THE ROLE OF MAD4, MNT AND C-MYC PROTEINS IN OX40 STIMULATED T CELLS

By

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CERTIFICATE OF APPROVAL

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List of Abbreviations

Ag- Antigen

AICD- Activation induced cell death

APC- Antigen presenting cell

BAD- BLC2-agonist of cell death

B cells- B lymphocytes

bHLHZ- Basic helix-loop-helix-leucine zipper

BiFC- bimolecular fluorescence complementation

BIM- BCL2-interacting mediator of cell death)

CDK- Cyclin-dependent-kinase

ChIP- Chromatin Immunoprecipitation

CK2- Casein kinase 2

CTLA-4- Cytotoxic T lymphocyte antigen 4

CRM1- Chromosome region maintenance 1

DC- Dendritic cell

DD- death domain

EAE- Experimental Autoimmune Encephalomyelitis

eIF4E- Eukaryotic inhibition factor 4E

FACS- Fluorescence Activated Cell Sorting

FOXO3a- forkhead box O3a

GSK- glycogen synthase kinase

HAT- Histone acetyltransferase

HDAC- histone deacetylase

HSV-1- Herpes Simplex Virus 1

IFN-γ- Interferron-gamma

Ig- immunoglobulin

Inr- Initiator

IRE- internal regulatory element

i.v.- intravenous

JNK-c-Jun N-terminal kinase

KO- Knockout

IL-2 (4,5,9,10,12,13)- Interleukin-2 or 4,5,9,10,12 or 13

LCMV- Lymphocytic choriomeningitis virus

LPS- lipopolysaccharide

MBI (II, III, IV)- Myc homology box I, II, III or IV

MEF- Mouse embryonic fibroblasts

MHC- Major-histocompatibility complex

Miz1- Myc-interacting Zn-finger protein 1

Mmip-1- Mad-member-interacting protein

MMP- matrix metalloproteinase

MTOR- mammalian target of rapamycin

NES- Nuclear export signal

NF-κB- Nuclear factor-kappa B

ODC- Ornithine decarboxylase

PCC- Pigeon cytochrome *c*

PI3K- phosphatidylinositol-3-kinase

PCR- Polymerase chain reaction

PKB- Protein kinase B

POZ- poxvirus and zinc finger

PP2- Protein phosphotase 2

PTEN- phosphatase and tensin homologue deleted on chromosome ten

RBC- Red blood cells

RING- Really interesting new gene

RSK- p90 ribosomal kinase

S6K- p70 S6 kinase

SAGE- Serial analysis of gene expression

s.c.- Subcutaneous

SEB- Staphylococcal Enterotoxin B

SID- mSin3 interaction domain

STAT4- Signal transducer and activator of transcription 4

T cells- T lymphocytes

TCR- T cell receptor

Th1- CD4 T helper 1

Th2- CD4 T helper 2

TLR- Toll-like receptor

TNF- Tumor necrosis factor

TRAF- Tumor necrosis family receptor-associated factor

URE- upstream regulatory element

WT- Wild type

Thesis Abstract

We sought to determine the molecular mechanisms by which OX40 stimulation enhances T cell function. Gene array analysis of OX40-activated Ag-specific T cells suggested a potential role for the transcription factor c-Myc and the antagonist Mad family proteins. Our model system was comprised of OVA-specific D011.10 T cells which were adoptively transferred into BALB/c recipients, which were then immunized with soluble OVA and an agonist anti-OX40 antibody or rat Ig control. Using this model system, we previously showed that the OX40 agonist antibody enhanced Ag-specific T cell proliferation and subsequent survival. Western blot analysis of Ag-specific T cells purified *ex vivo* from draining lymph nodes (DLNs) revealed that Mad4, Mnt and c-Myc proteins were all up-regulated at day 3 after OX40 engagement. Mad4 and Mnt protein levels peaked at 3-4 days after immunization and anti-OX40 engagement then decreased later as the cells contracted in size. In contrast, c-Myc protein levels remained unchanged over time following anti-OX40 stimulation. Anti-OX40 stimulation stabilized both Mad4 and Mnt proteins against post-translational degradation, and we demonstrated the presence of a serine residue in Mad4 that was essential for mediating accelerated protein degradation. We hypothesize that the up-regulation of c-Myc in activated T cells drives proliferation and subsequent activation-induced cell death, which is counteracted by Mad4 and Mnt proteins after anti-OX40 engagement. This increase in Mad4 and Mnt protein levels allowed for T cells to survive the “blast crisis” phase. Consistent with this hypothesis, siRNA knockdown of Mad4 and Mnt proteins led to decreased survival of anti-OX40-activated T cells. These data provide evidence that Mad4 and Mnt most likely play a role to enhanced memory T cell survival following anti-OX40 engagement.

Chapter 1

Introduction

The Immune System

The immune system is a body wide network composed of many interdependent cell types and tissues that collectively protect an organism from bacterial, parasitic, fungal and viral infections as well as from the growth of tumor cells. The organs of the immune system are classified as either primary or secondary lymphoid organs. The primary lymphoid organs include the bone marrow and the thymus, which are sites of lymphocyte differentiation; B lymphocytes (B cells) differentiate in the bone marrow, while T lymphocytes (T cells) differentiate in the thymus for positive and negative selection and eventual emigration to the periphery. The secondary lymphoid organs include the spleen, lymph nodes, tonsils, appendix and Peyer's patches within the small intestine. The immune system can mount an active "immune response" within these organs.

The immune system can be divided into two systems: innate and adaptive. Cells from the innate immune system have receptors that recognize a limited number of molecules, some of which are evolutionarily conserved and shared by many organisms such as lipopolysaccharides, peptidoglycans, non-methylated CpG and double-stranded RNA (1-4). Many of the innate recognition receptors are called pattern-recognition receptors and include the Toll-like receptor (TLR) family (1-5). Adaptive immunity refers to T cell and B cell mediated responses and includes "memory", that enhanced recall responses against a specific antigen.

The adaptive immune response is mediated by engagement of the T cell receptor (TCR). The TCR binds its ligand, a peptide bound to the major histocompatibility complex (peptide-MHC), which is a cell surface protein expressed on an antigen-presenting

cells (APCs). The specificity of ligand binding is determined by the clonotypic TCR, which arises from a process of recombination and is critical for generating an optimal and productive T cell response to antigen (Ag). The immunoglobulin (Ig) superfamily member CD28 is considered to be the primary costimulatory receptor. Engagement of CD28 by its receptors, B7-1 (CD80) and B7-2 (CD86), which are expressed on appropriately activated APCs, leads to enhanced expression of transcription factors, anti-apoptotic genes, cytokines and cytokine receptors that are necessary for survival, differentiation, avoidance of anergy and effector T cell function (11, 17). CD28 signals regulate early cytokine production; in particular IL-2 and IL-2 receptor (IL-2R) signals which may provide additional early anti-apoptotic signals. However, signaling through just CD28 alone is not sufficient for long-term T cell survival. Additional costimulatory signals are necessary for the development of long-term memory T cells.

Another molecule that is expressed after T cell activation is cytotoxic T lymphocyte antigen 4 (CTLA-4). CTLA-4 is a coinhibitory molecule and can also can bind to CD80 and CD86. Signaling through CTLA-4 provides a negative signal that counteracts the CD28 signal and limits T cell expansion (8, 9, 11, 12). In addition to the interaction between CD28 and B7, interaction between members of the tumor necrosis family receptor (TNFR) family with their ligands can play a role in T cell survival. There are three groups of TNFR family members: death domain (DD)-containing receptors, decoy receptors and TNF receptor-associated factor (TRAF) binding receptors (18-20). Signaling through TNFRs that contain a DD leads to downstream activation of caspase cascades and can induce apoptosis. On the other hand, TRAF binding receptors lack a DD and recruit the TRAF proteins and

activation of these receptors are associated with cellular activation, differentiation and survival (21). Members of the TNFR superfamily include OX40 and 4-1BB, amongst other molecules. Engagement of OX40 by an agonist antibody or its natural ligand (OX40L) leads to increased cytokine production, enhanced proliferation and development of increased numbers of long-term memory T cells. 4-1BB has highly similar functions to OX40; it is up-regulated after Ag and CD28 engagement and provides late-acting signals upon ligand binding (4-1BBL) that can also promote T cell survival (13, 22-25) (Figure 1.1).

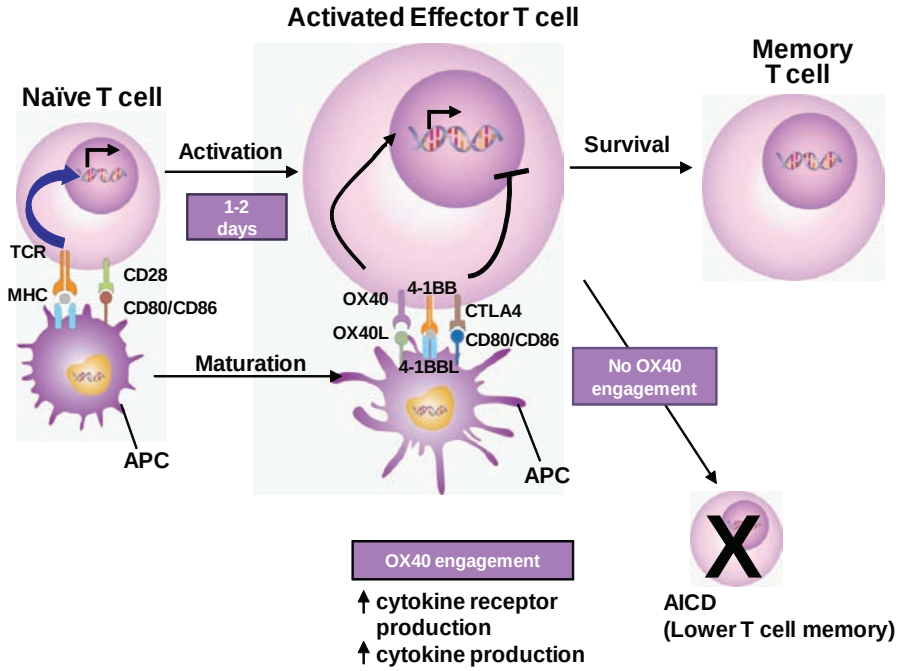


Figure 1.1: Role of OX40-OX40L interaction on T cell function. Optimal activation of naive T cells occurs after antigen recognition and signaling through early co-stimulatory molecules such as CD28-CD80/CD86. Activated T cells express OX40, 4-1BB and cytotoxic T lymphocyte antigen 4 (CTLA-4) on their cell surface. Engagement of OX40 on T cells by OX40L on APCs leads to increased proliferation and increased survival of memory T cels. In the absence of OX40 engagement, there is decreased cell survival and the majority of cells die by activation induced cell death (AICD) and fewer memorty T cells develop. Adapted from Sugamura et al (2004) Nat Rev Immunol. 4:420-431, with permission from Nature Publishing Group provided by Copyright Clearance Center.

Mechanisms of T cell death

Following T cell activation, the Ag-reactive T cells can undergo extensive rounds of proliferation and expansion, sometimes leading to a >1000-fold increase in the responding cells within a period of a few days (26). After the Ag has been cleared, the majority of expanded cells die by apoptosis, which counteracts the clonal expansion and only a few of these cells are left to survive and become long-lived memory T cells (27).

Apoptosis is characterized by a series of ordered morphological changes within the cell that ultimately lead to death (28). Apoptosis differs from necrosis which is involves organelle swelling and dissolution of the membranes. Apoptotic cells become rounded and shrink in size and there is blebbing of the plasma membrane. The nucleus and cytoplasm condense and the nuclear envelope and the nucleolus disintegrate and the chromatin is degraded at internucelosomal linker sites, resulting in multiple 180 base pair fragments. Subsequently, the mitochondria break down and release cytochrome c into the cytosol (29-34). The apoptotic cells are rapidly phagocytosed by adjacent cells or macrophages (35-39), which can make it difficult to detect the presence of apoptosis or apoptotic cells *in vivo.*

The molecular mechanisms leading to apoptosis of activated T cells is under debate and they are believed to be mediated by cell surface receptor proteins as well as intracellular proteins. Receptors that mediate apoptosis are cell surface proteins that are part of the TNFR family and contain an intracellular death domain. The family includes the Fas and TRAIL receptors (39). Early studies pointed to signaling through Fas (CD95, APO-1) via FasL (CD95L, APO-1L) engagement as a mediator of activation-induced T

cell death (31-44). Binding of Fas to FasL, induces the formation of an intracellular "death-inducing signaling complex" (DISC), which includes caspase 8, its adaptor/activator FADD, and its modulator c-FLIP (caspase-8 inhibitory protein) (31-44, 47-51). Caspases are part of a family of cysteine proteases that act as effectors of the cell death pathway (52). Caspase-8 is activated in the DISC upon Fas ligation and induces downstream caspase signaling cascades and subsequent cleavage of specific proteins which include lamins, topoisomerases, DNA-dependent protein kinase (DNA-PK), poly(ADP-ribose) polymerase (PARP) and some cell cycle regulators (53), which ultimately results in cellular apoptosis. In contrast to the Fas/FasL model, studies have shown T cells can die in the absence of Fas/FasL and signaling by regulation through intracellular protein (54-59).

Data has shown that activated CD4 and CD8 T cells can die due to Ag and cytokine withdrawal which leads to reduced expression of pro-survival proteins (15-16). The decline in Ag concentration leads to decreased stimulation of the TCR and therefore less activation of the REL-NF-κB and NF-AT transcription factors which are promote the expression of the pro-survival proteins Bcl-2 and Bcl-X_L (60). IL-2 and IL-7 are necessary for T cell survival and are also believed to up-regulate the pro-survival protein, Bcl-2 (59, 61-62). There are three subfamilies of the Bcl-2 family which include the pro-survival Bcl-2 family (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1), the pro-apoptotic Bax family (Bax, Bak and Bok) and the pro-apoptotic BH3 family (Bad, Bid, Bik, Blk, Hrk, BNIP3 and BimL). It has been proposed that the balance between the pro-survival protein Bcl-2 and the pro-apoptotic protein Bcl-2-interacting mediator of cell death (Bim) modulates survival and death of activated T cells. Bim is a member of the BH3-only proteins

due to the homology they have with Bcl-2 family members is within the BH3 domain (61). Bim was initially identified by its ability to bind the pro-survival protein Bcl-2 and inhibits its function (61).

Activated T cells express decreased levels of Bcl-2 at the peak of their response, just before they begin to contract and undergo apoptosis in vivo (63). The decrease in Bcl-2 is correlated with decreased survival *in vitro* and conversely, overexpression of Bcl-2 restores T cell survival (59). Decreased levels of Bcl-2 expression could be due to the cytokine withdrawal, as it is known that cytokines (such as IL-2 and IL-7) increase Bcl-2 expression (64-65). Decreased cytokine concentration leads to the reduction of the proteins PI3K and Akt, which are important for T cell survival. Supporting evidence came from *in vivo* studies which showed T cells could be rescued from apoptosis by injection of cytokines such as IL-7 after treatment with Staphylococcal Enterotoxin B (SEB) Ag (66-67). Pellegrini et al. (2003) demonstrated that Bim-induced apoptosis after T cell activation occurs in a Fas independent manner. They used a model examining the T cell response to herpes simplex virus (HSV-1) infection in Fas mutant lpr mice and Bim knockout mice. The results indicated that the kinetics of proliferation and contraction of the Ag-specific CD8 T cells was similar in WT, lpr and bim-/- mice in the DLN, however, there was an increased accumulation of CD8 T cells in the spleen of bim-/-mice. Their results indicated that Fas was dispensable but Bim was critical for T cell apoptosis after the Ag was cleared (59). The model predicts that cytokine withdrawal causes the balance between the pro-apoptotic levels of Bim versus the anti-apoptotic levels of Bcl-2 tend to shift in favor of driving apoptosis. The signals that control the induction of Bim in activated T cells are still unclear. Decreased

levels of Akt after cytokine withdrawal allows for increased transcriptional activation of forkhead box O3a (FOXO3a), which can induce expression of Bim (59, 68). In other cell types, it has been suggested that Bim is upregulated after growth factor withdrawal (69-71), however a report showed the levels of Bim were not different in activated versus resting T cells (54). Another potential mechanism for induction of Bim could be by through the transcription factor c-Myc (72). The exact mechanisms regulating Bim expression and the mechanisms that control activated T cell death still remain to be fully elucidated. These pathways may be important in mediating T cell survival after stimulation through anti-OX40.

OX40

OX40 (CD134) was identified as a cell surface marker on activated rat CD4 T cells (73). It is expressed on activated but not resting CD4 and CD8 T cells in mice and humans (74-78). OX40 is up-regulated on T cells 1-2 days after Ag and CD28 stimulation and is transiently expressed for up to 120 hours (79). The ligand for OX40 (OX40L) is a membrane-bound member of the TNF family. OX40L is expressed on activated antigen-presenting cells (APCs), such as activated B cells, dendritic cells, macrophages and Langerhans cells, as well as T cells and endothelial cells (80-87).

The extent of OX40 stimulation of T cells during an immune response is regulated at the level of OX40L expression on APCs. OX40 is expressed on all CD4 and CD8 T cells after TCR engagement, however, the expression of OX40L is more tightly controlled. T cell activation in the absence of a strong adjuvant leads to minimal

expression of OX40L (88). Therefore, in this circumstance, the majority of $OX40^+$ T cells will not be engaged by the natural ligand. Therefore, the extent of OX40 signaling *in vivo* is dependent on the level of OX40L available for binding to OX40.

Role of OX40 in promoting cell survival

OX40 stimulation has been shown to prolong T cell survival beyond the effector T cell stage and thus increases the generation of memory T cells. Initial studies showed that stimulation of OX40 *in vivo* with an agonist antibody or OX40L:immunoglobulin (Ig) fusion protein, in combination with a "danger" signal (LPS), rescued super-Ag-(a potent T cell mitogen) stimulated CD4 T cells from peripheral deletion (77). Increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-X_L have been observed following OX40 engagement (89-90). Conversely, these protein levels are decreased in activated OX40 KO T cells (89-90), which provides further evidence for the role of OX40 in T cell survival.

The effects of OX40 engagement on T cell survival have been studied in detail using the CD4 immunization model (Figure 1.2A). The use of an agonist Ab to OX40 promotes increased expansion and survival of CD4 memory T cells. This model uses soluble antigen (in the absence of adjuvant) to stimulate transgenic CD4 T cells (D011.10 model) or transgenic CD8 T cells (OT1 model). The Ag-specific T cells can be followed using an anti-idotypic or an allelic variant antibody, such as KJ1-26 or Thy1.1, respectively. Immunization with Ag and anti-OX40 led to a 15-fold increase in the long-term survival of Ag-specific CD4 T cells compared to Ag and control antibody. When animals were treated with a triple combination of Ag, anti-OX40 and LPS, a 60-fold increase in surviving cells

was demonstrated compared to treatment with Ag alone (77). Increased memory T cell development was also observed using an anti-OX40 monoclonal antibody in combination with two different adjuvants (complete Freud's adjuvant or alum with pertussis) (91). Peripheral migration of T cells was also enhanced after OX40 stimulation; increased numbers of Ag-specific T cells were detected in the peripheral blood and other peripheral organs and these cells were functional as they produced cytokines upon *ex vivo* stimulation with cognate Ag (92). Elevated levels of the Ag-specific peripheral blood T cells were maintained 196 days after immunization and OX40 treatment (7). These data provide evidence that OX40 stimulation leads to increased survival of long-term Ag-specific memory T cells.

The initial OX40 studies provided evidence that OX40 engagement enhanced CD4 T cell responses but recent data has shown that OX40-mediated signals can also augment CD8 T cell functions *in vitro* and *in vivo* (93-96). Antigen-specific CD8 T cells show increased expansion and interferon-γ (IFN-γ) production in response to OX40 stimulation (97). Treatment with the anti-OX40 agonist antibody significantly enhanced the effector differentiation of CD8 T cells *in vivo* in an IL-2 dependent manner (98). It has also been shown that OX40 engagement increases the generation of Ag-specific CD8 memory T cells surviving after the initial expansion. The OX40-mediated long-term maintenance and survival of CD8 T cells was dependent on the presence of CD4 T cells because depletion of CD4 T cells diminishes the response (98). CD8 T cell recall responses were also enhanced by OX40; treatment with anti-OX40 after re-challenge with Ag produced a robust recall response of Ag-specific CD8 T cells compared to mice treated with rat Ig control (97).

Proof for the role of endogenous OX40 in the absence of adjuvant antibody came from studies in OX40- and OX40L-deficient mice that lacked endogenous OX40/OX40L signaling in CD4 and CD8 T cells. OX40 deficient CD4 T cells secreted IL-2 and proliferated normally during the initial period of activation but developed fewer Ag-specific memory cells later. There was a delay in memory T cell development and there was a 10-20 fold reduction in the frequency of surviving, long-term memory T cells (91). In a lymphocytic choriomeningitis virus (LCMV) infection model, IL-2 production was decreased in OX40-/- mice and there was a 4-5 fold decrease in the number of IFN-γ-secreting CD4 T cells following infection (99). OX40L deficient mice have been shown to develop a lower frequency of Ag-specific Th1 and Th2 recall responses following immunization and showed a decrease in delayed-type hypersensitivity responses (99-100). The addition of OX40L-expressing B cells restored the defective Th2 responses (101). OX40 deficient CD8 cells showed similar proliferation to wild-type (WT) cells after initial Ag-stimulus but accumulation of primary effector cells was reduced 3-6 days after Ag-engagement (102).

The proinflammatory cytokine, interleukin-12 (IL-12) has been shown to play a role in OX40-enhanced T cell survival (103). Innate immune cells produce IL-12 after stimulation by viral or bacterial products through Toll receptors or CD40 stimulation. Signaling through IL-12 has been shown to enhance CD4 T cell differentiation, cytokine production, and survival (104). IL-12 is a heterodimeric cytokine, composed of the p35 and p40 subunits. Signaling through IL-12 requires the IL-12 receptor β2 subunit (IL-12Rβ2) and results in activation of downstream signaling molecules (104-106). Engagement of OX40 on Ag-activated CD4 T cells led to increased cell surface expression

of IL-12Rβ2. The expression of IL12Rβ2 was directly linked to OX40-mediated enhancement of T cell survival and was dependent on intracellular signaling through STAT4 (103). Beyond engagement of initial signaling cascades, the exact downstream signaling pathways that lead to OX40/IL-12 enhanced survival remains to be elucidated.

Signal transduction by OX40

OX40 signals through TRAF adaptor proteins, as has been shown with other TNFR family members that lack an intracytoplasmic death domain. The TRAF family is comprised of six genetically conserved adaptor proteins in mammals (TRAF1-6) (107-109), that can form heterotrimeric and homotrimeric signaling complexes (110-120). OX40 binds to TRAF2, TRAF3 and TRAF5 through the cytoplasmic tail of the receptor (121-122). TRAF proteins can signal through nuclear factor *kappa*-light-chain-enhancer of activated B cells (NF-κB) transcription factors and binding of OX40 to TRAF2 and 5 positively regulates NF-κB, whereas TRAF3 negatively regulates NF-κB (114, 117, 123). Others have shown that the NF-κB pathway is a principal target of OX40 signaling in primary T cells (124).

In mammalian cells, the NF-κB family consists of five members; c-Rel, p65/RelA, RelB, p50/p105 (NF-kB1) and p52/p100 (NF-κB 2). These proteins form homodimers and as well as heterodimers. Under resting conditions, NF-κB is inactive; it is sequestered to the cytoplasm and bound by IκB inhibitory molecules. The canonical NF-κB (NF-κB1) pathway is initiated by signal-dependent phosphorylation, ubiquitination and degradation of IκB. IκB phosphorylation is catalyzed by the IκB (IKK) complex that contains two homologous catalytic subunits, IKKα and IKKβ and the regulatory subunit IKKγ (125-

126). Activation of IKKβ subunits is essential for the NF-κB1 pathway in response to all pro-inflammatory stimuli (127-129). Release of NF-κB from IκB allows NF-κB complexes to translocate to the nucleus and activate downstream target gene transcription.

In addition to activating NF-κB target pathways, downstream signals from OX40 can lead to phosphorylation of phosphatidylinositol-3-kinase (PI3K) and the downstream activation and maintenance of protein kinase B (PKB, also known as Akt). Akt is believed to be a central mediator of cell survival and conversely, Akt activity is not maintained in OX40-deficient cells (90). OX40 signaling through Akt up-regulates the expression of the anti-apoptotic members of the Bcl-2 family such as BCL-X_L and BFL1 (89). These proteins negatively regulate pro-apoptotic members such as Bcl-2-antagonist of cell death (BAD) and Bcl-2-interacting mediator of cell death (BIM). There is evidence that there is a direct link between OX40 signaling and Akt and Bcl-2 family members (90), although the evidence is controversial, as studies have shown Bcl-2 and Bcl-XL to be dispensable for T cell function and survival in certain models (103, 130-131). Retroviral expression of active Akt in OX40-deficient T cells results in the up-regulation of expression of anti-apoptotic Bcl-2-family member proteins and completely reverses the defect in T cell survival, mimicking the action of forced expression of BCL-X_L or Bcl-2 (89). These studies provide evidence for some of the molecular events underlying the biological effects of OX40 engagement that are important for cellular proliferation, differentiation and survival (Figure 1.3).

Gene microarray studies

Signaling through OX40 enhances cell survival through regulation of anti-

apoptotic and pro-survival proteins. To further study the molecular mechanisms by which OX40 ligation influences T cell function, DNA microarray analyses (Affymetrix murine GeneChips) were performed to assess mRNA sequences that were differentially regulated in Ag-specific T cells activated by anti-OX40. Transcript levels were evaluated from *in vivo* Ag activated T cells and showed differential regulation of Mad4 and c-Myc mRNAs after anti-OX40 ligation in two different models: the primary immunization model (Figure 1.2) (7, 88) and a peripheral tolerance model (124-125).

In the primary immunization model, naïve D011.10 OVA-specific transgenic CD4 T cells were adoptively transferred into wild-type BALB/c mice and immunized with OVA in combination with agonist anti-OX40 or control (rat Ig) antibodies (Figure 1.2A). The effects of anti-OX40 engagement were compared a control antibody, as well as to anti-the monoclonal CTLA-4 antibody, which blocks the inhibitory function of CTLA-4, through mechanisms that are not yet understood. In contrast to the ability of anti-OX40 engagement to promote enhanced CD4 T cell proliferation and survival, CTLA-4 blockade leads to enhanced proliferation of CD4 T cells but did not promote long-term T cell survival. The microarray results showed a two-fold or greater increase in the expression of 44 genes after OX40 engagement *in vivo*, and a two-fold or greater downregulation of 112 genes (88). A 3-18-fold decrease in Mad4 transcript levels was observed in both CD4 and CD8 T cells after anti-OX40 engagement compared to either rat Ig or anti-CTLA-4 control in multiple experiments (Table 1.1). The transcript levels for c-Myc were slightly up-regulated in the CD4 T cell model (2-fold), but the levels were not changed in the CD8 T cells model. The transcript levels for the other

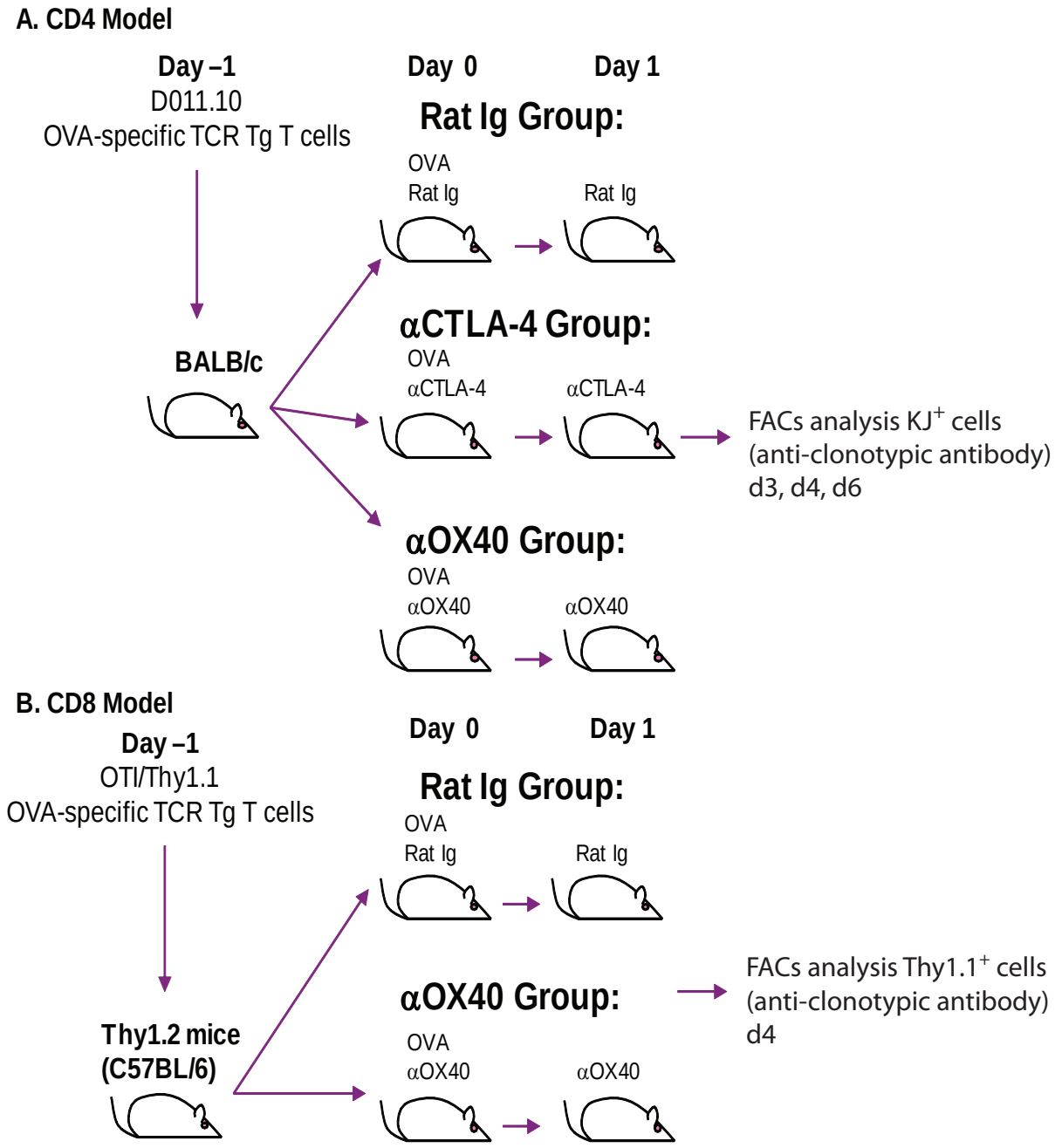


Figure 1.2: Immunization models. A. CD4 immunization model. 3 x 10^6 CD4/KJ^+ T cells were adoptively transferred i.v. into WT BALB/c recipients. One day later, mice were immunized s.c. with 500ug OVA and 50ug anti-OX40 (OX86) or 100ug anti-CTLA-4 (9H10) or rat Ig control. The following day, mice were given a second injection of anti-OX40 or anti-CTLA-4 or rat Ig. Cells were harvested from DLN at indicated times and Ag-specific cells were purified by magnetic bead sorting and analyzed using anti-CD4 and anti-KJ-126 antibodies. B. CD8 immunization model. 3 x 106 CD8/OTI^+ T cells were adoptively transferred i.v. into WT C57BL/6 recipients and treated as in (A). Cells were analyzed using anti-CD8 and anti-Thy1.1 antibodies.

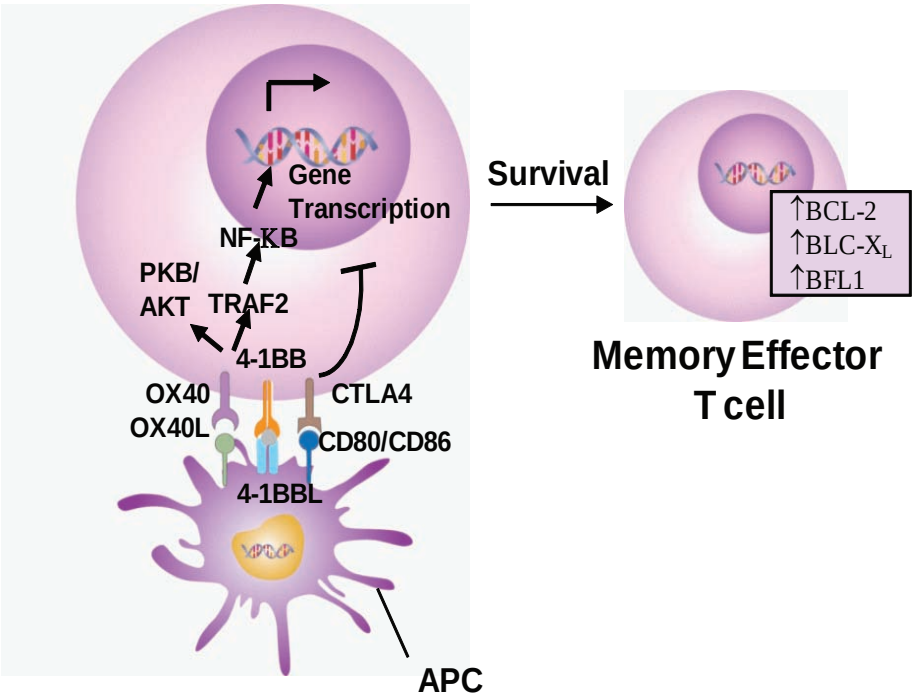


Figure 1.3: Signaling pathways after ligation of OX40. Ligation of OX40 on activated T cells leads to signaling through tumor-necrosis factor receptor-associated factor 2 (TRAF-2). Induction of TRAF-2 activates the transcription factor nuclear factor-kappa-B (NF-κB), which activates downstream target gene transcription. Engagement by OX40 also leads to sustained PKB/Akt activation and this has been shown to be important for cell survival. Additionally, OX40-mediated effects on survival are attributed to the increased levels of the anti-apoptotic proteins, Bcl-2 and Bcl-X_L and BFL1. Adapted from Sugamara et al., (2004) Nat Rev Immunol. 6:420-431, with permission from Nature Publishing Group provided by Copyright Clearance Center.

Mad family members were not different in either CD4 or CD8 T cells (Table 1.2).

Downregulation of Mad4 transcripts have also been described in a model of peripheral tolerance. Peripheral tolerance is a means of removing potentially autoreactive cells from the peripheral repertoire and can be induced through apoptosis, the induction of anergy (a state of immune nonresponsiveness), or suppression by regulatory T cells (132). Lathrop et al., (2004) showed that stimulation through anti-OX40 could break tolerance of T cells that would otherwise become anergic (132).

Table I: **Mad4 mRNA is downregulated after OX40 engagment.** Gene array analysis of anti-OX40 stimulated Ag-specific T cells.

Model	Ref	Cells	Timepoint	Treatment	Exp#	Fold Decrease Mad4
Primary Immunization Model	(37)	CD4	d3	Rat vs αOX40	1	-18.1
					2	-8.7
					3	-8.0
					4	-13.6
Primary Immunization Model	(37)	CD4	d3	αCTLA4 vs αOX40	1	-6.9
					2	-4.3
Peripheral Tolerance Model	(80)	CD4	d3	Rat vs αOX40	1	-6.9
					2	-4.3
Primary Immunization Model	(37)	CD8	d4	Rat vs αOX40	1	-2.6

Table II. **Transcripts from other Mad family members are not differentially regulated after OX40 engagement in Ag-specific T cells in the primary immunization model.** Gene array analysis of anti-OX40 stimulated Ag-specific T cells. Fold decrease is anti-OX40 compared to rat Ig. BD- below detection.

Gene	Cells	Timepoint	Rat Ig	anti-CTLA-4	anti-OX40	Fold Decrease Mad4
c-Myc	CD4	d3	314	327	694	2.0
	CD8	d4	1473		1987	1.5
Max	CD4	d3	783	1073	738	BD
	CD8	d4	441		464	BD
Mad1	CD4	d3	178	219	241	BD
	CD8	d4	BD		BD	BD
Mad2	CD4	d3	BD	BD	BD	BD
	CD8	d4	BD		BD	BD
Mad3	CD4	d3	203	216	318	BD
	CD8	d4	142		192	BD
Mnt	CD4	d3	277	309	364	BD
	CD8	d4	777		721	BD
Mga	CD4	d3	86	78	67	BD
	CD8	d4	104		92	BD

Gene array analysis from this model showed Mad4 transcripts were also down-regulated ~4-7 fold after OX40 engagement compared to control (Table 1) (131).

These results led to further study of the role of Myc and Mad proteins in T

cells after anti-OX40 engagement and is the focus of this thesis. Understanding the molecular mechanisms that mediate the biological effects of OX40 signaling could have clinical relevance in control of autoimmunity and cancer immunotherapy.

Clinical translation of OX40/OX40L

OX40 and OX40L have been used as targets in pre-clinical models. Blocking OX40-OX40L interaction or depleting $OX40^+$ T cells has been shown to reduce clinical signs of autoimmunity in animal models. In contrast, stimulation of OX40 through an agonist antibody in tumor-bearing mice can enhance T cell function and lead to increased tumor-specific T cell memory and enhanced anti-tumor immunity.

$OX40^+$ lymphocytes and $OX40L^+$ cells were detected at sites of inflammation in a number of T-cell mediated autoimmune, allergic and infectious diseases (133). $OX40^+$ T cells were first observed in sites of inflammation in a rat model for multiple sclerosis, termed experimental autoimmune encephalomyelitis (EAE). Therapeutic efficacy was demonstrated by depleting $OX40^+$ T cells (80, 134) or by blocking the OX40-OX40L interaction using *in vivo* agents that bind OX40L (recombinant OX40-immunoglobulin fusion proteins or an OX40L-specific antibody) (80, 134-146). Blocking OX40-OX40L interaction decreased clinical signs of disease in mouse models for diabetes, graft-versus-host-disease, inflammatory bowel disease and lung inflammation (80, 134-1146). OX40- or OX40L-deficient mice exhibited decreased disease symptoms in most autoimmune settings, implying a direct role for OX40-OX40L interactions in mediating these diseases (81, 100, 142, 147-153). In theory, the OX40-targeted therapy has the added benefit of avoiding

global immuno-suppression because it only targets recently activated T cells. Exploiting this target in human autoimmune disorders with therapeutic drugs are currently underway.

The ability of OX40 to promote survival of T cells has been used in the development of effective pre-clinical immunotherapies against tumors and/or chronic pathogens (91, 152-155). $OX40^+$ T cells have been detected within several human tumors including breast cancer, colon cancer, melanoma, head and neck cancer, prostate cancer and invasive bladder cancer (156). The tumor microenvironment can be immunosuppressive and induce Ag-specific T-cell tolerance but engagement of OX40 was shown to break T cell tolerance in mouse models (157-158). Treatment with OX40 agonists enhanced anti-tumor immunity and increased survival of tumor-bearing mice in several mouse models: melanoma, sarcoma, colon cancer, breast cancer and glioma (Figure 1.4) (154, 159-162). The anti-tumor immunity was dependent on the presence of both CD4 and CD8 T cells during tumor-specific priming. Ligation of OX40 created tumor-specific T cell memory populations; mice treated with anti-OX40 were resistant to subsequent challenge with the primary tumor, however, they

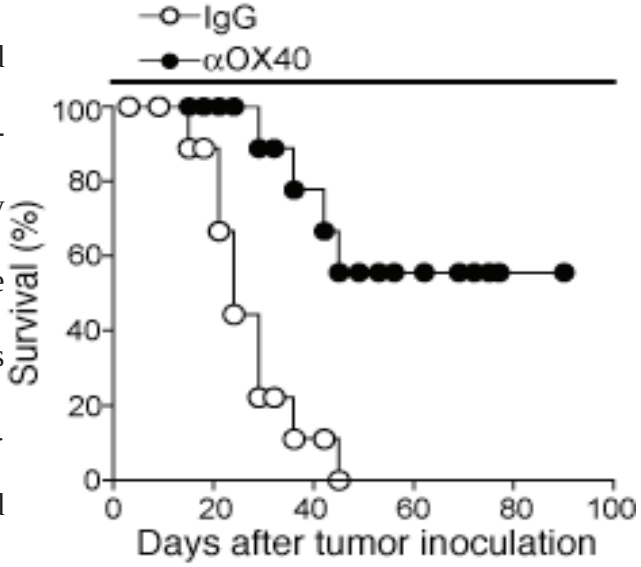


Figure 1.4: Antitumor effects of anti-OX40 engagement after s.c. inoculation with MCA205. Mice were inoculated with MCA205 sarcoma tumor cells s.c. Three to 7 days later, mice were injected with anti-OX40 or rat Ig i.p. The mice were then monitored for tumor growth. From Ruby and Weinberg (2009) J Immunol. 182:1481-1489. Copyright 2008 The American Association of Immunologists, Inc.

were susceptible to tumor growth when treated with a tumor of a different tissue origin (154). Our laboratory has produced a human-OX40-specific mouse monoclonal antibody and a human phase I clinical trial is currently underway in late-stage cancer patients.

The Max-interacting Network

T cell stimulation with anti-OX40 leads to increased cell size, proliferation and cell survival. The transcription factor c-Myc has been associated with each of these biological functions. Recent data from our lab indicated a role for the Myc antagonist, Mad4, in OX40 stimulated cells (7, 88). This led to the hypothesis that Mad4 and its family members are important for regulation of T cell function after OX40 engagement.

The Max-interacting network is a family of transcriptional regulators that play a key role in the regulation of cell growth, differentiation and apoptosis. The first *myc* gene was identified over 30 years ago as the transforming sequences of chicken retroviruses, which were able to transform myeloid cells, hence the name *myc* for myelomonocytic leukemia (163-164). Following the original discovery of c-Myc, the Max-interacting network has emerged. The network consists of the Myc family members (c-, N- and L-Myc), Max and the antagonist proteins which include the Mad family proteins (Mad1, Mxi1 (Mad2), Mad3, Mad4) and the related proteins Mnt and Mga (165-166). The Mad protein family members were originally identified by protein interaction screens testing their ability to bind to and form heterodimers with Max (167-169), and Max was initially identified by its ability to form heterodimers with Myc family proteins (170).

The Max-interacting proteins contain a basic region contiguous with a helix-

loop-helix-leucine zipper (bHLHZip) motif. The HLH and Zip motifs promote protein-protein interaction and the basic region mediates sequence-specific DNA binding. Mad1, Mxi1, Mad3 and Mad4 are closely related, while Mnt is distantly related to the Mad proteins and Mga is an unusual protein in that it contains both a bHLHZip domain and a T-domain DNA-binding motif (171). The Mad family members contain an alpha-helical amino-terminal domain termed the Sin3-interaction domain (SID) that is required for interactions with the transcriptional co-repressor Sin3a and Sin3b (172-174) (Figure 1.5).

The amino-terminus of the Myc protein sequence contains four domains called

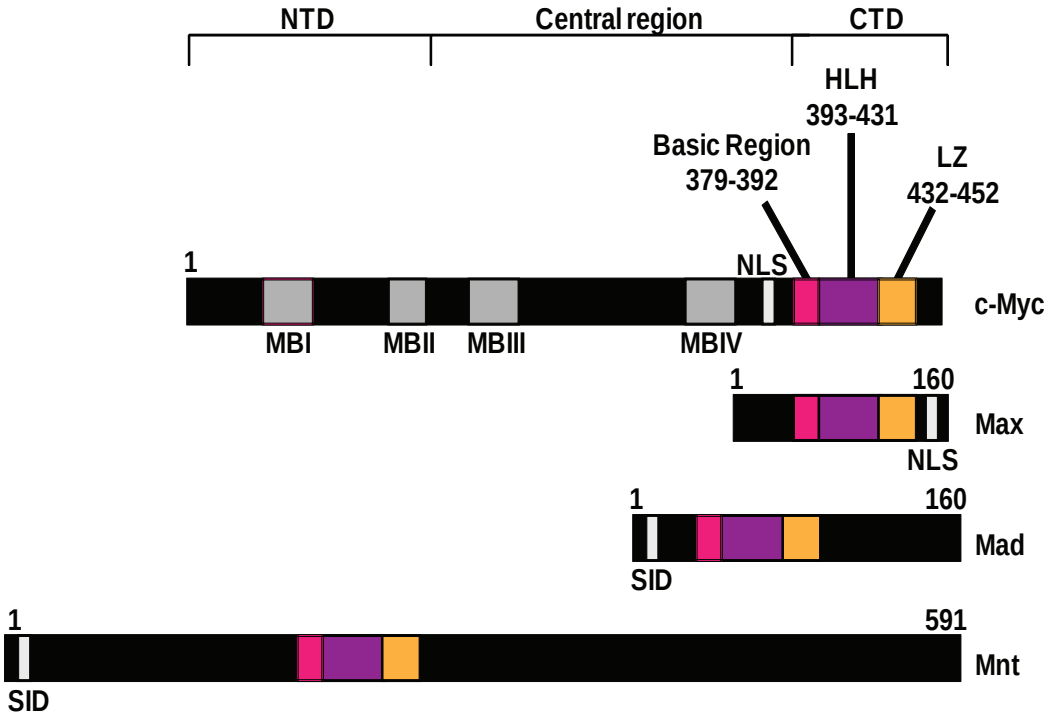


Figure 1.5: Structure schematic of components of the Max-interacting network. The known functional domains of different network components are summarized. MBI: Myc homology box I; MBII: Myc homology box II; MBII: Myc homology box III; MBIV: Myc homology box IV; NLS: nuclear localization signal; HLH: helix-loop-helix domain; LZ: leucine zipper domain; SID: mSin3-interaction domain. The number of anino acids refer to the amino of the human proteins. Adapted from Baudino and Cleveland (2001) Mol Cell Biol. 21:691--702, with permission from American Society for Microbiology.

Myc homology boxes (MBI, MBII, MBIII and MBIV), which are conserved in all Myc family members (168) (Figure 1.5). MBI has important functions in protein stability and MBII is also required for transcriptional activation and repression (176-177). MBIII has been studied less but is important for transcriptional repression and for transformation by c-Myc. Additionally, MBIII may function as a negative regulator of apoptosis (178). The MBIV domain is located in the central region, proximal to the bHLHZip domain and has been implicated in maximal DNA binding and gene expression and may also be important for apoptosis and transformation by c-Myc (179).

Max is constitutively expressed, whereas Myc and Mad family members are synthesized and rapidly degraded in response to extracellular stimuli. Max has a long half life (>24hours) (180) compared with the rapid turnover (15-20 minutes) of Myc, Mad and Mnt proteins (181-184). Unlike the other Mad family members, Mnt is expressed ubiquitously in proliferating and differentiating cells, but at varying levels (165, 185-186) . Myc, Mad and Mnt dimerize with Max through their bHLHZip domain. Myc and Mad proteins generally do not form homodimers or heterodimers with each other, however, Max can bind DNA as a homodimer (167-171, 184, 186-189). The Myc, Max, Mad-Max or Mnt-Max heterodimers bind specifically to a subclass of E-box elements, at the consensus sequence CACGTG.

Myc and Mad family proteins are believed to have antagonistic functions. Myc has a transcriptional activation domain and can activate transcription in a Max-dependent manner at promoter-proximal E-box sites (174, 187, 189). Mad-Max and Mnt-Max dimers appear to act as transcriptional repressors at these same sites (167). Myc proteins

induce cell proliferation, whereas Mad family proteins are associated with proliferative arrest and terminal differentiation (191-192). Genes encoding Myc family proteins are mutated or more commonly deregulated in many types of cancer and Mad family proteins can inhibit Myc-dependent cell transformation in cell culture experiments (193-194). Although Mad family members and Mnt exhibit similar biochemical properties, it has been suggested that Mad proteins have a specialized role as Myc antagonists, whereas Mnt probably serves a more general role antagonizing or regulating Myc activities (165).

Opposing regulation of chromatin structure and gene transcription

A model for transcriptional regulation by the Max-interacting network suggests that the activation state of shared target genes is mediated, at least in part, by opposing effects on histone acetylation and chromatin conformation (Figure 1.6). Repression by the Mad proteins is mediated by binding the co-repressor proteins Sin3A and Sin3B through their SID (165, 169, 195-196). The mSin3 proteins are part of a multi-subunit complex that contains histone targeting proteins and the histone deacteylases (HDAC), HDAC1 and HDAC2 (197-199). Deacetylase activity is crucial for Mad-mSin3 repression (199-200). The HDACs deacetylate the charged N-terminal tails of nucleosomal histones.

On the other hand, c-Myc interacts with co-activator proteins such as TRRAP and CBP/p300 that recruit histone acetyltransferases (HATs) (201-203). TRAPP binds to the MBII domain (204), and recruits the histone acetylase GCN5 (201). Additionally, INI1/SnF5 interacts with the bHLHZip domain which tethers the Swi-Snf complex and activates transcription through chromatin remodeling in an ATP-dependent manner (205-206).

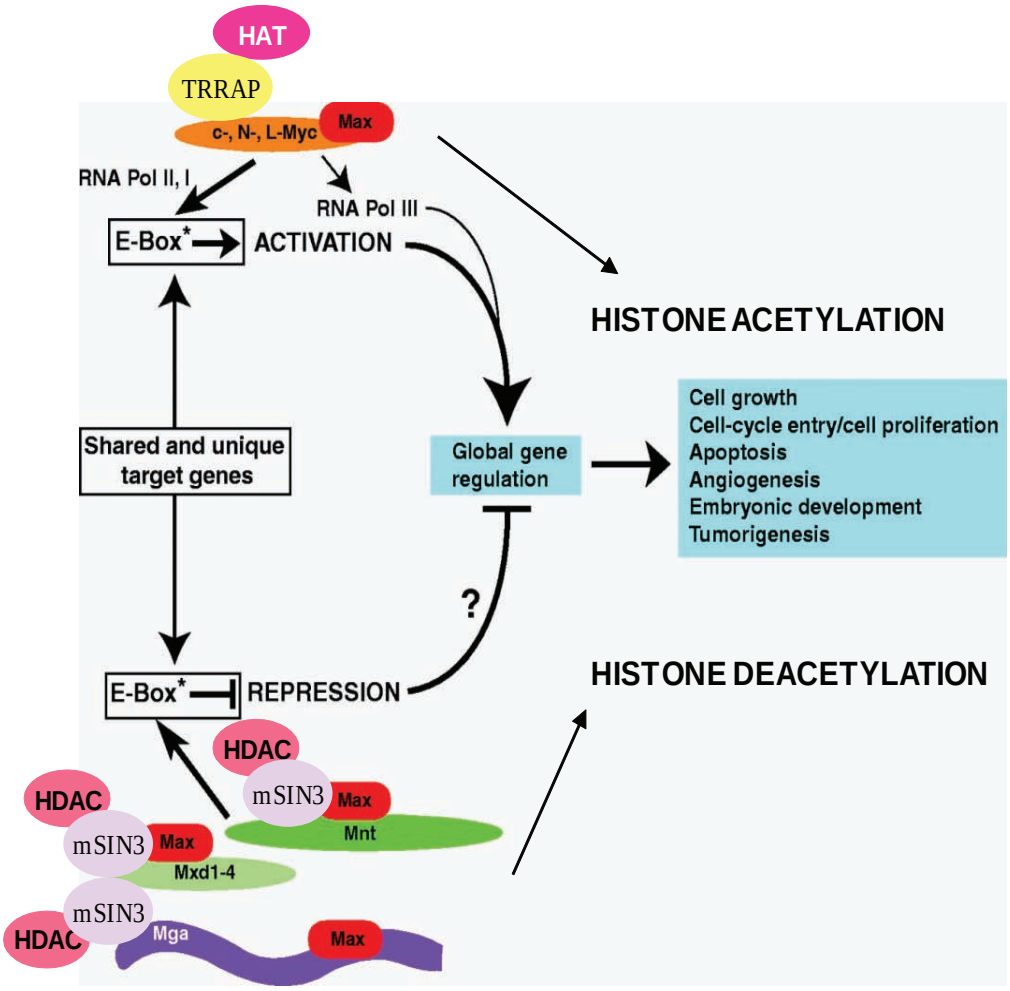


Figure 1.6: Working model for the relationship between activating Myc-Max complexes and repressive Mnt-Max, Mad-Max and Mga-Max complexes. Myc-Max complexes are thought to activate downstream gene targets by binding E box consensus sequences and recruiting chromatin modifying complexes such as TRRAP and histone acetyltransferases (HATs). Myc targets include gene that regulate cell growth and cell cycle and genes involved in cellular metabolism. Mnt-Max, Mad-Max, and Mga-Max complexes recruit mSin3 and histone deacetylase complexes (HDACs) and bind E-box sequences and repress transcription through histone deacetylation. Adapted from Hooker et al. (2006) J Cell Sci. 119:208-216. Adapted with permission from the Journal of Cell Science.

Reporter gene assays have demonstrated the importance of the recruitment of the SWI/SNF complex for the transactivation functions of c-Myc (193). Evidence has shown that Myc itself is also a substrate for these HATs (204, 208). Myc and Mad family proteins have opposing effects on chromatin regulation and they are believed to share similar target genes, however, it has not been definitively demonstrated that the HDAC-mediated repression by Mad family proteins is directed at all genes that are positively regulated by Myc (Figure 1.6).

Expression of Myc and Mad family proteins

Myc is virtually undetectable in quiescent cells in most cell types studied and mRNA and protein levels are up-regulated after mitogenic stimulation (182, 208). Forced expression of c-Myc in quiescent cells stimulates entry into the cell cycle (209). c-Myc is an immediate early gene and is rapidly induced upon mitogenic stimulation, to about 10-40-fold higher than in quiescent cells (210)**.** Its levels are highest at the G1 phase of the cell cycle, the levels rapidly decline by S phase and stay at a constant low level throughout the remainder of the cell cycle (181- 182, 208, 211-213). After mitogen withdrawal, c-Myc levels decline to very low levels and proliferating cells exit the cell cycle (182, 208, 212-213). c-Myc levels decrease to about 90% in differentiated cells (210). c-Myc is down-regulated in naïve quiescent T cells (214) but then up-regulated as part of the proliferative response after antigen-mediated TCR activation (215-216). To study the dynamic regulation of c-Myc in lymphocytes, Huang et al., (2008) generated a transgenic mouse that expressed an N-terminal GFP-c-Myc fusion protein (217). Naïve CD4 T cells expressed low levels of c-Myc-GFP and c-Myc-GFP levels were induced progressively after Ag-stimulation *in*

vivo between days 2 and 6, with the peak of expression at day 6 (217). These data provide evidence that c-Myc is part of T cell activation and differentiation.

Mad family gene expression is preferentially but not exclusively found in more differentiated cell populations *in vivo* and *in vitro* (169, 183, 217-219). There is extensive overlap in the expression patterns of the *Mad* genes during mouse embryonic development and in different adult tissues (171, 218-219). The expression of Mad3 is preferentially seen in proliferating cells that are in the S phase of the cell cycle (225, 219-221). Mnt-Max and Myc-Max complexes have been shown to coexist in a variety of proliferating cell types as demonstrated by co-immunoprecipitation and DNA-binding studies (165, 184, 223-224). The DNA-binding studies have suggested that Mnt-Max and Myc-Max complexes are the most abundant Max complexes in proliferating cells (166) (Figure 1.7).

Regulation of c-Myc expression

The expression of c-Myc is tightly regulated at several levels: transcription, elongation, translation, and the stability of the mRNA and protein (both which have a short half life of approximately 15-20 minutes) (181-185). Additionally, c-Myc is regulated at the post-translational level and through interaction with protein partners, such as by competition for dimerization with Max. After mitogenic stimulation, *c-myc* transcription is rapidly and transiently induced. In rapidly growing cells, the levels of c-Myc mRNA and protein are approximately equivalent. The regulation of the *c-myc* promoter is very complex and not well understood. Transcription of the *c-myc* gene is regulated at level of initiation as well as at the elongation and is positively and negatively regulated by multiple transcription factors

(210, 225).

Additional, *c-myc* is regulated by chromatin remodeling and through association of transcription factors with members of the SWI/SNF complexes and HATs that modulate the *c-myc* promoter (226). There are numerous signaling pathways that can transcriptionally activate the *c-myc* promoter. The tyrosine kinase Src has been identified as a key player in regulating *c-myc* expression (227-228) and may occur through signaling via the RhoGTPases or mitogen activated protein kinases (229). Additionally, Ras and PI3K pathways can target c-Myc transcription, translation and protein stability (230). Of particular relevance to this study, *c-myc* can be regulated by the NF-κB transcription factors. The *c-myc* gene contains two NF-κB binding sites, one within the upstream regulatory element (URE) and one within the internal regulatory element (IRE) (231-249). The induction of *c-myc* gene expression by NF-κB has been demonstrated in response to

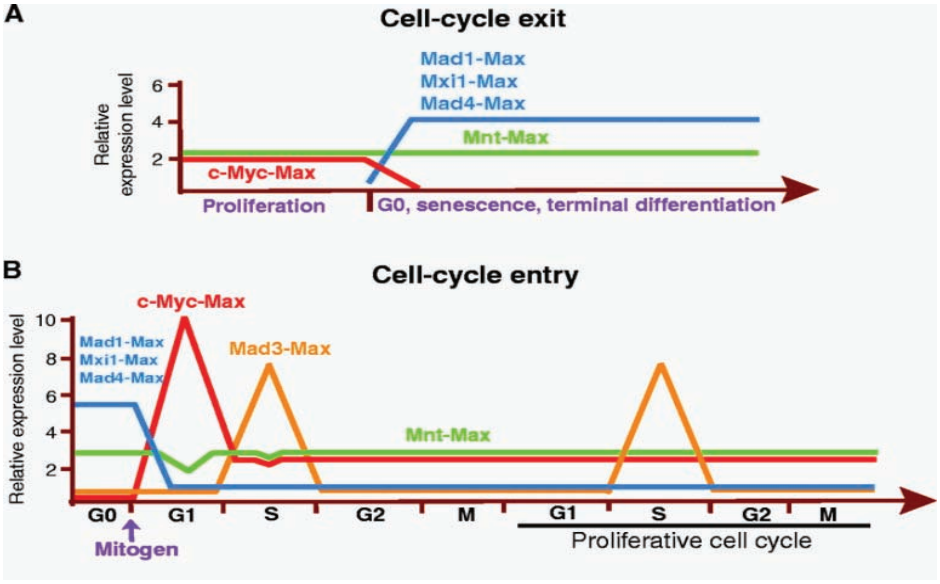


Figure 1.7: Schematic diagrams showing theoretical relationships between different Max complexes during (A) cell cycle exit and (B) cell-cycle entry and prolieration. From Hooker et al. (2006) J Cell Sci. 119:208-216. Adapted with permission from the Journal of Cell Science.

mitogen stimulation by anti-CD3/anti-CD28, anti-IgM/LPS and CD40/CD40L , amongst multiple other signaling pathways (245, 250-252). Signaling through cytokines such as IL-2 can induce *c-myc* expression by activating the downstream PI3K/Akt pathway; Akt is a negative regulator of GSK-3β, which modulates c-Myc protein stability (215-217). Transcription of the *c-myc* promoter can also be regulated by autosuppression. The c-Myc protein can repress its own promoter in a concentration dependent manner (218-226). The regulation of c-Myc is over complex and involves multiple levels of regulatory control.

Competition for dimerization with Max

Myc and Mad proteins are thought to counteract the effects on each other at least in part through competition for dimerization with Max (167, 246-247). Max has a long half-life but it is not a particularly abundant protein and it may be limiting when its dimerization partners are expressed at high levels, such as during cell-cycle entry. Dimerization is thought to be controlled by both the relative efficiencies of protein expression and by the relative efficiencies of dimerization between different family members. Since Myc and Mad proteins have been reported to be co-expressed in the same cell types (171, 218, 220, 254), the competition for dimerization with Max may regulate the balance between cell proliferation and differentiation.

Grinberg et al (2004) showed that Max is recruited to different subnuclear locations by interactions with Myc versus Mad family members (255). Mad4 was localized to the cytoplasm when expressed alone and was recruited to the nucleus by dimerization with Max. Cytoplasmic localization of Mad4 was mediated by chromosome region

maintenance 1 (CRM1)-dependent export from the nucleus. The other bHLHZ proteins, Mlx and MondoA, are also exported from the nucleus via a CRM1-dependent pathway (256). CRM1 is a member of the importin β superfamily of nuclear transport receptors and it binds proteins through the leucine rich nuclear export sequence (NES). CRM1 is the major receptor for the export of proteins out of the nucleus (257). Mad4 contains a NES signal, which is located near the amino terminus of the protein and it is not conserved in other Mad family members. The signal is immediately adjacent to a sequence that mediates interactions with the Sin3 transcriptional co-repressor that is conserved in all Mad family proteins (258). It has been suggested that the interactions between Mad4 and CRM1 and Sin3 could potentially affect each other and this could be a mechanism of coordination for the regulation of transcriptional repression and nuclear export.

By using the bimolecular fluorescence complementation (BiFC) analysis in live cells, a group showed that Max forms heterodimers with Myc more efficiently than it formed homodimers with itself and Myc competed more efficiently for heterodimer formation with Max than Mad3 (255). However, Mad4 competed more efficiently for heterodimerization with Max than Myc or Mad3. These results imply that Mad4 might be a more efficient repressor than Mad3, if the efficiency of repression is determined solely by the efficiency of dimerization with Max. While Mad4 proved to be the most efficient dimerization partner for Max, all Mad protein family members were capable of binding Max and mSin3 and they can all repress transcription and block proliferation of transformed cells to roughly the same extent (168-169, 193, 259-260).

Additional binding partners of Myc and Mad proteins

In addition to forming heterodimers with Max, Myc can also bind to protein Myc-interacting Zn-finger protein 1 (Miz1) through its bHLHZip domain. Miz1 is a poxvirus and zinc finger (POZ) domain zinc finger protein that activates transcription through initiator (Inr) elements and induces G1 cell cycle arrest (261). Myc binds to and inhibits Miz1 DNA binding, suggesting that Myc may repress transcription of genes via its effects on Miz 1 or Miz 1-like proteins (261).

Additional potential binding partners for Mad proteins have also been revealed. Mad-member-interacting protein (Mmip-1) is a protein that contains a Really Interesting New Gene (RING) finger and a Zip domain and has been shown to dimerize via the Zip domains of all the Mad family members but does not bind to c-Myc or Max. This interaction blocked the suppressive effects of Mad proteins and enhances c-Myc function (262-263). Another RING finger protein was identified, Mmip-2, which also blocked Mad functions through interactions with the Mad Zip domain and sequestered Mad1 into the cytoplasm when over-expressed (263-264). However, the interaction between Mad and Mmip proteins has not been verified *in vivo.*

A structurally and functionally related protein to Max was identified, termed Mlx (186, 265). Mlx is a long-lived and ubiquitously expressed bHLHZip protein and can form homodimers and bind to CACGTG elements. Mlx selectively forms heterodimers with Mad1, Mad4 and Mnt but does not dimerize with Mad2, Mad3, Myc or Max (186, 265). It has been demonstrated that Mad1-Mlx and Mnt-Mlx heterodimers can suppress transcription.

Downstream targets of Myc and Mad proteins

The biological effects of c-Myc expression are due to the large number of downstream target genes that are activated by c-Myc. Various studies, including several microarray expression analyses have revealed that c-Myc orchestrates the expression of 10-15% of all cellular genes (177, 191, 192, 266-268). A study using ChIP together with microchips containing DNA arrays representing entire chromosomes (ChIP-on-chip) suggested that Myc binds to many thousands of sites in the human genome (269-270). Specific classes of genes targeted by c-Myc include genes involved in cell cycle, protein synthesis, cell adhesion and cytoskeleton and metabolism. Some of the best defined Myc target genes involved in cell cycle regulation include cyclin D2, cyclin B1, cyclin-dependent-kinase (CDK) 4, CDC2-L1 and the CDK inhibitors p15INK4b and p21Cip1 (271-275). The induction of these genes provides evidence that c-Myc plays an activating role in cell cycle progression, in the G1/S phase, as well as the G2 phase. Additionally, genes that encode proteins that regulate metabolism, ribosome biogenesis and protein translation have been identified as Myc target genes (276). c-Myc regulates cell size through regulation of genes involved in cellular growth and metabolism. Recent studies showed that c-Myc can activate all three RNA polymerases (277). For example, c-Myc can engage the basal transcriptional machinery associated with RNA polymerase III to stimulate transcription of tRNAs and 5S rRNA and binds to and regulates rRNA genes in concert with RNA polymerase I (278-281). Two well characterized c-Myc targets that are involved in metabolism include carbamoyl phosphate synthetase, aspartate transcarbamylase, dihydroorotase (CAD) and ornithine decarboxylase (ODC), which

are necessary for nucleotide synthesis (282-283). Consistent with previous reports that the antagonist Mnt is coexpressed with c-Myc in many cell types (165), serial analysis of gene expression (SAGE) studies demonstrated that Mnt is in fact, induced by c-Myc (270). It is believed that the induction of Mnt serves as a negative feedback loop to restrict the transactivation activity of c-Myc. Hundreds to thousands of genes have been shown to be modulated by augmenting c-Myc expression (284), but it has not been definitively shown that c-Myc regulated genes are conversely antagonized by Mad family proteins.

Role of Myc in apoptosis

It was established in the early 1990s that c-Myc can activate events that lead to cellular apoptosis (285-287). Following growth factor withdrawal, c-Myc is down-regulated and cells exit the cell cycle. However, if c-Myc levels remain elevated, the cells undergo apoptosis (286). The molecular mechanisms leading to c-Myc-induced apoptosis have not been fully elucidated. The mitochondrial apoptotic pathway is involved in Myc-mediated apoptosis and Myc induced apoptosis is highly dependent upon changes in the relative levels of Bcl-2-family proteins (289-292) and availability of survival signals (293-294). In some cases, the downregulation of the anti-apoptotic protein Bcl-2 is important for Myc mediated apoptosis and conversely, it is inhibited by Bcl-2 protein expression (252, 289-290, 292). Myc over-expression has been shown to induce the expression and/or activation of the pro-apoptotic proteins Bax, Bak and Bim (72, 295). Myc can also modulate death receptor-mediated apoptosis, as well as survival pathways activated by death receptors.

Signaling through the death receptor, TNFR via TRAF2 in normal fibroblasts induced the cell survival-signaling-associated NK-κB transcription factor complex, which suppressed the cytotoxic action of TNF and promoted cell survival. However, in cells expressing deregulated c-Myc, this TNFR-induced activation of NF-κB was inhibited and TNFR signaling activated apoptosis in a p53-dependent manner (296). The mechanisms mediating c-Myc induced apoptosis may be a combination of both the extrinsic and intrinsic apoptotic pathways.

To determine the levels of c-Myc necessary to induce apoptosis in cells, Murphy and colleagues (2008) used a mouse model where they analyzed the effects of relatively low levels of deregulated c-Myc (297). In this transgenic mouse model, they were able to control the expression of c-Myc to levels that were either 1.5- or 2-fold increased over physiological levels. They showed a 2-fold over-expression of c-Myc in pancreatic cells stimulated proliferation but did not activate apoptosis. However, the apoptotic program was induced after administration of a cytotoxic agent, doxyrubicin. They interpreted that the 2-fold increase in c-Myc sensitized the cells to apoptosis but did not overtly activate it. When Myc levels were constitutively expressed 15-fold over endogenous levels, the cells underwent apoptosis. How c-Myc discriminates between targets involved in proliferation versus apoptosis is a question that remains to be elucidated. One hypothesis is c-Myc preferentially binds to proliferation gene targets when it is expressed at physiological levels and deregulated or over-expressed c-Myc will bind to both gene targets involved in proliferation and apoptosis. Alternatively, it has been suggested that low levels of c-Myc can bind all gene targets, however to a greater extent when c-Myc levels are increased. It

was shown that when c-Myc was expressed at low levels, it could induce apoptosis when cells were treated with a cytotoxic agent, implying low levels of c-Myc can sensitize cells to apoptotic programs but not without the cooperation of additional apoptotic stimuli (297). Therefore the level of c-Myc required to induce apoptosis may be dependent not just on the relative c-Myc levels in the cells but also on the internal or external stimuli that either promote or inhibit cell survival.

The ability of c-Myc to induce apoptosis has been shown to be both dependent and independent of p53, depending on the cell system studied (288, 292, 298). There is conflicting evidence for the role of Fas and FasL in c-Myc mediated apoptosis. Some studies have shown c-Myc mediated apoptosis to be related to an activated Fas death pathway, whereas another study showed that c-Myc induced apoptosis was independent of Fas, using FADD knockout cells (298-300).

c-Myc has been shown to induce Bim, which can trigger apoptosis (72). Bim levels were elevated in B cells overexpressing Myc (301). Bim was also acutely induced in MEFs and tumor cell lines in response to WT Myc and this was shown to be independent of p53 (72). The direct correlation between c-Myc induced Bim in activated T cell apoptosis has not been studied but the repressed transcription of Bim by Mad4 and Mnt in OX40 activated T cells may play a role in the OX40-mediated T cell survival.

Consequence of Myc/Mad knockouts

Gene targeting approaches have provided further insight into the function of the Max-interacting network of proteins. Both c-Myc and N-Myc are essential for murine

development (302-306); mice deficient for c-Myc die *in utero* at day E9.5 to 10.5 and are developmentally retarded; the embryos are small in size and have defects in the pericardium and neural tube closure. Additionally, the mice have severe developmental delays (307) and these defects in c-Myc knockout mice are associated with the failure of Myc-expressing cells to proliferate (308). Primary mouse embryo fibroblasts (MEFs) lacking c-Myc accumulate in G0 of the cell cycle and are rendered incompetent to proliferate in response to mitogenic stimulation (309-310). A similar phenotype was observed in activated T cells, c-Myc expression was necessary for T cells to enter the cell cycle following T cell stimulation (310). In contrast, primary cells, skin and gut epithelial cells continued to proliferate even after the conditional knock-out of c-Myc *in vivo* (311-312). Deletion of the *c-Myc* gene in the "immortal" Rat1A fibroblast cell line significantly slowed, but did not abrogate cell proliferation (313). This appeared to be the result of both a decreased rate at which cells traverse the cell cycle and a failure of some cells to enter a productive cell cycle (314-316). Loss of N-Myc or c-Myc caused a reduction in cell size in neuronal (317) and epidermal cells, as well as in *Drosophila* (308). In contrast, when c-Myc levels were reduced in mice, fibroblast cell size was normal but the cell number was reduced (309).

Max is crucial for development; Max deficiency resulted in the severely altered phenotype and early embryonic lethality at days E5.5 to 6.5 in mice (317). These defects were associated with a failure of the cells to divide. Mlx is structurally related to Max but it could not compensate for the loss of Max functions, implying that Mlx functions may be limited to regulation of Mad and/or Mnt proteins (265, 318).

Knockouts of the Mad family members have been generated for Mad1, Mad2 and

Mad3 but not Mad4. Knockouts of Mad1-3 show highly tissue specific defects, which may be due to the functional redundancy among the other family members leading to compensation for the loss of the respective proteins. Of note, primary splenic T cells from Mad2 knockout mice underwent accelerated entry into the cell cycle in response to mitogenic stimulation (220). In contrast, Mad3-deficient cells do not have defective cell-cycle entry or exit (221) but Mad3 deficiency sensitized thymocytes and neuronal cells to radiation-induced apoptosis.

Mice lacking Mnt are born runted and typically die within a few days of birth (185, 287). Mnt may have an important role in embryonic development, as Mnt deficient embryos are noticeably decreased in size as early as embryonic day 13.5. To investigate Mnt function, Hurlin et al (2003) studied mouse embryo fibroblasts (MEFs) from $Mnt^{-/-}$ mice and conditional Mnt knockout mice and showed they had a phenotype similar to that caused by Myc over-expression, including increased cell cycle entry, apoptosis and a predisposition towards tumorigenesis *in vivo* (185, 319-320). Mnt deficiency in T cells caused increased apoptosis and a partial block in T cell development in the thymus but later caused organomegaly, inflammatory lesions and lymphomagenesis (321). The anti-apoptotic proteins Bcl-2 and Bcl-X_L were decreased in Mnt-deficient T cells, implying a role for Mnt in cell survival (321). MEFs lacking both c-Myc and Mnt re-enter the cell cycle with kinetics similar to those of wild-type cells (320). Furthermore, several Myc and Mnt target genes are expressed with cell-cycle entry kinetics similar to those of wild-type cells following simultaneous deletion of *c-Myc* and *Mnt*. These results are consistent with data showing that RNAi-mediated knockdown of Mnt rescues the slow proliferative

phenotype of Rat1 fibroblasts that lack *Myc* and causes up-regulation of the Myc target gene ornithine decarboxylase (*Odc)* (319).

Posttranslational modifications of Myc and Mad proteins

Myc has been shown to undergo several types of post-translational modifications, including phosphorylation, ubiquitination, O-linked glycosylation and acetylation. There are three clusters of phosphorylation sites within c-Myc. These two clusters are phosphorylated by protein kinase casein kinase 2 (CK2) (322). One cluster resides in the central acidic domain, while the other is near the basic region. However, little is known about the regulation and function of these phosphorylation sites. The third cluster is localized in the transactivation domain in or near MBI. Two critical phosphoyrlation residues in this cluster are threonine 58 (T58) and serine 62 (S62), which are phosphorylated via several Ras effector pathways (323-324). Phosphorylation at these two residues has opposing affects on c-Myc protein stability; phosphorylation at S62 stabilizes c-Myc whereas T58 phosphorylation destabilizes c-Myc. T58 phosphorylation requires prior phosphorylation at S62 (324-326). c-Myc is transcriptionally induced after mitogenic stimulation, which also leads to downstream activation of PI3K by Ras. Activation of PI3K inhibits glycogen synthatase kinase-3B (GSK-3β), which is a negative regulator of c-Myc protein stability. As cells progress through the cell cycle, PI3K levels decrease, allowing for GSK-3β to become activated, which then phosphorylates c-Myc at T58. This dually phosphorylated form of c-Myc associates with the phosphorylation-directed prolyl isomerase, Pin1, which catalyzes a *cis*-to-*trans* conformational change in the phospho-S62-P63 peptidyl bond

of c-Myc. c-Myc is then dephosphorylated by protein phosphotase 2A (PP2A) at S62, resulting in the unstable, singly T58-phosphorylated form of c-Myc that is a substrate for ubiquitination by SCF^{FBW7} and degradation by the 26S proteasome (325, 327-328). Several lines of evidence suggest a link between Myc turnover and its transcriptional activity. Degradation of c-Myc through the Skp2 complex has been reported to stimulate Myc-dependent transcription (329-330). In addition, proteasome inhibition or knockdown of the Fbw7 ligase induces nucleolar accumulation of Myc, an organelle in which Myc is normally present at only very low levels (331-332). Myc functions in the nucleolus to directly stimulate RNA polymerase I transcription of rDNA (279-280). Evidence suggests that Myc is normally turned over very rapidly in the nucleolus in concert with the high rate of rRNA production. c-Myc stability can also be regulated by acetylation and this may be tightly connected with ubiquitination. Acetylation of c-Myc can decrease ubiquitination and increase its stability (203, 207, 333).

The post-translational modifications of Mad proteins have not been extensively studied but recent data showed that degradation of Mad1 is regulated by activation of the PI3K/Akt and MAPK pathways (334). Both p90 ribosomal kinase (RSK) and p70 S6 kinase (S6K) phosphorylated serine 145 of Mad1 upon mitogenic stimulation and phosphorylation at S145 led to ubiquitination and degradation of Mad1 through the 26S proteasome pathway. Post-translational modifications have not been reported for the other Mad proteins.

The phosphorylation state of Mnt is thought to directly regulate its function. Mnt is detectable by Western blot at two molecular weights: 72 kDa, which is the non-phosphorylated

form and a 74-kDa band that is phosphorylated Mnt. The non-phosphorylated form of Mnt is functionally active. In this state, Mnt is able to interact with and bind to mSin3 and recruit HDACs and thereby exert its effects as a transcriptional repressor at Myc target genes. Mnt is phosphorylated after serum stimulation and this disrupts the interaction with mSin3 proteins, which results in the relief of transcriptional repression.

Serum stimulation also results in increased levels of c-Myc and allows for binding of c-Myc/Max complexes to E-box sites and downstream activation of target genes. The exact mechanisms mediating Mnt phosphorylation and post-translational regulation have yet to be elucidated.

Summary

The studies summarized in this dissertation address the underlying molecular mechanisms occurring after OX40 stimulation of T cells, specifically the role of Mad4, Mnt and c-Myc proteins. We hypothesized that the up-regulation of c-Myc in activated T cells drives proliferation and consequently activation-induced cell death, which is counteracted by Mad4 and Mnt proteins after anti-OX40 engagement, ultimately allowing T cells to survive the "blast crisis" phase. This dissertation describes three aspects of Myc/Mad biology following OX40 mediated T cell co-stimulation. First, I describe the expression levels of Mad4, Mnt and c-Myc proteins in *ex vivo* isolated Ag-stimulated T cells following administration of anti-OX40 and Ag. In Chapter 2, I show that Mad4, Mnt and c-Myc proteins are up-regulated after OX40 engagement at various times post-immunization in T cells. The up-regulation of Mad4 and Mnt proteins is transient, peaking 3 and 4 days after anti-OX40 administration, when the cells are at the peak of their proliferative expansion. These two proteins decrease 6 days after OX40 engagement when the Ag-specific cells contract in size and number. On the other hand, c-Myc levels stayed elevated over the entire 6 day time course. Given that Mad4 and Mnt normally have a short half life, we hypothesized that signaling through OX40 stabilized these proteins through post-translational modifications. Indeed, as shown in Chapter 3, after anti-OX40 engagement, Mad4 and Mnt proteins were resistant to degradation after treatment with cycloheximide. Additionally, we demonstrated the presence of a serine residue in Mad4 that was essential for mediating accelerated protein degradation. Finally, the experiments performed in Chapter 4 demonstrate that Mad4 and Mnt proteins are necessary for survival of T cells after ligation by anti-OX40, as siRNA

knockdown of these proteins in Ag-activated effector cells led to decreased survival of OX40-stimulated T cells.

Chapter 2

Expression of Mad4, Mnt and c-Myc proteins in T cells after anti-OX40 engagement

Abstract

Myc and Mad proteins are thought to have antagonistic functions and are typically expressed at different stages of the cell cycle. Generally, Myc proteins are expressed in proliferating cells, whereas Mad proteins are expressed in differentiated, quiescent cells. Mnt, on the other hand, is expressed constitutively at all phases of the cell cycle and is co-expressed with Myc. Myc and Mad family proteins compete for dimerization with their binding partner, Max. The heterodimers are able to bind DNA at the consensus E-box sequences and activate or repress target genes, respectively. In this set of experiments, we show that Myc, Mad4 and Mnt proteins are all co-expressed in proliferating T cells after *in vivo* ligation by the costimulatory molecule, OX40. Mad4 and Mnt proteins are both up-regulated at days 3 and 4 after anti-OX40 treatment, at the peak of OX40-mediated T cell proliferation, and the levels decreased at day 6 as the cells decreased in cell size. On the other hand, c-Myc levels are up-regulated after anti-OX40 treatment and the levels persist from day 3 to day 6. Our data shows that OX40 stimulation leads to an up-regulation of Mad4, Mnt and c-Myc protein levels in T cells, compared to T cells isolated from rat Ig or anti-CTLA-4 control treated mice.

Introduction

Stimulation through the costimulatory molecule, OX40 (CD134) after TCR activation enhances CD4 and CD8 T cell responses (98, 102, 335-337). OX40 is up-regulated 1-2 days following activation of naïve T cells and is expressed on both CD4 and CD8 T cells (77-79). Subsequent engagement of OX40 by an agonist antibody or OX40L leads to increased cytokine production, enhanced proliferation, expansion, and development of increased numbers of long-term memory T cells (8, 338). A model to study gene regulation of anti-OX40 activated T cells was developed in which naïve OVA-specific transgenic T cells were adoptively transferred into wild-type mice and these mice were immunized with OVA in combination with agonist anti-OX40 or control (rat Ig) antibodies. Additionally, the effects of anti-OX40 engagement were compared to CTLA-4 blockade. CTLA-4 is a cell-surface receptor expressed by activated T cells (339-340) and CTLA-4 signaling down-modulates T cell responses, however, CTLA-4 blockade via anti-CTLA-4 monoclonal antibody leads to enhanced proliferation of CD4 T cells. In contrast to the ability of OX40 engagement to promote enhanced proliferation and CD4 T cell survival, CTLA-4 blockade enhances cellular proliferation but does not increase their long-term survival (7, 341). By comparing T cells treated with anti-OX40 versus anti-CTLA-4, we were able to examine differences at the molecular level that potentially could contribute to T cell survival.

To further define the molecular mechanisms by which OX40 engagement leads to enhanced survival and memory T cell development, microarray gene analysis of *in vivo* activated T cells was performed (7, 88). The results showed differential expression of

Mad4 and c-Myc transcripts after anti-OX40 ligation compared to rat Ig and anti-CTLA-4 controls (7, 88). The members of Max-interacting network are transcriptional regulators, which play a key role in the regulation of cell growth, differentiation and apoptosis. Myc and Mad proteins are proposed to have antagonistic functions; both form heterodimers with Max and bind to DNA consensus E box sequences where they activate or repress target genes through interaction with histone acetylases or histone deacteylases complexes, respectively (167, 175, 188, 190). Myc proteins induce downstream target genes that are associated with cell proliferation, cell growth or apoptosis, whereas Mad family proteins are associated with proliferative arrest and terminal differentiation (191-192).

The role of the Max-interacting network of proteins in T cell function has not been extensively studied. c-Myc is expressed at low levels in naïve quiescent T cells (214) but then up-regulated as part of the proliferative response after antigen-mediated TCR activation (215-216). T cells deficient for c-Myc display impaired proliferation (310, 342) and fail to progress through positive selection (343). Mad-family members are differentially expressed during T cell development but are typically expressed in terminally differentiated and quiescent lymphocytes (344). Mad2-deficient mice exhibited enhanced proliferative responses to mitogenic stimuli compared to control cells (345). Conversely, over-expression of Mad1 in T cells inhibited lymphocyte expansion, maturation and growth following pre-T-cell receptor (pre-TCR) and TCR stimulation (344). Mnt deficient T cells underwent increased apoptosis compared to control and partial block in T-cell development in the thymus and Mnt proved to be necessary for immune homeostasis (321). Thus, the Max-interacting network regulates proliferation, cell size, and quiescence of cells, all of

which are linked to the biological effects of OX40 engagement.

In this set of experiments, we investigated the expression of Myc, Mad4 and Mnt proteins in Ag-specific T cells after anti-OX40 stimulation *in vivo*. We report that Mad4 transcripts were down-regulated after engagement by anti-OX40. On the contrary, the protein levels of Mad4, Mnt and c-Myc proteins were up-regulated in proliferating Ag-specific T cells, 3-4 days after TCR engagement and anti-OX40 stimulation. The levels of Mad4 and Mnt protein expression were highest 3-4 days after OX40 stimulation and the levels decreased at day 6. In contrast, the levels of c-Myc protein increased early but remained unchanged later after anti-OX40 stimulation. The proteins were localized in the chromatin-bound, nuclear fraction after anti-OX40 engagement, implying that they are functionally active. We propose a model whereby the up-regulation c-Myc in activated T cells drives activation-induced cell death, and is counteracted by OX40-mediated up-regulation of Mad4 and Mnt proteins, thus, allowing T cells to survive the “blast crisis” phase (at approximately day 3), ultimately leading to an increase in the numbers of small resting memory cells.

Materials and Methods

Mice

Four- to 6-week-old male and female C57BL/6 and BALB/c mice were purchased from Charles River Laboratories and used at 6–10 weeks of age. BALB/c TCR-transgenic DO11.10 ($OVA_{323–339}$), C57BL/6 OT-I ($OVA_{357–364}$) which were bred onto the Ty1.1 background, and OT-I OX40-deficient mice (kindly provided by Dr. Michael Croft, La Jolla Institute for Allergy and Immunology, La Jolla, CA) were bred and maintained at the Earle A. Chiles Research Institute. An institutional animal care and use committee approved all animal studies. All mice were bred and maintained under specific pathogen-free conditions in the Providence Portland Medical Center animal facility. Experimental procedures were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Preparation of antibodies

Control ratIg antibody was purchased from Sigma (St. Louis, MO), whereas rat anti-OX40 antibody (OX86) and anti-CTLA-4 (9H10) were produced in the laboratory from hybridomas and affinity purified over protein G columns.

Adoptive transfer and immunization

Spleens and DLNs were harvested from DO11.10, OT-I or OT-I OX40 deficient mice and processed by crushing between two frosted glass microscope slides and red blood cell (RBC) lysed with Ammonium chloride-based RBC lysis buffer (ACK, Cambrex, East Rutherford,

NJ). The percentages of DO11.10 or OT-I T cells were identified by FACS using KJ1-26 or Thy1.1 antibodies, respectively prior to transfer (BD Biosciences, San Jose, CA). A total of 2–3x10^6 transgenic TCR T cells were adoptively transferred intravenously (i.v.) into BALB/c or C57BL/6 WT recipients. One day later, mice were immunized subcutaneously (s.c.) with 500µg of OVA (Sigma-Aldrich, St. Louis, MO) and 50µg of anti-OX40 (OX86), 100µg of anti-CTLA-4 (9H10), or rat IgG control (Sigma-Aldrich, St. Louis, MO). The following day, mice were given a second injection of anti-OX40, anti-CTLA-4 or rat Ig.

Purification of Ag-specific T cells

DLNs were collected at indicated times after immunization and processed into a single-cell suspension. Cells were stained for 0.5h on ice with biotinylated KJ1-26 antibody (0.5mg/10^6 cells) and then washed. Cells were harvested using anti-biotin microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity (>90%) was verified by flow cytometry.

Quantitative Real-time polymerase chain reaction (PCR)

DLNs were isolated 3 days after immunization and Ag-specific T cells were purified as described above. Total RNA from the donor D011.10 (>90%) was collected using a RNeasy kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. cDNA templates were prepared using random primers and reverse transcriptase (Invitrogen, Carlsbad, California). mRNA from Ag-specific CD4 T cells was quantitated by real-time PCR using the following primers: forward, 5'-TGCATCTTAGCACGCTTCAG, reverse and

5’-CCCGAACAACAGGTCTTCAC (Invitrogen, Carlsbad, California). Real-time PCR (Rotor-Gene 2000 Real Time Cycler, Corbett Research, N.S.W., Australia) was used for quantification purposes and SYBR green was used as the fluorophore (Molecular Probes, Eugene, OR).

Electroporation

T cells were transfected by means of the nucleofection technique from Amaxa (Cologne, Germany) using the mouse T-cell nucleofection kit, according to Amaxa’s protocol. In brief, DLN $CD4^+/KJ^+$ T cells were purified at day 4 after OX40 immunization were resuspended in mouse T cell nucleofection solution at a density of $2\text{-}3\times10^6$ cells per 100µL. 100µL of cell suspension were mixed with the respective amount of siRNA, transferred into a cuvette and pulsed in a Nucleofector I device using program X-01. Subsequently, the cells were diluted with 500µL of pre-warmed serum-free RPMI medium (37°C) and transferred into a microcentrifuge tube and incubated for 10 min at 37°C. Cells were then transferred to a 24-well-plate containing 1.5ml of pre-warmed Mouse T cell Nucleofector medium containing 5% FBS, 2mM glutamine and Medium Component A and B (Amaxa) (37°C) per well. Following transfection the cells were cultured at 37°C and 5% CO_2. The following day, 0.5ml of media was added to each well. Cells were analyzed by flow cytometry and lysed for Western blot 48hrs post transfection.

Immunoblot analysis

DLNs were isolated 3, 4 or 6 days after immunization and Ag-specific T cells were purified as described above. Cells were collected by centrifugation and resuspended in Laemmli

buffer and boiled at 98°C for 10 min. Lysates were quantitated using DC Protein Assay (BioRad, Hercules, CA), run on polyacrylamide gels (equivalent microgram quantities of protein), and transferred onto nitrocellulose or PVDF membranes. Antibodies for Mad4, Mnt, c-Myc, Lamin, PARP, GFP (Santa Cruz Biotechnology, Santa Cruz, CA), GAPDH (Millipore, Billerica, MA), Actin (Sigma) were used to detect these proteins.

Chromatin fractionation

To isolate chromatin, cells were resuspended (100×10^6 cells/ml) in buffer A (10 mM HEPES, [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 100X protease inhibitor (Sigma), 0.1 mM phenylmethylsulfonyl fluoride). Triton X-100 (0.1%) was added, and the cells were incubated for 5 min on ice. Nuclei were collected by low-speed centrifugation (4 min, 1,300 × g, 4°C). The supernatant was further clarified by high-speed centrifugation (15 min, 20,000 × g, 4°C) to remove cell debris and insoluble aggregates and the supernatant was collected as the cytoplasmic fraction. To fractionate the chromatin, nuclei suspension was resuspended in buffer A plus 1mM $CaCl_2$ and 0.2U micrococcal nuclease (Sigma, St. Louis, MO) at 37°C for 1 minute. The digestion was terminated by the addition of 1mM EGTA, the mixture was then centrifuged at 5,000 g for 3 min. Nuclei were lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, protease inhibitors as described above). Insoluble chromatin was collected by centrifugation (4 min, 1,700 × g, 4°C), washed once in buffer B, and centrifuged again under the same conditions. The final chromatin pellet (Chromatin fraction) was resuspended in Laemmli buffer and sonicated for 15 s. Proteins were analyzed by SDS-PAGE.

Results

Mad4 transcripts are down-regulated after OX40 engagement in vivo in Ag-specific CD4 T cells

Gene array data previously showed Mad4 transcripts were down-regulated in two models after engagement by anti-OX40: the primary immunization model and the peripheral tolerance model (Table 1.1). To verify these results, we performed quantitative real time PCR using RNA from Ag-specific CD4 T cells isolated at 3 days *ex vivo* after stimulation with Ag alone or Ag and anti-OX40 (Figure 1.2) Naive OVA-specific CD4 T cells were adoptively transferred into WT recipients and immunized with OVA and anti-OX40. Three days following immunization, the donor Ag-specific CD4 T cells were harvested from the DLNs and purified to approximately 90% purity and verified by flow cytometry (Figure 2.1). Mad4 transcript levels were examined by quantitative real-time PCR. The data were consistent with the gene array results: Mad4 transcripts were decreased approximately 10-fold in CD4 T cells treated with anti-OX40 compared to rat Ig control (Figure 2.2).

Specificity of Mad4 and Mnt antibodies

To determine if the protein levels correlated with the RNA levels, we examined the protein expression of Mad proteins in OX40 stimulated T cells by Western blot analysis. To verify the specificity of the antibodies, Ag-specific T cells were isolated from DLNs at day 4 after *in vivo* immunization with OVA and anti-OX40 and transfected by means of nucleofection with siRNA against Mad4 and Mnt and analyzed by Western blot. The results showed the bands at the expected molecular weights were decreased in cells transfected

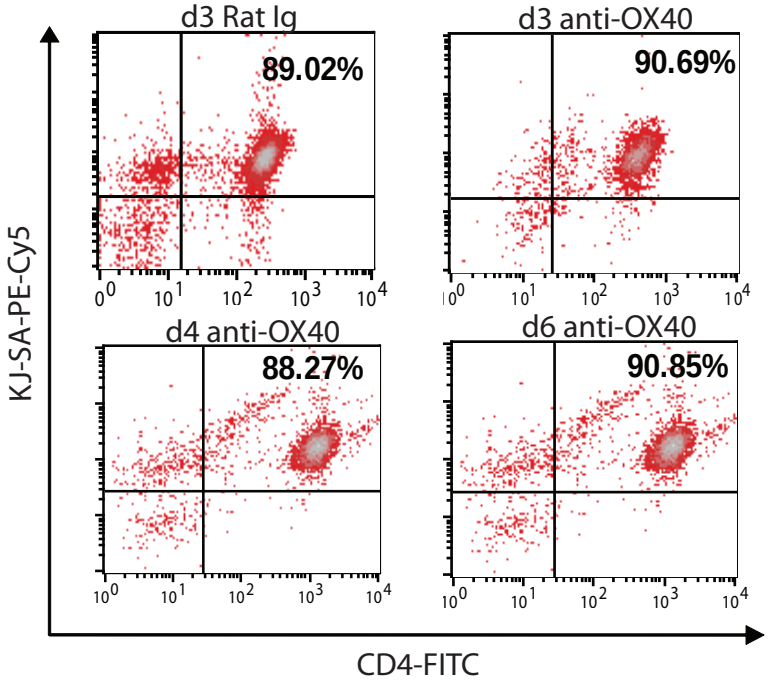


Figure 2.1: Purity of Ag-specific CD4 cells after magnetic bead separation. Cells were isolated from DLN at indicated times and stained with anti-CD4 and anti-KJ-126 antibodies and separated by magnetic bead separation using an Auto-MACS.

with the specific siRNA, verifying the specificity of these two antibodies and validating their use in the subsequent experiments (Figure 2.3).

Mad4, Mnt and c-Myc proteins are up-regulated after anti-OX40 engagement in vivo in Ag-specific CD4 T cells

In view of the changes in transcript levels of Mad4 in anti-OX40 stimulated T cells, we examined the protein levels of Mad4 and the related family member, Mnt, in Ag-specific T cells isolated from DLN at day 3 after treatment with Ag and anti-OX40. Mnt

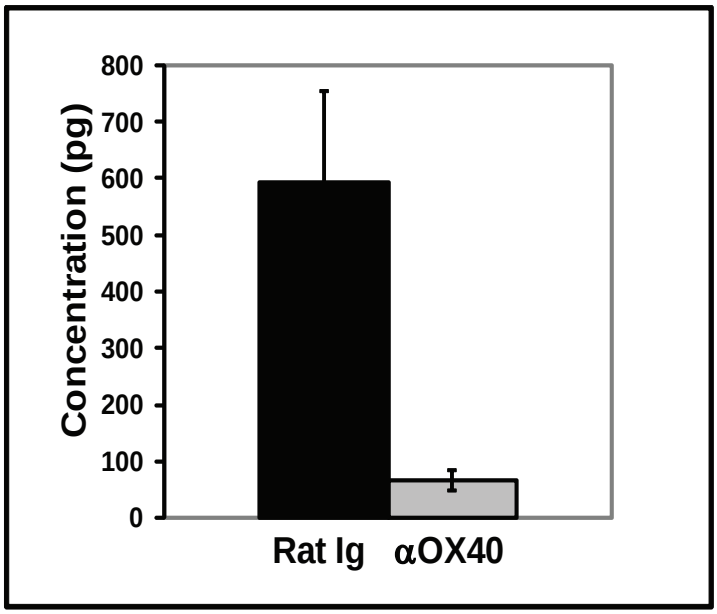


Figure 2.2: Mad4 transcripts are down-regulated in Ag-specific CD4 T cells after treatment with anti-OX40 compared to Rat Ig control. Cells were isolated from DLN at d3 after treatment with OVA and anti-OX40 or OVA and Rat Ig control. Lysates were analyzed by quantitative real-time PCR using primers for Mad4 and quantitated using a standard curve. Representative of 3 experiments with similar results.

transcript levels were not differentially regulated by gene microarray in cells engaged by anti-OX40 compared to controls in CD4 and CD8 T cells (Table 1.2). Quite opposite to our findings at the transcriptional level, we observed a 3-fold increase in Mad4 and Mnt proteins in Ag-specific CD4 T isolated from DLN *ex vivo* after treatment anti-OX40 vs. rat Ig (Figure 2.4).

Since we saw a change in Mad4 and Mnt proteins at day 3 after anti-OX40 engagement, we hypothesized these proteins might be temporally regulated. To test this hypothesis, we examined the protein levels of the Mad4, Mnt and c-Myc proteins over a six-day time course in Ag-specific T cells isolated *ex vivo* following anti-OX40 stimulation *in vivo* (Figure 2.5). The peak of the proliferative response after anti-OX40 engagement occurs between days 3 and 4 and the cells decrease in size and proliferation rate by day 6. Mad4 protein was undetectable in naïve cells and the protein was induced following OX40

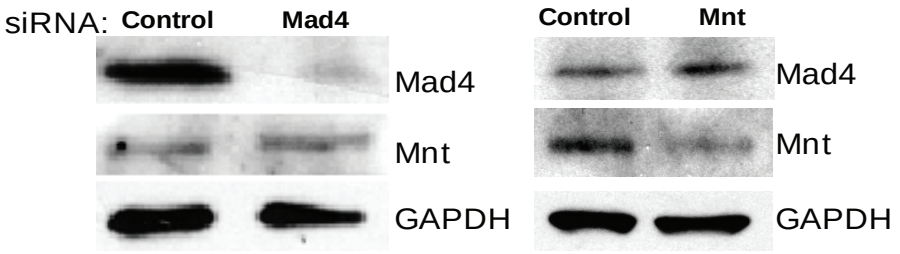


Figure 2.3: Specificity of Mad4 and Mnt antibodies. Ag-specific CD4 T cells were isolated from DLN at d4 after treatment with OVA and anti-OX40 and transfected with Mad4 and Mnt siRNA. Lysates were anlayzed by Western blot with the indicated antibodies. Representative of 4 experiments with similar results.

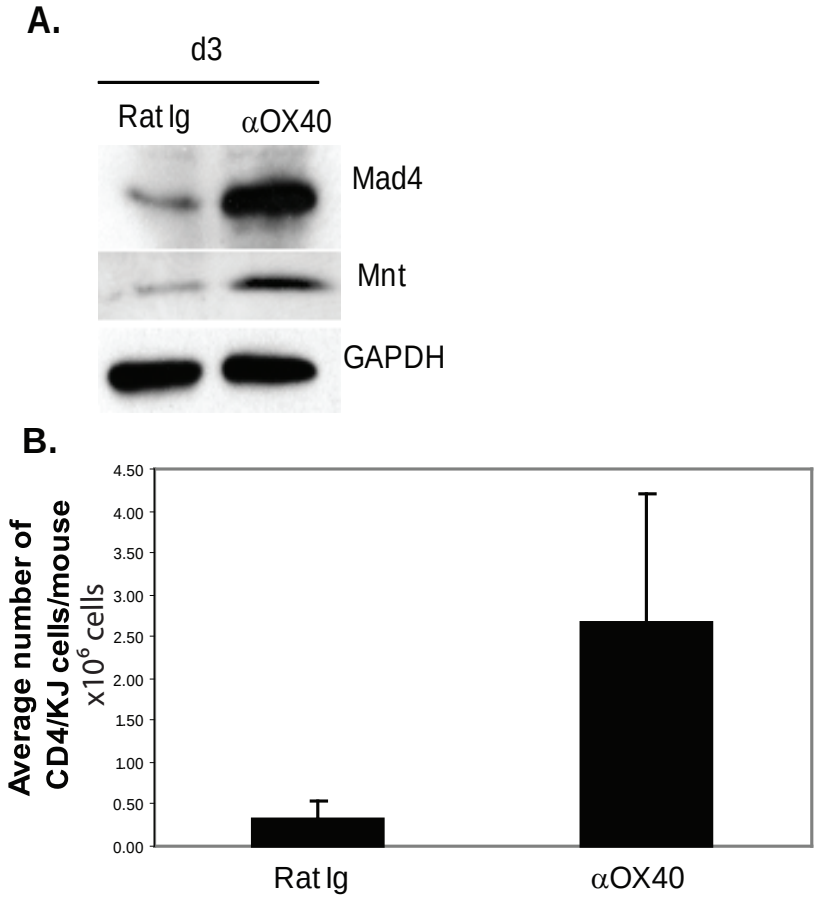


Figure 2.4: Antigen-specific CD4 T cells have increased levels of Mad4 and Mnt proteins after anti-OX40 treatment compared to isotype (Rat Ig) control. A. Ag-specific CD4 T cells were isolated from DLN at d3 after treatment with OVA and anti-OX40. Lysates were analyzed by Western blot with the indicated antibodies. B. Average number of cells recovered per mouse in each group. Representative of 3 experiments with similar results.

treatment at the days 3 and 4. Mad4 protein levels decreased on day 6 when the cells contracted in size and were in a more quiescent state. Mnt levels were increased on days 3 and 4 after Ag and anti-OX40 stimulation, decreasing at day 6. The protein levels of c-Myc were detected in naïve cells and increased on day 3 but thereafter the levels stay relatively constant over the time course (Figure 2.5). Following stimulation with anti-OX40, the cells are increased in size compared to rat Ig control at d3. At later time points, the cells start to contract and shrink in size (Figure 2.5B). As shown in Figure 2.5C and D, Mad4 and Mnt levels were the highest when the cells were at the peak of expansion and then declined as the cells decreased in number and became more quiescent. These data suggest that contrary to what we observed at the transcript level, Mad4 and Mnt proteins are up-regulated after stimulation through anti-OX40 and the levels peak at days 3 and 4 as the cells undergo their initial expansion and the levels decrease at day 6 when the cells contract. The levels of c-Myc, however, remain constant over time.

Subcellular localization of Mad4 and Mnt in Ag-specific CD4 T cells at various times after anti-OX40 engagement

The subcellular localization of transcription factors can regulate their transcriptional activities (346-348). Mad4 contains an N-terminal nuclear export signal (NES) that is not conserved in other Mad family proteins and Mad4 is actively exported from the nucleus (255). Mad4 is recruited to the nucleus by dimerization with Max, which contains a nuclear localization signal (NLS) in its protein sequence (255). Mnt and c-Myc also form heterodimers with Max but they do not have a NES. We sought to determine if Mad4,

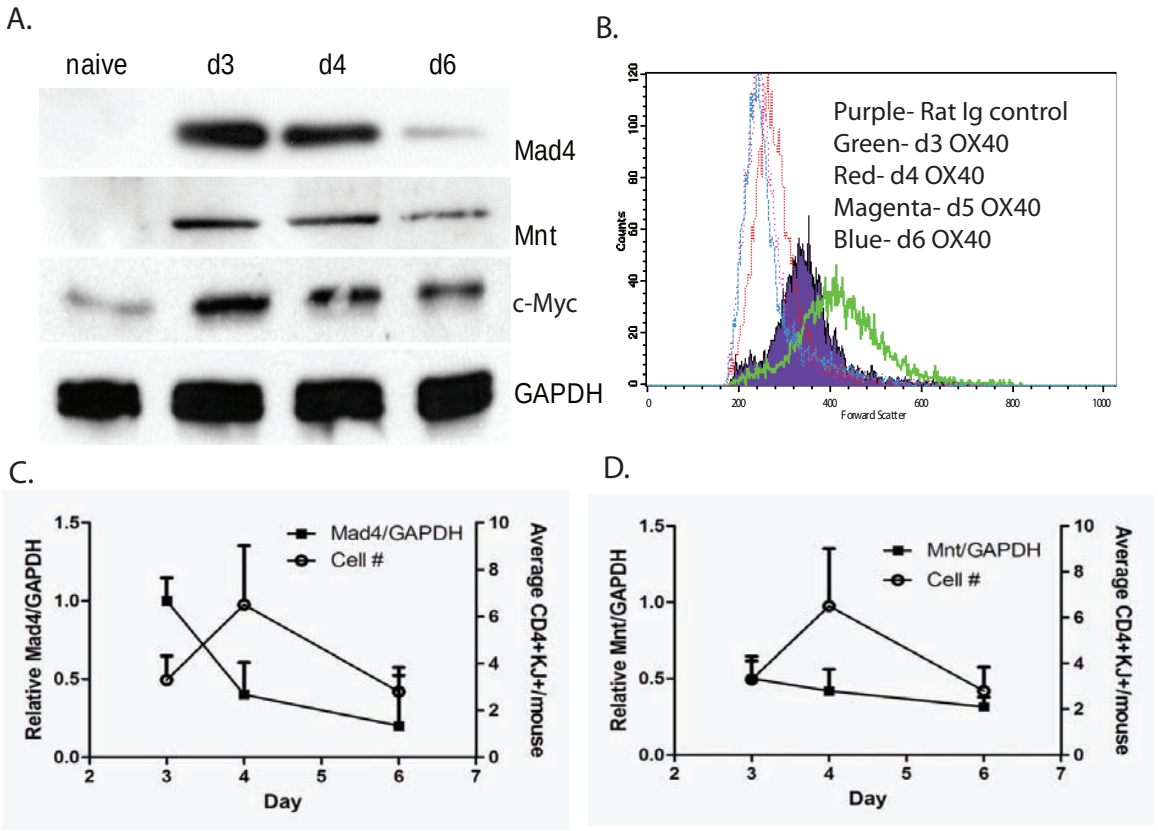


Figure 2.5: Levels of Mad4 and Mnt proteins increase over time in Ag-specific CD4 T cells after anti-OX40 treatment. A. Ag-specific CD4 T cells were isolated from DLN at indicated times after treatment with OVA and anti-OX40. Lysates were analyzed by Western blot with the indicated antibodies. B. Cell size for CD4/KJ^{+} T cells after anti-OX40 engagement as shown by forward scatter by flow cytometry. Filled purple- d3 Rat Ig control, Green- d3 anti-OX40, Red- d4 anti-OX40, Magenta- d5 anti-OX40, Blue- d6 anti-OX40. Average number of Ag-specific CD4 T cells from DLN per mouse (right axis) and relative Mad4 (C) and Mnt (D) protein levels (left axis) were graphed over time. Representative of 3 experiments with similar results.

Mnt and c-Myc proteins were transcriptionally active by determining whether they were localized within the chromatin-bound fraction within Ag-specific T cells after anti-OX40 treatment. Western blot analysis showed the Mad4 levels within the nuclear chromatin-bound fraction were elevated compared to the cytoplasm after anti-OX40 treatment at days 3 and 4. In contrast, on day 6, Mad4 protein was primarily localized in the cytoplasm. Mnt

protein was localized in the chromatin-bound fraction at all time points and the protein levels peaked on day 3 after T cell activation and decreased by day 6. c-Myc remained in the nuclear fraction over time and the levels peaked at day 3 but in contrast to Mad4 and Mnt, c-Myc levels were still elevated in the chromatin fraction on day 6 (Figure 2.6). Based on the subcellular localization, these data suggest that Mad4 and Mnt are functionally active during a discrete window after stimulation through anti-OX40.

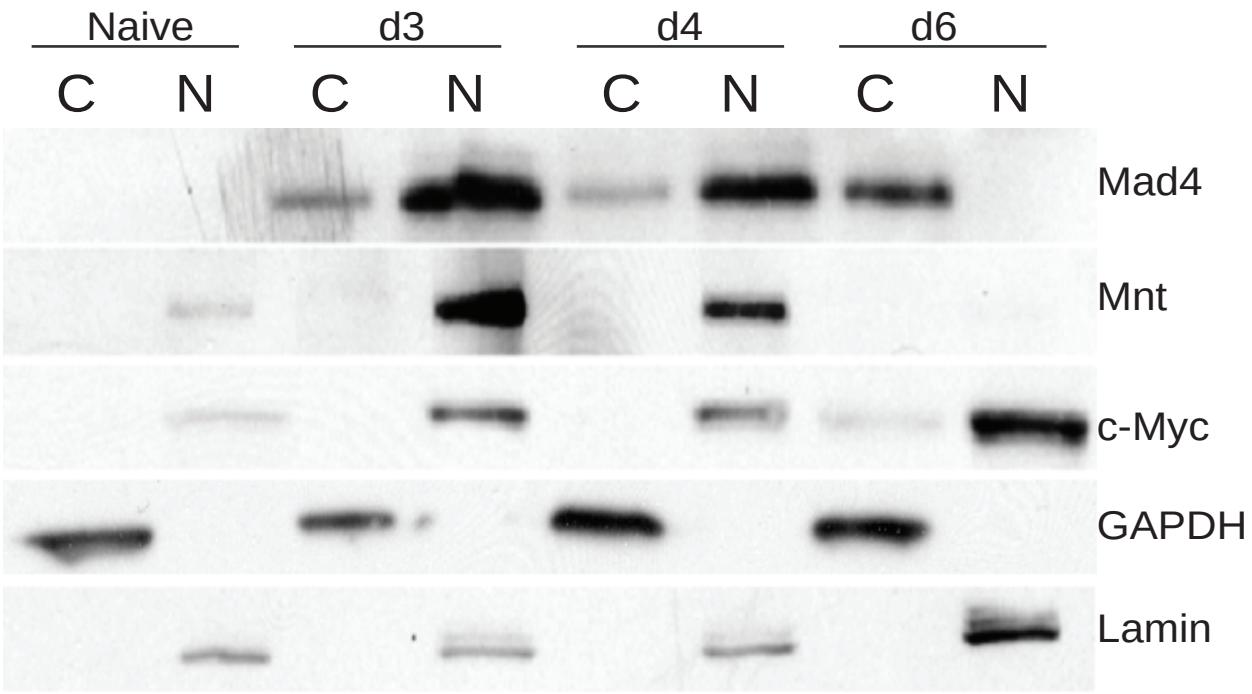


Figure 2.6: Subcellular localization of Mad4 and Mnt in Ag-specific CD4 T cells at various times after anti-OX40 engagement. Ag-specific CD4 T cells were isolated from DLN at indicated times after treatment with OVA and anti-OX40. The lysates were fractionated and analayzed by Western blot with the indicated antibodies. C, cytoplasm, N, nucleus. Representative of 3 experiments with similar results.

Mad4, Mnt and c-Myc proteins are up-regulated after anti-OX40 engagement in vivo in Ag-specific T cells when compared to anti-CTLA-4 blockade

Signaling through CTLA-4 provides a negative signal to T cells and "puts the brakes" on cellular proliferation (92). The anti-CTLA-4 antibody blocks this negative signal and hence increases proliferation and expansion of CD4 T cells (92). While both anti-OX40 and anti-CTLA-4 increase T cell proliferation, T cells isolated from hosts injected with anti-CTLA-4 do not show enhanced survival (92) (Figure 4.1B). To determine whether the increase in Mad4 and Mnt was specific for Ag stimulated CD4 T cells showing enhanced proliferation and survival versus proliferation alone, we compared the effects of anti-OX40 versus anti-CTLA-4 treatment. An increased percentage of Ag-specific CD4 T cells were recovered from cells treated with anti-OX40 compared to anti-CTLA-4 (Figure 2.7A). The protein levels for Mad4 were up-regulated in the Ag-specific CD4 T cells >2-fold at day 3 and 4 after OX40 engagement, compared to anti-CTLA-4 treatment. Mnt was slightly up-regulated at day 3 and was increased 2-fold at day 4 after anti-OX40 treatment. Anti-OX40 treatment also up-regulated c-Myc protein levels >2-fold at days 3 and 4 compared to anti-CTLA-4 (Figure 2.7B). Analysis of the protein levels at later time points was not performed because there are low numbers of surviving cells after treatment with anti-CTLA-4 at day 6. We next determined whether Mad4, Mnt and c-Myc proteins were up-regulated in the chromatin-bound fraction after anti-OX40 stimulation when compared to CTLA-4 blockade. The levels of Mad4 and Mnt were considerably increased in the chromatin fraction of anti-OX40 treated cells when compared to anti-CTLA-4 at days 3 and 4 (Figure 2.7C). The chromatin-associated levels of c-Myc were

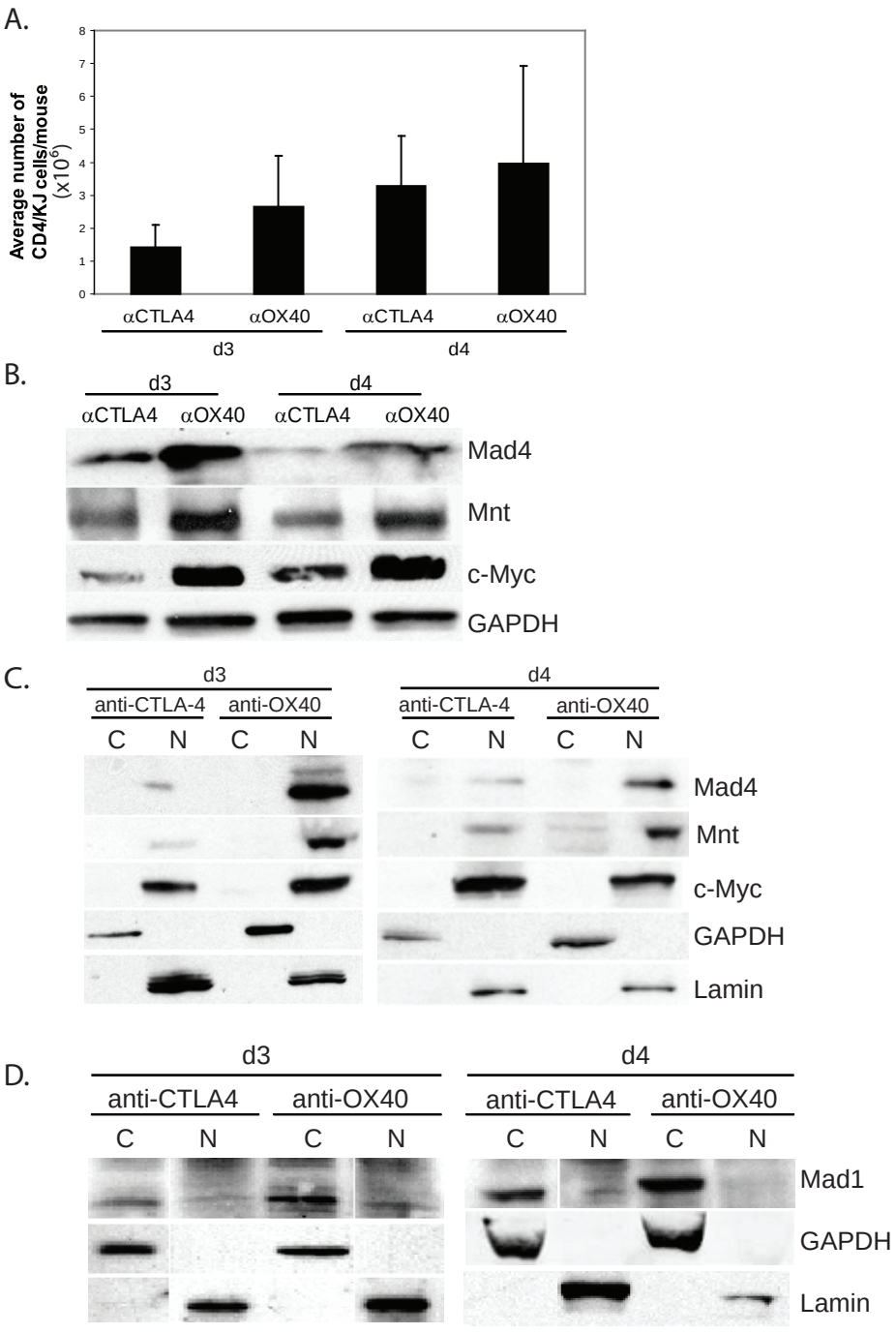


Figure 2.7: Mad4, Mnt and c-Myc proteins are up-regulated after anti-OX40 engagement in Ag-specific T cells when compared to anti-CTLA-4 blockade. A. Average number of cells recovered per mouse in each group. Representative experiment of minimum of 6 experiments. B. Ag-specific CD4 T cells were isolated from DLN at indicated times after treatment with OVA and anti-OX40 or OVA and anti-CTLA-4. Lysates were analyzed by Western blot with the indicated antibodies. C and D. The lysates were fractionated and analyzed by Western blot with the indicated antibodies. Representative of 3 experi-

approximately the same at day 3 and 4 compared to CTLA-4 blockade (Figure 2.7C). To determine if this result was specific to Mad4 and Mnt, we also examined protein expression of Mad1. We found that the Mad1 was sequestered in the cytoplasmic fraction, implying it is probably not functionally active after anti-OX40 treatment. The levels of Mad1 protein expression also did not greatly differ between the Ag-specific T cells isolated from anti-OX40 treated cells and anti-CTLA4 treated mice (Figure 2.7D). These data suggest that increased Mad4 and Mnt in the nuclear fraction are associated with the survival signal, rather than the proliferation signal after anti-OX40 engagement.

Anti-OX40 also increases Mad4 and Mnt levels in CD8 T cells isolated from anti-OX40 stimulated mice

The anti-OX40 antibody has been shown to have potent immune stimulating effects on CD8 T cells in similar adoptive transfer settings to those observed for CD4 T cells (Figure 1.2B) (93, 95-96). Naive C57BL/6 mice received adoptive transfer of CD8 TCR transgenic OTI/Thy1.1$^+$ T cells and were vaccinated with OVA in the presence or absence of anti-OX40. At day 4 following vaccination, Thy1.1$^+$ cells were bead purified from DLNs and harvested for analysis of Mad4 protein regulation. We analyzed Ag-specific CD8 T cells isolated from DLN 4 days after Ag priming to determine if Mad4 and Mnt proteins were also regulated by OX40 in the CD8 T cell model. Similar to the results obtained with CD4 T cells, Mad4 and Mnt levels were increased >4 fold and >2-fold, respectively in Ag-specific CD8 T cells after anti-OX40 treatment compared to control-treated mice (Figure 2.8A).

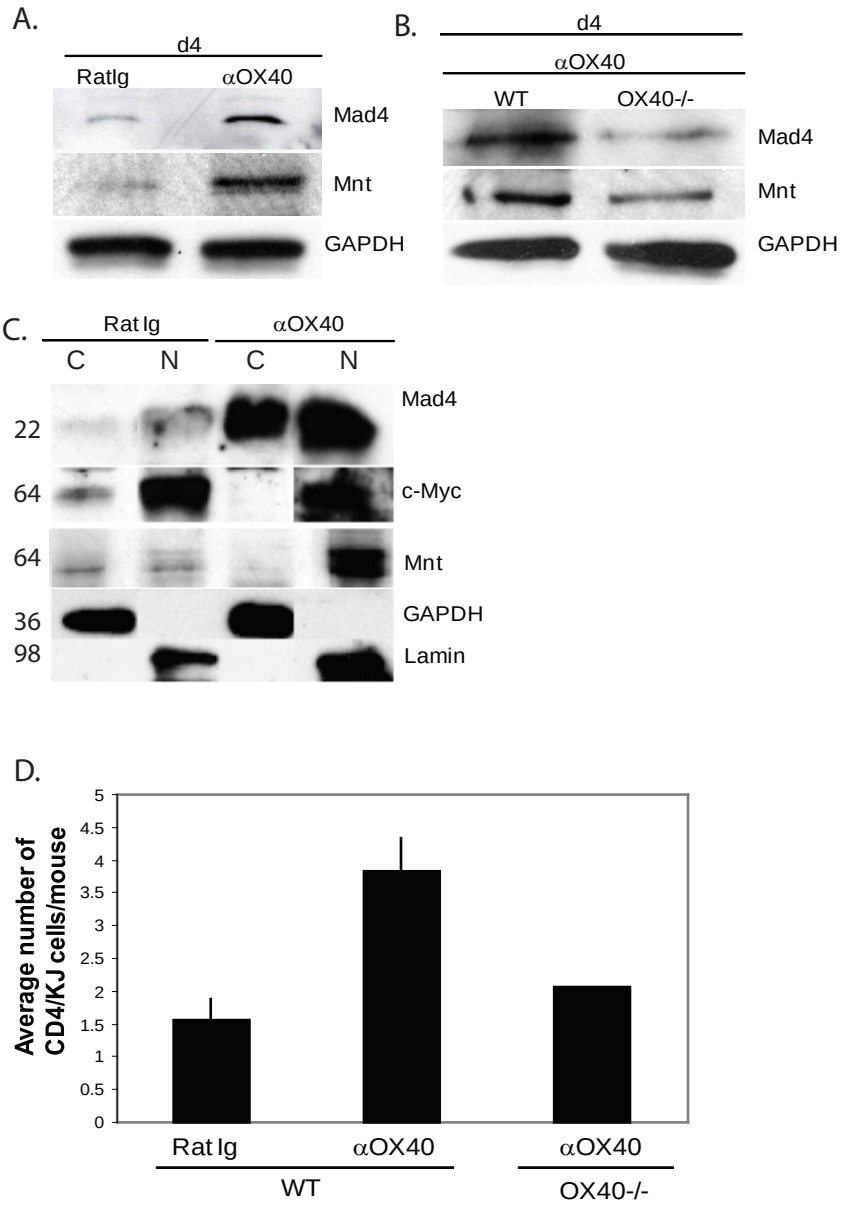


Figure 2.8: Treatment with anti-OX40 directly mediates an increase in Mad4 and Mnt proteins in Ag-specific CD8 T cells. A. Ag-specific CD8 T cells were isolated from DLN at day 4 after treatment with OVA and anti-OX40. Lysates were analyzed by Western blot with the indicated antibodies. B. Experiment was performed as in (A) using WT or OX40-/- Ag-specific CD8 T cells. C. The lysates were fractionated and analzyed by Western blot with the indicated antibodies. C, cytoplasm, N, nucleus. D. Average number of cells recovered from each mouse in each group. Each experiment was repeated twice for Rat Ig group, 3 times for anti-OX40 group with similar results and once for anti-OX40 treated OX40-/- group.

The data demonstrate that Mad4 and Mnt proteins are up-regulated after anti-OX40

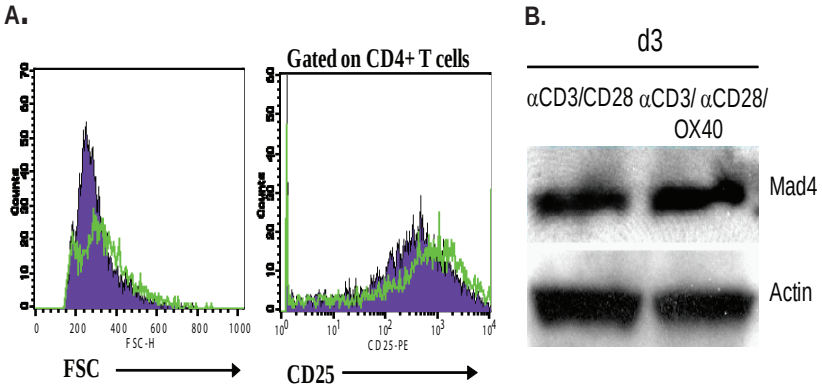
engagement *in vivo*. Since anti-OX40 could be acting directly via CD4 helper T cells, we aimed to determine whether anti-OX40 was acting directly on CD8 T cells or indirectly through other cell populations. To determine if this result was specific to direct signaling of OX40 expressed on the CD8 T cells, we examined Mad4 and Mnt protein expression in WT and OX40-/- OVA-specific CD8 T cells after adoptive transfer and administration of Ag and anti-OX40. We did not perform this experiment in the CD4 T cell model because the OX40-/- D011.10 mice were not currently available. Our results showed that Mad4 and Mnt protein levels did not increase and were >3- and 2-fold lower, respectively in OX40-/- CD8 T cells compared to WT control (Figure 2.8B). The levels of c-Myc were not examined in this experiment but we hypothesize the levels would not be different between the two groups because we did not see differences in the level of expressionbetween rat Ig and anti-OX40 treated mice. The up-regulation of c-Myc in CD8 T cells appears to be independent of signaling through OX40. Based on these results, we conclude that the anti-OX40-mediated increase in Mad4 and Mnt protein expression is in part mediated by direct OX40 signaling on the Ag-specific CD8 T cells.

To determine if Mad4 and Mnt proteins were also localized in the chromatin-bound fraction and up-regulated compared to control, as was observed in CD4 T cells, we examined cytoplasmic and chromatin-bound fractions from Ag-specific CD8 T cells for Mad4 and Mnt expression. Mad4 and Mnt levels were predominantly localized in chromatin-bound fraction of CD8 T cells after anti-OX40 treatment and were increased compared to Rag Ig control (Figure 2.8C). c-Myc was also localized in the chromatin bound fraction but the protein levels were not different between the two groups (Figure 2.8C). An increased

percentage of Ag-specific CD4 T cells were recovered from cells treated with anti-OX40 compared to rat Ig or from OX40-/- mice (Figure 2.8D). These data demonstrate that CD8 T cells, like CD4 T cells, upregulate Mad4 and Mnt in the chromatin-bound fraction following ligation of anti-OX40. This may represent an important common mechanism mediating survival of the blast crisis phase occurring at approximately day 3 in proliferating T cells.

Mad4 protein is not regulated after OX40 engagement in cell stimulated in vitro in CD4+ T cells

We also examined the levels of Mad4 protein in CD4 T cells stimulated *in vitro*, to determine if the OX40-mediated up-regulation of Mad4 protein was specific to *in vivo* activation of cells. CD4 T cells were purified from spleens and plated *in vitro* and stimulated with anti-CD3 and anti-CD28 or anti-CD3, anti-CD28 and anti-OX40 for 3 days. Cells were analyzed by flow cytometry for differences in cell size and the activation marker, CD25. Cells treated with anti-OX40 were slightly larger and had increased CD25 expression, demonstrating an OX40-specific effect on the cells, although the differences were not as great as seen *in vivo* (Figure 2.9A). However, Mad4 protein levels did not differ in samples treated with or without anti-OX40 *in vitro*, demonstrating that the model could not be recapitulated *in vitro* (Figure 2.9B). In the absence of stimulation, Mad4 protein was undetectable (naive fraction, Figure 2.5A and 2.6).



Purple- CD3/CD28 stim
Green- CD3/CD28/OX40 stim

Figure 2.9: Mad4 is not regulated in CD4 T cells after anti-OX40 stimulation *in vitro*. A. CD4 T cells were isolated from spleens and stimulation *in vitro* for 3 days with anti-CD3 and anti-CD28 in the presence or absence of anti-OX40. Cell size is depicted by foward scatter (FSC) and activated cells are depicted by CD25+. Filled purple- anti-CD3/anti-CD28 alone, Green- anti-CD3/anti-CD28/anti-OX40. B. Lysates were analyzed by Western blot with the indicated antibodies. Experiment was repeated 3 time with similar results.

Discussion

Mad family proteins are transcriptional repressors and are thought to antagonize c-Myc function. The expression of Mad4 proteins and c-Myc have been reported to be reciprocal in most cell systems studied, including lymphocytes (164). Mnt, on the other hand, is readily detected along with c-Myc in proliferating cells but has also been demonstrated to block the ability of Myc to transform cells in culture (165) and can directly repress some, but not all, Myc-Max target genes (185, 310, 320, 349). Here we show that engagement of OX40 by an agonist Ab led to a decrease in the transcript levels of Mad4 mRNA, but led to the up-regulation of Mad4 protein and its related family member, Mnt. Mad4 and Mnt proteins were temporally up-regulated at days 3 and 4 following engagement by anti-OX40 and the levels decreased at day 6 as the cells contracted and become more quiescent. c-Myc protein was co-expressed with Mad4 and Mnt at days 3 and 4, but on the contrary, the levels remained constant at 6 when the Mad4 and Mnt levels decreased. Mad4 and Mnt levels were increased in Ag-specific CD4 T cells treated with anti-OX40, compared to cells treated with anti-CTLA-4, implying a role for these proteins in cell survival. The Mad4 and Mnt paradigm also applied to anti-OX40 CD8 T cell survival, as we saw increases in the Mad4 and Mnt protein levels in Ag-specific CD8 T cells. Mad4 and Mnt proteins were not up-regulated in OX40-deficient Ag-specific CD8 T cells. Finally, we showed that this model was specific to cells activated *in vivo,* because we were not able to recapitulate this model *in vitro.*

Typically RNA levels are directly correlated with the level of protein expression. In this study, we observed a decrease in Mad4 transcripts in Ag-specific T cells after anti-OX40 engagement whereas the protein levels were increased. There are other gene

products that have been reported to have discrepancies between protein and mRNA levels. For example, a correlation between mRNA and protein levels was not observed in a study examining matrix metalloproteinase (MMP) proteins in human prostate cancers. MMP2 mRNA was decreased whereas the protein levels were significantly higher (350). Additionally, concordance between transcript and protein levels for certain genes in the LNCaP prostate cancer cell line were shown to vary greatly (351-352). These previous studies suggested that the discrepancy between RNA and protein levels could be due to a lag time for the changes at the RNA level to be reflected at the protein level. However this explanation is unlikely in our study because of the reported short half-life of Mad4 (181-184). Another possible reason for the inconsistency between Mad4 transcripts and protein is due to the inherent limitations of the gene microarray. The methodology and algorithms for Affymetrix data analysis are gene based and it cannot discriminate multiple mRNAs transcribed from the same gene (353). Mad4 transcripts have been detected as a doublet in muscle, kidney and lung tissue but not in brain, heart, liver (354) as well in P19 neuronal cells (220). The antibody used for detection in our experiments could detect a different isoform of the protein than was detected by the gene array and real-time PCR. The transcripts that were detected by gene microarray and real-time PCR could encode a non-coding message, whereby the transcript could have another function aside from encoding the protein. Additional reasons for poor correlation between gene array and protein expression could be due to post-transcriptional and post-translational modifications (355-356). Mad4 mRNA is very short-lived (357) and the RNA stability Mad4 could account for the differences observed at the transcript and protein level. It has been demonstrated that

c-Myc can be autosupressive, as the protein levels increase, it negatively regulates the rate of transcription (398-366). Mad4 transcripts may be regulated in a similar manner in anti-OX40 stimulated Ag-specific T cells. To further clarify the disparity between RNA and protein levels observed in this study, a more comprehensive analysis of Mad4 transcription to measure the rate of transcription by polysome fractionation or a Northern blot could be performed. Nevertheless, our initial gene regulation data has led us to an interesting observation in the regulation of Mad4, Mnt and c-Myc in activated T cells and the possible association between the expression of these genes and OX40-mediated T cell survival *in vivo.*

T cell activation induces expression of c-Myc (209-210) and its expression is required for T cell proliferation (367-368). This study is the first to report an increase in c-Myc protein expression in T cells after OX40 engagement. Previous studies looking at the molecular mechanisms downstream of OX40 signaling have pointed to NF-κB as a central mediator of the effects of OX40 on T cell survival. Several reports have shown that OX40 recruits TRAF 2, 3 and 5 to its cytoplasmic tail and the interaction of OX40 with TRAF 2 and 5 leads to activation of the NF-κB1 pathway (114, 117). The activation of NF-κB1 is a key event proceeding expression of specific genes responsible for proliferation and survival in T cells. c-Myc is a known target gene for NF-κB transcriptional activation (369-370), so c-Myc up-regulation after OX40 activation could in part be mediated via signaling through NF-κB.

We hypothesized that the up-regulation of c-Myc protein in activated T cells drives proliferation and consequently AICD, which is counteracted by Mad4 and Mnt

proteins after anti-OX40 engagement, ultimately allowing for T survival. In addition to signaling through TRAF2, OX40 can target the PI3K/Akt pathway and the sustained Akt signaling driven by OX40 leads to up-regulation of several anti-apoptotic protein Bcl-2 family members, including Bcl-x_L, Bcl-2 and Bfl-1. Studies have suggested that signaling via this pathway is necessary for T cell survival after OX40 engagement (89-90) and it has been proposed that the balance between the levels of the pro-apoptotic protein, Bim and the pro-survival protein, Bcl-2 can mediate T cell survival or death. Bim is a known target of c-Myc (72, 301) and Bcl-2 can block c-Myc induced apoptosis specifically without significantly affecting c-Myc induced proliferation (290, 292, 301) and Bcl-2 and Bcl-x_L have been shown to be down-regulated by forced c-Myc expression (372). The up-regulation of Bcl-2 after OX40 stimulation may potentially inhibit c-Myc induced Bim expression and apoptosis and therefore increase the survival of effector T cells. However, an increase in Bcl-2 was not been detected in our model (97). Hence the up-regulation of Mad4 and Mnt in anti-OX40 stimulated T cells may be a key to preventing c-Myc induction of Bim mediated apoptosis and allow for enhanced T cell survival.

A recent study showed that a 2- to 15-fold increase in transgentically expressed c-Myc protein over physiological levels was sufficient to induce apoptosis. The author proposed that the increased levels of c-Myc led to binding new sites, such as Myc target genes in the apoptotic pathway and an expansion of the transcriptional response (297). An alternative hypothesis was that the high levels of c-Myc were acting on the same target genes as low levels of c-Myc; however to induce apoptosis, additional pro-apoptotic signals were needed. The sustained increased in c-Myc protein after OX40

stimulation could risk targeting genes for the induction of apoptosis, such as Bim, which is important in mediating T cell death. The increased expression of Mad4 and Mnt could be compensating to repress apoptosis.

Evidence supporting the hypothesis that Mad4 and Mnt play a role in survival comes from studies using knockout mice. Granulocytes from *Mad1* knockout mice underwent increased apoptosis compared to control when grown in limiting amounts of cytokines (373-374). This phenotype was rescued when Mad1 was over-expressed in these cells. *Mad3* deficient mice also displayed decreased cell survival in the thymocyte population compared to control (221). Additionally, there was decreased T cell survival in Mnt deficient mice (321). This study is the first to report a role for Mad4 in T cell survival.

A recent study demonstrated that Mad1 could inhibit c-Myc induced apoptosis in response to a wide range of stimuli and by inhibiting the lipid phosphatase, phosphatase and tensin homologue deleted on chromosome ten (PTEN). PTEN regulates the PI3K/Akt pathway and Mad1 suppresses PTEN and thereby activates the Akt survival pathway (375). Mad4 and Mnt could be working via similar mechanisms as Mad1, whereby Mad4 and Mnt could repress PTEN or other pro-apoptotic gene targets and contribute to the sustained expression of the pro-survival pathways.

In this study, we provide evidence that both Mad4 and Mnt proteins are co-expressed with c-Myc in proliferating T cells after stimulation through anti-OX40 *in vivo*. There have been other reports where Mad proteins were detected along with Myc proteins in the same cell type. In developing neural tissue and P19 cells, Mad2 and Mad3

transcripts were predominantly detected in proliferating cells prior to differentiation and were co-expressed with c-Myc or N-Myc. The authors confirmed that both Myc and Mad proteins were expressed with Max within the same population (220). Studies examining the relative levels of Mnt and c-Myc after cell cycle induction have revealed that Mnt is induced shortly after c-Myc in cycling cells and the proteins are co-expressed at the same time (Peter Hurlin, personal communication). It is possible that c-Myc and Mad proteins are acting on different genes. Hundreds to thousands of genes are modulated by Myc (284), but it has not been definitively shown that Myc regulated genes are conversely regulated by Mad family proteins. Experiments examining the genomic binding sites of Drosophila Mnt and dMyc demonstrate that they recognize both unique and overlapping sites (267). Thus it remains to be determined whether Mad4 and Mnt act via different genes than c-Myc in anti-OX40-stimulated T cells or whether Mad4 and Mnt modulate expression of c-Myc regulated genes.

Our model demonstrating Mad4 and Mnt proteins were up-regulated in Ag-specific CD4 T cells activated by anti-OX40 also applied to the CD8 T cell model. Mad4 and Mnt protein levels were increased compared to rat Ig control, however c-Myc levels did not change, implying a common mechanism for Mad4 and Mnt in OX40-mediated T cell survival in Ag-specific CD4 T cells as well as CD8 T cells. Mad4 and Mnt levels were not increased in OX40-deficient Ag-specific CD8 T cells, implying that the increased levels of Mad4 and Mnt in OX40-stimulated T cells is, at least in part, mediated by direct signaling through OX40 on Ag-specific T cells.

Mad4 and Mnt proteins are up-regulated after stimulation through anti-OX40 in

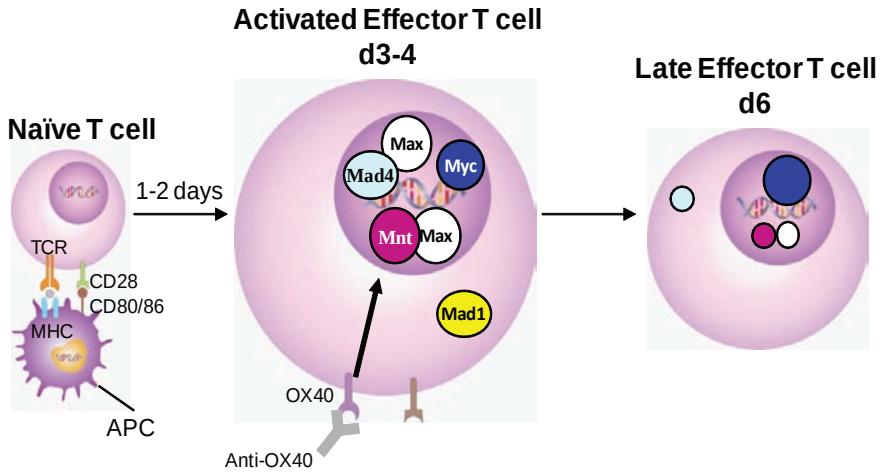
T cells *in vivo* but this effect was not recapitulated *in vitro*. Mad4 protein levels did not differ significantly in samples treated with or without anti-OX40 *in vitro,* suggesting that this finding is specific to cells activated *in vivo*. Many factors could contribute to the discrepancy between the *in vivo* and *in vitro* results including the cytokines concentrations and the levels of oxygen in the blood. The *in vivo* studies give a more accurate representation of the specific signaling pathways after stimulation through anti-OX40.

In contrast to the regulation of Mad4 and Mnt proteins in anti-OX40 stimulated T cells, Mad1 protein levels were not changed. Functionally the four Mad proteins appear to behave in a similar fashion but it has been previously shown that these proteins are differentially induced and expressed in response to distinct signals (220). Recent data showed that Mad4 competes more efficiently for heterodimerization with Max than other Mad proteins and Myc in live cells (255). These studies suggested that Mad4 could be the most efficient repressor of Myc function in the Mad family. The bHLHZip regions of Mad-family proteins are not functionally equivalent, suggesting that they might have unique and overlapping sets of target genes and biological activities (376-377). This could explain why there is specific regulation of Mad4 and Mnt proteins after OX40 engagement in T cells but not Mad1. The expression levels of Mad2 and Mad3 were not examined in this study, but the transcripts were not detected in microarray experiments (unpublished observation), suggesting these transcripts are not regulated by anti-OX40 in T cells.

The data presented here demonstrate that Mad4 and Mnt protein expression are up-regulated after stimulation through anti-OX40 in T cells, but not in rat Ig control or after

anti-CTLA-4 blockade in CD4 and CD8 T cells (Figure 2.10 and 2.11). We hypothesize that the discrepancy between the RNA and protein data could be due to post-translational modifications of the protein after stimulation through anti-OX40 in T cells. It is established that the half-life of Mad family proteins is very short, approximately 10-20 minutes, so these data could be explained if signaling through OX40 enhanced the post-translational stabilization of these proteins.

A. OX40 engagement:



B. No OX40 engagement or CTLA-4 blockade:

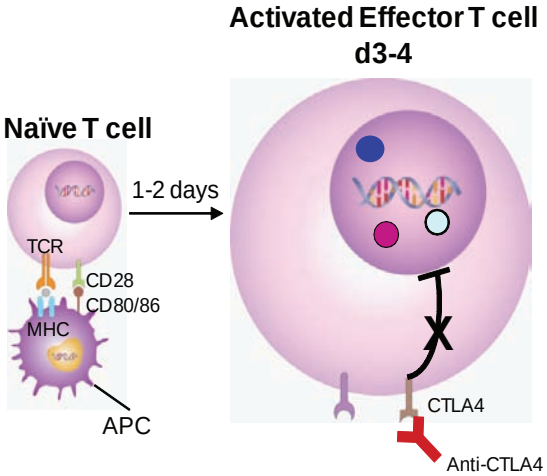


Figure 2.10: Proposed model for the role of Mad4 and Mnt proteins in anti-OX40 stimulated CD4 T cells. A. After anti-OX40 engagement on activated CD4 T cells, Mad4 and Mnt proteins are up-regulated and localized to the nucleus, as the cells undergo their initial expansion and increase in size. At day 6, when the cells have contracted and are decreasing in size, Mad4 is localized in the the cytoplasm and Mnt is barely detectable in the nucleus, while c-Myc levels remain elevated. B. In CD4 T cells engaged by anti-CTLA-4 (or in the absence of OX40 engagement), Mad4, Mnt and c-Myc levels are decreased.

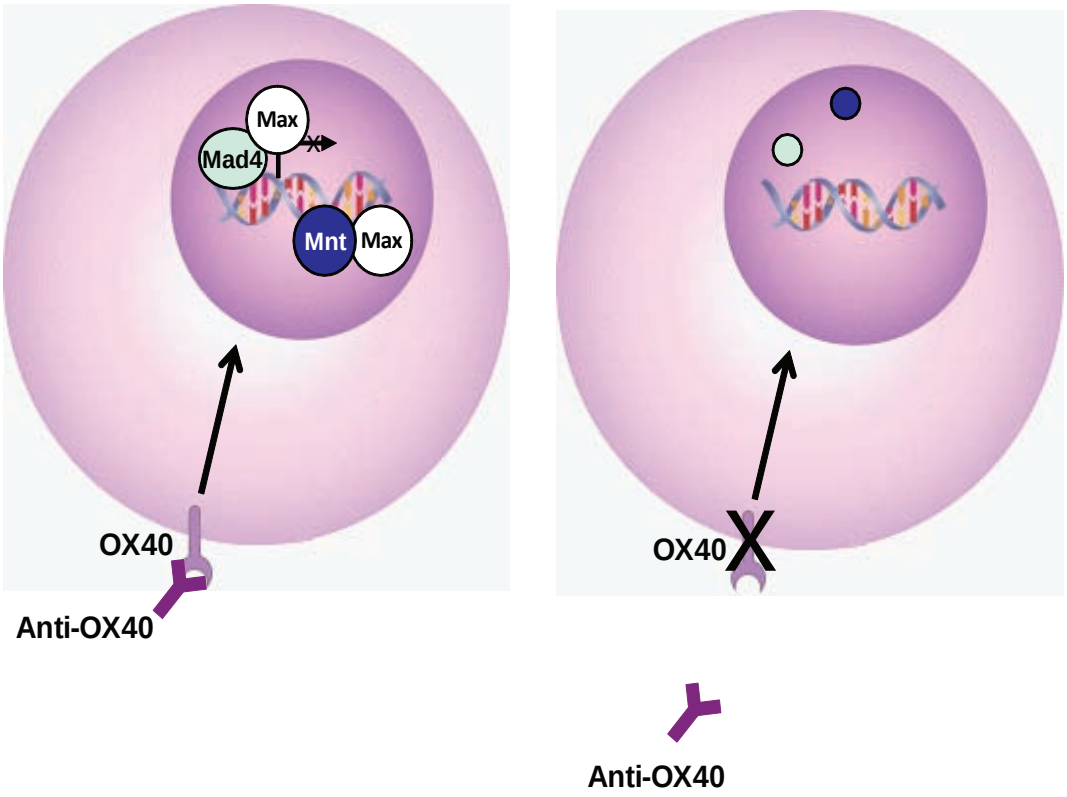


Figure 2.11: Proposed model for the role of Mad4 and Mnt proteins in anti-OX40 stimulated CD8 T cells. A. After anti-OX40 engagement on activated CD8 T cells, Mad4 and Mnt proteins are up-regulated and localized to the nucleus compared to Rat Ig control (not shown). In the absence of OX40, Mad4 and Mnt levels are not increased.

Chapter 3

Signaling through anti-OX40 stabilizes Mad4 and Mnt proteins

Abstract

Myc and Mad family proteins are short-lived proteins. There is clear evidence

demonstrating that c-Myc and Mnt proteins are regulated at the post-translational level, but the post-translational modifications of Mad proteins have been less extensively characterized. Our previous results showed that after T cell stimulation through anti-OX40 *in vivo*, Mad4 transcript levels were decreased whereas the protein levels were increased. Mnt transcript levels were similar by gene microarray, yet we observed an increase in the protein levels in Ag-stimulated T cells isolated from anti-OX40 treated mice. Therefore, we hypothesized that signaling through OX40 stabilizes Mad4 and Mnt proteins. The data presented here shows that after treatment with the *de novo* protein synthesis inhibitor cycloheximide for 60 minutes following *ex vivo* isolation of Ag-stimulated T cells from anti-OX40 treated mice, the levels of Mad4 and Mnt proteins remain relatively constant. In contrast, Ag-stimulated T cells isolated from anti-CTLA-4 treated mice at day 4 exhibit rapid protein degradation for both Mad4 and Mnt. To determine the mechanism of regulation, we show that a conserved serine residue within the Mad4 sequence was essential for mediating rapid degradation of Mad4 proteins. These results provide evidence that post-translational events occur to stabilize Mad4 and Mnt proteins in Ag-stimulated T cells isolated from mice treated with anti-OX40.

Introduction

Transcription factors are regulated at the post-translation level to ensure proper cellular function. Post-translational modifications include phosphorylation, acetylation, methylation and ubiquitination. The Max-interacting network can control many different aspects of cell behavior and fate and therefore are tightly regulated during cellular proliferation and differentiation to ensure proper cellular function (167, 378). For instance, c-Myc is regulated at multiple levels, which are important for controlling the stability and transactivation functions. c-Myc is often controlled by specific signaling pathways (192, 203, 207, 333, 379-384). Although the levels of Max protein remain constant throughout different conditions (385-3864), it also appears to be regulated at the post-translational level. Published data has shown that Max is constitutively phosphorylated *in vivo* by the protein kinase CK2 (387-391) and there is evidence that Max is acetylated by p300 *in vitro* and *in vivo* (333, 392). The post-translational modifications of Mad proteins have been less extensively studied, but phosphorylation of Mad1 and Mnt has been shown to be important for their functions.

A major mechanism regulating protein stability is the ubiquitin-dependent proteasomal pathways that lead to protein degradation. Ubiquitination is a process of conjugating ubiquitin to proteins that are targeted for proteasomal degradation in an ATP-dependent multi-step process that involves at least three additional enzymes: E1 activating, E2 conjugating and E3 ligase enzymes. There is extensive evidence demonstrating that the stability and ubiquitin-dependent degradation of c-Myc is controlled by phosphorylation and dephosphorylation events (393-395). The S62 and T58 sites within the Myc transactivation

domain (TAD) are targeted for phosphorylation and are believed to be important for Myc's stability and degradation. The two sites are interdependent because phosphorylation at S62 is a prerequisite for phosphorylation at T58. Phosphophorylation of S62 stabilizes Myc, whereas phosphorylation of T58 initiates a series of events that leads to Myc degradation. T58 phosphorylated Myc is ultimately polyubiquitinated and is degraded by the proteasome.

The post-translational modifications of Mad proteins have not been as extensively studied. A recent paper showed that Mad1 could be phosphorylated and degraded as a consequence of activation of the PI3K/Akt and mitogen activated protein kinase (MAPK) pathway, involving signaling through Ras/Raf/ERK (334). The Mad proteins contain the conserved phosphorylation consensus sequence, RXRXXS, which is a target for ACG family members, including Akt, S6K1 and RSK (Figure 3.1) (334). Akt is activated after mitogenic stimulation and can activate mammalian target of rapamycin (mTor), which signals to eukaryotic inhibition factor 4E (eIF4E) and the S6K1. eIF4E and S6K1 have been implicated in controlling cell size and G1 cell cycle progression (396-397). The authors demonstrated that serum stimulation led to ubiquitination and degradation of Mad1 and Mad1 was ubiquitinated in response to phosphorylation by RSK and S6K at serine 145 (334). They showed phosphorylation was inhibited by treatment with the combination of the PI3K inhibitor, LY294002 and the MEK inhibitor, PD98059. However, treatment with each individual inhibitor did not inhibit Mad1 phosphorylation, leading them to conclude that both the ERK/RSK and PI3K/Akt signaling molecules regulate Mad1 (Figure 3.1). Additionally, the E3 ligase, c-IAP1 has been shown to modulate Mad1 degradation (398). The role of these pathways in regulation of the other Mad family members has not been

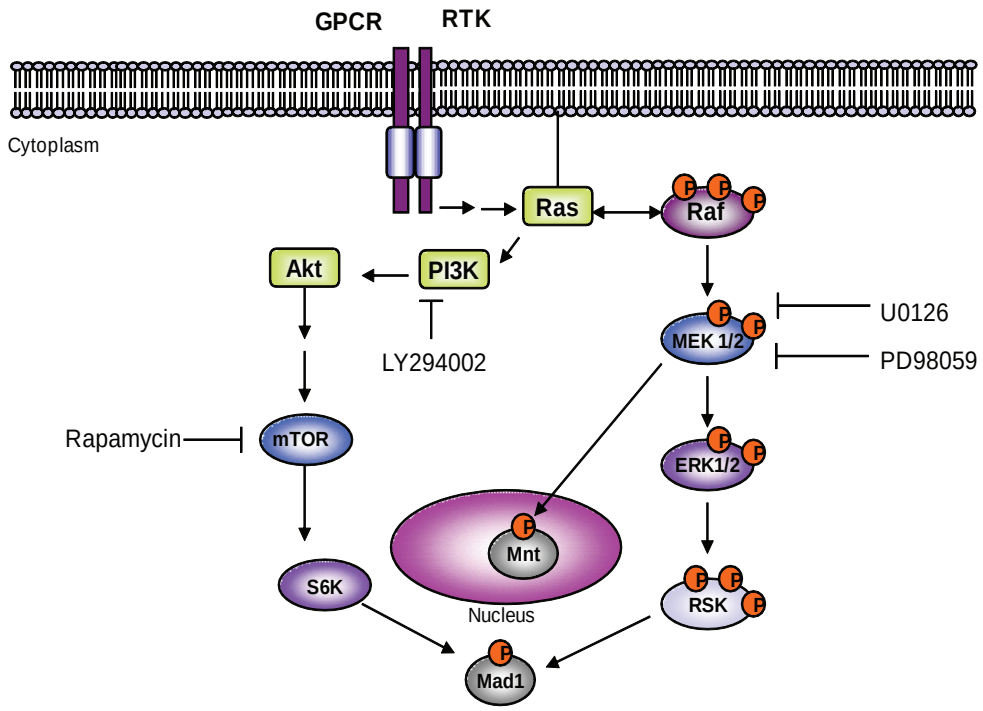


Figure 3.1: Potential signaling pathways mediating Mad and Mnt stability. Activation of receptor tyrosine kinases (RTKs) or G protein-coupled receptors (GPCRs) by growth factors or mitogens leads to the activation of Ras, which recruits Raf at the plasma membrane, which leads to phosphorylation at multiple sites. MEK1/2 is then phosphorylated and then phosphorylates RSK, which can inhibit Mad1 function by phosphorylating it and targeting it for degradation. Akt is activated by mitogen stimulation and can activate mTor, which signals to S6K1, which also phosphorylates Mad1.

studied.

Mnt is constitutively expressed in proliferating and differentiated cells but is expressed at different levels (165, 186). The phosphorylation state of Mnt is thought to directly regulate its function. Mnt is phosphorylated upon entry into the cell cycle and is dephosphorylated in differentiating cells (399). The hypophosphorylated form of Mnt is able to interact with mSin2 and recruit HDACs (399). It appears that Mnt is phosphorylated as a consequence of MKK/ERK signal transduction pathways, as

treatment of cells with the MEK inhibitor, U0126 prevented Mnt phosphorylation after serum stimulation in NIH3T3 cells and restored the Mnt-Sin3 interaction (399). However, the exact post-translational mechanisms of Mnt have not been fully elucidated.

In this chapter, we demonstrate that the OX40-induced increase in Mad4 and Mnt protein levels is due to increased stabilization of these proteins. Mad proteins have a short half-life and are rapidly degraded within 10-20 minutes (167, 175, 181-184, 188, 190). However, after treatment with anti-OX40, Mad4 and Mnt proteins were not degraded for at least 60 minutes after treatment with the *de novo* protein synthesis inhibitor, cycloheximide. The stabilization of Mad4 and Mnt protein levels was specific to anti-OX40 signaling when compared to anti-CTLA-4 blockade. The mechanism by which Mad4 is degraded has not been described, therefore we propose a potential mechanism of regulation via the putative RXXRXS phosphorylation consensus sequence, which was previously shown to be important for Mad1 protein stabilization (334). A plasmid containing a mutation in the putative phospho-serine residue, S145 led to increased levels of protein expression when compared to WT Mad4. The mutant Mad4 showed also increased protein stability, as demonstrated by a longer half-life after cells were incubated with cycloheximide. These studies identify a potential target for Mad4 stabilization mediated by anti-OX40 and potentially address the mechanism by which anti-OX40 regulates Mad4 protein levels.

Materials and Methods

Mice

Four- to 6-week-old male and female C57BL/6 and BALB/c mice were purchased from Charles River Laboratories and used at 6–10 weeks of age. BALB/c TCR transgenic DO11.10 ($OVA_{323–339}$) mice were bred and maintained at the Earle A. Chiles Research Institute. An institutional animal care and use committee approved all animal studies. All mice were bred and maintained under specific pathogen-free conditions in the Providence Portland Medical Center animal facility. Experimental procedures were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Preparation of antibodies

Control rat Ig antibody was purchased from Sigma (St. Louis, MO), whereas rat anti-OX40 antibody (OX86) and anti-CTLA-4 (9H10) were produced in the laboratory from hybridomas and affinity purified over protein G columns.

Adoptive transfer and immunization

Spleens and DLNs were harvested from DO11.10 mice and processed by crushing between two frosted glass microscope slides and RBC lysed with Ammonium chloride-based RBC lysis buffer (ACK, Cambrex, East Rutherford, NJ). The percentages of DO11.10 cells was identified by FACS using KJ1-26 antibodies before transfer (BD Biosciences, San Jose, CA). A total of 2–3x10^6 transgenic TCR T cells were adoptively

transferred i.v. into BALB/c recipients. One day later, mice were immunized s.c. with 500µg of OVA (Sigma) and 50µg of anti-OX40 (OX86) or 100µg of anti-CTLA-4 (9H10). The following day, mice were given a second injection of anti-OX40 or anti-CTLA-4.

Purification of Ag-specific T cells

DLNs were collected at indicated times after immunization and processed into a single-cell suspension. Cells were stained for 0.5h on ice with biotinylated KJ1-26 antibody (0.5mg/10^6 cells) and then washed. Cells were harvested using anti-biotin microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity (>90%) was verified by flow cytometry.

C*ycloheximide assay*

D011.10 T cells were purified from DLNs at day 4, as described above. 1-2x10^6 Ag-specific T cells were plated *ex vivo* in a 24 well plate and cells were treated with 50µg/ml cycloheximide for indicated times.

Immunoblot analysis

Cells were collected by centrifugation and resuspended in Laemmli buffer and boiled at 98°C for 10 min. Lysates were quantitated using DC Protein Assay (BioRad, Hercules, CA), run on polyacrylamide gels (equivalent microgram quantities of protein), and transferred onto nitrocellulose or PVDF membranes. Antibodies for Mad4, Mnt, GFP

(Santa Cruz Biotechnology, Santa Cruz, CA), GAPDH (Millipore, Billerica, MA) were used to detect these proteins.

Site-directed mutagenesis

Mad4 cDNA was kindly provided by Peter Hurlin (Shriners Research Institute, Portland, OR) and cloned into a TOPO vector (Invitrogen). S145A mutant Mad4 was generated by site directed mutagenesis (Stratagene, Agilent Technologies, Cedar Creek, TX) using the following primers: GTGTGCGCACAGACGCCACTGGCTCTGCTG and CAGCAGAGCCAGTGGCGTCTGTGCGCACAC and verified by sequencing (OHSU sequencing core, Portland, OR). The mutated Mad4 gene was subsequently cloned into the modified pWPI-GFP vector (Trono Lab, Ecole Polytechnique Federale de Lausanne and modified by Dr. Hong Ming Hu, EACRI, Portland, OR) using the USER enzyme. HEK-293 cells were plated at 1.5 $x10^5$ cells per well overnight in a 6 well plate and transfected with 2μg pWPI-GFP (empty vector), Wild-type pWPI-Mad4-GFP (WT) or S145A mutant pWPI-Mad4-GFP (S145A) using Metafectene (Biontex, Martinsried/ Planegg, Germany) for 48h under 5% FBS conditions. Cells were treated with cycloheximide (CHX; 100μg/ml) and harvested at the indicated times as described above. The cell lysates were analyzed by Western blot with the indicated antibodies.

Results

Mad4 protein is stabilized after OX40 engagement

Since the half-life of Mad family proteins is short, approximately 10-20 minutes, we hypothesized that signaling through anti-OX40 enhances the post-translational stabilization of these proteins. To address this hypothesis, we examined the stability of Mad4 and Mnt proteins in OX40 stimulated CD4 T cells incubated with cycloheximide, which inhibits *de novo* protein synthesis, hence protein stability can be observed in the absence of new protein translation. CD4 T cells were isolated from DLNs directly *ex vivo* four days after stimulation through Ag and anti-OX40 and incubated with the cycloheximide *in vitro* for the indicated time (Figure 3.2). The levels of Mad4 and Mnt protein levels remained relatively constant after cycloheximide treatment for 60 minutes after anti-OX40 treatment. However, anti-CTLA-4 treated cells exhibited rapid protein degradation for both Mad4 and Mnt starting at approximately 15 minutes after cycloheximide addition (Figure 3.2A). The average number of cells recovered from mice treated with anti-OX40 or anti-CTLA-4 is depicted in Figure 3.2B. These data support the hypothesis that signaling through anti-OX40 leads to stabilization of both the Mad4 and Mnt proteins in T cells.

Rapid degradation of Mad4 is conferred through the putative phosphorylation site, S145A

The post-translational mechanism by which Mad4 protein is rapidly degraded has not been previously described. It was previously shown that the Mad family member, Mad1 is phosphorylated at the serine 145 residue in the consensus sequence RXRXXS as a result of PI3K/Akt/mTOR and MAPK pathway activation and phosphorylation at this residue

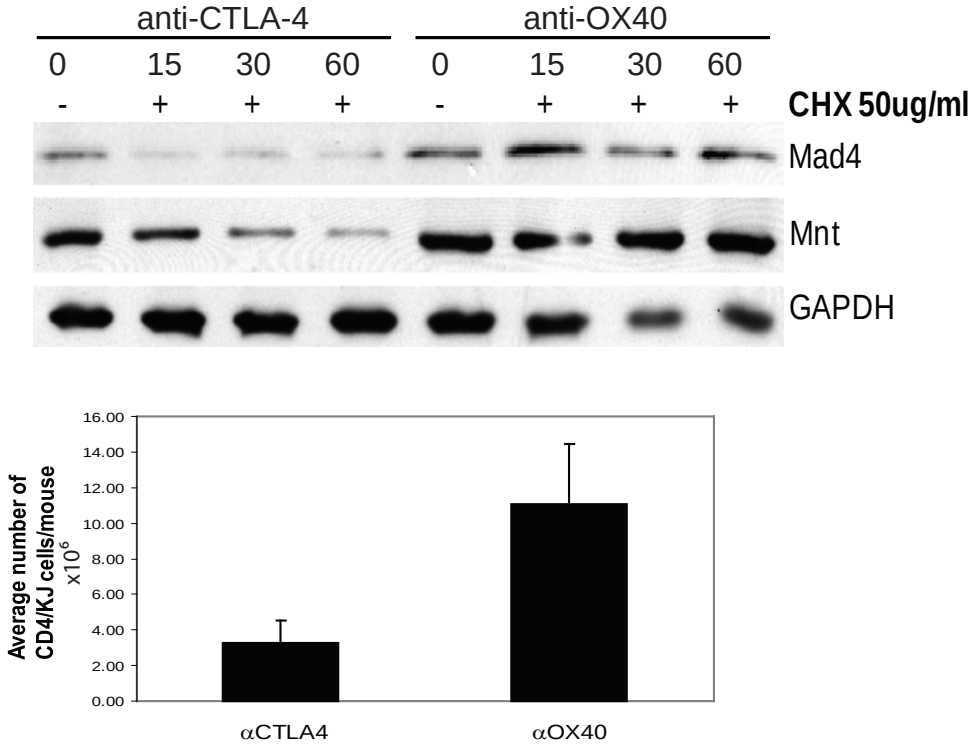


Figure 3.2: Mad4 and Mnt are stabilized in Ag-specific CD4 T cells after anti-OX40 stimulation. Ag-specific CD4 T cells were isolated from DLN at 4 days after treatment with OVA and anti-OX40. Cells were treated with cycloheximide (CHX; 50ug/ml) and harvested at the indicated time points. Lysates were analyzed by Western blot with the indicated antibodies. Experiment was repeated 3 times with similar results.

targets the protein for degradation (334). A mutation at this residue led to higher protein expression and stabilization of protein levels when compared to the WT Mad1 protein. Through comparative sequence analysis, we identified that the RXRXXS consensus sequence is conserved in the Mad4 protein sequence, therefore we hypothesized that this sequence regulated stabilization of Mad4 (Figure 3.3A). To test this hypothesis, using site-directed mutagenesis, we created a reporter plasmid containing WT Mad4 or S145A mutant Mad4 linked by IRES to GFP (Figure 3.3B). Stability was tested by transient transfection

of 293H cells with a plasmid that co-expresses Mad4 and GFP. The S145A mutant showed a higher level of Mad4 protein expression than that of WT Mad4 (Figure 3.3C). The levels of GFP expression remained constant between the groups as observed by Western blot (Figure 3.3C and D) and by flow cytometry (data not shown), ruling out the possible effect of differential transcription between the two plasmid constructs. To confirm that the reduced wild-type Mad4 protein levels in the proliferating cells was due to accelerated protein degradation, transfected cells were treated with cycloheximide to inhibit *de novo* protein synthesis at 48hrs post-transfection. Wild-type Mad4 was degraded rapidly, with a calculated half-life of <15 min; while Mad4 levels in the serine mutant remained stable after cycloheximide treatment for 60 min (Figure 3.3D). These results suggest that the rapid degradation of Mad4 is regulated by post-translational modifications at S145 of the RXRXXS consensus sequence and identify a potential mechanism exploiting the increased Mad4 protein stability in anti-OX40 stimulated T cells.

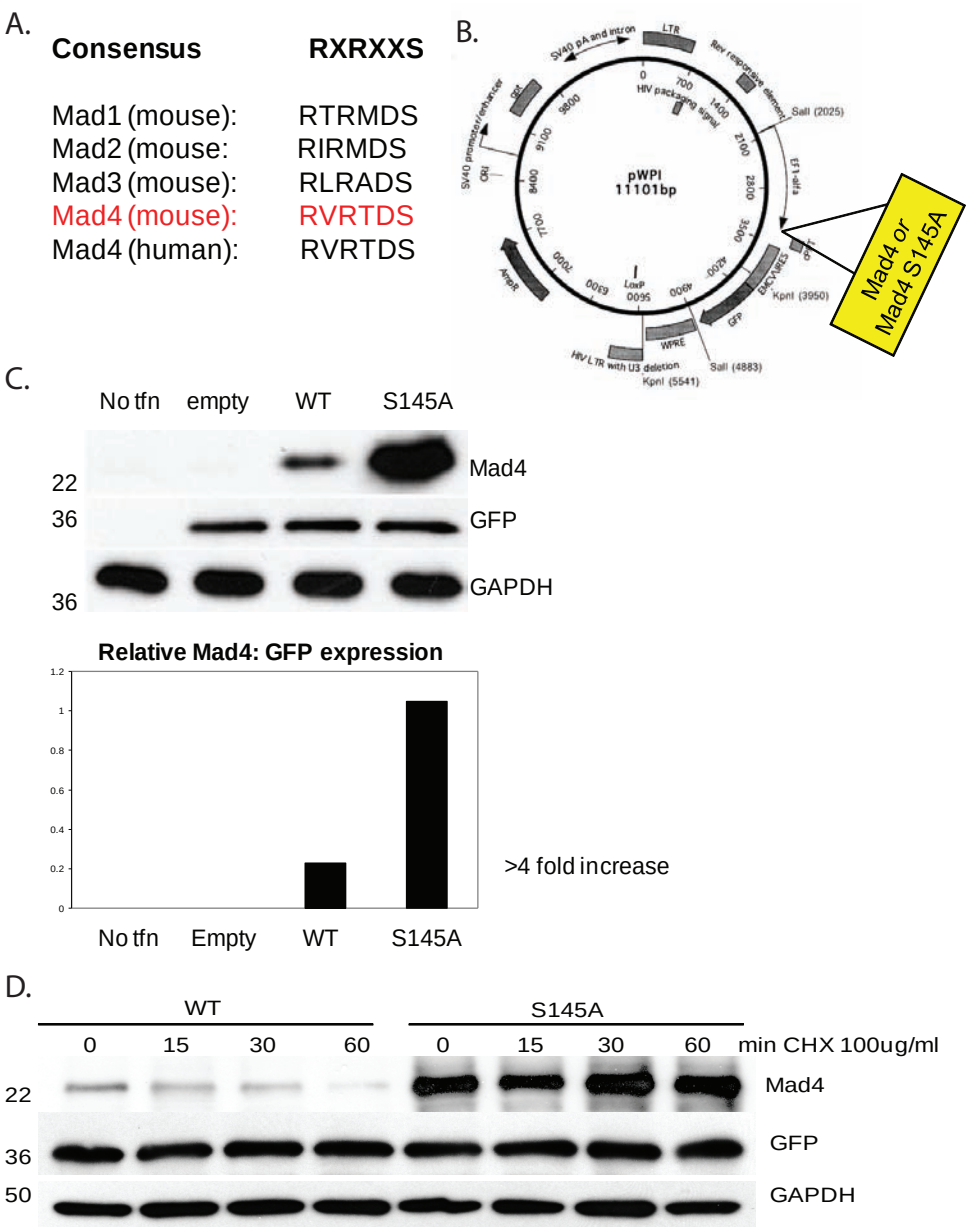


Figure 3.3: Mad4 degradation is conferred through the putative phosphorylation site, S145. A. Conserved consensus phosphorylation site in Mad famly members. B. Plasmid containing Mad4 and Mad4 S145A and GFP. C. Lysates were prepared from 293H cells transiently expressing empty vector (pWPI), wild-type Mad4 or S145A mutant Mad4 and analyzed by Western blot with the indicated antibodies. D. 293H cells expressing Mad4 and S145A mutant were treated with cycloheximide (CHX, 100ug/ml) and harvested at the indicated times. The cell lysates were analyzed by Western blot with the indicated antibodies. Experiment was repeated 3 times with similar results.

Discussion

Regulation of the Max-interacting network is important for cell cycle control. There is a great deal of evidence demonstrating that c-Myc protein is regulated not only at the transcriptional and translational level but also through post-translational mechanisms. c-Myc has been shown to be regulated through phosphorylation and ubiquitination by a number of signaling pathways and kinases. The mechanisms by which Mad4 and Mnt are regulated have been less extensively studied. In this study we showed that signaling through anti-OX40 stabilized both Mad4 and Mnt proteins against degradation. Additionally, we demonstrated the presence of a serine residue in Mad4 that was essential in mediating accelerated protein degradation. Mutation of this putative phosphorylation site led to increased stability of the protein.

Signaling through the PI3K and MAPK pathways has been implicated in cell growth and proliferation. It was previously shown that the PI3K and MAPK pathways mediate phosphorylation of Mad1 at S145 and the protein stability is regulated via these pathways (334). Cells that transiently over-expressed Mad4 were treated with PI3K or MAPK inhibitors to determine the effect on the stability of Mad4. We hypothesized that if the rapid turnover of Mad4 is regulated by phosphorylation at S145 and if phosphorylation was controlled by the PI3K and MAPK pathway, as previously demonstrated for Mad1, then treatment with specific inhibitors would prevent phosphorylation of that residue leading to increased levels of Mad4 protein. However, protein levels after treatment with these inhibitors were not changed, suggesting that Mad4 may not be specifically regulated via the MAPK or PI3K pathway (data not shown).

A recent report demonstrated that Mad1 is a target for phosphorylation by Akt at the S145 residue and this phosphorylation inhibited the repressive function of Mad1 by preventing it from binding to DNA target sequences but it did not affect the protein stability (400). Additionally, it was demonstrated that the S145A mutant Mad1, which is unable to be phosphorylated, rescued the DNA-binding ability of Mad1 and restored its repressive function, even in the presence of constitutively active Akt (400). This data is inconsistent with the previous report from Zhu et al. (2008), demonstrating that the S145 residue was phosphorylated by S6K and RSK and this phosphorylation targeted the protein for degradation. Additionally, the data from Zhu et al (2008), suggested that Mad1 was not a direct substrate for Akt phosphorylation (334).

In our studies, treatment with the Akt-specific inhibitor (Akt1,2 inhibitor) also did not affect Mad4 protein expression (data not shown), however these results need to be verified in T cells by treating activated T cells with these specific inhibitorsand assessing relative protein expression. Zhu et al. (2008) showed Mad1 stability was affected after treatment with a combination of both the PI3K and MAPK inhibitors (334). In our hands, treatment of the cells with a combination of both inhibitors caused cell death in the majority of the cells as early as after 1 hour of treatment and hence dual inhibition could not be correctly interpreted. These negative data are consistent with the biological function of OX40 which promotes activation of these kinases (90). Given that inhibition of PI3K and Akt did not affect that stability of Mad4, we hypothesize that OX40 is negatively regulating a yet unidentified kinase that is required for phosphorylation and degradation of Mad4.

In the study by Zhu et al. (2008), the authors showed phosphorylation of Mad1 was

inhibited by treatment with the combination of the PI3K inhibitor, LY294002 and the MEK inhibitor, PD98059. However, they reported that treatment with each individual inhibitor did not inhibit Mad1 phosphorylation, leading to the conclusion that both the ERK/RSK and PI3K/Akt signaling molecules are necessary for regulating Mad1. However, it has been reported that the MAPK inhibitor PD98059 only inhibits activation of the inactive form of MEK (401-402). The inhibitor U0126, on the other hand, inhibits both active and inactive MEK1,2 and is more potent and less toxic than PD98059. Treating the cells with the U0126 inhibitor as well would have provided more convincing evidence that Mad1 is not regulated by the MAPK pathway alone.

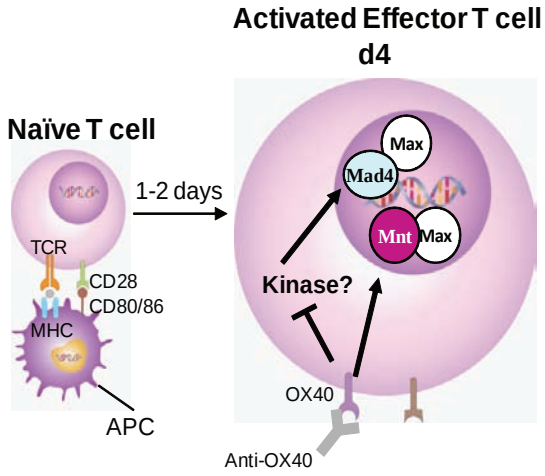
To further study the biological significance of the putative phospho-S145 residue in regulating the stability of Mad4 stability in OX40-stimulated T cells, a comparison between the phospho levels of Mad4 in cells treated with anti-OX40 or rat Ig or anti-CTLA-4 could be performed. We hypothesize that the levels of phospho-Mad4 would be decreased in cells treated with anti-OX40 compared to controls.

In addition to regulation at the post-translational level, the Max-interacting network are highly regulated at the transcriptional level as well. While the data suggest that Mad4 and Mnt are stabilized by post-translational mechanisms after stimulation through anti-OX40, the expression of these genes could also be regulated at the transcriptional level or by other mechanisms.

These results provide evidence that Mad4 and Mnt proteins are stabilized after anti-OX40 stimulation in Ag-specific CD4 T cells. Additionally, we demonstrated the presence of a putative phospho-serine residue in Mad4 that regulates its stability. Future studies will

determine whether anti-OX40 mediated phosphorylation of the S145 residue of Mad4 is necessary for the protein stability and will attempt to examine the exact signaling pathways involved in regulation of Mad4 and Mnt at the post-translational level (Figure 3.4).

A. OX40 engagement:



B. No OX40 engagement or CTLA-4 blockade:

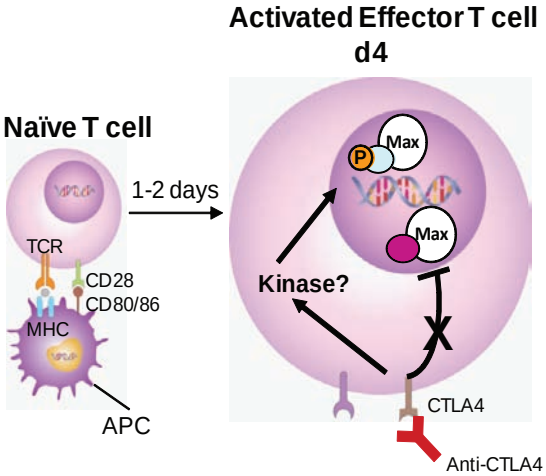


Figure 3.4: Proposed model for the role of Mad4 and Mnt proteins in anti-OX40 stimulated CD4 T cells. A. After anti-OX40 engagement on activated T CD4 T cells, Mad4 and Mnt proteins are stabilized. We hypothesize that signaling through OX40 inhibits a kinase that phosphorylates and leads to the degradation of Mad4. B. Mad4 and Mnt proteins are not stabilzed after signaling through anti-CTLA-4 (or in the absence of OX40 engagement) potentially because of the phosphorylation by a kinase that leads to the degradation of the proteins.

Chapter 4

Mad4 and Mnt proteins are important for cell survival after anti-OX40 engagement

Abstract

A major function of OX40 engagement on T cells is mediating increased cell survival leading to increased of memory T cell development. Although several candidate genes and pathways have been implicated in OX40-mediated cell survival, the exact molecular mechanisms that are essential in governing this effect have not been fully elucidated. In this chapter, we show that knockdown of Mad4 in combination with Mnt knockdown leads to a decrease in survival of OX40-activated Ag specific T cells. To further address the mechanisms that mediate Mad4 effects of anti-OX40-mediated survival, we examined Mad4 levels in IL-12 and STAT4 deficient Ag-specific T cells after anti-OX40 engagement. Recent data from our lab showed that the IL-12R signaling protein, IL-12Rβ2 was up-regulated after OX40 engagement and signaling through IL-12 via a STAT4 specific pathway was necessary for CD4 T cell survival. Mad4 levels were not up-regulated in IL-12 deficient Ag-specific T cells after ligation of OX40, suggesting Mad4 may play a role in survival of cells after OX40 engagement in an IL-12 dependent manner. These results show a role for Mad4 and Mnt proteins in the survival of effector CD4 T cells after OX40 stimulation.

Introduction

T cell activation *in vivo* leads to an initial expansion of Ag-specific T cells but the majority of stimulated T cells do not survive the initial activation event (391). Ligation of OX40 on T cells saves effectors from AICD, thus increasing the number of memory T cells that survive following immunization (338). Previous studies have shown that the proliferation of Ag-specific T cells from the DLN was greatly enhanced after stimulation through Ag and anti-OX40 compared to treatment with Ag and control antibody (rat Ig) at days 3 and 4 (Figure 4.1A) (87). Examination of the long-term survival of the Ag-specific T cells revealed that treatment with anti-OX40 enhanced the survival of Ag-specific T cells compared to treatment with Ag and rat Ig control or blockade using the anti-CTLA-4 antibody(Figure 4.1B) (87). Understanding the mechanisms that contribute to the ability of OX40 to enhance T cell survival could assist in the development of effective immuno-therapeutic strategies against tumors or chronic pathogens.

Several signaling pathways have been identified as key players in mediating OX40-enhanced T cell survival. Signaling through OX40 activates PI3K, which in turn activates Akt, a central mediator of cell survival (90). Studies have shown that the anti-apoptotic proteins, Bcl-2 and Bcl-X_L are elevated in OX40 stimulated cells, as a result of Akt activation and Akt, Bcl-2 and Bcl-X_L are not increased in OX40 deficient T cells (89-90). However, the role of Bcl-2 and Bcl-X_L in CD4 T cells is somewhat controversial because some studies have shown these proteins are dispensable for CD4 T cell survival (130-131).

We have shown that Mad4 and Mnt levels were increased in cells stimulated through anti-OX40 compared to CTLA-4 blockade, which led to the hypothesis that T cell survival

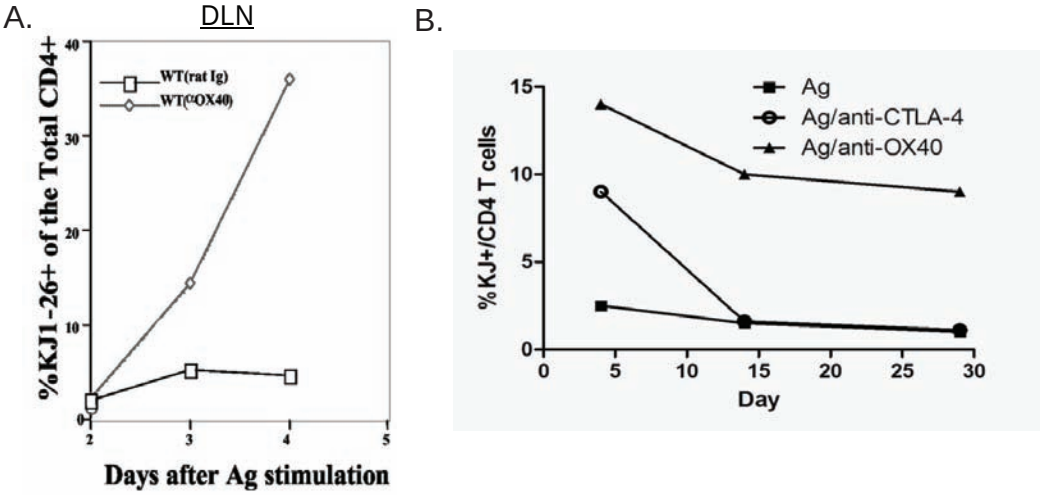


Figure 4.1: Costimulation by anti-OX40 leads to increased T cell proliferation and survival compared to Rat Ig and anti-CTLA-4. A. Increased proliferation of Ag-specific CD4 T cells after anti-OX40 engagement over time. B. Increased survival of Ag-specific CD4 T cells after anti-OX40 engagement compared to anti-CTLA-4 control. From: Weinberg et al. (2004) J Leukoc Biol. 75:962. Reproduced with permission from the FASEB Office of Publications.

following OX40 engagement could be mediated through a Mad4- and Mnt-dependent mechanism. Mad family members have been previously demonstrated to play a role in cell survival in various systems. Mad1 inhibited apoptosis when over-expressed in a tumor cell line (404). The study showed that over-expression of Mad1 in an inducible system prevented cell death apoptotic stimuli, including activation of the Fas or TRAIL receptor, and UV-induced DNA damage. The activation of caspase 8 was significantly reduced in cells over-expressing Mad1 after treatment with Fas specific antibodies. Additionally, exogenous expression of Mad1 rescued cells from apoptosis in cells using a microinjection approach. Cells were microinjected with c-Myc or E1A, which led to 3-fold decrease in surviving cells in culture. Co-expression of c-Myc with Mad1 in these cultures was sufficient to rescue most of the cells from apoptosis. Deletion of either the SID or Zip domain of Mad1

prevented rescue from c-Myc induced apoptosis, demonstrating the importance of these domains for the survival function. However this study was unable to determine if Mad1 was acting as a transcriptional repressor at c-Myc target genes that regulate apoptosis or through other means. Nevertheless, this study does provide evidence for a role of Mad proteins in cell survival, presumably by inhibiting c-Myc-mediated apoptosis (404).

Additional evidence for a role of Mad proteins in cell survival was observed in Mad3 knockout mice (221). Mad3 knockout mice displayed increased sensitivity to gamma irradiation in thymocytes and neuronal progenitor cells. The authors speculated that the deletion of the *Mad3* gene released the repression of Myc target genes, allowing transcriptional activation of Myc targets specific for apoptosis and therefore decreased cell survival. Consistent with this hypothesis, *Mad1* knockout mice exhibited decreased survival of granulocytic progenitor cells when grown in limiting amounts of cytokines, as well as increased sensitivity to induction of cell death using cytotoxic drugs (373-374). Conversely, over-expression of Mad1 led to increased survival of bone marrow derived myeloid cells in the presence of decreased cytokine levels (405). In view of these data, we hypothesized that increased Mad4 and Mnt protein expression in OX40-stimulated T cells was involved with the increased cell survival after anti-OX40 engagement. Our results show that loss of Mad4 and Mnt following siRNA knockdown led to decreased survival of Ag-specific CD4 T cells, supporting the hypothesis for a role for Mad4 and Mnt proteins in T cell survival after anti-OX40 engagement.

Recent data from our lab demonstrated that the cytokine IL-12 is required for anti-OX40-mediated CD4 T cell survival (103). IL-12 is a key cytokine secreted by APCs (104),

which primarily signals through the IL-12Rβ2 chain (104, 106) and induces phosphorylation of the Janus-family kinases JAK2 and TYK2, which in turn activates downstream STAT proteins, predominantly STAT4 (406-408). Signaling through IL-12 leads to enhanced CD4 T cell differentiation, cytokine production, and survival. Ruby et al (2008) demonstrated that the lifespan of IL-12 and STAT4 deficient Ag-specific CD4 T cells were decreased compared to wild type CD4 T cells after OX40 ligation (Figure 4.3) (103). To determine whether Mad4, Mnt and c-Myc were regulated by IL-12 and downstream pathways, we examined the levels of Mad4, Mnt and c-Myc proteins in IL-12Rβ2-deficient recipients and STAT4-deficient Ag-specific T cells after stimulation through anti-OX40. The levels of Mad4 were not up-regulated in T cells from IL-12Rβ2 KO animals but not in STAT4 KO T cells compared to WT controls. However, there was no difference in the levels of Mnt or c-Myc in STAT4 KO T cells or normal T cells in IL12Rβ2 recipients. These data suggest that signaling through IL-12 may regulate the stabilization of Mad4 and contribute to its function in cell survival through a STAT4 independent mechanism.

Materials and Methods

Mice

Four- to 6-wk-old male and female C57BL/6 and BALB/c mice were purchased from Charles River Laboratories and used at 6–10 weeks of age. BALB/c TCR transgenic DO11.10 ($OVA_{323–339}$) mice and BALB/c IL-12Rβ2-deficient mice were bred and maintained at the Earle A. Chiles Research Institute. The D011.10 STAT4-deficient mice were kindly provided by Dr. K. Murphy (Washington University, St. Louis, MO). An institutional animal care and use committee approved all animal studies. All mice were bred and maintained under specific pathogen-free conditions in the Providence Portland Medical Center animal facility. Experimental procedures were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Preparation of antibodies

Control rat Ig antibody was purchased from Sigma (St. Louis, MO), whereas rat anti-OX40 antibody (OX86) and anti-CTLA-4 (9H10) were produced in the laboratory from hybridomas and affinity purified over protein G columns.

Adoptive transfer and immunization

Spleens and DLNs from DO11.10 or DO11.10 STAT4-deficient mice were harvested and processed by crushing between two frosted glass microscope slides and RBC lysed with Ammonium chloride-based RBC lysis buffer (ACK, Cambrex, East Rutherford, NJ). The percentages of DO11.10 were identified by FACS using KJ1-26 before transfer (BD

Biosciences, San Jose, CA). A total of 2–3x10^6 transgenic TCR T cells were adoptively transferred i.v. into BALB/c or IL-12-deficient recipients. One day later, mice were immunized s.c. with 500µg of OVA (Sigma) and 50µg of anti-OX40 (OX86). The following day, mice were given a second injection of anti-OX40.

Purification of Ag-specific T cells

DLNs were collected at indicated times after immunization and processed into a single-cell suspension. Cells were stained for 0.5h on ice with biotinylated KJ1-26 antibody (0.5mg/10^6 cells) and then washed. Cells were harvested using anti-biotin microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity (>90%) was verified by flow cytometry.

Immunoblot analysis

DLNs were isolated 4 or 6 days after immunization and antigen-specific CD4 T cells were purified as described above. Cells were collected by centrifugation and resuspended in Laemmli buffer and boiled at 98°C for 10 min. Lysates were quantitated using DC Protein Assay (BioRad, Hercules, CA), run on polyacrylamide gels (equivalent microgram quantities of protein), and transferred onto nitrocellulose or PVDF membranes. Antibodies for Mad4, Mnt, c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA), GAPDH (Millipore, Billerica, MA), were used to detect these proteins.

Electroporation

T cells were transfected by means of the nucleofection technique from Amaxa (Cologne, Germany) using the mouse T-cell nucleofection kit, according to Amaxa's protocol. In brief, purified $CD4^+/KJ^+$ T cells taken *ex vivo* at day 4 after OX40 immunization were resuspended in mouse T-cell nucleofection solution at a density of 2×10^6 cells per 100µL. Per transfection, 100µL of cell suspension were mixed with the respective amount of siRNA (siRNA pool consisting of three to five target specific 19-25 nt siRNAs, Santa Cruz Biotechnology), transferred into a cuvette and pulsed in a Nucleofector I device using program X-01. Subsequently, the cells were diluted with 500µL of prewarmed serum-free RPMI medium (37°C) and transferred into a microcentrifuge tube and incubated for 10 min at 37°C. Cells were then transferred to a 24-well-plate containing 1.5ml of pre-warmed Mouse T cell Nucleofector medium containing 5% FBS, 2mM glutamine and Medium Component A and B (Amaxa) (37°C) per well. Following transfection the cells were cultured at 37°C and 5% CO_2. The following day, 0.5ml of media was added to each well. Cells were analyzed by flow cytometry at 48hrs post transfection.

FACS analysis of cells from DLN or spleens

Cells were incubated for 30 min on ice with a combination of the following antibodies: CD4-FITC (BD Bioscience/BD Pharmingen), Biotin-KJ1-26, Strepavidin-APC (BD Bioscience/BD Pharmingen). After washing three times with PBS containing 0.1% w/v BSA (Sigma-Aldrich) and 0.02% w/v sodium azide, cells were resuspended in FACS buffer. 7AAD was added 5 minutes prior to analysis. Harvested samples were run on a FACScalibur (Becton

Dickinson, Franklin Lakes, NJ).

Statistical analysis

Statistical significance was determined by the unpaired Student t test (for comparison between two groups), using Microsoft excel software; a $p < 0.05$ was considered significant. $_{*}$, $p<0.05$; $_{**}$, $p<0.001$; and $_{***}$, $p <0.0001$.

Results

Knockdown of Mad4 and Mnt decreases T cell survival

In view of our data and our interpretation of the Mad4 and Mnt literature, we hypothesized that Mad4 and Mnt up-regulation after anti-OX40 engagement is necessary for OX40-enhanced survival of activated T cells leading to increased memory T cell generation. To test this hypothesis, we used siRNA specific to Mad4 and Mnt proteins to knockdown the protein expression in CD4 T cells. Naive Ag-specific CD4 T cells were adoptively transferred into WT recipients, activated with Ag and anti-OX40 *in vivo* and purified from the draining lymph nodes 4 days after immunization. Cells were then transfected directly *ex vivo* with oligos containing Mad4 and Mnt siRNA by nucleofection and plated *in vitro* for 48 hours. Western blot analysis showed the specificity of the siRNA knockdowns (Figure 4.2A). We observed a 4-fold decrease in the number of cells surviving in the *in vitro* cultures after Mad4 and Mnt double knockdown compared to control, interestingly the Mad4 or Mnt single knockdown were not as effective (Figure 4.2B). Ag-specific cells were also stained with 7AAD, a marker that is excluded by live cells, and analyzed by flow cytometry. When transfected with Mad4 or Mnt siRNA alone, we did not observe a significant difference in the percentage of $7AAD^+$ cells compared to control. However, in cells transfected with siRNA for both Mad4 and Mnt, we found a two-fold increase in $7AAD^+$ cells compared to control (Figure 4.2C). When assessing Annexin V staining within these same samples we did not observe a difference in these groups (data not shown). These results provide evidence for the additive role of Mad4 and Mnt in CD4

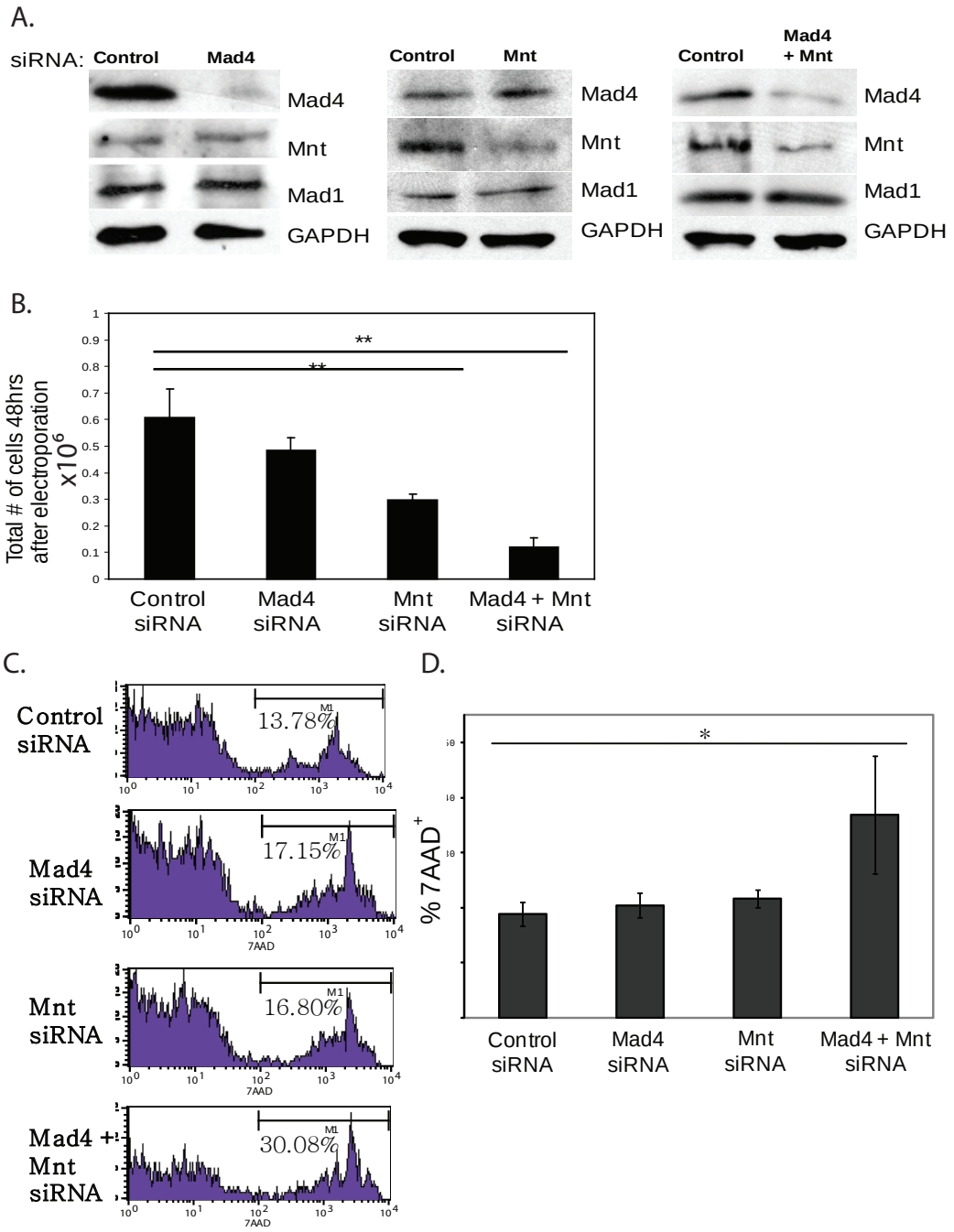


Figure 4.2: Knockdown of Mad4 and Mnt decreases T cell survival. A. Ag-specific CD4 T cells were isolated from DLN at d4 after treatment with OVA and anti-OX40. Cells were transfected by nucelofection (Amaxa) with Mad4 and Mnt siRNA. Lysates were analyzed by Western blot with the indicated antibodies. B. 2 x 10^6 cells were plated vitro for 48hrs post-transfection and the number of surviving cells was determined. C. Cells were stained with 7AAD and analyzed by flow cytometry, gating on CD4 and KJ^+ cells. D. Percent 7AAD positive cells. *p<0.05, **p<0.01. Figures 4.2A, C and D reproduced 4 times with similar results.

T cell survival after anti-OX40 stimulation.

Mad4 protein is down-regulated after anti-OX40 engagement in vivo in Ag-specific T cells transferred into IL-12 knockout recipients

Signaling through IL-12 was identified as a key regulator of OX40-mediated cell survival (103), which was partially mediated by intracellular signaling of STAT4. To determine if Mad4, Mnt or c-Myc played a role in OX40-mediated cell survival, we examined the levels of these proteins in the cytoplasmic and chromatin-bound fractions in IL-12 KO and STAT4 KO compared to WT control at d4 and d6. These time points were chosen because previous data had shown that the there was decreased survival of Ag-specific CD4 T cells WT in IL-12 KO hosts or a STAT4 KO T cells after OX40 ligation at these times (Figure 4.3) (103). Mad4 protein was expressed in both the cytoplasmic and chromatin-bound fraction in WT cells, but the levels were decreased in IL-12 deficient cells in the chromatin-bound fraction at d4 and in the cytoplasmic fraction at d6 (Figure 4.4A and B). The levels of Mad4 were not different in STAT4 deficient cells compared to WT (Figure 4.4C). The number of cells recovered from mice is depicted in Figure 4.4D. The levels of Mnt or c-Myc did not differ in the IL12 KO or STAT4 deficient background compared to controls (data not shown). These results suggest Mad4 may be regulated by signaling through IL-12 after OX40 engagement and may be involved with IL-12 mediated survival of OX40 stimulated T cells. However, this IL-12 specific effect appeared to be independent of signaling through STAT4.

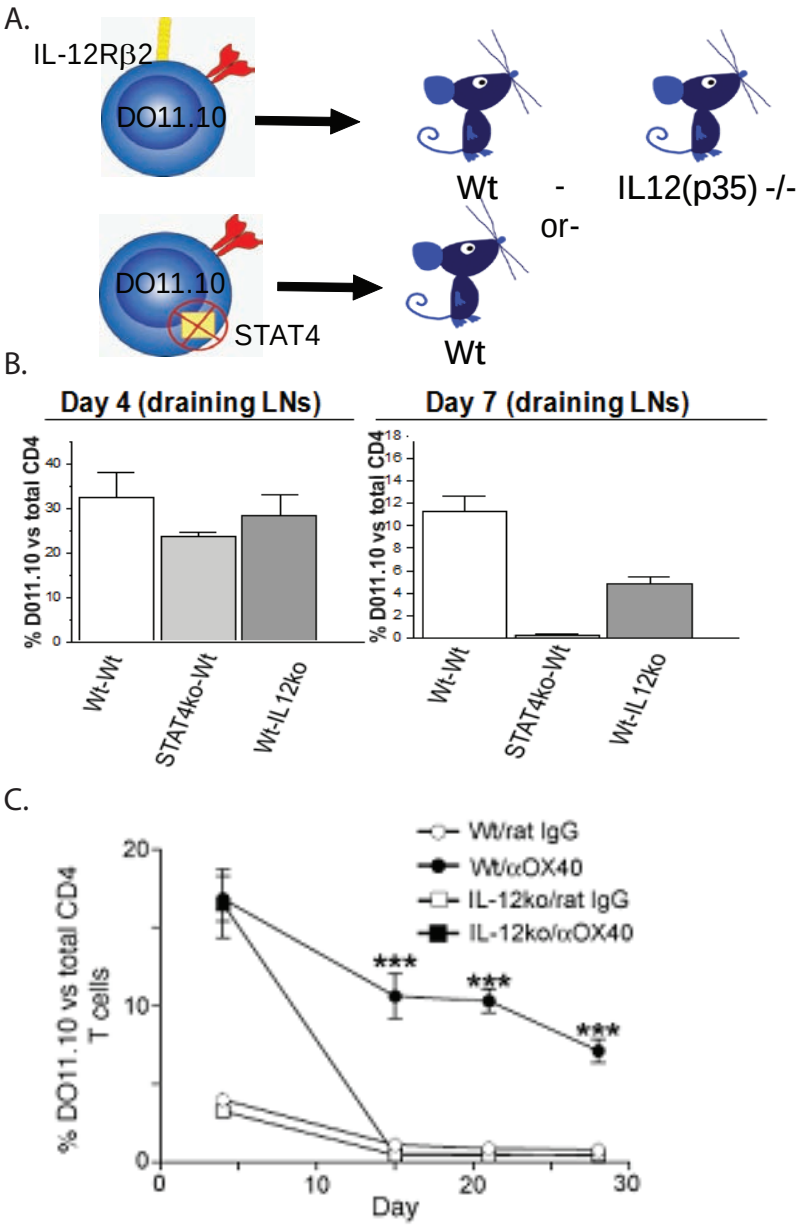


Figure 4.3: The effect of STAT4 and IL-12 on anti-OX40 stimulated Ag-specific CD4 T cell survival. A. Model for adoptive transfer: 3 x 10^6 WT OVA-specific D011.10 CD4 T cells were adoptively transferered into WT or IL-12-deficient mice. In addition, 3 x 10^6 D011.10-STAT-4-deficient CD4 T cells were adoptively transferred into WT mice. All mice were immunized with OVA and anti-OX40 as previously described. B. Four and 7 days after immunization, DLNs were harvested and the frequency of D011.10 CD4 T cells were determined (*n*=3-4). C. Four, 21, and 28 days after immunization, DLNs were harvested and the frequency of D011.10 CD4 T cells were determined (*n*=3-4). From Ruby et al. (2008) J Immunol 180:2140. Copyright 2008 The American Association of Immunologists, Inc.

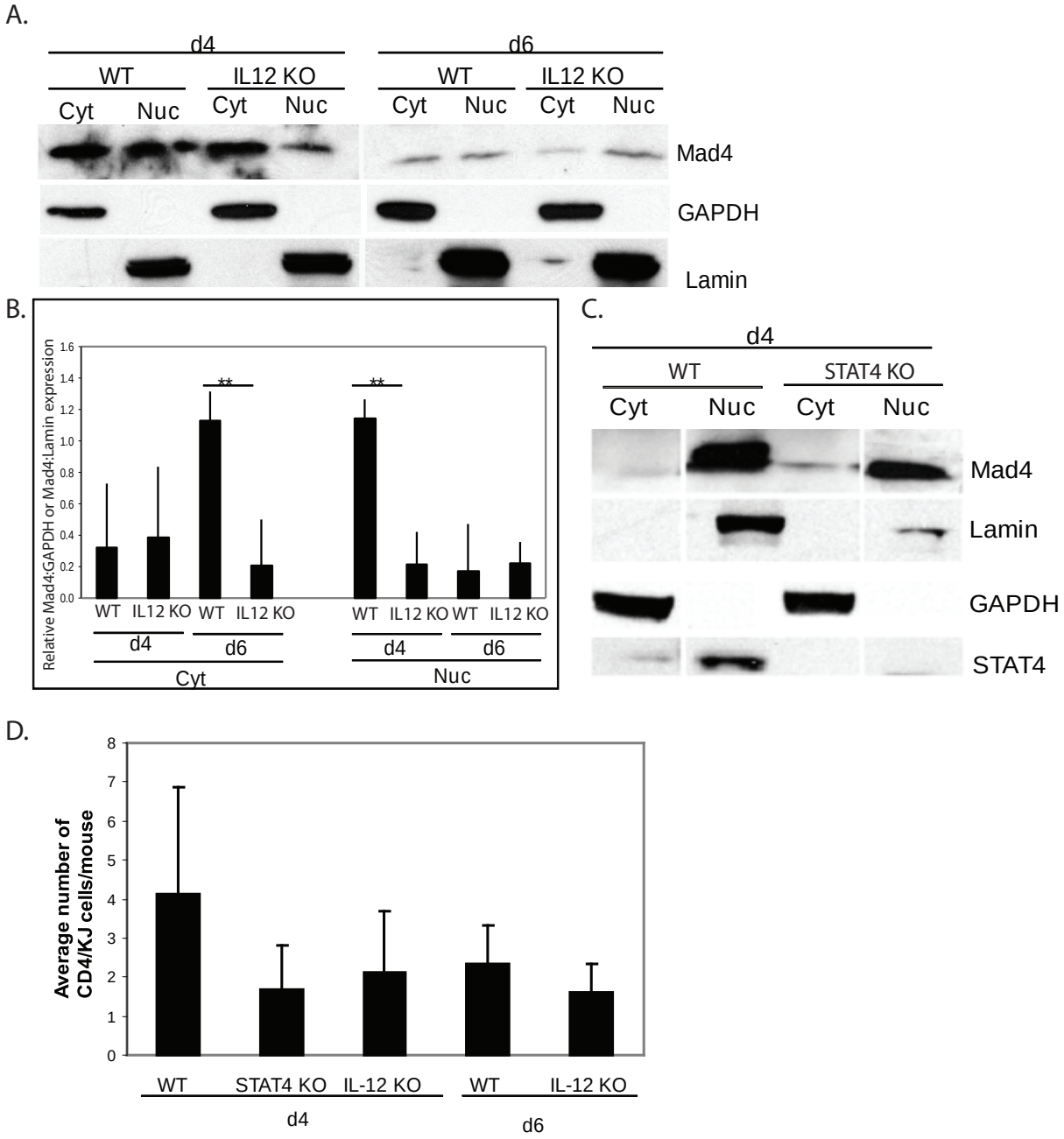


Figure 4.4: Mad4 protein is down-regulated in IL-12 KO cells after anti-OX40 engagement in Ag-specific CD4 T cells compared to WT. A. Ag-specific WT or IL-12 KO CD4 T cells were isolated from DLN at indicated times after treatment with OVA and anti-OX40 or OVA alone. Lysates were fractionated and analyzed by Western blot with the indicated antibodies. B. Quantification of Mad4 protein relative to GAPDH (cytoplasm) or Lamin (nucleus). Represents 3 experiments. *P value <0.05, **P value <0.01. C. Ag-specific WT or STAT4 KO CD4 T cells were isolated from DLN as described above and blotted with the indicated antibodies. D. Average number of cells recovered per mouse in each group. Representative of 3 experiments with similar results.

Discussion

We demonstrate that T cell survival after OX40 stimulation is in part regulated by Mad4 and Mnt. Knockdown of either one of these proteins alone did not have a significant effect on T cell survival, implying there is an additive effect between these two proteins. An important feature of OX40-mediated stimulation of T cells is decreased AICD leading to enhanced survival. Here we showed that engagement by anti-OX40 led to a significant up-regulation of Mad4 and Mnt proteins during the peak of OX40-mediated cell expansion and this up-regulation appears to be necessary for the survival of these T cells. A limitation of this study was it was performed *ex vivo*, ultimately performing these experiments in T cells deficient for Mad4 and Mnt expression *in vivo* would further corroborate our results.

The current model for regulation of the death of activated T cells points to Ag and cytokine withdrawal, which affects the balance between the levels of the pro-survival protein, Bcl-2 and the pro-apoptotic protein, Bim. c-Myc may play a role in controlling AICD, as c-Myc can induce transcription of Bim (72, 301) and Bcl-2 can inhibit c-Myc mediated apoptosis (252, 289-290, 292). The elevated levels of Bcl-2 in OX40-stimulated T cells could be a potential mechanism to overcome c-Myc induced apoptosis. However, an increase in Bcl-2 has not been detected in our model (103), hence the up-regulation of Mad4 and Mnt in anti-OX40 stimulated T cells may be necessary to prevent c-Myc driven apoptosis and allow for enhanced T cell survival.

A mechanism was recently described for Mad1 inhibition of c-Myc induced apoptosis (375). Mad1 repressed the expression of the tumor suppressor PTEN, which is a negative regulator of the PI3K/Akt pathway. The increased Mad1 expression thereby led to

increased levels of phosphorylated, active Akt. Activation of the PI3K/Akt pathway led to downstream activation of the NF-κB pathway and promoted cell survival (375). Signaling through these pathways has also been demonstrated in cells activated by OX40 (90, 114, 117, 123). A potential role for Mad4 in our model could be via a similar mechanism as Mad1, whereby Mad4 could repress PTEN or other pro-apoptotic gene targets and contribute to the sustained expression of the pro-survival pathways.

Mad1 was recently shown to be phosphorylated at S145 by Akt and this phosphorylation inhibited the repressive function of Mad1 (400). There could potentially be a negative feedback loop, whereby Mad1 could inhibit apoptosis by repressing apoptotic gene expression, as described above (375) and thereby allowing activation of the PI3K/Akt survival pathway. Akt could then phosphorylate Mad1 and target it for degradation, when the repressive function is no longer necessary. The levels of Mad4 and Mnt do not persist into later time points after anti-OX40 stimulation, although the levels of c-Myc remain elevated. The regulation of Mad4 and Mnt could potentially be working by a similar feedback mechanism and this could explain why there is the temporal expression of Mad4 and Mnt in OX40-activated T cells.

Our data showed that knockdown of Mad4 and Mnt proteins led to decreased survival of OX40-activated T cells. A redundant role for these proteins has been suggested because knockouts of Mad proteins show highly tissue specific defects, which is believed to be due to the functional redundancy among the other family members leading to compensation for the loss of the respective proteins (220-221). This correlates with previously published results showing that Mnt deficient T cells exhibited increased levels of apoptosis (321). The

anti-apoptotic proteins Bcl-2 and Bcl-XL were slightly down-regulated in Mnt-deficient T cells (320), which is consistent with data showing up-regulation of these anti-apoptotic proteins are necessary for T cell survival after OX40 engagement (89). However, we did not see a down-regulation of Bcl-2 protein after Mad4 and Mnt siRNA knockdown (data not shown) but perhaps this could be due to the incomplete knockdown of Mad4 and Mnt. Additionally, we did not see increased PARP cleavage by Western blot in cells transfected with siRNA against Mad4 and/or Mnt compared to control. This raises the issue of whether the observed differences in survival were due to increased apoptosis or increased cell death by other means such as necrosis or phagocytosis. These questions can be addressed in future studies which will attempt to cross Mad4 and Mnt deficient mice onto a transgenic mouse strain. These studies will further examine the proliferative and survival responses after treatment with anti-OX40. We hypothesize that long-term memory T cell survival after anti-OX40 engagement will be reduced in cells deficient for Mad4 and Mnt compared to WT control.

Another potential function of the increased levels of Mad4 and Mnt levels in OX40-stimulated T cells could be driving the differentiation and quiescent state of these cells. If this were the case, we would expect decreased levels of Mad4 and Mnt to affect the proliferation of the cells. We examined cell proliferation by thymidine incorporation in cells transfected with Mad4 and Mnt siRNA and did not observe a change in the rate of proliferation in Mad4 and/or Mnt deficient cells compared to control. This implies that the increased expression of Mad4 and Mnt in T cells engaged by anti-OX40 does not function to regulate cellular proliferation.

siRNA technology is a commonly used technique to study gene functions but there are several disadvantages to using siRNA. The most common issue with use of siRNA is potential off-target effects, which we partially controlled for by examining the protein levels of Mnt and Mad4, in cells transfected with Mad4 and Mnt siRNA, respectively and another Mad family member, Mad1 and demonstrated there were no off target effects for closely related family members. To fully control for off-target effects in experiments using siRNA, rescue experiments would be performed, whereby the phenotype could be recovered by experimentally overexpressing Mad4 and Mnt using plasmids that contained silent mutations that would confer resistance to siRNA degradation. Transfection with siRNA does not always lead to complete knockdown of the protein expression, however the observed phenotype with the partial knockdown suggested these proteins play an important role in the OX40-mediated biologic function. Another disadvantage is the duration of silencing depends on the rate of cell division and can be short. These issues will be addressed in future studies, where mice deficient for Mad4 and Mnt will be generated to further verify these results.

Signaling through IL-12 may be a potential mechanism for regulating T cell survival. It has been established that signaling through OX40 leads to the up-regulation of the signaling chain of the IL-12R, IL-12Rβ2, on CD4 T cells 4-6 days following OX40 engagement, when the levels of Mad4 and Mnt are decreasing (103). IL-12 signals primarily through JAK2 and TYK2, which activates downstream STAT proteins: STAT1, 3, 4 and 5 and STAT4 appears to be the major specific player of IL-12 signaling (408-409). Signaling through STAT4 partially regulates cell survival via the IL-12 pathway, however

the expression of Mad4 protein was not affected in STAT4 KO cells, implying the IL-12 mediated effects on Mad4 were independent of STAT4 signaling. In addition to signaling through the STAT proteins, IL-12 can also activate the Src family protein tyrosine kinase Lck (410) and the MAPK p38 (411-413). Interestingly, MAPK p38β transcripts were down-regulated in OX40 treated cells compared to cells stimulated with Rat Ig control and anti-CTLA-4 (unpublished observation). There could be a potential mechanism whereby signaling through IL-12 via the MAPK p38 pathway affects the stability of Mad4 and thereby affects protein survival. The role of this kinase on Mad4 stability and function will be an area of future study.

To more definitively study the function of Mad4 and Mnt in OX40 stimulated T cells, a transgenic mouse deficient for both Mad4 and Mnt in Ag-specific T cells could be generated for future studies. Studies in these mice could examine the proliferative and survival responses after treatment with OX40-specific stimuli. The cells will be followed out to later time points after Ag and anti-OX40 stimulation to determine if Mad4 and Mnt expression affects the development of long-term memory T cells and T cell recall responses. We hypothesize that survival of the Ag-specific T cells after treatment with anti-OX40 will be significantly reduced in cells deficient for Mad4 and Mnt compared to WT control. Additionally, we would expect that the loss of Mad4 and Mnt may also diminish the anti-tumor efficacy of anti-OX40, but will make cells less prone to causing autoimmune and inflammatory disorders. Taken together, these studies show a novel mechanism for the regulation of OX40 effects on T cell survival (Figure 4.5).

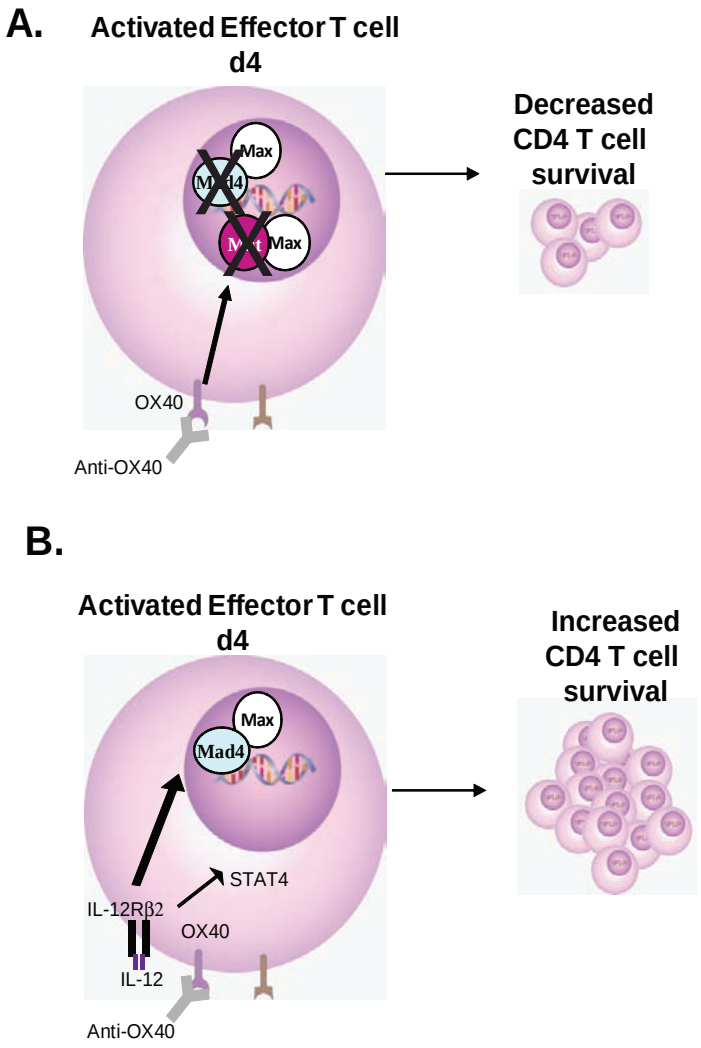


Figure 4.5: Proposed model for the role of Mad4 and Mnt proteins in anti-OX40 stimulated T cells. A. Knockdown of Mad4 and Mnt proteins in CD4 T cells engaged by anti-OX40 leads to decreased T cell survival. B. IL-12Rβ2 is up-regulated after engagement by anti-OX40 and is necessary for enhanced survival of memory T cells. Signaling through IL-12 may potentially regulate Mad4 via a STAT4 independent pathway.

Chapter 5

Conclusions and Future Directions

Immunological memory forms the basis for protective immunity and is orchestrated by long-lived memory T lymphocytes. The activation of naive T cells requires Ag recognition, which leads to expansion and differentiation of effector T cells (14-16). In addition to Ag recognition, optimal proliferation and acquisition of effector functions by naïve T cells requires engagement of costimulatory molecules provided by APCs. OX40 has been identified as a costimulatory receptor for CD4 and CD8 T cells. Signaling through OX40 dramatically enhances the function and survival T cells during the effector phase of a primary immune response and thereby increases the number of effector cells and memory T cells that are developed after Ag activation. Understanding the mechanisms that control memory T cell generation will have broad immunological and clinical implications. Such understanding will improve the development of more potent vaccine strategies against chronic pathogens and tumors and conversely may help in controlling autoimmune disorders.

The exact downstream signaling pathways that lead to enhanced T cell function and survival of Ag-responding memory T cells after OX40 engagement are not fully elucidated. Engagement of OX40 by its ligand or an agonist antibody leads to signaling through TRAF family members, which in turn activates NF-κB. Additionally, the kinase Akt is thought to play a role in the survival of T cells after OX40 stimulation and more recently a protein termed survivin, which belongs to the Inhibitor of Apoptosis (IAP) family was shown to play a role in OX40-mediated T cell proliferation and survival (90, 414). Data also suggest a role for the anti-apoptotic proteins from the Bcl-2 family such as Bcl-X_L, Bcl-2, and Bfl-1, as these proteins were reported to be increased after OX40 ligation in some models and

were down-regulated in OX40-deficient T cells (89-90).

Our results demonstrated that the enhanced survival of Ag-activated T cells following OX40 engagement involved the up-regulation and stabilization of Mad4 and Mnt proteins. Purified Ag-specific T cells exhibited up-regulated protein levels of Mad4, Mnt and c-Myc proteins after Ag and anti-OX40 engagement *in vivo*, compared to controls. The levels of Mad4 and Mnt peaked at days 3-4 and then decreased as the cells decreased in size, while c-Myc protein levels remained unchanged over time after OX40 stimulation. In addition to increased Mad4 and Mnt protein levels, we demonstrated that OX40 stabilized both Mad4 and Mnt proteins against degradation. Mad4 protein stability was dependent on the presence of a serine residue at the RXRXXS consensus sequence of Mad4, which mediated accelerated protein degradation. These data provide evidence that Mad4 and Mnt can play a role to enhance memory T cell survival after OX40 engagement. We propose a model whereby the up-regulation of c-Myc in activated T cells drives proliferation and consequently activation-induced cell death, which is antagonized by the OX40-mediated increases in Mad4 and Mnt proteins, allowing increased numbers of T cells to survive the "blast crisis" phase. Knockdown of Mad4 and Mnt by siRNA led to decreased survival of OX40-activated T cells, which supports this hypothesis (Figure 5.1 and 5.2).

It has been suggested that activated T cells die due to Ag and cytokine withdrawal and c-Myc may play a role in this process. The decline in Ag leads to decreased TCR stimulation and cytokine production. The lack of TCR stimulation diminishes the activation of the REL-NF-κB and NF-AT transcription factors. Activated T cells express decreased levels of Bcl-2 at the peak of their response, just before they begin to die in vivo (63). IL-2

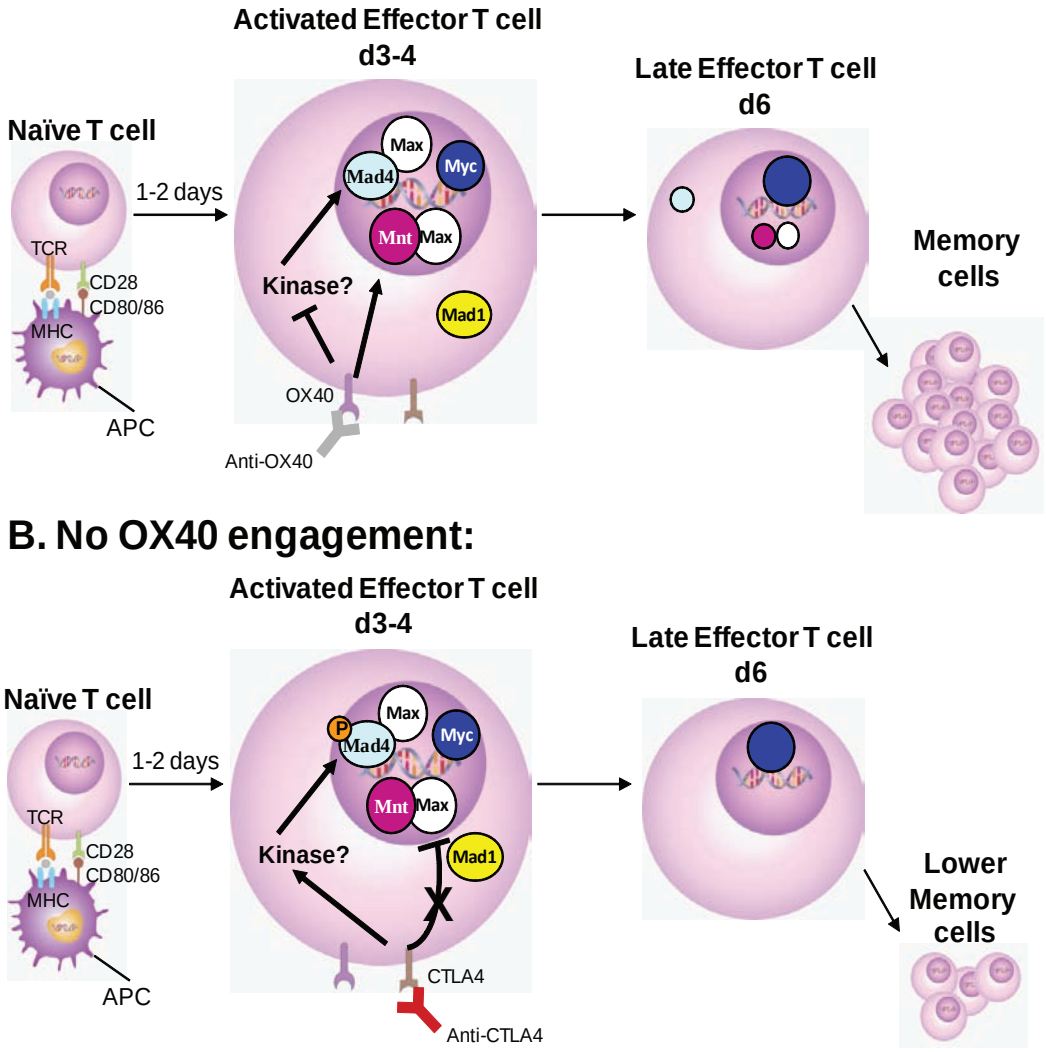


Figure 5.1: Proposed model for the role of Mad4 and Mnt proteins in anti-OX40 stimulated T cells. A. After anti-OX40 engagement on activated T cells, Mad4 and Mnt proteins are up-regulated and localized to the nucleus. We hypothesize that signaling through OX40 inhibits a kinase that phosphorylates and leads to the degradation of Mad4. The expresion and stabilization of these proteins is necessary for the survival of the T cells. At day 6, when the cells have contracted, Mad4 is localized in the the cytoplasm and Mnt is barely detectable in the nucleus, while c-Myc levels remain elevated. B. Signaling through anti-CTLA-4 (or in the absence of OX40 engagement), Mad4 and Mnt levels are decreased, potentially because of the phosphorylation by a kinase that leads to the degradation of the proteins. Hence there is increased cell death and decreased memory T cell development.

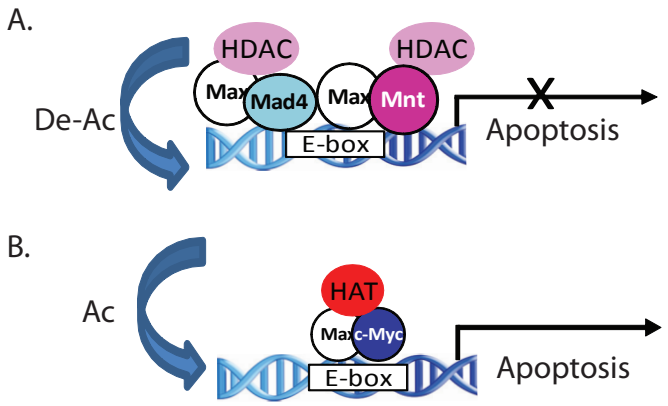


Figure 5.2: Proposed model for the gene regulation of Mad4, Mnt and c-Myc proteins in anti-OX40 stimulated T cells. A. In cells engaged by OX40, Mad4 and Mnt proteins heterodimerize with Max and recruit HDACs and deacetylate histones, thereby downregulating genes that induce apoptosis. B. In the absence of OX40 engagement, c-Myc successfully competes for heterodimerization with Max and binds to E-box sequences and recruits HAT complexes that acetylate histones and allow for transcription of downstream target genes that induce apoptosis.

and IL-7 have been implicated in activating the pro-survival protein Bcl-2 (37, 59, 62) and the cytokine withdrawal leads to decreased Bcl-2 and Bcl-XL levels (). Over-expression of Bcl-2 *in vitro* was able to restore T cell survival (58). It has been proposed that the balance between the levels of Bcl-2 and the pro-apoptotic Bim are what drives apoptosis after T cell activation. The elevated levels of Bcl-2 in OX40-stimulated T cells could be a potential mechanism to overcome c-Myc induced apoptosis. However, an increase in Bcl-2 has not been detected in our model, hence the up-regulation of Mad4 and Mnt in anti-OX40 stimulated T cells may be necessary to prevent c-Myc driven apoptosis and allow for enhanced T cell survival.

The induction of Bim may be due to the reduced levels of Akt after cytokine withdrawal, leads to activation of the transcription factor FOXO3A, which can in turn

activate Bim (60, 67-68). Bim is also a downstream target of c-Myc (272, 301). A potential mechanism in OX40-activated T cells is the up-regulated c-Myc induces Bim, which drives apoptosis, however, this is counteracted by the increased expression of Mad4 and Mnt, which repress the Bim target gene. The exact mechanisms regulating Bim expression and the mechanisms that control activated T cell death still remain to be fully elucidated.

Precedence for the role of Mad proteins in repressing c-Myc targets comes from a study describing Mad1 inhibition of c-Myc induced apoptosis in tumor cell lines (375). The expression of the tumor suppressor gene, PTEN was inhibited by Mad1 and this repression allowed for cell survival by activating PI3K/Akt and downstream activation of the NF-κB pathway (375). The PI3K/Akt and NF-κB pathways are important for the effects mediated by anti-OX40 stimulation (90, 114, 117, 123). Mad4 and Mnt could be functioning through similar mechanisms to repress pro-apoptotic target genes and contribute to the sustained expression of the pro-survival pathways.

Studies in knockout mice provide additional evidence for the role of Mad proteins promoting cell survival. An increased frequency of apoptosis was observed in granulocytes from *mad1* knockout mice when grown in limiting amounts of cytokines and this phenotype was reversed after adding back exogenous Mad1 (373-374, 405). Mad3-deficient thymocytes underwent increased apoptosis in response to radiation radiation (221). T cells from Mnt knockout mice showed increased levels of apoptosis and had a partial block in T cell development in the thymus (321). Additionally, the expression of the pro-survival proteins Bcl-2 and Bcl-XL were decreased, further demonstrating the role of Mnt in cell survival (321). Another study showed that knockdown of Mnt using siRNA

induced accelerated proliferation and apoptosis in fibroblasts (319). These data provide evidence for the role Mad family proteins in cell survival.

The exact mechanisms by which Mad4 and Mnt are regulated in OX40-stimulated T cells are unclear. Our data provided preliminary evidence that Mad4 is regulated by phosphorylation at the S145 residue and this targets it for degradation, as was previously demonstrated for Mad1 (334, 400). Mad1 was recently shown to be phosphorylated at S145 by Akt (400) and S6K and RSK (334). Phosphorylation of Mad1 by S6K and RSK decreased its stability and targeted it for degradation (334). However, another study showed that Mad1 is also targeted phosphorylation by Akt at the same residue, but phosphorylation by Akt inhibited the repressive function of Mad1 but did not affect its stability (400). The exact mechanisms regulating Mad1 *in vivo* remains to be resolved.

A negative feedback loop could exist for Mad1, as it had been previously shown that Mad1 represses PTEN and thereby activates Akt (375), which could then phosphorylate Mad1 and inhibit its function, when the repressive function is no longer necessary. The levels of Mad4 and Mnt do not persist into later time points after anti-OX40 stimulation, although the levels of c-Myc remain elevated. The regulation of Mad4 and Mnt could potentially be working by a similar feedback mechanism and this could explain why there is the temporal expression of Mad4 and Mnt in OX40-activated T cells.

However our preliminary studies treating cells with the Akt inhibitor did not affect the stability of Mad4 in cell lines but this experiment needs to be tested in primary T cells. A preliminary study in cell lines did point to the kinase RhoGTPase, as a potential modulator of Mad4 function (data not shown). Treatment with the RhoGTPase inhibit led

to increased levels of Mad4 protein in a cell line over-expressing Mad4. RhoGTPases or other mitogen kinases have been shown to activate the transcription factor Src, which is known to transcriptionally activate *c-myc* (230). Mad4 could potentially be regulated by RhoGTPase in OX40-activated T cells and this will be an area of future study.

Manipulation of the OX40-OX40L interaction has been proposed for the development of effective immunotherapy's for both cancer and autoimmune diseases. Targeting OX40 with anti-OX40 agonist antibodies leads to enhanced T cell function and survival of effector cells, and increases the pool of Ag-specific long-term memory T cells. It has been demonstrated that treatment with an anti-OX40 antibody or a soluble OX40L fusion protein can provide anti-tumor therapy in a number of mouse models. If Mad4 and Mnt proteins prove to be an essential part in the OX40-mediated survival of activated T cells, then enhancing the stabilization of Mad4 and Mnt might increase the anti-tumor effects of OX40 or completely replace the need for stimulation through OX40. For example, if a specific kinase governs the stability of Mad4 and/or Mnt, targeting the kinase with a specific inhibitor could enhance the function of these proteins and thereby enhance OX40-mediated anti-tumor efficacy. Additionally, blocking the signaling pathways that lead to stabilization of Mad4 and Mnt could provide a mechanism to block OX40 function and thereby temper autoimmune disease. Ultimately understanding the specific molecular mechanisms that regulate OX40 function could have important clinical implications in future OX40-specific therapies.

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