Overcoming Obstacles to Glioma Immunotherapy

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Dedication

This dissertation is dedicated to the memory of John Robert Ohlfest.

Abstract

Glioma is a type of malignant tumor of the non-neuronal cells of the central nervous system, the glia. These tumors are the most common malignant tumors of the central nervous system. The most aggressive and most prevalent of these, glioblastoma multiforme (GBM) is a deadly disease with a grim prognosis, with median survival at diagnosis of less than a year and a half. Standard treatment with irradiation and the DNA alkylating drug temozolomide yields incremental improvement in survival over irradiation alone but better therapies remain needed. Immune therapies are an emerging class of therapies that have shown great promise in the treatment of hematopoietic malignancies and solid tumors. These therapies harness the capability of the immune system to target and kill large numbers of tumor cells specifically, and it is has been suggested that most or all durable responses to treatment of solid tumors involve generation of an anti-tumor immune response. Several anecdotal reports of dramatic responses in GBM patients after receiving cancer vaccines (a type of immune therapy) suggest that immune therapies for glioma could yield substantial increases in survival of patients with these tumors. However, the overall record of vaccines for the treatment of this disease has been marked by failure, and substantial barriers remain to the implementation of other types of immune therapies in glioma patients. Several mechanisms by which tumors in general, and brain tumors in particular, evade the activity of the immune system have been outlined. These include accumulation of immune suppressive cell types, tumor intrinsic changes

that directly suppress the activity of infiltrating immune cells, and brain specific mechanisms of immune privilege. While these mechanisms are doubtless operative in many cases, accumulating evidence from clinical trials of adoptive transfer of T cells demonstrate that the accumulation of sufficient numbers of tumor-specific T lymphocytes at the tumor site can result in an overwhelming anti-tumor immune response and associated durable clinical responses. Therefore, my research over the past several years has focused on clinically relevant mechanisms in glioma patients that present obstacles to the development of a robust T cell mediated anti-tumor immune response. In this thesis, I outline experiments performed to understand and develop strategies for overcoming two obstacles to expanding large numbers of tumor specific cytolytic T lymphocytes in glioma patients: the anti-proliferative effect of the alkylating drug temozolomide on *in vivo* T cell expansion by cancer vaccination, and the differentiated phenotype of *ex vivo* expanded T cells for adoptive immunotherapy that is associated with diminished proliferative potential *in vivo.* A focus in these experiments is the targeting of tumors with T cells that are specific for antigenic determinants derived from tumor-specific mutations. Engineered T cell responses targeting individual patient-specific mutations may someday lead to significant improvements in the efficacy of immune therapy for glioma, and ultimately to improved outcomes for patients with these malignancies.

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Chapter 1: Introduction

It is by no means inconceivable that small accumulations of tumour cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction, with regression of the tumour and no clinical hint of its existence. It has also been suggested that the result of surgery for cancer may to a large extent be determined by the degree of resistance, presumably immunological in nature, against the tumor cells…

…What is to be sought is some means whereby the protective mechanism of the body has its reactivity against minor deviations from self-patterns made more sensitive—the converse of the effect of cortisone in dampening down immunological reactivity…

—Sir Macfarlane Burnet, 1957

Better treatments for glioma are needed

Glioma is a class of malignant diseases of the central nervous system, and accounts for the majority of morbidity and mortality from central nervous system tumors (Porter, McCarthy et al. 2010). The most common and most deadly of these, glioblastoma multiforme (GBM), has a median survival at diagnosis of less than a year and a half, even with the best current treatments (Stupp, Mason et al. 2005). Standard treatment for GBM includes whole brain irradiation and adjuvant chemotherapy with the alkylating drug temozolomide (McNamara, Lwin et al. 2014). Temozolomide was approved based on a seminal clinical trial conducted in the early 2000s (Stupp, Mason et al. 2005) which represented the first major advance in the treatment of the disease over standard radiotherapy in a quarter century (Walker, Green et al. 1980). Nonetheless, the prognosis of patients diagnosed with this disease remains dismal today and better treatments remain needed. Intense research effort has been devoted to developing new classes of treatments, including targeted inhibitors, anti-angiogenesis agents like bevacizumab, gene therapies and immune therapies (reviewed in Adamson, Kanu et al. 2009). Immunotherapy has been recognized as a clinically imporant new class of cancer treatments in recent years (Couzin-Frankel 2013), and glioma has been no exception to this trend (Okada, Kohanbash et al. 2009). In the remainder of this introduction, I outline some of the history and most important recent developments in the field of cancer immunotherapy, what these

experiments imply about how generally the immune system may be used to eradicate large solid tumors, discuss some of the attempts to harness the immune system to treat glioma, and explain how these studies influenced the design of the experiments described below.

The immune system as a treatment for cancer

The idea of stimulating the immune system as a treatment for cancer predates detailed knowledge of the nature of the immune response to pathogens, as William Coley injected patients' sarcomas with bacterial preparations at the end of the 19th century (Coley 1893). MacFarlane Burnet and Lewis Thomas developed the modern theory that cellular components of the immune system could recognize and destroy tumors in the 1950s (Burnet 1957, Thomas 1959). These theories were postulated at the dawn of the age of cellular immunology, as the anatomical origin and functional specialization of different classes of lymphocytes were only starting to be appreciated in the late 1950s and early 1960s (Glick G 1956, Good, Dalmasso et al. 1962). More knowledge and better techniques for understanding immunology would be needed before these theories could be tested, and they fell out of favor for several decades.

During this period, research in the field of tumor immunology focused on defining rejection antigens (targeted both by antibodies and T lymphocytes) in transplantable tumor lines in rodents; however, this line of inquiry led to neither

mechanistic understanding of the tumor rejection phenomenon or success in achieving this result in the clinic (reviewed in Old 1992). The main thrust of cancer research in the 1970s and 1980s was dedicated to understanding the genetic basis of the disease, with molecular biology advances allowing the identification of both viral and host derived oncogenes as key drivers of cellular transformation (Varmus 1989). A renaissance of tumor immunology has been in progress since the mid 1990s, with interest being renewed by new understanding of the mechanisms by which T cells could function as exogenous suppressors of tumor development (Shankaran, Ikeda et al. 2001, Dunn, Bruce et al. 2002). These theoretical advances have been followed in the last five years by the first successful clinical developments of immune based cancer treatments to the stage of FDA approval.

Indeed, the three main immune therapeutic modalities that are in clinical development—cancer vaccines, immune modulatory biologic agents and adoptive T cell transfer—have each seen prominent clinical successes occur since 2010. In 2010, Dendreon's Provenge, a dendritic cell based prostate cancer vaccine, became the first cancer vaccine to receive FDA approval (Karan, Holzbeierlein et al. 2012) after demonstrating improved overall survival in a Phase III clinical trial (Tanimoto, Hori et al. 2010). In 2011, ipilimumab, a human antibody against CTLA4 was approved for sale by the FDA (Lipson and Drake 2011). Marketed as Yervoy, ipilimumab became the first in what will likely be a

soon to expand new class of biologic drugs that modulate the activity of T cells to treat solid tumors, after improving survival of patients with metastatic melanoma (Hodi, O'Day et al. 2010). Most recently, adoptive transfer of chimeric antigen receptor (CAR) transduced T cells has garnered significant media attention (Grady 2012) after reports that dramatic remissions have been achieved in patients with B cell malignancies (Porter, Levine et al. 2011). Thus, cancer immunology and immunotherapy today are fields entering a new phase in their development, as theoretical advances are paving the way for new clinical successes and eventually routine clinical use (June, Rosenberg et al. 2012). These initial hints of success will be followed scientifically by further research into the mechanisms of tumor immunology, with the goal of improving the efficacy of immune therapies and expanding their use to different types of solid and hematopoietic tumors.

Lessons from CAR T cell therapy: requirements for killing a tumor

One of the most exciting recent developments in the field of cancer immunotherapy has been the success of chimeric antigen receptor (CAR) transduced T cells in treating B cell malignancies (Kalos, Levine et al. 2011). The excitement engendered by this approach has been driven by two main factors: First, the clinical responses achieved have been significant, with massive tumors shrinking and a dramatic tumor lysis syndrome observed in responding patients (Porter, Levine et al. 2011). Second, since the approach unites adoptive T cell

transfer with a genetic engineering approach that uses a rationally chosen target, it suggests that improved knowledge of immunology and tumor biology could lead to successful engineering of immune responses to other tumors. It is worthwhile, therefore, to consider why this strategy appears to be so successful, and what aspects of its success may be implemented for other tumors.

The idea of transferring T cells with an anti-tumor specificity into a tumor bearing host is not new, as the ability to induce immunity to a syngeneic tumor by adoptive transfer of spleen cells was first demonstrated over thirty years ago (Uyttenhove, Van Snick et al. 1980). This idea has been pioneered in its application in human cancer patients by the group led by Rosenberg and Restifo in the Surgery Branch of the National Cancer Institute, who began a series of clinical trials in the 1990s that demonstrated dramatic responses in patients adoptively transferred with T cells grown out of cultures of tumor infiltrating lymphocytes (TIL) derived from their own autologous tumor material (Rosenberg, Yang et al. 2011). While this approach has undoubtedly proved successful, most solid tumors do not bear TIL that can be expanded for adoptive transfer, and therefore alternate methods to expand or endow T cells with an anti-tumor specificity have been actively researched (Restifo, Dudley et al. 2012). The idea of engineering T cells with an artificial, non-MHC restricted, antibody type specificity was first implemented by the generation of chimeric genetic constructs fusing the genomic sequences encoding the heavy and light chains of an anti-

hapten immunoglobulin molecule with those encoding the constant regions of the T cell receptor alpha and beta chains (Gross, Waks et al. 1989). In subsequent years of development, technical improvements have lead to newer and better CAR designs, targeting a number of cell surface antigens as well as incorporating cytoplasmic signaling domains from a variety of co-stimulatory molecules to enhance the immunologic activity of the chimeric constructs (Kalos and June 2013).

The culmination of these efforts have been several promising early phase clinical trials showing dramatic regression of patients bearing CD19+ B cell malignancies treated with CAR-transduced autologous T cells (Kochenderfer, Wilson et al. 2010, Kalos, Levine et al. 2011, Porter, Levine et al. 2011). It is therefore worth considering what features of CAR T-cell therapy lead to such dramatic responses. The first noteworthy feature is the long term *in vivo* persistence and proliferative capacity of CAR-transduced T cells found after infusion in patients that exhibit tumor responses. For example, in one responding patient, six months after infusion, large numbers of CAR-expressing CD8 T cells were identified, with a variety of CAR expressing cells exhibiting heterogeneous surface immunophenotypes. This patient demonstrated subsets of cells expressing both memory markers like CD28 and CD127 as well as other cells with immediate *ex vivo* cytolytic capability (Kalos, Levine et al. 2011), suggesting that a correlate of success is the engraftment of both effector cells as well as cells with self-renewal

capability. Indeed, both greater numbers of infused cells as well as greater proliferative capacity of infused cells (as assessed by telomere length) have been associated with better outcomes in TIL based melanoma adoptive immunotherapy (Stupp, Mason et al. 2005, Rosenberg, Yang et al. 2011, Restifo, Dudley et al. 2012).

These phenotypes, that are thought to mark the capability for self-renewal (i.e. memory phenotype or long-telomere possessing cells), are associated with *in vivo* proliferation of immune cells, and it has been suggested that this *in vivo* proliferation is required to generate sufficient numbers of T cells at the tumor site (June, Rosenberg et al. 2012). Similarly, observations from trials of checkpoint blockade biologics, that inhibit T cell regulatory pathways bolster the idea that large numbers of tumor infiltrating cells are a prerequisite for successful immunotherapy (Pardoll 2012). CTLA-4 blockade is believed to work in proportion to the number of locally infiltrating T cells that can be activated (Lipson and Drake 2011, Postow, Callahan et al. 2012). PD-1 is another T cell regulatory pathway that has been targeted in melanoma (Topalian, Hodi et al. 2012), and the activity of PD-1 blockade is likely due to its inhibition of a negative feedback loop involving tumor-infiltrating T cell derived interferon gamma and STAT1-driven tumor expression of PD-L1 (Spranger, Spaapen et al. 2013).

A second noteworthy feature of CAR T cell therapy for B cell malignancies is the high activity of CD19 CAR-transduced T cells against CD19 expressing targets (Zhang, Snyder et al. 2007). This high activity is associated with a large amount of *in vivo* cytolysis, causing a tumor lysis syndrome, cytokine storm, and on-target off-tumor activity against normal B cells, which can become virtually undetectable in treated patients (Porter, Levine et al. 2011). This on-target off-tumor activity is noteworthy as it has important therapeutic implications. First, it suggests that successful immunotherapy may require an immune response so potent that it wipes out essentially all target-expressing cells. Indeed, CD19 CAR T cell treated patients have had prolonged extremely low levels of serum immunoglobulins associated with tumor remissions (Kochenderfer, Wilson et al. 2010). Second, it implies that CAR therapy will not be readily adaptable to the treatment of most solid tumors, at least not as presently constituted with a CAR that targets a single, highly expressed, tumor lineage defining cell-surface antigen (Maus, Fraietta et al. 2014). The pitfalls of the CAR approach to solid tumors are underscored, for example, by clinical trials of HER2-targeting CAR T cells that have led to serious, even fatal toxicities (Morgan, Yang et al. 2010, Lamers, Sleijfer et al. 2013) and a preclinical model where targeting of cancer associated fibroblasts via a CAR against fibroblast activation protein lead to targeting of the hematopoietic stem cell niche causing fatal cachexia (Tran, Chinnasamy et al. 2013).

So what rejection antigens can be targeted in most solid tumors? Studies of TIL therapy in melanoma provide the hints of an answer. It has been recognized for some time that mutated gene products can lead to novel CD8 T cell epitopes that are truly tumor specific (Wolfel, Hauer et al. 1995), and later studies have suggested that these types of mutation specific antigens, termed neo-antigens, make up the bulk of pre-existing anti-tumor immune responses in melanoma (Lennerz, Fatho et al. 2005). More recent, pioneering studies have shown that it is possible to prospectively identify such antigens by deep sequencing (Castle, Kreiter et al. 2012, Robbins, Lu et al. 2013)

Thus, it seems that at least two necessary components of an effective immunotherapy against a large, established solid tumor are the generation of an immune response consisting of large numbers of T cells at the tumor site, and the targeting of an antigen where all cells that express that antigen can be destroyed. As we turn to an examination of efforts at treating glioma-bearing patients by immunotherapy it is worthwhile to keep these goals in mind. Gliomas are typically not infiltrated by large numbers of lymphocytes (Parney, Waldron et al. 2009), and as we shall see, most clinical trials of immune therapy for glioma have not had defined antigenic targets or careful measurement of cellular correlates of immune response. For these reasons, it is perhaps unsurprising that most clinical trials of glioma immunotherapy have achieved underwhelming results (Okada, Kohanbash et al. 2009).

Immune privileged or immune specialized: Is it plausible to target central nervous system malignancies with immune therapy?

The unique anatomical localization of glioma as a solid tumor of the central nervous system (CNS) has led to some unique questions being asked of glioma immunotherapy that are not relevant to other solid tumors. Indeed, classic allogeneic tissue graft experiments by Medawar defined the CNS as a site of "immune privilege," that is, a site where immune responses are restrained (Medawar 1948). The main clinical manifestation of immune responses in the CNS was thought to be in autoimmune diseases like multiple sclerosis (Carson, Doose et al. 2006). Originally, the mechanism of immune privilege was assumed to be anatomical: the brain and eyes lack lymphatic drainage and the vasculature of these tissues is specialized to be relatively impermeable to cells and substances by a specialized structure called the blood brain barrier (Engelhardt and Coisne 2011). Later mechanistic studies revealed operant molecular mechanisms that suppress the accumulation and activity of lymphocytic infiltrates into the CNS, providing a rationale for the lack of T cell infiltrates in the CNS outside of pathophysiological circumstances (Griffith, Brunner et al. 1995).

Studies in glioma patients have also provided support for the idea that brain tissue itself so thoroughly suppresses T cell immune responses that

immunotherapy of glioma could be impractical. A particularly fascinating series of observations in patients receiving organ transplants from patients that died of glioma are relevant in this vein. Glioblastoma is noteworthy for spreading aggressively and distantly within the brain, but metastasizing to more distal sites extremely infrequently, almost always within the CNS (e.g., within the spinal cord) (Stark, Nabavi et al. 2005). However, after transplantation of a solid organ from a patient that died of GBM, a donor derived glial tumor was found in the organ recipient, with disseminated disease in several tissues outside of the CNS (Frank, Müller et al. 1998). Subsequent reports and retrospective analysis of earlier reports of tumors arising in patients receiving organ transplants from patients that died of glioma have suggested that this phenomenon is not unique, and call into question the suitability of patients that die of glioma as organ donors (Schiff, O'Neill et al. 2001, Armanios, Grossman et al. 2004). Furthermore, these reports raise several intriguing questions about the immunogenicity of glioma: The fact that multiple solid organs transplanted from patients that have died of glioma have led to transmissible disease suggests that disseminated but clinically undetectable micrometastases outside of the CNS are not uncommon in glioma patients. The observation that when transplanted within an organ that these micrometastases can lead to disease outside of the CNS, but only in immunosuppressed transplant recipients, suggests that the reason why extra-CNS metastasis of glioma are virtually unknown is because these disseminated cells are kept in check by the immune system. Indeed, taken together, it is

reasonable to speculate based on these reports that glioma is actually a rather "immunogenic" class of tumor, but that it is the specialized immunological milieu of the brain that suppresses anti-glioma immune responses.

Early attempts at immunotherapy for glioma were not successful, and reviews of the state of the field from the mid-1990s also ascribe the reason for these failures to the immune privileged status of the brain (Weller and Fontana 1995, Saas, Walker et al. 1997). However, in the last decade a profound shift in the understanding of immune privilege has taken place. It is now appreciated that non-pathological immune responses can take place in response to infections that occur in the CNS (Walker, Calzascia et al. 2003). The CNS, like all other tissues, is immunologically specialized, and tissue specific features of the CNS modulate the responses of infiltrating immune cells (Matzinger and Kamala 2011). These specializations include anatomical differences from most tissues, including the aforementioned lack of lymphatic drainage and specialized barriers around brain vasculature (Engelhardt and Coisne 2011). The role of these specializations is likely to limit the likelihood and extent of inflammation in the tightly spatially confined and sensitive post-mitotic tissues of the CNS. However, these specializations do not amount to an absolute impermeability of immune cells into brain tissue and are in fact immune regulatory specializations that can be overcome in order to resolve CNS infections while limiting tissue damage (Galea, Bechmann et al. 2007)

Of particular relevance for glioma immunotherapy are recent findings demonstrating that antigen specific T cells can bypass the blood-brain barrier (Galea, Bernardes-Silva et al. 2007), and that there appear to be additional layers of regulation to the process consisting of additional anatomical specializations, specialized antigen presenting cells and inflammation suppressive cell surface molecules and soluble factors. These specializations consist of a defined program that exists by which large numbers of antigen specific T cells can accumulate in the brain to eradicate target cells while limiting localized tissue disruption (Engelhardt and Ransohoff 2012, Ransohoff and Engelhardt 2012).

The tumors of patients with GBM are not typically infiltrated by large numbers of lymphocytes (Parney, Waldron et al. 2009). However, the density of infiltrating lymphocytes varies between patients, and above average numbers of infiltrating CD8 T cells are associated with longer survival (Yang, Tihan et al. 2010). Perhaps not coincidentally, the change in understanding of the nature of immune privilege has taken place simultaneously with the first convincing findings of individual patients benefiting from glioma targeting immunotherapies. Here, I review major recent findings in the field of glioma immunotherapy.

Glioma immunotherapy: a brief survey of the last ten years

A seminal finding that represented one of the first attempts to define a cellular correlate of response to glioma immunotherapy was described in 2001 by Prins and colleagues at Cedars Sinai (John, Wheeler et al. 2001). A noteworthy figure in this publication demonstrates a massive CD8 T cell infiltrate in a re-resected GBM following vaccination with dendritic cells (DC) pulsed with MHC-I eluted peptides derived from the autologous tumor (*ibid*). A later publication from the same group is also notable for containing one of the first clear descriptions of apparent clinical response in individual GBM patients after receiving immunotherapy. Specifically, in addition to recapitulating their finding of CD8 T cell infiltrate in re-resected GBM lesions after DC vaccination, they demonstrated patients responding to vaccination with dramatic pseudoprogressions (regions of increased enhancement in imaging studies) followed by tumor regressions that likely represent immunological infiltration and an anti-tumor immune response (Prins, Soto et al. 2011). Additionally, this study demonstrated that patients receiving DC vaccination whose tumors had the mesenchymal tumor gene expression profile as defined by microarray (which is typically associated with worse-than-average prognosis relative to all GBM) survived significantly longer than control patients with this tumor type, while this benefit was not seen in patients whose tumors had other gene expression profiles (*ibid*).

While these studies were notable for showing a cellular correlate of vaccine efficacy and for attempting to define molecular markers of prognostic significance, they were typical of many glioma vaccination approaches in that there is no molecularly defined antigenic target of the vaccination and relies on autologous tumor material as the source of vaccine antigen. Indeed, a number of similarly designed trials using autologous DC pulsed with autologous tumor material have been conducted (Rutkowski, De Vleeschouwer et al. 2004, Yamanaka, Homma et al. 2005, Fadul, Fisher et al. 2011), and these strategies are in active clinical development by firms seeking to commercialize the technology (Ottenhausen, Bodhinayake et al. 2013). To date, however, no prospective clinical study of autologous DC vaccination for GBM has demonstrated enhanced overall survival over control treated patients, and thus the answer to the question of whether this type of treatment will ever become a standard therapy is unclear.

A weakness of the strategy of autologous DC vaccination is the lack of a molecularly characterized antigenic target. This weakness makes it difficult to directly assess biomarkers of anti-tumor immune response (e.g., by peptide-MHC multimer staining of peripheral blood) and therefore to define reasons for treatment failure. An alternative strategy is to vaccinate patients with characterized CD8 T cell epitope peptides or with DC pulsed with these peptides.

These approaches have proved effective in the treatment of other types of tumors, and these strategies typically involve the targeting of commonly overexpressed antigens, using CD8 T cell epitopes known for common HLA alleles (Walter, Weinschenk et al. 2012). Enough of these overexpressed self-antigen derived peptides have been determined in glioma that this approach has been implemented in clinical trials of GBM patients (Okada, Kalinski et al. 2011). Similar to the outcomes reported for DC loaded with autologous material by Prins et al., in this study Okada et al. report modestly successful results, with circumstantial evidence of benefit in individual patients and a benign safety profile (*ibid*). Interestingly, the use of defined peptide antigens allowed the authors in this study to measure antigen specific immune responses in vaccinated patients and found increased numbers of antigen specific CD8 T cells in peripheral blood several weeks after the first vaccinations were given (*ibid*).

Another noteworthy example of a clinical trial that targets a defined antigen for vaccination in GBM patients was performed by Sampson and coworkers at Duke University, which targeted a protein derived from a tumor specific mutation called EGFRvIII (Sampson, Heimberger et al. 2010). This protein consists of an aberrant version of the EGFR protein caused by an in frame deletion of exons 2-7 within the *EGFR* gene, leading to a protein that has lost binding to EGF but which has an oncogenic constitutive activity (Gan, Cvrljevic et al. 2013). The

protein sequence at the junction of exons 1 and 8 contains an inserted glycine and represents a “non-self” antigenic target that can in principle be targeted both by antibodies and T cells, as the protein is a cell surface antigen not normally encoded in the human genome, and similarly the amino acid sequence spanning the deletion is not encoded by any normally expressed genes. Therefore, the authors targeted this protein by vaccinating glioma patients with EGFRvIII expressing tumors with a 15 amino acid peptide spanning both sides of the deletion, covalently linked to the adjuvant keyhole limpet hemocyanin (Sampson, Heimberger et al. 2010). Interestingly, this treatment led to detectable levels of anti-EGFRvIII antibodies in the serum of 6 of 14 patients analyzed as well as a specific loss of expression of EGFRvIII (but not wild type EGFR) in some patients upon recurrence of the tumor after vaccination (*ibid*).

However, only 3 of 16 patients in this study demonstrated a DTH response and no further data were presented characterizing T cell mediated immune responses (Sampson, Heimberger et al. 2010). These data, combined with previous reports showing that EGFRvIII specific peptides bind common HLA class I molecules with only moderate affinity relative to common pathogen derived CD8 T cell epitopes (Wikstrand, Reist et al. 1998, Wu, Xiao et al. 2006), suggest that EGFRvIII peptide may not represent a good target for T cells derived by peptide vaccination. EGFRvIII has an extracellular domain that differs from normal EGFR

and can be differentiated by specific monoclonal antibodies (Wikstrand, Hale et al. 1995), which has led to interest in EGFRvIII as a target of CAR-transduced adoptive T cell therapies. These studies are still in preclinical stages (Morgan, Johnson et al. 2012, Ohno, Ohkuri et al. 2013, Choi, Suryadevara et al. 2014), but suggest an exciting new avenue to try to translate some of the success that B cell targeting CARs have seen, to at least a subset of glioma patients. In this vein, CARs have also been developed targeting the IL-13Rα2, a tumor-associated antigen of unknown normal functional relevance over-expressed in some cases of glioma (Brown, Starr et al. 2013).

Overcoming obstacles to glioma immunotherapy

These studies highlight several problems that remain to be overcome before immunotherapy can become an effective, standard treatment for glioma patients. The emphasis that has been seen in targeting glioma by therapeutic vaccination using autologous tumor material is due to the relative ease with which this strategy can be implemented without knowledge of tumor specific antigens. However, vaccination strategies rely on T cell proliferation occurring *in vivo*, in the body of the glioma patient, which not only contains immune suppressive cell populations such as regulatory T cells and myeloid derived suppressor cells, but has also been exposed to potentially immunosuppressive treatments for the glioma, such as conventional chemotherapy with temozolomide, radiotherapy, and glucocorticoids like dexamethasone that provide symptomatic relief from

edema in the CNS. The latter treatment has been examined as regards the immune system of glioma patients and shown be associated with expanded populations of suppressive monocytes (Brown, Starr et al. 2013), but the role of standard chemotherapy remains unclear, with reports of both improved and suppressed immune responses in patients treated with temozolomide (Grossman, Ye et al. 2011, Sampson, Aldape et al. 2011).

Another key obstacle in glioma immunotherapy remains the identification and effective implementation of therapies that target tumor specific mutations as antigenic determinants. The experience of CAR transduced T cell immunotherapy of B cell malignancies demonstrates that a potent T cell response that can eliminate all host cells bearing its antigenic determinant can lead to remission of large solid tumors and sustained clinical response. While EGFRvIII-negative GBMs may be unlikely to have suitable targets for CARs, we believe it is likely that prospective identification of private, patient specific mutations can be targeted with similar efficacy. Numerous groups around the world are currently pursuing this strategy for a variety of solid tumors, and it seems likely that clinical trials will implement such an idea in the relatively near term future.

What are the *in* vivo effects of temozolomide on cancer vaccinations, and what do these effects mean for the design of cancer vaccines for glioma patients? How can tumor specific mutations be targeted without generating genetically engineered antigen receptors for each potential antigen? How can the proliferative potential of adoptively transferred CD8 T cells be maintained? Below, we present experiments suggesting that temozolomide suppresses *in vivo* T cell proliferation and discuss the implications of these experiments. We discuss below the implications of temozolomide treatment in glioma on the targeting of these types of mutations with peptide vaccination. Finally, we present data on the *in vitro* expansion of tumor antigen specific CD8 T cells with high proliferative potential using a cocktail of small molecules and cytokines. The aim of these latter experiments was to develop a relevant translational strategy for implementing personalized medicine to target patient specific tumor mutations in glioma and other solid tumors.

Chapter 2: Profound impairment of adaptive immune responses by alkylating chemotherapy

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Introduction

Immune mediated destruction of solid tumors requires the infiltration of adequate numbers of effector lymphocytes into the tumor site (Chen and Davis 2005). Tumors express mutant proteins termed “neo-antigens” that result from

frameshift, gene fusion, and missense mutations (Thomas, Baker et al. 2007). These neo-antigens rather than self-antigens tend to dominate the naturally occurring immune responses against cancer (Lennerz, Fatho et al. 2005, Sensi and Anichini 2006). The immunogenicity and tumor specificity of the neo-antigens provide a compelling rationale for their identification and targeting with therapeutic cancer vaccines. Recent bioinformatics advances make prospective identification of neo-antigens for personalized cancer vaccines feasible (Castle, Kreiter et al. 2012). Numerous analyses of individual patients suggest that naturally occurring T cell responses against neo-antigens can be associated with dramatic responses and long-term survival (Huang, El-Gamil et al. 2004, Lennerz, Fatho et al. 2005, Sensi and Anichini 2006). Indeed, it has been suggested that the generation of endogenous anti-tumor responses may be required for durable success of conventional therapies (Zitvogel, Apetoh et al. 2008). This hypothesis has led to much interest in combining immunotherapy with conventional modalities (Mitchell 2003), but the effect of conventional chemotherapy vis-à-vis immunotherapy is incompletely understood.

Numerous reports indicate a synergy between conventional chemotherapy and immune therapy. Synergy is mediated by diverse mechanisms including preferential depletion of regulatory T cells (Treg) (Machiels, Reilly et al. 2001, Ercolini, Ladle et al. 2005, Banissi, Ghiringhelli et al. 2009), liberation of homeostatic or inflammatory cytokines (Schiavoni, Mattei et al. 2000, Asavaroengchai, Kotera et al. 2002) and enhanced immunogenicity of

chemotherapy treated tumors (Ramakrishnan, Assudani et al. 2010, Michaud, Martins et al. 2011). In the context of vaccines targeting self-antigens, chemotherapy given prior to vaccination can yield synergy and enhanced survival (Machiels, Reilly et al. 2001, Walter, Weinschenk et al. 2012). Vaccination against tolerized self-antigens may require Treg depletion to access a latent pool of high avidity self-antigen specific CD8 T cells, whereas high avidity neo-antigen specific T cells can be generated by immunization without Treg depletion(Ercolini, Ladle et al. 2005).

The reported synergy between chemotherapy and vaccines is somewhat paradoxical given that the generation of an adaptive immune response is a highly proliferative process, and chemotherapeutic drugs are given for their selective toxicity to rapidly proliferating cells. The generation of a CD8 T cell response to an acute viral infection involves responder cells doubling ~14 times in a week (Blattman, Antia et al. 2002) and cancer vaccines that utilize neo-antigens with potent adjuvants can trigger similar levels of CD8 T cell proliferation (Wick, Martin et al. 2011). The number and proliferative potential of infused effectors have been associated with clinical response to adoptive immunotherapy of metastatic melanoma (Rosenberg, Yang et al. 2011), possibly due to a requirement for local proliferation of lymphocytes to generate sufficient effector: target ratios at the tumor site (Grange, Buferne et al. 2012, June, Rosenberg et al. 2012). In adoptive transfer protocols the transferred lymphocytes are cultured *ex vivo* and therefore are not exposed to chemotherapy(Rosenberg 2011). By contrast

cancer vaccines are administered to drive *in vivo* proliferation of lymphocytes in pre-treated patients, and the extent to which chemotherapy inhibits vaccine driven immune responses remains unclear.

The lack of understanding of the effect of chemotherapeutic drugs on cancer vaccines is particularly problematic with regard to alkylating chemotherapeutic drugs. Alkylating chemotherapies such as temozolomide and cyclophosphamide covalently modify DNA and inflict cytotoxic damage on exposed cells (Fu, Calvo et al. 2012). These drugs are commonly used for their anti-neoplastic effect to treat malignancies that are frequent targets of cancer vaccines such as glioblastoma multiforme (GBM) (Prins, Soto et al. 2011) and metastatic melanoma (Chapman, Einhorn et al. 1999), and additionally to deplete Treg prior to vaccination (Dudek, Mescher et al. 2008, Walter, Weinschenk et al. 2012). While case reports suggest that individual patients have benefited from cancer vaccines given after standard alkylating chemotherapy for GBM (Okada, Kalinski et al. 2011, Prins, Soto et al. 2011), overall cancer vaccines administered after temozolomide have had a record of failure (Okada, Kohanbash et al. 2009). Alkylating chemotherapeutics have immune inhibitory effects *in vitro*, specifically via selective toxicity to proliferating lymphocytes (Roos, Baumgartner et al. 2004) and inhibition of differentiation of immune effectors (Alvino, Pepponi et al. 1999). The applicability of these studies to human cancer patients remains unclear: the degree of lymphopenia in temozolomide treated glioblastoma patients is a negative prognostic factor (Grossman, Ye et al. 2011), but has also been

associated with greater vaccine induced antibody responses (Sampson, Aldape et al. 2011). To examine the impact of clinically relevant doses of alkylating chemotherapeutics on cancer vaccines, we used controlled animal experiments that minimized the numerous complicating factors encountered in human patients.

Materials and Methods

Cells and culture. GL261 and B16-F10 cells were maintained in DMEM supplemented with 10% FBS. The KM3M14 and O94M2 cell lines were derived from genetically engineered primary murine gliomas and were generated and maintained as described (Wiesner, Decker et al. 2009). Model antigen expressing tumors (Quad-GL261 and Quad-KM3M14) were generated by stable transfection with Quad antigen cassette (Ohlfest, Andersen et al.), a single coding sequence expressing the OT-I and OT-II epitopes of ovalbumin as well as human gp100 and mouse Ea.

Patient samples were thawed from aliquots frozen the day of collection and plated overnight before use. T cells were stimulated with IL-2 (R&D Systems) and CD3/CD28 activator beads (Invitrogen) as previously described (Trickett and Kwan 2003). Viable cell counts were assessed by trypan blue exclusion periodically after stimulation and are expressed proportionally relative to the

number of viable cells at the beginning of the assay. CD8 T cells were isolated to >90% purity by negative selection kit (Miltenyi).

Patient samples. GBM patient PBMC samples were obtained with informed consent and with approval from the University of Minnesota Institutional Review Board. Pre-temozolomide samples were collected the day of surgical resection, 4-6 weeks before the start of combined chemoradiotherapy. The patients' ages at time of treatment and sexes were as follows: Patient 1, 61 year old male; Patient 2, 73 year old female; Patient 3, 68 year old female; Patient 4, 46 year old male; Patient 5, 62 year old male. All patients had no pre-TMZ treatment besides surgery except for Patient 4 who was treated with dexamethasone. All patients were treated with 75 mg/m^2 of temozolomide daily for 42 days during chemoradiotherapy. Post-temozolomide samples were collected 4 weeks after the end of chemoradiotherapy (patients 1,4) or 7 weeks after the end of chemoradiotherapy, one week after a single adjuvant cycle of 5 days of 150 mg/m^2 temozolomide (patient 5).

Mice and animal models. Mouse experiments were performed in accordance with University of Minnesota Animal Care and Use Committee guidelines. C57BL/6J (B6) mice, C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) and B6.PL-Thy1a/CyJ (Thy1.1+) mice were purchased from the Jackson Laboratory and used at 6-10 weeks of age. Nur77^{GFP} reporter mice(Moran, Holzapfel et al. 2011) were courtesy of K. Hogquist (University of Minnesota, Minneapolis, MN). Gliomas were inoculated as described.(Ohlfest, Andersen et al.) Cell number

inoculated was 15,000 for GL261 and 30,000 for O94M2 and KM3M14. 75,000 B16-F10 cells were inoculated in the right flank. Glioma-bearing mice were euthanized when they became symptomatic; B16-F10-bearing mice were euthanized when tumors became >1000 mm^3. For adoptive transfer $2x10^6$ cells OT-I CD8 T cells were transferred into Thy1.1+ mice by retro-orbital injection and allowed to park for 24 hours before drug treatment.

Drug treatments. Temozolomide, carboplatin, doxorubicin and cyclophosphamide were obtained from Toronto Research Chemicals. Carboplatin, doxorubicin and cyclophosphamide were dissolved in PBS and administered by intraperitoneal injection. Temozolomide was well suspended in PBS immediately before being administered via oral gavage. g-irradiation was administered as a positive control for DNA strand breaks at a dosage of 15 Gy, 30 minutes before experiments. Mouse dosages of temozolomide and cyclophosphamide model relevant human pharmacokinetic exposures based on a calculated equivalence using published pharmacokinetic exposure data (Struck, Alberts et al. 1987, Genka, Deutsch et al. 1990) detailed in Table S2. Dosages of carboplatin and doxorubicin were selected based on the maximum anti-neoplastic dosages that were previously reported to have an immunostimulatory effect by a Treg depletion dependent mechanism (Machiels, Reilly et al. 2001).

Vaccinations. Ova vaccinations were performed with 100 mg of whole chicken ovalbumin protein (Fisher) and 10 mg of polyinosinic:polycytidylic acid stabilized

with poly-L-lysine(polyICLC, gift of A. Salazar, Oncovir, Washington, DC). All peptide vaccines were given with 50 mg of peptide and 10 mg polyICLC. Vaccinations were administered as subcutaneous injections at the base of the left hind leg.

Peptides and *in vitro* stimulation. SIINFEKL and variant peptides (Anaspec) were dissolved in sterile water. All other peptides (New England Peptide) were dissolved in minimal DMSO and diluted in sterile water. Splenocytes were incubated with peptides for 8 hours ($Nur77^{GFP}$ induction) or 24 hours (elaborated IFN-g). Elaborated IFN-g was measured by cytokine bead array (BD) and normalized to number of antigen specific T cells enumerated with BD counting beads. For B16-F10 stimulation B6 splenocytes were pulsed with 5 mg /mL of the mutant peptide cocktail or irrelevant control (16 and 18 amino acid peptides containing the OT-I and OT-II epitopes). Leukocytes from 100 mL of blood from B16-F10 bearing animals were incubated with 150,000 antigen pulsed splenocytes for 72 hours for IFN-g elaboration.

The peptide sequences are as follows: *GARC-1* RASAALLNKLYAMGL; B16-F10 mutant peptides: *Kif18b-mutant SKPSFQEFVDWENVSPELNSTDQP, Tubb3-mutant RRKAFLHWYTGEAMDEMEFTEAESN, Cpsf3l-mutant FKHIKAFDRTFANNPGPMVVFATPG, Tnpo3-mutant, DRNPQFLDPVLAYLMKGLCEKPLAS, Plod2-mutant, YNTSHLNNDVWQIFENPVDWKEK.* (Castle, Kreiter et al. 2012)

Flow cytometry and ELISA. All antibodies except as indicated were from eBioscience: Phosphorylated ATM (phosphor-Ser1981) antibody was from Millipore. An isotype control for phosphorylated ATM staining was included as a control for background staining in non-temozolomide treated lymphocytes and was the PE-conjugated murine IgG1 k isotype control from eBioscience. K^b-Ova peptide-MHC multimer staining was performed with dextramer (Immudex). PE conjugated D^b-GARC-1 tetramer was synthesized by NIH tetramer core facility (Atlanta, GA). Tetramer falloff assay was performed as described(Blattman, Antia et al. 2002) with the following modifications: K^b-Ova dextramer was used to stain and free dextramer was bound with biotinylated anti-K^b-Ova monoclonal antibody. Data were acquired using a BD FACSCanto II and analyzed using Cytobank.org software. Relative affinity and antibody titer were determined by ELISA as described (Re, Schiavone et al. 2008).

Results

An intrinsic proliferation defect of lymphocytes exposed to alkylating chemotherapy

To assess the proliferative potential of human lymphocytes exposed *in vivo* to alkylating chemotherapy, we examined the ability of T cells in GBM patient PBMC obtained ~1 month before the beginning of and ~1 month after the cessation of temozolomide chemoradiotherapy to proliferate. PBMC from healthy controls and pre-chemoradiotherapy GBM patients proliferated robustly upon stimulation, with viable cell counts increasing by day 4 and approximately doubling every other day thereafter (Figure 1A). Post-chemoradiotherapy PBMC 4 days after stimulation had smaller clusters of PBMC around artificial APC and lower numbers of viable cells relative to pre-chemoradiotherapy PBMC (Figure 1, A-C). This proliferative defect was not an artifact of altered suppressor cell number in the samples, which were similar in pre- and post-temozolomide patient samples (Figure S1). These data suggest that clinical exposure to temozolomide causes a proliferative defect in human T cells.

To choose clinically relevant doses of temozolomide with which to treat mice, we selected doses that yielded an equivalent pharmacokinetic exposure as obtained after oral dosing in humans (Newlands, Blackledge et al. 1992) (personal communication James Gallo, Mount Sinai School of Medicine, New York, NY). Low (25 mg/kg), intermediate (55 mg/kg), and high (80 mg/kg) dosages of temozolomide given daily for five days led to a transient reduction in lymphocyte counts similar in magnitude to that seen in patients treated with temozolomide

(Table S2). Medium and high doses inhibited proliferation of splenic T cells to a degree similar to the inhibition observed in human GBM patient PBMC cultures (Figure 1D). This proliferation defect is cell intrinsic because purified CD8 T cells from temozolomide treated mice demonstrated a similar inhibition of proliferation and activation of the DNA damage response as assessed by phosphorylated Ataxia Telangiectasia Mutated (pATM) staining (Figure 1, E and F).

Quantitative defects in immune responses to cancer vaccines after temozolomide treatment

To examine the effects of alkylating chemotherapy on neo-antigen cancer vaccines we vaccinated mice with the model antigen chicken ovalbumin (Ova) and polyICLC (Wick, Martin et al. 2011). A cluster of four daily subcutaneous vaccinations causes a robust CD8 T cell response, with the percentage of K^b-Ova-specific CD8 T cells in the blood expanding from essentially undetectable levels to ~6% of the CD8 compartment in a week (Figure 2A). This expansion from a precursor frequency of ~1/150,000 (Obar, Khanna et al. 2008) to a frequency of ~1/20 represents approximately 13 doublings. When followed by weekly boosters, similar to many established clinical protocols, a second peak in K^b-Ova-specific T cell percentage was observed a week after the third booster (Figure 2A). The magnitude of the CD8 T cell response was diminished in a dose dependent fashion by temozolomide treatment (Figure 2A). The levels of Ova-specific antibody circulating in the blood of temozolomide treated mice were also lower in a dose dependent fashion (Figure 2B).

Given that temozolomide and other alkylating drugs covalently modify DNA and that some of the directly produced DNA-alkyl adducts (Degan, Montesano et al. 1988) or indirectly produced DNA lesions (Hengstler, Hengst et al. 1997, Marchesi, Turriziani et al. 2007) are long lived, we measured the magnitude of immune responses to vaccines given several weeks after temozolomide treatment. B6 mice that were given intermediate dose temozolomide had significantly lower percentages of K^b-Ova-specific T cells elicited by a cluster of four vaccinations that began 80 days after the last dose of temozolomide relative to untreated, age matched controls, with a peak frequency of ~1% versus ~4.5% (Figure 2C).

Antigen experienced memory T cells have an intrinsic resistance to DNA intercalating chemotherapy with daunorubicin (Turtle, Swanson et al. 2009), and tumor specific T cell clones may be antigen experienced in cancer patients (Anichini, Molla et al. 2010). We therefore measured the effect of vaccine driven T cell expansion in an antigen experienced memory cell population in mice treated with alkylating chemotherapy prior to vaccination. Mice treated with intermediate or high dose temozolomide after an initial cluster of vaccines had a significant inhibition of antigen specific T cell proliferation when vaccinated with a second cluster of vaccines (Figure 2D). The peak percentage of K^b-Ova-specific CD8 T cells was approximately 10 fold lower (~2% versus ~20%) in intermediate dose temozolomide treated mice versus controls, and the percentage of K^b-Ova-

specific CD8 T cells decreased immediately following vaccination in high dose treated mice (Figure 2D).

We next quantified the impact of temozolomide on the efficacy of vaccines in tumor bearing animals using both model antigens and mutated tumor specific neo-antigens. B6 mice were implanted orthotopically with the syngeneic, antigen force-expressing Quad-GL261 glioma line (Ohlfest, Andersen et al.). The peak percentage of K^b-Ova-specific CD8 T cells in blood elicited by vaccination was significantly lower for high dose temozolomide pre-treated animals, and levels remained lower after numerous booster vaccines (Figure 3A). The GL261 cell line is sensitive to temozolomide (Zhu, Fujita et al. 2011) and therefore the median survival for all three treated groups was similar (Figure 3B). However, high dose temozolomide treatment abrogated the efficacy of vaccine treatment: i.e. mice treated with vaccine survived significantly longer than untreated controls, but the benefit of vaccination was lost in temozolomide treated mice. While temozolomide increases median survival in glioma patients (Stupp, Mason et al. 2005), benefit from treatment is not uniform, and patients with O-6-methylguanine-DNA methyltransferase (*MGMT*) promoter unmethylated tumors are less likely to benefit (Hegi, Liu et al. 2008). To model this clinical situation, we inoculated B6 mice with a Quad antigen expressing version of the B6 syngeneic KM3M14 glioma cell line (Quad-KM3M14), which is highly resistant to temozolomide treatment *in vitro* (data not shown). Intermediate dose temozolomide treatment abrogated the magnitude of K^b-Ova-specific CD8 T cell

response to a single cluster of four vaccinations with Ova and polyICLC (Figure 3C). In addition, a spontaneous K^b-Ova-specific CD8 T cell response observed in untreated mice was abrogated in temozolomide treated mice (Figure 3C). For this temozolomide insensitive, immunogenic cell line a single course of vaccination is a largely curative therapy (Figure 3D). This survival benefit is entirely abrogated by temozolomide treatment before vaccination, and median survival for temozolomide treated mice was shorter than non-treated controls (Figure 3D). Similarly, we found that inhibition of spontaneous immune responses by temozolomide treatment could lead to a failure to reject a highly immunogenic B6 glioma line expressing the SV40 Large T antigen (Figure S3).

To examine the impact of temozolomide treatment on mutated self neo-antigens we inoculated mice with the GL261 cell line. This cell line expresses an immunogenic mutant self protein, GARC-1, which forms a D^b-binding CD8 T cell epitope based on a single amino acid substitution due to a point mutation (Iizuka, Kojima et al. 2006). We vaccinated using a peptide containing the immunogenic amino acid substitution in glioma bearing mice, with or without temozolomide treatment. A significant reduction in the percentage of activated, D^b-GARC-1 specific CD8 T cells in blood elicited by vaccination was observed (Figure 3E).

DNA damage response induced by high intensity TCR stimulation following alkylating chemotherapy

Since we observed activation of the DNA damage response in T cells given a strong stimulation through the TCR (Figure 1), we hypothesized that the degree

of DNA damage response would correlate with TCR signal intensity. To dissect this question, OT-I mice were treated drugs and then their splenocytes were stimulated with altered peptide ligands that induce varying TCR signal strengths (Moran, Holzapfel et al. 2011). All three peptides induced similar levels of proliferation and minimal DNA damage response in OT-I cells from untreated control animals as assessed by Ki67 and pATM staining respectively (Figure 4A). In temozolomide treated animals the frequencies of Ki67+ OT-I cells were inhibited for all peptides, but interestingly, the percentage of proliferating cells that had activated the DNA damage response (i.e. were pATM+) increased with increasing strength of TCR stimulation (Figure 4, A and B).

To assess induction of DNA damage response in lymphocytes after treatment with other DNA damaging cancer therapies, we repeated the above experiments after treatment of OT-I mice with cyclophosphamide, carboplatin, doxorubicin and g-irradiation. Following treatment with the alkylating chemotherapy cyclophosphamide and g-irradiation, there were significantly more proliferating cells exhibiting DNA damage in response to the strong antigenic peptides SIIQFEKL and SIINFEKL than with the weak antigenic peptide SIIGFEKL or with no peptide (Figure 4C). The platinum drug carboplatin and the DNA intercalating agent doxorubicin did not lead to this effect, with similar pATM staining in proliferating cells stimulated with all peptides (Figure 4D). The induction of the DNA damage response upon stimulation after drug treatment was mirrored by the defect in CD8 T cell responses to vaccines in B6 mice. Temozolomide and

cyclophosphamide lead to markedly and significantly lower peak levels of antigen specific CD8 T cells in the blood following vaccination (Figure 4E). By contrast, in animals treated with carboplatin and doxorubicin the percentage of antigen specific CD8 T cells was incrementally lower and not significantly different from untreated controls. This defect is likely accounted for by responder lymphocytes both failing to enter cell cycle (as in Figure 4A) as well as undergoing apoptosis due to DNA damage response, since a fraction of adoptively transferred OT-I became apoptotic (Annexin V+7-AAD+) after vaccination with SIINFEKL peptide in temozolomide treated mice (Figure S4).

Lower affinity for antigen of vaccine responder lymphocytes after temozolomide treatment

Due to the greater DNA damage response we observed in OT-I T cells stimulated with stronger antigenic peptides, we hypothesized that *in vivo* vaccine responder cells would be skewed towards lymphocytes with antigen receptors with lower affinity for cognate antigen. We found that the median fluorescent intensity (MFI) of peptide-MHC multimer staining of memory CD8 T cells elicited by vaccination was lower in temozolomide treated mice than in controls (Figure 5, A and B). The higher initial rate of decay of staining in a multimer falloff assay (Blattman, Antia et al. 2002) in temozolomide treated animals also suggested a lower avidity of vaccine elicited CD8 T cells for antigen (Figure 5C). Similarly, we measured a lower relative affinity of anti-Ova serum Ig in temozolomide treated animals than in controls (Figure 5D).

Inferior functional characteristics of vaccine responder lymphocytes after temozolomide treatment

The lower affinity of responder lymphocytes for antigen after alkylating chemotherapy suggests that post-temozolomide vaccine responder cells are less sensitive towards antigenic targets. We hypothesized that these cells would receive lower intensity proliferative signals in temozolomide treated mice than controls and display inferior effector function upon stimulation. Using the Nur77^{GFP} TCR signal strength reporter mouse (Moran, Holzapfel et al. 2011), we directly tested this hypothesis by measuring GFP fluorescence intensity in vaccine expanded CD8 T cells upon antigenic stimulation. Vaccine expanded K^{b}-Ova-specific CD8 T cells from untreated control mice displayed high intensity GFP fluorescence upon culture with SIINFEKL peptide (Figure 6, A and B). In temozolomide pre-treated mice antigen specific GFP fluorescence upon stimulation was significantly lower (Figure 6B). We next measured IFN-g elaboration upon antigenic stimulation in culture and enumerated antigen specific cells per well. We calculate that in temozolomide pre-treated animals vaccine activated CD8 T cells elaborated 10 fold fewer IFN-g molecules on a per cell basis (Figure 6C).

Diminished efficacy of neo-antigen cancer vaccines after alkylating chemotherapy

To examine the efficacy of neo-antigen cancer vaccines after Treg depleting alkylating chemotherapy we used a peptide vaccine targeting five immunogenic

point mutations expressed by the B16-F10 melanoma cell line (Castle, Kreiter et al. 2012). Despite the fact that mice treated with low dose cyclophosphamide had a depletion of Treg cells as both a percentage of CD4 T cells and in absolute numbers (data not shown), we found that circulating leukocytes from mice vaccinated after cyclophosphamide elaborated significantly less IFN-g upon peptide stimulation than in mice receiving vaccine only (Figure 7A). Similarly, the survival benefit of vaccinated animals relative to untreated controls was lost in animals pre-treated with cyclophosphamide (Figure 7B). A similar finding with regard to overall survival was observed in a new follow up long-term survival analysis of a previously published clinical trial of an autologous cancer vaccine for metastatic melanoma (Dudek, Mescher et al. 2008). The ten patients who received only vaccinations had a median survival of 4.2 years, whereas the ten patients treated with low dose cyclophosphamide a week prior to their first vaccination had a significantly shorter median survival of 7.5 months (Figure 7C).

Discussion

We found that alkylating chemotherapy has a long lasting anti-proliferative effect on lymphocytes in mice and humans, and this effect leads to inferior responses to cancer vaccines targeting mutated self antigens. Animals pre-treated with alkylating chemotherapeutic drugs had lower peak numbers of vaccine responding CD8 T cells and lower antibody titers. This impairment corresponds to the activation of DNA damage responses in proliferating cells, and this

activation of DNA damage responses is greatest in responder cells receiving the strongest TCR signals from the vaccine. In turn, this selective toxicity in the cells with the highest affinity for cognate antigen leads to impairment of CD8 T cell and antibody responses. These responses consist of lymphocytes with on average lower affinity antigen receptors that have inferior effector function. Importantly, these effects occur were observed even at low, Treg depleting doses of alkylating chemotherapeutics (Machiels, Reilly et al. 2001).

The defects we observed are likely general to all populations rapidly proliferating immune responder cells expanded by vaccination (e.g. CD4 T cells, B cells, etc.). Activated lymphocytes implement a metabolic and anti-apoptotic program that allows for sustained synthesis of macromolecules and cell division (Frauwirth, Riley et al. 2002), dividing up to twice a day during the peak of adaptive immune responses (Blattman, Antia et al. 2002). Alkylating chemotherapy covalently modifies DNA with methyl adducts for methylating drugs like temozolomide or dacarbazine (Marchesi, Turriziani et al. 2007) or inter- and intra-strand alkyl crosslinks for nitrogen mustard derivatives like cyclophosphamide (Lawley and Brookes 1965). These lesions cause stalling of replication forks and double strand DNA breaks in proliferating cells (Roos and Kaina 2012). This DNA damage is detected by proteins such as ATM which binds to double strand DNA breaks and autophosphorylates, in turn activating numerous downstream effectors involved in cell cycle arrest and apoptosis such as Chk2 kinase (Matsuoka, Rotman et al. 2000) and p53 (Canman, Lim et al. 1998). Proliferation

driven toxicity in vaccine responder cells is therefore a side effect of alkylating chemotherapy that must be balanced against its reported immunomodulatory effects. In the case of neo-antigen vaccines for which Treg depletion is not required for efficacy, our data suggest that the negative anti-proliferative effect of chemotherapy is dominant over the immunomodulatory effect.

We observed that the immune inhibitory effect of alkylating chemotherapy was long lived, with significant defects in CD8 T cell priming persisting >10 weeks after cessation of temozolomide treatment (Figure 2C). The persistence of this effect could be due to the fact that DNA repair is induced by proliferative signals (Gupta and Sirover 1980), so quiescent naive lymphocytes may not fully repair DNA damage. This damage is then “activated” by replication fork read through during DNA synthesis in response to proliferative signals like vaccines.

Numerous studies have examined the effect of alkylating chemotherapy on immunotherapeutic modalities. The predominant finding reported has been depletion of Treg and induction of lymphopenia (Lutsiak, Semnani et al. 2005, Banissi, Ghiringhelli et al. 2009), although high doses have been associated with peripheral Treg expansion in rodents (Hirschhorn-Cymerman, Rizzuto et al. 2009) and humans (Sampson, Aldape et al. 2011). Several studies that have reported an immunostimulatory effect of alkylating chemotherapy due to Treg depletion have been conducted using transferred cells not exposed to drug (Ghiringhelli, Larmonier et al. 2004, Salem, Díaz-Montero et al. 2009, Mitchell, Cui et al. 2011). The clinical application of this strategy is complicated by the

difficulty of generating large numbers of tumor specific lymphocytes *ex vivo* for human patients (Yee 2005), and cancer vaccines are typically administered after standard chemotherapies (Zitvogel, Apetoh et al. 2008). Conversely, other studies of endogenous anti-tumor immune responses following Treg depletion have focused on self-antigens for which breaking tolerance is required (MacLean, Miles et al. 1996, Machiels, Reilly et al. 2001, Ercolini, Ladle et al. 2005, Walter, Weinschenk et al. 2012) or have not directly compared immune responses in exposed and non-exposed lymphocytes (Vaishampayan, Abrams et al. 2002, Hirschhorn-Cymerman, Rizzuto et al. 2009).

We have focused on tumor specific neo-antigens derived from mutated self proteins as well as exogenous model antigens, both of which are inherently immunogenic, i.e. can readily be targeted by vaccination without additional therapy to break tolerance. Such neo-antigens are technically challenging to predict from patient tumor samples, but have been retrospectively identified in clinically responding patients using tumor cell lines and patient lymphocytes in several studies (Sensi and Anichini 2006). Similarly, clinical experience from vaccination with idiotypic immunoglobulin for lymphoma suggests that non-germline encoded epitopes from hypervariable regions are more immunogenic and stimulate CD4 and CD8 cells preferentially over framework regions (Baskar, Kobrin et al. 2004). Due to their generation *de novo* in neoplastic cells, such mutant antigenic targets are less likely to cause autoimmune side effects and are not subjected to central tolerance that can cause negative selection of high-

avidity T cells (von Herrath, Dockter et al. 1994). Recent advances in bioinformatics have made prospective identification of immunogenic mutations possible, and is an active area of further research into personalized cancer vaccines (Castle, Kreiter et al. 2012). However, the experience of adoptive immunotherapy suggests that the proliferative potential of effector cells is a critical variable (Rosenberg, Yang et al. 2011). For personalized cancer vaccines targeting tumor specific mutations to be successful, they should be administered in a protocol designed to maximize the quality and proliferative ability of responder lymphocytes.

We demonstrated that the generation of T cell responses against mutated self proteins by cancer vaccines was inhibited by temozolomide in a mouse model of glioma (Figure 3E) and by cyclophosphamide in a mouse model of melanoma (Figure 7A). In addition, using the model antigen ovalbumin we found that T cell clones that did expand after alkylating chemotherapy had lower affinity for cognate antigen, and lower TCR signal strength and inferior effector function upon antigenic stimulation (Figures 5 and 6). These differences seem sufficient to account for the loss of survival benefit from vaccination that we observed in both temozolomide and cyclophosphamide treated mice (Figs. 3B, 3D and 7B). These data are suggestive of the possibility that a defect in immune effectors generated by vaccination accounts for the shorter overall survival observed in metastatic melanoma patients treated with low dose cyclophosphamide shortly before being vaccinated versus patients receiving vaccination alone (Figure 7C). While there

were no meaningful differences in responses to tumor associated self-antigens in these patients with or without cyclophosphamide pre-treatment (Dudek, Mescher et al. 2008), it is possible that the difference in survival is accounted for by undetected responses against tumor specific neo-antigens.

In conclusion, we found that vaccine driven and spontaneous adaptive anti-tumor immune responses were inhibited by the direct anti-proliferative effect of alkylating chemotherapy. These findings are particularly noteworthy since alkylating chemotherapy is a standard treatment for several malignancies that have been the target of vaccine immunotherapy, including temozolomide for GBM (Stupp, Mason et al. 2005) and dacarbazine for metastatic melanoma (Chapman, Einhorn et al. 1999). These findings suggest that easily implemented modifications of conventional clinical protocols for cancer vaccine trials, such as banking unexposed PBMC prior to chemotherapy for use in later immunotherapy, could yield improved results. It has been reported, for instance, that 500 mL of blood contains sufficient numbers of naïve precursor CD8 T cells to allow large numbers of T cells specific to multiple tumor and viral antigens to be expanded *in vitro* (Oelke, Maus et al. 2003). Thus, easily extracted quantities of lymphocytes could be frozen and stored, either as source material for the *in vitro* expansion of anti-tumor T cells or as a banked pool of non-drug exposed naïve T cells to be infused prior to vaccination.

Furthermore, future trials of immune therapy could use such prognostic markers to stratify patients based on their relative likelihood to benefit from conventional

alkylating chemotherapy versus cancer vaccines, and prioritize immune therapy over chemotherapy in those most likely to benefit. *MGMT* promoter methylation status in glioblastoma is prognostic of response to temozolomide and is widely measured clinically (Hegi, Liu et al. 2008), whereas tumors with the mesenchymal gene expression pattern have a poor survival prognosis but appear to be more sensitive to active immune therapy than glioblastomas with other gene expression patterns (Prins, Soto et al. 2011). Altering clinical protocols and basing patient treatment on known prognostic indicators of treatment response could minimize harm of conventional therapies to cancer vaccines and maximize efficacy, leading to improved outcomes for patients treated with these vaccines.

Figure 1. Clinically relevant doses of temozolomide inhibit T lymphocyte proliferation in humans and mice. PBMC were collected from healthy volunteers and GBM patients ~1 month before temozolomide (TMZ) chemoradiotherapy or ~1 month after chemoradiotherapy. Lymphocyte cultures were stimulated with CD3/CD28 beads and IL-2 and viable counts were assessed in 3 or 4 technical replicates. (A) Viable cell counts for Patient 1 PBMC samples obtained pre- and post-chemoradiotherapy are shown alongside those of a healthy volunteer as a control. Error bars indicate SEM. *, $p<0.05$. (B) Representative PBMC cultures are shown at 100x total magnification. (C) Viable cell counts 4 days post stimulation of PBMC are shown for three healthy volunteers, three pre-chemoradiotherapy GBM patients and three post-chemoradiotherapy patients. (D) C57BL/6 mice (n=3 per group) were treated with the indicated dosages of TMZ daily for 5 days and 2 days after the last dose mice were sacrificed and splenocytes plated with CD3/CD28 beads and IL-2. Representative of two independent experiments. Error bars indicate SEM. *, $p<0.05$; **, $p<0.01$. (E) Purified CD8 T cells from mice treated with the indicated dosages of TMZ (n=3 per group) were stimulated as above. (E) Viable cell counts were assessed. Data are pooled from two independent experiments. Error bars indicate SEM. ***, $p<0.001$. (F) Aliquots of CD8 T cells stimulated as in (E) were taken for FACS analysis of phosphorylated ATM on day 4 post-stimulation.

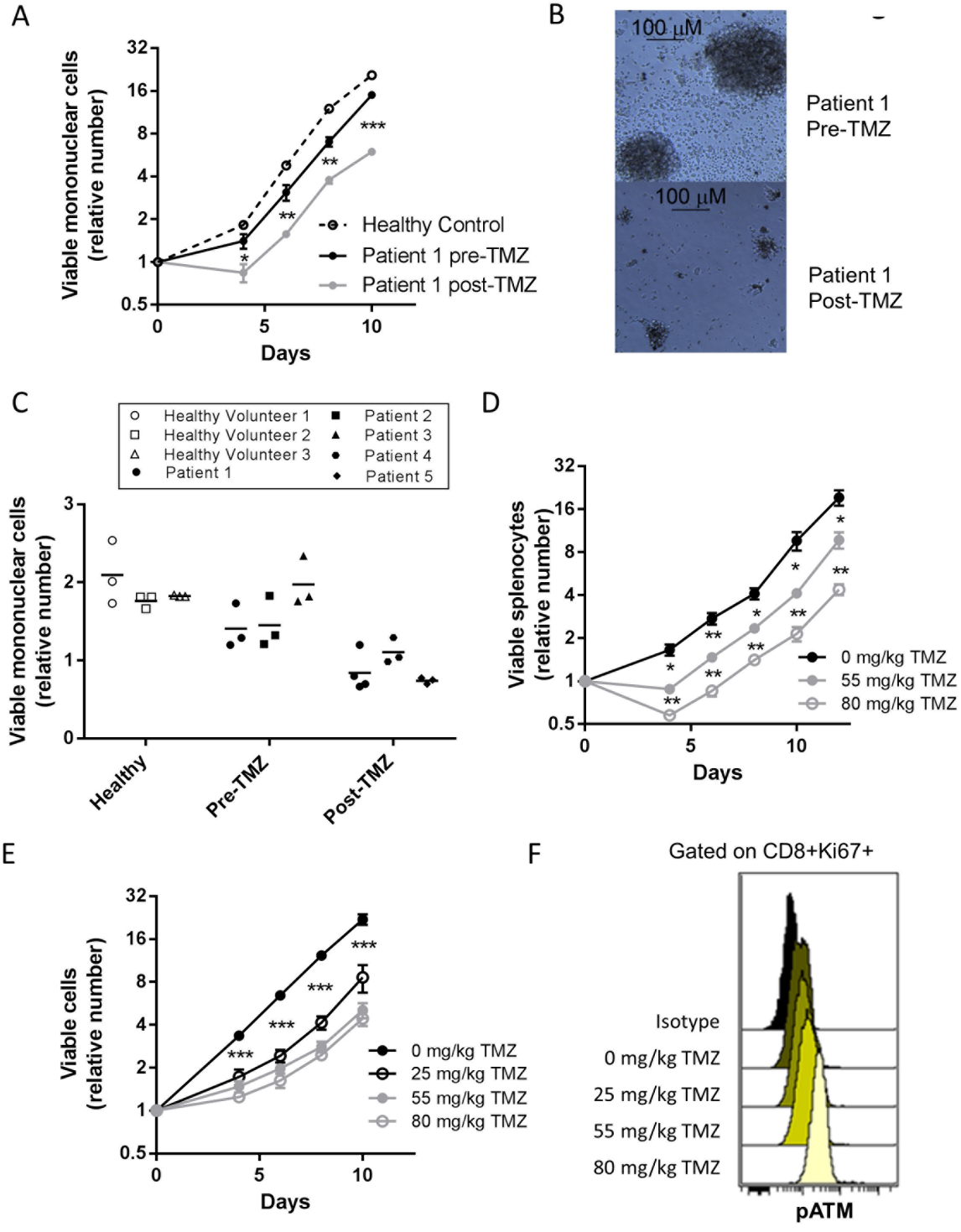

A
Viable mononuclear cells (relative number)
Days
Healthy Control
Patient 1 pre-TMZ
Patient 1 post-TMZ
B
100 μM
Patient 1 Pre-TMZ
100 μM
Patient 1 Post-TMZ
C
Healthy Volunteer 1
Healthy Volunteer 2
Healthy Volunteer 3
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Viable mononuclear cells (relative number)
Healthy
Pre-TMZ
Post-TMZ
D
Viable splenocytes (relative number)
Days
0 mg/kg TMZ
55 mg/kg TMZ
80 mg/kg TMZ
E
Viable cells (relative number)
Days
0 mg/kg TMZ
25 mg/kg TMZ
55 mg/kg TMZ
80 mg/kg TMZ
F
Gated on CD8+Ki67+
Isotype
0 mg/kg TMZ
25 mg/kg TMZ
55 mg/kg TMZ
80 mg/kg TMZ
pATM

Figure 2. Temozolomide exposure leads to a dose dependent inhibition of adaptive immune responses to vaccination. (A) C57BL/6 mice (n=7-8 per group) were treated with the indicated dosages of TMZ for 5 days, and 2 days after the last dose were vaccinated daily for 4 days with Ova and poly ICLC followed by weekly booster vaccinations as indicated. K^b-Ova specific CD8 T cells in the blood were assessed by flow cytometry. Error bars indicate SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. One representative experiment is shown; this experiment was performed independently three times with similar results. (B) The mice in (A) were terminally bled 70 days after the first vaccination and anti-Ova IgG/IgM was quantified by ELISA. Error bars indicate SEM. ***, $p<0.001$. (C) C57/BL6 mice (n=9 per group) were treated with TMZ at the indicated dosages for 5 days and 80 days after the last dose of TMZ were vaccinated daily for 4 days, with antigen specific CD8 T cells in blood assessed as above. Error bars indicate SEM. **, $p<0.01$; ***, $p<0.001$. The experiment shown is representative of two independent experiments with similar results. (D) C57BL/6 mice (n=5 per group) were vaccinated daily for 4 days and immunological memory was allowed to establish for 1 mo. at which point TMZ was administered daily for 5 days at the indicated dosages. The mice were again vaccinated daily for 4 days and given booster vaccinations weekly as indicated, and throughout antigen specific CD8 T cells in blood were assessed by flow cytometry. Error bars indicate SEM. **, $p<0.01$; ***, $p<0.001$.Representative of two independent experiments.

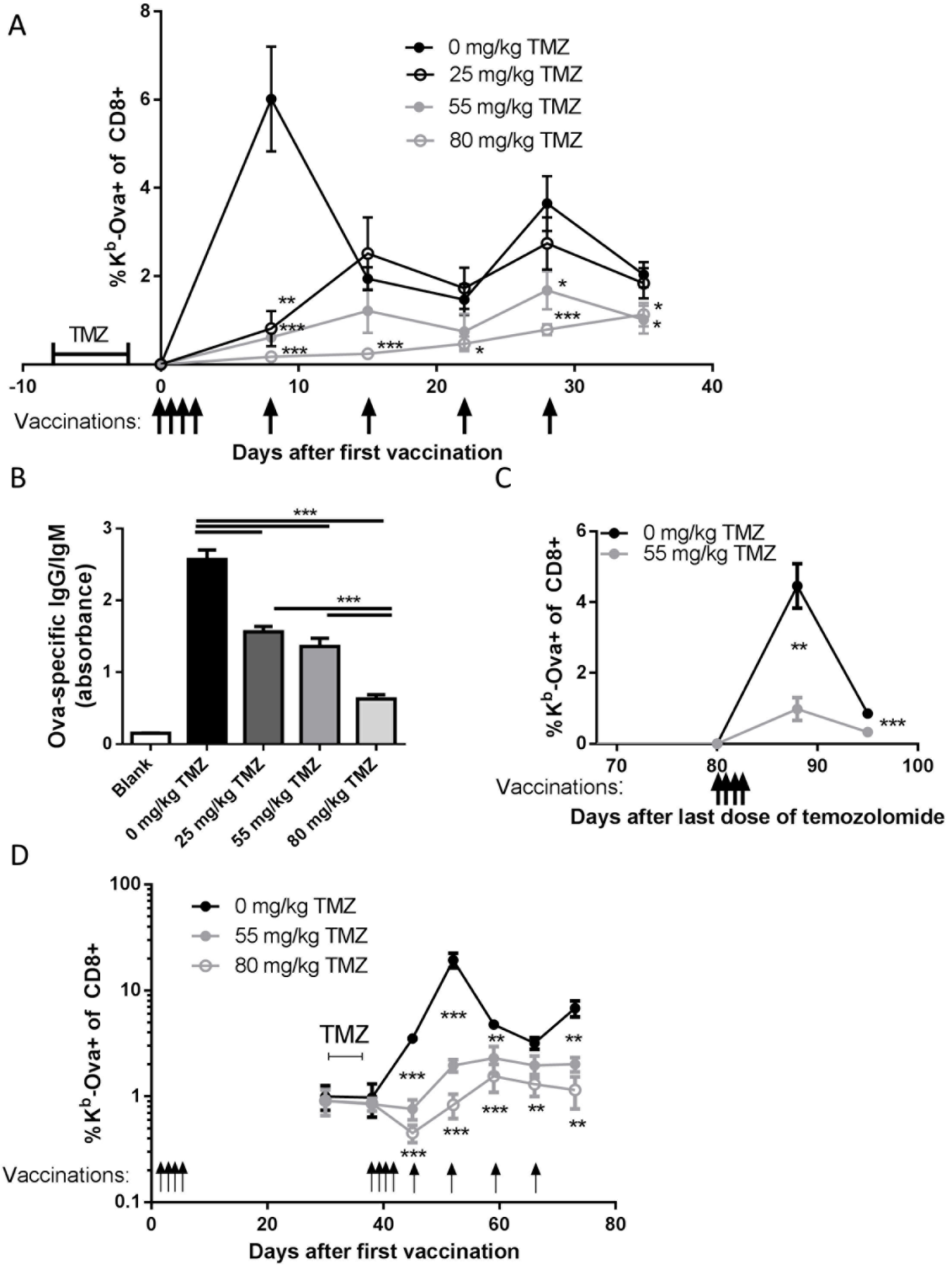

A
0 mg/kg TMZ
25 mg/kg TMZ
55 mg/kg TMZ
80 mg/kg TMZ
% K^b-Ova+ of CD8+
TMZ
Vaccinations:
Days after first vaccination
B
Ova-specific IgG/IgM (absorbance)
Blank
0 mg/kg TMZ
25 mg/kg TMZ
55 mg/kg TMZ
80 mg/kg TMZ
C
0 mg/kg TMZ
55 mg/kg TMZ
% K^b-Ova+ of CD8+
Vaccinations:
Days after last dose of temozolomide
D
0 mg/kg TMZ
55 mg/kg TMZ
80 mg/kg TMZ
% K^b-Ova+ of CD8+
TMZ
Vaccinations:
Days after first vaccination

Figure 3. Temozolomide exposure leads to inhibition of immune responses in tumor bearing animals. C57BL/6 mice (n=5-8 per group) were inoculated with Quad-GL261 cell line and in the indicated groups treated on days 6-10 after tumor inoculation with 80 mg/kg of TMZ and vaccinated with Ova and polyICLC on days 12-15, 19, 26, 33 and 40. (A) The percentage of K^b-Ova specific CD8 T cells in the blood was assessed at the indicated time points before control mice became moribund. Error bars indicate SEM. **, $p<0.01$; ***, $p<0.001$. (B) The percentage of mice in each group surviving is shown. P values shown are for log rank test. ns, not significant ($p>0.05$); ***, $p<0.001$. Representative of two independent experiments with similar results. (C) C57BL/6 mice (n=5-6 per group) were inoculated with Quad-KM3M14 and in the indicated groups were treated on days 3-7 after tumor inoculation with 55 mg/kg of TMZ and vaccinated with Ova and poly ICLC on days 10-13. K^b-Ova specific CD8 T cells were assessed by flow cytometry on day 17. Error bars indicate SEM. ns, not significant ($p>0.05$); *, $p<0.01$; **, $p<0.01$; ***, $p<0.001$. (D) The percentage of mice in each group surviving is shown. P values shown are for log rank test. ns, not significant ($p>0.05$); **, $p<0.01$. (E) C57BL/6 mice (n=6-8 per group) were inoculated with GL261 tumors and in the indicated groups treated with 55 mg/kg TMZ on days 6-10 after tumor inoculation and vaccinated with GARC-1 peptide and poly ICLC on days 12-15. D^b-GARC-1 specific activated CD8 T cells were assessed in blood by flow cytometry on day 19. Error bars indicate SEM. ns, not

significant (p>0.05); *, p<0.01. Representative of two independent experiments with similar results.

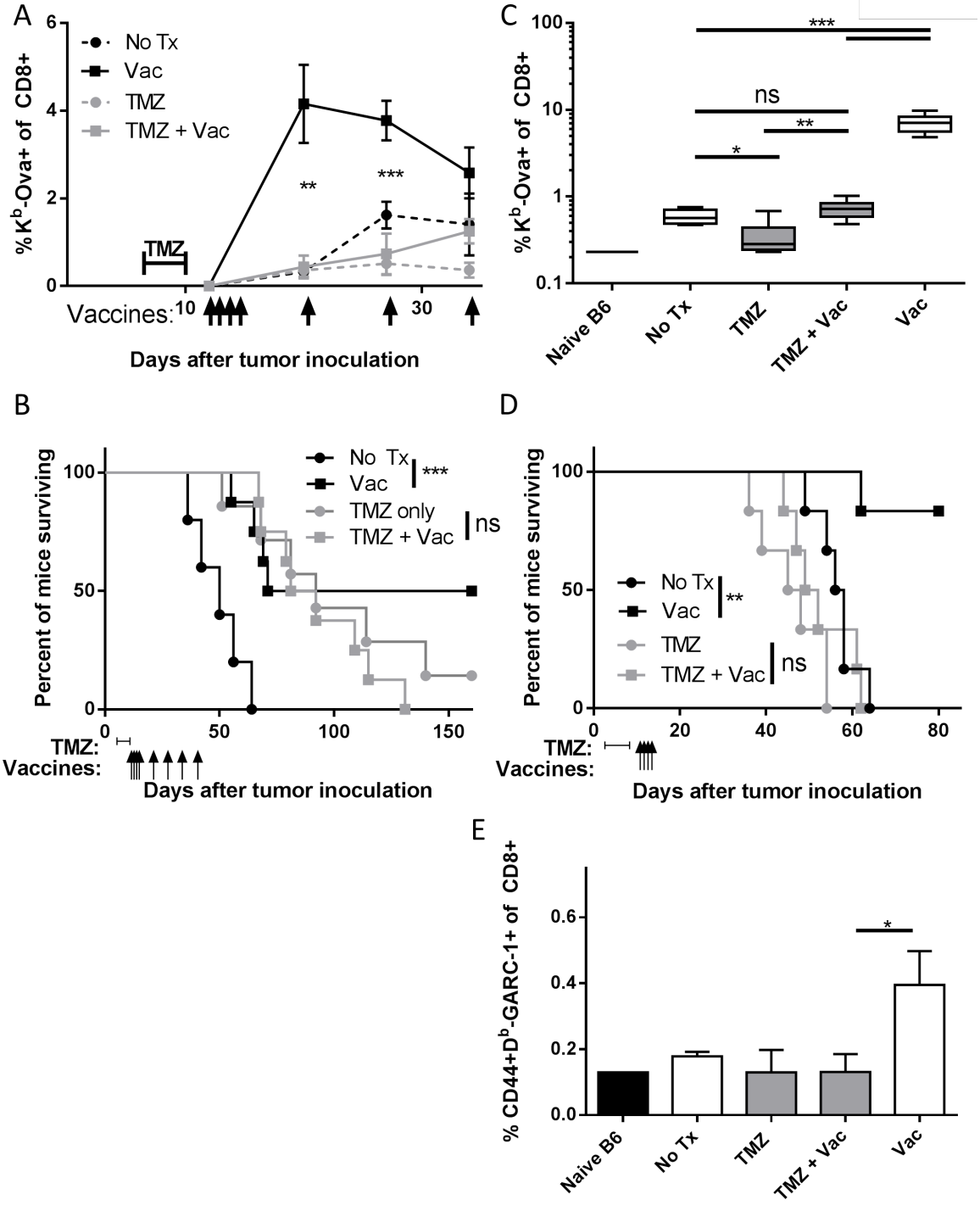

A
No Tx
Vac
TMZ
TMZ + Vac
% K^b-Ova+ of CD8+
TMZ
Vaccines:
Days after tumor inoculation
C
% K^b-Ova+ of CD8+
Naive B6
No Tx
TMZ
TMZ + Vac
Vac
B
Percent of mice surviving
No Tx
Vac
TMZ only
TMZ + Vac
TMZ:
Vaccines:
Days after tumor inoculation
D
Percent of mice surviving
No Tx
Vac
TMZ
TMZ + Vac
TMZ:
Vaccines:
Days after tumor inoculation
E
% CD44+D^b-GARC-1+ of CD8+
Naive B6
No Tx
TMZ
TMZ + Vac
Vac

Figure 4. Strong TCR signals induce DNA damage response following alkylating chemotherapy. (A) OT-I mice were treated with temozolomide for five days and two days later splenocytes were stimulated *in vitro* with peptide for 48 hours. Cells were stained for CD8, phosphorylated ATM and Ki67 and analyzed by flow cytometry. Representative plots are gated on CD8+ cells. (B) Aggregate data of flow cytometry as performed in (A). Percentage of proliferating (Ki67+) cells that were positive for phosphorylated ATM is plotted. Each experiment was performed with three technical replicates derived from the splenocytes of one mouse treated as indicated. Error bars indicate SEM. ns, not significant ($p>0.05$); **, $p<0.01$; ***, $p<0.001$. Data shown are pooled from two independent experiments with similar outcome. (C) and (D) Aggregate data of flow cytometry performed as in (A) for OT-I mice given the indicated treatments. Error bars indicate SEM. ns, not significant ($p>0.05$); **, $p<0.01$; ***, $p<0.001$. (E) C57BL/6 mice (n=6-9 per group) were given the indicated treatments then vaccinated daily for 4 days with ovalbumin and poly ICLC. 7 days after the first vaccine antigen specific cells in blood were assessed by flow cytometry. Error bars indicate SEM. ns, not significant ($p>0.05$); *, $p<0.05$; **, $p<0.01$. Data are pooled from three independent experiments with similar results.

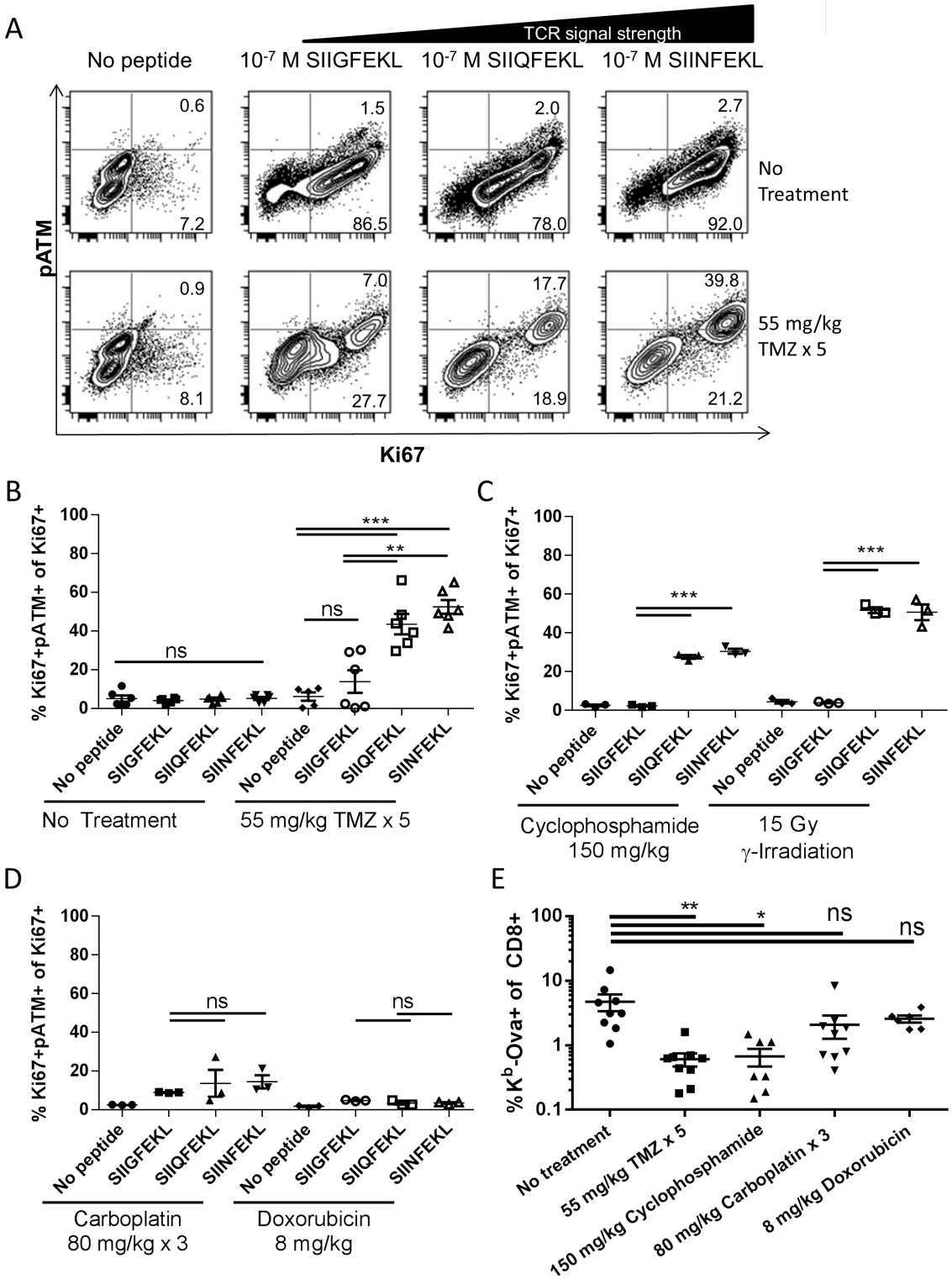
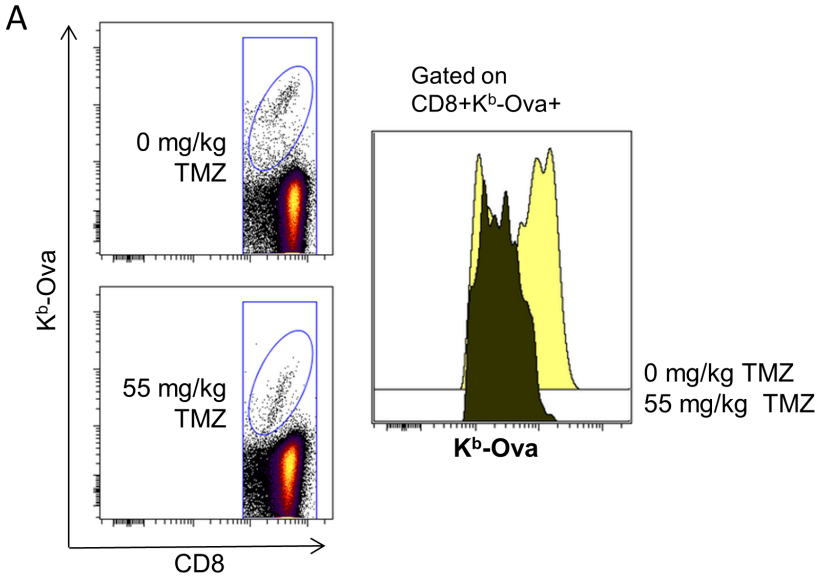
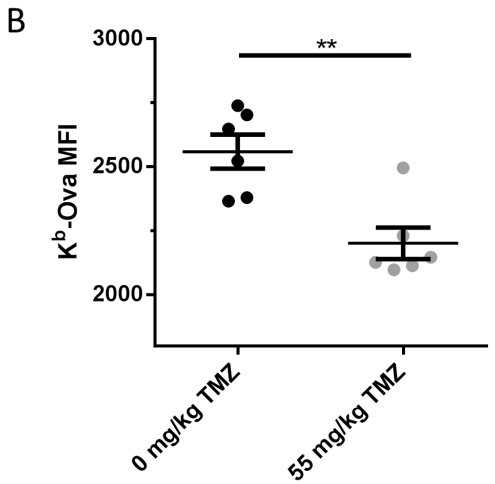
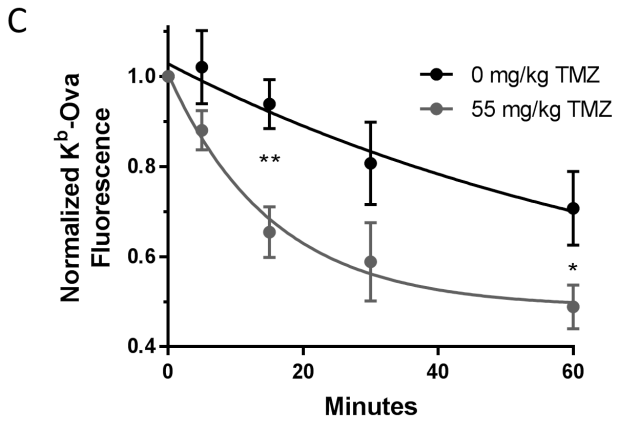

A
TCR signal strength
No peptide
10-7 M SIIGFEKL
10-7 M SIIQFEKL
10-7 M SIINFEKL
pATM
Ki67
0.6
7.2
1.5
86.5
2.0
78.0
2.7
92.0
No Treatment
0.9
8.1
7.0
27.7
17.7
18.9
39.8
21.2
55 mg/kg TMZ x 5
B
% Ki67+pATM+ of Ki67+
ns
***
**
No peptide
SIIGFEKL
SIIQFEKL
SIINFEKL
No Treatment
55 mg/kg TMZ x 5
C
% Ki67+pATM+ of Ki67+
***
Cyclophosphamide 150 mg/kg
15 Gy γ-Irradiation
D
% Ki67+pATM+ of Ki67+
Carboplatin 80 mg/kg x 3
Doxorubicin 8 mg/kg
E
% Kb-Ova+ of CD8+
**
*
ns
No treatment
55 mg/kg TMZ x 5
150 mg/kg Cyclophosphamide
80 mg/kg Carboplatin x 3
8 mg/kg Doxorubicin

Figure 5. The affinity of vaccine responding lymphocytes is lower following temozolomide. (A) C57BL/6 mice (n=5 per group) were treated with TMZ as indicated and vaccinated daily for 4 days with Ova and poly ICLC. 30 days later splenocytes were stained for K^b-Ova and CD8 and analyzed by flow cytometry. Antigen specific cells were gated as shown. (B) Aggregate data of a representative experiment performed as in (A). Error bars indicate SEM. **, $p<0.01$. Data shown are representative of two independent experiments with similar results. (C) C57BL/6 mice (n=3-4 per group) were treated with the TMZ as indicated and vaccinated daily for 4 days with Ova and poly ICLC. 7 days later splenocytes were stained for K^b-Ova and CD8. Relative affinity of vaccine responding CD8 T cells was calculated by observing decay in normalized total fluorescence by flow cytometry as described in Materials and Methods. Curves shown are first order exponential decays fit to data. Error bars indicate SEM. *, $p<0.05$; **, $p<0.01$. Data shown are pooled data of two independent experiments with similar results. (D) C57BL/6 (n=7-8 per group) mice were treated with the TMZ as indicated and vaccinated daily for 4 days with Ova and poly ICLC followed by 4 weekly boosters (as in Figure 2A) and terminally bled 70 days after the first vaccination. Relative affinity was calculated by dividing the absorbance of ELISA wells plated with PBS (control) by those plated with 1 M guanidinium chloride (chaotropic agent). Error bars indicate SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.


A
0 mg/kg TMZ
55 mg/kg TMZ
Kb-Ova
CD8
Gated on CD8+Kb-Ova+
0 mg/kg TMZ
55 mg/kg TMZ
Kb-Ova


B
Kb-Ova MFI
3000
2500
2000
**
0 mg/kg TMZ
55 mg/kg TMZ


C
Normalized Kb-Ova Fluorescence
1.0
0.8
0.6
0.4
0
20
40
60
Minutes
0 mg/kg TMZ
55 mg/kg TMZ
**
*

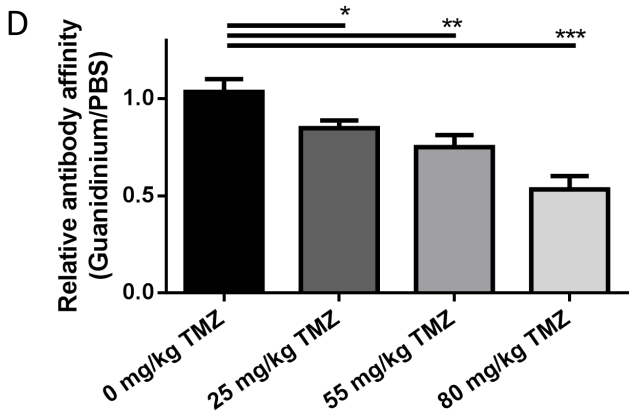

D
Relative antibody affinity (Guanidinium/PBS)
1.0
0.5
0.0
*
**
***
0 mg/kg TMZ
25 mg/kg TMZ
55 mg/kg TMZ
80 mg/kg TMZ

Figure 6. CD8 T cells expanded by vaccines following temozolomide have inferior functional characteristics. (A) Nur77^{GFP} TCR signal strength reporter mice (n=4 per group) were treated with the TMZ as indicated and vaccinated daily for 4 days with Ova and poly ICLC. 7 days later splenocytes were plated with the indicated amount of SIINFEKL peptide for 8 hours. Antigen specific T cells were identified by staining for K^b-Ova and CD8, and GFP intensity of antigen specific cells was assessed by flow cytometry. (B) Aggregate data of experiments performed as described in (A). Error bars indicate SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Data shown are pooled from two independent experiments with similar results. (C) C57BL/6 mice (n=3-4 per group) were treated with the TMZ as indicated and vaccinated daily for 4 days with ovalbumin and poly ICLC. 7 days later mice splenocytes were plated for 24 hours with the indicated amount of SIINFEKL peptide. Elaborated IFN-g was measured by cytokine bead array and normalized to antigen specific T cells as indicated in Materials and Methods. Error bars indicate SEM. *, $p<0.05$; **, $p<0.01$. Data shown are pooled data from two independent experiments with similar results.

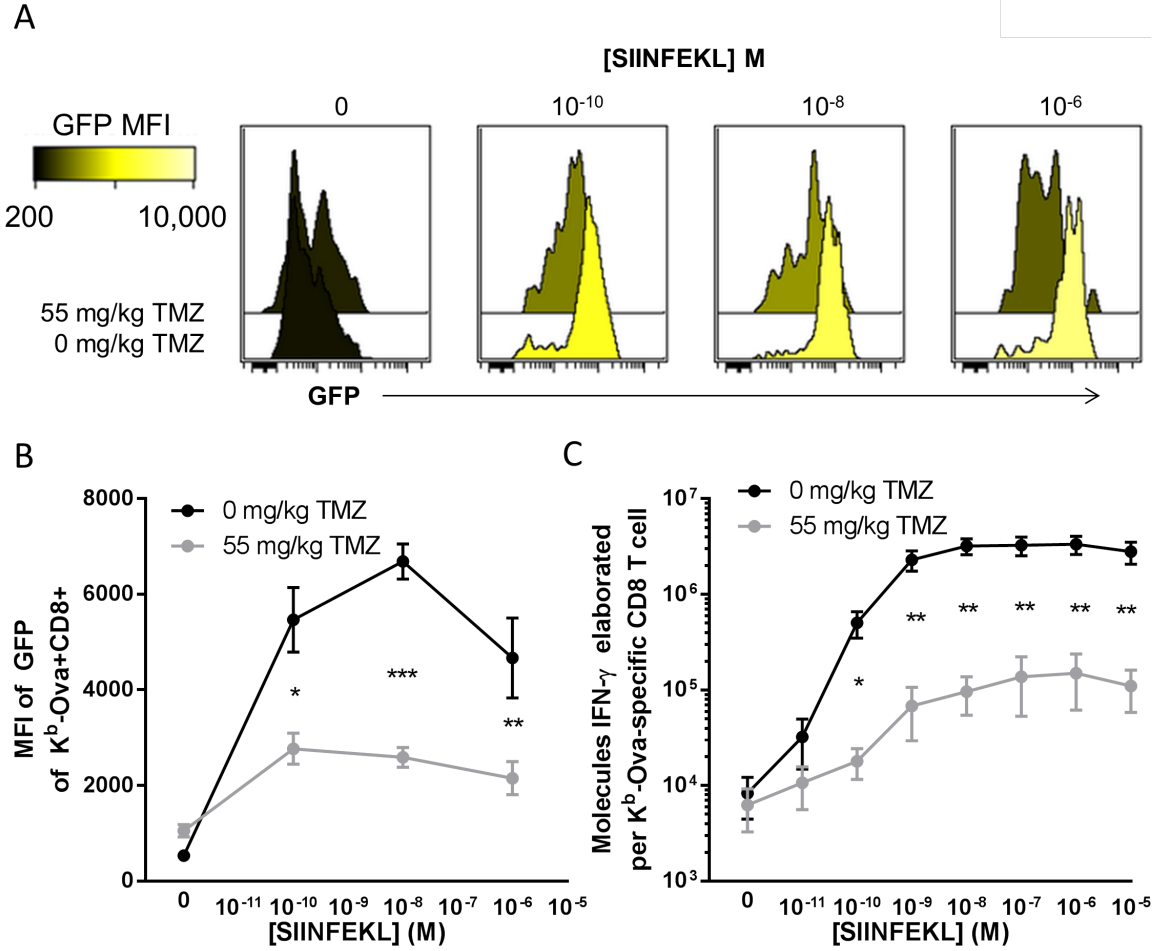
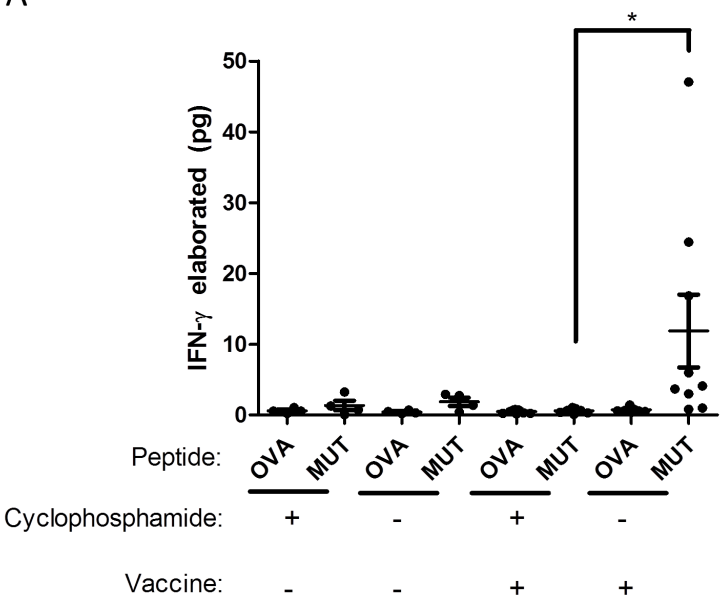
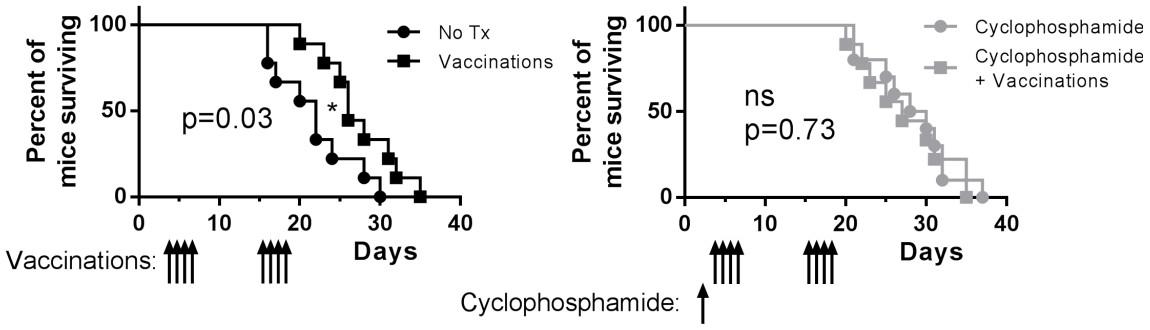

A
[SIINFEKL] M
0
10-10
10-8
10-6
GFP MFI
200
10,000
55 mg/kg TMZ
0 mg/kg TMZ
GFP
B
C
0 mg/kg TMZ
55 mg/kg TMZ
MFI of GFP of Kb-Ova+CD8+
Molecules IFN-γ elaborated per Kb-Ova-specific CD8 T cell
[SIINFEKL] (M)

Figure 7. Cyclophosphamide pre-treatment is associated with less survival benefit from melanoma vaccines. (A) C57BL/6 mice (n=9 per group) were implanted subcutaneously with $7.5*10^4$ B16-F10 melanoma cells. Mice were given cyclophosphamide as indicated day 2 after tumor inoculation and vaccinated with B16-F10 cell line mutant peptides and poly ICLC on days 3-6 and17-20 after tumor inoculation. On day 10 after tumor inoculation leukocytes from 100 mL blood were incubated with splenocyte APC pulsed either with irrelevant (Ova-derived) peptides or B16-F10 mutant peptide cocktail. Elaborated IFN-g was measured by cytokine bead array for all 9 mice per group for vaccinated groups, 4 per group for non-vaccinated groups. Error bars indicate SEM. *, $p<0.05$. (B) Survival of mice in (A). Mice were sacrificed when tumors reached >1000 mm^3 in volume, survival is depicted by Kaplan-Meier plot. P values shown are for log rank test. ns, not significant ($p>0.05$); *, $p<0.05$. (C) Patients with metastatic melanoma (n=10 per group) were enrolled in a clinical trial of the large multivalent immunogen and treated with autologous vaccinations either with or without a single dose of 300 mg/m^2 of cyclophosphamide one week beforehand. Overall survival is depicted by Kaplan-Meier plot. P values shown are for log rank test. *, $p<0.05$.

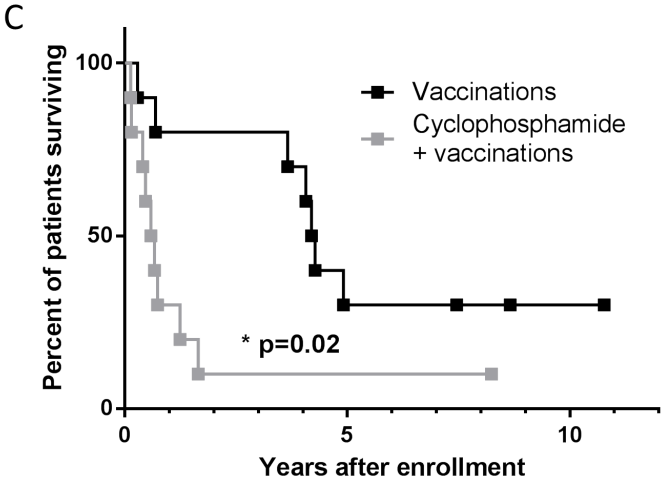
A


*
IFN-γ elaborated (pg)
50
40
30
20
10
0
Peptide:
OVA
MUT
OVA
MUT
OVA
MUT
OVA
MUT
Cyclophosphamide:
+
-
+
-
Vaccine:
-
-
+
+

B

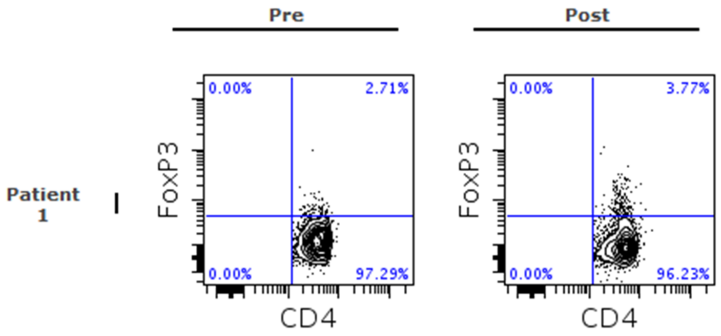

Percent of mice surviving
100
50
0
p=0.03
*
No Tx
Vaccinations
0
10
20
30
40
Days
Vaccinations:
Percent of mice surviving
100
50
0
ns
p=0.73
Cyclophosphamide
Cyclophosphamide + Vaccinations
0
10
20
30
40
Days
Cyclophosphamide:

C

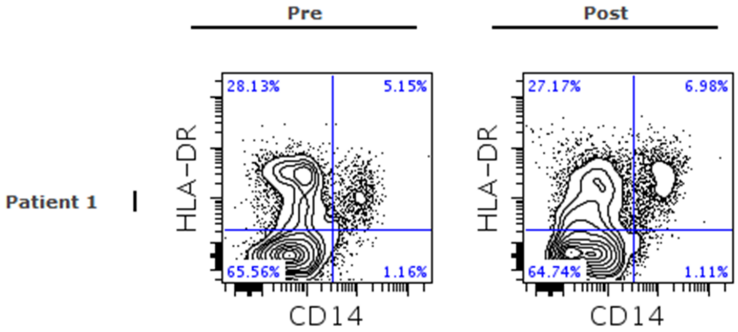

Percent of patients surviving
100
50
0
Vaccinations
Cyclophosphamide + vaccinations
* p=0.02
0
5
10
Years after enrollment

Supplementary Figure 1. Suppressive cell populations are similar in GBM patient PBMC before and after temozolomide treatment. GBM patient PBMC were treated identically to those input into proliferation assays and were assessed by flow cytometry. A representative patient sample is shown with (A) Treg defined as CD4+ FoxP3+ cells (B) monocytic myeloid derived suppressor cells defined as CD14+HLA-DR- and (C) granulocytic myeloid derived suppressor cells defined as CD15+CD33+.

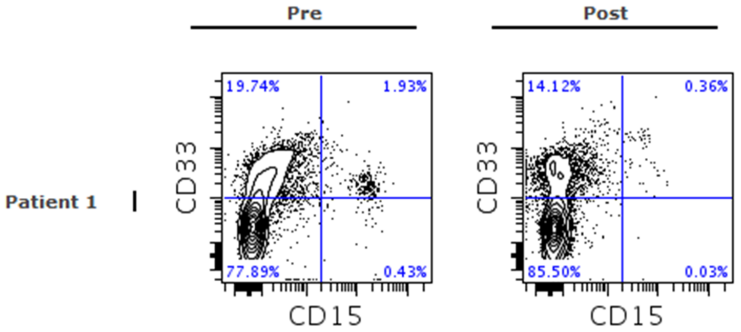
A


Pre
Post
Patient 1
FoxP3
CD4
0.00%
2.71%
0.00%
97.29%
0.00%
3.77%
0.00%
96.23%

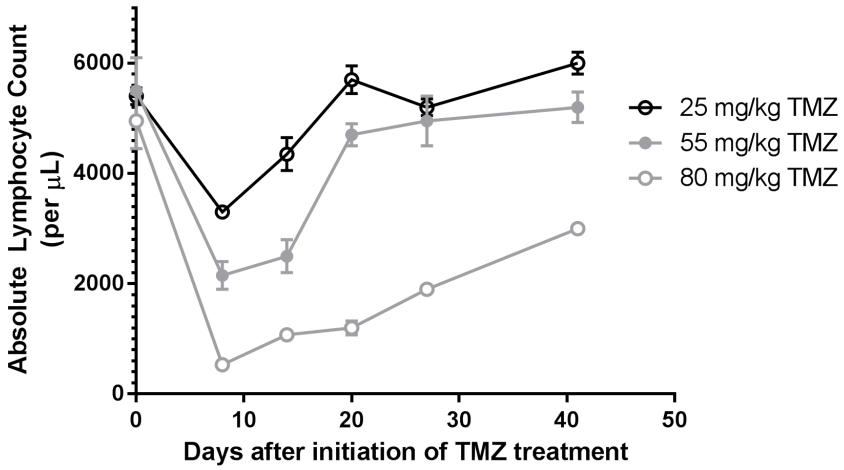
B


Pre
Post
Patient 1
HLA-DR
CD14
28.13%
5.15%
65.56%
1.16%
27.17%
6.98%
64.74%
1.11%

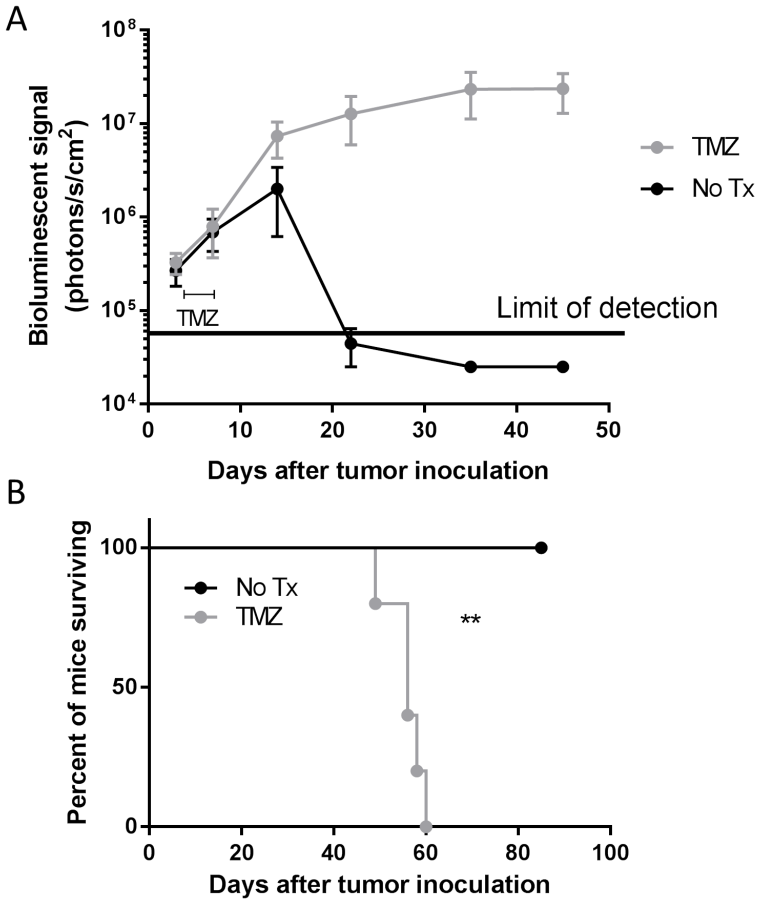
C


Pre
Post
Patient 1
CD33
CD15
19.74%
1.93%
77.89%
0.43%
14.12%
0.36%
85.50%
0.03%

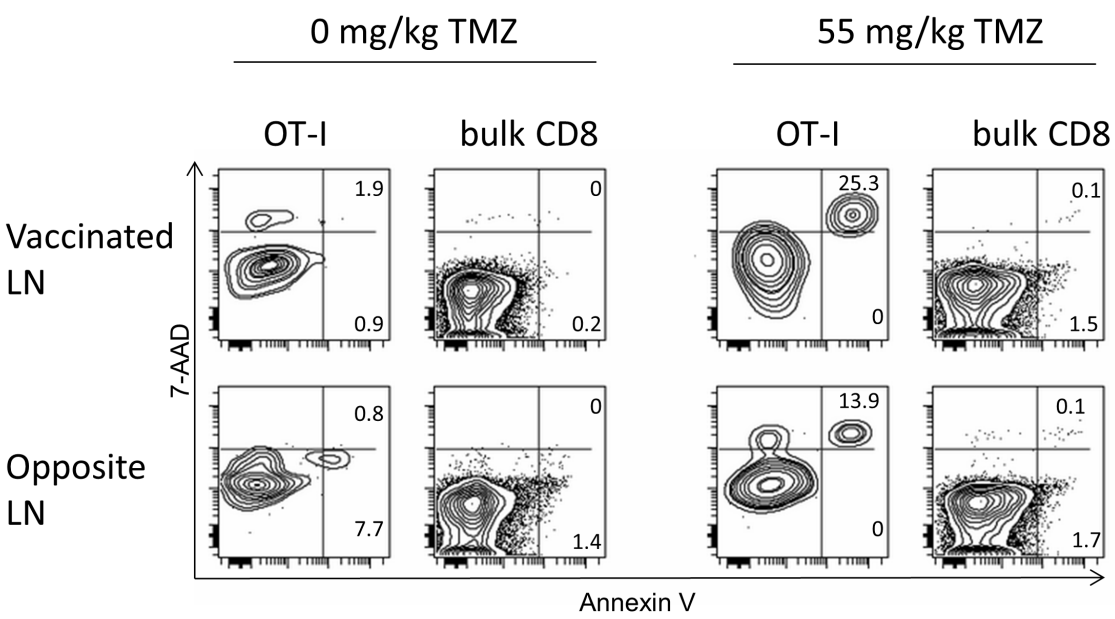
Supplementary Figure 2. Human dosages of temozolomide are modeled in mice. Transient lymphopenia is observed in C57BL/6 mice (n=4 per group) given indicated dosages of TMZ. Complete blood counts were obtained from 20 mL of blood using Hemavet machine (Drew Scientific), with absolute lymphocyte counts shown. Error bars indicate SEM.


Absolute Lymphocyte Count (per μL)
6000
4000
2000
0
0
10
20
30
40
50
Days after initiation of TMZ treatment
25 mg/kg TMZ
55 mg/kg TMZ
80 mg/kg TMZ

Supplementary Figure 3. Spontaneous anti-tumor immune responses are inhibited by temozolomide treatment. (A) C57/BL6 mice (n=5 per group) were implanted with 30,000 cells of the immunogenic syngeneic glioma cell line O94M2 which express the SV40 Large T antigen. Mice were treated as indicated with temozolomide on days 3-7 after tumor implantation and tumor growth was tracked with bioluminescent imaging. (B) Survival of mice in (A) is indicated with Kaplan Meier plot. P values shown are for log rank test. **, $p<0.01$.

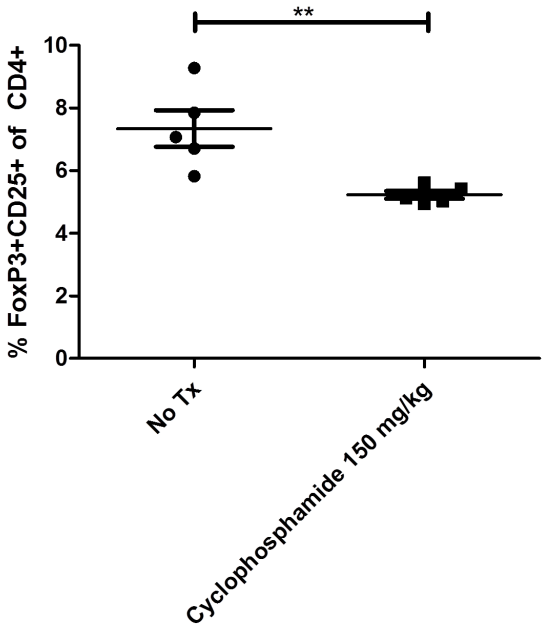

A
Bioluminescent signal (photons/s/cm2)
10^8
10^7
10^6
10^5
10^4
TMZ
Limit of detection
TMZ
No Tx
0
10
20
30
40
50
Days after tumor inoculation
B
Percent of mice surviving
100
50
0
No Tx
TMZ
**
0
20
40
60
80
100
Days after tumor inoculation

Supplementary Figure 4. Adoptively transferred OT-I undergo apoptosis following temozolomide exposure and vaccination. (A) $2x10^6$ Thy1.2+ OT-I CD8 T cells were transferred into Thy1.1+ host mice (n=3 per group). Mice were given the indicated treatment and then vaccinated in the left leg with SIINFEKL and polyICLC. 24 hrs later both the vaccinated (left) and opposite (right) inguinal lymph nodes were stained for Thy1.2, CD8, Annexin V and viability (7-AAD). Representative flow plots gated on CD8+Thy1.2+ (OT-I) or CD8+Thy1.2- (bulk CD8) are shown.


0 mg/kg TMZ
55 mg/kg TMZ
OT-I
bulk CD8
OT-I
bulk CD8
Vaccinated LN
Opposite LN
1.9
0.9
0
0.2
25.3
0
0.1
1.5
0.8
7.7
0
1.4
13.9
0
0.1
1.7
7-AAD
Annexin V

Supplementary Figure 5. Treg are depleted by low dose cyclophosphamide. C57BL/6 mice (n=5 per group) were implanted with 75,000 B16-F10 melanoma cells and treated with an intraperitoneal injection of 150mg/kg cyclophosphamide 2 days later. Four days after cyclophosphamide injection blood was stained for flow cytometry for CD3, CD4, CD8, CD25, and FoxP3. Treg (CD4+CD25+FoxP3+) in blood were assessed (A) as a percentage of CD4 T cells and (B) as absolute number of cells per microliter as assessed by flow cytometry counting beads. Error bars indicate SEM. **, $p<0.01$.

A


**
% FoxP3+CD25+ of CD4+
10
8
6
4
2
0
No Tx
Cyclophosphamide 150 mg/kg

B

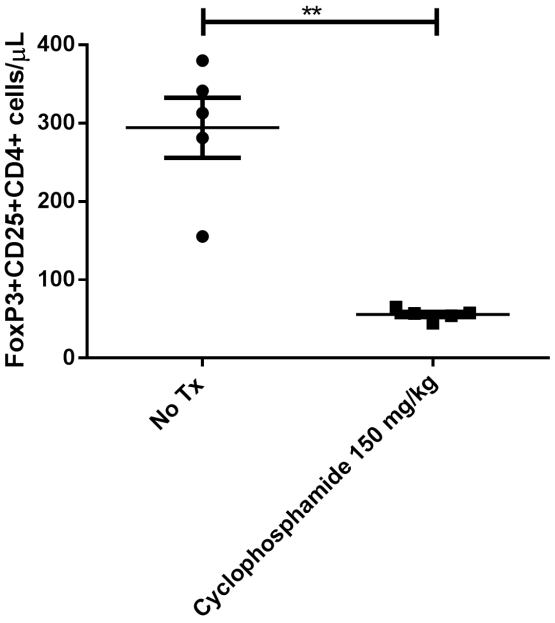

**
FoxP3+CD25+CD4+ cells/μL
400
300
200
100
0
No Tx
Cyclophosphamide 150 mg/kg

Table 1. Dosages of temozolomide in humans and mice.

Species / Dose	Time	Dose (human)	Dose(mice)	Lymphocytes rel. to normal
Human / Standard	42 days	3150 mg/m^2	n/a	~10-50%
Human / Adjuvant	5 days / cycle	1000 mg/m^2	n/a	~10-30%
Human / Intense Adjuvant	21 days / cycle	2100 mg/m^2	n/a	~10-20%
Mouse / High	5 days	~3000 mg/m^2 equiv.	80 mg/kg	~10-15%
Mouse / Medium	5 days	~2100 mg/m^2	55 mg/kg	~40-50%
Mouse / Low	5 days	~1000 mg/m^2	25 mg/kg	~55-65%

Chapter 3: Alkylating chemotherapy may exert a uniquely deleterious effect upon neo-antigen targeting vaccine strategies

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Abbreviations used: ATM, ataxia telangiectasia mutated; HLA, human leukocyte antigen; PBMC, peripheral blood mononuclear cell; TCR, T cell receptor; Treg, regulatory T cell;

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Alkylating chemotherapy and immunotherapy

Due to the hypothesis that sustained remissions of advanced cancer in response to conventional therapy may require endogenous immune responses to mediate their lasting effect, much interest has been generated by the prospect of combined chemo-immunotherapy.(Zitvogel, Apetoh et al. 2008) Using alkylating drugs as well as other cytotoxic chemotherapeutic agents, immune stimulatory effects have been reported by several mechanisms. These include the liberation of inflammatory and homeostatic cytokines by cytotoxic chemotherapy,(Asavaroengchai, Kotera et al. 2002) the immune stimulatory effect of the immunogenic death of tumor cells caused by chemotherapy,(Michaud, Martins et al. 2011) and the direct tumor sensitizing effect of chemotherapy to the cytolytic activity of immune cells.(Ramakrishnan, Assudani et al. 2010) Additionally, much interest has been directed to combination of conventional chemotherapy and active immunotherapeutic strategies such as cancer vaccines, to try to derive synergy between two complimentary approaches.(Brode and Cooke 2008)

This has particularly been the case for a widely used class of chemotherapeutic drugs, the alkylating chemotherapies. Alkylating chemotherapy refers to a class of DNA damaging chemotherapeutic agents that covalently modify DNA by either methylation or the generation of inter-strand or intra-strand alkyl

crosslinks.(Lawley and Brookes 1965, Marchesi, Turriziani et al. 2007) These agents include some of the oldest anti-neoplastic drugs known such as nitrogen mustard, and also many agents still in common clinical use such as cyclophosphamide, dacarbazine and temozolomide.(Marchesi, Turriziani et al. 2007) While these agents act non-specifically and alkylate many chemical species within the cell, their anti-neoplastic effect is mediated by accumulation of DNA damage, particularly in cells that proliferate rapidly, such as lymphocytes or transformed cells.(Roos, Baumgartner et al. 2004, Roos and Kaina 2012) Given their routine use for a number of common cancers, these drugs have been used in many clinical protocols of experimental immune therapies.(Dudek, Mescher et al. 2008, Prins, Soto et al. 2011, Walter, Weinschenk et al. 2012) Additionally, investigations into the utility of these drugs as conditioning regimens before adoptive transfer of immune cells(Restifo, Dudley et al. 2012) as well as of their immune modulatory effects(Machiels, Reilly et al. 2001) have led to interest in combination chemo-immunotherapy regimens using alkylating agents.

Previous work has demonstrated that in addition to their anti-neoplastic effects, alkylating chemotherapy selectively depletes certain immune populations. In particular, regulatory T cells (Treg) seem particularly susceptible to alkylating chemotherapy,(Banissi, Ghiringhelli et al. 2009) likely due to the fact that these cells proliferate in response to toleregenic stimuli in the steady state.(Seneschal, Clark et al. 2012) This has led to interest in the use of alkylating chemotherapy

as a conditioning regimen before vaccination in order to deplete Treg. While evidence exists that low dose alkylating chemotherapy can exert a stimulatory effect in the context of vaccinations targeting self-antigens which are normally suppressed by Treg, it is unclear how generalizable this finding is.(Ercolini, Ladle et al. 2005, Walter, Weinschenk et al. 2012) Indeed, given the profound proliferative burst that occurs during the initiation of an adaptive immune response,(Blattman, Antia et al. 2002) it is counter-intuitive that an anti-proliferative chemotherapeutic drug would be immune stimulatory. Our research group has recently published findings that indicate that, indeed, the anti-proliferative effect of alkylating chemotherapy exerts an immune suppressive effect upon subsequent vaccination that acts in a cell intrinsic manner in responder lymphocytes.(Litterman, Zellmer et al. 2013) This effect is particularly important in the case of tumor neo-antigens which are derived from “mutated-self” proteins that are immunogeneic even in non-Treg depleted hosts. Here we present a summary of the key findings of this paper and put forth a theoretical framework to explain how the quality of the T cells responsive to a given antigen could affect the outcome of alkylating chemotherapy given prior to vaccination. We conclude by discussing the implications of these data for clinical researchers, particularly for tumor immunotherapy targeting patient specific neo-antigens or using autologous tumor material from tumors harboring large numbers of mutations, such as melanoma or carcinoma of the lung.

Regulatory T cell depletion can be beneficial for immunization against self antigens

The immunological rationale for the combination of anti-proliferative alkylating chemotherapy with vaccination has been due to the depleting effect of alkylating chemotherapy on regulatory T cell populations. This finding has been reported for both conventional and metronomic dosing schedules in rodents and has also been observed in human patients treated with these drugs.(Ercolini, Ladle et al. 2005, Banissi, Ghiringhelli et al. 2009, Fadul, Fisher et al. 2011, Walter, Weinschenk et al. 2012) While at high doses alkylating drugs can cause a general leukopenia associated with a susceptibility to opportunistic infections(Grossman, Ye et al. 2011) (for instance, due to a profound neutropenia), at commonly used clinical doses these drugs have a relatively benign safety profile and are not commonly associated with lymphopenia as a dose limiting toxicity.(Wick and Weller 2005) Indeed, we and others have observed that it appears that Treg are semi-selectively depleted, decreasing both in absolute number and in relative proportion to other lymphocytes upon alkylating chemotherapy treatment.(Ercolini, Ladle et al. 2005, Litterman, Zellmer et al. 2013) We speculate that this is due to the fact that Treg are more likely to be undergoing cell division in the steady state than naïve T cells. Indeed, recent work examining the effect of toleregenic antigen presenting cells upon Treg in the steady state seems to support this idea. In the skin, for instance, the majority of

cycling T cells in normal individuals are Treg, which proliferate in an antigen specific way to local antigen presenting cells, presumably in response to self antigens.(Seneschal, Clark et al. 2012)

Experiments in mouse tumor models overexpressing tolerized self-antigens have demonstrated a mechanism to explain why regulatory T cell depletion by alkylating chemotherapy may have a beneficial effect in this context. Using human HER2-ovexpressing transgenic mice as hosts for a tumor driven by human HER2, Jaffee and colleagues found that diverse chemotherapeutic drugs could lead to synergy with vaccination, if administered before the vaccinations began.(Machiels, Reilly et al. 2001) This effect was mediated by an enhanced tumoricidal T cell priming. Later work demonstrated that this enhanced priming was due to depletion of Treg allowing recruitment into the immune response of self-specific T cells that were not activated in the presence of Treg.(Ercolini, Ladle et al. 2005) Interestingly, this beneficial effect of chemotherapy driven Treg depletion appears self antigen specific, as only human HER2 transgenic mice benefited from chemotherapy pre-treatment, as wild-type mice could generate anti-HER2 CD8 T cell responses without Treg depletion.(Ercolini, Ladle et al. 2005) The finding that only self-specific responses require Treg depletion is concordant not only with the fact that typical immune responses to pathogens occur routinely in Treg replete hosts, but also with the lymphoproliferative and autoimmune phenotype of individuals with an impaired regulatory T cell

compartment.(d'Hennezel, Ben-Shoshan et al. 2009, Hofer, Krichevsky et al. 2012) In addition to the above mentioned apparent self-specificity of Tregs themselves, it has been posited that Treg can act as a sink for stimulatory cytokines like IL-2 produced in response to antigenic stimulation of T cells. By sequestering the small amounts of IL-2 generated in response to weak self-antigens, Treg may raise the threshold for mounting a T cell response in order to delineate a sharp, all-or-none boundary between typically ineffective, weak antigens (“self”) and inherently immunogenic, strong antigens (“non-self”). (Hofer, Krichevsky et al. 2012) Interestingly, data from a recent clinical trial seem to provide experimental support in man for the viewpoint that alkylating chemotherapy pre-treatment can drive responses from a normally non-reactive, latent precursor population of T cells with self specificities. In an early phase clinical trial of a pool peptide vaccine consisting of highly tumor expressed HLA-A2 restricted self peptides, Walter and colleagues reported enhanced immune responses and overall survival upon low dose cyclophosphamide pre-treatment prior to vaccination.(Walter, Weinschenk et al. 2012)

Alkylating chemotherapy has an anti-proliferative effect on responder lymphocytes

In addition to their Treg depleting effects, alkylating chemotherapy also affects all exposed cells in the host, including any potentially tumor reactive lymphocytes

that would be expanded by a vaccine. For antigens that are inherently immunogenic, the effect of Treg depletion may be neglible, and therefore we sought to understand the responder cell intrinsic effect of alkylating chemotherapy on immune responses to this class of “non-self” antigens.(Ercolini, Ladle et al. 2005) We did indeed find a surprisingly long-lived anti-proliferative effect of alkylating chemotherapy on immune responses, with both the magnitude and quality of response to neo-antigens being impaired for at least 10 weeks after the administration of temozolomide.(Litterman, Zellmer et al. 2013) Both B and T cell responses had lower peak magnitudes, and both the antibodies and TCRs of the measured responses had lower affinity for cognate antigen. In all cases it seemed that these defects stem directly from the DNA damaging nature of the chemotherapy. Indeed, while temozolomide treated mouse splenocytes do not stain positively for phosphorylated ATM (a key marker of the DNA damage response) directly after isolation, upon *ex vivo* TCR stimulation a robust induction of phospho-ATM is observed. Our key finding (reproduced in Figure 1A) was that this proliferation-induced induction of DNA damage after alkylating chemotherapy is dependent on the strength of antigenic stimulation, with only a modest induction of phospho-ATM for a weak antigen-TCR pair (the altered peptide variant SIIGFEKL and the OT-I TCR, respectively) but strong induction for a high affinity antigen-receptor pair (SIINFEKL and OT-I).

We hypothesized that adaptive immune responses would be most impaired, therefore, for non-self antigens against which precursor populations with high affinity antigen receptors are present. We found this to be the case for both a model antigen (chicken ovalbumin) as well as for previously published neo-antigens identified in syngeneic mouse models of melanoma and glioma. After low dose cyclophosphamide or temozolomide treatment, respectively, neo-antigen reactive cells were undetectable in tumor bearing animals upon peptide vaccination.(Litterman, Zellmer et al. 2013)

In light of these new data from animal models, we performed a retrospective analysis of overall survival of patients enrolled on an early phase clinical trial of the large multivalent immunogen vaccine for metastatic melanoma.(Dudek, Mescher et al. 2008) In this clinical trial, cell surface proteins were extracted from each patient's tumor material and were adsorbed onto cell-sized silica beads. Interestingly, the clinical trial design was such that there were two cohorts of 10 patients each that received an identically prepared vaccine but where one cohort received low dose cyclophosphamide a week before the first vaccination and the other received just the vaccinations. In a new long-term overall survival analysis presented in Figure 1B, an intriguing difference in overall survival can be seen: median survival in the vaccine only group was over 4 years, compared to ~7 months for the cyclophosphamide and vaccine group. Immune monitoring conducted at the time of the trial revealed minimal detectable responses against

several common over-expressed tumor associated antigens in vaccinated patients.(Dudek, Mescher et al. 2008) While it is difficult to test the hypothesis, we speculate that the long term survival of the patients who received the vaccine only may have been due to the generation of immune responses against patient specific neo-antigens, and that this process was abrogated in the group that received cyclophosphamide pre-treatment. We note that multiple investigators have found that immune responses to patient specific mutations are prevalent among tumor infiltrating lymphocytes in metastatic melanoma, suggesting that peptides derived from these mutations may be the most “immunogenic” antigens present.(Lennerz, Fatho et al. 2005, Robbins, Lu et al. 2013) Additionally, we note that a follow up phase II clinical trial of the large multivalent immunogen vaccine that used an HLA-transfected allogeneic melanoma cell line failed to demonstrate the same long term survival of vaccinated patients,(Jha, Miller et al. 2012) providing additional circumstantial evidence that private mutations were the source of the responses seen in the original trial, which were inhibited by cyclophosphamide pre-treatment.

A hierarchy of tumor antigens and differential susceptibilities of associated immune responses to alkylating chemotherapy

Recent reviews summarizing new findings gleaned from genome wide analyses posit a hierarchy of tumor antigens: overexpressed self antigens are widely

shared among different patients but minimally immunogenic, whereas neo-antigens are highly immunogenic but unlikely to be shared by more than one patient.(Hacohen 2013, Kvistborg, van Buuren et al. 2013) In addition, we propose that the differing nature of the T cell repertoire reactive to these different types of tumor antigens leads to a spectrum of susceptibility to impairment by alkylating chemotherapy, as shown in Figure 2. At one end of the spectrum, normal self proteins are present in the thymus during T cell development and abundantly expressed in the periphery in the steady state, leading to minimally reactive T cells with low affinity TCRs that cannot be activated in the presence of Treg. The low intensity proliferative signals that these cells receive from cognate antigen and their requirement for Treg depletion for successful activation can lead to synergy between Treg depleting alkylating chemotherapy and vaccination against these antigens. At the other end of the spectrum, responses to non-self proteins like model antigens or tumor neo-antigens can be generated in Treg replete animals or patients and generate high intensity proliferative signals in responder lymphocytes. Responses to these antigens, therefore, are impaired by DNA damage induced upon vaccination after alkylating chemotherapy.

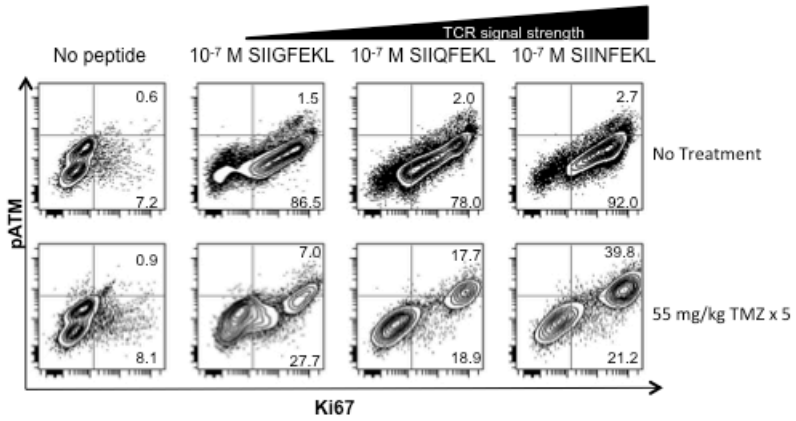
Recent clinical trials using chimeric antigen receptor transduced autologous T cells show the kinds of dramatic responses that are achievable when an overwhelming immune response targets every cell in the body that expresses a tumor antigen.(Porter, Levine et al. 2011) Given their high immunogenicity and

their restricted expression solely in tumor cells, neo-antigens represent extremely attractive targets for active immunotherapy. Recent advances in bioinformatics make the targeting of these antigens possible in principle, and are driving further research towards implementing this strategy as a clinical reality. We stress the potential pitfall for this strategy that conventional or immunomodulatory alkylating chemotherapy may pose, since it is used for several malignancies that would make desirable targets of these strategies including metastatic melanoma, carcinoma of the lung and glioblastoma. For cancers for which it is not feasible or preferable to dispense with this chemotherapy altogether, it is likely possible to modify clinical protocols to yield synergy between chemotherapy and immune therapy by sparing responder lymphocytes from the chemotherapy. This would entail extracting large numbers of PBMCs before chemotherapy and either expanding neo-antigen specific cells in culture or transducing them with an artificial neo-antigen specificity, perhaps using a TCR cloned from immunized HLA-transgenic mice.(Restifo, Dudley et al. 2012) More simply, re-infusing non-drug exposed naïve lymphocytes before immunization with peptide vaccinations or viral vectors could also yield an enhanced effect. Furthermore, for malignancies in which drugs other than alkylating agents can successfully be used for anti-neoplastic effect, targeted therapies or different classes of chemotherapeutic drugs with less impact on T cells are likely preferable. For instance, we examined both a DNA intercalating agent (doxorubicin) and a platinum agent (carboplatin) and did not observe nearly the same degree of anti-

proliferative effect on T cells upon vaccination.(Litterman, Zellmer et al. 2013) Targeted therapies, such as sorafenib or vemurafenib, should also be studied in this context. By developing clinical strategies that allow chemotherapy and immune therapy to synergize, improved results of experimental immune therapy trials may be achieved.

Figure 1. Deleterious effect on vaccination of alkylating chemotherapy A. High TCR signal strength leads to DNA damage in alkylating chemotherapy-exposed lymphocytes. OT-I mice were treated with temozolomide or left untreated, and their splenocytes were stimulated with the indicated SIINFEKL peptide variants. Stronger peptides (SIINFEKL, SIIQFEKL) lead to greater induction of DNA double strand breaks (measured with an antibody against phosphorylated ATM) in proliferating (Ki67+) cells than no stimulation or weaker peptide (SIIGFEKL). B. Shorter duration of overall survival upon vaccination with an autologous cancer vaccine for metastatic melanoma upon pre-treatment with cyclophosphamide. Patients enrolled in a clinical trial of the large multivalent immunogen (detailed in Dudek et al., 2008) were either treated with 300 mg/m^2 or left untreated a week before the first vaccination. Overall survival is depicted, 10 patients per group.

A


TCR signal strength
No peptide
10-7 M SIIGFEKL
10-7 M SIIQFEKL
10-7 M SIINFEKL
0.6
7.2
1.5
86.5
2.0
78.0
2.7
92.0
No Treatment
0.9
8.1
7.0
27.7
17.7
18.9
39.8
21.2
55 mg/kg TMZ x 5
pATM
Ki67

B

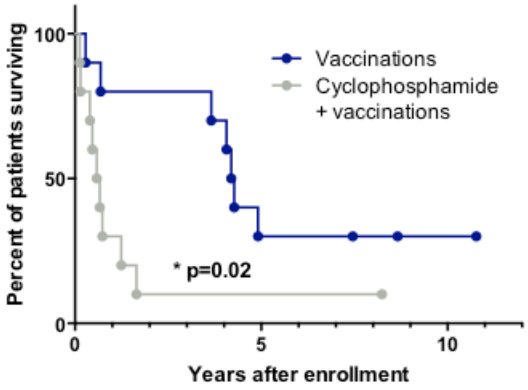
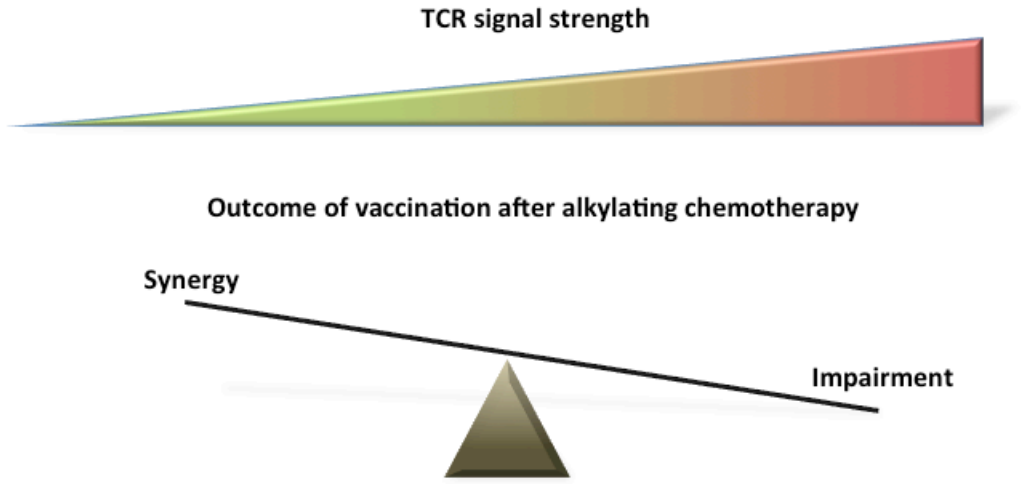

Percent of patients surviving
100
50
0
0
5
10
Years after enrollment
Vaccinations
Cyclophosphamide
+ vaccinations
* p=0.02

Figure 2. The outcome of vaccination after alkylating chemotherapy may depend upon the type of antigen targeted. Overexpressed self-antigens likely require depletion of regulatory T cells in order to generate robust responses, providing a rationale for “immunomodulatory” doses of alkylating chemotherapy. Mutated neo-antigens are recognized by T cells with high affinity antigen receptors and do not require regulatory T cell depletion. For these antigens, the DNA damage inducing nature of alkylating chemotherapy is deleterious.



Overexpressed tumor associated antigens

- Self antigens
- Low affinity receptors for cognate antigen
- Robust responses require Treg depletion
- Responder cells less sensitive to DNA damage upon vaccination

Mutated neo-antigens

- Recognized as "non-self"
- High affinity receptors for cognate antigen
- Immunogenic without Treg depletion
- Responder cells highly sensitive to DNA damage upon vaccination

Chapter 4: Antigen specific culture of memory-like CD8 T cells for adoptive immunotherapy

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Introduction

Adoptive immunotherapy is a promising treatment for hematopoietic malignancies and solid tumors that consists of infusion of *ex vivo* manipulated immune cells with an anti-tumor specificity.(Rosenberg 2011, Restifo, Dudley et al. 2012) This specificity is either an inherent property of cultured tumor infiltrating lymphocytes(Rosenberg, Yang et al. 2011, Restifo, Dudley et al. 2012) or is introduced by antigen specific expansion(Oelke, Maus et al. 2003) or transduction with an antigen receptor gene,(Porter, Levine et al. 2011, Robbins, Morgan et al. 2011) but in either case the T cells are typically stimulated with the potent T cell mitogen and growth factor IL-2. Repeated rounds of stimulation of CD8+ T lymphocytes in the presence of IL-2 lead to acquisition of effector function and enhanced *in vitro* killing of target cells but also to terminal differentiation and loss of proliferative capacity associated with inferior tumor control.(Gattinoni, Klebanoff et al. 2005, Gattinoni, Klebanoff et al. 2012) Numerous signaling pathways and transcriptional controllers have been identified as enhancing self renewal capability and memory formation of CD8 T cells, including signaling by common g-chain cytokines other than IL-2 (particularly IL-7 and IL-21)(Schluns, Kieper et al. 2000, Cui, Liu et al. 2011, Yang, Ji et al. 2012), the Wnt/b-catenin pathway,(Gattinoni, Zhong et al. 2009) and inhibition of cell growth and metabolism pathways.(Araki, Turner et al. 2009, Pearce, Walsh et al. 2009, Sukumar, Liu et al. 2013)

The coupling of proliferation and differentiation of CD8 T cells is particularly problematic for antigen specific cultures, which use multiple stimulations with autologous or artificial antigen presenting cells pulsed with specific peptides to massively expand antigen specific cells from very low precursor frequencies in peripheral blood.(Oelke, Maus et al. 2003, Weber, Caruana et al. 2013) This approach is appealing because it does not require tumor infiltrating lymphocytes or tumor-restricted expression of targets for chimeric antigen receptors, and can be adapted to use patient specific neo-antigens identified prospectively by deep sequencing.(Restifo, Dudley et al. 2012, Hacohen 2013, Heemskerk, Kvistborg et al. 2013, Kvistborg, van Buuren et al. 2013)

Results and Discussion

As a model system to investigate modifications to antigen specific cell culture conditions that would yield less differentiated, memory-like CD8 T cells from patient PBMC, we mixed congenically marked OT-I CD8 T cells with polyclonal C57BL/6 CD8 T cells at a ratio of ~1:100, approximating a frequency achievable from the naïve repertoire via peptide-MHC multimer enrichment.(Legoux and Moon 2012) We screened individual modifications to the culture protocol by mixing SIINFEKL pulsed bone marrow derived dendritic cells (BMDC) with the mixed CD8 T cells in media containing either alternate cytokines besides IL-2, or IL-2 and added drugs and assaying the percentage of memory phenotype

CD44+CD62L+ OT-I T cells after two weeks in culture. We identified numerous modified culture conditions that yielded small sub-populations CD62L+ cells **(Supplementary Fig. 1)**, and when these modifications were combined in a cocktail of memory inducing factors (IL-21 and IL-7 instead of IL-2, plus 2-deoxyglucose and the GSK3b inhibitor TWS119) we noted an additive effect, with sustained antigen specific proliferation of OT-I cells **(Fig. 1a)** where approximately half of the cells remained CD62L+ **(Fig. 1b)**. While the accumulation of Celltrace diluted cells is slower for cells cultured in this cocktail of factors **(Fig 1a.)**, this slower proliferation is balanced by enhanced survival of cells in this culture, as we observed a greater viability of cells cultured in the cocktail versus cells grown in IL-2 **(Supplementary Fig. 2)**. We enumerated the number of antigen specific cells grown by this method and observed roughly equivalent numbers of viable antigen-specific cells at various time points in cultures grown with this cocktail of factors as in cultures of cells grown in IL-2 **(Supplementary Fig. 3)**, indicating that this method could be used with similar amounts of starting material as in conventional expansion protocols.

To further characterize these cells, we performed RNAseq analysis on sorted OT-I cells derived from naïve ($CD44^{lo}$CD62L+) OT-I mouse spleens, cocktail cultured cells and differentiated ($CD44^{hi}$CD62L-) IL-2 cultured cells. Since the extent of differentiation of mouse CD8 T cells has been identified as a determinant of *in vivo* expansion and anti-tumor efficacy we sorted the

heterogeneous cocktail cultured cells into CD44^{hi}CD62L-, CD44^{hi}CD62L+ and CD44^{lo}CD62L+ before extracting RNA **(Fig. 1c)**. Principal component analysis and unsupervised non-hierarchical clustering revealed that all three populations cultured in the memory cocktail exhibited gene transcription profiles that clustered close to one another, were highly distinct from cells grown in IL-2 and clustered closer to naïve cells than IL-2 grown cells **(Fig. 1d)**. In order to compare our *in vitro* derived cells with bona fide memory cells differentiated in response to an infection, we compared the transcriptome of our cultured OT-I cells to OT-I CD8 T cells at various time points of an *in vivo* immune response to *L. monocytogenes* Ova described by the Immunological Genome Consortium (Best, Blair et al. 2013). This analysis revealed that cells grown in IL-2 *in vitro* and early effector OT-I cells *in vivo* (1-6 days after infection) showed similar transcriptional profiles, highly expressing genes involved in effector function (*Gzma, Ifng, Il2ra, Sema7a*). Cocktail grown cells and late memory cells (100 days after infection) also had transcriptional profiles that were similar to one another, showing levels of expression of effector genes that were intermediate between naïve cells and IL-2 grown cells, and higher levels of genes involved in self-renewal and survival like *Bcl2* and *Tcf7* than IL-2 grown cells or early effector cells. **(Fig. 1d, Supplementary Fig. 4)** Upstream regulator analysis identified numerous transcriptional regulators activated in cocktail grown cells versus IL-2 grown cells. These included factors previously described as master regulators of CD8 T cell memory including FOXO1(Hess Michelini, Doedens et al. 2013) and

BCL6,(Ichii, Sakamoto et al. 2002) as well as novel transcription factors that have not been described as having a role in CD8 T cell memory. Tentatively identified novel factors of biological interest are related to pathways of resistance to aging, stress response and metabolism such as SIRT1 and FOXM1(Brunet, Sweeney et al. 2004, Laoukili, Stahl et al. 2007) as well as factors that have proven critical to other aspects of immune function, such as FOXM1 and MYB in the regulation of proliferation in germinal center B cells (Lefebvre, Rajbhandari et al. 2010).

In order to assay the cocktail cultured OT-I cells for efficacy in treating a solid tumor, we transferred equal numbers of IL-2 or cocktail cultured OT-I cells intravenously into mice bearing established Quad-KM3M14 gliomas (that express the ovalbumin epitope SIINFEKL) after treatment with the conditioning chemotherapy temozolomide (Litterman, Zellmer et al. 2013). Upon *in vivo* restimulation with a vaccination consisting of the ovalbumin epitope SIINFEKL peptide and polyinosinic-polycytidylic acid stabilized with poly-L-lysine (poly ICLC) **(Fig. 2a)**, IL-2 cultured cells rapidly expanded and then quickly contracted, whereas the cocktail cultured cells expanded to a greater extent and maintained a significantly higher percentage of CD8 T cells in the blood for several weeks after vaccination **(Fig. 2b).** Even when normalized to the percentage of CD8 T cells present in blood 2 days after transfer to account for different rates of initial engraftment, the *in vivo* fold-expansion and persistence of the cocktail treated cells were greater **(Fig. 2c)**. This greater expansion and persistence was

associated with improved tumor control and enhanced overall survival of glioma bearing mice in mice that received cocktail grown cells versus mice that received IL-2 grown cells or control mice that received no transferred cells **(Fig. 2d,e).**

We next sought to test if this approach would be feasible using antigen specific CD8 T cells enriched from the polyclonal repertoire. We stained C57BL/6 splenocytes and lymph node cells with a K^b-Ova specific dextramer and performed magnetic enrichment with anti-PE microbeads. The bound fraction eluted after performing this enrichment contained naïve antigen specific cells at frequencies comparable to our OT-I experiments, which could be expanded when cultured with SIINFEKL pulsed BMDC in either IL-2 or cocktail containing media. **(Supplementary Fig. 5a).** Similarly to the OT-I system, IL-2 cultured cells upon expansion were almost uniformly negative for expression of CD62L, whereas cocktail cultured cells were heterogeneous with a large fraction remaining positive for CD62L after antigen specific expansion **(Supplementary Fig. 5b).** To test the applicability of this cocktail to human cells, we performed magnetic enrichment of antigen specific CD8 cells from naïve human PBMC by using HLA-A*0201 dextramers specific for both viral (Diamond, York et al. 1997) **(Fig. 3a)** and tumor neo-antigen (Wolfel, Hauer et al. 1995) epitopes **(Supplementary Fig. 6)** and cultured those cells with antigen pulsed autologous monocyte-derived dendritic cells (moDCs). In both culture conditions, antigen specific CD8 T cells preferentially expanded upon culture with antigen pulsed moDCs **(Fig. 3a).**

Antigen specific CD8 T cells cultured in the cocktail of memory inducing factors expressed high levels of cell surface molecules that were differentially expressed between IL-2 and cocktail treated mouse cells including CD95 (Fas receptor) and CCR7 and which serve as markers of a memory phenotype and enhanced *in vivo* persistence upon adoptive transfer in primates(Berger, Jensen et al. 2008) **(Fig. 3b)**. To test the applicability of the cocktail for applications requiring polyclonal expansion (e.g. before chimeric antigen receptor transduction) we used CD3/CD28 beads to expand polyclonal human CD8 T cells. We observed a similar degree of expansion in both groups as assessed by dilution of Celltrace dye, and found that the cocktail grown cells expressed memory marker cell surface proteins **(Fig. 3c).** The phenotype of these cells seems to correspond to a conventional T_{CM} phenotype and does not seem to be enriched for T_{SCM} as has been reported (Gattinoni, Lugli et al. 2011), as the CD8+ cells expanded with polyclonal stimulation were essentially all CD45RO+ within three days of stimulation **(Supplementary Fig. 7)**.

We have demonstrated that naïve CD8 T cells cultured with antigenic stimulation (either in the form of a specific peptide MHC complexes or via anti-CD3) in the presence of a cocktail of memory-formation associated small molecules and cytokines adopt a differential gene expression program relative to cells grown in IL-2. In mice, these cells have a greater proliferative potential and persistence *in vivo* and therefore have greater anti-tumor activity. Coupling this method with a

peptide-MHC multimer pulldown allows the rapid (2-3 week) culturing of antigen specific T cell pools with a less differentiated phenotype than cells grown in IL-2. While these cells retain some naïve like characteristics (for instance, greater *in vivo* expansion upon adoptive transfer than differentiated effector cells) the predominant phenotypic and transcriptional characteristics of these cells demonstrate activation and expansion by antigen, and resemble antigen-specific memory cells expanded after the resolution of a primary infection. The generation of such antigen-specific memory-like CD8 T cells could prove advantageous for cancer immunotherapy, particularly if coupled with prospective bioinformatics based identification of tumor specific mutation derived neo-antigens. This combination of factors may also be useful for expanding minimally differentiated T cells prior to transduction with antigen receptor expressing vectors, or as a jumping off point for the further optimization of T cell expansion protocols to limit differentiation. Finally, by analyzing the genetic program instantiated by these less differentiated cells, we have outlined some of the transcriptional controllers that could be useful targets of future genetic engineering approaches that would seek to enforce maintenance of proliferative capacity in T cells in extended culture prior to adoptive immunotherapy.

Materials and Methods

T cell culture

All immune cells were cultured in a T cell medium consisting of RPMI 1640 with 25 mM HEPES, supplemented with 10% heat-inactivated fetal bovine serum and 1:100 with penicillin streptomycin, non-essential amino acids and sodium pyruvate and 50 mM b-mercaptoethanol. Mouse CD8 T cells were isolated by pressing mouse spleen and lymph node cells through a 40 micron nylon mesh filter in RPMI followed by negative selection with a magnetic isolation kit for CD8a T cells (Miltenyi). For OT-I experiments mouse CD8 T cells were separately isolated from C57BL/6-Tg(TcraTcrb)1100Mjb/J (hereafter OT-I/Thy1.2) mice(Hogquist, Jameson et al. 1994) and B6.PL-Thy1a/CyJ (hereafter Thy1.1) mice and mixed at a ratio of 1:100. HLA-typed PBMC from CMV-seronegative donors were obtained from Precision Bioservices. Human CD8 T cells were isolated by separation from freshly thawed PBMC by negative selection with a magnetic isolation kit for CD8a T cells (Miltenyi). Antigen specific cells were enriched as previously described(Legoux and Moon 2012) from freshly isolated lymph node and spleen cells (mouse) or overnight incubated PBMC (human) after staining with dextramers according to manufacturer's instructions (Immudex). T cells were incubated mixed with peptide pulsed dendritic cells at a ratio of 2:1 or CD3/CD28 beads (Invitrogen) at a ratio of 1:1 and plated at a density of 10,000-20,000 T cells per well of round bottom 96 well plates in a volume of 150-200 mL per well. Fresh media containing the same concentration

of cytokines and drugs was added to each well at half the volume initially plated after 3-4 days. Cells were spun over a histopaque-1077 (Sigma-Aldrich) gradient to remove dead cells, counted and re-plated with fresh dendritic cells or CD3/CD28 beads once a week.

Generation of Dendritic Cells

Bone-marrow derived dendritic cells (BMDC) were cultured as previously described.(Muccioli, Pate et al. 2011) C57BL/6 femora, tibiae, humeri and pelves were rinsed with RPMI through a 40 micron nylson mesh, washed, red blood cell lysed with ACK buffer, washed again and plated in T cell media supplemented with murine GM-CSF for 7-9 days. BMDC were matured 24 hours before use by addition of 2 mg of polyinosinic:polycytidylic acid stabilized with poly-L-lysine(polyICLC, provided by Oncovir) per mL of culture medium. Human monocyte derived dendritic cells (moDC)(Oelke, Maus et al. 2003) were generated by isolating monocytes from freshly thawed PBMC with CD14 positive selection microbeads (Miltenyi) and culturing these monocytes for 8-10 days in T cell medium supplemented with human GM-CSF and human IL-4. moDC were matured 24 hours before use by addition of 2 mg of polyICLC per mL of culture medium. For both mouse BMDC and human moDC, dendritic cells were coated with cognate antigen peptide by adding peptide to matured dendritic cells at a concentration of 20 mg/mL and incubating at 37° C for 2 h. Dendritic cells were

washed 4 times in RPMI to remove excess peptide from media before being mixed with T cells.

Cytokines and small molecules

All cytokines except for human IL-2 were from Peprotech. Mouse cells were plated in T cell medium containing 1 ng/mL recombinant murine IL-2, or 10 ng/mL murine IL-7 and 20 ng/mL murine IL-21. Human cells were plated in T cell medium containing 80 IU/mL recombinant human IL-2 (R&D Systems), or 10 ng/mL human IL-7 and 20 ng/mL human IL-21. Human and mouse cells were incubated with 2-deoxyglucose (Sigma) at a concentration of 400 mM, and TWS119 (Selleck Chemical) at a concentration of 4 mM. For generation of bone-marrow derived dendritic cells, mouse bone marrow cells were plated in 20 ng/mL murine GM-CSF. For generation of monocyte-derived dendritic cells, human monocytes were plated in 100 ng/mL human GM-CSF and 50 ng/mL human IL-4.

Animals, tumor model, adoptive transfers, peptides and flow cytometry

Mouse experiments were performed in accordance with University of Minnesota Animal Care and Use Committee guidelines. C57BL/6J mice, OT-I and Thy1.1 mice were purchased from the Jackson Laboratory and used at 6-10 weeks of age.

Thy1.1 mice were inoculated with 30,000 cells of the SIINFEKL expressing syngeneic C57BL/6 glioma line Quad-KM3M14 in the ventral striatum as previously described. (Litterman, Zellmer et al. 2013) Tumor take and growth were assessed with bioluminescent imaging using a Xenogen IVIS 100 imager. Mice were treated with 75mg/kg temozolomide (Toronto Research Chemicals) suspended in sterile PBS by oral gavage daily for five days starting five days after the tumor was inoculated. One day after the last dose of temozolomide mice were given an adoptive transfer of 750,000 OT-I T cells as an intravenous injection into the retro-orbital venous sinus. Mice were vaccinated with 50 mg of Ova peptide (Anaspec) and 10 mg of polyICLC via a subcutaneous injection in the thigh near the inguinal lymph node 2 and 5 days after the adoptive transfer. Transferred T cell levels were measured by flow cytometry using 50 mL of blood extracted from the retro-orbital venous sinus. All antibodies used were from eBioscience. Ova (SIINFEKL) and CMV pp65 (NLVPMVATV) peptides were from Anaspec. CDK4 R24C (ACDPHSGHFV) peptide was from Genscript. Dextramers were from Immudex. Flow cytometry was performed using BD FacsCanto, LSRII and LSR Fortessa flow cytometers. Cell sorting for RNAseq was performed using a BD FacsAria II. Flow cytometry data was analyzed with Cytobank software.

RNA sequencing and Gene expression Data analysis

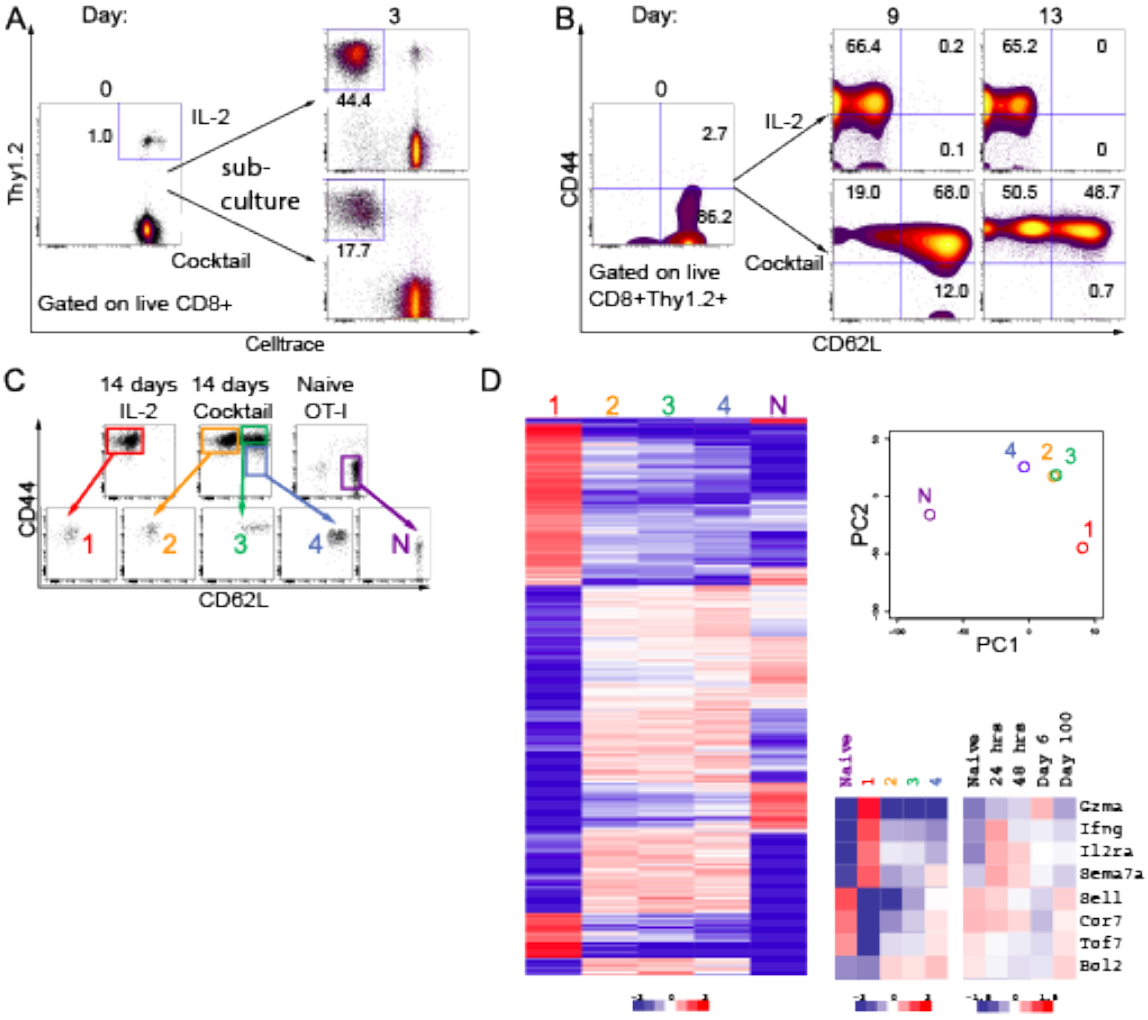
OT-I CD8 T cells were sorted as indicated in the text. Cells were pelleted and total RNA was extracted using the Arcturus PicoPure RNA Isolation Kit (Life

Technologies). Each sample was divided into three technical replicates and sequenced to a depth of ~40 million paired-end 50 base pair reads using the Illumina HiSeq 2000 sequencer (Illumina). Fastq files generated from RNA sequencing were mapped to the mm10 mouse genome using TopHat 2.0.(Trapnell, Pachter et al. 2009) Differential expression was calculated using Cuffdiff 2.1.1.(Trapnell, Williams et al. 2010)

Expression data for 28 mouse samples from the Immunological Genome Project Consortium (http://www.immgen.org/) was downloaded from the Gene Expression Omnibus (GEO) (GEO accession no series GSE15907: specific files GSM605891-6, GSM605898-605911, GSM920634-920641) for comparison to the gene expression data based in this study. These files were normalized according to methods described in Best et al., 2013. Expression data described in this study was compared to gene expression data described in the gene list and data provided in Supplementary Table 1 from Best et al., 2013 (44 mouse samples). In the microarray data described above, multiple probes for a gene were averaged. Data sets from each gene expression technique (RNA sequencing and Microarray) were separately normalized using the average value of each gene. All data sets were uploaded and analyzed using Genedata Analyst v7.6. Hierarchical clustering was performed with Cluster v3.0 (Eisen, Spellman et al. 1998) using the Euclidean distance metric and average linkage. Treeview v1.1.3(Heard, Kaufmann et al. 2009) was used to create heatmaps. To explore

functional networks, selected gene expression lists with both fold-change of >2 and direction of change were submitted to Ingenuity Pathway Transcriptional Regulators Analysis (www.ingenuity.com).

Figure 1. CD8 T cells cultured in a cocktail of cytokines and small molecules resemble memory cells. (a) Flow cytometric analysis of congenically marked OT-I CD8 T cells mixed ~1:100 with bulk CD8 T cells and expanded with SIINFEKL pulsed bone-marrow derived dendritic cells in IL-2 or cocktail of small molecules and cytokines (see methods). (b) Representative plots of expression of the phenotypic markers CD62L and CD44 on Thy1.2+ OT-I CD8 T cells. Plots in (a,b) representative of more than five independent experiments. (c) Plots of pre- and post-sort Thy1.2+ OT-I CD8 T cells grown as in (a) and purified by FACS for RNAseq analysis. (d) Unsupervised hierarchical clustering of RNAseq of populations sorted as in (c) showing all genes with a >2 fold difference between groups 1 and 3. Inset: principal component analysis of genes with >2-fold difference between any two groups for populations depicted in (c), and heatmap of selected biologically relevant genes in populations depicted in (c) and at selected time points of the OT-I response to *L. monocytogenes* Ova (from 21).


A
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Gated on live CD8+
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Celltrace
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Day:
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IL-2
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66.4
0.2
0.1
65.2
0
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19.0
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12.0
50.5
48.7
0.7
Cocktail
Gated on live
CD8+Thy1.2+
CD44
CD62L
C
14 days
IL-2
14 days
Cocktail
Naive
OT-I
1
2
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CD44
CD62L
D
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2
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4
N
PC2
PC1
Naive
1
2
3
4
Naive
24 hrs
48 hrs
Day 6
Day 100
Czma
Ifng
Il2ra
Sema7a
Sell
Cor7
Tof7
Bol2

Figure 2. Memory-like antigen-specific cultured CD8 T cells have greater anti-tumor efficacy than cells cultured in IL-2. (a) Schematic illustration of tumor treatment experiment. Mice were implanted with 30,000 Ova-expressing Quad-KM3M14 syngeneic glioma cells and treated with conditioning temozolomide. 750,000 OT-I cells grown as in (Figure 1a) were adoptively transferred and mice were boosted with two subcutaneous vaccinations with SIINFEKL peptide and polyICLC. (b) Thy1.2+ OT-I CD8 T cells as a percentage of total CD8 T cells in blood. **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$, IL-2 cultured cells versus cocktail cultured cells (Student's two-tailed t test). (c) Percentage of Thy1.2+ OT-I CD8 T cells of total CD8 T cells in blood, expressed as fold increase over the percentage of cells 2 days after transfer. **, $p<0.01$; ***, $p<0.001$, IL-2 cultured cells versus cocktail cultured cells (Student's two-tailed t test). (d) Bioluminescent tumor signal of mice adoptively transferred with no cells, IL-2 cultured cells, or cocktail cultured cells. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, for indicated comparisons. (e) Survival of mice in (d). *$p<0.05$; ***, $p<0.001$, for indicated comparisons (log-rank test). Data depicted are one representative experiment of two separate experiments with similar outcomes.

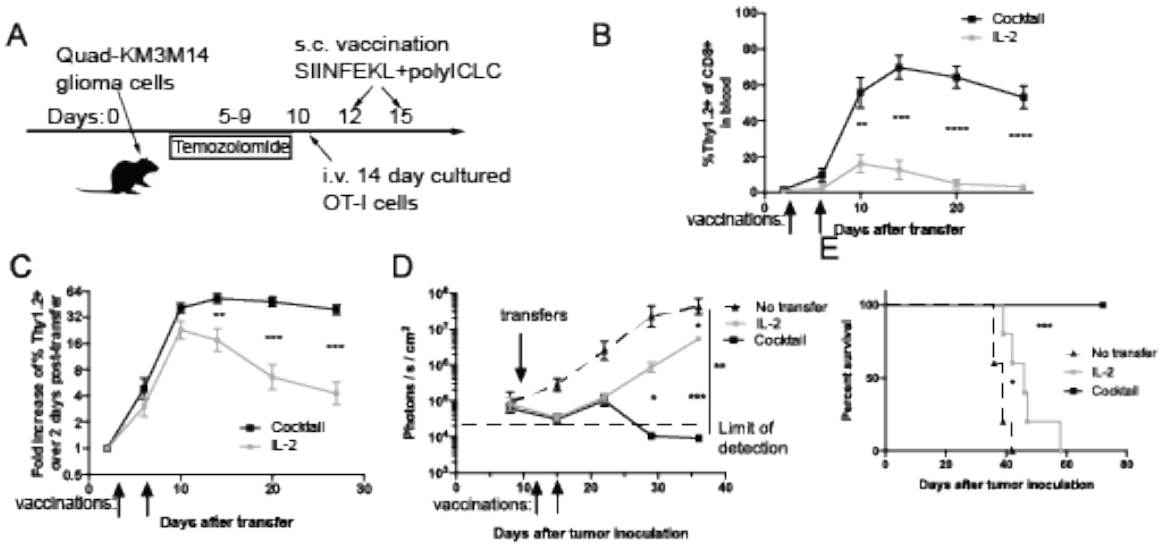
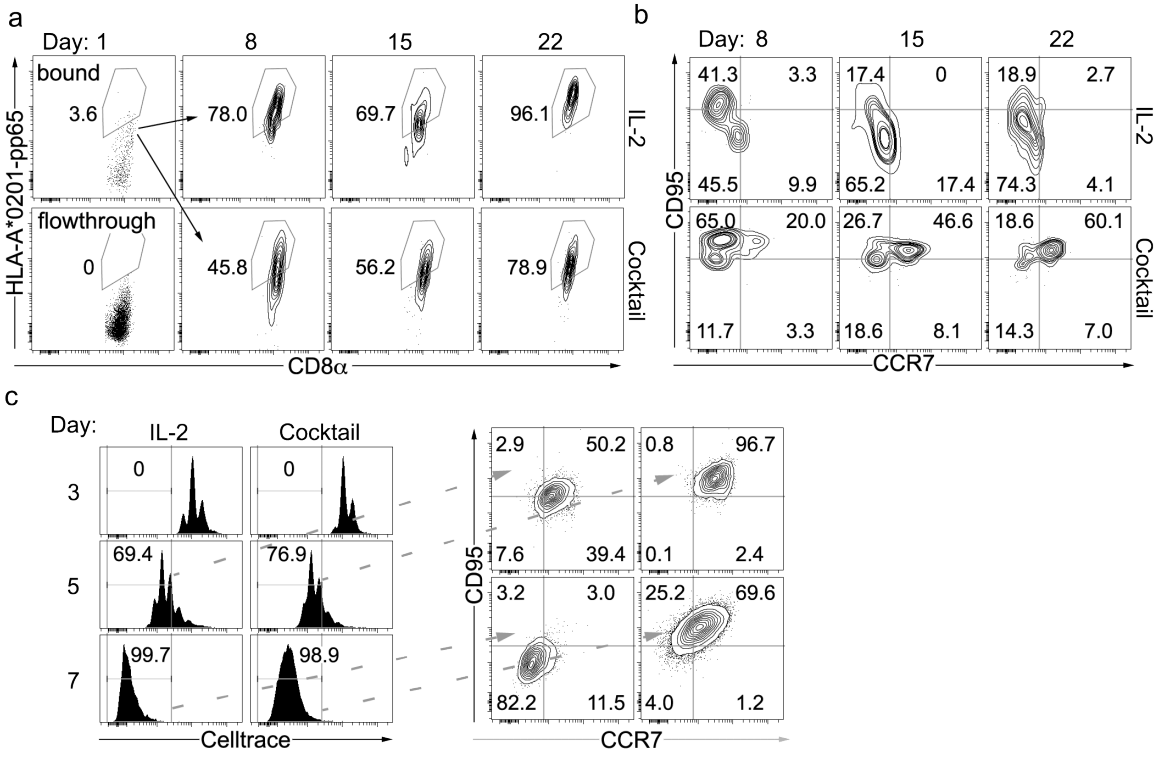

A
Quad-KM3M14 glioma cells
s.c. vaccination SIINFEKL+polyICLC
Days: 0
5-9
10
12
15
Temozolomide
i.v. 14 day cultured OT-I cells
B
Cocktail
IL-2
%Thy1.2+ of CD8+ in blood
vaccinations:
Days after transfer
C
Fold increase of% Thy1.2+ over 2 days post-transfer
Cocktail
IL-2
vaccinations:
Days after transfer
D
transfers
No transfer
IL-2
Cocktail
Photons / s / cm²
Limit of detection
vaccinations:
Days after tumor inoculation
E
Percent survival
No transfer
IL-2
Cocktail
Days after tumor inoculation

Figure 3. Antigen specific and polyclonal culture of memory-like CD8 T cells from human PBMC. (a) Flow cytometric analysis of CD8 T cells cultured from CMV-seronegative HLA-A*0201+ PBMC after pulldown with CMV-specific HLA-A*0201-pp65 dextramer. Cells were cultured in IL-2 or cocktail of memory-associated cytokines and small molecules. Plots depict dextramer-stained cells at pulldown and after 1, 2 or 3 weeks in culture with autologous antigen-pulsed monocyte-derived dendritic cells. (b) Representative plots of expression of the phenotypic markers of memory CCR7 and CD95 (Fas) on HLA-A*0201-pp65 dextramer positive gated cells. Plots in (a,b) representative of experiments performed with cells from two donors and two separate antigens. (c) Flow cytometric analysis of MACS isolated CD8 T cells from human PBMC expanded with CD3/CD28 antibody coated beads in IL-2 or cocktail containing media. Cells were labeled with Celltrace violet and divided cells were gated as shown for analysis of phenotypic markers CCR7 and CD95. Plots are representative of five independent experiments with PBMC from three donors.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IL-2
Cocktail
HLA-A*0201-pp65
CD8α
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22
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45.5
9.9
17.4
0
65.2
17.4
18.9
2.7
74.3
4.1
65.0
20.0
11.7
3.3
26.7
46.6
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8.1
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IL-2
Cocktail
CD95
CCR7
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7
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0
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98.9
Celltrace
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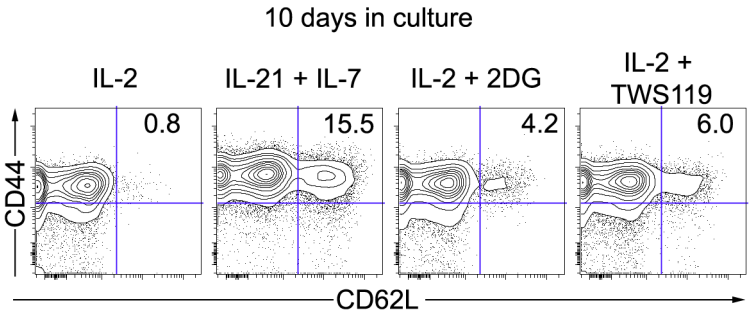
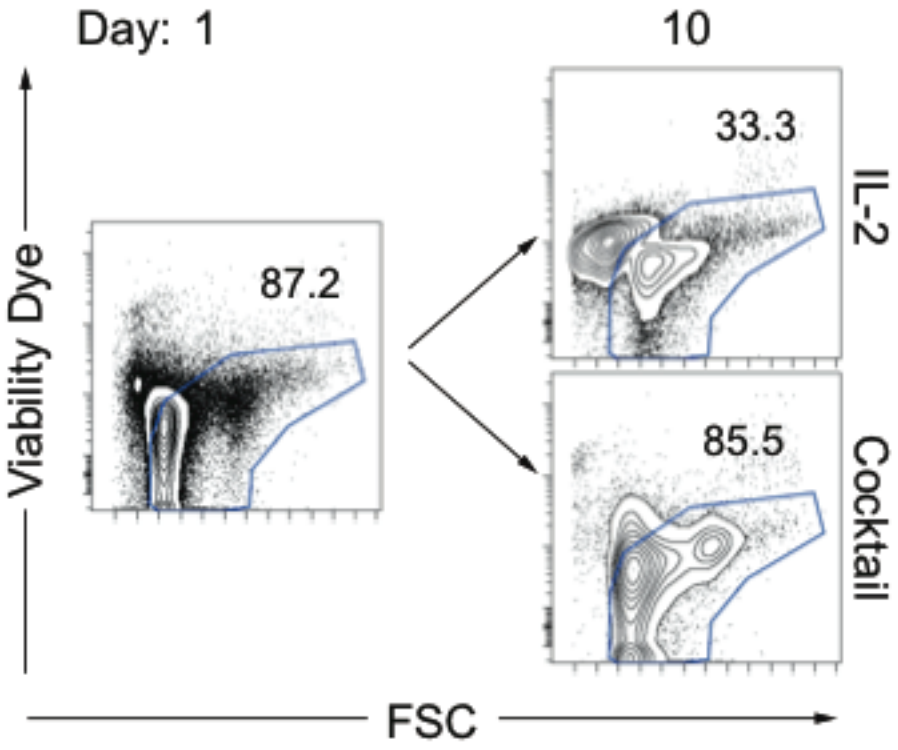
0.8
96.7
0.1
2.4
3.2
3.0
82.2
11.5
25.2
69.6
4.0
1.2
CD95
CCR7

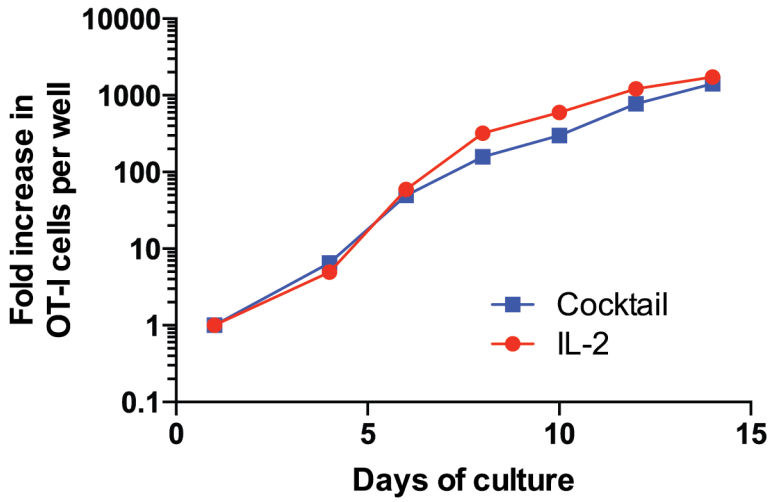
Supplementary Figure 1. Individual modifications to culture medium that yield a fraction of CD62L+ cells in extended culture. Thy1.2+ OT-I CD8 T cells were mixed with bulk Thy1.1+ CD8 T cells ~1:100 and cultured with the individual modification to an IL-2 containing culture medium indicated. Flow cytometric analyses of Thy1.2+ OT-I cells cultured for 10 days are shown.



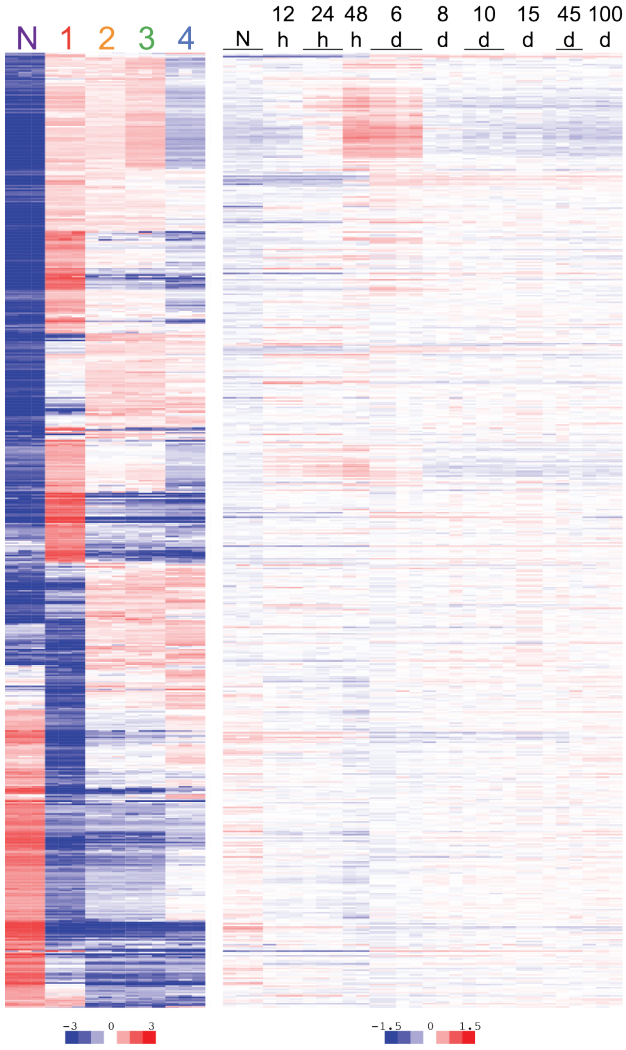
Supplementary Figure 2. Greater viability of cells cultured in cocktail containing medium. OT-I CD8 T cells were mixed with bulk CD8 T cells ~1:100 and cultured in IL-2 containing medium or cocktail containing medium in a 96 well round bottom plate. The percentage viable cells was assessed by flow cytometry after 10 days in culture (after being split on day 7) by staining with live cell impermeable viability dye and gating on viability dye and forward scatter.



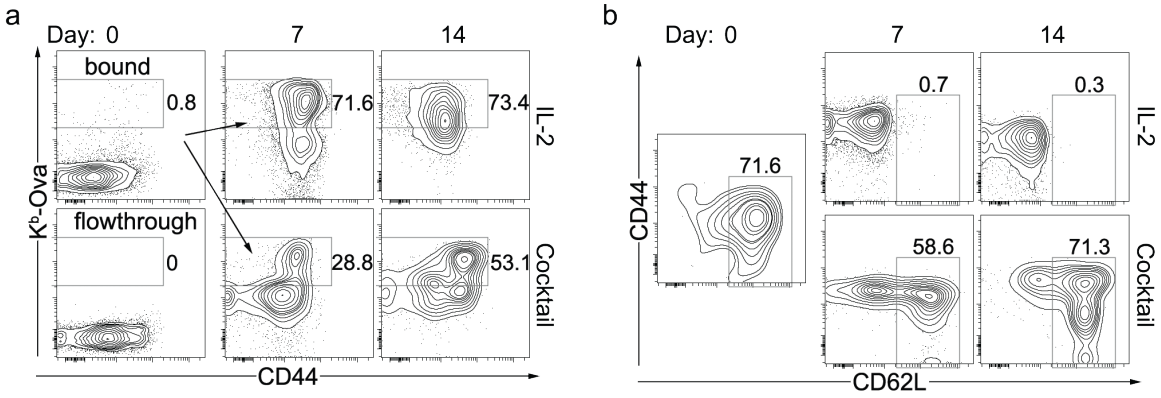
Supplementary Figure 3. Expansion of OT-I CD8 T cells in IL-2 and cocktail containing medium. OT-I CD8 T cells were mixed with bulk CD8 T cells ~1:100 and cultured in IL-2 containing medium or cocktail containing medium in a 96 well round bottom plate. The number of viable cells was enumerated by Trypan Blue exclusion and the percentage of OT-I CD8 T cells was measured by flow cytometry. The fold expansion of the absolute number of OT-I cells in culture is shown over time as the number of OT-I CD8 T cells per well at a given time divided by the number per well on the first day in culture. Each time point is shown as the mean of four biological replicates (wells) per time point.



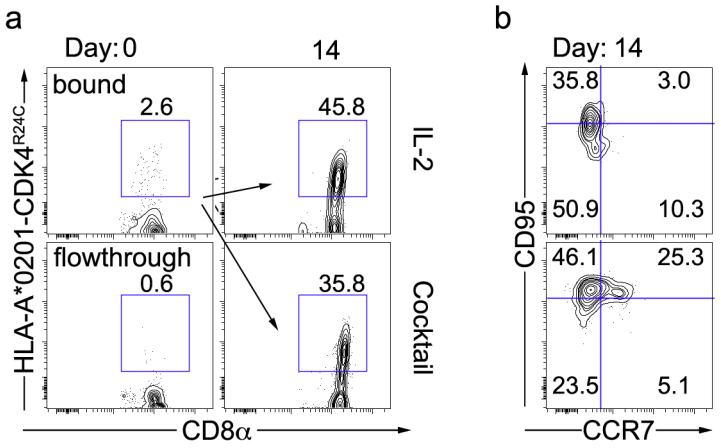
Supplementary Figure 4. Comparison of gene expression profiles between antigen-specific cell culture and a natural immune response. A heatmap of RNAseq gene expression levels of OT-I CD8 T cells cultured and sorted as in Figure 1c,d is shown side by side with timecourse data of microarray gene expression levels of OT-I CD8 T cells extracted from adoptively transferred C57BL/6 recipients infected with *L. monocytogenes* Ova from the Immunological Genome Consortium. Scale bars reflect differing sensitivities of RNAseq and microarray.


N 1 2 3 4
N 12 h 24 h 48 h 6 d 8 d 10 d 15 d 45 d 100 d
−3 0 3
−1.5 0 1.5

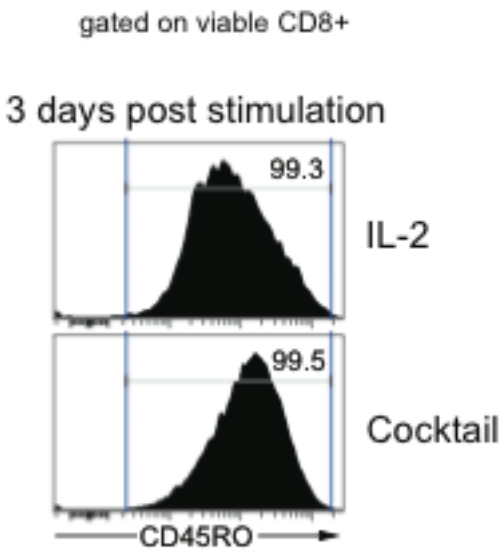
Supplementary Figure 5. Expansion of Ova-specific CD8 T cells from C57BL/6 mouse after dextramer pulldown. (a) Flow cytometric analysis of CD8 T cells cultured from C57BL/6 mouse spleen and lymph node cells after pulldown with K^b-Ova dextramer. Cells were cultured in IL-2 or cocktail of memory-associated cytokines and small molecules. Plots depict dextramer-stained cells at pulldown and after 1 or 2 weeks in culture with antigen-pulsed bone marrow-derived dendritic cells. (b) Representative plots of expression of the phenotypic markers CD62L and CD44 on K^b-Ova dextramer positive gated cells. Plots in (a,b) representative of two independent experiments.



Supplementary Figure 6. Expansion of neo-antigen-specific CD8 T cells from healthy human PBMC after dextramer pulldown. (a) Flow cytometric analysis of CD8 T cells cultured from HLA-A*0201+ PBMC after pulldown with HLA-A*0201- CDK4^{R24C} dextramer. Cells were cultured in IL-2 or cocktail of memory-associated cytokines and small molecules. Plots depict dextramer-stained cells at pulldown and after 2 weeks in culture with autologous antigen-pulsed monocyte-derived dendritic cells. (b) Representative plot of expression of the phenotypic markers of memory CCR7 and CD95 (Fas) on HLA-A*0201-CDK4^{R24C} dextramer positive gated cells.



Supplementary Figure 7. Polyclonal expanded CD8 T cells rapidly become CD45RO+. (a) Flow cytometric analysis of CD8 T cells cultured from PBMC three days after stimulation with CD3/CD28 beads and either IL-2 or cocktail of memory-associated cytokines and small molecules. Plots depict representative CD45RO staining of viable CD8+ T cells.



Chapter 5: Conclusions and Future Directions

Conclusions

The preceding three chapters have outlined a number of obstacles to successful immunotherapy of glioma. The treatment of glioma by standard therapies includes the use of known immunosuppressive drugs like glucocorticoids. Additionally, patients with GBM are treated with the cytotoxic chemotherapy temozolomide. Temozolomide is an alkylating chemotherapeutic drug that exerts its activity by inducing DNA methylation at guanine and adenine residues, which causes DNA double strand breaks and resultant cytotoxicity in rapidly dividing cells. While prior studies have defined the negative *in vitro* effects of temozolomide on proliferation in immune cells (Alvino, Pepponi et al. 1999), reports *in vivo* in patients receiving vaccines for GBM have been contradictory (Sampson, Aldape et al. 2011). Therefore, we set out to define the *in vivo* effect of clinically relevant doses of temozolomide on endogenous immune responses to vaccination following drug exposure. Collectively, our results show that temozolomide and other alkylating chemotherapeutic drugs have long lasting immunosuppressive effects, and argue that vaccination strategies for GBM should be designed to minimize the exposure of responder immune cells to temozolomide.

In addition, we considered the effect of alkylating chemotherapy on different vaccine target antigens. Chemotherapy has been reported to enhance the immune responses to vaccination, particularly in the case of over-expressed self-

antigens (Machiels, Reilly et al. 2001, Ercolini, Ladle et al. 2005). By contrast, our results using model antigens and mutated tumor neo-antigens showed impaired responses following alkylating chemotherapy. The difference in immunogenicity for the different classes of antigens are likely to be due to the nature of antigen receptors in the pre-immune repertoire that are specific to each. Antigen receptors on T lymphocytes which have high affinity for abundantly expressed self proteins are likely to lead to either negative selection in the thymus, development into the regulatory T cell lineage, or peripheral tolerance (Kyewski and Klein 2006). Antigens derived from tumor specific mutations are “non-self” antigens for which lymphocytes with high affinity antigen receptors are more likely to be present in the periphery. The mechanism of enhancement of immune responses by chemotherapy has been proposed to be due to the selective depletion of regulatory T cells, which are more likely than naïve lymphocytes to undergo cell cycle in the steady state (Ercolini, Ladle et al. 2005, Walter, Weinschenk et al. 2012). Normal immune responses to high affinity viral epitope antigens occur in regulatory T cell replete hosts, and the same is likely true for spontaneously occurring immune responses to mutated neo-antigens present in immunogenic tumors like melanoma (Lennerz, Fatho et al. 2005). Therefore, we propose a differential effect of alkylating chemotherapy on mutated neo-antigens, with the long lasting anti-proliferative effect on responder lymphocytes dominating and causing suppression of immune responses. This will be of

particular importance in the case of diseases like GBM where the only effective standard treatments are alkylating chemotherapies.

Finally, we present evidence showing that antigen specific CD8 T cells can be expanded in a cocktail of factors that allow numerical expansion while preserving a less differentiated phenotype. This type of strategy is likely to be useful for adoptive immunotherapy of solid tumors where pre-existing pools of tumor infiltrating lymphocytes are not present for *ex vivo* expansion, and in tumors where there are not good targets for chimeric antigen receptor transduced lymphocytes. Both of these conditions seem to be the case for the majority of cases of glioma, specifically the approximately 75-80% of cases that do not express the cell surface neo-antigen EGFRvIII. To conclude, I examine some of the future directions in glioma immunotherapy and solid tumor immunotherapy, and discuss the implications of the results presented in this work to these future developments.

Future Directions

Immune therapy is rapidly gaining acceptance as a standard tool in the treatment of disseminated solid tumors (Couzin-Frankel 2013). Due to their mechanism of action, which does not depend on a single type of genetic lesion (unlike targeted therapies), and the observation of dramatic individual responses to multiple metastatic tumor lesions (Postow, Callahan et al. 2012), immune checkpoint

inhibitors are being used to treat many patients with many types of solid tumors (Pardoll 2012). New checkpoint inhibitors will continue to receive FDA approval, most immediately inhibitors of the PD-1-PD-L1 pathway (Topalian, Hodi et al. 2012). Given the success of this new class of immune stimulatory drugs, it seems likely that this type of immune checkpoint blockade therapy will be added to trials of other types of immune therapy such as therapeutic vaccination or adoptive transfer of T cells; indeed, the trial that lead to the approval of ipilimumab was conducted with a design that paired the checkpoint inhibiting antibody with a therapeutic vaccination (Hodi, O'Day et al. 2010).

New pathways and technologies for unleashing T cell activity

Given the complexity of T cell signaling pathways, it seems likely that CTLA-4 and PD-1 are simply the first in a relatively large number of “druggable” T cell regulatory pathways. These include, for example, members of the TNF super-family of receptors (Croft, Benedict et al. 2013) some of which such as OX40 are in early stage clinical development (Curti, Kovacsovics-Bankowski et al. 2013). Further targets may be developed by genome scale interrogations. An interesting recent example of this type of screening, of T cell pathways that can be effective in improving T cell immunotherapy, used a genome wide shRNA lentiviral library to identify *Ppp2r2d* as a gene encoding a phosphatase that could be a useful

target in T cells to inhibit to improve cancer immunotherapy (Zhou, Shaffer et al. 2014).

An interesting facet of adoptive immunotherapy strategies is that the *ex vivo* manipulation of the T cell product to be infused allows the potential for complex genetic manipulations of the therapeutic cells. This ability to alter the function of just the anti-tumor T cells allows both the prospect of specifically and not globally inhibiting negative T cell pathways. For example, in the case of CTLA-4 blockade this could improve specificity and decrease toxicity, which can be significant for first generation immune checkpoint blockade therapies like ipilimumab (Weber 2009). T cell negative regulatory pathways that are not “druggable” due to a lack of a cell surface target or the existence of a suitable target of a small molecule inhibitor can in principle be targeted in the setting of adoptive immunotherapy by, for example, short hairpin RNAs (Zhou, Shaffer et al. 2014). Similarly, genetic circuits that modulate T cell differentiation can be manipulated in cells for adoptive transfer by exogenous overexpression or inhibition of specific microRNAs (Ohno, Ohkuri et al. 2013) or other genetic engineering techniques (Kalos and June 2013). Genome editing nucleases have seen proof-of-principle use to edit hematopoietic cells for adoptive transfer (Urnov, Rebar et al. 2010). More versatile modern nuclease technologies like TALENs and CRISPR/Cas9 nucleases are gaining traction (Gaj, Gersbach et al. 2013), and can be adapted for use in adoptive immunotherapy by targeting T cell regulatory pathways. Additionally, the creation of fusion protein effectors for technologies like CRISPR

that can target specific genomic loci have been used to modulate transcriptional activity or other epigenetic states (Konermann, Brigham et al. 2013). It seems likely that these technologies will be used in the near future to enforce advantageous states of T cell differentiation or even lead to super-physiological, *a la carte* implementation of T cell programs, for instance to enforce high levels of self renewal, cytotoxicity and resistance to tolerance or anergy.

New ways for T cells to find their targets

In addition to more advanced control of T cell responses through both new immune modulatory drugs and genetic engineering of adoptively transferred T cells, an area of immunotherapy that will likely change rapidly is the identify of the antigenic determinants of tumors that are targeted. The experience of targeting B-lineage defining markers like CD19 with CAR transduced T cells demonstrates that a sufficiently large immune response targeting a single antigen can have a dramatic effect, but unfortunately the B-cell lineage antigens seem to be rather exceptional in the field of cell surface cancer targets. It has been suggested that chimeric antigen receptors may be used to target a broader range of solid tumors by technical improvements that would allow chimeric antigen receptors to implement Boolean logic in target discrimination: i.e. only kill cells if they express target A and target B (Satta, Mezzanzanica et al. 2013). Some early designs of bi-specific chimeric antigen receptors (Grada, Hegde et al. 2013) or even more exotic methods for implementing this type of Boolean target discrimination

(Douglas, Bachelet et al. 2012) have been published. However, these strategies are in their infancy, and it remains unclear if these technologies and patterns of expression of cell surface antigens will be robust enough to allow widespread successful implementation of these strategies for most or many solid tumors.

The number of T cell "druggable" mutations

Given these limitations, it is possible that the most relevant new strategy being attempted to is to target solid tumors using T cell epitopes derived from tumor specific mutations. A number of recent reviews have outlined how this type of strategy may possibly be implemented in the clinic (Restifo, Dudley et al. 2012, Hacohen 2013, Heemskerk, Kvistborg et al. 2013). Significant questions remain unaddressed, however. Are there enough antigens to target? Early studies have demonstrated that only a minor fraction of mutations yield an epitope that can be targeted by a T cell response. In a mouse model of melanoma, only 16 of 50 predicted immunogenic epitope peptides derived from mutations determined by deep sequencing that were tested *in vivo* were verified as eliciting a T cell immune response, and the genes tested were those predicted to be most immunogenic of ~600 expressed mutations (Castle, Kreiter et al. 2012). In a study of melanoma patients, Rosenberg and colleagues found that about 10% of predicted immunogenic peptides identified by deep sequencing elicited responses from cultures of tumor infiltrating lymphocytes (Robbins, Lu et al. 2013). Thus, a reasonable estimate of the percentage of mutations that yield

immunological targets is on the order of 1%, although it may be somewhat higher or lower.

The number of mutations present in human cancers varies quite dramatically across tumor types, with some gliomas like pilocytic astrocytoma lying at the very lowest end with less than 1 mutation per 10 megabases (Alexandrov, Nik-Zainal et al. 2013). This compares unfavorably, from the perspective of finding immunologically targetable mutations, with melanoma, which on average contains 10 mutations per megabase, and highly mutated cases of which can contain 100 mutations per megabase (*ibid*). Both GBM and low grade glioma lie somewhere in the middle of this spectrum, with an observed range of about 1-10 mutations per megabase (*ibid*). While this would imply ~3,000-30,000 mutations across the genome in GBM, only ~2% of the human genome is coding, and of the 60-600 mutations in coding sequence that would be expected, only some fraction would be expressed. Thus, it seems likely that a typical GBM might only have one or a small handful, and at most perhaps 10 mutations that would generate T cell epitopes that could be targeted. However, these epitopes would more likely be targeted by T cell receptors that exist in the peripheral T cell repertoire due to their “non-self” nature. Again, the experience with B cell malignancies suggests that a sufficiently robust immune response targeting a single antigen can lead to the destruction of all or nearly all cells in the body expressing that antigen. Another data point arguing for the possibility of targeting

GBM with T cells is the previously discussed apparent immunogenicity of disseminated GBM cells outside of the CNS.

The future of strategies that target private, patient specific mutations is unclear. It is likely that their proving ground will be in the treatment of tumors that have heavy mutational burdens, like non-small cell lung cancer and melanoma (Alexandrov, Nik-Zainal et al. 2013). However, this strategy is exciting for a number of reasons: it promises to bring highly effective targeting of tumors to the setting of solid tumors; it promises a high therapeutic index and low toxicity relative to immunotherapies targeting self-antigens, since the targets would by definition only be expressed in tumor cells; and finally, this type of therapy would allow rapid next generation sequencing technologies to make a direct and dramatic impact on how patients are treated and their outcomes, a development that has thus far seen more hype than reality (Kurzrock, Kantarjian et al. 2014).

Towards personalized immunotherapy as a treatment for glioma

The potential use of next-generation sequencing to define personalized antigenic targets is one of many ways in which cancer immunotherapy is a field where cutting edge developments in biology and biotechnology are being rapidly translated into new clinical strategies. Similarly, in adoptive immunotherapy genetic engineering approaches and synthetic biology developments are rapidly changing what is possible (Kalos and June 2013, Maus, Fraietta et al. 2014).

Indeed, adoptively transferred immune cells can be considered a completely new paradigm in oncology, a type of living medicine that is different in kind from the small molecule drugs and biologic drugs that have come before (Fischbach, Bluestone et al. 2013).

Taken together, one can use the trends in the field, as well as the findings described in this thesis, to envision an ideal potential immunotherapeutic approach for GBM patients that could become practical to implement in the next five to ten years. Upon diagnosis, tumor resection could be used as a source of material for characterization of tumor antigens by deep sequencing. Both DNA and mRNA from tumor and matched normal tissue could be extracted and sequenced to determine highly expressed mutations, and mutations that generate likely neo-antigens could be predicted *in silico*. In parallel, these analyses would allow other clinically relevant traits like presence of the EGFRvIII generating mutation and *MGMT* expression levels or promoter methylation status to be assayed. Patient treatment could then be guided by the prognostic significance of these findings, for instance, by prioritizing immunotherapy over temozolomide treatment for patients whose tumors exhibit both the "immunogenic" mesenchymal gene expression profile (Prins, Soto et al. 2011) and the temozolomide resistant *MGMT* expressing phenotype (Hegi, Liu et al. 2008). Given likely advances in sequencing technology, it seems realistic that these types of analyses could be performed in the roughly six week recovery

period typically allowed between surgery and adjuvant treatment. For patients with EGFRvIII present, peripheral blood T cells could be transduced with a CAR targeting this antigen. For other patients, tumor specific neo-antigens could be determined and T cells specific for these epitopes could be expanded from PBMC prior to temozolomide treatment. Temozolomide treatment and whole brain irradiation could then be administered as they are today. In this case, however, the pre-existing immune response could amplify the effectiveness of conventional chemoradiotherapy by sensitizing tumor cells to T cell mediated cytolysis, as has been described (Ramakrishnan, Assudani et al. 2010)

This vision of a radically remodeled treatment of brain tumor treatment prioritizing the generation of effective, personalized immunotherapy is tremendously exciting. However, for these intellectually fascinating developments to be translated to the clinic, careful experiments are required to define the parameters that determine success or failure of these treatments. The experiments I have undertaken and described in this thesis were designed with these considerations in mind. Hopefully, better understanding of how to generate and manipulate immune responses against solid tumors will lead to better outcomes for cancer patients, particularly those with the deadliest types of solid tumors that are currently incurable, like glioblastoma multiforme.

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