Suppression of inflammation and tissue damage by a hookworm recombinant protein in experimental colitis

Ivana B Ferreira¹, Darren A Pickering¹, Sally Troy¹, John Croese¹,², Alex Loukas¹ and Severine Navarro¹

Gastrointestinal parasites, hookworms in particular, have evolved to cause minimal harm to their hosts when present in small numbers, allowing them to establish chronic infections for decades. They do so by creating an immunoregulatory environment that promotes their own survival, but paradoxically also benefits the host by protecting against the onset of many inflammatory diseases. To harness the therapeutic value of hookworms without using live parasites, we have examined the protective properties of the recombinant protein anti-inflammatory protein (AIP)-1, secreted in abundance by hookworms within the intestinal mucosa, in experimental colitis. Colitic inflammation assessed by weight loss, colon atrophy, oedema, ulceration and necrosis, as well as abdominal adhesion was significantly suppressed in mice treated with a single intraperitoneal dose of AIP-1 at 1 mg kg⁻¹. Local infiltration of inflammatory cells was also significantly reduced, with minimal goblet cell loss and preserved mucosal architecture. Treatment with AIP-1 promoted the production of colon interleukin (IL)-10, transforming growth factor (TGF)-β and thymic stromal lymphopoietin (TSLP), resulting in the suppression of tumour necrosis factor (TNF)-α, IL-13 and IL-17 A cytokines and granulocyte macrophage colony-stimulating factor (GM-CSF), CX motif chemokine (CXCL)-11 and cyclooxygenase synthase (COX)-2 mRNA transcripts. AIP-1 promoted the accumulation of regulatory T cells in the colon likely allowing rapid healing of the colon mucosa. Hookworm recombinant AIP-1 is a novel therapeutic candidate for the treatment of inflammatory bowel diseases that can be explored for the prevention of acute inflammatory relapses, an important cause of colorectal cancer.

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Inflammatory bowel diseases (IBD) are a group of chronic autoimmune diseases affecting the digestive track, primarily represented by Crohn’s disease (CD) and ulcerative colitis (UC). Both types of IBD are caused by an inappropriate immune response in genetically susceptible individuals to intestinal microbial species, however, the site and nature of inflammation differ between the two diseases. CD can affect the entire intestinal track from the mouth to the anus, whereas UC mainly affects the colon and the rectum. Although the role of innate cells is pivotal in CD, both conditions are T-cell-mediated and characterised by increased levels of interleukin (IL)-6, IL-17, interferon (IFN)-γ and tumour necrosis factor (TNF)-α. However, the immune response in UC appears to be more skewed towards a T-helper cell type 2 (Th2) response, with increased levels of IL-4 and IL-13 production in the tissue. The current treatments for IBD rely on nonspecific immunosuppressive drugs, such as steroids, antibiotics, and immunomodulators targeting the TNF pathway or the gut-homing integrin α4β7. Despite encouraging clinical trial end points, TNF-α inhibitors are not effective in all patients and do not prevent relapse. One of the major consequences of UC progression is the development of colorectal cancer, which is the third most common malignancy in humans. The rising incidence of IBD parallels the trend of other autoimmune and allergic diseases. However, for reasons that are still unclear, the rate of childhood-onset IBD has been the highest observed over the past two decades. In addition to the debilitating symptoms associated with the disease, children affected by early-onset IBD suffer significant malabsorption and nutritional deficiencies resulting in growth failure, skeletal impairment, and significant psychological and developmental delays. These recent observations underscore the urgent need for novel therapeutic approaches to be developed.

A promising new avenue of research using live helminth therapy has seen encouraging levels of success for the management of autoimmune diseases, such as IBD and Celiac disease. Indeed, experimental infection with ova of the pig whipworm Trichuris suis (TSO) successfully improved both UC and CD Disease Activity Index. However, because humans are not the natural host and infection resulted in rapid parasite clearance, repeated administrations were required and recent phase 2 clinical trials in IBD failed to meet clinical end points. Interestingly, hookworms, such as...
Necator americanus and Ancylostoma caninum, have co-evolved with their mammalian hosts where they establish chronic infections over many years.28 The tolerability of iatrogenic N. americanus infection has been assessed in patients with autoimmune gastrointestinal diseases. Hookworm infection coupled with escalating oral gluten challenge has been assessed in patients with autoimmune gastrointestinal diseases.32

Figure 1 Protective effects of AIP-1 against weight loss and colon damage in TNBS-induced colitis. (a-e) Mice received a single intraperitoneal injection (i.p) of 20 μg AIP-1 in PBS or vehicle, followed 5 h later by an enema with 2.5 mg of TNBS in 50% ethanol. (a) Body weight was recorded daily for the indicated groups. Data show means ± s.e.m. of a representative experiment out of five with n=5. Two-way ANOVA with Tukey’s comparisons test used to compare vehicle vs AIP-1 over time. (b, c) Colon length (cm) obtained from a representative mouse from each group. Histological score was performed by assessing epithelial changes (presence of goblet cells (1), hyperplasia (2), erosion (3)), cell infiltrate (4, 5), and mucosal architecture (6). Data show means ± s.e.m. of a representative experiment with n=5. Mann–Whitney U-test performed comparing naïve vs vehicle groups (#) or vehicle vs AIP-1 groups (*); *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

RESULTS
Recombinant Ac-AIP-1 protects against TNBS-induced intestinal inflammation

The immunoregulatory properties of Ac-AIP-1 have previously been explored using an in vitro T-cell suppression assay and protein-pulsed bone-marrow-derived dendritic cells.55 Suppression of inflammation using the related hookworm protein, AIP-2, was shown to be optimal at 1 mg kg−1 in a mouse model of asthma and colitis (unpublished). Therefore, the same dose for administering AIP-1 herein was used. To determine the therapeutic potential of AIP-1 in a model of acute inflammation, mice were treated with 1 mg kg−1 of AIP-1 or vehicle via the intraperitoneal (i.p) route. Five hours later, mice were administered 2.5 mg trinitrobenzoylsulfonic acid (TNBS) in 50% ethanol (b) Body weight was recorded daily for the indicated groups. Data show means ± s.e.m. of a representative experiment out of five with n=5. Two-way ANOVA with Tukey’s comparisons test used to compare vehicle vs AIP-1 over time. (b, c) Colon length (cm) obtained from a representative mouse from each group. Histological score was performed by assessing epithelial changes (presence of goblet cells (1), hyperplasia (2), erosion (3)), cell infiltrate (4, 5), and mucosal architecture (6). Data show means ± s.e.m. of a representative experiment with n=5. Mann–Whitney U-test performed comparing naïve vs vehicle groups (#) or vehicle vs AIP-1 groups (*); *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

A similar rationale was used earlier to investigate Ac-AIP-1 (also referred to as tissue inhibitor of metalloproteinase (TIMP)-1) as a potential modulator of dendritic and T-cell function.45 However, its efficacy as a therapeutic approach to suppress inflammatory disease was never tested. In this study, haptenating agent 2,4,6-trinitrobenzene sulfonic acid (TNBS) was used to evaluate the therapeutic validity of Ac-AIP-1 for treating acute colitis. Despite its limitations, the TNBS-model of colitis is T-cell mediated and skewed towards a Th2 phenotype comparable to human UC.46–48 Recombinant Ac-AIP-1 protected against all the hallmark parameters of inducible colitis and promoted a regulatory immune environment in treated mice.

Nematodes, and hookworms in particular, have been shown to ameliorate chronic inflammatory diseases by promoting regulatory immune circuits, particularly the induction of regulatory T cells (Treg) and the modification of the intestinal microbiota.28,32,34–37 We, and others, have shown that much of the immunomodulatory prowess of helminths can be attributed to the release of excretory/secretory (ES) products into host tissues.36,38–40 This complex mix of proteins and other molecules (unpublished) has been shown to ameliorate colitis in numerous mouse models,38,41 and denaturation of the protein component of ES products ablated the anti-colitic properties.38 Proteomic analysis of A. caninum excretory/secretory (ES) proteins revealed the relative abundance of two Tissue Inhibitor of Metallo protease (TIMP)-like proteins, anti-inflammatory protein (AIP)-1 and AIP-2,41 neither of which appear to have the protease inhibitory properties that characterise the TIMP family.44 We recently showed that AIP-2 induced the expansion of Treg that promoted long-term protection against allergic responses in both mice and humans.40

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ethanol via intrarectal injection, resulting in a 15 to 20% weight loss in the vehicle group over the course of the study (Figure 1a). Interestingly, mice treated with AIP-1 displayed <10% weight loss on the first day post-TNBS injection, and recovered most of their initial weight by day 3 (Figure 1a). Cellular enumeration of peritoneal lavages showed that i.p treatment with AIP-1 did not induce eosinophil infiltration at the site of injection (data not shown). In comparison to the naive control mice, colon lengths were significantly decreased in the vehicle group (P<0.0001), while AIP-1-treated mice remained unaltered by the administration of TNBS (Figure 1b). Macroscopic analysis of the colons revealed a significant reduction of tissue inflammation as seen by minimal adhesion, oedema, wall thickening and ulceration (Figure 1c). Haematoxylin and eosin (H&E) staining of distal colon sections from the vehicle group showed mucosal erosion and epithelial hyperplasia, pronounced cellular infiltration in the lamina propria and intraepithelial compartments, evidence of oedema and ulceration, and loss of healthy goblet cells (Figure 1d, top panels, Figure 1e). However, mice treated with AIP-1 displayed an overall mucosal architecture similar to that of healthy controls (Figures 1d and e). Mucin secretion and goblet cell numbers following periodic acid-Schiff-alcian blue (PAB) staining of the colons further illustrated that AIP-1 treatment suppressed inflammation both in experimental asthma and allergic inflammation, colons of mice exposed to TNBS were homogenised and analysed by ELISA (Figures 5a–c). In line with our previous observations, AIP-1Q119 treatment significantly suppressed inflammatory cytokines IL-13, IL-17 A and IFN-γ (Figures 5a–c). Interestingly, AIP-1Q119 administration also significantly increased the levels of thymic stromal lymphopoietin (TSLP) in the colon suggesting mucosal healing (Figure 5d). As seen previously in the spleen, IL-10 production in the colon of AIP-1Q119-treated mice was also markedly increased as well as TGF-β, suggesting the promotion of regulatory responses (Figures 5e and f). To reveal the potential involvement of regulatory T cells (Treg) in the protection against TNBS-induced colitis by AIP-1Q119, colons and peripheral tissues were collected and cells analysed by flow cytometry. While no significant differences were seen in the peripheral tissues, colons of mice treated with AIP-1Q119 displayed a significantly increased frequency of CD4⁺CD25⁺Foxp3⁺ cells (Figure 5g).

**AIP-1Q119 promotes colon immune regulation and tissue repair**

To address the impact of AIP-1Q119 treatment on the production of cytokines at the site of inflammation, colons of mice exposed to TNBS were homogenised and analysed by ELISA (Figures 5a–c). In line with our previous observations, AIP-1Q119 treatment significantly suppressed inflammatory cytokines IL-13, IL-17 A and IFN-γ (Figures 5a–c). Interestingly, AIP-1Q119 administration also significantly increased the levels of thymic stromal lymphopoietin (TSLP) in the colon suggesting mucosal healing (Figure 5d). As seen previously in the spleen, IL-10 production in the colon of AIP-1Q119-treated mice was also markedly increased as well as TGF-β, suggesting the promotion of regulatory responses (Figures 5e and f). To reveal the potential involvement of regulatory T cells (Treg) in the protection against TNBS-induced colitis by AIP-1Q119, colons and peripheral tissues were collected and cells analysed by flow cytometry. While no significant differences were seen in the peripheral tissues, colons of mice treated with AIP-1Q119 displayed a significantly increased frequency of CD4⁺CD25⁺Foxp3⁺ cells (Figure 5g).

**AIP-1Q119 affects the proinflammatory processes induced by TNBS in the colon**

To reveal suppression of inflammation by AIP-1Q119 in gut tissue, total RNA was extracted from colon sections of mice exposed to TNBS. Differentially expressed genes were identified by comparison of expression levels with vehicle-treated samples that served as baseline (Figure 6). Mice treated with AIP-1Q119 displayed a significant decrease in the expression of mRNAs in the colon encoding the proinflammatory mediators granulocyte macrophage colony-stimulating factor (GM-CSF)-, 2, cyclooxygenase (COX)-2, IL-6 and CXC-motif chemokine 11. Although not significant, mice treated with AIP-1Q119 also displayed increased expression of connective tissue growth factor (CTGF)-encoding mRNA, indicating potential enhanced tissue repair processes. Together, this profile suggests that AIP-1Q119 affects the expression of factors responsible for the migration of activated inflammatory cells such as neutrophils and lymphocytes, likely contributing to the reduction of tissue damage and promoting repair.

**DISCUSSION**

We have previously demonstrated that *A. caninum* excretory/secretory (AcES) products significantly alleviated intestinal pathology in a mouse model of UC. Protease digestion and heat denaturation showed the protective compound(s) of AcES were of protein moieties. Interestingly, despite AcES retaining characteristics of inducing Th2 cytokines, pro-regulatory and repair mechanisms were also observed. Indeed, proinflammatory cytokines IFN-γ, IL-6, IL-17 A and TNF-α were dramatically suppressed, and the recruitment of alternatively activated macrophages and IL-10/IL-4-producing cells were seen in the mucosa. As previously, shown by Mulvenna and colleagues, AcES is a complex mixture of over 100 proteins, the function of which remain mostly unknown. Recently, one of the most potent proteins produced by *A. caninum*, AIP-2, was shown to suppress allergen-induced inflammation both in experimental asthma and PBMCs from confirmed allergy patients. AIP-2 administration modified mesenteric dendritic cell function by enhancing...
retinaldehyde dehydrogenase activity, resulting in a significant accumulation of T_{reg} at mucosal sites. Interestingly, *A. cattinum* AIP-1 is also an abundant protein found in AcES; however, its function remains unclear. Ac-AIP-1, like Ac-AIP-2, contains a signal peptide followed by a TIMP-like domain. Ac-AIP-2 has a C-terminal tail that appears to be absent from Ac-AIP-1 (Figure 2), a feature previously observed in colonic mucosa in which the levels of IL-10 were significantly elevated in comparison to vehicle-treated mice but also in comparison with naive control. This further supports the notion that IL-10 production seems to be an important suppressive mechanism induced by AIP-1. A genetic-linkage analysis of patients with colitis revealed distinct mutations in the IL-10 gene, demonstrating a central role for this cytokine in the negative feedback regulatory mechanism induced by AIP-1. A genetic-linkage analysis of patients with colitis revealed distinct mutations in the IL-10 gene, demonstrating a central role for this cytokine in the negative feedback regulatory mechanism induced by AIP-1. A genetic-linkage analysis of patients with colitis revealed distinct mutations in the IL-10 gene, demonstrating a central role for this cytokine in the negative feedback regulatory mechanism induced by AIP-1.
trended towards elevated levels in AIP-1-treated mice, further supporting cell turnover, wound healing and tissue repair. While the direct action of AIP-1 on the epithelium, the production of TSLP and the inhibition of PGE-2 has yet to be fully demonstrated, AIP-1 seems to induce a multifactorial response beneficial for the suppression of inflammation and tissue damage induced by TNBS.

In like fashion to our findings with AIP-2 in a mouse model of asthma, AIP-1 seems to promote regulatory cells in the mucosa and suppress inflammation. However, the upregulation of genes involved in mucosal turnover observed herein for AIP-1 was not described for AIP-2. Indeed, AIP-1 seems to have a role in modulating local and systemic production of pro-regulatory cytokines, such as IL-10 and TGF-β, likely allowing tissue repair. Another cytokine important for mucosal repair in IBD is IL-22, which was significantly elevated in the colon of human volunteers experimentally infected with human hookworms. However, the involvement of IL-22 in AIP-1-induced protection in colitis has yet to be determined. Considering that both AIP-1 and AIP-2 are found abundantly in AcES, one can postulate that both proteins act concertedly to increase the number of regulatory cells and allow the tissue to rapidly heal from parasite-induced injury. While it would be pertinent to assess the combined therapeutic role of AIP-1 and AIP-2 in colitis, we have shown here that on its own, AIP-1 seems to be a good therapeutic candidate for the treatment of colitis by suppressing inflammatory responses, preventing tissue remodelling and promoting gut healing.

METHODS

Mice
Five-week-old male C57BL/6 were purchased from the Animal Resources Centre (Perth, Western Australia, Australia) and were housed according to Australian code for the care and use of animals for scientific purposes under specific pathogen-free conditions. Mice received food and water ad libidum. All procedures were approved by the James Cook University Animal Ethics Committee under projects A1484 and A2012.
Reagents and protein expression

Recombinant Ac-AIP-1 and the glycosylation mutant Ac-AIP-1Q119 were expressed as secreted proteins in the yeast *Pichia pastoris* using methods described elsewhere. Mutation of Asn-119 to Gln was achieved using PCR as described elsewhere. The cDNAs encoding the mature sequences of Ac-AIP-1 (amino acids 17-140) and Ac-AIP-1Q119 were cloned in frame into pPICZαA (Invitrogen, CA, USA) using *Xho*I and *Xba*I restriction sites. The recombinant plasmids were linearized by *Sac*I digestion and transformed into *P. pastoris* strain X-33 by electroporation according to the manufacturer’s instructions (Invitrogen). Transformants were selected on yeast extract-peptone-dextrose plates containing zeocin and assessed for expression of recombinant protein via western blot with monoclonal anti-6×His antibody. A western blot-positive clone for each protein was grown in a shaker flask, and expression of the recombinant 6×His tagged Ac-AIP-1 and Ac-AIP-1Q119 were induced with methanol, as per the manufacturer’s instructions (Invitrogen). The recombinant fusion proteins were purified with a nickel affinity column and eluates containing Ac-AIP-1 and Ac-AIP-1Q119 were concentrated using Amicon Ultra Centrifugal concentrators and buffer exchanged into phosphate-buffered saline (PBS) pH 7.4. Lipopolysaccharide contents in Ac-AIP-1 and Ac-AIP-1Q119 were below 5 ng mg⁻¹ as determined by the Limulus Amoebocyte Lysate (LAL) assay (Pierce Thermo Fisher Scientific, MA, USA).

Induction of colitis

Mice were randomly assigned to each group. Recombinant proteins were administered via the intraperitoneal (i.p) route in sterile phosphate-buffered saline at a dose of 1 mg kg⁻¹. Five hours later, mice were anaesthetised with xylazine (5 mg kg⁻¹, Rompun 2%, Bayer, Germany) and ketamine (50 mg kg⁻¹, Ketavesit; Pfizer Inc., NY, USA). 2,4,6-Trinitrobenzenesulfonic acid (TNBS; Sigma-Aldrich, MI, USA) was prepared by dissolving 2.5 mg in 50% ethanol. Once unresponsive, mice received an enema with a 125 mg kg⁻¹ dose of TNBS using a lubricated 20-G soft catheter (Terumo, Tokyo, Japan) as previously described. Animals were monitored daily for weight loss and general wellbeing over 4 days. Colitis experiments were repeated five times with a sample size (n) of five mice per experimental group.
accumulation as described previously. Morphology or periodic acid Schiff for detection of mucopolysaccharide containing 5% FBS, 400 U type I collagenase and 1 mg ml$^{-1}$ DNase I for扁平小鼠. Samples were homogenized in calcium- and magnesium-free Hank's Balanced Salt Solution and phosphatase and protease inhibitor cocktail (Roche, Basel, Switzerland). IFN-γ, TNF-α, IL-10, TGF-β (latent and active form), TSLP, IL-13 and IL-17 A were quantified by ELISA (BD Biosciences) from splenocyte supernatants and colon homogenates.

RNA extraction and gene array
A colon section (0.5 cm) was washed in PBS, placed in 1 ml of TRIzol and dissociated using a TissueLyser (Qiagen, Hilden, Germany) for 10 min with the use of metal beads. Total RNA extraction was performed by phenol–chloroform separation according to the manufacturer’s instructions. After treatment of RNA with RQ1 DNase (Promega, WI, USA), first-strand cDNA was produced with random hexamers and SuperScript III reverse transcriptase (Invitrogen). Samples were tested in 1:100 dilution using a custom wound healing RT2 Profiler PCR array and SYBR green (Qiagen). A Rotor-Gene 6000 (Qiagen) was used for real-time thermal cycling. Melting curve analysis was used to confirm that single products had been amplified. All genes were normalised for levels of transcription relative to the housekeeping genes beta-glucuronidase (Gusb), Hypoxanthine guanine phosphoribosyl transferase (Hprt), Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and beta-actin (Actb).

Cytokine quantification
Splenocytes were cultured in triplicate in flat-bottom 96-well plates (10$^6$ cells per well) either with complete RPMI 1640 medium alone or in medium supplemented with 1 μg ml$^{-1}$ anti-CD3 antibody (BD Biosciences) for 72 h at 37 °C and 5% CO$₂$. Colon samples were homogenized in calcium- and magnesium-free Hank’s Balanced Salt Solution and phosphatase and protease inhibitor cocktail (Roche, Basel, Switzerland). IFN-γ, TNF-α, IL-10, TGF-β (latent and active form), TSLP, IL-13 and IL-17 A were quantified by ELISA (BD Biosciences) from splenocyte supernatants and colon homogenates.

Clinical assessment of colitis
To eliminate bias, mice were assessed in a blinded fashion and de-identified at end point. Mice were weighed daily, and their overall appearance (piloerection), activity level and posture were recorded. No animals were excluded from the study. On day 3 following TNBS injection, mice were killed by CO$₂$ asphyxiation, and colons were collected for observation, characterisation by flow cytometry, cytokine measurements and RNA extraction. When dissecting, the level of tissue adhesion was scored from 0 to 3, with 0 corresponding to absence of adhesion and 3 corresponding to severe adhesion. Colons were measured, cut longitudinally, washed in saline, and observed under an Olympus SZ61 microscope (Notting Hill, VIC, Australia) ($×0.67–4.5$). Scoring of clinical pathology included adhesions (0–3), mucosal oedema (0–3), ulceration (0–3) and bowel wall thickening (0–3), for a maximum total score of 12 as previously described. Mesenteric lymph nodes (MLN), peripheral lymph nodes (brachial, inguinal, and popliteal) (PLN), spleens and colons were processed in RPMI 1640 media containing 2% foetal bovine serum (FBS), 400 U type I collagenase and 1 mg ml$^{-1}$ DNase I for扁平小鼠. Samples were tested in 1:100 dilution using a custom wound healing RT2 Profiler PCR array and SYBR green (Qiagen). A Rotor-Gene 6000 (Qiagen) was used for real-time thermal cycling. Melting curve analysis was used to confirm that single products had been amplified. All genes were normalised for levels of transcription relative to the housekeeping genes beta-glucuronidase (Gusb), Hypoxanthine guanine phosphoribosyl transferase (Hprt), Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and beta-actin (Actb).

Tissue preparation and cell culture
Mesenteric lymph nodes (MLN), peripheral lymph nodes (brachial, inguinal, and popliteal) (PLN), spleens and colons were processed in RPMI 1640 media containing 2% foetal bovine serum (FBS), 400 U type I collagenase and 1 mg ml$^{-1}$ DNase I for flat-bottom 96-well plates (10$^6$ cells per well) either with complete RPMI 1640 medium alone or in medium supplemented with 1 μg ml$^{-1}$ anti-CD3 antibody (BD Biosciences) for 72 h at 37 °C and 5% CO$₂$. Colon samples were homogenized in calcium- and magnesium-free Hank’s Balanced Salt Solution and phosphatase and protease inhibitor cocktail (Roche, Basel, Switzerland). IFN-γ, TNF-α, IL-10, TGF-β (latent and active form), TSLP, IL-13 and IL-17 A were quantified by ELISA (BD Biosciences) from splenocyte supernatants and colon homogenates.

Statistical analyses
All data were analysed with GraphPad Prism (version 7; San Diego, CA, USA). Sample size ($n=5$) was determined by using a power of 80%, one-sided test, representing the probability of finding significant differences between vehicle and AIP-1 or AIP-1Q119-treated groups, with an acceptable Type 1 error of 0.05 and an expected effect size of 1.8. Data are expressed as the mean ± s.e.m. Body weight values were analysed using two-way analysis of variance (ANOVA) followed by the Tukey’s post-hoc test. Comparisons for all pairs were performed by unpaired two-tailed Mann–Whitney U-test. Significance levels were set at a $P$ value of 0.05.

CONFLICT OF INTEREST
AL and SN have jointly invented a certain invention, entitled METHOD FOR TREATING INFLAMMATION, as described in the following patent applications: PCT Patent Application No. PCT/AU2013/000247, filed on 13 March 2013 with priority of 13 March 2012; and subsequent national phase patent applications in Australia, US, Europe, Canada, China, Japan, India, New Zealand and South Africa. The remaining authors declare no conflict of interest.

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Author contributions: IBF and SN performed the experiments. SN and AL conceived the study, designed the experiments and wrote the manuscript. DAP and ST expressed the proteins, generated the protein mutant and proofread the manuscript. JC gave advice on experimental design and proofread the manuscript.

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