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Suppression of cell proliferation and cytokine expression by HL-p36, a tick salivary gland-derived protein of *Haemaphysalis longicornis*

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Summary

Previously, a putative immunosuppressant-coding gene was identified from a complementary DNA library derived from the salivary glands of partially-fed *Haemaphysalis longicornis*. Using real-time polymerase chain reaction, the gene was shown to be predominantly expressed during blood feeding with the site of expression being mainly in the salivary glands; this was confirmed by Western blotting analysis. To investigate the function of this novel protein, in this study, we examined the proliferative responses of bovine mononuclear cells and murine splenic cells as well as the expression of profiles of several cytokines in these cells in the presence of the recombinant protein (*H. longicornis*-derived 36 000 molecular weight protein: rHL-p36). The addition of rHL-p36 at the beginning of the 72 hr cultivation period clearly inhibited proliferation of several mitogen-stimulated cells in a dose-dependent manner, with concomitantly significant down-regulation of messenger RNA levels for interleukin-2. The inhibitory response could be abrogated by blockage of HL-p36 with antibody, suggesting the direct involvement of rHL-p36 in the cell proliferation. Furthermore, the proliferative response of splenocytes isolated from rHL-p36-inoculated mice was significantly lower than for those from control mice, suggesting that rHL-p36 could also directly suppress immune responses *in vivo*. Interestingly, microarray analysis of the splenocytes showed that the expression of several immunomodulating genes was down-regulated by rHL-p36 inoculation. In conclusion, these results suggest that HL-p36 is an immunosuppressor that might play an important role in the modulation of host immune responses.

Keywords: cell proliferation; cytokines; immunosuppressant; salivary gland; tick

Introduction

Their saliva helps arthropod vectors to obtain a blood meal, and it has been widely observed that the saliva of arthropods enhances the infectivity of pathogens that are transmitted by the arthropods to vertebrate hosts. Several factors in arthropod saliva have been identified, and their functions have been demonstrated using various approaches. Knowledge of the salivary factors that are related to blood feeding in arthropod vectors is important in understanding the biology of arthropod-borne pathogens and in deriving new vector control strategies. Among all the factors, it seems likely that the immunosuppressant factor is one of the most important risk factors associated with infections caused by several arthropod-borne pathogens.[1,2] With regard to hard ticks, growing evidence has shown that immunomodulatory factors can interfere with the host immunity and pathogen transmission,[3–8] and in particular, the role of an immunosuppressant from the tick *Ixodes scapularis*, 'Salp 15', has been well established as important in the transmission of Lyme disease.[9] These observations raise the possibility that immunomodulatory factors can be important fundamental components to be considered in designing vaccines for preventing pathogen transmission, as suggested by Titus *et al.*[2]

In previous studies, a sequence (GenBank accession number: AB425235) homologous to the immunosuppressant protein, p36, derived from *Dermacentor andersoni* was found in a complementary DNA (cDNA) library derived from *Haemaphysalis longicornis*, a tick that is widely distributed in Eastern Asia and that is the major vector of *Theileria* spp., which are among the most economically important protozoan parasites affecting cattle in Asia.[10] Bergman and colleagues have shown that *D. andersoni*-derived p36 has suppressive activity against T-cell proliferation.[11–13] Homologous genes related to the *D. andersoni*-derived *p36* gene, such as *Ra-p36* and *Av-p36*, have also been isolated from *Rhipicephalus appendiculatus* and *Amblyomma variegatum*,[14] respectively. In the present study we have identified a gene from *H. longicornis* ticks that is homologous to the *D. andersoni*-derived *p36* gene, and herein named it as '*HL-p36*' for *H. longicornis*-derived 36 000 molecular weight protein. Information about the saliva factors in arthropod vectors, especially with regard to modulation of host immune responses, is important in defining adequate control strategies for arthropod-borne diseases.[2] However, there is limited information available about the role of host immunosuppressant factors from *H. longicornis*. In the present experiment, to investigate the role of a novel tick salivary gland-derived factor 'HL-p36' in host immunity, we examined the expression of the *HL-p36* gene and the effects of HL-p36 on cell proliferation and cytokine expression. We describe the characterizations of its properties and show that HL-p36 has the unique capacity to modulate the immunity of the vertebrate host.

Materials and methods

Sequence analyses

Alignment of the sequences was performed by the CLUSTAL W program[15] while phylogenetic analyses were performed using the neighbor joining method. The accession numbers for the genes analysed are: AF167171 (*D. andersoni*)[13] and AB164194 (*A. variegatum*) in the EMBL/GenBank, and TC183 (*A. variegatum*)[14] and TC3572 (*R. appendiculatus*)[16] in the Gene Indices page on the TIGR website (http://biocomp.dfci.harvard.edu/tgi/tgipage.html).

Ticks, RNA extraction and cDNA synthesis

Ticks were obtained from a colony of *H. longicornis* maintained on rabbits at the Graduate School of Veterinary Medicine, Hokkaido University as described previously.[10] Salivary glands, midguts and carcasses from 30 partially fed ticks (day 3 and day 6) were dissected in sterile phosphate-buffered saline (PBS) and RNA extracted by the TRIZOL™ reagent (Invitrogen, Carisbad, CA). Reverse transcriptase reaction was performed with 1 μg of the purified total RNA as described previously.[17] For gene expression analysis, RNA samples were also prepared from whole nymphal ticks at day 3 after the beginning of blood feeding as well as from the salivary glands of unfed nymphal ticks, initiated (sensitized to CO_2 and body temperature on the rabbits without blood feeding) nymphal ticks, engorged adult ticks and tick embryos (2 weeks postegg production).

RT-PCR and real-time PCR

The oligonucleotide primers used for reverse transcription–polymerase chain reaction (RT-PCR) were designed with restriction enzyme recognition sites to facilitate subcloning.[17] The HL-p36 cDNA were amplified by PCR using tick total cDNA as template and the primers HL-p36-F (*Eco*RI): 5′-CCG GAA TTC ATG AAC GGA CGA AGA ACT TGG-3′, (containing the ATG translation start codon and *Eco*RI restriction site), and HL-p36-R (*Not*I): 5′-ATA AGA ATG CGG CCG CTT ATA CTT TGC AGA GCG GGC-3′, (containing the TGA translation stop codon and *Not*I restriction site). The reaction mixtures contained 10 mM Tris–HCl, 50 mM KCl (pH 8·3), 0·1% Triton-X 100, 1·5 mM $MgCl_2$, each deoxynucleoside triphosphate (dNTP) at a concentration of 120 μM, 2·5 units of r*Taq* polymerase (Takara, Otsu, Japan), and each primer at a concentration of 0·5 μM. The PCR conditions were an initial 4-min incubation at 94°, followed by 25 cycles of incubation at 94° for 30 seconds, 56° for 30 seconds and 72° for 30 seconds, with the final extension at 72° for 10 min. To determine the presence of cDNA in the samples, PCR amplification of the tick actin gene was performed under conditions similar to those described above except that the annealing temperature was 60°. Primer sets specific for tick actin gene, ACT-for-A (5′-TGT GAC GAC GAG GTT GCC G-3′) and ACT-rev-A (5′-GAA GCA CTT GCG GTG GAC AAT G-3′), were used.[17] The products of amplification were subjected to electrophoresis on a 2% agarose gel.

Real-time RT-PCR using SYBER Green I was performed using a LightCycler™ (Roche Diagnostics, Mannheim, Germany). The cDNA template was added to a total volume of 20 μl containing PCR buffer, oligonucleotide primers (at 0·2 μM each of HL-p36-F (*Eco*RI) and HL-p36-R (180): 5′-CCA ATA TTG GTA GGA CTC TGT AA -3′), and 2 μl LightCycler-Fast Start DNA Master SYBER Green I (Roche Diagnostics). The tick actin gene in each sample was also amplified using primer pair ACT-108 (5′-TGGA TCG GCG GCT CCA TCC T-3′) and ACT-rev-A to check for loading of template cDNAs. The relative messenger RNA (mRNA) values were worked out as ratios by dividing the concentration of the PCR product from the HL-p36 cDNA by that from the actin cDNA.

Generation of recombinant protein and Western blotting

To generate the recombinant HL-p36 (rHL-p36), the amplified PCR product was digested with *Eco*RI (Takara) and *Not*I (Takara), and ligated into the expression vector pET43,1a(+) (Novagen, Inc., Madison, WI), which produces the desired recombinant product linked to (Nus) via a thrombin cleavage site and sub-cloned into *Escherichia coli* AD494 (DE3)/pLysS. Expression of the protein product by transformed *E. coli* was induced over 6 hr with 0·5 mM isopropyl-1-thio-beta-D-galactoside. The soluble rHL-p36 was purified to homogeneity from *E. coli* lysate supernatants by glutathione–Sepharose 4B resin column, according to the manufacture's protocol (Pharmacia Biotech Inc., Uppsala, Sweden), and was dialysed with sterile PBS. Nus was also expressed and purified in the same way as the control protein for each assay. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis were performed as described elsewhere. Rabbit anti-tick saliva sera against all tick stages (larva, nymph and adult ticks) were generated as described previously.[17] Recombinant HL-p36 was transferred from a 12% polyacrylamde gel to nitrocellulose. The nitrocellulose strips were incubated for 1 hr with normal or infected rabbit serum diluted 1 : 50 in PBS. After three 10-min washes, the strips were incubated for 1 hr with a 1 : 1000 dilution of peroxidase-labelled sheep anti-rabbit immunoglobulin G (IgG; ICN Biomedicals Inc., Costa Mesa, CA). After four 10-min washes, positive signals were visualized by using 3,3-diaminobenzidine tetrahydrochloride and cobalt chloride. Native HL-p36 in adult tick salivary glands or anti-HL-p36 antibodies in cattle infected with *H. longicornis*-borne theileriosis was detected as described above with some modifications.

Cell proliferation assay

To investigate the influence of the HL-p36 on cell proliferation, bovine peripheral blood mononuclear cells (PBMCs) or murine splenocytes (10^5 cells/well in flat-bottomed 96-well plates) were cultured at 37° for 72 hr with 5% CO_2 in the presence of concanavalin A (Con A; 5 μg/ml, Sigma, St Louis, MO), pokeweed mitogen (1 μg/ml, Gibco, Grand Island, NY), phytohaemagglutinin (1 μg/ml, Gibco) and lipopolysaccharide (50 μg/ml, Sigma), plus various concentrations (10, 1, 0·1 and 0·01 μg/ml) of rHL-p36. [^{3}H]Thymidine (0·5 μCi/well: ICN Biochemicals Ins) was added to the culture and cultured for an additional 6 hr, then the cells were harvested onto glass filters and incorporated radioactivity was measured by liquid scintillation counter (Aloka, Tokyo, Japan). All samples were tested in triplicate, and the data were presented as mean stimulation index (SI: defined as mean counts of test samples divided by mean counts of controls). The Nus protein was used as a control of the recombinant protein.

To confirm the suppressive activity against cell proliferation, in the same plate, Con A (5 μg/ml), rHL-p36 (10 μg/ml) and diluted anti-HL-p36 serum were added to splenocyte cultures at the beginning of culture, and the cells were harvested after 72 hr as described above. Normal mouse serum and sterile PBS were used as controls of the anti-HL-p36 serum.

Quantification of cytokine mRNA by real-time PCR

To quantify cytokine mRNA expression in 24 hr-cultivated bovine PBMCs with rHL-p36 (0·1 μg/ml), cytokine profiles. Interleukin-2 (IL-2), IL-4, IL-10, IL-12p40, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) were analysed by using real-time PCR. The isolation, RNA extraction, cDNA synthesis and real-time quantitative RT-PCR on PBMCs were conducted as described previously.[18]

In vivo *experimental design*

BALB/c mice (Japan SLC Inc., Shizuoka) were injected subcutaneously with 150 μg rHL-p36 at 10-day intervals three times. Nus protein and sterile PBS were used as controls of the rHL-p36 injection. Ten days after the last inoculation, splenocytes were isolated from the mice for cell proliferation assay and DNA microarray analysis. *In vitro* cell proliferation assay without rHL-p36 for the evaluation of direct immunosuppression was conducted as described above. Total RNA from fresh splenocytes was extracted using the TRIzol reagent as described above, and purified using an RNeasy mini kit (Qiagen, Germantown, MD). Labelling and hybridization of RNA for microarray analysis were performed using the Agilent low RNA input linear amplification kit (Agilent Technologies, Santa Clara, CA), and using an RNeasy purification kit (Qiagen) to clean up the cRNA. Equal amounts of RNA derived from rHL-p36-inoculated mice and of RNA from Nus-inoculated mice were assayed. The array was performed as described by the manufacturer. Microarray hybridizations were carried out on Agilent mouse oligonucleotide microarrays using 1 μg Cy3-labelled 'rHL-p36-inoculated' sample and 1 μg Cy5-labelled 'Nus-inoculated as control' sample. Hybridizations were carried out using the Agilent hybridization kit and a hybridization oven (Agilent Technologies). All microarrays were scanned using an Agilent DNA microarray Scanner (Agilent Technologies).

Statistical analysis

Data were analysed by one-way analysis of variance followed by Student's *t*-test. Differences between groups were considered significant if probability values of $P < 0{\cdot}05$ were obtained. All statistical analyses were performed with the statistical software STATCEL (OMS, Saitama).

(a)

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   1 ATGAACGGACGAAGAACTTGGTCGCTTTTGCTACTTTTGATGCTTGCTGCTGAAGTTCAT  60
     M  N  G  R  R  T  W  S  L  L  L  L  L  M  L  A  A  E  V  H
  61 ATGCAAGTTGTCAACCTAACTAAAGAAGCGGAGGATGTCGTGACCAGACTTGGAGAACGG 120
     M  Q  V  V  N  L  T  K  E  A  E  D  V  V  T  R  L  G  E  R
 121 TATACATCGTGGAAGAGTAAGATTGTGTCCTGGGGCCTTACAGAGTCCTACCAATATTGG 180
     Y  T  S  W  K  S  K  I  V  S  W  G  L  T  E  S  Y  Q  Y  W
 181 AAAAATGTAACTATAGGAGGAAACCGTCGCCCAATCTCTACATATGCCCAAGCTATGCAA 240
     K  N  V  T  I  G  G  N  R  R  P  I  S  T  Y  A  Q  A  M  Q
 241 GTAGGAAAGATGAAGGTTCTTCGGAAGCCAGTGAAATTCAGTCCCCCGCAGGACCTTGTT 300
     V  G  K  M  K  V  L  R  K  P  V  K  F  S  P  P  Q  D  L  V
 301 TGCCACCTGAACTTGACTTGGAATTTCACACGACACCTACTGAGTCCATTCCCGACCTAT 360
     C  H  L  N  L  T  W  N  F  T  R  H  L  L  S  P  F  P  T  Y
 361 TTAAACCTTAGTGTTCCAATGCTTGGTAGACTCGCAAGAATTGGAAACACCACGTTTCAA 420
     L  N  L  S  V  P  M  L  G  R  L  A  R  I  G  N  T  T  F  Q
 421 ATGAATCTCAGAGATGCCCCCTTTATAGAAGCTCATTTGGAATTTTATATTCCCCATGCT 480
     M  N  L  R  D  A  P  F  I  E  A  H  L  E  F  Y  I  P  H  A
 481 AACTTATATCCTGCTACCCTACTAATCGTTGCCTTGCTGCTGATACCGGCAAGGAGCAAC 540
     N  L  Y  P  A  T  L  L  I  V  A  L  L  L  I  P  A  R  S  N
 541 GCAGAACACCAGCAAGCGGAGGCGACGATCATGGTCAACGGCGCAGGCCCCCATATTGTG 600
     A  E  H  Q  Q  A  E  A  T  I  M  V  N  G  A  G  P  H  I  V
 601 TCCAACGCCCCCCGATCTGGGATAGCAACTGGAATCGTCACTACCACTCGTCGTCGGAGA 660
     S  N  A  P  R  S  G  I  A  T  G  I  V  T  T  T  R  R  R  R
 661 CATTCTGCGGCGGCGCGAAAACATGTGGCGCCCTGGCGGTGCCCGCTCTGCAAAGTATAA 720
     H  S  A  A  A  R  K  H  V  A  P  W  R  C  P  L  C  K  V  *
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(b)

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Av -p36 (AB164194) :   1 MHPKVAVGFLLLLASSREG-----ACTMVNLTEEANIYIKKMER----RLGEIDNWGLTE  51
Da -p36 (AF167171) :   1 MHHVIAVPLLLLLGGVLEAGALHKAAKIVNLTEEARKYVGRVNTT---ALGTIDSWGLTK  57
Ra -p36 (TC3572)   :   1 MSLKNVACLLFLMGTLSEG------AAKVNLTKKAEEYIKINAS------GNIDSWGMTG  48
Av -p36 (TC183)    :   1 MRSNLVGGLFVLTVILTEVS-----GEMVNLTRQAENYIKRLNQ----SSGNITDWGLTE  51
HL-p36 (AB425235)  :   1 MNGRRTWSLLLLLMLAAEVH-----MQVVNLTKEAEDVVTRLGERYTSWKSKIVSWGLTE  55
                           *    .  ::.*     *          ****.:*.  :           . * .**:*

Av -p36 (AB164194) :  52 NYTYWTRRH-FTDGERPVVAAVMKPECRLKEEQEIVQSV--ELN-DCNEIFTWNISHGIK 107
Da -p36 (AF167171) :  58 EYDYWTQQH-KRG--HPIKATVKRLSCVPGYKLDKPLRM--SQDYKCESELSLFIDKGML 112
Ra -p36 (TC3572)   :  49 SYGYWSGHQ-SRG-EYPVKTSVTQLKCTPLDGGNQVMRR--QRE--CDEGLDFFFTDGLV 102
Av -p36 (TC183)    :  52 DYSFWDTSQEDRPNVSPITVKVADVTCHPAPLEYERLDS--NFG-VKRIVYVWNITSRIW 108
HL-p36 (AB425235)  :  56 SYQYWKNVT-IGGNRRPISTYAQAMQVGKMKVLRKPVKFSPPQDLVCHLNLTWNFTRHLL 114
                           .* :*          *: . .                              :   :

Av -p36 (AB164194) : 108 SPFQLLVNVTLPMIRNGHAKHTKVVELDLSNLTIQKEIKNRVKHTGNLTQKVTMSCGFEA 167
Da -p36 (AF167171) : 113 SPFNLSATVKFPLIPSTHAEKTTVVTLDLN--GKMEDHKTIEKKSGNSEIRG---CNFFA 167
Ra -p36 (TC3572)   : 103 SPFNLPTEVNFPLIAGGQSTPHVRVQLDLS-----KDKTVVITKPGTPQMTT---CQFSV 154
Av -p36 (TC183)    : 109 SPFKLNINVRILLAAG-HEFKKQNFQFSVTTKPVIKATPPGKTPKYNRIYIER--CKLKA 165
HL-p36 (AB425235)  : 115 SPFPTYLNLSVPMLGRLARIGNTTFQMNLRDAPFIEAHLEFYIPHAN-LYPATLLIVALL 173
                           ***     : . :          . :.:      :         .

Av -p36 (AB164194) : 168 KTRFWGYFAFHVKRPRGDTPN--YNRVNIAALENKAKGLHKS---SGVLTYTIKGQYTQT 222
Da -p36 (AF167171) : 168 EVIFNGSFAYHSKKGR-------YYRVPVQYLKKYDEKLYAR---GHKLVYNITGAYTQK 217
Ra -p36 (TC3572)   : 155 NVAFDGKFAYHTKASNGMLEDGVYHSVNITALQDSTKGLIVK---EGKLQYELKGEVVQT 211
Av -p36 (TC183)    : 166 QVIFDGFFAYQSKSRERNVTK--YHTVHVGMLSNDSKRIVTENTYQQKLTYTLDGWHMRR 223
HL-p36 (AB425235)  : 173 LIPARSNAEHQQAEATIMVNGAGPHIVSNAPRSGIATGIVTT---TRRRRHSAAARKHVA 230
                                .  .:                *    .    :         :   .

Av -p36 (AB164194) : 223 LCRYQNNKRSKRSAGLQ 239
Da -p36 (AF167171) : 218 ICF-------------- 220
Ra -p36 (TC3572)   : 212 LCI-------------- 214
Av -p36 (TC183)    : 224 VLLSDSGSHKLIMIA-- 238
HL-p36 (AB425235)  : 231 PWRCPLCKV-------- 239
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(c)

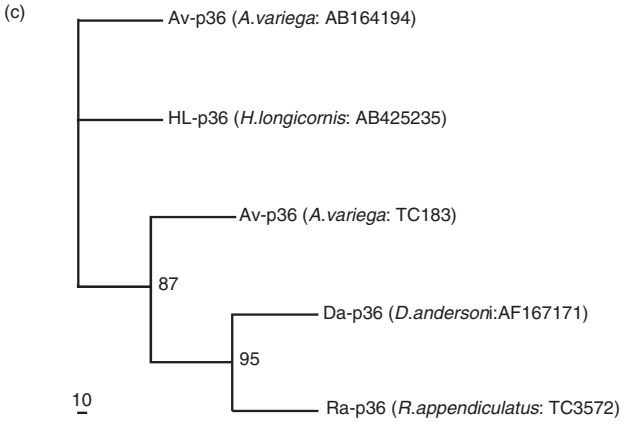

Figure 1. (a) DNA and putative amino acid sequence of HL-p36. The nucleotide sequence of HL-p36 has been submitted to the DDBJ, EMBL and GenBank nucleotide databases under accession no. AB425235. (b) Amino acid sequence alignment of 36 000 molecular weight immunosuppressive homologues from four tick species. The figure shows a CLUSTAL W[15] alignment of a HL-p36 with two from *Amblyomma variegatum* (Av-p36) (TC183)[14] and AB164194 in the EMBL/GenBank, *Dermacentor andersoni* (Da-p36) (AF167171)[13] and *Rhipicephalus appendiculatus* (Ra-p36) (TC3572).[16] The numbers indicate the amino acid position. Positions of amino acid sequence identity, amino acid residue similarity and highly conserved amino acid substitutions are marked by an asterisk, period and colon, respectively. The sequences in bold represent signal peptide sequences as predicted by the SIGNALP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/).[36] (c) A phylogenetic tree of nucleotide sequences of 36 000 molecular weight immunosuppressive homologues from four tick species. The unrooted tree was built using the neighbour-joining method by the CLUSTAL X program. Numbers indicate the bootstrap percentage (100 replicates). The scale indicates the divergence time.

Results

Sequence analysis of HL-p36

The full-length cDNA sequence of HL-p36 is shown in Fig. 1(a) (GenBank accession number: AB425235) and its putative amino acid with a predicted molecular mass of about 36 000 was compared with related immunosuppressant proteins of other species. Figure 1(b) shows an alignment of the p36 immunosuppressant proteins from four different tick species. In pairwise comparison, the HL-p36 exhibited higher similarity with a putative immunosuppressant protein from *A. variegatum* (TC183)[14] (20·5% sequence identity) than to the related proteins from

D. andersoni[13] and *R. appendiculatus*.[16] The phylogenetic tree also demonstrated that the HL-p36 coding nucleotide sequence is more closely related to the *A. variegatum* sequence than to the sequences of other ixodid ticks (Fig. 1c).

Expression analysis of HL-p36 mRNA

To determine the expression profiles of *HL-p36*, total RNA samples from various tick tissues and from ticks at different stages were subjected to RT-PCR. As shown in Fig. 2(a), *HL-p36* mRNA transcripts were detected in partially fed nymphal and adult ticks (3 days after the start of feeding), and found to be expressed specifically in salivary glands but not in midguts, skeleton and other organs as well as embryos (2 weeks after egg production). Based on real-time PCR analysis, whereas expression of *HL-p36* mRNA reached a peak at day 3 after blood feeding, its expression level was low or undetectable before blood feeding as well as during the initiation phase (exposure of ticks to rabbit but not blood feeding) and after engorgement (Fig. 2b).

Expression analysis of native HL-p36 in tick salivary glands

To confirm the expression of native HL-p36 in tick salivary glands, Western blot analysis was conducted. As shown in Fig. 2(c), the monoclonal antibody against HL-p36 reacted with a 36 000 molecular weight protein in the tick salivary gland extract prepared after blood-feeding on rabbits (day 3) but not from that prepared prefeeding. Furthermore, to investigate whether native

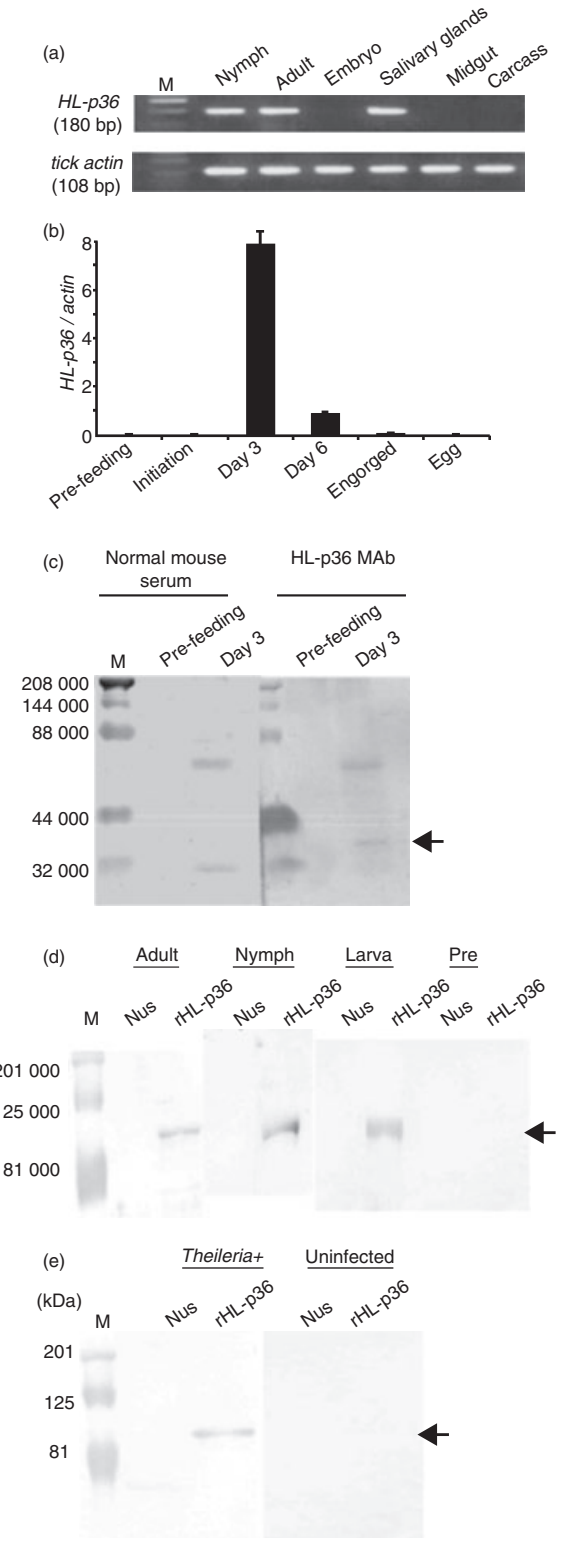

Figure 2. The expression of HL-p36 in tick salivary glands. (a) The expression of HL-p36 messenger RNA (mRNA) in each sample was analysed by reverse transcription–polymerase chain reaction (RT-PCR). The sample preparations are described in the *Materials and methods* section. The PCR products were resolved on 2% agarose gels. (b) HL-p36 expression analysed by real-time quantitative PCR. The HL-p36 mRNA level was normalized by the tick actin mRNA, and the relative mRNA values were worked out as ratios by dividing the concentration of the PCR product from the HL-p36 complementary DNA (cDNA) by that from the actin cDNA. Values represent means and error bars indicate the standard deviation. The results are means of three independent assays. (c) The expression of native HL-p36 protein as a 36 000 molecular weight protein (arrow) in tick salivary glands was detected by Western blot analysis with anti-HL-p36 monoclonal antibody. Lane M represents a molecular size marker, and numbers on the left indicate molecular weights (kDa). (d) Detection of HL-p36 antibody in experimentally *Haemaphysalis longicornis*-infested rabbits. Rabbit anti-tick sera and soluble recombinant HL-p36 were generated using the procedure described by Imamura *et al.*[17] The arrow indicates a specific 96 000 molecular weight band corresponding to the molecular mass of Nus-fusion HL-p36. (e) Detection of HL-p36 antibody in cattle naturally infected with *Theileria orientalis*, suggesting that the cattle had a history of exposure to the vector tick, *H. longicornis*. The *Theileria* infection was tested by PCR as previously described.[37]

HL-p36 is secreted into the host during tick feeding the reactivity of anti-sera raised against saliva of all tick stages (larva, nymph and adult) to rHL-p36 was tested by Western blotting. A specific signal against rHL-p36 was detected with each anti-tick serum, suggesting that native HL-p36 was secreted into the host during tick feeding through out all tick stages (Fig. 2d). As supplementary data, a specific signal against rHL-p36 was detected in the serum from *Theileria orientalis*-infected cattle suggesting that the cattle had a history of exposure to the vector tick, *H. longicornis*, infestation (Fig. 2e).

HL-p36 inhibits cell proliferation and cytokine expression *in vitro*

To investigate the causes underlying the effects of HL-p36 on cell proliferation, we examined the proliferation of the bovine PBMCs and mouse splenocytes in the presence of soluble rHL-p36 using [^{3}H]thymidine incorporation assays. As shown in Fig. 3(a,b), whereas the addition of rHL-p36 at the beginning of the 72-hr cultivation period clearly inhibited the proliferation of bovine PBMCs in a dose-dependent manner, the addition of Nus as a control protein had no effect on PBMC proliferation. Interestingly, the IL-2 mRNA levels were significantly decreased in PBMCs in the presence of rHL-p36 but not with Nus after 24 hr of culture (Fig. 3c). Both IL-12p40 and TNF-α were also significantly down-regulated in the PBMCs by rHL-p36.

We also compared the incorporation levels of [^{3}H]thymidine in proliferating murine splenocytes in the absence and presence of rHL-p36, and found that the presence of HL-p36 led to a decrease in [^{3}H]thymidine incorporation, indicating decreased cell proliferation (Fig. 4a). Furthermore, to determine if the inhibitory response can be blocked by anti-HL-p36 serum, mouse splenocytes were cultivated in the presence of Con A (5 μg/ml) and rHL-p36 (0·1 μg/ml) with serially diluted anti-HL-p36 sera. As shown in Fig. 4b, the addition of the anti-serum at the beginning of the 72-hr cultivation clearly reversed, in a dose-dependent manner, the inhibitory effect that rHL-p36 had on cell proliferation. This further underscored the finding that HL-p36 has a direct effect on host cell proliferation.

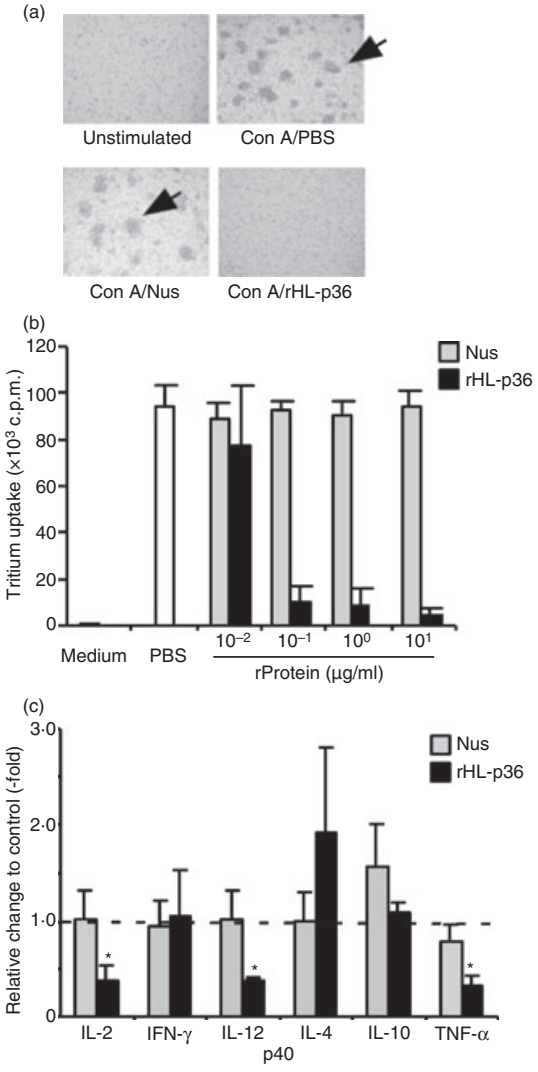

Figure 3. Soluble rHL-p36 inhibits concanavalin A (Con A)-stimulated cell proliferation and cytokine expression. (a) Photographs show the appearance of representatives of each condition after 72-hr cultivation. Bovine peripheral blood mononuclear cells (PBMCs) were cultured at 37° for 72 hr with 5% CO_2 in the presence of Con A (5 μg/ml) and rHL-p36 (0·1 μg/ml). Equal amounts of phosphate-buffered saline (PBS) with or without Nus protein (fusion protein for rHL-p36) (0·1 μg/ml) were used as negative controls. The arrow indicated blast formation by Con A stimulation, indicating proliferating cells. The blast formation was strongly inhibited by rHL-p36. (b) Effect of rHL-p36 on Con A-induced cell proliferation. PBMCs were incubated with various concentrations of rHL-p36 (0·01, 0·1, 1 and 10 μg/ml) or control Nus protein. The rProteins (rHL-p36 or Nus) were added at the beginning of culture. Cell proliferation was measured as incorporation of tritiated thymidine and expressed in counts per minute (c.p.m.) corresponding to DNA-incorporated radioactivity. The rHL-p36 exhibited a dose-dependent reduction in the proliferation. (c) Real-time polymerase chain reaction (PCR) quantification of messenger RNA (mRNA) expression levels for bovine cytokines in rHL-p36 treated-PBMCs. Bovine PBMC were cultivated with Con A (5 μg/ml) and rProteins (0·1 μg/ml) for 24 hr and harvested for RNA extraction. Each cytokine's mRNA level was normalized by the bovine β-actin mRNA as previously described,[18] and the relative index was determined in comparison to the cytokine mRNA level in the PBMCs without rProteins (1·0). Values represent means and error bars indicate the standard deviation. The results are means of three independent experiments using cells from three individual cattle.

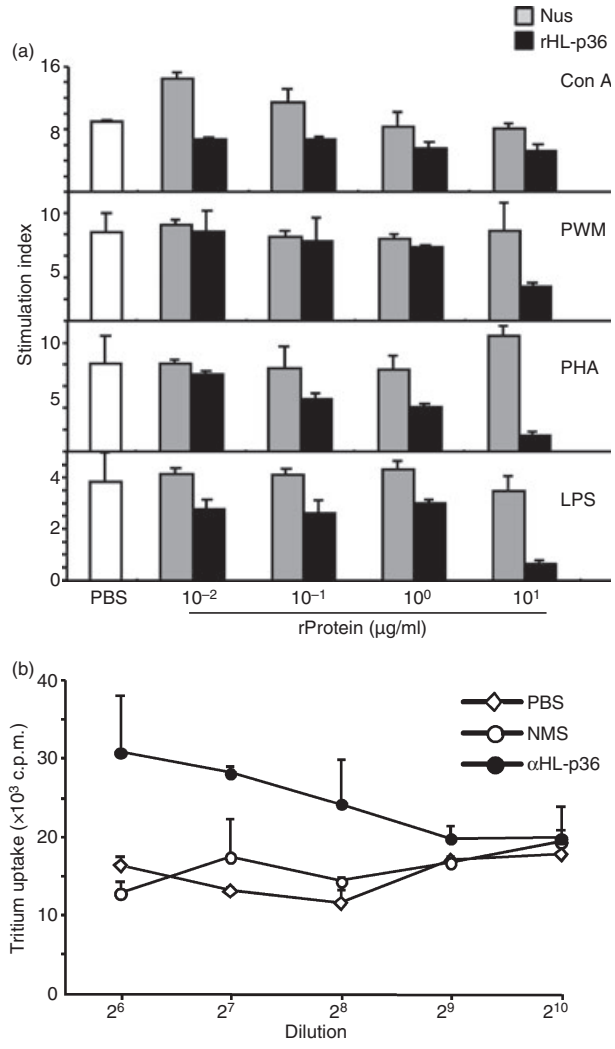

Figure 4. Inhibitory effect of rHL-p36 on mitogen-stimulated splenocyte proliferation. (a) Splenocytes from normal BALB/c mice were treated with the indicated concentrations of rHL-p36 for 72 hr with or without four mitogens. Data are means of stimulation index + SD from the triplicate cultures. (b) The inhibitory effect of rHL-p36 was abrogated upon the addition of anti-HL-p36 serum. Splenocytes were cultured for 72 hr in the presence of rHL-p36 (0·1 μg/ml) with concanavalin (Con A; 5 μg/ml). Inactivated serum from rHL-p36-inoculated mouse or normal mouse (NMS) and sterile phosphate-buffered saline (PBS) were added at the beginning of culture at various dilutions. LPS, lipopolysaccharide; PHA, phyto haemagglutinin; PWM, pokeweed mitogen.

The *in vivo* effect of HL-p36

To further evaluate the direct suppressive effect that HL-p36 has on host immunity, the cell proliferation assay and DNA microarray analysis were conducted using splenocytes. Inoculation of PBS or Nus in mice was found to have no notable effect on the proliferation of mitogen-stimulated mouse-derived cells. In contrast, the inoculation of rHL-p36 in mice was found to result in significant reduction of the proliferative ability of the mouse-derived cells (Fig. 5). Preliminary results from DNA microarray analysis revealed that whereas the expression of 2283 genes was significantly down-regulated (more than two-fold) by rHL-p36 injection, expression of 2838 genes was significantly up-regulated (more than two-fold) in murine spleen. The 10 most affected genes are listed in Table 1. Among the genes whose expression was affected by rHL-p36 injection, were the antigen presentation-related genes (*H2-Ea*: –3·6), IFN-related gene [interferon inducible proteins (*Ifi*): –3·5, –3·4 and –3·3] which is an important cytokine for intestinal immune systems, and CD8 antigen which is an essential marker for cytotoxic cells (cytotoxic T-lymphocyte and natural killer-cells) (–3·1). On the other hand, myeloperoxidase mRNA (21·7) and cathelicidin mRNA (21·0) related to infection were increased in the rHL-p36-injected mice.

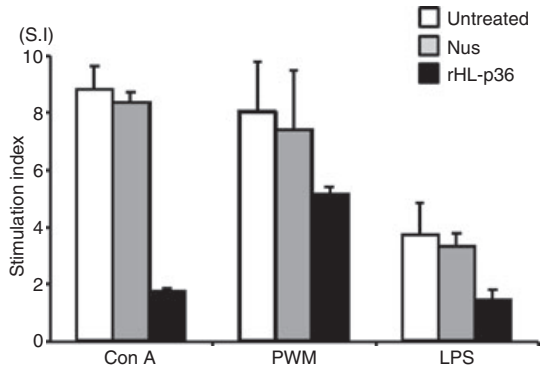

Figure 5. Decreased ability for cell proliferation in rHL-p36-inoculated mice. BALB/c mice were injected subcutaneously with 150 μg of rHL-p36 or Nus protein at 10-day intervals three times. Ten days after the last inoculation, splenocytes were isolated from the mice for cell proliferation assay using concanavalin A (Con A), pokeweed mitogen (PWM) and lipopolysaccharide (LPS), and then a fraction of the fresh cells was used for total RNA extraction for DNA microarray analysis (Table 1).

Discussion

The immunosuppressive functions of tick saliva or salivary gland extract are reportedly determined by several host immune responses.[1,2] However, there is very little information available on the specifically identified immunosuppressant molecule in tick saliva. The present study provides insights into the functions of tick saliva by demonstrating the ability of a tick saliva constituent protein (HL-p36) to suppress the host immune response. The cell proliferation suppressive effect of HL-p36 was demonstrated in two distinct models, *in vitro* and *in vivo*. The *in vitro* cell proliferation suppressive effect of HL-p36 was elicited in a dose-dependent manner. Interestingly, the expression of IL-2, which is one of the major cell growth factors, was found to be significantly reduced in the cells by HL-p36, consistent with the findings reported for

Table 1. Up-regulated or down-regulated genes in splenocytes from rHL-p36 inoculated mice[1]

Gene name	Accession no	Fold change
Down-regulated genes		
1. Activation-induced cytidine deaminase (Aicda), mRNA	NM_009645	−5·9
2. Regulator of G-protein signalling 13 (Rgs13), mRNA	NM_153171	−5·0
3. Growth arrest specific 6 (Gas6), mRNA	NM_019521	−4·7
4. Ras association (RalGDS/AF-6) domain family 6 (Rassf6), mRNA	NM_028478	−4·0
5. RIKEN cDNA 9030421J09 gene (9030421J09Rik), mRNA	NM_177744	−3·6
5. Histocompatibility 2, class II antigen E alpha (H2-Ea), mRNA	NM_010381	−3·6
6. Procollagen, type XIV, alpha 1 (Col14a1), mRNA	NM_181277	−3·5
6. cDNA clone MGC:41421 IMAGE:3371732, complete cds.	BC028540	−3·5
6. Adult male tongue cDNA, RIKEN full-length enriched library, clone:2310061N23 product: similar to interferon-α inducible protein (fragment) [*Mesocricetus auratus*], full insert sequence.	AK010014	−3·5
7. similar to immunoglobulin κ chain V region EV15 precursor (LOC383196), mRNA[XM484577]		−3·4
7. Interferon-α-inducible protein 27 (Ifi27), mRNA	NM029803	−3·4
7. Adult male testis cDNA, RIKEN full-length enriched library, clone: 4921530F17 product: hypothetical protein, full insert sequence.	AK029558	−3·4
7. RIKEN cDNA 5830458K16 gene (5830458K16Rik), mRNA	NM_023386	−3·4
7. Adult male testis cDNA, RIKEN full-length enriched library, clone:4933415F16 product: signalling lymphocyte activation molecule, full insert sequence.	AK016818	−3·4
8. Interferon-α-inducible protein 27 (Ifi27), mRNA	NM_029803	−3·3
9. Solute carrier organic anion transporter family, member 2bl, mRNA	NM_175316	−3·2
9. Interferon inducible GTPase 1 (Iigp1), mRNA	NM_021792	−3·2
9. G protein-coupled receptor 83 (Gpr83), mRNA	NM_010287	−3·2
10. Expressed sequence BB219290 (BB219290), mRNA	NM_145141	−3·1
10. cDNA clone MGC:41421 IMAGE:3371732, complete cds.	BC028540	−3·1
10. Late erythroblast-1-like mRNA, complete sequence	AY662646	−3·1
10. CD8 antigen, beta chain 1 (Cd8b1), mRNA	NM_009858	−3·1
Up-regulated genes		
1. Myeloperoxidase (Mpo), mRNA	NM_010824	21·7
2. Cathelicidin antimicrobial peptide (Camp), mRNA	NM_009921	21·0
3. Adult male hippocampus cDNA, RIKEN full-length enriched library, clone:2900019M05 product:unknown EST, full insert sequence.	AK013562	20·0
4. Cathepsin G (Ctsg), mRNA	NM_007800	19·6
5. Prominin 1 (Prom1), mRNA	NM_008935	19·0
6. *Mus musculus* chitinase 3-like 3 (Chi3l3), mRNA	NM_009892	18·7
7. Oxidized low density lipoprotein (lectin-like) receptor 1, mRNA	NM_138648	17·7
8. S100 calcium binding protein A8 (calgranulin A) (S100a8), mRNA	NM_013650	16·8
9. RIKEN cDNA 2900092M14 gene (2900092M14Rik), mRNA	XM_196763	16·5
9. Adult male corpora quadrigemina cDNA, RIKEN full-length enriched library, clone: B230208H11 product:unknown EST, full insert sequence	AK045519	16·5
10. Monoacylglycerol *O*-acyltransferase 2 (Mogat2), mRNA	NM_177448	16·1

[1]BALB/c mice were injected subcutaneously with 150 μg rHL-p36 at 10-day intervals three times. Nus protein was used as a control of the rHL-p36 injection. Ten days after the last inoculation, splenocytes were isolated from the mice for DNA microarray analysis.

assays using a homologous protein, Da-p36, which is an immunosuppressant saliva constituent protein from *D. andersoni*.[13] *In vivo*, the cell proliferative suppressive effects of HL-p36 appeared to be mediated by down-regulation of immunomodulating factors such as H2-Ea associated with the major histocompatibility complex class II molecule CD8, which is an essential marker for cytotoxic T lymphocytes or natural killer cells, and Ifi, which plays a critical role in host defence.

Our findings underscore earlier reports associating tick-associated immunomodulations in several species of hard ticks. As mentioned above, several immunomodulatory activities of tick saliva have been reported. In particular, cytokine modulations, such as inhibition of IL-2,[19] IL-12,[20] interferons[20–22] and TNF-α,[19,20] natural killer cell inhibition,[21–23] macrophage and dendritic cell inhibition,[20,24] and inhibition of surface expression of lymphocyte marker[25,26] have been well documented. In line with these

previous observations, it can be envisioned that HL-p36 is a specific immunosuppressant in *H. longicornis* tick saliva. Despite our preliminary microarray analysis data alluding to the idea that HL-p36 is indeed a negative immunomodulator, further detailed analyses will need to be conducted to confirm this. Moreover, contrary to a previous report on the inhibition of neutrophil activity,[27] several molecules including myeloperoxidase,[28] associated with host defence via neutrophils, and cathelicidin[29,30] were found to be elevated upon HL-p36 inoculation, although such changes may be secondary responses as the result of postimmunosuppression in the animals. At present, it is difficult to determine whether the genes whose expression was affected by HL-p36 could be directly or indirectly related to HL-p36-induced immunosuppression.

Although the exact mechanism of the suppression of cell proliferation and cytokine expression by HL-p36 remains unknown, HL-p36 can be added to the interesting list of immunomodulatory factors characterized in tick saliva-derived molecules (Table 2). Among these immunomodulatory factors, those of the ixodid tick, a well-known vector for Lyme borreliosis, have been well documented. In the case of *I. scapularis*, the identified immunomodulators such as Salp15,[3,4] Sialostatin L,[7] IL-2 binding protein,[5] and prostaglandin E_2[31] that are present in the ticks' saliva have been shown to have suppressive effects on lymphocyte proliferation, cytokine production, antigen presentation, lymphocyte and macrophage activation, dendritic cell maturation and neutrophil migration. In addition, Iris, an immunosuppressant from *I. ricinus*, has also been shown to modulate T-lymphocyte and macrophage responsiveness by inducing a T helper type 2 response and by inhibiting the production of proinflammatory cytokines.[8] Hannier *et al.* had indicated that in *I. ricinus* the 18 000 molecular weight protein could facilitate *Borrelia burgdorferi* transmission by preventing B-cell activation.[32] In particular, in the case of Salp15, the molecular mechanisms for the immnosuppression indicate that the molecule could bind to CD4, thereby inhibiting T-cell receptor ligation-induced signals resulting in impaired IL-2 production and impaired $CD4^+$ T-cell activation and proliferation.[3,4,6] Recently, Salp15 was also shown to inhibit Toll-like receptor- and *B. burgdorferi*-induced production of proinflammatory cytokines by dendritic cells and dendritic cell-induced T-cell activation via binding to DC-SIGN on dendritic cells.[33] These findings strongly suggest that local host immunosuppression by tick molecules assists *B. burgdorferi* in establishing an infection. In our pervious report, we have shown that *R. appendiculatus* ticks infected by *Theileria parva*, the causative agent of East Coast fever, started to transmit the parasites from 3 days post-tick attachment.[34] Interestingly, expression of HL-p36 mRNA reached a peak at day 3 post-blood feeding, corresponding to the beginning of the pathogen transmission. Although we could not directly compare the findings in *R. appendiculatus* to those in *H. longicornis*, one hypothesis is that HL-p36 could elicit suppression of both innate and acquired immunity at tick attachment sites, resulting in increased risk of *Theileria* transmission. In the present experiments, Con A, a lectin that induces T-cell proliferation by specific interaction with the T-cell receptor complex and a strong inducer of cell proliferation, was used to stimulate cell proliferatiion. Further, pokeweed mitogen, which is a B-cell mitogen, was used as the cell division stimulant, and we observed the suppression of cell proliferation by HL-p36 in both stimulations. However, our observations could not point to a specific HL-p36 target cell, although HL-p36 clearly

Table 2. List of specifically identified immunomodulatory factors in ticks

Tick species	Factor name	Immunomodulatory effects	Reference
Haemophysalis longicornis	HL-p36	Inhibit cell proliferation, cytokine expression and T-cell activation	
Dermacentor andersoni	Da-p36	T-cell inhibition	(11–13)
Ixodes scapularis	Salp15	T-cell inhibition via binding to CD4, impaired cytokine production	(3, 4, 6)
	Salp15	Dendritic cell inhibition via binding to DC-SIGN, impaired cytokine production	(33)
	Sialostatin L	Anti-inflammation, inhibits proliferation of cytotoxic T lymphocytes	(7)
	IL-2BP	Inhibit cell proliferation and T-cell activation	(5)
	PGE	Inhibit dendritic cell maturation and function	(31)
Ixodes ricinus	Iris	Inhibit production of proinflammatory cytokines, induce T helper type 2-type response	(8)
	18 000 BIP	B-cell inhibition	(32)

BIP, B-cell inhibitory protein; IL-2, interleukin-2; PGE, prostaglandin E.

affected the proliferation cells and modulation of cell immune responses *in vivo*. Further elucidation of the target cell(s) in HL-p36-induced immunosuppression is inevitable to fully understand the cell signalling pathways involved in the modulation of host immune responses.

In conclusion, we have shown that HL-p36 is a host immunosuppressant constituent protein in the saliva of *H. longicornis*, a widely distributed tick in Eastern Asia, which is the major vector of *Theileria* sp. and *Babesia* sp.[35] Information regarding the salivary factors of blood-feeding arthropods, especially in relation to immunomodulation is essential for the elaboration of adequate control strategies. In addition, the knowledge of the roles of the immunomodulators in pathogen transmission is vital for determining the disease transmission risks and appropriate control measures. Interestingly, the inhibitory effect of rHL-p36 on cell proliferation was abrogated by the addition of anti-HL-p36 serum in the present experiment. In general, it seems likely that the arthropod N-linked glycans are rather bulky structures so that antibodies directed to proteins expressed in bacterial systems may not detect the native protein. Fortunately, the monoclonal antibody against HL-p36 could recognize the native protein in tick salivary gland extracts, although HL-p36 was expected to be a highly glycosylated protein in tick saliva because it contains six N-linked sequons and two O-linked sequons. These data, although not showing a significant relationship between the anti-tick effects and the restoration of cell activation, suggest that HL-p36 might be a potential target for preventing tick-borne pathogen transmission.[2] Studies are underway to evaluate the possible application of HL-p36 as an anti-tick vaccine to control tick infestation and pathogen transmission.

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