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Splenic dendritic cells pulsed with *Ixodes ricinus* tick saliva prime naive CD4$^+$T to induce T$_h$2 cell differentiation *in vitro* and *in vivo*

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Abstract

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) in priming naive T cells. Using an *in vitro* priming system, we show that DCs incubated with *Ixodes ricinus* tick saliva initiate the T$_h$2 differentiation of CD4$^+$T cells. As determined with reverse transcription–PCR, the expression of IL-4 mRNA by these cells is higher than IFN-γ mRNA. Early endogenous production of IL-4 is thought to be important during the *in vitro* interaction of saliva-pulsed DCs with CD4$^+$T cells. Its neutralization with specific mAbs inhibits the development of IL-4-secreting T$_h$2 cells. Moreover, differentiated T$_h$2 cells proliferate only when saliva-pulsed DCs and IL-1β are added together early in the primary culture. As demonstrated by FACS analysis, the treatment *in vitro* of saliva-pulsed DCs by IL-1β enhanced the expression of B7 and mainly CD40 co-stimulatory molecules, which provide sufficient signals to stimulate sensitized CD4$^+$T cell proliferation. On the other hand, DCs treated with tick saliva only up-regulated mostly B7-2 co-stimulator expression and this was associated with differentiation of naive CD4$^+$T cells into T$_h$2 type of cells. The *in vitro* priming system is suitable to investigate the major elements implicated in the anti-tick immune response such as naive CD4$^+$T cells, whole DCs population and tick saliva, and it can provide the possibility to delimit further the saliva molecules, the DC subsets and the type of host cells involved in the T$_h$2 polarization. Corresponding *in vivo* experiments involving subcutaneous injection of tick saliva-pulsed DCs into BALB/c mice also elicited a T$_h$2 immune response. *Ex vivo* cultures of draining lymph node T cells stimulated with tick saliva produced higher IL-4 : IFN-γ ratios compared with controls, confirming the relevance obtained in the *in vitro* priming model. These experiments demonstrate the importance of tick saliva in priming DCs to initiate a T$_h$2-biased immune response *in vitro* and *in vivo*.

Introduction

Cytokines produced by CD4$^+$T cells polarize the immune response *in vivo* or *in vitro* in response to antigen challenge. T$_h$1 cells are characterized by the predominance of IFN-γ production while T$_h$2 cells produce predominantly IL-4 (1). This dichotomy in T helper activity is in part reported to be associated with susceptibility or resistance to pathogens. In experimental murine cutaneous leishmaniasis, selective activation of T$_h$1 cells is associated with resolution of the disease whereas selective activation of T$_h$2 cells with disease progression (2). Vectors such as tick or sand fly inject pathogens into skin along with saliva. Salivary gland lysate of the old world sand fly *Phlebotomus papatasi* exacerbates lesion size and enhances *Leishmania major* burden in disease-resistant CBA mice. This observation is correlated with inhibition of the production of T$_h$1, and enhancement of T$_h$2 cytokines (3). In BALB/c mice infested by *Ixodes ricinus* ticks infected with *Borrelia burgdorferi*, anti-borrelial IgG2a production is diminished in comparison with mice infected with a syringe (4). These examples reveal that ectoparasite saliva promotes a T$_h$2-type immune response. Pathogen-free *I. ricinus* instars elicit also a T$_h$2 immune response in BALB/c (H-2d) as well as other strains of mice such as DBA (H-2d), C57BL/6 (H-2b), C3H (H-2k), CBA (H-2k), SJL (H-2s) and FVB (H-2q) (5, 6). Many factors have been reported to be implicated independently or together in the polarization of the immune response *in vivo* (7). In mice infested with *I. ricinus*, neither the host genetic background nor the infestation intensity modified the orientation of the immune response (6). The molecular nature of tick saliva and the environment of the infested skin may finally determine the

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differentiation of naive CD4⁺T cells into T_h2 cells. The activation of CD4⁺T cells occurs mostly in secondary lymphoid organs and requires antigen-presenting cells (APCs). Dendritic cells (DCs) are widely distributed in the body and are the most important cells implicated in this activation (8). The original microenvironment of DCs including cytokines and antigens may influence their priming capacity to differentiate naive CD4⁺T cells along either a T_h1 or T_h2 pathway (9).

In our work an *in vitro* priming system has been set up. It reproduces the *in vivo* leukocyte reaction and brings out the importance of tick saliva for the polarization of the immune response. DCs pulsed with tick saliva induce the T_h2 differentiation of naive CD4⁺T cells *in vitro*, and when injected subcutaneously into BALB/c mice also *in vivo*. The treatment of *in vitro* cultures with neutralizing anti-IL-4 mAbs revealed the necessity of the early production of endogenous IL-4 to direct the immune response toward T_h2. Moreover, the early addition of exogenous IL-1β into the leukocyte reaction was found to be necessary for the proliferation, but not for the differentiation of T_h2 cells *in vitro*. IL-1β treatment of tick saliva-pulsed DCs enhanced the expression of the main accessory molecules B7-2 and CD40, which render them able to drive responder T_h2 cells proliferation. In the case of tick infestation, IL-1β appears to be necessary for the activation and the development of T_h2 immune response in the lymphoid organs.

Methods

Animals

Male rabbits (New Zealand) weighing an average 3 kg and BALB/c female mice 8- to 12-weeks old are purchased from Elevage des Dombes (Romans, France) and IFFA-CREDO (Arbresle, France), respectively. *Ixodes ricinus* adult female ticks were reared in our laboratory as previously described (10).

Tick saliva preparation

To collect saliva, adult *I. ricinus* ticks were allowed to feed for 5 days and 12 h on rabbit's ears. Partially engorged female ticks were removed and fixed by their dorsum on a sticky paper. To enhance the salivation, 5 μl of dopamine solution 0.2% (w/v) (Fluka, Buchs, Switzerland) in 50 mM PBS, pH 7.4, were injected into the haemocoele of the tick using a 0.3 × 13-mm needle (Becton Dickinson, Switzerland) (11). A finely drawn capillary tube of 20 μl of countenance was fitted over the mouthparts of each tick which was allowed to salivate for 20–40 min. The volume provided by each tick was 3 μl in average. Saliva from 100 partially fed ticks was pooled and stored at −20°C until use. Protein concentration of saliva was determined using BCA Protein Assay Kit (Pierce, Socochim, Switzerland).

DCs separation

A cellular suspension was obtained by teasing BALB/c mice spleens with a large striated forceps. The RBCs were removed by incubation in ACK hypotonic lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA) during 5–10 s. Cells/spleen (5×10^7) were washed and suspended in HBSS + 20% FCS. They were used to discard firstly macrophages that allowed to adhere to a Petri dish during 1 h at 37°C, and non-adherent cells were then re-suspended in the isolation buffer containing PBS 50 mM, pH 7.4, supplemented with 5% decomplemented FCS and 5% decomplemented normal rat serum and 1 mM EDTA. They were ready for DCs separation by a negative selection system, using the StemSep biotinylated antibody cocktail (Biocoba, Switzerland) in combination with StemSep magnetic colloid. The antibody cocktail contains biotinylated mAbs to the following mouse cell-surface antigens: CD2, CD3, CD19, TER 119, Ly-6G (gr-1) and neutrophils (7–4). These antibodies target and retain into the column, respectively: thymocytes, pre-B, B, NK myeloid and erythrocytes; T cells and NKT cells; B cells and follicular DCs; proerythroblast to mature erythrocytes; myeloid cells and neutrophils. Consequently, an enriched fraction of DCs flow through the column. To improve DCs enrichment, the harvested cells were subject of a second round of negative selection. Finally after discarding macrophages, the StemSep system eliminate unwanted cells to obtain a highly (>90) enriched DCs which are defined as $CD11c^+$, $I\text{-}A^+$, $F4/80^{low}$. DCs were finally washed and suspended in RPMI-1640 (GIBCO, Basel, Switzerland), supplemented with 10% FCS (v/v), 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids (Sigma, Switzerland), 0.05 mM mercaptoethanol, 100 U ml^{-1} penicillin/streptomycin (GIBCO) and 25 μg ml^{-1} fungizone (GIBCO).

CD4⁺T cells separation

RBCs-depleted spleen cell suspension prepared from BALB/c mice was washed twice in HBSS and suspended in the isolation buffer without EDTA. CD4⁺T cells were then separated using the StemSep biotinylated antibody cocktail (StemCell, Basel, Switzerland) for CD4⁺T cells in combination with StemSep magnetic colloid, according to the manufacturer's instructions. Recovered CD4⁺T cells (>90% purity) were washed and suspended in complete RPMI-1640.

CD4⁺T cell priming in vitro

Cell cultures were set up in 24-well plates (Falcon, Becton Dickinson, Switzerland). Purified splenic DCs per well (5×10^5) were pulsed *in vitro* with 15 μg of *I. ricinus* tick saliva (which has a concentration of 1 mg ml^{-1}) in 500 μl of complete RPMI-1640 during 24 h. Controls received RPMI-1640 alone. Thereafter, 5×10^6 purified CD4⁺T cells per well were added in 500 μl of complete RPMI-1640. In some experiments, complete RPMI-1640 medium was supplemented at the beginning of the culture with neutralizing anti-IL-4 mAbs (Becton Dickinson) at the concentration of 50 μg ml^{-1}. Cultures were maintained during 7 days at 37°C and 5% CO_2.

Secondary stimulation of primed CD4⁺T cells in vitro

Cultures were set up in 96-well plates (Falcon, Becton Dickinson). Purified splenic DCs per well (2×10^4) were pulsed with 4 μl of tick saliva overnight *in vitro* in 100 μl of complete RPMI-1640. Controls were maintained in RPMI medium alone. DCs were then cultivated with 2×10^5 of homologous CD4⁺T cells primed *in vitro* with saliva-pulsed DCs supplemented or not with 2 ng per well IL-1β (respectively,

$p_{s+IL\text{-}1\beta}$ $CD4^+$T cells and p_s $CD4^+$T cells). As controls, 2×10^4 purified splenic DCs per well pulsed or not with tick saliva were cultured with $CD4^+$T cells primed *in vitro* with DCs in the presence of 2 ng IL-1β per well only ($p_{IL\text{-}1\beta}$ $CD4^+$T cells). Cultures were maintained in 200 μl total volume of complete RPMI-1640 during 96 h at 37°C and saturated atmosphere and 5% CO_2. They were pulsed with 1 μCi per well of methyl [^{3}H]thymidine ([^{3}H]TdR) (specific activity 25 Ci mmol^{-1}) (Amersham, Bucks, UK) 24 h before harvesting the cells. [^{3}H]TdR incorporation was determined by liquid scintillation counting.

Competitive PCR analysis of cytokine messenger RNA production

RNA extraction. At the seventh day of primary culture, total RNA was extracted from collected 5×10^5 $CD4^+$T cells stimulated *in vitro* with tick saliva-pulsed DCs or DCs alone either in presence or absence of anti-IL-4. The prescript isolation kit (Gentra Systems, Minneapolis, MN, USA) was used according to manufacturer's instructions. Briefly following $CD4^+$T cell lysis, a protein-DNA precipitation solution was added to the cell lysate. After centrifugation, the supernatant containing the RNA was removed from the precipitated protein-DNA pellet. Total RNA was then precipitated with cold pure isopropanol. The pellet was washed in 70% ethanol, air dried, suspended in 20 μl of water treated with diethyl pyrocarbonate (DEPC) 1% (Sigma, Switzerland) and stored at −80°C until use. One microlitre containing 0.1–0.5 μg of total RNA was used as template for the reverse transcriptase reaction.

cDNA synthesis. Total RNA was heated to 57°C for 10 min, cooled on ice for 5 min and used as template for the cDNA synthesis. One microgram of total RNA contained in 5 μl of DEPC-treated H_2O was added to the master mix (kit Omniscript Qiagen) including 2 μl of reverse transcription buffer (100 mM Tris, 500 mM KCl, pH 8.3), 2 μl of mixed dNTP, 2 μl of oligo $(dT)_{15}$, 1 μl of omniscript reverse transcriptase and 8 μl of sterile H_2O. The reaction mixture was incubated for 60 min at 37°C, heated to 93°C for 5 min and chilled on ice for 5 min. The cDNA contained in 20 μl total volume was stored at −20°C until use.

Competitive PCR. The first-strand cDNA synthesis kit (HotStarTaq, Qiagen) was used in this competitive PCR. The semi-quantitative competitive PCR was carried out using a competitor construct (pPQRS) containing sequences for multiple cytokines including IL-4 and IFN-γ. Primers for IL-4 were 5′-CATCGGCATTTTGAACGAGGTCA-3′ (sense) and 5′-GCTACGGACCTAAGTAGCTATTC-3′ (anti-sense) and for IFN-γ were 5′-CATTGAAAGCCTAGAAAGTCTG-3′ (sense) and 5′-CTCATGAATGCATCCTTTTTCG-3′ (anti-sense). Sense and anti-sense primers were chosen on different exons separated by large intronic sequences which enabled unambiguous differentiation of cDNA from contaminating genomic DNA amplification products as described by Reiner *et al.* (12). Forty cycles were performed in this PCR. The thermal cycling conditions were as follows: 94°C for 40 s, 60°C for 20 s, 72°C for 40 s, followed by a final incubation at 72°C for 10 min. The simultaneous amplification of the cytokine gene in the first-strand cDNA and of a serial dilution of competitor of known concentration allowed the determination of the level of IL-4- and IFN-γ-specific transcripts. The point of equivalence in intensity between the competitor (upper band) and the cDNA (lower band) indicates the relative concentration of mRNA.

Subcutaneous injection of tick saliva-pulsed DCs. Purified splenic DCs (2×10^5) were pulsed or not with 15 μg of *I. ricinus* tick saliva *in vitro* overnight in 500 μl of complete RPMI-1640. Controls were maintained in RPMI-1640 medium. Cells were washed and re-suspended in 1 ml of PBS (50 mM, pH 7.4), and were then injected subcutaneously at the level of the shoulder into one flank of five BALB/c mice. Nine days after inoculation, mice were killed, and the axillary and brachial draining lymph nodes were removed. The draining lymph node cell suspensions were prepared and used in secondary stimulation *in vitro* with tick saliva. Results were assessed by the quantification of T cells proliferation and ELISA cytokine assay.

Secondary stimulation of draining lymph node T cells with tick saliva in vitro

In vitro proliferation. Draining lymph node cells per well (10^6) prepared from mice injected with tick saliva-pulsed DCs or control DCs were stimulated with 2, 4 and 8 μg of tick saliva. Cells were cultured in 200 μl total volume of complete RPMI-1640 during 96 h at 37°C and saturated atmosphere and 5% CO_2. Twenty-four hours before harvesting cells, 1 μCi per well of methyl [^{3}H]TdR was added. Methyl [^{3}H]TdR incorporation was determined by liquid scintillation counting.

In vitro IL-4 and IFN-γ production. Draining lymph node cells per well (10^6) prepared from mice injected with tick saliva-pulsed DCs or control DCs were stimulated with 4 μg of tick saliva. Culture supernatants were collected and used for IL-4 and IFN-γ quantification using an ELISA as previously reported (13). Dilutions of rIL-4 (12.5–400 U ml^{-1}) or rIFN-γ (4–125 U ml^{-1}) (PharMingen, Heidelberg, Germany) were used for construction of standard curves.

Flow cytometry. To assess the modification of adhesive and co-stimulatory molecules' expression on naive DCs that were either treated or not with tick saliva, or tick saliva plus IL-1β, flow cytometry analysis was performed. Cells were cultured during 7 days at 37°C and 5% CO_2. They were then harvested, washed and re-suspended in the staining buffer composed of PBS, 0.05% NaN_3 and 0.5% BSA. Aliquots of 10^5 cells/50 μl were incubated each with 1 μg of CD16/CD32 for 20 min in the dark, in order to block non-specific binding of antibodies to the FcγIII and FcγII receptors, and subsequently incubated for 30 min with 1 μg of the following FITC-labelled antibodies: anti-Ia^b, anti-CD80 (B7-1), anti-CD86 (B7-2), anti-CD40 or anti-CD54 (ICAM-1) (all from PharMingen). The corresponding primary-labelled isotype control antibodies were used for staining controls. Thereafter, cells were washed twice with the staining buffer and re-suspended in 500 μl of FACS buffer (0.15 M NaCl, 1 mM NaH_2PO_4 H_2O, 10 mM Na_2HPO_4 $2H_2O$ and 3 mM NaN_3). Cells were FACS analysed on a FACScan (Becton Dickinson, Heidelberg, Germany) using the corresponding CellQuest software.

Results

Expression of IL-4 and IFN-γ cytokines' mRNA during primary stimulation of CD4⁺T cells

Naive splenic CD4⁺T cells (5×10^5), primarily stimulated *in vitro* in growth medium in the presence of tick saliva-pulsed DCs or only DCs as control, were collected for mRNA analysis. IL-4 and IFN-γ mRNA levels were determined by competitive quantitative reverse transcription–PCR at day 7 of the culture. Cytokine mRNA concentrations of the T_h1 and T_h2 phenotype were compared (Fig. 1a). Splenic CD4⁺T cells primed *in vitro* with saliva-pulsed DCs expressed more IL-4 mRNA (100 pg μl^{-1}) than IFN-γ mRNA (20 pg μl^{-1}). These results indicate that tick saliva-pulsed DCs induce the differentiation of naive CD4⁺T cells into a T_h2 phenotype *in vitro*. When stimulated with non-pulsed control DCs, splenic CD4⁺T cells expressed high levels of both IL-4 and IFN-γ mRNA. IL-4 mRNA was not detectable in CD4⁺T cells primarily stimulated *in vitro* with tick saliva-pulsed DCs in the presence of neutralizing anti-IL-4 mAbs (Fig. 1b). Lower amount of IFN-γ mRNA (5 pg μl^{-1}) was expressed by CD4⁺T cells when cultures were treated with anti-IL-4 compared with that of untreated culture (20 pg μl^{-1}). *In vitro* T_h2 differentiation of CD4⁺T cells did not occur after treatment with neutralizing anti-IL-4. Nevertheless, this treatment did also not lead to the development of T_h1 cells.

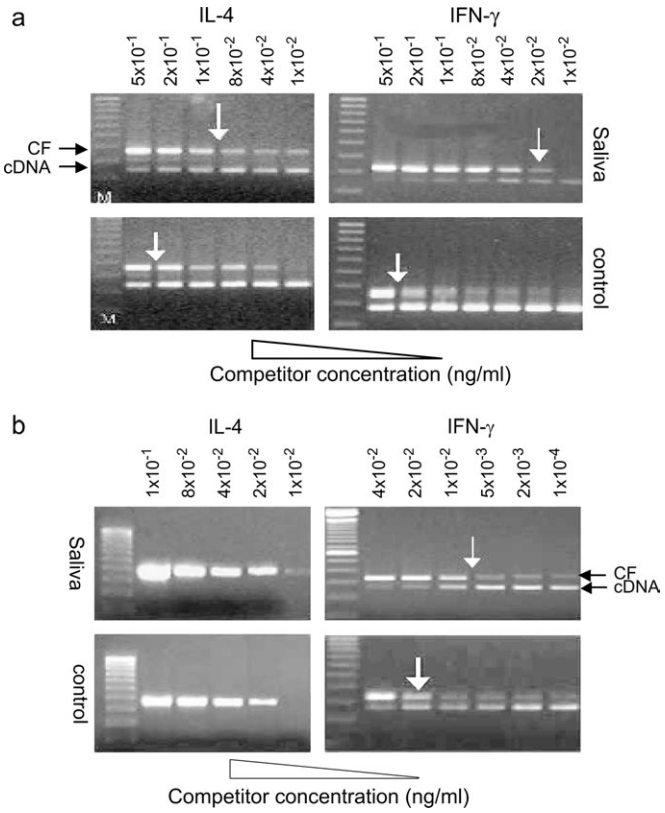

Fig. 1. Reverse transcription–PCR-based determination of the relative levels of IL-4 and IFN-γ mRNA, expressed by CD4⁺T cells primed *in vitro* with tick saliva-pulsed DCs or control DCs, performed either in the absence of anti-IL-4 (a) or in the presence of anti-IL-4 (b). The results presented are from a single experiment of three independent experiments showing virtually identical results. Ethidium bromide agarose-stained gels of representative PCRs using IL-4- and IFN-γ-specific primers in the presence of indicated serial dilutions of a competitor, for IL-4 and IFN-γ mRNA. Two microlitres of 2-fold diluted cDNA were used as template. The point of equivalence in intensity between the competitor fragment (CF) and the cDNA indicates the relative concentration of IL-4 and IFN-γ mRNA (see arrows) in CD4⁺T cells primed *in vitro*.

Effect of IL-1β addition during the primary culture on stimulated CD4⁺T cells proliferation in vitro

CD4⁺T cells primed *in vitro* with tick saliva-pulsed DCs or control DCs in the presence or absence of IL-1β were re-stimulated *in vitro* with tick saliva-pulsed DCs or control DCs (Fig. 2). Results showed that CD4⁺T cells primed in presence of IL-1β displayed a higher proliferation when secondary stimulated with tick saliva-pulsed DCs than with control DCs. CD4⁺T cells primed in the absence of IL-1β did not display a significant proliferation when secondary stimulated either with tick saliva-pulsed DCs or with control DCs. Likewise, no significant proliferation was recorded when CD4⁺T cells were primed with DCs alone even if IL-1β was present. This showed that in primary culture the presence of saliva was indispensable while IL-1β was necessary, but not sufficient, for CD4⁺T cells proliferation.

Effect of IL-1β addition during secondary in vitro stimulation on CD4⁺T cells proliferation

CD4⁺T cells were primed *in vitro* with tick saliva-pulsed DCs in the presence or absence of IL-1β. CD4⁺T cells primed in absence of IL-1β did not proliferate when co-cultured *in vitro* with tick saliva-pulsed DCs in the presence or absence of IL-1β. On the other hand, CD4⁺T cells primed in the presence of IL-1β displayed a similar high degree of proliferation no matter whether IL-1β was added or not to the culture medium in the secondary stimulation *in vitro* (Fig. 3). Results show that only CD4⁺T cells primed *in vitro* in the presence of IL-1β proliferate following the secondary stimulation. This finding indicates that the presence of IL-1β was required early during primary stimulation but not during secondary stimulation to induce proliferation of T_h2 cells primed *in vitro*.

Co-stimulatory molecules' expression by activated DCs

The expression of selected major co-stimulatory molecules involved in the accessory activity of DCs was analysed. As shown in Fig. 4, IL-1β treatment of ticks saliva-pulsed DCs up-regulated surface expression of B7-1 (CD80) and to a lesser extent B7-2 (CD86), but markedly CD40 co-stimulatory molecules. In the absence of IL-1β, tick saliva treatment of naive DCs increased drastically the expression of B7-2 (CD86) and to lesser extent CD40 molecules, while B7-1 expression remained very low. The class II molecules (Ia) were increased by DCs pulsed with tick saliva in the presence or absence of IL-1β. The surface expression levels of Ia molecules as well as co-stimulatory molecules remained very low in untreated DCs, while the expression of the adhesion molecule ICAM-1 (CD54) was significantly up-regulated in comparison with the treated DCs. The up-regulation of the co-stimulatory molecules, especially B7 molecules

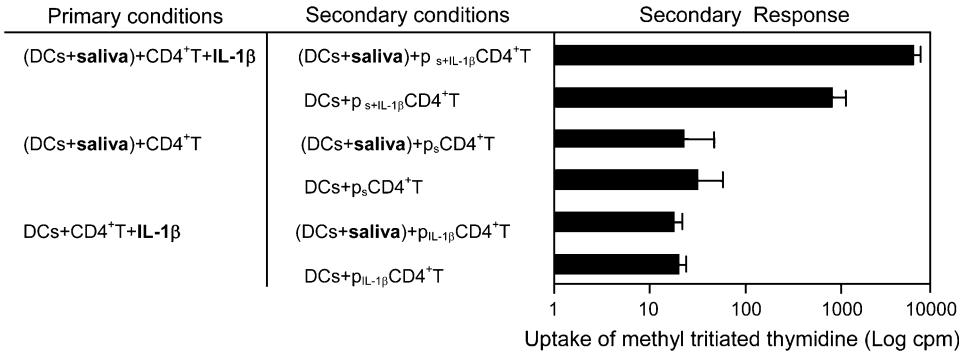

Fig. 2. Effect of the addition of IL-1β during stimulation on the proliferation of primed splenic $CD4^+$T cells following secondary stimulation with tick saliva-pulsed DCs or control DCs. Splenic $CD4^+$T cells cultured either with tick saliva-pulsed DCs in the presence or absence of IL-1β or control DCs plus IL-1β were secondarily stimulated with tick saliva-pulsed DCs or control DCs. Twenty-four hours before harvesting cells, 1 μCi per well of methyl [^{3}H]TdR is added, and incorporation of radioactivity was determined by liquid scintillation counting. Each value represents the mean of triplicate wells ± SD from a single experiment representative of three separate experiments. $p_{s+IL\text{-}1\beta}$ $CD4^+$T cells = $CD4^+$T cells primed in presence of saliva and IL-1β. p_s $CD4^+$T cells = $CD4^+$T cells primed in presence of saliva and absence of IL-1β. $p_{IL\text{-}1\beta}$ $CD4^+$T cells = $CD4^+$T cells primed in absence of saliva and presence of IL-1β.

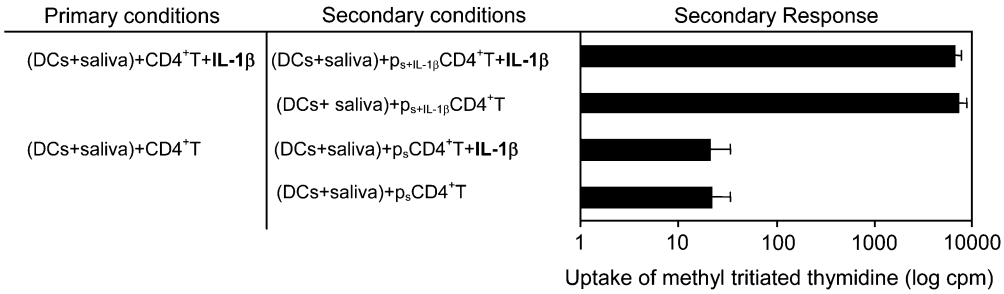

Fig. 3. Comparison of the effect of the addition of IL-1β on splenic $CD4^+$T cells proliferation during primary and secondary *in vitro* stimulation. Splenic $CD4^+$T cells primed *in vitro* with ticks saliva-pulsed DCs in the presence or absence of IL-1β were re-stimulated under the same conditions. Twenty-four hours before harvesting cells, 1 μCi per well of methyl [^{3}H]TdR is added. Incorporation of radioactivity was determined by liquid scintillation counting. Each value represents the mean of triplicate wells ± SD from a single experiment representative of three separate experiments.

and CD40, which is a prerequisite for full T cell activation, appeared to take place when tick saliva-pulsed DCs were treated in culture with IL-1β.

Activation of draining lymph node T cells primed in vivo with I. ricinus saliva-pulsed DCs

Nine days following subcutaneous injection of tick saliva-pulsed DCs into BALB/c mice, an increase of the size of axillary and brachial lymph nodes draining the site of inoculation was observed *in situ*. In contrast, the size of lymph nodes of the non-inoculated site has not changed (data not shown). Control mice received only DCs.

The activation of draining lymph node T cells primed *in vivo* with tick saliva-pulsed DCs or control DCs was assessed by the determination of the level of proliferation and cytokines (IL-4 versus IFN-γ) production. In secondary stimulation *in vitro*, primed draining lymph node T cells with the tick saliva-pulsed DCs exhibited, following stimulation with different protein–saliva concentration 2, 4 and 8 μg ml^{-1}, a high level of proliferation [>90 × 10^3 counts per minute (c.p.m.)]. The maximal of proliferation (145.213 × 10^3 c.p.m. ± 8.259 SD) was reached when primed T cells were stimulated with 4 μg ml^{-1} of protein–saliva. As a control, the proliferation of draining lymph node T cells primed *in vivo* with only DCs remained relatively very low (<25 × 10^3 c.p.m.) at different point of protein–saliva concentration.

Four micrograms per millilitre was the point of protein–saliva concentration that induce the higher level of proliferation used in the same cultures in view to collect the supernatant for the determination by ELISA of the amount of produced cytokines as IL-4 versus IFN-γ. Results revealed that draining lymph node T cells from mice injected with tick saliva-pulsed DCs produced higher level of IL-4 (16.2 U ml^{-1} ± 1.9 SD) than IFN-γ (0.3 U ml^{-1} ± 0.2 SD), while in the control, draining lymph node T cells from mice injected only with DCs secreted low amounts of both IL-4 (2 U ml^{-1} ± 0.5 SD) and IFN-γ (0.45 U ml^{-1} ± 0.15 SD).

The higher dose of IL-4 than IFN-γ produced by lymph node cells in secondary stimulation indicated that primarily tick saliva-pulsed DCs that reach the T cell area of the lymph node activate salivary antigen-specific T cells to differentiate into T_h2 cells; moreover, it appears that these cells react very fast to the low doses of saliva to proliferate and to produce IL-4.

Discussion

Pathogen-free Ixodidae vectors such as the prostriates, *Ixodes scapularis* and *Ixodes pacificus*, bias the immune response of C3H mice toward T_h2 (14). The metastriates, *Dermacentor andersoni* and *Rhipicephalus sanguineus*, are also able to induce T_h2 immune response into BALB/c mice (15) and C3H/HeJ mice (16), respectively. In our system, *I. ricinus* infestation of mice with different backgrounds, BALB/c (H-2^d), DBA (H-2^d), C57BL/6 (H-2^b), CBA (H-2^k), C3H (H-^{2k}), SJL (H-2^s) and FVB (H-2^q), produce always this type 2 immune response (6). *Ixodes scapularis* infected with *B. burgdorferi* induce a T_h2 immune response in Lyme disease susceptible C3H/HeJ mice (17). Exogenous delivery of the T_h1 cytokines, IFN-γ and IL-2, given at the time of tick feeding on mice, suppresses spirochetes transmission by *I. scapularis* (18). Christe *et al.* (4) show that BALB/c develops a mixed T_h1/T_h2 response after syringe inoculation of *B. burgdorferi* and a T_h2 immune response against the pathogen after tick inoculation. These results evidenced the crucial role of tick saliva in the T_h2 polarization of the mice immune response. The importance of tick saliva is revealed in their ability to modulate host immune response. Indeed, *R. sanguineus* tick saliva increases IL-10 and diminishes IFN-γ production by *Trypanosoma cruzi*-stimulated cells (11). Later, it showed to be able to reduce IL-12 and increase transforming growth factor-β production by murine cells cultured with trypomastigote forms of *T. cruzi* (16). These results support the view that *R. sanguineus* ticks may modulate the host's cytokine network and enhanced especially the production of type 2 cytokines. Moreover, IL-10 produced by tick-infested mice can interfere with functional maturation of DCs, and can selectively inhibit their capacity to induce the development of IFN-γ-producing T cells *in vivo*, favouring T_h2 differentiation.

DCs are specialized APCs required for the priming and activation of CD4⁺T cells (9). They are the most potent APCs in priming *L. major*-specific T cells, followed closely by B cells and finally by macrophages (19). In our work, the ability of *I. ricinus* saliva to direct the differentiation of IL-4-secreting naive CD4⁺T cells toward a T_h2 type of immune response was demonstrated using an *in vitro* priming system. Tick saliva-sensitized T_h2 cells were obtained by co-culturing naive CD4⁺T cells with tick saliva-pulsed DCs during 7 days. This period of time has also been considered to be optimal in other systems (19, 20). Primed CD4⁺T cells expressed higher levels of IL-4 than IFN-γ mRNA, and these levels remained lower than those observed in the control. This could be due to the relatively reduced number of naive T cell populations, responding specifically to saliva-pulsed DCs and differentiating into specific T_h2 cells. Suppressive effects of some saliva molecules on T cell activation are not excluded. In fact, *I. ricinus* saliva contains immunosuppressive as well as immunogenic molecules (21). However, the presence of suppressive molecules does not interfere with the establishment of T_h2 immune response *in vivo*. The high levels of IL-4 and IFN-γ mRNA measured in the control cultures can be

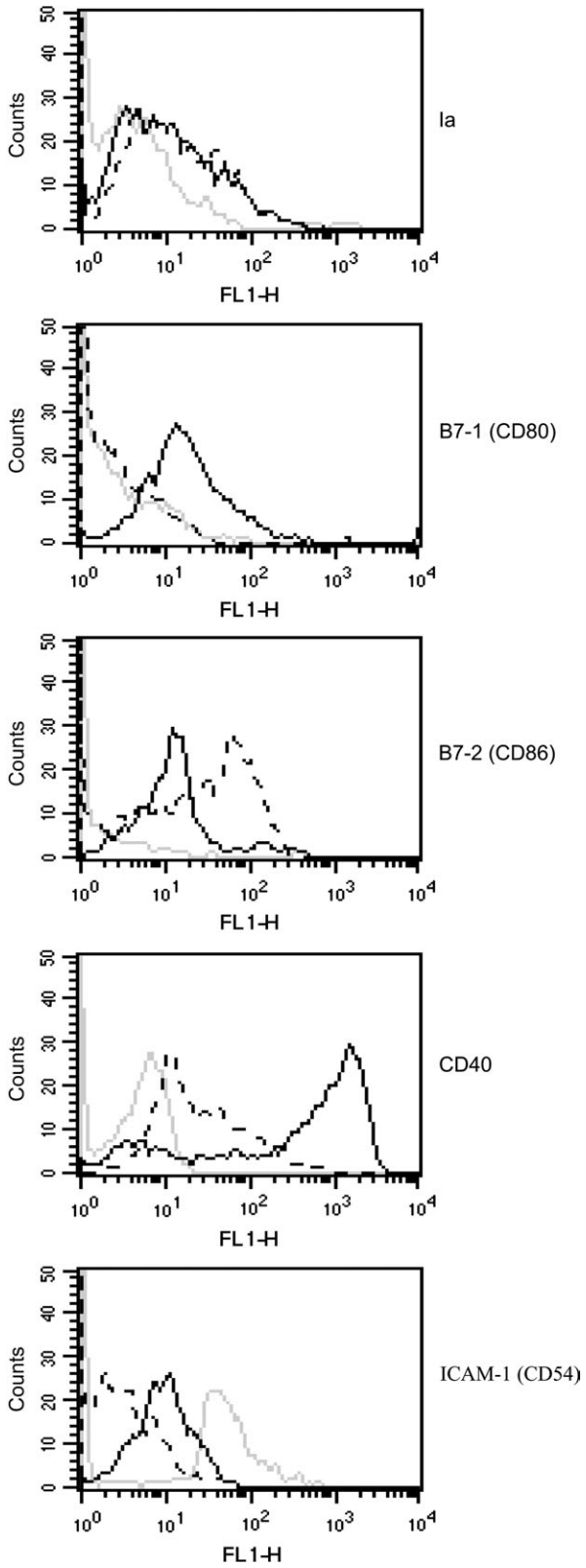

Fig. 4. Analysis of class II (Ia), B7-1 (CD80), B7-2 (CD86), CD40 and ICAM-1 (CD54) surface expression in DCs. The grey, the dotted and the black lines show the level of expression of different co-stimulators on (a) untreated DCs, (b) DCs pulsed with ticks saliva and (c) DCs pulsed with ticks saliva plus IL-1β, respectively. This experiment was repeated three times with virtually identical results.

explained by the stimulation of CD4⁺T cell clones that have an unrestricted profile producing IL-4 and IFN-γ simultaneously (22). Moreover, CD4 memory T cells present in the culture can simultaneously express IL-4 and IFN-γ (23).

To comparatively assess these results obtained *in vitro* with the *in vivo* situation, DCs were pulsed overnight with *I. ricinus* saliva and were injected subcutaneously into BALB/c mice. We found that this also induced the development of an anti-tick T_h2 immune response. The swelling of axillary and brachial draining lymph nodes indicates that saliva-pulsed DCs migrate from the site of inoculation to draining lymph nodes and activate responding cells triggering the enlargement of the secondary lymphoid organs. From the peripheral tissue, antigen-bearing DCs reach via lymph conducts the T area of lymph nodes (24). In our study, salivary antigens-bearing DCs prime T cells in the lymph nodes that differentiate into T_h2 type of cells and proliferate ensuring the increase of lymph node size. In this context, the activation of B cells is excluded since these cells reside in the follicular area where they respond to intact free antigens that exit the lymph or the blood and enter the lymph node. However, it was demonstrated recently an extrafollicular activation of lymph node B cells by especially particulate material as bacterial or viral antigen-bearing DCs (25). Taking into account this finding, an extrafollicular activation of lymph node B cells is excluding since in our work pathogen-free ticks reared in our laboratory were used to prepare tick saliva that did not contain any pathogen. Here evidence is available to indicate that T cells and especially T_h2 cells were activated by saliva-pulsed DCs *in vivo* into the lymph nodes. In agreement with our results, it was found that *in vitro* purified *I. ricinus* salivary gland protein with a molecular weight of 65 kDa (IrSG65) stimulated the proliferative activity of axillary and brachial lymph node cells prepared from mice infested with nymphal ticks. Depletion of CD4⁺T cells drastically reduced the ability of lymph node cells from infested mice to proliferate after (IrSG65) stimulation *in vitro* (26). Thus, the secondary stimulation of lymph node cell *in vitro* with different dose of saliva triggered the activation of T cells that proliferate and produce IL-4 cytokine. Our results confirmed the importance of tick saliva molecules in the polarization phenomenon, and on the other hand, demonstrate the usefulness of the *in vitro* priming model to assess different parameters of this immune response. Similar observations showing good correlation between *in vitro* and *in vivo* assays were made in other infection models. For instance, in the presence of phosphorylcholine-containing glycoprotein secreted by the nematode *Acanthocheilonema viteae*, DCs induce a T_h2 response while DCs maturing *in vitro* in the presence of LPS promote a T_h1 response (27).

IL-4 represents a reliable marker for differentiation of T_h2 type of cells and down-regulation of IFN-γ production (28). The addition of exogenous IL-4 to culture of naive CD4⁺T cells in the presence of mitogen (anti-CD3) (29) or alloantigen (30) directs the development of T_h2 effector cells, which on re-stimulation produce high levels of IL-4, IL-5 and IL-10 as well as IL-13 and granulocyte macrophage colony-stimulating factor. In our work, we showed that DCs pulsed overnight with tick saliva were able to trigger the *in vitro* differentiation of naive CD4⁺T cells into T_h2 cells expressing higher level of IL-4 than IFN-γ mRNA, without the addition of IL-4. Therefore, anti-IL-4 mAb applied in the culture inhibited the development of IL-4-secreting T_h2 cells while the expression of IFN-γ by CD4⁺T cells remained low.

DCs-derived factors determine T_h1 or T_h2 immune response. IL-12-secreting DCs1 have been shown to be required for differentiation of IFN-γ-secreting T_h1 cells *in vitro* (31). The low expression of IFN-γ mRNA following anti-IL-4 treatment in our culture system can be explained by the lack of IL-12 secretion due to an unknown tick saliva factor, or potentially by the secretion of IL-10 by DCs2, which could decrease the number of DCs1. Our experiments suggest that IL-4-dependent signalling pathway is indispensable to bias the cytokine secretion pattern toward the T_h2 phenotype. In contrast, the neutralization of any endogenous IL-4 with antibodies during primary culture of T cells from TCR transgenic mice specific for ovalbumin (OVA) and various types of APCs pulsed with OVA results in the appearance of T cells that produce IFN-γ and IL-2 upon stimulation (32). In our *in vitro* priming system, the endogenous IL-4 may be secreted by minor populations of splenic CD4⁺T cells such as memory T_h2 cells or naive αβ CD4⁺T cells. NK 1.1⁻ CD4⁺T cells express a high level of IL-4 transcripts 90 min after intravenous injection of *L. major* (33). In the natural infestation, IL-4 that sets the polarization toward T_h2 might be produced early by some cells of the skin such as mast cells, keratinocytes or infiltrating basophiles. Previously, in our group, Mbow *et al.* (34) showed by histological examination of skin biopsies that early during the first hours of the primary infestation, mast cells and degranulated mast cells did not vary in number. The number of infiltration eosinophils remained very low and the basophiles were rare or absent. However, it not exclude that mast cells and keratinocytes stimulated by tick saliva secrete the relatively little amount of IL-4 during the primary infestation. A higher amount of IL-4 registered in the skin during the secondary infestation may be assigned to infiltrating basophiles (34). Nevertheless, a member of our group attempted to segregate the different populations of cells from epidermis of ear's skin of mice primary infested by larva of *I. ricinus* and checked their ability to secrete IL-4. In this experiment, Tγδ cells were found to be the mean type of cells secreting IL-4. The pick of IL-4 production was reached at 18 h after infestation while the production of this cytokine by the other T cells (Tαβ, CD4⁺T and CD8⁺T cells) and CD3⁻ cells (keratinocytes and granulocytes) remained very low (L. Yvan, personal communication).

Tick saliva-specific T_h2 cells differentiated *in vitro* did not display any proliferation either during primary stimulation or during a secondary *in vitro* stimulation with tick saliva-pulsed DCs. However, they proliferated strongly when IL-1β was added as soluble co-stimulator in the *in vitro* priming culture (Fig. 2). In accordance with our finding, Chang *et al.* (35) showed that an optimal proliferation of T_h2 clones in response to IL-4 requires the addition of IL-1. Moreover, secreted IL-1β mostly acts as a co-stimulator for the proliferation of T_h2 lymphocytes (36, 37). In contrast, T_h1 clones secrete IL-2 and proliferate without any requirement for IL-1β. In our experiment, exogenous IL-1β was probably needed because IL-4 secreted by T_h2 cells inhibited the transcription and secretion of IL-1β (38). In TCR transgenic T cell

systems, neither IL-1β receptor antagonist nor antibodies to IL-1β diminish priming for IL-4 production mediated by antigen, APCs and IL-4 (39, 40). IL-1β is only required for the proliferation of some T_h2 clones in response to IL-4 but not for the differentiation of murine CD4⁺T cells into T_h2 clones. In our study, it appears that proliferative responses and lymphokine production by T_h2 cells exhibit distinct signalling requirements. Moreover, we show that IL-1β is not required in secondary stimulation of differentiated T_h2 cells either when CD4⁺T cells are primed in presence of IL-1β or in its absence (Fig. 3). This suggests that proliferation of differentiated T_h2 cells require an early IL-1β-dependent signalling pathway only during primary cell culture.

To provide further explanation to the activation of naive CD4⁺T cells, we compared the expression of the major accessory molecules, such as CD40, CD80 (B7-1) and CD86 (B7-2), by FACS analysis of tick saliva-treated DCs, DCs treated with saliva plus IL-1β, and control DCs, and explained their role in the stimulation of CD4⁺T cells at day 7 of *in vitro* culture. Tick saliva plus IL-1β-treated DCs exhibited mainly an increased CD40, and to a lesser extent also B7 receptor expression. CD40 had been shown to up-regulate the B7 co-stimulatory molecule, which stimulated T cells via the CD28 ligand and drove the cell to enter into the cell cycle and become fully active (41, 42). The high level of CD40 explains also the maintenance of the high expression of the class II molecules registered in our results. It had been reported that CD40 increased also the antigen-presenting functions of monocytes/DCs by maintaining high levels of MHC class II antigens and up-regulation of CD58 (leukocyte function-associated antigen-3) expression (43). Treatment of DCs only with tick saliva induced up-regulation of class II (Ia), CD40 and, markedly, B7-2 molecules' expression. The strength of these co-stimulators' expression appeared not sufficient to drive the proliferation of primary-stimulated CD4⁺T cells, but was strong enough to trigger their differentiation into T_h2 cells. The co-stimulation via B7-2 could promote the production of IL-4 by primed CD4⁺T cells; however, this needs to be experimentally clarified. Many studies showed the critical role of B7-2 in the preferential induction of a T_h2 immune response (44, 45). In the case of the *L. major* infection model, where a dichotomy in the immune response (T_h1 or T_h2) has been described to be dependent of the strain of the infected mice (C57BL/6 versus BALB/c), this hypothesis was supported by the finding that treatment of BALB/c mice with neutralizing anti-B7-2 mAb markedly reduce the levels of IL-4 produced in the lymph nodes draining leishmanial lesions (46). However, in different experimental models controversial results have been obtained concerning the implication of CD80 and CD86 as differential determinants of T_h1- versus T_h2-type cytokine profiles (47, 48). While DCs untreated with tick saliva increase slightly the class II (Ia) molecules but significantly the adhesion molecule ICAM-1 (CD54), these cells are not able to activate CD4⁺T cells in primary culture. ICAM-1 appeared thus to be inadequate for triggering T cell activation. Indeed, the selective contact of T cells with ICAM-1 plus MHC–peptides during priming can lead to a prominent hypo-responsive status (49). *Ixodes ricinus* saliva appears to be able to reduce DC expression of ICAM-1 (Fig. 4). According to our findings, salivary gland extracts of *D. andersoni* significantly down-regulated the expression of ICAM-1 by endothelial cells, which may alter leukocyte migration to the bite site (50). It has also been demonstrated that the ICAM-1–LFA-1 signalling pathway needs to be coupled to the B7–CD28 signalling pathway for the activation of lymphocytes (51).

In conclusion, the *in vitro* priming system employed in this study is restricted to major elements implicated in the anti-tick immune response such as naive CD4⁺T cells, whole DC populations, and tick saliva. However, the results obtained here are comparable to the situation *in vivo* with several strains of mice (6), including BALB/c. Thus, the *in vitro* model provides the possibility to delimit further the immunogenic saliva molecules that pulse DC subsets to induce T_h2 immune response. The neutralization of these saliva putative molecules may interfere with the establishment of T_h2 immune response, and subsequently with tick-borne pathogens' transmission.

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Abbreviations

APC	antigen-presenting cell
c.p.m.	counts per minute
DC	dendritic cell
DEPC	diethyl pyrocarbonate
[^{3}H]TdR	[^{3}H]thymidine
OVA	ovalbumin

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