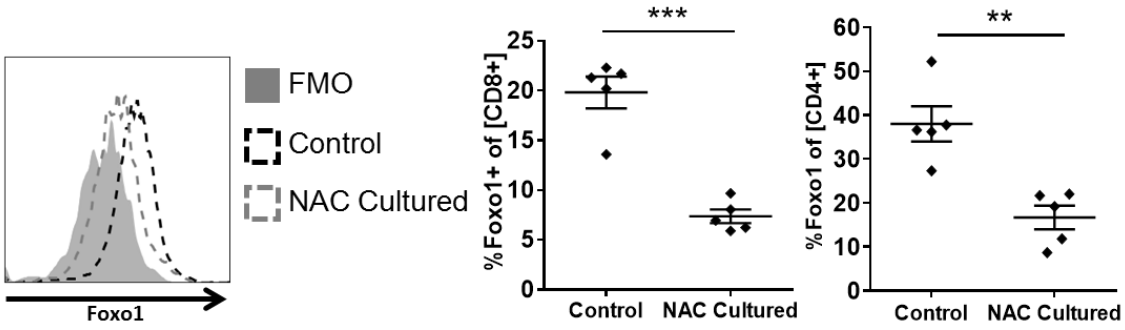

Figure 34. TIL1383I TCR transduced human T cells cultured in NAC have reduced expression of the Foxo1 transcription factor. TIL1383I TCR transduced human T cells were rapidly expanded for 10 days (± 2mM NAC) and were stained intracellularly for the expression of Foxo1. Left panels display representative histogram overlays of each indicated marker comparing NAC REPed and control cells to FMO control in CD8+ gated cells. Right panel displays percent positive Foxo1 for each individual donor in CD8+ and CD4+ gated cells as indicated. Bars denote mean ± SEM (n=5, **p<0.01, ***p<0.001).

for expression of p-Foxo1. Indeed, treatment of T cells with NAC induced the phosphorylation of Foxo1 with an approximately 20% increase in the MFI intensity of p-Foxo1 expression (Fig. 35a). Consistent with the role of phosphorylation in the degradation of Foxo1, there was also an observable rapid decline in the overall expression of total Foxo1 when cells were acutely treated with NAC (Fig. 35b).

We then investigated whether NAC had an effect of upstream factors established to be responsible for the phosphorylation of Foxo1. In particular, the activity of both mTOR and AKT have been implicated in the phosphorylation and subsequent repression of Foxo1 (648). Consistently, TIL1383I TCR transduced cells which have been REPed in NAC demonstrated an approximate 2.14-fold increase in the expression of phospho-S6 (Fig. 36a), a canonical marker of mTOR activity (677). Moreover, acute treatment of T cells with NAC enhanced the phosphorylation of AKT (Fig. 36b). Together, these results demonstrate that treatment of T cells with NAC represses the Foxo1 transcription factor via phosphorylation by upstream activation of mTOR and AKT pathways.

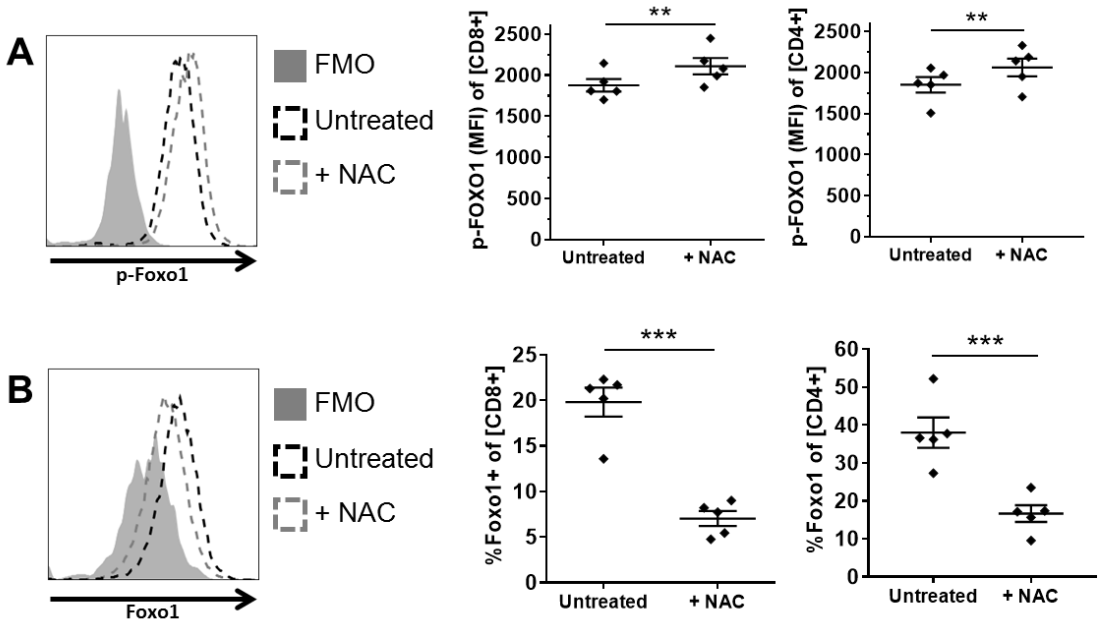

Figure 35. Acute treatment of T cells with NAC rapidly phosphorylates and attenuates the expression of Foxo1. Human TIL1383I T cells were acutely treated with NAC (25mM) for 60 min and then stained intracellularly for the expression of (A) p-Foxo1 and (B) total Foxo1. Left panels display representative histogram overlays of each indicated marker comparing NAC REPed and control cells to FMO control in CD8+ gated cells. Right panels display MFI or percent positive of the indicated marker for each individual donor in CD8+ and CD4+ gated cells as indicated. Bars denote mean ± SEM (n=5, **p<0.01, ***p<0.001).

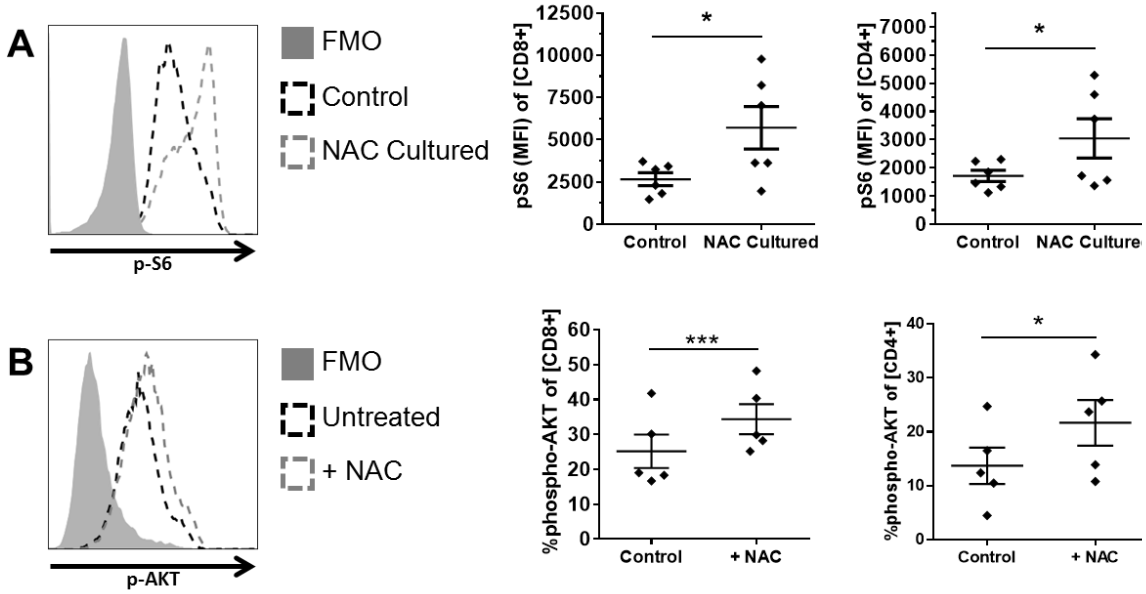

Figure 36. T cells cultured in NAC exhibit activation of mTOR and AKT pathways. (A) TIL1383I TCR transduced human T cells were rapidly expanded for 10 days (± 2mM NAC) and were intracellularly stained for the expression of pS6. (B) TIL1383I T cells were acutely treated with NAC (25mM) for 60min. Bars denote mean ± SEM (n=6, *p<0.05). Left panels display representative histogram overlays of each indicated marker comparing NAC treated and control cells to FMO control in CD8+ gated cells. Right panels display MFI or percent positive of the indicated marker for each individual donor in CD8+ or CD4+ gated cells as indicated. Bars denote mean ± SEM (n=5-6, *p<0.05, ***p<0.001).

CHAPTER 5 – DISCUSSION AND FUTURE DIRECTIONS

The adoptive cell transfer (ACT) of melanoma-specific TILs has generated curative responses in upwards of 40% of patients receiving this experimental therapy (219) which represents a significant improvement compared to the five-year survival rate of only 15.2% associated with standard-of-care regimens (189). Efficacy of treatment is independent of an individual patient's tumor or metastatic burden (155), but is highly dependent on the quality of the infused cell. Therapeutic efficacy of transferred cells is ultimately constrained by the durability of the cell to persist and to maintain anti-tumor functionality (166).

As a necessary mechanism of immune peripheral tolerance, activated T cells are programmed to undergo activation-induced cell death (AICD) upon repetitive stimulation of the T cell receptor (TCR). While indispensable for the prevention of autoimmune disorders, AICD can potentially be problematic in the clinical manipulation of T cells for ACT. As such, younger, less differentiated T cells, which are less susceptible to AICD, have consistently demonstrated superiority at *in vivo* tumor control (156,160,678). For instance, central memory cells, which have been well appreciated in the literature to be more efficacious in ACT based tumor control than effector memory cells (673), are less susceptible to AICD (653). Counterproductively, however, the rapid expansion protocol, necessary to generate a sufficient quantity of T cells for infusion, pushes the cells more towards a terminally differentiated effector phenotype (170), which ultimately makes them more susceptible to AICD (171). As such, novel manipulations of *ex vivo* culture are

needed to improve the phenotype of cells generated for ACT towards being more resistant to AICD.

AICD has previously been defined to be dependent on the accumulation of ROS and activation of JNK (366,370), and was, contemporaneously to this thesis project, reported to be inhibited by the p53 inhibitor pifithrin-μ (556). However, pifithrin-μ has been separately characterized as an inhibitor of the protein chaperone HSP70 inducing cell death by dysregulated protein aggregation (657). As such, data generated with pifithrin-μ cannot be directly attributed to p53. The use of the p53-knockout mouse in this thesis confirms that the presence of p53 is necessary for optimal AICD induced by TCR restimulation (Fig. 5). Furthermore, the relevance of p53 in AICD was demonstrated for both native and transduced TCRs with either p53 knockout mice that were crossbred with the TIL1383I TCR expressing h3T mouse, or with p53 knockout splenocytes that were transduced with the TIL1383I TCR (Fig. 6). However, the protection observed by p53 ablation was only partial suggesting that other pathways are concurrently activated during AICD, or potentially, some reports have suggested that p73, a paralogue of p53, can compensate for p53 in p53-null cell lines (679). Additional studies using RNA interference for the transient knockdown of p53 in wild type cells should be performed to distinguish protection from AICD from possible compensatory mechanisms in knockout cells.

Beyond demonstrating the requirement of p53 for the optimal onset of AICD, data from this thesis demonstrate that p53 is indeed activated upon TCR restimulation concurrent with the onset of cell death. TCR restimulation induces the phosphorylation

of p53 on Serine-15 (Fig. 7) which allows it to dissociate from the proteasomal degradation induced by its chief negative regulator HDM2 (572,573). Serine 15 is also located within a nuclear export signal motif on p53 which is deactivated upon Ser15 phosphorylation (578) facilitating the accumulation of p53 in the nucleus. Such nuclear accumulation, another hallmark of p53 activation (659), was also demonstrated by the co-localization of p53 with the Hoechst stained nucleus visualized on the Amnis ImageStream imaging flow cytometer (Fig. 8). Outside of the nucleus, p53 can function at the mitochondria to promote formation of the mitochondrial outer membrane permeabilization (569,570). However, there was no observable p53 co-localization with the mitochondria upon TCR restimulation (Fig. 8e), indicating that p53 likely augments AICD through its role as a transcription factor. As such, future investigations should interrogate what p53 pro cell death gene targets (i.e. BID, BAX, AIF) are upregulated upon TCR restimulation (575,680).

The role of p53 in AICD is consistent with the many reports demonstrating p53 to be an emerging factor in immune system regulation beyond simply maintaining genomic integrity. Watanabe *et al*. elegantly demonstrated that p53 enforces the requirement of TCR signaling for the proliferation of T cells as $p53^{KO}$ T cells were unrestrained in proliferation induced by IL-2 without antigen (582). p53 also constrains the development of autoimmune pathologies by promoting the differentiation of T regulatory cells (Tregs) (583,681). Additionally, the data of this thesis demonstrating an active role for p53 in AICD makes sense of the many reports where loss of p53 expression or function exaggerates autoimmunity and co-associates with many autoimmune conditions where

defective AICD is also problematic, including arthritis (584–588), multiple sclerosis, (589,590), and Crohn's disease (591,592).

Phosphorylation of p53 on Ser15 is indicative of a p53 response to DNA damage (665), and is moreover, required for subsequent cell death as an Alanine substitution on Ser15 thwarts cell death induced by genotoxic stress (579). Similarly, T cells from patients with ataxia telangiectasia, which have mutationally dysfunctional ATM, are also resistant to cell death induced by DNA damaging insults (594). Moreover, these cells are also resistant to phosphorylation of p53 on Ser15 (596). Consistently, data from this thesis demonstrate that ATM is also activated during AICD and, as demonstrated using the ATM inhibitors Caffeine and KU55933, activated p-ATM^{Ser1981} is also necessary for the phosphorylation of p53 on Ser15 (Fig. 9a). Additionally, cells were protected from AICD via ATM inhibition (Fig. 9b), demonstrating ATM to be a novel requisite factor in the onset of AICD.

Indeed, as activation of the p-ATM^{Ser1981}/p-p53^{Ser15} pathway, has been classically defined as a DNA damage response pathway (665), data from this thesis demonstrate the novel finding that TCR restimulation of activated cells likely induces damage to the DNA as evidenced by the upregulation in active DSB repair proteins: γH2AX and p-SMC1 (Fig. 10). This incursion of DNA damage happened very rapidly (≤15min) which is consistent with the kinetics of TCR restimulation induced oxidative stress accumulation (369). This accumulation of oxidative stress was demonstrated to be upstream of DNA damage as neutralization with the antioxidant N-acetyl cysteine (NAC) prevented an uptake in ROS expression as well as the onset of DNA damage during TCR restimulation (Fig. 14).

The data from this thesis, which demonstrate that TCR restimulation results in the upregulation of γH2AX and pSMC-1, indicative of DNA damage (599), is consistent with many reports which have indicated that DNA damage is problematic for proper immune cell function. Immune cells from individuals with advanced age present with an accumulation of DNA damage coupled with a diminished capacity in DNA double-strand break repair which correlates with a decline in immune function and increased senescence (682,683). Moreover, the accumulation of DNA damage and downregulation of DNA repair machinery can even be observed within the conserved time-frame of *in vitro* culture (684,685). Transferred T cells with eroded telomeres correlate with poor persistence and patient responses in ACT clinical trials (161). T cells with shorter telomeres, additionally, have higher baselines levels of γH2AX expression, a delayed DNA repair response, and are overall more susceptible to cell death (686,687). These reports suggest that cells with accumulated DNA damage are less fit to efficiently control tumor. Consistent with this premise, Sukumar *et al*. have recently demonstrated that T cells with less γH2AX expression are indeed superior at *in vivo* tumor control (688).

As the amount of DNA damage in lymphocytes has been shown to negatively correlate with the level of intracellular glutathione (689), we were successfully able to reduce the expression of the DNA damage marker γH2AX in Pmel-1 murine T cells when the glutathione pro-drug NAC was added to the culture media during expansion (Fig. 17). Affirming the role of DNA damage in the onset of AICD, these cells were also less susceptible to AICD when restimulated with cognate $gp100_{25\text{-}33}$ peptide (Fig. 17b). Importantly, resistance to DNA damage and AICD was durable as Vβ13+ cells recovered

from the spleens of mice receiving Pmel-1 cells expanded in NAC were more resistant to $gp100_{25\text{-}33}$ peptide induced γH2AX formation and AICD than recovered control cultured cells (Fig. 19). These previously NAC cultured cells, which continued to demonstrate reduced susceptibility to AICD, also exhibited increase persistence in the blood and spleens of recipient mice as well as a strikingly 33-fold improvement in recovered Vβ13+ from the tumors of B16-F10 challenged mice (Fig. 18). Consistent with the persistence of transferred cells being a key corollary of therapeutic success in the clinic (166), these more durable NAC cultured cells enhanced tumor control and survival of recipient mice (Fig. 20), which supports the central premise of this thesis that protecting T cells from AICD may bolster their therapeutic efficacy. Moreover, enhancement of tumor burden control and the survivability of recipient mice was observed in murine splenocytes transduced with the TRP-1 TCR that were cultured in NAC prior to transfer (Fig. 29), affirming that supplementation of culture media with NAC during lymphocyte expansion to be beneficial in both native and transduced TCR models.

Similarly, adding NAC to the culture during the rapid expansion of TIL1383I TCR transduced human T cells also attenuated susceptibility to DNA damage, alongside higher levels of surface thiols and reduced oxidative stress (Fig. 22). Even though there was not an adequate model to demonstrate an enhancement of *in vivo* tumor control by NAC (Fig. 26), these cells did consistently demonstrate themselves to be superior at *in vitro* anti-melanoma cytotoxicity than cells cultured without the addition of NAC (Fig. 23).

As the Pmel-1 model demonstrated that functional enhancements conferred by NAC culture were durable even after cells were removed from NAC and transferred *in*

vivo, we wanted to further investigate what phenotypic alternations may occur to therapeutic T cells cultured in NAC that would be indicative of better efficacy for ACT. Memory cells are more resistant to cell death induced by DNA damage than naïve cells (690) and central memory cells (T_{CM}) exhibit enhanced levels of surface thiols and resistance to TCR restimulation induced cell death compared to effector memory cells (T_{EM}) (653). Moreover, adoptive transfer of T_{CM} cells has demonstrated a superior ability to control tumor than T_{EM} cells (673). These studies prompted us to investigate whether the boosting of surface thiol levels and attenuation of DNA damage via NAC treatment would alter the percentages of T_{CM}/T_{EM} subsets. However, there was no alteration in CD62L and CCR7 expression amongst CD45RO+ cells (Fig. 30) which have been well established to define T_{CM} ($CD45RO^{+}CD62L^{HI}CCR7^{HI}$) and T_{EM} ($CD45RO^{+}CD62L^{LO}CCR7^{LO}$) memory subsets (691).

The effect of NAC on the expression of CD28 and 4-1BB co-stimulatory receptors was also examined. Addition of these signaling domains was one of the paramount improvements in CAR cell technology (105,106). CD28 co-stimulation alongside TCR restimulation can protect T cells from AICD (352), and moreover, CD28 expressing cells are more resistant to anti-CD3 induced AICD even without CD28 ligation (692). Similarly, ligation of 4-1BB also protects cells from AICD (171) and is being investigated in the clinic as a potential agonist therapy (693). However, TIL1383I TCR transduced human T cells cultured in NAC did not differ consistently in the expression of these markers compared to control cells; though, in our panel, we did observe an increase in the intensity of the costimulatory receptor ICOS (Fig. 31).

The exhaustion marker PD-1 is transiently upregulated during initial T cell activation, yet is sustained in chronically stimulated exhausted cells (609). Consistent with previous reports (170), TIL1383I TCR transduced human T cells rapidly expanded in our model system exhibited an increase in the exhaustion marker PD-1 as well as the senescence marker CD57 both of which associate with T cell dysfunction (674,675). However, the addition of NAC to the culture medium during rapid expansion significantly thwarted the expression of these markers (Fig. 32). Beyond receptor expression, NAC suppressed the development of an exhausted phenotype in these T cells at the transcriptional level by repressing the exhaustion associated transcription factor EOMES (Fig. 33). Attenuation in the development of T cell exhaustion or senescence by NAC is consistent with the reduction in DNA damage (γH2AX+ cells) in NAC cultured cells as γH2AX expression associates with cells exhibiting a senescent phenotype (694,695) and corresponds to a dysfunctional response to IFN-α, IL-2, and IL-6 cytokine stimulation in chronically stimulated T cells (696).

EOMES, which promotes the exhausted phenotype in T cells (697), has also been reported to be downstream of the transcription factor Foxo1 as repression of Foxo1 attenuates both EOMES and PD-1 expression (631). In agreement with this role of Foxo1 in the development of the exhausted phenotype, TIL1383I TCR transduced human T cells which were REPed in the presence of NAC additionally demonstrated reduced expression of Foxo1 in comparison to control cultured cells (Fig. 34).

The expression of Foxo1 is regulated primarily by phosphorylation mediated by activated Akt. Phosphorylation of Foxo1 results in cytoplasmic sequestration which

hampers its functionality as a transcription factor, and ultimately, in the cytosol, Foxo1 is ubiquitinated and then degraded in the proteasome (640,676). Consistently, when T cells were acutely treated with NAC there was an upregulation in the expression in both phosphorylated Foxo1 and phosphorylated (activated) Akt (Fig. 35-36). These data are consistent with other reports demonstrating the phosphorylation of Akt by NAC in other cell types such as neurons (698), cardiomyocytes (699), hepatocytes (700), and pancreatic islet cells (701).

Foxo1 can also be repressed by mTOR as demonstrated by a report from the Laboratory of Protul Shrikant which showed that T cells treated with the mTOR inhibitor rapamycin had enhanced Foxo1 activity as evidenced by an increase in total Foxo1 expression alongside a decrease in phosphorylated (inactive) Foxo1 (648). Consistently we observed the inverse, that TIL1383I TCR transduced human T cells expanded in NAC had an increase in the expression of phospho-S6 (pS6) (Fig. 36a) indicative of enhanced mTOR pathway activity (702), alongside a decrease in Foxo1 expression.

The restraint of Foxo1 activity by mTOR harmonizes many parallel reports in the literature which have demonstrated that either the repression of mTOR or promotion of Foxo1 achieves similar phenotypic outcomes in T cells (and vice-versa). For instance, Foxo1 activity is critical in the generation of Tregs (650,651) while, conversely, repression of mTOR also enhances Treg development (703,704). As CD62L and CCR7 are both direct target genes of Foxo1, activity of Foxo1 is critical for the development of T cell memory (647,649). On the other hand, repression of mTOR also promotes the formation of memory (705–707). Consistently, both the repression of mTOR and induction of Foxo1

enhance the expression of the memory-promoting EOMES transcription factor (648,649,706).

EOMES, also associating with T cell exhaustion, is likewise promoted by Foxo1 in chronically stimulated T cells to generate $EOMES^{HI}PD\text{-}1^{HI}$ exhausted T cells (631,697). Conversely, mTOR activity represses the development of T cell exhaustion. For instance, the Jeff Rathmell Laboratory has recently demonstrated that defective mTORC1 signaling, alongside metabolic deficiencies, drives T cell exhaustion induced by chronic stimulation from B cell leukemia (708). Conversely, Staron *et al*. has shown that rescuing T cells from exhaustion via αPD-L1 blocking antibody (610) coincides with an increase in mTOR activity as evidenced by an increase in pS6 expression (631). Moreover, inhibition of mTOR by rapamycin treatment counteracted the ability of αPD-L1 blockade to improve the functionality of exhausted T cells (631).

These reports compliment the finding in this thesis that T cells which are less exhausted via NAC treatment exhibit an enhancement of mTOR activity. However, this finding must be balanced in interpretation by reports which have demonstrated that repression of mTOR activity produces a more potent anti-tumor T cell. Rapamycin treatment has been shown to enhance memory development in T cells (705,706,709). Moreover, treatment of T cells with rapamycin prior to adoptive transfer enhances the tumor burden control of the T cells and the conferred survivability of treated mice (706). However, as rapamycin was originally characterized as a immunosuppressant (710), the appropriate dosage or schedule for mTOR inhibition can be critical for optimal anti-cancer benefit. Indeed, prolonging the course of rapamycin treatment *in vivo* can nullify the

memory generating benefit of a shorter course dosage schedule (711), inspiring Protul Shrikant to refer to rapamycin as a "rheostat" for T cell immunotherapy (712). Similarly, complete ablation of Foxo1 in therapeutic T cells would likely be detrimental to anti-tumor efficacy as Foxo1-deficient T cells present with defective homeostasis of naïve cells via impaired IL-7Rα expression and would likely have deficient development of long-term memory recall responses (646). Therefore, the exhaustion-limiting benefit of Foxo1 attenuation most be balanced against these more severe consequences from the complete knockout of Foxo1. As such, similar to mTOR, Susan Kaech (the senior author of Staron, *et al.*) said that the challenge in quelling T cell exhaustion via Foxo1 repression is to find the appropriate "sweet point" for optimal benefit (713). Treatment with NAC is likely a more moderate approach to Foxo1 modulation in comparison with previous reports which have relied on complete ablation. Moreover, NAC may have pleotropic effects that may positively counteract the negative consequences of Foxo1 attenuation. Further studies are needed to determine what effects of NAC are mediated by Foxo1 through the use of NAC treated Foxo1-null cells and/or co-treatment with the Foxo1 inhibitor AS1842856 (714).

There are several mechanisms whereby NAC may potentially modulate Foxo1 and mTOR expression. NAC could potentially aid Akt in Foxo1 repression by ensuring that the regulatory cysteines of Akt (Cys^{296}/Cys^{310}) are kept in their reduced form, which is critical for Akt activation (422). Furthermore, NAC has been shown to increase phospho-ERK levels during TCR signaling (715). Concurrently, phosphorylation of ERK has been shown to inversely correlate with the levels of sulfenic acid detected on ERK (716), and ERK

phosphorylation is blocked when cysteine modifications are prevented with dimedone treatment (430). Activated Erk has been shown to inactivate tuberous sclerosis 2 (TSC2) via phosphorylation (717). As TSC2 is a negative regulator of mTOR (718), potential ERK activation by NAC may facilitate an upregulation in mTOR. Follow-up studies should determine if NAC modulates the cysteine residues of AKT and if it is involved in ERK activation.

Beyond an attenuation of the exhausted phenotype, repression of Foxo1 could also provide an explanation for the enhanced levels of granzyme B observed in this thesis (Figs. 19, 25) as Foxo1 (and consistently rapamycin) has been demonstrated to repress granzyme B expression (648,706). Additionally, while we did not observe any modulation of CD28 or 4-1BB co-stimulatory receptors, from expanding T cells in NAC, there was an increase in ICOS receptor expression, of which ligation has been shown to repress Foxo1 (644).

Both CCR7 and CD62L are direct target genes of Foxo1, however there was no observable difference in the expression of these markers between NAC and control cultured cells. One potential explanation for this inconsistency may be that IL-15, which was also present in the cultures of rapidly expanding TIL1383I TCR transduced human T cells, may have bolstered the memory phenotype of the cells independent of modulation by Foxo1, as IL-15 is critical for the development and maintenance of long lived memory T cells (719,720). As such, the expression of these markers and other data in this thesis may have been different or more pronounced if the cells were cultured in IL-2 alone. Both IL-2 and IL-15 are common γ-chain receptor (CD132) cytokines who both additionally

share their respective β-chain receptor (CD122) subunits as well (721), differing only in their "high affinity" receptor subunits IL-2Rα (CD25) and IL-15Rα (CD215) (722). As such, they both similarly signal through JAK1/3 and STAT3/5 proximal signaling pathways (723). Both cytokines, being originally described as "growth factors," do induce the proliferation of T cells (222,724). However, despite their shared receptor and proximal signaling units, IL-2 and IL-15 have divergent effects on cultured T cells. IL-2 promotes the differentiation of effector T cells while also limiting persistence by priming cells to undergo AICD (343,344). The contribution of IL-2 in limiting T cell persistence is highlighted by the attenuation of proliferative control in IL-2 deficient mice (725,726).

In contrast, IL-15 protects T cells from AICD, as T cells from IL-15 overexpressing transgenic mice are resistant to AICD after being primed in IL-2 (357). The enhancement of durability of T cells by IL-15 is also important for its role in maintaining memory cells as IL-15 deficient mice have suboptimal levels of memory cells (727). Conversely, IL-2 signaling impedes IL-15 driven memory formation (728) suggesting a diametric influence over memory development between the two cytokines. Consistently, murine T cells cultured in IL-15 prior to adoptive transfer preferentially migrate to the lymph nodes of recipient mice while T cells cultured in IL-2 traffic to sites of induced inflammation (729). Studies in the Pmel-1 model, demonstrate that IL-15 cultured Pmel-1 cells have a greater expression of the central memory CD62L and CCR7 lymph-node homing receptors than cells cultured in parallel in IL-2 (730). Furthermore, IL-15 cultured Pmel-1 cells, while having reduced *in vitro* cytolytic functionality, were superior at tumor burden control and also enhanced the survivability of B16 melanoma challenged mice. As such, the addition

of IL-15 to the culture medium of TIL1383I transduced TCR transduced T cells may sustain the expression of CD62L and CCR7 central memory markers even in parallel with a decrease in Foxo1 expression, which may, in part explain while there was no alteration in these markers in NAC cultured cells. Studies which have shown expression of these markers to be influenced by Foxo1 have relied on the complete ablation of Foxo1 (645,649) which does not elucidate the consequence of moderating Foxo1 expression. Moreover, these studies make no attempt to rescue the central memory phenotype with IL-15 supplementation. As IL-15 and Foxo1 have contrasting effects on IL-17Rα expression (731), it is possible they may regulate CCR7 and CD62L expression through independent mechanisms.

In summation, the data presented in this thesis demonstrate that oxidative stress, previously defined to be a requisite factor for AICD induced by the TCR restimulation on activated T cells, augments into the onset of DNA damage as evidenced by an increase in two DNA damage response markers (H2AX, SMC-1) and subsequent activation of ATM and p53. These findings suggest an additional layer of regulation which likely escalates the commitment of an individual T cell to death upon TCR restimulation. Importantly, protection from DNA damage via culturing cells with NAC, associates with the reduced susceptibility to AICD and improved killing of melanoma cells both *in vitro* and *in vivo*. Additionally, the *in vivo* persistence of Pmel-1 cells was improved by NAC culture prior to adoptive transfer.

Adding NAC to the culture of TIL1383I TCR human transduced T cells benefited the cells in several ways by a reduction in DNA damage/AICD and T cell exhaustion with a

concurrent increase in the cytolytic capacity of the T cells (Fig. 37). TIL1383I TCR transduced cells currently being used in clinical trials were also susceptible to restimulation induced DNA damage, which was thwarted by both acute and long term culturing with NAC. Additionally, the addition of NAC to the culture medium during rapid expansion quelled upregulation of the exhaustion marker PD-1 which was found to be regulated at the transcriptional level via repression of EOMES and Foxo1. The effect of NAC on T cells bolsters their effector functionality as evidenced by enhanced control of tumors as well as the upregulation of granzyme B, likely through counteracting repression by Foxo1. However, increase in effector functionality does not seem to cause any decrease in the durability of the cells as evidenced by an increase *in vivo* persistence. Together this suggests that the effect of NAC is likely pleiotropic as NAC may enhance effector functionality, in part through the repression of Foxo1, but also enhances the persistence of the cell by other mechanisms which merit future investigations.

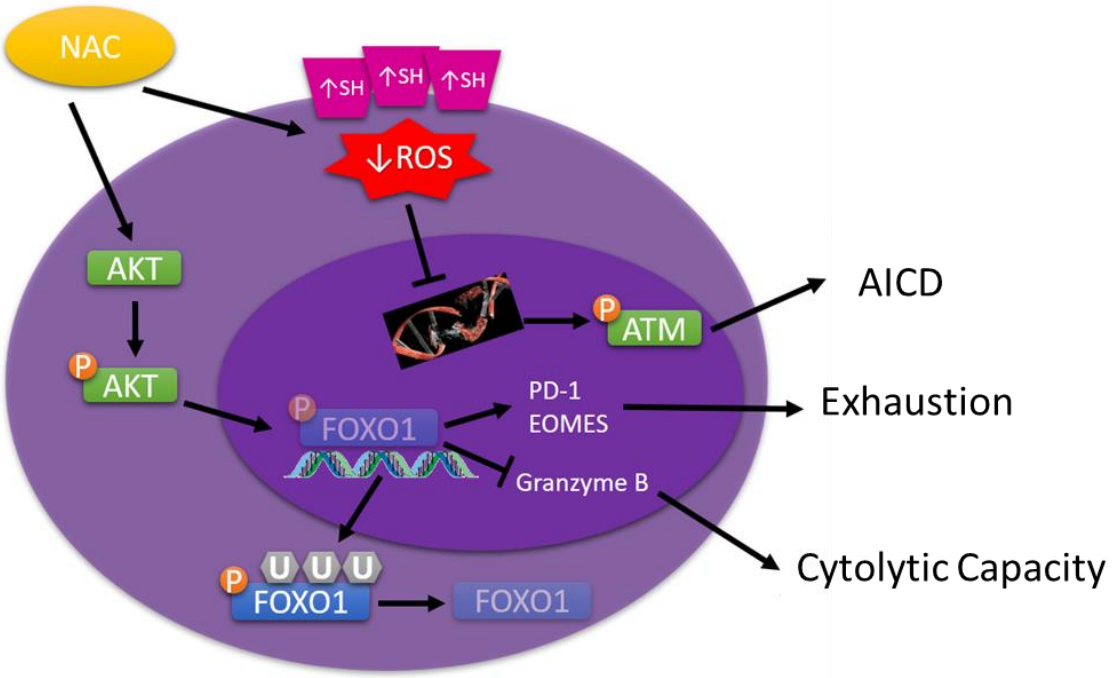

Figure 37. Addition of NAC to culture improves the phenotype of rapidly expanded TIL1383I TCR transduced human T cells via three distinct mechanisms. 1) NAC increases the antioxidant capacity of T cells as evidenced by an increase in the expression of surface thiols and a decrease in the detection of the oxidative stress sensitive dye DCFDA (Fig. 23). As DNA damage caused by TCR restimulation is dependent on ROS (Fig. 15), TIL1383I TCR transduced human T cells REPed in NAC have less γH2AX expression when restimulated with MEL624 cells (Fig. 23), and in parallel, are less susceptible to AICD (Fig. 24). 2) T cells acutely treated with NAC exhibit increase phosphorylation of AKT and Foxo1 (Fig. 36). As phosphorylation of Foxo1 results in its nuclear sequestration and ultimately, its proteasomal degradation (640,676), TIL1383I transduced human T cells REPed in NAC demonstrated a reduction in Foxo1 levels (Fig. 35). The rapid expansion of T cells results in an increase in the exhausted T cell phenotype [Fig. 33 and ref (170)]. As Foxo1 is upstream of EOMES and PD-1 in the promotion of the $EOMES^{HI}PD\text{-}1^{HI}$ exhausted T cells (631,697), these cells also exhibited a reduction in both EOMES and PD-1 (Figs 33,34), demonstrating that NAC restrains development of T cell exhaustion during rapid expansion. 3) As Foxo1 also restrains the expression of the cytotoxic molecule granzyme B (648), TIL1383I TCR transduced human T cells expanded in NAC also exhibited an increase In granzyme B (Fig. 26) which coincided with an increase in the *in vitro* anti-MEL624 cytotoxic functionality (Fig. 25).

CHAPTER 6 – MATERIALS AND METHODS

Cells, activation, and culture

All cells were maintained in a humidified incubator (Forma Scientific) at 37°C supplemented with 5% CO_2. Melanoma cell lines (human MEL624 and MEL624-28 and murine B16 cells) were obtained in 2013 from Drs. Michael Nishimura (Loyola University, Maywood IL) and Mark Rubinstein (Medical University of South Carolina, Charleston SC). All cells were periodically verified to be free of mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza) per vender protocol. Additionally, B16 cells were authenticated and confirmed to be free of rodent pathogens by Dr. Rubinstein.

Normal healthy donor apheresis cells were purchased from Key Biologics, Inc. or Research Blood Components. Cells from melanoma patients were obtained with consent as part of an IRB and FDA approved clinical trial (NCT01586403). PBMCs were isolated by Ficoll density gradient, activated with plate-bound anti-CD3 (5µg/mL, BioLegend #302902) and anti-CD28 (2µg/mL, BioLegend #317304) for 3 days, washed, and cultured in Iscove's Modified Dulbecco's Medium (Mediatech) supplemented with 10% FBS, 100 IU/mL rIL-2 (Peprotech), and 10ng/mL rIL-15 (Shenandoah), for at least 5 days prior to TCR restimulation.

For studies using h3T or Pmel transgenic mice obtained from Dr. Shikhar Mehrotra (Medical University of South Carolina, Charleston SC), splenocytes were prepared into single-cell suspensions from harvested spleens by mechanical isolation and removal of red blood cells by incubation with ACK buffer (Gibco) for 1-2 min. Cells are then washed

through a 70µm cell strainer (Fisher) and cultured in complete media supplemented with 100U/mL recombinant IL-2. Splenocytes from h3T mice were activated with plate-bound anti-CD3 (1µg/mL, BioLegend #100302) and anti-CD28 (2µg/mL, BioLegend #102102) for 2 days prior to use in assays. Pmel splenocytes were activated with 1µg/mL of $gp100_{25\text{-}33}$ peptide (AnaSpec) with or without supplementation with 10mM N-acetyl cysteine (NAC, Hospira).

Transduction and rapid expansion of TIL1383I transduced T cells

Transductions of TIL1383I Transduced T cells were performed at the Cell Therapy Core at Loyola University, Chicago. Isolated PBMCs were activated with 50ng/mL soluble anti-CD3 (OKT3) for 2-3 days. On the day of transduction, retroviral supernatants were collected from PG13 1383I A9 retrovirus producing cells and filtered through a 0.45µm cellulose acetate syringe filter. Filtered virus was then spun onto retronectin coated plates (30µg/mL) via centrifugation (2000G, 2hrs, 37°C, with no brake). Activated cells were then added to wells and centrifuged once more (2000G, 2hrs, 37°C, with no brake). Transduced cells were cultured for several days in AIM-V culture media (supplemented with 5% human AB serum, 300IU/mL recombinant human IL-,2 and 100ng/mL recombinant human IL-15) and then purified based on CD34 expression (CliniMACs) using CD34+ reagent. Subsequently, cells undergo a REP whereby $1x10^6$ transduced cells are co-cultured with $2x10^8$ irradiated feeder cells supplemented with 30ng/mL anti-CD3. Cells are left undisturbed for approximately 5 days where media and cytokines are

replenished and then continued in culture for an additional 5 days for use in assays or cryopreservation.

Generation of h3T/p53^{KO} mice

The generation of the *Trp53* knockout mouse has been described in (658) and is commercially available from the Jackson Laboratory (Cat# 002101). *Trp53* knockout mice were kindly gifted to us from the laboratory of Dr. Lisa Cunningham (formerly of the Dept of Pathology, MUSC). The development of the h3T mouse has been described in (660) and is maintained in the laboratory of our collaborator Dr. Shikhar Mehrotra. Selectively breeding of H3T+ mice and setting up mating pairs of h3T+/p53^{HET} and h3T+/p53^{KO} produced the most even ratio of h3T+/p53^{WT} and h3T+/p53^{KO} offspring. Genotypes were confirmed via PCR of digested tail clips using primers directed against h3T-α F: 5'-TCTCCCGGGCTTCTCACTGCCTAGCC-3' R: 5'-GTTAAGGGTATAGGATGTTAAGC-3'; h3T-β F: 5'-ATGGGCACAAGGTTGTTCTTCTATGTGGCCCTTTGTCTCC-3' R: 5'-GCACTAGACCGCGGGGCTCCGTCTGGATTCCAGCCC-3' and against p53 WildType: 5'-TATACTCAGAGCCGGCCT-3' AND p53 Knockout (neo): 5'-TCCTCGTCGTTTACGGTATC-3' (Fig. 38).

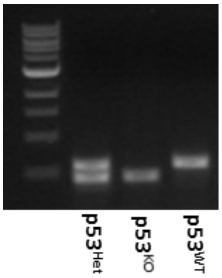

Figure 38. Genotype screening of p53 knockout mice. PCR was performed on tail clip digestions. PCR product was then evaluated by gel electrophoresis (2% low melting point agarose). Band indicating $p53^{WT}$ occurs at 430bp and $p53^{KO}$ occurs at 590bp with both bands occurring for heterozygotes.

Flow cytometry

Cell surface staining

For the staining of surface markers, cells were removed from assay and washed twice by pelleting by centrifugation (1,500rpm, 5min, 4°C) and resuspended in FACS Buffer (PBS w/ 5% FBS, 2mM EDTA, 0.05% NaN_3, pH 7.4). Cells were then stained with fluorochromes per vendor recommended concentration (diluted in FACS Buffer) for 30 min at 4°C protected from light. Subsequently, cells were then washed twice again in FACS Buffer where they were either acquired or processed further in other staining protocols.

Annexin staining

Cells were stained as in "Cell Surface Staining" with the exception that after antibody staining, cells were washed in Annexin V Binding Buffer (10mM HEPES, 140mM NaCl, 2.5mM CaCl2, pH 7.4). Fluorochrome conjugated Annexin V was then added directly to flow staining tube 15min prior to acquisition.

Intracellular staining

For the staining of intracellular proteins, cells were initially stained as described in "Cell Surface Staining." For the staining of secreted factors such as IFNγ, Protein Transport Inhibitor Cocktail (500X, eBioscience #00-4980) was added to assay medium at least 4 hrs prior to termination of experiment. Following surface staining, cells were processed using the Transcription Factor Staining Buffer Set (eBioscience #00-5523). Cells

were resuspended in Fixation/Permeabilization reagent and incubated for 20min. Following incubation, cells were washed twice in Permeabilization Buffer reagent. After washing, cells were resuspended in Permeabilization Buffer and incubated for 15 min followed by adding fluorochromes (diluted in Permeabilization Buffer) directly to flow staining wells/tubes and incubated for 1-2 hrs. Following incubation, cells were then washed in Permeabilization buffer and then FACS Buffer and then acquired.

Intracellular staining of phospho-specific proteins

For the staining of intracellular phospho-specific proteins, surface stain fluorochromes were added directly to assay media 20-30min prior to the conclusion of the assay. At the conclusion of the assay cells were fixed in 2% pre-warmed (37°C) paraformaldehyde for 20 minutes. Cells were then washed twice in FACS Buffer and then resuspended in 90% ice-cold methanol for 30min where they were then subsequently washed and incubated with phospho-specific fluorochromes (diluted in FACS Buffer) for 1-2hrs. Cells were then washed in FACS Buffer and acquired.

Acquisition and analysis

Cells were acquired on either the BD FACS Calibur, BD LSRFortessa, or the BD Fortessa X-20 cell analyzers in the Flow Cytometry Core of the Hollings Cancer Center. Analysis was performed using FlowJo Software (Tree Star, Inc.). Cells were first gated based on Forward-Scatter and Side-Scatter parameters to exclude debris. For human PBMC experiments, cells were then gated on either CD8+ or CD4+ cells. TIL1383I

transduced human cells were additionally gated on CD34+ cells. Murine Pmel cells were gated on Vβ13+CD8+ cells. TRP-1 transduced cells and h3T cells were gated on Vβ14+ or Vβ12+, respectively, prior to subsequent gating on CD8+ and CD4+ populations.

Amnis ImageStream

For Amnis ImageStream experiments, cells were stained with 250nM MitoTracker-DeepRed (Cell Signal) 30min prior to the conclusion of the assay where they were then fixed and stained as per "Intracellular Staining of Phospho-Specific Proteins." Cells were additionally stained with 250ng/mL Hoechst (Acros Organics) prior to antibody staining. Samples were acquired on the Amnis ImageStream at the Flow Cytometry Core Facility at Loyola University, Chicago. At least 10,000 events were acquired. Data were analyzed using the IDEAS analysis software (Amnis Corporation) using the co-localization module. Events with a similarity score of ≥1 were defined as positive for co-localization.

Recognition assay

TIL1383I expressing h3T splenocytes ($1x10^5$) were co-cultured with $1x10^5$ T2 cells pulsed with either 1μg/mL $hTyr_{368-376}$ cognate or Mart-1 irrelevant peptide for 16hrs. At the conclusion of the assay, cells were pelleted by centrifugation (1500rpm, 5min) and supernatants were harvested. Supernatants were then assayed to determine the amount of mouse-IFNγ using the DuoSet ELISA Kit (R&D Systems) according to vendor protocol and optical density was measured using the FLUOstart OPTIMA plate reader (BMG Labtech) under the absorbance configuration.

AICD assay

Cells were previously activated as denoted in "cell culture." TIL1383I expressing cells were plated at $2x10^5$ and co-cultured with $1x10^5$ T2 cells pulsed with 1µg/mL $hTyr_{368-376}$ cognate or Mart-1 irrelevant peptide or with $HLA\text{-}A2^+$ melanoma cell lines MEL624 or B16-A2 compared to $HLA\text{-}A2^-$ MEL624-28 or B16 cells as controls. Pmel cells were co-cultured with irradiated splenocytes pulsed with 1µg/mL $gp100_{25-33}$ peptide. TRP-1 transduced cells were co-cultured with irradiated splenocytes pulsed with 4µg/mL TRP-1 peptide. PBMCs were restimulated with 5µg/mL plate-bound anti-CD3. Cells were restimulated for 4hrs or as indicated in figure legends. Cell death was assessed by Annexin V staining via flow cytometry.

In vitro cytotoxicity assay

MEL624 and MEL624-28 cells were labeled with 0.1µM and 0.01µM CFSE (BioLegend) respectively according to vendor protocol. Each labeled cell type melanoma cell type was co-cultured ($2x10^4$ each) with TIL1383I TCR transduced T cells at various Effector:Target ratios achieved through serial dilution of the T cells. Cells were incubated overnight and then stained with Annexin V & 7AAD and acquired via flow cytometry. MEL624 and MEL624-28 cells were gated as either $CFSE^{HI}$ or $CFSE^{LO}$ cells and TIL1383I transduced cells were gated on the CFSE-negative CD34+ population. Cell death was analyzed as percent Annexin V/7AAD double-positive for each respective population.

Adoptive cell transfer

All animal experiments were performed with approval by the Institutional Animal Care and Use Committee at the Medical University of South Carolina. Eight to twelve week old female C57BL/6 wild type mice (NCI) were subcutaneously injected with $3x10^5$ B16F10 murine melanoma cells. Mice were randomized into treatment groups to ensure equal distribution in tumor size at initiation of the experiment. One day prior to adoptive cell transfer, mice were lymphodepleted through total body irradiation (5Gy, nonmyeloablative). Pmel cells ($2x10^6$) or TRP-1 transduced cells cultured in the absence or presence of 10mM NAC were adoptively transferred via retro-orbital injection. Twice weekly, tumors were measured using calipers and tumor area (mm^2) calculated by multiplying the length and width of two perpendicular measurements. Mice were sacrificed when tumor burden reach $400mm^2$ or when animals exhibited signs of distress.

Biodistribution analysis

Weekly blood samples were collected from the tail vein of mice into a microcentrifuge tube containing PBS w/ 2mM EDTA to prevent blood clotting. Blood samples were incubated with ACK buffer for 10 min to lyse Red Blood Cells. Cells were subsequently washed with PBS and then surface stained for flow cytometric analysis. A subset of mice where sacrificed on Day 6 post transfer and spleens and tumors were harvested. Spleens and tumors were processed into single cell suspensions by mechanical dissociation. Tumors were further digested in 5-10mL of Collagenase II (1mg/mL) incubated for 1 hr at 37°C with agitation of 250rpm. After incubation, digests were

washed and resuspended in 3mL of PBS and then gently layered onto 3mL of Histopaque 1083 (Sigma) in 15mL conical tube. Tubes were then centrifuged at 400G for 30min (with Brake set to '1'). Interface layer was then harvested, washed and processed for flow cytometric analysis.

Statistical methods

For experiments comparing stimulated and unstimulated or NAC treated and untreated samples from the same donor a two-tailed, paired student t-test was used to determine significance. Murine studies utilized an unpaired student t-test between treatment groups. For kinetic studies of expression of markers over a timecourse, a linear longitudinal regression was calculated utilizing generalized estimating equations with Wald tests used to calculate p-values between model coefficients at different observed time points. For murine in vivo studies, kinetics of tumor burden was modeled using longitudinal linear regression with p-values calculated using a likelihood ratio test based on a chi-square with 2 degrees of freedom. A log rank test was used to calculate p-value for differences in survival time. Statistical significance was determined as a p-value < 0.05.

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