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ORIGINAL ARTICLE

Anti-ANX A1 Antibody Therapy in MRL/lpr Murine Model of Systemic Lupus Erythematosus

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Abstract
Systemic lupus erythematosus (SLE) is a severe autoimmune disease characterized by dysfunction of immune regulation, overproduction of inflammatory cytokines and attack on normal tissues by self-reactive cells and antibodies. The main role in the pathogenesis plays the autoreactive tandem of B-T cells, responsible for lupus progression and acceleration. Both activated B and T cells express a phospholipid binding protein Annexin A1 and abnormal levels of the protein were found in murine and human autoimmune syndromes, potentiating its role as a therapeutic target. Here, using anti-annexin A1 antibody we explore its property to modulate the autoimmune response in MRL/lpr mouse model of lupus. Anti-ANX A1 antibody was tested in vitro using spleen cells from MRL/lpr mice to determine the effect on lymphocyte activation, plasma cells differentiation, apoptosis and proliferation by flow cytometry and ELISpot assays. Subsequently, several groups of young (disease-free) and old (sick) MRL/lpr mice were treated with the antibody to determine the levels of panel auto-antibodies and cytokines, T cell arrest and migration. Treatment of splenocytes with anti-ANX A1 antibody inhibited T-cell activation and proliferation, suppressed anti-dsDNA antibody-producing plasma cells and affected B cell apoptosis. Administration of the antibody to MRL/lpr mice resulted to decreased autoantibody levels to various lupus antigens, suppressed T cell migration from lymph nodes and increased the levels of IL4 mRNA compared to the control group. Anti-ANX A1 antibody therapy suppresses B and T cell over-activation and down- modulates disease activity.

Keywords Systemic lupus · Anti-annexin A1 antibody · MRL/Lpr mice · Antibody therapy

Abbreviations

SLE	Systemic lupus erythematosus
ANXA1	Annexin A1
ConA	Concanavalin
LPS	Lipopolysaccharide
ELISpot assay	Enzyme linked immunospot assay

Introduction

Systemic lupus erythematosus (SLE) is a polygenic autoimmune syndrome characterized by multiple abnormalities in the regulation of the immune response and loss of self-tolerance. The clinical picture of SLE is heterogeneous with multi-organ involvement (heart, joints, skin, lungs, blood vessels, liver, kidneys, and the nervous system). The cooperation between activated auto-reactive B and T lymphocytes with the participation of other immune cells such macrophages, NK cells and basophils leads to the formation of immune-complexes and inflammatory cells infiltration.

Silvya Bradyanova, Nikolina Mihaylova and Petroslav Chipinski authors contributed equally to the work.

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Current therapies include immunosuppression by cyclophosphamide and glucocorticosteroids, and cell inactivation by monoclonal antibodies (Dossybayeva et al. 2020; Shah et al. 2021). A specific and efficacious nontoxic therapy for SLE is still lacking.

Autoreactive B and T cell subsets play diverse roles in SLE pathology. Self-specific B cells are involved in the loss of T cell tolerance and could modify T cell cytokine production, while autoreactive T cells regulate B cell stimulation and autoantibody production. Moreover, $CD4^+$ T cells secrete pro-inflammatory cytokines which mediate disease progression by several B cell-independent mechanisms amplifying the lupus symptoms (Dörner et al. 2011; Foster 2007; Tai et al. 2018; Wilkinson and Rosser 2019).

Presently used and widely discussed B-cell based therapies are designed on the principle of general depletion by monoclonal antibodies against B-cell surface molecules such as CD19, CD20 (rituximab, ocrelizumab), and CD22 (epratuzumab), or selective elimination of some B-cell populations (Ahuja et al. 2007; Dörner et al. 2011; Kansal et al. 2019; Mei et al. 2012; Rovin et al. 2012; Wallace et al. 2014; Shah et al. 2021). Despite the successful introduction of clinical monoclonal therapies for a number of autoimmune diseases, total B-cell depletion remains inefficient and unspecific in many cases.

A number of inhibitory T-cell costimulatory molecules providing negative signals for activation and proliferation might have therapeutic properties for selective treatment of SLE (Crispín et al. 2010; Merrill et al. 2010; Tai et al. 2018; Zhang and Vignali 2016). Among them is ANXA1, a 37 kDa phospholipid-binding protein, also known as macrocortin, renocortin, and lipocortin-1 (Sheikh and Solito 2018). Its role in disease progression was reported in several autoimmune syndromes such as autoimmune encephalomyelitis and rheumatoid arthritis (RA) (Bruschi et al. 2018; D'Acquisto et al. 2007; Kretz et al. 2010; Paschalidis et al. 2010; Paschalidis et al. 2009; Perretti and D'acquisto 2009; Sheikh and Solito 2018; Yang et al. 2013a, b).

ANX A1 is expressed by many cell types such as circulating leukocytes, macrophages, fibroblasts and mast cells. It is preferentially located on the cytoplasmic surface of the plasma membrane, constituting 2–4% of total cytosolic protein, however, it is also detectable in the nucleus. It is involved in many physiological processes including cell growth and differentiation, membrane fusion, endocytosis and exocytosis (Perretti and D'acquisto 2009; Perretti and Dalli 2009; Yang et al. 2013a).

The endogenous ANX A1 plays a dual modulatory role in innate and adaptive immunity, but its function is not clear yet (D'Acquisto 2009; Perretti and D'acquisto 2009). The endogenous ANX A1 may restrict Th2 stimulation favoring the development of Th1- and Th17-mediated responses. Such an abnormal activity of Th1/Th17 subsets is typical for the autoimmune diseases raising the potential of ANX A1 as a therapeutic target (Bruschi et al. 2018; D'Acquisto et al. 2007; Paschalidis et al. 2010; Paschalidis et al. 2009; Yang et al. 2013a, b).

An important tool for understanding human autoimmune diseases, and specifically SLE, is the use of different mouse models such as $(NZBxNZW)F_1$ (New Zealand Black/White), MRL/lpr, pristane-treated Balb/c and BXSB mice (Richard and Gilkeson 2018; Rottman and Willis 2010). MRL/MpJ-$Tnfrsf6^{lpr}$/J (MRL/lpr) mice reproduce human SLE symptoms with the production of autoantibodies to nuclear components and multiple organ and systems damages (Richard and Gilkeson 2018).

We have previously used an experimental lupus-prone MRL/lpr model in our therapeutic approaches consisting of injection of test groups with a monoclonal anti-ANX A1 antibody to suppress SLE symptoms. Using MRL/lpr mice, we have successfully suppressed lupus progression, modulated IL10 secretion, prevented the appearance of anti-dsDNA IgG autoantibodies and prolonged the survival compared to the control groups (Mihaylova et al. 2020a). The aim of the present study was to explore in detail the T cell activation markers expression, suppression of T cell migration from lymph nodes, cell proliferation, apoptosis, autoantibody and cytokines production in vivo and ex vivo after the application of an anti-ANX A1 antibody in MRL/lpr mouse model of SLE.

Materials and Methods

Antibodies

Monoclonal mouse anti-ANX A1 (Human/Mouse/Rat) IgG1 antibody, clone 686122, was purchased from R&D Systems (Minneapolis, MN, USA). Unconjugated mouse IgG1 Isotype Control, clone P3.6.2.8.1 (eBioscience, Frankfurt, Germany) was used in all experiments. FITC (Fluorescein isothiocyanate)-conjugated anti-mouse CD3, PE (Phycoerythrin)-conjugated anti-mouse CD69, APC (Allophycocyanin)–conjugated anti-mouse CD25, eFlour450-conjugated anti-mouse CD3 and anti-mouse CD19 mAbs (eBioscience) were used for FACS (Fluorescence-activated cell sorting) experiments. Corresponding Isotype-matched IgG controls (eBioscience) were given for compensation and confirmation of antibody specificity.

AP (Alkaline phosphatase)-conjugated anti-mouse IgG (Sigma-Aldrich, Taufkirchen, Germany) were used for ELISpot assay. Unconjugated anti-mouse CD3 and CD28 antibodies (Sigma-Aldrich) were used for in vitro stimulation.

Mice

Female MRL/lpr (MRL/MpJ-Tnfrsf6^{lpr}/J) mice were purchased from The Jackson Laboratory, (Bar Harbor, Maine, USA). Groups of 7 weeks old and 16 weeks old female mice were randomly assigned to the respective groups (five animals per cage). The animals were maintained in barrier-type animal-house under specific-pathogen-free (SPF) conditions.

The study protocol, animal care and all procedures were approved by the Animal Care Commission at the Institute of Microbiology (N105/10072014) and were conducted in accordance with the International regulations (EU Directive 2010/63/EU).

Flow Cytometry

Spleens were taken from sacrificed 12 weeks old MRL/lpr mice ($n=6$ to 10) and monocellular suspension was prepared by grinding through sterile cell strainers (BD Biosciences, Erenbodegem, Belguim). The erythrocytes were lysed with a hypotonic ammonium chloride solution and the number of cells was counted by a haemocytometer. The splenocytes were washed with PBS (containing 2.5% FCS and 0.05% sodium azide) and stimulated for 16 h simultaneously with plate-bound anti-CD3/CD28 antibodies (2.5 µg/ml each) in the presence of different concentrations of anti-ANX A1 or Control antibodies (ranging from 5 ng/ml to 500 ng/ml). Later, the cells were incubated with anti-CD3-FITC, anti-CD25-APC and anti-CD69-PE (1 µg/10^6 cells) for 20 min at 4 °C, followed by two washes. Ten thousand cells were analyzed from each sample with a BD LSR II flow cytometer (BD Biosciences, San Jose, CA). Intact cells were gated using forward and side scatter and analyzed with Diva 6.1.1. software.

Proliferation Assay

Splenocytes from MRL/lpr mice were isolated and cultured (2×10^6 cells/ml) in complete RPMI (Roswell Park Memorial Institute Medium) 1640 medium (Gibco, Gaithersburg, MD) containing 10% FCS (fetal calf serum), 4 mM L-glutamine, and antibiotics. The cells were stimulated simultaneously with plate-bound anti-CD3/CD28 and co-cultivated for 5 days in the presence of different concentrations of anti-ANX A1 or Control antibodies (ranging from 5 ng/ml to 500 ng/ml) at 37 °C/5% CO_2. The control cells were stimulated either with 10 µg/ml lipopolysaccharide (LPS) (from *E. coli*, Sigma, L-2630), 10 µg/ml concanavalin A (ConA) (Sigma), or cultured in medium only. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide; thiazolyl blue) was added for additional 4 h at concentration of 5 mg/ml. Forward, the medium was decanted and 200 µL DMSO (dimethyl sulfoxide) was used to dissolve the formazan crystals. Proliferation was assessed by measuring the absorbance of converted dye at a wavelength of 590 nm corrected by subtracting the absorbance at 620 nm.

ELISpot (Enzyme-Linked Immunospot) Assay for Counting Specific Anti-dsDNA Antibody-Secreting Cells

ELISpot assay was used to assess how the anti-ANX A1 antibody treatment affects the activity of IgG anti-dsDNA antibody-producing plasma cells. Splenocytes from MRL/lpr mice were isolated (see above) and co-cultured (2×10^6/ml) in complete RPMI 1640 medium in the presence of different concentrations of anti-ANX A1 or Control antibodies (ranging from 5 ng/ml to 500 ng/ml), or medium only for 4 days at 37 °C/ 5% CO_2. Another set of cultured splenocytes was stimulated for 16 h either with plate-bound anti-CD3/anti-CD28 antibodies (2.5 µg/ml each), or with 10 µg/ml LPS, and incubated for 4 days under the same conditions in the presence of increasing concentrations of anti-ANX A1 or Isotype control antibodies.

Next, the ELISpot assay was performed as described (Mihaylova et al. 2017). Briefly, ethanol-activated ELISpot plates were coated with S1-treated calf thymus DNA. The splenocytes, pre-incubated in the culture plates with anti-ANX A1 Ab, were transferred into the DNA-coated ELISpot plates and further cultured for additional 4 h under the same conditions. The plates were then incubated with an AP-conjugated anti-mouse IgG and developed by 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium substrate (Sigma). The spots, corresponding to the number of anti-dsDNA IgG antibody-producing plasmocytes, were counted by C.T.L Immunospot S5 Versa Analyzer (Bonn, Germany).

Apoptosis Assay

Spleen cells from MRL/lpr mice were isolated as described above and co-cultured (2×10^6 cells/ml in complete RPMI-1640 medium) with anti-ANX A1 or Control antibodies (ranging from 5 ng/mL to 500 ng/mL) for 24 or 48 h at 37 °C/ 5% CO_2. The cells were then washed and tagged with eFlour450-conjugated anti-mouse CD19 or CD3 antibodies. Apoptosis of gated B (CD19+) or T (CD3+) cells was evaluated by flow cytometry using the Annexin V-FITC apoptosis detection Kit (eBioscience).

Treatment Schedule

Groups of 7 weeks old MRL/lpr mice with initial disease and 16 weeks old MRL/lpr mice with severe disease (20 animals each) were injected either with anti-ANX A1 or Control antibodies as described (Mihaylova et al. 2020a). Blood samples

were collected from the mice every two weeks and the sera were kept frozen at -70 °C for further analyses (Fig. 1).

Two other groups of 4 weeks old disease-free female MRL/lpr mice (n = 4 to 6) were treated under the same scheme with anti-ANX A1 or Control antibodies for a period of 6 weeks. After 4 weeks of treatment and at the end of the period (6th week), the animals were sacrificed and the axillar and the inguinal lymph nodes were isolated. Thereafter, monocellular suspensions were prepared as described above. Later, the cells were incubated with anti-CD3-FITC and eFlour450-conjugated anti-CD19 mAbs. The total number of cells was counted by an Automated Cell Counter (Countess™ II FL, ThermoScientific, Waltham, MA).

RNA Purification and Quantitative RT-PCR for Cytokine Detection

Two groups of 7 weeks old female MRL/lpr mice were treated for 7 weeks with anti-ANX A1 or Isotype control antibodies. At the age of 14 weeks, the animals were sacrificed and the spleens were isolated and single-cell suspensions were prepared. Real-time PCR was performed on total spleen mRNA. According to the manufacturer's requirements, RNA was isolated from 1×10^7 cells/ml using an RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). The concentration of purified RNAs was quantified spectrophotometrically at 260 nm (Quawell Q3000, UV Spectrophotometer). The RNAs were reverse transcribed using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommended protocol. For additional purification of DNA, a DNA-free kit (Ambion, Austin, TX, USA) was used. Quantitative RT-PCR was performed by primers and probes for measurement of IFNγMm01168134m1), IL4 (Mm00445259m1), IL6 (Mm00446190m1), IL10 (Mm00439614m1) and GAPDH (Mm99999915) using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) and TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, New Jersey, USA) on a CFX 96 multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Controls consisting of ddH_2O were negative for the target and housekeeper gene. Reactions were run in triplicates in at least three independent experiments. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as an internal control to normalize the variability in expression levels. The results were analyzed with CFX Manager using Relative Quantification method with standard curves.

Serological Measurements Using Autoantigen Microarrays

Pooled sera from each experimental group were analyzed by microarray assay. Protein microarrays with different SLE antigens and reference materials were printed by Scienion AG, Berlin, onto nitrocellulose-covered slides (Maine Manufacturing, Sanford, ME). Microarray measurements were carried out as described before (Papp et al. 2010). Briefly, slides were first incubated with 100 µl of 50-fold diluted pooled serum, then, following washing, were incubated in the mixture of 1:5 000 diluted secondary antibodies

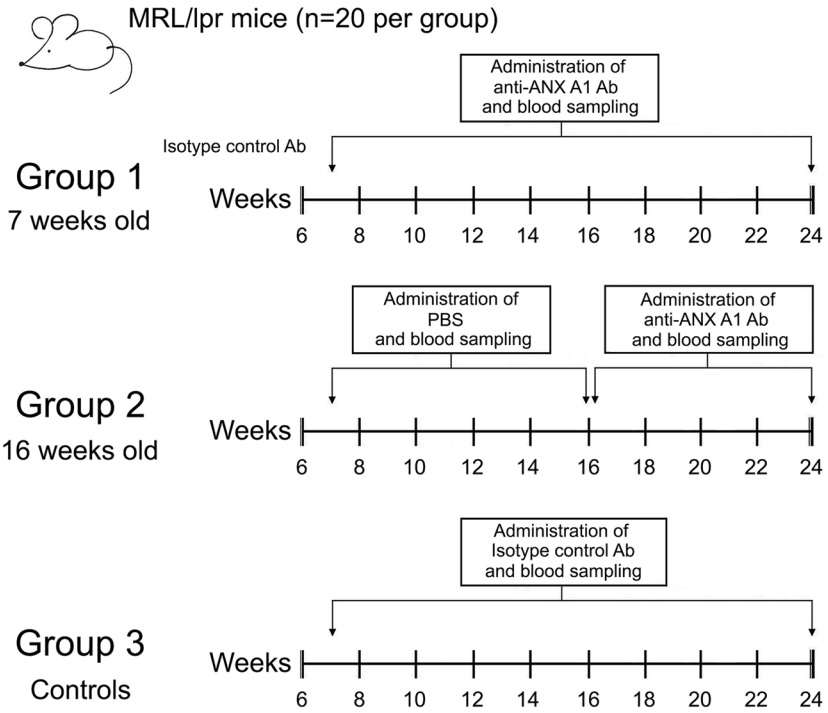

Fig. 1 The schedule of the experimental therapeutic design

(Molecular Probes). After a visual check of the image for errors, data were analyzed with GenePixPro 7 software (Molecular Devices). Specific signal intensities were obtained by subtracting the value of two standard deviations of local background; the final values displayed are relative fluorescent units (RFU). We normalized values by expressing change in reactivity relative to the starting value, to adjust for individual serological differences that are hidden in pooled serum but otherwise increase intergroup variability. Using this approach, the coefficient of methodological variation was in the range of 7 to 14% for the different antigens. Therefore, values showing more than 20% difference were considered as significantly different.

Statistical Analysis

Microarray assays were analyzed with GenePixPro 7 software. All other statistical analyses were performed with Prism software from GraphPad (San Diego, CA). Two-way ANOVA test was used to determine differences between each two groups. Values in the figures correspond to mean ± SD. All proliferation and ELISpot samples were triplicated. A value of $p < 0.05$ was considered as statistically significant.

Results

Modulation of T cell Activation Markers Expression by Anti-Annexin A1 Antibody

To investigate the role of ANX A1 on T-cell activation during disease progression, we tested the effect of anti-ANX A1 antibody on the modulation of the T cell activation markers CD25 and CD69. We performed FACS analyses using plate-bound anti-CD3/CD28 antibody-activated splenocytes from MRL/lpr mice co-cultured with different concentrations of the tested antibodies. Co-incubation with anti-ANX A1 antibody decreased the number of CD25 + /CD69 + splenocytes in a dose-dependent manner, registered by flow cytometry (Fig. 2A). Strongest suppression of T cell activation was achieved with an antibody concentration of 250 ng/ml (16%) compared with the control sample of activated cells (26.7%).

The control antibody did not affect T cell activation markers (data not shown).

Anti-ANX A1 Antibody Inhibits T-cell Proliferation in vitro

To study the ability of the anti-ANX A1 antibody to inhibit cell proliferation, splenocytes from MRL/lpr mice were treated in vitro with different concentrations of the tested antibodies with or without activation. Significant dose-dependent inhibition of cell proliferation was observed in anti-CD3/CD28 antibody pre-activated splenocytes, co-cultured with anti-ANX A1 antibody compared to the ANX A1 untreated cells (Fig. 2B, middle panel). The anti-ANX A1 antibody did not affect significantly T cell proliferation of non-activated T cells as well as LPS-stimulated MRL/lpr spleen cells (Fig. 2B, left and right panels). In addition, the incubation of MRL/lpr splenocytes with the control antibody did not influence cell proliferation (data not shown).

Anti-dsDNA IgG Antibody-Secreting Plasma Cells are Affected by Anti-Annexin A1 Antibody

The effect of increasing concentrations of the anti-ANX A1 or Control antibodies on the number of IgG anti-dsDNA antibody-producing plasmocytes was evaluated by ELISpot assay. Splenocytes from 8-week-old lupus-prone mice were co-cultured with anti-ANX A1 or Control antibodies with or without B and T cell stimulation. Cultivation of splenocytes in the presence of 250 ng/ml of the anti-ANX A1 antibody without stimulation resulted in a significantly reduced number of IgG anti-dsDNA antibody-producing plasma cells (Fig. 3A, left panel). The number of dsDNA-specific IgG secreting plasmocytes was also reduced in the presence of 500 ng/ml of the anti-ANX A1 antibody with LPS stimulation (Fig. 3A, right panel). No significant differences between anti-CD3/CD28 antibody stimulated cells were found (Fig. 3A, middle panel).

No reduction in the number of plasmocytes with the same specificity was found after the co-culturing with the control antibody (data not shown).

Effect of Anti-ANX A1 Antibody on the Apoptosis of T- and B- Lymphocytes

MRL/lpr mice carry a mutation of lymphoproliferation (lpr) resulting in lymphadenopathy and splenomegaly development. Animals with this mutation accumulate a large number of autoreactive T cells in the lymphoid organs. To study the role of anti-ANX A1 antibody treatment on apoptosis of both T and B lymphocytes, we cultured splenocytes from 8 weeks old female MRL/lpr mice in the presence of increasing concentrations of anti-ANX A1 or control antibodies for 24 and 48 h. The surface expression of phosphatidylserine on CD3 + or on CD19 + lymphocytes and cell permeability was monitored by flow cytometry. No modulatory effect on apoptosis was found after 24 h of incubation with the tested anti-ANX A1 antibody (data not shown). Subsequent analyses showed a concentration-dependent increase in the percent of annexin V positive cells (early apoptosis) within the CD19-gated cell population after 48 h of incubation with the anti-ANX A1 antibody (Fig. 3B, 3C, lower panels). In

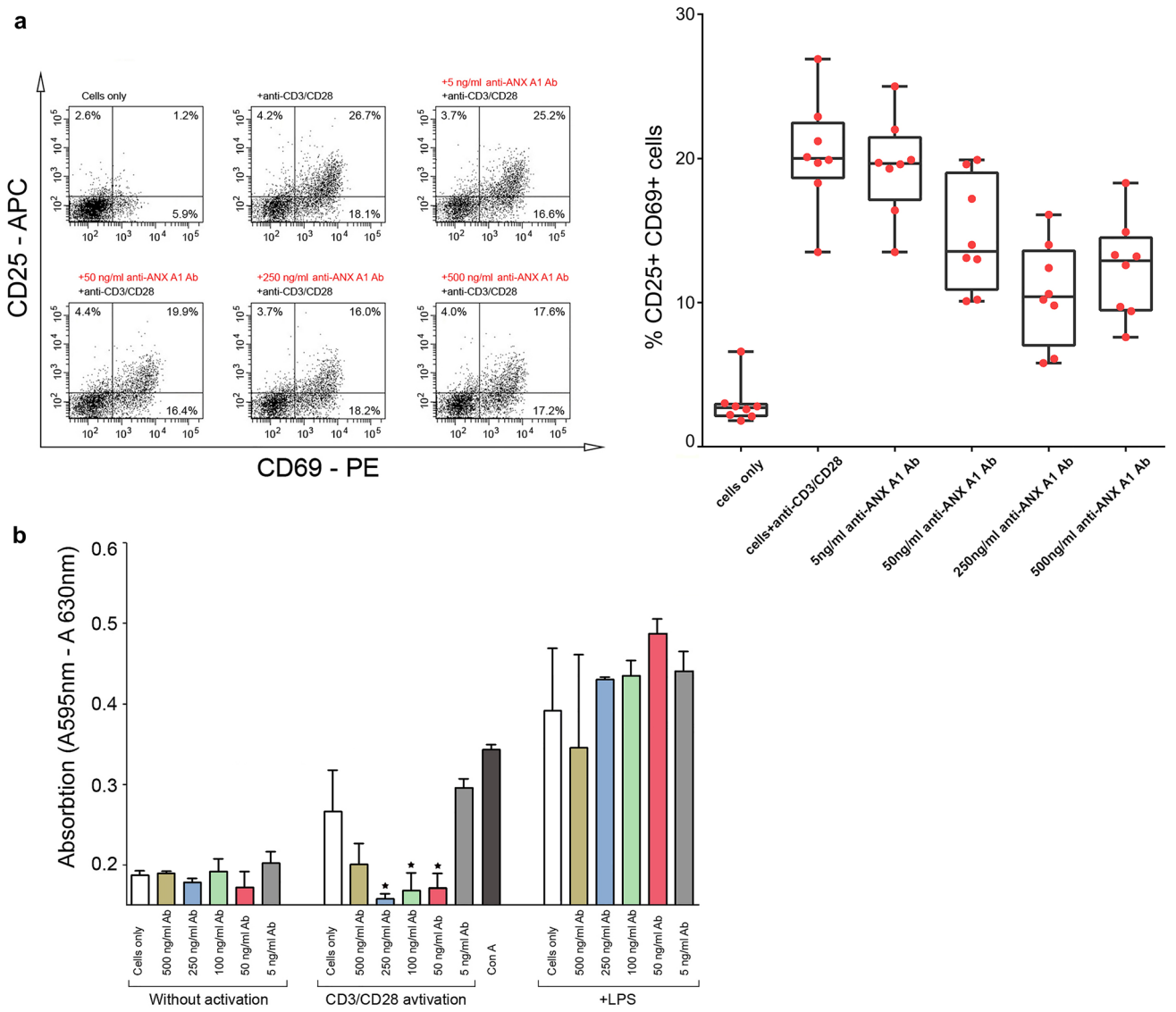

Fig. 2 Treatment of MRL/lpr splenocytes with anti-ANX A1 antibody suppresses activated T cells in vitro. **A** Modulation of T cells activation markers expression by anti-ANX A1 antibody. Anti-CD3/CD28 stimulated spleen cells from MRL/lpr mice were co-cultured with different concentrations of anti-ANX A1 antibody (upper part). The extracted results from all experiments are presented graphically (lower parts). **B** The anti-ANX A1 antibody inhibits cell proliferation in vitro. MRL/lpr splenocytes were stimulated with anti-CD3/CD28 antibodies or with LPS and were cultured in the presence of different concentrations of anti-ANX A1 antibody. The samples were compared to the untreated proliferating cells. All samples were triplicated and average values were used for analysis. Results are expressed as the mean value ± SD of triplicated assays (two-way ANOVA test; $*p < 0.05$). Data are representative of 5 independent experiments

contrast, a concentration-dependent decrease of percentage was obtained within the CD19 + cells in late apoptotic state (double positive for annexinV/propidium iodide) after 48 h anti-ANX A1 antibody therapy (Fig. 3B, lower panel, 3C), while the same treatment of splenocytes did not influence in dose-dependent manner the T cell apoptosis (Fig. 3B, 3C, upper panels). At the same time, the treatment of spleen cells with the control antibody did not affect the cell apoptosis (data not shown).

Anti-ANX A1 Antibody Suppresses T Cell Migration from the Axillar and the Inguinal Lymph Nodes

Two groups of animals (the start of the treatment with anti-ANX A1 or control antibodies, respectively, was at the 4th week of age) were sacrificed after 4 or 6 weeks of treatment. Their lymph nodes and spleens were taken and the lymphocytes within were isolated, labeled and counted by automated cell counter. A significant T cell accumulation was observed in the lymph nodes of ANX A1 antibody-treated

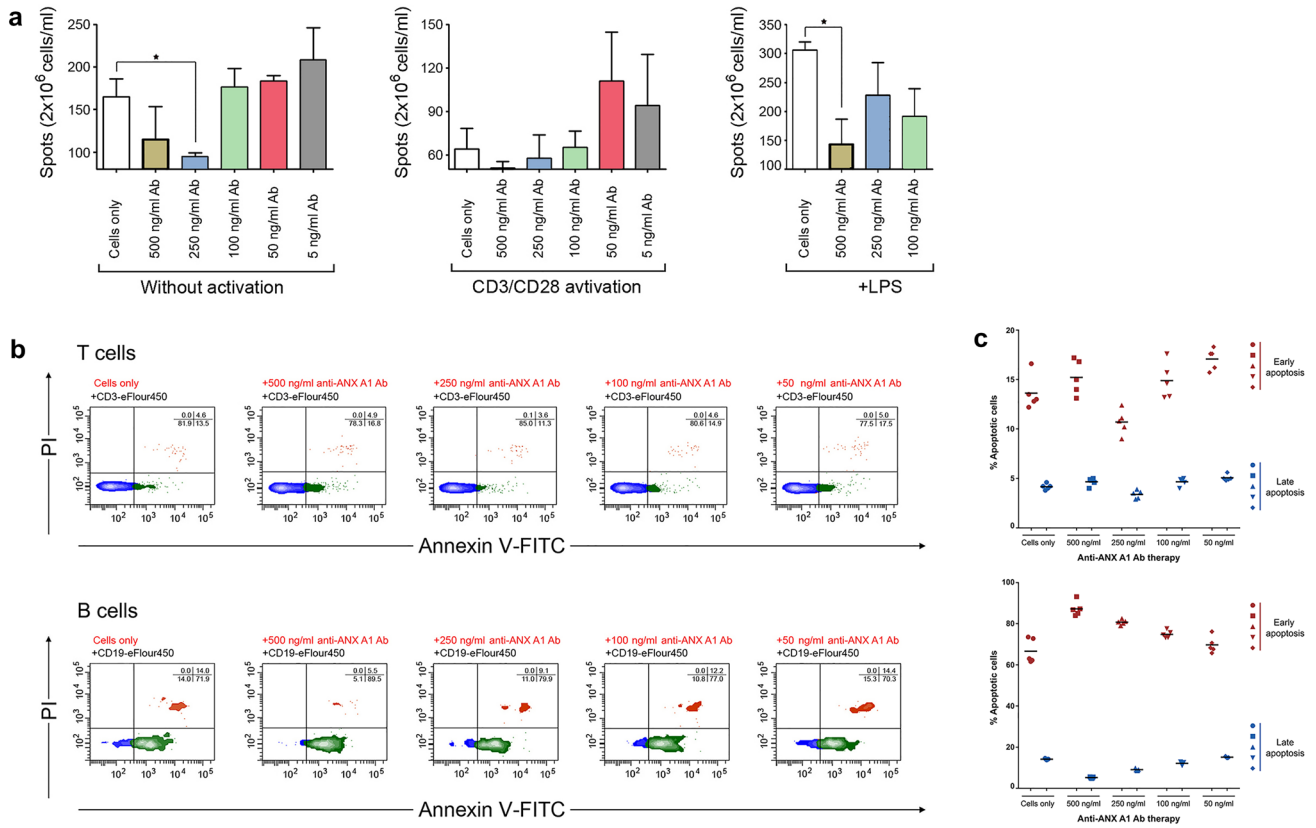

Fig. 3 Anti-ANX A1 antibody treatment decreases the number of anti-dsDNA antibody-secreting cells and induces T- and B- cell apoptosis. **A** Spleen cells from sick female MRL/lpr mice were cultured in the presence of different concentrations of anti-ANX A1 antibody. The cells were stimulated with anti-CD3/anti-CD28 antibodies or with lipopolysaccharide. Control samples were cultured in medium only. The number of plasma cells secreting anti-dsDNA specific IgG antibodies was determined using ELISpot assay. Results are expressed as the mean value ± SD of triplicates. The counted spots in the test-wells were compared with control wells containing medium only-cultured splenocytes (two-way ANOVA test; $*p < 0.05$). Representative data of 5 experiments are shown. **B** Spleen cells from MRL/lpr mice were cultured for 48 h in complete RPMI-1640 medium or in the same medium containing anti-ANX A1 antibody. Apoptosis was analyzed by flow cytometry. The percentage of stained cells is shown in each quadrant. Representative data of 5 experiments are shown. **C** The extracted results from all experiments are presented graphically and the percentage of apoptotic cells is shown. Each symbol represents an individual mouse; values are the mean ± SD for each group; horizontal lines show the median

mice after 6 weeks of therapy compared to untreated animals (88.5% to 45.3% from the total cell number) while no T cell accumulation in spleens were found (data not shown). No differences were found in B cell number in lymph nodes and spleens for both groups of animals. The counted total cell number in the lymph nodes of ANX A1 antibody injected mice was also significantly higher ($p < 0.01$) than control animals (Fig. 4).

Cytokine Measurements

To characterize the altered immune responses in MRL/lpr mice during the disease progression we examined the mRNA expression signature profiles of IL4, IL6, IL10, and IFNγ in splenocytes of 7-week-old animals treated for 7 weeks with anti-ANX A1 or Control antibodies. The quantitative RT-PCR analysis showed that MRL/lpr mice treated with anti-ANX A1 antibody expressed higher levels of IL4 mRNA than the animals treated with Control antibody. We have not observed significant differences in IL6, IL10, and IFNγ expression in anti-ANX A1 antibody—treated group compared to the control one (Fig. 5A).

The IgG Autoantibody Levels to Various Lupus Antigens were Affected by Anti-ANX A1 Antibody Treatment

The levels of autoreactive antibodies specific for lupus relevant antigens in MRL/lpr mouse serum were tested using autoantigen microarrays. Sera were collected every two weeks for the first 70 days of antibodies treatment and the samples from each experimental MRL/lpr group were pooled and further analyzed. The results for both the 7 weeks old (blue) and the 16 weeks old (red) groups which were treated

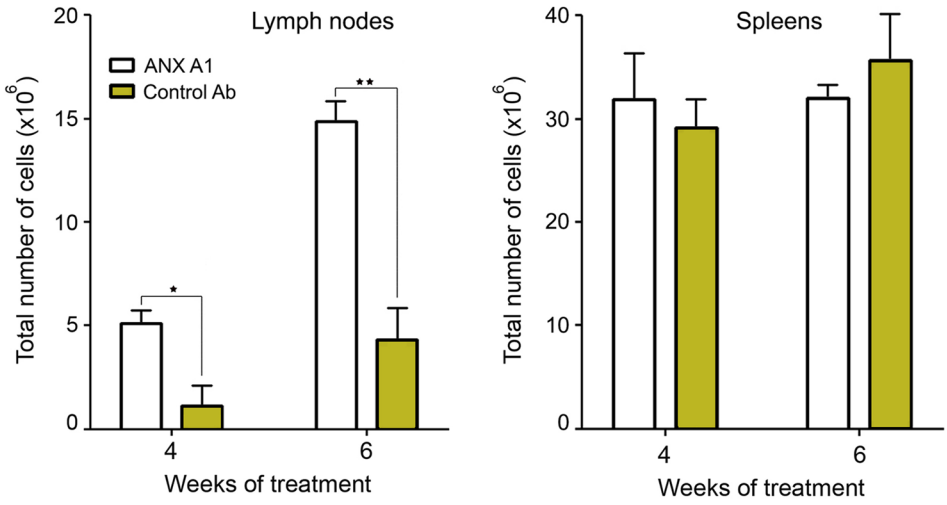

Fig. 4 Treatment of MRL/lpr mice with anti-ANX A1 antibody suppresses T cell migration from lymph nodes. Lymph nodes and spleens from anti-ANX A1 or Control antibodies-treated disease-free MRL/lpr mice were taken at the 4th or 6th weeks of treatment. The lymphocytes were isolated and the total number of cells was counted. Mean ± SD values were calculated for each group; p values were calculated using the two-way ANOVA test (*$p < 0.05$; **$p < 0.01$) in comparison to controls

either with the Control antibody (dashed line) or with ANX A1 antibody (solid line) are shown in Fig. 5B. No significant differences in the IgG values for all antigens were found between the ANX A1 treated and control 7 week-old groups, while a remarkable decline in dsDNA, RNA, histone, and chromatin specific IgG levels was observed in the anti-ANX A1 antibody-administered 16 week-old test group when compared to Control antibody—treated animals. Interestingly, the opposite result was found for Sm (RNA-binding proteins) antigen with increased auto-antibody levels in the anti-ANX A1-treated group. No significant differences were observed between the ANX A1-treated and control groups in IgG values specific for C1q, ssDNA, and Ro/SSA (Sjögren syndrome antigen).

The specific IgM values between the ANX A1 treated and control groups did not show significant differences (data not shown).

Discussion

In the current study, we observed that the treatment of T and B cells with anti-ANX A1 antibody in vitro and in vivo suppressed T-cell activation and proliferation, anti-dsDNA antibody-producing plasma cells and decreased autoantibody levels to various lupus antigens. B cell apoptosis and IL4 secretion were also affected, while T cell migration from lymph nodes was limited as a result of antibody therapy.

Several investigators have described that the cooperation between activated auto-reactive B and T lymphocytes in lupus-prone MRL/lpr mice leads to the formation of immune-complexes and inflammatory cells infiltration, overlapping the human lupus symptoms (Richard and Gilkeson 2018; Rottman and Willis 2010). We have previously applied anti-ANX A1 antibody in MRL/lpr, pristane-induced, and humanized NOD-SCID experimental models of SLE to down-modulate autoreactive T and B cells (Mihaylova et al. 2017, 2020a, b). Thus we achieved successful suppression of dsDNA-specific B cells and lupus progression, prevented the appearance of autoantibodies and significantly prolonged the survival. Our current results add to those observations by demonstrating the additional effect of anti-ANX A1 on other elements of the pathologic immune activation in SLE.

ANX A1 is not immunogenic without post-translational modifications and its increased expression in SLE is not a reason to expect anti-ANX A1 autoantibodies generation. Several authors, however, have observed the paradoxical high levels of anti-ANX A1 autoantibodies and concomitant high ANX A1 serum levels and increased cell surface expression in SLE patients. It has been speculated that the presence of high serum ANX A1 levels was a compensatory mechanism for anti-ANX A1 antibody over production, usually correlating with increased levels of other specific SLE markers, such as anti-dsDNA antibodies (Bruschi et al. 2018). To examine the T cell-mediated inflammation of the central nervous system, Paschalidis et al. used the myelin oligodendrocyte glycoprotein (MOG)—induced model of EAE and observed a mild protection from clinical disease in ANX A1 − / − mice, most prominently in the later phase of the disease (Paschalidis et al. 2009). This was associated with reduced antigen-specific recall proliferation and IL-2 production as well as reduced secretion of Th1 and Th17 type cytokines in ANX A1 − / − mice.

Here we explored further the properties of anti-ANX A1 antibody to down-modulate the over-activated autoreactive lymphocytes in a MRL/lpr mouse model. We have also demonstrated that both CD3 + and CD19 + MRL/lpr lymphocytes express surface ANX A1 (Mihaylova et al. 2020a), but the stimulation of the cells with anti-CD3/CD28 antibodies increased dramatically the expression of ANX A1 and induced the expansion and proliferation of T cells. As hypothesized, incubation of anti-CD3/CD28 stimulated

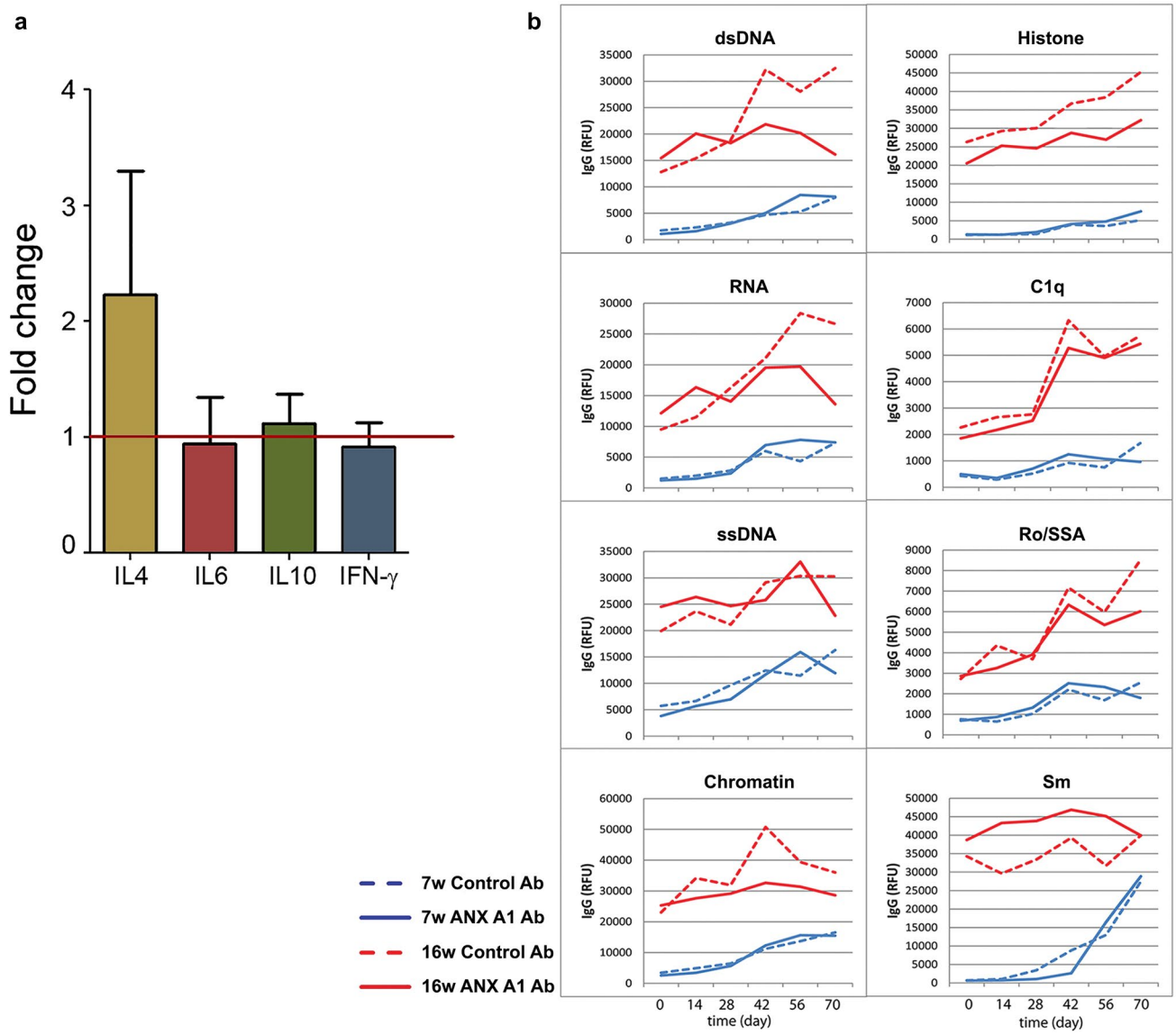

Fig. 5 Administration of anti-ANX A1 antibody to MRL/lpr mice suppresses the appearance of various lupus IgG antibodies and modulates IL4 production. **A** 7 week-old MRL/lpr mice were treated for 7 weeks with the anti-ANX A1 or Control antibodies. Quantitative RT-PCR of isolated mRNA from splenocytes was performed and the levels of IL4, IL6, IL10, and IFNγ mRNA expression was evaluated. The graphs depict the fold change in the expression in each respective cytokine. Mean ± SD values were calculated; p values were calculated using the two-way ANOVA test in comparison to controls. **B** The Anti-ANX A1 Ab administration to the 7 week-old (blue) and the 16 week-old (red) animals influenced various lupus IgG autoantibody levels measured by microarrays

spleen cells from MRL/lpr mice with different concentrations of the anti-ANX A1 antibody exhibited a significant dose-dependent decrease of CD25 + /CD69 + cells and inhibition of T-cell proliferation. However, no significant differences were observed in the number of IgG anti-dsDNA antibody-secreting plasma cells compared to untreated cells. In contrast, the addition of bacterial LPS increased the numbers of antibody producers, which were affected in the presence of 500 ng/ml of the anti-ANX A1 antibody. Moreover, we have obtained similar results in vitro and we observed a dose-dependent decrease of the number of differentiated antibody-producing cells with the same specificity using spleen cells from pristane-induced mice or PBMCs isolated from SLE patients cultured in the presence of increasing concentrations of the studied anti-ANX A1 antibody (Mihaylova et al. 2017, 2020b).

During the lupus progression, MRL/lpr mice develop enlargement of the lymphoid organs due to the accumulation of large numbers of autoreactive T cells. Culturing of splenocytes from lupus mice in the presence of anti-ANX A1 antibody increased the percentage of phosphatidylserine expression (early apoptosis) within CD19-gated cells,

while a concentration-dependent decrease of the percentage of cells in the late apoptotic state was found within the same lymphocytes. Similar increase of the percentage of B cells in late apoptosis was observed within PBMCs isolated from SLE patients after treatment with anti-ANX A1 antibody (Mihaylova et al. 2020b), probably as a result of decreasing of specific T cell activation and reduction of stimulation from auto-reactive T cells. In the same time, the anti-ANX A1 antibody treatment did not affect in dose-dependent manner the number of T cells in early or late apoptosis within CD3-gated splenocytes from MRL/lpr mice, which supports the hypothesis for apoptosis protection after over-activation reduction of these cells.

In vivo treatment with anti-ANX A1 antibody resulted in suppression of disease symptoms and proteinuria during the entire screening period as compared to the control group (Mihaylova et al. 2020a). In addition, the young (7 week-old) MRL/lpr animals treated with anti-ANX A1 antibody were protected from kidney mononuclear cell infiltrations, pathological glomerular immune-complex deposition and enlargement of the peripheral lymph nodes and spleens, while Control antibody-injected mice had inflammatory cell infiltrates, massive mesangial proliferation in the glomeruli, and severe lymphadenopathy and splenomegaly in the end of experiment.

During the SLE progression, a number of class-switched autoantibodies targeting nuclear antigens appears. ANX A1 protein is involved in two signal elements regulation (sphingosine-1-phosphate phosphatase and jagged 1 protein) engaged in T cell development, suggesting an indirect suppression of B cells via T cells deactivation, but we cannot exclude a direct effect on B cells also (Pham et al. 2008; Renshaw et al. 2010). In general, disease-free young animals are expected to have only a few plasmocytes producing auto-antibodies, but the 16 week-old mice with already developed lupus have high numbers of plasma cells producing antibodies with the same specificity. Interestingly, the treatment with anti-ANX A1 antibody reduced the autoantibody production specific to lupus relevant antigens in older sick MRL/lpr mice when compared to Control antibody-treated animals for the first 70 days of administration. Using microarrays, a remarkable decline in dsDNA, RNA, histone and chromatin-specific IgG levels was observed while the 7-week-old group was not affected by anti-ANX A1 treatment in the beginning of treatment. The results resemble the pictures obtained from humanized with PBMCs isolated from SLE patients NSG mice after administration of anti-ANX A1 antibody (Mihaylova et al. 2020b).

The in vivo treatment of young MRL mice with anti-ANX A1 antibody before the onset of the disease resulted in suppression of T cells expansion and differentiation which arrested the lymphocytes in the lymph nodes at the 4th and 6th week after the beginning of the administration. The same treatment did not affect significantly the cell number in the spleens. For instance, using ANX A1 antibody VJ-4B6 *Piras* has found an increase by 50% of the total cell counts either in lymph nodes and spleens at day 9 after treatment of mouse model of induced EAE (Piras 2014).

An immune system of healthy individuals manages a complex network of Cytokines providing a balance between regulatory and inflammatory mediators. Many of these biological elements are integrated into the pathological mechanisms associated with SLE development, but genetic polymorphisms and environmental factors complicate the role of individual cytokines involved in diseases progression (He et al. 2017; Juvet and Zhang 2012; Moudgil and Choubey 2011; Rojas et al. 2018). IFNγ produced by regulatory T cells suppresses autoreactive B cells in healthy individuals. However, these mechanisms do not seem to function in SLE patients and lupus-prone MRL/lpr mice as significantly increased IFNγ levels are detected, produced by T cells, natural killer cells, and Macrophages, as well as by accumulated CD3 + CD4-CD8- double-negative T cells (Juvet and Zhang 2012; Rojas et al. 2018). The administration of anti-ANX A1 antibody in MRL/lpr mice did not suppress either the serum or mRNA levels of IFNγ, but affected the cytokine production in a humanized SLE-NSG mice model (Mihaylova et al. 2020a, b).

IL-10 is a Th2 cytokine produced mainly by Tregs and IL-10-secreting B cells (Bregs), but its role in lupus progression is controversial. Several studies using MRL/lpr mouse model claimed reduced Bregs participation in autoimmunity regulation in vivo (Teichmann et al. 2012). IL-10 inhibits IFNγ production by both over-activated self-reactive CD4 + and CD8 + lymphocytes, but also it is responsible for B lymphocyte proliferation and increasing anti-dsDNA antibody secretion (Rojas et al. 2018). In our study, we found high serum levels of IFNγ and IL10 in Control antibody-treated MRL/lpr mice in both young and old animals, but anti-ANX A1 antibody administration resulted in IL10 down-regulation only (Mihaylova et al. 2020a). The same therapy did not affect the IFNγ and IL10 mRNA levels in splenocytes compared to the control group.

IL4 has an essential dual role for lupus manifestation and for the pathogenesis of SLE playing an opposing to IFNγ role, but also triggering an anti-dsDNA IgG antibody production. IL4-deficient mice develop autoantibodies and renal damages and it is not surprising that MRL/lpr mice have reduced IL4 levels, but the IL4 blockade leads to the prevention of lupus nephritis (Moudgil and Choubey 2011; Rojas et al. 2018). In our hands, splenocytes from MRL/lpr mice treated with anti-ANX A1 antibody expressed higher levels of IL4 mRNA than the animals treated with Control antibody, which supports the statement that IL4 could be involved in the restoration of the cytokine balance and down-modulation of the autoimmune response in MRL/lpr mice.

Anti-ANX A1 antibody treatment of MRL/lpr mouse splenocytes and its administration to animals had an inhibitory effect on lupus-associated B and T cell over-activation, leading to suppression of lupus symptoms and pathological development. It is another possible way to manage disease activity with the potential to down-regulate the autoimmunity.

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Declarations

Conflicts of interest None of the authors has any potential financial conflict of interest related to this manuscript.

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