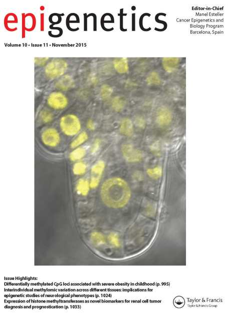

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Impaired T cell proliferation by *ex vivo* BET-inhibition impedes adoptive immunotherapy in a murine melanoma model

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Abstract

Activation of naïve $CD8^+$ T cells stimulates proliferation and differentiation into cytotoxic T-lymphocytes (CTLs). Adoptive T Cell Therapy (ACT) involves multiple rounds of *ex vivo* activation to generate enough CTLs for reinfusion into patients, but this drives differentiation into terminal effector T cells. Less differentiated CTL populations, such as stem cell memory T cells, are more ideal candidates for ACT because of increased self-renewal and persistent properties. *Ex vivo* targeting of T cell differentiation with epigenetic modifiers is a potential strategy to improve cytotoxic T-lymphocyte (CTL) generation for ACT. We established a pipeline to assess the effects of epigenetic modifiers on $CD8^+$ T cell proliferation, differentiation and efficacy in a preclinical melanoma model. Single treatment with epigenetic modifiers inhibited T cell proliferation *in vitro*, producing $CD44^{hi}CD62L^{hi}$ effector-like T cells rather than a stem cell memory T cell phenotype. Most epigenetic modifying agents had no significant effect on ACT efficacy with the notable exception of the bromodomain and extraterminal (BET)-inhibitor JQ1 which was associated with a decrease in efficacy compared to unmodified T cells. These findings reveal the complexity of epigenetic targeting of T cell differentiation, highlighting the need to precisely define the epigenetic targeting strategies to improve CTL generation for ACT.

Keywords: Cancer immunotherapy, Adoptive cell therapy, T cell differentiation, BET inhibition

Introduction

Adoptive cell therapy (ACT) involves the *ex vivo* expansion and reinfusion of tumour-specific $CD8^+$ T cells as potential treatment for different malignancies [1, 2]. ACT can induce durable responses in a subset of patients with melanoma [3, 4], but overall complete response rates remain relatively low. The persistence and survival of transferred $CD8^+$ T cells highly correlates with therapy success [5], and appears to be intrinsically associated with the T cell differentiation status. ACT with less differentiated memory $CD8^+$ T cell subsets results in superior persistence and anti-tumour immunity as compared to more differentiated effector T cell subsets [6]. However, to achieve the large numbers required for ACT, T cells undergo multiple rounds of expansion, which inevitably promotes T cell differentiation, resulting in a loss of proliferative potential, survival and multipotency [7].

It has been proposed previously that skewing the expansion of specific subsets of $CD8^+$ T cells during expansion may enhance ACT efficacy. For example, Gattinoni and colleagues have successfully altered $CD8^+$ T cell differentiation in mice using a Wnt-signaling inhibitor TWS119, resulting in T cells persisting in a less differentiated subgroup [8]. These less differentiated $CD8^+$ T cells, defined as stem cell memory T cells (T_{scm}), have increased self-renewal and multipotency capabilities. Importantly, $CD8^+$ T_{scm} cells were qualitatively superior compared to central memory (T_{cm}) and effector memory (T_{em}) $CD8^+$ T cells when used in ACT [8, 9].

As an alternative approach to manipulating T-cell phenotype by cell-signaling inhibition, the epigenetic features of T-cells could also be targeted during *ex vivo* activation and expansion. Epigenetic modifications (marks) such as DNA methylation

and histone modifications at gene-regulatory regions, are associated with the expression of key transcription factors controlling T cell differentiation and proliferation [10]. Importantly, different epigenetic marks can be manipulated by drugs that inhibit enzymes which covalently modify histones or DNA and are potential therapeutic targets to improve ACT. The effects of epigenetic modifiers on tumour cells has been extensively studied. Histone deacetylase inhibitors (HDACis), such as vorinostat and panobinostat, are approved for the treatment of cutaneous T-cell lymphoma and multiple myeloma, respectively [11, 12]. HDACis can enhance tumour immunity by upregulating MHC-I on tumour cells and are potentially synergistic with ACT [13]. Histone methyltransferase inhibitors (such as DOT1L, GSK343, and GSKJ4) inhibit proliferation and growth of leukemia, and glioblastoma cell lines [12, 14]. Bromodomain and extraterminal (BET)-domain readers of acetyl marks in histone tails can be also be targeted (for example by BET-inhibitors, JQ1, IBET and GSK2801), and has recently been demonstrated to improve the efficacy of ACT [15]. Whilst the effects of targeting different epigenetic marks on cancer cell proliferation has been reported [16], the effects of direct epigenetic modification on $CD8^+$ T cell proliferation and differentiation are not completely understood. $CD8^+$ T cell differentiation is tightly regulated by epigenetic events [10, 17, 18], and epigenetic targeting to reprogram T cells to overcome terminally differentiated cells used for ACT is a possible way of improving therapy.

Here we have further investigated the hypothesis that *ex vivo* treatment of activated $CD8^+$ T cells may reprogram T cells into a T_{scm} phenotype and improve ACT. The effects of single epigenetic modifiers, or the WNT-signaling inhibitor TWS119 on $CD8^+$ T cell activation was investigated in a pre-clinical murine model of ACT. We

found that most epigenetic modifiers promoted $CD44^{hi}CD62L^{hi}$ surface phenotype, but not into a T_{scm} $CD44^{lo}CD62L^{hi}$ phenotype. Surprisingly, cells treated with JQ1, the prototype BET-inhibitor, at high doses caused impaired proliferation and reduced treatment efficacy. We also report that we were unable to replicate changes to the T_{scm} phenotype by treatment with TWS119 during *ex vivo* T-cell expansion in our pre-clinical models.

Results

Epigenetic modifiers inhibited $CD8^+$ T cell proliferation, but did not alter expression of surface differentiation markers.

We utilised an *in vitro* $CD8^+$ T cell activation assay to assess the effects of epigenetic modifiers on T cell proliferation and differentiation. gBT-I are well characterised $CD8^+$ T cell receptor (TCR) transgenic mice specific for a H-$2K^b$-restricted Herpes Simplex Virus (HSV) glycoprotein B (gB) epitope, $gB_{498\text{-}505}$ [19]. We stimulated CFSE labeled gBT-I splenocytes with $gB_{498\text{-}505}$ peptide pulsed C57BL/6 splenocytes at a 1:1 ratio for 72 hours. In the absence of epigenetic modifiers, >95% of gBT-I $CD8^+$ T cells proliferated in response to cognate antigen (Figure 1A). *In vitro* activation of $CD8^+$ T cells leads to division dependent cell differentiation, and changes in expression of activation markers such as CD44 and CD62L [20]. Inactivated, naïve gBT-I $CD8^+$ T cells are $CD44^{lo}CD62L^{hi}$ [21], and differentiate into $CD44^{hi}CD62L^{hi}$ cells upon activation. We postulated that addition of epigenetic modifiers would inhibit T cell proliferation and alter expression of CD44 and CD62L.

We first screened the effects of 8 individual epigenetic modifiers on T cell proliferation. TWS119, a known inhibitor of T cell proliferation, was also tested [8].

The concentration of each drug that would reduce proliferation (determined by % CFSE dilution) to approximately 50%, but not affect cell viability was determined. The optimum range for TWS119 was between 3μM and 6μM (Figure 1B). With the exception of Panobinostat and GSK2801, all epigenetic modifiers inhibited T cell proliferation. Panobinostat and GSK2801 were highly toxic to T cells and were not tested further. The screen was repeated in 384 and 6 well plates, and the final optimal concentrations for each epigenetic modifier were determined (Table 2). Although there were varying standard deviations, measurements of T cell proliferation were a clear indicator of cellular changes and an effective way to ensure cells were still undergoing differentiation in the presence of epigenetic modifiers.

T cell differentiation was assessed by the expression of CD44 and CD62L [20]. TWS119 inhibited T cell differentiation, as 67.17 ± 0.85% of $CD8^+$ T cells were $CD44^{lo}CD62L^{hi}$ 72 hours post activation, consistent with previous published data [8] (Figure 2A). With the exception of vorinostat, activated gBT-I $CD8^+$ T cells treated with all epigenetic modifiers differentiated into $CD44^{hi}CD62L^{hi}$ T cells, similar to untreated cells. Activated gBT-I $CD8^+$ T cells exposed to vorinostat expressed a unique $CD44^{lo}CD62L^{lo}$ phenotype (Figure 2A, B). In summary, we did not observe differentiation into a different surface phenotype with most of our epigenetic modifiers, with the exception of vorinostat.

Inhibition of WNT/β-catenin signaling pathway did not form T stem cell memory in gBT-I TCR transgenic cells

It has been demonstrated previously that activation of $CD8^+$ T cells in the presence of TWS119 reduces proliferation and differentiates T cells towards a T_{scm} phenotype [8].

We sought to replicate this in our model, and similarly found that 3.5μM TWS119 inhibited gBT-I $CD8^+$ T cell proliferation (66.4% compared to 97.3% in untreated cells) (Figure 3A).

T_{scm} cells are proliferating ($CFSE^{lo}$) cells that uniquely retain the surface phenotype similar to a naïve T cell ($CD44^{lo}CD62L^{hi}$). However, we found that majority of $CFSE^{lo}$ proliferating gBT-I $CD8^+$ T cells had a $CD44^{hi}CD62L^{lo}$ or $CD44^{hi}CD62L^{hi}$ surface phenotype (Figure 3B). Less than 10% of proliferating cells displayed the reported T_{scm} ($CD44^{lo}CD62L^{hi}$) phenotype (Figure 3B), compared to 30% of proliferating cells as reported in the pMEL transgenic model [8]. The majority of the $CD44^{lo}CD62L^{hi}$ cells post TWS119 treatment remained $CFSE^{hi}$ (Figure 3B), suggesting that TWS119 inhibited gBT-I proliferation, with the majority of cells retaining a T_{naive} phenotype. We further tested TWS119 *in vitro* with two other $CD8^+$ TCR transgenic models (CL4 and OT-I) and were also unable to generate percentage of T_{scm} cells comparable to previously described literature (data not shown).

Even though we failed to observe changes in surface phenotype post TWS119 treatment, we tested if treated cells would recapitulate its self-renewal and stem like phenotype *in vivo*. We sorted $CD44^{lo}CD62L^{hi}$ and $CD44^{hi}CD62L^{hi}$ gBT-I $CD8^+$ T cells post TWS119 treatment and transferred them into lympho-depleted mice. However, we did not observe any evidences of self-renewal or stemness, as most transferred cells differentiated into $CD44^{hi}CD62L^{hi}$ T cells after 4 weeks, similar to untreated T_{naive} cells (Figure 3C, D). Taken together, our data suggested that although we were able to inhibit proliferation with TWS119, we did not observe any reprogramming of T cell phenotype.

ACT with JQ1 modified T cells reduced treatment efficacy in a preclinical cancer model

Although we did not observe changes to gBT-I $CD8^+$ T cells surface phenotype *in vitro*, we postulated that epigenetic modifying agents could have altered T cell function. We tested this with an established model of adoptive cell therapy whereby tumour-specific $CD8^+$ T cells are the key mediators of tumour cell death. We sought to determine if modifying gBT-I $CD8^+$ T cells *in vitro* would affect their anti-tumour effect *in vivo*. gBT-I $CD8^+$ T cells were activated *in vitro* in the presence of different epigenetic modifiers and administered to mice harboring a subcutaneous B16 melanoma expressing the gB antigen (B16-gB). B16-gB tumours grew rapidly in untreated mice (no ACT), with all tumours forming large palpable masses by 22 days. Administration of untreated, activated gBT-I T cells resulted in rapid tumour regression in the first 3 days, and tumour growth was suppressed for the next 7 days (Figure 4A). However, tumours eventually escape this control and relapse, growing exponentially. Mice within this group survive an average for 35 days, and there was a significant difference in survival between animals treated with unmodified T cells and untreated animals ($p < 0.001$). We examined the effect of modified gBT-I $CD8^+$ T cells on tumour growth and survival.

Treatment with gBT-I $CD8^+$ T cells modified with vorinostat, DOT1L inhibitor and GSKJ4 did not alter tumour growth differently from unmodified T cells (Figure 4A, C, and D). Interestingly, treatment with IBET and JQ1 modified T cells abrogated tumour regression in a proportion of animals, suggesting that $CD8^+$ T cell function was impaired (Figure 4B, E). Tumours were maintained at a mean volume of

approximately 200mm^3 – 400mm^3 for 20 days after treatment with IBET or JQ1 modified T cells before relapsing, and were significantly different from mice treated with unmodified T cells over time periods indicated in figure 4B, E (IBET: days 17-28; JQ1: days 19-25,27: *p<0.05, **p<0.01).There was no significant difference in overall survival between each treatment and unmodified T cells, with the exception of JQ1 treated T cells (p=0.0237) (Figure 4E). Although the effect was modest, modification with JQ1 and IBET inhibited tumour regression in our models of ACT.

Discussion

ACT can be effective in some individuals with advanced melanoma. However, a major hurdle for improving ACT efficacy is the development of T cell exhaustion during *ex vivo* expansion, which is necessary to generate sufficient numbers of cells for autologous infusion. In this study, we sought to manipulate CD8$^+$ T cell reprogramming by targeting epigenetic enzymes during the *ex vivo* T cell expansion step. Whilst multiple epigenetic modifiers demonstrated the capacity to inhibit proliferation *in vitro*, none showed evidence of differentiating cells into a T_{scm} surface phenotype or enhancing the efficacy of ACT when the treated cells were administered.

We successfully established an *in vitro* screen to examine the effects of epigenetic modifiers on proliferation and differentiation of TCR transgenic cells during antigen specific activation. T cell proliferation was inhibited to different extents by different epigenetic modifiers. High toxicity was observed when T cells were treated with low concentrations of GSK2801 and panobinostat, which is consistent with published observations where human lymphocytes are exposed to panobinostat long term (> 10

hours) [22, 23]. Although T cell proliferation is closely linked to its differentiation status, we did not observe measurable differences in differentiation, even though T cell proliferation was altered to varying extents by the epigenetic inhibitors. With the exception of vorinostat, all proliferating T cells differentiated into a $CD44^{hi}CD62L^{hi}$ phenotype either with or without treatment. In contrast, proliferating T cells exposed to vorinostat exhibited an unusual $CD44^{lo}$ surface phenotype post activation. Vorinostat can interfere with TCR signaling pathways and might be indirectly affecting the expression of activation molecules [24]. Another possible mechanism is that the HDAC1/miR-34a axis regulates CD44 expression in some tumour cell lines and inhibiting HDAC1 with vorinostat might have affected CD44 expression in this instance [25]. We were unable to confirm this finding with the other HDAC inhibitor, panobinostat due to its toxicity.

There are limited reports investigating the pharmacological manipulation of epigenetic mechanisms with respect to T cell differentiation in clinically relevant settings. A recent study has reported that T cells treated with the BET-inhibitor JQ1 in a xenograft model [15], demonstrated greater cell persistence, proliferation, enhanced formation of T_{cm}, T_{scm} populations and improved ACT efficacy. However, in the present study, JQ1 treatment during *ex vivo* expansion formed T cells with a T_{cm} like surface phenotype, but JQ1 treated T cell grafts demonstrated reduced efficacy *in vivo*. The disparity in findings could be attributed to differences in expansion protocols in both studies. Kagoya et al. treated their cells at a significantly lower concentration of JQ1 (0.15μM) over a longer period of time (14 days) compared to our study (8μM for 3 days). Furthermore, human studies utilises non-specific, polyclonal T cell activation to generate T cell grafts for ACT, whereas we

investigated antigen-specific $CD8^+$ T cell activation in our study. As TCR affinity affects the ability of $CD8^+$ T cells to differentiate into T_{scm} cells, our results might be influenced by our TCR transgenic model [26]. Lastly, JQ1 improved both $CD8^+$ and $CD4^+$ T cell function and engraftment [15], whereas our model is limited to the study of $CD8^+$ T cells. $CD4^+$ T cells play a crucial role in the maintenance of $CD8^+$ memory T cell function [27], and is another possible contribution to the disparity in findings.

In our study we examined expression of CD44 and CD62L as readouts for $CD8^+$ T cell *in vitro* differentiation. However, expression of these two markers did not correlate with observed functional impairment *in vivo* of JQ1- and iBET-treated T cells. In depth analysis of other T cell differentiation surface markers (such as CD25, CCR7, KLRG1, and transcription factors such as TCF-1 and EOMES) or transcriptome-wide surveys of *ex vivo* expanded T cells may provide additional insight into how broad based epigenetic targeting alters T cells differentiation and function in the context of ACT.

While we were unable to identify compounds that promote the generation of T_{scm} cells *ex vivo*, we note distinctive features of our model system which serve as important caveats for interpreting the results reported herein. We included the Wnt/β-catenin pathway inhibitor TWS119 as a positive control to promote T_{scm} *ex vivo* as reported previously [8]. However, despite the expected reduction in proliferation, we did not observe a coincident increase in T_{scm} cells in our TCR transgenic models. This inconsistency has been reported previously in a mouse model in which polyclonal T cells were activated and treated with TWS119 [28] resulting in reduced proliferation,

but no measurable change to the T_{scm} phenotype. Effects of Wnt/β-catenin signaling on *ex vivo* human T cell differentiation were investigated previously with either a combination of IL-7 and IL-15 [9], or TWS119 and IL-21 [29] and it therefore cannot be ruled out that differing protocols are associated with discrepant effects on T_{scm} phenotype. In addition, we note limitations of our model system that may impact upon the outcomes of modulating ACT efficacy. We recently reported that resistance to ACT in B16-OVA melanoma cells arises through multiple processes including transcriptional silencing of the targeted antigen genes [30]. In the present study, we observed down-regulation of GFP in relapsing tumour cell explants (data not shown) suggesting that this evolutionary process was likely operative. Therefore, enhanced ACT efficacy is expected to promote initial tumour regression, however the emergence of resistance in this model might not necessarily be delayed, for instance, if relapsing tumour cells are selected from pre-existing clonal outgrowths. Our data show that none of the *ex vivo* T-cell epigenetic treatments promoted survival and did not cause significant shrinkage to tumour volume arguing against these protocols as enhancing ACT. In contrast, both BET-inhibitors tested (JQ1 and IBET151) appeared to impair shrinkage of tumour volume, with JQ1-treated T cells also inducing significantly reduced survival.

Conclusions:

Our study demonstrates that single epigenetic modifiers can alter T cell proliferation, however at these doses we do not consistently observe measurable effects on differentiation. The epigenetic patterns regulating T cell differentiation are complex, with different epigenetic marks on multiple genes changing over the course of T cell activation[10]. Most studies currently expose T cells *ex vivo* with epigenetic

modifiers from the start of T cell activation. Further studies are required to identify how altering the timing, and combination of such modifiers can improve ACT and conversely to document protocols that inhibit efficacy.

Materials and Methods

Mice

gBT-I on a C57BL/6.SJL-PtprcaPep3b/BoyJ background (CD45.1) [19] were bred and maintained at Telethon Kids Institute (Perth, Australia). gBT-I are T cell receptor (TCR) transgenic mice specific for a H-2K^b-restricted Herpes Simplex Virus (HSV) glycoprotein B (gB) epitope, $gB_{498-505}$. Wild type C57BL/6 mice were obtained from Animal Resources Centre WA (Perth, Australia). The Telethon Kids Institute Animal Ethics Committee approved all experimental procedures.

In vitro T cell activation assay

gBT-I splenocytes were labeled with 2.5μM of carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes) as per manufacturer's instructions. C57BL/6 splenocytes were pulsed with 1μM of $gB_{498-505}$ peptide (Mimotopes) at 37^{o}C for 1 hour. CFSE labeled gBT-I splenocytes were incubated with $gB_{498-505}$ pulsed splenocytes at a 1:1 ratio, in the presence of varying concentrations of epigenetic modifiers. Concentrations were selected at doses that inhibited proliferation but caused minimal cell death thereby maintaining a viable pool of cells for infusion. Cells were diluted to a final concentration of $8.0x10^6$ cells/ml and transferred into a 384-well plate or 6-well plate. Cells were incubated in cRPMI media (RPMI media supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 2mM L-glutamine, 100U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), 3μg/ml LPS (Sigma, MO, USA) and 10U/ml IL-2 (Peprotech) in a 5% CO2 incubator at 37°C for 72 hours. Drugs used in this study are available from Selleckchem including SGC0946 (DOT1L-inhibitor; Catalog No.S7079), Vorinostat (SAHA, MK0683; Catalog No.S1047), Panobinostat (LBH589; Catalog No.S1030), GSK2801 (Catalog No.S7231), GSKJ4 (Catalog

No.S7070), GSK343 (Catalog No.S7164), IBET151 (GSK1210151A; Catalog No.S2780) and JQ1 (Catalog No.S7110).

Flow cytometry

Cell proliferation was analysed by flow cytometry. Activated cells were stained with anti-mouse CD45.1-V450 (clone A20), anti-mouse CD8-APC (clone 53-6.7), anti-mouse Va2-PE antibody (clone B20.1), anti-mouse CD44 (clone IM7) and anti-mouse CD62L (clone MEL-14) (BD Biosciences). Prior to acquisition cells were stained with propidium iodide (PI; Sigma) to exclude dead cells. Flow cytometry was performed on a BD LSR Fortessa (BD Biosciences). Flow cytometry analysis was performed using FlowJo (Treestar).

Tumour cell lines and inoculation

B16.F10 melanoma cells were purchased from the American Type Culture Collection (ATCC). B16 lines were transduced to express $gB_{498\text{-}505}$ and eGFP as previously described [31]. Transduction was confirmed by eGFP expression and $eGFP^+$ cells were sorted by flow cytometry to establish purified cell lines. B16.gB tumour cell lines were passaged routinely at 70–80% confluency and cultured in cRPMI media. 5 x 10^5 tumour cells were inoculated subcutaneously under the dermis of the left hand flank, and tumour growth was monitored daily with calipers.

Adoptive T cell therapy (ACT)

gBT-I T cells were activated *in vitro* as described above, in the presence and absence of epigenetic drugs. 8 days after B16.gB inoculation, mice with palpable tumours

were exposed to 550 Rad gamma irradiation, and received $1.0x10^7$ gBT-I T cells intravenously on the same day.

Statistics

All graphs and statistical analysis were performed using GraphPad Prism (GraphPad). Tumour growth curves were compared using an Mixed Model Type 3 Analysis of Variance (ANOVA), with Kenward-Roger Approximation using Estimated Marginal Means for multiple comparisons[32]. (http://doi.org/10.5281/zenodo.3341720)

List of abbreviations

ACT – Adoptive Cell Therapy

BET - Bromodomain and extraterminal

CFSE - Carboxyfluorescein succinimidyl ester

CTL - Cytotoxic T lymphocytes

HDAC - Histone deacetylase

TCR - T cell receptor

T_{em} - Effector Memory T cell

T_{cm} - Central Memory T cell

T_{scm} - Stem Cell Memory T cell

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Figure Legend

Figure 1. T cell proliferation inhibition by epigenetic modifiers *in vitro*. A) Representative FACS diagram depicting gBT-I T cell proliferation measured by CFSE dilution 72 hours post activation, in the presence of titrated concentrations of TWS119. Plots are gated on live, $CD8^+$ cells. B) Dot plots representing the percentage of proliferating ($CFSE^{lo}$) gBT-I T cells in the presence of titrated concentration of epigenetic modifiers, 72 hours after activation in a 384-well plate. Each graph represents treatment with one drug. Each data point represents the mean ± SD proliferation of three separate experiments.

Figure 2. T cell phenotype of activated T cells in the presence of epigenetic modifiers. A) Representative FACS diagram depicting expression of CD44 and CD62L on activated gBT-I T cells, in the presence or absence of different drugs. Plots are gated on live, $CD8^+$ cells, and are representative of three to four separate experiments. B) CD44 and CD62L expression represented at dot plots.

Figure 3. TWS119 treatment failed to generate a T_{scm} phenotype on gBT-I T cells. A) Dot plot representing $CFSE^{lo}$ proliferating gBT-I T cells in the presence of TWS119, 72 hours after activation in a 6-well plate. Each data point represents the mean ± SD proliferation. B) CD44 and CD62L expression of $CFSE^{lo}$ T cells post activation (top row). CFSE profile of $CD44^{lo}CD62L^{hi}$ cells demonstrate that most cells in this population remain naïve (bottom row). Plots are representative for 3 separate experiments. C) TWS119 treated cells were sorted into $CD44^{lo}CD62L^{hi}$, $CD44^{hi}CD62L^{hi}$ populations, and untreated $CD44^{lo}CD62L^{hi}$ populations (1 x 10^5

cells) were transferred into a lymphodepleted animal. CD44 and CD62L expression on transferred (CD45.1) cells 4 weeks post transfer are represented as FACS diagrams, and in D) a dot plot.

Figure 4. ACT with epigenetic modified gBT-I T cells did not improve tumour regression and survival. Tumour growth and survival curves of mice inoculated subcutaneously with B16.gB prior to receiving ACT consisting of activated gBT-I T cells treated with A)vorinostat, B)IBET, C)GSKJ4, D)DOT1L and E)JQ1. Graphs represent 3 separate experiments. Tumour growth between unmodified group and each modified group were compared with a Mixed Model ANOVA. Time points with significant differences in mean tumour volumes are represented with an asterisk (* <0.05, ** <0.01). Survival curves were compared with a Log-Rank (Mantel-Cox) test and there was a significant difference between unmodified T cells and untreated animals ($p < 0.001$). There was no significant difference between each treatment and unmodified T cells, with the exception of JQ1 treated cells. (JQ1 vs unmodified, $p = 0.0237$)

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Table 1. Epigenetic Modifiers Selected for Project

DOT1L inhibitor (DOT1-like histone H3K79 methyltransferase inhibitor)
Vorinostat (Histone deacetylase inhibitor)
Panobinostat (Histone deacetylase inhibitor)
GSK2801 (selective inhibitor of bromodomains BAZ2A/B)
GSKJ4 (histone demethylase inhibitor)
GSK343 (EZH2 selective inhibitor)
IBET (selective bromodomain inhibitor for BRD4)
JQ1 (selective bromodomain inhibitor for BRD2, BRD3, BRD4)

Table 2. Optimal dose of Epigenetic Modifiers used

Drug name	Optimal concentration
TWS119	3.5μM
DOT1L	2.5μM
Vorinostat	2.5μM
Panobinostat	-
JQ1	8μM
GSK343	6μM
GSKJ4	2.5μM
GSK2801	-
IBET	3μM

Figure 1

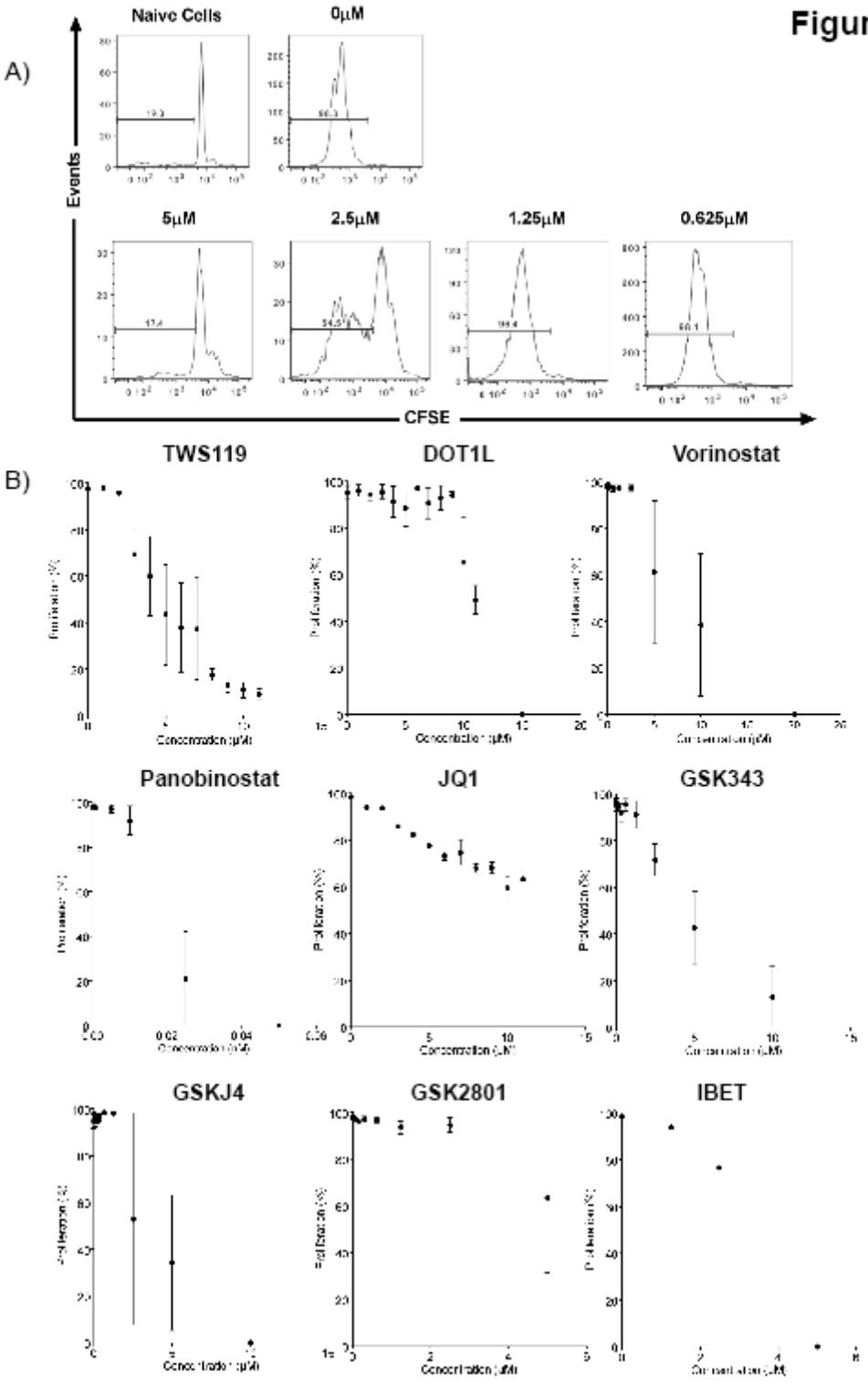
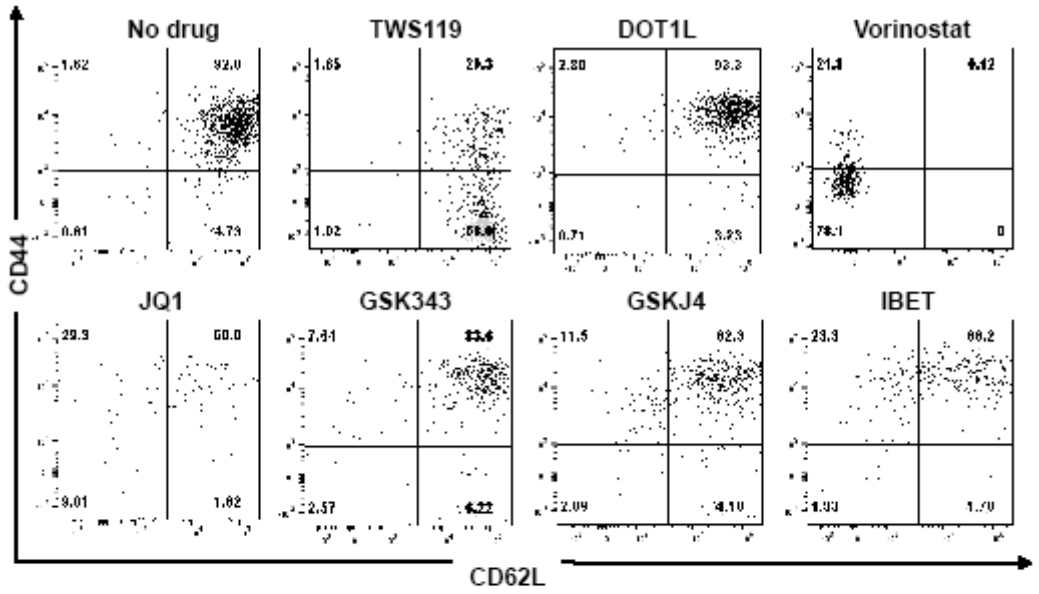

A)
Naive Cells
0μM
5μM
2.5μM
1.25μM
0.625μM
Events
CFSE
B)
TWS119
DOT1L
Vorinostat
Panobinostat
JQ1
GSK343
GSKJ4
GSK2801
IBET

Figure 2

A)


No drug
TWS119
DOT1L
Vorinostat
JQ1
GSK343
GSKJ4
IBET
CD44
CD62L

B)

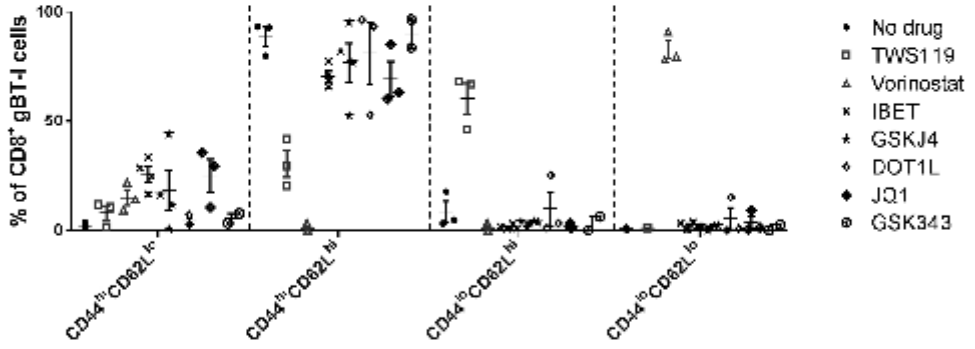

% of CD8+ gBT-I cells
100
50
0
CD44hiCD62Llo
CD44hiCD62Lhi
CD44loCD62Lhi
CD44loCD62Llo
No drug
TWS119
Vorinostat
IBET
GSKJ4
DOT1L
JQ1
GSK343

Figure 3

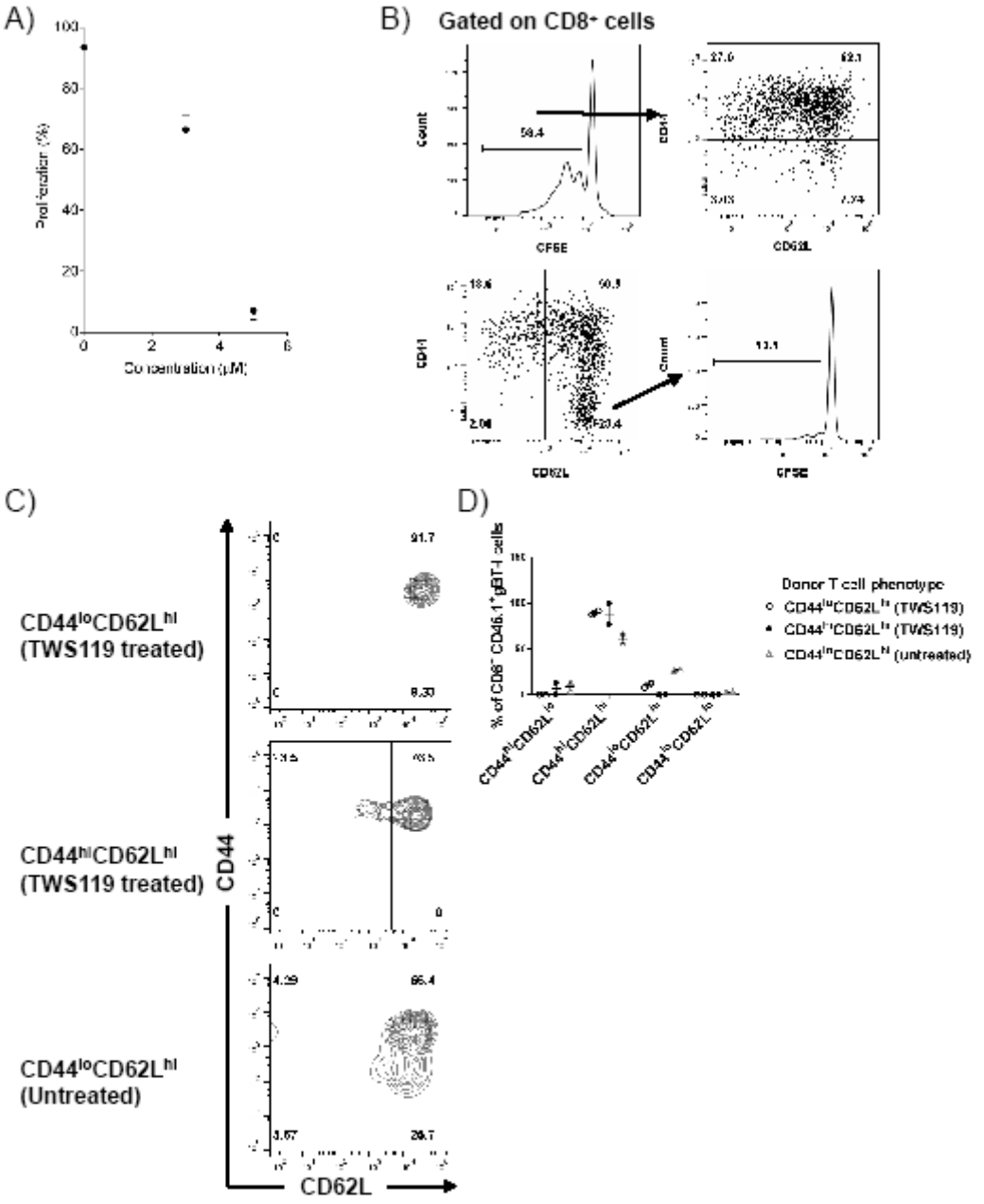
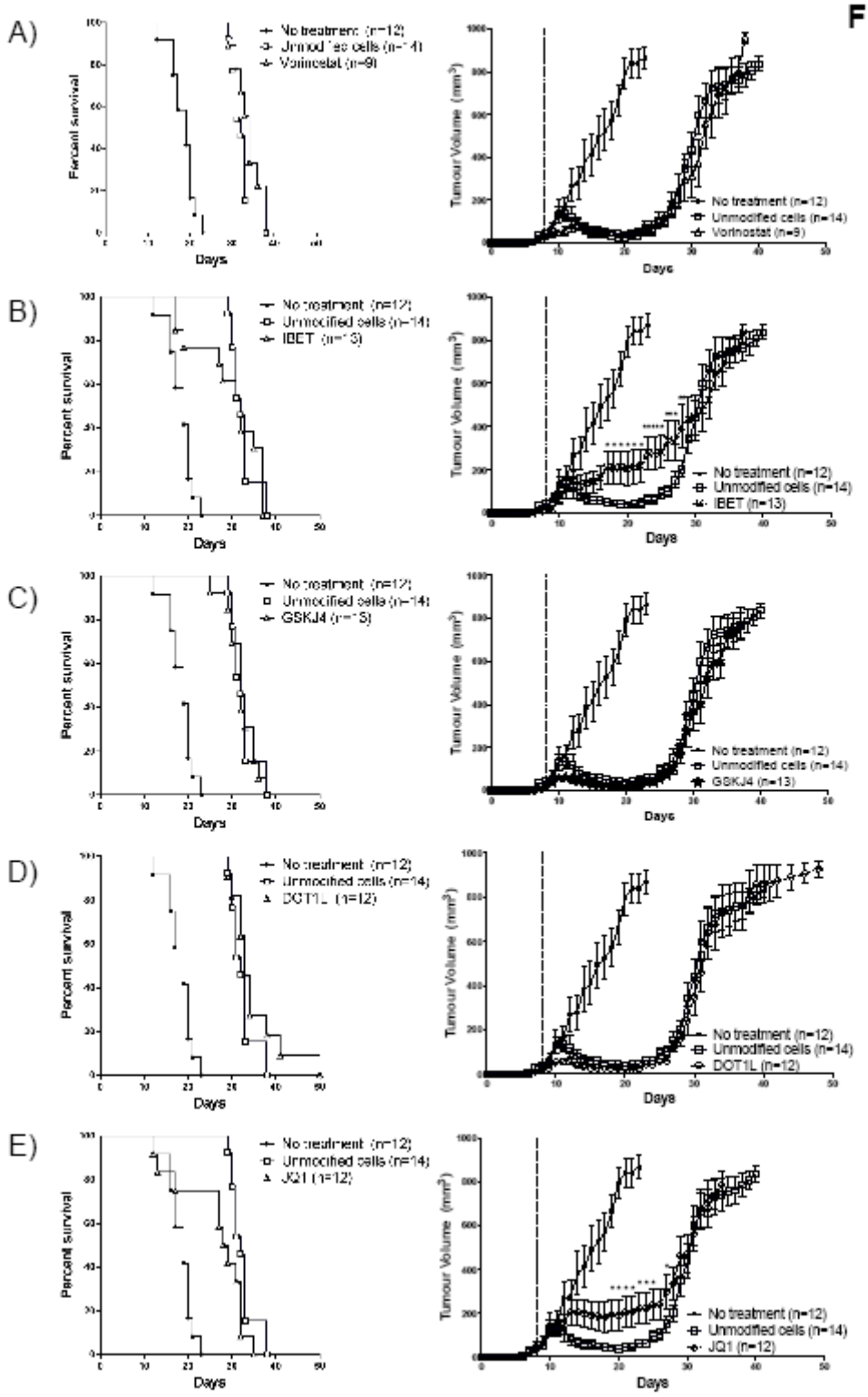

A)
B) Gated on CD8+ cells
Count
CFSE
CD44
CD62L
C)
CD44loCD62Lhi
(TWS119 treated)
CD44hiCD62Lhi
(TWS119 treated)
CD44loCD62Lhi
(Untreated)
CD44
CD62L
D)
% of CD8+ CD45.1+ gBT-I cells
Donor T cell phenotype
CD44loCD62Lhi (TWS119)
CD44hiCD62Lhi (TWS119)
CD44loCD62Lhi (untreated)

Figure 4


A)
B)
C)
D)
E)
Percent survival
Days
Tumour Volume (mm³)
No treatment (n=12)
Unmodified cells (n=14)
Vorinostat (n=9)
IBET (n=13)
GSKJ4 (n=13)
DOT1L (n=12)
JQ1 (n=12)