Prostaglandin E₂ Is a Major Inhibitor of Dendritic Cell Maturation and Function in *Ixodes scapularis* Saliva

Anderson Sá-Nunes, André Bafica, David A. Lucas, Thomas P. Conrads, Timothy D. Veenstra, John F. Andersen, Thomas N. Mather, José M. C. Ribeiro and Ivo M. B. Francischetti

*J Immunol* 2007; 179:1497-1505; doi: 10.4049/jimmunol.179.3.1497

http://www.jimmunol.org/content/179/3/1497

---

**References**
This article cites 52 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/179/3/1497.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Tick saliva is thought to contain a number of molecules that prevent host immune and inflammatory responses. In this study, the effects of *Ixodes scapularis* saliva on cytokine production by bone marrow-derived dendritic cells (DCs) from C57BL/6 mice stimulated by TLR-2, TLR-4, and TLR-9 ligands were studied. Saliva at remarkably diluted concentrations (<1/2000) promotes a dose-dependent inhibition of IL-12 and TNF-α production induced by all TLR ligands used. Using a combination of fractionation techniques (microcon filtration, molecular sieving, and reversed-phase chromatography), we unambiguously identified PGE2 as the salivary inhibitor of IL-12 and TNF-α production by DCs. Moreover, we have found that *I. scapularis* saliva (dilution 1/200; ~10 nM PGE2) marginally inhibited LPS-induced CD40, but not CD80, CD86, or MHC class II expression. In addition, saliva significantly suppressed the ability of DCs to stimulate Ag-specific CD4+ T cell proliferation and IL-2 production. Notably, the effect of saliva on DC maturation and function was reproduced by comparable concentrations of standard PGE2. These findings indicate that PGE2 accounts for most inhibition of DC function observed with saliva in vitro. The role of salivary PGE2 in vector-host interaction and host immune modulation and inflammation in vivo is also discussed. This study is the first to identify molecularly a DC inhibitor from blood-sucking arthropods. The *Journal of Immunology*, 2007, 179: 1497–1505.

Dendritic cells (DCs) are professional APCs that initiate the adaptive immune response to invading pathogens, and are found in two distinct functional states. Immature DCs are located in nonlymphoid tissues, such as skin and mucosa, and their primary function is to uptake Ags. In contrast, mature DCs are poorly phagocytic, but highly efficient stimulators of naive T cell responses (1). Accordingly, DCs produce IL-12 and TNF-α during the maturation process. These cytokines are involved in inflammation and differentiation of Th1 cells, as well as in the up-regulation of costimulatory molecules, including CD40, CD80, and CD86 (2, 3). DCs are probably one of the first immune defense cells activated by tick-transmitted pathogens during blood feeding. Failure to fully activate DCs may result in an unbalanced immune response, and consequently, in increased infection by tick-borne diseases.

In their adaptation to hematophagy, blood-sucking arthropods evolved salivary components that prevent their rejection and potentially enhance pathogen transmission. This is particularly true for hard ticks that remain attached to their hosts for several consecutive days (4). Therefore, ticks must modulate host inflammatory, immune, and vascular systems to successfully feed on blood and complete their life cycle. Several molecules have been described in tick saliva, including anticoagulants (5, 6), as well as vasodilator (7) and angiogenesis inhibitors (8). Immunomodulators (9, 10) such as inhibitors of neutrophil (11, 12) and T lymphocyte function (13, 14) in addition to an IL-2-binding protein (15) have been reported. Furthermore, in vivo and in vitro inhibition of cytokines has been demonstrated by *Ixodes scapularis* and other species of hard ticks. For example, macrophage production of IL-1 and TNF-α, as well as lymphocyte production of IL-2 and IFN-γ, were diminished in the presence of *Dermacentor andersoni* salivary gland extracts (16). In addition, splenocytes from C3H/HeJ mice infected with *I. scapularis* nymphs presented decreased production of IL-2 and IFN-γ and increased production of IL-4 and IL-10 when stimulated with Con A (17, 18). Also, saliva of *Rhipicephalus sanguineus* inhibited Con A-induced IL-2 production by splenocytes (19) and IL-12 production by LPS-stimulated bone marrow-derived DCs (20). Despite the potentially critical role of these molecules in the biology of tick feeding and host-vector interaction, their molecular identification has remained elusive to date.

**Prostaglandin E2 Is a Major Inhibitor of Dendritic Cell Maturation and Function in Ixodes scapularis Saliva**

Anderson Sá-Nunes,* André Bafica,‡‡ David A. Lucas,* Thomas P. Conrads,‡§ Timothy D. Veenstra,‡ John F. Andersen,* Thomas N. Mather,¶ José M. C. Ribeiro,* and Ivo M. B. Francischetti*‡

Tick saliva is thought to contain a number of molecules that prevent host immune and inflammatory responses. In this study, the effects of *Ixodes scapularis* saliva on cytokine production by bone marrow-derived dendritic cells (DCs) from C57BL/6 mice stimulated by TLR-2, TLR-4, and TLR-9 ligands were studied. Saliva at remarkably diluted concentrations (<1/2000) promotes a dose-dependent inhibition of IL-12 and TNF-α production induced by all TLR ligands used. Using a combination of fractionation techniques (microcon filtration, molecular sieving, and reversed-phase chromatography), we unambiguously identified PGE2 as the salivary inhibitor of IL-12 and TNF-α production by DCs. Moreover, we have found that *I. scapularis* saliva (dilution 1/200; ~10 nM PGE2) marginally inhibited LPS-induced CD40, but not CD80, CD86, or MHC class II expression. In addition, saliva significantly suppressed the ability of DCs to stimulate Ag-specific CD4+ T cell proliferation and IL-2 production. Notably, the effect of saliva on DC maturation and function was reproduced by comparable concentrations of standard PGE2. These findings indicate that PGE2 accounts for most inhibition of DC function observed with saliva in vitro. The role of salivary PGE2 in vector-host interaction and host immune modulation and inflammation in vivo is also discussed. This study is the first to identify molecularly a DC inhibitor from blood-sucking arthropods. The *Journal of Immunology*, 2007, 179: 1497–1505.

Dendritic cells (DCs) are professional APCs that initiate the adaptive immune response to invading pathogens, and are found in two distinct functional states. Immature DCs are located in nonlymphoid tissues, such as skin and mucosa, and their primary function is to uptake Ags. In contrast, mature DCs are poorly phagocytic, but highly efficient stimulators of naive T cell responses (1). Accordingly, DCs produce IL-12 and TNF-α during the maturation process. These cytokines are involved in inflammation and differentiation of Th1 cells, as well as in the up-regulation of costimulatory molecules, including CD40, CD80, and CD86 (2, 3). DCs are probably one of the first immune defense cells activated by tick-transmitted pathogens during blood feeding. Failure to fully activate DCs may result in an unbalanced immune response, and consequently, in increased infection by tick-borne diseases.

In their adaptation to hematophagy, blood-sucking arthropods evolved salivary components that prevent their rejection and potentially enhance pathogen transmission. This is particularly true for hard ticks that remain attached to their hosts for several consecutive days (4). Therefore, ticks must modulate host inflammatory, immune, and vascular systems to successfully feed on blood and complete their life cycle. Several molecules have been described in tick saliva, including anticoagulants (5, 6), as well as vasodilator (7) and angiogenesis inhibitors (8). Immunomodulators (9, 10) such as inhibitors of neutrophil (11, 12) and T lymphocyte function (13, 14) in addition to an IL-2-binding protein (15) have been reported. Furthermore, in vivo and in vitro inhibition of cytokines has been demonstrated by *Ixodes scapularis* and other species of hard ticks. For example, macrophage production of IL-1 and TNF-α, as well as lymphocyte production of IL-2 and IFN-γ, were diminished in the presence of *Dermacentor andersoni* salivary gland extracts (16). In addition, splenocytes from C3H/HeJ mice infected with *I. scapularis* nymphs presented decreased production of IL-2 and IFN-γ and increased production of IL-4 and IL-10 when stimulated with Con A (17, 18). Also, saliva of *Rhipicephalus sanguineus* inhibited Con A-induced IL-2 production by splenocytes (19) and IL-12 production by LPS-stimulated bone marrow-derived DCs (20). Despite the potentially critical role of these molecules in the biology of tick feeding and host-vector interaction, their molecular identification has remained elusive to date.
In this study, we have identified for the first time PGE₂ isolated directly from tick saliva as the main inhibitor of inflammatory and Th1-related cytokine production and of T cell stimulation by murine DCs. Remarkably, concentration-response assays performed using *I. scapularis* saliva and a commercial standard indicate that the amount of PGE₂ in saliva accounts for most of its immunosuppressive effects on DC maturation and function observed in vitro.

**Materials and Methods**

**Experimental animals**
Six- to 10-wk-old female C57BL/6 mice and DO11.10 mice that express transgenic TCR specific for OVA peptide 323–339 were purchased from Taconic Farms. Animals were bred and maintained at an American Association of Laboratory Animal Care-accredited facility at the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

*I. scapularis* saliva

*I. scapularis* saliva was obtained by inducing partially engorged adult female *I. scapularis* to salivate (3–4 days postattachment to a rabbit) into capillary tubes using the modified pilocarpine induction method (21).

**Purified TLR agonists**

Ultrapure *Escherichia coli* 0111:B4 LPS, peptidoglycan (PGN), and oligonucleotide CpG (1826) were purchased from InvivoGen.

**Bone marrow-derived DCs and CD11c⁺ cell cultures**

DCs were generated according to the method of Inaba et al. (22) with modifications (23). Briefly, bone marrow cells from the femurs of C57BL/6 mice were cultured in complete medium (RPMI 1640 medium with 10% heat-inactivated FBS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 mM 2-ME) and 20 ng/ml murine rGM-CSF (Sigma-Aldrich). At day 0, cells were seeded at 10⁶ per 100-mm petri dish (Falcon 1029 plates; BD Discovery Labware) in 10 ml of medium. At days 3 and 6, another 10 ml of complete medium containing 20 ng/ml murine rGM-CSF was added to the plates. In a setting of experiments, CD11c⁺ cells (enriched DCs) were obtained, as described elsewhere (24). In brief, DC suspensions were incubated with anti-CD11c MicroBeads (Miltenyi Biotec) for 15 min at 4°C, followed by a washing step in PBS/BSA, and then sorted using MACS columns (LS). Analysis of the sorted cells showed purity 80–90% CD11c⁺ cells (data not shown).

**FIGURE 1.** *I. scapularis* saliva inhibits IL-12p40 (A–C) and TNF-α (D–F) production by bone marrow-derived DCs stimulated by TLR ligands in a dose-dependent manner. DCs from C57BL/6 mice were generated by incubation with GM-CSF for 6–9 days. Then, cells were washed twice and preincubated with medium or different saliva dilutions. After 30 min, cells were stimulated overnight with TLR-4 (LPS, 100 ng/ml; A and D), TLR-2 (PGN, 10 μg/ml; B and E), or TLR-9 (CPG, 150 nM; C and F) ligands. Cytokine levels in culture supernatants were measured by ELISA. *, *p* < 0.05 vs LPS, CPG, and PGN groups without saliva dilutions.

**FIGURE 2.** *I. scapularis* saliva inhibits LPS-induced IL-12p70 (A) and stimulates IL-10 (B) production by bone marrow-derived DCs in a dose-dependent manner. DCs from C57BL/6 mice were generated by incubation with GM-CSF for 6–9 days. Then, cells were washed twice and preincubated with medium or different saliva dilutions. After 30 min, cells were stimulated overnight with LPS (100 ng/ml). Cytokine levels in culture supernatants were measured by ELISA. *, *p* < 0.05 vs LPS group without saliva dilutions.
Cytokine production assay

Six- to 9-day cultured bone marrow-derived DCs were gently collected, washed twice, and resuspended at 10⁶ cells/ml in complete medium. Cells were cultured at 10⁵ cells/well in round-bottom 96-well cluster plates (Costar, Corning Glass) and incubated with medium, saliva, saliva fractions, or PGE2 for 30 min before the addition of TLR2 (PGN, 10⁻⁹ g/ml), TLR4 (LPS, 100 ng/ml), or TLR9 (CpG ODN 1826, 0.15⁻⁶ M) ligands. Following overnight incubation at 37°C and 5% of CO₂, cell-free supernatants were collected and diluted accordingly to the IL-12p40, IL-12p70, TNF-α, and IL-10 determination by using BD OptEIA ELISA sets, according to manufacturer’s instructions (BD Biosciences). In some experiments, LPS-stimulated CD11c⁺ cells were used for cytokine production assay, as described above.

HPLC procedures

Molecular sieving HPLC of saliva was performed using a Superdex 75 column (3.2 × 300 mm) (Amersham Biosciences), and eluted at 0.05 ml/min with 10 mM HEPES buffer (pH 7.2), containing 0.15 M NaCl. The solvent was delivered using a CM-4100 pump (ThermoSeparation Products). The eluent was monitored for UV absorbance at 220 nm (SM-4100 UV Spectrophotometer; ThermoSeparation Products) with fractions being collected at 1-min intervals using a FC203-B fraction collector (Gilson). The activity of each fraction on LPS-induced IL-12p40 and TNF-α production was tested. The column was calibrated using BSA, OVA, carbonic anhydrase, myoglobin, cytochrome c, angiotensin I, tyrosine, and tryptophan.

Reversed-phase HPLC of Microcon YM-3 (Millipore) filtered saliva was performed on a C18 column (0.5 × 150 mm; ThermoSeparation Products) perfused at 25 µl/min using a PerkinElmer ABI 1400 pump. The eluent was monitored at 220 nm using a 785A detector (PerkinElmer). A gradient of 60-min duration from 5 to 90% acetonitrile in water, containing 0.1% trifluoroacetic acid, was imposed after injecting of the sample. Aliquots of these fractions were tested for cytokine-inhibitory activity. Sample loops of 20 or 100 µl were used.

Reversed-phase HPLC tandem mass spectrometry

Nanoflow reversed-phase liquid chromatography (RPLC) analyses were performed using an Agilent 1100 nano liquid chromatography system (Agilent) coupled online to a linear ion trap-Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FTICR; Thermo Electron) using
nanoelectrospray ionization. Separations were performed using 75-μm internal diameter × 360-μm outer diameter × 10-cm-long fused silica capillary columns (Polymicro Technologies) that were slurry packed in-house with 5-μm, 300 Å pore size C-18 silica-bonded stationary phase (Phenomenex). After injecting 6 μl of sample, the column was washed for 20 min with 98% mobile phase A (0.1% formic acid in water) at a flow rate of 500 nL/min. A linear gradient of 2% mobile phase B (0.1% formic acid in acetonitrile) to 90% mobile phase B in 25 min, then to 98% B in an additional 25 min, all at a constant flow rate of 200 nL/min, was used for all nanoLC-mass spectrometry (MS) analyses. The LTQ-FTICR was operated in a data-dependent tandem mass spectrometry (MS/MS) mode in which each full high resolution LTQ-FTICR scan is followed by five MS/MS scans in which the five most abundant molecular ions are dynamically selected for collision-induced dissociation using a normalized collision energy of 35%. Dynamic exclusion was used to minimize redundant acquisition of molecules previously selected for collision-induced dissociation. The heated capillary temperature and electrospray voltage were set at 160°C and 1.5 kV, respectively.

**PGE2 determination in saliva and in culture medium**

The amount of PGE2 in saliva samples as well as its concentration in the medium used for cell culture were determined by competition ELISA kit (R&D Systems), according to manufacturer’s instructions. The detection limit for the assay is 20 pg/ml.

**Flow cytometry**

Four million bone marrow-derived DCs were preincubated with medium, whole saliva, filtrate of Microcon YM-3, or different concentrations of PGE2. After 30 min, medium or OVA (100 μg/ml) was added and incubated overnight. The supernatant was collected for cytokine determination, and the cells were washed twice and stained with fluorochrome-labeled Abs against CD11c, CD40, CD80, CD86, and MHC class II (I-A^d; BD Pharmin-gen). A total of 100,000 live cell events as gated on forward and side scatter characteristics were acquired. Data were collected using a FACS Calibur (BD Immunocytometry Systems) with CellQuest (BD Biosciences) and analyzed with FlowJo software (Tree Star).

**Ag-specific CD4^+ T cell proliferation**

Bone marrow-derived DCs were preincubated with medium, whole saliva, filtrate of Microcon YM-3, or different concentrations of PGE2. After 30 min, medium and OVA (100 μg/ml) were added in the presence of LPS (100 ng/ml) and incubated overnight. After three washings, 25,000 cells were cocultured for 72 h in the presence of 100,000 CD4^+ T cells purified from spleens of congenic DO11.10 mice using Mouse CD4 Negative Isolation Kit (Dynal Biotech). Alamar Blue (Trek Diagnostic Systems) was added at 10% of incubation volume in the last 48 h of the culture. The cell proliferation was evaluated by reading the culture absorbance at 570 and 600 nm, as previously described (25).

**Statistical analysis**

Data are shown as mean ± SEM. Statistical differences were analyzed by ANOVA. A p value of 0.05 or less was considered statistically significant.

**Results**

I. scapularis saliva inhibits TLR-induced proinflammatory cytokine production by bone marrow-derived DCs

Following treatment with purified TLR agonists PGN (TLR2), LPS (TLR4), or Cpg (TLR9), DCs produced high levels of both IL-12p40 and TNF-α (Fig. 1). When cultures were preincubated

**FIGURE 5.** Identification of PGE2 in the positive inhibitory fraction. A, Base peak chromatogram of the predominant ion in the positive inhibitor fraction. B, Peaks observed from the molecular species eluting at 31.30 min, showing the consecutive loss of 352.22 Da. C, Tandem mass spectrometry spectra of the molecular ions shown in B (zoomed ×10 for detail).
with saliva for 30 min at 1/200 dilution, significant inhibition of LPS- or PGN-induced IL-12p40 production by DCs was observed, whereas CPG-induced IL-12p40 production was only marginally inhibited (Fig. 1, A–C). At 1/667 dilution, saliva only significantly inhibited IL-12p40 production induced by LPS, but no significant effect on CPG- or PGN-induced IL-12p40 synthesis was observed at this or higher dilutions (Fig. 1, A–C). In addition, saliva diluted at 1/200, 1/667, and 1/2000 strongly inhibited TLR ligand-induced TNF-α production by these cells. For example, saliva at 1/6667 dilutions produced 50% reduction in TNF-α levels induced by all three ligands (p < 0.001; Fig. 1, D–F). Cell death was not detected in cultures incubated with TLR ligands in the presence of saliva or medium (data not shown), and basal levels of IL-12p40 and TNF-α were not changed in cultures incubated with saliva only (data not shown).

Because IL-12p40 is a subunit shared by both IL-12 and IL-23 (26), we investigated whether saliva from I. scapularis influenced synthesis of IL-12p70 production, the bioactive IL-12. Fig. 2A shows that stimulation of DCs by LPS is accompanied by IL-12p70 production, which was completely inhibited by saliva at 1/200 dilutions (p < 0.001). Strong inhibition was also observed at dilutions of 1/667, 1/2000, and 1/6667 (98.6, 89.4, and 69.7% inhibition, respectively). Stimulation of DCs by LPS was also accompanied by production of IL-23, which was not diminished by saliva (data not shown). Anti-inflammatory cytokines such as IL-10 are also induced by LPS (27), and it may be regulated by I. scapularis saliva. We next measured this mediator in LPS-stimulated DCs pretreated with saliva. As shown in Fig. 2B, saliva at 1/200 and 1/667 dilutions significantly increases IL-10 production by DCs, reaching levels 2- to 3-fold higher than detected with LPS alone.

Molecular sieving chromatography of I. scapularis saliva

To identify molecule(s) responsible for the observed immunomodulatory effects, I. scapularis saliva was applied to a gel-filtration column and fractions were tested for inhibition of LPS-stimulated DCs. Fig. 3A depicts the chromatogram of I. scapularis saliva, and Fig. 3, B and C, respectively, shows the inhibition of IL-12p40 and TNF-α synthesis by DCs observed within a fraction eluting with a retention time of 22 min. These results suggest that the apparent molecular mass of the active molecule(s) is smaller than 1000 Da (Fig. 3A, inset). Because the pilocarpin (used for tick salivation) peak eluted at 34 min (~210 Da), it did not contaminate the fraction containing the inhibitory activity.

Fractionation of I. scapularis saliva by reversed-phase chromatography

To identify the molecule(s) involved in the inhibition of IL-12p40 and TNF-α synthesis, a 50-μL aliquot of saliva was filtered using a Microcon YM3. The filtrate (representing molecules with mass <3000 Da) was resolved by reversed-phase HPLC (Fig. 4A), and the activity of each fraction was tested on LPS-stimulated DCs (Fig. 4, B and C). Fraction 62, which eluted at the end of the gradient, produced the highest inhibition of both IL-12p40 (Fig. 4B) and TNF-α (Fig. 4C) production. The assay was repeated with fractions that induced stronger inhibition of these cytokines (OD ≤0.25 for IL-12p40 and OD ≤0.20 for TNF-α), and fraction 62 was consistently confirmed as the single active fraction (data not shown).

PGE_2 is the inhibitor of IL-12p40 and TNF-α production in I. scapularis saliva

Next, fraction 62 was analyzed by nano RPLC-MS, which revealed a single prominent peak at 31 min (Fig. 5A). A series of molecular ions was observed in the mass spectrum at regular intervals of mass-to-charge (m/z) 352.22 (Fig. 5B). The MS/MS spectra of the molecular ions shown in Fig. 4B were largely uninformative, arising from poor fragmentation (Fig. 5C). Comparison of the MS results with data contained within the METLIN Metabolite Database (http://metlin.scripps.edu/metabo_advanced. php) indicated that the molecular ion observed in these analyses was most likely PGE_2 (C_{20}H_{32}O_{5}). This result was subsequently validated using a PGE_2-specific ELISA. The amount of PGE_2 found by ELISA in a saliva sample was 505 ng/ml, representing a pharmacological concentration ~1.43 μM PGE_2.

Salivary PGE_2 most likely accounts for most inhibition of cytokine production by LPS-stimulated DCs

In an attempt to determine the relative contribution of salivary PGE_2 in the inhibition of IL-12p40 and TNF-α production by DCs, concentration-response curves were performed using a standard commercial PGE_2 and compared with the inhibition attained with given dilutions of saliva or YM-3 filtrate. An aliquot was taken from all these samples and used for estimation of PGE_2 concentration by ELISA. In these experiments, DC preparation was enriched with CD11c+ to rule out the contribution of macrophages or...
neutrophils in cytokine production. Fig. 6 shows the inhibitory curves for IL-12p40 and TNF-α production by DCs in the presence of whole saliva, YM-3 filtrate, or standard PGE2, all tested in a dilution designed to contain comparable concentrations of PGE2. Accordingly, the curves were superimposable, indicating that similar levels of inhibition were attained regardless of the preparation. We thus concluded that PGE2 most likely accounts for most, if not all, inhibition of IL-12p40 and TNF-α observed with whole saliva. Finally, the YM-3 retentate, which includes molecules with molecular mass greater than 3000 Da and did not contain any detectable PGE2, consistently had no inhibitory effect on IL-12p40 and TNF-α production (data not shown).

Effects of *I. scapularis* saliva on DC expression of CD40, CD80, CD86, and MHC class II

To further investigate the effects of saliva and PGE2 in DC maturation, bone marrow DCs were stimulated with LPS in the presence of medium (positive control), whole saliva, YM-3 filtrate, or PGE2. Subsequently, the expression of costimulatory molecules was analyzed by flow cytometry. Overnight incubation with LPS increased the surface expression of CD40, CD80, and CD86 in DCs (Fig. 7A). Preincubation with saliva or YM-3 filtrate at 1/200 dilution (both containing ~10 nM PGE2; Fig. 7B) slightly inhibited LPS-induced expression of CD40; however, no effects could be demonstrated for CD80 or CD86 (Fig. 7A). When higher concentrations of PGE2 (100 nM and 1 μM) were tested, CD80 or CD86 expression remained unchanged, but strong inhibition of CD40 expression in DCs was observed (data not shown). These findings suggest that PGE2 present in the saliva or YM-3 filtrate (1/200) dilution slightly suppresses LPS-induced CD40 expression by DCs.

The DCs used in this assay constitutively presented high expression levels of MHC class II molecules, and LPS only marginally affected its expression. This pattern remained the same in the

**FIGURE 7.** *I. scapularis* saliva and YM-3 filtrate marginally inhibit LPS-induced CD40, but do not affect CD80 and CD86 or MHC class II expression. Four million bone marrow-derived DCs were preincubated with medium, saliva (1/200), YM-3 filtrate (1/200), or PGE2 (10 nM) for 30 min prior to an overnight incubation in the presence of LPS (100 ng/ml). Cells were stained with fluorochrome-labeled Abs specific for CD11c, CD40, CD80, CD86, and MHC class II, and analyzed by flow cytometry. The data shown were gated for live CD11c+ cells (A). PGE2 content in the medium of each group was estimated by ELISA in the beginning of the incubation (B). Levels of IL-12p70 (C) and TNF-α (D) in the culture supernatant were estimated by ELISA at the end of the incubation period. *p < 0.05 vs LPS-stimulated group.
Tick modulation of the host’s innate and acquired immune response is recognized as a factor in the transmission of tick-borne diseases, such as Lyme borreliosis, babesiosis, and anaplasmosis (28–30). It has been suggested that I. scapularis saliva plays a role in infection, according to experimental model of guinea pig infection with Borrelia burgdorferi-infected ticks (31). In fact, tick saliva contains molecules that prevent complement activation (32) and T cell proliferation (13, 15, 33), and that polarize T cell responses to a Th2 profile (34). Tick saliva also modulates cytokine production by epidermal cells (35) and alters expression of adhesion molecules by skin-derived endothelial cells (36). More recently, a study demonstrated that saliva from R. sanguineus inhibits differentiation, maturation, and cytokine synthesis of bone marrow-derived DCs (20). Despite the critical role of immunomodulators in tick-host interaction, the molecular cloning and identification of most of these molecules have not been reported.

In the experiments described in this study, we have further studied the properties of I. scapularis saliva on DC maturation and function. Accordingly, we demonstrated for the first time that I. scapularis saliva down-modulates IL-12 and TNF-α as well as up-regulates IL-10 production by DCs stimulated with TLR ligands (Figs. 1 and 2). These findings are particularly important because both TNF-α and IL-12 are major products of maturing DCs and contribute to inflammation and to adaptive immune response, whereas IL-10 has an anti-inflammatory activity (3, 37). Furthermore, using a number of purification steps involving microcon filtration (cutoff 3 kDa), molecular sieving, and reversed-phase HPLC chromatographies, we have unambiguously identified, by mass spectrometry, PGE2 as the molecule present in the active salivary fraction (Figs. 4 and 5). Notably, superimposable inhibitory curves for inhibition of cytokine production by DCs were observed with whole saliva, YM-3 filtrate, and the corresponding concentration of PGE2 (Fig. 6). It was concluded that PGE2 accounts for most, if not all, DC-inhibitory activity on cytokine production found with I. scapularis saliva in vitro.

The finding that PGE2 is responsible for the inhibition of IL-12/TNF-α produced by LPS-stimulated DCs has been previously reported (27, 38) and has several implications in our understanding of tick-feeding behavior and vector-host interactions. PGE2 is known to modulate inflammatory response and the subsequent activation of the adaptive immune system (39, 40). Of note, these processes are interdependent and display an important cross-talk that is essential for normal homeostasis. For example, inflammatory cytokines (e.g., TNF-α) produced by DCs and macrophages induce the changes required for DC maturation and migration from the periphery to the lymph nodes. The presence of inflammatory cytokines also leads to up-regulation of adhesion molecule by the endothelium (e.g., E-selectins), and these molecules contribute to the recruitment of monocytes and other cell types to inflamed tissue (3).

Tick saliva contains pharmacological (μM range) amounts of PGE2 (9, 41–43). Therefore, it is plausible to suggest that blockade of DC-mediated cytokine production by this prostanoid may contribute to anti-inflammation and immunosuppression in vivo. This assumption is supported by the fact that inhibition of cytokine production by DCs (Fig. 6) or macrophages (44) was attained at remarkably low doses of PGE2. Finally, saliva increases DC production of IL-10, an anti-inflammatory cytokine whose expression is modulated by PGE2 (27). IL-10 also serves as a potent mechanism for limiting the maturation of DCs and their capacity to initiate Th1 response (1). Because maturation and differentiation of Th1 T cells are particularly dependent on IL-12 and TNF-α (1, 3),
FIGURE 9. Immunomodulators identified in *I. scapularis* saliva or salivary gland extract: putative and described functions. Pleiotropic PGE\(_2\) effects include inhibition of DC cytokine production (27, 38) (Fig. 6), Ag presentation (40) (Fig. 8), macrophage (51), and T lymphocyte activation (52). Sialostatin L, which affects neutrophil migration (11), is an inhibitor of cathepsins L and S that are involved in Ag processing, presentation, and consequently in T cell proliferation (53). Salp15 was described as an inhibitor of T cell activation (13) through binding to the CD4 TCR (14). IL-2-binding protein decreases IL-2 availability affecting T cell proliferation (15).

cytokines whose production was dramatically inhibited in our assays, it is plausible to hypothesize that saliva down-regulates inflammation and impairs Th1 responses.

Subsequent to activation by a given stimulus, DCs undergo a maturation process in which expression of the costimulatory molecules and cytokines necessary to activate and differentiate naïve T cells is up-regulated. To examine the effects of saliva in the maturation process of DCs, we tested its effects in the expression levels of CD40, CD80, CD86, and MHC class II and in the proliferation of CD4\(^+\) T cells. Our results demonstrate that saliva or YM-3 filtrate (both containing ~10 nM PGE\(_2\)) produced marginal inhibition of CD40 expression on one hand (Fig. 7A), but a significant attenuation of T cell proliferation and IL-2 production in contrast (Fig. 8). Of note, these findings were replicated with standard PGE\(_2\) at similar concentrations. In other words, the effects observed with saliva in DC maturation are most likely due to its PGE\(_2\) content. It is important to recognize that high concentrations of PGE\(_2\) completely inhibited the expression of CD40 (data not shown). Whether such concentration of PGE\(_2\) is achievable in vivo is not known, and it may also differ according to the stage of tick development (e.g., nymphs vs adults) and/or the time of feeding (e.g., hours vs days) (45). Nevertheless, our data support the view that *I. scapularis* PGE\(_2\) is the major in vitro inhibitor of maturation and function of DCs. It may operate alone or in combination with other molecules with immunomodulatory/anti-inflammatory function that remains to be identified in *I. scapularis* saliva.

The notion that salivary PGE\(_2\) affects DC function is particularly relevant because these cells are resident in the skin, and are probably one of the first cells to interact with salivary components at the tick attachment site. In this context, evidence for saliva-DC interaction comes from studies in which epidermal cell population containing Langerhans cells (the immature DC population of skin) was incubated in vitro with tick saliva. These cells, loaded with salivary Ags, were able to stimulate proliferation of T lymphocytes obtained from the lymph node of bite-sensitized tick-resistant guinea pigs (46). This indicates that DCs are presumably capable of priming an immune response in vivo during tick infestation.

That assumption is supported by an earlier study that identified the presence of Ags from ticks’ salivary glands associated with Langerhans cells (47). Another study showed that during primary infestations with *D. andersoni*, there is a decrease in the number of these cells around the sites of tick attachment. The authors have concluded that Langerhans cells migrate, probably to lymph nodes, after contact with components from saliva (48). In addition, murine splenic DCs or Langerhans cell-enriched epidermal cells that have been pulsed with live spirochetes mediate the production of anti-*B. burgdorferi* Abs in vitro (49). Finally, *B. burgdorferi* components are recognized by TLRs present in DCs (50). These results are consistent with the concept that DCs are active components in the immune response to tick saliva and potentially modulate pathogen transmission.

Therefore, PGE\(_2\) can be added to the increasing list of molecules characterized in *I. scapularis* saliva that modulate the immune system. As diagrammatically shown in Fig. 9, the putative and actual roles of these molecules on inflammation and adaptive immunity include inhibition of neutrophil accumulation (11), blockade of macrophage and T lymphocyte activation (51, 52), decrease in Ag presentation by DCs (40), and attenuation of IL-2 production by T lymphocytes (13–15). Therefore, inhibition of immune cell function by the tick saliva mixture provides further insights into the mechanism by which ticks successfully feed for extended periods of time. It also helps to explain how ticks regulate subsequent activation of acquired immune response to saliva components, tick mouthparts, and possibly, to the pathogens present in saliva.

Acknowledgments

We thank Drs. Thomas E. Wellemes, Robert W. Gwadz, Kathryn C. Zoon, and Alan Sher (National Institute of Allergy and Infectious Diseases/National Institutes of Health) for encouragement and support.

Disclosures

The authors have no financial conflict of interest.

References


The Journal of Immunology


