Salp15, an *Ixodes scapularis* salivary protein, inhibits CD4$^+$ T cell activation

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Abstract

Tick saliva has pleiotropic properties that facilitate persistence of the arthropod vector upon the host. We now describe a feeding-inducible protein in Ixodes scapularis saliva, Salp15, that inhibits CD4⁺ T cell activation. The mechanism involves the repression of calcium fluxes triggered by TCR ligation resulting in lower production of interleukin-2. Salp15 also inhibits the development of CD4⁺ T cell-mediated immune responses in vivo, demonstrating the functional importance of this protein. Salp15 provides a molecular basis for understanding the immunosuppressive activity of I. scapularis saliva and vector-host interactions.
Introduction

*Ixodes scapularis* ticks are the vector for several medically important pathogens, including the causative agents of Lyme disease and human granulocytic ehrlichiosis (Burgdorfer et al. 1982 and Chen et al. 1994). Tick-host interactions have been the subject of extensive investigation (Allen 1989 and Wikel and Bergman 1997). *I. scapularis* requires a 5 to 7 day attachment period on the mammalian host in order to feed to repletion (Binnington and Kemp, 1980). The long period of tick engorgement allows the host to generate immune responses directed toward secreted components of the vector. In turn, *I. scapularis* attempts to modulate these responses in order to effectively attach to and feed upon its host (Binnington and Kemp 1980; Kopecky et al. 1999; Ramachandra and Wikel 1992; Urioste et al. 1994 and Wikel 1999).

Tick salivary proteins that enter the host during feeding have multiple effects, including the ability to inhibit the complement cascade (Ribeiro, 1987, 1995; Valenzuela et al., 2000), impair NK cell function (Kopecky and Kuthejlova, 1998), reduce circulating antibody titers (Wikel and Bergman, 1997), repress production of cytokines, such as interleukin-2 (IL-2) and IFN-γ (Ferreira and Silva 1998; Ramachandra and Wikel 1992 and Schoeler and colleagues 1999), and inhibit the proliferation of T lymphocytes (Ramachandra and Wikel 1992 and Urioste et al. 1994). Immunosuppression of the host by tick saliva may result in more efficient transmission of several tick-borne pathogens (Wikel 1999 and Zeidner et al. 1996). Recently, a protein-associated IL-2 binding activity (Gillespie et al., 2001) has been described in tick saliva.

Several animal species acquire resistance to ticks after repeated exposure to the vector, a phenomenon known as tick immunity (Brown and Askenase 1985; Trager 1939; Wang and Nuttall 1999 and Wikel 1996). Altered cytokine expression levels (Schorderet and Brossard, 1994), acute basophil hypersensitivity at the site of the tick bite (Askenase et al., 1982), and circulating antibodies to several tick salivary gland proteins are all thought to contribute to this phenomenon (Girardin and Brossard 1989 and Worms et al. 1988). Tick-immunity results in impairment of attachment of adult and nymphal ticks, lower postengorgement weights, and increased levels of mortality. Therefore, it is not surprising that tick saliva contains components that modulate the host immune response. Assessing the components in tick saliva that are responsible for immunosuppression may lead to a further understanding of vector-pathogen-host interactions. We here report the characterization of a 15 kDa tick salivary protein that inhibits the activation of CD4+ T cells through the repression of calcium signals triggered by TCR engagement.

Results

**Salp15 inhibits CD4+ T cell proliferation**

At least 14 antigens in *I. scapularis* saliva that are recognized by the mammalian host during tick exposure were identified by probing an *I. scapularis* cDNA expression library with sera from rabbits...
that had been fed upon by ticks (Das et al., 2001). The sequence analysis of one of those antigens, Salp15, revealed weak similarities with two motifs of Inhibin A, a member of the TGF-β superfamily (FingerPRINTScan search tool at http://bioinf.man.ac.uk/cgi-bin/dbbrowser/fingerPRINTScan/muppet/FPScan.cgi) (Mason et al., 1985) (Figures 1A and 1B; E value: 1.9 × 103). Similar to tick saliva, TGF-β inhibits the production of cytokines, such as IL-2, and the proliferation of T cells (Wahl, 1994), suggesting that Salp15 may have immune-modulatory properties. Salp15 was expressed and purified in a Drosophila cell line-based system for use in functional assays (Figure 1C, lane 1). Tick-immune rabbit sera reacted with Salp15 in a Western blot assay (Figure 1B, lane 2), confirming that antibodies against tick saliva recognize the recombinant protein. Recombinant Salp15 migrated at 24–27 kDa in denaturing SDS-PAGE (Figure 1B), due to posttranslational modifications, consistent with other I. scapularis proteins (Das et al., 2000). The glycosylation of the protein was confirmed by staining with periodate-Schiff’s reagent (data not shown). Rabbit

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**Figure 1.** Characterization of Salp15. (A) Nucleotide and amino acid sequence of Salp15. The putative 21 amino acid signal sequence is highlighted in gray. The amino acid sequences highlighted in black represent regions that are similar to Inhibin A fingerprints. (B) Comparison of motifs 4 and 7 of the rat Inhibin A molecule and Salp15. Identical residues are linked with (|); similar residues are marked with (:). The * indicates the end of the sequence. (C) Recombinant Salp15 produced in Drosophila cells stained with Coomassie blue (1), Western blot analysis of the recombinant protein probed with tick-immune rabbit sera (2), and whole saliva probed with anti-Salp15 antibodies produced in rabbits (3). (D) Immunohistochemistry to detect Salp15 at the site of tick attachment. Skin biopsies of tick-infested mice were probed with Salp15 antisera (4) or normal rabbit sera (2) 2 days after tick detachment. Controls included the skin of naive mice (1 and 3).
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antisera raised against the recombinant protein recognized Salp15 in whole tick saliva (Figure 1C, lane 3). Some crossreaction was observed with proteins in the range of 50–55 kDa. Sera from naive rabbits did not react with proteins in tick saliva (data not shown). Salp15 could be readily detected in skin biopsies of mice 2 days after tick detachment (Figure 1D), indicating that the protein is present in vivo at the site of natural inoculation.

We examined the effect of Salp15 on the proliferation of purified CD4+ T cells in response to anti-CD3 and anti-CD28 monoclonal antibodies (mAb). Proliferative responses were impaired by Salp15 in a dose-dependent manner (Figure 2A). To rule out a cytotoxic effect of Salp15 on CD4+ T cells, their viability was tested by trypan blue exclusion at different time points of activation. No differences were observed in cell viability between control and Salp15-treated CD4+ T cells at 12, 24, and 42 hr of activation (Figure 2B). Salp15 activity was not due to induction of TGF-β during the activation process (data not shown). We then assessed the ability of Salp15 to bind CD4+ T cells. Purified CD4+ T cells were incubated with fluorescein-labeled Salp15 and analyzed by flow cytometry. Increasing concentrations of Salp15FITC bound to CD4+ T cells (Figure 2C). We also tested the binding capacity of Salp15 in a microtiter assay. Increasing concentrations of Salp15 bound to the membrane preparations (Figure 2D). The binding capacity of Salp15 was abolished

Figure 2. Salp15 inhibits CD4+ T cell proliferation. (A) 10⁶ naive CD4+ T cells per ml were activated with anti-CD3/CD28 mAbs for 78 hr in the presence of different concentrations of Salp15. The cells were pulsed with 1 μCi [³H]thymidine for the last 18 hr of the assay. (B) The viability of CD4+ T cells (10⁶ cells/ml) activated with anti-CD3/CD28 mAbs in the presence of Salp15 (50 μg/ml) (open squares) was analyzed by trypan blue exclusion at different times of activation. Closed squares represent control studies without Salp15. (C) Purified CD4+ T cells were incubated with Salp15FITC (4 μg, left line; 12 μg, middle line; and 30 μg per 10⁶ cells, right line). The cells were then analyzed by flow cytometry. (D) Membrane fractions were purified from CD4+ T cells and used in a microtiter assay to determine binding of Salp15 (closed squares). Preincubation of the membrane fractions with trypsin resulted in elimination of Salp15 binding (open squares). The results are representative of at least three independent experiments.
when the membrane preparations were preincubated with trypsin (Figure 2D), suggesting that Salp15 is binding to a protein component on the surface of CD4+ T cells.

**Salp15 inhibits IL-2 production and CD25 expression in CD4+ T cells**

We then examined the effect of Salp15 on IL-2 production by CD4+ T cells activated with anti-CD3 and anti-CD28 mAbs in the presence of Salp15. The level of IL-2 produced by CD4+ T cells stimulated in the presence of Salp15 was lower (Figure 3A) than in the control untreated cells. The inhibitory effect of Salp15 on CD4+ T cell IL-2 production was dose dependent (Figure 3A) and evident throughout the activation period (Figure 3B). The inhibition of IL-2 production was also observed at the mRNA level in purified CD4+ T cells that had been activated for 16 hr.

![Figure 3](image)

**Figure 3.** Salp15 inhibits IL-2 production and CD25 expression of anti-CD3/CD28 mAbs-activated CD4+ T cells. (A and B) 10^6 purified naive CD4+ T cells per ml were activated in vitro with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb in the presence of different concentrations of Salp15 for 44 hr (A) or 50 μg per ml of Salp15 (open squares) during different time periods (B). At the specified time points, IL-2 levels in the culture supernatants were measured by capture ELISA. Black squares represent controls without Salp15. (C) IL-2 mRNA was detected by RT-PCR in 16 hr stimulated CD4+ T cells in the presence of anti-CD3/CD28 mAbs ± 50 μg/ml of Salp15. β-actin was used as a control to ensure equal loads of RNA. (D) 10^6 purified CD4+ T cells were incubated with anti-CD3 + anti-CD28 in the absence or presence of 50 μg/ml of Salp15 or tick saliva (1/100 dilution) ± rabbit Salp15 antisera (1/500 dilution). Normal rabbit sera (NRS) was used as a control. The supernatants were analyzed at 48 hr of activation for IL-2. (E) 10^6 purified CD4+ T cells/ml were activated with anti-CD3/CD28 mAbs in the absence (thin line) or presence (thick line) of 50 μg/ml of Salp15. At 40 hr the cells were washed and stained for CD25 expression. (F) 10^6 CD4+ T cells/ml were activated with anti-CD3/CD28 mAbs in the presence of 50 μg/ml of Salp15 ± exogenous recombinant murine IL-2 (10 ng/ml). At 60 hr of activation, the cells were pulsed with [^3]H thymidine for 18 hr.
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The inhibition of IL-2 production was completely abolished by the addition of Salp15 antisera during the activation period (Figure 3D). Furthermore, saliva-mediated inhibition of IL-2 production by CD4+ T cells was reversed by Salp15 antisera (Figure 3D), suggesting that the proteins recognized by the antibodies are the primary inhibitors of IL-2 production by CD4+ T cells.

We then assessed whether the inhibitory effect of Salp15 on IL-2 production by CD4+ T cells influenced CD25 expression. Salp15 caused a substantial reduction in the level of CD25 expression at 40 hr of activation (Figure 3E). The reduction was evident as soon as 12 hr and persisted throughout the activation period (data not shown). These data show that Salp15 specifically inhibits IL-2 production and CD25 expression by CD4+ T cells activated by anti-CD3 and anti-CD28 mAbs. To further determine whether the lack of activation could be due to specific inhibition of IL-2 production, we assessed the effect of exogenously added recombinant IL-2 during activation. The proliferation of CD4+ T cells activated in the presence of Salp15 increased markedly with the addition of 10 ng/ml of recombinant murine IL-2 (rmIL-2) (Figure 3F). Furthermore, the expression of CD25 in the presence of Salp15 also increased to similar levels in the presence of recombinant IL-2 (data not shown). Interestingly, a stronger inhibitory effect of Salp15 on IL-2 production was observed at lower concentrations of anti-CD3 mAb (Figure 4A) or when CD4+ T cells were activated in the absence of costimulation provided by anti-CD28 (Figure 4B). These data suggested that Salp15 was interfering with T cell receptor (TCR) signals.
The initial production of IL-2 by CD4+ T cells that are undergoing activation provides strong signals that permit the activation process to continue and the cells to enter the proliferative cycle. We therefore tested whether the addition of Salp15 would have the same effect on CD4+ T cell activation when added at later time points, once IL-2 is already available to the cell. IL-2 production was decreased when the cells had been activated with anti-CD3 and anti-CD28 for 12 hr prior to the addition of Salp15 (Figure 4C). In contrast, the levels of IL-2 produced by CD4+ T cells incubated with anti-CD3 and anti-CD28 for 20 hr before the addition of Salp15 were less affected (Figure 4C), and no effect was found on IL-2 production when Salp15 was added at 40 hr of activation (Figure 4C). These data indicated that the inhibition of CD4+ T cell activation by Salp15 occurred in the initial period of activation. Overall, our results indicated that Salp15 inhibited CD4+ T cell activation by inhibiting IL-2 production, and suggested that Salp15 interfered with TCR-signaling.

Salp15 inhibits Ca2+ mobilization after TCR ligation

Several regulatory elements have been identified within the proximal IL-2 promoter, including the activator protein (AP)-1, nuclear factor of activated T cells (NF-AT), and nuclear factor κB (NF-κB) elements (Jain et al., 1995). We assessed the effect of Salp15 on the binding activity of these transcription factors in CD4+ T cells that had been activated with anti-CD3/CD28 mAbs. AP-1 DNA binding activity was not affected by Salp15 (Figure 5A). However, NF-AT and NF-κB DNA binding activities were substantially reduced in CD4+ T cells activated in the presence of Salp15 (Figure 5A). As a control, we also tested the DNA binding capacity of the cyclic AMP response element binding protein (CREB), which was not affected by the presence of Salp15 (Figure 5A). In agreement with these results, NF-AT transcriptional activity was compromised when CD4+ T cells from NF-AT-luciferase reporter transgenic mice were activated in the presence of Salp15 (Figure 5B). Salp15 did not induce AP-1 or NF-κB-DNA binding activity in unstimulated cells (data not shown), indicating that it exerted its effect through the inhibition of TCR-mediated transcriptional activation. Together, these results suggested that Salp15 was interfering with early signals induced by TCR crosslinking.

We hence assessed the effect of Salp15 on Ca2+ mobilization in response to TCR signals by flow cytometry using the fluorophore Indo-1. While anti-CD3 mAb caused a rapid increase of intracellular calcium levels, the presence of Salp15 delayed and reduced the Ca2+ flux induced by TCR ligation (Figure 5C). To determine whether the effect of Salp15 on Ca2+ mobilization was the cause of the inhibition in IL-2 production by the protein, we examined IL-2 production by CD4+ T cells activated with PMA and ionomycin. The levels of IL-2 produced by CD4+ T cells were equivalent in the absence or the presence of Salp15 (Figure 5D). Moreover, the addition of low doses of ionomycin (50 ng/ml) during activation of CD4+ T cells with anti-CD3 and anti-CD28 prevented Salp15-mediated inhibition of IL-2 production (Figure 4E) without affecting the viability of these cells (data not shown). These data imply that the inhibitory effect of Salp15 on CD4+ T cells is predominantly due to the inhibition of TCR-mediated calcium mobilization.
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Salp15 inhibits Cd4+ T cell activation in vivo

Activation of naive CD4+ T cells requires a rise in IL-2 production, whereas the activation of effector CD4+ T cells is less dependent on this cytokine (Yasui et al., 1998). To determine whether Salp15 inhibited the activation of effector CD4+ T cells, we performed restimulation assays with CD4+ T cells that had been differentiated in vitro for 4 days and from immunized mice. In vitro-generated effector CD4+ T cells produced similar levels of IL-2, IFN-γ, and IL-4 in response to anti-CD3 in the absence or presence of Salp15 produced in the Drosophila expression system (Figure 5).
6A). Similarly, Salp15 did not affect the proliferative capacity of restimulated effector cells (Figure 6B). For these experiments, we also cloned and expressed Salp15 as a fused partner to thioredoxin (TR) (Figure 6C) to specifically assess the effect of the immunomodulatory protein on CD4+ T cell activation and T cell-mediated antibody production in vivo. CD4+ T cells from TR-immunized animals responded similarly to TR, and TR fused to Salp15 (TR-Salp15) and produced high levels of IFN-γ (Figure 6D), suggesting that the stimulation of effector CD4+ T cells was not affected by the presence of Salp15. However, CD4+ T cells from TR-Salp15-immunized mice produced lower levels of IFN-γ in response to both TR and TR-Salp15 (Figure 6D), indicating that the presence of Salp15 inhibited the activation of antigen-specific CD4+ T cells in vivo. IL-4 production was below detectable levels in all cases (data not shown).

CD4+ T cells are also an important factor in the development of humoral antibody responses (Stavnezer, 1996). Therefore, we determined whether the inhibitory effect of Salp15 on CD4+ T cells influenced the generation of the murine antibody response. We found no difference in TR-specific IgM levels in the sera from TR- and TR-Salp15-immunized mice, indicating that T-independent B cell responses had not been affected by the presence of Salp15 (Figure 6E).

**Figure 6.** Salp15 inhibits CD4+ T cell activation in vivo. (A and B) In vitro-generated effector cells were activated for 24 hr with anti-CD3 in the absence (black bars) or presence (white bars) of Salp15 produced in *Drosophila* cells. The supernatants were collected after 24 hr and analyzed for IL-2, IL-4, and IFN-γ by capture ELISA (A) and proliferation (B). (C) Purified TR (1) and TR-Salp15 (2). (D) Balb/c mice were immunized with equimolar quantities of TR or TR-Salp15 (equivalent to 10 μg of TR-Salp15). Eleven days later, CD4+ T cells were purified and analyzed in recall responses to TR and TR-Salp15. IFN-γ levels were measured in 40 hr restimulation supernatants. (E) TR-specific IgM (1/160 dilution) and IgG (1/320 dilution) levels in the sera of the TR- (black bars) and TR-Salp15-immunized mice (white bars) were determined by ELISA using TR-bound plates. Experiments were repeated three times.
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Furthermore, Salp15 did not affect LPS-induced B cell proliferation (data not shown), agreeing with previous reports indicating that tick infestation does not affect, or slightly increases, B cell responses to LPS (Ganapamo et al., 1996). Similarly, Salp15 did not influence Borrelia burgdorferi-induced TNF-α production by CD11c+ cells (data not shown). In contrast, TR-specific IgG antibody levels were significantly reduced in sera obtained from mice immunized in the presence of Salp15 (TR-Salp15), compared to control immunized mice (Figure 6E). To ensure that Salp15 had not influenced the antigenicity of the fusion protein, we immunized mice with keyhole limpet hemocyanin (KLH) in the absence or presence of 50 μg of Salp15 produced in Drosophila cells. The levels of KLH-specific IgG antibodies in the sera of the mice that had been injected with Salp15 were significantly lower than in control animals (Figure 7A, p < 0.05). These data are in agreement with reports describing absent IgG responses in Balb/c mice after repeated exposures to tick feeding (Christe et al., 1998). Furthermore, delayed-type hypersensitivity reactions elicited by KLH administration were also impaired in mice that had been immunized with the protein in the presence of Salp15 at 24 hr (Figure 7B) and 48 hr postchallenge (not shown). At both time points, the difference in ear swelling between KLH− and KLH+ Salp15-immunized mice were statistically significant (Student's t test, P = 0.0017 at 24 hr; P = 0.0013 at 48 hr). The impaired development of a strong IgG antibody and DTH responses in the presence of Salp15 reinforced the idea of an inhibitory effect of the protein on CD4+ T cell activation.

Figure 7. Salp15 inhibits DTH induced by KLH. (A) Groups of five mice were immunized with 1 μg of KLH in the absence (black bar) or presence (white bar) of 10 μg of Drosophila-produced Salp15 in CFA. The animals were boosted after 2 weeks, and 10 days later their sera were analyzed for KLH-specific IgG antibodies by ELISA. (B) Groups of four mice were immunized with KLH in the absence or presence of Salp15. Animals immunized with Salp15 alone served as controls. Four days later the mice were challenged with KLH in the ears. Ear swelling was recorded at 24 and 48 hr after challenge. Results indicate increase in ear thickness at 24 hr postchallenge compared to time 0 (prior to immunization).
Discussion

Tick saliva is abundantly secreted into the host during feeding (reviewed by Wang and Nuttall, 1999) and contains anticoagulant, vasodilatory, and immunosuppressive activities (Ribeiro et al. 1985; Ribeiro et al. 1995; Urioste et al. 1994; Valenzuela et al. 2000 and Wikel 1996). The interaction between arthropods and their hosts is a dynamic process and the result of coevolution (Wang and Nuttall, 1999). Upon attachment of the vector, the host elicits both specific and nonspecific immune responses to the vector. In turn, ticks have developed adaptive mechanisms to modulate the host immune response (Ferreira and Silva 1999 and Wikel 1999). The equilibrium between the host immune responses and the vector’s immunosuppressive countermeasures partially determines the duration of attachment, the degree of engorgement, and the extent of pathogen transmission (Wikel and Bergman 1997 and Zeidner et al. 1996). In this study, we have characterized a 15 kDa *I. scapularis* saliva protein, Salp15, that inhibits CD4+ T cell activation.

Our results show that Salp15 has an inhibitory effect on CD4+ T cells in vitro and in vivo. IL-2 production, upregulation of the α-chain of the IL-2 receptor, and consequent proliferation are all readouts of CD4+ T cell activation (Theze et al., 1996). Salp15 inhibited all three in a dose-dependent fashion without exerting a toxic effect on CD4+ T cells. Moreover, the addition of exogenous IL-2 prevented the Salp15-mediated inhibition of CD4+ T cell activation, indicating that the inhibitory effect of Salp15 is due to repression of IL-2 production and not due to toxic effects or interference with the response of CD4+ T cells to IL-2.

Our results strongly suggest a quantitative effect of Salp15 that depends on the strength of the signal delivered through the TCR. Supporting this assertion, we have demonstrated that Salp15 activity is greatly increased when lower concentrations of anti-CD3 antibody are used to activate CD4+ T cells in the presence of anti-CD28 or in the absence of costimulation, which provides an increase of the signal delivered through the TCR (Michel et al., 2001). Furthermore, differences in the activity between native tick saliva and the recombinant protein produced in vitro may be due to the degree of appropriate folding in the recombinant protein. These alterations may not, however, influence binding capacity to the cell surface. These data strongly suggest that, under physiological conditions, Salp15 is likely to induce high levels of inhibition, even though it represents 0.1% of the total protein content of tick saliva (1 mg/ml, which corresponds to a concentration of 1 μg/ml of Salp15, data not shown).

Crosslinking of TCR complexes and CD28 molecules on naive T cells induces multiple intracellular signaling pathways that lead to the activation of specific nuclear transcription factors, including AP-1, NF-AT, and NF-κB. IL-2 is a key cytokine gene regulated by these transcription factors (Jain et al., 1995). Our data demonstrate that Salp15 inhibits IL-2 production upon TCR engagement by repressing calcium signals that result in decreased DNA binding activity of NF-AT and NF-κB. The effect of Salp15 on IL-2 production could be prevented by the activation of CD4+ T cells with PMA.
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plus ionomycin or the addition of ionomycin during activation of CD4+ T cells with anti-CD3/CD28. These data indicate that the inhibitory effect of Salp15 on IL-2 production of CD4+ T cells is due to impaired TCR signaling, resulting in the inhibition of transcription of the IL-2 gene.

Because of the potential immunosuppressive effect of the saliva protein in vivo, we tested whether the immune response against a foreign antigen was affected by the presence of Salp15. Our data suggest that the inhibitory effect of Salp15 on TCR-mediated CD4+ T cell activation has an effect on the murine immune response in vivo. Interestingly, Schoeler and colleagues (1999) showed that T lymphocytes from Balb/c mice repeatedly infested with I. scapularis nymphs in vivo do not produce high levels of IFN-γ in response to ConA restimulation ex vivo. We suggest that the inhibitory effect of Salp15 on IL-2 production affects the activation of naive CD4+ T cells in vivo and their differentiation to immune effector cells. On the other hand, CD4+ T effector cells were not inhibited when incubated with Salp15, probably because these cells are less dependent on IL-2 and do not or hardly produce this cytokine in response to antigen stimulation (Mannie et al., 1996). Most interestingly, TR-specific IgG but not IgM antibody responses were diminished in mice immunized with Salp15. IgM antibodies are produced by B lymphocytes, whereas switching to IgG subclasses is dependent on CD4+ T cell cooperation (Stavnezer, 1996). Indeed, Ganapamo and colleagues (1996) reported that tick infestation does not affect, or slightly increases, B cell responses to LPS. Consistent with these results, Salp15 did not exert any effect on LPS-induced B cell proliferation.

The results presented here argue against a relationship between Salp15 and the potential IL-2 binding protein activity described by Gillespie and colleagues (2001). First, the effects elicited by Salp15 on CD4+ T cells occur very early during the activation process, before IL-2 is produced by T cells. Second, the temporal frame of Salp15 activity as an immunosuppressor is limited, since the addition of Salp15 at 40 hr of activation does not affect the completion of the activation process, indicating that Salp15 does not influence IL-2–IL-2 receptor interactions. Third, Salp15 acts by interfering with TCR-mediated signaling events. Overall, our data indicate that Salp15 acts early during the activation process by interfering with TCR-mediated signals that lead to IL-2 production. The activity is eliminated when the levels of IL-2 and the increase of surface CD25 allow for a continuation of the activation process that is less dependent on TCR stimulation (Bajenoff et al., 2002). Tick saliva contains multiple activities that can influence CD4+ T and other cell type activation and effector functions. These include the inhibition of IFN-γ production by differentiating T cells in saliva of I. ricinus (Leboulle et al., 2002). Our data indicate that Salp15 has no effect on the commitment of these cells. Therefore, we can conclude that the overall activity of tick saliva on the immune system relies on the participation of several components, including Salp15.

Salp15 is the first I. scapularis protein associated with the immunosuppressive activity of tick saliva. Our data imply that the inhibitory effect of Salp15 on TCR-mediated activation of naive CD4+ T
cells results in decreased numbers of responding immune effector cells. Immune modulation is probably most important for the vector at the site of the tick bite, since the responses that the arthropod encounters during feeding occur locally. The inhibition of acquired immune responses may also be ecologically significant, helping to ensure that the host remains susceptible to future tick infestations. Moreover, microorganisms that rely on vectors to complete their natural life cycle may take advantage of these immunomodulatory strategies to promote their survival in the mammalian host and their adaptation to their new environment. Therefore, delineating the mechanisms that ticks use to influence host immune responses may lead to an understanding of competency of the vector for specific hosts, the prevention of arthropod-borne diseases, and the characterization of new molecules that can be used as anti-inflammatory or immunosuppressive agents.

**Experimental procedures**

**Purification of Salp15**

*salp15* was amplified from a pBluescript vector using specific primers: 5’-GAAAGCGGCCCAACT AAA-3’ and 5’-CTAACATCCGGAATGTG-3’. The PCR product was subcloned into the pMT/BiP/V5-His A vector (Invitrogen, Carlsbad, CA) and transfected into *Drosophila* S2 cells (Invitrogen) in combination with the hygromycin selection vector pCOHYGRO for stable transfection. The stable transformants were selected using 300 μg/ml hygromycin-B for 3–4 weeks. The resistant cells were grown as large spinner cultures, switched to DES serum-free medium for 2 days, and induced with copper sulfate to a final concentration of 500 μM for 4 days. The cells were then centrifuged at 1000 × g for 5 min. The supernatant was used to purify the protein using the Talon metal affinity resin (Clontech, Palo Alto, CA). The protein was eluted using 100 mM imidazole, extensively dialyzed against PBS (pH 7.8), and concentrated by centrifugal filtration through a 10 kDa filter (Millipore Corp., Bedford, MA). The PCR product was also subcloned into the pBAD/Thio-TOPO vector (Stratagene, La Jolla, CA) and transformed into TOP 10 *Escherichia coli* cells (Invitrogen). The protein, fused to thioredoxin (TR), was then induced with 2% arabinose and purified using Ni²⁺ columns, following the manufacturer's specifications (Invitrogen). The poli-His tagged fusion protein was eluted with imidazole, dialyzed, and concentrated. TR control protein was expressed and purified in an identical fashion. The purity of the proteins was checked on SDS-PAGE.

**Immunohistochemistry**

Female Balb/c mice were challenged with 15 nymphal ticks on the back. Two days after tick detachment, skin punch biopsies of 4–5 mm were obtained at the site of tick attachment in the experimental animals. The biopsies were embedded in Optimum Cutting Temperature (O.C.T.) compound (Sakura Finetek, U.S.A., Inc., Torrance, CA) and quick frozen in a dry ice-ethanol bath. Skin biopsies from naive mice were also obtained and frozen in O.C.T compound to serve as negative controls. Frozen O.C.T.-embedded skin specimens were cut into 4 μm thick serial sections in a cryostat, collected on Superfrost Plus glass slides (Gallard-Schlesinger Industries,
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Garden City, NY), and fixed with cold acetone for 20 min. Sections were allowed to air dry and were incubated with Tris-buffered saline (TBS) for 5 min. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ for 5 min at room temperature. Sections were rinsed with double distilled water (ddH₂O), followed by TBS for another 5 min, and then blocked with blocking buffer (5% normal goat serum and 1% triton-X 100 in TBS) at room temperature for 30 min. After the blocking step, sections were incubated overnight with Salp15 antisera (1:500 dilution). Normal rabbit sera were used as control. After three washes with TBS, the sections were incubated with peroxidase-conjugated anti-rabbit IgG (1:500 dilution) at 37°C for 30 min. Sections were washed with TBS followed by ddH₂O twice. The slides were developed with diaminobenzidine (Sigma Chemical Co., St. Louis, MO). The sections were rinsed in running water and mounted with Crystal/Mount (Biomed Corp. City State), followed by addition of a coverslip. The sections were then visualized under a light microscope.

Binding assays
Salp15 was equilibrated in carbonate buffer (pH 9.5) and labeled with fluorescein isothiocyanate (20 µg per mg of protein) for 2 hr at RT. Unbound fluorescein was then eliminated by size exclusion chromatography. The labeled protein was then buffer exchanged to a Tris-based buffer (Tris-HCl 50 mM and NaCl 150 mM [pH 7.0]). 10⁷ per ml purified CD4⁺ T cells were preincubated with 10 µg/ml of BSA for 10 min, incubated with Salp15-FITC for 30 min, washed, and analyzed by flow cytometry using a FACScalibur apparatus and the CellQuest software package (Becton Dickinson, Mountain View, CA). Negative controls were incubated in the same fashion with BSA-FITC (30 µg per 10⁶ cells). CD4⁺ T cell membrane fractions were obtained by passage of purified CD4⁺ T cells through a nitrogen bomb at 1000 psi. The membrane fraction was then collected by centrifugation, and the protein content was measured using the Bradford method. Complete cell disruption was assessed visually under the microscope. Four hundred nanograms of protein-containing membrane fractions were coated in 96-well plates in bicarbonate buffer (pH 9.6) overnight at 4°C, blocked with PBS plus 10% FCS, and preincubated with PBS or trypsin (20 µg/ml) for 1 hr, followed by extensive washing. The wells were incubated for 1 hr with 30 µg/ml of soybean trypsin inhibitor and washed. Salp15 was then added at different concentrations in PBS + 10% FCS followed by incubation with rabbit Salp15 antisera and a biotinylated anti-rabbit IgG and streptavidin bound to HRP. Salp15 antisera was prepared by immunizing a rabbit with 100 µg of recombinant Salp15 produced in the Drosophila expression system in complete Freund's adjuvant and boosting the animal with 100 µg of Salp15 in incomplete Freund's adjuvant at 2 and 4 weeks. The assay was developed with TMB substrate and stopped with TMB 1 component stop solution. The wells were read at 450 nm.

Tick saliva collection and determination of Salp15 concentration
Adult ticks were allowed to feed on the backs of guinea pigs for 3 days and were removed mechanically. The ticks were then immobilized, and a finely drawn capillary tube was fitted over their mouthparts. Two to five microliters of 5% pilocarpine (Sigma Chemical Co.) in methanol was
applied topically to their dorsa, and saliva was collected over periods of 1–2 hr, pooled, and stored at −70°C until use. The concentration of total protein present in tick saliva was determined by the Bradford method (Bio-Rad). The concentration of Salp15 in saliva was estimated by Western blot analysis using rabbit antisera and known concentrations of recombinant Salp15 produced in the Drosophila expression system as standards.

**CD4⁺ T cell purification and activation**

CD4⁺ T cells from naive or immunized mice were purified by negative selection as described (Anguita et al., 2001). 10⁶ CD4⁺ T cells per ml were activated with plate-bound anti-CD3 (5 μg/ml, except where indicated) plus soluble anti-CD28 (1 μg/ml). At the specified time points, the supernatants were collected and analyzed for cytokines by ELISA, as previously described (Anguita et al., 1996). For surface expression analysis of IL2R, the cells were recovered and incubated with phycoerythrin (PE) and cy-chrome conjugated antibodies specific for CD25 and CD4, respectively, and analyzed by flow cytometry. Total RNA was extracted using the Ultraspec RNA isolation reagent (Biotex Laboratory, Houston, TX) as recommended by the manufacturer. RT-PCR reactions were performed using the ProSTAR HF single-tube RT-PCR system (Stratagene), with primers specific for the murine IL-2 gene (Reiner et al., 1993). To ensure equal loads of RNA, β-actin primers were used (control). Lack of DNA contamination was assessed by running the same reactions in the absence of reverse transcriptase. Luciferase activity was analyzed as described (Rincón and Flavell, 1997). Effector CD4⁺ T cells were generated in vitro as described (Rincón and Flavell, 1997). In brief, purified CD4⁺ T cells were activated with plate-bound anti-CD3 + soluble anti-CD28 (5 and 1 μg/ml, respectively) for 4 days. The cells were extensively washed. 10⁶ effector cells/ml were restimulated with 5 μg/ml of plate-bound anti-CD3 in the absence or presence of 50 μg/ml Salp15 for 24 hr, and the supernatants were analyzed for IL-2, IL-4, and IFN-γ by capture ELISA. The cells were also analyzed for proliferation by the addition of [³H]-thymidine, as before.

**EMSA**

Nuclear extracts were obtained from 12 hr anti-CD3/CD28 mAbs activated and unstimulated CD4⁺ T cells in the presence (+) or absence (−) of Salp15 (Schreiber et al. 1989 and Tugores et al. 1992). Electromobility shift analysis was then performed using 2 μg of the extracts and 10⁴ cpm of the specific ³²P end-labeled double-stranded oligonucleotides representing the consensus binding sites for AP-1, NF-AT, NF-κB, and CREB (control) (Schreiber et al., 1989). The oligonucleotides used are as follows (sense strand): IL2-NFAT, 5’-GCCCAAGAGGAAAATTTGTTCATACAG-3’;AP-1,5’ GTCGACGTGAGTCAGCGCAGGTCAGCGGAGC-3’ (Rincón and Flavell, 1997); NF-κB, 5’-GATCAGAGGGACTTTTCCGAG-3’ (Millet et al., 2000); and CREB, 5’-AGAGATTGCCTGACGAGAGCTAG-3’.

**Intracellular calcium levels**

10⁶ CD4⁺ T cells were loaded with 7 μM Indo-1 for 45 min. Ten minutes before the assay, 2-aminoethoxydiphenyl borate (2-APB, 20 μM, a gift from M. Nelson) was added. The cells were
then transferred to a Standard Extracellular Solution (in mM: 140 NaCl, 4 KCl, 1 CaCl$_2$, 2 MgCl$_2$, 1 KH$_2$PO$_4$, 10 glucose, and 10 HEPES, [pH 7.4]). The ratio of bound Indo-1 fluorescence (395 nm) to unbound Indo-1 fluorescence (525 nm) was then determined for baseline, during preincubation with Salp15 (100 μg/ml), after anti-CD3 plus anti-hamster IgG treatment (40 and 60 μg/ml, respectively), and after ionomycin induction (500 ng/ml).

**Immunizations and DTH reactions**

Groups of four Balb/c mice were immunized with equimolar quantities (0.5 μM, corresponding to 50 μg of TR-Salp15) of TR or TR-Salp15 in complete Freund's adjuvant (CFA). Eleven days later, CD4+ T cells were purified and analyzed in recall responses to TR and TR-Salp15. IFN-γ levels were measured by capture ELISA in 40 hr restimulation supernatants (Anguita et al., 1996). TR-specific IgM (1/160 dilution) and IgG (1/320 dilution) levels in the sera of the immunized mice were determined by ELISA (Anguita et al., 1996) using TR-bound plates. In another set of experiments, groups of five mice were immunized with 1 μg of KLH in the absence or presence of 10 μg of Salp15. The mice were boosted with KLH ± Salp15 after 10 days in incomplete Freund's adjuvant (IFA). Ten days later, KLH-specific IgG levels in the sera were measured by capture ELISA, as before. Delayed-type hypersensitivity reactions were performed as follows. Groups of four mice were immunized with 10 μg of KLH in the absence or presence of 50 μg of Salp15. Control animals were injected with 50 μg of Salp15 without KLH. Four days later, all mice were challenged in both ears with 1 μg of KLH. Ear swelling was recorded at 24 and 48 hr after challenge. The difference between ear thickness before immunization and after challenge was recorded and tabulated for each mouse.
References


Salp15 inhibits T cell activation


