Μεταπτυχιακό Πρόγραμμα Σπουδών στην
''Κυτταρική & Γενετική Αιτιολογία, Διαγνωστική και Θεραπευτική των Ασθενειών του Ανθρώπου''

Graduate Program in
''The Molecular Basis of Human Disease''

Master Degree Thesis

The role of Tpl2 in the pathophysiology of Fulminant Hepatitis

Dimitra Virla

Scientific Committee:
Eliopoulos Aristeides, PhD
Tsatsanis Christos, PhD

Laboratory of Molecular and Cellular Biology
Department of Medicine
University of Crete

Heraklion 2008

Contents

Περίληψη

Η Κεραυνοβόλος ηπατίτιδα αποτελεί μια ηπατική νόσο με ιδιαίτερα υψηλά ποσοστά θνητότητας εξαιτίας της ανεπαρκούς θεραπείας. Πρόκειται για ένα κλινικό σύνδρομο χαρακτηριζόμενο από έντονη φλεγμονώδη διήθηση, μαζική απόπτωση, νέκρωση των ηπατοκυττάτων και εκταμένη ιστική βλάβη η οποία κυμαίνεται από ήπια ηπατίτιδα έως βοβαρή ηπατική ανεπάρκεια. Τα τελευταία χρόνια έχουν διεξαχθεί πολυάριθμες μελέτες με σκοπό να αποσαφηνιστούν οι μοριακοί μηχανισμοί οι οποίοι μεσολαβούν για τη Κεραυνοβόλο Ηπατίτιδα. Μολονότι δεν έχουν αποσαφηνιστεί πλήρως, ωστόσο έχει εδραιωθεί το γεγονός ότι η στρατολόγηση των ενεργοποιημένων Τ κυττάρων στο ήπαρ και η επακόλουθη ιστική βλάβη μεσολαβούμενη από σηματοδοτικά μονοπάτια παραγόντων νέκρωσης εμπλέκονται καταλυτικά. Η ενεργοποίηση των καταρρακτών μεταγωγής σήματος των MAPK (**M**itogen **A**ctivated **P**rotein **K**inases) παίζει κεντρικό ρόλο στην παραγωγή κυτταροκινών και χημειοκινών κατά τη διάρκεια μιας φλεγμονώδους αντίδρασης δρώντας καταλυτικά στην πρόκληση κυτταρικού θανάτου και ιστικής βλάβης. Πρόσφατες μελέτες στρέφονται στην περαιτέρω αποσαφήνιση των σηματοδοτικών μονοπατιών των MAP κινασών ενώ ένας αριθμός φαρμακολογικών αναστολέων τους δοκιμάζεται σε θεραπευτικές μελέτες που αφορούν ασθενείς με φλεγμονώδεις/αυτοάνοσες νόσους. Η Tpl2 κινάση, γνωστή και ως Cot είναι μια κινάση σερίνης/θρεονίνης η οποία ανήκει στην οικογένεια των ενεργοποιούμενων από μιτογόνα πρωτεϊνικών κινασών κινασών κινασών (MAPKKK). Λαμβάνοντας υπόψιν τη συμμετοχή της κινάσης στην ενεργοποίηση των λεμφοκυττάρων και των μακροφάγων από ερεθίσματα που έχουν σημασία στην παθογένεια φλεγμονωδών και αυτοάνοσων ασθενειών τόσο για την έναρξη όσο για την επίταση και την δημιουργία ιστικής βλάβης, η Tpl2 έχει αναγνωριστεί ως σημαντικός στόχος για την ανάπτυξη αντι-φλεγμονωδών φαρμάκων. Μελέτες έχουν δείξει ότι η έλλειψη της κινάσης σε ποντίκια που μολύνονται με LPS/D-galactosamine καθιστά ανθεκτικά τα ζώα στην ενδοτοξική καταπληξία, μέσω ελαττωμένης διαμεσολαβούμενης από την κινάση ERK, παραγωγής TNF-α ενώ η απαλοιφή αυτή δρα εξίσου προστατευτικά και σε περιπτώσεις αρθρίτιδας και επαγώμενης από TNF φλεγμονώδους νόσου του εντέρου (Inflammatory Bowel Disease, IBD). Εύλογα προέκυψε το βιολογικό ερώτημα της επίπτωσης της απαλοιφής του γονιδίου της Tpl-2 σε ποντίκια που νοσούν από κεραυνοβόλο ηπατίτιδα Συμπληρωματικά της αποσαφήνισης του ρόλου της Tpl2

στην μεταγωγή σημάτων που ρυθμίζουν την φυσική και ειδική ανοσία και τις φλεγμονώδεις αποκρίσεις, ελκυστικό αντικείμενο έρευνας αποτελεί η μελέτη της θεραπευτικής αξίας της Tpl2 για την ανάπτυξη αντι-φλεγμονωδών θεραπειών. Για τη ολοκληρωμένη συμπλήρωση των πειραμάτων, μελλοντικά θα χρησιμοποιηθούν διάφοροι μοριακοί αναστολείς της έκφρασης της Tpl2 κινάσης σε φυσιολογικά ζώα με κεραυνοβόλο ηπατίτιδα με σκοπό να αξιολογηθεί η δυνατότητα των μορίων αυτών να αναστείλουν τον φαινότυπο της νόσου.

Summary

Fulminant hepatitis is a clinical syndrome characterised by sudden and severe impairment of liver function, resulting from massive liver inflammatory infiltration with subsequent death of hepatocytes. Acute liver failure can be devastating and is associated with high mortality. However, the underlying pathophysiological mechanisms are not yet fully understood and therefore an effective therapeutic approach is difficult. With regard to novel therapeutic approaches of fulminant hepatitis, at the present time a significant number of research groups have been focused on the potential of gene silencing techniques for future therapeutic implementation. For this purpose, a promising candidate molecule is Tpl2, a serine/threonine protein kinase that has been classified as a member of the mitogen-activated protein kinase kinase kinase family (MAP3K). Based on its role in inflammation, Tpl2 has been recognized as an important target for the development of anti-inflammatory drugs. In this connection, Tpl2 protein was initially identified as a proto-oncogene that is activated by provirus insertion in retrovirus-induced T cell lymphomas and mammary adenocarcinomas in rodents. Since, Tpl2 has been classified into the mitogen-activated protein kinase kinase kinase (MAP3K) family, is a serine-threonine protein kinase and is known to have a pleiotropic role. Tpl-2 is implicated in the regulation of several cellular kinase pathways and has also been found to induce miscellaneous transcription factors. In addition, Tpl2 also plays an obligatory role in the transduction of Toll-like receptor and regulate the expression of cytokines, chemokines and other molecules involved in inflammation as well as in the regulation of innate and adaptive immunity. The finding that Tpl2 is involved in the Toll-like receptor 4 (TLR4) signalling pathway emerged by using Tpl2 knockout mice, revealed that after binding with TLR4, lipopolysaccharide (LPS) activates extracellular signal-regulated kinase (ERK) and induces the production of tumor necrosis factor-a (TNF-a) in a Tpl2-dependent manner. As a result, Tpl2-deficient mice are resistant to LPS-induced endotoxic shock because they lack ERK activation, resulting in lower TNF-a production. It should be noted that mice deficient to Tpl2 were studied in the context of an established inflammatory bowel disease (IBD) mouse model ($TNF^{\Delta ARE}$) showing that Tpl-2 kinase regulate the lymphocytic response and its absence caused a delayed onset and progression of IBD. Thus, above and beyond its role as a MAP3K kinase, Tpl2 mediates TNF-a and other cytokine

production, and is closely linked with the development of many inflammatory diseases, such as rheumatoid arthritis or inflammatory bowel disease. We will try to delineate the precise role of tpl2 protein in the context of inflammation and apoptosis in liver injury. Along these lines, we are going to investigate whether Tpl2 is critically involved in the pathophysiology of fulminant hepatitis and try to determine the molecular mechanism of its kinase action regarding inflammation and apoptosis-related signals. Finally, in course of defining the significant biological role and function of Tpl2 kinase in ConA-induced hepatitis we will investigate the value of Tpl2 inhibitor as potent hepatoprotective drug.

INTRODUCTION

1. Introduction

Considering the crucial role of inflammation and apoptosis in an elevating number of physiological and pathological conditions such as in Fulminant Hepatitis, it is interesting to speculate the mechanisms underlying these biological processes since have not been elucidated in detail yet. Inflammation is a complex reaction of innate immune system in vascularized tissues and involves the accumulation and activation of leukocytes and plasma proteins at a site of infection. Although inflammation serves as a protective function/mechanism in controling pathogens and promoting tissue repair, it can also cause tissue damage and disease [54]. Programmed cell death, or apoptosis, is controlled by many interactive signaling cascades initiated by various intracellular and extracellular stimuli to maintain normal cell turnover [30]. Analysis of the molecular mechanisms of cellular and cytokine-mediated mechanisms of inflammation and apoptosis has led to a better understanding of many human diseases.

Recently, it became clear that inflammation and apoptosis of hepatocytes is the critical cause of Fulminant Hepatitis. Since mitogenic lectins soon became tools and concanavalin-induced liver injury became an established animal model reflecting multiple molecular mechanisms involved in human acute viral and autoimmune hepatitis, it was temping for scientists, to investigate whether fulminant hepatitis is the culmination of vigorous cell-mediated immune attack on hepatocytes and study the biochemical events and elucidate signal transduction pathways that occur and are activated respectively during lymphocyte stimulation. In the last few years, the systematic analysis of immune cells in the liver and their interactions was extremely helpful for understanding immunopathogenesis of Fulminant Hepatitis. However, the additional characterization of the molecular mechanisms of the pathophysiology of Fulminant Hepatitis will provide new insights into the development of targeted and therapeutic agents such as siRNAs that appear to have enticing properties. As a consequence, investigators interested in this field have focused considerable attention on the potential of siRNAs for their future therapeutic implementation in clinical practice. In conclusion, this T cell-dependent model of inflammatory liver injury allow the investigation of basic principles of hepatic disorders associated with T cell activation and infiltration as well as pharmacological in vivo studies for the development of hepatoprotective drugs.

1.1. Fulminant Hepatitis

1.1.1. Pathophysiology of Fulminant Hepatitis

Fulminant hepatitis (FH) refers to a clinical syndrome resulting from inflammatory infiltrates, massive apoptosis, necrosis of liver and widespread lesions cells and ranges from mild hepatitis to severe failure of liver function. Hence, the severe form of fulminant hepatitis can be devasting, capable of debilitating many patients without liver transplantation worldwide and overall, associated with high mortality rate [79]. The aetiologies of fulminant hepatitis are of wide spectrum, including viral infection, autoimmune hepatitis, alcohol consumption, immunological insults and hepatotoxins. Whist the mechanisms underlying FH have not yet been elucidated in detail the recruitment of activated T cells in the liver and the subsequent hepatocyte death driven by the Fas ligand and TNF pathways appear to be critically involved in the development of the disease.The reason why some patients develop FH whereas others exposed to the same hepatic insults do not, remains still unknown. However immune responses, mediated mainly by aberrant antigen presentation and T cells, play a critical role in driving hepatocellular injury induced by the aforementioned causes [4].

At clinical level, fulminant hepatitis is also characterized by severe metabolic dearangements and neurologic aberrations which both can lead to encephalopathy and coagulopathy, followed ultimately by multi-organ failure. However, culmination depends on the etiology and as a consequence on the severity of FH. Recently was conducted a new classification represented by three subgroups of fulminant hepatitis that provides a better determination of etiology and estimate of prognosis. These subgroups have been defined as hyperacute, acute, and subacute. Hyperacute liver failure refers to encephalopathy that develops within 8 days of the onset of jaundice. Acute liver failure is defined as the onset of encephalopathy 8 to 28 days after the development of jaundice whereas subacute liver failure is defined as the development of encephalopathy 4 to 26 weeks after the onset of jaundice [8]. Then, according to the etiology and severity of FH, current remedy is based on improved intensive care, supportive treatment to retain the potential of liver regeneration and liver transplantation in irreparable cases. Fulminant hepatitis leads to death in over two-thirds of patients who do not receive liver transplants. Meanwhile, further

understanding of the molecular pathogenesis of fulminant hepatitis appears to be prerequisite for the development of more effective therapies [16].

1.1.2 Concanavalin A-induced hepatitis: A well-defined experimental model of inflammatory liver injury

In the beginning of 20th century, *James B. Sumner* at Cornell University originally extracted from the plant *Canavalia ensiformis* a crystalline protein that he named concanavalin A (Con A). Concanavalin A belongs to lectins, a galore class of proteins with agglutinating activity. In the course of defining the biological role and function of concanavalin A, *Werner G. Jaffe´* first reported that acts as a mitogen [55]. In the following years, there was an explonential growth in the use of mitogenic lectins in biological and clinical research. In 1992, *Tiegs and his colleagues* demonstrated and described a model of T cell-dependent hepatitis. This model of immune-mediated liver injury can be induced by intravenous injection of the T cell mitogenic plant lectin concanavalin A [64]. Thus, a significant number of research studies used the model of Con A-induced liver injury to further investigate pathophysiological mechanisms of the immune system, implicated in T cell mediated hepatitis since this field was poorly understood. To date, there is growing evidence suggesting that interaction among multiple immume cells and cytokine signaling, results in T-cell mediated fulminant hepatitis [54]. Since this novel model of severe hepatitis was, and retains to be, intensively used to study the immunopathogenesis of liver diseases, it recently became evident that inflammatory responses and hepatocellular apoptosis in this model are regulated by specific immune cells and cytokine signals.

The primary report by *Tiegs et al*, laid the foundation stones in the study of Con A-induced liver damage. In this connection, they demonstrated that mice with severe combined immunodeficiency syndrome (SCID), due to the total absence of both immunocompetent T and B cells, as well as athymic nude mice with immature and disfunctional T cells, are completely protected from Con A-induced hepatitis [64]. These results were subsequently confirmed and extended by using monoclonal antibodies against T cells. In particular, intravenous administration of a monoclonal antibody against Thy 1.2 antigen of T cells, protected mice from the hepatotoxic

properties of Con A. Similarly, mice pretreated with a monoclonal antibody directed against the $CD4^+$-glycoprotein of T lymphocytes, exhibited resistance to Con A-induced liver damage. This finding underlines the obligatory role of $CD4^+$ in the initiation of immunological response which ends to tissue damage. On the contrary, in an analogous experiment with a monoclonal antibody against the $CD8^+$ corecepror, mice proved to be susceptible to Con A-induced causal sequence leading to hepatitis [64]. These results indicated that cytokines released following $CD4^+$ T activation, rather than cytotoxic $CD8^+$ T cells, mediated liver injury.

Based on the aforementioned protection from Con A-hepatitis, provided either by anti-$CD4^+$ antibodies or by the in vivo destruction of macrophages, as well as on the micrograph revealed a direct lymphocyte-macrophage interaction, *Tiegs et al,* suggested a mechanism in which the recognition the concanavalin A-MHC –class II compex on macrophage surface by $CD4^+$ Th2 cells results in their activation and further proliferation [27]. Accordingly, this experimental disease is probably produced by both lymphocytes and macrophages. The protection by anti-CD4 antibodies against Con A suggests a mechanism in which $CD4^+$-bearing TH cells recognize the Con A-modified MHC-II structures of macrophages and become activated *(Figure 1)*.

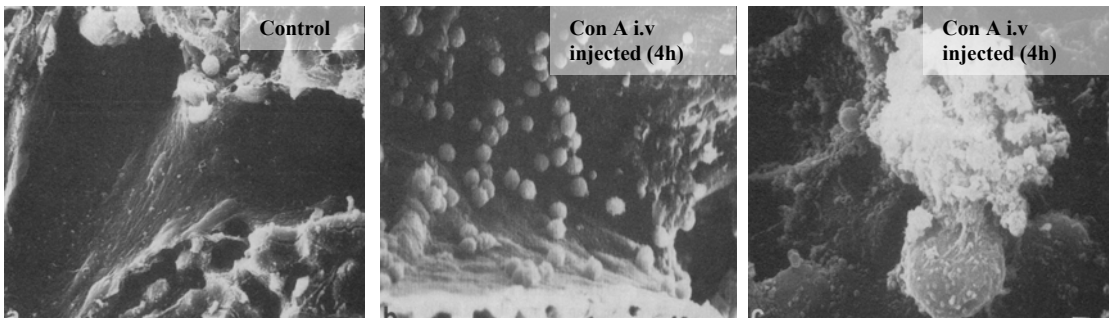


Figure 1*. Scanning electron micrograph of lobular blood vessels of mouse liver showing attachment of blood cells to the endothelium: (a) untreated, (b) 4 h after 20 mg/kg. (c) Detail 4 h after 20 mg/kg Con A given intravenously, showing the interaction between an activated macrophage (upper cell) and a lymphocyte (lower cell). Adopted from [64]*

hypothesis, was the discovery that Con A-hepatitis was accompanied by the release of IL-2 or else of T cell growth factor into the serum of the animals. Subsidiary evidence for the involvement of T cells came from the pharmacological intervention experiments with cyclosporine A or FK 506 whose immunosuppressive activity resulted in inhibition of T cell-dependent IL-2 production [64].

In summary, this study provided several lines of evidence and with much foresight emerged the effector role of T lymphocytes in vivo and established their crucial contribution in the pathophysiology of ConA-induced liver injury.

1.13. Critical contribution of innate immune cells in T-cell dependent liver injury

Recent studies provide some insights about a paradoxical nature of liver besides its significant metabolic function. Liver is constantly exposed to a plurality of invading pathogens due to its special anatomical location and blood supply as it occupies a watershed position between the gastrointestinal and the systemic venous circulation [47]. In order to face these diverse immunological challenges, liver is endowed with a unique defense system based on resident immune cells and their mechanisms for rapid immune responses. In particular, any pathologic stimulus for the liver will immediately activate the abundant **Kuppfer cells**, **natural killer** (NK) cells and **NK cells with T cell receptor** (NKT) in liver and will also trigger the recruitment of polymorhonuclear cells and complement components for an acute inflammatory response [30] [50]. Therefore, on account of the high proportion of immune cells and the rapid defense system in the liver many studies have referred to liver as a "lymphoid organ" with immunological properties, emerging a new scientific field of combined hepatology and immunology termed "hepatoimmunology" [47].

Tiegs et al., demonstrated and described ConA-induced fulminant hepatitis, a well established model that closely resembles the pathology of human autoimmune and viral hepatititis and implicates **T lymphocytes** as the key effector cells in Con A-induced hepatitis [64].

However, ConA-induced hepatitis is not exclusively a T cell- dependent model. Moreover, *Shaümann et al.,* examined the role of **Kuppfer cells** against the hepatotoxic effects of Con A, highlighted the importance of Kuppfer cells for T cell-mediated liver failure and addressed the question of how important are Kuppfer cells for TNF-dependent diseases. In particular, they injected mice intravenously with clodronate liposomes (Cl_2MBP) in order to deplete the abundant population of Kuppfer cells before ConA challenge and clearly showed that KCs contribute to T-cell-dependent liver injury induced by Con A [51]. Although, the identity of the TNF-

producing cells in this model remained unknown, *Shaümann et al.,* revealed by immunofluorescent staining of liver sections that Con A-induced intrahepatic TNF is mainly produced by KCs and to a lesser extent by some individual $CD4^+$ T cells [51]. In conclusion, T-cell- and TNF-dependent liver injury clearly depends on the activation of Kuppfer cells.

Other studies also focused on the intrahepatic immune environment in the context of inflammation revealed a pivotal role in first line immune defense is displayed by NK and NKT cells. *Takeda et al*, examined the possible contribution of **NK T cells** to the murine model of Con A-induced liver injury. Their study elucidated that the activation of NKT cells is fundamental for the development of T cell-mediated fulminant hepatitis since CD1d-deficient mice lacking NKT cells exhibited to be highly resistant to the hepatotoxic effects of concanavalin A [63]. Accordingly when NKT cells and Con A were coinjected in the liver of CD1d-deficiemt mice, was observed massive degenerative change comparable to that observed in Con A-injected wild-type mice [63]. Moreover, *Takeda et al.,* observed rapid depletion of hepatic NKT cell in the liver after Con A administration which appers to be physiological relevant to limit liver injury by these cells, because the continued presence of activated NKT cells would result in fatal liver damage. The fact that hepatic NKT cells may need to be eliminated quickly after they accomplish their effector functions in the liver, was interpreted by the data indicated that hepatic NKT cells constitutively expressed Fas at a high level and also up-regulated FasL expression rapidly after Con A administration. Hence, elimination of NKT cells may be caused via Fas/FasL-mediated suicide [52].

In the context of pathophysiology mechanims during liver inflammation, the hepatic infiltration of polymorphonuclear leukocytes such as neutrophils represents an early-stage response to tissue injury. In particular, this process involves priming and activation of neutrophils by inflammatory cytokines (e.g., TNF-α, IL-1β and IL-12) [40], chemokines (including macrophage-inflammatory protein (MIP)-1α, MIP-1β and IL-8) and complement components, followed by recruitment into the liver vasculature and hereupon neutrophils extravasate through chemotactic signal and finally contact to their target cells which in this instance are hepatocytes and trigger degranularion and oxidant stress [50]. Therefore, **neutrophils** can be induced to produce a variety of mediators that can manipulate inflammatory and immune responses. Besides the cytokines, chemokines and complement components, these

immune reactions include reactive oxygen species and proteases [47]. In 2004, *Bonder and his collegues* conducted a report where it was also emphasized the role of neutrophils as major initiators of lymphocyte recruitment and liver injury challenged by ConA. In that study, it was demonstrated by flow cytometry and immunochemistry that ConA binds directly to neutrophils, generates their activation, and triggers a significant increase in neutrophil recruitment into the murine liver. Notably, *Bonder et al,* also revealed that neutrophil depletion by a monoclonal antibody against murine granulocytes, diminishes extensively Con A-induced $CD4^+$ T lymphocyte recruitment to the liver and consequently ameliorates liver injury in this model of hepatitis [10].

1.1.4. Cytokines: major players that mediate and regulate inflammatory and immune responses in ConA-induced liver injury

Cytokines correspond to a family of proteins secreted by the cells of innate and adaptive immunity in response to pathogenic antigens in order to mediate and regulate immune and inflammatory responses. As a general rule, cytokines of innate and cytokines of adaptive immunity are produced by different immune cells and target different cells [43]. In general, multiple immune cells, including NK T cells and $CD4^+$ T cells cells and a variety of cytokines have been implicated in Con A–induced hepatitis, suggesting that T cell–mediated hepatitis results from the interactions of multiple cells and cytokines [27]. Following ConA administration in mice, T cells, macrophages and hepatocytes release cytokines such as TNF-α, IFN-γ, IL-6, IL-2, IL-4, IL-12, IL-1β, IL-18, IL-22, IL-27,CCR5, as well as nitric oxide that have been linked to the pathogenesis of hepatic lesions [12] [28] [41] [44] [48] [56] [2].

As it was mentioned previously, the interaction between the T cell antigen receptor of CD4+ Th2 cells and peptides presented by major histocompatibility complex (MHC) class II molecules on macrophages is known to induce the release of IL-1 from macrophages as well as the release of IL-2 from the CD4+ T cells during the initiation of TH cell proliferation. Moreover, mitogen-activated T_H lymphocytes secrete IL- 2 in the presence of IL- 1-producing cells [64].

Therefore, the action of T cells in the liver is partly activated through release of a variety of cytokines which target liver and immune cells via activation of multiple cascades. The JAK-STAT signaling pathways play a central role in liver

since a wide spectum of cytokines are expressed there in response to a liver invading pathogen. In general, binding of several of these cytokines (such as IFN-γ, IL-4, IL-6 e.t.c) to their receptors induce receptor dimerization, tyrosine phosphorylation of the receptor-associated JAKs, ensued by activation of the STATs. Phosphorylation of the STATs results in dimers formations and subsequent translocation to the nucleus in order to generate the transcription of many target genes, including a family of inhibitory proteins, suppressor of cytokine signaling (SOCS), which then turn off the JAK-STAT signaling pathway [43].

Inteferon-γ is a cytokine produced by T cell and NK cells whose principal function is to activate macrophages in both innate immune and adaptive-cell mediated immune responses. IFN-γ is a marker of activated $CD4^+$ T cells and is strongly elevated in mice post ConA challenge [12]. In order to investigate the role of IFN-γ/STAT1 signaling in T cell–mediated hepatitis, *Hong and coworkers* injected IFN-$\gamma^{-/-}$ and $STAT1^{-/-}$ mice with Con A and assessed the subsequent molecular and pathological changes in the liver [27] [57]. Following ConA administration, mice that carried germ line gene mutation either of IFN-γ or STAT-1 were protected against ConA-induced liver injury. Consistent with the above-mentioned data was the observation that these mice exhibited almost undetectable induction of the Inteferon Regulatory Factor-1 (IRF-1) protein and suppressor of cytokine signaling-1 (SOCS1) whereas induction of Bcl-XL protein was significantly enhanced. Taken together, it was demonstrated that IFN-γ/STAT1 signaling plays an essential role in inducing the expression of proapoptotic IRF-1 protein and ends to cover a detrimental role by activating $CD4^+$ and NK T cells and directly hepatocyte apoptosis and liver injury (Fig. 1) [27]. On the contrary, IL-6-deficient mice displayed dramatically increased liver inflammation, extesive apoptosis and necrosis compated to wild type mice who also exhibited signs of liver injury. Moreover, it was noted that IL-6 induced activation of STAT3 protects against Con A–induced liver injury by inhibition of IFN-γ and IFN-γ signaling and induction of Bcl-X_L and other antiapoptotic factors, which consequently protect against hepatic necrosis and apoptosis. In summary, *Hong et el* in their report, demonstrated that activation of multiple JAK-STATs is rapidly induced by ConA injection and based on their emerging findings advocated their initial hypothesis that Con A–mediated hepatitis is tightly regulated by STAT1 and STAT3, which trigger $CD4^+$ and NKT cell activation, and expression of apoptosis-associated genes [27] **(Figure 1)**.

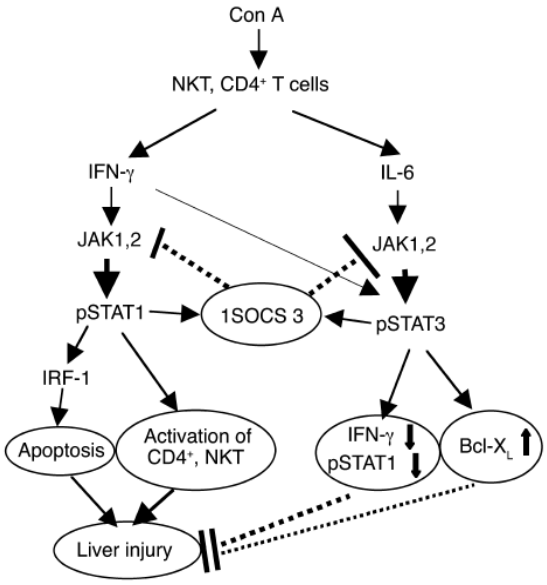


Figure 1 : *A proposed model of Con A–induced hepatitis suggest that T cell–mediated liver injury is tightly controlled by the opposing roles of STAT1 and STAT3(Adopted from [27].[27]*

Therefore, interleukin-6 is considered to be a pleiotropic, produced by many cell types, which function in both innate and adaptive immunity and has been also demonstrated to promote liver regeneration [60]. In addition, recent reports revealed that other membrers of the IL-6 family such as IL-11 and CT-1 dispayed protection against ConA-induced liver damage [11] [36]. In 2002, *Sun et al* demonstrated an additional mechanism that potentially is involved in the protective effect of IL-6 in T cell-mediated hepatitis. It has been well established that the critical step in the initiation of Con A-induced T cell hepatitis is the activation of NKT cells. Reliant on this well documented evidence, *Sun et al* revealed for the first time that IL-6, besides its antiapoptotic action, prevents T cell hepatitis via suppression of NKT cells through $CD4^+$ T cell- and STAT3-dependent mechanisms [60]. *Klein et al.,* used hepatocyte-specific gp130 knockout mice to determine the cell type that is responsible for IL-6-mediated liver protection and verified that IL-6 generates its protective effect through gp130-STAT3 signaling in liver cells [31].

IL-12 exacerbates murine ConA-induced hepatitis through an IFN-γ dependent mechanism. Recombinant murine IL-12 administered prior to ConA exacerbated both transaminase activities in plasma and histological signs of hepatitis. These markers of liver injury were significantly reduced by prophylactic, but not therapeutic treatment

with anti-IL-12 monoclonal antibody (mAb) [42]. The disease-modulatory effects of IL-12 and anti-IL-12 mAb were associated with profound and reverse modifications of a ConA-induced increase in the circulating levels of IL-4, IL-6, IFN-γ and TNF-α. Relative to control animals receiving ConA alone, the plasma levels of these cytokines were all augmented in IL-12/ConA-treated mice and diminished in anti-IL-12 mAb/ConA-treated mice. Anti-IFN-γ mAb also impeded the appearance of IL-12/ConA-induced hepatitis. Thus, IL-12-induced production of IFN-γ might play a role in mediating the hepatitis-inducing effect of ConA [42].

Recently, IL-18 has been shown to play a role in ConA- induced hepatitis. In general, IL-18 induces IFN-γ expression and secretion from IL-12-primed naive T cells to promote the differentiation of type 1 helper T cells. On the topic of IFN-γ synthesis, the IFN-γ promoter region has binding sites for NF-κB, AP-1, cyclosporine A-sensitive NFAT, intronic enhancer region (C3), and STAT-4. These regions are involved in the regulation of IFN-γ gene expression. IL-12 activates STAT-4 whereas IL-18 activates the IRAK/TRAF6 pathway, resulting in the activation both AP-1 via JNK and NF-κB. Mutation or deletion of the AP-1- and STAT-4-binding sites caused human $CD4^+$ T cells to fail in IFN-γ production upon combined stimulation with IL-12 and IL-18. Simultaneous activation of NF-κB and STAT-4 was also involved in the synergistic production of IFN-γ from a human myelomonocytic cell line stimulated by IL-12 and IL-18 [41] [85]. Therefore, the synergistic action of IL-12 and IL-18 results from the interaction between their corresponding signal-transduction pathways, leading to the activation of IFN-γ gene expression [25]. More recently, to our surprise, IL-18 has been shown to have the potential to induce IL-4 production. Therefore, IL-18 can induce both IFN-γ and IL-4 responses depending on its surrounding cytokine milieu. Indeed, with IL-12, IL-18 induces IFN-γ production, whereas without IL-12, IL-18 induces IL-4 and IL-13 production. Therefore, IL-18 should be seen as a unique cytokine that enhances innate immunity and both Th1- and Th2-driven immune responses [41].

The T cell derived cytokine IL-4 is primarily related to Th2 differentiation although it is characterised for its significant role in inflammation. The binding of IL-4 to IL-4R triggers $(JAK)^2$ 1 and JAK3 tyrosine kinase and specifically stimulates STAT6 which in turn translocates into the nucleus where it regulates gene transcription. In 2003 *Jaruga et al.* displayed that ConA-mediated liver injury is abolished in $IL\text{-}4^{-/-}$ and $STAT6^{-/-}$ mice without affecting IFN-γ/STAT1, IL-6/STAT3

or TNF-α/NF-κ signaling pathways or NK T cell activation. However, these mice exhibited markedly suppressed infiltration of neutrophils and eosinophils after ConA challenge. Moreover analysis of plasma levels of cytokines by elisa revealed diminished levels of eotaxins 1 and 2 and IL-5 in IL-$4^{-/-}$ and $STAT6^{-/-}$ mice compared to control suggesting that IL-4/STAT6 is responsible for in vivo production of eotaxin and IL-5 in ConA-induced liver injury [28]. As fas as the source of IL-5 production is concerned, *Louis et al*., reported that IL-5 is primarily secreted by NK T cells [35]. On the basis of aforementioned observations *Jaruga et al.,* proposed a model illustrating the pivotal role of IL-4 in Con A-induced hepatitis. In particular they supported that administration of Con A motivates NKT cells to produce IL-4 which then triggers hepatocytes and sinusoidal endothelial cells to secret eotaxins and enhances IL-5 production via a STAT6-dependent pathway, followed by attracting neutrophils. In general, Interleukin-5 is an activator of eosinophils and serves as the link between T cell activation and eosinophilic inflammation which ends to hepatitis [28].

Tumor necrosis factor (TNF-α) is a multifunctional cytokine primarily produced by macrophages and exerts a wide spectrum of cellular responses, including both inflammation and apoptosis. Intracellular pathways originating at the TNF receptor are either linked to apoptosis, nuclear factor (NF)-kappaB translocation, or Jun kinase (JNK) activation. Owing to its opposing roles as proinflammatory, antiapoptotic and apoptotic molecule it will be used as a connection for the next module and will be analysed below.

1.1.5. Considerations on signal transduction mechanisms of apoptosis in ConA-induced Fulminant Hepatitis

Aberrant apoptosis-mediated cell death is believed to eventuate in a wide range of different human diseases. Intensive analysis of the molecular mechanisms of apoptosis reliant on murine models confers a better understanding of many human diseases. As was mentioned in the introduction, fulminant and autoimmune forms of hepatitis are characterized by excessive inflammation, increased apoptosis, and necrosis. Apoptosis and necrosis may occur in parallel and high levels of apoptosis may result in secondary necrosis [30].

The biological process of apoptosis has been characterized as a mechanism involving death factors and death receptors which is controlled strictly. In the context of inflammation, apoptosis is triggered by the binding of ligands to death-inducing membrane receptors. Ligands such as TNF-a, FasL and TRAIL play an important role in ConA induced hepatitis.

1.1.5.1. TNFreceptor/TNF

Tumor necrosis factor (TNF-α) is a multifunctional cytokine primarily produced by macrophages and exerts a wide spectrum of cellular responses including inflammation and apoptosis and plays a predominant role in ConA-induced liver injury. In this pathologic instance, is mainly produced by Kuppfer cells and hepatocytes [51]. TNF-α plays a leading contribution in liver injury induced by ConA however it is noteworthy that it has dual role since TNF-α activates both apoptotic and antiapoptotic signaling pathways [33].

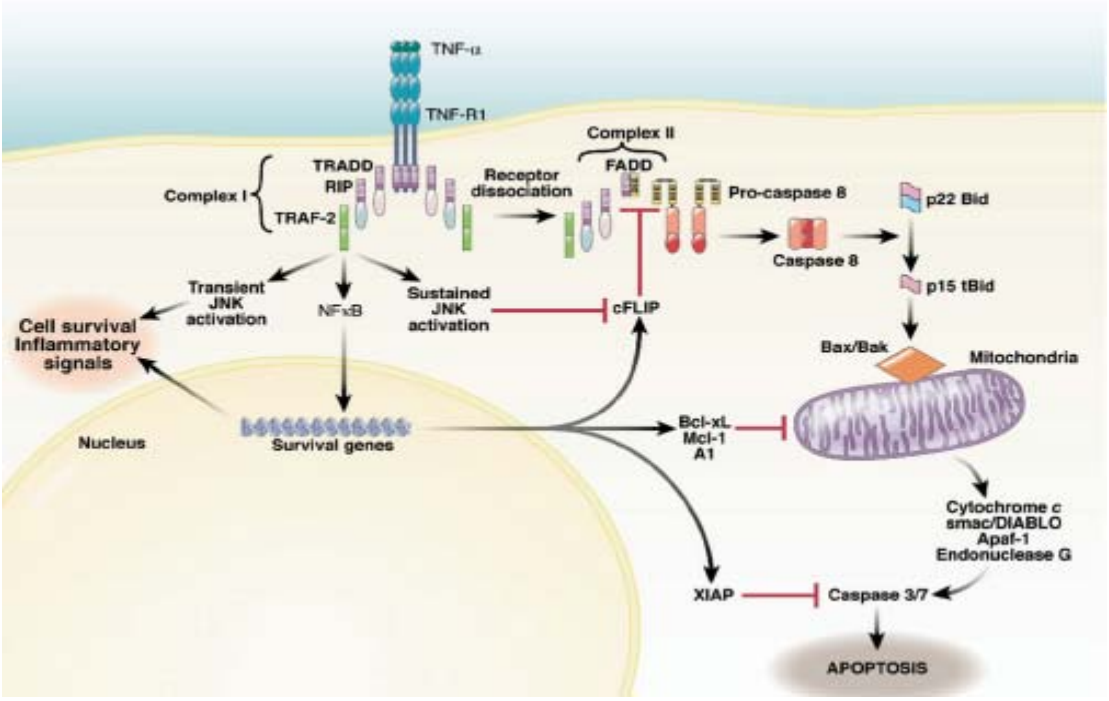


Figure 2: *Tumor necrosis factor a not only activates survival but also activates apoptosis. Adopted from [30]*

The role of TNF-α has been analysed in numerous reports. First, Gantner and his colleagues employed monoclonal antibodies directly against TNF-α in the context of ConA-induced hepatotoxic effects and demonstrated that pretreatment of mice with anti-TNF-a monoclonal antibody prevented Con A-induced hepatitis [24]. In addition

Trautwein et al. studied TNF-dependent pathways after con A injection in vivo. Specifically three groups of mice were injected with ConA, ConA plus anti-TNF, and control buffer respectively and subsequent to experimental analysis they observed elevated DNA fragmentation in hepatocytes 4-24 hours after con A injection. Analysis of their experiments displayed that all pathways were blocked by anti-TNF with the exception of c-Jun nuclear expression and DNA binding [65]. Hence, can be concluded that anti-TNF-a monoclonal antibody treatment abrogated not only hepatocyte damage but also NF-κB activation, which might be unfavourable for repair of liver damage liver regeneration. Therefore, an effective treatment of hepatitis may be achieved by selective inhibition of the apoptotic pathway without abolishing the beneficial effects of these proinflammatory cytokines, which are at least partially mediated by NF-κB activation. In this context, the blockade of FADD, an approach used by *Seino et al.,* whose report is further analysed below, could be an effective therapeutic strategy for liver diseases in which Fas and TNFR1 are implicated [53].

However, when *Tagawa et al.,* analyzed the role of TNF-α in the pathogenesis of Con A-induced hepatitis using TNF-α deficient mice they provided evidence revealed that susceptibility of TNF-$\alpha^{-/-}$ mice to fulminant hepatitis compared to control was not changed [62].

1.1.5.2. Fas receptor/Fas ligand

The Fas receptor is an essential mediator of liver injury since hepatocytes are highly susceptible to Fas-mediated apoptosis due to the increased Fas expression on hepatocytes. Simultaneously FasL is constitutively expressed on cytotoxic lymphocytes during fulminant hepatitis [71]. Therefore, as long as death factors are appropriately expressed they will be useful in maintaining tissue homeostasis and prevent the organism from liver disease.

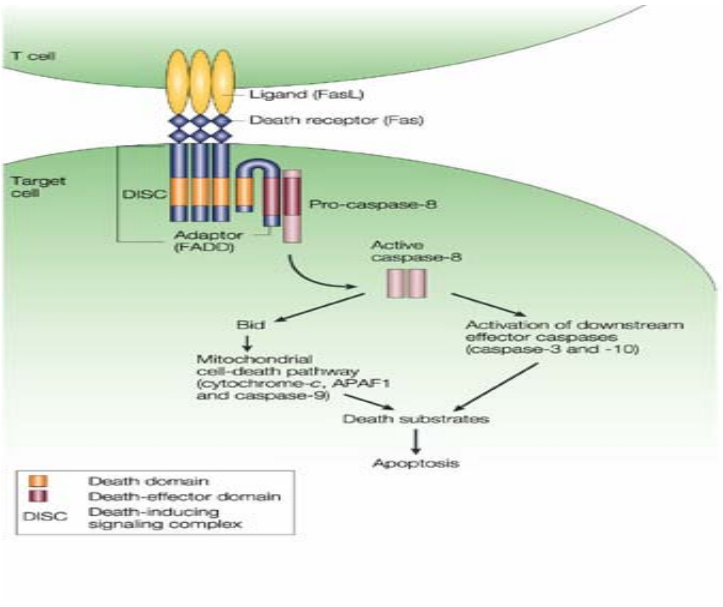


Figure 3: *Fas mediated signaling pathway of apoptosis, also called activation-induced cell death. Adopted from [69]*

On the contrary, intraperitoneal injection of agonistic anti-Fas antibody in normal mice, leads to activation of Fas in vivo and results to massive hepatocyte apoptosis and promptly causes animal death owing to fulminant hepatitis. The mechanism underlying lethal injury is a direct action of anti-Fas in inducing apoptosis in Fas-expressing liver parenchymal cells [70]. *Tagawa et al* used gld/gld mice in which the fas ligand gene is defective by a point mutation in exon 4 in order and displayed that development of the disease is completely suppressed by ConA challenge in gld/gld mice. Liver histology, TUNEL staining and DNA fragmentation analysis revealed severe apoptosis of the liver cells in the wild type mice but completely normal appearance in the mutant ones. The levels of IFN-γ mRNA in the liver and of C3H/HeJ-gld/gld mice increased similarly after Con A administration compared to organs of wild-type mice. In contrast, suppression of the disease in lpr/lpr mice in which the fas gene is disrupted by the insertion of a retroviral transposon was incomplete since a small amount of the fas mRNA was produced in these mice [61].

In order to study further the Fas/FasL signaling pathway of apoptosis, *Seino et al.,* examined whether the administration of Ad.FADD-DN could prevent Con A-induced hepatitis. Ad.FADD-DN carried a deletion mutant of FADD lacking 79 N-terminal amino acids which was able to interact with Fas and TRADD but did not initiate apoptosis [53]. Taken together, these results indicate that Ad.FADD-DN

treatment can block the T cell-mediated liver injury induced by Con A, in which both TNF-α and FasL are critically involved and could be an effective therapeutic strategy for liver diseases [53].

1.1.5.3. TRAIL receptor/TRAIL

The TNF-Related Apoptosis Inducing Ligand (TRAIL), also called Apo2 ligand, is a further member of the TNF superfamily [38] [49]. The aforementioned reports highlighted the essential roles of TNF-α and FasL in ConA model of hepatitis and raise the question whether TRAIL is implicated in apoptosis in the context of liver injury. To address this question, *Zheng et al.,* injected ConA in $TRAIL^{+/+}$ and $TRAIL^{-/-}$ deficient mice to induce hepatitis and discovered that $TRAIL^{-/-}$ were resistant to ConA-induced hepatitis since the serum transaminases and caspase-3 assay revealed significantly low levels of ALT and reduction of caspase-3 respectively. In addition, in vivo blocking of TRAIL by intraperitoneal injection of soluble DR5 into mice, protected animals against ConA-induced hepatitis. Furthermore, adoptive transfer of either hepatic or splenic mononuclear cells (MNCs) from $TRAIL^{+/+}$ and $TRAIL^{-/-}$ mice to $TRAIL^{-/-}$ mice, $TRAIL^{+/+}$ but not $TRAIL^{-/-}$ MNCs restored the sensitivity of recipient mice to ConA-induced hepatitis. These results indicate that TRAIL expression by immune cells is required for the development of ConA-induced hepatitis [78].

1.1.5.4. CD40/CD40L

Besides the aforementioned death factors, the TNF family also includes effector molecules such as CD154 which is commonly as CD40 ligand (CD40L). The binding of CD40 to CD154 activates the antigen presenting cells (APCs) and produced a variety of downstream effects through TRAF (**T**NF-**R**eceptor-**A**ssociated **F**actors) binding resulting in immune respone, inflammation and apoptosis. Therefore the CD40-CD40L interaction is a critical costimulatory parhway modulating the cellular immune response [70].

Recent studies have demonstrated the upregulated expression of CD40 on the surface of hepatocytes in the livers of patients with FH as well as the hepatic infiltration of activated CD154-bearing T cells [34]. Beyond these, *Afford et al.,* observed that enhanced expression of CD40 in human hepatocytes from patients with

chronic rejection after liver transplantation followed by CD40 activation which leads to triggering apoptosis via the Fas pathway [1]. On the basis of these puplished reports inspirited and *Zhou et al.,* documented the therapeutic efficacy of the inhibition of the CD40-CD154 pathway in treating fulminant hepatitis. Specifically, in their study they induced fulminant hepatitis by concanavalin A administration and odserved a noteworthy amelioration of ConA hepatitis in CD154-deficient mice compared with that in controls. Furthermore, hepatic levels of TNF-α and IL-4 were significantly lower in con A–treated $CD154^{-/-}$ mice compared with WT mice and was also noted a significant reduction in hepatic $CD4^{+}$ T-cell infiltration [79].

1.1.6. The powerful tool of gene silencing techniques as a novel therapeutic approach in liver injury

The idea of gene silencing techniques has been developed over the last 20 years with the discovery of antisense oligodeoxynucleotides (ASO) and their widespread use for gene regulation in cell culture experiments and in animal models. Indeed in 2000, *Zhang* and his colleagues supported this approach and showed that mice pretreated with an antisense oligonucleotide directed against Fas mRNA, exhibited reduced Fas expression in liver in a dose- and sequence-specific manner and therefore provided defense against agonistic Fas antibody-induced fulminant hepatitis and acetaminophen (AAP)-induced fulminant hepatitis [77].

However, a turning point was emerged for science by the discovery of RNA interference. Accordingly, RNAi seems to be more reliable as an effective and specific means of silencing gene expression [9]. It was of considerable interest the fact that the powerful tool of short interfering RNAs was soon applied on animal models of disease to “silence” specific gene activities [49] [58] [76]. Interestingly, the liver is an attractive target organ for the use of short interfering dsRNA because of the organ’s blood supply and propensity [47]. In this instance, hepatic influx of siRNA has been observed following their systemic administration [58] [76] [49]. Two scientific groups have recently used means of RNAi to examine whether Fas plays a pivotal role in fulminant hepatitis *in vivo* and both of these studies established a survival benefit of animal models using this approach of gene silencing. *Song and his*

colleagues provide the first *in vivo* evidence proved that infusion of Fas siRNA into an animal model of hepatitis can alleviate the disease [58].

These results were subsequently confirmed and extended by the study of *Zender* and his colleagues who have taken advantage of RNAi to "silence" caspase 8, a major downstream target of all known death receptors. In view of the crucial role of caspase 8 in Fas, in TNF-α as well as in TRAIL-mediated apoptosis, they demonstrated that systemic pretreatment with siRNAs targeting caspase 8, protected mice against Jo2 and AdFasL challenged fulminant hepatitis [76]. Significantly, they also assessed the valuable therapeutic efficacy of siRNA in mouse models resembling the clinical situation, since in that case caspase 8 siRNA would only be applied after the onset of fulminant hepatic failure. Therefore improvement survival obtained by siRNA treatment prior to as well as during ongoing liver damage highlights the RNAi as an attractive therapeutic strategy [76].

In addition to caspase 8 and Fas, other key molecules in various signal transduction pathways may also be attractive targets for therapeutic RNAi in the liver. For instance, osteopontin (OPN) is mainly known as a protein of an extracellular matrix, involved in biological processes such as cell adhesion and migration of inflammatory cells.. In 2004, *Diao et al,* verified the concept that osteopontin is involved in the pathogenesis of various inflammatory diseases and discovered that hepatic NKT cells are a major cellular source of OPN after ConA injection [18]. Reliant on previous data, a study conducted recently by *Saito et al,* revealed that intravenous hydrodynamic infusion of siRNA targeting OPN could be delivered successfully into hepatocytes in vivo where it inhibited endogemous OPN protein expression, thereby resulted in the amelioration of Con-A induced hepatic injury [49]. Altogether, these data suggest a siRNA-based treatment strategy can be applied for the treatment of various inflammatory diseases.

1.2 The Tpl2/Cot protein kinase

The *Tpl2/COT* has been classified into the mitogen-activated protein kinase kinase kinase (MAP3K) family, is a serine-threonine protein kinase and is known to have a pleiotropic role. Tpl-2 is implicated in the regulation of several cellular kinase pathways and has also been found to induce miscellaneous transcription factors. It is

expressed primarily in spleen, thymus, and lung tissue [37] and is a key component in activation of B cells, T cells and macrophages, resulting in TNF- production from these cell types [6] [19]. In tissue culture, overexpressed Tpl2 is constitutively active and can activate the ERK, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase pathways and the transcription factors NFAT and NF-κB in a variety of cell types [17]. The activation of NFAT and NF-κB, as well as the induction of IL-2 by T cells through these pathways indicate that Tpl-2 may play an important role in T cell activation and suggests a potential molecular mechanism that may contribute to its pathogenic potential in liver injury [66] [67].

1.2.1. Structural analysis of Tpl2/Cot gene

In 1991, transformation of Syrian Hamster Osaka Kanazawa (SHOK) cells with DNA extracted from a human thyroid cell line resulted in the identification of the ***"Cancer Ozaka Thyriod"***, commonly known as Cot gene. This product of gene rearrangement and encoded for an open reading frame (ORF) of 415 amonoacids (aa) that gave rise to two protein products (p52 & p46) owing to alternative initiation of translation on the same mRNA. However the physiological *cot* analogue (proto-oncogene) was found to encode for an ORF of 467 aa that generated two different protein products (58 & 52kDa) through alternative translation initiation sites. The structural differences between the physiological and the Cot oncoprotein concern replacement of 70 aa from the carboxyl-terminus of the former by 18 nonidentical aa in the Cot oncoprotein due to DNA rearrangement. This structural alteration led to higher kinase activity and transforming capacity of the Cot oncoprotein versus its physiological counterpart [3] [Aoki *et al*., 1993]. In parallel, the murine homolog of Cot is "***Tumor progression locus"*** (Tpl2) which is located on chromosome 18 and share ~93% identity at the aminoacid level. The Tpl-2 gene consists of eight exons, refered to a 35 Kb genomic DNA region. In this connection, Tpl2 protein was initially identified as a proto-oncogene that is activated by provirus integration in Moloney murine leukemia virus-induced T cell lymphomas and MMTV-induced mammary carcinomas in rodents [37] [39]. Provirus insertion always occurs in the last intron of gene and gives rise to mRNA transcripts that encode a carboxy-terminally truncated kinase with constitutive high kinase activity [45] [37]. Transgenic mice expressing the truncated form of Tpl2 under the control of a T cell-specific promoter develop T cell

lymphoblastic lymphomas by the age of 3 months [13]. Therefore the catalytic activity of truncated Tpl2 correlates with its oncogenic potential.

1.2.3. Regulation of Tpl2/Cot protein kinase

Despite these various important cellular functions, little is known about the precise molecular mechanism by which Tpl2 is regulated during cell signaling events. Foregoing studies have demonstrated that Tpl2 forms a stoichiometric complex of high avidity with the cytoplasmic NF-κB inhibitory protein p105 NF-κB and the inhibitory protein ABIN-2 [19] [23] [45] (8 –10syk). Through this molecular interaction, the NF-κB1 precursor protein p105 stabilizes Tpl2 and inhibits its kinase activity. Therefore, in unstimulated cells the action of Tpl2 in normally suppressed. Actually, the p105-bound Tpl2 is functionally inactive and its activation involves signal-induced (LPS and TNF) p105 degradation and the dissociation of Tpl2 from the complex. Liberated Tpl2 is active but unstable and consequently undergoes rapid degradation via the proteasome [73]. Strong evidence suggests that the signal-induced Tpl2/Cot activation requires IKKβ, which functions by phosphorylating p105 and triggering its proteolysis [73] [7]. Data presented by Cho et al., show that the release of Tpl2 from p105 and its subsequent activation and degradation in response to LPS depend on Tpl2 phosphorylation at Thr-290 [15]. The same group revealed that IKKβ is an obligatory upstream regulator of Tpl2 since the IKKβ catalytic subunit of the IKK signaling complex is also required for the direct phosphorylation of Tpl2 at Thr290, which regulates Tpl2 binding to NF-κB [45] [22].

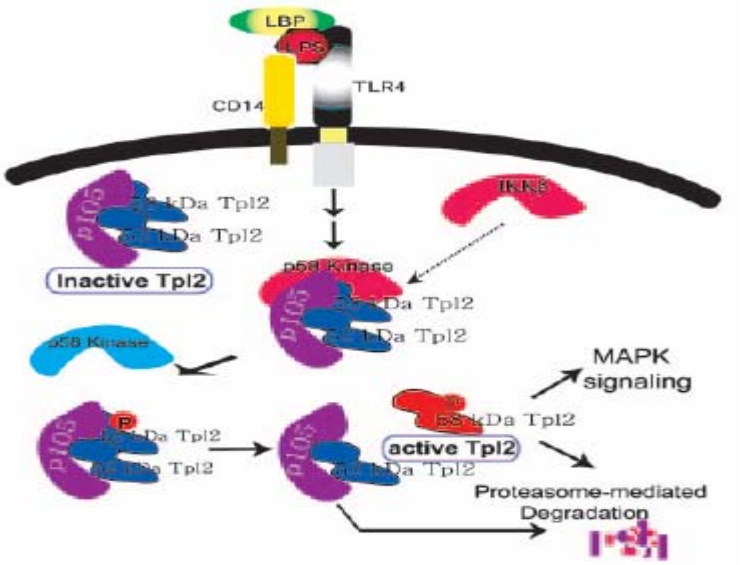


Figure 4: *A model of the role of Thr-290 phosphorylation in Tpl2 activation by LPS.Adopted from [15]*

Even though is established that IKKβ and NF-κB1 are required for the regulation of Tpl2 activation, additional molecules regulate Tpl2 as well in response to stimuli. For example, human kinase suppressor of Ras 2 (hKSR-2) negatively regulates Cot in the NF-κB and MEK/ERK pathways by direct binding. This group also provide evidence that Cot controls IL-8 production, possibly through the ERK and NF-κB signaling pathways which is inhibited dramatically by co-expression of hKSR-2 [14]. Recently, *Eliopoulos et al*., presented evidence showing that Tpl2 activation depends on the tyrosine kinase Syk, which is activated following TNF-α stimulation of macrophages [20]. In addition, the serine/threonine kinase Akt interacts with Tpl2, in order to regulate the function of Tpl2 in the NFkB signaling pathway in T cells although this phosphorylation event seems to have no regulatory effects [29].

1.2.3 The role of Tpl2 in the regulation of innate immunity

The generation of Tpl2 defective (Tpl2-/-) mice contribute considerably to the delineation of the role of Tpl2 in both innate and adaptive immune system. The Tpl2 knock-out (Tpl2-/-) mice are viable and have no obvious phenotypic defects [19]. However, a detailed analysis of these mice revealed a critical role for Tpl2 in the regulation of innate immunity and in the response to inflammatory signals through the modulation of the ERK MAPK signaling pathway. In terms of the innate immunity, the finding that Tpl2 is involved in the Toll-like receptor 4 (TLR4) signaling pathway emerged by genetic studies using Tpl2 knockout mice. This study revealed that after binding with TLR4, lipopolysaccharide (LPS) activates extracellular signal-regulated kinase (ERK) and induces the production of tumor necrosis factor-a (TNF-a) in a Tpl2-dependent manner. As a result, Tpl2-/- macrophages are defective in the induction of TNF-α and other pro-inflammatory molecules in response to such stimuli due to defective Tpl2/MEK/ERK signaling. Tpl2-deficient mice are resistant to LPS-induced endotoxic shock because they lack ERK activation, resulting in lower TNF-a production [19]. Therefore, Tpl2 is required for the expression of TNF-α in circulating plasma following the administration of LPS in vivo, and cultured macrophages from tpl2-/- mice displayed notably reduced TNF-α production upon LPS stimulation [19] [59]. In addition, LPS stimulated Tpl2-/- macrophages exhibited a restricted defect in ERK activation while the JNK and p38 MAPK signaling

pathways remained unaffected [19] [59]. It should be noted that mice deficient to Tpl2 were also studied in the context of an established inflammatory bowel disease (IBD) mouse model ($TNF^{\Delta ARE}$). These mice exhibited a robustly attenuated progression of the disease and completely ablated TNFα-induced ERK activation in tpl2-/- macrophages [17] [21, 32] showing that Tpl-2 kinase regulate the lymphocytic response and its absence caused a delayed onset and progression of IBD [32]. Taken together, Tpl2 kinase, above and beyond its role as a MAP3K kinase, mediates TNF-a and other cytokine production, and is closely linked with the development of many inflammatory diseases, such as rheumatoid arthritis or inflammatory bowel disease [26] [32].

1.2.4 Implication of Tpl2 in the regulation of adaptive immunity

As was mentioned above, the Tpl2-MEK-ERK module is considered to be a fundamental signaling component in cells of the innate immunity. However, Tpl2 signaling also plays an obligatory role in the regulation of adaptive immune system. To be more specific, *Eliopoulos et al.,* showed that MEK/ERK activation in response to CD40 stimulation is ablated in tpl2-/- B cells demonstrating that Tpl2 is required for the transduction of ERK activation signals initiated by CD40 engagement. This study also revealed that Tpl2 contributes to IgE synthesis in response to IL-4 and anti-CD40mAb stimulation in B lymphocytes [21]. Tpl2 also plays an important role in T cell function. Several puplished reports claim that Tpl-2 kinase plays an important role in T cell activation and interleukin-2 production by inducing the transcription of these genes [5] [45] [66] [67]. A recently published article presented evidence regarding Tpl2 ablation indicating that may affect the regulation of CTLA4 and revealed that TCR stimulated Tpl2-/- T cells exhibit a defect in ERK activation and CTLA4 induction and they hyperproliferate in response to antigen. Thus, Tpl2 is required for full activation of ERK in response to TCR signals, and the activation of ERK is the prerequisite for the induction of CTLA4 [68].

The significant role of Tpl2 in adaptive immunity is enhanced by the study of *Sugimoto et al.,* who demonstrated that marrow–derived DCs from Tpl2-/-mice produced significantly more IL-12 in response to CpG-DNA than those from WT mice. Consistently, Tpl2–/– mice showed Th1-skewed antigen-specific immune

responses upon OVA immunization and Leishmania major infection in vivo. These results indicate that Tpl2 is an important negative regulator of IL-12 production and Th1-type adaptive immunity since dendritic cell IL-12 is known to induce Th1-type T cell differentiation [59]

In this connection it should be remarked that *Wang et al*., presented evidence that OVA-immunized Tpl2-/- mice produce considerably higher levels of both OVA-specific and total IgE than Tpl2+/+ mice. It was proposed that the upregulation of IgE is correlated with increased secretion of Th2 cytokines sych as IL-4 and IL-5 and decreased secretion of IFN-γ by Tpl2-/- T cells exposed to ovalbumin [72]. The shift towards Th2 polarization of the T cell response to OVA in Tpl2-/- mice is in agreement with another study showing that the defence of Tpl2 knockout mice to Toxoplama gondii is impaired because of a T cell autonomous Th2 shift of the T cell response [74]. In this recently conducted study was demonstrated the Th2 polarization of the immune response in Tpl2 knockout mice suggesting that inhibition of Tpl2 may alleviate the symptoms of Th1-dependent autoimmune diseases [72].

1.2.5. The Tpl2 /Cot kinase as a target for therapeutic implementation

All of the foregoing studies were performed in Tpl2-deficient mice and cells derived from those mice and indicated that Tpl2 is an important regulator of both innate and adaptive immunity. At the same time, Tpl2 is also related to apoptosis since in an *in vitro* study, it was reported to participate in the apoptotic pathway by promoting caspase activation [46]. Consistent with the aforementioned apoptotic role of Tpl2 is a very recent report suggesting that Tpl2 plays a pathogenic role in a mouse model of renal ischaemic injury, especially on chemokine production and promotion of tubular epithelial cells apoptosis [75]. According to the above stated literature, Tpl2 has a pleiotropic role affecting inflammation and apoptosis. Therefore inhibition of Tpl2 seems to be an appropriate therapeutic target in these inflammatory diseases. In order to further validate the value of Tpl2 as a therapeutic agent for human inflammatory disorders is necessary to perform experiments targeting the down-regulation of Tpl2 in human cell types. One such report was conducted by *Hall et al.,* who demonstrated Tpl2 is essential for the LPS-induced activation of MEK and ERK in primary human monocytes and its inhibition blocks LPS- and IL-1β-induced TNF-

α production in primary human monocytes as well as in human blood. In this connection, was also shown that inhibition of Tpl2 block ERK activation, COX-2 expression, prostaglandin E2, the production of various soluble pro-inflammatory mediators (IL-6, IL-8), and the matrix metalloproteinases MMP-1 and MMP-3 in rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) [26]. Overall this report verified the physiological roles for Tpl2 in human inflammatory cell types, and laid the foundation stones regarding the value of Tpl2 as a target for therapeutic intervention in human.

1.3. Biological significance-Objectives

Fulminant hepatitis is a clinical syndrome characterised by sudden and severe impairment of liver function, resulting from massive liver inflammatory infiltration with subsequent death of hepatocytes. Acute liver failure can be devastating and is associated with high mortality. However, the underlying pathophysiological mechanisms are not yet fully understood and therefore an effective therapeutic approach is difficult. Consequently, further understanding of the molecular pathogenesis of fulminant hepatitis appears to be prerequisite for the development of more effective therapies. ConA induced liver injury is a novel model of severe hepatitis is intensively used to study the immunopathogenesis of liver diseases. This T cell-dependent model of inflammatory liver injury allow the investigation of basic principles of hepatic disorders associated with T cell activation and infiltration as well as pharmacological in vivo studies for the development of hepatoprotective drugs. With regard to novel therapeutic approaches of fulminant hepatitis, at the present time a significant number of research groups have been focused on the potential of gene silencing techniques for future therapeutic implementation. For this purpose, a promising candidate molecule is Tpl2, a serine/threonine protein kinase that has been classified as a member of the mitogen-activated protein kinase kinase kinase family (MAP3K). Based on its role in inflammation, Tpl2 has been recognized as an important target for the development of anti-inflammatory drugs. In view of the fact that the signals driving T cell activation in T-cell mediated fulminant hepatitis are not fully understood, we will investigate whether Tpl2 acts as a major pathogenic factor in a murine model of fulminant

hepatitis. The main aim of this study is to clarify the precise role of tpl2 protein in the context of inflammation and apoptosis in liver injury. Along these lines, we are going to investigate whether Tpl2 is critically involved in the pathophysiology of fulminant hepatitis and try to determine the molecular mechanism of its kinase action regarding inflammation and related signals. We also aim to pursue the characterisation the precise cytokine milieu which in turn shape and enhance inflammatory response and adaptive immunity in the context of the established model of ConA-induced fulminant hepatitis.

MATERIALS & METHODS

2. Materials and Methods

2.1. Animals

2.1.1. Animals manipulation

All animals received humane care according to the criteria outlined in Guide for the Care and Use of Laboratory Animals. Mice were housed in a temperature-controlled environment with a stable photoperiod of 12-hour light/12-hour dark cycle and allowed unrestricted access to standard mouse chow diet and tap water.

2.1.2. Genotyping of mice

Genomic DNA extracted from tail using phenol/chroroform and was analyzed with PCR to verify the deletion of the Tpl2 gene. A 188-bp PCR product using primers a and b corresponds to the tpl2 allele whereas a 400-kb PCR product using a and c is correlated to the deletion of tpl2 mice. The Polymerase Chain Reaction was performed using *Taq Polymerase system* (invitrogen*)*.

The primers used for the PCR reaction are the following:

	Sequence	Length	Tm
A. $Tpl2_{FW}$	5'-GGAGGTCCTTGGGAAGATAGA-3'	21b	54^{0} C
B. $Tpl2WT_{REV}$	5'-CCTTCCGTCCTGCTTGGAAC-3'	20b	56^{0} C
C. $Tpl2KO_{REV}$	5'-GCACGAGACTAGTGAGACGTG-3'	21b	56^{0} C

Table 1. Primers used for PCR genotyping

	94^{0} C	**5 min**	
Denaturation	**94^{0} C**	**40 sec**	**40 cycles**
Annealing	**56^{0} C**	**40 sec**	
Extension	**72^{0} C**	**40 sec**	
Final Extension	**72^{0} C**	**10 min**	
Hold	**4^{0} C**	∞	

Table 2. The conditions of the PCR reaction

2.1.3. Generation of Con A–Induced Hepatitis

The disease model of Con A-induced hepatitis is widely used as an animal model of T cell-mediated hepatitis as was mentioned previously. Female mice 10- to 12-week-old wild-type (Wt) C57Bl/6 and corresponding mice deficient in Tpl2 (Tpl2-/-) on a C57Bl/6 background were injected intravenously at a single dose of 10μg/gr of freshly prepared concanavalin A (Sigma, St. Louis, MO) reconstituted in sterile sodium chloride 0.9%. This dose was chosen to give severe hepatitis without mouse mortality. As a control, C57Bl/6 mice received sodium clroride 0.9% (NaCl 0.9%) mice in the tail vein. Mice were sacrificed 2, 4 and 8 hours following Con A or saline administration respectively.

2.1.4. Analysis of liver injury markers

The extent of hepatitis was initially determined biochemically. For this purpose, after we sacrificed 4, 8, and 24 hours after ConA or saline administration, we isolated blood at all times points respectively. Blood was incubated for 10 minutes to set in. We centrifuge the samples at 6000rpm for 15 minutes at room temperature. Serum was collected for determination of activities of liver injury markers after ConA administration. Measurement of serum plasma enzyme activities and particularly alanine aminotransferases (ALT) and aspartate aminotransferase (ASP) were assayed by standard enzymatic procedures in each sample serum in the Laboratory of Clinic Biochemistry, Teaching Hospital of Heraklion.

2.1.5. Histological Analysis.

Extent of hepatitis was also determined histologically. Therefore, we collected tissue for the assessments of liver damage.The isolated livers were fixed in 10% formalin. Tissues were embedded in paraffin, and consecutive 5-μm sections were mounted on slides. Sections were stained with hematoxylin/eosin (H&E) for histological examination

2.2. Cell Culture

2.2.1 Splenocyte Cultures

Cell culture media

- **RPMI 1640** - (Gibco)
  - L –Glutamine
  - 25mM Hepes

- Complete Media
  - RPMI 1640
  - 10% Fetal Bovine Serum (Gibco)
  - 1% Non Essential Aminoacids (Gibco)
  - 1% Sodium Pyruvate (Gibco)
  - 1% Penicillin/Streptomycin (Gibco)
  - 0,5% β-Mercaptoethanol (Sigma)

- 1X **P**hosphate **B**uffered **S**aline (PBS) pH 7.4:

  For 1 liter:
  - 8 gr NaCl
  - 0.2 gr KCl
  - 1.44 gr Na_2HPO_4
  - 0.24 gr KH_2PO_4

Spleens from naive WT and naïve Tpl2 deficient mice were removed and squeezed between sterile frosted slides and passed through a 40-μm cell strainer (BD PharMingen). Single-cell suspensions were washed with Phosphate Buffer Saline (PBS) and centrifuged for 10 minutes at 1400 rpm at room temperure. Cell suspensions were subsequently subjected to red blood cell (RBC) lysis, using sterile double destiled water for 4-5 seconds, and 10x HBSS (Hank's Balanced Salt Solution) to recuperate concentration. Cells are then washed with 10ml PBS and centrifuged for 10 minutes at 1400 rpm. Each cell pellet was resuspended in complete medium and 4 x 10^5 cells/well were plated in a 96-well plate for the thimidine assay

whereas 3 x 10^6 cells/well were plated in 6-well plates for western blot analysis. For Western Blot analysis, wild type and Tpl2-/- splenocytes cultured in compete medium (as above) were treated in vitro with Concanavalin A (sigma) at the concentration of 5μg/ml for 5´, 10´, 15´, 30´, 2h, 4h, 8h and 24h and whole cell protein extractions were prepared.

2.3. Techniques of Molecular Biology and Immunology

2.3.1. Thimidine assay

For the thimidine assay, wild type and Tpl2-/- splenocytes cultured in compete medium (as above) were treated in vitro with Concanavalin A (sigma) at the concentration of 3μg/ml or 5μg/ml. After 40 h of culture, cells were pulsed with [^{3}H] thymidine (Amersham Biosciences; 1 μCi/well) for 6 h. Following pulse with [^{3}H] thymidine cultures were harvested for counting.

2.3.2. Whole Cell Protein Extraction

Cells were collected from plates were immediately centrifuged at 1400rpm for 10 minutes. Supernatants were discarded and cell pellet was resuspended in 200μl of protein lysis buffer, which causes the rupture of the cell-membrane and the nuclear membrane.

We used a lysis buffer consisting of: RIPA solution, 10μg/ml Aprotinin, 10μg/ml, 1mM Sodium-Orthovanadate and 1mM PMSF and 1mM DTT. RIPA extraction buffer consists of:

10% glycerol	5% Na-deoxycholate
1M Tris-HCl pH 7,5	5M NaCl
10% NP-40	0,5M EDTA

Cells were incubated with the RIPA lysis buffer for 15 minutes on ice and then centrifuged for 15 minutes at 10000rpm at 4ºC. Supernatants, that are the protein extracts, were collected in a new eppendorf and stored at -80 ºC.

2.3.3. Quantification of protein extracts with Bradfort protein assay

- Biorad Protein assay Dye Reagent (Biorad Lab. Ltd., Hemel Hempsted, UK)
- Cuvettes

The quantification of proteins concentration is performed using the spectrophotometer and the Biorad Protein assay Dye Reagent that alters its colour from light to dark blue, depending on the concentration of proteins. We dilute Bradford reagent 1:4 with dH_2O and weadd the appropriate amount of protein extracts (1-20μl) to prepare a homogenous suspension that is transferred into cuvettes. Samples are counted in a spectrophotometer at 595nm and proteins concentration is calculated according the equasion of the standard curve, that links density (OD) (=y) and proteins amount in mg (=x). To be more accurate, we create new standard curve every time based on the lineae equation from the curve arises from the BSA standarts we estimate the concentration of protein samples.

2.3.4. SDS-PAGE Electrophoresis

Protein electrophoresis in a denaturating polyacrylamide gel results to the separation of proteins based on their molecular weight. It consists of two gels, a stacking gel, where protein samples are loaded, and a resolving gel, where proteins are separated. A concentration of 10% of the resolving gel is usually used, but depends on the molecular weight of the proteins to be studied.

The resolving gel is prepared first using the electrophoresis apparatus (Biorad), in 1.5mm thikness usually. Small layer of isopropanol is added on top, to bring into line the polyacrylamide gel, and it is left to rest for 10-15 minutes, in order to be polymerized. Then, the stacking gel is prepared and added on top of the running gel (isoporopanol has been removed) and combs are placed in the gel, quickly before it is thickened. The gel is left to gelatinate and after that it is transferred into the tank filled with 1lt of running buffer. Combs are removed and wells are thoroughly washed with 1x running buffer. Components of the gels and running buffer are listed below.

Resolving gel (10% polyacrylamide):

- ddH_2O
- 30% polyacrylamide mix (29:1)
- 1,5M Tris (pH 8,8)
- 10% SDS
- 10% ammonium persulfate
- TEMED

Stacking gel:

- ddH_2O
- 30% polyacrylamide
- 1M Tris (pH 6,8)
- 10% SDS
- 10% ammonium persulfate
- TEMED

Running Buffer (10x):

- 0.25M Tris-HCl, pH=8.3
- 1.92M glycine
- 1% (w/v) SDS

Protein extracts are supplemented with the gel sample buffer that helps to denaturate proteins.

Gel Sample Buffer:
- 187.5 mM Tris-HCl,
- 150 mM β-mercaptoethanol
- 6% (w/v) SDS
- 0.03% (w/v) bromophenol blue

Protein samples are then boiled in the Heat Block for 10 minutes at 100ºC, so as proteins to be denaturated in their primary structure, and immediately placed on ice, to reduce temperature. Samples are centrifuged at 14000rpm for 1 minute and are next

loaded in wells. A protein marker containing the following molecular weights: 181 KD, 115.5 KD, 82.2 KD, 64.2 KD, 48.8 KD, 37.1 KD, 25.9 KD, 19.4 KD (Invitrogen) is also loaded. The voltage of the apparatus is set at 100-120Volts and running takes place for 90-120 minutes, so as the proteins have run the gel and have been well separated. Molecular weight determination is based on the bands of the protein ladder.

2.3.5. Western Blot Analysis

Following the protein polyacrylamide gel electrophoresis, proteins must be blotted on a nitrocellulose transfer membrane. The gel is transferred from the previous apparatus into a different one consisting of a 'cassette' that includes two 3mm Whatmann papers and a nitrocellulose transfer membrane. Casettes are placed in a tunk full of 2lt Transfer buffer, in the correct direction, so as the proteins can move from the negative to the positive pole. Transfer of the proteins needs approximately 60-90 minutes. Then, the nitrocellulose membrane is placed in wash solution (TBST) supplemented with 10% milk, for blocking non-specific bindings, for 1 hour. Next, the membrane is washed three times with TBST, for 10 minutes and the primary antibody is added at the indicated concentration overnight, on a rocking apparatus, at 4ºC. The next day, the membrane is washed thoroughly, three times, for 10 minutes each, and then the secondary antibody is added, in the indicated concentration, for 1 hour at room temperature on a rocking apparatus. The washing steps are repeated and finally the membrane is ready to be developed, using Enhanced Chemo Luminescence system (Amersham), on an auto-radiography film (Fuji). The membrane can be re-used for the detection of a different protein, using another primary antibody. For this purpose, the membrane must be first subjected to 'stripping', for 15 minutes in a 55ºC pre-heated water-bath.

Buffers used for this step of Western blotting are:

Transfer buffer:

- 25mM Tris-HCl, pH 8,3
- 0,2M glycin
- ddH_2O up to 800ml
- 200ml methanol

TBST:

- 60ml NaCl 5mM
- 20ml Tris-HCl 1M, pH 8,0
- 2ml Tween
- ddH_2O up to 2lt

Strip solution:

- 100mM 2-mercaptoethanol
- 2% SDS
- 62,5mM Tris-HCl pH 6,7

Antibody	Animal source	Dilution	Molecular Weight (MW)	Manufacturer Supplier
p- ERK1/2 S**er217/221**	Mouse	1:500	42, 44 kD	Sigma
a-ERK1/2 (C-16)	Rabbit	1:1000	42, 44 kD	Santa Cruz
pY-STAT1	Rabbit	1:1000	84, 96 kD	Cell signaling
pY STAT3	Rabbit	1:1000	79, 86 kD	Cell signaling
Actin	Mouse	1:1000	43 kD	Cell signaling

Table 3. Primary antibodies

Antibody	Dilution	Molecular Weight (MW)	Manufacturer Supplier
a-mouse HRP	1:10000	42, 44 kD	Sigma
a-rabbit HRP	1:2000	42, 44 kD	Santa Cruz

Table 4. Secondary antibodies

2.3.6. Cytokine Determination via Enzyme-Linked Immunosorbent Assay (ELISA)

Secretion of serum and hepatic levels of cytokines were determined were determined by specific enzyme-linked immunosorbent assay (ELISA). In detail, TNF-

α, IL-6, IL-4, IFN-γ, IL-10 and IL-12 levels in serum and liver homogenates were estimated via ELISA (e Bioscience) and results are expressed as pg/ml.

Buffers

ELISA/ELISPOT Coating Buffer Powder: Reconstitute in 1L dH20; filter (0.22 uM).

- Assay Diluent (5X concentrate): Dilute 1/5 in distilled water.
- Substrate Solution: Ready to use (1X)
- Wash Buffer: 1X PBS- 0.05% Tween-20
- Stop Solution: 1N HCl

Regarding the experimental procedure, purified anti- mouse capture antibody is used at a dilution of 1:250. Coat Corning Costar 9018 96 well plate with 100 µl/well of capture antibody in Coating Buffer and was incubated overnight at 4°C. After washing five times with wash buffer (PBS-0.05% Tween) and aspirate wells, plates were blocked with 1X Assay Diluent room temperature for 1 hour. Following five washes with wash buffer, standard dilutions and samples were added to the appropriate wells respectively (200µl/well) and incubated at room temperature for two hours. A standard curve was constructed using recombinant mouse corresponding antibody. Each dilution of recombinant standard or sample was assayed in duplicate. After washing five times, wells were incubated with 100µl/well biotin-conjugated anti-mouse antibody (100µl/well diluted 1:250 in 1X Assay Diluent) for 1 hour at room temperature. Subsequently, five washes were performed and the detection enzyme Avidin-HRP (100µl/well diluted 1:250 in 1X Assay Diluent) was added. After repeating a total of seven washes, 100 µl/well of Substrate Solution to each well as added and the plate was sealed and incubated at room temperature for 15 minutes. The blue color produced by enzymatic activity was converted into yellow by adding 50 µl of Stop Solution to each well. The procedure is accomplished by the read of plate at 450 nm in an ELISA microplate reader (Biorad). The data were subsequently analysed to Excel.

RESULTS

3. Results

3.1. Tpl2 deficient mice

In our laboratory, Tpl2+/- mice on a C57BL/6 background were available. Tpl2+/- mice were apparently derived by mating of Tpl2+/+ and Tpl2-/-. Construction of the Tpl2 targeting vector and generation of Tpl2-/- mice is extensively described in the article by *Dumitru et al* [19]. Brother-sister mating of Tpl2+/- mice lead to generation of Tpl2+/+, Tpl2+/- and Tpl2-/- littermates. Wild type alleles of Tpl2 gene were determined by PCR using genomic DNA from tails. A 188-bp product corresponding to the nondeleted Tpl2 alleles (Fig. 5) was amplified by primer set of a and b from tail DNA of Tpl2 +/- mice (Materials and Methods, § 2.1.2, Table 1.). An approximately 400-bp fragment corresponding to the deleted allele of Tpl2 gene was amplified from Tpl2+/- and Tpl2-/- mice (Fig. 5). PCR genotyping of Tpl2+/+, Tpl2+/- and Tpl2-/- littermates lead to the determination of two Tpl2-/- mice (one male and one female) used for the propagation of Tpl2-/- mice that was followed by PCR for the next three generations. In general, all mice utilized in the experimenys reported here were derived by brother-sister mating of wild type (C57BL/6 backround) and Tpl2-/- mice respectively.

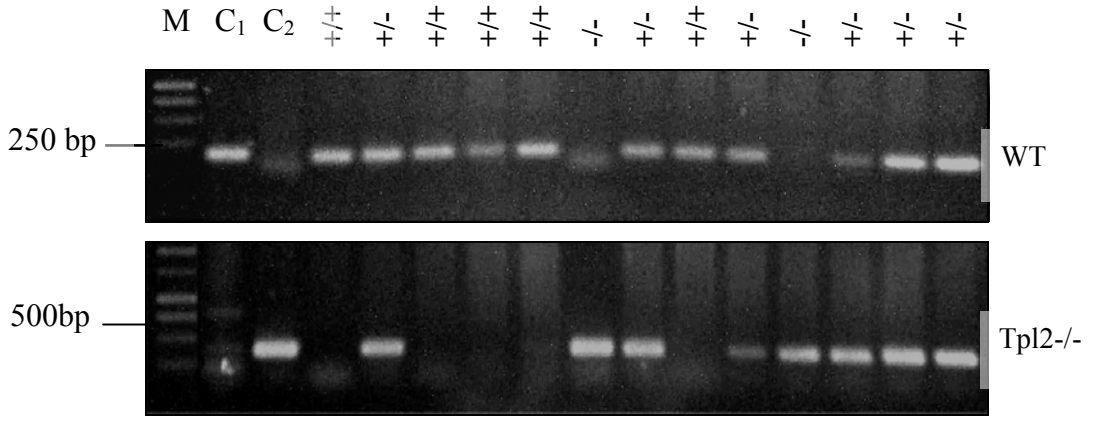


***Figure 5**: PCR genotyping of wild alleles (A) of Tpl2 using the primer set of a and b against tail genome and deleted allele sof Tpl2 using the primer set of a and c against genomic DNA from Tpl2+/- mice (M:DNA marker, C_1: positive control for WT and negative control for Tpl2-/-, C_2: negative control for WT and positive control for Tpl2-/-)*

3.2. Impact of Tpl2 kinase on ConA-induced liver injury

It was clearly stated in the introduction that intravenous administration of ConA is an excellent model resembling immune-mediated fulminant hepatic failure in humans. In this regard, ConA rapidly induces clinical and histological evidence of hepatitis reminiscent of the human disease, including elevation of transaminases, T cell infiltration and apoptosis of hepatocytes. Because of the major role of the Tpl2 in inflammation, we wished to test the hypothesis that Tpl2 is also involved in T cell-mediated hepatitis. To this end, we compared ConA-induced liver damage in Tpl2-/- and wild type mice. Thus, mice (6-8 animals per group, 8-10 weeks old) on the C57BL6 backround received intravenously 10 mg/kg ConA or saline as a vehicle control. In order to assess the progression of the disease, mice were sacrificed 2, 4, and 8 after ConA or saline administration. Blood serum was collected for determination of activities of hepatic injury markers. The alanine aminotransferases (ALT), aspartate aminotransferase (ASP), were assayed spectrophotometrically in serum in the Laboratory of Clinic Biochemistry, University Hospital of Heraklion. As shown in figure 6, both Tpl2-/- and wild type mice showed no significant increases in serum ALT or AST levels 2 hours post ConA injection. Interstingly, serum ALT and AST levels were significantly higher in Tpl2-/- mice 4 hours after ConA treatment compared with WT mice. However the estimated levels of ALT activity 8 hours after ConA treatment were consistent to our hypothesis. Indeed Tpl2-/- mice exhibited significantly lower serum ALT activity compared to wild type mice indicating augmented liver damage in wild type mice.

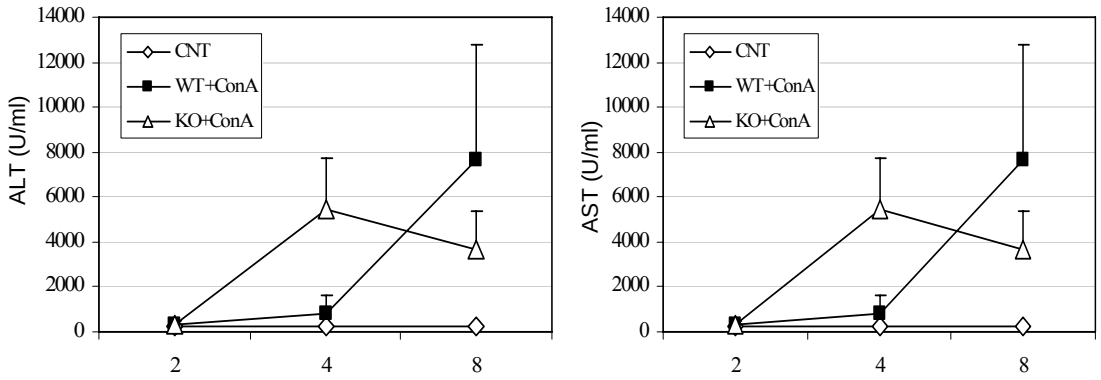


***Figure 6**: Assessment of AST/ALT levels in blood serum isolated from WT or Tpl2-/- mice following ConA administration. Note both the kinetics and levels of enzyme expression (n=4-8 mice per group).*

It is intriguing that Tpl2-/- mice produced increased ALT/AST levels 4 hours after ConA administration which are significantly attenuated eight hours post ConA injection. In contrast, wild type mice displayed a delay onset in the disease followed by severe liver damage 8 hours after ConA administratioon. Elevated ALT and AST levels in wild type mice were also confirmed by the histopathological findings 8 hours after ConA injection. A representative liver histology sample from ConA-injected wild type and Tpl2-/- after 8 hours is shown in figure 7.

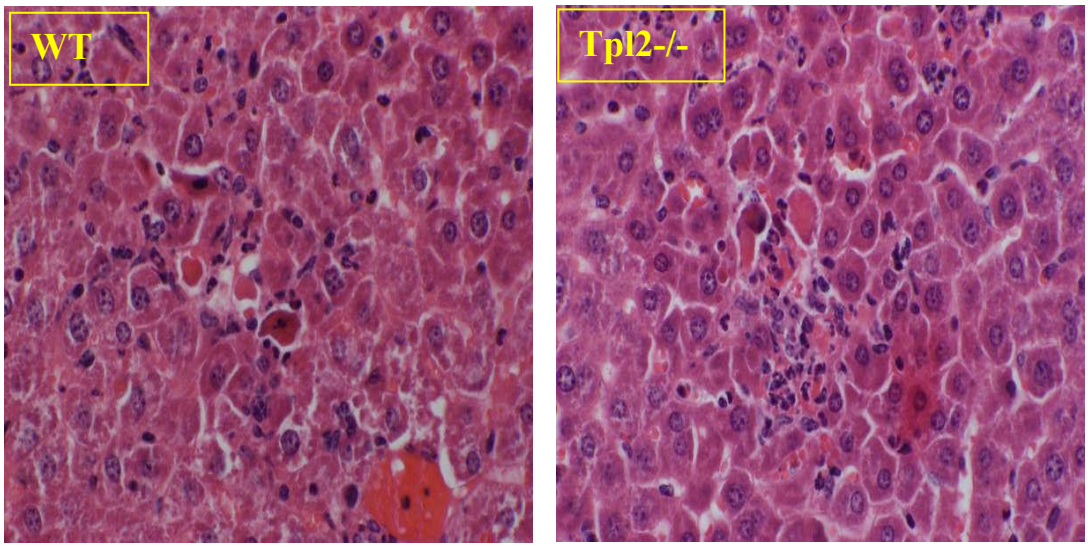


***Figure** 7: Photomicrographs of representative H&E mouse livers treated with ConA for 8 hours*

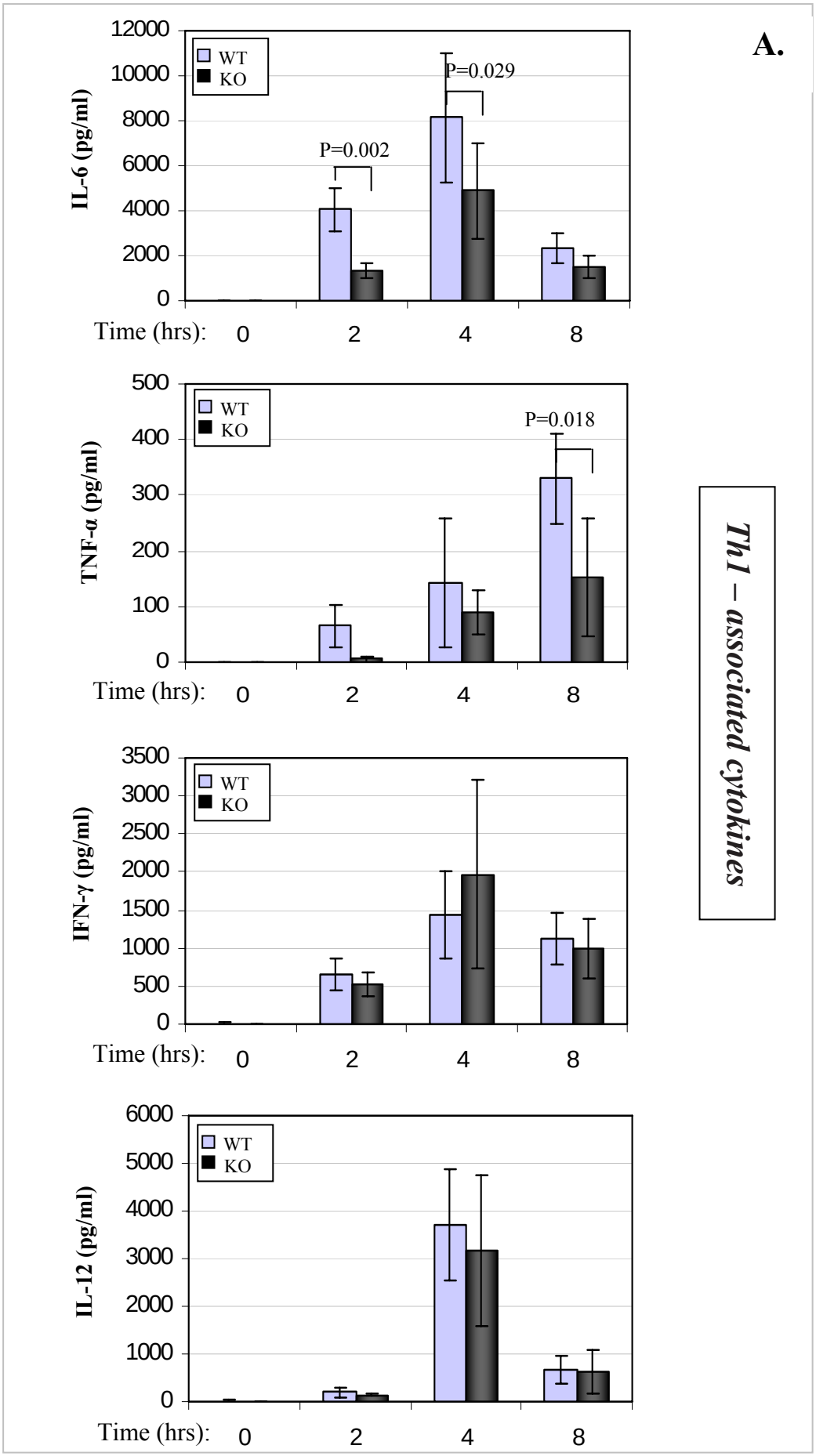
Therefore an evaluation of histopathology in Wt mice revealed areas of inflammation and necrosis whereas liver sections from Tpl2-/- displayed lower injury. Thus, Tpl2 ablation is related to the attenuated progression of the disease. Taken together these data demonstrate the importance of Tpl2 kinase for the development of ConA-induced liver injury.

3.3. Determination of the cytokine milieu in Wt and Tpl2-/- after ConA administration

ConA–induced liver injury is primarily driven by the infiltration of $CD4^+$ T cells and activation of hepatic NKT cells and macrophages, as well as the specific cytokines produced by these cells. Therefore, cytokine production is a key component of ConA-induced liver injury. We determined the plasma levels of various cytokines

in wild type and Tpl2-/- Con A–treated mice. Particularly, we isolated blood serum from untreated wild type, ConA-treated wild type and Tpl2-/- mice and we assessed using ELISA the production of the key T helper 1 cytokines TNF-α, IL-6, IFN-γ and IL-12, as well as the T helper 2–associated cytokines IL-4, and IL-10. As expected, we did not detect significant cytokine levels in the plasma of untreated-mice. It is demonstrated in figure 8 that two hours following ConA administration, both wild type and Tpl2-/- mice produced same amounts of IFN-γ, IL-12, IL-4 and IL-10 with the levels of IL-4 to be markedly high. At the same time point Tpl2-/- mice produced significantly lower levels of TNF-α and IL-6 ($p<0,05$) compared to wild type. It should be mentioned that the high levels of IL-4 and IL-6 are seen at an early point of ConA treatment. Four hours following ConA administration, wild type mice presented with significant increases in TNF-α and IL-6 compared to Tpl2-/- mice. Keeping in mind that IL-6 is protective through the induction of antiapoptotic effects, the difference in IL-6 levels at 2 hrs would thus be consistent with the lower liver damage (ALT/AST levels) observed in the WT versus KO mice at 4 hours. The opposite is true for IL-4 and more interestingly, IL-4 and IL-10 which were significantly elevated in Tpl2-/- mice four hours post ConA when compared wild type (Figure 8). Regarding IFN-γ and IL-12, as is indicated in Figure 8A, analysis of their production revealed increased levels compared to previous time points but no significant changes were observed between wild type and Tpl2-/- mice at any time point post ConA injection. Interestingly, eight hours post-injection, Tpl2-/- mice showed significant reduction in TNF-a production when compared to ConA-treated wild type mice at this time point. However, by 8 hours post ConA administration the assessed cytokines IL-6, IL-4 and IL-10 cytokines were similar in wild type and Tpl-/- mice (Figure 8).

Taken together, these data highlight the impact of Tpl2 signaling on the proinflammatory cytokine cascade associated with ConA administration, providing new insights into the mechanism(s) by which Tpl2 ablation may alleviate Th1-associated cytokine production and simultaneously favors Th2 response and attenuates ConA-associated, T cell–mediated liver injury. Futher analysis of the cytokines operating in ConA-treated wild type and Tpl2-/- is warranted.


A.
IL-6 (pg/ml)
12000
10000
8000
6000
4000
2000
0
WT
KO
P=0.002
P=0.029
Time (hrs): 0 2 4 8
TNF-α (pg/ml)
500
400
300
200
100
0
WT
KO
P=0.018
Time (hrs): 0 2 4 8
IFN-γ (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
WT
KO
Time (hrs): 0 2 4 8
IL-12 (pg/ml)
6000
5000
4000
3000
2000
1000
0
WT
KO
Time (hrs): 0 2 4 8
Th1 – associated cytokines

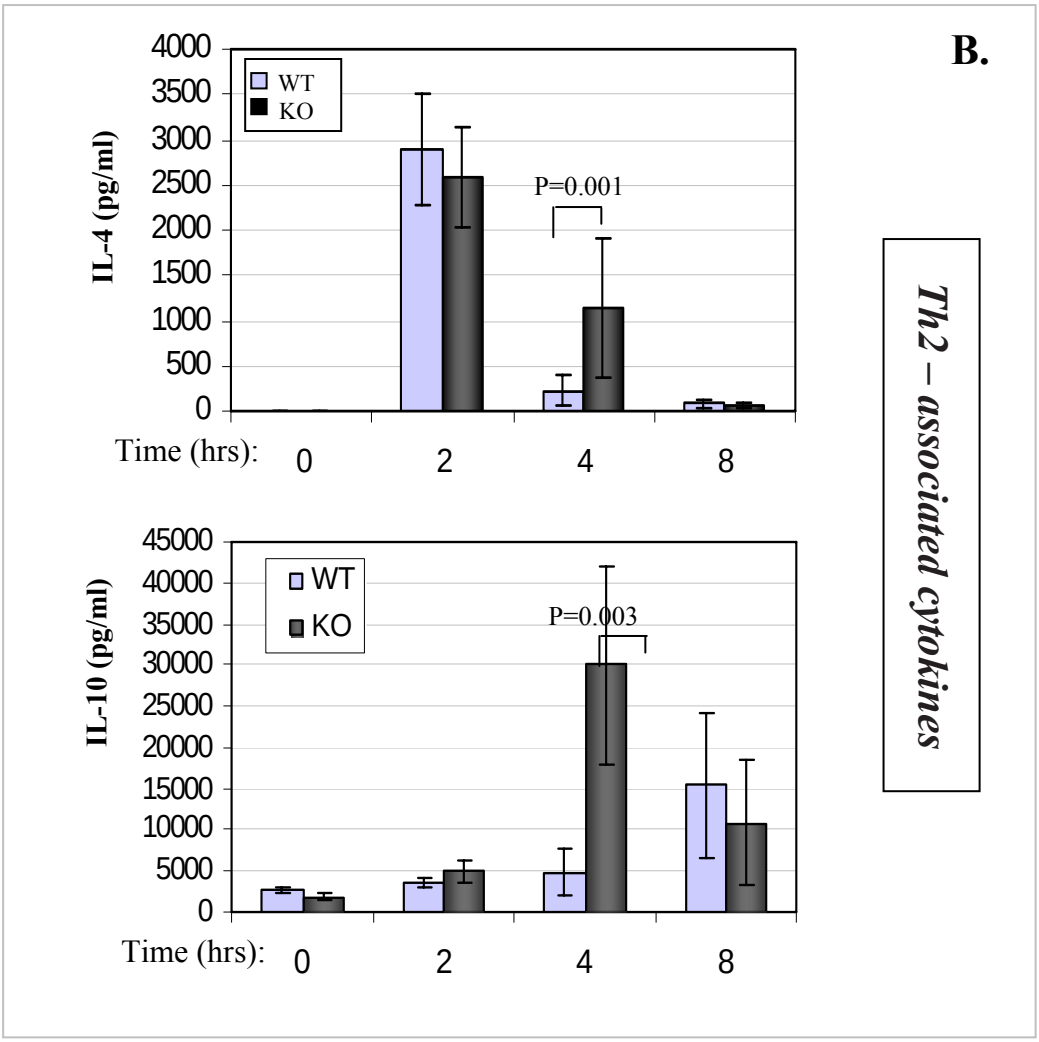


Figure 8: *Assessment of Th1 and Th2 cytokine levels in blood serum isolated from WT or Tpl2 KO mice following ConA administration.*

3.4. Tpl2 ablation affects IFNγ/STAT1 and IL-6/STAT3 signaling

The IFN-γ/STAT1 and IL-6/STAT3 signaling pathways play a central role in the liver since a wide spectum of cytokines are expressed in the site of inflammation in response to their activation [27]. It is well documented that activation of STAT1 by IFNγ is essential for the development of liver injury in ConA induced-hepatitis, whereas activation of STAT3 by IL-6 protects against liver injury [27]. Herein, we wanted to determine whether the activation of STAT1 and STAT3 is affected by abrogated Tpl2 signaling. For this purpose, we isolated slenocytes from wild type and Tpl2-/- mice, cultured and stimulated with 5μg/ml of ConA at various time points. Subsequently,

cells were harvested before and at sequential time points after stimulation with ConA. Cells were lysed with lysis buffer for 15 minutes on ice and whole protein extracts were collected. Protein extracts were quantified and 30μg of each sample were loaded on a 9% polyacrylamide gel. Activation of STAT1 and STAT3 was monitored by Western blot using the corresponding phospho-specific antibodies as shown in figure 9. In wild type, we observed that STAT1 is robustly activated 2 hours post ConA treatment which starts to be attenuated 4 hours upon ConA stimulation and is almost abolished at the following time points. Based on the slightly different phosphorylation status of STAT1 in Tpl2-/- we hypothesize that Tpl2 kinase is may implicated in STAT1 signaling. As shown in figure 9, there is a delay onset of the STAT1 activation in Tpl2-/- cells since is noted at 2h with the peak effect occurring at 4 hours. However, further experiments are required to verify this hypothesis. We also observed that the pattern of STAT3 phosphorylation between wild type and Tpl2-/- cells was similar to the phosphorylation of STAT1. In particular STAT3 is activated with significant peak at 2 hours in wild type while robust STAT3 phosphorylation in Tpl2-/- is significantly potentiated 4 hours upon ConA treatment. As a loading control, we used an anti-actin murine antibody in a 1:1000 dilution, developed from the same blot.

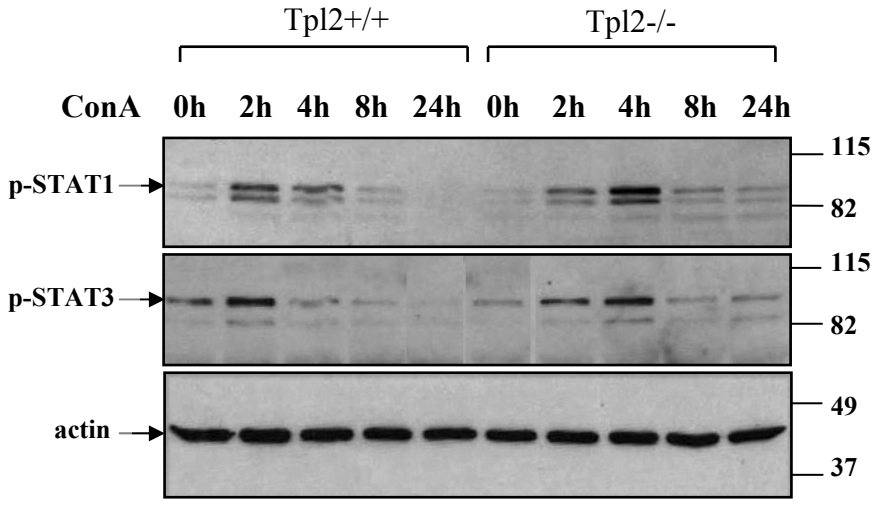


***Figure 9**: Tpl2 kinase is implicated in STAT1 and STAT3 signaling. Western blot of protein extracts (30μg) from lysates of wild type and Tpl2-/- slenocytes stimulated with 5μg/ml ConA at various time points (0,2,4,8 and 24 hours) in order to examine the phosphorylation status of STAT1 and STAT3 using anti-phospho (p)-STAT1 and anti-phospho (p)-STAT3 .*

Thus, we can assume that the defective liver injury in Tpl2-/- mice after ConA is favored by the promoted Th2 cytokine production and affected by the altetered activation signature of STAT3 due to abrogated Tpl2 signaling. Supplementary, we

should study whether Tpl2 kinase is implicated in IL-4/STAT6 signaling as IL-4 is a cytokine that plays significant role in ConA-induced hepatitis.

3.5. Deficiency in Tpl2 partially inhibits ERK activation and promotes T cell profileration upon ConA administration

Evidence, extensively presented in the introduction of this report, clarified that Tpl2 is required for full activation of ERK1/2 in response to various inflammatory signals. Concomitantly, we hypothesized that deficiency in Tpl2 kinase would affect ERK activation in response to mitogenic signals. In order to address this question, we isolated splenocytes from wild time and Tpl2-/- mice. Spleen cells were plated in the presence of 5μg/ml ConA and IL-2 for 0, 5, 10, 15 and 30 minutes (min). Cells were harvested and lysed to collect whole protein extracts. Following protein quantification, 30μg of each sample were loaded on a 10% polyacrylamide gel to proceed into immunoblotting. Activation of ERK1/2 was monitored by Western blot using the corresponding mouse phospho-specific (p)-ERK1/2. In order to control protein loading, we developed the same blot using a mouse anti-ERK1 antibody. As demonstrated in figure 10, we observed that the phosphorylation status of ERK1/2 that is clearly induced upon ConA stimulation for 30 min in wild type is significantly attenuated in absence of Tpl2 kinase. Therefore, in agreement to our hypothesis, inhibition of Tpl2 results in defective ERK signaling upon ConA stimulation of Tpl2-/- spleen cells. At this point, the functional aspect of this abrogated ERK signaling is of fundamental significance to emerge the dentrimental role of Tpl2 kinase in ConA-induced liver injury.

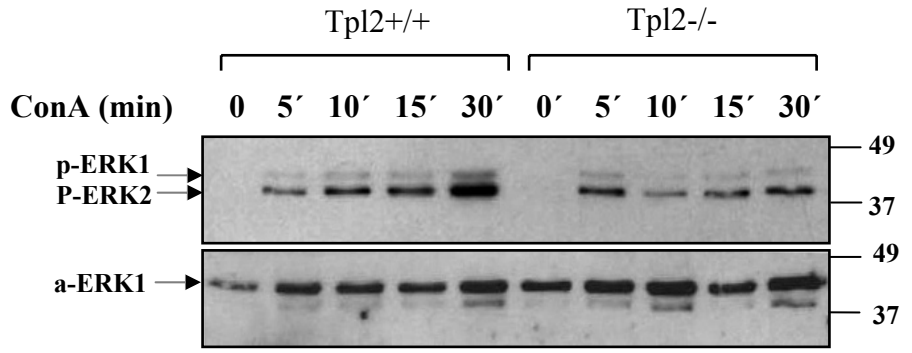


Figure 10: *Tpl2 ablation partially blocks the phosphorylation of ERK1/2. Western blot of protein extracts (30μg) from lysates of wild type and Tpl2-/- slenocytes stimulated with 5μg/ml ConA at various time points (0,5,10,15 and 30 min) in order to examine the phosphorylation status of ERK1/2 using anti-phospho (p)-ERK1 /2. Total ERK was used as a loading control.*

Based on the observation that Tpl2-/- mice excibited attenuated hepatic inflammation and damage we hypothesized that Tpl2 ablation is related to a defective T cell proliferation resulting in protection of mice from fulminant hepatitis. In order to evaluate this hypothesis splenocyte preparations isolated from wild type and Tpl2-/- mice were stimulated with two different concentrations of ConA (3μg/ml and 5μg/ml). [3H]Thymidine incorporation was measured 48 hours upon cells stimulation and is represented in figure 11. Contrary to our hypothesis and surprinigly enough, this experiment revealed enhancement of cell proliferation in Tpl2-/- cells 48 hours from the start of the ConA stimulation and especially at the lower concentration of 3μg/ml. Therefore, we concluded that Tpl2 ablation may enhance the proliferative capacity of the corresponding splenic T cells. In addition it should be mentioned that these data are in agreement to previous data demonstrated decreased phosphorylation of ERK1/2 in Tpl2-/- cells upon ConA stimulation. In this connection, *Tsatsanis et al.,* revealed that ERK activation inhibits T cell proliferation in response to TCR signals through the induction of expression of inhibitory molecules such as CTLA-4 [68].

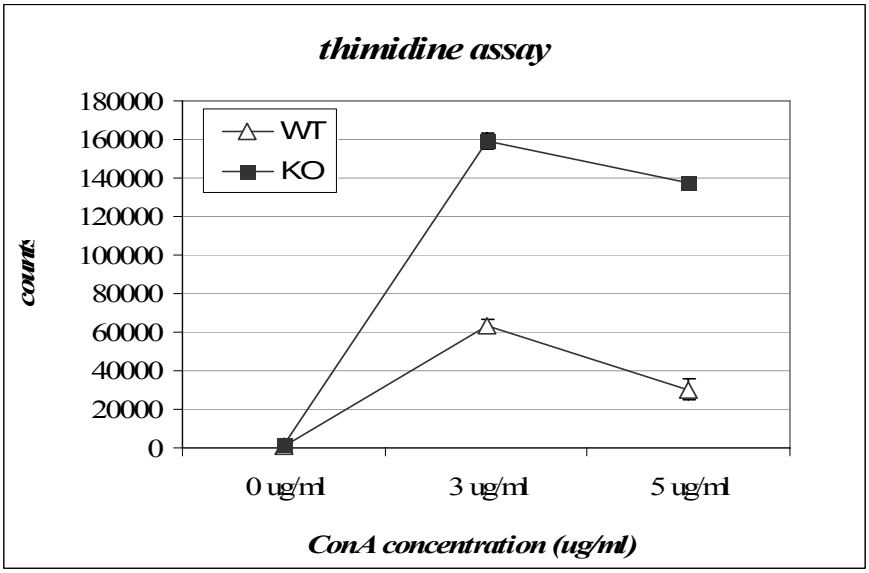


Figure 11: *Tpl2 ablation promotes T cell proliferation. Splenocytes isolated from WT and Tpl2-/- and stimulated with ConA (3μg/ml or 5μg/ml) were measured for thimidine incorporation 48h post ConA stimulation.*

The suggestion that Tpl2-/- cells respond to proliferative signals such as ConA more rapidly compared to wild type may explain the fact that Tpl2-/- mice display an earlier onset of the disease. However, the later events that mediate cell recruitment

and cytokine millieu allow almost completely the restoration to health. Overall, these experiments are preliminary and a lot of unresolved aspects remain to be elucidated.

DISCUSSION

4. Discussion

To date, the signals driving T cell activation in T cell-mediated fulminant hepatitis have not yet been elucidated in detail and there have been no reports concerning the involvement of Tpl2 in liver diseases. Several lines of evidence regarding the role of Tpl2 kinase in immune cell activation in vitro and in vivo, lead to the hypothesis that Tpl2 could be a major pathogenic factor in T cell-mediated hepatitis. To delineate the functional role of Tpl2 kinase in the development of ConA-induced liver injury, we took advantage of Tpl2-/-deficient mice. For this purpose C57BL6 and Tpl2-/- mice (8-10 weeks old) received intravenously 10 mg/kg ConA or saline as a vehicle control. Mice were sacrificed 2, 4 and 8 hours after ConA or saline administration in order to assess the progression of the disease. We estimated the liver enzyme kinetics during the progression of the disease by measuring the levels of amino transferases at various times points (2h, 4h and 8h) after ConA injection. No significant differences of ALT/AST were odtained 2 hours upon ConA administration for both wild type and knockout mice. Contrary to our expectations, determination of hepatic injury markers at 4 hours revealed that the levels of ALT and AST in ConA-treated Tpl2-/- mice were increased, whereas wild-type mice demonstrated significantly lower levels of liver enzymes ($p<0.001$) (Fig. 6). In contrast, administration of 10 mg/kg of ConA for 8 hours in Tpl2 deficient mice resulted in significantly lower serum liver values of ALT and AST as compared with those in wild-type mice ($p<0.05$) (Fig. 6). Consistently, the histology of liver sections from ConA-treated wild type mice showed marked areas of apoptosis, whereas mild degenerative changes were observed in Tpl2-/- mice upon injection of ConA (Fig. 7). Both kinetics and levels liver injury markers as well as the histological analysis represent intriguing findings and raise many questions regarding the role of Tpl2 in Fulminant Hepatitis as well as in the context of inflammation and apoptosis. So we investigated the impact of Tpl2 kinase deletion on the pathogenesis of ConA-associated T cell–mediated liver injury and identified that Tpl2 kinase seems to be protective at the earlier stages of the disease, whereas the absence of Tpl2 affects crucially the mechanism(s) mediating the ConA-mediated liver injury at the later stages of the disease. Hereupon we wanted to gain further insight into differential response of wild type and Tpl2-/- mice regarding soluble mediators of immune response during fulminant hepatitis. In order to determine the cytokine milieu during initiation and progression of ConA-induced

fulminant hepatitis we assessed the cytokine levels in blood serum isolated from wild type or Tpl2-/- mice following ConA administration.We found that Tpl2-/- mice treated with ConA for two hours produced significantly lower levels of TNF-α and IL-6 ($p<0,05$) compared to wild type animals. IL-4 was rapidly produced 2 hours upon ConA injection in wild type and Tpl2-/- mice. Our data demonstrating that Tpl2-/- mice produce significantly augmented levels of the Th2-associated cytokines IL-4 and IL-10, and lower levels of Th1-related cytokines TNF-α and IL-6 four hours post ConA administration compared to wild type were therefore surprising (Fig. 8). Regarding this cytokine-mediated model of ConA-induced hepatitis, IL-6 is known to result in protection whereas the opposite is has been proposed for IL-4 [27] [31] [28]. Interestingly, eight hours upon ConA injection, Tpl2-/- mice showed significant reduction in TNF-a production when compared to ConA-treated wild type mice which is in accordance with ALT/AST levels at this time points respectively (Fig. 6 and Fig.8). Therefore, the serum levels of Th1- and Th2- associated cytokines during the progression of the disease can explain ALT/AST levels at all sequential time points respectively and vise versa. In this study, we have displayed a key role of Tpl2 in controling T cell responses in ConA-mediated liver injury. Based on our preceding data we presume that Tpl2 deficiency either results in attenuated Th1 response which favors the shift to a Th2 response or gives rise to an intrinsic T cell defect that favors a Th2 response. The above-mentioned data are consistent with the data presented in a recently conducted study showing the Th2 polarization of the immune response in Tpl2 knockout mice and suggesting that inhibition of Tpl2 may alleviate the symptoms of Th1-dependent autoimmune diseases [72].The shift towards Th2 polarization of the T cell response to OVA in Tpl2-/- mice is in agreement with another study showing that the defence of Tpl2 knockout mice to Toxoplama gondii is impaired because of a T cell autonomous Th2 shift of the T cell response [74]. Nevertheless, the data in both these papers differ from the data in another report, showing that Tpl2 ablation promotes shift to the Th1 polarization of the T cell response in OVA-immunized mice which may reflect differences in the route of immunization

The action of T cells in the liver is in part mediated through release of a variety of cytokines, which target liver cells and immune cells via activation of multiple signaling cascades, including the signal transducers and activators of transcription factor (STATs) family members. For example, it is now established that

activation of STAT1 by IFN-γ is essential for the development of liver injury in Con A-induced hepatitis, whereas activation of STAT3 by IL-6 protects against liver injury [27]. Regarding this issue, we performed preliminary experiments on ConA-stimulated (0, 2, 4, 8, 24 hours) slenocytes from wild type and Tpl2-/- mice. As was shown in figure 9 there is a delay onset of the STAT3 activation in Tpl2-/- cells compared to wild type, since is noted at 2 hours with the peak effect occurring at 4 hours. We also observed that different pattern of STAT1 phosphorylation between wild type and Tpl2-/- cells. It is established that IFN-γ/STAT1 induce expression of proapoptotic IRF-1 protein, which mediates liver apoptosis and injury whereas IL-6/STAT3 activation is followed by induction of Bcl-XL and other antiapoptotic factors, protect against hepatic necrosis and apoptosis [27]. Thus, we can presume that T cell–induced hepatitis is tightly controlled by mutual functional antagonism of IFN-γ/STAT1 and IL-6/STAT3 signaling and both are affected by abrogated Tpl2 signaling. Our findings suggest that defective liver injury in Tpl2-/- mice after ConA is favored by the promoted Th2 cytokine production and affected by the altetered activation signature of STAT1 and STAT3 due to abrogated Tpl2 signaling. Administration of Con A also activates STAT4, STAT5, and STAT6 in the liver, but their precise roles remain obscure. The T cell-derived cytokine IL-4 specifically activates STAT6, which plays important role in Th2 response [28]. Complementary, we should study whether Tpl2 kinase is implicated in IL-4/STAT6 signaling as IL-4 is a cytokine that plays pivotal role in ConA-induced hepatitis. Beyond the afomentioned, Tpl2 is required for the transduction of signals that activate ERK signaling pathway in T cells [66] [67]. Hence, we wanted evaluate that Tpl2 deficiency causes defective ERK signaling upon mitogenic signals. Indeed, we demonstrated that ConA stimulation of Tpl2-/- spleen cells resulted in diminished ERK signaling as compared with ConA –treatd wild type splenocytes (Fig. 10). Our results are in agreement with the current literature. Recently, *Tsatsanis et al.,* revealed that inhibition of the MEK/ERK pathway is associated with enhanced proliferative capacity of T cells. In particular, they showed that activation of MEK/ERK signaling pathway by Tpl2-transduced TCR signals, inhibits T cell proliferation by promoting the induction of CTLA4 [68]. These data are in agreement with the [3H] thymidine incorporation assay we performed on ConA treated wild type and Tpl2-/- splenocytes since we discovered that Tpl2 deficiency favours T cell proliferation in response to ConA stimulation (Fig.11). Therefore, we propose that Tpl2 ablation on the one hand

enhances T cell proliferation resulting in an earlier onset of the disease but on the other hand attenuates Th1 response and promotes Th2 polarization leading to a shorter and alleviated progression of the disease. Consequently, the outcome of these experiments is of fundamental significance as on the basis of these results, we are a novel role of Tpl2 signaling in fulminant hepatitis is revealed. Inhibition of Tpl2 signaling may therefore prove extremely useful therapeutically in the treatment of autoimmune liver diseases and other fulminant liver pathologies. Preventing liver injury by either blocking or reducing Tpl2 signaling might prove to be a feasible approach to treating patients suffering from fulminant hepatitis. Certainly, more experiments are required and many questions remain to be elucidated.

References

1. **Afford, S.C., S. Randhawa, A.G. Eliopoulos, S.G. Hubscher, L.S. Young, and D.H. Adams**, CD40 activation induces apoptosis in cultured human hepatocytes via induction of cell surface fas ligand expression and amplifies fas-mediated hepatocyte death during allograft rejection. *J Exp Med*, 1999. **189**(2): p. 441-6.
2. **Ajuebor, M.N., Z. Wondimu, C.M. Hogaboam, T. Le, A.E. Proudfoot, and M.G. Swain**, CCR5 deficiency drives enhanced natural killer cell trafficking to and activation within the liver in murine T cell-mediated hepatitis. *Am J Pathol*, 2007. **170**(6): p. 1975-88.
3. **Aoki, M., F. Hamada, T. Sugimoto, S. Sumida, T. Akiyama, and K. Toyoshima**, The human cot proto-oncogene encodes two protein serine/threonine kinases with different transforming activities by alternative initiation of translation. *J Biol Chem*, 1993. **268**(30): p. 22723-32.
4. **Atillasoy, E. and P.D. Berk**, Fulminant hepatic failure: pathophysiology, treatment, and survival. *Annu Rev Med*, 1995. **46**: p. 181-91.
5. **Ballester, A., R. Tobena, C. Lisbona, V. Calvo, and S. Alemany**, Cot kinase regulation of IL-2 production in Jurkat T cells. *J Immunol*, 1997. **159**(4): p. 1613-8.
6. **Ballester, A., A. Velasco, R. Tobena, and S. Alemany**, Cot kinase activates tumor necrosis factor-alpha gene expression in a cyclosporin A-resistant manner. *J Biol Chem*, 1998. **273**(23): p. 14099-106.
7. **Beinke, S. and S.C. Ley**, Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. *Biochem J*, 2004. **382**(Pt 2): p. 393-409.
8. **Bernstein, D. and J. Tripodi**, Fulminant hepatic failure. *Crit Care Clin*, 1998. **14**(2): p. 181-97.
9. **Bertrand, J.R., M. Pottier, A. Vekris, P. Opolon, A. Maksimenko, and C. Malvy**, Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo. *Biochem Biophys Res Commun*, 2002. **296**(4): p. 1000-4.
10. **Bonder, C.S., M.N. Ajuebor, L.D. Zbytnuik, P. Kubes, and M.G. Swain**, Essential role for neutrophil recruitment to the liver in concanavalin A-induced hepatitis. *J Immunol*, 2004. **172**(1): p. 45-53.
11. **Bustos, M., N. Beraza, J.J. Lasarte, E. Baixeras, P. Alzuguren, T. Bordet, and J. Prieto**, Protection against liver damage by cardiotrophin-1: a hepatocyte survival factor up-regulated in the regenerating liver in rats. *Gastroenterology*, 2003. **125**(1): p. 192-201.
12. **Cao, Q., R. Batey, G. Pang, A. Russell, and R. Clancy**, IL-6, IFN-gamma and TNF-alpha production by liver-associated T cells and acute liver injury in rats administered concanavalin A. *Immunol Cell Biol*, 1998. **76**(6): p. 542-9.
13. **Ceci, J.D., C.P. Patriotis, C. Tsatsanis, A.M. Makris, R. Kovatch, D.A. Swing, N.A. Jenkins, P.N. Tsichlis, and N.G. Copeland**, Tpl-2 is an oncogenic kinase that is activated by carboxy-terminal truncation. *Genes Dev*, 1997. **11**(6): p. 688-700.
14. **Channavajhala, P.L., L. Wu, J.W. Cuozzo, J.P. Hall, W. Liu, L.L. Lin, and Y. Zhang**, Identification of a novel human kinase supporter of Ras (hKSR-2) that functions as a negative regulator of Cot (Tpl2) signaling. *J Biol Chem*, 2003. **278**(47): p. 47089-97.

15. **Cho, J. and P.N. Tsichlis**, Phosphorylation at Thr-290 regulates Tpl2 binding to NF-kappaB1/p105 and Tpl2 activation and degradation by lipopolysaccharide. *Proc Natl Acad Sci U S A*, 2005. **102**(7): p. 2350-5.
16. **Chu, H.C., Y.L. Lin, H.K. Sytwu, S.H. Lin, C.L. Liao, and Y.C. Chao**, Effects of minocycline on Fas-mediated fulminant hepatitis in mice. *Br J Pharmacol*, 2005. **144**(2): p. 275-82.
17. **Das, S., J. Cho, I. Lambertz, M.A. Kelliher, A.G. Eliopoulos, K. Du, and P.N. Tsichlis**, Tpl2/cot signals activate ERK, JNK, and NF-kappaB in a cell-type and stimulus-specific manner. *J Biol Chem*, 2005. **280**(25): p. 23748-57.
18. **Diao, H., S. Kon, K. Iwabuchi, C. Kimura, J. Morimoto, D. Ito, T. Segawa, M. Maeda, J. Hamuro, T. Nakayama, M. Taniguchi, H. Yagita, L. Van Kaer, K. Onoe, D. Denhardt, S. Rittling, and T. Uede**, Osteopontin as a mediator of NKT cell function in T cell-mediated liver diseases. *Immunity*, 2004. **21**(4): p. 539-50.
19. **Dumitru, C.D., J.D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J.H. Lin, C. Patriotis, N.A. Jenkins, N.G. Copeland, G. Kollias, and P.N. Tsichlis**, TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell*, 2000. **103**(7): p. 1071-83.
20. **Eliopoulos, A.G., S. Das, and P.N. Tsichlis**, The tyrosine kinase Syk regulates TPL2 activation signals. *J Biol Chem*, 2006. **281**(3): p. 1371-80.
21. **Eliopoulos, A.G., C.C. Wang, C.D. Dumitru, and P.N. Tsichlis**, Tpl2 transduces CD40 and TNF signals that activate ERK and regulates IgE induction by CD40. *Embo J*, 2003. **22**(15): p. 3855-64.
22. **Erny, K.M., J. Peli, J.F. Lambert, V. Muller, and H. Diggelmann**, Involvement of the Tpl-2/cot oncogene in MMTV tumorigenesis. *Oncogene*, 1996. **13**(9): p. 2015-20.
23. **Gandara, M.L., P. Lopez, R. Hernando, J.G. Castano, and S. Alemany**, The COOH-terminal domain of wild-type Cot regulates its stability and kinase specific activity. *Mol Cell Biol*, 2003. **23**(20): p. 7377-90.
24. **Gantner, F., M. Leist, A.W. Lohse, P.G. Germann, and G. Tiegs**, Concanavalin A-induced T-cell-mediated hepatic injury in mice: the role of tumor necrosis factor. *Hepatology*, 1995. **21**(1): p. 190-8.
25. **Gu, Y., K. Kuida, H. Tsutsui, G. Ku, K. Hsiao, M.A. Fleming, N. Hayashi, K. Higashino, H. Okamura, K. Nakanishi, M. Kurimoto, T. Tanimoto, R.A. Flavell, V. Sato, M.W. Harding, D.J. Livingston, and M.S. Su**, Activation of interferon-gamma inducing factor mediated by interleukin-1beta converting enzyme. *Science*, 1997. **275**(5297): p. 206-9.
26. **Hall, J.P., Y. Kurdi, S. Hsu, J. Cuozzo, J. Liu, J.B. Telliez, K.J. Seidl, A. Winkler, Y. Hu, N. Green, G.R. Askew, S. Tam, J.D. Clark, and L.L. Lin**, Pharmacologic inhibition of tpl2 blocks inflammatory responses in primary human monocytes, synoviocytes, and blood. *J Biol Chem*, 2007. **282**(46): p. 33295-304.
27. **Hong, F., B. Jaruga, W.H. Kim, S. Radaeva, O.N. El-Assal, Z. Tian, V.A. Nguyen, and B. Gao**, Opposing roles of STAT1 and STAT3 in T cell-mediated hepatitis: regulation by SOCS. *J Clin Invest*, 2002. **110**(10): p. 1503-13.
28. **Jaruga, B., F. Hong, R. Sun, S. Radaeva, and B. Gao**, Crucial role of IL-4/STAT6 in T cell-mediated hepatitis: up-regulating eotaxins and IL-5 and recruiting leukocytes. *J Immunol*, 2003. **171**(6): p. 3233-44.

29. **Kane, L.P., M.N. Mollenauer, Z. Xu, C.W. Turck, and A. Weiss**, Akt-dependent phosphorylation specifically regulates Cot induction of NF-kappa B-dependent transcription. *Mol Cell Biol*, 2002. **22**(16): p. 5962-74.
30. **Kanzler, S. and P.R. Galle**, Apoptosis and the liver. *Semin Cancer Biol*, 2000. **10**(3): p. 173-84.
31. **Klein, C., T. Wustefeld, U. Assmus, T. Roskams, S. Rose-John, M. Muller, M.P. Manns, M. Ernst, and C. Trautwein**, The IL-6-gp130-STAT3 pathway in hepatocytes triggers liver protection in T cell-mediated liver injury. *J Clin Invest*, 2005. **115**(4): p. 860-9.
32. **Kontoyiannis, D., G. Boulougouris, M. Manoloukos, M. Armaka, M. Apostolaki, T. Pizarro, A. Kotlyarov, I. Forster, R. Flavell, M. Gaestel, P. Tsichlis, F. Cominelli, and G. Kollias**, Genetic dissection of the cellular pathways and signaling mechanisms in modeled tumor necrosis factor-induced Crohn's-like inflammatory bowel disease. *J Exp Med*, 2002. **196**(12): p. 1563-74.
33. **Ksontini, R., D.B. Colagiovanni, M.D. Josephs, C.K. Edwards, 3rd, C.L. Tannahill, C.C. Solorzano, J. Norman, W. Denham, M. Clare-Salzler, S.L. MacKay, and L.L. Moldawer**, Disparate roles for TNF-alpha and Fas ligand in concanavalin A-induced hepatitis. *J Immunol*, 1998. **160**(8): p. 4082-9.
34. **Leifeld, L., C. Trautwein, F.L. Dumoulin, M.P. Manns, T. Sauerbruch, and U. Spengler**, Enhanced expression of CD80 (B7-1), CD86 (B7-2), and CD40 and their ligands CD28 and CD154 in fulminant hepatic failure. *Am J Pathol*, 1999. **154**(6): p. 1711-20.
35. **Louis, H., A. Le Moine, V. Flamand, N. Nagy, E. Quertinmont, F. Paulart, D. Abramowicz, O. Le Moine, M. Goldman, and J. Deviere**, Critical role of interleukin 5 and eosinophils in concanavalin A-induced hepatitis in mice. *Gastroenterology*, 2002. **122**(7): p. 2001-10.
36. **Mahboubi, K., B.C. Biedermann, J.M. Carroll, and J.S. Pober**, IL-11 activates human endothelial cells to resist immune-mediated injury. *J Immunol*, 2000. **164**(7): p. 3837-46.
37. **Makris, A., C. Patriotis, S.E. Bear, and P.N. Tsichlis**, Genomic organization and expression of Tpl-2 in normal cells and Moloney murine leukemia virus-induced rat T-cell lymphomas: activation by provirus insertion. *J Virol*, 1993. **67**(7): p. 4283-9.
38. **Marsters, S.A., J.P. Sheridan, R.M. Pitti, A. Huang, M. Skubatch, D. Baldwin, J. Yuan, A. Gurney, A.D. Goddard, P. Godowski, and A. Ashkenazi**, A novel receptor for Apo2L/TRAIL contains a truncated death domain. *Curr Biol*, 1997. **7**(12): p. 1003-6.
39. **Miyoshi, J., T. Higashi, H. Mukai, T. Ohuchi, and T. Kakunaga**, Structure and transforming potential of the human cot oncogene encoding a putative protein kinase. *Mol Cell Biol*, 1991. **11**(8): p. 4088-96.
40. **Nakamura, K., M. Okada, M. Yoneda, S. Takamoto, Y. Nakade, K. Tamori, K. Aso, and I. Makino**, Macrophage inflammatory protein-2 induced by TNF-alpha plays a pivotal role in concanavalin A-induced liver injury in mice. *J Hepatol*, 2001. **35**(2): p. 217-24.
41. **Nakanishi, K., T. Yoshimoto, H. Tsutsui, and H. Okamura**, Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. *Cytokine Growth Factor Rev*, 2001. **12**(1): p. 53-72.

42. **Nicoletti, F., R. Di Marco, P. Zaccone, A. Salvaggio, G. Magro, K. Bendtzen, and P. Meroni**, Murine concanavalin A-induced hepatitis is prevented by interleukin 12 (IL-12) antibody and exacerbated by exogenous IL-12 through an interferon-gamma-dependent mechanism. *Hepatology*, 2000. **32**(4 Pt 1): p. 728-33.
43. **O'Shea, J.J., M. Gadina, and R.D. Schreiber**, Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell*, 2002. **109 Suppl**: p. S121-31.
44. **Pan, H., F. Hong, S. Radaeva, and B. Gao**, Hydrodynamic gene delivery of interleukin-22 protects the mouse liver from concanavalin A-, carbon tetrachloride-, and Fas ligand-induced injury via activation of STAT3. *Cell Mol Immunol*, 2004. **1**(1): p. 43-9.
45. **Patriotis, C., A. Makris, S.E. Bear, and P.N. Tsichlis**, Tumor progression locus 2 (Tpl-2) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation. *Proc Natl Acad Sci U S A*, 1993. **90**(6): p. 2251-5.
46. **Patriotis, C., M.G. Russeva, J.H. Lin, S.M. Srinivasula, D.Z. Markova, C. Tsatsanis, A. Makris, E.S. Alnemri, and P.N. Tsichlis**, Tpl-2 induces apoptosis by promoting the assembly of protein complexes that contain caspase-9, the adapter protein Tvl-1, and procaspase-3. *J Cell Physiol*, 2001. **187**(2): p. 176-87.
47. **Racanelli, V. and B. Rehermann**, The liver as an immunological organ. *Hepatology*, 2006. **43**(2 Suppl 1): p. S54-62.
48. **Radaeva, S., R. Sun, H.N. Pan, F. Hong, and B. Gao**, Interleukin 22 (IL-22) plays a protective role in T cell-mediated murine hepatitis: IL-22 is a survival factor for hepatocytes via STAT3 activation. *Hepatology*, 2004. **39**(5): p. 1332-42.
49. **Saito, Y., S. Kon, Y. Fujiwara, Y. Nakayama, D. Kurotaki, N. Fukuda, C. Kimura, M. Kanayama, K. Ito, H. Diao, Y. Matsui, Y. Komatsu, E. Ohtsuka, and T. Uede**, Osteopontin small interfering RNA protects mice from fulminant hepatitis. *Hum Gene Ther*, 2007. **18**(12): p. 1205-14.
50. **Scapini, P., J.A. Lapinet-Vera, S. Gasperini, F. Calzetti, F. Bazzoni, and M.A. Cassatella**, The neutrophil as a cellular source of chemokines. *Immunol Rev*, 2000. **177**: p. 195-203.
51. **Schumann, J., D. Wolf, A. Pahl, K. Brune, T. Papadopoulos, N. van Rooijen, and G. Tiegs**, Importance of Kupffer cells for T-cell-dependent liver injury in mice. *Am J Pathol*, 2000. **157**(5): p. 1671-83.
52. **Seino, K., N. Kayagaki, K. Takeda, K. Fukao, K. Okumura, and H. Yagita**, Contribution of Fas ligand to T cell-mediated hepatic injury in mice. *Gastroenterology*, 1997. **113**(4): p. 1315-22.
53. **Seino, K., Y. Setoguchi, T. Ogino, N. Kayagaki, H. Akiba, H. Nakano, H. Taniguchi, Y. Takada, K. Yuzawa, T. Todoroki, Y. Fukuchi, H. Yagita, K. Okumura, and K. Fukao**, Protection against Fas-mediated and tumor necrosis factor receptor 1-mediated liver injury by blockade of FADD without loss of nuclear factor-kappaB activation. *Ann Surg*, 2001. **234**(5): p. 681-8.
54. **Serhan, C.N. and J. Savill**, Resolution of inflammation: the beginning programs the end. *Nat Immunol*, 2005. **6**(12): p. 1191-7.
55. **Sharon, N. and H. Lis**, History of lectins: from hemagglutinins to biological recognition molecules. *Glycobiology*, 2004. **14**(11): p. 53R-62R.

56. **Siebler, J., S. Wirtz, C. Frenzel, M. Schuchmann, A.W. Lohse, P.R. Galle, and M.F. Neurath**, Cutting edge: a key pathogenic role of IL-27 in T cell-mediated hepatitis. *J Immunol*, 2008. **180**(1): p. 30-3.
57. **Siebler, J., S. Wirtz, S. Klein, M. Protschka, M. Blessing, P.R. Galle, and M.F. Neurath**, A key pathogenic role for the STAT1/T-bet signaling pathway in T-cell-mediated liver inflammation. *Hepatology*, 2003. **38**(6): p. 1573-80.
58. **Song, E., S.K. Lee, J. Wang, N. Ince, N. Ouyang, J. Min, J. Chen, P. Shankar, and J. Lieberman**, RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat Med*, 2003. **9**(3): p. 347-51.
59. **Sugimoto, K., M. Ohata, J. Miyoshi, H. Ishizaki, N. Tsuboi, A. Masuda, Y. Yoshikai, M. Takamoto, K. Sugane, S. Matsuo, Y. Shimada, and T. Matsuguchi**, A serine/threonine kinase, Cot/Tpl2, modulates bacterial DNA-induced IL-12 production and Th cell differentiation. *J Clin Invest*, 2004. **114**(6): p. 857-66.
60. **Sun, R., Z. Tian, S. Kulkarni, and B. Gao**, IL-6 prevents T cell-mediated hepatitis via inhibition of NKT cells in CD4+ T cell- and STAT3-dependent manners. *J Immunol*, 2004. **172**(9): p. 5648-55.
61. **Tagawa, Y., S. Kakuta, and Y. Iwakura**, Involvement of Fas/Fas ligand system-mediated apoptosis in the development of concanavalin A-induced hepatitis. *Eur J Immunol*, 1998. **28**(12): p. 4105-13.
62. **Tagawa, Y., K. Sekikawa, and Y. Iwakura**, Suppression of concanavalin A-induced hepatitis in IFN-gamma(-/-) mice, but not in TNF-alpha(-/-) mice: role for IFN-gamma in activating apoptosis of hepatocytes. *J Immunol*, 1997. **159**(3): p. 1418-28.
63. **Takeda, K., Y. Hayakawa, L. Van Kaer, H. Matsuda, H. Yagita, and K. Okumura**, Critical contribution of liver natural killer T cells to a murine model of hepatitis. *Proc Natl Acad Sci U S A*, 2000. **97**(10): p. 5498-503.
64. **Tiegs, G., J. Hentschel, and A. Wendel**, A T cell-dependent experimental liver injury in mice inducible by concanavalin A. *J Clin Invest*, 1992. **90**(1): p. 196-203.
65. **Trautwein, C., T. Rakemann, D.A. Brenner, K. Streetz, L. Licato, M.P. Manns, and G. Tiegs**, Concanavalin A-induced liver cell damage: activation of intracellular pathways triggered by tumor necrosis factor in mice. *Gastroenterology*, 1998. **114**(5): p. 1035-45.
66. **Tsatsanis, C., C. Patriotis, S.E. Bear, and P.N. Tsichlis**, The Tpl-2 protooncoprotein activates the nuclear factor of activated T cells and induces interleukin 2 expression in T cell lines. *Proc Natl Acad Sci U S A*, 1998. **95**(7): p. 3827-32.
67. **Tsatsanis, C., C. Patriotis, and P.N. Tsichlis**, Tpl-2 induces IL-2 expression in T-cell lines by triggering multiple signaling pathways that activate NFAT and NF-kappaB. *Oncogene*, 1998. **17**(20): p. 2609-18.
68. **Tsatsanis, C., K. Vaporidi, V. Zacharioudaki, A. Androulidaki, Y. Sykulev, A.N. Margioris, and P.N. Tsichlis**, Tpl2 and ERK transduce antiproliferative T cell receptor signals and inhibit transformation of chronically stimulated T cells. *Proc Natl Acad Sci U S A*, 2008. **105**(8): p. 2987-92.
69. **van den Brink, M.R. and S.J. Burakoff**, Cytolytic pathways in haematopoietic stem-cell transplantation. *Nat Rev Immunol*, 2002. **2**(4): p. 273-81.

70. **van Kooten, C. and J. Banchereau**, CD40-CD40 ligand. *J Leukoc Biol*, 2000. **67**(1): p. 2-17.
71. **Wajant, H.**, The Fas signaling pathway: more than a paradigm. *Science*, 2002. **296**(5573): p. 1635-6.
72. **Wang, C.C., W. Watford , A. G. Eliopoulos, C. Tsatsanis, C. D. Dumitru, C. Daskalakis, J. O'Shea and P. N. Tsichlis**, Tpl-2 ablation promotes a type 2 T helper cell response, in ovalbumin-immunized mice. *Journal of Immunology*, 2008. **Submitted**.
73. **Waterfield, M.R., M. Zhang, L.P. Norman, and S.C. Sun**, NF-kappaB1/p105 regulates lipopolysaccharide-stimulated MAP kinase signaling by governing the stability and function of the Tpl2 kinase. *Mol Cell*, 2003. **11**(3): p. 685-94.
74. **Watford, W.T., B.D. Hissong, L.R. Durant, H. Yamane, L.M. Muul, Y. Kanno, C.M. Tato, H.L. Ramos, A.E. Berger, L. Mielke, M. Pesu, B. Solomon, D.M. Frucht, W.E. Paul, A. Sher, D. Jankovic, P.N. Tsichlis, and J.J. O'Shea**, Tpl2 kinase regulates T cell interferon-{gamma} production and host resistance to Toxoplasma gondii. *J Exp Med*, 2008. **205**(12): p. 2803-12.
75. **Yaomura, T., N. Tsuboi, Y. Urahama, A. Hobo, K. Sugimoto, J. Miyoshi, T. Matsuguchi, K. Reiji, S. Matsuo, and Y. Yuzawa**, Serine/threonine kinase, Cot/Tpl2, regulates renal cell apoptosis in ischaemia/reperfusion injury. *Nephrology (Carlton)*, 2008.
76. **Zender, L., S. Hutker, C. Liedtke, H.L. Tillmann, S. Zender, B. Mundt, M. Waltemathe, T. Gosling, P. Flemming, N.P. Malek, C. Trautwein, M.P. Manns, F. Kuhnel, and S. Kubicka**, Caspase 8 small interfering RNA prevents acute liver failure in mice. *Proc Natl Acad Sci U S A*, 2003. **100**(13): p. 7797-802.
77. **Zhang, H., J. Cook, J. Nickel, R. Yu, K. Stecker, K. Myers, and N.M. Dean**, Reduction of liver Fas expression by an antisense oligonucleotide protects mice from fulminant hepatitis. *Nat Biotechnol*, 2000. **18**(8): p. 862-7.
78. **Zheng, S.J., P. Wang, G. Tsabary, and Y.H. Chen**, Critical roles of TRAIL in hepatic cell death and hepatic inflammation. *J Clin Invest*, 2004. **113**(1): p. 58-64.
79. **Zhou, F., M.N. Ajuebor, P.L. Beck, T. Le, C.M. Hogaboam, and M.G. Swain**, CD154-CD40 interactions drive hepatocyte apoptosis in murine fulminant hepatitis. *Hepatology*, 2005. **42**(2): p. 372-80.