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Lymphotoxin, NF-κB, and Cancer: The Dark Side of Cytokines

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Abstract
Cytokines have been implicated in a variety of physiological processes involving lymphoid tissue development, lymphocyte activation, and control of regenerative processes such as wound healing. The first characterization of a cytokine implicated in abolishing or killing tumor cells – the tumor necrosis factor (TNF) – fostered and boosted a completely new field of research that in addition to cancer research started to generate an overwhelming amount of knowledge in immunology, various pathological processes, and other fields of research. Due to the complex networks and versatile functions of cytokines, it soon became clear that cytokines can possess diametric functions in various biological processes. As for tumor research it was shown that some cytokines – depending on the type of organ, the time of action, gender, and the cellular environment – can have either pro- or anticarcinogenic action. For those cytokines reported to be procarcinogenic, this could be accomplished by directly acting as oncogenes or generating an inflammatory environment that is procarcinogenic. Here we review a novel role for TNF family members – in particular lymphotoxin (LT) α and β – in physiology and in driving tumorigenesis, with special focus on the liver. We believe that recent findings on this particular cytokine might have strong implications for the therapy of liver cancer or other inflammation-induced cancer types.

A Brief History of Inflammation

Inflammation is classically defined as an immune response to tissue injury. The first description of inflammation was made by Aulus Cornelius Sensus in 47 BC. In his extant medical encyclopedia, 'de Medicina', Cornelius mentioned that redness and swelling with heat and pain (rubor et tumor cum calore et dolore) are the characteristic symptoms of inflammation. It was only until 1873 that Julius Cohnheim discovered the physiological basis of these symptoms to be leukocyte emigration from blood vessels and other vascular changes. A fifth cardinal sign, 'functio laesa' (loss of function), was added by Rudolph Virchow in 1858. All of these findings paved the way for establishing a cellular basis of pathol-

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ogy by deviating the study of inflammation from a traditional viewpoint which surrounds the four cardinal signs of inflammation [1].

The Inflammatory Types: Acute and Chronic Phases

In the recent past, there has been a tremendous increase in our understanding of inflammation, which has led to its division into acute and chronic phases.

Acute Phase

Typically, an inflammation is triggered either by a mechanical tissue injury or by recognition of pathogens like bacteria and viruses through pattern recognition receptors such as Toll-like receptors (TLRs) present on innate immune cells leading to the production of inflammatory cytokines and chemokines such as tumor necrosis factor (TNF)-α, IL-1, IL-6, IL-8, IL-11, CCL2, and CXCL8. Their activity in turn mediates inflammation by attraction of lymphocytes and monocytes. Among these, IL-1 and TNF are the most potent inflammatory molecules mediating acute inflammation, e.g. induced by lipopolysaccharide (LPS), and they are the primary cause of septic shock. In the liver, IL-1, IL-6, and TNF are shown to have systemic effects when secreted in sufficient amounts by inducing hepatocytes to produce acute-phase proteins such as C-reactive protein (CRP) and coagulation factors. These mediators activate brain endothelium to secrete prostaglandins such as PGE2 which in turn induce specific neuronal populations in the CNS to promote a sickness behavior characterized by fever, anorexia, fatigue, sleepiness, and social withdrawal [2].

Mechanical tissue injury in the absence of infection also leads to acute inflammation which promotes tissue repair and helps prevent colonization of damaged tissues by opportunistic pathogens. Tissue damage is recognized by nociceptors which induce a pain sensation by exudate formation and tissue swelling in the affected area. Prostaglandins lower the threshold of pain sensations by increasing the sensitivity of nociceptors. In this situation, the innate immune cells such as resident macrophages induce reparative responses. The sensing of the inflammatory milieu by the vagus nerve triggers an 'inflammatory reflex', which is involved in the negative control of inflammation [1, 3].

Chronic Phase

Failure to resolve acute inflammation resulting from immune responses to pathogens can lead to a persistent chronic inflammation which may last weeks or months and in some instances years. Cytokine interactions and infiltration of lymphocytes during this phase of inflammation lead to monocyte chemotaxis to the site of inflammation where resident macrophages are activated by interferon (IFN)-γ and MCP-1. These activated macrophages are restricted to the sites of inflammation by migration inhibition factors such as GM-CSF and IFN-γ which leads to an elevation in the levels of IL-1 and TNF. In case of chronic inflammation, cytokines such as IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-13, and TGF-β are known to promote humoral inflammation whereas IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, IFNs, IFN-γ-inducing factor (IGIF), TGF-β, TNF, and lymphotoxin (LT) are known to promote cellular inflammation [4].

These prolonged inflammatory signals can lead to the formation of granulomas and the generation of tertiary lymphoid organs (TLO) at the site of infection and in some cases might lead or contribute to certain diseases such as obesity, chronic obstructive pulmonary disease (COPD), autoimmune pancreatitis, type 2 diabetes, atherosclerosis, neurodegenerative diseases, and cancer [1].

Factors Influencing Inflammation Leading to Cancer

In 1863, Rudolf Virchow noticed the presence of leukocytes in neoplastic tissues and suggested that the 'lymphoreticular infiltrate' reflected the origin of cancer at sites of chronic inflammation. This was the first observation linking inflammation and cancer. A lot of evidence later supported Virchow's observations and we now know of a large set of factors ranging from bacteria, viruses, and environmental factors. Dietary components are also shown to promote a chronic inflammatory state which ultimately leads to cancer. In the recent past, molecular mechanisms linking inflammation and cancer have been established which led to the proposal of an inflammatory microenvironment as the seventh hallmark of cancer [5, 6].

Infectious Agents

In appreciation of the work involving the identification of infectious agents causing cancer, the Nobel Prize was awarded in 2005 to Barry Marshal and Robin Warren for elucidating the role of *Helicobacter pylori* in gastritis and peptic ulcers and to Harald zu Hausen for his discovery that human papilloma viruses are the caus-

ative agent for cervical cancer. There is substantial evidence showing that persistent *H. pylori* infection is associated with gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma. Infections with hepatitis B (HBV) or C (HCV) viruses increase the risk of hepatocellular carcinoma (HCC). Infections with *Schistosoma* or *Bacteroides* species are linked to bladder and colon cancer [7].

Noninfectious Agents

According to recent findings, up to 20% of cancers are linked to chronic infections. Inhaled pollutants such as silica and asbestos are linked to lung cancers as they are capable of efficiently triggering inflammation through effects on prointerleukin-1β processing by the inflammasome and this may mediate their tumorigenic activity. Evidence also suggests that lung cancer can promote inflammation through active secretion of molecules such as the extracellular matrix component versican which activates macrophages through TLR2 [7]. Tobacco smoke which has a high carcinogen content is thought to play a role not only as a tumor initiator but also as a tumor promoter, owing to its ability to trigger chronic inflammation [8]. Dietary factors are linked to 35% of cancers. Obesity, which is increasing rapidly in developed countries, can lead to chronic inflammation [8] which in turn can promote the development of HCC [9] and might increase the overall cancer risk by about 1.6-fold [10]. Sustained alcohol abuse and inflammatory bowel disease are also among the major noninfectious agents that are known to promote cancer, owing to their involvement in inducing chronic inflammation [11]. A massive tumor-associated inflammatory response can be initiated by cancer therapy. Radiation and chemotherapy cause massive necrotic death of cancer cells and surrounding tissues, which in turn triggers an inflammatory reaction analogous to a wound-healing response [12]. Accumulation of damaged DNA and cell senescence can also give rise to tumor-promoting chronic inflammation [13].

Why Does Inflammation Cause Cancer?

The tumor microenvironment contains stromal cells such as fibroblasts and endothelial cells, innate immune cells, and adaptive immune cells which secrete cytokines, growth factors, proteases, and other bioactive molecules that can act in an autocrine and/or a paracrine manner and generate a delicate balance between antitumor immunity, which is provided by the adaptive immune system, and tumor-promoting immune activity, which originates from the innate immune compartment. However, recent reports show that cytokines expressed by adaptive immune cells can also cause or drive cancer [14, 15]. During tumorigenesis, the host-mediated antitumor activity is thought to be suppressed and therefore proinflammatory actions prevail that ultimately support tumor growth, angiogenesis, invasion, and metastasis [11].

The cell types and cytokines expressed in the tumor microenvironment decide whether tumor growth suppression or progression occurs. The major cell types found in the tumor microenvironment are high numbers of T cells and tumor-associated macrophages (TAMs) [16]. TAMs are known to promote tumor growth and are necessary for invasion, migration, and metastasis [17]. Rather than the abundance of cell types prevailing in the tumor microenvironment, it is their activation and polarization profile shaped by cytokines that determines tumor suppression or progression. Cytokines, such as TNF-α, IL-1α, IL-1β, IL-6, IL-10, IL-12, IL-17, IL-23, IFN-γ, TGF-β, and TRAIL, are responsible for this polarization and are predominantly secreted by the respectively activated cells. Among these, IL-12, TRAIL, and IFN-γ are associated with cytotoxic T-helper 1 responses that mediate antitumor immunity, whereas TNF-α, IL-6, and IL-17 suppress such polarization and promote tumor growth [18].

The TNF Superfamily

The members of the TNFL/TNFR superfamily form a complex network of cytokines and receptors. Depending on different structural features within their cytosolic tail, the TNFR superfamily can be divided into 3 different subgroups with diverging cellular functions [19, 20].

The first group including, for example, TNFRI and FAS is characterized by mediation of downstream signaling via a so-called death domain (DD). By recruiting adaptors like the FAS-associated DD (FADD) or the TNFR-associated DD (TRADD), these receptors can induce caspase-8-dependent cell death [20–23] (see also fig. 1). Characteristic of the second group (e.g. TNFRII and LTβR) is the existence of TNF-receptor-associated factor (TRAF)-interacting motifs (TIMs) within their cytosolic domains. Recruitment of TRAFs upon receptor stimulation leads to the activation of downstream signal-

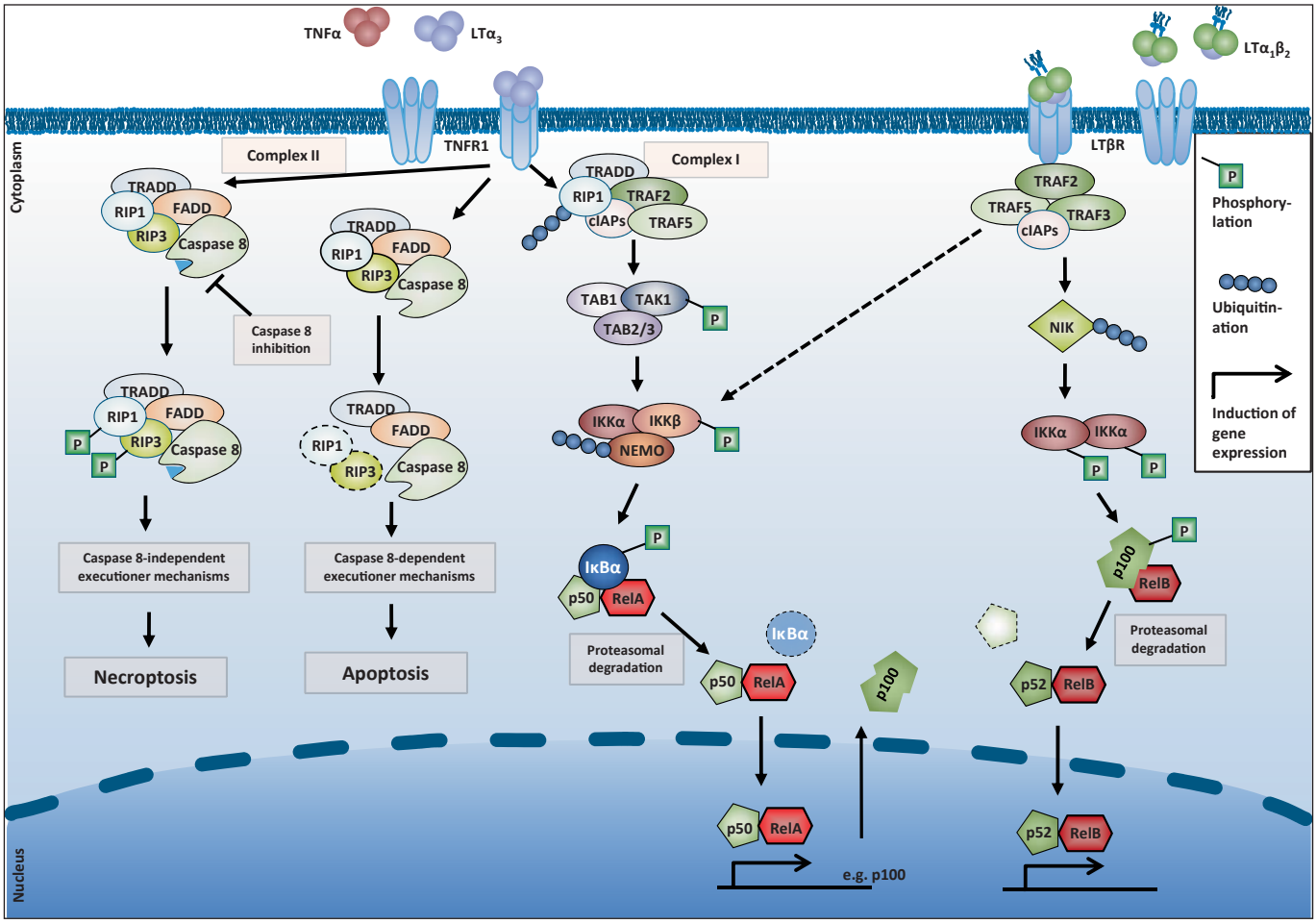

Fig. 1. Signaling cascades of TNFR and LTβR. Engagement of TNFRI can initiate canonical NF-κB signaling by recruiting complex I to its intracellular TRAF-binding domain. This leads to activation of the IKK complex via ubiquitination of NEMO and phosphorylation of IKKγ and the subsequent proteasomal degradation of IkBa and finally to nuclear translocation of the p50/RelA heterodimer. If the canonical NF-κB pathway is being disturbed, TNFRI multimerization induces the formation of complex II, which then mediates caspase 8-dependent cleavage of RIP1 and RIP3 leading to apoptosis. If caspase 8 is blocked, RIP1 and RIP3 become phosphorylated and activated by still not fully elucidated mechanisms finally leading to necroptotic cell death. The noncanonical NF-κB pathway becomes activated by binding of, for example, membrane-bound $LT\alpha_1\beta_2$ to LTβR causing ubiquitination of NIK and downstream activation of the homodimeric IKKα complex. This finally leads to phosphorylation and proteasomal cleavage of the p100 precursor to the p52 subunit, enabling the p52/RelB heterodimer to translocate into the nucleus and induce target-gene expression. Furthermore, it was shown that LTβR can also activate the canonical NF-κB pathway via TRAF5.

ing pathways, like for example nuclear factor-κB (NF-κB), extracellular signal-related kinase (ERK), p38 mitogen-associated kinase, or phosphoinositide 3-kinase (PI3K) [20]. Decoy receptors (e.g. DcR1 and DcR3) are the main components of the third group which do not exhibit any intracellular signaling functions but rather compete with the other two groups for their respective ligands [24]. Most members of the TNF superfamily are type II transmembrane proteins and, except for LTβ, mediate their receptor binding activity as noncovalently bound homotrimers or multimers of a higher number [25–27]. Some of the ligands, like for example TNF, can also be released from the cell membrane by proteolytic cleavage or even exist only as soluble molecules such as LTα.

Physiology

LT has been shown to exist in at least two different variants. It can either form membrane-bound LTαβ heterotrimers ($LT\alpha_1\beta_2$, $LT\alpha_2\beta_1$), where LTβ is exclusively anchored in the cell membrane, or soluble LTα homotrimers [28, 29]. Furthermore, it has been shown that LTαβ heterotrimers can also be shed from the cell surface by proteolytic cleavage and thus mediate their functions on more distant cells [30]. Similar to TNF homotrimers, LTα binds and activates TNFRI and TNFRII, as does the $LT\alpha_2\beta_1$ heterotrimer, whereas the $LT\alpha_1\beta_2$ heterotrimer is exclusively signaled via LTβR [31]. Stimulation of TNFRI and LTβR, like several other TNFR superfamily members via their ligands, has been shown to activate canonical/noncanonical NF-κB signaling and hence they play an important role in cell survival, proliferation, differentiation, and apoptosis.

The canonical NF-κB pathway is mainly activated via TNFRI and TNFRII in response to inflammatory cytokines such as TNF, IL-6, and IFN-γ during bacterial and viral infection. Engagement of TNFRI with one of its ligands (e.g. TNF or $LT\alpha_3$) leads to recruitment of the so-called complex I [consisting of TRADD, TRAF2, TRAF5, receptor-interacting protein (RIP)-1, and cellular inhibitor of apoptosis (cIAP)] [32]. This triggers the activation of the inhibitor of κB (IκB) kinase (IKK) complex (consisting of IKKα, IKKβ, and NEMO/IKKβ) by ubiquitination of the regulatory subunit NEMO leading to phosphorylation of IKKβ. IKKβ then in turn leads to the phosphorylation and proteasomal degradation of IκBa, hence allowing nuclear translocation of the p50/RelA (NF-κB1) heterodimer mediating proinflammatory and prosurvival signaling [33].

However, in case canonical NF-κB is disturbed or shut down, TNFRI stimulation induces the formation of complex II (consisting of TRADD, FADD, caspase 8, RIP1, and RIP3), leading to caspase 8-mediated apoptosis or, upon caspase blocking, to RIP1/3-mediated necroptosis [23, 32]. In addition to the canonical NF-κB pathway, the noncanonical NF-κB pathway is activated upon stimulation by a subset of TNFR superfamily members, e.g. LTβR and CD40, via their ligands [$LT\alpha_1\beta_2$ and CD40 ligand (CD40L), respectively] [34–37]. This pathway has been shown to be independent of IKKβ and NEMO [38]. Here, ligand engagement leads to a phosphorylation cascade activating first NF-κB-inducing kinase (NIK) and its downstream target $IKK\alpha_2$ [39]. The activated IKKα homodimer eventually marks p100 for proteasomal cleavage into p52, enabling the p52/RelB heterodimer to translocate into the nucleus and start target gene expression. This pathway has been shown to be crucial for inducing inflammation and for the development of thymus, secondary lymphoid organs (SLO), and TLO, as well as B-cell survival and maturation of follicular dendritic cells [40, 41].

Pathology

Under normal, physiological conditions LT expression can only be found in T, B, natural killer (NK), and lymphoid tissue inducer cells [42], whereas LTβR is mainly expressed on parenchymal and stroma cells of SLO and the thymus but also on myeloid cells [43], thus having the important role of LT signaling in the communication between lymphocytes and stromal cells during lymphoid organogenesis. The role of LT in lymphoid neogenesis and maintaining lymphoid microarchitecture has been under investigation for almost two decades. To dissect the exact role of LT signaling in these processes, several knockout mouse models, such as $LT\alpha^{-/-}$, $LT\beta^{-/-}$, and $LT\beta R^{-/-}$ mice, as well as transgenic mice overexpressing LT in certain tissues, have been generated and extensively studied. Already in 1994 it was shown that $LT\alpha^{-/-}$ mice, devoid of $LT\alpha_1\beta_2$, $LT\alpha_2\beta_1$, and $LT\alpha_3$, lack Peyer's patches (PPs), mature follicular dendritic cells, peripheral and mesenteric lymph nodes (pLNs; mLNs) and show a massively disturbed splenic microarchitecture [44, 45]. $LT\beta^{-/-}$ mice, being able to express $LT\alpha_3$, show similar but slightly less severe effects on splenic microarchitecture and still developing cervical lymph nodes (cLNs) and mLNs [46–48].

Similar to $LT\alpha^{-/-}$ mice, $LT\beta R^{-/-}$ mice showed a complete absence of lymph node and PP development, indicating that the noncanonical NF-κB pathway is the most important signaling cascade in these biological processes [49, 50]. These studies are further supported by the investigation of Aly/Aly (alymphoplasia) mice, which show the same phenotype due to a point mutation in NIK rendering it unable to mediate the activation of $IKK\alpha_2$ [51].

Role of LT in Pathogen-Associated Immune Responses and Therapeutics

LT signaling plays a beneficial role in combating pathogens by triggering innate immune responses. While neutralizing LT in autoimmune disease seems to

Table 1. Role of LT signaling in innate immunity

Pathogen	Role of LT signaling in infection	Reference
Mucosal pathogens (e.g. *Citrobacter rodentium*)	LTβR signaling recruits neutrophils for host protection	115
Mucosal pathogen *Citrobacter rodentium*	Regulates IL-22 production by RORgt+ innate lymphoid cells	77
Mycobacterium tuberculosis and *Listeria monocytogenes*	Mice lacking LTβ are more susceptible to infection due to deficient macrophage activation	116
Leishmania major	Membrane-bound $LT\alpha_1\beta_2$ is important for host resistance	117
Leishmania donovani	T-cell-generated LTα is required for the control of parasite growth	118
Disease	**Role of LT signaling in therapeutics**	**Reference**
Autoimmune diseases	Targeted depletion of LTα-expressing TH1 and TH17 cells inhibits autoimmune disease	119
Diabetes	Absence of lymph nodes in NOD mice treated with LTβR-Ig ensures protection from diabetes	120
LPS-induced systemic shock	TNF/$LT\alpha^{-/-}$ mice are resistant to lethal endotoxemia induced by intravenous LPS injection	121
DSS-induced intestinal inflammation	Blocking LTβR worsens intestinal inflammation	122
Cerebral malaria	$LT\alpha^{-/-}$ mice were protected against cerebral malaria and did not develop perivascular cerebral hemorrhage	123

provide beneficial results in some cases, there are many reports of disadvantages of blocking LT signaling (table 1).

LT and the Development of the Gastrointestinal Immune System

In the gastrointestinal tract, the relevance of the LT system begins with embryonic development. During this stage, LT signaling contributes to the formation of mLN or PP. Both of these organized intestinal lymphoid follicles are part of the gut-associated lymphoid tissue (GALT), i.e. the immune system of the digestive tract, which contains up to 70–80% of antibody-producing immune cells of the body. The GALT system is made up of SLO such as PP and mLN, tertiary lymphoid tissues [isolated lymphoid follicles (ILFs)], and dispersed lymphocytes which lie intraepithelially in the mucosa and in the lamina propria of intestinal villi.

PP were first described in 1677 by the Swiss anatomist Johann Conrad Peyer. They are highly specialized lymphoid nodules, located in the intestine, more precisely on the one hand in the human small intestine where they are located along the antimesenteric gut in an irregular pattern or, on the other hand, in the distal ileum where they appear in vast numbers forming a lymphoid ring. PP are surrounded by follicle-associated epithelium which, through its M cells, is able to communicate with the underlying immune cells via transcytosis of bacteria, viruses, or luminal antigens. Whereas for the human embryonic development of PP no data are available so far, PP formation in mice gives more information about the developmental process. Starting from embryonic day 15.5 (E15.5), stromal cells express LTβR, the ligand of the tyrosine kinase receptor RET [52], and are in contact with cells expressing the cell adhesion molecule (VCAM)-1 [53]. This compact cluster of cells is further colonized by $RET^+CD11c^+cKit^+LT^+$ cells and $IL7R^+LT^+CD3^-CD4^+$ lymphoid tissue inducer cells [52–54] and starts producing IL-7 and the chemokine CXCL13 [55]. As a result of CXCL13 production, more $IL\text{-}7Ra^+CD4^+$ cells get recruited to PP-organizing centers where they express and upregulate the membrane-bound complex $LT\alpha_1\beta_2$ [56], resulting in induction of LTβ-LTβR signaling and PP development. As shown by various studies of mouse models, PP development requires interaction between the membrane-associated ligand $LT\alpha_1\beta_2$ and LTβR. Mice in which

the LTα gene [44] is knocked out are not able to form PP or any other lymph nodes as are mice in which LTβR signaling is blocked by a functional inhibitor of LTβ-LTβR (LTβR-Ig) or by an anti-LTβ monoclonal antibody (mAb) [50]. However, mice deficient in the T-cell-derived ligand LTβ share a phenotype similar to that of LTα knockout mice but still possess both cLNs and mLNs [47].

The second lymphoid tissue formed in the gastrointestinal tract are mLNs. mLNs were already described in the second and first centuries BC by the Roman physician Rufus of Ephesus [57]. They are the largest lymphoid tissues among all GALT and develop before other gut-associated lymphoid aggregates at E12.5. Formation of mLNs is similar to PP development, except that the TNF superfamily member TRANCE rather than IL-7 is critical for the activation of LTi subpopulations [58]. Additionally, mice deficient in LTβ still develop mLN [47] and mice with transient blocking of the LTβ-LTβR signaling pathway by administration of LTβR-Ig still start initiating mLN formation at ~E11.5 [59].

In addition to SLOs, the gastrointestinal tract hosts tertiary lymphoid tissues that are environmentally induced and not programmed. ILFs were first described in 2002 by Hamada et al. [60]. Smaller than PP, they nevertheless play a crucial role due to their large number. About 100–200 ILFs are located in the murine small intestine, which form only in the first 2 weeks after birth as defined B cell follicles surrounded by dendritic cells. Similarity to cellular interactions necessary for PP and mLN development was shown using LTα- or LTβR-deficient mice [60, 61]. As was observed in BM chimeras, ILFs could only start developing when LTα was expressed on donor hematopoietic cells and LTβR on host cells. Some data suggest that ILF are subsequently formed from cryopatches (CP). CP, which are aggregates of immature T cell precursors, got their name through their localization between the crypts. Regarding the development of CP, only data from mice are available because clinical colon samples derived from patients are in general already exposed to commensal bacteria. The conversion of CP into ILFs seems to be easily comprehensible since it was suggested by Bouskra et al. [62] and Eberl et al. [63] that CP are clusters of LTi cells leading to an induction of ILF formation during intestinal colonization of bacterial commensals. The development of CP, which takes place in the second week after birth, was shown to be LTα dependent [63] and blocking of the LTβR signaling pathway by LTβR-Ig led to CP development failure and subsequent failure of IFL formation [62, 64].

LT and Gastrointestinal Immune Homoeostasis

The human gut hosts about 100 trillion microbial cells and thus has to handle an immense bacterial load which serves mostly beneficial functions in interaction with the host [65]. Still, it was shown that these commensal bacteria can also be correlated with the development of diseases such as inflammatory bowel disease or ulcerative colitis. Under normal conditions, IgA, the most abundant immunoglobulin isotype of the gut, provides protection against mucosal pathogens [66] by control of bacterial growth and prevention of adhesion to the intestinal epithelium [67, 68]. As a reaction to the pathogen, IgA B cells start migrating from the PP to the mLN and then, following differentiation to IgA-secreting cells, home under the influence of chemokines to the intestinal lamina propria [69]. The theory of a link between PP or mLN and therefore LT and IgA production has been extensively investigated [70–72]. $TNF^{-/-}$ mice develop only rudimentary PP but nevertheless are able to produce a normal IgA level. In contrast, $LT\alpha^{-/-}$ mice display a reduced amount of IgA, suggesting that the interaction between $LT\alpha_1\beta_2$ and LTβR is essential for IgA expression [73]. This theory was additionally supported by analysis of $LT\beta^{-/-}$ and $LT\beta R^{-/-}$ mice that also have lower IgA production. Further data from Kang et al. [73] and Fagarasan et al. [67] demonstrated the role of the LT-conditioned lamina propria in managing the support of IgA production independently of PP and mLN but in the presence of very recently IgA-switched B cells in the intestine.

LT also seems to play a role in the inflammatory process. Agyekum et al. [74] showed that patients with ulcerative colitis have strong expression of LTβ in colon samples. They could also show a similar pattern of $LT\beta^+$ cells in sections of ileum from patients with Crohn's disease. In both diseases LTβ expression was mostly increased on plasma cells and a subset of $CD4^+$ lymphocytes located in the lamina propria, whereas $CD8^+$ cells did not express LTβ. In a follow-up study they were able to show that LTβR activation by $LT\alpha_1\beta_2$ has a crucial effect on downregulation of the inflammatory process [75]. The Gram-negative bacterium *Citrobacter rodentium,* a natural mouse extracellular enteric pathogen, is widely used to analyze molecular mechanisms in gut immune responses. Since the infection mimics a human infectious colitis induced by enteropathogenic *Escherichia coli* or enterohemorrhagic *E. coli,* it is an attractive animal model. In the study of Spahn et al. [64] blocking of LTβR by the fusion protein LTβR-Ig led to increased severity of the

infection, indicating that signal transduction between membrane-bound $LT\alpha_1\beta_2$ and LTβR plays a critical role in the host defense against *C. rodentium*. This was further proven by LTβR-deficient mice in which early severe pathology was observed [64]. With respect to IL-22, which is essential for the host response after mucosal bacterial infection [76], Tumanov et al. [77] showed that control of intestinal IL-22 production is accomplished by LTβR stimulation. Concurrently, expression of IL-22 in WT mice was considerably stronger compared to that in $LT\beta R^{-/-}$ mice.

Whether signaling via the LTβR pathway in the host's immune response is actually of importance or not depends on the respective pathogens. Whereas *C. rodentium* infection showed a severe progress of disease in $LT\beta R^{-/-}$ mice, it was observed that, for example, *Salmonella enterica* serovar Typhimurium infection did not differ in pathogenesis when C57BL/6 mice were compared with $LT\beta R^{-/-}$ mice. This indicates that the progress of the disease is LTβR independent [78]. *S. enterica* leads to nonsystemic enterocolitis in cattle and humans but is not inducing the same pathogenesis in mice. Barthel et al. [78] used a streptomycin-pretreated mouse model that when infected with *S. enterica* colonizes the lower intestine and develops colitis. Analyzing streptomycin-pretreated $LT\beta R^{-/-}$ mice which lack GALT also indicated an initiation of systemic infection where PP and mLN are dispensable.

LT and Cancer

Besides its well-known function in the development and maintenance of lymphoid organs and its function in gastrointestinal immune function, LT-mediated signaling has been linked to cancer development in different studies (table 2).

Genetic Studies

Genetic studies in humans identified a polymorphism in the LTα gene locus associated with lower gene expression [79]. In a homozygous state this was associated with a lower risk for high-grade bladder tumors and a relative risk in human patients, whereas it was associated with a poor prognosis in diffuse large B-cell lymphoma [80, 81]. For lung cancer and endometrial cancer, a heterozygous state was associated with a lower incidence compared to individuals carrying the homozygous, low expressing allele [82, 83]. These variations may be explained by the pleiotropic functions of LT signaling and tissue-specific interactions with other intracellular signaling pathways. LTβR gene amplification was frequently found in nasopharyngeal carcinoma, a distinct type of head and neck cancer, due to an amplification of chromosome 12p13.3. In vitro studies showed a direct influence of LTβR-induced NF-κB signaling on tumor cell proliferation, indicating a potential oncogenic role of LTβR signaling in this cancer type [84].

LTβR-Mediated Signaling and Cancer

Deregulated NF-κB signaling due to mutations in its regulators can be found in different forms of B-cell lymphoma. For multiple myeloma, gain-of-function mutations in activators of NF-κB signaling like for example LTβR and inactivating mutations of negative regulators could be found [85]. Keats et al. [86] could show in patient samples as well as in cell lines that ligand-independent activation of the noncanonical NF-κB signaling pathway is the limiting step of malignant plasma cell transformation. Current findings in adult T-cell leukemia – a lymphoma characterized by constitutive NF-κB signaling activity – add further complexity by showing the involvement of micro-RNAs in the regulation of noncanonical NF-κB signaling in the transformation of these lymphoma cells but also in further cancer types [87].

The direct transforming activity of LTβR could be shown by Fujiwara et al. [88] in transformation assays using a cDNA library generated from a pancreatic ductal carcinoma. An N-terminal truncated LTβR form but also a full-length protein was able to induce in vitro cell growth of 3T3 cells in soft agar and tumor formation in nude mice.

In 2010, Ammirante et al. [14] could show that LT produced by tumor-infiltrating B cells induces IKKα activation and Stat-3 phosphorylation, leading to androgen-free survival in castration-resistant prostate cancer cells. In former studies, IKKα-driven downregulation of Maspin expression could be shown to be decisive for primary tumor growth and metastasis in prostate and breast cancer models [89]. In these studies, IKKα activation was linked to RANK-mediated NF-κB activation due to RANKL expression by infiltrating T lymphocytes [89, 90]. Nevertheless, castration resistance could be solely referred towards B-cell-derived LT as RANKL expression was not altered after castration and only LT knockout in B cells delayed the regrowth of prostate cancer cells after castration [14].

Receptors of the TNF superfamily not only activate NF-κB signaling but are also known to induce cytotoxicity in tumor cells. Therefore, also LT has been tested for this abil-

ity in different tumor cell lines. A cytotoxic effect of agonizing LTβR treatment could be shown in different adenocarcinoma cell lines alone or in combination with IFN-γ [91]. Lukashev et al. [92] showed not only a cytotoxic effect in vitro but also inhibition of tumor growth and enhanced sensitivity to chemotherapeutic agents in xenograft models. This may be due to the role of LTβR signaling in regulation of cell survival and apoptosis as well as its function in cellular proliferation and cell cycle regulation [93, 94]. Still, not all tumor cell lines or not all orthotopic xenografts derived from different surgical colorectal carcinoma samples respond to LTβR agonization [92].

Novel Effects of LT-Mediated Signaling in Cancer Promotion and Metastasis

It could be demonstrated by Ito et al. [95] that LT-defective NK cells show defects in maturation and homing leading to improper antitumor surveillance and more rapid tumor growth and metastasis. In contrast, Zhou et al. [96] showed in a spontaneous prostate cancer model that targeted LT ablation in T cells rescued the antitumor response by inhibiting clonal deletion of tumor-specific T cells. This decreased tumor incidence and inhibited metastasis. Due to these immune modulatory functions viruses may abuse LT-induced signaling pathways for their purposes. Human papillomavirus 16 E6 protein was recently proposed to induce LT and LTβR expression in cervical epithelial cell lines by downregulation of MHC class I molecules, thereby suppressing cytolytic antiviral responses [97]. Nevertheless, a clear demonstration of the underlying pathways is still lacking. A special role for LT as a niche-forming cytokine for T-cell lymphoma in the Eμ-Myc transgenic mouse model could be shown by Rehm et al. [98]. These lymphoma cells home via CCR7 into the bone marrow, where they alter stromal cellularity by activating LTβR signaling on residing stromal reticular cells. Abrogation of this interaction inhibits lymphoma growth in the bone marrow, making LT a potential drug target in lymphoma therapy.

LTβR signaling ablation in fibrosarcoma cells lead to tumor growth inhibition and a block in angiogenesis [99]. In further studies, it was shown that LTβR ligands LT and LIGHT expressed by tumor infiltrating lymphocytes lead to upregulation of the proangiogenic chemokine CXCL-2 in fibrosarcoma cells [100]. Therefore, LT also plays a tumor-intrinsic role for organization of tumor tissue during tumor development.

Tumor surveillance by the immune system is of the highest importance in the field of immunology. LT and its related downstream signaling via the NF-κB pathways have been shown to influence tumor growth and metastasis in a variety of models and via a variety of mechanisms. Still, the question remains of what role LT or other members of the TNF superfamily might have in tumor development and promotion. Kuprash et al. [101] therefore investigated whether complete abolishment of TNF and LT signaling in the $p53^{-/-}$ spontaneous tumor formation mouse model has an influence on the frequency of tumor formation. As $p53^{-/-}$ mice lacking TNF or LT or both signaling pathways do not show any significant difference in spontaneous tumor formation besides a slight delay in tumor-associated mortality, the authors concluded that inflammatory signaling has no protective role in tumor development and has only a minor role in tumor promotion. However, these experiments were performed with conventional and not with hematopoietic cell-specific knockout mice, leaving the question of tumor cell-intrinsic effects of TNF- or LT-mediated signaling.

LT Function in Liver Injury and Regeneration

LTβR signaling functions as a two-edged sword in the liver. It has been shown to deliver signals that regulate various liver functions in normal physiology but can also lead to chronic hepatitis and other severe forms of liver damage when chronically activated in a disease state. A model of liver regeneration after 70% partial hepatectomy showed that the T-cell-derived LT axis directly regulates liver regeneration [102, 103]. In addition, using a murine model of chronic liver injury, Ruddell et al. [104] found that LTβR on hepatic stellate cells is required for wound healing and regeneration. LTβR signaling has been demonstrated to be an important mediator of the pathogenesis of dyslipidemia in a LIGHT transgenic model, where hepatic gene expression was reprogrammed by T-lymphocyte-derived LIGHT [105].

Production of a variety of proinflammatory cytokines (e.g. TNF) plays an important role in hepatocellular damage. Accumulating evidence suggests that the activation of innate immunity also stimulates the production of LT during various liver injuries, such as chronic hepatitis B or C viral infections and alcohol abuse. LIGHT has recently been shown to be upregulated and to function as a proinflammatory cytokine in experimental hepatitis models, through interaction

with LTβR but not herpes virus entry mediator [106]. Moreover, pharmacological blockade of LTβR signaling by LTβR-Ig treatment significantly attenuates the progression of autoimmune hepatitis induced by intravenous administration of concavalin A (ConA), and reduces the production of inflammatory cytokines including TNF, IFN-γ, and LIGHT [107].

LT and Viral Liver Infections

Modulation of the LTβR signaling pathway by direct interaction of HCV core protein with LTβR was discovered 15 years ago [108, 109]. The role of LTβ in liver pathology was also identified in human patients with hepatitis C. In chronic HCV infection, LTβ expression is observed in multiple hepatic cell types, including oval cells, inflammatory cells, and small portal hepatocytes. LTβ expression is significantly increased especially when fibrosis or cirrhosis are present [110]. The role of LTβR signaling in HCV replication has further been confirmed by a high-throughput siRNA library screen performed by Ng et al. [111]. Silencing of gene members of the TNF/LT signaling pathway, including LTβ, TRAF2, RelA, and NF-κB2, resulted in inhibition of HCV replication by more than 60%. Besides, LTα was also shown to be upregulated under transient HBV X protein expression, mediated by the activation of NF-κB. In addition, treatment of LTα neutralizing antibodies reduced HBx-induced NF-κB activation in a dose-dependent manner, which indicates that upregulated LTα may be involved in HBx-induced NF-κB activation [112]. Most recently, using a GFP expressing adeno-associated virus (AAV) vector, Washburn et al. [113] found that forced expression of LIGHT by additional adenoviral transduction in the liver led to liver inflammation and clearance of the AAV infection, with enhanced CD8 effector T-cell levels. Interestingly, in LTβR$^{-/-}$ mice, expression of LIGHT still cleared AAV but caused no significant liver inflammation, which indicates that LIGHT interaction with LTβR plays a critical role in liver inflammation but is not required for LIGHT-mediated AAV clearance.

LT and HCC

Viral hepatitis and alcoholic liver disease can evolve into chronic disease, cirrhosis, and/or HCC. Chronic inflammation can lead to tissue remodeling through cell growth, apoptosis and/or necrosis and induction of oxidative stress. However, the molecular mechanisms of hepatitis-induced HCC remain unclear. Recently, our group uncovered the link between LTβR signaling and the development of chronic hepatitis and HCC [114]. We found aberrant hepatic expression of LTα and β and LTβR in human samples with HBV- or HCV-induced chronic hepatitis and HCC. On the contrary, TNF was only slightly increased in HBV-induced hepatitis but not in HCV-induced hepatitis and HCC. Upregulation of *LTα, LTβ,* and *LTβR* mRNA was not associated with the degree of liver inflammation, fibrosis, patient age, gender, HCV genotype, or type of virus infection. Moreover, upregulation of LT ligands and receptors seemed to be specific for HBV- or HCV-induced liver diseases, because transcript levels in nonviral liver diseases, including liver disorders with hepatitis (alcoholic steatohepatitis, cholestasis, primary biliary cirrhosis, and end-stage liver cirrhosis due to alcoholic liver disease), liver diseases without inflammation (steatosis and focal nodular hyperplasia), and other liver diseases (hemochromatosis, Wilson's disease, focal liver fibrosis, and nonviral HCC) showed significantly lower levels of LT ligands and receptors. This upregulation of components of the LTβR signaling pathway upon viral infection was further confirmed in a human hepatocyte cell line in vitro. After infection of HCVcc, the human hepatocyte cell line Huh-7.5 expressed elevated levels of LTα, LTβ, LIGHT, LTβR, and chemokines compared with noninfected Huh-7.5 cells. In order to identify which cell type is responsible for the upregulation of *LTα, LTβ, LTβR,* and *LIGHT* in human HCV-infected livers, liver cells were collected from HCV-induced hepatitis and HCC and sorted according to their CD45 surface expression, resulting in CD45-enriched (T and B cells; monocytes, macrophages, and Kupffer cells, and dendritic and NK cells) or CD45-depleted (hepatocytes, oval cells, and bile duct epithelial and endothelial cells) fractions. Surprisingly, similar to CD45-enriched cells, CD45-depleted cells showed an upregulation of *LTα, LTβ,* and *LIGHT* in HCV-induced hepatitis and HCC compared to healthy liver controls. Moreover, *LTβR* mRNA expression was significantly higher in CD45-depleted cells than in CD45-enriched cells. Immunohistochemical analysis for LTβ expression in healthy, HBV- or HCV-infected, and HCC-affected livers further corroborated these data. But, is sustained hepatic LTβR signaling causally linked to chronic hepatitis and HCC development? Two transgenic mouse lines that expressed LTα and β in a liver-specific manner were analyzed in our study. At the age of 4 months, no obvious

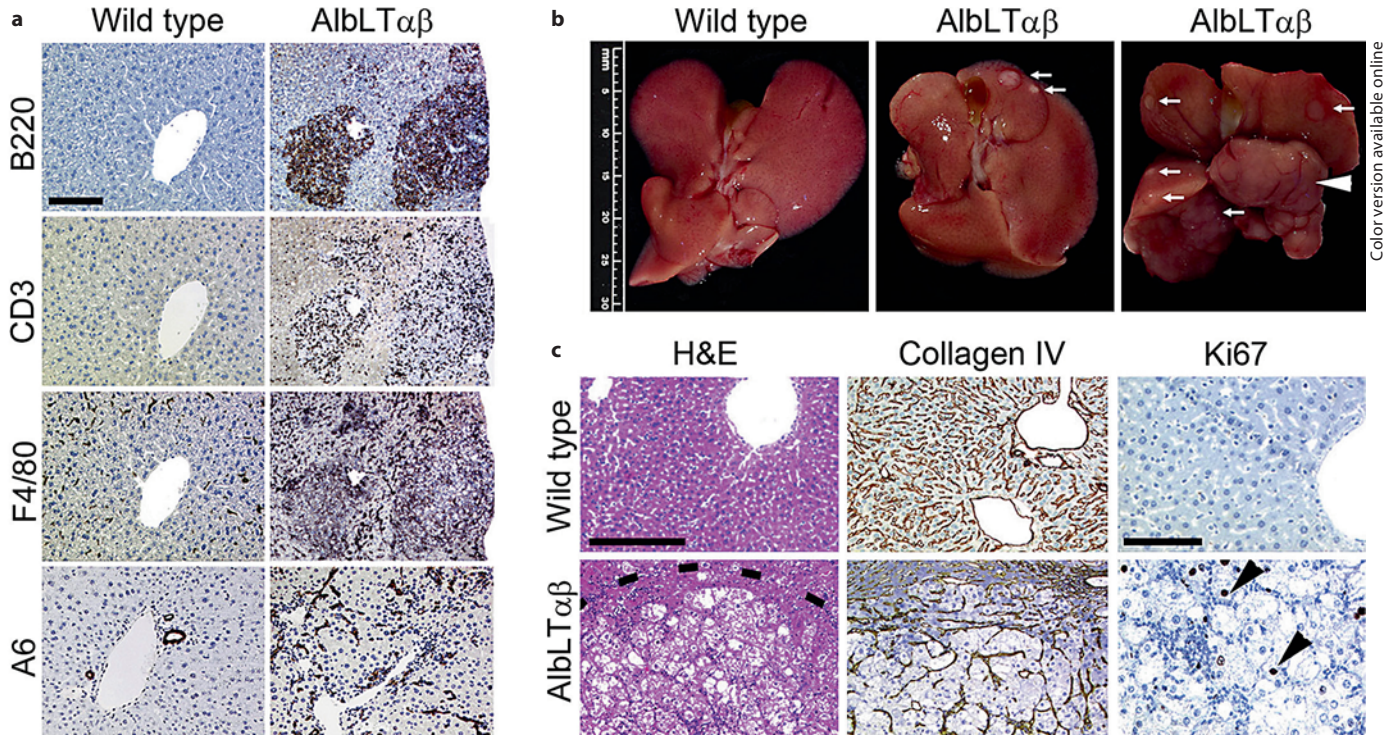

Fig. 2. Chronic inflammation and HCC development in AlbLTαβ mice. **a** Immunohistochemical analysis of representative 9-month-old C57BL/6 and AlbLTαβ livers. B220$^+$-stained B cells, CD3$^+$ T cells, F4/80$^+$ macrophages, Kupffer cells, and A6$^+$ oval cells. Scale bar = 150 μm. **b** Macroscopy of C57BL/6 (left panel) and AlbLTαβ livers at the age of 12 (middle panel) and 18 months (right panel). White arrows indicate tumor nodules. The white arrowhead indicates a liver lobe completely affected by HCC. The scale bar size is indicated. **c** Histological analysis of livers derived from C57BL/6 and AlbLTαβ mice at the age of 12 months. The dashed line depicts the HCC border. Collagen IV staining highlights the broadening of the liver cell cords and loss of collagen IV networks indicative of HCC in AlbLTαβ mice. Scale bar = 200 μm. High numbers of Ki67$^+$ proliferating hepatocytes (arrowheads) are only found in AlbLTαβ HCC (right column; scale bar = 100 μm). Adapted from Haybaeck et al. [114].

histological difference was found between AlbLTαβ transgenic mice and control littermates. However, at this stage, a cluster of genes showed dramatic expression changes, as revealed by DNA-microarray analysis and real-time PCR. For example, chemokines and genes involved in early growth response, cholesterol metabolism, and immediate early response (e.g. *c-Fos, Jun-b,* and *Socs-3*) were significantly elevated. Meanwhile, genes involved in cell cycle control, histone modifications, and cell metabolism were significantly downregulated. At the age of 4–6 months, portal and lobular inflammation, consisting of CD4$^+$, CD8$^+$ T cells, B220$^+$ B cells, and CD11c$^+$ dendritic cells, was found to be initiated in AlbLTαβ transgenic livers. From 9 months on, AlbLTαβ transgenic mice exhibited strong hepatitis, characterized by strong portal and lobular lymphocytic infiltrates and proliferation of A6$^+$ oval cells (see also fig. 2). Increased proliferation of Ki67$^+$ hepatocytes was also observed at this stage. Chronic inflammation coincided with elevated protein levels of IL-1β, IFN-γ, IL-6, and TNF. Chronic hepatitis in AlbLTαβ transgenic mice further leads to hepatotoxicity, characterized by significantly enhanced serum aminotransferase levels (ALT and AST) and more frequent apoptotic hepatocytes in the liver. Hepatitis persisted in the transgenic mice up to 18 months of age and approximately 35% of transgenic mice developed HCC, which is histologically characterized by broadening of liver cell cords, loss of collagen IV networks, and increased proliferative activity and is immunohistochemically characterized by evaluated expression of human liver tumor markers (Golgi protein 73, glutamine synthetase, and a-fetoprotein). We further analyzed the chromosomal aberrations by array comparative genomic hybridization analysis (aCGH). HCC from different

Table 2. Overview of lymphotoxin-associated cancer studies

Phenotype	Outcome	Disease	Reference
Human patients			
LTβR gene amplification	Procarcinogenic	Nasopharyngeal carcinoma	84
LTα (AA) polymorphism low-expression	Tissue-dependent		80–83
Constitutive-active noncanonical NF-κB	Procarcinogenic	Multiple myeloma; T-cell lymphoma	85–87
Mouse models			
Hepatocyte-specific overexpression of $LT\alpha_1\beta_2$ (AlbLTαβ mice)	Procarcinogenic	HCC	114
B-cell-derived LT in prostate cancer	Procarcinogenic	Promotion of castration resistance	14
IKKα-mediated Maspin suppression	Procarcinogenic	Enhanced tumor growth and metastasis	89, 90
Defective NK-cell maturation and homing in LTα-deficient mice	Procarcinogenic	Enhanced tumor growth and metastasis due to lack of tumor surveillance	95
LT-induced clonal deletion of tumor-specific T cells in Tag-I/TRAMP mice	Procarcinogenic	Enhanced tumor growth and metastasis due to lack of antitumor T-cell response	96
LT-induced formation of a lymphoma-permissive niche in the bone marrow	Procarcinogenic	Enhanced lymphoma cell survival and progression	98
LT-induced proangiogenic CXCL2 expression	Procarcinogenic	Promotion of tumor growth and tumor angiogenesis	99, 100

individual AlbLTαβ transgenic mice showed varied patterns of chromosomal aberrations.

Could overexpression of LT itself contribute to liver tumorigenesis? To investigate the mechanisms potentially involved in the progression of chronic hepatitis and HCC in AlbLTαβ transgenic mice, we intercrossed the mice with various knockout mice: (i) *Tnfr1*$^{-/-}$ mice, to investigate whether ablation of TNFR1 signaling would prevent chronic hepatitis and HCC formation in AlbLTαβ mice; (ii) *Rag1*$^{-/-}$ mice, which lack mature lymphocytes, and (iii) *Ikkβ*$^{\Delta hep}$ mice, which deplete functional NF-κB signaling in a hepatocyte-specific manner. As a result, intercrossing with *Rag1*$^{-/-}$ mice or *Ikkβ*$^{\Delta hep}$ mice prevented hepatitis and HCC, indicating that LT-induced HCC formation is dependent on both lymphocytes and NF-κB signaling. In contrast, AlbLTαβ/*Tnfr1*$^{-/-}$ mice displayed HCC incidence similar to that in AlbLTαβ mice, suggesting that TNFR1 signaling is not involved in the progress of LT-induced carcinogenesis.

To identify the major LT-responsive liver cell type, TNF or agonistic LTβR antibody (3C8) was administrated to IKKβ$^{\Delta hep}$ mice intravenously. Nuclear p65 (RelA) translocation was not detectable in hepatocytes under TNF treatment, although NF-κB target genes were found to be elevated, presumably through TNF-activated nonparenchymal cells (NPC). However, upon 3C8 administration, IKKβ$^{\Delta hep}$ livers were devoid of nuclear p65 translocation in hepatocytes and NPC, as well as upregulation of NF-κB target genes. This finding suggested that hepatocytes are the major cell type which integrates LTβR signaling in the liver. In contrast, treatment with 3C8 resulted in upregulation of selected NF-κB target genes in IKKα$^{AA/AA}$ livers, indicating that the canonical NF-κB pathway was involved in LTβR-induced hepatic signaling.

We further investigated whether inhibition of LTβR signaling would reduce chronic hepatitis and HCC formation. Administration of LTβR-Ig in 9-month-old AlbLTαβ transgenic mice for 2 months significantly reduced chronic hepatitis incidence and prevented chronic hepatitis-driven carcinogenesis. Our results introduce blocking LTβR signaling as a novel strategy for HCC chemoprevention and antifibrotic treatment in the context of sustained hepatic LTβR signaling. However, potential side effects of long-term LTβR treatment should also be evaluated in the future. The above pharmacologic study also confirmed LTβR signaling as key player in chronic hepatitis and HCC development.

Conclusions

The examples from human patients and mice described in this review indicate that cytokines can play an important role in the development of cancer. The exact underlying pathways and whether these cytokines induce a particular cellular environment (e.g. polarization of particular cell types) or directly act as oncogenes has still to be defined and analyzed.

Therefore, further experiments, establishment of novel mouse models, and analysis of human material with respect to particular organs will be needed to unambiguously identify those cancer types in which cytokines play a protumorigenic or an antitumorigenic role.

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