Down-regulation of survivin alleviates experimental arthritis

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ABSTRACT
Survivin is a proto-oncogene that regulates cell division and apoptosis. It is a molecular marker of cancer. Recently, survivin has emerged as a feature of RA, associated with severe joint damage and poor treatment response. The present study examined if inhibition of survivin affects experimental arthritis, which was induced in mBSA-immunized mice by an injection of mBSA in the knee joint or developed spontaneously in collagen type II-immunized mice. The inhibition of survivin transcription by a lentivirus shRNA construct alleviated joint inflammation and reduced bone damage. The inhibition of survivin reduced the levels of metalloproteinases, β-catenin, and vimentin, limiting the invasive capacity of synovia, while no inhibition of osteoclastogenesis could be found. The inhibition of survivin led to a p53-independent reduction of T cell proliferation and favored the transcription and activity of Blimp-1, which limited IL-2 production and facilitated formation of regulatory Foxp3+CD4+ and effector CD8+ T cells. The study shows that the inhibition of survivin is sufficient to reduce joint inflammation and bone damage in preclinical models of arthritis. Antiinflammatory effects of survivin inhibition are related to p53-independent control of lymphocyte proliferation. J. Leukoc. Biol. 97: 135–145; 2015.

Introduction
RA is an inflammatory joint disease affecting 1% of the world population, and the incidence of RA in Sweden shows no decrease [1]. Treatment of RA has been revolutionized recently by the use of biologic therapies, including drugs targeting cytokines (TNF and IL-6), cells (B and T cells), and signaling pathways (tyrosine kinases). However, up to 50% of RA patients are resistant to biologic therapies, which contributes to early mortality and indicates our still-restricted knowledge of RA pathogenesis.

Survivin, the smallest member of the inhibitor of apoptosis protein family, has emerged recently as a biomarker of RA [2]. It is a multifunctional protein that plays biologic roles in apoptosis and the cell cycle. Survivin accumulates in mitotic cells, where it regulates chromosome-microtubule attachment, activation of spindle assembly, and cytokinesis (reviewed in ref. [3]). In prophase, survivin regulates apoptosis via the cofactor-dependent inhibition of caspases [4]. Transcription of survivin is induced by growth factors insulin-like growth factor 1, epidermal growth factor, and vascular endothelial growth factor, via members of the Ras oncogene family, and JAK/STAT, NF-κB, and β-catenin signaling pathways [3–5]. Overexpression of survivin is associated with increased cell proliferation, which in tumors leads to invasive expansion and spreading, whereas in normal tissues it maintains regenerative potential and growth. In the context of RA, survivin is expressed in the inflamed synovial tissues and in RA synovial fibroblasts [6, 7], recognized by a reduced apoptosis and an increased ability to produce cytokines and growth factors. These proinflammatory factors favor survivin accumulation within the areas rich with macrophage and memory T cell [8, 9]. The expression of survivin in synovial tissue is suggested to promote the transition of synovial fibroblasts to an invasive phenotype, followed by proliferation of synovial tissue and pannus formation. The suppression of the survivin gene by use of small interfering RNA in fibroblasts prevented development of arthritis in the mice knee joints [10]. High levels of survivin are found in synovial fluid and blood of 28–60% of RA patients [11–13], where they are associated with the disease activity, production of autoantibodies [7, 8], and predicted development of joint damage [6, 12].

Clinical and experimental findings imply a role of survivin in the key processes in the pathogenesis of RA. A pilot study on the Medical Biobank of Northern Sweden showed that survivin was increased in individuals before clinical onset of RA. Importantly, survivin correlated with the cytokines driving formation of Th1

Abbreviations: 3D = 3-dimensional, Blimp-1 = B-lymphocyte-induced maturation protein 1, BS = bone surface area, BV = bone volume, CBA = cytometric bead array kit, CD52L = cluster of differentiation 62 ligand, CIA = collagen-induced arthritis, CT = X-ray computed tomography, CTSK = cathepsin K, DBA = dilute brown nonagouti, Foxp3 = forkhead box p3, gMFI = geometric mean fluorescence intensity, i.a. = intra-articular, IHC = immunohistochemistry, IRF = IFN regulatory factor, mBSA = methylated BSA, MMP = matrix metalloproteinase, MTP = metatarsosphingiel.

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and Th17 proinflammatory effector T cells at the initial stages of RA [2]. In the experimental setting, survivin is shown critical for the process of antigen presentation; the break point of immune responses in RA, as it is required for the fms-related tyrosine kinase 3-dependent maturation of dendritic cells; and expression of the MHCI receptor [14, 15]. Survivin is important for the formation of a functional TCR [16, 17] and for mounting of memory immune responses [18, 19]—processes that control accumulation of antigen-specific T cells in the arthritic synovia.

The present study addressed whether the inhibition of survivin is sufficient to reduce severity of RA and the degree of joint damage in an experimental setting. We used two widely studied models of antigen-induced arthritis [20, 21], which are characterized by progressive joint inflammation associated with infiltration of the joint tissues by immune cells, by damage to cartilage and bone, and by increased serum levels of antigen-specific antibodies and autoantibodies, a phenotype that resembles human RA. We used a single injection of a lentivirus to deliver a construct that incorporates in the host genome and produces shSurv. We showed that the inhibition of survivin, at the time of initial immunization with antigen or at the time at which clinical signs of disease were noted, alleviated the clinical and histologic features of arthritis. Furthermore, the skeletal damage in the inflamed joints was reduced. Importantly, the inhibition of survivin resulted in decreased cell proliferation. The present study provides experimental proof to the clinical association between survivin and the joint destructive course of RA.

MATERIALS AND METHODS

Arthritic mouse models

Balb/c mice (7-8 weeks old, female; Charles River, Scanbur, Karlsunde, Denmark) were subjected to mBSA-induced arthritis, as described earlier (ref. [22] and Fig. 1A). DBA/1 mouse (8 weeks old, male; Taconic Europe A/S, Ry, Denmark) were subjected to CIA, as described earlier (ref. [23] and Fig. 1B). Samples of serum, bone marrow, spleen, draining lymph nodes, and joints were collected for further analysis. All mice were housed at the animal facility at the Department of Rheumatology & Inflammation Research (University of Gothenburg, Sweden) under standard conditions of temperature and light and fed laboratory chow and water ad libitum. Experimental settings are approved by the Animal Ethics Committee of the University of Gothenburg (permits 319/2011 and 272/2010).

In vivo shRNA delivery

Lentiviral construct, MISSION TRCN0000054613 and TRCN0000054616 (Sigma-Aldrich, St. Louis, MO, USA), encoding shSurv16, was used separately and combined (shSurv13 + shSurv16). Both shRNAs target all three known isoforms of mouse survivin. The shNT was subjected to MISSION pLKO.1-puro small hairpin RNA controls, shRNA = small hairpin RNA, shSurv = small hairpin RNA targeting survivin, TCF = T cell factor, TRAP = tartrate-resistant alkaline phosphatase, Treg = regulatory T cell

Figure 1. Experimental design and feasibility of survivin down-regulation by shRNA. (A–C) Experimental setup for survivin targeting intervention in antigen-induced arthritis. (A) Balb/c mice were immunized with mBSA on Days 0 and 7. mBSA was then injected i.a. on Day 21. shRNA (shNT or shSurv) was administered once (i.a. or i.p.) on Day 0. (B) DBA/1 mouse was immunized with chicken-collagen II (CII) on Day –21 and on Day 0. shRNA (shNT or shSurv) was administered once (i.p.) on Day 0. (C) Experiments, vehicle*, Experiment 1, PBS; experiments 2–4, DMEM complete medium. (D) Percent change of survivin+ population in bone marrow and spleen of mice treated with shSurv (n = 10–16) compared with shNT (n = 7–9; dotted line), as measured by flow cytometry. (E) Flow cytometry histogram showing that treatment with shSurv16 or shSurv13 + 16 reduced the intensity of survivin expression (expressed as gMFI) on cells from bone marrow and spleen compared with shNT treatment.

RA = rheumatoid arthritis, RAGE = receptor for advanced glycation endproducts, RANK = receptor activator of NF-kB, RANKL = receptor activator of NF-kB ligand, Ror t = retinoid acid receptor-related orphan receptor 1, Runx2 = Runx-related transcription factor 2, shNT = nontargeting small hairpin RNA controls, shRNA = small hairpin RNA, shSurv = small hairpin RNA targeting survivin, TCF = T cell factor, TRAP = tartrate-resistant alkaline phosphatase, Treg = regulatory T cell

Evaluation of arthritis

All 4 paws of the CIA mice were examined by a blinded observer every 2nd day for the signs of swelling and/or redness and evaluated and scored from...
0 (no inflammation) to 4, as described earlier [23]. The arthritis index was constructed by adding the scores from the 4 limbs for each CIA mouse. No clinical evaluation was possible for the mBSA-injected knee joints as a result of a lack of visual changes.

At the end of experiments, CIA paws and mBSA-injected knee joints were fixed in 4% formaldehyde for 48 h, decalcified in Tris-EDTA buffer, embedded in paraffin, sectioned, and stained with H&E. Both sections of mBSA joints and CIA paws were coded and evaluated blindly for signs of synovitis and cartilage/bone destruction, according to the grading systems described previously [24]. Arbitrary scales from 0 to 3 (where 0 is a healthy joint) were used. The cumulative arthritis index was constructed for each mouse and included synovitis and erosive scores for all joints.

**Micro-CT imaging and analysis**

The left hind paw (from the tip of the toes and to the proximal part of tibia) of CIA mice was scanned and reconstructed into a 3D structure with SkyScan1176 micro-CT (Bruker, Antwerp, Belgium) with a voxel size of 9 μm. The scanning was done at 50 kV/467 mA, with a 0.5 mm aluminum filter. Exposure time was 765 ms. The X-ray projections were obtained at 0.3° intervals with a scanning angular rotation of 180°. The reconstructed dataset was segmented by an automated threshold algorithm. The projection images were reconstructed into 3D images by use of NRecon software (version 1.5.1; Bruker) and CT-Analyser (version 1.7; Bruker). The analysis was performed on the ankle joint and MTP II–IV joints chosen as regions of interests. The parameters measured and calculated were as follows: 1) BS and 2) BV were calculated by the marching cubes method; 3) specific Bv/BV ratio was given by BS density (BS/8V).

**Cell preparation**

Bone marrow cells from femur and tibia were flushed with PBS. Splenocytes and lymph nodes were washed through a 70 μm cell strainer, and dissolved in PBS. Cell lysates were prepared by adding 2-ME-supplemented Buffer RLT (Qiagen). In vitro lentivirus-transduced cells were used as positive control.

**Confirmation of lentiviral incorporation and survivin knockdown**

DNA was prepared from synovial tissue and spleen cells by use of the AllPrep DNA/RNA Mini Kit (Qiagen), according to the manufacturer’s instructions. The presence of lentivirus in the material was examined with PCR by use of the forward primer 5'-TGGACATCATATGCTTTACCGTA3' and the reverse primer 5'-GTATGTCGTGTGCTATTATGCT-3' and HotStar Taq (Qiagen). In vitro lentivirus-transduced cells were used as positive control. Survivin protein was measured in cells from bone marrow, spleen, draining lymph nodes, and synovia by flow cytometry, and the gene expression was quantified by use of quantitative PCR.

**Morphology and IHC**

Paraffin sections were deparaffinized and treated with heat-induced epitope retrieval in 2100 Retriever with BORG decloaker (Biocare Medical, Concord, CA, USA), endogenous peroxidase depleted with 0.3% H2O2 (Merck, Darmstadt, Germany), followed by blocking in serum solution (Vector Laboratories, Burlingame, CA, USA) and Fc Block (BD Biosciences, San Diego, CA, USA). After incubation with rabbit anti-mouse survivin antibody (AHP604; AbD Serotec, Kidlington, United Kingdom), rat anti-Foxp3 (145-5773; eBioscience, San Diego, CA, USA), or rabbit gamma globulins (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) as a negative control, the specimens were incubated with ImmPRESS anti-rabbit Ig polymer detection reagent and stained by use of ImmPRESS 5-amino-9-ethylcarbazole (Vector Laboratories), and Mayer’s hematoxylin (Histoloh, Göteborg, Sweden). TRAP staining was performed by use of Naphthol AS-BI phosphate (7-bromo-5-hydroxy-2-naphthoic-anisidine phosphate N2250; Sigma-Aldrich) on deparaffinized tissue sections. TRAP-positive cells were counted within 2 consecutive fields in the growth-plate areas by use of a Leica DMLB microscope and the Leica QWin software.

For immunofluorescence, the sections were stained with actin-staining BODIPY fluorescent (FL) phallacidin (Invitrogen, Carlsbad, CA, USA), followed by incubation with primary antibody and Alexa Fluor 555-conjugated goat anti-rabbit IgG (Invitrogen). Slides were mounted in ProLong Gold antifade reagent mounting medium with DAPI. Sections were visualized by use of a confocal laser-scanning microscope (LSM710; Zeiss, Oberkochen, Germany). The background fluorescence level was set as the negative control slides.

**In vitro proliferation assay and measurement of cytokine release**

Freshly prepared mouse spleen cells from arthritis mice were plated on a 96-well plate at 2 x 10^5 cells/well in Iscove’s complete medium and stimulated with Con A at 1.25 μg/ml or LPS at 10 μg/ml (both Sigma-Aldrich). Cell proliferation was measured by the incorporation of [3H]-thymidine (GE Healthcare, Uppsala, Sweden), as described previously [11]. A stimulation index was calculated by dividing stimulated values of proliferation (in counts per minute) by unstimulated values. Supernatants were collected after 48 h, and the cytokine levels were analyzed by a CBA kit (BD Biosciences), as described previously [25].

**Flow cytometry**

Single-cell suspensions from different tissues were preincubated with Fc Block (BD Biosciences), as described [22]. The cells were stained with antibodies, as indicated in Supplemental Table 1. Intracellular staining for survivin was performed, as described previously [15]. Analyses were performed on a FACS Canto II with FACS Flow software (BD Biosciences), and data were evaluated by use of FlowJo software (Tree Star, Ashland, OR, USA). Gating of the cells was based on the isotype control or on the fluorochrome-minus-one setting when needed. Data are shown as percentage or mFI, calculated in FlowJo, and compared with data acquired in the same run.

**Gene expression analysis**

RNA samples were prepared from cell lysates in Buffer RLT or from tissues in RNAlater solution (Qiagen) and processed as described earlier [15]. From synovia, tissue RNA was prepared with the RNeasy Fibrous Kit (Qiagen). RNA (400 ng) was used for cDNA synthesis by use of the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time amplification was performed, as described earlier [15], by use of primers, as indicated in Supplemental Table 1. The results are presented as a relative quantity compared with the expression level in the control cells with the delta-delta CQ (dCQ) method.

**Protein preparation and Western blotting**

Total protein was prepared from tissue by homogenization and sonication in the presence of protease and phosphatase inhibitors (Complete Mini and PhosSTOP; Roche Diagnostics GmbH, Basel, Switzerland). Protein concentrations were measured by use of the Biocinchonic Acid Protein Assay kit (Pierce, Rockford, IL, USA), according to the manufacturer’s protocol. Proteins were separated on SDS-PAGE, 4–12% Bis-Tris gels (NuPAGE; Invitrogen), and transferred to polyvinylidene difluoride membranes (NuPAGE; Invitrogen), which were blocked with 5% BSA and incubated with rabbit anti-pS253Y705 antibodies (ab76315; Abcam, Cambridge, United Kingdom) or rabbit antiactin antibodies (A2066; Sigma-Aldrich) at 4°C overnight. Detection was performed with peroxidase-conjugated anti-rabbit secondary antibody (NA954VS; GE Healthcare Life Sciences, Pittsburgh, PA, USA) and Amersham ECL Select substrate (GE Healthcare Life Sciences). Chemiluminescent signals were visualized and quantified by the ChemiDoc equipment and Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).
RESULTS

Down-regulation of survivin alleviates mBSA-induced arthritis

To test whether inhibition of survivin affects development of mBSA-induced arthritis, mice were treated with lentiviral constructs encoding shRNA silencing survivin gene expression. The shSurv16 construct was delivered separately or in combination with shSurv13 (shSurv13 + 16) via a single i.p. or i.a. injection (Fig. 1A). i.p. injection of shSurv induced 47–51% reduction in the survivin+ population in the spleen and 20–29% reduction in the bone marrow by Day 12 and sustained by Day 28 (Fig. 1D). This also coincided with the reduced intensity of survivin expression in the spleen and bone marrow cells of shSurv-treated mice (Fig. 1E). The i.a. injection of shSurv resulted in 17–43% reduction in the survivin+ population in spleen and in 7–8% reduction in the bone marrow, whereas the survivin+ populations in the draining lymph nodes and in synovia were similar in shSurv- and shNT-treated mice.

The safety data were obtained by comparing the shNT construct with vehicle. The injection of shRNA caused no signs of inflammation at the injection site and no weight loss as a consequence of the construct delivery. The degree of synovitis and bone erosion, and the cumulative arthritis index were similar in the shNT-treated mice and the mice injected with vehicle (Supplemental Table 2).

The i.p. injection of shSurv into mBSA-immunized mice reduced the arthritis frequency (shSurv16, 67%; shSurv13 + 16, 37.5%, vs. shNT, 76%; P = 0.024) and severity (arthritis index: shSurv16, 1.6 ± 1.1; shSurv13 + 16, 0.9 ± 1.4, vs. shNT, 1.8 ± 1.5; P = 0.04; Fig. 2A and B). shSurv treatment tended to reduce the frequency of joint damage (erosions: shSurv16, 33%; shSurv13 + 16, 12.5%, vs. shNT, 41%; P = 0.11). The i.a. injection of shSurv into the knee joints of mice on the day of immunization with mBSA reduced the arthritis frequency (shSurv13 + 16, 46%, vs. shNT, 77%; P = 0.11) and severity (arthritis index: shSurv13 + 16, 0.9 ± 1.2, vs. shNT, 1.5 ± 1.1; P = 0.18) with a pattern similar to that observed in the mice injected i.p. (Fig. 2C).

Down-regulation of survivin affects the cellular composition and invasive capacity of synovial tissue

The cellular composition of synovial tissue from the mBSA-injected knee joints was analyzed by flow cytometry and revealed that CD11b+CD18+ synovial macrophages were the major survivin+ cell subset in the inflamed synovia. shSurv treatment resulted in a reduced number of survivin+ cells within CD11b+CD18+ macrophages, reducing the level of their major adhesion molecule CD18 (Fig. 2D and E). No reduction was found in the number of survivin+ granulocytes or lymphocytes.

The survivin expression in synovial tissues correlated with transcription levels for regulators of invasive synovial growth: RAGE (r = 0.45, P = 0.031; Fig. 2F), vimentin (r = 0.44, P = 0.036; Fig. 2G), and β-catenin (r = 0.42, P = 0.047; Fig. 2H). We observed no significant difference in transcription of β-catenin, p53, or p21 in synovia of shRNA-treated mice (Fig. 2I–K), but the RNA levels of β-catenin correlated with survivin and p53 (Fig. 2H and L). shSurv treatment reduced gene expression of MMP13 (Fig. 2M) and MMP2 (Fig. 2N), which play an active role in cartilage degradation in RA (reviewed by Takaishi et al. [26]).

Therapeutic effect of survivin down-regulation in the CIA

We next examined if inhibition of survivin had a therapeutic effect on arthritis. To study this, collagen type II-immunized mice, which develop polyarticular inflammation resembling human RA in clinical and morphologic features, were treated with shSurv, delivered via a single i.p. injection on the 1st day of visible clinical arthritis (Fig. 1B). shSurv13 + 16-treated mice preserved weight during the progress of arthritis, whereas shNT-treated mice lost 8.6% of their body weight (P = 0.01; Fig. 3A). The levels of proinflammatory cytokines IL-6 and TNF were not changed in the shSurv-treated mice at Days 12 and 28 after survivin inhibition (Supplemental Fig. 1).

shSurv16-treated CIA mice tended to delay arthritis progression (Fig. 3B), whereas the shSurv13 + 16-treated group showed reduced arthritis severity compared with the shNT-treated group (Fig. 3C). The clinical score correlated with survivin expression in the bone marrow, as measured by flow cytometry (r = 0.48, P = 0.007), indicating a connection between these two processes (Fig. 3D).

Evaluation of the ankle and MTP joints of the left hind paw of the shNT-treated CIA mice by X-ray microtomography revealed severely eroded joint surfaces (Fig. 3E and G). The BV in the ankle and MTP joints of mice treated with shSurv13 + 16 was bigger compared with shNT-treated mice, suggesting that bone in the vicinity of inflamed joints was preserved by shSurv treatment (Fig. 3F and H). The BV in the foot correlated inversely with survivin expression in the bone marrow (r = -0.49, P = 0.007) and with the clinical arthritis score in the hind paw (r = -0.52, P = 0.004, and r = -0.50, P = 0.006, respectively; Fig. 3F and H). Thus, down-regulation of survivin prevented damage of the joint surfaces and inhibited bone loss in CIA mice.

Down-regulation of survivin predisposes to changes in the bone metabolism

As shSurv treatment inhibited bone resorption, and the bone-protective effect correlated with survivin expression in the bone marrow, we analyzed the effect of survivin inhibition on the formation of osteoclasts in the bone marrow. shSurv treatment reduced the survivin+ population within CD11b+CD3+B220− mononuclear osteoclast progenitors in the bone marrow (Fig. 4A) and the intensity of survivin expression on this population (Day 12: shSurv16, 1460 ± 137, shSurv13 + 16, 1661 ± 150, vs. shNT, 2082 ± 559; P = 0.0006 and 0.02, respectively; Day 28:

Statistics
Data were analyzed by use of GraphPad Prism (version 6.0 for Mac; GraphPad Software, San Diego, CA, USA) and expressed as mean ± so. Significance regarding differences between shSurv-treated and control groups was calculated by use of the Mann-Whitney U-test, and differences in frequency distributions between groups were calculated by use of χ² test. Two-tailed tests were used, and for the statistical evaluation of the results, P < 0.05 was considered significant.
The proportion of RANK+ osteoclast precursors and RANK+CD61+ preosteoclasts was increased within the CD11b+ population of shSurv-treated mice (Fig. 4C and D). The transcription of genes controlling bone metabolism (LDLR-related protein 5, Runx2, CTSK, IRF8, PU1, and the RANK-encoding TNFR superfamily 11a) showed no significant change with survivin inhibition nor was the activity of Stat3 affected (Supplemental Fig. 2A and B).

The number of TRAP+ mature osteoclasts in the femoral growth plate of mBSA-injected knee joints of shSurv-treated mice showed no differences from the control, shNT-treated mice (Fig. 4E). Consequently, no correlation was found between the number of TRAP+ cells and survivin expression in the bone marrow or the arthritis index. Taken together, these results provide evidence that the beneficial effect of the survivin inhibition on arthritis is not mediated primarily through inhibition of osteoclastogenesis.

Down-regulation of survivin reduces lymphocyte proliferation and promotes Blimp-1-dependent T cell maturation

shSurv-treated mice showed a reduction in the number of survivin+ cells within CD4+ and CD8+ T cell populations in the spleen (Fig. 5A), whereas the total size of these populations was the same as in shNT-treated mice. shSurv-treated mice showed reduced proliferative capacity of splenic T (Fig. 5B) and B cells (Fig. 5C).

p53 is a major regulator of the cell cycle and is controlled by survivin [16, 17, 27]. To study if survivin-dependent inhibition of lymphocyte proliferation in the arthritic mice was associated with changes in p53 transcription, we measured its mRNA levels in spleen, but no differences were seen (Fig. 5D). The transcriptional activity of p53 in shSurv-treated and control mice was compared by mRNA levels for the p53-target gene, p21. The levels of p21 mRNA were similar in shSurv- and shNT-treated
mice (Fig. 5E), indicating that the transcriptional activity of p53 was not changed by shSurv treatment and therefore, was a less probable mechanism of limited lymphocyte proliferation in these mice.

Another regulator of lymphocyte proliferation is the transcriptional repressor Blimp-1. Its antiproliferative effect is achieved through the transcriptional control of the IL-2 gene [28]. Indeed, shSurv-treated mice showed increased levels of Blimp-1 mRNA on Days 12 and 28 (Fig. 5F). This was associated with low IL-2 production by Con A-stimulated spleen cultures of shSurv-treated mice (Fig. 5G).

Blimp-1 polarizes T cells toward effector T cells through the repression of the \( \text{CD}62\text{L} \) gene [29, 30]. shSurv-treated mice showed a reduced CD62L+ T cell population in the spleen (shSurv16: 46.5 ± 5.0, shNT: 62.8 ± 2.6; \( P < 0.001 \)), supported by a reduction in the CD62L intensity/cell. This resulted in an increase in the effector (CD62L-CD44hi) and central memory (CD62L+CD44hi) T cell populations (Fig. 5H). Consequently, there was a reduction in the naïve (CD62LhiCD44lo) CD4+ and CD8+ T cell populations in the spleen (Fig. 5H).

The effect of shSurv treatment on the formation of pathogenic effector Th17 and Tregs was studied. Real-time PCR analysis of gene expression in the synovia showed that IL-17A mRNA levels correlated with the severity of arthritis, evaluated as arthritis score (Fig. 6A). shSurv-treated mice tended to decrease IL-17A mRNA in the knee synovia, whereas no change was observed in the spleen or in the Ror\(\text{g}\)t expression (Fig. 6B and C). Foxp3+ Tregs were accumulated in the knee synovia in proportion to arthritis (Fig. 6D and E). The shSurv-treated mice had a significant increase in Foxp3+ T cells in the spleen and draining lymph nodes (Fig. 6F), which was also associated with the increased IL-10 production in the T cell spleen cultures (Fig. 6G). Taken together, this might favor a reciprocal effect of survivin inhibition on the generation of effector Th17 and Tregs.

**DISCUSSION**

The present study shows that down-regulation of survivin is sufficient to reduce arthritis when introduced at the time of antigen exposure and at the overt stage of clinical arthritis. The antiarthritic effect observed in the present study was achieved as a result of partial down-regulation of survivin in leukocytes in the lymphoid organs, spleen, and bone marrow, where 30–60% reduction of the survivin+ population was reached. In the inflamed joints, down-regulation of survivin was...
achieved in the CD11b⁺CD18⁺ synovial macrophages, the major proinflammatory cell population within the rheumatoid synovial tissue contributing to early invasion and progression of erosive arthritis [31, 32]. Survivin-deficient macrophages had a reduction in the surface β₂-integrin (CD18), predisposing to poor leukocyte adhesion and transendothelial migration to the inflammation site [33, 34]. In the setting of experimental arthritis, this resulted in reduced leukocyte infiltration and in lower mRNA levels of RAGE and vimentin, regulators of synovial hyperplasia and invasive growth in RA [35–37]. The production of these proteins has been reported previously dependent on CD18 [38, 39].

We observed no reduction in the production of proinflammatory cytokines IL-6 and TNF as a consequence of survivin inhibition. This was partly unexpected, as shSurv-treated mice had better general health and had no weight loss during CIA. In this study we have not measured serum levels of these cytokines, but the levels are measured in the supernatants of cultured splenocytes stimulated with Con A for T cell activation and with LPS for B cell activation. The inhibition of survivin has little or no effect on the production of TNF and IL-6 in splenocytes, probably because survivin transcription is inhibited downstream of these cytokines. The slight increase of IL-6 and TNF supports the existence of a positive-feedback regulation of survivin transcription by these cytokines.

Survivin is the effector molecule downstream of the Wnt signaling pathway and is a transcriptional target gene for the β-catenin/TCF complex [40]. The Wnt/β-catenin signaling is essential for articular cartilage and bone development, renewal, damage reparation, and homeostasis [41]. The proteins of the Wnt family are enriched in hyperplastic rheumatoid synovial tissue compared with normal adult tissues [42]. Activation of β-catenin/TCF initiates sequential transcription of proteins required at each stage of joint development, balancing chondrocyte proliferation and homeostasis and also, osteoblast and osteoclast function in bone remodeling, which may contribute differently to inflammation and bone changes during arthritis [43–45]. Down-regulation of survivin resulted in transcriptional changes of several genes engaged in Wnt signaling, including a decrease of β-catenin and its downstream targets Runx2, indispensable trigger of osteoblast formation [46, 47], and MMP13 and MMP2, required for bone and cartilage homeostasis [48–50].

Consistent with previous reports [51, 52], down-regulation of survivin had an antiproliferative effect on the lymphocytes of the arthritic mice. In malignant tissues, the proliferative properties of survivin are dependent on the control of transcription and post-translational activity of the major cell-cycle regulator, p53 [53, 54]. Through physical interaction with p53, survivin acts as a transcriptional repressor for the

Figure 4. Down-regulation of survivin and the effect on osteoclasts. (A and B) shSurv treatment decreases the survivin⁺ population within CD11b⁺ bone marrow cells (A), as well as the intensity of survivin/cell (B), as measured by flow cytometry. ctr, Control. (C and D) Flow cytometry analysis of the effect of shSurv treatment on the RANK⁺ and RANK⁺CD61⁺ osteoclast precursor populations. (E) Osteoclastogenesis was assessed in the arthritic knee joints (mBSA arthritis, Day 28 after shRNA treatment i.p.). The number of mature osteoclasts, identified as TRAP⁺ cells (stained red; indicated by arrowheads) in the growth plate, was counted. The Mann-Whitney U-test was used to compare differences between groups. Original bar, 100 μm.
cyclin-dependent kinase inhibitors p21/p27 [27, 55]. The transcriptional analysis of the p53 and p21 genes in shSurv-treated arthritic mice was not able to identify changes in p53 mRNA levels or its transcriptional activity. β-Catenin is known to orchestrate an antiproliferative effect of p53 [56]; however, in shSurv-treated mice, the low protein levels of survivin corresponded to low transcription of β-catenin. Taken together, these observations indicate that limited lymphocyte proliferation in splenocytes of shSurv-treated mice does not occur as a consequence of enhanced function of p53. This is in agreement with earlier reports, suggesting a disparity between survivin-dependent thymocyte maturation and proapoptotic actions mediated by the activity of p53 [16].

Survivin inhibition resulted in a robust increase of the transcription of the gene coding for Blimp-1, a transcriptional repressor, essential for terminal differentiation of leukocytes. The functional activity of Blimp-1 in the shSurv-treated arthritic mice was recognized by the low production of IL-2 by lymphocyte cultures and by limited lymphocyte proliferation, as Blimp-1 is the only known repressor of IL-2 gene transcription, interacting with the IL-2 promoter to control the proliferation of T and B cells [28]. The up-regulation of Blimp-1 may provide an additional mechanism to p53 of survivin-dependent proliferation control in lymphocytes. This hypothesis is supported by p53-independent changes in T cell maturation, observed after the conditional knockout of the survivin gene [17, 18].

Other Blimp-1-related effects observed in shSurv-treated mice included persistent, low expression of CD62L on lymphocytes, which results in reduced leukocyte infiltration into tissues; limited the generation of the naïve T cell population; and induced a shift from naïve CD4+CD8- to effector CD4+/CD8+ populations. Blimp-1 is critical for the maturation of effector and memory CD4+ and CD8+ T cells [30, 57]. The increased expression of Blimp-1 in shSurv-treated mice was associated with an increase formation of the Foxp3+ Tregs in spleen and draining lymph nodes. Tregs are efficient in suppressing immune responses and restoring self-tolerance in RA. Indeed, transfer of Tregs slows down the progression of CIA [58], whereas the depletion of them enhances disease development [59]. Furthermore, Tregs protect against joint damage by suppressing osteoclastogenesis [60, 61]. Thus, the enhanced formation of effector CD8+ T cells, in combination with Tregs, and the low expression of CD62L on lymphocytes, limiting their migration capacity, create an anti-inflammatory T cell phenotype that could partly explain the reduced leukocyte influx into arthritic joints and inhibition of progressive bone loss in shSurv-treated mice.

Down-regulation of survivin efficiently prevented damage of the joint surfaces and inhibited bone loss in the arthritic mice. The CD11b/CD18 adhesion complex in combination with RANKL stimulation supports expression of CD61 and
transdifferentiation of monocytes into osteoclasts [62]. As survivin inhibition affected CD18, we expected this to limit osteoclastogenesis in the mBSA-arthritis mice, in parallel with the protein levels of survivin. The osteoclast formation in arthritis is facilitated by Th17 cells [63, 64]. We observed decreased levels of IL-17a mRNA in synovial tissue of shSurv-treated mice, which predisposed to a reduced Th17 support of osteoclast formation in the shSurv-treated mice. In contrast, we observed an increase, rather than a reduction, in RANK+CD61+ osteoclast progenitors in the bone marrow of shSurv-treated mBSA mice, and transcription of the osteoclast protease CTSK tended to be increased. The process of osteoclastogenesis seems to remain incomplete, as the number of TRAP+ mature osteoclasts in the growth plate of the inflamed knee joints was comparable in the shSurv- and shNT-treated groups. The evaluation of the RANK signaling pathway revealed no alteration of PU1 or IRF8 transcription or of STAT3 activity induced by down-regulation of survivin.

Blimp-1 has been reported recently to regulate bone formation. RANKL-stimulated bone marrow monocytes increase Blimp-1 expression, and repression of Blimp-1 activity is shown important for osteoclast differentiation [65]. Osteoclast-specific, Blimp-1-deficient mice had impaired osteoclast differentiation and increased bone mass [66]. The time frame of the Blimp-1 increase in shSurv-treated arthritic mice is limited to 12 and 28 days, which could explain the observed shift in osteoclastogenesis of the mice but was too short to change the bone mass.

In conclusion, survivin is an important biomarker of erosive RA. In the present study, we demonstrate that survivin controls molecular mechanisms essential for the development of the disease, as the inhibition of survivin is sufficient to alleviate experimental arthritis.

**AUTHORSHIP**

M.I.B. conceived of the project and designed experiments. K.M.E.A., M.N.D.S., M.C.E., and I-M.J. performed all experiments and analyzed the results. K.M.E.A. and M.I.B. wrote the manuscript.

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after lymphocyte activation and is found preferentially in memory T cells.


Survivin regulates experimental arthritis

Andersen et al.

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T cells · B limp-1 · p53 · Treg · erosions · synovitis
Down-regulation of survivin alleviates experimental arthritis


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