Characterization of surgery-induced vaccine dysfunction in a therapeutic murine melanoma model

by

Casey Lansdell

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Department of Biochemistry, Microbiology and Immunology
University of Ottawa
Faculty of Medicine

Supervisors

Dr. John C. Bell, PhD
Dr. Rebecca C. Auer, MD, MSc

Abstract

Surgical resection is the leading treatment of most solid tumours, however surgical stress creates an immunosuppressive environment that promotes metastases. A global decrease in T cell numbers and function post-surgery has been documented. However, the effect on tumour associated antigen (TAA)-specific T cells remains unclear. The objective is therefore to evaluate the impact of surgical stress on TAA-specific adaptive T cell immunity. Melanoma tumour-bearing C57BL/6 mice were vaccinated using AdhDCT, an adenovirus expressing dopochrome totaumerase (DCT), a melanoma TAA, and underwent abdominal nephrectomies to induce surgical stress. Surgical stress decreased the number of splenic cytotoxic T cells (CTLs) and their capacity to produce immunostimulatory cytokines (IFNγ and TNFα), as determined by flow cytometry. A perioperative accumulation in CTL-suppressive MDSCs was observed and demonstrated a direct suppression of CTL IFNγ and TNFα production and secretion. Understanding the mechanisms of perioperative T cell dysfunction will facilitate the development of targeted immunotherapies.

"We keep moving forward,
opening new doors,
and doing things
because we're curious
and curiosity keeps leading
us down new paths."

Walt Disney

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What a journey this has been!

I knew who I was this morning, but I've changed a few times since then.

-Lewis Carrol, *Alice's Adventures in Wonderland*

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List of Abbreviations

AdhDCT = adenovirus expressing human *DCT*

ANOVA = analysis of variance

APC = antigen presenting cell

CA = catecholamine

CAF = cancer-associated fibroblasts

CAR = chimeric antigen receptor

CTL = cytotoxic T cell

CMI = cell-mediated immunity

DAMPs = damage-associated molecular patterns

DC = dendritic cell

DCT = dopachrome tautomerase

DMEM = Dulbecco's Modified Eagle Medium

DPBS = Dulbecco's Phosphate-Buffered Saline

EGF = epidermal growth factor

ELISA = enzyme-linked immunosorbent assay

FBS = fetal bovine serum

HPA = hypothalamus-pituitary-adrenal

IFN = interferon

IGF-I = insulin-like growth factor I

IL = interleukin

IM = intramuscular

IP = intraperitoneal

MACS = magnetic activating cell sorting

MDSC = myeloid-derived suppressor cell

MHC = major histocompatibility complex

MRD = minimal residual disease

NK = natural killer cell

PAMPs = pathogen-associated molecular patterns

PDGF = platelet-derived growth factor

PFU = plaque forming units

PG = prostaglandins

PMA = phorbol 12-myristate 13-acetate

ROS = reactive oxygen species

RPMI = Roswell Park Memorial Institute media

SC = subcutaneous

STAT = signal transducer of activation and transcription

TAA = tumour-associated antigen

TCR = T cell receptor

TGF = tumour growth factor

Th = helper T cell

TLR = toll-like receptor

TNF = tumour-necrosis factor

T_{Regs} = regulatory T cells

VEGF = vascular endothelial growth factor

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1 – INTRODUCTION

1.1 Cancer

In Canada, it is estimated that almost half of the Canadian population will develop cancer in their lifetime and approximately a quarter will die from it. In 2015 alone, nearly 200,000 citizens will be diagnosed with cancer and 78,000 will die as a result of this burden[1]. Cancer is therefore the leading cause of death in Canada, distantly followed by heart disease and chronic lower respiratory disease[1]. The number of cancer-related incidences is expected to rise even further over the next few years due to an aging Canadian population[2].

Cancer is an incredibly complex disease that, despite many recent advances, is still poorly understood. Normal cells have the ability to maintain an equilibrium of life and death as needed by the body, however, a prominent and defining feature of cancer is the rapid development of abnormal cells that expand beyond their natural boundaries. Normal cells become altered in a multistep process as they undergo a series of genetic and epigenetic changes that transform them into uncontrollable growths, thus tipping the equilibrium[3,4]. Most cancers advance to form a solid tumour mass and can arise in several different ways including genetic inheritance, or exposure to viruses, carcinogens and radiation which can all cause DNA damage[5–7]. In many cases, tumour cells will migrate to other parts of the body and spread to other organs, a process termed as metastasis. Cancers that have metastasized elsewhere in the body severely dampen the chances of a good prognosis for the patient and are responsible for 90% of cancer-related mortality[8,9]. In 2000, Hanahan and Weinberg proposed the six hallmarks of cancer that contribute to tumourigenesis and metastatic dissemination which include self-sufficient cell growth, avoidance of programs that limit

cellular division, evasion of apoptosis, unlimited ability to replicate, induction of tumour-associated neovasculature and local invasion of tissues and distant metastasis[10]. In 2011, the list was updated to also include altered tumour cell metabolism, genomic instability to promote a selective advantage, ability to sustain a chronically inflamed environment and enhanced evasion of immune attacks[11–13]. These hallmarks have provided a platform upon which many targeted anti-cancer strategies and therapies have been based.

1.2 The Immune System

The immune system is comprised of a network of cells, tissues and organs that cooperate to protect the host from any "non-self" or foreign invaders such as bacteria, viruses, parasites and fungi in addition to transformed or aberrant cells[2]. The ability to successfully distinguish between "self" and "non-self" entities is the fundamental purpose of this network. The immune system can be divided into two parts – the innate or the adaptive immune response – although these distinctions are not considered mutually exclusive.

1.2.1 The innate immune response

The innate immune system is the first to respond to foreign antigens and provides a general, non-specific defense using a pattern recognition system. Pathogen or pathogen-associated molecular patterns (PAMPS) are exclusive to microbial pathogens and danger-associated molecular patterns (DAMPs) are endogenous molecules released from necrotic or dying cells, both can be recognized through pattern recognition receptors such as scavenger and toll-like receptors (TLRs) expressed on immune cells[14,15]. Recognition of

PAMPs and DAMPs upon infection or tissue damage initiates maturation and activation of innate immune cells such as granulocytes, dendritic cells (DCs), macrophages and natural killer (NK) cells[16].

1.2.2 The adaptive immune response

The adaptive immune system is slower to develop but generates an antigen-specific means of defense upon initial antigen exposure. In addition, the adaptive response provides protection against subsequent attacks from the same pathogen by maintaining a small pool of memory immune cells[13,16]. Upon secondary exposure to the same antigen, the adaptive immune cells respond much more rapidly. The adaptive immune cells are primarily T and B cells and their activation and activity is controlled by the primary innate immune response.

1.2.3 Interactions between the immune system and cancer

There is abundant evidence indicating that the human immune system can mount a powerful enough response that can eradicate cancers[17,18]. However, it is now understood that slow growing cancers have adapted complex mechanisms that enable them to evade the immune system[12,19], and instead use it to promote its tumour growth[4,20,21]. The dynamic role of the immune system as host protector and tumour growth advocate is a process known as immunoediting and consists of three phases: elimination, equilibrium and escape[13,22].

Theoretically, the threat of tumour development should be almost nil if the host has a highly functional, intact immune system leading to complete elimination of all tumour cells.

Unfortunately, not all individuals possess a highly functional immune system allowing a few surviving cancer cells to remain, despite being under immunosurveillance[13].

Over prolonged periods of time, the slow growth of these tumour cells is accompanied by repeated immune activation and elimination of some cells. Repeated cycles of tumour dormancy and antitumour activity by the immune system maintain an equilibrium phase[4].

In the escape phase, tumour cells that have acquired mutations allowing them to avoid immune recognition and elimination, begin to form a visible tumour mass. The mechanisms that facilitate the escape phase are complex and include selective pressures, immune suppression and promotion of tumour cell growth[4].

Alternatively, in some cases, generalized immune activation allowed the immune system to regain control and eliminate infected cells. As early as the late 1800's, physicians noted that in a few instances, patients injected with bacteria or bacterial products displayed some reduction in the size of their tumour[23]. Dr. William Coley, pioneer of cancer immunotherapy, was convinced that these infections stimulated a robust, generalized immune response capable of causing tumour regression[24]. Harnessing the power of the immune system in the context of cancer is therefore an intriguing focus of research.

1.2.3.1 The role of the innate immune system in cancer

Cellular stress, which is the case during tumour progression, often results in an inflammatory environment where there is an influx of innate immune cells such as

granulocytes, DC's, macrophages and NK cells which can then cooperate to activate the adaptive immune cells.

DC's and macrophages act as sentinel antigen presenting cells (APCs) that continuously patrol their microenvironment for signs of distress, such as a transformed tumour cell. When APCs encounter a distressed cell, its antigens are processed into peptide fragments which are then presented on the APCs surface. Additionally, APCs upregulate co-stimulatory molecules, release chemical mediators such as pro-inflammatory cytokines, chemokines, matrix remodelling proteases, reactive oxygen species (ROS) and promote the recruitment of additional immune cells to help restore tissue homeostasis[25].

APCs migrate to nearby lymphoid organs or the spleen where they present the antigens engulfed from the microenvironment to naïve adaptive immune cells as a peptide in the context of major histocompatibility complex (MHC) class I or class II molecules. MHC molecules are expressed on virtually every cell type and the MHC: peptide complex is critical for T cell receptor (TCR) recognition and proper activation along with costimulatory signaling molecules. However, virally-infected or cancerous cells often down-regulate their MHC expression to avoid T cell recognition. While often successful at evading the T cell response, infected cells are now highly susceptible to NK cell-mediated apoptosis. Inhibitory receptors on the NK cell recognize the MHC molecule, so a lack of engagement will activate the NK cell and initiate cytotoxicity. Additionally, cancer cells often upregulate stress ligands on their surface that interact with NK cell activating receptors[26].

1.2.3.2 The role of the adaptive immune response in cancer

The adaptive immune system is represented by B lymphocytes, $CD4^+$ helper T cells (Th) and $CD8^+$ cytotoxic T lymphocytes (CTLs), which differ from the innate system in that they possess receptors that are more diverse in their recognition potential and are antigen-specific.

If the antigen presented is considered foreign, only B and T cells that specifically recognize it will undergo clonal expansion and seek to destroy all cells expressing the particular antigen they are committed to. In the case of transformed cancer cells, the engulfed antigen is referred to as a tumour-associated antigen (TAA) that educates the adaptive immune cells to ensure that the newly made T and B cell clones will eradicate all cells expressing the TAA. B lymphocytes, with the aid of $CD4^+$ helper T cells, secrete antigen-specific antibodies that can neutralize their cellular targets and flag it for destruction by macrophages or NK cells. Similar to NK cell killing but now antigen-specific, CTLs can deliver a cytotoxic shock that rapidly induces apoptosis in their target cell. Upon contact with their target, CTLs and NK cells secrete perforin which creates pores in the target cell thereby allowing entry of Granzyme B, a protease that prompts immediate cell lysis[27,28]. Antigen-specific T cells will initiate cytokine secretions including interferon gamma (IFNγ), tumour necrosis factor alpha (TNFα) and a variety of interleukins (IL) in response to antigen recognition and other immune cells such as APCs, NK cells and B cells synergize with this antitumour activity[29–31].

Additionally, antigen stimulation of T cells reliably upregulates costimulatory molecules that promote T cell proliferation, differentiation and cytokine production[32]. 4-1BB

in particular, is a potent inducer of these activities and has previously been described as a definitive marker of tumour-reactive $CD8^+$ T cells [33]. The presence of tumour-infiltrating effector CTLs expressing 4-1BB has been strongly associated with improved survival in tumour models including ovarian cancer[34].

1.2.3.3 The role of the tumour microenvironment

Tumour-induced inflammation can promote tumour onset and development in the following ways: (1) it increases the amount of pro-angiogenic factors being produced in the tumour milieu such as vascular endothelial growth factor (VEGF), which supports neovascularization to the tumour and surrounding environment; (2) it heightens ROS production which can also contribute to tumour onset and development by instigating DNA damage, thus promoting genetic instability and uncontrolled proliferation[35]; and (3) it has been implicated in the generation of less immunogenic tumours due to the immune selective pressures that occur as a result of the intensive interactions between immune cells and cancer cells[4,14].

Host stromal cells in the tumour microenvironment have been shown to support tumour onset and subsequent growth. Primarily composed of cancer-associated fibroblasts (CAFs), they are characterized for their secretion of tumour-promoting factors such as transforming growth factor (TGF) -β and VEGF. These factors enhance tumour cell invasion, evasion of immune recognition and vasculature formation[36,37].

The release of cytokines into the tumour microenvironment can have a strong influence in the attenuation of disease progression. IFNγ and TNFα are two such cytokines.

IFNγ is the sole type II IFN and retains a range of functions. It is known to enhance MHC presentation, antigen processing, orchestrate leukocyte attraction, promote the growth maturation and differentiation of multiple cell type in addition to inducing anti-viral and anti-tumour mechanisms[30]. TNFα is a potent pro-inflammatory cytokine that aids in the recruitment of immune cells to the damaged or transformed tissue. While TNFα is paramount in host defence, dysregulation of this cytokine can lead to a chronically inflamed environment which has implications in tumour progression[26,38].

Furthermore, tumour progression generates an immunosuppressive environment by recruiting immune suppressive cells that significantly downregulates the effector immune response.

1.2.3.4 Mechanisms of immune suppression

Significant tissue damage is a regular consequence of chronic inflammation and therefore demands considerable repair in the aftermath. Immune homeostasis is generally regained by recruiting suppressive immune cells such as regulatory T cells (T_{Regs}) or myeloid-derived suppressor cells (MDSCs). These immune regulatory cells induce effector immune cell dysfunction through a variety of contact-dependent and soluble mechanisms thereby inhibiting their anti-tumour activity[14,15]. Tumour escape and metastasis can occur when the activity or number of suppressive cells and cytokines outweigh the effector functions of the TAA-specific immune cells.

1.2.3.4.1 The role of T_{Regs} in immune suppression

T_{Regs} can suppress most immune cells including $CD4^+$ and $CD8^+$ T cells, DCs, B cells macrophages and NK cells[41]. T_{Regs} are a subset of $CD4^+$ T cells expressing the IL-2 receptor (CD25) and Forkhead Box P3 (Foxp3), a transcriptional factor that regulates their immunosuppressive functions. T_{Regs} may deprive the microenvironment of IL-2 through their high-affinity IL-2 receptors thus hindering the activation of other T cell subsets. Additionally, IL-2 is known to upregulate Foxp3 expression thereby enhancing their suppressive function[42]. T_{Regs} also express ectoenzymes CD39/CD73 that convert adenosine triphosphate into adenosine which is lymphotoxic[42,43]. They also benefit from the excessive production of TGF-β which plays a central role in the proliferation and function of T_{Regs}[20,44] and when membrane-bound, TGF-β can also weaken CTL and NK cell responses[42].

1.2.3.4.2 The role of MDSCs in immune suppression

MDSCs are immature myeloid cells that limit the effector functions of CTLs by increasing production of TGF-β, ROS, peroxynitrates and enhancing L-arginine metabolism which is critical for TCR activation[35,45]. MDSCs are a heterogeneous population of cells that are classified as $CD11b^+Gr\text{-}1^{hi}$ granulocytic (g-) and $CD11b^+Gr\text{-}1^{dim}$ monocytic (m-) subtypes in mice. MDSCs express high levels of STAT-3 (signal transducer of activation and transcription), arginase and inducible nitric oxide synthase (iNOS) which effectively work to suppress T cell function[46]. Arginase activity involves the conversion of L-arginine into urea and L-ornithine and iNOS generates low levels of NO. Increased levels of ROS by MDSCs then combine with NO to produce peroxynitrates. These peroxynitrites can cause T cells to become unresponsive to antigen-stimulation by inducing nitration and nitrosylation of

different amino acids on the TCR and CD8+ molecules on the T cell surface[46,47]. Furthermore, the metabolism of L-arginine from the microenvironment impairs T cell proliferation, cytokine production and loss of CD3-ζ (zeta) chain expression on the TCR[48] and has been described in tumour models[49,50] and following physical injury[51]. Tumour-derived factors, such as TGF-β, promotes ROS production which is also involved in MDSC expansion and recruitment to the tumour[52].

Research has made it increasingly clear that there are a variety of mechanisms by which cancer cells exploit mechanisms of the immune system to block recognition and elimination and instead promote tumour formation. Although immunotherapy interventions have existed for quite some time, in the past, few trials have actually made it to the clinic and produced promising results. The clinic has therefore consistently relied on traditional therapies that although have off-target effects and toxicities, still often improve the patient's outcome. A better understanding of the interactions between cancer and the immune system will therefore lead to more innovative and efficacious cancer therapies that may be combined as an alternative to traditional treatments alone.

1.3 Current Cancer Treatments

Patients with cancer are evaluated for different treatment regimens based on their type of cancer, tumour characteristics, stage of cancer and personal wishes. Currently, the four leading treatment options to consider are chemotherapy, radiation, surgery and immunotherapy. These options can be delivered individually or in combination with one another to maximize the anti-tumour effects. A neoadjuvant treatment strategy involves an

initial step to shrink the tumour in advance of surgery while an adjuvant strategy is used afterwards to eliminate any residual cancer cells and reduce the risk of metastasis. In cases where a complete cure is not feasible, tumour shrinkage and control of growth and spread become the new objectives.

1.3.1 Chemotherapy

Chemotherapy is the use of chemical agents to block the growth of rapidly dividing cells which can be achieved via a variety of mechanisms. Damage to the DNA or interaction with critical components of the cytoskeleton such as microtubules can cause cell cycle arrest and lead to cell death[53]. Since rapid expansion is a hallmark of cancerous cells, chemotherapy drugs routinely interfere with cancer cell replication but also cause inevitable damage to other healthy cells that are also highly replicative. Chemotherapy drugs are generally distributed systemically in a course of treatments through intravenous injections or by oral delivery in pill form. A patient's regimen often includes one or more drugs delivered in a series of cycles in order to allow recovery and regeneration of intestinal epithelium and bone marrow between each dose due to high levels of toxicity[54]. Each cycle could last a few hours or a series of days depending on the drugs, type of cancer and the overall health of the patient. Since all rapidly dividing cells are attacked by these drugs, healthy tissues that demonstrate high proliferation under normal conditions are very susceptible. Affected areas during treatment include, but are not limited to, the lining of the digestive tracts which can cause nausea and loss of appetite, the skin which can become dry and irritated, nails that become brittle, hair that thins and importantly bone marrow toxicity leading to leukopenia and increased risk of infection[55]. Despite the successes of chemotherapy on tumour

shrinkage, it is very invasive for the patient and is not considered curative for most solid tumours. However, it is often used as an adjuvant to surgical resection or radiation therapy[56,57].

1.3.2 Radiation Therapy

Radiation therapy is another standard treatment option for cancer patients. It has been estimated that approximately half of all cancer patients will receive radiotherapy at some point during their care with varying degrees of success[58,59]. During this regimen, high-energy radiation is passed through the tumour in a targeted manner using a linear beam to focus on the affected area. This high energy can break chemical bonds and remove electrons from atoms inducing DNA damage thus causing cell cycle arrest and apoptosis. Due to the more precise deposition of treatment, damage to surrounding healthy tissue is minimized compared to chemotherapy[59]. Tumour characteristics play a significant role in determining cellular sensitivity to radiation therapy. Resistance to radiation therapy has been documented in patients with overly dense, hypoxic tumours. Additionally, elevated oxygen levels have been implicated in DNA repair mechanisms which can counteract the recent damage inflicted by the radiation[59,60]. Large tumour size and advanced stages of cancer also limit radiotherapy responsiveness which only reiterates its usefulness as a local therapy for palliation or as an adjuvant therapy to surgery[59,60].

1.3.3 Surgery

In cancer patients, surgical resection of the primary tumour is commonly the first step toward abrogating the disease and controlling tumour progression. Despite being an

indispensable treatment option, there is abundant evidence to demonstrate that an increase in tumour growth and metastases following surgical stress exists in a variety of different models[61–67]. Surgery as a risk factor for cancer metastasis is strengthened by data accumulated in the Auer lab using both pre-clinical and clinical models and mechanisms facilitating this are still under investigation[68,69]. Several potential concepts and mechanisms have been proposed such as tumour cell dissemination due to physical manipulation[70–72], minimal residual disease (MRD)[73] which is increased by neuroendocrine and paracrine signaling[62,66], neoangiogenesis[74], systemic release of growth factors[75] and by cell-mediated immune (CMI) suppression[69,72,76].

1.3.3.1 The perioperative period

Tumour tissue is highly non-cohesive and is generally surrounded and entwined by a network of blood vessels that have tumour cells embedded in the vessel walls. These properties can make tumour resection very difficult for the surgeon to control disseminating cells. The procedure leads to physical disruption of the tissue and its vascularization resulting in detached cells that can be easily released into the circulation[72].

Upon tumour resection, it is common for a few isolated tumour cells to remain in situ in microscopic deposits or as metastases, known as MRD. Complete elimination of MRD is of utter importance because the vast majority of cancer-associated morbidity and mortality is due to metastatic lesions that arise from these persistent cancer cells[4,73]. Surgical stress has long been thought to promote the development of pre-existing micro metastases and precipitate the formation of new ones in addition to facilitating local recurrences for many tumour types[76,77].

1.3.3.2 The effects of surgery on the tumour microenvironment

Wound healing is a complex process that is fundamental in the recovery from surgery. This process requires inflammation, angiogenesis and remodelling of the extracellular matrix. Following an influx of infiltrating lymphocytes, a whole host of growth factors are released and recruited to the injured tissue such as platelet-derived growth factor (PDGF), insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), TGF-β and VEGF[75]. While these growth factors are critical for the repair of damaged tissue, they have also been shown to promote neoangiogenesis and accelerate tumour growth. Angiogenic activity from wound healing may alert dormant metastases or promote regrowth from residual cells[67,74,75,78].

The perioperative period reveals heightened levels of stress biomarkers such as epinephrine and norepinephrine. These catecholamine (CA) neurotransmitters are considered mediators of the relationship between stress and cancer through the β receptors expressed by tumours[79]. They have been demonstrated to increase the invasive potential of tumour cells, influence migration and vascular growth and increase the activation of STAT-3, enabling tumour cell proliferation and survival[79,80]. Physiological and psychological pain, an obvious product of major surgery, can stimulate the release of CAs through the hypothalamus-pituitary-adrenal (HPA) axis, which also has implications in immune suppression[80]. Prostaglandins (PGs) are another example of a soluble factor that is commonly elevated perioperatively. CAs and PGs have been repeatedly shown to suppress CMI through CTLs, macrophages, dendritic cell and NK cell activity[66,76,79]. Inhibiting the effect of CAs and PGs using β-blockers and COX-2 inhibitors has successfully decreased

postoperative metastases and tumour progression in murine models and is under investigation in clinical trials[64–66,79].

1.3.3.3 Postoperative immune dysfunction

Tumour-free survival is dependent upon a highly functional immune system to eradicate all tumour cells from the body, however, surgical stress leads to suppression of the host's CMI activity. This impairment can be mediated through secreted soluble factors or through other immune cells that have assumed a suppressive phenotype. Surgery and the subsequent release of CAs and PGs have been correlated with a decrease the production of CMI-enhancing cytokines, such as IL-12 and IFNγ, and a reduction in the number and activity of circulating NK cells, CTLs and Th1 cells[66,81,82]. These compounds promote a transition from a Th1 cytokine response (IL-2, IFNγ and TNFα) to an anti-inflammatory Th2 cytokine response (IL-4,5,6,10 and 14) which in turn inhibits Th1 cytokines and prevents tumour rejection[72,79]. In addition, elevated immune suppressive hormones, such as cortisol, have been reported before and following surgery[81,83,84].

NK cells play a critical role in mediating tumour clearance postoperatively as demonstrated by previous work completed in the Auer lab. These studies have also shown that the effect of surgery significantly impairs NK cell function[68,69,85,86]. Platelet activation during the postoperative period promotes the formation of thrombin and fibrin clots which aggregate around the tumour cells and facilitate protection[87]. As a result of this coating, tumour cell adherence to endothelial cells has been described along with a decrease in NK cell-mediated destruction due to the induced physical barrier that blocks their contact[68,87]. Postoperative suppression of NK cell cytotoxicity toward tumour targets has been

established by the Auer lab and a partial recovery of this function can be obtained using preoperative vaccines[69,85,86]. Surgery was also shown to promote the migration of NK cells to the peritoneum, where the surgical trauma occurred, downregulate several NK cell activation/ maturation markers and induce MDSC accumulation in the spleen[69] which results in the suppression of NK function (unpublished data).

The role of T cells during the perioperative period has also been a focus of the Auer lab. We have previously demonstrated that anti-tumour immunity in TAA-vaccinated mice, mediated by T cells, is completely abrogated following surgery. Surgery also impairs TAA-specific T cell number and function including cytokine secretion, proliferation and cytotoxicity in a prophylactic murine model (unpublished data[88]). Other research groups have also described T cell dysfunction following trauma or surgery. An accumulation of MDSCs expressing arginase 1 (ARG1) postoperatively has been shown to reduce the level of circulating arginine which is central to TCR formation and lymphocyte proliferation[48,51,89–94].

The activation of the T cell immunoglobulin domain and mucin domain (TIM-3) and programmed death-1 (PD-1) pathway additionally promotes T cell dysfunction by blocking costimulation by APCs[91,95,96]. Cancer surgery has been shown to upregulate TIM-3 and PD-1/PD-ligand 1 (PD-L1) on immune cells and the level of expression correlates with the magnitude of surgical stress[95]. CD8+ T cells from colorectal surgery patients expressing TIM-3 and PD-1 inhibitory receptors simultaneously produced significantly less IFNy than those without expression[95]. Altogether, this provides strong evidence that T cells are critical to the anti-tumour response but are dysfunctional as a result of surgery.

All of the perioperative factors discussed above are rapidly initiated following surgery, occurring simultaneously in a short period of time. Together, these factors can temporarily render the patient vulnerable to progression of metastatic disease and possible local recurrence. The perioperative period therefore represents a critical window of opportunity to intervene with an alternative therapeutic strategy. Immune-based anti-cancer therapies have great potential during this period because the lowest tumour burden is immediately after resection of the primary tumour.

1.3.4 Cancer Immunotherapy

Immunotherapy involves a treatment regimen that capitalizes on the host's own immune system to fight diseases such as cancer. This can be accomplished by boosting the patient's existing immune system to become more activated and alert to cancer cells, or the patient can receive exogenous immune components to accomplish this. In some cases the treatment can provide very general immune activation, or it can be cancer-targeted. Strategies include the administration of check-point inhibitors and other monoclonal antibodies, cytokine-based vaccines and cancer vaccines.

1.3.4.1 Non-specific Cancer Immunotherapy

Non-specific cancer immunotherapies are aimed at stimulating a generalized immune response to enrich the anti-cancer efforts already in place. Strategies include administering immune stimulatory cytokines such as IL-2 and IFN which are currently used in the clinic to reduce recurrence and improve patient survival. Despite their success in promoting immune cell activation and differentiation, these cytokines are difficult to

tolerate at clinical doses and deliver toxicity to a majority of patients on the regimen[26,97]. Granulocyte-macrophage-colony-stimulating factor (GM-CSF) has also shown some promise as an promoter of DC differentiation and induces tumour antigen-specific CTLs by improving antigen presentation but is often used in conjunction with another therapy[98].

Checkpoint inhibitors are a novel class of antibody therapeutics that block immune checkpoints, which function to avoid overactivation. Most notably, inhibitory cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and PD-1 receptors are rapidly upregulated on T cells following antigen stimulation as a mechanism of control to negatively regulate their activation. Simultaneously, tumour cells notoriously upregulate PD-L1 which altogether dampens T cell immunity[91,99]. Clinical testing of monoclonal antibodies that block these receptors in order to unleash activated T cells have garnered significant attention in the treatment of cancer. Anti-CTLA-4 (Ipilimumab) has been FDA approved for use in the clinical treatment of patients with advanced stages of melanoma and renal cancer and FDA-approved anti-PD-1/PDL-1 treatments such as Nivolumab and Pembrolizumab, are also being investigated in advanced melanoma and lung cancer patients[17,99–102]. Although each therapy has demonstrated highly promising results in a subset of patients, a large percentage have remained unresponsive[103]. As one group suggests, elevated levels of MDSCs might be responsible for this resistance and elimination of MDSCs can lead to a cure for metastatic disease in a mouse model[104]. Common strategies to eliminate MDSCs in vivo include the use of antibodies and other cytotoxic agents.

1.3.4.2 Cancer-Specific Immunotherapy

Therapeutic cancer vaccines are administered to the patient in order to strengthen the response of the patient's own immune system to specifically target all cancer cells in the body, while sparing normal cells. These therapies are often used in conjunction with other cancer treatments. Several types of cancer-specific therapies exist such as tumour cell, DC, T cell, peptide and vector-based vaccines.

Tumour cell vaccines are generated from patient-derived tumour cells after the tumour has been surgically removed. These tumour cells are typically irradiated and then re-administered, to the patient from which they were isolated along with an immunostimulatory adjuvant. A significant limitation of this strategy is obtaining an optimal tumour specimen which limits its efficacy to only certain tumour types or stages[3].

The use of DC's in anti-cancer therapy involves first isolating DC precursors from the patient and differentiating them *in vitro* with a supply of cytokines and growth factors. DC's are then subjected to and loaded with TAA's derived from the patient's own tumour and forced into maturation[105]. When these mature, TAA-loaded DC's are injected back into the patient, they will present the antigens to endogenous lymphocytes, educate them against the TAA and stimulate an anti-tumour immune response.

T cells that have been genetically engineered to express a specific receptor for a TAA, known as a chimeric antigen receptor (CAR) have had recent success in the clinic with lymphoma and leukemia patients[106]. Evidence in murine models of B cell lymphoblastic leukemia demonstrated that CAR T cell therapy redirected CTL killing towards the associated

antigen, produced effector cytokines and induced long-term remission[107]. Similar efficacy, however, has not been shown for solid tumours[17,108].

Peptide-based vaccines employ short amino acid sequences derived from the tumour antigen to create an immunogenic peptide molecule that represents only a particular epitope of the antigen. However, their small size generally renders them weakly immunogenic by themselves so they are often accompanied by carrier molecules to increase chemical stability in favor of a more robust immune response[109]. Another consideration is the required MHC or HLA-typing (human version of MHC) of the host to ensure high-affinity binding of the peptide and a subsequent immune response. Successful activation of antigen-specific T cells depend on a well-fitted interaction between the MHC molecule expressed on their surface and the antigen peptide[109,110].

Vector-based vaccines utilize the backbone structures of either viruses or DNA plasmids to deliver TAA and elicit a humoral and anti-tumour immune responses that is more robust than peptide alone. These vectors are genetically altered to encode one or more tumour-specific antigenic sequences and upon patient injection, they infect the host where the antigens are then processed and presented to lymphocytes. Both strategies strive to elicit long-lasting protective anti-tumour immunity but can also induce vector tolerance into the host[111]. For safety precautions, viral vectors are usually genetically attenuated and non-replicative, however, a new wave of replicating oncolytic viruses has been recently highlighted[101]. Another advantage is that viruses for immune therapy are relatively easy to produce and inexpensive compared to other treatments.

A promising viral vector-based cancer vaccine known as AdhDCT, is a replication-deficient (E1/E3 deleted) type 5 adenovirus that expresses a human dopachrome tautomerase (hDCT) gene insert. Also known as tyrosine related protein-2 (Trp-2), DCT is a protein involved in the synthesis of melanin and is expressed in normal melanocytes and melanoma, making it a model melanoma TAA[112]. Interestingly, human melanoma TAA's are significantly homologous to their murine counterparts and are actually more immunogenic when injected into mice[113]. Several studies have shown that prophylactic and therapeutic vaccination with AdhDCT induces a DCT-specific T cell response in mice challenged with a B16 melanoma cancer cell line[114–116]. Furthermore, protection from B16 tumour challenge was conferred in mice prophylactically vaccinated with AdhDCT and was partially CTL-mediated[115]. Previous data accumulated from the Auer lab demonstrates that vaccination with AdhDCT prior to B16 tumour-challenge in a murine model can protect 70% of mice from recurrence, however strikingly, that effect is completely obliterated in mice undergoing additional surgical stress. A subsequent adoptive transfer experiment confirmed that T cells play a definitive role in this phenomenon[88].

Although there has been considerable progress in the way cancer immunotherapy is approached, the immune system is incredibly complex and there is still a plethora of information to be gained about how to appropriately harness its power to eradicate cancer. Some preclinical models suggest a cure for solid malignancies will employ a combination of two or more treatment options[66,67,117,118]. Extensive investigations in this regard are still under way in order to design and optimize the best strategy to achieve promising results.

Additional consideration must be given to the dose, administrative route, timing and sequence of vaccines in addition to other therapeutic treatments[119].

1.4 Rationale and Hypothesis

Surgical resection of a primary tumour is a mainstay of treatment, however, strong immunosuppression has been demonstrated during the post-operative period. The patient is therefore less able to clear any residual disease rendering them more susceptible to regrowth of the primary tumour and secondary metastases. An overall decrease in T cell number and function has been investigated following surgical stress in preclinical models and cancer patients[81]. The impact of surgery on TAA-specific T cells in a therapeutic cancer model however, has not been extensively studied.

AdhDCT is an viral vaccine that contains an adenovirus backbone and expresses DCT, a melanoma-TAA. Several studies have shown that prophylactic and therapeutic vaccination with AdhDCT induces a robust DCT-specific T cell response in mice challenged with a B16 melanoma cancer cell line[114–116] providing a strong rationale for its use in the study of TAA-specific immune responses. Furthermore, protection from B16 tumour challenge was conferred in mice prophylactically vaccinated with AdhDCT and was partially CTL-mediated[115]. Using a prophylactic model, previous data from the Auer lab confirmed that $CD8^+$ T cells play a role in the vaccine dysfunction we observe following surgery[88]. In a tumour-bearing model, AdhDCT vaccination significantly improved the survival of mice undergoing tumour resection alone while mice that received additional surgical stress

succumbed to their tumour burden more quickly. More simply, surgical stress completely abrogated the survival benefit conferred by the vaccination.

The effect of surgery on T cell immune responses in immunized mice is very intriguing. However, in the interest of moving towards a more therapeutically relevant model, we designed a new strategy to first implant a subcutaneous B16 tumour prior to AdhDCT vaccination and surgery in order to study the immune response in a tumour-bearing model. Our hypothesis is that TAA-specific CTL immunity is attenuated by surgical stress in TAA-immunized, tumour-bearing mice.

1.5 Objectives

1. Determine if the tumour-specific T cell immune response is diminished during the postoperative period in a tumour-bearing vaccine model of B16 melanoma.
2. Characterize surgery-induced mechanisms of immune suppression leading to tumour-specific $CD8^+$ T cell dysfunction.

2 – MATERIALS AND METHODS

2.1 Mice and cell lines

Age-matched female C57BL/6 mice (6-8 weeks old at initiation of each experiment) were purchased from Charles River Laboratories (Wilmington, MA) and housed under specific pathogen-free conditions. Isofluroane was used for anaesthesia. B16F10lacZ melanoma cells were received as a gift from Dr. Anne Chambers. Cells were cultured at 37°C in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) supplemented with 10% fetal

bovine serum (FBS) (Hyclone) and 1x of Penicillin/ Streptomycin (Invitrogen). All animal studies complied with the Canadian Council on Animal Care guidelines and were approved by the University of Ottawa's Animal Research Ethics Board.

2.2 Subcutaneous tumour model and immunization

Anesthetized mice were subcutaneously (SC) implanted with 3×10^5 B16F10lacZ cells reconstituted in 100µL Dulbecco's Phosphate Buffered Saline (DPBS) in their right hind flank on day 0. On day 7, mice were immunized intramuscularly (IM) with 1×10^8 PFU AdhDCT reconstituted in 100µL of PBS (50µL per thigh). As a control, some mice only received PBS.

2.3 Recombinant Virus

AdhDCT was provided by Dr. Brian Lichty of McMaster University (Hamilton, ON). AdhDCT is an E1/E3 deleted human type 5 adenovirus engineered to express the full-length *hDCT* gene. Adenovirus was generated in 293 cells and subsequently purified on a cesium chloride gradient as described previously[120].

2.4 Induction of surgical stress and sacrifice

All mice underwent anaesthesia and complete tumour resection (Res) on day 14. Some mice additionally underwent a full abdominal laparotomy and left nephrectomy (Nx) to induce surgical stress. On the day of surgery, all mice were given 3 doses of buprenorphine for pain management. Mice were sacrificed approximately 18 hours post-surgery by injecting 100µL Euthanyl (65 mg/mL) into the intraperitoneal cavity (IP).

2.5 Cell Harvesting

Mouse spleens were mashed through a 70μM nylon mesh strainer (BD Falcon) using a syringe plunger and washed in DPBS. Red blood cells (RBCs) were eliminated using an ammonium-chloride-potassium (ACK) lysis buffer prior to antibody staining for flow cytometry and magnetic activated cell sorting (MACS).

2.6 Peptides

The immunodominant DCT peptide that binds to H-2K^{b} ($DCT_{180\text{-}188}$ SVYDFFVWL; shared by human DCT and murine DCT) was synthesized by Biomer Technology (Pleasanton, CA).

2.7 Flow cytometry and antibodies

Cells were washed with cold fluorescence-activated cell sorting (FACS) buffer (PBS + 3% FBS + 1mM EDTA) then incubated with mouse CD16/CD32 (Fc Block^{TM}; BD Pharmingen) for 15 minutes at 4°C in the dark. For extracellular staining, cells were incubated for 30 minutes at 4°C in the dark with fluorochrome-conjugated antibodies (diluted 1:100) specific for cell surface antigens and fixed with 1% PFA. For intracellular staining, cells were permeabilized and fixed with Cytofix/Cytoperm (BD Pharmingen) for 20 minutes at 4°C in the dark prior to 30 minutes incubation with intracellular antibodies (1:200). The following antibodies were used for extracellular staining: CD3-PerCP (Biolegend), CD8α-FITC (BD Biosciences), CD4-PE (BD Pharmingen), Gr-1-FITC (eBioscience), CD25-FITC (Biolegend), CD137-PE (Biolegend) and Ly6C-APC (eBioscience). CD11c-PeCy7 (eBioscience) and CD11b-PE (eBioscience). The following antibodies were used for intracellular staining: IFNγ-PE (BD Biosciences), TNFα-PeCy7 (Biolegend) and FoxP3-APC (eBioscience). A Cyan-ADP 9 flow

cytometer was used to acquire data using Summit 4.3 software (Beckman Coulter) and Kaluza 1.2 software (Beckman Coulter) was used for the analysis.

2.8 *In vitro* peptide restimulation

1-2×10^6 spleen cells were resuspended in Roswell Park Memorial Institute (RPMI) medium (Hyclone) supplemented with 10% FBS and 1x Penicillin/Streptomycin (Invitrogen) (cRPMI). Cells were then stimulated with DCT peptide (2µg/mL) and pure CD28 (2µg/mL) (BD Pharmingen™) for co-stimulation or PMA (0.1µg/mL) and ionomycin (1µg/mL) for a total of 6 hours in the presence of brefeldin A (GolgiPlug™; BD Biosciences; 1µg/mL) which was added 1.5 hours into the total incubation time. Surface expression of CD137 was determined following a 24 hour incubation.

2.9 *In vivo* depletion of MDSCs

B16lacZ tumours were subcutaneously injected on day 0. Pure Gr-1 antibody (100µg) (eBioscience; clone: RB6-8C5), gemcitabine (120mg/kg) (Sagent Pharmaceuticals; NDC #25021-235-50) or 5-fluorouracil (50 mg/kg) (Accord Healthcare, Inc.; NDC #16729-276-38) was injected IP on day 13. Mice were sacrificed on day 15 to determine splenic T cell and MDSC proportion compared to mice that only received a tumour.

2.10 Isolation of T cells and MDSCs

T cells were isolated from the spleens of mice that received AdhDCT vaccination 7 days prior to harvest. MDSCs were isolated from the spleens of mice that were either surgically stressed 18 hours prior to harvest or untreated. Spleens were harvested and processed individually as previously mentioned. Erythrocyte-free single-cell suspensions

were washed and resuspended in MACS buffer (PBS + 3% FBS + 2mM EDTA). T cells were positively selected for using CD90.2 MicroBeads (Miltenyi Biotec; 130-049-101) and MDSCs were positively selected for using the Myeloid-Derived Suppressor Cell Isolation Kit (Miltenyi Biotec; 130-094-538) using the manufacturer's protocols. Cells were resuspended at 1×10^6 cells/50µL cRPMI.

2.11 T cell-MDSC coculture

Isolated T cells (1×10^6 cells/50µL cRPMI) from AdhDCT-vaccinated mice were plated in a round-bottom 96-well plate. MDSCs isolated from naïve or surgically stressed mice were added at different ratios (2.5×10^5, 5×10^5 or 1×10^6 cells/50µL cRPMI). CD3/CD28 Dynabeads (Life Technologies) were added to the culture in a 1:1 bead-to-cell ratio as well as 30 U/mL of mouse recombinant IL-2 as per manufacturer's recommendation. cRPMI was added to each well so that each well contained 200µL of media. Cells were then cultured for 4 days in a CO_2 incubator at 37°C. After 4 days cells were transferred to a V-bottom 96-well plate for re-stimulation with DCT peptide or PMA/Ionomycin using the protocol listed above for intracellular staining. After restimulation, supernatants were collected at stored in the -80°C freezer for later use.

2.12 Mouse IFNγ enzyme-linked immunosorbent assay (ELISA)

Supernatant from the T cell-MDSC coculture were collected after restimulating the cells with DCT peptide or PMA and Ionomycin. Supernatants were thawed on ice and the manufacturer's suggested protocol was followed for all subsequent steps. Briefly, the Mouse IFNγ ELISA Ready-Set-Go! (eBioscience) plate was coated with IFNγ capture antibody overnight at 4°C, then washed and blocked with the ELISA diluent for 1 hour at room

temperature (RT). 100µL of each standard and sample were then loaded onto the plate and incubated overnight at 4°C. A biotin-conjugated detection antibody was added to the plate and incubated for 1 hour at RT followed by a 30 minute RT incubation with Avidin-horseradish peroxidase, a detection enzyme. Each well was then coated with 100µL tetramethylbenzidine (TMB) substrate solution and allowed to develop for 15 minutes at RT before the addition of a highly acidic Stop Solution (Biolegend; 423001). The absorbance was detected at 450 nm.

2.13 Statistical analysis

Data was graphed and analyzed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA). A Student's two-tailed *t*-test was used to compare the mean between two groups and a one-way analysis of variance (ANOVA). Means are shown with standard error bars and the difference between means were considered significant at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

3 – RESULTS

3.1 Surgical stress decreases the absolute number of T cells, but not proportion, in tumour bearing mice therapeutically vaccinated with AdhDCT

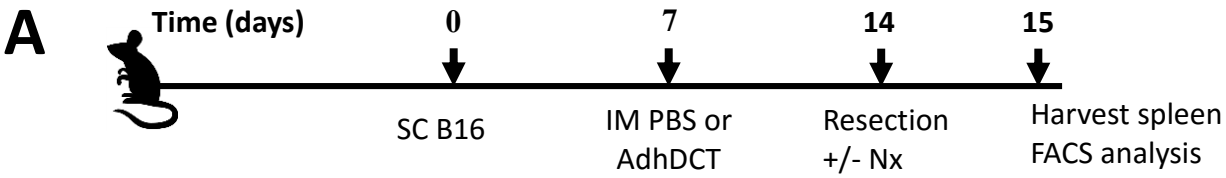
Previous work in the Auer lab demonstrated that 70% of mice undergoing therapeutic AdhDCT vaccination and partial resection of an established B16 tumour were protected from tumour outgrowth[88]. However, mice that endured further surgical stress, an abdominal laparotomy and nephrectomy, in addition to the above regimen succumbed to their tumour outgrowth much more quickly, similar to those that received PBS instead of AdhDCT

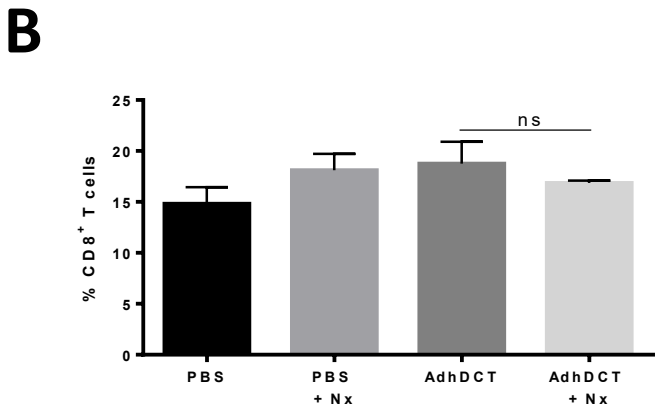
vaccination with 0% survival. Previous work also demonstrated that T cells play a critical role in generating an anti-tumour response that is capable of protecting AdhDCT vaccinated mice from tumour outgrowth in the post-operative period. This led us to the hypothesis that antigen-specific effector $CD8^+$ T cells may be dysfunctional during the post-operative period. Prior characterization of the effector $CD8^+$ T cell response revealed a peak response between 7 and 10 days following vaccination with AdhDCT. We therefore chose to assess the impact of surgical stress on the DCT-specific T cell response when it was most robust.

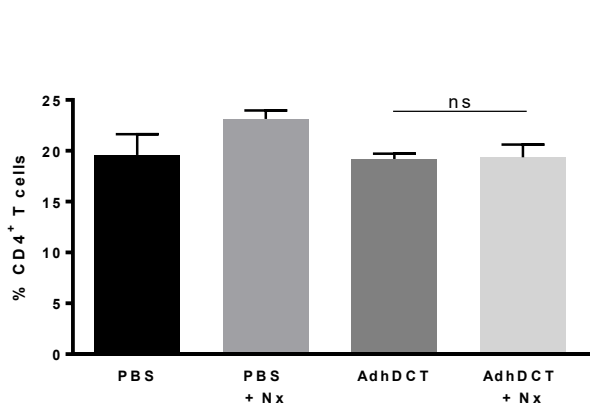
We challenged mice with B16 flank tumours on day 0, vaccinated them with AdhDCT or PBS on day 7 and performed complete resection with or without additional surgical stress in the form of laparotomy and left nephrectomy on day 14. Mice were then sacrificed approximately 18 hours following tumour resection, with or without surgical stress, and their spleens harvested for immune analysis by flow cytometry (**Figure 1a**). In our model, the proportion of both $CD8^+$ and $CD4^+$ T cells in the spleen was unaffected by surgical stress with or without vaccination (**Figure 1b, c**). However, the absolute number of $CD8^+$ T cells was significantly attenuated in tumour-bearing mice vaccinated with AdhDCT by the additional surgical stress ($p < 0.05$) in this model (**Figure 1d**). A similar trend was observed for the absolute number of $CD4^+$ T cells though it did not reach statistical significance (**Figure 1e**).

3.2 Surgical stress impairs cytokine production by DCT-specific $CD8^+$ effector T cells in the spleen within 18 hours

We then sought to determine if DCT antigen-specific $CD8^+$ T cells were also less activated and functional following surgical stress in tumour-bearing vaccinated mice. Since cytokine production is a significant parameter of $CD8^+$ T cell function, we designed a flow


A
Time (days)
0
7
14
15
SC B16
IM PBS or AdhDCT
Resection +/- Nx
Harvest spleen FACS analysis


B
% CD8+ T cells
25
20
15
10
5
0
ns
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx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20
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5
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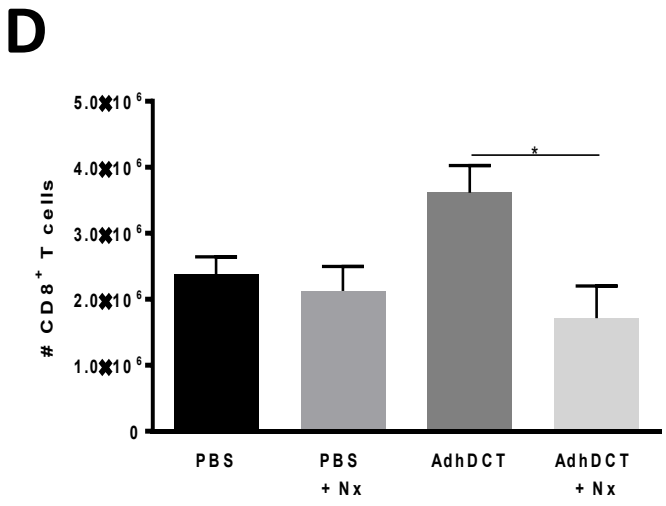
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx


D
CD8+ T cells
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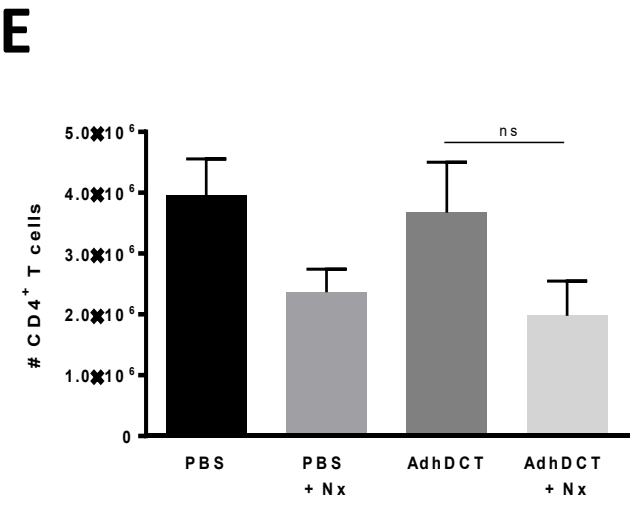
E
CD4+ T cells
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3.0✖10 6
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1.0✖10 6
0
ns
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx

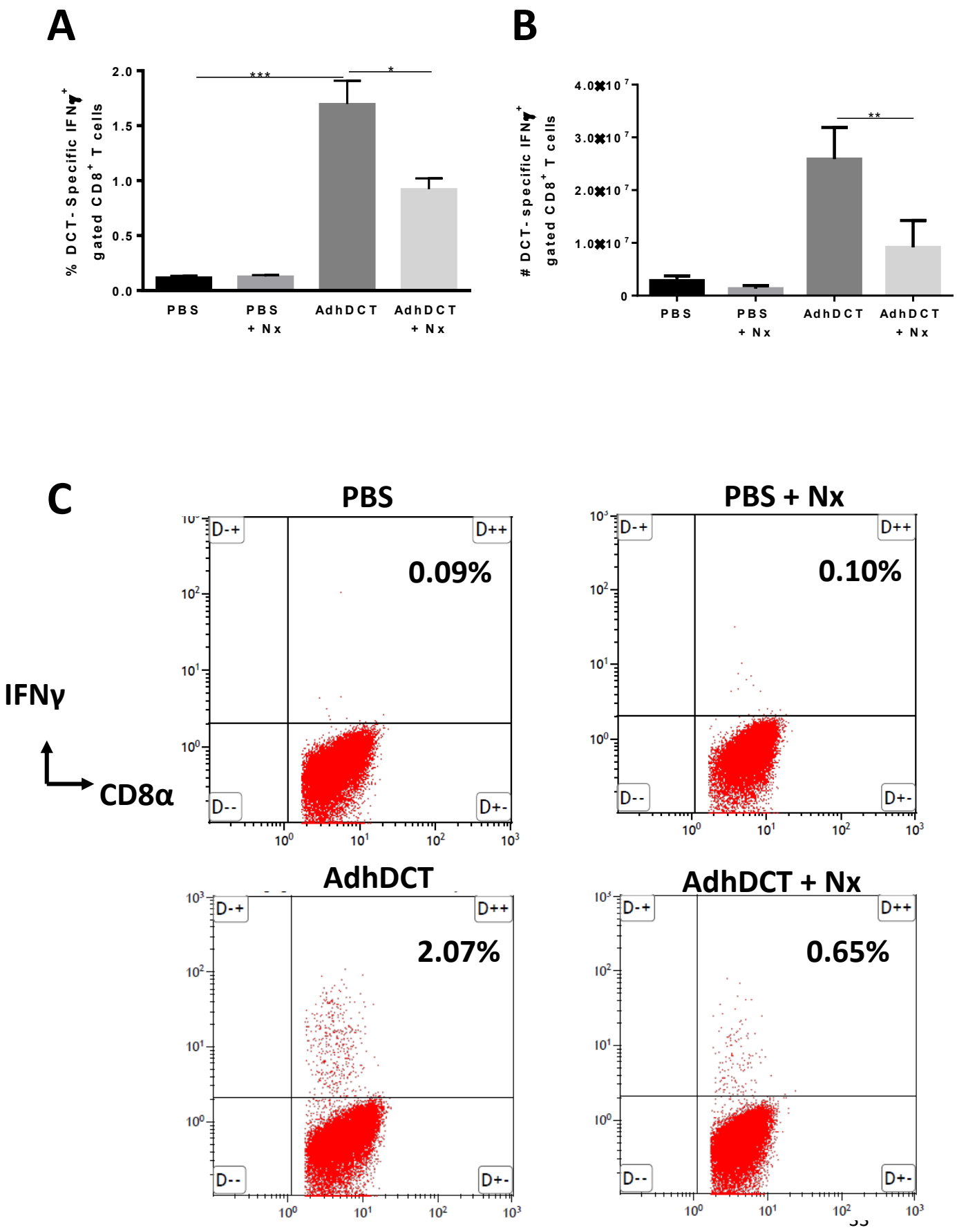
Figure 1. Proportion and absolute number of splenic T cells following therapeutic vaccination with AdhDCT in surgically-stressed mice. **a)** Experimental timeline. C57BL/6 mice were challenged SC with 3×10^5 B16F10lacZ cells on day 0 and then injected IM with PBS or 1×10^8 pfu AdhDCT on day 7. On day 14, all mice were anesthetized and underwent complete tumour resection. Some anesthetized mice additionally received a full laparotomy followed by a left nephrectomy (Nx) to induce surgical stress. All mice were euthanized 18 hours following surgery and their spleens were resected. Splenic lymphocytes were isolated and fluorescently labelled for FACS analysis. **b)** The proportion of $CD8^+$ T cells and **c)** $CD4^+$ T cells. **d) T**he absolute number of $CD8^+$ T cells and **e)** $CD4^+$ T cells per whole spleen. Statistical analysis was evaluated using a Student's t-test and a one-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

cytometry-based assay to measure the proportion and absolute number of DCT-specific $CD8^+$ T cells producing the cytokines IFNγ and TNFα in response to restimulation with the immunodominant DCT peptide. Using the same timeline as depicted in **Figure 1a**, the proportion and number of splenic DCT-specific $CD8^+$ T cells producing IFNγ and TNFα 18 hours post-surgery was determined following DCT peptide restimulation *in vitro*. $CD8^+$ T cells from mice that received the mock vaccination with PBS produced almost undetectable amounts of IFNγ while those vaccinated with AdhDCT produced significantly more ($p < 0.001$). However, mice that received AdhDCT vaccination and additional surgical stress demonstrated a significant decrease in the proportion of $IFN\gamma^+$ gated $CD8^+$ T cells ($p < 0.05$) (**Figure 2a, c**). The total number of splenic DCT-specific $IFN\gamma^+$ gated $CD8^+$ T cells of AdhDCT-vaccinated mice following surgical stress was also significantly decreased compared to those receiving the vaccination alone ($p < 0.01$) (**Figure 2b**).

This trend was also observed with TNFα cytokine production in response to DCT peptide restimulation. A heightened response in proportion (**Figure 3a, c**) and absolute number (**Figure 3b**) of $TNFa^+$ gated $CD8^+$ T cells was observed in tumour-bearing AdhDCT-vaccinated mice than those undergoing additional surgical stress ($p < 0.01$).

3.3 The upregulation of potent costimulatory molecule CD137 (4-1BB) on DCT-specific $CD8^+$ effector T cells is unaffected by surgical stress in tumour-bearing vaccinated mice

Furthermore, we explored whether or not surgical stress in tumour-bearing vaccinated mice impairs the upregulation of an important costimulatory molecule expressed on the surface of TCR-activated $CD8^+$ T cells. Splenocytes were harvested 18 hours post-surgery and restimulated for 24 hours with DCT peptide *in vitro*. The expression level of


A
% DCT-Specific IFNγ+ gated CD8+ T cells
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1.0
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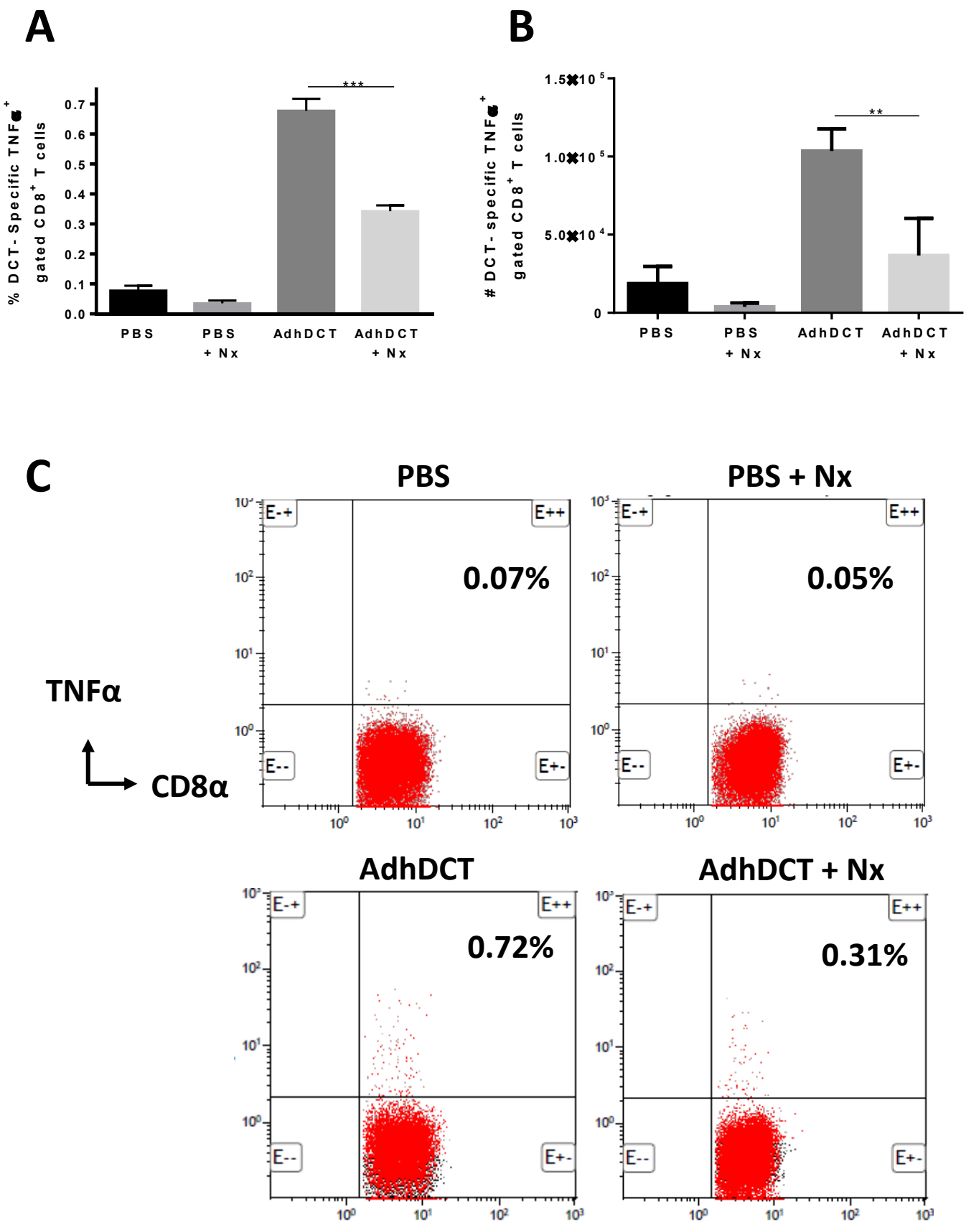
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
B
DCT-specific IFNγ+ gated CD8+ T cells
4.0✖10 7
3.0✖10 7
2.0✖10 7
1.0✖10 7
0
**
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
C
IFNγ
CD8α
PBS
D-+
D++
0.09%
D--
D+-
PBS + Nx
0.10%
AdhDCT
2.07%
AdhDCT + Nx
0.65%

Figure 2. Proportion and absolute number of IFNγ producing CD8+ T cells following therapeutic vaccination with AdhDCT in surgically-stressed mice. C57BL/6 mice were challenged SC with 3×10^5 B16F10lacZ cells on day 0 and then injected IM with PBS or 1×10^8 pfu AdhDCT on day 7. On day 14, all mice were anesthetized and underwent complete tumour resection. Some anesthetized mice additionally received a full laparotomy followed by a left nephrectomy (Nx) to induce surgical stress. All mice were euthanized 18 hours following surgery and their spleens were resected. Splenic lymphocytes were isolated and restimulated *in vitro* with DCT peptide for 6 hours in the presence of brefeldin A. Cells were then perforated and stained intracellularly for IFNγ with fluorochrome-conjugated antibodies for FACS analysis. **a)** The proportion of $CD8^+$ T cells producing IFNγ. **b)** The absolute number of $CD8^+$ T cells producing IFNγ. **c)** Representative flow dot plots. Statistical analysis was evaluated using a Student's t-test and a one-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.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% DCT-Specific TNF+ gated CD8+ T cells
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PBS + Nx
AdhDCT
AdhDCT + Nx
B
DCT-specific TNF+ gated CD8+ T cells
1.5✖10 5
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5.0✖10 4
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PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
C
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
TNFα
CD8α
E-+
E++
E--
E+-
0.07%
0.05%
0.72%
0.31%

Figure 3. Proportion and absolute number of TNFα producing CD8+ T cells following therapeutic vaccination with AdhDCT in surgically-stressed mice. C57BL/6 mice were challenged SC with 3×10^5 B16F10lacZ cells on day 0 and then injected IM with PBS or 1×10^8 pfu AdhDCT on day 7. On day 14, all mice were anesthetized and underwent complete tumour resection. Some anesthetized mice additionally received a full laparotomy followed by a left nephrectomy (Nx) to induce surgical stress. All mice were euthanized 18 hours following surgery and their spleens were resected. Splenic lymphocytes were isolated and restimulated *in vitro* with DCT peptide for 6 hours in the presence of brefeldin A. Cells were then perforated and stained intracellularly for TNFα with fluorochrome-conjugated antibodies for FACS analysis. **a)** The proportion of $CD8^+$ T cells producing TNFα. **b)** The absolute number of $CD8^+$ T cells producing TNFα. **c)** Representative flow dot plots. Statistical analysis was evaluated using a Student's t-test and a one-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

4-1BB on DCT-specific $CD8^+$ T cells was determined using flow cytometric methods. $CD8^+$ T cells from tumour-bearing AdhDCT-vaccinated mice displayed a significant upregulation of the 4-1BB molecule compared to those receiving the mock PBS vaccination ($p < 0.01$). However, AdhDCT-vaccinated mice undergoing the additional surgery displayed no significant difference in their 4-1BB expression profiles (**Figure 4**).

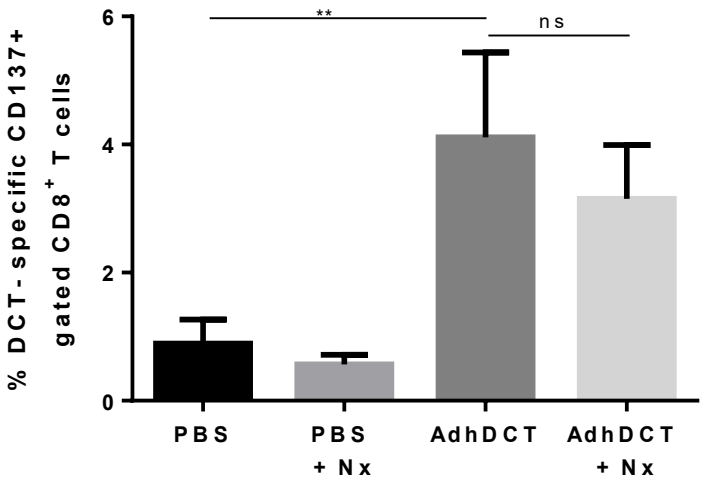
3.4 AdhDCT vaccination results in an accumulation of T_{regs} in the spleen but there is no additive effect 18 hours post-surgery

We then sought to determine if there were any immune cellular mechanisms that might play a role in the DCT antigen-specific $CD8^+$ T cell dysfunction that we have thus far observed in our therapeutic vaccination model of surgical stress. We first assessed which important immune regulatory cells expand within 18 hours of surgical stress, following the same timeline as in **Figure 1a**. T_{regs} are notorious for the role in maintaining immune cell homeostasis by suppressing effector cells. The proportion of T_{regs} in the spleen increase 7 days post AdhDCT vaccination ($p < 0.01$) compared to those receiving the mock PBS injection ($p < 0.01$). However, the proportion does not significantly change within the 18 hours post-surgery (**Figure 5a, b**).

3.5 $CD11b^+Gr\text{-}1^{hi}$ granulocytic MDSCs significantly expand within 18 hours of surgical stress

We then examined the proportion of $CD11b^+Gr\text{-}1^{hi}$ granulocytic (g-) and $CD11b^+Gr\text{-}1^{dim}$ monocytic (m-) MDSCs, which are immune cells that have potent capabilities to suppress the immune system. In this case, there was no significant differences in the

A


% DCT-specific CD137+ gated CD8+ T cells
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2
0
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PBS
PBS + Nx
AdhDCT
AdhDCT + Nx

B

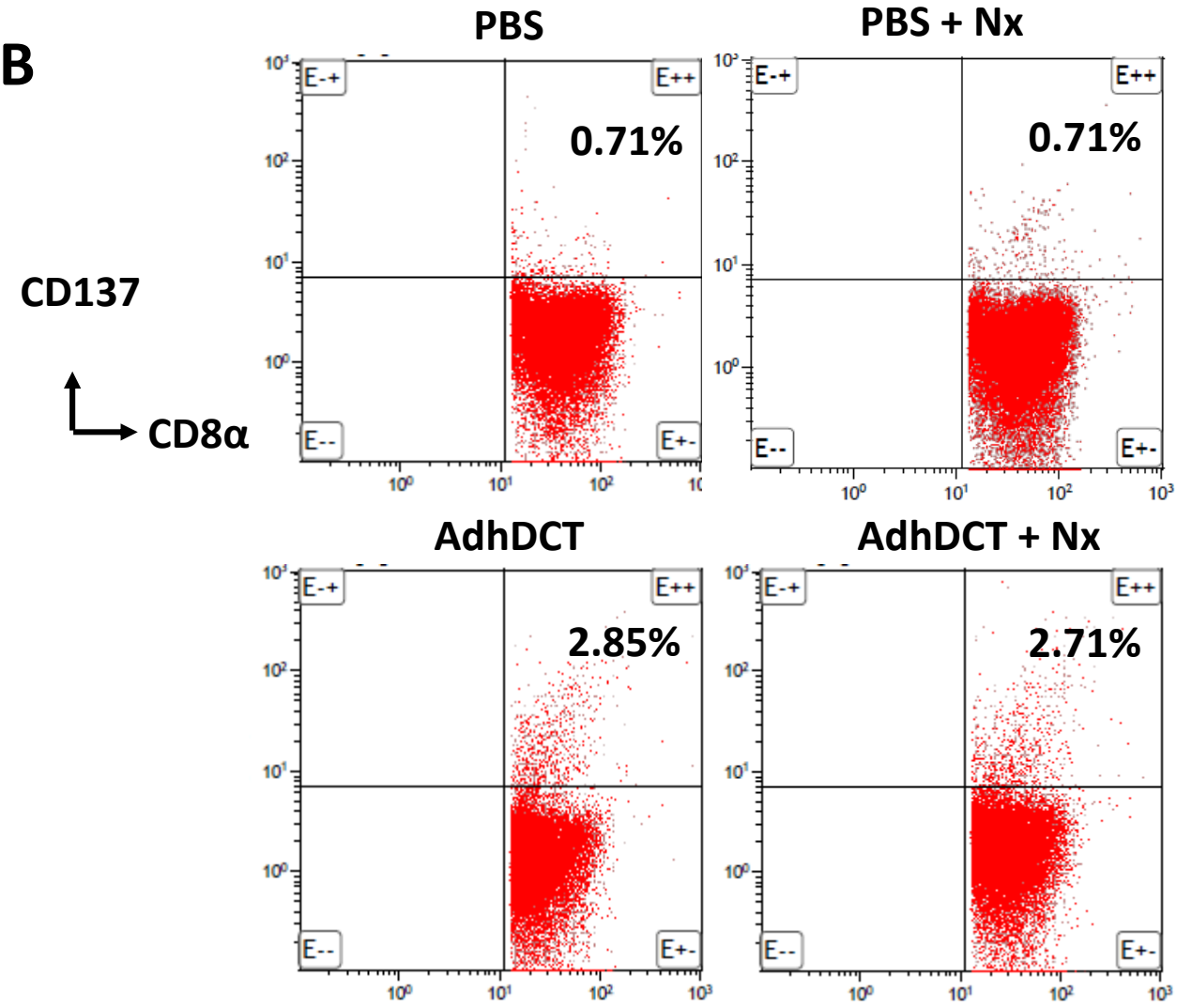
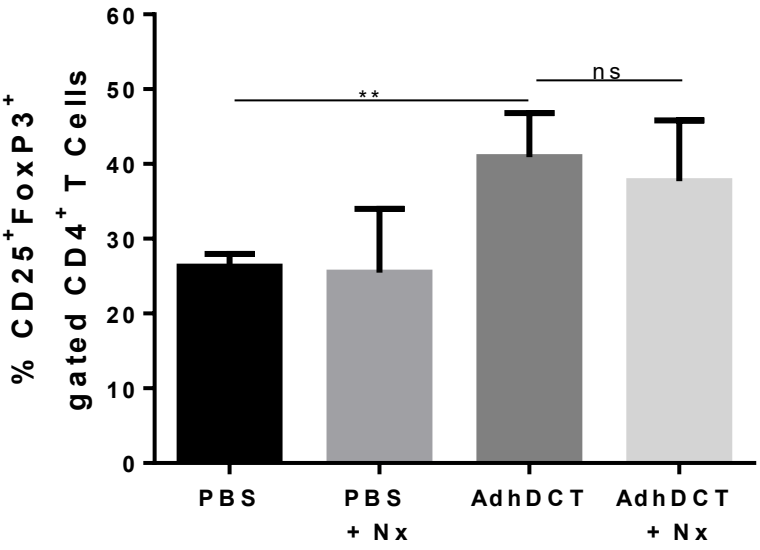

PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
CD137
CD8α
E-+
E++
E--
E+-
0.71%
0.71%
2.85%
2.71%

Figure 4. Proportion of splenic CD8 T cells expressing CD137 (4-1BB) following therapeutic vaccination with AdhDCT in surgically-stressed mice. C57BL/6 mice were challenged SC with 3×10^5 B16F10lacZ cells on day 0 and then injected IM with PBS or 1×10^8 pfu AdhDCT on day 7. On day 14, all mice were anesthetized and underwent complete tumour resection. Some anesthetized mice additionally received a full laparotomy followed by a left nephrectomy (Nx) to induce surgical stress. All mice were euthanized 18 hours following surgery and their spleens were resected. Splenic lymphocytes were isolated and restimulated *in vitro* with DCT peptide for 24 hours. Cells were stained with extracellular fluorochrome-conjugated antibodies for FACS analysis. **a)** The proportion of $CD8^+$ T cells expressing CD137. **b)** Representative flow dot plots. Statistical analysis was evaluated using a Student's t-test and a one-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

A


% CD25+FoxP3+ gated CD4+ T Cells
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50
40
30
20
10
0
**
ns
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx

B

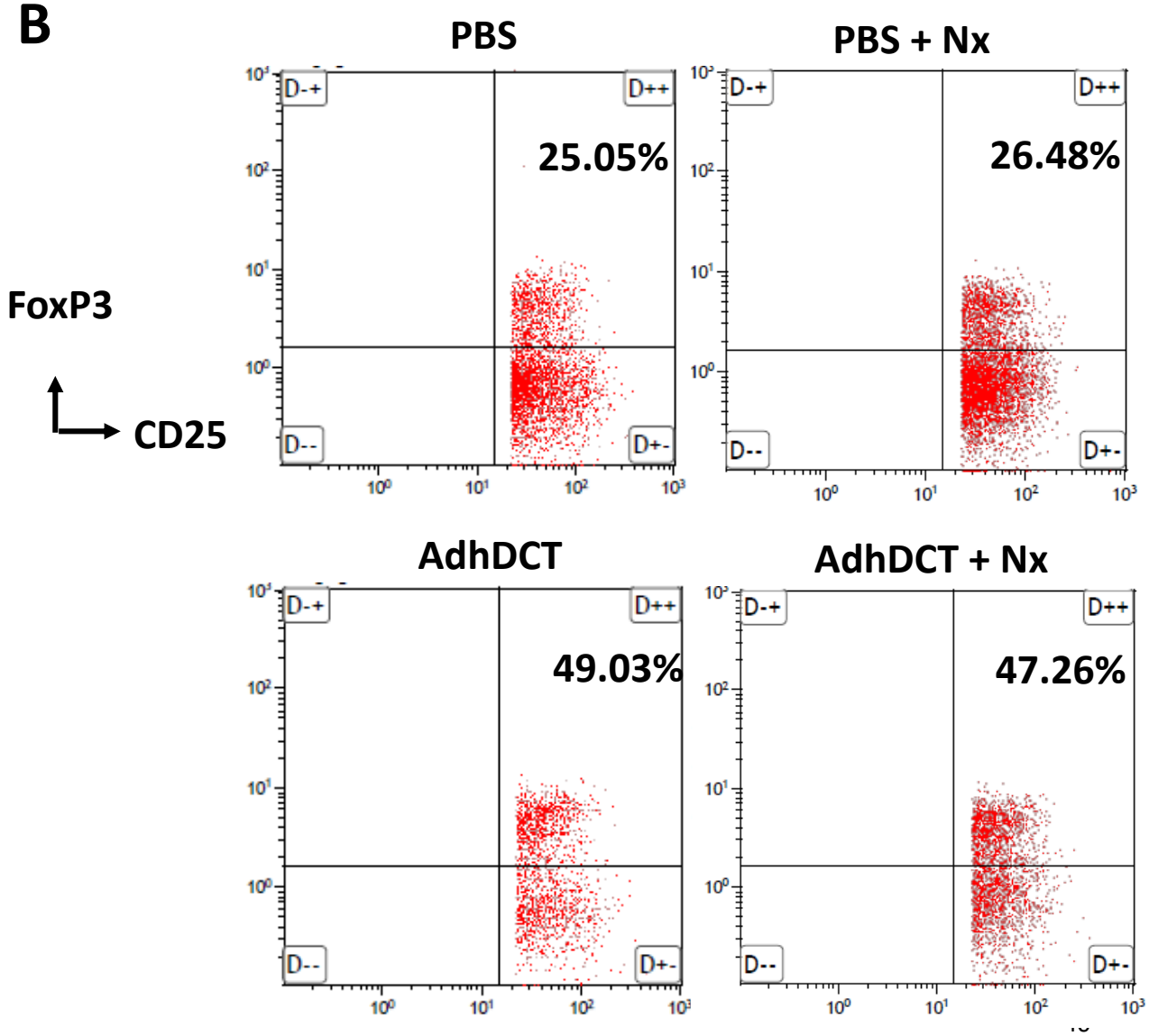

PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
FoxP3
CD25
D-+
D++
D--
D+-
25.05%
26.48%
49.03%
47.26%

Figure 5. Proportion of splenic Tregs following therapeutic vaccination with AdhDCT in surgically-stressed mice. C57BL/6 mice were challenged SC with 3×10^5 B16F10lacZ cells on day 0 and then injected IM with PBS or 1×10^8 pfu AdhDCT on day 7. On day 14, all mice were anesthetized and underwent complete tumour resection. Some anesthetized mice additionally received a full laparotomy followed by a left nephrectomy (Nx) to induce surgical stress. All mice were euthanized 18 hours following surgery and their spleens were resected. Splenic lymphocytes were isolated and stained with fluorochrome-conjugated antibodies for FACS analysis. **a)** The proportion of $CD25^+FoxP3^+$ gated $CD4^+$ T cells **b)** Representative flow dot plots. Statistical analysis was evaluated using a Student's t-test and a one-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

proportion of g-MDSCs in the spleen between tumour-bearing mice vaccinated with AdhDCT or PBS. The proportion of g-MDSCs did however significantly increase in tumour-bearing mice receiving AdhDCT and those receiving the addition of surgical stress ($p < 0.05$). Only a slight increase was observed between PBS vaccinated tumour-bearing mice and those receiving PBS and surgery. (**Figure 6a, c, 7a**).

There appeared to be a slight decrease in the proportion of m-MDSCs following surgical stress in AdhDCT vaccinated tumour-bearing mice compared to non-surgically stressed mice. This trend however, did not reach statistical significance (**Figure 6b, c**).

We then wished to determine if the additional surgery was responsible for the expansion of g-MDSC expansion and not the result of the tumour. We therefore repeated this experiment including a control group that received just AdhDCT vaccination alone with no implanted tumour. In this setting, the expansion of g-MDSCs was similar between mice receiving vaccination alone and those also implanted with a B16 tumour (**Figure 7b**). We additionally confirmed that the proportion of m-MDSCs do not significantly change with the addition of surgical stress in tumour-bearing AdhDCT-vaccinated mice (**Figure 7c**). Furthermore, m-MDSCs are also unaffected by the tumour alone (**Figure 7d**).

3.6 *In vivo* depletion of MDSCs using a Gr-1 antibody also partially depletes the $CD8^+$ T cell population

Our next objective was to determine if the expansion of MDSCs was impacting T cell function in our model and if their depletion during the perioperative period would translate to enhanced survival in AdhDCT vaccinated mice undergoing surgery. Before committing to a survival experiment, we conducted an experiment to ensure that MDSCs would be

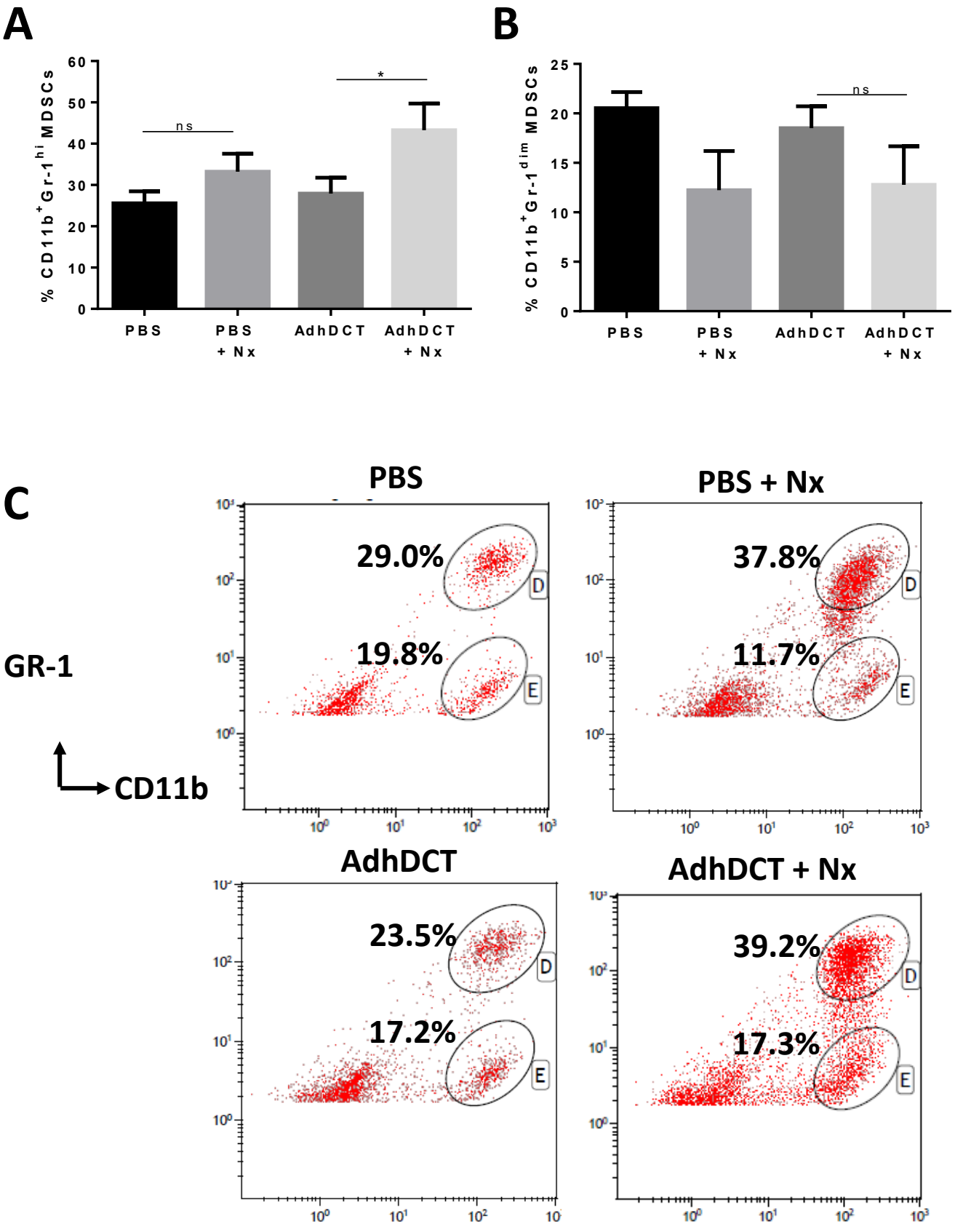
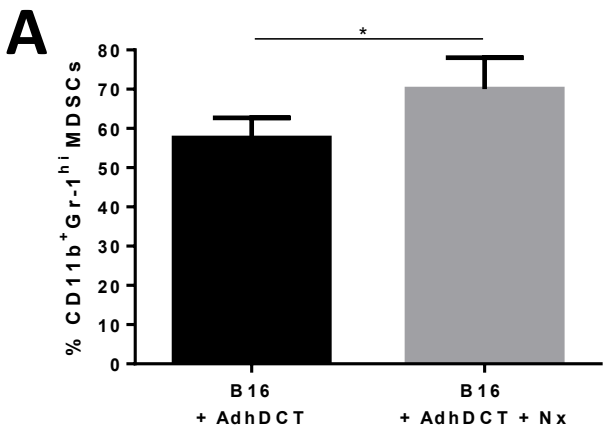
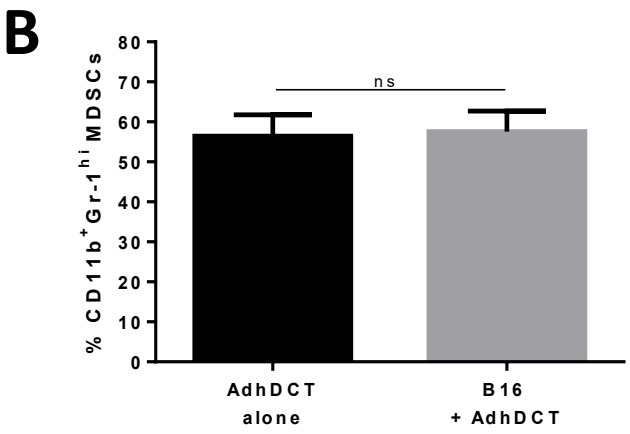
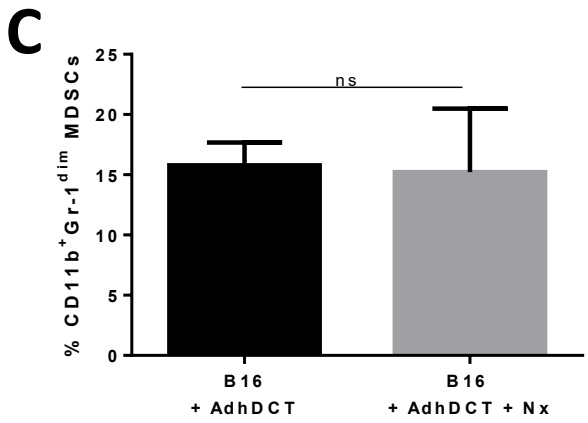

A
% CD11b+Gr-1hi MDSCs
60
50
40
30
20
10
0
ns
*
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
B
% CD11b+Gr-1dim MDSCs
25
20
15
10
5
0
ns
PBS
PBS + Nx
AdhDCT
AdhDCT + Nx
C
PBS
29.0%
19.8%
D
E
PBS + Nx
37.8%
11.7%
D
E
GR-1
CD11b
AdhDCT
23.5%
17.2%
D
E
AdhDCT + Nx
39.2%
17.3%
D
E

Figure 6. Accumulation of MDSCs in the spleen following therapeutic vaccination with AdhDCT in surgically-stressed mice. C57BL/6 mice were challenged SC with 3×10^5 B16F10lacZ cells on day 0 and then injected IM with PBS or 1×10^8 pfu AdhDCT on day 7. On day 14, all mice were anesthetized and underwent complete tumour resection. Some anesthetized mice additionally received a full laparotomy followed by a left nephrectomy (Nx) to induce surgical stress. All mice were euthanized 18 hours following surgery and their spleens were resected. Splenic lymphocytes were isolated and stained for $CD11b^+$ $Gr\text{-}1^+$ with extracellular fluorochrome-conjugated antibodies and detected by flow. **a)** The proportion of $CD11b^+$ $Gr\text{-}1^{hi}$ granulocytic MDSCs. **b)** The proportion of $CD11b^+$ $Gr\text{-}1d^{im}$ monocytic MDSCs. **c)** Representative flow dot plots. Statistical analysis was evaluated using a Student's t-test and a one-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.


A
*
% CD11b+Gr-1hi MDSCs
80
70
60
50
40
30
20
10
0
B16
+ AdhDCT
B16
+ AdhDCT + Nx


B
ns
% CD11b+Gr-1hi MDSCs
80
70
60
50
40
30
20
10
0
AdhDCT
alone
B16
+ AdhDCT


C
ns
% CD11b+Gr-1dim MDSCs
25
20
15
10
5
0
B16
+ AdhDCT
B16
+ AdhDCT + Nx

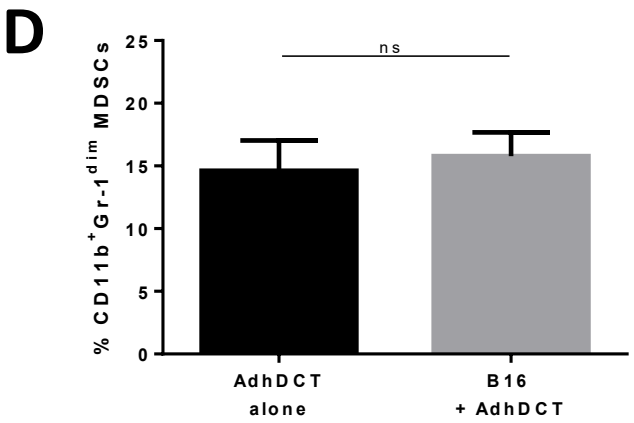

D
ns
% CD11b+Gr-1dim MDSCs
25
20
15
10
5
0
AdhDCT
alone
B16
+ AdhDCT

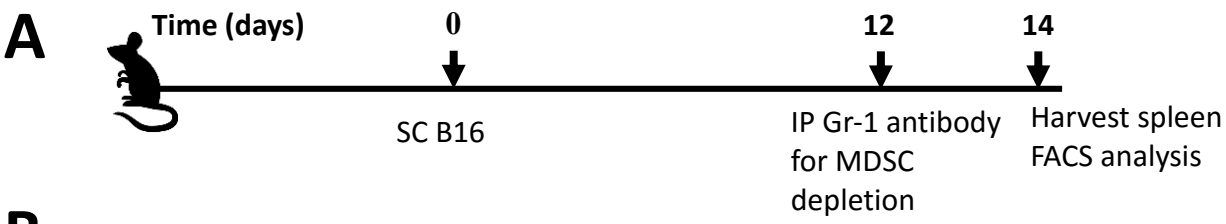
Figure 7. Surgical stress results in a further accumulation of MDSCs in the spleen than the tumour alone. C57BL/6 mice were either untreated or challenged SC with 3×10^5 B16F10lacZ cells on day 0. On day 7, all mice were injected IM with 1×10^8 pfu AdhDCT. On day 14, all tumour-bearing mice were anesthetized and underwent complete tumour resection. Some anesthetized mice additionally received a full laparotomy followed by a left nephrectomy (Nx) to induce surgical stress. All mice were euthanized 18 hours following surgery and their spleens were resected. Splenic lymphocytes were isolated and stained with extracellular fluorochrome-conjugated antibodies for FACS analysis. **a)** The proportion of $CD11b^+$ $Gr\text{-}1^{hi}$ granulocytic MDSCs compared between mice receiving resection alone, and those receiving additional surgical stress. **b)** The proportion of $CD11b^+$ $Gr\text{-}1^{hi}$ granulocytic MDSCs compared between AdhDCT vaccinated mice with and without a tumour. **c)** The proportion of $CD11b^+$ $Gr\text{-}1^{dim}$ monocytic MDSCs with and without additional surgery and **d)** with and without tumour. Statistical analysis was evaluated using a Student's t-test and a one-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

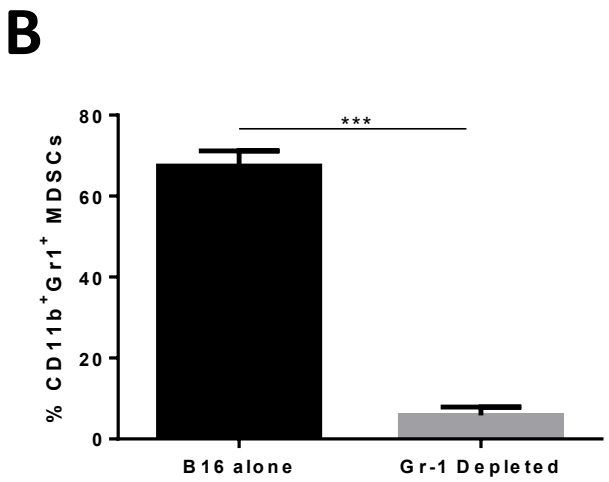
depleted on the day of surgery in our traditional timeline, which would be day 14. Mice were injected with B16 tumour cells on day 0 and then received a single IP injection with a pure Gr-1 antibody (100 μg) on day 12 (**Figure 8a**).

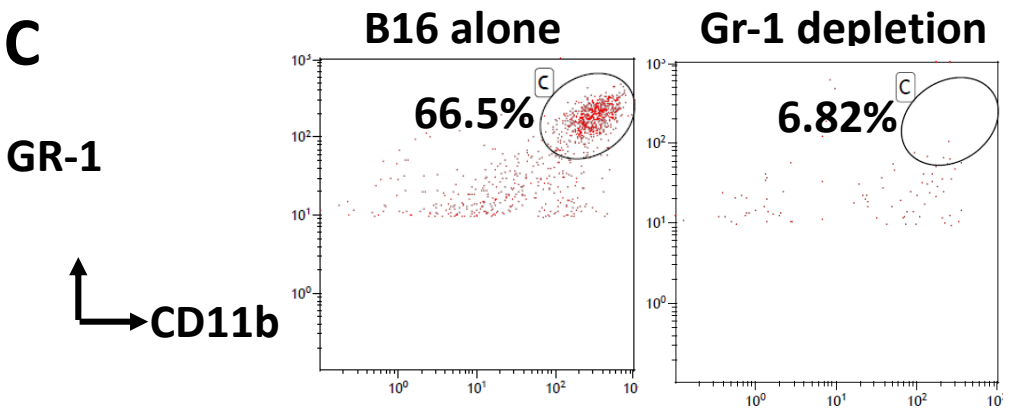
Mice were subsequently sacrificed on day 14 to evaluate the degree of MDSC depletion in their spleen. Tumour-bearing mice injected with Gr-1 antibody demonstrated a significant decrease the proportion of splenic $CD11b^{+}Gr\text{-}1^{+}$ MDSCs compared to those not injected with Gr-1 (**Figure 8b, c**). As a precaution, we additionally examined the proportion of splenic T cells. The addition of the Gr-1 antibody also significantly diminshed the proportion of $CD8^{+}$ T cells (**Figure 8d**) while the $CD4^{+}$ T cell population remained unchanged (**Figure 8e**).

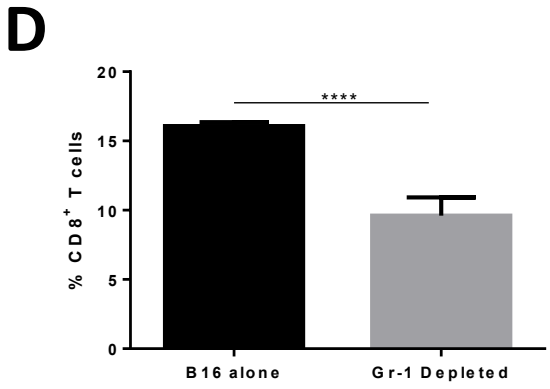
3.7 Depletion of MDSCs *in vivo* using common chemotherapeutic drugs Gemcitabine or 5-FU was unsuccessful

As an alternative to Gr-1 antibody to deplete MDSCs *in vivo*, we sought to use clinically relevant chemotherapeutic drugs Gemcitabine or 5-FU which have reported cytotoxic capabilities on MDSCs. On day 0, mice were injected with B16 tumour cells and then given a single injection of Gemcitabine (120mg/kg) or 5-FU (80 mg/mL) on day 12 (**Figure 9a**). When mice were sacrificed on day 14, we observed no significant difference in the proportion of splenic MDSCs between untreated tumour-bearing mice and those treated with either Gemcitabine or 5-FU (**Figure 9b**). Additionally, these chemotherapeutic drugs did not impact the proportion of $CD8^{+}$ or $CD4^{+}$ T cells in the spleen (**Figure 9c, d**).


A
Time (days)
0
12
14
SC B16
IP Gr-1 antibody for MDSC depletion
Harvest spleen FACS analysis


B
***
% CD11b+Gr1+ MDSCs
80
60
40
20
0
B16 alone
Gr-1 Depleted


C
B16 alone
Gr-1 depletion
GR-1
CD11b
66.5%
6.82%
C


D
****
% CD8+ T cells
20
15
10
5
0
B16 alone
Gr-1 Depleted

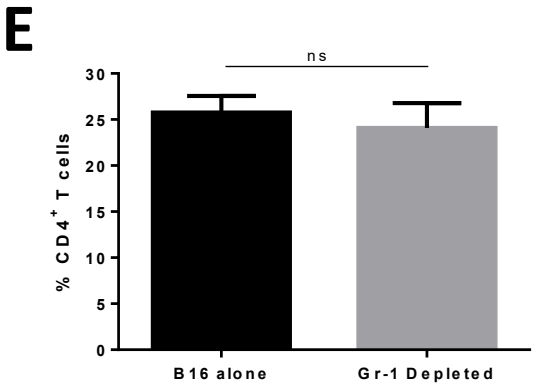
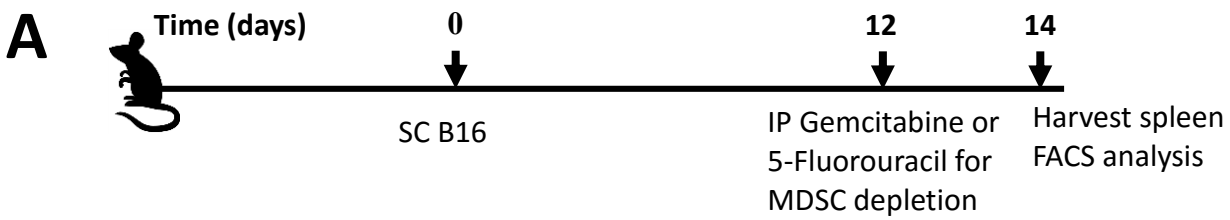
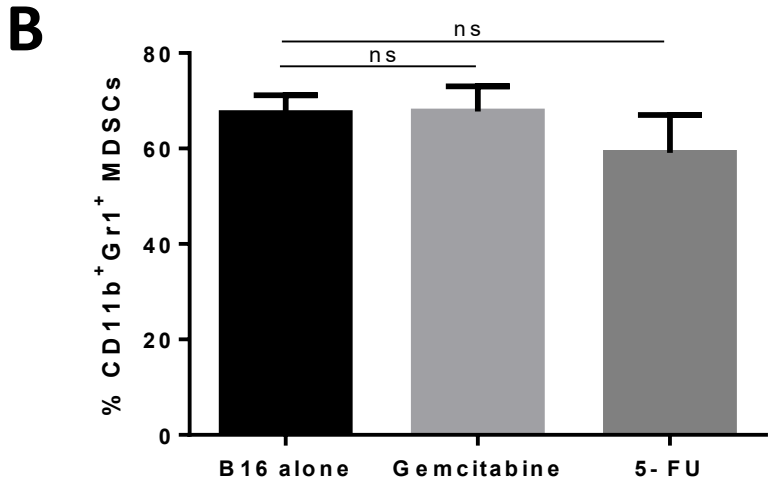
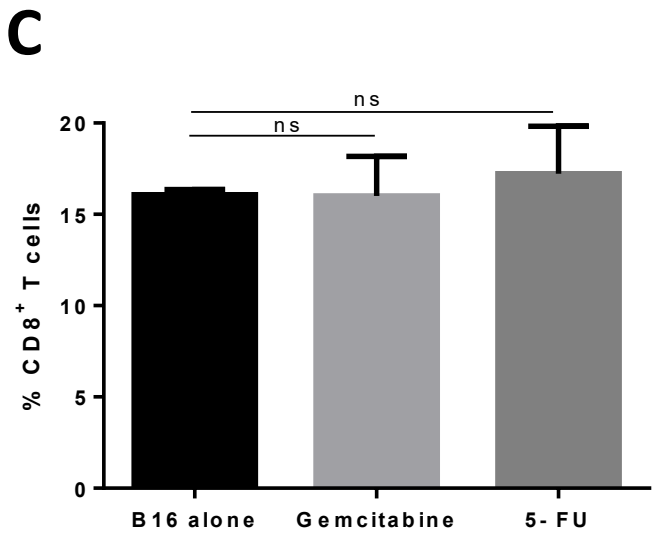

E
ns
% CD4+ T cells
30
25
20
15
10
5
0
B16 alone
Gr-1 Depleted

Figure 8. Perioperative depletion of MDSCs in vivo using a Gr-1 antibody. a) Experimental timeline. C57BL/6 mice were challenged SC with 3×10^5 B16F10lacZ cells on day 0. Some mice were then injected IP with Gr-1 anitbody on day 12. On day 14, all mice were euthanized and their spleens were resected. Splenic lymphocytes were isolated and fluorescently labelled for FACS analysis. **b)** The proportion of $CD11b^+$ $Gr\text{-}1^{hi}$ granulocytic MDSCs with and without Gr-1 antibody treatment. **c)** Representative flow dot plots. **d)** Proportion of $CD4^+$ T cells and **e)** $CD8^+$ T cells with and without Gr-1 antibody treatment. Statistical analysis was evaluated using a Student's t-test and a one-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.


A
Time (days)
0
12
14
SC B16
IP Gemcitabine or
5-Fluorouracil for
MDSC depletion
Harvest spleen
FACS analysis


B
ns
ns
80
60
40
20
0
% CD11b+Gr1+ MDSCs
B16 alone
Gemcitabine
5- FU


C
ns
ns
20
15
10
5
0
% CD8+ T cells
B16 alone
Gemcitabine
5- FU

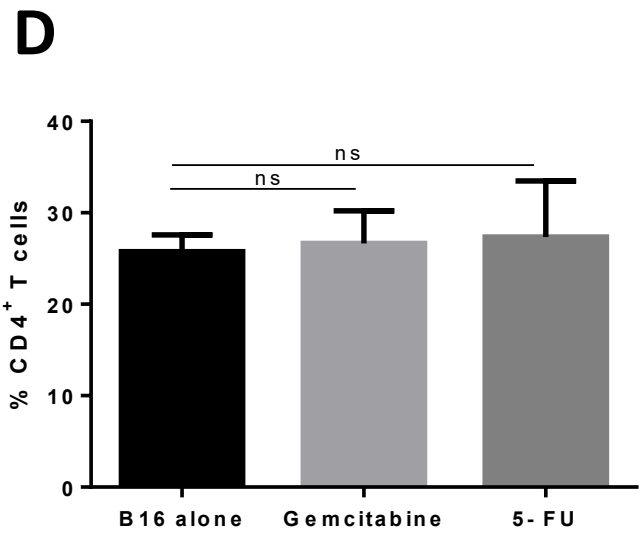
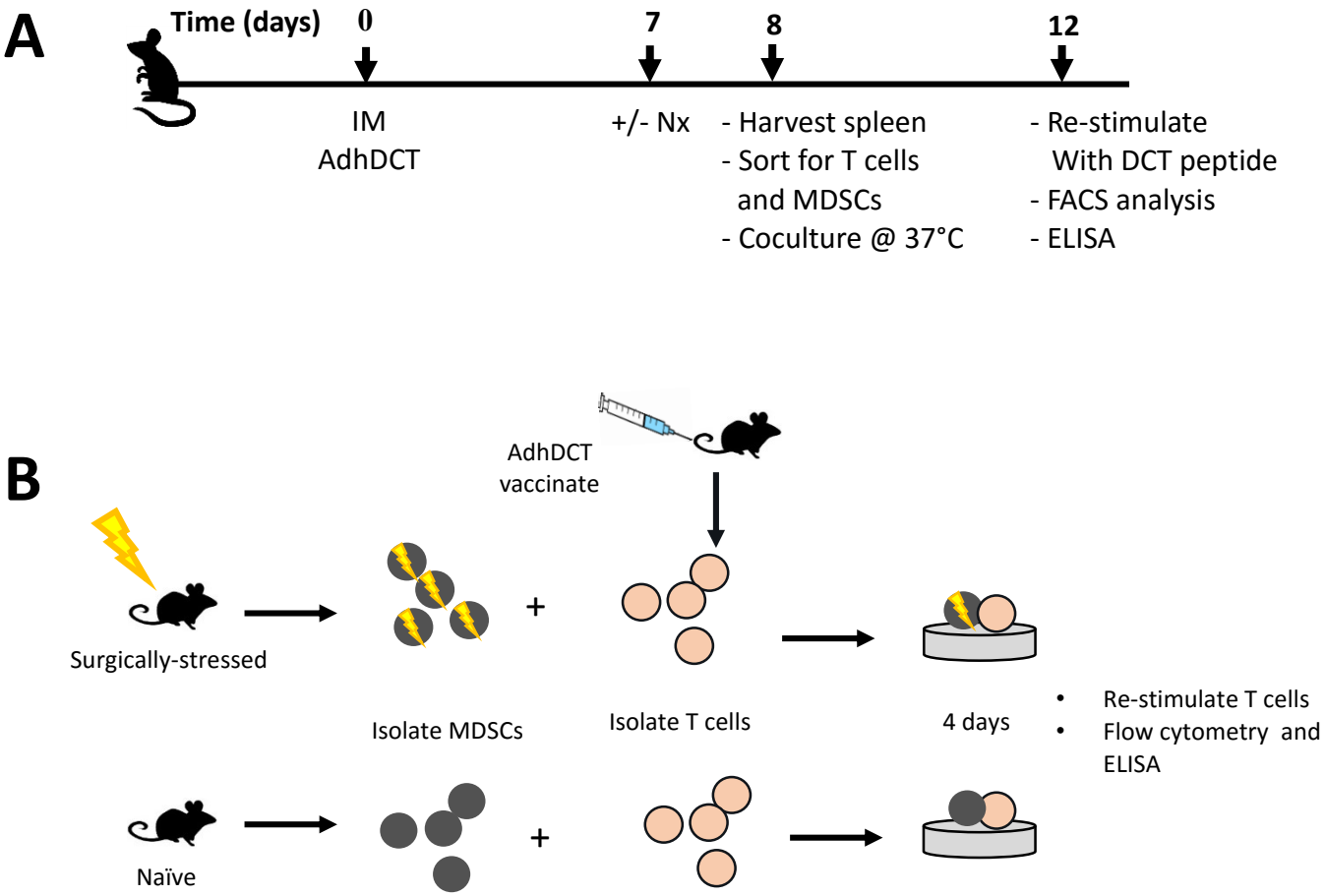

D
ns
ns
40
30
20
10
0
% CD4+ T cells
B16 alone
Gemcitabine
5- FU

Figure 9. Perioperative depletion of MDSCs in vivo using two common chemotherapeutics, Gemcitabine and 5-FU. a) Experimental timeline. C57BL/6 mice were challenged SC with 3×10^5 B16F10lacZ cells on day 0. Some mice were then injected IP with Gemcitabine or 5-FU on day 12. On day 14, all mice were euthanized and their spleens were resected. Splenic lymphocytes were isolated and fluorescently labelled for FACS analysis. **b)** The proportion of $CD11b^+$ $Gr\text{-}1^{hi}$ granulocytic MDSCs with and without chemotherapy treatment. **c)** Proportion of $CD8^+$ T cells and **d)** $CD4^+$ T cells with and without chemotherapy treatment. Statistical analysis was evaluated using a Student's t-test and a one-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

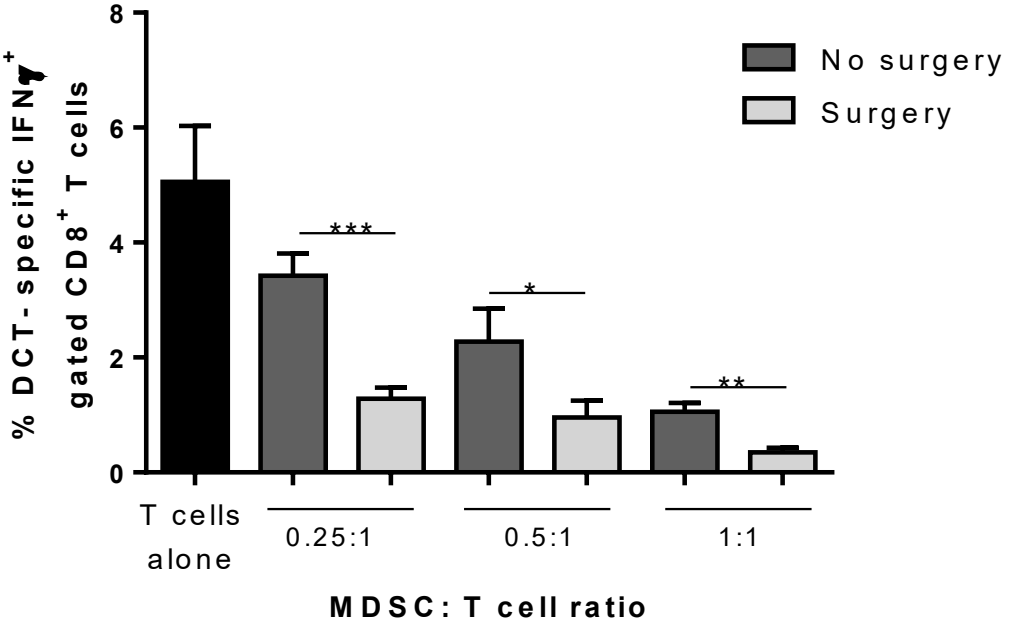
3.8 Non-specific and DCT-specific $CD8^+$ effector T cells have an impaired ability to produce and secrete cytokines when in direct contact with MDSCs from surgically stressed mice

Our project aim was to show that surgery-induced MDSCs were suppressive of $CD8^+$ T cells in our therapeutic model. Since we ran into some difficulty investigating the *in vivo* relationship between MDSCs and and $CD8^+$ T cells, we chose to approach the same objective in a different manner. We therefore performed an *in vitro* coculture using isolated T cells from AdhDCT vaccinated mice and MDSCs isolated from naïve or surgically-stressed mice. In this experiment, we chose not to tumour challenge these mice because, as in **Figure 7b and d**, we concluded that the presence of the tumour did not further enhance the proportion of MDSCs in AdhDCT-vaccinated mice.

The experiment was designed using 3 treatment groups of mice: AdhDCT-vaccinated T cell-donors, naïve MDSC-donors, and surgically-stressed MDSC-donors. On day 0, T cell-donor mice were injected IM with AdhDCT. Surgically-stressed MDSC-donors underwent our traditional regimen of a laparotomy and left nephrectomy on day 7, exactly the same as previous experiments. Naïve MDSC-donors remained untouched. On day 8, spleens were harvested from all 3 groups and either T cells or MDSCs were MACS-isolated from it depending on their group designation. T cells from AdhDCT-vaccinated mice were put into coculture with MDSCs from either naïve or surgically-stressed mice at different ratios in the presence of costimulation (CD3/CD28) and IL-2. After 4 days of coculture, T cells were restimulated with DCT peptide in order to elicit a DCT-specific response (**Figure 10 a, b**), or they were restimulated with PMA and Ionomycin to evoke a non-specific T cell response (**Figure 11a**). Supernatant was collected following restimulation and stored at -80°C for a


A
Time (days)
0
7
8
12
IM
AdhDCT
+/- Nx
- Harvest spleen
- Sort for T cells
and MDSCs
- Coculture @ 37°C
- Re-stimulate
With DCT peptide
- FACS analysis
- ELISA
B
AdhDCT
vaccinate
Surgically-stressed
Isolate MDSCs
Isolate T cells
4 days
Re-stimulate T cells
Flow cytometry and
ELISA
Naïve
C
No surgery
Surgery
% DCT-specific TNF+
gated CD8+ T cells
8
6
4
2
0
**
***
**
T cells
alone
0.25:1
0.5:1
1:1
MDSC: T cell ratio

D


% DCT- specific IFNγ+ gated CD8+ T cells
8
6
4
2
0
No surgery
Surgery
***
*
**
T cells alone
0.25:1
0.5:1
1:1
MDSC: T cell ratio

E

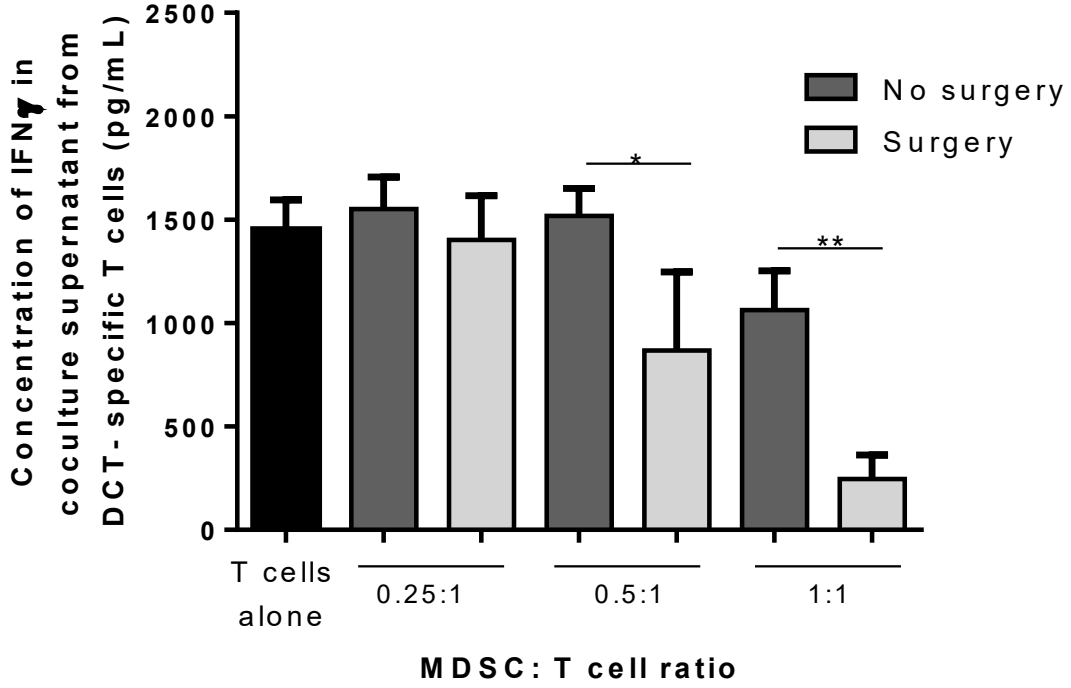
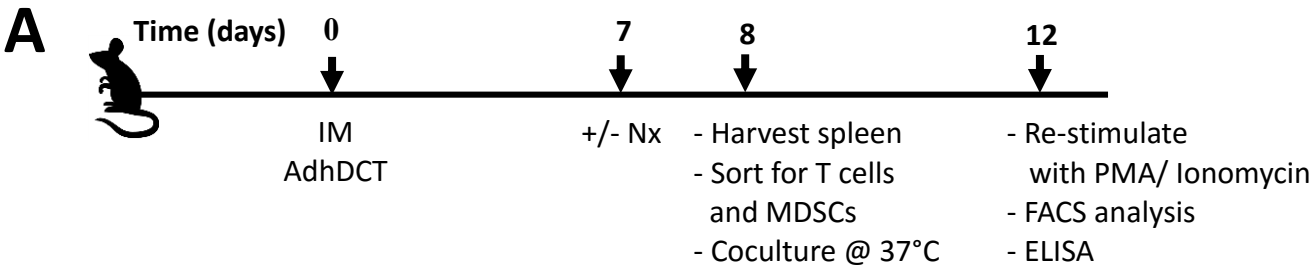
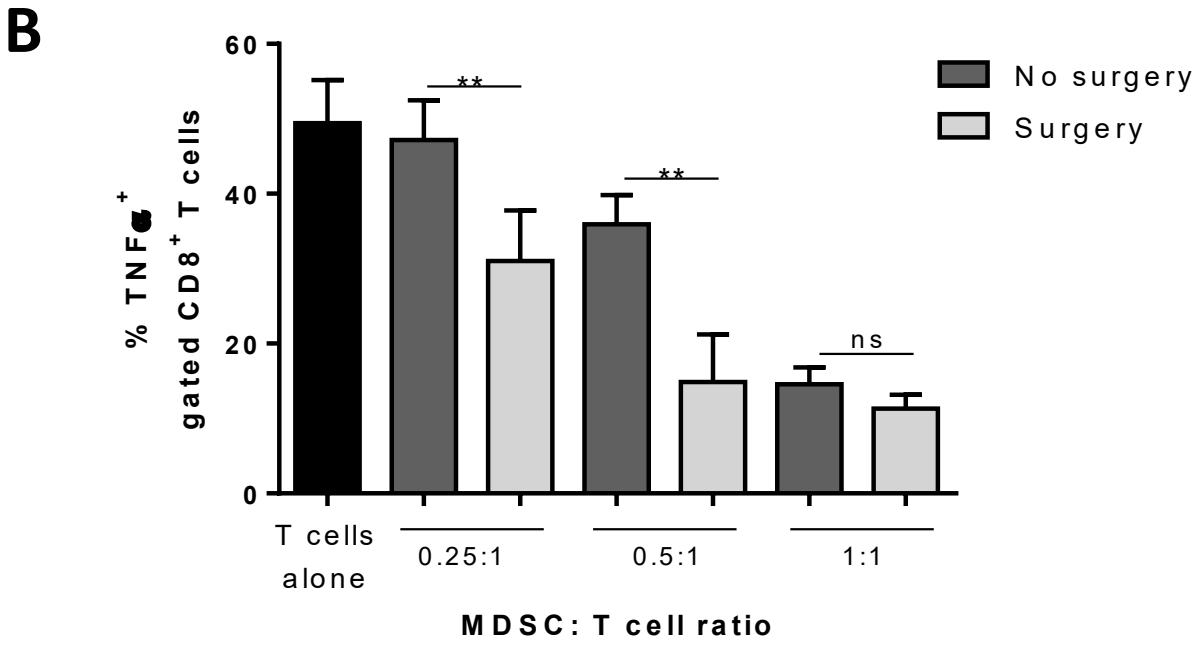
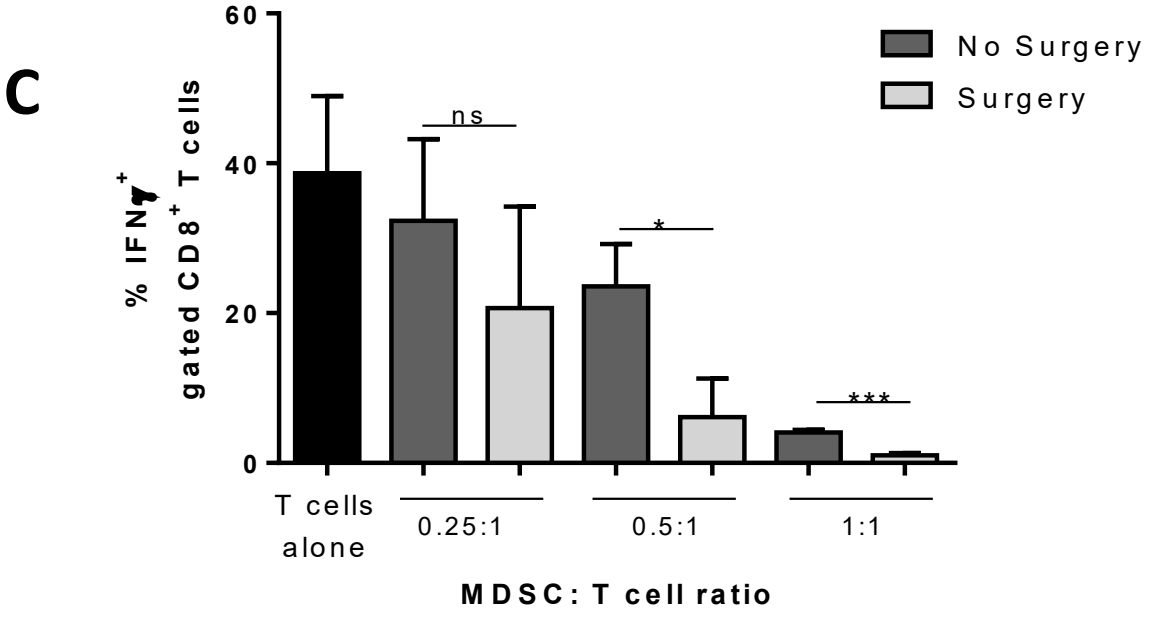

Concentration of IFNγ in coculture supernatant from DCT- specific T cells (pg/mL)
2500
2000
1500
1000
500
0
No surgery
Surgery
*
**
T cells alone
0.25:1
0.5:1
1:1
MDSC: T cell ratio

Figure 10. In vitro T cell coculture with MDSCs originating from naïve or surgically-stressed mice with DCT-specific IFNγ and TNFα readout. a) and b) Experimental timeline and design. C57BL/6 T cell donor mice were injected IM with AdhDCT on day 0. C57BL/6 MDSC donor mice were either untouched or subjected to a full laparotomy followed by a left nephrectomy (Nx) to induce surgical stress on day 7. On day 8, all mice were euthanized and their spleens resected so that either their T cells or MDSCs could be isolated using MACS separation. 1×10^6 T cells were then cocultured with different ratios of MDSCs isolated from naïve or surgically-stressed mice for 4 days. After 4 days, supernatants were collected and cells were restimulated with DCT peptide for 6 hours in the presence of brefeldin A. Supernatants were then collected again and cells were stained using extra-and intracellular fluorochrome-conjugated antibodies for FACS analysis. **c)** The proportion of DCT-specific $CD8^+$ T cells producing TNFα and **d)** IFNγ under different coculture conditions. **e)** ELISA to further evaluate the proportion of T cells secreting IFNγ to the supernatant following restimulation with DCT peptide. Statistical analysis was evaluated using a Student's t-test and a one-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$.


A
Time (days)
0
7
8
12
IM
AdhDCT
+/- Nx
- Harvest spleen
- Sort for T cells
and MDSCs
- Coculture @ 37°C
- Re-stimulate
with PMA/ Ionomycin
- FACS analysis
- ELISA


B
No surgery
Surgery
% TNFα+ gated CD8+ T cells
60
40
20
0
**
**
ns
T cells alone
0.25:1
0.5:1
1:1
MDSC: T cell ratio


C
No Surgery
Surgery
% IFNγ+ gated CD8+ T cells
60
40
20
0
ns
*
***
T cells alone
0.25:1
0.5:1
1:1
MDSC: T cell ratio

D

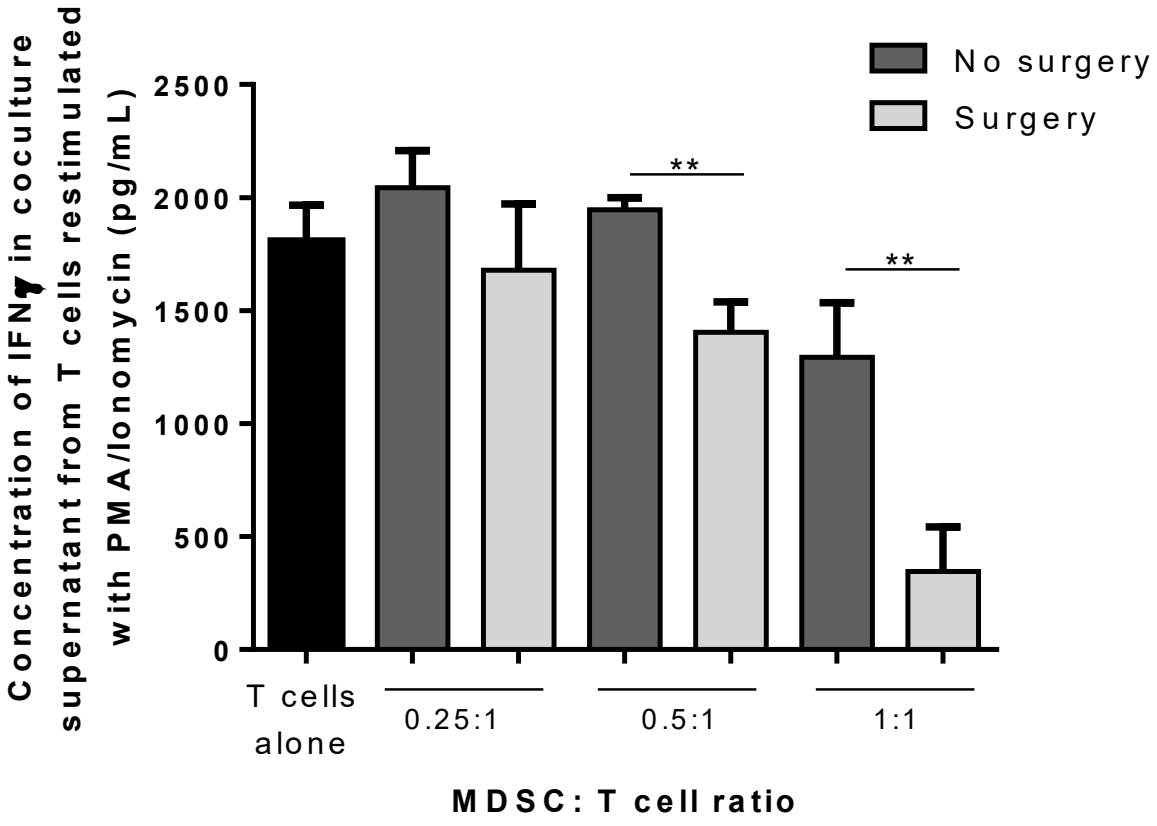

No surgery
Surgery
Concentration of IFNγ in coculture supernatant from T cells restimulated with PMA/Ionomycin (pg/mL)
2500
2000
1500
1000
500
0
**
**
T cells alone
0.25:1
0.5:1
1:1
MDSC: T cell ratio

Figure 11. In vitro T cell coculture with MDSCs originating from naïve or surgically-stressed mice with IFNγ and TNFα readout. a) Experimental timeline and design. C57BL/6 T cell donor mice were injected IM with AdhDCT on day 0. C57BL/6 MDSC donor mice were either untouched or subjected to a full laparotomy followed by a left nephrectomy (Nx) to induce surgical stress on day 7. On day 8, all mice were euthanized and their spleens resected so that either their T cells or MDSCs could be isolated using MACS separation. 1×10^6 T cells were then cocultured with different ratios of MDSCs isolated from naïve or surgically-stressed mice for 4 days. After 4 days, supernatants were collected and cells were restimulated nonspecifically with PMA and Ionomycin for 6 hours in the presence of brefeldin A. Supernatants were then collected again and cells were stained using extra-and intracellular fluorochrome-conjugated antibodies for FACS analysis. **b)** The proportion of $CD8^+$ T cells producing TNFα **c)** and IFNγ under different coculture conditions. **d)** ELISA to further evaluate the proportion of T cells secreting IFNγ to the supernatant following restimulation with PMA and Ionomycin. Statistical analysis was evaluated using a Student's t-test and a one-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

future ELISA assay. Cells were then stained for flow cytometry.

Previously, we had shown that $CD8^+$ T cells from vaccinated, surgically-stressed mice produce less IFNγ and TNFα cytokines in response to their specific antigen, a strong measure of $CD8^+$ T cell function (**Figure 2 and 3**). We therefore chose cytokine production and secretion as a measure of $CD8^+$ T cell function following direct contact with MDSCs. T cells alone, cultured in the presence of CD3/CD28 costimulation and IL-2, served as a positive control in these experiments to show their functional potential in the absence of suppressive cells. As seen by flow cytometry, there is a steady decline in the production of IFNγ and TNFα by DCT-specific $CD8^+$ T cells as the proportion of MDSCs in the culture increase giving an inverse relationship. This effect is even more pronounced when the MDSCs originate from surgically-stressed mice (**Figure 10 c, d**). At the MDSC: T cell ratio 0.25:1, DCT-specific $CD8^+$ T cells produce significantly less TNFα when surgically-stressed MDSCs are present compared to naïve MDSCs ($p < 0.01$). There was also a significant difference bewteen naïve MDSCs and surgically-stressed MDSCs at ratios 0.5:1 ($p < 0.001$) and 1:1 ($p < 0.01$) (**Figure 10c**).

In the case of DCT-specific $CD8^+$ T cells producing IFNγ, the same trend was revealed through flow cytometry. MDSCs from surgically-stressed mice were significantly more suppressive of IFNγ production by $CD8^+$ T cells than naïve MDSCs among all the MDSC: T cell ratios – 0.25:1 ($p < 0.001$), 0.5:1 ($p < 0.05$) and 1:1 ($p < 0.01$) (**Figure 10d**).

Furthermore, we wanted to verify this trend with an IFNγ ELISA assay using the supernatants after restimulation of the T cells with DCT peptide. As expected, there was an impairment in the ability of DCT-specific T cells to secrete IFNγ in the presence of MDSCs

from surgically-stressed mice. Significant differences were observed in the 0.5:1 ($p < 0.05$) and the 1:1 ($p < 0.01$) ratios between T cells cultured with naïve and surgically-stressed MDSCs (**Figure 10e**).

To evoke a non-specific cytokine response, $CD8^+$ T cells were restimulated with PMA and ionomycin following the coculture conditions and then stained for flow cytometry. A decline in the proportion of $TNF\alpha^+$ gated $CD8^+$ T cells was observed as the proportion of MDSCs in the coculture increased. When MDSCs originated from surgically-stressed mice compared to naïve, this suppressive effect was even more pronounced at 0.25:1 ($p < 0.01$) and 0.5:1 ($p < 0.01$) (**Figure 11a**). In the case of $IFN\gamma^+$ gated $CD8^+$ T cells the trend was very similar with significant differences between naïve and surgically-stressed MDSCs at ratios 0.5:1 ($p < 0.05$) and 1:1 ($p < 0.001$) (**Figure 11b**). Furthermore, the IFNγ ELISA generated data displaying the same trend where a higher proportion of MDSCs in contact with the T cells in culture resulted in a decrease of non-specific overall IFNγ secretions. The most significant differences between MDSCs from naïve versus surgically-stressed mice was observed with the 0.5:1 ($p < 0.05$) and 1:1 ($p < 0.05$) ratios (**Figure 11c**).

4 – Discussion

Currently, surgical resection is a leading option in the treatment of solid malignancies. However there is strong preclinical and clinical data to suggest that surgery promotes tumour growth and metastatic disease. This phenomenon has largely been attributed to an immunosuppressive environment induced by surgical stress. To counteract this suppression, cancer immunotherapy is becoming a more prominent area of research

and has delivered promising results in recent clinical trials[103]. Their ability to induce a humoral and tumour-specific immune response with fewer side-effects than traditional therapies make them ideal candidates[121,122]. There is an increasing need to understand how surgery, a mainstay of cancer treatment, impacts the immune system in order to facilitate the use of combination immunotherapies and mitigate the negative effects.

In this study, we characterized surgery-induced CTL dysfunction, despite vaccination with a TAA-expressing adenoviral vector. To evaluate the TAA-specific CTL response post-surgery we designed a clinically relevant, therapeutic tumour-bearing model. Melanoma tumour-bearing mice were vaccinated with AdhDCT, a melanoma TAA-expressing viral vector, followed by tumour resection and major surgical stress in the form of laparotomy and left nephrectomy. Previously, our lab demonstrated that neoadjuvant vaccination with AdhDCT can generate a robust DCT-specific T cell immune response that corresponds to a significant survival benefit in mice following the debulking of their tumour. When these mice were exposed to the additional major surgical stress imposed by our model, their survival is significantly decreased as they all succumbed to rapid local recurrence and death[88]. Surgical stress impairs the ability of the host's immune system to attack and defend against MRD leading to a poor prognosis, which is proportional to the amount of stress induced[76,91]. In the present study, we show that a decrease in the overall number of T cells and CTL-dysfunction in particular, occurs within 18 hours post-surgery. Furthermore, a post-operative accumulation of CTL-suppressive MDSCs simultaneously occurs and is partly responsible for this phenomenon.

Interestingly, by flow cytometry, there was no difference in the proportion of both $CD4^+$ and $CD8^+$ T cells between vaccinated mice that underwent resection and those that underwent resection and the additional surgical stress. A significant reduction in the total number of splenic T lymphocytes between these groups however, suggests that surgical stress is affecting both subsets equally. Previous results from our lab suggest that DCT-specific $CD8^+$ T cells are not undergoing apoptosis post-surgery but are significantly less proliferative upon DCT peptide stimulation. Migration of overall and antigen-specific T lymphocytes following trauma towards the inflammatory environment where tissue repair is occurring may also explain this phenomenon[123–125] but has yet to be investigated in this model.

Mediation of an anti-tumour response also requires the assistance of secreted soluble mediators from TAA-specific T cells. Since the number of CTLs was abrogated as a result of surgery, we proceeded to assess their ability to produce two important cytokines, IFNγ and TNFα by flow cytometry 18 hours after surgery. We observed a significant attenuation in cytokine production by DCT-specific CTLs in response to *ex vivo* DCT peptide stimulation, indicating that TAA-specific CTLs are significantly less activated post-surgery. A post-operative attenuation in the proportion and number of antigen-specific CTL production of IFNγ has been described in other models of trauma also[76,93,126]. Previous work in the Auer lab showed that in a prophylactic model, a postoperative attenuation of IFNγ production by DCT-specific CTLs lasted for a minimum of 3 days and fully recovered by postoperative day 28[88].

The expression of molecule 4-1BB (CD137) on the T cell surface is considered a reliable marker of highly reactive antigen-specific T cells. Resting T cells display no 4-1BB expression (<3%) but upon antigen-stimulation, 4-1BB is uniformly upregulated on all antigen-specific T cells between 12 hours and 5 days post-stimulation and its ligand expression is restricted to APCs [32,127–129]. It is a significant mediator of costimulation, anti-apoptotic functions, promoting T cell proliferation and T cell survival[127]. Recently, one study showed that activated 4-1BB CD8+ T cells constituted the majority of expanded tumour-reactive CTLs used in adoptive cell therapy[33]. Another study concluded that enhanced effector functions could be achieved if adoptive cell transfer of TAA-specific CTLs was used in conjunction with agonistic anti-4-1BB monoclonal antibodies[130]. In our model, significant upregulation of 4-1BB on the surface of CTLs isolated from AdhDCT-vaccinate mice was observed upon *ex vivo* stimulation with DCT peptide for 24 hours. Mice that were not vaccinated displayed very limited 4-1BB expression comparatively. We hypothesized that a decrease in 4-1BB expression would occur as a result of surgical stress, however no significant difference was observed suggesting that surgery-induced dysfunction occurs through a different pathway or further downstream. Several other studies have investigated other pathways in which CTL-related dysfunction has been induced following physical injury such as induced anergy and reduced arginine availabilty[21,48,131,132]. Following abdominal laparotomy in mice, increased arginine catabolism by ARG-1 expressed in MDSC thereby limiting the availability of arginine required for T cell function and disrupting TCR formation[48,94]. Patients suffering from burn or traumatic injury demonstrated T cell

unresponsiveness to mitogen activation and diminished proliferation and cytokine secretion[131].

To investigate the potential involvement of cellular mechanisms in the surgery-induced CTL dysfunction observed in our model, we sought to characterize host-derived immunosuppressive cell populations that are commonly assessed in other models of cancer and trauma. T_{Regs} are important in the maintenance of immune homeostasis and the control of inflammation. Elevated levels in the peripheral blood, lymph nodes and tumour microenvironment are associated with poor patient outcomes and depletion *in vivo* enhances vaccine-mediated anti-tumour immunity[133,134]. We therefore assessed if elevated levels were also associated with CTL-dysfunction during the immediate post-operative period. Vaccinated mice displayed a significant upregulation in T_{Reg} proportion, but similar proportions were observed in mice regardless of their surgical status. Vaccination induced T_{Reg} accumulation, but surgery did not further enhance this population. A recent study also established that human patients undergoing major surgery had low levels of circulating T_{Regs} 1 day after surgery compared to pre-operative levels, but was significantly elevated by day 6[135].

Alternatively, we observed a strong expansion of splenic MDSCs 18 hours following surgery, in particular the granulocytic population. In the majority of mouse models, MDSCs are commonly defined by the markers CD11b and Gr-1 which are used to identify the two major subpopulations -granulocytic (g-)($CD11b^{+}Gr\text{-}1^{hi}$) and monocytic (m-)($CD11b^{+}Gr\text{-}1^{hi}$). These immature myeloid cells are generated in the bone marrow and quickly differentiate into mature granulocytes, macrophages or DCs in healthy individuals. However, patients

suffering from a pathological condition such as cancer, infectious disease, bone marrow transplantation, sepsis or trauma, experience a partial block in the differentiation of these cells[51,136]. Various preclinical and clinical tumour models have described an accumulation of MDSCs in the bone marrow, blood and secondary lymphoid organs including the spleen[47,49,137,138].

In our model, the elevated level of splenic g-MDSCs was a direct effect of the surgery-induced trauma imposed on the mice and not a result of the tumour. It is possible that the presence of a tumour did not elevate MDSC levels because the tumour-burden was too small to have a significant impact at the time of endpoint. Levels of circulating MDSCs have been strongly correlated with the clinical stage of cancer[3,139]. Unfortunately, this may indicate that despite tumour resection at an early stage of the cancer, the consequences of surgery may lead to an accumulation of MDSCs and an increased risk of tumour recurrence if not all MRD is eliminated. This further highlights the need for perioperative therapies to boost the TAA-specific response following major surgical stress.

Multiple lines of evidence describe MDSCs as potent inhibitors of anti-tumour immunity. We rationalized that a depletion of surgery-induced MDSCs during the perioperative period would synergize with the boosting effect of AdhDCT vaccination to enhance anti-tumour immunity and prolong survival. Firstly, we performed a preliminary experiment where we administered a common Gr-1 antibody in vivo to eliminate MDSCs during the perioperative period. While successful at depleting the MDSC population, the Gr-1 antibody has been widely criticized for its non-specific depletion of a particular population. The Gr-1 antibody has been previously reported to deplete neutrophils, dendritic cells, and

subpopulations of monocytes and lymphocytes[140–143]. Not surprisingly, the use of Gr-1 antibody also decreased a significant portion of $CD8^+$ T cells in our model, hence why we did not pursue it as a reliable means to assess perioperative MDSC depletion because we are specifically assessing $CD8^+$ T cell responses. Two common chemotherapeutic drugs, Gemcitabine and 5-FU, have been routinely investigated for their capability to deplete MDSCs by triggering their apoptotic death in tumour-bearing mice[144–147]. However, using clinically relevant and previously published[144] doses of either drug did not affect the levels of splenic MDSCs in our tumour model.

The accumulation of MDSCs following surgery however, is not enough to indicate a functional suppression of $CD8^+$ T cells. Their notable ability to suppress T cell function distinguishes them from other more mature monocytes and neutrophils, which express similar markers[148]. We therefore measured the direct interaction between surgically-induced MDSCs and TAA-specific CD8+ T cell cytokine production and secretion. The *in vitro* coculture of MDSCs isolated from naïve or surgically-stressed mice with T cells isolated from AdhDCT-vaccinated mice at various cellular ratios led to a significant attenuation of DCT-specific $CD8^+$ T cell cytokines. Although many tumour models indicate a preferential expansion of the g-MDSC subset,[21,139,149,150] their ability to supress antigen-specific T cell responses is equivalent to that of m-MDSCs on a per cell basis despite different mechanisms of action[139]. We therefore did not distinguish between subsets in the in vitro coculture. Following restimulation with DCT peptide, IFNγ and TNFα production and secretion by $CD8^+$ T cells was significantly reduced on a cell-per-cell basis with MDSCs, even more so when the MDSCs originated from surgically-stressed mice. Additionally, overall $CD8^+$ T cell function,

using non-specific stimulation with PMA and ionomycin, resulted in significant deficits in the ability of $CD8^+$ T cells to produce and secrete cytokines when cultured in the presence of MDSCs. PMA and ionomycin directly induce an influx of calcium into the T cell which is required for activation thus bypassing the need for surface receptor stimulation. Since this mode of stimulation is not TCR-dependent, it suggests that MDSC-induced dysfunction affects a downstream target of calcium influx or is not TCR-mediated. This is interesting because several studies conclude that MDSCs interfere with TCR signaling events. Surgery has been shown to induce MDSCs to express arginase 1 (ARG1) leading to the metabolism of circulating arginine, which is an essential amino acid for TCR signaling[48,90,151] and modulation of the ζ-chain[49,93,152]. ROS and peroxynitrites from inducible nitric oxide synthase (iNOS) are largely produced by MDSCs and additionally give rise to TCR-associated dysfunction[3,148,153]. Further delving into these mechanisms will provide additional insight into the targeting mechanisms of the surgery-induced $CD8^+$ T cell dysfunction we observe. In our current model, we were able to conclude that surgically-induced MDSCs are potent inhibitors of critical anti-tumour responses, both overall and TAA-specific, initiated by $CD8^+$ T cells through their cytokine secretions.

Cancer patients routinely undergo surgical resection of their solid tumours to ensure their overall outcome, however, the perioperative period renders them susceptible to tumour escape due to elevated immunosuppressive mechanisms. During this time, one or more critical therapeutic perioperative interventions are needed to block suppressive mechanisms and enhance TAA-specific T cell responses with the goal of eliminating MRD and

improving long-term survival. Despite recent successes using immunotherapy adjuvants, advancements in the type and timing of treatment regimens remain under investigation.

Previous work in the Auer lab demonstrated that preoperative vaccination of tumour-bearing mice undergoing tumour resection prolonged their survival compared to mice that received additional major surgical stress in the same model. In the present study, we show that the surgery-induced vaccine dysfunction we previously observed is partially mediated by MDSC-induced suppression of T cells. A decrease in overall T cell numbers and attenuation of TAA-specific $CD8^+$ T cell cytokine production was observed within 18 hours following surgical stress. A postoperative accumulation of MDSCs demonstrated the ability to suppress TAA-specific $CD8^+$ T cell function through cytokine production and secretion. Importantly, cytokine activity was also abrogated when non-specific restimulation of $CD8^+$ T cells was used, bypassing early TCR signaling. These results may indicate a role for inhibitory receptors such as TIM-3 and PD-1 which have been shown to significantly increase expression in tumour and surgical stress models. Additionally blocking of these receptors in vivo enhanced TAA-specific $CD8^+$ T cell activation[95,96,99].

Surgery is a mainstay of treatment for solid tumours but surgical stress creates an immunosuppressive environment that promotes tumour recurrence and metastases. Understanding the mechanisms of T cell dysfunction, especially those that are TAA-specific, following surgery will facilitate the development of targeted immunotherapies to reverse this effect in the postoperative period.

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Contributions of Collaborators

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