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OX40 engagement stabilizes Mxd4 and Mnt protein levels in antigen-stimulated T cells leading to an increase in survival

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Abstract

OX40 engagement on activated T cells leads to increased proliferation, expansion and survival of Ag-specific T cells. Here we show that the Myc antagonists, Mxd4 and Mnt are transiently upregulated and translocated to the nucleus following OX40 engagement and may be involved in suppressing cell death. Both Mxd4 and Mnt are upregulated following OX40 stimulation through increased protein stability and we identify a critical phosphorylation site in Mxd4 that controls Mxd4 stability. The upregulation of Mxd4 and Mnt contributes to OX40-mediated T cell survival because siRNA knockdown of Mxd4 and Mnt led to increased cell death. We hypothesize the upregulation of c-Myc following OX40 engagement drives T cell proliferation and that upregulation of Mxd4 and Mnt suppresses Myc-dependent cell death. Thus, Mxd4 and Mnt upregulation following OX40 engagement most likely increases T cell survival.

Keywords

OX40; Mxd4; Mnt; T cells

Introduction

The T cell costimulatory molecule, OX40 is a member of the tumor-necrosis family receptor (TNFR) superfamily and is up-regulated 1-2 days following TCR stimulation of naïve T cells. OX40 is expressed on both CD4 and CD8 T cells [1-3]. Engagement of OX40 on activated T cells by its ligand (OX40L) or an agonist antibody leads to increased cytokine production, enhanced proliferation and development of increased numbers of long-term memory T cells [4-10]. In this study and past studies we have compared the effects of anti-OX40 engagement to CTLA-4 blockade [11-14]. CTLA-4 is also up-regulated after T cell activation, however engagement of CTLA-4 provides an inhibitory signal which down-modulates the immune response [15, 16]. The negative signal provided by CTLA-4 engagement can be blocked using an anti-CTLA-4 mAb, which leads to enhanced

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proliferation of CD4 T cells but in contrast to 2 stimulation through anti-OX40, CTLA-4 blockade does not promote their long-term survival in our model [13, 17]. Therefore, by comparing T cells treated with anti-OX40 versus anti-CTLA-4, we examined differences at the molecular level that could potentially contribute to T cell survival.

In this study, we examined the role of the Myc/Max/Mxd network of proteins in OX40-stimulated T cells. c-Myc is a transcription factor that plays an important general role in promoting cell proliferation [18, 19]. c-Myc is part of the Max-interacting network of transcription factors, which include a group of Myc antagonists, Mxd1-4 (formerly known as Mad1, Mxi1, Mad3 and Mad4) Mnt and Mga. The Myc and Mxd family proteins are extremely short-lived, with half-lives of 15-30 minutes and this unique property is important for their ultimate function in the cell [20-23]. Whereas Myc:Max complexes activate transcription, Mxd:Max, Mnt:Max and Mga:Max complexes repress transcription [24-27]. In addition, deletion of Mnt or siRNA knockdown of Mnt strongly sensitizes cells to apoptosis [28]. Mxd family proteins have also been implicated as suppressors of Myc-dependent apoptosis as deletion of the related Mxd3 in mice appears to sensitize some cell types, including T cells, to apoptosis and overexpression of Mxd1 can suppress apoptosis in some cell types [29-31].

In T cells, c-Myc levels are low in naïve quiescent cells [32] and upregulated following Ag-mediated TCR activation [33-35]. Gene deletion studies suggest that c-Myc upregulation in this setting plays a key role in promoting proliferation following TCR activation [35-38]. Whereas Myc upregulation promotes cell proliferation, it is also well-established that c-Myc upregulation can promote apoptosis of various cell types. Thus it is hypothesized that compensatory survival signaling is required to overcome this tendency and allow its proliferation-promoting activities to prevail. Given the demonstrated ability of Mxd and Mnt proteins in suppressing Myc-dependent apoptosis, one possibility is that they participate in suppressing cell death in activated T cells.

In this study, we show that Mxd4, Mnt and c-Myc proteins are upregulated in Ag-specific T cells purified *ex vivo* after OX40 engagement. Mxd4 and Mnt protein levels peaked 3-4 days after anti-OX40 administration and decreased as the cells contracted in size. In contrast c-Myc protein levels, which were also increased after OX40 stimulation, remained elevated over time. The increased levels of Mxd4 and Mnt proteins following OX40 engagement was caused by increased protein stability and we demonstrate the presence of a serine residue within the Mxd4 protein that is essential for mediating accelerated protein degradation. We also show that Mxd4 and Mnt protein expression is important for survival of OX40-stimulated T cells because knockdown these two proteins led to decreased T cell survival.

Results

Mxd4, Mnt and c-Myc proteins are up-regulated after anti-OX40 engagement in vivo in Ag-specific CD4 T cells

We examined the protein levels of Mxd4 and the related family member, Mnt, in Ag-specific T cells isolated from DLN at day 3 after treatment with Ag and anti-OX40. Naïve OVA-specific TCR transgenic CD4 T cells were adoptively transferred into wild-type BALB/c mice and the mice were stimulated with soluble OVA along with anti-OX40 or Rat Ig control Abs. Three days after immunization, the Ag-specific donor CD4 T cells were harvested from the draining lymph nodes (DLN) and Mxd4 and Mnt protein levels were examined by Western blot. Initially we analyzed cells for protein differences at the day 3 time point because several functional changes have been observed in Ag-specific T cells at day 3 after anti-OX40 engagement *in vivo* when compared to controls [13]. Our results showed a 3-fold increase in both Mxd4 and Mnt protein expression (normalized to GAPDH)

in Ag-specific CD4 T isolated from DLN *ex vivo* after anti-OX40 vs. Rat Ig treatment (Figure 1A).

Since we observed an increase in Mxd4 and Mnt proteins at day 3 after anti-OX40 engagement, we next tested if these proteins were temporally regulated. We examined protein levels of the Mxd4, Mnt and c-Myc proteins over a six-day period in Ag-specific T cells isolated *ex vivo* following anti-OX40 stimulation *in vivo* (Figure 1B). The peak of the proliferative response after anti-OX40 engagement occurs between days 3 and 4 [13] and the cells decrease in size and proliferation rate by day 6 [13, 39]. Mxd4 protein was undetectable in naïve cells and the protein was induced following Ag and anti-OX40 treatment and could be detected on days 3 and 4. Mxd4 protein levels decreased later, at day 6, when the cells contracted in size and become more quiescent. 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 remained relatively constant over time (Figure 1B).

The subcellular localization of transcription factors can regulate their activity [40-42]. Mxd4 contains an N-terminal nuclear export signal (NES) that is not conserved in other Mxd family proteins and Mxd4 is actively exported from the nucleus [43]. Mxd4 is recruited to the nucleus by dimerization with Max, which contains a nuclear localization signal (NLS) in its protein sequence [43]. Mnt and c-Myc also form heterodimers with Max but they do not have a NES. We sought to determine whether Mxd4, Mnt and c-Myc proteins were expressed in the nucleus by assessing chromatin-bound fractions within Ag-specific T cells after anti-OX40 treatment. Western blot analysis showed the Mxd4 levels within the nuclear chromatin-bound fraction were elevated compared to the cytoplasm by normalization to GAPDH after anti-OX40 treatment at days 3 and 4. In contrast, on day 6, Mxd4 protein was primarily localized within the cytoplasm. Mnt protein was localized in the chromatin-bound fraction and the protein levels peaked on day 3 after T cell activation and were undetectable by day 6. c-Myc remained in the nuclear fraction over time and the levels were high on at day 3 but in contrast to Mxd4 and Mnt, c-Myc levels were still elevated in the chromatin fraction as normalized to lamin expression on day 6 (Figure 1C). Based on the subcellular localization, these data suggest that Mxd4 and Mnt are functionally active during a discrete time frame after anti-OX40 stimulation.

Mxd4, Mnt and c-Myc are up-regulated after anti-OX40 engagement in Ag-specific T cells compared to CTLA-4 blockade

Ligation of CTLA-4 after TCR activation provides a negative signal to T cells and “puts the brakes” on cellular proliferation, leading to cell cycle arrest and decreased IL-2 production [44, 45]. The anti-CTLA-4 antibody increases proliferation and expansion of CD4 T cells by blocking a negative signal and by also providing increased costimulatory signaling through CD28 [46]. However, in contrast to anti-OX40 stimulation, T cells isolated from hosts injected with anti-CTLA-4 do not show enhanced survival [11]. While a subset of T cells stimulated through CTLA-4 have been shown to have increased survival in a PI3K dependent manner [47], in our model we observed decreased survival after CTLA-4 blockade when compared to ligation by anti-OX40 [11-13]. Stimulation through anti-OX40 stimulation exhibited a significant decrease in the amount of caspase activation in Ag-stimulated T cells isolated from the DLNs within the CD62L low population of cells compared to anti-CTLA-4 or Rat Ig stimulated mice (Figure 2A). To determine whether increased Mxd4 and Mnt was associated with Ag stimulated CD4 T cells that have increased proliferation and survival versus proliferation alone, we compared the effects of anti-OX40 versus anti-CTLA-4 treatment. We assessed whether Mxd4, 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 Mxd4 and Mnt were increased 4-fold in the chromatin

fraction of anti-OX40 treated cells when compared to anti-CTLA-4 at days 3 and 4 when normalized to lamin (Figure 2B). However, the chromatin-associated levels of c-Myc in T cells isolated from anti-OX40 treated mice versus anti-CTLA-4 were approximately the same at day 3 and 4 compared to CTLA-4 blockade (Figure 2B). To determine whether there were differences in the activation status of Ag-stimulated CD4 cells used in this study, purified Ag-specific CD4 T cells isolated from the DLNs from mice treated with Rat Ig, anti-CTLA-4 or anti-OX40 were analyzed for CD44, CD69, and CD62L. There were no significant differences observed in the activation status (using these three markers) of Ag-specific T cells isolated from these three groups (data not shown).

Mxd4 and Mnt levels are also increased in CD8 T cells isolated from anti-OX40 stimulated mice

The anti-OX40 antibody also has potent immune stimulating effects on CD8 T cells in a similar adoptive transfer setting as has been observed for CD4 T cells [48-50]. Therefore we tested whether Mxd4 and Mnt were upregulated in Ag-specific CD8 T cells following anti-OX40 administration in the OTI model. Four days following vaccination, the Ag-specific CD8 T cells were purified from DLNs and harvested for Mxd4 and Mnt protein expression. Similar to the results obtained with CD4 T cells, Mxd4 and Mnt levels were increased >4 fold and >2-fold, respectively in Ag-specific CD8 T cells after anti-OX40 treatment, when compared to control-treated mice (Rat Ig) when normalized to GAPDH (Figure 3A).

To determine if Mxd4 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 Mxd4 and Mnt expression. Mxd4 was localized in both the cytoplasmic and chromatin-bound fraction of CD8 T cells after anti-OX40 treatment and levels were increased 16-fold and 2-fold in the cytoplasm and nucleus, respectively, compared to Rat Ig control when normalized to GAPDH or lamin, respectively (Figure 3B). Mnt was localized in the chromatin-bound fraction after anti-OX40 treatment and the levels were also increased 2-fold in the nuclear fraction compared to Rat Ig control as normalized to lamin (Figure 3B). These data demonstrate that CD8 T cells, like CD4 T cells, up-regulate Mxd4 and Mnt in the chromatin-bound fraction following ligation of anti-OX40. Hence, this may represent an important common mechanism mediating anti-OX40-specific function in both CD4 and CD8 T cells.

The data demonstrate that Mxd4 and Mnt proteins were up-regulated after anti-OX40 engagement *in vivo*. Since anti-OX40 could be acting on CD4 or CD8 T cells, we aimed to determine whether direct OX40 stimulation on CD8 T cells was important for the upregulation of these proteins. Therefore, we examined Mxd4 and Mnt protein expression in WT compared to OX40-/- OTI CD8 T cells after adoptive transfer and administration of Ag and anti-OX40. Our results showed that Mxd4 and Mnt protein were >3- and 2-fold lower, respectively in OX40-/- OTI T cells compared to WT/OTI T cells when normalized to GAPDH (Figure 3C). Therefore, the anti-OX40-mediated increase in Mxd4 and Mnt protein expression is in part mediated through direct OX40 signaling of the Ag-specific CD8 T cells.

Mxd4 and Mnt are stabilized after OX40 engagement and putative phosphorylation site, S145A confers Mxd4 stability

The half-life of Mxd family proteins as well as c-Myc, is extremely short, approximately 10-20 minutes [20-22, 51], therefore signaling through anti-OX40 may increase the post-translational stability of Mxd4 and possibly Mnt. Therefore, we examined the stability of Mxd4 and Mnt proteins in CD4 T cells isolated from anti-OX40 treated mice by incubating

them with cycloheximide, which inhibits *de novo* protein synthesis. CD4 T cells were isolated directly *ex vivo* from the DLNs four days after mice were stimulated with Ag and anti-OX40 and the cells were incubated with cycloheximide for the indicated times (Figure 4A). Mxd4 and Mnt protein levels remained relatively constant over time after cycloheximide treatment in T cells isolated from anti-OX40 treated mice. However, cells isolated from anti-CTLA-4 treated mice exhibited rapid protein degradation for both Mxd4 and Mnt starting at approximately 15 minutes after cycloheximide incubation (Figure 4A). In contrast, GAPDH protein levels were not degraded differently in cells treated with anti-CTLA-4 or anti-OX40 (Figure 4A). These data support the hypothesis that signaling through anti-OX40 leads to stabilization of both the Mxd4 and Mnt proteins in T cells.

The post-translational mechanism by which Mxd4 protein is rapidly degraded has not been previously studied. However it was shown that the Mxd family member, Mxd1 is phosphorylated at the serine 145 residue in the consensus sequence RXRXXS. This phosphorylation event is a result of PI3K/Akt/mTOR and MAPK pathway activation and phosphorylation at this residue targets the protein for degradation [52]. Through comparative sequence analysis, we identified that an RXRXXS consensus sequence is conserved within the Mxd4 protein sequence; therefore this sequence may regulate stabilization of Mxd4 (Figure 4B). To test this hypothesis, we used site-directed mutagenesis and created a reporter plasmid containing WT Mxd4 or S145A mutant of Mxd4 linked to an IRES expressing GFP. Protein stability was tested by transient transfection of 293H cells with the plasmid that co-express Mxd4 or the mutant Mxd4 and GFP. The S145A mutant showed a 4-fold higher level of Mxd4 protein expression compared to WT Mxd4 when normalized to GAPDH (Figure 4C). The levels of GFP expression remained constant between the transfection groups as observed by Western blot and normalized to GAPDH (Figure 4C) and by flow cytometry (data not shown), ruling out the possible effect of differential transcription between the two constructs. To confirm that the reduced WT Mxd4 protein levels in the 293 cells was due to accelerated protein degradation, transfected cells were treated with cycloheximide to inhibit *de novo* protein synthesis, 48hrs post-transfection. WT Mxd4 was degraded quite rapidly, with a calculated half-life of <15 min; while Mxd4 levels in the serine mutant remained stable after cycloheximide treatment for at least 60 min (Figure 4D). These results suggest that the rapid degradation of Mxd4 is regulated by post-translational modifications at S145 of the RXRXXS consensus sequence and identify a potential target for Mxd4 protein stability via OX40 stimulation.

Knockdown of Mxd4 and Mnt decreases T cell survival

Stimulation through anti-OX40 leads to enhanced survival and a decrease in activation-induced cell death in this model system. In view of our data and our interpretation of the Mxd4 and Mnt literature, we speculated that Mxd4 and Mnt upregulation after anti-OX40 engagement may be part of a cascade of events leading to enhanced survival of activated T cells. To test this hypothesis, we transfected siRNA specific for Mxd4 and Mnt into T cells to knockdown their protein expression. Naive Ag-specific CD4 T cells were adoptively transferred into WT recipients, activated with Ag and anti-OX40 *in vivo* and purified from the DLNs 4 days after immunization. Cells were then transfected directly *ex vivo* with Mxd4 and Mnt siRNA by nucleofection and plated *in vitro* for 48 hours. Western blot analysis showed the specificity of the siRNA knockdowns (Figure 5A). To determine the efficiency of transfection, cells were transfected with pMax-GFP and gated on the live cells by FSC and SSC the percentage of GFP CD4/KJ+ cells was determined to be 76.10% (data not shown). On average, 40% of the cells survive 48 hours after electroporation. Upon siRNA administration, we observed a 4-fold decrease in the number of cells surviving in the *in vitro* cultures following Mxd4 and Mnt double knockdown compared to control, interestingly the Mxd4 or Mnt single knockdown were not as effective as the double knockdown (Figure 5B).

These cultures were also evaluated for 7AAD binding, a marker that is excluded by live cells, and the Ag-specific T cells were analyzed by flow cytometry. When transfected with Mxd4 or Mnt siRNA alone, we did not observe a significant change in the percentage of $7AAD^+$ cells compared to control. However, cells transfected with siRNA for both Mxd4 and Mnt showed a four-fold increase in 7AAD+ CD4 T cells compared to control siRNA (Figure 5C). This experiment was repeated four times and the average of four experiments is summarized in Figure 5D. These results provide evidence for an additive role of Mxd4 and Mnt in OX40-mediated enhancement of CD4 T cell survival.

Discussion

Signaling through OX40 on activated T cells leads to increased survival and memory T cell development. Here we show that engagement of OX40 by an agonist Ab leads to the upregulation and stabilization of the Mxd family proteins, Mxd4 and Mnt. Mxd4 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 became more quiescent. c-Myc protein was co-expressed with Mxd4 and Mnt at days 3 and 4, however, the levels remained constant at day 6 when Mxd4 and Mnt levels were decreasing. Signaling through anti-OX40 stabilized Mxd4 and Mnt proteins and we identified the presence of a putative phosphorylation residue in Mxd4, which was important for regulating protein stability. Additionally, Mxd4 and Mnt appear to play a role in T cell survival, as siRNA knockdown of Mxd4 and Mnt in OX40 stimulated T cells decreased their survival of effector T cells. We hypothesize that the upregulation of c-Myc in activated T cells drives proliferation and subsequent activation-induced cell death, which is counteracted by Mxd4 and Mnt proteins after anti-OX40 engagement. Therefore the OX40-induced increase in Mxd4 and Mnt protein expression may in part contribute to T cells survival and increased memory T cell generation observed after OX40 stimulation.

Comparison of T cells stimulated through anti-OX40 versus anti-CTLA-4 showed an increase in Mxd4 and Mnt in anti-OX40 treated cells. Previously published data from our lab showed that blockade of CTLA-4 in our model leads to increased proliferation but not increased survival [11-13]. Earlier reports showed that CTLA-4 function acted as a negative regulator of the immune response, hence blocking this signal releases this inhibition [44, 53-55]. However, recent evidence has shown that signals induced by CTLA-4 can activate a positive signaling pathway which allows for T cell survival and memory T cell formation [47]. These survival signals are mediated through PI3K via Akt, leading to upregulation of the antiapoptotic protein Bcl-2 or via another pathway leading to inhibition of the transcription factor FKHRL1, which is necessary to induce apoptosis.

T cell activation induces expression of c-Myc and its expression is required for T cell proliferation [56, 57]. This study is the first to report an increase in c-Myc protein expression in T cells after OX40 engagement. However, this is not just specific to anti-OX40 stimulation because the same increase in c-Myc levels were observed in mice receiving CTLA-4 blockade. c-Myc can play a dual role enhancing cellular proliferation as well as increasing apoptosis. A recent study showed that the transgenic over-expression of c-Myc at a 2- to 15-fold increase was sufficient to induce apoptosis [58]. The sustained increase of c-Myc protein after OX40 stimulation may target genes for the induction of apoptosis and this may be repressed by the upregulation of Mxd4 and Mnt proteins. In this model, the increase in Mxd4 and Mnt protein levels may allow for an increased percentage of Ag stimulated T cells to survive the “blast crisis” phase. A similar model has been proposed for Mxd3 expression in thymocytes [59]. Thymocytes from Mxd3 deficient mice displayed increased apoptotic sensitivity to gamma irradiation. Unlike other Mxd family members, Mxd3 was detected in proliferating thymocytes, during the S phase of the cell

cycle. The authors hypothesized that these S-phase cells were particularly vulnerable to c-Myc induced apoptosis and the increase in Mxd3 was orchestrating the transient down-regulation of apoptosis-related c-Myc target genes and rendering the cells less sensitive to c-Myc-induced apoptosis. The upregulation of Mxd4 and Mnt proteins in proliferating cells after OX40 engagement may be working through a similar mechanism.

Our data suggests that ligation of OX40 on T cells stabilizes Mxd4 and Mnt but the mechanisms leading to this stabilization is unclear. Previous studies showed that the PI3K and MAPK pathways mediate phosphorylation of Mxd1 at S145 and the protein stability is regulated via these pathways [52]. Future studies will be performed to determine if these kinase pathways are involved with Mxd4 phosphorylation in T cells. It is also unclear how Mnt stability is controlled, but recent studies indicate that ERK-directed phosphorylation of Mnt may be involved (C.W. Hooker, Wahstrom, T. Arsenian-Henriksson, P.J. Hurlin. Unpublished).

The upregulation and stabilization of both Mxd4 and Mnt appears to be important for the survival of T cells after OX40 engagement. This is because knockdown of both Mxd4 and Mnt proteins, but not the individual proteins, led to increased death of OX40-activated T cells. It is not known why both Mnt and Mxd4 proteins are stabilized following OX40 engagement, but one possibility is that they act to reinforce the repression of pro-apoptotic genes, like Bim, that are positively regulated by Myc [60, 61]. Alternatively, they may act to repress distinct proapoptotic genes that cooperate in promoting cell death. Mnt deficient T cells exhibited increased levels of apoptosis. In addition, the anti-apoptotic proteins Bcl-2 and Bcl-X_L were slightly down-regulated in Mnt-deficient T cells [62], which is consistent with data showing upregulation of these anti-apoptotic proteins are necessary for T cell survival after OX40 engagement [63]. However, we did not observe a decrease in Bcl-2 protein levels after Mxd4 and Mnt siRNA knockdown (data not shown). Stimulation through anti-OX40 leads to a decrease in caspase staining in the Ag-stimulated T cell population (Fig 2A), therefore, future studies will attempt address if Mxd4 and Mnt knockdown can increase caspase-specific stain in anti-OX40 treated CD4 T cells. To further study the function of these proteins in this model, crosses between Mxd4 or Mnt deficient mice onto a TCR receptor transgenic mouse strains will be generated. These studies will further examine the proliferative and survival status of T cells 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 Mxd4 and Mnt compared to WT T cells.

In conclusion, this study provides a novel role for the Myc/Max/Mxd network in Ag-stimulated T cells isolated from mice stimulated with anti-OX40. Identification of the molecular mechanisms that regulate Mxd4 and Mnt could potentially provide new strategies for the development of effective immunotherapies for enhancing vaccines as well as diminishing inflammation. If Mxd4 and Mnt proteins prove to be an essential part in the OX40-mediated survival of activated T cells, then enhancing the stabilization of Mxd4 and Mnt might further increase the anti-tumor effects of OX40 agonists or may partially replace the need for stimulation through OX40. Ultimately understanding the specific molecular mechanisms that regulate OX40 function could have important ramifications for future clinical trials that target immune-specific functions.

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 Thy1.1

background, and OT-I OX40-deficient mice on the C57BL/6 background (kindly provided by Dr. Michael Croft, La Jolla Institute for Allergy and Immunology, La Jolla, CA) [49] 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, 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 percentage of DO11.10 were identified by FACS using KJ1-26, which recognizes the specific DO11.10 T cell receptor [64] or Thy1.1 antibodies for identifying the OT1 specific T cells (BD Biosciences, San Jose, CA). A total of $2–3\times10^6$ 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 ofOVA (Sigma-Aldrich, St. Louis, MO) and 50μg of anti-OX40 (OX86), 100μg of anti-CTLA-4 (9H10), or 50μg Rat Ig 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. CD4 T 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 on an AutoMACS using anti-biotin microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. CD8 T cells were initially purified on an AutoMACS by negative selection, using a CD8 purification kit (Miltenyi Biotec, Auburn, CA), followed by negative selection with anti-Thy1.2 microbeads. The purity (>90%) of $KJ1\text{-}26^+$ (CD4) or $Thy1.1^+$ cells (CD8) was verified by flow cytometry FACsCalibur, Becton Dickinson, Franklin Lakes, NJ). The average numbers of $CD4/KJ^+$ T cells recovered per mouse from the DLN were as follows: d3 Rat, 0.5×10^6; d3 anti-OX40, 3×10^6; d4 OX40, 6×10^6; d6 OX40, 1.5×10^6; d3 anti-CTLA-4, 1.75×10^6, d4 anti-CTLA-4, 3×10^6. The average numbers of $CD8/Thy1.1^+$ T cells recovered per mouse from the DLN were as follows: d4 Rat, 1.5×10^6, d4 anti-OX40, 4×10^6.

Immunoblot analysis

DLNs were isolated 3, 4 or 6 days after immunization and Ag-specific T cells were purified as described above. Approximately $5\text{-}10 \times 10^6$ 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 12% polyacrylamide gels (equivalent microgram quantities of protein) (Ready Gel Tris-HCl Precast Gels, BioRad, Hercules, CA), and transferred onto nitrocellulose or PVDF membranes (Millipore, Billerica, MA). The following antibodies were using to detect these proteins: goat anti-Mxd4 (clone N-19, Santa Cruz Biotechnology, Santa Cruz, CA,), rabbit anti-Mnt (clone M-132, Santa Cruz Biotechnology), rabbit anti-c-Myc (clone N-262, Santa Cruz

Biotechnology), rabbit-Lamin A/C (clone H-110, Santa Cruz Biotechnology), rabbit anti-PARP 1/2 (clone H-250, Santa Cruz Biotechnology), mouse anti-GFP (clone B-2, Santa Cruz Biotechnology), mouse anti-GAPDH (clone 6C5, Millipore, Billerica, MA) or rabbit anti-Actin (A5060, Sigma, St. Louis, MO). For secondary detection, anti-mouse-HRP, anti-rabbit-HRP (Cell signaling, Danvers, MA) or anti-goat-HRP (sc-2060, Santa Cruz Biotechnology) were used. For chemilluminescent detection, blots were incubated in Lumiglo (Cell Signaling, Danvers, MA), exposed to film and developed. Blots were stripped using stripping buffer containing 62. 5 mM Tris, pH 6.8, 2% SDS and 100 mM 2-mercaptoethanol and reprobed for loading controls. Images were prepared using Adobe Photoshop and the ECL signals were quantitated using ImageJ software by normalizing protein expression to the respective loading control (National Institute of Health, Bethesda, MD).

Chromatin fractionation

To isolate chromatin, approximately 5-10 × 10^6 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 was added (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.

Cycloheximide assay

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

Site-directed mutagenesis

Mxd4 cDNA was cloned into a TOPO vector (Invitrogen). S145A mutant Mxd4 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 Mxd4 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 ×10^5 cells per well overnight in a 6 well plate and transfected with 2μg pWPI-GFP (empty vector), Wild-type pWPI-Mxd4-GFP (WT) or S145A mutant pWPI-Mxd4-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.

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 isolated *ex vivo* at day 4 after mice after treatment with OVA and anti-OX40. The cells 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, 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 at 48hrs post transfection. Transfection efficiency was assessed by transfection with pMax-GFP plasmid and gating on the live cells as determined by the FSC and SSC and determining the percentage of GFP positive $CD4^+/KJ^+$ cells by flow cytometry (FACs Calibur, Becton Dickinson, Franklin Lakes, NJ).

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 and analyzed for cell survival (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 ***, $p <0.0001$.

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Abbreviations used

CHX	cycloheximide
NES	nuclear export signal
NLS	nuclear localization signal, OX40L, OX40 ligand
DLN	draining lymph node
TNFR	tumor necrosis family receptor

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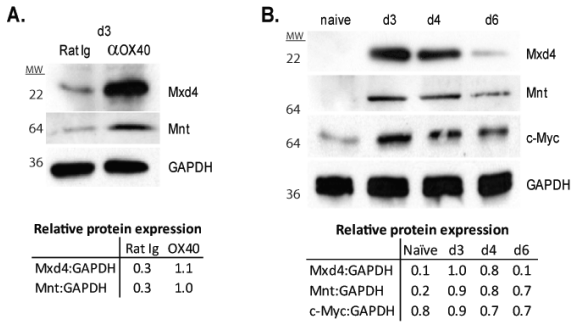
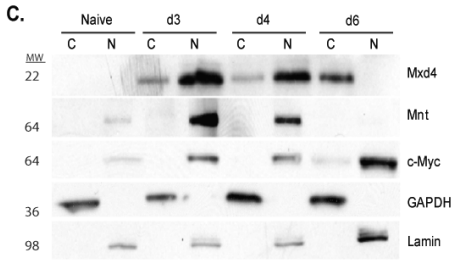


Relative protein expression

	Rat Ig	OX40
Mxd4:GAPDH	0.3	1.1
Mnt:GAPDH	0.3	1.0

Relative protein expression

	Naïve	d3	d4	d6
Mxd4:GAPDH	0.1	1.0	0.8	0.1
Mnt:GAPDH	0.2	0.9	0.8	0.7
c-Myc:GAPDH	0.8	0.9	0.7	0.7



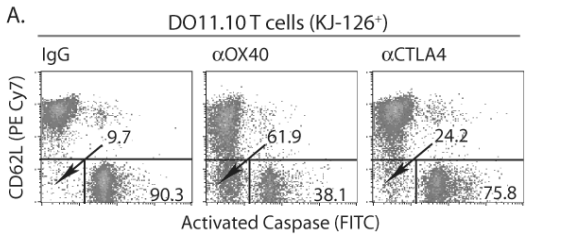
Relative protein expression

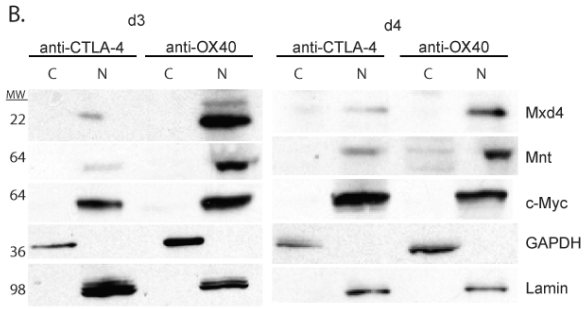
C: Relative to GAPDH, N: Relative to Lamin

	Naïve		d3		d4		d6	
	C	N	C	N	C	N	C	N
Mxd4	0.0	0.6	1.0	8.9	0.4	4.5	0.7	0.1
Mnt	0.1	0.6	0.2	5.7	0.2	2.2	0.0	0.0
c-Myc	0.0	0.6	0.0	2.6	0.0	1.6	0.1	1.5

Figure 1.

Antigen-specific CD4 T cells have increased levels of Mxd4 and Mnt proteins after anti-OX40 treatment. 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. Displayed is a representative of 3 experiments performed where all experiments showed similar results. B. 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. Displayed is a representative of 3 experiments performed where all experiments showed similar results. C. 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. Displayed is a representative of 3 experiments performed where all experiments showed similar results. Images were prepared using Adobe Photoshop and quantitated by normalizing protein expression to GAPDH or Lamin using ImageJ. The following numbers of mice were used for each group: Rat Ig: 17 mice, Naïve: 5 mice, d3 anti-OX40: 5 mice, d4 anti-OX40: 4 mice and d6 anti-OX40: 6 mice.





Relative protein expression

C: Relative to GAPDH, N: Relative to Lamin

	d3				d4			
	anti-CTLA-4		anti-OX40		anti-CTLA-4		anti-OX40	
	C	N	C	N	C	N	C	N
Mxd4	0.0	0.1	0.0	2.0	0.1	0.2	0.0	2.2
Mnt	0.0	0.1	0.0	2.0	0.0	0.7	0.0	2.7
c-Myc	0.0	0.7	0.0	2.0	0.0	2.3	0.0	3.0

Figure 2.

Mxd4, 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. OX40 stimulation decreases caspase activation in recently activated (CD62Llow) DO11.10 CD4 T cells. DO11.10 CD4 T cells were adoptively transferred into recipient mice and one day later mice were immunized s.c. with 500 μg ovalbumin and 50 μg anti-OX40, IgG, or 100 μg anti-CTLA-4. Four days after immunization lymph nodes were harvested and cultured for 24 hours. CaspACE FITC-VAD-FMK (Promega, Madison, WI) was added prior to flow analysis. Cells were gated on the ovalbumin-specific CD4 T cells (KJ-126+) and the analyzed for CD62L and caspase activation. The dot plot is a representative mouse/group, the numbers shown in the lower flow quadrants represent the average percent of the CD62L low/caspase$^{+/-}$ population in four mice per group. 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. The lysates were fractionated and analyzed by Western blot with the indicated antibodies. Each blot is representative of 3 experiments performed with similar results. Images were prepared using Adobe Photoshop and quantitated by normalizing protein expression to GAPDH or Lamin using ImageJ. The following numbers of mice were used for each group: d3 anti-CTLA-4: 8 mice, d4 anti-CTLA-4: 9 mice, d3 anti-OX40: 5 mice and d4 anti-OX40, 4 mice.

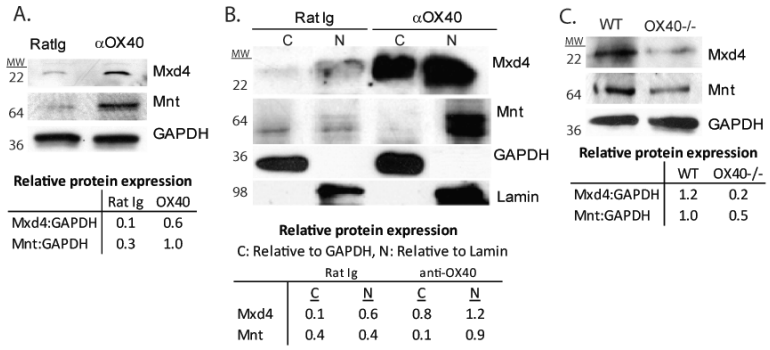


Figure 3.

Treatment with anti-OX40 directly targets Ag-specific CD8 T cells to increase in Mxd4 and Mnt proteins. A. Ag-specific CD8 T cells were isolated from DLN at day 4 after treatment with OVA and anti-OX40. CD8 T cells were purified on an AutoMACS by negative selection with anti-CD8 biotin beads, followed by negative selection with anti-Thy1.2 biotin microbeads. The purity of Thy1.1 CD8+ T cells was verified by flow cytometry. Lysates were analyzed by Western blot with the indicated antibodies. These blots are representative of 2 experiments performed with similar results. B. The lysates were fractionated and analyzed by Western blot with the indicated antibodies and blots were stripped. C, cytoplasm, N, nucleus. These blots are representative of 2 experiments performed with similar results. C. Experiment was performed as in (A) using WT or OX40-/- Ag-specific CD8 T cells. These blots are representative of 2 experiments performed with similar results. Images were prepared using Adobe Photoshop and quantitated by normalizing protein expression to GAPDH or Lamin using ImageJ. The following numbers of mice were used for each group: d4 Rat Ig: 15 mice, d4 anti-OX40 (WT): 7 mice and d4 anti-OX40 (OX40-/-): 12 mice.

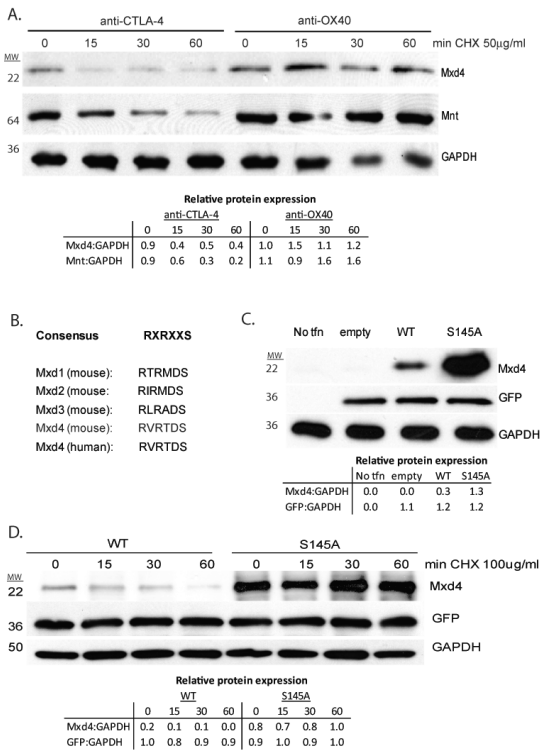


Figure 4.

Mxd4 and Mnt are stabilized in Ag-specific CD4 T cells after anti-OX40 stimulation. A. 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. These blots are one of 3 experiments performed with similar results. B. Conserved consensus phosphorylation site in Mxd family members. C. Lysates were prepared from 293H cells transiently expressing empty vector (pWPI), wild-type Mxd4 or S145A mutant Mxd4 and analyzed by Western blot with the indicated antibodies. These blots are one of 3 experiments performed with similar results. D. 293H cells expressing Mxd4 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. Images were prepared using Adobe Photoshop and quantitated by normalizing protein expression to GAPDH using ImageJ. These blots are one of 3 experiments performed with similar results.

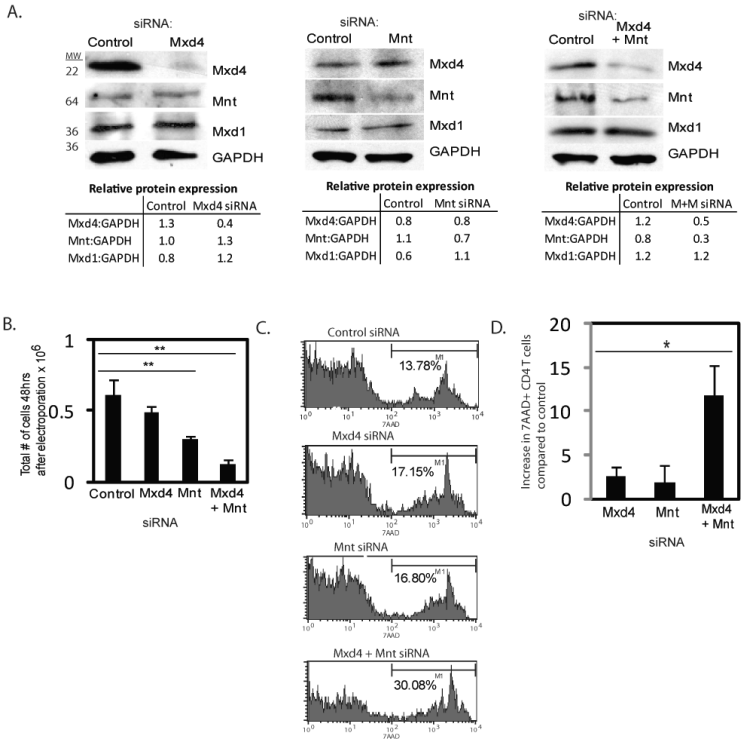


Figure 5.

Knockdown of Mxd4 and Mnt decreases T cell survival. A. Ag-specific CD4 T cells were isolated from DLN from 4 mice *ex vivo* at d4 after treatment with OVA and anti-OX40. Cells were transfected in triplicate by nucelofection (Amaxa) with Mxd4, Mnt, or scrambled (control) siRNA. Lysates were analyzed by Western blot with the indicated antibodies 48hrs post transfection. These blots are one of 3 experiments performed with similar results. Images were prepared using Adobe Photoshop and quantitated by normalizing protein expression to GAPDH using ImageJ. B. Cells were plated *in vitro* in triplicate for 48hrs post-transfection and the number of surviving cells was determined by counting cells on a hemocytometer using trypan blue exclusion. Representative of 2 experiments performed with similar results. C. Triplicate wells of cells were stained with 7AAD and analyzed by flow cytometry 48hrs post-transfection, gating on CD4+ and KJ-126+ cells. D. Average of four separate experiments as shown in C after transfection with indicated siRNA. Error bars represent SD. * $p<0.05$, ** $p< 0.01$.