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Helminth products modulate innate immune recognition of nucleic acids in systemic lupus erythematosus

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Abstract

Aim: Current treatment of Systemic Lupus Erythematosus (SLE) is suboptimal and causes broad immunosuppression. Therapeutic use of helminths or helminth products has been suggested for autoimmune diseases such as SLE. In the present study, we evaluated possible immunomodulating effects of adult body fluid (ABF) from *Ascaris suum* on peripheral blood mononuclear cells (PBMCs) from SLE patients in an ex vivo setup.

Methods: PBMCs from SLE patients and healthy controls (HC) were isolated and stimulated ex vivo with ABF and Toll-like receptor agonists or activators of the stimulator of interferon genes (STING) or mitochondrial antiviral signaling protein (MAVS) pathways. After 24 h of incubation, the cytokine profile was analyzed using ELISA and Meso Scale Discovery techniques.

Results: ABF suppressed production of IL-6, TNF-α, CXCL10, and IL-10 by PBMCs from SLE patients and HCs following stimulation with specific agonists. ABF also reduced IFN-u production by stimulated PBMCs from HCs.

Conclusions: Our data show that ABF has an immunomodulatory effect on the production of key cytokines in the pathogenesis of SLE. These results suggest that ABF or ABF components hold potential as a novel treatment option for SLE.

Keywords

Systemic lupus erythematosus, *Ascaris suum*, helminth products, autoimmunity, hygiene hypothesis

Introduction

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease that causes a broad spectrum of clinical manifestations in various organs. SLE has a female to male ratio of 9:1 and it predominantly affects women of child- bearing age.[1] It is also known as “the disease of a thousand faces.” The heterogeneous symptoms are caused by a break in self-tolerance, which creates autoreactive T- and B-cells. This is believed to be a central pathogenic factor.[2] Cellular inflammation and defective apoptosis of the patients’ cells create excess cell debris that contains nuclear antigens, such as DNA and RNA. These molecules are normally sequestered from the immune system due to their intracellular localization.[3] Some of the immune receptors involved in recognizing nucleic acids are endosomal Toll-like receptors (TLRs), for example TLR3, TLR7, TLR8, and TLR9 that recognize dsRNA, ssRNA, ssRNA, and unmethylated CpG DNA, respectively.[4,5] Other relevant receptors with ligands that are nucleic acids are the cytosolic DNA sensors and RIG-I-like RNA receptors.[6,7] The course of SLE is un-predictable with flares and remissions and symptoms span from mild rash to organ failure with a potentially fatal outcome. Unfortunately, treatment options continue to remain inadequate and SLE is associated with increased mortality compared to the general population.[3,8] Therefore, novel approaches to modulating the immune system are needed.

Through millions of years of co-evolution with their hosts, helminths have developed the ability to modulate host immunity and helminth colonization used to be the default state for humans. The hygiene hypothesis states that in- creased sanitation and decreased exposure to infections, such as those by helminths, increases the risk for inflammatory diseases.[9,10] The acute phase of a helminth infection is characterized by an increased type 2 helper T cell (Th2) response and inhibition of type 1 and type 17 helper T cell responses (Th1 and Th17). Persisting helminth infection modifies the Th2

response in favor of a regulatory environment with expansion of regulatory monocytes, alter- natively activated macrophages, regulatory dendritic cells (DCs), regulatory T- and B-cells (Tregs and Bregs), B-class switching to IgG4, and increased levels of anti-inflammatory cytokines.[11–13]

Ascariasis is one of the most prevalent helminth infections worldwide.[11] Studies have found that chronic *Ascaris suum* infection downregulates mucosal inflammatory pathways in pigs in vivo.[14] Also, adult body fluid (ABF) from the body cavity of *A. suum*, suppresses the secretion of pro-inflammatory cytokines from primary human macro-phages and DCs in vitro and reduces the capacity of DCs to induce IFN-γ production in naive T-cells.[14,15] Midttun et al. found that modulation of TLR-driven pathways is a primary mechanism by which *A. suum* exerts its regulatory effects, a quality that could therapeutically benefit SLE patients.[4,15]

Helminths or their derived products have been shown to decrease pro-inflammatory responses and ameliorate symptoms in animal models of multiple sclerosis, type 1 diabetes, inflammatory bowel disease, and rheumatoid arthritis.[16,17] Murine models of SLE have shown attenuated disease development after administration of helminth products or helminth infection. Specifically, reductions were observed in atherosclerosis, splenomegaly, proteinuria, and glomerulonephritis.[18–21] Furthermore, these studies reported increased IL-10 and TGF-β levels and decreased IFN-γ and IL-17A levels from splenocytes, expansion of Tregs, decreased activation of B-cells and T follicular helper cells and decreased production of antinuclear antibodies.[18–21] Shemer, et al. found that administering a helminth-based compound was as efficient as methylprednisolone in impeding the development of a model of lupus nephritis in mice.[22] Still, the effect of ABF in patients with SLE remains unknown.

Here, we show that the helminth product ABF down- regulates inflammatory responses in peripheral blood mononuclear cells (PBMCs) from SLE patients stimulated with intra- and extra-cellular ligands that are often present in these patients.

Methods

Preparation of adult body fluid from Ascaris suum

Adult A. *suum* were collected at a local abattoir (DAT- Schaub, Herning, Denmark) and the ABF was obtained by tail puncturing and collecting the released fluid from the body cavity. The ABF was centrifuged at 2,000g for 10 min, followed by centrifugation of the supernatant at 10,000g for 15 min. The resulting supernatant was aliquoted and stored at -80°C. Before cell stimulation, the ABF protein concentration was determined using a BCA Assay (Pierce).

Study participants and ethics

This study was approved by the National Committee on Health Research Ethics, Central Denmark Region (registration number: 1-10-72-244-18). All SLE patients provided written informed consent. Blood samples were collected from SLE patients (*n* = 10) during outpatient routine checkup visits at the Department of Rheumatology, Aarhus University Hospital. Clinical and serologic data were collected from their medical records. The data were stored using REDCap (Research Electronic Data Capture).[23] Blood samples from anonymous age- and sex-matched healthy controls (HC, *n* = 12) were provided by the Blood Bank at Aarhus University Hospital.

Peripheral blood mononuclear cell isolation and stimulation

Peripheral blood mononuclear cells (PBMCs) from SLE patients and HCs were isolated by use of Ficoll-Paque (GE Healthcare) density gradient centrifugation. Immediately following isolation and cell counting, PBMCs were cry-opreserved ($LiqN_2$ vapor phase) in heat-inactivated fetal bovine serum (FBS) containing 10% DMSO.

PBMCs were thawed, plated at a density of 1×10^6 cells/ mL in complete RPMI (Roswell Park Memorial Institute (RPMI) 1640 medium (Biowest) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin) and incubated in 5% CO2 at 37°C. After 24 h, the cells were stimulated with either 150 µg/mL ABF (final concentration) or an equal volume of cRPMI (see Figure 1). After one hour of incubation, PBMCs were further stimulated with different TLR agonists separately (final concentrations are reported here); TLR3 agonist: 50 ug/mL polyinosinic-polycytidylic acid sodium salt (poly(I:C), Sigma- Aldrich), TLR4 agonist: 10 ng/mL lipopolysaccharide (LPS, Sigma-Aldrich), TLR7 agonist: 1 ug/mL Gardiqui- mod (Sigma-Aldrich), TLR8

agonist: 1.5 ug/mL ssRNA40/ LyoVec (Invivogen) and TLR9 agonist: 0.25 µM CpG ODN2395 (Invivogen). Other PBMCs were transfected with Lipofectamine and either 0.1 ug/well deoxyribonucleic acid sodium salt from herring testes (HT-DNA, Sigma- Aldrich) or 0.1 ug/well High Molecular Weight (HMW) poly(I:C) (Invivogen). HT-DNA and HMW poly (I:C) activate the stimulator of interferon genes (STING) pathway and the mitochondrial antiviral signaling protein (MAVS) pathway, respectively. Lipofectamine alone and a CpG ODN2395 negative control (Invivogen) were also included. The cell-free supernatants were harvested after 24h of incubation and stored at -20°C until further analysis. PrestoBlue Cell Viability Reagent (Thermo Fischer Scientific) was used to evaluate the effect of ABF on the vi- ability of the PBMCs.

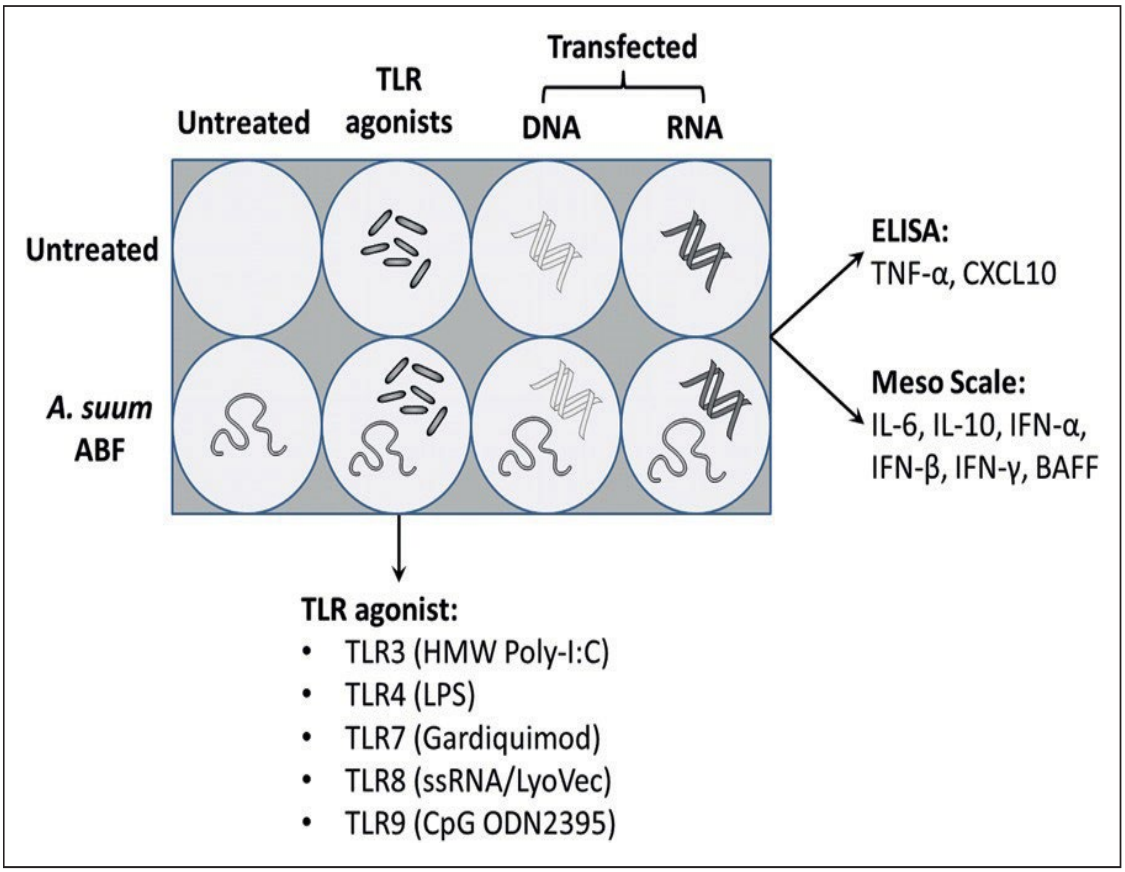

Figure 1. Peripheral blood mononuclear cells (PBMCs) were isolated from SLE patients or healthy controls. Cells were stimulated with or without *A. suum* adult body fluid (ABF) and with or without toll-like receptor (TLR) agonist (each TLR agonist separately) or transfected DNA or RNA. Secreted cytokines were quantified in culture supernatants after 24 hours of incubation using ELISA or Meso Scale. BAFF, B-cell activating factor; HMW Poly-I:C, high molecular weight polyinosinic-polycytidylic acid sodium salt; LPS, lipopolysaccharide.

Cytokine quantification

Levels of tumor necrosis factor-α (TNF-α) and C-X-C motif chemokine ligand 10 (CXCL10) were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) and read with a FLUOstar Omega Microplate Reader (BMG Labtech). Levels of interleukin-6 (IL-6), IL-10, B-cell activating factor (BAFF), interferon-α (IFN-α), IFN-β, and IFN-γ were measured using an electrochemiluminescent U-PLEX Metabolic Group 1 Assay (Meso Scale Discovery (MSD)) and read with a MESO QuickPlex SQ 120 Reader (MSD). All cytokine quantifications were performed according to the respective manufacturer's instructions.

Statistical analysis

Seven participants were the minimum number required for us to observe a 30% reduction (from 500 pg/mL +/- 95 pg/mL SD; alpha = 0.05) in TLR4 agonist-induced IL-6 at a power of 0.8. Data was analyzed using GraphPad Prism 8.0.0. Shapiro– Wilk normality and log-normality test was used to test for normality. As data were not normally distributed, non-parametric tests were used for all statistical analyses. Wilcoxon signed-rank test was used to analyze the effect of ABF on SLE-derived and HC-derived cells while Mann–Whitney U test was used to compare the effect of the different agonist on SLE-derived versus HC-derived cells. Mann–Whitney U test was also used to compare the age between the SLE and the HC group.

Results

Study population

Clinical data from the SLE patients and HCs are summarized in Table 1. At the time of sampling, 60% of the patients had increased anti-dsDNA antibody and lymphocytopenia (lymphocytes $<1.3 \times 10^9$/L). 60% received Prednisolone and 60% received other immunosuppressive medication, for example Hydroxychloroquine (30%), Methotrexate (20%), and Belimumab (10%).

Table 1. Characteristics of Systemic Lupus Erythematosus patients and healthy controls (HC).

Characteristics and clinical manifestations	SLE (n = 10)[a]	HC (n = 12)[b]
Age (years)	61.5 (54.5–65)	45.0 (38.0–62.3), *n.s.*[c]
Gender (female)	9 (90)	12 (100)
Duration of disease (years)	12.0 (5.8–23.0)	
SLEDAI-2K	4.0 (1.5–8.5)	
Prednisolone (mg/day)	2.5 (0.0–7.5)	
Other immunosuppressive	6 (60)	
Mb Sjo¨gren	2 (20)	
ANA positive	10 (100)	
dsDNA Ab (IU/mL)	16.5 (2.1–86.3)	
Hemoglobin (mmol/L) White blood	7.9 (7.4–8.6)	
cells (x 10^9/L) Lymphocytes (x 10^9/L)	5.4 (3.3–6.6)	
Platelets (x 10^9/L)	1.2 (0.7–1.6)	
	234 (167–279)	
P-Creatinine (μmol/L)	62.5 (49.5–68.3)	
CRP (mg/L)	4.0 (2.8–16.9)	
C3c (g/L)	0.8 (0.6–1.1)	
C4 (g/L)	0.07 (0.01–0.11)	
Rash	4 (40)	
Arthritis	3 (30)	
Mucositis	1 (10)	
Nephritis	1 (10)	
Serositis	0 (0)	

Data are presented as n (%) or median (IQR).
Ab: antibody; ANA: Antinuclear Antibodies; CRP: C-reactive protein; dsDNA: double-stranded DNA; SLE: systemic lupus erythematosus.
[a]SLE data represents the patients at the time of the blood sample.
[b]As we did not have information on the age of two of the HCs, they are not represented in this table.
[c]p = 0.11.

The effect of adult body fluid on cellular viability

The first thing we wanted to do was ensure that ABF alone did not impact the viability of the PBMCs. To test this, we performed a coincubation of PBMCs from HCs with ABF, as shown in Figure 1, but without any further stimulus. We observed that ABF did not negatively affect the viability of the PBMCs as measured by PrestoBlue Cell Viability Reagent (p = .45; n = 5).

Adult body fluid suppresses the secretion of cytokines following TLR3 and -4

stimulation

Following activation of the TLR3 and TLR4 pathways, the effect of ABF showed similar patterns. Specifically, TNF-α, IL-6, and IL-10 were all significantly reduced for both SLE- and HC-derived PBMCs. However, while CXCL10 levels were reduced in the cultures with PBMCs from HCs, this was not the case in cultures containing PBMCs derived from SLE patients (Figure 2 presents TLR3 agonist results and Supplementary Figure 1 presents TLR4 agonist results). TLR3 and -4 induced IFN-γ secretion by the HC-derived PBMCs was significantly suppressed by ABF in the case of TLR3 stimulation.

Adult body fluid has a mixed effect on cytokine production following TLR7, -8, and -9 stimulation

The effect of ABF on the TLR7 and TLR8 pathways also showed similarities. For both SLE and HC, the ABF significantly reduced secretion of TNF-α by PBMCs, while IL-6 and IFN-γ were not affected (Figure 3 presents TLR7 agonist results and Supplementary Figure 2 presents TLR8 agonist results). The presence of ABF was associated with an increased level of IL-10 production when PBMCs from SLE patients and HCs were stimulated with the TLR7 agonist, while production of IL-10 was suppressed in HC-derived PBMCs following activation of the TLR8 pathway. ABF increased IFN-α for both pathways in HC PBMCs. In contrast, ABF decreased CXCL10 production by HC-derived PBMCs following TLR7- and TLR8 stimulation as well as by SLE-derived PBMCs following TLR8 stimulation. When stimulating TLR9, ABF increased levels of IFN-α for the HC group, while secretion of CXCL10 was not affected (Figure 4). Cytokine levels were very low for SLE patients.

Adult body fluid decreases secretion of cytokines following stimulator of interferon genes and mitochondrial antiviral signaling protein stimulation

TLRs are not the only nucleic acid receptors present in innate immune cells. STING and MAVS are intracellular sensors which are activated by transfection with HT-DNA and HMW poly(I:C).[24–27] These pathways are also associated with SLE pathogenesis.[7,28] We therefore sought to assess the impact of ABF on the production of cytokines following stimulation of these pathways. We found that ABF reduced the

secretion of CXCL10 for both SLE- and HC-derived PBMCs following activation of the STING and MAVS pathways. In contrast, ABF exerted a suppressive effect only on the SLE-derived PBMCs production of IFN-α following activation of the MAVS pathway (Figure 4).

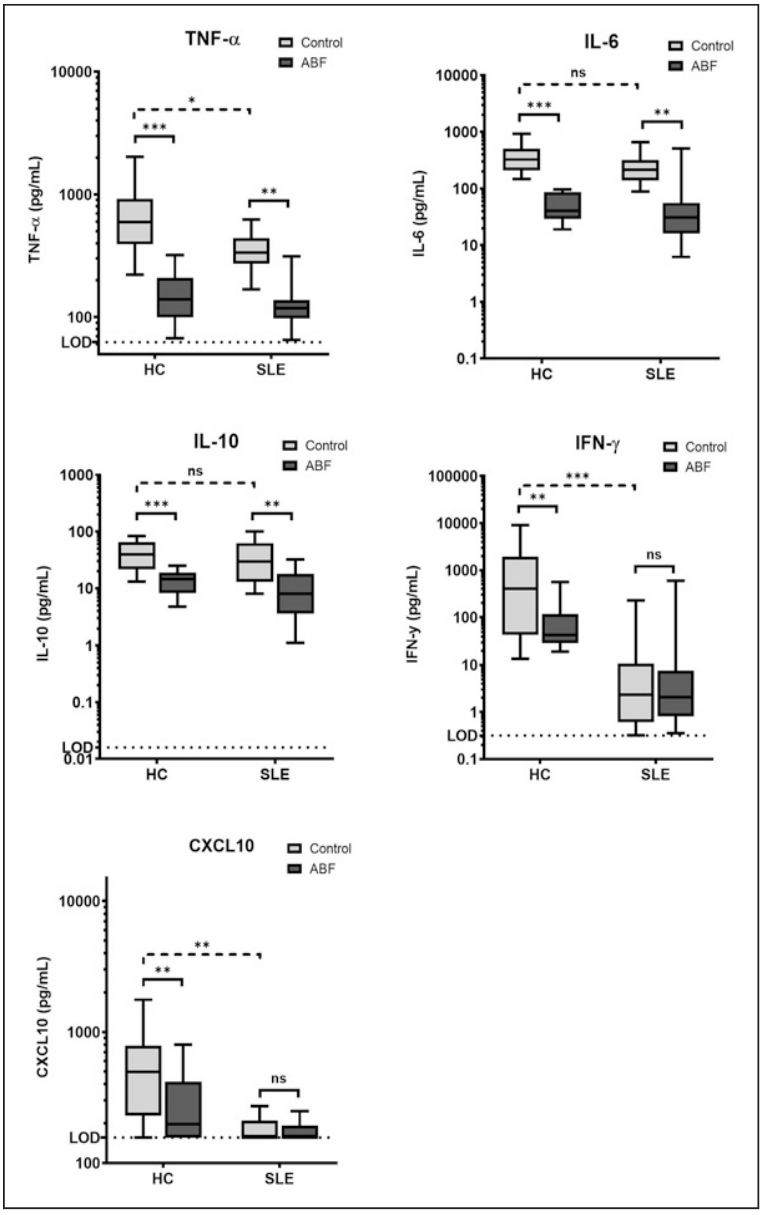

Figure 2. Effect of *Ascaris suum* adult body fluid (ABF) on cytokines induced by TLR3 agonist in healthy control (HC) and SLE-derived peripheral blood mononuclear cells. The box chart indicates the 25th percentile, median, and 75th percentile and the whiskers represent 10th–90th percentile. HC: n = 12, SLE: n = 10. Wilcoxon signed-rank test (solid lines) and Mann–Whitney U test (dashed lines) was used. *p < 0.05; **p < 0.01; ***p < 0.001. LOD, limit of detection.

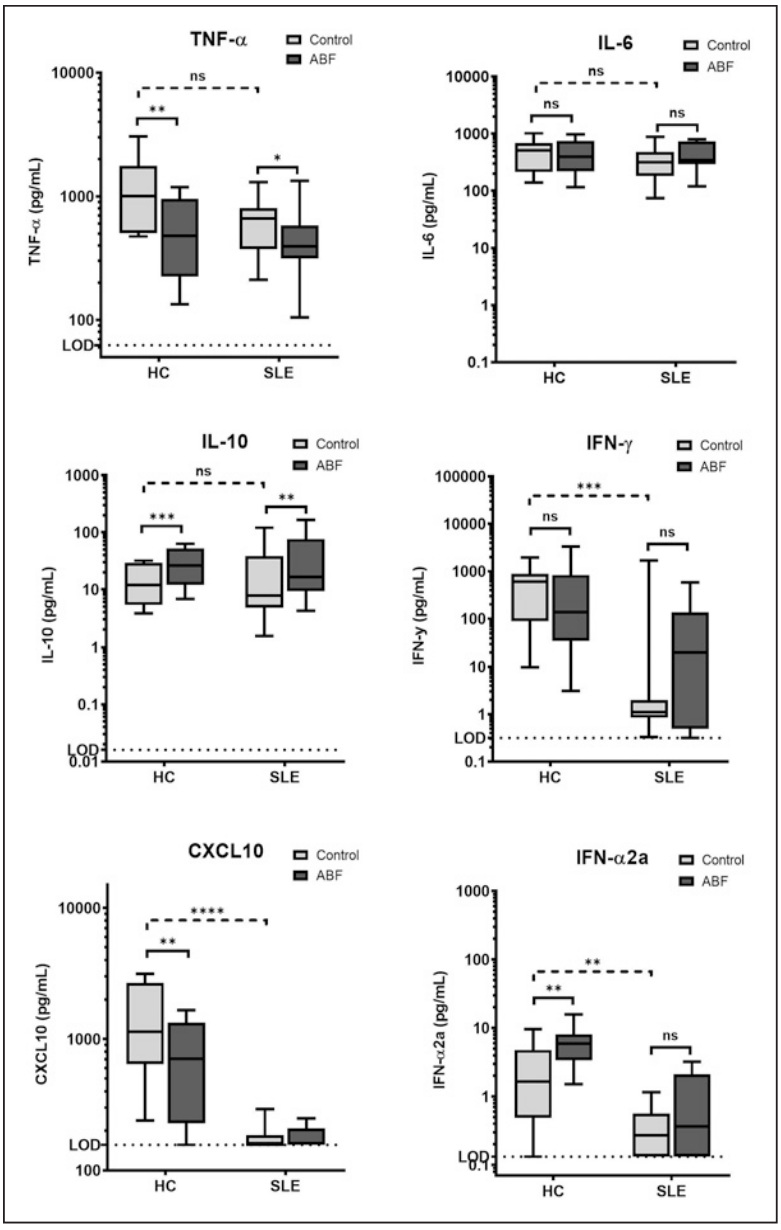

Figure 3. Effect of *Ascaris suum* adult body fluid (ABF) on cytokines induced by TLR7 agonist in healthy control (HC) and SLE-derived peripheral blood mononuclear cells. The box chart indicates the 25th percentile, median, and 75th percentile and the whiskers represent 10th–90th percentile. HC: n = 12, SLE: n = 10. Wilcoxon signed-rank test (solid lines) and Mann–Whitney U test (dashed lines) was used. *p < 0.05; **p < 0.01; ***p < 0.001. LOD, limit of detection.

BAFF levels were not affected by agonists or adult body fluid

None of the agonists we used induced a measurable increase of BAFF when compared to the untreated cells. Also, the ABF did not have any effect on the levels of BAFF (data not shown).

Discussion

In this study, we examined the effect of ABF from *Ascaris* on inflammatory responses from SLE-derived PBMCs stimulated with the endosomal receptors TLR3, -7, -8, and -9 and the cytosolic receptors upstream of STING and MAVS; receptors, that are all described to be associated with the lupus pathogenesis.[4,5,7,28,29] ABF was able to modulate cytokine secretion from the SLE-derived PBMCs by up- and downregulating several cytokines that are central to SLE pathogenesis.

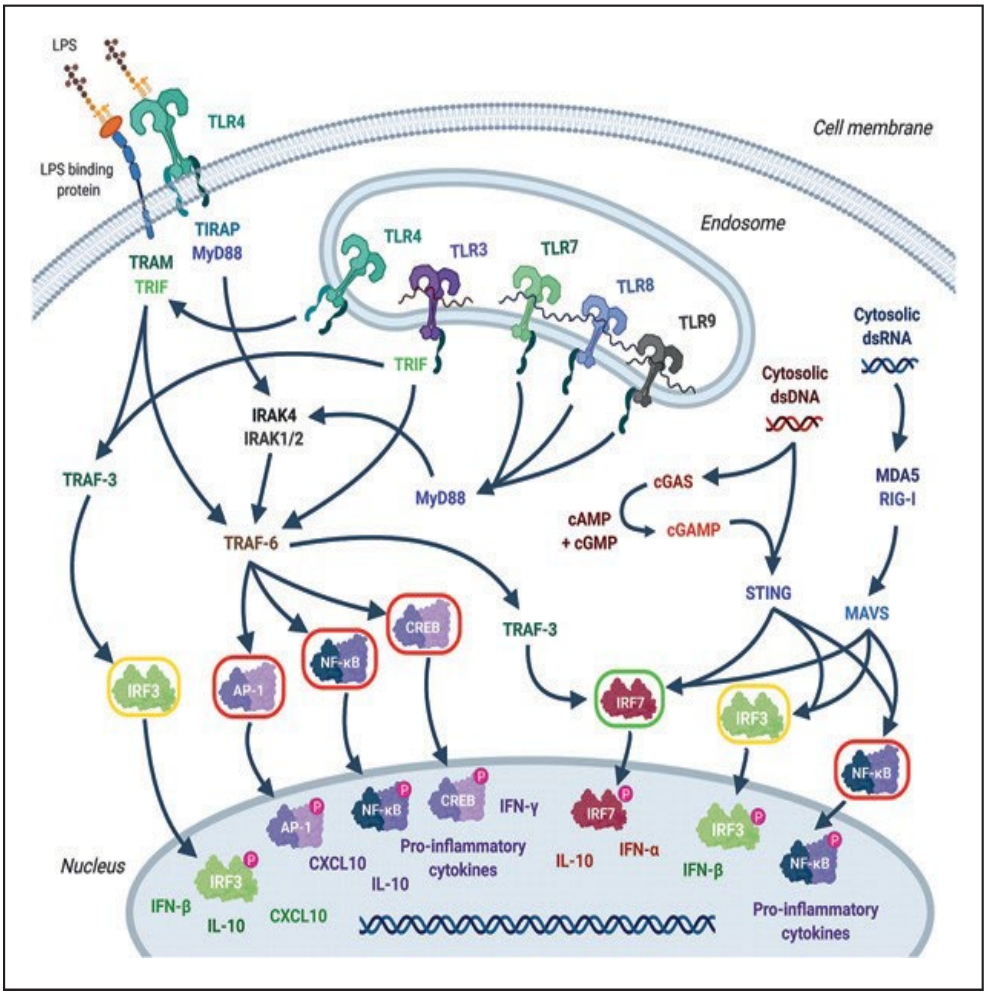

Figure 4. Effect of *Ascaris suum* adult body fluid (ABF) on cytokines induced by agonists of the TLR9 (a), stimulator of interferon genes (b), and mitochondrial antiviral signaling protein (c) pathways in healthy control (HC) and SLE-derived peripheral blood mononuclear cells. The box chart indicates the 25th percentile, median, and 75th percentile and the whiskers represent 10th–90th percentile. HC: n = 12, SLE: n = 10. Wilcoxon signed-rank test (solid lines) and Mann–Whitney U test (dashed lines) was used.
*p < 0.05; **p < 0.01; ***p < 0.001. LOD, limit of detection.

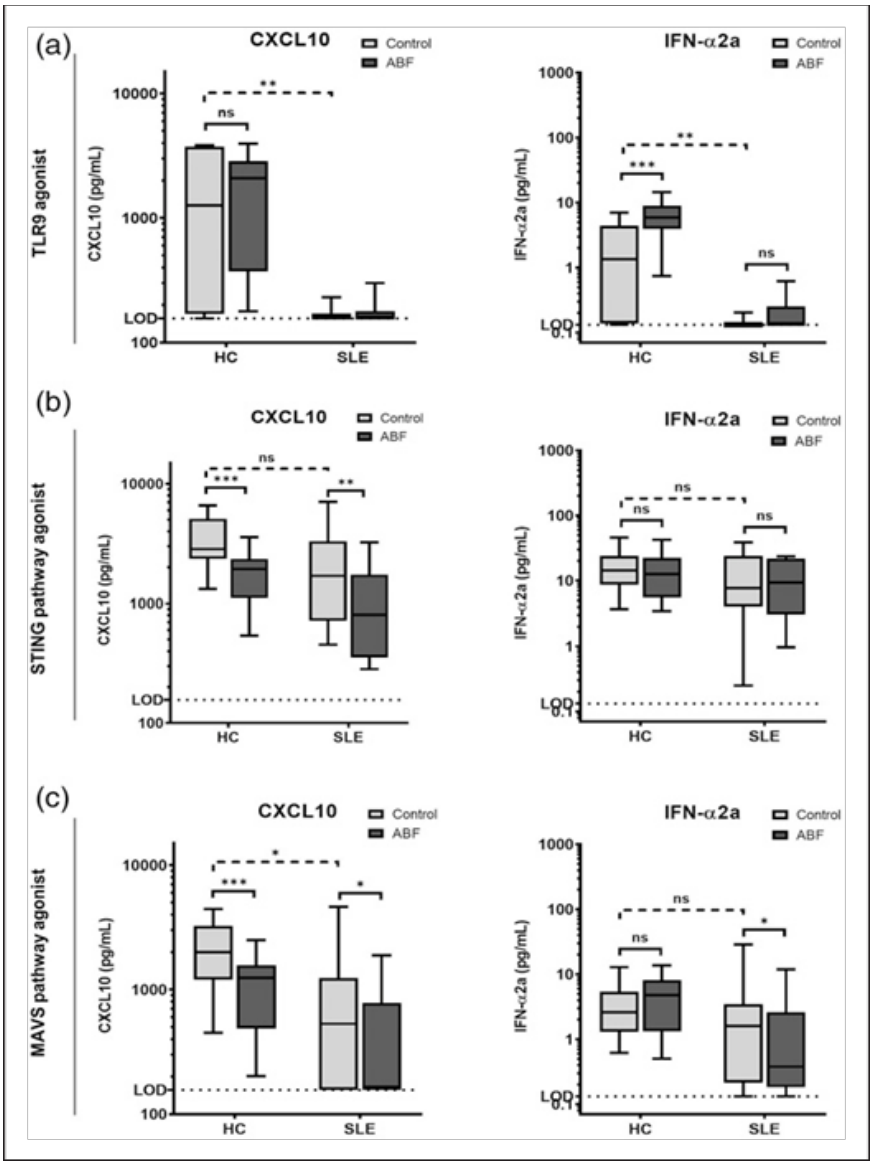

Figure 5. Model showing relevant intracellular pathways and the expected effect of *Ascaris suum* adult body fluid (ABF). Based on our data, we marked proteins we expect to be downregulated by the ABF with a red ring, proteins we expect to be upregulated with a green ring and proteins that we could not conclude on with a yellow ring (see discussion). This figure is based on figures and text from Brown et al. 33, Devarapu et al. 4, Blasius et al. 34, Kato et al. 28, and Motwani et al. 24. Adapted from “TLR4/5/7/8 Signaling Cascade” by BioRender.com (2019). Retrieved from https://app.biorender.com/biorender-templates. AP-1, activator protein 1; CREB, cAMP response element-binding protein; IRAK, interleukin-1 receptor-associated kinase; IRF, interferon regulatory factor; MAVS, Mitochondrial antiviral signaling protein; MyD88, myeloid differentiation factor 88; NF-κB, Nuclear Factor-κB; STING, stimulator of interferon genes; TIR, Toll- interleukin 1 receptor; TIRAP, TIR-domain-containing adapter protein; TRAF, TNF receptor-associated factor; TRAM, TRIF- related adaptor molecule; TRIF, TIR-domain-containing adapter- inducing interferon-β.

All evaluated cytokines have earlier been shown to be increased in SLE patient serum and are known to correlate with disease activity.[30–32] Yet, while the effect of ABF on the cytokine responses was conspicuous for both SLE-derived and HC-derived PBMCs, the effects were generally more pronounced for HC-derived cells. Overall, the different agonists induced a higher cytokine response in HC-derived PBMCs compared to cells from SLE patients. This difference may be explained by most of the SLE patients receiving immunosuppressive medication. However, even though patient cells were immunologically suppressed, their pro- duction of pro-inflammatory cytokines such as TNF-α, IL-6, IFN-α, and the interferon signature biomarker CXCL10 could still be further reduced by the ABF.

TLR3 and TLR4 share both common and distinct intracellular pathways (Figure 5). They both feed through the adaptor protein TIR-domain-containing adapter-inducing interferon-β (TRIF), which interacts with either TNF receptor-associated factor-3 (TRAF-3) for production of mainly type 1 IFN and CXCL10 or TRAF-6 for production of various pro-inflammatory cytokines.[4,33–35] As the TLR3 and -4 stimulations did not induce any type 1 IFN (data not shown), the production and suppression of CXCL10 must derive from either the TRAF-6/NF-κB pathway or indirect via TRAF-6/NF-κB induced IFN-u. IL-10 levels may also reflect stimulation of TRAF-6/NF-κB and/or TRAF-3/ IRF3 or reflect levels of, for example, IL-6, which stimulates the production of IL-10 as a regulatory negative feedback loop. TLR4 also feeds through MyD88, while TLR3 is a MyD88 independent pathway. The MyD88 pathway merges downstream with the TRIF pathway when activating TRAF-6.[4,33] Thus, as there is similar suppressive effect of ABF on both TLR3 and -4 induced pro- duction of TNF-α, IL-6, IL-10, IFN-u, and CXCL10, we hypothesize that the ABF does not exert its effect on MyD88, IRAKs, or TRIF, but more likely suppresses a protein in the common pathway from TRAF6 and downstream. Alternatively, ABF affects several molecules in the pathways with distinct functions.

The ABF also had a similar effect on TLR7 and TLR8 stimulation. These TLRs both signal via the MyD88 de- pendent pathway and activate either NF-κB/AP-1/CREB for induction of pro-inflammatory cytokines or IRF7 for pro- duction of IFN-α (Figure 5).[33] Interestingly, for PBMCs from both SLE patients and HCs the IL-6 and IFN-γ pro-duction were not suppressed by the ABF for these TLRs while the TNF-α and CXCL10

production were, except for SLE-derived PBMCs following activation of the TLR7 pathway, probably due to a very low agonist response. This confirms the hypothesis described above; that the ABF does not target upstream of MyD88 and IRAKs, but somewhere in the pathway downstream of TRAF-6. It also excludes TRAF-6 as a potential target for the ABF. The fact that TNF- α and CXCL10 were suppressed by the ABF while IL-6 and IFN-γ were not suggest that ABF does not affect the pathways downstream of TRAF-6 that induces IL-6 and IFN-γ production.

It is interesting that IL-10 increases when the TLR7 pathway is stimulated with ABF. This pattern is similar to the increase of IFN-α following TLR7 and -8 stimulation. Both of these cytokines can be induced by the pathway activating IRF7,[35] suggesting that proteins in this path- way are induced by the ABF. As MyD88, IRAKS, TRAF6, and TRAF3 are also involved in other pathways that seem to be suppressed, it is unlikely to be these proteins, but IRF7 could be a potential candidate for this inducing effect.

TLR9 stimulation activates a downstream cascade similar to TLR7 and -8, but no or low cytokine responses were observed. The low responses are probably due to low numbers of the TLR9-carrying pDCs in the PBMCs due to the freeze/thaw procedure.[36] However, some production of IFN-α and CXCL10 was induced. The increase of IFN-α is possibly due to the same mechanism as suggested for TLR7 and -8 with induction of IRF7.

Stimulation of STING and MAVS activates IRFs, primarily IRF3, and NF-κB (Figure 5).[24,28] In this study, the stimulation of STING and MAVS mainly induced interferons and CXCL10, indicating that the IRF pathways were the most activated. It is peculiar that ABF significantly suppresses CXCL10 for both STING and MAVS stimulation, while IFN-α levels were mostly unaffected. This suggests that ABF does not have an effect directly on STING or MAVS, but rather a complex effect on downstream proteins, for instance IRF3 and IRF7 as dis- cussed earlier.

As the worm product was added before agonist stimulation, the ABF may prevent the agonists from entering the cells, thereby giving rise to the observed suppressive effects. However, as ABF had both suppressive and stimulatory effects on different cytokines stimulated from the same TLR, for example TLR7, this suggests that the ABF exerts its effect on several intracellular pathways.

SLE is related to a dysregulated Th1/Th2 balance involving hyperfunction of Th2 cells which is why it could seem contradictory to consider helminths as treatment for this disease. However, a complex and powerful regulatory response is also initiated by helminths and our results indicate that an overall regulatory stimulus can prevail over the inflammatory ones. This is in line with the protective effect of helminths previously demonstrated in animal models of SLE.[18–22] This potential clinical benefit may partially be mediated by the induction of Tregs which are in low abundance in patients with active SLE.[37] Furthermore, helminth induced B-cell class switching to IgG4 may also be clinically beneficial as this antibody isotype has recently been correlated with attenuated SLE progression.[38]

In conclusion, our study suggests that the immuno- modulatory effect of ABF from *Ascaris suum* on SLE- derived PBMCs primarily consists of suppression of central pro-inflammatory cytokines. The SLE-derived PBMCs are already somewhat dysregulated and affected by immunosuppressive medication, yet, the ABF has an additive anti-inflammatory effect, which potentially can be ultilized therapeutically. This add-on effect was shown following stimulation with different intra- and extracellular DNA and RNA ligands mimicking endogenous stimulation of TLRs and cytosolic receptors.

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Declaration of conflicting interests

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Supplemental Material

Supplemental material for this article is available online.

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