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I, Jonathan P McNally , hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Immunology.

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The rational targeting of the DNA damage response pathway for the selective elimination of encephalitogenic T cells

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The rational targeting of the DNA damage response pathway for the selective elimination of encephalitogenic T cells

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Dissertation Abstract

The activation and expansion of self-reactive T cells from the immunologic repertoire can lead to detrimental autoimmunity. If these self-reactive T cells are specific for the auto-antigen myelin, the neurodegenerative disease multiple sclerosis can develop. Though it is well known that self-reactive T cells drive the development and persistence of MS, most of the current therapies that are available for the treatment of MS do not specifically target this important cell type. In general, treatments try to suppress the entirety of the immune system, as a means of suppressing self-reactive T cells, rather than directly removing them to prevent CNS damage. However, treatment has a high rate of associated co-morbidities as a result of off-target immunosuppressive effects of these therapies. We have proposed that a more effective approach to the treatment of autoimmune diseases, such as MS, is to directly remove the self-reactive T cells that induce pathology, rather than attempt to and modulate their activities. For this approach to be truly successful the therapy must be highly selective for pathogenic effector T cells with minimal off-target effects.

Here we present the use of a series of therapeutic strategies for the treatment of EAE, focused on the manipulation of the DNA damage response pathway. We have utilized drugs that target various stages of the DDR pathway, including directly damaging DNA with etoposide, enhancement of p53 signaling with nutlin, and inhibiting cell cycle arrest with MK-1775 and AZD7762, which can allow for a completely non-genotoxic treatment strategy. These strategies all take advantage of the intrinsic vulnerabilities that rapid division induces in activated T cells, thus allowing for their selective deletion. This results in an amelioration of symptoms in EAE due to the loss of the pathogenic T cells that drive disease. Since only rapidly dividing cells are vulnerable to the effects of our therapies, treatment is highly selective for pathogenic effector T

cells. This results in minimal off-target effects, which can positively impact treatment associated side effects. The use of combination DDR-targeting therapy for the treatment of MS has the potential to be a targeted, highly efficacious treatment with minimal side effects that could change the manner in which many autoimmune diseases are treated.

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List of Abbreviations

APC	Antigen presenting cell
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3 related
BBB	Blood brain barrier
CDK	Cyclin dependent kinase
CNS	Central nervous system
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DSB	Double stranded break
EAE	Experimental autoimmune encephalomyelitis
FDA	Food and Drug Administration
GM-CSF	Granulocyte macrophage colony stimulating factor
GWAS	Genome wide association study
HLA	Human leukocyte antigen
HLH	Hemophagocytic Lymphohistiocytosis
IFN	Interferon
IL	Interleukin
IGF-1R	insulin-like growth factor 1 receptor
LCMV	Lymphocytic Choriomeningitis Virus
mAb	Monoclonal antibody
MAG	Myelin associated glycoprotein
MBP	Myelin basic protein
MDR1	Multi-drug resistance type-1 transporter
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging

MRN	MRE11–RAD50–NBS1
MS	Multiple Sclerosis
PLP	Proteolipid protein
PML	Progressive multifocal leukoencephalophathy
Prf	Perforin
RNA	Ribonucleic acid
RPA	Replication protein A
RR-MS	Relapsing remitting Multiple Sclerosis
SP-MS	Secondary progressive Multiple Sclerosis
Stat	Signal transduced and activator of transcription
TCR	T cell receptor
Th	T helper cell
TNF	Tumor necrosis factor
WT	Wildtype

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Chapter 1

Introduction

Preamble

The immense breadth of pathogenic organisms in the environment provides evolutionary pressure on an organism for self-preservation. This evolutionary pressure led to the development of a variety of defensive mechanisms that combat these invasive threats. Initially, these defensive mechanisms were mainly of a chemical nature, as seen in the poly-phenolic compounds used by plants. Over time cellular responses co-evolved with chemical mediators into what we now refer to as the immune system. Vertebrates have further evolved their immune responses with cells that can adaptively respond to a given pathogenic challenge. However, the adaptive response can only be initiated after the detection of a new pathogen, and requires further time for optimal activation, thus, they are greatly outnumbered compared to the pathogen. The extremely rapid proliferation rate of invasive bacteria or viruses, which need only a fraction of the time to divide compared to a eukaryotic cell, furthers the differences in numbers. As a result these pathogen-specific immune cells must proliferate as fast as they safely can to reach a number that is able to keep an invading pathogen in check.

The diversity of the pathogenic assault on vertebrates applies strong evolutionary pressure on the vertebrate immune system to develop, yet economical, scheme by which a multitude of specific receptors, each clonal expressed on individual adaptive immune cells, and each with its own exact specificity, is produced to a near-limitless array of pathogens and their gene products. T lymphocytes (T cells) are one major cell population of this adaptive immune system. These lymphocytes are activated in response to stimulation of their T cell receptor (TCR). T cells express TCR that responds to a specific peptide sequence as presented by the histocompatibility complex (MHC). The antigen specificity of a TCR is randomly genertated, based on the order in which various TCR genes segments recombine together to form a TCR (1, 2). While this

recognition system is extremely economical, adaptive and robust, it comes at a specific cost. Namely, the generation of T cells with TCRs that are potentially reactive to self-antigens. The adaptive immune system has, however, several mechanisms to prevent the development and activation of self-reactive T cells, including deletion in the thymus and induction of peripheral tolerance (3). However, if these self-reactive T cells manage to survive thymic selection and become activated in the periphery, they can have devastating effects on the organism. This condition, termed autoimmunity, results from a lack of control of these self-reactive T cells. While much research has been done on the nature of auto-reactive T cells, to date there have been no therapeutics developed that can specifically target and remove only pathogenic self-reactive T cell populations in an autoimmune disease, while sparing other cellular populations. Here, we will discuss our investigation into the selective deletion of self-reactive T cells in the context of the autoimmune disease Multiple Sclerosis.

Clinical manifestation of Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic multifocal localized inflammatory disease in which immune mediated destruction of myelinated nerves leads to both physical and cognitive impairments of effected patients. Initial diagnosis of MS typically occurs between the ages of 20-40 (4), however, due to the chronic nature of the disease a majority of patients survive for multiple decades with symptoms (5). Thus, MS is very debilitating and has significant impacts on the patients quality of life, including impacts on overall lifespan (6). MS manifests in a variety of phenotypes (4). A majority of patients initially present with a relapsing-remitting (RRMS) disease which is typified by a series of disease flairs followed by periods of remission. The time

between flairs can be just a few days or be multiple years. In a smaller proportion of patients, ~20%, MS develops as a primary progressive (PPMS) disease where disability progresses without periods of disease remission. The third phenotype of MS is a progressive relapsing-remitting disease where patients have periods of remission but the level of pathology never returns to baseline. MS can progress in RRMS patients, ~40% of patients will develop a secondary progressive (SPMS) disease that results in a loss of periods of disease remission. The development of SPMS represents a severe escalation in MS pathology, and is associated with decrease in patient survival (6).

Evaluation of MS symptoms, including the initial diagnosis, is done through two different means, one, a series of tests to define disease-associated morbidities, and another which uses MRI to evaluate pathological changes to the central nervous system (CNS). To evaluate clinical symptoms various tests measure impairment of motor skills such as timed walking, hand/arm movement, and cognitive abilities (7). In addition to these tests direct detection of lesions by MRI is required for diagnosis (8). MRI scans do not necessarily correlate with symptoms but they do give an excellent non-invasive method to examine damage to the CNS. The type of symptom experienced - leg weakness, bladder dysfunction, optical impairment, etc. - has been attributed to the location of lesions in the CNS (4). Specifically, plaques on the cortex are associated with cognitive decline or dysfunction, and worsening prognosis (9, 10). MS plaques are classified by activity and degree of degradation. Active inflammatory plaques are defined by the presence of myelin-laden macrophages. This contrasts with an inactive plaque characterized by an absence of myelin, and only a few leukocytes present (10). T cells, B cells, microglia and macrophages have been found in found in lesions during autopsies of MS patients (11). Lesions primarily form in the myelinated white matter, though there is recent evidence of plaques

forming in the gray matter as well. The immune mediated destruction of myelinated tissue that forms CNS lesions manifests as severe debilitating disease in MS patients.

MS Etiology

The exact cause or cascade of events that leads to the development of MS is unknown. The potential etiology of MS involves a complex interplay between both genetic and environmental factors. These multi-factorial influents that lead to the development of MS are best demonstrated by studies evaluating the development of MS in concordant twins. Compston et al. found that there is only a 30-40% chance for an identical twin to develop MS after a diagnosis of their histo-identical sibling (12). This study demonstrates that while genetics do play a role, other non-genetic factors also make an important contribution to the etiology of MS.

Epitope mimicry is leading theory for how the neurodegenerative autoimmune response in MS develops. This theory states that the auto-reactive T cells that ultimately initiate MS express a dual specificity TCR. The primary high affinity peptide recognized by the TCR is for a foreign pathogenic antigen. The same TCR also has a low affinity for myelin derived peptides. The TCR's affinity for myelin is low enough for the T cells to have escaped deletion in the thymus during negative selection. These dual specificity T cells will become activated in response to their high affinity pathogenic antigen, but they will continue to respond to mimicked myelin peptides. Thus, MS may be induced as an unwanted side effect of a systemic immune response against a pathogen. The induction of myelin-specific T cells has been associated with Epstein Barr Virus (EBV), Human Herpes Virus-6 and Chlamydia infections (13, 14). A different proposal states that encephalitogenic T cells may actually express two separate TCRs, rather than

an expression of a single dual specificity TCR (15). One TCR recognizes a pathogenic antigen and the other recognizes a self-antigen. Such T cells are able to express two TCRs by successful recombining both copies of their TCR into functional receptors

Dietary influences may also correlate with disease susceptibility. Vitamin D is hypothesized to influence disease development. Decreased sun exposure and serum vitamin D levels have been associated with disease susceptibility (16). This is further correlates with the overwhelming percentage of MS patients in non-equatorial locations, predominantly Europe and North America (17). Other dietary factors that may affect the development of MS include a high salt diet that correlates with skewing towards a pathogenic T cell phenotype (18), and a high fat diet that is associated with an increased inflammatory cytokines (19, 20). Additionally increased dietary consumption of cinnamon and caffeine may play a protective role in preventing the development of MS (21, 22). Multiple correlations have been made between the development of MS and environmental influences, though no single stimulus, or series of stimuli, has been definitely shown to cause MS.

MS Genetics

Genetics analyses of MS patients have attempted to elucidate differences that predispose an individual to MS (4). The initial studies found that MS patients, like all other autoimmune diseases, tended to express a specific MHC class II allele (23). Expression of HLA-DR2b had the greatest association with the development of MS. Genome wide association study analysis found that all of the genetic variants associated with MS. These variants are critical genes in immune system development or governance, and are listed below grouped by function (24).

Cytokines: CXCR5, IL2Ra, IL7R, IL7, IL12Rb1, IL22Ra2, IL12a, IL12b, IRF8, TNFRSF1a, TNFRSF14, TNFSF14

Co-stimulatory molecules: CD37, CD40, CD58, CD80, CD86, CLECL1, VCAM1

Signal transduction pathways: CBLB, GPR65, MALT1, RGS1, STAT3, TAGAP, TYK2

Genetic analysis has helped to define many of the underlying mechanisms of MS, and has demonstrated the important role that antigen presentation and T cell activation plays in the development of MS pathology

Modeling MS with Experimental Autoimmune Encephalomyelitis

Observations from patients, in combination with genetic studies, have clearly defined a role for the immune system in MS. Much of the understanding of MS pathogenesis, however, has been elucidated using animal models. Experimental Autoimmune Encephalomyelitis (EAE) is the predominant animal model used to study autoimmune demyelination (25). EAE is initiated by immunization of CNS antigens in combination with adjuvant to induce an autoimmune disease that mirrors the pathology seen in MS patients. EAE is typically induced in mice and rats, however rabbit, guinea pig and non-human primate models have also been described (22, 23, 24).

MS is mainly a disease of the myelinated white matter of the CNS, therefore a majority of the antigenic targets that drive disease are located in myelin. The major cellular target in MS/EAE is the oligodendrocyte, a glial cell responsible for creating the myelin sheath around neurons. Originally EAE was induced by immunization with whole spinal cord homogenates to provide

the oligodendrocyte antigens. Early EAE models were initiated with the administration of whole spinal cord homogenates into rabbits (29). This method produced an immune-mediated disease that manifested with an ascending paralysis in the animal, demonstrated by ataxia, hind limb paralysis and finally forelimb paralysis, depending on the severity of the model. However, as understanding of the molecular composition of oligodendrocytes grew, specific immuno-dominant peptides were identified that were sufficient to induce disease.

Myelin basic protein (MBP) is the major protein component of the myelin sheath, by weight, and was the first purified protein to be utilized to induce EAE in Lewis rats (30). As new models of EAE were developed administration of purified MBP into PL/J mice produced disease became popular. The MBP_{89-99} peptide is the immuno-dominant epitope in the H-2^{u}-expressing PL/J mouse, and is used to induce disease. This is consistent with the detection of MBP_{89-99} peptide reactive T cells in the blood of MS patients, demonstrating that this MBP peptide is an epitope of interest in humans as well (31).

Proteolipid protein (PLP) was the first encephalitogenic component of the myelin sheath identified (29), and preparation of PLP were used to induce EAE in SJL mice (32). $PLP_{139-151}$ is the immuno-dominant peptide in PLP, and is used to induce EAE in SJL mice, (34–36). EAE in the SJL mouse causes a relapsing-remitting phenotype unlike the chronic disease seen in MBP induced disease in PL/J mice. Finding an animal model that demonstrated a relapsing-remitting phenotype presented a further correlation of EAE with classic MS pathology. Furthermore, SJL mice immunized with the $PLP_{139-151}$ peptide could develop T cells reactive to $PLP_{1781-190}$ and these T cells could induce EAE upon transfer (35). These experiments demonstrated that epitope spreading was associated with the development of disease relapse in the SJL model of EAE. Additional T cell spread epitopes were later described following $PLP_{139-151}$ immunization

including epitopes in both MBP and myelin oligodendrocyte glycoprotein (MOG) in the SJL mouse (37).

Myelin oligodendrocyte glycoprotein (MOG) was the last major protein antigen to be discovered in EAE. Mendel et al. first described the ability of MOG derived peptides to induce EAE in C57BL/6J mice. This study reported that pertussiss toxin could enhance disease onset and severity, the authors also reported that the toxin was not necessary for the induction of EAE (38). EAE induced by the MOG peptide in the C57BL/6J mouse is now the gold standard model for EAE (39). EAE in the C57BL/6J mouse induces a chronic progressive disease that plateaus over time. The popularity of the MOG model of EAE in C57BL/6J mice is due to the relative abundance of transgenic animals available on the background. The availability of transgenic mice has enabled a multitude of studies into disease development and the relative contributions of various biology proteins (40). A variation of the MOG peptide model of EAE in the C57BL/6J mouse uses whole purified MOG protein to induce disease. Immunization with whole MOG allows for the activation of MOG specific B cells and causes an exacerbated disease over the peptide model (41, 42).

The last major model of EAE uses the NOD (non-obese diabetic) mouse. The NOD mouse, expressing the I-A^{g7} MHC class II molecule, presents the same immuno-dominant MOG_{35-55} peptide that binds to I-A^{b} in the B6 mouse (43). This similarity allows for some crossover of reagents between the two models. Unlike other models of EAE where peptide immunization solely activates $CD4^+$ T cells, the NOD model activates a $CD8^+$ T cell clone reactive for the immunizing MOG_{35-55} peptide, at the MOG_{42-50} region. This allows for the study of endogenous MOG-specific $CD8^+$ T cells in EAE. Immunization with whole MOG protein allows for the activation of MOG-specific $CD4^+$ T cells, $CD8^+$ T cells and B cells all in the same mouse.

Though it is fairly underutilized the NOD mouse model of EAE has the potential to further define the interactions of all major leukocyte populations. An important consideration that must be factored into experimental design is that one EAE model cannot recreate all aspects of MS. Each model of EAE demonstrates a different component of MS and different types of models should be used to investigate the various aspects of disease pathogenesis (28).

The predominant antigen in MS patients is MOG, with antigenicity clustered around three main MOG amino acid regions (1-22 34-56 and 64-96). This second epitope, $MOG_{35-55,}$ is also the immuno-dominant peptide in C57BL/6J and NOD mouse, and is used to induce EAE in these mice. A study of myelin reactivate T cells in patients broke down T cell reactivity by protein component. Kerlero et. al demonstrated that 12/24 (50%) had T cells reactive to MOG while a much smaller fraction of patients had T cells that responded to PLP or MBP peptide (44). They showed in a larger cohort that 46% of patents mounted a T cell response to MOG peptides, vs 19% responding to MBP peptides, and an 8% response to PLP peptides (45). This study demonstrates that while MBP may have the greatest expression in oligodendrocytes, MOG is the predominant antigen of encephalitogenic T cells in MS patients.

Although MOG, MBP and PLP are the major antigens in MS, they are not the only antigens present. To investigate other antigens involved in MS, APCs were purified from the brains of MS patients. Peptides were eluted from both class I and class II MHC molecules of isolated APCs and analyzed. The sequenced peptides were not only from myelin proteins, but also included non-myelin proteins as well (46). In addition to MBP, PLP, and MOG, peptides were also present from MAG (myelin associated glycoprotein), S100b, GFAP, heat shock protein B-crystallin, transaldolase CNPase (2'-3'-cyclic nucleotide 3'-phosphodies-terase), oligodendrocytes specific (OSP) claudin-11, neurite outgrowth inhibitor (NogoA), and beta-synuclein (47–51).

These findings potentially implicate all of these proteins as possible antigens in MS/EAE. However, it is worth noting that only MBP, PLP, MOG and OSP/claudin-11 have ever been documented to induce any sort of clinically detectable disease in animal models. Thus, while present, the other peptides may represent spread epitopes for immune activation in the CNS.

Immunopathology of MS/EAE

$CD4^+$ T cells

Genetic analysis of MS patients has demonstrated that MHC class II has the highest association with the development of MS. This strongly implicated $CD4^+$ T cells in the development of MS, as $CD4^+$ T cell recognize antigen bound to MHC class II. Over the years research has demonstrated a dominant role for $CD4^+$ T cells in the pathogenesis of MS. $CD4^+$ T cells are both necessary to drive disease progression in both EAE and human disease, as well as sufficient to induce disease on their own in EAE. Depletion of $CD4^+$ T cells in mice, with an anti-CD4 monoclonal antibody, prevents the induction of EAE upon immunization, further demonstrating their importance in pathogenesis (52). Studies by Ben-Nun that described the first encephalitogenic T cell clone from Lewis rats, reactive towards MBP (53), demonstrated that adoptive transfer of these activated $CD4^+$ T cells induced disease in naive rats. These findings indicate that $CD4^+$ T cells alone are sufficient to cause disease with similar pathology to clinical MS.

The original thought was that a Th1 skewed phenotype was pathogenic, while a Th2 phenotype provided protection from disease development. Adorini et al. demonstrated that adoptive

transfer of *in vitro* skewed myelin-specific Th1 cells was able to induce disease, while Th2 skewed cells were unable to do so (37). The development of EAE is prevented in mice that lack T bet, the major transcription factor associated with the development of Th1 cells, (54). These studies in conjunction with the discovery of interferon (IFN)-γ in lesions of MS patients helped to set the paradigm of a pathogenic Th1 phenotype in MS. However, blockade or genetic depletion of IFN-γ did not provide further evidence for a pathogenic role of Th1 cells. Disruption of IFN-γ led to either more or less disease depending on the study and mechanism used to disrupt the cytokine (55–57). In fact, in one report, IFN-γ KO mice actually developed a more severe disease compared to wild type mice (58). This lead the authors to conclude that IFN-γ may be playing a protective regulatory role in EAE pathogenesis, a view that was completely contradictory to the original view that IFN-γ drove the pathogenesis of EAE (59). The genetic controversy surrounding the role for IFN-γ was further deepened by genetic loss of Stat1, the major upstream signaling kinase for IFN- γ, showed no effect on EAE (54). Oddly, the genetic deletion of IL-12 p40 in mice led to a resistance in the development of EAE (60). Since IL-12 signaling is associated with the induction of IFN-γ, this finding of disease resistance produced further confusion. However, with the discovery of IL-23, a cytokine in the IL-12 family that is a heterodimer of IL-12p40 and p19 (61) IL-12(p35) and its receptor IL-12R_{β}2 proved dispensable with regard to EAE development, but it helped resolve this controversy (62, 63). IL-23 proved to be required for disease development.

IL-23 is a critical cytokine in the development of the Th17 subset of T cells. Th17 T cells produce IL-17 as their signature cytokine. Th17 cells have been implicated in the pathogenesis of many autoimmune diseases, including MS (64, 65). Th17 differentiation is promoted by TGF-beta and IL-6, and is amplified by IL-21 (66) and the phenotype is stabilized by IL-23 (67).

In light of the roles IL-23 plays in the development of EAE and the maintenance of Th17 $CD4^+$ T cells, Th17 T cells became the prime focus of EAE investigation (68). IL-6 KO mice develop a mild EAE, similar to the IL-23 KO mouse (67). Unlike blocking IFN-γ, *in vivo* blockade of IL-17 with antibody reduced the severity of EAE (69, 70). Encephalitogenic $CD4^+$ T cells produced IL-17a during active EAE, however, CNS-infiltrating T cells in IL-23 p19KO mice did not produce IL-17, thus linking IL-23 to the induction of a Th17 response. Mice that are genetically deficient in IL-17, both IL-17a and IL-17f, developed a mild disease, but still developed some symptomology (72). In addition, IL-17 is insufficient to drive EAE pathology itself. Overexpression of IL-17 in mouse models does not lead to any increased pathology over WT mice, in EAE (72). The role of IL-17 in driving MS pathogenesis was as also investigated. Tissue analysis from autopsies of MS patient revealed IL-17 mRNA in MS lesions (71). A human anti-IL-17a monoclonal antibody, secukinumab, has been developed for the treatment of MS. Initial phase 2 clinical trials have shown promise in the treatment of RRMS (73). In summary, although IL-17 is involved in MS/EAE pathology, though it is not necessary for disease development.

Recently, onset of EAE has been connected with GM-CSF (granulocyte macrophage colony-stimulating factor) (74). GM-CSF promotes the maturation and activation of monocytes and DCs to exert pro-inflammatory functions, by increasing antigen presentation and release of inflammatory cytokines (75). Genetic loss of GM-CSF prevents the induction of EAE, demonstrating the critical role of GM-CSF in the development of EAE (76, 77). GM-CSF functions by activating myeloid cells and CNS resident microglial cells which are important as CNS resident APCs (76, 78). GM-CSF also mediates the production of IL-23 and IL-6, which induce and perpetuate the Th17 phenotype (79). Inactivation of the transcription factor RORγT,

by siRNA transfection, lead to decreased Th17 development, as measured by decreased IL-17 production, and decreased disease severity. However, there was no effect on GM-CSF production, supporting the position of GM-CSF as a critical upstream modulator of Th17 development (80). Noster et al. studied T cells isolated from the CSF and the peripheral blood of MS patients. They found a six-fold increase in the amount of GM-CSF in CSF of MS patients, thus proposing a similar role in human disease development that has been demonstrated in mice. Additionally they also showed GM-CSF-producing $CD4^+$ T cells in the CNS, though more often than not these GM-CSF-producing cells co-produced IFN-γ (Th1) rather than IL-17 (Th17) as they tend to in mice (81). Therefore this implies that GM-CSF is a relevant cytokine that drives the pathogenesis of EAE and MS, by recruiting and activating macrophages and DC in the CNS to drive the generation of pathogenic Th17 T cells (82, 83).

Schluesener and Wekerle found naive encephalitogenic T cell clones in unprimed Lewis rats, demonstrating that myelin-reactive T cells exist in the immune system at a normal static state (84). They demonstrated that the distribution and frequency of these cells differs between animals of different MHC backgrounds, and an increased frequency of myelin-specific T cells conferred susceptibility to EAE. Naïve MBP specific TCR transgenic T cells were adoptive transferred studies to study the effects of activation on naive auto-reactive T cells while migrating from periphery to CNS (85). T cells do not immediately infiltrate the CNS when activated. First, the MBP-specific T cells migrate to the lungs (86) then to the peri-thymic lymph node ~2 days after transfer, and then to the spleen on day 3, before finally making it to the CNS around day 4-5. CXCR3 and CCR6 expression on T cells has been reported to allow $CD4^+$ T cells to migrate into the inflamed CNS (87). After transmigration through the blood-brain barrier (BBB) the T cells require activation by CNS resident APCs. Microglial cells typically are the

major APC cell type in the CNS, though recruited DCs and B cells may also reactivate T cells. Once in the CNS, T cells migrated along the aluminal surface of the leptomeningeal vessels to establish contact with APCs, described as MHC class II^+, presumably microglial cells (88). GM-CSF is believed to be required at this stage to optimally activate the APCs. Upon reactivation these T cells penetrate into the CNS tissue and trigger disease (89). Intravital imaging of green fluorescent protein (GFP) labeled T cells shows that they migrate into the leptomeninges after reactivation by microglial cells (90). Administration of anti-α4 integrin antibody detached cells from the vascular surface and prevented further infiltration and disease development. The requirement for the reactivation of CNS-infiltrating T cells is furthered by the lack of disease development in both MHC class II KO and CD11c KO mice upon adoptive transfer of activated encephalitogenic T cells (91).

While GM-CSF is the only cytokine that has been demonstrated to be necessary for the development of EAE in mice, many others have been investigated to understand what role they may play in disease development. Naive myelin-specific transgenic $CD4^+$ T cells were activated *in vitro* under Th1, Th2, Th17 and Th9 skewing cytokine conditions, then adoptively transferred to assess the pathology induced by Th skewing of the T cells. Transfer of Th1 and Th17 cells produced disease, although with different pathogenic signatures. As originally described, transfer of Th2 skewed cells did not cause any disease. However, transfer of the recently defined Th9 cells did generate some pathology, demonstrating a potential role for IL-9 in EAE pathogenesis (92). A pathogenic role for tumor necrosis factor (TNF) is fairly well established in many autoimmune diseases, and it is a direct therapeutic target in some diseases including rheumatoid arthritis. In EAE both the genetic knockout and the antibody blockade of the TNFR leads to drastically decreased disease development (93, 94). This change appears to be due to

decreased microglial activation, and decreased recruitment of inflammatory leukocytes into the CSN, which may be a downstream effect of microglial activation. Suen et al demonstrated a role for both lymphtoxin α and β in the development of EAE (95). Thus a wide variety of inflammatory mediators all play a role in the pathogenesis of EAE.

The development of MS is a complicated interplay of immune mechanisms, predominately driven by $CD4^+$ T cells. $CD4^+$ T cells produce a series of immunologic mediators including GM-CSF, IL-17, IFN-γ and TNF that cause the recruitment and ultimately the demyelination of the CNS. Since $CD4^+$ T cells drive pathogenesis, and complete loss of $CD4^+$ T cells can ameliorate disease, they are the ideal target for the treatment MS. Many compounds have been used to target encephalitogenic $CD4^+$ T cells in MS/EAE with various success and specificity for their intended target, as discussed below.

$CD8^+$ T cells

It has been clearly demonstrated that $CD4^+$ T cells are more pathogenic than $CD8^+$ T cells in both MS and EAE (43). Unlike $CD4^+$ T cells, the transfer of MOG-specific transgenic $CD8^+$ T cells only induces a mild disease, demonstrating the diminished pathogenic potential of $CD8^+$ T cells in EAE development (96). However, CD8+ T cells still do play a role in the pathogenesis of MS/EAE. Large numbers of $CD8^+$ T cells have been found in the lesions of MS patients and they well outnumber the number of $CD4^+$ T cells in the lesions by 2-10 fold, depending on the report (96–98). The $CD8^+$ T cells in these lesions produce IL-17, demonstrating that they are potentially pathogenic (71). This IL-17 production has been demonstrated to support the development of Th17 $CD4^+$ T cells furthering pathogenesis. MBP-reactive $CD8^+$ T cells have

been found in the peripheral blood of MS patients (99). Upon *in vitro* co-culture with oligodendrocyte target cells, these MBP-specific $CD8^+$ T cells produce both IFN-γ and TNF, and can kill oligodendrocytes targets. Malipiero et al. investigated cytolytic properties of $CD8^+$ T cells in EAE with perforin (Prf) and Fas knockout (KO) mice. Prf KO mice developed a more severe disease then WT mice, demonstrating that it is dispensable in the induction of pathogenesis, or mediate some control of disease via $CD8^+$ T cell killing of APCs. However, both Fas KO mice and FasL KO mice developed an attenuated disease (100). $CD8^+$ T cells can play a role in the development of MS/EAE pathogenesis, though that does not seem to be their only role.

Mice that genetically lack $CD8^+$ T cells actually develop more severe disease than WT mice. This suggests the possibility of a protective role for $CD8^+$ T cells in MS/EAE (101–103). Further investigation into this potentially regulatory $CD8^+$ T cell subset in EAE has been conducted. $CD8^+$ T cells can secrete both TFG-β and IFN-γ to exert regulatory functions (104) . Furthermore, perforin may mediate a regulatory function in $CD8^+$ T cells by killing encephalitogenic $CD4^+$ T cells. These findings set up a possible dichotomy for effector $CD8^+$ T cells that can be cytotoxic in the CNS and exacerbate disease or they can be regulatory cells that may reduce disease.

B cells

B cells are neither necessary nor sufficient to induce disease in animal models of MS. μMT mice that genetically lack B cells develop normal EAE compared to WT mice. Additionally, anti-MOG B cell transgenic mice (IgH^{MOG}) do not spontaneously develop disease like the T cell

transgenic mice (105). For these reasons, very little attention was initially paid to B cells in the pathogenesis of MS. However, a role for B cells in disease development has become more apparent, particularly in patients with the RRMS form of the disease. Treatment with of RRMS patients with ritixumab (anti-CD20mAb), a B cell-depleting monoclonal antibody, reduced the rate of disease relapse, demonstrated a role for B cells in MS patients (106). Analysis of patient CSF shows that oligoclonal bands of antibodies were found in the CSF of MS patients, and the presence of these antibodies has become a useful biomarker for physicians (107). Analysis of both serum and CSF from MS patients revealed the presence of myelin-reactive auto-antibodies. Myelin-reactive auto-antibodies are present in the CSF, and deposits of myelin-reactive antibodies have been observed in the lesions of MS patients (108, 109). In EAE administration of anti-MOG antibodies increased disease severity (110). To understand the downstream actions of anti-MOG antibodies, disease development was compared in both FcRγ KO mice and C1q KO mice to WT mice. Loss of the FcRγ only had modest effects on disease reduction. In contrast, loss of C1q had a much more pronounced effect on disease development, demonstrating a role for complement activation as a mechanism for how antibodies can lead to demyelination (111). Anti-MOG, anti-MBP, anti-PLP and anti-MAG antibodies have all been found in the serum of MS patients (41, 112). However, only the anti-MOG antibodies have been shown to induce demyelination both in *in vitro* and *in vivo* animal models, probably due to complement activation, while anti-MBP and anti-PLP do not (44, 113). The ability of myelin-reactive antibodies to activate complement demonstrates a pathogenic role for B cells in MS.

Auto-antibody production by B cells does play a minor role in the pathogenesis of MS/EAE. However, the major role of B cells in disease development appears to be as antigen presenting cells. B cells have been found in the lesions and in the CSF of MS patients (114). Additionally,

tertiary lymphoid structures and B cell follicles have been described in the meninges of mice. While μMT mice that genetically lack B cells can still develop disease via peptide immunization, when they are immunized with whole MOG disease cannot be induced, demonstrating a role for B cells as APCs (105). B cell specific Class II KO mice do not develop disease either, which provides further evidence of B cells as APCs in EAE (115). Additionally, splenic B cells from mice with EAE showed increased IL-6 production, which increases their ability to induce pathogenesis as an APC by inducing Th17 $CD4^+$ T cells though the production of IL-6 (116). Though B cells are not the predominant drivers of pathogenesis in EAE, they still play an important role in disease development.

Current therapies and approaches to clinical management

While the etiology of MS remains elusive, studies have provided enough clues to define a target population of leukocytes that are responsible for disease pathology. Studies in EAE have clearly demonstrated that $CD4^+$ T cells are the major cell population responsible for MS pathogenesis. Therefore, a successful treatment strategy for MS should directly target this population. Most of the therapeutics that are currently used for the treatment of MS are aimed at pathogenic T cells, in theory, though the degree of specificity for this target varies widely between drugs. As a result of varying specificities, significant side effects on both hematopoietic and non-hematopoietic cells exist. (4). Most of the currently available therapeutics are designed for immune modulation in the early phases of disease, and are only efficacious in the treatment of RRMS. Therefore, there is still a need for therapies that are effective in patients with highly active MS (117). The ideal treatment would need to specifically target myelin-specific T cells

to prevent their pathogenic actions in the CNS (118). Unfortunately, a therapeutic strategy with this degree of specificity does not yet exist in the clinic. Below is a discussion of current therapeutics used to treat MS, including the side effects that limit the efficacy or use of each drug.

Immunomodulation with IFN-β

There are many therapies currently approved by the FDA for the treatment of MS, however, IFN-β has remained the standard of care, and first line treatment for the last 15 years. This is seen in most of the current clinical trials for MS where IFN-β treatment has replaced a placebo control as the standard to evaluate the efficacy of emerging therapies. Currently there are three formulations of IFN-β that are used, IFN-βa1 IFN-βb1 and IFN-βb2, though they are used interchangeably in the literature. IFN-β treated RRMS patients have a decreased lesion load compared to a placebo control, as well as a significant decrease in the rate of disease relapse. (24). However, IFN-β treatment of SPMS patients has little effects on disease progression (119). These results in patients have led to the belief that IFN-β modulates the immune system towards a more tolerant phenotype.

The precise mechanism of action for IFN-β treatment of MS is still unknown, though its effects can been deduced from observations in EAE, and in patients (120). As a whole IFN-β reduces antigen presentation, inhibits T cell proliferation, alters cytokine profiles and inhibits T cell migration across the BBB (121). Furthermore, the actions of IFN-β are predominantly mediated by myeloid cells that express the INFαR (122, 123). Mice deficient in IFNαR on APCs develop exacerbated symptoms in EAE, demonstrating a regulatory role for IFN-β (121). In patients IFN-

β decreases antigen presentation by microgial cells and B cells, by interfering with the actions of IFN-γ (124). In addition to decreasing MHC expression on APC, IFN-β decreases the expression of the co-stimulatory molecules CD80 and CD40 on APCs (125, 126).

IFN-β acts on APCs to modulate their interactions with T cells. Ultimately, the effects of IFN-β on autoreactive T cells is to constrain the development of Th17 $CD4^+$ T cells. This driven by a combination of decreased IL-12p40 secretion, believed to be IL-23, and increased IL-10 secretion (61). In patients treated with IFN-β the success of therapy directly correlates with increased serum IL-10 levels, and decreased IL-12p40 (127). Other documented effects of IFN-β on T cells include increased expression of CTLA-4 and Fas on $CD4^+$ T cells in patients treated with IFN-β, that may lead to increased apoptosis of auto-reactive T cells (128). However, the decreases in T cell proliferation and cytokine production can take upwards of two weeks for maximum effect to been seen after beginning treatment in patients (129).

Another theory for the efficacy of IFN-β relates to its antiviral effects. Biologically, the major producers of type 1 IFN (including both α and β) are plasmacytoid DCs. They produce type I IFN in response to TLR ligation from viral pathogens to prime the immune system. Since the viral infections have been associated with the induction of MS IFN-β may mediate is effects via its antiviral mechanisms (130, 14). However, no studies have been published to fully investigate this outcome or fully define the actions of IFN-β.

Therapeutic antibodies in the treatment of MS

Monoclonal antibodies (mAb) are a highly selective group of therapeutic agents that can target specific molecules expressed on the cell surface (131). Interestingly, these therapies in MS have

potent immunomodulatory effects both directly on their target population, but also indirect effects on other off-target immune populations. mAb are relatively large molecules that are unable to cross and intact healthy BBB, therefore they would require active transport across the BBB for function in the CNS. Studies have shown that only ~0.1% of systemically administered mAb can reach the CNS compartment under normal BBB function (132, 133). While there is documented disruption of the BBB during MS, including the presence of oligoclonal bands in the CSF, it is still unclear how much this disruption opens the CNS to a full scale diffusion of these therapies. However, it is unclear whether CNS infiltration of the mAbs is needed for their efficacy, or whether systemic actions are sufficient to reduce symptoms. Of the monoclonal antibody based therapies that are discussed below, only natalizumab is currently FDA approved for the treatment of MS. The other three drugs discussed all target various components of the immune system to treat the disease with various efficacies and side effects.

Natalizumab

Natalizumab is a humanized IgG4 that recognizes the α4 subunit of the integrin VLA-4. Currently it is the only antibody-based biologic drug that is FDA approved for the treatment of MS. Expression of the integrin α4β7 on T cells allows for trafficking and entry into the CNS in neuorinflammatory settings (134). Interaction with α4β7 expressed on T cells with VCAM on the epithelium of the BBB allows for firm adhesion of lymphocytes to blood vessels and promotes their extravasations out of the blood stream. Natalizumab blocks this interaction, and thus it functions by blocking the binding and ingress of leukocytes into the CNS. This mechanism gives it the potential to selectively target and inhibit inflammatory lymphocytes in

MS, because it prevents lymphocyte entry into the CNS. As an IgG4 subgroup natalizumab has low affinity for compliment activation as well as antibody-mediated cellular toxicity. Therefore, it can effectively block leukocytes expressing α4 without leading to their depletion and subsequent immune suppression (131). Intravital imaging of GFP labeled encephalitogenic T cells transferred into naive mice shows their migration into the leptomeninges upon reactivation. Administration of anti-α4 detached cells from this vascular surface and prevented further infiltration and disease development (90). By blocking entry into the CNS natalizumab can prevent neuroinflammation.

A series of clinical trials have investigated the efficacy of natalizumab in the treatment of RR-MS (8, 135, 136). While Polman et al. studied the efficacy of natalizumab in a placebo controlled study, the others, have all been compared to IFN-β as the standard of care. These studies have all demonstrated that natalizumab can effectively inhibit brain inflammation as measured by lesion formation on MRI. Treatment of RRMS patients prevented leukocyte infiltration into CNS for at least 6 months after termination of treatment, as determined by leukocytes in patient's CSF (137). This results in an estimated 42% reduction in risk of relapse over placebo and a 24% reduction is the risk of relapse over IFN-β treatment over a two year period. However, a *post-hoc* analysis of patients assessing treatment efficacy over the duration of disease revealed that natalizumab treatment is more effective in early stage patients, and that it has little effect in patients with developed chronic disease. They also found that natalizumab has very little effect on patients with secondary progressive disease. Combined, these studies show that natalizumab is most efficacious as an early intervention in the treatment of MS before long term neural damage has taken place.

The major issue with natalizumab is the inhibition of immune surveillance of the CNS. While the CNS was originally believed to be an immune privileged site, treatment with natalizamab has put this long held idea into question. Treatment with natalizumab can cause a potentially lethal condition known as Progressive Multifocal Leukoencephalopathy (PML), which is caused by the reactivation of the JC virus (138). After a subclinical acute infection with the JC virus, it can enter into a chronic latent phase in the bone marrow, spleen or brain (139). Under immune-compromised conditions, including HIV infection, organ transplantation and natalizumab therapy, the virus can reactivate in the brain causing encephalopathy. The estimated risk of an MS patient developing PML has been estimated at 1 in 1000 patients for a prolonged treatment period of over 18 months in duration (140). However, the risk for developing PML can be minimized by looking for anti-JC virus antibodies in patient serum and taking a different therapeutic approach if the patient is chronically infected with JC virus (141, 142).

Natalizumab treatment inhibits leukocyte entry into the CNS by blocking α4β7, however, it also affects leukocytes entry into other compartments. In addition to the CNS, α4β7 is used for entry into the gastrointestinal tract, as well as the genital mucosa. In fact, a similar antibody against the α4 subunit is used in the treatment of Inflammatory Bowel Disease to prevent ingress of leukocytes into the GI tract. An α4 antibody similar to natalizumab has even been tested for the prevention of simian immunodeficiency virus (SIV) infection in *rhesus macaques*. Furthermore the α4 subunit that natalizumab targets can pair with the β1 subunit to from α4β1, an integrin used by T cells to exit the blood stream and enter peripheral lymph nodes. While natalizumab appears to specifically target encephalitogenic leukocytes, it can affect a much wider breadth of cells than originally anticipated.

Daclizumab (Anti-CD25)

Daclizumab is a humanized IgG1 mAb that blocks CD25 from binding its ligand IL-2 (143). The expression of CD25, the α chain of the high affinity IL-2 receptor, has been shown to be a marker of activation on T cells. IL-2 drives the rapid proliferation of T cells upon activation, typically in an autocrine or paracrine manner. As such the rational for the use of daclizumab is to block the proliferation of effector T cells upon activation (144). Despite being an IgG1 antibody, daclizumab does not seem to lead to a complete deletion of $CD25^+$ T cells, only depleting ~60% of $CD25^+$ cells (131). Early clinical trials demonstrated that treatment with daclizumab results in a 72% reduction in CNS lesions by MRI in treated patients with RRMS. Naive and resting T cells typically express low levels of CD25, potentially increasing daclizumab's selectivity for effector T cells. However, regulatory T cells express high levels of CD25, since they require IL-2 for survival (145). Additionally the expression of CD25 has been shown to be part of their mechanism of regulation by acting as an IL-2 sink to regulate the proliferation of effector T cells. Tregs play a major role in tempering pathogenesis of MS, and the loss of this important population can be detrimental to not only MS progression but regulation of the immune system as a whole. Skin rashes, lymphadenopathy and CNS vasculitis are known side effects of daclizumab treatment (146), all of which have been associated with the loss of immune regulation by Tregs.

Rituximab (Anti-CD20)

Rituximab is a chimeric mouse/human $IgG1_\kappa$ that targets CD20 on mature memory B cells. While B cells are not sufficient or even necessary to cause disease, they can play a role in

pathogenesis. This is demonstrated by the depletion of auto-reactive B cells which leads to an improvement in disease (147). Treatment with rituximab leads to a rapid depletion of memory B cells *in vivo,* predominantly via a compliment-dependent mechanism, for up to six months after the termination of therapy. Plasma cells, are not depleted by rituximab therapy due to their lack of CD20 expression (148–150). This is touted as a benefit of the approach since it does not entirely destroy humoral immunity against previously encountered pathogens and vaccinations (149). However, plasma cells are relatively short lived for a memory immune population and need to be replenished by memory B cells. Since CD20-expressing memory B cells are depleted by rituximab, long term treatment will destroy humoral immunity, which is reflected by a decline in serum IgG levels over time (151).

In RRMS patients rituximab decreases lesion burden by 29% (106, 152). In addition 91% of patients had a decreased mean number of lesions compared to placebo control after a treatment. Interestingly rituximab therapy does not lead to a disappearance of oligoclonal bands in patient CSF (42). However, it does appear to affect T cell influx into the CNS. Rituximab-treated patients did have decreased numbers of T cells in their CSF. Since B cells can present antigen in the CNS their absence, due to treatment, may limit the available APCs that are needed to reactivate T cells in the CNS and cause damage.

Alemtuzumab (Anti-CD52)

Alemtuzumab is a humanized $IgG1_{\kappa}$ that targets CD52 on leukocytes and was originally used for the treatment of lymphocyte malignancies. Unlike the other antibody-based therapies that try to

specifically target encephalitogenic cells based on their expression of individual markers for CNS trafficking or activation, alemtuzumab targets nearly the entire immune system. CD52 is widely expressed on both lymphocytes as well as myeloid lineages; including T cells, B cells, NK cells, DC, monocytes, macrophage and some granulocytes (153). Depletion of leukocytes with alemtuzumab is associated with a dramatic decrease in cerebral lesions and a stabilization of the BBB (154). While the primary mechanism of action of alemtuzumab is the nearly complete obliteration of the immune system, and thus the prevention of immune-mediated pathology, a secondary mechanism of action has emerged as the immune repertoire starts to repopulate itself. As immune cells repopulate, the repertoire seems to skew towards a regulatory phenotype with an outgrowth of $CD25^{high}FoxP3^{+}$ regulatory T cells emerging first, creating a tolerance-enhancing environment.

Not surprisingly, the rate of secondary infection associated with alemtuzumab treatment is increased, as would be expected with this degree of immunosuppression. It has been reported that 66% of treated patients experienced an infection during treatment, as wells as an increased rate in the development of melanoma (155). Additionally, other adverse effects of treatment have been reported including the development of Grave's disease, Goodpasture syndrome and Idiopathic Thrombocytopenic Purpura (156). These adverse events, including the death of a patient, led to the termination of a trial for the use of alemtuzumab for the treatment of MS. While the use of alemtuzumab demonstrates the efficacy of immune suppression for the prevention of immune-mediated pathology, it also clearly demonstrates the major pitfalls of such a strategy with the multitude of side effects associated with a lack of proper immune functions.

Mitoxantrone in the treatment of MS

Mitoxantrone is a synthetic antineoplasitic agent that was developed as an analog of doxorubicin with the intent of decreasing the cardiac toxicity of the drug (157). It was originally, and is currently, used for the treatment of cancer, including acute myloid leukemia and prostate cancer (158). To date mitoxantrone is the only therapeutic that has shown any efficacy in treating secondary progressive MS (159). Mitoxantrone is a small flat molecule that can cross the BBB and enter the CNS. It functions by either intercalating into DNA or by inhibiting topoisomerase-2, both mechanisms result in DNA damage, and activation of the DNA damage response pathway (DDR) (see Figure 1), and ultimately the induction of apoptosis in the damaged cell. Mitoxantrone inhibits both DNA replication, as well as DNA-dependent RNA replication (160). *In vitro* it has been show to kill both proliferating and non-proliferating cells, demonstrating a mechanism of action that is cell cycle independent.

Studies of mitoxantrone's effects on the development of EAE, in both rats and mice, demonstrate that mitoxantrone is effective at inhibiting the development of disease when it is administered prior to the onset of symptoms, but it is also effective when administered after the development of clinical symptoms. Additionally, it is effective in preventing disease relapse in the SJL mouse model (161). Like most cytotoxic drugs that induce DNA damage, mitoxantrone works best on cells that are rapidly dividing. In the case of EAE these rapidly dividing cells are presumed to be the myelin-specific T cells that are induced upon immunization. The loss of these cells with mitoxantrone treatment is seen with a decrease in $CD4^+$ T cells that produce either IFN-γ, TNF or IL-2 upon *ex vivo* peptide restimulation. Additionally mitoxantrone has been shown to prevent macrophage dependent myelin degradation in the CNS, presumably due to the loss of upstream signals from $CD4^+$ T cells (162). In clinical trials, mitoxantrone decreased the rate of

relapse in RRMS patients. In secondary progressive MS patients mitoxantrone was able to decrease the lesion burden by MRI by >2 lesion over placebo control (159).

While mitoxantrone is the only therapeutic used for the treatment of escalating disease that is not managed by IFN-β or other drugs, it is only used as a last resort, as mitoxantrone has notable side effects that limit its use. The recommended dosage of mitoxantrone for MS is 12mg/M^2 every 3 months. This is notable since the recommended maximum lifetime dose for mitoxantrone is 120mg/M^2, which allows for treatment only over a 2.5 year period. The reason for this is that mitoxantrone accumulates in tissues. Autopsies of mitoxantrone treated patients demonstrated that the drug can persist in tissues for over 272 days after treatment. Accumulation of the drug was seen mainly in the heart, the thyroid and the liver (163). The persistence of mitoxantrone leads to one of the most notable side effects of treatment, cardiotoxicity. In one large scale phase 3 clinical trial of mitoxantrone in MS 5.6% of patients presented with therapy-induced cardiac complications, with 36% of these patient developing congestive heart failure (164). The other major side effect of mitoxantrone is the development of secondary leukemia, a problem seen with many DNA-damaging agents. The development of mitoxantrone-induced leukemia has been estimated at about 0.6% over a two year treatment regimen with mitoxantrone. Most reports document the development of Acute Myeloid Leukemia with mitoxantrone treatment, though a few case studies also report the development of Chronic Myeloid Leukemia as well as Acute Promyelocyte Leukemia (165, 166). Though mitoxantrone shows a high level of efficacy, these side effects limit the utility of mitoxantrone in the treatment of MS. Therefore, a treatment strategy that mimics the effects of mitoxantrone, but limits its side effects, would be very advantageous.

An approach to specifically target encephalitogenic T cells

MS is a chronic lifelong disease. It has been well described that T cells mediate pathogenesis and that removal of these pathogenic T cells resolves the disease. However, the current treatments for MS do not specifically target this known driver of disease; they are not a cure. We and others have proposed that the appropriate treatment for an autoimmune disease, such as MS, would remove the pathogenic cell population that induces damage without inhibiting protective immune responses. The current treatment strategies either fail to remove myelin-reactive T cells, in the case of IFN-β and natalizumab, or they target such a large portion of the immune system for deletion that it is unable to repel opportunistic infections or malignancy, in the case of daclizumab, rituximab, alemtuzumab and mitoxantrone. Since the current therapeutics are unable to meet our criteria for a successful treatment we need to investigate other options. Previous work from our collaborators has demonstrated that the drug etoposide may be able to meet our criteria for efficacy and selectivity in the clearance of pathogenic T cells.

Etoposide is a epipodophyllotoxin that is employed for the treatment of a wide variety of malignancies (167). The use of etoposide to kill rapidly dividing cells, such as tumors, has been applied for the treatment of other lymphoproliferative diseases. In particular etoposide has been documented to selectively kill pathogenic activated T cells in a mouse model of Hemophagocytic Lymphohistiocytosis (HLH), a disease were pathogenesis is mediated by a small population of activated effector T cells (168, 169). Johnson et al. demonstrated that etoposide is able to selectively kill pathogenic lymphocytic choriomeningitis viurs (LCMV)-specific T cells that induce pathology, while maintaining intact naive T cell populations. While the pathogenic effector T cells in HLH are not self-reactive, fundamentally they are very similar to auto-reactive

T cells, therefore, this targeted treatment for effector T cells should translate well for the treatment of MS. Interestingly, cytotoxic drugs are typically thought of as blunt immune suppressing agents. However, this report shows that etoposide can selectively target activated T cells *in vivo,* with the potential for limited off target cytotoxicity.

Killing activated T cells with etoposide

Etoposide is a DNA-damaging agent that targets topoisomerase II. Topoisomerase II is an essential enzyme that controls the topology of DNA during replication. Most cytotoxic drugs can be grouped as either poisons or inhibitors of their target molecule. Poisons, such as etoposide, mitoxantrone, and doxorubicin, function by stabilizing the topoisomerase II-DNA cleavage complex, unlike inhibitors which affect the enzyme while not affecting the DNA cleavage complex (170). Therefore, poisons become more effective with increasing topoisomerase II levels, and activated cells become more sensitive to their effects (171). The functional topoisomerase II is a homodimeric enzyme that interacts directly with supercoiled DNA. It passes an intact double helix of DNA though a transient enzyme-made double-stranded DNA break, an action that is required for chromosomal segregation during mitosis. Etoposide increases topoisomerse II mediated DNA cleavage by inhibiting the ability of the enzyme to detach from the DNA (172). The drug enters the complex through a direct interaction with the ATP-bound enzyme monomer so that each drug molecule only induces a single-stranded break. Though etoposide is typically thought to induce a double strained break (DSB), breaks in both strands of DNA are solely dependent on the molecular ratio of drug to enzyme (173, 174). The exact mechanism of how the etoposide-topoisomerase II-DNA complex is broken down is not

known since the 5' tyrosyl-DNA phosphodiesterase that would be able to remove topoisomerase II from a covalent complex has not been identified. Whatever mechanism is involved in the processing of this complex, the end result is DNA damage, which predominantly manifests as DSB throughout the genome (175). DSB in the genome are marked by γH2AX, phosphorylation of Ser-139 on histone H2A (176). γH2AX is thought to participate in DSB repair by holding broken DNA ends in close proximity and recruiting other DNA repair factors to the damaged area (177). Additionally, it can be used as a surrogate marker of cell killing by drugs that create DSB (178). DSB can be visualized by γH2AX foci after a few minutes of treatment with etoposide (176, 179).

Two potential mechanisms exist regarding the movement of the replication fork and etoposide-mediated DNA damage. The first mechanism involves the role of topoisomerase II in removing positive superhelical twists that accumulate ahead of the replication fork (180, 181). When etoposide binds topoisomerase II to DNA, progression of the replication fork would rip the DNA apart and cause a DSB. To test this theory the DNA synthesis inhibitors aphidicolin and hydroxyurea were used to inhibit the replication fork. DNA synthesis inhibitors were only partially effective in preventing etoposide-mediated cytotoxicity implying that DNA synthesis is not required for etoposide-induced DNA damage (182). The second mechanism is based on the role of topoisomerase II in unlinking daughter DNA molecules. This theory postulates that etoposide affects the dissociation of sister chromotids from each other by binding topoisomerase II to the DNA. This model relies on the association of topoisomerase II with DNA after the replication fork has transcribed the area (183). This idea is furthered by the ability of etoposide to induce a G2 blockade after DNA synthesis is complete. Replication protein A (RPA) foci that mark single-stranded DNA are detectable after etoposide treatment, and overlap replicated

chromatin rather than sites of ongoing DNA synthesis (25). Additionally, DSB have been seen behind the replication fork after etoposide treatment, further supporting this model (160).

Since topoisomerase II is not an integral component of the DNA replication machinery, etoposide does not lead to an immediate block of DNA synthesis. However, etoposide does induce a progressive inhibition of DNA replication that is evident from RPA foci that appear one hour following etoposide treatment (179). The term "cell cycle checkpoint" refers to signaling pathways that halt progression through the cell cycle until earlier processes such as DNA replication are complete (172). Cells that are damaged during their replication cycle face a particular challenge and must slow down, or fully halt replication until the damage is repaired. The two activating kinases from the phosphatidyl-inositol-3-OH family (PIKK) are ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3 related). These kinases sense DNA damage and activate the DDR pathway by phosphorylating p53. ATM responds to DSB while ATR responds to SSB and stalled replication forks.

Etoposide triggers activation of the ATM pathway with its downstream cell cycle checkpoint inhibitor Chk2 (179, 184). The MRE11–RAD50–NBS1 (MRN) complex forms on the end of a DSB and leads to the auto-phosphorylation of ATM. The MRN complex that forms in response to etoposide-mediated DNA damage is indistinguishable from that caused by other DSB such as ionizing radiation (185, 186). In contrast, activation of ATR requires extended single-stranded DNA regions covered in RPA, which plays a major role in loading ATR onto damaged DNA (187). In addition to the activation of ATM, mediated by DSB, etoposide can activated ATR and its downstream cell cycle checkpoint inhibitor CHK1. ATR is predominantly involved in DNA replication and is only active from late G1-G2 phase (188), which could explain the observation that cells are 2-3 times more susceptible to etoposide from mid-S to G2 phase, than cells in G1.

It is likely that the extended single stranded DNA segments needed for ATR activation represented a processing step in the repair of lesions generated by etoposide. ATR mediated Chk1 signaling appears to be responsible for the etoposide-induced inhibition of DNA replication by preventing the firing of new replication forks (179).

The aforementioned data suggests that whether etoposide causes a SSB or DSB does not ultimately matter for the induction of cytotoxicity, as both ATM and ATR are able to activate p53 and mediate apoptosis. In fact, the ability of etoposide to activate both kinases may give it an edge as a cytotoxic drug. The induction of apoptosis, by etoposide, functions through the cytochrome c-apaf-1-caspase-9 pathway. Studies to map out the etoposide-mediated cytotoxicity pathway have been conducted with a series of inhibitors (189). Large decreases in etoposide-mediated cell death were seen with the addition of I) wortmannin to prevent DNA-PK mediated phosphorylation of p53, II) cycloheximide to prevent *de novo* protein synthesis which the authors are largely attributing to the failure to induce Bax, Puma and Noxa, III) furosemide to prevent Bax translocation to the mitochondria, and IV) DUBQ to prevent mitochondrial permeability and cytochrome c release. This results in a pathway that requires transcription-dependent actions of p53, which results in the activation of the intrinsic apoptotic cascade. However, there is also evidence that Fas-mediated extrinsic death pathways may also play a role in cell death (190, 191). A threshold for etoposide-mediated apoptosis has been suggested, and it has been observed that this threshold is set at different levels in different cell types and cells lines. Therefore, the effects of etoposide are likely cell type specific, and dependent on factors that vary between cell types. This might allow for specific targeting of cells with a lower threshold for etoposide-mediated DNA damage, such as rapidly dividing T cells (192).

DNA Damage Response pathway and p53

Cells are constantly bombarded with an assortment of environmental and intrinsic factors that cause damage to the genome. While mild damage is repairable, extensive damage poses a potential oncogenic threat to the organism, and the cell must be removed for the health of the organism as a whole. Thus the cell has the choice of either A) live and repair or B) die when

faced with DNA damage. The transcription factor p53 assimilates a wide variety of cellular inputs (oncogene activation, DNA damage, mitotic impairment or oxidative stress) to initiate the appropriate output (193). The downstream gene targets of p53 vary widely in the functions. However, they can be broadly broken down into three categories I) genes involved in cell cycle arrest, II) genes involved in DNA damage repair, and III) genes involved in cell death, these are predominantly from the intrinsic apoptotic cascade. The ability of p53 to instruct towards growth-inhibitory genes in response to mild damage suggests the presence of a threshold Cell cycle arrest is the first endpoint of DDR pathway activation. The DDR pathway can induce cell cycle arrest through multiple mechanisms, including p53 gene products and direct activation of inhibitor kinases. A normal cycling cell requires a buildup of cyclins and cyclin-dependent mechanism that can control apoptosis with mild DNA damage (194). However, even a single DSB in the an essential gene can trigger the apoptosis signaling cascade (195, 196).

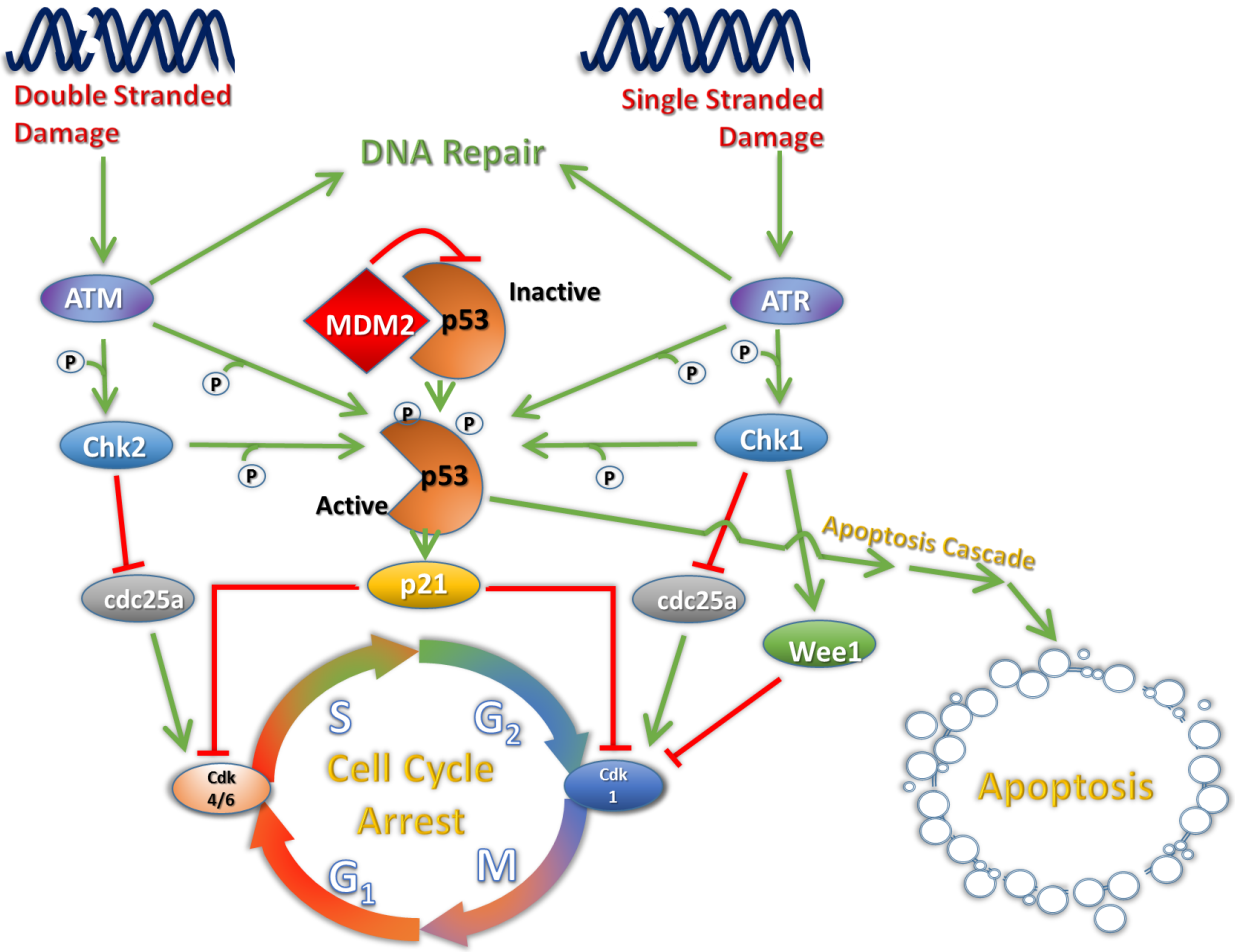

Figure 1: Model representation of the DNA damage response pathway.

kinase (CDK) conjugates to progress past cell cycle checkpoints in the cell cycle. These checkpoints exist at important cellular crossroads, including the transition point to start DNA replication (S phase) and entry into mitosis (G2/M phases). The p53 gene product $p21^{cip}$ is a non-specific CDK inhibitor that blocks the formation of the cyclin-CDK complex needed for progression through all of the checkpoints. Thus p53 can arrest cell cycle progression at most stages of cycling. The DDR pathway also activates more specific checkpoint inhibitors via ATM and ATR. Both Wee1 and Chk1 kinases function to inhibit G2/S cell cycle progression in a cyclin dependent manner. Cell entry into mitosis is driven by the cellular expression of the cyclin

B-CDK1 complex. The concentration of this complex starts off low at the beginning of S phase and accumulates throughout G2 until it peaks at a threshold concentration allowing for the end of G2 and the entry into mitosis (197). Wee1 phosphorylates CDK1 at Tyr 14 and 15, which inactivates CDK1 and prevents the interaction of CDK1 with cyclin B. This prevents cells from accumulating the CDK1-cyclin B complex and entering mitosis. These inhibitory phosphorylations can be removed by the phosphatase CDC25. Upon activation by ATR, Chk1 can phosphorylate CDC25 and inhibit it, thus perpetuating the inhibition of CDK1 (198–200). Therefore, both Chk1 and Wee1 function to inhibit cell cycle progression by preventing the accumulation of CDK1-cyclin B complexes.

The induction of apoptosis mediated by p53 is generally associated with the intrinsic apoptosis cascade. The BH3 only proteins Puma and Noxa as well as the effector protein Bax are all documented p53 gene products (201). Accumulation of all three proteins leads to mitochondrial outer membrane permeability, release of cyctochrome c and the activation of executioner caspase-3. Furthermore, cytosolic p53 has been reported to migrate to the mitochondria and associating with Bax and Bak in a pro-apoptotic manner (202). Additionally, p53 can induce the expression of the extrinsic apoptotic receptors Fas and TRAIL (203). Signaling through these receptors provides another mechanism for inducing apoptosis following the activation of the DDR pathway and p53.

The regulation of p53 is mediated by various post-translational modifications including phosphorylation, acetylation, ubiquitination and sumoylation. For example, phosphorylation of Ser46 favors activation of apoptotic genes (204). Mutation studies, where Ser46 on p53 is mutated to an Ala, reduced the ability of p53 to transactivate pro-apoptotic genes, including NOXA, PUMA, DR5 and PERP. However, cell cycle arrest genes were unaffected by this

mutation (205, 206). Several kinases have been demonstrated to preferentially phosphorylate Ser46 including HIPK2, DYRK2 and p38. Additionally, acetylation of lysine120 on p53 can lead to its accumulation on pro-apoptotic promoters such as BAX and PUMA. Acetylation may also help recruit other transcription factors that are able to overcome the transcriptional barriers of apoptotic genes. Another important site is lysine 320, ubquitination of this site leads to activation of proarrest genes p21 and cyclin G1 (207). Ubiquitination of p53 by MDM2 either leads to degradation or export of the protein into the cytoplasm, where it has been reported to interact with pro-apoptotic proteins at the mitochondrial surface (202). In either case ubiquitination decreases the available nuclear p53 that is available for DNA binding.

Growth arrest genes tend to have high affinity binding sites for p53, whereas pro-apoptotic genes tend to have lower affinity sites (208). Thus, the binding affinity for p53 could affect the promoter choice for p53 binding, when p53 is limiting. Whereas higher expression of p53 can override this, and triggers apoptosis (209). Supporting evidence for this is seen in delayed binding of p53 to pro-apoptotic promoters compared to cell cycle arrest promoters (210).

Project Aims

Multiple sclerosis is a chronic, debilitating autoimmune disease that results from immune mediated demyelination of the CNS. Multiple leukocyte populations have been associated with MS pathology, including T cells. Myelin-specific T cells orchestrate the neuroinflammation that results in MS, and are therefore the ideal target for a treatment. Currently, there are no effective treatments for MS that specifically target self-reactive T cells, and do not have major impacts other cellular populations. Our aim for a successful therapy for MS requires an approach that

can specifically treat and clear self-reactive immune responses while sparing protective beneficial naïve and memory responses, and minimizing off-target cellular and molecular damage. We aim from an approach that will I) specifically target pathogenic cells, II) not hinder protective immune responses, III) not cause off-target tissue damage and IV) be a systemic treatment that is antigen independent and broadly applicable. Incorporation of these aims as requisites for a treatment approach will yield a safer, more effective treatment for MS.

The major problem is that autoimmune T cells are remarkable similar, if not identical, to beneficial T cells that are vital in controlling infections. Thus, how can we specifically target myelin-specific T cells without radically altering protective immune responses. Initiation of the DDR pathway with the DNA damaging agent etoposide can target activated T cells, with specificity for activated T cells over naive T cells. Mitoxantrone can also kill activated pathogenic T cells in EAE, though its specificity is unknown. Therefore, we hypothesize that activation of the DDR pathway in activated T cells, initially with DNA damaging agents, will lead to their death. This approach can have specificity for activated T cells, over other cellular populations, based on the inherent vulnerabilities for rapidly dividing cells. We further hypothesize that downstream modulation of proteins in the DDR pathway with selective inhibitors will also preferentially kill activated T cells, even in the absence of a DNA damaging agent. We can utilize the killing of activated T cells through modulation of the DDR pathway to treat autoimmune diseases by killing activated self-reactive T cells. We believe that selective manipulation of the DDR pathway will be a successful means to clear self-reactive immune responses in EAE, without inhibiting protective naïve and memory responses.

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Chapter 2

Eliminating encephalitogenic T cells without undermining protective immunity

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Eliminating encephalitogenic T cells without undermining protective immunity

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Abstract

The current clinical approach for treating autoimmune diseases is to broadly blunt immune responses as a means of preventing autoimmune pathology. Among the major side effects of this strategy are depressed beneficial immunity and increased rates of infections and tumors. Utilizing the experimental autoimmune encephalomyelitis (EAE) model for human multiple sclerosis we report a novel alternative approach for purging auto-reactive T cells that spares beneficial immunity. The moderate and temporally-limited use of etoposide, a topoisomerase inhibitor, to eliminate encephalitogenic T cells significantly reduces the onset and severity of EAE, dampens cytokine production and overall pathology, while dramatically limiting the off-target effects on naïve and memory adaptive immunity. Etoposide-treated mice show no or significantly ameliorated pathology with reduced antigenic spread, yet have normal T cell and T-dependent B cell responses to *de novo* antigenic challenges, as well as unimpaired memory T cell responses to viral rechallenge. Thus, etoposide therapy can selectively ablate effector T cells and limit pathology in an animal model of autoimmunity, while sparing protective immune responses. This strategy could lead to novel approaches for the treatment of autoimmune diseases with both enhanced efficacy and decreased treatment-associated morbidities.

Introduction

Multiple Sclerosis (MS) is a neuro-inflammatory autoimmune disease in which T cell driven inflammation leads to demyelination and damage of axons in the central nervous system (CNS). MS manifests itself through a diverse array of clinical pathologies ranging from cognitive and ocular impairments to full paralysis (1, 2). MRI and patient necropsy studies reveal that actively demyelinating lesions are typified by infiltration of $CD4^+$ T cells and macrophages in the white matter of the CNS (3, 4). To date there is no known cure for MS, though there are treatments available that can ameliorate symptoms of the disease. However, they have limited efficacy, significant adverse effects or are broadly immunosuppressive. The standard first line treatment strategy for MS is the use of immunomodulating drugs: interferon-β, glatiramer acetate, and/or steroids (5). While the exact mechanism of action for these drugs is poorly understood, it is known that they all suppress or redirect immune activation. The next class of MS therapeutics are lymphocyte trafficking inhibitors, including natalizumab (6) and fingolimod (sphingosine 1-phosphate receptor analog (7, 8). These treatments inhibit lymphocyte migration, not only to the CNS, but also to sites of infection (9). As a final measure the chemotherapeutic drug mitoxantrone can be given in particularly severe and progressive cases, though its use is limited by cardiac toxicity (10, 11). Thus none of the current therapeutic strategies designed to prevent destruction of the CNS specifically target the encephalitogenic response. Reliance on agents which have a non-specific suppressive effect on the immune response leads to increases in secondary infections (12), and an increase in the outgrowth of tumors (13, 14). Moreover, the current therapeutic approaches do not stem the eventual progress of MS.

It is well established that damage to the CNS is mediated by a relatively small number of self-reactive T cells (15). We reasoned that instead of suppressing the immune system as a whole, a more logical and appropriate strategy to treat MS would focus on the selective targeting of these rogue encephalitogenic T cells. Therefore, we and others (16, 17), propose that selectively eliminating activated encephalitogenic T cells will effectively ameliorate the progression of MS while markedly reducing the off-target effects of therapy. To test the viability of this approach, we employed a mouse model of MS, experimental autoimmune encephalomyelitis (EAE). As reviewed by Gold et. al (18) EAE is induced by immunizing mice with neural antigens leading to CNS inflammation and damage, similar to what is seen in MS patients. EAE affords us a model that generates a tractable population of pathogenic T cells with defined epitopes and immunologic functions (19). Additionally, utilizing variations of EAE we can test our hypothesis under varying pathologic conditions including the generation of new encephalitogenic T cells to spread epitopes in the relapsing-remitting model of EAE.

As a means to selectively eliminate encephalitogenic T cells we used the cytotoxic drug etoposide. Etoposide is a topoisomerase inhibitor (20, 21) that is used clinically to treat a variety of cancers and hemophagocytic lymphohistiocytosis (HLH) (22), a primary immune deficiency where aberrant T cell responses lead to immune mediated pathology. In parallel studies by our group (Johnson et. al. 2013, "submitted for publication), we demonstrate that etoposide treatment in a mouse model of HLH decreases immune mediated pathology by selectively deleting pathogenic activated anti-viral T cells, demonstrating that etoposide is a useful tool to delete activated T cells that induce immune mediated damage. In addition this study provides a detailed mechanistic understanding of etoposide's action on activated T cells *in vivo*.

Here we report that utilizing etoposide as an agent to clear encephalitogenic T cells is effective in the treatment of the autoimmune disease EAE. Etoposide treatment reduced clinical symptoms as well as the number and function of encephalitogenic T cells. Notably etoposide treatment acts primarily against autoimmune effector T cells, and not naïve or memory T cells, thereby controlling EAE while maintaining beneficial immune responses to new and prior antigenic challenges.

Materials and Methods.

Mice: C57BL/6J and SJL/J mice were purchased from Jackson laboratories and *Xiap*$^{-/-}$ were bread in house (23). All mice were housed under specific pathogen-free conditions in an AAALAC-approved barrier facility at CCRF. All experiments were performed with prior IACUC approval and every attempt was made to reduce the numbers of animals used. Animals were under constant monitoring and care of the CCHMC veterinary staff.

EAE induction and treatment: 10 week old female C57BL/6 or SJL mice were immunized s.c. with 100μg MOG_{35-55} or $PLP_{139-152}$ emulsified in 5mg/mL CFA (Hooke Laboratories. Lawrence, MA). On Days 0 and 2 animals received i.p. injections of 250 ng pertussis toxin (Hooke Laboratories. Lawrence, MA). Disease severity was assessed every day beginning, on day 10 and assigned a value using the following scale: 1, tail flaccidity; 2, hind limb weakness; 3, hind limb paralysis; 4, hind and fore limb paralysis; 5, moribund. Immunization and pertussis toxin injections were formulated to maximize the disease severity to be ≤ 3 per the manufacturer's specifications (Hooke Laboratories, Lawrence, MA.) and recommendations of our IACUC. Etoposide was administered i.p. at 50mg/kg twice, four days apart (Sigma-Aldrich. St. Louis, MO) (24)

Histology: Mice were perfused with PBS followed by 10% formalin through the left ventricle. Fixed spinal cords were embedded in paraffin and sectioned into 6μm slices. Sections were either stained with luxol fast blue (Electron Microscopy Sciences. Hatfield, PA) and counter stained with eosin and hematoxylin or labeled with FluoroMyelin® green and DAPI (Life Technologies. Grand Island, NY). All sections were viewed on a Nikon Eclipse E600 microscope at a 10x objective and images were processed in ImageJ (NIH, Bethesda, MD).

Isolation of CNS mononuclear cells: Mice were perfused with PBS through the left ventricle prior to removing brain and spinal cord tissue was dissociated in a dounce homogenizer. Cells were suspended in a 30% percoll gradient (GE healthcare. Little Chalfont, UK) that was overlaid on a 70% percoll gradient and spun at 500 x G for 30 mins. at 18^0C. Lymphocytes were removed from the interface between percoll gradients and stained by flow cytometry.

MHC tetramer staining and flow cytometry: Spleens from individual mice were harvested and crushed through a 70μm cell strainer (BD Biosciences, San Jose, CA) to generate a single cell suspension. A total of 2 x 10^6 cells were stained with different combinations of the following cell surface antibodies anti-CD4, anti-CD8, anti-CD44, anti-CD62L, anti-CD16/32, anti-CD25, anti-CD11c, anti-CD11b, anti-NK1.1, anti-CD19, anti-F4/80, anti-FoxP3, anti-Bim, Anti-XIAP, and anti- Bcl-2 (Biolegend. San Diego, CA, eBiosciences. San Diego, CA, Miltenyi Biotec. Auburn, CA or Rockland Immunochemicals. Gilbertsville, PA). *Ex vivo* cytokine production was assessed by restimulation with 5ug peptide, MOG_{35-55}, $PLP_{178-191}$, $PLP_{139-152}$, MBP_{84-104}, GP_{33-41}, or GP_{61-80} (Synthetic Biomolecules. San Diego, CA) in the presence of golgi plug (BD Biosciences. San Jose, CA). Cells were permiableized with Cytofix/cytoperm kit (BD Biosciences. San Jose, CA) and stained with anti-IFN-γ, anti-IL17a or anti-TNF-α (Biolegend. San Diego, CA). The MOG_{35-55} I-A^b and LCMV GP_{61-80} I-A^b tetermers were provided by the NIH tetramer core. The LMCV GP_{33-41} K^b tetermer was made in house as previously described (25). Where noted, CD4+ T cells were isolated from spleen and inguinal lymph nodes and purified by negative selection using CD4+ T cells isolation kit II (Miltenyi Biotec. Auburn, CA) prior to staining. Flow cytometry data was acquired on an LSRFortessa (BD Biosciences. San Diego, CA) and was analyzed using FlowJo software version 7.6.5 (Tree Star Inc. Ashland, OR).

Proliferation assay: *1 x 10^5* total splenocytes were cultured in complete DMEM media supplemented with 5% FBS, 1uM HEPES, penicillin 1000 U/mL, streptomycin 1000 ug/mL, (Gibco. San Diego, CA) at 37 degrees with indicated antigen, peptides at 5μg/ml and ConA at 2μg/ml. After 48 hours in culture 1μCi of ^{3}H-thymidine was added to each culture and harvested 18 hrs later. Assays were read on one of two Top count NTX beta counter (Perkin Elmer. Waltham, MA).

LCMV infections: The Armstrong-3 strain of LCMV, a gift from R. Ahmed (Emory University, Atlanta, GA), was grown in BHK-21 cells; the number of plaque-forming units (pfu) was assayed on Vero cells as previously described (26). Mice were infected i.p. with 2 x 10^5 PFU lymphocytic choriomeningitis virus (LCMV Armstong) for primary infection and post etoposide treatment. Mice were re-challenged i.v. with 2 x 10^6 PFU Clone 13 LCMV to assess memory responses.

TNP-OVA antibody response: Protocol is derived from and detailed in Strait et. al. (27). In short mice were i.p. immunized with 200ul of conjugated TNP-OVA (1.25 mg/ml and 10 mg/ml). Serum was collected for antibody titers and determined by ELISA. Serial dilutions of serum were detected by anti-mouse IgG and IgM (BD Biosciences. San Jose, CA) bound to plate bound TNP-OVA. TNP and OVA were purchased from Sigma Chemicals (St. Louis MO).

Etoposide mediated apoptosis: Spleens and inguinal lymph nodes were harvested from mice 45 days post LCMV infection and 12 days post EAE induction. CD4+ T cells were purified by negative selection using CD4+ T cells isolation kit II (Miltenyi Biotec. Auburn, CA) and cultured in complete DMEM media supplemented with IL-7 (5 ng/ml) and IL-2 (50 ng/mL)

(PeproTech. Rocky Hill, NJ). Cells were cultured for 16 hrs. with etoposide, Q-VD-OPH at 20mg/ml (MP biomedical, Solon OH) and necrostatin at 30uM (Enzo Life Sciences, Farmingdale, NY) prior to staining with tetramers (MOG_{35-55} I-A^b and LCMV GP_{61-80} I-$A^{b)}$ antibodies (anti-CD4, anti-CD16/32 and anti-CD44) and viability dye efluor 506 (eBiosciences. San Diego, CA)

Statistics: Where appropriate, results are given as the mean ± standard error (SE) with statistical significance determined by two-tail t-test, using either paired or unpaired (assuming equal variance) according to the data characteristics. Survival curves were assessed by Wilcoxon-Gehen test for differences among groups. Significance was defined as $p<0.5$. Statistical analysis was performed using the GraphPad Prism 5.04 software (GraphPad Software Inc. La Jolla CA)

Study approval: All animal studies were approved by the Cincinnati Children's Research Foundation institutional animal care and use committee.

Results

Our basic premise is that activated auto-reactive T cells are more susceptible to directed ablation than naïve or memory T cells, *in vivo*. Thus, to test our ability to target auto-reactive T cells we needed a well-established disease model with a distinct pathology, and a defined auto-antigen eliciting a tractable population of encephalitogenic T cells; therefore, we chose the myelin-oligodendrocyte glycoprotein (MOG) model in C57BL/6 (B6) mice (28). The MOG model gave us a population of tractable encephalitogenic T cells that 1) can be enumerated by tetramer staining, 2) has well-defined functional cytokine profiles and 3) has a defined spread epitope. As a means to induce clearance of auto-reactive cells we chose etoposide since it targets rapidly dividing cells (21), such as activated effector T cells.

Given the known clinical kinetics of etoposide in the treatment of HLH and its mouse model, along with our knowledge of the kinetics of EAE pathology, we found that two treatments (at 50mg/kg) four days apart yielded optimal effects. Treatments starting on day five post-induction of EAE followed four days later on day nine yielded the greatest amelioration of symptoms; however, etoposide is effective at other time points, both earlier and later (Figure 1).

Etoposide treatment significantly reduces the onset of EAE pathology. Mice treated with etoposide, on day 5 and 9 post-disease induction, showed a significantly delayed onset of disease with decreased mean clinical disease scores when compared to vehicle-treated mice (Figure 2A). Importantly, treatment with etoposide was able to completely prevent EAE in over 60% of treated mice and prolong the mean time to disease in the rest, 20.4 days for treated mice vs. 16.1 for vehicle (Figure 2B).

The immune-mediated destruction of oligodendrocytes in the CNS is a hallmark of MS/EAE (29). We investigated the ability of etoposide treatment to limit the immune-mediated damage by assessing the demyelination of the white matter in the CNS. Using quantitative histological analysis for myelin (luxol fast blue and FluoroMyelin® green) we found that control vehicle-treated mice showed severe demyelination in all anatomical regions, cervical to coccygeal. Strikingly, etoposide-treated mice showed little to no demyelination, having well defined borders between white matter and grey matter as seen in healthy mice (Figure 3A). Cellular infiltration into the spinal cord was determined by enumerating DAPI staining for nucleated cells in the white matter of the spinal cord. Here again, the numbers of infiltrating cells residing in the white matter of etoposide-treated mice was up to a 5 fold less than in the CNS infiltrates in vehicle-treated mice in all anatomical regions studied (Figure 3B). This is consistent with the lack of demyelination that we observed in etoposide treated mice where fewer infiltrating cells can cause less damage. To gain a further understanding of cellular infiltrates into the CNS, we homogenized brains and spinal cords from mice 15 days after disease induction to analyze infiltrating leukocyte populations by flow cytometry (Figure 3C). The greatest differences in cellular populations after etoposide treatment were seen in T cells with a full log decrease in infiltrating $CD4^+$ T cells after treatment. While etoposide treatment significantly decreased the total number of T cells in the CNS, we wanted to assess the number of MOG specific $CD4^+$ T cells. To test this we employed a MOG_{35-55} $I\text{-}A^b$ MHC class II tetramer, which allowed us to track antigen specific $CD4^+$ T cells regardless of their functional state. Etoposide treatment significantly decreased both the percentage (Figure 3D) and the total number (Figure 3E) of MOG specific $CD4^+$ T cells in the CNS as well as in the spleen (Figure 3F). These data

demonstrate that etoposide treatment is able to prevent CNS damage by systemically limiting the number of encephalitogenic T cells in EAE.

Etoposide treatment eliminates encephalitogenic T cells. Treatment with etoposide significantly mitigated symptoms of EAE. We postulated that etoposide worked by killing encephalitogenic effector T cells that would normally mediate damage. To test this, we evaluated the effects of etoposide treatment on numbers and function of MOG_{35-55} specific $CD4^+$ T cells. First we enumerated the total number of MOG_{35-55} specific $CD4^+$ T cells by tetramer staining, and second, we analyzed the *ex vivo* cytokine production of MOG_{35-55} specific $CD4^+$ T cells throughout the course of EAE (30, 31). etoposide treatment resulted in a significant decrease in the number of cytokine producing MOG_{35-55} specific $CD4^+$ splenic T cells throughout the course of the experiment (Figure 3 E,F). Similar to the results we observed with tetramer staining, etoposide decreased the frequency of IL-17A, IFN-γ and TNF producing MOG_{35-55} specific $CD4^+$ T cells throughout the course of disease, immediately following treatment (day 10) and at the peak of disease (day 15). As disease plateaued at day 30 and inflammation subsided a difference was only noticeable in TNF levels (Figure 4B, C, D). Taken together, these results demonstrate that etoposide treatment is able to alleviate the symptoms of EAE by eliminating encephalitogenic $CD4^+$ T cells that would otherwise induce pathology.

Etoposide treatment does not cause nonspecific lymphopenia. Etoposide is regularly used as part of a high dose, multi-drug cocktail used to treat leukemia. A common side effect of this clinical cocktail is marrow suppression (32). However, when etoposide is used alone to treat EAE, we found no cytopenias following treatment. The total number of splenocytes was not

significantly reduced after treatment (Figure 5). Moreover, when leukocyte subsets were delineated no difference was seen in the absolute number of splenic T cells (either $CD4^+$or $CD8^+$), regulatory T cells (T reg), B cells, or NK cells after etoposide treatment. However, a transient decrease in the total number of antigen presenting cells, both dendritic cells and macrophages, was observed. These differences recovered within five days indicating that there is no long-term change in the output of hematopoietic cells or antigen presentation (data not shown).

Etoposide treatment decreases pathology in established disease. Having demonstrated the efficacy of prophylactic treatment, we next investigated the effectiveness of etoposide therapy on established disease. Here again B6 mice were immunized with MOG_{35-55} peptide to induce disease and once symptoms were evident, with a mean clinical score of 1, we began etoposide therapy. When etoposide was administered at days 12 and 16 during an established course of disease, etoposide significantly decreased disease severity compared to vehicle treated controls (Figure 6A). This demonstrates that etoposide treatment can effectively diminish the severity of an established course of EAE.

Etoposide treatment decreases the rate of disease relapse. The most common presentation of MS is the relapsing-remitting form (7), which is well modeled using SJL mice immunized with the $PLP_{139-151}$ peptide (33). Thus, to access the impact of our treatment design on disease relapse, we induced EAE and allowed the mice to develop a full primary course of disease prior to treatment. Once all mice were in remission and had a disease score of 0, we randomize them into two groups, etoposide or vehicle, and began treatment, day 17 and 21, after initial induction.

Mice were then monitored for disease relapse. Etoposide treatment decreased the percentage of mice that relapsed, 46% vs. 73% of vehicle mice (Figure 6C). Additionally, in the mice that did relapse etoposide treatment delayed the mean time to relapse, 27 days vs. to 21 days for vehicle controls. Moreover, the mice that did relapse showed less severe disease compared to vehicle treated mice. These data demonstrate that etoposide treatment can effectively reduce the rate and severity of relapse.

Etoposide treatment decreases the breadth of epitope spread. In both the acute-progressive and the relapsing-remitting forms of MS/EAE a hallmark of disease progression is epitope spread (34, 35). To determine if etoposide treatment was preventing disease relapse by effecting epitope spread, we assessed the effect of etoposide treatment on the development of epitope spread in both the B6 and SJL models. As seen in Figure 5A, etoposide treatment decreased the recall response to the $PLP_{178-191}$ spread epitope as well as the recall response to the immunizing epitope MOG_{35-55} in the B6 mouse. In contrast to the B6 mouse, SJL mice have an increased number of defined EAE spread epitopes, including both intra- and inter-molecular spread epitopes. Etoposide treatment during disease remission had no effect on the immunizing $PLP_{139-151}$ epitope, likely, due to the fact that an initial course of disease has already taken place prior to treatment. However, etoposide treatment during remission prevented the activation of novel neural antigen specific T cells, including the $PLP_{178-191}$ intra-molecular spread epitope and both the MOG_{92-106} and MBP_{84-104} intermolecular spread epitopes. The deceased relapse seen in Figure 4B correlated with markedly reduced spread to both intra- and inter-molecular epitopes (Figure 7B). Together these data suggest that etoposide therapy act to reduce epitope spread, providing a molecular basis for the observed deceases in clinical pathology.

Etoposide does not inhibit naïve T cell responses to viral infections. Having demonstrated that etoposide had no major effects on splenic cellularity (Supplemental Figure 2), we wanted to further test the specificity of etoposide treatment by investigating the effects of etoposide treatment of naïve T cell function. To investigate this, B6 mice were pretreated with etoposide ten and six days prior to infection with lymphocytic choriomeningitis virus (LCMV) and their primary responses were compared to vehicle treated controls. On day ten post infection, the T cell response to LCMV was analyzed by enumerating the LCMV specific $CD8^+$ and $CD4^+$ T cell responses using LCMV GP_{33-41} K^b MHC class I tetramer and LCMV GP_{61-80} I-A^b MHC class II tetramer, respectively (Figure 8A-C). Mice pretreated with etoposide generated an equally robust anti-viral $CD4^+$ and $CD8^+$T cell response, with no significant difference compared to vehicle treated mice. To confirm that etoposide pre-treatment did not inhibit functional anti-viral responses of naïve T cells we assessed their cytokine production by *ex vivo* peptide restimulation (Figure 8D,E). Both $CD8^+$ and $CD4^+$ T cells from etoposide pre-treated mice produced a robust response including the anti-viral cytokines IFN-γ and TNF. Etoposide treatment did not reduce the total number of cytokine producing T cells when compared to vehicle mice. Importantly the LCMV-specific T cells maintained their poly-functionality, which has been shown to be vital in maintaining viral immunity (36, 37). As a final test of functionality LCMV viral load was assessed by qRT-PCR for LCMV nucleoprotein. No differences were observed in viral RNA levels between etoposide and vehicle treated mice (data not shown). Clearance of LCMV confirms that naïve T cells remain functional after treatment with etoposide.

Pretreatment with etoposide does not inhibit T cell-dependent B cell responses. In addition to anti-viral responses, $CD4^+$ T cells are vital for driving B cell activation and Ig class switching. To further investigate the effects of etoposide on naïve immune responses we immunized mice with the hapten-carrier TNP-OVA to investigate both helper $CD4^+$ T cell and B cell responses. Mice were pretreated with etoposide, as above, and then immunized with TNP-OVA in alum, followed by a TNP-OVA boost on day twelve. Serum was serially collected to assess antibody production against the immunizing antigens. Etoposide pretreatment did not inhibit the generation of either anti-TNP IgG or IgM (Figure 9). At each time point, both etoposide- and vehicle-pretreated mice generated comparable amounts of anti-TNP antibodies. These data confirm that etoposide treatment does not impair the activation of naive $CD4^+$ T cells or B cell responses to neo-antigens.

Etoposide does not alter protective T cell memory. The moderate use of etoposide to eliminate encephalitogenic T cells results in a significant reduction of EAE severity and overall pathology, without impairing naïve T and B cell responses. It thus became critical to assess if etoposide treatment affected protective T cell memory. To this end, we set up the model depicted in Figure 10A. B6 mice were infected with the Armstrong strain of LCMV, allowed to recover and develop long-term anti-viral memory T cells, both $CD8^+$ and $CD4^+$ T cell populations which can be tracked by tetramer staining (25). Next, we randomized the mice and induced EAE in half of them while the remainder were left uninduced, and then both cohorts were randomized into etoposide or vehicle treatment groups; this approach allowed us to track and target encephalitogenic effector cells in mice with pre-existing anti-viral memory. To assess the persistence of a functional anti-LCMV memory T cells, the mice, including an age-matched naïve cohort, were then challenged with the Clone 13 strain of LCMV. This will allow us to

confirm functional memory since only mice with an established memory population to LCMV can rapidly clear a Clone 13 infection, in the absence of memory, Clone 13 induces long term persistent infection and clonal exhaustion of LCMV specific $CD8^+$ T cells (36, 38).

Using this system, we found that the memory T cell responses to viral re-challenge were unaffected by either etoposide treatment and/or a course of EAE (Figure 10B-E), and that etoposide treatment of EAE is unaffected by prior LCMV infection (Figure 11). Etoposide treatment did not alter the pronounced expansion of LCMV GP_{33-41} specific $CD8^+$ T cells and LCMV GP_{61-80} specific $CD4^+$ T cells, when compared to vehicle control mice, regardless of EAE. All groups showed similar multiple-fold expansion of memory cells, as assessed by antigen-specific tetramer staining and compared to resting B6 mice with established anti-LCMV memory or naïve B6 mice responding to a *de novo* Clone 13 infection (Figure 10B,C). These results clearly show that mice treated for EAE and purged of MOG-specific T cells by etoposide still possess memory T cells that can undergo comparable secondary antigen-driven expansion to viral re-challenge. Furthermore, the expanded memory T cells from all of the treatment groups demonstrated equivalent IFN-γ production following *ex vivo* peptide re-stimulation (Figure 10D,E). In fact, the LCMV-specific $CD8^+$ T cells from mice that had EAE and were treated with etoposide showed enhanced IFN-γ production (Figure 10D). As expected the age-matched, untreated, naïve B6 mice infected with Clone 13 showed reduced IFN-γ production from both $CD8^+$ and $CD4^+$ T cells.

Importantly, the expanded LCMV-specific memory T cells from all of the treatment groups showed equal functionality in the clearance of a LCMV Clone 13 infection (Figure 10F), thus

demonstrating that even upon targeted ablation of encephalitogenic T cells from mice with EAE, a robust functional memory subset persists that is capable of clearing the normally persistent Clone 13. As expected, naïve mice were unable to clear a primary Clone 13 infection. Taken together, these data clearly demonstrate that preexisting functional memory T cells capable of inducing sterilizing immunity to viral re-challenge do survive in mice ablated of auto-reactive T cells by etoposide.

Etoposide selectively induces apoptosis of activated encephalitogenic T cells. We have demonstrated that etoposide treatment decreases the total number of encephalitogenic T cells in mice with EAE while not affecting the numbers of memory or naïve T cells. We next wanted to confirm that the decreases we observed in EAE pathology were from etoposide-mediated cell death of encephalitogenic T cells. *In vitro* treatment with etoposide has previously been shown to induce death in encephalitogenic T cells (39); however, the specificity of etoposide mediated death was not investigated. To test this, we cultured purified $CD4^+$ T cells from mice that were 12 days post-EAE induction and 45 days post LCMV Armstrong infecting, allowing us to have MOG specific encephalitogenic $CD4^+$ T cells, memory LCMV $GP61^+$ specific $CD4^+$ T cells and naïve $CD4^+$ T cells all in the same mouse. $CD4^+$ T cells were cultured *in vitro* with increasing concentrations of etoposide to determine the survival of each T cell population. When normalized to the spontaneous death in culture for untreated cells the encephalitogenic $CD4^+$ T cells showed substantial apoptosis induction with etoposide treatment that increased with dosage, 37-41% death (Figure 12A). In contrast, both memory and naïve T cells showed minimal death in culture regardless of the concentration of etoposide that they were cultured in. These data confirms that etoposide selectively induces apoptosis in activated encephalitogenic T cells while

sparing both memory and naïve T cells at the same time. One likely explanation of the enhanced sensitivity of encephalitogenic T cells to apoptosis is dysregulation of Bcl-2 family members.

Activated encephalitogenic T cells are primed for apoptosis. Previous work from our group has demonstrated that activated T cells express disequilibrated members of the Bcl-2 protein family as a means of undergoing cellular contraction at the conclusion of a T cell response; specifically that effector T cells express higher levels of the proapoptotic BH3 protein Bim (40, 41). To investigate the apoptotic potential of encephalitogenic T cells, we induced EAE in mice 45 days after the clearance of an LCMV infection allowing for the presence of both memory LCMV specific T cells and MOG specific encephalitogenic T cells in the same mouse. We stained purified $CD4^+$ T cells for Bcl-2 and Bim directly *ex vivo*. Encephalitogenic $CD4^+$ T cells had diminished expression of the anti-apoptotic protein Bcl-2 compared to memory and naive $CD4^+$ T cell populations in the same mouse (Figure 12C). This is consistent with previously published reports on Bcl-2 expression in EAE (42). Additionally, encephalitogenic $CD4^+$ T cells expressed higher levels of Bim (Figure 12E). This disequilibrium of survival factors in encephalitogenic T cells increases their potential for apoptosis as compared to either memory or naive T cells. Furthermore, Moore et. al. reported that bulk T cells from B6 mice at 21 days post EAE induction express elevated levels of the inhibitor of apoptosis XIAP (43). We furthered this observation by finding elevated XIAP expression in MOG specific $CD4^+$ T cells at day 12 as compared to naïve $CD4^+$ T cells in the same mouse (Figure 8F). However, XIAP expression does not protect activated $CD4^+$ T cells from etoposide-mediated cell death. Purified, and *in vitro* activated $CD4^+$ T cells from wild type and *Xiap*$^{-/-}$ mice are equally susceptible cell death mediated by etoposide (Figure 12G). This demonstrates that XIAP expression does not

confer protection against etoposide mediated cell death. In fact, even when XIAP is overexpressed under a ubiquitin promoter in an EAE model it only provides marginal, a fraction of a fold, protection against etoposide mediated cell death (39).

The DNA damage response stemming from the effects of etoposide have been well described (44–47). This includes the activation of p53, and the production of the pro-apoptotic BH3 proteins PUMA and NOXA. When the effects of etoposide are paired with Bcl-2 family disequilibrium it suggests a specific mechanism for the selective clearance of encephalitogenic T cells that we observe *in vivo*. To test if apoptosis or necroptosis is the primary mechanism of etoposide-mediated cell death in encephalitogenic T cell we utilized the pan-caspase inhibitor Q-VD-OPH to prevent apoptosis (48, 49), and the RIP-1 inhibitor necrostatin to prevent necroptosis (50). We found that when encephalitogenic $CD4^+$ T cells were treated with etoposide in the presence of Q-VD-OPH cell death decreased from the level of etoposide only to the level of vehicle treated cells (Figure 8H). Conversely, when cells were treated with etoposide in the presence of necrostatin no effect on cell death was observed, as compared to etoposide alone. This supports that apoptosis is the primary mechanism for etoposide- mediated cell death of encephalitogenic $CD4^+$ T cells in EAE.

Discussion.

Here we have tested our concept of using targeted chemotherapeutics to ablate auto-reactive T cells while sparing naïve and memory T cell populations. We demonstrated that the cytotoxic drug etoposide can effectively treat EAE by preferentially targeting encephalitogenic T cells, to the exclusion of other protective lymphocytes. Etoposide treatment of EAE significantly decreased the total number of encephalitogenic T cells in treated mice, as determined by both MOG-specific tetramer staining and effector cytokine production. This ablation of encephalitogenic T cells led to a substantial decrease in clinical and tissue-associated pathology. Our analysis of spinal cord cross-sections reveals that etoposide treatment decreases cellular infiltration resulting in decreased demyelination and neuronal damage. Additionally, we demonstrated that etoposide treatment of mice with ongoing EAE decreased the overall magnitude and numbers intra- and inter-molecular spread epitopes. This suggests that the diminished severity of EAE, in both the B6 and SJL models, is due in part to limiting the breadth of additional effector T cells that become activated. In the end, etoposide-based therapy resulted in markedly reduced severity of or complete absence of EAE pathogenesis.

The true benefit of our approach is its selectivity. Importantly, the specificity resides not so much in etoposide itself, but in the approach of targeting the activation state of auto-reactive effector cells. In other words, etoposide targets for removal only those T cells that are currently undergoing active antigen-driven expansion and that are primed for apoptosis by their Bcl-2 family member expression profile. Etoposide purges encephalitogenic T cells in the context of EAE. Likewise if etoposide is given during the acute phase of a viral infection the anti-viral effector T cells would be purged. Thus, if treatment is limited to periods during or immediately following overt autoimmune pathology, etoposide shows highly specific ablation of

encephalitogenic T cells while sparing the more quiescent naïve and memory T cell compartments. Moreover, this approach is likely to result in a long-term "hole" in the T cells repertoire to the activating auto-antigen. As we demonstrated in (Johnson et al., 2013, "submitted for publication"), etoposide can equally ablate highly activated T cell during a LCMV infection. Since etoposide mediated apoptosis of effector T cells is not specified for, nor limited, to encephalitogenic or autoimmune T cells, it suggesting that this approach may have more applicability in other T cell-specific autoimmune diseases.

This represents a significant and novel approach in the treatment of autoimmune diseases. Moreover, this approach alleviates many of the complications associated with broad-spectrum immunosuppressant drugs currently in use. In addition, by focusing therapy on the rogue auto-reactive subset of T cells at the time they are most highly active, etoposide-based therapy significantly limits off-target effects. For example, etoposide treatment may reduce reactivation of JC virus, which can lead to the fatal progressive multifocal leukoencephalopathy in MS patients (12), by sparing protective memory T cell populations as well as decreasing the need for global immuno-suppression. Having the ability to treat MS without the risks associated with traditional immune suppression may represent a real and meaningful advance in patient care.

There is a consensus that the current protocols for the treatment of MS are insufficient. As the debate over new therapeutics is waged, the continual issue with the use of cytotoxic agents has been their lack of specificity. Here we have demonstrated that cytotoxic drugs, such as etoposide, can be used with increased specificity towards activated encephalitogenic T cells. Others have proposed the use of monoclonal antibody treatment; while these treatments show promise in the treatment of MS, they target immune cells for elimination based on surface antigen expression, not by function. Drugs newly approved or under investigation for MS

include alemtuzumab (anti-CD52, which is expressed on most lymphocytes as well as DCs and monocytes) and rituximab (anti-CD20, expressed on B cells), these drugs have no specificity for the encephalitogenic T lymphocytes that drive the neural pathology. In contrast to our use of etoposide, alemtuzumab is the antithesis of functional specificity because it depletes all lymphocytes. Further studies have been done using antibodies against CD25, the α-chain of the IL-2 receptor which is designed to target expression on activated T cells (51). However, this molecule is expressed in high concentrations on regulatory T cells, and as a result these antibodies may deplete not only activated T cells but also the regulatory compartment. Our data show that etoposide primarily clears activated effector T cells, while sparing regulatory T cells (Supplemental Figure 2) allowing for a better semblance of immune homeostasis.

The use of cytotoxic agents do carry toxicity risks, and etoposide is no different (52), yet toxicity tends to be dosage dependent. By decreasing the number of times that etoposide is administered, since it will not be needed long term to ablate encephalitogenic cells, we can decrease the risks associated with cytotoxic drugs. Decreasing treatment can be accomplished by proper timing which can mitigate epitope spread. Increasing the breadth of immunological activation to new epitopes and new proteins has been shown to propagate disease progression, and be responsible for disease relapse. Etoposide treatment, even under limiting dosage, is able to prevent the activation of T cells to new neural epitopes thus preventing disease progression and future relapse. It is not yet known whether etoposide is unique among cytotoxic agents for its immune selectivity. The cytotoxic drugs mitoxantrone and, rarely, cyclophosphamide are currently used to treat MS. Their mechanisms of action remain poorly defined, but they are likely to have some similarities to etoposide. Ongoing studies in our group are investigation this possibility. Notably, mitoxantrone is used in continuous dosing cycles in some cases until the lifetime

maximum dosage has been reached. We have demonstrated that treatment with etoposide is most effective while auto-reactive T cells are in an activated effector state, and that treatment while they are in a more quiescent naïve or memory state is ineffective. As a result, treatment at the start of a disease flare will yield the greatest effect of ablating encephalitogenic T cells. This study reveals new insights into the mechanism of action of etoposide, and potentially other cytotoxic drugs including mitoxantrone and cyclophosphamide, in the treatment of MS.

A future alternative to cytotoxic drugs for the deletion of self-reactive T cells could be small molecule inhibitors of anti-apoptotic Bcl-2 family members. Studies have shown that small molecule inhibitors that bind to Bcl-2, as well as its homologs Bcl-w and Bcl-x_L (53), may have efficacy in animal models of autoimmunity (46, 54, 55).

Further studies will need to be conducted to determine if other pharmaceutical compounds, including other cytotoxic drugs (e.g., mitoxantrone or cyclophosphamide) or Bcl-2 family inhibitors, can be used in a similar manner to selectively eliminate encephalitogenic T cells. Additionally, studies will need to be conducted in other models of autoimmune disease, particularly where pathology may be reversed through the removal of self-reactive T cells. This study reveals new insight and potential for ablative T cell therapy of autoimmunity.

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Authorship Contributions

J.P.M. designed and performed experiments, interpreted data and wrote the manuscript. J.D.K. designed experiments, interpreted data and wrote the manuscript. D.A.H. and M.B.J. designed experiments and edited the manuscript. E.E.E., C.E.T. and E.G. preformed experiments and edited the manuscript.

Conflict of Interest

None of the authors have conflicts to disclose.

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Figure Legends:

Figure 1. Etoposide treatment decreases the severity of EAE when administered at different times. MOG35-55 induced EAE in B6 mice was treated with either etoposide (50mg/kg) or vehicle control four days apart starting on either day 3, 5 or 9 after the induction of EAE. Clinical scores for etoposide and vehicle control mice are shown.

Figure 2: Prophylactic etoposide treatment of EAE reduces the severity and incidence of disease. MOG_{35-55} induced EAE mice were treated with either etoposide (50mg/kg) or vehicle control 5 and 9 days after induction of EAE. A) Clinical scores for etoposide and vehicle control mice. B) Cumulative incidence for disease presentation in etoposide and vehicle treated mice. Results are shown as cumulative data from 5 independent experiments.

Figure 3: Etoposide treatment decreases damage to neurological tissue. Thirty days after induction of EAE mice were sacrificed and neural tissue was assessed for damage. A) Paraffin embedded spinal cord sections were stained with FluoroMyelin® green and DAPI to assess demyelination and cellular infiltration into the spinal cord. B) Enumeration of DAPI positive events for cell infiltration into specific regions of the spinal cord. Images viewed at 10X objective, yellow bar represents 500 μm. C) Fifteen days after induction of EAE brain and spinal cord homogenates were assessed for differential leukocyte populations by flow cytometry. D) Representative flow plot of CNS cells stained with the MOG_{35-55} $I\text{-}A^b$ MHC class 2 tetramer, gated on $CD4^+CD16/32^-$ population of CNS infiltrating leukocytes. E) with cumulative data at day 15. F) Splenocytes stained with the MOG_{35-55} $I\text{-}A^b$ MHC class 2 tetramer at days 10, 15 and 30 post-disease induction. Data are cumulative of 3 independent experiments, n=8 in each

group. * denotes $p<0.05$; ** denotes $p<0.01$; *** denotes $p<0.001$, Two-way ANOVA with Bonferroni posttests.

Figure 4: Etoposide treatment decreases the number of MOG_{35-55} reactive $CD4^+$ T cells. EAE was induced in B6 mice and treated with etoposide or vehicle 5 and 9 days later. On days 10, 15 and 30 total splenocytes were assayed for MOG_{35-55} reactivity by *ex vivo* peptide restimulation. A) Representative flow plot of IL-17a production by $CD4^+$ T cells restimulated *ex vivo* with MOG_{35-55} peptide. Cumulative data for B) IL-17a production, IFN-γ production and TNF-α production from 3 – 5 independent experiments, n=10 per group. * denotes $p<0.05$; ** denotes $p<0.01$; Two-tailed t-test.

Figure 5: Etoposide treatment does not induce population specific cytopenias. MOG35-55 induced EAE in B6 mice was treated with either etoposide or vehicle on day 5 and 9 after the induction of EAE. Splenocytes were harvested on day 10 and leukocytes populations were assessed by flow cytometry. Representative flow plots (above) and total splenic numbers (below) and shown. N=8, *$p<0.05$

Figure 6: Etoposide treatment of EAE reduces disease severity and incidence of relapse. A) EAE induced in B6 mice was treated with etoposide or vehicle after the presentation of clinical symptoms at day 12 and 16. Clinical scores are depicted for etoposide and vehicle mice. Cumulative data of 2 independent experiments, n=10 in each group, Two-way ANOVA. EAE was induced in SJL mice. After remittance of the primary course of disease mice were randomized and treated with etoposide or vehicle at day 17 and 21. B) Clinical scores are

depicted for etoposide and vehicle treated mice along with C) the cumulative rate of relapse, n=15 per group, depicted for etoposide and vehicle treated mice, Kaplan-Meier with Gehan-Breslow-Wilcoxon test. Fifteen mice per group, 4 (26.7%) mice from vehicle did not relapse and 8 (53.3%) mice from etoposide treated mice did not relapse.

Figure 7: Etoposide treatment prevents epitope spread. A) Mice were pretreated with etoposide or vehicle at day 5 and 9 after the induction of EAE in B6 mice. Splenocytes were harvested at day 30. Antigen specific proliferation was determined by *in vitro* incorporation of ^{3}H-thymadine. B) EAE was induced in SJL mice. After remittance of the primary disease course mice were treated with etoposide or vehicle at day 17 and 21. Splenocytes were harvested on day 35. Antigen specific proliferation was determined by *in vitro* incorporation of ^{3}H-thymadine. Cumulative of 3 independent experiments, n=15 per group (MOG) and n=9 or 15 per group (PLP). * denotes $p<0.05$; ** denotes $p<0.01$; two-way t-test. Dashed line denotes minimum level of significance for SI ($\geq$ 95% CI) mean background ^{3}H incorporation level of 1011 cpm, with a mean conA stimulated foreground of 7995 cpm.

Figure 8: Generation of a naïve immune response after treatment with etoposide. B6 mice were pretreated with etoposide (50mg/kg) 14 and 10 days prior to infection with LCMV Armstrong. Ten days post infection spleens were harvested to assess T cell responses. Total number of LCMV specific T cells was assessed by tetramer staining, A) GP_{33-41} specific $CD8^{+}$ T cells, gated on a $CD8^{+}CD16/32^{-}$ population, B) and GP_{61-80} specific $CD4^{+}$ T cells, gated on a $CD4^{+}CD16/32^{-}$ population. Total splenocytes were re-stimulated *ex vivo* with viral peptide C) GP_{33-41} or D) GP_{61-80} to assess cytokine production, 3 independent experiments, n=8 per group.

No statistical difference between vehicle and etoposide treated groups was observed, two-tailed t-test, and both groups were highly significant compared to uninfected controls, two-way ANOVA, $p<0.001$.

Figure 9: Etoposide treatment does not inhibit humoral immune responses. Mice were pretreated with either etoposide or vehicle control four days apart, then immunized with TNP-OVA in alum six days later. Blood was drawn for serum starting at the day of immunization (Day 0). Serum titers of IgG (a) and IgM (b) were determined by ELISA. n=8

Figure 10: Treatment of EAE with etoposide is effective with a memory anti-viral response. Six week old B6 mice were infected with LCMV Armstrong. At day 35 EAE was induced and treated with etoposide or vehicle control 5 and 9 days later. The clinical scoring of etoposide and vehicle treated mice is depicted. Cumulative of 2 independent experiments, n=15 per group

Figure 11: Treatment of EAE with etoposide does not inhibit memory responses to viral re-challenge. A) Six week old B6 mice were infected with LCMV Armstrong. At day 35 EAE was induced and treated with etoposide or vehicle control 5 and 9 days later. Mice were sacrificed 5 days after rechallenged with LCMV Clone 13 at day 56. Splenocytes were analyzed for total number of LCMV specific T cells using tetramers. B) GP_{33-41}-specific $CD8^+$ T cells and C) GP_{61-80}-specific $CD4^+$ T cells. Total splenocytes were restimulated *ex vivo* with LCMV peptide D) GP_{33-41} or E) GP_{61-80} to assess IFN-γ secretion. F) Total RNA was extracted and reversed transcribed from liver biopsies. qPCR for LCMV viral load was assessed from copy numbers of LCMV nuclear protein normalized to S14. (n=6-10 per group). Dashed line indicates

minimum level of detection. No statistical difference between vehicle and etoposide treated groups was observed, two-tailed t-test, and all four groups were highly significant compared to age-matched, naïve controls infected with Clone 13, two-way ANOVA, $p<0.001$.

Figure 12. Etoposide selectively mediates apoptosis of encephalitogenic T cells. $CD4^+$ T cells were purified by negative selection from the spleen and inguinal lymph nodes of mice 12 days post-EAE induction. MOG-effector $CD4^+$ T cells are defined as $CD44^+$ MOG_{35-55} $tetramer^+$, GP61-memory $CD4^+$ T cells are defined as $CD44^+$ GP_{61-80} $tetramer^+$ and naïve $CD4^+$ T cells are defined as $CD44^-$ $CD62L^+$, all three populations are defined in a $CD4^+CD16/32^-$ gate. A) CD4+ T cells were cultured for 16 hours with varying concentrations of etoposide. Cells were co-stained to differentiate cellular populations and death was assessed by positivity for flow cytometry viability dye. Cell death was normalized to vehicle control B) Representative histogram of Bcl-2 staining for MOG-effector, GP_{61}-memory and naïve $CD4^+$ T cells C) with cumulative data. D) Representative histogram of Bim staining for MOG-effector, GP_{61}-memory and naïve $CD4^+$ T cells E) with cumulative data. F) $CD4^+$ T cells were purified from day 12 mice with EAE, cultured in vitro with etoposide (10uM) and stained for XIAP expression. XIAP MFI is depicted between naïve and encephalitogenic $CD4^+$ T cells with and without etoposide treatment. G) Purified $CD4^+$ T cells from wild type and *Xiap*$^{-/-}$ B6 mice were activated with anti-CD3 and anti-CD28 in vitro. Following activation $CD4^+$ T cells were treated with etoposide and assessed for cell death by a flow cytometry viability dye. H) $CD4^+$ T cells were purified from day 12 mice with EAE and cultured *in vitro* with etoposide (10uM), Q-VD-OPH (20mg/mL) or necrostatin (30uM) for 16 hours. Cells were co-stained to differentiate cellular populations and death was assessed by positivity for flow cytometry viability dye. Cumulative of 2 independent experiments, n=6 per group. * denotes $p<0.05$; ** denotes $p<0.01$; two-way t-test.

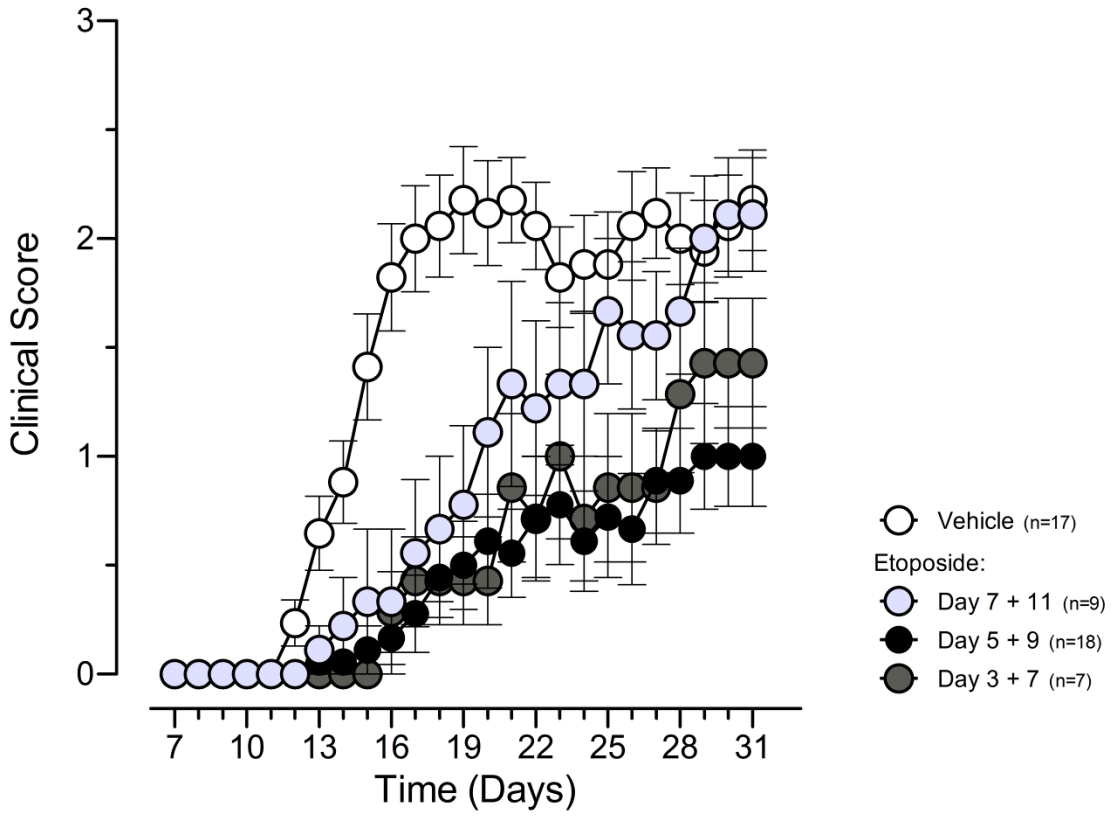
Clinical Score
0
1
2
3
7
10
13
16
19
22
25
28
31
Time (Days)
Vehicle (n=17)
Etoposide:
Day 7 + 11 (n=9)
Day 5 + 9 (n=18)
Day 3 + 7 (n=7)

Figure 1

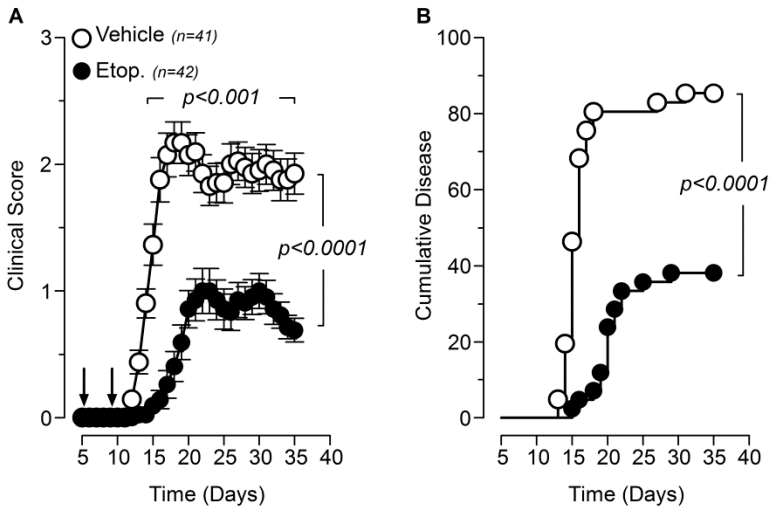
A
Vehicle (n=41)
Etop. (n=42)
p<0.001
p<0.0001
Clinical Score
Time (Days)
B
Cumulative Disease
p<0.0001
Time (Days)

Figure 2

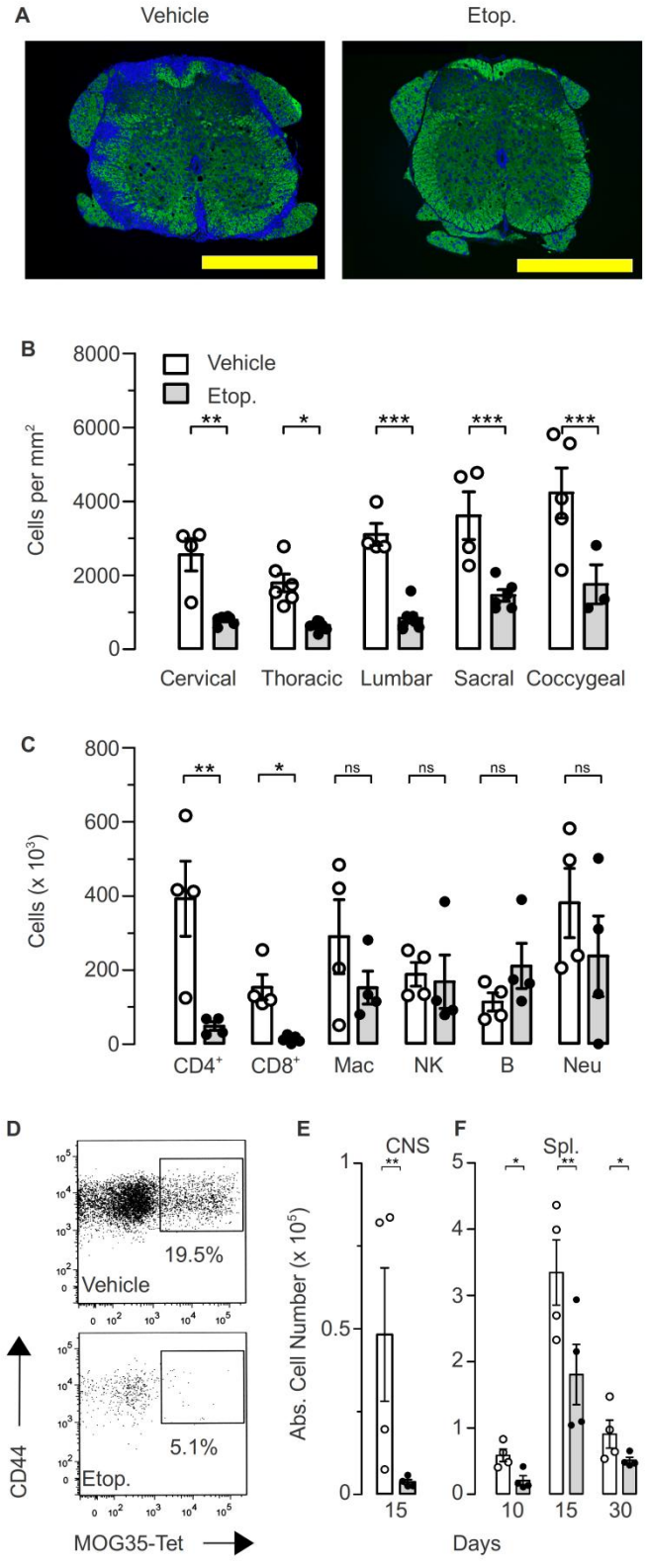
A
Vehicle
Etop.
B
Vehicle
Etop.
Cells per mm²
8000
6000
4000
2000
0
**
*
***
***
***
Cervical
Thoracic
Lumbar
Sacral
Coccygeal
C
Cells (x 10³)
800
600
400
200
0
**
*
ns
ns
ns
ns
CD4⁺
CD8⁺
Mac
NK
B
Neu
D
19.5%
Vehicle
5.1%
Etop.
CD44
MOG35-Tet
E
CNS
Abs. Cell Number (x 10⁵)
1
0.5
0
**
15
F
Spl.
5
4
3
2
1
0
*
**
*
10
15
30
Days

Figure 3

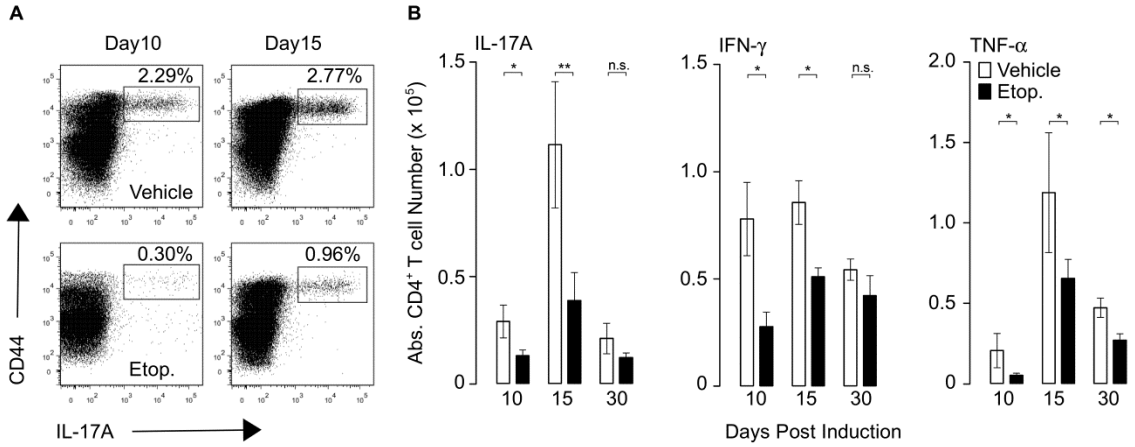
A
Day10
Day15
2.29%
2.77%
Vehicle
0.30%
0.96%
Etop.
CD44
IL-17A
B
IL-17A
IFN-γ
TNF-α
Vehicle
Etop.
Abs. CD4+ T cell Number (x 10^5)
n.s.
10
15
30
Days Post Induction

Figure 4

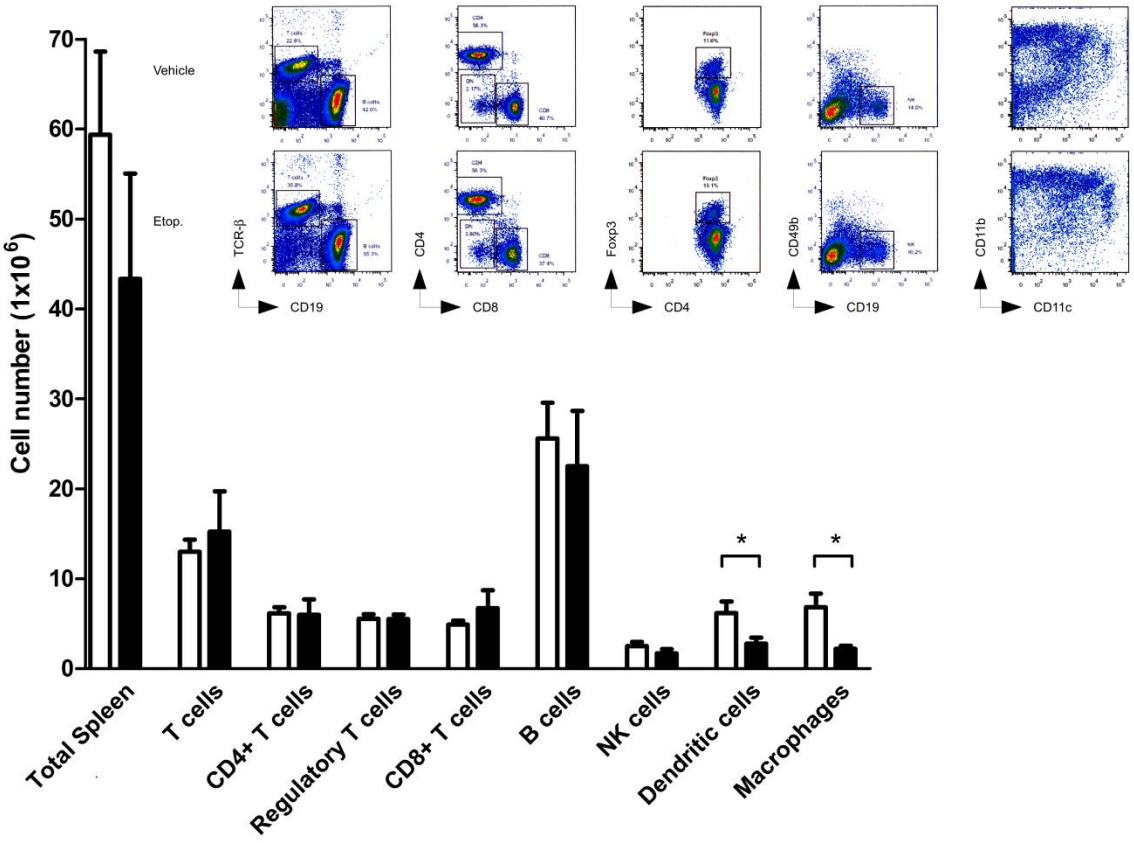
Vehicle
Etop.
Cell number (1x10^6)
70
60
50
40
30
20
10
0
TCR-β
CD19
CD4
CD8
Foxp3
CD4
CD49b
CD19
CD11b
CD11c
*
*
Total Spleen
T cells
CD4+ T cells
Regulatory T cells
CD8+ T cells
B cells
NK cells
Dendritic cells
Macrophages

Figure 5

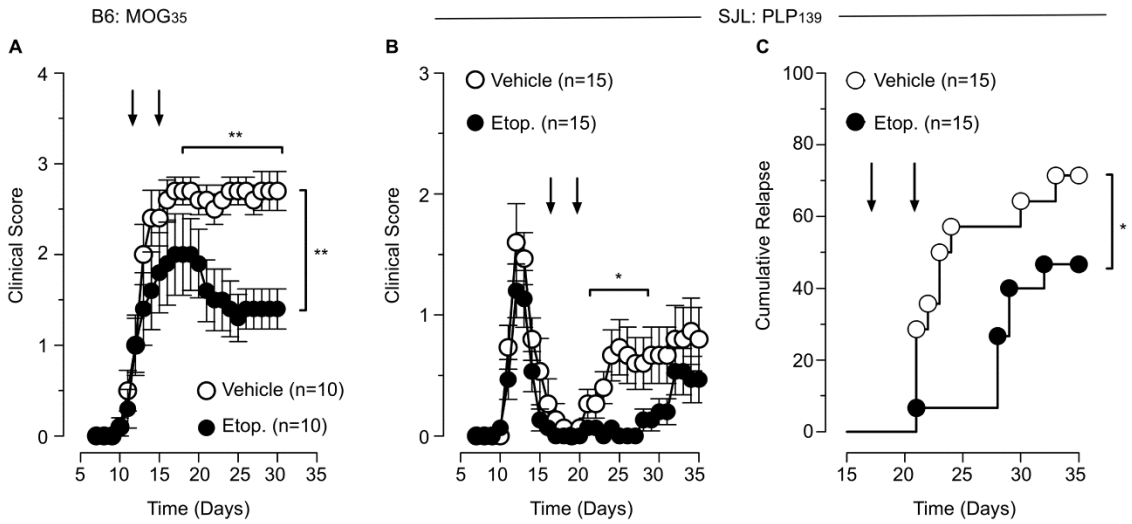
B6: MOG35
SJL: PLP139
A
B
C
Clinical Score
Time (Days)
Vehicle (n=10)
Etop. (n=10)
Vehicle (n=15)
Etop. (n=15)
Cumulative Relapse
**
*

Figure 6

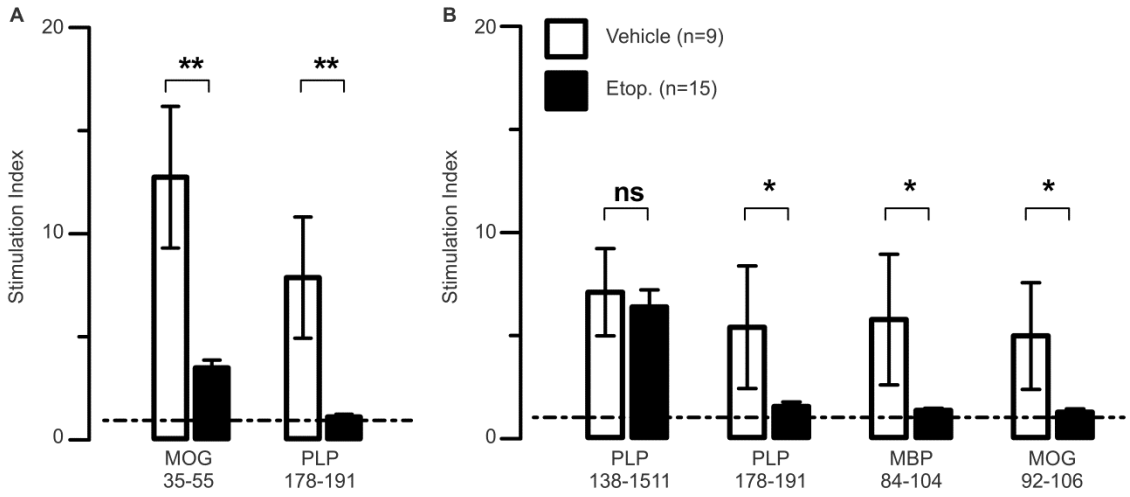
A
20
10
0
Stimulation Index
**
**
MOG
35-55
PLP
178-191
B
Vehicle (n=9)
Etop. (n=15)
ns
*
*
*
PLP
138-1511
PLP
178-191
MBP
84-104
MOG
92-106

Figure 7

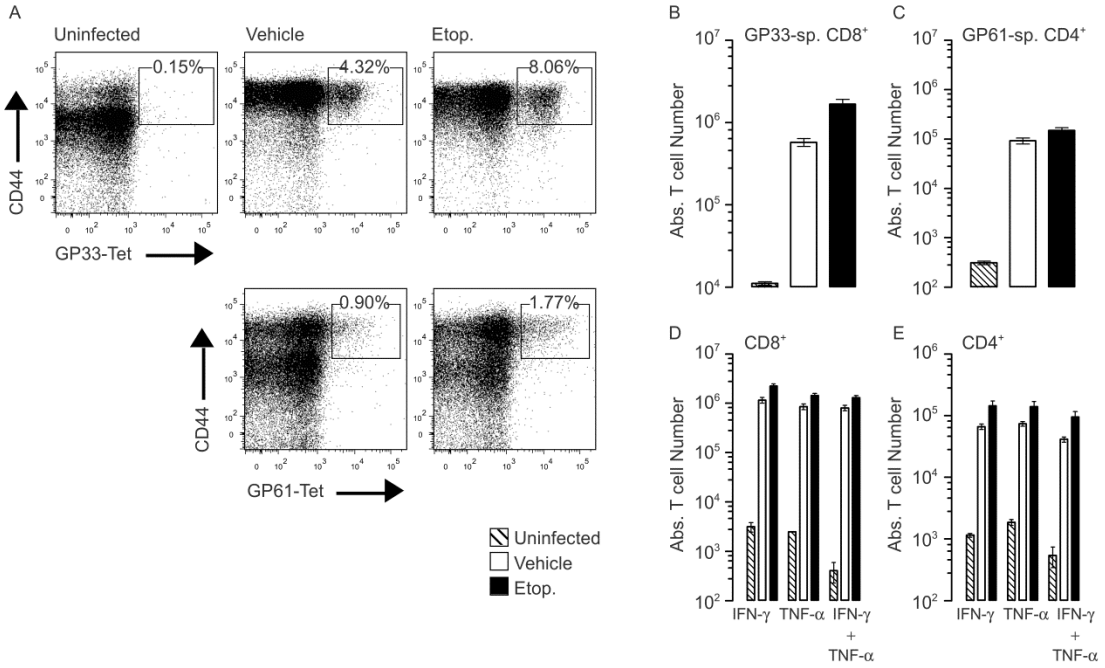
A
Uninfected
Vehicle
Etop.
0.15%
4.32%
8.06%
CD44
GP33-Tet
0.90%
1.77%
CD44
GP61-Tet
Uninfected
Vehicle
Etop.
B
GP33-sp. CD8+
Abs. T cell Number
C
GP61-sp. CD4+
Abs. T cell Number
D
CD8+
Abs. T cell Number
IFN-γ
TNF-α
IFN-γ + TNF-α
E
CD4+
Abs. T cell Number
IFN-γ
TNF-α
IFN-γ + TNF-α

Figure 8

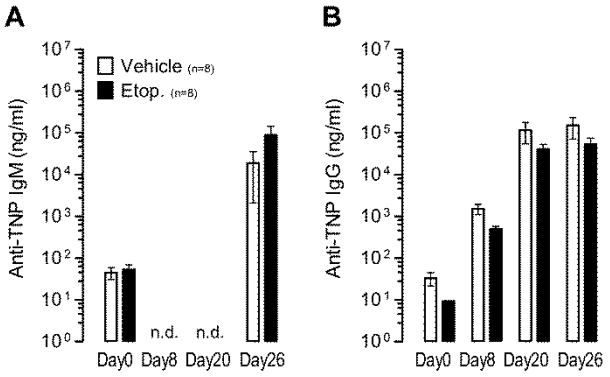
A
B
Vehicle (n=8)
Etop. (n=8)
Anti-TNP IgM (ng/ml)
Anti-TNP IgG (ng/ml)
n.d.
n.d.
Day0 Day8 Day20 Day26
Day0 Day8 Day20 Day26

Figure 9

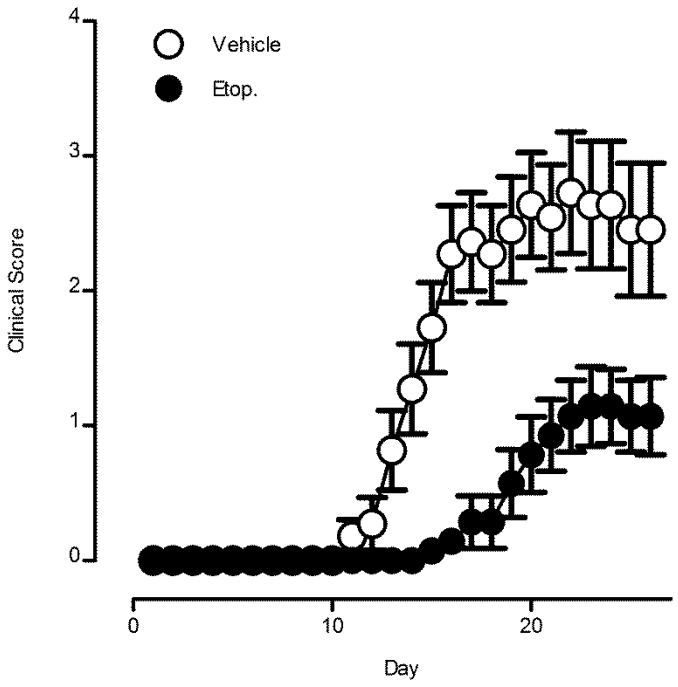

Figure 10

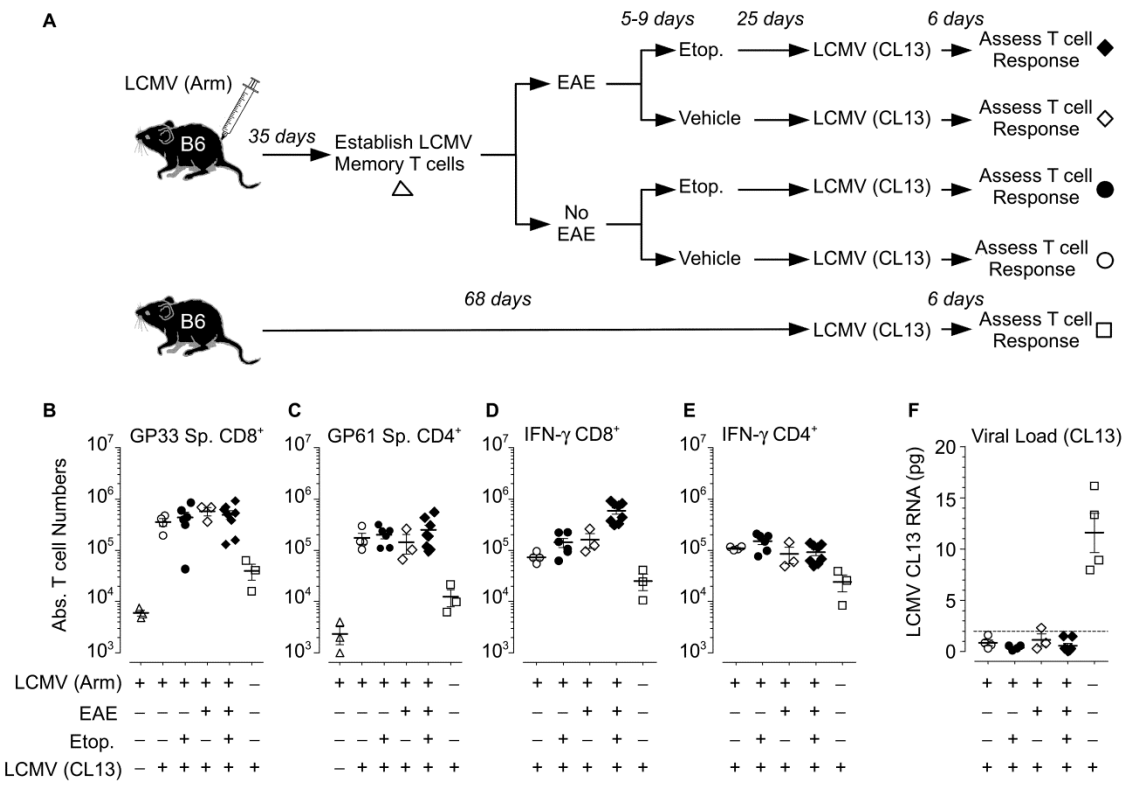
A
5-9 days
25 days
6 days
LCMV (Arm)
B6
35 days
Establish LCMV Memory T cells
EAE
No EAE
Etop.
Vehicle
LCMV (CL13)
Assess T cell Response
68 days
B
GP33 Sp. CD8+
C
GP61 Sp. CD4+
D
IFN-γ CD8+
E
IFN-γ CD4+
F
Viral Load (CL13)
Abs. T cell Numbers
LCMV CL13 RNA (pg)
LCMV (Arm)
EAE
Etop.
LCMV (CL13)

Figure 11

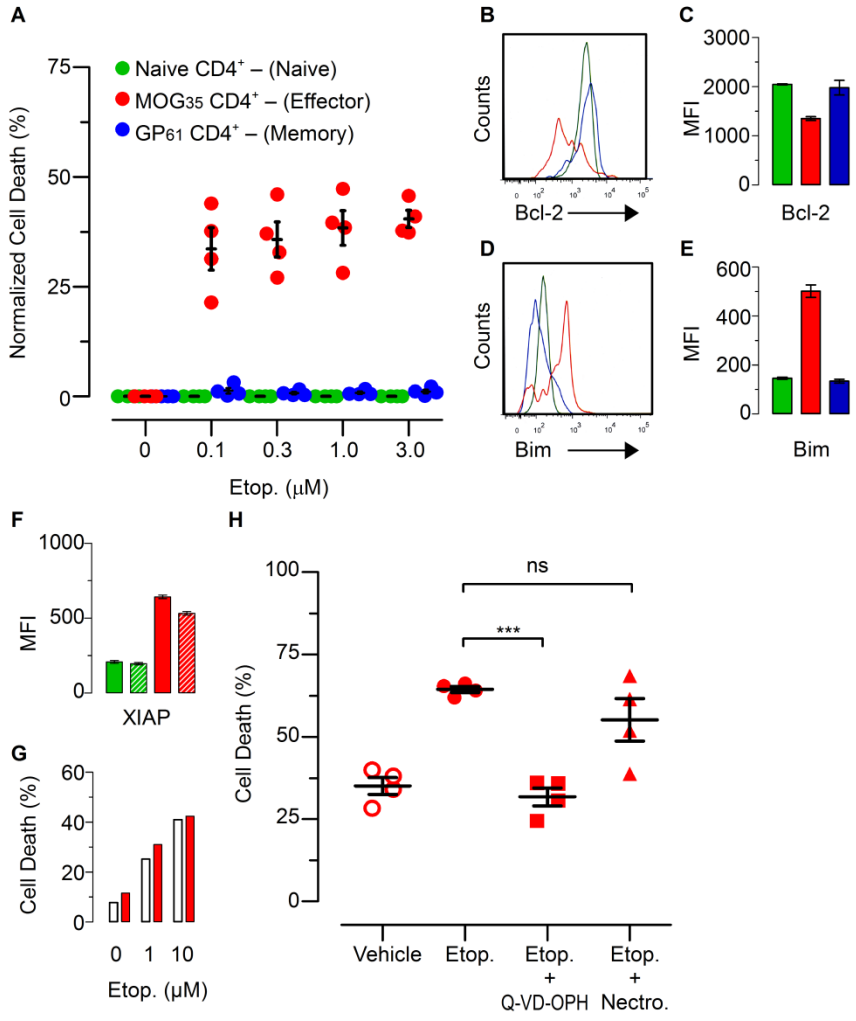
A
Naive CD4+ – (Naive)
MOG35 CD4+ – (Effector)
GP61 CD4+ – (Memory)
Normalized Cell Death (%)
75
50
25
0
0
0.1
0.3
1.0
3.0
Etop. (μM)
B
Counts
Bcl-2
C
MFI
3000
2000
1000
0
Bcl-2
D
Counts
Bim
E
MFI
600
400
200
0
Bim
F
MFI
1000
500
0
XIAP
G
Cell Death (%)
60
40
20
0
0
1
10
Etop. (μM)
H
Cell Death (%)
100
75
50
25
0
ns
***
Vehicle
Etop.
Etop. + Q-VD-OPH
Etop. + Nectro.

Figure 12

Chapter 3

Manipulating DNA damage response pathways for the treatment of immune diseases

Manipulating DNA damage response pathways for the treatment of immune diseases

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Abstract

The race between pathogens and the immune response drives rapid expansion of lymphocyte populations, suggesting genomic stress in responding lymphocytes. We have previously reported the unique apoptotic sensitivity of activated T cells to DNA damaging drugs in diverse therapeutic contexts. We now report that activated T cells display a pronounced DNA damage response (DDR) *in vitro* and *in vivo*. We hypothesized that this intrinsic DDR could be manipulated with targeted small molecules for the elimination of pathologic T cells in a therapeutic context. We found that potentiation of p53 (via inhibition of MDM2) or abrogation of the G2/M cell-cycle checkpoint (via inhibition of CHK1/2 or WEE1) synergized with DNA damaging drugs for the elimination of acutely activated, pathologic T cells. Moreover, a combination of p53 potentiation and checkpoint abrogation displayed therapeutic benefits in preclinical disease models (Hemophagocytic Lymphohistiocytosis, and immune regulatory disorder; and Experimental Autoimmune Encephalitis, a model of Multiple Sclerosis), without the concurrent use of genotoxic chemotherapeutics or significant off target toxicities. Thus, targeted manipulation of p53 and cell cycle checkpoints may represent a new therapeutic modality with significant translational potential for diverse immune mediated diseases.

Introduction

The adaptive arm of the immune system has evolved under pressure from invasive pathogens that proliferate at rates higher than that of eukaryotic cells. Due to this pressure on lymphocytes they have evolved to divide at a very rapid rate, upon activation, in an attempt to catch a pathogenic invader. However, activation of the immune system requires tight regulation to minimize immune mediated damage to the host. In T cells inappropriate immune activation can become pathogenic due to genetic deficiencies, as seen in Hemophagocytic Lymphohistiocytosis (HLH), or from self-antigens in the case of autoimmune disease including, Multiple Sclerosis. The current clinical approach to manage inappropriate immune activation is to inhibit the progression of disease through the use of broad spectrum immune suppression, rather than specifically targeting the pathogenic T cells that induce disease. This non-specific approach uses the blanket application of cytotoxic drugs, steroids or biologic drugs to disrupt or remove entire immune populations. The resulting non-specific immune suppression can lead to opportunistic infections, the reactivation of latent infections and the outgrowth of malignancies that are normally kept in check by a functional immune response. While immunosuppressive treatment strategies are able to manage disease symptoms, the cost of their side effects can be very high.

We have previously reported on the efficacy of etoposide treatment of HLH-like disease and EAE in mice (1, 2). In both disease contexts, treatment led to a loss of activated T cells that drive pathogenesis. Most notably the treatments were selective towards activated T cells, and led to no functional loss of naïve or memory immune responses. Due to the increased specificity of etoposide treatment, many of the detrimental side effects of broad spectrum immune suppression are mitigated. However, since etoposide functions by inducing DNA damage, there is still potential for inducing malignancies and other off-target side effects (3, 4). Therefore, creating a

treatment that maintains the selectivity of etoposide, while not causing DNA damage is an imperative.

Conventional chemotherapeutics are designed to cause DNA damage and activate the DNA damage response (DDR) pathway. This ultimately leads to p53 activation and results in either cellular arrest of the damaged cell for repair or apoptosis (5, 6). However, recent studies have investigated a new generation of non-DNA damaging agents for the treatment of cancer that act at various points in the DDR cascade (7), including drugs that disrupt cellular arrest by inhibiting checkpoints that control cell cycle entry. A majority of the studies utilizing these new drugs use them in combination with DNA damaging agents and have seen a high success rate with multiple compounds entering into clinical trials (8, 9). One of the major reasons that inhibitors of cell cycle have seen success is the rapid rate of proliferation of cancer cells. Since an activated T cell undergoes cell division approximately every 6-8 hours following activation, a rate faster than most tumors, we propose the extension of this non-genotoxic cell cycle checkpoint inhibitor therapy to the treatment of T cell mediated lymphoproliferative disorders.

Here we report a novel treatment for T cell mediated lymphoproliferative disorders, utilizing a MDM2 inhibitor (nutlin-3) and cell cycle checkpoint inhibitors (MK-1775 and AZD7762). We demonstrate that activated T cells have spontaneous, intrinsic DNA damage that activates the DDR pathway. With the use of selective inhibitors in the DDR pathway to disrupt normal cell cycle progression and potential p53 signaling, this combination therapy leads to the clearance of activated T cells without the use of an exogenous DNA damaging agent. Treatment with this non-genotoxic combination inhibitor therapy was effective in the treatment for the mouse models of both HLH and EAE.

Materials and Methods.

Mice: C57BL/6J, *prf*$^{-/-}$ and *p53*$^{-/-}$ mice were purchased from Jackson Laboratories and breed in house . TCR-transgenic P14 mice were a gift from P. Marrack (University of Colorado/Howard Hughes Medical Institute, Denver, CO). All mice were housed under specific pathogen-free conditions in an AAALAC-approved barrier facility at CCRF. All experiments were performed with prior IACUC approval and every attempt was made to reduce the numbers of animals used. Animals were under constant monitoring and care of the CCHMC veterinary staff.

LCMV infections and treatments: LCMV-WE viral stocks were generated and tittered as described. Mice were infected via i.p. injection with 200 PFU. The Armstrong-3 strain of LCMV, a gift from R. Ahmed (Emory University, Atlanta, GA), was grown in BHK-21 cells; the number of plaque-forming units (pfu) was assayed on Vero cells as previously described (36). Mice were infected i.p. with 2 x 10^5 PFU lymphocytic choriomeningitis virus (LCMV Armstong) for primary infection and post etoposide treatment. Mice were re-challenged i.v. with 2 x 10^6 PFU Clone 13 LCMV to assess memory responses. All chemotherapeutics were obtained from the Cincinnati Children's Hospital Medical Center clinical pharmacy: etoposide (50mg/kg or 10mg/kg), methotrexate (70mg/kg), cyclophosphamide (100mg/kg or 200mg/kg). Nutlin-3 (50mg/kg) and MK-1775 (40mg/kg or 60mg/kg) (Cayman Chemicals, Indianapolis IA) additionally AZD6672 (25mg/kg, Selleck chemical)

MHC tetramer staining and flow cytometry: Spleens from individual mice were harvested and crushed through a 70μm cell strainer (BD Biosciences, San Jose, CA) to generate a single cell suspension. A total of 2 x 10^6 cells were stained with different combinations of the following

cell surface antibodies anti-CD4, anti-CD8, anti-CD44, anti-CD62L, anti-CD16/32, anti-CD25, anti-F4/80, anti-FoxP3, (Biolegend. San Diego, CA, eBiosciences. San Diego, CA, Miltenyi Biotec. Auburn, CA or Rockland Immunochemicals. Gilbertsville, PA). The MOG_{35-55} I-A^b and LCMV GP_{61-80} I-A^b tetramers were provided by the NIH tetramer core. The LMCV GP_{33-41} K^b tetermer was made in house as previously described (37). Phospho staining for γH2Ax, phos-p53, phos-ATM and phos Chk2

Ex vivo cytokine production was assessed by restimulation with 5ug peptide, MOG_{35-55}, GP_{33-41}, or GP_{61-80} (Synthetic Biomolecules. San Diego, CA) in the presence of golgi plug (BD Biosciences. San Jose, CA). Cells were permiablized with Cytofix/cytoperm kit (BD Biosciences. San Jose, CA) and stained with anti-IFN-γ or anti-IL17a (Biolegend. San Diego, CA. Where noted, $CD4^+$ T cells were isolated from spleen and inguinal lymph nodes and purified by negative selection using CD4+ T cells isolation kit II (Miltenyi Biotec, Auburn, CA) prior to staining. Flow cytometry data was acquired on FACScaliber or a LSRFortessa (BD Biosciences. San Diego, CA) and was analyzed using FlowJo software version 7.6.5 (Tree Star Inc. Ashland, OR).

Comet assay: Naïve and in *vitro* activated P14 $CD8^+$ T cells were assessed for DNA damage per manufacture's recommendations for neutral Comet Assay (Trevigen, Gaithersburg, MD). In short $1x10^5$ T cells were mixed with 37^0 agarose plated on slides. Cells are lysed overnight and rinsed prior to electrophoresis. Slides are dried at 370 then stained with SYBR gold and viewed on a Leica ST-2 fluorescent microscope

In vitro cell death assay: Splenocytes were harvested from either C57BL/6J or P14 TCR transgenic mice and activated in vitro with the cognate peptide (LCMV GP_{33-41}, 5ug/ml) and cultured in complete DMEM media for two days. Activated cells were then expanded in vitro with IL-2 (50 ng/mL) (PeproTech. Rocky Hill, NJ) for three days. Cells were cultured for 16 hrs. with indicated treatments and cell death way evaluated by MFGD89 binding (38) and viability dye efluor 506 (eBiosciences. San Diego, CA)

HLH induction: HLH was induced in $prf^{-/-}$ mice with LCMV-WE infection as described (ref). Mice were examined longitudinally, typically three times/week, for development of HLH-like disease symptoms. Moribund was defined as >20% loss of starting weight.

EAE induction and treatment: 10 week old female C57BL/6 mice were immunized s.c. with 100μg MOG_{35-55} or $PLP_{139-152}$ emulsified in 5mg/mL CFA (Hooke Laboratories. Lawrence, MA). On Days 0 and 2 animals received i.p. injections of 250 ng pertussis toxin (Hooke Laboratories. Lawrence, MA). Disease severity was assessed every day beginning, on day 10 and assigned a value using the following scale: 1, tail flaccidity; 2, hind limb weakness; 3, hind limb paralysis; 4, hind and fore limb paralysis; 5, moribund. Immunization and pertussis toxin injections were formulated to maximize the disease severity to be ≤ 3 per the manufacturer's specifications (Hooke Laboratories, Lawrence, MA.) and recommendations of our IACUC. Etoposide was administered i.p. at 50mg/kg twice, four days apart (Sigma-Aldrich. St. Louis, MO) (39)

Tissue processing for toxicity: WT C57BL/6J were treated as described 24 hrs apart. Tissue was harvested 24 hrs later. Bone marrow was harvested from crushed femurs. Small infesting was harvested and run on Ussing chamber for permeability assay as previously described (40)

Statistics: Where appropriate, results are given as the mean ± standard error of the mean (SEM) with statistical significance determined by two-tail t-test, using either paired or unpaired (assuming equal variance) or one way ANOVA with Dunnet post-hoc test, according to the data characteristics. Survival curves were assessed by Wilcoxon-Gehen test for differences among groups. Significance was defined as $p<0.5$. Statistical analysis was performed using the GraphPad Prism 5.04 software (GraphPad Software Inc. La Jolla CA)

Study approval: All animal studies were approved by the Cincinnati Children's Research Foundation institutional animal care and use committee.

Results

Selective destruction of activated effector T cells with DNA damaging agents

We have previously reported on the efficacy of the topoisomerase inhibitor etoposide for the selective elimination of pathogenic, activated T cells in both HLH and EAE (1, 2). While etoposide is often used clinically to treat HLH (10), there is only one other report of it being used to treat autoimmunity (11). There are however, other DNA damaging agents that are used clinically to treat autoimmunity. To further our study on the selective clearance of activated T cells we investigated other classes of DNA damaging agents to determine whether they too are able to functionally clear activated T cells. In addition to etoposide this study included the DNA alkylating agent cyclophosphamide and the inhibitor of folic acid synthesis, methotrexate. To evaluate the ability of these DNA damaging agents to effectively eliminate activated T cells wild type (WT) mice were infected with LCMV to produce activated antiviral T cells in response to the viral infection. Mice were treated with the indicated DNA damaging agent at 5 days post infection and sacrificed eight days post infection to examine the remaining T cell populations. Total splenic activated LCMV-specific $CD8^+$ T cells were enumerated by the MHC class 1 D^b tetramer bound to the LCMV GP_{33-41} peptide (figure 1a). All three classes of DNA damaging agents were able to significantly decrease the total number of LCMV specific $CD8^+$ T cells, to various degrees. The greatest loss of activated T cells was seen by the agents that directly damaged the DNA themselves, etoposide and cyclophosphamide. Our previous reports of etoposide demonstrated its specificity towards activated T cells while sparing other protective immune populations. To determine if these DNA damaging agents had specificity for activated T cells total splenic naive $CD8^+$ T cells were enumerated. Consistent with our reports, etoposide treatment did not reduce the naive T cell population after treatment (figure 1b). Similarly,

treatment with methotrexate had no measureable effect on naive T cell numbers. In contrast treatment with cyclophosphamide decreased the naive $CD8^+$ T cell population by over 70%, from a carrier-treated mean of $3.03x10^6$ to $0.88x10^6$ naive $CD8^+$ T cells, demonstrating that not all DNA damaging agents are equally effective nor equally selective for the clearance of activated T cells.

Activated T cells display spontaneous DNA damage response

Etoposide has been reported to induce double strand DNA breaks which activate the DNA damage response (DDR) pathway (12). To confirm that etoposide treatment of activated T cells was inducing double strand DNA breaks a single cell gel electrophoresis COMET assay was performed on activated T cells treated with etoposide. Splenocytes from P14 TCR transgenic mice were activated with the cognate LCMV $_{GP33-41}$ peptide *in vitro*. As expected etoposide-treated activated T cells displayed large tails which included an average of 26.4% of cleaved DNA in the tail (figure 2a). However, in an unexpected finding carrier-treated activated T cells also displayed significant DNA damage as compared to non-activated naive T cells. To follow up on this initial observation we investigated other makers for DNA damage. Typically cells mark DNA damage by phosphorylation of histone 2A (γH2AX)(13, 14). Multiple sites of DNA damage were observed by confocal microscopy, as indicated by ϒH2AX staining in activated T cells as compared to naive T cells (figure 2b). To further investigate our finding of endogenous DNA damage in activated T cells the activation of key points in the DDR pathway was examined using phos-flow staining to determine markers of protein activation by site-specific phosphorylation. Activated T cells exhibited significant activation of the multiple members of

the DDR pathway (figure 2c). This includes activation of ATM, ATR, CHK1/2, and p53 as (indicated by protein phosphorylation). DNA damaging agents are well documented to activate the DDR pathway and activate p53. However, here we have demonstrated that activated T cells display endogenous DNA damage, which implies that DNA damaging agents are not necessary to activate p53.

Nutlin potentiates p53 signaling and function

p53 has been shown to be the master regulator for cell fate decisions in the event of damage (15). Its initial activation leads to cell cycle arrest and attempts at repairing the cellular damage that prompted its activation (16). However, if p53 signaling continues or there is an overwhelming amount of cellular damage, apoptotic pathways takeover and the cell undergoes apoptosis. Knowing that there is a threshold p53 signal required for apoptosis we postulated that enhancement of p53 signaling would drive cell fates towards apoptosis. To test this we used the MDM2 inhibitor nutlin-3 (17). Nutlin has been shown to enhance the signaling activity of p53 by binding and inhibiting the ubiquitnator MDM2, preventing the degradation of p53. Thus nutlin is able to augment p53 signaling, impelling cells towards apoptosis without causing any DNA damage.

Our initial investigation in purging activated T cells with an $MDM2_i$ is focused on its ability to potentiate p53 signaling. If p53 signaling is amplified through MDM2 inhibition, the magnitude of the DDR response necessary to meet the p53-induced apoptosis threshold should be reduced. This led us to postulate that treatment with an $MDM2_i$ would allow for a decreased dosage of etoposide necessary to effectively delete activated T cells. To test this, we treated

activated T cells with a dose curve of etoposide *in vitro*, with and without MDM2 inhibition (figure 3a). The addition of an $MDM2_i$ shifted the curve to the left demonstrating that its addition resulted in equivalent killing of activated T cells with approximately a log decrease in etoposide concentrations.

To evaluate the functionality of nutlin on p53 signaling activated T cells were stained with phospho(S15)-p53 to define the amount of phosphorylated p53 (figure 3b). $MDM2_i$ treatment by itself had no effect on p53 functionality. As expected etoposide treatment lead to an increase in active p53. However, the addition of nutlin to the etoposide treatment caused a shift in phospho-p53 staining indicating an increase in the amount of functionally active p53 in the T cells after nutlin co-treatment with etoposide.

To evaluate the *in vivo* effects of co-administration of etoposide with an $MDM2_i$ we employed the LCMV infection model to activate T cells. Infected mice were treated with a combination nutlin and of low dose etoposide, 10mg/kg, an 80% reduction in DNA damaging agent. This treatment resulted in a nearly a two log decrease in LMCV-specific activated $CD8^+$ T cells resulting in levels near the limit of detection for the assay (figure 3c). In contrast, treatment with either low dose etoposide or the $MDM2_i$ alone had negligible effects on the total numbers of LCMV specific activated T cells. To test the specificity of the $MDM2_i$ combination for activated T cells the total number of naive T cells was examined (figure 3d). Combination treatment with the $MDM2_i$ and low dose etoposide had minimal, albeit significant decrease in the total number of naive T cells. This data demonstrates that nutlin in combination with etoposide-induced activation of the DDR pathway is able to synergistically increase killing of activated T cells with a significantly decreased dose of DNA damaging agent.

Nutlin is non-genotoxic

Nutlins effects on p53 have been shown to be down stream of DNA damage in the DDR pathway (ref). To confirm that nutlin does not cause any DNA damage, activated T cells were treated with the $MDM2_i$ and /or etoposide for four hours to allow for any DNA damage to occur. The treated cells were stained for ϒH2AX phosphorylation, indicating DNA damage, and 7-AAD, to determine DNA content and cell cycle phase. Carrier-treated activated T cells showed measurable DNA damage in the population of cells in S or G2/M phases (figure 4), further corroborating our findings described in figure 1. As previously reported, treatment with etoposide results in significant DNA damage, regardless of the presence of an $MDM2_i$. However, nutlin treatment did not cause any increase in ϒH2AX staining over the carrier-treated cells, demonstrating that nutlin treatment is indeed non-genotoxic.

Nutlin functions in a p53-dependant manner.

Nutlin has been reported to act on MDM2 allowing for longer term molecular survival of p53. To determine if the functions of an $MDM2_i$ are p53-dependant activated T cells from WT ($p53^{+/+}$), p53 heterozygous ($p53^{+/-}$), and p53 KO ($p53^{-/-}$) mice were cultured with a combination of etoposide and MDM2 inhibition (figure 5). Loss of a single allele of p53 had no effect on the clearance of activated T cells either by etoposide, or on the synergistic effects of MDM2 inhibition. However, the loss of both p53 alleles resulted in minimal clearance of activated T cells and the loss of the $MDM2_i$'s synergistic effects with etoposide. This demonstrates that nutlin functions in a p53-dependant manner.

Nutlin increases the efficacy of etoposide treatment of EAE.

Following the demonstration that nutlin can synergize with etoposide and increase it efficacy for the killing of activated T cells in vivo. We wanted to investigate this synergy in a disease setting. We utilizing the prophylactic EAE model that we have previously used to evaluate the effects of etoposide on activated cells (1). Following the induction of EAE mice were treated with either full strength etoposide (50mg/kg), low dose etoposide (20 mg/kg) plus or minus the addition of nutlin. The combination of low dose etoposide with nutlin decreased the clinical severity of EAE (figure 3e) Though there is an 80 percent reduction in the amount of drug administered with low dose etoposide, when administered in combination with nutlin, it was more effective in the treatment of EAE than full strength etoposide. We next investigated the effects of low dose etoposide in combination with nutlin on the clearance of encephalitogenic T cells (figure 3f). Similar to the effects on clinical scoring low dose etoposide in combination with nutlin significantly reduced the number of MOG tetramer^{+} CD4^{+} T cells, with equal or greater efficacy than full strength etoposide.

Combination therapy with etoposide and cell cycle checkpoint inhibitors selectively kills activated T cells.

Ultimately p53 activation leads to either cell cycle arrest to allow for the repair of damage or apoptosis. The first transcript that is upregulated by p53 activation is the cell cycle inhibitor $p21^{cip}$ (18). $p21^{cip}$ is responsible for inducing cell cycle arrest by binding and inhibiting CDK activation leading to arrest in either G_1 or G_2. Evolutionarily this is a mechanism to prevent the proliferation of cells with damaged DNA and enable the cell to correct the damage in order to

prevent malignancies. However, if we are able to inhibit cell cycle arrest through pharmaceutical means then the default cell fate becomes apoptosis in cells that have p53 activation.

To test this hypothesis we used two different cell cycle inhibitors, the Wee-1 inhibitor MK-1775 (19) and the pan CHK1/2 inhibitor AZD7762 (20). While both compounds inhibit at different points in the pathway, both drugs ultimately function by preventing the inhibition of CDK activation, thereby allowing for unregulated cell cycle transition (ref). We treated activated T cells with an etoposide dose curve and the cell cycle inhibitors (figure 6a). Similar to the effects that we saw with the $MDM2_i$, co-treatment with either a CHK_i or a $WEE1_i$ with etoposide shifted the dose curve, resulting in an increased efficacy for killing activated T cells, with a decreased dose of etoposide. Importantly, when we evaluated the selectivity of MK-1775 and AZD6672 we observed a preferential killing of activated T cells over naïve T cells. We cultured *in vitro* activated T cells and naive T cells with dose curves of either the CKH_i or $WEE1_i$ (figure 6b). Both inhibitors alone resulted in significant killing of activated T cells. The $WEE1_i$ did not kill naive T cells at any dose on the curve, while only at very high doses of the CHK_i was there some death of naïve T cells.

Treatment with cell check point inhibitors promotes premature cell cycle entry.

Both Chk1 and Wee1 function as inhibitors of cell cycle entry into G_2/M by either directly or indirectly effecting the activation of CDK1 (ref). Therefore chemical inhibition of either kinase should lead to deregulation of G_2/M entry. When *in vitro* activated T cells were cultured with the CHK_i cells accumulated in G_2 (figure 7a). Similarly when activated T cells were cultured with the $WEE1_i$ cells accumulated in M phase over time (figure 7b). Treatment with the cell

cycle inhibitors manipulated the cycling of activated T cells. Since the ultimate target of the CHK_i and $WEE1_i$ is CDK1 we used the CDK inhibitor SU-9516 to verify the mechanism of action figure 8). Treatment of activated T cells with either inhibitor led to cell death, as previously reported (21, 22). However, the addition of the CDK_i significantly decreased the kill efficiency of both cell cycle checkpoint inhibitors. In contrast the CDK1 inhibition had a minimal effect on the efficacy to etoposide mediated killing of activated T cells, which is driven by the activation of p53.

To determine if manipulation of cycling leads to premature G2/M entry activated T cells were cultured with inhibitors and pulsed with EdU to determine cells that were actively synthesizing there genome (figure 6c). Normal cycling cells (carrier treated) had a majority of the EdU^+ cells in the G_2/M phase with fully replicated genome, as defined by 7-AAD staining. In contrast the Edu^+ proliferating cells treated with either inhibitor failed to fully duplicate their genome, presumably due to premature entry into G2/M. To further investigate the effects of disregulated cell cycle control we examined the marker of DNA damage γH2AX. Activated cells treated with either inhibitor displayed significant DNA damage by γH2AX staining. Damage was most evident in cells that had entered into M phase (phospho-$H3^+$) but were not a full 2N. Therefore inhibition of CHK or WEE1 leads to an accumulation of cells that have prematurely entered into mitosis without a fully duplicated genome. This lack of a completely duplicated genome leads to the activation of DDR pathways.

The rate of cell division positively correlates with the efficacy of cell cycle checkpoint inhibitors.

We postulate that the selectivity of cell cycle checkpoint inhibitors for activated T cells is due to their rapid rate of proliferation. To test the role that proliferation rate has on the efficacy of cell cycle checkpoint inhibitors on *in vitro* activated T cells were labeled with CFSE and cultured for 24 hours in IL-2 to measure proliferation. Cells were subdivided into groups by the number of cell division per 24 hours, as measured by CFSE dilution (figure 9). Nominal cell death was seen in cell that did not divide, but as the rate of proliferation increased rate of cell death increased as well. This demonstrates that the efficacy of cell cycle checkpoint inhibitors correlates with the rate of cell division.

Cell cycle checkpoint inhibitors synergize with etoposide to kill activated T cells *in vivo*. Having demonstrated that cell cycle checkpoint inhibitors can efficiently kill activated T cells *in vitro* by disrupting cell cycle entry into G_2/M, we wanted investigate there efficacy in our *in vivo* LCMV model. Both CHKi and WEE1 inhibitors are well documented to synergize with DNA damaging agents such as gentamycin in many tumor models. Following these results we investigated the synergistic effects of the cell cycle checkpoint inhibitor treatment in conjunction with low dose etoposide (10mg/kg). Single drug treatment with either low dose etoposide, CHK_i or $Wee1_i$ had minimal effects on the clearance of LCMV specific activated T cell (figure 6d). However, treatment with cell cycle checkpoint inhibitors in combination with low dose etoposide lead to increased clearance of LCMV specific activated T cells to levels below the limit of detection in the assay. When naive T cell numbers were enumerated combination treatment with

etoposide and cell cycle checkpoint inhibitors did lead to a significant loss of naive T cell numbers (figure 6e). Similar to the results published in tumor models treatment with cell cycle checkpoint inhibitors and the DNA damaging agent etoposide were able to clear activated T cells *in vivo*. While this combination is very effective for the killing of activated T cells, it also leads to the loss of a significant population of naive T cells as well, decreasing the selectivity of this approach.

Combination therapy has minimal off targets effects in vivo

Conventional DNA damaging agents are known for their off target side effects by non-specifically inducing damage in other rapidly dividing tissue compartments, including the bone marrow and gastrointestinal tract. To investigate off target toxicities of combination inhibitor therapy we first looked in the GI tract. Mice were treated with either combination inhibitor therapy, etoposide or cyclophosphamide twice, similar to the schedule for our therapeutic treatment of inflammatory diseases. The following day the small intestine was harvested to assess the effects of treatment on intestinal permeability. Small intestines were placed into an Ussing chamber with a FITC dextran gradient to determine if defects in permeability occurred. Mice given combination inhibitor therapy had no increased FITC dextran cross over, as compared to the healthy carrier treated mice (figure 10a). In contrast mice given DNA damaging agents, either etoposide or cyclophosphamide, showed a marked increase in intestinal permeability, as seen by increased FITC dextran levels. Additionally, trans-epithelial resistance (TER) was measured for the treated intestines. Similar to the permeability results combination inhibitor therapy with $WEE1_i$ and $MDM2_i$ had no loss of TER as compared to carrier treatment

(figure 10b). Treatment with CHK_i and $MDM2_i$ did show a decrease in TER, however, this resistance was still higher than the levels seen with DNA damaging agents.

In addition to the GI track we looked at the effects of combination inhibitor therapy on hematopoietic stem cells (HSC) in the bone marrow. To determine if therapy caused damage to HSC mice were treated, and bone marrow was harvested 5 hours later. HSC were stained for γH2AX to look for damage. No damage was seen with in the $lineage^- sca1^+ckit^+$ (LSK) with any of the treatments (figure 10c). In the rapidly dividing $lineage^-sca1^-ckit^+$ (LK) population combination inhibitor therapy induced no visible damage. In contrast cyclophosphamide, and to a lesser extent etoposide, caused damage to the LK HSCs. We followed this up by investigating the repercussions of treatment induced bone marrow damage. Bone marrow was harvested 48 hours after treatment with combination inhibitor therapy or DNA damaging agents and the total numbers of LK and LKS HSCs were enumerated (figure 10d). In the LK population there was not a loss in total cell numbers after combination inhibitor therapy or etoposide. However, cyclophosphamide treatment resulted in a large loss of LK cells. This is consistent with the damage seen by γH2AX staining. No DNA damage was seen in the LSK population after 5 hours, and this correlates with no significant loss of LSK cell numbers at the later time point.

Regulator T cells have also been reported to be a rapidly dividing T cell population *in vivo*. Additionally, DNA damaging agents including cyclophosphamide are reported to killing Tregs (ref). To test the effects of combination inhibitor therapy on Tregs mice were treated with either combination inhibitor therapy, etoposide or cyclophosphamide twice, similar to the schedule for our therapeutic treatment of inflammatory diseases. Total splenic regulatory T cells ($CD4^+CD25^+FoxP3^+$) numbers were enumerated one day after the second treatment (figure 10e).

Combination inhibitor therapy did not lead to a decrease in Treg numbers, though cyclophosphamide treatment did significantly decrease total Treg numbers.

Combination therapy treats HLH-like disease by eliminating pathogenic T cells

To test the therapeutic effects of the combination inhibitor therapy with a $MDM2_i$ and cell cycle checkpoint inhibitors we utilized the mouse model of HLH-like disease. $prf^{-/-}$ mice were infected with LCMV, which results in a $CD8^+$ T cell-mediated inflammatory disease that mirrors most of the symptoms of patients with HLH (23). Mice were treated at day five and six post-disease induction to purge the pathogenic T cells activated by the viral infection. At day eight post infection splenocytes were harvested to assess the survival of pathogenic LCMV specific $CD8^+$ T cells after combination inhibitor therapy (Figure 11a). Etoposide treatment has become the standard of care for treatment of HLH (10), and is able to effectively decrease the number of pathogenic $CD8^+$ T cells. Combination inhibitor therapy with a $MDM2_i$ and a CHK_i or $WEE1_i$ was equally as effective at purging pathogenic $CD8^+$ T cells in the mouse model of HLH as etoposide. When we investigated the effects of treatment on naive $CD8^+$ T cells combination inhibitor therapy with CHK_i had no effect on naïve T cells, though $WEE1_i$ treatment did decrease the total number of naive T cells (figure 11b), though to a lesser extent that the DNA damaging agents described in figure 1. IFN-γ has been shown to be the major pathogenic mediator produced by activated $CD8^+$ T cells in the mouse model of HLH (24). Therefore we next investigated the effects of combination inhibitor therapy of serum IFN-γ levels. Similar to the results seen with the clearance pathogenic $CD8^+$ T cells, IFN-γ levels were decreased back to WT levels after combination inhibitor therapy (figure 11c). Finally mice were followed

longitudinally for survival (figure 4d). Combination therapy was able to significantly increase survival rates. All carrier treated mice were declared moribund by 25 days post-infection. In contrast, most treated mice survived for the duration of the experiment, presumably due to the clearance of pathogenic $CD8^+$ T cells lost during treatment. While etoposide treatment is the clinical mainstay for the treatment of HLH, combination inhibitor therapy proves to be as equally effective as etoposide.

Combination therapy with nutlin and cell cycle checkpoint inhibitors treats EAE by eliminating encephalitogenic T cells.

We have demonstrated that combination inhibitor therapy is able to effectively treat HLH, a $CD8^+$ T cell-mediated inflammatory disease. To investigate the effects of combination inhibitor therapy in a $CD4^+$ T cell-mediated disease we used EAE, the animal model of the autoimmune disease MS. EAE was induced in WT C57BL/6J mice and treated with the $WEE1_i$ and the $MDM2_i$ on days five and nine post-immunization. Clinical scores were monitored throughout the course of disease (figure 12a). Combination inhibitor therapy significantly increased the time to the onset of symptoms, as well as decreased the overall severity of disease. To understand the effects of combination inhibitor therapy on activated T cells in EAE splenocytes were harvested from mice at day 15 of disease to evaluate the clearance of pathogenic T cells. Encephalitogenic $CD4^+$ T cells were enumerated by MHC tetramer staining with the MOG_{35-55} peptide bound to the $I\text{-}A^b$ MHC. Combination inhibitor therapy significantly decreased the number of activated MOG specific T cells, similar to the results of etoposide treatment (figure 12b). We next evaluated the total number of functionally pathogenic $CD4^+$ T cells after treatment by

determining the number of IL-17 producing $CD4^+$ T cells after MOG_{35-55} peptide restimulation. Combination inhibitor therapy significantly decreased the number of IL-17 producing $CD4^+$ T cells, similar to the levels of etoposide treatment (figure 12c). This data demonstrates that combination inhibitor therapy with MK-1775 and nutlin is able to effectively purge pathogenic activated $CD4^+$ T cells during acute EAE. To investigate the specificity of the combination of WEE1 and MDM2 inhibition the absolute number of splenic naïve T cells was enumerated (figure 12d). Neither combination therapy with the inhibitors or with etoposide had any effect on the total population of naïve T cell, consistent with our previous reports. To further define the specificity of combination therapy we investigated the effects of treatment on the regulatory T cell population. Regulatory T cells have been described as a key immune population that can establish peripheral tolerance and keep autoimmunity in check. We evaluated the effects of treatment of Treg populations during EAE (figure 5e). Treatment with the $WEE1_i$ and the $MDM2_i$ does not decrease Treg numbers as compared to carrier treated mice. Etoposide treatment of EAE did not lead to a decrease in total Treg numbers as compared to naive mice, however, Treg numbers were decreased as compared to carrier treatment.

Combination therapy can effectively treat new onset EAE.

We have demonstrated that combination inhibitor therapy with MK-1775 and nutlin can effectively purge pathogenic $CD4^+$ T cells in EAE with a prophylactic treatment. However, for a therapy to truly be clinically relevant for the treatment of MS it has to be effective after the presentation of symptoms. To test the effectiveness of combination inhibitor therapy on new onset EAE we induced disease and waited to treat until the symptoms of disease developed.

Once all mice developed symptoms, and were scored at a minimum of one, we administered treatment. Mice with new onset EAE that were treated with combination inhibitor therapy developed a less severe pathology with a mean peak disease score of 1.9, vs 2.6 of carrier treated mice (figure 12e). Carrier treated mice maintained a clinical scoring around their peak score. In contrast after a less severe peak pathogenesis, combination inhibitor therapy treated mice regained mobility as the study progressed. Combination inhibitor therapy selectively purged encephalitogenic CD4+ T cells in EAE and has proven to be a viable treatment strategy not only prophylactically, but also as a therapeutic intervention.

Combination therapy with nutlin and cell cycle checkpoint inhibitors treats EAE while sparing memory T cell function

We have demonstrated that combination therapy is able to purge activated T cells *in vitro* and *in vivo* in both the disease models of HLH and EAE. Additionally we have shown that combination therapy has minimal effects on naive T cells populations demonstrating that the treatment has specificity for activated T cells . However, if the true goal of this study is to define a treatment strategy with minimal broad spectrum immune suppression it is critical to assess the effects on protective memory T cell functions. To this end, we utilized the LMCV memory model of EAE that we have previously published (1). C57Bl/6J mice were infected with the LCMV, and were allowed to clear the virus and develop a population of long-term anti-viral memory T cells. Sixty days post-infection EAE was induced in the mice and treated as indicated five and nine days later. Finally the mice were re-challenged with the Clone 13 strain of LCMV to assess the persistence of a functional anti-LCMV memory T cells. A Clone 13 rechallenge allows for

confirmation of a functional memory response, as only mice with an established LCMV specific memory population can rapidly clear a Clone 13 infection. In the absence of a memory T cell response Clone 13 induces long term persistent infection and induced clonal exhaustion of LCMV-specific $CD8^+$ T cells (25, 26).

Using this system, we found that the memory T cell responses to viral re-challenge were unaffected by combination inhibitor therapy (figure 13). Treatment did not alter the pronounced secondary expansion of LCMV GP_{33-41} specific $CD8^+$ T cells and LCMV GP_{61-80} specific $CD4^+$ T cells, when compared to carrier treated mice or naïve B6 mice responding to a *de novo* Clone 13 infection (figure 13a,b). These results clearly show that mice treated for EAE and purged of MOG-specific T cells by treatment with $WEE1_i$ and $MDM2_i$ still possess memory T cells that can undergo comparable secondary antigen-driven expansion to viral re-challenge. Furthermore, the expanded memory T cells demonstrated equivalent IFN-γ production following *ex vivo* peptide re-stimulation to carrier treatment, both of which were enhanced as compared to, naïve *de novo* response (figure 13c,d). Importantly, the expanded LCMV-specific memory T cells from all of the treatment groups showed functionality in the clearance of a LCMV Clone 13 infection (figure 13e), further demonstrating that even upon targeted ablation of encephalitogenic T cells from mice with EAE, a robust functional memory subset persists that is capable of clearing the normally persistent Clone 13 infection. Taken together, these data clearly demonstrate that combination inhibitor therapy does not hamper the functionality of memory T cells capable of inducing immunity to viral re-challenge while ablating of auto-reactive T cells for the treatment of EAE.

Combination inhibitor therapy kills reactivated memory T cells.

We have shown that clearance of activate T cells with either combination inhibitor therapy or etoposide based therapy is highly selective for activated T cells, having minimal effects on naïve, quiescent memory and regulatory T cell populations. While treatment is highly effective during the acute phase of disease, this phase is not always a viable time point for therapy in a clinical setting. A truly viable treatment strategy for most autoimmune diseases, including MS, requires inducing the clearance of memory T cells populations after their initial activation. To test for the clearance of memory T cells by combination inhibitor therapy, mice were infected with LCMV to generate a tractable memory T cell population. After a 6 week period, to insure a quiescent memory population, mice were boosted with a LANAC liposomal peptide vaccine containing LCMV glycoprotein peptides (27). Following this reactivation of the memory T cells the mice were treated with combination inhibitor therapy to purge the reactivated memory T cells (figure 14). Treatment with MDM2i or lower dose etoposide had minimal effects on purging the reactivated memory T cells. However, when the $MDM2_i$ was used in combination with either the checkpoint inhibitors, or lower dose etoposide the reactivated memory T cell populations were decreased to levels near or below the limit of detection. This demonstrates that combination inhibitor therapy has the potential to not only be effecting during the acute phase of disease but also during disease remission.

Combination inhibitor therapy selectively kills activated human T cells. Having demonstrated both the efficacy and the specificity of combination inhibitor therapy in killing activated murine T cells, we next investigated its effects on primary human T cells to determine

the translational applications of the therapy. PBMCs from normal donors were activated *in vitro* to generate a rapidly dividing effector state. Activated and naïve human T cells were stained with phospho-specific antibodies against p53, ATM, CHK1 and CHK2 to define the endogenous activation of the DDR pathway in activated T cells (figure 15a). Similar to what we observed in activated mouse T cells (figure 2), activated human T cells displayed considerable activation of the DDR pathway starting with γH2AX expression and ultimately leading to the phosphorylation, and activation of p53. Having confirmed the endogenous activation of p53 in activated human T cells we wanted to verify the efficacy of combination inhibitor therapy. Activated T cells along with purified naïve T cells from normal human donors were co-cultured with dose curves of etoposide (figure 15b), CHK_i (figure 15c) and WEE1*i* (Figure 15d) +/- $MDM2_i$. Consistent with our observations with mouse cells, all three compounds are able to selectively kill activated human T cells while inducing little death of naïve T cells. Combination inhibitor therapy holds promise. These findings demonstrate that combination inhibitor therapy is equally effective and specific at purging activated human T cells as it is in mice.

Discussion

We have described a novel treatment strategy for lymphoproliferative disorders that is designed to exploit normal life cycles of T cells. This treatment results in improved clinical outcomes in multiple models of lymphoproliferative diseases by specifically targeting activated, pathogenic T cells. Activated T cells undergo rapid proliferation upon activation, as an evolutionary mechanism that is necessitated by the rate of viral and bacterial replication. For the immune system to effectively deal with an infection, a massive expansion of antigen specific lymphocytes is necessary. Inherent in this rapid proliferation DNA replication errors are made. This results in the spontaneous activation of the DDR pathway. While this has been noted previously in cell lines, this is the first time it has been documented in primary T cells (28, 29, 6). Normally when such replication errors arise, the cell would arrest, and repair the damage. However, in the presence of cell cycle inhibitors, such as AZD7762 and MK-1775, arrest and repair are prevented, ultimately resulting in the death of the cell.

The initial study that demonstrated the efficacy of inhibiting cell cycle checkpoints used RNAi to deplete Chk1 (Morgan 2006). The loss of Chk1 function forced cells into premature mitosis following treatment with gemcitabine and decreased tumor survival. Zabludoff et. al. discovered AZD7762 by screening protein libraries for a Chk inhibitor. They discovered AZD7762 as a small molecule that is able bind and inhibit Chk1, but also Chk2 to a lesser degree. The normal function of Chk1 is to phosphorylate CDC25 and induce G2/S arrest (20). The original studies of AZD7762 investigated its effects on killing tumors in culture and in xenograft tumor model in conjunction with DNA damaging agents, predominantly gentamycin (21, 30, 8). The effectiveness of AZD7762 in combination with etoposide is similar to the multiple reports of cell death induced by AZD7762 in combination with a variety of DNA damaging against.

Hirai et. al discovered MK-1775 by screening of protein libraries for an inhibitor of the kinase activity of Wee1 (31). Wee1's function is to inhibit the G2 checkpoint by phosphorylating CDK1 and preventing its interaction with cycle-B. Similar to AZD7762, MK-1775 has been shown to work in conjunction with a variety of DNA damaging agents, including gemcitabine, carboplatin and cisplatin, for the treatment of colorectal, cervical and ovarian cancers in cell culture and xenograft models (31–33). Importantly when used in conjunction with 5-fluorouracil it can decrease the amount of DNA damaging agent needed to induce cell death. The authors reported an IC_{50} of >100uM for 5-FU alone. However, with the addition of 300nM MK-1775 the IC_{50} decreased to 2uM. On its own MK-1775 showed little anti-proliferative activity. In addition to forcing cells through the G2/M checkpoint, MK-1775 has been shown to force cells arrested in S phase to enter mitosis (34).

Non-genotoxic combination therapy does not induce DNA damage as conventional treatments do. In contrast it amplifies endogenous DDR activation and disrupts normal cell cycle control. By allowing premature entry into mitosis, cells with an incomplete genome cannot be repaired and undergoes apoptosis mediated by p53 activation. In contrast double stranded DNA breaks cause by conventional DNA damaging agents can be repaired, although not necessarily in the correct conformation, which has been shown to lead to malignancies (35, 3). In the past cycle cell inhibitors were designed to be used in combination with DNA damaging agents to maximize their effects. While a few studies have removed the DNA damaging agent from the protocol we are the first to utilize the intrinsic DDR pathway activation in activated T cells for the induction of apoptosis with cell cycle inhibitors.

A further benefit to non-genotoxic combination therapy is its selectivity. We have demonstrated that therapy primarily targets activated T cells with little effect on hematopoietic, naïve, memory

or regulatory subsets. This outcome results from the rapid proliferation rate of 6-8 for activated T cells hours (Braciale PLOS one 2010) vs. the proliferation rate of regulatory T cells or hematopoetic stem cells which divide once every 8 days or 2.5 weeks, respectively (abkowitz blood 2000) (Vukmanovic-stejic JCI 2006), both on which are cells that are considered to rapidly divide *in vivo*.

A vital finding from this study is that non-genotoxic combination therapy is able to kill activated human T cells. While further studies will need to be conducted prior to human use there is existing evidence for efficacy. Additionally, further studies are ongoing to investigate other applications of non-genotoxic combination therapy in the treatment of other autoimmune diseases, as well for allo-responses in organ transplantation. Since this mechanism does not depend on the nature of the antigenic stimulation, meaning that the same mechanisms are in play regardless of the nature of T cells activation e.g. viral infection or endogenous auto-antigens. This allows for the treatment of a wide variety of immune mediated disease, and holds the potential to be a more specific, less toxic alternative to broad spectrum immune suppression for the treatment of lymphoproliferative diseases.

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Figure Legends:

Figure 1. Chemotherapeutics acts via selective destruction of activated effector T cells in LCMV-infected prf-/- mice. LCMV-infected *prf*$^{-/-}$ mice were treated with carrier, etoposide, cyclophosphamide 5 d after LCMV infection. Spleens were harvested at 8 d post-infection for quantification of LCMV-specific T cells . (A) Absolute numbers of virus-specific (D^b-GP_{33} tetramer$^+$) $CD8^+$ T cells were quantified in each treatment group. Dotted line represents average background staining in a naïve animals. (B) Absolute numbers of naive ($CD44^{lo}$) CD8+ T cells were quantities in each treatment group.; the dotted line represents average from the carrier treated group. *p <0.05, **p <.001 and ***p <0.001. Results represent 3 independent experiments, N= 6-8

Figure 2. Activated T cells display a spontaneous DDR. (A) Comet Assay of P14 $CD8^+$ T cells which were either naïve or activated in vitro with peptide. Activated cells were cultured with vehicle or etoposide(1μM) for 4 4hrs. (B) Representative micrographs of CD8+ cells stained for γH2AX with DAPI counterstain (C) P14 D8+ T cells were transferred into recipient mice (C57BL6/J) which remained naive or were infected with LCMV the following day to generate activated T cells. Spleens were harvested at 6 d post-infection and stained for DDR markers as indicated. Percentages of cells in the upper, right quadrant are shown. expression analysis comparing. Data are mean +/- SE. Significance was determined by one way ANOVA with Bonferroni Post-Hoc test. *p <0.05, **p <.001 and ***p <0.001. Results represent 3 independent experiments, N=8

Figure 3. Nutlin-3 potentiates p53 signaling and function. (A) Naïve and *in vitro* activated P-14 $CD8^+$ T cells were co-cultured with increasing concentrations of etoposide and/or nutlin-3 for 18 hrs *in vitro*. Apoptosis is defined as percent $phosphatidylserene^+$ as defined by MFGD8 staining. (B) Representative flow plots from *in vitro* activated P-14 transgenic $CD8^+$ T cells cultured with etoposide and/or nutlin-3 for 4 hrs. Cells were stained for Phospho-p53 expression and DNA content (7-AAD). (C) Wild type C57Bl/6 mice were infected with LCMV-WE and treated as described on day 5 post-infection. Splenocytes were harvested on day 8 post-infection and LCMV specific (K^b-$GP_{33}{}^+$ tetramer $CD44^+$) $CD8^+$ T cells and (D) naïve ($CD44^{lo}$) $CD8^+$ T cell were enumerated. (E) EAE clinical scoring for wild type C57Bl/6 and treated as indicated on days 5 and 9. (F) Splenocytes were harvested on day 30 and stained for MOG_{35} specific $CD4^+$ T cells ($CD4^+$, $CD44^+$, I-A^b MOG_{35} $tetramer^+$). Data are mean +/- SE. Significance was determined by one way ANOVA with Bonferroni Post-Hoc test. *p <0.05, **p <.001 and ***p <0.001. Results represent 3 independent experiments, N=6-10

Figure 4. Nutlin does not induce DNA damage. Representative flow plots of *in vitro* activated P-14 transgenic CD8+ T cells cultured with etoposide and/or nutlin-3 for 4 hrs. Cells were stained for DNA content (7-AAD and phospho-ϒH2Ax expression,

Figure 5. Nutlin is p53 dependent. PHA stimulated T cells from WT, $p53^{+/-}$ or $p53^{-/-}$ mice were cultured with etoposide (1uM) or etoposide and nutlin-3 (5uM) for 18 hrs. Apoptosis is defined as percent $phosphatidylserene^+$ as defined by MFGD8 staining. . Data are mean +/- SE.

Significance was determined by one way ANOVA with Bonferroni Post-Hoc test. *p <0.05, **p <.001 and ***p <0.001. Results represent x independent experiments, N=4

Figure 6. Combination treatment with etoposide and checkpoint inhibitors selectively kills activated T cells. (A) Naïve and *in vitro* activated P-14 transgenic $CD8^+$ T cells were co-cultured with increasing concentrations of etoposide and MK-1775, or AZD7762 for 18 hrs *in vitro*. Apoptosis is defined as percent $phosphatidylserene^+$ as defined by MFGD8 staining. (B) Naïve and *in vitro* activated P-14 transgenic CD8+ T cells were cultured with increasing concentrations of MK-1775 or AZD7762 for 18 hrs *in vitro.* (C) Representative flow plots from *in vitro* activated P-14 transgenic CD8+ T cells were cultured with indicated treatment for 4 hrs. Cells were stained for phospho-H2Ax expression, DNA content (7-AAD) and Phos-H3 for mitosis (D) Wild type C57Bl/6 mice were infected with LCMV-WE and treated as described on day 5 post-infection. Splenocytes were harvested on day 8 post-infection, and total splenic LCMV specific (K^b-$GP_{33}{}^+$ tetramer $CD44^+$) $CD8^+$ T cell and (E) naïve ($CD44^{lo}$) $CD8^+$ T cells were enumerated. Data are mean +/- SE. Significance was determined by one way ANOVA with Bonferroni Post-Hoc test. *p <0.05, **p <.001 and ***p <0.001. Results represent 3 independent experiments, N= 8

Figure 7. Cell cycle checkpoint inhibitors induce premature mitosis (A) P-14 transgenic CD8+ T cells activated with peptide stimulation *in vitro* and cultured with AZD7762 or (B) MK1775 for 4 hr with Edu added after 3 hours. Cells were stained for Edu, DNA content (7AAD), and Phos-H3 for mitosis. (C) Representative flow plots form the 4hr time point in

panels C and D. Cells in G1, S, and G2 are represented in grey with gates shown. Mitotic phospho-H3+ cells are shown in black.

Figure 8. Cell cycle inhibition with MK-177 and AZD7762 act on CDK1 to propel cell cycling. *in vitro* activated P-14 transgenic CD8+ T cells were co-cultured with etoposide (3uM), MK-1775(10uM), or AZD7762(1uM)for 18 hrs *in vitro*. Apoptosis is defined as percent phosphatidylserene^{+} as defined by MFGD8 staining. Data are mean +/- SE. Significance was determined by one way ANOVA with Bonferroni Post-Hoc test.. N=4

Figure 9. Cell death by combination inhibitor therapy is correlated with the rate of cellular division. CFSE labeled P14 CD8+ T cells were activated *in vitro*. They were cultured with etoposide, AZD7762 or MK-1774. Rate of cellular division was assessed by CFSE dilution over course of culture. Data are mean +/- SE. Significance was determined by one way ANOVA with Bonferroni Post-Hoc test.. N=4

Figure 10. Combination inhibitor therapy does not cause off target tissue damage. WT mice were treated as described twice, 24 hours apart and tissues was harvested 24 hours following the second treatment. (A) FITC-dextran migration was measured through isolated ileum in a Ussing chamber with (B) that addition of trans-epithelial resistance measurements. (C) The MFI of ϒH2AX staining of LK (lin^{-} c-kit^{+}) and LSK (lin^{-} sca1^{+} c-kit^{+}) hematopoietic

cells harvested from the bone marrow of the femur, with (D) total cell counts per femur. (E) Total cell count per spleen of regulatory T cells ($CD4^+CD25^+FoxP3^+$) after treatment.

Figure 11. Combination therapy with nutlin and checkpoint inhibitors treats HLH by eliminates pathogenic T cells. $Prf^{-/-}$ mice were infected with LCMV-WE and treated as described on day 5 and 6 post-infection. (A) Splenocytes were harvested on day 8 post-infection and LCMV specific (K^b-GP_{33}^+ tetramer, $CD44^+$) $CD8^+$ T cell cell and (B) naïve ($CD44^{lo}$) $CD8^+$ T cells were enumerated. (C) Serum IFN-ϒ levels from 8 days post-infection. (D)HLH Survival curve from treated wild type and $Prf^{-/-}$ miceN= 8

Figure 12. Combination therapy nutlin and checkpoint inhibitors treats EAE by specifically eliminating pathogenic T cells. EAE was in induced in wild type C57Bl/6 and treated as indicated on days 5 and 9. Splenocytes were harvested on day 30 and stained for A) MOG_{35} specific $CD4^+$ T cells ($CD4^+$, $CD44^+$, I-A^b MOG_{35} $tetramer^+$) B) CD4+ T cells producing IL-17 by *ex vivo* MOG_{35-55} peptide restimulation, and C) regulatory T cells ($CD4^+$, $CD25^+$, $Foxp3^+$). D) EAE was induced in C57Bl/6 mice and treated as indicated on day 12 and 16 after the presentation of clinical symptoms. Clinical scores are depicted for treated mice. Data are mean +/- SE. Significance was determined by one way ANOVA with Bonferroni Post-Hoc test. *p <0.05, **p <.001 and ***p <0.001. Results represent 3 independent experiments, N= 8-12

Supplemental Figure 13. Non-gentoxic combination therapy does not impair memory immune responses. Six week old C57Bl/6J mice were infected with LCMV. At day 60 EAE was induced and treated as indicated 5 and 9 days later. Mice were sacrificed 5 days after rechallenged with LCMV clone 13 at day 56. A)Splenocytes were analyzed for total number of LCMV specific GP_{33-41}^{+} memory $CD8^{+}$ T cells B) and of LCMV specific GP_{61-80}^{+} memory $CD4^{+}$ T cells. C) Splenocytes were evaluated for IFN-ϒ production after *ex vivo* peptide restimulation with GP_{33-41} and D) GP_{61-80}. E) Total RNA was extracted and reversed transcribed from liver biopsies. qPCR for LCMV viral load was assessed from copy numbers of LCMV nuclear protein normalized to s14. Data are mean +/- SE. Significance was determined by one way ANOVA with Bonferroni Post-Hoc test. *p <0.05, **p <.001 and ***p <0.001. Results represent 3 independent experiments, N= 8-12

Figure 14. Combination inhibitor therapy kills reactivated memory T cells. WT mice were infected with LCMV to generate memory T cells. Six weeks post infection the mice were immunized with LANAC peptide vaccine to reactivated memory T cells and treated as described two days post vaccination. Splenocytes were harvested on day 8 post-infection, and total splenic LCMV specific (K^{b}-GP_{33}^{+} tetramer $CD44^{+}$) $CD8^{+}$ T cell and (E) naïve ($CD44^{lo}$) $CD8^{+}$ T cells were enumerated. Data are mean +/- SE. Significance was determined by one way ANOVA with Bonferroni Post-Hoc test. *p <0.05, **p <.001 and ***p <0.001. Results represent 2 independent experiments, N= 5-7

Figure 15. Non-genotoxic combination therapy kills activated human T cells. Normal donor PBMCs were activated in vitro with concavalin A and IL-2. Activated T cells were co-cultured with increasing concentrations of (B) etoposide (C) AZD7762 or (D) MK-1775 +/- nutlin-3 (3μM) for 18 hrs *in vitro*. Apoptosis is defined as percent phosphatidylserene^{+} as defined by MFGD8 staining. Results represent 5 independent experiments, N=3

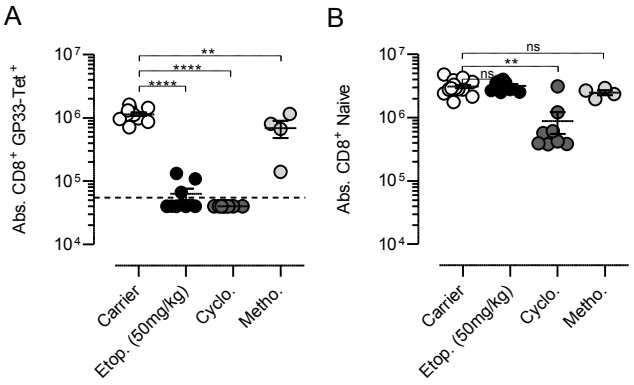
A
B
Abs. CD8+ GP33-Tet+
Abs. CD8+ Naive
10^7
10^6
10^5
10^4
**
****
****
ns
ns
**
Carrier
Etop. (50mg/kg)
Cyclo.
Metho.

Figure 1

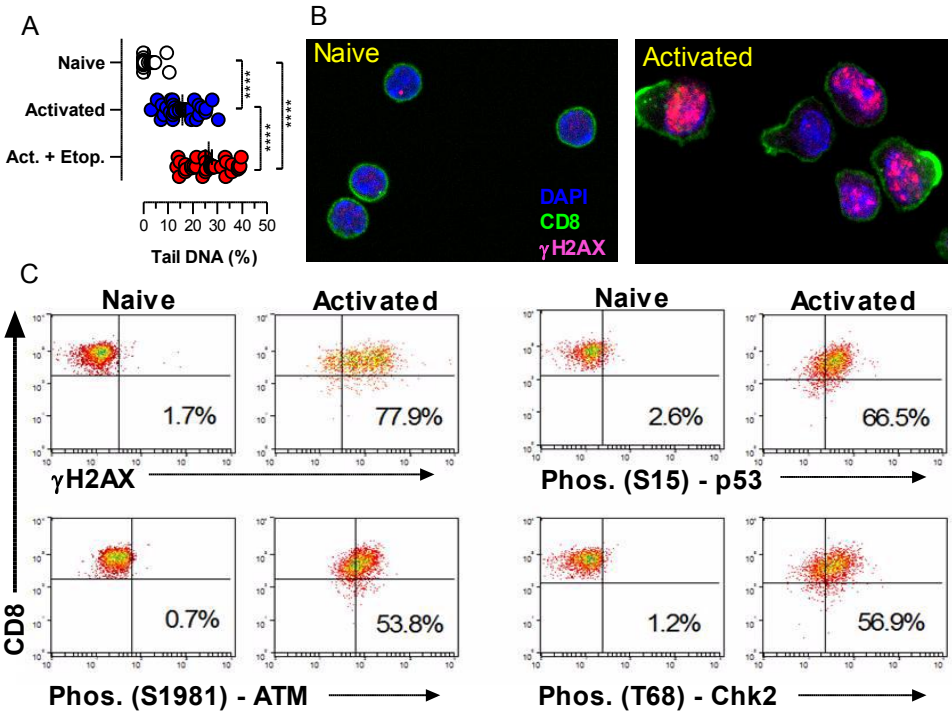
A
Naive
Activated
Act. + Etop.
****
****
****
0 10 20 30 40 50
Tail DNA (%)
B
Naive
Activated
DAPI
CD8
γH2AX
C
Naive
Activated
Naive
Activated
1.7%
77.9%
2.6%
66.5%
γH2AX
Phos. (S15) - p53
CD8
0.7%
53.8%
1.2%
56.9%
Phos. (S1981) - ATM
Phos. (T68) - Chk2

Figure 2

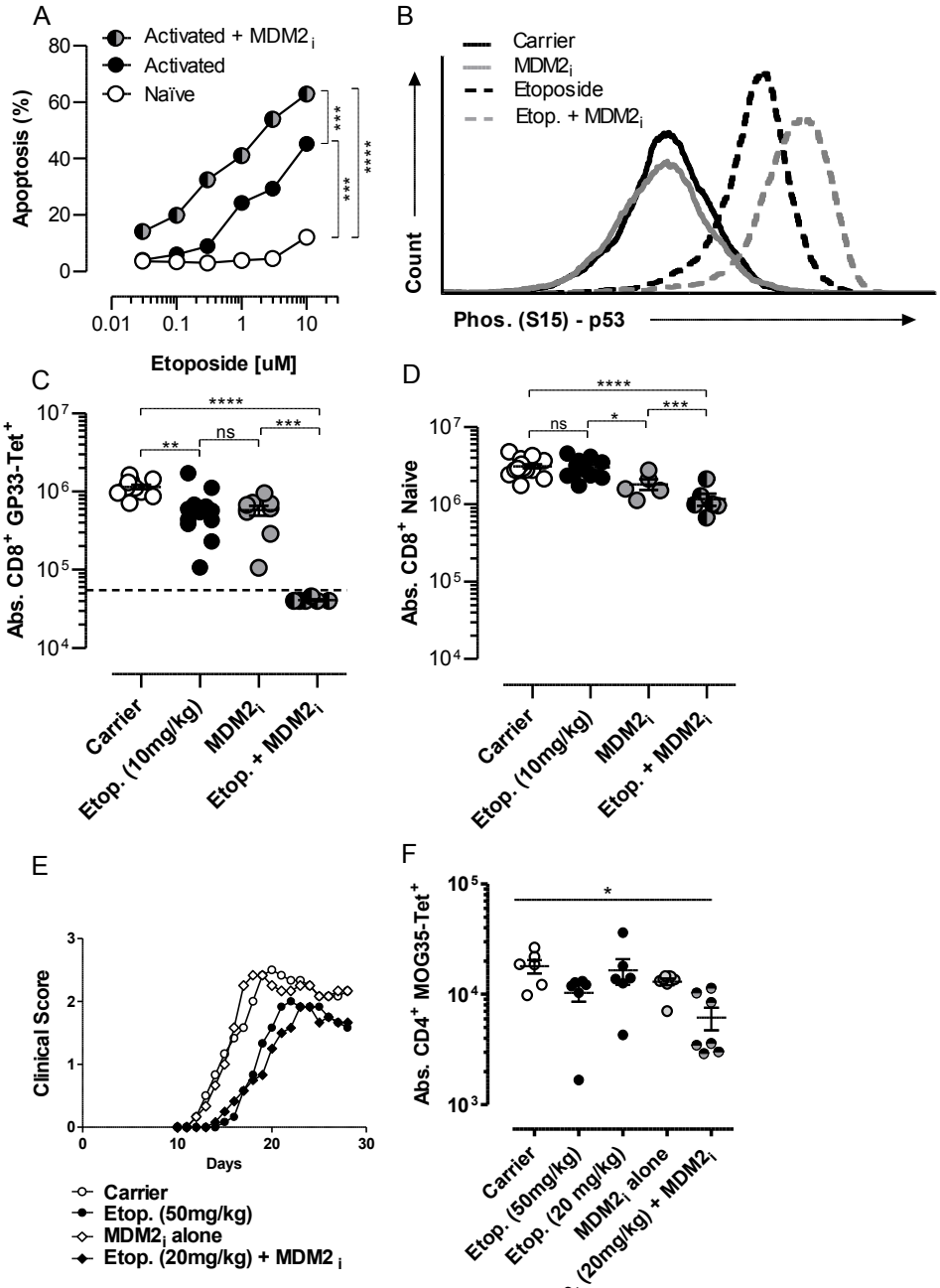
A
Activated + MDM2i
Activated
Naïve
Apoptosis (%)
Etoposide [uM]
B
Carrier
MDM2i
Etoposide
Etop. + MDM2i
Count
Phos. (S15) - p53
C
Abs. CD8+ GP33-Tet+
Carrier
Etop. (10mg/kg)
MDM2i
Etop. + MDM2i
D
Abs. CD8+ Naive
E
Clinical Score
Days
Carrier
Etop. (50mg/kg)
MDM2i alone
Etop. (20mg/kg) + MDM2i
F
Abs. CD4+ MOG35-Tet+
Etop. (20 mg/kg)

Figure 3

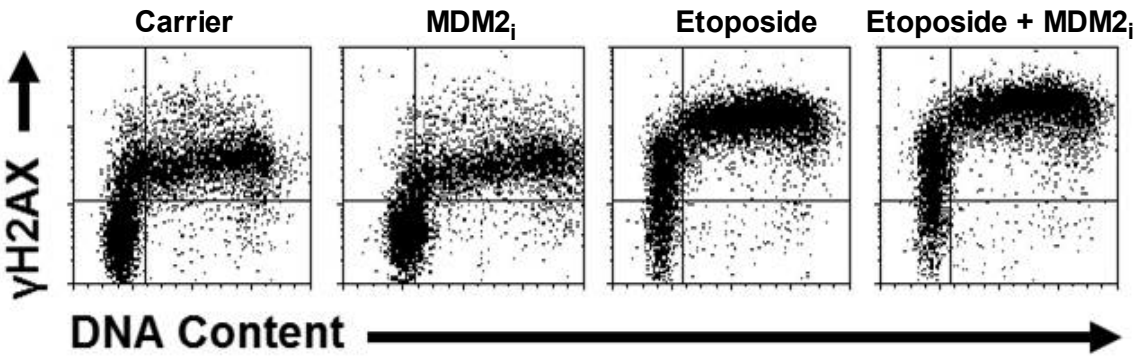
Carrier
MDM2i
Etoposide
Etoposide + MDM2i
γH2AX
DNA Content

Figure 4

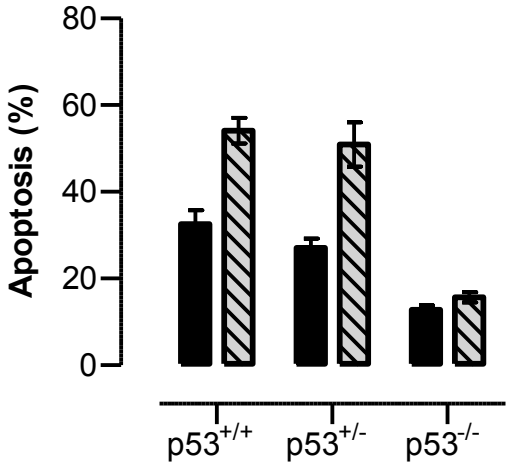

Figure 5

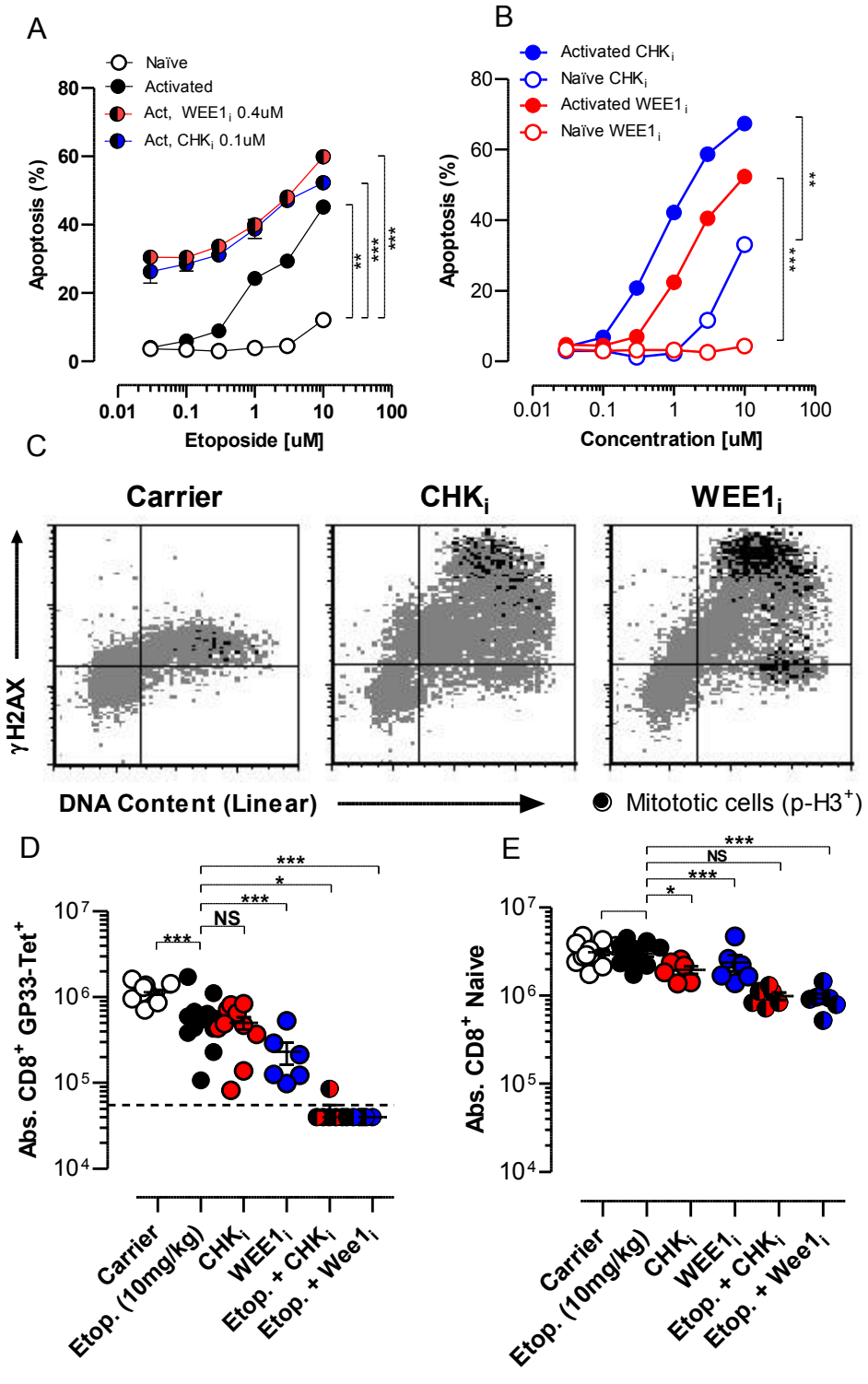
A
Naïve
Activated
Act, WEE1i 0.4uM
Act, CHKi 0.1uM
Apoptosis (%)
Etoposide [uM]
B
Activated CHKi
Naïve CHKi
Activated WEE1i
Naïve WEE1i
Apoptosis (%)
Concentration [uM]
C
Carrier
CHKi
WEE1i
γH2AX
DNA Content (Linear)
Mitototic cells (p-H3+)
D
Abs. CD8+ GP33-Tet+
NS
Carrier
Etop. (10mg/kg)
CHKi
WEE1i
Etop. + CHKi
Etop. + Wee1i
E
Abs. CD8+ Naive
NS
Carrier
Etop. (10mg/kg)
CHKi
WEE1i
Etop. + CHKi
Etop. + Wee1i

Figure 6

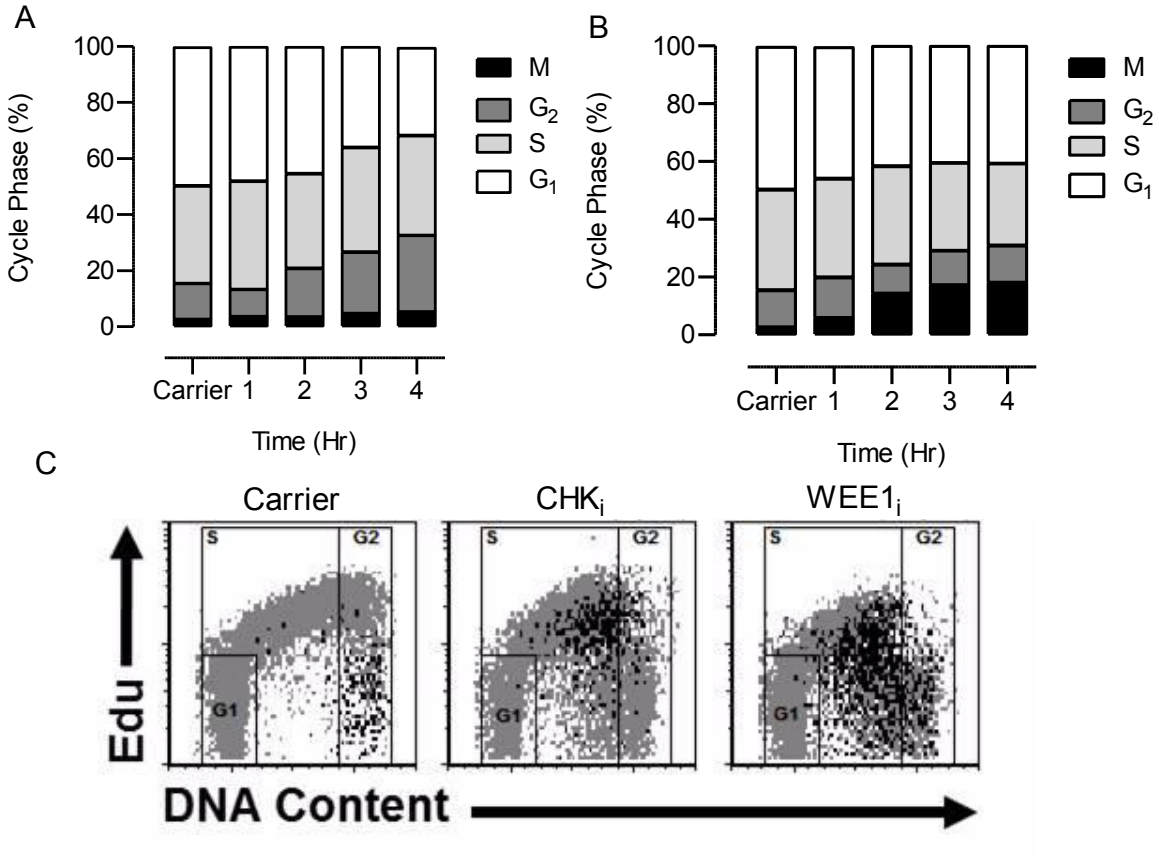
A
100
80
60
40
20
0
Cycle Phase (%)
M
G2
S
G1
Carrier 1 2 3 4
Time (Hr)
B
100
80
60
40
20
0
Cycle Phase (%)
M
G2
S
G1
Carrier 1 2 3 4
Time (Hr)
C
Carrier
CHKi
WEE1i
S
G2
G1
Edu
DNA Content

Figure 7

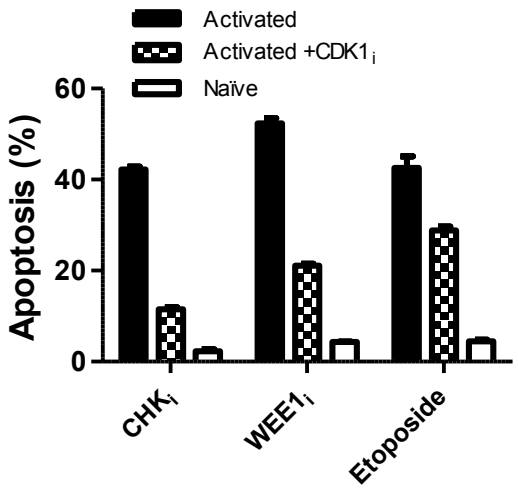
Activated
Activated +CDK1i
Naïve
Apoptosis (%)
60
40
20
0
CHKi
WEE1i
Etoposide

Figure 8

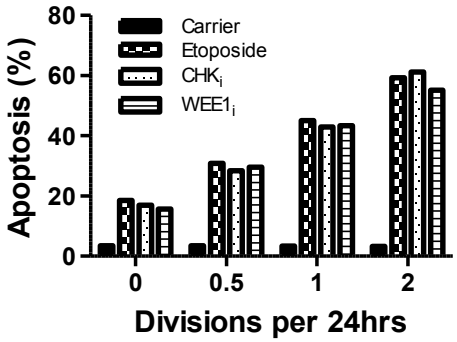
80
60
40
20
0
Apoptosis (%)
Carrier
Etoposide
CHK$_i$
WEE1$_i$
0
0.5
1
2
Divisions per 24hrs

Figure 9

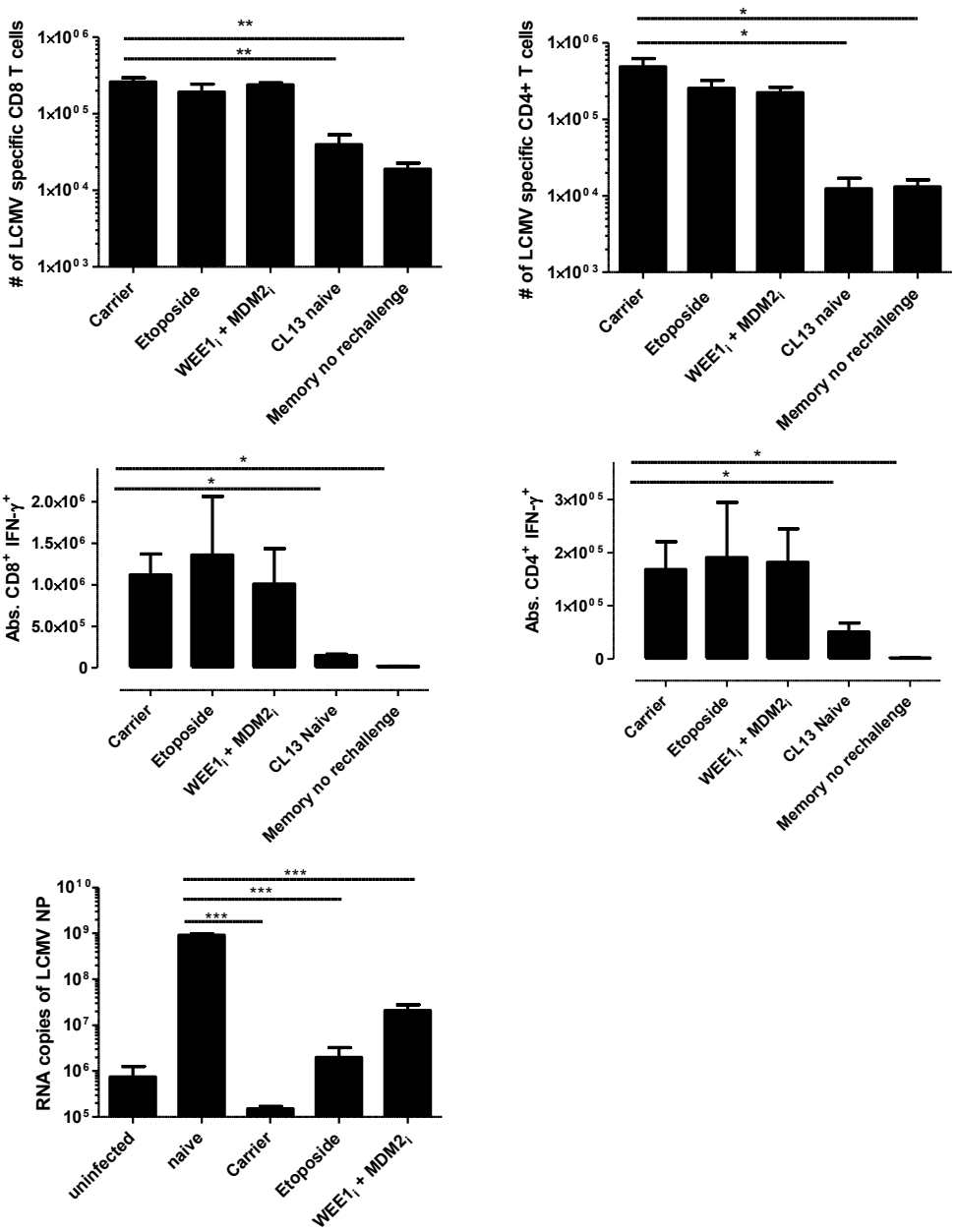
# of LCMV specific CD8 T cells
1x10^6
1x10^5
1x10^4
1x10^3
**
**
Carrier
Etoposide
WEE1i + MDM2i
CL13 naive
Memory no rechallenge
# of LCMV specific CD4+ T cells
1x10^6
1x10^5
1x10^4
1x10^3
*
*
Carrier
Etoposide
WEE1i + MDM2i
CL13 naive
Memory no rechallenge
Abs. CD8+ IFN-γ+
2.0x10^6
1.5x10^6
1.0x10^6
5.0x10^5
0
*
*
Carrier
Etoposide
WEE1i + MDM2i
CL13 Naive
Memory no rechallenge
Abs. CD4+ IFN-γ+
3x10^5
2x10^5
1x10^5
0
*
*
Carrier
Etoposide
WEE1i + MDM2i
CL13 Naive
Memory no rechallenge
RNA copies of LCMV NP
10^10
10^9
10^8
10^7
10^6
10^5
***
***
***
uninfected
naive
Carrier
Etoposide
WEE1i + MDM2i

Figure 10

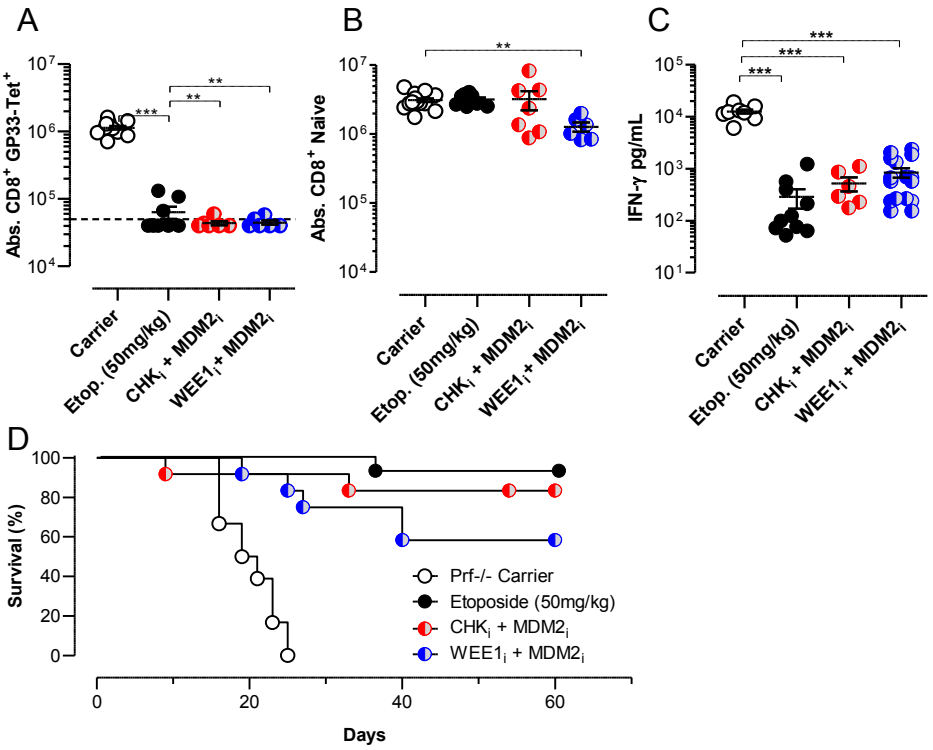
A
B
C
D
Abs. CD8+ GP33-Tet+
Abs. CD8+ Naive
IFN-γ pg/mL
Carrier
Etop. (50mg/kg)
CHKi + MDM2i
WEE1i + MDM2i
Survival (%)
Days
Prf-/- Carrier
Etoposide (50mg/kg)
CHKi + MDM2i
WEE1i + MDM2i

Figure 11

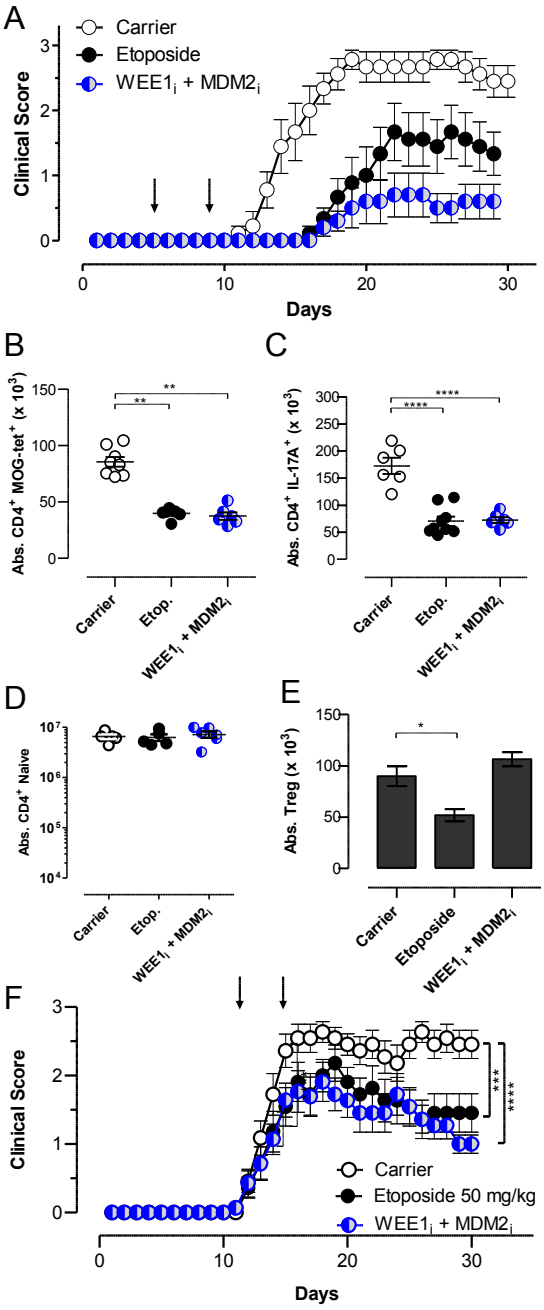
A
Carrier
Etoposide
WEE1i + MDM2i
Clinical Score
Days
B
Abs. CD4+ MOG-tet+ (x 10^3)
Carrier
Etop.
WEE1i + MDM2i
C
Abs. CD4+ IL-17A+ (x 10^3)
D
Abs. CD4+ Naive
E
Abs. Treg (x 10^3)
Etoposide
F
Etoposide 50 mg/kg

Figure 12

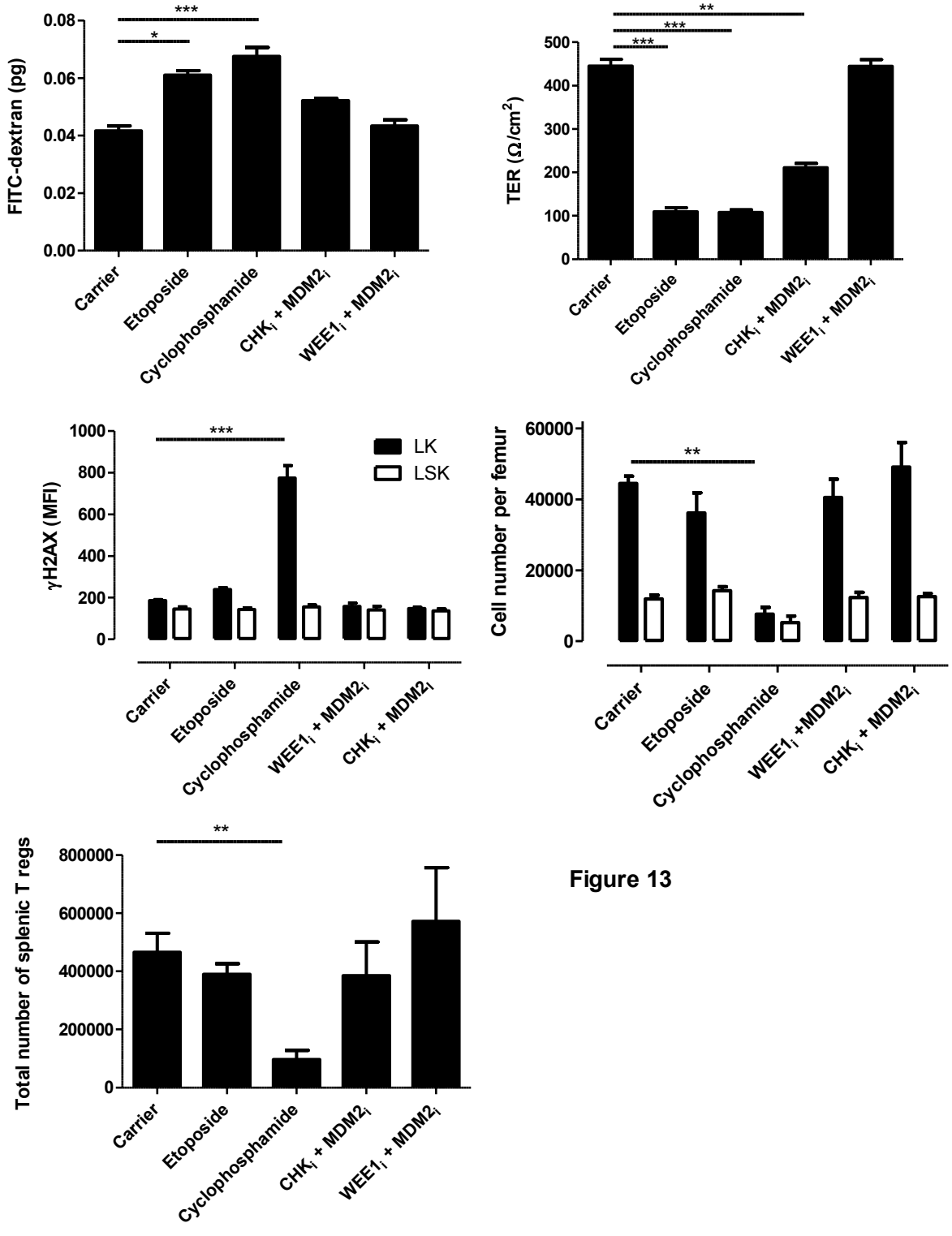
FITC-dextran (pg)
0.08
0.06
0.04
0.02
0.00
***
*
Carrier
Etoposide
Cyclophosphamide
CHK$_i$ + MDM2$_i$
WEE1$_i$ + MDM2$_i$
TER (Ω/cm^2)
500
400
300
200
100
0
**
***
***
γH2AX (MFI)
1000
800
600
400
200
0
LK
LSK
Cell number per femur
60000
40000
20000
0
Total number of splenic T regs
800000
600000
400000
200000
0

Figure 13

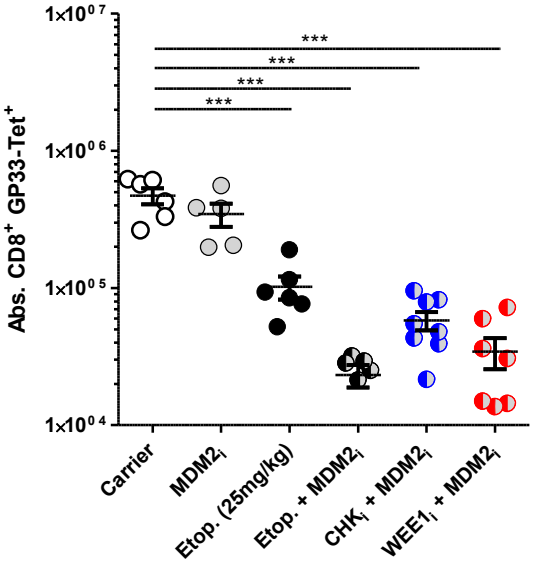
1×10^07
1×10^06
1×10^05
1×10^04
Abs. CD8+ GP33-Tet+
***
***
***
***
Carrier
MDM2i
Etop. (25mg/kg)
Etop. + MDM2i
CHKi + MDM2i
WEE1i + MDM2i

Figure 14

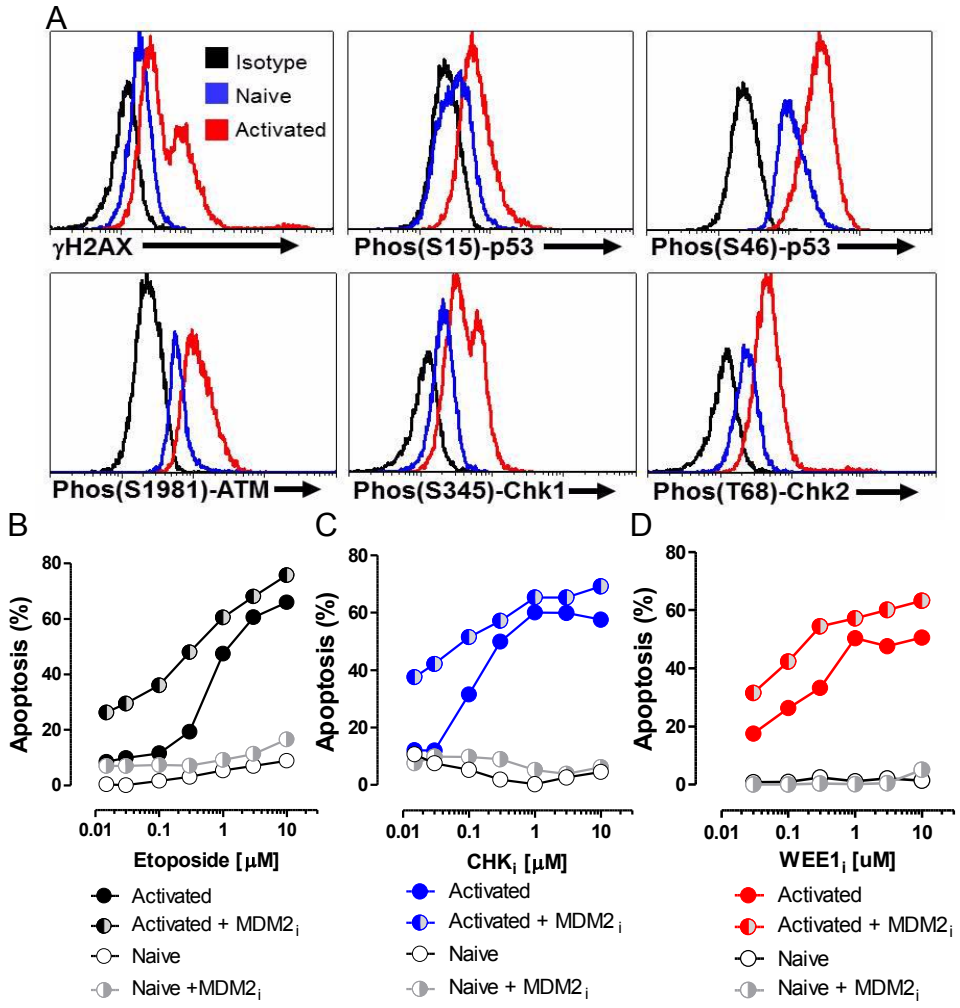
A
Isotype
Naive
Activated
γH2AX
Phos(S15)-p53
Phos(S46)-p53
Phos(S1981)-ATM
Phos(S345)-Chk1
Phos(T68)-Chk2
B
C
D
Apoptosis (%)
Etoposide [μM]
CHK$_i$ [μM]
WEE1$_i$ [uM]
Activated
Activated + MDM2$_i$
Naive
Naive +MDM2$_i$
Naive + MDM2$_i$

Figure 15

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Chapter 4

Discussion

Summary of data

Here, we presented the use of a series of therapeutic strategies for the treatment of EAE, focused around the manipulation of the DNA damage response pathway. We have utilized drugs that target various stages of the DDR pathway, from directly damaging DNA with etoposide (Chapter I), to enhancement of p53 signaling with nutlin, to inhibiting cell cycle arrest with MK-1775 and AZD7762 (Chapter II). These strategies all take advantage of the intrinsic vulnerabilities that rapid division induces in activated T cells thus allowing for their selective deletion. This results in an amelioration of symptoms in EAE due to the loss of the pathogenic T cells that drive disease.

Killing activated T cells by manipulating the DDR pathway is effective in EAE

Etoposide is a topoisomersase II inhibitor that functions similarly to the current MS drug, mitoxantrone. It is a cytotoxic drug that induces DNA damage in rapid proliferating cells which makes it a likely drug choice for the treatment of autoimmune diseases, such as MS/EAE. We demonstrated that the prophylactic use of etoposide was effective in the treatment of EAE. Etoposide treatment dramatically decreases the severity of EAE, it extended the time to disease onset, and decreased the cumulative rate of disease. More importantly, treatment of clinically apparent, ongoing disease was also significantly decreased in magnitude in the progressive C57BL/6J mouse model, as well as decreasing the rate of disease relapse, in a relapsing-remitting disease model. Etoposide treatment significantly decreased the total number of MOG-specific $CD4^+$ T cells both in the CNS, as well as in the periphery. Treatment also decreased the total number of MOG specific $CD4^+$ T cell producing IFN-γ, TNF and IL-17a. Therefore

etoposide was able to deplete MOG specific $CD4^+$ T cells, a prerequisite that we set for an effective treatment for MS. This demonstrates that directly damaging DNA with etoposide to activate the DDR pathway decreases disease progression.

We next investigated the effects of manipulating downstream effector proteins in the DDR pathway. Etoposide induces apoptosis of activated T cells via a p53-dependent mechanism, thus, we investigated a mechanism to enhance p53 signaling which should enhance the induction of apoptosis. p53 expression is regulated by MDM2, therefore, inhibition of MDM2 should enhance p53 functions by preventing its destruction. We tested the enhancement of p53 signaling with the MDM2 inhibitor nutlin. Hypothetically, this should allow for decreased DDR input to achieve the same endpoint of apoptosis, if there is an amplification of p53 activity. We demonstrated that co-treatment with nutlin amplified DDR signaling to an extent that allowed for an 80% reduction in the concentration of etoposide needed to kill activated T cells leading to a decreased severity of EAE.

The major drawback associated with using etoposide or mitoxantrone for the treatment of MS is that they both are genotoxic, functioning by initiating DNA damage, which can lead to malignancies. In our investigation of manipulating the DDR pathway we examined the use of cell cycle checkpoint inhibitors MK-1775 and AZD7762 as a means of creating a completely non-genotoxic treatment strategy. MK-1775 inhibits Wee1, while AZD7762 inhibits Chk1/2. Both Wee1 and Chk1 function to inhibit G2/M cell cycle progression in a cyclin-dependent manner. Thus, combination inhibitor therapy with nutlin-3 and MK-1775 or AZD7762 leads to premature cell cycling and apoptosis of activated T cells by blocking the inhibitory actions of Wee1 and Chk1. As with etoposide treatment, combination inhibitor therapy was able to purge MOG specific $CD4^+$ T cells and decrease the production of IL-17, with comparable efficacy to

treatment with etoposide. Additionally, combination inhibitor therapy proved effective in the treatment of EAE at both prophylactic and therapeutic time points.

Combination inhibitor therapy causes minimal off-target damage

As discussed in the introduction, all of the current treatments for MS have severe side effects which generally result from off-target effects. We demonstrated that etoposide and combination inhibitor therapy was effective as a treatment for EAE. However, to improve upon the current state of therapies these treatments need to be truly selective for encephalitogenic T cells. Analysis of total immune cell populations by number one day following etoposide treatment reveals that there are no significant changes in total cells counts at a population level, including no significant loss of naïve T cells following treatment. Most notably, all three treatment approaches did not delete regulatory T cells. We next investigated treatment-mediated immune suppression at a functional level. Following a course of etoposide treatment, naive T cells responded to an LCMV infection at a similar magnitude as vehicle-treated control animals. Similarly, naive B cells were able to produce similar antibody titers to immunization with TNP-OVA following etoposide treatment. We next investigated possible effects of treatment of EAE on memory T cell functionality. EAE was induced in mice that contained tractable memory T cells from a previously cleared LCMV infection, and then treated. Treatment did not affect memory T cells functions in purging a LCMV clone 13 viral rechallenge, thus validating the survival of functional memory T cells, even during the time point that activated T cells are removed. Additionally, combination inhibitor therapy had minimal effects on intestinal permeability and on hematopoietic cells in the bone marrow. Combination inhibitor therapy

allows for a highly selective method to kill pathogenic activated T cells in MS/EAE. Unlike treatment with etoposide or cyclophosphamide, combination inhibitor therapy does not act by damaging DNA, which prevents the chance for secondary malignancies developing from treatment. Therefore due to the selectivity of combination inhibitor therapy and its lack of off target effects, it has the potential to be a new highly effective treatment strategy for MS.

Etoposide's selectivity for activated T cells has been alluded to previously. The relationship between etoposide-induced DNA damage, the activation of p53 and apoptosis in human cells was investigated by Korvek et al. (1). In a comparison of resting human T cells, purified from PBMCs, and rapidly proliferating Jurkat T cells, they demonstrated that a concentration of 1μM etoposide will kill proliferating T cells but does not kill resting T cells This concentration of 1uM is similar to what the *in vivo* concentration of etoposide of 50mg/kg. While Jurkats are not really analogous to non-transformed effector T cells, this study does demonstrate that quiescent T cells are more resistant to etoposide that rapidly dividing "T cells". Additionally, studies need to be conducted to determine if the selectivity seen with etoposide is broadly applicable to other DNA damaging agents or if etoposide is unique in this regard. In particular, studies need to be conducted focusing on mitoxantrone, the only analogous drug currently approved for the treatment of MS.

Activated T cells display intrinsic DNA damage

All proliferating cells have inherent DNA breakage as part of their replication. Such breaks can result from the transient DSB induced by the normal function of topoisomerase II or from the Okazaki fragments during replication of the 3' DNA strained. What is notable is that rapidly

expanding effector T cells undergo pronounced DDR pathway activation, which is an important component for the success of combination inhibitor therapy. It is well documented that treatment of proliferating cells with DNA damaging agents, such as etoposide, activates the DDR pathway (2, 3). This starts with damage foci indicated by γH2AX, followed by activation of signaling kinases ATM and ATR, and finally the activation of p53 and its downstream transcripts. We have detected a similar activation on the DDR pathway in activated, rapidly proliferating T cells, albeit to a lesser degree than what is observed with etoposide treatment. We believe that this activation of the DDR in activated T cells is due to errors made during DNA replication, which is further amplified due to the extremely rapid rate of division during T cell expansion. Activated T cells can divide every 6-8 hours (4) vs. the proliferation rate of regulatory T cells or hematopoietic stem cells which divide once every 8 days or 2.5 weeks, respectively (5, 6). This non-lethal DDR pathway activation in proliferating cells is designed to activate the growth arrest function of p53 and cell cycle checkpoints to stop the damaged cell from entering mitosis, to allow for repair of the damaged genome (7). However, with the proper manipulation through the use of combination inhibitor therapy, which is designed to inhibit cell cycle arrest and enhance p53 signaling towards the apoptotic threshold, this minor, non-lethal DDR pathway activation provides enough of a stimulus, without the addition of exogenous DNA damaging agents to kill activated T cells.

DDR pathway activation has been observed in a series of both primary tumors and cell lines. γH2AX foci, phos-ATM, phos-Chk2, and phos-p53 staining have all been seen in a variety of untreated malignant tissue types, with the intensity of DDR pathway activation has correlated with the progression of a tumor (8–10). Furthermore, this phenomenon of intrinsic p53 activation has also been observed in EAE. Examination of spinal columns from mice with EAE

demonstrated an increase in total p53 and Bax expression by western blot and immunohistochemistry (11, 12). This increased expression correlates with T cell activation. Though the activation of p53 is not directly observed, it can be inferred due to the increased expression of its downstream gene product Bax. While we are the first to show DDR pathway activation in primary activated T cells, the true novelty of this finding is our ability to manipulate this activation for the selective killing of activated T cells *in vivo*.

MDM2$_i$ enhances p53-mediated apoptosis of encephalitogenic T cells and allows for a decrease in the amount of DNA damaging drugs

The use of small molecules to influence the endpoint decisions of p53 has been previously proposed and demonstrated (13, 14). p53 activity can be enhanced by blocking its interactions with MDM2, as nutlin does (15), or by inhibiting MDM2's E3 ligase activity, its ability to ubiquitinate and destroy p53. Ultimately, the real target of nutlin is to overcome the duality of p53 functions, and point the cell towards apoptosis in spite of p53's function as a cell redeemer. Nutlin has been shown to induce apoptosis in SJSA cells and overcome elevated p21 levels by enhancing p53 signaling to a point that breaks the apoptotic threshold (16). Nutlin's ability to induce apoptosis is further defined by Best et al. who demonstrated that it can be used in combination with etoposide, in a manner similar to our protocol, to effectively kill tumor cells at a greater rate than etoposide alone (17).

Nutlin's primary mechanism of action is the inhibition of MDM2, leading to the enhancement of p53 signaling. However, there are reports that nutlin also inhibits the multi-drug resistance type-1 transporter (MDR1) (18, 19). MDR1 has been documented to transport many different DNA

damaging agents, including etoposide and mitoxantrone, out of the target cell decreasing their effectiveness. If nutlin is able to inhibit the export of etoposide this provides an additional mechanism for the observed synergy between etoposide and nutlin. Inhibition of MDR1 is particularly important when dealing with encephalitogenic T cells, since they are believed to express high levels of MDR1. Ramesh et al. recently described the phenotype of "pathogenic" Th17 $CD4^+$ T cells as IL-17^+MDR1^+IL-$23R^+$ (20). While it has not yet been confirmed this phenotype of "pathogenic" Th17 cells matches the description of our target encephalitogenic T cell population in EAE. Nutlin's ability to inhibit MDR1 and retain drugs within cells may prove vital for the killing of self-reactive T cells, particularly in MS/EAE by further enhancing the synergistic effects of combination therapy.

Working model

The induction of apoptosis in activated T cells by combination inhibitor therapy is a multistep process (Figure 1). The apoptotic cascade is initiated by DNA damage. This damage can be induced though via DNA damaging agents, such as etoposide which causes both double and single stranded breaks. Or, as we have shown, this damage can come as a side effect of rapid,

imperfect DNA replication in activated T cells. Regardless of how the DNA damage happens,

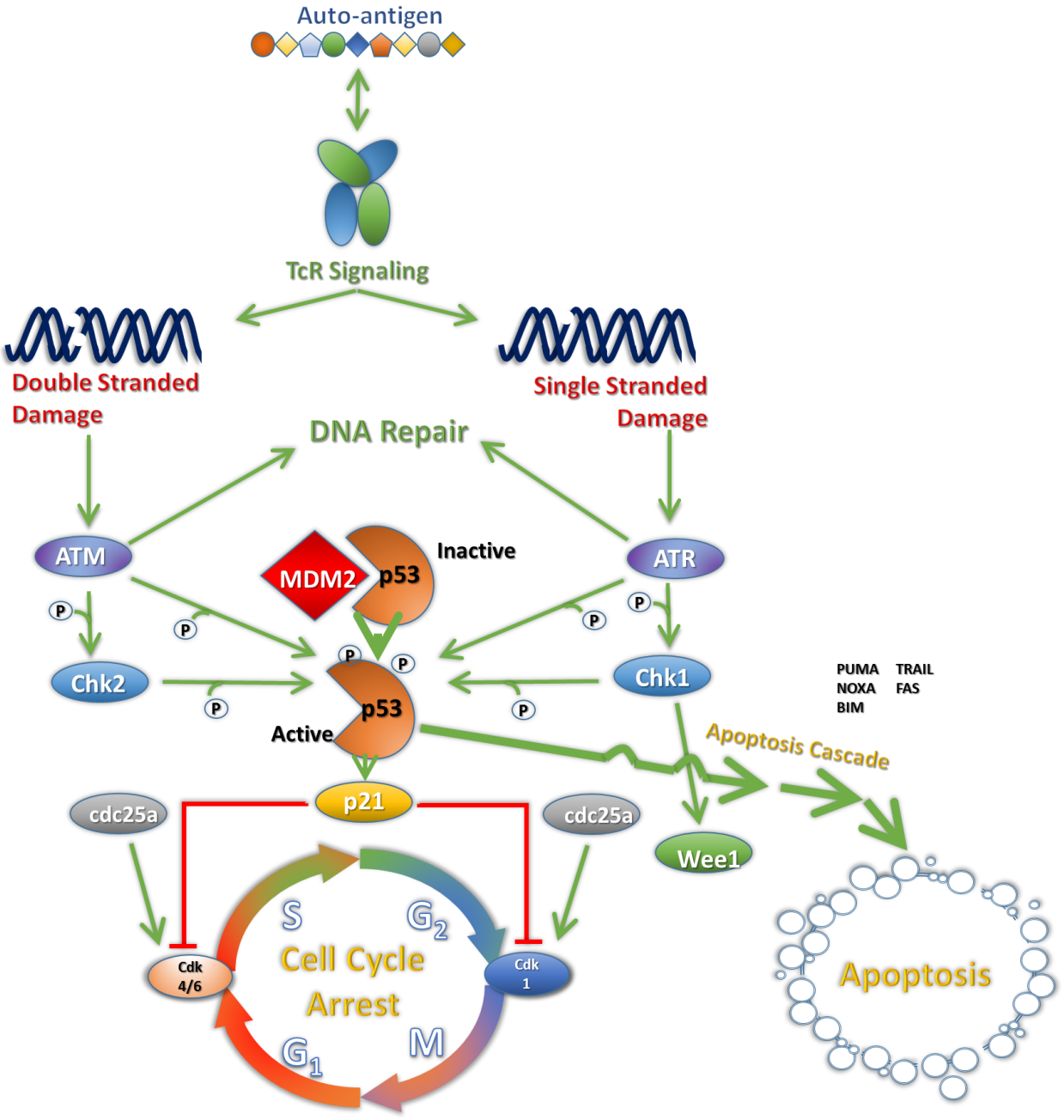

Figure 1: Model summary of T cell death via the DNA damage response pathway.

the sites of DNA damage recruit a series of damage response proteins that ultimately result in the activation of the signaling kinases and ATR. These kinases activate both p53, as well as the cell

cycle inhibitors Chk1, which function by inhibition CDC25. However, with the addition of AZD7762 Chk mediated repression of cell cycle progression in removed. This results in the continued cycling of damaged cells, and ultimately entry of cells, prior to the generation of 4N DNA, into mitosis, resulting in a mitotic catastrophe.

The exact molecular events that initiate apoptosis in the absence of Chk1 are still not clearly defined (21). However, Vance et al. has demonstrated that cell death is caspase-3-dependent, but not dependent on p53 (22, 23). Additional studies inducing cell death with ionizing radiation demonstrated that cell death following Chk1 depletion is caspase-2-dependent, but is not affected by over expression of Bcl-2 or Bcl-xl (24). Regardless of the exact mechanism of cell death, it is well documented to be p53-independent. Therefore, the combination of cell cycle checkpoint inhibitors with nutlin utilizes two independent pathways to induce cell death, unlike the combination of etoposide and nutlin which simply amplifies p53 signaling. Furthermore, utilizing a combination of drugs that can synergize and allow for a minimal use of either compound provides a rationale for creating effective drugs combinations at doses which produce minimal side effects, as we have demonstrated here.

The combination of ATR and ATR also activate p53 and its downstream gene products. In the absence of an extrinsic DNA damaging agent activated T cells display minor, non-lethal activation of p53, which biologically is intended to lead to the induction of DNA repair responses towards the damage. However, with the addition of nutlin, which prevents the destruction of p53, the p53 response is amplified. Due to the increase in available p53 pro-apoptotic gene products are also produced, including Puma, Noxa, Bax, Fas and TRAIL. Once a threshold of pro-apoptotic Bcl-2 family proteins are expressed the apoptosis cascade is initiated via cytochrome C release from the mitochondria and caspase-3 activation. Thus through

manipulation of the DDR pathway we are able to prevent cell cycle arrest and induce cell death in activated T cells without the addition of a DNA damaging agent.

Future directions

Selectively removing encephalitogenic T cells with mitoxantrone

Mitoxantrone is currently FDA approved for the treatment of MS, therefore any improved understanding of possible uses of mitoxantrone may have an immediate clinical benefit. Studies of mitoxantrone in EAE show similar results of decreased clinical scoring and cytokine-producing T cells following treatment. However, no studies to date have investigated the specificity of mitoxantrone in the same manner that we have done with etoposide. Additionally, if mitoxantrone has the greatest effect on killing encephalitogenic T cells while they are rapidly dividing following activation, then its use in the clinic should reflect this. Currently mitoxantrone is administered every 3 months in highly progressive cases. Our data would suggest that this may not be the most effective use of the drug. Instead, mitoxantrone may be more effective if it is administered when a patient experiences a disease flare, which would correspond to the recent activation of encephalitogenic T cells. These studies need to be conducted to determine if mitoxantrone can be used in a more efficient manner.

A direct comparison of our study of etoposide in combination with nutlin and mitoxantrone in combination with nutlin needs to be conducted. Because mitoxantrone is already clinically approved for the treatment of MS the addition of nutlin would only require an additional agent to study in clinical trials, rather than etoposide as well. Etoposide and mitoxantrone have similar mechanism of action, acting on topoisomerase II to initiate DNA damage and thus the addition of

nutlin should have similarly synergistic effects. Additionally, since the major drawback to the use of mitoxantrone is the side effects which mandate a lifetime maximum dose, the use of nutlin to decrease the amount of mitoxantrone administered could dramatically change its use, and allow for treatment with mitoxantrone in severe cases of MS to last for decades rather than years.

Additional DDR pathway inhibitors of interest

SCH99725 is another Chk1 inhibitor of interest (25). Our preliminary studies utilizing SCH99725 have shown promise in killing not only activated T cells, with similar results to AZD7762, but also affecting the development of EAE (data not shown). Notably SCH99725 has shown efficacy when used in conjunction with MK-1775 in the absence of any additional DNA damaging agents (26, 27). These studies showed synergy between the two drugs, which demonstrates that there are different roles for Wee1 and Chk1 in the induction of cell cycle arrest. Thus AZD7762 and MK-1775 are not necessarily interchangeable in combination inhibitor therapy. Further investigations will be needed to discern these differences in the functions of Wee1 and Chk1 in relation to the induction of cell death.

We have shown that nutlin is able to enhance apoptosis in activated T cells, however, there are other small molecules described in the literature that act on the p53-MDM2 axis and should be investigated in the future. For example, MI-43 is another MDM2 inhibitor that has been shown to enhance p53 signaling (28), and though nutlin works well to induce apoptosis in activated T cells, MI-43 or similar drugs may further enhance p53 signaling in activated T cells. Another approach to altering p53 signaling is to directly target p53 itself. RITA is a compound that works by binding p53 and inducing a conformational change (29). It can induce p53-dependent

apoptosis in cancer cells, including carcinoma of colon, lung, breast, skin melanomas and various lymphomas (29–31). RITA functions by significantly and selectively affecting the upregulation of pro-apoptotic genes. It has been proposed that the conformational changes induced by RITA allow for an increased affinity of p53 for pro-apoptotic transcription factors and DNA binding sites. Since nutlin does not alter the affinity of p53 for gene targets it will not change p53's default of initially favoring the upregulation of cell cycle arrest genes. Nutlin initially leads toward an upregulation of $p21^{cip}$ while RITA leads to a downregulation of $p21^{cip}$ (14). Additionally MDM2 acts to degrade $p21^{cip}$ as well as p53, and thus inhibiting MDM2 might not be the best approach to inhibiting cell cycle arrest (32, 33).

Preventing cell cycle arrest by targeting $p21^{cip}$

$p21^{cip}$ is the major effector molecule involved in p53-mediated cell cycle arrest. It induces cell cycle arrest by inhibiting a variety of cyclin-CDK complexes preventing cell cycling though the G1, S and G2/M checkpoints. Since $p21^{cip}$ is one of the first gene transcripts to appear after p53 activation the induction of cell cycle arrest becomes the default cell fate of p53 activation. Due to this pharmacologic inhibition or down regulation, $p21^{cip}$ has been hypothesized as a target that would help with the eradication of tumors through the induction of p53-mediated apoptosis (13). Unfortunately, there are no small molecule inhibitors of $p21^{cip}$ currently available. However, there are a few published mechanisms that can inhibit or down regulate $p21^{cip}$.

Despite the lack of an existing small molecule inhibitor of $p21^{cip}$, we can learn about the potential therapeutic benefits of targeting $p21^{cip}$ through viral proteins. The Adenovirus E1A protein increases the sensitivity of tumor cells to DNA-damaging agents. It was demonstrated

that this increase in apoptosis is a result of the direct interactions of E1A with $p21^{cip}$, preventing cell cycle arrest (34). The adenovirus has evolved E1A to prevent cell cycle arrest as a mechanism to keep host cells actively proliferating, which furthers the propagation of the virus. E1A can bind with $p21^{cip}$, and inhibit it's interactions with the cyclin-CDK complex. With the addition of a DNA-damaging agent this results in a blockade of $p21^{cip}$-mediated cell cycle arrest, and the progression of cells with damaged DNA through both the G1 and G2/M checkpoints. To date the only therapeutic uses of E1A to induce apoptosis have been through its incorporation into cytolytic viral therapies (35, 36). While E1A has been demonstrated to increase the sensitivity of tumors to DNA damaging agents, all of these studies have been conducted in infected or transected cells. Therefore, getting E1A into target cells is the major problem that limits the potential of E1A as an inhibitor of $p21^{cip}$ for the therapeutic treatment of pathogenic effector T cells. The $p21^{cip}$ binding region of E1A has been identified and specific polypeptides have been shown to be functional (36). Though it still remains to be seen if the administration of the soluble protein, or the addition of some other vehicle will allow for E1A entry into the target cell to inhibit the actions of $p21^{cip}$.

In addition to the E1A viral inhibitor of $p21^{cip}$, cytokine signaling has been shown to modulate $p21^{cip}$ expression. The addition of the growth factor IL-3 has been shown to limit apoptosis induced by DNA-damaging agents (37). It was later demonstrated that removal of IL-3 from culture media made tumor cells more sensitive to DNA-damaging agents (38, 39). The loss of IL-3 resulted in decreased expression of $p21^{cip}$, which in turn leads to a decrease in cell cycle arrest and an increase in fatal mitotic events. While this cell death is reported to be cytochrome c-dependent, no differences in Bcl-2, Bcl-xL or Bax levels were seen, similar to the reports on AZD7762. Thus, IL-3 dictates the choice between p53-dependent cell cycle arrest or apoptosis

(40). An additional target involved in p53 induced apoptosis resulting from IL-3 withdrawal. Overexpression of the insulin-like growth factor 1 receptor (IGF-1R) can protect cells from apoptosis during IL-3 withdrawal. Fully functional p53 leads to decreased IGF-1R expression and the induction of apoptosis (41). However, if IGF-1R is not diminished due to a loss of function mutation in p53 or if it is over expressed IGF-1R can protect against p53-mediated apoptosis induced by etoposide (42).

This mechanism of IL-3 withdrawal provides for two different possibilities for increasing the effectiveness of p53-mediated apoptosis of pathogenic effector T cells. First the addition of a neutralizing antibody against either IL-3 or the IL-3R alpha chain to our current therapeutic strategies may enhance death, as activated T cells are the major producers of IL-3 which acts in an autocrine manner. Moreover, only transient loss of IL-3 at the time of DNA damage was necessary to increase apoptosis (38) so long term blockade of IL-3 should not be necessary to inhibit the effects of $p21^{cip}$ on cell cycle arrest. There have been recent investigations using an anti-CD123 (IL-3Rα) antibody in the treatment of malignancies to inhibit cell survival (43), thereby demonstrating the potential effects of blocking IL-3 signaling in inducing apoptosis in activated T cells. Second is targeting IGF-1R, downstream of IL-3 signaling. There are currently a number of IGF-1R inhibitors that are commensally available that have demonstrated efficacy in the induction of apoptosis in tumor cells (44, 45). The addition of an IGF-1R inhibitor to a combination inhibitor therapy protocol could provide an even broader inhibition of cell cycle arrest than MK-1775 or AZD7762 currently do.

The effects of therapy on memory T cells

Memory T cells are important in chronic diseases like MS when the timing of the initial activation of self-reactive T cells cannot be predicted. We do not know if the continued presence of self-antigen, as seen in autoimmunity, allows for the generation of classically described memory T cells. However, we do know that self-reactive T cells persist throughout the course of the disease, and for many years. The clinical use of combination inhibitor therapy for the treatment of MS has to be able to kill self-reactive "memory" T cells. Therefore, understanding the nature and susceptibility of self-reactive "memory" T cells will be very important. It is possible that self-reactive "memory" T cells may not be as quiescent as classical memory T cells, due to the persistence of stimulatory antigen. If this is true, self-reactive "memory" T cells may be more susceptible to treatment than other resting populations. As a direct comparison of self-reactive "memory" T cells and quiescent memory T cells can be done by comparing diabetogenic transgenic BDC2.5 $CD4^+$ T cells following diabetes and LCMV specific transgenic SMARTA $CD4^+$ T cells following LMCV infection. Analysis of the T cells activation profiles, proliferative rates and baseline activation of the DDR pathway should be conducted to determine the susceptibility of self-reactive "memory" T cells to combination inhibitor therapy.

Should self-reactive "memory" cells prove to be truly quiescent, purging them following reactivation may be a viable approach. We have demonstrated that we can kill memory T cells, and that memory T cells are susceptible to combination inhibitor therapy, if they are reactivated. Therefore, it may be possible to reactivate memory myelin-specific T cells during remission and remove them with treatment. Admittedly, reactivation of myelin-specific T cells in patients could be very detrimental, with the potential to cause not only a relapse of MS, but also further progression of the disease. Utilizing natalizumab to block trafficking to the CNS in conjunction

with the reactivation of self-reactive memory T cells may mitigate these risks by keeping myelin-reactive T cells in the periphery. This would keep the myelin-reactive T cells out of the CNS were they could cause damage. Extensive animal modeling would need to be conducted before such a procedure could be attempted. Also, the risk of such a procedure would need to be carefully evaluated to determine a cost/benefit ratio for the patient.

Reactivation of self-reactive memory T cells may prove to be to detrimental as a means of removing encephalitogenic T cells from MS patients. If they cannot be killed after they have been activated we may be able to remove them before they become activated and pathogenic. Since relapse and disease progression is contingent upon epitope spreading (46), we could attempt to "pre-purge" T cells responding to spread epitopes prior to their activation to prevent the progression of disease. Our preliminary data suggest that immunization of myelin peptides in a liposome vaccine will activate myelin-specific T cells, but it does not induce EAE. Since the self-antigens in MS are well defined it should be possible to activate T cells specific for spread epitopes via vaccination (47). Following activated myelin specific T cells should be susceptible to combination inhibitor therapy, and removed from the patient, before they can cause damage. Once again natalizumab may be needed to safeguard the CNS during this procedure. We could test this approach in the SJL model of EAE. The mice would be vaccinated with peptides from the MOG, MBP and PLP_{178} spread epitopes. Once activated, these cells will be removed with combination inhibitor therapy. We would then induce EAE, via the standard protocol with PLP_{139}, and look at the rate of disease relapse. If our hypothesis is correct, mice that have been pre-purged of their spread epitope specific T cells will initially undergo normal disease progression since the first phase is mediated by PLP_{139} specific T cells. However, they should

not have any relapse after the primary phase of disease since we have removed the mediators of disease relapse and progression.

Applying the selective ablation of auto-reactive T cells to Type 1 Diabetes

The use of etoposide and combination inhibitor therapy to kill pathogenic activated T cells is not solely applicable to the treatment of MS/EAE. Our protocol is designed to kill activated effector T cells regardless of their specificity. This opens the use of our therapeutic approach to a wide variety of diseases where pathogenesis is mediated by activated T cells. Type 1 diabetes (T1D) is another T cell-mediated autoimmune disease where the removal of pathogenic self-reactive T cells would be a promising therapeutic approach. Unlike MS there are no immune modulating treatments currently FDA approved for the treatment of T1D. This is mainly due to the fact that insulin replacement has proven effective in the management of symptoms. However, administration of exogenous insulin does nothing to prevent immune-mediated damage of islets, and in some cases can further pathogenesis through the induction of insulin specific auto-antibodies (48). Studies in NOD/LtJ mouse, a model for spontaneous induction of T1D, have demonstrated that T cells are both necessary and sufficient to cause disease (51). Transfer of islet-specific TCR transgenic $CD4^+$ T cells is able to induce diabetes in both Rag KO and SCID mice that contain no endogenous lymphocytes. Since self-reactive $CD4^+$ T cells are the main drivers of pathogenesis in T1D, as they are in EAE, we postulate that our treatment protocols to purge these pathogenic T cells with etoposide or combination inhibitor therapy will be equally effective in the treatment of T1D.

Our preliminary data indicates that our treatment protocols to purge diabetogenic T cells in NOD/LtJ mice are effective for the treatment of T1D. As previously discussed transfer of islet-specific TCR transgenic $CD4^+$ T cells into Rag KO mice will induce diabetes. However, if the recipient mice are treated with either full strength etoposide (50mg/kg) or a combination of AZD7762 and nutlin following T cell transfer, these treatments will prevent the onset of diabetes and the mice will remain at a normal glycemic level (data not shown). The onset of diabetes is prevented in this model through the deletion of the transferred diabetogenic T cells, which are undetectable after treatment in either the spleen or the pancreatic lymph node. These results predict that our protocol can kill activated T cells in the context of diabetes.

Islet transplantation has been proposed as a means to revive endogenous insulin production in diabetic patients (49, 50). In our transplantation protocol islets are isolated from a non-diabetic donor mouse and placed under the kidney capsule of a diabetic mouse. From the kidney these new islets can reestablish proper blood glucose regulation. Unfortunately, in patients given transplanted islets average longevity for the islet graft is approximately 2 years (51). Thus immune-mediated rejection can occur by two different mechanisms: first is the islet-specific T cell response that initiated the original islet destruction. DDR pathway intervention has the potential to deal with this immune response.

An additional application is in allo-graft rejection. Humans can express six different class I MHC molecules on a graft. Variation in any of these can induce an allo-reactive response capable of inducing graft rejection. To prevent the rejection of, for example an allo-islet graft, our therapeutic approach may be extended for the removal of allo-reactive effector T cells.

Applying treatment to the ablation of allo-reactive T cells in transplantation

The use DDR pathway intervention therapy to remove allo-reactive lymphocytes in organ transplantation could have a major effect on the transplantation field. The current therapeutic protocols to prevent graft rejection following transplantation are broad immune suppression with corticosteroids and calcineurin inhibitors. Additionally alemtuzumab and anti-thymocyte globulin are used to suppress the immune system (52). The use of these therapies for organ transplantation results in the exact same adverse effects, including increased susceptibility to infections and malignancies as seen with MS treatment (see introduction for full discussion). As we have demonstrated DDR pathway intervention therapy is not broadly immuno-suppressive, and can function against a polyclonal T cell response.

Our preliminary investigations into purging allo-reactive immune responses have focused on the use of etoposide in a model of skin grafting. Skin from the ear of BALB/C mice is transplanted onto the flank of C57BL/6J mice to test the effects of etoposide treatment on allo-graft rejection (Figure 2A). Treatment with three doses of etoposide every other day following surgery has been shown to significantly delay graft rejection (Figure 2B). At this point treatment with etoposide does not lead to long term graft survival. Future studies will need to be conducted with combination inhibitor therapy to see if this protocol can further graft survival. Another tactic would be to attempt to remove the allo-reactive response prior to surgery, which may prevent any damage to the transplanted graft.

Unlike the development of autoimmunity, which happens spontaneously, there is generally prior indication that an organ will be transplanted into a patient. This prior warning does allow for preventative measures to be taken prior to surgery to prevent an allo-response from the immune

repertoire. We have demonstrated that DDR pathway intervention therapy can kill T cells following activation *in vivo*. This works against T cells reactive towards self-proteins, such as

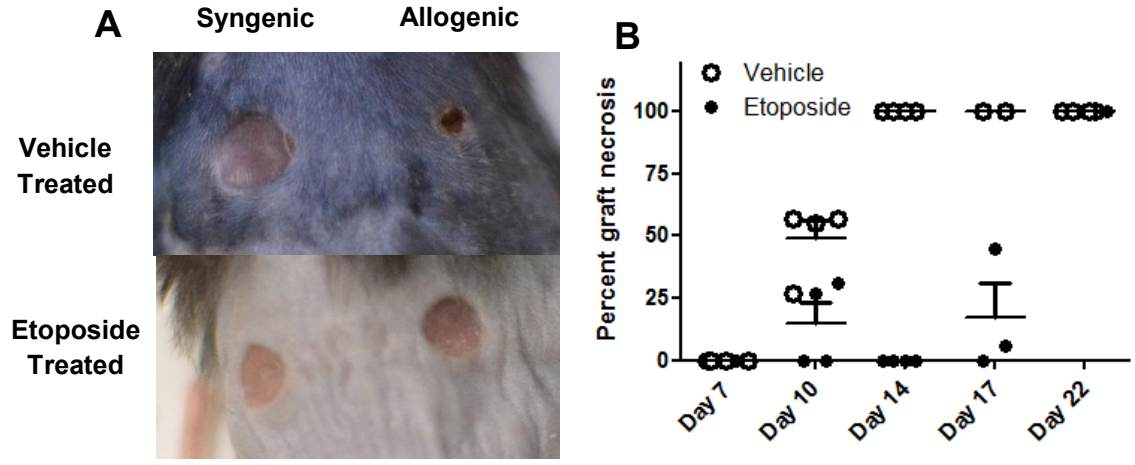

Figure 2: Etoposide treatment delays the rejections of allogenic skin grafts. Two skin grafts were implanted into the back of recipient WT C57BL/6J mice, an allogenic graft from a BALBC/J mouse was place on the right, and a syngenic control graft from a B6 mouse was place on the left side. The recipient mice were treated with etoposide (50mg/kg) at days 2 and 5 following surgery. A) Representative images from recipient mice at 14 days following surgery. B) Percent necrosis of allogenic graft over time. Statistical analysis was performed using students T test. n=4

MOG, or exogenous proteins, such as the LCMV glycoprotein. This strategy may also be an effective approach to remove allo-reactive T cells prior to surgery, and prevent graft rejection. Immunization with irradiated PMBCs from the organ donor will induce an allo-response, which would lead to graft rejection after surgery. However, once these allo-reactive T cells are activated they are susceptible to DDR pathway intervention therapy. Immunization of C57BL/6J mice with irradiated BALB/cJ splenocytes will induce K^d and D^d reactive T cells. Treatment with etoposide can reduce the frequency of BALB/cJ reactive T cells to frequencies

below the unprimed naive repertoire (data not shown). Pre-purging with DDR pathway intervention therapy can reduce the frequency of potentially detrimental allo-reactive T cells, however, further investigations need to be conducted to determine how pre-purging allo-reactive T cells prior to transplantation affects long term graft survival. The caveat to this approach is that it would only be applicable in cases where the mismatched MHC alleles are known with a few days advanced notice. But, treatment does have the potential to limit the use of broad-spectrum immune suppression following organ transplantation.

Investigation of the effects of therapy on B cells

This study focused on the removal of self-reactive T cells that are pathogenic in an autoimmune context. We have demonstrated that the various therapeutic approaches, which are described above, are effective for the removal of activated T cells that cause damage in a predominately T cell-mediated autoimmune disease. While T cells play a major role in many autoimmune diseases, many other autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, myasthenia gravis and Grave's disease, pathogenesis is driven by auto-reactive B cells. Therefore, for the successful treatment of this category of autoimmune diseases auto-reactive B cells would need to be eliminated. We have demonstrated that etoposide does not kill naïve B cells, however, its effects on activated B cell still need to be investigated. Activated B cells induce recombination activating gene (RAG) mediated DSB in the DNA to edit their B cell receptor in the periphery, during both affinity maturation as well as Ig class switching (53). RAG mediated DSB can activate the DDR pathway in B cells (54, 55). Further activation of the

DDR pathway in B cells may make activated B cells even more susceptible to combination inhibitor therapy than T cells.

In the context of MS/EAE the effects of DDR pathway intervention therapy on B cells can be investigated in the MOG protein model of EAE. This generates MOG-specific B cell clones which can be monitored following DDR pathway intervention therapy. However, since B cells play a minor role in EAE, it is probably not the best model to investigate the effects of DDR pathway intervention therapy on effector B cells. Instead the collagen induced arthritis (CIA) model of rheumatoid arthritis (RA), which is an inducible B cell driven model of autoimmunity, would be a more suitable model to study the effects of DDR pathway intervention therapy on effector B cells. Treatment of CIA with topoisomerase inhibitors have been shown to be effective, giving further credence for the treatment of RA with combination inhibitor therapy (56). Following our studies in EAE, the effects of DDR pathway intervention therapy on RA development can be investigated following treatment both after disease induction, after the development of collagen specific auto-antibodies and after the development of symptoms, such as joint swelling. As a control we can verify the efficacy of treatment by assessing the loss of collagen specific $CD4^+$ T cells using the I-A^q bovine collagen $II_{271\text{-}285}$ MHC class II tetramer, to determine the clearance of activated T cells. This model will allow for the simultaneous investigation of combination inhibitor therapy on both self-reactive T cell and B cells in the same disease model.

Concluding remarks

A common criticism that we have received about combination inhibitor therapy is that it does not specifically target encephalitogenic T cells but rather activated T cells. We are targeting the inherent vulnerabilities of rapidly dividing T cells, therefore all activated T cells become targets of the therapy. Other groups are trying to further increase the specificity of their therapies by directly targeting a specific, antigen-specific T cell clone, i.e. MOG_{35-55}-specific T cells. While combination inhibitor therapy does not target via antigen dependence, we would argue that this approach adds utility to our treatment strategy rather than being a flaw. Targeting MOG_{35-55}-specific T cells in a mouse model may be a viable approach in EAE. Human autoimmune diseases, including MS, are mediated by a polyclonal T cell response. The ability to target all activated T cells greatly increases the utility of combination inhibitor therapy to kill any pathogenic T cell clone. This is independent of whether the T cell is reactive to MOG, PLP, or MBP in the context of MS, collagen, insulin, or EBV. It is the utility of combination inhibitor therapy that gives it so much promise for the treatment of multiple diseases, including RA, T1D and HLH, as well as organ transplantation. Combination inhibitor therapy provides a method to target pathogenic T cells in relevant disease models that is non-genotoxic and specific for its target, resulting in a treatment with minor off-target effects.

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