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RESEARCH ARTICLE

cFLIP overexpression in T cells in thymoma-associated myasthenia gravis

Djeda Belharazem[1], Berthold Schalke[2], Ralf Gold[3], Wilfred Nix[4], Mario Vitacolonna[5], Peter Hohenberger[5], Eric Roessner[5], Torsten. J. Schulze[6], Güher Saruhan-Direskeneli[7], Vuslat Yilmaz[7], German Ott[8], Philipp Ströbel[9] & Alexander Marx[1]

[1]Institute of Pathology, University Medical Centre Mannheim, University of Heidelberg, Mannheim, Germany
[2]Department of Neurology, University of Regensburg, Regensburg, Germany
[3]Department of Neurology, University of Bochum, Bochum, Germany
[4]Department of Neurology, University of Mainz, Mainz, Germany
[5]Department of Thoracic Surgery, University Medical Centre Mannheim, Mannheim, Germany
[6]Institute for Transfusion Medicine and Immunology, German Red Cross Blood Service, University Medical Centre Mannheim, Mannheim, Germany
[7]Department of Physiology, School of Medicine, Istanbul University, Istanbul, Turkey
[8]Department of Pathology, Robert-Bosch Hospital, Stuttgart, Germany
[9]Institute of Pathology, University of Goettingen, Goettingen, Germany

Correspondence
Djeda Belharazem, Institute of Pathology, University Medical Centre Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, D-68135 Mannheim, Germany.
Tel: (+)49 621-383 5953; Fax: (+)49 621-383 2005;
E-mail: d.belharazem@gmail.com

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Abstract

Objective: The capacity of thymomas to generate mature CD4+ effector T cells from immature precursors inside the tumor and export them to the blood is associated with thymoma-associated myasthenia gravis (TAMG). Why TAMG (+) thymomas generate and export more mature CD4+ T cells than MG(−) thymomas is unknown. **Methods**: Unfixed thymoma tissue, thymocytes derived thereof, peripheral blood mononuclear cells (PBMCs), T-cell subsets and B cells were analysed using qRT-PCR and western blotting. Survival of PBMCs was measured by MTT assay. FAS-mediated apoptosis in PBMCs was quantified by flow cytometry. NF-κB in PBMCs was inhibited by the NF-κB-Inhibitor, EF24 prior to FAS-Ligand (FASLG) treatment for apoptosis induction. **Results**: Expression levels of the apoptosis inhibitor cellular FLICE-like inhibitory protein (c-FLIP) in blood T cells and intratumorous thymocytes were higher in TAMG(+) than in MG(−) thymomas and non-neoplastic thymic remnants. Thymocytes and PBMCs of TAMG patients showed nuclear NF-κB accumulation and apoptosis resistance to FASLG stimulation that was sensitive to NF-κB blockade. Thymoma removal reduced cFLIP expression in PBMCs. **Interpretation**: We conclude that thymomas induce cFLIP overexpression in thymocytes and their progeny, blood T cells. We suggest that the stronger cFLIP overexpression in TAMG(+) compared to MG(−) thymomas allows for the more efficient generation of mature CD4+ T cells in TAMG(+) thymomas. cFLIP overexpression in thymocytes and exported CD4+ T cells of patients with TAMG might contribute to the pathogenesis of TAMG by impairing central and peripheral T-cell tolerance.

Introduction

Thymomas are thymic epithelial tumors, comprising WHO type A, AB, B1, B2, B3, and rare other histological subtypes.[1–3] They maintain intratumorous thymopoiesis to a variable extent and this correlates with a variable frequency of associated myasthenia gravis (MG).[4,5] MG is an autoimmune muscle fatigability that is elicited by autoantibodies to various targets at the neuromuscular junction.[5–7] In thymoma-associated MG (TAMG) this target is almost always the acetylcholine receptor (AChR).[6] Anti-AChR autoantibody production is a $CD4^+$ T cell-dependent process.[7–9] Of note, the production of anti-AChR autoantibodies in TAMG does not occur inside the

thymoma. Instead, the intratumorous generation of autoreactive CD4(+) effector T cells and their export from the thymoma to the blood is a key feature of almost all TAMG(+) thymomas.[10] By contrast, MG(−) thymomas typically generate and export much fewer or no mature CD4(+) effector T cells.[10] The mechanisms underlying this dichotomy are unknown. We report here that cellular FLICE-like inhibitory protein (cFLIP), an inhibitor of the extrinsic, TNFa-, FAS-Ligand- (FASLG) and TRAIL-driven apoptosis pathway[11] is overexpressed in TAMG(+) thymomas, in thymocytes derived thereof and in blood T cells but not B cells.

Patient Characteristics and Experimental Procedures

Patients, tissues, and isolation of lymphocytes

Clinico-pathological characteristics of the studied thymomas (WHO type AB, B2, B3; $n = 72$; 32 MG(+), 40 MG (−)) are given in Table 1. Adequate material of the rare type A and B1 thymomas[12] was not available. Thymocytes were mechanically released from unfixed minced thymoma tissue, using a cell culture sieve and filtered through a cell strainer (40 μm). Thymocytes and peripheral blood mononuclear cells (PBMCs) ($n = 44$) were purified by Ficoll density gradient centrifugation (Biochrom, Berlin, Germany). CD4+, CD4+, CD45A+, CD8 and CD8+CD45A+ T-cell subsets were isolated from PBMC by either positive (CD4) or negative (CD8) immunoselection, using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) (Fig. S1). CD4+CD45A+ and CD8+CD45A+ T cells were isolated by using the CD4+CD45A+ T-cell isolation kit and MIDI MACS Separator from Miltenyi Biotec. B cells were positively selected using CD19 microbeads (Miltenyi Biotec). The purity of T-cells subsets (CD4 (95%) and CD8 (91%)) and B cells (97%) was analysed by flow cytometry using FITC-conjugated mouse anti-human-CD4 (clone RPA-T4) or CD8 (clone LT8) or CD19 (clone MB19-1) (Abcam, Cambridge, UK), PBMCs of blood donors ($n = 30$; 20–80 years of age) were provided by the local blood bank. T and B cells percentages in PMBCs were estimated by flow cytometry (Guawa Merck-Millipore, Hessen, Germany). PBMCs from nine early onset MG (EOMG) patients Table 1) were investigated in this study. Ethical approval was obtained from the institutional review board (approval #2009-290N-MA/2010).

Table 1. Characteristics of 72 patients and tissues studied for cFLIP expression and function: thymoma type AB, B2, and B3 (WHO classification); MG+ (%), percentage of patients with myasthenia gravis; stage, according to Masaoka-Koga (WHO, 2004).[1]

Diagnosis	*N*	Age range (y)	Sex (m:f)	Stage (I–IV)	MG+ (%)
Type AB	26	33–79	9:17	I ($n = 15$) II ($n = 10$)	10 (38.46)
Type B2	29	27–81	15:14	I ($n = 12$) II ($n = 7$) III ($n = 6$) IV ($n = 4$)	16 (55.17)
Type B3	17	45–76	10:7	I ($n = 7$) II ($n = 4$) III ($n = 4$) IV ($n = 2$)	7 (41.17)
EOMG	9	25–39	6:3		100

cFLIP, cellular FLICE-like inhibitory protein; m, male; f, females; MG, myasthenia gravis; EOMG, early onset MG.

QRT-PCR, Western blot

RNA isolation from unfixed thymoma tissue, thymocytes derived thereof, PBMCs, T-cell subsets, and B cells; cDNA synthesis; qRT-PCR (relative quantification was calculated using the ΔΔCt method with Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH) standard: TaqMan; FAST SYBR Green; Applied Biosystems, Darmstadt, Germany), and western blot were performed as described.[13] Cytosolic and nuclear proteins were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermoscientific, Karlsruhe, Germany). Mouse anti-cFLIP (G11 D16A8; Cell Signaling, Francfort, Germany), rabbit anti-p65 (C-20, sc-372; Abcam, UK), rabbit anti-ß-actin (New England Biolabs, Frankfurt, Germany), and PARP (poly (ADP ribose) polymerase (H-250) Santa Cruz, Heidelberg, Germany) were used for western blot.

Primers for cFLIP quantification were as follows: 5′-CA CTGAAAGTCCCCGTCAAC-3′ forward and 5′-CGTGCT GTGTACCTGCCCAAT-3′ reverse and for GAPDH: 5′ TC GACAGTCAGCCGCATCT 3′ forward and R: 5′ CCGT TGACTCCGACCTTCA 3′ reverse.

Survival and apoptosis detection, FACS, NF-κB/p65 localization

Survival of PBMCs was measured by MTT assay (Sigma Aldrich, Munich Germany)[13] For apoptosis induction, 10^5 PBMCs from thymoma patients and blood donors were treated with 5–10 ng/mL FASLG (Sigma Aldrich, Germany) for 24 h, followed by MTT assay. FAS expression levels on PBMCs were quantified by flow cytometry, using FITC-conjugated mouse anti-human-CD95 (clone DX2; BD Pharmingen, Heidelberg Germany).[14] Allophycocyanin/Phycoerythrin (APC/PE-labeled) mouse anti-human CD4 and CD8 were used for characterizing PBMCs subsets. To study the impact of NF-κB on the resistance toward FAS-mediated apoptosis, PBMCs were pretreated either with the

NF-κB inhibitor, EF24 (24 h, 2 μmol/L), or solvent Dimethyl sulfoxide (DMSO) followed by FASLG (10 ng/mL for an additional 6 h). Subsequently, part of the PBMCs were studied in parallel (1) for apoptosis by flow cytometry as described[4]; and (2) for nuclear and cytosolic NF-κB localisation by western blot of nuclear and cytosolic protein extracts, using rabbit anti-human p65 (clone E379, Abcam, UK)[15] and goat anti-rabbit antibodies (Cell Signaling, Germany). Nuclear and cytosolic fractions of PBMCs for NF-κB localization were separated using NE-PER Extraction Reagents (ThermoScientific, Karlsruhe, Germany) according to the manufacturer's recommendations.

Detection of the functional +49A/G CTLA4 polymorphism

Total genomic DNA was extracted from frozen tissue of 31 TAMG(+) (nine AB, 16 B2 and six B3) and 39 MG(−) thymomas (15 AB, 13 B2 and 11 B3), using the DNA tissues core kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To characterize the +49A/G polymorphism of the *CTLA-4* gene, we used the TaqMan Universal PCR Master Mix, DNA (20 ng/μL) and the TaqMan probes rs231775 in Step ONE Plus Fast Real-Time PCR System, using TaqMan 5′ allelic discrimination assay (Applied Biosystems, Grand Island, NY, USA) according to the manufacturer's protocol.

Statistical analysis

All statistical tests were performed with GraphPad Prism V6.0 (GraphPad Software Inc, La Jolla, USA). Two-tailed Student's *t*-test was used with $\alpha < 0.05$ and a confidence level of 95% ($P < 0.05$ was considered as significant) when comparing cFLIP gene expression levels in different groups and among EOMG, TAMG(+), and MG(−) thymoma patients. A subsequent *F* test was used to compare variances with $\alpha < 0.05$ at a confidence level of 95% ($P < 0.05$ was considered significant). The CTLA4 genotypic frequencies between TAMG and MG(−) thymomas were compared using the chi-square test (χ^2).

Results

cFLIP is overexpressed in thymoma-derived thymocytes in TAMG

By qRT-PCR and western blot, we found significant overexpression of cFLIP mRNA and protein in whole thymoma tissue extracts and thymoma-derived thymocytes of TAMG(+) compared to MG(−) thymoma patients in WHO type AB, B2, and B3 thymomas (Figs. 1, 2). In snap frozen tissue of 12 available pairs of thymoma and adjacent non-neoplastic residual thymus, cFLIP mRNA expression levels were significantly lower in residual thymuses than in the adjacent MG(−) and TAMG(+) thymomas (Fig. 3).

cFLIP is overexpressed in PBMCs and decreases after thymoma removal

Expression levels of cFLIP were higher in PBMCs of TAMG(+) compared to MG(−) thymoma patients, and both were higher than in PBMCs of blood donors ($n = 58$) and patients with early onset MG (EOMG; $n = 9$) ($P < 10^{-4}$ and $P < 10^{-4}$, respectively) (Fig. 4A) The age-related decline of cFLIP levels in PBMCs from blood donors (Fig. 4A) was not obvious in thymoma patients (not shown). CD4(+) and CD8(+) blood T cells, but not B cells of TAMG(+) patients showed increased cFLIP expression (Fig. 4B–D). Overexpression was most prominent in the CD4(+)CD45A(+) subset of blood T cells of patients with TAMG ($P = 0.0006$) (Fig. 4E).

One follow-up blood sample was available in each of the six patients at follow-up periods of 2 months ($n = 1$), 6 months ($n = 4$), and 12 months ($n = 1$). In these follow-up samples (of two patients with type AB and four patients with type B2 thymomas), cFLIP mRNA levels in PBMCs were significantly lower than in blood samples at the time of surgery (Fig. 5A). cFLIP protein levels had also declined in the PBMCs of the three tested patients with B2 (one TAMG(+) and two MG(−) thymoma patients) (Fig. 5B).

PBMCs in thymoma patients are resistant to FAS-mediated apoptosis

On treatment with FASLG (5 ng and 10 ng/mL for 24 h), the PBMCs of all 16 blood donors showed decreased survival, while the PBMCs of all 10 tested thymoma patients (four MG(−): two AB and two B2; six TAMG(+): three AB, two B2, and one B3) were resistant (Fig. 6), in spite of almost identical expression levels of CD95 in the two cohorts of PBMCs (Fig. S2). TAMG(+) and MG(−) thymoma PBMCs showed no significant difference in terms of apoptosis resistance (not shown; see Discussion).

Inhibition of NF-κB leads to cFLIP repression in PBMCs from TAMG patients and restores sensitivity to FASLG

Treatment of PBMCs with FASLG alone reduced cFLIP mRNA and protein levels only in PBMCs of four blood donors but not in five tested thymoma patients (middle columns and bands in Fig. 7A and B, respectively). However, after pretreatment of PBMCs with the NF-κB inhibitor, EF24, and cFLIP levels were repressed almost equally

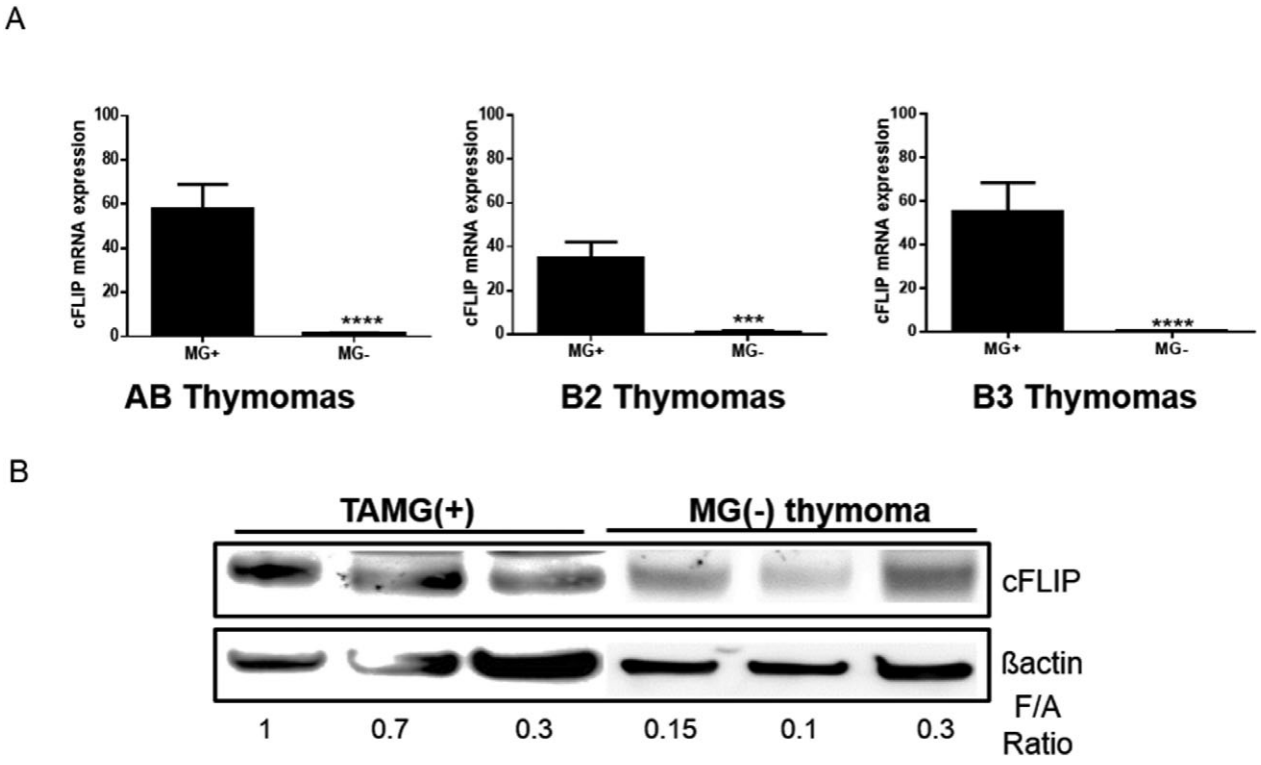

Figure 1. cFLIP mRNA and protein expression analysis by Q-RT-PCR and western blot. (A) mRNA expression in whole tissue extracts of AB thymomas (10 TAMG(+) and 16 (MG(−)), B2 thymomas (16 TAMG(+) and (13 MG(−)) and B3 thymomas (seven TAMG+ and 10 MG(−)) (+)); ****$P < 10^{-4}$, ***$P = 0.0002$). (B) Protein expression in three TAMG(+) and three MG(−) AB thymomas. F/A denotes the ratio of the intensity values of cFLIP band and the respective actin (loading control) band as measured by densitometry using ImageJ program. cFLIP, cellular FLICE-like inhibitory protein; TAMG, thymoma-associated myasthenia gravis.

by FASLG treatment in PBMCs of blood donors and thymoma patients (right columns in Fig. 7A; right bands in Fig. 7B). Treatment of PBMCs from nine thymoma patients (four with AB, two with B2, and three B3 subtypes) with EF24 alone had little effect on survival, while EF24 pretreatment sensitized PBMCs in a dose-dependent manner for subsequent FASLG treatment with decreased survival (Fig. 8A). Furthermore, PBMCs from four blood donors but not from four thymoma patients showed massive apoptosis on FASLG treatment alone. Again, EF24 pretreatment lead to comparable apoptosis in PBMCs of blood donors and thymoma patients on subsequent FASLG treatment (Fig. 8B).

By western blot analysis of nuclear and cytosolic protein extracts, PBMCs showed nuclear p65 localization (i.e. NF-κB activation) only in preoperatively obtained PBMCs of thymoma patients ($n = 8$) but not in blood donors ($n = 4$). Nuclear expression was slightly higher in PBMCs of TAMG(+) than MG(−) thymoma patients (Fig. S3A and B). In each of three different thymoma patients, a single follow-up PBMC sample had enough cells to study protein expression. After variable periods after thymoma surgery (i.e. at 2, 6, and 12 months), nuclear p65 expression was strongly reduced, while cytoplasmic expression was increased in PBMCs (Fig. S4).

The +49 CTLA4 genotype is associated with cFLIP expression levels in TAMG(+) thymomas

TAMG was previously shown to be uniquely associated with the +49A/A CTLA4 genotype that confers protection in a variety of other autoimmune diseases.[16] In the present cohort of patients, we confirm this finding, since the CTLA4+49A/A genotype was significantly more prevalent in TAMG(+) than MG(−) thymoma patients ($P = 0.0003$ by χ^2 Test; Table 2). Surprisingly, cFLIP mRNA levels in thymoma tissue extracts were higher in TAMG patients with the +49A/A genotype than in TAMG with other genotypes, while no such association was obvious in MG (−) thymoma patients (Fig. S5).

Discussion

Export of CD4+ CD45RA+ effector T cells from thymomas to the blood is typical of TAMG(+) but not MG(−)

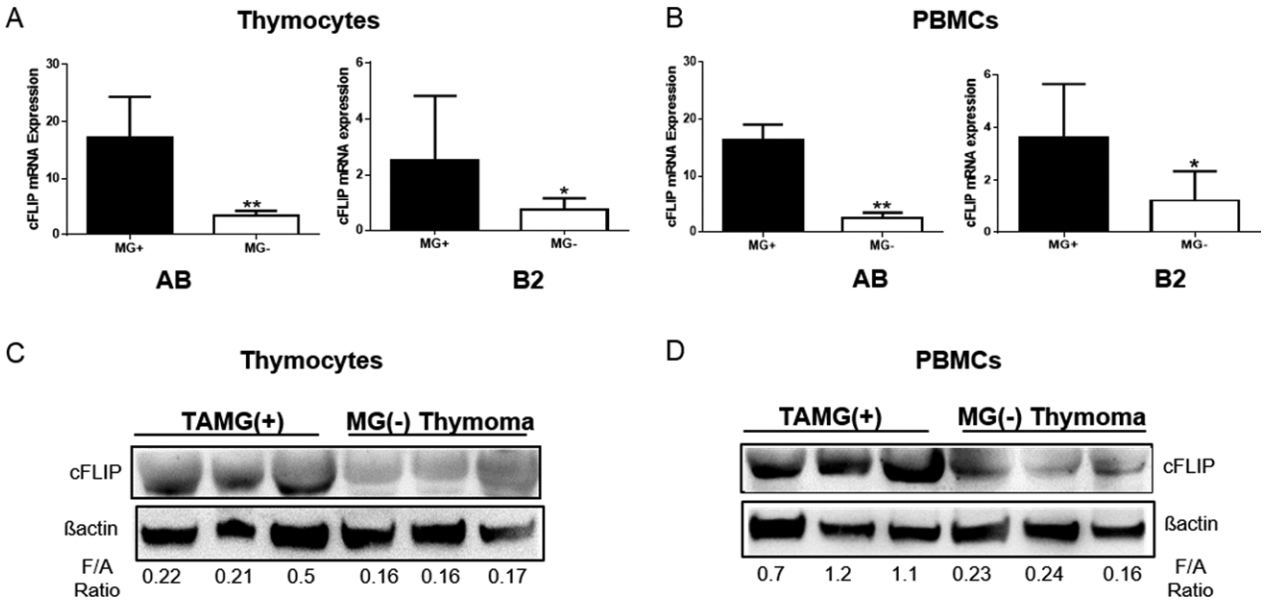

Figure 2. cFLIP mRNA and protein expression in thymocytes and PBMCs from TAMG(+) and MG(−) type AB and B2 thymomas. (A) Expression of mRNA in thymocytes from three TAMG(+) and three MG(−) type AB thymomas and five TAMG(+) and four MG(−) type B2 thymomas. (B) Expression of mRNA in PBMCs from patients with eight TAMG(+) and nine MG(−) type AB thymomas and five TAMG(+) and five MG(−) type B2 thymomas. (C and D) Protein expression in thymocytes and PBMCs from patients with type AB thymomas (three TAMG(+) and three MG(−) thymomas). F/A denotes the ratio of the intensity values of cFlip band and the respective actin (loading control) band as measured by densitometry using ImageJ program. cFLIP, cellular FLICE-like inhibitory protein; PBMCs, peripheral blood mononuclear cells; TAMG, thymoma-associated myasthenia gravis. *p = 0.020, 0.045, **p = 0.0077, 0.0071.

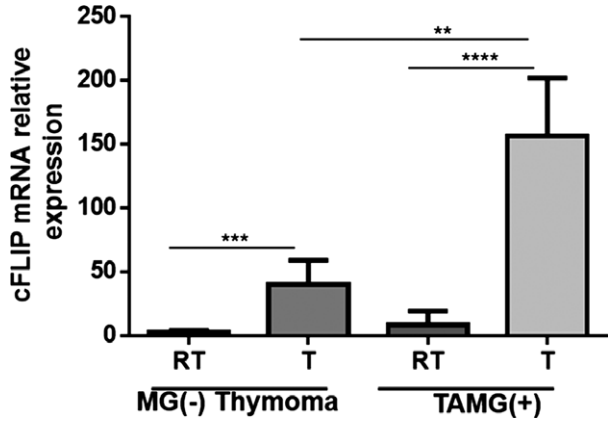

Figure 3. Intrapersonal comparison of cFLIP mRNA expression in thymoma (T) and the adjacent, non-neoplastic residual thymus (RT): six pairs each of TAMG(+) and MG(−) type AB thymomas and the respective RT from the same patients studied by qRT-PCR (**P = 0.0018, ***P = 0.0001 and ****P < 0.0001). cFLIP, cellular FLICE-like inhibitory protein; TAMG, thymoma-associated myasthenia gravis.

thymomas, while export of CD8(+) T cells from MG(−) and TAMG(+) thymomas is similar.[17,18] The reason for the export of CD4+CD45RA+ T cells from TAMG(+) thymomas is poorly understood. However, the lower number of apoptotic thymocytes in TAMG(+) thymomas,[19] the unique CTLA4$^{high/gain\text{-}of\text{-}function}$ genotype +49A/A association with TAMG[16] that we confirm here (Table 2), and the recently shown role of CTLA4 as attenuator of thymic negative selection,[20] have been clues that attenuated intratumorous deletion of poorly tolerized thymocytes might contribute to TAMG.[5,12,19] We now add two new mechanistic facets to this hypothesis, suggesting that (1) cFLIP overexpression in intratumorous thymocytes may contribute to central tolerance failure, while (2) cFLIP overexpression in the PBMCs derived from the intratumorous cFLIP^{high} thymocytes may contribute to the observed loss of peripheral tolerance.

The higher cFLIP expression in TAMG(+) than MG(−) thymomas suggests that signals delivered by the stroma of TAMG(+) and less so of MG(−) thymomas might increase cFLIP expression in thymocytes (Fig. 2); thereby oppose thymocyte apoptosis,[21] and let potentially intolerant, naïve mature T cells "escape" to the blood and peripheral immune system.[19] The alternative possibility that TAMG patients may show systemic, T cell autonomous overexpression of cFLIP is unlikely for two reasons: first, because cFLIP expression levels are low in non-neoplastic remnant thymuses adjacent to thymomas and, second, because the cFLIP^{high} state in PBMCs is reversible

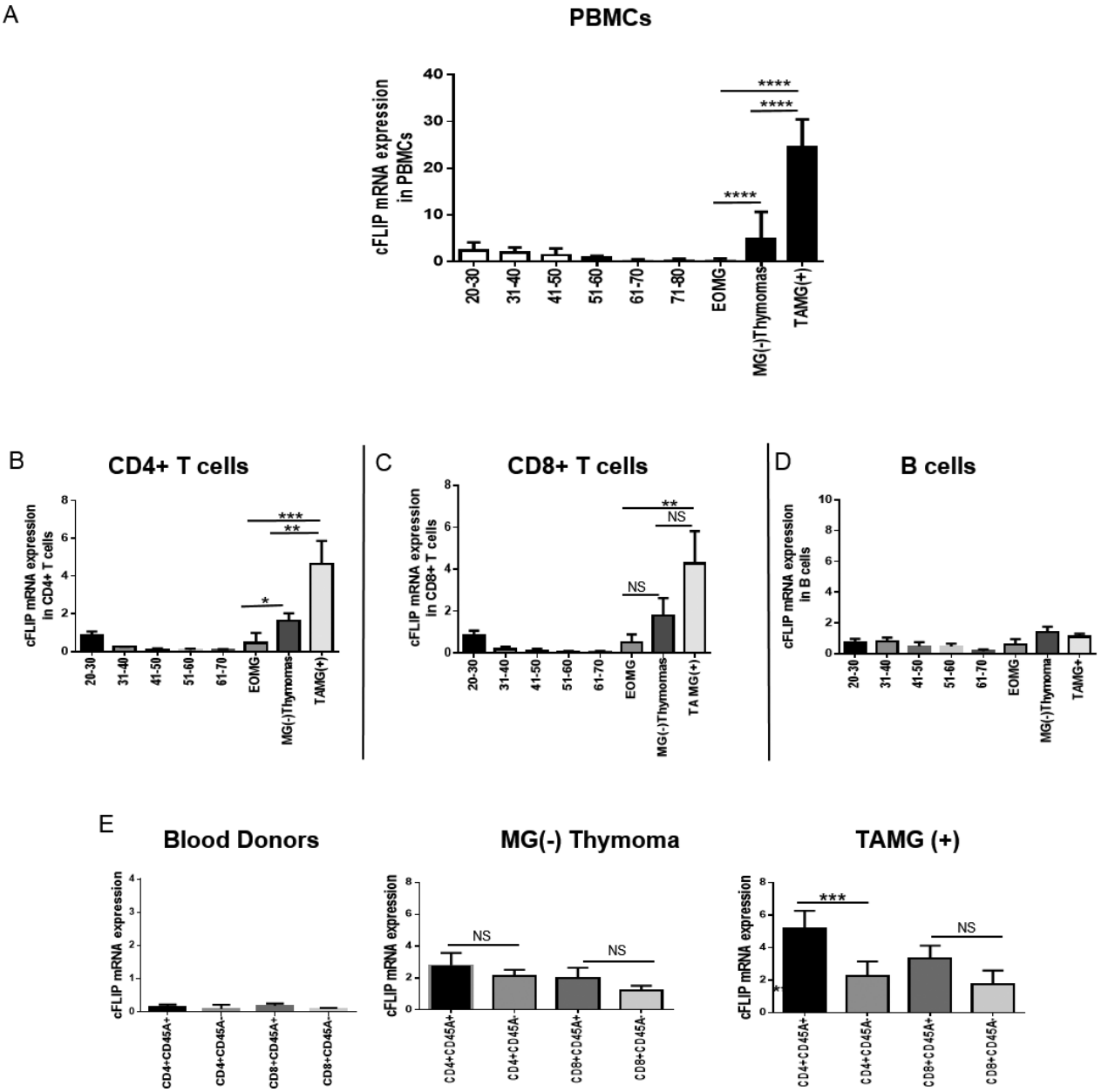

Figure 4. Expression of cFLIP in PBMCs and blood T-cell subsets. (A) PBMCs of blood donors (20–80 year-old people) (n = 20 in each decade), of patients with EOMG (anti-AChR-positive MG patients [25–39] years old, n = 9), MG(−) thymomas (n = 6; five type AB, one type B2) and TAMG (+) thymomas (n = 6; three AB, and three B2 [****p < 0.0001]). (B–D) cFLIP mRNA expression in blood-derived CD4(+) and CD8 (+) T cells and B cells from blood donors and patients with EOMG, MG(−) thymomas, and TAMG(+) thymomas (***P = 0.0010, **P = 0.0019 and 0.0095) and *P = 0.0286. (E) cFLIP mRNA levels in different T-cell subsets, CD4+CD45A(+), and CD45A(−) versus CD8+CD45A(+), and CD45A(−) from three blood donors (51–78 years old), six AB thymomas (three MG(−) and three TAMG(+) [55–71 years old]). (***P = 0.0006, **P = 0.0029, *P = 0.018 or P = 0.025, DP, double-positive; NS, not significant). GAPDH was used as reference for cFLIP mRNA analysis by real-time PCR. cFLIP, cellular FLICE-like inhibitory protein; PBMCs, peripheral blood mononuclear cells; EOMG, early onset myasthenia gravis; TAMG, thymoma-associated myasthenia gravis.

after thymoma removal. On the other hand, the lower cFLIP levels observed in MG(−) compared to TAMG(+) thymomas may increase the efficacy of negative T-cell selection and elicit the observed reduced export of CD4+ T cells from MG(−) thymomas.[17] A comparable impact of cFLIP on T-cell export has been observed in cFlip$^{-/-}$ mice: Similar to MG(−) thymomas, cFlip^{low} murine thymuses show inefficient "late" thymopoiesis due to

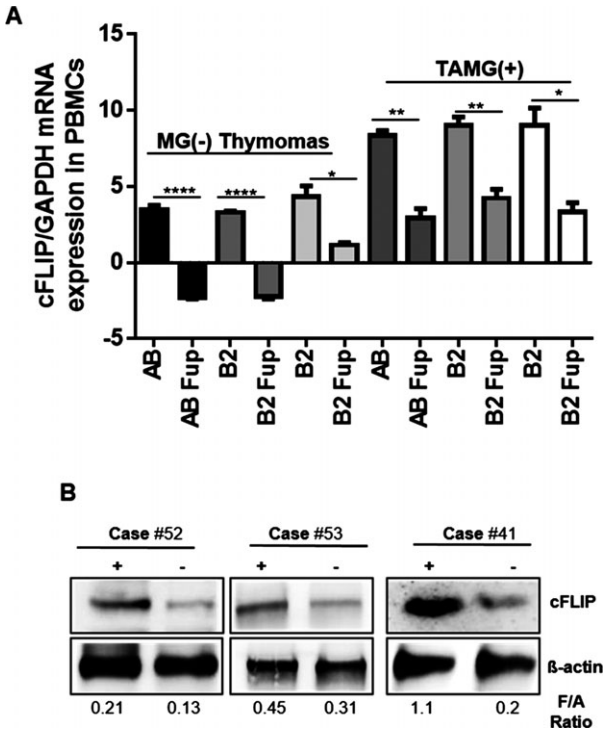

Figure 5. (A) cFLIP mRNA expression analysis by real-time PCR in PBMCs from two patients with type AB (#10 and #25) and four type B2 thymomas (#39, #41, #52, and #53) before thymectomy and on follow-up (Fup). $^{****}P < 10^{-4}$ and $^{**}P = 0.0015$, and 0.0044, $^{*}P = 0.012$. (B) Analysis of cFLIP protein expression by western blot in two MG(−) type B2 thymomas (cases #52 and #53) and one TAMG (+) type B2 thymoma (#41). F/A denotes the ratio of the intensity values of the cFLIP band and the respective actin (loading control) band as measured by densitometry using ImageJ program. Fup was 2 months in case #41, 6 months in cases #10, #25, #52, and (#53); and 12 months in case #39. cFLIP, cellular FLICE-like inhibitory protein; PBMCs, peripheral blood mononuclear cells; TAMG, thymoma-associated myasthenia gravis.

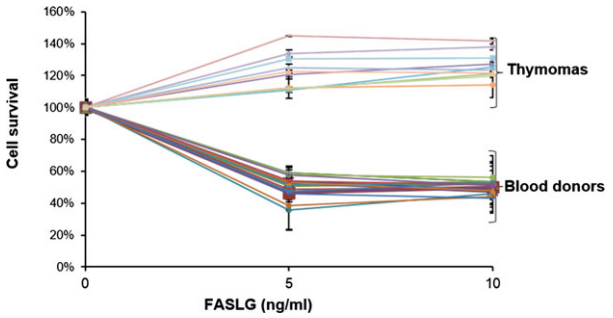

Figure 6. Survival analysis of PBMCs from blood donors (n = 16, age range 20–80 years) and thymoma patients (n = 10; four MG(−) [two AB and two B2 subtype] and six TAMG(+) [three AB, two B2, and one B3 subtypes]) by MTT assay after treatment with FASLG (5 and 10 ng/mL for 24 h). There was a significant difference between thymoma patients and blood donors but not between TAMG(+) and MG(−) thymoma patients. Shown is one representative experiment (of two independent investigations; each assay was run in triplicate). PBMCs, peripheral blood mononuclear cells; TAMG, thymoma-associated myasthenia gravis; FASLG, Fas Ligand.

increased apoptosis of single-positive (SP) thymocytes and reduced export of T cells to the periphery.[21] However, in contrast to cFlip$^{-/-}$ murine thymuses that show a loss of both SP CD4+ and SP CD8+ thymocytes,[21] most MG(−) thymomas show a preferential deficiency of CD4+ SP thymocytes, while their export of SP CD8+ T cells is similar to the SP CD8+ T-cell export of TAMG(+) thymomas.[17,22] The reason for the latter observation is not clear; since cFLIP expression levels are increased in both CD8+ and CD4+ SP thymocytes of thymomas (Fig. 4). Therefore, we assume that cFLIP-independent, thymocyte subset-specific apoptotic mechanisms are operative: While such mechanisms are largely unknown in MG(−) thymomas,[10] they have been described in mice with knockouts of various stromal genes, such as CD80, CD86, and CD40.[23] Interestingly, CD40 deficiency has recently been observed in thymomas.[24] Furthermore, thymocyte subset-specific differences also occur between CD4+CD8+ double positive (DP) and SP thymocyte: although cFLIP levels are similar in DP and SP thymocytes,[21] DP thymocyte subsets in thymomas and cFlip$^{-/-}$ mice are protected from apoptosis while SPs are not.[21,25]

General impairment of central tolerance through cFLIP overexpression in thymocytes may explain the broad spectrum of autoimmune diseases that occur in a minority of thymomas,[20,26] but does not explain the strong bias for the AChR, other striational muscle antigens and cytokines as autoimmune targets in the majority of thymoma patients.[27–31] (Table S1) bias has been thought to be caused by abnormalities of the neoplastic microenvironment,[5,30,32] including absence of myoid cells; abnormal expression of MHC genes, cytokines and tissue-specific autoantigens (e.g. AChR subunits); and lack of (autoimmune regulator) AIRE.[5,10,33,34] In concert, these abnormalities are assumed to "prime" developing intratumorous thymocytes for the AChR and other autoantigens,[35] but is has been enigmatic why autoantigen-specific "priming" does not induce negative selection. Our data now suggest that overexpression of cFLIP in TAMG(+) thymocytes during "priming" could be a mechanism to counteract negative selection and allow "primed" T cells to escape from the thymoma to the blood.

How effector T cells escape from *peripheral* tolerance once they are released from thymomas is unclear. The failure of thymomas to generate FoxP3(+) regulatory T cells[36] is a likely candidate mechanism that may become relevant only when AChR-directed, potentially autoreactive effector T cells are released from a given thymoma (i.e. in the TAMG(+) setting). In addition, overexpression

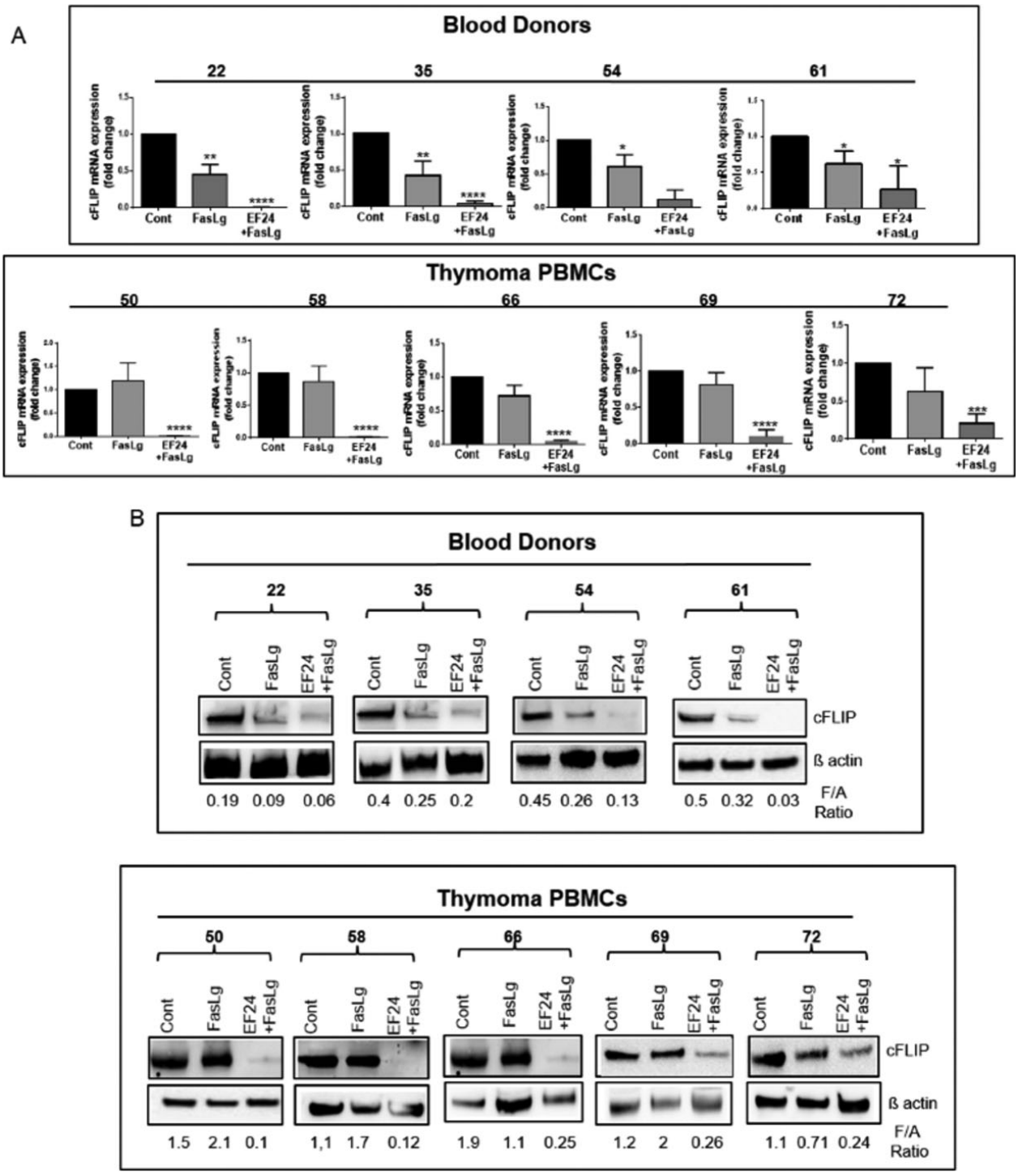

Figure 7. Impact of the NF-κB inhibitor EF24 on cFLIP expression of PBMCs from thymoma patients and blood donors. (A) QRT-PCR analysis of cFLIP expression in PBMCs from blood donors ($n = 4$) and five thymoma patients (two type AB, two B2, and one B3 thymoma). Left columns represent cFLIP mRNA levels in untreated PBMCs (cont); middle colums represent cFLIP levels in PBMCs treated with FASLG (10 ng/mL); right colums represent levels after pretreatment with EF24 for 24 h and subsequent FASLG treatment (10 ng/mL) for an additional 6 h. (B) cFLIP western blot analysis of whole PBMCs protein extracts (same samples and treatments as in [A]). 22, 35, 54, 61 50, 58, 66, 69, and 72 denote the ages of the patients (in years) from whom blood samples were obtained $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 10^{-4}$. cFLIP, cellular FLICE-like inhibitory protein; PBMCs, peripheral blood mononuclear cells; FASLG, Fas Ligand.

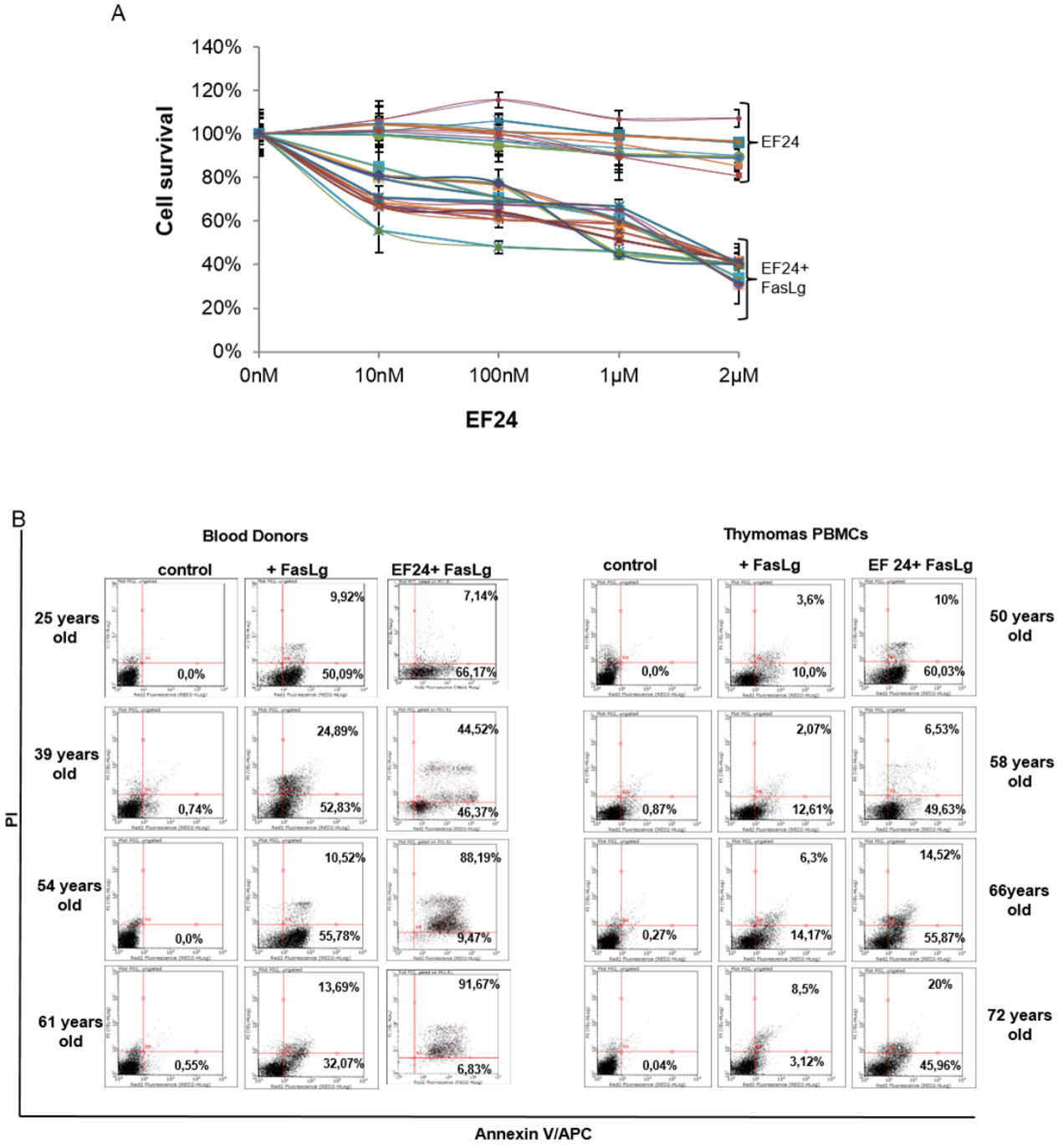

Figure 8. Impact of the NF-κB inhibitor EF24 on survival of PBMCs from thymoma patients and blood donors. (A) Cell survival analysis using the MTT assay of PBMCs from nine thymoma patients (four with AB, two with B2, and three B3 subtypes) treated either with EF24 alone or with EF24 followed by FASLG). (B) Apoptosis measurement by AnnexinV/APC/PI flow cytometry (FACS, Fluorescence-Activated Cell Sorting Guawa Millipore) in PBMCs of four blood donors and four thymoma patients. PBMCs were treated either with FASLG (10 ng/mL) or with EF24 for 24 h followed by FASLG (10 ng/mL) for an additional 6 h. 25, 39, 54, 61 50, 58, 66, and 72 denote the ages of the patients (in years) from whom blood sample were obtained. PBMCs, peripheral blood mononuclear cells; FASLG, Fas Ligand.

of cFLIP can block the FAS/FASLG-dependent death of TH1 and TH17 cell. This escape mechanism from peripheral tolerance is operative in several autoimmune diseases and mouse models,[37] including experimental autoimmune myasthenia gravis.[33] Therefore, the increased resistance of cFLIP^{high} PBMCs of thymoma patients to FASLG-induced

Table 2. Genotypic frequencies of 49A/G SNPs of the CTLA4 gene in TAMG(+) and MG(−) thymomas.

	n	A/A	A/G	G/G	*P* value for chi-square test
TAMG(+)	31	18 (58.06)	10 (32.26)	3 (9.68)	0.0003
MG(−) thymoma	39	5 (12.82)	25 (64.10)	9 (23.08)	ns

The frequency of the $+49^{\text{gain-of-function}}$ AA genotype is significantly higher in TAMG(+) than MG(−) thymoma patients. SNPs, single-nucleotide polymorphisms; TAMG, thymoma-associated myasthenia gravis.

apoptosis (Fig. 6), and the overcoming of this resistance by pharmacological cFLIP downregulation (Fig. 8A and B) strongly suggest that cFLIP overexpression in PBMCs of thymoma patients plays a role in the escape of these potentially autoreactive T cells from peripheral tolerance. This hypothesis is not in conflict with the observation that PBMCs of both TAMG(+) and MG(−) thymoma patients are resistant to FASLG-induced apoptosis (Fig. 6). The resistance of PBMCs in MG(−) thymoma patients is likely due to the high number of thymoma-derived CD8+ T cells in the blood[17,18,22] that we now find to have increased levels of cFLIP (Fig. 4). While export only of CD8+ T cells from thymomas is insufficient to elicit TAMG,[17] CD8+ T cells exported from MG(−) thymomas may not be "innocent," since autoimmune diseases other than TAMG are very common in both MG(−) and TAMG (+) thymoma patients.[34]

The mechanism underlying the overexpression of cFLIP in intratumorous thymocytes in TAMG is unknown. Furthermore, the observed association between CTLA4 genotypes and cFLIP expression levels in TAMG(+) thymomas remain enigmatic since no direct linking of CTLA4 signaling to modulation of cFLIP expression has been reported.[38] By contrast, the nuclear accumulation of p65/NF-κB in PBMCs of TAMG(+) patients in vivo, the downregulation of cFLIP by pharmacological NF-κB inhibition in PBMCs from thymoma patients, and the sensitization of $cFLIP^{high}$ PBMCs by NF-κB inhibition to FAS-mediated apoptosis imply that increased NF-κB signaling is a driver of cFLIP overexpression in PBMCs. Since both cFLIP and nuclear NF-κB expression decline in PBMCs after thymoma resection, it is tempting to speculate that NF-κB signaling is initiated in thymocytes inside the thymoma. Notwithstanding this open question, the impact of NF-κB on cFLIP expression in PBMCs justifies the consideration of pharmacological NF-κB inhibition as therapeutic strategy for TAMG. Furthermore, the elucidation of the currently unknown activators of NF-κB in PBMCs of TAMG(+) patients may offer additional therapeutic perspectives.

The decline of cFLIP expression in PBMCs after thymoma removal resembles the postoperative drop of recent thymic emigrants as defined by T-cell receptor excision circles (TRECs).[18,39] The fact that cFLIP expression was highest in CD4+CD45RA+ blood T cells at the time of surgery and dropped thereafter hints to their derivation from the thymoma and shows that the $cFLIP^{high}$ phenotype is tumor dependent. Whether the disappearance of $cFLIP^{high}$ PBMCs after thymoma surgery reflects absence of adequate stimuli in the extratumorous microenvironments or homeostatic elimination of $cFLIP^{high}$ cells from the repertoire through lack of appropriate niches[22] cannot be decided. Nevertheless, the normalization of cFLIP levels after thymoma removal warrants testing cFLIP overexpression in PBMCs as adjunct tool for the diagnosis of primary and recurrent thymomas, and for the delineation of thymic hyperplasia in EOMG patients, in whom cFLIP levels were adequate for age (Fig. 4A). This differential diagnosis may be further facilitated in the future by measuring the expression of another anti-apoptotic protein, survivin, that was recently described as being overexpressed in PBMCs in EOMG.[40]

In summary, the current findings suggest that thymoma-dependent cFLIP overexpression in thymocytes and peripheral T cells derived thereof may be a new mechanism that interferes with central tolerance as well as peripheral tolerance of potentially autoreactive T cells that have "escaped" from thymomas. The findings have the perspective to test the quantification of cFLIP levels as the diagnostic marker for primary and recurrent thymomas (vs. other mediastinal masses) and consider NF-κB blockade as a therapeutic strategy for TAMG.

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Author Contribution

The study was conceived and designed by D. B. and A. M. The experiments were conducted by D. B. Clinical data and material was provided by B. S., R. G., W. N., P. H., E. R., T. S., G. S.-D., V. Y., G. O. and P. S. Data were analyzed by D. B., M. V. and A. M. The manuscript was written by D. B. and A. M., and reviewed and approved by all coauthors.

Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Representative results of FACS analysis of supposedly naïve (CD45RA+) CD4+ and CD8+ blood T-cell subsets in a blood donor and a WHO type AB thymoma patient with MG.

Figure S2. CD95 receptor (FAS) determination in PBMCs by flow cytometry in a 66-year-old blood donor, a 66-year-old MG(−) thymoma and a 54-year-old TAMG(+) patient, using a FITC-labeled anti-CD95 antibody. The figure is representative of 10 blood donors, five MG(−) thymoma, and six TAMG(+) patients.

Figure S3. Identification of p65 in nuclear and cytosolic protein extracts of PBMCs by western blot. (A) Four blood donors (20, 23, 41, 55 years old), one non-myasthenic patient (50 years old) after resection of a normal thymus (NT) and two patients with type AB thymomas (one MG(−) thymoma and one TAMG(+)). (B) Six additional patients with type AB thymoma patients (three MG (−) thymoma and three TAMG(+)). As loading controls poly (ADP ribose) (PARP) polymerase for (nuclear protein extract) and ß-actin for cytosolic protein extract are used. NF-κB /PARP (P65/PA) denotes the ratio of the intensity values of the NF-κB/p65 band and the respective PARP band, while NF-κB/actin (P65/A) denotes the ratio of the intensity values of the NF-κB/p65 band and the respective β-actin band, as measured by densitometry, using ImageJ program.

Figure S4. Identification of NF-κB/p65 in nuclear and cytosolic protein extracts by western blot in 3 preoperatively obtained PBMCs of thymoma patients (cases #10 (TAMG AB), #39, and #41 (TAMG B2)) and in their follow-up PBMCs obtained 2 (#41), 6 (#10), and 12 (#39) months, respectively, after thymoma surgery**.** As loading controls poly (ADP ribose) (PARP) polymerase for (nuclear protein extract and ß-actin for cytosolic protein extract) are used. NF-κB/PARP (P65/PA) and NF-κB/actin (P65/A) ratios as in Figure S3.

Figure S5. Correlation between cFLIP mRNA expression and +49 CTLA-4 SNP in 31 TAMG(+) and 39 MG(−) thymomas (Table 2).

Table S1. Detailed clinical information about each individual patient included in this study (n = 72).

