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Survey

Biology and signal transduction pathways of the Lymphotoxin-αβ/LTβR system

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ABSTRACT

This review focuses on the biological functions and signalling pathways activated by Lymphotoxin α (LTα)/Lymphotoxin β (LTβ) and their receptor LTβR. Genetic mouse models shed light on crucial roles for LT/LTβR to build and to maintain the architecture of lymphoid organs and to ensure an adapted immune response against invading pathogens. However, chronic inflammation, autoimmunity, cell death or cancer development are disorders that occur when the LT/LTβR system is twisted.

Biological inhibitors, such as antagonist antibodies or decoy receptors, have been developed and used in clinical trials for diseases associated to the LT/LTβR system.

Recent progress in the understanding of cellular trafficking and NF-κB signalling pathways downstream of LTα/LTβ may bring new opportunities to develop therapeutics that target the pathological functions of these cytokines.

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1. Genomic organization and tissue specific gene expression

In the human genome, Lymphotoxin α (LTα) and Lymphotoxin β (LTβ) genes are located on both sides of the gene encoding TNFα (Fig. 1). These genes form a cluster within the major histocompatibility complex class III region on chromosome 6 [1]. This genetic organization and linkage is conserved on mouse chromosome 17. Only 1 kb separates the polyadenylation site of LTα mRNA from the 5′ end of TNFα mRNA in the mouse genome. The close proximity of these genes and the targeting vector strategy used by different research group may explain the phenotypic discrepancies they observed (see chapter 5). TNFα mRNA is expressed in hematopoietic as well as in non-hematopoietic tissues. Two main mechanisms account for the synthesis and stability of TNFα mRNA. On one hand, putative cis-regulatory elements in the 5′ untranslated region (UTR) like NF-κB, PU.1 (purine-rich box), a cyclic AMP

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Ligands

Receptors

Fig. 1. Human genomic organization of genes coding for LTα, LTβ, TNFα and LIGHT and their receptors.

response element (CRE), ATF-2, c-jun/AP-I, AP-2, SP-1, Krox-24 and NF-AT (nuclear factor-activated T cells), contribute to its level of expression. On the other hand, the 3′ UTR contains an AU-rich element (ARE), which is known to control posttranscriptional regulation of TNF gene expression by destablizing the mRNA and interfering with translation.

As opposed to TNFα, LTα, LTβ and LIGHT mRNA display a more restricted pattern of expression. LTα and LTβ mRNA are expressed in hematopoietic cells, such as mature B, T and NK cells, whereas LIGHT is mainly expressed on activated T cells [2]. Characterization of the regulatory regions of LTα revealed several NFAT binding site that allow PBMC to upregulate the transcript in response to PMA/ionomycin or anti-CD3/CD28 [3]. Early studies identified TNFα and phorbol ester PMA as inducers of LTβ in human T cells. Mutational analysis revealed a role of the transcription factors Egr-1, Sp1, NF-κB and Ets for the induction of LTβ mRNA [4,5]. Both LTα and LTβ have also been detected in hepatocytes. In hepatocytes, LTα is upregulated upon HBV infection through activation of NF-κB [6,7]. In addition, LTβ is also upregulated through IL-6 and IL-1β-induced NF-κB and Ets-1 activation [8].

LTβ is also expressed in developing hair follicle in response EDA signalling [9].

Some negative regulatory mechanisms have also been described. For instance, in B cells, LTα transcription is repressed in an allelic-specific manner by B cell-factor 1 (ABF1) [10]. Likewise, recruitment of the transcription factor YY1, CREB and CTCF to a regulatory region of the fourth exon of LTβ is associated with transcriptional repression [11].

TNFR1 expression is ubiquitous and constitutive. However, type I and type II interferons, particular interleukins and TNFα itself are, among others, known inducers of TNFR1 expression. In contrast, TNFR2 expression is more restricted to cells of the immune system, such as B and T lymphocytes, macrophages, but also epithelial cells of the gut, neurons and some cancer cell lines. Cis-regulatory elements in the 5′ UTR of TNFR2 such as T cell factor 1 (TCF-1), Ikaros, AP-1, IL-6 receptor E (IL-6RE), ISRE, GAS, NF-κB, and SP-1 integrate most of the signalling pathways regulating TNFR2 inducibility [12,13]. The gene encoding LTβR is located near TNFR1 on human chromosome 12. The promoter region of LTβR resembles that of a housekeeping-like gene and lacks TATA and CAAT sequences. LTβR is constitutively expressed in the stroma of secondary lymphoid organ and of the thymus, and in myeloid cell lineage [14]. However, during mouse embryogenesis its mRNA level of expression is elevated in epithelial layers of various tissues [15]. HVEM mRNA, as opposed to LTβR, is constitutively expressed on B and T cells and its level of expression can be further increased upon PMA or TNF activation [16]. TROY is expressed in developing hair follicles and acts as a co-receptor with NgR1/LINGO1 in repression of axonal regeneration [17].

2. Protein structure and cross reactivity of LTαβ/TNFα and their receptors

2.1. Structure of the ligands

Most members of the TNF ligand family are type II transmembrane proteins (N-terminal side facing the intracellular compartment). Full-length TNFα is expressed at the cell surface (mTNFα) but can be shed by proteases to secrete soluble TNFα (sTNFα) (see chapter 3). LTα is devoid of transmembrane domain as opposed to its homolog LTβ. LTα self-associates to form homotrimers $LT\alpha_3$, or binds one or two LTβ ligand to form $LT\alpha_2\beta_1$ and $LT\alpha_1\beta_2$ heterotrimers, respectively (Fig. 2). However, $LT\alpha_2\beta_1$ is a minor form that only represents less than 2% of total LTβ in activated T cells [18]. LIGHT is also exclusively expressed at the cell surface.

The primary structure of TNFα and LTα contains eight β strands (A to H) that fold into an antiparallel β sheet sandwich, which allow the formation of trimeric TNFα or LTα [19–21]. There are also conserved residues preceding the loops connecting the β strands. Two of these loops, A–A″ and D–E, contain residues important for receptor binding that are D50 and Y108, respectively.

2.2. Structure of the receptors

TNFR1, TNFR2, TROY, HVEM and LTβR are type I transmembrane glycosylated proteins consisting of an extracellular domain (ECD), a transmembrane domain (TMD), and an intracellular domain (ICD). They all contain four cysteine-rich domain (CRD) in the ECD domain that are more or less conserved. These CRD confer the specificity and the affinity for cognate ligands. X-ray crystal structure of the ECD of TNFR1 revealed that three receptor molecules bind a trimer of LTα [22].

These receptors diverge essentially in their ICD domain and are subdivided as death and the non-death TNF receptor families. TNFR1 is the prototype of the TNF death receptor family and displays a single death domain that is important to mediate NF-κB activation as well cell death (see chapter 4). TNFR2, TROY, HVEM and LTβR are non-death receptors. They do not contain a death domain but rather one or two TRAF (TNF Receptor Associated Factor) binding sites [23–28] (Fig. 2). These sites mediate activation of downstream signalling cascades controlling the transcription factors NF-κB or AP1.

TNFR1 and TNFR2 contain in their ECD a pre-ligand-binding assembly domain (PLAD) that is distinct to the domain that mediates the binding of TNFα or LTα [29]. This domain confers the ability to TNFR1 and TNFR2 to preform a trimer in the absence of ligand. Deletion of the PLAD domain interferes with the binding of TNFα, indicating that the ligand cannot assemble a trimer of TNFR1 or TNFR2. TROY, HVEM and LTβR are devoid of PLAD domain and require their ligand to assemble as functional trimers.

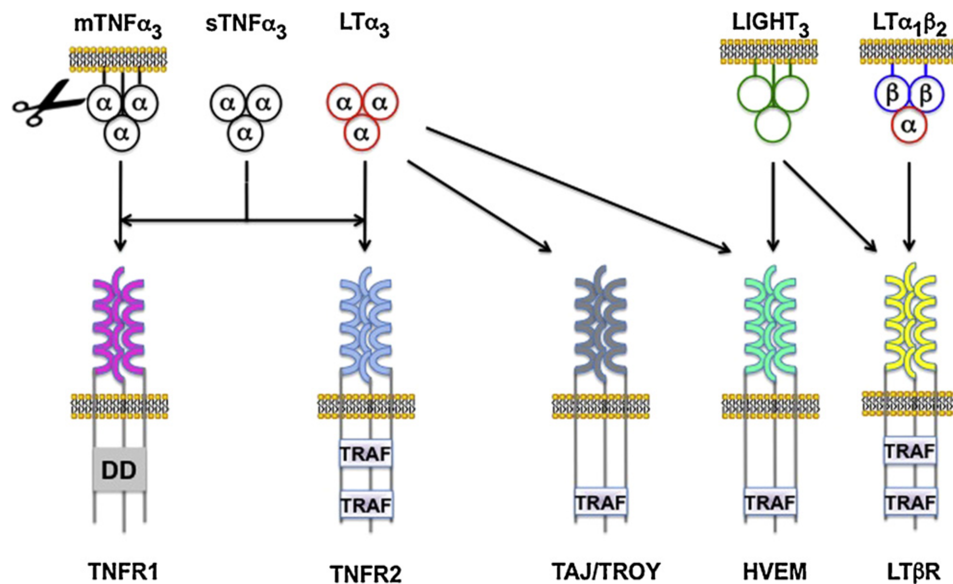

Fig. 2. Structure and binding of LTα, LTβ, TNFα and LIGHT and their receptors. Membrane bound trimeric TNFα ($mTNF\alpha_3$) binds TNFR2 with higher affinity than TNFR1, whereas soluble trimeric TNFα ($sTNF\alpha_3$) displays a higher affinity for TNFR1 than TNFR2 [30]. $LT\alpha_3$ exists only as soluble form and binds TNFR1 and TNFR2 with equal affinity. TROY is a recently identified LTα receptor [23,28]. LIGHT binds HVEM and LTβR, while $LT\alpha_1\beta_2$ is restricted to the binding of LTβR. DD depicts a death domain, and TRAF, a TRAF binding site.

2.3. Specificity of the interaction between ligands and receptors

Soluble TNFα (sTNFα) and membrane anchored-TNFα (mTNFα) bind to TNFR1 and TNFR2. However, mTNFα preferentially binds TNFR2 [30]. Specificity can be also modulated by mutagenesis of critical residues either in the amino-terminal or carboxy-terminal part of TNFα. Indeed, mutated R32W/S86T TNFα binds to TNFR1, whereas mutated D143N/A145R exclusively binds to TNFR2, suggesting that different subdomains of TNFα modulate its specificity towards TNFR1 or TNFR2 [31,32].

Similarly, LTα displays some specificity towards its receptors TNFR1, TNFR2 and LTβR. Indeed, mutation D50N in the A–A″ loop or Y108F in the D–E loop of LTα does not prevent the generation of $LT\alpha_3$ but abrogates its binding to TNFR1 and TNFR2. In contrast, these mutant forms assemble into an active $LT\alpha_1\beta_2$ and triggers LTβR biological functions [33].

TROY also binds LTα but its affinity regarding mutants LTα has not been addressed [17]. LIGHT binds two receptors, HVEM and LTβR [2,34]. Again, specificity is achieved by specific amino acids located in different loops of the ligand [35]. Tyrosine Y173 is the analogue of Y108 of LTα. Mutation Y143F causes a decrease of binding of HVEM and LTβR. In contrast, mutant G119E is fully altered for the binding to LTβR whereas it conserved a residual affinity for HVEM.

In contrast to LIGHT, $LT\alpha_1\beta_2$ heterotrimers have, so far, a unique partner that is LTβR [36]. Interactions between mouse and human TNF ligands and specie specific TNFR have been extensively studied in vitro and in vivo and revealed some cross-reactivity for the lymphotoxin system [37].

3. Shedding and exocytosis: two important steps for the control of LTα/TNFα and TNFR1/2 biological activity

Shedding and exocytosis of TNFL and TNFR are important mechanisms involved in developmental processes, inflammatory responses and apoptosis. Shedding is the task of a family of metalloproteases, named sheddases. ADAM 17 (a disintegrin and metalloproteinase) was first identified to shed membrane-associated TNFα (mTNFα) into soluble TNFα (sTNFα), giving the alternate name TACE (TNF-α converting enzyme) [38,39] (Fig. 2). The rate of TNFα shedding must be tightly controlled to avoid accumulation of detrimental sTNFα over beneficial mTNFα (see Van Hauwermeiren et al., in this issue). Shedding of $LT\alpha_1\beta_2$ was reported in patients suffering of rheumatoid arthritis and release of $LT\alpha_1\beta_2$ was associated with pro-inflammatory cytokines that contribute to synovitis. In this case, $LT\alpha_1\beta_2$ is shed by TACE but also MMP8 [40]. Conversely, shedding of membrane anchored-LIGHT was proposed to be rather a mechanism of inactivation because the truncated form seems relatively unstable [41].

The density of TNFR at the cell surface contributes likely to the strength and duration of downstream signalling pathways. Shedding, but also exocytosis, are two processes that control their biological functions. Like mTNFα, TNFR1 and TNFR2 are shed from the cell surface by TACE, releasing their ectodomain in the extracellular compartment [42–44] (Fig. 3). sTNFR1 and sTNFR2 are able to bind to mTNFα, sTNFα and LTα, but with a lower affinity than full-length anchored-TNFR1/2. TNFα, PMA, inhibition of the proteasome and TLR3 were reported to induce TNFR1 shedding [45–47]. In addition, the type II integral membrane protein ARTS-1 (aminopeptidase regulator of TNFR1 shedding) and NUCB2 (nucleobindin 2) were shown to bind TNFR1, but not TNFR2, and to potentiate its shedding [48,49]. Another mechanism accounts to dampen LTα/TNFα bioactivity. Indeed, TNFα can be sequestered by released full-length TNFR1 in exosome-like vesicles [50]. This extracellular form of TNFR is devoid of complex I (see chapter 4) and is able to bind recombinant TNFα. Mechanistically, cAMP-dependent activation of PKA allows the pre-complex ARTS-1/NUCB2/TNFR1 to be redirected through the exosome-like vesicles transport. The recruitment of the proteins BIG2 and ARF1/3 to TNFR1 regulates the release of TNFR1-containing vesicles in the extracellular compartment [49,51]. Interestingly, exosome TNFR1-containing vesicles have been detected in the blood of human patients [52]. Thus, the complex ARTS-1/NUCB2/TNFR1 is at the crossroad of two paths, TNFR1 shedding and/or exosome-like vesicles formation.

Of note, missense mutations in the ectodomain of TNFR1 were detected in patients affected by a periodic fever syndrome, called TRAPS (TNFR1-associated periodic syndromes). These patients

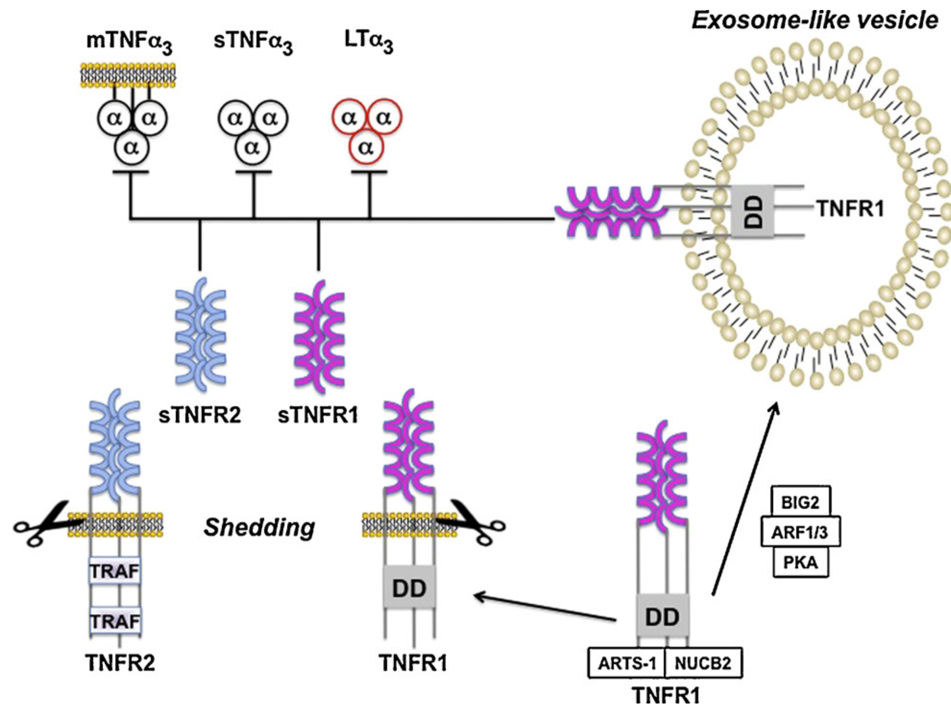

Fig. 3. Shedding and exocytosis of TNFR1/2. TNFR1 and TNFR2 are shed from the cell surface by TNF-α converting enzyme (TACE, depicted by scissors) to generate soluble TNFR1 (sTNFR1) and soluble TNFR2 (sTNFR2). TNFR1-interacting complex ARTS-1/NUCB2 potentiates the shedding of TNFR1. cAMP-dependent activation of PKA triggers the recruitment of the complex BIG2/ARF1/3 to TNFR1 to release exosome-like TNFR1-containing vesicles. sTNFR2, sTNFR1 and exosome-like TNFR1-containing vesicles capture free mTNFα_3, sTNFα_3 or LTα_3.

display impaired downregulation of membrane TNFR1 and reduced shedding [53]. Therefore, there is a possibility that particular TRAPS mutations inactivate ARTS-1 recruitment and/or TACE protease activity.

4. Trafficking of TNFR: more than simply recycling or degradation; cell death and activation of the alternative NF-κB pathway enter into the game

Endocytosis of TNFR has been considered for a long time as a mechanism of recycling or degradation to shut down downstream signalling pathways. TNFR2 is a good candidate whose endocytosis limits TNFα activity. Indeed, while mTNFα activates TNFR2, sTNFα induces its internalization [30,54]. The endocytic route probably involves AP adaptor family proteins, such as AP1 or AP2, and clathrin. Indeed, AP1 and AP2 adaptor proteins recognize tyrosine-based (YXXΦ) and di-leucine ([D/E]XXXL[L/I]) consensus motif [55]. TNFR2 contains within its tail one di-leucine motif. Mutation of both leucine prevents internalization and lysosomal degradation of TNFR2, but not activation of NF-κB.

In contrast, internalization of TNFR1 has a different fate and is rather associated to cell death. Indeed, upon TNFα stimulation, TNFR1 recruits at the plasma membrane a large complex of proteins called complex I. This complex contains the death domain protein TRADD, which binds to TNFR1 and allows the recruitment of the RING domain proteins TRAF 2/5 and c-IAP1/2 [56]. The latter induce the formation of K63-polyubiquitin chain on particular lysine residue of the kinase RIP1. The latter triggers a cascade of ubiquitination on the scaffold protein NEMO (IKKγ) that allows the activation of IKKβ, the phosphorylation and degradation of IκBα and the nuclear translocation of NF-κB. This pathway has been defined as the classical, or canonical, NF-κB pathway, which mediates the pro-inflammatory and pro-survival activity of TNFα. However, when the activation of the classical NF-κB pathway is compromised, a second complex forms, named complex II. This complex contains the death domain containing proteins FADD, deubiquitinated RIP1 and caspase-8 [57]. The formation of complex II is dependent on a tyrosine-containing region YXXW in the cytosolic tail of TNFR1, named TRID domain. Disruption of this domain prevents clathrin-dependent internalization of TNFR1 and cell death [58]. Thus, TNFα-induced endocytosis of TNFR1 is mandatory to induce cell death when the pro-survival NF-κB pathway is shut off. Interestingly, some viruses have evolved to inhibit TNFR1 internalization as a mechanism to escape immune surveillance. Indeed, the adenoviral protein 14.7 K inhibits the recruitment of key regulators of TNFR1 endocytosis, such as dynamin 2 and clathrin [59].

LTβR, like TNFR1, activates the classical NF-κB pathway from the plasma membrane. However, as opposed to TNFR1, LTβR activates a second pathway called the alternative, or non-canonical, NF-κB pathway that leads to the processing of the NF-κB precursor p100 into its active form p52 [60,61]. Activation of both the classical and the alternative NF-κB pathways relies on the ability of LTβR to recruit TRAF proteins. However, separate pools of TRAF proteins control the activation of both NF-κB pathways and diverged by their function and cellular location (Fig. 4). Ligation of LTβR induces its trimerization and a fast recruitment of TRAF2/5 via two distinct TRAF binding sites within the tail of LTβR [24,25]. This pool of TRAF2/5 activates the classical NF-κB pathway through phosphorylation and ubiquitination of the IKK complex, which induces the phosphorylation and degradation of IκBα and the release of p50/p65. Another pool of TRAF proteins is linked to the constitutive proteasomal degradation of the NF-κB-inducing kinase (NIK), a key kinase acting downstream of LTβR for the phosphorylation-mediated processing of p100 [62]. Through a non-conventional TRAF3 binding site, NIK recruits the negative regulatory complex TRAF3/TRAF2/c-IAP1/2 allowing the E3 ligase c-IAP1/2 to mediate its K48-linked polyubiquitination towards NIK [63–65]. Stabilization of NIK in response to ligation of LTβR requires a displacement of TRAF3 from NIK to interrupt its K48-linked polyubiquitination [66]. However, the pool of TRAF3/NIK is localized into intracellular bodies implying that, either TRAF3 is

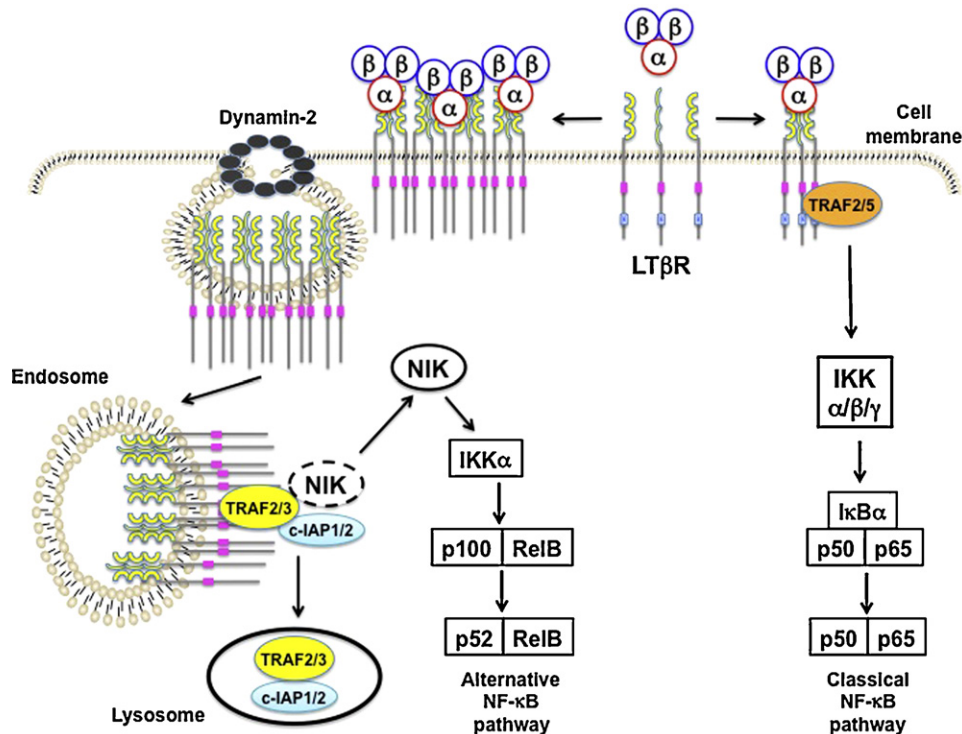

Fig. 4. Trafficking of LTβR uncouples the induction of the classical and the alternative NF-κB pathways. The heterotrimer $LT\alpha_1\beta_2$ induces the trimerization of LTβR. Early recruitment of TRAF2/5 at cell membrane anchored-LTβR occurs through two TRAF binding sites within the cytosolic tail (pink and blue rectangle). This step allows the activation of the IKK complex (IKKα/β/γ) and the release of p50/p65 NF-κB dimers from the inhibitor IκBα (classical NF-κB pathway). Aggregates of trimeric LTβR are internalized through a dynamin-2-dependent route that requires a particular region of the receptor (pink rectangle). LTβR is brought into endocytic vesicles with the cytosolic tail facing the intracellular compartment. This configuration allows to competing out TRAF2/3-c-IAP1/2 away from NIK and to interrupt its constitutive degradation (dashed circle NIK). The negative regulatory complex is degraded in the lysosomal compartment and NIK is stabilized (plain circle NIK). NIK activates IKKα and both induce the processing of p100/RelB into p52/RelB (alternative NF-κB pathway).

displaced from NIK and recruited to cell surface LTβR, or that LTβR is internalized and in close vicinity of TRAF3/NIK. A recent study showed that activated LTβR is internalized through at least two routes. One is AP2/clathrin-dependent and is not linked to activation of NF-κB, the other is clathrin-independent but dynamin-2-dependent and is required for the activation of the alternative, but not the classical, NF-κB pathway [25]. Thus, these studies shed light on the role of trafficking for the spatio-temporal activation of the two NF-κB signalling pathways downstream of LTβR [25].

5. Secondary lymphoid organ developmental and architectural defects in knock-out mice models

To determine the biological roles of LTα, conventional LTα-deficient mouse strains were generated [67,68]. $LT\alpha^{-/-}$ mice exhibited a broad spectrum of abnormalities associated with an absence of most secondary lymphoid organs (SLO) development, such as lymph node (LN) and Peyer's patches (PPs) (Table 1). These phenotypic abnormalities were also seen in an ENU-induced

Table 1
Summary of immune developmental defects of mice deficient for the LT/TNF system and signalling proteins involved in the activation of NF-κB. Conv (conventional), LN (lymph node), mes (mesenteric), cer (cervical), PPs (Peyer's patches), ND (not determined).

Mouse strain	LN			PPs	Splenic T & B zone	References
	Mes	Cer	Others			
Corw, $LT\alpha^{-/-}$	+/–	–	–	–	–	[67,68]
Conv. $LT\alpha^{-/-}$ xTgTNFα	+/–	–	–	–	+/–	[68,94]
ENU LTα	–	–	–	–	ND	[69]
LoxP-$LT\alpha^{-/-}$	–	–	–	–	+/–	[96]
$LT\beta^{-/-}$	+	+	–	–	+/–	[70,71]
Conv. $TNF\alpha^{-/-}$	+	+	+	+	+/–	[73]
LoxP-$TNF\alpha^{-/-}$	+	+	+	–	+/–	[74]
$TNFR1^{-/-}$	+	+	+	–	+	[75]
$TNFR1^{-/-}$	+	+	+	+/–	ND	[76]
$LT\beta R^{-/-}$	–	–	–	–	–	[77]
aly/aly	–	–	–	–	–	[84]
$NIK^{-/-}$	–	–	–	–	–	[85]
$p65^{-/-} \times TNFR1^{-/-}$	–	–	–	–	–	[83]
$RelB^{-/-}$	–	–	–	–	–	[86,87]
$p52^{-/-}$	+	+/–		–	+/–	[88,89]
ENUp52^{LYM1}	+	–	+/–	–	–	[93]
ENU p52^{Xdr}	+	+/–	+/–	ND	–	[90]
$p50^{-/-}$	+	+	+	+	+	[92]
$p50^{-/-} \times p52^{-/-}$	–	–	–	–	–	[91]

mouse strain carrying a frame-shift generating a premature stop codon within the coding region of LTα [69]. Inactivation of LTβ revealed developmental defects for some, but not all, LN and PPs indicating that LTα may have functions independent of LTβ, despite the ability of LIGHT to bind LTβR [70–72]. A role for TNFα in PPs development was first dismissed based on the generation of conventional TNFα-deficient mice [73]. However, a more advanced approach based on Cre-LoxP technology showed that TNFα was absolutely indispensable for PPs organogenesis [74]. More intriguingly, TNFR1-deficient mice were generated by different research groups and concluded an absolute or a mild role of this receptor in PPs organogenesis [75,76]. Thus, the role of TNFα/TNFR1 axis in PPs development is still unclear and needs further characterization.

Absence of all lymph node and Peyer's patches development were fully recapitulated in LTβR-deficient mice and in embryos of wt mice injected in utero with recombinant LTβR-Ig fusion proteins, supporting the idea that $LT\alpha_1\beta_2$/LTβR axis is crucial for these biological events [77,78]. At the earliest stage of development, $LT\alpha_1\beta_2$-expressing LTi, which derived from foetal liver precursors, establish a tight interaction with LTβR positives lymphoid tissue organizer cells (LTo), also called embryonic stromal, or mesenchymal, organizer cells [79–81]. RANKL and IL7 regulate the expression of $LT\alpha_1\beta_2$ on lymphoid tissue inducer cells (LTi) involved in LN and PPs organogenesis, respectively [82]. Likewise, LTo upregulate the expression of adhesion molecules, such as ICAM-1, VCAM-1 and MAdCAM-1, and secrete B and T cell chemoattractants, like CXCL13 (BLC), CCL19 (ELC) and CCL21 (SLC). These chemokines feedback on LTi cells via CXCR5 and CCR7 receptors and further induce the expression of $LT\alpha_1\beta_2$ and integrins involved in recognition of VCAM-1 and MAdCAM-1. Because these genes are NF-κB targets it was expected that mice deficient for this transcription factor display developmental defects of lymphoid organs. Indeed, double knock-out mice p65 (RelA)/TNFR1 lack all LN and PPs. Reconstitution experiments with wt or p65/TNFR1-deficient foetal liver cells into irradiated wt or p65/TNFR1-deficent mice allowed to conclude that the defect arose from the stromal compartment [83]. The SLO development defects observed in p65/TNFR1 and LTβR knock-out mice are phenocopied in aly (alymphoplasia) mice. This mouse strain carries a single point mutation in the carboxy-terminal region of the NF-κB-inducing kinase (NIK) [84]. Altogether, the phenotypic similarities of LTβR, NIK and p65/TNFR1 double knock-out mice suggested that these proteins are part of an unique pathway downstream of LTβR. Analysis of NIK-deficient mice confirmed this hypothesis and demonstrated that NIK was required to potentiate the transcriptional activity of p65 [85]. In addition, LTβR acts through NIK for the induction of the processing of p100 into p52/RelB [61,62]. RelB-null mice revealed grossly similar defects observed in p65/TNFR1 knock-mice indicating that RelB and p65 control distinct set of genes, with no obvious compensatory mechanisms, for SLO development [86,87]. However, conventional p100/p52 knock-out mice, as well as ENU-induced xander (xdr) mice carrying a point mutation leading to aberrant mRNA splicing and premature stop codon within the Rel homology domain of p52, exhibit less severe developmental defects (mesenteric and cervical LN present, Table 1) [88–90]. This is the consequence of compensatory mechanisms between p50 and p52 for some LN development, which are abrogated in p50/p52 double knock-out mice [91,92]. Interestingly, another ENU-induced mouse strain, called LYM1, was characterized and displays a missense point mutation between the two serine that mediate NIK-induced p100 processing [93]. This mouse strain revealed a more severe phenotype than p52-null mice (lack all LN), besides an intact expression of p50. These observations indicate that accumulation of unprocessed p100 acts as a super-inhibitor that prevents compensatory mechanisms of p50 for LN development.

In the spleen, T and B cells positioning was altered in conventional $LT\alpha^{-/-}$ mice. However, these defects were restored by crossing conventional $LT\alpha^{-/-}$ mice with transgenic (Tg) TNFα mice, but not with Tg LTα mice [94,95]. It was found that conventional $LT\alpha^{-/-}$ mice were deficient in TNFα. Because of the close proximity of LTα and TNFα coding regions, it is likely that transcription of TNFα was disturbed by the presence of the neo cassette from the targeting vector used to generate conventional $LT\alpha^{-/-}$ mice. To solve this problem, neo-free $LT\alpha^{-/-}$ mice were generated with Cre-LoxP technology, in which TNFα production was intact [96]. These mice shared several splenic defects reminiscent to LTβ-deficient mice, as opposed to conventional $LT\alpha^{-/-}$ mice, indicating that TNFα plays non-redundant roles in these settings [67,70,71,96]. Further detailed analyses of other splenic developmental defects, such as FDC network, marginal zone B and germinal center formation have been reported elsewhere for TNF/Lymphotoxin and NF-κB family members [97,98].

6. Tissue-specific transgenic mouse models as tools to study the pathological roles of LTα/LTβ

Induction of LTα and LTβ gene transcription is tightly regulated by specific transcription factors in hematopoietic and non-hematopoietic cells. One could expect that uncontrolled activation of these genes lead to inflammatory-associated disorders through exacerbated $LT\alpha_3$ and/or $LT\alpha_1\beta_2$-mediated downstream signalling pathways. Several mouse models have been generated and brought further insights into the role of LTβR and TNFR1 in pathological disorders.

6.1. Inflammation and tertiary lymphoid organ

Tertiary lymphoid organs (TLO), also called ectopic lymphoid organs, are lymph node-like structures that form in certain chronically inflamed tissues. TLO share some structural similarities with SLO, like T and B cells areas, germinal centers and high endothelial venules (HEV). In human, TLO are frequently observed in autoimmune and inflammatory diseases (rheumatoid arthritis, ulcerative colitis), in infectious diseases (chronic hepatitis C) and in cancer (ductal breast carcinoma) [99,100]. The first mouse strain that showed that LTα was associated with chronic inflammation and TLO development was the RIP-LTα mice [101,102]. In this transgenic mouse strain, LTα is under the control of rat insulin promoter (RIP) allowing expression of the transgene in pancreatic islets of Langerhans, but also in kidney and skin. These mice develop signs of early stage of insulitis with peri-islet accumulation of lymphocytes and inflammation in the kidney. RIP-LTα/$LT\beta^{-/-}$ mice develop a similar phenotype indicating that $LT\alpha_3$ is sufficient to induce insulitis but also lymphoangiogenesis [95,103]. Generation of RIP-LTα on TNFR1- or TNFR2-deficient background revealed that $LT\alpha_3$ mediates its pro-inflammatory activity through TNFR1 [104]. Bitransgenic RIP-LTα/LTβ develop a more severe phenotype with infiltrating lymphocytes into pancreatic islets [105]. The cellular phenotypes and compartmentalization of RIP-LTα/LTβ pancreatic infiltrates are similar to LN.

Altogether, $LT\alpha_3$/TNFR1 and $LT\alpha_1\beta_2$/LTβR signalling pathways cooperate to induce gene transcription of adhesion molecules, chemokines and lymphokines necessary to build a TLO. However, this inflammatory environment does not lead to tissue destruction and type I diabetes, unless an additional co-stimulatory signal is provided in the form of B7.1 co-expression in the islets, which activates infiltrating T cells resulting in beta cells destruction [106].

6.2. Inflammation-induced cell death

The thymus is the primary lymphoid organ for T cell development and selection. The thymus is structured into an outer cortex and an inner medulla, containing cortex thymic epithelial cells (cTEC) and medulla epithelial cells (mTEC), respectively. The space between cTEC and mTEC is filled by thymocytes at different stages of development. cTEC provide a proliferative signal to thymocytes, while mTEC participate to the process of negative selection. Acute inflammation or chronic infections are conditions that lead to the shrinkage of the thymus, a process called thymic involution. Knowing the fact that LTα and LTβ are upregulated during inflammation, two transgenic mouse models were generated to address whether elevated expression of these two cytokines in thymocytes was sufficient to induce thymic involution. A first bitransgenic mouse strain was obtained using a targeting vector with the lck promoter upstream of either mouse LTα or mouse LTβ [107]. T cell specific expression of the transgenes was detected in different tissue, like the thymus, mesenteric lymph nodes and Peyer's patches. To achieve expression of ectopic LTα and LTβ under physiological conditions, another group cloned the locus containing the human regulatory regions and genes coding for LTα and LTβ into the targeting vector [108]. However, this approach brought human TNFα into the game because its coding region is located between LTα and LTβ. Despite these differences, both transgenic strains developed an accelerated thymic involution characterized by massive cell death of thymocytes and stromal cells [107,108]. In these transgenic strains, two main complexes form, which are $LT\alpha_3$ and $LT\alpha_1\beta_2$. Surprisingly, bitransgenic LTα/LTβ backcrossed on TNFR1- or LTβR-deficient background recovered thymus cellularity, indicating that TNFR1 and LTβR played non-redundant roles in cell death of thymic cells. Reciprocal bone transfer experiments revealed that the pro-death activity of $LT\alpha_3$ and $LT\alpha_1\beta_2$ was mediated by TNFR1 and LTβR within the stromal compartments. Histological analyses of thymi from bitransgenic LTα/LTβ mice indicated an enlargement of the medulla over the cortex, suggesting the cTEC are the primary target cells undergoing apoptosis [107].

How TNFR1 and LTβR activate cell death and what is the timeline of activation of these receptors is unknown and needs further characterization.

6.3. Inflammation-induced cancer

Acute and chronic inflammations are important biological settings contributing to hallmarks of cancer development [109]. HBV- or HCV-infected patients suffer from chronic hepatitis that is frequently associated to hepatocellular carcinoma (HCC) development. LTα and LTβ are target genes upregulated in HBV- or HCV-infected hepatocyte cell lines, as well in human HCV-infected livers [6,7,110]. Of note, in HCV-infected hepatocytes, the viral core protein has the ability to bind LTβR and TNFR1 and to modulate the activation of NF-κB [111–114]. These findings suggested that activation of the classical NF-κB pathway, by $LT\alpha_1\beta_2$/LTβR and $LT\alpha_3$/TNFR1 axis, and/or activation of the alternative NF-κB pathway, by $LT\alpha_1\beta_2$/LTβR axis, may contribute to HCC development. To address this hypothesis, bitransgenic liver specific (albumin promoter) LTα/LTβ mice were generated [115]. Ectopic expression of LTα and LTβ was observed in hepatocytes, but absent from the spleen, thymus, mesenteric lymph node, pancreas and kidney. Transcriptomic analyses of hepatocytes from 3 months aged Tg-Abl- LTα/LTβ versus control littermates mice revealed an upregulation of several chemoattractant (MCP-1, MCP-3, GROα and IP-10) for monocytes, macrophages (Kupffer cells), dendritic and T cells [110]. Between 4 to 9 months of age, Tg-Abl-LTα/LTβ mice display T, B and DC cells, and latter, macrophages infiltrates in the liver forming organized TLO [110,115]. At this stage, this inflammatory environment induces expression of IL1-β, IFNγ, IL-6 and to a lower extend TNFα, altogether inducing proliferation of oval cells. In other mouse models of chemical-induced carcinogenesis, IL-6R and TNFR1 signalling are mandatory to generate HCC [116,117]. In contrast, TNFR1 is fully dispensable to mediate LTα/LTβ-induced HCC, leaving the possibility that $LT\alpha_3$ expressed on hepatocytes might be dispensable for HCC development. However, we cannot rule out that $LT\alpha_3$ induces oval cells proliferation and HCC development towards activation of other TNFR members.

Tg-Abl-LTα/LTβ/TNFR2$^{-/-}$ mice fail to develop HCC, leaving the possibility that HVEM-expressing infiltrating lymphocytes would be targeted by ectopic $LT\alpha_3$. This is particularly relevant in light of the phenotype of Tg-Abl-LTα/LTβ/RAG1$^{-/-}$ mice that neither develop hepatitis nor HCC. Thus, ectopic $LT\alpha_3$ and $LT\alpha_1\beta_2$ on hepatocytes are not sufficient to mediate HCC development but require a signal from infiltrating immune cells. Conversely, activation of LTβR on hepatocytes is indispensable to mediate HCC development.

As previously reported, LTβR induces both the classical and the alternative NF-κB pathways [61]. However, Tg-Abl-LTα/LTβ/IKKβ$^{\Delta hep}$ mice, but not Tg-Abl-LTα/LTβ/IKKα^{AA} mice, do not display sign of hepatitis or HCC. Thus, the classical NF-κB pathway is pro-carcinogenic in Tg-Abl-LTα/LTβ/ mice through chemoattractant expression by hepatocytes [110].

7. Concluding remark

The LT/TNF/LIGHT triad and their cognate receptors constitute a complex network of cytokines and receptors involved in the development and homeostasis of the immune system, inflammation and cell death. These cytokines and receptors act locally through cell–cell interactions or at distant sites through shedding or exocytosis.

These biological processes are important to limit the half-life of ligands or receptors at the cell surface and to interrupt signalling pathways. It also modifies the biochemical properties of ligand and receptors, exemplify by the switch from membrane-bound TNFα to soluble TNFα. When this intricate network is compromised inflammatory diseases and cancer development can occur.

Too much sTNFα is deleterious, but generation of soluble TNFR1/TNFR2 is one way the nature has created to prevent this to happen. Men have tried to reproduce this approach with more or less success in mouse models and biological molecules are in clinical trials to treat inflammatory disorders and cancers. A first generation of TNF blockers (Ethanercept, Remicade) emerged a decade ago to treat inflammatory disorders, like rheumatoid arthritis or Crohn's disease. However, side effects (lymphoma, tuberculosis) arose in some patients, probably due to the lack of discrimination between mTNFα and sTNFα from these molecules. Ethanercept is a decoy-TNFR2 antibody and Remicade is an antibody directed to TNFα. However, it is hazardous and challenging to find the right therapeutic window that does not target membrane-bound TNFα. Similar approaches with biological molecules were undertaken to neutralize $LT\alpha_1\beta_2$, $LT\alpha_3$ or LIGHT (anti-LTα and decoy-LTβR). These molecules have some advantages to target multiple ligands (decoy-LTβR targets LIGHT and $LT\alpha_1\beta_2$ but not -$LT\alpha_3$, and anti-LTα target $LT\alpha_1\beta_2$, and $LT\alpha_3$ but not LIGHT) but suffer from a lack of specificity.

Novelty may come from small molecules that target the TNF receptors and either prevents the binding of the ligand, or the recruitment of signalling proteins.

Alternatively, preventing TNFR to activate the classical NF-κB and not the alternative NF-κB pathway, or the opposite, might be a more adapted response to particular inflammatory pathologies or cancer development. The next decade will tell us whether these approaches are relevant.

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