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The Role of Tpl2 Protein Kinase in Carcinogenesis and Inflammation

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1. Introduction

The mitogen activated protein kinase (MAPK) cascade is a family of serine-threonine protein kinases controlling a vast array of cellular responses. Among the oldest signaling pathways known, the MAPK cascade is evolutionarily conserved, being found in species ranging from yeast to humans (Widmann *et al*., 1999). All eukaryotic cells possess multiple MAPK signaling pathways. In mammals, four major subfamilies (termed classical MAPKs) have been identified including extracellular signal-regulated kinase 1/2 (ERK1/2), c-jun N-terminal protein kinases (JNK/SAPK), p38 isoforms (p38 α, β, γ, or δ), and ERK5 (Dhanasekaran and Johnson, 2007). These multiple parallel MAPK cascades all operate through a three tiered system characterized by consecutive phosphorylation events. Each of the pathways is equipped to respond to a remarkably diverse array of stimuli, including cytokines, growth factors, irradiation, hormones, injury, and stress (Cargnello and Roux, 2011). The binding of stimuli to receptors triggers the initial phosphorylation events. These events transform inactive MAP3K into active MAP3K. Subsequently, active MAP3K phosphorylates, and thereby activates, downstream MAP2K (MEK) proteins. The active MAP2K proteins then phosphorylate MAPK (ERK) molecules. These MAPK proteins then regulate the transcription of a diverse group of genes involved in inflammation, cell survival, cell division, development, metabolism, differentiation, motility, and repair (Cargnello and Roux, 2011).

Additionally, over the last two decades atypical pathways including ERK 3/4, ERK 7/8 and Nemo-like kinase (NLK) have been discovered (Cargnello and Roux, 2011). Similar to classical MAPK family members, these proteins phosphorylate target substrates on serine or threonine residues. However, they are not organized into the classical three-tiered model and contain a less well conserved motif on the activation loop of the kinase domain.

Due to the diverse effects that MAPK signaling pathways have on cellular processes, the classical MAPK pathways have been intensely studied to assess their role in human disease. Detailed reviews for each pathway were previously published (Weston and Davis, 2007; Wagner and Nebreda, 2009; Cargnello and Roux, 2011; Schindler *et al*, 2007; Nishimoto and Nishida, 2006). Therefore, this chapter will focus on one protein kinase, *Tpl2,* within the

MAPK family and how altered expression (either underexpression or overexpression) of this kinase is linked to inflammation, cancer and other disease states.

2. The MAP3K8/Tpl2 gene

MAP3K8 (known as *Cot* or Tpl2 in rodents) is a MAP3K protein first isolated from thyroid tumors as a gene capable of inducing morphological transformation of NIH3T3 and SHOK cells (Miyoshi *et al*., 1991). Structurally, the open reading frame of the *Tpl2* gene encodes a 467 amino acid polypeptide (Aoki *et al*., 1991). Provirus integration occurring in the last intron of the *Tpl2* gene in rodents generates a mutant protein 36 amino acids shorter than the full length version and also is an activating mutation (Patriotis *et al*., 1993; Erny *et al*., 1996; Ceci *et al*., 1997). This truncated *Tpl2* gene has enhanced kinase activity (Gandara *et al*., 2003). Additionally, analysis of the N-terminal region of this gene reveals three alternate translation start sites. Consequently, the polypeptides translated from the *Tpl2* gene will contain 467, 464, or 438 amino acids (Aoki *et al*., 1991). The 467- and the 464-amino acid proteins are detected as a single 58-kDa band by Western Blot analysis and the 438 amino acid protein is detected as a 52-kDa band.

Functionally, some differences exist between these two forms of the protein. The 58-kDa protein has a shorter half-life (10 minutes) than the 52-kDa protein (30 minutes). Thus, the amino-terminal domain contains sequences important for Tpl2 stability. Additionally, cell lines transformed with constructs only expressing the 58-kDa Tpl2 protein have higher tumor incidence and reduced latency when compared to cell lines transformed with the 52-kDa protein indicating that the N-terminal region of the protein is important in regulating function of this gene (Aoki *et al*., 1991).

Tpl2 has the ability to cross-talk with numerous signaling pathways. In unstimulated cells Tpl2 forms a ternary complex with Nuclear factor kappa B p105 (NF-κB p105) and A20-binding inhibitor of NF-κB2 (ABIN-2) (Lang *et al*., 2004). This complex increases Tpl2 stability and renders Tpl2 inactive towards its main downstream substrate MEK, the ERK kinase. Upon activation by inflammatory stimuli, Tpl2 becomes phosphorylated by IκB kinase (IKK), releasing both Tpl2 and NF-κB to participate in downstream signaling. In addition to participating in ERK signaling, Tpl2 can cross-talk with multiple MAPK signaling pathways including p38, JNK, ERK 1/2, and ERK 5 (Salmeron *et al*., 1996, Patriotis *et al*., 1993, Chiariello *et al*., 2000) (Figure 1). Additionally, wildtype and mutant Tpl2 activates the transcription factor NFAT in response to stimulation through the T cell receptor (TCR) and causes heightened activation of NF-κB (Tsatsanis *et al*., 1998, Belich *et al*., 1999).

3. Tpl2 regulation involves NF-κB

Our understanding of Tpl2 regulation was greatly enhanced by the identification of the interaction between p105 and Tpl2 (Belich *et al*., 1999). p105 is a NF-κB precursor protein which also acts as a NF-κB-inhibitory protein by sequestering NF-κB complexes in the cytoplasm of cells (Rice *et al*., 1992). Stoichiometric binding of Tpl2 to the C-terminus of p105 occurs within two distinct regions of Tpl2. The C–terminal region of Tpl2 (residues 398-467) binds to a region adjacent to the ankyrin repeats on p105 (residues 497 to 534), while the kinase domain of Tpl2 binds to the death domain on p105 (Beinke *et al*., 2003). In steady state conditions, the entire pool of Tpl2 is associated with p105, irrespective of the cell type, whereas only about one third of the p105 pool is occupied by binding to Tpl2 (Belich, et al., 1999).

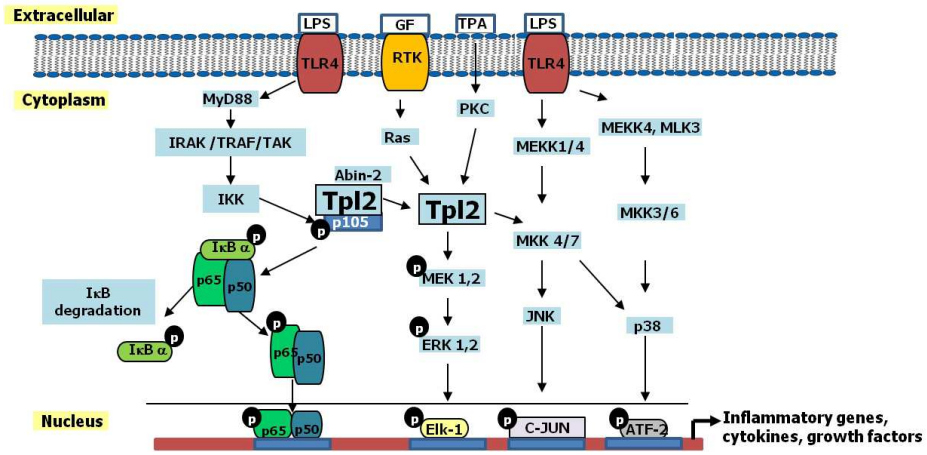

Fig. 1. Tpl2 signaling pathways. Tpl2 interacts with numerous signaling pathways including ERK, JNK, p38, and NF-κB. Tpl2 is normally held in complex with Abin-2 and p105. Upon activation with a stimulus, IKK phosphorylates p105, freeing Tpl2 to participate in ERK, JNK and p38 signaling and p50 to heterodimerize with other NF-κB family members.

Functionally, this interaction between Tpl2 and p105 is significant. p105 binding to Tpl2 inhibits Tpl2 kinase activity and thus prevents Tpl2 from being able to activate downstream signaling cascades. Despite this ability to inhibit Tpl2 kinase activity, p105 improves Tpl2 solubility and stability (Beinke, *et al.*, 2003; Jia, *et al.,* 2005). Tpl2, when not bound to p105, is unstable, appears to have difficulty folding and can form insoluble aggregates (Jia, *et al.,* 2005). Despite the fact that Tpl2 binds p105, p105 is not necessary for *Tpl2* gene expression. Bone-marrow derived macrophages (BMDMs) and splenocytes that lack p105 have normal *Tpl2* gene expression levels (Waterfield, *et al.,* 2003). However, Tpl2 protein levels in these cells are significantly reduced. This reduction in Tpl2 protein expression is correlated with a subsequent reduction in LPS induced ERK signaling (Waterfield *et al.*, 2003). This indicates that binding to p105 not only confers Tpl2 stability, but it also renders it unable to phosphorylate MEK, possibly by blocking access to MEK (Waterfield *et al.*, 2003; Bienke *et al.*, 2003). The inactivity of Tpl2 in the complex may function as a mechanism to prevent unregulated Tpl2 activity in unstimulated cells.

As discussed later in this chapter, truncation of Tpl2 in its C-terminus greatly enhances the catalytic activity and oncogenic potential of Tpl2 in T cell lymphomas (Ceci, et al., 1997). Since the interaction of Tpl2 with p105 requires the C terminus of Tpl2 in vitro, truncation of Tpl2 could theoretically change the association between Tpl2 and p105. In 2003, Beinke addressed this issue and found that truncation of Tpl2 did not hinder the ability of this protein to bind to p105 (Beinke, *et al.,* 2003). However, it did block the ability of p105 to negatively regulate Tpl2 kinase activity. The heightened Tpl2 kinase activity found in Tpl2 truncated cells can result in persistent phosphorylation of targets such as MEK, ultimately leading to increased gene expression of numerous genes involved in cell cycle progression (Matsumura, *et al.*, 2009). Additionally, increased Tpl2 kinase activity can be oncogenic, as aberrant MEK/ERK activity has been identified in several cancer types (Matsumura, *et al.*, 2008).

In 2004, a third protein, ABIN-2, was reported to associate with the Tpl2/p105 complex (Lang, *et al.,* 2004). Tpl2 and ABIN-2 bind to similar regions on the C-terminal region of p105. Both Tpl2 and ABIN-2 bind to the death domain and bind to residues 497-538. However, ABIN-2 also needs to bind to the PEST region of p105 for optimal association. It is reported that in order to maintain steady state protein levels of Tpl2, ABIN-2 is necessary (Lang, *et al.*, 2004). However, it is not involved in maintaining protein levels of p105. Whereas, the functional significance of the role of ABIN-2 in the Tpl2/p105 complex has not been fully elucidated, it has been shown that the solubility of ABIN-2 is increased by binding to p105. Therefore, similar to what was described above for Tpl2, it appears that ABIN-2 binding to p105 is required to ensure correct protein folding.

Tpl2 is activated in response to various proinflammatory stimuli such as lipopolysaccharide (LPS), tumor necrosis factor (TNF) and CD40 ligand. LPS stimulation results in IKK-induced p105 proteolysis, releases Tpl2 from p105, renders Tpl2 enzymatically active, and increases downstream MEK kinase activity (Beinke *et al*., 2004; Waterfield *et al*., 2003). This newly liberated Tpl2, which is now susceptible to rapid degradation by the proteosome, will phosphorylate substrates in the ERK and JNK pathways. Additionally, p105 is subsequently degraded into p50 by the proteosome (Beinke, *et al.,* 2004). The p50 subunit can now dimerize with other NF-κB family members and translocate to the nucleus where the active NF-κB complex can regulate over 400 genes. The overall result is an upregulation of diverse genes involved in growth, differentiation, and inflammation.

4. Tpl2 knockout mice

Despite the significant function Tpl2 has in eliciting cellular responses, Tpl2$^{-/-}$ animals appear phenotypically normal (Dumitru *et al*., 2000). Immune organs including bone marrow, thymus, spleen, and lymph nodes all develop normally and production of T and B cell subsets are unaltered. Additionally, when subjected to challenge by T cell-dependent and independent antigens such as KLH and LPS-TNP, Tpl2$^{-/-}$ mice produce normal antibody responses (Dumitru *et al*., 2000).

Over the last decade, numerous laboratories have used a variety of disease models to assess the response of Tpl2$^{-/-}$ mice to bacterial, viral, or carcinogenic challenge. However, the results have shown differential responses to challenge, with the absence of Tpl2 sometimes promoting a disease state and sometimes protective against the disease. These differing responses are most likely due to variation in stimuli used and is cell-type specific. As described below, most laboratories have used lipopolysaccharide (LPS), which binds through TLR4, as the major stimulus in Tpl2$^{-/-}$ mice. However, targeting different receptors through the use of other stimulators such as 12-O-tetradecanoylphorbol-13-acetate (TPA), Poly IC, or CpG receptors can yield different responses. Thus, because Tpl2 can signal through numerous pathways, analysis of the effects of underexpression or overexpression of this protein kinase is quite convoluted.

4.1 Evidence supporting that Tpl2$^{-/-}$ mice exhibit lower levels of inflammatory markers

Several laboratories have reported a reduction in pro-inflammatory cytokines in Tpl2$^{-/-}$ mice. Dumitru reported that TNF-α is markedly lower in Tpl2$^{-/-}$ macrophages stimulated with LPS, providing Tpl2$^{-/-}$ mice with resistance to LPS/D-Galactosamine stimulated

endotoxic shock (Dumitru *et al*., 2000). Others have reported similar findings, deficits in TNF- α, Cox-2, PGE_2, and CXCL1 in LPS-stimulated macrophages (Eliopoulos *et al*., 2002; López-Peláez *et al*., 2011). In LPS-stimulated Tpl2$^{-/-}$ macrophages, this impaired secretion of TNF- α and PGE_2 is triggered by a defect in ERK activation. Additionally, in bone marrow-derived dendritic cells (BMDCs) and macrophages, Tpl2$^{-/-}$ mice have significantly lower expression of IL-1β in response to LPS, poly IC and LPS/MDP (Mielke *et al*., 2009). Consequently, these mice displayed significantly higher mortality and bacterial burden when infected with *L. monocytogenes*. Recently, it was reported that obese Tpl2$^{-/-}$ mice have a significant reduction in TNF- α and monocyte chemoattractant protein-1 (MCP-1) in adipose tissue compared to WT controls (Perfield, *et al*., 2011). This reduction in inflammatory markers in Tpl2$^{-/-}$ mice was associated with an improvement in whole-body insulin resistance. Because inhibition of *Tpl2* is associated with a reduction in inflammatory markers in LPS-stimulated cells, several pharmaceutical companies have developed small molecule inhibitors of Tpl2 to assess whether inhibition of this protein can be beneficial for chronic inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease, and pancreatitis. Details on these studies are outlined later in this chapter.

4.2 Tpl2$^{-/-}$ mice can display enhanced pro-inflammatory profiles

Contradictory to the details above, our laboratory and others have reported heightened inflammatory responses in Tpl2$^{-/-}$ mice. López-Peláez found that Tpl2 deficiency in LPS-stimulated macrophages was associated with an increase in Nitric Oxide Synthase 2 expression (López-Peláez *et al*., 2011). Similarly, Zacharioudaki found that expression of IRAK-M, whose function is to compete with IL-1R-associated kinase (IRAK) family of kinases, is decreased in Tpl2$^{-/-}$ macrophages (Zacharioudaki *et al*., 2009). This deficit in IRAK-M in Tpl2$^{-/-}$ macrophages was associated with elevations in TNF and IL-6 and a hyperresponsiveness to LPS.

Our laboratory examined the inflammatory response in TPA-treated Tpl2$^{-/-}$ or wild type mouse skin (DeCicco-Skinner, *et al*., 2011). We found significantly higher numbers of neutrophils in Tpl2$^{-/-}$ mice, achieving levels 35 times greater than control animals within 12 hours (DeCicco-Skinner *et al*., 2011). Additionally, the skin of Tpl2$^{-/-}$ mice exhibited significant increases in the number of mast cells, $CD3^+$ cells and the pro-inflammatory cytokines IL-12 and IL-1 β.

The heightened inflammation found in the skin of Tpl2$^{-/-}$ mice may be initiated by aberrant inflammatory signaling pathways. Numerous inflammatory pathways are associated with Tpl2 activity including ERK, JNK, p38 and NF-κB. We used selective inhibitors specific to each of these pathways both *in vitro* and *in vivo* to assess differential responses in keratinocytes from Tpl2$^{-/-}$ or wildtype mice and found that a dysregulation in NF-κB activation and signaling is the primary defect in Tpl2$^{-/-}$ keratinocytes (DeCicco-Skinner, *et al*., 2011). We confirmed that keratinocytes from Tpl2$^{-/-}$ mice have basal and TPA-treated NF-κB activity levels significantly greater than wildtype mice using an NF-κB reporter assay. Moreover, whereas treatment with an NF-κB inhibitor could block edema and neutrophil infiltration in wildtype mice, the inhibitor was unable to block the exaggerated NF-κB response in the Tpl2$^{-/-}$ mice. Additionally, using confocal microscopy we found high levels of active p65 NF-κB present in the nucleus of Tpl2$^{-/-}$ keratinocytes prior to TPA

treatment that dramatically increased within 15-30 minutes of TPA treatment. The increase in active NF-κB may be explained by lower levels of inhibitory proteins that normally sequester NF-κB in the cytoplasm of cells. In this regard we found that wildtype mice had significantly higher protein levels of the NF-κB inhibitors IκB alpha and IκB beta compared to Tpl2$^{-/-}$ animals. Others have reported similar findings as our laboratory (Mielke *et al*., 2009; López-Peláez *et al*., 2011). Mielke reported that Tpl2$^{-/-}$ bone marrow-derived dendritic cells (BMDCs) displayed increased p65 NF-κB both basally and after stimulation with LPS when compared to wildtype controls (Mielke, *et al*., 2009). Similarly, they found that increased IκB degradation may be partially responsible for the heightened NF-κB levels. Moreover, López-Peláez demonstrated that while degradation of the NF-κB inhibitor IκB alpha was similar between Tpl2$^{-/-}$ and wildtype BMDMs, Tpl2$^{-/-}$ cells exhibited a slower recovery in IκB alpha protein levels, remaining low at 4 hours post LPS treatment (López-Peláez *et al*., 2011).

The data associating the absence of *Tpl2* with a heightened NF-κB activation, opposes what others have found. Previous reports have shown LPS stimulation leads to equal induction of NF-κB in Tpl2$^{-/-}$ and control macrophages (Dumitru *et al*., 2000). Others have shown that Tpl2$^{-/-}$ mouse embryo fibroblasts and Jurkat T cells with kinase-deficient Tpl2 have a reduction in NF-κB activation but only when certain stimuli are administered (Lin *et al*., 1999; Das *et al*., 2005). Thus the relationship between Tpl2 and NF-κB still remains an enigma and more work is needed to clarify the nuances of this interaction.

It is possible that whether Tpl2 serves more of a pro-inflammatory or anti-inflammatory role may depend on cell type and stimuli. As previously mentioned, Tpl2 exhibits broad specificity because it interacts with numerous signaling pathways. Most of the previous reports that found Tpl2 to be proinflammatory used stimuli functioning through TLRs. It should be noted that the two stage skin carcinogenesis model used in our model employs TPA as a tumor promoter signaling through protein kinase C (PKC). PKC is a multigene family of serine/threonine kinases (Griner and Kazanietz, 2007). Six isoforms of PKC are expressed in mouse keratinocytes, namely, α,δ, ε, μ, η, ζ (Reddig *et al*., 1999). Each isoform contributes differently to normal keratinocyte growth and differentiation, and, if inappropriately regulated, certain isoforms are associated with disease pathogenesis. It is currently unknown if one or more PKC isoforms are dysregulated in Tpl2$^{-/-}$ mice.

4.3 Tpl2$^{-/-}$ mice have alterations in T cell polarization

Tpl2 is reportedly activated in rodents by provirus integration in retrovirus-induced T cell lymphomas (Ceci *et al*., 1997). Therefore, due to this connection with T cells, several laboratories have investigated if Tpl2$^{-/-}$ mice have alterations in T cell profiles in response to antigenic challenge. Watford, *et al*. reported that *Tpl2* was induced by IL-12 in human and mouse T cells in a Stat4-dependent manner (Watford *et al*., 2008). Tpl2$^{-/-}$ mice have impaired host defense against *Toxoplasma gondii,* reduced parasite clearance, and decreased IFN-γ production. Additionally, ovalbumin-immunized Tpl2$^{-/-}$ mice challenged with ovalbumin developed more severe bronchoalveolar eosinophilic inflammation than wildtype controls (Watford *et al*., 2010). Tpl2$^{-/-}$ mice were polarized toward a Th2 response, exhibiting heightened IL-4 and IL-10 signaling. In contrast, Sugimoto, *et al*. reported a significant increase in IL-12 and a decrease in CpG-DNA treated Tpl2$^{-/-}$ macrophages and dendritic

cells, polarizing the mice toward a Th1 profile (Sugimoto *et al*., 2004). Additionally, it has been reported that IL-23, a cytokine necessary for expansion and stabilization of Th17 cells, is reduced in Tpl2$^{-/-}$ macrophages indicating Tpl2 may be involved in balancing Th1/Th17 differentiation (Kakimoto, *et al*., 2010).

5. Tpl2 and carcinogenesis

The exact role of Tpl2 in carcinogenesis is also convoluted. Early reports demonstrated *Tpl2* truncation of the C-terminus, resulting from provirus insertion, to be associated with T-cell lymphoma (Ceci *et al*., 1997). Additionally, overexpressing the truncated form of this gene was associated with rodent T-cell lymphomas and an accelerated cell cycle in T lymphocytes.

Subsequently, several reports found elevated MAP3K8 activity in a number of human cancers including breast, endometrial, thymomas, lymphomas, lung, Hodgkin's disease, and nasopharyngeal carcinoma (Salmeron *et al*., 1996; Sourvinos *et al*., 1999; Patriotis *et al*., 1993,; Tsatsanis *et al*., 2000; Eliopoulos *et al*., 2002; Clark *et al*., 2004). In lymphomas the enhanced MAP3K8 activity could modulate ERK1/2 effector proteins such as p70 S6K and Jun B, resulting in an increased progression through the cell cycle (López-Peláez *et al*., 2011; Fernández *et al*., 2011). Recently it was reported that overexpression of Tpl2 induced androgen-dependent prostate cancer growth, and that MAP3K8 was upregulated in prostate cancers in mouse models and clinical specimens (Jeong *et al*., 2011). Additionally, heightened *MAP3K8* expression levels are correlated with acquired resistance to drug therapy in melanoma (Johannessen *et al*., 2010). The physiological role of Tpl2 in the etiology of human cancers remains a mystery. Overexpression of *Tpl2/MAP3K8 in vivo* is weakly oncogenic and *Tpl2/MAP3K8* mutations in human cancers are rarely found (Ceci *et al*., 1997; Clark et al. 2004, Vougioukalaki *et al*., 2011).

In contrast to the oncogenic role of *Tpl2,* recent evidence suggests that under certain conditions Tpl2 may serve a tumor suppressor role. Using Tpl2$^{-/-}$ mice crossed with the T cell receptor transgene, Tsatsanis found that 81% of mice developed T cell lymphomas within 6 months whereas lymphomas were absent from wildtype mice (Tsatsanis *et al*., 2008). This heightened carcinogenesis was due to chronic stimulation of splenic T cells in Tpl2$^{-/-}$ mice, for anti-CD3/anti-CD28 stimulated Tpl2$^{-/-}$ splenocytes have increased proliferation and reduced expression of a negative regulator of T cell activation, CTLA4 .

Moreover, our laboratory recently reported that *Tpl2* can act as a tumor suppressor gene in chemically-induced skin cancer (DeCicco-Skinner *et al*., 2011). In our model, Tpl2$^{-/-}$ or wildtype mice were subjected to a two-stage dimethylbenzanthracene/ 12-O-tetradecanoylphorbol-13-acetate (TPA) mouse skin carcinogenesis model. We found that Tpl2$^{-/-}$ mice developed a significantly higher incidence of tumors (80%) than wildtype mice (17%), as well as reduced tumor latency and a significantly higher number of total tumors (113 vs 6). Additionally, keratinocytes from Tpl2$^{-/-}$ mice were found to have an accelerated cell cycle as demonstrated *in vivo* through heightened Ki67 staining.

A skin grafting protocol has been used by our laboratory to further determine the *in vivo* phenotype of keratinocytes and fibroblasts from Tpl2$^{-/-}$ and wildtype mice. In agreement with our two-stage skin carcinogenesis model, we found that nude mice grafted with

fibroblasts and v-ras^{Ha} -transduced keratinocytes from Tpl2$^{-/-}$ mice developed tumors nine times larger than mice grafted with fibroblasts and v-ras^{Ha} -transduced keratinocytes from wildtype mice. Additionally, mice grafted with mixed genotypes (WT fibroblasts/KO keratinocytes or KO fibroblasts/WT keratinocytes) developed tumors that were intermediate in size suggesting a defect in Tpl2 signaling in both fibroblasts and keratinocytes was responsible for the increased tumor formation.

Despite the fact that *Tpl2* can serve as an oncogene, and sometimes a tumor suppressor gene in mice and rats, the physiological response of *Tpl2* in the etiology of human cancers remains a mystery. Our laboratory has previously identified a *Tpl2* truncation mutant in a primary human lung adenocarcinoma, but analyses of other lung cancers suggest this isn't a common occurrence (Clark *et al*., 2004). Additionally, others have searched the Sanger Institute COSMIC database for *Tpl2* gene mutations and identified only one human tumor sample (out of 477) that contained a point mutation in the *Tpl2* gene (Gantke *et al*., 2011; Parsons *et al,* 2008). The significance of this point mutation in the development or progression of the patient's glioblastoma multiforme is unknown.

6. Tpl2 inhibitors

As described above, *Tpl2* can interact with several inflammatory pathways. It activates the MEK/ERK pathway and plays a pivotal role in the production and signaling of TNF. Tpl2 also regulates TNF-R signaling and the production of type 1 interferon and other pro-inflammatory cytokines such as IL-1β. In macrophages stimulated by LPS, Tpl2 activates the MAPK pathway. Overexpression of Tpl2 activates the MAPK signaling pathways, NFAT, and induces IL-2 production which further activates NF-κB via the NF-κB inducing kinase (NIK) (Patriotis *et al*., 1994; Salmeron *et al*., 1996; Tsatsanis *et al*., 1998a and 1998b; Lin *et al,* 1999). Analysis of Tpl2$^{-/-}$ mice revealed a role for Tpl2 in TLR, IL-1, CD40, TCR, and also reduction of LPS-induced TNF-α production (Dumitru *et al*., 2000; Tsatsanis *et al*., 2008; Eliopoulos *et al*., 2003). TNF-α, a pro-inflammatory cytokine, is involved in the initiation and progression of inflammatory diseases such as rheumatoid arthritis (RA), psoriasis, inflammatory bowel disease, and multiple sclerosis (Feldmann *et al*., 2005). TNF-α also stimulates the production of other proinflammatory cytokines such as interleukin-1 (IL-1) and interleukin- 6 (IL-6), and induces the activity of enzymes such as the matrix metalloproteases (MMPs), all of which contribute to RA. Inhibition of TNF-α with a number of biological agents that bind and neutralize TNF-α has offered a significant therapeutic advantage in treating RA (Jarvis & Faulds, 1999,; Elliot *et al*., 2008; Bang & Keating, 2004). The therapeutics included soluble TNF receptor and monoclonal antibodies for the neutralization of TNF-α, such as etanercept (soluble TNFRII-Fc; Enbrel), infliximab (Remicade), and adalimumab (anti-TNFα antibodies; Humira) and have improved the quality of life of RA patients.

The success of these drugs has validated the use of anti-TNF-α therapies for treating arthritic and inflammatory diseases. It has also necessitated the search for additional small molecule inhibitors with similar or related mechanisms of action. Major pharmaceutical companies have focused on the inhibition of TNF-α production through either inhibiting TNF-α converting enzyme or the disruption of signal transduction pathways that include several members of the MAPK family. However, the therapeutic agents described above

require administration via injection or infusion. Therefore, identification of an orally available small molecule therapy would provide an additional benefit to patients. Since Tpl2 inhibition can decrease TNF-α levels, it has become an attractive target for treating RA and an orally available small molecule inhibiting TNF-α synthesis and/or signaling could have widespread therapeutic potential. Since Tpl2 ablation protects mice from TNF-α -induced inflammatory bowel disease and pancreatic and pulmonary inflammation, Tpl2 inhibition is an attractive strategy for treating these diseases.

Research studying the effect of Tpl2 inhibition on disease outcome is still in its infancy because only a handful of small molecule inhibitors of Tpl2 have been identified. A few factors contribute to the paucity of Tpl2 inhibitors. First, no crystal structure is available for Tpl2. Second, Tpl2 has low homology to other kinases. Although this is an advantage in selecting unique targets, it also presents significant challenges for structure-based drug design, especially when coupled with the lack of crystal structure. On the other hand, Tpl2 is not inhibited by staurosporine, a nonspecific kinase inhibitor, and it is the only known human kinase that has a proline instead of a conserved glycine in the glycine-rich ATP binding loop (Luciano *et al.,* 2004; Patriotis, *et al.,* 2003). These factors may lead to the discovery of highly selective inhibitors. Nonetheless, several strategies have been used to synthesize and/or isolate Tpl2 inhibitors.

One natural product inhibitor of Tpl2 has been identified. Luteolin [2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-chromenone] is a flavonoid found abundantly in green peppers, perilla, celery, and chamomile tea. Luteolin has been shown to inhibit TNF-α-induced COX-2 expression in mouse skin epidermal cells by inhibiting Tpl2 activity (Kim *et al.,* 2011).

Several synthetic inhibitors of Tpl2 have also been characterized with 1,7-Naphthyridine-3-carbonitriles 1 and quinoline- 3-carbonitrile 2 as the earliest classes of compounds reported as reversible and ATP-competitive Tpl2 kinase inhibitors. These Tpl2 inhibitors demonstrate a good correlation between cell free Tpl2 inhibition with p-MEK and TNF inhibition in cell-based assays. Thieno [2,3-c]pyridines and pyrimidines are the other two classes of Tpl2 inhibitors studied recently. 1,7-napthyridine- 3-carbonitriles and quinoline-3-carbonitriles, indazoles, and thienopyridines are the most common Tpl2 inhibitors. These classes of compounds will be discussed below.

Gavrin et al., (2005) first described a series of 6-substituted-4-anilino-[1,7]-naphthyridine-3-carbonitriles as Tpl2 inhibitors evaluated in cell-free, cellular, and blood environments. One of these compounds, 1,7-naphtyridine- 3-carbonitrile, was used successfully to inhibit RANKL-induced osteogenesis through the induction of transcription factors c-Fos and NFATc1 in RANKL treated cells, unraveling the pivotal role played by Tpl2 in osteoclastogenesis (Hirata *et al.,* 2010). The same inhibitor subsequently demonstrated that LPS-induced TNF-α production via the Tpl2-MEK signaling is regulated by modulating the levels of tip-associated protein (Hirata *et al.,* 2010a). It appears that Tpl2 might be involved in not only the post-transcriptional but also the translational stages of TNF-α production. Some of the carbonitriles were further tested in primary human monocytes and blood. Inhibition of Tpl2 led to a decrease in production of TNF-α and other pro-inflammatory factors. In synoviocytes, the inhibitors blocked ERK activation, COX-2 expression, and the production of IL-6, IL-8, PGE_2, MMP-1 and MMP-3, further validating the role of these inhibitors as therapeutics (Hall *et al.,* 2007).

A series of quinoline-3-carbonitriles were synthesized and tested in cell-based phosphorylation assays in LPS-treated human monocytes. Compounds were identified that selectively inhibited pMEK formation and LPS/D-Gal-induced TNF-α release (Hu *et al.,* 2006). Another series of compound synthesis involved studying 8-chloro-4-(3-chloro-4-fluorophenylamino)-6-((1-(1-ethylpiperidin-4-yl)-1*H*-1,2,3-triazol-4-yl)methylamino)quinoline-3-carbonitriles that inhibited TNF-α production in an LPS-stimulated rat inflammation model. This was an important step in Tpl2 inhibitor identification since this compound was orally efficacious and demonstrated potential for oral treatment for RA (Wu *et al.,* 2009).

Structure based design efforts yielded a series of novel Indazole-based compounds. These compounds showed promising results as Tpl2 inhibitors as tested in LANCE based and cell-based pERK assays. Some of the top compounds inhibited pERK activity in monocytes and had an IC_{50} of 3.2μM. They exhibited good kinase selectivity against certain kinases but not against others. Efforts to improve their kinase selectivity profile are under way (Hu *et al.,* 2011).

Another novel series of 7-amino substituted thieno[2,3-*c*]pyridines was identified as Tpl2 inhibitors with an IC_{50} of 1μM. These compounds were studied for their Tpl2 inhibition and selectivity against kinases. They successfully and selectively inhibited the Tpl2 pathway following LPS stimulation in macrophages and BMDM cells in a dose-dependent manner and showed good selectivity over MEK and ERK (Cusac *et al.,* 2009). More recently, a high through-put screening of a compound library yielded thieno[3,2-d]pyrimidines as promising Tpl2 kinase inhibitors. They had an IC_{50} as low as 0.18 μM, exhibited good binding to the Tpl2 protein as shown by biacore-based competitive binding studies, and showed good selectivity against kinases (Ni *et al.,* 2011).

7. Conclusion

There has been an explosion of interest in Tpl2 inhibitors to treat RA and other inflammatory and autoimmune diseases. However, similar to the description above for the role of Tpl2 in carcinogenesis, it may be an oversimplification of the situation to assume that inhibition of Tpl2 would reduce inflammation. We have found thirty times higher neutrophil numbers in TPA-treated skin from Tpl2 knockout mice when compared to wildtype TPA-treated mouse skin. Additionally, we have found higher edema, inflammatory enzymes and cytokines in Tpl2 knockout mice (DeCicco-Skinner *et al.,* 2011). Moreover, in contrast to the description above in RA-FLS cells, we have identified significantly elevated COX-2, PGE_2 and PGE_2 receptor levels in Tpl2 knockout keratinocytes with the elevated COX-2 contributing to increased skin tumorigenesis (unpublished findings).

In summary, more research needs to be conducted to clarify the role that *Tpl2* plays in immune responses, carcinogenesis, and inflammatory conditions. We caution that *Tpl2* gene regulation needs to be better understood before inhibitors are used systemically. *Tpl2* appears to have divergent roles in different cells and tissues, and therefore drugs used to selectively inhibit *Tpl2* in the hopes of controlling inflammation may actually exacerbate inflammation and carcinogenesis in certain tissues such as skin.

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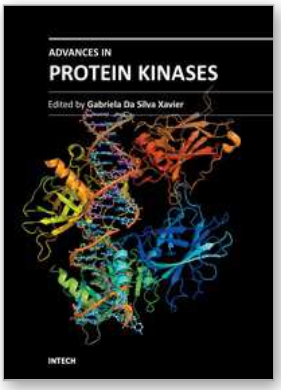

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Proteins are the work horses of the cell. As regulators of protein function, protein kinases are involved in the control of cellular functions via intricate signalling pathways, allowing for fine tuning of physiological functions. This book is a collaborative effort, with contribution from experts in their respective fields, reflecting the spirit of collaboration - across disciplines and borders - that exists in modern science. Here, we review the existing literature and, on occasions, provide novel data on the function of protein kinases in various systems. We also discuss the implications of these findings in the context of disease, treatment, and drug development.

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