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TITLE: TAM receptor tyrosine kinase function and the immunopathology of liver disease

Short title: TAM receptor tyrosine kinases in liver disease

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Abstract

TAM receptor tyrosine kinases are implicated in the regulation of the innate immune response through clearance of apoptotic cellular debris and control of cytokine signaling cascades. As a result they are pivotal in regulating the inflammatory response to tissue injury. Within the liver, immune regulatory signaling is employed to prevent the over-activation of innate immunity in response to continual antigenic challenge from the gastrointestinal tract. In this review we appraise current understanding of the role of TAM receptor function in the regulation of both innate and adaptive immunity, with a focus on its impact upon hepatic inflammatory pathology.

Introduction

The TAM receptor tyrosine kinases (Tyro3, Axl, MERTK) are a relatively recently discovered family of signaling molecules with diverse biological roles. Initially cloned from leukaemic cancer cell lines, they are expressed in a variety of tissues including the hematopoietic, nervous and reproductive systems (21, 28, 29, 31, 55). Axl is widely expressed in the human body(1), whilst MERTK is found in hematopoietic cells and in specialized epithelia including retinal pigmental epithelium and Sertoli cells (12, 21, 77). Tyro 3 is strongly expressed in central nervous system (33, 41). In common with other receptor tyrosine kinase (RTK) families, downstream signaling involves interaction with growth factor pathways, making them proto-oncogenic and over-expressed in many human cancers (4, 23, 35). The family is distinctive in a number of ways, including a unique ligand-receptor interaction and important regulatory roles in innate and adaptive immunity (74). In this review we appraise the current understanding of TAM receptor signaling in inflammatory pathologies, highlighting our current understanding of their role in the immunopathology of liver disease.

TAM receptor function in tissue development and homeostasis

TAM signaling plays a role both in tissue embryogenesis and homeostasis through clearance of apoptotic cells (52). TAM receptors expressed on specialized epithelial cells and phagocytes bind to phosphatidylserine (PtdSer) on the outer phospholipid membrane of apoptotic cells via an intermediary association with their respective

ligands (61). This interaction enables selective engulfment and uptake of apoptotic cells. Recent studies in rodents implicate MERTK in this process. Retinal pigment epithelial cells in MERTK knockout mice fail to clear apoptotic cells and cellular debris, resulting in prolonged inflammation, fibrosis and retinal degeneration (49) (13). MERTK has also been reported to be important in mammary epithelial glandular involution after lactation (59). In a similar manner, TAM signaling in Sertoli cells is required to help clear apoptotic remnants of meiosis in the testes: these accumulated in male TAM knockout mice, resulting in inflammatory damage to seminiferous tubules and infertility (68) (77). Within the central nervous system of mice, microglial cells lacking MERTK were unable to clear ineffective synaptic connections, impairing hippocampal development and propagating neuronal damage (30).

TAM receptor ligands

The two most studied ligands of TAM receptors are Gas6 and Protein S (Pros1). They share over 40% sequence homology and depend upon vitamin K for binding to TAM receptors (40). Protein S is a regulatory component of the coagulation cascade; however this function does not involve TAM receptors (6) (26) (58). It is produced by hepatocytes, endothelial cells and in those tissues mentioned above which utilize MERTK mediated clearance of apoptotic cells (6). Gas6 is expressed primarily in vascular smooth muscle and endothelial cells. *In vitro* studies have shown that Gas6 can bind and activate Axl without PtdSer, indicating a function distinct from apoptotic cell clearance (72). In steady state, serum concentrations are low (<0.2nM) but rise dramatically during acute stress or tissue injury such as sepsis (14, 47, 73).

Galectin-3 has recently been identified as a TAM receptor ligand. Amongst diverse roles in an array of cellular processes, its expression is elevated following tissue damage, including in cardiac myocytes after myocardial infarction and in both acute and chronic liver injury (24) (27) (43) (70). It is produced by macrophages and

contributes to fibrogenesis through recruitment of fibroblasts to sites of tissue damage. Galectin-3 employs a number of downstream signaling cascades and the distinct role of TAM signaling within this repertoire is unclear; at present it is known to facilitate phagocytosis via MERTK (7). That Gas6 and Galectin-3, both TAM ligands, are frequently up-regulated after tissue injury is noteworthy, suggesting a role for TAM signaling in response to tissue damage (14, 24).

TAM signaling in immune regulation

Perhaps the most prominent aspect of TAM receptor function is in regulation of immunity. TAM receptor loss results in exaggerated activation and ineffective resolution responses, resulting in excessive inflammatory tissue damage. This has been demonstrated in experimental models of both sterile and pathogen induced inflammation. In endotoxemia models, MERTK knockout mice almost uniformly succumbed to septic shock and died as a result of tissue damage mediated by excessive levels of TNF-α and IL-1 (9). In mice, bleomycin induced lung injury was attenuated when surface MERTK expression on macrophages was enhanced. Anti-inflammatory mediators (TGF-β and hepatocyte growth factor HGF) are more abundant whilst TNF-α and IL-1β expression is reduced (34).

It is therefore evident that TAM signaling regulates innate immune responses through the modulation of cytokine production. Rothlin et al. demonstrated that pro-inflammatory cytokine production by murine dendritic cells after Toll-like receptor (TLR) activation is attenuated by TAM receptor signaling, specifically MERTK and Axl. This is mediated by SOCS1 and 3 (suppressors of cytokine signaling); inhibitory proteins that act at various points in the TLR signaling cascade. Increased SOCS1 and 3 expression occurs downstream of TAM receptor activation. The authors demonstrate a dynamic feedback loop in which the initial burst of cytokines produced

as a product of TLR signaling bind to their respective receptors and activate transcription factor STAT1. As well as promoting further pro-inflammatory cytokine production, STAT1 also induces Axl. In association with Gas6 or Pros1, Axl interacts directly with cytokine receptor interferon associated receptor (IFNAR). This complex of proteins appears to differentially activate STAT1, redirecting its downstream genetic targets towards SOCS1 and 3 and acting as a 'brake' for cytokine production after TLR activation by pathogens (56).

TAM signaling in macrophages skews the cytokine profile in favor of wound healing and resolution of inflammation after uptake of apoptotic cells. MERTK mediated efferocytosis promotes expression of 'Th2' like cytokines including IL-4, IL-10 and TGF-β (15). An *in vitro* study in mice demonstrated that this is dependent upon inhibition of NF-κB and activation of the PI3K pathway (62). The ingested products of apoptosis themselves induce further MERTK expression: cholesterol metabolites from cell wall fragments activate the liver X receptor, which binds and activates the MERTK promoter (45). In addition, IL-10 acts in an autocrine manner to induce further MERTK expression and propagate an anti-inflammatory response to tissue damage (79). Gas6 and Pros1 are secreted in an autocrine manner by macrophages and dendritic cells in response to both MERTK and Axl activation, helping to amplify TAM signaling at sites of inflammation (56).

Differential expression of TAM receptors in different immune cell types may indicate specificity in biological function. Zagorska et al. noted more abundant expression of Axl in murine dendritic cells, whilst MERTK was more commonly expressed in macrophages. They report an increase in Axl expression in response to TLR ligands lipopolysaccharide and poly I:C, whereas MERTK expression was induced by the uptake of apoptotic cells and IL-10 as described above. These observations may

support a model in which MERTK signaling enables phagocytic clearance in homeostatic settings, whilst Axl signaling functions in sentinel antigen presenting cells in response to acute inflammatory insults (76).

These immune regulatory functions are exploited by pathogens in order to evade immune recognition. Enveloped viruses such as dengue and Ebola express PtdSer on their outer membranes in a process termed 'apoptotic mimicry' (44), hijacking MERTK and Axl signaling pathways to enable their uptake by antigen presenting cells and suppress the innate anti-viral response (42, 65). In a mouse model of respiratory syncytial virus (RSV) and H1NI influenza infection, both MERTK and Axl expression was increased following viral exposure. Their increased expression directly attenuated IFN-β production whilst promoting Th2 type responses. Similarly, in response to fungal (*Aspergillus*) infection, Axl up-regulation in macrophages resulted in an inhibition of interferon-γ mediated NK and T cell responses(63).

TAM signaling and autoimmunity

Autoimmunity is the result of inappropriate activation of adaptive immunity in response to self antigen, characterized by hyperactive inflammatory responses in antigen presenting cells and a failure to inhibit the formation of autoreactive T and B cell clones (57). It is perhaps unsurprising that TAM signaling has been implicated in autoimmunity in view of the established significance of TAM receptors in both antigen presenting cells and clearance of self-antigen in the form of apoptotic cell remnants.

Support for this association can be found in mouse models. A profound poly-autoimmune syndrome resembling systemic lupus erythematosus (SLE) develops in TAM triple ($MERTK^{-/-}$, $AxL^{-/-}$, $Tyro3^{-/-}$) knockout mice, characterized by elevated titers of autoantibody, uncontrolled B and T cell proliferation and accumulation of lymphocytes in secondary lymphoid organs (38). In humans with SLE there is

defective clearance of autoreactive lymphocytes in the germinal centers of lymph nodes (18) by tingible body macrophages that are, in mice, known to express MERTK (51). Furthermore Pros1 is frequently deficient in SLE (and in other autoimmune pathologies including ulcerative colitis), suggesting a role for reduced TAM signaling in its pathogenesis (20) (67) (32).

Recent work has highlighted a further role for TAM signaling at the interface of innate and adaptive immunity. Cytotoxic T cells in mice express Pros1 and externalize patches of PtdSer, thereby activating MERTK on the surface of antigen presenting cells to dampen pro-inflammatory cytokine production and antigen specific responses (10).

TAM receptors and anti-tumor immunity

TAM mediated immune regulation is also important in the context of anti-tumor immunity. Classically this is facilitated by NK cells, which are primed to delete neoplastic cells indiscriminately (66). In addition, tumor associated antigens exposed early in tumor development can generate effector CD8+ T cells (16). With time, however, neoplasms evolve to evade host immunity, a process which involves employing a number of mechanisms including pro-resolution, regulatory signaling cascades of the TAM receptor kinase family (60).

Work by Paolino et al has demonstrated the inhibitory role of TAM signaling in NK cell activation. *In vitro* assays of NK cell proliferation and production of interferon-gamma were attenuated by stimulation with Gas6. *In vivo*, the addition of an unselective TAM inhibitor restored the cytotoxic activity of NK cells and reduced both tumor and metastatic burden (48).

Within the tumor microenvironment, associated-associated macrophages interact intimately with tumor cells to promote tumor growth, invasion and systemic spread. This is achieved through evasion of host immunity. There is evidence that TAM signaling plays a key role in this harmful process: MERTK knockout mice display reduced tumor burden and fewer metastases in a xenograft model (11). Furthermore, Gas6 expression is elevated in a number of solid tumors (22, 75). A number of different micro-environmental cues stimulate MERTK expression in associated-associated macrophages. These include ingested phagocytic material, the autocrine secretion of Gas6 and IL-10 and macrophage colony stimulating factor secreted by tumor cells. This promotes the production of anti-inflammatory cytokines including TGF-β and IL-10, which not only attenuate adaptive anti-tumor T cell immunity but also directly stimulate tumor cell survival (22).

TAM receptor tyrosine kinase function in liver disease

Steady state hepatic immunity

The concept of liver 'tolerance' has been acknowledged since early observations in animal transplant models of spontaneous acceptance of donor allograft despite MHC class mismatch (8). Immune tolerance is advantageous for the liver, allowing it to manage the large antigen load received from the gastrointestinal tract. Hepatic tolerance is orchestrated by the resident population of antigen presenting cells, adapted epithelial cells and an enriched natural killer cell population (71).

Given that TAM receptors are expressed in all of these cell types and contribute to immune regulation, their role in hepatic immunity warrants further investigation. All three TAM receptors have been identified in the livers of wild type mice. MERTK is expressed in Kupffer cells and sinusoidal endothelial cells but not in hepatocytes. Axl is expressed in all three cell types while Tyro 3 is restricted to resident macrophages (50).

The most informative data on the role of TAM RTKs in hepatic immunity comes from the TAM triple knockout mouse. By six months of age it spontaneously develops an autoimmune hepatitis with rising transaminases and increasing titers of autoantibodies to smooth muscle antigen and antinuclear antigen. Histological analysis reveals an infiltration of autoreactive CD4+ T cells and circulatory macrophages. Hepatocytes have elevated pro-inflammatory cytokine expression, including IL-6, IL-1β, TNF-α and interferons through up-regulation of NF-κβ and interferon regulatory factor 3 (IRF3). This autoimmune phenotype was not seen when TAM knockout mice bone marrow was transplanted with wild type stem cells (50).

These observations suggest that TAM receptors are vital for maintaining immune tolerance in the liver. Inappropriate activation of innate immunity by effective clearance of 'self-antigen' by efferocytosis and by dampening of pro-inflammatory cytokine cascades appears to prevent autoreactive T cell clone formation. It is not clear, however, if there is a direct effect on T cell activation and proliferation.

Acute inflammation and liver injury

MERTK may be protective in acute liver injury. In a murine model of hepatic ischemia, serum Gas-6 levels rose shortly after arterial ligation. Western blot analysis of homogenized liver extracts after ischemic insult showed a selective increase in phosphorylated MERTK over phosphorylated Axl, indicating preferential MERTK mediated signaling in this context. Gas-6 knockout mice showed higher mRNA levels of pro-inflammatory cytokines (IL-1A, TNFα) and more frequently succumbed to fulminant hepatic failure after only partial ischemic insult. Administration of recombinant Gas-6 restored protection from fulminant disease. It is not clear if this protective effect is mediated by TAM signaling in hepatic immune cells or in

parenchyma, but the regulatory effect of Gas 6 administration on cytokine production was replicated *in vitro* in a surrogate Kupffer cell line (36).

MERTK signaling has been studied in humans with both acute liver failure syndromes and acute on chronic liver failure (ACLF). A significant cause of morbidity in these patients is sepsis. Work undertaken by Bernsmeier et al. shows an expansion of MERTK positive circulating monocytes compared to healthy and cirrhotic controls. There is a concomitant increase in Gas-6, Pros-1 and galectin-3 as well as phosphorylated MERTK, indicating active MERTK signaling. This MERTK positive phenotype was reproduced in healthy monocytes incubated in plasma from ACLF patients. MERTK positive monocytes exhibit an attenuated response to endotoxin challenge, as previously described. Blockade of MERTK with a small molecule inhibitor in these monocytes restored TNFα and IL-6 production in response to lipopolysaccharide(5).

The authors demonstrate that MERTK positive monocytes are more prone to transendothelial migration and propose a dynamic model in which monocytes are recruited to the inflamed liver, resulting in increased MERTK expression in response to hepatic injury. However in the setting of a systemic inflammatory response, endothelial dysfunction enables reverse transmigration of these monocytes into peripheral blood and local lymph nodes, potentially contributing to immune paresis and vulnerability to sepsis (5).

Chronic inflammation and liver injury

Although beneficial in the steady state and perhaps in response to acute liver injury, in models of chronic liver disease TAM receptor signaling is potentially deleterious. Activation of hepatic stellate cells (HSCs) is pivotal in the progression of liver injury (39). These cells secrete collagen and other extracellular matrix proteins in chronic

liver disease, promoting fibrogenesis and cirrhotic transformation (3). Murine experimental models of chronic liver injury have confirmed the role of TAM receptor signaling in this process. HSC activation relies upon Gas-6 mediated activation of Axl, leading to up-regulation of signaling via AKT and NF-κβ in mice exposed to carbon tetrachloride. Transcription and translation of Axl was increased as well as activation of the downstream signaling in both liver macrophages and stellate cells (2, 17).

In another mode of chronic liver injury, mice fed a choline deplete, ethionine supplemented diet developed steatohepatitis. Gas-6 deficient mice fed this diet showed a reduction in HSC activation and expression of TGF-β. Furthermore, onset of necroinflammation and steatosis was delayed compared with wild-type mice. Expression of TNF-α, IL-1β and macrophage chemotactic protein 1 (MCP-1) mRNA was reduced, with a concordant reduction in macrophage infiltration at 7 days (17).

TAM receptor signaling has recently been studied in the context of chronic hepatitis C virus (HCV) infection. A strong interferon (IFN) response is predictive of viral eradication (19). Chronically infected HCV patients with prolonged activation of type I/III IFN signaling pathways and high baseline expression of downstream IFN-stimulated genes (ISGs) *prior* to treatment are less likely to achieve sustained virological response (SVR). This is thought to be due to less vigorous further induction of ISGs upon commencing treatment. The mechanism for this phenomenon is not fully understood, however work by Read et al. suggests a role for Axl. In *in vitro* models and *in vivo*, Axl expression was up-regulated in chronically infected hepatocytes; furthermore those hepatocytes from patients with a 'non-responder' phenotype in chronic HCV showed higher Axl expression than 'responders'. Axl expression was potently induced by interferons, and is mediated by a number of

transcription factors including STAT1. *In vitro* hepatocyte Axl overexpression resulted in reduced STAT1 phosphorylation and subsequent ISG expression. This is illustrated in Figure 1. Taken together these data suggest that IFN induced Axl expression mediates a negative feedback loop, down-regulating IFN signaling in a similar manner to that elucidated previously by Rothlin et al. in dendritic cells (56). In hepatocytes this does not appear to be via SOCS1 and 3 but may be a direct effect of Axl on IFN signaling pathways (53) (54).

In summary, TAM receptors and their ligands are widely expressed in the liver and contribute to hepatic immune regulation by preventing autoreactive T cell development in steady state. In response to injury, Gas-6 and MERTK mediate down-regulation of acute inflammatory cascades. However in the context of chronic inflammation, Axl signaling results in smoldering inflammation, fibrosis and reduced viral clearance. A schematic of these processes is summarized in Figure 2.

Future prospects in hepatic TAM receptor research

Current research into TAM receptor function is focused upon their roles within immune regulation and tumor biology. TAMs are widely over-expressed in most human cancers and their expression is associated with an aggressive phenotype and a higher burden of metastasis (46) (64) (69) (78). Evidence indicates activation of MERTK signaling is a mechanism that suppresses host anti-tumor immunity. Within the liver, Axl is overexpressed in murine hepatocellular carcinoma cell lines and is associated with a higher propensity to metastasize *in vivo* (25). Further understanding of TAM signaling in immune regulation in hepatocellular carcinoma (HCC) is required. Recent work has shown that hepatic tumor associated macrophages are ‘tolerized’ in vivo by tumor up-regulation of CD47 (37). It is possible that TAM receptor ligation by circulating Gas-6 in the tumor microenvironment may have similar, yet distinct effects upon tumor associated

macrophages, as well as roles in modulating NK cells, CD4 + and CD8+ T cells to promote tumor progression. These adaptations may present opportunities for therapeutic intervention in HCC by restoring host anti-tumor immunity.

Recent work has demonstrated the importance of TAM signaling following acute tissue injury, however its impact upon other hepatic inflammatory liver diseases remains unexplored. Further work to validate this in humans is required. In addition, studies investigating the role of TAM signaling in other immune-mediated hepatic inflammatory diseases are warranted. With an array of molecular inhibitors to individual TAM receptors and their ligands currently available, the possibility of targeted therapy for aberrant TAM signaling in liver disease is an exciting prospect.

Acknowledgments

Figure 1 and 2 were constructed from images made available by Servier Medical Art, which is licensed under a Creative Commons Attribution 3.0 Unported License. http://www.servier.com/Powerpoint-image-bank.

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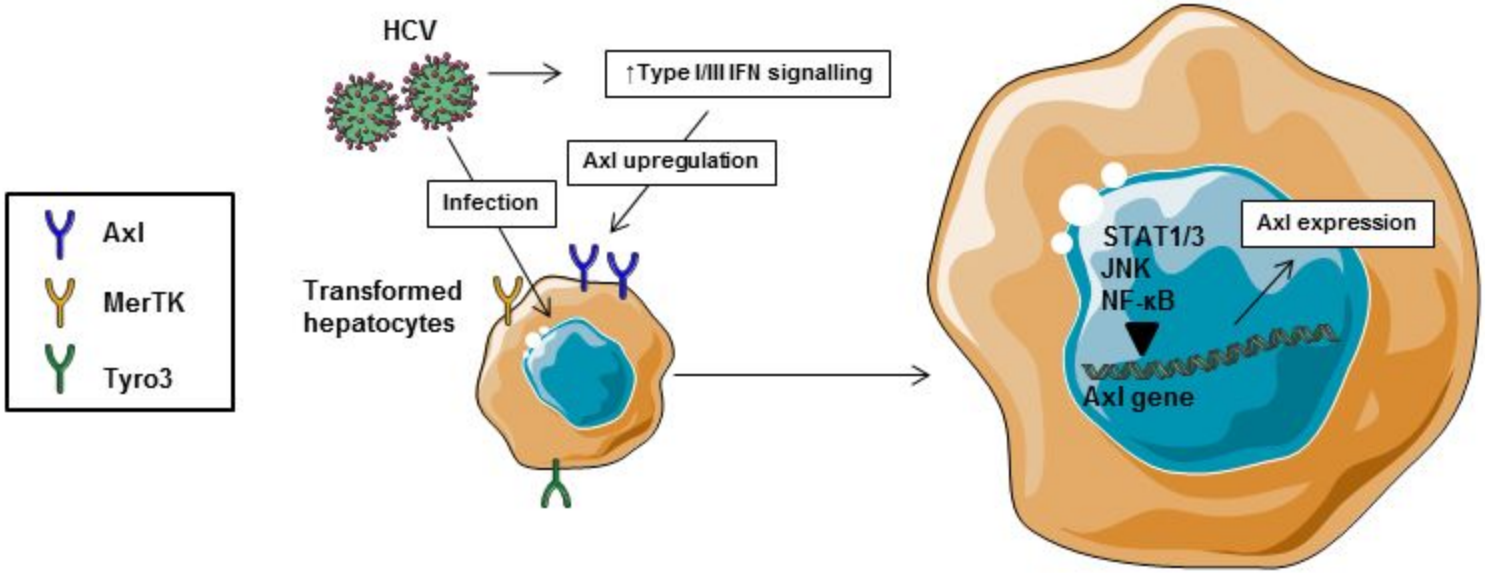
Figure Legends

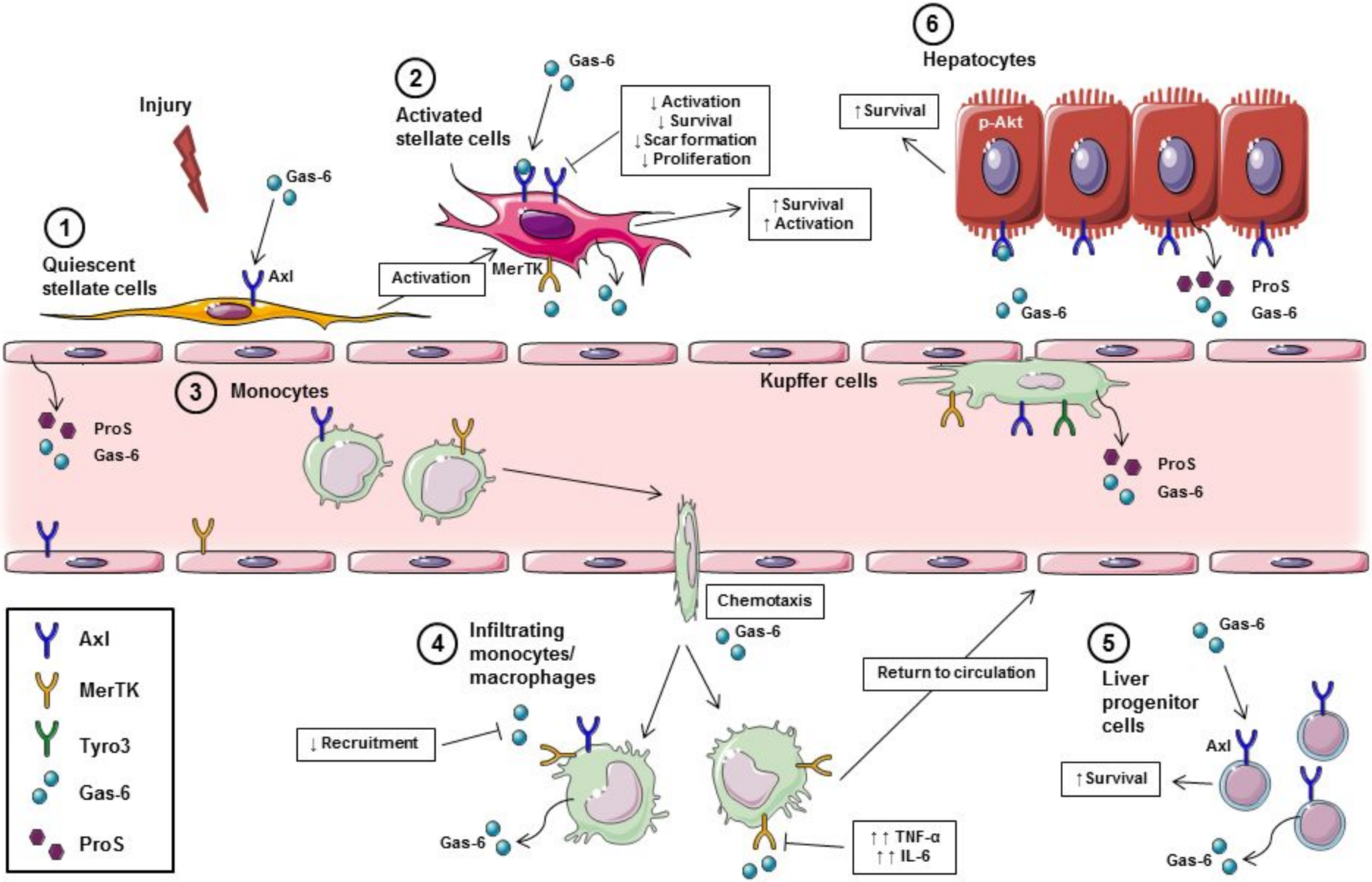
Figure 1: Schematic representation of Axl regulation during HCV infection

Axl is upregulated following HCV infection potentially through upregulation of IFN type I/III inflammatory signalling pathways in transformed hepatocytes. HCV-mediated Axl expression is mediated through a variety of transcription factors including STAT1/3, JNK and NF-κB.

Figure 2: Schematic representation of TAM receptors and ligands in liver inflammatory pathologies

1. Axl is found on quiescent hepatic stellate cells (HSC) and becomes upregulated along with Gas-6 during HSC activation following liver injury. **2.** Gas-6 leads to phosphorylation of Axl and MerTK in HSC and promotes HSC survival and activation. Inhibition of Axl in HSC reduces activation, survival, scar formation and proliferation. **3.** Circulating monocytes express MerTK and Axl and Kupffer cells express all TAM receptors and are the main producers of Gas-6 in normal livers. **4.** Upon injury monocytes migrate across the endothelium into tissue, promoted by Gas-6. Gas-6 reduces LPS-induced secretion of TNF and IL-1β in macrophages and inhibition of MerTK in monocytes leads to a significant increase in LPS-induced TNF and IL-6 production. **6.** Liver progenitor cells express Axl and Gas-6, which is a survival factor for liver progenitor cells. **7.** Hepatocytes express Axl but not MerTK or Tyro3. Gas-6 induces phosphorylation of Akt and protects hepatocytes from cell death.


Axl
MerTK
Tyro3
HCV
↑Type I/III IFN signalling
Axl upregulation
Infection
Transformed hepatocytes
STAT1/3
JNK
NF-κB
Axl gene
Axl expression


6
Hepatocytes
↑ Survival
p-Akt
Gas-6
ProS
Gas-6
2
Activated
stellate cells
Gas-6
↓ Activation
↓ Survival
↓ Scar formation
↓ Proliferation
↑ Survival
↑ Activation
Injury
Gas-6
1
Quiescent
stellate cells
Axl
Activation
MerTK
3
Monocytes
Kupffer cells
ProS
Gas-6
ProS
Gas-6
Chemotaxis
Gas-6
4
Infiltrating
monocytes/
macrophages
Return to circulation
↓ Recruitment
Gas-6
↑↑ TNF-α
↑↑ IL-6
5
Liver
progenitor
cells
Gas-6
Axl
↑ Survival
Gas-6
Axl
MerTK
Tyro3
Gas-6
ProS