MODULATION OF CD40 LIGAND (CD40L) EXPRESSION BY

POLYPYRIMIDINE TRACT-BINDING PROTEIN

by

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Abstract of the Thesis

Modulation of CD40 ligand (CD40L) expression by polypyrimidine tract-binding protein.

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CD40 ligand (CD40L or CD154) is a protein expressed on activated CD4+ T cells, which is crucial for antibody-dependent and cell-mediated immunity. The expression of CD40L is tightly regulated at multiple levels throughout a time course of T cell activation. At the post-transcriptional level the CD40L message is rapidly degraded at early time points of activation followed by a significant increase in message stability at later times of activation (24-48 hr). Previous work from our lab revealed that a cytoplasmic polypyrimidine tract binding protein (PTB)-containing-complex binds to the CD40L 3'UTR at later times of T cell activation. To understand the direct relationship between PTB and CD40L mRNA stability and subsequently CD40L expression, we used viral RNA interference against PTB and scrambled shRNA (Control) sequence in model CD40L mRNA stability T cell line, Jurkat/D1.1. Downregulation of PTB resulted in dramatic decrease in half-life of the CD40L mRNA. The downregulation of PTB did not significantly change the percentage of CD40L+ cells, but caused an approximate 2-fold decrease in the mean fluorescence (MFI) of CD40L. Cellular fractionation of CD40L mRNA from shCTRL- and shPTB-infected cells revealed a novel role for nuclear PTB in retaining the

CD40L mRNA in the nucleus. In addition, cytoplasmic PTB is important for optimal association of CD40L message with translating polysomes. Analysis of PTB cellular distribution during a time course of CD4+ T cell activation revealed cytoplasmic and nuclear localization in all resting and activated cells. However, there was an increase in cytoplasmic PTB expression at late times of activation. Binding studies revealed that CD40L mRNA is bound by *nuclear* PTB at all times of activation indicating that the requirements for binding of CD40L message by nuclear versus cytoplasmic PTB is highly distinct. Finally, the binding of CD40L message corresponded to a post-translational modification of cytoplasmic PTB that appears to correlate with a change in phosphorylation status of PTB. Confocal microscopy analysis of CD4+ T cells with activation-induced CD40L mRNA stability revealed co-localization of PTB and CD40L mRNA at distinct foci, suggesting a role of PTB in the localization of the stable CD40L message during T cell activation.

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Introduction

The immune system plays an important role in maintaining homeostasis of an organism. The primary function of the immune system is to encounter and eliminate microbes; and this function is carried out by two distinct but overlapping parts, the innate and adaptive immune systems. The innate system is the first line of defense whereas the adaptive immune system includes the cell-mediated immune (CMI) response and humoral immune response. CD4+ "helper" T cells play an important role in both responses through their ability to secrete regulatory cytokines and provide cognate "help" through constitutive and activation-induced co-stimulatory molecules [1]. A critical activation-regulated molecule expressed by CD4+ T cells is CD40 Ligand (CD40L) also known as CD154. This molecule is important for CMI and pivotal for humoral responses [2].

The CD40L gene is located on the X-chromosome at position Xq26.3-27.1 [3]. The gene encodes a type II transmembrane glycoprotein of molecular size between 34-39 kDA [4] that is mainly expressed on CD4+ T cells, but also to be expressed on platelets and eosinophils [5-6]. CD40L belongs to the tumor necrosis (TNF) superfamily because of protein structure homology with other TNF proteins. The receptor CD40 is constitutively expressed on macrophages, dendritic cells, and B cells [7-9]. Ligation of CD40 on B cells by CD40L induces: (1) the upregulation of co-stimulatory molecules and cytokines, (2) proliferation, (3) the rescue from antigen-induced apoptosis, (4) differentiation into memory and plasma cells, (5) the generation of germinal centers, (6) isotype class switching of immunoglobulins, and (7) somatic hypermutation of the

immunoglobulin genes [9-12]. CD40 ligation on monocytes and dendritic cells causes:
(1) an increase in survival, (2) upregulation of co-stimulatory molecules, (3) secretion of
cytokines and metalloproteinases (MMP), (4) increased tumoricidal activity, (5) nitric
oxide production, and (6) cross-priming of CD8^{+} cytotoxic T cells (CTLs) [7-8, 13-15].
The clinical importance of CD40-CD40L interaction is demonstrated in patients
diagnosed with X-linked Hyper-IgM (HIM) syndrome, an immunodeficiency disease
characterized by low or absent IgG, IgA, and IgE antibodies and normal or elevated
levels of IgM antibodies in the serum. These patients throughout their lifetime are
susceptible to opportunistic organisms, including *cryptosporidium* and *Pneumocystis
carinii* indicating that a cell-mediated immune response is also jeopardized. Mutations in
the CD40L gene underlie X-Linked Hyper IgM syndrome and mutations in the CD40
gene or signaling molecules associated with CD40 are defined as Non-X-Linked Hyper
IgM syndrome [16].

Given the multifunctional roles of CD40L in the CMI and humoral immune
responses it is not surprising that the gene is tightly regulated at multiple levels in
activated CD4+ T cells [17-, 37-40]. At the transcriptional level, the CD40L promoter
contains multiple binding sites for nuclear factor of activated T cells (NFATs), nuclear
factor κB (NF-κB), AP-1, and μE3; all transcription factors activated after engagement of
TCR-peptide-MHC co-stimulatory molecules [17-19]. At the post-transcriptional level
CD40L expression is controlled by the stability of its mRNA [20-21]. Our lab has shown
that at early times after CD3-TCR activation its mRNA is very unstable, half life ($t_{1/2}$) <
40 minutes, however at later stages of CD3-TCR activation CD40L message becomes

very stable with 24 hours post activation ($t_{\frac{1}{2}}$) of 84 minutes and at 48 hours post

activation ($t_{\frac{1}{2}}$) of 132 minutes [20]. In contrast, when T cells are activated by

pharmacological agents such as PMA and ionomycin the CD40L mRNA is stabilized

very rapidly, ($t_{\frac{1}{2}}$) ~2 hours [22]. Furthermore, activation via PMA and Ionomycin

induces the highest levels of CD40L on the surface of CD4+ T cells [22], suggesting a

role of CD40L mRNA stability in the expression of high levels of the protein.

The regulation of expression of CD40L through an activation-induced process of

mRNA stability is unique compared to mechanisms regulating the mRNA stability of

other cytokines. Although the TNF superfamily members, TNF-alpha and TNF-beta (also

known as lymphotoxin) are highly homologous to CD40L, their mRNAs are regulated

through an AU-rich element (ARE) decay mechanism (AMD) [23-27]. Cytokine and

growth factor transcripts that are regulated through this mechanism contain canonical

adenine and uridine-rich sequences (AUUUA or UUAUUUAUU) in their 3' untranslated

region (UTR) that are recognized by ARE-binding proteins (ARE-BPs). These proteins

target the mRNA for degradation [23-24], translational regulation [25-26], or stability

[27]. The CD40L mRNA 3'UTR contains potential AU-rich elements, however no

protein binding or function has been found in associated with these regions [20-21]. Co-

stimulatory signaling can also modulate the mRNA stability of AMD targeted transcripts

[28-29]. For example, LFA-1 signaling recruits HuR, instead of TTP to the ARE of TNF-

alpha for increased mRNA stability [28,23]. In contrast, co-stimulatory signaling does not

modulate the CD40L activation-induced mRNA stability [20]. The activation-induced

mRNA stability has been reported in beta cells of the pancreas in regulating the mRNA

stability of genes required for insulin production after a time course of glucose stimulation [30-31], suggesting that this mechanism of mRNA stability is not unique to the immune system.

Over the decade the appreciation for the importance of post-transcriptional regulation of genes involved in the inflammatory response has grown [see reviews 32-33]. Many of these factors are regulated through AMD, however, other cytokines, for example, VEGFA (Vascular Endothelial Growth Factor A), are regulated at the translational level by a complex that binds to GAIT (IFNγ activated inhibitor of translation) element in its 3'UTR induced by IFN-γ signaling that inhibits the eIF4F translation initiation complex [34]. The genome encoded small interfering microRNAs also play an important role in the post-transcriptional regulation of inflammatory and co-stimulatory genes [35-37]. The co-stimulatory molecule ICOS (Inducible T Cell Costimulator) is regulated by miR-101 that by unknown mechanism requires a RING-type ubiquitin ligase family member, Roquin, to degrade ICOS [35]. The increased expression of ICOS in homozygous mutant mice for Roquin is thought to be one of the mechanisms that lead to autoimmunity in these mice, because they produce large quantities of self-reactive antibodies [35,38]. Furthermore, mutation in another gene, ZC3H12A, also develop autoimmunity in mice, because this gene encodes a zinc-finger protein with a riboendonuclease function [39], suggesting that post-transcriptional regulation of immune genes is far more diverse and complex.

At the post-translational level CD40L expression is regulated by: (1) down-regulation of CD40L surface expression by receptor-mediated endocytosis and lysosomal degradation [40], (2) proteolytic cleavage to release a soluble form [41], and (3) ubiquitination by the transmembrane E3 ligase GRAIL during T cell anergy [42]. CD40L expression is also intrinsically transient after CD3-TCR activation where it is barely detectable 1-2 hours after activation, rising to maximal levels 6-24 hours post-activation, and decreasing to basal levels by 48 hours [20]. The CD40L protein can be sustained on the surface by co-stimulation through CD28, ICOS, and IL-2 [43-44].

Our lab has identified two RNA-binding proteins complexes that bind to 3' untranslated region (3'UTR) (nucleotides 1349 to 1609) of the CD40L message at later times of CD3-TCR activation. The ribonucleoproteins involved in complex I are polypyrimidine tract-binding protein (PTB) and nucleolin [45-46]. The ribonucleoproteins of complex II are PTB and hnRNP L [47] (**see Figure 1**). Complex I binds to a heterogeneous region consisting of "islands" of canonical PTB binding sites (UCUU) [45-47]. Within the human stability element there exists a bona fide region (nts 1410-1473) that stabilizes the entire CD40L mRNA [47]. Complex II binds to a region that contains two canonical PTB binding sites and a polymorphic CA-repeat sequence that interacts with hnRNP L [47] (**see Figure 1**). Our lab has recently shown that the mouse CD40L mRNA is also regulated by PTB-containing complex that stabilizes the mRNA with prolonged CD3-TCR activation. The mouse CD40L mRNA was shown to become stabilized in *in vivo* primed T cells following secondary exposure to KLH-

antigen *ex vivo* [48]. This work demonstrated for the first time that regulated CD40L mRNA stability occurs in response to activation during an ongoing immune response.

Polypyrimidine tract-binding protein (PTB also known as heterogenous nuclear ribonucleoprotein I) is an abundant RNA binding protein that shuttles between the nucleus and cytoplasm [49]. PTB consists of three different isoforms that arise from alternative splicing of exon 9. PTB1 is a 55 kDa protein encoded by an mRNA that lacks exon 9 entirely and i. PTB4 contains the entire exon 9 and is approximately 57 kDa, while PTB3 contains the last 57 base pairs of exon 9 [50]. PTB2 is reported to be an isoform that arises from alternative splicing of exons 3-10 to give a 25 kDa protein [51], however PTB has also been shown to be proteolytically cleaved to generate a 25 kDa protein [52]. In the nucleus PTB forms a complex with PTB-associated factor (PSF) and spliceosome machinery for inclusion or exclusion of exons through polypyrimidine tract sequences in introns [50]. Cytoplasmic PTB functions: (1) in the mRNA stability of many mRNAs (preproinsulin mRNA in β-cells, vinculin mRNA in fibroblasts, Rab8A mRNA in B cells [53-55]), (2) IRES (Internal Ribosome Entry Site)-mediated translation of viral proteins [56-57], and (3) localization of mRNAs [54, 58]. PTB is regulated by phosphorylation of Serine-16 by protein kinase A (PKA) that transports PTB from the nucleus to the cytoplasm [49], and this regulation is important in the mRNA stability of the preproinsulin mRNA [30-31] and vinculin mRNA [53].

In my thesis work I examined the role and regulation of PTB in modulating the expression of CD40L through regulating mRNA stability. Our lab has previously shown

that in vitro depletion of PTB from cytoplasmic extracts results in the rapid decay of radiolabeled CD40L 3'UTR probe [45]. My work extended these observations by using lentiviral vectors to express shRNA against PTB to demonstrate the role of PTB in both stabilizing CD40L mRNA and regulating the expression of CD40L on the T cell surface. This work also led to increased understanding of PTB-specific mechanisms for CD40L expression. Specifically, in resting, early-activated and late activated T cells, Complex I is consistently present in the nucleus and capable of complexing with CD40L mRNA. Only after prolonged activation does binding occur in the cytoplasm. Surprisingly, PTB is present in both nucleus and cytoplasm at all times prior to and after activation with a modest increase of PTB at the 48 h time point. Furthermore, dramatic post-translational modifications of cytoplasmic PTB occurred during time course of activation, while nuclear PTB remained modestly unchanged, which might be due to phosphorylation of PTB.

Figure 1.

Complex I
Complex I
Complex II
Nucleolin
Nucleolin
PTB hnRNPL
PTB
PTB
Stop codon
AAAA
Xba
E1
E5
Hae
A
B
C

Xbal
cuagaacgucuaacacaguggagaaccgaaa*ccccccccccccccccccc*gccacccucucggacaguuauuc

E1
auucucuuucaaucucucucucuccaucucucucuuucagucucucucucucaaccucuuucuuccaauc

E5
ucucuuucucaaucucucuguuucccuuugucagucucuucccucccccagucucucuucucaauccc

ccuuucuaacacacacacacacacacacacacacacacacacacacacacacacacacacacacacacac

HaeIII
acagagucagg

Figure 1: Schematic of the CD40L mRNA stability-complex.

A diagram of the CD40L mRNA 3'UTR bound by the stability-complex showing the complex consists of a lower molecular weight complex (Complex I) and a higher molecular weight complex (Complex II). Complex I consists of the RNA binding proteins, PTB and nucleolin, while Complex II consists of PTB and hnRNP L. Complex I binds to sites A (black, first black nucleotide is nucleotide1349) and B (blue) in the CD40L mRNA 3'UTR, while Complex II binds to site C (red, last red nucleotide is nucleotide 1609). PTB is able to bind to all three sites because of the presence of canonical polypyrimidine tracts sequences, UCUU (underlined).

Material and Methods

Part I: Regulation of CD40L expression by PTB in Jurkat/D1.1 cells.

Cell culture:

The human Jurkat/D1.1 T cell line were maintained in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (Gemini Biologicals), 100 μg/ml streptomycin, 100 μg/ml penicillin, and L-Glutamine (RPMI complete). 293T cells were maintained in DMEM/F12 (50:50) medium supplemented with 10% Fetal Bovine Serum (Gemini Biologicals), 100 μg/ml streptomycin, 100 μg/ml penicillin, and L-Glutamine (DMEM complete). The cells were incubated at 37°C, 5% CO_2.

Lentiviral production, concentration, and infection:

The lentiviral plasmids, VSV-G, psPAX2 and pLVTHM-U6-shCTRL and pLVTHM-U6-shPTB were generated and used as previously described [ref]. Viral particles were packaged in 293T cells by using FuGENE HD Transfection Reagent (Roche) to transiently transfect in combination the VSV-G, psPAX2 and pLVTHM plasmids. Culture supernatants were collected every 24 hrs. 293T cells were checked by flow cytometry for GFP expression to confirm lentiviruses were being produced 48 hrs post-transfection. Culture supernatants were transferred to Beckman tubes and ultracentrifuged at 4°C in Beckman SW28 rotor for 1.5 hrs at 25,000 rpm. Concentrated lentiviruses were incubated with 5 million Jurkat/D1.1 cells and 10 μg/ml Polybrene at 37°C, 5% CO_2. 2 days after incubation infection was check by flow cytometry for GFP expression.

Staining of CD40L surface expression:

5×10^5 infected Jurkat/D1.1 cells were collected and washed once in FACS wash (3% FBS and 0.1% Sodium Azide in 1X PBS). The cells were stained with 1:100 dilution of either a biotinylated anti-human CD40L mAb (Biolegend) or a corresponding biotinylated mouse IgG1 isotype control (eBioscience). Cells were incubated for 45 min at 4°C. After washing once with FACS wash the cells were stained with a 1:100 dilution of PE-conjugated streptavidin and incubated for 45 min at 4°C in the dark. Cells were washed one final time in FACS wash and subsequently fixed with 1% paraformaldehyde in FACS wash. Fluorescence was measured using a BD FACSCalibur flow cytometer.

Intracellular staining for PTB expression:

5×10^5 infected Jurkat/D1.1 cells were collected and washed once in FACS wash (3% FBS and 0.1% Sodium Azide in 1X PBS) followed by intracellular staining with BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences).

Cytoplasmic and Nuclear extracts:

1×10^7 infected Jurkat/D1.1 cells were collected and washed once with 1X PBS. The cells were resuspended in 150 microliters of complete Buffer A (10mM HEPES (7.9), 10mM KCl, 0.1mM EDTA (8.0), 0.1mM EGTA, before use: 1mM DTT, 1mM PMSF, and 1X Protease Inhibitor Cocktail) and kept on ice for 15 minutes. After 15 minutes 10 microliters of 10% NP-40 was added and cells vortexed for 10 seconds. The extract was spun at 4°C for 10 minutes at 12,000 rpm. The supernatant representing the cytoplasmic extract was collected and protein concentration measured using Bradford reagent and spectrophotometer. The pellet (nuclei) was washed once with Buffer A and resuspended in

50 microliters of complete Buffer C (25% Glycerol, 20mM HEPES (7.9), 0.4mM NaCl, 1mM EDTA (8.0), 1mM EGTA, before use: 1mM DTT, 1mM PMSF, and 1X PIC). The nuclei was incubated on ice with 10 second vortexes every 5 minutes. After 30 minutes on ice the nuclei was spun at 4°C for 5 minutes at 14,000 rpm. The supernatant representing nuclear extracts was collected and protein concentration measured.

Western immunoblotting:

Ten micrograms of cytoplasmic and two micrograms of nuclear extracts were resolved on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (PVDF) (Amersham Biosciences). After transfer, the membranes were blocked in 5%milk/1X PBS+0.1% Tween-20 (T-PBS) at room temperature for one hour. Membranes were incubated overnight at 4°C with primary antibody, then washed three times with 1X T-PBS and incubated at room temperature for one hour with a 1:3000 dilution of either goat anti-mouse or donkey anti-goat HRP-conjugated secondary antibodies (Bio-Rad). The blots were washed three times with 1X T-PBS and then treated with ECL luminol reagent to visualize the protein bands (Pierce).

DRB (5,6-dichloro-α-D-ribofuranosylbenzimidazole) treatment and RNA Isolation:

5×10^6 exponentially growing Jurkat/D1.1 shCTRL and shPTB cells were resuspended in 4 mL of their own growth media. DRB was added to the cell suspension at 50 μg/ml and incubated for 10 minutes at 37°C, 5% CO_2. 1 ml aliquots were removed at the allotted time points with the initial 10 minutes of DRB treatment representing time point, 0 minutes. The cells were spun down at 1200 rpm and the pellets were lysed in 1 ml of

Trizol reagent (Invitrogen) and total RNA was extracted following the manufacturer's protocol.

RT-quantitative PCR:

Reverse transcription reactions were carried out in a 30 μl volume containing 1 μg of total RNA, 50mM Tris-HCl pH (8.3), 75mM KCl, 3mM MgCl$_2$, 10 mM DTT, 50 U Supercript II RT (Invitrogen), 20U RNasin (Promega), 1mM dNTPs, and 500 ng oligo(dT)$_{15}$ primer (Promega). The reactions were incubated at 43°C for 50 minutes hour followed by 70°C for 15 minuntes to inactivate the RT.

Quantitative PCR was performed in a 20 μl reaction using 2 μl of cDNA, 1X Fast-Start Roche Master Mix containing Sybr green, dNTPs, and Taq, with 250 nM of exon-spanning CD40L 5' primer (5'-TCATGAAAACGATACAGAGATGC-3') and CD40L 3' primer (5'-CTTCGTCTCCTCTTTGTTTAACATT-3') or 250nM of exon-spanning β-actin 5' primer (5'-CCAACCGCGAGAAGATGA-3') and β-actin 3' primer (5'-TCCATCACGATGCCAGTG-3'). qPCR reactions were amplified on ABI 7900HT Real-Time PCR machine, with the following parameters: 95°C for 10 min, followed by 40 cycles of 10 seconds at 95°C and 30 seconds at 60°C. Data was analyzed on ABI 7900HT SDS2.2 software analysis program. Standard curve containing 10-fold dilutions from 1×10^8 PCR amplicon molecules to 1×10^3 molecules was used to quantify copy numbers from cDNA samples. Time points were normalized to β-actin and the fraction of mRNA remaining against time point zero plotted in Excel or Prism.

Part II: The impact of PTB on the cellular distribution of CD40L mRNA:

Cell fractionation:

Cell fractionation was performed essentially as described [61] with minor changes. $5x10^7$ Jurkat/D1.1 cells infected with lentivirus carrying shPTB or shCTRL were homogenized in a Dounce homogenizer in 5 ml of homogenization buffer (HB) (10 mM KOAc, 10 mM K-HEPES pH7.5, 1.5 mM Mg(OAc)$_2$, 2 mM DTT, 200 U/mL RNAsin (Promega) with protease inhibitors (Roche). To generate the free ribosomal, polysomal and S130 fractions, 2 ml of the cell homogenate was centrifuged for 5 min at 1,500 rpm to sediment nuclei and unbroken cells. One ml of resulting crude cytoplasmic supernatant was centrifuged for 20 min at 25,000 rpm in a SW 55 Ti to sediment contaminants with the supernatant representing the free ribosome fraction. The remaining 1 ml of the crude cytoplasmic extract was overlaid over a 30% sucrose cushion in HB buffer and spun at 36,000 rpm for 150 min to pellet the translating polysomes fraction, which was then resuspended in 250 μl of HB buffer. The top layer S130 (polysome-free) fraction was also collected after centrifugation. Nuclear and cytoplasmic fractions were generated as described earlier. RNA from each fraction was isolated using TRIzol LS (Invitrogen) and RT-quantitative PCR analysis of CD40L mRNA was performed as mentioned earlier.

Distribution of cytoplasmic *Renilla* luciferase mRNA:

The pRL-ABC construct was generated previously [47]. The analysis of *Renilla* luciferase mRNA into polysomes and S130 fractions were performed as described above. The primers used for quantitative PCR were forward primer (5′-

GAAGTTGGTCGTGAGGCACT-3′) and reverse primer (5′-

TCCGTTTCCTTTGTTCTGGA-3′).

CD4+ T cell isolation, stimulation and RT-qPCR:

Primary human CD4$^+$ T cells were isolated from whole blood (provided by NBAH Blood

Center) by negative isolation using Dynalbeads Untouched Human CD4 T cells

(Invitrogen). 2 x 10^7 CD4+ T cells were kept resting or stimulated with 1µg/mL

Phytohaemagglutinin (PHA) and 10ng/mL Phorbol 12-myristate 13-acetate (PMA) for 2

hours. After activation the cells were treated with DRB for the indicated times and

resuspended in TRIzol (Invitrogen). RNA isolation, RT, and qPCR were performed as

mentioned earlier.

RNA-protein colocalization by in situ hybridization:

2 x 10^5 CD4+ T cells either resting or activated were seeded onto Poly-L-Lysine coated

cover slips. The cells were washed twice with 1X PBS, followed by fixation with 4%

Paraformaldehye in 1X PBS for 20 minutes at room temperature. After fixation, the cells

were washed twice with 1X PBS, followed by permeabilization with 0.1% Triton X-100

in 1X PBS for 15 minutes at room temperature. The cells were washed once with 1X PBS

then twice with 2X SSC and incubated overnight at 4°C in 70% Ethanol. The cells were

hybridized with complementary anti-sense biotinylated CD40L oligo (5'-

AGGACTCTCTGGATGTCTGC-3') or control sense biotinylated oligo

(5'GCAGACATCCAGAGAGTCCT-3') at 25ng/µL in hybridization buffer (300mM

NaCl, 30mM Sodium Citrate, 10mM EDTA, 25mM NaH$_2$PO$_4$ (7.4), 10% Dextran

Sulfate, 250ng/ul denatured sheared salmon sperm DNA) for 16 hours at 45°C. After

hybridization, the cells were washed three times with 2X SSC. Following wash steps, the

cells were incubated at room temperature with 1:2000 dilution of Alexa 488-Streptavidin

(Molecular Probes) in 4X SSC with 0.1% Triton X-100 for 45 minutes. The cells were

washed three times with 4X SCC, then probed with anti-PTB antibodies at 1:1000 dilution

in 2X SCC with 0.1% Triton X-100 for 1 hour at room temperature. The cells were

washed three times with 2X SCC, then probed with 1:200 dilution of rabbit anti-mouse

IgG (H+L) conjugated Cy5 antibodies (Invitrogen) in 2X SCC/0.1% TX-100 at room

temperature for 1 hour. The cells were washed three times with 2X SCC, then probed with

1ug/mL of DAPI in 2X SCC for 2 minutes, then washed twice with 2X SCC and mounted

onto microscope slides with antifade Fluoromount-G (Southern Biotech). The slides were

visualized using Carl Zeiss LSM 510 Meta confocal microscope. Images were analyzed

using NIH ImageJ software.

Part III: Regulation of cytoplasmic PTB-CD40L mRNA complexing by post-translational modifications of PTB:

RNA probes

DNA templates were generated by PCR using specific forward primers containing a T7

promoter sequence and specific reverse primers for CD40L nucleotides 1300 to 1609.

RNA probes were synthesized using 0.5-1μg of template DNA, 0.4mM each of rATP,

rCTP, and rGTP, 0.04mM of rUTP, 30mM DTT, 20U RNasin, 1X T7 transcription buffer

(40 mM Tris-HCl (pH 7.9), 6mM MgCl$_2$, 2mM spermidine, 10mM NaCl), 25-40 uCi of

[32]P rUTP and 4.25 U T7 RNA polymerase (Promega) at 37°C for 1 hour. Reactions were treated with RQ1 DNase for 15 min. and centrifuged through G-25 columns (Amersham Biosciences) to remove unincorporated nucleotides.

RNA-EMSA (REMSA)

Twenty microliter reactions were prepared in RNA-binding buffer (40 mM KCl, 10 mM HEPES (pH 7.9), 3 mM $MgCl_2$, 1 mM DTT, and 5% glycerol), 4 ng *Escherichia coli* tRNA, 5 μg of CD4+ T cell cytoplasm or nuclear extracts or Jurkat/D1.1 total, cytoplasmic or nuclear extracts and 4 x 10^4 CPM of in-vitro transcribed RNA probes. The binding reactions were incubated at room temperature for 30 min., followed by the addition of 2 μl of a RNase mix containing 40 U RNase T1, 100 pg RNase A and 0.015 U RNase V1 and incubation at 37°C for 30 min. After RNase treatment, 100 μg of heparin was added and the reactions were incubated on ice for 10 min. The samples were separated on a 7% native acrylamide gel in 0.25X Tris-borate-EDTA at 200-250V for 2-4 hrs and visualized by autoradiography.

CD4+ T cell activation and Western immunoblotting:

CD4+ T cell isolation was performed as previously mentioned. 2x107 CD4+ T cells were activated with plate-bound anti-CD3 (4 ug/mL clone HIT3a) for 0, 2, and 48 hours. Extracts were performed as mentioned earlier. Western immunoblotting was also performed as mentioned earlier using 10 ug of cytoplasmic and 2 ug of nuclear extracts.

2 Dimensional Gel Electrophoresis (2D GE) analysis:

Twenty micrograms of cytoplasmic and five micrograms of micrograms from differentially activated CD4+ T cells (or Jurkat/D1.1 cells) were dialyzed against DeStreak Rehydration Solution (GE Healthcare) for 2-4 hours. The samples were then subjected to isoelectric focusing (IEF) using 7 cm Immobiline DryStrip Gels with immobilized pH gradient range 6–11 (GE Healthcare). IEF was performed in an IPGphor (GE Healthcare) according to the following protocol: rehydration for 16 h at 20°C with 50µA per strip; step 1: step-n-hold 300V for 2hrs; step 2: gradient 1000V for 0:30hrs; step 3: gradient 5000V for 4000Vhrs; step 4: step-n-hold 5000V for 0:20hrs. After IEF, strips were equilibrated in 50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol with 1% DTT for 15 min, and then for 15 min in the same buffer without DTT but with 4% iodoacetamide. Equilibrated strips were placed on top of 4-20% linear gradient polyacrylamide gels and SDS–PAGE performed. After electrophoresis, the proteins were transferred to PVDF membranes and immunoblotted against PTB with anti-PTB antibodies. The membranes treated with ECL luminol reagent to visualize the protein bands (Pierce) by film or LAS Fujifilm machine.

Lambda Phosphatase treatment and 2D GE:

Twenty micrograms from Jurkat/D1.1 cytoplasmic extracts were mock-treated or treated with 800U Lambda Phosphatase (NEB) in 20uL final volume of 50mM HEPES (7.5), 100mM NaCl, 2mM DTT, 0.01% Brij 35, 2mM MnCl$_2$, and 1X PIC. Samples were incubated at 30°C for 3 hours and dialyzed against DeStreak Rehydration Solution (GE Healthcare) for 4 hours and 2D GE protocol followed as described earlier.

Results

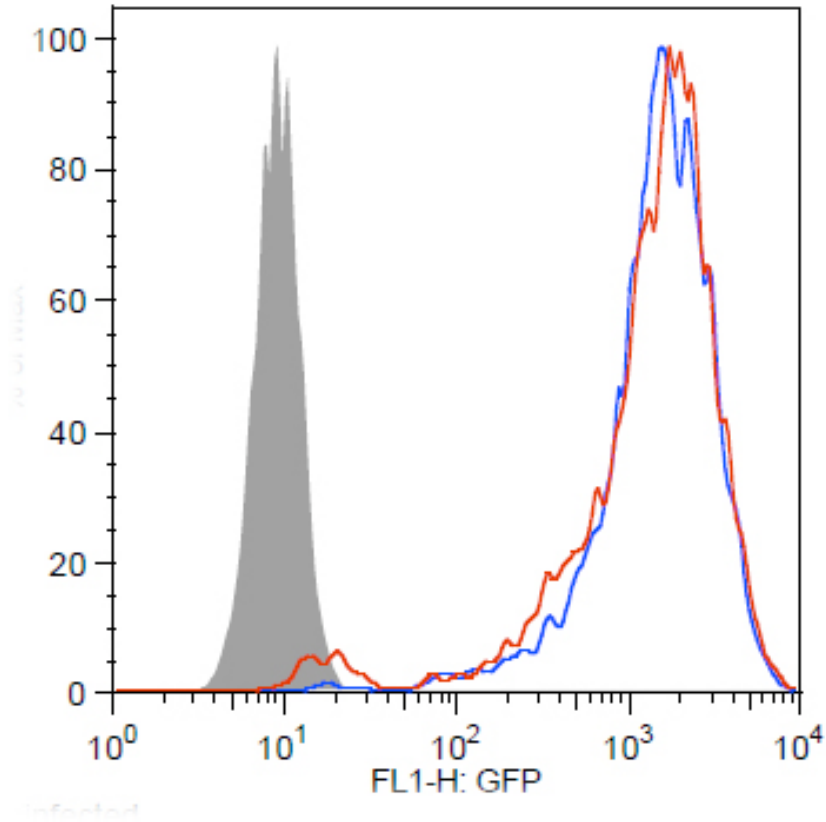**Regulation of CD40 ligand expression by polypyrimidine tract-binding protein (PTB)**.

Polypyrimidine tract-binding protein (PTB) regulates CD40L mRNA stability.

Previous work in our lab showed that a heterologous (*Renilla* luciferase) construct containing the minimal binding site (E1-E5) of the CD40L mRNA stability element conferred increased mRNA stability on the transcript [47]. This region contains the largest number of consensus PTB binding sites, although 5' and 3' regions of E1-E5 region also contain some binding sites for PTB [45]. In contrast, *in vitro* ^{32}P-labelled CD40L mRNA stability element probe lacking the E1-E5 minimal region decayed faster than the full-length probe [45]. These results suggested that the PTB binding sites were a bona fide stability element by conferring enhanced stability on a heterologous transcript. To test role of PTB in directly regulating the stability of CD40L mRNA we infected Jurkat/D1.1 cells with a lentivirus containing a U6 promoter in the pLVTHM vector that expresses GFP and either shRNA against PTB (shPTB) or scrambled sequence (shCTRL). Our infection efficiency was consistently very high, between 90-100% (GFP positive), and a representative profile is shown in **Figure 2**.

After 2 days, infected cells were collected and cytoplasmic and nuclear extracts analyzed by Western immunoblotting with anti-PTB antibody (BB7) to check the downregulation of PTB by shPTB. Cells expressing shPTB showed a decrease in PTB in both the cytoplasmic and nuclear fractions (**Fig. 3A**). The downregulation of PTB in

shPTB expressing cells was further confirmed by intracellular staining with anti-PTB

measured by flow cytometry (**Fig. 3B**).

　　　　To analyze the effect of downregulating PTB on the expression of CD40L

mRNA, shPTB- and shCTRL-expressing Jurkat cells were treated with the transcriptional

inhibitor, DRB (5,6-dichloro-a-D-ribofuranosylbenzimidazole) for 0, 30, 60, and 120

minutes. RNA was collected at each time point and the amount of RNA remaining was

analyzed by quantitative RT-PCR. Results showed a decrease in the CD40L mRNA

stability with the downregulation of PTB (**Fig. 4A**). A 50% fold decrease was also

evident at the steady-state level of CD40L mRNA (**Fig. 4B**). These results supported our

previous in vitro work by demonstrating that PTB is critical for maintaining the steady

state level of CD40L mRNA by directly regulating its decay rate.

Figure 2.

Figure 2. Jurkat/D1.1 cells are highly infectable with lentiviruses.

Flow cytometry profile of the infection efficiency of pLVTHM-shPTB (red) and pLVTHM-shCTRL (blue) viruses in Jurkat/D1.1 cells showing greater than 95% of the population expressing GFP. The grey peak indicates uninfected cells. Representative histogram from three independent experiments.

Figure 3.

Figure 3. PTB is downregulated in cells infected with shPTB viruses.

(A) Western blot analysis of PTB expression in Jurkat/D1.1 shPTB and shCTRL cells. 10 ug of cytoplasmic and 2 ug of nuclear extracts were separated by SDS-PAGE and immunoblotted with anti-PTB antibodies (lanes 1, 2, 5 and 6). Membranes were stripped and re-probed with anti-Actin (lanes 3, 4, 7 and 8) for loading control. Representative blot from three independent experiments. (B) Intracellular staining profile for PTB in shPTB (red) and shCTRL (blue) cells. Grey peak represents isotype antibody control for the anti-PTB antibody. Representative histogram profile from two independent experiments.

Figure 4.

Figure 4. PTB stabilizes the CD40L mRNA and maintains steady-state levels.
(A) After 2 days the Jurkat/D1.1 cells infected with shPTB and shCTRL were treated with the transcriptional inhibitor, DRB, for 0, 30, 60 and 120 minutes. RNA was collected at each time point and reverse transcribed and CD40L amounts quantified by quantitative PCR (qPCR). All time points were normalized to β-actin. Representative graph came from three independent experiments. (B) Steady-state analysis of CD40L mRNA at the initial time point of DRB treatment (0 minutes) between shPTB and shCTRL cells. Samples were normalized to MLN51. Representative graph came from three independent experiments.

PTB affects the surface expression of CD40L.

        To address if downregulation of PTB modulates the surface expression of CD40L, infected cells were stained with anti-CD40L antibodies and analyzed by flow cytometry. The results showed an approximate 40% reduction in the mean fluorescence intensity (MFI) per cell of cells infected with shPTB compared to shCTRL. A representative profile is shown in **Figure 5** with CD40L expression on the y-axis and GFP expression on the x-axis. These results demonstrate a direct correspondence between PTB expression, stabilization of CD40L mRNA and CD40L expression in activated T cells.

Figure 5.

shCTRL cells   shPTB cells

Figure 5. Downregulation of PTB affects the surface expression of CD40L.
Surface staining for CD40L on shPTB and shCTRL cells and analyzed by flow
cytometry. Shown is a representative profile with GFP expression on the x-axis and
CD40L expression on the y-axis. The mean fluorescence intensity (MFI) for CD40L of
the double positive (GFP+CD40L+) population is indicated. The change in MFI for
CD40L expression is indicated underneath each profile. Representative dot plot from
three independent experiments.

The impact of PTB on the cellular distribution of CD40L mRNA:

<u>PTB regulates the distribution of the CD40L mRNA.</u>

Our lab has previously shown that PTB binds to the ribonucleoparticle (RNP) of the CD40L mRNA and also associates with the translating polysomes [46,51]. Given that PTB functions in both the nucleus and cytoplasm it is possible that PTB influences the fate of transcribed and processed CD40L mRNA in the nucleus to be destined for translation in the cytoplasm. To test this hypothesis RNA from the nuclear and cytoplasmic fractions of cells expressing either shPTB or shCTRL was reverse transcribed and analyzed by quantitative PCR for the amount of CD40L RNA in each fraction. The results showed that in shCTRL cells there is more CD40L mRNA present in the nucleus compared to the cytoplasm, while downregulated PTB gave the opposite result with more CD40L in the cytoplasm than the nucleus (**Fig. 6**). Overall there was substantially more total RNA present in the cytoplasm than the nucleus in both shPTB and shCTRL cells (data not shown). These results suggest the CD40L mRNA is being held in the nucleus by PTB.

Figure 6.

Figure 6. Downregulation of PTB re-distributes the CD40L mRNA in Jurkat/D1.1 cells.

The distribution of CD40L mRNA between the nucleus and cytoplasm was measured in normal amounts (shCTRL) and limiting amounts (shPTB) of PTB. 1ug of total RNA isolated from the nucleus and cytoplasm was reverse transcribed and the amount of CD40L RNA quantified by qPCR. The results are shown in percentage of CD40L mRNA present in the nucleus and cytoplasm. Representative graph from three independent experiments.

<u>PTB regulates the CD40L mRNA association with translating polysomes</u>.

To evaluate the fate of the CD40L mRNA in the cytoplasm and association with polysomes, the cytoplasmic fraction was separated into translating polysomes, S130 (polysomes-free), and free ribosome sub-fractions. The presence of CD40L mRNA in each fraction was measured by quantitative PCR. The results showed that downregulation of PTB decreased the amount of CD40L mRNA in the polysomes with an increase in both the S130 and free ribosome fraction. The presence of the CD40L mRNA in shCTRL showed a greater amount in the translating polysomes with a lesser amount in the S130 and free ribosomes fraction (**Fig. 7**).

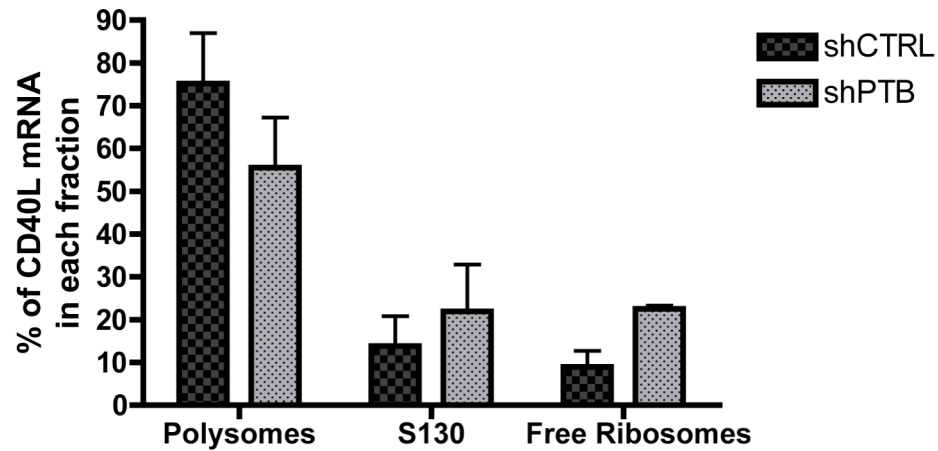Previous results in our lab revealed that the CD40L mRNA stability element (nucleotides 1300-1609) was capable of stabilizing a heterologous transcript (*Renilla* luciferase) and increasing the luciferase activity [47]. We wanted to know if the CD40L mRNA stability element (nucleotides 1300-1609) would also contribute to the increased luciferase activity at the translational level. To evaluate this hypothesis the *Renilla* transcript with and without the stability element was stably selected in uninfected Jurkat/D1.1 cells. The cytoplasm of cells was then sub-fractionated into polysomes and S130 fractions, followed by RNA isolation. The RNA in each fraction was reverse transcribed and amount of *Renilla* transcript in each fraction measured by quantitative PCR. Analysis of the *Renilla* mRNA with (pRL-ABC) and without (pRLSV40) the stability element did not reveal any change in associations to the polysomes (**Fig. 8)**,

suggesting other elements in the CD40L mRNA are contributing to the association with translating polysomes.

Figure 7.

Figure 7. Cytoplasmic PTB associates the CD40L mRNA to translating polysomes.
The CD40L mRNA distribution in the cytoplasm was measured in normal (shCTRL) and
limiting amounts (shPTB) of PTB. 1ug of total RNA from fractionated cytoplasmic
fractions (polysomes, S130 and free ribosomes) were reverse transcribed and CD40L
amounts quantified by qPCR. The results are shown in percentage of CD40L mRNA
present in each fraction. Representative graph from three independent experiments.

Figure 8:

Renilla luciferase

Figure 8: The CD40L mRNA stability element does not increase the association of _Renilla_ transcript with translating polysomes.

Renilla luciferase transcript with the CD40L mRNA stability element was compared to an unmodified transcript for the distribution into translating polysomes and S130 fractions in uninfected Jurkat/D1.1 cells. 1 ug of total RNA from each fraction was reverse transcribed and _Renilla_ mRNA amounts quantified by qPCR. Shown is percentage of _Renilla_ luciferase mRNA in each fraction. Representative graph from two independent experiments.

PTB and CD40L mRNA co-localize with PHA/PMA stimulation for 2 hours in CD4+ T cells.

Besides sites of translating polysomes messenger RNA molecules may be recruiting to two other RNA-protein interacting sites: Processing bodies (P Bodies) and Stress Granules (SGs) [59]. P bodies are thought to be sites of mRNA degradation because they contain many decapping enzymes and exonucleases [59]. Stress granules are sites where mRNAs are protected from degradation but are not actively being translated; many proteins of translation initiation machinery localized at SGs after an environmental stress [59]. We wanted to test the hypothesis that CD40L mRNA and PTB may be localized to P bodies and/or SGs during differential CD4+ T cell activation. In order visualization if the CD40L mRNA and/or PTB localize to these sites a dual RNA hybridization and immunoflourescent protocol was performed [the protocol was a kind gift from Dr. Kiledjian's lab]. This protocol employs the use of a complementary DNA oligo (with a biotin moiety conjugated to the 5' end) to the CD40L mRNA to detect the RNA molecule. Following hybridization immunofluorescence with anti-PTB antibodies is used to visualize PTB. In a preliminary experiment CD4+ T cells were either not activated (naïve) or activated with PHA and PMA for 2 hours. The cells were treated with DRB for 15, 30, and 60 minutes to demonstrate that this method of activation stabilizes the CD40L mRNA very rapidly (**Fig. 9A**) and induces high levels of the CD40L mRNA (**Fig. 9B**). Confocal analysis of the activated cells demonstrated co-localization of PTB and CD40L mRNA at distinct foci in CD4+ T cells (**Fig. 10**). Unfortunately in unactivated cells the CD40L mRNA signal was too weak to visualize any co-localization between PTB and the CD40L mRNA. Increasing the number biotin moieties on the oligo

probe could increase the signal in unactivated cells to visualize the localization of the CD40L mRNA. Future experiments will look for the co-localization of CD40L and/or PTB with known proteins associated with P bodies and SGs in differentially activated CD4+ T cells.

Figure 9.

Figure 9. Short stimulation with PHA/PMA stabilizes the CD40L mRNA.
(A) CD4+ T cells were kept resting (naïve) or activated with PHA/PMA for 2 hours and cells were treated with DRB for 0, 15, 30 and 60 minutes. RNA was collected at each time point, reverse transcribed and CD40L mRNA amounts quantified by qPCR. (B) Steady-state levels of CD40L mRNA were analyzed at time point 0 minutes of the DRB treatment from resting (naïve) and activated cells. Graphs are from one preliminary experiments.

Figure 10.

2hr PHA/PMA cells

CD40L mRNA   PTB   Merge

Figure 10. Stimulation with PHA/PMA for 2 hours co-localizes PTB and CD40L mRNA at distinct foci in CD4+ T cells.

CD4+ T cells were stimulated with PHA/PMA for 2 hours and hybridized with complementary 5' biotin-conjugated oligo to detect the CD40L mRNA. After hybridization the cells were stained with anti-PTB antibodies to detect PTB. Confocal images showing focal points of co-localization between CD40L mRNA (green) and PTB (red) with PHA/PMA stimulation. Top panel represents one cell and the bottom panel represents another cell. Images are from one preliminary experiment.

Regulation of cytoplasmic PTB-CD40L mRNA complexing by post-translational modifications of PTB:

PTB containing-complexes constitutively binds to the CD40L mRNA in the nucleus, but only bind to the mRNA in the cytoplasm following prolonged CD4+ T cell activation.

Previous work in our lab showed that the CD40L mRNA is only bound by complex I in the cytoplasm with prolonged CD3-TCR activation in CD4+ T cells corresponding to an increase in mRNA stability [60]. To ask whether nuclear PTB is capable of binding to the CD40L mRNA during a time course of activation, RNA-EMSA was performed with cytoplasmic and nuclear extracts from differentially activated CD4+ T cells. The results showed that in the nucleus complex I/II are present and can bind to the CD40L mRNA regardless of activation state (**Fig. 11, lanes 6-9**). Complex I/II binding to the CD40L mRNA only occurs with prolonged T cell activation in the cytoplasm, confirming previous results (**Fig. 11, lanes 10-12**). The mRNA stability of CD40L therefore is influenced by a differential regulation of complex I/II formation during time course of T cell activation.

Figure 11.

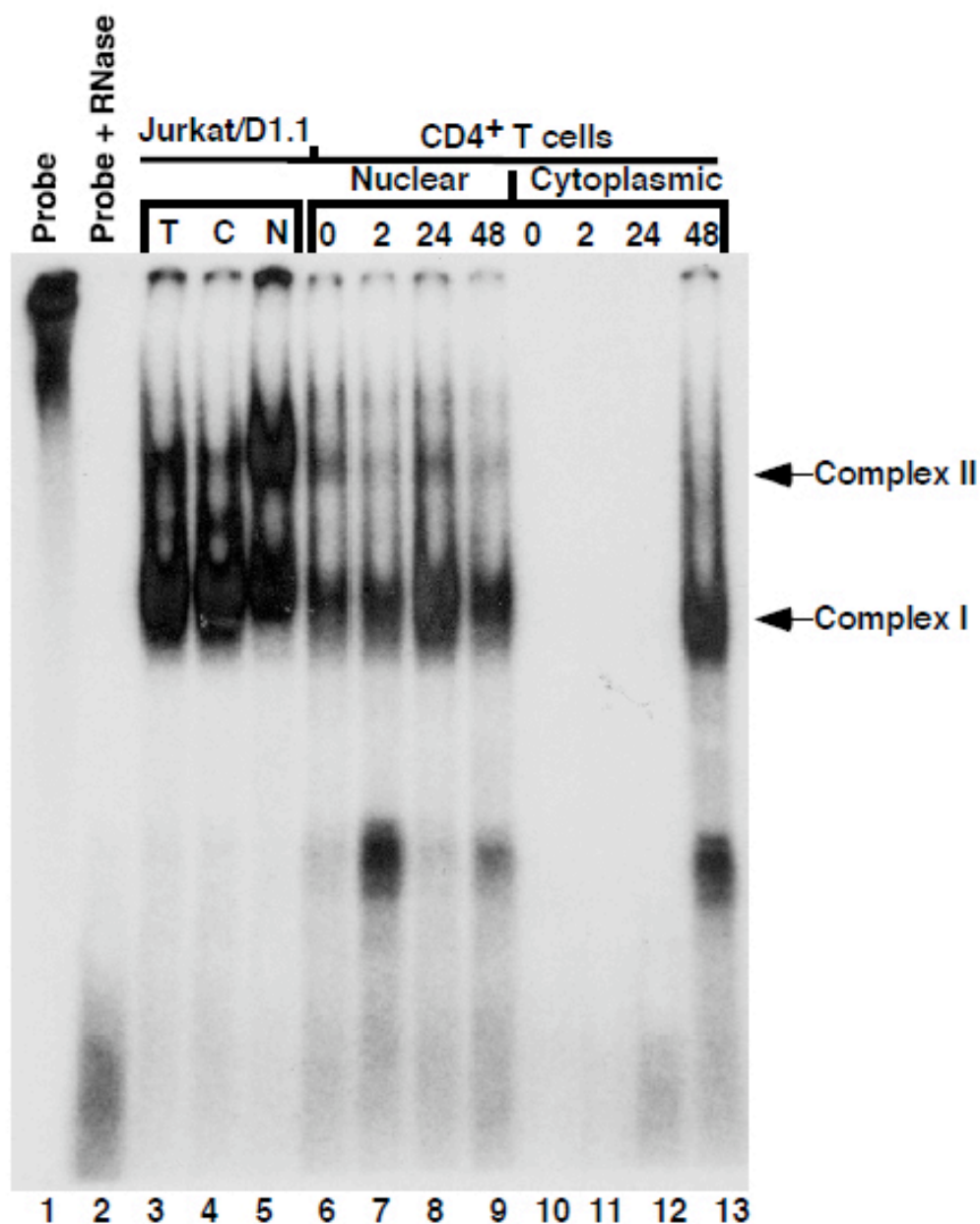**Figure 11. Nuclear complex-forming PTB is capable of binding to the CD40L mRNA independent of the activation state of CD4+ T cell**.

RNA-EMSA from cytoplasmic and nuclear extracts from differentially activated CD4+ T cells with P^{32}-labelled 3'UTR stability element from the CD40L mRNA. Total, cytoplasmic and nuclear extracts from Jurkat/D1.1 were also used. The CD40L mRNA stability complex I and complex II are indicated.

PTB is present in both the cytoplasm and nucleus; and its expression remains relatively
constant within time course of CD4+ T cell activation.

      The results from the binding experiment carried out with extracts from
differentially activated CD4+ T cells revealed that complex I/II binding to the CD40L
mRNA to be an important factor for stabilizing the message with prolonged T cell
activation. The major protein of complex I/II is PTB. Nucleolin is present in complex I
[46], while hnRNP L is present in complex II [47]. To observe if the distribution of the
CD40L stability complex proteins: PTB, nucleolin, and hnRNP L changed within a time
course of T cell activation, cytoplasmic and nuclear extracts from differentially activated
CD4+ T cells were analyzed by Western immunoblot with anti-PTB antibodies, anti-
nucleolin antibodies and anti-hnRNP L antibodies. PTB and nucleolin were detected in
both the cytoplasm and nucleus throughout the time course of activation, without a
dramatic change in expression in both fractions (**Fig. 12A**). Fractionation of the
cytoplasm was analyzed by probing for Actin and fractionation of nucleus by probing for
PARP (**Fig. 12A**). The distribution of hnRNP L also did not change in either the
cytoplasm or nucleus during time course of activation (**Fig. 12B**), however only one
isoform of hnRNP L was detected in the cytoplasm (**lanes 11-13**), indicating a possible
role of cytoplasmic hnRNP L in regulating the CD40L mRNA.

Figure 12.

Figure 12. PTB, nucleolin, and hnRNP L expression levels remain relatively constant throughout time course of CD4+ T cell activation.

(A) CD4+ T cells were activated for 0 (naïve), 6, 12, 24, and 48 hours with plate-bound anti-CD3 antibodies. Cytoplasmic (lanes 1 to 5) and nuclear (lanes 6 to 10) extracts were analyzed from each time point by Western immunoblotting with anti-PTB antibodies and anti-nucleolin antibodies. Fractionation was verified by probing with anti-Actin antibodies (cytoplasmic) and anti-PARP (nuclear). (B) In a separate membrane cytoplasmic and nuclear extracts from naïve, 2 hour and 48 hour activated CD4+ T cells with plate-bound anti-CD3 antibodies were probed for hnRNP L (lanes 11 to 16).

Cytoplasmic PTB is modified over time course of CD4+ T cell activation.

Cytoplasmic PTB was detected in differentially activated CD4+ T cells, however this does not address the question of why and how nuclear PTB is capable of binding the CD40L while cytoplasmic PTB only binds with prolonged activation. This observation suggested that either the PTB was differentially modified between the two compartments or that other associated factors were required for complexing in the cytoplasm. It is known that post-translational modifications of RNA binding proteins modulate their ability to stabilize or decay their targeted mRNA [32]. Also, nucleocytoplasmic shuttling of PTB by phosphorylation at Serine-16 stabilizes the preproinsulin mRNA in β cells stimulated for prolonged period of time with glucose [30]. To address the possibility that cytoplasmic PTB is modified during a time course of activation, nuclear and cytoplasmic extracts from differentially activated CD4+ T cells were subjected to 2-dimensional gel electrophoresis (2D-GE) and immunoblotted with anti-PTB antibodies. Cytoplasmic PTB is modified during a time course of activation to resemble nuclear PTB (**Fig. 13, panels 1-4**). Nuclear PTB does not change dramatically during the time course of activation (**Fig. 13, panels 5-8**).

To further demonstrate that the modification of cytoplasmic PTB corresponds to differential binding to the CD40L mRNA in activated CD4+ T cells, cytoplasmic and nuclear extracts from Jurkat/D1.1 cells were subjected to 2D GE analysis and immunoblotted for PTB. This T cell line has a stable CD40L mRNA and cytoplasmic PTB capable of complexing with the mRNA [60], many characteristics similar to primary

CD4+ T cells. The migration of cytoplasmic and nuclear PTB in Jurkat/D1.1 cells was similar to that of the nuclear and 48 hour post-activated CD4+ T cell profiles (**Fig. 14**). These findings further demonstrate that post-translational modifications of PTB are important in its ability to bind to the CD40L transcript and to ultimately regulate CD40L mRNA stability.

Figure 13.

Figure 13. Cytoplasmic PTB is modified over time course of CD4+ T cell activation. Cytoplasmic and nuclear extracts from CD4+ T cells activated over a time course were subject to 2 dimensional gel electrophoresis and immunoblotted for PTB. The different isoforms of PTB are indicated.

Figure 14.

Nucleus

Cytoplasm

Figure 14. Cytoplasmic and nuclear PTB in Jurkat/D1.1 cells have similar isoforms.
Jurkat/D1.1 cytoplasmic and nuclear extracts were subjected to 2D GE analysis and
immunoblotted for PTB. PTB has a theoretical pI of 9.6. The different isoforms of PTB
are indicated.

Cytoplasmic PTB is a phosphorylated protein in Jurkat/D1.1 T cells.

  To test the hypothesis that the modification of cytoplasmic PTB in Jurkat/D1.1 cells represented distinct phosphorylation events, cytoplasmic extracts were untreated or treated with Lambda phosphatase, an enzyme that removes phosphate groups from Serine, Tyrosine and Threonine amino acids. These extracts were subjected for 2D GE analysis for any change in PTBs' isoelectric charge. The results showed that treatment with the phosphatase changed the isoelectric charge of PTB toward the anode (negative) side of the pH gradient strip compared to untreated samples (**Fig. 15**). These results indicate that cytoplasmic PTB is phosphorylated in Jurkat/D1.1 cells.

Figure 15.

Figure 15. Cytoplasmic PTB is a phosphoprotein in Jurkat/D1.1 cells.

(A) Cytoplasmic extract from Jurkat/D1.1 cells were treated or mock treated (untreated) with Lambda phosphatase, an enzyme that removes phosphates from Serine, Tyrosine and Threonine amino acids. The samples were subjected to 2D GE and immunoblotted for PTB. (B) Higher magnification of 2D GE with arrows indicating the highest negatively charged form of PTB.

Discussion

CD40 ligand is a molecule expressed on CD4+ T cells, which is critical for both humoral and cell-mediated immunity [2]. In my thesis work, the current knowledge of CD40L expression was extended by establishing the *in vivo* requirement for PTB in regulating CD40L mRNA stability in activated T cells. Furthermore, this work provides new insights into the regulation of PTB during a time course of CD4+ T cell activation and suggests a mechanism for regulating the late phase of CD40L mRNA stability.

Regulation of CD40 Ligand expression by polypyrimidine tract-binding protein (PTB).

The CD40L mRNA stability element contains three distinct PTB consensus-binding sites [45]. Previous *in vitro* studies from our lab revealed that PTB bindings sites between nucleotides 1410 and 1473, designated E1 to E5, are required for CD40L mRNA stabilization [45]. Downregulating PTB using a lentiviral RNA interference approach was highly effective at demonstrating that CD40L mRNA stabilization is dependent on PTB. In addition, the PTB-mediated destabilization of the CD40L transcript directly affected the surface expression of CD40L establishing the relationship between appropriate levels of surface CD40L and message stability. PTB has also been shown to increase the levels of insulin by stabilizing the preproinsulin mRNA with prolonged stimulation with glucose in β-cells of the pancreas [53]. Our lab has also shown in B cells that a PTB-dependent activation-induced mRNA stability pathway exists with prolonged TLR9 activation. For example, TLR9 activation with unmethylated CpG stabilizes the Rab8A mRNA through PTB [55].

The next question we asked regarding the post-transcriptional regulation of CD40L by PTB was the role of this protein on CD40L mRNA stability and surface expression of CD40L in CD4+ T cells over a time course of activation. Activation of CD4+ T cells through the TCR induces transient expression of CD40L that peaks between 6-24 hours and beyond 24 hours returns to baseline levels [20]. CD4+ T cells activated through engagement of the TCR and the co-stimulatory molecule, CD28 expresses CD40L over a period of 4 days [43], suggesting CD28 has a role in preventing the return of CD40L to baseline levels at later times of activation. Whether this regulation during the late phase of CD40L expression occurs at the post-transcriptional or post-translational level is a question that will be examined in future experiments. Co-stimulation of CD4+ T cells does stabilizes the ARE regulated IL-2 mRNA [29,62]. Ligation of CD28 translocates NF90 from the nucleus to the cytoplasm to stabilize the IL-2 mRNA [62]. However, how CD28 ligation modulated the instability trans-acting factor TTP of the IL-2 mRNA is unknown.

The impact of PTB on the cellular distribution of CD40L mRNA:

Our lab and others have previously reported the association of PTB with translating polysomes [46,51]. PTB cytoplasmic function is largely to regulate stability messenger RNAs [53-55]. We demonstrate a new function of PTB in that it is required to increase the association of the CD40L mRNA to translating polysomes. Whether PTB plays a direct role in stabilizing the mRNA on translating polysomes to increase the CD40L expression is not yet determined. PTB has been shown to regulate the c-myc and

insulin receptor expression at the translational level by promoting translation through an Internal Ribosome Entry Site (IRES) in the 5'UTR of both mRNAs [63-64]. In both reports the IRES element was inserted into bicistronic *Renilla* and firefly luciferase reporter and saw an increase in the expression of cap-independent firefly luciferase activity in cell lines. These observations were confirmed by *in vitro* translation reactions using the bicistronic reporter mRNA and reticulocyte extracts [63-64]. Previous work from our lab found that insertion of the CD40L mRNA stability element into the *Renilla* luciferase reporter construct increased luciferase activity. However, this increase was caused by an increase in mRNA stability of Renilla mRNA and not enhanced of association with translating polysomes [47]. Whether the 5'UTR and other 3'UTR elements of the CD40L mRNA are also required to increase the association with translating polysomes by PTB is under investigation. Interestingly, the inhibitor of the mTOR/S6K pathway, rapamycin, inhibits the expression of insulin by decreasing the PTB-dependent preproinsulin mRNA stability in β-cells [65]. This pathway requires the recognition of a poly CU-rich tract in the 5'UTR for increased translation [66]. Whether PTB plays a role in mTOR/S6K pathway in regulating translation has not been addressed.

The observation that the CD40L mRNA requires PTB for both stabilization and optimal recruitment to polysomes raises the question as to whether CD40L mRNA and PTB are associated in sites known to foster RNA-protein interactions. Processing bodies (P bodies) are sites of nontranslating mRNA and contain an abundance of mRNA decapping enzymes (DCP1/2, Hedls) and the exonuclease Xrn1 [59]. Therefore, P bodies are categorized as sites of mRNA decay. On the other hand, stress granules (SGs) are

sites of mRNAs that are translationally stalled by environmental stress. They contain

many components required for translation (eIF4E, 40S ribosomal unit, PABP) [59].

Messenger RNAs in SGs can be modified to re-initiate translation or be recruited for

degradation [59]. In preliminary experiments CD40L mRNA and PTB were visualized in

co-localized foci within the cytoplasm of activated CD4+ T cells. Experiments are

ongoing to determine if P bodies and/or SGs markers are also present in these foci.

Information from these experiments will enhance understanding the regulatory

mechanisms of CD40L mRNA decay/stability and PTB-mediated regulation of mRNAs

in CD4+ T cells.

Regulation of cytoplasmic PTB-CD40L mRNA complexing by post-translational modifications of PTB:

In defining the activity of Complex I in CD4+ T cells we demonstrated that

complex activity was only present in the cytoplasm with prolonged TCR activation.

However, nuclear Complex I is constitutively present regardless of the extent of T cell

activation. A striking observation that was also made is that PTB is present in the

cytoplasm and nucleus of differentially activated CD4+ T cells. Nuclear PTB expression

remains constant throughout the time course of activation. Cytoplasmic PTB expression

is constant throughout the time course with a modest increase at 48 hours post-activation.

We are currently investigating if there is also an increase of PTB mRNA during time

course of activation to correlate with the expression of PTB and complex formation.

These results did suggest that both nuclear and cytoplasmic PTB are differentially

modified or that additional activation-induced cytoplasmic proteins are required for

complexing. We identified by 2D gel electrophoresis experiments that cytoplasmic PTB is modified during the time course of T cell activation. In contrast, nuclear PTB does not get modified. Many hnRNP proteins are regulated by post-translational modifications, which in turns regulates the post-transcriptional control of their target messages [32]. For example, the Small Ubiquitin-like Modifier (SUMO) is a modification of hnRNP M and hnRNP C, which in turn increases the nuclear export of mRNAs from the nucleus [67]. It has also been reported that phosphorylation of nuclear PTB at Serine-16 is directly linked to its ability to shuttle into the cytoplasm in 293/HEK cell line and pancreatic β-cells [49,30]. The nucleocytoplasmic translocation of PTB has also been reported to be important in the mRNA stability of preproinsulin by prolonged glucose stimulation [30-31]. The phosphatase treatment assay in Jurkat/D1.1 cells did modify the isoelectric point of cytoplasmic PTB, indicating that cytoplasmic and nuclear PTB are differentially phosphorylated. Identifying if upon activation of CD4+ T cells PTB is phosphorylated at Serine-16 or another residue and if these modifications are required for PTB binding to the CD40L transcript will reveal a mechanism underlying the activation-induced PTB-mediated regulation of CD40L expression.

Conclusions and future directions:

The PTB-dependent regulation of mRNA stability through prolonged activation or stimulation is emerging to be a new regulatory post-transcriptional mechanism. We demonstrated here CD40 ligand expression is dependent on the mRNA stability by PTB. In our first model (**Figure 16**) PTB directly regulates the mRNA stability of CD40L and

the increase in transcript stability is essential for optimal expression of CD40L. PTB

regulates the CD40L mRNA by distributing it between the nucleus and cytoplasm, thus

raising the possibility the mRNA is protected from degradation in the nucleus or is being

retained there for a reason not yet known. Interestingly, cellular mRNA distribution plays

an important role in animal development, for example the distribution of mRNA away

from the germ plasm in *Drosophila* protects certain mRNAs in the early embryo from

degradation [68].

We also demonstrated here a potentially new regulatory mechanism of the CD40L

mRNA stability-complex formation in activated CD4+ T cells. In our second model

(**Figure 17**) PTB binding and complex-formation to the CD40L mRNA seems dependent

on post-translational modifications of PTB in differentially activated CD4+ T cells. In

contrast to other cell types were nucleo-to-cytoplasmic shuttling of PTB regulates mRNA

stability [30,31,49,58], differentially activated CD4+ T cells constitutively have PTB in

the cytoplasm and nucleo-to-cytoplasmic shuttling does not seem to regulate the CD40L

mRNA stability. Prolonged TCR activation modulates the post-translational regulation of

PTB and thus allow it bind to the CD40L mRNA in late-activated CD4+ T cells. Whether

the post-translational modification is due to hyperphosphorylation of PTB is under

investigation. Furthermore, post-translational modifications of other RNA binding

proteins modulates post-transcriptional regulation of their target trancripts, for example

unphosphorylated TTP destabilizes the TNF-α mRNA, on the other hand phosphorylated

TTP stabilizes the TNF-α mRNA by interacting with 14-3-3 adaptor proteins [69].

There are plenty of more work to be done in understanding the regulation of

CD40L mRNA stability and CD40L expression. It would be of interest to look at the

other stability-complex components, nucleolin and hnRNP L, because they might also be post-translationally modified in order to regulate complex formation to the CD40L mRNA. Nucleolin is a phosphoprotein that gets phosphorylated by Cdc2, CK2 and PKC-zeta [70]; and hnRNP L is phosphorylated by CaMKIV [71]. Both these phosphorylation events occur in the nucleus, therefore looking at cytoplasmic modifications of these proteins opens up new knowledge to the function of nucleolin and hnRNP L.

Optimal expression of CD40L on CD4+ T cells depends on transcriptional and post-transcriptional regulation (mRNA stability) [22]. However, activation only through the TCR induces a transient expression of CD40L [20]. The initial expression is dependent on the transcription of CD40L mRNA, however prolonged activation that stabilizes the CD40L mRNA coincides with reduced expression of CD40L [20]. Therefore, based on these reports there is a discrepancy in the regulation of CD40L expression by the mRNA stability of CD40L. Interestingly, ligation of CD28 stabilizes the expression of CD40L with prolonged activation [43], thus providing an attractive avenue to look at in understanding the regulation of CD40L by its post-transcriptional regulation. Finally, increased expression of CD40L has been implicated in many autoimmune diseases such as, rheumatoid arthritis and systemic lupus erythematosus (SLE) [70-72]. Whether the increased expression in these diseases is due to de-regulation of CD40L mRNA stability is unknown. Although, being able to modulate CD40 ligand expression through PTB will be a potential promising pharmaceutical target in treating autoimmune diseases as well as other immune-related conditions.
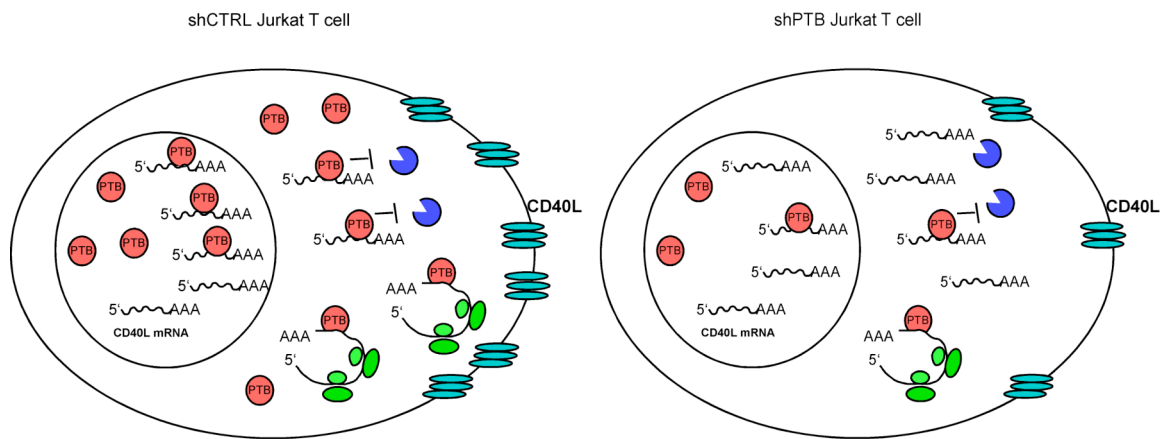
Figure 16.

Figure 16: Model of PTB-dependent mechanisms regulating CD40L expression in Jurkat T cells.

Under normal conditions (shCTRL Jurkat T cells) PTB is highly expressed in both the nucleus and cytoplasm of the Jurkat T cell (a model cell line of late activated T cells). In the nucleus PTB appears to retain the CD40L mRNA away from the cytoplasm, possibly being a site where the CD40L mRNA could be stabilized. Once in the cytoplasm the CD40L mRNA is also able to be bound by PTB and possibly protect the transcript from ribonucleases. Protected from ribonucleases the CD40L transcript increases its association to translating polysomes; therefore lead to optimal expression of CD40L on the plasma membrane. Under limiting levels of nuclear PTB (shPTB Jurkat T cells) the CD40L mRNA transports out of the nucleus to the cytoplasm. In the cytoplasm the CD40L mRNA is highly susceptible to degradation by ribonucleases (and the mRNA decay machinery) because of the downregulated expression of cytoplasmic PTB. The CD40L transcript thus is less able to associate with translating polysomes and the expression of CD40L on the plasma membrane decreases.
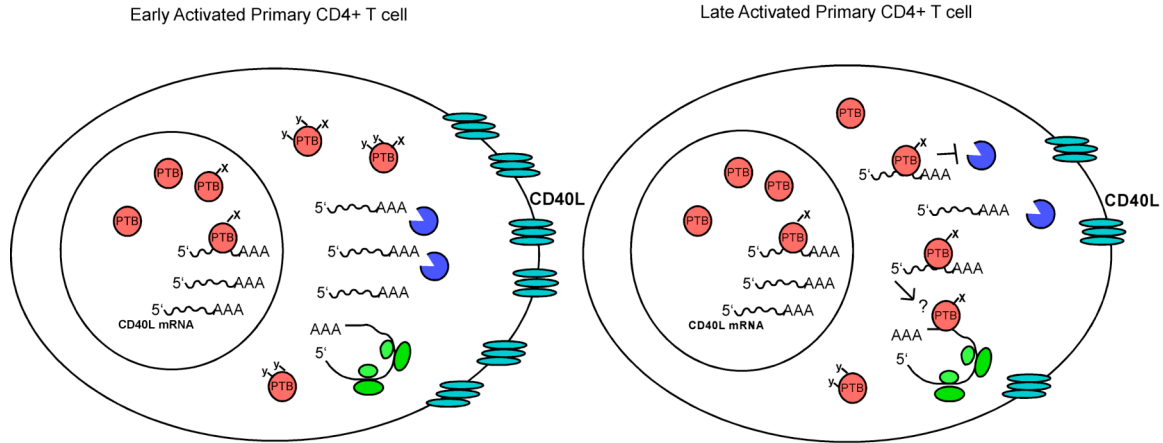
Figure 17.

Figure 17: Model of regulation of CD40L mRNA stability by post-translational regulation of PTB in differentially activated primary CD4+ T cells.

In early-activated primary CD4+ T cells transcription of the CD40L mRNA is greater than the stability of the transcript. This inverse relationship increases the surface expression of CD40L in early-activated cells. The fast decay of the CD40L transcript coincides with the inability of PTB (and stability complex) from binding to the message. However, nuclear PTB in early-activated cells is able to bind to the CD40L mRNA. This differential ability of nuclear and cytoplasmic PTB to bind to the CD40L mRNA is due to differential post-translational modifications of PTB in both compartments. With prolonged activation the post-translational modification (possibly hyper-phosphorylation) of cytoplasmic PTB is removed and the CD40L mRNA becomes bound by PTB (and stability complex). Nuclear PTB in late-activated cells does not get modified due to prolonged activation and is still able to bind to the CD40L mRNA. In the late-activated state the transcription of the CD40L mRNA is diminished and the mRNA stability greatly increased. However, in late-activated cells the surface expression of CD40L also diminishes, thus raising the question whether: (1) the mRNA stability of CD40L is affecting the translation of the mRNA or (2) is the diminished surface CD40L expression due entirely to the post-translational regulation of CD40L.

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