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Research Article

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Research Article

Splenectomy modulates the immune response but does not prevent joint inflammation in a mouse model of RA

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Abstract

The spleen is the largest secondary lymphoid organ which is involved in the development of B cells and also in systemic (auto)immune responses. Using the recombinant human G1 domain-induced arthritis (GIA) model in splenectomized and control BALB/c mice, we investigated the role of the spleen in the induction and pathogenesis of autoimmune arthritis. Splenectomized mice developed GIA with a similar clinical picture to the control group. However, we observed significant alterations in the humoral and cellular immune responses in splenectomized mice. In the sera of the splenectomized mice, we found lower pro-inflammatory cytokine and anti-rhG1 IgM levels, but higher IL-4, anti-rhG1 IgG1 and anti-CCP and RF antibodies. The arthritis induction in the splenectomized group was associated with a significant expansion of activated helper T cells and an increase in the proportion of the circulating B1 and marginal zone B cell subsets. Importantly, immunization of the splenectomized mice with rhG1 induced the formation of germinal centers in the inguinal- and mesenteric lymph nodes (i/mLNs) which showed an active immune response to rhG1. Finally, both B and T cells from the mLNs of the splenectomized mice showed decreased intracellular Ca^{2+} signaling than those of the control group. Collectively, these findings indicate that the presence of the spleen is not critical for the induction of GIA, and in its absence the autoimmune arthritis is most likely promoted through the compensatory activity of the i/mLNs. However, our data implies the immunological role of the spleen in arthritis which could be further assessed in human RA.

Keywords: autoimmune arthritis, splenectomy, T- and B cell activation

Abbreviations: a-CCP, anti-cyclic citrullinated peptide antibody; AIRE, autoimmune regulator gene; APCs, antigen-presenting cells; Ca^{2+}, calcium; CD, cluster of differentiation; CIA, collagen-induced arthritis; DDA, dimethyl-dioctadecyl-ammonium bromide; DMEM, Dulbecco's modified Eagle's medium; DTH, delayed type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FSC, forward scatter; GC, germinal center; GIA, recombinant human G1-induced arthritis; i.p., intraperitoneal; IFNγ, interferon gamma; Ig, immunoglobulin; IL, interleukin; iLN, inguinal lymph node; MADCAM-1, mucosal vascular addressin cell adhesion molecule 1; mLN, mesenteric lymph node; MZ, Marginal zone; NFDM, nonfat dry milk; Nkx2-3, Nirenberg-Kim (NK) 2 homeobox 3; Nkx2-$3^{-/-}$, Nkx2-3-deficient mouse (homozygous); OPD, orthophenylene-diamine; PBS, phosphate buffer solution; PGIA, proteoglycan-aggrecan induced arthritis; PI3K, phosphoinositide 3-kinases; PKB, protein kinase B; PTPN2, tyrosine-protein phosphatase nonreceptor type 2; RA, rheumatoid arthritis; RF, rheumatoid factor; rhG1, recombinant human G1; RPMI, Roswell Park Memorial Institute Medium; SLE, systemic lupus erythematosus; SSC, side scatter; TD, T cell-dependent immune reaction; TF, transcription factor; Th, T helper cell; TNF-α, tumor necrosis factor alfa; VEGF, vascular endothelial growth factor.

Introduction

Rheumatoid arthritis (RA) is a common systemic autoimmune disease that affects approximately 1% of the world's population, with a higher prevalence in females than in males [1–3]. The trigger(s) of RA is still unknown; however, the interplay of genetic and environmental factors is thought to contribute to the development of RA [4–6]. Among the environmental factors, smoking may increase the risk of RA by modifying post-transcriptional regulation and inducing epigenetic modifications [4, 5, 7]. These, in turn facilitate the formation of autoreactive B and T helper (Th) cells, the latter being essential for orchestrating and amplifying the systemic (auto)immune reaction [8, 9]. These cells, in combination with innate immune cells like dendritic cells and macrophages target self-antigens in the joints leading to inflammation of the synovial membrane, cartilage damage, and bone erosions [10]. As a result, RA patients develop symmetrical joint swelling, severe pain, and morning stiffness, which ultimately leads to impaired movement and general weakness [11, 12]. Moreover, RA is often associated with extra-articular manifestations, such as accelerated atherosclerosis, Sjorgen's syndrome, rheumatoid nodules, and rarely Felty syndrome [13, 14].

The spleen is the largest secondary lymphoid organ, which plays essential roles in both innate and adaptive immunity, and it is crucial for B-cell maturation, too, including the differentiation of B1, B2, and marginal zone B cells [15, 16]. Moreover, the spleen is also involved in the development of autoimmune diseases, as e.g. splenomegaly is common in RA, systemic lupus erythematosus (SLE), and systemic sclerosis [17–20]. Interestingly, in 1% of RA patients Felty syndrome

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develops, which is defined by the presence of splenomegaly and neutropenia, wherein splenectomy can be an effective therapy of choice in some cases to improve the neutrophil count [21, 22]; however, the potential effect on the arthritis itself was not reported specifically. Although RA is a common systemic autoimmune disease that has been thoroughly studied, the currently available pharmacological therapies are restricted to reduce the symptoms and delay the disease pathogenesis [1, 23].

Autoimmune arthritis animal models, such as collagen-induced arthritis (CIA) and recombinant human G1 domain-induced arthritis (GIA), have proven to be extremely useful in studying the pathophysiology and immunological processes of RA [24, 25]. The translational significance of the GIA arthritis model lies in its ability to mimic many clinical and immunological aspects of human RA [22, 24, 25]. In the GIA mouse, model there is a predominant Th1 and Th17 polarization during arthritis induction, which is accompanied by significant autoantibody production including anti-rhG1- and anti-cyclic citrullinated peptide antibodies (anti-CCP) [24, 26, 27].

In our previous study, we investigated the effect of the Nkx2-3 transcription factor (TF) genetic deficiency in autoimmune arthritis [26]. Nkx2-3 is a homeodomain TF which is crucial for the development of the spleen, and the expression and regulation of mucosal addressin cell adhesion molecule-1 (MADCAM-1) [28, 29]. Nkx2-3 deficient mice (Nkx2-3$^{-/-}$) hence have a severely disturbed spleen structure with the lack of the marginal zone and the red pulp, and thus they are incapable of providing the necessary microenvironment for B-cell maturation and T-cell-dependent (TD) immune reactions [30]. Since GIA is a T-cell-dependent autoimmune process [24, 31], we tested if we can induce autoimmune arthritis in Nkx2-3$^{-/-}$ mice [26]. Surprisingly, Nkx2-3$^{-/-}$ mice developed autoimmune arthritis despite their severely disturbed spleen structure, however, with lower incidence and severity, less joint damage and fewer osteophyte formation revealed by micro-CT scan images compared to the normal BALB/c control group [26]. Moreover, the rhG1-induced immune response of the cultured spleen cells of the Nkx2-3$^{-/-}$ had lower proliferation and decreased cytokines release compared to those of the control mice. In the blood sera of the Nkx2-3$^{-/-}$, we measured lower levels of anti-CCP IgG2a, IL-17, IL-23, and IFN-γ, but higher IL-4 than in control mice indicating an altered Th1/Th17 immune response in the Nkx2-3$^{-/-}$ mice. Finally, we found that the Ca^{2+} signal in B cells of Nkx2-3$^{-/-}$ mice had a lower amplitude compared to control mice after stimulation with either anti-IgM or anti-IgG, while stimulation of T cells with anti-CD3 showed similar Ca^{2+} signals in both mouse groups. We concluded that the ameliorated autoimmune arthritis in the Nkx2-3$^{-/-}$ mice might be due to the spleen structural defects, and also to the weaker B-cell activation [26]. Since in the Nkx2-3$^{-/-}$ mice the spleen is not completely missing, here we wanted to decipher how the complete absence of the spleen after surgical removal (splenectomy), could impact the induction of autoimmune arthritis.

Surgical removal of the spleen has been already studied in another model of autoimmune arthritis, CIA [32, 33]. Both reports showed that CIA could be induced after splenectomy in mice [32] or rats [33]. Based on this, they concluded that the collagen-specific immune response was independent from the spleen [32, 33]. However, results were different concerning the antibody production [32, 33]. In the first study, the antibody levels and DTH responses were similar in splenectomized and control mouse groups [32]. In the second study, increased levels of CII-specific antibodies were measured in the splenectomized rats which was attributed to the compensatory mechanism by the bone marrow [33].

In the current study, we aimed to investigate further the significance of the spleen in the development of autoimmune arthritis. To that end, we induced autoimmune arthritis in splenectomized- and spleen-preserved (control) BALB/c mice using the GIA model. We monitored clinical parameters, inflammatory markers, and histological changes, as well as their cellular and humoral immune responses. Here, we found that splenectomized mice developed autoimmune arthritis similarly to the controls. However, we observed significant alterations in the cellular and humoral immune responses of splenectomized mice. Based on these results, we conclude that the absence of the spleen was compensated by other peripheral lymphoid organs, primarily the inguinal and mesenteric lymph nodes (LNs) in the splenectomized mice, which facilitated GIA.

Materials and methods

Mice

We used 2–6-month-old female BALB/c mice. Mice were kept under conventional conditions at 24 ± 2°C with a controlled 12/12 h light/dark cycle at the Department of Immunology and Biotechnology's Transgenic Mouse Facility. The mice were housed in groups of five and received acidified water and food ad libitum. All animal experiments were conducted following the University of Pécs, Animal Welfare Committee regulations (BA02/2000-23/2020).

Antibodies and reagents

All chemicals were purchased from Sigma–Aldrich unless otherwise stated.

For flow cytometry we used: washing buffer, PBS containing 0.1% NaN_3; staining buffer, PBS containing 0.1% BSA, and 0.1% NaN_3; fixation buffer: PBS containing 0.1% paraformaldehyde.

For histological staining, we used: washing buffer, PBS; blocking buffer, PBS containing 5% BSA.

For ELISA we used: blocking buffer, PBS containing 1.5% non-fat dry milk; washing buffer, PBS containing 0.05% or 0.5% Tween-20 was used as for cytokine- or antibody ELISA.

The following monoclonal antibodies were used for flow cytometry (also see Supplementary Table S1): anti-CD3-FITC, anti-CD4-PE, anti-CD8a-PE-Cy5, anti-CD25-APC, anti-CD38-PE, anti-CD73-Alexa Fluor 647, anti-IgD-FITC, anti-IgM-PerCP-Cy5.5, anti-CD138-APC-R700, anti-B220-PE-Cy7, CD23-BV421, all from BD Bioscience (San Jose, CA, USA). For immunofluorescence: anti-B220-Alexa Fluor 647 and anti-CD3-FITC. For immunohistochemistry: rat anti-mouse IgD (BD Bioscience) and PNA-Biotin (Vector Laboratories, BioMarker, Gödöllő, Hungary). Extravidin-alkaline phosphatase (Sigma-Aldrich) or ImmPRESS goat anti-rat IgG-HRP polymeric conjugate (Vector Laboratories, BioMarker, Gödöllő, Hungary) was used to detect the PNA, or IgD antibody, respectively. For ELISA: HRP rat anti-mouse-IgG1, HRP rat anti-mouse IgG2a and HRP rat anti-mouse-IgM (BD Bioscience) were used.

Surgical procedures

The 2–3-months-old female BALB/c mice were divided into two groups: in the first group mice were splenectomized, whereas the second group served as control. Mice were splenectomized as follows: we anesthetized the mice using 100 mg/kg ketamine (Calypsol, Gedeon Richter, Budapest, Hungary) and 10 mg/kg xylazine (Sedaxylan, Eurovet Animal Health, Bladel, The Netherlands) i.p. before the operation. Then the abdomen was shaved and disinfected, after which a skin incision was made laterally on the left side of the mice, followed by the opening of the abdominal cavity. First, the spleen vascular pedicles were ligated using a 6-0 silk suture, then the spleen was freed from the surrounding tissues and removed. The skin and abdominal incisions were closed using a 6-0 silk suture. To avoid hypothermia, the surgeries were carried out at 37°C controlled temperatures. After the surgeries, each mouse was provided with paracetamol (bene-Arzneimittel GMbH Munich, Germany) mixed with water for 7 days. Splenectomy did not influence the general health status, behavior, or the body weight of the mice.

Induction and assessment of recombinant human G1-induced arthritis

GIA was induced 5 weeks after the splenectomy and in the control mice at the age of 4–5 months as described earlier [24, 25]. Briefly, the mice were injected three times (once every three weeks) intraperitoneally with a mixture of 40 μg rhG1 antigen and dimethyl-dioctadecyl-ammonium (DDA) adjuvant dissolved in PBS. After the second immunization, arthritis severity and clinical signs were examined using a clinical scoring system as described earlier [24, 25], where each mouse limb received a score between 0 and 4 based on swelling, redness, and ankylosis of the joints of the paws (0 = no swelling, 1 = mild swelling and redness, 2 = moderate swelling, 3 = severe swelling and redness without joints' ankylosis and 4 = ankyloses of the joints of the paws), thus the highest severity score is 16 [24, 25]. The diameter of inflamed limbs was measured using a digital caliper throughout the experiment. Mice were sacrificed three weeks after the third immunization, and blood sera, mesenteric and inguinal lymph nodes (LNs), and spleens were collected and later used for *in-vitro* studies.

Histology and immunohistology

Hind legs of arthritic mice were collected after sacrifice and fixed in 10% formalin. Then the specimens were decalcified in EDTA solution at 37°C for one day. Following embedding in paraffin, 4 μm thick slides were made and stained with Mayer's hematoxylin and eosin (HE) solution using a Leica ST 4040 linear automatic stainer (Leica Biosystems, Germany). Finally, slides were scanned using Pannoramic MIDI Scanner (3DHistech, Hungary) and the images were analyzed using the Pannoramic View Software (3DHistech, Hungary).

The spleens of the control mice, and mesenteric- and inguinal-LNs of both control and splenectomized mice were isolated and embedded in a cryostat embedding medium, then kept at –80°C. Three different planes (150 μm apart) of 8 μm thick cryostat sections were cut. The slides were incubated overnight at room temperature, then fixed with cold acetone for 5 min. For immunofluorescence, sections were blocked for 20 min. After that, a mixture of anti-B220-Alexa Fluor 647 and anti-CD3-FITC antibodies was added to the sections and incubated for 45 min at room temperature, followed by washing three times with PBS. For immunohistochemistry, sections were incubated with phenylhydrazine hydrochloride in PBS for 20 min, then washed with PBS, followed by blocking for 20 min. Next, the sections were incubated with anti-IgD and biotinylated PNA for 45 min, followed by three times washing with PBS. After that, sections were incubated with extravidine alkaline phosphatase and goat anti-rat IgG-HRP for 45 min, then washed three times with PBS. For color development, DAB was used for HRP, and NBT with BCIP and levamisole (1 mg/ml) were used for AP detection, respectively. Sections were analyzed using an Olympus BX61 fluorescent microscope. Digital pictures were acquired with a CCD camera using the ZEN software. Images were analyzed using ImageJ Software to determine the average follicle size and germinal center size and number.

In-vitro cell culture for rhG1 antigen-specific proliferation assay and cytokine production

Cells were isolated from the mesenteric LNs of the splenectomized and control mice and the spleen of the control mice and cultured in DMEM supplemented with 10% fetal calf serum (FCS). For the proliferation assay, 3×10^5 cells/well were cultured with/without the rhG1 antigen in triplicates on 96-well plates for 5 days. The proliferation rate was measured using the Promega CellTiter96® Nonradioactive Cell Proliferation Assay (Promega, Madison, WI, USA) following the manufacturer's instructions. For cytokine production, 1.8×10^6 cells/well were seeded on a 48-well plate with or without the rhG1 antigen. After 5 days of culturing, supernatants were collected and kept at –20°C and later used for cytokine ELISA measurements.

Cytokine- and antibody-ELISA measurements

Blood sera and the supernatants of the *in vitro* cultured spleen and mesenteric LN cells were used to measure the level of the cytokines IL-1β, IL-4, IL-6, IL-17, IL-23, IFNγ, and TNFα using sandwich ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

The serum levels of the rhG1 antigen-specific antibodies was measured using indirect ELISA as described previously [1]. Briefly, 96-well ELISA plates were coated overnight with the rhG1 antigen at room temperature. On the next day, plates were blocked for 1 h then washed 5 times. Diluted sera were added and incubated for 2 h at room temperature, then washed 5 times. Finally, plates were incubated with peroxidase-conjugated anti-mouse-IgG1- or anti-mouse-IgM (BD Bioscience, San Jose, CA, USA) secondary antibodies for 2 h at room temperature. Orthophenylenediamine (OPD) chromogen and H_2O_2 (Dako, Kromat, Budapest, Hungary) were used to develop the plates. Optical density values were read at 492 nm with iEMS reader (MF Thermo Labsystems).

Levels of the sera anti-CCP IgG1 and IgG2a antibodies were measured using the Immunoscan CCP Plus ELISA kit (SVAR, Malmö, Sweden) according to the manufacturer's instructions with slight modification. The reaction was developed using peroxidase-conjugated anti-mouse-IgG1 or IgG2 (BD Bioscience, San Jose, CA, USA) as secondary antibodies.

Levels of the sera rheumatoid factor (RF) IgG and IgM levels were measured using the FineTest Mouse RF-IgG or -IgM ELISA Kits (FineTest, Wuhan, China) according to the manufacturer's instructions.

Flow cytometric analysis

Blood was collected from splenectomized and control groups of healthy- and arthritic-mice. The blood was hemolyzed, then the cells were counted. Briefly, 10^6 cells/sample were washed twice with flow cytometry washing buffer and then incubated with different cocktails of fluorochrome-conjugated monoclonal antibodies diluted in flow cytometry staining buffer for 30 min, at RT in the dark. Finally, the samples were washed twice and resuspended in flow cytometry fixation buffer. Data acquisition was performed using a FACS Canto II flow cytometer and FACS DIVA software (BD Biosciences) for data analysis. We defined the following cell subsets based on surface markers: $CD3^+$, T cells; $CD3^+CD4^+$, $CD4^+$ T cells; $CD3^+CD8^+$, $CD8^+$ T cells; $CD3^+CD4^+CD25^+$, activated $CD4^+$ T cells; $IgD^{hi}IgM^{low}CD23^+$: follicular B cells; $IgD^{low}IgM^{hi}CD23^-$: B1 and MZ B cells; $Ig^+CD73^+CD38^+$, memory B cells; $B220^-CD138^+$, plasma cells.

Ca^{2+} signaling measurements

To measure the intracellular calcium levels, single-cell suspensions of the inguinal- or mesenteric-LNs were prepared and suspended in RPMI supplemented with 5% FCS and 4 mM $CaCl_2$ (1×10^6 cells/ml). Next, cells were loaded with Fluo-3-AM intracellular Ca^{2+} indicator fluorescence dye at 37°C in humidified air with 5% CO_2 for 30 min. Using a BD FACS Calibur flow cytometer, the intracellular Ca^{2+} change was detected in the FL-1 channel, where the Fluo-3 fluorescence is proportional to the intracellular Ca^{2+} level. The baseline was measured for 1 min, then the B cells were activated with anti-IgM or anti-IgG, while the T cells with anti-CD3 cross-linking. Each sample was measured for 5½ min. Cell Quest software (BD Biosciences, San Jose, CA, USA) was used to analyze the data. Ca^{2+} signal measurements are shown as FL-1 change, calculated by dividing the mean fluorescence intensity (MFI) of each time point with the corresponding baseline MFI.

Statistical analysis

Data analyses were performed with MS Excel. Data in the diagrams were presented as mean ± SEM. Student's *t*-test was used to compare the experimental groups, *P*-values ≤ 0.05 were considered statistically significant.

Results

Autoimmune arthritis developed in splenectomized mice similarly to the control mice

In our previous study, we showed that the structural defects of the spleen as a consequence of Nkx2-3 transcription factor deficiency led to a decrease in the severity and incidence of autoimmune arthritis in mice [26]. Since the Nkx2-3 mutant mice still have a spleen, although with severely disturbed histological structure and function, we were curious whether the complete absence of the spleen would affect the development of autoimmune arthritis. To investigate this, we surgically removed the spleen and then induced autoimmune arthritis by immunizing the mice with rhG1 antigen and followed the clinical course (severity, incidence) and immunological responses in BALB/c mice. Following immunization GIA developed in both experimental groups (splenectomized and control) with similar kinetics (Fig. 1). The first signs of inflammation and swelling appeared already after the second immunization (day 25); however,

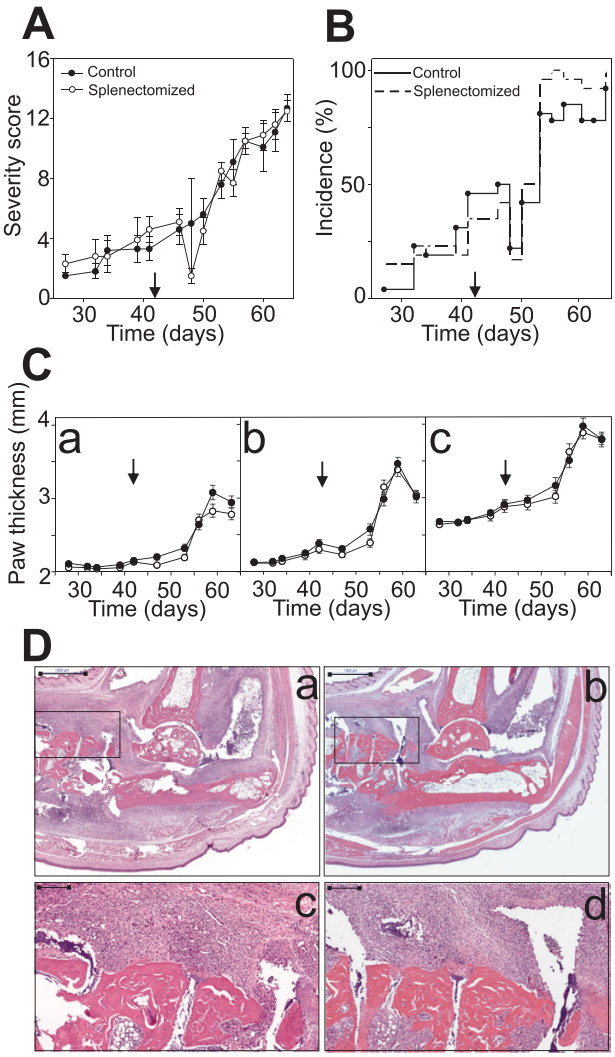

Figure 1: Development of rhG1-induced arthritis (GIA) in splenectomized- and control mice. Diagrams show the comparison of clinical severity score (A), incidence (B) and paw thickness (C) of mice. Autoimmune arthritis was induced in 4–5 months old female BALB/c mice using the rhG1 mixed with DDA adjuvant, *n* = 26 splenectomized (white circles) and *n* = 26 control (black circles) mice. Black arrows show the time of the third immunization. The paw thickness was measured on (Ca) front paws, (Cb) hind paws and (Cc) ankles using a digital caliper in splenectomized (*n* = 12, white circles) and control (*n* = 11, black circles) mice. Results are presented as mean ± SEM. Histology of the inflamed hind legs from splenectomized (Da and Dc) or control (Db and Dd) arthritic animals. Representative HE-stained images show the ankle joint and some of the small joints in the feet of the mice at 2× (Da and Db) and 10× (Dc and Dd) magnification. Rectangular areas in Da and Db are shown at higher magnification in Dc and Dd, respectively. Scale bars in the panels show 1000 μm (Da and Db) or 200 μm (Dc and Dd), respectively.

similarly to earlier studies, the clinical severity score and incidence showed a robust increase after the third immunization in both groups (Fig. 1A and B). At the end of the experiment, we observed similar severity scores in both groups (splenectomized group: 12.5 ± 0.7; control group: 12.7 ± 0.9) (Fig. 1A), and approximately 90% of the mice in both groups developed arthritis (Fig. 1B). To complement the clinical scoring, mice paw thickness was measured using a digital caliper (Fig. 1C). In accordance with the scoring, we observed similar progression of the inflammation on the

front- and hind-paws, as well as the ankles (Fig. 1Ca–c). Three weeks after the third immunization, we sacrificed the mice and collected their blood sera, mesenteric- and inguinal-LNs, and spleens of the control group for further *in vitro* measurements. To confirm the clinical picture, we also made histology of the legs from the splenectomized- (Fig. 1Da and c) and control (Fig. 1Db and d) arthritic mice. There was no significant difference between the histological picture of the splenectomized- and control-sections: signs of intensive inflammation were observed in both cases around the ankle joint and other small joints of the legs (Fig. 1Da and c vs. Db and d, respectively). Similar to earlier studies [24], heavy infiltration with neutrophils and lymphocytes in the synovial- and the neighboring-tissues (Fig. 1Da and b) could be observed with severe cartilage damage and bone invasion (Fig. 1Dc and d).

Changes in the serum cytokine and anti-rhG1 and anti-CCP antibody levels in the arthritic splenectomized mice

In the GIA model, similarly to human RA, significant cytokine concentrations and anti-rhG1- and anti-CCP autoantibody levels are detectable in the serum of the mice [24, 26, 27]. So, after following the clinical parameters, we went on to measure the serum parameters to check if the surgical removal of the spleen affected the levels of pro- and anti-inflammatory cytokines and autoantibodies in the serum. Despite the similar clinical picture in both mouse groups, we observed a significantly higher IL-1β level in the blood sera of the control than in splenectomized mice (P = 0.012) (Fig. 2Aa). We also observed slightly higher levels of the inflammatory cytokines IL-6 and TNFα in the control group (Fig. 2Aa and b). Moreover, the Th1 and Th17 cytokine (IFNγ, IL-17, and IL-23) levels were also higher in the control than splenectomized mice (Fig. 2Aa and b), while the IL-4 level was slightly increased in the splenectomized group (Fig. 2Aa). None of the above described serum cytokine concentration differences were statistically significant with the exception of IL-1β (as mentioned above).

Next, to characterize the humoral immune response, we measured the specific anti-rhG1 antibodies (IgG1 and IgM isotypes), the anti-CCP antibodies (IgG1 and IgG2a isotypes), and the RF (IgM and IgG isotypes) (Fig. 2B–D), as these autoantibodies have been shown to correlate with the disease severity in the GIA model [24]. Surprisingly, we found a higher (but not statistically significant) level of anti-rhG1 IgG1 autoantibody in splenectomized (OD_{492} = 3.67 ± 0.49) than in control mice (OD_{492} = 2.74 ± 0.4) (Fig. 2Ba), but a significantly lower anti-rhG1 IgM in the splenectomized (OD_{492} = 0.9 ± 0.03) than the control mice (OD_{492} = 1.22 ± 0.06) (P = 0.0001) (Fig. 2Bb). Both anti-CCP IgG1 and -IgG2a were significantly higher in the splenectomized (OD_{450} = 2.32 ± 0.19; 0.35 ± 0.02, respectively) than in control mice (OD_{450} = 1.67 ± 0.18; 0.28 ± 0.02, respectively) (P = 0.019 and 0.02, respectively) (Fig. 2Ca and b). Finally, both RF-IgM and -IgG were elevated (but not statistically significantly) in splenectomized mice compared to the controls (308 ± 30 IU/ml vs. 242 ± 28 IU/ml and 463 ± 29 IU/ml vs. 412 ± 30 IU/ml, respectively) (Fig. 2Da and b). Overall, these findings demonstrate that following arthritis induction the level of IgG isotype autoantibodies consistently increased in splenectomized mice.

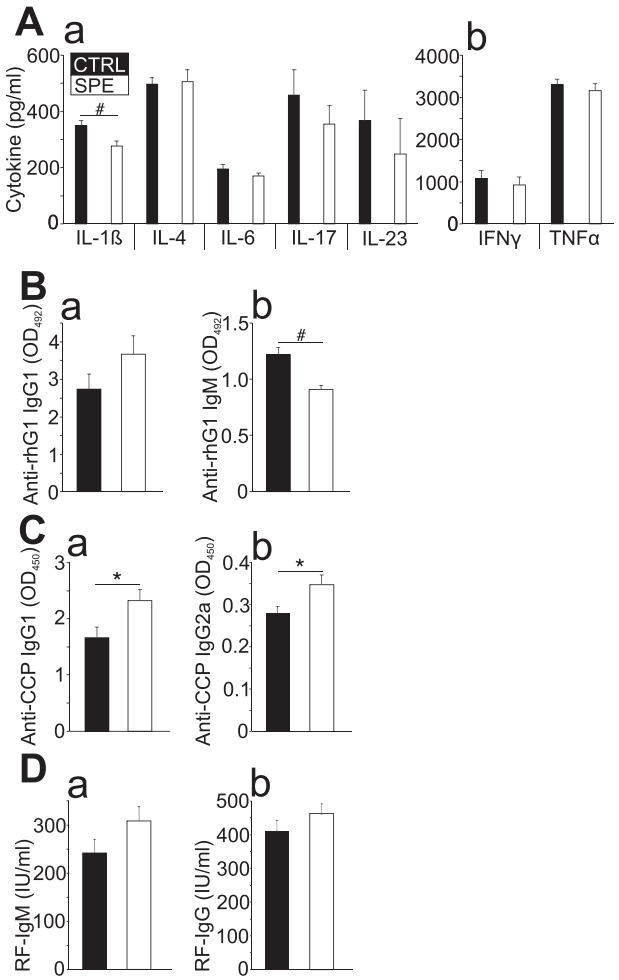

Figure 2: Comparisons of the levels of serum cytokines, anti-rhG1-specific antibodies, anti-CCP, and RF levels in the arthritic splenectomized (n = 25, white bars) and control (n = 24, black bars) mice. A: Diagram shows the concentration of cytokines (pg/ml) in the sera of the arthritic splenectomized and control mice. Note: IFNγ and TNFα levels are shown in a separate diagram (Ab) because their concentrations were markedly higher than the other measure cytokines (IL-1β, IL-4, IL-6, IL-17, and IL-23) shown in panel Aa. B: Diagrams show the level of rhG1-specific antibodies in the sera of arthritic splenectomized and control mice; Ba: the level of anti-rhG1 IgG1 (serum dilution 16 000×), Bb: the level of anti-rhG1 IgM (serum dilution 100×). C: Diagrams show the level of anti-CCP antibodies in the sera of arthritic splenectomized- and control mice; Ca: the level of anti-CCP IgG1 (serum dilution 50×), Cb: the level of anti-CCP IgG2a (serum dilution 50×) is shown. D: Diagrams show the level of RF in the sera of arthritic splenectomized- and control mice; Da: the level of RF-IgM (serum dilution 200×), Db: the level of RF-IgG (serum dilution 20×) is shown. Results are presented as mean ± SEM. Statistically significant ($P \leq 0.05$) differences are indicated: decreased (#) or increased (*) in splenectomized mice compared to the control, respectively.

Arthritic splenectomized mice displayed alterations in B-cell proportions and increased ratio of activated T helper cells

After measuring the serum parameters, we next investigated the effect of splenectomy on the circulating T and B cells in control and splenectomized mice.

Since the spleen plays an important role in B cell development and function, first we analyzed the B cell subpopulations (Fig. 3). We found that splenectomy caused a shift in the ratio between the follicular- ($IgD^{high}IgM^{low}CD23^{+}$) and

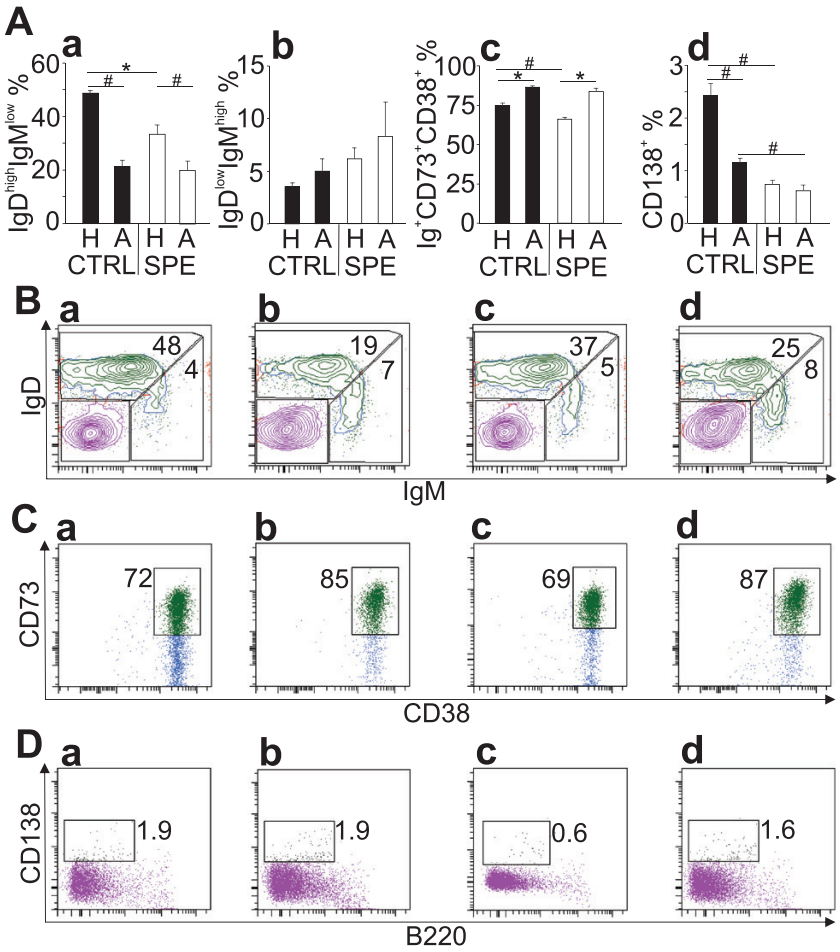

Figure 3: Comparison of the circulating B cell subsets in splenectomized (SPE) healthy (H) and arthritic (A) (n = 5, white bars) and control (CTR) (healthy [H] and arthritic [A]) (n = 5, black bars) mice assessed by flow cytometry. A: The diagrams show the ratios of the following cell populations gated on lymphocytes according to FSC/SSC: follicular B cells ($IgD^{high}IgM^{low}CD23^{+}$) (Aa); non-follicular B cells, comprising mostly B1 and marginal zone B cells ($IgD^{low}IgM^{high}$ $CD23^{-}$) (Ab); memory B cells ($Ig^{+}CD73^{+}CD38^{+}$) (Ac) and plasma cells ($CD138^{+}$) (Ad). Results are presented as mean ± SEM. Statistically significant ($P \leq 0.05$) differences are indicated: decreased (#) or increased (*) in splenectomized mice compared to the control, respectively. Representative flow cytometry contour- and dot-plots show the distribution of blood lymphocytes isolated from control healthy (panels "a") or arthritic (panels "b") and splenectomized healthy (panels "c") and arthritic (panels "d") mice based on their IgD/IgM- (B), CD38/CD73- (C) or B220/CD138 (D) staining, respectively. Numbers in the plots show the percentage of the gated cell population(s).

non-follicular ($IgD^{low}IgM^{high}CD23^{-}$) B cells (comprising mostly B1 and MZ B cells) towards the latter population (Fig. 3Aa,b and 3Ba,c). The percentage of the memory B cells (Ig^{+} $CD73^{+}CD38^{+}$) (Fig. 3Ac and 3Ca,c) and plasma cells ($CD138^{+}$) (Fig. 3Ad and 3Da,c) decreased significantly in the splenectomized mice. In arthritic mice, the shift in B cells to the non-follicular population was even more pronounced (Fig. 3Aa,b and 3Bb,d), there was a significant elevation in the memory B cell population (Fig. 3Ac and 3Cb,d) and a significant decrease in plasma cell percentage (Fig. 3Ad and 3Db,d), regardless of the splenectomy.

Splenectomy increased significantly the percentage of circulating T cells (Fig. 4Aa and 4Ba,c), but did not influence the ratio of $CD4^{+}/CD8^{+}$ T cells (Fig. 4Ab,c and 4Ca,c). In control arthritic mice, there was an expansion of the total T-cell pool but not in the splenectomized mice (Fig. 4Aa and 4Bb,d). In arthritic mice, the frequency of $CD4^{+}$ T cells increased while the $CD8^{+}$ T cells' frequency decreased (the latter change was statistically significant), which was not influenced by the splenectomy (Fig. 4Ab,c and 4Cb,d). Importantly, there was a clear expansion of the activated ($CD25^{high}$) $CD4^{+}$ T cells in arthritic mice, which was significantly higher in the splenectomized mice (Fig. 4Ad and 4Db,d).

Significance of the spleen in rhG1-induced autoimmune arthritis (GIA) model

Unexpectedly, our results showed that the splenectomy did not alter the clinical picture of GIA significantly, although there were clear differences in the serum parameters and the circulating lymphocyte composition in the blood. This prompted us to investigate the exact immunological role of the spleen in GIA. Although historically, the immune reactions (lymphocyte proliferation and cytokine production) were always assessed from isolated spleen cell cultures in GIA and its predecessor, PGIA (proteoglycan aggrecan induced arthritis) models, here we wanted to verify the immunological activation of the spleen in the control mice after induction of autoimmune arthritis with rhG1 antigen both histologically and functionally [24, 26, 34]. To that end, first, we compared the spleens of arthritic to non-arthritic (healthy) BALB/c mice using histological techniques (Fig. 5A). Using

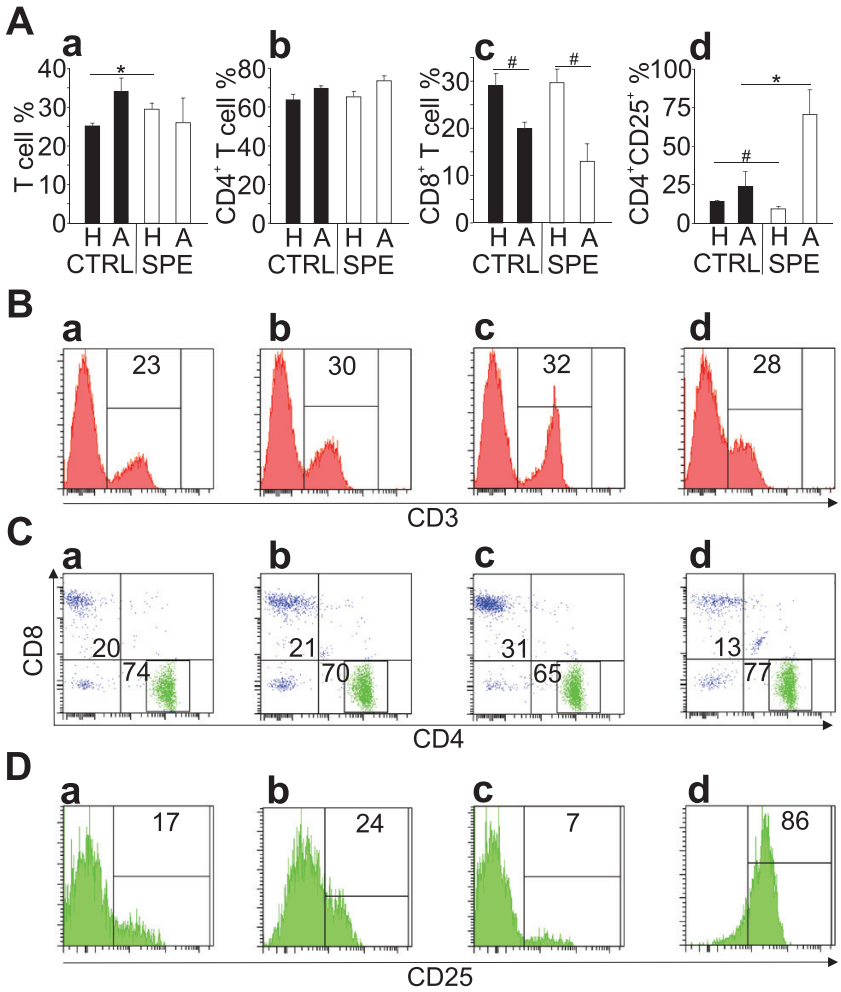

Figure 4: Comparison of the circulating T cell subsets in splenectomized- (healthy and arthritic) (n = 5, white bars) and control (healthy and arthritic) (n = 5, black bars) mice assessed by flow cytometry. A: Diagrams show the ratios of the following cell populations: T cells ($CD3^+$) (Aa); helper T cells ($CD3^+CD4^+$) (Ab); cytotoxic T cells ($CD3^+CD8^+$) (Ac); activated helper T cells ($CD3^+CD4^+CD25^+$) (Ad). Results are presented as mean ± standard error of the mean (SEM). Statistically significant ($P \leq 0.05$) differences are indicated: decreased (#) or increased (*) in splenectomized mice compared to the control, respectively. Representative flow cytometry histogram- and dot-plots show the distribution of blood lymphocytes isolated from healthy- (panels "a") or arthritic- (panels "b") control- and healthy- (panels "c") and arthritic- (panels "d") splenectomized mice based on their CD3- (B), CD4/CD8- (C) or CD25 (D) staining, respectively. Numbers in the plots show the percentage of the gated cell population(s).

immunofluorescence (IF) with anti-CD3 and anti-B220 staining, we could distinguish the B- and T cell zones (follicles and PALS, respectively) of spleen sections from healthy (Fig. 5Aa) and arthritic mice (Fig. 5Ba). As expected, we observed significantly larger follicles in the spleens of the arthritic (Fig. 5Ac and 5Ba) compared to healthy mice (Fig. 5Aa and 5Ba). Next, we wanted to investigate whether these follicles were actively engaged in ongoing immunological reactions, thus we sought to identify the germinal centers (GCs) in these sections using the specific GC marker peanut agglutinin (PNA) with IgD counterstaining in immunohistochemistry (IHC) (Fig. 5Ab and Ad). We found that in the spleens of arthritic mice there were significantly more numerous and larger PNA^+ GCs than in healthy mice (Fig. 5Ad-Ab and 5Bb-c).

To examine the specific cellular immune response against the rhG1 antigen, the spleens of the arthritic mice were also cultured with or without the rhG1 antigen to observe their proliferation levels, and their cytokine production in response to the rhG1 antigen. Confirming previous observations [24, 26, 34, we found a definitive proliferation response of spleen cells stimulated *in vitro* with the rhG1 antigen (Fig. 5C). We measured significant amounts of IL-4, IL-6, IL-17, IFNγ, and TNFα in the supernatant of *in vitro* rhG1-stimulated spleen cells (Fig. 5D–H). These results underline that the cells of the spleen were directly engaged in the systemic immune response against the rhG1 antigen, and so promoted the development of autoimmune arthritis in the GIA model.

The rhG1-specific immune response shifted towards lymph nodes in arthritic splenectomized mice

Although the spleen is affected during the induction of arthritis, the splenectomized mice still developed autoimmune arthritis with an onset and incidence similar to those in mice with spleens. We hypothesized that the immune system compensated for the absence of the spleen and other peripheral lymphoid organs may have "taken over," at least partially, the role of the spleen in the autoimmune response. Therefore, next we set out to investigate the possible role of the

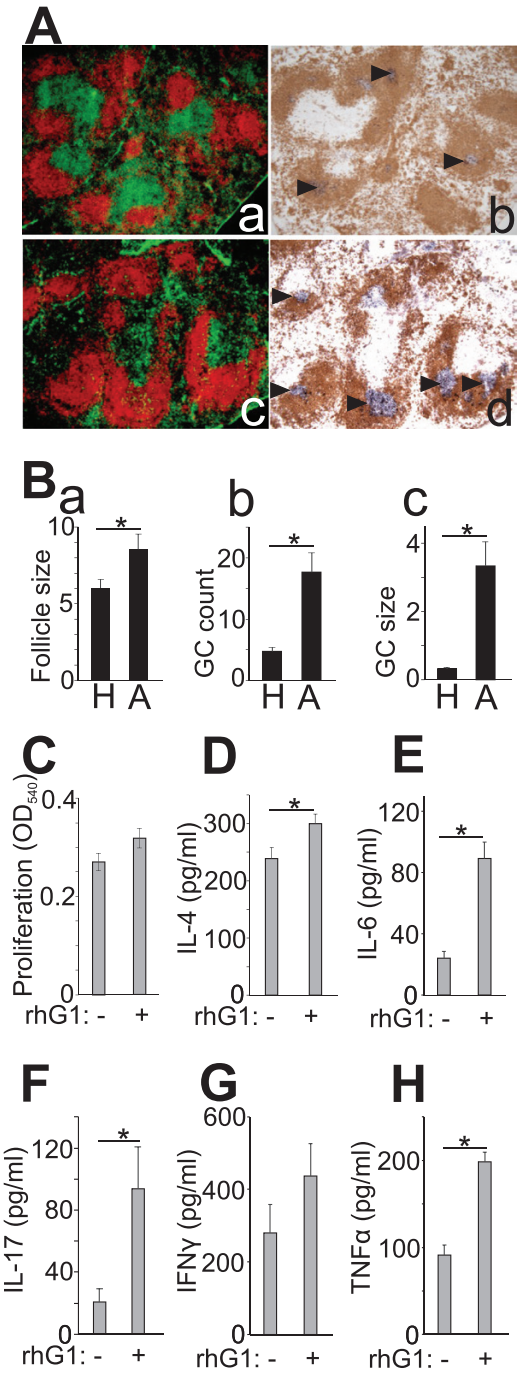

Figure 5: Comparison of the immune-histology and the rhG1-induced immune response in the spleens of arthritic and healthy BALB/c control mice. A: Representative immunohistology panels show the immunofluorescence (IF) and immunohistochemistry (IHC) of the spleens of healthy mice (n = 3) (Aa and Ab), and arthritic mice (n = 3) (Ac and Ad), respectively. For IF (Aa and Ac), cryosections were stained with anti-CD3 (green) and anti-B220 (red). For IHC (Ab and Ad), we used the germinal center (GC) (indicated by black arrowheads) marker PNA (blue) and anti-IgD (brown) for mature follicular B cells. Images were taken at 10x magnification, scale bars are 200 μm. B: Diagrams show the comparisons of the average follicle size (Ba), GC count (Bb), and GC size (Bc), respectively. In case of the average follicle size we measured at least three section planes from each mouse in the IF preparations. In case of the GCs, for the average GC count we counted all GCs from all sections from each mouse, and then measured the average GC size of the detected GCs/section from each mouse in the IHC preparations. Results are presented as mean ± SEM. C: Diagram shows the rhG1-induced *in vitro* proliferation of spleen cells. Results are presented as mean ± SEM calculated from the data of n = 26 mice. D–H: Diagrams show the cytokine concentrations measured with ELISA from the *in vitro* cultured spleen cell supernatants with or without rhG1. Results are presented as mean ± SEM. Statistically significant differences are indicated (*$P \leq 0.05$).

mesenteric and inguinal lymph nodes (LNs) in GIA. Already during the isolation, it was obvious that in splenectomized arthritic mice the inguinal LNs (iLNs) were macroscopically larger than in the control arthritic group, which also correlated with the significantly elevated (P = 0.009) cell numbers ($6.63 \pm 0.67 \times 10^6$ vs. $4.9 \pm 1.09 \times 10^6$ cells). As a first step, we compared the iLNs and mesenteric LNs (mLNs) of the control and splenectomized groups of arthritic and healthy mice by immunohistology, similar to the analysis of spleen. We found that the iLNs of arthritic splenectomized mice (Fig. 6Bc and d) had noticeably larger follicles as well as larger and more abundant GCs than the arthritic control (Fig. 6Ac and d), healthy control (Fig. 6Aa and b), and healthy splenectomized (Fig. 6Ba and b) mice (quantitative analysis shown in Fig. 6Ca and c). These numerous large GCs in the iLNs of the arthritic splenectomized mice indicated an ongoing immune response.

In the mLNs, we observed slightly more GCs in the arthritic splenectomized (Fig. 6Ec and d) than in the arthritic control mice (Fig. 6Dc and d), and they both had more (although not significantly) GCs than healthy control (Fig. 6Da and b) and healthy splenectomized mice (Fig. 6Ea and b) (analysis shown in Fig. 6Fa and c). However, the average follicle size was slightly larger in the healthy splenectomized mice than in other mouse groups (Fig. 6Fa). Nevertheless, the average GCs size of all mouse groups was similar (Fig. 6Fc). In contrast to the iLNs, in the mLNs, the effect of the splenectomy was not so clear, however, the slightly increased GC count in the arthritic mice indicated that the mLNs might be also activated in GIA.

After the morphological analysis, next, we examined the cellular immune response of the mLN cells to the rhG1 antigen in arthritic splenectomized and control mice *in vitro*. We cultured the mLN cells in the presence or absence of the rhG1 antigen to measure the lymphocyte proliferation and cytokine production. We found no significant difference in the rhG1-induced proliferation of the mLN cells isolated from the arthritic splenectomized or control mice (Fig. 7A).

In the GIA model, IFNγ and IL-17 are of special importance among other cytokines that can be detected in the supernatants of cultured spleen cells in response to rhG1 antigen, as shown here (Fig. 7D–H) and in previous studies [24, 26, 34, 35]. Hence, we were curious to investigate whether the *in vitro* production of those cytokines in mLN cell cultures in response to rhG1 was affected by the absence of the spleen (Fig. 7B-F). Remarkably, mLN cells of the arthritic splenectomized mice (even without rhG1 antigen stimulation) produced markedly higher levels of IL-4, IL-6, and IFNγ, and significantly more IL-17 (P = 0.038) and TNFα (P = 0.036) than those of the control group (Fig. 7B–F). The production of these cytokines increased only slightly after stimulation by the rhG1 antigen (Fig. 7B–E), except for TNF-α (Fig. 4F). In comparing the cytokine patterns produced by the mLN cells to those of the spleen cells (Fig. 7B–F and Fig. 5D–H), we found characteristic differences. We observed very low IL-17 and IFN-γ levels in the supernatant of the mLN cell cultures (rhG1-stimulated and -non-stimulated) of the

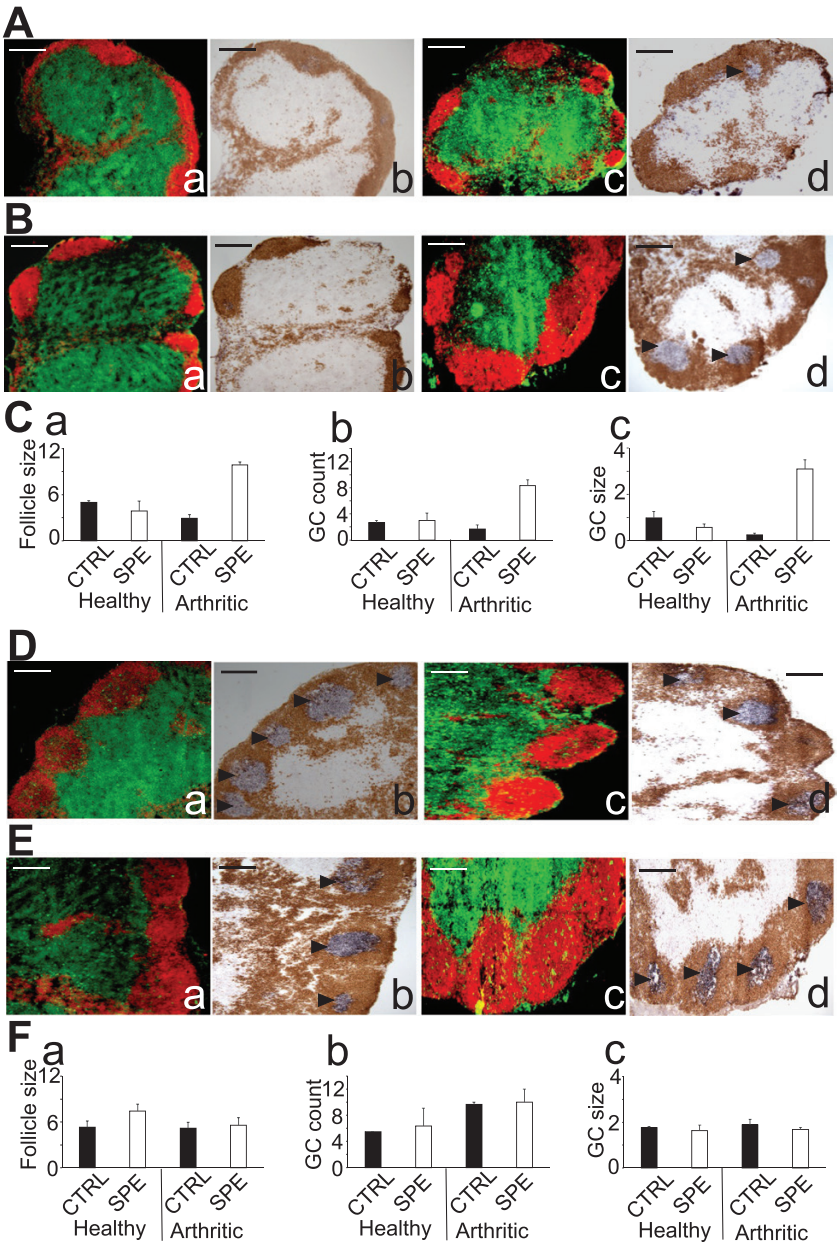

Figure 6: Histological comparison of the inguinal and mesenteric LNs from control or splenectomized mice before and after arthritis induction (healthy and arthritic, respectively). For immunofluorescence (IF) cryosections were stained with anti-CD3 (green) and anti-B220 (red) mAbs. For immunohistochemistry (IHC) cryosections were stained with PNA GC marker (blue) and anti-IgD (brown) for mature follicular B cells. Images are representative of at least two mice from each group. A: Inguinal LNs form healthy (Aa,b) and arthritic (Ac,d) control mice. B: Inguinal LNs from healthy- (Ba,b) and arthritic- (Bc,d) splenectomized mice. Images were taken at 10× magnification, scale bars are 200 μm. C: Diagrams show the statistical analysis of inguinal LNs: Average follicles size (Ca) of at least three follicles from/section from each mouse was measured in IF preparations. Average GC count (Cb) was calculated from all IHC sections from each mouse. Average GC (Cc) size was measured from the detected GCs/section from each mouse in IHC preparations. Results are presented as mean ± SEM. D: Mesenteric LNs form healthy (Da,b) and arthritic (Dc,d) control mice. E: Mesenteric LNs from healthy- (Ea,b) and arthritic (Ec,d) splenectomized mice. Images were taken at 10× magnification, scale bars are 200 μm. F: Diagrams show the statistical analysis of mesenteric LNs: Average follicles size (Fa) of at least three follicles from/section from each mouse was measured in IF preparations. Average GC count (Fb) was calculated from all IHC sections from each mouse. Average GC (Fc) size was measured from the detected GCs/section from each mouse in IHC preparations. Results are presented as mean ± SEM.

arthritic control mice (Fig. 4D, E), while both IL-4 and IL-17 were produced robustly in the mLN cell cultures isolated from splenectomized arthritic mice (Fig. 7B, D versus Fig. 5D, E). The production of IL-6, IFNγ and TNF-α by mLN-derived lymphocytes were comparable to the spleen cell cultures of arthritic mice (Fig. 7C, E and F versus Fig. 5E, G, H).

Taken together, the morphological and functional data from the iLNs and mLNs of splenectomized mice (Figs. 6 and 7) supported our hypothesis that the systemic immune reaction against the rhG1 antigen in splenectomized mice shifted from the spleen to other peripheral lymphoid organs like mLNs and iLNs.

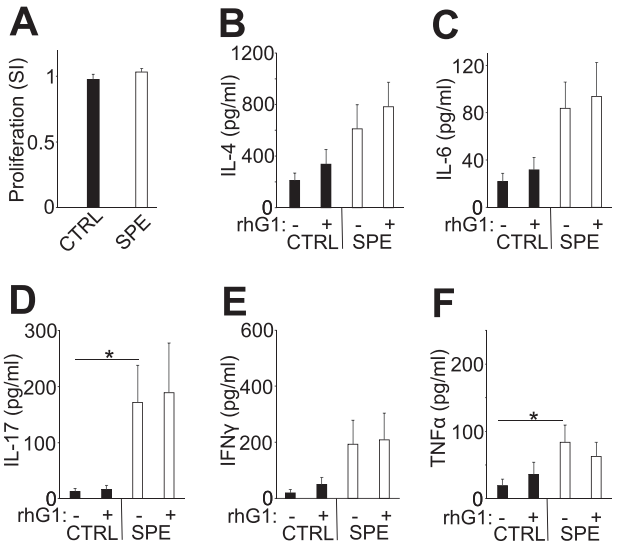

Figure 7: Comparison of the rhG1-induced immune response of the mesenteric LN cells isolated from arthritic control (n = 26, black bars) or -splenectomized (n = 26, white bars) mice. A: Proliferation of the *in vitro* cultured mesenteric LN cells stimulated with rhG1, presented as stimulation index (SI) (calculated as the ratio of stimulated to non-stimulated values). B-F: Cytokine levels were detected in the supernatants of the *in vitro* cultured mesenteric LN cells isolated from arthritic control or splenectomized mice in the presence or absence of the rhG1 antigen using sandwich ELISA. Results are presented as mean ± SEM. Statistically significant differences are indicated (*$P \leq 0.05$).

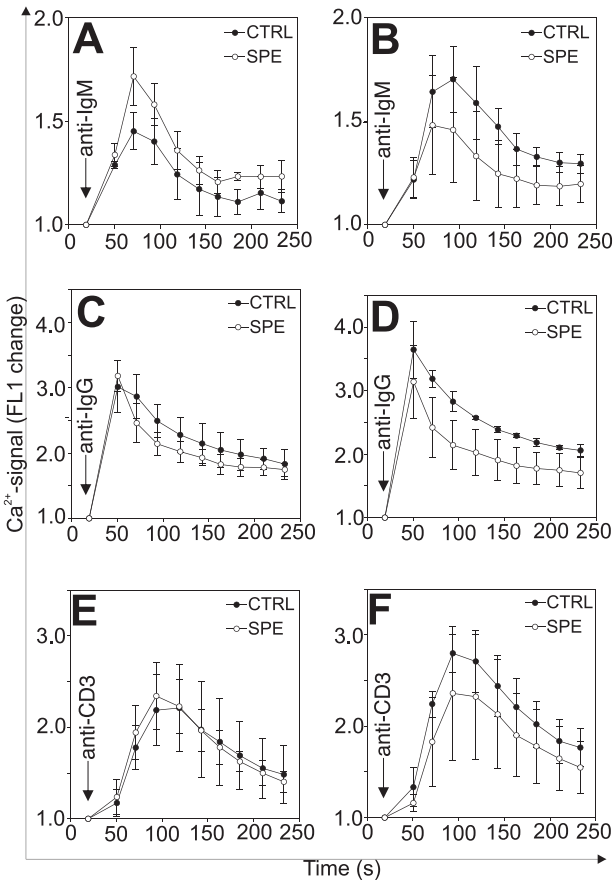

Figure 8: Comparison of the intracellular Ca^{2+} signaling in B and T cells from inguinal and mesenteric LNs of the arthritic control (n = 3) and -splenectomized (n = 3) mice. Cells of the inguinal (A, C, E) and mesenteric (B, D, F) LNs were isolated and loaded with the Ca^{2+}-specific indicator Fluo-3, then stimulated with selective activators for B or T cells. The intracellular Ca^{2+} change was detected as time-dependent FL-1 fluorescence change for 5 min using a flow cytometer. The baseline was measured for 1 min, and then the cells were stimulated and measured for 5 min. B cells were activated using anti-IgM- (A, B) or anti-IgG (C, D) antibodies, while T cells (E, F), were activated using anti-CD3 antibody cross-linking. Values are presented as mean ± SEM.

Comparison of Ca^{2+} signaling in the lymph nodes of the arthritic splenectomized and control mice

Previously, we demonstrated that in Nkx2-3 knock-out mice, the splenic B-cell Ca^{2+} signaling showed significant alterations [26]. In this work, we wanted to investigate whether the surgical removal of the spleen affected the Ca^{2+} signaling of T and B cells in iLNs of arthritic mice. We compared the intracellular Ca^{2+} signal in the iLN cells of control and splenectomized mice after selectively crosslinking the B- and T-cell receptors. We found a higher (but not statistically significant) anti-IgM-induced Ca^{2+} signal in the B cells isolated from iLNs of splenectomized mice compared to those of the control mice (Fig. 8A). On the contrary, there was no difference in the Ca^{2+} signal between the splenectomized and the control mice when we activated the iLN B cells with anti-IgG or the iLN T cells with anti-CD3 (Fig. 8C, E). However, the B and T cells isolated from the mLNs showed different activation patterns than those isolated from the iLNs. The Ca^{2+} signal of B cells after stimulation by either anti-IgM or anti-IgG showed a decreased (but not statistically significant) amplitude of Ca^{2+} signal in the splenectomized mice compared to the control (Fig. 8B, D). Interestingly, the Ca^{2+} signal in T cells stimulated with anti-CD3 also showed a decreased (but not statistically significant) amplitude in the splenectomized mice compared to the control (Fig. 8F).

Discussion

The spleen is an essential component of the immune system: one of its unique functions is to contribute to the homeostasis of the peritoneal cavity through regulation of the B and T cells migration between the peritoneal cavity and the circulatory system [36, 37]. During the induction of autoimmune arthritis, using the GIA mouse model and its parent model, the proteoglycan-aggrecan induced arthritis (PGIA), we inject the antigen/adjuvant mixture into the peritoneal cavity, which leads to the local activation and expansion of Th1, Th17, and conventional B cells in the peritoneal cavity and mLNs, which ultimately results in a systemic (auto)immune response where the spleen is highly involved [27].

Previously, we found lower incidence and milder severity of the GIA in Nkx2-3 deficient mice, which might be due to the disturbed spleen structure and the alteration in B-cell maturation and activation [26]. To further study the function(s) of the spleen in autoimmune arthritis, in this work we studied the effect of the surgical removal of the spleen on the immunopathology of autoimmune arthritis in normal BALB/c mice. Therefore, we induced autoimmune arthritis in splenectomized and control BALB/c mice using the GIA mouse model. There is a significant difference between the two approaches: in mice genetically deficient for the Nkx2-3 transcription factor, there is still some spleen, although with severely disturbed structure and function, whereas in the case of splenectomy the spleen is a completely absent.

In line with previous reports in the CIA model using splenectomized mice [32] or rats [33], here, mice developed GIA despite the absence of the spleen. In fact, all splenectomized mice developed arthritis and their severity score was similar to that of the control (spleen preserved) group. Moreover, the progress of inflammation of the front- and hind paws and ankles was similar in both groups. Based on these clinical data, the spleen seemed to be dispensable in GIA. Therefore, our first goal here was to elucidate the exact role of the spleen in GIA. It is also of note that in most previous works on GIA and PGIA [24, 26, 34], some immune response markers (e.g. lymphocyte proliferation and cytokine production) were determined using *in vitro* spleen cell cultures, suggesting that the spleen was indeed involved in autoimmune arthritis. In support of this scenario, we found that the spleen of the arthritic control mice had significantly larger follicles and GCs than the healthy BALB/c mice. Moreover, we found a strong cellular immune response of the *in vitro* cultured spleen cells stimulated with the rhG1 antigen, as it was shown in the increased level of proliferation and cytokine production. These results indicate that the spleen is indeed involved in the induction of autoimmune arthritis in the GIA model. Based on this, we hypothesized that, in splenectomized mice, other peripheral lymphoid organs may have taken over the role of the spleen and compensated for its absence to initiate autoimmune arthritis.

Due to the site of antigen administration, or the proximity to affected joints, mesenteric and inguinal LNs, respectively, seemed obvious candidates for this "compensatory" effect. This was already supported by the fact that splenectomized mice had remarkably larger iLNs with significantly increased lymphocyte numbers, and larger follicles, and more abundant GCs, indicating that the iLNs of the arthritic splenectomized mice were engaged in the ongoing immunological reactions, unlike the arthritic control or the healthy groups. We found similar histological changes in the mLNs of the arthritic splenectomized mice, although the changes were less prominent than in the case of the iLNs. These findings suggest that even though the mLNs may have served as the lymphoid tissues for initiating immune responses after immunization, by the appearance of joint inflammation and autoantibody production, the main site for anti-rhG1 immune response may have shifted to the iLNs. The relatively minor difference between splenectomized- and control mLN in terms of GC number may be due to their drainage of the intestines and their active participation in the immune regulation of the intestinal mucosa [38, 39].

Our functional testing of the rhG1-specific immune response of the mLN cell cultures also confirmed that in the splenectomized arthritic mice there was a significant lymphocyte activation, as shown by the lymphocyte proliferation response and the cytokine production. We observed that the mLN cells of arthritic splenectomized mice produced larger quantities of Th1 (IFN-γ), Th2 (IL-4), and Th17-type (IL-17) cytokines together with the pro-inflammatory cytokines (IL-6 and TNF-α) than the control group. These functional data also confirmed that, in the absence of the spleen, the mLNs have an important compensatory role in GIA. A comparison of the cytokine production by spleen cells or the mLN cells revealed that there was a stronger Th2 and Th17 immune response in the mLN of splenectomized mice. On the contrary, the splenic immune reaction in the control group was shifted more toward the Th1 direction (in line with previous works [24, 26, 34]). Furthermore, the higher level of the pro-inflammatory cytokine TNF-α in the spleen cell cultures of the control compared to the mLN cell cultures of the splenectomized group probably reflects the substantial macrophage content of spleen, in addition to other potential TNF-α producer innate cell subsets [15]. These results suggest that the autoimmune arthritis induction was probably concentrated in the spleen. However, in the absence of the spleen the activation and homing of the immune cells shifted to other peripheral lymphoid organs including mLNs and iLN.

Previously, in the Nkx2-3 knock-out model, we found a correlation between altered Ca^{2+} signaling in B lymphocytes and ameliorated autoimmune arthritis [26]. Here, the spleen's surgical removal affected the B- and T cell activation similarly to what we found in the Nkx2-3 knock-out mice. However, it seems that this weaker lymphocyte activation due to the absence of the spleen is still sufficient to generate a robust autoimmune response and support the development of GIA.

In BALB/c mice, the immune responses are typically biased toward the Th2 immunity [35, 40]; however, in autoimmune arthritis induction using the GIA model leads to a significant shift toward the Th1- and Th17 immune responses [24, 35, 41]. Since the surgical removal of the spleen appears to impact the B- and T-cell activation in the mLNs of the arthritic mice, we analyzed whether the serum cytokine and autoantibody profiles mirrored these changes. The serum cytokine levels confirmed that the Th1 immune response was more prominent in the control group than in the splenectomized group.

One of the main characteristics of the GIA model is the strong humoral pro-arthritic immune response, coupled with the production of high-affinity autoantibodies against the rhG1 antigen, anti-CCP, and RF in the sera [24, 35]. Anti-CCP is now considered a specific laboratory marker for RA, and it is correlated with the disease severity and the level of joint erosions [4, 42]. Interestingly, in this study, we found that the levels of anti-rhG1 IgG1, anti-CCP (IgG1 and IgG2a), and RFs were markedly higher in the sera of the splenectomized group than in the control group despite the similar severity score. These differences might be caused by the alterations in T- and B-cell activation in the arthritic splenectomized mice as described above. It is also of note, that in an earlier study in splenectomized rats with CIA, similarly elevated antibody levels were reported, which was explained by the compensatory antibody production from the bone marrow [33] not assessed here.

The association of high levels of anti-rhG1-, anti-CCP-, and RF-antibodies in the arthritic splenectomized with the decreased production of IgM-type anti-rhG1 antibodies may reflect altered B-cell composition and tissue distribution after splenectomy, particularly affecting MZ B cells and B-1 cells from the peritoneal cavity [43, 44]. The spleen is essential for the development and maturation of B cells, where immature B cells arriving from the bone marrow will complete their maturation [44–46]. In our study, we observed that the ratio of follicular- to non-follicular (MZ and B-1) B cells in circulation shifted toward the latter group in the splenectomized mice, which was further enhanced by arthritis induction. One of the possible explanations for this could be that after the removal of the spleen, the MZ B

and B-1 cells cannot home to the spleen and thus we detect more of them in the circulation. B-1 cells mainly reside in the pleural- and peritoneal serous membranes requiring splenic influences for survival and are responsible, together with the MZ B cells, for the production of most of the circulating IgM [44, 47–50]. However, the disturbed recirculation of the B cells due to the absence of the spleen impairs IgM production by MZ B and B-1 cells. In addition, IgM plays a crucial regulatory role in the immune response by binding to IgG autoantibodies as part of their regulatory function [48, 51]. Based on this, at least hypothetically, the significant decrease in the rhG1-specific IgM level in the sera of the arthritic splenectomized mice, might have led to less efficient clearance of the IgG isotype autoantibodies, which was reflected in the high-affinity autoantibody (IgG1 and IgG2a) levels. Our findings are in agreement with a previous study by Boes and colleagues [52], where they introduced the IgM mutation to B cells, in which B cells were not able to secrete IgM but they could still express surface IgM and IgD to undergo class switching and produce other Ig isotypes in lupus-prone lymphoproliferative (lpr) mice [52]. They reported that these mice lacking soluble IgM produced unexpectedly high levels of IgG autoantibodies against the double-stranded DNA and histones [52].

Besides the effects on B cells, splenectomy leads to an alteration in T cell homeostasis, too [53, 54]. Not only because the spleen plays a role in the immune response to TD antigens but also in promoting self-tolerance and T-cell homeostasis by inducing the formation of the regulatory T cells and the expression of the autoimmune regulator gene (AIRE) [53, 54]. Kim *et al*. reported a significant increase in the circulating memory CD8 T cells in splenectomized mice after lymphocytic choriomeningitis virus infection [54]. In our present work, on the contrary, immunization of splenectomized mice with the rhG1 antigen resulted in a significant increase in the circulating Th population, and a decrease in the CD8 T cells.

Conclusion

The absence of the spleen did not abrogate the development of autoimmune arthritis in the GIA model. We confirmed that the iLNs and mLNs took part in the development of the rhG1-induced immune response leading to enhanced T helper activation and elevated serum autoantibody levels. The complex reorganization of the cellular components of the immune network provided sufficient compensation for the absence of the spleen in the development of autoimmune arthritis.

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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Conflict of interests

None.

Author contributions

Conceptualization, F.B, P.B., and T.B.; methodology, E.K., O.K, E.G., X.J., and F.B.; data analysis, E.K. and F.B.; investigation, E.K., O.K., E.G., and X.J.; resources, F.B., P.B., and T.B.; writing—original draft preparation, E.K. and F.B.; writing—reviewing and editing, F.B., P.B., and T.B.; supervision, F.B.; project administration, F.B.; funding acquisition, F.B., P.B., and T.B. All authors have read and agreed to the published version of the manuscript.

Ethical approval

All animal experiments were conducted following the University of Pécs, Animal Welfare Committee regulations (BA02/2000-23/2020). The animal research adheres to the ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines).

Data availability

There are no new data associated with this article.

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