REGULATION OF THE NF-KB PATHWAY BY NEDDYLATION IN CHRONIC LYMPHOCYTIC LEUKEMIA: BEYOND IKB

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INTRODUCTION

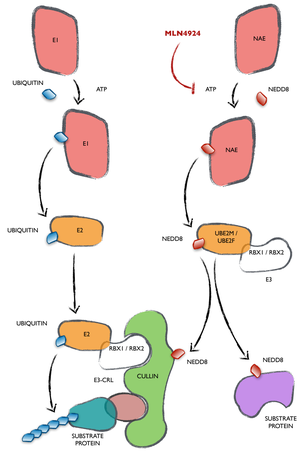
Chronic lymphocytic leukemia (**CLL**) is the most prevalent lymphoproliferative disorder in western countries. At present time, it is an incurable disease and its molecular etiology is still largely unknown.

NF-kB is a transcription factor controlling the expression of many genes involved in inflammation and cell survival, and is constitutively active in many tumors, including CLL. NF-kB can be activated by canonical and non-canonical pathways. The canonical pathway is switched on and off by controlling the degradation of its inhibitor, the protein **IkB**, that binds and sequesters the NF-kB transcription factor in the cytoplasm. The NF-kB transcription factor is a dimer that can be constituted by different proteins. The dimer activated by the canonical pathway is formed by the proteins **p65** (RelA) and p50. Several signaling pathways activated by extracellular stimuli can induce the phosphorylation of IkB at serines 32 and 36 that triggers its ubiquitination and degradation at the proteasome, releasing the NF-kB transcription factor free to translocate to the nucleus and activate survival genes.

Ubiquitin is a small protein that can be conjugated to target proteins, mainly in lysine residues, altering their functionality. As ubiquitin contains lysine residues itself, it can also be ubiquitinated, thus forming polyubiquitination chains that can be recognized as degradation tags for the target proteins. However, ubiquitin can also be conjugated as a single residue (monoubiquitination) in a reversible way, functioning as a regulatory mechanism. Moreover, ubiquitin-like proteins is a family of proteins homologous to ubiquitin that have been involved in the regulation of different cellular processes. **NEDD8**, SUMO, ISG15 and others belong to these so-called ubiquitin-like (**UBL**) proteins. UBLs are conjugated to target proteins in a three-step process catalyzed by enzymes generally termed as E1, E2 and E3. E1 proteins are responsible for the activation of the UBLs using ATP, an are specific for them. On the other hand, E3s define the target proteins, so there is a great variety of them, grouped in different structural families. The biggest family of E3s ubiquitin ligases are the Cullin-RING ligases that need to be conjugated to NEDD8 (**NEDDylated**) in their Cullin module to be functional. The regulatory role of NEDDylation is underlined by its reversibility catalyzed by de-NEDDylation enzymes like **DENP1**, as well as by the existence of NEDD8-degradation proteins like **NUB1**, that target NEDD8 and its conjugates to the proteasome.

As the inhibitor of the NF-kB pathway, IkB, is ubiquitinated by a Cullin-RING ligase, several groups have explored its inhibition by modulating the NEDDylation of its Cullin module. Indeed, an inhibitor molecule of the E1 of NEDD8 (**MLN4924-** Pevonedistat) has been used to block NF-kB in different tumor cells (referencia) and is under clinical trials. MLN4924 has been demonstrated to reduce the viability of B-CLL cells and to synergize with inhibitors of BCR-activated pathways. Somehow puzzlingly, MLN4924 induces an increment in IkBα phosphorylation accompanied by a decline in its protein levels. A better knowledge of the molecular mechanisms by which NEDDylation control NF-kB pathway will uncover new combinational and more effective treatments as well as prevent undesirable side effects.

The ubiquitination and NEDDylation pathways



RESULTS

1. IkB IS NOT OVER-MODIFIED BY UBL IN CLL, BUT OTHER MEMBERS OF THE PATHWAY ARE

Protein Name	Site	Average basal	Average MLN	Average sensitivity	Peptide
TRAF2	27	-1,5	-4,6	-3,1	TLLGTK*LEAK
TRAF2	119	-5,7	-8,6	-2,9	GTLK*EYESCHEGR
TRAF2	176	-0,9	-4,6	-3,7	APCCGADVK*AHHEVCPK
TRAF2	277	-3,0	-3,3	-0,2	CESLEK*K
TANK	189	-2,3	-4,2	-1,9	LNIPDTATETQCSVPIQCTDKTDK*QEALFKPQAK
TANK	189+195	9,0	3,8	-5,2	LNIPDTATETQCSVPIQCTDKTDK*QEALFK*PQAK
TANK	195	3,8	2,2	-1,7	LNIPDTATETQCSVPIQCTDKTDKQEALFK*PQAK
TANK	195+199	7,9	3,7	-4,2	LNIPDTATETQCSVPIQCTDKTDKQEALFK*PQAK*DDINR
TANK	199	3,9	2,7	-1,2	LNIPDTATETQCSVPIQCTDKTDKQEALFKPQAK*DDINR
TANK	308	7,5	3,2	-4,3	TTDK*TKPSNLVNTCIR
TANK	310	3,3	1,6	-1,7	TK*PSNLVNTCIR
IKKß	106	3,6	7,6	4,0	K*YLNQFENCCGLR
NEMO	143	6,6	4,8	-1,7	ASVK*AQVTSLLGELQESQSR
NEMO	285	-3,6	-1,1	2,5	QEVIDKLK*EEAEQHK
NEMO	344	3,6	5,4	1,8	LK*ASCQESAR

Fold respect to healthy untreated: <0; 0 to 2.5; 2.5 to 5; 5 to 7.5; 7.5 to 10; >10

Fold variation in response to MLN4924: < -2.5; >-2.5, <2.5; >2.5

Protein Name	Site	Average basal	Average MLN	Average sensitivity	Peptide
CUL3	292	3,4	-16,5	-19,9	NGK*TEDLGCMYK
CUL1	410	4,0	-2,0	-6,0	FINNNAVTK*MAQSSSK
CUL5	724	8,2	-4,8	-13,0	TQEAIIQIMK*M#R

Table 1. Proteins involved in NF-kB activation with altered ubiquitin-like post-translational modifications in chronic lymphocytic leukemia.
Highlights: TANK, IKKß and NEMO show an average increase in their post-translational modifications in CLL. However, while TANK modifications could be reverted by MLN4924, those of IKKß and NEMO could not, or even increase. TRAF2 modifications are low in CLL and decrease even more with MLN4924.

2. NEDDYLATION CONTROLS NOT ONLY THE STABILITY OF IkB BUT ALSO ITS PHOSPHORYLATION IN CLL

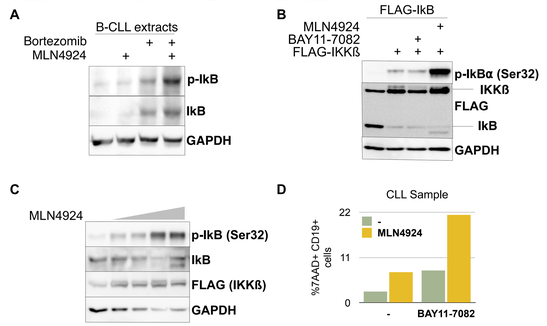


Figure 1. IkBα stability and phosphorylation are regulated by NEDDylation in CLL.
A. Due to the results on the di-Gly screening we decided to study the effect of MLN4924 over the phosphorylation of IkB. MLN4924 slightly stabilizes IkBα over the action of bortezomib, but increases its phosphorylation.
B. IKKß induces the phosphorylation and degradation of IkB. MLN4924 potentiates IkB phosphorylation.
C. When IkBα and IKKß are co-expressed, MLN4924 stabilizes IKKß, inducing the phosphorylation of IkBα and its decline.
D. MLN4924 potentiates the cell death induced by the IKKß inhibitor BAY11-7082.

3. NEDDYLATION CONTROLS THE STABILITY OF IKKß

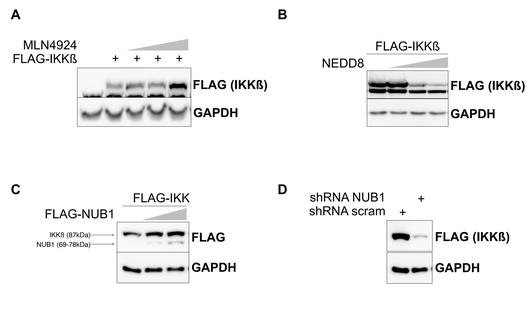
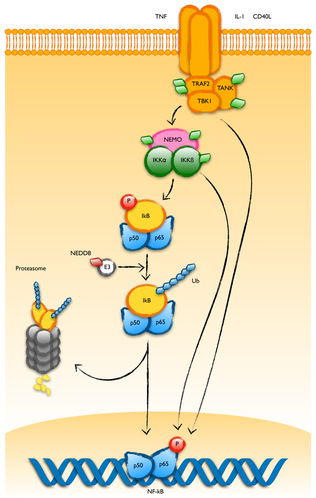


Figure 2. NEDDylation affects the stability of IKKß.
A. MLN4924 induces de accumulation of IKKß in a dose dependent manner.
B. Overexpression of increasing ammounts of NEDD8 induces a decline in IKKß .
C. The NEDD8-degrader protein NUB1 increments the amount of IKKß protein.
D. Knocking down of NUB1 results in a destabilization of IKKß.

The NF-kB pathway showing the UBL post-translational modifications altered in CLL (green diamonds)



4. MLN4924 INDUCES THE DECLINE OF p65

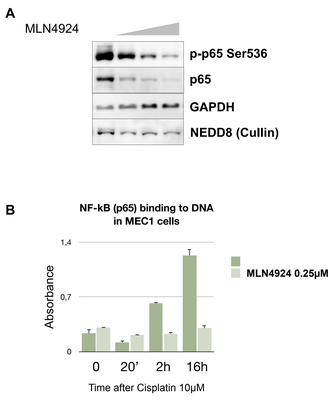


Figure 3. MLN4924 induces the degradation of p65.
A. NF-kB signaling is turned off by a negative feedback loop through the transcriptional control of the NFKBIA gene (coding for IkBα). We studied the role of NEDDylation in the stability of p65. Treatment of HEK293 cells with MLN4924 reduces p65 levels exogenously expressed.
B. Using a DNA binding assay kit for p65, we observed that MLN4924 interferes with the binding of p65 to DNA induced by cisplatin.

5. NEDDYLATION CONTROLS THE TRANSCRIPTION OF THE NFKBIA GENE (IkBα)

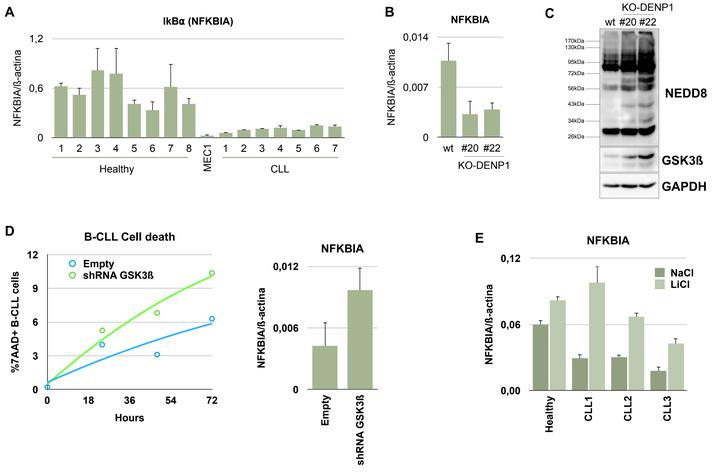


Figure 4. NEDDylation controls the transcription of the NFKBIA gene (IkBα) through the modulation of GSK-3ß.
A. We observed that the levels of NFKBIA mRNA expression were significantly low in CLL
B. When the de-NEDDylation protein NEDP1 was knocked-out by two different CRISPR/Cas9 constructs (#20 and #22) in HEK293 cells, we observed a reduction in NFKBIA mRNA expression.
C. NEDP1 KO was accompanied by a stabilization of GSK-3ß protein, an inhibitory kinase of RelA/p65.
D. Interference of GSK3ß in B-CLL cells promotes the accumulation of NFKBIA mRNA and an increment in basal cell death.
E. Inhibition of GSK-3ß with Lithium reverts NFKBIA mRNA repression in B-CLL lymphocytes.

CONCLUSIONS

NF-kB pathway is a bona fide target of the action of MLN4924, as we and others have demonstrated. As IkB is regulated by the ubiquitin-proteasome system, the NEDDylation pathway is a plausible target to block cell survival in CLL. However, our data show that the regulation of this pathway is complex, affecting the stability and functionality of IKKß and p65, in addition to IkBα. Other proteins upstream in the pathway also show alterations in their UBL modification pattern in CLL.

The data presented here demonstrates a post-translational and transcriptional regulation of different members of the NF-kB pathway:

- NEDDylation controls the stability of the IkB kinase IKKß, probably promoting its degradation by the ubiquitin-proteasome system.
- MLN4924 induces a robust phosphorylation of IkB accompanied by a decline in its protein levels.
- MLN4924 also induces a decrease in the protein level of p65.
- Finally, NEDDylation also plays a role in the stability of GSK-3ß that negatively controls the mRNA expression of the NFKBIA gene, coding for IkBα.

These data provide new insights into the role of UBL post-translational modifications in the physiopathology of CLL, unveiling some non desirable effects of MLN4924, thus opening the way to new combinational treatments to overcome them.

This work has been funded by project PI17/01688 of the Spanish National Institute of Health Carlos III and project CI20/30 of Foundation “Luchamos por la Vida”