Original Paper

Regulation of Arthritis Severity by the Acid Sphingomyelinase

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Key Words

Rheumatoid arthritis • Acid sphingomyelinase • Ceramide • Inflammation

Abstract

Background/Aims: Rheumatoid arthritis is a chronic autoimmune disease hallmarked by inflammation in synovial joints. Treatment is hampered by the lack of a cure and current disease-modifying drugs are associated with potentially severe toxicities. **Methods:** We investigated arthritis severity by measuring joint swelling and pro-inflammatory cytokine production in a murine experimental model of inflammatory arthritis (antigen-induced arthritis). We analyzed acid sphingomyelinase knock-out mice and wild-type littermates, as well as mice treated with the pharmacological acid sphingomyelinase inhibitor amitriptyline. **Results:** Genetic ablation or pharmacological inhibition of acid sphingomyelinase reduced joint swelling and levels of pro-inflammatory cytokines in the arthritic joint. **Conclusion:** We identified acid sphingomyelinase as a novel druggable target in rheumatoid arthritis. Functional inhibitors of acid sphingomyelinase have been clinically used for decades, are well tolerated and suitable for long-term treatment. They would be immediately available for clinical development as a novel rheumatoid arthritis therapy.

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder with an intermittent course that is characterized by an inflammation of synovial joints. It results in painful cartilage loss and bone erosions [1]. The disease progresses continuously, but the speed and severity varies. The local joint inflammation is followed by deformation and disability

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and can spread systemically, affecting i.e. lungs, eyes and skin [2]. Up to 1 % of the adult population is affected by RA worldwide [3]. Whereas some risk factors have been identified, the underlying mechanisms remain incompletely understood.

RA is typically treated with immunosuppressive disease modifying anti-rheumatic drugs (DMARDs) such as methotrexate. However, major toxicity and tolerability concerns (i.e. cytopenia, hepatotoxicity pulmonary interstitial disease, teratogenic and abortifacient properties) can occur with an incidence rate of up to 3-5 % [4]. Several new therapeutic strategies are being pursued, i.e. anti-TNF biologics and anti-IL-6 receptor antibodies [5]. However, an unmet clinical need for a new, well-tolerated and effective treatment for RA persists.

Acid sphingomyelinase (human ASM, murine Asm; gene *Smpd1*) is a lysosomal enzyme. Upon activation, ASM is translocated to the plasma membrane and hydrolyses the abundant membrane lipid sphingomyelin to ceramide. Ceramide generation results in a reorganization of the plasma membrane through self-association of ceramide molecules, leading to the formation of ceramide-enriched plasma membrane platforms [6]. This reorganizes receptors and signaling molecules, i.e. by clustering receptors, spatial focusing of activated receptors with intracellular molecules and/or exclusion of inhibitory molecules, facilitating and enhancing the transmission of a given signal [7]. Various stimuli have been reported to activate ASM in this fashion and to induce a variety of different cellular effects (reviewed in [8-10]).

ASM and/or ceramide have also been implicated in autoimmunity: ASM has been shown to be important for mast cell function [11], ceramide is increased in a chronic colitis model [12] and in a murine model of multiple sclerosis, Asm-deficient mice were protected from disease manifestations [13] and in an *in vitro* study, endothelial ASM was reported to regulate T cell transmigration across brain endothelial cells [14]. Concerning RA itself, ceramide has been reported to be increased in the synovial fluid of RA patients [15]. The functional implications, however, are unknown and the role of acid sphingomyelinase has not been studied so far.

Given these previously reported findings, we chose to investigate the role of ASM in a murine model of inflammatory arthritis, which shares some features with the human disease. We investigated the susceptibility of Asm-deficient mice (*Smpd1*^{-/-}) to murine antigen-induced arthritis (AIA). Briefly, in the AIA model mice are immunized subcutaneously with a foreign antigen, i.e. methylated bovine serum albumin (mBSA). After immunization, this antigen is then injected directly into the joint cavity, where it is recognized by the primed immune system and elicits a local inflammatory response similar to the inflammation seen in RA. Lack of Asm reduced arthritis severity. Pharmacological targeting of Asm with the functional inhibitor amitriptyline [16, 17] also effectively reduced arthritis symptoms, establishing Asm as a potential new therapeutic target in RA.

Materials and Methods

Mice

Asm-deficient mice (*Smpd1*^{-/-}) [18] and syngenic wild-type littermates were bred on the C57BL/6 Jackson background. All mice were bred and housed under pathogen-free conditions as recommended by the Federation of European Laboratory Animal Science Associations (FELASA) in the vivarium of the University of Duisburg-Essen, Germany. Mice had *ad libitum* access to food and water and were kept on a 12 h/12 h light/dark cycle. Mice were genotyped routinely by PCR. All procedures were approved by State Agency for Nature, Environment and Consumer Protection (LANUV NRW) in Düsseldorf, Germany.

Induction and assessment of antigen-induced arthritis

Methylated bovine serum albumin (mBSA, Sigma-Aldrich, Steinheim, Germany) was diluted to 2 mg/ml in 5 % glucose in phosphate-buffered saline (PBS: 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium phosphate, 2.0 mM, pH 7.2) and emulsified in a 1:1 (v/v) ratio with complete

Freund's adjuvant solution (Sigma) using lubricant- and rubber free syringes (BD Bioscience, Heidelberg). The completed emulsion was injected subcutaneously into the axillary and inguinal lymph node regions on experimental day -21 and day -14. On experimental day 0, arthritis was induced by injecting 10 µl of a 10 mg/ml mBSA 5 % glucose solution into one knee joint cavity using a 30 G needle. The contralateral joint was injected with 10 µl vehicle (5 % Glucose) solution. Knee joint diameter was assessed with a digital caliper immediately after induction and once daily on all subsequent days.

Histopathological assessment

Mice were sacrificed on experimental day 4 after arthritis induction, whole knee joints (including synovium, adjacent tissues and bone) were removed and fixed with 4 % paraformaldehyde, de-calcified with Decalcifier Soft (Carl-Roth, Karlsruhe, Germany), dehydrated with ethanol, embedded in paraffin and trimmed to 4-6 µm thin sections. These were stained with the Goldner-Elastica technique and analyzed on a Leica microscope (Leica Microsystems, Wetzlar, Germany).

Joint cytokine measurements

Mice were sacrificed on experimental day 2 or 4 after arthritis induction as indicated in the respective figure legends. Joint homogenates were prepared as previously described [19]: Whole knee joints (including synovium, adjacent tissues and bone) were removed, snap-frozen and pulverized using a pestle and mortar, filled with liquid nitrogen. The organ powder was transferred to pre-cooled 15 mL reaction tubes and resuspended in 1 mL cold PBS containing 10 µg/mL aprotinin (Sigma), 10 µg/mL leupeptin (Sigma) and 1x Complete Protease Inhibitor Cocktail (Roche, Freiburg, Germany). Samples were vortexed, spun down at 300 x g for 5 min at 4 °C and then sonicated for 10 sec three times. Homogenates were centrifuged at 400 x g for 10 min at 4 °C. The supernatants were transferred to 1.5 mL reaction tubes, centrifuged at 15 000 x g and the supernatants were collected for cytokine analysis and protein quantification. Levels of IL-1β, IL-6 and IL-17 were assessed using R&D Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. For IL-1β and IL-6 analyses, samples were routinely diluted with the respective calibrator diluent to prevent samples from exceeding the linear range. Data are expressed as pg cytokine/mg protein.

Analysis of lymphocyte subsets

Mice were sacrificed and inguinal lymph nodes were resected on experimental day 0 (21 days after the initial immunization). Single cell suspensions were prepared by carefully pushing cells through a 70 µm filter. Follicular B cells were stained using anti-B220 (Thermo Fisher Scientific, Waltham, MA, USA), anti-CD95 (BD), anti-GL7 (Thermo Fisher), anti-IgM and anti-IgG (both BD). T cell subsets were stained using anti-CD4 (BD), anti-CD8 (Thermo-Fisher), anti-TCRβ (Thermo Fisher), anti-CD62L (BD) and anti-CD44 (BD). Cells were analyzed by flow cytometry using a LSR III flow cytometer (BD). Regulatory T cells were stained using the Affymetrix Mouse Regulatory T Cell Staining Kit #1 (Thermo Fisher) and analyzed on a FACSCalibur flow cytometer (BD) and using FLOWJo software (FLOWJo, Ashland, OR, USA).

In vitro restimulation

Mice were sacrificed and inguinal lymph nodes were resected under sterile conditions on experimental day 0 (21 days after the initial immunization). Single cell suspensions were prepared in serum-free RPMI medium (Gibco, Invitrogen) as described above and seeded at 5 x 10⁶ cells/ml in microtiter plates. Stimulation media were added at a ratio of 1:1 (v/v) to achieve a final concentration of 5 % Asm-free mouse serum and 0.5 % glucose in all samples. Asm-free mouse serum was obtained by drawing blood via tail vein puncture from *Smpd1*^{-/-} mice and centrifuging at 2 000 x g for 10 min. When indicated, samples also contained a final concentration of either 1 or 2 mg/mL mBSA or 10 µg/ml PHA-P (Sigma) and 10 U/mL mouse recombinant IL-2 (Thermo Fisher). Cell proliferation was assessed after 48 h using the BrdU Cell Proliferation ELISA (colorimetric) kit (Roche) according to the manufacturer's instructions. The BrdU labeling reagent was added for the final 24 h of incubation. The supernatants from these cultures were collected and levels of IL-2, IL-4, IL-6 and IL-17 were assessed using Quantikine ELISA kits (R&D) according to the manufacturer's instructions.

Amitriptyline treatment

Amitriptyline hydrochloride ($\geq 98\%$, Sigma) was freshly diluted in 0.9 % sodium chloride. Mice received 9.375 mg amitriptyline/kg bodyweight intraperitoneally every 6 h starting 2.5 days prior to arthritis induction. The total daily dose of amitriptyline was chosen based on the dosages previously reported in studies using *in vivo* mouse models [20, 21], but adapted to fit our four-times daily treatment schedule. The latter was chosen to ensure a constant plasma level of the drug based on the published half life time of the drug [22]. Treatment was continued until the mice were sacrificed. The control group received 0.9 % sodium chloride injections.

Statistical analyses

Data are presented as arithmetic means $\pm$ standard deviation. For multiple comparisons, we first confirmed normal distribution of the data with D'Agostino & Pearson omnibus normality test. We then used analysis of variance (ANOVA) with Bonferroni posttests to test for significant differences between selected pairs. All data were obtained from independent measurements.

Results

Acid sphingomyelinase deficiency ameliorates arthritis severity

To define the role of Asm in RA, we utilized the murine AIA model (Fig. 1 A). *Smpd1*^{-/-} mice and wild-type littermates were first immunized subcutaneously with an antigen, methylated bovine serum albumin (mBSA). One week later, the immunization was repeated to boost the immune system. Arthritis was then induced 14 days after the second immunization by injecting the antigen directly into the joint cavity to induce a local inflammatory response. We monitored joint swelling over the course of 5 days and observed a marked reduction in joint swelling upon Asm-deficiency (Fig. 1 B). To further show a reduction in arthritis severity upon Asm knock-out, we analyzed the joints histologically. Histopathological signs of arthritis were reduced upon Asm-deficiency (Fig. 1 C). We also quantified the levels of three of the hallmark cytokines in human RA – IL-1 β , IL-6 and IL-17. These cytokines are pro-inflammatory and pro-erosive drivers in RA [23]. IL-1 β and IL-6 are secreted by synovial fibroblasts and macrophages. IL-1 β can also be released by platelets. IL-6 can additionally be produced by various other cell types, including T- and B-cells. IL-17 is produced by Th17 cells. As expected, arthritis induction in the mBSA-injected knee resulted in a significant increase in the levels of these pro-inflammatory cytokines compared to the vehicle-injected control joint (Fig. 1 D-F). Cytokine levels in arthritic joints of *Smpd1*^{-/-} mice, however, were significantly lower than in the arthritic joints of wild-type littermates (Fig. 1 D-F).

B- and T-Cell responses to the immunization are not affected by acid sphingomyelinase deficiency

To exclude the possibility that the observed effects on arthritis severity were due to a different response to the immunization in *Smpd1*^{-/-} mice, we analyzed lymphocyte subsets in immunized mice prior to arthritis induction.

To gain some insight into a potential role of T helper cells in the resistance of *Smpd1*^{-/-} mice, we analyzed the total number of CD4⁺ cells, naïve CD4⁺ cells, central memory and central effector T helper cells. Effector memory T (T_{EM}) cells are the subset generally associated with autoimmune- and other chronic inflammatory diseases [24-31]. Specifically in RA, T_{EM} cells have been reported to be increased in the synovial fluid [32] and recently an inhibitor of the Kv1.3 channel (HsTX1[R14A]) was reported to reduce inflammation in the pristane-induced arthritis model by inhibiting T_{EM} cell activation [33]. Central memory T cells (T_{CM}) cells do not migrate to inflamed tissues and have little to no effector function, but proliferate and differentiate to effector cells in response to antigenic stimulation. In this study, they thus serve as a control to compare the efficacy of immunization in *Smpd1*^{-/-} mice. However, we did not see any differences in any of the T helper cell subsets (Fig. 2 A) in lymph nodes of immunized *Smpd1*^{-/-} mice compared to immunized wild-type littermates.

Next, we studied the role of CD8⁺ T cells in our model. CD8⁺ effectors can contribute to autoimmunity through the secretion of inflammatory cytokines or inappropriate induction of apoptosis, but they can also have ameliorative effects, such as elimination of self-reactive cells and self-antigen sources [34]. CD8⁺ T cells seem to have mainly destructive functions in inflammatory joint diseases: In a collagen-induced arthritis model, CD8⁺ T cell deficiency partially prevented the disease [35]. In a human synovium-SCID mouse chimera model, CD8⁺ T cells were shown to be required for the formation of ectopic germinal centers that typically form in RA and drive immunoglobulin production [36]. CD8⁺ T cells were also suggested to contribute to cartilage damage through lysis of chondrocytes under inflammatory conditions [37]. In contrast to this, increased numbers of CD8⁺ T_{EM} cells were reported in the synovial fluid of RA patients, which were less cytotoxic and produced more anti-inflammatory IL-10 and were thus suggested to function as negative regulators of the disease [38].

Despite the still undefined role of CD8⁺ in RA, we determined the total number of CD8⁺ cells as well as the cell numbers in the naïve, central memory and effector memory subsets

Fig. 1. Acid sphingomyelinase deficiency ameliorates experimental arthritis severity (A) The time-course of the AIA model is schematically shown. Methylated bovine serum albumin (mBSA) is first injected subcutaneously (s.c.) as an emulsion with complete Freund's adjuvant (CFA). 7 days later, the immunization is repeated. 14 days after the second immunization, mBSA is injected intraarticularly (i.a.) to induce arthritis. (B-F) AIA was induced in *Smpd1*^{-/-} mice and wild-type littermates. (A) Joint swelling was monitored over 5 days by measuring joint diameter with a digital caliper. Data are presented as the ratio of the arthritic joint to the vehicle-injected control joint as mean ± SD of n = 11 mice per group. (C) On day 4 after arthritis induction, knee joints were dissected and Masson-Goldner stained sections were prepared. Representative images of n = 4-5 mice are shown. Black bars indicate 1 mm. Arrow-heads indicate inflammatory infiltration, arrows pannus formation and asterisks exudation into the joint cavity. Levels of IL-1β (D), IL-6 (E), IL-17 (F) were quantified by ELISA. Data are presented as the amount of the respective cytokine

per mg protein ± SD of n = 9-10 mice per group. Data passed the D'Agostino & Pearson omnibus normality test and asterisks indicate significance as assessed by 2-way ANOVA with Bonferroni posttests: *** p < 0.001.

Fig. 2. Acid sphingomyelinase deficiency has no effect on B- and T-Cell numbers after immunization (A-D) Mice were immunized with mBSA and lymphocytes were isolated from inguinal lymph nodes on experimental day 0. Different lymphocyte subsets were analyzed by flow cytometry. Data are presented as the mean of the absolute cell numbers per lymph node of $n = 4-8$ mice $\pm$ SD. Significance as assessed by 2-way ANOVA with Bonferroni posttests, but no significant differences were detected. (A-B) T-helper (A) and cytotoxic T-Cells (B) cells: Naive: TCR β^+ , CD62L $^+$, CD44 $^-$; central memory: TCR β^+ , CD62L $^+$, CD44 $^+$; effector memory: TCR β^+ , CD62L $^-$, CD44 $^+$. (C) Regulatory T-Cells: CD4 $^+$, FoxP3 $^+$; activated regulatory T-Cells: CD4 $^+$, FoxP3 $^+$, CD25 $^+$. (D) B cells: B220 $^+$, follicular (FO) B cells: B220 $^+$, CD95 $^+$, Gl7 $^+$; class-switched (IgM $\rightarrow$ IgG) B cells: IgM $^+$, IgG $^+$.

central memory: TCR β^+ , CD62L $^+$, CD44 $^+$; effector memory: TCR β^+ , CD62L $^-$, CD44 $^+$. (C) Regulatory T-Cells: CD4 $^+$, FoxP3 $^+$; activated regulatory T-Cells: CD4 $^+$, FoxP3 $^+$, CD25 $^+$. (D) B cells: B220 $^+$, follicular (FO) B cells: B220 $^+$, CD95 $^+$, Gl7 $^+$; class-switched (IgM $\rightarrow$ IgG) B cells: IgM $^+$, IgG $^+$.

in our model to monitor immunization efficacy in *Smpd1*^{-/-} mice, as CD8 $^+$ T cell responses are induced in response to foreign antigens. We did not detect any differences in cytotoxic T cells (Fig. 2 B) in lymph nodes of immunized *Smpd1*^{-/-} mice compared to immunized wild-type littermates.

Regulatory T (T_{reg}) cells have also been reported to be involved in the pathogenesis of autoimmune diseases, including RA [39-42]. Previously, Asm-deficiency or Asm-inhibition has been linked to increased T_{reg} frequencies in naïve mice [43, 44]. Here, we studied immunized mice and did not detect any differences in T_{reg} cell number in lymph nodes of immunized *Smpd1*^{-/-} mice compared to immunized wild-type littermates (Fig. 2 C). The activation status of the T_{reg} cells was also similar in both groups (Fig. 2 C).

B cells are also integral to RA pathogenesis due to auto-antibody production, auto-antigen presentation and cytokine production [45-47]. Follicular (FO) B cells are mature peripheral B cells that participate in T cell-dependent immune responses to protein antigens and are thus particularly well suited to monitor immunization efficacy. Isotype-switching from IgM to IgG also serves as a successful immunization marker in this study. However, we also did not detect a difference in the number of follicular B cells in immunized *Smpd1*^{-/-} mice compared to immunized wild-type littermates (Fig. 2 D). The percentage of IgM to IgG class switched cells was equivalent as well (Fig. 2 D).

To address any potential differences in the function of lymphocytes from *Smpd1*^{-/-} mice, we also re-stimulated lymphocytes from immunized mice with the mBSA-antigen *in vitro*. We did not detect any significant differences in either proliferation of lymphocytes from *Smpd1*^{-/-} mice (Fig. 3 A), nor in their cytokine response (Fig. 3 B-E) compared to wild-type lymphocytes.

Acid sphingomyelinase inhibition with amitriptyline ameliorates arthritis severity

To investigate whether the unknown role of Asm in mediating arthritis severity could be exploited clinically, we treated wild-type mice with the functional Asm inhibitor amitriptyline

Fig. 3. Acid sphingomyelinase deficiency has no effect on proliferation and cytokine release upon antigen re-stimulation (A-E) Lymphocytes were left untreated, re-stimulated with 1 mg/mL mBSA or stimulated with 10 µg/mL PHA and 10 U/mL IL-2 in vitro and proliferation was monitored (A) or release of IL-2 (B), IL-4 (C), IL-6 (D) or IL-17 (E) was quantified by ELISA. Data are presented as means ± SD of n = 8 mice per group. No significant differences were detected by 2-way ANOVA with Bonferroni posttests.

Fig. 4. Pharmacological acid sphingomyelinase inhibition ameliorates arthritis severity (A-D) Immunized mice were treated with 9.375 mg/kg bodyweight amitriptyline hydrochloride (AMI) i.p. every 6 hours or received placebo. Treatment commenced two days prior to arthritis induction and was continued until the mice were sacrificed. (A) Joint swelling was monitored over 3 days after arthritis induction by measuring joint diameter with a digital caliper. Data are presented as the ratio of the arthritic joint to the vehicle-injected control joint as mean ± SD of n = 9-11 mice. (B-D) On experimental day 2 after arthritis induction, knee joints were dissected and analyzed by ELISA for the levels of IL-1β (B), IL-6 (C) and IL-17 (D). Data are presented as the amount of the respective cytokine per mg protein ± SD of n = 8-11 mice. Asterisks indicate significance as assessed by 2-way ANOVA with Bonferroni posttests: *** p<0.001.

[16, 17]. Joint swelling (Fig 4 A) and levels of pro-inflammatory cytokines (Fig. 4 B-D) were significantly decreased upon Asm-inhibition.

Discussion

Our study identifies a novel function of Asm and ceramide in inflammatory arthritis: Upon either genetic Asm-deficiency or pharmacological inhibition, disease manifestations are reduced in an experimental model of inflammatory arthritis.

So far, it is unclear how Asm controls arthritis severity. We analyzed different lymphocyte subsets in the lymph nodes after immunization to test if *Smpd1*^{-/-} mice can recognize the foreign antigen appropriately. We did not detect any differences between the wild-type and *Smpd1*^{-/-} mice in any of the analyzed subsets (Fig. 2), which are markers of immunization efficacy and/or reflect important mediators of RA. We also tested whether these cells respond normally to antigen-stimulation by isolating lymphocytes from lymph nodes of immunized mice and stimulating with the foreign antigen used for the immunization (mBSA) *ex vivo*. Again, we could not detect differences between wild-type and *Smpd1*^{-/-} lymphocytes with regards to proliferation and cytokine production (Fig. 3). Thus, a functional defect of lymphocyte stimulation/antigen-recognition in *Smpd1*^{-/-} lymphocytes as the cause of the observed reduction in arthritis severity is unlikely, given that both the proliferative- and cytokine responses to the antigen were unaffected by *Smpd1*-knock-out.

This apparently contrasts another study that has reported a role of ASM in modulating T-helper cell responses [48]: The study by Bai et al. showed CD4⁺ T cell activation upon stimulation with anti-CD3/CD28 antibody-beads *ex vivo*, which was blunted upon treatment with functional ASM inhibitors. However, the *ex vivo* stimulation using defined and crosslinked antibodies may not reflect the *in vivo* situation with the involvement of many more receptors that may compensate for downregulation of one signaling pathway.

As described above, an effect due to an imbalance of effector- and regulatory lymphocyte populations upon Asm-deficiency seems unlikely as well, as T- and B-cell populations in response to the immunization regimen were similar in both wild-type and *Smpd1*^{-/-} mice (Fig. 2). Our study also included the analysis of regulatory T cells, whose frequency was not altered in Asm-deficient mice after immunization. Hollmann et al. and Zhou et al. have previously reported an increase of regulatory T cells upon Asm-deficiency in untreated mice [43, 44]. Zhou et al. identified ASM as a negative regulator of T_{reg} development, but also noted opposing effects based on the composition of the cytokine cocktail used for experimental T_{reg} induction in their own and in another study by Kue et al. [44, 49]. Our data show no differences in T_{reg} cell number and activation status in *Smpd1*^{-/-} mice after immunization and thus suggest that the immunization regimen is a strong enough stimulus to overcome the reported baseline differences in untreated mice.

A possible explanation for the reported findings is a defect in lymphocyte adhesion and/or emigration upon Asm-deficiency. Endothelial Asm has been reported to regulate T cell transmigration across brain endothelial cells [14]. Similarly, endothelial Asm may regulate lymphocyte extravasation in synovial micro-vessels. Enhanced cell adhesion upon sphingomyelinase- or ceramide treatment has been reported in different cell types, hinting at a general mechanism [50-52]. Particularly relevant to this study are reports on hematogenous metastasis, indicating that platelets secrete Asm to regulate tumor cell extravasation [51]. Subsequently, this process was shown to involve P-selectin, p38 MAPK and β 1-Integrin [53, 54]. These molecules have previously been ascribed significant roles in RA [55-60]. Consequently, it is conceivable that lymphocyte extravasation is regulated by a similar, platelet-ASM-dependent mechanism. Given that platelets have been reported to accumulate in the synovial microcirculation in the antigen-induced arthritis model, they might provide an ideal platform for lymphocyte recruitment into the joint [60]. Platelets are newly recognized for their significant role in inflammatory diseases, including arthritis [61] and platelet depletion has been reported to ameliorate experimental arthritis [62]. Thus, lack of Asm secretion by platelets in *Smpd1*^{-/-} mice may be responsible for amelioration of arthritis severity reported in this study.

Currently, it remains unclear how Asm-deficiency ameliorates arthritis severity and further studies are necessary to elucidate the mechanism. Nevertheless, the effectiveness

of pharmacological Asm-inhibition in reducing disease manifestations identify ASM as a potential new druggable target in RA. It remains an open question whether ASM-inhibition will be able to ameliorate an acute clinical episode of RA. In our experimental models, amitriptyline treatment only commenced before arthritis induction to achieve steady Asm-inhibition by the onset of arthritis. If inflammation in general is blunted by amitriptyline, the drug may also be effective during an acute phase of RA. However, further studies are necessary to investigate this. The advantages of ASM inhibitors, such as amitriptyline, are that several of these inhibitors are well-known antidepressants that are safe, cheap and have been clinically used for decades. Their favorable properties include good absorption (all are orally active), distribution (extensive tissue binding, most can also cross the blood-brain barrier), metabolism and excretion, activity across different cell types, no habituation, reversible inhibition and no rebound effects [63]. They are also well tolerated and very suitable for long-term therapy. Additionally, the favorable properties of the functional ASM inhibitors provide a definite benefit compared to glucocorticoids, which are frequently used in RA and which should only be administered for the shortest possible time due to the potentially severe adverse effects [64, 65]. The toxicity and tolerability of the current gold standard of care in RA – methotrexate – also leaves a lot to be desired, given that the incidence rate of severe adverse events is up to 3-5 % and given its additional abortifacient and teratogenic properties [4]. While new biological agents for the treatment of RA are making progress on their way into the clinic, these new treatments – if they are successful – will be quite expensive and their administration may also require intravenous injections (e.g. Rituximab). Thus, amitriptyline or other functional inhibitors of ASM do not only have the advantage of being well tolerated, but are also easy to administer orally and inexpensive.

Conclusion

The results of this study demonstrate a protective effect of Asm-inhibition on arthritis manifestations: Both genetic deficiency and pharmacological inhibition of Asm reduce joint swelling and levels of pro-inflammatory cytokines in the joint. Amitriptyline is a safe drug that has been used clinically for years and would be immediately available for clinical development as a novel RA therapy.

Disclosure Statement

The authors declare that they have no conflict of interest.

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