<u>A</u> Novel Sphingomyelinase-Like Enzyme in <u>Ixodes scapularis</u> Tick Saliva Drives Host CD4+ T cells to Express IL-4

F. J. Alarcon-Chaidez[1,3,4], **V. D. Boppana**[1,3,4], **A.T. Hagymasi**[1,2], **A. J. Adler**[1,2], and **S. K. Wikel**[1,3]

[1]Department of Immunology of Cancer and Infectious Diseases, School of Medicine, University of Connecticut Health Center, Farmington, CT 06030, USA

[2]Center for Immunotherapy of Cancer and Infectious Diseases, School of Medicine, University of Connecticut Health Center, Farmington, CT 06030, USA

Abstract

Tick feeding modulates host immune responses. Tick-induced skewing of host CD4+ T cells towards a Th2 cytokine profile facilitates transmission of tick-borne pathogens that would otherwise be neutralized by Th1 cytokines. Tick-derived factors that drive this Th2 response have not previously been characterized. In the current study, we examined an *I. scapularis* cDNA library prepared at 18-24 hours of feeding and identified and expressed a tick gene with homology to *Loxosceles* spider venom proteins with sphingomyelinase activity. This *I. scapularis* sphingomyelinase-like (IsSMase) protein is a Mg^{+2}-dependent, neutral (pH 7.4) form of sphingomyelinase. Significantly, in an in vivo TCR transgenic adoptive transfer assay IsSMase programmed host CD4+ T cells to express the hallmark Th2 effector cytokine IL-4. IsSMase appears to directly program host CD4 T cell IL-4 expression (as opposed to its metabolic by-products) because induced IL-4 expression was not altered when enzymatic activity was neutralized. TCR transgenic CD4 T cell proliferation (CFSE-dilution) was also significantly increased by IsSMase. Furthermore, a Th2 response is superimposed onto a virally-primed Th1 response by IsSMase. Thus, IsSMase is the first identified tick molecule capable of programming host CD4+ T cells to express IL-4.

Keywords

Ixodes scapularis; BALB/c; CD4; immune modulation

INTRODUCTION

Ticks transmit a variety of disease-causing infectious agents of medical and veterinary importance (1). Established, resurging and emerging tick-borne diseases of human health importance include spotted fevers, ehrlichioses, Lyme borreliosis, babesiosis, tick-borne encephalidides and hemorrhagic fevers (2,3). Additionally, tick toxicoses and tick-

transmitted theileriosis, babesiosis, cowdriosis and anaplasmosis are major constraints on livestock production (1).

Ticks feed on the blood of mammals over the course of several days, and have therefore evolved complex strategies to facilitate blood feeding. Tick saliva contains numerous pharmacologically active molecules that modulate host haemostasis, wound healing, pain and itch responses and inflammation (4,5,6,7,8). These tick-induced changes in host defenses that facilitate extended blood feeding also enhance pathogen transmission (4,9). The salivary gland transcriptome of the tick *Ixodes scapularis* contains over 800 gene clusters of putatively secreted proteins, and many genes within a given cluster display high levels of polymorphism and are differentially expressed during the course of blood feeding (7).

The ability of ticks to modulate host immune responses in particular is thought to be central in facilitating pathogen transmission. In vitro studies have shown that *Ixodes scapularis* salivary gland extract contains an IL-2 binding protein (10) as well as the protein Salp15 that inhibits T cell proliferation (11,12) and dendritic cell production of IL-6, IL-12p70 and TNF (13). In vitro studies have also shown that tick saliva can polarize CD4+ T cells towards a Th2 cytokine profile marked by IL-4 expression (4,14,15,16,17). It is likely that this Th2 response and concomitant suppression of Th1 responsiveness (marked by IFN-γ expression) facilitates the transmission of pathogens that would otherwise be neutralized by Th1 cytokines.

We recently developed an in vivo TCR transgenic adoptive transfer system to study the influence of *I. scapularis* feeding on host CD4+ T cell responses (18). Although this model system is normally strongly Th1 biased (19,20), *I. scapularis* infestation or salivary gland extract (SGE) induced host CD4 T cells to express IL-4 (18). This tick-induced host CD4 T cell IL-4 expression is likely relevant to pathogen transmission because repeated infestation resulted in partially reduced IL-4 expression (18), and repetitive tick infestation is correlated with a reduced ability to modulate host immunity and to transmit pathogens (21). Furthermore, *I. scapularis* nymphs infected with *Borrelia burgdorferi* also display a partially reduced ability program host CD4 T cells to express IL-4 (18), illustrating that ticks and their transmitted pathogens drive antagonistic host immune responses.

Identification and characterization of the factor(s) present in tick saliva that drive host CD4 T cell IL-4 expression is of critical importance as this could inform strategies to prevent pathogen transmission. In fact, although factors that drive Th1 responsiveness have been extensively characterized, very few molecules have been identified that can drive Th2 responses (22,23,24). In the current study we examined an *I. scapularis* salivary gland cDNA library prepared at 18-24 hour of feeding and characterized a clone with homology to the dermonecrotic protein LiD1 found in the venom gland of the spider *Loxosceles intermedia* (25). LiD1 belongs to a family of venom proteins with sphingomyelinase (SMase) activity (26). The *I. scapularis* SMase-like clone was selected for study because of its expression early in blood feeding and due to the fact that membrane lipid rafts are specialized membrane microdomains containing sphingomyelin that are involved in T cell receptor signalling upon antigen engagement (27). Lipid rafts differ for Th1 and Th2 CD4 T cells, making T cell membrane lipids a target for modulation of T cell responses (28). Importantly, this *I. scapularis* SMase-like protein (IsSMase) can program host CD4 T cell IL-4 expression independently of its enzymatic activity.

MATERIALS AND METHODS

Mice and adoptive transfer

6.5 TCR transgenic mice that express a TCR transgenic TCR specific for an I-E^d - restricted epitope deriving from hemagglutinin (HA) of influenza virus PR8 ([110]SFERFEIFPKE[120]) (29) were maintained on a BALB/c (H-2^d) Thy1.1 background. Lymph node preparations from 6.5 TCR transgenic mice were depleted of CD8$^+$ cells using magnetic beads, and the naive Thy1.1$^+$ HA-specific TCR transgenic CD4 T cells were labelled with CFSE prior to adoptive transfer in Thy1.2$^+$ BALB/c recipients (purchased from the Jackson Laboratory) that had been exposed to various forms of HA antigen as well as ticks or tick products, and subsequent functional analyses were performed as previously described (18,19,20). In short, adoptive transfer recipients were treated intradermally with either a bolus of soluble HA peptide (200 ug) or 10^5 PFU of a recombinant vaccinia virus expressing HA (viral-HA) at the site of tick feeding or injection of tick product. Four days post-transfer, adoptively transferred TCR transgenic CD4 T cells (identified as CD4$^+$Thy1.1$^+$) were recovered from the draining brachial and axillary lymph nodes, and the ability of the divided (CFSE-diluted) TCR transgenic CD4 T cells to express intracellular cytokines was measured by FACS following in vitro restimulation with 100 μg/ml HA peptide plus 5 μg/ml Brefeldin A (Sigma-Aldrich). All quantitative data are expressed as the mean ± SEM, and differences in cytokine expression levels between experimental groups were analyzed using an unpaired two-tailed Students t-test.

The University of Connecticut Health Center Institutional Animal Care and Use Committee approved all protocols used in this study.

Ticks and tick infestation

A pathogen-free *I scapularis* colony was maintained as previously described (30). In short, all life cycle stages are kept in sterile glass vials with mesh tops in desiccators over a saturated solution of potassium sulfate to maintain 97% relative humidity at 22°C with a 14 hour light: 10 hour dark photoperiod. Adult ticks blood feed on New Zealand white rabbits and nymphs and larvae are fed on mice.

Tick exposed mice were infested with pathogen-free *I. scapularis* nymphs confined within a capsule consisting of top half of a 1.5 ml microcentrifuge tube (Fisher Scientific) secured to clipped fur on the shoulder region with a 4:1 mixture (w/w) of calophonium (rosin, Sigma-Aldrich) and beeswax. Engorged nymphs were removed from the capsule as they detached from the mouse.

Salivary gland extract (SGE)

Adult *I. scapularis* fed for 4 days on white New Zealand rabbits (30), at which time females were removed for collection of salivary glands. Prior to dissection, ticks were washed with sterile distilled water and then surface sterilized with 70% ethanol. Salivary glands were removed and placed together into a minimal volume of 0.15 M Dulbecco's phosphate buffered saline, pH 7.2 (1x PBS, Gibco) held on ice. Pooled salivary glands were sonicated at 55 kHz for 1 minute while held in an ice water bath followed by centrifugation at 14,000 g for 20 minutes at 4°C. Supernatant was collected as salivary gland extract (SGE) and protein concentration determined by bicinchoninic acid assay (31). SGE was divided into 50 μl aliquots and stored at -30°C. All SGE samples used in this study were frozen and thawed once.

Cloning and expression

A cDNA from the salivary glands of *I. scapularis* (7) encoding a putative sphingomyelinase-like (IsSMase) protein (NCBI accession Q202J4) was amplified by PCR using the forward primer 5′-GGGGGACAACTCTCTTGGGTTTTT-3′ and the reverse primer 5′-TATGTAGAAGGGGCTCAAAGGATT-3′. Amplified sequence was cloned into the insect cell expression vector pIB/V5-His-TOPO (Invitrogen), which contains a blasticidin resistance gene, a 14-amino acid epitope (V5) and a polyhistidine (6xHis) sequence at the C-terminus to facilitate purification. Integrity of the resulting construct was assessed by sequencing in both directions.

Endotoxin-free recombinant plasmids were purified using the EndoFree Plasmid Maxi Kit (Qiagen) and transfected into High 5, *Trichopulsia ni*, cells (Invitrogen) according to the manufacturer's protocol. Stably transfected cells were grown in a 2 L Wave Bioreactor (GE Healthcare) at 27°C and maintained in the presence of blasticidin at a final concentration of 10 μg/ml. Cultures reaching densities $5\text{-}8 \times 10^6$ cells/ml were processed by centrifugation at 6,000xg to remove cells and particulate matter. Cell culture supernatants were buffer-exchanged with 1x PBS and concentrated at least 10X with a Vivaflow 200 tangential flow ultrafiltration system (Vivascience) fitted with low protein-binding regenerated cellulose membranes (10 kDa MWCO). Concentrates were loaded onto a Ni+2-nitriloacetic acid (NiNTA, Qiagen) column that was pre-equilibrated and washed with 50 mM NaH_2PO_4, 500 mM NaCl, 10-15 mM Imidazole, pH 8.0, and the His-tagged proteins were eluted with 50 mM NaH_2PO_4, 500 mM NaCl, 250 mM Imidazole, pH 8.0. Eluted fractions were extensively dialyzed against PBS and concentrated, using Ultrafree-4 concentrators (10 kDa MWCO, Amicon). Protein was quantified by the bicinchoninic acid (BCA) assay (31). Purity was assessed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (32) and gels were stained with Coomassie blue.

Sphingomyelinase activity assay

Functional activity of recombinant IsSMase in neutral (pH 7.4) or acidic (pH 5.0) conditions was determined by monitoring the rate of sphingomyelin hydrolysis at 37°C, using Amplex Red Sphingomyelinase Assay Kit (Invitrogen). Briefly, recombinant IsSMase (12.5 μg/ml) or controls were mixed in triplicate with 10 mM Amplex Red reagent (Invitrogen), 2 U/ml horseradish peroxidase, 0.2U/ml choline oxidase, 8 U/ml alkaline phosphatase, 0.5 mM sphingomyelin, and reaction buffer (100 mM Tris-HCl, pH 7.4) containing 10 mM $MgCl_2$, 10 mM $MnCl_2$, or 10 mM $ZnCl_2$ in a final volume of 300 ul/well in 96-well microtiter plates. For each test, reaction buffer containing no enzyme was used as a negative control while sphingomyelinase from *Staphylococcus aureus* (0.02 U/ml, where one unit is defined as the amount of sphingomyelinase that will hydrolyse 1 μmole of TNPAL-sphingomyelin per minute at pH 7.4 at 37°C) was a positive control. Sphingomyelinase induced conversion of the Amplex Red reagent to red-fluorescent resorufin was detected using excitation and emission wavelengths of 530 nm and 590 nm, respectively. Fluorescence was recorded at 10 min intervals over a period of 3 h to follow the kinetics of the reaction. For each point, background fluorescence was corrected by subtracting the negative control values. For testing under acidic conditions, 100 mM Tris-HCL buffer was substituted for 50 mM sodium acetate, pH 5.0, and enzyme and substrate were mixed and incubated for 1 h before addition of Amplex Red reagent. The effects of using different metal ions was determined under similar conditions by substituting Mg^{+2} with Mn^{+2} or Zn^{+2} in the reaction buffer. In both instances, end-point measurements were taken after an additional 2 hr incubation period. To neutralize IsSMase enzymatic activity the protein was subjected to two freeze (-80°C) and thaw cycles.

RESULTS

Molecular cloning of a SMase-like protein from *I. scapularis*

A BLAST search analysis of a cDNA library from the salivary glands of partially fed (18-24 h) *I. scapularis* females (7) revealed the presence of a cDNA clone with 39% nucleotide and 59% amino acid homology to the dermonecrotic protein LiD1(Accession No. AAQ16123) found in the venom gland of the spider *Loxosceles intermedia* (25). LiD1 is a 31 kDa protein belonging to a group of dermonecrotic proteins that cause severe skin lesions and other systemic effects through intense sphingomyelinase (SMase) activity against cellular matrix (26).

The putative protein product of the *I. scapularis* gene was named IsSMase. Predicted translation of the full-length sequence of IsSMase revealed an open reading frame (ORF) of 365 amino acids. A predicted signal peptide cleavage site was identified by the SignalP 3.0 program (33) and shown to reside between amino acids 17 (C) and 18 (Q) of the translated ORF, resulting in a theoretical 39.7 kDa mature protein with an isoelectric point of 6.03. Comparison of the translated sequence of IsSMase against the Conserved Domain Database (CDD) revealed motifs conserved in the glycerophosphoryl diester phosphodiesterase super-family UgpQ (COG0584). Alignment of spider and tick SMases is shown in Fig. 1 with conserved motifs indicated.

Primary sequence comparison suggested that residues involved in the catalysis mechanism by spider SMases may be conserved in the tick protein. Tick SMase could display an $(\alpha/\beta)8$ barrel fold as described for *Loxosceles* SMase D (34) with the active site formed by His29 and His70 and the amino acid residues Glu40, Asp51, and Asp109 involved in metal ion coordination. In addition, the catalytic loop could be stabilized by a disulfide bridge formed between residues Cys69 and Cys75 (arrows) as described for the spider SMase D (Fig. 1).

Protein expression and purification

The final yield of recombinant IsSMase secreted by High 5 cells was in the range of 200-500 μg/L in harvested medium. SDS-PAGE analysis of recombinant IsSMase purified from insect cell culture supernatants (Fig. 2*A*) indicated an apparent size, including the 6x His and V5 tags, of ~45 kDa that closely matched the size predicted from the cDNA sequence.

Sphingomyelinase activity

In an in vitro sphingomyelinase assay, a concentration of 12.5 μg/ml of recombinant IsSMase resulted in a gradual increase in fluorescence intensity over the time interval assayed indicating hydrolysis of sphingomyelin which was similar to that observed when using 0.02 U/ml *S. aureus* sphingomyelinase (positive control) (Fig. 2*B*). Analysis of IsSMase in acidic conditions (50 mM sodium acetate, pH 5.0) resulted in significantly reduced fluorescence intensity (Fig. 2*C*). In addition, substitution of Mg^{+2} ions by Mn^{2+} resulted in 81.0 % decrease in sphingomyelinase activity while the use of Zn^{2+} ions almost completely abolished this activity (<1.0%) (Fig. 2D). These results suggest that IsSMase is likely a Mg^{+2}-dependent, neutral (pH 7.4) form of sphingomyelinase.

IsSMase programs CD4 T cells to express IL-4

As previously observed in our TCR transgenic adoptive transfer model (18), intradermal injection of soluble HA peptide into control mice resulted in significant proliferation of TCR transgenic CD4 T cells as measured by CFSE dilution (Fig. 3, *A* and *B*). These divided TCR transgenic CD4 T cells, however, neither gained the ability to express the Th1 cytokine IFN-γ nor the Th2 cytokine IL-4 (Fig. 3*C*). This non-immunogenic response is consistent with

the absence of pathogen-associated molecular patterns (PAMPs) or other adjuvant-like molecules (35). Intradermal injection of 5 μg SGE programmed a fraction of the divided TCR transgenic CD4 T cells to express IL-4 (Fig. 3 C and D). Although this fraction was relatively small (~5%), this effect was highly reproducible (P<0.0001 compared to control) and similar in magnitude to our previous studies (18). A roughly similar level of IL-4 expression was induced by 5 μg recombinant IsSMase (P<0.0001 compared to control) (Fig 3 C and D). TCR transgenic CD4 T cell proliferation (CFSE-dilution) was also significantly increased by both SGE (P<0.004) and IsSMase (P<0.0001) over background (Fig. 3, A & B). Importantly, a recombinant *Aedes aegypti* salivary gland Kazal serine protease inhibitor protein expressed and purified in the same way as IsSMase lacked IL-4 polarizing activity (data not shown), indicating that IsSMase possesses specific Th2-skewing activity.

IsSMase superimposes a Th2 response onto a virally-primed Th1 response

As previously observed, TCR transgenic CD4 T cells primed by a recombinant vaccinia virus expressing HA (viral-HA) in the absence of tick infestation or intradermal injection of either IsSMase or SGE proliferated more vigorously (Fig. 4, A and B) and developed a greater capacity to express IFN-γ (Fig. 4C) compared to TCR transgenic CD4 T cells from soluble HA peptide administered mice. Similar to tick infestation and injection of SGE, injection of IsSMase did not alter either proliferation or IFN-γ in response to viral-HA but did increase IL-4 expression (P=0.004). The fraction of TCR transgenic CD4 T cells expressing IL-4 with IsSMase + viral-HA was somewhat lower than with peptide + IsSMase (Fig 4 C and D), suggesting a degree of antagonism in the activities of virus and IsSMase. Taken together, IsSMase is the first salivary gland molecule of any tick species to be demonstrated to cause Th2 polarization of host CD4+ T cells.

IsSMase enzymatic activity is not required to program CD4 T cell IL-4 expression

To test whether SMase enzymatic activity is required to program host CD4 T cell IL-4 expression, we first tested bacterial SMase in an attempt to rule out sphingomyelinase activity as the sole reason for this effect (Fig. 2*B*). Intradermally administered 10 U bacterial SMase did not program TCR transgenic CD4 T cells to express IL-4 (Fig. 5*A*). Furthermore, IsSMase that was subjected to two freeze-thaw cycles and lost detectable enzymatic activity in the Amplex Red Sphingomyelinase Assay (data not shown) retained its capacity to drive CD4 T cells to express IL-4 (P=0.004) (Fig 5, *A* and *B*) whereas expression of IFN-γ was not significantly altered.

DISCUSSION

Ticks remain attached, inject saliva and feed on host blood for 4 to 15 days with a typical adult female tick consuming approximately 4,000 mg of host blood (36). This intimate host contact resulted in evolution of differentially expressed salivary gland transcriptomes encoding hundreds of putatively secreted proteins that modulate or act as counter measures to multiple physiological components of host haemostasis, wound healing, and innate and specific acquired immune defences (4,7,8).

A broadly recurrent theme among ixodid tick biology is that host T cell responses are redirected towards a Th2 profile marked by IL-4 expression, which often is accompanied with suppression of Th1 cytokines (4,14,15,16,17,18). Furthermore, this Th2 response appears to facilitate pathogen transmission (37 38,39,40), while repeated infestations reduces the extent of both the degree of tick-induced host Th2 response and pathogen transmission (18,21). Here, we report the cloning, expression and characterization of the first tick salivary gland molecule responsible for programming IL-4 expression in host CD4+ T cells.

Discovery of a cDNA clone with amino acid homology to *L. intermedia* dermonecrotic protein (25) was of interest due its presence in a salivary gland cDNA library prepared early in tick attachment and blood feeding. We hypothesized that the product of this gene contributed to formation of the hemorrhagic pool from which the tick feeds. IsSMase shares an identical glycerphosphoryl diester phosphodiesterase domain catalytic site with venom SMases of *L. intermedia* (26). This relationship is not surprising since spiders and ticks are both members of the class Arachnida. Tick recombinant IsSMase shows homology to a spider dermonecrotic protein; however, intradermal injection of 5 μg recombinant IsSMase did not induce any visually detectable cutaneous changes (data not shown) suggesting little if any dermonecrotic activity. Intense dermonecrosis at the tick attachment site would not be desirable due to potential disruption of tick feeding and creation of a site favorable for secondary bacterial and fungal infections. However, we cannot rule out the possibility that IsSMase enzymatic activity contributes to feeding lesion formation in combination with the many other enzymes in *I. scapularis* saliva (7).

SMase biological properties are relevant to tick feeding and pathogen transmission. SMases catalyze hydrolysis of sphingomyelin into phosphorylcholine and ceramide (41). The latter is then catabolized to sphingosine by ceramidase, and sphingosine kinase converts sphingosine to sphingosine-1-phosphate (42). Neutrophils are a significant component of the histopathological response to tick feeding (43,44); however, ceramide suppresses neutrophil spreading and respiratory burst in the presence of TNF (45) which is known to be induced by *I. scapularis* feeding (15). Additionally, *I. scapularis* saliva reduces neutrophils, β2-integrin expression, and uptake and killing of *B. burgdorferi* spirochetes (46). Finally, sphingosine-1-phosphate acts on T cell differentiation during activation to decrease Th1 and enhance Th2 responses by reducing dendritic cell TNF and IL-12, which act to initiate Th1 differentiation (47).

The current study is the first to identify a tick molecule responsible for the programming of an IL-4 response by host CD4+ T cells. IsSMase enzymatic activity is not required to program IL-4 expression, therefore it is less likely that ceramide and sphingosine-1-phosphate are responsible for the observed IL-4 programming. We speculate that a freeze-thaw stable structure within IsSMase might bind a TLR or other receptor on APC or innate immune cells that in turn facilitate Th2 differentiation. It cannot be ruled out; however, that IsSMase enzymatic activity might contribute to modulation of certain facets of host immune responses such as lipid rearrangements involved in T cell receptor signalling (27). IsSMase may not be the only factor contained within SGE that can program host CD4 T cells to express IL-4, and it might be possible that multiple factors synergize to mediate this activity. An additional point to consider is that ticks obtain a blood meal over a period of approximately five days for a nymph and up to 14 days for an adult. The amount of saliva introduced varies during the course of feeding as does the amount of blood consumed (36). We also know that differential expression of salivary gland genes occurs during feeding (7), which could change the amount of a saliva protein introduced.

Development of a Th1 or Th2 CD4+ T cell response is primarily driven by pathogen-associated molecular patterns (PAMPs) from microbes and metazoan parasites that interact with specific TLRs on dendritic cells (23,48). PAMP-TLR combinations that drive Th1 responses are well characterized, however, knowledge of combinations that elicit Th2 responses is extremely limited (49). The synthetic lipopeptide Pam3cys can drive Th2 responses via TLR2 (48), and other molecules that can elicit host IL-4 expression include: *Schistosoma mansoni* soluble egg antigen, the filarial nematode phosphorylcholine-containing glycoprotein ES-62, *Toxoplasma gondii* stimulated lipoxins, certain forms of *Candida albicans*, *Porphyromonas gingivalis* LPS and *Nippostrongylus brasiliensis* chitin

(24,48,49,50,51). To this limited group of factors, we add *I. scapularis* sphingomyelinase (IsSMase).
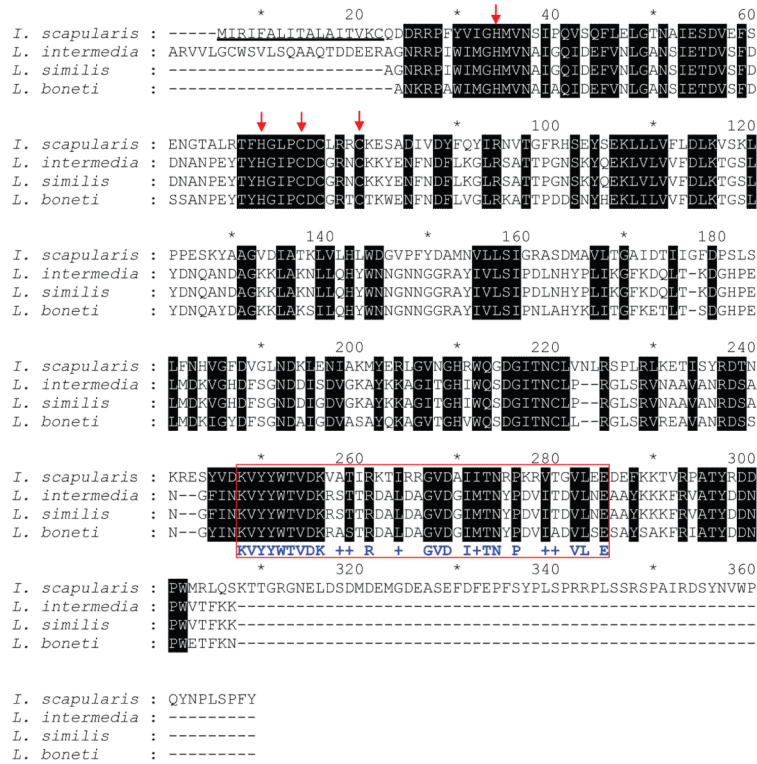
Acknowledgments

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                      *        20         *       40         *        60
I. scapularis : -----MIRIFALITALAITVKCQDDRRPFYVIGHMVNSIPQVSQFIELGTNAIESDVEES
L. intermedia : ARVVLGCWSVLSQAAQTDDEERAGNRRPIWIMGHMVNAIGQIDEFVNLGANSIETDVSFD
L. similis    : ---------------------AGNRRPIWIMGHMVNAIGQIDEFVNLGANSIETDVSFD
L. boneti     : ---------------------ANKRPAWIMGHMVNAIAQIDEFVNLGANSIETDVSFD


                      *        100        *       120
I. scapularis : ENGTALRTFHGLPCDCLRRCKESADIVDYFQYIRNVTGFRHSEYSEKLLLVFLDLKVSKL
L. intermedia : DNANPEYTYHGIPCDCGRNCKKYENFNDFLKGLRSAGTPGNSKYQEKLVLVVFDLKTGSL
L. similis    : DNANPEYTYHGIPCDCGRNCKKYENFNDFLKGLRSAGTPGNSKYQEKLVLVVFDLKTGSL
L. boneti     : SSANPEYTYHGIPCDCGRTCTKWENFNDFLVGLRKATTPDDSNYHEKLILVVFDLKTGSL


                      *        140        *       160        *        180
I. scapularis : PPESKYAAGVDIATKLVLHLWDGVPFYDAMNVLLSIGRASDMAVLTGAIDTIIGFDPSLS
L. intermedia : YDNQANDAGKKLAKNLLQHYWNNGNNGGRAYIVLSIPDLNHYPLIKGFKDQLT-KDGHPE
L. similis    : YDNQANDAGKKLAKNLLQHYWNNGNNGGRAYIVLSIPDLNHYPLIKGFKDQLT-KDGHPE
L. boneti     : YDNQAYDAGKKLAKSILQHYWNNGNNGGRAYIVLSIPNLAHYKLITGFKETLT-SDGHPE


                      *        200        *       220        *        240
I. scapularis : IFNHVGFDVGLNDKLENIAKMYERLGVNGHRWQGDGITNCLVNLRSPLRLKETISYRDTN
L. intermedia : LMDKVGHDFSGNDDISDVGKAYKKAGITGHIWQSDGITNCIP--RGLSRVNAAVANRDSA
L. similis    : LMDKVGHDFSGNDDIGDVGKAYKKAGITGHIWQSDGITNCIP--RGLSRVNAAVANRDSA
L. boneti     : LMDKIGYDFSGNDAIGDVASAYQKAGVTGHVWQSDGITNCIL--RGLSRVREAVANRDSS


                      *        260        *       280        *        300
I. scapularis : KRESYVDKVYYWTVDKVATIRKTIRRGVDAIITNRPKRVTGVLEEDEEKKTVRPATYRDD
L. intermedia : N--GFINKVYYWTVDKRSTTRDALDAGVDGIMTNYPDVITDVLNEAAYKKKFRVATYDDN
L. similis    : N--GFINKVYYWTVDKRSTTRDALDAGVDGIMTNYPDVITDVLNEAAYKKKFRVATYDDN
L. boneti     : N--GYINKVYYWTVDKRASTRDALDAGVDGIMTNYPDVIADVLSESAYSAKFRIATYDDN
                       KVYYWTVDK ++ R  +  GVD I+TN P  ++ VL E


                      *        320        *       340        *        360
I. scapularis : PWMRLQSKTTGRGNELDSDMDEMGDEASEFDFEPFSYPLSPRRPLSSRSPAIRDSYNVWP
L. intermedia : PWVTFKK-----------------------------------------------------
L. similis    : PWVTFKK-----------------------------------------------------
L. boneti     : PWETFKN-----------------------------------------------------


I. scapularis : QYNPLSPFY
L. intermedia : ---------
L. similis    : ---------
L. boneti     : ---------
```
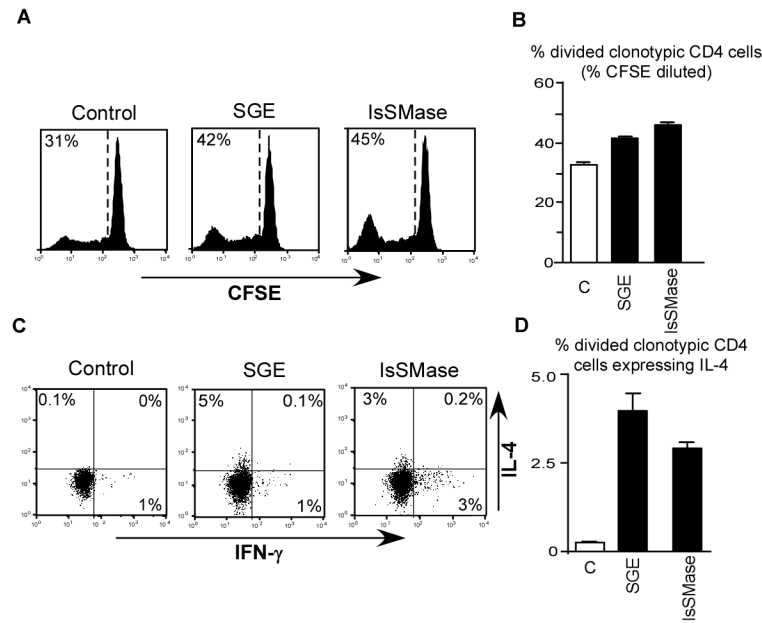
Figure 1.
Alignment of *I. scapularis* SMase-like protein (Q202J4) and the sphingomyelinase proteins from <u>*Loxosceles*</u> spp. spiders. Searches against the conserved domain databases (CDD) revealed motifs conserved in the glycerophosphoryl diester phosphodiesterases family UgpQ (COG0584). An alignment of the tick protein and the reported Loxosceles sp. SMases shows the conserved motif (red square) and the catalytically important residues H34, H70, C76, and C80 (arrows). *Loxosceles intermedia* (AAQ16123), *L. similis* (Q56JA9), *L. boneti* (Q5YD77). The predicted leader peptide for IsSMase is underlined.
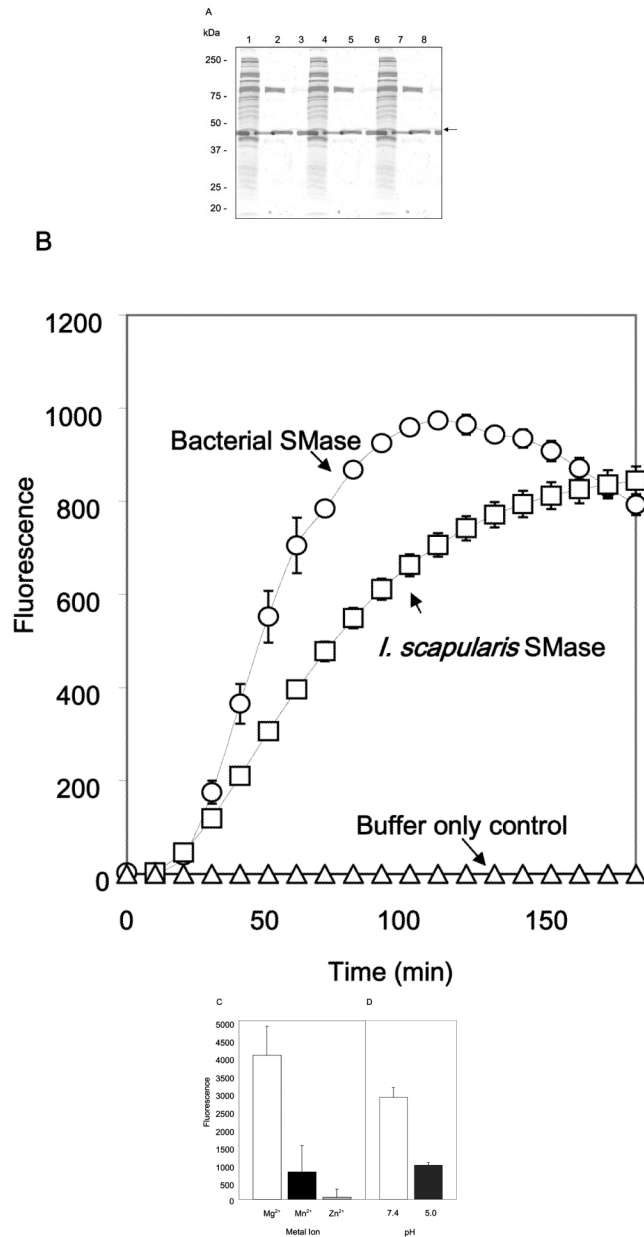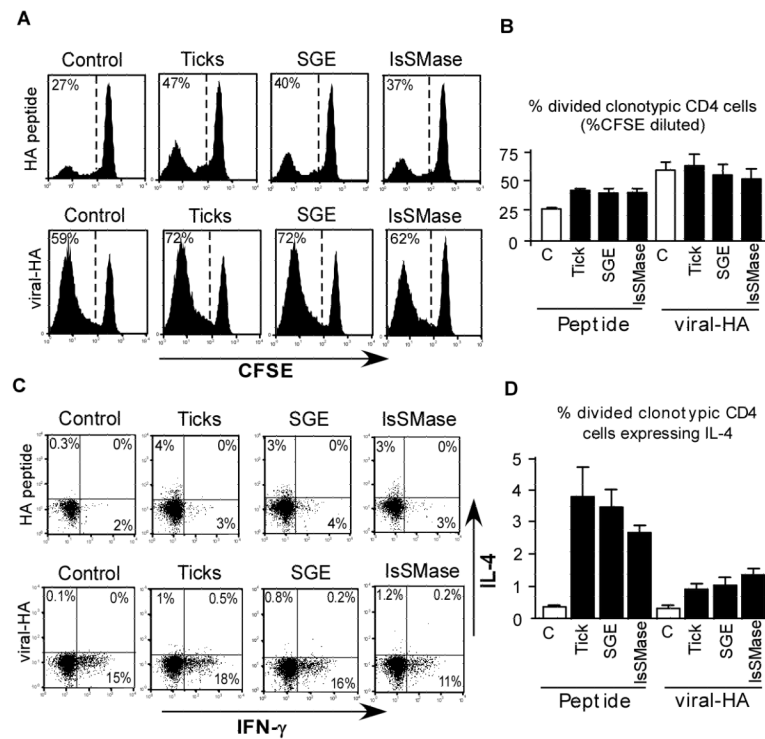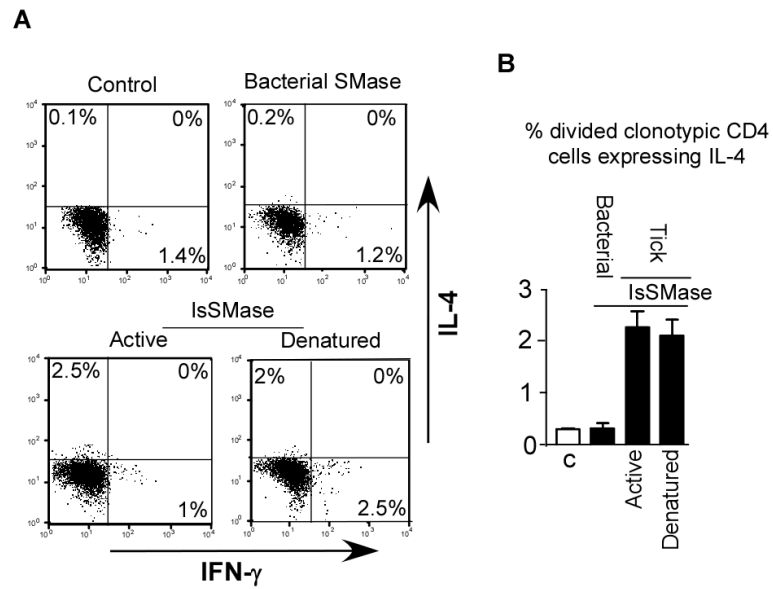
Figure 2.
Expression and functional activity of IsSMase. *A*, Purified recombinant IsSMase <u>was resolved</u> by 10% discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) <u>and stained with Coomassie blue dye</u>. Lane 1, molecular weight standard (kDa); Lane 2, flow-through; Lane 3, final wash; Lanes 4-8, eluates containing purified IsSMase (indicated by arrow). *B*, Functional activity of IsSMase was assessed by monitoring the rate of sphingomyelin hydrolysis at 37°C under standard conditions. C and D, effect of metal ions and pH on IsSMase activity. The sphingomyelinase activity of the IsSMase was recorded at multiple time points using excitation in the range of 530-560 nm and emission detection at 590 nm. Data plotted represent means ± SE (n=3). *C*, Activity of IsSMase at different pH.

Figure 3.
Recombinant IsSMase programs CD4 T cells responding to associated soluble antigen to develop the capacity to express IL-4. Adoptively transferred CFSE-labeled BALB/c HA-specific TCR transgenic CD4 T cells were recovered following adoptive transfer into BALB/c recipients that were simultaneously treated intradermally with soluble HA peptide and either SGE, IsSMase or PBS (Control or C). *A*, Representative CFSE-dilution histograms (left side, with the percentage of TCR transgenic CD4 T cells that have diluted CFSE-fluorescence shown) and *B*, quantitative data (mean ± SEM) corresponding to panel A. *C*, Representative IFN-γ vs IL-4 intracellular staining plots corresponding to the CFSE-divided TCR transgenic CD4 T cells in panel A. *D*, Quantitative analysis of intracellular IL-4 expression. *N*=4 to 6 for each group.

Figure 4.
Recombinant IsSMase superimposes IL-4 expression potential in CD4 T cells undergoing
Th1 differentiation in response to viral immunization. Adoptively transferred CFSE-labeled
BALB/c HA-specific TCR transgenic CD4 T cells were recovered 4 days following
adoptive transfer into BALB/c recipients that were simultaneously injected intradermally
with either soluble HA peptide or viral-HA at the site either infested with 10 nymphs on day
-1 or intradermally injected with SGE, IsSMase or PBS (Control, C). *A*, Representative
CFSE-dilution histograms and *B*, quantitative analysis. *C*, Representative IFN-γ vs IL-4
intracellular staining plots and *D*, quantitative analysis of IL-4 intracellular cytokine
expression. *N*=4 for both groups.

Figure 5.
IsSMase enzymatic activity is not required to program CD4 T cell IL-4 expression. IL-4 expression potential in CD4 T cells exposed to bacterial SMase (10 U of *Bacillus cereus* SMase) and active vs denatured IsSMase. *A*, representative IFN-γ vs IL-4 intracellular staining plots and *B*, quantitative analysis. *N*=4 to 6 for each group.