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MTADV 5-MER peptide suppresses chronic inflammations as well as autoimmune pathologies and unveils a new potential target-Serum Amyloid A

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ABSTRACT

Despite the existence of potent anti-inflammatory biological drugs e.g., anti-TNF and anti IL-6 receptor antibodies, for treating chronic inflammatory and autoimmune diseases, these are costly and not specific. Cheaper oral available drugs remain an unmet need. Expression of the acute phase protein Serum Amyloid A (SAA) is dependent on release of pro-inflammatory cytokines IL-1, IL-6 and TNF-α during inflammation. Conversely, SAA induces pro-inflammatory cytokine secretion, including Th17, leading to a pathogenic vicious cycle and chronic inflammation. 5- MER peptide (5-MP) MTADV (methionine-threonine-alanine-aspartic acid-valine), also called Amilo-5MER, was originally derived from a sequence of a pro-inflammatory CD44 variant isolated from synovial fluid of a Rheumatoid Arthritis (RA) patient. This human peptide displays an efficient anti-inflammatory effects to ameliorate pathology and clinical symptoms in mouse models of RA, Inflammatory Bowel Disease (IBD) and Multiple Sclerosis (MS). Bioinformatics and qRT-PCR revealed that 5-MP, administrated to encephalomyelytic mice, up-regulates genes contributing to chronic inflammation resistance. Mass spectrometry of proteins that were pulled down from an RA synovial cell extract with biotinylated 5-MP, showed that it binds SAA. 5-MP disrupted SAA assembly, which is correlated with its pro-inflammatory activity. The peptide MTADV (but not scrambled TMVAD) significantly inhibited the release of pro-inflammatory cytokines IL-6 and IL-1β from SAA-activated human fibroblasts, THP-1 monocytes and peripheral blood mononuclear cells. 5-MP suppresses the pro-inflammatory IL-6 release from SAA-activated cells, but not from non-activated cells. 5-MP could not display therapeutic activity in rats, which are SAA deficient, but does inhibit inflammations in animal models of IBD and MS, both are SAA-dependent, as shown by others in SAA knockout mice. In conclusion, 5-MP suppresses chronic inflammation in animal models of RA, IBD and MS, which are SAA-dependent, but not in animal models, which are SAA-independent.

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1. Introduction

Serum Amyloid A (SAA)1 and SAA2 are most prominently induced in liver during sepsis and/or inflammation, with concentrations in serum increased by as much as a 1000-fold over basal levels [1–6]. The secreted proteins form hexamers and monomers, the last are associated with serum with high density lipoprotein (HDL) and are thought to be involved in the role of HDL in maintenance of lipid homeostasis [7]. SAA can substitute for TGF-β in the induction of Th17 cells yet, it engages a distinct signaling pathway that results with a pro-inflammatory differentiation program [8]. Therefore, SAA contributes *in vivo* to Th17-mediated pathogenesis, revealed in inflammatory bowel disease (IBD) and experimental autoimmune encephalomyelitis (EAE) by loss- and gain-of function models. Collectively, SAA contributes selectively to Th17 cell functions *in vivo* and suggest strategies for therapeutic modulation in Th17-mediated inflammatory disease [8].

In an earlier study we reported that a unique variant of human CD44 (called CD44vRA; [9]) is found in cells from the inflamed joints of rheumatoid arthritis (RA) and psoriatic arthritis patients. The CD44vRA variant contains the amino acid sequence methionine-threonine-alanine-aspartic acid, valine (MTADV) at the splicing junction between variant exons 4 and 5 [9]. This differs from the parental CD44 variant (CD44v3-v10), which contains the same CD44vRA sequence, but lacks alanine in the corresponding splicing junction, and instead contains the MTDV at the same site. This small difference generates a substantial change, because cells expressing CD44vRA stimulate a significant growth enhancement of inflammatory cells, whereas cells expressing CD44v3-v10 do not [9]. Furthermore, the sequence MTADV is extremely rare in proteins. A NCBI BLAST RefSeq protein sequence database, that contains 81,347 human protein sequences, reveals that only one protein, Isoleucyl-tRNA synthetase, shares with CD44vRA the MTADV sequence and, surprisingly, is also involved in rheumatoid diseases [10]. We proposed, therefore, that the MTADV sequence is associated with pro-inflammatory activities such as cell growth. Indeed, anti-CD44vRA monoclonal antibodies (mAbs) attenuated the joint inflammation in collagen-induced arthritis (CIA), the mouse model of RA [11]. Therefore, we suggested that MTADV sequence plays a pivotal role in RA and possibly in other chronic inflammations. If so, a synthetic MTADV 5-MER peptide (5-MP) could potentially provide therapeutic benefit by competing on a shared target with the built-in MTADV sequence of CD44vRA, thus reducing its pathological activity. While this hypothesis seeks an experimental evidence, we independently found that the 5-MP (called also Amylo-5MER) interferes with SAA assembly and bioactivity e.g., enhancing release of pro-inflammatory cytokines. Moreover, we found that the peptide reduced the pathological activities in mouse models of RA, Inflammatory Bowel Disease (IBD) and Multiple Sclerosis (MS) when delivered by IP/SC injection or oral administration. The pathology of these maladies, as well as other chronic inflammations, is associated with SAA [1–6, 12–14]. The 5-MP (MTADV) thus provides a novel spear to combat chronic inflammation and autoimmunity, possibly linked to its ability to target SAA.

2. Materials and methods

2.1. Peptides

The 5-MP (MTADV) as well as 7-MP (RMTADVD) and 9-MP (TRMTADVDR) were produced by Sigma-Aldrich Rehovot, Israel and Sigma-Aldrich Woodlands, TX, USA (>95% purity). 5-MP and the scrambled peptide (TMVAD) were produced by PepTech Corp. Burlington, MA, USA (>98% purity) as well. The peptides were acetylated at the N-terminus and amidated at the C-terminus by the manufacturers to improve stability. The same capped- peptides were used for *in vitro* experiments (except in the experiments described in Fig. 4, in which non-capped MTADV was used).

2.2. Induction of collagen-induced arthritis (CIA)

2.2.1. Mice

Generation of CIA in C57BL/6 mice and assessment of inflammation by measuring footpad swelling or volume using plethtysmometer (Ugo Basile, Gemonio, VA Italy) were performed as described previously [11, 15]; type II collagen is a gift from Dr Richard O. Williams, The Kennedy Institute of Rheumatology, University of Oxford, UK). PBS and 5-MP at a dose of 3.5 mg/kg (about 70 μg peptide/injection/mouse), or as otherwise indicated, were IP injected at onset of disease (when the footpad swelling measurement was >1.7 mm, designated day 0). 5-MP at a dose of 3–3.5 mg/kg (about 70 μg peptide/injection/mouse), was standardly used in all *in vivo* experiments described in this article, unless otherwise indicated. Then, the peptide was given every day for 10 days and the footpad swelling measurements were followed on the same dates. Measurements were count when one or both (average was recorded) hind footpads showed increment (Δ) over the measurement of day 0. When the second hind footpad did not show increased volume on the same day as the first footpad, it was excluded from results if appeared later. Yet, the second hind footpad measurement was included in CIA clinical score (Fig. 1A). CIA scoring as follows: 0- Normal, no signs of involvement; **1**- Swelling and redness of one joint; redness, red spots on paw; **2**- Two joints involved, partial swelling, difficulties with stretching paw-limping; **3**- More than two joints involved in swelling, no loading on paw; **4**- Severe arthritis in the entire paw; **5-** Maximally swollen paw. The institute's Animal Ethics Committee (MD-12-13460-4) approved this protocol.

Ten arthritic mice, subjected to the 5-MP (5 mice) or PBS (5 mice), were killed at the end of the standard experiment (10 days after the first peptide injection). Their joints were removed, fixed with formaldehyde and sent under blind manner to a pathological laboratory (LEM laboratories, Science Park, Rehovot, Israel) to prepare joint sections and staining with Hematoxylin-Eosin. The pathologist's histological evaluation criteria (under blind manner): **0**- Normal; no signs of involvement; **1**- Cartilage ulcerations mild diffuse inflammatory infiltrates and minimal focal; **2**- Moderate inflammatory infiltrates and mild focal or minimal multifocal cartilage ulcerations; **3**- Marked inflammatory infiltrates and marked multifocal cartilage ulcerations; **4**- Severe inflammation with necrosis and edema and severe extensive areas of cartilage ulcerations.

2.2.2. Rats

Bovine Type II Collagen (CII; MDBiosciencies, Oakdale, MN, USA) in emulsion (CII/IFA, 2 mg/ml) was subcutaneously injected into the base of the tail (400 μg CII/rat) of Lewis female rats (Janvier Labs, Le Genest-Saint-Isle, Franch). At day 7 the hind paw volumes (basal data) were quantified using a digital water plethysmometer (Ugo-Basile). At days 12–27 5-MP was daily administered at the different dosages (0.0008, 0.004, 0.02, 0.1, 0.12, 0.6, 3 and 15 mg/kg) by SC injection (5 ml/kg). Dexamethasone (0.1 mg/kg dissolved in 0.1% Tween-80 + 99% CMC; 0.5% w/v in water at a concentration of 0.01 mg/ml) was administered by oral gavage (10 ml/kg). Arthritis progression at days 12, 14, 18, 21, 24 and 27 was followed by measuring the volume of hind paws. The protocol was approved by the Animal Experimentation Commission of the Generalitat of Catalunya (DAAM: 8822).

2.3. Generation of TNBS-induced IBD

C57BL/6J (21–25g) male mice (Charles River's laboratories, France) received at day 0 an intra-rectal administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Sigma Aldrich) 40 μl at 150 mg/kg; dissolved in a 1:1 mixture of 0.9% NaCl with 100% ethanol or the vehicle only (control). 5-MP at two doses (3 mg/kg and 15 mg/kg) was daily SC injected, starting 5 days before TNBS administration (day 0) up-to one day after TNBS administration. On completion of the experiment, animals were euthanized on day 2 by cervical dislocation. Anti-TNF antibody (0.1 mg/

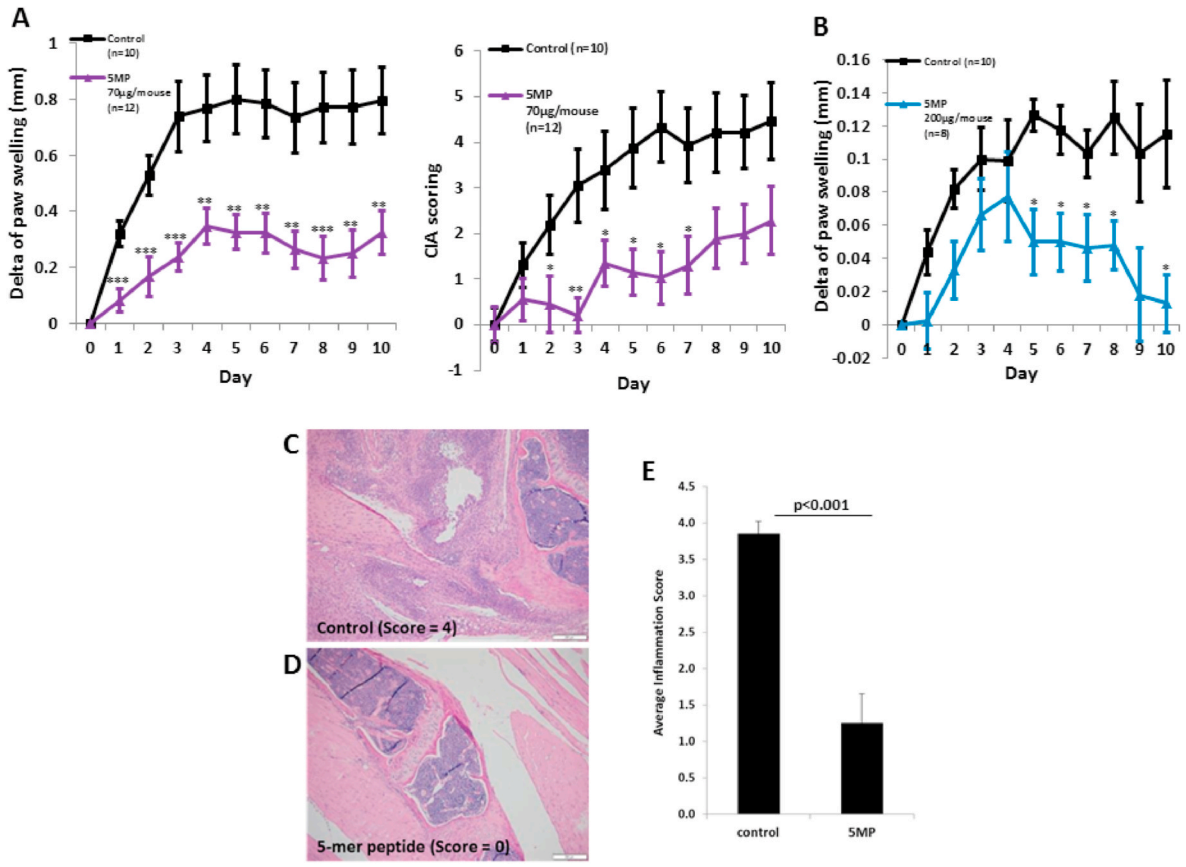

Fig. 1. (A and B) 5-MP inhibits joint inflammation in mice with CIA. C57BL/6 mice were IP administrated at onset of CIA with PBS or with 3.5 mg/kg (~70 μg/mouse; **A** and supplementary **1A**) or 10 mg/kg (~200 μg/mouse; **B**) and then every day for the next 10 days. The daily increase of joint inflammation was measured by changes in footpad swelling, using a micro-caliper (Δ). Tree different measurement assays, footpad swelling (**A**, left panel), footpad volume, using plethysmometer (Supplementary Fig. 1A) and CIA scoring (see M&M; **A**, right panel), that were used in the same experiment, demonstrate similar results, indicating the reproducibility of our data. Results shown by±SEM. Statistical analysis by two-tailed Student's t-test equal variance, analyzing the differences between the groups at each time point,*p < 0.05, **p < 0.01, ***p < 0.001. Altogether, the peptide therapeutic effect was detected in 7 independent experiments using the standard CIA protocol, but employing two different mouse strains (DBA/1 and C57BL/6) and two different routes of peptide administration (IP and SC). (**C to E)** Histopathological analysis. Intraperitoneally injected 5-MP protects against damage mediated by invading inflammatory cells. Joint sections from arthritic mice treated with PBS (**C**) or 5-MP (**D**) were stained with Hematoxylin-Eosin and evaluated under blind manner by a pathologist. The average (+/− SEM) inflammation intensity (**E**) is scored, as indicated in M&M (N = 5 for both groups). P < 0.001 by two-tailed Student's t-test equal variance. 5 MP = 5-MER peptide.

mouse; lot B218621 Biolegend, San Diego, CA, USA) was IP injected at day 3 and day 0, as positive control. Histopathological Ameho's scores [16] was used to evaluate the histochemical pathology at the end of the experiment, employing hematoxylin-Eosin staining: **0**- No alterations; **1**- Middle mucosal and/or submucosal inflammatory infiltrates with edema. Few mucosal erosions. Integrity of muscularis mucosa; **2**- Same criteria as score 1, but >50% of the section; **3**- Large inflammatory infiltrate with ulcerations area through all the colonic wall; **4**- Same criteria as score 3, but >50% of the section; **5**- Wide ulcerations with cellular necrosis; **6**- Same criteria as score 5, but >50% of the section. The protocol was approved by the by Institute Pasteur in Lille (License n° B59-35009).

2.4. Induction of EAE

EAE was generated in C57BL/6 (Supplementary Fig. 7) or SJL (Fig. 7) mice with an emulsion containing 200 μg of MOG35–55 (Supplementary Fig. 7) or PLP (Fig. 7A and B) (both from Sigma-Aldrich, Rehovot, Israel) as described in Ref. [17]. 5-MP was orally delivered by Gavage five days after EAE induction and then every day, as indicated in the figures, at peptide concentration of 35 mg/kg (x10 higher concentration than IP/SC injection in CIA mouse model). Mice were observed daily for the appearance of neurological symptoms, using the following evaluation score: **0**- asymptomatic; **1**- partial loss of tail tonicity; 1.5, limp tail; **2**- hind limb weakness (right reflex); **3**- ataxia; **4**- early paralysis; and **5**- full paralysis. The protocol was approved by the institute's Animal Ethics Committee (MD-17-15375-5).

2.5. Western Blot (AB) analysis

For the WB analysis and for all *in vitro* assays we used Recombinant Human Apo SAA1 from PeproTech, Parkway Short Hills, NJ, USA. This Apo SAA1 is a non-glycosylated protein with at least 98% purity, contains less than 0.1 ng/μg Endotoxin and reconstituted in water to 1.0 mg/ml. A quantity of 100 μg of human SAA1, diluted in medium without serum, was pre-incubated in the absence or the presence of 100 μg 5-MP for 24h. After incubation, the SAA1 protein was determined by AB, as follows: the samples were run on SDS-PAGE 12.5% gel with molecular-weight markers. The proteins were transfer to PVDF 0.45 μM membranes (Merck Millipore, Darmstadt, Germany). SAA was detected, using human SAA mAb (Abcam, Cambridge, UK). Immunoreactive bands were visualized by chemiluminescence with ECL-Plus (Biological Industries, Beit Haemek, Israel) and imaging by a CCD camera-based imager (Bio-Rad, Nobel Drive Hercules, CA, USA).

2.6. ELISA assessment of IL-6 and IL-1β in human cells

2.6.1. Fibroblasts

Fibroblast cell line (a gift from Dr I. Bank Sheba Medical Center, Tel Hashomer, Israel) was obtained from synovial fluid removed from the knee of an RA patient and cultured as described in Ref. [18]. P2/A1 Cell line Fibroblasts of no more than 10 passages, were seeded into 24-well NUNC plates (1–2x10^5/well) in serum-free Dulbecco's modified Eagle's medium (DMEM) (Biological Industries, Beit Haemek, Israel) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (Biological Industries) and incubated alone or with 7 μg/ml SAA1 in the absence or present of 200 μg/ml 5-MP peptide or TMVAD scrambled peptide for 48h at 37 °C in 5% CO_2 in air. Cell supernatants were collected for ELISA at the end of the incubation.

2.6.2. THP-1 cells

Monocytoid cells from THP-1 cell line (American Type Tissue Culture Collection, Manassas, VA; TIB-202) were maintained in RPMI 1640 (Biological Industries) supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamin (Biological Industries). For stimulation, THP-1 cells (0.5x10^6/well) were seeded into 24-well NUNC plates in serum-free RPMI 1640 supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine either alone or with 2.5 μg/ml SAA1 in the absence or presence of 200 μg/ml (or other concentration, as indicated) of 5-MP for 24h at 37 °C in 5% CO2 in air. Cell supernatants were collected for ELISA at the end of incubation.

2.6.3. PBMCs

Venous blood was obtained from healthy volunteers (student and stuff from our Faculty of Medicine) and drawn into heparin-containing tubes. Whole blood was diluted 1:1 in Dulbecco's PBS (DPBS) overlaid with Lymphoprep (Stemcell Thchnologies, Vencouver, Kanada) gradient. Following centrifugation at 600×*g* for 30 min at RT, cells from the interface were collected and washed twice in PBS by centrifugation. The PBMC's were maintained in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine in 50 ml flasks for 24h equilibration. For stimulation, PBMC were seeded into 24-well NUNC plates (0.5x10^6/well) in serum-free RPMI 1640 supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine and were incubated with 0.01 or 0.1 μg/ml of SAA1 in the absence or presence of 50, 200 or 500 μg/ml 5-MP 24, 48 or 72 h at 37 °C in 5% CO2 in air. PBMCs incubated in culture medium only, served as background control. After the incubation the supernatants were collected for ELISA.

2.7. ELISA

The release of IL-6 and IL-1β to cell supernatant were measure by ELISA MAXTM kit (BioLegend, San Diego, CA, USA), according the manufacturer instructions.

2.8. SAA aggregation assays

The SAA1-12 peptide RSFFSFLGEAFD (molecular mass 1422.6; TFA-free, hydrochloride salt, purity ~95%) and the 5-MP (molecular mass 535.6, hydrochloride salt, purity ~98%) were synthesized by Genscript, Piscataway, NJ, USA. SAA1-12 peptide was dissolved in 1 M NH4OH at 20 mg/ml, then diluted with cold deionized water and 5X Tris-saline buffer (0.2 μm pre-filtered), and neutralized with 1 M HCl to pH 7.5, on ice. The resulting SAA1-12 stock solution was 2 mg/mL (1.4 mM), in 1X Tris-saline (50 mM Tris, 150 mM NaCl, pH 7.5). 5-MP was dissolved at 4 °C overnight in 1X Tris-saline buffer at 2.67 mg/ml (5 mM), and was 0.45 μm filtered before use. For aggregation experiments, an aliquot of the 1.4 mM SAA1-12 stock solution was diluted with either Tris-saline buffer or 5-MP in Tris-saline buffer, such that the final concentrations of SAA and 5-MP were each 1 mM. Mixtures were incubated at 37 °C for the times indicated. For the Thioflavin T (ThT, Sigma Aldrich, Woodlands, TX, USA) fluorescence assay, 5 μL aliquots of sample were diluted with 150 μL of Tris-saline buffer and 20 μL of 0.1 mM ThT solution, and transferred to a 96-well NBS treated solid black microplate (Corning Inc., NY, USA). Fluorescence was measured using a Synergy H1 microplate reader (BioTek Instruments Inc., Winoski, Vermont, USA). For nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM), sample aliquots were removed at the indicated times, stored at −20 °C and defrosted immediately prior to analysis. NTA measurements were performed using a Malvern Nanosight LM10 (Malvern Instruments., Malvern, UK). For TEM, 5 μL sample aliquots were pipetted onto copper grids and negatively stained with 1% uranyl acetate solution. Samples were imaged on a Phillips CM12 Transmission Electron Microscope (FEI Corp, Waltham, MA, USA) at 120 kV with a 4k x 2.67k GATAN digital camera (courtesy of the Skirball Institute for Biomolecular Medicine at NYU Langone Health).

2.9. Quantitative qRT-PCR

RNA was extracted from regional lymph nodes of peptide treated and control mice using Bio Tri RNA (Biolab, Jerusalem, Israel). Samples of 0.5 μg total RNA were reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA, USA). Quantitative real time RT-PCR (qRT-PCR) for CD14, Gadd45b, Irf-1 and Trail was performed with ABI PRISM 7900HT Sequence Detection System using the TaqMan Gene Expression Assay (Applied Biosystems). All samples were analyzed in triplicate; Rplpo1 (Applied Biosystems) was used as internal control for normalization.

2.10. Assessment of colonic gene expression by qRT-PCR

Total RNA was isolated from colonic tissues using Rneasy kit (Macherey Nagel, Gutenberg, France) according to the manufacturer's instructions. RNA quantification was performed using spectrophotometry. After treatment at 37 °C for 30 min with 20–50 units of RNase-free DNase I (Roche Diagnostics corporation, Indianapolis, IN, USA), oligo-dT primers (Roche Diagnostics), the "high capacity cDNA reverse transcription kit" (Applied Biosystems) was used to synthesize single-stranded cDNA. Primers sequence are depicted in Supplementary Table 2. mRNA was quantified using SYBR green Master Mix (Applera, Courtaboeuf, France) with mice specific oligonucleotides in a GeneAmp Abiprism 7000 (Applera). In each assay, calibrated and no-template controls were included. Each sample was run in triplicate. SYBR green dye intensity was analyzed using the Abiprism 7000 SDS software (Applera). All results were normalized to the unaffected housekeeping gene GAPDH.

2.11. Transcriptomic analysis by RNA-Seq

Messenger RNA was extracted from regional lymph nodes of four MTADV-treated and four PBS-treated mice, using using Bio Tri RNA (Biolab, Jerusalem, Israel). The RNA from each mouse was individually prepared for RNA sequencing. Quality and concentration of the RNA were evaluated by TapeStation (RNA ScreenTape kit; Agilent, Santa Clara, CA, USA) and libraries were prepared with the KAPA Standard mRNA-Seq kit (Roche Diagnostics, Basel, Switzerland), according the manufacture's recommendations. Libraries were sequenced on NextSeq 500 system machine (NextSeq 500/550 High Output v2 kit; Illumumina, San Diego, CA, USA), with 75 cycles and single-read sequencing setting. Libraries preparations and sequencing were performed at the center for Genomic Technologies, Faculty of Medicine, Hebrew University of Jerusalem. Raw reads were processed to remove low quality and technical bases, using in-house scripts and cutadapt (version 1.12; [19]). Processed reads were aligned to the mouse genome, GRCm, using TopHat (version 2.1.1; [20]), allowing for 6 mismatches. HTseq-count (version

0.6.0; [21]) was used for counting reads per gene with gene annotations from Ensembl, elease 89, then differential expression between MTADV- and PBS-treated mice was calculated with DESeq2 (version 1.14.1; [22]), using default parameters. The Broad Institute's GSEA [23] was used with the whole transcriptomic expression data (cut-off independent) to perform the gene set enrichment (Fig. 7C). Gene sets from the hallmark collection of the molecular signatures database (MSigDB) were used for the GSEA [23]. Bioinformatics analysis was performed by the Bioinformatics Unit of the I-CORE at the Hebrew University and Hadassah Medical Center.

3. Results

3.1. 5-MER peptide (5-MP) attenuates collagen-induced arthritis (CIA)

CD44vRA, expressing the MTADV sequence, is detected in inflamed joint cells of RA patients and much less in the blood of the same patients or normal donors or in the joints of osteoarthritis patients [9]. Therefore, we focused efforts on examining the anti-inflammatory effect of the 5-MP in CIA, a mouse model of RA. The 5-MP (N-acetylated and C-amidated to improve its stability) was injected IP at the onset of CIA (when joint inflammation is already detected by the naked eye) and then at daily doses for the next 10 days. From preliminary studies we learned that daily doses of 3.5 mg/kg (~70 μg peptide/mouse) or 10 mg/kg (~200 μg/mouse) significantly reduced the footpad swelling or volume reflecting joint inflammation. In contrast, daily peptide injection at doses lower than 3.5 mg/kg or higher than 10 mg/kg peptide could not significantly reduce the joint inflammation, thus providing a window-like therapeutic effect, typical to multiple drugs e.g., Ref. [24]. Indeed, it was confirmed, following analyzing the data by a blind manner, that, daily peptide injection at doses of 3.5 mg/kg (~70 μg/mouse; Fig. 1A and Supplementary Fig. 1A) and 10 mg/Kg (~200μg/mouse; Fig. 1B) significantly reduced the joint inflammation when compared to PBS control mouse groups. Injection of 3.5 mg/kg was selected as a standard dose (unless otherwise is indicated). Next, we asked whether extension of MTADV by two or four amino acids (one or two residues at each end), to generate 7-MER and 9-MER peptides, respectively (including alanine in the center), could preserve the therapeutic effect detected by 5-MP. Supplementary Fig. 1B shows that the 5-MP displays the strongest anti-inflammatory effect, while similar, but not identical, peptides are less effective, suggesting a specific peptide effect by 5-MP. Therefore, 5-MP was selected as the leading therapeutic agent. To show that the footpad swelling measurement can be confirmed by additional joint inflammation assays, inflammation was assessed in the same experiment and the same mouse groups by 3 different methods: footpad swelling (Fig. 1A, left panel), footpad volume, using Plethysmometer (Supplementary Fig. 1A) and clinical scoring (Fig. 1A, right panel; see M&M). The 5-MP shows almost the same anti-inflammatory effect by all 3 parameters.

To confirm this finding histologically (Fig. 1C to 1E), we used the standard peptide injection protocol. Mice were killed at the end of the experiment (10 days after the first peptide injection), joint sections from arthritic and control mice were stained with Hematoxylin-Eosin, and analyzed under blind manner by a pathologist. The joint spaces and capsules from a mouse that received vehicle only were heavily invaded by inflammatory cells, hypertrophy was identified and severe damage was detected in bone and cartilage. Inflammation Score was marked by 4 (Fig. 1C). On the other hand, the joint spaces and capsules from a mouse that received the peptide were free of cell invasion, hypertrophy was not observed, and damage in bone and cartilage was barely detected. Inflammation Score was marked by 0 (Fig. 1D). Quantifying this finding (see M&M), the average inflammation score of 5 mice treated with PBS was 4, whereas the average inflammation score of 5 mice treated with the 5-MP was 1, and the difference is highly significant (Fig. 1E). The PK analysis suggests that that the 5-MP therapeutic effect is generated short time after the peptide administration, when the peptide concentration in serum is sufficient to systematically target SAA (indirect PK), playing as extracellular chaperone. Further, the PK data of the peptide clarifies that the peptide is stable (not degraded immediately by peptidases) in the blood, and have similar PK profile following SC and IP modes of administration (Supplementary Fig. 2).

3.2. 5-MP does not interfere with normal immune responses

While the 5-MP suppresses the chronic inflammation it does not interfere (unlike anti-TNF [25]) with normal immune responses, such as delayed type hypersensitivity and normal response to a foreign antigen. Further, the 5-MP does not generate neutralizing antibodies (Supplementary Fig. 3).

3.3. 5-MP targets acute phase protein SAA

Next we asked what is the target molecule of the 5-MP? Cell lysates from synovial fluid cells of an RA patient were incubated overnight with biotinylated 5-MER peptide at 4 °C and isolated with streptavidin-coated Sepharose beads (schematic presentation in Supplementary Fig. 4). The proteins bound to the peptide were identified by HPLC system coupled to the Mass Spectrometry (MS; Supplementary Fig. 4). Of 380 proteins identified in the inflamed synovial fluid cell lysates only 17 proteins were detected in the peptide-bound protein population, but not in the peptide unbound protein population (Supplementary Table 1 and Supplementary Data file 1, Excel). Transthyretin, Apolipoprotein B and SAA, all are amyloidogenic proteins, attracted our attention owing to their association with chronic inflammations [3,26–30]. A special attention was focused on SAA, because it stimulates cell migration, cell growth and angiogenesis, all three are hallmarks of chronic inflammation [12].

3.4. 5-MP diminishes SAA hexamer assembly and cytokine release from SAA-stimulated fibroblasts

SAA hexamers of 75 kDa [26,31] (the top bend of Fig. 2A) or two times (160 kDa) longer polymers [32], but not SAA monomers or dimers (the bottom bends), are the native forms of SAA [26,31]. It appears that formation of these SAA polymers is dependent on amino acids residues located in the N-terminal first 12 amino acids of human SAA, that contains a total of 104 amino acids [26]. Our working hypothesis is that SAA hexamer formation (as well as amyloid aggregation, see below) is inhibited by a specific interaction of 5-MP with the SAA N-terminal sequence of the SAA monomer, thus interfering with the formation of SAA polymers and the subsequent aggregate formation (see section 3.7). Indeed, Western Blot with anti-SAA antibody shows, that the small quantity (but reproducible in 4 experiments) of SDS-resistant [33] SAA1 hexamer (but not SAA1 monomers or dimers) is almost entirely absent after SAA co-incubation with the peptide (Fig. 2A). It should be noted that typical amyloid fibrils do not enter the gels because of large size and high SDS resistance. So, this hexamer, which still enter the gel, may represent early soluble SAA, subjected to aggregation at a later time (see below).

To further decipher the 5-MER peptide mechanism of action, we asked how the 5-MER peptide affects various biological functions of RA fibroblasts? P2/A1 Fibroblasts (derived from no more than 10 cell passage [18]) were selected, because they are highly involved in chronic inflammations [34]. Moreover, SAA is upregulated in cytokine-stimulated fibroblasts [35,36] and conversely SAA stimulates chemokine and cytokine production in fibroblasts, including IL-6 and IL-1β, which are predominantly produced by these cells following SAA stimulation [37,38], generating uncontrolled vicious inflammatory cycle. SAA1 enhanced the release of IL-6 from the SAA-stimulated human fibroblasts, but inclusion of the 5-MP inhibited IL-6 (Fig. 2B and C) and IL-1β (Fig. 2D) release from these cells. Further, MTADV peptide, but not the scrambled peptide (TMVAD), inhibited the release of IL-6 from SAA-activated fibroblasts (Fig. 2C).

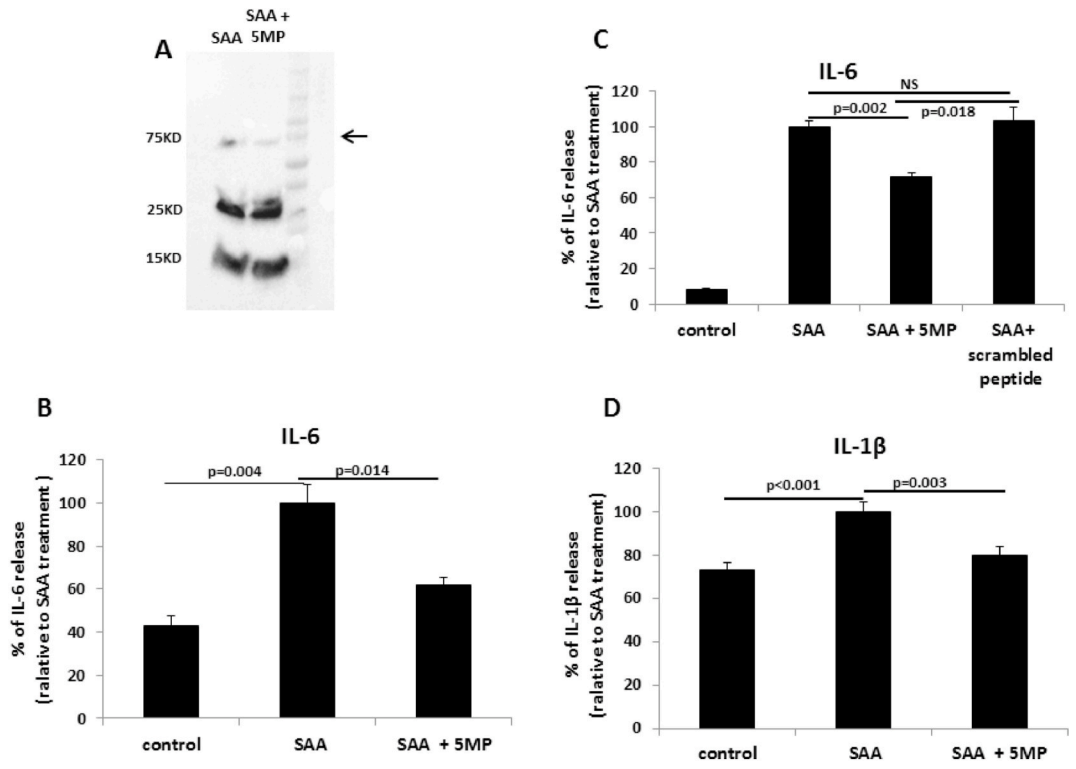

Fig. 2. 5-MP inhibits release of pro-inflammatory cytokines from RA fibroblasts stimulated with SAA1. **(A)** 5-MP reduced assembly of SAA. Western blot analysis of SAA in solution with anti-SAA antibody in the absence (left) or presence (right) of 5-MP. The molecular weight scale is shown on the left hand side. SAA MW – 12 kDa. The peptide interferes with the generation of SAA hexamer. One of four similar experiments. (**B** and **C)** 5-MP inhibits the releases of IL-6. 5-MP (MTADV; **B and C**), but not the scrambled peptide (TMVAD; **C**) inhibits IL-6 release from SAA-stimulated RA fibroblasts after 48h of incubation. The fibroblasts, that were treated as indicated on the X- axis of the figures, were analyzed by ELISA for % of the maximal IL-6 release (134 pg/ml in **B** and 325 pg/ml in **C**), measured in fibroblasts incubated with SAA only. (**D**) 5-MP inhibits the releases of IL1-β. RA fibroblasts, incubated as in **B** and **C,** were treated as indicated on the X-axis of the figure and analyzed by ELISA for % of the maximal IL1-β release (24 pg), measured in fibroblasts subjected to SAA only. Results are shown by±SEM (n = 3) Statistical analysis by two-tailed Student's t-test equal variance. One of six (**B**) and one of two (**C**) experiments showing similar results. (**D**) is an average of 4 experiments, showing similar results. 5 MP = 5-MER peptide.

Given that the SAA is active as assemblies of polymers or aggregates, these findings suggest that reducing the self-assembly of SAA by MTADV peptide (Fig. 2A) restrains its ability to enhance the inflammatory response (Fig. 2 B to D). In conclusion, the 5-MP plays in this experiment as an extracellular chaperone [39].

3.5. 5-MP is not toxic under in vitro conditions, but reduces viability of SAA-stimulated fibroblasts

We showed by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] (MTT) assay [40] that 5-MP (but not the scrambled peptide) is not toxic by itself for fibroblasts, but inhibits the enhanced viability of these cells when stimulated with SAA. We further found that these effects cannot be attributed to endotoxin (LPS) contamination of the SAA (Supplementary Fig. 5). Additionally, *In vitro* pharmacological profiling off-target bioanalysis [41] by Eurofins Panlabs Discovery Services, revealed that no one of 87 core proteins was off-targeted by the 5-MP (Supplementary Document 1).

3.6. 5-MP diminishes cytokine release from SAA-stimulated THP-1 monocytes and-peripheral blood mononuclear cells

Next we asked whether 5-MP can inhibit pro-inflammatory cytokine release from SAA-stimulated THP-1 human monocyte cell line and primary peripheral mononuclear cells (PBMCs) of normal human donors. These cell phenotypes, like fibroblasts, are also activated under chronic inflammation [42]. Like in Fibroblasts, the peptide inhibits the IL-6 release from SAA1-stimulated THP-1 monocytes (Fig. 3A) and this inhibition effect was significantly stronger than the effect of identical doses of scrambled TMVAD peptide (Fig. 3B). However, no inhibition effect was detected by either one of these peptide on the basic background release of this pro-inflammatory cytokine (Fig. 3B). Similarly, the 5-MP inhibited IL-6 (Fig. 3C) and IL-1β (Fig. 3D) release from SAA1-sensityzed primary human PBMCs of healthy donors, but not from non-sensitized corresponding cells (3C). This finding suggests that only the pathological over-sensitized IL-6 is inhibited by the 5-MP, while the normal release of this cytokine, which is possibly required for homoeostasis is not affected by the peptide, yet it slightly, but significantly (Fig. 3B and C) enhances its release. The time-dependent inhibition of IL-6 release by 5-MP was recorded 24 (Figs. 3C), 48 and 72 (Supplementary Fig. 6) hours after addition of 5-MP to SAA-stimulated PBMCs. Note that In primary cell culture, 72 h is a relatively long time and may be considered a "chronic stimulation". Altogether, the suppressive effects of this human-derived peptide is detected in both mouse (Fig. 1) and human (Fig. 3) experimental models.

3.7. 5-MP inhibits amyloid aggregation of SAA N-terminal sequence

Full length SAA, SAA (1–104), is hexameric in its native state [26]. A C-terminal proteolytic cleavage converts hexameric SAA (1–104) to SAA (1–76) following the dissociation of the former to a monomer. Monomeric SAA (1–76) is prone to amyloid aggregation [43,44]. Given that SAA is aggregated into amyloid assemblies that further enhance chronic inflammatory activities, we explored the peptide effect on SAA aggregation, which can generate amyloidosis in 1–7% of RA patients [45,46]. We found that 5-MP interferes with aggregation of a peptide comprised

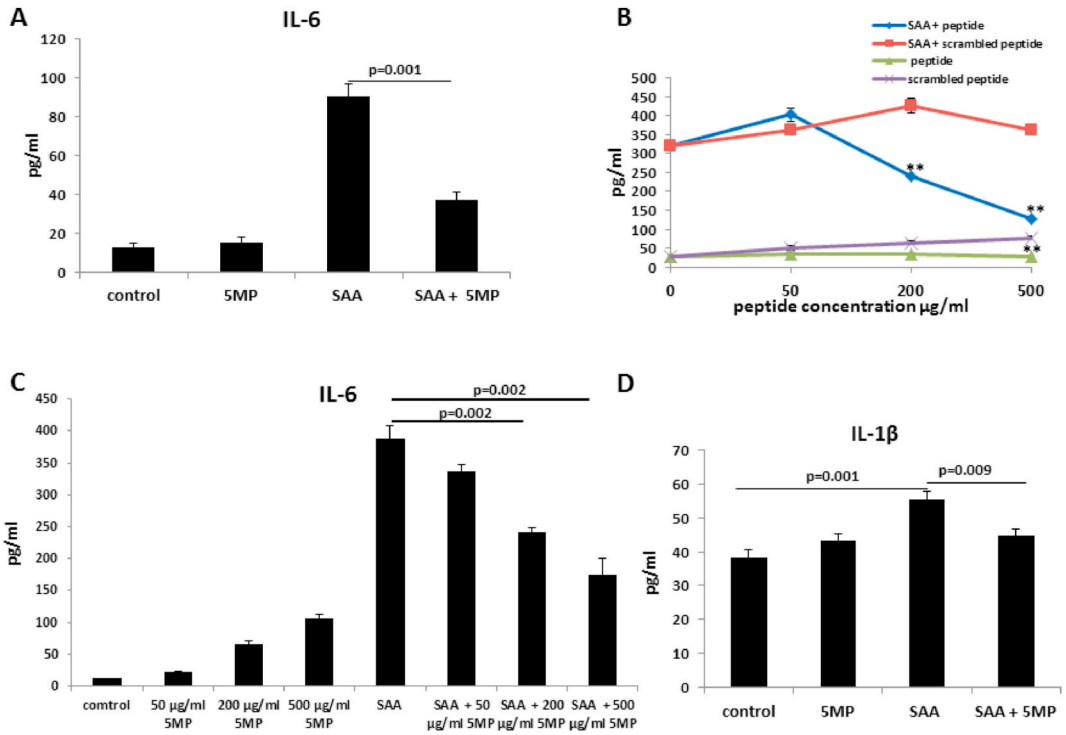

Fig. 3. (**A to D**) 5-MP inhibits release of pro-inflammatory cytokines from human SAA1-stimulated THP-1 and-PBMC cells. (**A** and **B**) THP-1 cells.Non-stimulated (control) or SAA-stimulated THP-1 cells were incubated for 24h in the absence and presence of a standard dose of 5-MP (200 µg/ml) and their ability to release IL-6 was analyzed by ELISA. One of 4 similar experiments. **(B)** SAA (200 µg/ml) -stimulated top lines) or non-stimulated (bottom lines) THP-1 cells were incubated in the absence and presence of increasing doses of MTADV 5-MP (blue line) or identical doses of TMVAD scrambled peptide (red line). The ability of the peptides to inhibit IL-6 release from the cells was assessed by ELISA. **(C** and **D)** PBMCs. SAA-non-stimulated or SAA-stimulated PBMCs were incubated in the absence and presence of increasing doses of 5-MP (**C**) or 200 µg/ml 5 MP (D) and the ability of the peptides to inhibit IL-6 (**C**) and IL-1β (**D**) release from the cells was assessed by ELISA after 24h and 4h hours of incubation, respectively. (**C**) One of 8 and (**D**) one of 3 similar experiments. Results are presented by±SEM (n = 3). Statistical analysis by two-tailed Student's t-test equal variance. n = 3. **P < 0.01. 5 MP = 5-MER peptide.

of the first 12 N-terminal amino acids of SAA, which is the nucleus of SAA amyloid fibril formation [27]. We employed nanoparticle tracking analysis (NTA) (Fig. 4A) and Thioflavin T (ThT) fluorescence (Fig. 4B) for quantitative evaluation of SAA1-12 aggregation with and without the 5-MP. Aggregate morphology of samples was examined by transmission electron microscopy (TEM) (Fig. 4C). Under neutral physiological ionic strength solution, SAA1-12 peptide incubated alone for 4h showed many particles with apparent hydrodynamic diameters of ca. 300 to >1000 nm in NTA analysis (Fig. 4A). By TEM, the same samples showed the formation of thin wormlike protofibrils up to a few hundred nm in length (Fig. 4C, left panel). However, addition of equimolar 5-MP strongly retarded the extent of SAA aggregation, i.e., the total particle concentration as well as particle sizes were substantially reduced (Fig. 4A) and no protofibrils was detected after 4h (Fig. 4C, right panel). More prolonged incubation of SAA1-12 alone led to the formation of amyloid fibrils, as judged by ThT assay (Fig. 4B). The addition of the 5-MP significantly reduced the extent of aggregation (Fig. 4B). Thus the 5-MP acts primarily to retard the early stage of SAA1-12 aggregation. Further, this experiment demonstrates direct interaction between the SAA N-terminal 1–12 amino acids sequence and MTADV.

3.8. 5-MP does not inhibit CIA in rats, that do not express SAA

SAA protein is expressed in multiple mammalian species, including human, mouse, hamster, rabbit, dog, mink, cow, sheep and horse [1]. However, rats do not produce SAA protein [47], although SAA mRNA can be detected in these animals [48]. It appears that the rat mRNA lacks a highly conserved coding region for portions of two amphipathic helical domains and the joining sequence [48]. It should be further stressed that amyloidosis is hardly detected in rats [49]. Therefore, a prediction was made: if rats do not express the SAA protein, the 5-MP, that targets SAA in *in vitro* models, would not be able to inhibit CIA in rats, owing to lack of this target *in vivo*. Although the 5-MP attenuated the mouse CIA (Fig. 1), the same peptide, injected at wide spectrum of both high (Fig. 5A) and low (Fig. 5B) concentrations (including the equivalent peptide range effective in mouse anti-CIA peptide concentration), failed to inhibit CIA in rats. In contrast, dexamethasone (non-specific anti-inflammatory drug) substantially displayed such an inhibitory effect (Fig. 5). This finding implies that once SAA is missing from the animal the peptide cannot exert its therapeutic activity. Collectively, 5-MP neither influences an immune response (including autoimmunity), which is not SAA- dependent, nor normal immune responses (Supplementary Fig. 3), stressing its specificity.

3.9. 5-MP attenuates IBD

Next question was whether the therapeutic activity of 5-MP can be extended to additional inflammatory diseases aside of CIA (Fig. 1), focusing on those associated with pathological SAA, such as IBD [8,50,51] and MS [8,14,52]. To this end, SAA knockout mice displayed attenuated IBD and EAE [8], indicating the SAA-involvement in these maladies. Our *in vitro* and *in vivo* studies further support the concept that SAA is the 5-MP Target. It should be stressed that our IBD model, unlike the CIA model, is a preventive rather than therapeutic model. However, like the RA [12] and MS [8] mouse models, IBD pathology is associated with SAA [8], providing a potential target for 5-MP treatment. Furthermore, our findings show (see below) that in the IBD mouse model the disease attenuation effect of 5-MP is at least as good and possibly better than anti-TNF drugs, which are widely used in in human IBD treatment.

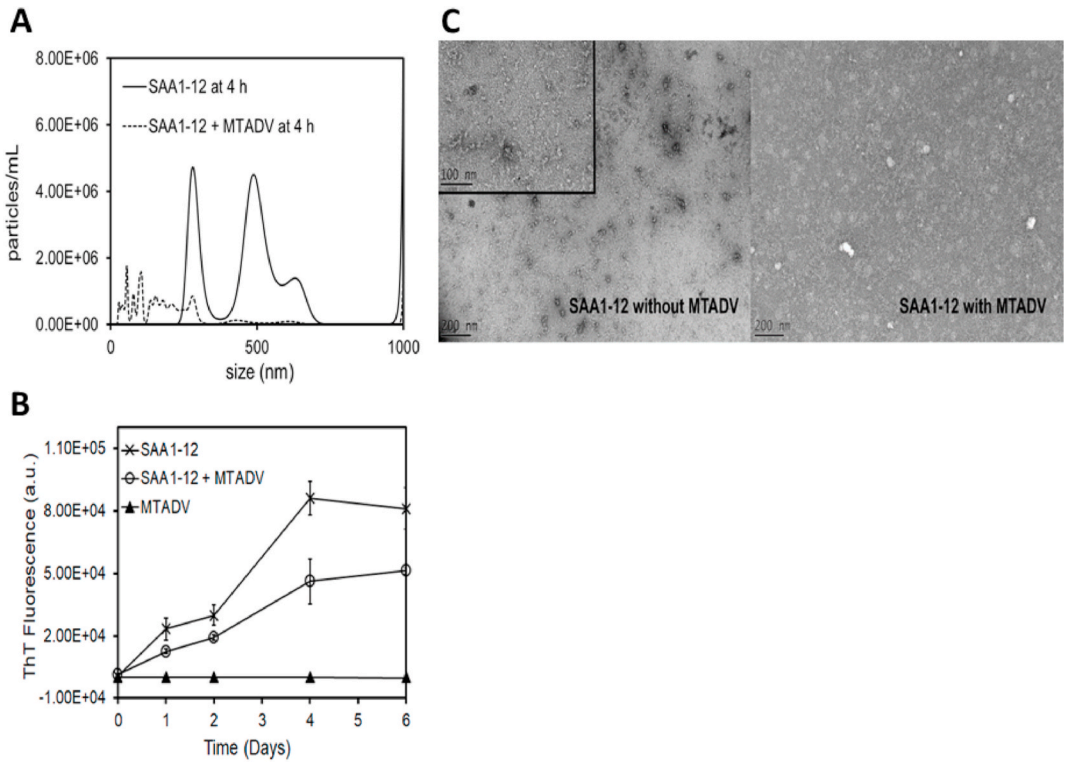

Fig. 4. (**A to C**) The aggregation of SAA1-12 to amyloid-type fibrils is slowed in the presence of 5-MP. (**A**) Nanoparticle Tracking Analysis of SAA1-12 incubated for 4h in the absence or presence of equimolar 5-MP, that shows early stage of aggregation to particles with apparent hydrodynamic diameters of several hundred nm, is inhibited by the 5-MP. (**B**) Thioflavin T fluorescence detects increasing amyloid-type fibril content in a solution of SAA1-12 incubated over a period of days. In the presence of equimolar 5-MP, SAA1-12 fibril content is reduced at all time points. The values were statistically analyzed (*$p < 0.05$), using Student's *t*-Test and a one tailed hypothesis. (**C**) Transmission electron micrographs monitor the time-dependent formation of protofibrillar and fibrillar aggregates of SAA1-12 in the absence (left) or presence (right) of equimolar 5-MP, after incubation for 4h (inset-2x magnification). The experiment was repeated twice with similar results. 5-MP alone does not form amyloid fibrils.

Using Tri Nitrobenzene Sulfonic Acid (TNBS) induced-IBD in C57BL/6 mice, it was found that daily SC injection of the 5-MP attenuated IBD, as indicated by histopathological score (Ameho; Fig. 6A) of the inflamed colon, when compared to control mice. Note that the therapeutic effect of the 5-MP was at least as good, if not better, when compared with that of anti-TNF treatment (Fig. 6A). Furthermore, mRNA analysis by qRT-PCR revealed down-regulation of the pro-inflammatory IL-1β, IL-6 and Keratinocyte Chemoattractant (KC) mRNAs in colon extracts of mice treated with 15 mg/kg 5-MP (Fig. 6, B1 to B3). Transcript changes were not significant in mRNAs of TNFα, IL-10, FoxP3 and ICAM-1. In contrast, the mRNA of IFNγ was significantly upregulated ($p < 0.001$ by Anova's) after 5-MP injection (Fig. 6B4). This finding is not surprising, because IFNγ can pleiotropically display pro- or anti- inflammatory activities [53]. This IBD suppression was confirmed by bio-luminescence analysis of C57BL/6 mice subjected to TNBS-or dextran sulfate sodium (DSS)-induced IBD, using s.c. or oral delivery routes for the 5-MP administration [54].

3.10. 5-MP attenuates experimental autoimmune encephalomyelitis (EAE)

Following preliminary studies, showing the ability of i.p. 5-MP injection to inhibit limb paralysis in EAE, the peptide was daily orally delivered under the standard protocol, but its concentration was increased 10x (from 3.5 mg/kg to 35 mg/kg). The peptide was administered by gavage into C57BL/6 mice 5 days after EAE induction with Myelin Oligodendrocyte Glycoprotein (MOG35-55) + pertussis toxin and then daily orally delivered (as indicated in the Figure) with 5-MP (Supplementary Fig. 7). Using more aggressive protocol, the EAE was induced in SJL mice with proteolipid protein (PLP) + pertussis toxin and then daily orally delivered with 5-MP for the next 10 days (Fig. 7A and B). Orally delivered peptide significantly inhibited the limb paralysis, when compared with LPS orally administered corresponding control mice (Supplementary Fig. 7 and Fig. 7A and B). Higher concentration of 5-MP was used under oral delivery protocol than under IP/SC protocols, because the peptide must confront against enzyme digestion, which may reduce the concentration of the peptide below the therapeutic effect, if standard peptide doses (3.5 mg/kg) are given. Notably, the 5-MP attenuation effect is less impressive in the EAE model than in the other models, because drug oral delivery must confront with the digestion system of the intestine. However, altogether, the EAE experiments were similarly repeated 5 times (including the experiments described in the Supplementary Fig. 7), and they are statistically significant. The principle importance of the EAE model is related to the oral delivery of the peptide, which is more practical and more acceptable by patients than injections. Further, the mice were subjected to transcriptome analysis, when the peptide showed a significant anti-paralytic effect (day 15) in the experiment described in Fig. 7B.

3.11. 5-MP upregulates genes protecting against chronic inflammation and neuron degeneration

We next explored the transcriptomic changes induced by 5-MP. Messenger RNA was extracted from reginal lymph nodes of encephlitogenic mice, orally treated with 5-MP or vehicle only (Fig. 7B) and then subjected to mRNA-Seq and Gene Set Enrichment Analyses (Supplementary Data file 2, Excel). The non-cutoff dependent Gene Set Enrichment Analysis (GSEA) [23] was then used to investigate the possibility that specific gene sets are over- represented in the up/down regulated genes upon treatment with 5-MP. This analysis revealed that

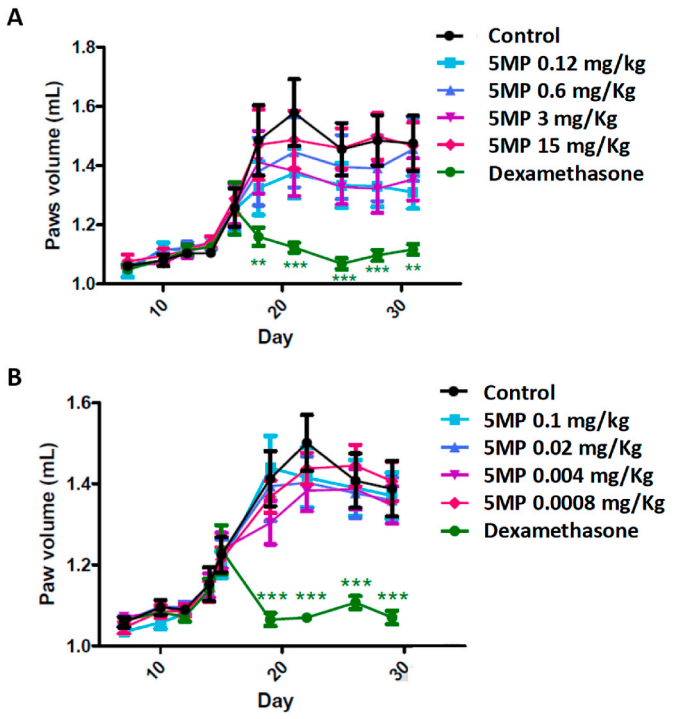

Fig. 5. (A and B) 5-MP failed to inhibit CIA in SAA-deficient rats. Lewis Rats were SC injected at onset of CIA with PBS or the indicated high (**A**) and low (**B**) concentrations of 5-MP or dexamethasone and then every day for the next 30 days. The daily size of footpad volume, reflecting joint inflammation, was automatically measured, using plethysmometer. Results are expressed as mean ± SEM (n = 6 for **A** and n = 10 for **B**). Statistics by two ways ANOVA **p < 0.01***p < 0.001 vs. CIA Control Group. 5 MP = 5-MER peptide.

MSigDB Hallmark gene sets of IFN-γ response (Fig. 7C, Top), apoptosis (Fig. 7C, Middle) and inflammatory response (Fig. 7C, Bottom) were significantly enriched (FDR<0.0002) in the up-regulated genes of the 5-MP orally-treated mouse group. Among the genes that highly contributed to the enrichment of the above-mentioned gene sets, we found significant number of anti-neurodegeneration and anti-chronic inflammation genes, represented, for example, by Batf2 [55], IL-18bp [56] and Atf3 [57]. A few other genes pleiotropically code for additional or other functions (the entire gene set is recorded in Supplementary Data file 2). To validate the GSEA results, the mRNA of four genes that highly contributed to enrichment analysis were subjected to qRT-PCR. Indeed, upregulation of these mRNAs, following peptide treatment, was statistically confirmed (Fig. 7D).

4. Discussion

The studies presented here unveil the interaction between the 5-MP MTADV (Amilo-5MER) and SAA, and the outcome of this interaction on inflammatory models. The 5-MP displays specific binding to SAA *in vitro* and therapeutic activity in RA, IBD and MS mouse models, which share the SAA target and/or SAA dependency [8,12]. Each one of 5-MP route of administrations was repeated in two different mouse models, indicating that the suppressive effect of 5-MP is independent of route of administration. The oral bioavailability of 5-MP in EAE (which is more useful in practical terms) could be attributed to fact that 10X higher peptide dose was used in this mouse model. In contrast, the 5-MP could not display therapeutic activity in rats [47], which are SAA deficient owing to a translation defect [48]. Unlike rats, man, mouse, and other mammals do express SAA [1]. Furthermore, the rat study shows that SAA-independent immune response is not affected by the 5-MP, stressing its specific inhibition effect and supporting the view that acute inflammation (DTH) is not influenced by the peptide (Supplementary Fig. 3A). On the other hand, we have not yet evidence that MTADV peptide competes with CD44vRA on CD44vRA targets, a concept that motivated this research project. While this possibility cannot be excluded and should be explored, we independently revealed that SAA is an MTADV target, simply because the RA synovium cell extract, the object of our search for identifying the peptide targets, contains SAA (Supplementary Fig. 4). It is possible, that one of the 17 proteins, bound to biotinylated 5-MP (supplementary Table 1) is also a target of CD44vRA. Verification of this prediction, which may allow affinity comparison between the two ligands as well, is a matter of a special study.

SAA, especially its predominant isoforms, SAA1 and SAA2, are a pro-inflammatory acute phase protein (reviewed in Refs. [1–6]) associated with inflammation under pathological conditions such as RA [12,13], IBDs [8,13], MS [8,14] and Alzheimer's disease (AD) [4]. SAA can spontaneously generate functional hexamers, which may subsequently initiate a chronic inflammatory cascade. Indeed, we found that SAA1 in solution generates SDS-resistant hexamers [33], which almost vanish in the presence of 5-MP (Fig. 2A). However, whether SAA hexamers are native hexamers, dissociation products from aggregates or other form of SDS-tolerated oligomers have yet to be determined. Although it is well established that SAA-deficient mice showed reduced ability to generate IBD or EAE [8], the SAA phenotypes (monomer hexamers, other oligomers) and isotypes (SAA1, SAA2), which potentiate susceptibility to develop chronic inflammation, should be identified. On the other hand, potentially pathological fibrillary SAA assemblies can be formed by C-terminal cleavage that converts SAA (1–104) to SAA (1–76) following the dissociation of the former to a monomer. Monomeric SAA (1–76) is prone to amyloid aggregation [44], leading to life threatening amyloidosis [3]. The amyloidogenic core of SAA is included in the N-terminal 10–15 amino acid segment [27]. Therefore, this short N-terminal segment can be used to validate potential drugs that may function by modulating SAA aggregation pathways. Furthermore, the fact that 5-MP interferes with SAA1-12 aggregation indicates that the peptide interacts with amino acids located in this region, which is partially conserved in mouse and human SAA (BLAST) and shared by human SAA1 and SAA2, the functional isoforms of SAA [4]. This fact explains why the human 5-MP is effective in both mouse and human models and why our findings related to SAA1 can be extrapolated to SAA2.

We hypothesized that the binding of 5-MP (Amilo-5MER) to SAA (or SAA1-12) could affect the aggregation process leading to the formation of protofibrils and fibrils. The 5-MP MTADV sequence has an alternating sequence of hydrophobic (M, A, V) and hydrophilic (T, D) amino acid side chains. Longer polypeptides with such an alternating sequence have been reported to assemble into fibrillar amyloid-like structure [58]. Thus, 5-MP has a high potential of association with other amyloidogenic sequences. Once bound to SAA1-12, the 5-MP could disrupt the normal self-aggregation process of SAA segments, potentially poisoning the growing ends of a protofibril or fibril. The binding of 5-MP to SAA (or SAA1-12) could affect both SAA hexamerization (Fig. 2), and amyloid aggregation (Fig. 4). Both structures could be important crossroads in the development of chronic inflammatory maladies and amyloidosis, respectively. We show that 5-MP significantly retarded the early stage aggregation of SAA1-12 and interfered with protofibril formation. At longer time periods, amyloid fibrils were formed by SAA1-12 regardless of co-incubation with 5-MP, but the extent of amyloid fibril formation was less in the presence of 5-MP. The effect of 5-MP on SAA1-12 aggregation suggests that 5-MP may function by delaying pathological SAA aggregation in tissues, allowing increased time for the normal clearance of SAA from tissue before it becomes aggregated to pathological insoluble fibrillary deposits.

It was found that IBD and EAE were suppressed in SAA knockout mice [8] and that the suppression is similar to the 5-MP inhibition effect in IBD (Fig. 6) and EAE (Fig. 7) mouse models. In both experimental models SAA could be a target. Notable, the IBD model, unlike the CIA

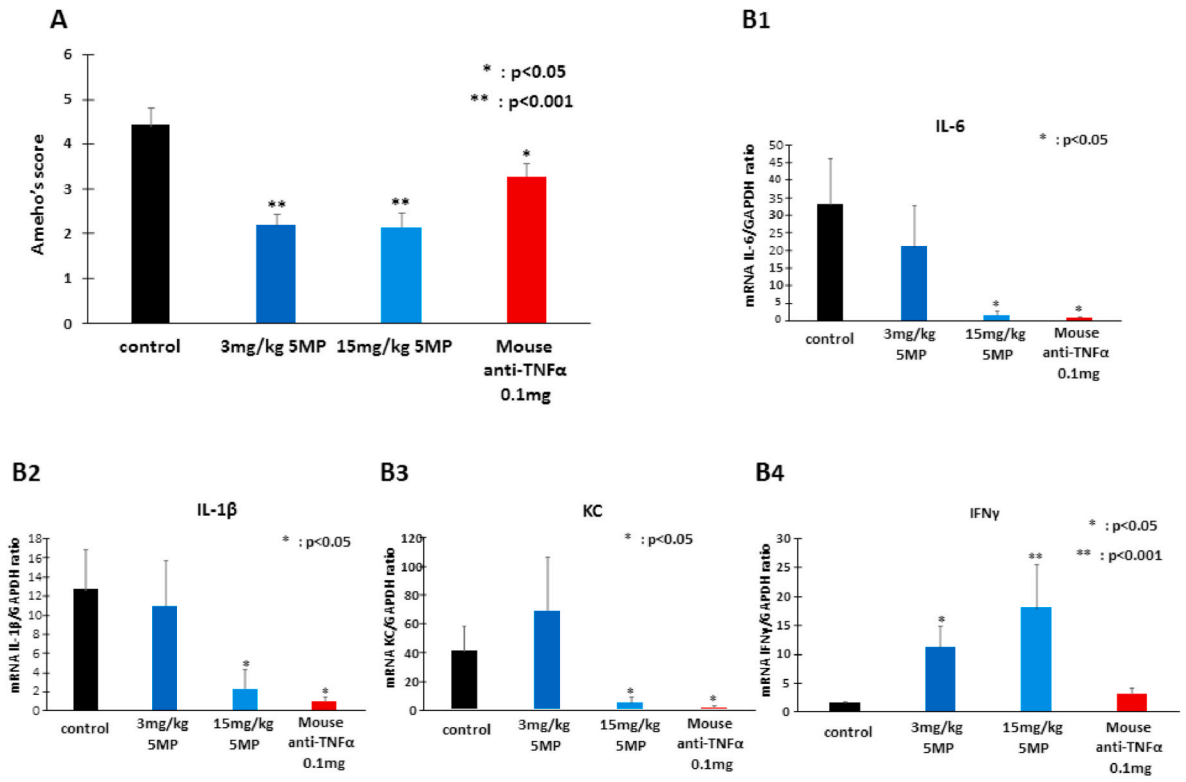

Fig. 6. (A and B). Injection of 5-mp attenuated IBD. TNBS-IBD-induced mice were left untreated (control) or daily SC-treated with 5 MP or anti-TNF as shown in figure. (**A**) Clinical analysis by histopathological Ameho's score. (**B1-4**) Transcriptom analysis. *Ex vivo* mRNA analysis of IL-6, IL-1β, keratinocyte chemoattractant (KC) transcripts and IFNγ. Total RNA was isolated from colonic tissues of each mouse at the end of the experiment (described in **A**) and qRT-PCR (in triplicates) was performed for IL-6, IL-1β, KC and IFNγ transcripts (shown in the figure) as well as for TNFα, IL-10, FoxP3 and ICAM-1 (described in Results). n = 14 to 17 mice per group. Results are shown as±SEM. Statistical Analysis: All comparisons were analyzed using the Anova's Test for two independent samples. 5 MP = 5MER peptide.

model, is preventive model. However, anti-IBD attenuation effect of 5-MP, described in Fig. 6A, could justify, in our next study, the establishment of advanced therapeutic model such as anti-IL-10RA with H. hepaticus infection [8]. In the EAE model, the 5-MP was injected 5 days after the induction of disease, which is in the midway between preventive and therapeutic models and may represent asymptomatic MS. This asymptomatic phase is typical to MS under remission, but need still medical treatment to avoid or delay relapse. As pathological SAA is involved in both human IBD [8] and MS [4,14], a question is raised whether the 5-MP, which inhibits cytokine release from SAA-sensitized PBMCs, but not from non-sensitized PBMCs, could show an efficient therapeutic effect in relevant human diseases as well? How the peptide discriminates between sensitized and non-sensitized PBMCs is a matter of further research. Interestingly, it was previously found that hexapeptides fibrils derived from amyloidogenic proteins show, like 5-MP, both therapeutic activity in EAE and suppression of IL-6 production [59]. Both SAA [60] and IL-6 [61] are substantially elevated in COVID-19 patients and both are affected by 5-MP, raising a possibility for 5-MP suppressive activity in COVID19.

The extent to which the unique peptide MTADV might exert beneficial therapeutic activity on other diseases characterized by amyloid-type proteins accumulation has not been identified yet. In addition to SAA accumulation in RA [12,13], IBD [13] and MS [4,14,52] or the corresponding mouse models of IBD and MS [8], a wide variety of diseases are associated with amyloidogenic proteins. For example, SAA in AA amyloidosis [3], Alzheimer's Disease (AD) [4] and COVID19 [60]; Amyloid β in Alzheimer's Disease [3]; Amylin in type 2 diabetes [3]; Apolipoproteins A-1 [3] and B [30] in atherosclerosis; and Transthyretin in various transthyretin amyloidosis [28]. We already have evidence that 5-MP binds to transthyretin and preliminary studies with 5-MP in a mouse model of AD are promising. This is hardly surprising because SAA is involved in Alzheimer's [4]. The broad application of this peptide-based approach to modulate pathological amyloid protein accumulation in general should be under investigation.

The GSEA [23] provided, an additional perspective for this study. The 5-MP suppresses not only chronic inflammation, but also upregulates genes that contribute to resistance to this pathology as well as to protection against neurodegeneration (Supplementary data file 2). We suggest that transcription of genes inducing resistance to inflammation and neurodegeneration are downregulated during the pathological process, owing to the attenuation of the homeostasis system, that keeps this process under control. Once 5-MP reduces the pathological effect, the homeostasis is partially or completely restored, resulting with re-upregulation of these genes. The linkage between these two basic findings (gene upregulation and inflammation inhibition) is a matter for a further investigation.

Our concept, schematically described in Fig. 8, is expanded and supported by additional findings. June-Yong Lee and colleagues [8] showed that inflammation-derived SAA and IL-6 induce differentiation of pathogenic Th17 cells, that further fuel the inflammatory destructive process. Additionally, it has been reported that the pro-inflammatory cytokine IL-1β stimulate SAA secretion from intestinal epithelial cells [62]. It was further implied that RA synovial fibroblasts are major IL-6 producers (as confirmed in Fig. 2), stressing that the effect is associated with genetic polymorphism [63].

In conclusion, our study unveils a novel potential drug with a potency to combat chronic inflammations. The study elucidates also a unique mechanism of action underling peptide-induced upregulation of disease- resistant genes, as well as a novel SAA target that can be an object for therapeutic strategies in SAA-associated pathologies. 5-MP (Amilo-5MER) is currently under development for IBD in patients and exhibits an excellent safety profile.

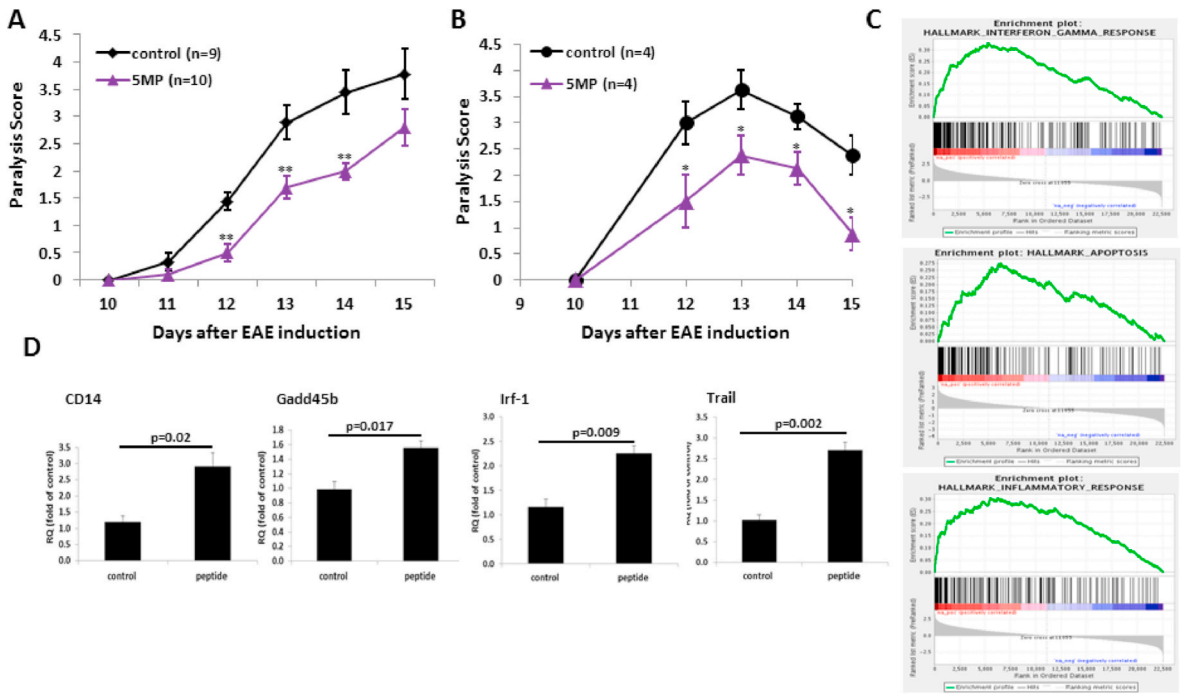

Fig. 7. (**A** to **D**). **Orally-delivered 5-MP inhibits limb paralysis and up-regulates inflammatory and EAE/MS resistance-involved genes in mice with EAE.** (**A** and **B**) Peptide inhibitory effect on limb paralysis. EAE was induced in SJL mice with PLP/pertussis. Five days later they were orally delivered by gavage with vehicle only or 5-MP. The peptide was given every day for the next 10 days, and limb paralysis was daily scored (as indicated in M&M; two of 3 similar experiments). Results are shown as±SEM. Statistical analysis by Mann and Whitney *U* test. *p < 0.05; **p < 0.005. (**C** and **D**). Transcriptome analysis. mRNA was collected from the mice described in **B** at day 15 after EAE induction and subjected to Gene Set Enrichment Analysis (GSEA) (**C**) or qRT-PCR (**D**). **C. GSEA.** GSEA of the whole transcriptomic data of 5-MP orally delivered mice vs control mice. MSigDB Hallmark of IFN-γ response (Top), apoptosis (Middle) and inflammatory response (bottom) gene sets (A,B and C respectively in Data file S2) were significantly enriched in the up-regulated genes of the peptide treated mice (FDR <0.0002). (**D**) Transcriptome analysis. qRT-PCR confirmation of some the genes contributing to up-regulation of GSEA-assessed gene sets shown in **C**. Results are shown as±SEM (n = 3). One of two similar experiments. Statistical analysis by two-tailed Student's t-test equal variance. RQ = relative quantification. 5 MP = 5MER peptide.

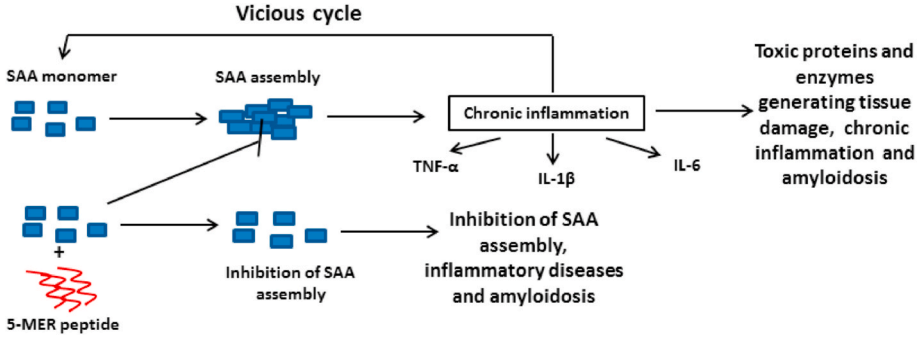

Fig. 8. Schematic presentation of the 5-MP therapeutic effect. Activated fibroblasts [35,36], epithelial cells [8] or monocytes [64] produce Serum Amyloid A (SAA), that can be accumulated as monomers, self-associated oligomers or aggregated amyloid fibrils. The assembled or self-associated SAA enhances sterile chronic inflammation, resulting with accumulation of toxic proteins and enzymes as well as pro-inflammatory cytokines, including IL-1β and IL-6 (Figs. 2, 3 and 6B and ref [5]), which at high concentration present a "cytokine storm". The IL-1β and IL-6 cytokines re-stimulate the activated cells to assemble more SAA, that generates more destructive cytokines. This snowball growing SAA inflammatory perpetuum mobile (or vicious cycle) is targeted by 5-MP, which interferes with SAA assembly, resulting with inflammation or amyloidosis amelioration.

Author statement

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2021.102713.

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