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The alternative NF-κB pathway from biochemistry to biology: Pitfalls and promises for future drug development

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Abbreviations:
BAFF, B cell activating factor belonging to the TNF family
BLC, B lymphocyte chemoattractant
ELC, Epstein-Barr virus induced molecule 1 ligand chemokine
FDC, follicular dendritic cells
FL B cells, follicular B cells
GC, germinal center
IKK, inhibitor kappa B kinase
KO, knock out
LIGHT, homologous to lymphotoxin exhibits inducible expression and competes with HSV glycoprotein D for Herpes virus entry mediator a receptor expressed by T lymphocytes
LMP1, latent membrane protein 1
LN, lymph node
LTβR, lymphotoxin-beta receptor

ABSTRACT

The past two decades have led to a tremendous work on the transcription factor NF-κB and its molecular mechanisms of activation. The nuclear translocation of NF-κB is controlled by two main pathways: the classical and the alternative NF-κB pathways. The classical NF-κB pathway activates the IKK complex that controls the inducible degradation of most IκB family members that are IκBα, IκBβ, IκBε and p105. The alternative NF-κB pathway induces p100 processing and p52 generation through the activation of at least two kinases, which are NIK and IKKα. Genetic studies have shown that IKKγ is dispensable for the alternative pathway, which suggests the existence of an alternative IKKα-containing complex. It is noteworthy that activation of particular p52 heterodimers like p52/RelB requires solely the alternative pathway while activation of p52/p65 or p52/c-Rel involves a "hybrid pathway". Among others, LTβR, BAFF-R, CD40 and RANK have the ability to induce the alternative pathway. The latter plays some roles in biological functions controlled by these receptors, which are the development of secondary lymphoid organs, the proliferation, survival and maturation of B cell, and the osteoclastogenesis. Exacerbated activation of the alternative pathway is potentially associated to a wide range of disorders like rheumatoid arthritis, ulcerative colitis or B cell lymphomas. Therefore, inhibitors of the alternative pathway could be valuable tools for the treatment of inflammatory disorders and cancers.

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MALT, mucosa-associated l
ymphoid tissue
MEFs, mouse embryonic fibroblasts
MZB, marginal zone B cell
NALT, nasal-associated lymphoid
tissue
NF-κB, nuclear factor-kappa B
NIK, NF-κB-inducing kinase
PP, Peyer's patch
RANK, receptor activator of NF-κB
RANKL, RANK ligand
TNF, tumour necrosis factor
TNFR, TNF receptor
TRAF, TNF receptor associated factor
SLC, secondary lymphoid tissue
chemokine
SLO, secondary lymphoid organ
TLO, tertiary lymphoid organ
wt, wild type

1. Introduction

Since its discovery nearly 20 years ago, NF-κB has emerged as one the most studied mammalian transcription factor. Although initially identified in activated B cells, it rapidly appeared that this transcription factor is essential for both innate and adaptive immunity, cell survival and inflammation, among other biological functions [1,2]. In mammals, the Rel/NF-κB family is comprised of p65 (RelA), c-Rel (Rel), RelB, p50 and p52. These structurally related proteins share extensive sequence similarities within their N-terminal Rel Homology Domain (RHD) that enables them to dimerize, to translocate into the nucleus and to bind to specific DNA sequences named κB sites. Among the Rel/NF-κB family, only p65, c-Rel and RelB contain a C-terminal transcriptional activation domain and therefore are able to directly activate the transcription. The other two members, p50 and p52, are synthesized as large precursors called p105 and p100, respectively. Only upon dimerization with p65, c-Rel, RelB or Bcl3 can p50 and p52 behave as transcriptional activators [3].

In most cells, NF-κB homodimers and heterodimers are maintained latent in the cytoplasm in association with inhibitors of the IκB family. The rapid and transient activation of NF-κB complexes (e.g. p50/p65), in response to a wide range of stimuli such as pro-inflammatory cytokines (TNFα, IL-1β, IL-6, CD40L), DNA damaging agents (camptothecin, daunomycin), Toll-like receptors (TLRs) agonists or viruses (HTLV1, EBV), is generally regulated by the classical NF-κB pathway (or canonical pathway) [4]. The latter involves the activation of the IKK complex, which contains the catalytic subunits IKKα and IKKβ, the regulatory subunits NEMO/IKKγ and ELKS, and the heat shock protein Hsp90/Cdc37 chaperone complex [1,5,6]. The activated IKK complex phosphorylates IκB members (IκBα, IκBβ, IκBε and p105) on a consensus motif DSGΦxS and phosphorylated serine act as binding site for β-TrCP, the substrate recognition subunit of an E3 ligase named $SCF^{\beta\text{-}TrCP}$ [7]. This process, then, leads to the ubiquitination on specific lysine and ubiquitinated IκBs are directed to the proteasome for full degradation, leaving free NF-κB complexes to enter into the nucleus. Among the inducers of the classical NF-κB pathway, few of them are able to trigger an additional pathway through the activation of the NF-κB-inducing kinase (NIK) and IKKα [8,9]. This pathway has been named the alternative NF-κB pathway (or non-canonical) and drives the post-translational processing of p100 to mature p52 [10]. Strikingly, IKKγ is not absolutely required for the activation of the alternative NF-κB pathway, which suggests the existence of an alternative IKKα-containing complex [10,11]. Although, p100 is also an $SCF^{\beta\text{-}TrCP}$ E3 ligase substrate, ubiquitinated p100 is not completely degraded by the proteasome but rather cleaved to generate active DNA-binding p52 product. This process is generally slower than the activation of the classical pathway and leads to a delayed activation of nuclear p52-containing complexes, such as p52/RelB. The mechanisms of generation of p52 are, either constitutive or inducible, either co-translational or post-translational, and take place in different cellular compartments. The activation of the alternative pathway appears to be restricted to some particular TNFR members, which are involved in secondary lymphoid organ development, in B cell survival and homeostasis, and in osteoclastogenesis (LTβR, CD40, BAFFR, RANK). Moreover, the alternative pathway is also activated by some oncogenic viruses, such as EBV or HTLV1 [12–17]. Such inducers always generate two waves of activated NF-κB complexes that result from the sequential activation of the classical pathway followed by the activation of the alternative NF-κB pathway. Thus, according to the cell type and the nature of the stimulus, the classical NF-κB pathway and/or the alternative NF-κB pathway control the fine-tuning of their own and/or common NF-κB target genes, whose expression contributes to the pleiotropic biological functions of this ubiquitous transcription factor.

2. The alternative NF-κB pathway

2.1. Nomenclature and biochemical features of p100/p52

According to the species and the research group that cloned the *nfkb2* gene, the precursor p100 has been given additional names like NF-κB2, p98, p97, LYT-10, H2TF1 or KBF2, and similarly for the cleaved product p52 as p49, p50B or p55. Along this review I will use p100 for the precursor and p52 for the cleaved product.

The precursor p100, like its homolog p105, has the particularity to belonging to both the NF-κB family and the IκB family. Indeed, p100 displays in its N-terminal part the conserved Rel homology domain (RHD) found in all NF-κB family members, which is required for dimerization, nuclear translocation and DNA-binding. In addition, p100 has, within its C-terminal part, a stretch of ankyrin repeats (seven), which is a common feature shared by all IκB family members (IκBα, IκBβ, IκBε, p105, p100, Bcl3) (Fig. 1). The folding of the ankyrin repeats domain allows p100 to mask its nuclear localization signal (NLS), and therefore p100 is mainly cytosolic in most unstimulated cells. Between the RHD and the ankyrin repeats is located a glycine-rich region (GRR) that is essential for determining the site of cleavage, and for preventing full degradation of p100 through the proteasome [18,19]. The precursor p100 contains a phosphorylation site for NIK that is required for inducible p100 processing, and phosphorylation sites for IKKα that are involved in both constitutive and inducible p100 processing [9,20]. p100 is the main inhibitor of RelB and generation of p52/RelB results from proteolytic cleavage of a unique pool of p100/RelB [21]. Under physiological conditions, produced p52/RelB is only poorly sequestered by other IκB molecules and is free to modulate the rate of transcription of its specific target genes. Other p52-containing complexes like p52/p65 or p52/c-Rel are generated from p100/p65 and p100/c-Rel pools [22]. However, cytosolic retention of p52/p65 and p52/c-Rel dimers is controlled by multiple IκB inhibitors (mainly IκBα and IκBβ), whose degradation are induced through activation of the IKK complex. Thus, these dimers are at the crossroads of the alternative and the classical NF-κB pathways, which I propose to refer as the hybrid pathway (Fig. 2). Indeed, these dimers are produced through a straight activation of the alternative pathway (NIK- and IKKα-dependent) but are activated by the classical pathway (IKKβ- and IKKγ-dependent). In addition, dimerization of p52 with p52, or with p50, leads to transcriptionally inactive nuclear complexes, but association with nuclear Bcl3 converts p52/p52 homodimers to transcriptionally active p52/p52/Bcl3 trimers [23]. Moreover, p100 can form a trimeric complex with p50/p65 or p50/RelB but the function of these complexes is still not fully understood [21,24–26].

2.2. Mechanisms of activation of the alternative NF-κB

2.2.1. Post-translational processing of p100

2.2.1.1. Induction by TNFR family members. Although the last decade of research on IκBs molecules allowed the establishment of a quite detailed molecular mechanism for the degradation of IκBα, IκBβ, IκBε and p105, conversely p100 was still the lost IκB member and the mechanisms of its partial proteolytic cleavage were poorly characterized. Research over the last 4 years has really opened a new avenue in the field of NF-κB. In fact, the first biochemical experiments that identified a signalling protein triggering the induction of p100 processing was reported in 2001 by Sun and co-workers [9]. They showed that overexpression of NIK in 293 cells was sufficient to induce p100 processing independently of IKKα and IKKβ. Moreover, splenocytes derived from *aly/aly* (alymphoplasia) mice, which carry an inactivating point mutation in the gene encoding NIK, display a stronger reduction in the level of p52 than that of control *aly/+* heterozygous cells. However, the role of IKKα and IKKβ has been revisited in a more accurate manner using IKKα- and IKKβ-deficient MEFs. It turned out that overexpression of NIK in wt MEFs, or in IKKβ-deficient MEFs, induce p100 processing but does not in IKKα-deficient MEFs [8]. Thus, from these studies it appeared that IKKα acts downstream of NIK for p100 processing. However, the first studies that reconstituted a full picture of an inducible p100 processing *in vivo* were realized by studying signalling downstream of three independent members of the TNFR family, that are LTβR, BAFFR and CD40 [10,11,27,28]. Indeed, it was shown that treatment of LTβR-expressing MEFs and

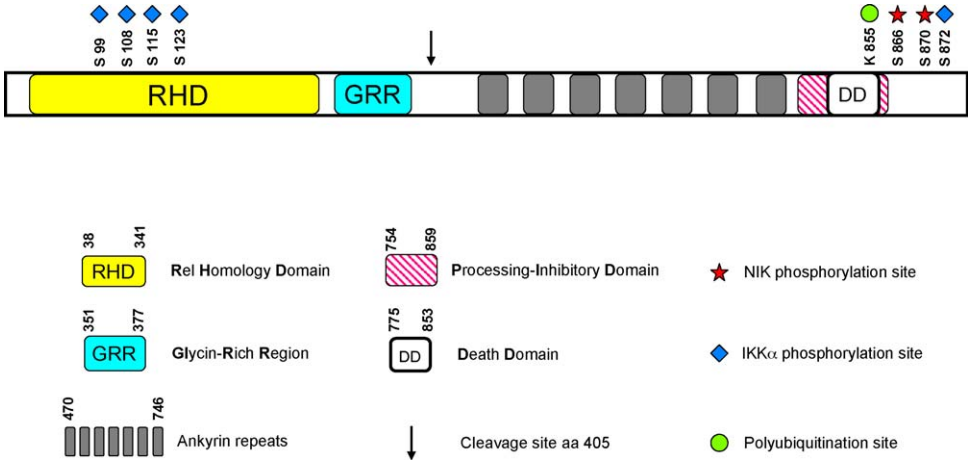

Fig. 1 – Structural features of the human p100 protein (amino acid numbering is based on GenBank accession no. S76638).

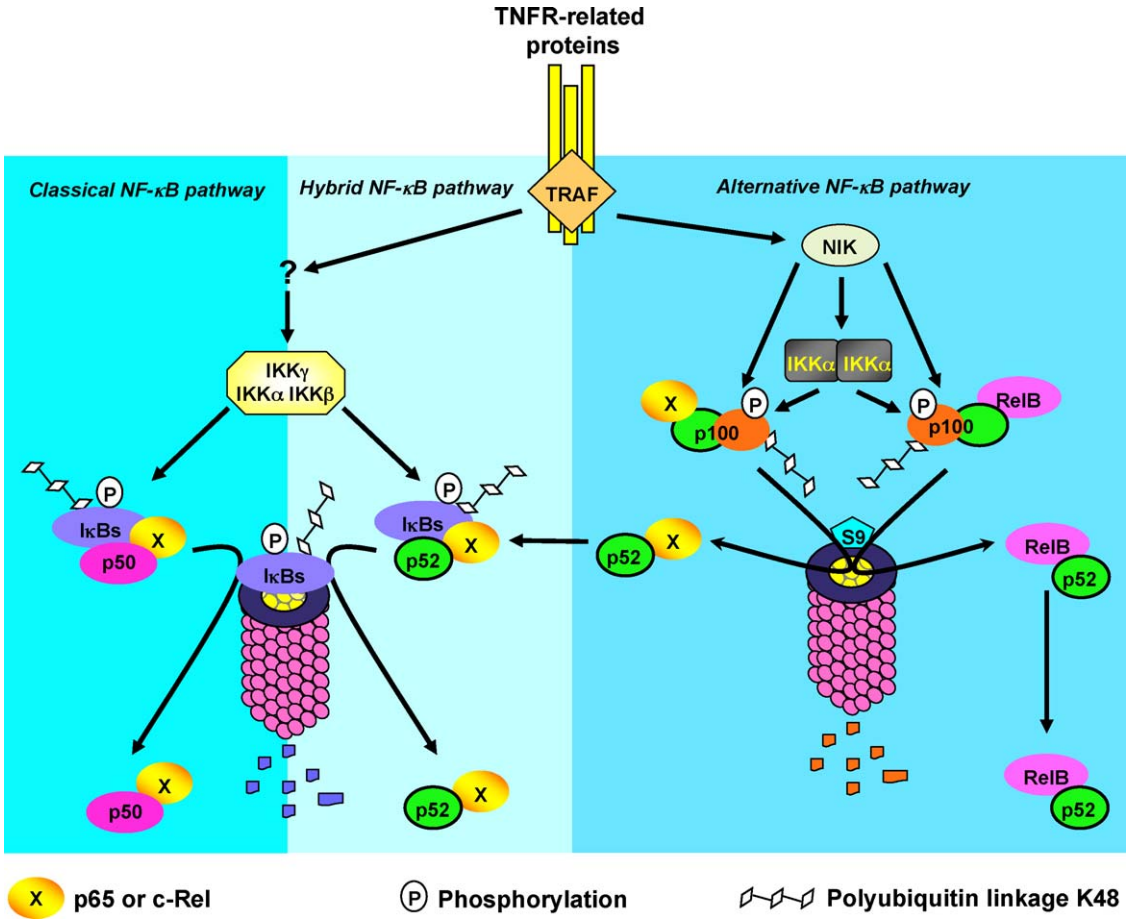

Fig. 2 – Model for the activation of the classical and the alternative NF-κB pathways by TNFR-related proteins (LTβR, BAFF-R, CD40, Fn14, RANK, CD27, CD30 and LMP1) and upon *H. pylori* infection. Upon receptor activation, TRAF proteins mediate the activation of the classical NF-κB pathway probably via the requirement of an upstream kinase (still unknown), which activates the IKK complex. The latter phosphorylates IκBs (IκBα and IκBβ), which become ubiquitinated and then fully degraded through the 26 S proteasome. Thus, NF-κB complexes p50/X and p52/X (X = p65 or c-Rel) are freed and translocate into the nucleus for modulating transiently the expression of their target genes. While the classical pathway is turned on within minutes, activation of the alternative pathway takes about a few hours. According to the receptor, TRAF proteins are either activated, or inhibited, in order to trigger NIK and IKKα kinase activities. NIK mediates p100 phosphorylation/ubiquitination in a IKKα-independent and IKKα-dependent manners. The phospho-p100 is recognized and polyubiquitinated by the E3 ligase $SCF^{\beta\text{-}TrCP}$ (not pictured here). Then, the phospho-ubiquitinated p100 interacts with the S9 subunit of the 19 S proteasome lid and is partially degraded through the proteasome for generating p52/RelB and p52/X dimers. Once generated, p52/RelB dimers are free to move to the nucleus whereas p52/X dimers are first captured by IκBs for their cytosolic retention and then activated through the classical pathway. Thus, while p52/RelB complexes are generated and activated through the alternative pathway, pre-bound p52/X dimers are generated via the alternative pathway but activated through the classical pathway. Therefore, these particular dimers are controlled by a "hybrid pathway".

BAFFR/CD40-expressing B cells with their cognate natural ligands induce p100 processing in wt cells but not in *aly/aly* cells [10,11,27]. Thus, these biochemical data clearly underlined a role for NIK downstream of LTβR, BAFFR and CD40. Further studies showed that other stimuli are also capable of inducing both the classical and the alternative NF-κB pathway (Table 1). Surprisingly, it was observed that LTβR and BAFF-R induce p100 processing independently of IKKγ [10,11]. Thus, these results suggest that an alternative IKKα-containing complex might transmit the signal for p100 processing. Although it is speculated that this complex is an IKKα homodimer, we cannot rule out that it contains additional scaffold proteins. The blockade of induced p100 processing by a pre-treatment with cycloheximide has led to the conclusion that either a protein neo-synthesis is required prior to the induction of the alternative pathway or/and that a pre-existing protein has a short half-life [11,27,29]. Among other proteins, NIK is likely to be a candidate altered by the use of cycloheximide because its endogenous level of expression is very low in most cell types [11] (Dejardin, personal communication). In addition, it has been shown that induction of the alternative pathway by BAFF or CD40L leads to an elevated level of NIK protein. The mechanism that accounts for the increase of NIK protein is rather based on an inhibition of

Table 1 – Genetic evidences for the involvement of TNF ligands members and their cognate receptors, viral proteins and bacteria, in post-translational p100 processing

Ligand	Receptor	NIK	IKKα	IKKγ	References
TNF/TNFR members					
LTα1β2	LTβR	+ (≠)	+	–	[10]
BAFF/BLys	BAFFR	+ (≠)	ND	–	[11,28]
CD40L	CD40	+ (≠)	ND	ND	[27]
Tweak	Fn14	+ (≠)	+	–	[47]
RANKL	RANK	+	ND	ND	[91]
CD70	CD27	+ (*)	+ (*)	– (*)	[146]
CD30L	CD30	ND	ND	ND	[147]
Virus	**Viral protein**	**NIK**	**IKKα**	**IKKγ**	**References**
Virus and viral proteins					
EBV	LMP1	+ (≠)	+	–	[13,14,16]
HTLV-1	Tax	ND	ND	+	[15,37]
KHSV	v-FLIP/K13	–	+	ND	[38]
Bacteria	**Strain**	**NIK**	**IKKα**	**IKKγ**	**References**
Helicobacter pylori	TN2	+ (≠)	+ (*)	ND	[39]

Symbols used for signalling proteins requirement: (–) not required; (+) required and (ND) not determined. These results are based on experiments NIK-, IKKα- and IKKγ-deficient cells, *aly/aly* cells (≠) and down-modulation of signalling proteins expression through the use of siRNA (*).

TRAF3-mediated NIK degradation [30]. Beside the stabilization of NIK, its molecular mechanisms of activation are still poorly understood. Nevertheless, biochemical analyses of truncated p100 allowed to target the phosphorylation site that mediates NIK-induced p100 processing [9]. NIK phosphorylates p100 at serines 866 and 870, which permits to alleviate the intrinsic inhibition mediated by the PID domain (processing-inhibitory domain) (Fig. 1). The phosphorylation of serines 866 and 870 is a prerequisite for two molecular events. First, in addition to phosphorylating both serines 866 and 870, NIK serves as a docking molecule for the recruitment of IKKα to p100 [17]. Once recruited, IKKα phosphorylates serines at both N-terminal (serines 99, 108, 115 and 123) and C-terminal regions (serine 872) of p100. Second, similar to IκBα, phosphorylated p100 leads to the recruitment of the E3 ligase $SCF^{\beta\text{-}TrCP}$, polyubiquitination of lysine 855 and subsequent processing to p52 [17,31–33]. Thus, NIK and IKKα phosphorylate distinct phosphoacceptor sites within p100 (although none of the serine phosphorylated by IKKα match up with the consensus phosphorylation site for IKKs) that are located along both N- and C-terminal arm of p100. Using the C-terminal region of p100 as bait, Fong et al. identified the protein S9, a non-ATPase subunit associated to the lid of the 19 S proteasome [34]. Analyses of truncated mutants revealed that the death domain (DD) of p100 interacts with S9. As the proposed mechanisms above have been elucidated by over-expressing NIK and IKKα, it remains to demonstrate that they account for the endogenous processing of p100 induced by membrane anchored proteins like, LTβR, CD40, BAFF-R, RANK, Fn14, CD30 or CD27.

2.2.1.2. *Induction by viral and bacterial products*. Back to 1994, the first evidence that p100 processing could be induced came from two studies on the viral proteins Tax and LMP1 and their role in the activation of NF-κB [35,36]. Moreover, the mechanisms of LMP1-and Tax-mediated p100 processing were unknown. Recent studies have shed light on the strategy adapted by some viral proteins for triggering p100 processing. In HTLV-1-infected T cells, Tax appears to specifically target IKKα via NEMO to p100, triggering phosphorylation- and ubiquitination-dependent p100 processing [15,37]. Thus, unlike NIK-induced p100 processing, Tax-induced p100 processing requires IKKγ (Fig. 3). Moreover, dominant negative NIK, either a kinase dead or a truncated C-terminal NIK, is not able to inhibit Tax-induced p100 processing, which suggests a mechanism that is NIK-independent. However, some issues have not been addressed yet. Indeed, the mutations S866A–S870A within p100 abolish NIK-mediated and Tax-mediated recruitment of IKKα [15,17]. Thus, either these mutations disturb the conformation of p100 and prevent the binding of IKKα, or they prevent specifically the phosphorylation on both serine 866 and 870. In the latter case, it is not known which kinase mediates Tax-induced p100 phosphorylation. In addition, although Tax targets IKKα to p100, the way Tax activates IKKα's kinase activity is still unknown. Another virus, the Kaposi's Sarcoma associated Herpes Virus (KSHV) has quite functional similarities with HTLV-1. Through the expression of its viral protein K13/v-FLIP, KSHV induces p100 processing through the recruitment of IKKα to p100 in a NIK-independent fashion (Table 1) [38]. Thus, HTLV-1 and KSHV have developed a strategy that bypass NIK but rather involves an IKKγ-containing complex as a source of activated IKKα for inducing p100 processing (Fig. 3). However, the Epstein-Barr virus (EBV) rather mimics TNFR-mediated p100 processing via the expression of its viral protein LMP1 (Fig. 2) [12–14,16]. LMP1 is a six membrane-spanning domains with a long C-terminal cytoplasmic tail that oligomerizes in the plasma membrane without ligand binding. Along the cytoplasmic tail of LMP1, the subdomain carboxyl-terminal activation region 1 (CTAR1) is the start point for the alternative pathway [16]. Genetic models allowed to demonstrating the requirement of NIK and IKKα, but not IKKβ and IKKγ, for the induction of the

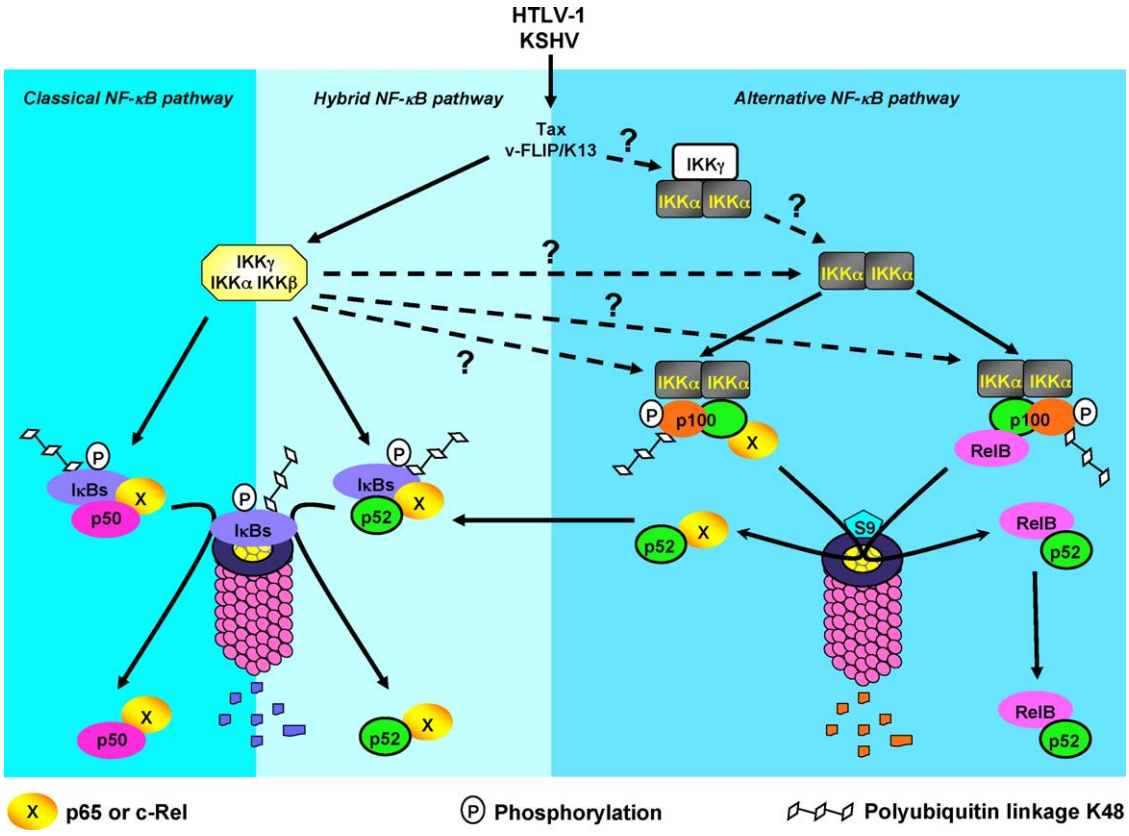

Fig. 3 – Model for the activation of the classical and the alternative NF-κB pathways by viral proteins like Tax and v-FLIP/K13. In this model, the viral proteins bypass NIK and rather activate p100 processing through either the high molecular weight IKK complex or might use a different IKK complex containing solely IKKγ and IKKα. In either case, the viral infection leads to the activation and to the recruitment of IKKα to p100 for its $SCF^{\beta\text{-}TrCP}$-dependent processing. Although not pictured here, Tax induces also p100 processing through an $SCF^{\beta\text{-}TrCP}$-independent mechanism that would occur in the nucleus [15,148].

alternative pathway, suggesting that LMP1 mimics TNFR members (Table 1).

The Gram-negative bacterium *Helicobacter pylori* is, so far, the only prokaryotic pathogen known to inducing the alternative pathway [39]. Using co-culture experiments, Omata and co-workers have shown that the *H. pylori* strain TN2 activates the alternative pathway in B lymphocytes, but not in gastric epithelial cells. However, the induction of p100 processing is blocked in *aly/aly* B cells and in siRNA IKKα transfected B cells (Table 1). Thus, *H. pylori* infected B cells seem to follow the same rules as TNFR members for p100 processing (Fig. 2). In order to determine how *H. pylori* triggers the activation of p100 processing, heat-inactivated TN2 bodies and *H. pylori* LPS were incubated with the IM-9 B cell line. It appeared that both agents induced the processing of p100. The nature of the heat-resistant agent that requires NIK and IKKα for the induction of p100 processing is unknown, but LPS might not be the good candidate since another study claims that LPS induces the generation of p52 through a co-translational mechanism (see Section 2.2.3) [40].

2.2.2. *Constitutive processing of p100*

Constitutive processing of p100 has been found in various lymphomas associated to *nfkb2* rearrangements. Such genetic alterations always result in generation of C-terminal truncated p100 mutants that lack the PID domain (see Keutgens et al., this issue). Thus the mechanisms that control the constitutive processing of truncated oncogenic p100 and full-length p100 appear to be different in certain aspects. First, mutations within the nuclear localization signal (NLS) of C-terminal truncated p100 mutants (such as HUT 78 or LB40) abolish drastically their constitutive processing while they only marginally affect NIK-induced full-length p100 processing [41]. Thus, nuclear translocation of truncated p100 seems to be required for their constitutive processing. The constitutive processing of truncated p100 does not require IKKβ, IKKγ and NIK but seems to involve IKKα, although the difference between IKKα-deficient cells and IKKα-deficient cells reconstituted with wt IKKα is rather weak [20]. The function of IKKα would be to phosphorylate the serines 99, 108, 115 and 123 within the N-terminal region of truncated p100 while the nuclear shuttling of truncated p100 seems to be an IKKα-independent event [20]. However, it is unknown whether the cleavage of truncated p100 occurs in the nucleus or whether truncated p100 needs to acquire post-translational modifications in the nucleus, which are required for the cleavage in the cytoplasm. As expected, β-TrCP is dispensable for the constitutive processing of truncated p100 mutants since *nfkb2* rearrangements remove its target site [32]. Surprisingly, in overexpression system truncated p100

mutants do not display significant ubiquitination [9]. Therefore, from these observations, one could question the putative use of proteasome inhibitors for the treatment of diseases associated to aberrant expression of truncated p100 proteins.

2.2.3. *Co-translational processing of p52*

Although, most studies support a model involving a major role for post-translational processing of p100 via the ubiquitin-proteasome system, a few studies claimed that, like for the *nfkb1* gene product p105/p50, p52 is generated in a co-translational manner involving proteosomal processing. The generation of p52 is dependent on the GRR and its location determines the site of processing in nascent p52 [19]. Analyses of a series of nested C-terminal truncated mutants of the *nfkb2* gene indicated that sequences located downstream of the amino acid 454 were dispensable for p52 production *in vivo*. In other respects, the limiting rate of the co-translational generation of p52 is conferred by the peptide sequence located between the GRR and the cleavage site of p52. How inhibitors of the proteasome prevent the processing of nascent p52 is still puzzling. A piece of the puzzle came out from a study by Mordmüller et al. The authors showed that, while treatment of wild type cells with LIGHT or LPS results in the co-translational production of p52, the same treatment applied to IKKγ-deficient cells, or cells expressing the IκBα super-repressor, fails to increase the level of p52 [40]. These results showed that co-translational production of p52 depends on the activation of the classical NF-κB pathway, which is implicated in *de novo* protein synthesis, in particular p100. Thus, the first conclusion that can be drawn is that proteasome inhibitors block the degradation of IκBα and, therefore, the induced *nfkb2* gene transcription and subsequently of the co-translational production of p52. Yet, it does not rule out that the proteasome is not directly involved in the cleavage of nascent p52. Analyses of motifs involved in ubiquitin-mediated processing of p105 revealed that two lysine residue located downstream of the GRR are targeted for ubiquitination-mediated proteolytic cleavage of nascent p50 [42]. Intriguingly, no lysine residue is present within the minimal region (from the end of the GRR to amino acid 454) required for the co-translational generation of p52. From this observation, it is hard to conceive how the proteasome could be directly involved in the processing of nascent p52. Interestingly, it has been shown that LPS-induced Relish (the drosophila p100/p105 homolog) cleavage does not require the proteasome, but rather the caspase protease Dredd [43]. Whether a caspase-like activity is involved in p100 processing remains to be determined. Whatever cleaves p100 to p52, are NIK and IKKα involved in the co-translational generation of p52 *in vivo*, and what are the biological functions of this mechanism, are certainly two important questions. Although, $nik^{-/-}$ and $ikk\alpha^{-/-}$ mice are useless to address these questions, recently the use of genomewide ENU mutagenesis screen allowed Starr and co-workers to obtain a mouse strain that harbours a premature stop codon between the two serine within the NIK phosphorylation sites at the C-terminal part of p100 (Tucker et al., personal communication). These mice display the same defects of secondary lymphoid organ development than that of $ltbr^{-/-}$ and $nik^{-/-}$ mice. These results reveal that the co-translational generation of 52 is not sufficient to compensate the LTβR-NIK axis involved in post-translational processing of p100 required for proper secondary lymphoid organ development. It does not rule out that NIK and IKKα play a role in the co-translational generation of p52 but future studies are required to clarify this particular point. Moreover, it remains to determine under which physiological conditions the co-translational generation of p52 is relevant *in vivo*.

2.2.4. *Positive and negative regulators of the alternative NF-κB pathway*

One of the common features of inducers of the alternative NF-κB pathway described above is their property to co-induce the classical NF-κB pathway. An additional degree of complexity exits at the level of the cross talk linking both pathways, which has been first described for the LTβR [10]. In wild type MEFs, the first cascade activated is the classical pathway that leads to the translocation of dimers like p50/p65 and transcription of its numerous target genes, one of them being *nfkb2* [10,44]. Indeed, LTβR-activated p65- or IKKβ-deficient MEFs display a lower level of p100 and subsequently of p52 proteins than that of LTβR-activated wt MEFs. Therefore, activation of the classical NF-κB pathway may feed the alternative pathway to ensure sufficient synthesis of p100 and production of its cleaved product p52 (Fig. 4). Conversely, p52 homodimers could potentially downregulate, in a dose-dependent manner, the transcription of the *nfkb2* gene under the control of p50/p65 [45].

De novo synthesis and/or stabilization of positive regulators of the alternative pathway would be a way to ensure the production of p52. Indeed, a recent study has demonstrated that stimulation of M12 B cells with either an agonist anti-CD40 antibody or with recombinant BAFF induces the stabilization of NIK [30]. In this model, NIK would be rescued from TRAF3-mediated degradation through the proteasome. These results could be one explanation, among others, for the inhibitory effects of cycloheximide on receptor-induced p100 processing described earlier [11,27]. Thus, TRAF3 appears to be a negative regulator of CD40- and BAFF-induced p100 processing. On the other hand, TRAF2 and TRAF5 behave as positive regulators for TWEAK and CD40-induced p100 processing in MEFs and in CD40-expressing 293T cell line, respectively [46,47]. Surprisingly, conditional TRAF2-deficient B cells display an elevated basal p100 processing [48]. Thus, the differences between primary B cells and transformed cell lines clearly emphasize the importance of *in vivo* models to understand the regulation of TRAF-mediated p100 processing. Other intriguing results emerged from the generation of TRAF3/p100 double knock out mice. While TRAF3 disruption leads to postnatal lethality, $Traf3^{-/-}p100^{-/-}$ progeny live into adulthood [49,50]. How the absence of p100 can rescue TRAF3-mediated lethality is puzzling. While it has been demonstrated, in cultured cell lines, that TRAF3 has a negative regulatory function on the mechanism leading to p100 processing, one could argue that $Traf3^{-/-}$ mice die due to a hyper-activation of p52-containing complex. However, mice lacking the C-terminal ankyrin repeats domain of p100 do not share major phenotype similarities with $Traf3^{-/-}$ mice and die around 10 weeks of age from gastric hyperplasia [51]. Interestingly, p100, but unlikely p52, would participate in a deleterious process generated by the absence of TRAF3. Future studies are awaited to clarify the fine tuning between TRAF3

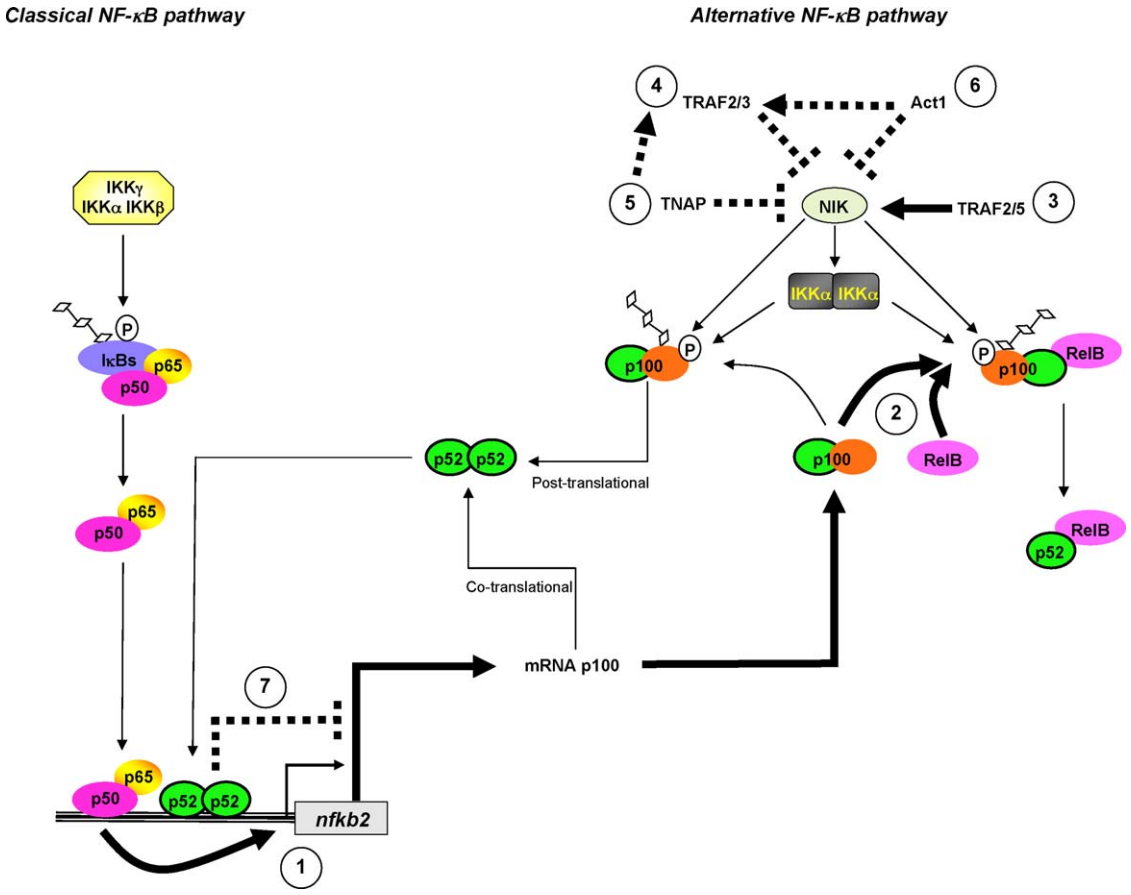

Fig. 4 – Putative positive and negative regulatory mechanisms controlling p52/RelB generation. Positive regulations are drawn as thick arrows (numbered 1–3) and negative regulation as thick dashed line (numbered 4–7). (1) Activation of the classical NF-κB pathway controls the level of transcription of the *nfkB2* gene through activation of IKKβ and p50/p65 [10]. (2) RelB requires the phosphorylation of its Ser 368 for its dimerization with p100 and stabilization of the latter, leading to a pool of p100/RelB intended to the generation of p52/RelB [58]. (3) TRAF2 and TRAF5 regulate positively the alternative pathway through NIK and IKKα for the production of p52/RelB, at least in MEFs expressing Fn14 [47]. (4) In other settings such as BAFF-R and CD40-expressing B cells, TRAF2 and/or TRAF3 inhibit p100 processing by targeting NIK for proteasomal degradation [30,48]. (5) TNAP (TRAFs and NIK-associated protein) binds TRAF2 and TRAF3 and could potentiate TRAF's inhibitory function and thus NIK turn-over, or could inhibit NIK kinase activity independently of TRAF proteins [55]. (6) The absence of Act1 leads to an elevated p100 processing. Act1 preferentially binds TRAF3 and both might cooperate to dampen CD40- and/or BAFF-R-mediated NIK kinase activity. Alternatively, Act1 could mediate its negative function in a TRAF-independent manner [54]. (7) Processing of free p100 generates transcriptionally inactive p52 homodimers that could downregulate *nfkb2* transcription by competing with p50/p65 for the same κB site.

and p100. Thus, the role of TRAF proteins in the alternative NF-κB pathway seems to be specific for each TNFR-related proteins and probably relies on the nature of particular TRAF oligomers.

Again, unexpected results came from the Act1-deficient mice. Although Act1/CIKS has been first described as a JNK- and NF-κB -activating protein in overexpression systems, Act1-deficient B cells show a stronger activation of both the classical and the alternative NF-κB pathway [52–54]. The increase of peripheral B cells survival, which culminates in lymphadenopathy, splenomegaly, hypergammaglobulinemia and autoantibodies production, observed in *Act1*$^{-/-}$ mice can be rescued by generating Act1/CD40 or Act1/BAFF-R double knock out mice. This result would imply that Act1 acts as a negative regulator in CD40- and BAFF-mediated B cell survival. Yet, it is not clear which NF-κB signalling contributes to the phenotype of Act1-deficient mice. Whatever the regulatory function of Act1 might be, future studies need to be carried out to resolve the discrepancies between the early biochemical studies on Act1/CIKS and the phenotype of *Act1*$^{-/-}$ mice.

Recently, a novel NIK-interacting protein called TNAP (TRAFs and NIK-associated protein) has been discovered through a yeast two-hybrid screening [55]. TNAP overexpression suppresses NIK kinase activity, and subsequently p100 processing, p65 phosphorylation, and IκBα degradation. In addition, lentivirus TNAP shRNA-infected cells stimulated with either CD40L, $LT\alpha_1\beta_2$ or LPS display a higher rate of p100 processing than that of control cells. Besides this interesting finding, it remains to determine what physiological role TNAP might play.

On the other hand, NF-κB2/p100 is known to be the main inhibitor of RelB [21,56]. In most resting wild type cells, p100 has a very long half-life [57]. Nevertheless, in the murine RelB-deficient S107 plasmacytoma cell line p100 has a very short half-life but p52 is highly expressed [58]. Reintroduction of wt RelB reduces strongly the pool of p52 and stabilizes p100. Moreover, RelB-mediated p100 stabilization requires the Ser 368 of RelB. Thus, the level of RelB expression seems to directly correlate with the amount of stable p100 protein but inversely correlates with the level of p52. These results would favour a model in which, in the absence of RelB, the processing of p100 is co-translational and give rise mainly to p52, whereas when RelB is present it may associate with the nascent p100, thus preventing the premature cleavage into p52. Therefore, RelB seems to behave as a double-edge sword for the generation of p52. Indeed, free p100 precursors would produce high amount of p52 through ~~the~~ a co-translational processing, whereas RelB-p100 complexes would generate p52/RelB through the NIK-IKKα axis.

3. Biological functions of the alternative NF-κB pathway

The biological functions controlled by the alternative NF-κB pathway are truly a difficult task to analyze because redundancies between p50-containing complexes and p52-containing complexes occur frequently for the control of NF-κB target genes expression. Nevertheless, some particular biological functions seem to be strictly dependent on the activation of the alternative NF-κB pathway and the generation of p52. Herein are described the main biological functions that are regulated, to some extent, by the alternative NF-κB pathway.

3.1. *Thymic organogenesis and self-tolerance*

The thymus is the primary lymphoid organ for the establishment of self-tolerance and therefore it is important to understand how this process is regulated [59]. The development and organization of the thymus are the result of an interplay between maturing thymocytes and epithelial and dendritic cells. Within the thymic stroma, distinct subsets of antigen-presenting cells (APCs), such as cortical thymic epithelial cells (cTECs) and medullary TECs (mTECs), thymic dendritic cells (DCs) and macrophages, each presenting unique sets of self-peptides, contributes to the diversity of self-antigen. The expression of self-antigens is regulated through an unorthodox phenomenon, termed promiscuous gene expression, and is a particular and possibly unique feature of TECs, especially mTECs. The latter can be further divided into subsets according to their phenotype and their level of autoimmune regulator (AIRE) expression, with the most mature mTECs expressing the highest level of AIRE protein. The differentiation of TEC precursors to immature mTECs, and then to mature mTECs requires, among other players, inducers and intermediates of the alternative NF-κB pathway. Indeed, *ltbr*$^{-/-}$ mice display a disturbed thymic microenvironment with malformed mTECs, which results in the retention of mature thymocytes and autoantibody production, suggesting key roles for LTβR signalling in thymic lymphocyte homeostasis and central tolerance induction [60]. These mice have a reduced expression of AIRE that seems to be proportional to the reduced number of mTECs, although another study claimed that the reduced level of AIRE expression is directly linked to a reduced LTβR-mediated *Aire* gene transcription [61]. Surprisingly, the phenotype of *ltbr*$^{-/-}$ mice is more severe than that of the *ltb*$^{-/-}$*light*$^{-/-}$ mice that lack both known LTβR ligands. This would indicate that additional, so far unknown, ligand(s) partially compensate for the absence of LTβ and LIGHT in the thymic medulla [60]. Because NIK mutant *aly/aly* mouse strain and IKKα-deficient mice share similar defects than that of *ltbr*$^{-/-}$ mice, we could speculate that the NIK-IKKα axis downstream of the LTβR might constitute an essential step in thymic organogenesis [62,63]. However, NIK$^{aly/aly}$ mice show more severe phenotypes within thymic structures than LTβR-deficient mice do, suggesting that it would be reasonable to speculate that the axis NIK-IKKα acts downstream of an additional receptor(s) beyond LTβR [60]. Although expected, it is not proven yet that p52/RelB functions downstream of the cascade LTβR-NIK-IKKα along the differentiation process of mTECs. Indeed, whereas mature mTECs are highly reduced in number in the case of LTβR- and NIK-deficient mice, inactivation of RelB apparently results in a complete blockade of mTECs differentiation (and Aire expression) [60,64–66]. Nevertheless, we cannot rule out a role for RelB in mature mTECs downstream of LTβR, or that other p52-containing complexes play in the game as well, such as p52/p52/Bcl3. At the present time, it is not known whether RelB and Bcl3 compensate for each other for the maturation of mTECs, through the association with their main partners that are p52 and p50. The pleiotropic effects of LTβR, NIK and RelB on *Aire* gene expression, the formation of the thymic microenvironment and the peripheral lymphoid system, make it difficult to determine the effects exerted at each level for the establishment of self-tolerance. Further studies will certainly bring new insights on the role of each player acting downstream of the alternative NF-κB pathway.

3.2. *Secondary lymphoid organ development*

The development of lymphoid tissues, in particular lymph nodes (LNs) and Peyer's patches (PPs), involves at early stage the interaction between haematopoietic progenitor cells (called inducer cells LT$\alpha_1\beta_2^+$RANKL$^+$RANK$^+$IL7Rα$^+$CXCR4$^+$CXCR5$^+$CCR7$^+\alpha_4\beta_1^+$CD45$^+$CD4$^+$CD3$^-$) and mesenchymal progenitor cells (called organiser stromal cells LTβR$^+$VCAM1$^+$ICAM1$^+$MadCAM1$^+$IL7$^+$) [67,68]. These cells express on their cell surface TNFR/TNFL family members that are able to induce both the classical and the alternative NF-κB pathway (Fig. 5) [69]. The first signalling pathway that has been shown to be crucial for lymphoid organogenesis was related to the LTβR [70]. LTβR-deficient mice lack all LNs, PPs and display a disturbed splenic architecture [71–73]. So far, two ligands can trigger LTβR signalling, LT$\alpha_1\beta_2$ and LIGHT. LIGHT-deficient mice develop all LNs, which indicates that LT$\alpha_1\beta_2$ signalling through LTβR has a dominant role in lymphoid organogenesis. However, *light*$^{-/-}$*ltb*$^{-/-}$ mice are still able to develop mesenteric and cervical LNs, suggesting that an additional ligand binds to LTβR [74]. All

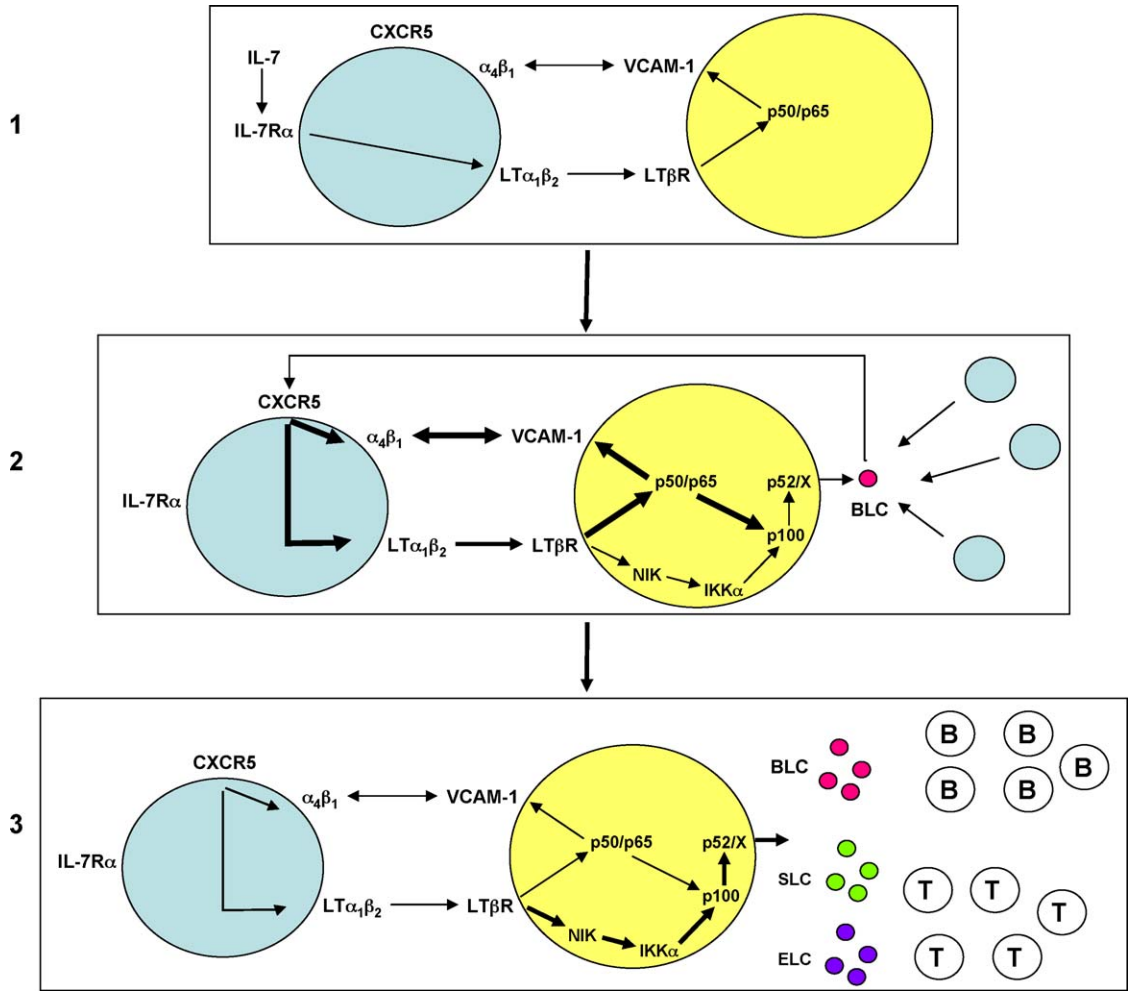

Fig. 5 – Model for lymph nodes and Peyer's patches development. (1) IL7-Rα$^+$ CXCR5$^+$ $\alpha_4\beta_1^+$ inducer cells stimulated via IL-7 express LT$\alpha_1\beta_2$, which binds LTβR on organizer cells activating the classical pathway and p50/p65. The latter stimulates VCAM-1 expression and contributes to the interaction with $\alpha_4\beta_1^+$ inducer cells. (2) Overtime the activation of the LTβR turns on the alternative pathway that allows to expressing BLC. A positive-feedback mechanism takes place via BLC and its receptor CXCR5 on inducer cells. Signalling via CXCR5 leads to the upregulation of $\alpha_4\beta_1$ and LT$\alpha_1\beta_2$ reinforcing the cellular attachment to VCAM-1 expressing organizer cells while inducing further production of BLC. Focal sites of elevated BLC concentrations probably attract additional inducer cells. (3) At latter stage of organogenesis, the alternative pathway controls the expression of other chemokines such as ELC and SLC. BLC, and both SLC and ELC, are responsible for the recruitment of mature B and T cells, respectively, into the developing lymphoid organ, where they segregate into distinct B- and T-cell zones.

LNs, PPs and splenic microarchitecture fail to develop properly in *aly/aly* and *nik*$^{-/-}$ mice [75,76]. These results provided genetic evidences for a role of LTβR upstream of NIK and were recently corroborated through biochemical studies on LTβR signalling [10]. Apart from LTβR, *rank*$^{-/-}$ and *rankl*$^{-/-}$ mice develop PPs and normal splenic microarchitecture, but LNs do not develop due to a reduced number of inducer cells and a downmodulation of LT$\alpha_1\beta_2$ expression [77–80]. RANKL-dependent LNs formation depends on the expression of LT$\alpha_1\beta_2$ because transgenic expression of RANKL cannot rescue LNs formation in *lta*$^{-/-}$ mice [81]. Because the RANKL-RANK axis has been shown to induce NIK, and thereby the alternative NF-κB pathway, in addition to acting downstream of LTβR in organizer cells, NIK might act upstream of LTβR-expressing organizer cells by providing sufficient numbers of inducer cells bearing LT$\alpha_1\beta_2$. However, induction of LTβR by injection of a specific agonistic monoclonal antibody does not induce LNs formation in RANK-deficient mice. This indicates that RANK and LTβR are required for LNs development and that they regulate two independent developmental processes. Because RANK and LTβR activate both the classical and the alternative NF-κB pathway, the question is who plays first in the cascade of events that leads to the formation of secondary lymphoid organ (SLO). Analyses across phenotypes of various mice deficient for particular NF-κB subunits reveal that p65 might act at early stage of LNs development. A number of potential roles for p65 can be envisioned in the development of LNs. Although the latter do not develop in *rela*$^{-/-}$*tnfr1*$^{-/-}$ mice, they have normal numbers of inducer cells, which suggest that the defect is not downstream of RANK [82]. Biochemical studies have shown that

LTβR-induced p50/p65 is regulated through the classical NF-κB pathway [10,29]. In addition, p65 is absolutely required for LTβR-induced VCAM1 transcription [10]. In this scenario, the absence of p65 would not allow the induced expression of adhesion molecules (e.g. VCAM1) required for a strong interaction between organizer cells and $\alpha_4\beta_1^+$ inducer cells, which leads to aggregation of inducer and organizer cells prior full SLO development (Fig. 5). In addition, activated p65 induces p100 production and feeds the alternative NF-κB pathway. At this point, another actor, RelB, plays its specific roles along the alternative NF-κB pathway downstream of LTβR. Indeed, although RelB-deficient mice have normal numbers of inducer cells, at least a subset of LNs, which can vary in the extent of cellularity, are detectable shortly after birth, as in $ikk\alpha^{AA}$ knock-in mice [64,65,68,83,84] (Paul Rennert, personal communication). Thus, activation through IKKα of RelB-containing complexes seems to be required for full development of most LNs. Indeed, we have shown that the axis LTβR-NIK-IKKα in stromal cells controls the induction of chemokines such as, BLC (CXCL13), ELC (CCL19) and SLC (CCL21) [10]. Focal sites of elevated BLC concentrations attract additional $CXCR5^+$ inducers cells and at later stage, ELC and SLC are responsible for the recruitment and the positioning of mature T cells into the developing lymphoid organ. RelB orchestrates its genetic program with its DNA-binding partners p50 and p52. While p50-deficient mice do not display LNs developmental defects, inguinal and popliteal LNs are strongly reduced in size in p100/p52-deficient mice, whereas they have relatively normal axillary LNs [85,86]. Thus, p50/RelB could compensate for axillary LNs development in $nfkb2^{-/-}$ mice. Indeed, $nfkb1^{-/-}nfkb2^{-/-}$ mice lack all LNs [87]. Although BLC, SLC and ELC are important for fulfilling lymphoid organ formation downstream of the alternative NF-κB pathway, they cannot support by themselves the full developmental process because their combined absence does not completely block the formation of all LNs [88,89].

At this point, the differences in LNs development between, on one hand $nik^{-/-}$ and $p65^{-/-}$ mice, and on the other hand, $relb^{-/-}$ and $ikk\alpha^{AA}$ mice, could suggest that NIK acts through the straight pathway LTβR-NIK-IKKα-p52/RelB, as well as to a separate LTβR-NIK-p65 pathway. A few scenarios might be envisioned to explain the similar phenotype of NIK-deficient mice and p65-deficient mice. First, NIK could control p65 nuclear translocation through the classical pathway but most studies have demonstrated that IκBα degradation is not altered in LTβR-activated NIK-deficient cells or in *aly*/*aly* MEFs [10,29,76]. Rather, it has been proposed that NIK mediates LTβR-induced transactivating activity of p65 [76]. In this study, mRNA expression of IκBα and MCP1 have been analyzed in wt and NIK-deficient MEFs 8 h after LTβR stimulation. It is known that rapid activated p50/p65 dimers bound to a subset of target promoters are gradually replaced by slower activated p52/RelB dimers, as observed for IκBα ([90] and Dejardin, unpublished results). Therefore, the absence of detection of IκBα and MCP1 mRNA at late time points could result from the inability of NIK-deficient cells to activate p52/RelB in response to LTβR stimulation. In order to resolve this issue, expression of those two target genes should be carefully analyzed at early time points in order to confirm or to rule out the role of NIK in p65 transcriptional activity. Alternatively, the absence of NIK could prevent the generation of p52/p65. Since p52-deficient mice have a milder LNs developmental defect, it is assumed that p50/p65 compensates. In this situation, this would imply that inactivation of NIK leads to a blockade of p50-containing complexes. So, it is likely possible that when NIK-deficient organizer cells are induced by $LT\alpha_1\beta_2$, only the classical NF-κB pathway is rapidly triggered and p50/p65 allow to turn on target genes like VCAM1 and p100. Because p100 cannot be processed, the precursor accumulates overtime into the cytosol and becomes a super-repressor by competing with other IκB-like molecules for their pre-bound NF-κB complexes. It is noteworthy that, in different settings, it has been shown that elevated p100 can take away p50/p65 complexes from IκBα to form a triple complex p100/p50/p65 [21,91]. In addition, it has been recently shown that p100 can act as a regulatory brake for the activation of naïve T cells by limiting nuclear translocation of p50/p65 [92]. Thus, in $nik^{-/-}$ cells, p50/p65 complexes associated to p100 could become refractory to LTβR activation. As a consequence, the inability to sustain NF-κB-induced VCAM1 expression would block further interactions between $\alpha_4\beta_1^+LT\alpha_1\beta_2^+CXCR5^+$ inducer cells and $VCAM1^+LT\beta R^+BLC^+$ organizer cells, and thus would prevent further LTβR activation. Therefore, the absence of NIK in stromal cells could lead to a supra-accumulation of p100, which culminates in a complete blockade of NF-κB.

The involvement of NF-κB in the development of mucosa-associated lymphoid tissues (MALT) is slightly different. The lack of PPs in LTβR-, NIK-, IKKα-, p52- and RelB-deficient mice further emphasizes the role of the alternative NF-κB pathway in SLO formation downstream of LTβR, with the particularity that RANK signalling is not involved. Similar to LNs development, inactivation of particular intermediates of the alternative NF-κB pathway impact on different stages of PPs development. However, for nasal-associated lymphoid tissue (NALT) development, the alternative NF-κB pathway is not required for the initiation but rather for the maturation [93] (see Ref. [69] for further details).

While none of the NF-κB subunits are required for the development of the spleen, almost all of them play, to some extent, a role in the maintenance of its microarchitecture [69]. It is noteworthy that, p50 is dispensable for the formation of most splenic microdomains like germinal centers, FDC networks, but required for marginal-zone B cells, whereas the axis LTβR-NIK-IKKα-p52/RelB is absolutely required [69,94]. Another level of complexity is reached in splenic microarchitecture development because an additional activator, that is Bcl3, can associate with its DNA-binding partners p50 or p52 and fulfils functions that cannot be compensated by other NF-κB dimers [95–97]. However, while some LTβR-induced p52/RelB target genes have been identified in the spleen, such as, BLC, ELC and SLC, so far no p52/p52/Bcl3 target genes have been formally characterized by chromatin immunoprecipitation assays [10,98]. Finally, the role of p65 in spleen development is not well characterized because rescued $p65^{-/-}tnfr1^{-/-}$ mice and $tnfr1^{-/-}$ mice have similar defects [82,99].

Altogether, we can conclude that inducers and intermediates of the alternative NF-κB pathway play key roles in SLO formation, but further studies are required to better understand how they regulate the fine-tuning of gene expression programs necessary to build up a functional lymphoid organ.

3.3. *B cell development, survival and homeostasis*

B lymphocytes develop in bone marrow in a series of steps going from precursor B cells to pro- and pre-B cells, which finally lead to BCR-expressing immature B cells. When immature B cells leave the bone marrow to reach the spleen they mature through different stages. They start at a transitional stage T1 to progress to T2 into follicules. From there, they become either resident marginal zone B cells (MZB) or mature follicular B cells (also called conventional B cells or B_2 cells), which enter the circulation and migrate to secondary lymphoid organs to become activated B cells [94,100].

In order to address the role of NF-κB in B cell development from mice progeny that die *in utero*, bone marrow chimeras transfer of NF-κB-deficient foetal liver cells into irradiated wt recipient mice had to be carried out [101]. It turned out that haematopoietic precursors from p50-p65 double KO mice failed to generate $B220^+$ B cell precursors (pro/pre B cells) after transfer (Fig. 6) [102]. However, this defect is not cell autonomous because p50-p65-deficient $B220^+$ cells can be detected in the periphery of mice co-reconstituted with normal haematopoietic precursors. This implies that NF-κB might regulate the development and/or survival of B cell precursors through regulation of an extracellular factor. Although still unidentified, this factor could counteract TNF-induced apoptosis as IKKβ-TNFR1 double KO mice are rescued for lymphopoiesis [103]. The intrinsic roles of IKKβ and IKKγ in B cells development have been also investigated by conditional gene targeting and by the use of an *in vitro* differentiation system as well [104,105]. Specific ablation of IKKβ or IKKγ does not alter the development of bone marrow B cells but leads to a profound reduction in numbers of subsets of splenic B cells, like follicular B cells and marginal zone B cells. Therefore, these results confirm a B cell-extrinsic role for the classical NF-κB pathway in survival of bone marrow precursors, while they highlight a B cell-intrinsic role for the classical NF-κB pathway in survival of specific splenic B cells. Intermediates of the alternative pathway are also involved in the control of survival and maturation of splenic B cells. Analyses of *aly* mice revealed an autonomous B cell defect that resulted in a strong reduction (but not total) of MZB and follicular B cells [106–108]. Similar conclusions have been drawn from experiments with bone marrow chimeras from $ikk\alpha^{-/-}$ or $ikk\alpha^{AA}$ mice [8,109]. Thus, both the classical and the alternative NF-κB pathway seem to contribute to the survival and maturation of transitional T2 cells.

Splenic B cell survival is mainly dependent on signalling pathways downstream of two receptors that are BCR (B cell receptor) and BAFF-R [94]. While both of them have the ability to activate the classical pathway, BAFF-R can activate the alternative NF-κB pathway as well [11]. The work of Siebenlist and co-workers nicely shed light on the genetic link between BAFF-R and the alternative pathway. Indeed, using *aly* mice and $nfkb2^{-/-}$ mice they showed that NIK and activated p52-containing complexes mediate BAFF-induced transitional T2 B cell survival and maturation (Fig. 6). In addition, analyses of $nfkb1^{-/-}$, $nfkb2^{-/-}$ and $nfkb1^{-/-}nfkb2^{-/-}$ bone marrow cells cultured *in vitro* with recombinant BAFF revealed that its pro-survival activity is either mediated through the classical and the alternative NF-κB pathway or solely via the alternative pathway through a compensatory mechanism involving p50 [11]. One mechanism regulating BAFF-mediated transitional B cell survival is the up-regulation of the anti-apoptotic protein Bcl-2. At later stage, other anti-apoptotic NF-κB target genes, such as A1 and $Bcl\text{-}_{XL}$, are required for BAFF-mediated mature B cells survival [110]. The nature of p50 and/or p52 dimers that mediate the anti-apoptotic activity of BAFF has not been formally identified but there is a likely possibility that these dimers contain c-Rel and/or p65 since mice deficient for both c-Rel and p65 exhibit a relative complete blockade in T1 [111]. While both the classical and the alternative pathways are required for progression of transitional B cells, it is neither

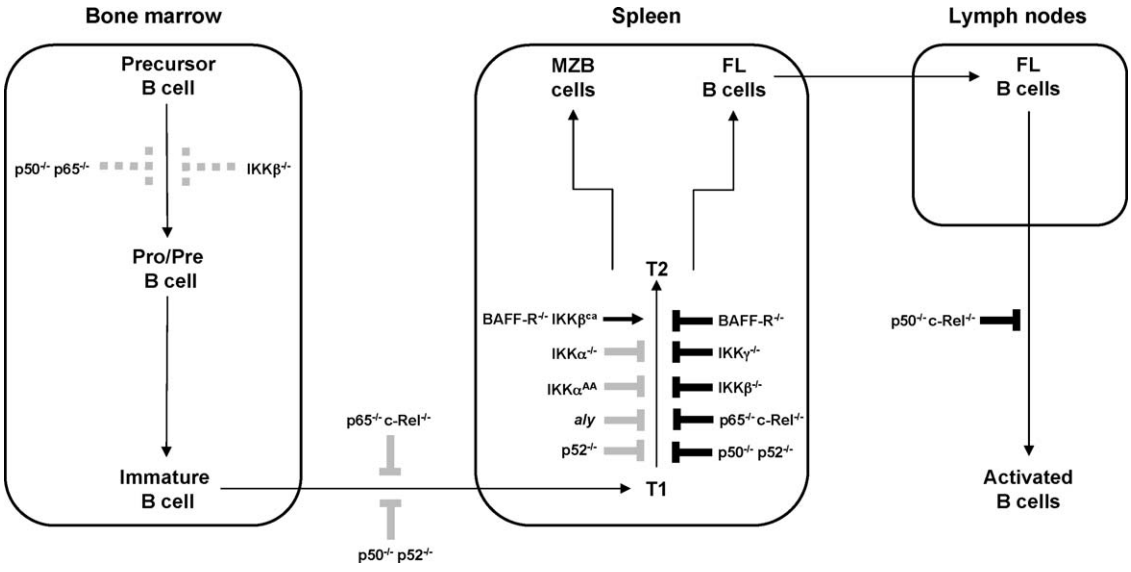

Fig. 6 – Role of the classical and the alternative pathway in NF-κB-mediated B cell development, maturation and survival *in vivo*. Plain bars represent a B cell autonomous defect and dashed bars a non-B cell autonomous defect. Black bars mean that maturation beyond a particular stage does not occur and grey bars mean that maturation can occur but is markedly decreased. Thick arrows display the recovery of B cells maturation at a particular stage.

clear which pathway acts first, nor it is obvious which receptors control the activation of the classical pathway. Recently, an attempt for evaluating the role of the classical pathway in developing B cell has been conducted by creating a mouse strain with a conditional knock-in of a constitutive active IKKβ (IKKβ^{ca}), for which both phosphoacceptors within the kinase loop have been substituted by glutamic acid (IKKβ^{EE}) [112]. When these mice are crossed on a *baffr*$^{-/-}$ genetic background, T1 to T2 transition is restored, so are MZB and follicular B cells. However, p100 processing is not elevated in *ikkβ*^{ca} B cells. Thus, under this forced and constant activation of the classical NF-κB pathway, the alternative pathway requirement is likely circumvented. Thus, the classical pathway downstream of BAFF-R is likely to be important but in these conditions its biological functions are probably overestimated. Knock-in mice with a BAFF-R that bears specific point mutations that abrogate either the classical or the alternative NF-κB pathway would certainly be an elegant way for assigning the precise biological functions of each pathway downstream of BAFF-R. The role of CD40 in B cell biology and the intricate connection between both NF-κB pathways are still poorly characterized [27]. Through the use of mice deficient in one or more NF-κB subunits it has been shown that particular NF-κB subunits control cell survival, proliferation and homotypic aggregation downstream of CD40 [113]. Yet, it is not known precisely how the classical and the alternative pathway contribute to CD40's biology. Further studies are certainly required to determine to what extent the alternative NF-κB pathway has a role in governing B cell survival, maturation and homeostasis.

3.4. *Osteoclastogenesis*

Bone remodelling involves synthesis of organic matrix by osteoblasts (derive from mesenchymal stem cells) and bone resorption by osteoclasts (originate from haematopoietic monocyte/macrophage precursors). Any disturbance between these two developmental processes leads to skeletal abnormalities characterized by increased (osteopetrosis) or decreased (osteoporosis) bone mass [114]. Increased osteoclasts activity is also observed in many disorders, including primary bone tumours, multiple myeloma or rheumatoid arthritis. Osteoclastogenesis requires contact between osteoblasts and osteoclasts. Regulation of osteoclast formation and function is dictated by a sequential gene expression program, which involves pro-survival cytokines (M-CSF), TNFR/TNFL family members (RANK/RANKL, TNFRI/TNF), signalling adaptor proteins (TRAF6), kinases (IKKs, MPAK) and transcription factors (PU.1, AP1, NF-κB). Among these proteins, the OPG/RANK/RANKL triad is of particular interest. Basically, osteoclasts express RANK (also called TRANCE-R or ODAR) and osteoblasts express RANKL (also called TRANCE, ODF or OPGL) and its soluble decoy receptor OPG (osteoprotegerin, also called OCIF) whose function is to prevent the binding of RANKL to RANK [115]. Any disequilibrium of this tight regulation impacts on bone remodelling. Indeed, mice deficient for RANK or RANKL show normal macrophage development, but precursor cells do not commit to osteoclast causing osteopetrosis [78,79]. A similar osteopetrotic phenotype has been observed with p50/p52 double KO mice. In the process of osteoclast differentiation, the mononuclear precursors undergo a fusion that results in the formation of multinucleated osteoclasts. In p50-p52 double KO mice the differentiation appears to be arrested before fusion [116,117]. This unexpected phenotype was not visible in single KO mice, which suggests that functional redundancies between p50 and p52 occur during osteoclast differentiation [85,118,119]. Because *c-rel*$^{-/-}$ mice and *relb*$^{-/-}$*rag1*$^{-/-}$ mice do not display osteopetrotic phenotype, it is tempting to speculate that p50/p65 and/or p52/p65 complex would be master NF-κB dimers in osteoclast development [64,120,121]. Based on the general anti-apoptotic function of p65-containing complexes, this hypothesis would be in agreement with the results obtained from *ikkβ*$^{\Delta}$ mice for which interferon-responsive cells are deficient in IKKβ, including myeloid cells [122]. Indeed, *in vivo*, in the absence of IKKβ, the classical NF-κB pathway is inhibited and osteoclast precursors die from TNF-induced apoptosis. The generation of *ikkβ*$^{\Delta}$ *tnfr1*$^{-/-}$ mice allows to rescuing TNF-induced depletion of osteoclast precursors, nonetheless these cells do not commit to multinucleated osteoclast and mice still display an osteopetrotic phenotype [122]. The role of NF-κB for regulating osteoclast development and function is relevant not only in mice but also in humans. Indeed, patients with X-linked osteopetrosis carry a X420W point mutation in NEMO/IKKγ [123]. Therefore, the absolute requirement of IKKβ and IKKγ strongly suggests that the classical NF-κB pathway is crucial for osteoclasgenesis. *In vivo*, the alternative pathway seems to be dispensable for basal osteoclastogenesis because *nik*$^{-/-}$, *aly/aly* and *ikkα*^{AA} knock-in mice are not osteopetrotic [75,76,124]. However, treatment of isolated bone marrow cells from *ikkβ*$^{\Delta}$, *ikkα*^{AA} or *nik*$^{-/-}$ mice, or isolated foetal liver cells from *ikkα*$^{-/-}$ mice, with M-CSF and RANKL fails to induce osteoclastogenesis *in vitro* [91,122,125]. Thus, there is a possibility that the axis NIK-IKKα downstream of RANK plays a role in basal osteoclastogenesis *in vivo* but its function could be overcome by other osteoclastogenic signalling pathways. Indeed, cytokines like TNF or IL-1β in combination with RANKL completely rescue the osteoclastogenic defect of *ikkα*^{AA} bone marrow cells [122]. Therefore, it could be informative to cross *nik*$^{-/-}$ or *ikkα*^{AA} mice with *tnfr1*$^{-/-}$ mice for assessing the putative role of the alternative NF-κB pathway in basal osteoclastogenesis *in vivo*.

Although RANK stimulation leads to the activation of both the classical and the alternative NF-κB pathway, the role of the alternative pathway *in vivo* is not obvious and could be masked due to redundancy between NF-κB subunits (p50 versus p52) and/or compensation through other osteoclastogenic signalling pathways.

4. Diseases potentially linked to a deregulated activation of the alternative NF-κB pathway and of p100 processing

4.1. *Inflammatory disorders*

There is no doubt that cytokines and chemokines inducing or driven by the alternative NF-κB pathway are involved in inflammatory disorders. While it has been demonstrated that LTβR is a master receptor involved in the development of SLO,

there are strong evidences that the molecular mechanisms controlling tertiary lymphoid organ (TLO, also called tertiary lymphoid tissues or ectopic lymphoid structure) formation required the LTβR as well. TLO are organized lymphocytic aggregates (B- and T-cell areas) that form at sites of chronic inflammation via a process called lymphoid neogenesis (or lymphoid neo-organogenesis) [126,127]. Unlike SLO, TLO are not connected to afferent lymph vessels and are not encapsulated, which implies that they are directly exposed to stimulating antigens and pro-inflammatory cytokines. TLO arise typically in non-lymphoid locations but the identity of stromal cells initiating their development is unknown. Nevertheless, TLO formation has been observed in several mouse models of chronic inflammatory pathologies (*H. pylori*-induced gastritis, collagen-induced arthritis) but also in transgenic mice by ectopic expression of inducers or target genes of the alternative pathway [127]. For instance, constitutive tissue specific expression of LTβ, BLC, or ELC into pancreatic islets or kidney is sufficient to generate TLO [127]. It is noteworthy that TLO have been detected in a significant percentage of patients suffering of divers chronic inflammatory diseases like rheumatoid arthritis, Sjogren's syndrome, multiple sclerosis, ulcerative colitis or chronic hepatitis C [126]. Thus, inhibition of LTβR function in SLO and/or TLO could be beneficial for the treatment of chronic inflammatory pathologies. Indeed, administration of LTβR-Ig fusion proteins (acting as a decoy receptor for $LT\alpha_1\beta_2$ and LIGHT) has been successfully used in rodents disease models of collagen-induced arthritis or inflammatory bowel disease, and this approach is being tested in clinical trials [128].

Is there some room for chemical inhibitors targeting specifically signalling proteins (e.g. NIK) triggering the alternative NF-κB pathway? Although the decoy LTβR strategy seems promising, the systemic administration could have limitations for the treatment of multiple sclerosis. Specific chemical inhibitors might have a better efficiency for crossing the haematoencephalic barrier than that of decoy LTβR. On the other hand, Hepatitis C virus (HCV) core protein is able to bind the cytoplasmic tail of the LTβR and modulate its signalling [129,130]. At the present time, it is not known whether the HCV core protein can force LTβR to form multimers but if so HCV-induced LTβR activation would be ligand-independent and therefore decoy LTβR therapy would be useless.

Beside TLO formation, another hallmark of most autoimmune diseases is the exacerbated expression of the pro-survival cytokine BAFF and in these conditions it allows the survival of unwanted autoantibody producing B cells [131]. For instance, BAFF levels are elevated in the synovial fluid of patients with rheumatoid arthritis, in salivary glands of patients in Sjögren's syndrome and in the central nervous system (CNS) of mice with experimental autoimmune encephalomyelitis (EAE). Recently, tremendous efforts have been accomplished for the development of biological antagonists of BAFF, such as anti-BAFF antibody (belimumab) or decoy receptors for BAFF and APRIL (TACI-Fc) [132]. The rational for using specific inhibitors of the alternative pathway would be their power to inhibit simultaneously several TNFR mediating pathologic conditions, like LTβR and BAFF-R. In the case of the rheumatoid arthritis, putative inhibitors of the alternative pathway could also prevent inflammation-induced osteoclastogenesis. Indeed, NIK has been shown to be critical for antigen-mediated induction of bone erosion in several mouse models [133]. Thus, blockade of the alternative pathway with chemical inhibitors could have the advantage to spread the inhibition at levels of multiple effectors of chronic inflammation.

4.2. *Tumourigenesis*

There are some evidences that deregulation of p100 processing is associated to the emergence of haematopoietic and solid tumours. Indeed, truncations in the C-terminal region of the *nfkb2* gene are associated with developments of various haematopoietic tumours, including chronic lymphocytic leukaemia, multiple myeloma and cutaneous T-cell lymphoma [134–138]. Many of these *nfkb2* rearrangements encode abnormal proteins that lack part of the ankyrin repeats domain, and thereby, these proteins loose their IκB-like inhibitory properties, become mainly nuclear and give rise to a strong production of p52 [139]. Additional insights into the oncogenic role of p100/p52 have stemmed from the phenotype of $nfkb2^{\Delta CT/\Delta CT}$ mice that develop spontaneous gastrointestinal tumours [51]. Thus, at least, two mechanisms may account for the oncogenic phenotype of truncated p100 proteins: first, the overexpression and elevated DNA-binding activity of p52-containing complexes, and second, the lack of inhibitory function of truncated p100. Moreover, one of the main partners of p52, that is Bcl3, has been also originally discovered in a subset of chromosomal translocations associated to various haematopoietic tumours (see Keutgens et al., this issue). Because Bcl3 does not bind DNA by itself, p52 homodimers might associate with overexpressed Bcl3 and form an oncogenic DNA-binding complex p52/p52/Bcl3. Such a complex could play a role in breast tumourigenesis as well [140]. Indeed, p52 and Bcl3 have been found being overexpressed in human clinical breast tumour samples [21,141]. It is noteworthy that one additional task of the tumour suppressor gene p53 could be to control the putative oncogenic property of p52/p52/Bcl3. Indeed, wild type p53 can induce the association of p52 homodimers with histone deacetylase HDAC1 and the downregulation of Bcl3 protein, thus preventing the accumulation of p52/p52/Bcl3 [142]. Conversely, some tumour-derived p53 mutants induce *nfkb2* gene expression, which results in upregulation of anti-apoptotic genes and chemoresistance [143].

Interestingly, a few viruses have the ability to hijack the host's alternative NF-κB pathway for their replication and/or for mediating their oncogenic properties. The viral proteins LMP1, Tax and v-FLIP/K13 encoded by the Epstein-Barr virus (EBV), the human T cell leukaemia virus 1 (HTLV1) and the Kaposi's sarcoma herpesvirus (KHSV), respectively, can induce co-translational production of p52 and/or post-translational p100 processing [13,14,16,38]. The sustained activation of the alternative NF-κB pathway could lead to an uncontrolled proliferation and an elevated anti-apoptotic activity in HTLV1-infected T cells, KHSV-infected cells (primary effusion lymphoma), or EBV-infected cells (Hodgkin's disease, nasopharyngeal carcinoma, Burkitt's lymphoma). It is noteworthy that LMP1 signals through the NIK-IKKα axis for which IKKγ is

dispensable, while viral proteins Tax and v-FLIP/K13 signal through the IKK complex but bypass NIK. Therefore, inhibitors of NIK should not be effective for the treatment of HTLV-1- or KHSV-derived haematopoietic tumours. Moreover, among the viral proteins encoded by EBV that achieve immortalization of B cells, LMP1 is capable of constitutively signalling by mimicking CD40 pathway which culminate in the production of the anti-apoptotic cytokine BAFF [144,145].

Thus, there is a likely possibility that, in conjunction with other factors, abnormal or subverted activation of the alternative NF-κB pathway contributes to cellular transformation.

5. Concluding remark

The information provided by KO mice and derived cell lines described herein has revealed many insights into the morphogenetic and cell fate decision regulated by inducers and intermediates of the alternative NF-κB pathway. However, the results have to be interpreted with caution. Indeed, it has to be demonstrated that observed phenotypic abnormalities result from the inactivation of the gene studied and are not the consequence of an indirect disequilibrium of the intricate NF-κB/IκB network. Conditional knock-in of intermediates of the alternative NF-κB pathway should ease the understanding of their role in specific tissues or cell types. In the future, these mice will prove to be crucial reagents for developing new cellular models, for examining the roles of p52-containing complexes in different diseases and for determining which target genes are regulated by particular p52-containing dimers. It seems that targeting the alternative pathway has some therapeutic promises but much remains to be learned about the appropriate clinical use of these putative drugs, including their safety profile and their ability to synergize with other conventional or biological agents.

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