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Suppressed diversity of survivin splicing in active rheumatoid arthritis

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Abstract

Introduction: Alternative splicing distinguishes normal and pathologic cells. High levels of oncoprotein survivin recognise patients with severe rheumatoid arthritis (RA). Here, we assess clinical relevance of alternative splicing of survivin in leukocytes of peripheral blood (PBMC) and bone marrow (BM) in RA patients.

Method: Transcription of survivin wild-type (survivin-WT), survivin-2B and survivin- Δ Ex3 was measured in 67 randomly selected RA patients and in 23 patients before and after B cell depletion with rituximab. Analysis was done in relation to disease activity, anti-rheumatic treatment and serum levels of rheumatoid factor (RF) and survivin.

Results: Survivin-WT was the dominant splice variant equally expressed in T and B cells, while survivin-2B and survivin- Δ Ex3 were higher in B cells. High disease activity (DAS28 $>$ 5.1) was associated with an excess of survivin-WT and low ratios between survivin-2B/WT ($p=0.035$) and survivin- Δ Ex3/WT in PBMC. Depletion of B cells by rituximab caused a decrease in survivin-WT ($p=0.005$) in PBMC, increasing the ratio between survivin-2B/WT ($p=0.009$) and survivin- Δ Ex3/WT ($p=0.001$) in BM. This increase in survivin-2B/WT was associated with reduction in CD19+ BM cells ($r=0.929$, $p=0.007$), RF (IgM, $r=0.857$, $p=0.024$; IgA, $r=0.739$, $p=0.021$), and DAS28 (0.636, $p=0.054$). The increase in survivin- Δ Ex3 in BM was associated with a reduction of CD19+ BM cells ($r=0.714$, $p=0.058$) and DAS28 ($r=0.648$, $p=0.049$), while survivin- Δ Ex3/WT was associated with RF (IgG, $r=0.882$, $p=0.016$).

Conclusion: This study demonstrates that the suppressed diversity of survivin splicing in leukocytes may attribute to adverse self-recognition in RA. Depletion of autoantibody producing B cells improves the balance of survivin splicing.

Introduction

Survivin is a multifunctional protein that belongs to the inhibitor of apoptosis (IAP) family and is encoded by the *BIRC5* gene, which is found at chromosome 17q25 in humans [1]. Survivin is a marker of malignant cell growth expressed in a vast range of cancers (reviewed by [2]). In normal tissues, survivin is essential for fetal development and for regeneration and repair of damaged tissues [3].

Survivin has been identified in cytoplasm, nucleus and mitochondria and has different functions within these cellular localisations [4]. Nuclear survivin plays a part in regulation of cell division, whereas mitochondrial and cytoplasmic survivin inhibits apoptosis and promotes

cell proliferation [5, 6]. Survivin is upregulated during the G2/M phase in mitosis and forms a chromosomal passenger complex together with inner centromere protein, Aurora B and borealin, aiding formation of microtubules and their attachment of kinetochores during cytokinesis [7]. When released from the nucleus, survivin displays anti-apoptotic functions. Cytoplasmic survivin forms a complex with the X-linked IAPs (XIAP), which enhances its stability against ubiquitin-dependent degradation [8]. The XIAP-survivin complex binds caspase-3, preventing its pro-apoptotic functions. In the mitochondrial compartment, survivin binds pro-apoptotic protein Smac/Diablo that inhibits its release and activation of caspase-9 [9].

The mRNA of human survivin has six different splice variants of which wild-type survivin (survivin-WT, 142 amino acids), survivin with an insert of additional exon 2 (survivin-2B, 165 amino acids) and survivin with deletion of exon 3 (survivin- Δ Ex3, 137 amino acids)

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(Fig. 1) are the most frequent [10, 11] and comprise 98 % of mRNA expression from the *BIRC5* gene. All splice variants are identical in the N-terminus containing the BIR domain and differ in the carboxyl region. Survivin-WT and survivin-2B are actively moved out from the nucleus binding the carboxyl region to an exportin-1 [12, 13]. Survivin-ΔEx3 lacks the export signal, which is thought to keep it in the nucleus and in the mitochondria [5, 14].

Survivin-WT can form homodimers in solution and the balance between the dimer and monomer forms of survivin seems to regulate its ability to translocate and function in cellular compartments [15]. Additionally, survivin-WT may form heterodimers with survivin-2B and survivin-ΔEx3, which disrupts their normal function in cell death regulation and cell proliferation control [10, 14, 16]. Survivin-2B has a pro-apoptotic function [12, 17, 18] interfering with and blocking tubulin polymerisation and inducing mitochondria-dependent apoptosis [12, 17]. Survivin-ΔEx3 has dual functions. Similar to survivin-WT, it may inhibit apoptosis by preventing a XIAP-dependent activation of caspases in the cytoplasm and a release of Smac/Diablo from mitochondria [16, 19, 20]. In non-cancer cells, survivin-ΔEx3 mediates cell spreading, migration and stability [21]. If overexpressed, survivin-ΔEx3 also has a pro-apoptotic function and inhibits cell growth and proliferation in cell cultures [2, 22–24].

Overexpression of survivin in non-cancerous processes has been linked to inflammation, presumably contributing to the decreased apoptosis in the T cells of cerebrospinal fluid in multiple sclerosis [25], in skin lesions of patients with psoriasis [26] and in synovial tissue [27, 28] of patients with rheumatoid arthritis (RA). Reports on the role of survivin in the function of pluripotent stem cells [29, 30] and in the development of mature T cells [31] reserve a place for survivin-dependent mechanisms in immune responses. Our recent studies have shown an association between survivin and three key stones in the

pathogenesis of RA: smoking [32], carriage of HLA-DRB1 antigen [33] and production of the RA-specific autoantibodies, rheumatoid factor (RF) and antibodies to citrullinated peptides (ACPA) [33–35]. High levels of survivin are associated with poor prognosis in RA predicting progressive joint damage and low responsiveness to anti-rheumatic treatment [35, 36].

In the present study we ask whether differential splicing of the survivin gene is of clinical relevance in RA. The measurement of three major splice variants of survivin showed that the proportional composition between survivin-WT and survivin-2B or survivin-ΔEx3 rather than the quantity of individual variants in leukocytes of peripheral blood (peripheral blood mononuclear cells (PBMCs)) and bone marrow (BM) is clinically important. An excess of survivin-WT and low survivin-2B/WT and survivin-ΔEx3/WT ratios in PBMCs distinguished patients with high disease activity, while anti-rheumatic treatment led to a gradual increase of the survivin-2B/WT ratio. Therapeutic B-cell depletion changed the profile of survivin splice variants in leukocytes of RA patients by reducing survivin-WT and favouring splicing of pro-apoptotic survivin-2B and survivin-ΔEx3. This change in survivin composition correlated to the reduction of B cells in BM, reduction in serum levels of RF and reduction in the disease activity. The association between survivin splicing and the autoreactive nature of B cells revealed a connection of survivin with the functional immune processes in RA.

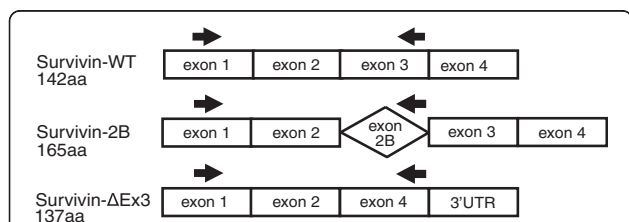


Fig. 1 Survivin splice variants. Exon organisation in mRNA of human most frequent survivin splice variants, which comprise 98 % of mRNA expression from the *BIRC5* gene. Splice variants were measured using the same forward primer located in the N-terminus. The reverse primers were located within exon 3 (for survivin-WT), within the inserted sequence derived from intron 2 (for survivin-2B) and within the unique sequence of exon 4 (for survivin-ΔEx3). Arrows indicate positions of PCR primers. Sequences for each primer can be found in Methods. UTR untranslated region, WT wild type

Table 1 Clinical characteristic of patients with rheumatoid arthritis

	Cohort (n = 67)	Rituximab-treated group (n = 23)
Age (years)	56.6 (24–79)	59.4 (29–79)
Female/male	65/2	21/2
Disease duration (years)	10.2 (1–40)	13.1 (2–33)
RF positive (%)	83.6	96.0
ACPA positive (%)	71.7	83.0
DAS28	3.86 (1.38–6.77)	5.89 (4.6–6.8)
Methotrexate (n)	55 (82.1 %)	18 (78.3 %)
Dose (mg/week)	18.2 (7.5–25)	18.0 (10–25)
Anti-TNFα	10 (14.9 %)	0
Rituximab	13 (19.4 %)	9 (39.1 %)
Anti-IL-6R	2 (3.0 %)	0
CTLA4	1 (1.5 %)	0
Serum survivin (ng/ml)	18.5 (0.02–306.21)	2.07(0.10–14.9)

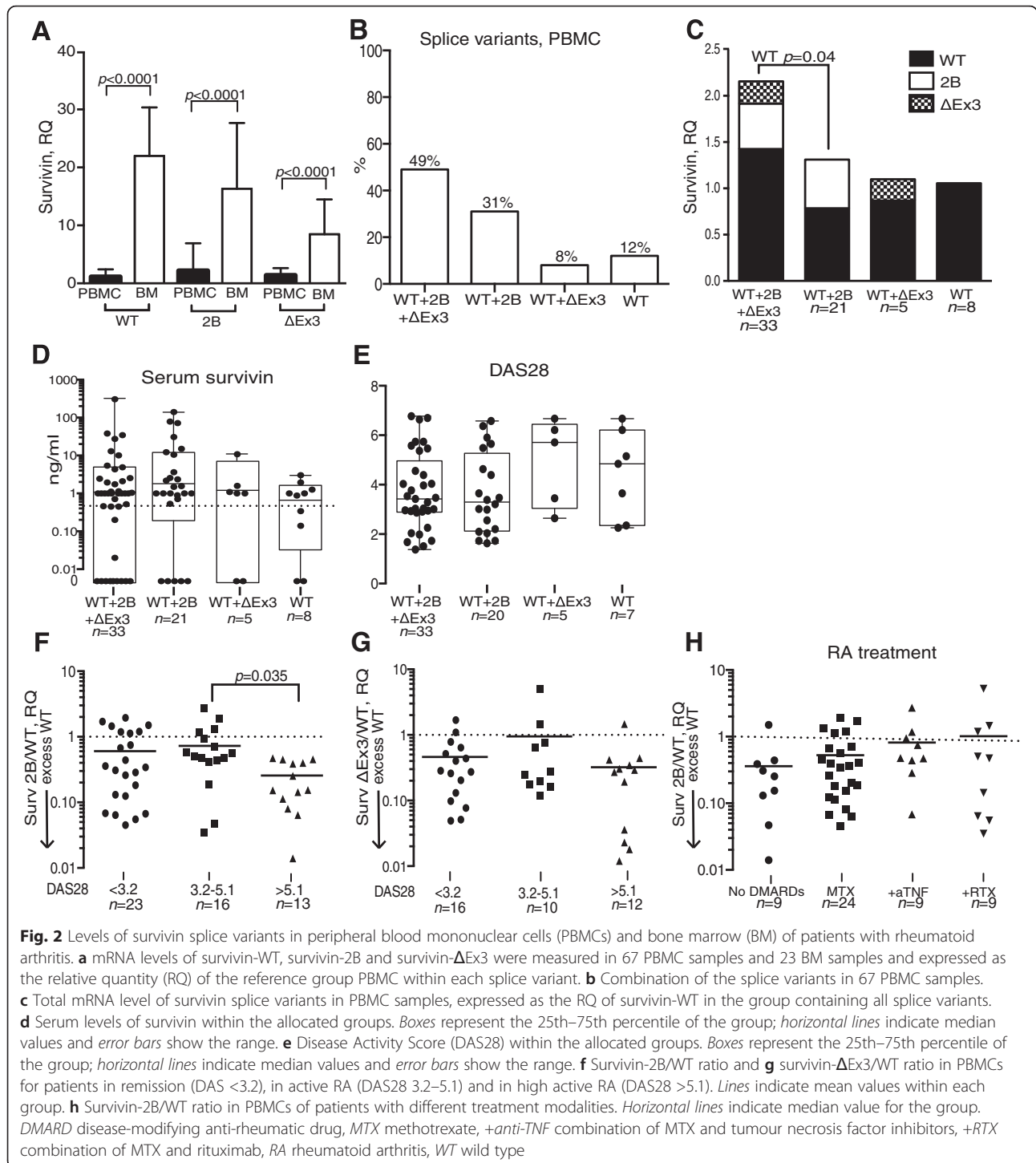
Values are mean and minimum and maximum are shown
ACPA antibodies against cyclic citrullinated peptides, CTLA4 cytotoxic T lymphocyte associated protein, DAS28 Disease Activity Score based on the evaluation of 28 joints, IL-6R interleukin 6 receptor, RF rheumatoid factor, TNFα tumour necrosis factor alpha

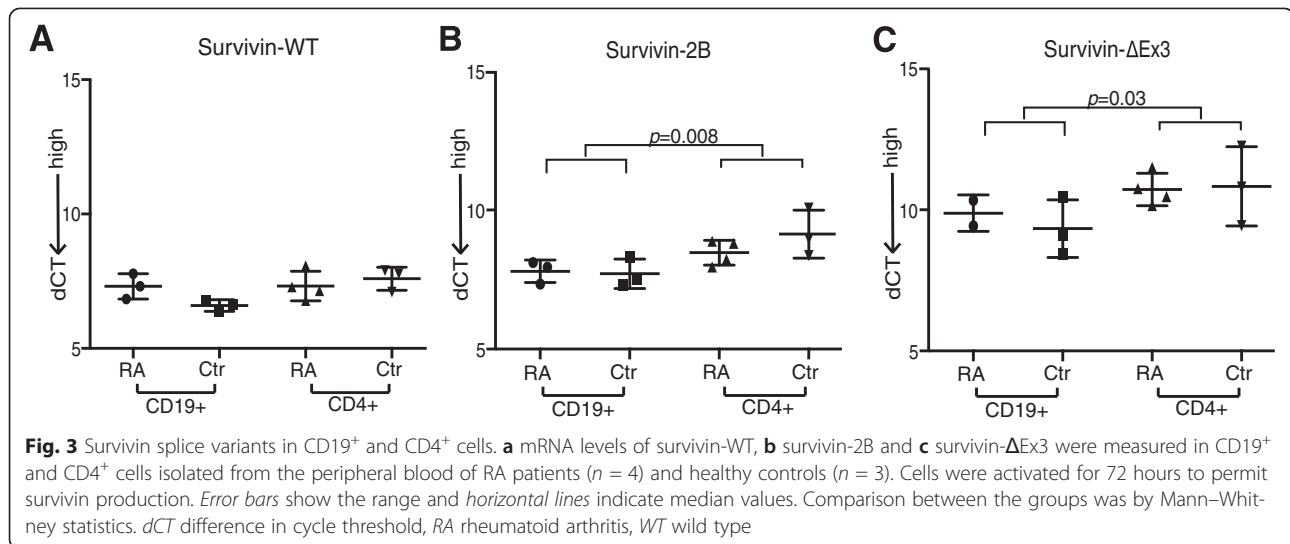
Methods

Patients

A total of 67 randomly selected patients with established RA who attended the Rheumatology Clinic at Sahlgrenska University Hospital, Gothenburg, were included in the study. All patients fulfilled the 1987 Rheumatoid Arthritis Classification Criteria [37]. Clinical and demographic

characteristics of the patients are presented in Table 1. At the time of blood sampling, 55 patients (82 %) were treated with methotrexate (MTX), and 23 of 55 patients combined MTX with biologics (tumour necrosis factor- α (TNF α) inhibitors, 10 patients; rituximab (RTX), 13 patients). One patient was treated with abatacept as monotherapy, and two with tocilizumab. The remaining nine





patients had no treatment with disease-modifying anti-rheumatic drugs (DMARDs) at the time of the study. Another 23 RA patients were treated with 1000 mg B cell depleting monoclonal anti-CD20 antibodies (RTX; Hoffman-La Roche Ltd, Basel, Switzerland) provided intravenously on days 1 and 15 as described elsewhere [38]. Disease activity was assessed at the day of blood sampling

by evaluation of 28 joints for swelling and tenderness, and the Disease Activity Score (DAS28) was calculated.

Ethical approval

The study was approved by the Ethical Committee of the Sahlgrenska University Hospital. All patients gave written informed consent before giving blood and bone marrow samples.

Table 2 Effect of rituximab treatment in patients with rheumatoid arthritis

	Before RTX	After RTX ^a
DAS28 score	5.89 ± 0.6	4.34 ± 0.7
Rheumatoid factor (IU/ml)		
IgG	41.5 ± 29.3	22.7 ± 19.5*
IgM	67.2 ± 31.1	50.2 ± 38.3
IgA	41.0 ± 38.3	25.0 ± 19.5
WBC count (×10 ⁹ /l)		
PBMCs	6.5 ± 3.1	7.8 ± 3.2
BM	2.1 ± 1.4	2.6 ± 1.6
CD19 ⁺ (%) ^b		
PBMCs	7.7 ± 3.8	0
BM	9.4 ± 4.3	1.3 ± 1.2 [†]
CD3 ⁺ (%) ^b		
PBMCs	45.9 ± 15.8	42.5 ± 12.9
BM	28.0 ± 9.8	29.4 ± 16.5

Values presented as mean ± standard deviation, n = 23

^a Samples 1–3 months after RTX treatment

^b From the lymphocyte population

* p = 0.008

[†] p < 0.0001

BM bone marrow, DAS28 Disease Activity Score based on evaluation of 28 joints, PBMC peripheral blood mononuclear cells, RTX rituximab, WBC white blood cell

Blood and bone marrow sampling

Serum was prepared by centrifugation for 15 minutes at 800 × g, aliquoted and stored at -20 °C until analysed. PAXgene Blood RNA tubes (PreAnalytix, Hombrechtikon, Switzerland) were used for mRNA preparation according to the manufacturer's recommendations. BM samples were obtained from 23 patients before RTX treatment by aspiration from the crista iliaca as described previously [38]. Blood and BM sampling was repeated 1 month or 3 months after the RTX treatment.

Cell isolation and stimulation

Mononuclear cells from the peripheral blood (PBMCs) and BM were isolated by density gradient separation on Lymphoprep™ (Axis-Shield PoC As, Oslo, Norway). For in-vitro experiments, CD19⁺ B cells and CD4⁺ T cells were separated from the isolated PBMCs using Dynabeads® (Invitrogen, Carlsbad, CA, USA). The separated cell populations were resuspended in Dynal buffer (2 % bovine serum albumin (BSA) and 1 mM ethylenediamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS)) at a concentration of 2 × 10⁶ cells/ml. CD19⁺ cultures were stimulated with Pam3Cys (10 µg/ml; EMC Microcollections, Tübingen, Germany) and CD4⁺ cultures were stimulated with Concanavalin A (5 µg/ml; Sigma, St. Louis,

MO, USA). Cells were collected after 72 hours, washed and lysed in RLT lysis buffer for RNA analysis.

Serological measurements

Serum levels of survivin were measured using a matched antibody pair by a sandwich enzyme-linked immunosorbent assay (ELISA) (DYC647; R&D Systems, Minneapolis, MN, USA) as described previously [39]. Samples were diluted 1:10 and the detection limit was 0.1 ng/ml. Values above 0.45 ng/ml were defined as positive. Total levels of RF and ACPA antibodies were measured at the Laboratory of Clinical Immunology at Sahlgrenska University Hospital. RF of IgG, IgM and IgA subclasses were analysed from serum, diluted 1/1000, using an ELISA assay [38].

RNA isolation and SYBR Green-based real-time PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and Paxgene Blood miRNA kit (Qiagen) according to the manufacturer's recommendations. The RNA concentration and quality were measured using a Nanodrop spectrophotometer (ND1000 Spectrophotometer) and Experion™ RNA StdSens Analysis chip (Bio-Rad Laboratories, Hercules, CA, USA). cDNA was synthesised using the Applied Biosystems (Foster City, CA, USA) High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's recommendations. Transcription of survivin was assessed using SYBR Green qPCR Mastermix (SABiosciences, Qiagen) and ViiA™ 7 Real-Time PCR (Applied Biosystems). All samples used the identical forward primer GACCACCGCATCTCTACATTC and splice variant specific reverse primers (Fig. 1): survivin-WT, TGCTTTTATGTTCTCTATGGG; survivin-2B, AAGTGCTGGTATTACAGGCGT; and survivin-ΔEx3, ATTGTTGGTTTCCTTTCATG [40] (Sigma). Melting curves for each PCR were performed between 60 and 95 °C to ensure specificity of the amplified product. All samples were run in duplicate with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene and with a negative control. Expression levels of target genes were normalised to GAPDH to obtain the difference in cycle threshold (dCt). Six samples from healthy subjects were used as the reference group. The relative quantity (RQ) was calculated using the ddCt method.

Statistical evaluation

Correlation between variables was analysed by Spearman test. Differences between two groups were tested by Mann–Whitney test and chi-square analysis. All tests were two-tailed and conducted with a 95 % confidence interval (CI) and $p < 0.05$ was considered significant. Statistical analyses were performed using the Prism programme (v.6.x; GraphPad software, San Diego, CA, USA).

Results

Diversity of survivin splicing in leukocytes of the peripheral blood and BM

Transcription of survivin-WT, survivin-2B and survivin-ΔEx3 was analysed in 67 PBMC samples and in 23 BM samples of randomly selected RA patients. Clinical characteristics of the patients are presented in Table 1. The RQ of each survivin splice variant was much higher in the BM compared with PBMCs and showed approximately 20 times excess for survivin-WT, 15 times for survivin-2B, and seven times for survivin-ΔEx3 (Fig. 2a). Survivin-WT was the predominant splice variant present in all PBMC and BM samples. Survivin-2B and survivin-ΔEx3 were undetectable in 19–43 % of PBMC samples and 4–9 % of BM samples, which resulted in a diversity of survivin splicing in RA leukocytes (Fig. 2b). The level of survivin-WT was significantly higher ($p = 0.04$) in the samples combining expression of all three splice variants (Fig. 2c). The univariate analysis revealed no significant association between the levels of individual survivin splice variants and clinical parameters of RA (all $r < 0.3$; data not shown). Serum levels of survivin (Fig. 2d), DAS28 (Fig. 2e) and disease duration (data not shown) were similar in the groups with different combinations of survivin splicing.

Composition of survivin splicing and clinical signs of RA

Since survivin-WT may form dimers with survivin-2B and survivin-ΔEx3 opposing its functions, we studied the survivin-2B/WT ($n = 54$) and survivin-ΔEx3/WT ($n = 38$) ratios in PBMCs where both splice variants were present. We found that survivin-2B/WT was low and reflected a significant excess of survivin-WT in the patients with high disease activity (DAS28 > 5.1) (Fig. 2f). Similarly, low survivin-ΔEx3/WT ratio with a relative excess of survivin-WT was found in PBMC samples irrespectively of DAS28 (Fig. 2g).

To study whether anti-rheumatic treatment affects the survivin-2B/WT ratio, we compared survivin splicing in patients with different treatment modalities. The survivin-2B/WT ratio increased gradually with the intensification of immunosuppression. This ratio was lowest in the patients having no DMARD treatment, and tended to increase in the patients treated with a combination of methotrexate with TNF inhibitors (Fig. 2h). The patients treated with a combination of MTX and RTX had further increase of the survivin-2B/WT ratio.

Difference of survivin splicing in T and B lymphocytes

To study composition of survivin splice variants produced by T and B lymphocytes, we separated CD4⁺ T cells and CD19⁺ B cells from PBMCs of four randomly selected RA patients (two women, two men) and of

three healthy women. The separated cells were activated *in vitro* to permit survivin production, and mRNA levels of survivin-WT, survivin-2B and survivin- Δ Ex3 were measured. The levels of survivin-WT were similar in CD4⁺ and CD19⁺ cells (Fig. 3a), while survivin-2B and survivin- Δ Ex3 were significantly higher in CD19⁺ B cells (Fig. 3b and c, respectively). No significant difference in survivin-2B/WT and survivin- Δ Ex3/WT ratio was found between RA patients and controls.

B-cell depletion modifies survivin splicing in BM and PBMCs

To assess whether B-cell depletion changed the splicing of the survivin gene we analysed the paired PBMC and BM samples of 23 RA patients before and after RTX treatment (Table 2). The RTX treatment resulted in the complete depletion of B cells from the PBMCs and a significant reduction of the B-cell population in the BM [40]. Consequently, a decrease of RF and of the disease activity (Fig. 4a) was observed. Low survivin-2B

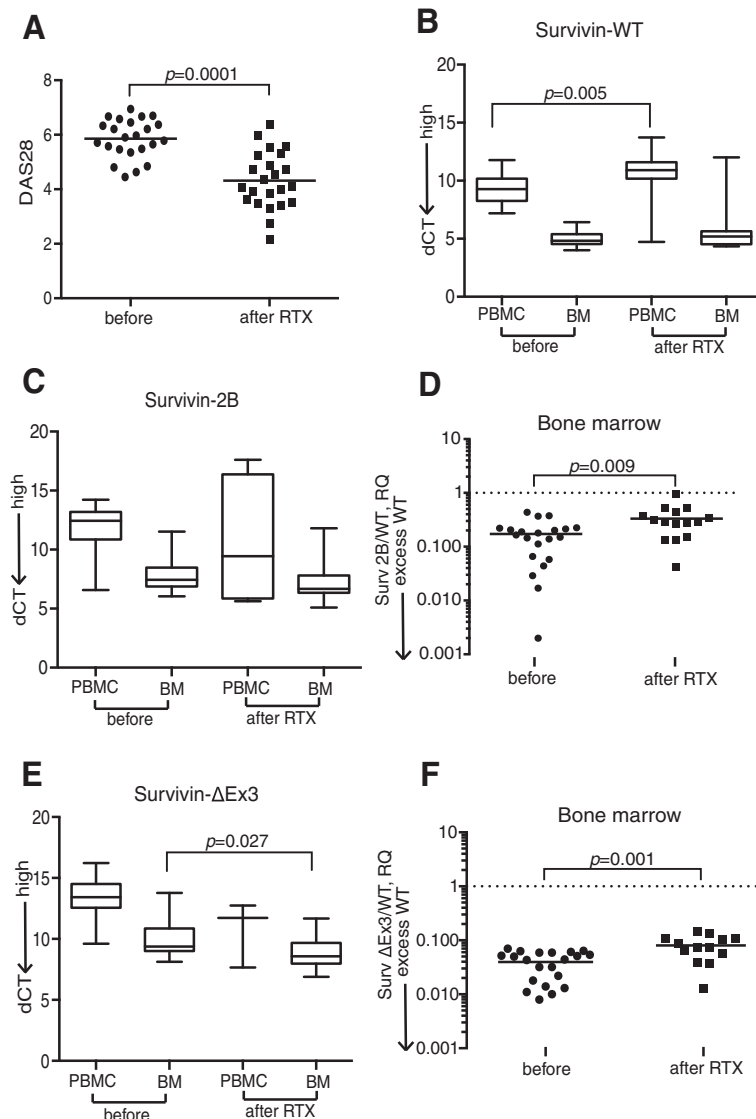


Fig. 4 Survivin splicing in blood and bone marrow leukocytes of RA patients treated with rituximab (RTX). **a** Disease Activity Score (DAS28) before and after RTX treatment. mRNA level of **b** survivin-WT and **c** survivin-2B in peripheral blood mononuclear cell (PBMC) and bone marrow (BM) leukocytes of 23 patients before and 1–3 months after RTX treatment. **d** Survivin-2B/WT ratio in BM before and 1–3 months after treatment. **e** Survivin- Δ Ex3 in PBMCs and BM before and 1–3 months after RTX treatment. **f** Survivin- Δ Ex3/WT ratio in BM before and after RTX treatment. Boxes represent the 25th–75th percentile of each group, horizontal lines indicate median value and error bars show the range. Comparison between groups performed with Mann-Whitney statistics. dCT difference in cycle threshold between survivin and glyceraldehyde 3-phosphate dehydrogenase, RQ relative quantity

in the BM before RTX treatment was associated with higher numbers of BM CD19⁺ B cells ($r = 0.857$, $p = 0.024$).

The B cell depletion in PBMCs was followed by a decrease of survivin-WT (Fig. 4b). The individual expression of survivin-2B in PBMCs and BM showed no statistical change (Fig. 4c). The change was, however, sufficient to attain a significant increase of the survivin-2B/WT ratio in BM (Fig. 4d). The reduction in survivin-WT and the change of the survivin-2B/WT ratio correlated to the reduction of the BM CD19⁺ B cells (Fig. 5a) and of RF levels (Fig. 5b). Remarkably, the association was strong for low-affinity RF-IgM antibodies and for the high-affinity RF-IgA. The reduction in survivin-WT and the increase of the survivin-2B/WT ratio was in direct proportion to the decrease of clinical activity of arthritis (Fig. 5c).

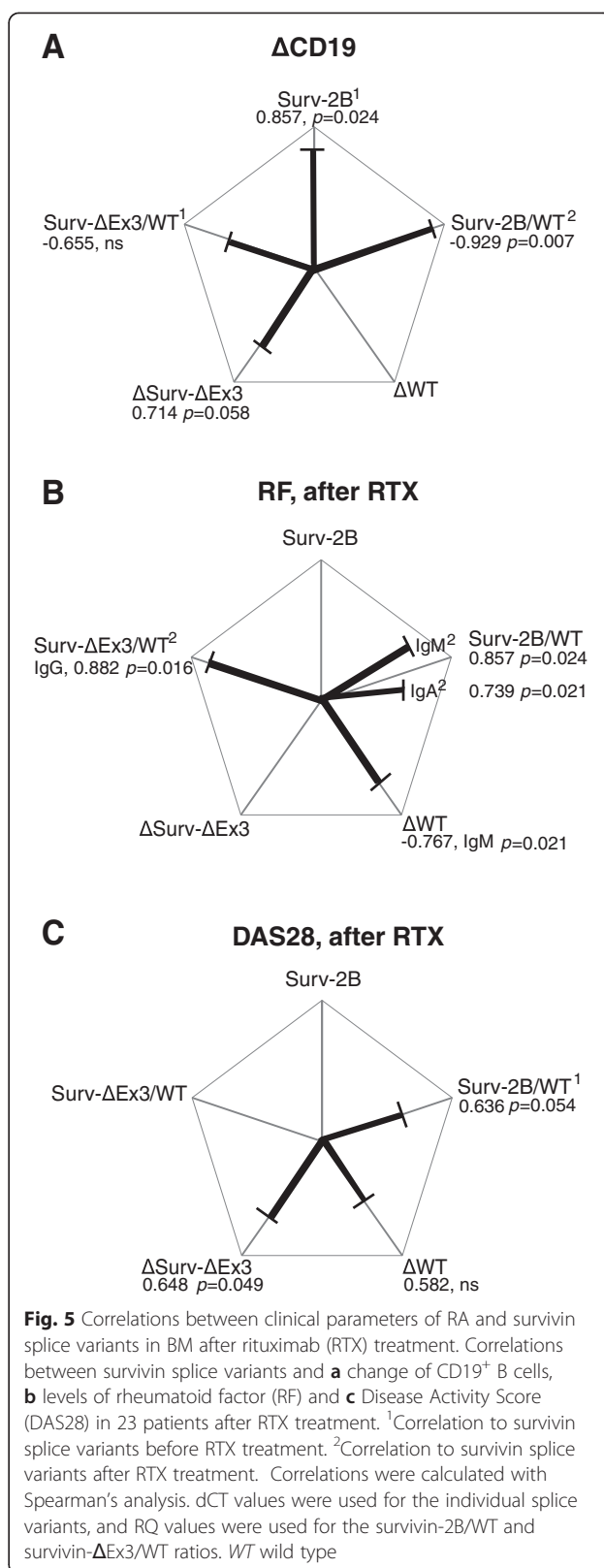
Following RTX treatment, survivin-ΔEx3 was increased in BM (Fig. 4e), which caused a significant increase of the survivin-ΔEx3/WT ratio (Fig. 4f). Analogously with survivin-2B, the change in survivin-ΔEx3 after RTX treatment correlated to a decrease in the BM CD19⁺ B cells (Fig. 5a) and in the serum survivin levels ($r = 0.928$, $p = 0.008$). The survivin-ΔEx3/WT ratio showed a correlation to the reduction of the high affinity RF-IgG antibodies (Fig. 5b).

Discussion

This study shows that transcription of survivin in the leukocytes of PBMCs and BM has a tight connection to the pathogenesis of RA and to the functional autoimmune processes in these compartments. We present evidence that changes in the pattern of survivin splicing may belong to the clinically important events in RA.

Survivin-WT is the dominant survivin splice variant in PBMCs and BM of RA patients, followed by survivin-2B and survivin-ΔEx3, which confirms the observations in malignant cells and in pluripotent stem cells [29, 30, 41–43]. In the observational and in the B cell depletion parts of this study, the excess of survivin-WT is connected to high clinical activity of RA, suggesting that survivin-WT favours inflammatory and aggressive profile of leukocytes. Previous studies reported high expression of survivin in lymphocytes infiltrating tissue lesions in several non-cancer pathologies [25–28, 44]. However, splicing composition of this expression was not assessed.

This study showed that B cells from peripheral blood of RA patients and from healthy controls were recognised by high production of survivin-2B and survivin-ΔEx3. The low survivin-2B before RTX treatment and the increased production of survivin-2B and survivin-ΔEx3 thereafter was in direct relation to B cell numbers in the BM, and occurred in parallel with a decrease in survivin-WT. This observation leads us to suggest that IgD⁺ B cells depleted



Conclusions

Alternative splicing is generally viewed as a mechanism distinguishing healthy and pathologic cells. This study extends this perspective and demonstrates that suppressed diversity of survivin splicing occurred in active RA as a part of autoimmune inflammation. Depletion of autoreactive B cells contributes to changes of the survivin splicing profile and decrease of autoantibody production (Fig. 6).

Abbreviations

ACPA: Anti-citrullinated protein antibody; BM: Bone marrow; BSA: Bovine serum albumin; CD19: Cluster of differentiation 19; CI: Confidence interval; CT: Cycle threshold; DAS28: Disease Activity Score; Δ Ex3: delta exon 3; Diablo: Direct IAP binding protein with low pI; DMARD: disease-modifying anti-rheumatic drug; EDTA: Ethylenediamine tetraacetic acid; ELISA: Enzyme-linked immunosorbent assay; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IAP: Inhibitor of apoptosis; MTX: Methotrexate; PBMC: Peripheral blood mononuclear cell; PBS: Phosphate-buffered saline; RA: Rheumatoid arthritis; RF: Rheumatoid factor; RQ: Relative quantity; RTX: Rituximab; SMAC: Second mitochondria-derived activator of caspases; TNF α : tumour necrosis factor alpha; WT: Wild type; XIAP: X-linked IAP.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MT carried out the experiments, analysed and interpreted the data and drafted the manuscript. KMEA carried out the experiments, analysed and interpreted the data. SA collected the material, carried out the experiments and analysed and interpreted the data. MB participated in the study design, carried out the experiments and analysed and interpreted the data. MCE carried out the experiments and analysed and interpreted the data. STS coordinated the collection of study material and the acquisition of patient data. MIB conceived and designed the study, interpreted the data and drafted the manuscript. All authors contributed to the creation of important intellectual content, to the preparation of the manuscript and gave their final approval to the manuscript.

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