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Katarzyna Skórka[1 C, D, F], Nupur Bhattacharya[3, B, F], Paulina Własiuk[1, B, F], Małgorzata Kowal[2, A, B, F], Daniel Mertens[3, 4, A, B, E, F], Anna Dmoszyńska[2, E, F], Krzysztof Giannopoulos[1, 2, A–C, E, F]

Thalidomide Regulation of NF-κB Proteins Limits Tregs Activity in Chronic Lymphocytic Leukemia

[1] Department of Experimental Hematooncology, Medical University of Lublin, Poland
[2] Department of Hematooncology and BMT Unit, Medical University of Lublin, Poland
[3] Department of Internal Medicine III, University of Ulm, Germany
[4] "Mechanisms of Leukemogenesis" Cooperation Unit, German Cancer Research Center DKFZ, Heidelberg, Germany

A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Thalidomide may represent a novel therapeutic strategy in the treatment of chronic lymphocytic leukemia (CLL). Since the activation of nuclear factor kappa B (NF-κB) causes not only malignant transformation and tumor progression, but also allows tumor cells to evade immune surveillance, NF-κB signaling components might constitute a potential target for future therapy in CLL.
Objectives. The current study is an attempt to characterize proteins regulated by thalidomide. Thalidomide's influence on NF-κB proteins and on regulatory *T* cells (Treg) in CLL was investigated.
Material and Methods. A total of 15 patients with CLL were treated with a combined thalidomide/fludarabine regimen. Peripheral blood mononuclear cells were separated by Ficoll density gradient centrifugation. To evaluate glucocorticoid-induced tumour-necrosis-factor-receptor-related protein (GITR) expression in regulatory *T* cells, cells incubated with anti-CD3, ani-CD4 and anti-CD25 were permeabilized and then stained with anti-FOXP3 and analyzed using flow cytometry. Human TNF enzyme-linked immunosorbent assay (ELISA) was used to determine the tumor necrosis factor (TNF) levels in the serum. To evaluate NF-κB activity, chemiluminescent oligonucleotide-based ELISA was performed.
Results. It was found that thalidomide regulates NF-κB activity differentially, and the activity of certain NF-κB components correlated with TNF levels and *T* regulatory cell ($CD4^+CD25^{high}GITR^+$).
Conclusions. These results might indicate that thalidomide not only regulates TNF but also directly interferes with NF-κB components (**Adv Clin Exp Med 2014, 23, 1, 25–32**).

Key words: nucelar factor kappa B (NF-κB), thalidomide, chronic lymphocytic leukemia (CLL), regulatory *T* cells (Treg), tumor necrosis factor (TNF).

Immunomodulatory drugs including thalidomide [1] represent a novel and promising therapeutic option in chronic lymphocytic leukemia (CLL). It has been shown that thalidomide modulates the tumor microenvironment by inhibiting angiogenesis, as well as modulating the immune system by co-stimulating immune effector cells [1–3]. Moreover thalidomide therapy in CLL reduces the numbers of regulatory *T* cells (Tregs). In CLL patients the numbers of regulatory *T* cells (Treg) are increased, which constitutes a crucial mechanism of immunosuppression [4].

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Furthermore, it is known that thalidomide blocks nuclear factor kappa B (NF-κB) activation by suppressing the activity of the inhibitor of NF-κB IκB kinase (IKK) [5]. NF-κB is a family of structurally related transcription factors that play a major role in the inflammation and immune responses. Moreover, NF-κB inhibits apoptosis, induces proliferation and angiogenesis, suggesting that NF-κB has a pivotal role in oncogenesis and tumor progression [6, 7]. In many solid tumors and hematological malignancies, including CLL, NF-κB dimers are located in the cell nucleus, where they are constitutively active [8]. This constitutive activation of NF-κB causes not only malignant transformation and tumor progression, but also enables the tumor cells to evade immune surveillance [7].

The mechanisms involved in the process of NF-κB activation in leukemic cells are mainly associated with microenvironmental stimuli including cells and cytokines [9, 10]. Functional interactions between CD40 (located on CLL cells) and CD154 (located on lymphocytes, T helper lymphocytes and sometimes, to some extent, on leukemic cells), stimulation by cytokines including IL4 (interleukin 4), BAFF (B cell activating factor), a proliferation-inducing ligand (APRIL) and vascular endothelial growth factor (VEGF) could be responsible for NF-κB activation in CLL [9, 11, 12]. Tthe prognostic importance of the RelA component of NF-κB in CLL has been noted [13]. Hence it has been suggested that NF-κB could be a potential target for future CLL therapy.

Since immunomodulatory drugs could be an effective therapeutic option for CLL patients and the mechanism of thalidomide's action is not fully understood, the present study aimed to define thalidomide's regulation of NF-κB proteins and its influence on Tregs in CLL.

Material and Methods

The Patients

The current study involved 15 patients with CLL (median age 63, range 49–73) who were treated in a clinical study with thalidomide and fludarabine [14]. The patients were treated at the Department of Hematooncology at the Medical University of Lublin, Poland. Informed consent was obtained from all patients and the study was approved by the local ethics committee. The clinical characteristics of the patients are shown in Table 1. The patients' molecular characteristics, including genomic aberrations, were detected using fluorescence in situ hybridization and the *IgVH* mutational status, which was determined by sequencing as previously described [15].

Table 1. Clinical and molecular characteristics of chronic lymphocytic leukemia patients

Characteristic	Patiens (n)
Median age (years)	63
Range	49–73
Gender	
Female	6
Male	9
Response to thalidomide	
Yes	11
No	4
Rai stage	
0	0
1	4
2	6
3	1
4	4
IgVH mutation status	
Mutated	1
Unmutated	12
Not available	2
Cytogenetics	
del 17p13,+12q13	1
del 11q22-q23, del13q14	1
del11g22-q23, del14q(IgH)	1
del13q14 (sole abnormality)	5
+12q13	1
normal karyotype	3
other:	
(del14q(IgH),del13q14,+11q(CCND1))	1
not available	2
ZAP-70 (cut off 20%)	
Positive	11
Negative	4
CD38 (cut off 30%)	
Positive	3
Negative	12

IgVH – Immunoglobulin heavy chain variable region. ZAP-70 – Zeta-Chain-Associated Protein Kinase.

Study Protocol

Thalidomide (100 mg p.o. per day) was started at day 0. Every 28 days fludarabine was given for 5 subsequent days (25 mg/m2 i.v. per day), starting at day 7, for up to 6 cycles. In order to prevent thrombosis, acetylsalicylic acid (100 mg) was administered. The patients' clinical response was assessed after six cycles of thalidomide/fludarabine therapy according to International Workshop on CLL (IWCLL) response criteria [16].

Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were separated by Ficoll (Biochrom AG, Berlin, Germany) density gradient centrifugation. After isolation, the cells were stored frozen in liquid nitrogen until the time of the analyses.

Flow Cytometry

The PBMC were thawed. To evaluate glucocorticoid-induced TNF receptor (GITR) expression in Tregs, the GITR were stained after incubation of $1*10^6$ cells with antibodies according to the manufacturer's protocols. To characterize Tregs, the cells were incubated with anti-CD3, ani-CD4 and anti-CD25 permeabilized, then stained with anti-FOXP3 (eBiosciences, San Diego, CA, USA) and analyzed as previously described [4]. The difference in the percentage of Treg GITR+ was calculated as a change in the frequency of Treg GITR+ after thalidomide therapy.

ELISA

Serum was separated and stored at –80˚C. To determine the level of tumor necrosis factor (TNF) in the serum, human TNF-alpha Quantikine HS enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN, USA) was used in accordance with the manufacturers protocols. The difference in TNF levels was calculated as serum TNF level after thalidomide therapy/serum TNF level before therapy.

Chemiluminescent Oligonucleotide-based ELISA

To evaluate the activity of different NF-κB components, chemiluminescent oligonucleotide-based ELISA (co-ELISA) was performed. After coating single-stranded oligonucleotides, binding NF-κB and detecting NF-κB-oligo complexes on 96-well plates, chemiluminescent and colorimetric detection were performed as previously described [17]. The changes in NF-κB proteins were calculated as the average activity of certain proteins after thalidomide therapy/average activity of certain proteins before thalidomide therapy.

Statistical Analysis

All results are presented as median values with the range. To assess correlations among the variables Spearman's rank correlation test was used. All the tests reported were two-sided, and results were considered significant if the P-value was 0.05 or less.

Results

Thalidomide Regulates NF-κB Proteins Activity Differentially

To characterize thalidomide regulation of NF-κB proteins, the activity of all DNA-binding NF-κB proteins before and after therapy was determined by co-ELISA. NF-κB proteins were regulated differentially after thalidomide therapy: p50 was downregulated in 8 patients and upregulated in 7 patients; p52 was downregulated in 6 patients and upregulated in 4 patients; p65 was downregulated in 7 patients and upregulated in 7 patients; RelB was downregulated in 11 patients and upregulated in 4 patients; c-Rel was downregulated in 6 patients and upregulated in 4 patients (Fig. 1). Before the therapy the average activity of certain NF-κB subunits, like p50, p52, RelA, RelB, were correlated with each other, with the exception of c-Rel (Table 2). Correlations were observed between the average activity of p50 and p52 ($R = 0.82$, $p < 0.05$), between p50 and p65 ($R = 0.87$, $p < 0.05$), between

Table 2. Correlations between average activity of certain components of NF-κB before thalidomide therapy

	p50	p52	p65	RelB	c-Rel
p50		0.81*	0.86*	0.64*	0.38
p52	0.81*		0.79*	0.93*	0.31
p65	0.86*	0.79*		0.62*	0.39
RelB	0.64*	0.93*	0.62*		0.44
c-Rel	0.38	0.31	0.39	0.44	

* $p < 0.05$.

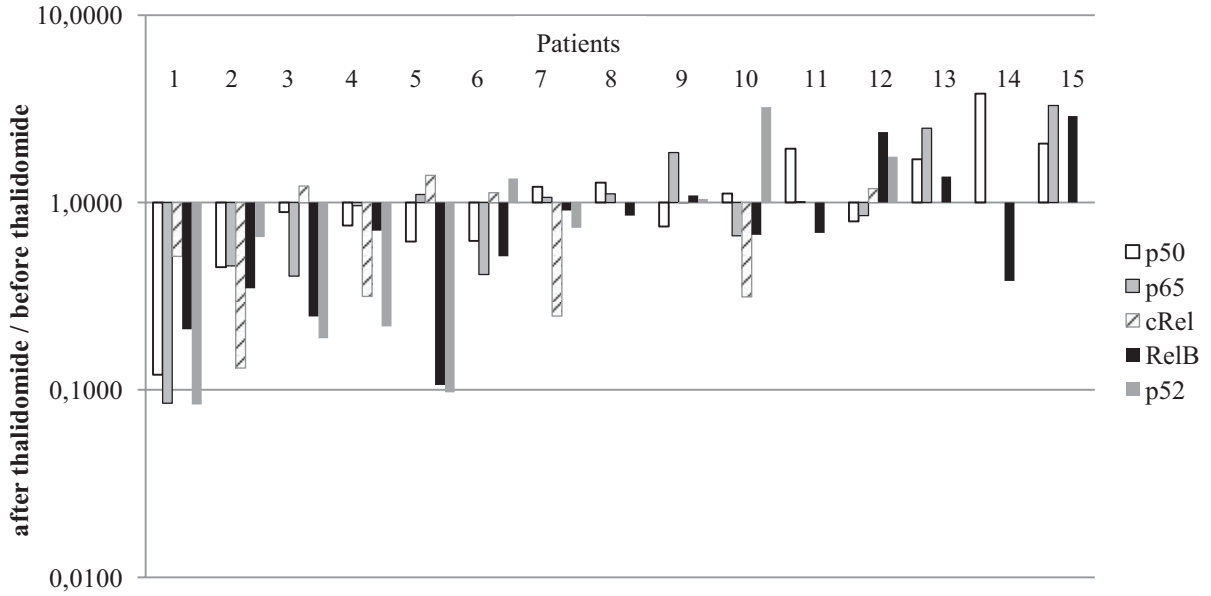

Fig. 1. Thalidomide's differential regulation of NF-κB activity in 15 CLL patients. Ratios were calculated as the avarage activity of particular NF-κB proteins after thalidomide therapy in reference to the avarage activity of particular NF-κB proteins before thalidomide therapy, assessed by chemiluminescent oligonucleotide-based ELISA (co-ELISA)

p50 and RelB (R = 0.65, p < 0.05), between p52 and p65 (R = 0.80, p < 0.05), between p52 and RelB (R = 0.93, p < 0.05) and between p65 and RelB (R = 0.62, p < 0.05).

Changes in NF-κB Activity do not Differ in Clinical and Molecular Subgroups of CLL Patients

No differences in the activity of NF-κB proteins were observed in different clinical and molecular subgroups. The activity of p52 was higher in patients who responded to the therapy, but the difference did not reach statistical significance (Table 3). Interestingly, both c-Rel and p52 showed the largest differences in patients with and without a flare reaction (Table 4), in patients with and without mutated *IGHV* (Table 5) and in patients with unfavorable (+12, 11q-, 17p-, complex karyotype) and favorable (13q-, normal karyotype) cytogenetic aberrations (Table 6), although statistical significance was not reached here either.

Changes in NF-κB Activity did not Correlate with ZAP-70 and CD38 Expression

To characterize the association of NF-κB proteins with prognostic markers, their activity was correlated with ZAP-70 as well as with CD38 expression. Changes in p50 did not correlate with ZAP-70 (R = –0.01, p > 0.05) and CD38 expression R = –0.32, p > 0.05). Changes in p52 did not correlate with ZAP-70 (R = –0.07, p > 0.05) and CD38 expression (R = 0.05, p > 0.05). Changes in p65 did not correlate with ZAP-70 (R = 0.24, p > 0.05) and CD38 expression (R = 0.07, p > 0.05). Changes

Table 3. Median of changes in the activity of NF-κB components in responders and non-responders after thalidomide therapy

NF-κB component	Responders to thalidomide therapy	Non-responders to thalidomide therapy	Statistical significance (p)
p50	1.16	0.75	No (p = 0.55)
p52	0.88	0.15	No (p = 0.13)
p65	1.01	1.10	No (p = 0.45)
RelB	0.68	0.70	No (p = 1.00)
c-Rel	0.99	0.85	No (p = 0.40)

Table 4. Median of changes in the activity of NF-κB components in CLL patients with or without flare reaction

NF-κB component	Patients with flare reaction	Patients with no flare reaction	Statistical significance (p)
p50	0.86	0.88	No (p = 0.51)
p52	0.37	0.88	No (p = 0.45)
p65	0.83	1.01	No (p = 0.74)
RelB	0.51	0.85	No (p = 0.21)
c-Rel	0.41	0.06	No (p = 0.59)

Table 5. Median of changes in the activity of NF-κB components in CLL patients with mutated *IgVH* and unmutated *IgVH*

NF-κB component	Patients with mutated IgVH	Patients with unmutated *IgVH*	Statistical significance (p)
p50	1.11	1.04	No (p = 1)
p52	3.22	0.65	No (p = 1)
p65	0.66	1.06	No (p = 1)
RelB	0.67	0.69	No (p = 1)
c-Rel	0.31	0.99	No (p = 1)

IgVH – Immunoglobulin heavy chain variable region.

Table 6. Median of changes in the activity of NF-κB components in CLL patients with unfavorable and favorable cytogenetic aberrations

NF-κB component	Patients with unfavorable cytogenetic aberrations	Patients with favorable cytogenetic aberrations	Statistical significance (p)
p50	0.68	1.21	No (p = 0.39)
p52	0.21	0.73	No (p = 0.55)
p65	1.03	1.03	No (p = 0.93)
RelB	0.61	0.68	No (p = 0.93)
c-Rel	1.13	0.31	No (p = 0.23)

Unfavorable cytogenetic aberrations: +12q, 11q-. 17p-, complex karyotype.
Favorable cytogenetic aberrations: 13q-, normal karyotype.

in RelB did not correlate with ZAP-70 (R = 0.27, p > 0.05) and CD38 expression (R = 0.37, p > 0.05). Changes in c-Rel did not correlate with ZAP-70 (R = –0.72, p > 0.05) and CD38 expression (R = 0.29, p > 0.05) (Table 7).

Changes in c-Rel Activity Correlated with Lymphocyte Doubling Time (LDT)

Interestingly, changes in c-Rel showed a significant inverse correlation with lymphocyte doubling time (LDT), (R = –0.83, p < 0.05). However, changes in p50 (R = 0.04, p > 0.05), in p52 (R = 0.08, p > 0.05), in p65 (R = –0.10, p > 0.05) and in RelB (R = –0.27, p > 0.05) did not correlate with LDT.

The Effect of Thalidomide Modulation of NF-κB Proteins on TNF Regulation of *T* Cells

Correlations in the changes in the average activity of NF-κB subunits with the levels of TNF were investigated, as well as the expression of the specific TNF receptor GITR, which characterizes Treg. There was a strong correlation between changes

Table 7. Correlations between the average activity of certain components of NF-κB and ZAP-70 and CD38 expression

ZAP-70	–0.01	–0.07	0.24	0.27	–0.72
CD38	–0.32	–0.42	0.07	0.37	0.29
	p 50	p 52	p 65	Rel B	c Rel

P > 0.05, ZAP-70 – Zeta-Chain-Associated Protein Kinase.

in NF-κB (p50) and differences in TNF concentration (before and after treatment) (R = 0.69, p < 0.05), and a strong correlation between changes in NF-κB (p52) and percentages of Treg GITR+ (R = 0.97, p < 0.05).

Discussion

The NF-κB family includes five proteins that can be divided into two groups according to differences in their structure, function and posttranscriptional modification. One group includes the RelA, RelB and c-Rel proteins, while the second group comprises NF-κB1 proteins (p50/p105) and NF-κB2 proteins (p52/p100) [18]. These components can form homo- or heterodimers, which are active forms that bind to their target site on DNA. The most common dimer present in the human organism is dimer p50/RelA [19]. Based on the structure of the dimers, NF-κB regulates the expression of different genes caused by their diverse affinities to specific gene promoter binding sites. The regulation of gene transcription is also dependent on the time of the dimers' diffusion into the nucleus [19]. Moreover, the type of gene whose expression is regulated depends on the microenvironment and the cell type, since the NF-κB signaling pathway is integrated with many other signaling pathways [20].

According to recent data, the level of NF-κB activity in CLL cells is highly diverse, and the constitutive activity of NF-κB in unstimulated CLL cells is always higher than the activity in normal B cells. In CLL cells, prevailing NF-κB components include p50, RelA and c-Rel proteins [9, 13]. Moreover, it has been shown that NF-kB activity is increased in CLL patients and correlates with the survival of CLL cells *in vitro* [13]. Interestingly, CLL cells with higher NF-κB activity undergo apoptosis upon NF-κB inhibition more easily, which may suggest that blocking NF-κB could be a therapeutic option for CLL. CLL cells are even more sensitive to pharmacological inhibitors of NF-κB signaling pathway than normal lymphocytes [19].

Keifer et al. [5] demonstrated that thalidomide can block NF-κB DNA binding through a mechanism that involves the suppression of IKK activity. The current study showed that thalidomide regulates the activity of NF-κB differentially.

Thalidomide is a drug with pleiotropic activities, but it is known to have an anti-TNF and anti-Treg effect [14]. It has been shown that Treg cells, whose numbers are increased in CLL patients and correlate with serum levels of TNF, constitute a crucial mechanism of immunosupression in CLL patients [4]. Hence the present study investigated correlations between changes in the average activity of NF-κB subunits before and after thalidomide therapy and changes in immunological parameters connected with T cells, including the frequency of TregGITR+ and TNF serum level. The results showed a strong correlation between changes in NF-κB (p52) and differences in Treg-GITR+, and a strong correlation between changes in NF-κB (p50) and differences in TNF. Why this correlation is limited only to certain components of NF-κB remains unclear.

This study represents the first report of a new possible mechanism of thalidomide action associated with reducing regulatory *T* cells expressing GITR, which is a TNF receptor, through the modulation of NF-κB proteins. It is possible to speculate that along with the regulation of TNF levels, thalidomide might modulate the activity of the p50 and p52 components of NF-κB and subsequently reduce Treg GITR+. It has been demonstrated that GITR is a member of the TNFR superfamily, and one of Treg's markers can inhibit the suppressive function of Treg *in vitro* [21, 22]. Interestingly, Zhan et al. showed that NF-κB (Rel A, c-Rel, NF--κB1) acts as a positive regulator of GITR expression on CD4+ and CD8+ lymphocytes/cells [23]. Moreover, it has also been proved that GITR signaling can activate NF-κB, which promotes *T* cell survival [24].

The function of Treg could be correlated with levels of TNF [14, 27]. Elevated levels of TNF have been shown in CLL patients, and an association with progression of the disease has been found [26]. The current authors' recent results demonstrated a correlation between TNF serum level and Treg in CLL patients and an inability to block the suppressive activity of Treg [26]. Nevertheless, in rheumatoid arthritis, TNF regulates Treg function differentially and can downmodulate their suppressive

activity [25]. Different mechanisms are probably responsible for TNF regulation on Treg activity in autoimmunological disorders and neoplasia.

To conclude, through the regulation of TNF levels, thalidomide might modulate the activity of the p50 and p52 components of NF-κB and subsequently reduce regulatory T cells expressing GITR, which is a TNF receptor. These results point to another hitherto unknown mechanism of action for thalidomide, not only by the regulation of TNF, but also through subsequent regulation of NF-κB.

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Address for correspondence:

Krzysztof Giannopoulos
Department of Experimental Hematooncology
Medical University of Lublin
Chodzki 4a
20-950 Lublin
Poland
Tel.: +48 81 756 48 12
E-mail: giannop@tlen.pl

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