Expression, regulation and modulation of Fas Ligand during T lymphocyte activation

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Abstract

Auto-immune diseases stem from an inability to eliminate immune cells that react to "self" peptides. In particular, when T lymphocytes react to self peptides, or become activated, they undergo elimination in a process of programmed cell death or apoptosis. In T lymphocytes one of the key effectors of apoptosis is a membrane protein, Fas ligand, since it engages Fas, the caspase cascade, and ultimately cell death. Therefore the biosynthesis of Fas ligand is tightly controlled to maintain immune homeostasis. HuR is an RNA binding protein that has been shown to have an important role in all aspects of T lymphocyte maturation and function. HuR is known to be important in the posttranscriptional regulation of many cytokines by stabilizing, relocalizing and promoting the translation of their mRNAs. The effects of HuR are mediated through binding to AU rich elements in the untranslated regions of these mRNAs. We find that HuR posttranscriptionally regulates Fas ligand through AU rich and U rich motifs in its mRNA and explore the contexts in which this regulation is important.

Résumé

Les maladies auto-immunes découlent d'une incapacité d'éliminer les lymphocytes T qui reconnaissent l'hôte. En général, l'apoptose suit lors qu'un lymphocyte T devient activé ou répond aux peptides dérivés de l'hôte. Une des molécules principales qui induit l'apoptose dans les lymphocytes T est une protéine membranaire nommée Fas ligand qui se lie au récepteur Fas pour activer la cascade de caspases ce qui entraine la mort cellulaire. En conséquence, la production de Fas ligand est strictement contrôlée pour entretenir l'homéostase immunitaire. HuR est une protéine qui lie l'ARN qui est important pour les fonctions des lymphocytes T. HuR agit par des effets post-transcriptionels sur les ARNm de plusieurs cytokines par stabilisation, relocalisation et l'efficacité de traduction. HuR promeut ses fonctions en liant des régions riches en AU dans les parties non-traduites des ARNm. Nous démontrons que Fas ligand est régulé de façon post-transcriptionel par HuR via des régions AU riches et U riches, et nous examinons dans quels contextes cette régulation est importante.

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List of commonly used abbreviations

ActD	Actinomycin D
AICD	Activation induced cell death
AMD	ARE mediated decay
APC	Antigen presenting cell
APRIL	Acidic protein rich in leucines
ARE	AU rich element
AUBP	AU rich element binding protein
AUF1	AU-rich element/poly(U)-binding/degradation factor
BRF1	TFIIB related factor
CHX	Cycloheximide
ConA	Concanavalin A
COX	Cyclo-oxygenase
(E)GFP	(Enhanced) green fluorescent protein
ELAV	Embryonic lethal abnormal vision
ENL	Erythema nodosum leprosum
ERK	Extracellular signal receptor kinase
FADD	Fas associated death domain
FasL	Fas ligand
FLIP	FLICE like inhibitory protein
GM-CSF	Granulocyte macrophage colony stimulating factor
IFN-γ	Interferon-γ
IL	Interleukin
IRF	Interferon regulatory factor
KSRP	KH type splicing regulatory protein
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
miRNA	Micro RNA
NF-AT	Nuclear factor activated T cells

NF-KB	Nuclear factor kB
NK	Natural killer
PARP	Poly ADP ribose polymerase
PB	processing body
PHA	Phytohemagglutinin
PI	Propidium iodide
PI3K	Phosphoinositide 3 kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
RAR	Retinoic acid receptor
RBD	RNA binding domain
RBP	RNA binding protein
RNP	Ribonuclear protein
ROR	Retinoid related orphan receptor
RXR	Retinoid x receptor
SEB	Staphylococcal enterotoxin B
SG	stress granule
SLE	Systemic lupus erythematosus
TCR	T cell receptor
THD	Thalidomide
TIA	T cell intracellular antigen
TIAR	TIA related
TNF	Tumour necrosis factor
TRAIL	TNF related apoptosis inducing ligand
TTP	Tristetraprolin
UTR	Untranslated region

Chapter 1: Introduction

1.1 The physiological roles of Fas ligand

The immune system is responsible for the elimination of pathogens and tumours from the host. The immune response acts via two branches, namely the innate host defenses that identify pathogens as foreign entities by the recognition of common motifs, and the adaptive response that generates a pathogen specific response. The effector cells from these two compartments communicate between each other and with other tissues by means of cytokines. Cytokines can be membrane bound or released into the extracellular space as soluble molecules. Their effects are due to ligation with cognate receptors on the cell membrane, which activates signal transduction cascades and provokes responses such as chemotaxis, adhesion, cytokine release, differentiation, and proliferation. Defects in cytokine regulation and signaling can have deleterious consequences and cause autoimmune disease, decrease resistance to infection, trigger cytokine storms in response to infection etc. One of the major groups of cytokines is the TNF superfamily which includes TNFα, FasL, CD40L and TRAIL and that has been linked to many chronic and deadly diseases. For example, defects in the Fas-FasL receptor-ligand pair are implicated in autoimmune lymphoproliferative syndrome and systemic lupus erythrematosis both of which are autoimmune diseases (Strasser et al., 2009). Therefore there is currently much interest in the development of immunomodulatory drugs that can target cytokine production. TNF superfamily is of particular interest as therapeutic targets since they are involved in a variety of immune pathologies such as: sepsis, Crohn's disease, ENL, multiple myeloma. Although some drugs directly target TNF such as humanized anti TNF monoclonal antibodies, there is also interest in drugs such as thalidomide and its analogues which appear to target several members of the TNF superfamily including

TNFα, FasL and TRAIL. Numerous studies highlighted the importance of such treatments in combating acute and chronic diseases that involve the immune system. However, effective treatments are still lacking for many of these illnesses. This is probably due to the fact that our understanding of the molecular mechanisms that modulate the function and the expression of some members of this family of cytokines is still poor.

One such member is the Fas-FasL signalling machinery. While several studies have indicated that targeting the Fas-FasL signaling pathways may be a useful strategy for therapeutic intervention in certain diseases, these treatments so far showed limited effects or in some cases led to deadly consequences. It has been shown that the administration of agonistic Fas-specific antibodies (Ogasawara et al., 1993) or FASL (Huang et al., 1999) can trigger massive apoptosis in hepatocyte cells leading to hepatitis and death in mice. One of the main reasons of these failures could be a lack of clear understanding of all the mechanisms and the players that are involved in regulating the expression and the function of the Fas-FasL pair. Therefore, defining all these mechanisms and identifying the regulators that modulate the Fas-FasL expression and function could open the door to new strategies to combat many of the deadly diseases mentioned above. The aim of this thesis is to delineate the posttranscriptional regulatory mechanisms that regulate the expression of *fasl* gene and explore the possibility of using a drug such as thalidomide and its analogues as a means to prevent FasL expression/function in one of the main cellular components of the immune system, T lymphocytes.

1.1.1 Fas ligand is a member of the TNF superfamily of cytokines

Fas is considered a prototypical "death receptor", as Fas engagement by FasL triggers a cascade of caspase activation, which culminates in apoptotic death of the Fas-bearing cell. Fas is best known as a "death receptor" able to induce apoptosis when ligated by FasL, and Fas and FasL have been most studied in the immune system, where they regulate lymphocyte cell death (Nagata and Golstein, 1995). Indeed, Fas and FasL are co-expressed on activated T cells, and Fas/FasL interactions mediate activation-induced cell death (AICD), the physiological process that regulates the magnitude and duration of an immune response by triggering the death of antigen-activated T cells (Strasser et al., 2009). Defects in Fas and FasL cause a progressive accumulation of lymphocytes, which may be accompanied by autoimmune symptoms. In mice, mutations of Fas or FasL give rise to the *lymphoproliferative* (*lpr*) and *generalized lymphoproliferative disease* (*gld*) phenotypes, and in patients, dominant-negative mutations cause the Autoimmune Lymphoproliferative Syndrome (ALPS). Fas (CD95, APO-1) and FasL (CD95L, CD178) are a receptor – ligand pair belonging to the TNF-R / TNF superfamily (see Figure 1). In mice, Fas and FasL are exclusive high affinity binding partners. In humans, FasL can also bind Decoy Receptor 3 (DcR3), a soluble molecule (Pitti et al., 1998). Mouse and human FasL are 77% homologous at the amino acid level (Takahashi et al., 1994).

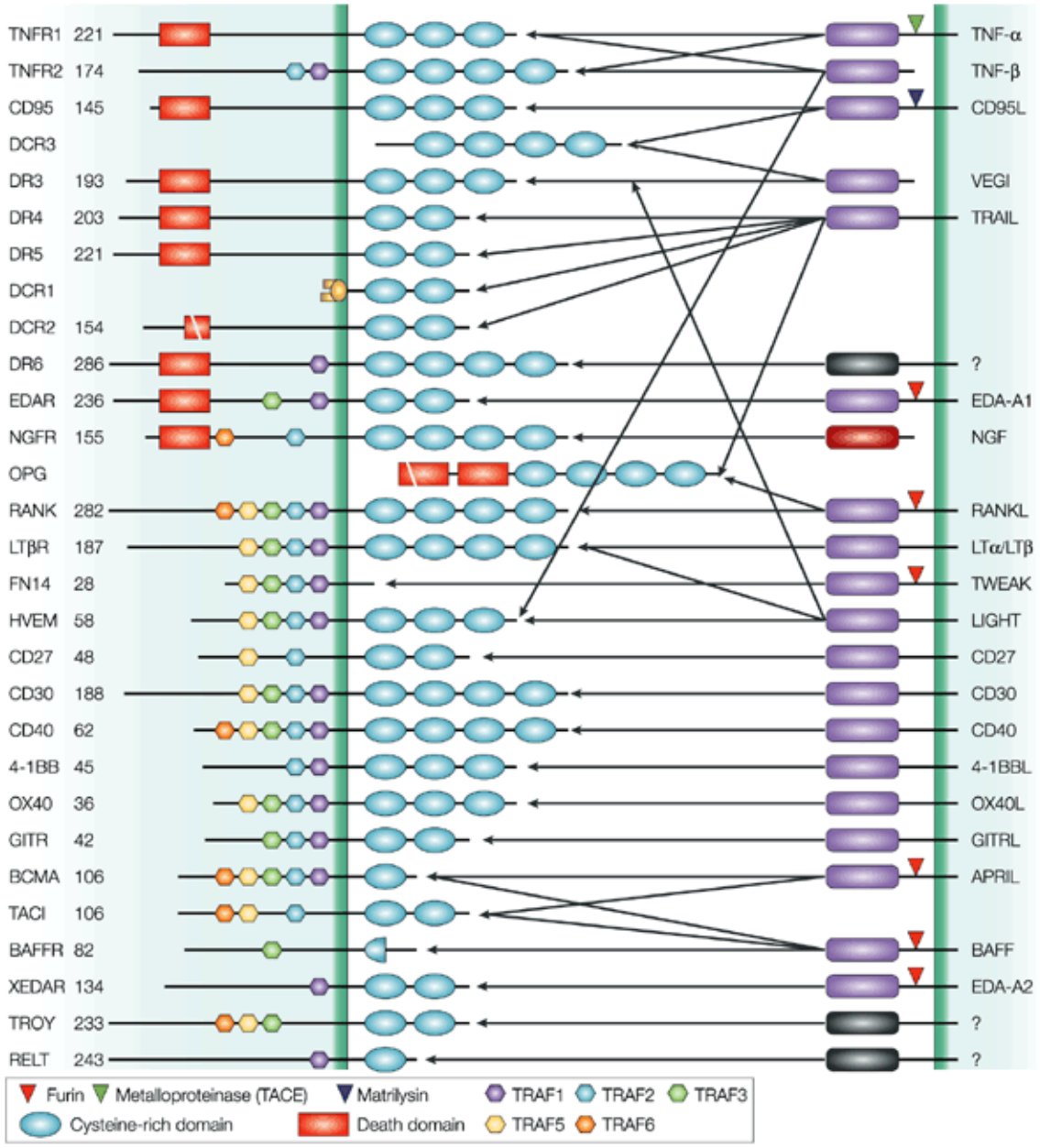

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Figure 1: Schematic representation of the TNF (right) and TNF receptor (left) superfamily members.

Common motifs are indicated; triangles indicate proteolytic cleavage sites and TRAF are sites that bind TNF Receptor Associated Factors. In addition, black arrows indicate the receptor and ligand specificities (Aggarwal, 2003).

Fas is a 45 kDa transmembrane homotrimeric member of the TNF receptor superfamily (Itoh et al., 1991). The intracellular tail of Fas contains a death domain, which is capable of recruiting and activating the apoptotic machinery in response to engagement by FasL (Chinnaiyan et al., 1995). Fas Ligand (FasL), which is the focus of this thesis, is a homotrimeric member of the TNF superfamily. It is a 37-41 kDa (depending on glycosylation) type II transmembrane protein that binds Fas (Suda et al., 1993). Thus, Fas and FasL are the prototypical "death" receptor ligand pair involved in extrinsic apoptosis. More recently, non-apoptotic functions such as proliferation and differentiation in a variety of contexts have been attributed to Fas and FasL (Alderson et al., 1993; Desbarats et al., 2003; Desbarats and Newell, 2000).

FasL is flanked to the 3' by two other TNF family members, GITRL and OX40L, on chromosome 1 in both mice and humans. Like Fas ligand, the rest of the TNF family is involved in immune homeostasis though not exclusively via apoptosis (TNF, TRAIL) (Chinnaiyan et al., 1996a; Chinnaiyan et al., 1996b; Pan et al., 1998; Sheridan et al., 1997). Other members of the TNF family impact the immune system through co-stimulation (CD40L), proliferation (APRIL) and differentiation (CD27). Interestingly, NGF is also a TNF superfamily member and mediates similar processes in the nervous system. Similarly, EDA-A1 and EDA-A2 are involved in differentiation of epithelial tissues. All of these proteins trimerize via an extracellular trimerisation domain. In addition, they have conserved extracellular domain structures, which bind cysteine rich domains (CRD) of their cognate receptors. Most TNF family members are type II

membrane anchored, others are secreted. Due to their physiological functions, many of these TNF superfamily ligands are exquisitely regulated at every level of biosynthesis. The importance of this superfamily of ligands is underscored by the fact that mutations in several of its members such as FasL are linked to severe pathological defects.

1.1.2 Mouse mutants of Fas and FasL

Defects in Fas and FasL result in unrestrained proliferation of T lymphocytes and autoimmunity. Both Fas and FasL were originally characterized by mapping the loci responsible for lymphoproliferative (lpr), lymphoproliferative-complements gld (lpr-cg) and generalized lymphoproliferative disease (gld) phenotypes in natural mouse mutants (Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992b). These natural mouse mutants have strikingly similar phenotypes characterized by the expansion of CD3+B220+CD4-CD8- T lymphocytes, enlarged spleen and lymph nodes, increased immunoglobulin titers and presence of autoimmune anti-nuclear antibodies. Targeted mutations of Fas and Fas ligand lead to more severe phenotypes than the natural mutations (Karray et al., 2004; Senju et al., 1996). The most severe defects cause renal disease by immunoglobulin complex formation, which deposit in the glomeruli of the kidney. Similar mutations in Fas and FasL have been found in human patients with autoimmune lymphoproliferative disorder (ALPS) (Bi et al., 2007; Fisher et al., 1995) and systemic lupus erythromatosis (SLE) is tied to defects in apoptotic signaling through Fas (Chen et al., 2006a; Lee et al., 2001; Xue et al., 2006).

1.1.3 Fas, Fas Ligand and apoptosis

While Fas seemed to be expressed in many tissues including heart, liver and lymphoid organs (Watanabe-Fukunaga et al., 1992b), FasL expression was restrained to tissues of immune-privilege and lymphoid organs including the gut associated lymphoid tissue (GALT) (Suda et al., 1993). Over-expression of FasL in cell culture, resulted in apoptosis, and it was hypothesized that Fas was a death receptor and that engagement by FasL triggered programmed cell death (Suda et al., 1993). The importance of the FasL-Fas signaling was highlighted by the fact that spontaneous mutations in both humans and mice were linked to severe autoimmune diseases. Indeed, mice bearing a homozygous defect in the genes encoding Fas (*Fas*$^{lpr/lpr}$ or *Fas*$^{lprcg/lprcg}$) (Watanabe-Fukunaga et al., 1992a) or FasL (*Fasl*$^{gld/gld}$) (Takahashi et al., 1994) develop lymphadenopathy and SLE-like autoimmune disease. Additionally, a heterozygous mutation in the *fas* gene was found in a large fraction of ALPS (Autoimmune lympho-proliferative syndrome) patients (Strasser et al., 2009). These phenotypes were explained in part by a failure to eliminate autoreactive T lymphocytes by apoptosis and consequent perturbation of T lymphocyte homeostasis (Strasser et al., 2009; Watanabe-Fukunaga et al., 1992a). These observations demonstrated that Fas-FasL-mediated signal transduction pathways play a key role in controlling the immune system.

1.1.4 The prototypical extrinsic cell death pathway

The apoptotic pathway engaged by Fas signaling is known as the caspase cascade. Fas exists as a trimer at the cell surface and recruits a death inducing signaling complex

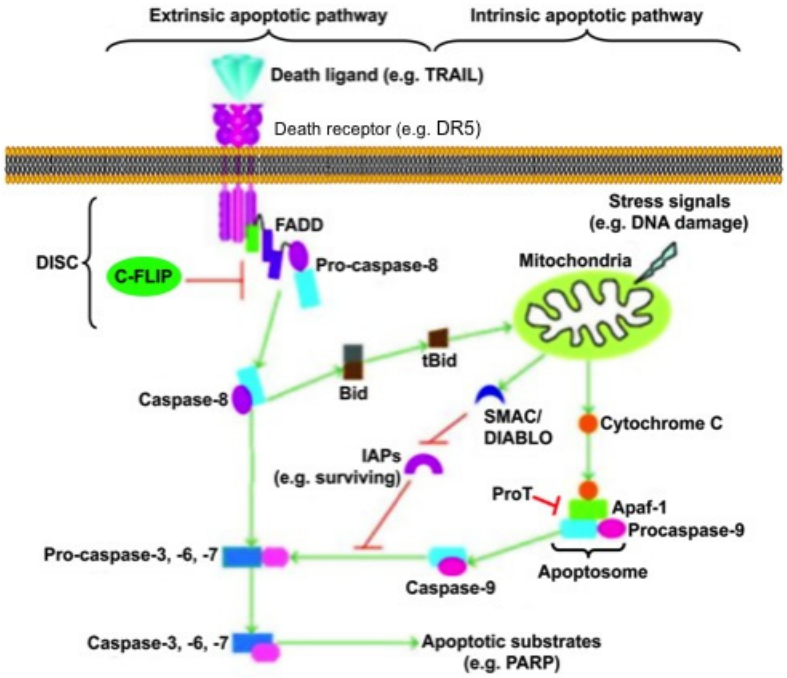
Extrinsic apoptotic pathway
Intrinsic apoptotic pathway
Death ligand (e.g. TRAIL)
Death receptor (e.g. DR5)
FADD
DISC
C-FLIP
Pro-caspase-8
Stress signals
(e.g. DNA damage)
Mitochondria
tBid
Bid
Caspase-8
SMAC/
DIABLO
Cytochrome C
IAPs
(e.g. surviving)
ProT
Apaf-1
Procaspase-9
Pro-caspase-3, -6, -7
Apoptosome
Caspase-9
Caspase-3, -6, -7
Apoptotic substrates
(e.g. PARP)

Figure 2: Schema for basic apoptotic signaling pathways.

Ligation of death ligands (e.g., TRAIL, Fas ligand) with their receptors (e.g., DR5, Fas) results in formation of the death-inducing signaling complex (DISC), in which procaspase-8 will be recruited through the death adaptor protein FADD and cleaved to generate activated caspase-8. Certain stress signals can target mitochondria and induce cytochrome C release from the mitochondria into the cytosol leading to caspase-9 activation by forming an apoptosome via binding to Apaf-1. Both caspase-8 and caspase-9 activate downstream procaspases-3, -6, and -7, leading to cleavage of their target death proteins such as PARP. In addition, truncated Bid (tBid), activated by caspase-8 via cleavage, facilitates insertion of Bax into the mitochondrial membrane leading to cytochrome C release. Therefore, tBid may serve as a link between the extrinsic and intrinsic apoptotic pathways. Inhibitors of apoptosis proteins (IAPs) such as survivin can bind to activated caspase-9 and prevent its action on effector caspases.

(DISC) which is composed of FADD, FLIP and procaspase 8 or 10. FADD is recruited to Fas via homotypic death domain (DD) interactions upon receptor engagement (Chinnaiyan et al., 1995). Similarly, procaspase 8 and 10 interact with FADD via homotypic death effector domain (DED) and are activated by cleavage (Muzio et al., 1996). Procaspases 8 and 10 undergo autocatalytic cleavage upon recruitment to the DISC. This cleavage is modulated by FLIP, which also contains a DED domain and is recruited to FADD. FLIP can either promote or repress caspase cleavage depending on splice variant and expression levels. Cleaved caspases 8 and 10 can then activate executioner caspases 3, 6 and 7 and also cleave Bid, which leads to mitochondrial release of cytochrome c, reviewed in (Yin, 2000). The requirement for Bid cleavage in the induction of Fas dependent apoptosis depends on cell type. Both caspase cleavage and mitochondrial release of cytochrome c lead to the activation, via cleavage, of apoptotic substrates such as the inhibitor of caspase activated DNase and PARP, resulting in the destruction of essential cellular machinery and cell death (Figure 2). Apoptosis is one of the main biological consequences of Fas ligation. The different cellular contexts in which Fas exerts pro-apoptotic functions are described below.

1.1.5 T lymphocyte apoptotic contexts dependent on Fas ligand

1.1.5.1 Negative selection

Fas/FasL were strongly suspected to control T lymphocyte homeostasis by apoptosis due to the phenotype of the mouse mutants, this was confirmed in 1995 by 3 back to back articles in the February issue of Nature which indicate that FasL is

upregulated in response to T lymphocyte stimulation and subsequent Fas engagement induced cell autonomous apoptosis (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995). This literature has defined the role of FasL until recently. There are two major processes that are important to T lymphocyte homeostasis. Both of these rely upon the recognition of peptides presented on MHC to the T cell receptor (TCR). There are 2 types of MHC: Class I and class II. All cells express MHC class I, and the peptides presented by this MHC are derived from the intracellular milieu. Under normal circumstances, all of the peptides presented will be derived from the host. In contrast, during intracellular bacterial infection or viral infection, some pathogen-derived peptides will be presented on MHC class I (Vyas et al., 2008). Only cytotoxic CD8+ T lymphocytes can engage MHC class I with the TCR. The class II MHC expression is restricted to professional antigen presenting cells (APCs) (Vyas et al., 2008). There are several types of APC including, macrophages, dendritic cells and B lymphocytes. These cells are capable of sampling the extracellular environment by endocytosis, and presenting the peptides derived on MHC class II (Vyas et al., 2008). Thus MHC class II usually presents self-peptides from the extracellular space, or from extracellular pathogens. MHC class II can only bind to the TCR on T lymphocytes that are CD4+ (Murphy et al., 2008).

The first of these processes required for T lymphocyte homeostasis is the selection of T lymphocytes in the thymus. This serves to develop a pool of T lymphocytes which do not respond to self-antigen but which can recognize foreign peptides bound to MHC. In this process, T lymphocytes that cannot engage MHC

efficiently with their TCR are removed by neglect (no growth signal). Cells whose TCR binds MHC class I with moderate affinity will acquire CD8 expression. Cells which engage MHC class II moderately will acquire CD4 expression and cells which engage MHC-self peptide complexes with high affinity *are negatively selected by apoptosis*. This process is absolutely essential for self-tolerance and the prevention of autoimmunity. Although Krammer and colleagues found that Fas ligand is not involved in selection of MHC class I binding T lymphocytes and thus the development of non self-reactive CD8+ T lymphocytes (Muller et al., 1995), a more global study of thymic apoptosis indicates that Fas-Fas ligand are important in thymic selection (Castro et al., 1996).

T lymphocyte homeostasis requires AICD to remove expanded effector populations. Proliferation, cytokine production and/or cytotoxic effector functions are consequent to TCR recognition of MHC-peptide when co-stimulatory signals are present. The presence of foreign peptides on MHC leads to the rapid expansion of T lymphocytes capable of recognizing the pathogen. However, most of these cells are eliminated after pathogen clearance and only a small pool remain for the maintenance of immunological memory. Thus activation and proliferation are usually followed by apoptosis, which is termed AICD. There is evidence that Fas/FasL is involved in both AICD and thymic selection which are necessary for the development and maintenance of self-tolerance in T lymphocytes (reviewed in (Murphy et al., 2008)).

1.1.5.2 Immunological privilege

In addition, sites of immune privilege are specific tissues that are less accessible to the adaptive immune response. In these sites, there is expression of antigens which cannot be expressed in the thymus (thus refractory to thymic selection) and/or the result of an active immunological response would be maladaptive (Niederkorn, 2006). These sites include the testes, portions of the nervous system including the eye, the placenta etc. It was found that sites of immune privilege, which constitutively express FasL, caused Fas induced apoptosis of invading T lymphocytes (Griffith et al., 1996). Thus a model was proposed where Fas engagement was responsible for the elimination of infiltrating self-reactive T lymphocytes and that sites of immune privilege, which express FasL, would cause apoptosis of invading Fas expressing immune effectors (Bellgrau et al., 1995). An interesting corollary to this model was that any tissue expressing FasL should be protected from immune destruction. This would have had far reaching implications for the treatment of autoimmune diseases and in transplantation. Although corneal grafts expressing FasL were not rejected by the host (Griffith et al., 1995; Griffith et al., 1996), other experiments involving over-expression of FasL in pancreatic islet cells in autoimmune diabetic mice were not successful (Kang et al., 1997). Thus the role of FasL in the induction and maintenance of immune privilege is still unclear.

1.1.6 Non-apoptotic roles of Fas-FasL signaling

In addition the functions described above, Fas also has clearly defined non-apoptotic roles. Most notably, Fas is responsible for neuroprotection in mouse models of Parkinson's disease (Landau et al., 2005), neurite outgrowth in dorsal root ganglia

(Desbarats et al., 2003), liver regeneration after partial hepatectomy (Desbarats and Newell, 2000), and T lymphocyte stimulation in certain contexts (Alderson et al., 1993; Kennedy et al., 1999). Recently, caspase cleavage has been found to promote differentiation, which is in contrast to its better-defined role in apoptosis reviewed in (Lamkanfi et al., 2007). Caspase inhibition and removal of the Fas DD does not abrogate either neuroprotection or neurite outgrowth, and ERK activation appears to be responsible for neurite outgrowth (Desbarats et al., 2003). This suggests that the intracellular tail of Fas signals not only via the DD but probably via other pathways which remain to be elucidated.

Similarly, FasL has been found to "reverse signal" in both CD4+ and CD8+ T lymphocytes presumably via a polyproline region and serine residues in its intracellular tail upon Fas engagement (Desbarats et al., 1998; Desbarats et al., 1999; Sun et al., 2006; Suzuki et al., 2000). In CD4+ cells, there is induction of cell cycle arrest via an unknown mechanism. In contrast, in cytotoxic CD8+ cells, the polyproline region binds several SH3-containing proteins including Fyn, the p85 subunit of PI3K, and Grb2, which leads to localization of FasL in lipid rafts and enhances T cell receptor (TCR) signals, while the serine residues are phosphorylated by casein kinase 1 and this leads to NF-kB and AP-1 activation (Sun et al., 2006). FasL reverse signaling is currently accepted as a major co-stimulatory signal for CD8+ T lymphocytes (Lettau et al., 2008; Sun and Fink, 2007).

1.1.7 Roles of Fas and Fas ligand in specific immune compartments

Finally, with the advent of floxed Fas and FasL alleles in mice, several groups have started to dissect the lymphoproliferative phenotype of these mice by deleting Fas or FasL in specific immune lineages. The results of these studies strongly suggest that the lymphoproliferative phenotype in Fas/FasL defective mice are not solely due to autonomous T lymphocyte defects and loss of immune privilege (Hao et al., 2008; Hao et al., 2004; Karray et al., 2004; Mabrouk et al., 2008; Stranges et al., 2007).

Although, the deletion of FasL in T lymphocytes leads to a loss of self-tolerance and expansion of CD3+B220+CD4-CD8- T lymphocytes, the deletion of FasL in B lymphocytes also promotes autoimmunity (Karray et al., 2004). Surprisingly, when Fas is deleted from T lymphocytes, the lpr phenotype is not recapitulated (Hao et al., 2004). However, when Fas is absent from monocytes/macrophages the autoimmune phenotype is present (Stranges et al., 2007). These findings indicate that the expression of FasL and Fas in APCs is also very important to the maintenance of self-tolerance.

Taken together, the discovery of non-apoptotic signaling by Fas, the reverse signaling by FasL, the clearly defined role for Fas and FasL outside of the T lymphocyte compartment indicate that the lymphoproliferation observed in the natural mouse mutants is not simply explained by a disturbance in autonomous T lymphocyte apoptosis. Although autonomous AICD in T lymphocytes may occur in specific contexts, there is now evidence that suggests this is not the most important role of Fas and FasL. There is a complex interaction between the different cell types of the immune system in

conditions where either Fas or FasL are absent (Hao et al., 2008; Hao et al., 2004; Karray et al., 2004; Mabrouk et al., 2008; Stranges et al., 2007). Presently, it seems likely that FasL expressed on T lymphocytes is important for the deletion of Fas expressing APCs. Furthermore, expression of FasL in CD8+ T cells allows for deletion of infected host cells by apoptosis (Bossi and Griffiths, 1999; Vignaux and Golstein, 1994). Conversely, Fas expression on APCs is important to co-stimulate CD8+ T cells and to allow for their deletion after T lymphocyte engagement. In addition, Fas expression on T lymphocytes is required for efficient activation of the CD4+ subset. Though, the immune phenotype has been the focus of investigation, there are evidently roles for Fas and FasL in other systems, such as the nervous system, which have not yet been adequately explored.

1.2 Biosynthetic regulation of Fas ligand

1.2.1 Regulation of Fas ligand expression and function

The expression of most cytokines is tightly regulated to ensure precise and appropriate responses to immunogenic stimuli. In particular, the members of the TNF superfamily tend to be transiently expressed upon stimulation. In the case of the pro-apoptotic family members such as TNFα, FasL and TRAIL, this transient and directed release allows for effector function without causing widespread non-specific apoptosis and tissue damage. Thus in consequence to these functions of FasL, there are multiple levels of biosynthetic regulation that allow for this directed expression in sites of immune privilege and at the immunological synapse.

1.2.2 Post-translational modifications

1.2.2.1 Glycosylation

FasL is glycosylated on 3 asparagine residues (Schneider et al., 1997). These residues are required for expression of Fas ligand protein and are all found in the extracellular portion of fas ligand: N184, N250 and N260. Mutation of any of these sites allows for active cell surface expression of FasL. In contrast, abolishing all 3 sites by PCR mutagenesis drastically reduces cell surface expression. All of the glycosylation mutants retain their ability to bind Fas (Orlinick et al., 1997). FasL expression is increased in the placenta during gestation. This increase in expression correlates with increased FasL glycosylation. In addition, in malignant ovarian epithelia, there is the

secretion of heavily glycosylated FasL that coincides with microvesicular release. This hints that context specific regulation of cell surface expression of Fas ligand by glycosylation may be important to its biological functions (Abrahams et al., 2003; Runic et al., 1996).

1.2.3 FasL expression on cell surface

1.2.3.1 Sorting

Once the FasL protein has been synthesized its trafficking to the cell membrane is tightly regulated. Currently, it appears that the sorting of FasL into a distinct endosomal compartment is regulated by protein partners that interact with the polyproline region. These ligands have been studied in a variety of cell types and surprisingly the results have been concordant. It appears that several proteins involved in microtubule nucleation called Fes/CIP4 homology, FCH proteins: FBP17 (Ghadimi et al., 2002; Qian et al., 2006), CIP4 (Qian et al., 2006), PACSIN2 (Ghadimi et al., 2002; Qian et al., 2006), and Rho GAP C1 (Qian et al., 2006) bind to the FasL intracellular tail via their SH3 domains to promote lysosomal localization. In contrast, another FCH protein, CD2BP binding to the cytoplasmic tail of FasL allows for intracellular localization (Baum et al., 2005). In addition, it has been shown that the mono-ubiquitination of FasL on lysines flanking the polyproline domain are required for sorting Fas ligand to the multivesicular bodies (Zuccato et al., 2007). And, the same group also found that tyrosine phosphorylation is similarly requisite for localisation of FasL to secretory lysosomes (see Figure 3).

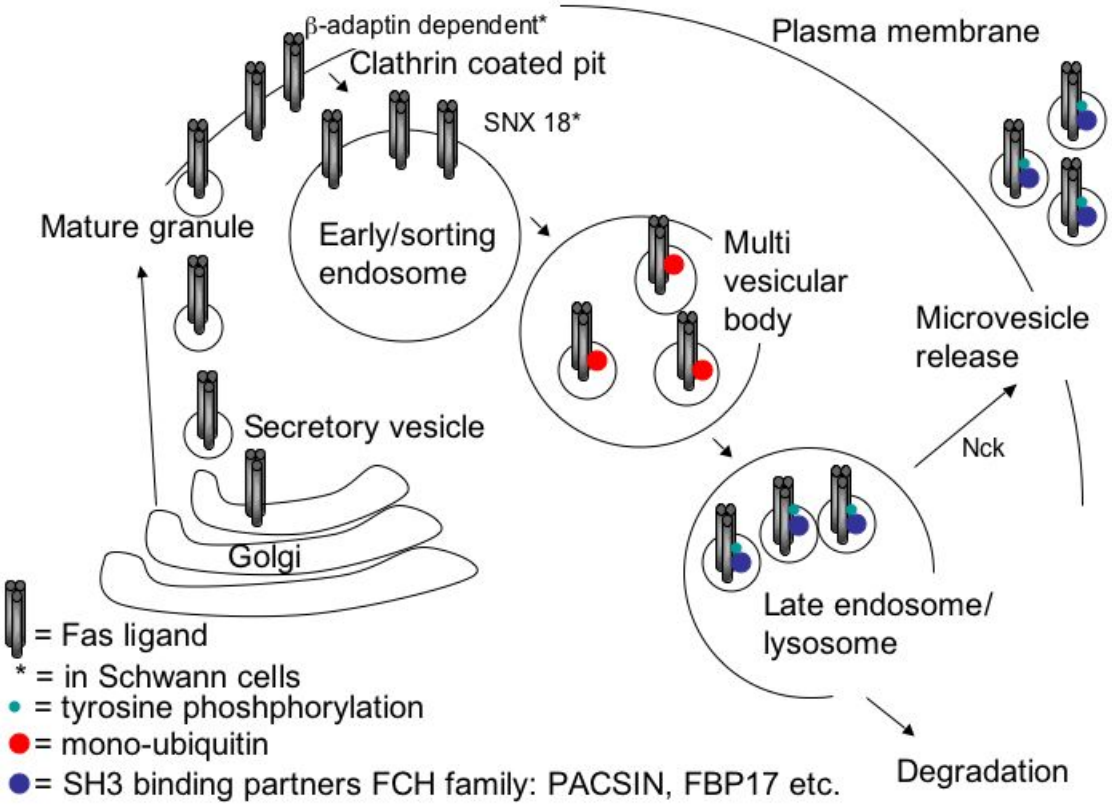
β-adaptin dependent*
Clathrin coated pit
Plasma membrane
SNX 18*
Mature granule
Early/sorting endosome
Multi vesicular body
Microvesicle release
Secretory vesicle
Nck
Golgi
Late endosome/ lysosome
= Fas ligand
* = in Schwann cells
= tyrosine phoshphorylation
= mono-ubiquitin
= SH3 binding partners FCH family: PACSIN, FBP17 etc.
Degradation

Figure 3: Representation of Fas ligand sorting.

Fas ligand matures through the secretory pathway. Upon cell surface expression it is rapidly re-internalized into endosomes. These vesicles can be stored for release upon cell stimulus, for example $CD8^+$ T lymphocytes, or it can be targeted for lysosomal degradation (Baum et al., 2005; Blott et al., 2001; Ghadimi et al., 2002; Lettau et al., 2006; Linkermann et al., 2009; Qian et al., 2006; Thornhill et al., 2007; Wenzel et al., 2001; Zuccato et al., 2007).

1.2.3.2 Lipid rafts

Vesicle fusion is also tightly controlled during T lymphocyte activation (Bossi and Griffiths, 1999). FasL containing vesicle fusion with the cell membrane occurs preferentially at lipid rafts during activation. Lipid rafts recruit many surface receptors and associated signaling molecules which allows for more efficient activation of transduction cascades (Kabouridis, 2006). Thus FasL reverse signaling capacity from lipid rafts is probably increased. In addition, the localization in lipid rafts is known to increase the pro-apoptotic activity of FasL (Cahuzac et al., 2006).

1.2.3.3 Cleavage

Once released on to the cell surface FasL is rapidly cleaved (Kayagaki et al., 1995) by metalloproteinases 3 and 7 (Powell et al., 1999; Vargo-Gogola et al., 2002) and also by ADAM-10 (Kirkin et al., 2007). There are 3 MMP cleavage sites on the ECD of FasL (Vargo-Gogola et al., 2002). This releases a soluble form of FasL similar to the soluble splice product. Since soluble FasL less effectively cross-links Fas, it does not strongly induce apoptosis and may in fact block apoptosis by inhibiting interactions between Fas and membrane bound FasL (Tanaka et al., 1998). The intracellular cleavage of FasL has also been documented and allows for the uncoupling of reverse and Fas signaling. In addition, there are reports that the intracellular cleavage product of ADAM 10 can be processed by signal peptide peptidase, SPP2a, translocate to the nucleus and act as a negative regulator of transcription (Kirkin et al., 2007).

1.2.4 Control at the gene expression levels

In higher eukaryotes, the regulation of gene expression can be achieved at many different levels such as transcription, RNA modification (including splicing), RNA transport, RNA stability control, translation efficiency and post-translational events. It is well established that the genes encoding the TNF superfamily of cytokines are regulated not only at the transcription level but are also are modulated posttranscriptionally (Abdelmohsen et al., 2008; Barreau et al., 2005; Jacobson and Peltz, 1996; Katsanou et al., 2006; Katsanou et al., 2009; Katsanou et al., 2005). Unlike transcriptional regulation, the posttranscriptional mechanisms and the factors as well as the players involved in Fas and FasL expression are still elusive.

1.2.4.1 Transcription

FasL expression has been studied in detail in T lymphocytes. The expression of FasL is intricately controlled at the level of transcription, splicing, translation and post-translation. FasL is known to be induced by TCR, lectin, ionophore or mitogen stimulation and its mRNA expression has been shown by several groups to peak 4 hours after activation (Li-Weber and Krammer, 2002).

Usually transcriptional efficiency correlates with the accessibility of the genomic region encoding the gene to the transcriptional machinery. Thus DNA methylation and histone deacetylation reduce chromatin accessibility, and indicate transcriptional repression (Cedar and Bergman, 2009). Prior to stimulation, T lymphocytes have heterochromatic structure around the FasL locus. Transcription is repressed since there is

heavy CpG methylation, histone deacetylation and resistance to DNaseI digestion (Castellano et al., 2006). Subsequent to stimulation, the levels of CpG methylation and histone acetylation do not change, however, the level of DNaseI sensitivity increases indicating that the accessibility to transcription is increased (Castellano et al., 2006). The region remains DNaseI sensitive up to 24 hours post-stimulus and indicates a novel mechanism of chromatin regulation (Castellano et al., 2006).

In the periphery, the physiological stimulus for T cells is the TCR, which normally engages foreign peptides presented on major histocompatibility complex (MHC) (Vyas et al., 2008). In addition, T lymphocytes only become activated if they concurrently receive signals from the TCR, CD4 or CD8, and CD28 (Sharpe, 2009). A complex signaling cascade ensues which activates calcineurin, Ras-MEK/ERK, PKCθ and PI3K-Akt pathways (see Figure 4). The key transcription factors activated during TCR stimulation are nuclear factor of activated T cells (NF-AT), AP-1 and NF-kB (Villalba et al., 1999). The FasL promoter contains 3 sites for NF-AT and Egr3 at –120, -180 and –680 from the cap site. These sites are required for FasL mRNA expression and are occupied up to 16 hours after activation (Li-Weber et al., 1999). In addition, there are, 2 NF-kB sites at –50 and –530 that promote FasL expression (Hsu et al., 1999a), 3 IRF-1 sites at +65, -65 and –120 (Chow et al., 2000; Kirchhoff et al., 2002) which are essential for FasL expression. AP-1 driven transcription of FasL is also important for AICD (Baumann et al., 2003). Most cell types can upregulate FasL upon stress such as hypoxic or genotoxic insult, and this expression is usually driven by NF-kB and AP-1 (Eichhorst et al., 2000; Kasibhatla et al., 1998). There are other pathways and therefore

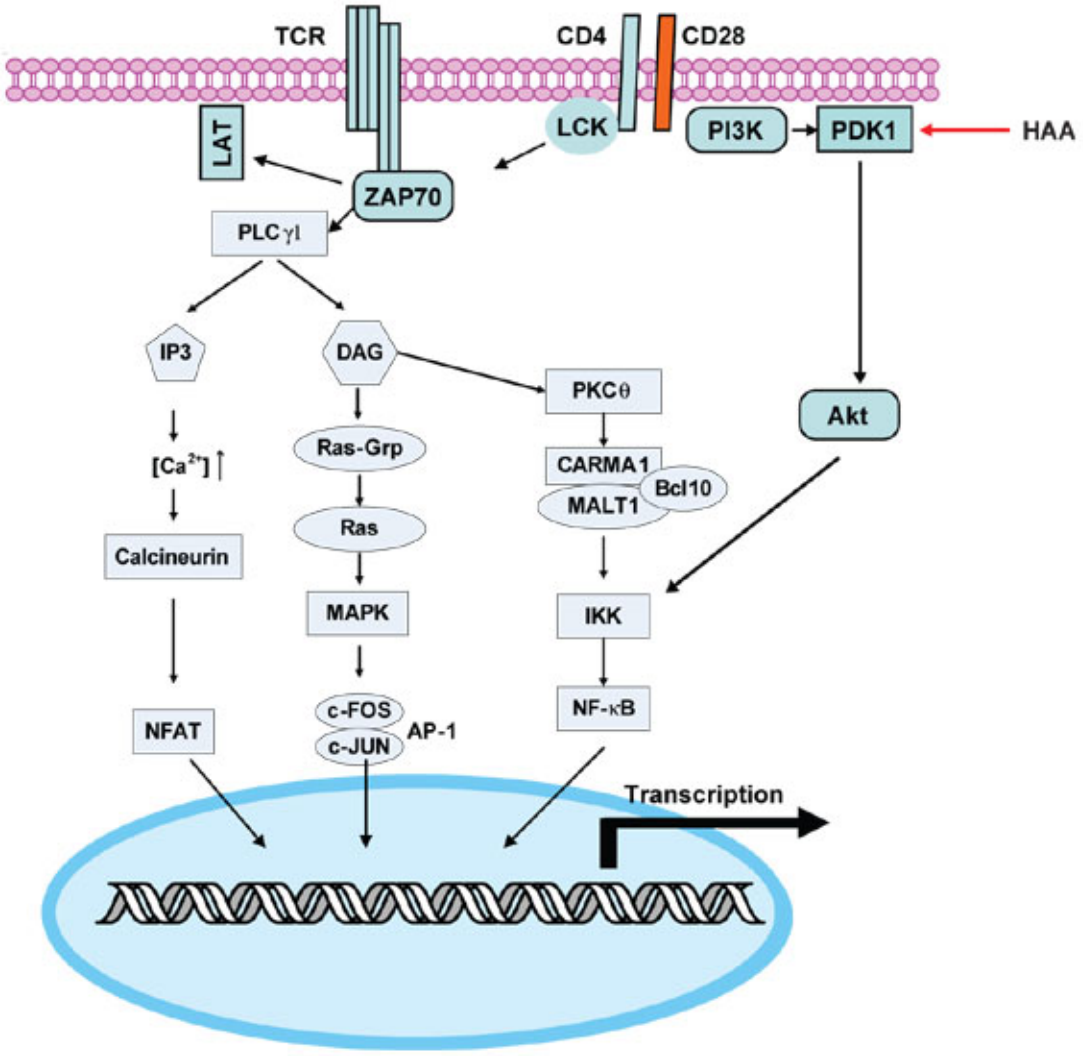
TCR
CD4
CD28
LAT
LCK
PI3K
PDK1
HAA
ZAP70
PLCγl
IP3
DAG
PKCθ
Akt
[Ca2+]↑
Ras-Grp
CARMA1
Bcl10
MALT1
Calcineurin
Ras
MAPK
IKK
NFAT
c-FOS
c-JUN
AP-1
NF-κB
Transcription

Figure 4: Schematic representation of major TCR signaling pathways.

Key pathways for the transcription of Fas ligand are the activation of calcineurin by calcium flux, which causes NFAT activation, PKCθ activation that activates NF-kB, and diacyl glycerol that activates AP-1 (Hayashi et al., 2007).

transcription factors which modulate FasL transcription, notably the p38-JNK pathway (Hsu et al., 1999b; Zhang et al., 2000).

At first, many of the studies delineating the transcriptional regulation of FasL revolved around nuclear receptors. There is a thymus specific version of retinoid related orphan receptor, RORγ which is expressed in CD4+CD8+ thymocytes and is capable of abrogating AICD in a T cell line (He et al., 1998). It also appeared that retinoic acid receptor, RAR and retinoid x receptor, RXR activation with 9-cis retinoic acid decreased AICD (Bissonnette et al., 1995; Yang et al., 1995). More specifically, in the presence of active RARα, FasL is repressed, and apoptosis is inhibited. Conversely, in the presence of active RARγ, transcription of FasL is activated and AICD is enhanced. When both RARα and γ are active, RARα and the apoptosis inhibitory effect outcompete the effects of RARγ (Toth et al., 2004; Toth et al., 2001). Interestingly, Szondy and colleagues also noticed that Nur77, another nuclear receptor, was upregulated by active RARγ. Nur77 was already implicated in AICD (Woronicz et al., 1995). Expression of dominant negative versions of Nur77 caused defects in AICD. However, the most highly expressed Nur77 family member in activated T cells is Nor-1 and Nor-1 mutations do not interact with the gld (FasL natural mutant) allele (Cheng et al., 1997). Thus the role of retinoids in the expression of FasL remains elusive.

In addition to the retinoids, there is evidence that the glucocorticoids are also capable of regulating FasL expression. There is a glucocorticoid receptor (GR) binding site at –980 in the FasL promoter that represses FasL transcription and AICD (Baumann

et al., 2005). Heinzel and colleagues have found that NF-kB competes for binding at a site overlapping the –980 GR binding site, thus the GR reduces FasL transcription by preventing NF-κB mediated transcriptional activation (Novac et al., 2006).

Interestingly, the transcriptional control of FasL differs in sites of immune privilege. This correlates with the constitutive expression of FasL at these sites in opposition to the inducible expression in T lymphocytes and during stress. Tight junctions between Sertoli cells allow for immunological privilege in the testes though they also provide trophic support for gametogenesis. In Sertoli cells, transcription is constitutively driven by a site –299 from the cap site which binds Sp1 (McClure et al., 1999). Sp1 is a zinc finger transcription factor which has the distinctive ability to promote the transcription of housekeeping genes in a TATA-box independent manner due to its interactions with components of the transcriptional pre-initiation complex (Tan and Khachigian, 2009). In the cornea and the anterior chamber of the eye, the same site at –299 is required for FasL expression, however it is bound by an unknown transcription factor other than Sp1 (Zhang et al., 1999a). At present, it appears that the occupation of this site allows for constitutive expression of FasL.

1.3 Posttranscriptional regulation by AU rich elements and their binding partners.

1.3.1 Post-transcriptional regulation

The expression of the TNF superfamily of cytokines is also regulated post-transcriptionally involving cis and trans-acting factors that collaborate to modulate the turnover, the cellular distribution and/or translation of messages encoding many members of this family. In general, a direct association between the *cis*-element located within the 3' untranslated region (3'UTR) of these mRNAs and the *trans*-acting factors is required to affect the fate of these mRNAs. Therefore, understanding this level of regulation is crucial to define how and whether a given message is expressed in response to extracellular stimuli. In the following section I will describe these *cis*- and *trans*-acting factors and highlight their importance and functional relevance on mRNA expression.

1.3.1.1 mRNA stability and mRNA decay mechanisms

The intrinsic stability and half-life of a target mRNA is dictated by both *cis*-factors (specific RNA sequence) and *trans*-factors (RNA-binding proteins and micro-RNAs) (Bhattacharyya et al., 2006; Brennan and Steitz, 2001; Wilusz and Wilusz, 2004). The decay of mRNAs is most often initiated by the shortening of the poly(A) tail, followed by degradation of the transcript achieved in a 3'-5' manner by the exosome, a large enzymatic complex of six proteins. Alternatively, the cap structure of the RNA can be removed and the nucleic acid degraded from the 5' end towards the 3'-terminus.

Apart from these two principal decay mechanisms, more unusual forms of decay exist, which degrade specific targets. Among them, the deadenylation-independant decapping, which has been described only in *S. cerevisiae,* as well as the endoribonucleolytic decay, a mechanism where there is cleavage of the transcript while still located on the polysomes (Schneider et al., 2001; Vasudevan and Peltz, 2003; Vasudevan et al., 2002). This cleavage is achieved within the coding region of the RNA to generate two parts that can be further degraded by one of the mechanisms presented above. Finally, other forms of decay exist, which are mainly associated with surveillance mechanism and degradation of faulty transcripts. Nonsense mediated decay (NMD) recognizes mRNAs with premature stop codons and selectively degrades them. In mammalian cells, this recognition is often achieved by the presence of an exon junction complex that has not been removed and a premature termination codon located 50-to-55 nucleotide upstream the stop codon (Conti and Izaurralde, 2005; Maderazo et al., 2003; Mitchell and Tollervey, 2003; Shyu et al., 2008; Singh et al., 2007; Wilkinson, 2005; Wilkinson and Shyu, 2002). This mechanism is also used for abnormal mRNAs such as those containing a 3'-UTR in their introns, frameshifts or any other irregularities mimicking a premature termination codon. The category of surveillance mRNA decay mechanisms also encompasses the less well-defined non-stop decay which targets transcripts lacking a stop codon as well as the no-go decay for mRNAs that cannot be translated (Garneau et al., 2007).

1.3.1.2 AU-rich elements (AREs) and their effect on gene expression

One of the main cis-factors regulating mRNA stability in eukaryotes is found in the 3'UTR of the transcript. This element is typically an RNA sequence rich in adenosine and uridine and called the AU-rich elements or AREs (Figure 5). Both of the main decay pathways discussed above are involved in the degradation of ARE-containing messages (Garneau et al., 2008).

The first description of AREs came from the analysis of a 51 nucleotides sequence in the 3'UTR of granulocyte-macrophage-colony-stimulating factor (GM-CSF). Transfer of this sequence to the 3'-UTR of any stable mRNA caused an increase in the degradation rate (Shaw and Kamen, 1986). AREs are often found in short-lived mRNAs such as those coding for differentiation factors, cytokines, cell-cycle regulators and growth factors (Barreau et al., 2005). The presence of one or many AREs in a message does not directly destabilize the RNA but rather creates a binding site for many RNA-binding proteins (RBPs) involved in targeting the mRNA for decay. While the consensus sequence for AREs is subject to many divergent opinions, it appears that the presence of the pentamer AUUUA located in a U-rich region would be largely responsible for AU-rich elements mediated decay (AMD) (Abdelmohsen et al., 2008; Barreau et al., 2005; Ma et al., 1996; von Roretz and Gallouzi, 2008). However, different studies have also shown that the presence of such a sequence does not necessarily confer instability to its message and conversely, that some destabilizing AREs do not contain the pentamer AUUUA (Chen et al., 1994; Chen and Shyu, 1994; Ma et al., 1996).

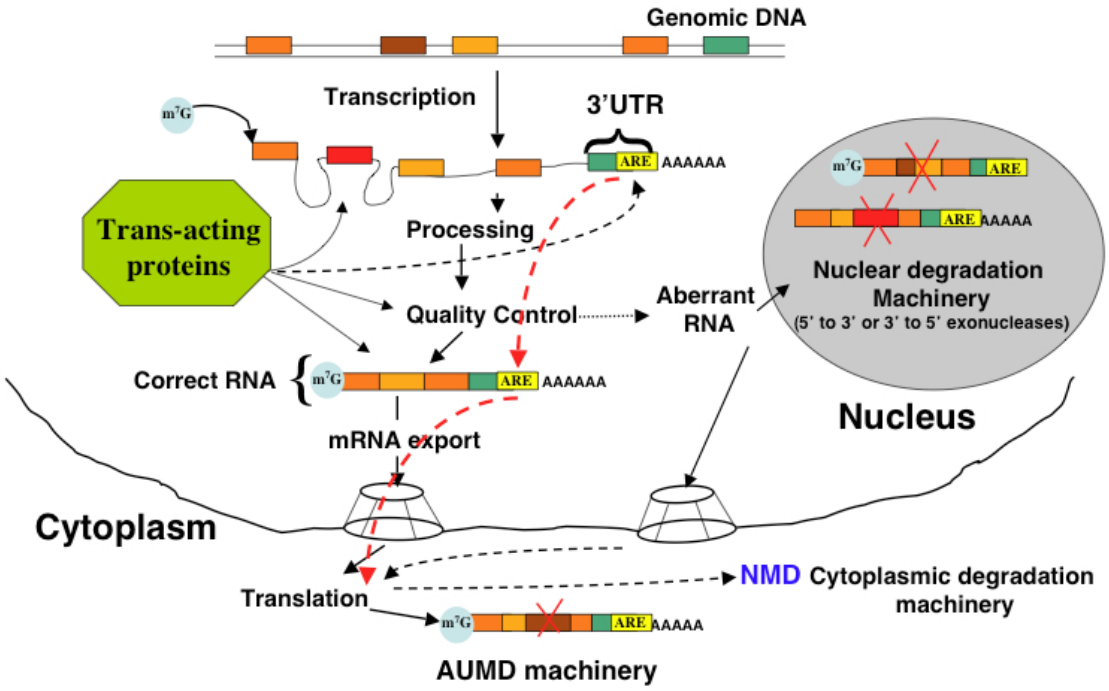
Genomic DNA
Transcription
3'UTR
m7G
ARE
AAAAAA
Trans-acting proteins
Processing
Quality Control
Aberrant RNA
Nuclear degradation Machinery
(5' to 3' or 3' to 5' exonucleases)
Correct RNA
Nucleus
mRNA export
Cytoplasm
Translation
NMD
Cytoplasmic degradation machinery
AUMD machinery

Figure 5: The processing and the translation of Pol II messages involve the quality control machinery.

To ensure the translation of a correct protein, the processing of Pol II transcripts implicates several steps, capping, splicing, polyadenylation, nuclear transport and mRNA turnover. The nuclear RNA degradation machinery consists largely of exonucleases (that are not represented here). The aberrant messenger mRNA will be degraded either in the nucleus or in the cytoplasm after export. After scanning by the quality control machinery, the mRNA is exported to the cytoplasm for translation. The location of ARE-containing mRNA degradation is not fully understood, however some evidence suggests that it could occur in both the nucleus and/or in the cytoplasm. For example, mRNAs of early response genes (ERGs) that encode key growth-regulatory proteins (e.g. cytokines, lymphokines, and protooncogenes) are expressed immediately upon cell stimulation, however they are eliminated shortly afterwards. Thus, although regulation of ERG expression occurs at the transcriptional level, the rapid nuclear export and translation of these mRNAs, as well as their rapid degradation, are prominent mechanisms in the expression of their genes. These processes are tightly regulated and highly coordinated to ensure proper cell growth and differentiation.

1.3.1.3 Classification of the ARE-containing messages

The ARE-containing messages were originally classified based on the mechanisms of degradation and their nucleotide structure (Chen and Shyu, 1995). Here we will describe the existing classifications and discuss them based on the new observations implicating the AREs-containing 3'UTR in other cellular functions.

By looking to the databases, we realize that many mRNA, which contain an AU-rich element in their 3'UTR are either stable or do not play a function in cell cycle or development. These observations are suggestive of a role for AREs other than decay. For example, the regulation of the nuclear export of some mRNAs might be an explanation for the presence of an ARE (Barreau et al., 2005; Gallouzi and Steitz, 2001; Veyrune et al., 1996). These sequences were also shown to regulate the deadenylation rate of some mRNAs in *Xenopus* oocytes (Voeltz et al., 2001; Voeltz and Steitz, 1998). This raises the question, how can we classify ARE-containing messages? What is the simple way to identify them?

In 1995 Chen and Shyu categorized the ARE-containing mRNAs in three groups (Chen and Shyu, 1995). This classification is widely accepted and was based on sequence features, deadenylation and degradation kinetics of AU_3A-conatining AREs (Chen and Shyu, 1995; Shyu et al., 1991; Xu et al., 1997). Class I AREs, such as the c-*fos,* c-*myc*, cyclin A, cyclin B1, cyclin D1 and $p21^{waf1}$ AREs, contain one to three copies of the

pentanucleotide AUUUA within U-rich regions. Class II AREs, like the GM-CSF, IL-3, TNFα (Tumor Necrosis Factor-α) AREs, also exist in a U-rich background and consist of at least two overlapping copies of a critical nonamer UUAUUUA(U/A)(U/A) (Lagnado et al., 1994) and direct asynchronous deadenylation. This suggests a processive nucleolytic digestion of the poly A^+ tail followed by mRNA decay (Chen et al., 1995a; Lagnado et al., 1994; Xu et al., 1997). Class III, for example the c-*jun*, Neurofilamin, β-adrenergic receptor and VEGF (Vascular endothelial growth) AREs, contain a U-rich sequence lacking the typical AUUUA element, but nevertheless signal mRNA degradation. In this classification we note that the pentanucleotide AUUUA is not required in all cases to have an ARE functional in rapid mRNA decay. Conversely, it was observed that not all mRNAs that contain AU-rich sequences regardless of the AU_3A repeat undergo degradation (Bakheet et al., 2001). For example, the 3'UTR of MDR1 (human Multidrug resistance gene which encodes the P-glycoprotein) mRNA is very AU-rich (70%) presenting similar sequence features of c-*myc* and c-*fos* 3'UTR, yet this mRNA presents a half-life of 8 h (Prokipcak et al., 1999). The fact that the presence of the pentanucleotide AUUUA and/or the nonamer UUAUUUA(U/A)(U/A) sequences in an ARE does not guarantee the rapid degradation of the message (Chen and Shyu, 1994), suggests that the functional relevance of these sequences goes beyond their involvement in the degradation pathway. On the other hand, proteins that are known to specifically interact with the AREs *in vitro*, can be associated with very stable messages. Using cDNA microarrays combined with immunoprecipitation experiments, the Keene group observed that some proteins such as HuR (HuA) and HuB (HelN1), members of the ELAV-family (embryonic lethal, abnormal vision (Campos et al., 1985; Robinow et al.,

1988)) of RNA-binding proteins known to interact specifically with AU_3A sequences in vitro (Myer et al., 1997), are associated with very stable mRNAs such as β-actin and YB1 (Tenenbaum et al., 2000).

A classification of the AREs, preformed by Bakheet et al., 2001, allowed the inclusion of many messages based solely on the number of AU_3A repeats in the 3'UTR, without necessarily belonging to the short-lived mRNA family (Bakheet et al., 2001). They applied a bioinformatic approach to define the full repertoire of ARE-containing mRNAs (Bakheet et al., 2001). They used a computational derivation of a 13-bp ARE pattern combined with multiple expectation maximization for motif elicitation (MEME) and consensus analysis. Interestingly, they found that 8% of the human mRNA sequences analyzed belong to the ARE-mRNA database (ARED). Based on the total genes predicted in the human genome (35000-120 000) (Aparicio, 2000), they suggested that the number of ARE-containing genes is between 2800-9600 (Bakheet et al., 2001). They categorized the ARE-mRNAs in five groups containing respectively, five, four, three and two AUUUA repeats while the last group contains only one pentamer within the 13-bp ARE pattern. The list of ARE-mRNA defined by this method, contain the majority of the messages previously described as short-lived. Of note, the ARED database defined FasL mRNA as a novel ARE-containing message. However it also presents many new members (Bakheet et al., 2001), some of which do not have a short half-life, which is consistent with the hypothesis that AUUUA sequences may play an important role in other processes. These observations indicate that much more effort and experiments are needed to determine the subtleties of this cis-element in mRNA metabolism.

1.3.1.4 AREs and mRNA export

The majority of messenger RNAs contain elements that are required either for targeting the message for export from the nucleus, or for targeting it for degradation. However, analysis of these components reveals the existence of some elements, such as the AU_3A sequence, that are involved in both export and turnover, suggesting a link between these two processes (Barreau et al., 2005; Doller et al., 2008b; Gallouzi et al., 2001; Veyrune et al., 1996; Veyrune et al., 1997). Indeed, it was observed that the 3'UTRs of many mRNAs direct their movement from the nucleus to the cytoplasm. For example, the β-actin 3'UTR can direct a reporter sequence to the cell periphery of fibroblasts. Two separate 54 nt and 43 nt regions of the β-actin 3'UTR are required for this cytoplasmic relocalization (Kislauskis et al., 1993; Kislauskis et al., 1994). The mRNA localization in neurons of the microtubule-associated protein tau, which is connected with neurofibrillary tangle formation in Alzheimer's disease, is dependent on its 3'UTR (Behar et al., 1995; Sadot et al., 1994). The sequences responsible for the movement of these mRNAs contain AU-rich elements. Furthermore, Veyrune and his colleagues showed that the AUUUA pentanucleotide directs the c-*myc* mRNA to the cytoplasm (Veyrune et al., 1996). The mechanism by which these elements regulate mRNA export is still unclear. The involvement of RNA-binding proteins such as HuR in the nuclear export of some ARE-containing messages (Barreau et al., 2005; Doller et al., 2008b; Gallouzi et al., 2001; Gallouzi and Steitz, 2001) suggests that the AREs could serve as an anchor to other adaptor proteins that would regulate the cellular movements of their host messages. The fact that HuR is also known to stabilize and modulate the

translation of ARE-containing mRNA targets (Abdelmohsen et al., 2008; Abdelmohsen et al., 2007; von Roretz and Gallouzi, 2008), indicates that coordination between all these processes must exist to allow the expression of ARE-containing messages at the right timing.

The tight link between mRNA stabilization and export could be an indication that mRNAs define their final destination before they exit the nucleus. RNA-binding proteins such as HuR could ensure the integrity of its associated mRNAs while guiding them to the translation machinery (Barreau et al., 2005; Doller et al., 2008b). It has been established that AREs could play a role in regulating the movement of some messages from the cell body of neurons to the synapses, allowing their translation locally in response to an external signal (Alvarez et al., 2000; Bassell et al., 1998; Brittis et al., 2002; Eng et al., 1999; Koenig et al., 2000). Work from the Flanagan laboratory demonstrated that axons do not receive all of their proteins by anterograde transport from the cell body while growing toward their target neurons (Tanaka and Sabry, 1995). Indeed, they observed that axons translate some of the proteins required for growth and guidance locally, within the synaptic areas (Alvarez et al., 2000; Bassell et al., 1998; Brittis et al., 2002; Eng et al., 1999; Koenig et al., 2000). For example, when an axon reaches the spinal cord midline, the Ephrin receptor (Eph) is expressed in the distal segment and exported to the cell surface. The Eph family of receptor tyrosine kinases (RTKs), and their ephrin ligands such as EphA2, are important in cell segregation and axon targeting during the development of the nervous system (Flanagan and Vanderhaeghen, 1998; Hattori et al., 2000). EphA2 upregulation is controlled by a 67 nt

sequence in the 3'UTR of its mRNA. This sequence is highly conserved between mouse and human EphA2 mRNA, and contains two AUUUA sequences (Brittis et al., 2002). The sequence responsible for this translational control is the cytoplasmic polyadenylation element (CPE) (UUUUAAU), which is located a short distance upstream of the AAUAAA polyadenylation signal. When the CPE is mutated to GGCGGAG, the translation of a reporter cDNA (GFP), fused to an altered EphA2 3'UTR, is abolished in the distal axon segment, but not the cell body (Brittis et al., 2002). This local translation, far away from the cell body (the distance can be greater than one meter) has been explained by the active export of the mRNA along the axon to the synapses. Although the RNA-binding proteins involved in this movement are not known, it is likely that some shuttling proteins that interact specifically with AUUUA and the AU-rich sequences, such as HuR (Fan and Steitz, 1998a), can be involved in this process (Brennan and Steitz, 2001; Perrone-Bizzozero and Bolognani, 2002; Quattrone et al., 2001). Identifying these factors and their regulation mechanisms will help to understand neuronal flexibility and plasticity. Additionally, work from several laboratories suggests that HuR could be implicated in the stabilization and the nuclear export of Tumor necrosis factor alpha (TNFα) mRNA in response to LPS treatment (Dean et al., 2001; Di Marco et al., 2001; Dumitru et al., 2000; Jacob et al., 1996; Kontoyiannis et al., 1999). The TNFα mRNA contains AREs in its 3'UTR, with a repeat of AU_3A element, which is responsible for the interaction with HuR (Dean et al., 2001; Di Marco et al., 2001; Dumitru et al., 2000; Jacob et al., 1996; Kontoyiannis et al., 1999). TNFα association with HuR could explain how message exit the nucleus to reach rapidly the translation machinery in response to LPS.

These observations suggest that the movement of these mRNAs from the nucleus to the cytoplasm plays a crucial role in their expression in response to external stimuli. The link between the ARE mediated decay (AMD) process and ARE-dependent cellular localization remains unclear. The only explanation, so far, comes from the involvement of some RNA binding proteins such as HuR in both the stabilization and export of ARE-containing mRNAs (Barreau et al., 2005; Fan and Steitz, 1998a, b; Gallouzi and Steitz, 2001; Peng et al., 1998).

The vast majority of the AU binding proteins are either shuttling proteins and/or can regulate their localization by interactions with other proteins (Diaz-Moreno et al., 2009; Fan and Steitz, 1998a; Pinol-Roma and Dreyfuss, 1992; Taylor et al., 1996; Zhang et al., 2005; Zhang et al., 1993). Changes in localisation of AU binding proteins is associated with post-translational modifications due to extracellular signaling events, such as nuclear to cytoplasmic movement of HuR after phosphorylation by PKC (Doller et al., 2008a). Shuttling of HuR can also be affected by certain drugs, such as thalidomide (Jin et al., 2007).

Many of the post-translational modifications to AU binding proteins affect not only localization but also binding affinity for the ARE containing mRNA species. Although, many studies may confound localization and affinity of AU binding proteins, there are still clear indications that AU binding proteins can relocalize ARE mRNA from the nucleus to the cytoplasm (Doller et al., 2008a). In addition, some of the AU binding proteins such as TIA1 and TIAR can inhibit translation by recruiting bound ARE

containing mRNA to cytoplasmic foci known as stress granules (SGs) (Kedersha et al., 1999), while other ARE containing messages can be targeted to cytoplasmic foci containing exosomes (distinct from stress granules and processing bodies) (Lin et al., 2007). This indicates that AU-binding proteins can direct ARE bearing mRNAs to discrete cytoplasmic compartments.

1.3.1.5 AREs and mRNA translation

Although AREs were initially linked to mRNA stability, it has become evident that AREs allow for translational regulation as well. Several *trans*-acting factors that bind AREs, exert their post-transcriptional effects by interfering with translation. For example, TIA-1 (Piecyk et al., 2000) and TIAR (Gueydan et al., 1999) are ARE binding proteins that cause translational silencing of TNFα mRNA. It has also been shown that ARE containing transcripts are directed to translationally silent SGs via TIA-1 and TIAR (Kedersha et al., 1999). HuR, another ARE binding protein has been reported to increase translation in the case of p53 mRNA (Galban et al., 2003; Mazan-Mamczarz et al., 2003) as well as in the case of the mRNA encoding the apoptotic inhibitor protein, prothymosin α (Lal et al., 2005). Paradoxically, HuR can also decrease translation of some ARE mRNAs such as c-myc, TNFα and COX2 (Katsanou et al., 2005). In the case of p27, translation is repressed by HuR since its binding impedes access of the ribosome to the IRES in the 5'UTR (Kullmann et al., 2002). In addition, translational repression of c-myc by HuR has been recently shown to be mediated by the recruitment of miRNAs let-7b/c to an adjacent site in the 3'UTR (Kim et al., 2009). It remains to be seen which of these mechanisms predominate in different cellular contexts.

1.3.2 The role of the ARE-binding proteins (AUBPs) in mRNA turnover

As mentioned above, AREs need to associate with protein in *trans* to be able to target their messenger RNA for decay (Anderson et al., 2006). These proteins are commonly known as AU-rich Binding Proteins (AUBPs). Some of them act by helping recruiting decay machinery such as the exosome to the mRNA, while others can target the message to specific cytoplasmic decay foci, processing bodies (PBs) (Abdelmohsen et al., 2008; Abdelmohsen et al., 2007; Barreau et al., 2005; Kedersha et al., 2005; Laroia et al., 2002; Mukherjee et al., 2002; von Roretz and Gallouzi, 2008). Among these AUBPs we find AU-rich binding factor-1 (AUF1), KH splicing regulatory protein (KSRP), RNA helicase associated with AU-rich elements (RHAU), heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) and tristetraprolin (TTP) (Garneau et al., 2007; Gherzi et al., 2004; Hamilton et al., 1997; Malter, 1989).

If AREs associate with proteins that target these messages for decay, other AUBPs associate with the same region of the RNA, but exert an opposing effect, stabilizing the ARE-containing mRNA. While the precise mechanism mediating this stabilizing function is not clear, their action is thought to be mediated either by the relocalization of mRNAs away from the site of decay or by competing with destabilizing factors for the same binding site. Either way, these actions would lead to the same effect, i.e. a longer half-life for ARE-containing mRNAs. The RNA-binding protein HuR is one of the main AUBP proteins stabilizing mRNAs and competes for binding to AREs with three of the destabilizing factors mentioned above, namely AUF1, KSRP and TTP (Lal et

al., 2004; Linker et al., 2005). Thus, in a situation where binding of HuR is favoured, the target mRNA is stabilized. In addition, HuR has been described as a key player in the transfer of some of its target transcripts from PBs (a site of mRNA decay) to polysomes (a site of translation) (Bhattacharyya et al., 2006).

1.3.3 Trans-acting factors which destabilize ARE mRNAs

Since AU rich elements were first characterized as mRNA destabilizing elements, it is not surprising that many *trans* factors can bind ARE-containing mRNAs and promote decay (Figure 6). Although many factors promote AU-rich Mediated mRNA Decay (AMD), they all impinge on the same pathways (Abdelmohsen et al., 2008; Abdelmohsen et al., 2007; Barreau et al., 2005; von Roretz and Gallouzi, 2008). There are 3 main pathways which cause mRNA decay: The first is the removal of the 7meG CAP by Dcp1a/Dcp2 followed by 5'-3' exonucleolyic processing by Xrn1 and Lsm1 (Lin et al., 2007). The second is deadenylation of the 3' polyA tail by PARN (although PAN2 and CCR4 are possibly involved) followed by 3'-5' exonucleolysis by the Rrp4, Rrp41, Rrp46 and Csl4 exosomal components (Lin et al., 2007). A third pathway involving targeting of AU rich messages for degradation by miRNA and TTP has also been shown to act on TNFα mRNA. This may engage the previous 2 pathways or may allow for endonucleolytic degradation via the RISC (Jing et al., 2005). Please refer to table 1 for AU binding proteins that modulate ARE bearing messages.

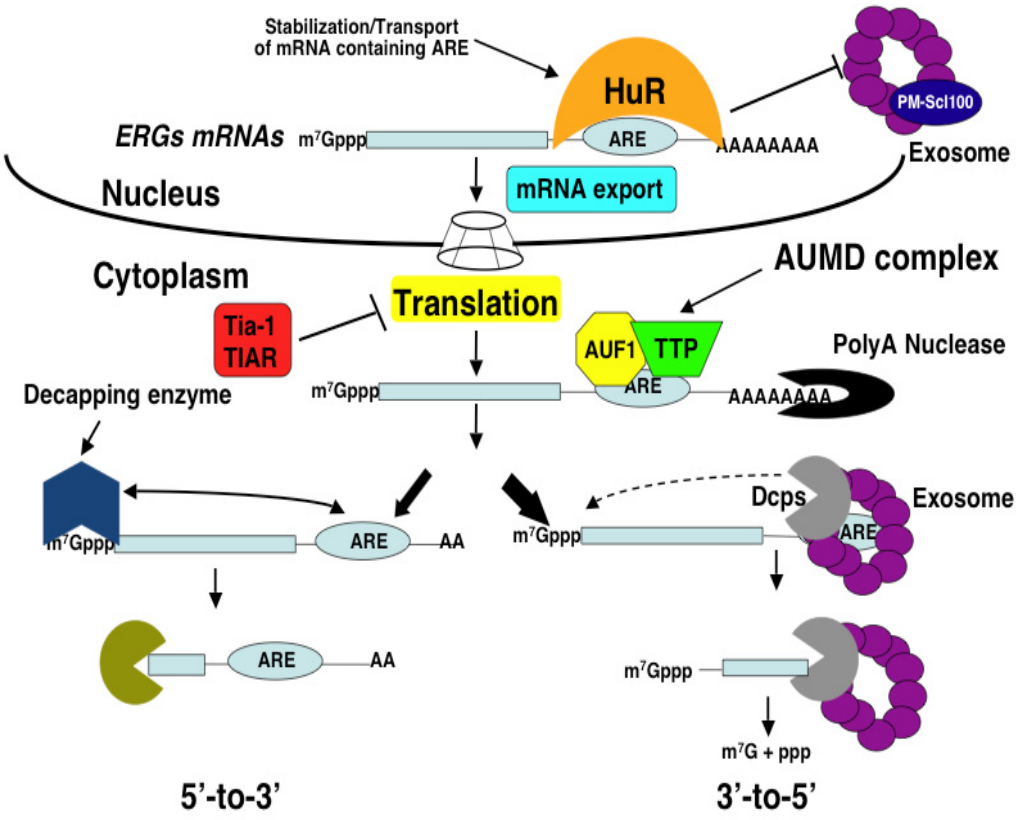
Stabilization/Transport
of mRNA containing ARE
HuR
PM-Scl100
ERGs mRNAs
m7Gppp
ARE
AAAAAAAA
Exosome
Nucleus
mRNA export
Cytoplasm
AUMD complex
Translation
Tia-1
TIAR
AUF1
TTP
PolyA Nuclease
Decapping enzyme
m7Gppp
ARE
AAAAAAAA
Dcps
Exosome
m7Gppp
ARE
AA
ARE
AA
m7Gppp
m7G + ppp
5'-to-3'
3'-to-5'

Figure 6: AUMD pathway prevents the over expression of early response genes (ERGs).

The mRNA expression of early response genes occurs immediately after cells are activated with extracellular stimuli. Upon processing of the pre-mRNA, transacting factors, such as HuR, bind to the AU rich elements (ARE) in the 3'UTR of these messages and mediate the transport of the mRNA from the nucleus to the cytoplasm. The binding of these factors to the ARE is thought to stabilize these messages likely by inhibiting the recruitment of the exosome. These mRNAs are then transported to polysomes where they are translated into proteins. Under certain cellular conditions, the translation of these messages can be repressed by the binding of the TIA-1/TIAR proteins to the ARE. Once these messages are translated and the stabilization/export factors dissociate from the mRNA, the AUMD complex binds to the ARE and target the mRNA for degradation. The major degradative pathway involves recruitment of the exosome to the mRNA via the AUMD complex after deadenylation of the poly A^+ tail by Poly (A) Nuclease. The mRNA is then subsequently decapped by DcpS and degraded 3' to 5' by exonucleases. Alternatively, the mRNA once decapped could also be degraded by exonucleases in a 5' to 3' direction.

AUBPs	Localization	Target *cis*-element	Function(s) on target messages
HuR	Nucleocytoplasm	ARE and U-rich-sequences	Stabilization; affects localization; translation regulator
KSRP	Nucleocytoplasm	GU-rich elements	Promotes mRNA decay
AUF1	Nucleocytoplasm	ARE and U-rich-sequences	Increases or decreases stability
TTP	Nucleocytoplasm	ARE and U-rich-sequences	Promotes decay
TIA-1	Nucleocytoplasm	ARE and U-rich-sequences	Inhibits translation
TIAR	Nucleocytoplasm	ARE and U-rich-sequences	Inhibits translation
hnRNPA1	Nucleocytoplasm	ARE and U-rich-sequences	Promotes decay and splicing
hnRNPC	Nucleus	Not known	Splicing
BRF1		ARE and U-rich-sequences	Promotes decay

Table 1: Summary of AUBPs expressed in T lymphocytes.

This non-exhaustive table of AUBPs expressed in T lymphocytes also indicates the cellular localization, the canonical binding sequence and the effects mediated on the bound transcripts.

1.3.4 Trans-factors which stabilize ARE mRNAs

Decay of ARE-containing mRNAs is a tightly regulated process. In addition to the multitude of AU binding proteins that promote mRNA decay, there are some AU

binding proteins that can compete for ARE binding and allow for ARE dependent mRNA stabilization. Compared to the AU binding proteins that promote AMD, there are relatively few which stabilize AU rich messages.

AUF-1 is an ARE binding protein implicated in AMD. However, AUF1 has several splice isoforms some of which retain their ability to bind ARE mRNAs but appear not to target mRNAs for degradation. Thus some AUF1 isoforms preferentially stabilize these mRNAs (Xu et al., 2001). The effect of AUF1 is also dependent on cell type (Loflin et al., 1999) and the number of AUUUA repeats in the ARE (Xu et al., 2001).

The ELAV proteins are also ARE binding proteins that promote mRNA stability. There are 4 family members of which, HelN1, HuC and HuD are preferentially expressed in the nervous system and gonads. HuR is more ubiquitously expressed (Atasoy et al., 1998; Ma et al., 1996). HuR is known to stabilize both transcription factors (Fan and Steitz, 1998b; Peng et al., 1998; Wang et al., 2000) and cytokines (Di Marco et al., 2001; Fan and Steitz, 1998b) regardless of ARE class. TNFα mRNA and TNF family member, CD40L, are of particular interest since they are both stabilized by HuR (Dean et al., 2001; Di Marco et al., 2001; Sakai et al., 2003). The tissue distribution of HuR combined with its ability to bind all ARE classes make HuR an important determinant of ARE mRNA stability.

1.3.5 How an ARE-containing mRNA escapes AMD to complete its mission: implication of the HuR protein

ARE-containing messages are degraded in both the nuclear and cytoplasmic compartments (Wilusz and Wilusz, 2004, 2007; Wilusz et al., 2001) (Mukherjee et al., 2002). However. the observations supporting this conclusion do not explain how these messages get exported without being degraded. Interestingly, it is a fact that when the cell cycle is initiated, the ARE-containing mRNAs escape nuclear degradation and safely reach the cytoplasm, where they can be translated (Moore, 2002). One explanation could come from the fact that some RNA-binding proteins interact very early with these messages in the nucleus, such as many AU-rich binding proteins as well as some hnRNP proteins (Barreau et al., 2005; Hamilton et al., 1997; Hamilton et al., 1993). In studying the regulation of ARE-containing messages, the Steitz laboratory identified HuR (Myer et al., 1997) as a *trans*-acting protein which interacts with its mRNAs before they exit the nucleus (Gallouzi et al., 2000; Gallouzi and Steitz, 2001). The other neuron specific Hu proteins, namely Hel-N1 (or HuB), HuC, and HuD, have previously been identified as target antigens in paraneoplastic encephalomyelitis sensory neuropathy (the Hu syndrome); a disease characterized by diverse neuronal degeneration that is associated with small cell lung cancer (SCLC) (Dalmau et al., 1991; Levine et al., 1993; Szabo et al., 1991). All four Hu proteins contain three highly conserved RNA binding domains belonging to the RBD (RNA binding domain; also called RRM: RNA recognition motif) superfamily (Burd and Dreyfuss, 1994); however, their auxiliary regions (the sequence N-terminal to RBD1 and the hinge region between RBDs 2 and 3) differ. All Hu proteins exhibit high affinity for ARE sequences (Brennan and Steitz, 2001; Perrone-Bizzozero and Bolognani, 2002).

Deletion studies conducted on HuC, HuD, and HuR have suggested that RBDs 1 and 2 are responsible for ARE binding, while RBD3 binds simultaneously to the poly A^+ tail of an mRNA (Abe et al., 1996; Ma et al., 1997). It appears that HuR acts by protecting the body of the message from degradation rather than by slowing the rate of deadenylation (Peng et al., 1998). HuR may initially bind mRNAs in the nucleus and accompany them into the cytoplasm, providing ongoing protection from the degradation machinery.

It has been observed that HuR shuttles between the nucleus and the cytoplasm using two different alternative pathways; one of which is CRM1 dependent while the other is CRM1-independent (Gallouzi et al., 2001). By doing so HuR serves as an adaptor for the export of many of its mRNA targets (Doller et al., 2008a). These observations and the fact that a fraction of cytoplasmic HuR localizes to heavy polysomes (Gallouzi et al., 2000), indicates that HuR not only plays a role in the protection of RNA during its export, but HuR could also affect its translation. Overall, all these observations suggest that proteins such as HuR help some messenger RNAs to escape the nuclear or cytoplasmic degradation machinery to safely reach their final destination.

1.3.6 Role of HuR in T lymphocytes

HuR is known to be overexpressed in many secondary lymphoid organs such as the spleen, thymus and the gut. In addition, stimulation via LFA1 in T lymphoma cell lines, causes cytoplasmic relocalization of HuR (Atasoy et al., 1998) and a coincident increase in the stability of several cytokines such as GM-CSF, IL-3 and TNFα (Wang et al., 2006). Furthermore HuR is known to be overexpressed in activated T lymphocytes

(Atasoy et al., 1998). Recently, T lymphocyte lineage specific HuR knock out mice were generated by Kontoyiannis and colleagues (Papadaki et al., 2009). The T lymphocytes from these mice have severe maturation defects. The thymic T lymphocytes have proliferative defects that remain unexplained by any of the known cell cycle targets of HuR such as p53, p21, and p27. These mice also do not delete auto-reactive thymocytes from the repertoire and thus have defects in thymic negative selection. Again, currently defined targets of HuR, such as Fas do not explain this effect. In addition, the T lymphocytes deficient in HuR do not undergo positive selection due to a defect in TCR signaling, and the chemokines required for thymic egress rely on HuR for stabilization and are not present in sufficient quantity to allow emigration of mature thymocytes into the periphery (Papadaki et al., 2009). Interestingly, the unexplained aspects of this phenotype are reminiscent of the Fas and Fas ligand mutant mice.

1.3.7 Summary

HuR is involved in the posttranscriptional regulation of cytokines and other transiently expressed mRNA species at the level of mRNA localization, stability and translation efficiency. Based on the role of HuR in the regulation of cytokines, cell cycle proteins, and signal transduction proteins, it is not surprising that HuR has recently been found to be of primordial importance in the maturation, expansion, migration and effector functions of T lymphocytes. HuR functions by binding mRNA at AU rich sequences thereby relocalizing messages, delivering them to the translational machinery, or preventing the binding of other AUBPs which target the mRNA for decay. Thus HuR is essential for the expression of many transiently expressed transcripts for example TNFα in macrophages. FasL is a member of the TNF superfamily and like the other TNF

family members, it mediates a variety of immune processes including apoptosis in activated T lymphocytes, and proliferation and co-stimulation in $CD8^+$ T lymphocytes. Fas ligand is known to be a key immune regulator due to the effect of mutations on immune homeostasis. The biosynthesis of the TNF superfamily members is exquisitely controlled. This also applies to FasL since uncontrolled production of a pro-apoptotic protein could result in tissue damage. Strangely, though most levels of the biosynthesis of FasL have been investigated, its post-transcriptional regulation has never been described despite a clear role for this level of regulation in other TNF family members.

1.3.8 Rationale

Many TNF family members are regulated post-transcriptionally by motifs in 3'UTR of their mRNAs. These elements can affect the half-lives, the cellular localization and/or the translation of these messages (Abdelmohsen et al., 2008; Abdelmohsen et al., 2007; Barreau et al., 2005; Chen et al., 1995b; Jacobson and Peltz, 1996, 1999). For example, the expression of TNFα and CD40L mRNAs is regulated both at the level of mRNA stability and by the accessibility of the mRNA to the translational machinery (Barnhart et al., 2000; Ford et al., 1999a; Han and Beutler, 1990; Han et al., 1990; Wang et al., 1997). All these messages contain AU rich elements (AREs) that are responsible for the rapid decay and/or the translational regulation of these transcripts and they are also targets of HuR (Dean et al., 2001; Di Marco et al., 2001; Ford et al., 1999b; Sakai et al., 2003).

Likewise, the expression of the FasL protein was also shown to be regulated at the translational level. Xiao and colleagues (Xiao et al., 2004) have observed that the full length FasL mRNA contains structures that inhibit translation. They noticed that although the mRNA abundance was comparable to the full length, the amount of protein produced was vastly increased when the 5'region was truncated. Interestingly, as for TNFα, CDL40, and FasL contain an ARE consensus pentamer (database). In addition, the 3' extremity of the FasL 3'UTR contains a large proportion of U's (37%) and shares 64% nucleotide identity with the TNFα ARE (see Figure 8). Furthermore, the computed secondary structures of both FasL and TNFα mRNA are strikingly similar (see Figure 9).

Based on these similarities we investigated whether FasL mRNA and HuR interact and found that this is indeed the case. It is also known that HuR is relocalized to the cytoplasm upon p38 MAPK activation, a process that is inhibited by immunomodulatory drugs such as thalidomide (Jin et al., 2007). In fact, levels of TNF family members TNFα, TRAIL and FasL have been shown to be affected by thalidomide in multiple myeloma patients (Grzasko et al., 2006). Even more interestingly, thalidomide exerts its effects on TNFα by decreasing the stability of its mRNA (Moreira et al., 1993; Sampaio et al., 1991). We explore the possibility that thalidomide exerts its effects via HuR and consequently affects mRNA targets such as FasL.

Chapter 2: FasL expression in activated T Lymphocytes involves HuR-mediated stabilization

A substantial portion of this chapter will be submitted as a manuscript.

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G.L.D performed the experiments, analyzed data and wrote the paper.
Dr. Sergio DiMarco designed research, performed some of the experiments, analyzed data, helped writing the paper.
Dr. Julie Desbarats designed research and analyzed some of the data.
Dr. Imed Gallouzi designed the experiments, analyzed the data and wrote the paper
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2.2 Abstract

A prolonged activation of the immune system is one of the main causes of hyperproliferation of lymphocytes leading to defects in immune tolerance and autoimmune diseases. Fas ligand (FasL), a member of the TNF superfamily, plays a crucial role in controlling this excessive lymphoproliferation by inducing apoptosis in T cells leading to their rapid elimination. Here we establish that posttranscriptional regulation is part of the molecular mechanisms that modulate FasL expression and show that in activated T cells FasL mRNA has a short half-life (~2h). Our sequence analysis indicates that the FasL 3'untranslated region (UTR) contains two AU rich elements (ARE) that are similar in sequence and structure to those present in the 3'UTR of TNFα mRNA. Through these AREs, the FasL mRNA forms a complex with the RNA-binding protein HuR both *in vitro* and *in ex-vivo*. Knocking down HuR in HEK 293 cells prevented the PMA-induced expression of a GFP reporter construct fused to the FasL 3'UTR. Collectively, our data demonstrate that the posttranscriptional regulation of FasL mRNA by HuR represents a novel mechanism that could play a key role in the maintenance and proper functioning of the immune system.

2.3 Introduction

Fas and Fas ligand (FasL) are a transmembrane receptor ligand pair of the TNF receptor and TNF family, primarily involved in maintaining the homeostasis of the immune system by eliminating antigen-activated lymphocytes that consequently limits the magnitude and duration of the immune response (Strasser et al., 2009). Fas/FasL mediate this effect by triggering an apoptotic response in these cells by recruiting the adaptor protein FADD to the intracellular tail of Fas by death domain interactions. In turn, the FasL-Fas-FADD complex recruits pro-caspases 8 and 10 via homotypic death effector domain interactions leading to caspase cleavage and apoptosis (Strasser et al., 2009). The importance of Fas and FasL is evidenced by the fact that defects in their expression trigger excessive lymphoproliferation resulting in loss of immune tolerance and autoimmune diseases (Karray et al., 2004; Senju et al., 1996; Strasser et al., 2009; Watanabe-Fukunaga et al., 1992a). While the Fas receptor is constitutively expressed in most tissues, FasL is restricted to activated lymphocytes and sites of immune privilege (Strasser et al., 2009). This supports the idea that FasL but not Fas is the limiting factor in the Fas/FasL-induced signalling pathways. The disruption of Fas-FasL signalling pathways by spontaneous mutations in mice or in human patients has been associated with diseases such as SLE (systematic lupus erythematosus) or ALPS (autoimmune lympho-proliferative syndrome) (Strasser et al., 2009; Watanabe-Fukunaga et al., 1992a). Likewise, an increase in FasL-mediated apoptosis of normal, Fas bearing, bystander cells causes certain immunopathologies such as hepatitis that is linked to excessive T cell activation (Strasser et al., 2009). Hence, a tight regulation of FasL expression during T

cell activation is critical to maintain the homeostasis and the proper functioning of the immune system.

During the past decade, the majority of studies have focused on delineating the molecular mechanisms that modulate FasL expression at the transcriptional level. Several factors, such as NF-AT, NF-κB and IRF-1 have been shown to directly activate the transcription of the *fasl* gene (Chow et al., 2000; Hsu et al., 1999a; Li-Weber et al., 1999). It is well established for other TNF family members such as TNFα, TRAIL and several interleukins, that though transcription is tightly regulated, the amount of mRNA produced does not correlate with the protein expression levels (Beutler et al., 1986). In many cases, while the steady state levels of these messages remains unchanged, the protein levels significantly increase in response to extracellular stimuli (Jacob et al., 1996; Jacobs et al., 1996). This increase is due to the fact that the expression of these mRNAs is also regulated posttranscriptionally at the level of subcellular localization, mRNA turnover, and translational efficiency. These posttranscriptional effects are mainly mediated by AU rich elements (AREs) in the 3' untranslated regions (3'UTR) of many cytokine mRNAs (Antic and Keene, 1998; Asirvatham et al., 2009; Barreau et al., 2005). AREs are known to regulate a variety of transiently expressed cytokines during T cell activation. These include IFNγ, GM-CSF, CD83, TNFα and CD40L (Chemnitz et al., 2009; Ford et al., 1999a; Tobler et al., 1988). This regulation is due to the stabilization of these messages by a mechanism that involves their association with ARE-binding proteins such as HuR (Barreau et al., 2005; Di Marco et al., 2001; Sakai et al., 2003).

HuR belongs to the ELAV (Embryonic lethal Abnormal Vision) family of RNA binding proteins that contain three other members, HuB, HuC and HuD (Keene, 1999). Of the four ELAV family members, only HuR is ubiquitously expressed, and it is particularly well expressed in primary and secondary lymphoid tissues such as the thymus, spleen and the gut (Atasoy et al., 1998; Mukherjee et al., 2009). Recently, it has been shown that disrupting HuR expression in T lymphocytes causes a severe defect in their maturation (Papadaki et al., 2009). Indeed, though these mice have a wildtype thymic microenvironment, the HuR -/- thymocytes of these mice are unable to undergo positive selection, negative selection and thymic egress (Papadaki et al., 2009). The defect in positive selection is attributed to an alteration in the TCR-signalling pathway. Likewise, the defect in thymic egress can be explained by defects in chemokine signaling required in this process such as the TNF receptor family members including Fas (Papadaki et al., 2009).

The observations described above and the fact that FasL belongs to the TNFα family of cytokines raised the possibility that the expression of FasL could depend on posttranscriptional events involving ARE-binding proteins such as HuR. In this study we addressed this question and showed that the 3'UTR of FasL mRNA contains AREs strikingly similar in structure to those of TNFα. Our data demonstrate that via these AREs, FasL mRNA associates with HuR and that this association is absolutely required for its expression. We also discuss the functional relevance of posttranscriptional regulation of FasL and its impact on T lymphocytes maturation.

2.4 Materials and methods

Constructs:

The human FasL 3'UTR and FasL 3'UTR fragments were PCR amplified and a 5' BamHI site followed by stop codon and a 3' HindIII site were introduced. These PCR fragments were inserted into a pEGFP-C2 vector (Clontech) between the BglII and HindIII sites downstream of GFP. The GST and GST-HuR constructs were previously described (Brennan et al., 2000). All plasmids were prepared using the plasmid maxiprep kit (Qiagen) according to manufacturer's instructions.

The fusion proteins were purified as described in (Brennan et al., 2000; Frangioni and Neel, 1993) with the following modifications. The proteins were eluted from the glutathione-agarose beads with three applications of 500 µl glutathione elution buffer (10 mM for the first elution, and 20 mM for the second and third elutions). Proteins were then dialyzed overnight against phosphate-buffered saline at 4 °C. The 20 mM glutathione eluates were the most pure (as determined by SDS-PAGE) and were used in all experiments.

Cell culture, treatments and transfections:

Jurkat cells (E6 clone) (ATCC) were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 (Invitrogen) augmented with 10% FBS (Invitrogen). Jurkat cells were stimulated with PHA-M (Sigma) at 1µg/ml or PMA (Sigma) at 50ng/ml for times indicated. RNA stability curves were generated by treatment of cells with actinomycin D (Sigma) at 5 µg/ml. Transfections were performed in 12 well plates using

1μg plasmid DNA and TransPass RV (New England Biolabs) according to manufacturer's instructions.

HEK 293 cells (ATCC) were maintained in Dulbecco's modified Eagle medium (Multicell) supplemented with 10% FBS (Sigma). HEK 293 cells were stimulated with 50ng/ml PMA (Sigma) for times indicated. Transfections were performed in $10cm^2$ dishes with 8-16μg of plasmid DNA and lipofectamine reagent (Invitrogen) according to manufacturer's instructions. Transfections of siRNA were performed with 60nM of duplexes (siHuR or siCtrl) per $10cm^2$ cell culture dish, using lipofectamine (Invitrogen) and plus reagent (Invitrogen) according to manufacturer's instructions (van der Giessen et al., 2003). HEK 293 cells were transfected at 20% confluency, then retransfected with siRNA 24 hours later. Knockdown was assessed 52 hours after the first transfection.

RNA immunoprecipitation and RT-PCR

Preparation of mRNA (mRNP) Complexes: Immunoprecipitation and RNA preparation were performed as previously described (Tenenbaum et al., 2002) with several modifications. Briefly, cell extracts were prepared from PHA stimulated Jurkat cells or PMA stimulated HEK 293 cells. mRNP lysate (400 μl) was precleared with 8μl protein-G-Sepharose and the precleared lysate was subsequently divided for each immunoprecipitation. Immunoprecipitations were performed using antibodies to HuR (3A2) and IgG1 isotype control antibody (Sigma). Specific messages associated with HuR were defined using RT-PCR.

RT-PCR: RNA was isolated from the immunoprecipitated mRNP complexes using the ChargeSwitch RNA isolation kit (Invitrogen) scaled to 20% of manufacturer's

instructions. Purified RNA was eluted in 30 µl of water, and 4 µl was reverse-transcribed using the Sensiscript reverse transcription kit (Qiagen) according to the manufacturer's protocol in 20 µl of final volume. Subsequently, 2 µl of cDNA was PCR-amplified using HotStarTaq (Qiagen) using actin, FasL, or GFP cDNA-specific primers (see supplemental materials and methods). The sequences of the primers, as well as the PCR conditions, are described in the supplemental materials and methods.

Gel shift

The FasL RNA probes were produced by in vitro transcription as previously described (Gallouzi et al., 1998). Regions of FasL 3'UTR were PCR amplified with sense primers containing the T7 promoter in 5' (see supplemental materials and methods for primer sequences). The purified PCR fragment was used as a template for transcription using ^{32}P UTP with T7 RNA polymerase (Promega) according to manufacturer's instructions. The RNA binding assay was performed with purified 300ng GST and GST-HuR as previously described (Di Marco et al., 2001).

Northern blot

Northern blot analysis was performed as described (Wojciechowski et al., 1999). 15µg of RNA was used and was isolated with the RNeasy Plus extraction kit (Qiagen) according to manufacturer's instructions. After transferring to a Hybond-N membrane (Amersham Biosciences) and UV-cross-linking, the blot was hybridized with GFP or 18S rRNA probes prepared with ^{32}P dCTP by random priming with Ready-to-go DNA labelling beads (GE healthcare) according to the manufacturer's instructions. PCR-amplified fragments of GFP and 18S rRNA were used to generate labeled probes. After

hybridization, the membranes were washed and subsequently exposed on BioMax films (Kodak).

FACS

Cells were counted by using a hemocytometer and adjusted to a concentration of $1x10^6$ cells/ml. 0.5 ml of cells was washed 3 times in 1.5ml PBS and then resuspended in 0.5 ml PBS with 2% FBS. Acquisition of data was done on a FACScan (Becton Dickinson), and the analysis of these data was performed with FlowJo software.

Western blot

Total cell extracts were prepared as described (Gallouzi et al., 1998). Western blotting was performed as described previously (Brennan et al., 2000). The blots were probed with antibodies to HuR (3A2), tubulin (Developmental Studies Hybridoma Bank), GFP (JL8, Clontech).

Immunofluorescence

Immunofluorescence was performed as previously described (Fan and Steitz, 1998a). Anti-HuR (3A2) was used at a 1:1500 dilution in 1% goat serum/phosphate-buffered saline. HuR was detected using a 1:500 diluted AlexaFluor 488 conjugated goat anti-mouse IgG polyclonal antibody. DAPI staining 1:20000, was performed after secondary antibody. A Zeiss Axiovision 3.1 microscope was used to observe the cells using a 63x oil objective, and an Axiocam HR (Zeiss) digital camera was used for immunofluorescence photography.

q-PCR

RNA was isolated for RT q-PCR by using the ChargeSwitch RNA isolation kit (Invitrogen) scaled to 20% of manufacturers instructions. RNA was quantitated using the Ribogreen kit (Molecular Probes) and 150ng was reverse transcribed using the Sensiscript reverse transcription kit (Qiagen). Subsequently, the cDNA was PCR amplified with primers described in supplemental materials and methods, using the Quantitect SYBR green kit (Qiagen) in a Corbett Rotor Gene real time thermocycler. The Ct value was used to calculate the amount of the cDNA of interest by extrapolation from a standard curve.

q-PCR and PCR conditions

GFP and FasL Q-PCR

Samples were incubated 15 minutes at 95°C to activate hotstart enzyme, followed by cycling for 40 seconds at 94°C, 30 seconds at 63°C, and 30 seconds at 72°C. Specificity of amplified PCR product was tested by subsequent melt curve.

18S Q-PCR

Samples were incubated 15 minutes at 95°C to activate hotstart enzyme, followed by cycling for 35 seconds at 95°C, 35 seconds at 60°C, and 30 seconds at 72°C. Specificity of amplified PCR product was tested by subsequent melt curve.

RNA-IP:

FasL PCR

Samples were incubated 15 minutes at 95°C to activate hotstart enzyme, followed by cycling for 5 minutes at 94°C, 40 seconds at 63°C, and 35 seconds at 72°C for 5 cycles, then for 30 cycles of 60 seconds at 94°C, 40 seconds at 63°C, and 35 seconds at 72°C for. Final extension was done for 10 minutes at 72°C.

GFP PCR

Samples were incubated 15 minutes at 95°C to activate hotstart enzyme, followed by cycling for 40 seconds at 94°C, 30 seconds at 63°C, and 30 seconds at 72°C for 22 cycles. Final extension was done for 10 minutes at 72°C.

β-actin PCR

Samples were incubated 5 minutes at 95°C, followed by cycling for 30 seconds at 94°C, 30 seconds at 54°C, and 60 seconds at 72°C for 22 cycles. Final extension was done for 10 minutes at 72°C.

Cloning of FasL3'UTR and T7 probes:

Cloning FasL 3'UTR:

Samples were incubated 5 minutes at 95°C, followed by cycling for 60 seconds at 94°C, 40 seconds at 56°C, and 40 seconds at 72°C for 25 cycles. Final extension was done for 10 minutes at 72°C.

T7 probes:

Samples were incubated 5 minutes at 95°C, followed by cycling for 60 seconds at 94°C, 40 seconds at 56°C, and 40 seconds at 72°C for 35 cycles. Final extension was done for 10 minutes at 72°C.

Primers for gel shift probes:

1

sense:

GAATTGTAATACGACTCACTATAGGGCGATAAGAGAAGCACTTTGGGAT

antisense: ACATTTTGAACCCTGTGGT

2

sense: GAATTGTAATACGACTCACTATAGGGCGATTCAAAATGTCTGTAGCTCC

antisense: ACTAAGATGAGTGTAGAACAC

3

sense: GAATTGTAATACGACTCACTATAGGGCGATCATCTTAGTGCCTGAGAG

antisense: CTCTAGTCTTCCTTTTCCAT

4

sense:

GAATTGTAATACGACTCACTATAGGGCGAAAGACTAGAGGCTTGCATAA

antisense: TTAGGAATGAAATGAGTCCC

5

sense:

GAATTGTAATACGACTCACTATAGGGCGATCATTCCTAACACAGCATGT

antisense: TCAAAATCTTGACCAAATGC

6

sense:

GAATTGTAATACGACTCACTATAGGGCGATCATTCCTAACACAGCATGT

antisense: TTTGTATCCTAACATATACTTTTTA

5.1

sense:

GAATTGTAATACGACTCACTATAGGGCGAGATCTGATTCCTAACACAGCATG

TG

antisense: ACAAAGCTTCACACACCCCTACAATTGCA

5.2

sense:

GAATTGTAATACGACTCACTATAGGGCGAGATCTAGGGGTGTGTGTGTGTGT

GTG

antisense: AATAAGCTTTTCACAATATCTACATTCTC

5.3

sense:

GAATTGTAATACGACTCACTATAGGGCGAGATCTAGATTGTGAAGTACATAT

TA

antisense: AGGAAGCTTCAAAATCTTGACCAAATGCA

6.1

sense:

GAATTGTAATACGACTCACTATAGGGCGAGATCTAGATTTTGAATGCTTCCTG

A

antisense: AGTAAGCTTCAATCAATGATTTTTAAGCA

6.2

sense:

GAATTGTAATACGACTCACTATAGGGCGAGATCTAGATTGATTGTCAGCTACT

A

antisense: TTTAAGCTTAATGCACATCTACATAAATA

6.3

sense:

GAATTGTAATACGACTCACTATAGGGCGAGATCTGATGTGCATTTTTGTGAAA

T

antisense: TTTAAGCTTTGTATCCTAACATATACTTT

Q-PCR, RNA-IP and cloning primers:

FasL PCR/Q-PCR:

sense: AGAGAGGGAACCACAGCACA

antisense: CACTCCAGAAAGCAGGACAAT

GFP PCR/Q-PCR:

sense: AGTGCTTCAGCCGCTACCC

antisense: GCTTGTGCCCCAGGATGTT

18S rRNA Q-PCR:

sense: GGTGACGGGGAATCAGGGTT

antisense: CGGGTCGGGAGTGGGTAAT

β-actin PCR

sense: GCGCGGATCCGCGGACTATGACTTAGTTGCG

antisense: GCGCGCGGCCGCCCACATTGTGAACTTTGGGGG

FasL 3'UTR cloning:

sense: ATAAGATCTAAGAGAAGCACT

antisense:TTTTATTACAAGCTTTCATTTCACA

Mice and ex vivo cell culture:

LckCre^{+}Elavl$^{fl/fl}$ mice and tissue preparation were previously described (Papadaki et al., 2009). Mixed or purified T cell populations were maintained in RPMI 1640 supplemented with 2.5% FBS and 1% penicillin/streptomycin. T lymphocytes were stimulated with PMA (50ng/ml) and ionomycin (2μM).

2.5 Results

2.5.1 FasL mRNA has a short half-life in activated T cells

Previous reports have shown that FasL mRNA is rapidly induced in T lymphocytes upon T cell receptor engagement and mitogen stimulation (Strasser et al., 2009). To define whether this up-regulation was associated with a stabilization of the FasL mRNA, we first assessed its steady state levels in T cells exposed to various activators. Jurkat T cells were treated with either phytohemagglutinin-M (PHA), a lectin that non-specifically aggregates cell surface receptors and PKC activator or phorbol myristyl acetate (PMA), a PKC agonist (Chien et al., 2001; Strasser et al., 2009). We observed a rapid increase in the level of FasL mRNA at 3 hours of PHA treatment. In contrast, PMA treatment had a much smaller effect on the steady state levels of FasL mRNA (Figure 7A). Interestingly, levels of FasL mRNA return to baseline within 6-12 hours of PHA stimulus, indicating that FasL is transiently expressed in response to lectins similarly to other cytokines. In the absence of PHA or PMA, however, the FasL mRNA was not detected (Figure 7A). Next, we determined the half-life of FasL mRNA under these conditions. We performed actinomycin D (Act D) pulse chase experiments (Dormoy-Raclet et al., 2007) and determined the half-life of the FasL mRNA using q-RT-PCR. Jurkat cells were treated with PHA for 3 hours to induce maximal FasL mRNA expression and then treated with 5μg/ml ActD for different periods of time. We observed that upon PHA treatment the half-life of FasL mRNA in Jurkat cells was slightly over 2 hours (Figure 7B). Of note,

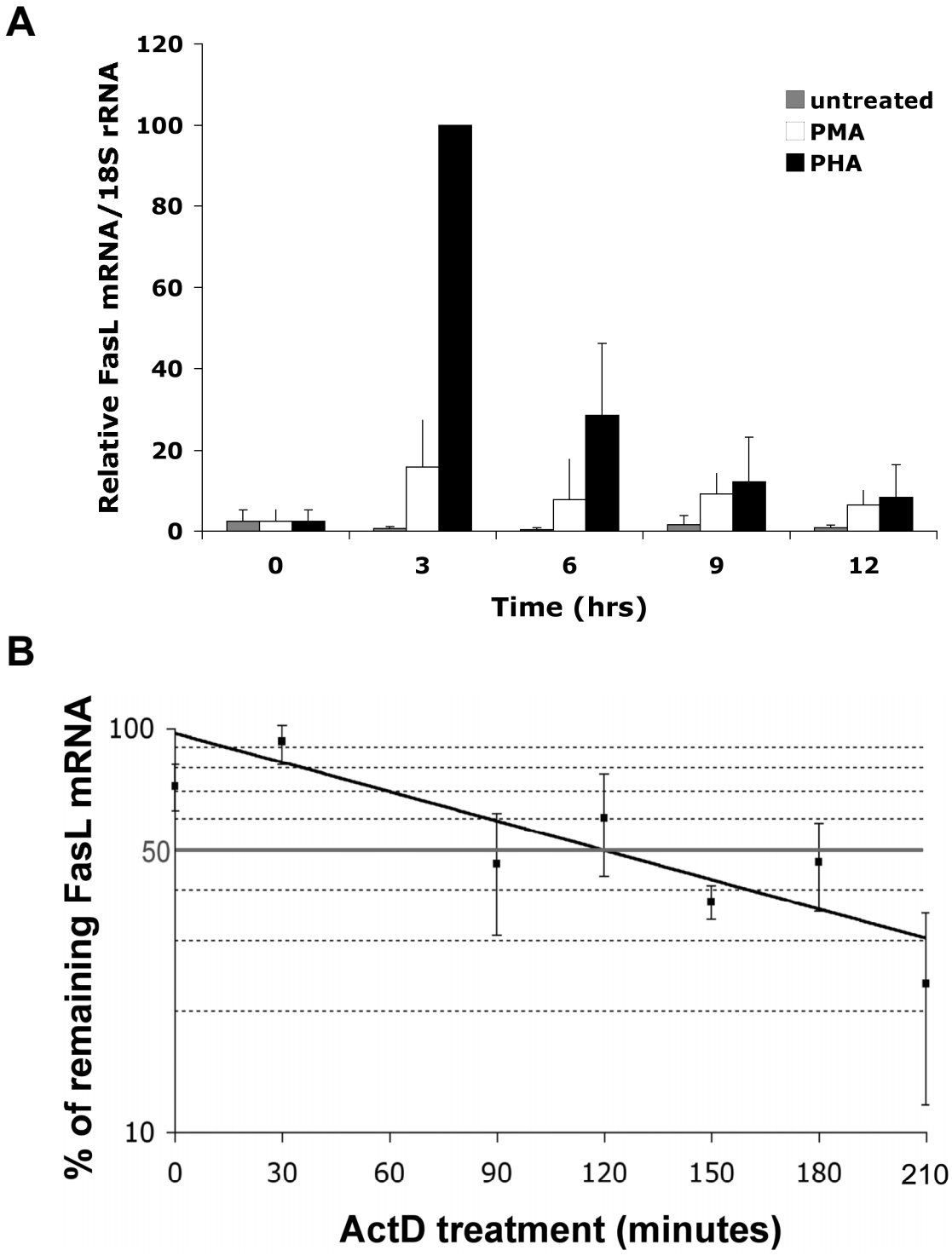

A
120
100
80
60
40
20
0
Relative FasL mRNA/18S rRNA
untreated
PMA
PHA
0
3
6
9
12
Time (hrs)
B
100
50
10
% of remaining FasL mRNA
0
30
60
90
120
150
180
210
ActD treatment (minutes)

Figure 7: FasL mRNA has a short half-life in activated T cells.

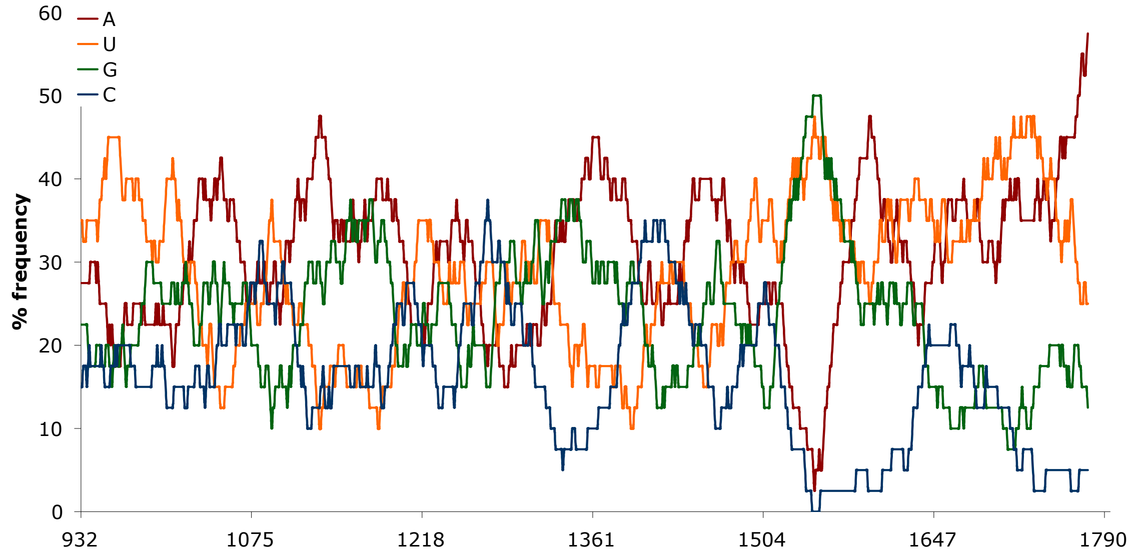
(A) RNA was isolated from Jurkat cells which were stimulated for 0, 3, 6, 9, and 12 hours with 50ng/ml PMA or 1μg/ml PHA-M. Fas ligand mRNA expression was determined by qRT-PCR and was normalized to 18S rRNA expression. All values are relative to peak expression at 3 hours of PHA treatment and plotted as the percentage +/- the S.D. of three independent experiments. (B) FasL mRNA stability was determined by treatment of Jurkat T cells with PHA for 3 hours followed by 5 μg/ml actinomycin D (ActD) treatment for 0, 30, 60, 90, 120, 150, 180 and 210 minutes. The half-life of FasL mRNA is indicated by the 50% line and corresponds to approximately 120 min. Fas ligand mRNA was detected by qRT-PCR and was normalized to 18S rRNA expression as described above.

since in untreated Jurkat cells FasL mRNA is not detectable, it was not possible to assess its half-life under these conditions. Therefore, the rapid increase and the relatively short half-life of the FasL mRNA indicate that for this message to be properly expressed, stabilization mechanisms, similar to those reported for other TNF family members, are activated at least for a short period of time.

2.5.2 The 3'UTR of FasL mRNA binds to HuR in an ARE-dependent manner

The stabilization of cytokine mRNAs is usually mediated by U-rich elements such as AREs located in their 3'UTR (Barreau et al., 2005; Jacobson and Peltz, 1996). Our initial analysis of the primary sequence of FasL 3'UTR indicated that it contains the typical AUUUA consensus sequence as well as a 300nt U-rich region (Figure 8). The mFold prediction software for mRNA folding showed that both TNFα and FasL mRNAs could fold and form a highly similar secondary structure (Figure 9A). This prediction method has highlighted the existence in the extreme 3' end of the FasL 3'UTR of two conserved U-rich sequences that are similar in structure and localization to the TNFα ARE-1 and ARE-2 (Figure 9B) (Fialcowitz et al., 2005a). These observations suggest that similarly to TNFα (Di Marco et al., 2001), the expression of FasL mRNA could involve the association of these U-rich sequences (AREs 1 or 2) with the HuR protein. To test this possibility, we first assessed whether HuR can interact with the FasL mRNA in Jurkat T cells. To ensure the expression of FasL mRNA and reduce FasL-induced apoptosis, Jurkat cells were treated for only 2 hours with PHA. These cells were then used to perform an immunoprecipitation experiment with the anti-HuR antibody (Gallouzi et al., 2000) followed by RT-PCR analysis. We observed that in PHA treated Jurkat cells the FasL mRNA specifically associated with HuR when compared to an

A



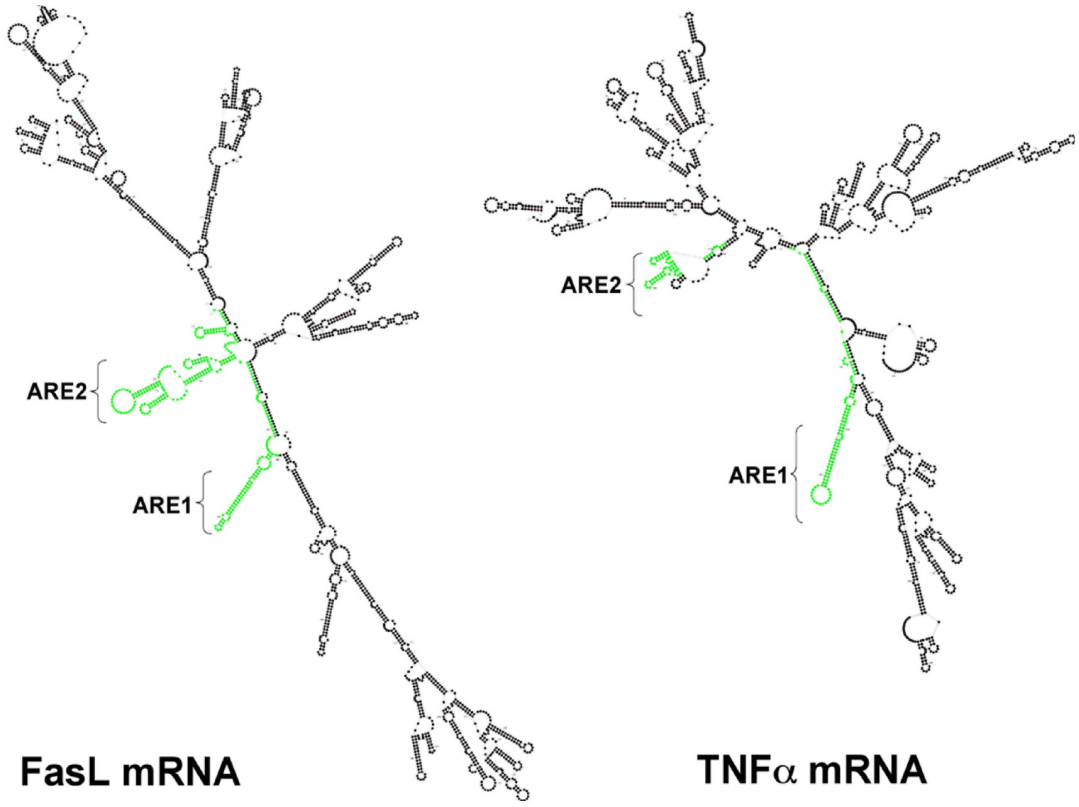
B

```
                   10        20        30        40        50
huFasL TAAGA--GAAGCACTTTGGGATTCTTTCCATTATGATTCTTTGTTACAGGCACCGAGAAT
        : ::   :: : :: :      :  ::: :::       : :  ::   ::  : :     ::
huTNFa TGAGGAGGACGAACATCC--AACCTTCCCAAACGCCTCCCCTGCCCCAATCCCTTT--AT
                 10        20        30        40        50

         60        70        80        90       100       110
huFasL GTTGTATTCAGTGAGGGTCTTCTTACATGCATTTGAGGTCAAGTAAGAAGACATGAACCA
              ::  : ::    :  :: :     : : :: : ::::  ::  :::  ::
huTNFa TACCCCCTCCTTCAGACACC-CTCAACCTCTTCTG-GCTCAA--AAAGAGAATTGGGG--
           60        70        80        90          100       110

        120       130       140       150       160       170
huFasL AGTGGACCTTGAGACCACAGGGTTCAAAATGTCTGTAGCTCCTCAACTCACCTAATGTTT
        :   :   :  :: : :: : ::  :: : :   : : :     : :: :::       ::
huTNFa -GCTTAGGGTCGGAACCCAAGCTTAGAACTTTAAGCAACAAGACCAC-CAC-------TT
                 120       130       140       150                 160

        180       190       200       210       220       230
huFasL ATGAGCCAGACAAATGGAGGAATATGACGGAAGAACATAGAACTCTGGGCTGCCATGTGA
          : :: :  :  :  :::::: ::  :    : :::  ::: :     :::: ::     :
huTNFa CGAAACCTGGGA--TTCAGGAATGTGTGGCCTGCACAGTGAAGT----GCTGGCA----A
               170         180       190       200            210

        240       250       260       270       280       290
huFasL AGAGGGAGAAGCATGAAAAAGCAGCTACCAGGTGTTCTACACTCATCTTAGTGCCTGAGA
         :   ::::   : :::  :  ::  ::::            :::::   : : :::: : :
huTNFa CCACTAAGAAT--TCAAACTGGGGCCTCCAGA--------ACTCA--CTGGGGCCT-ACA
               220         230       240                  250

        300           310        320       330       340       350
huFasL GTATTTA-----GGCAGATTGAAAA-GGACACCTTTTAACTCACCTCTCAAGGTGGGCCT
        :  :: :     : ::  : :::   ::: :::      :       ::: :     ::: :
huTNFa GCTTTGATCCCTGACATCTGGAATCTGGAGACCAGGGA----GCCTTTGGTTCTGGCCAG
        260       270       280       290           300       310

                360       370       380       390       400       410
huFasL --TGCTACCTCAAGGGGGACTGTCTTTCAGATACATGGTTGTGACCTGAGGATTTAAGGG
          :::: :   :   : ::    ::  :  : : ::   ::  ::  : :::  : :::
huTNFa AATGCTGCAGGACTTGAGAAGACCTCACCTAGAAAT---TGACACAAGTGGACCTTAGGC
           320       330       340       350          360       370

                420       430       440       450       460       470
huFasL ATGGAAAAGGAAGACTAGAGGCTTGCATAATAAGCTAAAGAGGCTGAAAGAGGCCAATGC
         :            : ::: : :: :: : :   ::  :::  : ::     :::     :
huTNFa CTTCCTC----TCTCCAGATGTTTCCAGACTTC-CTTGAGACACGGAGCCCAGCCCT--C
                 380       390       400        410       420

                480       490       500       510       520       530
huFasL CCCACTGGCAGCATCTTCACTTCTAAATGCATATCCTGAGCCATCGGTGAAACTAACAGA
       :::: :::   :: :: :   : :  ::: :   ::       :   :   : : :   :
huTNFa CCCA-TGGAGCCAGCTCCCTCTATTTATGTTTGCACTTGTGATTATTTATTATTTATTTA
          430       440       450       460       470       480

                540       550       560       570       580       590
huFasL TAAGCAAGAGAGATGTTTTGGGGACTCATTTCATTCCTAACACAGCATGTGTATTTCCAG
       : :    :    :  : :    :  :: :    :: :::     : :: :   :: :  :    :
huTNFa TTATTTATTTA--TTTACAGATGAATGTATTTATTTGGGAGACCGGG-GTATCCTGGGGG
          490         500       510       520       530       540

                600       610       620       630       640       650
huFasL TGCAATTGTAGGGGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTATGACTAAAGAGAGA
         : : :::::: :        ::  :   :        : :  :::    :  ::  :   ::
huTNFa ACCCAATGTAGGAGCTGCCTTGGCTCAGACATGTTTTCCGTG---AAAACGGAGCTGAAC
                550       560       570       580          590

                660       670       680       690       700       710
huFasL ATGTAGATATTGTGAAGTACATATTAGGAAAATATGGGTTGCATTTGGTCAAGATTTTGA
       :    : : ::    : :::         ::     : ::  :: : :::: : : : :::: :
huTNFa AATAGGCTGTTCCCATGTAGCCCCCTGGCCTCTGTGCCTT-CTTTTGATTATGTTTTTTA
        600       610       620       630        640       650

                   720       730       740       750       760
huFasL A--TGCTTC-CTGACAATCAACTCTAAT-AGTGCTTAAAAA-TCATTGATTGTCAGCTAC
       :  :  ::  ::::  :      ::::: : :::: :       : :    : :::::     :
huTNFa AAATATTTATCTGATTAAGTTGTCTAAACAATGCTGATTTGGTGACCAACTGTCACTCAT
         660       670       680       690       700       710

          770       780       790       800       810       820
huFasL TAATGATGTTTTCCTATAATATAATAAATATTTATGTAGATGTGCATTTTTGTGAAATGA
       :  :::   : : ::     :    :  : : : :::: ::  ::   :  : :  : ::
huTNFa TGCTGAGCCTCTGCTCCCC-AGGGGAGTTGTGTCTGTA-ATC-GCCCTACTATTCAGTGG
         720       730       740       750         760       770

          830       840       850
huFasL AAACATGTAATAAAAAGTATATGTTAGGATAC
         : :   ::::::   :    :    :  :  :
huTNFa CGAGA---AATAAAGTTTGC-TTAGAAAAGAA
               780       790       800
```

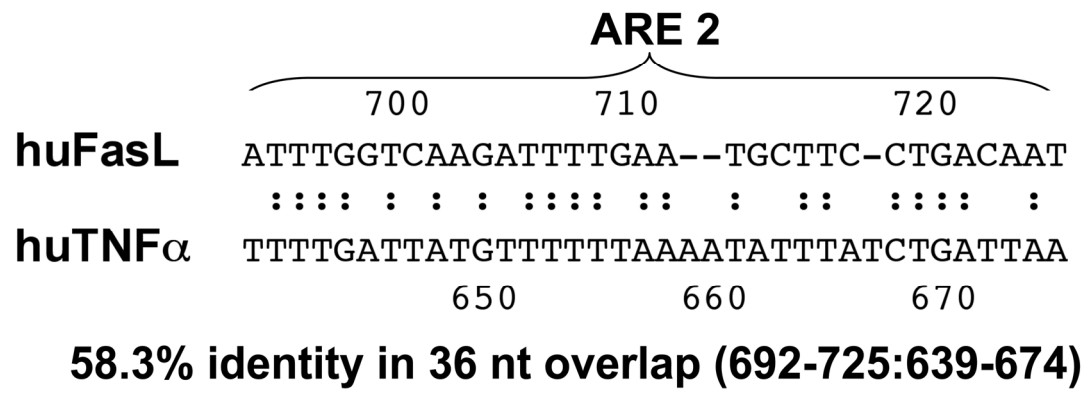
Figure 8: Features of the FasL 3'UTR

(A) Plot of nucleotide density over the length of the FasL 3'UTR. (B) Global alignment of the TNFα 3'UTR with AREs in bold and the FasL 3'UTR (Huang and Miller, 1991). There is 44.3% nucleotide identity between the FasL and TNFα 3'UTRs which is the same degree of homology between their coding regions.

A



B



58.3% identity in 36 nt overlap (692-725:639-674)

Figure 9: **Both TNFα and FasL mRNAs contain two AREs that are similar in location and secondary structures.**

(A) Predicted secondary structures of the full-length FasL mRNA (left) with U rich region (ARE1 and ARE2) highlighted in green. The full-length mRNA of TNFα (right) is shown with previously described AREs highlighted in green (Zuker, 2003). (B) Alignment of the TNFα ARE2 with the U rich regions of the FasL 3'UTR (Huang and Miller, 1991).

isotype matched control antibody (Figure 11A). In order to map the HuR binding sites in the FasL-3'UTR and to prove that the interaction between HuR and FasL mRNA is direct, the FasL-3'UTR was divided into six 150nt regions which were used as probes for RNA electromobility shift assay (REMSA) (Figure 11B). We observed that all regions other than region 2 bind purified GST-HuR (Figure 11C). However, regions 5 and 6, which are particularly U rich (Figure 10A), bind HuR most strongly (Figure 11C, compare lanes 15, 18 to lanes 3, 6, 9 and 12). We also showed that this interaction is competed away by an excess of the same unlabeled probes (Figure 11D).

Next, we mapped regions 5 and 6 more precisely, to determine the number of HuR binding sites in these regions. Regions 5 and 6 of the FasL 3'UTR were further sub-divided into six 50nt sub-regions (Figure 12A). REMSA experiments as described above, showed strong interactions between HuR and some of the sub-regions, notably sub-regions 5.2, 5.3, 6.1, 6.2 and 6.3 (Figure 12B). Therefore, our results indicate that there are at least four distinct U-rich HuR binding sites in regions 5 and 6 in addition to the weaker binding sites in regions 1, 3 and 4 (Figure 11C). Furthermore, since the binding of HuR to these fragments can be competed by the same unlabeled probes (Figure 12C), these interactions seem to be specific. Together these observations argue that HuR directly binds to the 3'UTR of FasL in an ARE-dependent manner.

We ascertained the minimal sequence from the FasL 3'UTR required to mediate the reduction in mRNA levels. Is it possible to recapitulate the effect of the FasL 3'UTR with one of the gel shift regions which is known to bind HuR? We cloned each of the

A

Probe sequences for REMSA

Overlapping 150nt probes:

1: TAAGAGAAGCACTTTGGGATTCTTTCCATTATGATTCTTTGTTACAGGCACCGAGAATGTTGTATTCAGTGAGG
GTCTTCTTACATGCATTTGAGGTCAAGTAAGAAGACATGAACCAAGTGGACCTTGAGACCACAGGGTTCAAAATGT

2: TCAAAATGTCTGTAGCTCCTCAACTCACCTAATGTTTATGAGCCAGACAAATGGAGGAATATGACGGAAGAACA
TAGAACTCTGGGCTGCCATGTGAAGAGGGAGAAGCATGAAAAAGCAGCTACCAGGTGTTCTACACTCATCTTAGT

3: CATCTTAGTGCCTGAGAGTATTTAGGCAGATTGAAAAGGACACCTTTTAACTCACCTCTCAAGGTGGGCCTTGC
TACCTCAAGGGGGACTGTCTTTCAGATACATGGTTGTGACCTGAGGATTTAAGGGATGGAAAAGGAAGACTAGAG

4: AAGACTAGAGGCTTGCATAATAAGCTAAAGAGGCTGAAAGAGGCCAATGCCCCACTGGCAGCATCTTCACTTC
TAAATGCATATCCTGAGCCATCGGTGAAACTAACAGATAAGCAAGAGAGATGTTTTGGGGACTCATTTCATTCCTAA

5: TCATTCCTAACACAGCATGTGTATTTCCAGTGCAATTGTAGGGGTGTGTGTGTGTGTGTGTGTGTGTGTGTG
TATGACTAAAGAGAGAATGTAGATATTGTGAAGTACATATTAGGAAAATATGGGTTGCATTTGGTCAAGATTTTGA

6: AAGATTTTGAATGCTTCCTGACAATCAACTCTAATAGTGCTTAAAAATCATTGATTGTCAGCTACTAATGATGTTTTC
CTATAATATAATAAATATTTATGTAGATGTGCATTTTTGTGAAATGAAAACATGTAATAAAAAGTATATGTTAGGATACA

B

Probe sequences for REMSA

Overlapping 50nt probes derived from regions 5 and 6 above:

5.1: TCATTCCTAACACAGCATGTGTATTTCCAGTGCAATTGTAGGGGTGTGTG

5.2: GGGGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTATGACTAAAGAGAGAATGTAGATATTGTGAA

5.3: ATATTGTGAAGTACATATTAGGAAAATATGGGTTGCATTTGGTCAAGATTTTGA

6.1: AAGATTTTGAATGCTTCCTGACAATCAACTCTAATAGTGCTTAAAAATCATTGATTG

6.2: TCATTGATTGTCAGCTACTAATGATGTTTTCCTATAATATAATAAAT**ATTTA**TGTAGATGTGCATT

6.3: GATGTGCATTTTTGTGAAATGAAAACATGTAATAAAAAGTATATGTTAGGATACA

Figure 10: FasL 3'UTR probes for REMSA.

(A) Sequences of the 6 probes spanning the entire FasL 3'UTR with overlap excluding T7 promoter. (B) Sequences of the 6 probes spanning regions 5 and 6 of the FasL 3'UTR with overlap excluding T7 promoter. The AUUUA pentamer in the ARE is shown in bold and is located in region 6.2.

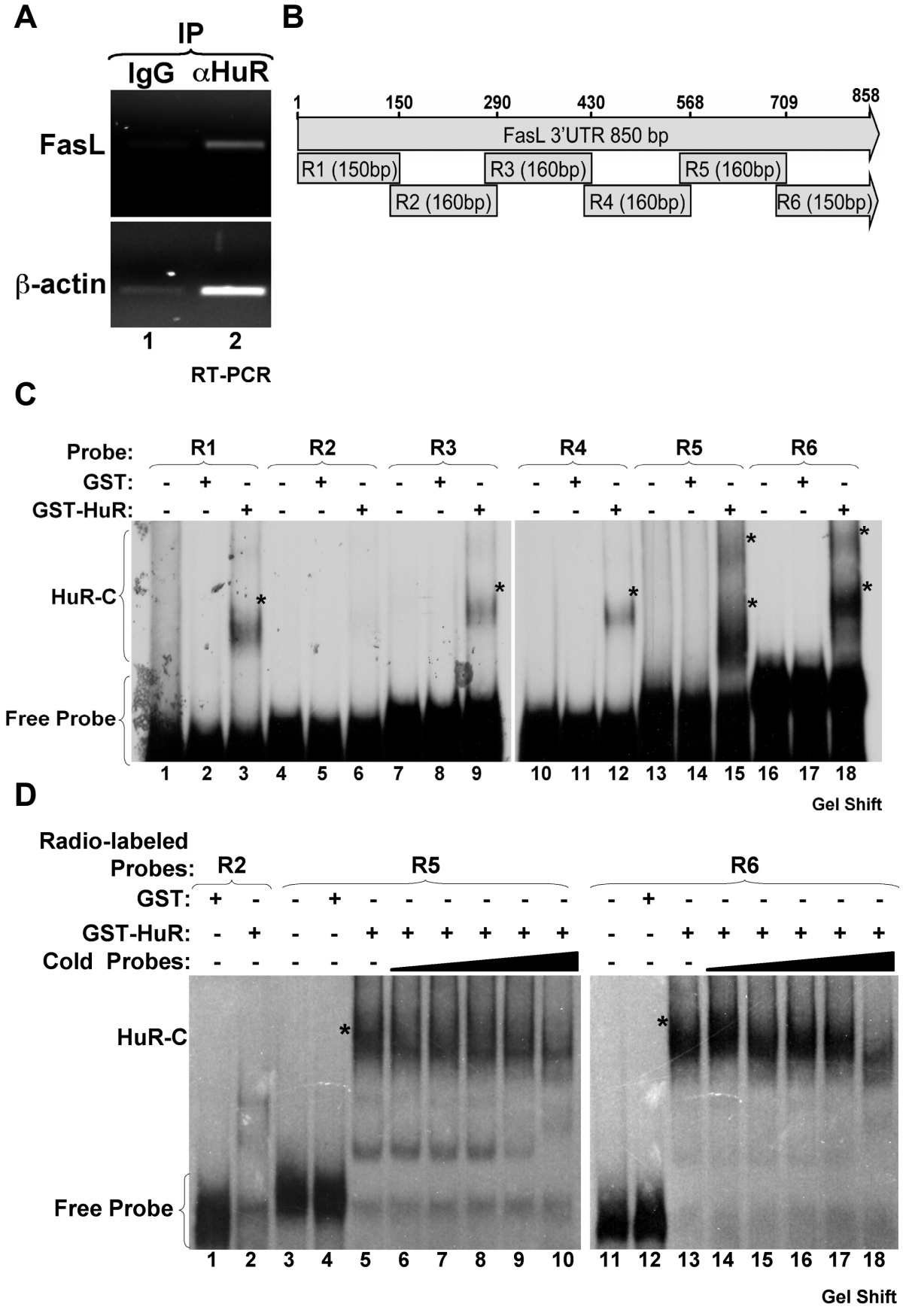

A
IP
IgG
αHuR
FasL
β-actin
1
2
RT-PCR
B
1
150
290
430
568
709
858
FasL 3'UTR 850 bp
R1 (150bp)
R2 (160bp)
R3 (160bp)
R4 (160bp)
R5 (160bp)
R6 (150bp)
C
Probe:
R1
R2
R3
R4
R5
R6
GST:
GST-HuR:
HuR-C
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Gel Shift
D
Radio-labeled Probes:
R2
R5
R6
GST:
GST-HuR:
Cold Probes:
HuR-C
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Gel Shift

Figure 11: Fas ligand mRNA associates with HuR via U rich sequences in the 3'UTR.

(A) HuR was immunoprecipitated from Jurkats stimulated with PHA-M 1μg/ml for 2 hours with the anti-HuR (3A2) antibody (lane 2). A mouse IgG1 antibody was used as an isotype matched specificity control (lane 1). Immunoprecipitation was followed by RT-PCR for FasL (upper) and β-actin (lower) was used as a positive control. Representative blots of two independent experiments are shown. (B) Diagram indicating the location and length of probes for gel shift assay. (C) HuR associates with regions 1 (lane 3), 3 (lane 9), 4 (lane 12), 5 (lane 15), and 6 (lane 18) of the FasL 3'UTR. Gel shift binding assays were performed by incubating 150 ng of purified GST or GST-HuR protein with radiolabeled probes (see B). Radiolabeled probe-GST-HuR complexes (HuR-C) are indicated with an asterisk. (D) Increasing concentration of unlabeled probes for the regions 5 and 6 were incubated with GST or GST-HuR in the presence of radiolabeled probes from the same regions. HuR-C are indicated by asterisks. These gel shifts were performed with 300ng purified GST or GST-HuR and with 0.01X (lanes 6, 14), 0.1X (lanes 7, 15), 1X (lanes 8, 16), 10X (lanes 9, 17) and 100X (lanes 10, 18) amounts of unlabeled probe. (C and D) Representative gel shift blots of two independent experiments are shown.

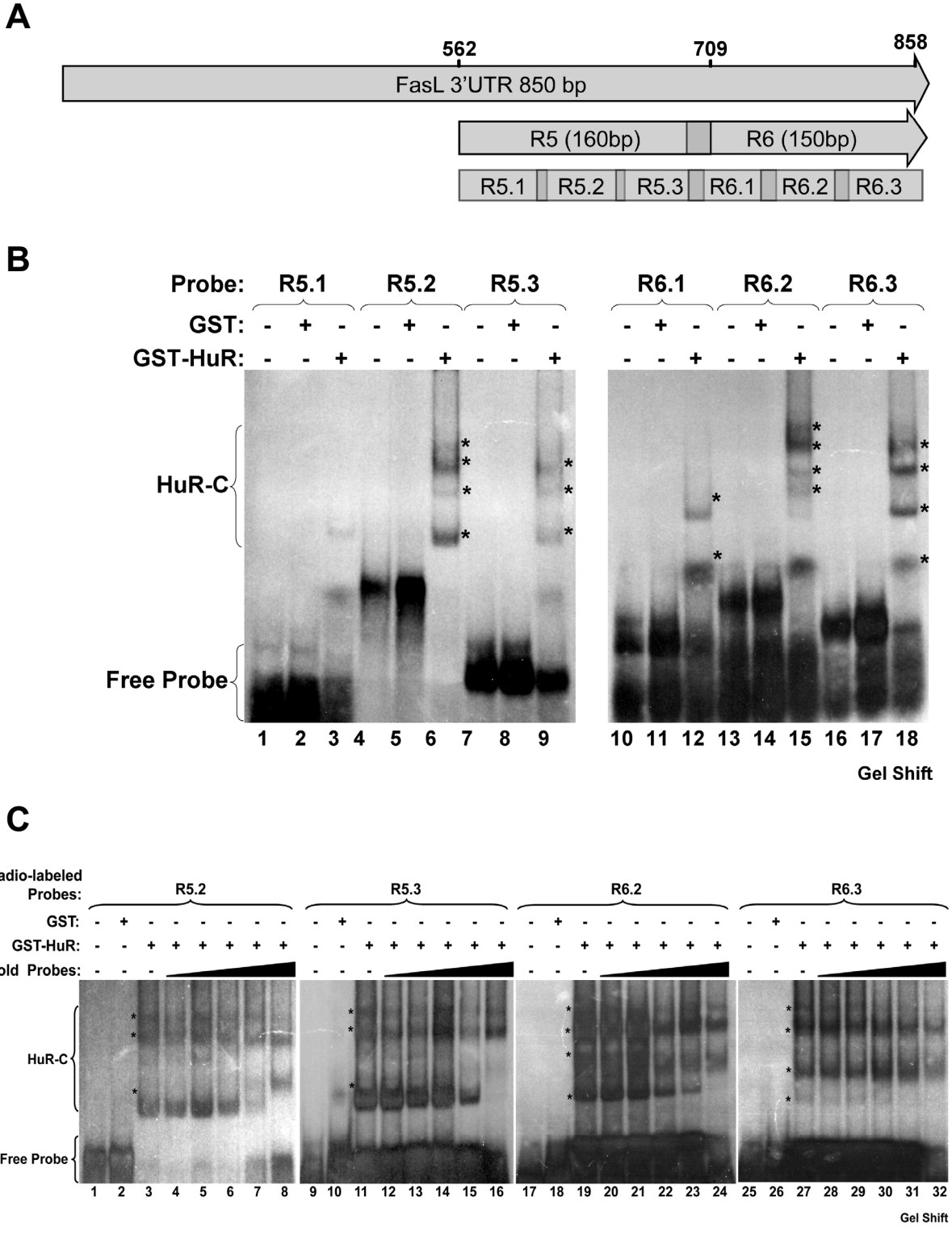

A
562
709
858
FasL 3'UTR 850 bp
R5 (160bp)
R6 (150bp)
R5.1
R5.2
R5.3
R6.1
R6.2
R6.3
B
Probe:
GST:
GST-HuR:
HuR-C
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Gel Shift
C
Radio-labeled Probes:
Cold Probes:
HuR-C
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
Gel Shift

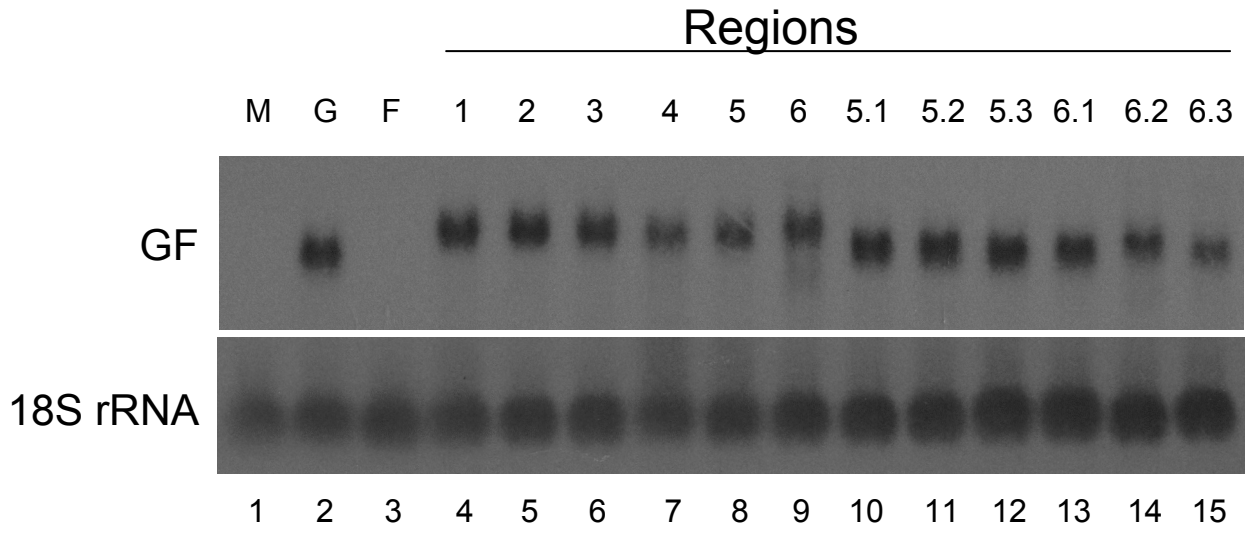
Figure 12: HuR binds to specific U-rich regions of the FasL mRNA.

(A) Diagram indicating the location and length of probes for gel shift assay. (B) HuR forms complexes (HuR-C) with regions 5.2 (lane 6), 5.3, (lane 9), 6.1 (lane 12), 6.2 (lane 15), and 6.3 (lane 18), indicated by asterisks. Gel shift binding assay performed by incubating 300 ng of purified GST or GST-HuR protein with radiolabeled probes. (C) Competition of HuR-probe interactions (HuR-C), indicated by asterisks, with unlabeled probe for the regions with shifts as shown in B. Regions 5.2, 5.3, 6.2 and 6.3. These gel shifts were performed with 300ng purified GST or GST-HuR and with 0.01X, (lanes 4, 12, 20, 28) 0.1X (lanes 5, 13, 21, 29), 1X (lanes 6, 14, 22, 30), 10X (lanes 7, 15, 23, 31) and 100X (lanes 8, 16, 24, 32) amounts of unlabeled probe. (B and C) Representative gel shift blots of two independent experiments are shown.

fragments from the gel shift experiments (Figure 11) and the smaller fragments from regions 5 and 6 (Figure 12) downstream of a stop codon in the pEGFP-C2 vector. We transfected these constructs into HEK 293 cells with the GFP and GFP-FasL 3'UTR (full length) controls and performed Northern blotting to determine the expression of GFP reporter mRNA. To our surprise, all of these GFP constructs were expressed at similar levels to the GFP control vector (Figure 13). The lowered expression of the GFP-FasL 3'UTR relative to GFP is most probably due to destabilizing RNA binding proteins. The inability of any fragment to recapitulate the effect of the full length FasL 3'UTR indicates that there is an interaction between these regions either by the formation of secondary or tertiary folded mRNA structures or by cooperative protein binding which is necessary for the destabilization of the transcripts bearing the FasL 3'UTR.

2.5.3 HuR protein is required for the expression of an mRNA containing FasL 3'UTR

The data described above suggest that ARE sequences could collaborate with HuR to ensure the rapid expression of FasL mRNA during T cell activation. Hence, we assessed whether the expression of FasL mRNA depends on HuR in cells treated with activators such as PMA or PHA. Ideally, we would have liked to test this possibility in the context of the full-length FasL message by following its expression in the presence or absence of HuR. However, expressing the full-length FasL mRNA caused massive cell death in different cell lines including Jurkat regardless of stimulus (data not shown). Therefore we used GFP reporter constructs in which we fused the FasL 3'UTR to the GFP coding sequence. The entire FasL 3'UTR was included in the reporter construct since any or all of the HuR binding sites described in Figures 11 and 12 could mediate



Northern blot ***blot***

Figure 13: Fragments of the FasL 3'UTR do not recapitulate the effect of the full length FasL UTR on GFP expression.

HEK 293 cells were mock transfected (lane 1), transfected with GFP control vector (lane 2), GFP FasL 3'UTR vector (lane 3) or GFP with the indicated FasL 3'UTR fragments (lanes 4-15) used in gel shift assays to map HuR interactions. The cells were harvested for RNA extraction 16 hours post transfection for Northern blot.

regulatory effects. Surprisingly, flow cytometry experiments showed that the level of expressed GFP was reduced by > 55% in Jurkat cells transfected with GFP-FasL-3'UTR when compared to GFP alone (Figure 14A). This is probably due to other factors which in the absence of any stimulus bind the FasL 3'UTR in trans and promote its rapid decay. To eliminate the possibility that poor transfection efficiency in Jurkat cells led to the evaluation of a selected population, we transfected HEK 293 cells with the same constructs and obtained similar results by flow cytometry (Figure 14B). In agreement with GFP protein levels, Northern blot analysis showed that in the absence of any treatment, the amount of GFP-FasL3'UTR mRNA in HEK 293 cells was significantly decreased compared to the GFP control (Figures 14C, D). These experiments suggest that in the absence of extracellular stimulus, the FasL-3'UTR mediates the rapid decay of the GFP reporter mRNA.

Due to the effects of PMA or PHA treatment on FasL expression, we investigated whether these actions were recapitulated on the expression of the GFP-conjugated FasL-3'UTR. The GFP-FasL-3'UTR or GFP plasmids were transfected into HEK 293 cells which were then treated or not with PHA or PMA for 4 hours. Since the HEK 293 cells have been shown to activate the PKC pathway in response to PMA (Bol et al., 1997), we expected that the PMA treatment of these cells will mimic the effect seen in activated Jurkat cells. Indeed, by flow cytometric analysis we observed that though PHA had no effect on GFP-FasL 3'UTR protein levels (data not shown), PMA rescued GFP protein expression in these cells (Figure 15A). Using Northern blot analysis we observed a

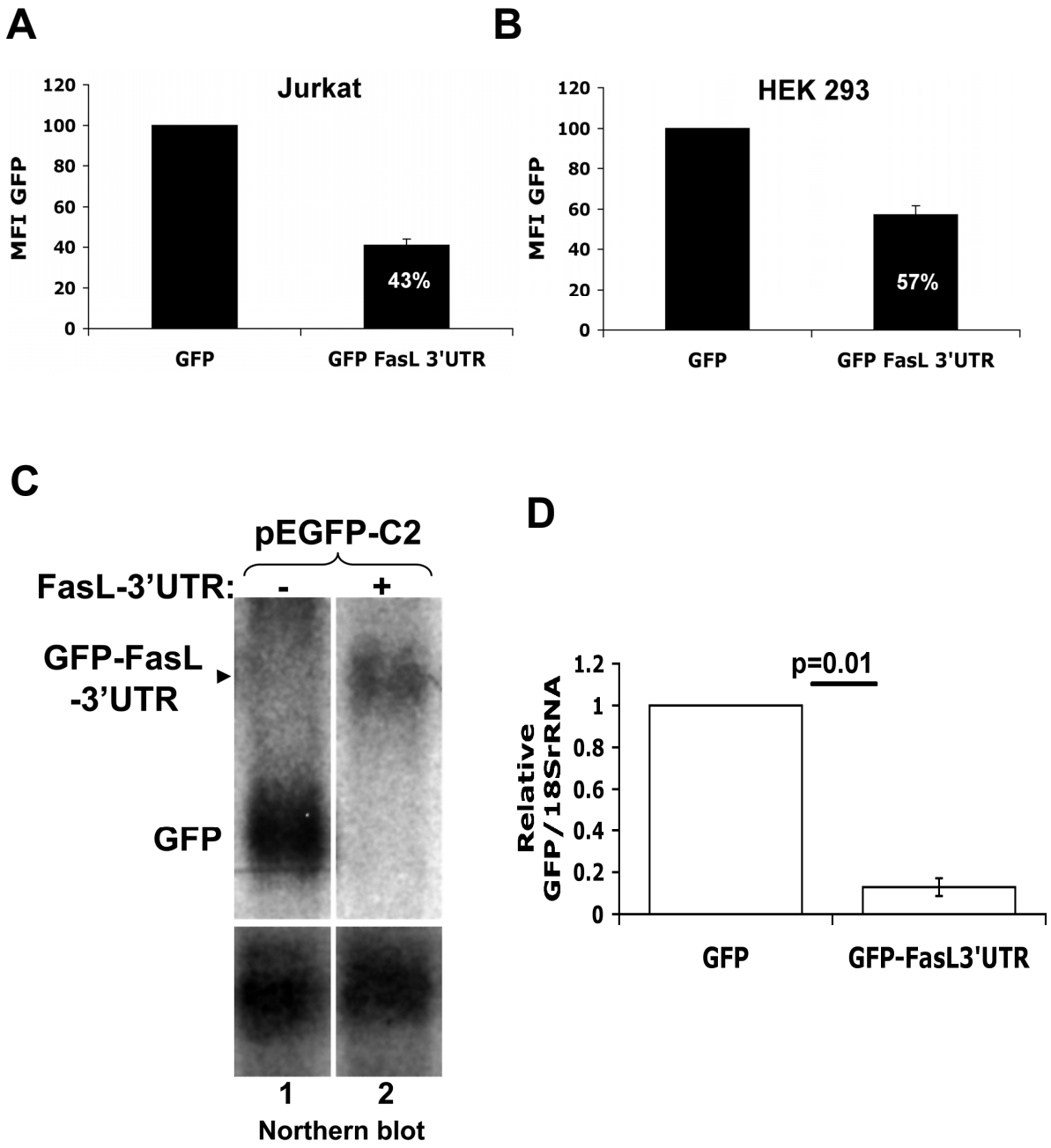

A
Jurkat
MFI GFP
120
100
80
60
40
20
0
43%
GFP
GFP FasL 3'UTR
B
HEK 293
MFI GFP
120
100
80
60
40
20
0
57%
GFP
GFP FasL 3'UTR
C
pEGFP-C2
FasL-3'UTR:
-
+
GFP-FasL
-3'UTR
GFP
1
2
Northern blot
D
p=0.01
Relative
GFP/18SrRNA
1.2
1
0.8
0.6
0.4
0.2
0
GFP
GFP-FasL3'UTR

Figure 14: Fas ligand 3'UTR decreases the mRNA and protein levels of a GFP reporter in both Jurkats and HEK 293 cells.

(A-B) Jurkats (A) and HEK 293 (B) cells were transfected with pEGFP-C2 control and pEGFP-C2-FasL3'UTR. Flow cytometric analysis of GFP expression was performed 24 hours post transfection. (C) RNA was isolated from the pEGFP-C2 (lane 1) and pEGFP-C2-FasL3'UTR (lane 2) transfected HEK 293 cells for Northern blot for GFP and 18S rRNA control. (D) The levels of GFP mRNA were quantified using the ImageQuant software program (Molecular Dynamics, USA). Levels were then standardized against 18S levels and plotted as the percentage +/- the standard deviation of three independent experiments.

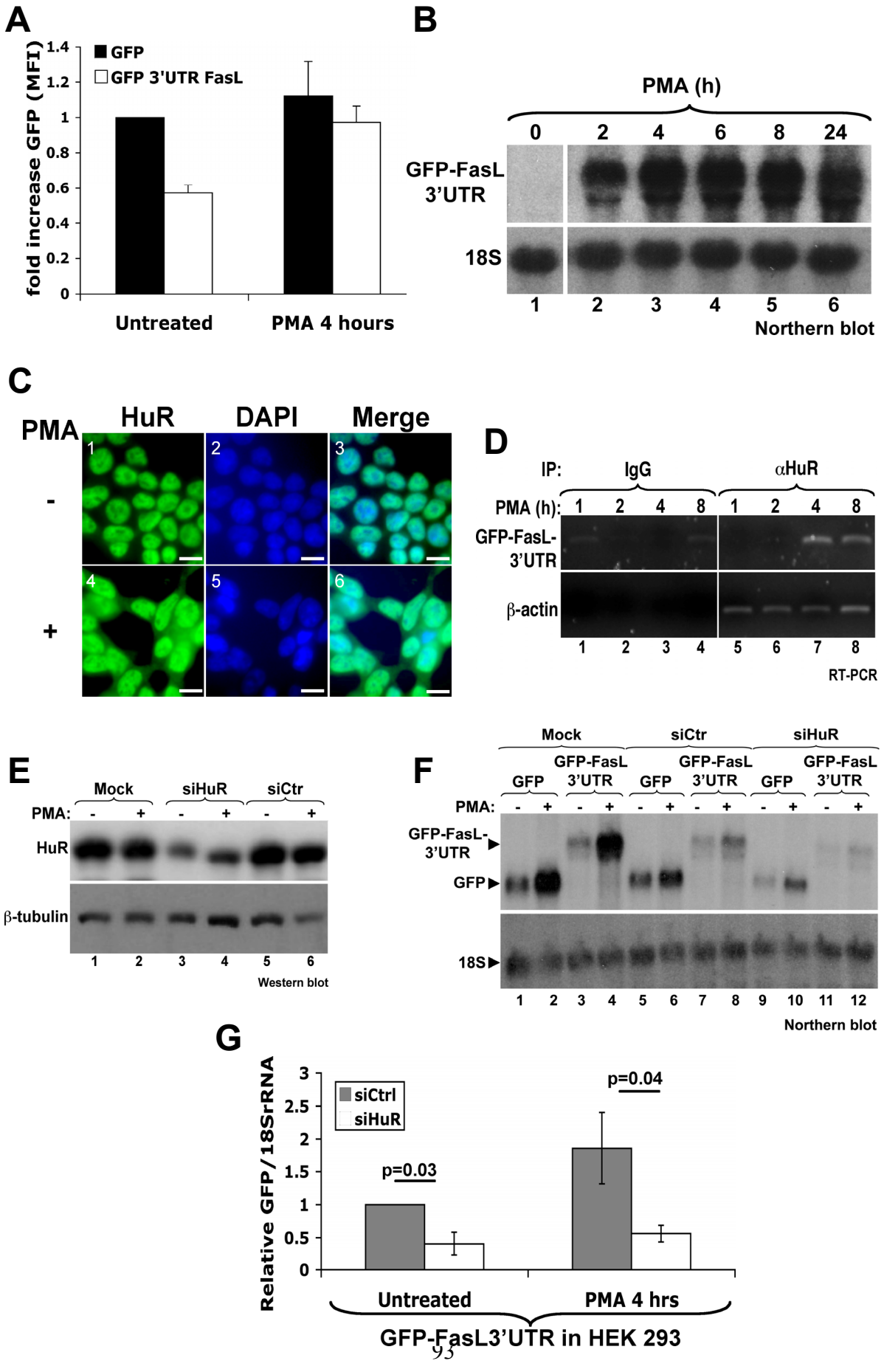

A
fold increase GFP (MFI)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
GFP
GFP 3'UTR FasL
Untreated
PMA 4 hours
B
PMA (h)
0
2
4
6
8
24
GFP-FasL 3'UTR
18S
1
2
3
4
5
6
Northern blot
C
PMA
HuR
DAPI
Merge
-
+
1
2
3
4
5
6
D
IP:
IgG
αHuR
PMA (h):
1
2
4
8
1
2
4
8
GFP-FasL-3'UTR
β-actin
1
2
3
4
5
6
7
8
RT-PCR
E
Mock
siHuR
siCtr
PMA:
-
+
-
+
-
+
HuR
β-tubulin
1
2
3
4
5
6
Western blot
F
Mock
siCtr
siHuR
GFP
GFP-FasL 3'UTR
GFP
GFP-FasL 3'UTR
GFP
GFP-FasL 3'UTR
PMA:
-
+
-
+
-
+
-
+
-
+
-
+
GFP-FasL-3'UTR
GFP
18S
1
2
3
4
5
6
7
8
9
10
11
12
Northern blot
G
Relative GFP/18SrRNA
3
2.5
2
1.5
1
0.5
0
siCtrl
siHuR
p=0.03
p=0.04
Untreated
PMA 4 hrs
GFP-FasL3'UTR in HEK 293

Figure 15: The expression of the GFP-FasL-3'UTR mRNA and protein in conditions of PKC activation is mediated by HuR.

(A) HEK 293 cells were transfected with either GFP (filled) or GFP-FasL3'UTR (open). These cells were treated for 4 hours with 50ng/ml PMA 16 hours post-transfection. GFP protein expression was assessed by flow cytometry. (B) HEK 293 cells were transfected with plasmids expressing GFP or GFP-FasL3'UTR as described in A. Total mRNA from untreated (0) or cells treated with 50ng/ml PMA for 2, 4, 6, 8, and 24 hours (lanes 2 -6) were harvested and used for Northern blot analysis with specific radioloabeled DNA probes against GFP mRNA and 18S as loading control. Representative blots of three independent experiments are shown. (C) HEK 293 cells were left untreated (panels 1-3) or treated for 4 hours with 50 ng/ml PMA, (panels 4-6) fixed, permeabilized and stained with 3A2 (anti-HuR) followed by anti-mouse conjugated to Alexafluor488 (panels 1, 4) and DAPI (panels 2, 5). Merged HuR and DAPI images are shown in panels 3 and 6. A single representative field for each cell treatment of two independent experiments is shown. Bars, 20μm. (D) RNA immunoprecipitation of extracts from HEK 293 cells stimulated with PMA 50ng/ml for 1 (lanes 1, 5), 2 (lanes 2, 6), 4, (lanes 3, 7) and 8(lanes 4, 8) hours with 3A2 (anti HuR) (lanes 5-8) antibody or mouse IgG1 isotype control (lanes 1-4). Immunoprecipitation was followed by RT-PCR for GFP (upper) and β-actin (lower) as a positive control. (E-F) HEK 293 cells were mock, siHuR or control siRNA (si ctrl) transfected at 0 and 24 hours. This was followed by GFP or GFP-FasL3'UTR transfection and PMA treatment for 4 hours. (E) Total cell extracts from HEK 293 cells treated with siRNA and transfected with GFP-FasL3'UTR and treated or not with PMA as described above were collected and used for Western blot with the anti-HuR and

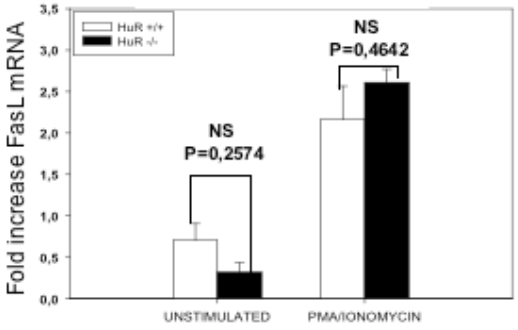
tubulin antibodies. (F) Total mRNA were prepared from the same samples described in (E) or from cells transfected with GFP plasmid and were used for Northern blot analysis using GFP and 18S rRNA radiolabeled probes. (G) The levels of GFP mRNA were quantified and standardized as described in (Figure 14D). Levels for each treatment were plotted as the percentage +/- the standard deviation of two independent experiments.

significant increase in GFP-FasL-3'UTR mRNA expression after 2 to 24 hours of PMA stimulation (Figure 15B).

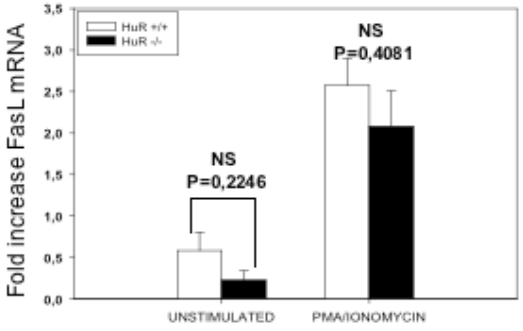
HuR has previously been reported to translocate to the cytoplasm in order to stabilize target mRNAs in a PKC dependent manner (Doller et al., 2008a). In agreement with these findings, we observed a rapid translocation of HuR from the nucleus to the cytoplasm in HEK 293 cells that became visible after 4 hours of PMA treatment (Figure 15C). Therefore, we tested whether the interaction of GFP-FasL-3'UTR with HuR is dependent on PKC activation. Cells transfected with this reporter gene were treated for various periods of time with PMA and then used to perform an immunoprecipitation experiment with the anti-HuR antibody that was followed by RT-PCR analysis. The data showed that GFP-FasL-3'UTR associated with HuR only after 4 to 8 hours of PMA treatment (Figure 15D). Next, we tested whether HuR is required for PMA-mediated expression of GFP-FasL-3'UTR mRNA. We depleted HuR expression in HEK 293 cells containing GFP-FasL-3'UTR or GFP control using specific siRNA duplexes (Mazroui et al., 2008) in the presence or absence of PMA. We were able to deplete HuR expression by >50% (Figure 15E). Our experiments showed that in HuR knockdown cells, PMA treatment did not affect GFP expression however, it failed to induce the expression of GFP-FasL-3'UTR mRNA (Figures 15F-G). This indicates that HuR is indeed responsible for the increased expression of GFP-FasL-3'UTR mRNA during (PMA-mediated) PKC activation.

In addition, we tested the effect of HuR depletion on FasL mRNA in *ex vivo* T lymphocytes. Briefly, mixed or CD8+ T lymphocytes were isolated from the thymi and spleens from T cell specific HuR knockout animals, $LckCre^{+}Elavl^{fl/fl}$, and wild type control mice. The mixed and purified T cell populations were left unstimulated as controls or were stimulated with 50ng/ml PMA and 2μM ionomycin to induce FasL expression. The expression of FasL mRNA relative to 18S rRNA control by qPCR indicates that there is a non-significant decrease in FasL expression in unstimulated $LckCre^{+}Elavl^{fl/fl}$ thymocytes and splenocytes compared to wild type (Figure 16A and B). This trend is abolished upon PMA and ionomycin stimulation of these mixed populations (Figure 16A and B). In contrast, the CD8+ sorted splenic and thymic populations show an increase in FasL expression in the PMA and ionomycin stimulated $LckCre^{+}Elavl^{fl/fl}$ cells (Figure 16C). Interestingly, the stimulated thymic T cells as a mixed population show very little difference in FasL mRNA expression between $LckCre^{+}Elavl^{fl/fl}$ and wild type controls (Figure 16B). However, there is a clear trend in the CD8+ thymocyte population to increase FasL expression in the absence of HuR. Corollary to this finding is that the non-CD8+ thymocytes probably show an equal an opposite effect of HuR on FasL mRNA expression. Unfortunately, the effect of HuR ablation in $CD4^{+}$ thymocytes was not investigated.

A **FasL mRNA expression in total splenocytes**



B **FasL mRNA expression in total thymocytes**



C **FasL mRNA expression in PMA/ionomycin activated CD8+ T lymphocytes**

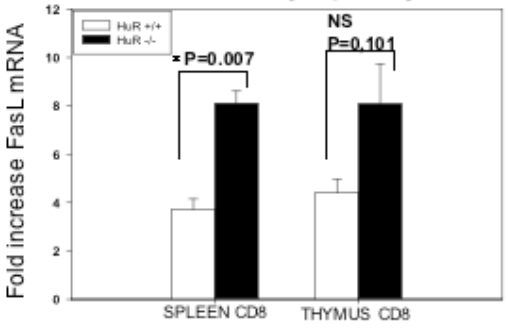


Figure 16: Effects of HuR depletion on ex vivo splenocyte and thymocyte cultures. Splenocytes (A), thymoctes (B), $CD8^+$ thymocytes and $CD8^+$ splenocytes (C) were harvested and purified from wild type and $LckCre^+Elavl^{fl/fl}$ mice. These cells were stimulated for 4 hours with PMA (50ng/ml) and 2 μM ionomycin to induce FasL expression. Cells were harvested for RNA extraction and qRT-PCR for FasL and 18S rRNA control.

2.6 Discussion

Regulation of cytokine mRNAs by posttranscriptional mechanisms at the level of splicing, subcellular localization, stability and translational efficiency is requisite for their appropriate expression (Katsanou et al., 2006). However, this level of regulation has never been investigated for FasL. In this study we demonstrate that the expression of the FasL mRNA is regulated posttranscriptionally by a mechanism that involves the HuR protein. The FasL 3'UTR harbours two ARE sequences that are similar in structure and U-content to the TNFα AREs. Similarly to the TNFα message, HuR directly binds to FasL mRNA in an ARE-dependent manner. This binding depends on the activation of PKC-induced pathways that in turn trigger the rapid cytoplasmic accumulation of HuR leading to the expression of FasL mRNA. Therefore, collectively, our data support a model whereby HuR protein plays a key role in the transient expression of FasL mRNA in response to PKC activators. This suggests that HuR could be involved in T lymphocyte activation and selection which directly affects the homeostasis of the lymphoid system and the duration of the immune response.

HuR is typically involved in relocalizing, stabilizing and modulating the translational efficiency of ARE containing mRNAs. In the case of TNFα, there are two AREs in the 3'UTR. Interestingly, these AREs mediate distinct posttranscriptional effects on TNFα message (Di Marco et al., 2001; Fialcowitz et al., 2005a). The first ARE binds HuR and is responsible for LPS mediated TNFα expression. Though normally this region mediates the destabilization and the translational repression of

TNFα mRNA in macrophages, upon LPS stimulus, this ARE also allows for the stabilization of the TNFα message via HuR (Di Marco et al., 2001; Ford et al., 1999b). Although, it has been suggested that ARE2 modulates TNFα mRNA expression and protein abundance (Di Marco et al., 2001; Ford et al., 1999b), the molecular mechanisms behind these effects are still unknown. Our observations suggest that though FasL and TNFα mRNAs have little sequence similarity in the first ARE, the secondary structure of the hairpin required for the recruitment of ARE binding proteins (Fialcowitz et al., 2005a) is conserved in addition to considerable sequence and structural homology in the second ARE (Figure 9). This indicates that these two TNF superfamily members could be posttranscriptionally regulated by similar mechanisms. Indeed, consistent with TNFα, our study shows that HuR associates strongly and specifically with two regions of the FasL 3'UTR which span both putative AU rich regulatory regions, including the fragment (6.2) which contains the AUUUA pentamer. Although this suggests that HuR regulates the half-life of FasL mRNA via association with these two AREs, the fact that HuR also interacts with other U-rich elements in the FasL 3'UTR (Figure 11), argues that association with multiple regions could also be required for the HuR-mediated activation of FasL mRNA expression. The expression of the TNFα mRNA in macrophages (Katsanou et al., 2006) may likewise parallel the up regulation of FasL mRNA expression mediated by HuR driven by mitogenic stimulus that activates posttranscriptional mechanisms such as mRNA relocalization and/or stabilization.

Due to the multitude of HuR binding sites that we found by gel shift assay on fragments of the 3'UTR, we wondered whether all of these regions were involved in the

regulation of the FasL 3'UTR and tested each region with GFP reporter constructs. We were expecting that some of the regions which bound especially strongly to HuR, for example regions 5 and 6, would be able to recapitulate the decreased expression observed when the full length FasL 3'UTR is used with a GFP reporter. Surprisingly, this was not the case since all of these constructs were expressed as well as the GFP control vector. The decreased mRNA expression observed with the GFP FasL 3'UTR, is not due to HuR binding to AU rich sequences because HuR exclusively mediates mRNA stabilization and not decay. In fact, AMD is regulated in part by the preferential recruitment of either stabilizing or destabilizing AUBPs. The segmentation of the FasL 3'UTR seems to preferentially allow for the recruitment of stabilizing AUBPs, discourages the association of the destabilizing AUBPs, or both. This may be due to either the importance of folded mRNA structures which cannot form in the truncated portions of the FasL 3'UTR and thus do not allow AUBP recruitment. Conversely, some AUBPs, notably HuR have been shown to bind cooperatively to mRNA (Fialcowitz-White et al., 2007). Therefore it is possible that several weak AUBP binding sites through out the FasL 3'UTR are required to effectively recruit AUBPs. It is also possible, that the segmentation of the FasL 3'UTR could disrupt the recruitment of AUBPs via miRNA binding. Though the mechanisms are unknown, there is some evidence which points to recruitment of AUBPs by miRNA binding (Jing et al., 2005). The subtleties of FasL 3'UTR regulation by AUBPs in T lymphocytes in quiescence or TCR stimulation should be further investigated. These results suggest that the regulation of this transcript may be complex.

Here we show that PKC activation correlates with the transient expression of FasL mRNA (Figure 15). Cell treatments with the PKC agonist PMA leads to the expression of FasL or the GFP-Fasl-3'UTR mRNAs in a mechanism that involves their association with HuR in an ARE-dependent manner. This is consistent with previous reports showing that HuR is phosphorylated by PKCα at serines (Ser) 158 and 221 (Doller et al., 2007). Interestingly, Ser 221 is located within the hinge region of HuR that is known to regulate its nucleocytoplasmic shuttling (Fan and Steitz, 1998a). The activation of PKC has been recently tied to the relocalization of HuR to the cytoplasm and the stabilization of target mRNAs in this subcellular compartment (Doller et al., 2008b). This is concordant with previous findings, showing that T cell activation via PKC pathways triggers the HuR-mediated cytoplasmic translocation of the CD83 mRNA and this involves the phosphorylation of the HuR protein ligand APRIL (Chemnitz et al., 2009). Our results raise the possibility that FasL mRNA could be regulated the same way, since PKC activation causes HuR to relocalize to the cytoplasm and this correlates with an increase in its association with a reporter mRNA fused to the FasL 3'UTR (Figures 15C, D). Indeed, protein ligands such as pp32 and APRIL have been shown to regulate the export of HuR and some of its mRNA targets in different cell systems (Doller et al., 2008b). Hence, the PKC-mediated phosphorylation of HuR may potentially provide a mechanism for FasL mRNA not only to be stabilized but also to be rapidly translocated to the translation machinery in the cytoplasm for protein synthesis. Exploring the implication of HuR protein ligands such as pp32 and APRIL in regulating the cellular movement of FasL mRNA in activated T cells could help us better understand how the

expression of this message is modulated during normal conditions and during the activation of an immune response.

Though the results we obtained in HEK 293 cells were compelling, we tested the effect of HuR on Fas ligand expression in a more physiological paradigm. Recently, the floxed HuR mice have been produced and have been used to generate T lymphocyte specific HuR knock out animals (Papadaki et al., 2009). Using this system, we tested the effect of HuR on FasL mRNA expression in mixed splenocytes, thymocytes and $CD8^+$ purified populations from each of these tissues. Interstingly, in the resting splenic and thymic cultures there was a decrease in FasL expression that did not reach statistical significance, which correlates well with our results in HEK 293 cells with the GFP reporter constructs and with the association that we observed between FasL mRNA and HuR in stimulated Jurkat cells. This difference in FasL expression was not apparent in the stimulated cultures. A different picture emerges when the $CD8^+$ cells are stimulated: the expression of FasL is increased when HuR is not present. Normally we would have expected a decrease in FasL mRNA if HuR was not able to stabilize the transcript. This unexpected finding could be explained either by differences in post-transcriptional regulation of FasL in our experimental systems and in CD8+ T cells or by an indirect mechanism. It has been shown in the T cell specific HuR knock out mice that TTP, which is a destabilizing AUBP, is significantly over expressed compared to control mice (Papadaki et al., 2009). In addition, the effect of HuR depletion on FasL mRNA in $CD8^+$ T cell populations must be counterbalanced by an opposing response if the mixed cultures from the same tissues show no effect. Therefore it is probable that the HuR

depleted CD4+ T cells in the thymus, and the CD4+ T cells, NK cells, B cells, or macrophages in the spleen exhibit a decrease in FasL expression in response to PMA and ionomycin stimulation compared to wild type animals. Again, this level of regulation needs to be further investigated to dissect the exact posttranscriptional regulatory mechanisms acting on FasL mRNA.

Though there is currently some controversy towards the functions of FasL in the immune system (Strasser et al., 2009), there is a consensus that the Fas-FasL signalling pathway is required for immune tolerance and homeostasis as evidenced by natural mouse mutants and autoimmune proliferative syndrome (ALPS) patients (Strasser et al., 2009). In addition, there have been several studies linking defects in Fas induced apoptosis to auto-immune diseases such as systemic lupus erythrematosis (SLE). One study in particular links the number of microsatellite repeats in the FasL 3'UTR to SLE (Chen et al., 2006b). Interestingly, this microsatellite repeat is located in region 5.2, which we show binds HuR very strongly (Figure 12). Thus it is possible that HuR-mediated posttranscriptional regulation of FasL is important for the maintenance of immune tolerance.

Our observations indicate that despite the high level of expression of HuR protein in unstimulated cells (Figure 15E), FasL or the GFP-FasL-3'UTR messages are rapidly degraded (Figure 14). It is possible, that under these conditions, the FasL mRNA remains in the nucleus where it associates with factors known to promote the AU-rich Mediated mRNA decay (AMD) pathway (von Roretz and Gallouzi, 2008). There are several well

characterized activators of AMD which are members of the CCCH zinc finger protein family that include proteins such as tristetraprolin (TTP), Butyrate response factor 1 (BRF1) and KH-type splicing regulatory protein (KSRP) (Cao et al., 2007; Dean et al., 2004; Gherzi et al., 2006; Stoecklin et al., 2002). It has been shown that under different growth conditions HuR competes for binding to AREs with KSRP and TTP proteins (Doller et al., 2008b). In addition, HuR has been described as a key player in the transfer of some of its target transcripts from processing bodies (PB) (a site of mRNA decay) to polysomes (a site of translation) (Bhattacharyya et al., 2006). Thus, it is possible that in stimulated T cells, where PKC pathways are activated, binding of HuR to the FasL mRNA is favoured leading to its stabilization and rapid translation. Therefore, assessing the implication of proteins such as TTP, BRF1 and KSRP in regulating FasL mRNA expression before and after stimulation and defining how their function could be counter balanced by HuR, could provide new strategies to control T lymphocyte responses under normal and or pathological conditions. This may lead to the identification of novel therapeutic targets/tools for the treatment of autoimmune diseases.

Chapter 3: Thalidomide as potential modulator of Fas-FasL pathway

3.1 Introduction

Thalidomide is best known for the teratogenic effects it caused while being used to treat morning sickness in pregnant women during the late 1950's to early 1960s (Lenz, 1988). Though it was initially developed as a sedative, more recently it has been shown to have important immunomodulatory effects and is approved for the treatment of multiple myeloma, a plasma B cell malignancy (Hicks et al., 2008), and erythrema nodosum leprosum (ENL), an inflammatory complication of leprosy (Van Veen et al., 2009). Currently, thalidomide and its analogues are under review as a treatment for a variety of other autoimmune diseases such as Crohn's disease (Akobeng and Stokkers, 2009; Srinivasan and Akobeng, 2009) and Behçets disease (Direskeneli et al., 2008; Green et al., 2008).

Thalidomide is thought to mediate immunomodulatory effects through a number of targets. These include a number of cytokines including several interleukins, interferons, TGFβ, and VEGF, which are mostly downregulated in pro-inflammatory settings upon thalidomide treatment (reviewed in (Paravar and Lee, 2008)). Exceptions to this are IL-2, IL-12, and IFNγ, (Davies et al., 2001; Haslett et al., 2005) which are induced by thalidomide treatment. Other cytokines such as IL-8 and IL-10 are either up or down regulated depending on cellular context (Corral et al., 1999; Moreira et al., 1997; Nasca et al., 1999; Ye et al., 2006).

Despite the pleiotropic effects of thalidomide on the immune system, the prototypical target of thalidomide is TNFα. TNFα mRNA levels in LPS activated monocytes are significantly reduced by thalidomide treatment (Sampaio et al., 1991). TNFα mRNA in LPS activated monocytes is rapidly degraded upon treatment with

thalidomide or its analogues, leading to lower steady state levels of TNFα (Moreira et al., 1993; Zhu et al., 2003). Thus TNFα is regulated at a post-transcriptional level by thalidomide. In addition, thalidomide has been shown to act on a number of other transiently expressed cytokines such as TRAIL, FasL as well as TNFα in multiple myeloma patients though the mechanism has not yet been investigated (Grzasko et al., 2006).

Interestingly, thalidomide also modulates inducible cyclo-oxygenase, COX-2, whose enzymatic activities are necessary for the biosynthesis of pro-inflammatory prostaglandins (Hla and Neilson, 1992). The mechanism by which COX-2 is downregulated by thalidomide is like TNFα, at the level of mRNA stability (Jin et al., 2007). Normally, HuR stabilizes COX-2 mRNA and the induction of COX-2 mRNA is dependent on its association with HuR (Sengupta et al., 2003). There is evidence that HuR is phosphorylated upon PKC activation, which causes shuttling from the nucleus to the cytoplasm with the associated COX-2 mRNA. Cytoplasmic localization of COX-2 mRNA promotes its stabilization and expression (Doller et al., 2009; Doller et al., 2007). The decrease in COX-2 mRNA stability caused by thalidomide treatment is due to a decrease in p38MAPK activation, which causes HuR to remain in the nucleus upon IL-1β stimulus. Thus HuR cannot relocalize the COX-2 mRNA to the cytoplasm and prevent AMD resulting in diminished mRNA stability (Jin et al., 2007).

FasL is regulated by thalidomide, in that its production is decreased in the serum of multiple myeloma patients receiving thalidomide therapeutically (Grzasko et al., 2006) and FasL protein abundance are decreased in the spinal cords of mouse models of amyotrophic lateral sclerosis receiving thalidomide or lenalidomide (Kiaei et al., 2006).

However, there are also reports that in NK cells, FasL production is increased in response to the thalidomide analogue lenalidomide (Wu et al., 2008). Therefore, the fact that FasL mRNA levels and p38 MAPK induced HuR re-localization are both targeted by thalidomide(Jin et al., 2007), and that based on our data showing an HuR-mediated regulation of FasL mRNA stability, we investigated the possibility of using thalidomide as a tool to modulate FasL expression in T Lymphocytes.

3.2 Materials and methods

Cell culture

Cell lines:

Jurkat cells (E6 clone) (ATCC) were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 (Invitrogen) augmented with 10% FBS (Invitrogen). TT24.8.A.1 NK T cells (gift from Dr Joyce Rauch) were maintained in DMEM (Invitrogen) supplemented with 1% non essential amino acids (Invitrogen), 1% L-glutamine (Invitrogen), 1% HEPES (Invitrogen) and 0.1% β-mercaptoethanol (Sigma).

T5 ex-vivo cells:

T5 cells were prepared according to the protocol of Baumann et al.(Baumann et al., 2005). Briefly, lymph nodes were harvested from 6-8 week old C57BL/6 mice, mechanically disaggregated and cultured 16 hours in RPMI 1640 supplemented with 10% FBS, 1% penicillin streptomycin (Invitrogen) and 1μg/ml concanavalin A. Cells were washed and cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin streptomycin and 2.5U/ml IL-2 (Peprotech) for 5 days.

Treatments:

Cells were stimulated with PHA-M (Sigma) at 1μg/ml or PMA (Sigma) at 50ng/ml and concanavlin A (2μg/ml) for times indicated. Stimulations with anti-CD3 (OKT3) were done with polystyrene cell culture plates precoated for 16 hours at 4°C with indicated concentrations of antibody, anti-CD28 (15E8) (Pelicluster) was used at concentrations and times indicated. Anti-FasL (NOK-1) was used at 10μg/ml, FasFc (Sigma) was used at 0.05μg/ml, membrane bound FasL (Upstate) was used at 1:2000 dilution, anti-Fas (Upstate) (CH11) was used at 1:5000 dilution. Thalidomide (Sigma) was a mixture of R and S enantiomers solubilized in DMSO, and all treatments were performed at 50μg/ml. Cycloheximide (Sigma) was solubilized in ethanol and used at a final concentration of 10μg/ml for times indicated.

FACS

Cells were counted by using a hemocytometer and adjusted to a concentration of $1x10^6$ cells/ml. 0.5 ml of cells was washed 3 times in 1.5ml PBS and then resuspended in 50 μl binding buffer (0.01 M HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM $CaCl_2$) with Annexin V-FITC (Biolegend) according to manufacturers instructions and PI (Sigma) (20μg/ml) for 15 minutes. Samples were diluted to 350 μl with binding buffer prior to acquisition of data on the FACScan (Becton Dickinson). For tracking AICD, 50 μl peripheral blood was washed 3 times in 1.5 ml PBS, followed by lysis in 1x PharmLyse (BD Pharmingen) for 5 minutes at room temperature. The lysis buffer was neutralized and the cells resuspended in 50 μl PBS 2% FBS FACS buffer with anti Vβ8-PE (F23.1) (Santa Cruz Biotechnology) and CD4-FITC (BD Pharmingen) according to manufacturers instructions. Cells were washed in FACS buffer 3 times prior to data acquisition. The

same staining protocol was used for sorting $CD4^{+}V\beta8^{+}$ and $CD4^{+}V\beta8^{-}$ populations on the FACS ARIA. The analysis of these data was performed with FlowJo software.

q-PCR

RNA was isolated for RT q-PCR by using the ChargeSwitch RNA isolation kit (Invitrogen) scaled to 20% of manufacturers instructions. RNA was quantitated using the Ribogreen kit (Molecular Probes) and 150ng was reverse transcribed using the Sensiscript reverse transcription kit (Qiagen). Subsequently, the cDNA was PCR amplified with primers described in supplemental materials and methods, using the Quantitect SYBR green kit (Qiagen) in a Corbett Rotor Gene real time thermocycler. The Ct value was used to calculate the amount of the cDNA of interest by extrapolation from a standard curve.

SEB model of AICD:

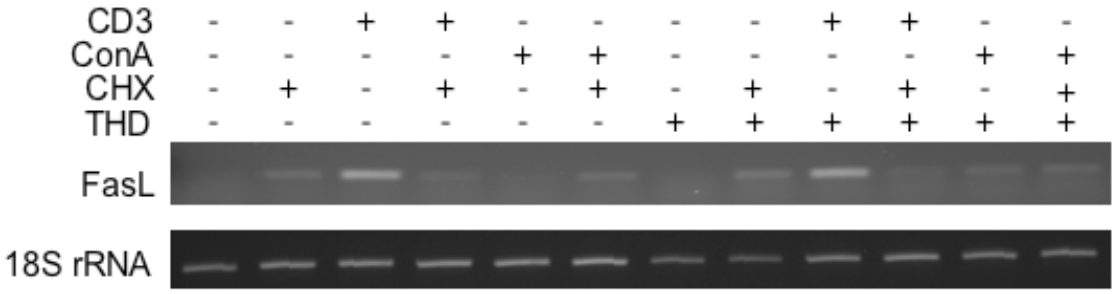
Peripheral blood collection (approximately 50 μl) was performed by tail venipuncture at days –3, 2, 4, and 7 relative to SEB administration. AICD was induced in 6-8 week old female C57/BL6 mice by i.p. administration of 20μg SEB (Sigma). Thalidomide (Sigma) 1.5 mg/ml (final dose 480 μg) or 30% DMSO vehicle control was administered i.p. 6 hours prior to SEB for the first dose, then every 12 hours for 9 days. On day 9 mice were sacrificed, blood was collected by cardiac puncture, lymph nodes and spleen were also harvested for analysis.

3.3 Results

3.3.1 FasL mRNA expression is not affected by thalidomide in CD3 and ConA stimulated NK T cells and T5 cells.

We first set out to determine whether we could recapitulate the effect of thalidomide on FasL mRNA expression in NK cells. We selected NK T cells for these experiments due to their well characterized role in autoimmunity (Yamamura et al., 2007). For these experiments we used TT24.8.A.1 NK T cell line which is a hybridoma derived from the fusion of C57/BL6 splenocytes stimulated with CD1d loaded peptide and a TCR- thymoma. Several of these hybridoma clones were expanded and characterized and the TT24.8.A.1 line was found to express NK1.1 as well as TCRα/β which categorizes these cells as NK T cells (Behar et al., 1999; Gumperz et al., 2000). To begin with, we stimulated theTT24.8.A.1 cells with plate bound 10ug/ml anti-CD3 antibody (1452C11) or with concanavalin A, an APC dependent T cell mitogen for 16 hours. To determine whether steady state levels of FasL mRNA were affected by thalidomide, and whether any effects were dependent on *de novo* protein synthesis, we concurrently treated these NKT cells with or without 50ug/ml thalidomide and/or cycloheximide (100ug/ml). This concentration of thalidomide was used since it is well above the 1-2 μg/ml serum concentrations attained with therapeutic dosage in human patients (Teo et al., 1999). We observed approximately a four fold increase in FasL mRNA abundance relative to 18S rRNA by semi-quantitative RT-PCR upon CD3 stimulation, in contrast concanavalin A had no effect on FasL expression due to the

NK T cells


CD3 - - + + - - - - + + - -
ConA - - - - + + - - - - + +
CHX - + - + - + - + - + - +
THD - - - - - - + + + + + +
FasL
18S rRNA

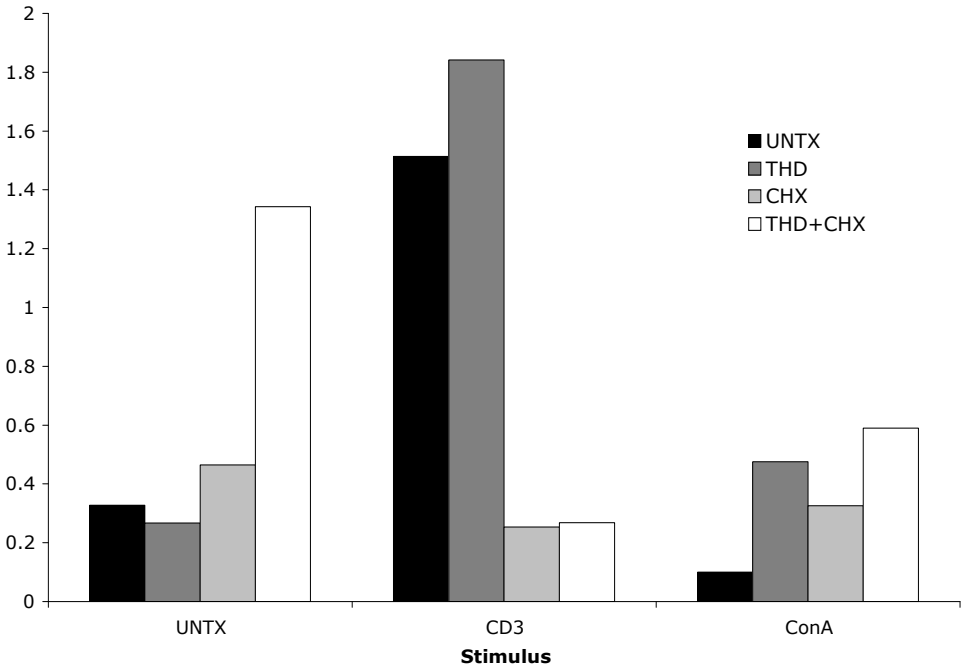

2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
UNTX
THD
CHX
THD+CHX
UNTX
CD3
ConA
Stimulus

Figure 17: The effect of thalidomide on activation induced FasL mRNA expression in murine NKT cells.

(A) NKT cells were stimulated with either anti CD3 antibody (1452C11) coated wells (10ug/ml) or 2ug/ml concanavalin A (ConA). Cells were concurrently treated with thalidomide (THD) (50ug/ml) and/or cycloheximide (CHX) (10ug/ml) for 16 hours, then RNA was extracted, reverse transcribed and PCR amplified. (B) Densitometric quantitation of the PCR amplified fragments on agarose gel.

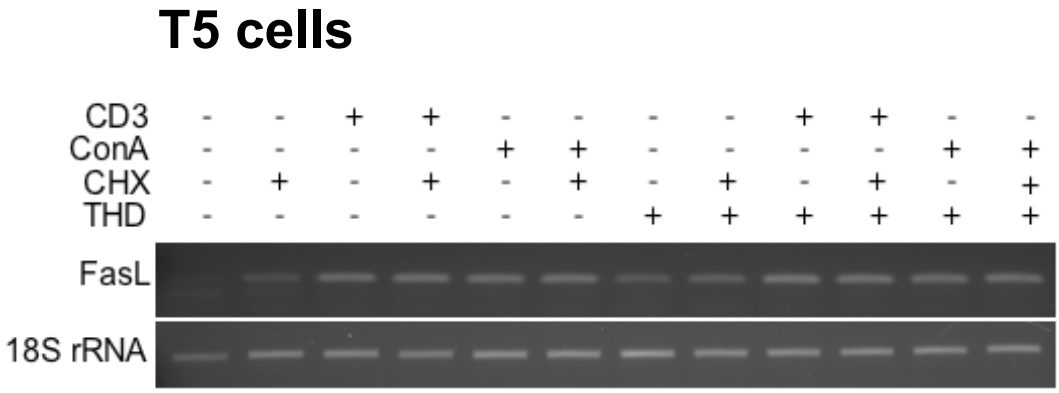
absence of APCs (Figure 17). This indicates that there are no residual APCs in this NK T cell line. Interestingly, the expression of FasL in NK T cells appears to be dependent on new protein synthesis since its induction is abrogated by cycloheximide treatment (Figure 17). However, we observed no increase in FasL mRNA expression due to thalidomide in the resting or CD3 stimulated NK T cells, which was expected based on the findings of Wu and colleagues (Wu et al., 2008). Our contrasting results may be due to differences in stimulus and cell type. Wu and colleagues stimulate NK cells with antibody bound tumour cells and thus engage the Fc receptors, conversely, we stimulated via the TCR. In addition, though NK and NK T cells share many features such as the rapid release of cytotoxic granules upon stimulation and the recognition of antibody bound targets via CD16, these cell types have clear functional differences in that NK T cells have been shown to respond to lipid antigens presented on CD1 while NK cells do not. Though we do not observe any differences in FasL mRNA expression in CD3 stimulated NK T cells attributable to thalidomide, this does not exclude a possible effect of thalidomide in other contexts.

Activated T lymphocytes are also robust and well characterized expressers of FasL. Therefore we decided to test whether FasL mRNA expression on *ex vivo* T5 T lymphocytes was affected by thalidomide treatment. Briefly, lymph nodes were harvested from 6 week old C57/BL6 mice and disaggregated into single cell suspensions. These cells were the stimulated with concanavalin A an APC dependent T cell mitogen. Concanavalin A was used since there are residual APCs from the lymph node cell preparation and it selectively expands the T cell populations.Subsequently, the expanded T cell population is maintained for 5 days in IL-2 containing media allowing for the T

lymphocytes to further out-compete the other cell types in the culture. Thus these T5 cells are highly enriched for T lymphocytes. These cells were then restimulated with either plate bound anti-CD3 antibody (1452C11) or concanavalin A for 16 hours to induce FasL mRNA expression. The T5 cells were concurrently treated with and without 50ug/ml thalidomide and/or cycloheximide. Similarly to the NK T cells we observed an increase in the amount of FasL mRNA relative to 18S rRNA control by semi-quantitative RT-PCR with TCR stimulation (Figure 18). In addition, we notice the induction of FasL mRNA with concanavalin A treatment since there must be residual APCs despite the T cell specific trophic support given during the isolation of these cells. Again, there are no differences in FasL expression attributable to thalidomide though in the case of T lymphocytes, FasL mRNA induction after mitogenic stimulus appears to be independent of new protein synthesis. It is possible that the p38 MAPK pathway by which thalidomide affects HuR localization (Jin et al., 2007) is not engaged by restimulated T5 cells. In addition, T5 cells are a mixed T cell population with a relevant persistent APC population. Therefore, it is possible that thalidomide exerts effects in a subset of T5 cells but that the rest of the population masks the result.

3.3.2 FasL mRNA expression is not induced by SEB therefore the effect of thalidomide cannot be tested.

Since thalidomide reduced FasL protein levels in a mouse model of amyotrophic lateral sclerosis (Kiaei et al., 2006), we decided to ascertain the effect of thalidomide on AICD *in vivo*. AICD is a FasL dependent process that deletes activated T cells from the periphery (Alderson et al., 1995; Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995). The staphylococcal enterotoxin B (SEB) mouse model of AICD was chosen since it leads


T5 cells
CD3 - - + + - - - - + + - -
ConA - - - - + + - - - - + +
CHX - + - + - + - + - + - +
THD - - - - - - + + + + + +
FasL
18S rRNA

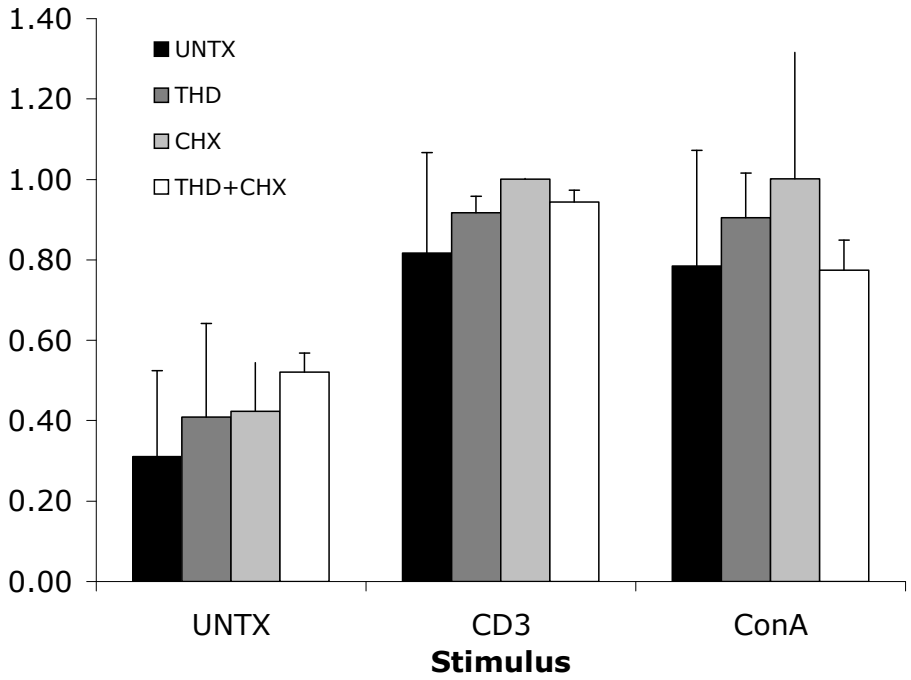
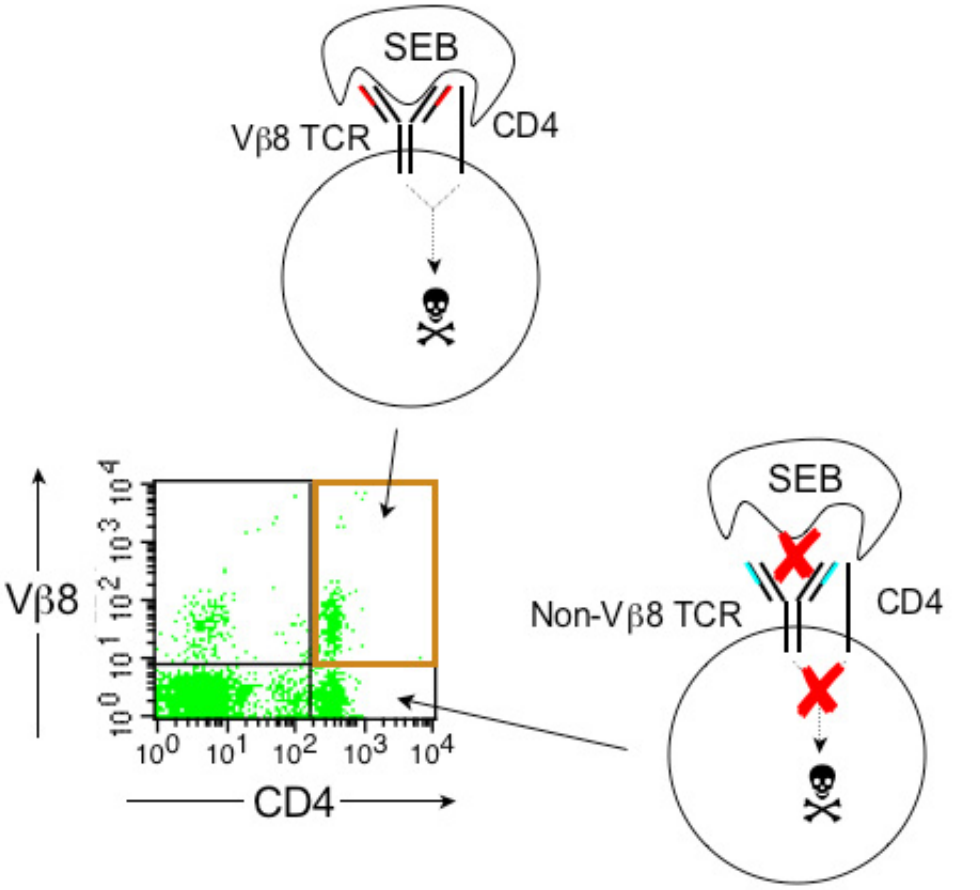

1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
UNTX
THD
CHX
THD+CHX
UNTX
CD3
ConA
Stimulus

Figure 18: The effect of thalidomide on activation induced FasL mRNA expression in murine T5 cells.

(A) T5 cells were stimulated with either anti CD3 antibody (1452C11) coated wells (10ug/ml) or 2ug/ml concanavalin A (ConA). Cells were concurrently treated with thalidomide (THD) (50ug/ml) and/or cycloheximide (CHX) (10ug/ml) for 16 hours, then RNA was extracted, reverse transcribed and PCR amplified. Picture shown is representative of 3 experiements. (B) Densitometric quantitation of the PCR amplified fragments on agarose gel.

to a traceable decrease in specific T lymphocyte subsets. SEB is a super-antigen, which allows it to activate T lymphocytes via the TCR regardless of co-stimulatory signals. SEB binds specifically to T lymphocytes bearing CD4 and the Vβ8fragment in the rearranged TCR. In this model of AICD, the introduction of SEB causes the rapid deletion of $CD4^{+}V\beta8^{+}$ T cells in the periphery (Kawabe and Ochi, 1991). Other T cell subsets remain unperturbed by SEB treatment and can serve as control unstimulated populations (summarized in Figure 19).

We tracked SEB mediated deletion of $CD4^{+}V\beta8^{+}$ T cells in the peripheral blood over 9 days with or without thalidomide treatment in 6-8 week old female C57/BL6 mice. We harvested 50μl of peripheral blood 3 days prior to the experiment to determine the baseline levels of $CD4^{+}$ $V\beta8^{+}$ T cells in each mouse by flow cytometry. SEB (20μg) was injected intra peritoneally into 9 mice, 5 of these received DMSO vehicle injections every 12 hours for the following 9 days, while 4 mice received 480μg thalidomide every 12 hours for the following 9 days. A further increase in thalidomide dose was not possible due to limitations of the solubility of thalidomide and the maximum volume which can be injected into the peritoneal cavity. We harvested 50 μl of peripheral blood on days 2, 4, and 7 from each mouse to track the changes in $CD4^{+}$ $V\beta8^{+}$ T cells by flow cytometry. On day 9, blood was collected by cardiac puncture and the spleen and lymph nodes were harvested from these mice for flow cytometric analysis of the $CD4^{+}V\beta8^{+}$ and control $CD4^{+}V\beta8^{-}$T lymphocyte populations. Peripheral blood samples show a significant decrease in the proportion of $CD4^{+}V\beta8^{+}$ T lymphocytes by 4 days after SEB injection, indicating that these cells have been deleted by AICD (Figure 20). Although, mice receiving thalidomide were visibly sedated, one of the side-effects of this drug, they


SEB
Vβ8 TCR
CD4
Vβ8
CD4
SEB
Non-Vβ8 TCR
CD4

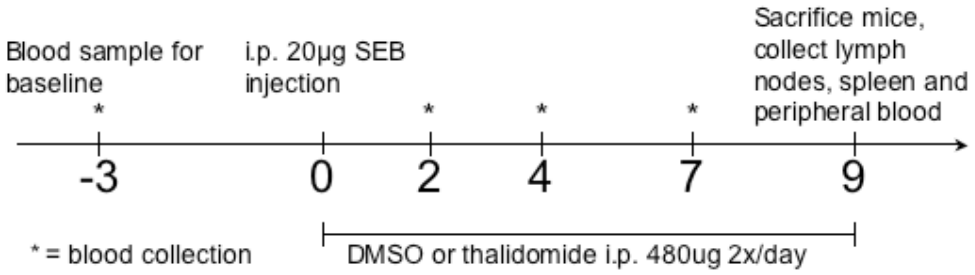
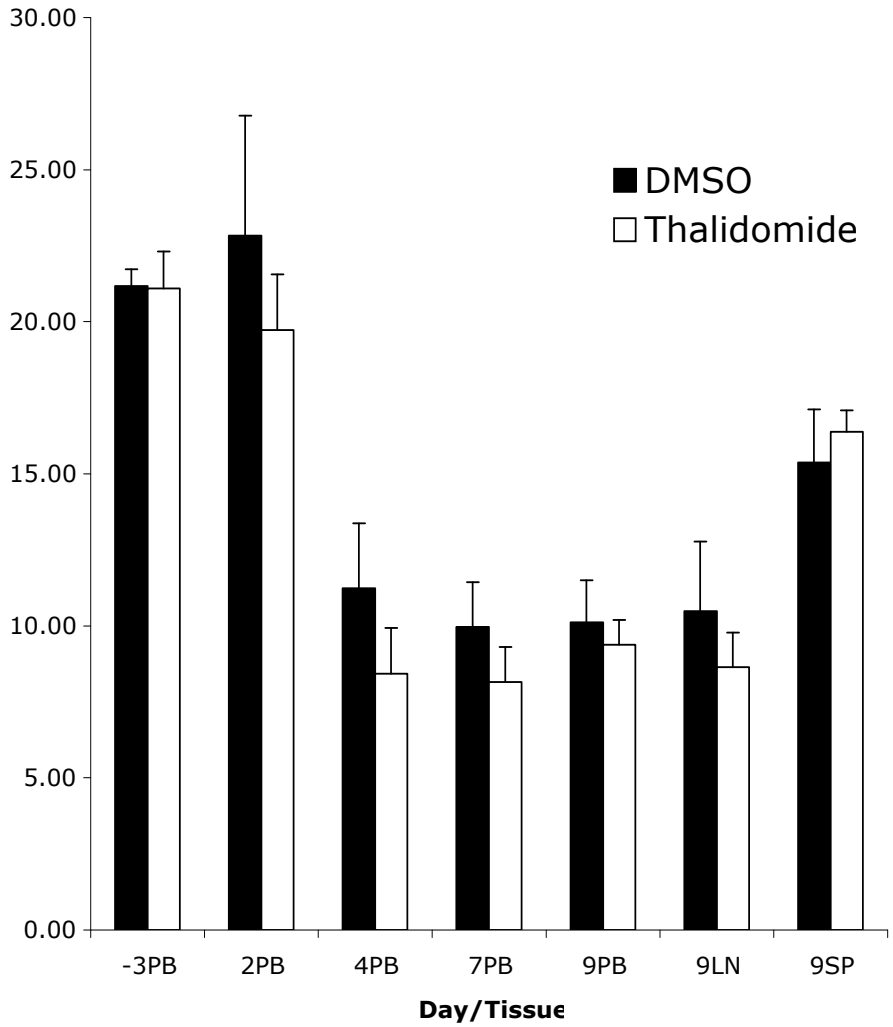

Blood sample for baseline
i.p. 20µg SEB injection
Sacrifice mice, collect lymph nodes, spleen and peripheral blood
-3
0
2
4
7
9
* = blood collection
DMSO or thalidomide i.p. 480ug 2x/day

Figure 19: The SEB mouse model of AICD.

SEB is a bacterial superantigen that causes the transient activation and subsequent apoptosis of CD4+ T cells bearing the Vβ8 segment in their rearranged TCR. (A) Targeted T cells express Vβ8 and undergo apoptosis in response to SEB. Control T cells express TCRs containing V fragments other than Vβ8 and do not undergo apoptosis in response to SEB. The ratio of $CD4^+$ $V\beta8^+$ to $CD4^+$ $V\beta8^-$ T cells in peripheral blood and secondary lymphoid organs tracks AICD after SEB administration. (B) Graphic representation of the *in vivo* paradigm used to determine the effect of thalidomide on SEB induced AICD. Briefly, peripheral blood samples were collected from 6-8 week old female C57/BL6 mice 3 days prior and at days 2, 4, 7 and 9 after SEB injection. After SEB injection, mice were given 480ug thalidomide every 12 hours. On day 9, at the peak of AICD observed in the peripheral blood, the mice were sacrificed to observe thalidomide dependent differences in the T cell populations of the lymph nodes and spleen.



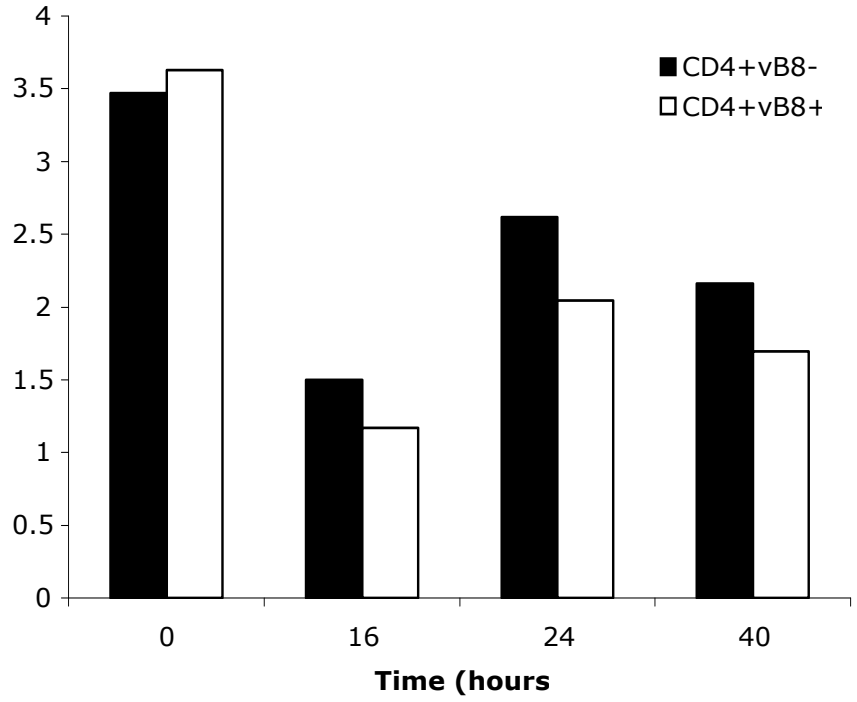
N=5 control DMSO treated mice
N=4 thalidomide treated mice

Figure 20: There is no significant difference in SEB induced AICD in thalidomide treated mice.

Peripheral blood (PB), lymph nodes (LN), and spleen (SP) were immunostained with anti CD4-FITC and anti Vβ8-PE antibodies, then analyzed by flow cytometry. The percentage of CD4 T cells bearing the Vβ8 TCR was assessed at days -3, 2, 4, 7, and 9 for peripheral blood, and day 9 for spleen and lymph node.

showed no differences in number of $CD4^+V\beta8^+$ T cells at any time point in the peripheral blood, or in the lymph nodes and spleen 9 days after SEB injection, indicating that thalidomide does not exert an effect in this model of AICD (Figure 20). Since thalidomide is known to exert effects on cytokines in C57/BL6 mice at lower doses than we used (Choe et al., 2009; Rocha et al., 2006), this raised the possibility that the AICD that we observed in the SEB model was not dependent on FasL synthesis. To verify that FasL was being preferentially expressed on the T lymphocytes activated by SEB, we performed semi-quantitative RT-PCR on sorted $CD4^+V\beta8^+$ (SEB activated) and $CD4^+V\beta8^-$ (quiescent) T cell populations from the SEB treated mice. Since the decrease in $CD4^+V\beta8^+$ cells is apparent in peripheral blood and secondary lymphoid organs by 4 days after SEB treatment, we harvested spleen and lymph nodes at 0, 16, 24 and 40 hours after injection since they provided ample material for cell sorting. Surprisingly, the amount of FasL mRNA was virtually identical in both spleen and lymph nodes at all time points in the $CD4^+V\beta8^+$ and control $CD4^+V\beta8^-$T lymphocyte populations when normalized to 18S rRNA levels which suggests that SEB treatment does not induce FasL mRNA expression (Figure 21). This indicates that SEB mediated AICD proceeds in a manner independent of FasL expression on activated T lymphocytes. Since SEB does not induce FasL expression, this model of AICD is not an appropriate model system for testing the physiological effects of thalidomide on FasL expression.

Splee



Lymph node

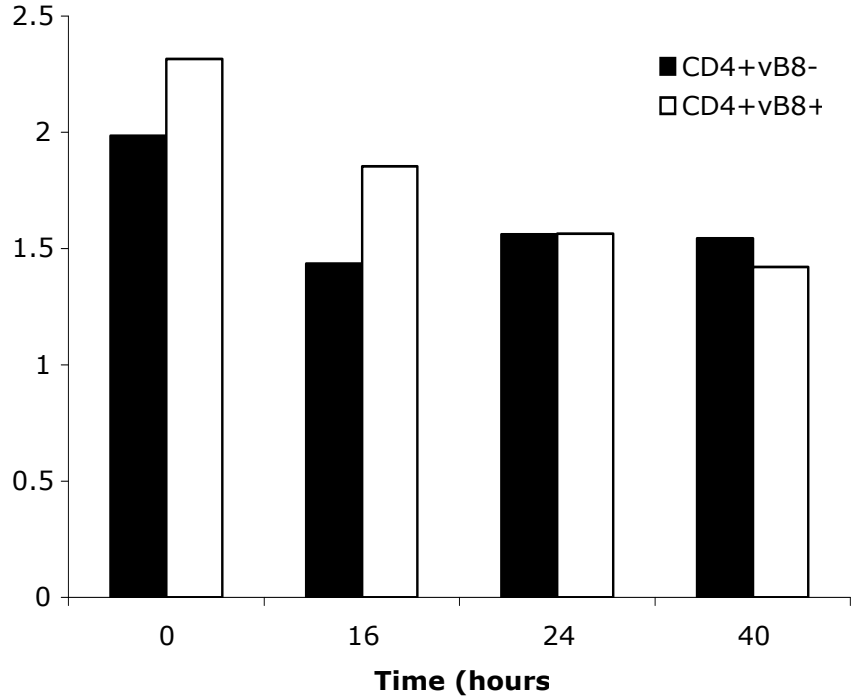


Figure 21: There is no significant effect of SEB on FasL mRNA expression.

C57/BL6 mice were injected i.p. with saline or 20ug SEB. Spleen (A) and lymph node (B) were immunostained with anti CD4-FITC and anti Vβ8-PE antibodies, then FACS sorted. RNA was extracted from sorted CD4+ T cells bearing the Vβ8 TCR or control Vβ8 negative cells from spleen and lymph node followed by RT-PCR. Densitometric quantitation of the PCR amplified fragments on agarose gel is shown.

3.3.3 FasL mRNA expression is significantly decreased with thalidomide treatment in PMA and PHA stimulated Jurkat T cells.

Our previous steady state data for FasL expression in activated PHA or PMA and PHA activated Jurkat T cells provided us with a fully optimized, readily available system in which to test the effect of thalidomide on FasL expression. Jurkat cells were pre-treated with or without 50ug/ml thalidomide and subsequently activated with 3 hours of PHA treatment or 4.5 hours of PHA and PMA treatment. Though there is no effect of thalidomide on the PHA stimulated cells, there is a significant decrease in FasL mRNA levels with thalidomide treatment in the PHA and PMA stimulated Jurkats (Figure 22). To test whether this decrease in FasL mRNA expression has any functional consequences, we first tested whether Jurkat cells undergo AICD subsequent to activation and whether apoptosis is FasL driven. We incubated Jurkat T cells for 16 hours with membrane bound FasL (1:2000), PHA, PMA and PHA and CH11 anti-Fas IgM (1:5000), in the presence of NOK-1, a Fas ligand neutralizing antibody or FasFc, a chimeric immunoglobulin Fc-Fas extracellular domain construct that can also block Fas-FasL interactions. These Jurkats were then stained with propidium iodide, to reveal cells that had lost membrane integrity and Annexin V-FITC, which detects phosphatidyl serine. One of the hallmarks of apoptosis is the phosphatidyl serine flip from the inner leaflet of the cytoplasmic membrane to the extracellular side thus the cells showing annexin V staining but no PI staining were in the early stages of apoptosis. To our surprise, less than 10% of Jurkat cells enter apoptosis with membrane bound FasL, PHA or PMA and PHA. In contrast, incubation with anti Fas (CH11), caused a huge increase in apoptotic cells (Figure 23). Blocking with NOK-1 (anti-Fas ligand) or FasFc only had an effect on

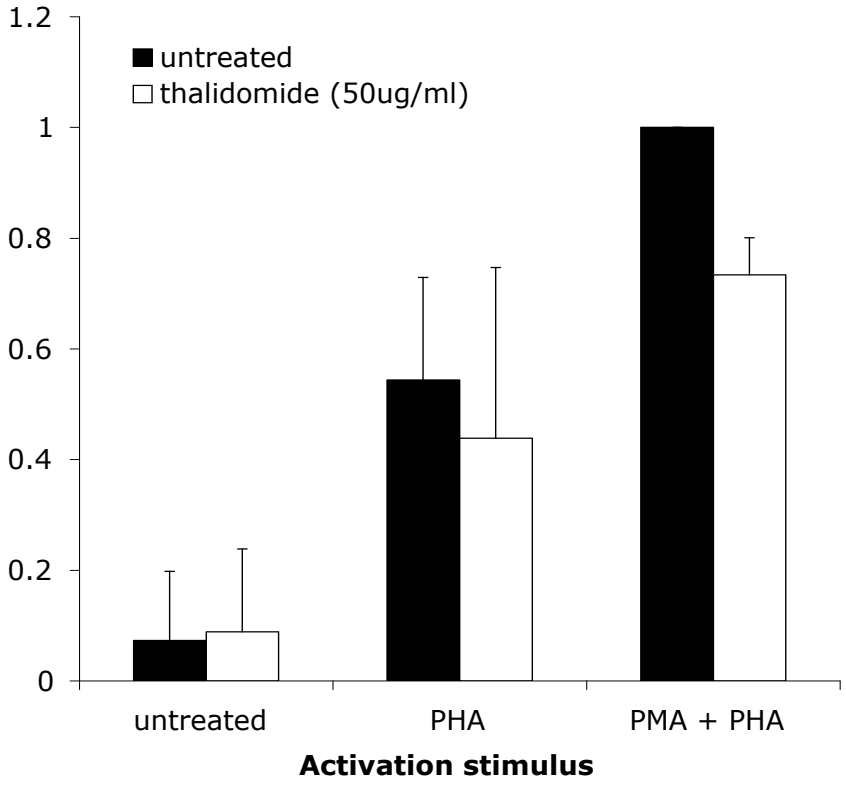
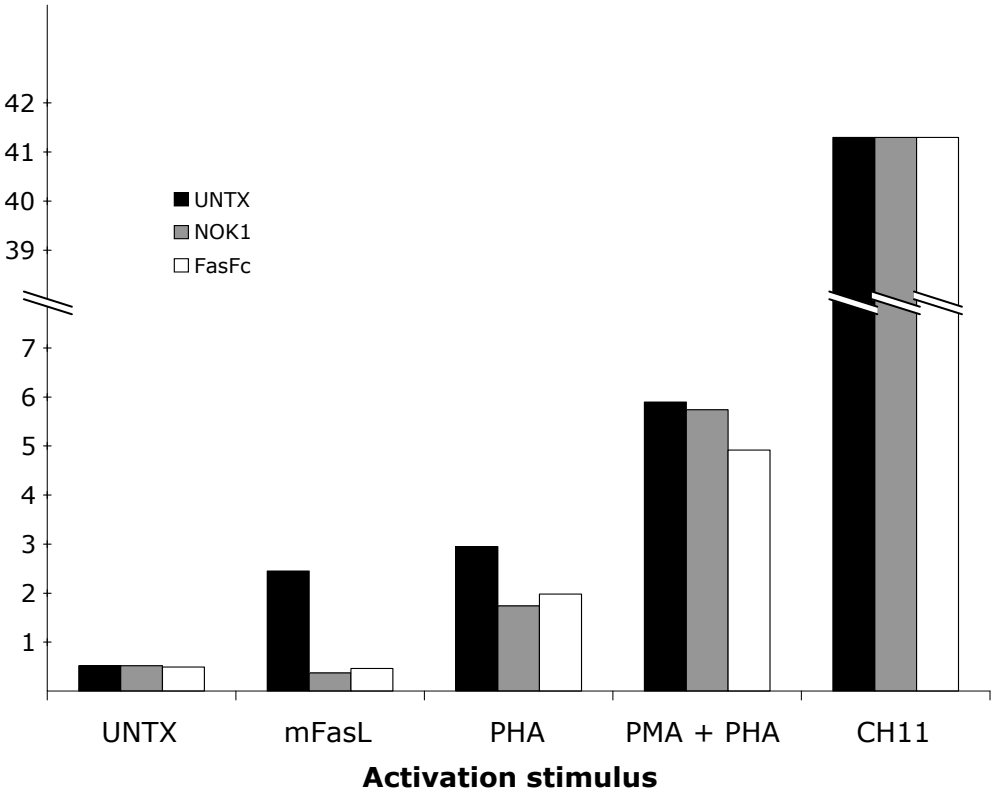

1.2
1
0.8
0.6
0.4
0.2
0
untreated
thalidomide (50ug/ml)
untreated
PHA
PMA + PHA
Activation stimulus

Figure 22: Thalidomide reduces FasL mRNA expression in PHA and PMA stimulated Jurkat cells.

Jurkat T cells were stimulated 3 hours with PHA (1ug/ml) or 4.5 hours with PMA (50ng/ml) + PHA (1ug/ml) to induce peak expression of FasL mRNA. Cells were pretreated 30 minutes with DMSO (untreated control) or thalidomide (50ug/ml). RNA was extracted from these cells followed by Q-RT-PCR for FasL normalized to 18S rRNA loading control.

the membrane bound FasL and PHA induced apoptosis (Figure 23). Thalidomide treatment was not attempted in this paradigm since the effect of thalidomide on FasL mRNA was exclusively on T cells stimulated with both PMA and PHA. The inability of FasL antibodies to block AICD induced by PMA and PHA indicates that thalidomide could not exert an effect on AICD via FasL.

In an attempt to induce apoptosis more robustly, we stimulated Jurkat T cells with more physiological stimuli, plate bound anti-CD3 antibodies (OKT3) and anti CD28 antibodies to induce AICD via TCR and costimulatory pathway engagement respectively. Similar to the previous findings, only CD3 at high antibody concentration in the absence of co-stimulation was sufficient to induce apoptosis. Again, the amount of apoptosis as determined by Annexin V staining and the absence of PI staining indicated that approximately 7% of CD3 stimulated cells enter apoptosis (Figure 23). This population of apoptotic cells seems to be reduced with anti-FasL antibody (NOK-1) indicating that a proportion of this activation induced apoptosis is attributable to Fas-FasL signaling (Figure 23). Conversely, there is no obvious effect of 50 μg/ml thalidomide treatment on the number of Jurkat cells undergoing AICD (Figure 23).


42
41
40
39
7
6
5
4
3
2
1
UNTX
NOK1
FasFc
UNTX
mFasL
PHA
PMA + PHA
CH11
Activation stimulus

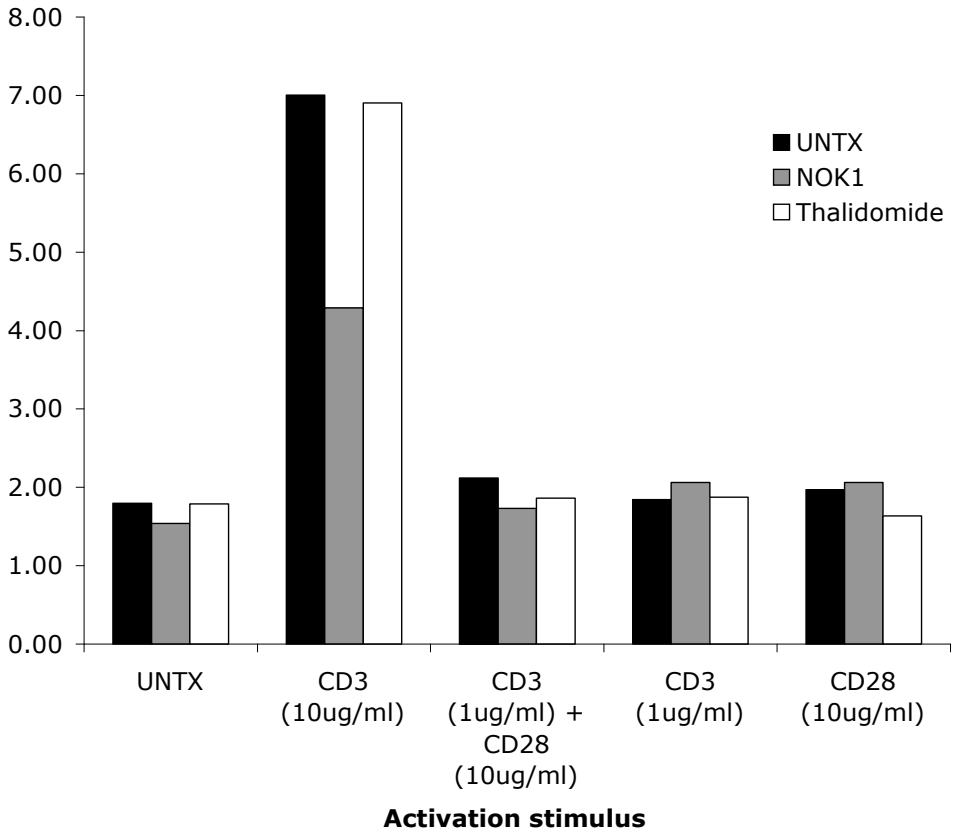

8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
UNTX
NOK1
Thalidomide
UNTX
CD3 (10ug/ml)
CD3 (1ug/ml) + CD28 (10ug/ml)
CD3 (1ug/ml)
CD28 (10ug/ml)
Activation stimulus

Figure 23: PHA and PMA do not cause FasL dependent AICD.

(A) Jurkat T cells were treated with membrane bound FasL (1:2000), PHA (1ug/ml), PMA (50ng/ml) + PHA (1ug/ml) or IgM anti Fas (CH11) (1:2000) for 16 hours. FasL induced AICD was blocked by concurrent treatment with anti FasL (NOK1) and FasFc. Cells were stained with AnnexinV-FITC and propidium iodide (PI) and analysed by flow cytometry. The percentage of cells in early apoptosis (Annexin V+, PI-) is shown. (B) AICD in Jurkat cells stimulated by the TCR and CD28 co-stimulatory pathway. Cells were stimulated with plate bound anti CD3 (OKT3) (10ug/ml), plate bound CD3 (1ug/ml) + soluble anti CD28 (15E8) (10ug/ml), plate bound anti CD3 (1ug/ml) or soluble anti CD28 (10ug/ml). FasL induced AICD was blocked with anti FasL antibody (NOK1) but not with thalidomide (50ug/ml) treatment.

3.4 Discussion

Our results show that thalidomide can provoke a reproducible decrease in FasL mRNA abundance in mitogen and lectin stimulated Jurkat T cells (Figure 22). In the context of p38 MAPK activation, thalidomide has been shown to affect the localization of HuR and associated COX-2 mRNA transcripts. Given that PKC agonism by PMA and TCR engagement are known to activate p38 MAPK in T cells (Morley, 1997), this raises the possibility that FasL mRNA is regulated by thalidomide in a similar p38 MAPK dependent manner. We set out to demonstrate the physiological consequences of reduced FasL mRNA expression due to thalidomide treatment during T cell activation. Unfortunately, the physiological significance of these findings is unclear since the AICD induced by concurrent PHA and PMA treatment is not dependent on FasL.

In discordance with previously published results (Wu et al., 2008), we find that there is no effect of thalidomide on the expression of FasL mRNA in activated the NK-T cell line. Possible explanations for this discrepancy are that the cells we used were NK-T cells and not NK cells. Consequently, we stimulated these cells via the TCR and not via CD16. Similarly, we found no *in vivo* effect of thalidomide on AICD using the SEB model. However, we also confirmed that AICD in this model is independent of T cell expressed FasL (Bonfoco et al., 1998) therefore the physiological effect of thalidomide on FasL expression in activated T cells cannot be assessed in this system. Interestingly, the deletion of $CD4^{+}V\beta8^{+}$ T cells after exposure to SEB is dependent on FasL expression in the periphery (Bonfoco et al., 1998; Ettinger et al., 1995; Renno et al., 1998). Given that the dose of thalidomide given to these mice was substantial, this might indicate that

thalidomide does not affect FasL expression significantly in this peripheral compartment. To verify this hypothesis, AICD should be induced by antigen presented on MHC instead of super-antigen, such as the pigeon cytochrome c transgenic system (Singer and Abbas, 1994), or the ovalbumin transgenic system (Watanabe et al., 1997). Both of these models of AICD are reported to be Fas ligand dependent. Alternatively, co-stimulation by FasL in CD8+ T lymphocytes could be used as a gauge of physiological relevance.

The studies demonstrating an effect of thalidomide on FasL protein expression (Kiaei et al., 2006; Wu et al., 2008) do not mention which antibodies were used to detect FasL. This omission is not trivial, since many of the commercially available antibodies to FasL, recognize irrelevant proteins especially in immunohistochemistry (Fiedler and Eibel, 2000; Herr et al., 2000; Strater et al., 2001). In agreement with this, the seemingly increased expression of cell surface FasL observed in lenalidomide (an analogue of thalidomide) treated NK cells has very little effect on the cytotoxicity of activated NK cells. In addition, FasL is usually sequestered in secretory lysosomes and vesicles in NK cells and is specifically released at the immunological synapse, therefore very little is available at the cell surface for detection (Bossi and Griffiths, 1999). Conversely, the lenalidomide treatment increases perforin expression and cytotoxic activity by a perforin dependent mechanism (Wu et al., 2008).

Ultimately, though there may be a small effect of thalidomide on Jurkat T cells, the physiological consequences of this decrease has been difficult to determine due to experimental limitations. The observation of a decrease in steady state levels of FasL mRNA in activated T cells treated with thalidomide is congruent with our hypothesis that HuR may be sequestered in the nucleus by thalidomide treatment. Our model of action

would be similar to previously published findings on the actions of thalidomide on the p38 MAPK induced relocalization of HuR from the nucleus to the cytoplasm (Jin et al., 2007). Though PKC agonism and TCR stimulation are documented to activate p38 MAPK in Jurkat cells, it is possible that TCR stimulation in NK T cells and in previously stimulated T5 cells does not allow for p38 MAPK activation due to negative feedback or differential expression of components in the signaling cascade. This provides an explanation for the lack of p38 MAPK dependent effects of thalidomide on HuR in the NK T cells and in the T5 cells. It remains to be seen whether p38 MAPK or other pathways are responsible for the increase in HuR association with FasL mRNA during PKC activation in T lymphocytes. Also, the p38 MAPK dependent effects of thalidomide on HuR localisation do not exclude the modulation of other RNA binding proteins or HuR by other pathways.

At present, thalidomide is used for the palliative treatment of multiple myeloma and for ENL. Recently, thalidomide has been proposed as a long term treatment for a variety of auto-immune conditions. Though thalidomide and its analogues are considered to be promising treatments for auto-immune disease, its actions are pleiotropic. The increased and decreased levels of many different cytokines and growth factors indicate that this class of immunomodulatory drug may have different effects on various immune pathologies. It is therefore very important to determine not only the effect of thalidomide on individual cytokines, but develop an understanding of its mechanism of action. For example, could the effect of thalidomide on HuR localization explain the differences in cytokine expression? With a mechanism of action, it may be easier to predict which diseases and patient populations would benefit from thalidomide therapy and which

groups would see their auto-immune disease deteriorate. Likewise, humanized monoclonal antibodies which neutralize TNFα are currently used as treatments for Crohn's disease and have been linked to tumourigenesis since TNFα normally suppresses tumour growth. If the thalidomide based immunomodulatory drugs modulate their effects on auto-immunity by acting on TNFα, then these side effects are also likely to occur.

Chapter 4: Discussion

4.1 FasL mRNA is labile

Cytokines are transiently expressed and tightly regulated proteins that maintain an intricate regulation of the immune system. Many cytokines are strictly regulated at every level of biosynthesis. In particular, cytokines mRNAs are controlled not only by transcriptional throughput but also at the level of mRNA turnover and translational efficiency. Here we found that similarly to many other cytokines, FasL is a transiently expressed mRNA species following T cell activation. In addition, the FasL mRNA has a half life of 2 hours at peak expression after stimulus. This half-life is relatively short compared to other mRNA species (Sharova et al., 2009). If peak expression of FasL requires stabilization of its mRNA, the calculated half life of 2 hours is likely much longer than the FasL mRNA half life under quiescent conditions. This hints that FasL mRNA may be regulated at the level of mRNA turnover.

4.2 HuR interacts with FasL mRNA

Due to homology with the first ARE of TNFα 3'UTR, we were prompted to investigate whether HuR could associate with FasL mRNA. Interestingly, we found that HuR does indeed associate with FasL mRNA by immunoprecipitation of mRNP complexes and also by RNA electromobility shift assay. Virtually the entire FasL 3'UTR binds HuR when the interaction was mapped by gel shift. However, the distal 300nt of the UTR appeared to have much stronger binding to HuR than any other region. Further mapping of the HuR binding site by gel shift identified four 50nt regions within the distal 300nt which bind HuR strongly. The interaction between HuR and these regions could

be competed with an excess of unlabelled probe, indicating that these interactions are reversible and specific. HuR is known to bind mRNA in a cooperative manner (Fialcowitz-White et al., 2007). The number of HuR binding sites identified in the FasL 3'UTR suggests that the cooperativity of HuR binding may be important for the regulation of FasL.

4.3 HuR and autoimmunity

Predictably, one of the regions that binds HuR contains the Class V ARE (database), and all of the other sequences are U rich. Interestingly, the most proximal binding site that binds HuR forms a long U rich hairpin. These structures have previously been identified as necessary for HuR binding (Fialcowitz et al., 2005b). Of particular interest, this hairpin contains a (TG)n microsatellite which has been implicated in SLE (Chen et al., 2006a). This indicates that the length of the hairpin and the ability of this hairpin to recruit RNA binding proteins may impact protein expression and have physiological relevance in auto-immunity. Such is the case in the mouse TNFα 3'UTR. The NZW mouse strain has a polymorphism which inserts a GAU trinucleotide in the hairpin region of the first ARE of the 3'UTR of TNFα. This reduces HuR binding to the 3'UTR of TNFα and is associated with a decrease in TNFα production. The reduction of TNFα production is correlated with an autoimmune phenotype in these mice. Therefore the relationship between the number of microsatellite repeats in the FasL 3'UTR, the ability of this region to bind HuR and SLE should be further investigated.

4.4 Effects of PKC on HuR binding to FasL mRNA

Due to a dearth of reliable FasL protein detection reagents, we decided to create GFP constructs with the FasL 3'UTR. The UTR was included as a whole to preserve the structural integrity and the numerous HuR binding sites in this sequence. In both Jurkat cells and HEK 293 cells, we noticed that the GFP-FasL3'UTR construct was less expressed, at the level of mRNA and protein. Given that most ARE sequences compete for a variety of AUBPs which mediate opposing effects, this result was not surprising. The effect that did surprise us was the apparent rescue of GFP-FasL3'UTR expression when HEK 293 cells are treated with the PKC agonist, PMA. Further investigation led us to observe the relocalisation of HuR to the cytoplasm upon PKC activation, as well as an increased association between GFP-FasL3'UTR mRNA and HuR after PKC activation by mRNP immunoprecipitation. Finally, we also noted that the PKC driven increase in GFP-FasL3'UTR expression is dependent on HuR since this increase in expression is abolished when HuR is knocked down.

Our last findings are in accordance with the recent results of other groups. In general, they find that stimuli which lead to the relocalization of HuR, impact the expression of target messages. For example, in the case of COX-2 mRNA, upon stimulus HuR becomes phosphorylated by PKC at 2 serine residues in the hinge domain involved in shuttling. Phosphorylation of HuR leads to cytoplasmic relocalization of COX-2 mRNA and promotes the stabilization of this transcript (Doller et al., 2008a; Doller et al., 2009; Doller et al., 2007). A similar regulatory mechanism may also function for vascular endothelial growth factor (VEGF) mRNA (Amadio et al., 2008). In a slight variation on this theme, CD83 mRNA is efficiently exported from the nucleus and

stabilized by HuR during casein kinase II activation (CK2). CK2 phosphorylates APRIL on threonine 244 and promotes shuttling of APRIL to the cytoplasm with its binding partner HuR and associated CD83 mRNA (Chemnitz et al., 2009; Fries et al., 2007).

To this end, it would be interesting to know whether the subcellular localization, stability and translational efficiency of FasL mRNA are affected by PKC activation. The mechanism that causes HuR to relocalize from the nucleus to the cytoplasm upon PKC stimulus would also be appealing to investigate. Is the phosphorylation of the hinge region sufficient for relocalization, or are there protein binding partners that are also involved? Since FasL is predominantly expressed in activated T lymphocytes, is the PKC isoform which is activated downstream of the TCR, PKC theta, capable of phosphorylating HuR and mediating the same effects as PKC alpha and beta? I could hypothesize that upon TCR stimulus, PKC theta becomes activated and phosphorylates the hinge region of HuR and causes shuttling from the nucleus to the cytoplasm possibly affecting the interaction of HuR with its protein ligands such as pp32. Concurrently, the activation of NF-kB, NF-AT and IRF-1 by TCR signaling events would cause an increase in the transcription of FasL. The FasL mRNA would bind to HuR, be protected from AMD and be shuttled to the cytoplasm and made available to the translational apparatus by HuR.

4.5 p38 MAPK and HuR

In addition to its direct effects on HuR, PKC agonism and TCR stimulation in T lymphocytes is known to activate p38 MAPK (Salojin et al., 1999; Zhang et al., 1999b). Interestingly, p38 MAPK can be activated by the phosphorylation of non-canonical

residues in activated T lymphocytes and thus is independent of MKK3 and MKK6 (Salvador et al., 2005). The activation of the p38 MAPK pathway is also known to impinge on the functions of HuR (Jin et al., 2007; Song et al., 2005; Subbaramaiah et al., 2003). Furthermore, p38 MAPK activation is required for FasL expression, AICD and is involved in SLE (Hsu et al., 1999b; Wong et al., 2009; Zhang et al., 2000). Hypothetically, it is possible that p38 MAPK activation allows for HuR to stabilize FasL mRNA upon T cell stimulation.

4.6 Thalidomide and its effects on p38 MAPK and HuR

Thalidomide is an immunomodulatory drug that can repress the cytoplasmic shuttling of HuR induced by p38 MAPK activation (Jin et al., 2007). We have found that thalidomide can reduce the abundance of FasL mRNA in T cells stimulated with both PMA and PHA but not PHA alone. This raises the possibility that thalidomide exerts its effects on FasL through p38 MAPK and HuR. Therefore, during PHA stimulus, p38 would not be activated, HuR would not relocalize to the cytoplasm and the effect of thalidomide on nucleocytoplasmic trafficking of HuR would be latent. However, under conditions of PKC agonism, for example PMA treatment, thalidomide would inhibit HuR's normal p38 MAPK dependent shuttling to the cytoplasm to exert stabilizing, relocalizing and translational effects on target mRNAs. Thus the effect of thalidomide would only be observed under conditions where p38 MAPK dependent HuR shuttling is required.

4.7 Mechanism of action for thalidomide

AREs regulate many cytokines at the post-transcriptional level (Hao and Baltimore, 2009). Is it possible that the pleiotropic effects of thalidomide are due to the same mechanism? For instance, since thalidomide abolishes the ability of HuR to shuttle to the cytoplasm during p38 MAPK activation (Jin et al., 2007), this could potentially effect the localization, stability and translation of many ARE containing cytokine mRNAs. If the effects of thalidomide are due to the block in the cytoplasmic shuttling of HuR, understanding the mechanism of action could also lead to the development of more specific drugs which inhibit HuR shuttling but which do not carry the risks and side effects of thalidomide. This could also explain why some HuR binding mRNAs are differentially regulated by thalidomide. Perhaps, thalidomide only affects p38 MAPK dependent processes, thus HuR mediated stabilization via pathways other than p38 MAPK would remain unaffected.

4.8 Future studies:

4.8.1 Regulation of FasL mRNA by other factors

More importantly, this is the initial description of the post-transcriptional regulation of FasL by its 3'UTR. Though we have found that HuR interacts with the 3'UTR of FasL, we have not described which functions of HuR such as sub-cellular localization, stability, and translation, pertain to this mRNA target. Furthermore, AUBPs other than HuR almost certainly mediate the reduced expression of the GFP-FasL3'UTR constructs and warrant supplementary investigation. Recently, TNFα mRNA has been found to be targeted for degradation by the recruitment of miRNAs by TTP, an AUBP

(Jing et al., 2005). Interestingly, the FasL 3'UTR is predicted to contain several paired miRNA binding sites. The trans-factors that bind to the FasL 3'UTR may mediate important effects on expression and regulation of FasL which could in turn, impact autoimmune diseases such as SLE. SLE is specifically linked to defects in Fas-FasL mediated apoptosis and it appears that the number of microsatellite repeats in the HuR binding region is strongly correlated with the presence of the discoid rash and other symptoms of SLE (Chen et al., 2006a; Xue et al., 2006).

4.8.2 Physiological effects of HuR binding to FasL mRNA

During the course of these experiments, it has become clear that AICD is difficult to recapitulate in both Jurkat cells and *in vivo*. Recent reports involving the conditional, tissue specific ablation of Fas and FasL expression also indicate that the original model of T cell autonomous AICD via FasL is at best, an incomplete representation of FasL functions (Hao et al., 2008; Hao et al., 2004; Mabrouk et al., 2008; Stranges et al., 2007). It is clear from both human and mouse mutations in the Fas-Fas ligand receptor pair that these molecules have an enormous effect on immune homeostasis and that their appropriate regulation is essential (Bi et al., 2007; Fisher et al., 1995; Karray et al., 2004; Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992a). Though the physiological relevance of these molecules is clear, the functions of Fas and FasL need to be redefined in terms of which tissues express FasL, during which cellular contexts and with which physiological outcomes. The development of specific antibody reagents that enable the reliable detection of FasL protein are also necessary for the delineation of FasL's biological functions. Other TNF superfamily members have diverse roles in the immune system, the skin and the nervous system. Their roles involve proliferation,

differentiation, and apoptosis. It is interesting that although the role of FasL has been best described in terms of apoptosis, its roles extend into differentiation and proliferation. In addition, the physiological role of FasL outside of the T cell compartment is also slowly coming to light. Despite these limitations, the work presented here contributes to the understanding of which cellular contexts allow for FasL expression.

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